Supporting Information

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SI Materials and Methods

PDB Preparation. The full latest protein database was downloaded using <rsync -rlpt -v -z–delete–port = 33444 rsync.wwpdb.org:: ftp_data/structures/divided/pdb/ ./pdb>. Each file in the database was cleaned to remove any nonpeptide components such as water molecules, nucleic acid molecules, metal ions, and small molecule drug molecules. For NMR solution structures, only the first model in conformer ensembles was used. Individual helices were then extracted from each of the remaining 111,867 files resulting in 2,819,149 files, one for each helix in the PDB containing a helix plus one nonhelix flanking residue at each end. Helices were defined according to information in the PDB file header.

Hotspot Identification. All necessary hotspot information for GLP-1 and PTH was readily available in the literature from alanine scanning mutagenesis experiments.

Structural Alignment. Structural alignments were carried out using a program called Click (1). Click was chosen because unlike the majority of structural alignment software, it does not consider sequence order or use sequence alignment. Instead, it uses the molecule Cartesian coordinates to align constellations of points independent of residue order. This is important for identifying the closest matching D-peptide hotspot constellations because their sequence order and/or direction is very often different to the L-peptide query.

Target Compatibility. Helix matches were assembled using Chimera (2) on the surface of the GLP-1R and PTH1R ECDs (PDB ID codes: 3IOL and 3C4M). Matched (D) hotspots were aligned with their corresponding (L)-hotspots. The central linker region was constructed using chimera and the saved coordinate file converted to (D). The linker was also then assembled on the surface of GLP-1R and PTH1R such that it lined up with helix junction residues. Residues that clashed with the target were mutated accordingly.

Helix Integrity Checking. PSI-PRED (3) was used to predict the likely secondary structure of each candidate. Recalculation was carried out following each mutation to remove target clash and mutations were accepted on the basis that helical structure was predicted. Deviation from helical would have led to mutation to different residues types until helix was maintained and clash removed. Failure to do both means the candidate would be demoted. The web tool PepCalc (4) was used to predict peptide solubility. If poor solubility was predicted, the mutations would be revised, secondary structure checks repeated, and solubility checks rerun. This process would be repeated until all clash, secondary structure, and solubility requirements are satisfied.

Peptide Synthesis. Both (L)- and (D)-peptides were obtained from Lifetein LLC. The peptides were produced by chemical synthesis and supplied with HPLC-MS quality data (Fig. S2).

Cell Lines and Reagents. HEK293 cell line was obtained from the ATCC. HEK293 cell line was tested for mycoplasma contamination. HEK293 cells were maintained in DMEM (ATCC) supplemented with 10% FBS and 1% pen/strep/glutamine, and the appropriate selection antibiotics when required.

Library Construction, Amplification, and Lentiviral Plasmid Construction. Gaussia Luciferase vector was generated by PCR amplification of the Gaussia Luciferase gene from the pTK GLuc (provided by the Stagljar laboratory, Donnelly Centre, University of Toronto, Toronto) using primer for insertion of restriction sites (EcoRI and XmaI): Primer forward 5′-GGAACTAACCGGTCGCCACCATGGGAGTCAAAGTTCTGTTTGCC-3′, primer reverse 5′-CAATGCCGAATTCTTAGTCACCACCGGCCCCCTT-GATC-3′. The PCR product was digested and cloned into pLJM17 lentiviral vector. The pLJM17 vector contains a CMV promoter and hygromycin for the selection marker.

Luciferase Assay. HEK293 cells stably expressing hGLP1R and reporter CRE-Gaussia Luciferase construct were trypsinized from subconfluent culture and seeded in a 96-well plate at a density of 5,000 cells per well. Cells were incubated overnight at 37 °C in 5% CO_2. Cells were treated with different concentrations of L-GLP1 peptide, D-GLP1 peptide, and forskolin. After 6 h of incubation, 20 μL of cell medium was transferred to a black flat-bottomed 96-well plate. Fifty microliters of Working solution (Pierce Gaussia-Firefly Luciferase Dual Assay Kit; Thermo Scientific no. 16181) was added into each well containing cell medium. Immediately after adding the reagent, samples were read using a luminometer with a 480-nm filter.

Western Blot. HEK293 cells stably expressing hGLP1R were treated with different concentrations of L- or D-GLP1 peptides for different time points. Cells were lysed with lysis buffer [50 mM Tris·HCl pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 mM $Na3VO_4$, 10 mM sodium pyrophosphate, 25 mM NaF, 1× protease inhibitor mixture (Sigma)] for 30 min at 4 °C. Protein samples were separated on a NuPage Bis·Tris 10% SDS/PAGE gel (Invitrogen) and transferred to PVDF membranes. Transferred samples were immunoblotted with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and detected using enhanced chemiluminescence (GE Healthcare).

Protease Stability Assay. Stocks of 20 μM peptide in 200 μL of total volume (10 mM Tris-base, 10 mM NaCl, pH 7.4) were supplemented with 5 μM $CaCl_2$, and 30 μL were removed for the untreated T0 sample. Proteinase K (ProtK; Bioshop) was then added to a final concentration of 100 μg/mL Samples were incubated at 37 °C, and 30 μL was removed after each time point and protease activity was blocked by the addition of 10 mM PMSF (200 mM stock dissolved in isopropanol). Protease inactivated samples were frozen at −20 °C until further use. Digestions were repeated three times. Frozen samples were supplemented with 8 μL of sample loading buffer (4× NuPAGE; ThermoFisher Scientific), boiled (50 °C) for 10 min, and centrifuged (16,128 × *g*, 10 min) before loading the gel [12% NuPAGE Bis-Tris (ThermoFisher Scientific)] with Mes running buffer). Gels were run at 200 V for ∼35 min and stained using Coomassie Brilliant Blue dye. Densitometry of bands was determined using ImageJ software (5) with background subtraction. All samples were normalized to their respective untreated sample (T0).

Circular Dichroism. Secondary structure determination was carried out using a Jasco J-720 spectropolarimeter. Lyophilized peptide powders were dissolved in pure water, and CD spectra was read immediately. Peptide concentrations were 20 μM for L-GLP1 and 150 μM D-GLP1 in water. Concentrations varied between peptides to enable collection of clear spectra, as peptides generally lacked strong CD signals. Samples were read using a 0.1-cm cuvette pathlength with three accumulations per run, 50 nm/min scanning speed. All spectra were background subtracted and converted to mean residue molar ellipticity using standard formulas

to allow direct comparison between samples of varying concentration and amino acid length. Spectra are reported in Fig. S3. The D-GLP1 peptide spectra has been inverted to allow for visual comparison with the L-GLP1 peptide spectra.

1. Nguyen MN, Tan KP, Madhusudhan MS (2011) CLICK–topology-independent comparison of biomolecular 3D structures. *Nucleic Acids Res* 39:W24–W28.
2. Pettersen EF, et al. (2004) UCSF Chimera–A visualization system for exploratory research and analysis. *J Comput Chem* 25:1605–1612.
3. Buchan DW, Minneci F, Nugent TC, Bryson K, Jones DT (2013) Scalable web services for the PSIPRED protein analysis workbench. *Nucleic Acids Res* 41:W349–W357.
4. Lear S, Cobb SL (2016) Pep-Calc.com: A set of web utilities for the calculation of peptide and peptoid properties and automatic mass spectral peak assignment. *J Comput Aided Mol Des* 30:271–277.
5. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675.

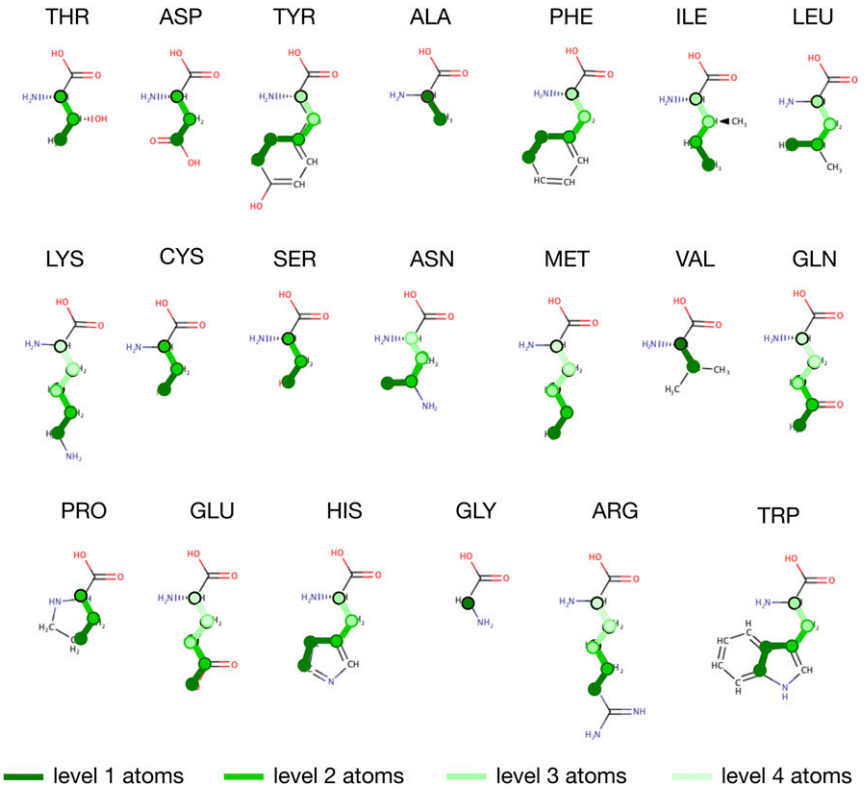


Fig. S1. Full atom level definitions for all 20 amino acids. Atom levels are defined in order of priority as the pairs most distal to the backbone, through to the pair most proximal.

L-GLP1

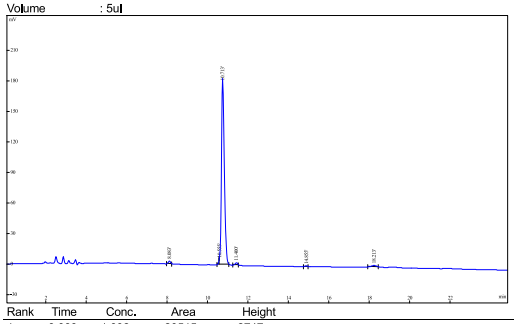
HPLC REPORT

Sample Description:

Structure	: #1 HR-30
Number	: 0200046
Lot #	: LT170116-LT051462
Column	: 4.6mm*250mm, SinoChrom ODS-BP
Mobile Phase	: A=0.1% TFA/Acetonitrile,
	: B=0.1%TFA/water,

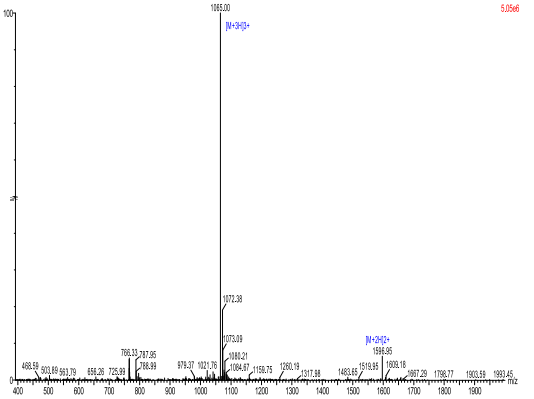
Gradient :	A	B
0.01min	30%	70%
25min	55%	45%
25.1min	100%	0%
30.0min	STOP	

Flow rate	: 1.0ml/min
Wavelength	: 220nm
Volume	: 5ul



Rank	Time	Conc.	Area	Height
1	8.083	1.092	20515	2747
2	10.555	0.5939	11156	5972
3	10.713	95.82	1799902	183655
4	11.400	1.009	18963	2200
5	14.855	0.2744	5155	794
6	18.213	1.213	22777	1377
Total		100	1878468	196745

Mass Spectrometry Report



Sample: 540410 **MW: 3189.69**

Item	Parameter	Item	Parameter
Probe:	ESI	Probe bias:	+4.5kv
Nebulizer Gas Flow:	1.5L/min	Detector:	1.5kv
CDL:	-20.0v	T.Flow:	0.2ml/min
CDL Temp:	250°C	B.conc:	50%H2O50%ACN
Block Temp:	200°C		

D-GLP1

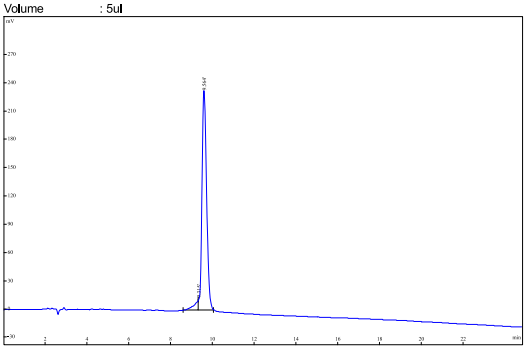
HPLC REPORT

Sample Description:

Structure	:#1 RH-29
Number	: 0200046
Lot #	:LT161017-LT540410
Column	: 4.6mm*250mm, SinoChrom ODS-BP
Mobile Phase	: A=0.1% TFA/Acetonitrile,
	: B=0.1%TFA/water,

Gradient :	A	B
0.01min	52%	48%
25min	77%	23%
25.1min	100%	0%
30.0min	STOP	

Flow rate	: 1.0ml/min
Wavelength	: 220nm
Volume	: 5ul



Rank	Time	Conc.	Area	Height
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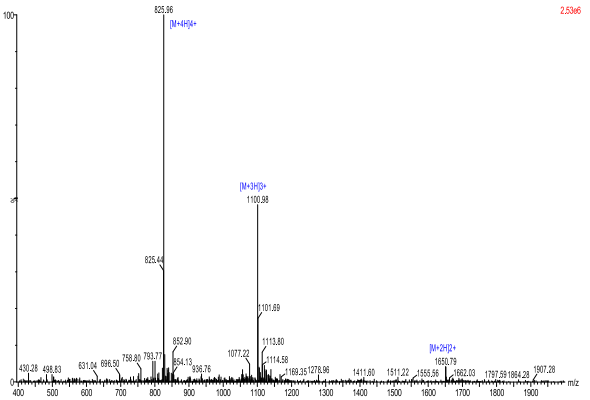
2	9.564	95.26	3572556	233031
Total		100	3750343	244435

Mass Spectrometry Report



Sample: 051462 **MW: 3298.69**

Item	Parameter	Item	Parameter
Probe:	ESI	Probe bias:	+4.5kv
Nebulizer Gas Flow:	1.5L/min	Detector:	1.5kv
CDL:	-20.0v	T.Flow:	0.2ml/min
CDL Temp:	250°C	B.conc:	50%H2O50%ACN
Block Temp:	200°C		

Fig. S2. HPLC and mass spectroscopy reports. These were carried out by Lifetein LLC and supplied along with the synthesized peptide.

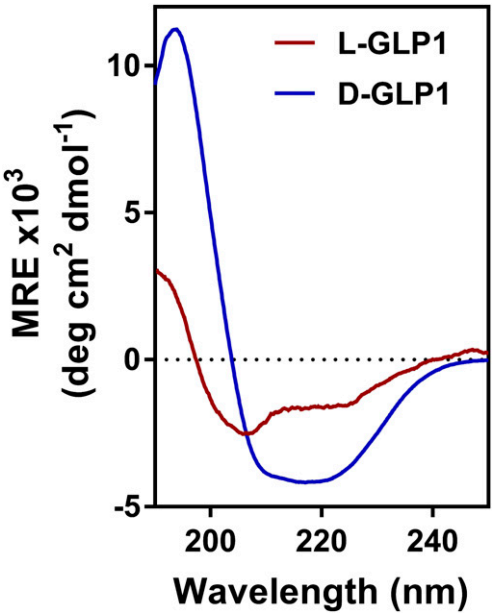


Fig. S3. CD spectra of L-GLP1 and D-GLP1 in water. Peptides were dissolved in water, and CD spectra was recorded. The D-GLP1 peptide spectrum has been inverted to allow for visual comparison with the L-GLP1 peptide spectrum.

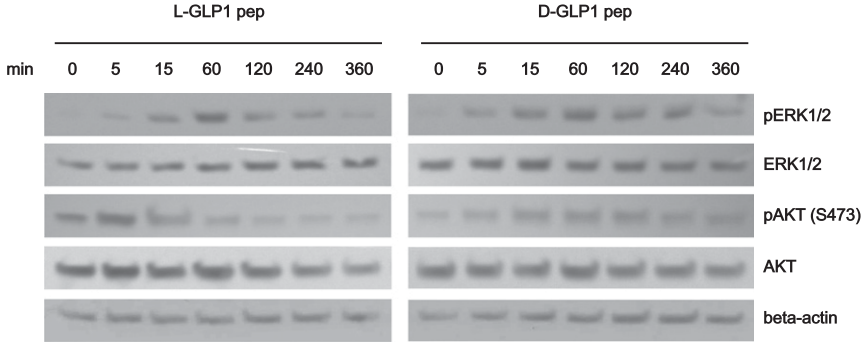


Fig. S4. GLP1 Western blots. HEK293 cells stably expressing GLP1R were stimulated with 10 μM of L- or D-GLP1 peptide at different time points. Proteins were resolved by SDS/PAGE and Western blotted using anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-AKT, or anti-AKT antibodies. The experiments were performed in triplicate. A representative blot is shown for each antibody.

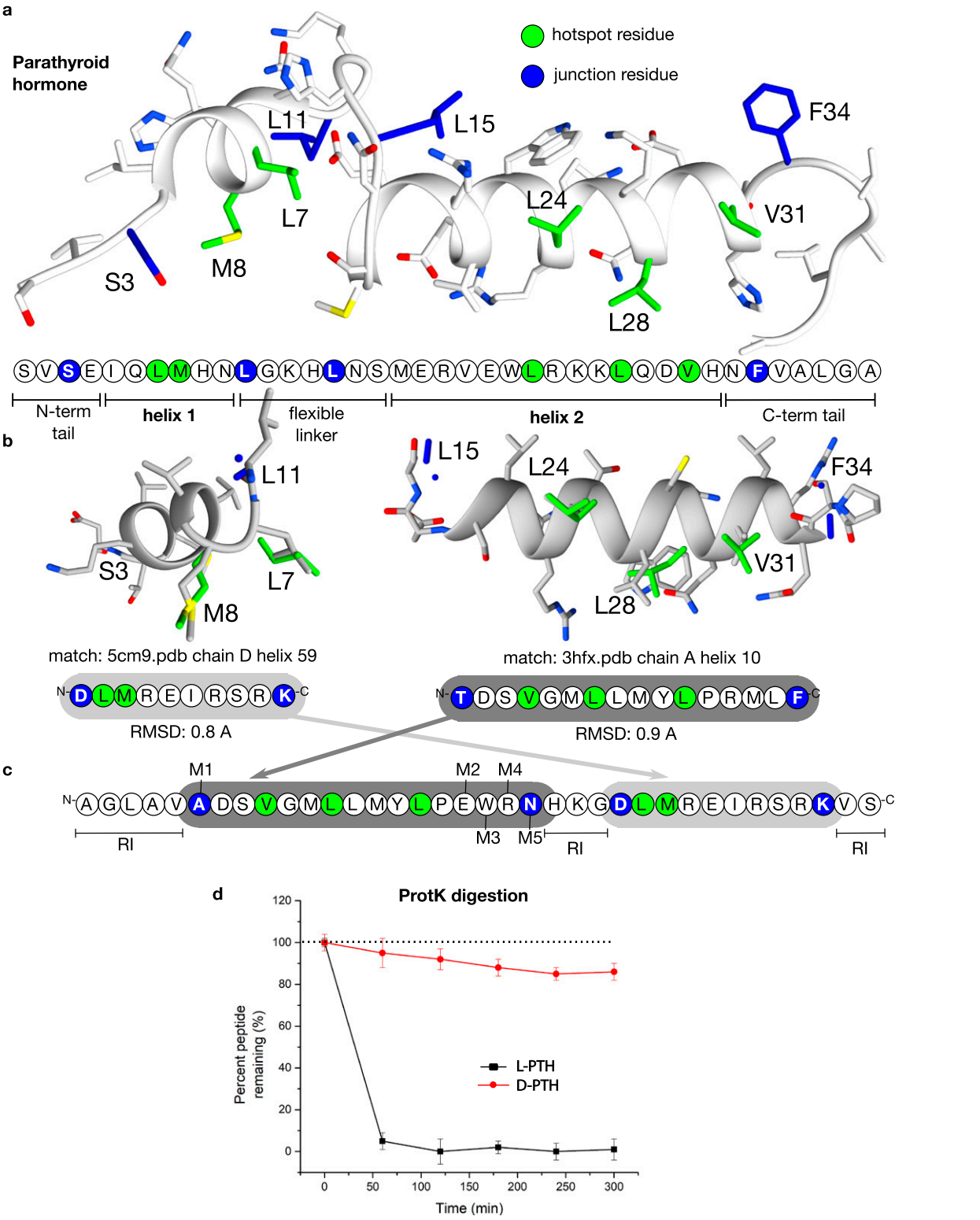


Fig. S5. Construction and protease degradation of (L)- and (D)-PTH peptides. (*A*) (L)-PTH structure and sequence with hotspots highlighted in green and junctions in blue. (*B*) (D)-PDB match structures and sequences. (*C*) Final (D)-PTH construction from match sequences and RI. (*D*) Quantification of remaining peptide post-ProtK treatment in 50-min intervals. Intensity of peptide bands were normalized to the intensity of the untreated peptide (T0) and converted to a percentage relative to T0. The (L)-enantiomeric form undergoes rapid degradation while the (D)-enantiomer persists after 5 h of treatment with ProtK. Error bars are reporting SE. Data represent the average of three independent experiments.