Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supporting information for Activation of a G protein-coupled receptor through indirect antibody-mediated tethering of ligands

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Antibody	Manufacturer	Clone	Cat No.
Mouse anti- HLA-A,B,C	Biolegend, San Diego, CA, USA	W6/32	311402
Mouse anti- CD63	Abcam, Cambridge, MA, USA	MEM-259	Ab8219
Mouse anti- GFP	RnD Systems, Minneapolis, MN, USA	#454505	MAB42401-R
Rabbit-anti MHC1	Cell Signaling Technologies	Polyclonal	12851
Mouse anti-CD81	RnD Systems, Minneapolis, MN, USA	#454720	MAB4615

Supporting Table 1. Commercial antibodies used in this study.

Conjugate or peptide	m/z observed	m/z calculated
VHH_{MHC-II}	15620	n/a
VHH_{6E}	13479	n/a
VHH_{MHC-I}	14445	n/a
VHH_{rIg}	14525	n/a
VHH_{GFP}	14215	n/a
VHH_{mIg}	15162	n/a
$VHH_{\alpha5\beta1}$	15016	n/a
VHH_{MHC-II} -biotin-azide	15492	15492
VHH_{6E}-biotin-azide	13333	13461
VHH_{MHC-I}-biotin-azide	14300	14428
VHH_{rIg}-biotin-azide	14374	14502
VHH_{GFP}-biotin-azide	14080	14208
VHH_{mIg}-biotin-azide	15012	15140
$VHH_{\alpha5\beta1}$-biotin-azide	14875	15003
VHH_{6E}-PTH_{1-11}	15165	15172.3
VHH_{MHC-I}-PTH_{1-11}	16140	16139.3
VHH_{rIg}-PTH_{1-11}	16214	16213.3
VHH_{GFP}-PTH_{1-11}	15920	15919.3
VHH_{mIg}-PTH_{1-11}	16852	16851.3
$VHH_{\alpha5\beta1}$-PTH_{1-11}	16718	16714.3
VHH_{MHC-II} -PTH_{1-34}	20346	20339.7
VHH_{GFP} -PTH_{1-34}	18910	18927.7
PTH(1-11)-dbco	1839.3	
PTH(1-34)-PEG3-dbco	4847.7	
PTH(1-11)-Cys	1411.2	

Supporting Table 2: Summary of mass spectrometric characterization of VHH-PTH conjugates.

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hPTHR1                  MGTARIAPGL ALLLCCPVLS SAYALVDADD VMTKEEQIFL LHRAQAQCEK
hPTHR1-GFP              MGTARIAPGL ALLLCCPVLS SAYALVDADD VMTKEEQIFL LHRAQAQCEK
rPTHR1-delNT26-181_     MGAARIAPSL ALLLCCPVLS SAYAL..... ~~~~~~~~~~ ~~~~~~~~~~
rPTHR1-delNT-HA         MGAARIAPSL ALLLCCPVLS SAY....PYD VPDYAGGGG. ~~~~~~~~~~
hPTHR1_6E               MGTARIAPGL ALLLCCPVLS SAYALVDADD VMTKEEQIFL LHRAQAQCEK

                        51                                                  100
hPTHR1                  RLKEVLQRPA SIMESDKGWT SASTSGKPRK DKASGKLYPE SEED......
hPTHR1-GFP              RLKEVLQRPA SIMESDKGWT SASTSGKPRK DKASGKLYPE SEEDKMSKGE
rPTHR1-delNT26-181_     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
rPTHR1-delNT-HA         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
hPTHR1_6E               RLKEVLQRPA SIMESDKGWT QADQEAKELA RQISGKLYPE SEED~~~~~~

                        101                                                 150
hPTHR1                  .......... .......... .......... .......... ..........
hPTHR1-GFP              ELFTGVVPIL VELDGDVNGH KFSVSGEGEG DATYGKLTLK FICTTGKLPV
rPTHR1-delNT26-181_     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
rPTHR1-delNT-HA         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
hPTHR1_6E               ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                        151                                                 200
hPTHR1                  .......... .......... .......... .......... ..........
hPTHR1-GFP              PWPTLVTTLS YGVQCFSRYP DHMKQHDFFK SAMPEGYVQE RTIFFKDDGN
rPTHR1-delNT26-181_     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
rPTHR1-delNT-HA         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
hPTHR1_6E               ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                        201                                                 250
hPTHR1                  .......... .......... .......... .......... ..........
hPTHR1-GFP              YKTRAEVKFE GDTLVNRIEL KGIDFKEDGN ILGHKLEYNY NEHLVYIMAD
rPTHR1-delNT26-181_     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
rPTHR1-delNT-HA         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
hPTHR1_6E               ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                        251                                                 300
hPTHR1                  .......... .......... .......... .......... ..........
hPTHR1-GFP              KQKNGTKAIF QVHHNIEDGS VQLADHYQQN TPIGDGPVLL PDNHYLHTQS
rPTHR1-delNT26-181_     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
rPTHR1-delNT-HA         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
hPTHR1_6E               ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

                        301                                                 350
hPTHR1                  .......... .......... .......... ..KEAPTGSR YRGRPCLPEW
hPTHR1-GFP              ALSKDPNEKR DHMVLLEFVT AAGITHGMDE LYKEAPTGSR YRGRPCLPEW
rPTHR1-delNT26-181_     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
rPTHR1-delNT-HA         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
hPTHR1_6E               ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~KEAPTGSR YRGRPCLPEW

                        351                                                 400
hPTHR1                  DHILCWPLGA PGEVVAVPCP DYIYDFNHKG HAYRRCDRNG SWELVPGHNR
hPTHR1-GFP              DHILCWPLGA PGEVVAVPCP DYIYDFNHKG HAYRRCDRNG SWELVPGHNR
rPTHR1-delNT26-181_     ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ .......... ..........
rPTHR1-delNT-HA         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ .......... ..........
hPTHR1_6E               DHILCWPLGA PGEVVAVPCP DYIYDFNHKG HAYRRCDRNG SWELVPGHNR

                        401                                                 450
hPTHR1                  TWANYSECVK FLTNETRERE VFDRLGMIYT VGYSVSLASL TVAVLILAYF
hPTHR1-GFP              TWANYSECVK FLTNETRERE VFDRLGMIYT VGYSVSLASL TVAVLILAYF
rPTHR1-delNT26-181_     ~~~~~~~~~~ .........E VFDRLGMIYT VGYSMSLASL TVAVLILAYF
rPTHR1-delNT-HA         ~~~~~~~~~~ .........E VFDRLGMIYT VGYSMSLASL TVAVLILAYF
hPTHR1_6E               TWANYSECVK FLTNETRERE VFDRLGMIYT VGYSVSLASL TVAVLILAYF
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                         451                                                500
hPTHR1                   RRLHCTRNYI HMHLFLSFML RAVSIFVKDA VLYSGATLDE AERLTEEELR
hPTHR1-GFP               RRLHCTRNYI HMHLFLSFML RAVSIFVKDA VLYSGATLDE AERLTEEELR
rPTHR1-delNT26-181_      RRLHCTRNYI HMHMFLSFML RAASIFVKDA VLYSGFTLDE AERLTEEELH
rPTHR1-delNT-HA          RRLHCTRNYI HMHMFLSFML RAASIFVKDA VLYSGFTLDE AERLTEEELH
hPTHR1_6E                RRLHCTRNYI HMHLFLSFML RAVSIFVKDA VLYSGATLDE AERLTEEELR

                         501                                                550
hPTHR1                   AIAQAPPPPA TAAAGYAGCR VAVTFFLYFL ATNYYWILVE GLYLHSLIFM
hPTHR1-GFP               AIAQAPPPPA TAAAGYAGCR VAVTFFLYFL ATNYYWILVE GLYLHSLIFM
rPTHR1-delNT26-181_      IIAQVPPPPA AAAVGYAGCR VAVTFFLYFL ATNYYWILVE GLYLHSLIFM
rPTHR1-delNT-HA          IIAQVPPPPA AAAVGYAGCR VAVTFFLYFL ATNYYWILVE GLYLHSLIFM
hPTHR1_6E                AIAQAPPPPA TAAAGYAGCR VAVTFFLYFL ATNYYWILVE GLYLHSLIFM

                         551                                                600
hPTHR1                   AFFSEKKYLW GFTVFGWGLP AVFVAVWVSV RATLANTGCW DLSSGNKKWI
hPTHR1-GFP               AFFSEKKYLW GFTVFGWGLP AVFVAVWVSV RATLANTGCW DLSSGNKKWI
rPTHR1-delNT26-181_      AFFSEKKYLW GFTIFGWGLP AVFVAVWVGV RATLANTGCW DLSSGHKKWI
rPTHR1-delNT-HA          AFFSEKKYLW GFTIFGWGLP AVFVAVWVGV RATLANTGCW DLSSGHKKWI
hPTHR1_6E                AFFSEKKYLW GFTVFGWGLP AVFVAVWVSV RATLANTGCW DLSSGNKKWI

                         601                                                650
hPTHR1                   IQVPILASIV LNFILFINIV RVLATKLRET NAGRCDTRQQ YRKLLKSTLV
hPTHR1-GFP               IQVPILASIV LNFILFINIV RVLATKLRET NAGRCDTRQQ YRKLLKSTLV
rPTHR1-delNT26-181_      IQVPILASVV LNFILFINII RVLATKLRET NAGRCDTRQQ YRKLLRSTLV
rPTHR1-delNT-HA          IQVPILASVV LNFILFINII RVLATKLRET NAGRCDTRQQ YRKLLRSTLV
hPTHR1_6E                IQVPILASIV LNFILFINIV RVLATKLRET NAGRCDTRQQ YRKLLKSTLV

                         651                                                700
hPTHR1                   LMPLFGVHYI VFMATPYTEV SGTLWQVQMH YEMLFNSFQG FFVAIIYCFC
hPTHR1-GFP               LMPLFGVHYI VFMATPYTEV SGTLWQVQMH YEMLFNSFQG FFVAIIYCFC
rPTHR1-delNT26-181_      LVPLFGVHYT VFMALPYTEV SGTLWQIQMH YEMLFNSFQG FFVAIIYCFC
rPTHR1-delNT-HA          LVPLFGVHYT VFMALPYTEV SGTLWQIQMH YEMLFNSFQG FFVAIIYCFC
hPTHR1_6E                LMPLFGVHYI VFMATPYTEV SGTLWQVQMH YEMLFNSFQG FFVAIIYCFC

                         701                                                750
hPTHR1                   NGEVQAEIKK SWSRWTLALD FKRKARSGSS SYSYGPMVSH TSVTNVGPRV
hPTHR1-GFP               NGEVQAEIKK SWSRWTLALD FKRKARSGSS SYSYGPMVSH TSVTNVGPRV
rPTHR1-delNT26-181_      NGEVQAEIRK SWSRWTLALD FKRKARSGSS SYSYGPMVSH TSVTNVGPRA
rPTHR1-delNT-HA          NGEVQAEIRK SWSRWTLALD FKRKARSGSS SYSYGPMVSH TSVTNVGPRA
hPTHR1_6E                NGEVQAEIKK SWSRWTLALD FKRKARSGSS SYSYGPMVSH TSVTNVGPRV

                         751                                                800
hPTHR1                   GLGLPLSPRL LPTATTNGHP QLPGHAKPGT PALETLETTP PAMAAPKDDG
hPTHR1-GFP               GLGLPLSPRL LPTATTNGHP QLPGHAKPGT PALETLETTP PAMAAPKDDG
rPTHR1-delNT26-181_      GLSLPLSPRL P.PATTNGHS QLPGHAKPGA PATET.ETLP VTMAVPKDDG
rPTHR1-delNT-HA          GLSLPLSPRL P.PATTNGHS QLPGHAKPGA PATET.ETLP VTMAVPKDDG
hPTHR1_6E                GLGLPLSPRL LPTATTNGHP QLPGHAKPGT PALETLETTP PAMAAPKDDG

                         801                           832
hPTHR1                   FLNGSCSGLD EEASGPERPP ALLQEEWETV M*
hPTHR1-GFP               FLNGSCSGLD EEASGPERPP ALLQEEWETV M*
rPTHR1-delNT26-181_      FLNGSCSGLD EEASGSARPP PLLQEGWETV M*
rPTHR1-delNT-HA          FLNGSCSGLD EEASGSARPP PLLQEGWETV M*
hPTHR1_6E                FLNGSCSGLD EEASGPERPP ALLQEEWETV M*
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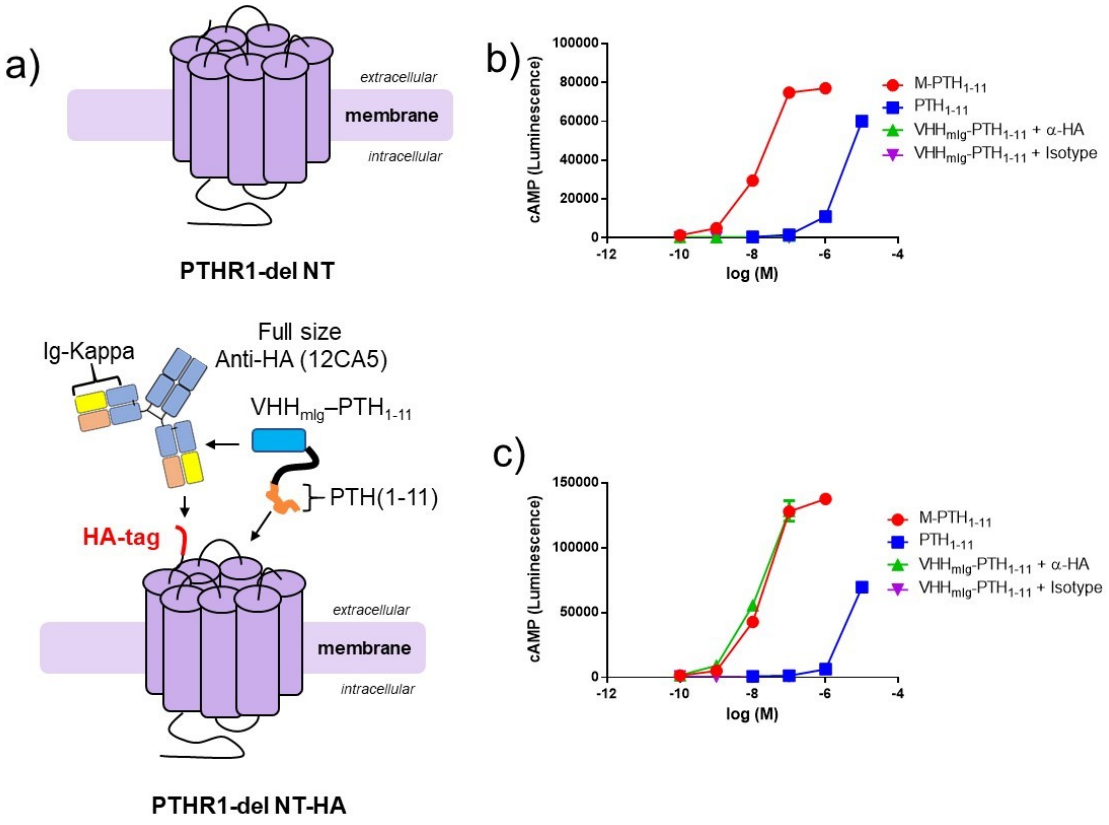
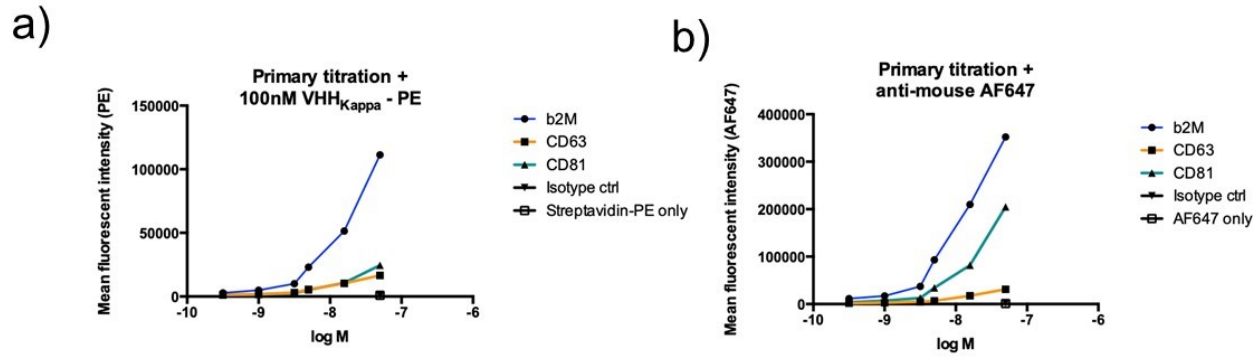
Supporting Figure 1. Sequence alignment of PTHR1 constructs used in this study. Alignment was performed using ClustalOmega. Constructs for hPTHR1 GFP[1], rat PTHR1 lacking extracellular domain residues 26-181 (rPTHR1-delNT26-181)[2], and rat PTHR1 lacking extracellular domain residues 26-181 with HA-tag incorporated (rPTHR1-delNT-HA)[3] were described previously. The GFP insert is shown in green and the HA tag in blue. Residues 1-22 in hPTHR1 correspond to the signal peptide.



Supporting Figure 2. Targeting PTHR1 lacking extracellular domain. HEK293 cells stably expressing cAMP-responsive luciferase were transiently transfected with either rat PTHR1 lacking extracellular domain (rPTHR1-delNT) or a construct with an HA tag inserted in place of the extracellular domain (rPTHR1-delNT-HA). See below for sequences. (**a**) Schematic of receptor constructs and targeting strategy. (**b**) Representative dose-response curves (one of four independent experiments) for cells transfected with rPTHR1-delNT. Data points represent mean ± SD. X-axis concentrations refer to that of VHH-PTH constructs. Lines on the graph are not from the fitting of a model and only serve to guide the eye. PTH_{1-11} is the same sequence as listed in Figure 2a. The sequence of M-PTH(1-11) in this assay is YVUELQLMHQX where Y is 1-aminocyclopentane-1-carboxylic acid , U is Aib, and X is homoarginine. cAMP response assays were performed as described in methods. The difference in activity between M-PTH(1-11) and PTH(1-11) is in line with previously noted structure-activity relationship studies[4].



Supporting Figure 3. Assessment of antibody staining of HEK293 cells. HEK293 cells were stained with the mouse monoclonal antibodies at the indicated concentrations. Staining protocols are described in methods and antibodies used are detailed in Supporting Table 1. Data represent mean fluorescent intensity readouts in the indicated channels. Data shown are from a single representative experiment from three replicates. (**a**) Primary conventional antibodies were mixed at the doses indicated on the x-axis with VHH$_{mIg}$-biotin (here titled VHH$_{Kappa}$) conjugate (100 nM final concentration) and used to stain HEK293 cells. Antibody staining was detected using streptavidin-PE. Isotype control indicates polyclonal mouse IgG was used in place of a monoclonal mouse antibody. Streptavadin-PE indicates no primary antibody was used. (**b**) Primary conventional antibodies were used at the indicated concentration to label HEK293 cells. Primary antibody binding was detected with a secondary anti-mouse-Alexafluor647 conjugate.

References

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4 N. Shimizu, J. Guo and T. J. Gardella, *J Biol Chem*, 2001, **276**, 49003–49012.