

COMPOSITIONS AND METHODS FOR DEREPRESSING RE1 SILENCING
TRANSCRIPTION FACTOR TARGET GENES

CROSS-REFERENCE TO RELATED APPLICATIONS AND CLAIM TO PRIORITY

[0001] This application claims the priority of U.S. Provisional Patent Application Nos. 62/939,149, filed November 22, 2019, and 63/086,248, filed October 1, 2020, the disclosures of which are incorporated herein by reference.

FIELD OF THE INVENTION

[0002] Methods, compounds, and compositions for derepressing RE1 silencing transcription factor (REST) target genes are provided. In particular, a peptide having the sequence TEDLEPPEPPLPKEN (SEQ. ID NO: 1) and EDLEPPEPPLPK (SEQ. ID NO: 15) , or the reversed sequences made of D-amino acids (retro inverted, RI) nekplppeppeldet (SEQ ID NO: 16) and kplppeppelde (SEQ ID NO: 17), are disclosed for inhibiting REST activity. The peptides are useful to treat, prevent, or ameliorate conditions such as traumatic brain injury, epilepsy, dementia, Huntington's Disease (HD), chronic pain, brain cancer (including glioblastoma multiforme), pancreatic cancer; diabetes, and peripheral nerve injury.

BACKGROUND

[0003] The repressor element 1 (RE1) silencing transcription factor (REST) is a repressor of hundreds of neuronal genes¹. Its targets represent genes required for the terminally differentiated neuronal cell phenotype, including genes encoding voltage and ligand dependent ion

channels, their receptors, growth factors, and axonal-guidance proteins (Bruce AW et al, Proc Natl Acad Sci U S A 101, 10458-10463 (2004); Conaco C et al, Proc Natl Acad Sci U S A 103, 2422-2427 (2006); Mortazavi A et al, Genome Res 16, 1208-1221 (2006); and Otto SJ et al, J Neurosci 27, 6729-6739 (2007); all of which are incorporated by reference herein). Thus, during neurogenesis, REST is progressively down regulated to allow elaboration of the mature neuronal phenotype (Ballas et al, 2005 supra). The importance of this event is demonstrated by gain-of-function studies that indicate the persistence of REST impedes terminal neuronal differentiation (Mandel G et al, Proc Natl Acad Sci U S A 108, 16789-16794 (2011) and Gao Z et al, J Neurosci 31, 9772-9786 (2011); both of which are incorporated by reference herein).

[0004] Relatively little is known either about the transcriptional or post-transcriptional regulation of REST (Ballas N et al 2005 supra; Ballas N et al, Neuron 31, 353-365 (2001); and Kojima T et al, Brain Res Mol Brain Res 90, 174-186 (2001); all of which are incorporated by reference herein). However, two phosphorylation sites on REST (serines 861 and 864) regulate neuronal differentiation by their interaction with C-terminal domain small phosphatase-1 (CTDSP1)². When CTDSP1 removes phosphates from serine 861 and 864, REST protein is stabilized and neuronal differentiation is inhibited²⁻⁴.

SUMMARY OF THE INVENTION

[0005] Disclosed herein are methods, compounds, and compositions for developing peptides with high affinity to CTDSP1.

[0006] Disclosed herein are methods, compounds, and compositions for binding C-terminal domain small phosphatase 1 (CTDSP1).

[0007] Disclosed herein are REST phosphomimetic peptides, TEDLEPPEPPLPKEN (SEQ ID NO: 1), EDLEPPEPPLPK (SEQ. ID NO: 15), nekplppeppeldet (SEQ ID NO: 16) and kplppeppelde (SEQ ID NO: 17), which bind CTDSP1 to inhibit REST activity. The lowercase letters represent D-amino acids, which are resistant to degradation and therefore increase peptide half-life, without compromising binding affinity⁵.

[0008] Disclosed herein are ninety eight (98) REST phosphomimetic peptide variants (RPPv) (SEQ IDS: 18 through 117), which inhibit CTDSP1 activity on REST to varying degrees (Figure 25).

[0009] Disclosed herein are intracellular transport peptides, such as cell penetrating peptides (CPP) and/or endosomal release sequences, (SEQ ID NOS: 118 through 137 and 140 through 159) that may be fused to an RPP (SEQ ID NOS: 1 and 15 through 17) or an RPPv (SEQ ID NOS: 18 through 117) at the N- or C-terminus to improve intracellular transport. Also disclosed are linkers (SEQ ID NOS: 138, 139, 160 and 161) that may be inserted between an RPP or RPPv and one of the peptides listed in Table 7, to further improve intracellular transport.

[0010] Disclosed herein is that RPP (SEQ ID NO: 1 and 15 through 17) or RPPv (SEQ ID NOS: 18 through 117) fused at the N- or C-terminus to an intracellular transport peptide (SEQ ID NOS: 118 through 137 and 140 through 159) and cyclized to further improve binding affinity and stability (increase peptide half-life). Examples of cyclized fusion proteins are SEQ ID NOS: 2, 5, 12, 13, and 14.

[0011] Disclosed herein is that RPP (SEQ ID NO: 1 and 15 through 17) or RPPv (SEQ ID NOS: 18 through 137 and 140 through 159) fused at the N- or C-terminus to an intracellular transport peptide (SEQ ID NOS: 118 through 137 and 140 through 159), promotes degradation of REST protein. Examples of the fusion protein are SEQ ID NOS: 2, and 4 through 14.

[0012] Disclosed herein is that REST phosphomimetic peptide of SEQ ID NO: 1 and 15-17 or RPPv SEQ ID NOS: 18 through 117 fused at the N- or C-terminus to an intracellular transport sequence (SEQ ID NOS: 118 through 137 and 140 through 159), promotes expression of REST target genes. Examples of the fusion protein are SEQ ID NOS: 2, 4 through 14.

[0013] Disclosed herein are RPP SEQ ID NOS: 1 and 15 through 17 or RPPv (SEQ ID NOS: 18 through 117) fused at the N- or C-terminus to an intracellular transport peptide (SEQ ID NOS: 118 through 137 and 140 through 159), is useful for treating animals having a disease or condition associated with REST or CTDSP1 by administering to the animal a therapeutically effective amount of the compound so that expression of REST target genes are increased or that expression of BDNF is increased. Examples of the fusion protein are SEQ ID NOS: 2 and 4 through 14.

[0014] Disclosed herein are RPP SEQ ID NOS: 1 and 15 through 17 or RPPv (SEQ ID NOS: 18 through 117) fused to an intracellular transport peptide (SEQ ID NOS: 118 through 137 and 140 through 159), is useful for treating traumatic brain injury, chronic pain, peripheral nerve injury, epilepsy, diabetes, Alzheimer's disease, Huntington's disease, brain tumors (including glioblastoma multiforme), or pancreatic cancer in an animal. Examples of the fusion protein are SEQ ID NOS: 2 and 4 through 14.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The accompanying drawings are incorporated in and constitute a part of the specification. The drawings, together with the general description given above and the detailed description of the exemplary embodiments and methods given below, serve to explain the principles of the invention. In such drawings:

[0016] Figure 1. (A) Full length REST protein showing the location of the CTDSP1 binding site. (B) The retro inverted (RI) REST phosphomimetic peptide integrated with a cell penetrating peptide in a cyclic format.

[0017] Figure 2. Binding affinity of the REST phosphomimetic peptide (RPP) for CTDSP1 was measured using the Monolith (NanoTemper). The affinity of RPP (Table 4; SEQ ID NO: 165) for CTDSP1 (Table 2; SEQ ID NO: 163) is approximately 130 pM. Binding was assessed by fluorescently labelling the His tag on CTDSP1 (40 nM) with RED-tris NTA dye (20 nM) and measuring the change in fluorescents when RPP (up to 0.5 μ M) is bound.

[0018] Figure 3. Binding affinity of the RPP for CTDSP1 was measured using the Biacore (GE Healthcare Life Sciences). CTDSP1 (Table 2; SEQ ID NOS: 163) was immobilized on a CM5 sensor chip (GE Healthcare Life Sciences) and binding affinity of (A) GST control or (B) RPP (Table 4; SEQ ID NO: 165) was measured.

[0019] Figure 4. RPP is cell permeable and accumulates in the nucleus. (A-D) 100 nM FLAG-RPP-CPP (CAQKDYKDDDDKTEDLEPPEPPLPKENGRKKRRQRRRG (SEQ. ID NO: 4)) was added to mesenchymal progenitor cells (MPCs) and incubated for 4 hours. After 6 days in culture, the RPP concentrated in the nucleus. (E, F). Over a 6-day period, the peptide translocates from the cytoplasm to the nucleus (standard deviation, n=3). (A) cytoskeleton (phalloidin); (B) nucleus (hoechst dye); (C) RPP (FLAG Ab); (D) composite image.

[0020] Figure 5. RPP accumulates in the nuclei of the sciatic nerve in spinal cord. A 0.7 cm coronal spinal cord section from the L3 and L4 region of a Sprague Dawley (SD) male rat showing the surrounding nuclei of the sciatic nerve, harvested 48 hours after 1 mg (1 mg/mL) of peptide was injected into the site of a mid-thigh sciatic nerve defect injury. A. Hoechst stain shows nuclei. B. FLAG antibody shows FLAG-RPP-CPP (SEQ. ID NO: 4) localization.

[0021] Figure 6. RPP reduces REST protein levels. (Left) Western blot showing exogenously expressed REST protein levels in HEK293 cells after treatment with vehicle or 100 nM FLAG-RPP-CPP (SEQ. ID NO: 4) for 4 hours. The blot was probed with anti-REST (Millipore-Sigma, 07-579) and anti-GAPDH. (Right) Bar graph showing 100 nM FLAG-RPP-CPP decreased REST protein levels 58%. Band intensity was calculated by measuring the area of gel peaks using ImageJ.

[0022] Figure 7. RPP increases BDNF expression. mRNA levels in HEK 293 cells measured with qPCR after a 4-hour incubation with FLAG-RPP-CPP (SEQ. ID NO: 4, 100 nM) or vehicle (PBS) 24 hours in culture. The mean fold change in expression of BDNF ($2^{-\Delta\Delta C_T}$) was 2.401 ± 0.885 (standard deviation, $n=3$). A mean fold change of 1 = no change. $2^{-\Delta\Delta C_T}$ calculated as described by Livak et al., 2001.

[0023] Figure 8. mRNA levels of Kv4.3 in NBFL cells 48 h after CTDSPI inhibition. Cells were transfected with a plasmid expressing RPP (SEQ ID NO: 1) with GFP (+) or a control peptide with GFP (-). 24 hours after transfection, cells were sorted based on fluorescence intensity. Error bars represent standard deviation, $n=2$.

[0024] Figure 9. Comparison of linear and cyclic RPP. NBFL cells were dosed with (1 μ M) RPP (linear: SEQ ID NO: 4; cyclic: SEQ ID NO: 2, Table 8) or control peptide (SEQ ID NO: 3, Table 9) for 24 or 48 hours. Efficacy on was assessed by measuring changes in (A) BDNF, (B) NGF, and (B) Kv4.3 transcript levels. $N=1$

[0025] Figure 10. BDNF and NGF mRNA expression (normalized to actin) in mesenchymal progenitor cells (MPCs), from two patients, after a 48 hour incubation with water (Ctrl) or 3 μ M RPP (SEQ. ID NO: 9). mRNA levels are shown relative to control (standard deviation, $n=2$).

[0026] Figure 11. iPSCs (neural stem cells (NSC)-NL5) were dosed with one of the RPP sequences (3 μ M) or water control shown below. After 7 days in culture, cells were assessed for neuronal differentiation using the Microtubule Associated Protein 2 (MAP2) neuronal marker (normalized to DAPI). Media and variants were replaced on Day 3. MAP2 levels are relative to control in the bar graph (standard deviation n=4).

Sequence ID number	Peptide
Control	Water
5	CTEDLEPPEPPLPKENSGDIMGEWGNEIFGAIAGFLGYGRKKRRQRRRG ^{cyclic}
6	TEDLEPPEPPLPKENRRWRRRWRr
7	EDLEPPEPPLPKRWRRRWRr
8	AGDLEQPEPPVAKKKKNRRWRRWRr
9	rrrrwrrwrrwrrnekplpeppeldet
10	TEDLEPPEPPLPKENrrrrwrrwrrwrr
11	TEDLEPPEPPLPKENRRRRRRC ₁₄ RRWRRr

Lowercase = D-amino acids
C₁₄ = 2-amino-tetradecanoic acid

[0027] Figure 12. iPSCs (neural stem cells (NSC)-NL5) were with 1 μ M RPP (SEQ ID NO. 9) or water control. After 7 days in culture, cells were assessed for neuronal differentiation using neuronal markers TUJ1 (class III beta-tubulin) and MAP2 (both normalized to DAPI). Media and RPP were replaced on Day 3. (standard deviation, n=6).

[0028] Figure 13. iPSCs (neural stem cells (NSC)-NL5) were with 1 μ M RPP (SEQ ID NO. 13) or water control. After 7 days in culture, cells were assessed for neuronal differentiation using neuronal markers TUJ1 and MAP2 (both normalized to DAPI). Media and RPP were replaced on Day 3. (standard deviation, n=6).

[0029] Figure 14. RPP (SEQ. ID NO: 12) increases expression of K_v4.3, K_v7.2, Nav1.8 and OPRM1. qRT-PCR analysis of mRNA expression (normalized to β -actin), relative to water control, after incubation of adult Sprague Dawley (SD) rat L5 DRGs with various

concentrations of rpp for 48 h. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, one-way ANOVA and Dunnett's test, error bars = standard error of the mean.

[0030] Figure 15. 3 μ M RPP for 48 hours (SEQ ID NOS: 13 and 14) increased expression of Nav1.8. qRT-PCR analysis of mRNA expression (normalized to β -actin), relative to water control, after incubation of RPP with adult Sprague Dawley (SD) rat L5 DRGs. Error bars = standard error of the mean.

[0031] Figure 16. RPP (SEQ. ID NO: 12) increases expression of BDNF and NGF. qRT-PCR analysis of mRNA expression (normalized to β -actin), relative to water control, after incubation of adult Sprague Dawley (SD) rat L5 DRG neurons with various concentrations of RPP for 48 h. * = $p < 0.05$, one-way ANOVA and Dunnett's test, error bars = SEM.

[0032] Figure 17. RPP (SEQ. ID NO: 12) does not cause necrosis in DRG neurons. LDH toxicity assay in SD rat incubated with water, RPP (1, 3, or 10 μ M), or triton for 48 hours. *** = $p < 0.001$, one-way ANOVA and Dunnett's test, error bars = SD.

[0033] Figure 18., RNA Levels of Chronic Pain Genes After SNI. RNA fold change relative to β -actin on Day 0 (sham, $n=3$), 7 ($n=5$), or 28 ($n=2$) after SNI (mean $\pm$ SD, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with Dunnett's multiple comparison test. RNA was isolated from sciatic nerve DRG (L5).

[0034] Figure 19. REST mRNA levels increase after TBI. (A) Normal levels of REST mRNA expression in the ipsilateral cortex of uninjured mouse. (B) REST levels increase dramatically in the mouse ipsilateral cortex 7 days after cortical controlled impact injury⁶. mRNA visualized by RNAscope with DAPI counterstain.

[0035] Figure 20. (a) Schematic of AAVS1-Nanoluc-Halotag Knock-in (KI) construct used to engineer NCRM-1 iPSCs. The CMV-driven construct was inserted into the safe harbor

AAVS1 locus of Chr. 19q by transcription activator-like effector nucleases (TALENs). AAVS1 KI was confirmed by sequencing and junction PCR (not shown). (b) Schematic of MAP2-Nanoluc-Halotag Knock-in (KI) construct used to engineer NCRM-1 iPSCs. Nanoluciferase-Halotag was knocked-in to the MAP2 transcriptional start site (TSS) (Chr. 2) using zinc finger nucleases (ZFN) and MAP2-in frame KI confirmed by sequencing and junction PCR (not shown). CMV-NLHT and MAP2-NLHT iPSCs express pluripotency markers OCT4/NANOG/TRA 1-81/ TRA 1-60 (not shown). (c) Luciferase assay of CMV-NLHT iPSCs and MAP2-NLHT neural stem progenitor cells (NSPC) and neurons. Note the increasing luciferase activity with neuronal differentiation of MAP2-NLHT cells consistent with increased MAP2 expression with differentiation.

[0036] Figure 21. Rat sciatic nerve injury model: (Left) Surgical approach of sciatic nerve transection. (Right) Immunohistochemistry-Immunofluorescence staining from section of sciatic nerve. Tissues were stained for β III Tubulin, Laminin I, and DAPI. The scale bars = 100 μ m.

[0037] Figure 22. Rat sciatic nerve harvest: (Left) En Bloc section of rat spinal column and associated peripheral nerves. (Right) Image of sciatic nerve injury site with associated lumbar nerve roots

[0038] Figure 23. A. Compared to baseline (BASE), SNI induces sensitization of mechanical pain threshold (MT) 4-days post-injury ($n=16$); paired t-test, $*p<0.05$, SHAM shows no significant difference in mechanical threshold 4-days post-surgery. B. Analgesic effect of single S.C. injection of morphine on MT post-injury (SNI+MOR, $n=4$). Mean $\pm$ SEM; $F=4.62$, $*p<0.05$ by one-way ANOVA before and after treatment; mean difference and 95% CI= -7.3, -14.5 to -0.135, $p<0.05$ by Bonferroni's Multiple Comparison test. 20 mg/Kg of MOR ($n=4$; $F=19.03$, $**p<0.001$ by one-way ANOVA).

[0039] Figure 24. Conditioned Place Preference (CPP) after conditioning with saline

(control) and 4 doses of morphine (MOR) in SNI mice. MOR induced CPP in a dose-dependent manner (one-way ANOVA, $F=4.167$, $p<0.05$); 10 mg vs control ($p<0.001$) and 20 mg ($p<0.05$) by Tukey's multiple comparisons test.

[0040] Figure 25. *In vitro* CTDSP1 phosphatase activity (relative to GST control) on the REST peptide (TEDpSPPpSPPLPKEN) as a substrate after exposure to 1 μ M of RPP (SEQ ID NO: 1) or one of its variants (Table 3).

[0041] Figure 26. *In vitro* on target (CTDSP1) and off target phosphatase activity (1=PPA1, 2=PPM1H, 3=PPM1A, 4=PP3CA, 5=PPP1CA, 6=PP5CA), relative to water control, on the phosphorylated REST peptide (TEDpSPPpSPPLPKEN) as a substrate after exposure to RPP (SEQ ID NO: 12). N=5, error bars are standard deviation.

[0042] Figure 27. Building RPP libraries. A) Scheme of the experiment; B) Nucleotide Sequence of the REST construct (before mutagenesis); C) Protein Sequence of the REST construct (before mutagenesis). RPP, CPP and His tag are underlined.

[0043] Figure 28. RPP (SEQ ID NO: 13) structure. Along the bottom of the structure is the CPP containing a poly arginine sequence with two 2-aminotetradecanoic acids that are integrated with the REST RI phosphomimetic (starting with the lysine at the bottom right of the structure).

[0044] DETAILED DESCRIPTION OF THE EMBODIMENTS In an embodiment, the present invention provides the methods (described in examples 1 through 3) for developing peptides with high affinity to CTDSP1. Importantly, the method uses peptide evolution techniques to generate peptides with high affinity to CTDSP1.

[0045] In an embodiment, the present invention provides a peptide of SEQ ID NOS: 1 and 15 through 17 or a peptide having at least 50%, more preferably at least 60%, yet more preferably at

least 70%, still more preferably at least 80%, even more preferably at least 90%, or even yet more preferably at least 95% similarity to SEQ ID NO: 1 and 15 through 17. These peptides or similar peptides thereof are referred to herein as REST phosphomimetic peptides (RPP). Importantly, however, the glutamic acids in the RPP must be maintained, particularly the glutamic acid at positions 5 and 8 of SEQ ID NO: 1, positions 4 and 7 of SEQ ID NO: 15, positions 8 and 11 of SEQ ID NO: 16, and positions 6 and 9 of SEQ ID NO: 17.

[0046] In an embodiment, the present invention provides RPP_v (SEQ ID NOS: 18 through 117), or a peptide having at least 50%, more preferably at least 60%, yet more preferably at least 70%, still more preferably at least 80%, even more preferably at least 90%, or even yet more preferably at least 95% similarity to an RPP_v (SEQ ID NOS: 18 through 117). The peptide or similar peptide thereof is referred to herein as the REST phosphomimetic peptide variant (RPP_v) (SEQ ID NOS: 18 through 117). Importantly, however, the glutamic acids in SEQ ID NOS: 18 through 67 must be maintained, particularly the glutamic acid at positions equivalent to 5 and 8 of SEQ ID NO: 1. For the retro inverted sequences (RPP_v^{RI}) (SEQ ID NOS: 68 through 117), positions 8 and 11 of SEQ ID NO: 16 must be maintained.

[0047] In an embodiment, the present invention a fusion protein between RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) and an intracellular transport peptide (SEQ ID NOS: 118 through 137 and 140 through 159) or a peptide having at least 50%, more preferably at least 60%, yet more preferably at least 70%, still more preferably at least 80%, even more preferably at least 90%, or even yet more preferably at least 95% similarity.

[0048] The identity/similarity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the

more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are.

[0049] Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, Adv. Appl. Math. 2:482, 1981; Needleman & Wunsch, J. Mol. Biol. 48:443, 1970; Pearson & Lipman, Proc. Natl. Acad. Sci. USA 85:2444, 1988; Higgins & Sharp, Gene, 73:237-44, 1988; Higgins & Sharp, CABIOS 5:151-3, 1989; Corpet et al., Nuc. Acids Res. 16:10881-90, 1988; Huang et al. Computer Appls. in the Biosciences 8, 155-65, 1992; and Pearson et al., Meth. Mol. Bio. 24:307-31, 1994. Altschul et al., J. Mol. Biol. 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

[0050] The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., J. Mol. Biol. 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, MD 20894) and on the Internet, for use in connection with the sequence analysis programs BLASTP, BLASTN, BLASTX, TBLASTN AND TBLASTX. Additional information can be found at the NCBI web site (www.ncbi.nlm.nih.gov).

[0051] BLASTN is used to compare nucleic acid sequences, while BLASTP is used to compare amino acid sequences. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences.

[0052] Once aligned, the number of matches is determined by counting the number of

positions where an identical nucleotide or amino acid residue is presented in both sequences. The percent sequence identity is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence, or by an articulated length (such as 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100. For example, a nucleic acid sequence that has 1166 matches when aligned with a test sequence having 1554 nucleotides is 75.0 percent identical to the test sequence ($1166/1554 \times 100 = 75.0$). The percent sequence identity value is rounded to the nearest tenth. For example, 75.11, 75.12, 75.13, and 75.14 are rounded down to 75.1, while 75.15, 75.16, 75.17, 75.18, and 75.19 are rounded up to 75.2. The length value will always be an integer. In another example, a target sequence containing a 15-nucleotide region that aligns with 20 consecutive nucleotides from an identified sequence as follows contains a region that shares 75 percent sequence identity to that identified sequence (that is, $15/20 \times 100 = 75$).

[0053] For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost 5 of 1). Homologs are typically characterized by possession of at least 70% sequence identity counted over the full-length alignment with an amino acid sequence using the NCBI Basic Blast 2.0, gapped blastp with databases such as the nr or swissprot database. Queries searched with the blastn program are filtered with DUST (Hancock and Armstrong, 1994, Comput. Appl. Biosci. 10:67-70). Other programs use SEG. In addition, a manual alignment can be performed. Proteins with even greater similarity will show increasing percentage identities when assessed by this method, such as at least about 75%, 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to a protein. When aligning short peptides (fewer than around 30 amino acids), the alignment is performed using the Blast 2 sequences

function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequence will show increasing percentage identities when assessed by this method, such as at least about 60%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% sequence identity to a protein. When less than the entire sequence is being compared for sequence identity, homologs will typically possess at least 75% sequence identity over short windows of 10-20 amino acids, and can possess sequence identities of at least 85%, 90%, 95% or 98% depending on their identity to the reference sequence. Methods for determining sequence identity over such short windows are described at the NCBI web site.

[0054] One indication that two nucleic acid molecules are closely related is that the two molecules hybridize to each other under stringent conditions, as described above. Nucleic acid sequences that do not show a high degree of identity may nevertheless encode identical or similar (conserved) amino acid sequences, due to the degeneracy of the genetic code. Changes in a nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid molecules that all encode substantially the same protein. An alternative (and not necessarily cumulative) indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

[0055] In certain embodiments, the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) may be fused to another peptide, e.g., to allow for cell penetration. Preferably, the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) is fused to a peptide sequence that allows cell penetration and escape from endosomes. More preferably, the cell penetration and escape peptide sequence is one SEQ ID NOS: 118 through 137 and 140 through 159. RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through

117) and cell penetration peptide (CPP) (SEQ ID NOS: 118 through 137 and 140 through 159) may be fused directly or contain a linker (e.g., SEQ ID NOS: 138, 139, 160, or 161) sequence connecting them. It has been shown that when cells or tissues are dosed with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), it localizes in the nucleus (FIG. 3, SEQ ID NO: 4 and Fig. 4, SEQ ID NO: 4), causes REST degradation (FIG. 5, SEQ ID NO: 4), increases the expression of genes targeted by REST (FIG. 7, SEQ ID NO: 4, FIG. 8, SEQ ID NO: 1, FIG. 9, linear_SEQ ID NO: 4 and cyclic_SEQ ID NO: 2, FIG. 10, SEQ ID NO: 9, FIG. 14, SEQ ID NO: 12, FIG. 15, SEQ ID NOS: 13 & 14, and FIG. 16, SEQ ID NO: 12), and causes neural differentiation (FIG. 11, SEQ ID NOS: 5 through 11, FIG. 12, SEQ ID NO: 9, and FIG. 13, SEQ ID NO: 13).

[0056] The present inventor has discovered that the RPP (SEQ ID NOS: 1 and 15 through 17) binds CTDSP1 to inhibit REST activity. The RPP has low pM affinity for CTDSP1, as demonstrated by the binding curves shown in figures 2 and 3. In cells and animals, the RPP binds to CTDSP1, which prevents CTDSP1 from binding to REST.

[0057] Increased REST and associated neural gene repression underlie the pathology of the following diseases or disorders:

[0058] Traumatic Brain Injury - Traumatic brain injury (TBI) results in loss of cognitive ability due to neuronal death. Two issues should be addressed to improve cognitive recovery after TBI, 1) reduce the neuronal death that continues after injury and 2) regenerate lost neurons.

[0059] REST levels increase dramatically in the brain after TBI (Fig. 19). Consistently, several studies have shown that brain injury caused by acute ischemia induces REST expression in neurons resulting in their death⁷⁻⁹. These studies demonstrate that neuron survival improves when REST is removed⁷⁻⁹. The clinical implications are profound. For example, elevated levels of REST

are correlated with an increased frequency of seizures, which commonly occur after brain injury¹⁰⁻¹². In animal models of brain injury, the rate of seizure decreases significantly when REST is inhibited^{13,14}. Also, chronically elevated levels of REST after TBI are likely to play a role in neurodegenerative diseases attributed to a history of brain injury¹⁵⁻¹⁷. Both *in vitro* and *in vivo* studies show that removing REST improves neuronal survival⁷⁻⁹, function^{14,18}, and regeneration^{2,19-24}. Therefore, targeting REST after TBI should mitigate the effects of brain damage and offset the risks of developing associated age-related neurodegenerative diseases, such as Alzheimer's^{17,25}.

[0060] The basis for neurogenesis improving cognitive function in TBI patients are studies associating increased neurogenesis with improved learning, memory, and other cognitive functions²⁶⁻³⁰ and studies showing the converse, that inhibition of neurogenesis via antimetabolic agents, radiation, or genetic manipulations impairs hippocampal dependent forms of memory²⁹⁻³³. Defects in neurogenesis are also linked with many diseases with cognitive etiologies including developmental disorders (e.g., microcephaly³⁴, megalencephaly³⁵, and autism³⁵) and neurodegenerative diseases (e.g., dementia and Alzheimer disease)³⁶. Experimental treatments (including blood transfusions, growth and neurotrophic factors, and stem cell transplantation^{10,37-49}) have focused on regenerating neurons. These attempts have failed to stimulate the neurogenesis required to restore cognitive function, at least in part because they are not capable of terminal differentiation of neural progenitor cells in the context of TBI^{26,28,50-57}. Terminal differentiation of neural progenitors into neurons is blocked at a single checkpoint by the RE1 silencing transcription factor known as REST.

[0061] Peripheral Nerve Injury - Neuronal regeneration in peripheral nerve injury (PNI) patients is inhibited in at least two ways. First, there is a reduction in neurotrophic factors (NTFs) that support the growth, survival, and differentiation of both developing and mature neurons⁵⁸.

Second, there is a decrease in the expression of genes required for synaptic plasticity including axonal growth, vesicular transport, and ionic conductance⁵⁹. Both phenomena occur coincident with injury-induced expression of REST⁶⁰. Therefore, eliminating REST accelerates recovery from a PNI by reversing a block on nerve regeneration. Inhibition of CTDSP1 with the RPP promotes REST degradation (Fig. 6), increases neuronal differentiation (Fig. 11, 12, and 13), and increases NTF expression (Fig. 7, 9, 10, and 16), which demonstrates the potential of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) to stimulate nerve regeneration.

[0062] Chronic Pain - Many of the genes that are repressed in the central and peripheral circuits during chronic pain are either direct or indirect targets of the repressor element-1 (RE1)-silencing transcription factor (REST), an inhibitor of neuronal gene expression in stem cells, neural progenitors, and non-neuronal cells⁶¹⁻⁶³. REST levels are normally kept low in healthy neurons through active degradation to keep the chromatin clear of this powerful neuronal gene repressor⁶⁴⁻⁶⁹. Before the transition to neuropathic pain, REST levels significantly increase in the neurons of the peripheral nerves of mice and rats after peripheral nerve injury followed by a spike in neurons in the central nervous system where pain stimuli are processed and experienced^{60,70,71}. The activation of REST after nerve injury results in decreased expression of several genes required for normal excitability of sensory neurons, including the potassium channels K_v4.3 (Kcnd3) and K_v7.2 (Kcnq2), the sodium channel Nav1.8 (Scn10a), and the mu opioid receptor Oprm1 (Fig. 18)^{60,72-74}. The basis for blocking REST to alleviate chronic pain are published studies using mouse and rat peripheral nerve injury (PNI) models^{60,72-74}. In mice, genetic knockdown of REST in sensory neurons after PNI restores gene expression of the mu opioid receptor^{60,72,73}, Nav1.8^{60,72,73}, K_v4.3^{72,73,75}, and K_v7.2^{72,73} in the dorsal root ganglion, restores normal K⁺M-type current in C

nerve fibers⁶⁰, reduces hyperalgesia and allodynia⁷²⁻⁷⁴, and restores morphine analgesia^{60,74} in chronic pain models (Table 12). Disruption of the REST repressor complex, using an optimized REST mimetic of the mSin3 binding site, an adaptor protein which contributes to transcriptional repression of many genes including REST targets, restored C-fiber function, reduced hyperalgesia and allodynia, and restored morphine analgesia in mouse models of chronic pain (Table 12)⁷⁴. In rats, knockdown of REST controls the transition from acute to chronic pain, demonstrated by a reduction in hyperalgesia and allodynia, and restored muscarine analgesia (Table 12)^{72,73}.

Table 12							
Summary of chronic pain studies using REST blocking methods							
REST block	Day post injury	PNI	Assessment	Result	Species	Number	Ref.
Antisense Oligo	1, 3, 5, & 6	pSNL	Kv4.3	Recovered [+2x (mRNA) compared to vehicle control on Day 7]	Mouse	5 M	75
Antisense Oligo	1, 3, 5, & 6	pSNL	Oprm1	Recovered [+3x (mRNA) compared to Vehicle control on Day 7]	Mouse	3 M	60
			Nav1.8	85% recovered [+2x (mRNA) compared to control on Day 7]			
			C-fiber	Recovery of function, compared to sham [PWT on Day 7]			
			Morphine analgesia	85% recovery, compared to sham [PWT on Day 7]			
mSin3 decoy	5-11	pSNL	C-fiber	75% reversal of hyposensitivity, compared to Sham [PWT on Day 12]	Mouse	2-6/sex	74
			Morphine analgesia	Reversal of loss [PWT (ICS, IPS) on Day 12 & 19]			
		Fibromyalgia (IPS, ICS)	Hyperalgesia/allodynia	Reverse (Day 12 & 19)			
Over expression AAV-REST (DRG)	-	-	Hyperalgesia (mechanical, hot, & cold)	Induction (PWT) on Day 20, 25, 30	Mouse	3-5/sex	72,73
cKO	Pre	CFA, SNI, pSNL-	Hyperalgesia (mechanical, hot, & cold)	None (PWT, Day 5, 10, 15)			
		SNI	Kv7.2, Nav1.8, Oprm1, Kv4.3	Prevent down regulation			
siRNA	21-26	SNL	Allodynia (tactile)	Reduce (up to 45%, Day 21 to 26)	Rat	6-9 M	72,73

			Hyperalgesia (mechanical)	Reduce (up to 65%, Day 21 to 26)			
			Charm2	Restores mRNA [2x compared to control siRNA on Day 5]			
			Muscarine analgesia (tactile & mechanical)	Restores (PWT)			
Intermediate psychological stress (IPS); Intermittent cold stress (ICS); Paw withdrawal threshold (PWT)							

[0063] We recognize the studies presented in Table 12 reveal a discrepancy in the reduction in hypersensitivity between the mouse conditional knockout (cKO) study (complete knockout), which showed no hypersensitivity and no downregulation of REST target genes after injury (Table 12), and the rat siRNA knockdown study, which only partially reduced allodynia (up to 45%) and hyperalgesia (up to 65%) after injury. We believe the knockout effect is more potent than siRNA knockdown, because the bioavailability of siRNAs is limited by their size (>13,000 kD) and poor cellular trafficking, due to endosomal entrapment.

[0064] We are aware that the studies in Table 12 lack rigor in that power analysis was not done to establish the number of animals per group and the animal numbers were low which makes statistical analysis questionable. Some of the studies used the less predictive partial sciatic nerve ligation (pSNL) model, versus the preferred spared nerve injury (SNI) chronic pain model. The majority of these studies only assess males. However, despite these shortcomings, the outcomes of the studies are consistent and, therefore, support our proposed *in vivo* efficacy assessment of drug for the treatment of chronic pain. Furthermore, because REST activity is highly conserved in mammals, we believe these studies provide strong proof-of-concept support to test modulation of REST in humans.

[0065] We discovered that REST is targeted for degradation through phosphorylation of its serines 861 and 864. REST is stabilized by dephosphorylation of these sites by the C-terminal domain small phosphatase 1 (CTDSP1)²⁻⁴. Like REST, CTDSP1 is expressed in nonneuronal tissues where it contributes to neural gene silencing via its interaction with REST. We concluded

that inhibition of CTDSP1 would be sufficient to promote REST degradation and stimulate gene expression. In support of this conclusion, we have demonstrated that knockdown of CTDSP1 in mesenchymal progenitor cells (MPCs) and nerves causes expression of REST target genes and axonal regeneration⁷⁶. We leveraged our mechanistic understanding of the REST-CTDSP1 interaction to develop a cyclic phosphomimetic peptide encompassing the relevant regulatory region of REST⁷⁷. The REST phosphomimetic peptide (RPP) has several pharmacologic attributes. It is highly stable due to its cyclic structure and D-amino acid composition (e.g., SEQ ID NO: 9, 12, 13, 14, 16, 17, and 68-117). It has low pM binding affinity exceeding that of most antibodies (Fig. 2 & 3), suggesting a low risk of off-target effects. Its small size (<3.5 kD) indicates it should have bioavailability approaching that of a small molecule drug. Our preliminary data shows that our mimetic inhibits the activity of CTDSP1, reduces REST protein levels, and results in the expression of neural genes needed for proper neuronal activity⁷⁷.

[0066] Most importantly, our approach is predicted to be safe and effective for the following reasons: 1) Our drug is selective for nuclear targets primed for regenerative response, such as after nerve injury. The basal organization of chromatin does not permit the epigenetic “writing” of a new set of instructions; nerve injury however, triggers a concerted, widespread, and transient nucleosome reorganization, a Genomic Transient Intermediate State (GTIS): a temporary nucleosomal architecture favorable for epigenetic reprogramming for the necessary response⁷⁸⁻⁸¹. 2) Our drug has very high binding affinity (low pM) and therefore will not have off target effects (Fig. 2&3). 3) We are proposing a brief treatment period of less than 1 month duration (~~Example 16, Aim 2~~). The basis for our short treatment period is that REST is the gate keeper for terminal differentiation of neurons^{64,82}, a process which irreversibly commits a cell to that lineage⁸³. Neural progenitors, which have epigenetic similarities to neurons in a chronic pain state⁶³, because they are

also in a GTIS, terminally differentiate after a few days of induction. Consistently, we have shown the RPP can induce neuronal differentiation (measured by MAP2 positive cells, a marker of neuronal differentiation) within 7 days (Fig. 11, 12, and 13). Once the process has been started and the cell has been committed, there is no need for further treatment. Therefore, we expect the pain relief achieved through this early treatment period to be durable. 4) We have shown our drug is not toxic to neurons in an *ex vivo* DRG neuron toxicity assay measuring necrosis (Fig. 17). 5) REST and CTDSP1 are primarily involved in neurodevelopment, therefore, we predict inhibiting their activity in injured, adult cells will be well tolerated. 6) The safety of peptide drugs has made them an attractive therapeutic strategy. Currently, there are more than 68 marketed peptide drugs with global sales exceeding \$14.7 billion, and 140 are in clinical development^{84,85}.

[0067] INNOVATION

[0068] First-in-class drug candidate. We have developed the first drug to target a transcriptional repressor of neuronal genes, REST. The published literature show that REST expression contributes to chronic pain (Table 12). After PNI, REST and CTDSP1 expression is increased in both the peripheral (Figure 18) and central nervous systems resulting in dysfunction⁷⁻⁹. Several published studies in rodents have shown that blocking REST alleviates chronic pain^{60,72-74}.

[0069] Innovative drug design. We have developed a cyclic phosphomimetic cell penetrating peptide that destabilizes REST by inhibiting CTDSP1³, the phosphatase that protects REST from degradation, allowing for the expression of neural-specific genes. Like REST, expression of CTDSP1 is restricted to nonneuronal cell types, except after neuronal injury. Our inhibitor is novel in that it directly targets a transcriptional checkpoint that immediately regulates the genes necessary to induce regeneration. It overcomes challenges to targeting transcription factors and to creating therapeutics against serine phosphatases. Regarding the latter, other

approaches have focused on phosphoprotein phosphatases (PPPs), and the metal-dependent protein phosphatases (PPMs)⁸⁶. However, CTDSP1 is a haloacid dehalogenase (HAD) which uses two catalytic aspartic acids instead of relying solely on a metal ion for activity⁸⁷. This catalytic site is amenable to specific inhibition as demonstrated by the success in developing therapeutics against similar enzymes, such as HIV-1 protease⁸⁸⁻⁹¹. Finally, CTDSP1 has a proline dependent substrate preference, not found in other serine phosphatases, which we have exploited to improve the binding and stability of our drug.

[0070] PRELIMINARY DATA. REST represses gene expression by binding to the chromatin at repressor element-1 (RE-1) sites near the regulatory regions of neuronal genes, including ion channels, growth factors, and axonal-guidance proteins¹. Therefore, before stem cells, such as neural progenitor cells (NPCs), can terminally differentiate, they must first target REST for degradation to express the required neuronal genes⁶⁴. REST is protected from degradation by CTDSP1, which is both necessary and sufficient to prevent neuronal gene expression². Dominant-negative CTDSP1, which can bind phosphorylated targets but cannot catalyze dephosphorylation, induces terminal differentiation of P19 stem cells⁹².

[0071] We have previously shown that the interaction between CTDSP1 and REST is dependent on phosphorylation of REST serines 861 and 864 by the MAP kinase, ERK2, and that mutation of these serines to alanine increases REST stability²⁻⁴. We surmised that a non-hydrolysable phosphomimetic of this REST regulatory region could slow down CTDSP1 activity and promote REST degradation. To test our hypothesis, we developed a REST Phosphomimetic Peptide or RPP⁷⁷ to assess dose-dependent effects on REST and REST-targets. The RPP contains amino acids 858 to 870 from REST with serine 861 and 864 mutated to glutamates, which can mimic both the shape and overall charge of phosphoserine. To the C-terminus, we fused an

arginine-rich cell-penetrating peptide and endosomal exit sequence derived from HIV-Tat and HA2, respectively, which are used to deliver peptides and proteins into cultured mammalian cells and live organisms and facilitate crossing of the blood-brain barrier (BBB)⁹³⁻⁹⁶.

[0072] We investigated if RPP could decrease REST protein (Fig.6). In the left panel, the Western blot (WB) shows that RPP decreased REST (exogenous + endogenous) levels in HEK cells. In the right panel, quantification of total REST protein (exogenous + endogenous) levels by WB analysis shows an overall decrease of 58.3% in the RPP dosed HEK cells (Fig.6).

[0073] In an *in vitro* binding assay, RPP had low pM affinity for CTDSP1, with a slow off rate (Fig.2 &3). In an *in vitro* assay of phosphatase inhibition, RPP inhibited its target, CTDSP1, at low nM concentrations, but did not inhibit off target PP5, PP1, PPM1H, PPM1A, or PP3CA activity at 10 μ M (Fig.26). In a cell permeability and stability assay in MPCs, RPP translocated into the nucleus (Fig.4 and Fig.5), which is consistent with the localization profile of both REST and CTDSP1^{92,97}. RPP levels remained stable after 6 days in culture.

[0074] It has been established by that blocking REST in rodent chronic pain models, prevents downregulation of Nav1.8, Kv4.3, Kv7.2, and Oprm1 which are implicated as the cause of hyperalgesia and allodynia (Table 12). Based on these studies, we tested whether blocking CTDSP1 could stimulate the expression of REST target genes associated with chronic pain. We have also shown that NBFL cells transfected with RPP (without HIVTAT-HA2) or dosed with linear or cyclic RPP evoke an increase (2-fold and up to 4-fold, respectively) in Kv4.3 mRNA (Fig. 8&9c).

[0075] We found that 3 μ M RPP (SEQ ID NOS: 5-11) increased human neural progenitor cell (iPSC) differentiation by 2- to 2.7-times as measured by MAP2 (mature neuron marker) expression normalized to DAPI (nucleus), compared to control (Fig.11), and 1 μ M RPP (SEQ ID NO: 9 and 13) increased human neural progenitor cell (iPSC) differentiation as measured by TUJ1

(up to 36% and 3-times respectively) and MAP2 (up to 33% and 2-times respectively) (neuronal markers) expression normalized to DAPI (nucleus) (Fig. 12 and 13). There was no increase in cell death compared to control (data not shown). The basis for using this screen is that eliminating REST in neural progenitor cells has been shown to induce neuronal differentiation²², which is a readout for ensemble derepression of neuronal genes.

[0076] In a preliminary assessment of efficacy and toxicity, RPP was assessed at 0, 1, 3, or 10 μ M for 48 hours in an *ex vivo* culture of whole DRG neurons for its potential to induce expression of Kv4.3 (Fig. 14), Kv7.2 (Fig. 14), Nav1.8 (Fig. 14 and 15) and OPRM1 (Fig. 14) and cause neurotoxicity (Fig. 17). At 3 μ M SEQ ID NO: 12 induced Kv4.3, Kv7.2, Nav1.8, and OPRM1 approximately 0-, 6-, 3-, and 3-times, respectively, compared to control (Fig. 14). At 3 μ M SEQ ID NOS: 13 and 14 induced Nav1.8 approximately 6- and 13-times, respectively (Fig. 15). At 10 μ M SEQ ID NO: 12 induced Kv4.3, Kv7.2, Nav1.8, and OPRM1 approximately 2-, 7-, 5-, and 4-times, respectively (Fig. 14). In a lactate dehydrogenase LDH cytotoxicity assay, RPP sequence ID NO:12 showed no toxicity at 0, 1, 3, or 10 μ M (Fig. 16). As expected, the positive control (triton, n=6)) was toxic (Fig. 17).

[0077] Epilepsy - Epilepsy is uncontrolled electrical activity in the brain which causes confusion, loss of consciousness, and uncontrolled movements⁹⁸. It is the result of dysregulation of ion channels and receptors including SCN1A (Nav1.1), SCN2A (Nav1.2), SCN1B (Nav beta subunit 1), KCNQ2 (Kv7.2), and KCNQ3 (Kv7.3), which are targets of REST repression^{14,99}. The underlying cause of epilepsy is an increase in REST levels in the nucleus of neurons¹⁰⁰.

[0078] Diabetes - Pancreatic beta cells and neurons share similar transcriptional pathways during their differentiation program, which involve the elimination of REST¹⁰¹. Down regulation of REST target genes by over expression of REST in beta cells decreases insulin secretion^{102,103}.

[0079] Alzheimer's disease - In Alzheimer's disease, acetylcholine and choline acetyltransferase (ChAT), a transferase enzyme necessary for the synthesis of acetylcholine, are very low. The decrease in concentration of this enzymes contributes to the memory and cognition deficits associated with Alzheimer's. Increased REST levels in the brain suppress ChAT¹⁶.

[0080] Huntington's disease - In Huntington's disease, the translocation of REST from the cytoplasm to the nucleus of neurons is thought to be the cause of neuronal degeneration associated with the disease¹⁰⁴⁻¹⁰⁶.

[0081] Brain cancers, including glioblastoma multiforme - Brain cancers, including glioblastoma multiforme (GBM), originate from brain tumor initiating cells (BTICs), which are cancerous stem cells that display a robust capacity for self-renewal and can become cancerous tumors. These cells are resistant to radiation and chemotherapy and become proliferative months after treatment¹⁰⁷⁻¹⁰⁹. The RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) has the potential to prevent tumors from forming by terminally differentiating BTICs. Once cells have terminally differentiated they can no longer proliferate and seed new tumors¹¹⁰. It is important to state that terminal differentiation is not the same as the differentiation achieved by other methods, such as by targeting chromatin remodeling with inhibitors of histone deacetylases (HDACs). Inhibition of these enzymes alters gene expression indiscriminately and cannot achieve the permanent terminal differentiation that is required to prevent recurrent GBM. Terminal differentiation of BTIC into neurons is blocked at a single checkpoint by REST. The importance of this repressor in maintaining oncogenicity is highlighted by the observation that increased levels of REST correlate with both recurrence of GBM¹¹¹⁻¹¹³ and shorter periods of disease free survival^{112,114}. The reliance of BTICs on REST to maintain their oncogenicity creates an

opportunity for therapeutic intervention. REST is targeted for degradation through phosphorylation of serines 861 and 864, and that these serines are kept in a dephosphorylated state by the C-terminal domain small phosphatase, CTDSP1². Therefore, inhibition of CTDSP1 with the RPP (SEQ ID NOS: 1 and 15-17) or RPP_V (SEQ ID NOS: 18 through 117) that is fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) should release the REST-brake on terminal differentiation and prevent the recurrence of GBM and other brain tumors.

[0082] Pancreatic cancer - REST levels are high in advance stage, metastatic positive pancreatic cancer cells¹¹⁵. Pancreatic cancer patients with high REST levels in their tumors have worse survival rates¹¹⁵. *In vitro* functional experiments show that knockdown of REST suppressed proliferation, migration, invasion and epithelial-mesenchymal transition of pancreatic cancer cells (AsPC-1 and PANC-1)¹¹⁵. *In vivo* experiments (a subcutaneous BALB/c nude mouse model and a superior mesenteric vein injection BALB/c nude mouse model) show that knockdown of REST suppressed growth and metastasis of xenografted tumors¹¹⁵. Therefore, inhibiting REST improves patient outcomes for those disorders. Accordingly, the present invention also relates to methods for inhibiting REST in a cell. In general, the method comprises contacting a cell with the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159). For example, it can be exposing a cell for a sufficient amount of time for the RPP- (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) to enter the cell and have an effect on REST activity. The method can be practiced either *in vitro* or *in vivo*. Where practiced *in vitro*, the method can be used to study the activity of REST, to test other compounds for the ability to supplement or antagonize the effects of the RPP- (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through

137 and 140 through 159) on REST, or for any other reason of importance to a researcher. When practiced *in vivo*, the method can be used as a method of treating a subject for one or more diseases or disorders associated with REST. According to the method of this aspect of the invention, preferably, activity of REST is decreased. The step of contacting a cell can be any action that causes the agent to physically contact one or more target cells. Thus, it can be by way of adding the agent directly to an *in vitro* culture of cells to be contacted, and allowing the agent sufficient time to diffuse through the media and contact at least one cell. Likewise, it can be through addition of the agent to cells in an aqueous environment. Alternatively, it can be by way of administering the agent to a subject via any acceptable administration route, and allowing the body of the subject to distribute the agent to the target cell through natural processes. Thus, the *in vivo* methods can be methods of localized or systemic delivery of the agent to a cell in animals, including all mammals and humans in particular. According to this aspect, the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) can be used to treat a subject therapeutically or prophylactically, and to prepare a composition for use in treating.

[0083] In yet another embodiment, the invention provides a method of treating a subject suffering from or at risk of suffering from a disease or disorder involving REST. In general, the method comprises administering the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), in an amount sufficient to affect the amount or activity of REST in the subject. In certain aspects, the binding of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) to CTDSP1 results in inhibition of REST activity in a cell. In general, the method comprises administering a sufficient

amount of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) for a sufficient time to inhibit REST activity. Often, the amount administered and the amount of time is adequate to see a change in one or more clinical symptoms of a disease or disorder, or to stop progression of a disease or disorder from reaching a stage where one or more clinical symptoms are seen. According to this aspect, agent can be used to treat a subject therapeutically or prophylactically, and to prepare a composition for use in treating.

[0084] In one embodiment, the present invention provides methods for treating, alleviating, or ameliorating traumatic brain injury, chronic pain, peripheral nerve injury, epilepsy, diabetes, Alzheimer's disease, Huntington's disease, brain tumors (including glioblastoma multiforme), or pancreatic cancer in a subject. The terms "treating" or "alleviating" or "ameliorating" and similar terms used herein, include prophylaxis and full or partial treatment. The terms may also include reducing symptoms, ameliorating symptoms, reducing the severity of symptoms, reducing the incidence of the disease, or any other change in the condition of the patient, which improves the therapeutic outcome. The methods involve administering to a subject suffering from traumatic brain injury, chronic pain, peripheral nerve injury, epilepsy, diabetes, Alzheimer's disease, Huntington's disease, brain tumors (including glioblastoma multiforme), or pancreatic cancer in an animal, or a subject in need of treatment for traumatic brain injury, chronic pain, peripheral nerve injury, epilepsy, diabetes, Alzheimer's disease, Huntington's disease, brain tumors (including glioblastoma multiforme), or pancreatic cancer in an animal, the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159).

[0085] The administration of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ

ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) to the subject can be through any known and acceptable route. Such routes include, but are not necessarily limited to, oral, via a mucosal membrane (e.g., nasally, via inhalation, rectally, intrauterally or intravaginally, sublingually), intravenously (e.g., intravenous bolus injection, intravenous infusion), intraperitoneally, and subcutaneously. Administering can likewise be by direct injection to a site (e.g., organ, tissue) containing a target cell (i.e., a cell to be treated). Furthermore, administering can follow any number of regimens. It thus can comprise a single dose or dosing of the drug, or multiple doses or dosings over a period of time. Accordingly, treatment can involve repeating the administering step one or more times until a desired result is achieved. In embodiments, treating can continue for extended periods of time, such as weeks, months, or years. Those of skill in the art are fully capable of easily developing suitable dosing regimens for individuals based on known parameters in the art. The methods thus also contemplate controlling, but not necessarily eliminating, the disease or disorder. The preferred routes of administration in accordance with the present invention are oral and via a mucosal membrane.

[0086] The amount to be administered varies depending on the subject, stage of the disease, age of the subject, general health of the subject, and various other parameters known and routinely taken into consideration by those of skill in the medical arts. As a general matter, a sufficient amount of the agent will be administered in order to make a detectable change in the symptom of the subject. Suitable amounts are disclosed herein, and additional suitable amounts can be identified by those of skill in the art without undue or excessive experimentation.

[0087] The RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) is administered in a form that is acceptable, tolerable, and effective for the subject. Numerous pharmaceutical forms and

formulations for biologically active agents are known in the art, and any and all of these are contemplated by the present invention. Thus, for example, the agent can be formulated in oral solution, a caplet, a capsule, an injectable, an infusible, a suppository, a lozenge, a tablet, a cream or salve, an inhalant, and the like.

[0088] Those of ordinary skill in the art will readily optimize effective dosages and administration regimens as determined by good medical practice and the clinical condition of the individual subject.

[0089] The frequency of dosing will depend on the pharmacokinetic parameters of the compounds and the routes of administration. The optimal pharmaceutical formulation will be determined by one of skill in the art depending on the route of administration and the desired dosage. Such formulations may influence the physical state, stability, rate of *in vivo* release and rate of *in vivo* clearance of the administered agents. Depending on the route of administration, a suitable dose is calculated according to body weight, body surface areas or organ size. The availability of animal models is particularly useful in facilitating a determination of appropriate dosages of a given therapeutic. Further refinement of the calculations necessary to determine the appropriate treatment dose is routinely made by those of ordinary skill in the art without undue experimentation, especially in light of the dosage information and assays disclosed herein as well as the pharmacokinetic data observed in animals or human clinical trials.

[0090] Typically, appropriate dosages are ascertained through the use of established assays for determining blood levels in conjunction with relevant dose response data. The final dosage regimen will be determined by the attending physician, considering factors which modify the action of drugs, e.g., the drug's specific activity, severity of the damage and the responsiveness of the patient, the age, condition, body weight, sex and diet of the patient, the severity of any infection,

time of administration and other clinical factors. As studies are conducted, further information will emerge regarding appropriate dosage levels and duration of treatment for specific diseases and conditions. Those studies, however, are routine and within the level of skilled persons in the art. Typical dosage may be about 0.6 mg (0.01 mg/kg) to about 60 g (1 g/kg) weekly (in human), more preferably monthly.

[0091] It will be appreciated that the peptides, compositions and treatment methods of the invention are useful in fields of human medicine and veterinary medicine. Thus, the subject to be treated is a mammal, such as a human or other mammalian animal. For veterinary purposes, subjects include for example, farm animals including cows, sheep, pigs, horses and goats, companion animals such as dogs and cats, exotic and/or zoo animals, and laboratory animals including mice, rats, rabbits, guinea pigs and hamsters.

[0092] The RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) may be administered to a subject animal, preferably mammals, such as humans, in need thereof as a pharmaceutical or veterinary composition, such as tablets, capsules, solutions, or emulsions. The RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) can be present in compositions containing other ingredients. Non-limiting examples of compositions appropriate for the present invention are pharmaceutical compositions, such as in the form of tablets, pills, capsules, caplets, multiparticulates (including granules, beads, pellets and micro-encapsulated particles); powders, elixirs, syrups, suspensions, and solutions. Pharmaceutical compositions will typically comprise a pharmaceutically acceptable diluent or carrier. Pharmaceutical compositions are preferably adapted for administration parenterally (e.g., orally). Orally administrable compositions may be in solid or liquid form and

may take the form of tablets, powders, suspensions, and syrups, among other things. Optionally, the compositions comprise one or more flavoring and/or coloring agents. In general, therapeutic and nutritional compositions may comprise any substance that does not significantly interfere with the action of the agent on the subject.

[0093] Pharmaceutically acceptable excipients or carriers suitable for use in such compositions are well known in the art of pharmacy. The compositions of the invention may contain 0.01-99% by weight of the agent. The compositions of the invention are generally prepared in unit dosage form. The excipients used in the preparation of these compositions are well-known in the art.

[0094] Further examples of product forms for the composition are food supplements, such as in the form of a soft gel or a hard capsule comprising an encapsulating material selected from the group consisting of gelatin, starch, modified starch, starch derivatives such as glucose, sucrose, lactose, and fructose. The encapsulating material may optionally contain cross-linking or polymerizing agents, stabilizers, antioxidants, light absorbing agents for protecting light-sensitive fills, preservatives, and the like.

[0095] In general, the term carrier may be used throughout this application to represent a composition with which the agent may be mixed, be it a pharmaceutical carrier, foodstuff, nutritional supplement or dietary aid. The materials described above may be considered carriers of the agent for the purposes of the invention. In certain embodiments of the invention, the carrier has little to no biological activity, particularly on REST.

[0096] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following examples are

given to illustrate the present invention. It should be understood that the invention is not to be limited to the specific conditions or details described in the examples.

Example 1 - His-CTDSP1 Plasmid construction

[0097] The codon optimized (using IDT codon optimization tool) CTDSP1 gene was cloned into the pBAD-HisA plasmid (Thermo Fisher Scientific). First, pBAD-HisA plasmid was amplified with primers P33 and P34 (Table 1) introducing *HindIII* and *XhoI* restriction sites. The PCR reaction (20 μ L) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 6 min. Following amplification, the PCR fragment was gel-purified by the QIAGEN gel-band purification kit and digested with *HindIII* and *XhoI* restriction enzymes. All digested fragments were purified with a QIAGEN kit and ligated in appropriate combination using T4 DNA ligase (NEB) according to recommendations of the manufacturer.

Table 1 - Primers used for plasmid construction

Primers		
Name	Sequence	SEQ ID NO:
P12	AAATAAGCTTGTATATCTCCTTCTTAAAGTTAAACA	269
P14	AATAACTCGAGAGATCCGGCTGCTAACAAAGC	270
P17	ATAAGCTTTAATACGACTCACTATAGGGTTAACTTTAGTAAGGAGGACAGCTAA	271
P19	GTGGTGATGGTGATGGTGACCTA	272
P20	ATTATTCTCGAGTTAATAGCCGGTGCCGTGGTGATGGTGATGGTGACCTA	273
P21	AAATACTCGAGTCGTTTTATCTGTTGTTTGTCTGGT	274
P22	AAATAAAGCTTCTCTGAATGGCGGGAGTATGAAAA	275
P23	CCGCGAATGGTGAGATTGAGAA	276
P24	ACGCAAAAAGGCCATCCGTCAG	277
P28	ATTATTCTCGAGTTAATAGCCGGTGCCTAAGCCGCTACCACCACGCCGACGCTGACGG	278
P31	AAATAAGCTTATGGATAGTAGTGCGGTGATCA	279
P32	ATTATTCTCGAGCTAGTGGTGATGGTGATGGT	280

P33	AACTAAGCTTTTCCTCCTGTTAGCCCCAAAAAAC	281
P34	AATACTCGAGGCTGTTTTGGCGGATGAGAGAA	282
P35	AAATCCATGGATAGTAGTGCGGTGATCA	283
P36	AAATCATATGGATAGTAGTGCGGTGATCA	284
P37	AATACTCGAGCTAGTGGTGATGGTGATGGTGAG	285
P38	AATACTCGAGAGAGCCAGAACCAGATCCCGGA	286
P39	TAAGCCGCTACCACCACGCCGAC	287
P40	GGTGCAATTCCTAAACTCCAGTACAATACTTACTGC	288
P41	GTATTGTAAGTGGAGTTTTAGGAATTGCACCATTTTCCTC	289
P108	AATACTCGAGTTATTTTGGAGGATGGTCGCCACCA	290
P109	AATAAAGCTTATGGGATCCGGTTCTGGCTCAGGTTCTTCC	291
Degenerate Primers		
Name	Sequence	
11F	NAANCANCANTGNCANAGNAANATTGTGGTTCTGGCTCTGGTCGTAAAA	292
12F	GNACNACNACNGCNAANGGNAANTTGTGGTTCTGGCTCTGGTCGTAAAA	293
13F	GNANCACNANTGCNANAGGNANATTGTGGTTCTGGCTCTGGTCGTAAAA	294
14F	NAACNANCACNGNCAANGNAAANTTGTGGTTCTGGCTCTGGTCGTAAAA	295
15F	GAANCANCANTGNCANAGNAANATTGTGGTTCTGGCTCTGGTCGTAAAA	296
16F	GAACNACNACNGCNAANGGNAANTTGTGGTTCTGGCTCTGGTCGTAAAA	297
17F	GAANCACNANTGCNANAGGNANATTGTGGTTCTGGCTCTGGTCGTAAAA	298
18F	GAACNANCACNGNCAANGNAAANTTGTGGTTCTGGCTCTGGTCGTAAAA	299
19F	GAANCANCACTGCCANAGNAAAATTGTGGTTCTGGCTCTGGTCGTAAAA	300
20F	NAACCACCANTGNCAAAGGAANATTGTGGTTCTGGCTCTGGTCGTAAAA	301
21F	GAANCACNACTGCCANAGNAAAATTGTGGTTCTGGCTCTGGTCGTAAAA	302
22F	GNACCACCANTGCNAAAAGGAANATTGTGGTTCTGGCTCTGGTCGTAAAA	303
11R	TGNTGNTTNCANATNTTNGGNACACATTTAGCTGTCCTCCTTACTAAAGTT	304
12R	TNGTNGTNCCNGANCTNCGNTACACATTTAGCTGTCCTCCTTACTAAAGTT	305
13R	TGNTNGTTNCNGATNTNCGGNACACATTTAGCTGTCCTCCTTACTAAAGTT	306
14R	TNGTNGTNCCANANCTTNGNTACACATTTAGCTGTCCTCCTTACTAAAGTT	307
15R	TGNTGNTTCCANATNTTNGGNACACATTTAGCTGTCCTCCTTACTAAAGTT	308
16R	TNGTNGTTCCNGANCTNCGNTACACATTTAGCTGTCCTCCTTACTAAAGTT	309
17R	TGNTNGTTCCNGATNTNCGGNACACATTTAGCTGTCCTCCTTACTAAAGTT	310
18R	TNGTNGTTCCANANCTTNGNTACACATTTAGCTGTCCTCCTTACTAAAGTT	311
19R	TGGTNGTTNCAGATCTTNGGNACACATTTAGCTGTCCTCCTTACTAAAGTT	312
20R	TGNTGGTTCCANATNTTCGGTACACATTTAGCTGTCCTCCTTACTAAAGTT	313
21R	TGGTNGTTNCAGATCTNCGGNACACATTTAGCTGTCCTCCTTACTAAAGTT	314
22R	TGNTGGTTCCNGATNTTCGGTACACATTTAGCTGTCCTCCTTACTAAAGTT	315
N = any nucleotide		

[0098] Ligated fragments were transformed in 10G chemically competent cells (Lucigen) according to manufacturer's protocol. Transformed cells were plated on LB plates containing 50 µg/mL ampicillin and incubated overnight at 37°C. Colonies were tested for the presence of the insert by colony PCR. Colonies were picked and resuspended in 20 µl of sterile 0.9% sodium

chloride solution. One μ L of this solution was transferred to the PCR tube and amplified with Taq polymerase (New England Biolabs, cat# M0482S) and 30 pM of the flanking primers. Each PCR reaction (20 μ L) was initially heated to 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealed at 55°C for 15 sec., and extended at 72°C for 1 min. Amplification products were visualized by agarose electrophoresis. Clones with the correct inserts were inoculated into the culture tubes containing 5 mL of LB with the appropriate antibiotic and incubated overnight at 37°C. Constructs were then purified using the Monarch Plasmid miniprep kit (NEB).

Example 2 - Expression and Purification methods for His-CTDSP1

[0099] His-CTDSP1 (Table 2; SEQ ID NO: 162): *E. Coli* (10G strain Lucigen) with pBAD-CTDSP1 construct were incubated overnight at 37°C with vigorous shaking. Then 4 mL of the night culture was added to 500 mL of LB media with 50 μ g/mL ampicillin in a 1 L flask and incubated at 37°C with shaking. When the culture's OD600 reached 0.4, we added arabinose to the final concentration of 0.02% and incubated 16 hours at 30°C with shaking. Next morning, cells were spun down (Eppendorf centrifuge 5810R) at maximum speed and frozen at -80°C. When needed, cell pellets were removed from the freezer, incubated at room temperature, lysed with 4 mL of BPER protein lysis reagent (ThermoFischer) and purified protein with HisPur Cobalt Purification kit (ThermoFischer, Cat # 90091) as described by the manufacturer.

Table 2 - CTDSP1 Nucleotide and Protein Sequences

Nucleotide Sequence (SEQ ID NO: 162)

ATGGATAGTAGTGCGGTGATCACACAAATCTCCAAGGAGGAAGCCCGTGGGCCGCT
GCGGGGGAAGGGTGATCAAAAATCGGCAGCTAGTCAAAAACCTCGCTCTCGTGGGA
TACTTCATTCGCTGTTTTGCTGCGTCTGCCGCGATGACGGAGAAGCATTGCCTGCGCA
TTCAGGGGCGCCTTTACTTGTTGAGGAAAATGGTGCAATTCCTAAACAAACTCCAGT
ACAATACTTACTGCCGGAGGCAAAGGCACAAGACAGTGATAAGATATGTGTAGTAA
TAGACTTAGATGAAACACTGGTACATTTCGTCATTCAAACCTGTTAATAATGCGGATT

TCATCATACCTGTAGAAATCGACGGGGTTGTCCATCAGGTTTACGTCCTGAAGCGGC
 CTCATGTAGATGAATTTTACAGCGGATGGGCGAGTTATTTGAATGTGTGCTGTTTAC
 AGCTAGTCTTGCCAAGTACGCGGATCCTGTCGCGGATTTGCTTGATAAGTGGGGTGC
 GTTTCGGGCGAGATTATTTTCGCGAATCTTGCCTTTTTCACAGAGGTAACACTACGTGAA
 GGACCTTAGTCGTCTGGGTAGAGATCTTAGAAGAGTGCTGATCCTTGACAACAGCCC
 AGCCAGCTATGTCTTTCATCCGGATAACGCAGTACCCGTGGCGTCTTGGTTCGACAA
 TATGTCGGACACGGAGCTGCATGACCTGTTGCCGTTCTTTGAGCAGTTGAGTCGCGTT
 GATGACGTTTACTCGGTTTTGCGTCAACCCCGTCCGGGATCTGGTTCTGGCTCTCACC
 ATCACCATCACCCTAG

Protein Sequence (SEQ ID NO: 163)

MDSSAVITQISKEEARGLRGKGDQKSAASQKPRSRGILHSLFCCVCRDDGEALPAHSGA
 PLLVEENGAIPKQTPVQYLLPEAKAQDSDKICVVIDLDELTHVSSFKPVNNADFIIPVEIDG
 VVHQVYVLKRPHVDEFLQRMGELFECVLFTASLAKYADPVADLLDKWGAFRARLFRES
 CVFHRGNYVKDLSRLGRDLRRVLILDNSPASVVFHPDNAVPVASWFDNMSDTELHDLPL
 FFEQLSRVDDVYSVLRQPRPGSGSGSHHHHHH

Linker and His tag are underlined

Example 3 - Developing peptides with high affinity to CTDSP1

[00100] Peptide candidates were generated using RNA display and protein evolution methods.

[00101] **Construction of RNA display libraries.** We've built 3 different libraries of RPP variants. In all libraries serines 861 and 864 were replaced by glutamates. Library 1 was built using primers P11 through P14 (Table 1). In library 1 each position of the REST peptide was mutated. It was estimated to have 1×10^9 variants. In library 2 (primers P14 through P18, Table 1), glutamates were not touched by the degenerate oligos. This library was expected to have 65×10^6 variants. In library 3, only one of the glutamates and every second codon was mutated. This library was expected to generate only 65000 variants.

[00102] In all 3 libraries, the RPP sequence (SEQ. ID NO: 1) was diversified by synthetic degenerate oligoes. Only one degenerate base was introduced per codon: either first or the second position which gave a choice or 4 amino acids. REST cassettes were amplified from pBAD constructs as two fragments: left and right, which were reunited by ligation (Figure 27). The left

fragment was amplified with flanking forward primer P23 (Table 1) and one of the reverse primers (11R through 22R, Table 1).

[00103] The right fragment was amplified with one of the forward primers (11F through 22F, Table 1) and the reverse primer P24 (Table 1). The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 40 sec. Following amplification, PCR fragments were gel-purified by the QIAGEN gel-band purification kit, mixed and ligated with T4 DNA ligase. The ligation reaction contained 20 µl of 10x ligation buffer, 100 ng of fragment mix, 0.5 µl of 100 mM ATP, 1 µl of T4 DNA ligase (NEB cat# M0202S) and 1 µl of T4 polynucleotide kinase. The reaction mix was incubated at room temperature and used as a template for PCR with flanking primers P17 and P20 (Table 1) using program described above. PCR fragment was gel-purified by the QIAGEN gel-band purification kit and used as a template in the mRNA display experiments.

[00104] For quality control purposes, we cloned and sequenced a fraction of this library. An aliquot of this library was digested by *HindIII* and *XhoI* restriction enzymes, purified by QIAGEN kit and cloned in pBAD vector as described above for CTDSP1 gene. Individual clones were sequenced by GeneWiz to confirm that REST cassettes were successfully mutated and that majority of clones were ligated without frame-shift mutations.

[00105] ***In vitro* transcription.** RNA was translated from amplified libraries using RiboMAX Large Scale RNA Production System T7 (Promega, Cat# P1300) according to the manufacturers protocol and purified by RNeasy Mini Kit (Qiagen, Cat# 74104)

[00106] **Ligation of mRNA to DNA linker with puromycin.** XL-PSO oligonucleotide was synthesized by IDT. The sequence of the oligonucleotide was: 5'-PsoC6-(uagccggug)₂-OMe-AAAAAAAAAAAAAAAAA-Spacer9-Spaser9-ACC-Puro-3'. For ligating this

oligonucleotide to mRNA we mixed the following reagents in a PCR tube: 29.5 µl of RNase-free water, 1 µl of 1 M HEPES-KOH, pH 7.6, 5 µl of 1 M KCl, 2 µl of 25 mM spermidine, 0.5 µl of 125 mM EDTA, 8 µl of mRNA from previous step and 4 µl of 100 mM of XL-PSO oligonucleotide. PCR tube was placed in the PCR machine, heated to 70°C for 5 min and cooled to 25°C at 0.1°C/s speed. Then we transferred the mixture to the 96-well plate on ice, put the 365-nm handheld UV lamp on top and irradiated the plate for 20 min. After that cross-linked RNA was purified by RNeasy Mini Kit (Qiagen, Cat# 74104).

[00107] *In vitro* translation. Translation was performed using PUREexpress in vitro Protein Synthesis Kit (NEB, Cat# E6800S). We mixed the following reagents in a 1.5 ml tube: 20 µl of Solution A, 15 µl of solution B, 0.5 µl of RNasin Plus, 4.5 µl of water and 10 µl of cross-linked RNA (1 µg/µl). The mixture was incubated at 37°C for 2 h.

[00108] Purification of Peptides with His-tag. We purified RNA-peptide complexes using Ni-NTA magnetic beads (Qiagen, Cat# 36111). 100 µl of beads was washed with 300 µl of wash buffer (50mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 0.005% Tween 20), separated on the magnetic stand and suspended in 300 µl of wash buffer. 25 µl of RNA-peptide complexes from previous step was added to the washed beads and incubated on the end-over-end shaker for 30 min at room temperature. Beads were washed 3 times with the wash buffer followed by separation on the magnetic stand and eluted with 50 µl of the elution buffer (50mM NaH₂PO₄, 300 mM NaCl, 500 mM imidazole, 0.005% Tween 20).

[00109] Purification with Oligo-d(T)₂₅ magnetic beads. Oligo-d(T)₂₅ magnetic beads were purchased from New England Biolabs (Cat# S1419S). 100 µl of the bead suspension was washed with 500 µl of wash buffer I (20mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA) by spending them in the buffer and separating on the magnetic stand followed by suspension in 50

μl of wash buffer I. 50 μl of the RNA-peptide complexes from the previous step were mixed with 50 μl of binding buffer (100mM Tris-HCl, pH 7.5, 1 M NaCl, 2 mM EDTA), heated at 65°C for 2 min, placed on ice for 1 min and mixed with the washed beads. This mixture was incubated at room temperature for 5 min, then washed twice with 500 μl of wash buffer I (20mM Tris-HCl, pH 7.8, 500 mM NaCl, 1 mM EDTA) and once with 500 μl of wash buffer II (20mM Tris-HCl, pH 7.8, 200 mM NaCl, 1 mM EDTA).

[00110] Peptide Cyclization. RNA-peptide complexes bound by the Oligo-d(T)₂₅ magnetic beads were incubated for 30 min in the cyclization buffer (20mM Tris-HCl, pH 7.8, 0.00 M NaCl, 3 mM α,α'-dibromo-m-xylene (Sigma-Aldrich), 33% acetonitrile) with periodic shaking. Following incubation, beads were washed with the washing buffer III (20 mM Tris-HCl, pH 7.8, 0.3 M NaCl, 5 mM 2-mercaptoethanol), then with washing buffer IV (20 mM Tris-HCl, pH 7.8, 0.3 M NaCl, 0.5 mM TCEP) followed by washes with wash buffer I (20mM Tris-HCl, pH 7.8, 500 mM NaCl, 1 mM EDTA) and wash buffer II (20mM Tris-HCl, pH 7.8, 200 mM NaCl, 1 mM EDTA). Purified products were eluted from the beads by adding 30 ul of elution buffer (20mM Tris-HCl, pH 7.8) and incubating at 65°C for 2 min followed by immediate separation on the magnetic bead stand.

[00111] Affinity Selection. Affinity selection was performed in NUNC Maxisorp plates (Thermo Fischer Scientific). His-CTDSP1 (Table 2; SEQ ID NOS: 163) was dissolved in 100 μl of PBS, transferred to Maxisorp plate well and incubated on orbital shaker for 2 h at room temperature. Plate wells were washed twice with PBS and blocked with casein (PBSC buffer or PBS with 1% casein) at room temperature for 1 h with shaking and then washed 3 times with PBS. Negative selection wells were coated with casein only by incubating with 300 μl of the PBSC solution and washed 3 times with PBS. Purified RNA-peptide complexes were first added to these

negative selection wells containing 100 μ l of PBS and incubated with shaking at room temperature for 20 min. Next, this solution was transferred to the positive selection wells (covered by His-CTDSP1) containing 125 μ l of PBSC and incubated with shaking at room temperature for 1 h. Off-target selection was performed by adding 25 μ l of purified His-CTDSP1 to each well and incubating for 3 min. Following incubation wells were washed 3 times with PBS and used for cDNA synthesis.

[00112] cDNA Synthesis and PCR. We used SuperScript III First-Strand Synthesis System (Invitrogen, Cat# 18080-051) for the cDNA synthesis. First, 16 μ l of water was mixed with 2 μ l of 50 mM primer P19 (Table 1) and 1 μ l of the dNTP solution and added this mix to the well in the Maxisorp plate. Next, this plate was incubated at 65°C for 5 min followed by cooling down at 4°C for 1 min. Then we transferred 20 μ l mixture from the plate to the PCR tube and added 20 μ l of the reaction mixture containing 4 μ l of 10x buffer, 8 μ l of 25 mM MgCl₂, 4 μ l of 0.1M DTT, 2 μ l of RNaseOUT and 2 μ l of Superscript III reverse transcriptase. This mixture was incubated at 50°C for 50 min. Then we added 2 μ l of RNase H and incubated tubes at 37°C for 20 min.

[00113] DNA corresponding to the strong binders surviving the selection was amplified with primers P17 and P20 (Table 1). Amplifications was carried out using Vent DNA polymerase. The PCR reaction (20 μ L) was initially heated at 95°C for 2.5 min followed by 20 or 25 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 30 sec. Following amplification, PCR fragment was run on the agarose gel, purified by the QIAGEN gel-band purification kit according to manufacturer's protocol.

[00114] Analysis of the NGS Data. PCR reactions from each cycle were submitted for Next Generation Sequencing. We used Amplicon-EZ service GeneWiz. Unique sequences were quantified and the most abundant sequences were selected for further testing (Tables 3 and 3a)

Table 3 - Protein and Nucleotide Sequences of RPP Variants (SEQ ID NOS are identified in Table 19)

RPP Variant	Protein Sequence
v1	MCTEDLEPPEPLPKENC
v2	MCTEAPAPPEPALPKKKKKNC
v3	MCTEDLQPPTAVPQENC
v4	MCTEAPAPPEPALPKKKKKNC
v5	MCTADLEPPEPRMEKKKVDC
v6	MCTGDLQPPKTTVSKKDC
v7	MCTEDLQSPKTTMTKENC
v8	MCTEDLEPPEPLPKEDC
v9	MCTEDQEQEEQLPEENC
v10	MCTADLKPPKTTMTKQNC
v11	MCPGDLQPEPPMPKEYC
v12	MCTEDLEPPKATMTKKDC
v13	MCTEDQERPPVTKEDC
v14	MCIADPEPPEAQLPEGNC
v15	MCTGVQEPPEATLPKKNC
v16	MCSEAQEPPEPRLPQVNC
v17	MCTKHLEPPGPPLPQENC
v18	MCTAAPEPPEPPVSKEYC
v19	MCTEDLQPKTTMTKEYC
v20	MCSVDLQPPARLRPMVNC
v21	MCTGDLQPPEPRLPQVNC
v22	MCTGDLQPPEAQVIEVNC
v23	MCTEDLQPPPEQLPEVNC
v25	MCTEDMEPRKTTMTKKYC
v26	MCTEAPAPPEPALPKKKKKKNC
v27	MCTEAPAPPEPALPKKKKKKNC
v28	MCTEDLQSPKTTMTKENC
v29	MCTGDLKLPEPPMSKKKKKNC
v30	MCTEDLQPPKTTMAKEYC
v31	MCSEDPEPPKTTMTKKNC
v32	MCTEDLKPPPEASLPEENC
v33	MCAGDLEQPEPPVAKKKKKKNC
v34	MCNGDLERPEPPVAKEYC
v35	MCTEDLKPPPEPLPKENC
v36	MCNEALEPPPLRKEHC
v37	MCPEDLERPPLTKEHC
v38	MCTEDLEPPERPLPREIC
v39	MCAGDLKPPETTMSKKNC
v40	MCTEDLQPPERSQPMESC
v41	MCPEDLQPPPEPALPEKKKKKIVVLALV VKSASVGVV
v42	MCTEVLVPRTTSGKGRLWFWLWSQKG HPSASACGS

v43	MCAEDLQPPPLLEAHCGSDSGRKKRRQ C
v44	MCTAAPEPPEPQLPQANC
v45	MCPADLQQPETSLEENC
v46	MCSVDLQPPARLRPMVNC
v47	MCTEALEPPEPPLTKENC
v48	MCTEAMEPPEPPLARESC
v49	MCTADLQPPEASLPQQNC
v50	MCTAAPEPPEPRLPEGNC
v51	MCTKDLAPQAPLLKENC

Table 3b - Protein Sequences of RPP^{RI} Variants* (SEQ ID NOS are identified in Table 19)

RPP ^{RI} Variant	Retroverted (RI) Protein Sequence
v1	cneklppeppeldetcm
v2	cnkkkkkplapeppapaetcm
v3	cneqpvatppqldetcm
v4	cnkkkkkplapeppapaetcm
v5	cdkksvttkppqldgtcm
v6	cdkksvttkppqldgtcm
v7	cnektmttkpsqldetcm
v8	cdeklppeppeldetcm
v9	cneplqeelleldetcm
v10	cnqktmttkppkldgpcm
v11	cyekpmppeppqldgpcm
v12	cdektmttakppeldetcm
v13	cdektvppreqdetcm
v14	cngeplqaeppepdaicm
v15	cnkkpltaeppeqvgcm
v16	cnvqplppgppelhkcm
v17	cneqplppgpppepaatcm
v18	cyeksvppeppepaatcm
v19	cyektmttkplqldetcm
v20	cnvmprlrappqldvscm
v21	cnvqpqrseppqldgtcm
v22	cnveivqaeppeqldgtcm
v23	cnveplqpeppqldetcm
v25	cykktmttkppemdcm
v26	cnkkkkkkkplapeppapaetcm
v27	cnkkkkkplapeppapaetcm
v28	cnektmttkpsqldetcm
v29	cnkkkkksmppeplkldgtcm
v30	cykeamttkppqldetcm
v31	cnkktmttkppepdscm
v32	cneplsaeppkldetcm
v33	cnkkkkkavppepgeldgacm
v34	cyekavppepreldgncm
v35	cneklppeppkldetcm

v36	chekrlpppelaencm
v37	chektlppreldepem
v38	cierplpreppeldetcm
v39	cnkksmtteppkldgacm
v40	csmqqsrepqqledetcm
v41	cvvgvsvaskvvlalvvikkkkkkeplapeppqldepem
v42	csgcasasphgkqswlwfvlrgkgsttrpvlvetcm
v43	cqrrkrgsdsgchaellpppqldeacm
v44	cnaqplqpeppepaatcm
v45	cneepstepqqldapcm
v46	cnvmprlrappqldvscm
v47	cnektlpppeppelaetcm
v48	cseralppeppemaetcm
v49	cnqqplsaeppqlatcm
v50	cngeplrpeppepaatcm
v51	cneklppaqpaldktcm

*the lowercase letter depicts D-amino acids.

Example 4 - Construction of the Peptide-GST fusions and their expression

[00115] As a first step we cloned GST protein in pET29 vector. GST was codon optimized, flanked by *HindIII* and *XhoI* sites and synthesized by IDT. It was amplified by Phusion DNA polymerase (NEB, cat#M0530S) with primers P109 and P108 (Table 1). The PCR reaction (20 μ L) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 1 min. Following amplification, PCR fragment was gel-purified by the QIAGEN gel-band purification kit according to manufacturer's protocol.

[00116] pET29 plasmid was amplified with primers P12 and P14 (Table 1) introducing *HindIII* and *XhoI* restriction sites. The PCR reaction (20 μ L) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 6 min. Following amplification, PCR fragment was gel-purified by the QIAGEN gel-band purification kit and digested with *HindIII* and *XhoI* restriction enzymes. All digested fragments were purified with QIAGEN kit and ligated in appropriate combination using T4 DNA ligase (NEB) according to recommendations of the manufacturer. Ligated fragments were

transformed in 10G chemically competent and sequenced as described above. Expression construct for GST-RPP is shown in Table 4 (SEQ ID NO: 164)

Table 4 - Nucleotide and Protein Sequences of RPP-GST fusion

<u>Nucleotide Sequence (SEQ ID NO: 164)</u> ATGTGTACCGAAGATCTGGAACCACCAGAACCACTGCCAAAGGAAAATTGTggat ccGGTTCTGGCTCAGGTTCTTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGT GCAACCCACTCGACTTCTTTTGAATATCTTGAAGAAAAATATGAAGAGCATTGTGA TGAGCGCGATGAAGGTGATAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGT TTCCCAATCTTCCTTATTATATTGATGGTGTGTTAAATTAACACAGTCTATGGCCAT CATACTGTTATATAGCTGACAAGCACAAACATGTTGGGTGGTTGTCCAAAAGAGCGTGC AGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATACGGTGTTCGAGAAT TGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATTTTCTTAGCAAGCTACCTGA AATGCTGAAAATGTTTGAAGATCGTTTATGTCATAAAACATATTTAAATGGTGATCA TGTAACCCATCCTGACTTCATGTTGTATGACGCTCTTGATGTTGTTTATACATGGAC CCAATGTGCCTGGATGCGTTCCCAAAATTAGTTTGTGTTTAAAAAACGTATTGAAGCT ATCCCACAAATTGATAAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAG GGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCTCCAAAATAA
<u>Protein Sequence (SEQ ID NO: 165)</u> MCTEDLEPPEPPLPKENCGSGSGSGSSPILGYWKIKGLVQPTRLLEYLEEKYEEHLYERD EGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMMLGGCPKERAESML EGAVLDIRYGVSR IAYS KDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDM LYDALDVVLYMDPMCLDAFPKLVCFKKRIEAI PQIDKYLKSSKYIAWPLQGWQATFGGG DHPPK
RPP Sequence is underlined

[00117] Selected sequences were re-created by PCR using primers shown in Table 5, gel-purified by the QIAGEN gel-band purification kit, digested by *HindIII* and *BamHI* restriction enzymes and cloned in pET vector described above in-frame with the GST tag. Ligated fragments were transformed in 10G chemically competent cells (Lucigen) according to manufacturer's protocol. Transformed cells were plated on LB plates containing 25 µg/mL kanamycin and incubated overnight at 37°C. Next morning colonies were tested for the presence of the insert by colony PCR. Colonies were picked and resuspended in 20 µl of sterile 0.9% sodium chloride

solution. One μL of this solution was transferred to the PCR tube and amplified with Taq polymerase (New England Biolabs, cat# M0482S) and 30 pmoles of the flanking primers. Each PCR reaction (20 μL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 1 min. Amplification products were visualized by agarose electrophoresis. Clones with the correct inserts were inoculated into the culture tubes containing 25 $\mu\text{g/mL}$ kanamycin and incubated overnight at 37°C. Next morning constructs were purified by Monarch Plasmid miniprep kit (NEB). Constructs were transformed into BL21(DE3) competent cells (Lucigen) as described above.

[00118] Purification for Peptide-GST fusions. *E.coli* cells (BL21 strain) with the selected constructs were incubated overnight at 37°C with vigorous shaking. Next morning, we added 1 ml of the night culture to 100 ml of LB media with 25 $\mu\text{g/mL}$ kanamycin in 1 L flask and incubated it at 37°C with shaking. When culture's OD600 reached 0.4 we added IPTG to the final concentration of 1 mM and incubated 16 h at 30°C with shaking. Next morning, cells were spun down in the Eppendorf centrifuge 5810R at maximum speed and frozen at -80°C. When needed, cell pellets were removed from the freezer, incubated at room temperature and lysed with 3 ml of BPER protein lysis reagent (ThermoFischer). Peptide-GST fusions were purified using glutathione agarose (ThermoScientific cat # 16100) as described by the manufacturer.

Table 5 –Primers used for cloning RPP variants in-frame with GST

RPP Variant	Primer	Primer Sequence	SEQ ID NO:
v1 (RPP)	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGGAACCACCAGAACCA CCACTGCCAA	166
	reverse primer	AATAGGATCCACAATTTTCCTTTGGCAGTGGTGGTTCTGGTGGT TCCA	167
v2	forward primer	AATAAAGCTTATGTGTACCGAAGCTCCGGCACCACCAGAACCA GCACTGCCAAAG	168
	reverse primer	AATAGGATCCACAATTTTTTTTTTTTCTTTGGCAGTGCTGGTT CTGGTGGTGC	169
v3	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGCAACCACCAACAGCA GTGCCAC	170

	reverse primer	AATAGGATCCACAATTTTCCTGTGGCACTGCTGTTGGTGGTTGC AGATCTTC	171
v4	forward primer	AATAAAGCTTATGTGTACCGAAGCTCCGGCACCACCAGAACCA GCACTGCCAAAG	172
	reverse primer	AATAGGATCCACAATTTTTTTTTTTTCTTGGCAGTGCTGGTTCTG GTGGTGC	173
v5	forward primer	ATGTGTACCGCAGATCTGGAACCACCAGAACACGAATGGAA	174
	reverse primer	AATAGGATCCACAATCTACCTTTTTTTTTTCCATTCTGTTCTG GTGGTTCCA	175
v6	forward primer	AATAAAGCTTATGTGTACCGGAGATCTGCAACCACCAAAAAACA ACAGTGTCAG	176
	reverse primer	AATAGGATCCACAATCTTCTTTGACACTGTTGTTTTTGGTGGT TGCAGA	177
v7	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGCAATCACCACCAAAAAACA ACAATGACAA	178
	reverse primer	AATAGGATCCACAATTTTCCTTTGTCATTGTTGTTTTTGGTGATT GCAGA	179
v8	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGGAACCACCAGAACCA CCACTGCCAA	180
	reverse primer	AATAGGATCCACAATCTTCTTTGGCAGTGGTGGTTCTGGTGG TTCCAGA	181
v9	forward primer	AATAAAGCTTATGTGTACCGAAGATCAGGAACAACAAGAAGA ACAACCTG	182
	reverse primer	AATAGGATCCACAATTTTCCTCTGGCAGTTGTTCTTCTGTTGTT CCTGATC	183
v10	forward primer	AATAAAGCTTATGTGTACCGCAGATCTGAAACCACCAAAAAACA ACAATGACAA	184
	reverse primer	AATAGGATCCACAATTTTGCTTTGTCATTGTTGTTTTTGGTGGTT TCAGA	185
v11	forward primer	AATAAAGCTTATGTGTCCCGGAGATCTGAAACAACCAGAACCA CCAATGCCAA	186
	reverse primer	AATAGGATCCACAATATTCCTTTGGCATTGGTGGTTCTGGTTGT TTCAGA	187
v12	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGGAACCACCAAAAAGCA ACAATGACAA	188
	reverse primer	AATAGGATCCACAATCTTCTTTGTCATTGTTGCTTTTGGTGGTT CCAGA	189
v13	forward primer	AATAAAGCTTATGTGTACCGAAGATCAGGAACGACCACCAGTG ACAAAG	190
	reverse primer	AATAGGATCCACAATCTTCTTTGTCAGTGGTGGTCTTCTGA TCTTC	191
v14	forward primer	AATAAAGCTTATGTGTATCGCAGATCCGGAACCACCAGAAGCA CAACTG	192
	reverse primer	AATAGGATCCACAATTTCCCTCTGGCAGTTGTGCTTCTGGTGGT TCCGGATCTG	193
v15	forward primer	AATAAAGCTTATGTGTACCGGAGTTCAGGAACCACCAGAAGCA ACACTG	194
	reverse primer	AATAGGATCCACAATTTTCTTTGGCAGTGTGCTTCTGGTGGT TCTGAAC	195
v16	forward primer	AATAAAGCTTATGTGTAGCGAAGCTCAGGAACCACCAGAATCA CGACTG	196
	reverse primer	AATAGGATCCACAATTTACCTGTGGCAGTCGTGATTCTGGTGGT TCTGAG	197
v17	forward primer	AATAAAGCTTATGTGTACCAAACATCTGGAACCACCAGGACCA CCATTGC	198
	reverse primer	AATAGGATCCACAATTTCTGTGGCAATGGTGGTCTGGTGGT TCCAGATG	199
v18	forward primer	AATAAAGCTTATGTGTACCGCAGTCCGGAACCACCAGAACCA CCAGTGT	200
	reverse primer	AATAGGATCCACAATATTCCTTTGACACTGGTGGTCTGGTGGT TCCGGAG	201

v19	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGCAACTACCAAAAAACA ACAATGACA	202
	reverse primer	AATAGGATCCACAATATTCCTTTGTCATTGTTGTTTTGGTAGT TGCAGA	203
v20	forward primer	AATAAAGCTTATGTGTTCCGTAGATCTGCAACCACCAGCACGA CTACGGCCAA	204
	reverse primer	AATAGGATCCACAATTTACCATTTGGCCGTAGTCGTGCTGGTGG TTGCAGAT	205
v21	forward primer	AATAAAGCTTATGTGTACCGAGATCTGCAACCACCAGAATCA CGACAGCCA	206
	reverse primer	AATAGGATCCACAATTTACCTGTGGCTGTCGTGATTCTGGTGGT TGCAGAT	207
v22	forward primer	AATAAAGCTTATGTGTACCGAGATCTGCAACCACCAGAAGCA CAAGTGA	208
	reverse primer	AATAGGATCCACAATTTACCTCTATCACTTGTGCTTCTGGTGGT TGCAGAT	209
v23	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGCAACCACCAGAAACCA CAACTGCCA	210
	reverse primer	AATAGGATCCACAATTTACCTCTGGCAGTTGTGGTTCTGGTGGT TGCAGAT	211
v25	forward primer	AATAAAGCTTATGTGTACCGAAGATATGGAACCACGAAAAACA ACAATGA	212
	reverse primer	AATAGGATCCACAATTTTCTTTGTCATTGTTGTTTTCTGGTGGT CCATAT	213
v26	forward primer	AATAAAGCTTATGTGTACCGAAGCTCCGGCACCACCAGAAACCA GCACTGCCAAAG	214
	reverse primer	AATAGGATCCACAATTTTTTTTTTTTTTTTCTTTGGCAGTGCTG GTTCTGGTGGT	215
v27	forward primer	AATAAAGCTTATGTGTACCGAAGCTCCGGCACCACCAGAAACCA GCACTGCCAAAG	216
	reverse primer	AATAGGATCCACAATTTTTTTTTTTTTTTTCTTTGGCAGTGCTGGT CTGGTGGTG	217
v28	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGCAATCACCAAAAAACA ACAATG	218
	reverse primer	AATAGGATCCACAATTTTCTTTGTCATTGTTGTTTTGGTGATT GCAGA	219
v29	forward primer	AATAAAGCTTATGTGTACCGAGATCTGAAACTACCAGAAACCA CCAATGTCAAAG	220
	reverse primer	AATAGGATCCACAATTTTTTTTTTTTTTTTCTTTGACATTGGTGGT CTGGTAG	221
v30	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGCAACCACCAAAAAACA ACAATG	222
	reverse primer	AATAGGATCCACAATTTCTCTGCCATTGTTGTTTTGGTGGT TGCAGAT	223
v31	forward primer	AATAAAGCTTATGTGTAGCGAAGATCCGGAACCACCAAAAAACA ACAATGAC	224
	reverse primer	AATAGGATCCACAATTTTCTTTGTCATTGTTGTTTTGGTGGT CCGGAT	225
v32	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGAAACCACCAGAAGCA TCACTGC	226
	reverse primer	AATAGGATCCACAATTTTCTCTGGCAGTGATGCTTCTGGTGGT TTCAGAT	227
v33	forward primer	AATAAAGCTTATGTGTGCCGAGATCTGGAACAACCAGAAACCA CCAGTGGCAA	228
	reverse primer	AATAGGATCCACAATTTTTTTTTTTTTTTTCTTTGCCACTGGTGGT CTGGTTGTTT	229
v34	forward primer	AATAAAGCTTATGTGTAACGGAGATCTGGAACGACCAGAAACCA CCAGTG	230
	reverse primer	AATAGGATCCACAATATTCCTTTGCCACTGGTGGTTCTGGTCGT TCCAGAT	231
v35	forward primer	AATAAAGCTTATGTGTACCGAAGATCTGAAACCACCAGAAACCA	232

		CCACTGC	
	reverse primer	AATAGGATCCACAATTTTCCTTGGCAGTGGTGGTTCTGGTGGT TTCAGAT	233
v36	forward primer	AATAAGCTTATGTGTAAACGAAGCTCTGGAACCACCACCACTG CGAAAG	234
	reverse primer	AATAGGATCCACAATGTTCTTTTCGCAGTGGTGGTGGTTCCAG AGCTTC	235
v37	forward primer	AATAAGCTTATGTGTCCCGAAGATCTGGAACGACCACCATTG ACAAAG	236
	reverse primer	AATAGGATCCACAATGTTCTTTGTCAATGGTGGTCGTTCCAGA T	237
v38	forward primer	AATAAGCTTATGTGTACCGAAGATCTGGAACCACCAGAACGA CCACTGC	238
	reverse primer	AATAGGATCCACAAATTTTCCTTGGCAGTGGTCGTTCTGGTGGT TCCAGA	239
v39	forward primer	AATAAGCTTATGTGTGCCGAGATCTGAAACCACCAGAAACA ACAATGTC	240
	reverse primer	AATAGGATCCACAATTTTCTTTGACATTGTTGTTTCTGGTGGT TTCAGA	241
v40	forward primer	AATAAGCTTATGTGCACCGAAGATCTGCAACAACCAGAACGA TCACAGC	242
	reverse primer	AATAGGATCCACAATTTCCATTGGCTGTGATCGTTCTGGTTGT TGCAGA	243
v41	forward primer	AATAAGCTTATGTGTCCCGAAGATCTGCAACCACCAGAACCA GCACTGCCAGAGAAAAA	244
	reverse primer	AATAGGATCCGACCACGCGACGCTGACGGCGCTTTTACGAC CAGAGCCAGAACCAAA	245
	additional primer	ACCAGAACCACTGCCAGAGAAAAAAAAAAAAAAAAAATTGT GGTTCTGGCTCTGGTCTG	246
v42	forward primer	AATAAGCTTATGTGTACCGAAGTTCTGGTACCACGAACCACC AGTGGCAAAGGAAG	247
	reverse primer	AATAGGATCCGAGCCACACGCCGATGCTGACGGATGGCCTTT TTGCGACCAGAGCCAGAA	248
	additional primer	ACCAGAACCACTGGCGCAAAGGAAGATTGTGGTTCTGGCTC TGGTCGCAAAAAGGCCA	249
v43	forward primer	AATAAGCTTATGTGTGCCGAGATCTGCAACCACCACCACTG CTAGAGGCA	250
	reverse primer	AATAGGATCCACACTGACGGCGCTTTTACGACCAGAGTCAGA ACCACAATGTGCCTCT	251
	additional primer	ATCTGCAACCACCACCTGCTAGAGGCACATTGTGGTTCTGA CTCTGGTCGTAAAAAG	252
v44	forward primer	AATAAGCTTATGTGTACCGCAGTCCGGAACCACCAGAACCA CAACTG	253
	reverse primer	AATAGGATCCACAATTTGCCTGTGGCAGTTGTGGTTCTGGTGGT TCCGGAG	254
v45	forward primer	AATAAGCTTATGTGTCCCGCAGATCTGCAACAACCAGAAACA TCACTG	255
	reverse primer	AATAGGATCCACAATTTCTCTGGCAGTGATGTTCTGGTTGT TGCAGAT	256
v46	forward primer	AATAAGCTTATGTGTTCCGTAGATCTGCAACCACCAGCACGA CTACG	257
	reverse primer	ACAATTTACCATTGGCCGTAGTCGTGCTGGTGGTTGCAGAT	258
v47	forward primer	AATAAGCTTATGTGCACCGAGCTTTGGAACCACCAGAACCA CACTG	259
	reverse primer	AATAGGATCCACAATTTCTTTGTCAAGTGGTGGTTCTGGTGGT TCCAAAG	260
v48	forward primer	AATAAGCTTATGTGTACCGAAGCTATGGAACCACCAGAACCA CCACTG	261
	reverse primer	AATAGGATCCACAATTTCTCTGCCAGTGGTGGTTCTGGTGGT TCCATAG	262
v49	forward primer	AATAAGCTTATGTGTACCGCAGATCTGCAACCACCAGAAAGCA	263

		TCACTG	
	reverse primer	AATAGGATCCACAATTTTGCTGTGGCAGTGATGCTTCTGGTGGT TGCAGA	264
v50	forward primer	AATAAAGCTTATGTGTACCGCAGCTCCGGAACCACCAGAACCA CGACTGCCA	265
	reverse primer	AATAGGATCCACAATTTCCCTCTGGCAGTCGTGGTCTGGTGGT TCC	266
v51	forward primer	AATAAAGCTTATGTGTACCAAAGATTGGCACCACAAGCACCA CCATTGCT	267
	reverse primer	AATAGGATCCACAATTTTCCTTTAGCAATGGTGGTGGTCTGTGGT GCCAAA	268

Example 5 - Inhibition of the CTDSP1 phosphatase activity by RPP and RPP variants

[00119] Results: Figure 25 - The top 51 most abundant RPP variants identified in the RNA display/protein evolution screen are listed in-Table 3 in the order of most (V1) to least (V51) abundant peptides. The primers used to make these peptides are listed in-Table 5. A phosphatase activity screen (Table 6, Figure 25) was used to assess the ability of the top 51 RPPv to inhibit CTDSP1 phosphatase activity at amino acids 861 and 864 on the endogenous phosphorylated REST peptide (TEDpSPPpSPPLPKEN).

[00120] The phosphorylated REST peptide (TEDpSPPpSPPLPKEN) was synthesized by GeneScript. Phosphatase reactions were performed with 10 mM Tris pH 8, 10 mM MgCl₂, 100 nM CTDSP1, 0.5 μ M phosphate sensor (Thermo Fischer), 50 μ M of REST peptide and various concentrations of peptide-GST fusions at room temperature for 10 min. All assays were performed in 96-well plates (Corning P/N 3686) rinsed with water 10 times. Fluorescence was measured by BioTek Synergy HTX using kinetic read with excitation at 420/27 nm and emission at 485/20 nm.

[00121] The screen showed RPP (V1) inhibited phosphatase activity by approximately 60%, relative to control (GST), at 1 μ M (Table 6, Figure 25). Several variants inhibited phosphatase activity better than RPP (Table 6, Figure 25). The most potent inhibitors were v33, with 100% inhibition, and v35, with approximately 90% inhibition of phosphatase activity (Table 6, Figure 25).

Table 6 - Inhibition of CTDSP1 phosphatase activity by RPP variants

RPP variant	Relative Phosphatase activity	Standard deviation, P< 0.05	Number of replicas
RPP	0.42	0.25	5
v2	0.66	0.64	6
v4	0.67	0.28	3
v5	0.49	0.25	6
v6	0.77	0.30	4
v7	0.24	0.21	5
v9	0.65	0.32	4
v10	0.84	0.45	4
v11	0.13	0.19	3
v12	0.41	0.14	4
v13	0.20	0.20	4
v14	0.54	0.19	2
v15	0.70	0.41	4
v16	0.79	0.43	4
v17	0.24	0.22	6
v18	0.43	0.30	4
v19	0.76	0.00	1
v20	0.13	0.13	2
v21	0.26	0.17	4
v22	0.59	0.37	2
v23	0.60	0.42	4
v25	0.63	0.45	4
v26	0.46	0.20	2
v27	0.19	0.19	2
v28	0.33	0.33	2
v29	0.17	0.30	4
v30	0.62	0.63	4
v31	0.43	0.35	3
v32	0.89	0.56	4
v33	0.00	0.00	3
v34	0.74	0.48	4
v35	0.07	0.09	4
v36	0.68	0.31	3
v37	0.16	0.15	5
v38	0.28	0.30	3
v39	0.59	0.07	3
v40	0.29	0.19	3
v41	0.46	0.33	6
v42	0.48	0.24	7
v43	0.97	0.00	1
v44	0.53	0.47	3
v46	0.37	0.32	7
v47	0.20	0.22	3

v49	0.58	0.37	4
v51	0.71	0.46	3

Figure 26, Table 13 - RPP, SEQ ID NO: 12, inhibited CTDSP1 activity with an EC₅₀ of approximately 20 nM, but did not affect the activity of several other phosphatases.

Assay: The ability of RPP SEQ ID NO: 12 to inhibit CTDSP1 activity at amino acids 861 and 864 on the endogenous phosphorylated REST peptide (TEDpSPPpSPPLPKEN) was assessed as described for Figure 26 above.

Table 13 - CTDSP1 and off target phosphatase acclivity after RPP (SEQ ID NO: 12) dosing			
Phosphatase	RPP SEQ ID NO: 12 nM	Average N=5 % Activity	Standard Deviation
CTDSP1	0	100	0
CTDSP1	10	89.97	15.64
CTDSP1	20	48.7	14.65
CTDSP1	40	34.46	11.58
CTDSP1	60	31.17	21.23
CTDSP1	80	22.67	10.2
CTDSP1	100	13.72	13.35
PPA1	10000	101.125	1.611
PPM1H	10000	98.279	2.406
PPM1A	10000	102.786	2.559
PP3CA	10000	100.222	2.897
PPP1CA	10000	101.136	3.711
PP5CA	10000	97.902	5.245

Example 6 - Binding affinity of RPP assessed using Monolith (NanoTemper) and Biacore (GE Healthcare Life Sciences).

[00122] Expression constructs for His-CTDSP1 (Table 2; SEQ ID NO: 162) and GST-RPP (Table 4; SEQ ID NO: 164) were constructed and purified as described in **Example 2**. The His tag on CTDSP1 (40 nM) was labelled with RED-tris NTA dye (20 nM), with a 3:1 dye to CTDSP1 ratio, and unbound dye was removed by gravity-flow size exclusion. Binding affinity of

the linear GST-RPP (Table 4; SEQ ID NO: 165) to His-CTDSP1 (Table 2; SEQ ID NO:163) was determined using Monolith (NanoTemper), following manufacturer recommendations. Results of the binding assay are presented in Figure 2: The RPP (Table 4; SEQ ID NO: 165) was assessed for binding to His-CTDSP1 at several concentrations ranging from low pM up to 0.5 μ M. K_D corresponding to the binding of linear RPP to CTDSP1 was calculated at 130 pM.

[00123] Binding affinity of the linear GST-RPP (Table 4; SEQ ID NO: 165) to His-CTDSP1 (Table 2; SEQ ID NO: 163) was measured using Biacore by GE Healthcare Life Sciences. 20 μ L (5 μ g/mL) of His-tagged CTDSP1 in 10 mM sodium acetate, pH 5.0 was immobilized on a CM5 chip (GE Healthcare Life Science) using an amine coupling following a protocol recommended by Biacore (cat# BR-1000-50, GE Healthcare Life Science). This purification yielded approximately 1300 RU of protein bound to the CM5 chip. The CM5 chip was then washed with HBS-EP buffer (pH 7.4), 120 μ L of the analyte: GST-RPP or GST alone (negative control) was injected at a concentration of 500 nM in HBS-EP buffer (pH 7.4) at flow rate of 30 μ L/min. The association time was 2 minutes and dissociation time is 3 minutes. Finally, bound protein was washed with 10 mM glycine-HCl, pH1.5 (BR-1003-54, GE Health science) for 25 second at 50 μ L/min flow rate. The binding of linear RPP to CTDSP1 resulted in: K_D = 1.7 pM (Figure 3B, the negative control is shown in Figure 3A).

Example 7 - RPP and RPPv fused with the cell-penetrating peptides (CPPs) and/or peptides facilitating endosome escape

[00124] Several RPP and RPPv were synthesized with attachments of the cell-penetrating peptides (CPPs) and/or peptides facilitating endosome escape. The list of CPPs and linkers we used is shown in Table 7.

Table 7 –Additional peptide allowing cell penetration and escape from endosomes

Name	Protein Sequence (a)	SEQ ID NO:	RI sequence (b)	SEQ ID NO:
CPP	GRKKRRQRRR	118	rrrqrkkrg	140
CPP	GDIMGEWGNEIFGAIAGFLGYGR KKRRQRRR	119	rrrqrkkrgyglfgaiagfiengwegmgmidg	141
CPP	RRRRRRRR	120	rrrrrrrr	142
CPP	KKKKKKKK	121	kkkkkkkk	143
CPP	DIMGEWGNEIFGAIAGFLG	122	glfgaiagfiengwegmid	144
CPP	CHHHHHHRKKRRQRRRRHHHHHC	123	chhhhhrrrrqrkkrrhhhhhc	145
CPP	CHHHHHRRRRRRRRRRHHHHHC	124	chhhhhrrrrrrrrrrhhhhhc	146
CPP	FFLIPKGRRRRRRRRGC	125	cgrrrrrrrgkplffc	147
CPP	FΦRRRR	126	rrrrΦf	148
CPP	RRWRRRWRRRRWWRr	127	rrwwrrrrrrrrrrrr	149
CPP	RWRRRRRWRRRWWRr	128	rrwwrrrrrrrrrrrr	150
CPP	RRWRRRWRRRRWWRr	129	rrwwrrrrrrrrrrrr	151
CPP	RRWRRRWRRRr	130	rrrrrrrrrrrrrr	152
CPP	RRWRRRWWRr	131	rrrrrrrrrrrr	153
CPP	RRWRRRWRRR	132	rrrrrrrrrrrr	154
CPP	RRRRRRC ₁₄ RRWRRr	133	rrrrrrrrrrrrrrrrrrrr	155
CPP	YALTSAISRIITHHHHHH	134	hhhhhhhtirsiasltay	156
CPP	RRRRRC ₁₄ RRWRR-	135	rrrrrrrrrrrrrrrrrrrr	157
CPP	RRC ₁₄ RRC ₁₄ RRRRR	136	rrrrrrrrrrrrrrrrrrrr	158
CPP	RRC ₁₄ RRR	137	rrrrrrrrrrrr	159
Linker 1	GS	138	sg	160
Linker 2	GSGS	139	sgsg	161
Φ is L-2-naphthylalanine				
Lower case = D-amino acids				
C ₁₄ = 2-amino-tetradecanoic acid				

Example 8 - RPP is internalized by mesenchymal progenitor cells and sciatic nerve.

[00125] Mesenchymal progenitor cells (MPCs)

[00126] **Result.** After a 4 hour incubation with RPP (SEQ ID NO.: 4), followed by 6 days in culture, RPP remains internalized in mesenchymal progenitor cells (MPCs) (Figure 4, C and D) and localizes in the nucleus over the 6 day culture period (Figure 4, E and F).

[00127] Materials and Methods

[00128] Dosing and Cell Culture. MPCs were cultured as previously described¹¹⁶, plated at 1.0×10^4 cells/cm² onto glass coverslips in a 24-well plate, incubated overnight at 37°C, dosed with 100 nM RPP (SEQ ID NO: 4) for 4 hours, followed by a change in media. Cell were cultured for 6 days with a media change every two days. Interim MPC harvesting occurred at 24 and 72 hours to assess intracellular RPP localization.

[00129] Immunohistochemistry. Phalloidin (Invitrogen) was used for cytoskeletal staining, Hoechst 333429 (Calbiochem) dye was used to visualize the nucleus, and FLAG antibody (Sigma-Aldrich) was used to visualize the RPP. Images were taken with a confocal laser scanning microscope.

[00130] Sciatic nerve

[00131] Result. Linear RPP accumulates in the nucleus of sciatic nerve tissue (Figure 5, A and B).

[00132] Materials and Methods

[00133] Dosing and Procedure. Figure 5: The sciatic nerve of a Sprague Dawley rat (L3 and L4 region) was exposed (Schmitz and Beer, 2001)¹¹⁷ and a 0.7 cm section remove to create an approximately 1 cm segmental defect following retraction of the nerve stumps (Hems and Glasby, 1993)¹¹⁸. 1 mg of RPP (SEQ ID NO: 4) in PBS was injected into the site of injury and the surgical incision was closed. After 48 hours, the animal was sacrificed and the spinal cord from the L4 to L6 region was sectioned coronally.

[00134] Rational for dose levels. Maximum feasible dose.

[00135] Test Article Identification. RPP (SEQ ID NO: 4)

[00136] Purity. $\geq 95\%$

[00137] **Immunohistochemistry.** Hoechst 333429 (Calbiochem) dye was used to visualize the nucleus, and FLAG antibody (Sigma-Aldrich) was used to visualize the RPP. Images were taken with a light microscope.

Example 9 - RPP induced degradation of REST protein.

[00138] **Result.** RPP (SEQ ID NO: 4) decreased REST protein levels by 58% compared to vehicle control (Figure 6).

[00139] **Materials and Methods**

[00140] **Test Article Identification.** RPP (SEQ ID NO: 4)

[00141] Purity $\geq 95\%$

[00142] **Dosing and Cell Culture.** HEK 293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum. Transfection of HEK 293 cells, with 100 nM of full-length human REST protein², was performed in 80 to 90% confluent cultures in 35 mm dishes using lipofectamine2000 (Life Technologies), following manufacturer recommendations. 24 hours post transfection, cells were dosed with 100 nM RPP (SEQ ID NO: 4) or vehicle control (PBS) overnight.

[00143] **Western Blot Analysis.** Whole-cell lysates were prepared following the procedures in Ballas et. al., 2001¹¹⁹. Western blots were performed by standard procedures using anti-REST-C⁶⁴, anti-GAPDH (abcam [6C5]), and anti-IgG conjugated to infrared dyes (Thermo Fisher), and analyzed on an Odyssey infrared fluorescence imager (LiCor). The bar graph is a quantification of the Western Blot using ImageJ (<https://imagej.nih.gov/ij>).

Example 10 – RPP can be used to induce expression of BDNF, NGF, Kv4.3, Kv7.2, Nav1.8, and OPRM1 mRNA and is not neurotoxic.

Result. RPP increases expression of BDNF, NGF, Kv4.3, Kv7.2, Nav1.8, and OPRM1 (Fig. 7, 8, 9, 10, 14, 15, and 16; Table 15). RPP (SEQ ID NO: 12) does not cause necrosis in DRG neurons (Fig. 17), as demonstrated by an LDH cytotoxicity assay in DRG neurons incubated with various concentrations of RPP for 48 h. *** = $p < 0.001$, $n = 4$, one-way ANOVA and Dunnett's test, error bars = SD.

Table 15. RPP induced expression of BDNF, NGF, Kv4.3, Kv7.2, Nav1.8, and OPRM1 mRNA						
Target	Figure	Increase	Cell Type	RPP SEQ ID NO	Dose	Duration
BDNF	7	2.4x	HEK 293	4	100 nM	24 h
BDNF	9	5x	NBFL	2	1 μ M	16 h
BDNF	9	2.3x	NBFL	2	1 μ M	48h
BDNF	9	2.3x	NBFL	4	1 μ M	48 h
BDNF	10	2x	MPC	9	3 μ M	48 h
BDNF	16	1.4x	DRG	12	0.3 μ M	48 h
BDNF	16	1.3x	DRG	12	1 μ M	48 h
BDNF	16	1.3x	DRG	12	3 μ M	48 h
BDNF	16	1.7	DRG	12	10 μ M	48 h
NGF	9	3.2x	NBFL	2	1 μ M	16 h
NGF	9	1.7x	NBFL	2	1 μ M	48 h
NGF	9	1.9x	NBFL	4	1 μ M	48 h
NGF	10	2.9x	MPC	9	3 μ M	48 h
NGF	16	-	DRG	12	0.3 μ M	48 h
NGF	16	0.9x	DRG	12	1 μ M	48 h
NGF	16	1.25	DRG	12	3 μ M	48 h
NGF	16	1.9	DRG	12	10 μ M	48 h
Kv4.3	8	3x	NBFL	1	2 μ g (transfected)	48 h
Kv4.3	9	5x	NBFL	2	1 μ M	16 h
Kv4.3	9	5x	NBFL	2	1 μ M	48 h
Kv4.3	9	19.5x	NBFL	4	1 μ M	48 h
Kv4.3	14	0x	DRG	12	1 μ M	48 h
Kv4.3	14	0x	DRG	12	3 μ M	48 h

K _v 4.3	14	1.9x	DRG	12	10 μ M	48 h
K _v 7.2	14	0	DRG	12	1 μ M	48 h
K _v 7.2	14	6.7x	DRG	12	3 μ M	48 h
K _v 7.2	14	7.5x	DRG	12	10 μ M	48 h
Na _v 1.8	13	0.8x	DRG	12	1 μ M	48 h
Na _v 1.8	14	2.6x	DRG	12	3 μ M	48 h
Na _v 1.8	14	4x	DRG	12	10 μ M	48 h
Na _v 1.8	15	6x	DRG	13	1 μ M	48 h
Na _v 1.8	15	13x	DRG	14	1 μ M	48 h
OPRM1	14	1.2x	DRG	12	1 μ M	48 h
OPRM1	14	3x	DRG	12	3 μ M	48 h
OPRM1	14	3.9x	DRG	12	10 μ M	48 h

[00144] Figure 7 Materials and Methods

[00145] Test Article Identification. RPP (SEQ ID NO: 4), purity. $\geq 95\%$

[00146] Dosing and Cell Culture. 100 nM RPP; HEK 293 cells,

[00147] mRNA extraction and quantification protocol. Cells were lysed in QIAzol

(Qiagen) and total RNA was extracted using RNeasy Midi Kit (Qiagen) according to manufacturer's instructions. Purified RNA was quantified with the NanoDrop 2000 (ThermoFisher), and reverse transcription was run using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 2 ng/ μ L RNA per reaction. For qRT-PCR, 5 μ L of cDNA (corresponding to 10 ng of RNA) were mixed with to 10 μ L SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) and 1 μ L of each primer (final primer concentration 500 nM each). Reactions were run in triplicates in a QuantStudio™ 7 Flex Real-Time PCR system (Applied Biosystems). Amplification data were analyzed using the comparative cycle threshold ($\Delta\Delta C_t$) method and β -actin as calibrator. The primers used were as follows: β -actin forward: 5'-AGAGCACGAGCTGCCTGAC-3', β -actin reverse: 5'-GGATGCCACAGGACTCCA-3', BDNF forward: 5'TATTAGTGAGTGGGTAACGGCG3', and BDNF reverse: 5'GAAGTATTGCTTCAGTTGGCCTT3'.

[00148] Figure 8 Material and Methods

[00149] Transient Transfections and Cell Culture. NBFL cells were grown as previously described¹²⁰. 0.5 M NBFL cells were seeded on a 35 mm dish, incubated at 37°C overnight, and transfected with 2 µg of REST (SEQ. ID NO: 1)-IRES-GFP cDNA² using lipofectamine2000 (Life Technologies), following manufacturer recommendations. After a 48 hour incubation period, cell were sorted by fluorescence activated cell sorting (FACS),

[00150] mRNA extraction and quantification. mRNA was extracted, as described in Example 10 , from GFP + and – cells; mRNA levels of Kv4.3 were determined using real-time RT-PCR¹¹⁶. Gene expression was normalized using β-actin (*ACTB*) as an internal housekeeping control. The β-actin primers used are the same as Example 10 . The Kv4.3 primers were: forward: CTCCTACCACCTGCTGCTC and reverse: TCAGTCCGTCGTCTGCTTTC.

[00151] Figure 9: Material and Methods

[00152] Plasmids. RPP (SEQ ID NOS: 2 and 4): One segment of this construct containing T7 promoter, CPP, and His tag was synthesized as a gBlock (Table 8) by IDT and amplified with primers described in Table 8. The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec., and extension at 72°C for 30 seconds. Following amplification, PCR fragment was gel-purified by the QIAGEN gel-band purification kit according to manufacturer's protocol.

[00153] pET29 plasmid was amplified with primers: forward: P12 and P14 (Table 1) introducing *SbfI* and *XhoI* restriction sites. The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 6 min. Following amplification, PCR fragment was gel-purified by the QIAGEN gel-band purification kit and digested with *SbfI* and *XhoI* restriction enzymes. All

digested fragments were purified with a QIAGEN kit and ligated in appropriate combination using T4 DNA ligase (NEB) according to the manufacturer's recommendations. Ligated fragments were transformed in 10G chemically competent cells and sequenced.

[00154] The nucleotide sequence of RPP (Table 8) was assembled and digested by *HindIII* and *BamHI* restriction enzymes. This fragment was cloned in pET vector described above in-frame with the CPP and His tag.

[00155] Control peptide: The control peptide (CP) was incorporated into the gBlock sequence shown in Table 9 and then cloned into the pET vector. The CP sequences are shown in Table 9.

[00156] ***In vitro* Expression.** RPP (Table 8) or CP (Table 9) were expressed *in vitro* using the PURE Express *In Vitro* Protein Synthesis Kit (New England Biolabs). The RPP (SEQ ID NOS: 2 and 4) or CP sequence was amplified from the RPP (SEQ ID NOS: 2 and 4) or CP expression constructs (Table 8 and 9) with primers listed in Table 8, and gel-purified by the QIAGEN gel-band purification kit. The PCR reaction (20 μ L) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec., and extension at 72°C for 30 sec. Then, 15 μ L of the purified PCR fragment was mixed with 10 μ L of Solution A and 7.5 μ L of Solution B of the PURE Express *In Vitro* Protein Synthesis Kit (New England Biolabs) and incubated 2 hours at 37°C. Next, 2 μ L of DNase I was added and incubated for 20 min at 37°C.

Table 8 - RPP-CPP-His_pET cassette

Nucleotide Sequence (SEQ ID NO: 316)

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cctgcaggTAATACGACTCACTATAGGGTTAACTTTAGTAAGGAGGACAGCTAAaagcttAT
GTGTACCGAAGATCTGGAACCAACCAGAACCACTGCCAAAGGAAAATTGTggatcc
GGCTCTGGTCGTAAAAAGCGCCGTCAGCGTCGGCGTGGTGGCTCCGGTAGCTTAGGT
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CACCATCACCATCACACGGCACCGGCTATTAAActcgag
<u>Coding Sequence (SEQ ID NO: 317)</u> ATGTGTACCGAAGATCTGGAACCACCAGAACCACCACTGCCAAAGGAAAATTGTggatccGGCTCTGGTCGTAAAAAGCGCCGTCAGCGTCGGCGTGGTGGCTCCGGTAGCTTAGGTCACCATCACCATCACACGGCACCGGCTATTAA
<u>Protein Sequence (SEQ ID NO: 2)</u> MCTEDLEPPEPLPKEN <u>CGSGSGRKKRRQRRRGSGSLGHHHHHH</u> GTGY
<u>Amplification Primers</u> Forward: aaatcctgcaggTAATACGACTCACTATAGGGTTAAC (SEQ ID NO: 318) Reverse: ATTATTctcgagTTAATAGCCGGTGCCGTGGTGATGGTGATGGTGACCTA (SEQ ID NO: 319)
<u>gBlock (SEQ ID NO: 320)</u> tatacctgcaggTAATACGACTCACTATAGGGTTAACTTTAGTAAGGAGGACAGCTAAaagcttATGTGTGAAGACGCCAAAAACATAAAGAAAGGCCCGGCCATTCTATCCGTGTggatccGGCTCTGGTCGTAAAAAGCGCCGTCAGCGTCGGCGTGGTGGCTCCGGTAGCTTAGGTCACCATCACCATCACACGGCACCGGCTATTAAActcgagagatc
The restriction sites: small font RPP, CPP and His tag: underlined

Table 9 - Control-CPP-His pET cassette

<u>Nucleotide Sequence (SEQ ID NO: 321)</u> cctgcaggTAATACGACTCACTATAGGGTTAACTTTAGTAAGGAGGACAGCTAAaagcttATGTGTGAAGACGCCAAAAACATAAAGAAAGGCCCGGCCATTCTATCCGTGTggatccGGCTCTGGTCGTAAAAAGCGCCGTCAGCGTCGGCGTGGTGGCTCCGGTAGCTTAGGTCACCATCACCATCACACGGCACCGGCTATTAAActcgag
<u>Coding Sequence (SEQ ID NO: 322)</u> ATGTGTGAAGACGCCAAAAACATAAAGAAAGGCCCGGCCATTCTATCCGTGTggatccGGCTCTGGTCGTAAAAAGCGCCGTCAGCGTCGGCGTGGTGGCTCCGGTAGCTTAGGTCACCATCACCATCACACGGCACCGGCTATTAA
<u>Protein Sequence (SEQ ID NO: 3)</u> MCEDAKNIKKGPAPFYPCGSGSGRKKRRQRRRGSGSLGHHHHHHGTGY
<u>Amplification Primers</u> Forward: aaatcctgcaggTAATACGACTCACTATAGGGTTAAC (SEQ ID NO: 323) Reverse: ATTATTctcgagTTAATAGCCGGTGCCGTGGTGATGGTGATGGTGACCTA (SEQ ID NO: 324)

324)

The restriction sites: small font

Control peptide, CPP and His tag: underlined

[00157] Purification. RPP (SEQ ID NOS: 2 and 4) and CP: The final solution from the *in vitro* expression step was diluted to 100 μ L and filtered through the Amicon Ultracel 0.5 mL-100K columns (Sigma) to remove ribosomes. The flow through was added to 100 μ L of washed Ni-NTA Magnetic Agarose Beads (Qiagen) and was washed with wash buffer as described by the manufacturer.

[00158] Cyclization. The RPP (SEQ ID NO: 2) or was cyclized by mixing the beads with 500 μ L of the cyclization solution which was made by mixing 2.65 mL of the 1.33x PBS (66.5 mM phosphate buffer, 400 mM NaCl) with 1.32 mL of dibromo-m-xylene solution in acetonitrile (2.5 mg/mL). Following incubation with the cyclization solution beads were washed once with wash buffer I (PBS with 25 mM imidazole, 0.5 μ L/mL of mercaptoethanol), once with wash buffer II (PBS with 25 mM imidazole, 0.5 mM TCEP), twice with wash buffer III (PBS with 25 mM imidazole) and eluted with 50 μ L of the elution buffer (PBS with 500 mM imidazole). Imidazole was removed using Bio-Rad Micro Bio-Spin Chromatography columns.

[00159] Dosing and Cell Culture. NBFL cells (Fig. 9) grown in 6 well were dosed with 1 μ M of linear (SEQ ID NO: 2) or cyclic (SEQ ID NO: 2) RPP (Table 8) or control peptide (Table 9) for 16 or 48 hours.

[00160] Mesenchymal progenitor cells (MPCs) (Fig 10) from two patients were plated on 12 well plates and cultured in general medium (GM) (DMEM, 10% FBS, PSF). Lyophilized RPP (SEQ ID NO: 9) was dissolved in water at a concentration of 5 mM (1 mg/34.89, diluted in medium to 3 μ M, and then incubated with the cell for 48 hours. The control was water.

[00161] mRNA Extraction and Quantification.

[00162] After dosing, cells were lysed and assessed for BDNF, NGF, and K_v4.3 mRNA levels (Figure 9). All of the primers used have been described except for NGF: forward: 5'TATCCTGGCCACACTGAGGT3' and reverse 5'TCCTGCAGGGACATTGCTC3'.

[00163] **Figure 10 Materials and Methods**

[00164] MPCs were cultured as described in Gervasi et al., 2020⁷⁶.

[00165] **Figure 14, 15, and 16 Material and Methods.**

[00166] L5 DRGs were dissected from adult male rats and plated in 12-well plates pre-coated with poly-D-Lysine (PDL) and laminin in a minimal volume of medium (250 μ l Neurobasal™ -A medium (GIBCO) supplemented with B27 (GIBCO), 50 ng/ml NGF (Sigma-Aldrich) and penicillin streptomycin) to allow attachment to the culture plate. One day after plating, DRGs were incubated with RPP (SEQ. ID NO: 12: 1 μ M, 3 μ M or 10 μ M in culture medium, Fig. 14 and 16; SEQ ID NOS: 13 and 14: 3 μ M, Fig. 15) for 48 h. For RNA expression analysis, DRGs were lysed in 300 μ l of Qiazol in a 1.5 ml tube containing Bullet Blender Pink Beads using a Bullet Blender Tissue Homogenizer (NextAdvance). Total RNA was extracted using RNeasy Midi Kit (Qiagen) according to manufacturer's instructions. Purified RNA was quantified with NanoDrop 2000 (ThermoFisher), and reverse transcription was run using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 2 ng/ μ l RNA per reaction. For qRT-PCR, 5 μ l of cDNA (corresponding to 10 ng of RNA) were mixed with to 10 μ l SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) and 1 μ l of each primer (final primer concentration 500 nM each). Reactions were run in triplicates in a QuantStudio™ 7 Flex Real-Time PCR system (Applied Biosystems). Amplification data were analyzed using the comparative cycle threshold ($\Delta\Delta$ Ct) method and β -actin as calibrator. Primers were as follows: β -actin: F- AGAGCTATGAGCTGCCTGAC, R- GGATGCCACAGGACTCCA; K_v4.3: F-

AGCTGTGCCTCAGAACTAGGCTTT, R-TACCAGAAAGACGCAGGGATGCTT; Kv7.2 F-CCGGCAGAACTCAGAAGA AG, R- TTTGAGGCCAGGGGTAAGAT; OPRM1: F-TTCCTGGTCATGTATGTGATTGTA, R- GGGCAGTGTACTG GTCGCTAA.

[00167] Figure 17 Materials and Methods

[00168] Cytotoxicity Assay: Cytotoxicity of RPP (SEQ. ID NO: 12) was assessed with LDH-Glo™ Cytotoxicity Assay (Promega) according to the manufacturer's instructions. Medium was collected 48 h after treatment with RPP or 15 min incubation with 2% triton X-100 (which induces necrosis). Medium was diluted 1:50 in LDH storage buffer (200 mM Tris-HCl (pH 7.3), 10% Glycerol, 1% BSA). To verify the linear range of the assay, LDH titration curve was also run together with the experimental samples. 50 µl of diluted medium or LDH serial dilutions were incubated with 50 µl of LDH Detection Reagent (50 µl LDH Detection Enzyme Mix, 0.25 µl Reductase Substrate) for 40 min. Luminescence was recorded with an Infinite M200 Pro (Tecan) instrument.

Example 11 – RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) **can be used to Induce Neurodifferentiation**

[00169] Approach 1. To determine if RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) are the most effective in promoting human neurogenesis and differentiation, an *in vitro* screening assay using neural stem progenitor cells (NSPCs) from the NCRM-1/XCL-1 iPS cell line will be used. NCRM-1/XCL-1 iPS cells were generated from CD34+ human cord blood cells by episomal plasmid reprogramming¹²¹ and differentiated to neural stem progenitor cells

(NSPCs) through an embryoid body (EB) method¹²². NCRM-1/XCL-1 NSPCs are positionally naïve NSPCs that can be rapidly differentiated to neurons¹²³ making them ideal for broad-based toxicology and phenotypic screening platforms¹²⁴⁻¹²⁶.

[00170] We will use two engineered NCRM-1/XCL-1 lines for high-throughput screening in 96-well plate assays. To determine if the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) is cytotoxic, we will use an NCRM-1/XCL-1 line that constitutively expresses NanoLuc® luciferase under the control the CMV promoter. In this line, a CMV-Nanoluciferase-Halotag (CMV-NLHT) construct was inserted into the safe harbor AAVS1 locus of Chr. 19q by transcription activator-like effector nucleases (TALENs) (Fig. 20a). We will use luciferase activity as a rapid surrogate readout of CMV-NLHT cell number. Using this approach, we will collect time-course data to measure toxicity over prolonged exposures to the drug. If the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) shows toxicity levels resulting in $\geq 10\%$ loss of luciferase signal, this will be confirmed using CellTox™ Green Cytotoxicity Assay.

[00171] To assess the pro-neuronal differentiation effect of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), we will use a second engineered NCRM-1/XCL-1 line. In this line, Nanoluciferase-Halotag (NLHT) is knocked-into the MAP2 transcriptional start site (TSS) (Chr. 2) using zinc finger nucleases (ZFN) (Fig. 20b). MAP2 expression increases with neuronal differentiation in NCRM-1/XCL-1 cultures¹²⁷ and this is replicated by increasing luciferase activity in MAP2-NLHT culture media (Fig. 20c). We will use luciferase activity in media as a surrogate readout of MAP2-NLHT neurodifferentiation and collect time-course data to track RPP (SEQ ID

NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) differentiation effects over real-time. This approach will allow us to rapidly compare the neurodifferentiation rates in cultures treated with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) or control peptide. Positive neurodifferentiation effects will be independently validated by qRT-PCR expression screening of two cohorts of cell fate marker genes¹²¹; a cohort of NSPC and multipotency marker genes of LIN28A, RPS27L, IFITM2, IGFBP3, ANXA1; and neuronal marker genes C1ORF61, IGLON5, IGSF11, CHL1, SOX9. Luciferase activity and qRT-PCR values will be reported as mean $\pm$ standard error of the mean (SEM). Statistically significant drug effects on the rate of neuronal differentiation will be determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

[00172] PREDICTED RESULT & ALTERNATIVE STRATEGY. We predict that dosing of human NSPCs with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will accelerate neuronal differentiation as measured by increased MAP2-luciferase activity, decreased expression of NSPC/multipotency markers and increased expression of neuronal markers when compared to vehicle controls.

[00173] If the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) effects shift the temporal profile NCRM-1/XCLs marker expression, this may necessitate data collection at alternate time-points. If the luciferase screen lacks the sensitivity to assess the pro-differentiation

effects of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), qRT-PCR data can be used to assess neural differentiation.

[00174] Material and methods

[00175] Plasmids. CMV-Nanoluciferase-Halotag (CMV-NLHT) is commercially available from Promega.

[00176] Cell lines and cell culture. NCRM-1/XCL-1 iPS cells were generated from CD34⁺ human cord blood cells by episomal plasmid reprogramming¹²¹ and differentiated to neural stem progenitor cells (NSPCs) through an embryoid body (EB) method¹²² and neurons¹²³ as previously described. The method for inserting a CMV-Nanoluciferase-Halotag (CMV-NLHT) construct into the safe harbor AAVS1 locus of Chr. 19q (NCRM-1/XCL-1 cell line) by transcription activator-like effector nucleases (TALENs) was previously described by Papapetrou et al., 2016¹²⁸. The method for knocking in Nanoluciferase-Halotag (NLHT) into the MAP2 transcriptional start site (TSS) (Chr.2, of the NCRM-1/XCL-1 cell line) using zinc finger nuclease (ZFN) has been previously described¹²⁷.

[00177] Luciferase assay. The methods for determining CMV-NLHT cell number and neurodifferentiation (MAP2-NLHT) have been previously described by Fritz et al., 2017¹²⁷ and He et al., 2011¹²⁹.

[00178] qRT-PCR screen. The qRT-PCR methods for screening cell fate marker genes has been previously described by Chou et al., 2011¹²¹.

[00179] Approach 2: Induced pluripotent stem cells (neural stem cells (NSC)-NL5) differentiate after 7 Days of RPP dosing (Fig. 11 (SEQ ID NOS: 5-11, 3 μ M); Fig. 12 (SEQ ID

NO: 9, 1 μ M,) Fig. 13 (SEQ ID NO: 13 (Fig. 27), 1 μ M,)) using the following “Neuron to Blank” protocol:

1. 24 well culture plates (Black Visiplate) were coated with Matrigel (12 mL/well for 30 minutes at 37°C).
2. NSCs-NLS (see media below) were plated at 0.5×10^5 per well.

Component	Final Concentration	Amount
DMEM/12 Medium	1X	97 mL
N2 (100 x)	1X	1 mL
B27 Supplement	2%	2 mL
bFGF (prepared as 100 ug/mL stock)	10 ng/mL	10 μ L

3. After 24 hours (cells should be >70% confluent), the media was changed to neuronal differentiation medium (below) with various concentrations of test peptides or water control added (Fig. 11, 12, and 13, Table 14). The neuronal differentiation medium, with new test peptides and control, was changed every 2 days for 5 days.

Component	Final Concentration	Amount
D-MEM/F-12	1X	88.6 mL
GlutaMAX™ -I Supplement	2mM	1 mL
Bovine Serum Albumin (at 25%)	1.8%	7.2 mL
hESC Supplement	2%	2 mL
BDNF (prepared as 25 ug/mL stock)	10 ng/mL	40 μ L
GDNF (prepared as 100 ug/mL stock)	10 ng/mL	10 μ L

4. On Day 7 cells were fixed 4% formaldehyde in 1x PBS for 30 minutes.
5. Cells were immunolabeled with mouse anti-TUJ1 (1:1000) and rabbit anti-Map2 (1:500)

overnight. Then label with 2nd antibody (anti-mouse488, anti-rabbit568 and DAPI) for 1 hour.

6. Data was collected on a BioTek plate reader
 - a. Dapi: Excitation 360/40; Emission 460/20, Gain 35;
 - b. Alexa 488: Excitation 485/20; Emission 528/20, Gain 50;
 - c. Alexa 568: Excitation 560/20; Emission 620/10, Gain 75).

[00180] Results: In Figure 11, RPPs SEQ ID NOS: 5 through 11 at 3 μ M increased (1.7- to 2.7-times) NSC-NL5 differentiation after 7 days, as measured by MAP2 (mature neuron marker) expression normalized to DAPI (nucleus) and relative to control. In Figure 12, RPP SEQ ID NO: 9 at 1 μ M increased NSC-NL5 differentiation approximately 35% after 7 days, as measured by TUJ1 and MAP2 neuronal markers (normalized to DAPI (nucleus)) and relative to control. In Figure 13, RPP SEQ ID NO: 13 at 0.1 and 1 μ M increased NSC-NL5 differentiation after 7 days, as measured by TUJ1 (increased 60% and 3-times, respectively) and MAP2 (increased 27% and 2-times, respectively) neuronal markers (normalized to DAPI (nucleus) and relative to control). The basis for using this screen is that eliminating REST in neural progenitors cells has been shown to induce neuronal differentiation²², which is a readout for ensemble derepression of neuronal genes.

Example 12 - RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) can be used to improve motor and cognitive function after traumatic brain injury.

[00181] Approach. We will determine whether treatment with the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), in a rat model of TBI alters neuronal survival and

the amount of neurogenesis. We will correlate these measures with histopathological and functional recovery outcomes to determine the effectiveness of the REST peptide after TBI. We have shown that REST mRNA increases around the lesion after injury (Figure 19). We will use a well-characterized rat model of controlled cortical impact injury (CCI)^{6,130-134}.

[00182] Male and female Sprague-Dawley rats (approximately 250-300 g males, 150-200 g females) will be injured by CCI unilaterally over the right parietal cortex (5 mm diameter, 4 m/s, 2 mm depth). Sham animals will undergo identical procedures without impact. Six hours after CCI, rats will have a cannula implanted in their right ventricle that will attach to an osmotic minipump for intraventricular catheter (ICV) delivery of the control or RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) (0.17 µg/h). Six hours is a realistic initial therapeutic window with translational relevance to determine the efficacy of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will be infused until sacrifice. We will sacrifice rats at two different time points – the first cohort at 3 dpi to examine acute effects of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) on neuronal death and proliferation, and the second cohort at 30 dpi to determine effects of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) on functional and morphological recovery and neurogenesis. For each cohort, there will be 12 groups in a 3 (control peptide, two doses of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 159 through 143) x 2 (sham, CCI) x 2 (male/female) design. Male and female rats will be run on different days

to enable identification of any sex-specific differences in injury, behavior or response to the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159). Rats will be injected intraperitoneal (i.p.) with BrdU (50 mg/kg) daily during the first week to determine proliferation and maturational cell fate (1st cohort – daily injections 1 – 3dpi, sacrifice 2 h after final injection; 2nd cohort – daily 1-7dpi). Our prior experience suggests that we will need 16 rats/group for significant behavioral data (2nd cohort), 8 rats/group for immunohistochemistry (1st cohort).

[00183] In the second cohort, to determine the functional consequence of treatment with the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) compared to controls, we will examine motor and cognitive behavior at different time points post-CCI or sham injury. We will train rats before injury to obtain baseline readings for motor function on the beamwalk, rotorod, and open field tests. Rats will be tested for recovery of motor function on both beamwalk and rotorod on days 1, 3, 7, and 10 days post-injury (dpi). Open field testing will be conducted on days 4, 12, and 22 dpi. Recognition memory will be assessed with a novel object recognition test performed at 20 dpi. Data will be analyzed by repeated measure two-way ANOVA with Dunnett's post hoc correction. Spatial memory and learning will be determined by the Morris Water Maze (MWM) assay starting at 25 dpi. Swim speed and latency to find a hidden platform will be recorded for all trials. The probe trial, conducted on the fifth day of training, will determine the time the rat spends in the quadrant that previously contained the hidden platform. A visible platform test will also be performed on each rat to ensure that there is little difference in visual acuity between the animals. A two-way ANOVA with Tukey's multiple comparison correction will determine if the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to

a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) significantly alters cognitive function in the MWM in either the sham or injured rats in comparison with control peptide¹³⁵.

[00184] After the functional assessments, 8 rats/group will be sacrificed by transcardial perfusion, and the brains removed and processed. We will determine lesion size at both 3 and 30 dpi by staining sections with cresyl violet and measuring 12 sections spaced at 500 μ m intervals through the lesion. Lesion volume will be expressed as a percentage of the volume of the ipsilateral hemisphere. Serial coronal brain sections through the frontoparietal cortex and dorsal hippocampus containing the subventricular zone (SVZ) and the dentate gyrus (DG) will be examined. The total number of BrdU positive cells (detected with anti-BrdU) in different hippocampal regions and the SVZ will be counted using stereology to determine whether the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) alters the survival of newly proliferated cells after injury. Sections will be co-stained with different cell-specific markers to determine the identity of the proliferated cells at 3 dpi and their maturational fate at 30dpi. Cell specific markers include NeuN (mature neurons), SOX2 (neural stem cells), doublecortin (DCX) (neuronal progenitors), GFAP or ALDH1L1 (astrocytes), Iba1 (microglia), NG2 (oligodendrocyte precursor cells, OPCs), and APC or GSTpi (mature oligodendrocytes). The number of BrdU/ NeuN double positive cells in the DG and olfactory bulb at 30dpi will be compared between the different treatment groups to determine whether the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) increases post injury neurogenesis. We will also determine whether there is migration of BrdU+ neuronal progenitors or mature neurons to different regions of the hippocampus – away from the DG, or from the SVZ towards the lesion area or in the rostral migratory stream (RMS). Examination of the total number

of each BrdU⁺ cell type within the perilesional area in addition to the neurogenic niches will determine whether the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) alters pathology after injury. As we expect that the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will increase neuronal survival, we will quantify the number of degenerating neurons (Fluorojade C), and the number of surviving neurons (NeuN) in the peri-lesional area at both time points. We will examine the corpus callosum with Luxol fast blue to indicate any differences in the amount of myelin. The unbiased optical fractionator method will be used to count cells in the neurogenic areas, peri-lesional area, and the rostromigratory stream and olfactory bulb to obtain accurate cell specific and proliferating cell counts. All data will be analyzed by two-way ANOVA with Dunnetts post hoc correction. The distribution of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will be examined in sections by staining with the FLAG antiserum. The remaining 8 rats per group (2nd cohort) will be sacrificed and their brains quickly removed. Brain regions that are both ipsilateral and contralateral to the lesion will be punched out and snap frozen for RNA and protein isolation. We will determine REST levels by western blot and BDNF expression by qRT-PCR around the lesion or in neurogenic niches in comparison to control treated rat brains.

[00185] PREDICTED RESULT & ALTERNATIVE STRATEGY. We expect the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will reduce the lesion volume after CCI at both 3dpi and 30dpi by enhancing neuronal survival. Degenerating and surviving neurons will be

examined at both time points to directly assess the effect of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) treatment on neuronal survival. A significant early neuroprotective effect of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) in the perilesional area may result in improved motor function in the first week. Although we have previously found that the greatest neuronal death was at 3 dpi after CCI in the mouse¹³⁶, it is possible that we may need to examine a different time point in the rat. We also expect that animals treated with the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will show increased neurogenesis in comparison to those treated with control peptide. Our data will show whether treatment with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) results in an increase in DCX+ precursors in different brain regions at 3 dpi or an increase in BrdU+/ NeuN + mature neurons in the hippocampus, olfactory bulb or around the lesion at 30dpi. We will be able to correlate these data with behavioral outcomes, to see whether treatment with the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will result in improved performance in the MWM assay or novel object recognition assay¹³⁵. In this way, we will be able to determine whether the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) has any negative effect on inflammation or cell proliferation.

[00186] Materials and Methods

[00187] Test System.

[00188] Species. Male and female Sprague-Dawley rat.

[00189] Age at Study start. Approximately 22 to 29 weeks

[00190] Weight at study start. Approximately 250 to 300 g for males and 150 to 200 g for females.

[00191] **Study Design.** For each cohort (1 and 2), there will be 12 groups in a 3 (control peptide, two doses of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) x 2 (sham, CCI) x 2 (male/female) design. In cohort 1 (immunohistochemistry), there will be 8 rats/group and in cohort 2 (behavior), there will be 16 rats/group.

[00192] **Rational for dose levels.** The high dose will be the maximum feasible dose (approximately 0.17 µg/h) and the lower dose will be the estimated efficacious dose based on cerebral spinal fluid (CSF) drug levels that approximate the efficacious dose determined in Example 13.

[00193] **Route of Administration.** A cannula implanted in the right ventricle attach to an osmotic minipump for intraventricular catheter (ICV) delivery.

[00194] **Frequency of Administration.** Continuous infusion until sacrifice.

[00195] **Period of Dosing.** Cohort 1: 3 dpi; Cohort 2: 30 dpi.

[00196] **Environmental Conditions.** 2 Rats/cage, with food and water provided *ad libitum*.

[00197] **Test Article Identification.** RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159)

[00198] Purity. ≥95%

[00199] Preparation of Dose Formulation. In bulk every 4 days.

[00200] Dose Formulation Assay and Stability. Dose formulations will be assessed for stability and concentration on Day 1 and 7 in the first week of the study. The acceptable concentration range is $\pm 10\%$ of nominal.

[00201] **Controlled Cortical Impact (CCI) Model.** The CCI model has been previously described^{6,130-134}. RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) or control (vehicle only) administration will initiate 6 h after CCI.

[00202] **Immunohistochemistry.** Methods have been previously described by Xiong, Y et al., 2007 and 2008^{135,137}.

[00203] **Morris Water Maze.** Methods have been previously described by Choi et al., 2006¹³⁸.

Example 13 - RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) **can be used to improve regeneration after peripheral nerve injury.**

[00204] **Approach.** Nuerotrophic factors (NTFs) are needed for functional recovery after a peripheral nerve injury (PNI) and their presence, or lack thereof, are biomarkers of the strength of the regenerative response^{139,140}. Several NTF genes, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), pleitrophin (PTN), and neurotrophin-3 (NTF-3), are known to be repressed by injury-induced expression of REST and we have demonstrated that RPP (SEQ ID NO: 2) can reverse this transcriptional repression, at least in the case of BDNF and NGF (Fig. 7, 9, 10, and 16)^{141,142}.

[00205] 1) Real-time quantitative reverse transcription PCR (qRT-PCR) analysis showed that NBFL cells dosed with 1 μ M linear (SEQ ID NO: 4) or cyclic (SEQ ID NO: 2) RPP for 16 or 48 hours, evoke an increase in BDNF and NGF mRNA expression (Fig.9, A and B and table below).

NTF response to 1 μ M RPP in Figure 9			
NTF	RPP	Increase	
		16 h	48 h
BDNF	Linear	5x	2.25x
BDNF	Cyclic	NA	1.75x
NGF	Linear	3.2x	1.66x
NGF	Cyclic	NA	1.87
NA= Not assessed			

[00206] 2) RPP (SEQ ID NO: 9, 3 μ M) increased NTFs BDNF and NGF approximately 2- and 3-times, respectively, relative to water control, after 48 hours. This finding confirmed that RPP is active in MPCs (Figure 10). MPCs were cultured in general medium as previously described by Gerevasi et al., 2020⁷⁶.

[00207] 3) We will quantify the effect of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) on the expression of NGF, BDNF, PTN, and NTF-3 in mesenchymal progenitor cells (MPCs) isolated from male and female patients with musculoskeletal trauma. MPCs are present at the site of peripheral nerve injury and are extensively characterized¹⁴³. MPCs will be harvested during the course of normal and pre-planned surgical treatments using a standard isolation protocol developed by Dr. Leon Nesti^{116,143}. MPCs from 3 different subjects will be expanded in culture. First, we will passage cells four times without neuronal induction to establish a baseline for the expression of REST and NTFs transcript and protein levels by qRT-PCR and

Western Blot (WB). Next, we will repeat expansion with neuronal induction as described by Bulken-Hoover, *et al.*¹⁴⁴. Briefly, MPCs are plated in pre-induction media, for 2 days, then augmented with all-trans-retinoic acid (RA) for 1 day, followed by 7 days in the neuroinductive media. The neuroinductive media will be changed every third day. On day 7-post induction, REST and NTFs levels will be assessed. We expect that neuronal induction should result in decreased REST expression with a corresponding increase in expression of NTFs.

[00208] To assess the potency of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 159 through 143) induction of NTFs expression in MPCs, we will first need to establish dosing by assessing RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) cytotoxicity. Briefly, MPCs will be plated in a modified pre-induction media substituting RA with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) at 4 dose levels up to 10 μ M or pre-induction media (with RA) for 1-day. There will be three technical and three biological replicates for each dosing group and cytotoxicity will be determined using the MTT assay¹⁴⁵. To determine the optimal concentration of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) for decreasing levels of REST and increasing levels of NTFs, we will perform a dose-response (3 dose levels) in neuroinductive media with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) replacing RA at 1, 3, and 6-days post-treatment (dpt). RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and

140 through 159) doses will be based on the findings of the MTT assay¹⁴⁵. Our negative and positive controls will be neuroinductive media - or + RA, respectively. We will quantify the gene expression and protein levels of REST and NTFs with qRT-PCR and WB^{139,146}. Cells will be harvested at 1, 4, and 7-dpt and RNA and protein will be isolated from the cell lysates. A two-way ANOVA analysis, followed by a Tukey's post hoc test, will be performed to make comparisons between - and + controls and RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) dosing groups for indicated time points. Means of populations will be reported as mean $\pm$ SD, with a p-value of 0.05 or less considered statistically significant. Statistical analysis will be performed using the SAS statistical package (SAS Institute, Cary NC).

[00209] 4) RPP (SEQ ID NO: 12) was assessed at 0, 0.3, 1, 3, 10 μ M in an *ex vivo* culture of whole DRG neurons (L5, from male SD rats) for its potential to induce expression of BDNF and NGF (Fig. 16). RPP increased BDNF expression approximately 25% at 0.3, 1, and 3 μ M and 75% at 10 μ M. RPP increased NGF approximately 50% and 2-times at 3 and 10 μ M, respectively (Fig. 15). No affect was observed at 1 μ M, and 0.3 μ M was inconclusive due to an n=1 (Fig. 16) .

[00210] 5) RPP (SEQ ID NO: 12) was assessed in an LDH cytotoxicity assay conducted on *ex vivo* cultured whole DRG neurons (L5, from male SD rats). RPP showed no toxicity at 0, 1, 3, or 10 μ M (Fig. 17). As expected, the positive control (2% triton) was neurotoxic, as demonstrated by an increase of 90,000 RLU.

[00211] 6) We will assess the ability of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) to induce NTF expression and stimulate regeneration in both motor and sensory *ex vivo* nerve models through the respective use of spinal cord and isolated dorsal root ganglion

(DRG) explants¹⁴⁷. Both models are used extensively to study neuroprotective and trophic properties of growth factors¹⁴⁸. A 10 mm segment of intact DRG will be extracted (from 6 rats) from both sides of the spinal column (L4-L6) and cultured (Figure 21 & 22)^{149,150}. The remaining spinal column material will be preserved for motor-neuron linked spinal cord slice cultures. These spinal columns will be sectioned transversely at 300 μ m intervals with a microtome and the slices will be transferred to culture inserts with semipermeable membranes and allowed to acclimatize in culture conditions for a week^{149,150}. The culture inserts with week-old organotypic spinal cord slices, having a stable population of surviving motor neurons, will then be transferred to 6-well plate for an additional 7 days in culture.

[00212] Both *ex vivo* explant cultures will be maintained for 2 weeks in neurobasal/B27 medium to allow extensive elongation of neuronal processes¹⁴⁷. To induce a physical injury, a glass Pasteur pipette will be used to create a scratch through the elaborated neuronal processes 6 mm from the perimeter of the DRG ganglia and from the gray-white matter junction of the ventral spinal cord slice. The *ex vivo* cultures will be treated with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) (0, 1, 3, or 10 μ M) starting 24 hours post-injury.

[00213] The regenerative capacity of injured neurites (motor and sensory) will be determined by monitoring cell viability and measuring axonal outgrowth. Cell viability will be assessed 7-dpt by labeling live and dead cells respectively with calcein-AM and ethidium homodimer-1. Neurite extension, an indicator of neuron regeneration, will be measured at 1, 4, and 7-dpt. Gray-scaled micrographs of the scratch region will be acquired with a Zeiss AxioObserver microscope coupled to a monochrome digital camera¹⁵¹. The number, length, and total area of regenerated sprouts within the scratch region will be analyzed with ImageJ¹⁵².

[00214] NGF exerts much of its functional activity through its receptor, the TrkA receptor in the DRG¹⁵³, while PTN has been found to cause increased axonal outgrowth primarily in motor neurons^{150,154}. BDNF and NTF-3 are present in both motor and sensory neurons. We will quantify the gene expression and protein levels of NTFs with qRT-PCR and WB^{139,146}. Tissue from each *ex vivo* system harvested at 1, 4, and 7-dpi and RNA and protein lysates isolated. A two-way ANOVA analysis, followed by a Tukey's post hoc test, will be performed to make comparisons between control and RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) dosing groups for indicated time points. Means of populations will be reported as mean $\pm$ SD, with a p-value of 0.05 or less considered statistically significant. Statistical analysis will be performed using SAS software (SAS Institute, Cary NC).

[00215] 7) RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will be assessed for the ability to improve regeneration and functional recovery after sciatic nerve defect *in vivo* in 100 rats. We have demonstrated that RPP accumulates in the nuclei of the sciatic nerve neurons in the spinal cord 48 hours after RPP was injected at the sciatic nerve injury site (Fig. 4). RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will be administered intravenously (IV), which will allow us to test a higher maximum feasible dose (MFD) as compared to intramuscular (IM) or subcutaneous (SC) administration. Administration by IV also avoids first-pass metabolism associated with oral and intraperitoneal (IP) administration. Dosing concentrations for the regeneration and functional recovery study will be based on the findings of a one-month single IV dose range-finding toxicity and toxicokinetic study in Sprague-Dawley rats. The dose

range-finding toxicity study will be initiated upon completion after completion of 1). Briefly, the study will use four dosing levels up to 1000 mg/kg or max tolerated dose or MFD, as recommended by FDA guidance (M3(R2), 2009). The number of animals allocated per group will be as follows: Main Study: 10/sex/group; TK: 3/sex/control group and 9/sex/dosing group. Animals will be assessed for body weight, food consumption, clinical chemistry, hematology, urinalysis, organ weights, histopathology (8 core tissues), and toxicokinetics.

[00216] In the regeneration and functional recovery study, Sprague-Dawley rats will be administered RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) weekly, by IV using a long-term venous catheter¹⁵⁵, for 6-weeks in 3 dosing groups (TBD) + control (10/sex/group). All animals will be anesthetized, prior to exposing the sciatic nerve¹⁵⁶ and removing a 0.7 cm section to create an approximately 1 cm segmental defect following retraction of the nerve stumps¹⁵⁷. The nerve will be repaired using the decellularized nerve graft. The surgical incision will then be closed, and the nerve will be allowed to regenerate for 6 weeks. Only the dosing groups will be given RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159). During the 6-week period, we will evaluate functional recovery and tolerance to RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) dosing. Each week the rats will be weighed, temperature recorded, and toe-spreading reflex assay (to determine the maximum footprint width of the injured leg testing motor nerve function) will be performed¹⁵⁶. Walking track analysis (Digigait) will be performed every 2 weeks, and electrophysiological studies¹⁵⁸ will be conducted. Briefly, electrophysiology testing will be performed on the rat sciatic nerve. Electrical stimuli are applied using single-pulse shocks (1mA,

0.1ms) to the native sciatic nerve trunk at the point 5 mm proximal to the graft suturing point. Compound muscle action potentials (CMAPs) are recorded on the gastrocnemius belly from 1V to 12V or until a supramaximal CMAP is reached. Normal CMAPs from the un-operated contralateral side of sciatic nerve are also recorded for comparison. The recovery rate will be determined by the ratio of injured hindlimb's CMAP to contralateral normal hindlimb's CMAP^{159,160}.

[00217] At the end of week 6, the sciatic nerve as well as the ventral horn (VH) of the spinal cord and DRG associated with the L4 to L6 nerve roots will be collected^{161,162}. Nerve regeneration will be evaluated histologically. The proximal and distal ends and the center of the grafts will be prepared for transmission electron microscopy (TEM) through the cross section to evaluate myelination along the graft^{163,164}. Briefly, ultrathin sections of ~70 nm will be cut from the nerve using an ultramicrotome, then stained with uranyl acetate and lead citrate. 10-15 fields from random sections will be selected for analysis and the number, and the size of myelinated fibers will be quantified. Mean fiber density will be calculated as previously described⁷³. In addition to TEM, more sections from the same nerve will be prepared for IHC to visualize β -tubulin III in order to measure the direct growth of the axons, S100 to measure the penetration and growth of Schwann cells into the graft, von Willebrand factor to assess for angiogenesis and capillary infiltration of the healing nerve, and Luxol blue staining^{165,166} to measure myelin formation within the graft. The ventral horns and DRG will be assessed for RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) associated changes within the cell bodies of the motor and sensory nerves that were injured. Images of the stained tissues will be taken with a light microscope and quantification of intensity and area fraction of the positive reactions in anatomically matched tissues will be performed using the ImageJ software (<https://imagej.nih.gov/ij/>), to compare nerve regeneration rates between the

groups¹⁶⁵. DRG neurons of the injured nerve will be counted and compared to the contralateral uninjured DRG as previously described¹⁶⁷. NTFs (BDNF, NGF, PTN, and NTF-3), CTDSF1 and REST will be examined via qPCR and WB.

[00218] PREDICTED RESULT & ALTERNATIVE STRATEGY. We expect that mesenchymal progenitor cells (MPCs) treated with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) + neuroinductive media (- retinoic acid, RA) will increase the expression of neurotrophic factors (NTFs) and decrease the levels of REST, compared to neuroinductive media - RA, but similar to neuroinductive media + RA. We expect that the *ex vivo* models treated with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will show an increase in neurite outgrowth in comparison to the no treatment group due to decreased REST levels. It is possible that the regenerative effects of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) may not be pronounced in the absence of support cells, which can secrete neurotrophic factors *in vivo* following neuronal injury. An alternative experiment will include the co-culture of neuro-supportive cells (e.g., Schwann cells and mesenchymal stem cells) with the dorsal root ganglion (DRG) and spinal cord explants, enriched with motor and sensory neurons, prior to injury and the subsequent RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) treatment. Successively we will analyze the ability of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) to help the regeneration of the severed nerve in a rat model. We expect that the rats

treated with RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will have improved recovery as compared to control rats as determined by histological, electrophysiological, and functional assessments. We also expect RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) to increase NTFs expression and decrease REST protein levels. If we do not observe signs of recovery and removal of the REST blockade, we will increase the local concentration of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) by direct injection to the target site (i.e., graft site or intrathecal).

[00219] Materials and Methods

[00220] Cell Culture. MPC harvesting, culturing, and neurotrophic induction have been previously described¹⁶⁸. Briefly, MPCs are plated in pre-induction media, for 2 days, then augmented with all-trans-retinoic acid (RA) for 1 day, followed by 7 days in the neuroinductive media. The neuroinductive media will be changed every third day. On day 7-post induction, REST and NTFs levels will be assessed.

[00221] qRT-PCR. Methods described in Example 10.

[00222] Western Blot Analysis. Whole-cell lysates were prepared following the procedures in Ballas et. al., 2001¹¹⁹. Western blots will be performed by standard procedures using anti-REST-C⁶⁴, anti-NTFs (using commercially available antibodies), anti-GAPDH (abcam [6C5]), and anti-IgG conjugated to infrared dyes (Thermo Fisher), and analyzed on an Odyssey infrared fluorescence imager (LiCor).

[00223] Test Article Identification. RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), Purity. $\geq 95\%$

[00224] Preparation of Dose Formulation. In bulk every 4 days.

[00225] Dose Formulation Assay and Stability. Dose formulations will be assessed for stability and concentration on Day 1 and 7 in the first week of the study. The acceptable concentration range is $\pm 10\%$ of nominal.

[00226] Vertebrate Animals. Male and female adult Sprague-Dawley rats, (approximately 250-300 g males, 150-200 g females) will be used to determine the efficacy of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) in improving recovery from PNI. The studies will require 100 rats, 8 -11 weeks old. Rats will be obtained from Charles River Laboratories.

[00227] Description of Procedures:

[00228] Surgery: Rats will be anesthetized with isoflurane (4% induction, 3% maintenance) prior to all surgical procedures. In 2) organotypic spinal cord cultures will be prepared from the lumbar spinal cord of postnatal-day-8 rats (Sprague Dawley) using techniques as described previously (Rothstein et al., 1993; Corse et al., 1999). Briefly, rats will be quickly sacrificed, and the lumbosacral spinal cord will be removed and placed in Gey's balanced salt solution (Gibco) containing glucose (6.4 mg/L). In a laminar flow hood using sterile technique, the meninges will be carefully removed under magnification, and the lumbar spinal nerve roots will be transected. The cord will be placed on Aclar film and sectioned at 300 μm intervals from L2 to L5 with a McIlwain tissue chopper. Individual cord sections will be carefully transferred, using the Gey's balanced salt/glucose solution, to Millicell-CM (Millipore) permeable membranes in 6-well

culture plates. Five cord sections will be placed on each membrane. Each well contained 1 mL of medium consisting of 50% minimal essential medium plus 25 mM Hepes; 25% Hanks balanced salt solution with D-glucose (25.6 mg/L); 25% heat-inactivated horse serum; and 2 mM L-glutamine. Cultures will be maintained at 37°C under 5% CO₂ in a humidified incubator for a week with medium being changed every 3 days. After a week, the slices will be transferred to another 6-well culture plates and allowed to stabilize and extend neurites for another 7 days in culture prior to any treatments.

[00229] In 3) we will expose the sciatic nerve¹¹⁷ (Figure 21 & 22) and remove a 0.7 cm section to create an approximately 1 cm segmental defect following retraction of the nerve stumps (Hems and Glasby, 1993). The nerve will be repaired using the decellularized nerve graft. The surgical incision will then be closed, and the nerve will be allowed to regenerate for 6 weeks. Group one (control) will undergo approximately 1 cm sciatic nerve resection and will be grafted with a 1 cm decellularized allograft nerve. Group two through five will undergo a 1 cm sciatic nerve resection, grafted with a 1 cm decellularized allograft and given weekly IV injections of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) at drug concentrations to be determined from the dose range-finding study. All animals will be sacrificed at week 6.

[00230] Behavioral Assessments: We will monitor the animals for up to 6 weeks to evaluate functional recovery and tolerance to peptide treatment. Each week the rats will be weighed, temperature recorded and toe-spreading reflex assay (to determine the maximum footprint width of the injured leg testing motor nerve function) will be performed¹¹⁷. Walking track analysis will be performed every 2 weeks. and Electrophysiological studies will be conducted prior to sacrifice at 6 weeks.

[00231] Electrophysiology Assessments: Electrophysiology testing will be performed following previous methods¹⁵⁸. In brief, the rat sciatic nerve is re-exposed and electrical stimuli (single-pulse shocks, 1 mA, 0.1 ms) are applied to the native sciatic nerve trunk at the point 5 mm proximal to the graft suturing point. CMAPs are recorded on the gastrocnemius belly from 1V to 12V or until a supramaximal CMAP is reached. Normal CMAPs from the un-operated contralateral side of sciatic nerve are also recorded for comparison. Grass Tech S88X Stimulator (Astro-Med Inc.) is used for the test and PolyVIWE16 data acquisition software (Astro-Med, Inc.) is used for recording. Recovery rate is the ratio of injured hindlimb's CMAP to contralateral normal hindlimb's CMAP of a rat¹⁵⁸.

[00232] Sacrifice: Rats will be sacrificed at 6 weeks post injury. After sacrifice, we will use histology to evaluate axon growth through the graft, remyelination, MPC cell activity at the site of injury, anterior horn cell and DRG activity and vascularization of the regenerating nerve. We will harvest the gastrocnemius and tibialis anterior muscle of each rat after sacrifice to assess gross weight to quantify atrophy.

[00233] Justification: After PNI, there is a complex interplay between cells and molecules in the vasculature, the immune system and all of the cells endogenous to the peripheral nervous system. We do not completely understand the sequence nor the interactions between the different cells, signaling molecules, cell – cell interactions and circuitry that occurs after traumatic injury, so it is impossible to model it *in vitro* at the current time. Additionally, the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) that is the subject of investigation in this proposal may impact neural precursor cells, adult neurons as well as other cell types, and so it is critical to evaluate it *in vivo* in the context of PNI. The FDA also requires *in vivo* proof of efficacy before

allowing IND status for a biologic, so this provides further justification for the *in vivo* nature of these experiments. Rats are used because of a significant body of behavioral research after PNI has indicated their reliability in these types of experiments. Thus, there is an extensive literature that provides a detailed framework for design and execution of experiments. Rats are more intelligent than mice, and are larger, enabling a greater ability to discern changes in functional recovery after injury. Rat physiology has more similarities to humans than the mouse, allowing for better translational relevance of experimental data. Further, the REST pathway we are targeting is conserved in Rats.

[00234] Minimization of Pain and Distress: Rats will be anesthetized with isoflurane (3-4%) for the segmental peripheral nerve defect. Following surgery animals will be allowed to recover on a heat pad until mobile. Rats will be administered acetaminophen (6 mg/ml in drinking water) for 2 days after the surgery, and additionally if signs of pain or distress are observed. Rats will be observed daily by both laboratory and veterinary staff, and animals deemed to be in distress will be treated or euthanized according to veterinary recommendations.

[00235] Euthanasia: The euthanasia used is consistent with the recommendations of the American Veterinary Medical Association Guidelines for the euthanasia of animals.

Example 14 - RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) **can be used for the prevention and treatment of chronic pain.**

Completed assessments:

The activation of REST after nerve injury results in decreased expression of several genes required for normal excitability of sensory neurons, including the potassium channels K_v4.3 (Kcnd3) and

Kv7.2 (Kcnq2), the sodium channel Nav1.8 (Scn10a), and the mu opioid receptor Oprm1^{60,72-74}. The basis for blocking REST to alleviate chronic pain are published studies using mouse and rat peripheral nerve injury (PNI) models^{60,72-74}.

[00236] 1) Real-time quantitative reverse transcription PCR (qRT-PCR) analysis showed that NBFL cells dosed with 1 μ M linear (SEQ ID NO: 4) or cyclic (SEQ ID NO: 2) RPP for 16 or 48 hours, evoke an increase in Kv4.3 mRNA expression (Fig.9, C and Table 16 below).

Table 16 Kv4.3 response to 1 μ M RPP in Figure 9		
RPP	Increase	
	16 h	48 h
Linear	5x	5x
Cyclic	NA	25x
NA= Not assessed		

[00237] 4) RPP (SEQ ID NO: 12; 0 (water), 1, 3, or 10 μ M) was incubated for 48 hours in an *ex vivo* culture of whole DRGs (L5, from male SD rats) and assessed for its potential to induce expression of chronic pain associated genes Kv4.3, Kv7.2, Nav1.8, and OPRM1 (Fig. 14). RPP increased Kv4.3 expression 2-times at 10 μ M, compared to control; increased Kv7.2 expression 7.5- and 9-times at 3 and 10 μ M, respectively; increased Nav1.8 expression 2.9- and 4.8-times at 3 and 10 μ M, respectively; increased OPRM1 expression at 3 and 10 μ M respectively (Fig. 14). RPP sequence ID numbers 13 and 14 were assessed at 3 μ M after a 48 hour dosing period in an *ex vivo* culture of whole DRGs (L5, from male SD rats) and assessed for Nav1.8. Sequence 13 and 14 increased Nav1.8 expression 6- and 13-times, respectively, compared to control (Fig. 15).

[00238] 5) RPP (SEQ ID NO:12) was not neurotoxic in a LDH assay conducted on *ex vivo* cultured whole DRGs neurons (L5, from male SD rats). RPP did not increase RLU levels

compared to control. As expected the positive control (2% triton) increased RLU 90,000-times (Fig. 17).

Planned assessments:

AIM 1: DETERMINE THE PHARMACOKINETICS, DISTRIBUTION, AND DOSING OF RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159)

[00239] SUBAIM 1.1 Development and validation of liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for detecting RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), (in a range between 10 and 10,000 ng/mL) in rat and monkey blood plasma, CSF, and tissue.

[00240] SUBAIM 1.2. To determine the route of administration in the *in vivo* animal studies and the clinic, an exploratory pharmacokinetics (PK) study in SD rats will be conducted. Blood plasma samples will be collected after IV, SC, or PO administration of 1000 mg/kg RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) at 5 min, 30 min, 4 h, 8 h, 24 h, and 48 h (2/sex/time point) and assessed for peak drug concentration (C_{max}), area under the curve (AUC), and half-life ($t_{1/2}$). To assess RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), penetration to the central and peripheral nervous system, target tissues (CSF, brain, lumbar spinal cord and dorsal root ganglia (DRG)) will also be collected at euthanasia and measured for drug concentrations. RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), will be detected

using validated LS-MS/MS methods (SUBAIM 1.1)

[00241] SUBAIM 1.3. To determine dosing in the rat efficacy study (Aim 2), a single dose range-finding study in SD rat using the optimal route of administration identified in SUBAIM 1.1 will be conducted. Four dose levels + vehicle control (3/sex/group) will be selected, based on the PK data from SUBAIM 1.1. The high dose (HD) should identify the limit dose (maximum feasible dose (MFD)), maximum tolerated dose (MTD), and/or saturation of exposure). The lower doses will be spaced in 1/3 increments. Animals will be assessed daily for survival and clinical signs (abnormalities and signs of pain or distress). Blood will be collected post dose at 6 time points selected based on the PK data from SUBAIM 1.1 and assessed for C_{max} , AUC, and $t_{1/2}$. Tissues that will be assessed for gene changes in the efficacy study (DRGs, lumbar spinal cord, brain) will be collected at euthanasia and assessed for RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), concentration.

[00242] AIM 2: ASSESSING THE EFFECT OF RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) ON CHRONIC PAIN.

[00243] We have demonstrated that RPP accumulates in the nuclei of rat motoneurons in the lumbar (L4 – L6) spinal cord 48 hours after administration at the site of sciatic nerve transection (Fig.4). To assess the effects of our drug on pain, we will use the preclinical SNI model using the sciatic nerve. SNI is a well-established animal model for neuropathic pain that produces robust and prolonged changes in thermal sensitivities, peripheral and central morphine analgesia, and c-fiber hypoesthesia⁷⁴. Furthermore, SNI has previously been shown by us and others to induce expression of REST and its target genes implicated in chronic pain (Fig.18 and Table 12,

respectively)^{60,72-74}. Additionally, we observed increased CTDSP1 levels, as we predicted (Fig.18).

[00244] Efficacy of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) to decrease chronic pain will be done in Phase 1 and durability of the pain reduction will be determined in Phase 2. The SNI sciatic nerve rat model will be used to test if RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), decreases chronic neuropathic pain. Administration of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), or vehicle or a standard of care drug used to treat neuropathic pain, oxycodone will be done. The experimental procedure for phase 1 is summarized in Table 17 below:

Table 17

SNI and Sham Surgery Groups		Rat #	Euthanasia Day Post-Start of rpp dosing	Assessment
1	rpp LD	12 SNI	14	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
		12 SNI	28	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
2	rpp MD	12 SNI	14	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
		12 SNI	28	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
3	rpp HD	12 SNI	14	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
		12 SNI	28	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
4	Oxycodone	12 SNI	14	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
		12 SNI	28	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
5	Vehicle	12 SNI	14	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
		12 SNI	28	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		

[00245] A total of 312 2-month old SD rats (equal numbers of males and females) will be needed for this aim. A sample size of 12 rats per group will have 80% power to detect a difference of 1.20 standard deviations in pain assessments. Power analysis is based on a t-test for independent samples with a 5%, 2-sided significance level. For the efficacy study, the rats will be randomly assigned to the experimental groups (n = 12/group) designated into the table on the right. Rats from each of the ten randomized groups will be examined with stimulus evoked and non-stimulus evoked behavior tests, morphological, immuno-histochemical analysis, quantitative RT-PCR (qRT-PCR) analysis, and WB analysis. At euthanasia, blood will be drawn at 2 time points (14 and 28 days) post-start of drug delivery and assessed for standard PK parameters. The DRG and brain will be extracted to determine Oprm1, Nav1.8, Kv4.3, and Kv7.2 levels. In addition, tissue will be collected for LC-MS/MS analysis to determine rpp concentration.

[00246] *Surgery:* SNI or sham surgery (surgery which exposes the nerve branches without nerve injury) will be performed as described previously¹⁶⁹. Briefly, the rats will be anesthetized and the common peroneal and tibial nerves ligated. A 2-4 mm segment of each nerve distal to the ligation will be cut and removed, leaving the third branch of the sciatic nerve, the sural nerve intact. The sural nerve is spared to produce an increased response to noxious and non-noxious stimuli in the ipsilateral innervated region beginning at 4-days post-injury, stabilizing at 7 days and maintained up to 6 months. 7-days post injury is the accepted time point for development of chronic neuropathic pain and is translationally relevant.

[00247] *Drug/Vehicle:* Administration will begin on day 5 post-surgery. For RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), the route, frequency, and drug concentrations to be tested will be based on the PK studies in Specific Aim 1. For oxycodone, the route of administration and efficacious drug exposure in the rat that approximates efficacious exposure in the humans is known: (oxycodone at a dose of 0.56 mg/kg and 0.2 mL vehicle)¹⁷⁰.

[00248] *Behavior Tests:* The effects on pain transmission and motor function will be assessed one day prior to surgery and every other day beginning on day 4 post-surgery. Both stimulus evoked and non-stimulus evoked behavior tests will be used to determine the functional effectiveness of the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) compared to oxycodone and vehicle. In recent years concerns over the translatability of some stimulus evoked behavior tests has led to the increasing use of stimulus evoked, non-stimulus evoked, and operant or voluntary behavior tests. For this proposal we will use the most widely used and translatable stimulus evoked behavior tests: the electronic Von Frey and heat hyperalgesia tests, a non-stimulus

evoked gait analysis, and a place escape/avoidance paradigm to measure the aversive state of pain¹⁷¹. Recent studies have indicated the value in including gait analysis to detect subtle improvements and deteriorations in animal models¹⁷². The rat handlers and assessors performing the behaviors tests will be blinded to which experimental/control group the rats were randomly assigned. Each animal will have a unique animal number. A secret color code will be used to designate control and experimental groups. Only after analysis will this code be revealed.

[00249] *Stimulus Evoked Behavior Tests: Hypersensitivity to mechanical stimulation:*

An electronic von Frey (Bioseb, Chaville, France) consisting of a hand-held force transducer fitted with a plastic tip will be used to test the mechanical withdrawal threshold of the left and right hind paws. The plastic tip of the force transducer will be applied to the medial plantar surface of the paw for testing the saphenous nerve and to the lateral plantar surface of the paw to test the sciatic (peroneal nerve).

[00250] Heat hyperalgesia will be measured based on a published method that we modified to use a laser as the heat source¹⁷³. An 808 nm wavelength laser will be used as the heat source (2W output power, the beam diameter on the skin = 3 mm) and will be applied to the relevant dermatome area for the nerve being tested. The small beam size allows the involved dermatomes to be specifically targeted. For the sciatic nerve and saphenous nerves, limb withdrawal latency induced by thermal stimulation will be recorded with a cutoff point of 10 seconds.

[00251] *Non-Stimulus Evoked Behavior Test: Gait Analysis:* Sciatic nerve motor function will be assessed using the Digigait and Sciatic Nerve Index¹⁷². Briefly, utilizing the Digigait equipment available in the Rodent Behavioral Core, which has been characterized and optimized by our laboratory, sciatic nerve function will be determined pre-injury and on post-

surgical days 7, 14 and 28. Rats will be placed on the motorized treadmill within the Digigait holder and a speed of 20cm/sec set for recording of all animals. The Sciatic Nerve Index program will be used to analyze the function of the affected hind limb.

[00252] Place Escape/Avoidance: This procedure is designed to test the hypothesis that rats will avoid the environment that is associated with mechanical stimulation to the hyperalgesic area. It has been reported that a sensitivity to change in escape/avoidance behavior occurs in injured rats after treatment with analgesics (Baastrup et al 2010)¹⁷⁴. The cerebral-dependent place escape/avoidance paradigm test has been described by LaBuda and Fuchs (2000)¹⁷¹ and depends on escape/avoidance learning to a novel aversive environment. The animals will be tested only once 3 to 5 weeks post-injury by the same trained investigator. Testing will be done in the USU Rat Behavior Core. The animals will be allowed to habituate to ambient light and noise conditions in the test room for a minimum of 1 hour. For this test, the rats will have free access between the ‘non-aversive’ dark and ‘aversive’ light side of an enclosed chamber with a mesh floor which is readily accessible by a von Frey filament from below. Either the injured or non-injured hind paw will be routinely stimulated if the rat is in the dark or light area, respectively. A mechanical stimulus (von Frey filament) that is adequate to elicit a withdrawal response on the injured paw will be applied every 15 sec to the lateral plantar surface of one of the hind paws of the animal, depending on the location of the animal during that time, for a period of 30 min. Escape/avoidance behavior will be defined as a shift from the dark to the light area. The percentage of time spent in the white side of the box and the number of crossings between the black and white sides will be recorded. The cumulated time in the white side and the total number of crossings will be used as an indication of escape/avoidance learning.

[00253] *Euthanasia*: For morphological and immunohistochemical analysis, the rats

will be anesthetized with ketamine/xylazine (80 to 100 mg/kg + 10 mg/kg, i.p., 21 gauge needle) and perfused transcardially with 300 ml phosphate buffered saline (pH 7.4) followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, nerves, DRG and spinal cord will be dissected, post-fixed for 24 hours in 4% paraformaldehyde and cryoprotected for 24 hours in 30% sucrose. The tissue samples will be cut into 10 μ m sections using a cryostat (Leica CM3050 S, Leica Biosystems, Wetzlar, Germany).

[00254] *Histology:* After euthanasia and nerve sampling, sections will be stained with Hematoxylin and Eosin. The expression levels and distribution of the REST-target genes Oprm1, Nav1.8, Kv4.3, and Kv7.2 will be assessed by immunofluorescence.

[00255] *Gene Expression Analysis:* For gene expression analysis of the sciatic nerve and its associated DRG and spinal cord segments, REST-target genes Oprm1, Nav1.8, Kv4.3, and Kv7.2 and control genes (Hprt, Gapd, Rn18s) will be assessed by qRT-PCR.

[00256] *Protein Expression Analysis:* Protein expression analysis of the sciatic nerve associated DRGs, will be assessed by SDS-PAGE immunoblotting of Oprm1, Nav1.8, Kv4.3, and Kv7.2. GAPDH will be used as loading control.

[00257] *Statistical Approach:* For behavior data, results will be presented as mean $\pm$ standard error of the mean (SEM). A 2 factor ANOVA will be used to assess groups of animals based on time post-treatment. The Bonferroni-Holm method will be used to adjust for multiple comparisons. Two-tailed statistical significance will be established as $P < 0.05$. For immunohistochemical analysis, Two-way ANOVA will be used to compare means for interaction effects as well as effect of group and time. For qRT-PCR, values will be reported as mean $\pm$ standard error of the mean (SEM). Statistically significant drug effects on the gene expression will be determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

[00258] The experimental procedure for Phase 2 is outlined in Table 18 below:

Table 18

SNI and Sham Surgery Groups		Rat #	Euthanasia Day Post-Start of rpp dosing	Assessment
1	rpp (TBD)	12 SNI	14	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
		12 SNI	28	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
4	Oxycodone	12 SNI	14	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
		12 SNI	28	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
5	Vehicle	12 SNI	14	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		
		12 SNI	28	All rats behavior testing 8 rats morphological analysis (4 SNI/ 4 sham) 16 rats qPCR analysis (4 SNI/ 4 sham)
		12 Sham		

[00259] In Phase 2, the durability of the pain reduction of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will be examined. For this study, the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) dose (low, medium, or high) and the period of drug delivery (14 or 28 days) that was determined in Phase 1 to be most efficacious will be used. The same methodology described above for the Phase 1 efficacy study will be followed. The difference being that after the drug administration period (14 or 28 days) the rats will be monitored and behaviorally assessed for pain response and motor function every other day for 30 days. The rats will be euthanized on day 30. A total of 72 2-month old, SD rats (equal numbers of males and females) will be needed for this study. A sample size of 12 rats per group will have 80% power to detect a difference of 1.20 standard deviations in pain assessments. Power analysis is based on a t-test for independent samples with a 5%, 2-sided significance level. The rats will be randomly assigned to

the experimental groups designated in the table on the right. Rats from each of the ten randomized groups will be examined with stimulus evoked and non-stimulus evoked behavior tests, morphological, immuno-histochemical analysis, quantitative RT-PCR (qRT-PCR) analysis, and WB analysis.

[00260] Aim 3. TOXICOLOGY AND SAFETY PHARMACOLOGY ASSESSMENTS OF RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159).

To support first-in-human dosing, we will assess *in vitro* cardiac and genetic toxicity and *in vivo* toxicology, safety pharmacology, and toxicokinetics of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) in two species, as recommended by FDA guidances M3(R2), S2B, S6(R1), S7A, and S7B¹⁷⁵⁻¹⁷⁹. In both species, the route of administration will be selected based on the results of the PK assessment in AIM 1.

[00261] SUBAIM 3.1. Single dose range-finding study in monkey: Four dose levels + vehicle control will be assessed in 3 monkeys/sex/group. The HD should identify the MTD, MFD, and/or saturation of exposure. The lower doses will be spaced in 1/3 increments. Blood will be collected at 6 time points (TBD base on PK data collected in Aim 1) and assessed for C_{max}, AUC, and t_{1/2}. There will be daily assessments for survival and clinical signs.

[00262] SUBAIMS 3.2 & 3.3. Toxicology studies in rat (3.2) and monkey (3.3). The dosing period in both species will be 4 weeks, to support dosing in humans of the same duration + a 6-week recovery period, selected because of the high stability of rpp observed in the cellular assays⁷⁷. There will be three dose levels + vehicle control. The high doses will be the

maximum tolerated doses identified in the dose range-finding studies (rat AIM 1.2 and monkey Aim 3.1). The MD will be 1/3 lower than the HD, based on AUC in the range-finding studies. The LD will approximate the efficacious dose in the *in vivo* efficacy study (Aim 2). The dosing interval will be the $t_{1/2}$ of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) in each species. In the rat study, there will be 15/sex/group. 10/sex/group will be sacrificed at the end of the Dosing Period and 5/sex/vehicle will be sacrificed at the end of the Recovery Period. Additionally, 9/sex/rpp groups and 3/sex/vehicle group will be assigned to the toxicokinetic portion of the study. In the monkey study, all animals will be assessed for toxicology and TK (3/sex/group in the Main Study and 2/sex/group in the Recovery Period). The following toxicological parameters will be assessed in both species: clinical observations, body weight, food consumption, ophthalmoscopy (fundoscopy and slit lamp examinations), and a standard battery of clinical chemistry, organ weights, and histological parameters will be assessed. Monkeys will undergo assessments of respiratory (e.g. tidal volume and hemoglobin oxygen saturation) and cardiac electrocardiogram recordings measured using non-surgical telemetry functions¹⁷⁹, as recommended by FDA guidance S7A¹⁷⁹. The cardiovascular assessment will occur, twice during the pre-treatment, after the first dose, and at the end of the Recovery Period (if needed). The recordings will be evaluated qualitatively by a consultant board certified cardiologist. All waveforms will be qualitatively evaluated to detect rhythm or conduction disturbances, including evaluations of PR and QRS intervals. Rats will be assessed for CNS functions using the modified Irwin's functional observational battery^{179,180}. TK assessments (C_{max} , AUC, and $t_{1/2}$) from blood plasma will be made at 6 time points (determined by the rang-finding studies).

[00263] SUBAIM 3.4. *In vitro* hERG assay¹⁷⁷. Inhibition of hERG channels is a

common cause of long QT syndrome and is also correlated with arrhythmias that can lead to ventricular fibrillation and sudden cardiac death. To test whether RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) inhibits hERG channel current, we will contract an electrophysiological evaluation on hERG channel current in CHO cells treated with a high concentration of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) (10 μ M) or positive control (cisapride, 0.03 μ M). Each cell will serve as its own positive control. Whole cell recordings will be conducted using conventional voltage clamp techniques.

[00264] SUBAIM 3.5. Genotoxicity. RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will be assessed in *in vitro* OECD compliant Ames and *in vitro* chromosomal aberration assays (S2B, November 1997)¹⁷⁶ to determine its genotoxic potential.

[00265] AIM 4. RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) ABUSE POTENTIAL ASSESSMENT. Because opioid receptor gene expression is inhibited by REST, it is possible that RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) could augment the effects of endogenous opioid receptor ligands such as endorphins^{181,182}. Additionally, recent studies do show an increase in expression of REST in the DRG and the periaqueductal gray area (PAG) during conditions involving pain^{183,184}. To determine if RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) produces reward-seeking behavior we will

evaluate its effects on rats using conditioned place preference (CPP), a widely used behavioral assay to test the rewarding properties of drugs¹⁸⁵⁻¹⁸⁹. CPP is a well-established method that has demonstrated morphine reward-seeking behavior in an SNI animal model.

[00266] First, RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will be administered (route TBD) to uninjured rats and then CPP will be performed. Oxycodone will be used in place of morphine as a positive control for consistency with Aim 2 and saline will be injected into separate rats as a negative control.

[00267] The apparatus consists of a three-compartment chamber: the two outer compartments are designed to have different characteristics (*e.g.* white vs. black walls), and the central compartment has no special characteristics. Each experiment consists of three phases. Phase I Pre-test (Day 1): After administration of saline, rats will be placed in the center compartment with gates open to provide access to both the other compartments. Rats will be monitored for 15 min to determine baseline preference by recording the time spent in each chamber. The mean spontaneous preference times for the compartments will be determined. Rats that spend more than 60% of the test time in any one chamber will be considered biased and will be removed from the experiment. Phase II Conditioning (Days 2–7): Rats will be administered vehicle or RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), and then they will be randomly assigned/conditioned to receive RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) in one compartment and vehicle in the other. The dose of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and

140 through 159) used will be based on the concentration that produces measurable effects on mechanical and thermal hyperalgesia determined in AIM 2. Treatment compartment and the order of drug and vehicle administration will be counterbalanced for all groups to avoid biases. This phase consists of six consecutive conditioning sessions 30 min in length. Conditioning will also be done with oxycodone for a separate group. Phase III Test (Day 8): rats will be injected with saline and placed in the center of the CPP box and monitored for 15 min. The difference between the time spent in the drug-paired compartment during post-conditioning and that spent during pre-conditioning will be used to assess the degree of place conditioning elicited by RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159)¹⁹⁰.

[00268] RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will be assessed using 8-groups of 12 injured rats (SNI vs. SHAM, conditioning with oxycodone, RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159), and saline). A sample size of 12 rats per group will have 80% power to detect a difference of 1.20 standard deviations in pain assessments. Power analysis is based on a t-test for independent samples with a 5%, 2-sided significance level. The rats will be randomly assigned to the experimental groups. Rats will be assigned to undergo SNI or SHAM surgery randomly. The von Frey test will be performed one day before and 4 and 8-days post-injury to monitor the pain state. At 8-days post-injury the CPP test will be conducted as previously described. The research associate performing the behavior tests will be blinded to which experimental group/control group the rat was randomly assigned. Each animal will have a unique animal number. A secret color code will be used to designate control and

experimental groups. Only after analysis will this code be revealed. Animals will be euthanized, and samples analyzed as described in AIM 2.

[00269] PREDICTED RESULTS & ALTERNATIVE STRATEGIES. We predict that the RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) will reduce REST levels in injured neurons and restore proper expression of ion channels required for excitability. At the behavioral level, RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) should return the stimulus or non-stimulus evoked behavior tests to baseline in SNI rats. Furthermore, given its mode of action, we do not expect RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) to have addictive properties. However, if drug administration shows abuse potential, we will confirm this positive result with a progressive ratio (PR) schedule of reinforcement during RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) administration.

Example 15 - RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) can be used to prevent relapse of glioblastoma multiforme (GBM)

[00270] Assess the neurogenic and anti-oncogenic potency of RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) on brain tumor initiating cells (BTICs) and *in vivo*. Test article (RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through

117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) activity will be assessed in primary cultured BTICs derived from 10 grade IV GBM patients obtained at the Mayo Clinic using methods we have established¹⁹¹⁻¹⁹³. Cells (0.25×10^4) will be plated (3.8 cm² TC dish) in media (250 μ L) dosed with 5 μ L vehicle or with 3 dose levels of test article at the optimal dose range (determined in the neuronal differentiation study described in Example 13) for 0, 2, 4, or 8 days (Main Study) and then allowed to recover after wash out of test article for 8 days (Recovery Period). Markers for pluripotency (Sox2 and Nestin), proliferation (Ki67), cell death (caspase 3), and differentiation (MAP2, neuron and GFAP, glia) will be monitored on Days 0, 2, 4, and 8 during the dosing period (Main Study) and on Days 10, 13, and 16 during the Recovery Period (3/TC wells/group for each day) by WB and RT-PCR.

[00271] Primary cultured BTICs derived from 10 grade IV GBM patients will be used in an intracranial xenograph tumorigenicity nude mouse model as described previously by us and others^{191,192,194}. Briefly, cells (5×10^4) will be suspended in 5 μ L PBS (control) or PBS containing one of 3 TBD (based on BTIC neurogenic potency assay above) test article dose levels and delivered using an automated syringe pump with a guide-screw system^{112,195}. The injection coordinates will be X:1.5; Y:1.34; Z: -3.5 targeting the mouse striatum; a highly reliable site for tumor engraftment¹⁹⁶. Mice will be assayed for survival during weeks 4, 8, 16, and 20 using the Kaplan-Meier method¹⁹⁷ followed by postmortem histopathology to evaluate tumor size and invasiveness (H&E stain), cell death (TUNEL), and proliferation (anti-human nuclei) (3/sex/group for all procedures).

[00272] Statistical approach: Values will be reported as mean $\pm$ standard error of the mean (SEM) or standard deviation (SD) as indicated. Statistical significance will be determined by applying appropriate parametric or non-parametric variations of the student's t test or one-way

analysis of variance (ANOVA). Briefly, the ratio of total REST to phosphorylated REST at different control and drug candidate treatments will be statistically tested using the Kruskal-Wallis test. Differences in marker expression between BTICs control and drug candidate cultures and orthotopic xenographs will be statistically tested using the Tukey multiple comparison test. Differences in tumor size and invasiveness between mice injected with BTICs treated with control or test article will be statistically tested using the Mann-Whitney U test, and differences in survival will be statistically tested using the log-rank test and Cox proportion hazard test¹⁹⁸.

[00273] Predicted results: At every time point, we expect mice with xenografts of test article dosed BTICs to present tumor free or with smaller less invasive tumors, with more cell death (TUNEL), and less GBM proliferation (anti-human nuclei) as compared to control. Additionally, we expect increased survival time in mice injected with test article and BTICs compared to BTICs alone.

[00274] Materials and methods

[00275] Test Systems

[00276] Cells. Primary cultured brain tumor initiating cells (BTICs) were derived from 10 grade IV GBM patients. The methods for extracting and propagating the BTICs have been previously described by Dr. Alfredo Quinones-Hinojosa and his research team¹⁹¹⁻¹⁹³.

[00277] Species/Strain. Nu/nu Harlan Sprague Dawley mice

[00278] Age: At study start: 6 weeks

[00279] Weight: At study start: Males: 20-30 g; Females: 18-35 g

[00280] Test Article Identification.

[00281] RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159)

[00282] **Purity:** $\geq 95\%$ HPLC

[00283] **Dose Formulation preparation and stability:** RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159) and vehicle control will be prepared in bulk every week. Dose formulation concentrations will be assessed on at the beginning of every week. The acceptable concentration range is $\pm 10\%$ of nominal.

[00284] **Vehicle:** Water

[00285] **Study Design**

[00286] **Neurogenic potency of test article Study:**

[00287] **Dose:** 3 test article dose levels + vehicle control. The test article doses will be determined based on the results of the neuronal differentiation study described in Example 11.

[00288] **Administration:** Doses will be administered every 2 days with media change.

[00289] **Duration:** Main study: 0, 2, 4, or 8 days; recovery period: 2, 5, or 8 days.

[00290] **Number of replicates.** 3 tissue culture (TC) wells per group.

[00291] **Dosing volume.** 5 μL

[00292] **Parameters analyzed.** Markers for pluripotency (Sox2 and Nestin), proliferation (Ki67), cell death (caspase 3), and differentiation (MAP2, neuron and GFAP, glia) will be monitored on Days 0, 2, 4, and 8 during the dosing period (Main study) and on Days 10, 13, and 16 during the recovery period (3/TC wells/group for each day) by WB and RT-PCR (Table 10).

Table 10								
Assessment of neurogenic potency of test article on BTICs								
Test Article* Group No.	Dose	No. of replicates (TC wells (3.8 cm²))						
		Main Study				Recovery Period		
		Day 0	Day 2	Day 4	Day 8	Day 10	Day 13	Day 16
1	0	3	3	3	3	3	3	3

2	TBD	3	3	3	3	3	3	3
3	TBD	3	3	3	3	3	3	3
4	TBD	3	3	3	3	3	3	3
*The test article will be RPP (SEQ ID NOS: 1 and 15 through 17) or RPP _V (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159)								

[00293] Anti-oncogenic potency of test article on BTICs in mice:

[00294] Dose: 3 doses of test article + vehicle control. Test article doses will be based on the results of the neurogenic potency study.

[00295] Frequency of administration: Single dose

[00296] Route: Automated syringe pump with a guide-screw system^{112,195}. The injection coordinates will be X:1.5; Y:1.34; Z: -3.5 targeting the mouse striatum; a highly reliable site for tumor engraftment¹⁹⁶.

[00297] Vehicle: Phosphate-buffered saline (PBS)

[00298] Number of animals. 3/sex/group

[00299] Dosing volume. 5 μ L

[00300] Parameters analyzed. Mice will be assayed for survival during weeks 4, 8, 16, and 20 using the Kaplan-Meier method¹⁹⁷ followed by postmortem histopathology to evaluate tumor size and invasiveness (H&E stain), cell death (TUNEL), and proliferation (anti-human nuclei) (Table 11).

Table 11							
Assessment of anti-oncogenic potency of test article on BTICs in mice							
Test Article* Group No.	Dose	Injection Solution		Mouse No. (M/F)			
				Weeks			
		BTIC No.	PBS μL	4	8	16	20
1	0	5 x 10 ⁴	5	3/3	3/3	3/3	3/3
2	TBD	5 x 10 ⁴	5	3/3	3/3	3/3	3/3
3	TBD	5 x 10 ⁴	5	3/3	3/3	3/3	3/3
4	TBD	5 x 10 ⁴	5	3/3	3/3	3/3	3/3

* RPP (SEQ ID NOS: 1 and 15 through 17) or RPP_v (SEQ ID NOS: 18 through 117) peptide fused to a CPP (SEQ ID NOS: 118 through 137 and 140 through 159)

[00301] **Environmental Conditions**

[00302] **Housing.** 2 animals/cage, 12 hour light/dark cycle.

[00303] **Diet.** *Ad libitum* food and water.

[00304] Table 19 below shows the sequences for SEQ ID NOS: 1 through 324:

Table 19.		
Sequence ID	Sequence	Name
1	TEDLEPPEPPLPKEN	-
15	EDLEPPEPPLPK	-
16	nekplppeppeldet	-
17	kplppeppelde	-
18	MCTEDLEPPEPPLPKENC	v1
19	MCTEAPAPPEPALPKKKKNC	v2
20	MCTEDLQPPTAVPQENC	v3
21	MCTEAPAPPEPALPKKKKNC	v4
22	MCTADLEPPEPRMEKKKVDC	v5
23	MCTGDLQPPKTTVSKKDC	v6
24	MCTEDLQSPKTTMTKENC	v7
25	MCTEDLEPPEPPLPKEDC	v8
26	MCTEDQEQEEQLPEENC	v9
27	MCTADLKPPKTTMTKQNC	v10
28	MCPGDLKQPEPPMPKEYC	v11
29	MCTEDLEPPKATMTKKDC	v12
30	MCTEDQERPPVTKEDC	v13
31	MCIADPEPPEAQLPEGNC	v14
32	MCTGVQEPPEATLPKNC	v15
33	MCSEAQEPPEPRLPQVNC	v16
34	MCTKHLEPPGPPLPQENC	v17
35	MCTAAPEPPEPPVSKEYC	v18
36	MCTEDLQLPKTTMTKEYC	v19
37	MCSVDLQPPARLRPMVNC	v20
38	MCTGDLQPPEPRLPQVNC	v21
39	MCTGDLQPPEAQVIEVNC	v22
40	MCTEDLQPPEPQLPEVNC	v23
41	MCTEDMEPRKTTMTKKYC	v25
42	MCTEAPAPPEPALPKKKKKNC	v26
43	MCTEAPAPPEPALPKKKKKNC	v27
44	MCTEDLQSPKTTMTKENC	v28
45	MCTGDLKLPEPPMSKKKKKNC	v29

46	MCTEDLQPPKTTMAEKYC	v30
47	MCSEDPEPPKTTMTKKNC	v31
48	MCTEDLKPPEASLPEENC	v32
49	MCAGDLEQPEPPVAKKKKNC	v33
50	MCNGDLERPEPPVAKEYC	v34
51	MCTEDLKPPEPPLPKENC	v35
52	MCNEALEPPPLRKEHC	v36
53	MCPEDLERPPLTKEHC	v37
54	MCTEDLEPPERPLPREIC	v38
55	MCAGDLKPPETTMSKKNC	v39
56	MCTEDLQQPERSQPMESC	v40
57	MCPEDLQPPEPALPEKKKKKIVVLALVVKSAVSVGVV	v41
58	MCTEVLVPRTTSGKGRLWFWLWSQKGHPSASACGS	v42
59	MCAEDLQPPPLLEAHCGSDSGRKKRRQC	v43
60	MCTAAPEPPEPQLPQANC	v44
61	MCPADLQQPETSLEENC	v45
62	MCSVDLQPPARLRPMVNC	v46
63	MCTEALEPPEPPLTKENC	v47
64	MCTEAMEPPEPPLARESC	v48
65	MCTADLQPEASLPQQNC	v49
66	MCTAAPEPPEPRLPEGNC	v50
67	MCTKDLAPQAPLLKENC	v51
68	cnekplppeppeldetcm	v1 ^{RI}
69	cnkkkkkplapeppapaetcm	v2 ^{RI}
70	cneqpvatppqldetcm	v3 ^{RI}
71	cnkkkkkplapeppapaetcm	v4 ^{RI}
72	cdkksvttkppqldgtcm	v5 ^{RI}
73	cdkksvttkppqldgtcm	v6 ^{RI}
74	cnektmttkpsqldetcm	v7 ^{RI}
75	cdekplppeppeldetcm	v8 ^{RI}
76	cneplqeelleldetcm	v9 ^{RI}
77	cnqktmttkppkldgpcm	v10 ^{RI}
78	cyekpmppepqlgpcm	v11 ^{RI}
79	cdekktmtakppeldetcm	v12 ^{RI}
80	cdektvppreqdetcm	v13 ^{RI}
81	cngeplqaeppepdaicm	v14 ^{RI}
82	cnkkpltaeppeqvgtcm	v15 ^{RI}
83	cnvqplppgppelhkcm	v16 ^{RI}
84	cneqplppgppepaatcm	v17 ^{RI}
85	cyeksvppeppepaatcm	v18 ^{RI}
86	cyektmttkplqldetcm	v19 ^{RI}
87	cnvmprlrappqldvscm	v20 ^{RI}
89	cnvqpqrseppqldgtcm	v21 ^{RI}
89	cnveivqaeppeqldgtcm	v22 ^{RI}
90	cnveplqeppeqldetcm	v23 ^{RI}

91	cykktmttkppemdetc	v25 ^{RI}
92	cnkkkkkpklaeppapaetcm	v26 ^{RI}
93	cnkkkkkplaeppapaetcm	v27 ^{RI}
94	cnektmttkpsqldetc	v28 ^{RI}
95	cnkkkkksmppeplkldgcm	v29 ^{RI}
96	cykeamttkppqldetc	v30 ^{RI}
97	cnkktmttkppepdescm	v31 ^{RI}
98	cneplsaeppkldetc	v32 ^{RI}
99	cnkkkkkavppepqldgacm	v33 ^{RI}
100	cyekavppepreldgncm	v34 ^{RI}
101	cnekplppeppkldetc	v35 ^{RI}
102	chekrlpppelaencm	v36 ^{RI}
103	chektlpppreldpc	v37 ^{RI}
104	cierplpreppeldetc	v38 ^{RI}
105	cnkksmtteppkldgacm	v39 ^{RI}
106	csmqpsrepqqledetc	v40 ^{RI}
107	cvvgvsvaskvvlalvvikkkkkeplaeppqldpc	v41 ^{RI}
108	csgcasasphgkqswlwfwrkgsttrpvlvetcm	v42 ^{RI}
109	cqrrkrgsdsgchaellpppqldacm	v43 ^{RI}
110	cnaqplqpeppapaetcm	v44 ^{RI}
111	cneplstepqqldapcm	v45 ^{RI}
112	cnvmprlrappqldvscm	v46 ^{RI}
113	cnektlpppeppelaetcm	v47 ^{RI}
114	cseralppeppemaetcm	v48 ^{RI}
115	cnqqplsaeppqldatcm	v49 ^{RI}
116	cngeplrpeppapaetcm	v50 ^{RI}
117	cneklppaqpaldktcm	v51 ^{RI}
118	GRKKRRQRRR	-
119	GDIMGEWGNEIFGAIAGFLGYGRKKRRQRRR	-
120	RRRRRRRR	-
121	KKKKKKKK	-
122	DIMGEWGNEIFGAIAGFLG	-
123	CHHHHHRKKRRQRRRRHHHHHC	-
124	CHHHHHRRRRRRRRRHHHHHC	-
125	FFLIPKGRRRRRRRRGC	-
126	FΦRRRR	-
127	RRWRRWRRRRWRRr	-
128	RWRRRRWRRWRRr	-
129	RRWRRWRRRRWRRr	-
130	RRWRRWRRRRr	-
131	RRWRRWRRr	-
132	RRWRRWRRR	-
133	RRRRRC ₁₂ RRWRRr	-
134	YALTSAISRIITHHHHHH	-
135	RRRRRC ₁₄ RRWRR	-

136	RRC ₁₄ RRC ₁₄ RRRRR	-
137	RRC ₁₄ RRR	-
138	GS	-
139	GSGS	-
140	rrrqrrkkr	-
141	rrrqrrkkrvglfgaiagfiengwegmgmidg	-
142	rrrrrrr	-
143	kkkkkkkk	-
144	glfgaiagfiengwegmid	-
145	chhhhhrrrrqrrkrhhhhc	-
146	chhhhhrrrrrrrrhhhhc	-
147	cgrrrrrrgkplffc	-
148	rrrΦf	-
149	rrwwrrrrwrrwrr	-
150	rrwwrrrrrrwrr	-
151	rrwwrrrrwrrwrr	-
152	rrrrwrrwrr	-
153	rrwrrwrr	-
154	rrwrrwrr	-
155	rrwrrC ₁₄ rrrrr	-
156	hhhhhhrrrrrsiastlay	-
157	rrwwrrC ₁₄ rrrrr	-
158	rrC ₁₄ rrC ₁₄ rrrrr	-
159	rrC ₁₄ rrr	-
160	sg	-
161	sgsg	-
162	ATGGATAGTAGTGCGGTGATCACACAAATCTCCAAGGAGGAA GCCCGTGGGCCGCTGCGGGGAAGGGTGATCAAAAATCGGCA GCTAGTCAAAAACCTCGCTCTCGTGGGATACTTCATTCGCTGT TTTGCTGCGTCTGCCGCGATGACGGAGAAGCATTGCCTGCGCA TTCAGGGGCGCCTTTACTTGTTGAGGAAAATGGTGCAATTCCT AAACA <u>AA</u> ACTCCAGTACAATACTTACTGCCGGAGGCAAAGGCA CAAGACAGTGATAAGATATGTGTAGTAATAGACTTAGATGAA ACACTGGTACATTTCGTCATTCAAACCTGTTAATAATGCGGATT TCATCATACCTGTAGAAATCGACGGGGTTGTCCATCAGGTTTA CGTCCTGAAGCGGCCTCATGTAGATGAATTTTACAGCGGATG GGCGAGTTATTTGAATGTGTGCTGTTACAGCTAGTCTTGCCA AGTACGCGGATCCTGTCGCGGATTGCTTGATAAGTGGGGTG GTTTCGGGCGAGATTATTCGCGAATCTTGCGTTTTTCACAGA GGTAACTACGTGAAGGACCTTAGTCGTCTGGGTAGAGATCTTA GAAGAGTGCTGATCCTTGACAACAGCCCAGCCAGCTATGTCTT TCATCCGGATAACGCAGTACCCGTGGCGTCTTGGTTCGACAAT ATGTCGGACACGGAGCTGCATGACCTGTTGCCGTTCTTTGAGC AGTTGAGTCGCGTTGATGACGTTTACTCGGTTTTGCGTCAACC CCGTCCGGGATCTGGTTCTGGCTCTCACCATCACCATCACCAC	-

	TAG	
163	MDSSAVITQISKEEARGPLRGKGDQKSAASQKPRSRGILHSLFCC VCRDDGEALPAHSGAPLLVEENGAIKQTPVQYLLPEAKAQDSD KICVVIDLDETLVHSSFKPVNNADFIIPVEIDGVVHQVYVLKRPHV DEFLQRMGELFECVLFTASLAKYADPVADLLDKWGAFRARLFR SCVFHRGNYVKDLSRLGRDLRRVLILDNPSASYVFHPDNAVPVA SWFDNMSDTELHDLLPFFEQLSRVDDVYSVLRQPRPGSGSGSHH HHHH	-
164	ATGTGTACCGAAGATCTGGAACCACCAGAACCACCACTGCCA AAGGAAAATTGTGGATCCGGTTCTGGCTCAGGTTCTTCCCCTA TACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCG ACTTCTTTTGGGAATATCTTGAAGAAAAATATGAAGAGCATTG TATGAGCGCGATGAAGGTGATAAATGGCGAAACAAAAAGTTT GAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGATGG TGATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATA GCTGACAAGCACACATGTTGGGTGGTTGTCCAAAAGAGCGT GCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGAT ACGGTGTTTTGAGAATTGCATATAGTAAAGACTTTGAAACTCT CAAAGTTGATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATG TTCGAAGATCGTTTATGTCATAAAACATATTTAAATGGTGATC ATGTAACCCATCCTGACTTCATGTTGTATGACGCTCTTGATGTT GTTTTATACATGGACCCAATGTGCCTGGATGCGTTCCCAAAAT TAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGA TAAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAG GGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAAT AA	-
165	MCTEDLEPPEPPLPKENCGSGSGSGSSPILGYWKIKGLVQPTRLLL EYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDGDVKL TQSMARIYIADKHNMLGGCPKERAISMLEGAVLDIRYGVSR IAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDF MLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAIQIDKYLKSSK YIAWPLQGWQATFGGGDHPPK	-
166	AATAAAGCTTATGTGTACCGAAGATCTGGAACCACCAGAACC ACCACTGCCAA	v1f
167	AATAGGATCCACAATTTTCTTTGGCAGTGGTGGTTCTGGTGG TTCCA	v1r
168	AATAAAGCTTATGTGTACCGAAGCTCCGGCACCACCAGAACC AGCACTGCCAAAG	v2f
169	AATAGGATCCACAATTTTTTTTTTTTTTTCTTTGGCAGTGCTGGT TCTGGTGGTGC	v2r
170	AATAAAGCTTATGTGTACCGAAGATCTGCAACCACCAACAGC AGTGCCAC	v3f
171	AATAGGATCCACAATTTTCTGTGGCACTGCTGTTGGTGGTTG CAGATCTTC	v3r
172	AATAAAGCTTATGTGTACCGAAGCTCCGGCACCACCAGAACC	v4f

	AGCACTGCCAAAAG	
173	AATAGGATCCACAATTTTTTTTTTTCTTTGGCAGTGCTGGTTCT GGTGGTGC	v4r
174	ATGTGTACCGCAGATCTGGAACCACCAGAACCACGAATGGAA	v5f
175	AATAGGATCCACAATCTACCTTTTTTTTTTCCATTCGTGGTTCT GGTGGTTCCA	v5r
176	AATAAAGCTTATGTGTACCGGAGATCTGCAACCACCAAAAAC AACAGTGTCAA	v6f
177	AATAGGATCCACAATCTTTCTTTGACACTGTTGTTTTTGGTGGT TGCAGA	v6r
178	AATAAAGCTTATGTGTACCGAAGATCTGCAATCACCAAAAAC AACAATGACAA	v7f
179	AATAGGATCCACAATTTTCCTTTGTCATTGTTGTTTTTGGTGAT TGCAGA	v7r
180	AATAAAGCTTATGTGTACCGAAGATCTGGAACCACCAGAACC ACCACTGCCAA	v8f
181	AATAGGATCCCACAATCTTCCTTTGGCAGTGGTGGTTCTGGTG GTTCCAGA	v8r
182	AATAAAGCTTATGTGTACCGAAGATCAGGAACAACAAGAAGA ACAACCTG	v9f
183	AATAGGATCCACAATTTTCCTCTGGCAGTTGTTCTTCTTGTTGT TCCTGATC	v9r
184	AATAAAGCTTATGTGTACCGCAGATCTGAAACCACCAAAAAC AACAATGACAA	v10f
185	AATAGGATCCACAATTTTGCTTTGTCATTGTTGTTTTTGGTGGT TTCAGA	v10r
186	AATAAAGCTTATGTGTCCCGGAGATCTGAAACAACCAGAACC ACCAATGCCAA	v11f
187	AATAGGATCCACAATATTCCTTTGGCATTGGTGGTTCTGGTTG TTTCAGA	v11r
188	AATAAAGCTTATGTGTACCGAAGATCTGGAACCACCAAAAGC AACAATGACAA	v12f
189	AATAGGATCCACAATCTTTCTTTGTCATTGTTGCTTTTGGTGGT TCCAGA	v12r
190	AATAAAGCTTATGTGTACCGAAGATCAGGAACGACCACCAGT GACAAAG	v13f
191	AATAGGATCCACAATCTTCCTTTGTCAGTGGTGGTCGTTCCCTG ATCTTC	v13r
192	AATAAAGCTTATGTGTATCGCAGATCCGGAACCACCAGAAGC ACAACCTG	v14f
193	AATAGGATCCACAATTTCCCTCTGGCAGTTGTGCTTCTGGTGG TTCCGGATCTG	v14r
194	AATAAAGCTTATGTGTACCGGAGTTCAGGAACCACCAGAAGC AACAACCTG	v15f
195	AATAGGATCCACAATTTTTCTTTGGCAGTGTGCTTCTGGTGGT TCCTGAAC	v15r

196	AATAAAGCTTATGTGTAGCGAAGCTCAGGAACCACCAGAATC ACGACTG	v16f
197	AATAGGATCCACAATTTACCTGTGGCAGTCGTGATTCTGGTGG TTCCTGAG	v16r
198	AATAAAGCTTATGTGTACCAAACATCTGGAACCACCAGGACC ACCATGTC	v17f
199	AATAGGATCCACAATTTTTCCTGTGGCAATGGTGGTCCTGGTGG TTCCAGATG	v17r
200	AATAAAGCTTATGTGTACCGCAGCTCCGGAACCACCAGAACC ACCAAGTG	v18f
201	AATAGGATCCACAATATTCCTTTGACACTGGTGGTTCTGGTGG TTCCGGAG	v18r
202	AATAAAGCTTATGTGTACCGAAGATCTGCAACTACCAAAAAC AACAATGACA	v19f
203	AATAGGATCCACAATATTCCTTTGTCATTGTTGTTTTTGGTAGT TGCAGA	v19r
204	AATAAAGCTTATGTGTTCCGTAGATCTGCAACCACCAGCACGA CTACGGCCAA	v20f
205	AATAGGATCCACAATTTACCATTGGCCGTAGTCGTGCTGGTGG TTGCAGAT	v20r
206	AATAAAGCTTATGTGTACCGGAGATCTGCAACCACCAGAATC ACGACAGCCA	v21f
207	AATAGGATCCACAATTTACCTGTGGCTGTCGTGATTCTGGTGG TTGCAGAT	v21r
208	AATAAAGCTTATGTGTACCGGAGATCTGCAACCACCAGAAGC ACAAGTGA	v22f
209	AATAGGATCCACAATTTACCTGTGGCTGTCGTGATTCTGGTGG TTGCAGAT	v22r
210	AATAAAGCTTATGTGTACCGAAGATCTGCAACCACCAGAACC ACAAC TGCCA	v23f
211	AATAGGATCCACAATTTACCTCTGGCAGTTGTGGTTCTGGTGG TTGCAGAT	v23r
212	AATAAAGCTTATGTGTACCGAAGATATGGAACCACGAAAAAC AACAATGA	v25f
213	AATAGGATCCACAATATTTCTTTGTCATTGTTGTTTTTCGTGGT TCCATAT	v25r
214	AATAAAGCTTATGTGTACCGAAGCTCCGGCACCACCAGAACC AGCACTGCCAAAG	v26f
215	AATAGGATCCACAATTTTTTTTTTTTTTTTTCTTTGGCAGTGCT GGTTCTGGTGGT	v26r
216	AATAAAGCTTATGTGTACCGAAGCTCCGGCACCACCAGAACC AGCACTGCCAAAG	v27f
217	AATAGGATCCACAATTTTTTTTTTTTTTTTTCTTTGGCAGTGCTGGT TCTGGTGGTG	v27r
218	AATAAAGCTTATGTGTACCGAAGATCTGCAATCACCAAAAAC AACAATG	v28f

219	AATAGGATCCACAATTTTCCTTTGTCATTGTTGTTTTTGGTGAT TGCAGA	v28r
220	AATAAAGCTTATGTGTACCGGAGATCTGAAACTACCAGAACC ACCAATGTCAAAG	v29f
221	AATAGGATCCACAATTTTTTTTTTTTTCTTTGACATTGGTGGT TCTGGTAG	v29r
222	AATAAAGCTTATGTGTACCGAAGATCTGCAACCACCAAAAAC AACAATG	v30f
223	AATAGGATCCACAATATTTCTCTGCCATTGTTGTTTTTGGTGGT TGCAGAT	v30r
224	AATAAAGCTTATGTGTAGCGAAGATCCGGAACCACCAAAAAC AACAATGAC	v31f
225	AATAGGATCCACAATTTTTCTTTGTCATTGTTGTTTTTGGTGGT TCCGGAT	v31r
226	AATAAAGCTTATGTGTACCGAAGATCTGAAACCACCAGAAGC ATCACTGC	v32f
227	AATAGGATCCACAATTTTCCTCTGGCAGTGATGCTTCTGGTGG TTTCAGAT	v32r
228	AATAAAGCTTATGTGTGCCGGAGATCTGGAACAACCAGAACC ACCAAGTGGCAA	v33f
229	AATAGGATCCACAATTTTTTTTTTTTTCTTTGCCACTGGTGGT TCTGGTTGTTT	v33r
230	AATAAAGCTTATGTGTAACGGAGATCTGGAACGACCAGAACC ACCAAGT	v34f
231	AATAGGATCCACAATATTCCTTTGCCACTGGTGGTTCTGGTCG TTCCAGAT	v34r
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233	AATAGGATCCACAATTTTCCTTTGGCAGTGGTGGTTCTGGTGG TTTCAGAT	v35r
234	AATAAAGCTTATGTGTAACGAAGCTCTGGAACCACCACCACTG CGAAAG	v36f
235	AATAGGATCCACAATGTTTCCTTTGCGAGTGGTGGTGGTTCCAG AGCTTC	v36r
236	AATAAAGCTTATGTGTCCCGAAGATCTGGAACGACCACCATTG ACAAAG	v37f
237	AATAGGATCCACAATGTTTCCTTTGTCAATGGTGGTCGTTCCAG AT	v37r
238	AATAAAGCTTATGTGTACCGAAGATCTGGAACCACCAGAACG ACCACTGC	v38f
239	AATAGGATCCACAAATTTCCCTTGGCAGTGGTCGTTCTGGTGG TTCCAGA	v38r
240	AATAAAGCTTATGTGTGCCGGAGATCTGAAACCACCAGAAAC AACAATGTC	v39f
241	AATAGGATCCACAATTTTTCTTTGACATTGTTGTTTCTGGTGGT TTCAGA	v39r

242	AATAAAGCTTATGTGCACCGAAGATCTGCAACAACCAGAACG ATCACAGC	v40f
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244	AATAAAGCTTATGTGTCCCGAAGATCTGCAACCACCAGAACC AGCACTGCCAGAGAAAAA	v41f
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246	ACCAGAACCAGCACTGCCAGAGAAAAAAAAAAAAAAAAAATTGT GGTTCTGGCTCTGGTCGT	v41
247	AATAAAGCTTATGTGTACCGAAGTTCTGGTACCACGAACCACC AGTGGCAAAGGAAG	v42f
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250	AATAAAGCTTATGTGTGCCGAAGATCTGCAACCACCACCACTG CTAGAGGCA	v43f
251	AATAGGATCCCACTGACGGCGCTTTTTACGACCAGAGTCAGA ACCACAATGTGCCTCT	v43r
252	ATCTGCAACCACCACCACTGCTAGAGGCACATTGTGGTTCTGA CTCTGGTCGTAAAAAG	v43
253	AATAAAGCTTATGTGTACCGCAGCTCCGGAACCACCAGAACC ACAACCTG	v44f
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257	AATAAAGCTTATGTGTTCCGTAGATCTGCAACCACCAGCACGA CTACG	v46f
258	AATAGGATCCACAATTTACCATTGGCCGTAGTCGTGCTGGTGG TTGCAGAT	v46r
259	AATAAAGCTTATGTGCACCGAgGCTTTGGAACCACCAGAACCA CCACTG	v47f
260	AATAGGATCCACAATTTTCCTTTGTCAGTGGTGGTTCTGGTGG TTCCAAAG	v47r
261	AATAAAGCTTATGTGTACCGAAGCTATGGAACCACCAGAACC ACCACTG	v48f
262	AATAGGATCCACAACCTTTCCCTTGCCAGTGGTGGTTCTGGTGG TTCCATAG	v48r
263	AATAAAGCTTATGTGTACCGCAGATCTGCAACCACCAGAAGC ATCACTG	v49f
264	AATAGGATCCACAATTTTGCTGTGGCAGTGATGCTTCTGGTGG TTGCAGAT	v49r

265	AATAAAGCTTATGTGTACCGCAGCTCCGGAACCACCAGAACC ACGACTGCCA	v50f
266	AATAGGATCCACAATTTCCCTCTGGCAGTCGTGGTTCTGGTGG TTCC	v50r
267	AATAAAGCTTATGTGTACCAAAGATTTGGCACCACAAGCACC ACCATTGCT	v51f
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269	AAATAAGCTTGTATATCTCCTTCTTAAAGTTAAACA	P12
270	AATAACTCGAGAGATCCGGCTGCTAACAAAGC	P14
271	ATAAGCTTTAATACGACTCACTATAGGGTTAACTTTAGTAAGG AGGACAGCTAA	P17
272	GTGGTGATGGTGATGGTGACCTA	P19
273	ATTATTCTCGAGTTAATAGCCGGTGCCGTGGTGATGGTGATGG TGACCTA	P20
274	AAATACTCGAGTCGTTTTATCTGTTGTTTGTCGGT	P21
275	AAATAAAGCTTCTCTGAATGGCGGGAGTATGAAAA	P22
276	CCGCGAATGGTGAGATTGAGAA	P23
277	ACGCAAAAAGGCCATCCGTCAG	P24
278	ATTATTCTCGAGTTAATAGCCGGTGCCTAAGCCGCTACCACCA CGCCGACGCTGACGG	P28
279	AAATAAGCTTATGGATAGTAGTGCGGTGATCA	P31
280	ATTATTCTCGAGCTAGTGGTGATGGTGATGGT	P32
281	AATAAAGCTTTTCCTCCTGTTAGCCCCAAAAAAC	P33
282	AATACTCGAGGCTGTTTTGGCGGATGAGAGAA	P34
283	AAATCCATGGATAGTAGTGCGGTGATCA	P35
284	AAATCATATGGATAGTAGTGCGGTGATCA	P36
285	AATACTCGAGCTAGTGGTGATGGTGATGGTGAG	P37
286	AATACTCGAGAGAGCCAGAACCAGATCCCGGA	P38
287	TAAGCCGCTACCACCACGCCGAC	P39
288	GGTGCAATTCCTAAAACCTCCAGTACAATACTTACTGC	P40
289	GTATTGTACTGGAGTTTTAGGAATTGCACCATTTTCCTC	P41
290	AATACTCGAGTTATTTTGGAGGATGGTCGCCACCA	P108
291	AATAAAGCTTATGGGATCCGGTTCTGGCTCAGGTTCTTCC	P109
292	NAANCANCANTGNCANAGNAANATTGTGGTTCTGGCTCTGGT CGTAAAA	11F
293	GNACNACNACNGCNAANGGNAANTTGTGGTTCTGGCTCTGGT CGTAAAA	12F
294	GNANCACNANTGCNANAGGNANATTGTGGTTCTGGCTCTGGT CGTAAAA	13F
295	NAACNANCACNGNCAANGNAAANTTGTGGTTCTGGCTCTGGT CGTAAAA	14F
296	GAANCANCANTGNCANAGNAANATTGTGGTTCTGGCTCTGGT CGTAAAA	15F
297	GAACNACNACNGCNAANGGNAANTTGTGGTTCTGGCTCTGGT	16F

	CGTAAAA	
298	GAANCACNANTGCNANAGGNANATTGTGGTTCTGGCTCTGGT CGTAAAA	17F
299	GAACNANCACNGNCAANGNAAANTTGTGGTTCTGGCTCTGGT CGTAAAA	18F
300	GAANCANCACTGCCANAGNAAAATTGTGGTTCTGGCTCTGGTC GTAAAA	19F
301	NAACCACCANTGNCAAAGGAANATTGTGGTTCTGGCTCTGGTC GTAAAA	20F
302	GAANCACNACTGCCANAGGNAAAATTGTGGTTCTGGCTCTGGTC GTAAAA	21F
303	GNACCACCANTGCNAAAGGAANATTGTGGTTCTGGCTCTGGTC GTAAAA	22F
304	TGNTGNTTNCANATNTTNGGNACACATTTAGCTGTCCTCCTTA CTAAAGTT	11R
305	TNGTNGTNCNGANCTNCGNTACACATTTAGCTGTCCTCCTTA CTAAAGTT	12R
306	TGNTGNTTNCNGATNTNCGGNACACATTTAGCTGTCCTCCTTA CTAAAGTT	13R
307	TNGTNGTNCANANCTTNGNTACACATTTAGCTGTCCTCCTTA CTAAAGTT	14R
308	TGNTGNTTCCANATNTTNGGNACACATTTAGCTGTCCTCCTTA CTAAAGTT	15R
309	TNGTNGTTCNGANCTNCGNTACACATTTAGCTGTCCTCCTTA CTAAAGTT	16R
310	TGNTGNTTCCNGATNTNCGGNACACATTTAGCTGTCCTCCTTA CTAAAGTT	17R
311	TNGTNGTTCANANCTTNGNTACACATTTAGCTGTCCTCCTTA CTAAAGTT	18R
312	TGGTGNTTNCAGATCTTNGGNACACATTTAGCTGTCCTCCTTA CTAAAGTT	19R
313	TGNTGGTTCCANATNTTCGGTACACATTTAGCTGTCCTCCTTAC TAAAGTT	20R
314	TGGTNGTTNCAGATCTNCGGNACACATTTAGCTGTCCTCCTTA CTAAAGTT	21R
315	TGNTGGTTCCNGATNTTCGGTACACATTTAGCTGTCCTCCTTAC TAAAGTT	22R
316	CCTGCAGGTAATACGACTCACTATAGGGTTAACTTTAGTAAGG AGGACAGCTAAAAGCTTATGTGTACCGAAGATCTGGAACCAC CAGAACCACCACTGCCAAAGGAAAATTGTGGATCCGGCTCTG GTCGTAAAAAGCGCCGTCAGCGTCGGCGTGGTGGCTCCGGTA GCTTAGGTCACCATCACCATCACCACGGCACCGGCTATTA CGAG	-
317	ATGTGTACCGAAGATCTGGAACCACCAGAACCACCACTGCCA AAGGAAAATTGTGGATCCGGCTCTGGTCGTAAAAAGCGCCGT	-

	CAGCGTCGGCGTGGTGGCTCCGGTAGCTTAGGGTCACCATCACC ATCACCACGGCACCGGCTATTAA	
318	AAATCCTGCAGGTAATACGACTCACTATAGGGTTAAC	-
319	ATTATTCTCGAGTTAATAGCCGGTGCCGTGGTGATGGTGATGG TGACCTA	-
320	TATACCCTGCAGGTAATACGACTCACTATAGGGTTAACTTTAG TAAGGAGGACAGCTAAAAGCTTATGTGTGAAGACGCCAAAAA CATAAAGAAAGGCCCGGCCATTCTATCCGTGTGGATCCGGC TCTGGTCGTAAAAGCGCCGTCAGCGTCGGCGTGGTGGCTCCG GTAGCTTAGGTCACCATCACCATCACCACGGCACCGGCTATTA ACTCGAGAGATC	-
321	CCTGCAGGTAATACGACTCACTATAGGGTTAACTTTAGTAAGG AGGACAGCTAAAAGCTTATGTGTGAAGACGCCAAAAACATAA AGAAAGGCCCGGCCATTCTATCCGTGTGGATCCGGCTCTGG TCGTAAAAGCGCCGTCAGCGTCGGCGTGGTGGCTCCGGTAG CTTAGGTCACCATCACCATCACCACGGCACCGGCTATTAATC GAG	-
322	ATGTGTGAAGACGCCAAAAACATAAAGAAAGGCCCGGCCA TTCTATCCGTGTGGATCCGGCTCTGGTCGTAAAAGCGCCGTC AGCGTCGGCGTGGTGGCTCCGGTAGCTTAGGTCACCATCACC TCACCACGGCACCGGCTATTAA	-
323	AAATCCTGCAGGTAATACGACTCACTATAGGGTTAAC	-
324	ATTATTCTCGAGTTAATAGCCGGTGCCGTGGTGATGGTGATGG TGACCTA	-
2	<u>MCTEDLEPPEPPLPKENCGSGSGRKKRRQRRRGSGSLGHHHHH</u> <u>HGTGY</u>	-
3	<u>MCEDAKNIKKGPAPFYPCGSGSGRKKRRQRRRGSGSLGHHHHH</u> <u>HHGTGY</u>	Control
4	CAQKDYKDDDDKTEDLEPPEPPLPKENGRKKRRQRRRG	-
5	CTEDLEPPEPPLPKENSGDIMGEWGNEIFGAIAGFLGYGRKKRRQ RRRG ^{cycli(head to tail)}	-
6	TEDLEPPEPPLPKENRRWRRRWRWRr	-
7	EDLEPPEPPLPKRWRRRRWRWRr	-
8	AGDLEQPEPPVAKKKKNRRWRRWRr	-
9	rrrrwrrwrrrrnekplppeppel ^{det}	-
10	TEDLEPPEPPLPKENrrrrwrrwrrwrr	-
11	TEDLEPPEPPLPKENRRRRRRC ₁₄ RRWRRr	-
12	rrwrrC ₁₄ rrrrkplppeppel ^{cyclic (head to tail)}	-
13	rrC ₁₄ rrC ₁₄ rrrrkplppeppel ^{cyclic (head to tail)}	-
14	rrC ₁₄ rrrkplppeppel ^{cyclic (head to tail)}	-
Lower case = D amino acids; C ₁₄ = 2-aminotetradecanoic acid; Φ is L-2-naphthylalanine; N = any nucleotide		

[00305] Although certain presently preferred embodiments of the invention have been specifically described herein, it will be apparent to those skilled in the art to which the invention pertains that variations and modifications of the various embodiments shown and described herein may be made without departing from the spirit and scope of the invention.

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