

Extended data

The second extracellular loop gates *Stachel*-mediated activation of ADGRG6/GPR126

Franziska Wiechert ^{1,*}, Michal Szczepek ^{2,*}, Gunnar Kleinau ^{2,*}, Lisa Kupsch ^{3,*}, Amal Hassan Ali ², Monique Gallandi ², Andrea Schmidt ², David Speck ², Anja Koch ², Justus Loerke ¹, Thiemo Sprink ^{4,5}, Tarek Hilal ^{6,7}, Christian M.T. Spahn ¹, Torsten Schöneberg ^{3,8,§}, Ines Liebscher ^{3,§}, and Patrick Scheerer ^{2,§}

¹ Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Institute of Medical Physics and Biophysics, 10117 Berlin, Germany

² Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Institute of Medical Physics and Biophysics, Group Structural Biology of Cellular Signaling, 10117 Berlin, Germany

³ Rudolf Schönheimer Institute of Biochemistry, Molecular Biochemistry, Medical Faculty, Leipzig University, 04103 Leipzig, Germany

⁴ Max Delbrück Center for Molecular Medicine in the Helmholtz Association (MDC), Technology Platform for cryo-Electron Microscopy, Robert-Rössle Str. 10, 13125 Berlin, Germany

⁵ Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Core Facility for cryo-Electron Microscopy, Robert-Rössle-Str. 10, 13125 Berlin, Germany

⁶ Research Center of Electron Microscopy and Core Facility BioSupraMol, Freie Universität Berlin, Berlin, Germany

⁷ Freie Universität Berlin, Institute of Chemistry and Biochemistry, Laboratory of Structural Biochemistry, 14195 Berlin, Germany

⁸ School of Medicine, University of Global Health Equity, Kigali, Rwanda

* Equal contribution

§ To whom correspondence:

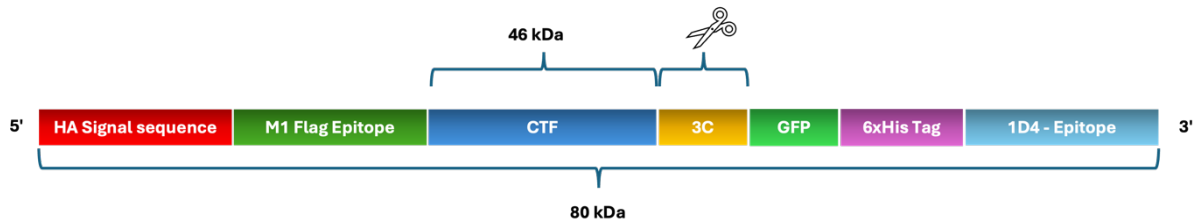
Patrick Scheerer (patrick.scheerer@charite.de)

Ines Liebscher (Ines.Liebscher@medizin.uni-leipzig.de);

Torsten Schöneberg (schoberg@medizin.uni-leipzig.de);

Extended data Figure 1: Sequences and construct design GPR126-CTF complex

Construct of Gpr126-eGFP (DNA:ID373)



Sequences:

GPR126-CTF

MKTIIALSY**IFCLVFA****DYKDDDDK****HFGVLMDL**PR**SA**SQ**LDARNTKVLTFISYIGCGISA**
IFSAATLLTYVAFEKLRRDYPSKILMN**LSTALLFLNLLFLLDGWITSFNVDGLCIAVAV**
LLHFFLLATFTWMGLEAIHMYIALVKVFNTYIRRYILKFCIIGWGLPALVVSVVLASR
NNNEVYGKESYGKEKGDEFCWIQDPVIFYVTCAGYFGVMFFLNIAMFIV**VMVQICGR**
NGKRSNRTLREEVLRNLRSVVSL**TFL**LGMTWGFA**FAFWGPLNIPFMYLFSIFNSLQGL**
GIFIFHCAMKENVQKQWRRHLCCGRFRLADNSDWSKTATNIIKKSSDNPGKSLSSSSI
GSNSTYLTSKSKSSSTTYFKRNSHTDSASMDKSLSKLAHADGDQTSIIPVHQVIDKVK
GYCNAHSDNFYKNIIMSDTFSHST**KF****LEVL****FQGP****MVSKGEELFTGVVPILVELDGDVN**
GHKFSVSGEGEGDATYGKLTL**KFI**CT**TGKL**PVPW**PTLVTT**LT**YGVQCFSRYPDHMKQ**
HDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILG
HKLEYNNYNSHNVYIMAD**KQKNGIKVNF**KIRHNIEDGS**VQLADHYQQNTPIGDGPVLL**
PDNHYLSTQSALSKDPNE**KRDH**MV**LLEFVTAAGITLGMDEL****YKGRPHHHHHHTETS**
QVAPA*

Human dominant-negative $G\alpha_s$ protein (pFB1) ¹

MGCLGNSKTEDQRNEEKAQREANKKIEKQLQKDKQVYRATHRLLLLGAGESGKSTI
VKQMRILHVNGFNGDSEKAT**KVQDIKNNLKEA**IETIVAAM**SNLVPPVELANPENQFR**
VDYILSVMNVPDFDFPPEFYEHAKALWEDEGVRACYERSNE**YQLIDCAQYFLDKIDV**
IKQADYVPSDQDLLRCRVLTSGIFET**KFQVDKVN**FHMFDVGG**QRDERRKWIQCFND**
VTAIFVVASSYN**MVIREDNQTNRLQEALNLFKSIWNNRWLRTISVILFLNKQDLLAE**
KVLAGK**SKIEDYFPEFARYTTPEDATPEPGEDPRV**TRAKY**FIRDEF**LRISTASGDGRHY
CYPHFTCAVDTENIRRVFNDCRDIIQRMHLRQYELL

Human $G\beta 1$ protein in dual expression vector (pFastBacDual)

MHHHHHHH**LEVL****FQGP**GSSGSELDQLRQEAEQLKNQIRDARKACADATLSQITNNIDP
VGRIQMRTRRTLRGHLAKIYAMHWG**TDSRL**LV**SA**SQDGKLIWDSYTTNKVHAIP**LR**
SSWVMTCAYAPSGNYVACGGLDNICSIYNLKTREGNVRVSREL**AGHTGYLSCCRFL**
DDNQIVTSSGDTTCALWDIETGQQTTTFTGHTGDVMSLSLAPDTRLFVSGACD**ASAK**
LWDVREGMCRQTFTGHESDINAICFFPN**GNAFATGSDDATCRLFDLRADQELMTYSH**
DNIICGITSVSFSKSGRLLLAGYDDFNCNVWDALKADRAGVLAGHDNRVSCLGVTD
DGMAVATGSWDSFLKIWN

Human $G\gamma 2$ protein in dual expression vector (pFastBacDual) ¹

MASNNTASIAQARKLVEQLKMEANIDRIK**VSKAAADLMAYCEAHAKEDPLLTPVPA**
SENPFREKKFFCAIL

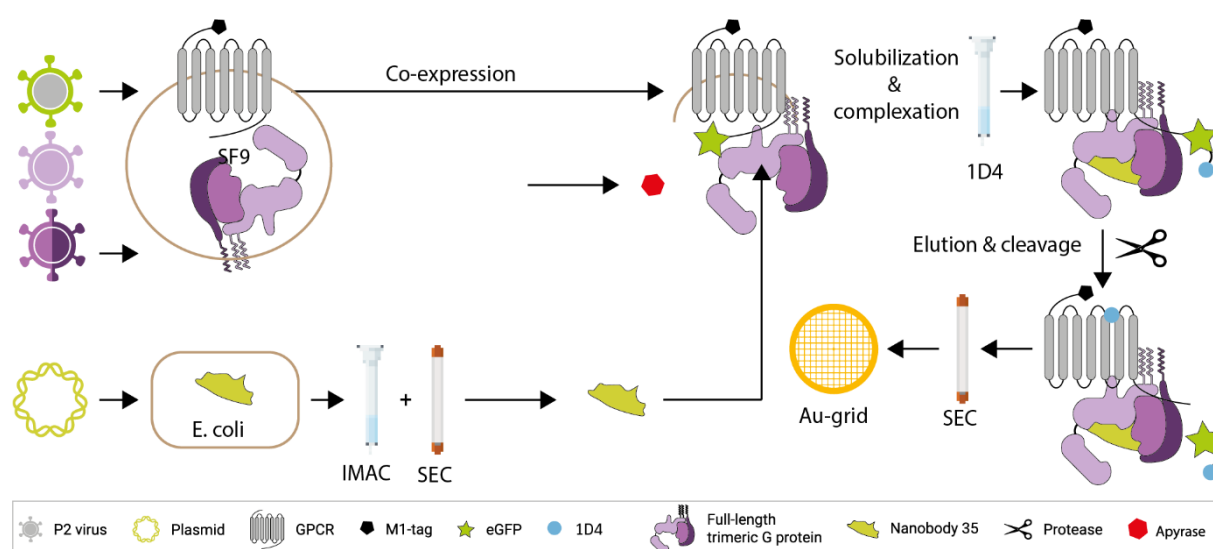
Nanobody - Nb35 ²

MKYLLPTAAAGLLLLAAQPAMAQVQLQESGGGLVQPGGSLRLSCAASGFTFSNYKM
NWVRQAPGKGLEWVSDISQSGASISYTGSVKGRFTISRDNKNTLYLQMNSLKPEDT
AVYYCARCPAPFTRDCFDVTSTTYAYRGQGTQVTVSSHHHHHEPEA

Sequence alignment Gpr126-CTF (DNA:ID373) vs. adhesion G protein-coupled receptor G6 [Homo sapiens] (KAI2544095.1)

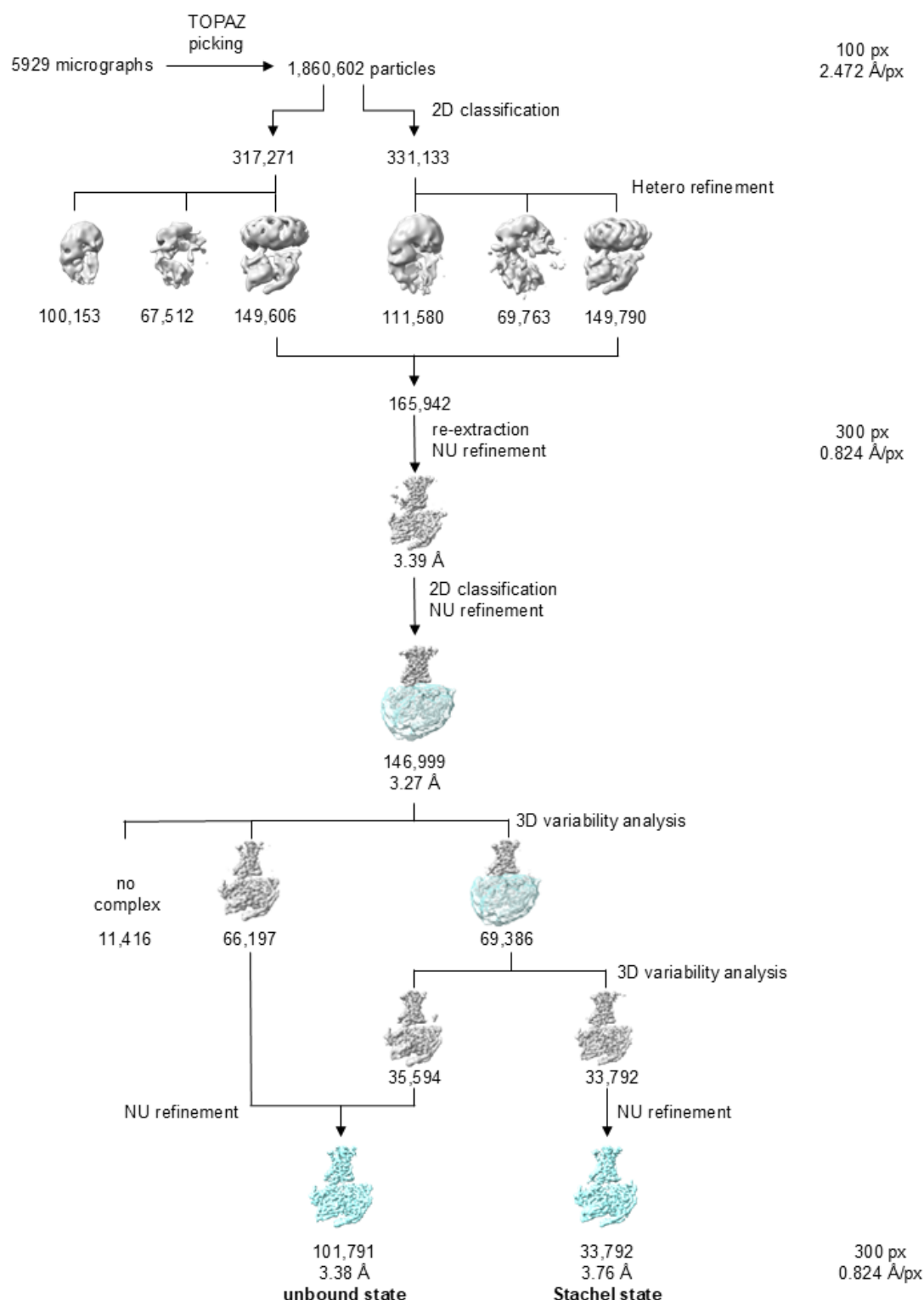
KAI2544095.1	MMFRSDRMWSCHWKWKPSPLLFLFALYIMCVPHSVWGCANCRVVLSPNSGTFSTPCYPNDYPNSQACMWTLRAPT	75
GPR126	-----	0
KAI2544095.1	GYIIQITFNDFDIEEAPNCIYDSLSDNGESQTKFCGATAKGLSFNSSANEMHVSFSSDFS IQKKGFNASYNRVA	150
GPR126	-----	0
KAI2544095.1	VSLRNQKVILPQTSDAYQVSVAKSISIPELSAFTLCFEATKVGHEDSDWTAFSYSNASFTQLLSFGKAKSGYFLS	225
GPR126	-----	0
KAI2544095.1	ISDSKCLLNALPVKEKEDIFAESFEQLCLVWNNLSLGSIGVNFKRNYETVPCDSTISKVIPGNGKLLLSGNQNEI	300
GPR126	-----	0
KAI2544095.1	VSLKGD IYNFRLWNFTMNAKILSNLSCNVKGNVVDWQNDFWNIPNLALKAESNLSCGSYLIPLPAAELASCADLG	375
GPR126	-----	0
KAI2544095.1	TLCQATVNSPSTTPPTVTNNMPVTNRIDKQRNDGIIYRISVVIQNILRHPEVKVQSKVAEWLNSTFQNWNYTVYV	450
GPR126	-----	0
KAI2544095.1	VNISFHL SAGEDKIKVKRSLEDEPRLVLWALLVYNATNNTNLEGKIIQKLLKNNESLDEGLRLHTVNVRLGHC	525
GPR126	-----	0
KAI2544095.1	LAMEEPKGYWPSIQPSEYVLPKPKPGFSASRICFYNATNPLVTYWGPDISNCLKEANEVANQILNLTADGQN	600
GPR126	-----	0
KAI2544095.1	LTSANITNIVEQVKRIVNKEENIDITLGSTLMNIFSNILSSSDSDLLESSEALKTIDELAFKIDLNSTSHVNIT	675
GPR126	-----	0
KAI2544095.1	TRNLALSVSSLLPGTNAISNFSIGLPSNNESYFQMDFESGQVDPLASVILPPNLENLSPEDSVLVRRQAQFTFFN	750
GPR126	-----	0
KAI2544095.1	KTGLFQDVGPQRKTLVSYVMACSIGNITIQNLKDPVQIKIKHTRTQEVHHPICAFWDLNKNKSFGGWNTSGCVAH	825
GPR126	-----	0
KAI2544095.1	RDSDASETVCLCNHFTHFGVLMDLPRSA	900
GPR126	-----SQLDARNTKVLTFISYIGCGISAI	47
KAI2544095.1	LMNLSTALLFLNLLFLLDGWITSFNVDGLCIAVAVLLHFFLLATFTWMGLEA IHMYIALVKVFNTYIRRYILKFC	975
GPR126	LMNLSTALLFLNLLFLLDGWITSFNVDGLCIAVAVLLHFFLLATFTWMGLEA IHMYIALVKVFNTYIRRYILKFC	122
KAI2544095.1	IIGWGLPALVVSVVLASRNNNEVYGKESYGKEKGDEFCWIQDPVIFYVTCAGYFGVMFFLNIA MFIVVMVQICGR	1050
GPR126	IIGWGLPALVVSVVLASRNNNEVYGKESYGKEKGDEFCWIQDPVIFYVTCAGYFGVMFFLNIA MFIVVMVQICGR	197
KAI2544095.1	NGKRSNRTLREEVLRNLRSVVSLTFL LGMTWGF AFAFWGPLNIPFMYLFSIFNSLQGLG IIFIFHCAMKENVQKQW	1125
GPR126	NGKRSNRTLREEVLRNLRSVVSLTFL LGMTWGF AFAFWGPLNIPFMYLFSIFNSLQGLG IIFIFHCAMKENVQKQW	272
KAI2544095.1	RRHLCCGRFRLADNSDWSKTATNIIKKSSDNLGKSLSSSSISGSNSTYLTSSKSSSSSTTYFKRNSHTDSASMDKSL	1200
GPR126	RRHLCCGRFRLADNSDWSKTATNIIKKSSDNLGKSLSSSSISGSNSTYLTSSKSSSSSTTYFKRNSHTDSASMDKSL	347
KAI2544095.1	SKLAHADGDQTSIIPVHQVIDKVKGVCNAHSDNFYKNIIMSDTFSHSTKF	1250
GPR126	SKLAHADGDQTSIIPVHQVIDKVKGVCNAHSDNFYKNIIMSDTFSHSTKF	397

Extended Data Figure 2



Extended data Fig. 2: Schematic workflow illustrating the production and purification of the target complex. Recombinant P2 baculovirus encoding GPR126–eGFP, $G\alpha_s$ protein, and $G\beta/\gamma$ protein is used for simultaneous co-infection of *Sf9* insect cells. Complex formation is accelerated by the addition of Nb35 and apyrase. After cell lysis, the protein is solubilized and purified using 1D4–agarose affinity chromatography. The target protein is captured, washed, and finally eluted by the addition of 1D4 peptide and protease. The eluted, cleaved sample is then subjected to size-exclusion chromatography for the final purification step. The resulting complex-enriched fractions are subsequently used for EM grid preparation.

Extended Data Figure 3

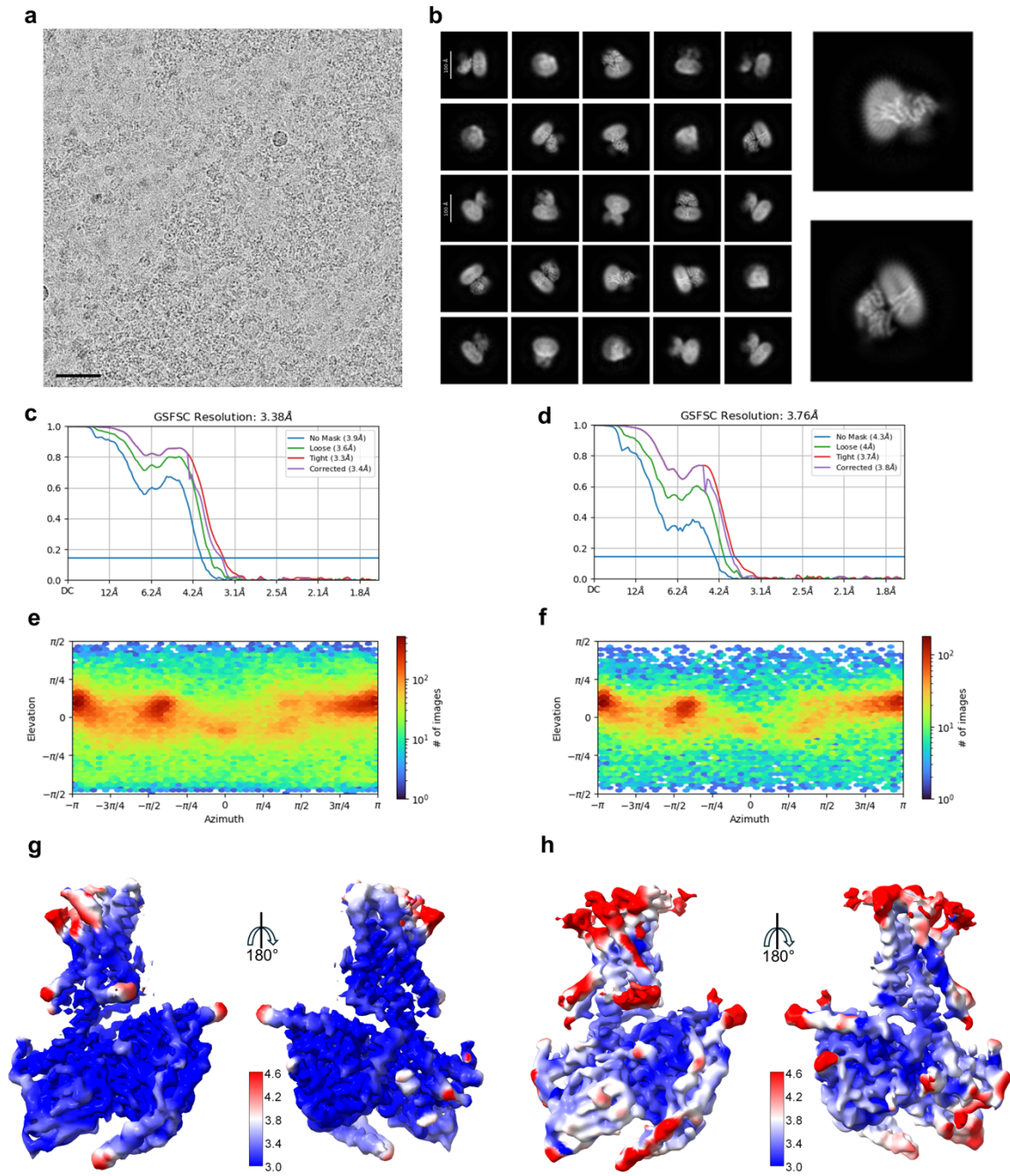


Extended data Fig. 3: Cryo-EM processing, sorting and refinement scheme for the GPR126-CTF-Gs-Nb35 complexes. Processing of the dataset was done in CryoSPARC³. Using a pre-trained TOPAZ⁴ model 1.86 million particles were picked from 5,929 micrographs and extracted with a box size of 300 px (Fourier cropped to 100 px, 2.472 Å/px).

All picked particles underwent two independent sortings. Complex particles were chosen using 2D classification to identify complex particles, followed by 3D hetero refinement. Positively assigned particles of either sorting were combined and used for further analysis resulting in a bigger dataset of 165,942 particles. These were re-extracted at full resolution (0.824 Å/px) and refined. Another round of 2D classification was applied to remove junk particles, followed by NU refinement with correction of CTF parameters resulting in a consensus structure of 146,999 particles with a global resolution of $FSC_{0.143}=3.27$ Å. 3D variability analysis in principal component analysis mode was applied to this structure using a focus mask on G protein (mask shown in light blue) at a filter resolution of 4 Å. Results were clustered into two structures, while 11,416 particles showed no complex and were discarded. The resulting structures were one without bound *Stachel* density (66,197 particles) and one of 69,386 particles that showed further variability in the *Stachel* region.

To these particles another round of 3D variability was applied in the same manner, resulting in 35,594 particles that show the previously identified unbound state and 33,792 particles that display *Stachel* density in the binding pocket. The separated states were subjected to a final round of NU refinement. Resulting in a reconstruction of an unbound state of 101,791 particles with a global resolution of $FSC_{0.143}=3.38$ Å and a reconstruction of a *Stachel* bound state of 33,792 particles with a global resolution of $FSC_{0.143}=3.76$ Å.

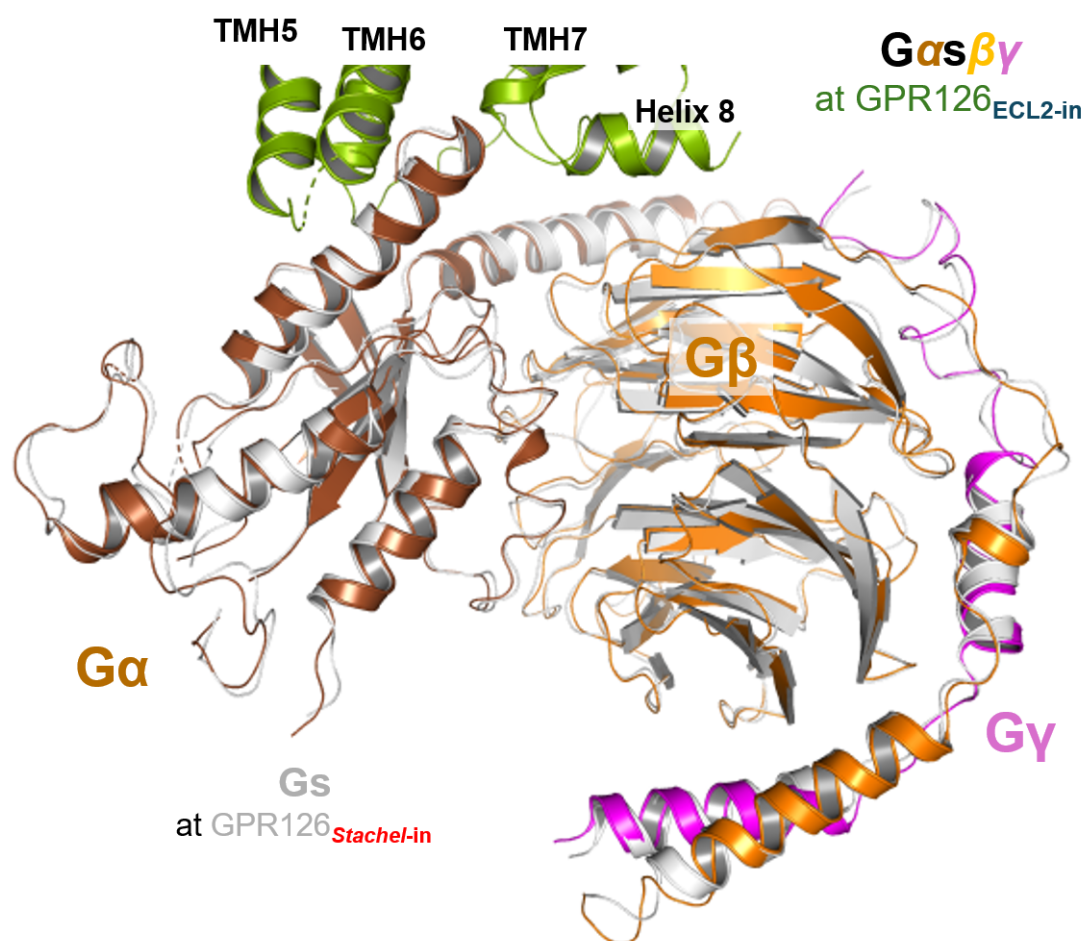
Extended Data Figure 4



Extended data Fig. 4: Cryo-EM data analysis of active GPR126-CTF-Gs-Nb35 complexes. (a) Representative cryo-electron micrograph with scale bar representing 50 nm in the image. (b) Representative 2D class averages of the complex confirming the presence of GPR126-CTF, Gs $\alpha\beta\gamma$ protein and nanobody 35. Different projection directions are present. Two exemplary classes are shown to demonstrate how details of complex and transmembrane domains are visible. (c, d) Global resolution estimation by Fourier shell correlation calculations (FSC = 0.143 cut-off) after "gold standard" refinement. The light blue curve was calculated without masking, the red and green curves by applying spherical masks and the purple one after phase randomization using a soft-mask for (c) the GPR126-CTF-Gs-Nb35 complex without bound *Stachel* (GPR126-CTF_{ECL2-in}) and (d) the GPR126-CTF-Gs-Nb35 complex with bound *Stachel* (GPR126-CTF_{Stachel-in}). (e, f) Distribution of projection directions as estimated of the

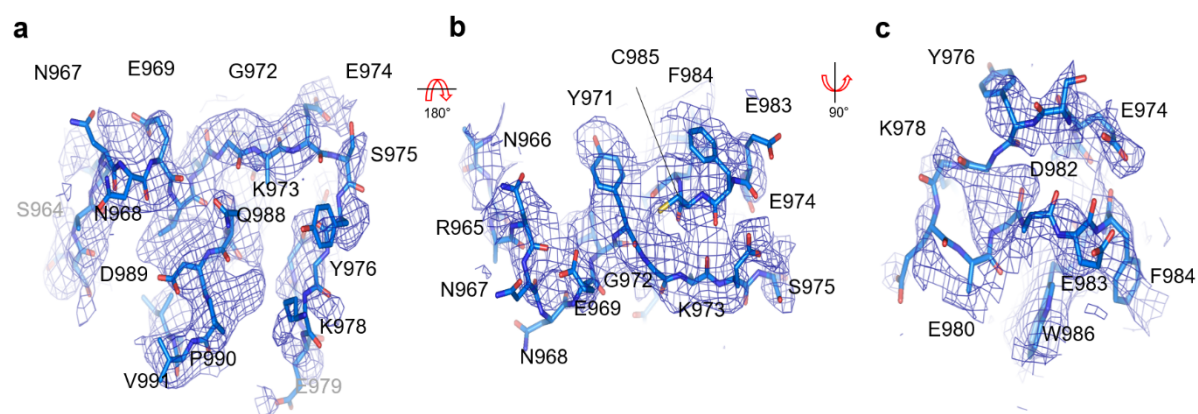
final homogeneous non-uniform (NU) refinement for **(e)** the GPR126-CTF_{ECL2-in}-Gs-Nb35 complex and **(f)** the GPR126-CTF_{Stachel-in}-Gs-Nb35 complex. **(g)** Representation of local resolution estimation for GPR126-CTF_{ECL2-in} with the final cryo-EM density coloured according to local resolution value from blue (3.0 Å) to red (4.6 Å). **(h)** Representation of local resolution estimation for the GPR126-CTF_{Stachel-in} with the final cryo-EM density according to the local resolution value from blue (3.0 Å) to red (4.6 Å).

Extended Data Figure 5



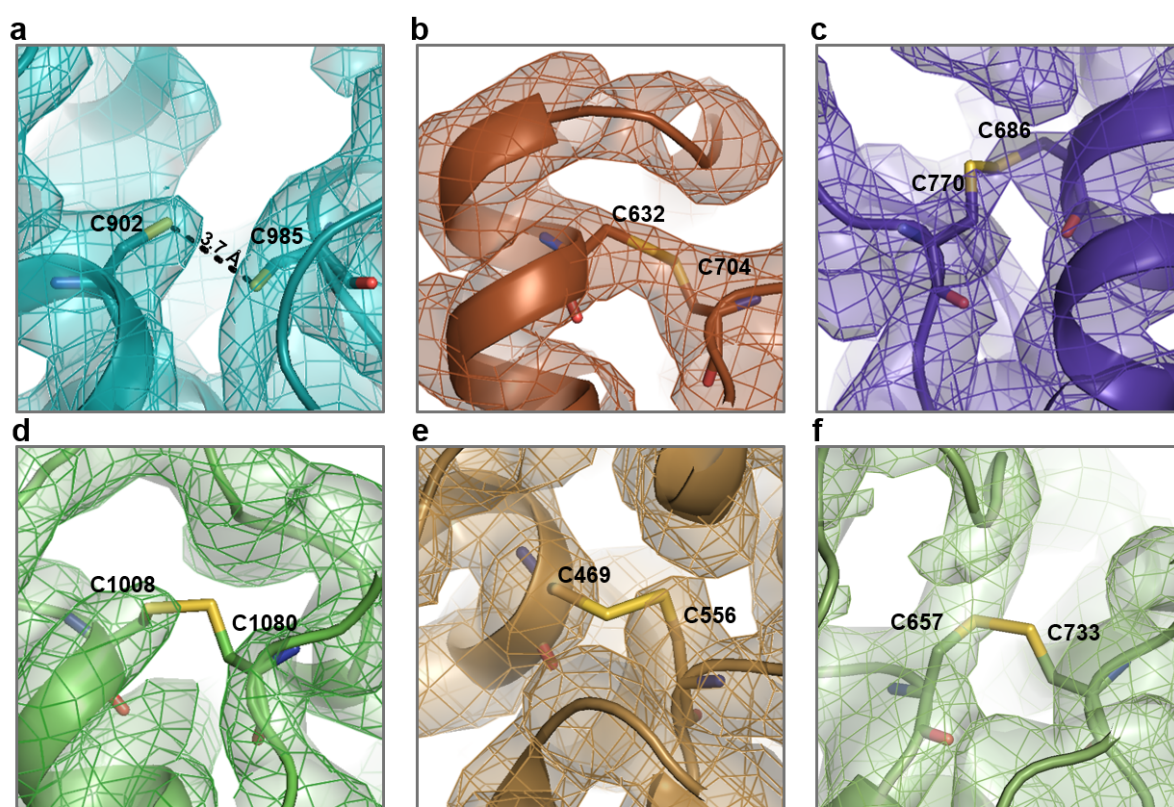
Extended data Fig. 5: Backbone superposition between the G proteins bound at the two solved GPR126-CTF structures (GPR126-CTF_{ECL2-in} and GPR126-CTF_{Stachel-in}). Superposition of two bound heterotrimeric Gs protein molecules shows strong overlap in the binding mode and orientation towards the respective receptors, specifically in the core elements with secondary structure.

Extended Data Figure 6



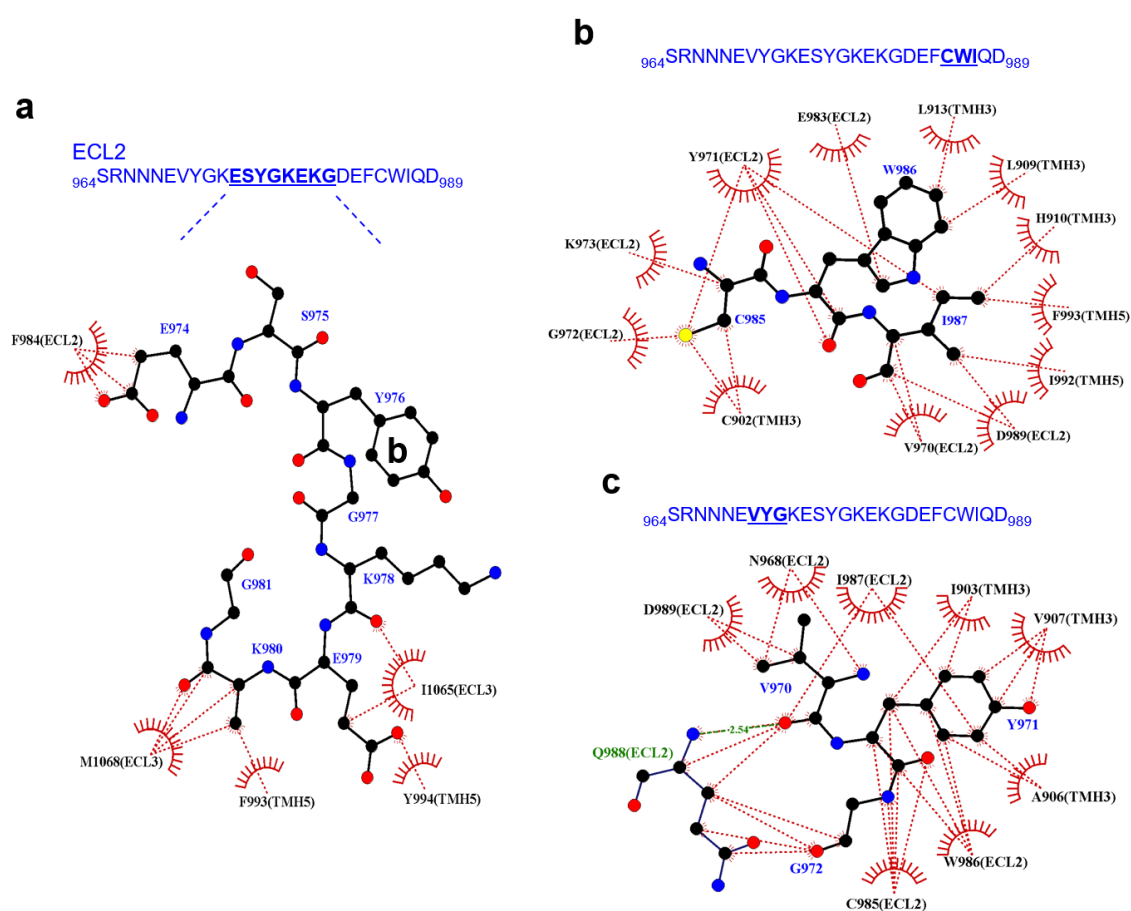
Extended data Fig. 6: Quality of the cryo-EM density map of ECL2 in the GPR126-CTF ECL2-in-Gs-Nb35 complex. Three different views (a-c) are shown to illustrate the ECL2. The cryo-EM density is shown as blue mesh at a contour level of 7σ , while the model of ECL2 is depicted as sticks.

Extended Data Figure 7



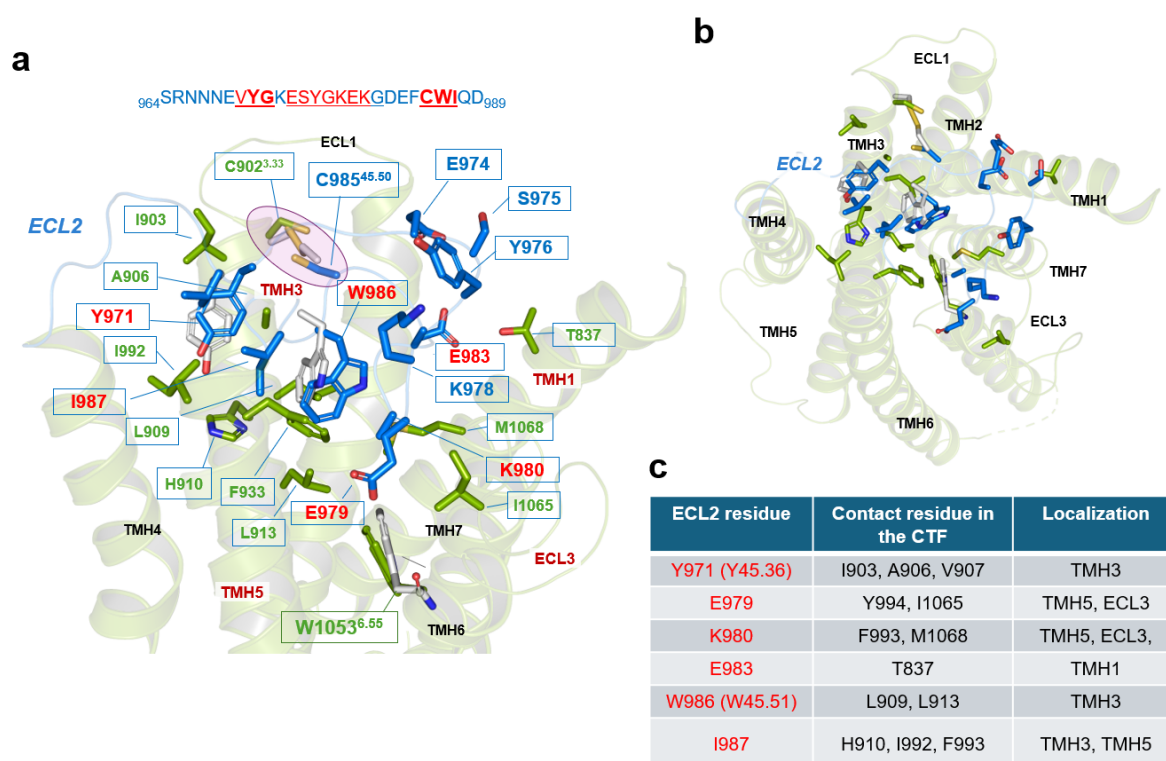
Extended data Fig. 7: Details of the disulfide bonding between ECL2 and TM3 in different published cryo-EM structures of aGPCR. (a) GPR126-CTF_{ECL2-in} does not show a disulfide bond between C902 in TM3 and C985 in ECL2. The distance between the sulphur-atoms being 3.7 Å. (b) For a GPR133-CTF structure (PDB-ID 7ept / EMDB-31232) clear density for a disulfide bond between C632 and C704 is visible. (c) For C686 and C770 in the GPR64-CTF structure (PDB-ID 7wuq / EMDB-32388) as well as for structures of (d) LPHN3-CTF (PDB-ID 7wy8 / EMDB-32887), or (e) GPR56-CTF (PDB-ID 7sf8 / EMDB-25077) and (f) GPR110-CTF (PDB-ID 7wy0 / EMDB-32883) the cryo-EM maps show no continuous density for a covalent bond between the conserved cysteines in TM3 and ECL2, even though the models insinuate one. Of note, the interpretative analysis was conducted across the entire contour level, with only clearly interpretable examples from high-quality maps being considered. In cases where an unambiguous assignment was not possible due to uncertainty or ambiguity, the corresponding bond was classified as present. All figures display cryo-EM densities as light-coloured surface mesh and models in cartoon representation with the cysteines emphasized in stick representation colored according to their elements.

Extended Data Figure 8



Extended data Fig. 8: Interactions between parts of the ECL2 to other residues in GPR126-CTF_{ECL2-in}. (a) Potential hydrogen bonds and hydrophobic interactions between the central part of ECL2 (ECL2 sequence above the picture and analyzed amino acids are underlined) and the TMD were analyzed using HBPLUS with a maximum donor acceptor distance of 3.5 Å as implemented in LigPlot+ v.2.2.9⁵. Potential hydrogen bonds are indicated by green dashed lines and labelled with their distance in Å. Residues closer than 3.9 Å were deemed to be in van der Waals contacts. They are shown as dashed red segments of a circle, with possible points of interactions indicated by red dashed lines. (b) Interactions of highly conserved W986 and adjacent residues. (c) Interactions of highly conserved residue Y971 and adjacent residues.

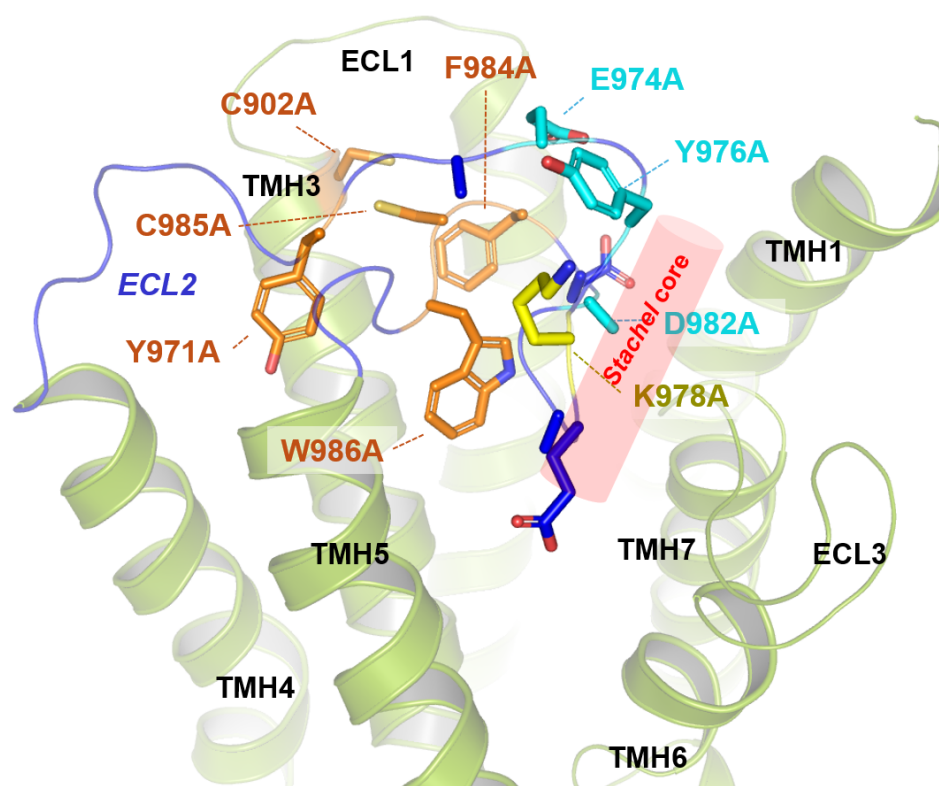
Extended Data Figure 9



Extended data Fig. 9: Spatial arrangement of GPR126 ECL2 and interactions within the 7TMD. (a) ECL2 residues contacting the 7TMD are underlined in the sequence and highlighted in red in the structure (see also (c)). Contact residues in the 7TMD of the CTF are shown as green sticks. Intramolecular ECL2 interactions are not depicted. The absent disulfide bridge in contrast to the recently published GPR126 structure (PDB-ID 21DU ⁶) and other aGPCR CTF structures is marked by a magenta circle. In addition, W⁹⁸⁶ in ECL2 shows a distinct orientation in our GPR126-CTF_{ECL2-in} structure compared to the CTF structure with a *Stachel*-in conformation (white stick). In contrast, W¹⁰⁵³ in TMH6 is similar in both structures (white stick from the *Stachel*-in structure). (b) Top view of ECL2 and 7TMD residues in contact distance. (c) Table of major ECL2–7TMD contacts color-coded as in (a).

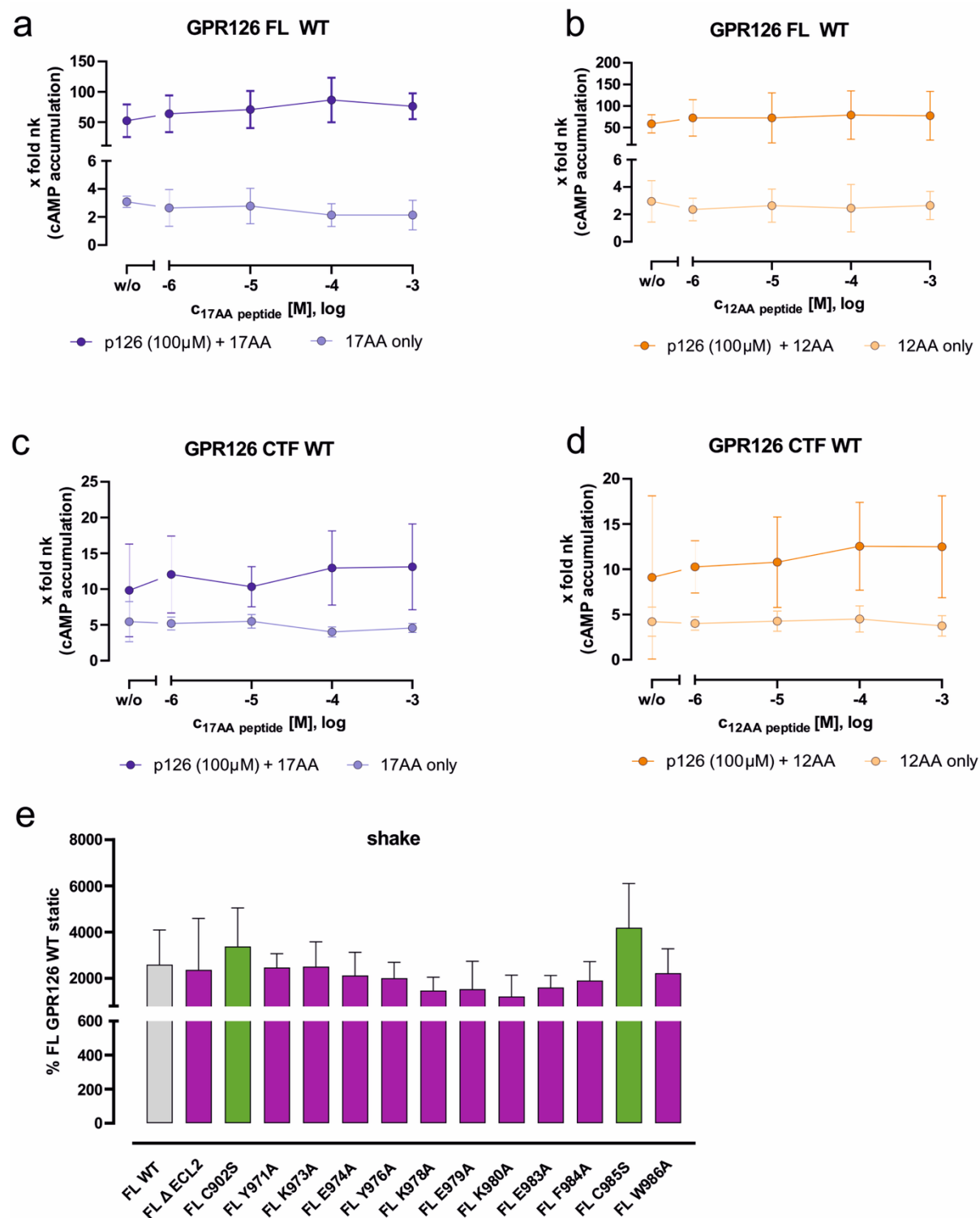
Extended Data Figure 10

CTF_{ECL2-in} with CAM positions and inactivation and hyperstimulation by mutants for p126 activation



Extended data Fig. 10: Spatial arrangement of the GPR126-CTF ECL2 in the GPR126-CTF_{ECL2-in} structure, interactions with the 7TMD, and mutant positions with functional impact. Functionally distinct ECL2 mutants mapped onto the GPR126 structure using color codes and stick representations. This analysis reveals two main outcomes: *i*. Inactivating variants (orange) cluster between ECL2 and TMH2/TMH3 and are located in close proximity to the highly conserved residues Y⁹⁷¹ and W⁹⁸⁶; *ii*. in contrast, the three CAMs (cyan) are positioned in a region that likely interfaces with the bound *Stachel*, as indicated by superimposition (inset, rotated view) with the recently determined GPR126 structure (PDB-ID 21DU ⁶) (*Stachel* core shown in red). Of note, an increased efficacy of p126 was observed at two mutants (E⁹⁷⁴A and K⁹⁷⁸A, in yellow, hyperstimulation), which are either also CAMs or are positioned at the center of ECL2.

Extended Data Figure 11



Extended data Fig 11: Functional data of GPR126 constructs. (a, b) stimulation of wild-type (WT) GPR126-FL with peptides corresponding to the ECL2 (17AA, 12 AA) alone or co-stimulated with the p126 peptide, (c, d) stimulation of GPR126-CTF with peptides corresponding to the ECL2 (17AA, 12AA) alone or co-stimulated with the p126 peptide, (e) shake assay of GPR126 FL ECL2 mutants in HEK293T cells; basal activity to 100%. One-way ANOVA * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Group	Receptor name	PDB-ID	Species	External ligand	Complex binder/ Interaction protein	Overall resolution (Å)	Disulfide-bond TMH3-ECL2	Reference
I	LPHN3/ ADGRL3	7sf7	human		mini-G13	2.90	Yes (927-999)	7
		7wy5	mouse		Gq	2.75	No (1008-1080)	8
		7wy8	mouse		Gs	2.80	No (1008-1080)	
		7x10	mouse		G12	2.93	Yes (1008-1080)	
		7wyb	mouse		Gi	2.97	? (1008-1080)	9
II	CD97/ ADGRE5	8jmt	human		apo	3.30	No (927-999)	
		7ydh	human		miniG13	3.10	No (612-684)	10
		7ydj	human		miniG12	3.03	No (612-684)	
		7ydm	human		Gq	2.89	Yes (612-684)	
		7ydp	human		miniGs	3.10	No (612-684)	11
		8ikl	human		mini-G13	2.30	Yes (612-684)	
V	GPR133/ ADGRD1	8ikj	human		apo	2.90	Yes (612-684)	
		7ept	human		Gs	3.00	Yes (632-704)	12
		7wu2	human		mini-Gs	3.00	Yes (632-704)	13
		8x9s	human	DHT	Gs	3.49	Yes (632-704)	14
		8x9t	human	YNH	Gs	2.75	Yes (632-704)	14
		8x9u	human	metenolone	Gs	2.88	Yes (632-704)	14
		9iv1	human	DHT	mini-Gs	2.98	Yes (632-704)	14
		9iv2	human	DHT	Gs	3.53	Yes (632-704)	14
VI	GPR110/ ADGRF1	9v0u	human		miniG13	3.51	No (632-704)	15
		7wu3	human		mini-Gs	3.10	No (657-733)	13
		7wu4	human	LPC	mini-Gi	3.40	? (657-733)	
		7wu5	human	LPC	mini-Gi	3.00	No (657-733)	
		7wxu	human		Gq	2.85	Yes (657-733)	16
		7wxw	human		Gs	2.84	Yes (657-733)	
		7x2v	human		Gi	3.09	? (657-733)	
		7wz7	human		G12	2.8	? (657-733)	
VIII	GPR112/ ADGRG4	7wy0	human		G13	2.66	No (657-733)	17
		8g2y	human	LPC	mini-G _{s/q}	3.44	Yes (657-733)	
	GPR64/ ADGRG2	7wuj	human		Gs	3.30	Yes (2809-2886)	18
		7wui	mouse	IP15	Gs	3.10	Yes (686-770)	
		7wuq	mouse		Gs	2.90	Yes (686-770)	
		7yp7	mouse		Gs	3.10	Yes (686-770)	19
		7xkf	mouse		BT-Gs	2.40	Yes (686-770)	
		7xke	mouse		Gs	2.90	Yes (686-770)	
	GPR114/ ADGRG5	7xkd	mouse		BT-Gs	2.40	Yes (686-770)	12
		7eq1	human		Gs	3.30	No (314-404)	
IX	GPR56/ ADGRG1	7sf8	human		mini-G13	2.70	No (469-556)	7
	GPR97/ ADGRG3	7d76	human	BCM	mini-Go with palmitoylation	3.10	Yes (338-420)	20
		7d77	human	cortisol	mini-Go with palmitoylation	2.90	Yes (338-420)	
	ADGRG6/ GPR126	21du	human	apomorphine	Gs	2.90	Yes (930-1013)	6
IX	ADGRV1/ VLGR1	9fte	mouse		apo	3.82	Yes (5963-6045)	https://doi.org/10.2210/pdb9fte/pdb

Extended data Table 1: Overview of determined aGPCR-CTF structures including visibility of a disulfide bond between TMH3 and the ECL2 (red – no disulfide bond identified). “?” indicates difficulties to clearly define or to exclude a disulfide bond. DHT denotes 5-alpha-dihydrotestosterone, YNH denotes methyl 3-[2-(2,4-dimethylphenoxy)ethanoylamino]-4-(4-ethylpiperazin-1-yl)benzoate.

Receptor construct (COS-7)	Whole cell expression		Surface expression (total DNA 500 ng per well)		Titration of surface expression (total DNA 1000 ng per well)		
	%	SD	%	SD	ng DNA of receptor construct	%	SD
GPR126-FL WT	21.01	7.13	31.46 *	11.41	500	9.04	1.65
GPR126-FL C902S	36.97	5.90	12.45	7.67	500	2.69	1.46
					750 *	9.23	2.02
					1000	19.53	7.81
GPR126-FL Y971A	40.67	4.43	7.67	2.90	500	3.17	2.48
					750	4.97	1.93
					1000 *	12.34	3.42
GPR126-FL K973A	33.88	4.55	29.59 *	5.61			
GPR126-FL E974A	21.42	11.43	35.06 *	11.27			
GPR126-FL Y976A	30.81	4.01	29.60 *	3.93			
GPR126-FL K978A	22.31	7.29	25.22 *	7.48			
GPR126-FL E979A	24.11	7.07	36.01 *	2.61			
GPR126-FL K980A	26.96	8.80	38.24 *	14.09			
GPR126-FL D982A	-1.61	0.42	0.09	0.09			
GPR126-FL E983A	6.51	3.75	11.60	4.65	500	0.26	0.26
					750	1.58	0.71
					1000 *	15.18	4.20
GPR126-FL F984A	30.59	11.12	12.26	4.77	500	1.06	0.81
					750	2.89	1.98
					1000 *	12.80	4.13
GPR126-FL C985S	32.89	7.41	13.37	9.08	500	0.58	0.46
					750	2.06	2.41
					1000 *	6.27	3.83
GPR126-FL W986A	31.90	4.55	29.50 *	6.90			
GPR126-FL Δ ECL2	23.66	3.49	29.29 *	12.16			

Receptor construct (HEK293T)	Whole cell expression		Surface expression (total DNA 500 ng per well)		Titration of surface expression (total DNA 1000 ng per well)		
	%	SD	%	SD	ng DNA of receptor construct	%	SD
GPR126-CTF WT	101.15	8.92	27.47 *	3.60	500	13.25	5.83
GPR126-CTF C902S	112.91	15.80	11.52	2.34	500	8.55	2.23
					750	14.76	8.44
					1000 *	21.68	12.70
GPR126-CTF Y971A	97.89	7.73	12.62	4.53	500	2.94	1.95
					750	5.28	1.86
					1000 *	16.20	2.23
GPR126-CTF K973A	111.36	14.53	17.85	8.35	500	13.43	4.22
					750	17.33	7.55
					1000 *	20.29	10.05
GPR126-CTF E974A	104.26	12.29	39.33 *	6.27			
GPR126-CTF Y976A	99.50	9.51	20.70	1.07	500	15.31	2.31
					750	19.75	4.68
					1000 *	26.60	8.54
GPR126-CTF K978A	101.91	20.97	31.83 *	8.55			
GPR126-CTF E979A	106.16	14.76	37.27 *	7.82			
GPR126-CTF K980A	98.95	6.44	33.28 *	7.30			
GPR126-CTF D982A	106.31	13.34	24.14 *	4.30			
GPR126-CTF E983A	106.87	21.23	28.83 *	7.29			
GPR126-CTF F984A	103.61	12.65	13.03	4.93	500	9.71	4.12
					750	14.75	1.32
					1000 *	17.26	6.00
GPR126-CTF C985S	106.45	10.94	11.24	4.34	500	9.55	3.12
					750	13.11	5.20
					1000 *	16.11	5.32
GPR126-CTF W986A	105.04	8.92	17.13	7.08	500	12.13	3.18
					750	17.23	4.00
					1000 *	24.76	5.91
GPR126-CTF Δ ECL2	101.12	11.80	16.33	5.37	500	13.86	1.93
					750	17.73	4.27
					1000 *	23.89	7.17
					750	10.65	2.53
					1000 *	14.69	4.54
GPR126-CTF ΔStachel	103.54	6.43	52.54 *	11.97			
GPR126-CTF M819A	105.70	18.81	37.39 *	14.83			
GPR126-CTF ΔStachel	105.25	10.34	32.37 *	6.24			

Y976A							
GPR126-CTF ΔStachel D982A	102.64	15.43	35.46 *	6.30			
GPR126-CTF ΔStachel ΔECL2	103.68	7.02	27.97 *	7.61			
GPR126-FL WT	37.92	6.48	52.36 *	7.23	500	10.26	2.58
GPR126-FL C902S	76.39	12.00	54.30 *	10.20			
GPR126-FL Y971A	43.11	2.19	38.02	9.05	500	10.53	1.04
					750	19.76	1.83
					1000 *	50.81	10.74
GPR126-FL K973A	37.60	4.44	85.91 *	15.71			
GPR126-FL E974A	29.25	2.83	93.33 *	19.51			
GPR126-FL Y976A	35.82	1.63	89.19 *	18.94			
GPR126-FL K978A	32.33	3.82	98.29 *	28.15			
GPR126-FL E979A	32.92	2.84	96.38 *	26.64			
GPR126-FL K980A	33.39	3.88	93.87 *	21.61			
GPR126-FL D982A	-4.41	5.38	3.03	1.17			
GPR126-FL E983A	24.15	1.16	75.89 *	16.16			
GPR126-FL F984A	42.13	5.75	47.71 *	12.21			
GPR126-FL C985S	49,35	4,38	23,58	4,00	500	3,21	2,06
					750	9,89	3,79
					1000 *	34,69	9,17
GPR126-FL W986A	38,31	3,08	82,30 *	16,14			
GPR126-FL Δ ECL2	39,10	4,43	95,77 *	26,07			

Extended data Table 2: Overview of here designed and used GPR126-CTF and GPR126-FL receptor constructs including whole cell expression and surface expression in COS-7 and HEK293-T cells. * and colored table cells visualize the used amounts in stimulation assays.

	GPR126-CTF_{ECL2-in}-Gs-Nb35	GPR126-CTF_{Stachel-in}-Gs-Nb35
Data Collection	PDB-ID: 34HS EMD-59856	PDB-ID: 35CL EMD-100357
Magnification	96,000x	96,000x
Voltage (kV)	300	300
Electron dose (e ⁻ /Å ²)	43	43
Nominal defocus range (μm)	-1 to -2	-1 to -2
Pixel size (Å/px)	0.824	0.824
Map sharpening B factor (Å ²)	132.4	107.0
Resolution (Å, 0.143 FSC)	3.27	3.76
Symmetry imposed	<i>C1</i>	<i>C1</i>
Initial number of micrographs	5929	5929
Initial particle images (no.)	1,860,602	1,860,602
Final particle images (no.)	101,791	33,792
Final map pixel size (decimated, Å/px)	0.824	0.824
Model refinement		
Initial model used (PDB ID)	7eq1	7eq1
Model resolution (Å)	3.3	3.7
FSC threshold = 0.143		
Model resolution (Å)	3.7	4.1
FSC threshold = 0.5		
Model vs. map correlation coefficient (cc_mask)	0.77	0.77
Model composition		
Non-hydrogen atoms	7,629	7641
Protein residues	978	977
B factors (Å ²) (mean)	132.4	107.0
Validation (proteins)		
R.m.s. deviations bond length (Å)	0.005	0.005
R.m.s. deviations bond angle (°)	0.858	0.890
Poor rotamers (%)	0.38	0.37
Ramachandran plot		
Outliers (%)	0.00	0.00
Allowed (%)	9.00	7.26
Favored (%)	91.00	92.74
Clash score, all atoms	4.67	5.16
Molprobity score	1.76	1.73

Extended data Table 3: Data collection and refinement statistics.

References Extended Data

- 1 Liang, Y. L. *et al.* Phase-plate cryo-EM structure of a biased agonist-bound human GLP-1 receptor-Gs complex. *Nature* **555**, 121-125, doi:10.1038/nature25773 (2018).
- 2 Garcia-Nafria, J., Lee, Y., Bai, X., Carpenter, B. & Tate, C. G. Cryo-EM structure of the adenosine A(2A) receptor coupled to an engineered heterotrimeric G protein. *Elife* **7**, doi:10.7554/eLife.35946 (2018).
- 3 Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat Methods* **14**, 290-296, doi:10.1038/nmeth.4169 (2017).
- 4 Bepler, T. *et al.* Positive-unlabeled convolutional neural networks for particle picking in cryo-electron micrographs. *Nat Methods* **16**, 1153-1160, doi:10.1038/s41592-019-0575-8 (2019).
- 5 Laskowski, R. A. & Swindells, M. B. LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *J Chem Inf Model* **51**, 2778-2786, doi:10.1021/ci200227u (2011).
- 6 Qiu, N. *et al.* Extra-helical allosteric binding site of apomorphine in ADGRG6. *Cell Discov* **12**, 9, doi:10.1038/s41421-025-00866-1 (2026).
- 7 Barros-Alvarez, X. *et al.* The tethered peptide activation mechanism of adhesion GPCRs. *Nature* **604**, 757-762, doi:10.1038/s41586-022-04575-7 (2022).
- 8 Qian, Y. *et al.* Structural insights into adhesion GPCR ADGRL3 activation and G(q), G(s), G(i), and G(12) coupling. *Mol Cell* **82**, 4340-4352 e4346, doi:10.1016/j.molcel.2022.10.009 (2022).
- 9 Guo, Q. *et al.* A method for structure determination of GPCRs in various states. *Nat Chem Biol* **20**, 74-82, doi:10.1038/s41589-023-01389-0 (2024).
- 10 Wang, N. *et al.* Structural basis of CD97 activation and G-protein coupling. *Cell Chem Biol* **30**, 1343-1353 e1345, doi:10.1016/j.chembiol.2023.08.003 (2023).
- 11 Mao, C. *et al.* Conformational transitions and activation of the adhesion receptor CD97. *Mol Cell* **84**, 570-583 e577, doi:10.1016/j.molcel.2023.12.020 (2024).
- 12 Ping, Y. Q. *et al.* Structural basis for the tethered peptide activation of adhesion GPCRs. *Nature* **604**, 763-770, doi:10.1038/s41586-022-04619-y (2022).
- 13 Qu, X. *et al.* Structural basis of tethered agonism of the adhesion GPCRs ADGRD1 and ADGRF1. *Nature* **604**, 779-785, doi:10.1038/s41586-022-04580-w (2022).
- 14 Yang, Z. *et al.* Identification, structure, and agonist design of an androgen membrane receptor. *Cell* **188**, 1589-1604 e1524, doi:10.1016/j.cell.2025.01.006 (2025).
- 15 Pu, X. *et al.* Cryo-EM structural elucidation and molecular mechanism of the GPR133-G13 signaling complex. *Biochem Biophys Res Commun* **777**, 152165, doi:10.1016/j.bbrc.2025.152165 (2025).
- 16 Zhu, X. *et al.* Structural basis of adhesion GPCR GPR110 activation by stalk peptide and G-proteins coupling. *Nat Commun* **13**, 5513, doi:10.1038/s41467-022-33173-4 (2022).
- 17 Jones, D. T. D. *et al.* Tethered agonist activated ADGRF1 structure and signalling analysis reveal basis for G protein coupling. *Nat Commun* **14**, 2490, doi:10.1038/s41467-023-38083-7 (2023).
- 18 Xiao, P. *et al.* Tethered peptide activation mechanism of the adhesion GPCRs ADGRG2 and ADGRG4. *Nature* **604**, 771-778, doi:10.1038/s41586-022-04590-8 (2022).
- 19 Lin, H. *et al.* Structures of the ADGRG2-G(s) complex in apo and ligand-bound forms. *Nat Chem Biol* **18**, 1196-1203, doi:10.1038/s41589-022-01084-6 (2022).
- 20 Ping, Y. Q. *et al.* Structures of the glucocorticoid-bound adhesion receptor GPR97-Go complex. *Nature* **589**, 620-626, doi:10.1038/s41586-020-03083-w (2021).