Supporting Information

Directed evolution identifies high affinity cystine-knot peptide agonists and antagonists of Wnt/β-catenin signaling

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Supporting Information Text

Materials and Methods.

Protein expression and purification

LRP6 constructs were expressed and purified as described previously (1). In short, histidine (His)-tagged proteins were secreted from *Trichoplusia ni* cells and purified by Ni NTA affinity and size exclusion chromatography with 50mM Tris pH 8.0, 300 mM NaCl as running buffer. Constructs containing an avi-tag were co-expressed with a plasmid containing BirA. Proteins for crystallization were isolated with an affinity column of immobilized anti-His-tag mAbs and eluted in 50 mM sodium acetate pH 4.6, 300 mM NaCl followed by size-exclusion chromatography (SEC) in 10 mM MES pH 5.5, 300 mM NaCl.

Peptide synthesis

CKPs were synthesized using standard 9-fluorenylmethoxycarbonyl protocols as described earlier (2). Unless stated otherwise N and C termini are unprotected. Disulfide linkages were established by dissolving 150-300 mg crude peptide in 20 ml DMSO, adding it to 450 ml H2O with 150 mg glutathione (reduced) and 75 mg oxidized glutathione, pH value was adjusted to 9.0 with NH_4OH. Mixture was incubated in a closed container for 24 h with stirring. After purification by RP-HPLC, peptide quality was checked by LC-MS, peptide identity and presence of 3 disulfide bonds were confirmed by MS. Most CKPs were shown to have >95% purity, only five CKPs (R77, R19, Lr-EET-3.5 F30dTyr, Lr-EET-3.5 H63Py, Lr-EET-3.5 N28dHis) showed lower purity. All peptides with low purity were not crucial to the outcome of this study as they were only assessed once and not followed upon. Compiled data for the peptide quality can be found in **Extended Data File 1**.

Bivalent CKP synthesis

Bivalent CKPs were synthesized by Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction using Tris(3-hydroxypropyltriazolyl-methyl)amine (THPTA) as Cu(I) stabilizing ligand. Reactions were carried out by dissolving the homobivalent linker bis-propargyl-PEG5/bis-propargyl-PEG13/bis-propargyl-PEG18 (1.0 equiv., Broadpharm BP-20660, BP-222514 and BP-22842) and Azidohomoalanine modified CKP (Lr-EET-3.5 NahA R12MeR, 2.0 equiv., about 6.5 mg) in 1-2 mL ammonium bicarbonate buffer solution (100 mM, pH = 9) containing copper(II) sulfate (10.0 equiv.), THPTA (20.0 equiv.) and ascorbic acid (25.0 equiv.). Conditions were optimized by varying solvent system, amount of copper catalyst and incubation time to yield the desired bivalent products. Reactions were stirred at room temperature and completed in 3-5 h as monitored by

LC-MS. After purification by RP-HPLC, bi-3.5-PEG5/bi-3.5-PEG13/bi-3.5-PEG18 purity was confirmed by LC-MS and lyophilized to obtain a white powder (about 2 mg each).

Phage Display

Display of EETI-II on M13 phage: EETI-II was displayed on the surface of M13 bacteriophage by modifying a previously described phagemid pS2202b. Standard molecular biology techniques were used to replace the fragment of pS2202d encoding Erbin PDZ domain with a DNA fragment encoding for EETI-II. The resulting phagemid (p8EETI-II) contained an open reading frame that encoded for the maltose binding protein secretion signal, followed by a gD tag and EETI-II, and ending with M13 major coat protein p8. *E. coli* harboring p8EETI-II were co-infected with M13-KO7 helper phage and cultures were grown in 30 ml 2YT medium supplemented with 50 μg/ml Carbenicillin and 25 μg/ml Kanamycin at 30 °C overnight. The propagated phage was purified according to standard protocols and re-suspended in 1 ml PBT buffer (PBS, 0.5% BSA and 0.1% Tween 20), resulting in the production of phage particles that encapsulated p8EETI-II DNA and displayed EETI-II. The display level was analyzed using a phage ELISA.

Library Construction and Sorting: Three libraries were constructed: Library 1, in which loop 1 (residues 3 - 8) was randomized with the degenerate codon encoding all natural amino acids except Cys at 6, 8 or 10 amino acids in length; or Library 2, in which loop 5 (residues 22 - 26) was randomized with the same set of degenerate codon with fixed length of 5 amino acids; or Library 3, in which both loop 1 (randomized with 6, 8, and 10 amino acids) and loop 5 (randomized with 5 amino acids) were simultaneously mutated with degenerate codon encoding for 19 amino acids. Oligonucleotides for mutagenesis were synthesized using custom mixes of trimer phosphoramidites encoding for 19 amino acids at equimolar concentration (Glen Research, Sterling, VA). The stop template is the single strand DNA of p8EETI-II containing three stop codons in the region spanning positions 3 - 26 and was used to construct all three libraries. The pool of three libraries contained ~3 x 10^{10} unique members.

Affinity maturation: A new library based on the most potent hit from the initial selections R1 was created by “soft-randomization” of loop 1. Soft randomization means that for each randomized nucleotide a mixture of 70% wild-type (wt) nucleotide and 10% of each of the other nucleotides was used in oligosynthesis, this will result in a library that is biased to carry roughly 50% wt amino acids at each position. Randomized oligos were ordered from IDT and the library was cloned on gene8 (g8) and gene3 (g3) of M13 bacteriophages as described previously (3). The library quality was assessed by sequencing of several random clones and found to be 74% (g8) and 58% (g3)

in frame with the expected distribution of nucleotides at randomized positions. The total diversity was determined to be $>10^{10}$ for both libraries. Phage pools were applied to four rounds of standard binding selections against avi-tagged LRP6 E1 (3). After the fourth selection round with the g3 library a very stringent off-rate selection was performed by preincubating the phage pool with biotinylated LRP6 E1 (10 nM, 30 min), then a 500-fold molar excess of unbiotinylated LRP6_E1 was added (1 h). After pull down LRP6 E1-phage complexes were washed for a total of 1 h. Since the enrichment disappeared after this round an additional "recovery round" with less stringent conditions (20 nM LRP6 E1 and 30 min total washing) and no off-rate selection was performed. 96 random clones after rounds 3 and 4 of the g8 library and rounds 3-6 of the g3 library were screened in a phage ELISA against LRP6 E1 and sequenced. Two to three CKPs from each screen with the highest signal over noise ratio were selected for synthesis.

Surface plasmon resonance (SPR)

A Biacore S200 instrument was used for all measurements in 50 mM Tris pH 7.5, 300 mM NaCl, 0.05% Triton X, 5% Glycerol. 300-500 RU of biotinylated LRP6 E1 was immobilized on a Streptavidin (SA) functionalized sensor chip (GE Healthcare). Different peptide concentrations were injected either in a multiple or single cycle experiment. All data were double-referenced by subtracting the signals from buffer injections and blank sensor spots and fitted to a 1:1 binding model in the instrument's software.

Different truncations of LRP6 extracellular domains were immobilized on SA sensor chips. DKK1 (R&D systems) and SOST were injected starting from 100 and 500 nM, respectively, with a series of 2-fold dilutions. For the competition experiments the chip surface was saturated with injection of Lr-EET-3.5 (500 nM) prior to the first injection and 500 nM CKP was added to every DKK1 or SOST dilution. Data were double referenced and fitted to a two state model in the instrument software.

Interaction of Lr-EET-3.5 with LRP5 was measured by immobilizing Lr-EET-3.5 on a CM5 chip using the amide coupling kit (both GE Healthcare) according to manufacturer's instructions. Data was recorded by injecting a dilution series of LRP5 (R&D Systems, 7344-LR), data processing and analysis was the same as above.

X-ray Crystallography

Purified protein was concentrated to 10 mg/ml and mixed with 5-fold molar excess of peptides in in 10 mM MES pH 5.5, 300 mM NaCl. Commercial crystallization screens (Hampton Research and Qiagen) were used to identify crystallization conditions. Crystals grew from a 1:2 mixture of

protein and 0.2 M DL-Malic acid pH 7.0, 20% w/v Polyethylene glycol 3,350 (Hampton Research), 12% glycerol was added to the mother liquor for cryo protection and crystals were snap frozen in liquid nitrogen. Diffraction data was collected at the Stanford Synchrotron Radiation Lightsource (SSRL) beamline 12-2 on a Pilatus 6M detector (Dectris). Data was processed with programs XDS (version 0.71), XSCALE (version Nov 1, 2016) and XDSCONV (version Nov 1, 2016) (4). Initial phases were obtained by molecular replacement with PHASER (version 7.0.021) (5) using chain A of PDBID 3SOV as search model. The structure was iteratively refined by model-building in COOT (version 0.8.9) (6, 7) followed by refinement with REFMAC5 (version 5.8) (8), BUSTER (version 2.11.6) (9) or Phenix-Refine (version 1.9_1692) (10). The data collection and refinements statistics are given in **SI Table 2**.

Wnt reporter assays

HEK293 cells with stably integrated firefly-luciferase-based Wnt reporter (TOPbrite, TB) (11) and pRL-SV40 Renilla luciferase (Promega) were maintained in a 5% CO_2 humidified incubator at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with nutrient mixture F12 (50:50), 10% FBS, 2 mM Glutamax (Gibco) and 40 µg/ml hygromycin (Cellgro). Cells were grown for at least 24 h before any experiments. 20,000 cells/well in 50 µl medium were seeded in each well of clear-bottom white polystyrene 96-well plates (Falcon) and incubated for 24 h. Cells were then transfected with Wnt-expressing constructs and Fugene HD (Promega; cDNA and Fugene were -glyceromixed in 10 µl OptiMEM (Gibco)) for 24-48 h, then peptide was added for 6 h. Alternatively, cells were treated simultaneously with recombinant proteins (WNT3a (5036-WN-010/CF, R&D systems) and/or RSPO2 (3266-RS-025/CF, R&D systems) and peptides and incubated for 6 h. For the LGK974 treatment, cells were pre-incubated with 100 nM LGK974 for 24 h. All peptide stock solutions were prepared in DMSO, the final DMSO concentration on cells was <1%. Read out was obtained with 50 µl of Dual-Glo Luciferase Assay system (Promega, E2940) according to the manufacturer's instructions on a Perkin Elmer EnVision multilabel reader. The ratios of firefly luminescence to Renilla luminescence were calculated, background-subtracted (in reference to untransfected samples) and normalized untreated samples. Cell lines were obtained from the Genentech gCell laboratory and were tested for mycoplasma contamination and authenticated by single-nucleotide polymorphism (SNP) analysis.

Activation of WNT3a signaling by bivalent Lr-EET-3.5 were assessed by stimulating HEK293 TB cells with 0, 10 and 50 ng/ml recombinant WNT3a (R&D Systems) and dilution series of bivalent CKPs and monovalent Lr-EET-3.5 R12MeR control for 6h at 37 °C. Synergy between bivalent Lr-EET-3.5 and RSPO2 was measured in HEK293 TB cells stimulated with 0, 5, 10 and 20 ng/ml

recombinant WNT3a and a dilution series of bi-3.5-PEG5 in the presence of 20 ng/ml recombinant RSPO2 (R&D Systems) for 6h at 37 °C. Luciferase read-out was obtained as described above. For each experiment, three independent assays were performed with 5 technical replicates of each datapoint.

RNA sequencing

RNA-seq libraries were prepared using TruSeq stranded Total RNA library prep kit (Illumina, CA). The libraries were sequenced on Illumina HiSeq 2500 sequencers to obtain on average 34 million single-end reads (50 bp) per sample. RNA-seq reads were first aligned to ribosomal RNA sequences to remove ribosomal reads. The remaining reads were aligned to the mouse reference genome (NCBI build 38) using GSNAP (version 2013-10-10) (12), allowing a maximum of two mismatches per 50-b sequence (parameters: '-M 2 -n 10 -B 2 -i 1 -N 1 -w 200000 -E 1–pairmax-rna = 200000–clip-overlap'). Transcript annotation was based on the RefSeq database (NCBI annotation release 104). To quantify gene expression levels, the number of reads mapped to the exons of each RefSeq gene was calculated. Read counts were scaled by library size and quantile-normalized, and precision weights were calculated using the 'voom' R package (13). Subsequently, differential expression analysis on the normalized count data was performed using the 'limma' R package (14) by contrasting antibodies, CKPs or LGK974 treated samples with DMSO-treated samples at 24 h. Gene expression was obtained in the form of normalized reads per kb gene model per million total (nRPKM) values, as described earlier (15).

Intestinal organoids

Mouse organoids were established from isolated crypts collected from the entire length of the small intestine and maintained as previously described (16). Mouse handling was performed according to the animal use guidelines of Genentech, a member of the Roche Group, and the Institutional Animal Care and Use Committee, conforming to California State legal and ethical practices. Organoids were passaged at least twice per week and grown using IntestiCult Organoid growth medium (cat. no. 06005; StemCell Technologies) or advanced DMEM/F12 supplemented with EGF, Noggin and Rspondin. Organoids were treated with the corresponding antibodies (1ug/ml), CKPs (10uM) or LGK974 (1uM) for 24h and then imaged or processed for downstream analysis. Organoids were imaged using a Nikon Eclipse Ti scope with a Nikon Plan Fluor 10 × Ph1 DLL objective lens using an Andor Neo camera (1 × 1 binning; 200-ms exposure), and images were acquired using NIS Elements (v. 4.50 64-bit; Nikon).

Osteogenic assays

MC3T3-E1 (subclone 14) were obtained from ATCC (ATCC® CRL-2594™). Cells were propagated in Alpha Minimum Essential Medium (Gibco, A1049001) supplemented with 10% fetal bovine serum and 1x Antibiotic-Antimycotic (Gibco, 15240062) (referred to as om- condition) in a 37°C incubator with 5% CO_2. Cells were seeded into 24 well plates in a volume of 500 µl at a density of 10,000 cells/cm^2. To induce differentiation, 50 µg/ml ascorbate-2-phosphate and 10 mM β-glycerophosphate were added to the medium (om+). Treatments with LRP6 binding reagents were performed in om+ medium and medium was changed every 2-3 days. Staining of the cell was performed every 7 days by fixing monolayers with PBS + 4% paraformaldehyde for 20 min at room temperature. After washing with PBS, 1% alizarin red S (in water, pH 4.2) was applied for 30 min at room temperature and washed 3 times with 50% ethanol. After air drying, stained cell monolayers were imaged on a scanner. Quantification of stain for calcified nodules was done by ImageJ. The total nodule area above a threshold defined by the background from om- wells was measured.

Quantitative RT-PCR analysis

MC3T3-E1 cells were treated as described above and RNAs were isolated using the RNeasy kit (Qiagen). The real-time PCR reactions were performed with the TaqMan RNA-to-C_T 1-step kit (Applied Biosystems) by preparing samples as follows (total 10 µl reaction): 5 µl of TaqMan RT-PCR mix, 0.5 µl of TaqMan gene expression probe, 0.25 µl of TaqMan RT enzyme mix, 50 ng of RNA, 2.25 µl of nuclease-free water. The reaction was initiated at 48 °C for reverse transcription for 15 min, followed by 45 amplification cycles (activation of AmpliTaq Gold DNA polymerase at 95 °C for 10 min, denaturation at 95 °C for 15 sec, and anneal/extend at 96 °C for 1 min). The assay was run on QuanStudio 7 Flex Real-Time PCR systems. Relative RNA levels were calculated using the $\Delta\Delta C_T$ method and normalized to mouse actin level within the same sample and further normalized to the sample from om- cells. Probe (*Bglap* (Mm03413826_mH) used for the assay was obtained from Thermofisher.

Human duodenal organoids

Human primary organoids experiemnts were performed at Ocello, B.V. Briefly, cells were seeded as 100 organoid fragments per well in 384 wells plates in a hydrogel in 3D in organoid media. Immediately after seeding, cultures were exposed to different combinations of WNT3a and RSPO3 and CKP. At day two, medium and treatments were replaced. After five days, cultures

were fixed, stained for nuclei and actin cytoskeleton and imaged with 4x magnification. Image analysis of the 3D stacks was performed using Ominer® image analysis software.

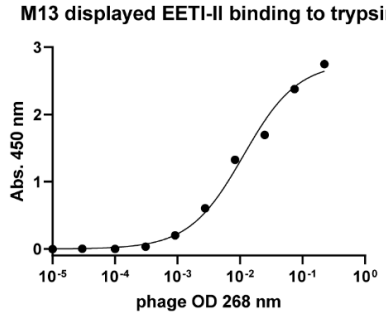
Isolating lung cells

Lungs were isolated from 4-6 week old *Sftpc Cre ERT2; LSL TdTomato* animals and digested 400 µg/ml Liberase DL (Sigma 5401127001) and 100 µg/ml elastase (Worthington LS006365) in RPMI (Gibco 72400120) in a gentleMACS c-tube (Miltenyi 130-096-334) for 20 min. The cells were then filtered through a 100 µm filter, pelleted (300 x g, 5 min, 4 °C), and resuspended in ACK red blood cell lysis buffer (Gibco A1049201) for 3 min, after which the buffer was inactivated by adding PBS with 2% fetal bovine serum. Cells were then filtered through a 70 µm strainer (Fisherbrand 22363548), pelleted again (300 x g, 5 min, 4°C), and resuspended in magnetic activated cell sorting (MACS) buffer (0.5% BSA, 2 mM EDTA in PBS) with mouse FcR Blocking Reagent (Miltenyi 130-059-901) to block non-specific binding of antibodies. Endothelial and immune cells were depleted by incubating cells with anti-CD31 (Miltenyi 130-097-418), anti-CD45 (Miltenyi 130-052-301) and anti F4/80-beads (Miltenyi 130-110-443) and passed through a LS magnetic column (Miltenyi 130-042-401). Mouse handling was performed according to the animal use guidelines of Genentech, a member of the Roche Group, and the Institutional Animal Care and Use Committee, conforming to California State legal and ethical practices.

Lung alveolospheres

Cells were then resuspended in growth factor-reduced Matrigel (Corning 354230) at a concentration of 1 million cells/ml then seeded in 35 µl domes in 24-well culture plates. After 30 min at 37 °C to solidify the Matrigel. 500uL of culture medium was added to each well (ADMEM/ F12 supplemented with 1% penicillin/streptomycin, HEPES, GlutaMAX), with 2% B27 supplement (Gibco 17504044), 100 ng/ml Noggin (Peprotech 250-38), 50 ng/ mL EGF(Peprotech 315-09), 100 ng/mL FGF-10 (Preprotech 100-26), 100 ng/ml FGF-7 (Preprotech 450-60), 1.25 mM N-acetylcysteine (Sigma A9165-5G), 5 µM A8301 (Tocris Bioscience 2939) and 1 µg/ml JAG peptide (Anaspec AS-61298), 20 nM LGK974 (Selleck Chem S7143) and the indicated CKP (48 nM) or WNT3a ligands (50 ng/ml). Cells were cultured for 2 weeks and media was changed biweekly. At the end of the experiment, the 24-well culture plate was imaged using an ImageXpress system (Molecular Devices).

a



b

campaign 1

peptide	Sequence (loop1 / loop5)	n	s/n ratio	ELISA	IC_{50} (Wnt1)	IC_{50} (Wnt3a)
	loop1 loop5					
EETI	GCPRILMR CKQDSDCLAGCVCGPNGFCG	-	-	-	n.i.	n.i.
	GC RTNRVKGG CKQDSDCLAGCVCGPNGFCG	1	45.5	3.2		
	GC GSNKIRPR CKQDSDCLAGCVCGPNDFCG	1	43.5	3.2		
	GC ANRVKRTS CKQDSDCLAGCVCGPNGFCG	1	42.0	3.4		
R77	GC SNRVKVRA CKQDSDCLAGCVCGPNGFCG	1	42.0	3.3	23 µM	n.i.
	GC VNRVRG CKQDSDCLAGCVCSGGRDCG	1	41.6	3.4		
	GC MNHVKARR CKQDSDCLAGCVCGPNGFCG	1	40.2	2.9		
	GC ENRTKG CKQDSDCLAGCVCGFRGTCG	1	38.7	3.1		
	GC VNSVKR CKQDSDCLAGCVCSRGVNCG	1	37.7	3.3		
	GCRSGNTIRKRE CKQDSDCLAGCVCGGPGGCG	1	37.6	3.0		
	GC RGNIIK CKQDSDCLAGCVCNESRGCG	1	37.6	3.2		
	GCASSNSIRQGW CKQDSDCLAGCVCGPKSNCG	1	37.4	3.3		
R19	GC RNSIKR CKQNSDCLAGCVCSVGHGCG	1	37.4	3.1	5 µM	n.i.
	GC RSNRIR CKQDSDCLAGCVCYGHGDCG	1	36.8	2.6		
	GC SNYVKR CKQDSDCLAGCVCSPGGRCG	1	35.9	3.1		
	GC VNKIKG CKQDSDCLAGCVCGVEGRCG	1	35.7	3.0		
	GC GNKIRA CKQDSDCLAGCVCRDRVGCG	1	33.7	2.8		
	GC SRNSIK CKQDSDCLAGCVCASGSSCG	1	31.1	3.4		
	GC VSNRVNKG CKQDSDCLAGCVCGPNGFCG	1	29.0	3.3		
	GC NRIRNS CKQDSDCLAGCVCGRGDYCG	1	26.3	2.0		
	GCRSVNKI CKQDSDCLAGCVCGSSRNCG	1	25.4	2.8		
	GC RANRVSGR CKQDSDCLAGCVCGPNGFCG	1	18.3	1.7		
	GC RSNKLREARG CKQDSDCLAGCVCGSRQDCG	1	6.8	0.6		

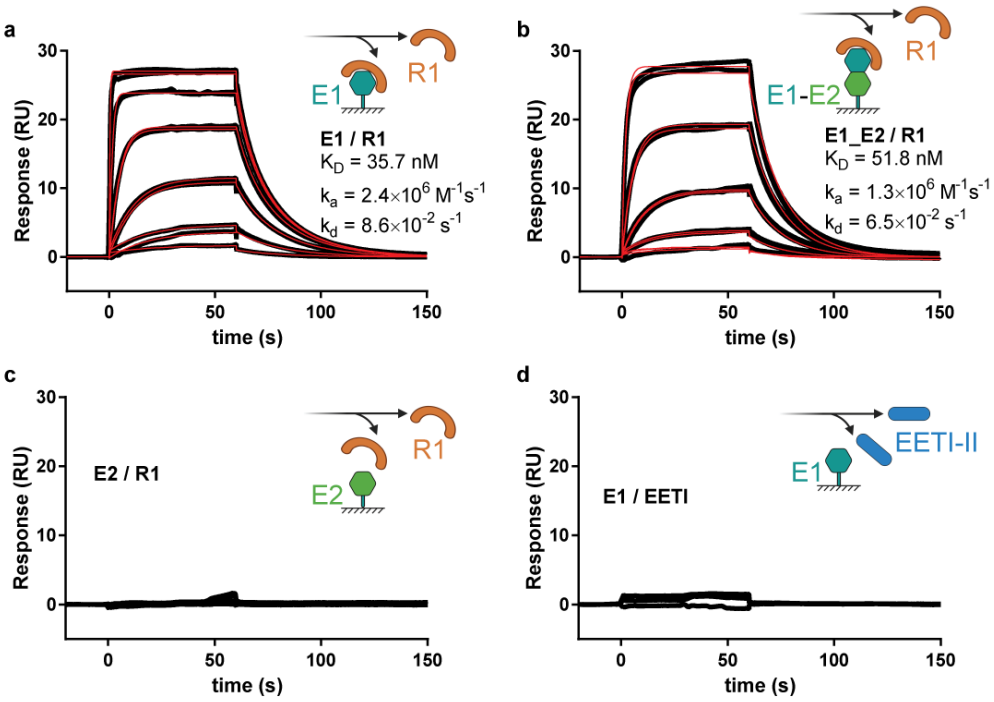
campaign 2

peptide	Sequence (loop1 / loop5)	n	s/n ratio	ELISA	IC_{50} (Wnt1)	IC_{50} (Wnt3a)
	loop1 loop5					
R1	GCQAINRVKRQR CKQDSDCLAGCVCGPNGFCG	91	56.5	3.2	217 nM	n.i.

c

SI Fig. 1

Analysis of initial CKP phage display selections. (a) EETI-II displayed on M13 bacteriophages binds to trypsin as measured by phage ELISA. (b) Sequence alignment and ELISA results of phage clones isolated after round four of two phage display campaigns. n: number of clones with identical sequences isolated from 96 random clones. s/n ratio: ratio between ELISA signal of wells coated with E1-E2 and blank wells. ELISA: absolute ELISA signal in positive wells. (c) Sequence logo obtained from aligned loop 1 sequences from campaign 1. Peptide names are included for the CKPs that were synthesized and characterized for cellular potency in Wnt1- and Wn3a-mediated signaling assay in HEK293-TB cells. IC_{50} values were derived from three independent experiments, each run with technical triplicates. n.i., no inhibition at 100 µM concentration.



SI Fig. 2

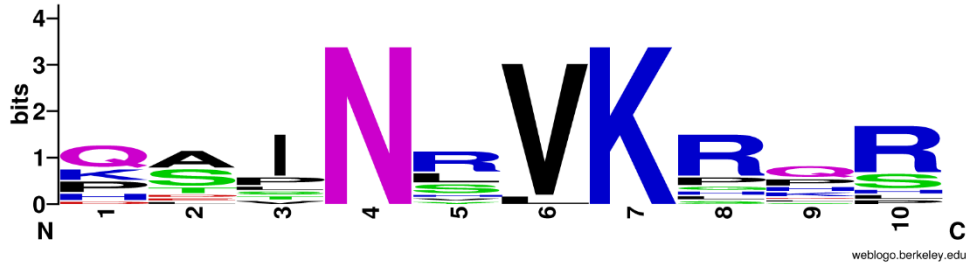
LRP6 domain specificity determination of R1 CKP by surface plasmon resonance. Different concentrations of R1 were injected over surfaces with immobilized E1 (a), E1-E2 (b) and E2 (c). EETI-II was used as negative control and injected over immobilized E1 (d). The maximum concentration of EETI-II and R1 used is 1 µM. Responses are shown in black and fits to a 1:1 model in red where applicable.

a

Round 3, gene 8

peptide	Sequence	n	s/n ratio
	loop1 loop5		
EETI	G C P R I L M R C K Q D S D C L A G C V C G P N G F C G	-	-
R1	G C Q A I N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	-	-
Lr-EET-6	G C K A I N L L K P Q R C K Q D S D C L A G C V C G P N G F C G	1	23.6
Lr-EET-7	G C Q S P N R V K H P R C K Q D S D C L A G C V C G P N G F C G	1	22.8
Lr-EET-8	G C Q S V N V V K R P R C K Q D S D C L A G C V C G P N G F C G	1	22.4
	G C P S I N R V K G Q S C K Q D S D C L A G C V C G P N G F C G	1	16.6
	G C P S L N R V K L Q P C K Q D S D C L A G C V C G P N G F C G	1	14.0
	G C H T I N S V K R S S C K Q D S D C L A G C V C G P N G F C G	1	13.8
	G C Q A I N R V K R P H C K Q D S D C L A G C V C G P N G F C G	1	13.5
	G C K D I N S V K R L R C K Q D S D C L A G C V C G P N G F C G	1	13.0
	G C P T I N H V K R K R C K Q D S D C L A G C V C G P N G F C G	1	11.7
	G C Q L S N L V K R Q S C K Q D S D C L A G C V C G P N G F C G	1	11.6
	G C Q A I N R V K R D R C K Q D S D C L A G C V C G P N G F C G	1	11.6
	G C K E I N Y V K P H R C K Q D S D C L A G C V C G P N G F C G	1	11.4
	G C Q S I N R V K S Q R C K Q D S D C L A G C V C G P N G F C G	1	11.0
	G C H A P N S V K R H R C K Q D S D C L A G C V C G P N G F C G	1	10.8
	G C D A T N L V K R K L C K Q D S D C L A G C V C G P N G F C G	1	10.6
	G C H A Y N R V K G R P C K Q D S D C L A G C V C G P N G F C G	1	10.5
	G C H E I N R V K R R N C K Q D S D C L A G C V C G P N G F C G	1	10.4
	G C P E N N R V K S Q R C K Q D S D C L A G C V C G P N G F C G	1	10.4
	G C Q D D N R M K L R R C K Q D S D C L A G C V C G P N G F C G	1	10.2
	G C H S I N S T K R Q R C K Q D S D C L A G C V C G P N G F C G	1	9.9
	G C Q S I N Q V K H H L C K Q D S D C L A G C V C G P N G F C G	1	9.9
	G C H A V N R V K R P L C K Q D S D C L A G C V C G P N G F C G	1	9.1
	G C Q V I N T I K R T R C K Q D S D C L A G C V C G P N G F C G	1	8.6
	G C Q S Q N S V K R K G C K Q D S D C L A G C V C G P N G F C G	1	8.5
	G C H A I N A V K R Q S C K Q D S D C L A G C V C G P N G F C G	1	8.4

b



SI Fig. 3

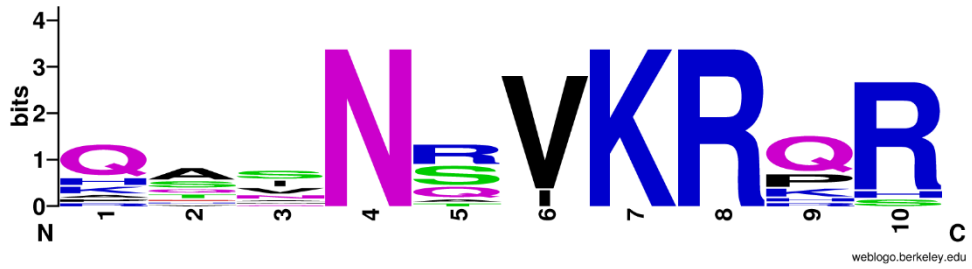
Sequencing results of round 3 (gene8) affinity maturation of R1 CKP. (a) Sequence alignment and ELISA results of phage clones isolated after round 3 of affinity maturation on gene8. n: number of clones with identical sequences isolated from 96 random clones. s/n ratio: ratio between ELISA signal of wells coated with E1-E2 and blank wells. ELISA: absolute ELISA signal in positive wells. (b) Sequence logo obtained from aligned loop 1 sequences

a

Round 4, gene 8

peptide	Sequence	n	s/n ratio
	loop1 loop5		
EETI	G C P R I L M R C K Q D S D C L A G C V C G P N G F C G	-	-
R1	G C Q A I N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	-	-
Lr-EET-1	G C Q S I N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	2	28.7
Lr-EET-2	G C K Q M N Q V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	23.9
Lr-EET-3	G C R A S N A V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	21.4
	G C K E V N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	20.3
	G C P T V N T I K R K R C K Q D S D C L A G C V C G P N G F C G	1	18.8
	G C A Q S N Q V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	17.6
	G C Q A I N S I K R H R C K Q D S D C L A G C V C G P N G F C G	1	16.6
	G C Q S Q N S V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	15.2
	G C Q A S N Q V K R R H C K Q D S D C L A G C V C G P N G F C G	1	13.6
	G C Q A N N S V K R P R C K Q D S D C L A G C V C G P N G F C G	1	13.4
	G C H T A N S V K R K S C K Q D S D C L A G C V C G P N G F C G	1	12.9
	G C H A V N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	12.8
	G C Q V N N R V K R P R C K Q D S D C L A G C V C G P N G F C G	1	12.6
	G C Q H S N S V K R P R C K Q D S D C L A G C V C G P N G F C G	1	12.5
	G C Q D I N R I K R H R C K Q D S D C L A G C V C G P N G F C G	1	12.4
	G C K M S N H V K R K P C K Q D S D C L A G C V C G P N G F C G	1	12.3
	G C Q A I N R V K R Q L C K Q D S D C L A G C V C G P N G F C G	1	12.3
	G C Q A T N H V K R H R C K Q D S D C L A G C V C G P N G F C G	1	12.2
	G C Q A I N R V K R D R C K Q D S D C L A G C V C G P N G F C G	1	12.0
	G C P D I N R V K R P R C K Q D S D C L A G C V C G P N G F C G	1	11.6
	G C Q A S N S V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	11.4
	G C Q A S N R V K R E H C K Q D S D C L A G C V C G P N G F C G	1	11.2
	G C D S I N R V K R P R C K Q D S D C L A G C V C G P N G F C G	1	10.8
	G C L T S N R V K R H H C K Q D S D C L A G C V C G P N G F C G	1	10.5
	G C P S L N R V K R H I C K Q D S D C L A G C V C G P N G F C G	1	10.2

b



SI Fig. 4

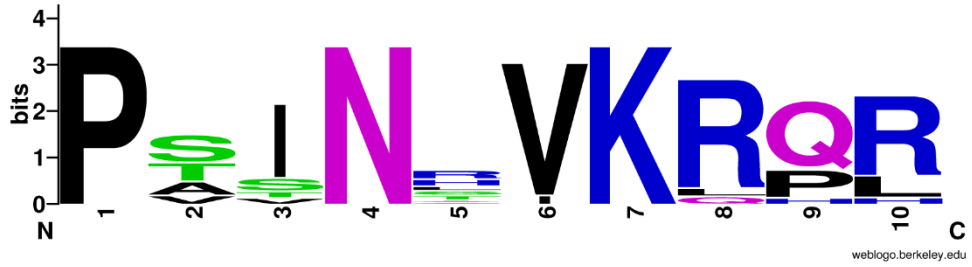
Sequencing results of round 4 (gene8) affinity maturation of R1 CKP. (a) Sequence alignment and ELISA results of phage clones isolated after round 4 of affinity maturation on gene8. n: number of clones with identical sequences isolated from 96 random clones. s/n ratio: ratio between ELISA signal of wells coated with E1-E2 and blank wells. ELISA: absolute ELISA signal in positive wells. (b) Sequence logo obtained from aligned loop 1 sequences

a

Round 3, gene 3

peptide	Sequence	n	s/n ratio
	loop1 loop5		
EETI	G C P R I L M R C K Q D S D C L A G C V C G P N G F C G	-	-
R1	G C Q A I N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	-	-
Lr-EET-3.6	G C P S I N S V K R H H C K Q D S D C L A G C V C G P N G F C G	1	14.8
Lr-EET-3.7	G C P T I N H V K R Q R C K Q D S D C L A G C V C G P N G F C G	2	13.2
	G C P T I N R V K Q P R C K Q D S D C L A G C V C G P N G F C G	1	12.1
	G C P S I N H V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	12.0
	G C P V I N R V K R Q L C K Q D S D C L A G C V C G P N G F C G	1	11.8
	G C P A T N A I K R Q R C K Q D S D C L A G C V C G P N G F C G	1	11.5
	G C P S I N Q V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	10.9
	G C P A V N T V K R P R C K Q D S D C L A G C V C G P N G F C G	1	10.7
	G C P S I N R V K L P R C K Q D S D C L A G C V C G P N G F C G	1	10.3
	G C P S S N L V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	9.7
	G C P V S N S V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	9.6
	G C P S I N T V K R P R C K Q D S D C L A G C V C G P N G F C G	1	9.5
	G C P T I N L V K R Q L C K Q D S D C L A G C V C G P N G F C G	1	9.1
	G C P A I N R V K R Q L C K Q D S D C L A G C V C G P N G F C G	1	9.0
	G C Q A V N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	8.8
	G C Q A T N R V K R Q H C K Q D S D C L A G C V C G P N G F C G	1	8.7
	G C P L D N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	8.6
	G C Q V D N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	8.5
	G C Q A I N L V K R P R C K Q D S D C L A G C V C G P N G F C G	1	8.5
	G C P A S N H V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	8.2
	G C Q A I N R I K R Q A C K Q D S D C L A G C V C G P N G F C G	1	8.2
	G C Q S V N R V K R Q H C K Q D S D C L A G C V C G P N G F C G	1	8.0
	G C Q A I N R V K R Q L C K Q D S D C L A G C V C G P N G F C G	1	7.4
	G C P T I N R V K R D H C K Q D S D C L A G C V C G P N G F C G	1	7.4
	G C P A I N R V K R Q V C K Q D S D C L A G C V C G P N G F C G	1	7.3

b



SI Fig. 5

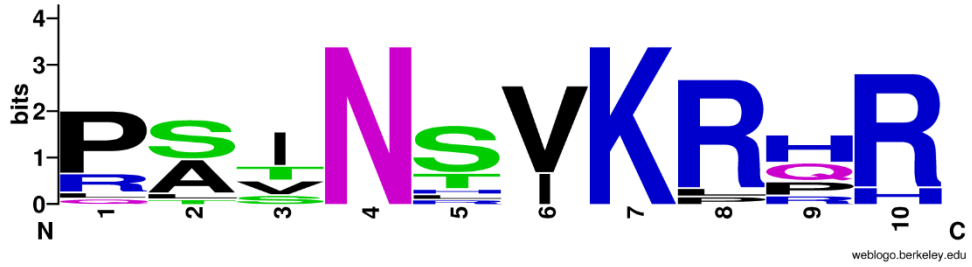
Sequencing results of round 3 (gene3) affinity maturation of R1 CKP. (a) Sequence alignment and ELISA results of phage clones isolated after round 3 of affinity maturation on gene3. n: number of clones with identical sequences isolated from 96 random clones. s/n ratio: ratio between ELISA signal of wells coated with E1-E2 and blank wells. ELISA: absolute ELISA signal in positive wells. (b) Sequence logo obtained from aligned loop 1 sequences

a

Round 4, gene 3

peptide	Sequence	n	s/n ratio
	loop1 loop5		
EETI	G C P R I L M R C K Q D S D C L A G C V C G P N G F C G	-	-
R1	G C Q A I N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	-	-
Lr-EET-3.8	G C P T I N S V K R H R C K Q D S D C L A G C V C G P N G F C G	1	11.9
	G C P S I N S V K R H R C K Q D S D C L A G C V C G P N G F C G	3	10.8
Lr-EET-3.9	G C P S I N T V K R P R C K Q D S D C L A G C V C G P N G F C G	1	10.8
	G C R S V N S V K R P R C K Q D S D C L A G C V C G P N G F C G	1	10.6
	G C Q A T N R V K L R R C K Q D S D C L A G C V C G P N G F C G	1	10.3
	G C P S I N H V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	9.8
	G C P L V N T V K R P R C K Q D S D C L A G C V C G P N G F C G	1	9.3
	G C R A V N S I K R Q H C K Q D S D C L A G C V C G P N G F C G	1	8.9
	G C P A I N S I K R H R C K Q D S D C L A G C V C G P N G F C G	1	7.5
	G C P A S N L V K P R R C K Q D S D C L A G C V C G P N G F C G	1	7.2
	G C P S S N S V K R H R C K Q D S D C L A G C V C G P N G F C G	1	7.2
	G C R A T N S I K R Q H C K Q D S D C L A G C V C G P N G F C G	1	7.2
	G C L A T N T I K R Q R C K Q D S D C L A G C V C G P N G F C G	1	7.1
	G C P M V N S V K R R H C K Q D S D C L A G C V C G P N G F C G	1	6.9
	G C P S I N S V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	6.9
	G C P M I N H I K R P R C K Q D S D C L A G C V C G P N G F C G	1	6.8
	G C Q A A N R V K R Q L C K Q D S D C L A G C V C G P N G F C G	1	6.6
	G C Q A V N R V K R Q Y C K Q D S D C L A G C V C G P N G F C G	1	6.4
	G C P T I N V V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	6.3
	G C Q S I N Q I K R Q R C K Q D S D C L A G C V C G P N G F C G	1	6.2
	G C P V I N H V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	6.0
	G C P A I N Y V K R H R C K Q D S D C L A G C V C G P N G F C G	1	6.0
	G C P A K N L V K R H L C K Q D S D C L A G C V C G P N G F C G	1	5.9
	G C P L N N L V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	5.9
	G C P S I N L V K R P R C K Q D S D C L A G C V C G P N G F C G	1	5.8

b



SI Fig. 6

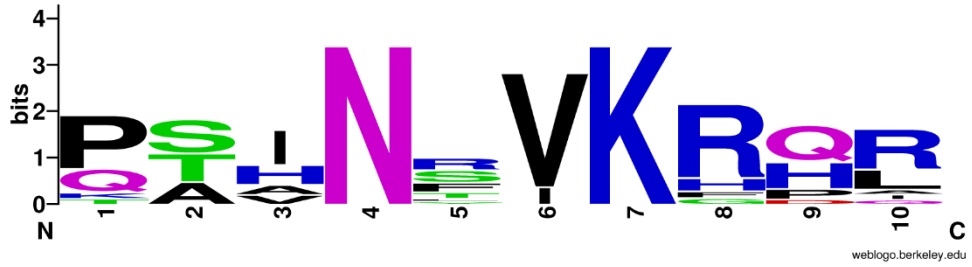
Sequencing results of round 4 (gene3) affinity maturation of R1 CKP. (a) Sequence alignment and ELISA results of phage clones isolated after round 4 of affinity maturation on gene3. n: number of clones with identical sequences isolated from 96 random clones. s/n ratio: ratio between ELISA signal of wells coated with E1-E2 and blank wells. ELISA: absolute ELISA signal in positive wells. (b) Sequence logo obtained from aligned loop 1 sequences

a

Round 5, gene 3

peptide	Sequence	n	s/n ratio
	loop1 loop5		
EETI	G C P R I L M R C K Q D S D C L A G C V C G P N G F C G	-	-
R1	G C Q A I N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	-	-
Lr-EET-3.1	G C K T I N R V K R D R C K Q D S D C L A G C V C G P N G F C G	1	20.9
Lr-EET-3.2	G C P T I N T I K R Q R C K Q D S D C L A G C V C G P N G F C G	1	12.1
Lr-EET-3.3	G C P S I N R V K R Q A C K Q D S D C L A G C V C G P N G F C G	1	11.5
	G C P S I N R V K R Q Q C K Q D S D C L A G C V C G P N G F C G	1	10.6
	G C T T H N F V K H H L C K Q D S D C L A G C V C G P N G F C G	1	9.4
	G C P S I N T V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	9.2
	G C P S I N S V K R P R C K Q D S D C L A G C V C G P N G F C G	1	9.2
	G C Q A A N L V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	9.1
	G C P S I N S I K R P R C K Q D S D C L A G C V C G P N G F C G	1	8.9
	G C P T V N S V K R Q R C K Q D S D C L A G C V C G P N G F C G	2	8.8
	G C Q A H N F V K H H I C K Q D S D C L A G C V C G P N G F C G	1	8.5
	G C P A A N R V K R H L C K Q D S D C L A G C V C G P N G F C G	1	8.5
	G C Q A H N Y V K F H L C K Q D S D C L A G C V C G P N G F C G	1	8.5
	G C Q S H N F V K G H L C K Q D S D C L A G C V C G P N G F C G	1	8.4
	G C P V S N S V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	8.3
	G C P S V N L V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	8.3
	G C P S I N H V K R H H C K Q D S D C L A G C V C G P N G F C G	1	8.0
	G C P T I N T V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	7.7
	G C P S I N R V K R Q S C K Q D S D C L A G C V C G P N G F C G	1	7.6
	G C P V I N S V K R H R C K Q D S D C L A G C V C G P N G F C G	1	7.6
	G C P S I N S V K R H R C K Q D S D C L A G C V C G P N G F C G	1	7.5
	G C P T I N R V K R H L C K Q D S D C L A G C V C G P N G F C G	1	7.5
	G C E N H N Y V K Y H R C K Q D S D C L A G C V C G P N G F C G	1	7.5
	G C P S T N S V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	7.3
	G C P S I N S V K R Q L C K Q D S D C L A G C V C G P N G F C G	1	7.3

b



SI Fig. 7

Sequencing results of round 5 (gene3) affinity maturation of R1 CKP. (a) Sequence alignment and ELISA results of phage clones isolated after round 5 of affinity maturation on gene3. n: number of clones with identical sequences isolated from 96 random clones. s/n ratio: ratio between ELISA signal of wells coated with E1-E2 and blank wells. ELISA: absolute ELISA signal in positive wells. (b) Sequence logo obtained from aligned loop 1 sequences

a

Round 6, gene 3

peptide	Sequence	n	s/n ratio
	loop1 loop5		
EETI	G C P R I L M R C K Q D S D C L A G C V C G P N G F C G	-	-
R1	G C Q A I N R V K R Q R C K Q D S D C L A G C V C G P N G F C G	-	-
Lr-EET-3.4	G C Q S H N Y V K H H L C K Q D S D C L A G C V C G P N G F C G	2	12.6
	G C Q V H N Y V K H H L C K Q D S D C L A G C V C G P N G F C G	1	11.8
	G C Q A H N Y V K H H L C K Q D S D C L A G C V C G P N G F C G	3	11.7
	G C P T I N Q V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	11.5
	G C P S D N R V K R P R C K Q D S D C L A G C V C G P N G F C G	1	11.5
Lr-EET-3.5	G C Q S N H I L K H N R C K Q D S D C L A G C V C G P N G F C G	3	11.3
	G C Q A H N F V K H H I C K Q D S D C L A G C V C G P N G F C G	2	10.9
	G C P L S N S V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	10.3
	G C P A I N K V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	9.9
	G C Q A H N F V K H H L C K Q D S D C L A G C V C G P N G F C G	3	9.6
	G C P T I N R V K R Q L C K Q D S D C L A G C V C G P N G F C G	1	9.6
	G C P M I N S V K R H R C K Q D S D C L A G C V C G P N G F C G	1	9.5
	G C Q A H N H V K H H I C K Q D S D C L A G C V C G P N G F C G	1	9.4
	G C P A S N A V K R R H C K Q D S D C L A G C V C G P N G F C G	1	9.2
	G C P S I N S V K R R L C K Q D S D C L A G C V C G P N G F C G	1	9.1
	G C P S I N S I K R K F C K Q D S D C L A G C V C G P N G F C G	1	9.0
	G C K S V N S V K R K Q C K Q D S D C L A G C V C G P N G F C G	1	9.0
	G C Q A H N Y V K F H L C K Q D S D C L A G C V C G P N G F C G	2	8.9
	G C P V I N S V K R Y K C K Q D S D C L A G C V C G P N G F C G	1	8.8
	G C E N H N Y V K Y H R C K Q D S D C L A G C V C G P N G F C G	1	8.4
	G C P T I N H V K R Q R C K Q D S D C L A G C V C G P N G F C G	1	8.3
	G C P T E N R V K R V R C K Q D S D C L A G C V C G P N G F C G	1	8.3
	G C Q T H N Y I K Y H L C K Q D S D C L A G C V C G P N G F C G	1	8.2
	G C P T I N Q V K R K R C K Q D S D C L A G C V C G P N G F C G	1	8.1
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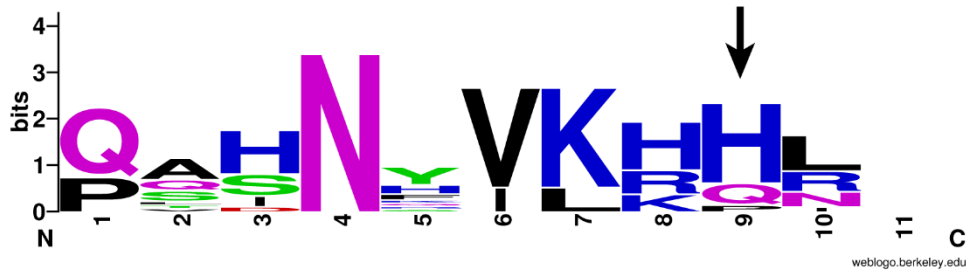
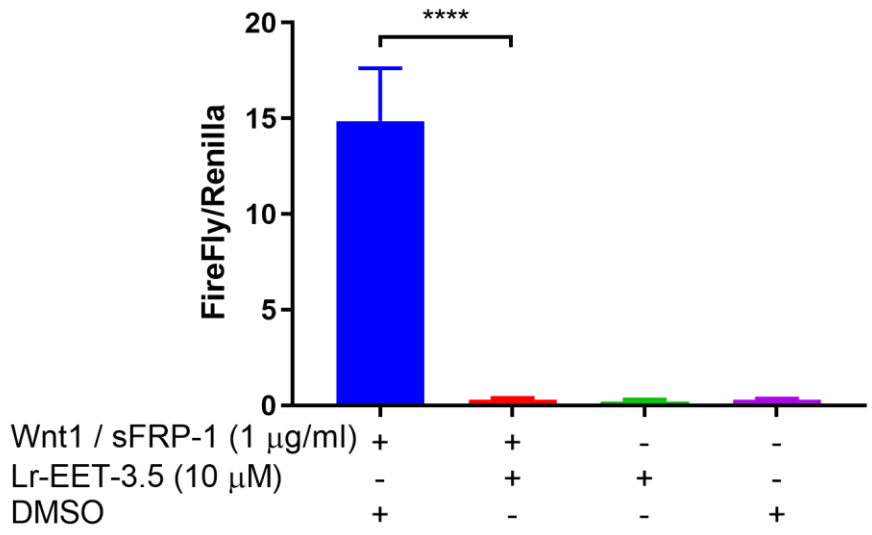
b



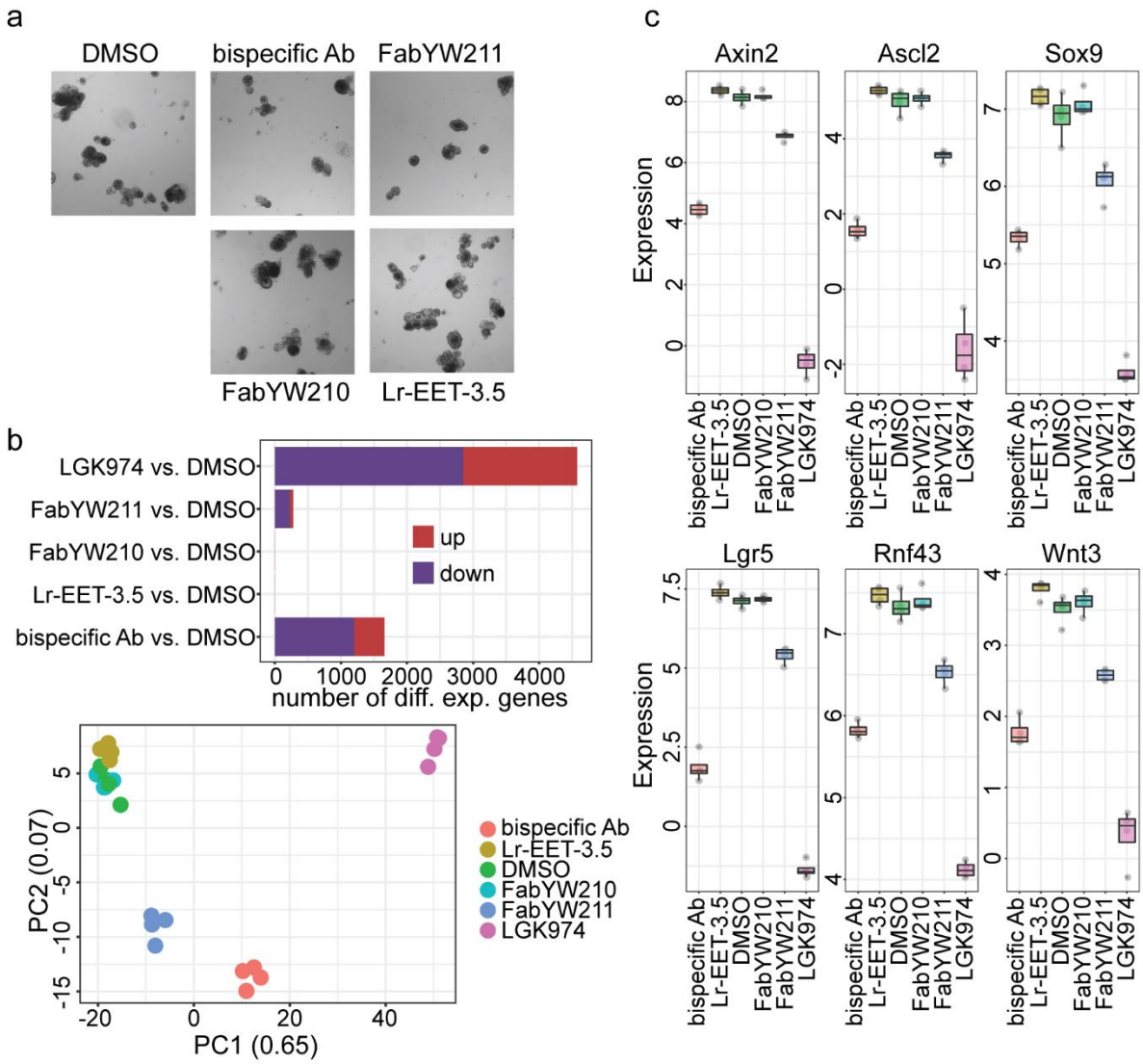
SI Fig. 8

Sequencing results of round 6 (gene3) affinity maturation of R1 CKP. (a) Sequence alignment and ELISA results of phage clones isolated after round 6 of affinity maturation on gene3. n: number of clones with identical sequences isolated from 96 random clones. s/n ratio: ratio between ELISA signal of wells coated with E1-E2 and blank wells. ELISA: absolute ELISA signal in positive wells. (b) Sequence logo obtained from aligned loop 1 sequences. Arrow indicates enrichment of histidine at position 11 in the clones.



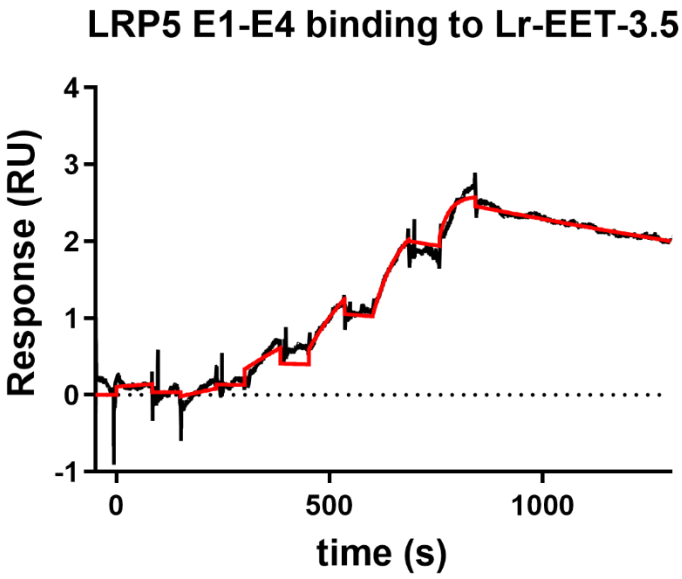
SI Fig. 9

Inhibition of Wnt/β-catenin signaling mediated by exogenous Wnt1-sFRP1 complex. HEK 293 TB cells were treated with recombinant Wnt1/sFRP-1 complex in the presence or absence of Lr-EET-3.5 and incubated overnight. The ratio of the firefly signal to the Renilla signal is plotted.



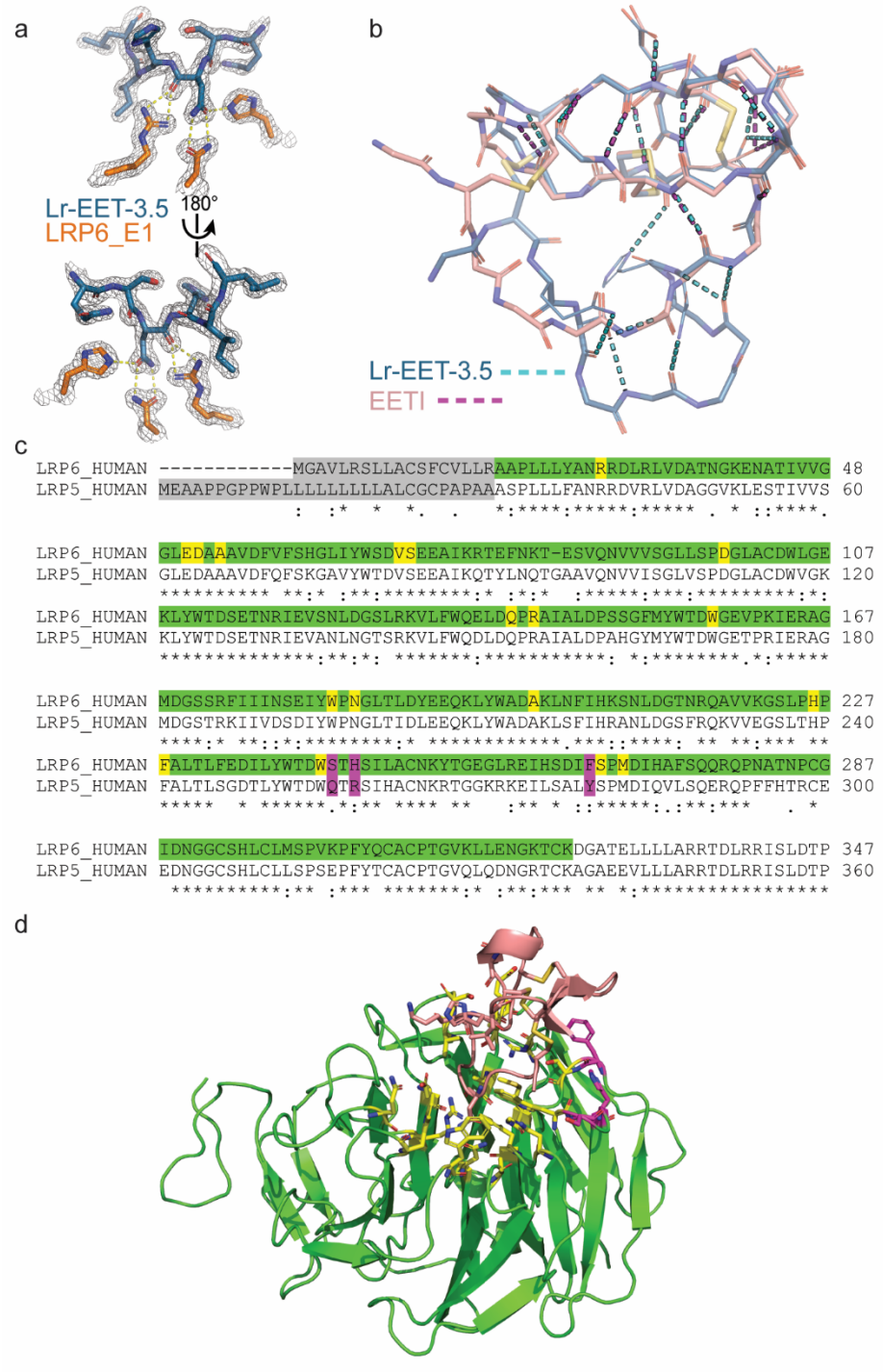
SI Fig. 10

(a) Representative images of SI organoid cultures 48h after treatment with depicted antibodies or CKPs. (b) Number of differentially expressed genes (up-regulated in red, down-regulated in blue) between various treatment is depicted. Principal component analysis of most variable genes across treatments. (c) Select transcripts related to intestinal Wnt signaling activity are depicted across treatments. See Methods section for statistical analysis.



SI Fig 11

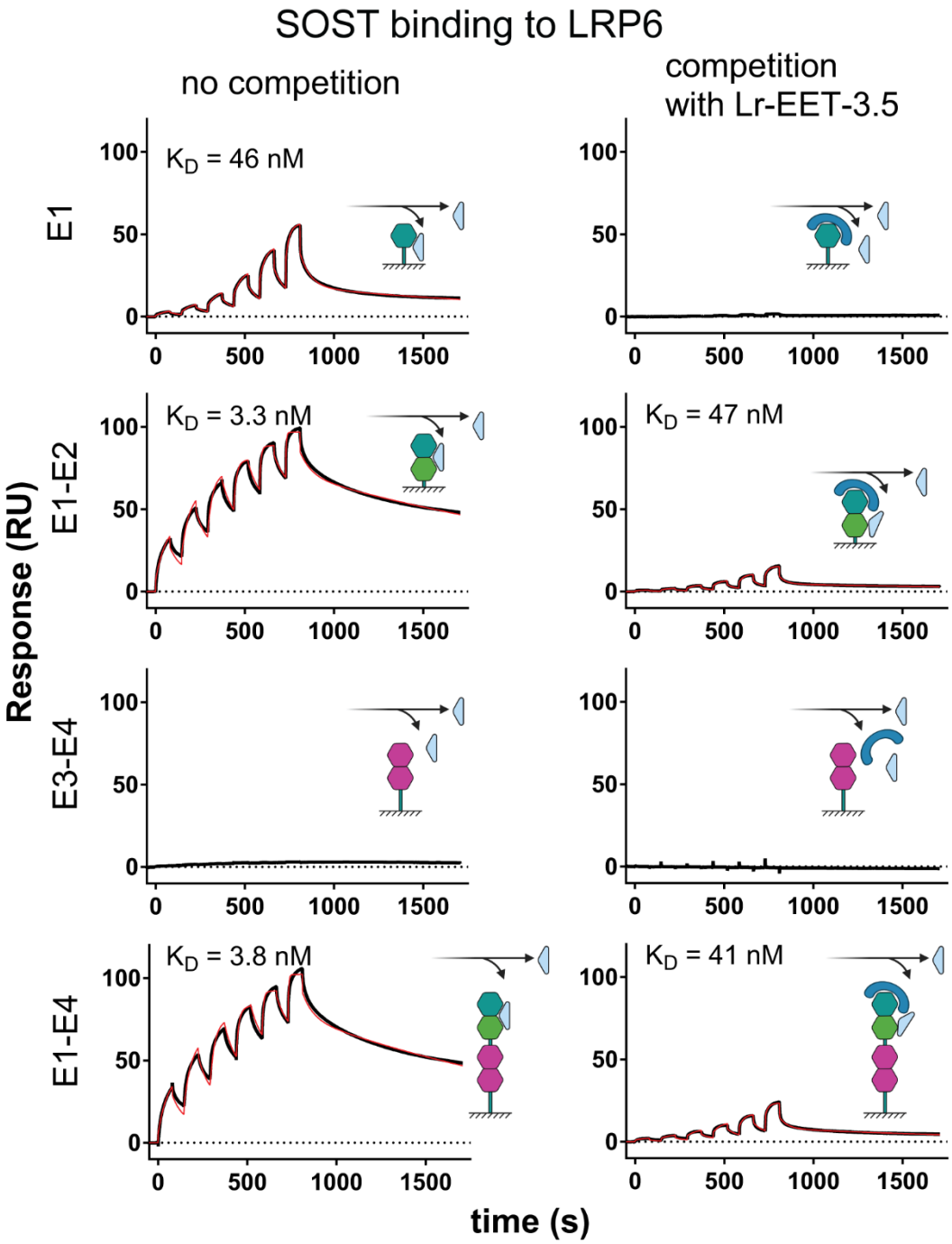
Binding of Lr-EET-3.5 CKP to LRP5 E1-E4 domains. Lr-EET-3.5 is immobilized on a sensor chip by amide chemistry, and increasing concentrations of LRP5 E1-E4 are injected. Data is fitted to a 1:1 interaction model. One representative repetition of the experiment is shown. The experiment was independently repeated twice. K_D values of 1.07 and 0.65 nM were obtained.



SI Figure 12

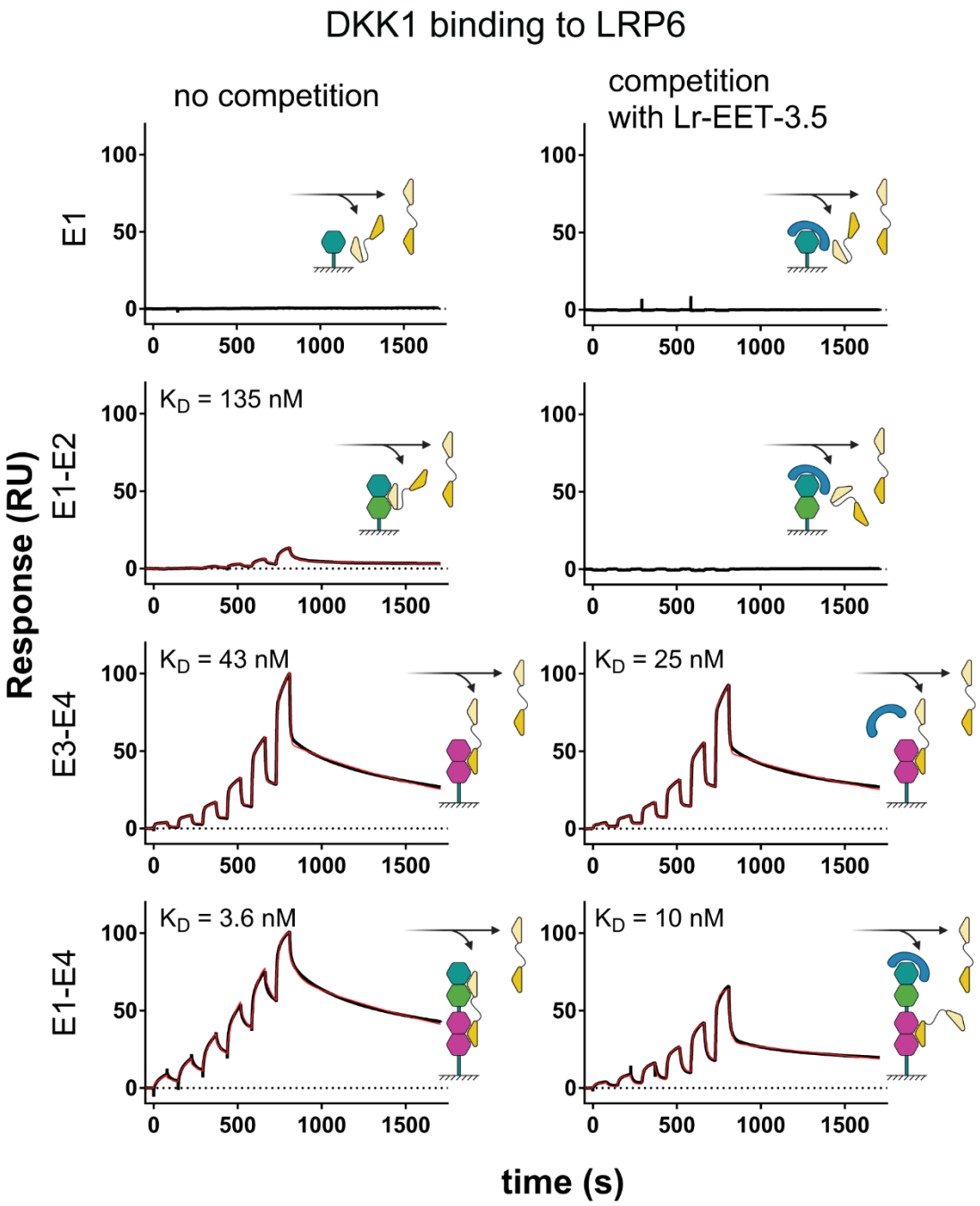
Co-crystal structure of LRP6 CKP and LRP6 E1. (a) Final 2Fo-Fc map contoured at 1.5 δ shown as grey mesh around the NHI motif of Lr-EET-3.5 (blue) with selected interacting residues of E1 (orange) viewed from two angles. Hydrogen bonds are shown in yellow. (b) Intramolecular hydrogen bonds in EETI and Lr-EET-3.5. Superposition of EETI (salmon) and Lr-EET-3.5 (blue)

only backbone atoms, cysteine side chains and side chains involved in polar contacts are shown as sticks. Hydrogen bonds are shown as dashes in cyan for Lr-EET-3.5 and pink for EETI. (c) sequence alignment of the E1 domain (green background) of human LRP6 and human LRP5, signal sequences are highlighted in grey. Residues that form the Lr-EET-3.5 epitope (within 4 Å of Lr-EET-3.5) and are identical between LRP5 and LRP6 are highlighted in yellow; residues of the binding epitope that are different between LRP6 and LRP5 are highlighted in magenta. (d) Structural view of the LR-EET-3.5 epitope, coloring scheme as in (c), Lr-EET-3.5 is shown in salmon.



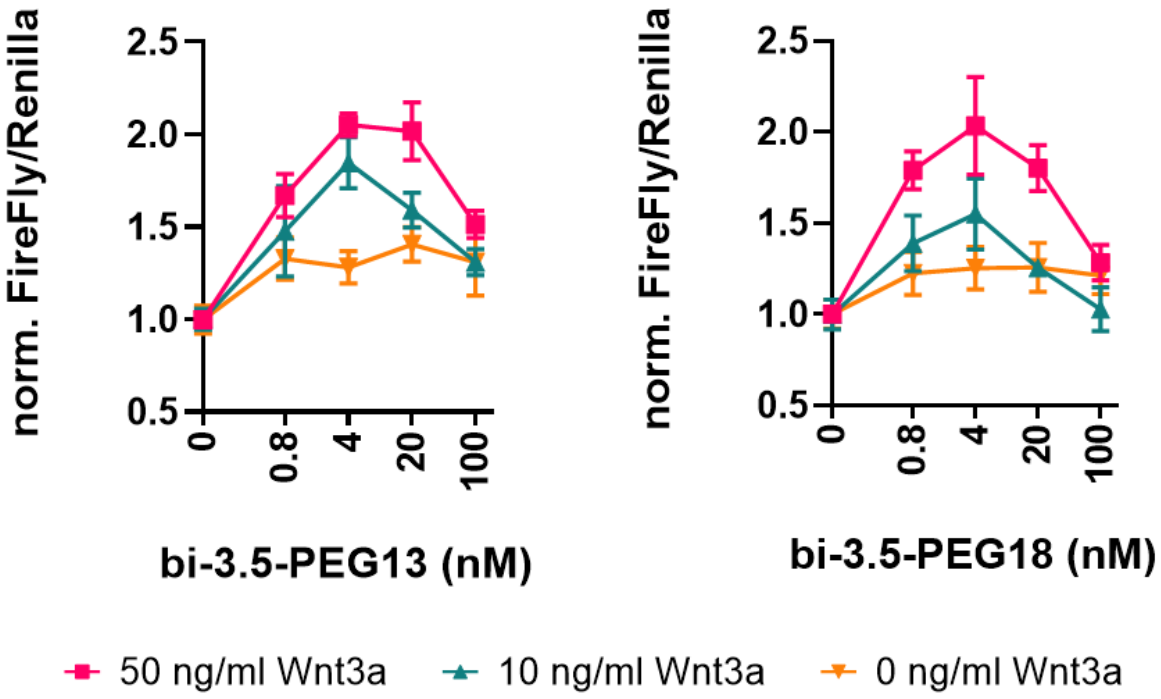
SI Figure 13

Binding of SOST to different domains of LRP6. SPR experiments were conducted with different LRP6 constructs as indicated on the left. 15.6 – 500 nM SOST was injected with (right column) or without (left column) competition with Lr-EET-3.5. Sensograms are shown in black overlaid with fits in red.



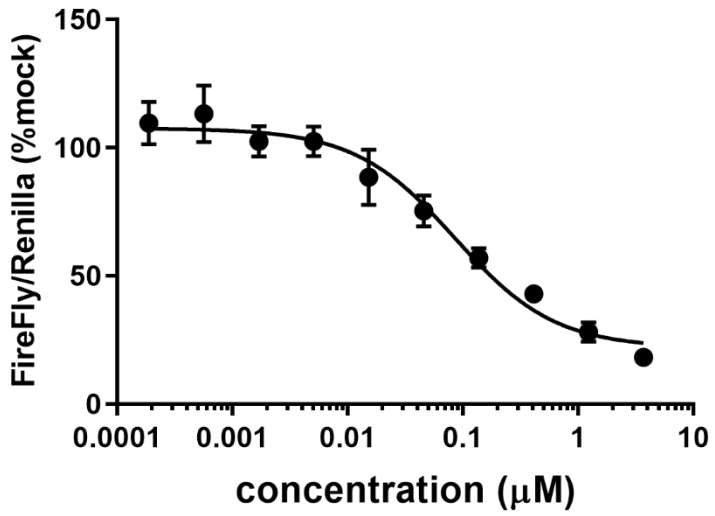
SI Figure 14

Binding of DKK1 to different domains of LRP6. SPR experiments were conducted with different LRP6 constructs as indicated on the left. 3.1 – 100 nM DKK1 was injected with (right column) or without (left column) competition with Lr-EET-3.5. Sensograms are shown in black overlaid with fits in red.



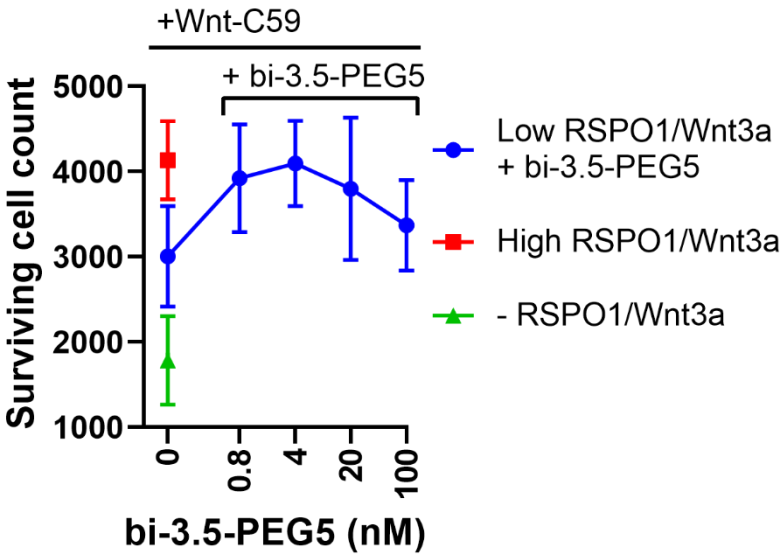
SI Figure 15

Activation of Wnt/β-catenin signaling by bivalent LRP6 CKPs with PEG13 and PEG18 linkers. Activation of Wnt signaling by bivalent CKPs with PEG13 linker (left) and PEG18 linker (right). A representative example from 3 biological replicates with 5 technical replicates is shown, error bars represent SD. All data were normalized to the control with no added CKP in each group.



SI Fig. 16

Inhibition of Wnt1 signaling by a bivalent CKP. Representative example of a reporter cell line assay (IC_{50} = 85nM). HEK293-TB cells were transfected with Wnt1 expression constructs and treated with increasing amounts of bi-3.5-PEG5. An IC_{50} of 98 ± 22 nM was determined from 3 independent assays. Symbols represent means with SD from 4 technical replicates.



SI Fig. 17

Quantification of nuclei counts per live organoid per indicated treatment measured in four biological replicates. Error bars represent SD. Human duodenal organoid cultures were treated for five days with 500 nM of Wnt-C59 in the absence of presence of Wnt3a conditioned media, RSPO1 and varying concentrations of bi-3.5-PEG5 as indicated. High RSPO1/Wnt3a = 250 ng per ml/50% Wnt3a conditioned medium. Low RSPO1/Wnt3a = 2.5 ng per ml/25% Wnt3a conditioned medium. After five days, cultures were fixed, stained for nuclei and actin cytoskeleton and imaged by fluorescence microscopy. Image analysis of the 3D stacks was performed using Ominer® image analysis software.

SI. Table 1

		Surface plasmon resonance				TOPbrite assays IC_{50} ± SD (nM)							
		k_a ($\times 10^5$ Ms^{-1})	k_d ($\times 10^{-2}$ s^{-1})	K_D (nM)	n	Wnt1 (transfection)	n	Wnt1/sFRP-1 (exogenous)	n	Wnt 3a (transfection)	n	Wnt 3a (exogenous)	n
R3/R4 gene8	R1	24.3 ± 4.7	8.1 ± 1.1	33.5	3	217 ± 130	5	-	-	n.i.	4	n.i.	3
	Lr-EET-1	21.8 ± 2.6	5.9 ± 0.6	27.2	2	-	-	-	-	-	-	-	-
	Lr-EET-2	19.1 ± 2.2	3.9 ± 0.3	20.7	2	160 ± 39	8	-	-	n.i.	3	n.i.	3
	Lr-EET-3	22.6 ± 3.0	4.8 ± 0.4	21.2	2	-	-	-	-	-	-	-	-
	Lr-EET-6	3.2 ± 0.3	11.6 ± 2.3	366	2	-	-	-	-	-	-	-	-
	Lr-EET-7	10.1 ± 1.5	64.6 ± 1.3	647	2	-	-	-	-	-	-	-	-
	Lr-EET-8	23.1 ± 4.0	13.6 ± 1.1	59.2	2	-	-	-	-	-	-	-	-
R3-R6 gene3	Lr-EET-3.1	18.0 ± 1.4	6.9 ± 0.5	38.1	2	-	-	-	-	-	-	-	-
	Lr-EET-3.2	13.5 ± 1.8	3.3 ± 0.3	24.7	2	-	-	-	-	-	-	-	-
	Lr-EET-3.3	11.3 ± 0.8	2.5 ± 0.1	21.8	2	-	-	-	-	-	-	-	-
	Lr-EET-3.4	2.2 ± 0.3	0.065 ± 0.001	2.96	3	92 ± 15	5	-	-	n.i.	4	n.i.	3
	Lr-EET-3.5	2.5 ± 0.3	0.025 ± 0.0003	1.01	3	51 ± 21	7	c.i.	4	n.i.	4	n.i.	3
	Lr-EET-3.6	10.2 ± 0.2	3.9 ± 0.8	38.4	2	-	-	-	-	-	-	-	-
	Lr-EET-3.7	14.5 ± 2.9	2.9 ± 0.3	20.7	2	-	-	-	-	-	-	-	-
	Lr-EET-3.8*	n.a.	n.a.	n.a.	2	-	-	-	-	-	-	-	-
	Lr-EET-3.9	14.7 ± 1.2	9.4 ± 0.2	64.6	2	-	-	-	-	-	-	-	-
	Lr-EET-3.10	27.2 ± 6.3	3.6 ± 0.4	13.3	2	108 ± 28	5	-	-	n.i.	3	n.i.	3
	Lr-EET-3.11	17.9 ± 3.3	6.0 ± 0.4	34.0	2	n.i.	4	-	-	-	-	-	-
	Lr-EET-3.5.N5A.I7G	n.i.	n.i.	n.i.	3	n.i.	4	-	-	-	-	-	-
	Lr-EET-3.5.N5A.I7G.L8A	n.i.	n.i.	n.i.	3	n.i.	4	-	-	-	-	-	-
	Lr-EET-3.5.F30A	n.i.	n.i.	n.i.	3	n.i.	4	-	-	-	-	-	-
	Lr-EET-3.5.I7K	n.i.	n.i.	n.i.	3	n.i.	4	-	-	-	-	-	-
	Lr-EET-3.5.R12MeR	2.6 ± 0.3	0.017 ± 0.003	0.65	3	37 ± 12	4	-	-	n.i.	3	-	-
Controls	dFz7-21	-	-	-	-	151 ± 86	3	-	-	32 ± 10	4	50.8 ± 36.2	4
	Fz7-21S	-	-	-	-	n.i.	4	-	-	n.i.	3	n.i.	3
	DKK peptide	-	-	-	-	n.i.	3	-	-	n.i.	3	n.i.	3
	bispecific LRP6 Ab	-	-	-	-	2.4 ± 1.1	3	-	-	0.29 ± 0.06	3	0.20 ± 0.08	4
	EETI-II	n.i.	n.i.	n.i.	1	n.i.	3	-	-	n.i.	3	n.i.	3

Affinities were determined by SPR, E1 domain of LRP6 was immobilized and concentration series of peptides were injected. Data were fitted to a 1:1 binding model. IC_{50} values are determined from peptide treatment of HEK293-TB cells that were either transfected with Wnt1 or Wnt3a encoding plasmids or treated with recombinant Wnt1/sFRP-1 or Wnt3a; n: number of independent assays

n.a.: not applicable, n.i.: no interaction observed (SPR), no inhibition observed (TOPbrite) at 100 µM, -: not tested, c.i.: complete inhibition at 10 µM, no IC_{50} determined

* interaction observed, but not quantified because it could not be fitted to 1:1 interaction model

SI. Table 2

Protein complex	**E1:Lr-EET-3.5**
Crystalization condition	20% w/v PEG 3350 0.2 M Na malate, pH 7.0
Data collection	
Resolution range (Å)	42.12-1.60
Completeness	97.63
R_{merge}	0.05 (1.05)
$\langle I \rangle / \sigma I$	10.54 (2.01)
CC(1/2)	1.0 (0.64)
Unit cell parameters	
Space group	C2
a, b, c (Å)	108.89, 46.94, 87.79
α, β, γ (°)	90, 124.34, 90
Refinement	
Molecules/AU	2 (1 complex)
R_{work}(%)	17.4
R_{free}(%)	19.2
RMSD of bond lengths	0.01
RMSD of bond angles	1.153
Ramachandran plot (%)	
favored	93.7
allowed	6.3
outliers	0.0

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