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Supplementary Information for

Epigenetic regulator UHRF1 inactivates REST and growth suppressor gene expression via DNA methylation to promote axon regeneration

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SI Materials and Methods

Surgeries and tissue preparations

Sciatic nerve injury experiments were performed as described (1). The sciatic nerve was exposed by skin incision at mid-thigh level, a crush injury performed with constant forceps pressure for 10 seconds. Injured and contralateral uninjured sciatic nerves were dissected at the indicated time after surgery.

For Spinal Cord Injury, dorsal column hemisection was performed at the thoracic 9/10 (T9/10) vertebral segment. A small (1.0–1.5 cm) midline incision was made over the thoracic vertebrae at the T9−T10 level, the paraspinal muscles freed from the T9 and T10 vertebrae, and the vertebral column stabilized with metal clamps placed under the T9/10 transverse processes. A dorsal laminectomy was performed to expose the dorsal surface of the spinal cord at T9/10. The dura was then removed with fine forceps and the dorsal column transversely cut using fine iridectomy scissors. Afterwards, the musculature was sutured with absorbable sutures and the skin with wound clips.

For Dorsal Root Injury, the dorsal root of lumbar 4 (L4) DRG was crushed at the lumbar 2 (L2) vertebral segment. A small (1.0–1.5 cm) midline incision was made over the lumbar vertebrae at the L2-L3 level, the paraspinal muscles freed from the L2/3 vertebrae, and the vertebral column stabilized with metal clamps placed under the L2/3 transverse processes. Next, a dorsal laminectomy was performed to expose the dorsal roots, and fine forceps used to crush the right L4 dorsal root for 10 seconds. Afterwards, the musculature was sutured with absorbable sutures and the skin with wound clips.

For western blot analysis, mouse DRG tissues or DRG spot cultures were collected at the indicated time after injury, homogenized in lysis buffer as described (1). Protein concentration

was quantified by Bio-Rad protein assay kit (Bio-Rad). Western blots were scanned with a Bio-Rad ChemiDoc MP System and quantified in ImageJ.

In vivo regeneration assay

In vivo regeneration assay was performed as described (1) with minor modifications. To quantify sciatic nerve regeneration, we counted the number of SCG10-positive regenerating axons at multiple distances from the crush site. The crush site was defined as the position along the sciatic nerve length with maximal SCG10 intensity. Axons were counted at 1 mm intervals starting at 1 mm from the crush site and averaged from 3 sections per animal. The cross-sectional width of the nerve was measured at the point at which the counts were taken and was used to calculate the number of axons per 100 μm of nerve width.

RG108 was dissolved in 100 μl dimethyl sulfoxide (DMSO) from a 10 mg/ml DMSO stock. An equivalent volume of DMSO was used for vehicle control. Intraperitoneal injection was performed 2 h before sciatic nerve crush and was repeated 1 day later. Animals were sacrificed, and DRGs and nerves were dissected 3 days following nerve crush.

Five animals per group were examined, and measurements were taken with the observer blind to treatment. RG108 (Sigma, R8279) was delivered by intraperitoneal injection at 9 mg/kg body weight. Averages were taken for each nerve section and were compared between control and RG108 injected groups for corresponding distances from the crush sites.

Embryonic DRG neuron spot culture and *in vitro* regeneration assay

Embryonic DRG neurons were cultured as previously described (1). Mouse E13.5 DRG neurons were dissected, trypsinized and dissociated. Neurons from two or more littermate embryos were pooled for each biological replicate. For spot culture, 10,000 DRG neurons were resuspended in 5 μl of medium and plated on poly-d-lysine/laminin-coated culture dishes and incubated for 10 minutes before adding additional medium. To knock down or overexpress

UHRF1, miR-9-5p or miR-9-5p inhibitor, DRG neurons were infected with lentivirus at DIV1. To knock down REST and PTEN, DRG neurons were infected with lentivirus at DIV4. *In vitro* regeneration assays were performed as previously described (1). DRG spot cultures were axotomized with a blade (FST, 10035-10) at DIV7 under a dissection microscope (Nikon, SMZ645). To assess axon regeneration, axotomized spot cultures were fixed at the indicated time and immunostained for SCG10. Images were acquired using a Nikon, TE2000E microscope at 10x. Regenerative axon growth was measured by measuring the length from the axotomy line to the axon tip and averaged from three technical replicates per biological replicate. Measurements were taken with the observer blind to treatment.

To assess the role of DNA methylation inhibition on *in vitro* axon regeneration, DRG spot cultures were treated with 100 μM RG108 or DMSO (vehicle control) 10 mins prior to axotomy. The treatment was repeated 24 hours later. Spot cultures were fixed 40 h after axotomy and analyzed as described above; Measurements were taken with the observer blind to treatment.

Adult DRG neuron culture and Neurite Length Analysis

Adult DRG neurons were cultured as previously described (2). For preconditioning injury, L4 DRGs were dissected from DMSO or RG108 treated mice that received or not a prior (3 days) sciatic nerve injury in Hanks' balanced salt solution with 10 mM HEPES (HBSS-H). DRG were treated at 37°C with consecutive applications of papain (15 U/ml) and collagenase (1.5 mg/ml) in HBSS-H. After washes, DRG were dissociated by trituration, passed through a 100 μm cell strainer, resuspended in Neurobasal A media with Pen/Strep, B27 and Glutamax and plated on poly-d-lysine/laminin-coated culture dishes. Adult DRG neurons were cultured for 20 h and stained with the neuronal specific marker TUJ1.

For quantification of average axon length, total neurite length stained by TUJ1 was measured by a custom neuron skeletonization program in Nikon Elements (V4.6) and was

normalized to the number of neuronal cell soma. The values are averaged from four technical replicates per biological replicate.

Antibodies and lentiviruses

The following antibodies were used in this study: anti-UHRF1 (Santa Cruz, sc-373750), anti-SCG10 (Novus Cat# NBP1-49461 RRID:AB_10011569), anti-βIII tubulin (Covance Research Products Inc Cat# MMS-435P RRID:AB_2313773; Sigma-Aldrich Cat# T8660 RRID:AB_477590), anti-Histone H3K9Me2 (Abcam Cat# ab1220 RRID:AB_449854), anti-Histone H3K9Me3 (Abcam Cat# ab8898 RRID:AB_306848), anti-DNMT1 (Santa Cruz Biotechnology Cat# sc-20701 RRID:AB_2293064), anti-DNMT3a (Santa Cruz Biotechnology Cat# sc-20703 RRID:AB_2093990) and anti-REST (rabbit polyclonal p73 3106, generous gift from Gail Mandel (3) . To express UHRF1 and REST in cultured DRG neurons, human cDNA of UHRF1 and REST was sub-cloned into pLenti6.2/V5-DEST vector (DNASU, HsCD00329484) and N174 (Addgene, 60859) for constitutive overexpression. To knock down UHRF1, REST or PTEN or overexpress miR-9-5p, miR-9-3p or miR-9-5p inhibitor in cultured DRG neurons, UHRF1 MISSION shRNAs (Sigma, TRCN0000311096), REST MISSION shRNAs (Sigma, TRCN0000321488), PTEN MISSION shRNAs (Sigma,TRCN0000322421), LentimiRa-mmu-miR-9-5p Vector (ABM Inc., mm40662), LentimiRa-Off-mmu-miR-9-5p Vector (ABM Inc., mm31039) and Lenti mmu-miR-9-3p Lentivector (ABM Inc., mm42109) were used and lentiviruses were generated as previously described (1).

RNA preparations and RT-qPCR

Total RNA from DRG spot cultures was extracted at the indicated time after axotomy using PureLink RNA mini kit (Life Technologies) or miRNeasy Micro Kit (Qiagen). RNA was isolated from 6 or more spots for each condition per each biological replicate. To perform quantitative PCR, iTaq Universal SYBR Green Supermix was used (Bio-Rad) with validated primer sets from

PrimerBank (http://pga.mgh.harvard.edu/primerbank/). Quantitative PCR analysis was done with the following primer set specific for UHRF1, RPL13a, REST, PTEN and CDKN1A : UHRF1; 5′-CCACACCGTGAACTCTGTC-3′ and 5′-GGCGCACATCATAATCGAAGA-3′, RPL13a; 5′-AGCCTACCAGAAAGTTTGCTTAC-3′ and 5′-GCTTCTTCTTCCGATAGTGCATC-3′, REST; 5'-CATGGCCTTAACCAACGACAT-3' and 5'-CGACCAGGTAATCGCAGCAG-3', PTEN; 5'-TGGATTCGACTTAGACTTGACCT-3' and 5'-TGTGACAAAAGTGACACAGATCA-3', CDKN1A; 5'-CCTGGTGATGTCCGACCTG-3' and 5'- CCATGAGCGCATCGCAATC-3'. All qPCR genes were normalized to RPL13a. For the verification of mature miRNA-9-5p, the TaqMan probes for miR-9-5p, miR-361-5p and snoRNA135 were purchased from Life Technologies.

Bioinformatics analyses

For miR-9 target analysis, validated miR-9 targets were included from MetaCore. Only targets that displayed minimal proper expression directionality (10% fold change) at either 3 or 8 hours post injury in our previously published microarray (2) were used for downstream analysis in MetaCore.

For REST target gene analysis**,** REST target genes were retrieved from (4) and converted to Ensembl ID. Additionally, validated targets from MetaCore were included, generating a list of 951 REST target genes. RNA-seq data from Naïve Dorsal Root Ganglia (DRG) and 5 day post sciatic nerve crush DRG neurons obtained from CAST/Ei mouse (5) were downloaded from Gene Expression Omnibus (GSE67130). Sequences were quality-controlled using PRINSEQ and aligned to mm10 using STAR. Reads in features were counted using HTseq, and variance stabilized normalized counts were calculated using DESeq2. RNA-seq data from e12.5 and e17.5 DRG neurons (6) were downloaded from Gene Expression Omnibus (GSE66128) and processed as described above. Normalized gene counts were converted to Z-scores (average of counts in each row divided by standard deviation of the row). Heatmap were generated using

heatmap.2 function of the *gplots* R package. Gene clusters were extracted using *cutree* function in the *dendextend* R package. DAVID was used for Gene Ontology enrichment analysis.

DNA Methylation Assay

Genomic DNA was prepared from mouse embryonic DRG spot cultures using AllPrep DNA/RNA Micro Kit following the manufacturer's instructions (Qiagen, 80284). Genomic DNA was isolated from 18 or more spots for each condition per each biological replicate. CDKN1A (EPMM104782, Qiagen), PTEN (EPMM105761, Qiagen) and REST (EPMM108620-1A, Qiagen) gene methylation levels were analyzed by the EpiTech Methyl II PCR assay. Unmethylated or methylated DNA is selectively digested by methylation-sensitive (cuts unmethylated and partially methylated DNA, leaving only hypomethylated DNA) and methylation-dependent restriction enzymes (cuts any methylated DNA, leaving only unmethylated DNA) according to the manufacturer's instructions. The remaining DNA after digestion is quantified by real-time PCR. The relative concentrations of differentially methylated DNA are determined by comparing the amount of each digest with that of mock digest (no enzyme added).

For measurement of global DNA methylation, genomic DNA was prepared from mouse DRG tissues using AllPrep DNA/RNA Micro Kit following the manufacturer's instructions (Qiagen, 80284). The global DNA methylation assay was performed using the MethylFlash Methylated DNA 5-mC Quantification Kit (Epigentek, #P-1034) with 50 ng genomic DNA per well following the manufacturer's instructions.

ChIP–qPCR analysis

The SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling, #9005) was used as described, with a few modifications, for ChIP assays. Embryonic DRG neurons from 60 or more spots were fixed for 10 min with 1% formaldehyde, and the reaction was quenched by the

addition of 125 mM glycine. Crude nuclei were isolated in SONIC buffer (50mM HEPES (pH7.6), 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na-deoxyxhilate and 0.1% SDS) and were sonicated with probe-based sonicator. The sheared chromatin samples were immunoprecipitated overnight at 4 °C under gentle rotation with indicated antibodies and then add ChIP-grade protein G magnetic beads to each IP reaction and incubate for 2h at 4 °C with rotation. After extensive washing of samples, immunocomplexes were eluted for 30 min at 65 °C, then were treated for 2h at 65 °C with proteinase K for reversal of crosslinking. After extraction of DNA, quantitative PCR analysis was done with the following primer set specific for the CpG region of CDKN1A, PTEN, REST, CALB1, GLRA1, SYT4 and NMDA1 : CDKN1A; 5′-GACGAGGGTCAGGCTATGTC-3′ and 5′-CCCTAGAAACATTGGCAACC-3′, PTEN; 5′-GGCTCGTGGATGCTATTCTC-3′ and 5′-CCAAATTCCCATTCCCCTAT-3′, REST; 5'-CGGCACATAGGAATTCGGTG-3' and 5'- AAACGGCTCTCCCCTAGTTC-3', CALB1 RE1; 5'-GCTCCGCGCACTCTCAAA-3' and 5'-GAGATGACTGCAGGTGGGATTC-3', GLRA1 RE1; 5'-GCTCTGACACCTCGTCCCTCTA-3' and 5'-GAAGCCAGAGAGCGCCACT-3', SYT4 RE1; 5'-AGACAAGCTTTTCAGAAGAGCCA-3' and 5'-CCGCGCTCTCTATCAGCAAT-3', NMDA1 RE1; 5'-GCAGCTGTCTTTTTCGCCTT-3' and 5'-CTGAAGCGTATTGGGCGC-3'

Immunohistochemistry

DRG neurons dissected from mice that received or not a prior (1 or 3 days) sciatic nerve injury (SNI). Following a brief post-fixation in 4% paraformaldehyde and several washes in PBS, sections were blocked using 5% donkey serum dissolved in PBS. Subsequently, sections were incubated overnight at 4°C in the following primary antibodies diluted in blocking reagent: chicken anti-βIII tubulin (1:500, Abcam, Cat# ab107216 RRID:AB_10899689). UHRF1 antibody directly conjugated to Alexa Fluor 594 (Thermo Fisher, Cat# A10474) was incubated with primary antibodies. Tissue was washed several times with PBST, incubated with fluorescent-conjugated secondary antibodies (1:500; Thermo Fisher Scientific) and diluted in blocking

reagent, washed, and mounted in ProLong Gold antifade mountant with DAPI (Thermo Fisher Scientific). Images were taken with a Nikon TE-2000E microscope.

For quantification of percentage of DRG neurons expressing UHRF1, the total number of L4 DRG neurons was quantified by counting βIII tubulin-positive profiles (TUJ1). DRG neurons expressing UHRF1 were counted as TUJ1-positive cells that co-labeled with UHRF1 in the nucleus. The values are averaged from 3 DRG sections per animal.

To assess apoptotic cells, DRG neurons infected with the indicated lentivirus at DIV1, fixed at DIV7 and immunostained for cleaved-caspase3 (Cell signaling, #9664). Images were acquired using a Nikon, TE2000E microscope at 10x. The DRG neurons stained by cleaved caspase3 or DAPI were automatically counted by Nikon. For cell death index, the number of DRG neurons stained by cleaved-caspase3 was normalized to the number of DRG neurons stained by DAPI. The values are averaged from three spots per biological replicate.

Coimmunoprecipitation

Embryonic DRG neurons from 60 or more spots were solubilized in lysis buffer containing protease inhibitors and phosphatase inhibitors. The cell lysates were sonicated with probe-based sonicator and centrifuged at 14’000rpm, 4'C for 30 min. Protein concentration was determined by BCA method. The supernatants incubated with the indicated antibody overnight. Protein-G Dynabeads were added for 2h. The beads were washed with lysis buffer and bound proteins eluted and separated by SDS–PAGE, followed by immunoblotting with the indicated antibodies.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism (v7.02). All data are presented as mean ± SEM.

Figures 2A, 2C, 2H, 3C, 4A, 4B, 4D, 4E, 4F, 5C, 6C, 7D, 7E, 8A, 8C and 8E were analyzed using one sample T-tests. Figure 1G, 1H, 2E, 3E and 6B was analyzed by an unpaired T-test. Figures 1B, 1D, 1E, 3B, 3G, 3H, 4I, 4J, 6F, 6G, 7F, 7H, 8B, 8D and 8F were analyzed using One-way ANOVAs with post-hoc Dunnett tests. All axon regeneration measurements were taken with the observer blind to treatment. Asterisks indicate P values: * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ and **** $p < 0.0001$.

References for SI reference citations

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