Cellular/Molecular

Epigenomic Regulation of Schwann Cell Reprogramming in Peripheral Nerve Injury

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The rapid and dynamic transcriptional changes of Schwann cells in response to injury are critical to peripheral nerve repair, yet the epigenomic reprograming that leads to the induction of injury-activated genes has not been characterized. Polycomb Repressive Complex 2 (PRC2) catalyzes the trimethylation of lysine 27 of histone H3 (H3K27me3), which produces a transcriptionally repressive chromatin environment. We find that many promoters and/or gene bodies of injury-activated genes of mature rat nerves are occupied with H3K27me3. In contrast, the majority of distal enhancers that gain H3K27 acetylation after injury are not repressed by H3K27 methylation before injury, which is normally observed in developmentally poised enhancers. Injury induces demethylation of H3K27 in many genes, such as *Sonic hedgehog* (*Shh*), which is silenced throughout Schwann cell development before injury. In addition, experiments using a Schwann cell-specific mouse knock-out of the Eed subunit of PRC2 indicate that demethylation is a rate-limiting step in the activation of such genes. We also show that some transcription start sites of H3K27me3-repressed injury genes of uninjured nerves are bound with a mark of active promoters H3K4me3, for example, *Shh* and *Gdnf*, and the reduction of H3K27me3 results in increased trimethylation of H3K4. Our findings identify reversal of polycomb repression as a key step in gene activation after injury.

Key words: chromatin; injury; myelin; polycomb; regeneration; Schwann cell

Significance Statement

Peripheral nerve regeneration after injury is dependent upon implementation of a novel genetic program in Schwann cells that supports axonal survival and regeneration. Identifying means to enhance Schwann cell reprogramming after nerve injury could be used to foster effective remyelination in the treatment of demyelinating disorders and in identifying pathways involved in regenerative process of myelination. Although recent progress has identified transcriptional determinants of successful reprogramming of the Schwann cell transcriptome after nerve injury, our results have highlighted a novel epigenomic pathway in which reversal of the Polycomb pathway of repressive histone methylation is required for activation of a significant number of injury-induced genes.

Introduction

The remarkable plasticity of Schwann cells underlies the extensive regenerative capacity of peripheral nerves (Kim et al., 2013; Jessen and Mirsky, 2016). After injury, Schwann cells proliferate and launch the transcriptional signature of immature stages with concomitant repression of myelin genes (Nagarajan et al., 2002; Patodia and Raivich, 2012a, 2012b; Kim et al., 2013; Brosius Lutz and Barres, 2014; Jessen and Mirsky, 2016), so the transition was historically viewed as dedifferentiation. However, Schwann cells in injured nerves also activate genes that are never expressed during embryonic Schwann cell development and are unique to the injury condition, such as *Shh* and *Olig1* (Arthur-Farraj et al., 2012; Lin et al., 2015). Such changes allow Schwann cells to promote axon regeneration and therefore have been termed "repair" Schwann cells. For example, although dispensable in differentiation, the injury-induced transcriptional factor c-Jun was highlighted as a critical regulator of the repair process (Arthur-Farraj et al., 2012). Nerves with c-Jun-deficient Schwann cells displayed delayed demyelination, greater neuronal death, reduced macrophage migration and myelin clearance, and failure of reinnervation and functional recovery. These observations reflect the

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 0270-6474/16/369135-13$15.00/0

Table 1. Primer sequences used for qRT-PCR and ChIP-qPCR experiments

Rat qRT-PCR primer sequence	
Igfbp2	
Forward	CCCCCAGTGGCAGTTAACCT
Reverse	CAGTGCCACCACCCTCTCTATTA
Thy1	
Forward	TACTCTAGCCAACTTCACCACCAA
Reverse	GCCCGAGACTCGAAGTTCAC
Esm1	
Forward	GCTGATCCTGGCTGAGATTTG
Reverse	TGTTGGCTGAAAGTTGGTCACT
Shh	
Forward	GCGGGCATCCACTGGTACT
Reverse	TCGGACTTCAGCTGGACTTGA
Fgf5	
Forward	GGGCTGGGTGGCTTTTTC
Reverse	CGAGCAACCGGAACTTTCC
Vegfa	
Forward	AATCCAGAAAGCCTGACATGAAG
Reverse	GCCCTCCGGACCCAAA
Vgf	
Forward	TCTCCTCCACCTCTTCGATGAT
Reverse	TAGTCTCATTGAACTGTCCACCAAAC
Gdnf	
Forward	ACTGACTTGGGTTTGGGCTA
Reverse	CCTGGCCTACCTTGTCACTT
Mmp13	
Forward	CTGGAACCACATTGAAGAATTGTAA
Reverse	GCACCCAAGAATCAGGTGATC
Cxcl2	
Forward	TTCCTGGGTGCAGTTCGTTT
Reverse	CATGGGCTCCTGTACCTCAAC
Foxq1	
Forward	GTAGCTGCCCCGGAAAAAG
Reverse	ACGCTGGCCGAGATCAAC
Parvb	
Forward	GTCCACTCTGCGGGTCCTT
Reverse	GCTCTGTCACTCGACGTCCTT
Runx2	
Forward	CCGTAGAGAGCAGGGAAGACA
Reverse	ACGATGGCAGACACCAACTG
Hmga2	
Forward	GGAAATGGCCACAACAAATTG
Reverse	CGGACTCTTGCGAGGATGTC
Mouse qRT-PCR primer sequence	
Igfbp2	
Forward	GCGGGTACCTGTGAAAAGAG
Reverse	CCTCAGAGTGGTCGTCATCA
Runx2	
Forward	ACCAAGTAGCCAGGTTCAAC
Reverse	GAGGATTTGTGAAGACTGTTATGG
Olig1	
Forward	AGCGATGTAGTTGCTTGGGAT
Reverse	CTGGCTCTAAACAGGTGGGAT
Pou3f1/Scip	
Forward	CTGAGCTTCAAGAACATGTGCAA
Reverse	GCGATCTTGTCCAGGTTGGT
Mmp13	
Forward	ACCTGATTCTTGCGTGCTATGA
Reverse	GCAGATGGACCCCATGTTTG
Thy1	
Forward	TTCCTCCCTGCCAACCAA
Reverse	GCACAGTCCAACTTCCCTCATC
Bdnf	
Forward	GGTATCCAAAGGCCAACTGA
Reverse	GCAGCCTTCCTTGGTGTAAC

(*Table Continues*)

Table 1. Continued

Hmga2	
Forward	CAAGAGGCAGACCTAGGAAATG
Reverse	CTCTTGCGAGGATGTCTCTTC
Wif1	
Forward	ACCCCTACCCCACCATCTG
Reverse	CACGCGAAAGGTTAACAAAGG
Esm1	
Forward	GAAATGGTTAAATCCACGCTGAT
Reverse	GGTCACTAAAATGGAGCCTTCTCT
Fgf5	
Forward	AAAAGCCACCGGTGAAACC
Reverse	TCACTGGGCTGGGACTTCTG
Shh	
Forward	CAGCGACTTCCTCACCTTCCT
Reverse	AGCGTCTCGATCACGTAGAAGAC
Gdnf	
Forward	TCTCGAGCAGGTTCGAATGG
Reverse	AAGAACCGTCGCAAACTTTACC
Gfap	
Forward	GACTGTGGAGATGCGGGATGGTGA
Reverse	GTGCTGGTGTGGGTGGGAACTGAG
Vgf	
Forward	TCTCCTCCACCTCTTCGATGAT
Reverse	TAGTCTCATTGAACTGTCCACCAAAC
Rat ChIP-qPCR primer sequence	
Igfbp2	
Forward	CACAGCAAGTTGCAGGTAATGC
Reverse	CCCGCTGAGCTACGAGTTTC
Runx2	
Forward	CACCCCTTCATCTCTCAACCA
Reverse	TTGTTTCCCGGCGTTTTG
Thy1	
Forward	GGCAATGGTGGATAGAAAGCA
Reverse	CCGTGAGTACTCTCCCATTTGG
Bdnf	
Forward	TGAAAAACGGGCAGTTGGA
Reverse	TCCCTTTGTGTGGCGAACA
Hmga2 (1)	
Forward	ACTGTGCGGTCAGAAAGCAA
Reverse	GCTGAGTGCCCCACTGGAT
Hmga2 (2)	
Forward	GGAAAGACACATCTGTGCAGACA
Reverse	TTTTCGGAGCCCACGGTAT
Esm1 (1)	
Forward	AAGCAGCCTGCGTTGCA
Reverse	CCGCAGCGCACACTTG
Shh	
Forward	CCCCACCCCCACTTTTG
Reverse	GTCATTCTGGTTCCCAACACAA
Fgf5	
Forward	CGGCCAGGGCATTCTAGAA
Reverse	GGTAGCAGGCATGGCTAACC
Gdnf	
Forward	GCGGGCGACTGAGTGTAAAG
Reverse	GCCTCCCTCCGGTTCAAC
Vgf (1)	
Forward	CATTGCCGCATAGATGAGTCA
Reverse	CAATTAGCTGTCTCTCGGTGTCTCT
Vgf (2)	
Forward	ACGCCCAAGGACGACTTG
Reverse	CCCACACCGCAGATCCA
Foxq1	
Forward	GACCGCTCCTGCGTCTTTC
Reverse	CCAGCGGAGGACAACCTTAC

(*Table Continues*)

Table 1. Continued

Sqle	
Forward	CACCCCTCTGCCCAGTACA
Reverse	CACACTTCTTCATTCAGCCAACTAA
Mouse ChIP-qPCR primer sequence	
Igfbp2	
Forward	CCGCTAGCTCGCTGCACTA
Reverse	CCAATCTCGGCAGCATGTT
Runx2	
Forward	GTTGTTTGCGCCTCTTTGC
Reverse	AGCACAGCGATCCACTTTTTG
Thy1	
Forward	TTTCCCTCAGCCTCCGATT
Reverse	GCTGAAGCCCGCAGTTTTC
Bdnf	
Forward	GGGAAGACCTCTCCACACTCTTAC
Reverse	AGCTCCTGGCCTGAACAAAG
Hmga2	
Forward	GGGTTGCTCTCTCCGCAAA
Reverse	GGGACACAATTCACTCCAAGTCT
Esm1	
Forward	GCTGACCACACTCCTGGTACCT
Reverse	GCAATCCACCGCATATTTGG
Fgf5	
Forward	TGCGGGTGCTGGGTAAA
Reverse	CCGTCCTCTGTGGTCACATG
Shh (H3K27me3)	
Forward	GGAAGCGCAGACAGACACTCT
Reverse	CACAACAGCCTGGCACTCTCT
Shh (H3K4me3)	
Forward	GGTAGCAAGGCTGGAGAGCTT
Reverse	CGCCCCTGCCAGCTATAATA
Gdnf	
Forward	CCCCTGGATTGCGTGCTC
Reverse	GGACATTAACTCCAAGTGGCCC
Vgf	
Forward	CCTTCCCCTTTCAAACCCTTA
Reverse	GGGCGCCAGTAAAAGATCAG
Sqle	
Forward	CCTGTAGCTCTCTTGCGTTTGA
Reverse	GCTCGCTCTGGAGGAACTCTT
Tekt3	
Forward	GCCTTGGAATGGATAGGGAGTT
Reverse	GATAGCGGGTGTTGGGAGACT

importance of the functional repair Schwann cells in nerve regeneration and other studies have shown that aging-associated impairment of peripheral nerve regeneration is due to deficient Schwann cell responses (Verdú et al., 2000; Kang and Lichtman, 2013; Painter et al., 2014).

Epigenomic regulation is an important aspect of genetic reprogramming in differentiation of diverse cell lineages. The roles of several epigenomic modulators have been characterized in Schwann cell differentiation and myelination (Chen et al., 2011; Jacob et al., 2011; Hung et al., 2012; Weider et al., 2012; Ma et al., 2015), but the injury-induced epigenomic reprograming that leads to the activation of genes involved in the nerve repair program has not been characterized.

Polycomb Repressive Complex 2 (PRC2) represses target genes through dimethylation and trimethylation of histone H3 Lys27 (H3K27me2 and H3K27me3; Pasini et al., 2004; Montgomery et al., 2005; Boyer et al., 2006; Ezhkova et al., 2009; Ezhkova et al., 2011; Aldiri and Vetter, 2012). The complex consists of EZH2, with methyltransferase activity, and the nonredundant core subunits, suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED). H3K27 methylation represses transcription by recruiting other factors such as PRC1, which in turn catalyzes ubiquitination of H2A, blocking transcriptional elongation, and RBP2 demethylase, which reverses the trimethylation of H3 Lys4 (H3K4me3), a modification associated with active promoters (Viré et al., 2006; Mikkelsen et al., 2007; Stock et al., 2007; Ku et al., 2008; Pasini et al., 2008; Blackledge et al., 2015).

As a first step in understanding the role of the polycomb pathway and H3K27 trimethylation in peripheral nerves, we analyzed a Schwann-cell-specific deletion of the *Eed* (Eed cKO) subunit of the PRC2 complex. Early development of myelin was largely unaffected, but older mice developed progressive hypermyelination and morphological changes affecting both myelinated and unmyelinated nerve fibers of adult nerves (Ma et al., 2015). Interestingly, loss of H3K27me3 caused an induction of a number of repressed genes and, in particular, 30% of derepressed genes were a subset of genes that are normally upregulated after peripheral nerve injury. This included the S*onic hedgehog* (*Shh*) gene, which is not normally expressed in Schwann cells, but is activated upon nerve injury (Arthur-Farraj et al., 2012; Lin et al., 2015). Because depletion of H3K27me3 was sufficient for the activation of a number of injury-induced genes in Schwann cells, removal of H3K27me3 may be required in conjunction with injury-induced transcription factors to activate a larger subset of injury response genes. The results described below indicate how injury genes are normally repressed before injury and describe the epigenetic changes that are involved in reprogramming Schwann cells after nerve injury.

Materials and Methods

Primer sequences and antibodies. The antibodies targeting H3K27me3 (Active Motif, 39155), H3K27ac (Active Motif, 39133), and H3K4me3 (Millipore, 04–745) were used in the study. Primers are listed in Table 1.

Experimental animals and nerve injury surgery. All animal experiments were performed according to protocols approved by the University of Wisconsin Graduate School. Eed-floxed mice were generated by backcrossing the *flox* allele for seven generations against the C57BL/6 genetic background and mated to mP0TOTA-Cre [B6N.FVB-Tg(Mpz-cre)26Mes/J; Jackson Laboratory]. 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. Sciatic nerves of adult Sprague Dawley rats or mice were cut by following the surgery protocol described previously (Hung et al., 2015). As a control, the contralateral limb also received a sham operation consisting of only a skin incision. The nerve tissue distal to the transection and contralateral (sham) nerves were isolated for use in gene expression analysis and ChIP experiments. Both male and female mice were used individually per sample at a similar ratio between the floxed *Eed* and *Eed* cKO genotypes of gene expression analysis and ChIP experiments. Male rats were used in ChIP experiments after nerve injury surgery.

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 GSK-J4 (Tocris Bioscience, 4594) or DMSO at 37°C for 1 or 3 d.

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 μg 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). Statistical analyses were evaluated by one-way ANOVA.

In vivo *ChIP.* Sciatic nerves were subjected to ChIP with anti-H3K27me3 and anti-H3K27 acetylation antibodies as described previously (Hung et al., 2015).

Micrococcal nuclease (MNase)-aided in vivo *ChIP.* Sciatic nerves were subjected to MNase-ChIP with anti-H3K4me3 antibody as described previously with minor modifications (Ma et al., 2015). After incubation

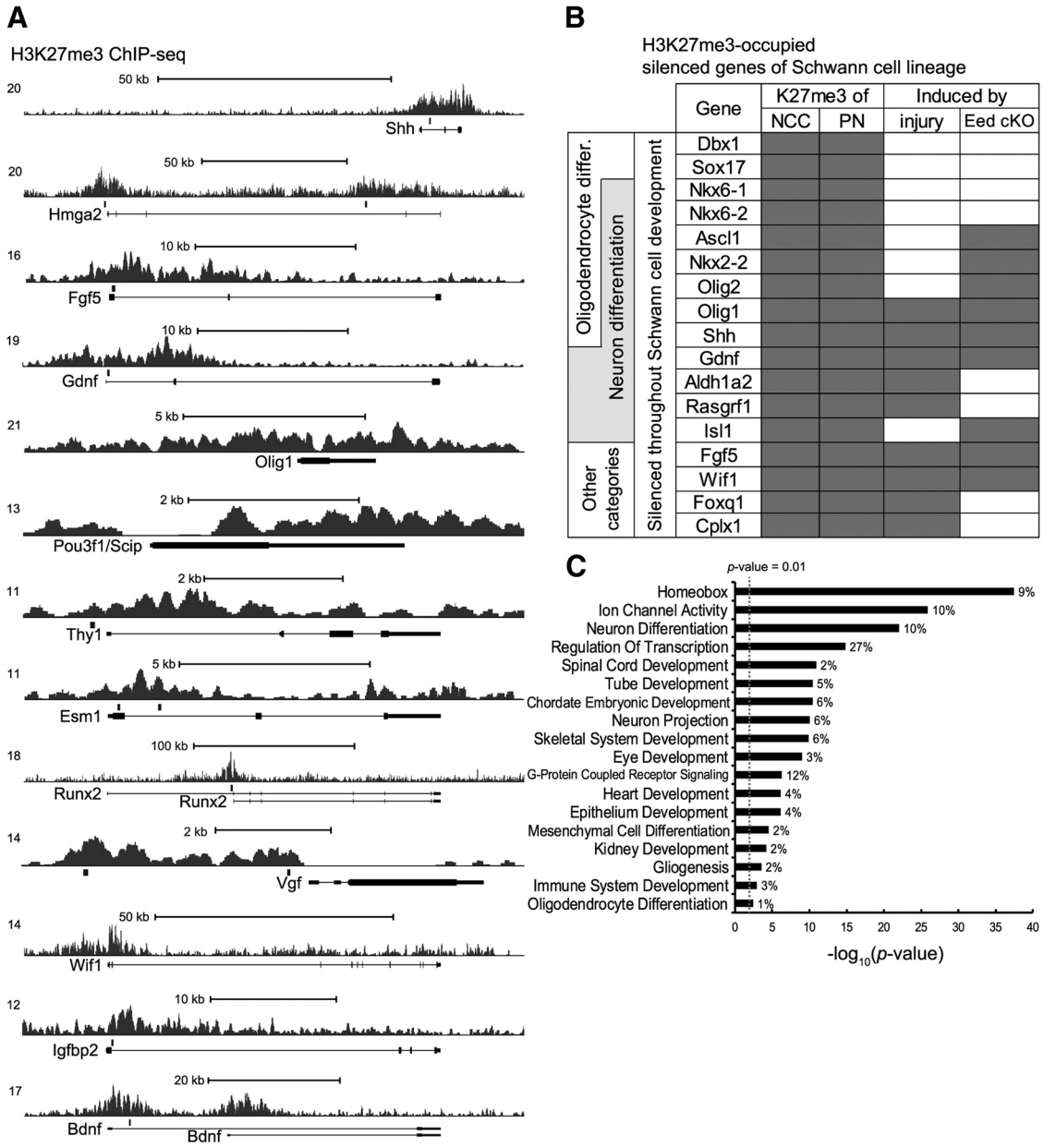

Figure 1. Analysis of H3K27me3-occupied genes in peripheral nerve. ***A***, ChIP-seq mapping of H3K27me3 was performed in intact rat sciatic nerve. The transcription start site (TSS) is on the left. The locations of primer sets used in the ChIP-qPCR analysis (Fig. 4) are indicated with gray rectangles above the gene bodies. ***B***, Representative genes with H3K27me3 that are not detectably expressed in expression profiles of human neural crest and mouse embryonic Schwann cells are listed (Buchstaller et al., 2004; Rada-Iglesias et al., 2012). Gray fillings indicate H3K27me3 occupancy of genes in NCCs and peripheral nerves (PNs), with postinjury induction ≥5-fold, and genes tested by qRT-PCR analysis and identified derepressed by the Schwann-cell-specific deletion of *Eed* (Eed cKO) compared with control, with $p < 0.05$. Injury-induced genes were identified from microarray analysis of peripheral nerves 1, 3, or 7 d after injury (Barrette et al., 2010; Arthur-Farraj et al., 2012; Kim et al., 2012). Other injury-induced genes that are associated with H3K27me3 and are not detectably expressed in neural crest/embryonic Schwann cells are as follows: Pcdh20, Gch1, Runx2, Runx1, Tlr2, Ikzf1, Irf8, Nptx1, Slc32a1, Arhgap9, Fosl1, Il10ra, Mcoln3, and C1ql2. ***C***, Enriched gene ontology categories for the silenced genes in Schwann cell lineage with H3K27me3 present at NCC and peripheral nerves. Categories with $p < 0.01$ are listed.

with Dynabeads Protein G (Invitrogen, 10004D), ChIP samples were washed once with washing buffer 1 (WB1; containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 125 mM NaCl), once with WB2 (containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 250 mM NaCl), and twice with WB3 (containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 500 mM NaCl). The samples were then eluted at 65°C with elution buffer containing 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1% SDS for 15 min. DNA was purified by phenol chloroform extraction and subjected to qPCR or sequencing. Statistical analyses were evaluated by one-way ANOVA.

ChIP-seq. Library preparation and sequencing was performed by the University of Wisconsin Biotechnology Center as described previously (Hung et al., 2015). Base calling was performed using the standard Illumina Pipeline. Reads were mapped to the *Rattus norvegicus* genome rn5 using Bowtie to produce SAM files for further analysis. From the two biological replicates, 14,043,249 and 27,142,309 reads were obtained in input samples, 25,903,222 and 20,923,482 reads in H3K27me3 ChIP samples, 11,943,112 and 10,652,537 reads in H3K4me3 samples (Sham), and 8,034,552 and 13,272,596 in H3K4me3 samples (cut). Hypergeometric optimization of motif enrichment (HOMER; Heinz et al., 2010) was used to determine enriched binding regions for H3K27me3-ChIP relative to sequencing of an input chromatin sample. The raw data files are deposited in National Center for Biotechnology Information Gene Expression Omnibus under accession numbers GSE84265 and GSE84272.

Bioinformatic analysis. K27me3-occupied genes were defined by the presence of H3K27me3 around the transcriptional start site (±7 Kb) with a HOMER peak score ≥10. A previous microarray analysis of embryonic Schwann cell development [neural crest to postnatal day 0 (P0)] was used to identify expressed genes, which were called present on the majority of replicate expression arrays for at least one developmental stage by Buchstaller et al. (2004). We also used neural crest cell (NCC) RNA-seq analysis by Rada-Iglesias et al. (2012) to identify nonexpressed gene (Reads Per Kilobase of transcript per Million mapped reads [RPKM] <1.5). Nonexpressed genes in both experiments were designated as the silenced genes in Schwann cell lineage. Genes with RPKM ≥1.5 of the NCC RNA-seq analysis were referred to as NCC genes. Genes with fold reduction ≥2 of any embryonic time points from embryonic day 9.5 (E9.5) relative to P0 (Buchstaller et al., 2004) and between P0 and P56, the time point of myelination at maturity identified by Verdier et al. (2012), were identified as downregulated genes of embryonic and postnatal Schwann cells, respectively. Gene ontology analysis was performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID; da Huang et al., 2009a, 2009b).

Results

In our previous study, we found that depletion of H3K27me3 by a Schwann-cell-specific deletion of a PRC2 component, *Eed*, leads to derepression of a group of genes that are normally expressed after injury (Ma et al., 2015). This suggested that the removal of this repressive histone modification is a critical step for the activation of a subset of injury response genes in Schwann cell repair program. To understand the role of H3K27 trimethylation in gene repression before injury, we first generated a genome-wide map of H3K27me3 in mature rat sciatic nerve. The ChIP-seq analysis revealed 2622 H3K27me3-occupied genes of peripheral nerves that have H3K27me3 peaks within gene bodies (*Shh*, *Hmga2*, *Fgf5*, and *Gdnf*) and/or at promoters (*Runx2* and *Vgf*) (Fig. 1*A*). Approximately 10% of H3K27me3-associated genes become induced ≥2-fold after nerve injury.

Two genes that promote axonal survival and regeneration after injury are *Gdnf* and *Shh* (Oppenheim et al., 1995; Yan et al., 1995; Boyd and Gordon, 2003; Hashimoto et al., 2008; Fontana et al., 2012; Martinez et al., 2015). A recent study found that Sonic hedgehog is not expressed at any stage of Schwann development before its *de novo* induction after injury (Lin et al., 2015). Therefore, we also analyzed the genes associated with H3K27me3 compared with the H3K27me3-map of human NCCs (Rada-Iglesias et al., 2012) and performed a comparison analysis. Approximately 30% of peripheral nerve H3K27me3-associated genes are silenced and occupied with H3K27me3 in NCCs (Rada-Iglesias et al., 2012). Many of these genes are not detectably expressed in microarray analysis of embryonic Schwann cell development (Buchstaller et al., 2004) and some were induced in Eed-deficient Schwann cells, including *Nkx2–2*, *Olig1*, *Wif1*, and *Fgf5* (see Fig. 6*B*; Ma et al., 2015). The comparison analysis of H3K27me3-map of peripheral nerves and NCC suggests that the mark was established at an early stage of Schwann cell development and representative genes of this type are shown in Figure 1*B*. Gene ontology analysis was used to characterize H3K27me3-occupied genes (Fig. 1*C*). Consistent with previous studies, many homeobox genes are subject to polycomb silencing (Schwarz et al., 2014). Another large group of these silenced genes are specifically associated with neuron differentiation and another subset (including *Ascl1*, *Nkx2–2*, *Olig1*, *Olig2*, and *Shh*) are also critical regulators of oligodendrocyte differentiation and gliogenesis (Orentas et al., 1999; Fogarty et al., 2005; Vallstedt et al., 2005; Fig. 1*B*,*C*).

Discovering injury-induced genes such as *Sonic hedgehog* is important because these appear to be unique markers of the repair Schwann cell phenotype (Jessen and Mirsky, 2016). If one assumes that repression by H3K27me3 in neural crest is a feature of such genes, our results identify a number of other candidate markers that may uniquely mark the repair Schwann cell phenotype in mature nerves. For example, both *Gdnf* and *Fgf5* are highly induced in Schwann cells after nerve injury. Altogether, we identify ~24 H3K27me3-occupied genes that are silenced in NCC and then become strongly activated (induction ≥5-fold) in Schwann cells after nerve injury, including *Olig1*, *Shh*, *Gdnf*, *Aldh1a2*, *Rasgrf1*, *Fgf5*, *Wif1*, *Foxq1*, and *Cplx1* (examples listed in Fig. 1*B*). However, it should be noted that, in contrast to microarray data, *Gdnf* expression has been detected in embryonic Schwann cells (Piirsoo et al., 2010).

Using previous microarray analysis of embryonic and postnatal Schwann cell development (Buchstaller et al., 2004; Verdier et al., 2012), we found that ~8% of H3K27me3-occupied genes are expressed in embryonic and/or promyelinating stages, but then are downregulated before mature myelination (reduction ≥2-fold), including genes that were induced in Eed-deficient peripheral nerve, such as *Igfbp2*, *Pax3*, *Hmga2*, *Tbx2*, and *Pou3f1/Scip* (see Fig. 6*B*; Ma et al., 2015). Many of these genes lacked H3K27me3 in NCCs and therefore likely gain the mark at later stages of Schwann cell development. This analysis suggests that the polycomb pathway plays a role in Schwann cell fate determination by silencing genes of other lineages, particularly neuronal and oligodendrocyte transcriptional regulators, and in Schwann cell differentiation by downregulating genes that are expressed in embryonic and promyelinating stages of development.

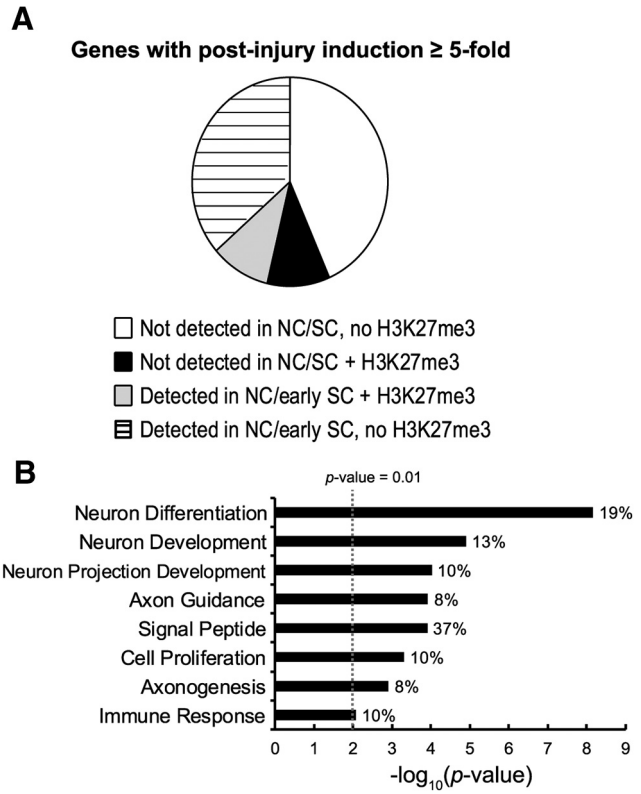

Figure 2. H3K27me3-occupied injury-activated genes are involved with critical roles of repair Schwann cells. ***A***, Injury-induced genes (Barrette et al., 2010; Arthur-Farraj et al., 2012; Kim et al., 2012) were divided by their embryonic expression and the presence of H3K27me3 around the transcription start site (±7 Kb). The expression is determined by RNA-seq analysis of human NCCs (Rada-Iglesias et al., 2012) and microarray analysis of embryonic Schwann cell (SC) development (Buchstaller et al., 2004). ***B***, Enriched gene ontology categories for H3K27me3-occupied genes with postinjury induction ≥5-fold. Categories with *p*-value <0.01 are listed.

Polycomb-repressed genes can be activated by H3K27 demethylases (Agger et al., 2007; Blackledge et al., 2015). We supposed that injury-activated genes regulated by an epigenetic on-off switch may have silenced or a low basal level expression and therefore likely show a high induction after injury (≥5-fold). H3K27me3-occupied genes constituted ~20% of the more highly induced nerve injury genes (Fig. 2*A*) and the gene ontology analysis indicated that known functions of injury-induced genes associated with H3K27me3 were particularly associated with the important roles of Schwann cells in injury responses, such as secreted/membrane proteins (signal peptides, e.g., Fgf5, Gdnf, and Shh), immune responses (Tlr2), guiding axons/axonogenesis and promoting neuronal development (e.g., Ngfr and Nrcam) (Fig. 2*B*). The latter categories include Shh and Gdnf, which provide axon guidance cues and promote axonal regeneration and neuronal survival after injury (Hashimoto et al., 2008; Schuster et al., 2010; Fontana et al., 2012; Martinez et al., 2015), representing such repair Schwann cell genes with H3K27me3 that play critical roles in nerve regeneration.

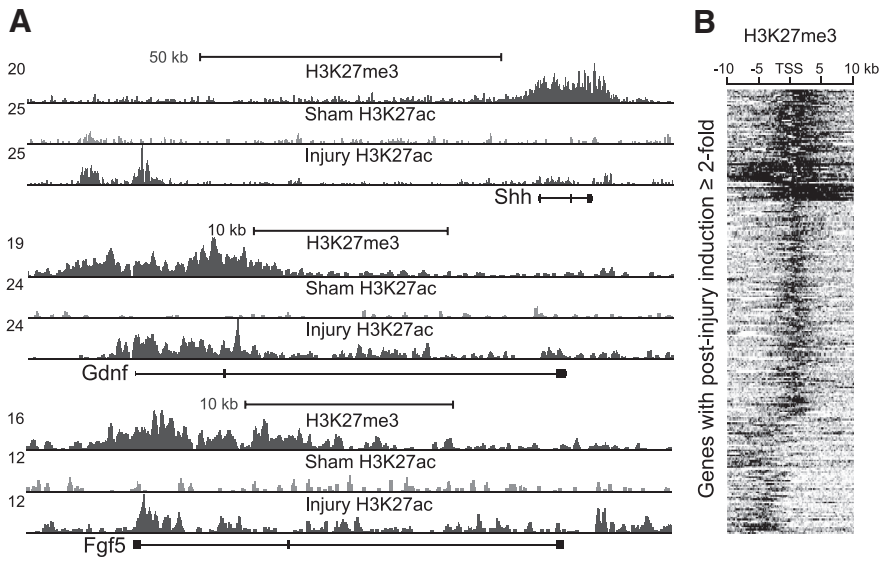

Figure 3. Injury-induced enhancers are mostly devoid of H3K27me3 before injury. ***A***, ChIP-seq mapping of H3K27 acetylation (Hung et al., 2015) was performed in rat sciatic nerves of sham (light gray) and 3 d after injury (dark gray). The transcription start site (TSS) is on the left. ***B***, Heat map showing enrichment of H3K27me3 sequencing tags aligned to a 20 Kb window around the TSS of genes upregulated 1, 3, or 7 d after injury with postinjury induction ≥2-fold.

Defining the repression state of injury-activated genes

We have previously identified injury-induced enhancers by performing ChIP-seq for H3K27acetylation before and after nerve injury and found that these enhancers were generally enriched with motifs of injury-induced transcription factors such as c-Jun and Runx2 (Hung et al., 2015). We supposed that the injury-induced enhancers may be marked or poised for activation after injury. Previous studies have characterized developmentally poised enhancers as those that have open chromatin, histone H3K4 monomethylation, and binding of histone acetylases CBP/p300, yet are occupied by the repressive H3K27me3 rather than H3K27 acetylation (Rada-Iglesias et al., 2011; Rada-Iglesias et al., 2012; Calo and Wysocka, 2013). In contrast, actively engaged enhancers are marked by H3K27 acetylation. We therefore tested the hypothesis that enhancers of injury-induced genes are in the poised state, which would prepare them for rapid activation upon injury by switching from trimethylation to acetylation of H3K27. However, our analysis found that distal enhancers that gain H3K27 acetylation after injury are almost universally not premarked by H3K27me3 (e.g., Shh; Fig. 3*A*). Most of the injury-induced enhancers that gain H3K27 acetylation are distal to the promoter and the only sites in which injury induced an apparent local change from H3K27 trimethylation to acetylation were the promoter regions of a few genes, such as *Hmga2*, *Fgf5*, *Gdnf*, and *Runx2*, in which injury-induced sites of H3K27 acetylation overlap with repressive H3K27me3 before injury. Therefore, the distal enhancers of injury-activated genes are largely not preprogrammed as poised enhancers before injury.

Because H3K27me3 apparently does not repress enhancers before injury, we examined the distribution of H3K27me3 in injury-induced genes and the resulting heat map shows that the repressive histone modification is generally found in the promoter regions and/or gene bodies (Fig. 3*B*). Therefore, H3K27 trimethylation does not appear to be associated with enhancers that are induced after injury, but rather proximal promoters and/or gene bodies.

Loss of H3K27 trimethylation after nerve injury

We performed nerve injury experiments to determine whether there was loss of this repressive histone mark at an early time point (1 d) after nerve injury. We chose 1 d after nerve injury to minimize contamination from immune cells, which normally infiltrate injured nerves beginning at 3 d after injury (Perkins and Tracey, 2000; Mueller et al., 2003). ChIP-qPCR analysis showed a reduced H3K27me3 level in injured nerves compared with sham nerves, suggesting denervation-induced demethylation of H3K27 (Fig. 4). For several of these genes, such as *Shh*, *Gdnf*, and *Fgf5*, Schwann-cell-specific deletion of *Eed* leading to depletion of H3K27me3 was sufficient to activate their expression even in the absence of injury (see Fig. 6*B*).

To determine whether the demethylation is required for gene activation after injury, we used GSK-J4, which binds the catalytic pocket and inhibits both H3K27 demethylases, JMJD3 and UTX (Kruidenier et al., 2012). Unfortunately, this inhibitor has not been shown to pass the blood–brain or blood–nerve barrier. Therefore, to deliver the inhibitor effectively and control the environment of injured nerves, we used a nerve explant protocol in which some injury responses are activated upon transfer of nerves to culture (Banner and Patterson, 1994; Mårtensson et al., 2007; Shin et al., 2013; Blom et al., 2014). In this system, we observed induction of a number of injury-activated genes, including *Shh*, *Igfbp2*, *Fgf5*, and *Gdnf*, even after 24 h in culture. To test the importance of H3K27 demethylation, explanted sciatic nerves were cultured in the presence of either GSK-J4 or DMSO vehicle. The induction of injury-activated genes by explant could be inhibited by GSK-J4, as shown by *Shh*, *Fgf5*, *Gdnf*, *Runx2*, *Hmga2*, and others in a 1 or 3 d incubation (Fig. 5). As a control, another injury-induced gene that is not associated with H3K27me3, *Timp1*, was induced ~15-fold in explant culture, but this level was unaffected by GSK-J4 inhibition.

We used ChIP and gene expression analysis of nerves from the Schwann-cell-specific knock-out of Eed to determine whether genes are regulated by H3K27me3. Ultrastructural analysis

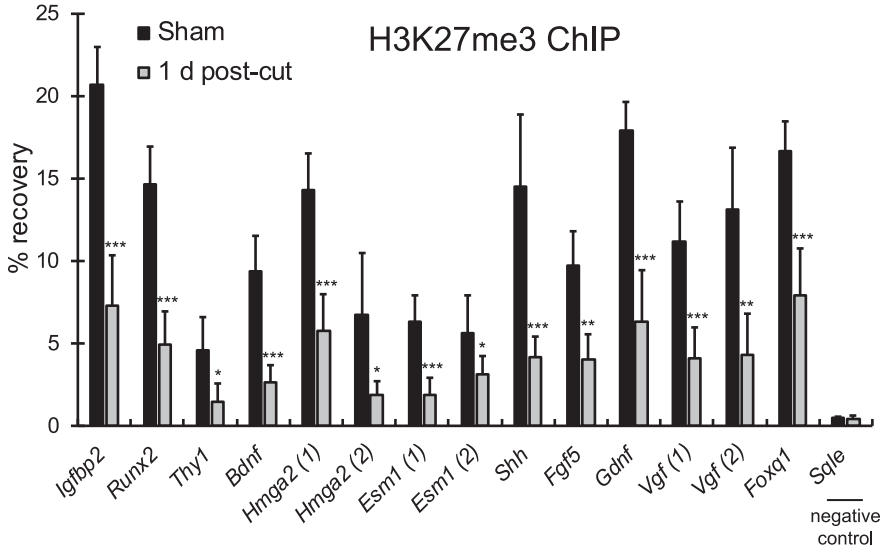

Figure 4. Injury induces demethylation of H3K27. H3K27me3-ChIP assays were performed with distal stumps of rat sciatic nerves 1 d after injury or sham surgery and the percentage recovery relative to input was calculated by qPCR analysis. H3K27me3-bound genes identified by ChIP-seq are shown and the primer set numbers represent their location from the left. *Sqle* is a negative control not associated with H3K27me3. Data are shown as mean ± SD. $^{**}p < 0.005$, $^{***}p < 0.0005$. $n = 5$ for sham and $n = 6$ for 1 d after injury.

showed no apparent developmental abnormalities or pathologies that might induce injury response in Eed-deficient nerves at 2 months (Ma et al., 2015), the time point of this study. There was a significant loss of H3K27me3 at genes in Eed-deficient nerves compared with control nerves (Fig. 6*A*) and many of GSK-J4 affected genes were correlatively derepressed, including *Igfbp2*, *Runx2*, *Fgf5*, *Shh*, and *Hmga2* (Fig. 6*B*). This analysis also expands our previous list of Eed-regulated genes, which was obtained through microarray analysis (Ma et al., 2015), to include other injury-induced genes such as *Runx2*, *Gdnf*, *Fgf5*, *Olig1*, *Pou3f1/Scip*, and *Bdnf*. Interestingly, the promoter of *Bdnf*, in particular, was recently reported as a PRC2 target in mature neurons and becomes activated upon JMJD3-mediated demethylation (Palomer et al., 2016).

Only a subset of injury-activated genes were derepressed in peripheral nerves in

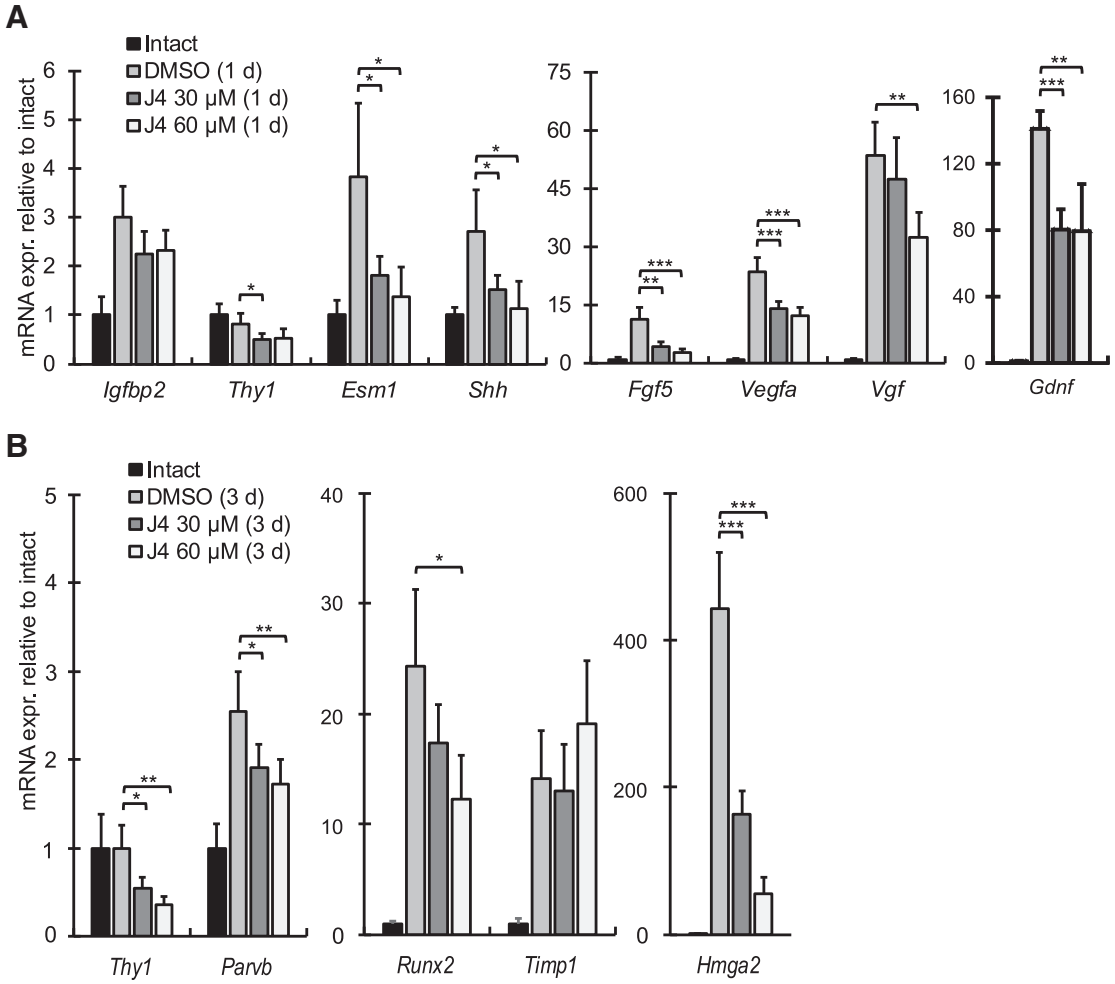

Figure 5. Inhibition of H3K27 demethylation attenuates the induction of injury-activated genes. ***A***, ***B***, Rat sciatic nerve explants were cultured for 1 or 3 d with the presence of GSK-J4 at indicated concentrations or DMSO vehicle and subjected to qRT-PCR together with immediately frozen nerve segments after dissection (indicated as intact). The intact level of each gene is set as 1. Expression levels were normalized to 18S rRNA. Data are shown as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. $n = 5$ per condition.

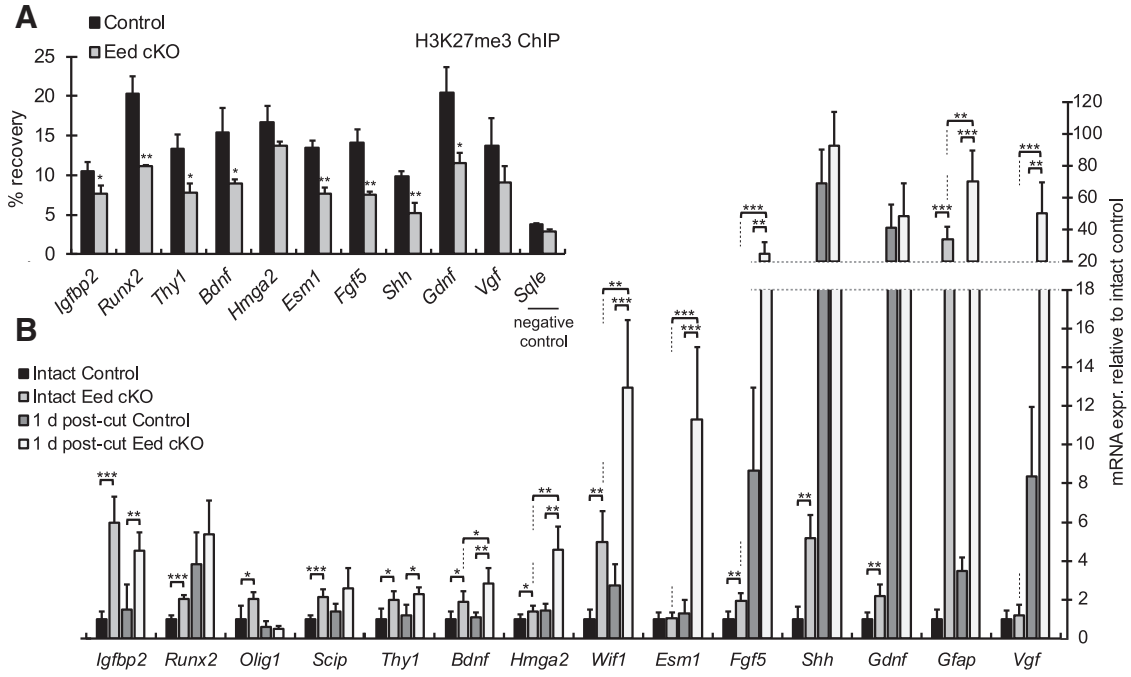

Figure 6. H3K27me3 controls the expression of injury-activated genes of Schwann cells. ***A***, ChIP assays showing changes of H3K27me3 in 1 month Eed cKO sciatic nerves compared with control. An actively transcribed gene *squalene epoxidase* (*Sqle*) in Schwann cells displays low levels of H3K27me3. Data are shown as mean ± SD. $*p < 0.05$, $**p < 0.005$. $n = 3$ per genotype. ***B***, qRT-PCR analysis was used to identify the expression level of injury-activated genes from 2-month Eed cKO and control sciatic nerves in intact condition or 1 d after injury. Expression levels were normalized with *Gapdh*. Data are shown as mean ± SD. $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$. $n = 5$ per genotype and condition.

Eed cKO peripheral nerves (Ma et al., 2015). This suggested that the loss of H3K27me3 is sufficient for activation of some genes, but we wished to determine whether H3K27me3 loss may be required for a greater proportion of injury-activated genes. This model would suggest that some injury-activated genes may be expressed prematurely (or more highly) if H3K27me3 was depleted. Therefore, we investigated how Eed-deficient nerves primed with the reduced level of H3K27 methylation responded to the injury in gene activation. Although *Esm1* and *Vgf* were not derepressed in intact Eed cKO nerves, their levels were dramatically higher in Eed-deficient nerves 1 d after injury, along with other genes such as *Bdnf*, *Hmga2*, *Wif1*, and *Fgf5* (Fig. 6*B*). Notably, *Bdnf* and *Hmga2* become fully induced at 7 and 3 d after injury, respectively (Barrette et al., 2010; Arthur-Farraj et al., 2012), but Eed-deficient nerves showed premature induction of these genes at 1 d after injury. In contrast, Olig1 was modestly induced in the Eed cKO, but its expression did not increase above baseline after injury in either control or Eed-deficient nerves. Based on previous microarray data (Barrette et al., 2010; Arthur-Farraj et al., 2012), the peak time point of Olig1 induction after nerve injury is 7 d and our own control data indicate only a marginal increase at day 1. The inability of Eed loss to increase Olig1 expression after injury may reflect the requirement of additional factors in addition to c-Jun that are absent at this early time point. However, the premature/higher induction of several genes in nerves primed with the reduced methylation suggests that demethylation of H3K27 is required and is a rate-limiting step in the activation of a larger subset of injury-activated genes beyond those that we have described previously (Ma et al., 2015), in which H3K27me3 loss appeared to be necessary and sufficient.

Injury regulation of the H3K4me3 promoter mark

To gain insight into how H3K27 demethylation could activate genes after nerve injury, we also examined the injury-induced changes of H3K4me3, which is most often associated with active promoters (Mikkelsen et al., 2007; van Ingen et al., 2008; Lauberth et al., 2013). The trimethylation of H3K4 is often inversely correlated with trimethylation of H3K27 (Agger et al., 2007; Issaeva et al., 2007; Pasini et al., 2008). Surprisingly, the ChIP-seq mapping revealed the presence of H3K4me3 at 54% of injury-activated genes (≥5-fold induction after injury) of uninjured nerves (sham condition). The somewhat high percentage of H3K4me3-bound transcription start sites may be due to basal expression levels of some genes before injury, such as $p75^{NTR}$/*Ngfr* (Cosgaya et al., 2002). However, it has been shown that H3K4me3 is found on poised promoters that are also associated with H3K27me3 (Mikkelsen et al., 2007; Paige et al., 2012).

Consistent with this possibility, the mapping also displayed both H3K27me3 and H3K4me3 at promoters of 7% of injury-activated genes that are silenced in NCC and throughout Schwann cell development, including *Shh*, *Fgf5*, *Gdnf*, *Olig1*, and *Wif1* (Fig. 7*A*). In particular, *Shh*, *Olig1*, and *Gdnf* were reported to be silenced in mature myelinated nerves and immature Schwann cells (Arthur-Farraj et al., 2012; Lin et al., 2015), so the H3K4me3 signal was less likely from any active promoters. In contrast to these, the transcription start sites of silenced genes insensitive to injury, such as *Tekt3* and *Barhl2*, lack H3K4me3. The map from 1 d after injury showed that the H3K4me3 was increased and/or broadened to a certain degree at some injury-activated genes such as *Shh*, *Hmga2*, *Fgf5*, and *Gdnf*. Overall, our data indicate that distal enhancers of injury-induced genes are not poised or "premarked" by characteristics of enhancers nor are they marked with H3K27me3. However, many injury-induced genes have promoters that appear to be primed with H3K4me3 before injury.

Because H3K27me3 recruits RBP2 demethylase, which removes H3K4me3 (Pasini et al., 2008), we investigated whether demethylation of H3K27 is sufficient to induce trimethylation of

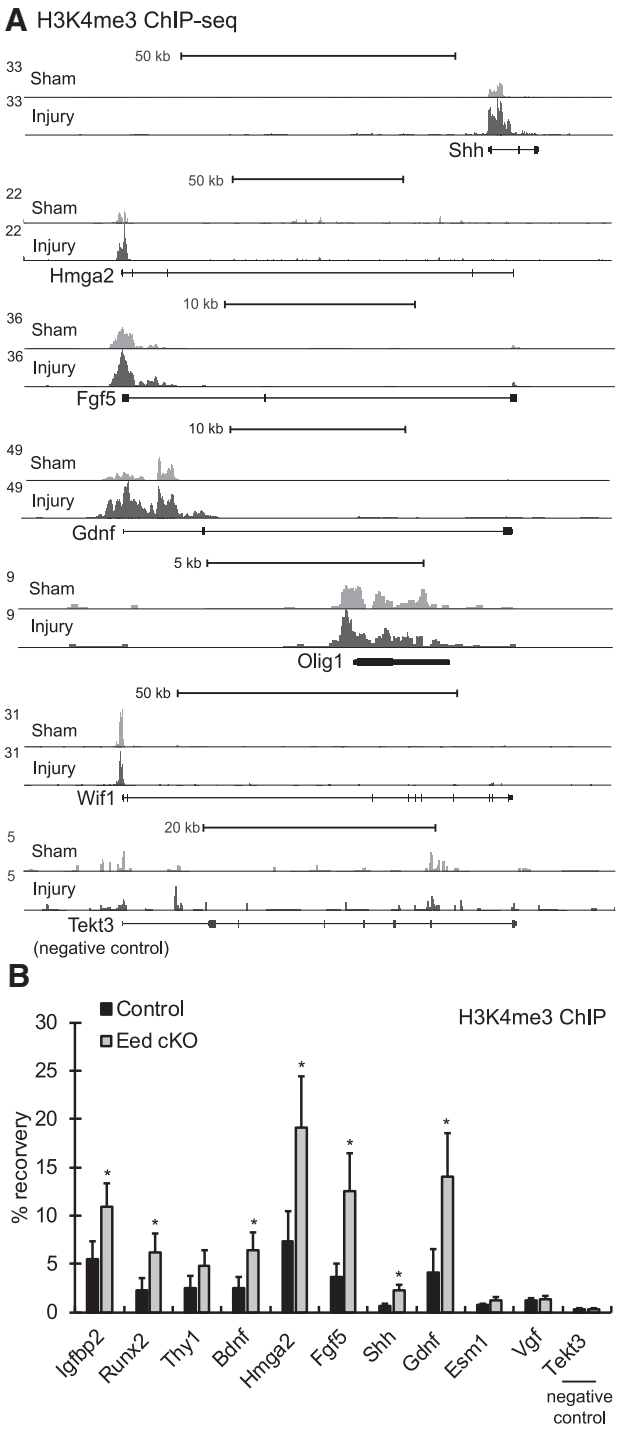

Figure 7. The active histone mark H3K4me3 premarks the transcriptional start sites of injury-activated genes of mature myelinated nerves and increases after injury. ***A***, ChIP-seq mapping of H3K4me3 was performed in rat sciatic nerves of sham (light gray) and 1 d after injury (dark gray). The transcription start site (TSS) is on the left. ***B***, ChIP-qPCR showing changes of H3K4me3 in 1-month Eed cKO sciatic nerves compared with control. A silenced gene *Tekt3* in Schwann cells displays a lack of H3K4me3.

H3K4 in Schwann cells. ChIP-qPCR analysis identified an increase of H3K4me3 at nearby transcriptional start sites of Eed-deficient nerves, in which H3K27me3 level is reduced compared with control nerves (Fig. 7*B*), and the induction was correlated to derepression of genes (Fig. 6*B*). Consistent with the above ChIP-seq analysis, the analysis showed a significant level of H3K4me3 at injury-activated genes compared with a silenced gene, *Tekt3*, in control nerves. This result would explain our observations that demethylation of H3K27 after injury leads to the induction of the H3K4me3 active promoter mark at some injury-activated genes.

Discussion

Studies of Schwann cell responses after nerve injury have demonstrated the extensive reprogramming of the Schwann cell transcriptome (Nagarajan et al., 2002; Bosse et al., 2006; Barrette et al., 2010; Arthur-Farraj et al., 2012). The observed changes include repression of the myelin program, but also activation of several genes that are not normally expressed in Schwann cell development. Studies of the c-Jun transcription factor and the Wlds mutant have shown that the rapid reprogramming is vital to the regenerative response to nerve injury (Arthur-Farraj et al., 2012). Although it was expected that transcriptional reprogramming would also require epigenomic changes, the specific mechanisms have not been characterized. In previous work, an important clue to one of the mechanisms became apparent when we found that depletion of H3K27 methylation by a Schwann-cell-specific knock-out of the Eed subunit of the PRC2 complex was sufficient to activate several injury genes (Ma et al., 2015). Profiling of H3K27me3 in mature nerve *in vivo* revealed that ~20% of injury-activated genes are associated with the repressive H3K27me3 mark and this includes several well known injury-induced genes that play a role in nerve regeneration, such as *Gdnf*, *Bdnf*, and *Shh* (Zhang et al., 2000; Pepinsky et al., 2002; Calcutt et al., 2003; Song et al., 2006; Hashimoto et al., 2008; Fontana et al., 2012; Wilhelm et al., 2012; Martinez et al., 2015). The functions of other genes, such as the *Runx* transcription factors and *Fgf5*, have not been characterized, although they are strongly induced after nerve injury (Scarlato et al., 2001; Barrette et al., 2010; Hung et al., 2015).

Although c-Jun is required for full activation of several genes after nerve injury, many of these genes are partially induced by injury even in the absence of c-Jun (Arthur-Farraj et al., 2012). In addition, c-Jun is required for full induction of ~100 genes after nerve injury, indicating that other mechanisms are involved in the reprogramming of Schwann cells. To understand the mechanism of Schwann cell reprogramming after injury, it is important to identify the mechanisms by which many injury-inducible genes are repressed or silenced before injury. In this effort, we have been aided by our recent identification of injury-inducible enhancers that were identified through analysis of an active enhancer mark, H3K27ac, in injured nerve (Hung et al., 2015). Previous studies had indicated that many enhancers in stem cell development are at least temporarily in a "poised" state in which there is open chromatin and association with an active enhancer mark (H3K4me1), along with repressive H3K27me3 (Rada-Iglesias et al., 2011; Rada-Iglesias et al., 2012; Calo and Wysocka, 2013). Therefore, we had anticipated that enhancers of injury-induced genes would follow a similar pattern in which the H3K27me3 mark in poised enhancers would be replaced with H3K27ac as the enhancers were activated. Contrary to our expectations, the ChIP-seq analysis shows that the previously described injury-induced enhancers are largely not premarked by H3K27me3 before injury. Instead, H3K27me3 is generally found in the promoters and around transcription start sites of the gene body. Therefore, the reversal of polycomb repression after nerve injury is not generally associated with the formation or activation of distal enhancers of injury genes, but instead is focused on reversal of H3K27me3 at promoters and/or gene bodies of injury genes.

Therefore, our overall model is that full activation of many injury genes requires reversal of polycomb repression at promoters/gene bodies in concert with binding of injury-induced transcription factors such as c-Jun and Runx2 at distal enhancers. For example, there are some common downstream genes of c-Jun and polycomb pathways, such as *Shh*, *Fgf5*, *Gdnf*, and *Runx2*. Because injury-induced enhancers are not premarked, this would suggest that some injury-induced transcription factors act as pioneer factors to open up the chromatin structure at enhancers of injury-induced genes. Accordingly, a recent study showed that the AP-1 (Jun/Fos) transcription factor can make chromatin accessible for other transcription factors (Biddie et al., 2011).

H3K27 methylation was thought to be relatively stable and therefore a mechanism of long-term transcriptional memory. However, the identification of the H3K27-specific demethylases JMJD3 and UTX demonstrated that polycomb repression can be reversed (Agger et al., 2007; Lee et al., 2007). Therefore, polycomb repression of many injury genes predicts that demethylation of H3K27 contributes directly to activation of a significant number of injury genes. Peripheral nerve injury induces an increase in the protein level of the Jmjd3/Kdm6b H3K27 demethylase (Gomez-Sanchez et al., 2013). We therefore tested the requirement of H3K27 demethylation using GSK-J4 treated nerve explants. The results showed substantial reduction of injury genes that are normally activated in this model. In addition, we also tested whether H3K27me3 depletion resulted in premature and/or augmented induction of nerve injury genes. Although loss of H3K27me3 is sufficient for activation of certain injury genes, our results indicate that it is a rate-limiting step for other genes because activation of other injury genes was premature and/or augmented in the Eed cKO. Several genes listed in our analyses, *Igfbp2*, *Fgf5*, *Shh*, *Runx2*, and *Hmga2*, were found to be deregulated in other cell types with altered expression of PRC2 components or Jmjd3 (Boyer et al., 2006; Ezhkova et al., 2011; He et al., 2012; Yang et al., 2013; Perrigue et al., 2015), suggesting that H3K27me3 is a commonly used epigenetic block that needs to be removed for their activation.

In this study, we have also shown that the reduction of H3K27me3 leads to the induction of trimethylation of H3K4 at the promoter that promotes the binding of general transcription initiation factor TFIID (van Ingen et al., 2008; Lauberth et al., 2013) in peripheral nerves. This was to some extent anticipated because polycomb repression is associated with the RBP2 demethylase, which demethylates H3K4me3 (Pasini et al., 2008). There have been a number of studies describing the bivalent H3K27me3/H3K4me3 state in stem cell analysis (Mikkelsen et al., 2007; Stock et al., 2007; Ku et al., 2008). This bivalent chromatin signature was thought to repress promoters while keeping them poised, which then resolves upon ES cell differentiation into a H3K4me3-dominant state through H3K27 demethylation (Bernstein et al., 2006; Mikkelsen et al., 2007; Voigt et al., 2012). We do observe overlap of H3K27me3 and H3K4me3 at some genes, including some with the same bivalent mark in neural crest analysis. Although our *in vivo* analysis does not allow us to conclude that a given promoter is associated with both histone modifications in the same cell, the induction of H3K4me3 and derepression of repair Schwann cell genes such as *Shh* is caused by the Schwann-cell-specific deletion of Eed. In addition, the presence of the bivalent mark at some silenced genes such as *Olig1*, *Gdnf*, and *Fgf5* in NCCs is consistent with an early establishment of the mark in Schwann cell development (Rada-Iglesias et al., 2012).

Although we have performed *in vivo* chromatin analysis to retain the important axonal signals affecting Schwann cell development, a limiting factor of our analysis is that sciatic nerve contains some endothelial and fibroblast nuclei, although the majority of sciatic nerve nuclei (>75%) are Schwann cells (Salonen et al., 1988; Joseph et al., 2004). In addition, ~50–80% of Schwann cells are myelinating and the rest are nonmyelinating Schwann cells (Zorick et al., 1996; Topilko et al., 1997). For those genes expressed at very low levels in mature nerve, this would suggest, although not prove, that their expression is repressed in both Schwann cell types. Nonetheless, the Schwann-cell-specific deletion of Eed leads us to conclude that removal of polycomb repression leads to changes in Schwann cell gene expression.

Aging slows myelin clearance and the onset of functional recovery after injury (Vaughan, 1992) and such delay appears primarily due to a deteriorating response of transcriptional mechanisms of Schwann cells rather than those of neurons (Ceballos et al., 1999; Verdú et al., 2000; Kang and Lichtman, 2013; Painter et al., 2014). Age-dependent epigenetic change can play a role because it was shown that a decline in the brain remyelination with aging is an outcome of the progressive loss of HDAC-mediated repression (Shen et al., 2008). Our analyses showed that several of injury-activated genes affected by aging, including *Fgf5*, *Bdnf*, *Gdnf*, and *Igfbp2* (Painter et al., 2014), are regulated by H3K27me3, which implies that there may be age-related impairment in the demethylation dynamics. Interestingly, aging also causes derepression of some H3K27me3-regulated genes (*Shh*, *Thy1*, *Wif1*, etc.) in intact nerves and *Shh* appears to be the second-highest overexpressed genes in old nerves compared with young nerves (Verdier et al., 2012). It is therefore worth investigating the alterations brought by aging in H3K27me3 landscape of uninjured nerves and demethylation after injury.

The dramatic plasticity of Schwann cell lineage can be observed also in the enteric and parasympathetic nervous systems where Schwann cell precursors (SCPs) adopt a neuronal fate and lose the Schwann cell marker SOX10 (Dyachuk et al., 2014; Espinosa-Medina et al., 2014; Uesaka et al., 2015). Enteric SCPs, in particular, contribute to 20% of myenteric and submucosal neurons in the large intestine such that SCP-derived neurogenesis is required for the postnatal maintenance of the neuron population of the enteric nervous system (ENS; Uesaka et al., 2015). In addition, enteric glia retain neurogenic potential and are able to become enteric neurons in response to ENS injury (Laranjeira et al., 2011). The observation that neuron differentiation genes are highly enriched among silenced genes throughout Schwann cell development, with H3K27me3 found in both NCCs and peripheral nerves or H3K27me3-occupied injury-activated genes, raises an intriguing possibility: that demethylation of H3K27 takes a place during the parasympathetic nervous system and ENS neurogenesis derived from SCPs or enteric glia after injury. Importantly, *Nkx2–2*, *Olig1*, *Olig2*, *Shh*, *Isl1*, *Pax3*, and *Pax6* are among the critical regulators of neuron differentiation and are silenced by H3K27me3, as we have demonstrated using Eed-deficient peripheral nerves.

Our study illustrates H3K27me3 dynamics and regulation of injury-activated genes. Because patients with peripheral nerve injury often face a long rehabilitation period, the findings are of clinical importance and may lead to the identification of tools for sustainable expression of the repair genes that effectively facilitate the process.

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