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Vitamin C regulates Schwann cell myelination by promoting DNA demethylation of pro-myelinating genes

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Abstract

Ascorbic acid (vitamin C) is critical for Schwann cells to myelinate peripheral nerve axons during development and remyelination after injury. However, its exact mechanism remains elusive. Vitamin C is a dietary nutrient that was recently discovered to promote active DNA demethylation. Schwann cell myelination is characterized by global DNA demethylation in vivo and may therefore be regulated by vitamin C. We found that vitamin C induces a massive transcriptomic shift (n = 3,848 genes) in primary cultured Schwann cells while simultaneously producing a global increase in genomic 5-hydroxymethylcytosine (5hmC), a DNA demethylation intermediate which regulates transcription. Vitamin C up-regulates 10 pro-myelinating genes which exhibit elevated 5hmC content in both the promoter and gene body regions of these loci following treatment. Using a mouse model of human vitamin C metabolism, we found that maternal dietary vitamin C deficiency causes peripheral nerve hypomyelination throughout early development in resulting offspring. Additionally, dietary vitamin C intake regulates the expression of myelin-related proteins such as periaxin (PRX) and myelin basic protein (MBP) during development and

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AUTHOR CONTRIBUTIONS

G.W. conceived, designed, and supervised the study; T.C.H., V.C., N.S.A, and S.M. executed cell-based experiments. D.W.S. and T.C.H analyzed sequencing data. P.M. designed and executed Schwann cell assays used in sequencing experiments. T.C.H. and G.W. drafted the manuscript. All authors edited, commented, and approved the final manuscript.

CONFLIC T OF INTEREST

The authors declare no competing interests.

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remyelination after injury in mice. Taken together, these results suggest that vitamin C cooperatively promotes myelination through 1) increased DNA demethylation and transcription of pro-myelinating genes, and 2) its known role in stabilizing collagen helices to form the basal lamina that is necessary for myelination.

Keywords

5-hydroxymethylcytosine; DNA demethylation; myelin; Schwann cell; vitamin C

1 | INTRODUCTION

Schwann cells are the myelinating glia of the peripheral nervous system responsible for saltatory conduction of peripheral nerve axons (Salzer, 2015). Schwann cells sort and ensheathe axons late in embryonic development before initiating myelination postnatally (Jessen & Mirsky, 2005; Monk, Feltri, & Taveggia, 2015). After peripheral nerve injury, Schwann cells help promote axonal regeneration and remyelinate axons which cross the injury site and rein-nervate distal targets (Stassart et al., 2013). Numerous molecular mechanisms have been implicated in controlling Schwann cell myelination throughout development and after peripheral nerve injury. Ascorbic acid (Vitamin C) has long been known to be necessary for Schwann cell myelination of peripheral axons both in vitro and in vivo: Schwann cell dorsal root ganglion (DRG) co-cultures fail to produce myelinated segments without the presence of vitamin C in the culture medium (Eldridge, Bunge, Bunge, & Wood, 1987). Similarly, mice deficient of Sodium-dependent Vitamin C transporter 2 (SVCT2), the principal vitamin C transporter in Schwann cells, exhibits hypomyelination of peripheral nerves and deficient remyelination after injury (Gess, Lohmann, Halfter, & Young, 2010; Gess et al., 2011; Rohr, Halfter, Schulz, Young, & Gess, 2017). The pro-myelinating effect of vitamin C is thought to be mediated by its role in collagen production and cross-linking. Vitamin C is a cofactor for Prolyl 4-hydroxylase (P4) which stabilizes the collagen triple helix, a necessary extracellular matrix component of the basal lamina which is essential to Schwann cell myelination (Eldridge et al., 1987; Gorres & Raines, 2010). However, vitamin C also promotes myelination in oligodendrocytes which do not synthesize a basal lamina (Guo, Suo, Cui, Yuan, & Xie, 2018). This suggests that vitamin C promotes Schwann cell myelination independently of P4 activity and that the molecular mechanism underlying its pro-myelinating effect is more complex than initially understood.

We recently found that vitamin C promotes the activity of Ten-eleven translocation (TET) dioxygenases that are responsible for active DNA demethylation and 5hmC generation (Dickson, Gustafson, Young, Zuchner, & Wang, 2013; Minor, Court, Young, & Wang, 2013). TET enzymes convert methylated cytosine (5mC) into 5-hydroxymethylcytosine (5hmC), a stable epigenetic mark that recruits a unique set of binding partners compared with 5mC to regulate transcription (Spruijt et al., 2013). 5hmC can remain in the genome to enable transcriptional changes or can be further oxidized by TET enzymes into transient intermediates that are excised by the base excision repair pathway to produce unmodified cytosine and complete active DNA demethylation (He et al., 2011; Ito et al., 2011; Maiti & Drohat, 2011). Alterations in promoter and gene body 5hmC content drive changes in gene

transcription (Shi, Ali, Tang, & Yang, 2017). TET enzymes and 5hmC have numerous roles in neuronal function, development, and synaptic plasticity in vivo (Li et al., 2014; Rudenko et al., 2013; Xu et al., 2012; Yu et al., 2015). However, despite their well-described function in neuronal processes, the epigenetic role of vitamin C, TET enzymes, and 5hmC in Schwann cell myelination has yet to be elucidated.

Methylation dynamics of DNA and histone lysine residues play important roles in Schwann cell myelination and function (Fuhrmann, Mernberger, Nist, Stiewe, & Elsasser, 2018; Gomez-Sanchez et al., 2013; Ma, Hung, & Svaren, 2016). Most notably, Schwann cell myelination is characterized by global loss of DNA methylation and demethylation of myelin-related genes throughout development (Varela-Rey et al., 2014). Additionally, mice lacking the *Gnmt* gene which encodes the enzyme responsible for metabolizing S-Adenosylmethione, the methyl donor responsible for DNA methylation exhibits rampant global methylation and hypomyelination of peripheral nerves (Varela-Rey et al., 2014). This suggests a negative correlation between methylation and myelination whereby hypermethylation results in hypomyelination. Conversely, molecular agents which promote DNA demethylation, like vitamin C, may subsequently thwart methylation levels to promote myelination.

In this study, we investigated the epigenetic role of vitamin C in Schwann cell myelination. Vitamin C treatment of primary cultured Schwann cells induced massive changes in transcription and a global increase in genomic 5hmC. Vitamin C up-regulated the transcription of 10 myelin-related genes which exhibited concurrent changes in both promoter and gene body 5hmC content. Using a mouse model relevant to human vitamin C metabolism, we found that dietary vitamin C deficiency impaired peripheral myelination throughout early development. Finally, we found that dietary vitamin C intake was necessary for the expression of periaxin (PRX) and myelin basic protein (MBP), critical components of the myelin sheath, throughout development, and during remyelination after injury. Overall, these results suggest that vitamin C promotes Schwann cell myelination not only through collagen stability but also through direct epigenetic regulation of myelin-related genes.

2 | METHODS

This study was not pre-registered with OSF Registries.

2.1 | Materials

The following primary antibodies were used in this study: PRX (Sigma cat#HPA001868, RRID:AB_2172440, 1:1,000), MBP (Novus Biologicals cat#MAB42282, 1:500), 5hmC (Active Motif cat#39769, RRID:AB_10013602, 1:1,000). Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, RRID:AB_2768317, 1:1,000), and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invtirogen, RRID:AB_2768316, 1:1,000) secondary antibodies were used for all immunofluorescence-based experiments.

2.2 | Animals

C57BL/6 background mice containing a functionally inactive Gulonolactone oxidase (*Gulo*) gene were obtained from the Mutant Mouse Resource & Research Centers (MMRRC cat #000015-UCD, RRID:MGI:3583115, NIH) and were handled as approved by the Institutional Animal Care and Use Committee (IACUC). Mice were maintained as previously described (Maeda et al., 2000). Briefly, homozygous $Gulo^{-/-}$ mice of both sexes were weaned at 3 weeks of age and fed ad libitum with irradiated mouse chow (Teklad, diet #2918). Mice were allowed free access to water which was supplemented with vitamin C. During colony expansion and maintenance, mice were provided water containing 330 mg L-Ascorbic acid/liter and 0.01 mM EDTA which was prepared fresh weekly. For developmental studies, breeding pairs of homozygous $Gulo^{-/-}$ mice were provided water upon mating supplemented with either 330 mg/L or 99 mg/L L-ascorbic acid (to reflect vitamin C sufficiency and deficiency, respectively). The resulting pups mice of both sexes from ascorbate sufficient and deficient breeding pairs were killed by CO_2 asphyxiation followed by cervical dislocation at postnatal days P4, P9, and P17. Pup sciatic nerves were then collected, fixed, and maintained in 30% sucrose until processed for further histological analysis. Multiple mothers at different times were provided either sufficient or deficient doses of vitamin C. Therefore, resulting pups were not born at the same time and were not from the same litter. Mice were allocated to the appropriate studies arbitrarily without randomization. No sample size calculation was performed. Instead, sample size conventions from the field were used. Three mice per group were used for developmental studies; thus 18 mouse pups were used for these experiments. Mice for developmental experiments were housed as breeding pairs with their resulting litter. No exclusion criteria were pre-determined. Animals were given access to food and water at all times. For histochemical and immunofluorescent analysis, animal name and group identification were removed by one experimenter and analyzed by another experimenter. Therefore, analyses were performed by a blinded experimenter ignorant of animal identities and their associated experimental group.

The experimenter was blind to the identities of animals and their respective groups. The experimental timeline and sample size of each experiment are described in Figure 1.

2.3 | Cell culture

Primary cultured Schwann cells were isolated from 3-month-old Fisher rat (RRID:RGD_734478) sciatic nerves as previously described (Bacallao & Monje, 2015). Rats were of both sexes, of normal weight (200g), had access to food and water at all times, and were house in a cage containing five rats. Rats were sacrificed by CO_2 asphyxiation followed by cervical dislocation and sciatic nerve collection. Nerves were cut into small segments, incubated in vitro for 10 days in DMEM medium containing 10% heat-inactivated FBS, and allowed to degenerate. Degenerated nerve explants were dissociated using a 0.25% dispase/0.05% collagenase solution and the resulting cell suspension was plated on poly-L-lysine (PLL)-coated dishes (Sigma). Purified primary Schwann cells were expanded up to passage one in DMEM media containing 10 nM neuregulin (recombinant heregulin-β1) and 2 μM forskolin. Experiments were performed on Schwann cells between 3 and 6 passages and plated on glass coated in PLL and laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane. Schwann cells were isolated from at least five different rats

and used for all downstream experiments. Schwann cells were grown for experiments in DMEM supplemented with 10% FBS and without neuregulin or forskolin. In vitro experiments using vitamin C were performed using 50 μM sodium ascorbate unless otherwise stated in the text.

2.4 | Semithin sectioning

Sciatic nerves were dissected from Vitamin C sufficient and deficient Gulo$^{-/-}$ mice and resin embedded for semithin sectioning as described previously (Rebelo et al., 2018). Nerves were fixed in 3% glutaraldehyde 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C and subsequently placed in 30% sucrose until processed. Nerves were post-fixed with 2% osmium tetroxide and dehydrated using a graded ethanol series (50%, 75%, 95%, and 100%). After dehydration, nerves were embedded in Spurr's resin (EMS, Cat# 14,300), polymerized at 80°C overnight, and sectioned at 1,000 nm for light microscopy using an EM UC6 ultramicrotome (Leica). Sections of 1,000 nm semithin were transferred to glass slides, stained with 1% alkaline toluidine blue solution, and photographed with the Nikon Eclipse TE2000-U. G-ratio, axon diameter, and fiber diameter measurements were executed using FIJI (Schindelin et al., 2012).

2.5 | Nerve crush

2.5-month-old homozygous Gulo$^{-/-}$ mice of both sexes and normal weight (20–28g) were provided water containing either 330 mg/L or 16.5 mg/L L-ascorbic acid for 2.5 months to induce vitamin C sufficiency and deficiency, respectively. For nerve crush surgeries, mice were anesthetized by 3% isofluorane, the fur from the right hindlimb was removed, and the sciatic nerve was exposed. The sciatic nerve was crushed with Dumont #5 forceps for 30 s and the surgical wounds were sealed using nylon thread. Sham surgery was also performed on the contralateral leg but without nerve crush. Surgeries were performed midday. Following injury mice were given 1 mg/ml acetaminophen analgesic as described previously (Danzi, Motti, Avison, Bixby, & Lemmon, 2016). The dose of L-ascorbic acid given to vitamin C deficient mice was then doubled to 33 mg/L after surgery to assist wound healing but to also maintain global vitamin C deficiency. Mice were killed by CO_2 asphyxiation followed by cervical dislocation 14 days post-injury (dpi) and both sham and crushed nerves were collected, fixed overnight, and maintained in 30% sucrose until processed for further histological analysis. For histochemical and immunofluorescent analysis, animal name and group identification were removed by one experimenter and analyzed by another experimenter. Therefore, analyses were performed by a blinded experimenter ignorant of animal identities and their associated experimental group. The experimental timeline and sample size of each experiment are described in Figure 1. Three animals were used per condition; thus, six total mice were used for the experiment. Mice for nerve crush experiments were housed as before surgery in cages containing five animals. Animals were placed in individual cages following surgery to recover from injuries. Animals were given access to food and water at all times.

2.6 | Sciatic nerve sectioning and immunofluorescence

Sciatic nerves used in immunofluorescence experiments were fixed in 4% paraformaldehyde overnight and maintained in 30% sucrose until further processing. Nerves were embedded in

OCT solution (VWR), snap frozen in liquid nitrogen, cut longitudinally into 12 μm frozen sections, and then mounted onto glass slides. Sections were permeabilized using 0.4% Triton X-100, blocked in 3% bovine serum albumin (BSA), and then left to incubate overnight at 4°C with primary antibody in blocking buffer solution. Slides were then incubated in fluorophore-conjugated secondary antibody for 1 hr at 20°C, washed in PBS, and then counterstained with DAPI before mounting.

2.7 | IncuCyte ZOOM live cell imaging

Live cell imaging was performed using an IncuCyte ZOOM (Essen BioScience). Schwann cells were seeded into 24-well plates at 10,000 cells per well coated in PLL and laminin and allowed to settle overnight at 37°C. Cells were treated with vitamin C (50 μM, 0 hr), placed at 37°C, and then imaged every 2 hr for a total of 90 hr. High-throughput analysis of process length (mm/cell body cluster), well confluence and total cell body clusters (a machine mask measurement of total cell numbers) was performed at every time point following image capture.

2.8 | 5hmC immunocytochemistry

Cells were seeded onto 24-well plates containing glass 12 mm coverslips at 10,000–50,000 cells per well. After treatments were performed, cells were fixed with 4% paraformaldehyde and incubated with 2N HCl at 37° for 20 min before neutralization with 100 μM Tris-HCl for 10 min. Cells were then washed with PBS, blocked with 3% bovine serum albumin, and 0.4% Triton X-100 in PBS for 1 hr, then incubated with anti-5hmC primary antibody (Active Motif cat#39769, 1:1,000) at 4° overnight. Cells were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000) and counterstained with DAPI.

2.9 | Image acquisition

Images from immunofluorescence experiments were captured using a Zeiss LSM 710 confocal microscope at a bit depth of 16 and into a 512 × 512 frame size. Fluorescence intensity was quantified using Fiji software (Schindelin et al., 2012). Pixel intensity values were averaged eight times and measured from every cell within the image field from a minimum of five fields per condition (~500 cells total).

2.10 | RNA-seq analysis

Schwann cells were cultured to 75% confluency and treated with or without sodium ascorbate (50 μM) for 7 days. The RNA-seq experiment was performed from one rat line with three independent cell culture preparations in each condition and completed in 1 day. Total RNA was extracted from cells using the RNEasy Mini Kit from Qiagen (Hilden, Germany). A Bioanalyzer 2000 was used to measure the quality of RNA. The RNA integrity numbers (RIN) of every sample was above 9. Whole transcriptome sequencing (also known as RNA-seq) was carried out at the Sequencing Core of John P. Hussman Institute of Human Genomics at the University of Miami using the TruSeq Stranded Total RNA Library Prep Kit from Illumina. Briefly, after ribosomal RNA (rRNA) was depleted, sequencing libraries were ligated with standard Illumina adaptors and subsequently sequenced on a Hiseq2000 sequencing system (125 bp paired-end reads, four samples per lane; Illumina). RNA-seq

analysis was performed using a previously published pipeline (Camarena et al., 2017; Sant et al., 2018). Raw read data were first run through quality control metrics using FastQC. Reads were trimmed with trim_galore to remove low-quality bases from reads (scores < 20 in Phred + 33 format) and Illumina adapters. After quality control was checked and trimming performed, sequence reads were aligned to the rat transcriptome (Rnor_6.0, Ensembl.org) and quantified using the STAR aligner (Dobin et al., 2013). Statistical significance was determined by edgeR and DESeq2. Differential genes were those with an adjusted P value below 0.05 by both algorithms (Love, Huber, & Anders, 2014; Robinson, McCarthy, & Smyth, 2010).

2.11 | hMeDIP-seq analysis

Schwann cells were cultured to 75% confluency and treated with or without sodium ascorbate (50 μM) for 7 days. The hMeDIP-seq experiment was performed from one rat line with three independent cell culture preparations in each condition and completed in 1 day. Genomic DNA was extracted from Schwann cells using QIAamp DNA mini kits from Qiagen (Hilden, Germany) according to the manufacturer's instructions. A Qubit Fluorometer from Life Technologies was used to quantify the concentration of DNA. A Bioanalyzer 2000 was used to measure the quality of DNA. DNA was submitted for hydroxymethylated DNA immunoprecipitation sequencing (hMeDIP-seq) at the Epigenomics Core at the University of Michigan. Briefly, DNA was sonicated to approximately 100 bp and then ligated with Illumina adaptors. A portion of the DNA was set aside for sequencing as un-precipitated input DNA and the remaining DNA was incubated overnight at 4°C with an antibody against 5hmC (Active Motif cat#39769). Antibodies containing immunoprecipitated DNA were then pulled down using Protein G magnetic beads (Invitrogen). Beads were re-suspended in proteinase K buffer and incubated for 3 hr at 55°C to remove the antibodies from the DNA. Unprecipitated input DNA was incubated with proteinase K buffer alongside the precipitated DNA. After proteinase K treatment, DNA was purified using AMPure beads (Beckman Coulter). Efficiency of immunoprecipitation was evaluated using the 5hmC, 5mC, and cytosine DNA standard pack (Diagenode). DNA was then sequenced on a HiSeq4000 sequencing system (50 bp single-end reads, three samples per lane; Illumina).

hMeDIP-seq analysis was performed using a previously published pipeline (Camarena et al., 2017; Sant et al., 2018). Reads were trimmed with trim_galore to remove low-quality bases from reads (scores < 20 in Phred + 33 format) and Illumina adapters. After quality control was checked sequence reads were aligned to the rat genome (Rnor_6.0, Ensembl.org) using BWA (Li & Durbin, 2010). Multi-mapped reads were removed using Samtools and duplicate reads were removed using PicardTools (https://broadinstitute.github.io/picard/) (Camarena et al., 2017; Li et al., 2009; Sant et al., 2018). To identify regions of the genome with substantial levels of 5hmC, peaks were called and filtered using the Irreproducible Discovery Rate (IDR) method developed for the ChIP-seq portion of the ENCODE project. Briefly, peak calling was performed with MACS2 using the narrow peak mode and a relaxed threshold of 0.001 (Zhang et al., 2008). Peaks were ranked from strongest to weakest peaks in each individual sample, and the rank order of common peaks among samples was compared with filter for only peaks with similar strength across all samples using the R-

scripts provided for the IDR pipeline with a threshold of 0.02. Reads within peak regions were quantified using HT-Seq-count (Anders, Pyl, & Huber, 2015). To minimize false positives, we considered only peaks with a minimum of 2X fold change and below an adjusted P-value of 0.05 by edgeR to be significant.

2.12 | Sequencing data

ChIP-seq and RNA-seq data have been uploaded to the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/, GSE13 7139).

2.13 | Statistical analysis

Quantitative data are presented as the mean and standard error of mean. GraphPad Prism 6 was also used to analyze data and generate graphs. Differential gene expression from RNA-seq and peak differences from hMeDIP-seq was determined by edgeR and DESeq2. For G-ratio calculations, G-ratios were plotted using linear regression and group means were used for statistical comparisons using Student's *t* test. Data were assessed for normality using D'Agostino & Pearson normality test within Graphpad Prism. For image analysis, pixel intensity values from every cell within the image field were plotted and analyzed by Kruskal–Wallis test with multiple comparisons test and Dunn correction. GraphPad Prism outlier calculator was used to identify outliers. No outliers were identified. Data were not assessed for normality.

3 | RESULTS

3.1 | Vitamin C deficiency hinders Schwann cell myelination throughout development

Mice with depleted SVCT2, the principal vitamin C transporter in Schwann cells, exhibit peripheral nerve hypomyelination as early as postnatal day 15 (P15) (Rohr et al., 2017). However, mice endogenously produce vitamin C in the liver starting from birth unlike humans who must consume vitamin C via the diet or supplementation (Wheeler, Ishikawa, Pornsaksit, & Smirnoff, 2015). Surprisingly to date, the effect of dietary vitamin C on peripheral myelination during early development has yet to be determined. To test whether dietary vitamin C is necessary for Schwann cell myelination, we assessed sciatic nerve myelination in Gulonolactone oxidase knockout ($Gulo^{-/-}$) mice who, like humans, lack the ability to endogenously synthesize vitamin C and must consume it through the diet (Maeda et al., 2000). Schwann cell myelination in rodents begins postnatally while pups solely consume vitamin C through the mother's milk. Therefore, we assessed myelination throughout development in pups reared from $Gulo^{-/-}$ parents provided a sufficient (330 mg/L) or deficient (99 mg/L) vitamin C diet through the water supply. Pups raised with deficient vitamin C exhibited a hypomyelinating phenotype as early as postnatal day 4 (P4) compared with pups raised with sufficient vitamin C (Figure 2a). This hypomyelinating phenotype persisted through P17 after a bulk of developmental myelination has occurred (Figure 2b and c). G-ratio quantifications of myelinating fibers (axon diameter/myelinated fiber diameter) further corroborate these findings. Additionally, there were no apparent differences in the percentages of myelinated axons between sufficient and deficient mice regardless of axon caliber or developmental time point (Fig. S1). Overall, our results show that vitamin C is necessary for proper Schwann cell myelination throughout development.

3.2 | Vitamin C promotes global DNA hydroxymethylation in Schwann cells

Vitamin C is thought to indirectly promote Schwann cell myelination through its function in collagen crosslinking. We and others previously reported that vitamin C also serves as a cofactor for ten-eleven translocation (TET) methylcytosine dioxygenases which promote DNA hydroxymethylation, thus making vitamin C an important epigenetic regulator of transcription (Young, Zuchner, & Wang, 2015). We reasoned that vitamin C may also promote DNA hydroxymethylation and alter transcription in Schwann cells. Vitamin C treatment of primary cultured rat Schwann cells induces 5-hydroxymethylcytosine (5hmC) generation in a dose-dependent manner. Treatment at 10 μM, the average concentration in human plasma corresponding to vitamin C deficiency (Institute of Medicine (US) Panel on Dietary Antioxidants and Related Compounds 2000), modestly increased global nuclear 5hmC content in Schwann cells ~18% above baseline. However, increasing the treatment to 50 μM, the average concentration of vitamin C in healthy human plasma, further elevated 5hmC ~ 70% above the level of control (Figure 3a and b). We then sought to quantify global 5hmC changes following vitamin C treatment using hydroxymethylated DNA immunoprecipitation sequencing (hMeDIP-seq). In total, 131,849 5hmC peaks were up-regulated and 53,090 peaks were down-regulated by vitamin C treatment (Figure 3c). These changes occurred in both promoter and gene body regions while being largely excluded from the transcription start site (TSS) (Figure 3d,e). Since vitamin C is also a cofactor for JmjC domain-containing histone demethylases, we investigated histone methylation changes in major trimethylated histone marks following vitamin C treatment in Schwann cells. We found that vitamin C induced demethylation of H3K9me3, H3K27me3, and H3K36me3 in Schwann cells while H3K4me3 remained unaffected (Fig. S2). Overall, our results show that vitamin C induces global DNA hydroxymethlation changes in the Schwann cell genome.

3.3 | Vitamin C promotes pro-myelinating gene expression

5hmC is a known regulatory mark of transcription which recruits a unique set of binding partners compared with 5-methylcytosine (5mC). We therefore sought to quantify transcriptional changes resulting from vitamin C treatment and alterations in 5hmC. Using RNA-seq, we found that vitamin C treatment induced differential expression of 3,848 transcripts, accounting for over 25% of all expressed transcripts (Figure 4a). Of the differential transcripts, 2,028 transcripts were up-regulated while 1,820 transcripts were down-regulated by vitamin C treatment, consistent with the bi-directional transcriptional regulation of 5hmC (Wu et al., 2011). We then investigated 5hmC profiles within differentially transcribed genes. Genes up-regulated by vitamin C treatment contained higher 5hmC content in promoter and gene body regions compared with down-regulated genes (Fig. S3). Additionally, up-regulated genes exhibited increased 5hmC content at the TSS while down-regulated genes did not. 5hmC is not only an intermediate of DNA demethylation but is also an epigenetic mark that recruits a unique set of binding partners compared with 5mC to regulate transcription (Spruijt et al., 2013). Since vitamin C induces massive changes in both transcription and global levels of 5hmC, we investigated gross biological pathways which were altered upon vitamin C treatment. Using Gene Ontology (GO) Biological Processes analysis, we discovered that a majority of pathways up-regulated by vitamin C are relevant to myelination during development including extracellular matrix (ECM) organization, myelin assembly, and axon guidance (Figure 4b). Simultaneously, the

majority of pathways down-regulated by vitamin C treatment are related to Schwann cell immune function, which is classically thought to be antagonistic to myelin formation and maintenance during development (Lindborg, Mack, & Zigmond, 2017; Mayo, Quintana, & Weiner, 2012). This shows that vitamin C activates pathways relevant to myelin formation and development while suppressing pathways which are antagonistic to myelination.

Since pathways relevant to myelination are activated upon vitamin C treatment, we then investigated transcriptional changes in pro-myelinating genes. Our RNA-seq data showed that vitamin C treatment up-regulated the transcription of 10 well-characterized pro-myelinating genes (Jessen & Mirsky, 2005) in primary Schwann cells, including prominent myelin sheath components such as Pmp22 (peripheral myelin protein 22), PRX (periaxin), and MBP (myelin basic protein), as well as enzymes of myelin lipid metabolism such as UGT8 (UDP Glycosyltransferase) (Figure 4c). Myelin genes such as Mpz (myelin protein zero) and MAG (myelin-associated glycoprotein) remained unaffected by vitamin C treatment. Notably, the top up-regulated gene following vitamin C treatment was Egr2 (Krox20), a transcription factor considered the master regulator of peripheral myelination (Svaren & Meijer, 2008). In contrast, vitamin C altered the transcription of only a few genes associated with immature and/ or mature (non-myelinating) Schwann cells such as L1cam and GFAP (Fig. S4a). It is worth noting that vitamin C did not affect transcription of c-Jun, a negative regulator of the myelin program in Schwann cells (Jessen & Mirsky, 2008), even in the presence of increased Krox20, which is considered an antagonist of c-Jun expression. We then probed our hMeDIP dataset to assess 5hmC content in the loci of the abovementioned genes. We found that all pro-myelinating genes assessed exhibited differential changes in 5hmC within their promoter or gene body regions following vitamin C treatment (Figure 4d). Differential changes in 5hmC rarely correlated with transcriptional changes in non-myelinating genes (Figure S4b and c). Overall, our results show that vitamin C up-regulates numerous pro-myelinating which may be driven, in part, by changes in 5hmC content along promoter and gene body regions of these loci.

3.4 | Vitamin C promotes the transcription and hydroxymethylation of ECM genes

Vitamin C supplementation up-regulates collagen IV and laminin expression in Schwann cell cultures (McGarvey, Baron-Van Evercooren, Kleinman, & Dubois-Dalcq, 1984). Vitamin C deficiency has been linked to an ablation of collagen I and IV expression in the sciatic nerve (McGarvey et al., 1984; Rohr et al., 2017). We found that Gulo$^{-/-}$ pups raised on a deficient vitamin C diet (99 mg/L) exhibited reduced expression of both collagen I and IV by P17 compared with pups raised on a sufficient vitamin C diet (Figure S6). DNA hypermethylation of collagen genes suppresses collagen expression in numerous cell types (Kopp, Winterhalter, & Trueb, 1997; Rhodes et al., 1994; Sengupta & Smith, 1998; Thompson, Simkevich, Holness, Kang, & Raghow, 1991). Therefore, vitamin C may promote collagen and laminin expression in Schwann cells via DNA demethylation. Given this evidence, we first assessed transcriptional changes following vitamin C treatment in Schwann cell collagen types I and IV. In parallel, we determined changes in the subunits for laminin-2, a heterotrimeric ECM protein known for its role in Schwann cell differentiation and myelination (Chen & Strickland, 2003; Yu, Chen, North, & Strickland, 2009). RNA sequencing analysis of vitamin C–treated Schwann cells showed an elevated transcription of

all three laminin-2 subunits (Lama2, Lamb1, and Lamc1) as well as all collagen I and IV alpha chains expressed in Schwann cells (Col1a1, Col4a1, Col4a2, and Col4a5) following 50 μM vitamin C treatment (Figure 5a). We next assessed 5hmC in the promoter and gene body regions of these loci. hMeDIP-seq revealed that vitamin C altered 5hmC in the promoter regions of collagen genes but did not alter promoter levels of 5hmC in laminins (Figure 5b). Similarly, vitamin C treatment altered gene body levels of 5hmC in all Schwann cell collagens and laminins assessed (Figure 5c). Interestingly, vitamin C up-regulated the transcription of nearly every collagen gene expressed in Schwann cells and 75% exhibited concurrent changes in promoter and gene body 5hmC following treatment (Table S1). These data show that vitamin C up-regulates the transcription of ECM genes and promotes simultaneous changes in promoter and gene body 5hmC content at these loci in Schwann cells. These findings are in agreement with prior observations in numerous cell types showing that DNA hypermethylation of collagen genes suppresses collagen expression (Kopp et al., 1997; McGarvey et al., 1984; Rhodes et al., 1994; Sengupta & Smith, 1998).

The increased transcription of laminin and collagen by vitamin C is expected to have an impact on Schwann adhesion and process extension in culture. Prolonged treatment with vitamin C (50 μM, 5 days) produced a morphological change and alignment of Schwann cells in culture. In contrast, untreated cells formed star-like clusters and exhibited no alignment of cell body or cell processes (Figure 5d). This observation prompted us to further investigate the changes in Schwann cell process length by using IncuCyte live cell imaging, which allows a temporal monitoring of the cultures by phase contrast microscopy and the quantitative assessment of parameters related to cell growth. Indeed, we found that a single treatment with vitamin C (50 μM) increased Schwann cell process length which persisted up to 90 hr at the conclusion of the experiment (Figure 5e*, right panel*). It is well-established that Schwann cells exhibit strong anchorage dependency to the ECM to proliferate and differentiate. We then assessed changes in Schwann cell proliferation following vitamin C treatment. Treatment with vitamin C at 50 μM, significantly increased the incorporation of EdU into nuclear DNA 5-fold above control levels (Fig. S5a and b). To corroborate this result, we then used IncuCyte live cell imaging to track proliferation of Schwann cells following treatment in real time. We found that a single treatment with vitamin C increased Schwann cell confluence percentage and total cell numbers, both measures of proliferation, beginning ~40 hr following treatment, and persisting up to 90 hr at the conclusion of the experiment (Fig. S5c). Overall, these data show that one way in which vitamin C can affect Schwann cell morphology and adhesion is by directly increasing the transcription of assorted collagen and laminin isoforms, most likely by increasing the content of 5hmC in promoter and gene body regulatory regions of these loci.

3.5 | Vitamin C regulates PRX and MBP expression throughout development and following injury in Gulo$^{-/-}$ mice

Vitamin C treatment up-regulated myelin proteins such as PRX and MBP in *in* vitro experiments (Figure 4c). PRX and MBP are both integral components of the myelin sheath necessary for proper myelination. PRX is predominantly expressed in myelinating Schwann cells and interacts with the dystroglycan complex to link the Schwann cell cytoskeleton to the basal lamina, a process that is essential to myelination (Dytrych, Sherman, Gillespie, &

Brophy, 1998; Gillespie et al., 2000). Similarly, MBP is one of the most abundant proteins present in peripheral myelin sheaths (Muller, Hochhaus, Fontana, Luhmann, & White, 2015). As vitamin C induces DNA demethylation and up-regulates Schwann cell PRX and MBP expression in vitro, we then investigated whether vitamin C is necessary for PRX and MBP expression during sciatic nerve myelination throughout early development in Gulo$^{-/-}$ mouse pups. In pups raised on a sufficient vitamin C diet (330 mg/L), PRX expression increased across development, sharply elevating at P17 as Schwann cells in the nerve transition into the mature myelinating phenotype (Figures 2c and 6a, c). However, pups raised on a deficient vitamin C diet (99 mg/L) exhibited ablated PRX expression at all developmental time points through P17 (Figure 6a and c). Dietary vitamin C deficiency also ablated expression of MBP, although only at P17 (Figure 6b and d). Overall, these data demonstrate that vitamin C is necessary for the expression of PRX and MBP during myelination throughout development and may underlie its effect to promote myelination in vivo.

PRX and MBP are critical for Schwann cell remyelination of regenerated axons following peripheral nerve injury (Gillespie et al., 2000; Kim, Maynard, Strickland, Burlingame, & Milbrandt, 2018). As vitamin C regulates the expression of PRX and MBP during developmental myelination, we investigated whether vitamin C is also necessary for their expression during remyelination following crush injury. Gulo$^{-/-}$ mice given a sufficient vitamin C diet (330 mg/L) and subjected to nerve crush exhibit a marked decrease in PRX expression 14 days post-injury (dpi) at the crush site and distal to the injury, consistent with Schwann cell death and myelin debris clearance during Wallerian degeneration (Lindborg et al., 2017). However, mice provided a deficient vitamin C diet (16.5 mg/L) display further impairment of PRX expression 14 dpi at all sites assessed (Figure 7a and c). However, MBP expression was unaffected by vitamin C deficiency 14 dpi, although may be altered at later time points during remyelination (Figure 7b and d). It has been shown that mice lacking SVCT2, the principal vitamin C transporter in Schwann cells, exhibit impaired remyelination of regenerated axons at the distal stump following nerve crush, thus suggesting that vitamin C promotes Schwann cell remyelination following injury (Rohr et al., 2017). It is plausible that the pro-myelinating effect of vitamin C may be mediated by its role in promoting the expression of myelin proteins like PRX and MBP. Overall, our data show that dietary vitamin C promotes the expression of PRX during remyelination after injury.

4 | DISCUSSION

Schwann cells are integral components of peripheral nervous system development and function. During embryonic development, Schwann cell precursors of neural crest origin flood peripheral nerves to regulate synaptic targeting and neuronal survival (Jessen, Mirsky, & Lloyd, 2015; Riethmacher et al., 1997). Postnatally, myelinating Schwann cells propagate saltatory conduction of peripheral nerves to enhance synaptic transmission (Salzer, 2015). After injury, Schwann cells release from degenerating axons to proliferate and take on a repair phenotype which recruits macrophages, initiates myelin breakdown, and secretes neurotrophic factors to promote nerve regeneration (Jessen et al., 2015). The plasticity of

Schwann cells to perform numerous functions within peripheral nerves is critical to neuronal function, yet the mechanisms that drive these states remain poorly elucidated.

It is becoming increasingly clear that the plasticity of Schwann cells during nerve development and repair is driven, in part, by epigenetic mechanisms. Histone acetylation and methylation dynamics play critical roles in developmental myelination and remyelination following injury (Brugger et al., 2017; Chen, Wang, & Yoon, 2011; Fuhrmann et al., 2018; Jacob et al., 2011; Ma et al., 2016). Methylation of DNA was recently discovered to regulate myelination, as DNA hypermethylation results in hypomyelination in vivo (Varela-Rey et al., 2014). We initiated this study under the premise that molecular agents which promote DNA demethylation may therefore promote Schwann cell myelination and function. We previously discovered that vitamin C promotes DNA demethylation by acting as a cofactor for TET enzymes. TETs convert 5mC into 5hmC and transient intermediates that are excised by the base excision repair pathway to produce unmodified cytosine (Young et al., 2015). 5hmC is a stable epigenetic mark which recruits readers such as MeCP2 and Uhrf2 to mediate neuronal gene expression and nervous system function (Chen et al., 2017; Mellen, Ayata, Dewell, Kriaucionis, & Heintz, 2012). Therefore, vitamin C–induced DNA demethylation may drive Schwann cell myelination and function. Vitamin C is already known to be critical for peripheral myelination due to its role in collagen crosslinking and basal lamina formation (Eldridge et al., 1987; Gess et al., 2011; Rohr et al., 2017). This study reveals a simultaneous mechanism whereby vitamin C promotes the expression of basal lamina components and pro-myelinating genes directly via DNA demethylation of these loci, thereby mediating Schwan cell function. Further studies are now needed to elucidate the relative contributions of these mechanisms through embryonic and early development. Transcriptionally induced DNA demethylation has been observed in previous studies, thus the role of peripheral transcriptional mechanisms in 5hmC generation resulting from vitamin C treatment cannot be excluded (Feldmann et al., 2013). Mammalian cells have been reported to exhibit dramatic loss of 5hmC following their adaptation from tissue into culture which could partially be rescued by the addition of vitamin C (Nestor et al., 2015). This is likely due to the absence of vitamin C in FBS and in culture media formulations (Michels & Frei, 2013; Young et al., 2015). Therefore, future studies should consider these concerns and the use of vitamin C in their culture system when evaluating 5hmC in culture.

ECM organization and basal lamina formation are critical for Schwann cell myelination and function (Eldridge et al., 1987; Gess et al., 2011). Laminin 2 and collagens I and IV are major components of the Schwann cell basal lamina and are critical for Schwann cell myelination (Chen & Strickland, 2003; Rohr et al., 2017; Uziyel, Hall, & Cohen, 2000). Vitamin C is classically thought to promote myelination by promoting post-translational crosslinking of collagen via P4 hydroxylase to form the basal lamina (Eldridge et al., 1987; Gorres & Raines, 2010). This illustrates the classical mechanism whereby vitamin C indirectly promotes myelination by regulating basal lamina assembly. Indeed, it is notable that hypomyelination in a mutant lacking an ascorbic acid transporter could be rescued by exogenous collagen (Rohr et al., 2017). Here, we show that vitamin C promotes the transcription of Laminin 2 and collagens I and IV in primary cultured Schwann cells, confirming results from previous studies (McGarvey et al., 1984). Most importantly, we

found that these transcriptional changes correlated with alterations in 5hmC content in the promoter and gene body regions of these loci, which is known to regulate transcription. Vitamin C may therefore promote the expression of ECM molecules important for Schwann cell myelination via its epigenetic function in TET-mediated DNA demethylation. Vitamin C also promoted the expression of pro-myelinating genes which correlated with altered promoter and gene body 5hmC content. Interestingly, vitamin C deficiency ablated baseline expression of PRX in adult animals, suggesting that vitamin C may be necessary during adulthood for myelin maintenance and the expression of myelin proteins. For the first time, we have shown that vitamin C induces 5hmC generation within myelin-related genes which correlates with their transcription, thus exploring a novel mechanism which potentially underlies Schwann cell myelination. These findings suggest the mechanism of action for vitamin C in Schwann cell myelination to be more complex than previously thought. We therefore propose that vitamin C may potentiate Schwann cell myelination by 1) P4-mediated collagen crosslinking to promote basal lamina formation, and 2) inducing TET-mediated DNA demethylation of collagens, laminins, and pro-myelinating genes to regulate their transcription and subsequently promote myelination (Figure 8). Vitamin C exerts pleiotropic effects on Schwann cells and how its epigenetic function drives myelination should therefore be further elucidated.

Humans lack the ability to endogenously synthesize vitamin C and must acquire it through dietary consumption or supplementation. Human infants consume vitamin C via maternal breast milk before switching to other dietary sources by early childhood, making the vitamin C levels of children contingent upon environmental resources and maternal nutrition. Mothers from low-income populations have a much higher risk to develop vitamin C deficiency, as do their children (Mosdol, Erens, & Brunner, 2008). Additionally, behaviors such as cigarette smoking and chronic alcohol abuse further reduce plasma vitamin C concentrations (Faizallah, Morris, Krasner, & Walker, 1986; Lykkesfeldt et al., 2000; Schectman, Byrd, & Gruchow, 1989). These and other various nutritional and behavioral determinants can influence the levels of vitamin C in breast milk, therefore affecting infant vitamin C consumption and possibly developmental myelination. Previous studies using SVCT2$^{\pm}$ mice exhibit hypomyelination and a reduced capacity for remyelination following nerve injury (Gess, Rohr, & Young, 2013; Rohr et al., 2017). This model demonstrates proof of concept that vitamin C is necessary for Schwann cell myelination in vivo and avoids the need to consider vitamin C intake. However, mutations affecting SVCT2 function are rare, and unlike mice humans are unable to endogenously synthesize vitamin C and therefore must acquire it through the diet. These considerations prompted the need for a new model relevant to human vitamin C consumption to be implemented to study the effect of vitamin C on myelination. Therefore, we used the Gulo mouse model in our experiments which allows us to examine the effect of dietary vitamin C on Schwann cell myelination throughout development and after peripheral nerve injury. Using this model, this study is the first to show that sufficient maternal vitamin C consumption is necessary for peripheral myelination in early developing offspring and may therefore have a lasting impact on neurological function. Since myelination occurs postnatally, nutritional practices in young children may also influence this process. While baby formula contains higher levels of vitamin C than human breast milk, cow milk's vitamin C content is nearly negligible (Camarena & Wang,

2016). Additionally, childhood reluctance to eating green vegetables eliminates high sources of vitamin C from a child's diet. Childhood nutrition may therefore influence Schwann cell myelination and more work is needed to assess its lasting impact on nervous system function throughout development.

Due to its pro-myelinating effect, the use of vitamin C as a therapy for demyelinating neuropathies such as Charcot-Marie-Tooth disease (CMT) has been of great interest for many years. Dysregulated expression of pro-myelinating genes such as Krox20, PRX, and Pmp22 leads to CMT onset with varying form and severity (Bird, 1993a, 1993b; Kijima et al., 2004). Our study found that vitamin C regulates pro-myelinating gene expression in Schwann cells and may therefore be a viable therapy for CMT. Previous work had shown that vitamin C had ameliorated symptoms in a CMT1A mouse and was proposed to act by decreasing *Pmp22* expression (Passage et al., 2004). This stirred great interest in forming clinical trials to treat CMT. Unfortunately, multiple clinical trials have found no benefit for vitamin C in the treatment of CMT1A, the most common demyelinating CMT subtype (Burns et al., 2009; Pareyson et al., 2011). Additionally, we were unable to replicate the previously published effect of vitamin C on *Pmp22* expression, instead finding that treatment with vitamin C up-regulated *Pmp22* in primary cultured Schwann cells. However, CMT1A is a slowly progressive neuropathy and previous clinical trials lasted between 12 and 24 months, a duration that may be too short to detect differences in treatment outcomes. Despite the discrepancies brought about from clinical trials, the epigenetic role of vitamin C in promoting myelination in development, after injury, and therapeutically during disease is nonetheless relevant. Previous work has shown that pro-myelinating genes such as Krox20, PRX, Pmp22, and MBP undergo DNA demethylation throughout development as their expression increases and myelination proceeds, which may be impacted by dietary vitamin C (Varela-Rey et al., 2014). Additionally, vitamin C promotes oligodendrocyte generation and remyelination after cuprizone-mediated demyelination, suggesting vitamin C could therefore play a role in CNS myelination and in treating CNS demyelinating diseases such as multiple sclerosis (Guo et al., 2018). Although its therapeutic value in CMT remains elusive, the role of vitamin C in the epigenetic regulation of pro-myelinating genes and other Schwann cell functions during disease states may be crucial to its therapeutic potential in other demyelinating neuropathies and should not be overlooked.

In conclusion, our study suggests that vitamin C promotes Schwann cell myelination and developmental function, in part, through TET-mediated DNA demethylation. These findings present a novel mechanism by which vitamin C regulates Schwann cell function and further elucidates how epigenetic mechanisms underlie glial function in the peripheral nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

5hmc	5-hydroxymethylcytosine
5mC	5-methylcytosine
Cadm4	cell adhesion molecule 4
Col1a1	collagen 1 alpha 1
Col4a1	collagen 4 alpha 1
Col4a2	collagen 4 alpha 2
Col4a5	collagen 4 alpha 5
Egr2	early growth response 2
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
Lama2	laminin subunit alpha 2
Lamb1	laminin subunit beta 1
Lamc1	laminin subunit gamma 1
Mag	myelin-associate glycoprotein
MBP	myelin basic protein
Mpz	myelin protein zero
Nfkb1	nuclear factor kappa beta subunit 1
Pmp22	peripheral myelin protein 22
PRX	periaxin
RRID	Research Resource Identifier
TES	transcription end site
TSS	transcription start site

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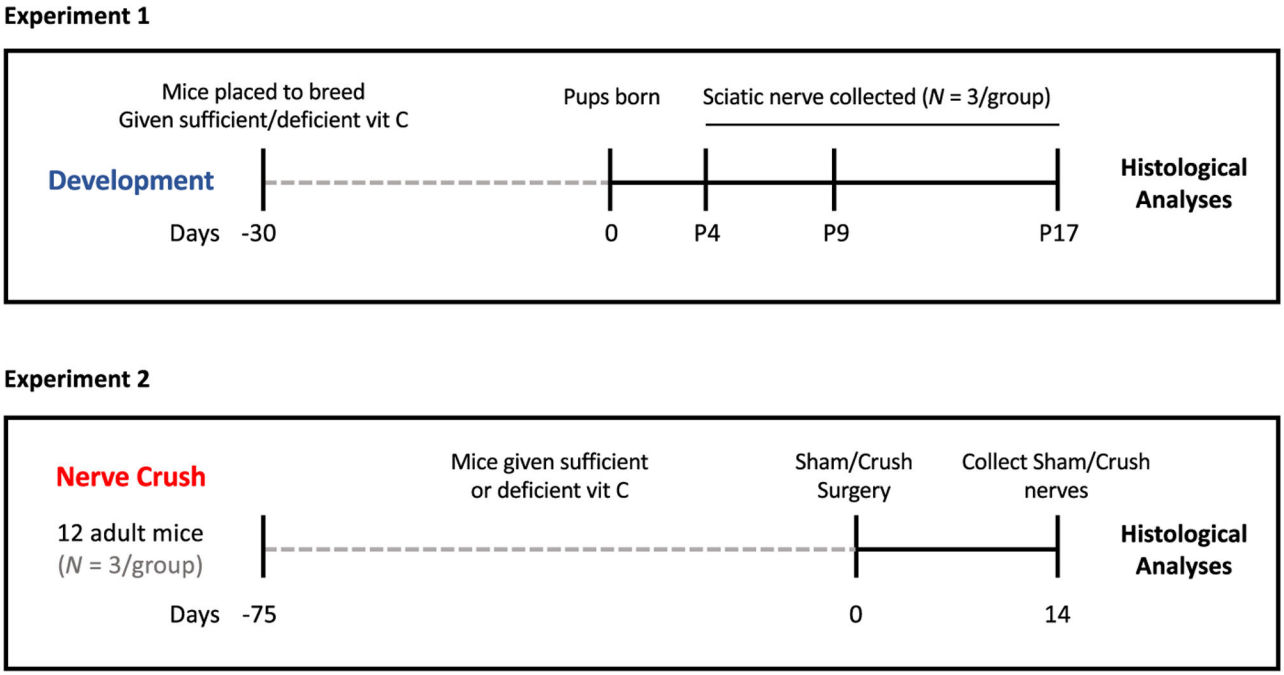

FIGURE 1.

Experimental timeline and design for mouse experiments. In Experiment 1, adult Gulo$^{-/-}$ mice breeding pairs were provided either sufficient (330 mg/L) or deficient (99 mg/L) vitamin C in the drinking water. Sciatic nerves of the resulting pups from the breeding pairs were collected at postnatal day 4 (P4), P9, and P17. For Experiment 2, adult Gulo$^{-/-}$ mice were provided either sufficient (330 mg/L) or deficient (16.5 mg/L) vitamin C for 2.5 months. Mice were then subject to sciatic nerve crush surgery on the lateral nerve while the contralateral nerve was subject to sham surgery. Sham and crush nerves were then collected 14 days post-injury and subjected to histological analysis. $N = 3$ animals were used in each condition and time point assessed in both experiments

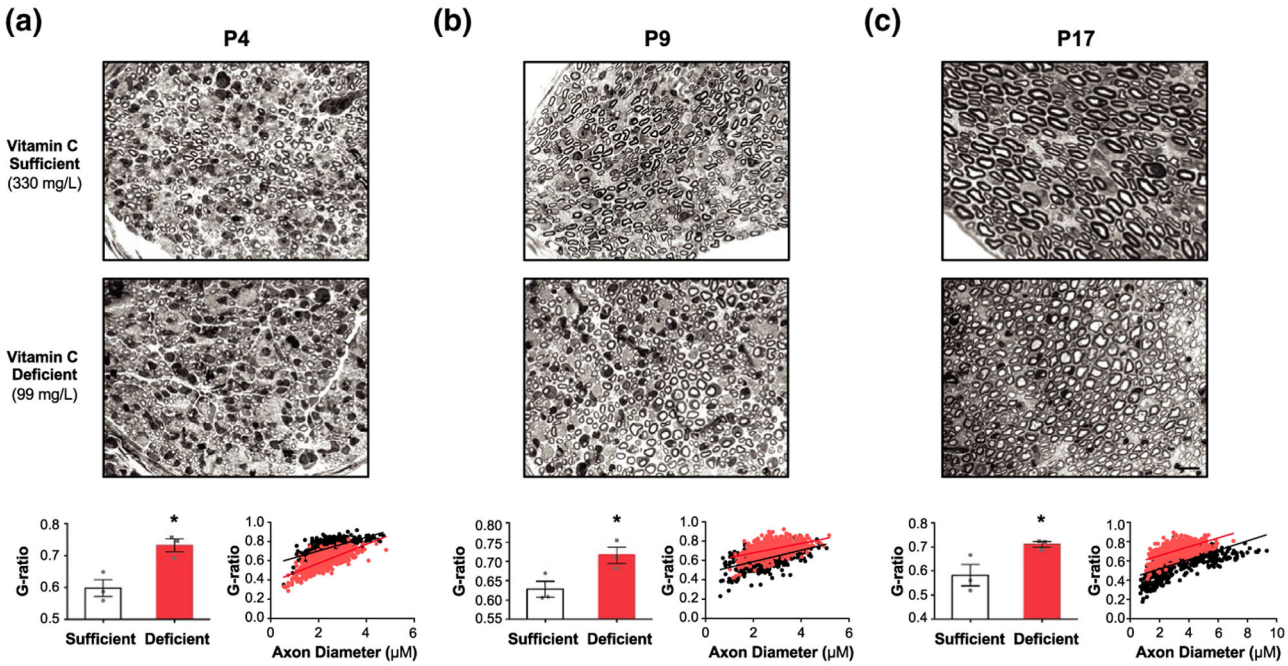

FIGURE 2.

Vitamin C deficiency induces persistent hypomyelination in Gulo$^{-/-}$ mice throughout development. (a–c) Semithin sections of sciatic nerves from Gulo$^{-/-}$ mouse pups reared on a vitamin C sufficient (330 mg/L) or deficient (99 mg/L) diet and collected at postnatal day 4 (P4), P9, and P17. G-ratio averages (axon diameter/fiber diameter) are represented by bar graphs with plotted averages for each animal (grey dots, $N = 3$ animals per time point). Pooled g-ratio calculations from each condition ($N > 500$) are represented by scatterplot including line of best fit. *$p < .05$. All data are means ± *SEM*. Scale bar = 10 μm

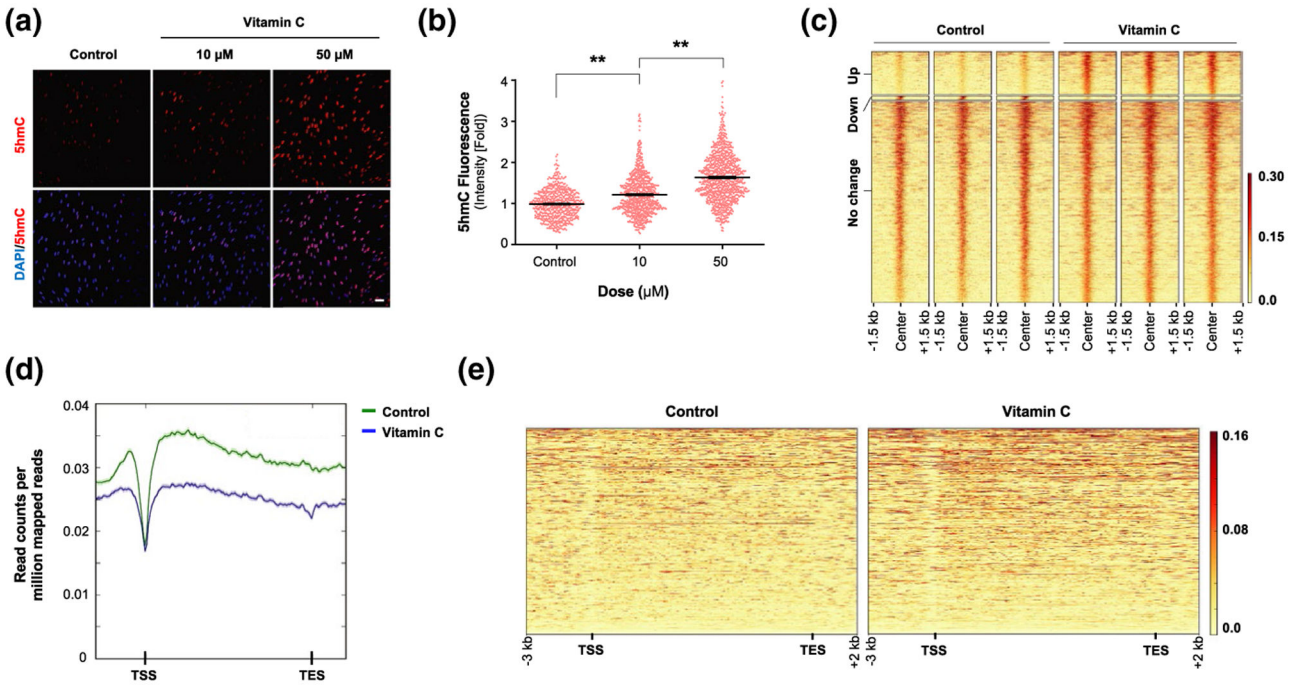

FIGURE 3.

Vitamin C augments global levels of 5hmC. (a) Immunofluorescence of 5hmC in primary cultured Schwann cells after vitamin C treatment. (b) Quantification of immunofluorescence shows that 10 μM vitamin C treatment modestly increases 5hmC, while 50 μM treatment greatly elevates 5hmC 70% above control levels ($N > 250$ data points per condition). (c) Heatmap of global 5hmC peaks following vitamin C treatment (7 days, 50 μM) as determined by ChIP-seq. Units are in read counts per million mapped reads. (d) Read counts per million mapped reads across promoter and gene body regions following vitamin C treatment. (e) Heatmap of the relative abundance of reads across promoter and gene body regions following treatment. $^{**}p < .01$. $N = 3$ independent cell culture preparations for ChIP-seq experiment. All data are means ± *SEM*. Scale bar = 20 μm. TSS = transcription start site; TES = transcription end site

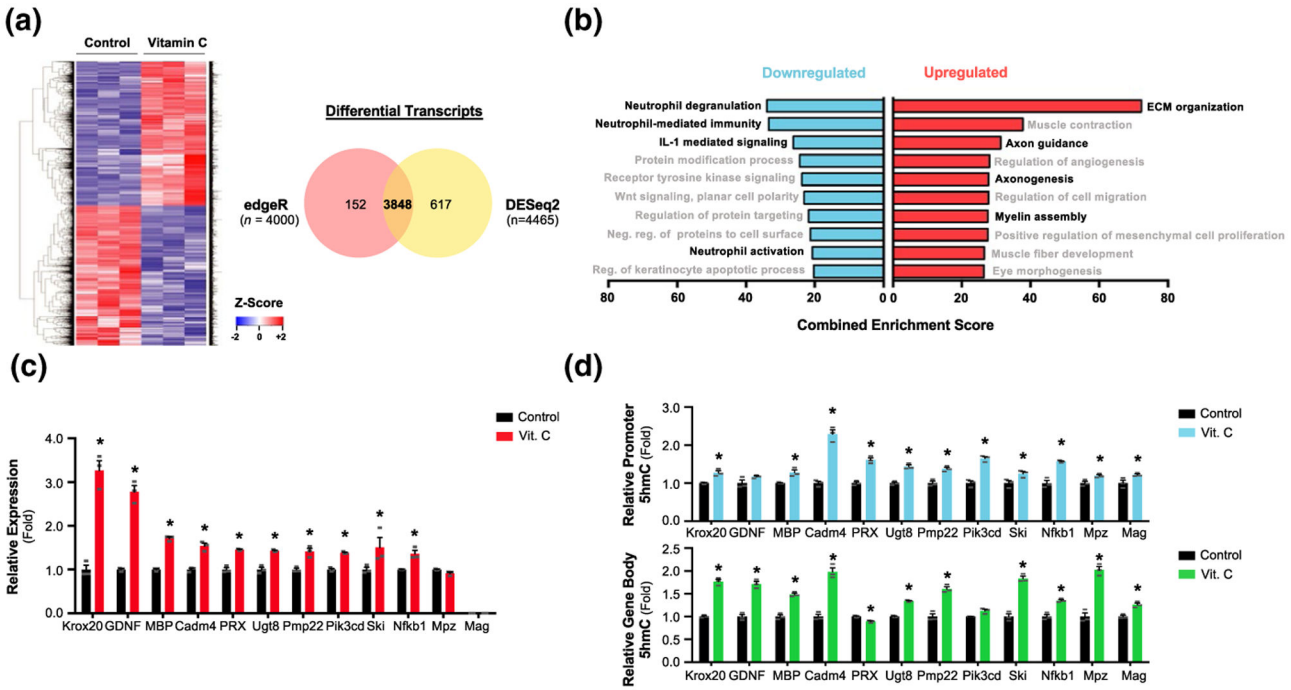

FIGURE 4.

Vitamin C alters the Schwann cell transcriptome and up-regulates pro-myelinating gene expression and 5hmC content. (a) Heatmap of the relative abundance of reads for all transcripts after vitamin C treatment (7 days). The Venn Diagram represents of the number of differential transcripts called by statistical algorithms edgeR (red) and DESeq2 (yellow). A total of 3,848 transcripts were considered differential by both algorithms. (b) Gene Ontology (GO) pathway analysis of RNA-seq data showing top up-regulated and down-regulated pathways in Schwann cells following vitamin C treatment. Annotations are ranked by combined p value and z scores (Combined Enrichment Score). (c) Relative expression of pro-myelinating genes following vitamin C treatment. Mag expression was below detectable levels. (d) Relative abundance of 5hmC in the promoter and gene body regions of pro-myelinating genes following vitamin C treatment. Data from bar graphs (c and d) represent average fragments per kilobase per million mapped reads (FPKM) expressed as fold change from control. $*p < .05$, $**p < .01$. $N = 3$ independent cell culture preparations for RNA-seq experiment. All data are means ± *SEM*

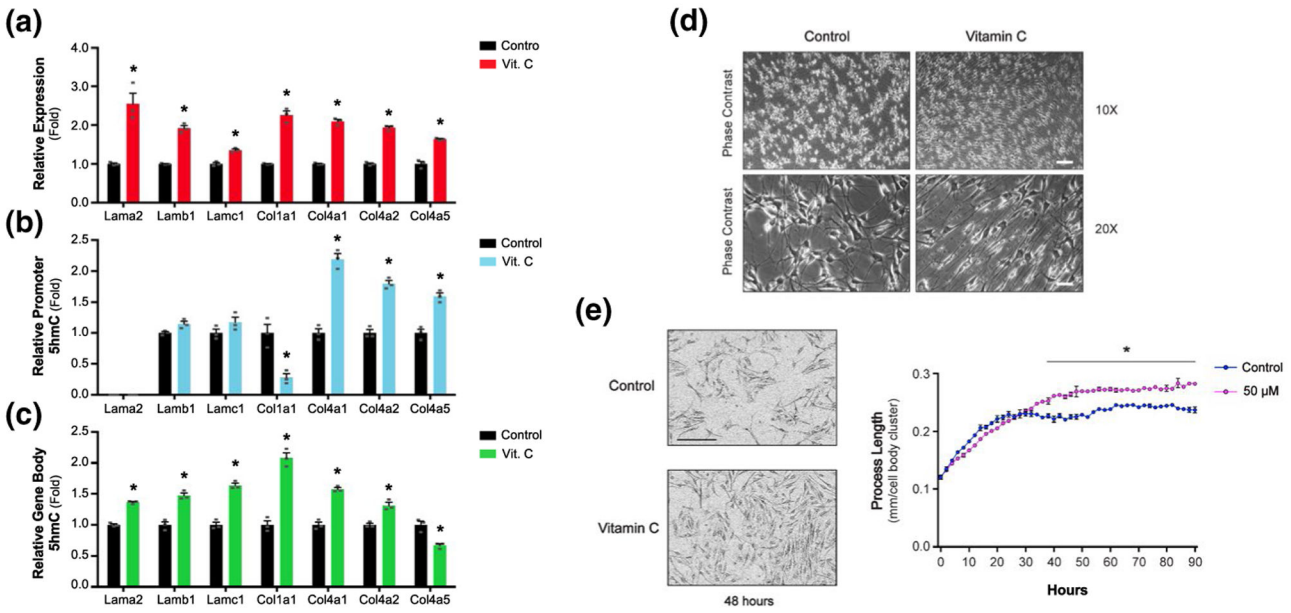

FIGURE 5.

Vitamin C up-regulates the transcription and 5hmC content of ECM genes. (a) Relative expression of laminin and collagen genes following vitamin C treatment. (b) Relative abundance of 5hmC in the promoter regions of laminin and collagen genes following vitamin C treatment. Lama2 promoter 5hmC was below detectable levels. (c) Relative abundance of 5hmC in the gene body regions of laminin and collagen genes following vitamin C treatment. (d) Phase contrast images of Schwann cells in culture treated with vitamin C (50 μM, 5 days treatment). Lower panels are high-magnification (20X) images showing morphological changes following treatment (Scale bar = 100 μm for top panels. Bottom panels = 50 μm). (e) Live imaging and high-throughput quantification of Schwann cell process length following vitamin C treatment. Images show Schwann cells 48 hr following treatment (Scale bar = 200 μm). Data from bar graphs represent average fragments per kilobase per million mapped reads (FPKM) expressed as fold change from control. *$p < .05$. $N = 3$ independent cell culture preparations used for RNA/ChIP seq and Incucyte experiments. All data are means ± *SEM*

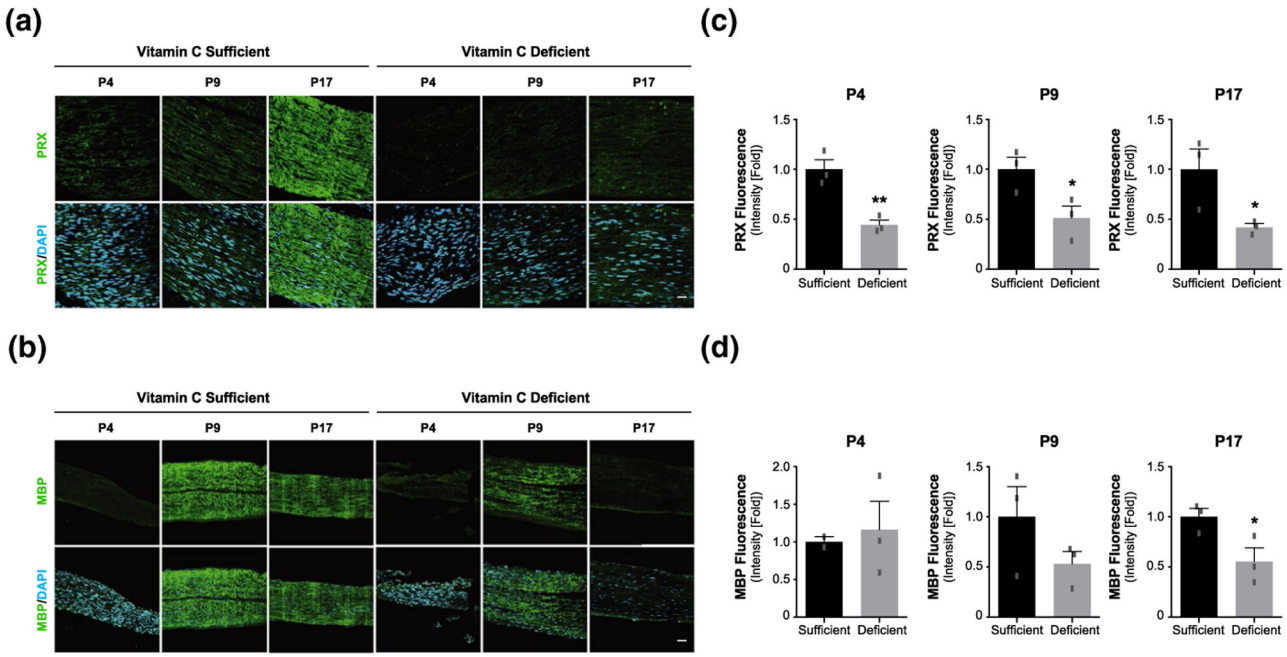

FIGURE 6.

Vitamin C regulates the expression of PRX and MBP throughout developmental myelination. (a) Immunofluorescence of PRX in sciatic nerves collected from Gulo$^{-/-}$ mouse pups reared on a vitamin C sufficient (330 mg/L) or deficient (99mg/L) diet and collected at postnatal day 4 (P4), P9, and P17. Scale bar = 10 μm. (b) Immunofluorescence of MBP in sciatic nerves collected from Gulo$^{-/-}$ mouse pups reared on a vitamin C sufficient (330 mg/L) or deficient (99mg/L) diet and collected at postnatal day 4 (P4), P9, and P17. Scale bar = 20 μm. (c) Quantification of PRX expression in the sciatic nerve throughout developmental myelination at three time points assessed. (d) Quantification of MBP expression in the sciatic nerve throughout developmental myelination at three time points assessed. $N = 3$ animals per time point and condition. *$p < .05$; **$p < .01$. All data are means ± *SEM*

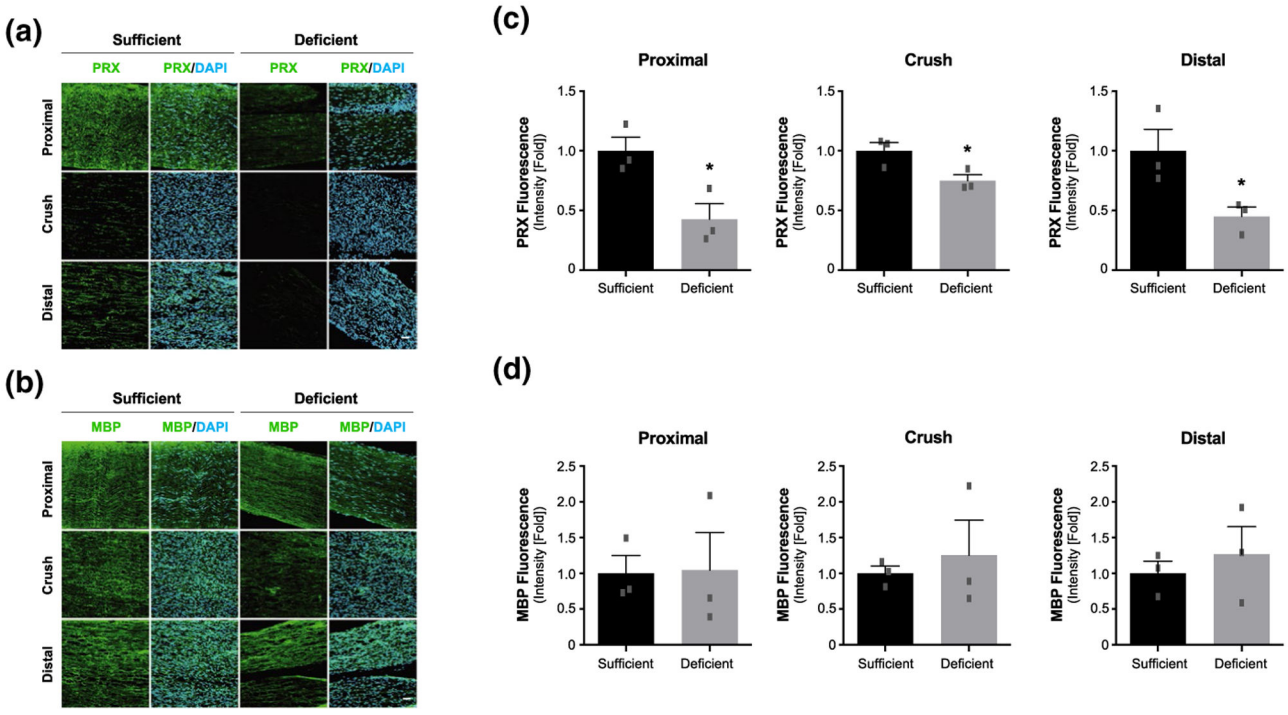

FIGURE 7.

Vitamin C regulates the expression of PRX but not MBP during remyelination following injury. (a) Immunofluorescence of PRX in crushed sciatic nerves collected from Gulo$^{-/-}$ mice provided a vitamin C sufficient (330 mg/L) or deficient (16.5 mg/L) diet for 2.5 months and collected at 14 days post-injury (dpi). (b) Immunofluorescence of MBP in crushed sciatic nerves collected from Gulo$^{-/-}$ mice provided a vitamin C sufficient (330 mg/L) or deficient (16.5 mg/L) diet for 2.5 months and collected at 14 days post-injury (dpi). (c) Quantification of PRX expression following sciatic nerve crush in three regions assessed. (d) Quantification of MBP expression following sciatic nerve crush in three regions assessed. $N = 3$ animals per time point and condition. *$p < .05$. All data are means ± *SEM*. Scale bar = 10 μm

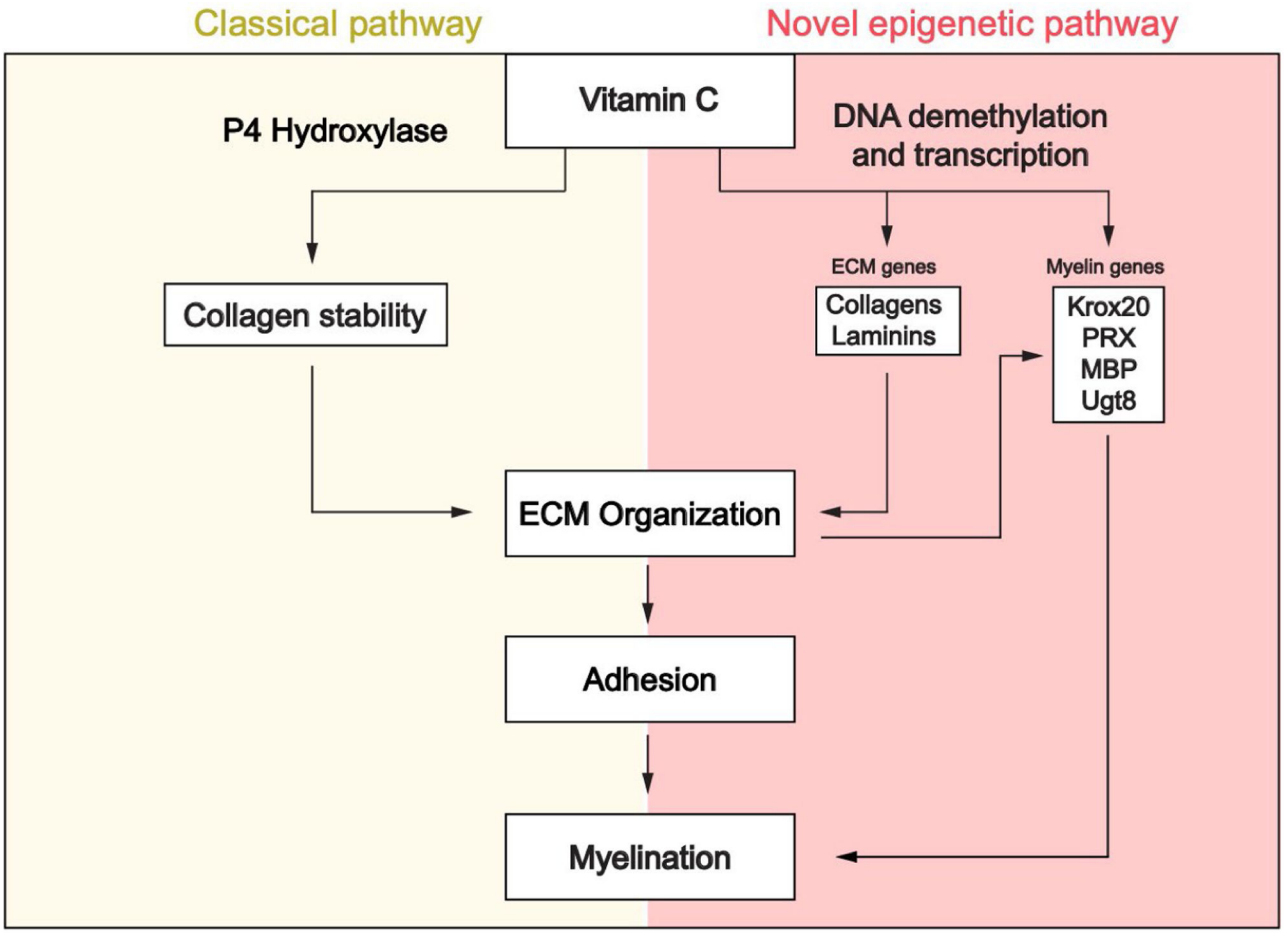

FIGURE 8.

Working model of the role of vitamin C in Schwann cell myelination. Vitamin C is classically known to promote myelination by serving as a cofactor for P4 hydroxylase which stabilizes collagen during basal lamina formation and is necessary for myelination. Therefore, vitamin C is thought to indirectly promote myelination. Here, we present a novel epigenetic pathway whereby vitamin C promotes myelination via TET-mediated demethylation. Vitamin C induces demethylation in promoter and gene body regulatory regions of genes involved in Schwann cell myelination (Krox20, PRX, and MBP) and ECM organization (Laminin-2 subunits, Collagens I and IV). Demethylation correlates with increased transcription of these genes and promotes their relevant functions. Therefore, vitamin C may promote myelination 1) indirectly through P4 hydroxylase activity, and 2) directly via active demethylation and transcriptional regulation of genes relevant to myelination