Regulation of Polycomb Repression in Schwann Cells.

By

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Regulation of Polycomb Repression in Schwann Cells.

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Schwann cells perform the important task of myelinating axons and providing metabolic support during the development of peripheral nerves, enabling high velocity nerve conduction. In situations where the nerves become damaged, Schwann cells undergo transcriptional reprogramming into a state called repair cells that initiate a series of regenerative events involving myelin debris clearance, axon regeneration, and remyelination. Here we describe the epigenomic regulation by histone demethylases and deubiquitinase in Schwann cells during the development and the nerve injury response. The demethylase proteins JMJD3/UTX contain the Jumonji catalytic domain that actively removes H3K27me3 created by Polycomb repressive complex 2. Likewise, the deubiquitinase BAP1 also contains the catalytic domain UCH that specifically removes the H2AK119ub1 created by Polycomb repressive complex 1. Schwann-cell specific knockout of the EED subunit of PRC2 was previously observed to undergo premature induction of nerve injury genes. In addition to the finding, many nerve injury genes not only underwent the loss of H3K27me3 but also the loss of H2AK119ub1 in EED-KO. Therefore, we developed the Schwann cell-specific double knockouts of JMJD3/UTX and single knockout of the BAP1 respectively in attempt to prevent the induction of Polycomb-repressed, pro-regenerative genes. However, we found that demethylases are dispensable for the development of Schwann cell and have a minor role in the early induction of certain nerve injury genes after injury. Interestingly, we found that loss of BAP1 has significant phenotypes including the myelin deformities, thinner myelin of larger caliber axons, and premature induction of nerve injury genes, indicating that loss

of BAP1 causes activation of Polycomb-repressed genes. The developmental phenotype is similar to that of Schwann cell-specific of O-linked N-Acetylglucosamine Transferase knockout model. OGT is known to interact with BAP1 and was reported to inhibit activation of a key transcription factor (JUN) required for nerve injury responses by Schwann cells. However, *c-Jun* was not prematurely elevated in *Bap1*-KO as observed in OGT-KO. Altogether, we hypothesized BAP1 is required to maintain Polycomb repression of the nerve injury program in Schwann cells. My research revealed the varying importance of demethylases and deubiquitinase in maintaining the Polycomb repression in development and nerve injury response.

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List of Acronyms

Bap1	BRCA1 associated protein-1
Bdnf	Brain-derived neurotrophic factor
ChIP	Chromatin immunoprecipitation
cKO	Conditional knockout
CMT	Charcot-Marie-Tooth disease
CNS	Central nervous system
Eed	Embryonic ectoderm development
Egr2/Krox20	Early growth response protein 2
ErbB2	Erb-B2 receptor tyrosine kinase 2
Gdnf	Glia-derived neurotrophic factor
H2AK119ub1	Histone H2A lysine 119 ubiquitination
H3K27me3	Histone H3 lysine 27 trimethylation
Hdac	Histone Deacetylases
Hmga2	High mobility group AT-hook 2
HOMER	Hypergeometric Optimization of Motif EnRichment
KDM6A	lysine demethylase 6A, also known as Utx
KDM6B	lysine demethylase 6B, also known as Jmjd3
Mbp	Myelin basic protein
Mpz	Myelin protein zero
PNS	Peripheral nervous system
PRC1/2	Polycomb repressive complex ½
PR-DUB	Polycomb repressive deubiquitinase
qRT-PCR	Quantitative real-time reverse transcription PCR
RING1	Ring finger protein 1
Runx2	Runt related transcription factor 2
Shh	Sonic hedgehog
Sox10	Sex-determining region Y-box containing gene 10

Suz12	Suppressor of zeste 12
Tead	TEA domain family member

Chapter 1

Introduction and Dissertation Plan

Epigenetic Regulation of Polycomb Repression in Schwann Cells After Injury

ABSTRACT

Schwann cells derived from the neural crest cells form myelin by wrapping the plasma membranes around the axons in the peripheral nervous system. Through the complex regulatory interactions between transcriptional and chromatin layers, many aspects of Schwann cell development are initiated by Schwann cell-axon interface signaling and NRG1/ERBB2/3-mediated downstream pathways to promote the radial sorting and myelination of the peripheral nerves. In addition, reprogramming of Schwann cells after nerve injury to promote nerve regeneration is sophisticated and involves injury-induced transcription factors and chromatin regulators such as *c-Jun* and Polycomb complexes. Transcription factors, intracellular pathways, and epigenetic modulators critical to Schwann cell development and responses to injury will be discussed here in detail. Demethylases and deubiquitinase are some of the potential mechanisms that activate the nerve injury genes that warrant significant characterization and are discussed here.

Schwann cell Structure

Myelinating Schwann cells are glial cells in the peripheral nervous system that produce myelin sheaths to encase the axons that innervate muscles and other tissues (1). Myelin produced by Schwann cells increases conduction speed of action potentials, which is particularly important for neuromuscular communication. Schwann cell cells play varying roles in the peripheral nervous system including somatic and autonomic components. In the somatic component, sensory nerves project from the neurons in the dorsal ganglion whereas the motor axons extend from motor neurons in the spinal cord (2,3). Larger caliber axons of >1 micrometer are typically myelinated by Schwann cells in postnatal development while others of smaller diameter (mostly sensory) are clustered into Schwann cells to form non-myelinating Remak bundles. First characterized in animal tissues by German physiologist Theodor Schwann in the 19th century, Schwann cells were originally recognized as a mere supporting cell type but later discovered to have multiple substantial roles such as trophic support and repair activities (1,4,5).

The structure of the fully myelinated axon contains the compacted layers of plasma membranes and enriched myelin proteins produced by mature Schwann cells. The adaxonal side of Schwann cells forms the axoglial interface, but abaxonal side is covered by a basal lamina, which completely encases the Schwann cell with the extracellular matrix. Axon bundles are encased within a perineurium containing fibroblasts and blood vessels, and this organization is termed a fascicle. The large nerves are composed of many fascicles that are surrounded by the outer layer called the epineurium (6). During development, Schwann cells specifically select larger caliber axons in a highly regulated process called radial sorting and form myelin around the axons (7). Myelination leads to clustering of the sodium channels at the nodes of Ranvier where the ion channels are densely grouped, allowing for action potentials to be rapidly regenerated when traversing over distance (8). Myelin compaction promotes the speed of saltatory conduction across

the axons and is therefore essential for processing signals in the peripheral nervous system. Schwann cells likely evolved to complement the central nervous system by promoting the action potentials across the longer axonal paths in larger vertebrates.

Prior to myelination, Schwann cells develop from neural crest cells that specialize into many PNS cell types (9). This process is distinct from the differentiation of neural tube cells into neurons oligodendrocytes, and astrocytes in CNS (10). Neural crest cells activate cell type specific transcription factors, undergoing the change into Schwann cell precursors and then immature Schwann cells. Each stage of Schwann cell differentiation expresses some common Schwann cell protein markers such as S100 and GFAP (1). The immature Schwann cell continues to rapidly proliferate until individual Schwann cells begin to perform radial sorting by specifically selecting one larger caliber axon (usually (>1 micrometer). Then they produce the myelin proteins in very large quantity needed to wrap the axon. Complex interactions of adhesions and extracellular matrix proteins are involved at the axon/Schwann cell interface since the selection and compaction occur depending on the axonal diameter. Some Schwann cells become non-myelinating Remak bundles containing multiple sensory axons <1 micrometer in diameter (7).

In contrast to oligodendrocytes in the CNS, Schwann cells always myelinate the axons in a 1:1 ratio. During the myelination of the axons, the myelin layers become compacted in the internodes between nodes of Ranvier. Vesicles are required to transport the materials as the myelination is a highly demanding process. Along the compacted plasma membranes, there are membrane channels containing gap junctions within Schwann cell myelin, which are present in areas of noncompact myelin known as Schmidt-Lanterman incisures, which are routes for nutrient flow to the axons (11). When the axons are fully myelinated, Schwann cells undergo the shift from

the developmental mode to homeostatic stage, which is characterized by an intermediate level of myelin gene expression (12).

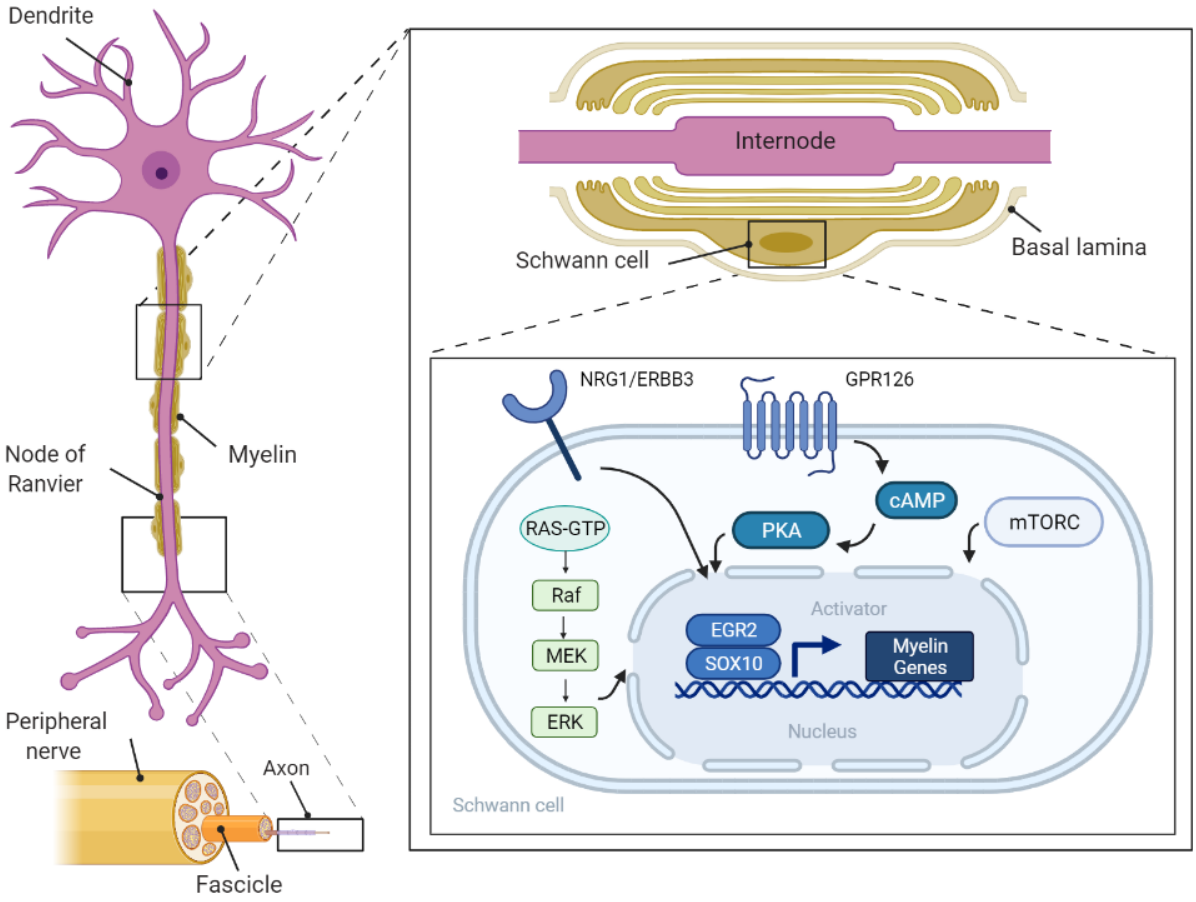

Figure 1. Schwann cells from myelin on axons, which are encased in fascicles of sciatic nerves. With myelin encasing the axon, the high velocity of action potentials become possible, allowing for the communication between the motor nerve to the neuromuscular junction. The sciatic nerve is the largest nerve in mammalian vertebrates and Schwann cells are the most abundant cell type.

Developmental transcription factors and signaling pathways

Schwann cell development from neural crest cells to Schwann cell progenitors and subsequent immature Schwann cells involves differentiating transcription factors such as NOTCH, SOX10, and PAX3. For instance, NOTCH has multiple roles by promoting the differentiation of Schwann cells and also regulating the number of the Schwann cell pool through maintaining proliferation (13). PAX3 is expressed at an early stage in Schwann cell precursors and prevents premature induction of myelin genes (14). SOX10 is a high-mobility group transcription factor that binds to many regulatory elements of developmental genes and is required for the entirety of developmental timeline including the myelinating stage (15). The further development involves stage-specific transcription factors during the transition from the Schwann cell progenitors to immature, highly proliferating Schwann cells. Several of the transcription factors at this stage are: TEAD1 and SOX2 (16,17). Alongside SOX10 which is still required at this stage, multiple transcription factors coordinate radial sorting during development. SOX2 controls the self-assembly process of Schwann cells through cytoskeletal reorganization (18). The YAP/TAZ coactivators of the TEAD1 transcription factor are needed for the radial sorting of axons during the development through the mechanosensitive Hippo kinase pathway that controls phosphorylation of YAP and TAZ co-activators (16). It should be noted that the commonly used *Mpz*-cre driver used in this dissertation becomes active at E14, which corresponds to the Schwann cell precursor stage, meaning that early neural crest stages of differentiation are unaffected.

Upon reaching the threshold in the immature Schwann cell number, pro-myelinating transcription factors begin to express transcription factors such as *Yy1*, *Oct6*, and *Egr2*. POU3F1/OCT6 defines the pro-myelinating stage, and it works with YY1, SOX10 and TEAD/YAP/TAZ to activate *Egr2* prior to the myelination (19,20). *Egr2* is considered as a master regulator of myelination and binds to regulatory elements in major myelin genes and also lipid

biosynthetic genes along with *Srebp1* which is an important transcription factor for initiating lipid synthesis (21,22). Sterol regulatory element binding proteins 1 and 2 (*Sbrep1/2*) system essentially couples neuregulin/mTOR signaling to lipid metabolism promoting the highly acute production of myelin lipid in Schwann cells. Inhibiting the SREBP system causes loss of lipid gene expression accompanied with a severe hypomyelination phenotype (22).

Major myelin genes that are induced in Schwann cells include Myelin basic protein (MBP), Myelin Protein Zero (MPZ), and Peripheral Myelin Protein 22 (PMP22), which are highly enriched in the myelin sheath of peripheral nerves, and the deficiency/altered dosage of these myelin proteins leads to several peripheral neuropathies. In particular, Charcot Marie Tooth (CMT) is a group of diseases that negatively affect the peripheral nerves, resulting in sensory and mobility issues for afflicted patients (23). A substantial fraction of these cases is caused by the duplication of the *PMP22* gene at chromosome 17, classified as CMT1A (24). *MPZ* mutations (CMT1B) causes the wide variety of neuropathy phenotypes in CMT cases (25). Considering that EGR2 is a master transcription factor that drives lipid biosynthesis and myelination in Schwann cells, several severe CMT subtypes were attributed to the mutations in the *EGR2* gene (26).

Since myelination is sensitive to the balance of pro-myelinating regulators and negative regulators, many negative regulators are downregulated during myelination and then upregulated during nerve injury (see below). For instance, the *Zeb2* transcription factor recruits the deacetylases HDAC1/2 as part of the NuRD complex to counteract the negative regulators of myelination prior to *Egr2* activation (27,28). NOTCH and SOX2 are downregulated because they promote proliferation and inhibit the myelination (13,17). Additional negative regulators such as neural crest transcription factors *Tfap2a* and *Pax3* are also downregulated considering their proliferative roles in the development and would otherwise delay myelination (14,29-31).

The neuregulin signaling (NRG1-ERBB2/3) axis is at the axonal and Schwann cell interface and is a central pathway with numerous downstream pathways like ERK and mTOR (32,33). The signaling pathways lead to the survival, migration, proliferation and differentiation of Schwann cells. Membrane bound Type III NRG1 on the axon activates the ERBB2/3 receptors and promotes the differentiation of Schwann cell progenitors into mature developmental stages. Specifically, SHP2 is downstream of the ERBB2 receptor and regulates the ERK pathway (34). The magnitude of ERK activation affects the myelin thickness. In addition, mTOR is regulated by the PI3K/AKT pathway and in turn is controlled by ERBB2 (32). NRG1 has multiple isoforms or ligands secreted by axons that have differing effects on ERBB2/3 signaling. Mice deficient in NRG1 Type III display hypomyelination or even the outright failure of myelination, and the thickness of myelin is also dependent on the concentration of NRG1 Type III signaling (35). The secreted form of neuregulin (NRG1 Type I) does not normally regulate the thickness of myelin but becomes active after nerve injury (36). In general, NRG signaling regulates most of Schwann cell development and is a positive regulator.

At the maturation when the axons are fully wrapped, Schwann cell program undergoes the change in transcriptome stabilizing the myelin production turnover and entering a less metabolically active and more homeostatic maintenance phase. At this point, some positive and negative regulators are no longer needed. Surprisingly, NRG signaling is not required for myelin maintenance. Co-activators YAP and TAZ are also not required (16). GPR126 regulates the cAMP levels in Schwann cells and is required for the radial sorting during the development, but it turns out to be dispensable for myelin maintenance (37). However, SOX10 and EGR2 are still required

in Schwann cells as their ongoing expression help maintain the myelin gene expression required for Schwann cells (38).

Signaling pathways in nerve injury

Beyond promoting the structural insulation through myelination, Schwann cells and non-myelinating Remak bundles also have an ability to promote axon regeneration when they are damaged (4). Nerve injury is normally modeled in rodents by sciatic nerve crush or transection, which causes degeneration of axons distal to the site of injury. Prior to the regeneration in peripheral nerves, various components of the repair program involve the elaborate coordination of many events including macrophage infiltration, and myelin clearance, which all then lead to guided axon innervation and remyelination (4). Within a few days after injury, Wallerian degeneration of axons and the lack of axonal contact triggers reprogramming of Schwann cells into repair cells that begin to secrete cytokines (39). The cytokines simulate the recruitment of macrophages and additional immune cells to perform phagocytosis of axonal/myelin debris (40). In addition, Schwann cells also have a significant role in myelin clearance by autophagy (also called myelinophagy) as well as phagocytosis mechanisms (41,42).

Schwann cells distal to the injury are transformed into the elongated shape called Bungner bands that provide a track for axonal guidance as a part of major events leading to axon regeneration and remyelination (43). Additionally, other Schwann cells are both proliferative and motile, involved in the axonal guidance. While the regenerative capacity of the PNS is considerably greater than the CNS, axon regeneration is often slow and incomplete in humans (44). Remyelination is the final phase of the regeneration program when the pro-myelinating transcriptional factors are reactivated to promote lipid biosynthesis and myelin formation. The internodes in remyelinated nerve are usually thinner and shorter compared to the original state,

likely due to inefficiency of growth factors and molecular signaling in complex microenvironments (45).

Reprogramming of injured Schwann cells to the repair state requires substantial changes in transcriptome and signaling pathways*: c-Jun, Stat3* and mTOR pathways are some of the major examples (46-48). The molecular mechanisms do not necessarily mirror those in the developmental program, in fact many of them are not highly expressed in healthy nerves. For instance, JUN, which combines with *Fos* to form the AP1 heterodimer, is not required for myelination but is required for the full repair program (46). In fact, the absence of *c-Jun* resulted in the outright failure of any functional recovery and remyelination due to lack of axonal reinnervation and slowed myelin clearance. JUN is one of the earliest transcription factors activated in Schwann cells after injury and binds to regulatory elements of many nerve injury genes required for nerve regeneration. In addition, *c-Jun* is a negative regulator of *Egr2* and myelinating genes, which likely contributes to downregulation of the myelin program in Schwann cells of injured nerve. Several signaling pathways such as OGT1 and mTORC have been proposed to regulate *c-Jun* expression (48,49). As a complement to loss-of-function studies, overexpression of *c-Jun* in transgenic studies is sufficient to activate a significant portion of the injury program and causes demyelination (43). Other transcription factors have not yet been studied to the same degree, but inactivation of STAT3 in Schwann cells after nerve injury results in decreased autocrine Schwann cell survival signaling and a significant loss of Schwann cells from chronically denervated stumps. (47).

Mechanisms of Wallerian degeneration of axons have come to light through study of a spontaneous mouse mutant (Wlds), which showed prolonged survival of axons after nerve injury and delayed regeneration (50). Further elucidation of this pathway showed that axon degeneration

is dependent upon a pathway involving *Sarm1*, which is the receptor that triggers the Wallerian degeneration and eventual axonal fragmentation through the conserved NAD pathway (51). SARM1 hydrolyzes NAD leading to accumulation of ADPR and cADPR, activating the calpain, calcium-regulated cysteine proteases, which in turn cleaves neurofilaments and microtubules. As a result, the Schwann cell responses to axonal injury including myelin clearance and regeneration are delayed in myelinated axons of a Wlds or SARM1 mutant.

During Wallerian degeneration, numerous cytokines are secreted by Schwann cells to summon the waves of immune cells (macrophages and neutrophils) in assisting the clearance of axonal fragments and myelin debris. CCL2/MCP-1 is among the most prominent secreted molecules (40,52). Protein levels of cytokines such as IL-1β and TNFα, peak within 1 day of injury, and macrophages infiltrate injured nerve within 3 days (40). In addition, Schwann cells also contribute to the myelin clearance breaking down their own myelin by myelinophagy. Roughly half of total myelin clearance is due to Schwann cells and the rest is attributed to macrophage and other phagocytic cells (53).

Several genes produced in Schwann cells after injury play important roles in nerve regeneration. For instance, growth derived neurotrophic factor (GDNF) is a growth factor that supports axonal survival/regeneration and boosts the functional recovery in mouse models (54). GDNF protein levels peak at about 1 day after injury. SHH protein is a hedgehog agonist that is neuroprotective and it also peaks at 1 day and it was also found to help regulate the level of the pro-regenerative *c-Jun* transcription factor in Schwann cell after nerve injury (55,56). Recent single-cell sequencing studies suggests that the coordinated action of different cell types is needed to promote the overall regeneration program, although Schwann cells play a central role in nerve regeneration (57).

Axonal guidance is another transcriptionally complex element of the regeneration program where denervated Schwann cells undergo radically phenotypic changes into Bungner bands that secrete the signaling substrates needed to mobilize the injured nerves (43). For instance, *Gdnf*, *Vegf*, and *Fgf5* are also critical to promoting the survival of axonal elongation (58,59). Depending on the severity of injury, axons do not always reach their destinations for reinnervation due to insufficient production of growth factors resulting in the failure of regeneration. Furthermore, axon pathfinding during the regeneration is controlled by families of axon guidance molecules including Ephrins and Semaphorins (60,61). Significant amounts of components in extracellular matrix including collagen genes such as *Col4a5* also are involved in supporting regrowing axons (62).

Transcriptional regulation of remyelination involves the transcriptional factors like *Sox10*, *Oct6*, *Egr2*, which are reactivated to promote the lipid biosynthesis. However, this transition is not necessarily the mirror of developmental program. NRG1 and ERBB2/3 pathway likely are also the center of signaling pathways that integrate and coordinate a number of nerve injury responses (63). Studies showed that artificially boosting *Nrg1* expression in mouse models can result in increased remyelination and other elements of regeneration (36). Lastly, some other of the overarching signaling pathways during the timeline are ERK1/2 and the mTORC1 pathways. A report shows the mTORC1 pathway is transiently reactivated in reprogramming of SCs (48,64). By genetic deletion of the mTORC1-subunit RAPTOR in mouse SCs, the authors found that *c-Jun* failed to upregulate and that mTORC1 reactivation is required for timely myelin clearance and remyelination.

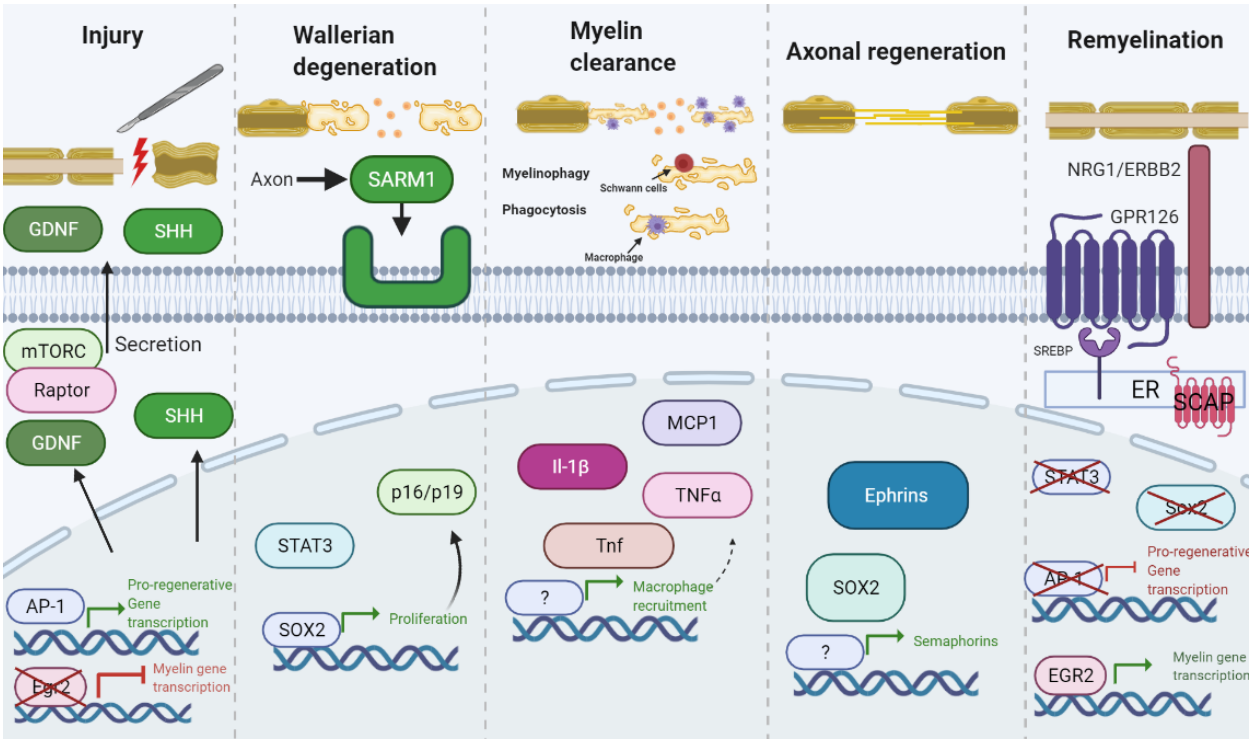

Figure 2. The timeline of prominent pathways involved in Schwann cell plasticity, Wallerian degeneration, axonal regeneration, and remyelination. Schwann cells secrete important growth factors to maintain their survival and reprogram into repair cells when the peripheral nerve injury occurs. The repair Schwann cells supporting regeneration are derived from reprogramming of mature Schwann cells rather than relying on a stem cell niche. The debris and fragments surrounding the injury site will be cleared out to support axon growth. Remyelination is typically thinner in myelin thickness compared to the original state.

Epigenetics in Nerve Injury Response

A number of epigenomic pathways play a role in the transition of Schwann cells to their unique state after nerve injury. For example, many injury-activated genes are associated with repressive histone H3K27 trimethylation (H3K27me3) in mature nerve prior to injury (65). This histone modification is formed by Polycomb Repressive Complex 2 (PRC2), which represses transcription through recruitment of other repressive chromatin complexes. Canonical PRC2 is composed of core subunits including EZH2, a methyltransferase responsible for di/tri-methylation of H3K27, EED, and SUZ12 (66). Additional PRC2-associated proteins, such as AEBP2 and JARID, influence how the H3K27me3 is deposited (67). In our laboratory, a Schwann cell-specific knockout of EED was dispensable for myelin formation, but analysis of nerve injury in the EED knockout indicated that removal of H3K27 methylation is a rate-limiting step in reprogramming Schwann cells after nerve injury (65).

The Schwann cell-specific knockout of EED showed relatively normal myelin structure, although there was some degree of hypermyelination and Remak bundle disruption at 7 months of age. RNA-seq analysis revealed that key nerve injury genes *Gdnf*, *Shh* and brain derived neurotrophic factor (*Bdnf*) are repressed by PRC2 and are associated with repressive H3K27me3 (68). A broader analysis showed premature activation of a subset of the injury program in the *Eed* KO, and ChIP analysis showed that the activation of these injury genes was associated with decreased H3K27me3. Moreover, loss of *Eed* accelerates and amplifies the induction of several nerve injury genes including a soluble form of neuregulin (NRG1 Type I) that is secreted by Schwann cells after injury (65,69). The Schwann cell-specific *Eed* knockout rodent model also showed reduced proliferation after injury indicating that H3K27me3 is an important regulator of the SC injury program (69). The altered proliferation was associated with elevated expression of the p16 and p19 transcripts of the *Cdkn2a* gene, and it was previously proposed that demethylation

of the *Cdkn2a* gene eventually curtails Schwann cell proliferation after injury (70). Therefore, it was hypothesized that H3K27me3 demethylation controls some aspects of the nerve injury response, which we have tested in Chapter 2

The role of the PRC2 complex in polycomb repression is intimately involved with the PRC1 complex. Generally, canonical PRC1 is an epigenetic reader for H3K27me3 through Cbx2/4/6/7/8 and catalyzes an additional monoubiquitination of histone H2A on K119 (H2AK119ub1) through Ring1A/B E3 ligase that represses transcription (71). Canonical and noncanonical (or variant) PRC1 complexes vary in subunit composition, depending on the presence of RYBP, PCGF and CBX isoforms, that differ in targeting mechanisms (72). For example, BMI/PCGF4 is a major subunit of canonical PRC1.4, but there are five other PCGF family members. PCGF2/4 are part of canonical PRC1 complexes, while all six PCGF proteins are constituents of non-canonical PRC1 complexes that lack CBX subunits (73). RYBP is also associated with non-canonical PRC1 complexes and help target H2A ubiquitination to gene loci in the absence of H3K27me3 (74,75). Noncanonical forms of PRC1 have higher activity and are responsible for the majority of H2A ubiquitination, although CBX-containing canonical PRC1 is nonetheless involved in targeting H2A ubiquitination to pre-existing sites of H3K27me3.

While PRC1 and PRC2 complexes and their histone modifications are largely colocalized, they can also be regulated independently (75-77). Genes may be transcriptionally silenced by H2AK119ub1 alone, often deposited by non-canonical PRC1 which can be targeted by associated DNA-binding proteins, or through interactions of subunits such as RYBP (73,74). H2AK119ub1 can also stimulation of H3K27 methylation through binding of the JARID2 subunit of PRC2.2 (78). Therefore, polycomb repression could be initiated by either PRC2-mediated H3K27 trimethylation or PRC1-mediated H2AK119 ubiquitination. Accordingly, we found that many

injury genes are also associated with repressive ubiquitination (H2AK119ub1) in mature nerve prior to injury (79). ChIP profiling revealed significant changes in levels of H3K27me3 and H2AK119ub1 modifications on several genes that become activated upon nerve injury, such as *Shh* and *Gdnf* (65,79).

Moreover, because levels of H3K27me3 and H2AK119ub1 decreased within 1 day of the transection of sciatic nerve, this implied that reversal of polycomb repression could function as the master switch for induction of the nerve injury genes in Schwann cells through the active removal of such histone marks. In this dissertation, we have proposed that H3K27 demethylases and/or H2A deubiquitinases would be required for injury gene activation. Specifically, H3K27 demethylases are the erasers of PRC2-dependent histone mark (80). UTX and JMJD3 are H3K27 demethylases (KDM6A and KDM6B) that belong to the Jumonji family containing the catalytic JMJC protein demethylase domain (81). In a pilot study, we found that GSKJ4, an inhibitor of JMJD3/UTX, was able to delay the induction of key nerve injury genes in nerve explants (65), and it was also reported that JMJD3 protein becomes induced in Schwann cells after nerve injury. Therefore, we hypothesized that the development of conditional knockout *Jmjd3/Utx* would block the induction of nerve injury genes, and the results are described in Chapter 2.

Since H2AK119ub1 is also reduced at one day after injury (79), a parallel hypothesis is that removal of H2A ubiquitination after nerve injury is necessary for reprogramming of Schwann cells to enact the nerve injury response. It is possible that both H3K27 demethylation and H2A deubiquitination are required to overcome polycomb repression in activation of injury genes, or alternatively, that H2A deubiquitination is the primary trigger. To determine the mechanism responsible for loss of H2AK119, we decided to investigate the role of BAP1, a known deubiquitinase (DUB) for H2AK119, and characterize its role in Schwann cells. BAP1 is a subunit

of the Polycomb repressive deubiquitinase complex (PR-DUB), and characterization of the CALYPSO ortholog of BAP1 in *Drosophila* showed its association with Polycomb repression (82).

Recent studies have highlighted some paradoxical results in terms of BAP1 regulation of polycomb repression (83,84). *Bap1* deletion in hematopoietic cells resulted in increased Polycomb repression and levels of H3K27me3and a myeloproliferative phenotype. However, when bred with EZH2 knockout, some of the phenotypes observed in the mouse model with double mutations are partially rescued including the restoration of normal proliferation. These results are consistent with a model in which increased H2AK119ub1 may recruit the PRC2 through JARID2, promoting increased Polycomb repression, since the loss of *Bap1* would have promoted increased H2AK119ub1. However, it was also reported that *Bap1* loss led to increased expression of PRC2 subunits. Another study employing *Bap1* deletion in the human HAP1 cell line showed similar increase of H3K27me3 but also the increased level of H2AK119ub1 accompanied by principally gene downregulation (85). Genes affected by *Bap1* deletion had only limited overlap with Polycomb-mediated silencing by the comparative analyses with the transcriptome datasets from of the RING1B and EZH2 knockouts, indicating that *Bap1* may target significantly different sets of genes independent from Polycomb regulation. Therefore, the main hypothesis guiding studies in chapter 3 is that the loss of BAP1 in Schwann cells could result in stronger Polycomb repression, and deficient induction of nerve injury genes.

In contrast, other studies have found that the function of BAP1 protein is to safeguard polycomb-repressed genes by limiting the spread of both H2AK119ub1 and H3K27me3 (86,87). Otherwise, the loss of BAP1's active removal function would result in weakened Polycomb repression due to titration of the readers of H2AK119ub1 and H3K27me3 readers that silence

transcription (86,88). While this idea is somewhat counter-intuitive, it is clear that H2AK119ub1 is not only concentrated on promoters but is also more diffusely localized across the genome at lower levels. Therefore, BAP1 could safeguard transcriptionally silent genes by delimiting H2AK119 modifications and preventing inappropriate spread of JARID2-dependent H3K27 methylation (86,87). Accordingly, the *Drosophila* homolog of BAP1, CALYPSO, was originally classified as a polycomb group gene (82).

In addition to BAP1, other subunits of the mammalian PR-DUB complex are O-GlcNac Transferase (OGT), FOXK1 and HCF1 in addition to previously mentioned ASXL1/2. OGT has known functions in both Schwann cell development and nerve injury response (49,89). OGT catalyzes the addition of a single N-acetylglucosamine through an O-glycosidic linkage to serine or threonine and an S-glycosidic linkage to cysteine residues. A Schwann cell-specific OGT-KO led to the elevated JUN levels, and premature activation of nerve injury genes, causing a significant degree of tomacula and thinner myelination of larger caliber axons and eventual axonal degeneration. In addition, remyelination was delayed after injury, but remyelination could be rescued by deleted one allele of *c-Jun*, indicating significant roles in development and nerve injury response (49). Our analysis in chapter 3 allows us to compare the Schwann cell-specific knockouts of two components of the PR-DUB complex, and the results indicate overlapping yet distinct roles of both components in actively repressing the nerve injury program in Schwann cells.

Overall, it is important to note that there have been only our own investigations of PRC2 function in Schwann cells, and no other publications of PRC1 function in this cell types have emerged. Investigating the role of polycomb regulators in the postnatal development of Schwann cells provides an opportunity to understand how polycomb regulation affects myelin homeostasis, as well as Schwann cell responses to nerve injury, which are a unique system in which terminally

differentiated cells can be reprogrammed to a pro-regenerative phenotype. We have deliberately chosen not to focus on the genesis of polycomb repression in Schwann cell development, which might require manipulation at the neural crest stage, but rather on how polycomb repression affects myelination and the epigenomic reprogramming associated with nerve injury in Schwann cells.

Thesis Plan

The JMJD3, UTX, and BAP1 proteins play important roles in Polycomb regulation and are relatively unknown in Schwann cells throughout development and nerve injury response. Many nerve injury genes are Polycomb-repressed and active removal of such histone marks might be required to initiate the regeneration program. My graduate work has focused on characterizing the Polycomb regulation in Schwann cells by developing the knockouts of *Jmjd3/Utx* demethylases and *Bap1* deubiquitinase in vivo.

In Chapter 2, we tested the hypothesis that *Jmjd3/Utx* are required to derepress the nerve injury genes in Schwann cells by using CRE Lox to delete the domain in Schwann-cell specific mouse model. We observed that *Jmjd3/Utx* are dispensable for both Schwann cell development and nerve injury response, though *Jmjd3* knockout resulted in a minor delay in the induction of nerve injury genes first day after injury. The inductions eventually recover to the level similar to control. These findings allow us to explore the alternative models of how the Polycomb regulation occurs in Schwann cells during the development and nerve injury response.

In Chapter 3, we also tested the hypothesis that *Bap1* is required to derepress the nerve injury genes in Schwann cells by utilizing the similar approach of CRE Lox to delete the domain in Schwann-cell specific mouse model. BAP1 KO mouse model exhibited a number of phenotypes including abnormal myelinations and premature activations of nerve injury genes, though its behavior was largely normal. These findings lead us to investigate the possible downstream

pathways from *Bap1* and their places in the overall Polycomb regulation in Schwann cells during the development.

Altogether, these data have further enhanced our understanding of how Polycomb regulation is achieved in Schwann cells. In Chapter 4, I summarize my findings and discuss possible future directions.

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Chapter 2

H3K27 demethylases are dispensable for activation of Polycomb-regulated injury response genes in peripheral nerve.

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ABSTRACT

The induction of nerve injury response genes in Schwann cells depends upon both transcriptional and epigenomic reprogramming. The nerve injury response program is regulated by the repressive histone mark H3K27 trimethylation (H3K27me3), deposited by Polycomb repressive complex 2 (PRC2). Loss of PRC2 function leads to early and augmented induction of the injury response gene network in peripheral nerves, suggesting H3K27 demethylases are required for derepression of Polycomb-regulated nerve injury genes. To determine the function of H3K27 demethylases in nerve injury, we generated Schwann cell-specific knockouts of H3K27 demethylase Kdm6b and double knockouts of *Kdm6b/Kdm6a* (encoding JMJD3 and UTX). We found that H3K27 demethylases are largely dispensable for Schwann cell development and myelination. In testing the function of H3K27 demethylases after injury, we found early induction of some nerve injury genes was diminished compared to control, but most injury genes were largely unaffected at 1 and 7 days post-injury. Although it was proposed H3K27 demethylases are required to activate expression of the cyclin-dependent kinase inhibitor *Cdkn2a* in response to injury, Schwann cell-specific deletion of H3K27 demethylases affected neither the expression of this gene nor Schwann cell proliferation after nerve injury. To further characterize the regulation of nerve injury response genes, we found injury genes are associated with repressive histone H2AK119 ubiquitination catalyzed by PRC1, which declines after injury. Overall, our results indicate H3K27 demethylation is not required for induction of injury response genes and that other mechanisms likely are involved in activating polycomb-repressed injury genes in peripheral nerve.

INTRODUCTION

Myelination of peripheral nerve axons by Schwann cells provides multiple important functions including trophic support, structural integrity, and enabling saltatory conduction (1,2). These attributes allow robust transmission of action potentials and maintain axon homeostasis. However, the intrinsic plasticity of Schwann cells to promote repair of peripheral nerves involves several regenerative processes in response to Wallerian degeneration of axons, including both macrophage recruitment and clearance of myelin debris (3-5). Schwann cells reprogram themselves to support axon regeneration and undergo a transformation from a highly quiescent state to active repair cells that elongate to form Bungner bands that facilitate axon regeneration (6-8). These elaborate cellular behaviors do not depend on a stem cell niche (9), but rather reflect an innate ability of terminally differentiated Schwann cells to undergo a dramatic transdifferentiation to a more proliferative, pro-regenerative state called the repair Schwann cell (6).

The reprogramming is supported by an array of epigenetic changes converging into the overall response to simulate axon regeneration. After the nerve damage, substantial reshaping of the transcriptome occurs through dramatic changes in transcription factors as well as dynamic changes in acetylation and methylation of histones in Schwann cells (10-14). Among the most important transcription factors essential for nerve repair in Schwann cells is JUN, an AP-1 component that is required and sufficient for activation of many injury genes (8,15). For many JUN target genes and other genes activated by injury, the basal levels of these aforementioned genes are low or absent in mature Schwann cells prior to injury due to repressive H3K27me3 made by the Polycomb repressive complex 2 (PRC2) (8,12-14,16). For instance, glial derived neurotrophic factor (*Gdnf*) is one of the PRC2-regulated genes important for nerve regeneration (17). Sonic hedgehog (*Shh*) is another highly induced gene that promotes regeneration (18). In our

previous studies, we found that derepression of many nerve injury genes is accompanied by H3K27 demethylation after injury (12-14).

Canonical PRC2 is composed of core subunits including the lysine methyltransferases EZH1/2, suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED) (19,20), and there are a number of accessory subunits that play important roles. A Schwann cell-specific *Eed* conditional-knockout mouse model exhibited premature induction of nerve injury genes in uninjured nerves (12). Moreover, the nerve injury experiment showed that *Eed* cKO mice had premature and/or augmented induction of nerve injury genes after injury (13), demonstrating the importance of H3K27me3 in repression of the injury related program. In line with these findings, ChIP studies indicated the promoters and gene bodies of many nerve injury genes in Schwann cells showed loss of H3K27me3 after injury (12), indicating that this histone mark acts as a switch for transcriptional induction.

Given that the Schwann cell responses to nerve injury are accompanied by H3K27me3 reprogramming, this provides a novel system to test the requirements for activation of PRC2 repressed gene networks that support axonal regeneration and proliferation. We hypothesized that active removal of H3K27me3 by H3K27 demethylases JMJD3/KDM6B and UTX/KDM6A, is required for induction of the nerve injury network in Schwann cells after injury. JMJD3 and UTX proteins belong to the Jumonji family containing the catalytic JMJC protein demethylase domain (21). These H3K27 demethylases have been shown to be required for activation of Polycomb-repressed genes in other systems, including neural progenitor cell development and T cell differentiation (22-24). However, other demethylase-independent mechanisms for derepression

have been characterized (25) and PRC2 repression is linked with PRC1 repression, which involves monoubiquitination of H2A (H2AK119ub1) (19). Therefore, we have also more fully characterized the repressed state of nerve injury genes by profiling H2AK119ub1 made by Polycomb repressive complex 1 (PRC1).

Previous studies suggested that JMJD3-mediated demethylation of H3K27 limits Schwann cell proliferation after injury by activation of the *Cdkn2a* gene (26). *Cdkn2a* encodes both INK4A/p16, which is a cyclin-dependent kinase inhibitor and tumor suppressor, and p19/ARF, an important regulator of p53 activation. Consistent with this model, the Schwann cell specific knockout of *Eed* led to persistent *Cdkn2a* expression and a failure of Schwann cells to proliferate after injury (13). Interestingly, studies showed that neurofibromas caused by *NF1* mutation most often transition to malignant peripheral nerve sheath tumor through the co-mutation of PRC2 subunit genes and *CDKN2A (27-29)*. Therefore, we sought to test the hypothesis that *Jmjd3/Kdm6b* and *Utx/Kdm6a* are required to activate *Cdkn2a* (p16 and p19) and other injury genes after injury.

MATERIALS AND METHODS

Mouse Nerve Injury Surgery. Animal experiments were performed according to protocols approved by the University of Wisconsin, Madison School of Veterinary Medicine. *Kdm6b/Jmjd3*-floxed mice (31) and *Kdm6a/Utx* floxed mice [Jax#024177] (35) were maintained on the C57BL/6 genetic background (24) and mated to mP0TOTA-Cre (*Mpz*-cre) (30). Double *Jmjd3/Utx* floxed mice were generated and homozygous floxed *Jmjd3* and *Jmjd3/Utx* alleles with and without the Mpz-cre transgene were used for experiments. Prior to surgery, animals were anesthetized with isoflurane (Piramal Healthcare), and an injection of 5 mg/kg ketoprofen was given for analgesia. A 5 mm long incision was made through the skin and muscle exposing the sciatic nerve. The nerve was either cut as close to the proximal lateral region of the femur as possible or crushed 1 minute using fine forceps. As a control, the contralateral leg also received a sham operation consisting of only a skin and muscle incision. The skin wound was sutured with rodent surgical staples. Six wildtype and knock-out nerve tissues distal to the transection or sham site were isolated with epineurium removed and frozen immediately in dry ice and stored at -80º for further processing in ChIP experiments.

Chromatin Immunoprecipitation. Six freshly dissected mouse sciatic nerves per condition were minced in 1% formaldehyde for 8 min and then quenched for 10 min with glycine to a final concentration of 0.125 M. Samples were sequentially lysed in buffers LB1, LB2, and LB3 + 0.03% SDS (11). DNA was fragmented to an average size of 0.5–2 kb using 5× for 10 min Bioruptor (Diagenode) cycles on the medium setting. Each aliquot of sonicated chromatin (150 µg) was incubated overnight at 4 °C with 5 µg of antibody. A 10% aliquot was saved for input analysis. An 80-µl aliquot of protein G Dynabead (Invitrogen) slurry was added to each ChIP sample, rotating overnight at 4 °C. Immunoprecipitations were washed three times in RIPA buffer and then eluted

at 65 °C in reverse cross-linking buffer (50 mM Tris, 10 mM EDTA, 1% SDS). ChIP DNA was purified by phenol chloroform extraction and resuspended in 10 mM Tris, pH 8.0. Antibodies used in the study are normal rabbit IgG (Millipore, 12-370) and H3K27me3 (Active Motif, 39155). Statistics were calculated using Student's t test. Error bars represent standard deviation, and asterisks denote p value (*, $p \leq 0.05$; **, $p \leq 0.005$). The samples were generated from independent chromatin pools (n = 3 for H3K27me3 ChIP) and were analyzed using quantitative PCR primers listed in (Table 1).

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 4º. The nerves were then postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature. Following OsO4 postfixation, the nerves were dehydrated in a graded ethanol series, and then further dehydrated in propylene oxide and embedded in Epon 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.

Immunofluorescence. Freshly dissected nerves were embedded in Tissue-Tek OCT compound (Sakura Finetek) and snap frozen with liquid nitrogen. Longitudinal or transverse cryostat sections (10 μm) were air-dried for 5 min and fixed in 4% paraformaldehyde for 10 min. The sections were then blocked in PBS containing 5% donkey serum/1% BSA/0.5% Triton-X 100 for 1 hr 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 hr. Hoechst 33342 (1:5,000 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 Nikon A1R confocal and quantitated by both Columbus imaging software and manual curation.

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 Na3VO4 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 polyvinylidene fluoride membrane, proteins were blocked in TBST containing 5% nonfat dry milk for 1 hr at room temperature. Primary and Secondary antibody incubations were performed in TBST containing 5% nonfat dried milk at 4 C for overnight and at room temperature for 1 hr, 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.

Quantitative RT-PCR. RNA was isolated from sciatic nerves using the Trizol/chloroform RNA extraction protocol. To prepare cDNA, 250 ng or 1 μg of total RNA of mouse, respectively, was used from each sample. qRT-PCR and data analysis were performed as described previously. qPCR was performed with two replicates per sample, and statistical analyses were evaluated by one-way ANOVA.

RNA seq. 500-1000 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. Illumina sequencing data were mapped to the GRCm38/mm10 genome. Data were analyzed using DESeq2 (61) to determine differentially regulated genes between uninjured and injured nerves in wild type and double cKO mice (p-value < .05).

ChIP-seq. Sham and injured sciatic nerves of 2 adult male Sprague–Dawley rats were used in ChIP-seq analysis after micrococcal nuclease digestion of peripheral nerve chromatin as described (13) using an antibody for H2AK119ub1. Library preparation and sequencing was performed by the UW Biotechnology Center as described previously (11). 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. Hypergeometric optimization of motif enrichment (HOMER, RRID:SCR_010881) (62) was used to determine enriched binding regions for H2AK119ub1 relative to sequencing of an input chromatin sample.

RESULTS

Conditional inactivation of *Jmjd3* and *Utx* in Schwann cells

To test the involvement of H3K27 demethylases in activation of the Schwann cell injury program, we made a conditional deletion of *Jmjd3/Kd*m6b specifically in Schwann cells using the *Mpz*-cre driver (30). The conditional allele (*Jmjd3* f/f) has exons 14-20 of the *Jmjd3* gene flanked by loxP sites (31) (Figure 1A). This would result in deletion of the catalytic Jumonji domain, at E13.5-14.5 stage in Schwann cells, causing a frameshift in the C terminus of the protein. We validated the knockout of *Jmjd3* gene using quantitative RT-PCR with primers located within the deleted exons. The results showed ~76% loss in *Jmjd3* expression in mutant intact nerves compared to control intact nerves (Figure 1C), which corresponds to the proportion of Schwann cells in peripheral nerve (12).

In order to test for potential redundancy in H3K27 demethylation, we also knocked out the other major H3K27 demethylase, *Utx/Kdm6a*, which is also expressed in RNA-seq profiles of peripheral nerve (13,32,33). *Utx* is an X-linked gene composed of 29 exons (34), and the conditional allele contains loxP sites surrounding exon 24 coding for the catalytic domain of *Utx* (35) (Figure 1B). After breeding this allele to the Mpz-cre driver line, we found a similar loss of ~70% of *Utx* expression in peripheral nerve from single and double knockouts (Figure 1C). As seen in previous studies using Mpz-cre (14), the residual *Jmjd3* and *Utx* expression is likely due to the presence of non-Schwann cell types in peripheral nerve.

Developmental effects of inactivating H3K27 demethylases

Before testing the involvement of *Jmjd3* and *Utx* in nerve injury, we evaluated whether deletion of *Jmjd3* and *Utx* affect Schwann cell development. In our previous analysis of the *Eed*

knockout, peripheral nerve development was relatively normal up to 2 months of age, with development of hypermyelination and Remak bundle fragmentation at later time points (14). However, in the *Jmjd3/Utx* conditional knockouts, there are no obvious behavioral phenotypes typically found in peripheral neuropathy such as hindlimb clasping even up to 7 months of age, and visual inspection showed the normal opaque appearance of myelinated nerve. Despite the apparent lack of such behavioral phenotype, we examined nerve ultrastructure by performing electron microscopy analysis of sciatic nerve in the Schwann cell-specific double knockout of *Jmjd3* and *Utx*.

The electron microscopy images of mature myelin in sciatic nerves of ~2 months old mutant mice showed a normal distribution of axon diameters and myelin sheaths compared to controls (Figure 2A). Myelin thickness was measured using the g-ratio, which is axon diameter divided by outer diameter of myelin sheath, which is plotted against the axon diameter. The linear regressions of both distribution plots for both control and mutant mice are virtually aligned, indicating no significant difference in myelin thickness compared to control (Figure 2B). The number of myelinated fibers is also comparable in both genotypes.

Our previous analysis of the *Eed* knockout identified progressive defects in hypermyelination and Remak bundle integrity at later time points (14). Therefore, we also harvested the sciatic nerves of 7 month old double knockout mice and processed for electron microscopy imaging. The morphology is similar between mutant and control genotype as no deformities were observed in the mutant nerve (Figure 2A). Abnormalities like myelin outfolding, and development of tomacula caused by excess myelin membranes were observed in the *Eed*

knockout (14). However, no myelin pathology is seen in Schwann cell-specific knockout of the two H3K27 demethylases. While we had observed some difference in Remak bundles in *Eed* cKO mice (14), they appear to be intact in mutants, similar to those of controls and no major deformity is consistently observed in the double knockout nerves. Therefore, H3K27 demethylase activity is not required for myelin development or maintenance.

Control of proliferation after injury

It had been reported that JMJD3 is induced after injury (26), which was proposed to regulate Schwann cell proliferation after injury by removing H3K27 methylation from the *Cdkn2a* gene that encodes both p16 and p19 tumor suppressor proteins. However, examination of nerve injury RNA-seq data sets did not show that *Jmjd3* is induced at the transcript level (13,33). While there could be post-transcriptional induction of JMJD3, we found that JMJD3 protein is expressed even before injury in Schwann cells in contrast to the earlier findings (Figure 3A). We tested the specificity of our antibody using siRNA for *Jmjd3* in the cultured S16 cells (Supplementary Figure 1). In addition, the protein level of JMJD3 did not appear to significantly increase after nerve injury, as measured by the immunofluorescence and western blot. Nonetheless, *Jmjd3* could regulate nerve injury gene induction at 1 day post injury (dpi) even in the absence of induced protein levels.

The normal induction of *Cdkn2a* (p16 and p19 transcripts) occurs at 3-7 days after injury, and earlier data suggested that JMJD3 would be required for this induction (26). In partial support of this model, we had previously investigated the effect of H3K27me3 depletion in Schwann cell specific *Eed*-cKO mice and found constitutively high *Cdkn2a* transcript expression (p19 and p16) along with reduced proliferation of Schwann cells after injury (13). Therefore, this model would

predict that loss of H3K27 demethylases in Schwann cells would result in increased proliferation due to the inability to derepress *Cdkn2a* after injury. To test if *Jmjd3* is required for *Cdkn2a* induction and regulation of cell proliferation after peripheral nerve injury, we performed immunofluorescence for p19 and Ki67 at 4 days after nerve crush. We quantitated the number of cells positive for p19 and Ki67 along with Schwann cell marker SOX10. In the *Jmjd3/Utx* double knockout we found that there was no difference from control at 4 dpi timepoint for both p19 induction and proliferation (Figure 3B,C). Since proliferation normally subsides by 14 days after injury, it is possible that the proliferation would be maintained longer in the absence of *Jmjd3* and *Cdkn2a* induction. However, the level of proliferation at longer timepoints after injury, at both 7dpi and 14dpi, was not significantly different from control (Figure 3D,E,F and Supplementary Figure 2). In addition, we also determined the transcript levels of p16 and p19 transcripts of *Cdkn2a* using qRT-PCR at 3/4 dpi and 7 dpi. We found no significant difference between genotypes (Figure 3G). Therefore, neither *Jmjd3* nor *Utx* is required for *Cdkn2a* induction, and their deletion has no major effects on proliferation after injury.

H3K27 demethylases are not required for macrophage infiltration after nerve injury

Another major component of the nerve injury response involves Schwann cell production of chemokines (e.g. *Mcp1*/*Ccl2*) that recruit macrophages that clear out the myelin debris that can inhibit axonal regeneration (36-39). To test whether the demethylase knockout may have effects on the activities of immune cells, we examined the CD68 macrophage marker by immunofluorescence to assess macrophage infiltration and observed no significant difference between genotypes at 4 and 14 dpi (Figure 4A,B), indicating that demethylases are not required for macrophage recruitment by Schwann cells in injured nerve.

Regulation of nerve injury genes by H3K27 demethylases.

We had found that a significant subset of nerve injury genes is regulated by PRC2, including *Shh* (Figure 5A), *Gdnf*, and *Runx2* (12-14). Several of these genes are rapidly induced after injury within 24 hours whereas the levels of other injury-induced transcripts become induced at later timepoints (3-7 days) (13). Therefore, we hypothesized that the *Jmjd3* and *Utx* knockouts would block the transcriptional induction of many of these genes after nerve injury. We utilized quantitative RT-PCR analysis of sciatic nerve RNA in sham and injured nerve from both the single knockout of *Jmjd3* and double knockout of *Jmjd3/Utx* mouse lines to measure the injury induction of selected PRC2-repressed genes. While there are other cell types in nerve, the downstream response of nerve injury genes like *Shh* and *Gdnf* is specific to Schwann cells (8,33). In the Jmjd3/Utx conditional knockout, qRT-PCR experiments with 1 dpi and 4 dpi RNA samples of DKO nerve were conducted (Fig. 5B and 5C). We observed that the induction of *Shh, Gdnf,* and *Fgf5* was modestly impacted in double knockouts at 1 dpi compared to control. However, the induction of these genes recovered to normal injury-induced levels at 4 dpi.

We observed no significant changes in the induction of several nerve injury genes such as *Shh*, *Gdnf*, *Fgf5*, *Runx2*, and *Hmga2* in the *Jmjd3* knockout at 1 dpi (Supplementary Figure 3A). In contrast, the induction of *Fgf5* and *Hmga2* was slightly reduced at 3dpi (Supplementary Figure 3B). At 7dpi in *Jmjd3* cKO, all of the genes with the exception of *Fgf5* recovered to the levels observed in the wild type mice (Supplementary Figure 3C).

We assessed H3K27 demethylase dependence of global gene expression by performing RNA-seq at 1 and 7 days post nerve transection. We identified differentially expressed genes by

comparing two datasets (1 dpi DKO vs 1dpi control, 7 dpi DKO vs 7dpi control). After injury, there were only a few genes that were significantly lower in the DKO (<0.05 p-value, FDR corrected). Several studies have characterized the nerve injury program in Schwann cells using RNA-seq (13,32,33), and ChIP-seq analysis identified 4091 genes associated with H3K27me3 in intact nerves (12-14). Using these data sets, we had identified injury-induced genes that are associated with H3K27me3 resulting in 902 genes and refined them using RNA-seq analysis of sorted Schwann cells after nerve injury (33) to ensure that their induction occurs in Schwann cells. Of these, there were 343 injury-induced genes that were found to be regulated by PRC2, since they were induced in the *Eed* cKO before and/or after injury (Figure 5D).

At 1 dpi, there are only 6 genes that are significantly lower in the double knockout, and three of them are previously defined as PRC2-regulated injury genes (*Fgf5*, *Sfrp1*, *Erbb4*). *Fgf5* is previously studied as an important growth factor for Schwann cell migration and promote tissue regeneration (36). *Erbb4* belongs to a family of important ErbB receptor family that interacts with neuregulin-1. Similarly, at 7 dpi, there are only four genes that are significantly lower. One of them had also been defined as PRC2-regulated injury gene: *Igfbp5, which* is a modulator of IGF signaling and is previously characterized to have a role in peripheral axon regeneration (40,41), and two others (*Pappa* and *Sez6*) are injury-induced genes associated with H3K27me3. Therefore, the demethylase activity of JMJD3 and UTX appears to be largely dispensable for the induction of the injury program except for a few relevant injury genes (*Fgf5*, *Sfrp1*, *Erbb4*).

Although relatively few injury genes are affected by loss of H3K27 demethylase activity, we identified 146 genes that are significantly altered in the DKO prior to injury (sham). Out of the

54 genes that are downregulated, 23 genes are associated with H3K27me3. This analysis highlighted a potential discrepancy with the injury timepoints, where much fewer genes were lower in the DKO. However, it turns out that 31 of these 54 genes are regulated by injury, and most are significantly downregulated after injury, which explains why most of these are not differentially regulated in the DKO after injury. Since they are downregulated after injury, they are associated with the myelination program in Schwann cells, and their decrease in the DKO indicates that demethylases are required for their developmental induction in Schwann cells. Many of the genes induced during Schwann cell myelination are dependent upon the EGR2 transcription factor (42,43), and several of the downregulated genes had been identified as EGR2 target genes: (*Frzb, Hcn1 Fgl2, Slc6a15, Spp1*). However, most of the EGR2 regulatory network including major myelin genes was unchanged, consistent with the normal nerve morphology described above.

Overall, both RNA sequencing datasets showed that a majority of PRC2-regulated genes (13) remained unchanged in double cKO nerves under both cut and sham conditions (Supplementary Figure 4A,B). In addition, the lack of any morphological defects and the small number of deregulated genes suggest that demethylases are not essential for Schwann cell development, although their absence leads to a delayed induction of some injury genes.

Histone modification changes in nerve injury genes

In previous studies, we had found that the levels of H3K27me3 at the promoters of nerve injury genes such as *Shh and Gdnf* decreased within 24 hours after peripheral nerve injury. To test that the H3K27me3 mark at such sites is actually being targeted by demethylase, we utilized the Schwann cell specific knockout of *Jmjd3* and performed ChIP in sham and cut nerves with H3K27me3 antibody. In contrast to the previously described reduction of H3K27me3 levels after

injury, the level of H3K27me3 in several injury gene promoters remained elevated after injury in the *Jmjd3* cKO, indicating that the injury-induced reduction of H3K27me3 is *Jmjd3* dependent (Supplementary Figure 5).

While H3K27me3 had been implicated as the bottleneck to the activation of injury gene network in previous reports (12-14,26), there are demethylation-independent mechanisms that could overcome polycomb repression after nerve injury (19,25). It is possible that increased levels of H3K4me3 alone could be sufficient to activate Polycomb-repressed genes. We had previously found that H3K4me3 marks are associated with promoters of *Shh, Gdnf, Hmga2*, and other PRC2-regulated genes (12), which indicates that these promoters correspond to the previously defined bivalent state of promoters associated with both H3K27me3 and H3K4me3 (44,45). Using our H3K4me3 ChIP-seq data, we examined distribution of H3K4me3 marks on H3K27me3-associated injury genes in peripheral nerve in intact and injured conditions. A distribution plot of H3K4me3 on the 343 H3K27me3-associated injury genes shows a narrow peak at the TSS that increases at 1day post-injury compared to that of intact condition (Figure 6A). The increased H3K4me3 is a plausible explanation for some of the most dramatic early changes in injury gene activation, which can occur several days in advance of detectable increases in transcript levels. As controls, we observed no significant changes in the level of H3K4me3 for randomly selected 300 injury genes lacking H3K27me3 and another 300 randomly selected uninduced H3K27me3-associated genes after injury)

Injury-induced depletion of H2A ubiquitination.

Studies of Polycomb repression have elucidated an important role of the PRC1 complex which modifies H2AK119 by ubiquitination. The traditional model has been that Polycomb

repression occurs sequentially where H3K27me3 is first deposited to the site of gene loci and then attracts Polycomb repressive complex 1 that will in turn deposit the H2A119ub1 to further repress the gene activity (19). However, while H3K27 methylation does often overlap with H2AK119ub1, H2A ubiquitination can affect PRC2 recruitment and activity (46), and it has been shown that these modifications can be established and regulated independently of each other (19,47).

This raises the possibility that H2AK119ub1 by PRC1 is involved in the repression of nerve injury genes, and furthermore that deubiquitination of this histone mark is required for their induction (48,49). We therefore performed the nerve injury experiment followed by ChIP using an H2AK119ub1 antibody. We found that many of the previously defined PRC2-repressed injury genes are associated with H2AK119ub1 at the promoter sites and they underwent the losses after injury (Figure 6B,C). Following this, we also performed the ChIP sequencing study for H2AK119ub1 in sham and injured nerve. In sham nerve, we found a large overlap of H2AK119ub1 with the H3K27me3 on injury-induced promoters (Figure 6D). We plotted the average distribution of H2AK119ub1 at the promoters of 343 Polycomb regulated genes and found significant enrichment flanking the transcription start sites. In addition, the peak distribution is lower in injured condition compared to that of intact condition (Supplementary Figure 6). This can be seen for two individual genes (*Gdnf* and *Fgf5*), which show changes in their H2AK119ub1 profiles in sham vs. injured nerve.

Our results suggest a new model in which H2A deubiquitination would play a key role in reversing Polycomb repression rather than H3K27 demethylation. However, our previous observations of injury gene derepression in the *Eed* cKO, would predict that loss of *Eed* may

trigger loss of H2A ubiquitination. We tested that by performing H2Aub1 ChIP assays in the *Eed* cKO, and we did indeed find a significant loss of H2AK119ub1 in uninjured nerve of the *Eed* cKO (Figure 6E). Therefore, our data indicate that both H2A ubiquitination and H3K27 demethylation may be required for proper regulation of injury genes.

DISCUSSION

Many nerve injury genes are repressed by H3K27me3 and their expression remains low or absent in mature peripheral nerve. Previous studies had suggested that demethylation is required for induction of nerve injury and cell cycle genes such as *Shh*, *Gdnf*, and *Cdkn2a*, promoting the regeneration program to proceed in an appropriate and timely manner (26). This idea was supported by previous studies of a Schwann cell-specific deletion of *Eed* (12-14), showing that preventing PRC2 repression can exert significant effects on the injury program. Therefore, we determined whether demethylases are required for gene activation after injury.

In development of Schwann cell-specific knockout of H3K27 demethylases, we first examined if they were required for Schwann cell development before assessing their roles in the nerve injury response. The H3K27 demethylase activity of JMJD3 and UTX are not essential for Schwann cell development and myelination. No abnormal developmental phenotype is seen at the maintenance stage of Schwann cells and the timepoint beyond 2 months of age in the mouse models. Therefore, we could focus on the early gene induction events in the aftermath of injury. Our primary hypothesis was that PRC2-regulated genes required H3K27 demethylases to be induced in the early phase of regeneration, many of which are induced 24 h after injury. Any epigenetic changes required for induction should occur at this early timepoint, and we had previously observed decreased H3K27 methylation at this time (12-14). However, for most injury genes, the demethylase activity of JMJD3 and UTX are not required for their induction with the exception of a few genes like Fgf5. Some injury genes were still lower at 4 dpi but RNA-seq from DKO 7 dpi nerves showed that there were only four significantly altered genes across the injury-induced transcriptome. Given the relatively subtle effects of nerve injury gene induction and their

recovery by 7 d, it is not likely that extended analysis of nerve regeneration will reveal a phenotype following the nerve injury in the DKO.

It had been reported that JMJD3 is induced in mouse model after 5 days post nerve transection by immunofluorescence and had proposed that JMJD3 has a significant role in Schwann cell proliferation after injury through demethylation of the *Cdkn2a* gene (26). Our earlier studies had confirmed the presence of H3K27me3 on the *Cdkn2a* promoters, and we also had observed overexpression of both p19 and p16 transcripts and reduced proliferation after injury with a Schwann cell specific knockout of *Eed* (13). The regulation of *Cdkn2a* gene by PRC2 has relevance to the Schwann cell-derived tumors in Neurofibromatosis caused by mutation of the *NF1* tumor suppressor gene (50), The progression from neurofibromas in *NF1* to the more malignant form called malignant peripheral nerve sheath tumor (MPNST) is often accompanied by co-mutation of the *CDKN2A* gene and genes encoding subunits of the PRC2 complex (e.g. *EED* and *SUZ12*) (27-29). Indeed, *NF1* microdeletions are more predisposed to MPNST due to the deletion of both *NF1* and the neighboring *SUZ12* gene (51).

We tested the proliferation in double knockout of demethylases expecting there would be increased and/or prolonged Schwann cell proliferation after injury if H3K27 demethylases were required for induction of p19 and p16. However, despite the evidence for PRC2 regulation of *Cdkn2a* and Schwann cell proliferation after injury, our double knockout results showed that H3K27 demethylases are not required for *Cdkn2a* induction nor regulation of Schwann cell proliferation after injury. There is no significant difference between genotype in terms of proliferation at 7 and 14 dpi. In addition, our immunofluorescence data using independent antibodies for JMJD3 indicates that it is expressed in Schwann cells prior to nerve injury. Given

the early changes in H3K27me3 at one day post-injury, this is likely due to targeting/activation of pre-existing JMJD3 protein.

While previous studies have shown that demethylase activity regulates H3K27 methylation status, it has been documented that JMJD3 and UTX have demethylation-independent activities, and they are constituents of larger complexes with MLL proteins and also associated with BRG1-containing complexes (21). In the conditional mutants used here, the loxP sites remove exons containing the catalytic domain, with a resulting frameshift leading to loss of the entire C-terminus. Nonetheless, JMJD3 and UTX could be involved in the injury gene regulation in a demethylase-independent manner. The previously described association of H3K27 demethylases with MLL complexes (21) is consistent with the increased H3K4me3 at injury genes (12).

Most of the genes that were found to be JMJD3/UTX dependent were found in the sham condition. This gene set was much larger than found in the 1 dpi and 7 dpi RNA-seq data sets, but most of the downregulated genes are ones that naturally decrease after nerve injury based on several data sets (13,32,33), which explains why they were not significantly different after injury in the DKO. Their decrease after injury implies that they are co-regulated with the rest of the myelination program that is dependent upon Schwann cell contact with axons. Indeed, several of this set are among the genes regulated by the pro-myelinating EGR2 transcription factor (43). In turn, this suggests that H3K27 demethylase activity is required for full induction of a subset of the injury program. However, we did not detect any overt myelination defects, and many of these genes are decreased in the range of 40-75%. Evaluation of the entire EGR2-regulated gene network shows that most genes are unchanged in the DKO. Nonetheless, many of these genes are associated

with H3K27me3. We speculate that genes that increase in the Sham DKO samples are mechanistically linked to the downregulated genes, perhaps involving a transcriptional repressor such as WT1.

There are several possible mechanisms by which Polycomb repression can be bypassed without demethylation. For example, the proliferation of Schwann cells after injury, typically beginning at 3-4 days after injury, can lead to the passive reduction of H3K27me3 marks if H3K27 methylation is not maintained after DNA replication (52). This could explain the lack of effect at 7 d post-injury, but likely does not explain the early induction of injury genes at 1 d given the time course of Schwann cell division after injury. A second potential model is the H3K4me3 histone mark which is commonly associated with active promoters. Many nerve injury genes are associated with bivalent modification of H3K27me3 and H3K4me3 (12), and increased H3K4me3 is consistent with the traditional framework of activation through rebalancing of Polycomb and Trithorax-like mechanisms (44,45,53). Therefore, mechanisms to increase H3K4 methylation could be sufficient to activate Polycomb-repressed injury genes. Finally, many Polycomb-repressed genes contain both H3K27me3 and H2AK119ub1 formed by PRC1. PRC1 modifications were thought to be entirely dependent upon H3K27 methylation, but more recent studies indicate that PRC1 and PRC2 can be regulated independently, and that PRC1 activity may stimulate recruitment of PRC2 (19,54). Using ChIP-seq analysis, we found a general colocalization of H3K27me3 and H2AK119ub1 consistent with previous studies, but some injury genes only have evidence of PRC1 repression. However, the ChIP-seq data suggest a fairly significant decrease of H2AK119ub1 at 1 day after injury. Therefore, it may be that antagonizing PRC1 repression is the primary means of activation, and removal of H3K27me3 may simply be a consequence of H2A

deubiquitination. Several different H2A deubiquitinases have been identified, which could play a role in injury gene activation, including MYSM, USP16 and BAP1 (49,55).

The activation of the nerve injury program is regulated by transcription factors like JUN, but coordinate activation of this regenerative program requires definition of the mechanisms by which the nerve injury program is repressed. Some nerve injury genes are active in early neural crest and Schwann development (56) and become repressed in differentiated Schwann cells, and others (like Shh) appear to be induced de novo without having been expressed in their differentiation from neural crest (16). The repressed status of nerve injury genes involve not only polycomb repression, but also repression by transcription factors like *Zeb2* (57,58) and epigenomic remodelers such as histone deacetylases and the NuRD complex (10,59,60). We have not observed derepression of all H3K27me3-associated genes in the *Eed* cKO (13), so it is possible that derepression will involve multiple epigenomic complexes to maintain repression of the nerve injury program while allowing for its rapid induction after injury. Our studies highlight for the first time the involvement of PRC1 repression in this program, which is the focus of ongoing studies

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Primer sequences. Information on primer seq and antibodies used in the paper are provided in Table 1.

Table 1. qRT-PCR primers (mouse)		
Runx2	Forward	ACCAAGTAGCCAGGTTCAAC
	Reverse	GAGGATTTGTGAAGACTGTTATGG
Fgf5	Forward	AAAAGCCACCGGTGAAACC
	Reverse	TCACTGGGCTGGGACTTCTG
Shh	Forward	CAGCGACTTCCTCACCTTCCT
	Reverse	AGCGTCTCGATCACGTAGAAGAC
Gdnf	Forward	TCTCGAGCAGGTTCGAATGG
	Reverse	AAGAACCGTCGCAAACTTTACC
Hmga2	Forward	CAAGAGGCAGACCTAGGAAAT
	Reverse	CTCTTGCGAGGATGTCTCTTC
Cdkn2a/p16	Forward	GAATCTCCGCGAGGAAAGC
	Reverse	TGTCTGCAGCGGACTCCAT
Cdkn2a/p19	Forward	CACCGGAATCCTGGACCAGG
	Reverse	CACCGTAGTTGAGCAGAAGAGCT
Kdm6b/Jmjd3	Forward	CATGAACACCGTGCAGCTAT
	Reverse	CTCATGTACCGCGAACCACT
Kdm6a/Utx	Forward	AATATTGGCCCAGGTGACTG
	Reverse	TCACAGAAGTCATTCAAAACACC
ChIP primers		
Shh +3307	Forward	GGAAGCGCAGACAGACACTCT
	Reverse	CACAACAGCCTGGCACTCTCT
Gdnf	Forward	CCCCTGGATTGCGTGCTC
	Reverse	GGACATTAACTCCAAGTGGCCC
Antibodies	**Catalog number**	**Company**
SOX10	AF2864	R & D systems

Ki67	Ab16667	Abcam
p19/ARF	Sc-32748	Santa Cruz
CD68	Ab125212	Abcam
JMJD3	#A9780	Abclonal
ACTB	#AC004	Abclonal
IgG	12-370	Upstate/Millipore
H2AK119ub1	8240	Cell Signaling Technology
H3K27me3	AM39155	Active motif

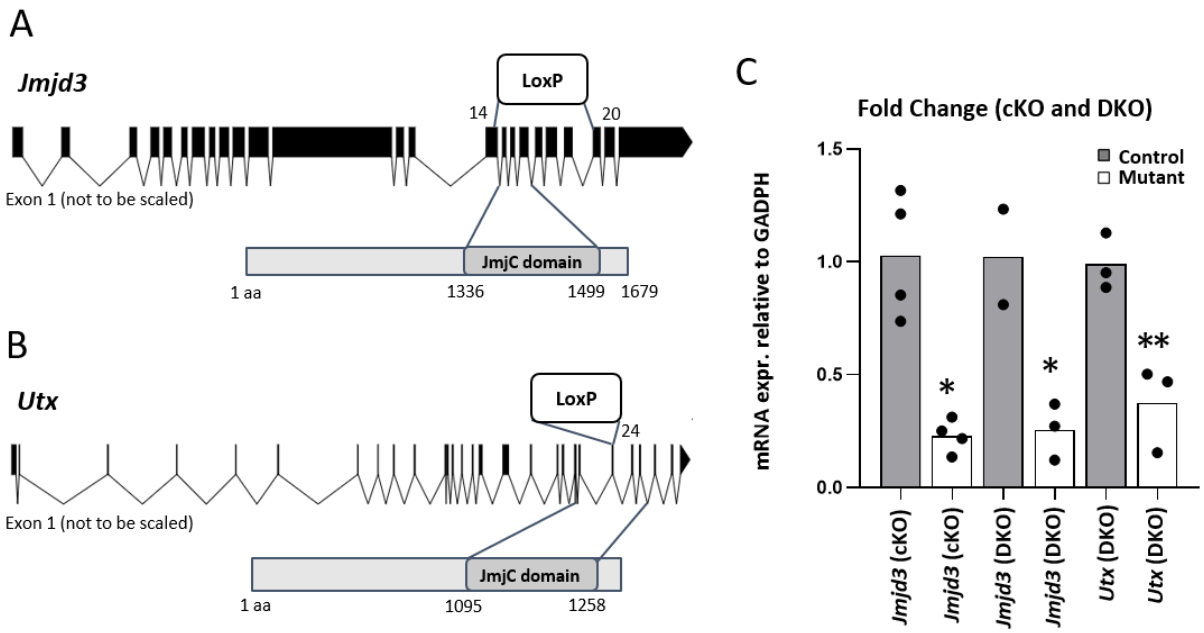


Figure 1. Schwann-cell specific knockout of H3K27 demethylases. A. In the *Jmjd3* locus, the catalytic domain in exons 14-20 is flanked by loxP sites. **B.** Similarly, the UTX locus contains the floxed 24th exon that would impair the catalytic function upon excision. **C.** qRT-PCR analysis of RNA extraction from distal stumps of control and DKO sciatic nerves 1 d after cut was performed. N=4 for control and n=4 for single *Jmjd3* cKO. n=2 for control and n=3 for DKO nerves. Data: mean ± STDEV; $**p < 0.005$, $*p < 0.05$ (one-way ANOVA).

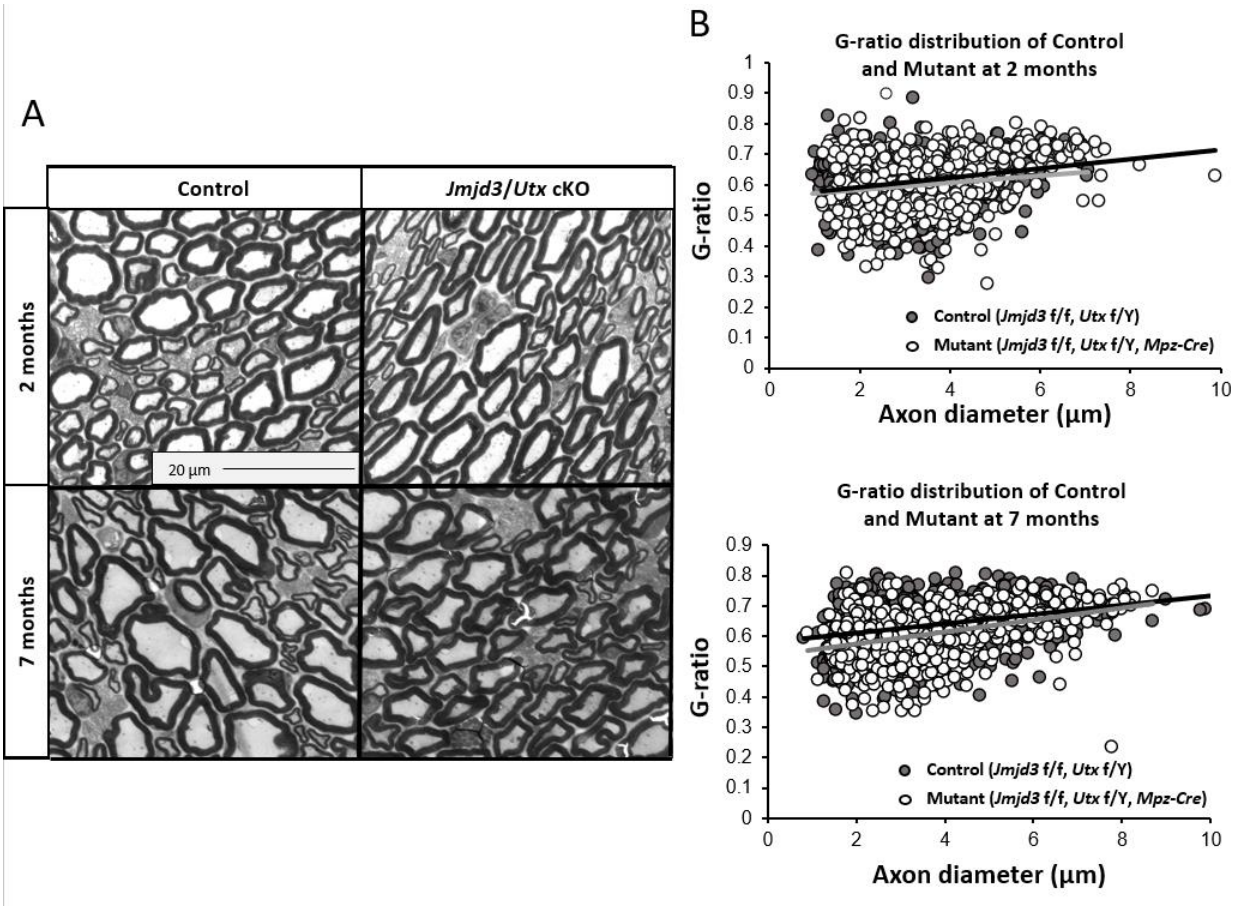


Figure 2. Schwann cell H3K27 demethylase activity is not required for development. A. Electron micrographs of the sciatic nerves at the indicated developmental time points of nerves of *Jmjd3/Utx* DKO mice and littermate controls. Scale bars 20 µm. **B.** For g-ratio analysis (axon diameter/diameter of myelinated fiber), the diameter of axon and outer diameter of myelinated fiber were measured on over 900 randomly selected fibers per genotype. Data: n=3 per genotype and age.

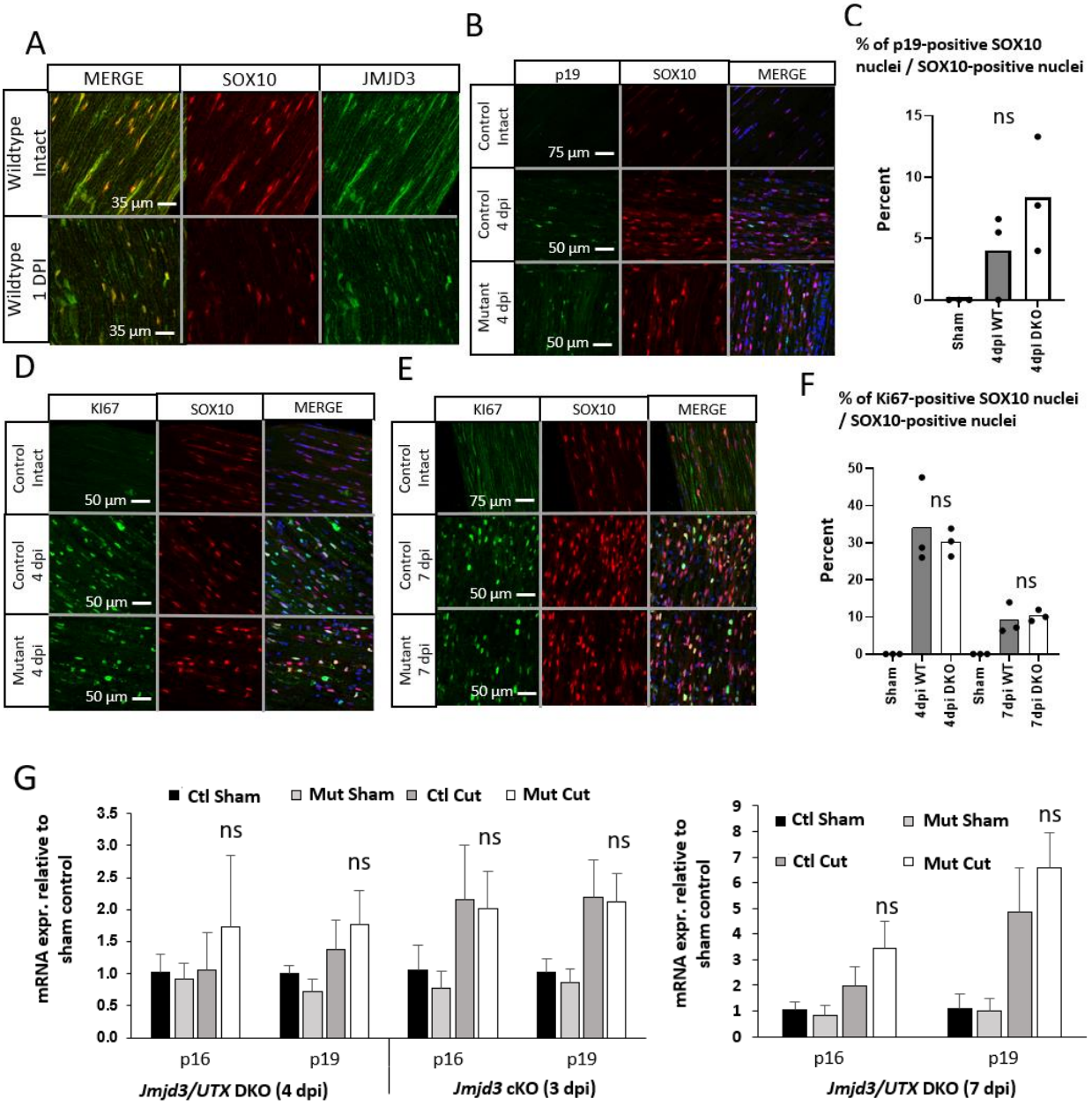

A
MERGE
SOX10
JMJD3
Wildtype Intact
Wildtype 1 DPI
35 µm
35 µm
B
p19
SOX10
MERGE
Control Intact
Control 4 dpi
Mutant 4 dpi
75 µm
50 µm
50 µm
C
% of p19-positive SOX10 nuclei / SOX10-positive nuclei
Percent
ns
Sham
4dpi WT
4dpi DKO
D
KI67
SOX10
MERGE
Control Intact
Control 4 dpi
Mutant 4 dpi
50 µm
50 µm
50 µm
E
KI67
SOX10
MERGE
Control Intact
Control 7 dpi
Mutant 7 dpi
75 µm
50 µm
50 µm
F
% of Ki67-positive SOX10 nuclei / SOX10-positive nuclei
Percent
ns
ns
Sham
4dpi WT
4dpi DKO
Sham
7dpi WT
7dpi DKO
G
Ctl Sham
Mut Sham
Ctl Cut
Mut Cut
mRNA expr. relative to sham control
ns
p16
p19
p16
p19
Jmjd3/UTX DKO (4 dpi)
Jmjd3 cKO (3 dpi)
Ctl Sham
Mut Sham
Ctl Cut
Mut Cut
mRNA expr. relative to sham control
ns
ns
p16
p19
Jmjd3/UTX DKO (7 dpi)

Figure 3. H3K27 demethylase activity is dispensable for Schwann cell proliferation after nerve injury. A. Immunofluorescence analysis of the longitudinal sections from distal stumps of wildtype sciatic nerves 1 day post-injury (dpi) was performed using the indicated antibodies. n=3 for control and n=3 for 1dpi nerves. **B.** Immunofluorescence analysis to measure p19, a cell cycle inhibitor, was performed in the sections of control and DKO sciatic nerves at 4 days aftercrush. **C.** The quantification of p19 at 4dpi. **D, E.** Immunofluorescence analysis to measure Ki67, a cell proliferation marker, was performed in the sections of control and DKO sciatic nerves at various timepoints after injury utilizing both transection and crush. **F.** The quantification of Ki67. **G.** qRT-PCR analysis was used to identify the expression level of p19 *and* p16 transcripts of *Cdkn2a* from *Jmjd3* cKO and DKO and control sciatic nerves of uninjured condition or 3 day after cut/4 day after transection/crush or 7 day after cut. Expression levels were normalized with *Gapdh*. Data: mean ± SD;; n=6 for sham and n=6 for 3 dpi *Jmjd3* cKO, n=4 for sham and n=4 for 4dpi DKO, and n=3 control and n=5 for 7 d post-cut dKO (one-way ANOVA). Ns=not significant

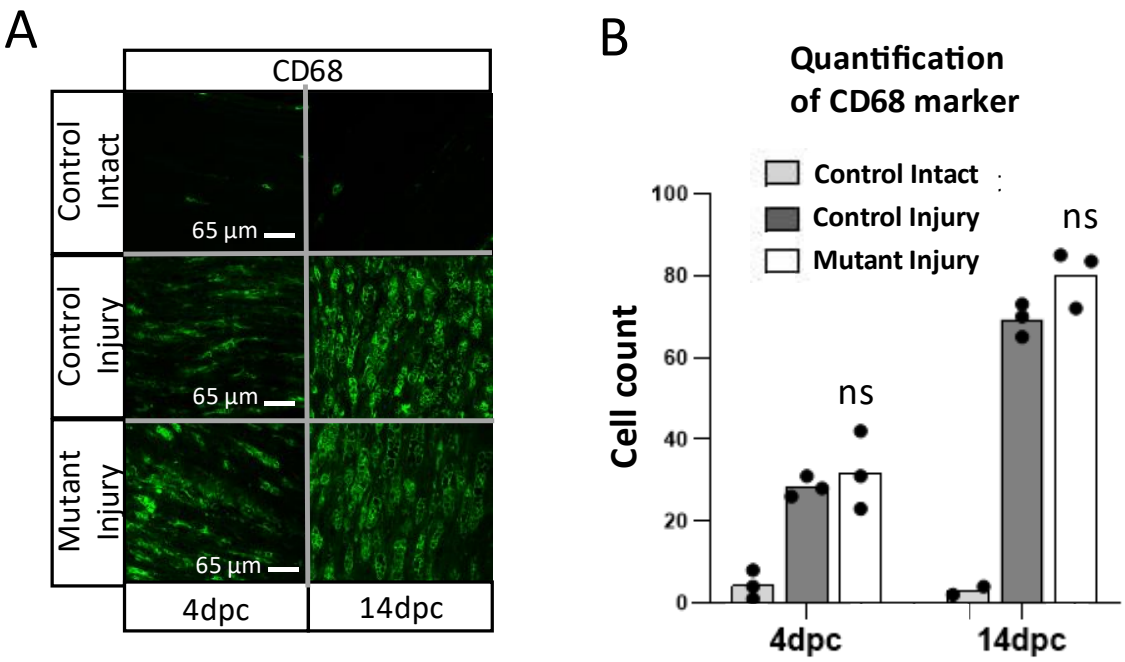
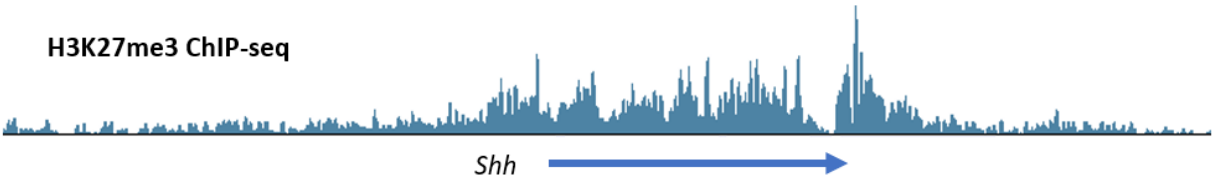
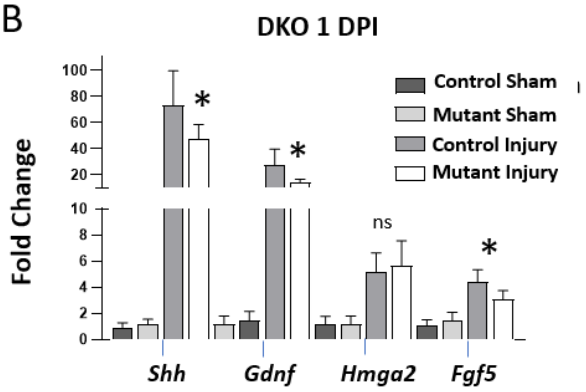


Figure 4. H3K27 demethylase activity is not required for macrophage recruitment after nerve injury. A. Immunofluorescence analysis of the longitudinal sections from distal stumps of control and *Jmjd3/Utx* cKO sciatic nerves at various timepoint after crush were performed using the indicated antibodies. **B.** Quantification of CD68. Data: n=3 for control and n=3 for DKO nerves at 4dpc and n=2 for control and n=3 for 14dpc.

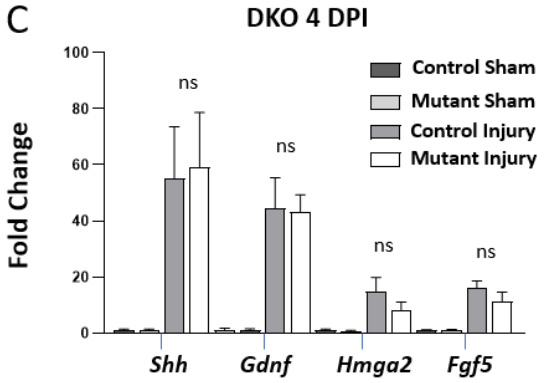
A



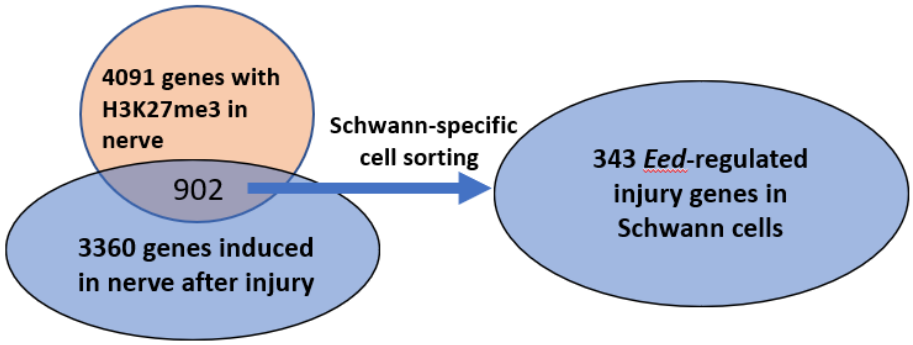
B



C



D



E

	Sham	1 dpi	7 dpi
Genes up in demethylase dcKO:	92	2	1
Downregulated genes:	54	6	4
Injury:	31	3	3
Contain H3K27me3:	23	4	2
Regulated in *Eed*-cKO:	14	2	1

Sham			
B3gnt5	*Enpp1*	***Ephb1***	*Erbb4*
Fgl2	*Frzb*	*Hcn1*	*Nrn1*
Plch1	*Scn8a*	*Slc1a2*	*Slc6a15*
Spp1	***Tmem229a***	***Wt1***	***Zdhhc2***

1 dpi	
Errb4	*Fgl2*
Fgf5	*Gad1*
Lrrc2	*Sfrp1*

7 dpi	
Igfbp5	*Nrn1*
Pappa	*Sez6*

Figure 5. H3K27 demethylase activity is not absolutely required for the induction of nerve injury genes. A. ChIP track of Sonic hedgehog (*Shh*) locus shows the association of repressive H3K27me3. **B.** qRT-PCR analysis was used to identify the expression level of injury-responsive genes from 2-month *Jmjd3* cKO and control sciatic nerves in uninjured condition or 1 day after transection. Expression levels were normalized with *Gapdh*. Data: mean ± SD; Asterisks indicate p-value between genotypes in the respective condition. $*p < 0.05$; n=5 for control and n=7 for DKO (one-way ANOVA). **C.** qRT-PCR analysis displays the expression of indicated nerve injury genes in distal stumps of nerves, indicated genotypes 4 d after crush. Data: mean ± SD; Asterisks indicate p-value between genotypes in the respective condition. $*p < 0.05$; n=6 for control and n=6 for DKO (one-way ANOVA). **D.** The Venn diagram shows intersection of gene sets with H3K27me3 and known injury genes. The list of 4091 genes with H3K27me3 was filtered by >10 peak score (13). The combined 1 and 7 dpi list of 3360 unique injury genes were obtained from the RNA-seq datasets (13,32), filtered by <0.05 p-value and >2-fold change. The gene list was further filtered for Schwann cell specific expression using published cell sorting data (33). **E.** Tables summarize and highlight the downregulated genes from DKO samples compared to control with significant p-values across sham, 1, and 7 days after injury.

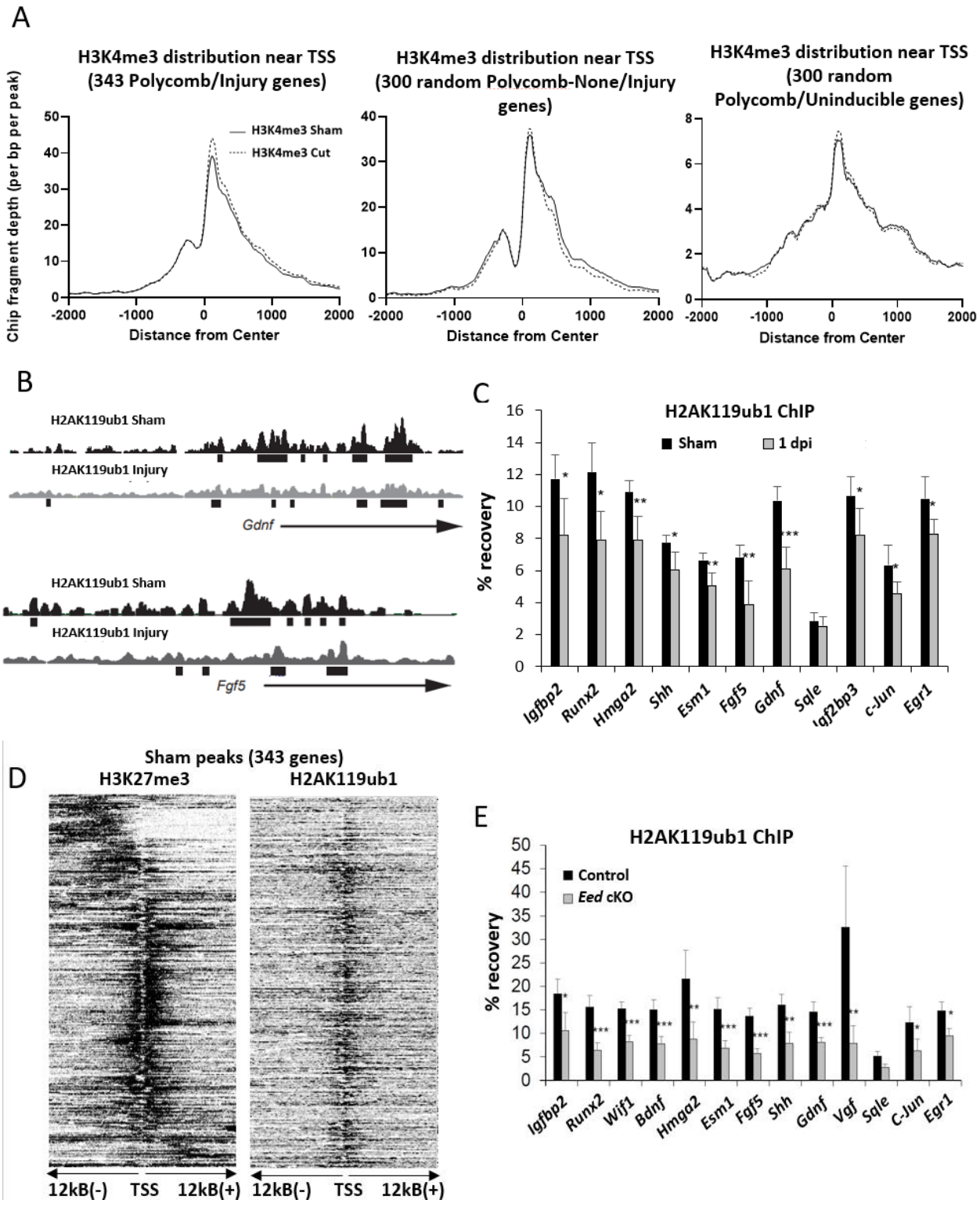
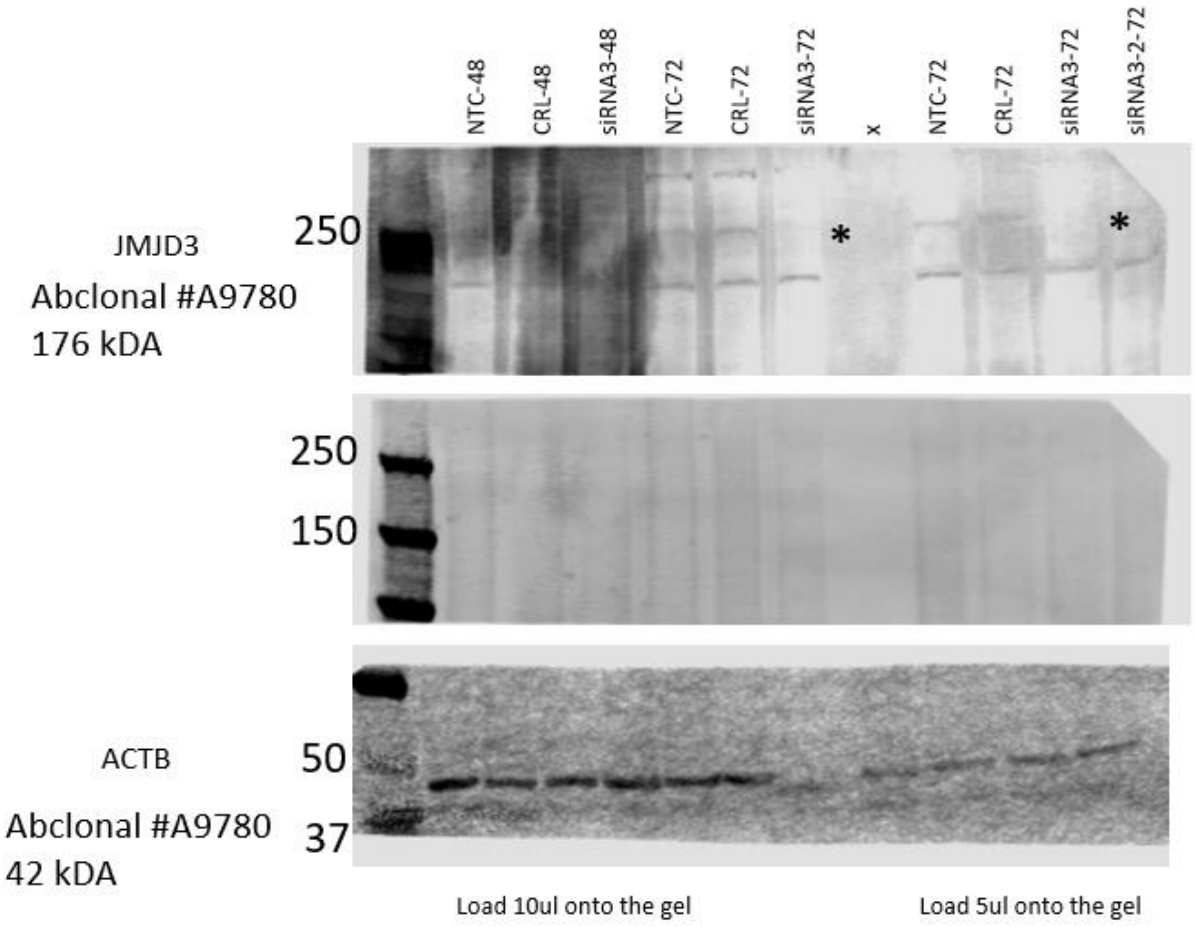
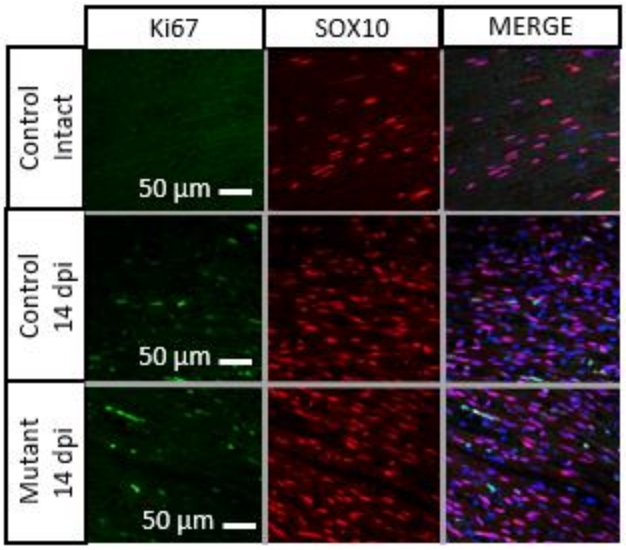

A
H3K4me3 distribution near TSS
(343 Polycomb/Injury genes)
H3K4me3 distribution near TSS
(300 random Polycomb-None/Injury genes)
H3K4me3 distribution near TSS
(300 random Polycomb/Uninducible genes)
H3K4me3 Sham
H3K4me3 Cut
Chip fragment depth (per bp per peak)
Distance from Center
B
H2AK119ub1 Sham
H2AK119ub1 Injury
Gdnf
H2AK119ub1 Sham
H2AK119ub1 Injury
Fgf5
C
H2AK119ub1 ChIP
Sham
1 dpi
% recovery
Igfbp2
Runx2
Hmga2
Shh
Esm1
Fgf5
Gdnf
Sqle
Igf2bp3
c-Jun
Egr1
D
Sham peaks (343 genes)
H3K27me3
H2AK119ub1
12kB(-)
TSS
12kB(+)
12kB(-)
TSS
12kB(+)
E
H2AK119ub1 ChIP
Control
Eed cKO
% recovery
Igfbp2
Runx2
Wif1
Bdnf
Hmga2
Esm1
Fgf5
Shh
Gdnf
Vgf
Sqle
C-Jun
Egr1

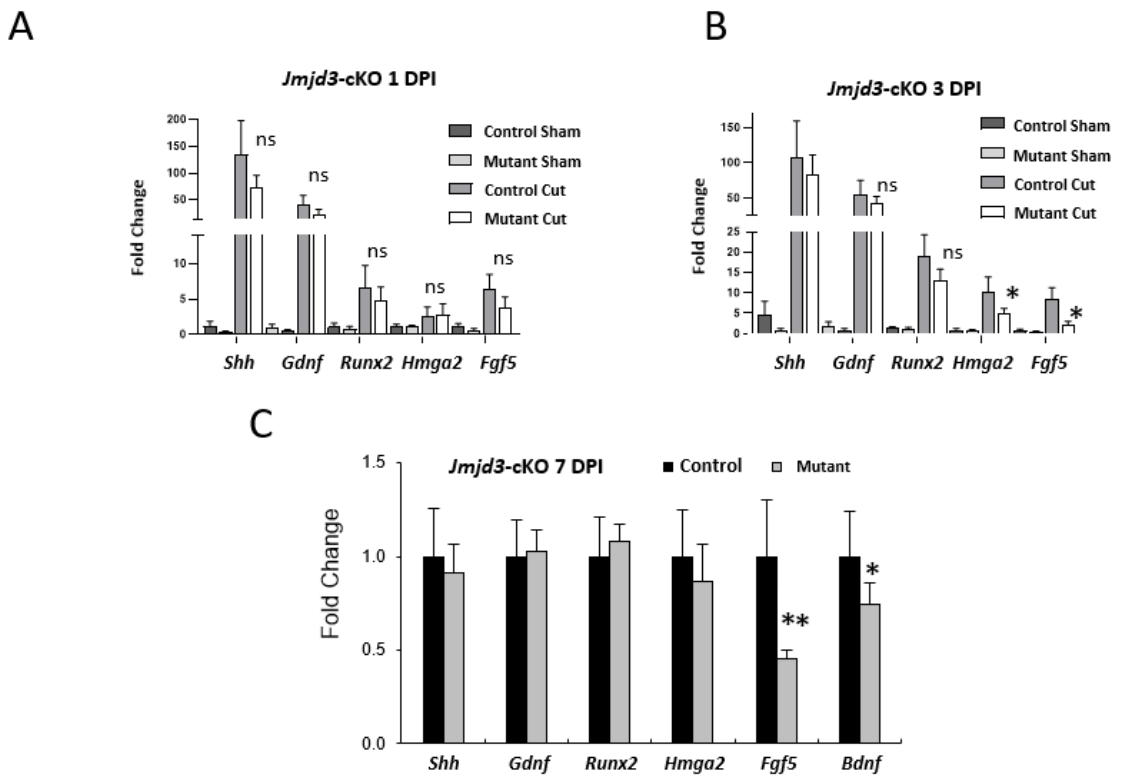
Figure 6. H2AK119ub1 is dynamically regulated during injury gene induction. A. The average distribution plots of histone mark H3K4me3 at TSS were based on the respective lists of Polycomb-regulated genes, 300 randomly selected injury genes lacking H3K27me3, and 300 randomly selected uninduced Polycomb genes after injury. The H3K4me3 ChIP seq data were generated using two replicates. **B**. ChIP-seq tracks of Growth-derived neural factor (*Gdnf*) and Fibroblast growth factor 5 (F*gf5*) loci show the association of repressive H2AK119ub1 mark at the promoter sites and the loss of such mark after injury. **C**. ChIP analysis of lysates from distal stumps of control and wildtype sciatic nerves 1 d after cut was performed using the H2AK119ub1 antibody. n=5 for control and n=5 for cut nerves. Data: mean ± STDEV; $**p < 0.005$, $*p < 0.05$ (one-way ANOVA). **D.** The heatmaps display the distribution of H3K27me3 and H2AK119ub1 centered at TSS based on 343 Polycomb-regulated gene list. The H3K27me3 and H2Ak119ub1 ChIP seq data were generated using two replicates. **E.** ChIP analysis of lysates from intact control and *Eed* cKO sciatic nerves was performed using the H2AK119ub1 antibody. n=5 for control and n=5 for *Eed* cKO nerves. Data: mean ± STDEV; $**p < 0.005$, $*p < 0.05$ (one-way ANOVA).



Supplementary Figure 1. JMJD3 Antibody Specificity Testing. Western blot analysis of lysates from S16 cell culture that is knock down with siJMJD3 for 2 d or 3 d after was performed using the indicated antibodies

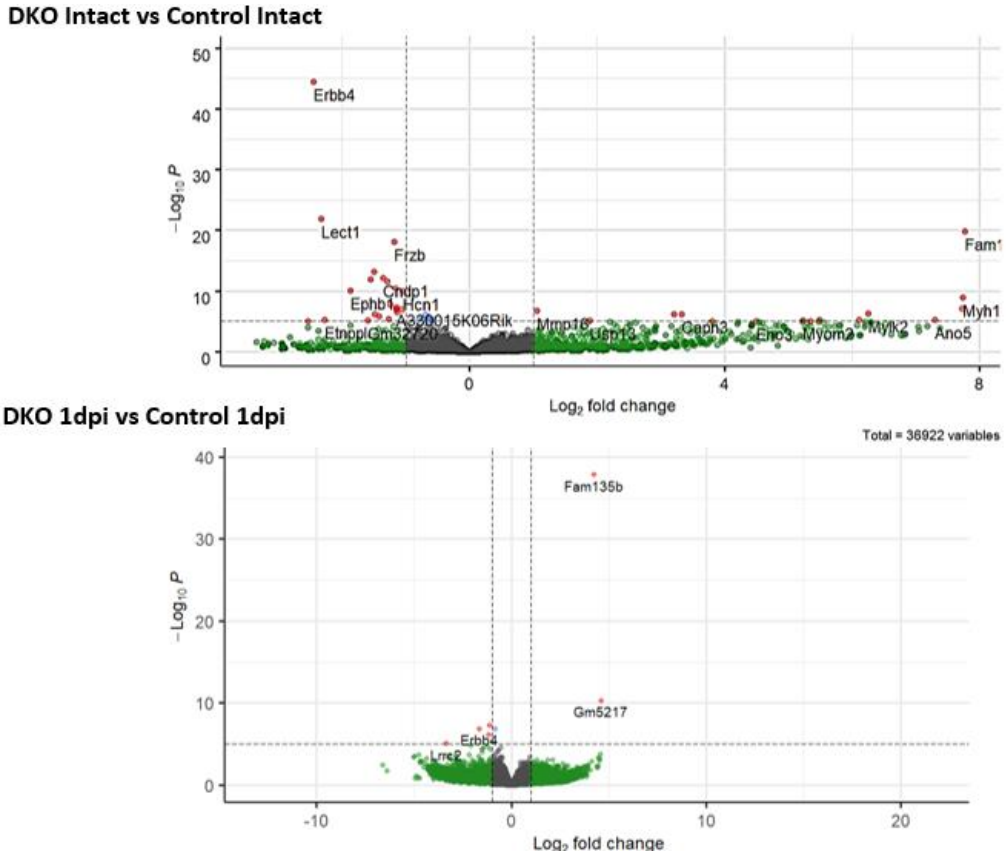


Supplementary Figure 2. No hyperproliferation nor prolonged proliferation is detected in DKO after 2 weeks nerve injury. Ki67, a cell proliferation marker, was used to probe the sections of control and DKO sciatic nerves at 14d after injury.



Supplementary Figure 3. Expression analysis of control and *Jmjd3* cKO mice at 1 3, and 7 dpi. A and B. *Jmjd3* cKO injury dataset indicate no significant differences besides *Fgf5* at 3 dpi, which is the only gene found in that of DKO. At 3 dpi, they have recovered in 4 dpi DKO which is similarly seen here in 3 dpi *Jmjd3* cKO data. **C.** No significant difference is seen for many nerve injury genes in *Jmjd3* cKO at 7 DPI with the exception of *Fgf5*. Data: 1 dpi *Jmjd3 cKO* control n=5 and mutant n=5. 3 dpi *Jmjd3 cKO* n=6 and mutant n=6. 7dpi *Jmjd3* cKO control n=6 and mutant n=6. mean ± STDEV; $^{**}p < 0.005$, $^{*}p < 0.05$ (one-way ANOVA).

A



B

Myelin genes

	DE in intact?	DE in 1dpi?	DE in 7dpi?
Pmp22	No	No	No
Mpz	No	No	Yes
Mag	No	No	No
Mbp	No	No	No
Plp1	No	No	No
Egr2	No	No	No
Ndrg1	No	No	No
Cntf	No	No	No
Sox10	No	No	No
Dhh	No	No	No
Il16	No	No	No
Aatk	No	No	No

Polycomb genes

	DE in intact?	DE in 1dpi?	DE in intact?
Gdnf	No	No	No
Olig1	No	No	Yes
Shh	No	No	No
Bdnf	No	No	No
Runx2	No	No	No
Hmga 2	No	No	No
Fgf5	No	Yes	No
Tmpr5 ss	No	No	No
Ccl2	No	No	No
Gfap	No	No	No
Ucn2	No	No	No

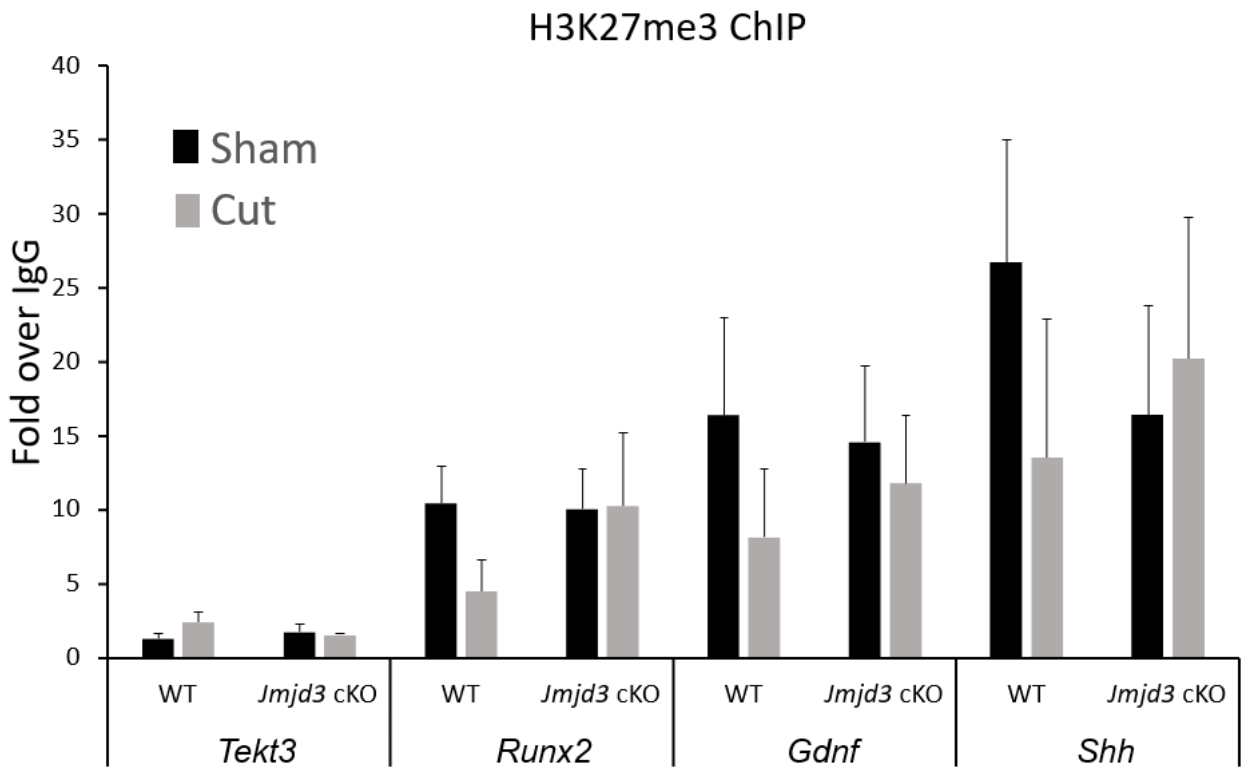
Proliferation genes

	DE in intact?	DE in 1dpi?	DE in 7dpi?
Cdkn2a	No	No	No
PCNA	No	No	No
MCM2	No	No	No
Ki67	No	No	No
Pax3	No	No	No
ErbB2	No	No	No
Tgf-beta	No	No	No
Notch	No	No	No
Gpr126	No	No	No
Lamin	No	No	No

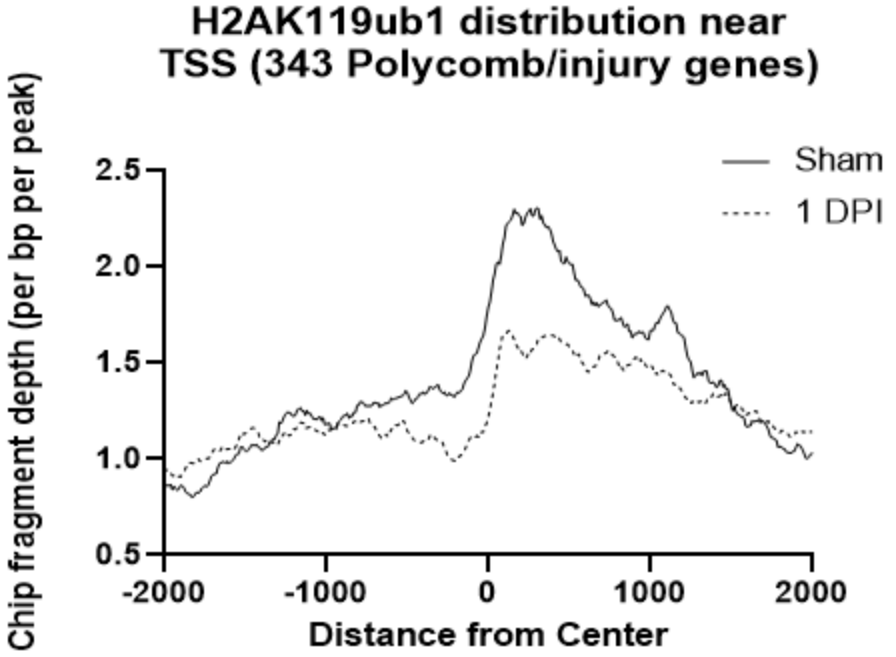
Immune genes

	DE in intact?	DE in 1dpi?	DE in 7dpi?
Cd68	No	No	No
Cd11b	No	No	No
Iba1	No	No	No
Arg1	No	No	No
TNF-alpha	No	No	No
Il4	No	No	No
Il1-beta	No	No	No
IL10	No	No	No

Supplementary Figure 4. RNA-seq analysis of control, DKO mice at 1 and 7dpi after nerve injury. A. Volcano plots generated from DEseq2 for intact and injury dataset indicate no overall changes at both 1 and 7 dpi after filtering with cell sort data. **B**. Tables summarize the changes of nerve repair and other genes between intact, 1, and 7 days after injury.



Supplementary Figure 5. *Jmjd3* mutant mice resulted in a reduced loss of H3K27me3 levels after nerve injury compared to control. ChIP analysis was performed using lysates from distal stumps of control and *Jmjd3* cKO sciatic nerves 1 day post cut. 6 pooled nerves for control and *Jmjd3* cKO were used for each of 3 replicate assays. Error bars=S.E.M.



Supplementary Figure 6. Plotting of the average H2AK119ub1 distribution between sham and 1dpi in wildtype based on the list of 343 Polycomb-regulated injury genes. The plot showed that the average distribution is lower in 1dpi compared to that of sham.

Chapter 3

H2AK119 Deubiquitinase Bap1 is Required to Silence the Nerve Injury Program in Schwann Cell

In preparation

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ACKNOWLEDGEMENTS

Seongsik Won performed Western blots and Ki Ma performed in vivo ChIP-seq for H3K27me3 and H2AK119ub1. The authors thank Benjamin August and Randall Massey from Electron Microscopy Facility for processing the samples, the University of Wisconsin Biotechnology Center Gene Expression Center for providing RNA library preparation, and the DNA Sequencing Facility for their sequencing services. For comparative analysis in transcriptomes, the statistical significance for the overlap between potential gene lists was tested at Nemaotes bioinformatic tools by Jim Lund which utilizes the Fisher's Exact Test. This work was supported by the National Institutes of Health: R01 NS100510 to JS, and in part by a core grant to the Waisman Center from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (P50 HD105353).

ABSTRACT

Schwann cell programming during myelination involves transcriptional networks that activate gene expression but also repression of genes that are active in neural crest/embryonic differentiation of Schwann cells. We previously found that a Schwann cell-specific deletion of the EED subunit of the Polycomb Repressive Complex (PRC2) complex led to inappropriate activation of many such genes. Moreover, some of these genes become re-activated in the pro-regenerative response of Schwann cells to nerve injury, and we found premature activation of the nerve injury program in the conditional knockout of *Eed*. The activation of the pro-regenerative program in response to peripheral nerve injury is associated with the removal of H3K27 trimethylation formed by PRC2 and H2AK119 monoubiquitination (H2AK119ub1), deposited by Polycomb repressive complex 1 (PRC1). Therefore, we hypothesized that H2AK119 deubiquitinases may be involved in modulating polycomb repression of the genes involved in embryonic development/nerve injury. To determine the role of H2AK119 deubiquitination, we generated a Schwann cell-specific knockout of the H2AK119 deubiquitinase *Bap1*. We found that loss of *Bap1* causes myelin pathology that is similar to the phenotype of *Eed* deletion. A significant proportion of genes associated with H2AK119ub1 were prematurely activated compared to control in intact nerves. Although BAP1 interacts with OGT in the PR-DUB complex, Schwann cell-specific deletion of *Bap1* does not seem to activate the *c-Jun* pathway as seen in OGT-KO model. Overall, our results indicate *Bap1* is required for polycomb repression of the injury response genes in Schwann cells.

INTRODUCTION

Schwann cells play an important role in the stability, integrity, and functions of the peripheral nervous system. In their terminally differentiated state, the myelin formed by Schwann cells enables saltatory conduction (1), and several types of inherited neuropathy are caused by impaired Schwann cell function (2-4). Several studies have identified molecular determinants of a homeostatic phase upon myelin maturation, and several mouse mutants of neuropathy-associated genes have demonstrated progressive myelin thickening and deformations that eventually impacts peripheral nerve function (5-11).

In our previous studies, we had determined the role of the polycomb repressive complex 2 (PRC2) that forms repressive histone H3K27 trimethylation (H3K27me3) by creating a Schwann cell-specific knockout of the *Eed* subunit of PRC2. We observed a progressive hypermyelination phenotype, which was associated with increased activation of the PI3 kinase activity (12). Moreover, gene expression analysis revealed that EED had a role in maintaining repression of a significant proportion of the injury program that is normally activated only after peripheral nerve injury (12,13). The injury-induced reprogramming of Schwann cells allows transformation to pro-regenerative cells, which support growth and survival of axons, clear myelin debris by autophagy/phagocytic responses, and form elongated Bungner bands to pave the way for nerve regeneration, before eventually myelinating any regenerated axons (14). Activation of the injury program was associated with reduction of the H3K27me3 on these injury genes (12,13). Overall, these studies suggested that Polycomb repression was important for the homeostatic phase of myelination, and also for repressing the injury program in mature Schwann cells (15).

The actions of PRC2 are highly correlated with that of PRC1, which catalyzes monoubiquitination of histone H2A (H2AK119ub1) through RING1A/B subunits (16). The canonical forms of PRC1 containing CBX subunits are epigenetic readers of H3K27me3 formed

by PRC2, leading to further repression through histone H2A ubiquitination and chromatin compaction (17). Accordingly, the level of H2AK119ub1 is often associated with H3K27me3, and we found that H2AK119ub1 also decreases upon activation of polycomb-repressed injury genes (18). While PRC1 repression was originally thought to be secondary to PRC2, there can be independent regulation of PRC1 to establish H2AK119ub1 that can subsequently trigger H3K27 methylation (16,19,20).

To test the role of PRC1 repression in development and injury, we created a Schwann cell-specific deletion of an H2AK119 deubiquitinase, BAP1 (21). BAP1 is part of the PR-DUB complex, containing also O-Linked N-Acetylglucosamine (GlcNAc) Transferase (OGT) (22). Based on the loss of H2AK119ub1 on injury-induced genes (18), we originally hypothesized that BAP1 may be required to activate injury genes, but also proposed that polycomb repression prior to injury would be enhanced. While loss of BAP1 has been shown in some cases to stabilize polycomb repression, we found that Schwann cell-specific deletion of BAP1 led to hypermyelination and premature activation of the injury program, suggesting that positive and negative regulators of polycomb-associated histone modifications play mutually reinforcing roles in myelin homeostasis.

MATERIALS AND METHODS

Mouse Colony. Animal experiments were performed according to protocols approved by the University of Wisconsin, Madison School of Veterinary Medicine. *Bap1* floxed mice [Jax# 031874] were maintained on the C57BL/6 genetic background and mated to mP0TOTA-Cre (*Mpz*-cre) (23).

Morphometric quantification of myelination. 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 4º. The nerves were then postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature. Following OsO4 postfixation, the nerves were dehydrated in a graded ethanol series, and then further dehydrated in propylene oxide and embedded in Epon resin. Ultrathin transverse sections were either contrasted with Reynolds lead citrate and 8% uranyl acetate in 50% ethanol or stained with toluidine blue. Images were obtained either with a Philips CM120 electron microscope with an AMT BioSprint side-mounted digital camera at the UW Medical School Electron Microscope Facility or with Nikon Ti2 microscopy. 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.

Immunofluorescence. Freshly dissected nerves were embedded in Tissue-Tek OCT compound (Sakura Finetek) and snap frozen with liquid nitrogen. Longitudinal or transverse cryostat sections (10 μm) were air-dried for 5 min and fixed in 4% paraformaldehyde for 10 min. The sections were then blocked in PBS containing 5% donkey serum/1% BSA/0.5% Triton-X 100 for 1 hr 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 hr. Hoechst 33342 (1:5,000 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 Nikon A1R confocal and quantitated by both Columbus imaging software and manual curation.

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 Na3VO4 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 polyvinylidene fluoride membrane, proteins were blocked in TBST containing 5% nonfat dry milk for 1 hr at room temperature. Primary and Secondary antibody incubations were performed in TBST containing 5% BSA (Sigma, A7906) at 4 C for overnight and at room temperature for 1 hr, 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. The following antibodies were used in Table 1.

Quantitative RT-PCR. RNA was isolated from sciatic nerves using the Trizol/chloroform RNA extraction protocol following the purification with Zymo's RNA Clean and Concentrator kit. To prepare cDNA, 250 ng or 1 μg of total RNA of mouse, respectively, was used from each sample. qRT-PCR and data analysis were performed as described previously (24). qPCR was performed with two replicates per sample, and statistical analyses were evaluated by one-way ANOVA.

RNA seq. For each sample, at least 1000 ng total RNA was purified with no DNAse treatment, and sent to Genewiz (South Plainfield, NJ) for library preparation after PolyA selection and

Illumina sequencing (Illumina HiSeq 2x150bp). Illumina sequencing data were mapped to the GRCm38/mm10 genome. Data were analyzed using DESeq2 (25) to determine differentially regulated genes between uninjured and injured nerves in wild type and *Bap1* KO mice (p-value < .05).

Bioinformatic Analysis. We employed previously ChIP-seq data sets (GSE159265) of H2AK119ub1 in rat peripheral nerve (18) to create the heat maps for the deregulated genes. The data matrix for each heatmap was generated by HOMER software (26) using the tag directories based on ChIP-seq data sets and then clustered with Cluster 3.0 (27) and visualized with Java Tree View (28). The gene ontology enrichment analyses were conducted with PANTHER software to identify the enriched biological processes based on respective upregulated and downregulated lists of *Bap1* KO genes. (29). For comparative analysis in transcriptomes, the statistical significance for the overlap between potential gene lists was tested at Nemaotes bioinformatic tools by Dr. Jim Lund which utilizes the Fisher's Exact Test.

RESULTS

Conditional inactivation of *Bap1* in Schwann cells

To test the role of BAP1 in controlling the induction of developmental and nerve injury genes, we developed a Schwann cell specific conditional knockout of *Bap1* using the *Mpz*-cre driver, which is active in embryonic Schwann cell development (23). The allele contains the loxP sites that surround exons of 6-14 of *Bap1* including part of the catalytic UCH domain at E13.5-14.5 (Figure 1A) (21). Furthermore, the excision also results in a frameshift 3' of the deletion, leading to loss of the C-terminal sequence. The mouse knockout was validated by quantitative RT-PCR analysis of peripheral nerve cDNA using primers located at 7th and 8th exons in the deleted region, showing loss of *Bap1* in the conditional knockout (Figure 1A,B). As is commonly observed with the *Mpz*-cre driver, the residual Bap1 expression is attributed to other cell types found in sciatic nerve. We also further confirmed the loss of BAP1 protein through immunostaining with an antibody targeting an epitope in the C-terminus of BAP1. Coexpression of BAP1 and SOX10, a marker of Schwann cells (30), was absent in the knockout whereas there are cells positive for such markers in control samples (Figure 1C).

Myelin abnormalities in the *Bap1* knockout

The mice were overtly normal with no evident motor impairment. To determine if *Bap1* knockout affects Schwann cell development, we performed toluidine blue staining of semthin sections to analyze the nerve ultrastructure in detail (Figure 2A). At 6 weeks, the sciatic nerves of mutant mice were examined and many axons of varying caliber were observed to contain tomacula, and myelin infolding/outfolding (Figure 2B,C) (31). We quantified axons associated with myelin tomacula for wild type and the *Bap1* KO and found that there are a significantly greater percentage of abnormally myelinated axons in the knockout compared to control (Figure 2D). Myelin thickness is assessed using the g-ratio, which is the axon diameter divided by outer diameter of

myelin sheath (24). The myelin sheaths of some larger caliber axons are noticeably thinner in the knockout, but myelin sheaths of smaller caliber axons are hypermyelinated. The mutant mice displayed a skewed distribution of axon diameter compared to controls with a notable deficit of the higher caliber axons. The unusual myelination was significant to enough to affect the g-ratio distribution which is indicated by the trendlines. (Figure 2E,F).

Derepression of injury genes in the BAP1 knockout

Given the myelin defects observed in the *Bap1* KO, we decided to perform RNA-seq of peripheral nerve to compare *Bap1* cKO nerves and controls (n=6) at ~6 weeks of age. As shown in the volcano plot, there were widespread changes in gene expression (Figure 3A), and we identified 2446 upregulated and 2788 downregulated genes ($p<0.05$, FDR corrected). Gene ontology analysis revealed that upregulated genes are associated with proliferation and gliogenesis (29). Enriched categories for downregulated genes included processes of Schwann cell differentiation and lipid synthesis (Supplementary Figure 1).

For further analysis, we used a >2-fold cutoff, yielding 708 upregulated genes and 604 downregulated genes in the *Bap1* cKO (Figure 3B). Within the upregulated dataset, we observed several significant nerve injury genes that were prematurely activated in our analysis of PRC2 function using the *Eed* cKO, including *Shh, Gdnf, Fgf5, Hmga2, Vgf, Igfbp2*, which are among the classic nerve injury genes (13). Upregulated genes include cell cycle regulators (*Cdkn2a*, *Prc1*) (32), growth factors (*Nrg1*, *Btc*) (33), cytokines (*Cxcl10*) , and transcription factors (*Tfap2a*, *Runx2*, and *Pou3f1*) (12,24,34). Since PRC1 and PRC2 regulation is connected at multiple levels, we compared the deregulated genes with those obtained our previous RNA-seq profiling of a Schwann cell-specific deletion of *Eed* (15). Although we had speculated that knocking out *Bap1* could result in strengthened Polycomb repression, we observed derepression of many polycomb-

regulated genes, there are 105 upregulated *Bap1*-KO genes that are in common with those upregulated in the *Eed* conditional knockout. Additionally, there are 52 downregulated genes that are regulated by EED.

The increase in cell cycle-associated genes revealed by RNA-seq suggested that there may be changes in proliferation. However, we did not detect any increased proliferation by assessing KI-67, which could in part be due to the increased level of *Cdkn2a* that was observed in the *Bap1* KO as well as the *Eed* KO (15). In the downregulated dataset, there was modest downregulation of several myelin genes with *Mbp* being the most significant. These appear to be consistent with the ontology analysis that indicates downregulation of lipid biosynthesis. *Epha4* is also one of the notable genes for downregulated list. Ironically, *Epha4* was proposed to have a role that negatively regulate myelination (35).

Polycomb-associated histone modifications in BAP1-regulated genes

Since we had observed loss of polycomb repression, we also measured the levels of H2AK119ub1 and H3K27me3 by Western blot of sciatic nerve of the *Bap1* conditional knockout and found significantly increased levels of both histone marks (Figure 4A,B). Elevated H2AK119ub1 is consistent with the loss of H2A deubiquitinase activity, and increased levels of H3K27me3 are consistent with studies showing that PRC1 activity can attract H3K27me3 deposition by PRC2 (20,36,37).

We assessed the correlation of gene expression changes with polycomb-associated histone marks that we had previously characterized, since previous studies show that the local levels of H2AK119ub1 and H3K27me3 at the promoters of many nerve injury genes decrease as they are activated following peripheral nerve injury (13,15,18). Using ChIP-seq profiles of H3K27me3 and H2AK119ub1 in rat sciatic nerve (13,15,18), we generated the separate heatmaps for distribution

of H3K27me3 and H2AK119ub1 reads for *Bap1* KO upregulated and downregulated genes. We found that more than a third of the upregulated genes are associated with H3K27me3 and H2AK119ub1 (Figure 4C,D). Specifically, at least 216 *Bap1*-dependent upregulated genes are associated with both or either of histone marks (Figure 4E), which includes many key nerve injury genes (e.g. *Shh, Gdnf,* and *Runx2*) which had been found to be regulated by *Eed* (13,15,18). However, at least 187 Bap1-dependent genes, which is a significant proportion of the downregulated genes, are also associated with H2AK119ub1 and/or H3K27me3, suggesting that polycomb repression may be strengthened for some genes in the Bap1 knockout.

Since our previous studies had indicated that loss of EED led to upregulation of a significant proportion of the nerve injury program in Schwann cells, we focused on the overlap between the nerve injury genes and associated histone marks – H3K27me3 and H2AK119ub1 (Figure 4A)**.** Using the called peaks from the histone modification analysis, we found that around 577 out of 1860 injury-induced genes are associated with either or both histone marks (Figure 5A,B). Just around 129 genes carry H2AK119ub1 alone and another 243 genes with H3K27me3 alone. 205 genes carry both histone marks. Not all of these genes are deregulated in the *Eed* or *Bap1* knockout, although it is possible that the regulation of PRC1 and PRC2 is somewhat redundant for injury genes.

Focusing on the set of 577 injury genes associated with H3K27me3 and/or H2AK119ub1, a significant set are affected by BAP1 KO. At least 70 *Bap1*-dependent upregulated genes that are nerve injury induced are also associated with either or both histone marks. Several of them are prominent nerve injury genes including *Gdnf, Igfbp2, Pou3f1, Fgf5, Shh, Runx2,* and *Hmga2* (38,39). In contrast, only at least 10 *Bap1*-dependent downregulated genes associated with either or both histone marks that are nerve injury induced are affected (Figure 5C).

Comparison of the roles of BAP1 and OGT

BAP1 interacts with OGT as subunits of the PR-DUB complex. In previous studies, a Schwann-cell specific *Ogt* KO mouse model was created that exhibited myelin defects that were traced to the premature activation of JUN (41). The mutant mouse exhibited a significant number of tomacula and thinner myelination of larger caliber axons, although the phenotype was more severe than what was observed in the *Bap1* KO. We determined if a common PR-DUB target gene set could be defined by comparative analysis of genes regulated by OGT and BAP1, we compared the RNA seq data of the *Bap1* KO to the published RNA-seq analysis of the *Ogt* KO (41). Out of 1298 upregulated genes with significant p-values in OGT-KO datasets, and there are 153 matches with upregulated *Bap1* KO genes and 54 matches with downregulated *Bap1* KO genes (Figure 6C).

Interactions of polycomb-regulated genes with the JUN-regulated network of injury genes.

A significant proportion of dysregulated target genes in the *Ogt* KO are unchanged in the *Bap1* KO. However, one key difference between the *Bap1* and *Ogt* knockouts is the activation of JUN in the latter (41). JUN is a major regulator of nerve injury program in Schwann cell and is required for efficient regeneration (40). In healthy nerve, JUN is normally not expressed at high levels prior to injury. It was possible that part of the induction of injury genes could have been mediated by JUN, since there is a modest 1.7-fold induction of the *c-Jun* transcript in our RNA-seq analysis of the Bap1 knockout. Therefore, we examined the JUN by western blot but observed no significant difference in JUN expression between genotypes at 6 weeks of age (Figure 6A,B).

It is still possible that JUN activity is stimulated post-transcriptionally, and RNA seq indicates that *Fos*, a component of AP-1, is upregulated 2.8-fold. Therefore, we compared the RNA seq profile of the BAP1 KO to RNA-seq analysis of a mouse model with Schwann cell-specific JUN overexpression (42). Using this data set, there are only 11 common genes that are upregulated in both BAP1 KO and the JUN overexpression model, such *as Shh, S100a4, Btc,* and *Cxcl10*. We found that the statistical significance for overlap between upregulated *Bap1* KO list and c-JUN cKO list is $p < 1.763e-10$. While there is overlapping regulation of injury genes by JUN and polycomb repression, the evidence indicates that they also play in dependent roles since we do not see induction of the entire JUN target gene network in Schwann cells. Therefore, we conclude that BAP1's active role in regulating nerve injury genes is not mediated by JUN. There is also an overlap of JUN-regulated genes and those that were found to be deregulated in the EED KO, but we also did not detect measurable induction of JUN in the EED cKO (15).

DISCUSSION

The function of polycomb repression in Schwann cell development has a number of facets related to the diverse complexes that impinge on this type of regulation. In particular, there are multiple assemblies of PRC2 and PRC1 complexes that can play unique roles in establishing polycomb repression in different genes (43,44). Our previous study of the *Eed* knockout revealed roles in the regulation of myelin homeostasis and also in repression of the Schwann cell injury program (12,13,15). However, PRC2 regulation is intertwined in several respects with PRC1, and we had found changes in PRC1-mediated H2A ubiquitination in the *Eed* knockout and also dynamic changes in this modification in Schwann cells after injury (18).

To understand how dynamics of H2AK119 ubiquitination affect polycomb repression in Schwann cells, we focused on BAP1 which is a known component of PR-DUB complex that also includes other subunits like OGT and ASXL1/2 (21). Loss of BAP1 in Schwann cells causes an abnormal developmental phenotype that is evident at six weeks. Specifically, the smaller caliber axons contain thicker myelin where the larger caliber axons contain thinner myelin in the mutant mouse. Numerous types of tomacula are also observed in the mutant. We noted that there are significantly fewer number of larger caliber axons compared to the number in control. These developmental phenotypes are similar to those seen in the Schwann cell-specific knockout of *Eed* (12).

To determine the role of BAP1 in myelin homeostasis, an RNA-seq analysis revealed significant changes in gene regulation. Importantly, we found that several key nerve injury genes are prematurely activated in *Bap1* KO including *Shh, Gdnf, Runx2, Hmga2*, Cdkn2a, and *Fgf5,* which were also activated in the *Eed* ckO (15), but this analysis revealed a broader range of polycomb-repressed genes, many of which are associated with H2AK119 ubiquitination. It is possible that these caused the myelination disorders seen in the numerous tomacula in mutant mice.

Several candidate genes were derepressed in both the *Eed* and *Bap1* cKO, including *Igfbp2* which was shown to increase PI3 kinase signaling (12). However, a leading candidate is the *Nrg1* gene, since previous studies have found that efficient remyelination of injured nerves requires the de novo activation of *Nrg1* type I in denervated Schwann cells (12). We had found that H3K27me3 mediates repression of *Nrg1* and there is increased expression of the type I *Nrg1* transcript in the uninjured Eed cKO nerves (15). A recent study showed that transgenic overexpression of NRG1 type 1 resulted in hypermyelination of small caliber axons (45). Another potential mechanism could involve BAP1 regulation of the PTEN lipid phosphatase since a Schwann cell-specific deletion of *Pten* caused hypermyelination (8,46). It has been reported that BAP1 could repress *Pten* at the transcriptional and post-transcriptional levels (47,48). However, *Pten* levels were not elevated in our RNA-seq analysis and Western blot showed no change in PTEN protein levels (data not shown).

Although one of enriched gene ontology categories was proliferation/cell cycle genes, we did not see any increased proliferation in knockout nerves at this timepoint. This could be due to the derepression of the *Cdkn2a* gene (encoding p19 and p16), which we had previously identified as an *Eed*-regulated gene (15). The RNA-seq analysis did not suggest any increase in other cell populations such as macrophages.

Several studies have characterized the role of BAP1 and divergent results have been obtained. In several cases, loss of BAP1 leads to increased polycomb repression which is associated with increased levels of H2A ubiquitination (21,49). In contrast, other studies have shown that loss of BAP1 leads to loss of polycomb repression (50,51), which has been attributed to a function of BAP1 to safeguard transcriptionally silent genes by delimiting (or bookending) H2AK119 and H3K27me3 modifications that would titrate out polycomb repression if allowed to

spread (50,51). Accordingly, the Drosophila homolog of BAP1, calypso, was originally classified as a polycomb group gene (52). These mechanisms are highly relevant to the observed mutation of BAP1 in several types of cancer since both gain- and loss-of-function models of polycomb repression can contribute to malignancy (43,49,50,53). In our case, we observed derepression of a number of genes associated with H2AK119ub1 and/or H3K27me3, despite the fact that there were global increases in both H2AK119ub1 and H3K27me3 by Western blot. However, some genes were also decreased as well, suggesting that there may have been increased polycomb repression of some genes.

Our studies allow us to compare the consequence of deletion of two components of the PR-DUB complex. BAP1 interacts with OGT in the PR-DUB complex, and OGT has known functions in suppressing the nerve injury response. Specifically, OGT catalyzes the addition of a single N-acetylglucosamine through an O-glycosidic linkage to serine or threonine residues of the JUN protein (41,54). The Schwann cell-specific OGT-cKO leads to the elevated JUN activity, resulting in the premature activation of the nerve injury response during development and exhibiting significant degree of tomacula and thinner myelination of larger caliber axons. Therefore, it was possible that loss of *Bap1* could phenocopy the myelination defects seen in *Ogt*-KO (at 3 months), and the myelin defects showed some similarities as noted above. A comparison of the RNA-seq data sets revealed that 153 out of 708 upregulated genes in *Bap1* KO overlaps with those of *Ogt*-KO genes. Some of these 153 genes have relevance to the Schwann cell nerve injury genes, which could explain the abnormal myelination in *Bap1* KO mouse model.

We had identified the presence of H2AK119ub1 (but not H3K27me3) on the *Jun* promoter, but *Jun* was only slightly elevated (1.7-fold) in the RNA-seq analysis. Moreover, we did not observe any change in JUN protein in the Schwann cell specific knockout of *Bap1*. One of the

striking findings of the OGT analysis was that the failed regeneration after injury could be rescued by deletion of one allele of *Jun* (41). Using *Ogt*-KO RNA seq data, there were 11 matches out of 28 upregulated JUN-targeted genes with *Bap1* KO genes, indicating the possibility of JUN's significant role, but there are also quite significant differences in the effect of BAP1 loss, since the phenotype at later stages is less severe compared to the *Ogt* cKO. Our results support a model in which PR-DUB is a multifunctional complex that prevents premature activation of the nerve injury response. BAP1 and OGT appear to play functionally distinct roles within this complex by maintaining polycomb repression and inhibiting JUN, respectively. These roles are mutually reinforcing since there is considerable overlap of JUN-regulated and polycomb-regulated genes in the nerve injury response. It remains to be determined how PR-DUB activity is altered in Schwann cells after nerve injury. There is no apparent change in *Ogt* and *Bap1* transcripts (or those of other PR-DUB subunits) after injury although protein levels or modifications may change.

Numerous pro-regenerative genes in Schwann cells are repressed by both H2AK119ub1 and H3K27me3 and their basal expression can be virtually undetectable in intact, healthy peripheral nerve. Previous studies had suggested that removal of polycomb repression is required for induction of nerve injury genes such as *cJun*, *Shh*, *Gdnf*. This idea was supported by previous studies of a Schwann cell-specific deletion of *Eed* showing that preventing PRC2 repression causes loss of H2AK119ub1 and injury gene derepression (12). PRC2 is commonly known to work cooperatively with PRC1, but recent studies had indicated that PRC1-mediated repression can be mediated independently of PRC2 (44). Therefore, future experiments will be used to determine if deubiquitinase(s) play a role in regulation of injury genes in Schwann cells.

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Primer sequences. Information on primer seq and antibodies used in the paper are provided in Table 1.

Table 1. qRT-PCR primers (mouse)		
Bap1	Forward	CAAGAGTCACAGCTGCCTGA
	Reverse	GAACCAGCCACCTCCTCTG
Cdkn2a/p16	Forward	GAATCTCCGCGAGGAAAGC
	Reverse	TGTCTGCAGCGGACTCCAT
Cdkn2a/p19	Forward	CACCGGAATCCTGGACCAGG
	Reverse	CACCGTAGTTGAGCAGAAGAGCT
Antibodies	**Catalog number**	**Company**
SOX10	AF2864	R & D systems
Ki67	Ab16667	Abcam
p19/ARF	Sc-32748	Santa Cruz
ACTB	#AC004	Abclonal
IgG	12-370	Upstate/Millipore
H2AK119ub1	8240	Cell Signaling Technology
H3K27me3	AM39155	Active motif
H3	14269	Cell Signaling Technology
c-JUN	9165	Cell Signaling Technology
PTEN	9188	Cell Signaling Technology

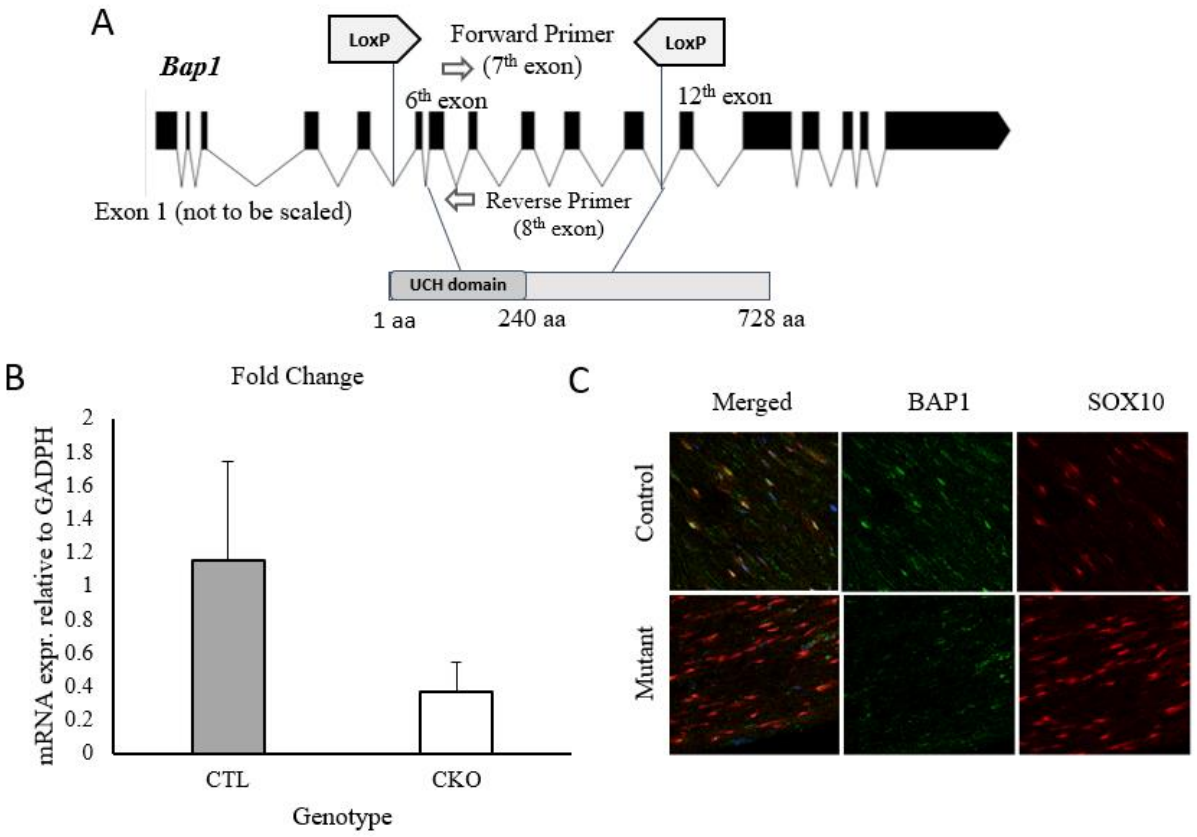
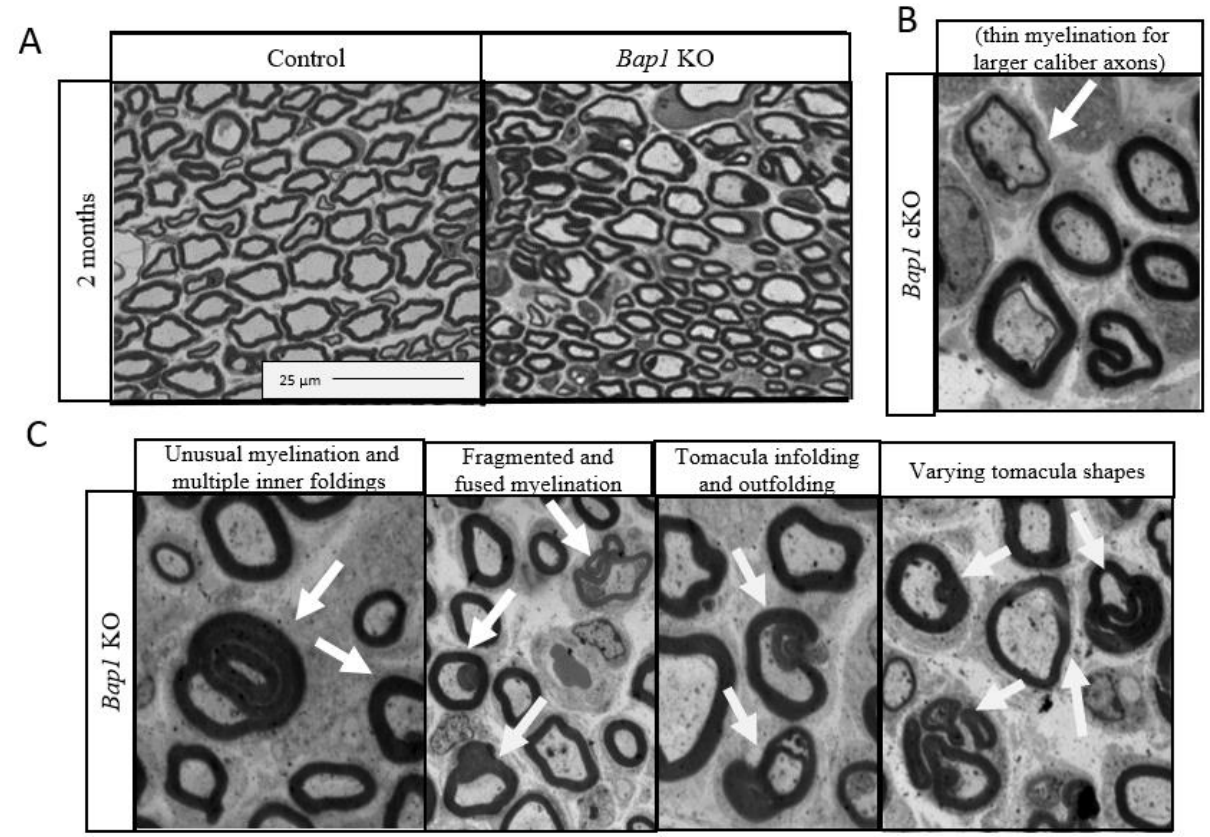


Figure 1. Schwann-cell specific knockout of H2AK119 deubiquitinase. A. In the *Bap1* locus, the catalytic domain in exons 6-12 is flanked by loxP sites. **B.** qRT-PCR analysis of RNA extraction from control and KO sciatic nerves was performed. N=4 for control (*Bap1* fl/fl) and n=4 for *Bap1* fl/fl/Mpz-cre. n=4 for control and n=4 for KO nerves. **C.** Immunofluorescence analysis of the longitudinal sections from the control and *Bap1* KO sciatic nerves were performed using the indicated antibodies.


A
Control
Bap1 KO
2 months
25 µm
B
(thin myelination for larger caliber axons)
Bap1 cKO
C
Unusual myelination and multiple inner foldings
Fragmented and fused myelination
Tomacula infolding and outfolding
Varying tomacula shapes
Bap1 KO

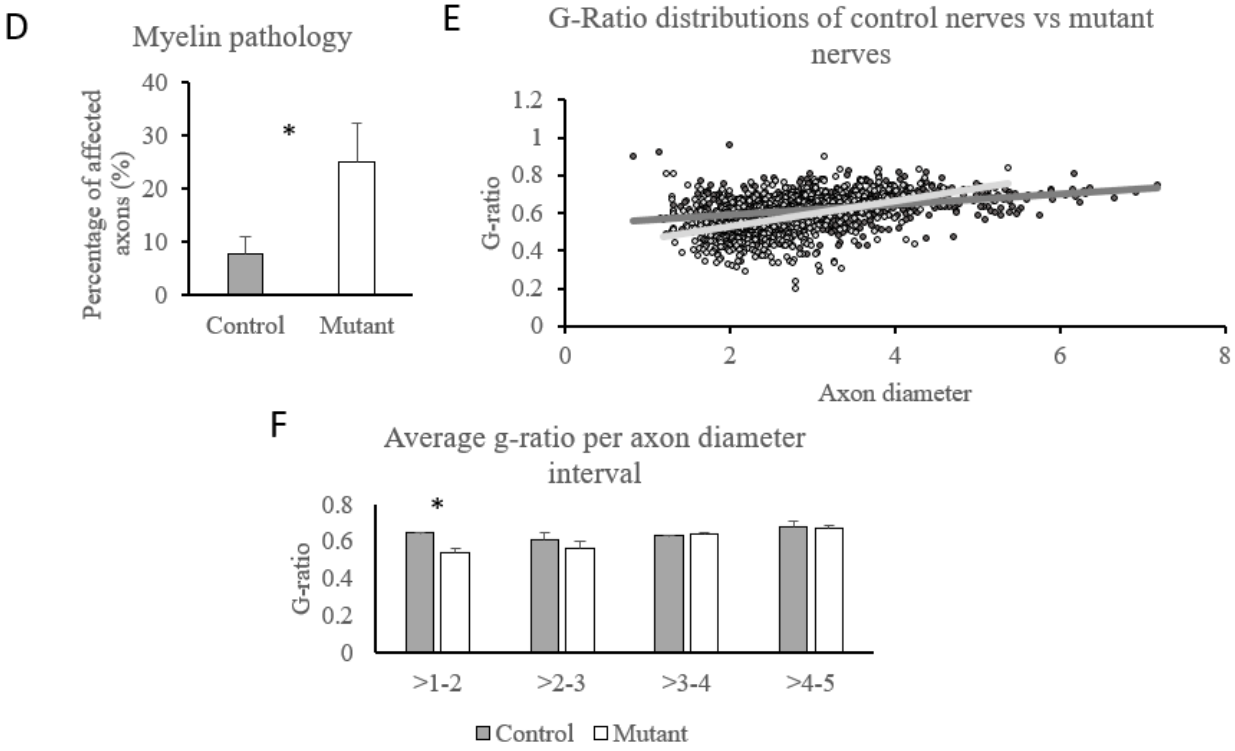
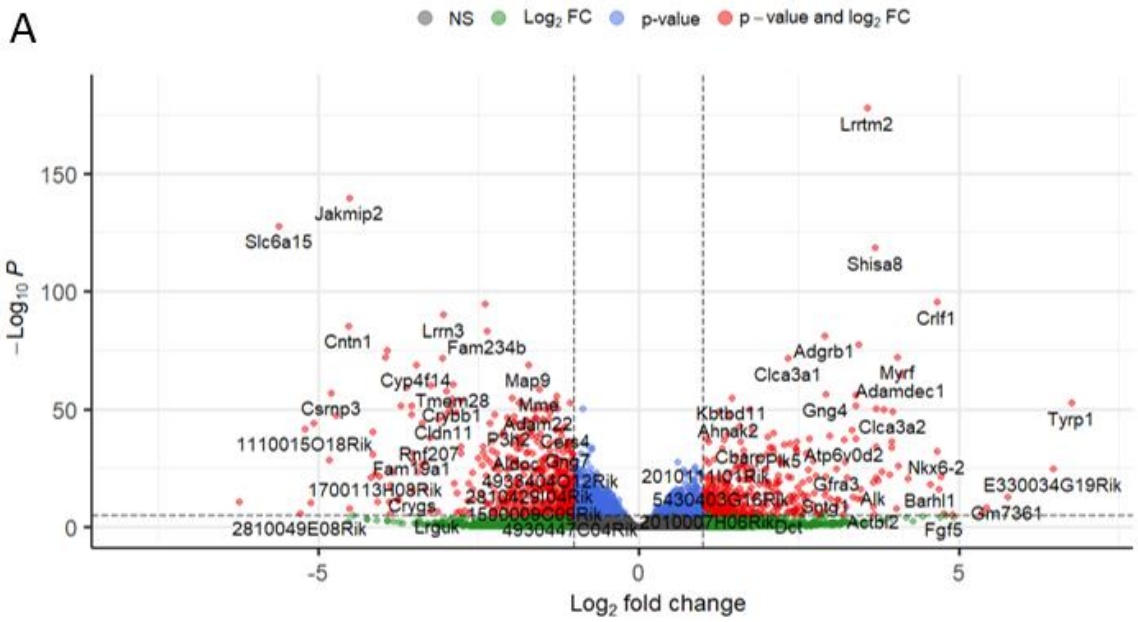

D
Myelin pathology
Percentage of affected axons (%)
40
30
20
10
0
*
Control
Mutant
E
G-Ratio distributions of control nerves vs mutant nerves
G-ratio
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
Axon diameter
F
Average g-ratio per axon diameter interval
G-ratio
0.8
0.6
0.4
0.2
0
*
>1-2
>2-3
>3-4
>4-5
Control
Mutant

Figure 2. Schwann cell H2AK119 deubiquitinase activity is required for development. A. Electron micrographs of the sciatic nerves at 2 months of nerves of *Bap1* KO (*Bap1* fl/fl/*Mpz*-cre) mice and littermate controls. Scale bars 25 μm. **B,C.** Electron micrographs indicating the abnormal myelination in specific axons, some contain tomacula and myelin infolding/outfolding. **D.** The average percentage of abnormally myelinated axons per sample for each genotype. **E.** For g-ratio analysis (axon diameter/diameter of myelinated fiber), the diameter of axon and outer diameter of myelinated fiber were measured on over 700 randomly selected fibers per genotype. Data: n=2 per genotype. Filled circles and dark gray line are control, and open circles/light gray line are mutant. **F.** The average g-ratio is presented for axons within the specified diameter bins.

A

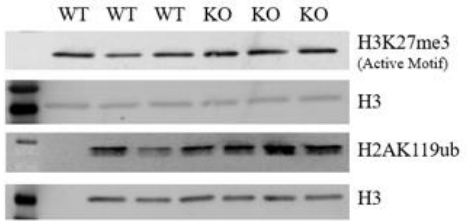


B *Bap1* KO:

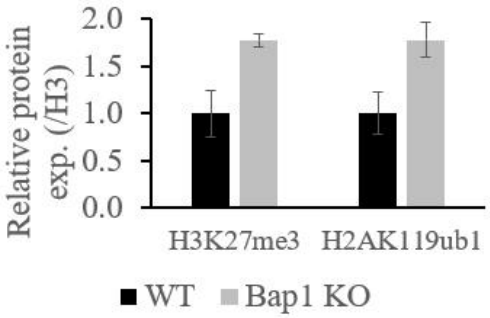
	Upregulated	Downregulated
Total genes:	2446	2788
>2.0 fold filter:	708	604
Regulated in *Eed*-cKO (960):	105*	52*

Figure 3. RNA-seq analysis of BAP1-deficient peripheral nerve. A. Volcano plot generated from DEseq2 for mutant over control in log2 scale that indicate significant overall changes. **B.** Table summarizes the upregulated and downregulated genes from KO samples compared to control with significant p-values and at least 2 fold change and the overlap with genes that are deregulated in the Schwann cell-specific knockout of *Eed*. The statistical significance for overlap between upregulated *Bap1* KO list and *Eed* cKO list is $p < 4.041e\text{-}32$. The statistical significance for overlap between downregulated *Bap1* KO list and *Eed* KO list is $p < 1.732e\text{-}07$. The asterisk indicates the statistical significance for the selected overlap.

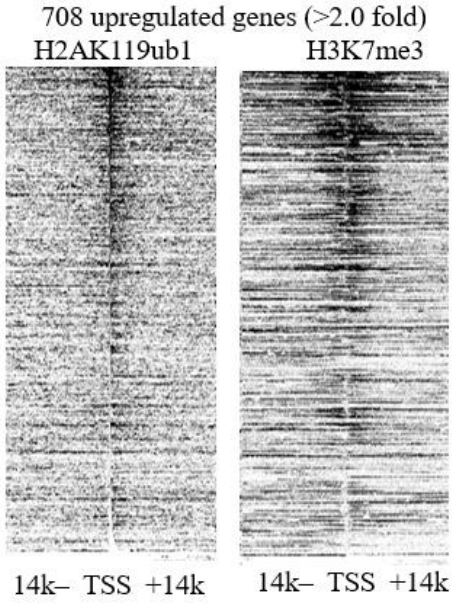
A



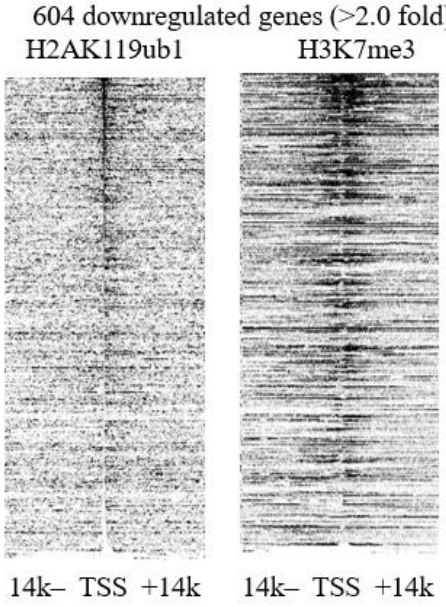
B



C



D



E

Bap1 KO:

	Upregulated	Downregulated
Total genes:	2446	2788
>2.0-fold filter:	708	604
H2AK119ub1:	172*	106*
H3K27me3:	216*	187*

Figure 4. *Bap1*-regulated genes are associated with both H2AK119ub1 and H3K27me3. A. Western blot analysis of lysates from *Bap1* KO and control was performed using the indicated antibodies. **B**. Bar graphs indicated the significantly higher global levels of H3K27me3 and H2AK119ub1 in Bap1 KO compared to control, after normalization to total histone H3 (n=3). **C.** The heatmaps display the distribution of H3K27me3 and H2AK119ub1 in rat sciatic nerve centered at TSS based on 708 upregulated *Bap1*-regulated gene list. The H3K27me3 and H2Ak119ub1 ChIP seq data were generated using two replicates. **D.** The heatmaps display the distribution of H3K27me3 and H2AK119ub1 in rat sciatic nerve centered at TSS based on 604 downregulated *Bap1*-regulated gene list. **E.** Table summarizes the upregulated and downregulated genes from KO samples associated with either or both H2AK119ub1 and H3K27me3 peaks proximal to TSS in rat sciatic nerve. The statistical significance for overlap between upregulated *Bap1* KO list and H2AK119ub1-regulated list is $p < 2.195e-42$. The overlap between upregulated *Bap1* KO list and H3K27me3-regulated list is $p<3.476e-40$. The overlap between downregulated *Bap1* KO list and H2AK119ub1-regulated list is $p < 2.282e-15$. The overlap between downregulated *Bap1* KO list and H3K27me3-regulated list is $p < 7.008e-36$. The asterisk indicates the statistical significance for the selected overlap.

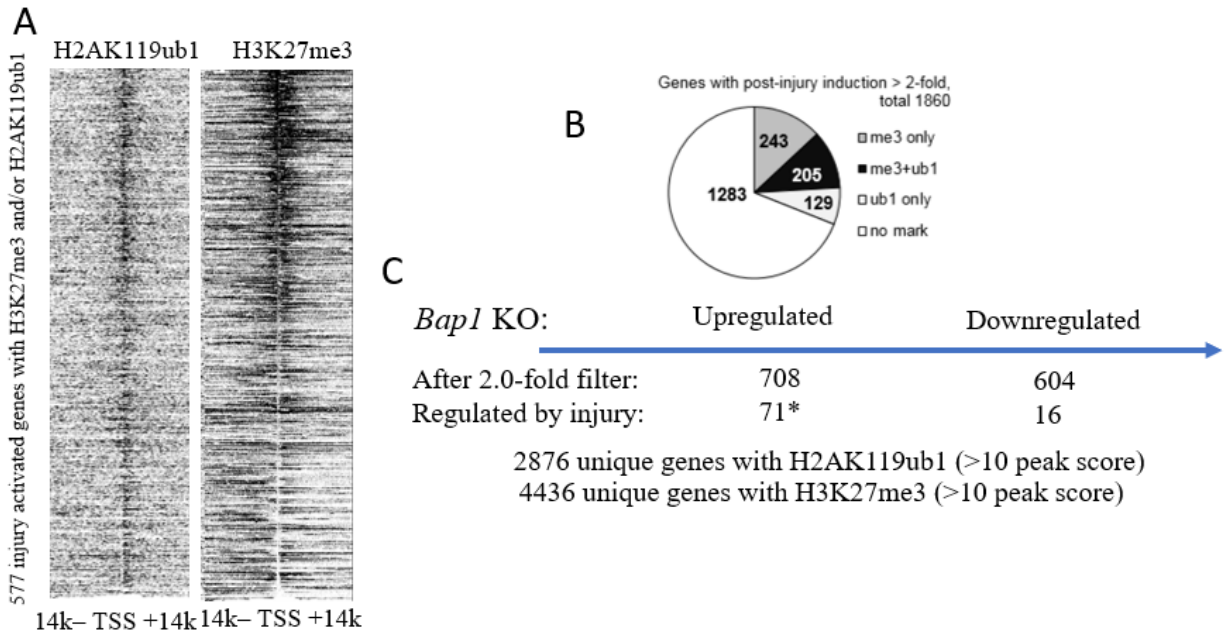


Figure 5. BAP1 deletion regulates genes induced by nerve injury. A. The heatmaps display the distribution of H3K27me3 and H2AK119ub1 in rat sciatic nerve centered at TSS based on 577 injury-activated genes. **B.** Piechart summarizes the numbers of injury-induced genes associated with either H2AK119ub1 and/or H3K27me3. **C.** Table summarizes the upregulated and downregulated genes from KO samples compared to control with significant p-values and at least 2 fold change, and the number that are also injury-regulated genes. The statistical significance for overlap between upregulated *Bap1* KO list and 577 Polycomb-injury list is $p < 7.520e-25$. The statistical significance for overlap between downregulated *Bap1* KO list and 577 Polycomb-injury list is $p < 0.375$. The asterisk indicates the statistical significance for the selected overlap.

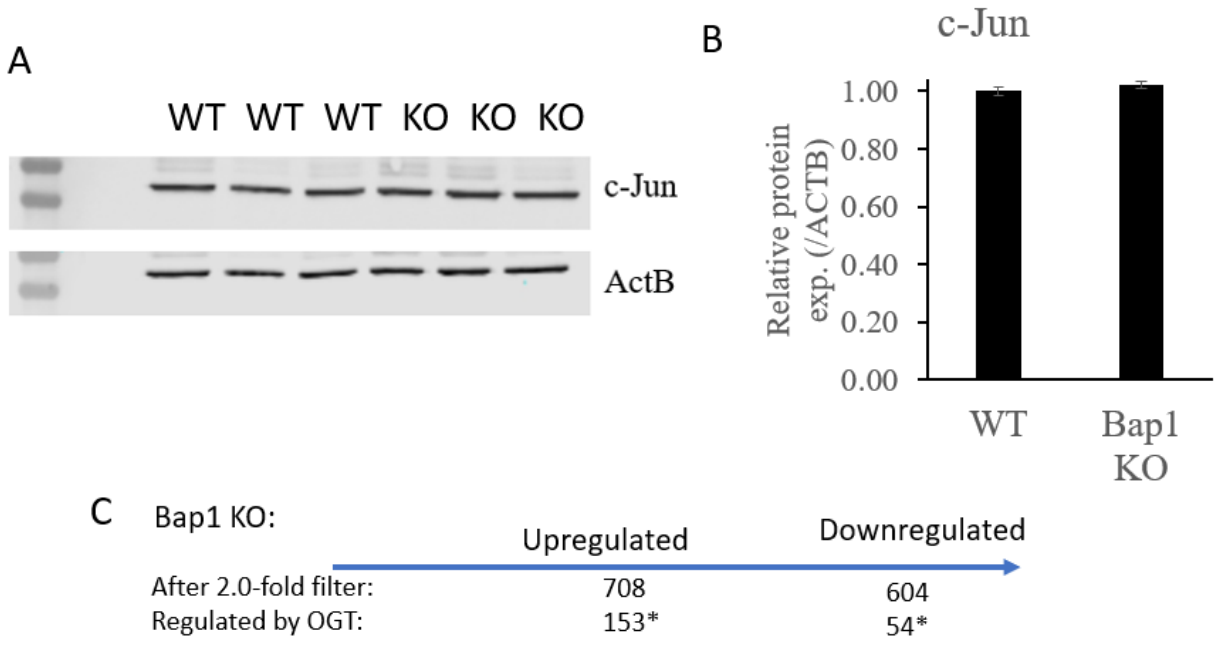


Figure 6. Overlap of genes regulated by BAP1 and OGT. A. Western blot analysis of lysates from *Bap1* KO and control was performed for JUN. **B**. Bar graph shows levels of JUN normalized to ACTB between *Bap1* KO and control (n=3). **C.** Table summarizes the upregulated and downregulated genes from KO samples compared to control with significant p-values and at least 2 fold change, and the number that also deregulated in the Schwann cell-specific knockout of OGT. The statistical significance for overlap between upregulated *Bap1* KO list and OGT KO list is $p < 1.262e-51$. The statistical significance for overlap between downregulated *Bap1* KO list and OGT cKO list is $p < 1.895e-04$. The asterisk indicates the statistical significance for the selected overlap.

GO Enrichment Analysis of Upregulated Genes

positive regulation of G2/M transition of mitotic cell cycle	27	12	2.62	4.57	+	9.13E-05	3.94E-03
↳positive regulation of mitotic cell cycle phase transition	87	28	8.46	3.31	+	6.18E-07	4.62E-05
↳regulation of mitotic cell cycle phase transition	287	72	27.89	2.58	+	5.53E-11	1.16E-08
↳regulation of mitotic cell cycle	456	100	44.32	2.26	+	7.21E-12	1.75E-09
↳regulation of cell cycle phase transition	371	80	36.06	2.22	+	1.62E-09	2.28E-07
↳positive regulation of cell cycle phase transition	108	32	10.50	3.05	+	4.70E-07	3.66E-05
↳positive regulation of cell cycle process	246	56	23.91	2.34	+	1.08E-07	9.69E-06
↳positive regulation of cell cycle	332	74	32.27	2.29	+	2.93E-09	3.98E-07
↳positive regulation of cellular process	5825	813	566.13	1.44	+	1.43E-28	5.62E-25
↳positive regulation of biological process	6350	863	617.15	1.40	+	2.98E-27	7.81E-24
↳positive regulation of mitotic cell cycle	122	32	11.86	2.70	+	4.84E-06	2.82E-04
↳positive regulation of cell cycle G2/M phase transition	30	13	2.92	4.46	+	5.76E-05	2.62E-03
↳regulation of cell cycle G2/M phase transition	105	33	10.20	3.23	+	1.01E-07	9.10E-06
↳regulation of G2/M transition of mitotic cell cycle	97	31	9.43	3.29	+	1.80E-07	1.56E-05
positive regulation of gliogenesis	78	19	7.58	2.51	+	8.95E-04	2.73E-02
↳positive regulation of neurogenesis	291	52	28.28	1.84	+	1.17E-04	4.88E-03
↳positive regulation of nervous system development	352	61	34.21	1.78	+	7.42E-05	3.30E-03
↳positive regulation of developmental process	1453	230	141.22	1.63	+	1.41E-11	3.18E-09
↳positive regulation of multicellular organismal process	1628	226	158.22	1.43	+	4.82E-07	3.74E-05
↳positive regulation of cell development	371	67	36.06	1.86	+	1.22E-05	6.60E-04
↳positive regulation of cell differentiation	956	158	92.91	1.70	+	2.25E-09	3.13E-07

GO Enrichment Analysis of Downregulated Genes

fatty acid beta-oxidation	55	25	6.36	3.93	+	2.71E-07	4.32E-05
↳fatty acid catabolic process	80	29	9.26	3.13	+	1.38E-06	1.75E-04
↳cellular lipid catabolic process	186	44	21.52	2.04	+	7.14E-05	5.41E-03
↳cellular lipid metabolic process	885	198	102.39	1.93	+	8.96E-16	7.06E-13
↳lipid metabolic process	1135	239	131.32	1.82	+	4.04E-16	3.54E-13
↳primary metabolic process	6405	935	741.06	1.26	+	5.62E-15	3.54E-12
↳metabolic process	7571	1076	875.96	1.23	+	7.80E-15	4.55E-12
↳organic substance metabolic process	7141	1015	826.21	1.23	+	1.23E-13	5.70E-11
↳cellular metabolic process	6518	966	754.13	1.28	+	2.28E-17	2.39E-14
↳cellular catabolic process	1573	262	182.00	1.44	+	4.53E-08	8.39E-06
↳catabolic process	1810	303	209.42	1.45	+	2.09E-09	5.67E-07
↳lipid catabolic process	274	63	31.70	1.99	+	3.50E-06	4.09E-04
cholesterol biosynthetic process	39	16	4.51	3.55	+	9.82E-05	7.23E-03
↳sterol biosynthetic process	45	18	5.21	3.46	+	4.79E-05	4.06E-03
↳organic hydroxy compound biosynthetic process	165	38	19.09	1.99	+	2.95E-04	1.91E-02
↳organic hydroxy compound metabolic process	441	87	51.02	1.71	+	1.31E-05	1.31E-03
↳steroid biosynthetic process	105	28	12.15	2.30	+	2.67E-04	1.78E-02
↳alcohol metabolic process	294	60	34.02	1.76	+	1.41E-04	9.90E-03
↳secondary alcohol biosynthetic process	39	16	4.51	3.55	+	9.82E-05	7.26E-03
↳small molecule biosynthetic process	390	80	45.12	1.77	+	8.18E-06	8.77E-04
intracellular lipid transport	30	12	3.47	3.46	+	8.54E-04	4.61E-02
↳lipid transport	287	56	33.21	1.69	+	6.43E-04	3.67E-02
↳organic substance transport	1831	332	211.85	1.57	+	4.89E-14	2.41E-11
↳lipid localization	323	61	37.37	1.63	+	7.03E-04	3.96E-02
smoothened signaling pathway	80	30	9.26	3.24	+	5.14E-07	7.29E-05
↳signal transduction	4962	487	574.10	.85	-	6.85E-05	5.26E-03
↳signaling	5282	535	611.12	.88	-	7.40E-04	4.14E-02
↳response to stimulus	8592	889	994.09	.89	-	4.80E-05	4.04E-03
Schwann cell development	35	13	4.05	3.21	+	9.19E-04	4.87E-02
↳cell development	1837	274	212.54	1.29	+	6.33E-05	5.06E-03
↳cell differentiation	3632	506	420.22	1.20	+	2.39E-05	2.20E-03
↳cellular developmental process	3685	517	426.35	1.21	+	9.73E-06	1.04E-03
↳developmental process	5627	793	651.04	1.22	+	2.49E-09	6.54E-07
↳anatomical structure development	5233	732	605.46	1.21	+	5.09E-08	9.22E-06
↳neurogenesis	1514	249	175.17	1.42	+	2.99E-07	4.58E-05
↳nervous system development	2114	355	244.59	1.45	+	4.13E-11	1.33E-08
↳system development	4223	607	488.60	1.24	+	4.82E-08	8.82E-06
↳multicellular organism development	4842	686	560.22	1.22	+	2.94E-08	5.87E-06
↳Schwann cell differentiation	40	14	4.63	3.03	+	9.50E-04	4.97E-02

Supplementary Figure 1. Gene ontology enrichment analyses of the upregulated and downregulated genes in *Bap1* KO. A. 2788 upregulated genes and 2466 downregulated genes significantly affected by *Bap1* KO were analyzed with the GO Enrichment Analysis, which is powered by PANTHER, by selecting the Biological Process and *Mus musculus* as criteria at Gene Ontology Resource website.

Chapter 4

Summary and Future Directions

Summary

Schwann cells produce a vast amount of lipid to myelinate the axon. The timing of myelination, the amount and composition of lipid substrates, and the selection of axons by Schwann cells are all carefully regulated by a number of epigenetic and genetic mechanisms (1). Polycomb repression complexes (PRC) are the major chromatin players for the development and nerve injury response in Schwann cells. The Schwann cell specific knockout of *Eed* in mouse model inactivates PRC2 and prematurely induces a number of nerve injury genes (2). Furthermore, this mutant model displayed a hypermyelination phenotype along with the increased level of proliferation after nerve injury. When measured by ChIP analysis, both H3K27me3 and H2AK119ub1 levels at the promoters of nerve injury genes were reduced in the *Eed* knockout (3,4). Normally, the levels of such histone marks are maintained in intact nerves and decrease after the peripheral nerve injury. We hypothesized that the active removal of H3K27me3 and/or H2AK119ub1 is the master switch for initiating the regeneration program in Schwann cells after nerve injury.

Here we focused on the potential mechanisms that regulate the H3K27me3 and H2AK119ub1 in Schwann cells respectively. The mechanism that involves the H3K27 demethylation appears to be the bottleneck for activating the nerve injury genes, and we hypothesized that *Jmjd3* and *Utx* would be required for injury gene activation. Both demethylases could have redundant functions, or they could target different sets of H3K27me3 occupied genes, especially the set of nerve injury genes. *Uty* is also a demethylase candidate, but it contains the catalytically dead domain which makes it less likely to play a significant role (5). According to the hierarchical model, existing H3K27me3 deposition attracts PRC1 to also deposit H2AK119 at the same genomic sites to co-repress the genes. We hypothesized that a number of deubiquitinase

candidates including *Bap1*, *Mysm1*, and *Usp16* (6-8) may have important roles in Schwann cells. However, *Bap1* is part of the mammalian PR-DUB complex that performs the deubiquitinating function (9). The PR-DUB complex also contains an OGT subunit which was reported to regulate the development and nerve injury response in Schwann cells (10,11). We sought to develop Schwann cell specific conditional knockouts of both demethylase and deubiquitinase candidates to test their roles in regulation of nerve injury genes.

H3K27 demethylases in Nerve Injury

The Schwann cell-specific double knockout of *Jmjd3* and *Utx* genes in mouse model exhibited a relatively small set of affected genes even though the level of H3K27me3 at key nerve injury genes was maintained even after nerve injury (4). However, for the most part, the mouse model underwent normal development and displayed no abnormal behavior from birth to maturity. Prior to the nerve injury, *Jmjd3* and *Utx* were initially thought to be expressed at a low basal level and would increase after injury. Based on the report of Gomez Sanchez et al., Jmjd3 was proposed to activate the polycomb-repressed CDKN2A which is an important regenerative element of Schwann cell repair (12). However, RNA seq and western blots indicate that *Jmjd3* does not change much after injury, and the level of proliferation stays relatively similar after injury in sciatic nerve of mutant mice compared to control. Most of the gene set affected by the double knockout has little or no known role in neither Schwann cell development nor nerve injury response. However, we noted that some of the nerve injury genes such as *Shh* and *Gdnf* are slightly delayed from induction at 1 dpi in mutant mice. Since the nerve injury genes are highly induced in the control, we were interested to test the longer timepoint after nerve injury. The level of induction was eventually restored to be similar as that of control after 3 dpi, indicating that there are other mechanisms that bypass Polycomb repression. It is possible that *Jmjd3* and *Utx* catalytic activity

is required for full injury gene induction at 1 dpi. Then other mechanisms would have compensated the losses of *Jmjd3* and *Utx* at later timepoint. However, overall the demethylases are not required for the derepression of nerve injury genes.

H2AK119 deubiquitinases and Polycomb Repression

The alternative model of H2AK119ub1 was proposed to regulate the nerve injury genes. H2AK119ub1 is associated with many of the nerve injury genes at the promoter sites and also affected by *Eed* KO (4). The Schwann cell-specific knockout of *Bap1* was developed to test the hypothesis that active removal of H2AK119 is required to activate the nerve injury genes. However, the mouse knockout displayed the abnormal Schwann cell development as well as the premature transcriptional induction of some key nerve injury genes. Thinner myelination was observed for some axons of larger caliber in the mutant mice. A significant number of other axons contain tomacula or outfoldings/infoldings of myelin. It was not clear whether the deubiquitination was required to activate the genes, especially before the actual injury.

While it was also not clear how the lipid synthesis was affected by the loss of *Bap1*, the gene ontology study indicated some of the downregulated genes are involved with myelin production that plausibly explain the myelin defects (13). Despite the phenotypes, the mouse behavior appears to be largely normal in terms of gait and hindlimb clasping. It is likely that *Bap1* has some non-catalytic functions or alternative mechanisms that may indirectly activate the nerve injury genes considering the original hypothesis that the loss of Bap1 should result in strengthened Polycomb repression of nerve injury genes. Interestingly, the gene ontology study also points out that some molecular processes from the upregulated gene list from *Bap1* cKO are related to the cell cycle and proliferation. We attempted to test this by measuring p16/19 transcriptional levels and KI-67 protein level but none of the significant differences were observed (3).

One alternative model shows that *Bap1* primarily operates as a safeguard by limiting the spreading of H3K27me3 beyond normal limits. *Bap1* specifically removes H2AK119ub1 to help maintain the high concentration of Polycomb repression along with H3K27me3 at specific genomic sites, otherwise such repression can be lost (14,15). This mechanism could explain why some of the key nerve injury genes marked with both H3K27me3 and H2AK119ub1 are prematurely activated in *Bap1* KO mouse model. However, some of the other genes derepressed in *Bap1* KO have little relevance to nerve injury response in Schwann cells. Additionally, there are numerous genes associated with H2AK119ub1 that are not derepressed despite the loss of *Bap1*. Plausibly, this can be explained the work of Fursova et al., where the loss of *Bap1* resulted in simultaneous derepression of some sets of genes and repression of other sets of genes in same system (15).

We also attempted to test another alternative model that *Bap1* could potentially interact with OGT as subunits in PRC-DUB complex. OGT was previously studied in Schwann cells and is implicated in many aspects of Schwann cell's physiology (9-11,16). Schwann cell-specific knockout of OGT1 displayed abnormal development which was similar to that of BAP1 KO. Thinner myelination of larger caliber axons, thicker myelination of smaller caliber axons, and some degree of tomacula were all observed in both OGT KO and *Bap1* KO respectively. Therefore, we hypothesized that *Bap1* KO could have phenocopied OGT KO in terms of transcriptome. OGT KO displayed the elevated level of *c-Jun*, implying that *c-Jun* is repressed by OGlncAc post-translational modification. We measured *c-Jun* in *Bap1* KO at both transcriptional and protein levels. *Fos*, one of JUN components, was elevated by 2.8 fold. However, JUN protein level did not significantly differ from control. We have not fully concluded that BAP1 does not interact with

OGT yet, and we believe this side project is still worth pursuing for further characterization of the relationship between OGT and BAP1.

Future Directions

Currently there are no other known H3K27me3 demethylases but there are additional H2AK119 deubiquitinases that are untested in Schwann cells such as Usp16 and Mysm1 (7,8). It is possible that each of the DUB regulates different sets of nerve injury genes or even perform the redundant role. It is of interest to engineer the conditional knockouts of each gene in mouse models and observe the impacts on both transcriptional levels and developmental phenotypes. Specifically, we would want to see if the knockout may cause aberrant mouse behaviors or any kind of abnormality in myelination and nerve conductance. If the phenotype is observed, then the proposal models will be further tested with various biochemical, molecular, and bioinformatic approaches to delineate their roles in the existing upstream or downstream pathways that regulate myelin thickness and regeneration program. At chromatin level, it would be interesting to see how H2AK119ub1 genome-wide distribution is affected for each genotype in different conditions – sham, various timepoints after injury, and exposure to pharmacological drugs. These would result in a much better understanding of how the Polycomb regulation takes the place in Schwann cells.

Bap1 also warrants further testing especially for distributions of H3K27me3 and H2AK119ub1 utilizing ChIP-seq or Cut & Run tool in Schwann cells. Additionally, we would be very interested in determining the effect of BAP1 KO on the nerve injury response in Schwann cells but would require the inducible knockout rather than conditional knockout. Since the premature induction of nerve injury genes were observed, it is likely the nerve injury would result in further augmentation of the nerve injury genes possibly leading to the delayed regeneration, which is seen in OGT KO (10). Evolving technologies will make it possible to profile the histone

distributions at single cell level. Such data will allow for us to be able to better distinguish the Schwann cell-specific gene changes and others from sciatic nerves considering that fibroblasts, axonal nerve cells and macrophages also play important roles in regeneration (17).

It is also possible that deubiquitination alone is not sufficient to activate the nerve injury genes since many nerve injury genes are associated with H3K27me3, H2AK119, and H3K4me3. One model involves the hierarchical co-repression where H3K27me3 recruits PRC1 to deposit H2AK119ub1 to the same site (18). Another model is similar but PRC1 takes the greater priority and PRC2 in turn is being recruited afterward to deposit the H3K27me3 (19). This hypothesis can be tested by developing a PRC1 knockout through ablation of RING1A/B in mouse model and profiling its nerves to see whether the H3K27me3 and H2AK119ub1 distributions change between intact and injury conditions. The expectation is that we would see greater premature inductions of the key nerve injury genes such as *Shh* and *Gdnf* compared to EED KO. Additionally, better understanding of the Polycomb regulation would require further studies of PRC1 repression including its many noncanonical forms. PRC1 contains greater diversity of subunits and accessory proteins compared to PRC2 (20). The compositions and functions of variant PRC1s such as PRC1.2 and PRC1.6 are relatively unknown in Schwann cells. It may be challenging and tedious to develop the knockout model for each composition, therefore we should focus on employing the similar approaches with canonical forms first like we did with Schwann cell-specific *Jmjd3*/*Utx* DKO and *Bap1* KO mouse lines.

It is entirely possible that Polycomb derepression may be only secondary to the unknown mechanisms in activating the nerve injury genes. Cell division is not fast enough to dilute the repressive marks (21). Mechanisms that involve PRC1 and PRC2 depletion or inhibition may be plausible, which would explain that demethylases and deubiquitinases may be not needed at all. It

would require long non-coding RNAs or proteins that inhibit or deplete PRC1/2. Alternatively, *Bap1* or other chromatin regulator could recruit COMPASS complex and in turn activate the nerve injury genes through the deposition of H3K4me3, a typically activating histone mark (22). It was already observed that many of the nerve injury genes are associated with the higher level of H3K4me3 after nerve injury. The implication of this proposal is that Polycomb-repressed genes can be still activated without demethylation or deubiquitination, as H3K27me3 and H2AK119ub1 are not necessarily always repressive in some contexts (15). Therefore, we propose to knock out the catalytic components, specifically SET1, of COMPASS complex in Schwann cells in rodent models as well. However, COMPASS complex also contains a high diversity of subunits like PRC1 does. These approaches will pose a significant challenge especially if some subunits have redundant functions (22).

Ultimately, further examination of Polycomb regulation in Schwann cells requires extensive characterization of PRC1 noncanonical forms, Bap1's role in nerve injury context and its relationship with OGT, and additional DUB candidates. The role of H3K4 methyltransferase Compass and its relationship with *Bap1* in Schwann cells would be also worthwhile as a topic for deeper investigation. The findings may extend to the oligodendrocytes which are known to have some Polycomb repressed nerve injury genes as well. Oligodendrocytes have relatively limited regenerative capacity compared to that of Schwann cells (23), but it would be interesting to make comparison and see which nerve injury genes are revealed to be cell type specific. The proposed epigenomic profiling of Schwann cells here may help answer some of the very broad questions for many cell types in various organism models and cellular processes including higher order organization in nucleus, stem cell proliferation, and cancer development (24). For instance, Schwann cells are somatic and have an unusual ability to dedifferentiate after nerve injury which

we have already identified to involve extensive Polycomb regulation (3,25). This could provide potentially new understanding for studying the physiologies of embryonic or induced pluripotent stem cells. In other cases, Schwann cells can develop into malignant form of cancer called MPNST, which is partly driven by Polycomb regulation dysfunction (26). Further characterization of how the histone distributions change and affect the key genes in MPNST may help unlock some insights for other kinds of cancer.

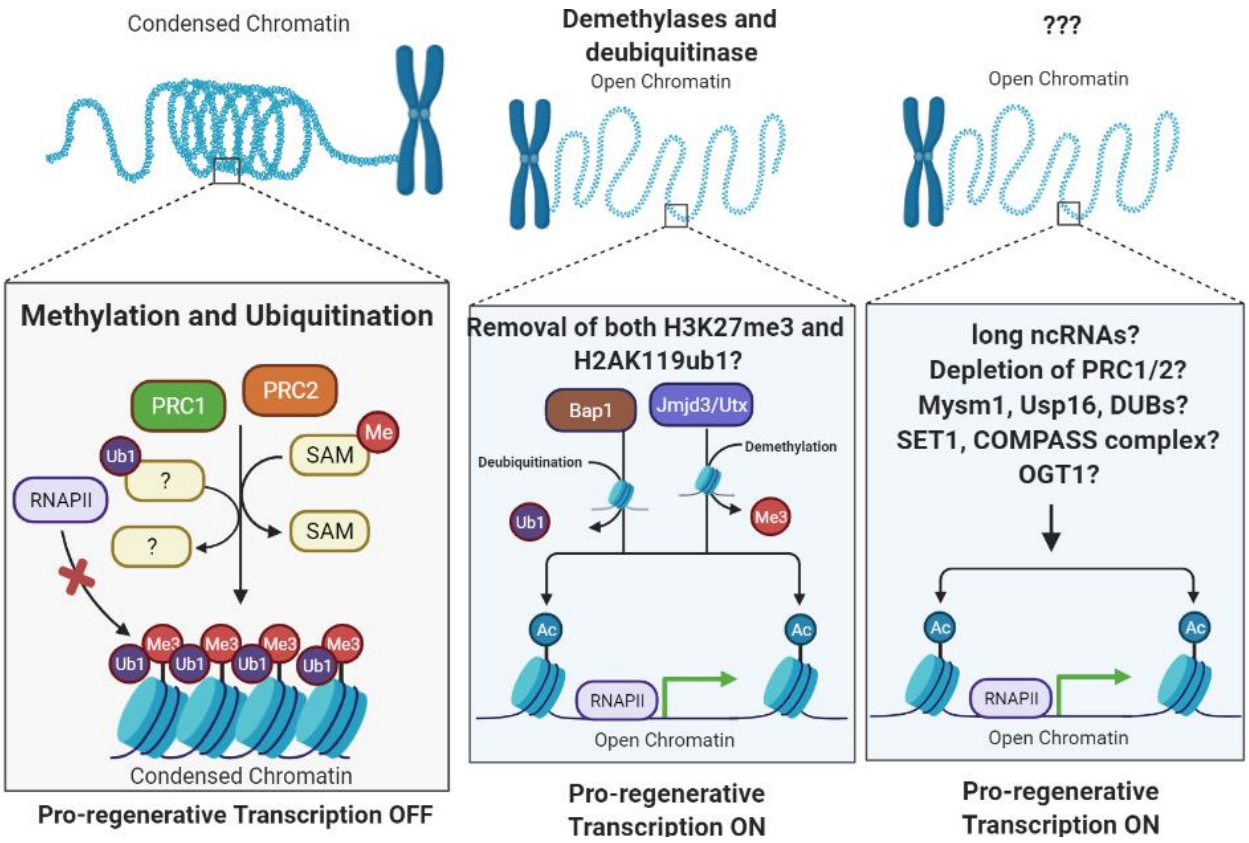


Figure 1. Alternative molecular models in activating the nerve injury genes in Schwann cell. The master switches that initiate the reprogramming of SCs potentially involve the Polycomb regulation. The Polycomb repression needs to be reversed for the nerve injury genes to become activated but there are a number of possible mechanisms that can remove such repression. Both demethylation and deubiquitination by *Jmjd3/Utx* and *Bap1* might be required. It is possible that long ncRNAs or other mechanism that deplete PRC1/2 are sufficient to remove the repression. Additional DUBs such as *Mysm1* and *Usp16* could be also involved. COMPASS complex and OGT subunit of PRC-DUB may mediate the pathways within the Polycomb regulation and should be further investigated.

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Appendix

Publications with Co-authorship

DUNCAN, I. D., BUGIANI, M., RADCLIFF, A. B., MORAN, J. J., LOPEZ-ANIDO, C., **DUONG, P.,** AUGUST, B. K., WOLF, N. I., VAN DER KNAAP, M. S. AND SVAREN, J. "**A Mutation In The Tubb4a gene Leads To Microtubule Accumulation With Hypomyelination And Demyelination**". *Annals Of Neurology*, vol 81, no. 5, 2017, pp. 690-702.

ABSTRACT

Objective: Our goal was to define the genetic cause of the profound hypomyelination in the taiep rat model and determine its relevance to human white matter disease.

Methods: Based on previous localization of the taiep mutation to rat chromosome 9, we tested whether the mutation resided within the Tubb4a (β-tubulin 4A) gene, because mutations in the TUBB4A gene have been described in patients with central nervous system hypomyelination. To determine whether accumulation of microtubules led to progressive demyelination, we analyzed the spinal cord and optic nerves of 2-year-old rats by light and electron microscopy. Cerebral white matter from a patient with TUBB4A Asn414Lys mutation and magnetic resonance imaging evidence of severe hypomyelination were studied similarly.

Results: As the taiep rat ages, there is progressive loss of myelin in the brain and dorsal column of the spinal cord associated with increased oligodendrocyte numbers with accumulation of microtubules. This accumulation involved the entire cell body and distal processes of oligodendrocytes, but there was no accumulation of microtubules in axons. A single point mutation in Tubb4a (p.Ala302Thr) was found in homozygous taiep samples. A similar hypomyelination associated with increased oligodendrocyte numbers and arrays of microtubules in oligodendrocytes was demonstrated in the human patient sample.

Interpretation: The taiep rat is the first animal model of TUBB4 mutations in humans and a novel system in which to test the mechanism of microtubule accumulation. The finding of microtubule accumulation in a patient with a TUBB4A mutation and leukodystrophy confirms the usefulness of taiep as a model of the human disease.

MA, K. H., **DUONG, P.**, MORAN, J. J., JUNAIDI, N. AND SVAREN, J. "**Polycomb Repression Regulates Schwann Cell Proliferation And Axon Regeneration After Nerve Injury**". *Glia*, vol 66, no. 11, 2018, pp. 2487-2502.

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.