



## The tethered peptide activation mechanism of adhesion GPCRs

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1	The tethered peptide activation mechanism of adhesion GPCRs
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19	Adhesion G protein-coupled receptors (aGPCRs) are characterized by the presence of
20	auto-proteolysing extracellular regions (ECRs) involved in cell-cell and cell-extracellular
21	matrix interactions <sup>1</sup> . Self-cleavage within the aGPCR auto-proteolysis-inducing (GAIN)
22	domain produces two protomers, N-terminal and C-terminal fragments (NTF and CTF),
23	that remain non-covalently attached after receptors reach the cell surface <sup>1</sup> . Upon NTF
24	dissociation, the C-terminus of the GAIN domain acts as a tethered agonist (TA) peptide
25	to activate the 7-transmembrane (7TM) domain with a mechanism that has been poorly

26 understood<sup>2-5</sup>. Here we provide cryo-EM snapshots of two distinct members of the

27 aGPCR family, GPR56 and Latrophilin-3 (LPHN3). Low resolution maps of the receptors 28 in their NTF-bound state indicate that the GAIN domain projects flexibly towards the 29 extracellular space, keeping the encrypted TA peptide away from the 7TM. High 30 resolution structures of GPR56 and LPHN3 in their active, G protein-coupled states, 31 reveal that after ECR dissociation, the decrypted TA peptides engage the 7TM core with a 32 remarkable conservation of interactions that also involve extracellular loop 2 (ECL2). TA 33 binding stabilizes breaks in the middle of TM6 and TM7 that facilitates aGPCR coupling 34 and activation of heterotrimeric G proteins. Collectively, these results enable us to propose a general model for aGPCR activation. 35

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37 Adhesion GPCRs (Family B2) contain extracellular regions (ECRs) that participate in cellular 38 communication to regulate cell size, shape, polarity, adhesion, migration, cycle, death and 39 differentiation<sup>6</sup>. The ECRs include diverse sets of adhesion domains and a conserved GAIN 40 domain responsible for self-cleaving the receptor into the NTF that binds to extracellular 41 components and the CTF or 7TM domain that couples to G proteins. Cleavage occurs 42 intracellularly during protein maturation, and the two fragments remain non-covalently attached after presentation at the cell surface<sup>7</sup>. ECR dissociation through prospective force-based 43 44 mechanisms mediated by binding to extracellular partners is followed by 7TM rearrangements that lead to G protein activation<sup>8</sup>. 45

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GPR56 (ADGRG1) and LPHN3 (ADGRL3) are two distinct aGPCRs involved in diverse physiological processes<sup>9</sup>. GPR56 is widely distributed and implicated in immune system functions, hemostasis, brain development, muscle function and male fertility <sup>10</sup>. Besides the GAIN domain, the GPR56 ECR consists of a PLL (Pentraxin/Laminin/neurexin/sex-hormonebinding-globulin-Like) domain critical for protein ligand binding during oligodendrocyte development<sup>11</sup>. Dysregulation of GPR56 is associated with cancers<sup>12-14</sup> and cortical brain

malformation disorders, including bilateral frontoparietal polymicrogyria (BFPP)<sup>11,15</sup>. In neural 53 54 stem cells and platelets, GPR56 is activated by its extracellular matrix ligand, collagen III, and signals through the G<sub>12/13</sub> family of G proteins<sup>16,17</sup>. In contrast, LPHN3 is primarily abundant in 55 56 the central nervous system (CNS), where it interacts with several trans-cellular signaling 57 proteins, including teneurins (TENs) and fibronectin-like domain containing leucine-rich transmembrane proteins (FLRTs)<sup>9,18,19</sup> that are critical for the maintenance of the synaptic 58 59 architecture. Besides its GAIN domain, the LPHN3 ECR consists of a lectin binding domain (LEC), an olfactomedin-like domain (OLF) that contribute to its cell-cell adhesive properties<sup>9,18,19</sup>, 60 61 and a hormone binding domain (HormR) resembling the typical hormR domain of Family B1 GPCRs. LPHN3 is proposed to mediate its neuronal functions mainly through G<sub>12/13</sub> protein 62 coupling and signaling through the Rho/Rac pathway, leading to actin cytoskeletal 63 changes<sup>1,20,21</sup>. Notably, LPHN3 has been linked to substance abuse and attention deficit 64 hyperactivity disorder (ADHD), elevating its interest as a potential pharmacological target<sup>22</sup>. 65

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Crystal structures have shown the architecture of the LPHN1 GAIN and HormR domains<sup>23</sup>, the 67 LPHN3 OLF and LEC domains in complex with FLRT<sup>24-26</sup>, and the entire ECR of GPR56<sup>11</sup>. A 68 69 recent cryo-EM study showed the structure of the aGPCR GPR97/miniG<sub>o</sub> complex as stabilized by partial agonist glucocorticoids<sup>27</sup>. Notably, however, the mechanism of aGPCR activation by 70 71 the native tethered agonist (TA) peptide remains elusive. Here we describe the structures of 72 GPR56 and LPHN3 in their fully active, G protein-coupled states bound intramolecularly to their 73 native TA peptides. Complemented by low-resolution cryo-EM visualization of NTF-bound 74 holoreceptors, functional assays, and molecular dynamics (MD) simulations, the results provide a structural framework for aGPCR activation and G protein recruitment. 75

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#### 77 GPR56 and LPHN3 display flexible ECRs

78 We first sought to examine the NTF-bound (TA-encrypted; Fig. 1a) structures of human GPR56 and LPHN3 by cryo-EM. For LPHN3, we purified recombinant receptor including the N-terminal 79 80 HormR and GAIN domains bound non-covalently to the 7TM after self-cleavage at the native G 81 protein proteolytic site (GPS) (Extended Data Fig. 1). Because purified full-length auto-82 proteolysed GPR56 underwent spontaneous ECR dissociation, we employed an 83 autoproteolytically-deficient GPR56 H381S GPS mutant holoreceptor (Extended Data Fig. 2). 84 For both GPR56 and LPHN3, cryo-EM visualization allowed us to obtain 3D reconstructions 85 only at low resolution (Fig. 1b-c; Extended Data Figs. 1 and 2), primarily due to the continuous 86 flexibility of the ECRs. This flexibility was particularly evident in LPHN3 (Fig. 1b and Extended 87 Data Fig. 1f), presumably because the stalk connecting the GAIN domain to the 7TM is extended by two amino acids compared to the GPR56 stalk, accounting for a ~6Å increase in 88 89 length. LPHN3 embedded in lipid nanodiscs exhibited the same behaviour (Extended Data Fig. 90 1q-i), indicating that the detergent micelle did not perturb putative interactions between the 91 GAIN and 7TM domain. Collectively, these results showed that in the NTF-bound state, the 92 GAIN domain is not ordered against the 7TM, presumably allowing the ECR the flexibility to 93 sample protein/ligand binding partners in the extracellular space.

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## 95 Structures of active-state GPR56 and LPHN3 in complex with G<sub>13</sub>

96 For structural studies of activated GPR56 and LPHN3 in their TA-bound states coupled to G 97 protein, we created constructs consisting of the unencrypted peptide agonists followed by the 98 stalk linker and the 7TM domain, thus mimicking aGPCR CTFs (Figs. 1d, 2a and Extended Data Fig. 3a). Inspired by the engineering of a thermostable mini $G_{\alpha 12}^{28}$ , we designed a mini-G protein 99 100 variant of the  $G_{13} \alpha$  subunit (Extended Data Fig. 3a-b). Co-expression of receptors and mini $G_{13}$ 101 heterotrimer enabled us to isolate stable complexes (Extended Data Fig. 3c) and obtain cryo-102 EM maps of GPR56/miniG<sub>13</sub> and LPHN3/miniG<sub>13</sub> (Fig. 1e-f). Combinations of local refinements of the 7TM domain and G protein yielded maps with nominal resolutions of 2.7 Å and 2.9 Å for 103

GPR56, and 2.9 Å and 3.1 Å for the LPHN3 complexes, respectively (Extended Data Table 1
and Extended Data Figs. 4 and 5).

## 106 **Tethered-peptide-agonist interactions with the 7TM**

The stalks of aGPCRs are ~20-24 residue N-terminal extensions of TM1, with the first ~7 amino acids comprising the TA (Fig. 2a). Both GPR56/ and LPHN3/G protein complex maps revealed well-resolved densities for the native TA peptides bound within the orthosteric site of the 7TM bundle (Fig. 2), in agreement with the proposed tethered-peptide-agonist model<sup>1</sup>. In this configuration, the stalks bend nearly 180° downward into the core of the 7TM (Figs. 2b-d and 3 a-b), permitting the TAs to engage a remarkable set of conserved interactions, predominantly with TMs 1, 2, 6, 7 and extracellular loop 2 (ECL2) (Figs. 2 and 3, Extended Data Table 2).

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115 The observed interactions were assessed with G protein activation assays using plasma 116 membrane-enriched isolates from cells overexpressing mutant receptors (Fig. 3c-d). We note 117 that relative receptor levels were measured using the same membrane isolates and do not 118 represent direct measurement of cell surface receptor levels. To assess surface expression, we 119 employed a cell surface biotinylation/pulldown experiment for two of the most activity-defective mutants (W<sup>7.42</sup>A and F<sup>2.64</sup>A, described below), which showed that targeted mutations did not 120 121 substantially impact receptor trafficking to the plasma membrane (Fig. 3, Extended Data Figs. 6-122 8, Extended Data Table 3 and Supplementary Data Table 1).

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The third residue of most aGPCR TAs is a highly conserved phenylalanine (F385 in GPR56 and F844 in LPHN3) required for TA-stimulated G<sub>13</sub> activation (Fig. 3a-d; Extended Data Figs. 6 and 7)<sup>4</sup>. GPR56 F385 interacts with C411<sup>1.47</sup> and forms a hydrophobic interaction with F454<sup>2.64</sup> (Fig. 3a) (Wooten numbering in superscript <sup>29</sup>, equivalent to the Ballesteros-Weinstein numbering for Family A GPCRs <sup>30</sup>). Likewise, LPHN3 TA residue F844 interacts with I872<sup>1.47</sup> and F914<sup>2.64</sup> (Fig. 3b and 3d). The interaction with C411<sup>1.47</sup> was dispensable for G protein activation by GPR56,

but the hydrophobic interactions of either TA phenylalanine with  $F^{2.64}$  are essential, as demonstrated by the near complete loss of G<sub>13</sub> activation by GPR56 F454<sup>2.64</sup>A or LPHN3 F914<sup>2.64</sup>A mutants (Fig. 3c-d).

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134 Another set of critical interactions were observed between the TA and ECL2 that reaches into 135 the interior of the 7TM domain to form a wedge-like plug structure (Fig. 3e-f; Extended Data Fig. 8a-b). ECL2 residues GPR56 W557<sup>45.51</sup> and LPHN3 W1000<sup>45.51</sup> reside within hydrophobic 7TM 136 patches (L476<sup>3.36</sup> and I558<sup>45.52</sup> in GPR56 or L934<sup>3.36</sup>, L1001<sup>45.52</sup> and I1008<sup>5.36</sup> in LPHN3) and 137 138 interact with the sixth TA residue, a conserved leucine (GPR56 L388 or LPHN3 L847) (Fig. 3ef). Interestingly, W421<sup>45.51</sup> in the ECL2 of GPR97 (ADGRG3; PDB ID: 7D76, 7D77) also reaches 139 140 down into the 7TM interior close to the bound glucocorticoid ligand<sup>27</sup>. Our assays show that these interactions are essential for tethered agonism, as the GPR56 W557<sup>45.51</sup>A and LPHN3 141 W1000<sup>45.51</sup>A mutants had negligible ability to activate G<sub>13</sub> (Fig. 3c-d; Extended Data Figs. 6 and 142 143 7). Notably, ECL2 assumes a stable configuration due, in part, to the presence of a disulfide 144 bond between TM3 residue C<sup>3.29</sup> and ECL2 residue C<sup>45.50</sup>, adjacent to W<sup>45.51</sup> that coordinates the 145 binding of TA L388/L847. We postulate that the decrypted TA peptide needs to be flexible to thread through a relatively narrow opening at the extracellular face of the receptor and interact 146 147 with multiple residues of the orthosteric binding site. In support of this notion, MD simulations of 148 the TA peptide alone in solution showed that it did not assume the conformation observed in the 149 orthosteric binding site, but was instead conformationally variable without adopting a secondary 150 structure (Extended Data Fig. 9a-b).

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Our examination of the activity of GPR56 M389A and LPHN3 M848A mutants (Fig. 3c-d) reaffirmed that mutation of this conserved seventh TA residue critically reduced G protein stimulation by GPR56 or GPR110<sup>4</sup>. In the TA-bound structures, GPR56 M389 and LPHN3 M848 interact with GPR56 I620<sup>6.56</sup> and LPHN3 L1072<sup>6.57</sup>, respectively, (Fig. 3e-f), and mutation of

156 either residue moderately reduced receptor-stimulated G<sub>13</sub> activation (Fig. 3c-d). More importantly, in both LPHN3 and GPR56, the TA seventh methionine interacts with W<sup>6.53</sup> 157 158 (Extended Data Fig. 8c-d), a conserved residue that interacted with the bound steroid ligand in the GPR97 partial agonist-activated receptor structure<sup>27</sup>. The function of the aGPCR W<sup>6.53</sup> 159 seems to parallel the Family A GPCR "toggle switch" residue W<sup>6.48 31</sup>, which rearranges upon 160 161 agonist binding and drives the opening of the cytoplasmic end of TM6, thereby enabling G protein engagement. In agreement with this role, mutation of W<sup>6.53</sup> in GPR56 (W617A) or 162 163 LPHN3 (W1068A) strongly abrogated receptor-dependent G protein activation (Fig. 3c-d).

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#### 165 Active state conformation of the GPR56 and LPHN3 7TM bundles

166 Even though GPR56 and LPHN3 belong to different aGPCR subfamilies, their TA-bound 7TM 167 conformations were remarkably similar (Extended Data Fig. 8e). On the extracellular side of 168 both active-state receptors, TM1 is bent towards the transmembrane bundle presumably by TA 169 agonist stabilization within the 7TM domain (Fig. 3g). TM7 is bent outwards, accommodating 170 both the TA and the portion of ECL2 that reaches down into the orthosteric binding site (Extended Data Fig. 8a-b). Residue G<sup>7.50</sup> (G645 of GPR56 and G1094 of LPHN3) acts as a 171 172 pivot point to kink TM7, which parallels the reported kinked TM7 arrangements of active Family 173 B1 GPCRs<sup>32-34</sup> (Extended Data Fig. 10a-b). Accompanying TM7, TM6 is kinked outwards at the hinge residue G<sup>6.50</sup>, three residues from toggle switch W<sup>6.53</sup>. The intracellular halves of GPR56 174 175 and LPHN3 TM5 and TM6 are in an open conformation, as expected for fully activated G 176 protein-bound states. Notably, the degree of TM6 opening and the kink at residue 6.50 is 177 distinct compared to the glucocorticoid-bound GPR97/miniG<sub>o</sub> complex (Extended Data Fig. 178 10c,q), where TM6 appeared to open more modestly without a kink<sup>27</sup>. This difference, along with 179 the lack of cytoplasmic opening of TM7 in the GPR97/miniG<sub>o</sub> structure, may reflect that the 180 tethered-peptide acts as a full agonist while the corticoids are partial agonists that stabilize an 181 intermediate active state<sup>35</sup>.

Interestingly, residue Q<sup>7.49</sup> establishes an electrostatic interaction with the indole nitrogen of the toggle switch W<sup>6.53</sup>, stabilizing the joint extracellular opening of the transmembrane helices and the hydrophobic core of the receptors (Extended Data Fig. 8c-d). The core of both receptors comprises a network of residues that interact with the methionine TA seventh residue through W<sup>6.53</sup>, which is coordinated by M487<sup>3.47</sup> and F637<sup>7.42</sup> in GPR56 or M945<sup>3.47</sup>, F942<sup>3.44</sup> and F1086<sup>7.42</sup> in LPHN3, as well as the aforementioned electrostatic interactions involving Q<sup>7.49</sup> (Fig. 3e-f, Extended Data Fig. 8c-d, f).

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## 190 aGPCR interactions with G<sub>13</sub>

191 GPR56 and LPHN3 engage the G protein somewhat differently, as evidenced by the 12° 192 rotation between the N-terminal  $\alpha$ -helices ( $\alpha$ N) in the coupled G<sub>13</sub>  $\alpha$  subunit (Extended Data Fig. 193 10d). Superposition of the C $\alpha$  atoms of G $\alpha$  of miniG<sub>13</sub> bound to GPR56 with miniG<sub>0</sub> coupled to 5HT1B (PDB ID: 6G79)<sup>36</sup> shows an RMSD of 1.54 Å, reflecting an overall conformational 194 195 similarity between these receptor-coupled G proteins (Extended Data Fig. 10e). As observed in 196 Family A and B1 GPCRs, the intracellular ends of TM5 and TM6 in the GPR56/miniG<sub>13</sub> and 197 LPHN3/miniG<sub>13</sub> complexes are present in open conformations that accommodate binding of the 198 G protein C-terminal α5 helix (Figs. 2 and 4a). While many side chain interactions between 7TM 199 elements and the  $\alpha$ 5 helix are conserved between GPR56 and LPHN3 (Fig. 4b-c), we observed 200 a notable difference with GPR56 TM2, which is closer to the  $\alpha$ 5 helix, resulting in the positioning of TM7 closer to TM6 (Fig. 4a). In effect, GPR56 residue D434<sup>2.44</sup> of TM2 establishes a 201 hydrogen bond interaction with  $G\alpha_{13}$  residue Q226<sup>H5.22</sup> not observed at the equivalent LPHN3 202 203 TM2 position (Fig. 4b-c). Consistent with this observation, the GPR56 D434A mutant had 204 markedly reduced receptor-stimulated G<sub>13</sub> activation (Extended Data Figs. 6e and 7a).

206 The structures also reveal interactions between aGPCR ICL2 residues and the G protein Nterminal  $\alpha N$  helix.  $G_{\alpha 13}$  residues K27<sup> $\alpha N.51$ </sup>, T28<sup> $\alpha N.52$ </sup> and R32<sup>hns1.03</sup> engage Y505<sup>ICL2</sup> of GPR56. 207 whereas T28<sup> $\alpha$ N.52</sup> and R32<sup>hns1.03</sup> establish polar interactions with backbone  $\alpha$ -carbonyls of LPHN3 208 ICL2 E961 and E963, respectively. Additionally, GPR56 F502<sup>ICL2</sup> and the conserved LPHN3 209 210 residue F960<sup>ICL2</sup> establish hydrophobic interactions with multiple G protein residues, including a Pi-Pi interaction with miniG<sub> $\alpha13$ </sub>  $\alpha5$  helix residue F212<sup> $\alpha5.08$ </sup> (Fig. 4d-e). The substantially reduced 211 abilities of GPR56 F502A and LPHN3 F960A mutants to activate G<sub>13</sub> supports the importance of 212 213 these interactions (Extended Data Figs. 6e-f and 7).

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Our MD simulations of active state LPHN3 without bound G protein provide a glimpse into the dynamics of ICL2. Comparing the difference in root mean square fluctuations (RMSF) in the final 100 ns of 1  $\mu$ s trajectories between five replicate simulations with and without the TA reveals that in the absence of the TA, the half of ICL2 near TM5 becomes more flexible (Extended Data Fig. 9c-d), suggesting that tethered agonist binding to the 7TM domain stabilizes the ICL2 conformation.

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## 222 Model for tethered agonist mechanism of activation of aGPCRs

223 Dissociation of NTFs from aGPCRs unveils the TA peptide so that it may activate the 7TM by a 224 mechanism that has been poorly understood<sup>1</sup>. Numerous studies have detected isolated 225 aGPCR NTFs in a variety of tissues, suggesting that their presence is a remnant of an activation event or a result of spontaneous NTF shedding<sup>1,7</sup>. An activation mechanism with parallels has 226 227 been described for protease-activated GPCRs (PAR1-4)<sup>37,38</sup>, in which cleavage of the N-228 terminal leader sequences by exogenous proteases exposes a tethered peptide that serves to 229 activate the receptor, although PAR TAs do not share sequence similarity with aGPCR TAs. Crystal structures of GPR56<sup>11</sup> and LPHN1<sup>23</sup> ECRs in complex with their cleaved TAs showed 230

231 that the peptides fold as  $\beta$ -strands encrypted within the core of the GAIN<sub>B</sub> subdomain. Our NTF-232 bound structures illustrate that in the context of the holoreceptor, the GAIN domain is not 233 anchored to the 7TM, thus keeping the TA encrypted and at a distance from the 7TM bundle 234 (Extended Data Fig. 1e-f). In the TA-activated state structures, the decrypted TA penetrates the 235 7TM orthosteric binding pocket where it adopts a partial  $\alpha$ -helical fold to stabilize an active 236 receptor conformation (Fig. 2). Our MD simulations show that the peptide on its own is flexible 237 and adopts minimal secondary structure (Extended Data Fig. 9a-b), indicating that the TA 238 conformations observed in the X-ray studies and our current cryo-EM study are stabilized by 239 interactions with the GAIN domain or the 7TM, respectively. This conformational adaptability 240 appears to be a key component underlying the encryption/decryption of the TA and its agonistic 241 properties for aGPCRs. The remarkable conservation of TA peptide interactions and 7TM 242 conformation, including the role of ECL2, observed for two active-state aGPCRs from distinct 243 subfamilies suggests that the structural determinants of receptor activation by the tethered 244 agonist may be universal to aGPCRs, a hypothesis that will be further tested in future studies of 245 additional aGPCRs in complex with different G protein partners.

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## 338 Figure legends

339 Fig. 1 | Cryo-EM reconstructions for GPR56 and LPHN3. a, Cartoon representation of a self-340 cleaved NTF-bound aGPCR. The encrypted tethered agonist (TA, orange) resides in a  $\beta$ -strand 341 conformation within the core of the GAIN domain. b, c, Low resolution maps for NTF-bound 342 state receptors showing flexibility of the ECR. Side and top views of **b**, LPHN3 (magenta) and **c**, 343 GPR56 (blue). Maps are the result of 3D classification, with three distinct classes of LPHN3 344 superimposed. Dashed arrows indicate NTF mobility. d, Interactions of the NTF with 345 extracellular partners are proposed to result in its dissociation from the CTF, thereby decrypting 346 the TA peptide in the extracellular space. Binding of the TA within the 7TM domain (CTF) 347 orthosteric site stabilizes an active receptor conformation that induces G protein nucleotide 348 exchange and elicits intracellular signaling. e, f, High resolution maps for active GPR56 and

LPHN3 complexes with G protein. The micelle-embedded 7TM domains are coupled to miniG<sub>13</sub>
 heterotrimer. The cryo-EM maps have been assigned and colored accordingly. In **b-f**, scale bars
 are indicated.

352

353 Fig. 2 | Structures of active GPR56 and LPHN3 complexes with bound tethered agonist 354 (TA) peptide. a, Decrypted TA sequence for GPR56 on the left (cyan) and LPHN3 on the right 355 (pink), stalk linker sequences are underlined in black and followed by the 7TM starting with the first TM1 residue V<sup>1.34</sup>. **b**, Model for active tethered agonist-bound GPR56/miniG<sub>13</sub> complex with 356 357 box around the tethered agonist binding site (left) and cryo-EM density and model for the TA peptide (right). c, Model for active tethered agonist-bound LPHN3/miniG<sub>13</sub> complex with box 358 359 around the tethered agonist binding site (right) and cryo-EM density and model for the TA 360 peptide (left). d, Top-down views of active GPR56/ (left) and LPHN3/miniG<sub>13</sub> (right) complexes. 361 Black arrows point to the tethered agonist bound to the 7TM domains and to the stalk linkers 362 emanating from TM1.

363

364 Fig. 3 | Tethered-peptide-agonist interactions. a, b, Tethered agonist (TA) interactions with 365 TM1 and TM2 in **a**, GPR56 and **b**, LPHN3. **c**, **d**, Activation of reconstituted  $G_{13}$  via GTPyS 366 binding activity assay by TA and TA-interacting mutants for c, GPR56 and d, LPHN3. Data 367 displayed as average of n = 3 biologically-independent reactions with error bars representing  $\pm$ 368 S.D. Statistical significance between mutant and wild-type receptors was calculated using RM 369 one-way ANOVA analysis, n.s. = not significant, \* = p < 0.05, \*\* = p < 0.01. Further detailed 370 statistical information can be found in Supplementary Data Table 1. e, f, TA interactions with 371 ECL2 and the hydrophobic core in e, GPR56 and f, LPHN3. GPR56 7TM in blue and TA in 372 cyan, LPHN3 7TM in magenta and TA in pink. g, Top view of superposition with GPR97 (PDB 373 ID: 7D77, in white) showing helix rearrangements for TM1, TM6 and TM7 when compared to 374 TA-bound-GPR56 (blue) and -LPHN3 (magenta) and cortisol ligand (carbon atoms in green)- bound GPR97, showing that the cortisol or TA ligands occupy a common orthosteric site withinthe three receptors.

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378 Fig. 4 | G protein binding by GPR56 and LPHN3. a, G protein binding through the mini-G<sub>13</sub> α5 379 helix ( $\alpha$ 5) with superimposed GPR56 (blue) and LPHN3 (magenta). **b**, mini-G<sub>13</sub>  $\alpha$ 5 helix ( $\alpha$ 5) 380 interactions with GPR56 and c, LPHN3. Many of these interactions are conserved. TM3 (L494<sup>3.54</sup> and L497<sup>3.57</sup>), TM5 (M586<sup>5.57</sup>, I590<sup>5.61</sup> and R592<sup>5.63</sup>), TM6 (T605<sup>6.41</sup>), and TM7 381 (M655<sup>7.60</sup>) of GPR56, and TM3 (M955<sup>3.57</sup> and L956<sup>3.58</sup>), TM5 (M1033<sup>5.61</sup>), TM6 (S1052<sup>6.37</sup> and 382 I1055<sup>6.40</sup>) and TM7 (Q1105<sup>7.61</sup>) of LPHN3 establish hydrophobic or polar interactions with the 383 384  $G\alpha_{13} \alpha 5$  helix. **d**, ICL2 interactions with the mini- $G_{13}$  N-terminal helix ( $\alpha N$ ) for GPR56 and e, 385 LPHN3. G protein residues labeled in gold, receptor residues labeled in black, hydrogen bond 386 interactions are dashed grey lines.

387

## 388 Methods

#### 389 **Construct design, cloning and virus production of aGPCRs**

390 For the structural and biochemical studies of aGPCRs in their NTF-bound form, the full-length 391 sequence of human GPR56 (ADGRG1) (Isoform 2, Uniprot ID: Q9Y653-2) and LPHN3 392 (ADGRL3) (Isoform 1, Uniprot ID: Q9HAR2-1, residues 495-1138) were cloned into 393 pFastBac1<sup>™</sup> (ThermoFisher) following an N-terminal hemagglutinin (HA)-membrane targeting 394 signal peptide and a FLAG-tag (for LPHN3, the tag was also flanked by a Tobacco Etch Virus 395 nuclear-inclusion-a endopeptidase (TEV) protease - cleavage site). For GPR56, a cleavage-396 deficient GPR56 H381S GPS mutant was employed to prevent autoproteolysis and dissociation 397 of the N- and C-terminal GPR56 fragments during purification.

398 For the active-state constructs, truncated versions comprising the 7TM domain and the tethered 399 agonist peptide sequences corresponding to residues 383-687 and 842-1138 of GPR56 and

400 LPHN3, respectively, were cloned into pFastBac1<sup>™</sup>. The ORFs were inserted following an N-401 terminal HA-membrane targeting signal peptide and supplemented with an additional 402 methionine residue that was found to be important for the efficient signal peptide cleavage in our 403 preliminary studies (data not shown). The GPR56 expression constructs included either a 6X or 404 a 10X C-terminal histidine tag preceded by a Human Rhinovirus (HRV) 3C Protease cleavage 405 site. The LPHN3 expression constructs included a green fluorescent protein (GFP) module 406 located between the 3C cleavage site and the C-terminal 6X histidine tag. A schematic 407 description of the constructs used in this study is provided in Extended Data Figs. 1-3.

408 Baculovirus production was conducted using the Bac-to-Bac system (ThermoFisher). Viruses 409 were prepared according to manufacturer's instructions in *Spodoptera frugiperda* 9 (*Sf9*) cells 410 grown in ESF921 medium (Expression systems LLC).

411

## 412 **Construct design and cloning of the miniG<sub>13</sub> heterotrimer**

413 The miniG<sub>q13</sub> generated in this study was inspired by the design of miniG<sub>q12</sub> described in Nehmé *et al*<sup>28</sup>. The alpha helical domain of human  $G_{a13}$  (residues D253<sup>S4H3.05</sup> through T262<sup>S4H3.14</sup>) was 414 replaced by a GGSGGSGG linker, and the stabilizing mutations G57D<sup>S1H1.03</sup>, E58N<sup>S1H1.04</sup>, 415 S248D<sup>S4.07</sup>, E251D<sup>S4H3.03</sup>, I271D<sup>H3.08</sup>, I355A<sup>H5.04</sup> and V358I<sup>H5.07</sup> were introduced (Extended Data 416 417 Fig. 3b). In addition, residues 1-30 were replaced by the first 15 N-terminal residues of G<sub>ai2</sub> to 418 improve expression and purification while maintaining interaction with the receptor (Extended Data Fig. 10f). As described for miniG<sub>q</sub><sup>39</sup>, the miniG<sub> $\alpha$ 13/i</sub> sequence was cloned into the P10 419 420 promoter cloning site in pFastBac<sup>TM</sup> Dual (Invitrogen) and fused downstream of a human  $G_{\gamma 2}$ 421 gene that was separated by a 3X GSA (Gly/Ser/Ala) linker. Human G<sub>B1</sub> including an N-terminal 422 6X histidine tag and Human rhinovirus 3C protease signal sequence was inserted into the 423 second cloning site after the polyhedrin promoter (Extended Data Fig. 3a). The pFastBac™ 424 Dual vector allows for the bicistronic gene expression, and was utilized to form the 425 heterotrimeric G protein complex in situ.

426

#### 427 **Protein expression and purification**

#### 428 Purification of NTF-bound aGPCR holoreceptors in detergents

429 Proteins were expressed in Sf9 cells at 27°C and were harvested 48 h post viral infection. Cell 430 pellets were lysed in a buffer containing 20 mM Tris pH 7.5, 1 mM EDTA, 15% v/v glycerol, 1 431 mM PMSF, 160 µg/mL benzamidine, 2.5 µg/mL leupeptin and 2 mg/mL iodoacetamide for 1h at 432 4°C. Membranes were harvested through centrifugation at 37,000 x g for 30 min and 433 homogenized in 0.5% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace) and 0.1% (w/v) 434 cholesteryl hemisuccinate (CHS, Anatrace) in solubilization buffer (20 mM HEPES pH 7.5, 150 435 mM NaCl, 15% v/v glycerol, 1 mM PMSF, 160 µg/mL benzamidine, 2.5 µg/mL leupeptin, 436 benzonase and 1 mg/mL iodoacetamide). Insoluble material was removed by centrifugation at 437 37,000 x g for 30 min. Detergent solubilized receptors were purified by affinity capture, with Ni 438 chelate resin capture for the uncleavable GPR56, and double anti-FLAG / Ni chelate resin 439 capture for LPHN3 that was applied to ensure the purification of the intact protein containing 440 both the N- and C- termini, which are not covalently attached.

441 Solubilized GPR56 was supplemented with 10 mM imidazole and loaded onto a TALON Metal 442 Affinity Resin (Takara) column. The column was washed with 20 mM HEPES, pH 7.5, 150 mM 443 NaCl, 10% v/v glycerol, 2 mM MgCl<sub>2</sub>, 10 mM imidazole, 0.1% (w/v) LMNG, 0.02% (w/v) glyco-444 diosgenin (GDN, Anatrace), 0.01% (w/v) CHS. The protein was eluted with 20 mM HEPES, pH 445 7.5, 150 mM NaCl, 10% v/v glycerol, 2 mM MgCl<sub>2</sub>, 200 mM imidazole, 0.01% (w/v) LMNG, 446 0.002% (w/v) GDN, 0.001% (w/v) CHS. HRV 3C Protease was added and incubated at 4°C for 447 16h while the sample was simultaneously dialyzed against low imidazole buffer. The cleaved 448 sample was then loaded onto a TALON resin and the flow through was collected and 449 concentrated using an Amicon Ultra Centrifugal Filter (MWCO 100kDa, Merck-Millipore).

For LPHN3 the clarified supernatant was incubated with an Anti-DYKDDDDK G1 Affinity Resin
(Genscript) by batch binding for 2 h at 4°C. The resin was packed into a gravity flow column and

452 washed with a wash buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl, 0.075% (w/v) 453 LMNG, 0.025% (w/v) GDN, and 0.01% (w/v) CHS. Protein elution was achieved by the addition 454 of FLAG-elution buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.075% (w/v) LMNG, 0.025% 455 (w/v) GDN, and 0.01% (w/v) CHS and 0.25 mg/mL FLAG peptide). Eluent was then loaded into 456 a pre-washed TALON Metal Affinity Resin (Takara) column and was washed with wash buffer 457 supplemented with 30 mM imidazole. HRV 3C protease was added to the bead slurry and 458 incubated at 4°C for 16h to allow for on-column cleavage. The cleaved sample was collected in 459 wash buffer and concentrated as described above for GPR56.

460 For the removal of oligomeric fragments, the samples were resolved over a Superose 6 461 Increase 10/300 GL column (GE Healthcare) with running buffer containing 20 mM HEPES pH 462 7.5, 150 mM NaCl, 0.00075% (w/v) LMNG, 0.00025% (w/v) GDN and 0.0001% (w/v) CHS. EM 463 fractions containing monomeric receptor were pooled, concentrated and utilized for the cryo-EM 464 experiments. Samples were resolved by SDS-PAGE and immunoblotted utilizing a monoclonal anti-FLAG antibody (THE<sup>™</sup> DYKDDDDK Tag Antibody- HRP, mAb, Mouse; GenScript, 1:1000) 465 466 to confirm the presence of the LPHN3 FLAG-tagged ECR. Further experimental details are 467 provided in Extended Data Figs. 1 and 2.

468

469 NTF-bound LPHN3 purification and encapsulation in nanodiscs

Brain polar lipids (BPL, Avanti Polar Lipids, 141101P) were dissolved in chloroform:MeOH (3:2),
dried and kept under vacuum overnight. Lipids were hydrated by the addition of 20 mM Hepes,
pH 7.5, 150 mM NaCl, 60 mM sodium cholate (30 mM final concentration, assuming molecular
weight of 650 gr/mol) and subjected to 10 freeze/thaw cycles with liquid nitrogen. Lipids were
sonicated, flash frozen and stored in -80°C until use.

Membrane Scaffold Protein 1D1 (MSP1D1) was prepared as described <sup>40</sup>. In brief, *Escherichia coli* BL21 (DE3) harboring plasmid pET-28a(+) with MSP1D1 was grown overnight at 37°C in
Terrific Broth (TB) medium supplemented with 30 µg/mL Kanamycin. Cultures were diluted 1:33

478 in TB, supplemented with 30 µg/mL Kanamycin, grown at 37°C to 2.3-2.5 A<sub>600</sub> units, induced by 479 addition of 1 mM IPTG and grown for an additional 3.5 h at 37°C. Cells were harvested, 480 resuspended in MSP lysis buffer (40 mM sodium phosphate, pH 7.4), flash-frozen in liquid 481 nitrogen and stored in -80°C. For MSP1D1 purification, cells were thawed in MSP lysis buffer 482 and supplemented with protease inhibitor (cOmplete mini EDTA-free, Roche) and 1 mM 483 phenylmethanesulfonyl fluoride (PMSF). Cells were sonicated, and the lysate was centrifuged at 484 30,000 g for 30 min, at 4°C. The supernatant was mixed with Ni-NTA resin (Takara), incubated 485 with agitation for 1 h, at 4°C, packed into a gravity column and the flow-through was discarded. 486 The resin was washed with 4 column volumes (CVs) of MSP wash 1 buffer (40 mM Tris-HCl pH 487 8.0, 300 mM NaCl), 4 CVs of MSP wash 2 buffer (40 mM Tris-HCl pH 8.0, 300 mM NaCl, 50 488 mM sodium cholate), 4 CVs of MSP wash 1 buffer and 4 CVs of MSP wash 3 buffer (40 mM 489 Tris-HCl pH 8.0, 300 mM NaCl, 30 mM imidazole). The protein was eluted with MSP elution 490 buffer (40 mM Tris-HCl pH 8.0, 300 mM NaCl, 300 mM imidazole). The eluted protein was 491 concentrated to ~20 mg/mL using a 10 kDa concentrator (Amicon Ultra Centrifugal Filter MWCO 492 10 kDa, Merck-Millipore) and dialyzed at 4°C against buffer containing 20 mM Hepes, pH 7.5, 493 150 mM NaCl.

494 pMSP1D1 was a gift from Stephen Sligar (Addgene plasmid #20061;
495 http://n2t.net/addgene:20061 ; RRID:Addgene\_20061) <sup>41</sup>.

496 The expression of LPHN3 and membrane preparation were performed in a similar manner to 497 the detergent-based purification schemes described above with some exceptions. Membranes 498 were homogenized in 1% (w/v) n-Dodecyl- $\beta$ -D-Maltopyranoside (DDM, Anatrace) instead of 499 LMNG and the affinity purification steps were performed in a buffer containing 0.1% (w/v) DDM 500 and 0.01% (w/v) CHS. Protein was concentrated to ~150 µM using a 100 kDa concentrator and 501 reconstituted into MSP1D1 using the following molar ratios: LPHN3 : 7H-MSP1D1 - 1 : 3 ; 7H-502 MSP1D1 : BPL Lipids – 1:70, in the presence of 33.8 mM sodium cholate, and 3.7% v/v 503 glycerol. The mixture was incubated in the dark, for 1 h at 4°C with gentle stirring, followed by

504 the addition of Bio-Beads SM-2 Resin (Bio-Rad) (1 gr / 1 mL mixture) and an incubation for an additional 1 h at 4°C. Bio-Beads were then added and incubated for 16 h at 4°C. Bio-Beads 505 506 were removed by centrifugation, and the mixture was incubated with Anti-DYKDDDDK G1 507 Affinity Resin (Genscript) for 1 h, at 4°C. The resin was packed into a gravity flow column and 508 washed with FLAG Wash Buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% v/v glycerol). The 509 protein was eluted with FLAG Elution Buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% v/v 510 glycerol, 0.25 mg/mL FLAG peptide). Eluted nanodiscs were then incubated with Ni-NTA resin 511 for 1 h, at 4°C with gentle stirring. The resin was packed into a gravity column, washed with 512 nanodisc Ni-NTA Wash Buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 10% v/v glycerol, 10 mM 513 imidazole) and eluted with nanodisc Ni-NTA Elution Buffer (20 mM Hepes, pH 7.5, 150 mM 514 NaCl, 10% v/v glycerol, 250 mM imidazole). The sample was concentrated to ~500 µl using a 515 100 kDa protein concentrator and loaded onto Superose 6 Increase 10/300 GL column that was 516 resolved using size exclusion chromatography (SEC) buffer (20 mM Hepes, pH 7.5, 150 mM 517 NaCl). Fractions were analyzed by fluorescence (Em: 295 nm, Ex: 330 nm) and by SDS-PAGE. 518 Fractions containing both the N- and C- termini of LPHN3 and MSP1D1 were combined, 519 concentrated and utilized for the cryo-EM studies.

520

521 Expression and purification of active-state receptor complexes

522 Sf9 cells were co-infected with 7TM-GPR56 or 7TM-LPHN3 and miniG<sub>13/i15</sub> dual baculoviruses 523 and incubated for 48 h at 27 °C. Cells were harvested by centrifugation and resuspended by 524 Dounce homogenization in buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, 10 mM 525 MqCl<sub>2</sub>, 10% v/v glycerol, 0.1 mM tris(2-carboxyethyl) phosphine (TCEP), protease inhibitors 526 cocktail (20 µM leupeptin, 5.2 µg/mL aprotinin, 1.4µg/mL pepstatin, 0.023 mg/mL PMSF and 1 527 mM benzamidine) and benzonase nuclease (Millipore). Following the addition of 25 mU/mL 528 apyrase (Sigma), the cell suspension was incubated at room temperature for 2 h with gentle 529 stirring. Detergent was added to reach a 1% (w/v) concentration (0.8% lauryl maltose neopentyl

530 glycol (LMNG, Anatrace), 0.2% (w/v) glycol-diosgenin (GDN, Sigma Aldrich), 0.1% (w/v) 531 cholesteryl hemisuccinate (CHS, Anatrace)), and incubated for 1.5 h at 4 °C. Solubilized 532 proteins were clarified by centrifugation at 40,000 g for 30 min at 4 °C and the supernatant was 533 batch bound to pre-washed HisPur Ni-NTA affinity resin (Thermo Scientific) in the presence of 534 30 mM imidazole for 1 h at 4 °C with gentle stirring. Beads were packed into a gravity column 535 and washed with Ni-NTA buffer (20 mM HEPES pH: 7.5, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 30 mM 536 imidazole, 20% v/v Glycerol, 20 µM leupeptin, 1 mM benzamidine, 0.1 mM TCEP, while slowly 537 decreasing the detergent concentration of the wash to 0.01% (w/v) (0.008% (w/v) LMNG, 538 0.002% (w/v) GDN, 0.001% (w/v) CHS). Protein was eluted using the final wash buffer 539 containing 250 mM imidazole and concentrated to ~200 µL. The concentrated eluate was 540 incubated O/N with 1 mg of HRV-3C protease (Sigma-Aldrich) per 50 mg of protein. Following 541 3C digestion, the sample was resolved over an Enrich SEC 650 column (Bio-Rad) using SEC 542 running buffer containing 20 mM HEPES: pH 7.5, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.1 mM TCEP 543 and 0.001% (w/v) detergent mix. Fractions corresponding to receptors with complexed G protein 544 were pooled and concentrated for the preparation of cryo-EM grids.

545

## 546 Cryo-EM data acquisition and processing for the NTF-bound LPHN3 in detergents and 547 nanodiscs

548 3.5 µL of purified samples at 8-10 mg/mL were applied on glow-discharged (90 sec, 15 mA, 549 PELCO easiGlow<sup>™</sup>, TED PELLA Inc.) holey carbon gold grids (Quantifoil R1.2/1.3, 200 mesh). 550 The grids were blotted using a Vitrobot Mark IV (FEI) with 3 s blotting time at 22°C in 100% 551 humidity, and plunge-frozen in liquid ethane. A total of 10,870 movies were recorded on a Titan 552 Krios electron microscope (ThermoFisher Scientific - FEI) operating at 300 kV at a magnification 553 of x105K and corresponding to a magnified pixel size of 0.86 Å. A BioQuantum energy filter 554 (Gatan) was operated with an energy slit width of 20 eV. Micrographs were recorded using a K3 direct electron camera (Gatan) with an exposure rate of ~30.6 electrons/Å<sup>2</sup>/s and defocus 555

556 values ranging from  $-0.8 \,\mu\text{m}$  to  $-2.3 \,\mu\text{m}$ . The total exposure time was 1.49 s, and intermediate 557 frames were recorded in 0.033 s intervals resulting in an accumulated dose of ~45.5 electrons per Å<sup>2</sup> and a total of 45 frames per micrograph. Automatic data acquisition was done using EPU 558 559 (ThermoFisher Scientific - FEI). For the nanodisc sample, the micrographs were recorded using 560 a Falcon 3 direct electron detector (FEI, ThermoFisher Scientific) with an exposure rate of ~1.17 electrons/Å<sup>2</sup>/s and defocus values ranging from  $-0.8 \mu m$  to  $-2.3 \mu m$ . The total exposure 561 562 time was 35 s, and intermediate frames were recorded in 0.875 s intervals resulting in an accumulated dose of ~41 electrons per  $Å^2$  and a total of 40 frames per micrograph. A total of 563 564 3053 micrographs were collected. Micrographs were subjected to beam-induced motion correction using RELION 3.1 MotionCor2<sup>42</sup>. CTF parameters for each micrograph were 565 determined by CTFFIND4<sup>43</sup>. Initial particle selection in RELION 3.1<sup>42</sup> was done manually 566 567 followed by particle extraction and 2D classification for a template guided particle picking that 568 resulted in an initial set of 6,246,443 particle projections (32,494 for the nanodisc data). The 569 particles were subjected to reference-free two-dimensional classifications and three-dimensional (3D) classifications in RELION 3.1 <sup>42</sup>. An *ab-inito* model low pass filtered to 40 Å was used as 570 571 an initial reference model for maximum-likelihood-based 3D classifications. A total of 187,766 572 particles were subjected to 3D refinement and contributed to the map presented in Fig. 1c. A 573 flowchart describing data processing steps is in Extended Data Fig. 1.

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# 575 Cryo-EM data acquisition and processing for FL-GPR56 and the active state complexes 576 of GPR56 and LPHN3

577 3  $\mu$ l of purified samples at concentrations of 5 mg/mL, 7.5 mg/mL or 4.5 mg/mL of the FL-578 GPR56, GPR56/miniG<sub>13/i</sub> or LPHN3/miniG<sub>13/i</sub> complexes, respectively, were applied to glow-579 discharged (50 seconds at 10 mA) UltrAuFoil gold grids (Quantifoil, Au300-R1.2/1.3) in 100% 580 humidity at 4°C. Samples were blotted for 1 second and plunged-frozen in liquid ethane using a 581 Vitrobot Mark IV (Thermo Fisher Scientific). Cryo-EM imaging was performed on Titan Krios 582 (ThermoFisher) electron microscopes operated at 300 kV with a K3 Summit direct electron detector (Gatan) at a magnification of 55,000 X (0.8677 Å/pixel) for GPR56/miniG<sub>13/i</sub> complex 583 584 and 57,050 X (0.8521 Å/pixel) for the FL-GPR56 and LPHN3/miniG<sub>13/i</sub> complex in counting 585 mode. For FL-GPR56 4,718 movies, dose fractioned over 79 frames, were recorded for 0.0255 sec/frame for a total dose of 65.25 electrons/Å<sup>2</sup> in super-resolution mode with a defocus range 586 587 of 0.6-1.4 µm. For the GPR56/miniG<sub>13/i</sub> complex, 6,653 movies, dose fractioned over 57 frames, were recorded for 0.05 sec/frame for a total dose of 60.79 electrons/Å<sup>2</sup> in super-resolution mode 588 589 with a defocus range of 0.8-1.8 µm. For the LPHN3/miniG<sub>13/i</sub> complex, 4,667 movies, dose 590 fractioned over 63 frames, were recorded for 0.04 sec/frame for a total dose of 68.95 electrons/Å<sup>2</sup> in super-resolution mode with a defocus range of 0.6-1.8 µm using SerialEM <sup>44</sup>. 591 592 Cryo-EM data processing was performed with cryoSPARC<sup>45</sup>. For FL-GPR56, an initial set 593 2,318,547 particles were selected and subjected to 2D and 3D classification with a total of 594 13,965 particles contributing to the map presented in Fig. 1d. A total of 6,794,073 particles 595 were extracted from the corrected 6,653 micrographs for the GPR56/miniG<sub>13/i</sub> complex. Multiple 596 2D and 3D classification rounds were performed. A subset of 541,279 particles were subjected 597 to homogeneous refinement followed by local refinements of the active GPR56 transmembrane domain and G protein with resolutions at 2.7 Å and 2.9 Å, respectively. A total of 4,242,031 598 599 particles were extracted from the corrected 4,667 micrographs for the LPHN3/miniG<sub>13/i</sub> complex. 600 After 2D and 3D classification, a subset of 440,914 particles were subjected to homogeneous 601 and non-uniform refinements followed by local refinements of the active LPHN3 transmembrane 602 domain and G protein with resolutions at 2.9 Å and 3.1 Å, respectively. Maps resulting from the 603 refinements sharpened DeepEMhancer local were using 604 (https://doi.org/10.1101/2020.06.12.148296) and combined in Chimera <sup>46</sup> contributing to the 605 maps presented in Fig. 1f-g. Flowcharts describing data processing steps are presented in the 606 Extended Data Figs. 2d, 4 and 5.

607

## 608 Model building and refinement for active complexes

Homology models prepared with Phyre2<sup>47</sup> for the 7TM domains of LPHN3 and GPR56, the Ras 609 domain of  $G_{\alpha 13}$  (PDB ID: 1ZCB <sup>48</sup>), and coordinates for  $G_{\beta 1}$  and  $G_{\nu 2}$  subunits (PDB ID: 7MTS <sup>49</sup>) 610 611 to build the miniG<sub> $\alpha$ 13/i</sub>, were used as initial models for docking into the EM density maps using Chimera <sup>46</sup>. The models were subjected to iterative rounds of manual refinement in Coot <sup>50</sup> and 612 real-space refinement in Phenix <sup>51</sup>. Validation of cryo-EM maps and models was performed with 613 Phenix <sup>51</sup> comprehensive cryo-EM validation. Model statistics were validated with Molprobity <sup>52</sup>. 614 Final refinement statistics are provided in Extended Data Table 1. UCSF Chimera <sup>46</sup> and 615 ChimeraX <sup>53</sup> were used for map/model visualizations and figure preparation. 616

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## 618 Molecular dynamics simulations for LPHN3 and analysis

619 Starting from our cryo-EM structure of LPHN3-miniG<sub>13</sub> complex, the G protein was removed, and the receptor alone was oriented in a lipid bilayer with the OPM webserver <sup>54</sup>. PDB files were 620 621 prepared both with and without the first 7 N-terminal residues of the tethered agonist sequence. The CHARMM-GUI <sup>55</sup> was used to prepare the system in a 1-palmitoyl-2-oleoyl-sn-glycero-3-622 phosphocholine POPC/CHS lipid bilayer solvated in TIP3P water <sup>56</sup> with 150 mM NaCl. Input 623 files were generated with the CHARMM36m forcefield (CHARMM36m) with hydrogen mass 624 repartitioning. Simulations were run in the NAMD2.14 <sup>57</sup> software using a Langevin thermostat 625 626 and a Nosé-Hoover Langevin piston barostat at 1 atm with a period of 50 fs and decay of 25 fs. Nonbonded interactions were smoothed starting at 10 Å to 12 Å with long-range interactions 627 628 treated with particle mesh Ewald (PME) and periodic boundary conditions were employed. The system was restrained with 5 kcal/mol/Å<sup>2</sup> harmonic restraints on all non-water, non-ion, and 629 630 non-hydrogen atoms, minimized for 1500 steps, and gradually heated from 0 to 303.15 K in 631 increments of 20 K simulating for 0.4 ns at each increment. An additional 10 ns of equilibration 632 was run before restraints were removed from lipid atoms for an additional 10 ns of equilibration. 5 kcal/mol/Å<sup>2</sup> harmonic restraints were then applied to only protein CA atoms for 10 ns 633

634 increments while the force constant of the restraints was gradually reduced to 2.5, 1.0, and 0.5 kcal/mol/Å<sup>2</sup>. The first 30 ns of unrestrained simulation was also discarded as equilibration and 1 635 us of MD simulations were run using a 4 fs timestep with SHAKE and SETTLE <sup>58,59</sup>. For 636 637 simulations of the tethered agonist peptide alone, setup and simulation were almost identical, 638 except for the absence of a lipid bilayer, the lack of harmonic restraints used during 639 equilibration, and the fact that three replicates were performed as that was sufficient for 640 convergence of the calculated quantities. Pymol (https://pymol.org/2/) was used for visualization 641 of MD simulations experiment results.

642

#### 643 **Preparation and Quantification of aGPCR membrane homogenates.**

644 Wild type or alanine substituted GPR56 or LPHN3 mutant receptors (achieved through site-645 directed mutagenesis) were expressed in 50 mL Sf9 cultures through baculoviruses infection. 646 Cells were harvested 48 h post infection and lysed by nitrogen cavitation in a lysis buffer 647 containing 20 mM Hepes pH 7.4, 1 mM EGTA, and protease inhibitor cocktail (23 µg/mL 648 phenylmethylsulfonyl fluoride, 21 ug/mL L-1-p-tosylamino-2-phenylethyl-chloromethyl ketone, 21 649 µg/mL Na-p-tosyl-L-lysine-chloromethyl ketone, 3.3 µg/mL leupeptin and 3.3 µg/mL soy bean 650 trypsin inhibitor). Cell debris was cleared by centrifugation at 1000 g and membranes were 651 precipitated at 100,000 g. The membranes were Dounce homogenized in lysis buffer, collected 652 at 100,000 g, and homogenized in lysis buffer supplemented with 12% w/v sucrose. Total 653 protein content of membrane homogenates was measured by Bradford assay and samples 654 containing ~5-10 mg total protein were stored at -80°C. For western blotting, 10 µg of 7TM/CTF 655 membranes were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) 656 membranes. Membranes were blocked in 5% w/v bovine serum albumin (BSA) in PBS for 30 657 min, and incubated at 4 °C overnight with 0.1 µg/mL pentaHis antibody (Qiagen). Membranes 658 were washed with TBST, incubated with 1:5000 IR-800 donkey anti-mouse antibody (LiCor) in

659 BLOTTO (TBS with 5% w/v milk and 0.1% NP-40) for 1 hr at 22 °C. After incubation, 660 membranes were washed twice in TBST, twice in TBS, and imaged using an Invitrogen iBright 661 system. For full-length GPR56 membranes, blots were blocked in BLOTTO and probed using 662 antibodies specific for the NTF (R&D Systems, Cat. No. AF4634) and the CTF (EMD Millipore, 663 Cat. No. ABS1028) and processed with 1:5000 IR-800 donkey anti-rabbit antibody (LiCor) or 664 1:5000 Alexa-Fluor 647 donkey anti-sheep antibody (ThermoFisher Scientific) as described 665 above. Triplicate western blot lanes were quantified via pixel densitometry using Adobe 666 Photoshop, and relative receptor levels were plotted using GraphPad Prism version 9.0.2 667 (GraphPad Prism) for Windows, GraphPad Software, San Diego, California USA, 668 www.graphpad.com (Extended Data Fig. 7, Supplementary Table 1 and Supplementary Figure 669 1).

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### 671 [<sup>35</sup>S]-GTPγS Binding Activity Assays

Membrane homogenates (5  $\mu$ g / assay time point) were reconstituted with 200 nM purified G $\alpha_{13}$ and 500 nM purified G $\beta_1$ G $\gamma_2$  in binding buffer containing 50 mM Hepes pH 7.4, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 3  $\mu$ g/mL purified BSA (NEB). Heterotrimeric G<sub>13</sub> proteins (wild type G<sub>13</sub>, G<sub>13/i15</sub>, G<sub>13/i29</sub>) were expressed in *Trichoplusia ni* (High-Five<sup>TM</sup>) insect cells and purified as described <sup>60</sup>.

Kinetic GTP<sub>γ</sub>S binding assays were initiated by the addition of an equal volume of binding buffer containing 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 20 μM GDP, 4 μM [ $^{35}$ S]-GTP<sub>γ</sub>S (25-50,000 cpm / pmol). Endpoint assays or aliquots withdrawn from kinetic assays were quenched with 20 mM Tris pH 7.7, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM GTP, 0.08% w/v lubrol C12E10 and filtered through Whatman GF/C filters using a Brandel Harvester. The filters were washed, dried, and subjected to liquid scintillation counting. 683 Testing of G protein partners for the structural studies was carried out similarly, with the 684 following exceptions: membrane homogenates (5 µg) were reconstituted with 250 nM purified 685  $G\alpha_{13}\beta_1\gamma_2$ ,  $G\alpha_{13i15}\beta_1\gamma_2$ , or  $G\alpha_{13i29}\beta_1\gamma_2$  heterotrimer in binding buffer with 20 µM GDP, then preincubated for 10 min at 25 °C. The assay was initiated by the addition of an equal volume of 686 687 binding buffer supplemented with 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 20 µM GDP, and 4 µM [<sup>35</sup>S]-688 GTPyS (25-50,000 cpm/pmol). Following a 10 min incubation at 25°C, reactions were quenched 689 and filtered through Protran BA85 nitrocellulose filters (GE Healthcare) using a Millipore vacuum 690 manifold. Filters were then processed as described above. Data analysis and representation 691 was performed using GraphPad Prism.

692

## 693 Measurement of aGPCR relative cell surface levels

694 Log phase 10 ml Sf9 cultures were infected with 1/100 volume of amplified aGPCR virus for 36 695 h. Cells were washed twice at 4 °C with PBS and protease inhibitor cocktail prior to incubation 696 with 2 mM Sulfo-NHS-LC-Biotin (ThermoFisher) in PBS for 15 min at 22 °C. Cells were 697 guenched and washed twice with TBS and lysed at 4 °C for 30 min in lysis buffer (25 mM Hepes 698 pH 7.4, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 1% w/v Triton X-100, 2% v/v glycerol, 699 protease inhibitor cocktail). Lysates were clarified by centrifugation at 21,000 g, incubation with 700 a 50 µl bed volume of G25 Sephadex and reclarification at 21,000 g. The supernatant was 701 tumbled at 4 °C for 1 h with a 40 µl bed volume of Streptavidin Sepharose HP (Cytiva). The 702 resin was washed two times with lysis buffer and eluted with reducing SDS-PAGE sample buffer 703 at 42 °C for 5 min. AGPCRs were resolved by SDS-PAGE, immunoblotted with the pentaHis 704 antibody (Qiagen).

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#### 772 Author contributions

773 X.B.-A. and G.G.T. designed GPR56 and G protein constructs. D.M., M. S.-B and G.G.T. 774 designed LPHN3 constructs. X.B.-A. expressed and purified active GPR56 and LPHN3 775 receptors, G proteins and complexes, prepared cryo-EM grids, oversaw data collection, 776 processed cryo-EM data for complexes, modeled the structures, analyzed the structural data 777 and prepared the manuscript. R. M. N. performed mutagenesis experiments, cloned G protein 778 constructs, purified G proteins, assisted in the purification of complexes, analyzed data and 779 prepared the manuscript. A.L.V. and H.S. conducted G protein activation experiments. D.M. 780 expressed and purified NTF-bound LPHN3 receptors, prepared cryo-EM grids, collected and 781 processed cryo-EM data and assisted in figure preparation. F. H. purified and processed cryo-782 EM data for FL-GPR56. O. P. prepared cryo-EM grids, performed data collection. M.M.P.-S. 783 performed G protein activity assays and assisted in figure preparation. M. J. R. performed and 784 analyzed molecular dynamic simulations. E.H.Y. Purified LPHN3 and reconstituted in

- nanodiscs. A. B. S. assisted in cryo-EM data processing. M. C. P. assisted in protein
  purifications and mutagenesis. J. G. M. assisted in biochemical assays and cloning. A.L.V. F.K.,
  and G.G.T. purified G proteins and prepared aGPCR membranes. X. B.-A., R. M. N., M. S.-B,
  G. G. T. and G. S. wrote the manuscript. G. S. supervised the project.

## **Competing interests**

791 The authors declare no competing interests.

#### 793 Data availability

All data generated or analyzed in this study are included in this article and the Supplementary Information. The cryo-EM density maps and corresponding coordinates have been deposited in the Electron Microscopy Data Bank (EMDB) and the Protein Data Bank (PDB), respectively, under the following accession codes: EMD-25077 and 7SF8 (7TM GPR56-miniG<sub>13</sub>) and EMD-25076 and 7SF7 (7TM LPHN3-miniG<sub>13</sub>).

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## 800 Extended Data Figure Legends

801 Extended Data Figure 1. a, Design for the NTF-bound LPHN3 construct used for structural 802 studies. Human LPHN3 (V1, residues 495-1138) was cloned into pFastBac containing a 803 hemagglutinin signal peptide tag (HA) and a cleavable N-terminal FLAG-tag. The construct 804 included a C-terminal cleavable GFP followed by a His<sub>6</sub> tag. **b**, Size exclusion chromatography 805 (SEC) profile of LPHN3. Samples corresponding to the main monomeric (grey) fractions were 806 combined and used for cryo-EM studies. c, Coomassie stained SDS-PAGE of the pooled 807 protein sample visualized by cryo-EM showing the presence of the N-terminal ECR and the 7TM 808 domain. d, Anti-FLAG Western-blot analysis of the LPHN3 sample purified in detergent. e, Cryo-809 EM reference-free 2D class averages of LPHN3 purified in detergent and f, processing flow 810 chart of the NTF-bound LPHN3 sample, including particle selection, 2D and 3D classifications. 811 g, Size exclusion chromatography (SEC) of LPHN3 embedded in lipid nanodiscs composed of 812 MSP1D1 and brain polar lipids (BPL). Yellow bars indicate fluorescence of lipids (Ex: 295 nm, 813 Em: 330 nm). Arrowed bars indicate signal overload. Grey shaded area shows fractions that 814 were used for the cryo-EM analysis. h, SDS-PAGE stained with InstantBlue, showing purified 815 MSP1D1, purified LPHN3 in DDM, reconstituted LPHN3 in lipid nanodiscs before SEC, and 816 selected SEC fractions of the nanodiscs. Bold fractions were used for cryo-EM. i, 817 Representative reference free 2D class averages of LPHN3 embedded in lipid nanodiscs.

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819 Extended Data Figure 2. a, Design for the full-length cleavage deficient (CD, H381S) GPR56 820 construct used for structural studies. Human GPR56 (V2-FL) was cloned into pFastBac 821 containing a hemagglutinin signal peptide tag (HA) and a cleavable N-terminal FLAG-tag. The 822 construct included a C-terminal cleavable His<sub>6</sub> tag. **b**, Size exclusion chromatography (SEC) 823 profile of full-length GPR56. Fractions corresponding to the monomeric peak (grey) were 824 collected and used for structural studies. c, Coomassie stained SDS-PAGE of the FL-GPR56 825 sample used for cryo-EM analysis. d, Cryo-EM data processing workflow of the FL-GPR56 826 sample. e, f. Low resolution 3D maps of NTF-bound receptor conformations with docked 827 structures of the ECR of e, GPR56 and f, LPHN3. The GAIN domains and TA peptides are 828 colored in blue and cyan for GPR56 and in magenta and light pink for LPHN3. Domains are 829 labeled. Docked available ECR crystal structures corresponding to PDB 5KVM and 4DLQ for 830 GPR56 and LPHN1, respectively. Scale bars are provided in the left bottom corner.

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832 Extended Data Figure 3. a, Design for the tethered agonist complex constructs used in the 833 study. Receptors are presented on top, where sequences corresponding to the TA and 7TM 834 regions of GPR56 and LPHN3 were inserted after a hemagglutinin signal peptide (HA) and a 835 methionine residue. Expression vector for the mini $G_{13}$  heterotrimer presented at the bottom. **b**, 836 The MiniG<sub>q13/15</sub> sequence. Residues at the N-terminus corresponding to  $G_{q12}$  sequence are in 837 grey. The linker replacing the alpha helical domain is in yellow. Residues corresponding to the stabilizing mutations G57D<sup>S1H1.03</sup>, E58N<sup>S1H1.04</sup>, S248D<sup>S4.07</sup>, E251D<sup>S4H3.03</sup>, I271D<sup>H3.08</sup>, I355A<sup>H5.04</sup>. 838 839 V358I<sup>H5.07</sup> are underlined and presented in bold. **c**, Size-exclusion chromatography (SEC) 840 profiles of purified miniG<sub>13</sub>-coupled GPR56 (left) and -LPHN3 (right) with insets showing 841 Coomassie-stained SDS-PAGE of the SEC complex peaks.

843 Extended Data Figure 4. a, Workflow of cryo-EM data processing for the active tethered 844 agonist bound 7TM-GPR56/miniG<sub>13</sub> complex. b, Angular distribution heat map of particle 845 projections for 7TM-GPR56/miniG<sub>13</sub> reconstruction. c, Gold standard Fourier shell correlation 846 (FSC) curve for receptor and miniG<sub>13</sub> reconstructions. Dashed line represents the overall 847 nominal resolution of each reconstruction at 0.143 FSC calculated by CryoSPARC. d, Overall 848 composite cryo-EM map for the 7TM-GPR56/miniG<sub>13</sub> complex with chain assignments for its 849 components. **e**, Cryo-EM density for TMs 1-7, the  $\alpha$ 5 helix of miniG $\alpha_{13/i}$  and the bound tethered 850 agonist for the 7TM-GPR56/miniG<sub>13</sub> complex.

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852 Extended Data Figure 5. a, Workflow of cryo-EM data processing for the active tethered 853 agonist bound 7TM-LPHN3/miniG<sub>13</sub> complex. b, Angular distribution heat map of particles for 854 7TM-LPHN3/miniG<sub>13</sub> reconstruction. **c**, Gold standard Fourier shell correlation (FSC) curve for 855 receptor and miniG<sub>13</sub> reconstructions. Dashed line represents the overall nominal resolution of 856 each reconstruction at 0.143 FSC calculated by CryoSPARC. d, Overall composite cryo-EM 857 map for the 7TM-LPHN3/miniG<sub>13</sub> complex with chain assignments for its components. e, Cryo-858 EM density for TMs 1-7, the  $\alpha$ 5 helix of miniG $\alpha_{13/i}$  and the bound tethered agonist for the 7TM-859 LPHN3/miniG<sub>13</sub> complex.

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Extended Data Figure 6. Kinetic measurements of receptor-stimulated G protein 13 [35S]-861 862 GTPyS binding in membranes normalized to the activities of wild type (WT) GPR56 or LPHN3. 863 7TM/CTF-only truncated receptors with a, b, point mutations at the TA residues. c, d, TA-864 interacting point mutants. e, f, G protein interaction site point mutants. g, h, 7TM core-stabilizing 865 point mutants. Note: GPR56 Q644A and LPHN3 E948A were found at low abundance, thus potentially explaining their reduced activities. i, Equivalent amounts of WT, W617<sup>6.53</sup>A, 866 F637<sup>7.42</sup>A, and F454<sup>2.64</sup>A full-length GPR56 holoreceptors were activated by ice-cold urea 867 868 treatment to dissociate NTFs from CTFs prior to measurement of G13 initial GTPyS binding 869 rates at 20 °C. The urea-dependent changes in approximated initial linear rates demonstrate 870 that wild type GPR56 was activated by urea significantly more than each mutant, indicating that 871 the mutations impart reduced functional activity and that the mutant receptors are not 872 completely dysfunctional or mis-folded. Data represent the average of each kinetic reaction 873 measured as technical triplicates with error bars representing +/- S.D. Unpaired, two-tailed 874 student's t tests were used to determine significance between initial rates. \* = p < 0.05, \*\*\*\* = p875 < 0.0001. Further detailed statistical information can be found in Supplementary Data Table 1. 876 j, Relative aGPCR cell surface levels for selected mutants and WT receptors were measured by 877 intact cell biotinylation, streptavidin pulldown and anti-His tag immunoblotting.

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879 Extended Data Figure 7. a, Relative abundances of CTF-only truncated GPR56 receptors in 880 membrane homogenates determined by immunoblotting for anti-His tag. b, Relative 881 abundances of CTF-only truncated LPHN3 receptors in membrane homogenates determined by 882 immunoblotting for anti-His tag. c, Relative abundances of holoreceptor GPR56 NTFs and CTFs 883 before and after treatment of membrane homogenates with ice-cold 6M urea. CTF was 884 immunoblotted for via a GPR56-specific CTF antibody, and NTF was immunoblotted for via a 885 GPR56-specific NTF antibody. \*Multiple glycosylated NTF bands. Data represent the mean 886 band intensity of western blots performed in triplicate with error bars representing +/- S.D. 887 Unpaired, two-tailed student's t tests were used to determine significance between wild type and 888 mutant receptors with reduced abundances. \* = p < 0.05. Further detailed statistical information 889 can be found in Supplementary Data Table 1.

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Extended Data Figure 8. a, b, Density corresponding to the TA peptide and ECL2 in GPR56 (a) and LPHN3 (b), indicating that the TA and loop are penetrating the 7TM cavity. c, d, Residues surrounding the toggle switch residue (W<sup>6.53</sup>) in GPR56 (c) and LPHN3 (d). Electrostatic interactions are shown as dotted grey lines. e, Superposition of GPR56 and

LPHN3, showing similarities in 7TM domain conformation. f, G<sub>13</sub> GTPγS binding activity for
mutants LPHN3 (magenta) and GPR56 (blue) that interact with W<sup>6.53</sup>. Data represent mean of
biologically independent reactions performed in triplicate with error bars representing +/- S.D.
RM one-way ANOVA was used to determine significance between mutants and WT. Further
detailed statistical information can be found in Supplementary Data Table 1.

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901 Extended Data Figure 9. Molecular dynamics simulations for the LPHN3 tethered agonist 902 and its binding to the 7TM domain. a, Four snapshots of the LPHN3 TA peptide MD 903 simulations in solution spaced 200 ns apart. b, Average secondary structure percentages of 904 LPHN3 peptide from MD simulations. ' $\alpha$ ' refers to the very broad range of -160  $\leq \varphi \leq$  -20; -120  $\leq$ 905  $\psi \leq 50$ ; ' $\beta$ ', beta-sheet; 'PP2', polyproline 2. **c**, **d**, Cryo-EM structure of LPHN3 colored by the 906 difference in RMSF values between wild-type MD simulations and simulations with the tethered 907 agonist (TA) region (dark gray) removed. Positive values indicate an increase in flexibility when 908 the TA is deleted. **a** and **b** correspond to two different color scales.

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910 Extended Data Figure 10. a-b, Overall structural comparison of 7TM domains of G protein 911 coupled GPR56 (blue) with active state Family B1 receptors: GLP1R (PDB ID: 5VAI; receptor in 912 brown, GLP1 peptide in tan), GCGR (PDB ID: 6WPW; receptor in dark green, glucagon 913 derivative ZP3780 in light green) and calcitonin receptor (PDB ID: 5UZ7; orange). a, side view 914 showing similarities in 7TM domain topology and **b**, top view with superposition of B1 agonists 915 with GPR56 TA in the orthosteric site. c, Superposition of glucocorticoid ligand-bound GPR97 916 (PDB ID: 7D77, light grey) with GPR56 (blue) and LPHN3 (magenta). Arrows are indicating 917 differences in TM1, TM6 and TM7 between the ligand and to TA-bound structures. **d**, Top view 918 of superimposed GPR56 and LPHN3 complexes showing positioning of mini-G<sub>13</sub> N-terminal 919 helix ( $\alpha N$ ) with respect to the receptor TMs. **e**, Superposition of GPR56 bound mini-G<sub>a13</sub> (gold) 920 vs. 5HT1A (PDB ID: 6G79) bound mini- $G_{\alpha 0}$  (green). f, GTP $\gamma$ S binding assay for recombinant  $G_{13}$ 

921 proteins in which the authentic N-terminus of  $G_{\alpha 13}$  was replaced with 15 or 29 residues of the 922  $G_{\alpha i2} \alpha N$  to improve expression, stability and ability to interact with receptor. Stimulation of  $G_{13/29}$ 923 nucleotide exchange by both receptors GPR56 (blue) and LPHN3 (magenta) was reduced 924 substantially when compared to wild type  $G_{13}$  or  $G_{13/15}$ . Receptor constructs used in this assay 925 are the TA-decrypted GPR56 and LPHN3. Data displayed as mean of reactions (n=18 for all 926 except GPR56 + G<sub>13/i29</sub>, n=17, and LPHN3 + G<sub>13/i29</sub>, n=16) with error bars representing +/-927 S.E.M. Statistical significance between experimental condition and corresponding control group 928 was calculated using Mann-Whitney analysis, n.s. = not significant, \*\*\*\* = p < 0.0001. Further 929 detailed statistical information, including exact p values, can be found in Supplementary Data 930 Table 1. **g**, G protein binding through  $\alpha$ 5 helix of mini-G<sub> $\alpha$ 13</sub> (gold) by GPR56 (blue) and mini-G<sub> $\alpha$ 0</sub> 931 (red) by GPR97 (PDB ID: 7D77, in white) showing substantially greater opening of TM5-6 in the 932 GPR56 TA-bound structure.



**V L M** V S S V E V D A V<sup>1.34</sup> - TM1 ТҮ











