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## Polycomb Repression regulates Schwann Cell Proliferation and Axon Regeneration after Nerve Injury

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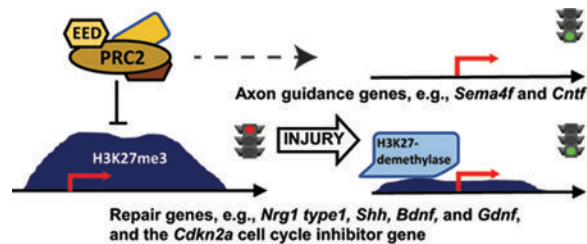
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### Abstract

The transition of differentiated Schwann cells to support of nerve repair after injury is accompanied by remodeling of the Schwann cell epigenome. The EED-containing polycomb repressive complex 2 (PRC2) catalyzes histone H3K27 methylation and represses key nerve repair genes such as *Shh*, *Gdnf* and *Bdnf*, and their activation is accompanied by loss of H3K27 methylation. Analysis of nerve injury in mice with a Schwann cell-specific loss of EED showed the reversal of polycomb repression is required and a rate limiting step in the increased transcription of *Neuregulin 1* (type I), which is required for efficient remyelination. However, mouse nerves with EED-deficient Schwann cells display slow axonal regeneration with significantly low expression of axon guidance genes, including *Sema4f* and *Cntf*. Finally, EED loss causes impaired Schwann cell proliferation after injury with significant induction of the *Cdkn2a* cell cycle inhibitor gene. Interestingly, PRC2 subunits and *CDKN2A* are commonly co-mutated in the transition from benign neurofibromas to malignant peripheral nerve sheath tumors (MPNST's). RNA-seq analysis of EED-deficient mice identified PRC2-regulated molecular pathways that may contribute to the transition to malignancy in neurofibromatosis.

### Abstract



### Keywords

Schwann myelin chromatin nerve injury axon neurofibromatosis

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## Introduction

Schwann cells in the peripheral nervous system not only create myelin, but also become reprogrammed after nerve injury to support nerve regeneration. Soon after injury, repair cells derived from both myelinating and non-myelinating (Remak) Schwann cells create a structural and trophic environment that stimulates axon regeneration (Brosius Lutz and Barres 2014; Gomez-Sanchez et al. 2017; Jessen and Mirsky 2016). Repair Schwann cells activate autophagy and phagocytosis mechanisms to remove myelin debris, which inhibit axon regrowth and branching (Brosius Lutz et al. 2017; Gomez-Sanchez et al. 2015; Mukhopadhyay et al. 1994; Shen et al. 1998), and promote recruitment of macrophages that further facilitate myelin removal and regeneration (Cattin et al. 2015; Fischer et al. 2008; Niemi et al. 2013). Elongated Schwann cells distal to the injury site form Bands of Bungner, which serve as tracks for axonal regeneration (Arthur-Farraj et al. 2012; Gomez-Sanchez et al. 2017).

Such injury-responsive changes are mediated by a distinct transcriptional program that involves the transcription factor JUN (Arthur-Farraj et al. 2012). Many injury-induced genes encode intercellular signaling molecules such as *sonic hedgehog (Shh)*, which is silenced throughout Schwann cell development prior to injury (Lin et al. 2015). Schwann cells in injured nerve induce genes encoding factors that promote axon survival and regeneration: e.g. *brain- and glial-derived neurotrophic factors, Bdnf* and *Gdnf*, *nerve growth factor (Ngf)*, and *leukemia inhibitory factor (Lif)* (Boyd and Gordon 2003; Cafferty et al. 2001; Fontana et al. 2012; Li et al. 2015; Widenfalk et al. 2009). While Schwann cells are normally dependent on axonal neuregulin type III signaling in normal development (Michailov et al. 2004; Taveggia et al. 2005), neuregulin 1 (NRG1) type I is also secreted by Schwann cells shortly after injury, and is required for efficient remyelination (Stassart et al. 2013). In many cases, levels of these genes are low or absent in mature Schwann cells prior to injury.

Despite the striking adaptability of Schwann cells to damage, the clinical outcomes of human patients generally exhibit only partial recovery in many cases (Höke 2006; Lundborg 2000). One of the main reasons is that axons must regenerate over a relatively long distance, and Schwann cells more distal to injury sites gradually lose their ability to foster nerve regeneration (Jonsson et al. 2013; Ronchi et al. 2017; Sulaiman and Gordon 2009; Sulaiman and Gordon 2013), which could be in part due to reduced expression of neurotrophic factors like GDNF and BDNF (Eggers et al. 2010; Fontana et al. 2012; Höke et al. 2002; Li et al. 1997; Michalski et al. 2008; Sulaiman and Gordon 2009). Therefore, identifying the molecular mechanisms that enable rapid axon regeneration is important for improving therapeutic strategies for peripheral nerve damage.

While many studies of gene expression changes after nerve injury have been performed (Arthur-Farraj et al. 2012; Arthur-Farraj et al. 2017; Barrette et al. 2010; Clements et al. 2017; Nagarajan et al. 2002), somewhat less is known about the mechanisms governing the epigenomic transition of mature to repair Schwann cells. It has become clear that epigenomic changes are important for such reprogramming events, employing mechanisms of gene activation and derepression (Brügger et al. 2017; He et al. 2018; Hung et al. 2015;

Jacob 2017; Ma and Svaren 2018). Our previous studies identified the association of trimethylation at Lys27 of histone H3 tail (H3K27me3) at promoters of many genes that become activated after peripheral nerve injury, and we found that the demethylation is required for full activation of some repair genes (Ma et al. 2016). H3K27 methylation is catalyzed by Polycomb Repressive Complex 2 (PRC2), comprising the lysine methyltransferase EZH1/2 and the nonredundant core subunits, suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED). EED is not required during Schwann cell development and myelination (Ma et al. 2015), although there is Remak bundle disruption and hypermyelination in older EED deficient mice. Gene expression analyses, however, revealed a premature derepression of some injury-response genes in uninjured *Eed* cKO nerves. The findings suggested that Schwann cell EED and PRC2 normally repress the injury responsive-transcriptional program and therefore PRC2 could affect nerve regeneration. In addition to its role in injury, loss of PRC2 has been associated with disease progression of the Schwann cell-derived tumors in neurofibromatosis, caused by loss of the NF1 tumor suppressor. Inactivation of PRC2 occurs in the malignant form, known as malignant peripheral nerve sheath tumors (MPNST's), as *SUZ12* or *EED* genes encoding PRC2 subunits are mutated or deleted in a high proportion of MPNST's (Cleven et al. 2016; De Raedt et al. 2014; Lee et al. 2014; Pekmezci et al. 2017; Zhang et al. 2014).

Given that PRC2 has been identified as a regulator of Schwann cell repair genes after injury (Ma et al. 2015; Ma et al. 2016), we used the *Eed* cKO model to determine how lack of PRC2 activity would affect nerve injury responses and the gene expression reprogramming that occurs in Schwann cells after injury.

## Materials and Methods

### Primer sequences and Antibodies.

The primers and antibodies are listed in Tables 1 and 2, respectively.

### Experimental animals and Nerve injury surgery.

All animal experiments were performed according to protocols approved by the University of Wisconsin Graduate School Animal Care and Use Committee. *Eed*-floxed mice (B6;129S1-*Eed*<sup>tm1Sho</sup>/J, RRID:IMSR\_JAX:022727) were generated by backcrossing the *fllox* allele for seven generations against the C57BL/6 genetic background and mated to mP0TOTA-Cre (B6N.FVB-Tg(Mpz-cre)26Mes/J, obtained from Jackson Laboratory, RRID:IMSR\_JAX:017927). Mice were genotyped as described previously (Feltri et al. 1999; Xie et al. 2014). Samples collected from mice homozygous for floxed *Eed* served as control in this study. The sciatic nerves of adult Sprague-Dawley rats or mice at the age of 2 months were exposed and transected at the sciatic notch (Hung et al. 2015) or crushed 1 min using fine forceps. As a control, the contralateral limb also received a sham operation consisting of only a skin incision. The nerve tissues distal to the transection or crushed lesions, which were labeled with sterile black ink, and contralateral (sham) nerves were isolated for use in gene expression analysis, Western blotting, immunohistochemistry or ChIP experiments. For electron microscopy analysis, the sciatic nerve was analyzed 4 mm distal to the crushed lesion. Both male and female mice were used individually per sample at similar ratio

between the floxed *Eed* and *Eed* cKO genotypes. Male rats were used in ChIP experiments after nerve injury surgery.

### **Electron microscopy and morphometric quantification.**

Freshly dissected sciatic nerves were immersion fixed in a solution of 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight at 4°C. The nerves were then postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature. Following OsO<sub>4</sub> postfixation, the nerves were dehydrated in a graded ethanol series, and then further dehydrated in propylene oxide and embedded in Epon or Durcupan epoxy resin. Ultrathin transverse sections were contrasted with Reynolds lead citrate and 8% uranyl acetate in 50% ethanol. Images were obtained with a Philips CM120 electron microscope with an AMT BioSprint side-mounted digital camera at the UW Medical School Electron Microscope Facility. Densitometric quantification was performed using NIS-Elements 4.0. Three mice per genotype were analyzed, and statistical analyses were evaluated by one-way ANOVA in all the experiments.

### **Immunohistochemistry.**

Freshly dissected nerves were embedded in Tissue-Tek OCT compound (Sakura Finetek) and snap frozen with liquid nitrogen. Longitudinal or transverse cryostat sections (14 μm) were air-dried for 5 min and fixed in 4% paraformaldehyde for 15 min. The sections were then blocked in PBS containing 5% donkey serum/1% BSA/3% Triton-X 100 for 1 h at room temperature. Primary antibody incubation was performed overnight at 4°C in PBS containing 5% donkey serum/1% BSA/1% Triton-X 100 and secondary incubation was performed in PBS at room temperature for 1 h. Hoechst 33342 (1:5000 in PBS, Sigma) was applied to stain nuclei for 1 min. Three 4 min washes were performed in PBS after fixation and blocking, and in PBS containing 0.1% Tween20 after primary antibody incubation and nuclear staining. After coverslips were mounted using Fluoromount-G™ (SouthernBiotech), sections were examined on a confocal microscope (Nikon A1R-Si). Statistical analyses were evaluated by one-way ANOVA.

### **Western blot.**

Freshly dissected nerves were snap frozen with liquid nitrogen and crushed. The nerves were then homogenized in lysis buffer (50 mM Tris-HCL at pH 6.8, 10% glycerol, 2% SDS, 10% β-mercaptoethanol, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub> and Protease Inhibitor Cocktail (Sigma, P8340)) using a motorized pellet pestle. Cells in culture were homogenized in 3X lysis buffer. After a 15 min incubation in ice, lysates were boiled at 95°C for 3 min and centrifuged at 4°C for 15 min. Subsequently, supernatants were collected and subjected to SDS-PAGE. After transfer to nitrocellulose membrane, proteins were blocked in TBST containing 5% nonfat dry milk for 1 h at room temperature. Primary and Secondary antibody incubations were performed in TBST containing 5% non-fat dried milk at 4°C for overnight and at room temperature for 1 h, respectively. Three 5 min-washes were performed in TBST after the incubations. The membranes were scanned and quantitated with the Odyssey Infrared Imaging System (Li-Cor Biosciences). Statistical analyses were evaluated by one-way ANOVA.

### Nerve explant cultures.

Adult male Sprague-Dawley rat sciatic nerves were cut into 3 mm segments and cultured in serum-free RPMI-1640 medium supplemented with penicillin/streptomycin in the presence of GSKJ4 (Tocris, Cat. No. 4594) or DMSO at 37 °C for 1 d. RNA was purified from the explanted nerves at the indicated timepoints.

### Micrococcal nucleases (MNase) aided Chromatin immunoprecipitation (ChIP) in vivo.

Sciatic nerves were subjected to MNase-ChIP with an antibody targeting H3K27me3 as described previously (Ma et al. 2016) with a few changes. Instead of washing with RIPA buffer after the immunoprecipitation, ChIP samples were washed with washing buffer 1 (WB1, 50 mM Tris-HCl, pH7.5; 10 mM EDTA; 125 mM NaCl) once, WB2 (50 mM Tris-HCl, pH7.5; 10 mM EDTA; 250 mM NaCl) once, and WB3 (50 mM Tris-HCl, pH7.5; 10 mM EDTA; 500 mM NaCl) twice.

### ChIP-seq.

Library preparation and sequencing was performed by the UW Biotechnology Center as described previously (Hung et al. 2015). Basecalling was performed using the standard Illumina Pipeline. Reads were mapped to the *Rattus norvegicus* genome rn5 using Bowtie (RRID:SCR\_005476) to produce SAM files for further analysis. From the two biological replicates, we obtained 24,145,063 and 30,080,399 reads in input and 28,792,879 and 30,462,059 reads in H3K27me3 ChIP samples. Hypergeometric optimization of motif enrichment (HOMER, RRID:SCR\_010881) (Heinz et al. 2010) was used to determine enriched binding regions for H3K27me3-ChIP relative to sequencing of an input chromatin sample. H3K27me3-occupied genes were defined by the presence of the histone modification around the transcriptional start site ( $\pm 7$  Kb) with HOMER peak-score  $\geq 10$  (Ma et al. 2016). The raw data files are deposited in National Center for Biotechnology Information Gene Expression Omnibus (GEO) under accession number GSE106994.

### qRT-PCR.

RNA was isolated from sciatic nerves using RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's directions. To prepare cDNA, 250 ng or 1  $\mu$ g of total RNA of mouse or rat nerves, respectively, was used from each sample. qRT-PCR and data analysis were performed as described previously (Hung et al. 2012). qPCR was performed with two replicates per sample, i.e., two technical replicates. Statistical analyses were evaluated by one-way ANOVA.

### RNA-seq.

>500 ng total RNA was used to generate RNA-seq libraries using the Illumina TruSeq Stranded total RNA sample preparation kit according to the manufacturer's instructions. An average of 14 million reads per sample was obtained and mapped to the GRCm38/mm10 genome. Data were analyzed using CLC Bio Workbench to determine differentially regulated genes between uninjured and injured nerves in wild type and *Eed* cKO mice (p-value, < 0.05). The raw data files are deposited in National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE106994.

## Results

### Analysis of Nerve Injury Responses in the *Eed* cKO

To explore the potential importance of EED in Schwann cell responses to nerve injury, we examined the repair process by electron microscopy at 14 d following sciatic nerve crush at the sciatic notches of 2 month old mice. Regenerating nerves initiate a program that includes myelin debris removal, axon regeneration and remyelination (Arthur-Farraj et al. 2012; Jessen and Mirsky 2016). Since our previous studies showed precocious activation of injury genes in the *Eed* cKO (Ma et al. 2016), we had anticipated that there may be accelerated regeneration. However, the ultrastructural analysis revealed that there were relatively fewer axons at the transverse sections 4 mm distal to the injury site in *Eed* cKO nerves compared to sections of control mice, which exhibited a substantial number of myelinated axons at 14 d after injury (Figure 1A, B). Importantly, the number of regenerated axons greater than 1  $\mu\text{m}$  in diameter, which should eventually become remyelinated, were significantly reduced in *Eed* cKO nerves.

Clearance of myelin debris is a critical step because myelin, particularly myelin-associated glycoprotein (MAG), inhibits axon growth (Filbin 2003; Mukhopadhyay et al. 1994; Shen et al. 1998). Soon after injury, Schwann cells downregulate a myelin gene network and activate autophagy and phagocytic programs to carry out myelin clearance, which is further facilitated by recruited macrophages (Brosius Lutz et al. 2017; Gomez-Sanchez et al. 2015; Hirata and Kawabuchi 2002). Quantitation of myelin debris at 14 d post-injury did not reveal a significant difference in *Eed* cKO nerves compared to control nerves (Figure 1C). The number of infiltrating macrophages and engulfed myelin debris were also comparable. In addition, myelin breakdown was apparent 3 d post injury in *Eed* cKO nerves at levels comparable to control nerves (not shown), along with the expected decrease in myelin gene expression such as *Mag*, *peripheral myelin protein 22* (*Pmp22*) and an essential transcription factor of myelination, *Egr2/Krox20* (Figure 1-figure supplement 1).

To assess the regeneration affected by *Eed* deletion, we examined nerves at one month after crush injury (Figure 1A). Myelination in *Eed* cKO nerves appeared largely similar to control nerves at this later timepoint. The number of myelinated axons and, more importantly, the total number of axons greater than 1  $\mu\text{m}$  in diameter were comparable between the two groups (Figure 1B), indicating that delayed axon regeneration did eventually recover. The analysis of g-ratios, the ratio of the axonal diameter to the diameter of outer myelin sheath, however, revealed persistently thinner myelin sheaths around axons greater than 3  $\mu\text{m}$  in *Eed* cKO nerves (Figure 1D). In summary, the lower number of axons (> 1  $\mu\text{m}$ ) at 4 mm distal to crush sites at 14 d indicated that *Eed* cKO nerves have delayed axonal regeneration after injury.

Nerve remyelination involves re-activation of a number of myelin genes that are controlled by the EGR2/Krox20 transcription factor (Decker et al. 2006; Le et al. 2005; Topilko et al. 1994). To determine if there was a deficit in remyelination responses of Schwann cells, we examined levels of *Egr2* and found that its level in the *Eed* cKO had recovered to a similar level as in control nerves (Figure 1E). However, the expression levels of myelin-associated genes such as *Pmp22*, *Mbp*, and *Mpz* of *Eed* cKO nerves were lower than that of control

nerves, which reflects the overall reduced number of myelinated axons at 14 d after injury. Nonetheless, sciatic functional indexes and responses to the toe pinch test, which evaluate functional recovery of motor and sensory nerves, respectively, were comparable between control and *Eed* cKO mice, measured at 2 d and every week till the 4th week after crush injury (data not shown). In summary, these data indicate that PRC2 activity is required for timely axon regeneration during nerve repair process. Although remyelination eventually occurred in the *Eed* cKO, a lower level of myelin gene expression and thinner myelin sheaths (> 3  $\mu$ m) at 14 days after injury is likely due to a delay in axon regeneration and subsequent myelination.

### Control of Neuregulin-regulated Pathways by PRC2

We tested if EED affects activation of the ERK (extracellular signal-regulated kinase) pathway 1 d after injury, which becomes highly upregulated within few hours and regulates myelin breakdown at an early stage of nerve repair (Napoli et al. 2012), and lasts over 2 weeks after injury (Guertin et al. 2005; Sun et al. 2013; Yamazaki et al. 2009). The level of ERK activation was not statistically different in the *Eed* cKO compared to control (p value, ~ 0.09). Accordingly, RNA-seq analysis (described below) did not identify changes in ERK-dependent injury genes. Our previous analysis of uninjured nerves had revealed an increase in p-AKT in the cKO (Ma et al. 2015), and at 1 d after injury, there was a similar increase in AKT phosphorylation at Thr308 and Ser473, which are catalyzed by PDK1 and mTORC2, respectively (Andjelkovi et al. 1997; Sarbassov et al. 2005) (Figure 2). At 1 d after injury, there is little change in p-AKT in wild type mice (Norrmén et al. 2018; Ronchi et al. 2016).

The AKT pathway is induced by neuregulin binding to the ERBB2/3 receptor in Schwann cells (Atanososki et al. 2006b; Carroll et al. 1997; Fledrich et al. 2014; Guertin et al. 2005). Therefore, we decided to examine regulation of *Neuregulin1* (*Nrg1*) type I, which is induced in Schwann cells within 24 hours after injury, and increases remyelination efficiency (Ronchi et al. 2016; Stassart et al. 2013). Interestingly, the ChIP-seq mapping of H3K27me3 in mature nerve (Ma et al. 2016) indicated that transcription start sites of *Nrg1* type I and III are occupied by this repressive histone mark (Figure 3A), and ChIP-qPCR assays showed a decrease in H3K27me3 in the type I promoter after injury (Figure 3B), showing a correlation between methylation dynamics of H3K27 and *Nrg1* type I gene activation. Gene expression analysis of *Eed* cKO nerves, which display a Schwann cell-specific loss of H3K27me3 (Ma et al. 2015), revealed derepression of *Nrg1* type I in *Eed* cKO nerves at 2 months of age in the absence of injury (Figure 3C). In contrast, the *Nrg1* type III transcript was not induced by injury in either control or *Eed* cKO nerves, as expected (Stassart et al. 2013). The increased level of *Nrg1* type I in *Eed* cKO nerves was also observed by Western blot in uninjured nerves (Figure 3E), and could contribute to elevated AKT phosphorylation in the *Eed* cKO before and after injury (Ma et al. 2015).

In order to independently test if H3K27 trimethylation regulates induction of mRNA encoding NRG1 type I, we tested if the activity of H3K27 demethylases is required for the *Nrg1* type I gene activation after injury. Peripheral nerve injury increases the protein expression of a H3K27 demethylase JMJD3/KDM6B in Schwann cells (Gomez-Sanchez et al. 2013). We employed a nerve explant model in which there is activation of injury-

responsive signals and repair genes by incubation of nerve segments in culture media (Arthur-Farraj et al. 2012; Gomez-Sanchez et al. 2015; Ma et al. 2016; Shin et al. 2013). In this system, we did observe increased mRNA for *Nrg1* Type I, but the GSK-J4 inhibitor of H3K27-demethylases JMJD3/KDM6B and UTX/KDM6A (Kruidenier et al. 2012), blocked the induction (Figure 3D). Our results collectively suggest that peripheral nerve injury promotes the activity of H3K27 demethylases that is required for full and timely activation of the Schwann cell *Nrg1* type I transcript, which is repressed by H3K27me3 in uninjured nerves.

### EED-mediated Transcriptional Regulation in Schwann Cells

To further investigate how PRC2 represses injury induced gene expression, we performed RNA-seq analysis of mouse sciatic nerves at 2 months of age. The use of RNA-seq compared to our previous microarray data (Ma et al. 2015) identified a significantly larger group of PRC2-regulated genes due to the inherent sensitivity of the RNA-seq analysis. We compared these expression data to a genome wide ChIP-seq profile of H3K27me3 in wildtype mature sciatic nerves to identify genes regulated by the EED-containing PRC2 complex. Our chromatin immunoprecipitation protocol was optimized to employ micrococcal nuclease digestion to produce mononucleosomal fragments (< 200 bp), to increase the resolution of ChIP-seq analysis. The analysis identified 4091 genes of peripheral nerves that were occupied by H3K27me3 around the transcription start site ( $\pm$  7 kb), including 532 genes that are normally induced (> 2 fold) after injury, and revealed the H3K27me3 enrichment at silent or low-expressed genes (RPKM = < 1) (Figure 4A and Supporting Information Table 1). For example, H3K27me3 was highly enriched at a silenced gene of peripheral nerves, *sonic hedgehog* (*Shh*) (Arthur-Farraj et al. 2012; Lin et al. 2015), and over 73% of H3K27me3-occupied genes were expressed at very low levels (RPKM < 5). Interestingly, H3K27me3 was also abundant at some highly expressed genes, such as *desert hedgehog* (*Dhh*), a signaling molecule required for the structural and functional integrity of the peripheral nerves (Parmantier et al. 1999; Sharghi-Namini et al. 2006). This may be due to cell heterogeneity in sciatic nerves including endothelial cells and fibroblasts, although the majority of sciatic nerve (>75%) are Schwann cells (Joseph et al. 2004; Salonen et al. 1988).

Comparing the *Eed* cKO to wild type littermate, there were 471 upregulated genes, including 85 injury genes, with greater than 2-fold change (p-value, < 0.05) (Figure 4B and Supporting Information Table 2). Approximately half (234) of the upregulated genes were associated with H3K27me3, including 48 injury genes. Among 196 genes with a high fold change (> 5-fold), the most highly induced genes were quite low in control nerves (RPKM < 1, 145 genes). Some of the more highly derepressed genes in *Eed* cKO nerves include *fatty acid binding protein 7* (*Fabp7/Bfabp*), a marker of Schwann cell precursors and immature Schwann cells (Jacob et al. 2014; Kurtz et al. 1994), and *protein tyrosine phosphatase receptor type Z 1* (*Ptprz1*), which regulates oligodendrocyte differentiation (Harroch et al. 2002; Kuboyama et al. 2015). 130 genes were decreased by the deletion (<2-fold), including 29 H3K27me3-associated genes. The function of H3K27me3 in gene activation has not been reported, however. In contrast, major myelin genes were largely not affected in *Eed* cKO nerves.



## EED regulation of Injury-induced genes

Together with the ultrastructural analysis, the results showed that EED-mediated repression controls hundreds of genes, but appears dispensable for primary myelination as the phenotype of peripheral nerve is essentially normal at this age (Ma et al. 2015). In addition, *Eed* cKO resulted in derepression of only a subset of H3K27me<sub>3</sub>-associated injury genes. However, we reasoned that there may be other injury-induced genes in which loss of polycomb repression is not sufficient for activation but is nonetheless required. In other words, some polycomb-repressed genes may require additional injury responsive pathways, such as the transcription factor JUN-mediated regulation that is critically involved in reprogramming of Schwann cells for nerve repair (Arthur-Farraj et al. 2012; Hung et al. 2015).

To provide a more comprehensive view of injury responsive gene regulation and identify more EED-regulated genes, we performed RNA-seq analysis of control and *Eed* cKO nerves 1 d after nerve transection. This timepoint was chosen to show the transcriptome of injured nerves before significant infiltration by immune cells, such as macrophages, which migrate into peripheral nerve beginning at 3 d after injury (Hirata and Kawabuchi 2002). Previous studies have shown that the early repair response of Schwann cells promotes survival of injured neurons and axon regeneration through activation of neurotrophic factors and surface proteins, and elicits an innate immune response to further facilitate nerve regeneration (Boyd and Gordon 2003; Cafferty et al. 2001; Fontana et al. 2012; Hashimoto et al. 2008; Henderson et al. 1993; Martinez et al. 2015; Perrin et al. 2005; Rotshenker 2011).

In addition to the genes induced prior to injury in the *Eed* cKO (described above), the analysis revealed an additional 116 genes were upregulated in *Eed* cKO nerves only after injury, including 46 H3K27me<sub>3</sub>-associated genes, therefore identifying more EED-regulated genes in injury-induced pathways (Figure 5A). The list included 50 genes that normally become activated after injury, and 22 genes, such as *Gap43*, *Sox2*, *Hmga2*, *Vgf*, *Wif1*, and *Esm1*, were associated with H3K27me<sub>3</sub>. Many of this set normally become activated at later timepoints after injury (at 3–7 days, referred to as late injury genes), but are unchanged or even reduced at 1 d after injury in control mice (Figure 5C and Supporting Information Table 4) as previously reported for *Bdnf* (Ma et al. 2016). As discussed above, removal of PRC2 repression is presumably not sufficient for their activation, but their activation at this early time is accelerated by removal of H3K27me<sub>3</sub>. Therefore, loss of EED leads to augmented or premature induction of a number of genes in addition to those that are elevated in the uninjured *Eed* cKO nerves.

Our analysis of wildtype transcriptome identified 845 genes that were induced greater than 2-fold (p-value, < 0.05) in distal stumps 1 d after injury, compared to contralateral uninjured nerves (Supporting Information Table 3). Consistent with previous studies, induction included a significant number of genes encoding secreted proteins, such as neurotrophic factors GDNF, FGF5, and NGF, in addition to molecules mediating signaling pathways such as TGF- $\beta$ 1 (Boyd and Gordon 2003; Cafferty et al. 2001; Fontana et al. 2012; Henderson et al. 1993; Meyer et al. 1992). Genes that promote macrophage recruitment and myelin debris clearance (e.g. *Mcp1/Ccl2*, *Il1a*, *Il1b*, *Lif*, *Il11*) were also induced at this time point (Napoli et al. 2012).

While we have previously demonstrated reduction of H3K27me3 on injury induced genes already at 1 day (Ma et al. 2016), we hypothesized that loss of EED could augment or accelerate induction of injury genes at this time point. Among genes activated at 1 d after injury, 38 genes were further upregulated in the *Eed* cKO (> 2 fold; p-value, < 0.05), including 18 genes associated with H3K27me3 (Figure 5B). For example, *Vgf* and *Ecel1* became highly activated (> 50 fold, relative to uninjured control nerves) 1 d after injury. *Eed* cKO further elevated the induction of such genes by greater than 3-fold compared to injured control nerves. There are also genes that become repressed after injury, and the analysis showed that EED is required for injury-induced downregulation of a subset of such genes. However, this did not include myelin genes that normally are reduced after injury.

### Polycomb Regulation of Axon Regeneration Genes

While we have initially focused on the effects of EED on early gene induction after injury, we also wished to analyze potential mechanisms for the delayed axon regeneration. Therefore, RNA-seq analysis of wildtype nerves was performed 14 d after crush injury, which revealed 1020 genes upregulated greater than 2 fold (p-value, < 0.05), compared to uninjured nerves. Comparison to the *Eed* cKO at 14 d after injury showed that a lack of EED led to a further upregulation of 203 genes (> 2 fold) and a downregulation of 100 genes (< -2 fold) in the overall transcriptome (Supporting Information Table 5). Only a small fraction of such genes was, however, part of the injury-responsive transcriptome of control nerves (Figure 6A).

To better understand the phenotype of *Eed* cKO nerves, we compared our RNA-seq data with data from the analysis of injured nerves, in which *Jun* was deleted specifically in Schwann cells (Arthur-Farraj et al. 2012). The loss of the JUN transcription factor led to failures in axon regeneration but also in Bungner band formation and myelin debris clearance. Importantly, the *Jun* cKO array analysis identified a subset of injury genes implicated in nerve regeneration and provided a list of genes critical for axon regeneration. We also compared with gene expression analysis of injured nerves of *Wld<sup>S</sup>* mice, in which axons degrade slowly, and Schwann cells therefore remain differentiated and incompetent to support repair process (Barrette et al. 2010). The comparison analysis did not yield a large overlap of genes that were commonly downregulated or upregulated between *Eed* cKO and *Jun* cKO studies. Such an observation was consistent with facts that the induction of JUN protein and mRNA in the *Eed* cKO was very similar to control, measured at 5 d and 14 d after injury, respectively (Figure 6B, C). Instead, we identified only 10 genes that were commonly downregulated in the *Eed* cKO and *Jun* cKO in injured nerves, including *Sema4f*, *Epha5*, *Fgf5*, *Olig1*, *Btc*, *Mmp17*, and *Runx2* that were lower also in *Wld<sup>S</sup>*-injured nerves compared to wildtype injured nerves (Figure 6C). Importantly, the critical role of semaphorin 4F transmembrane protein (SEMA4F) in axoglial interactions was demonstrated in a dorsal root ganglion neuron and Schwann cell co-culture system (Parrinello et al. 2008). EPHA5 has not been studied in the context of peripheral nerve injury, but it does affect axon guidance in retinal ganglion cells and spinal cord (Wang et al. 2016; Yue et al. 1999). The transcript level of other genes known for promoting axon regeneration and neuronal survival such as *Shh*, *Bdnf* and *Gdnf* (Boyd and Gordon 2003; Fontana et al. 2012; Hashimoto et al. 2008; Ma et al. 2016; Martinez et al. 2015) was largely unaffected at 14 d post-crush injury,

even though EED loss caused derepression in uninjured nerves or premature induction (Figure 6C).

Furthermore, the analysis identified a significant decrease in *ciliary neurotrophic factor* (*Cntf*) (Figure 6D). The neurotrophic cytokine CNTF is primarily expressed by Schwann cells and substantially mediates axonal growth, demonstrated by markedly decreased number of regenerated axons in mouse models with genetic ablation of *Cntf* (Masu et al. 1993; Selvaraj et al. 2012; Simon et al. 2010).

### EED Supports Injury-induced Schwann Cell Proliferation

One gene controlled by H3K27 trimethylation is the *Cdkn2a* tumor suppressor gene encoding the INK4A/p16 (cyclin-dependent kinase inhibitor) and ARF/p19 proteins (Bracken et al. 2007; Gomez-Sanchez et al. 2013). The p16 and p19 proteins are implicated in controlling Schwann cell over-proliferation during nerve regeneration after injury-induced expression (Atanasoski et al. 2006a; Gomez-Sanchez et al. 2013). Using transcript-specific primer sets for p16 and p19 transcripts in qRT-PCR, there is not much induction at 1 day after injury in control mice, but there is a premature induction of both transcripts 1 d after injury in the *Eed* cKO (Figure 7A). In addition, the two transcripts are modestly induced at 7d in control mice, but are greatly increased in the *Eed* cKO and remain elevated at 14 d after crush. Consistent with the regulation of *Cdkn2a* by PRC2, we also observed H3K27me3 over the promoters of both the *p16* and *p19* transcripts (not shown).

Since p16 and p19 control Schwann cell proliferation, we tested if injury-induced proliferation of Schwann cells after injury is affected in the *Eed* cKO by assessing the number of denervated Schwann cells with Ki67 expression 5 d after injury. Double labeling of Ki67 and the Schwann cell specific marker SOX10 showed that there was a significantly lower number of proliferating Schwann cells in *Eed* cKO nerves (Figure 7B, C). In addition, as predicted by the mRNA analyses, we see enhanced expression of the p19/ARF protein at the same timepoint. These data indicate that EED promotes proliferation of Schwann cells in the early response to injury by limiting induction of the p16 and p19 transcripts of the *Cdkn2a* gene, as predicted by earlier studies of H3K27 demethylation (Gomez-Sanchez et al. 2013). Previous studies have shown that Schwann cell proliferation after injury is offset by increased apoptosis, and that proliferation per se is not required for regeneration (Yang et al. 2008).

### Comparison to NF1-associated MPNST gene expression profiles

PRC2 subunit mutations are often associated with *CDKN2A* mutations in development of malignant peripheral nerve sheath tumors. In one study, ~80% of ~50 MPNST's lost *CDKN2A* expression, and ~75% of those tumors have mutations in either the *EED* or *SUZ12* genes (Lee et al. 2014). To identify other EED-regulated genes that may be relevant to pathogenesis of MPNST, we compared genes upregulated in the *Eed* cKO with differentially expressed genes between PRC2-deficient and other MPNST's. An RNA-seq analysis of MPNST's identified 449 genes with a 3 fold or higher expression in the absence of *SUZ12* or *EED* (Lee et al. 2014). The comparison analysis revealed that 40 such genes were upregulated in *Eed* cKO nerves either in uninjured or injured conditions (Figure 7D

and Supporting Information Table 6). This includes *Igf2* and homeobox transcriptional regulators, *Hoxa13* and *Hoxd13*, which are highly overexpressed in cancers such as esophageal and gastric cancers (Gu et al. 2009; He et al. 2017). Our ChIP-seq analysis found that 193 genes among the 449 genes activated in PRC2-deficient human MPNST's were occupied with H3K27me3 in rat peripheral nerve.

## Discussion

Our studies of the *Eed* conditional knockout show that PRC2 function is dispensable for early postnatal myelination by Schwann cells, and that myelin is largely normal at two months of age (Ma et al. 2015). However, there is premature derepression of injury genes in the *Eed* cKO nerves, indicating that loss of H3K27me3 is an epigenomic switch involved in regulation of a subset of injury genes (Ma et al. 2016). Therefore, we decided to test if loss of EED may alter the nerve injury process and found that many of the early steps of repair process, termed Wallerian degeneration, appear to proceed normally in the *Eed* cKO. We found no significant differences in the amount of myelin debris and the number of infiltrating macrophages, suggesting that PRC2 was not required for clearance of myelin debris that can inhibit axon regeneration (Mukhopadhyay et al. 1994; Shen et al. 1998). Accordingly, our analysis of EED regulated genes did not identify known genes involved in autophagy or phagocytosis (with the notable exception of *Megf10*), nor did we see altered expression of genes required for macrophage infiltration (e.g. *Ccl2/Mcp1*). In addition, there was no impact on induction of the JUN transcription factor, which is important for several facets of nerve injury responses (Arthur-Farraj et al. 2012).

Given the augmented and early induction of some nerve injury genes, we did look for evidence of accelerated nerve injury processes, but early stages after nerve injury were apparently unaffected. We speculate the normal demethylation of H3K27, at least in young mice, may be sufficient to drive expression of adequate amounts of critical nerve repair genes. However, the ultrastructural analysis revealed that the function of Schwann cell EED is critical for timely axon regeneration, a function likely to be critical for efficient nerve regeneration since the survival and capacity of Schwann cells decrease at more distal sites that are chronically denervated (Benito et al. 2017; Eggers et al. 2010; Höke et al. 2002; Jessen and Mirsky 1999; Jonsson et al. 2013; Li et al. 1997; Michalski et al. 2008; Ronchi et al. 2017; Sulaiman and Gordon 2009).

The RNA-seq analysis together with H3K27me3-mapping revealed many injury-activated genes targeted by the PRC2 complex. Loss of polycomb repression was sufficient for a subset of injury genes to be activated in uninjured nerves (e.g., *Shh*, *Gdnf*, and *Ngf*). Activation of genes presumably involves coordination of loss of polycomb repression with activation of specific transcription factor pathways, and a substantial number of EED-repressed genes were identified only after injury. Some displayed a premature induction in injured *Eed* cKO nerves (*Bdnf*, *Sox2*, *Artemin*), indicating the needs of injury-signaling pathways in addition to loss of H3K27me3. It is striking that many injury-induced genes with H3K27me3 are efficiently induced within 24 hours of injury, and therefore the total number of PRC2-regulated genes may only become apparent from ongoing experiments examining the role of H3K27 demethylases in the injury response.

The slowed axon regeneration at 14 d prompted an examination of genes that could explain this phenotype. One potential cause is the substantial decrease in *ciliary neurotrophic factor* (*Cntf*). The neurotrophic cytokine CNTF is highly expressed by Schwann cells at the end of the first postnatal week, and studies demonstrated its neuroprotective and axon-growth promoting effect using animal models of neuropathy and peripheral nerve injury with genetic ablation of *Cntf* (Homs et al. 2011; Masu et al. 1993; Sendtner et al. 1991; Sendtner et al. 1992). In addition, the inhibition of CNTF by antibody-mediated receptor blocking significantly decreased the growth rate and number of regenerating axons (Vega-Meléndez et al. 2014). Likewise, exogenous CNTF improved axon regeneration after central and peripheral nerve injury (Homs et al. 2011; Hoyng et al. 2014; Müller et al. 2009; Vega-Meléndez et al. 2014). CNTF binding to neuronal receptors activates STAT3 that stimulates regeneration-associated genes and microtubule assembly, which play an important role in axonal stability and growth (Gu et al. 2016; Leibinger et al. 2013; Pellegrino and Habecker 2013; Selvaraj et al. 2012; Vigneswara et al. 2014). Furthermore, *Eed* cKO resulted in a significant downregulation of *semaphorin 4F* (*Sema4f*) that was greatly induced in injured control nerves. Depletion of SEMA4F in co-culture assays caused disruption of Schwann cell-axonal interactions (Parrinello et al. 2008). Injured *Jun* cKO nerves also exhibited a significant decrease in *Sema4f* expression and manifested slow axon regeneration (Arthur-Farraj et al. 2012). Therefore, deficient expression of *Cntf* and *Sema4f* may be responsible for delayed in axon regeneration in *Eed* cKO nerves during nerve repair.

Efficient remyelination of injured nerves requires the de novo activation of *Nrg1* type I in denervated Schwann cells (Stassart et al. 2013). By analyzing the promoters of the type I and type III transcripts of *Nrg1*, we found that H3K27me3 mediates repression of *Nrg1* and there is increased expression of the type I *Nrg1* transcript in the uninjured *Eed* cKO nerves, indicating a mechanism by which type I *Nrg1* is repressed in uninjured nerve. Interestingly, NRG1 increases the expression of nuc-ErbB3 (a nuclear variant of the NRG1 receptor, ErbB3), which has transcriptional activity by modulating H3K27me3 level in Schwann cells (Adilakshmi et al. 2011; Ness et al. 2016). However, nuc-ErbB3 likely has other activities since the phenotype and gene expression changes that we observe in the *Eed* knockout differ in several respects.

As has been shown in other systems (Chen et al. 2009; Conway et al. 2015; Ezhkova et al. 2011; He et al. 2012), PRC2 activity prevents inappropriate activation of lineage-defining transcription factors, as there was a low level activation of neuronal transcription factors (*Pax6*, *Isl1*, etc.). The derepressed level of these transcription factors was not sufficient to drive significant levels of neuronal gene expression, however. A number of homeobox genes (*Hoxa7*, *Hoxa10*, *Hoxd13*) and neural crest transcription factors (*Pax3*, *Tfap2a*, *Tfap2b*) that are expressed in early SC development (Balakrishnan et al. 2016; Doddrell et al. 2012) were also induced at a low level. Interestingly, a number of transcription factors involved in oligodendrocyte development (*Nkx6.2*, *Nkx6.1*, *Myrf*) were also somewhat elevated (Mitew et al. 2013), but did not drive induction of unique oligodendrocyte genes (Lopez-Anido et al. 2015) in the *Eed* cKO.

The polycomb pathway represses the *Cdkn2a* gene encoding cell cycle inhibitors INK4a/p16 and ARF/p19 (Biehs et al. 2013; Bracken et al. 2007; Chen et al. 2009; He et al. 2012;

Voncken et al. 2003), and loss of EED or other PRC2 subunits resulted in derepression of *Cdkn2a* that was correlated to the proliferation-defective phenotype. Importantly, INK4a/p16 and ARF/p19 are implicated in controlling Schwann cell over-proliferation during nerve regeneration after injury-induced expression (Atanasoski et al. 2006a; Gomez-Sanchez et al. 2013). *Eed* cKO nerves display a slower rate of Schwann cell proliferation after injury with a premature and augmented expression of both transcripts for p16/Ink4a and p19/Arf, although this did not impair the regenerative process. Our observations are consistent with previous reports that nerve regeneration is not dependent upon Schwann cell proliferation (Kim et al. 2000; Yang et al. 2008).

Neurofibromatosis is a syndrome with Schwann cell-derived tumors caused by loss of either the NF1 or NF2 tumor suppressors (Carroll 2016). Loss of NF1 is associated with an increased risk that the disease progresses from benign neurofibromas to malignant peripheral nerve sheath tumors (MPNST's). Interestingly, a very high percentage of MPNST's have lost function of PRC2 through deletion/mutation of *EED* or *SUZ12*, or less frequent mutations of PRC2-associated proteins: e.g. *AEBP2* and *RBBP7* (De Raedt et al. 2014; Lee et al. 2014; Zhang et al. 2014). As shown here, proliferation in the absence of PRC2 function would be inhibited by induction of *CDKN2A*, but the PRC2 subunit mutations in MPNST's are often associated with loss of function mutations in *CDKN2A* (Lee et al. 2014). The predominant co-occurrence of PRC2 alterations and *CDKN2A* mutation in MPNST's with *NF1* mutation suggests that these mutations coordinate to drive development of the malignant form of the disease. Furthermore, the findings of our study address some molecular pathways to MPNST's mediated by the dysregulated polycomb repression. Previous studies have identified gene expression changes in MPNST's with loss of PRC2 function (Lee et al. 2014), and we found some of the same changes in our *Eed* cKO mice. More specifically, *Eed* cKO led to an upregulation of *Nrg1*, which is also observed in neoplastic Schwann cells within human neurofibromas and MPNST's (Stonecypher et al. 2005), and aberrant NRG1 signaling contributes to the pathogenesis of neurofibromas and MPNST's in mouse models (Brosius et al. 2014; Gomez-Sanchez et al. 2013; Huijbregts et al. 2003; Kazmi et al. 2013). In addition, SEMA4F-deficiency was also identified in models of Neurofibromatosis type 1 and human neurofibromas, and appears to be a mechanism of loss of Schwann cell-axonal interaction, which contributes to tumorigenesis (Parrinello et al. 2008). It is interesting to note that mature myelinating Schwann cells are normally resistant to tumor formation in NF1 deficient mice, but after injury, neurofibromas develop at the wound site (Ribeiro et al. 2013). Thus, loss of polycomb repression after injury may contribute to neurofibroma development, but permanent loss of PRC2 may be required for transition to the MPNST stage. We did not find any evidence of neoplasia in EED deficient mice even after injury, presumably due to the induction of *Cdkn2a* and the lack of deregulated Ras signaling. A recent study used a shRNA screen to identify driver genes for MPNST development (Patel et al. 2016). Seven such genes were identified, and *Meis1* was functionally validated. Another gene in this set, *Pitx2*, is highly upregulated in EED deficient nerves, and three of the seven have moderate to high levels of H3K27me3 on the promoters (*Meis1*, *Pitx2*, *Prrx1*), suggesting that loss of PRC2 function would play a role in their induction in MPNST's.

Our study elucidated a novel regulation of repair Schwann cell genes mediated by the polycomb pathway and biological implications of EED deficiency in nerve regeneration and Schwann cell proliferation. The findings are of clinical importance and may lead to identification of therapeutic measures to facilitate peripheral axon regeneration and also to inhibit MPNST-pathogenesis with loss of the PRC2 subunits SUZ12 or EED.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Main Points**

Polycomb repressive complex 2 (PRC2) prevents premature induction of several Schwann cell injury genes, including neuregulin 1, type I.

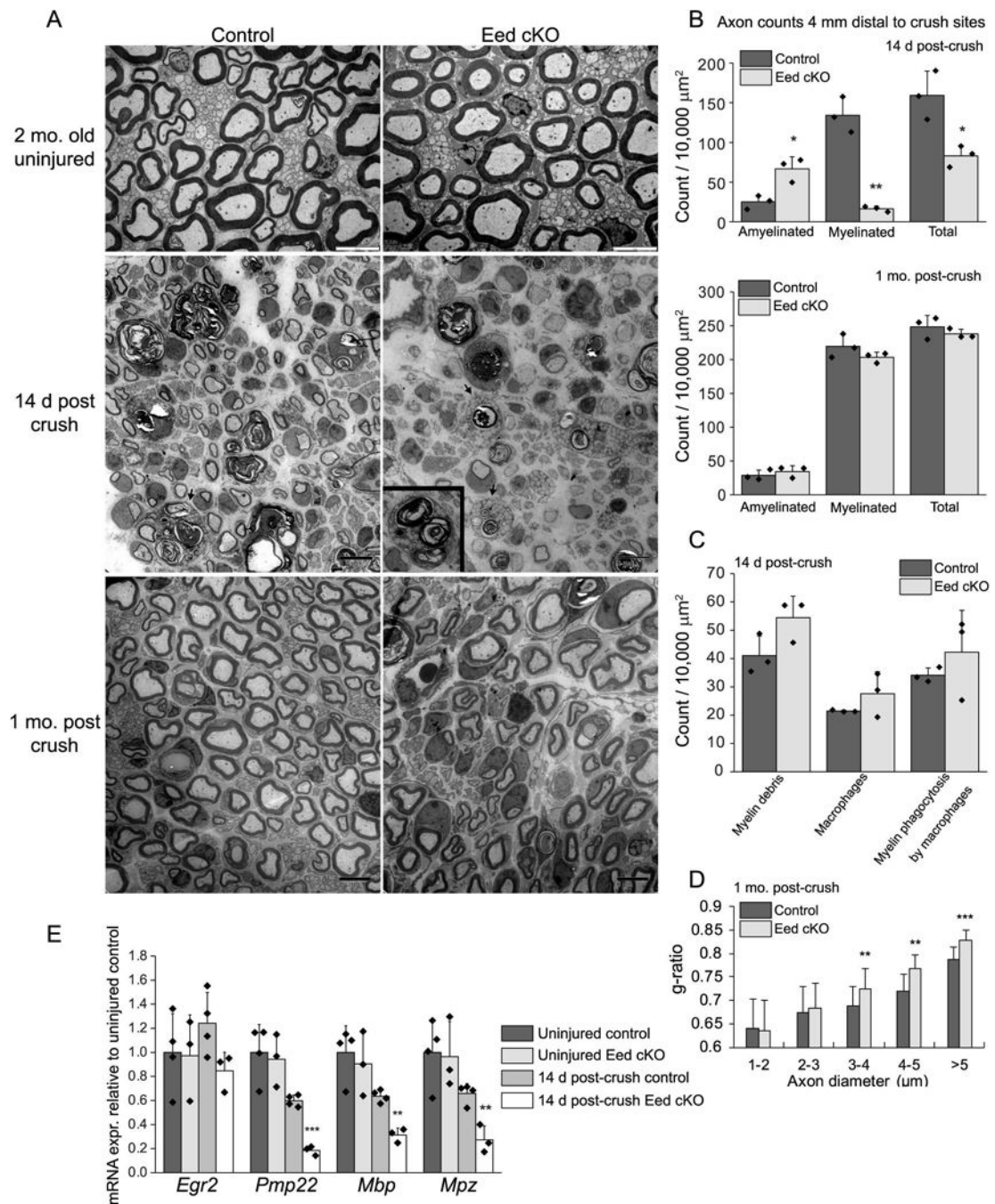
Loss of PRC2 impairs Schwann cell proliferation after injury, and also leads to a delay in axon regeneration.

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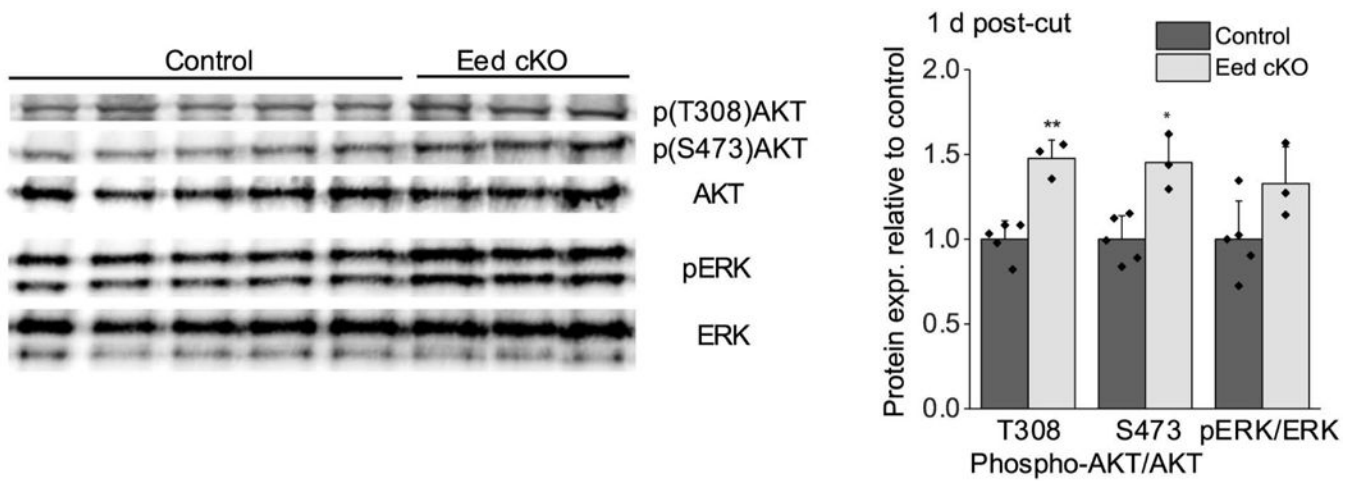


**Figure 1. Schwann cell EED is required for timely axon regeneration.**

**A**, Electron micrographs of the sciatic nerves at the indicated time points after crush and uninjured nerves of *Eed* cKO mice and littermate controls. The arrows and inset show macrophages engulfing myelin debris. Scale bars of uninjured and injured nerve images are 5 μm and 8 μm, respectively. **B**, **C**, Myelinated and amyelinated axons (> 1 μm in diameter), myelin debris, macrophages, and engulfed myelin by macrophages were counted in randomly selected fields that accounted for over 40% of an entire sciatic nerve cross section from each animal and normalized per surface area (10,000 μm<sup>2</sup>). Data: mean ± STDEV; \*\**p*

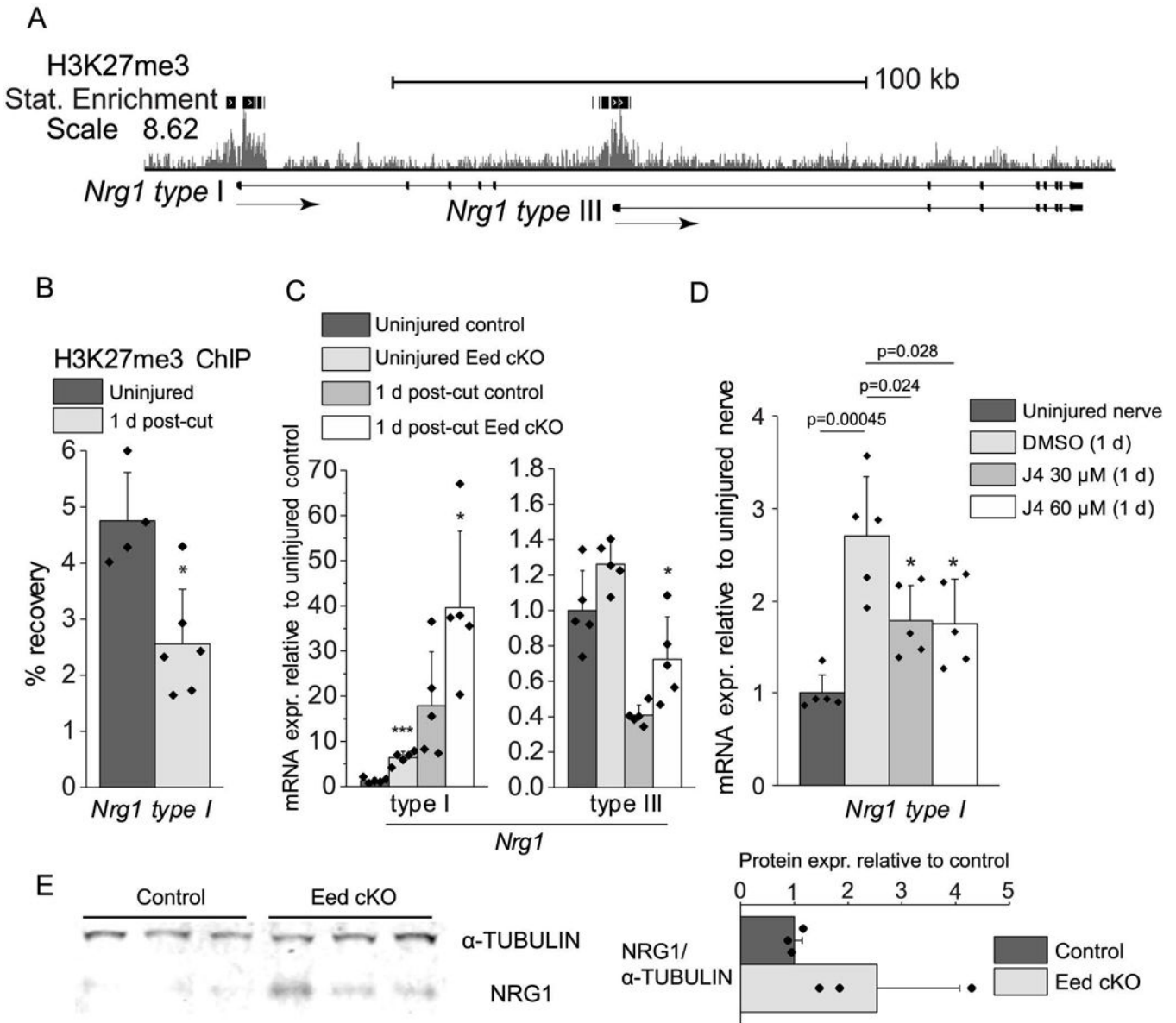


< 0.005, \* $p$  < 0.05; n=3 per genotype (one-way ANOVA). **D**, For g-ratio analysis (axon diameter/diameter of myelinated fiber), the diameter of axon and outer diameter of myelinated fiber were measured on over 500 randomly selected fibers per genotype. Data: weighted mean  $\pm$  pooled STDEV; \*\*\* $p$  < 0.0005, \*\* $p$  < 0.005, \* $p$  < 0.05; n=3 per genotype and age (one-way ANOVA). **E**, qRT-PCR analysis was used to identify the expression level of myelin genes from 2 month *Eed* cKO and control sciatic nerves in uninjured condition or 14 day after crush. Expression levels were normalized with *Gapdh*. Asterisks indicate p-value between genotypes in the respective condition. Data: mean  $\pm$  SD; \*\* $p$  < 0.005, \*\*\* $p$  < 0.0005; n=4 for control and n=3 for *Eed* cKO (one-way ANOVA).



**Figure 2. Increased activation of AKT in *Eed* cKO nerves after injury.**

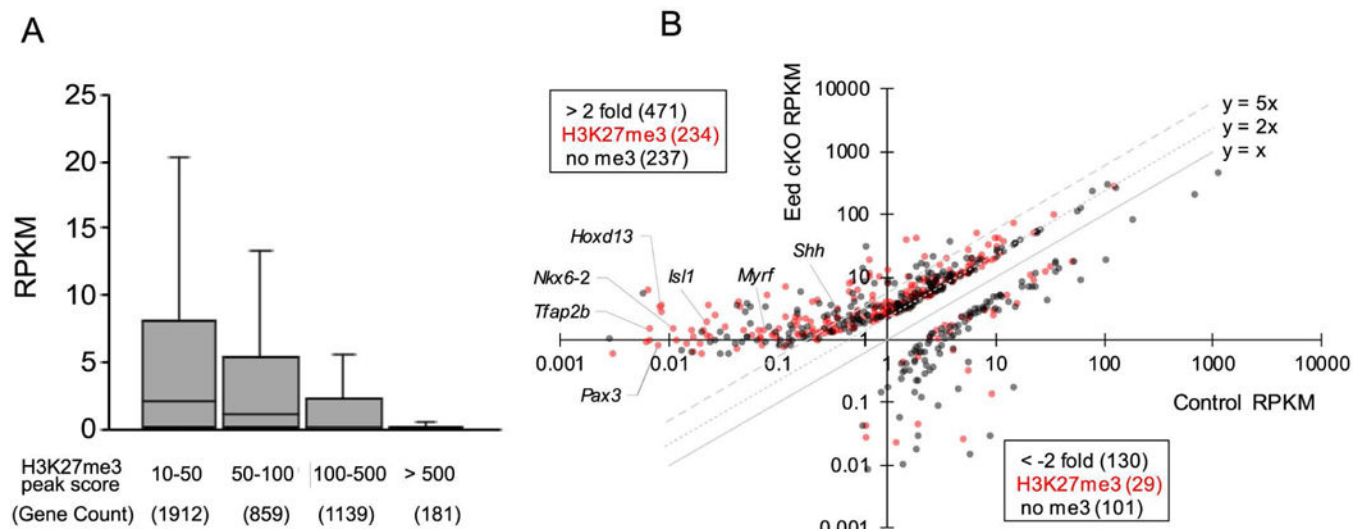
Western blot analysis of lysates from distal stumps of control and *Eed* cKO sciatic nerves 1 d after cut was performed using the indicated antibodies. n=5 for control and n=3 for *Eed* cKO nerves. Data: mean ± STDEV; \*\* $p < 0.005$ , \* $p < 0.05$  (one-way ANOVA).

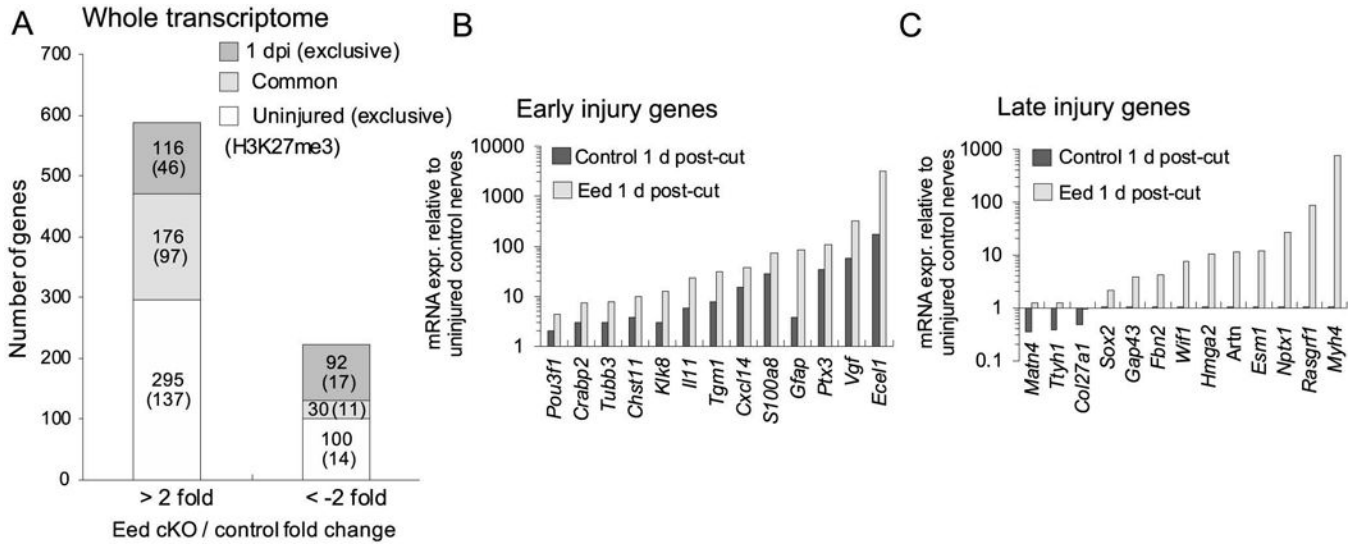


**Figure 3. Schwann cell *Nrg1* type I is regulated by PRC2.**

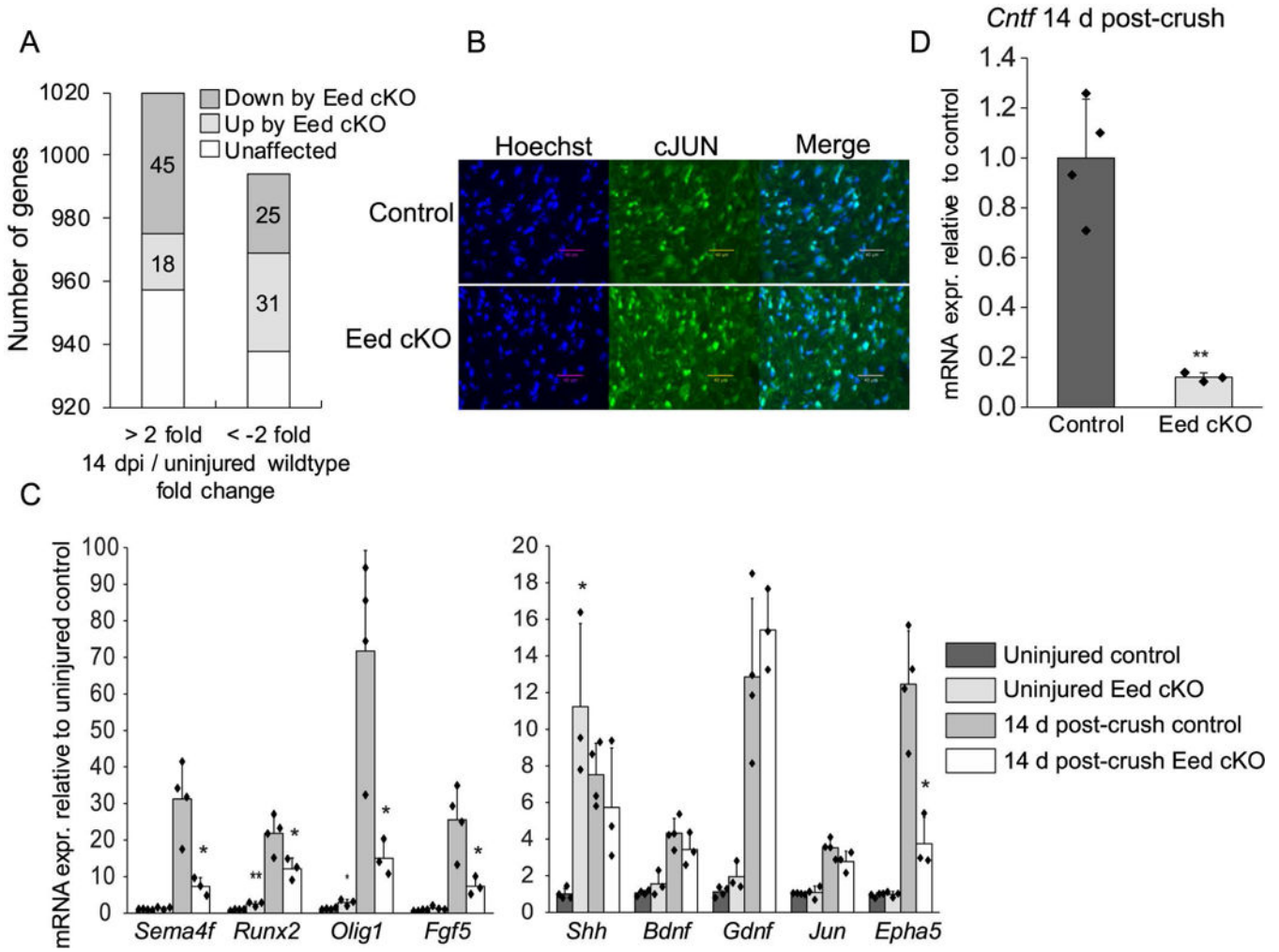
**A**, ChIP-seq mapping of H3K27me3 was performed in uninjured rat sciatic nerves. The transcription start site (TSS) is on the left. Statistical (Stat) Enrichment indicates regions with more sequencing reads than random chance. **B**, H3K27me3-ChIP assays were performed with distal stumps of rat sciatic nerves 1 d post-cut or sham surgery, and percent recovery relative to input was calculated by qPCR analysis. Data: mean  $\pm$  SD; \* $p$  < 0.05;  $n=5$  for sham and  $n=6$  for 1 d post-cut (one-way ANOVA). **C**, qRT-PCR analysis was used to identify the expression level of *Nrg1* type I and type III from 2 month *Eed* cKO and control sciatic nerves of uninjured condition or 1 day after cut. Expression levels were normalized with *Gapdh*. Data: mean  $\pm$  SD; Asterisks indicate p-value between genotypes in the respective condition. \* $p$  < 0.05, \*\*\* $p$  < 0.0005;  $n=5$  per genotype and condition (one-way ANOVA). **D**, Rat sciatic nerve explants were cultured for 1 d in the presence of GSK-J4

at indicated concentrations or DMSO vehicle and subjected to qRT-PCR together with immediately frozen nerve segments after dissection (indicated as uninjured). Uninjured level of *Nrg1* is set as 1. Expression levels were normalized to 18S rRNA. Data: mean  $\pm$  SD; n=5 per condition (one-way ANOVA). **E**, Nerve lysates were obtained from uninjured control and Eed cKO nerves, and were blotted with an antibody to the extracellular domain of NRG1. The 30 kd band was normalized to  $\alpha$ -tubulin, and the bar graph shows the average of the 3 replicates per condition.



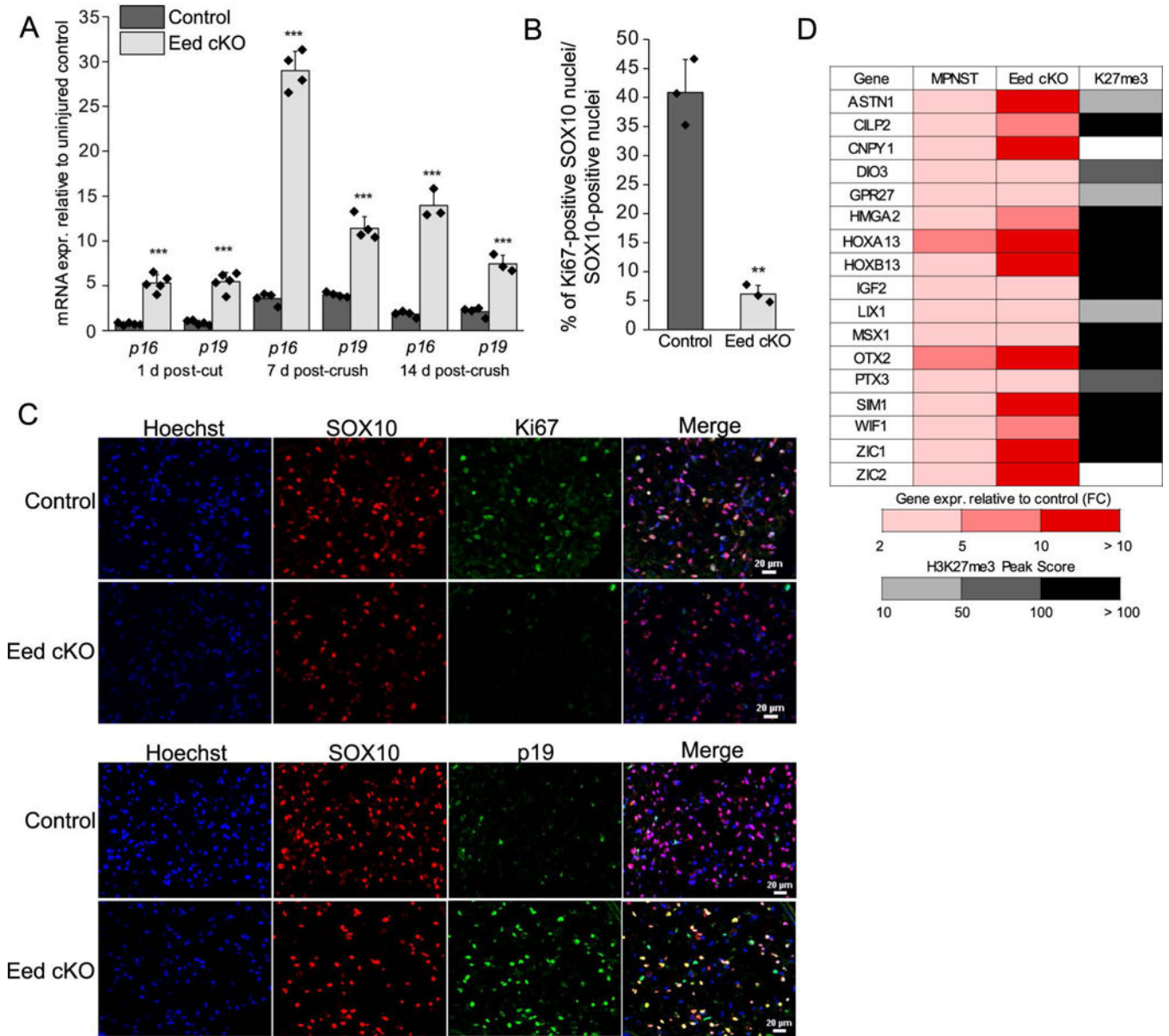


**Figure 5. Polycomb activity regulates early transcriptional response after nerve injury.** **A**, RNA-seq analysis identified a number of genes dysregulated by *Eed* cKO in uninjured or 1 d after injury conditions. Gene expression was determined by RNA-seq analysis of 3 samples per genotype and condition ( $p$ -value,  $< 0.05$ ). The brackets indicate the number of genes associated with H3K27me3 around the transcription start site ( $\pm 7$  Kb). **B**, **C**, RNA-seq analysis was used to identify the expression level of early and late injury genes in *Eed* cKO nerves and control nerves at 1 d post-cut relative to uninjured control nerves. Note that the y-axis is on a log scale. Data: mean of  $n=3$  with  $p$ -value,  $< 0.05$  per genotype. The late injury genes were identified from microarray analysis of peripheral nerves 3, 5 or 7 d after injury ( $> 2$  fold) (GEO accession: GSE22291, GSE38693, GSE33454) (Barrette et al., 2010; Arthur-Farraj et al., 2012; Kim et al., 2012)(Kim et al. 2012). See Supporting Information Table 3 and 4 for the complete list of genes analyzed in Figure 5.



**Figure 6. The expression of critical axonal growth genes *semaphorin 4F* and *ciliary neurotrophic factor* is dependent on EED-mediated transcriptional regulation during nerve repair.**

**A**, RNA-seq analysis identified a number of genes dysregulated by *Eed* cKO among genes differentially expressed during nerve regeneration at 14 d post-crush compared to uninjured nerves. See Supporting Information Table 2 and 5 for the list of dysregulated genes by *Eed* cKO in overall transcriptome and in the injury responsive transcriptome 14 d post-crush, respectively (n=3 per condition for control and n=2 for *Eed* cKO 14 d post-crush). **B**, Immunohistochemistry on transverse sections of distal stumps displays the JUN expression in nerves of indicated genotypes 5 d after cut. Scale bars, 40  $\mu$ m. **C**, **D**, qRT-PCR analysis was used to identify the expression level of injury-responsive genes from 2 month *Eed* cKO and control sciatic nerves in uninjured condition or 14 day after crush. Expression levels were normalized with *Gapdh*. Data: mean  $\pm$  SD; Asterisks indicate p-value between genotypes in the respective condition. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ ; n=4 for control and n=3 for *Eed* cKO (one-way ANOVA).



**Figure 7. *Eed* cKO exhibits impaired proliferation in injured nerves.**  
**A**, mRNA expression relative to uninjured control level set as 1 (not shown) at indicated time points was assessed by qRT-PCR with primer sequences specific to p16/Ink4a and p19/Arf transcripts of *Cdkn2a*. Expression levels were normalized with *Gapdh*. Data: mean  $\pm$  SD; \*\*\* $p < 0.0005$ ;  $n=5$  and  $n=4$  per genotype and condition at 1 d post-cut and 7 d post-crush, respectively, and  $n=4$  for control and  $n=3$  for *Eed* cKO at 14 d-post crush (one-way ANOVA). **B**, **C**, The expression of a proliferation marker Ki-67 and p19/ARF among SOX10-positive nuclei at 5 d after denervation was assessed by immunohistochemistry on transverse sections of indicated genotypes. Scale bars, 20  $\mu$ m.  $n=3$  per genotype. Data: mean  $\pm$  STDEV; \*\* $p < 0.005$  (one-way ANOVA). **D**, Representative genes that were commonly upregulated in PRC2-deficient MPNSTs relative to non-deficient MPNSTs (> 3 fold, RNA-seq) (Lee et al., 2014) and *Eed* cKO nerves relative to wildtype nerves of uninjured or 1d,



14d post-injury conditions (> 2 fold, RNA-seq) are listed (FC, fold change). Gray shading indicates H3K27me3 occupancy of genes in peripheral nerves of uninjured or post-injury conditions. See Supporting Information Table 6 for expression fold changes by *Eed* cKO among PRC2-deficient MPNST genes and H3K27me3 peak score.

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**Table 1.**

Primer sequences used for qRT-PCR and ChIP-qPCR experiments

qRT-PCR primer sequence (rat)		
Nrg1 type I	Forward	CCGCGTAGAGCGCTCATC
	Reverse	CTTGCCCTCTGCCTTCTTTGC
qRT-PCR primer sequence (mouse)		
<i>Nrg1 type I</i>	Forward	GGAAGGGCAAGAAGAAGG
	Reverse	TTTACACCGAAGCACGAGC
<i>Nrg1 type III</i>	Forward	ACTCAGCCACAAACAACAGAAAC
	Reverse	GAAGCACTCGCCTCCATT
<i>Egr2</i>	Forward	TGCTAGCCCTTCCGTTGA
	Reverse	TCTTTCCGCTGTCTCGAT
<i>Mpz</i>	Forward	CCCTGGCCATTGTGGTTTAC
	Reverse	CCATTCACCTGGACCAGAAGGAG
<i>Pmp22</i>	Forward	CACGGTCGGAGCATCAGG
	Reverse	TCCTTGGAGGCACAGAACACT
<i>Mbp</i>	Forward	GAGGAAGAGACAGCCGCTCTG
	Reverse	CAGGATTCGGGAAGGCTGAG
<i>Sema4f</i>	Forward	TGCTGACGGCGACCAAT
	Reverse	TGGCTTTTCTGGGTGTTT
<i>Runx2</i>	Forward	ACCAAGTAGCCAGGTTCAAC
	Reverse	GAGGATTTGTGAAGACTGTTATGG
<i>Olig1</i>	Forward	AGCGATGTAGTTGCTTGGGAT
	Reverse	CTGGCTCTAAACAGGTGGGAT
<i>Fgf5</i>	Forward	AAAAGCCACCGGTGAAACC
	Reverse	TCACTGGGCTGGGACTTCTG
<i>Shh</i>	Forward	CAGCGACTTCTCACCTTCTT
	Reverse	AGCGTCTCGATCACGTAGAAGAC
<i>Gdnf</i>	Forward	TCTCGAGCAGGTTTCAATGG
	Reverse	AAGAACCCTCGAAACTTTACC
<i>Bdnf</i>	Forward	GGTATCCAAAGGCCAACTGA
	Reverse	GCAGCCTTCTTGGTGTAAAC
<i>Jun</i>	Forward	CGGCTACAGTAACCCTAAGATCCT
	Reverse	GCCAGGTTCAAGGTCATGCT
<i>Epha5</i>	Forward	TGGTCAACAGCCAATTATTCTGA
	Reverse	GCCCCATCCACACATACC
<i>Cntf</i>	Forward	TGCTGAGATTCCCATGTGATG
	Reverse	TTGGAGATGGTGGCCTCTTT
<i>Ink4a/p16</i>	Forward	GAATCTCCGCGAGGAAAGC
	Reverse	TGTCTGCAGCGGACTCCAT

qRT-PCR primer sequence (rat)		
<i>Arfp19</i>	Forward	CACCGGAATCCTGGACCAGG
	Reverse	CACCGTAGTTGAGCAGAAGAGCT

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**Table 2.**

Antibodies used for immunohistochemistry, Western blots and CHIP

Antibodies	Catalog number	Company
p(T308)-AKT	13038	Cell Signaling
p(S473)-AKT	4060	Cell Signaling
AKT	4691	Cell Signaling
pERK 1/2	4370	Cell Signaling
ERK 1/2	4695	Cell Signaling
H3K27me3	AM39155	Active Motif
JUN	SC-1694, H-79	Santa Cruz
SOX10	AF2864	R & D Systems
Ki67	Ab16667	Abcam
NRG1	MABN42	Millipore
p19/ARF	sc-32748	Santa Cruz

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