Reevaluating GPR30: A Paradigm Shift from Estrogen Receptor to Unique Hydrophilic Ligand Activation

Authors: Heng Liu, Shimeng Guo, Antao Dai, Peiyu Xu, Xin Li, Sijie Huang, Xinheng He, Kai Wu, Xinyue Zhang, Dehua Yang, Xin Xie, H. Eric Xu, Shimeng Guo, Dehua Yang, Xin Xie, H. Eric Xu

Date: 2024-02-24T00:00:00+00:00

Abstract

孤儿受体 GPR30，此前被归类为 G 蛋白偶联雌激素受体（GPER），其配体特异性一直存在争议。通过结合结构解析、生化结合和细胞信号检测的综合方法，我们证明雌激素并不直接结合或激活 GPR30。GPR30 的冷冻电镜结构揭示了一个意外的亲水性配体结合口袋，与经典的疏水性类固醇结合位点存在显著差异，这与雌激素结合不一致。我们进一步确认了 Lys05 等亲水性激动剂是 GPR30 的真正激活剂，并为其结合机制和受体激活提供了结构见解。我们的发现有必要重新定义 GPR30 在雌激素信号传导中的作用，表明其激活通过不依赖于雌激素结合的机制发生。本研究为开发靶向 GPR30 配体和重新解释其在雌激素介导过程中的作用开辟了新途径。

Full Text

Preamble

Reevaluating GPR30: A Paradigm Shift from Estrogen Receptor to Unique Hydrophilic Ligand Activation

Heng Liu[1,8], Shimeng Guo[1,8], Antao Dai[2,8], Peiyu Xu[1,8], Xin Li[1,3], Sijie Huang[1], Xinheng He[1,3], Kai Wu[1,4], Xinyue Zhang[1], Dehua Yang[2,3], *Xin Xie*[1,3,5,6], H. Eric Xu[1,3,7*]

[1]The State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
[2]The State Key Laboratory of Chemical Biology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
[3]University of Chinese Academy of Sciences, Beijing 100049, China
[4]The Shanghai Advanced Electron Microscope Center, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

[5]School of Pharmaceutical Science and Technology, Hangzhou Institute for Advanced Study, University of Chinese Academy of Sciences, Hangzhou, China
[6]Shandong Laboratory of Yantai Drug Discovery, Bohai Rim Advanced Research Institute for Drug Discovery, Yantai, China
[7]School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China
[8]These authors contributed equally

*Correspondence: dhyang@simm.ac.cn (D.Y.), xxie@simm.ac.cn (X.X.), eric.xu@simm.ac.cn (H.E.X.)

Abstract

The orphan receptor GPR30, previously classified as a G protein-coupled estrogen receptor (GPER), has been a subject of debate regarding its ligand specificity. Through an integrative approach combining structure elucidation, biochemical binding, and cell signaling assays, we demonstrate that estrogen does not directly bind to or activate GPR30. Cryo-EM structures of GPR30 reveal an unexpected hydrophilic ligand-binding pocket with striking differences from classical hydrophobic steroid-binding sites, making it inconsistent with estrogen binding. We further confirmed that hydrophilic agonists like Lys05 serve as true activators of GPR30, providing structural insights into their binding mechanism and receptor activation. Our findings necessitate a paradigm shift in defining GPR30' s role in estrogen signaling, indicating that its activation occurs through mechanisms independent of estrogen binding. This study opens new avenues for developing targeted GPR30 ligands and reinterpreting its role in estrogen-mediated processes.

Introduction

Estrogen is a pivotal hormone in human physiology that orchestrates a wide range of biological processes, from reproductive functions to cardiovascular health and bone integrity[1–3]. Its effects are traditionally understood to be mediated through nuclear estrogen receptors ERα[4] and ERβ[5],[6], which function as transcription factors regulating gene expression[1–4]. However, the rapid, non-genomic actions of estrogen imply the existence of membrane-associated estrogen receptors that enable swift cellular responses[7–9]. The G protein-coupled estrogen receptor (GPER), also known as GPR30[10–14], was identified as one such candidate over 18 years ago[15],[16]. Initial studies reported that GPR30 binds estrogen[15],[16] and mediates rapid estrogen signaling through various pathways[15–18], including downstream pathways mediated by the G protein subtype Gq[19], which induces generation of the second messenger inositol phosphate IP1, leading to activation of PKC and calcium signaling. This discovery sparked great interest and debate over the role of GPR30 in estrogen physiology[20],[21].

The identification of GPR30 as a novel estrogen receptor provided a mecha-

nism for the rapid action of estrogen and facilitated the development of GPR30 modulators[20]. While the endogenous ligand 17β-estradiol (E2) reportedly activates GPR30[15],[16], selective estrogen receptor antagonists like tamoxifen[22],[23] and raloxifene[24], as well as the ER-degrading agent fulvestrant[16], have also been reported to display GPR30 agonism. Additionally, several synthetic ligands, including G-1, were reported to be selective GPR30 agonists.

However, subsequent studies have produced inconsistent results regarding whether GPR30 truly serves as an estrogen receptor with independent signaling capacity from nuclear estrogen receptors[19],[26–37]. A recent unbiased screen found no activation of GPR30 by estrogen, 4-hydroxytamoxifen, or the selective GPR30 agonist G-1 in cells overexpressing GPR30, challenging its classification as a bona fide estrogen receptor. Instead, this screen identified chlorhexidine, Lys05, and 9-aminoacridine as potent GPR30 activators[19].

Furthermore, knocking down GPR30 in MCF-7 cells expressing both estrogen receptors (ERs) and GPR30 showed no impact on E2-induced generation of cyclic AMP (cAMP), the second messenger downstream of Gs activation, whereas E2-induced cAMP production was affected by double knockout of ERα and ERβ[26]. The presence of membrane ERs (mERs)[38], albeit at a limited level of about 3-10% of classical nuclear ERs, which are reported to cooperate with GPR30[29], further complicates the rapid non-genomic signaling events mediated by E2[33]. There is also evidence that G-1 may signal through estrogen receptor variants ERα-36 and ERα-46 instead of GPR30[31],[39–41]. In addition, rapid signaling by estrogen at the plasma membrane is absent in ER-negative but GPR30-positive cells[26]. Clearly, definitive evidence for direct estrogen binding and signaling by GPR30 remains lacking and controversial[28],[34].

In this paper, we combined biochemical, structural, and functional approaches to directly test whether GPR30 interacts with and signals in response to estrogen and estrogen-related compounds. We determined cryo-EM structures of GPR30 bound to Gq in the presence of high concentrations of estrogen and related ligands G-1 and fulvestrant; however, none of these compounds were found in these GPR30 structures. Functional and radioligand binding assays were consistent with the structural data, showing no direct GPR30-estrogen interaction. In contrast, the hydrophilic ligand Lys05 was found to bind the orthosteric pocket of GPR30, revealing a unique mechanism of GPR30 activation independent of estrogen binding. By definitively demonstrating that GPR30 is not a membrane estrogen receptor, our integrative study fundamentally shifts current understanding and necessitates reinterpretation of GPR30' s contributions to estrogen biology.

Cryo-EM Structures Contradict GPR30 as a Direct Estrogen Receptor

GPR30 was hypothetically conceived as a G protein-coupled estrogen receptor that directly binds estrogen, offering mechanisms to explain the non-genomic

effects and tissue-selectivity of estrogen signaling. This provocative idea has profoundly impacted our comprehension of rapid estrogen biology. We therefore initially aimed to use structural biology, specifically cryo-EM—a technique successfully applied in numerous GPCR-ligand studies[42–47]—to visualize estrogen binding directly to GPR30.

For our cryo-EM studies, we engineered human GPR30 by appending a hemagglutinin (HA) signal peptide, FLAG, and His tags for expression and purification, and introduced a BRIL fusion tag at the N-terminus to enhance complex stability (Fig. S1). We utilized an engineered Gαq (see Methods) that has proven effective in mimicking Gαq-coupled GPCR activation in various receptors, such as the growth hormone secretagogue receptor (GHSR)[48]. Co-expression of GPR30 with this engineered Gαq, along with rat G$ 1, *bovineG* $2, and scFv16 in Hi5 insect cells, allowed us to form stable GPR30-Gq complexes in the presence of E2, G1, or fulvestrant (Fig. S1).

We determined structures of GPR30-Gq complexes with saturated concentrations of E2, the selective agonist G-1, and the ER-degrader fulvestrant at 3.1-3.2 Å resolution (Fig. S1, Fig. S2, Table S1). In all structures, GPR30 adopts an active-like state typical of GPCR-G protein coupling. Surprisingly, despite the relatively high resolutions achieved, no ligand density was observed in the GPR30 binding pocket (Fig. 1A [Figure 1: see original paper]-B).

We additionally solved an apo-GPR30-Gq structure at 2.9 Å resolution (Fig. 1C, Fig. S2, Table S1). Structural alignments reveal striking similarity between GPR30 conformations in the presence or absence of added ligands, with root mean square deviation (RMSD) values of just 0.373 Å, 0.378 Å, and 0.368 Å when comparing structures solved with E2, G-1, or fulvestrant to the apo-GPR30-Gq complex, underscoring the highly analogous states regardless of these compounds' presence (Fig. S3A). A comparison of the EM structures with the AlphaFold[49] structure showed overall concordance yet revealed notable local discrepancies in positioning of transmembrane helices, particularly 3-4 Å differences in the extracellular regions of TM1, TM2, and TM3 (Fig. 1D), indicating that even for recurrent folds such as GPCRs, AlphaFold predictions can still deviate substantially from experimental structures[50].

Notably, examination of GPR30' s pocket architecture reveals a striking hydrophilic nature enriched with negatively charged residues (Fig. 1E-F). This clashes with the accommodation of hydrophobic estrogen ligands (Fig. S3B), in contrast to related steroid hormone receptors with hydrophobic pockets as seen in ERα, ERβ, and several steroid-binding GPCRs (Fig. S3C). Additionally, the GPR30 pocket is much larger than corresponding pockets for steroid-related ligands (Fig. S3D). This spacious GPR30 cavity also appears unsuitable for smaller estrogen binding. Together, these inconsistencies argue that estrogen is unlikely to be the cognate ligand as originally hypothesized, based on the cryo-EM structural evidence we established. In the following sections, we provide further biochemical and functional evidence that estrogen is not a direct ligand of GPR30.

Functional Assays Challenge GPR30 as a Direct Estrogen Receptor

Our structural findings challenge the proposed concept of GPR30 as a membrane estrogen receptor. We further investigated this through binding experiments and signaling analyses.

First, we performed competitive radioligand binding assays using [^{3}H]17β-estradiol ([^{3}H]-E2) (Fig. 2A [Figure 2: see original paper]) with CV1 cells expressing GPR30 or ERα. As anticipated and in alignment with existing literature[51], ERα-transfected cells exhibited high-level binding to [^{3}H]-E2 that could be specifically competed by addition of unlabeled E2 (Fig. 2B). Conversely, [^{3}H]-E2 showed low basal-level binding to GPR30-transfected cells (similar to empty cells) in the presence or absence of increasing concentrations of unlabeled E2 (Fig. 2B). E2-related ligands such as tamoxifen and raloxifene (Fig. 2A) could also effectively compete with [^{3}H]-E2 in binding to ERα but did not affect the nonspecific binding of [^{3}H]-E2 on GPR30 (Fig. 2C-D). We recapitulated these results with [^{3}H]-E2 binding to membranes from Hi5 cells overexpressing GPR30 and ERα, and the consistent results further reinforce the lack of direct GPR30-estrogen interaction (Fig. S4A).

In cell-based functional assays, E2 elicited no changes in cAMP or IP1 in GPR30-expressing cells at concentrations up to 100 µM, indicating no stimulation of Gs or Gq pathways (Fig. 2E, F). E2 did not reduce cAMP levels promoted by forskolin either, indicating no activation of the Gi pathway (Fig. 2G). In contrast, the recently identified GPR30 agonist Lys05 (Fig. 2A) induced dose-dependent accumulation of IP1 without affecting cAMP levels (Fig. 2E-G), consistent with knowledge that GPR30 dominantly couples to Gq[19]. Lys05 also induced calcium flux in GPR30-expressing cells with an EC_{50} value of ~462 nM, a response not observed with E2-related compounds or in parental HEK293 cells (Fig. S4B-C), consistent with activation of the Gq-IP1-calcium pathway. Together, our integrated binding and functional data clearly indicate that GPR30 is not an estrogen receptor.

Structure of GPR30 Bound to Lys05

Our functional assays established that Lys05, a molecule characterized by multiple positively charged nitrogen atoms (Fig. 2A), effectively activates GPR30, initiating downstream signaling through the Gq pathway (Fig. 2E, Fig. S4B-C). Intrigued by this interaction, particularly given GPR30' s negatively charged ligand-binding pocket (Fig. 1E), we embarked on a detailed structural investigation.

We successfully determined the high-resolution structure of the Lys05-GPR30-Gq complex at an overall resolution of 2.6 Å (Fig. 3A [Figure 3: see original paper], Fig. S5). This resolution enabled us to resolve the entire structure, including the transmembrane helices, intracellular loops, and the Gq heterotrimer.

Most notably, we observed distinct EM density for Lys05 within the GPR30 binding site (Fig. 3A), in stark contrast to our previous GPR30 structures solved in the presence of E2 and related compounds (Fig. 1A-B).

In the Lys05-bound state, the ligand is nestled within a pocket formed by TM2, TM3, TM6, and TM7. This pocket is enriched with negatively charged residues, aligning perfectly with Lys05' s structure (Fig. 3B), which features two hydrophobic ends linked by a positively charged segment (Fig. 2A). This spatial arrangement highlights the specificity of Lys05 binding to GPR30.

A comparative analysis between the apo-GPR30 and Lys05-activated GPR30 structures revealed a similar activated conformation (Fig. S5G). The RMSD values for the entire complex were 1.223 Å and 0.326 Å for the receptor alone, indicating a high degree of structural similarity. Upon closer inspection, we noted that TM3 and TM5 in the Lys05-bound structure underwent inward shifts compared to their positions in the apo structure. Specifically, TM3 shifted by approximately 0.9 Å (Cα of T131$^{3\ 26}$) and TM5 by around 2.5 Å (Cα of E213$^{5\ 37}$), suggesting ligand-induced conformational changes (Fig. S5G).

Structural analysis reveals a unique distribution of the transmembrane region of GPR30. Notably, TM1 and TM4 arrangements in GPR30' s 7TM bundle deviate from amine/steroid receptors such as the serotonin receptor 5HT1A[52] and G protein-coupled bile acid receptor GPBAR[53] (Fig. S6A), but match better with peptide-activated GPCRs like GHSR[48] and neuromedin U receptor 2 (NMUR2)[54] (Fig. S6B). This architecture likely accommodates Lys05' s linear amine linker. Overall, the Lys05-bound GPR30 structure provides molecular insights into ligand interactions enabling pharmacological targeting of GPR30' s distinctive ligand-binding pocket.

Molecular Recognition of Lys05 by GPR30

The relatively high-resolution structure of Lys05 coupled with an activated GPR30 conformation has elucidated the ligand recognition mechanisms in detail (Fig. 4A [Figure 4: see original paper]). For analysis, we designated the nitrogen atoms in Lys05' s symmetrical structure as N1 through N5, from the bottom to the top of the ligand structure. Lys05 inserts into the orthosteric pocket lined by TM2, TM3, TM6, and TM7, engaging with the receptor through a tri-layered interface (Fig. 4A).

At the bottom of the binding pocket, Lys05 insertion rearranges L137$^{3\ 32}$ and M141$^{3\ 36}$. Specifically, L137$^{3\ 32}$ flips toward TM2 while M141$^{3\ 36}$ rotates approximately 50° away (Fig. S7A). This rearrangement gives rise to a hydrophobic sub-binding pocket consisting of L108$^{2\ 53}$, L137$^{3\ 32}$, M141$^{3\ 36}$, W272$^{6\ 48}$, A313$^{7\ 42}$, and F314$^{7\ 43}$ (Fig. 4A-B). To underscore the importance of these hydrophobic contacts, we introduced alanine mutations at these sites and assessed their impact on Lys05 activity. Strikingly, alanine substitution at any of these residues—including L108$^{2\ 53}$A, L137$^{3\ 32}$, M141$^{3\ 36}$, W272$^{6\ 48}$, and F314$^{7\ 43}$—nearly abolished activation by Lys05 (Fig. 4C, Fig. S7) without affecting

GPR30 surface expression (Table S2). These data suggest the crucial role of the hydrophobic sub-pocket in stabilizing Lys05.

At the center of the pocket is a hydrophilic layer, where the positively charged N2 and N3 amines of Lys05 form polar contacts with E115$^{2\ 60}$, Q138$^{3\ 33}$, E275$^{6\ 51}$, and N310$^{7\ 39}$ (Fig. 4A, 4D). Specifically, E115$^{2\ 60}$ forms polar interactions with the N2 amine of Lys05, while N310$^{7\ 39}$ and E275$^{6\ 51}$ form polar interactions with the N3 amine of Lys05 (Fig. 4D). Critically, the N2 and N3 amines provide essential positive charges for accommodation of the surrounding negatively charged pocket (Fig. 4A). As expected, E115$^{2\ 60}$A and Q138$^{3\ 33}$A mutations eliminated activation, while E275$^{6\ 51}$A reduced potency by approximately ten-fold (Fig. 4C, Fig. S7, Table S2).

The top of the pocket forms a hydrophobic layer involving weaker interactions between the top tryptamine moiety of Lys05 and residues from TM6 and ECL3 of GPR30, engaging H300 ^{3}, H282$^{6\ 58}$, and P303$^{7\ 32}$ (Fig. 4E). Mutations in these amino acids mildly impacted activation (Fig. 4C, Fig. S7, Table S2), suggesting their auxiliary role in Lys05 recognition.

Activation Mechanisms of GPR30

In the cryo-EM map of the apo-GPR30-Gq structure, several structured waters clearly form hydrogen-bonding interactions within the orthosteric pocket. Specifically, two well-organized water molecules, denoted as W1 and W2, solved to a local resolution of approximately 3.0 Å (Fig. S2K), are hypothesized to potentially play a role in GPR30' s self-activation. These water molecules, positioned in proximity to the negatively charged pocket core, actively engage in hydrogen bonds with key residues (Fig. 5A [Figure 5: see original paper]-B). W1' s positioning and interactions, particularly with E115$^{2\ 60}$, E275$^{6\ 51}$, and N310$^{7\ 39}$, coincide with the tryptamine group of Lys05 (Fig. 5C). Meanwhile, W2 establishes hydrogen bonds with E275$^{6\ 51}$ and E218$^{5\ 42}$ (Fig. 5A-C). These water-mediated contacts appear to mimic ligand interactions, promoting GPR30' s adoption of an active-like state and offering insights into potential basal activation mechanisms of GPR30.

In the absence of an inactive GPR30 structure, we conducted a comparison with the inactive -opioid receptor (KOR)[55], which displays one of the highest sequence similarities among all GPCRs to GPR30. This comparison between active and inactive GPCR states illuminated the activation mechanism of GPR30. Notably, the active GPR30 structures demonstrate typical rearrangements characteristic of activated GPCRs, such as the agonist-induced outward movement of TM6 at the cytoplasmic side, similar to changes observed in KOR[56] (Fig. 5D).

In the Lys05-GPR30-Gq complex, Lys05 binding exerts pressure on the W272$^{6\ 48}$ toggle switch, triggering a cascade of conformational shifts (Fig. 5E). This interaction induces alterations in the F268$^{6\ 44}$-V145$^{3\ 40}$ of the PIF motif and R155$^{3\ 50}$ of the DRY motif, ultimately disrupting the ionic lock between

TM3 and TM6. As a result, TM6 moves outward by approximately 6.2 Å at the R254$^{6\ 30}$ Cα position. Concurrently, TM7' s cytoplasmic portion shifts inward by about 2.7 Å (Fig. 5D). This inward movement allows Y324$^{7\ 53}$ of the NPxxY motif and Y234$^{5\ 58}$ to form hydrogen bonds with R155$^{3\ 50}$ of DRY (Fig. 5F-H). These coordinated structural changes facilitate opening of the cytoplasmic pocket, priming GPR30 for effective coupling with downstream G protein.

Gq Coupling of GPR30

Structural comparisons of the GPR30-Gq complex with other Gq/11-coupled class A GPCRs[46],[54] reveal a broadly parallel coupling mechanism. However, our analysis identified a distinctive feature in GPR30' s coupling with Gαq, particularly in the positioning of the αH5 helix of the Gαq protein (Fig. 6A [Figure 6: see original paper]). In the GPR30-Gq complex, the N-terminus of αH5 is positioned closer to the junction of TM5 and TM6 in GPR30, diverging by approximately 15° compared to other Gq-coupled receptors such as GSHR[48], NMUR2[54], and CCKAR[57] (Fig. 6A). This unique orientation may reflect the specific activation characteristics of GPR30, which induces a comparatively smaller displacement of TM6 (Fig. 6A).

The interaction between GPR30 and Gαq involves two critical regions of the receptor: engagement of the Gαq $ $5 helix by TM3, TM5, TM7, and H8 of GPR30, and interactions mediated by GPR30' s intracellular loops (ICLs) (Fig. 6B). The major interface is formed by the distal αH5 region of Gαq and the intracellular receptor cavity. Key hydrophobic and polar interactions at this interface include: (1) a hydrophobic stack between V394 . ^{5}.^{26}, L393 . ^{5}.^{25}, L388 . ^{5}.^{20}, L384 . ^{5}.^{16}, I383 . ^{5}.^{15} on the Gαq $ $5*helix and GPR30 residues* $L159^{\{3\}^x}\{54\}$, $L241^{\{5\}^x}\{65\}$, $A356^{\{6\}^x}\{33\}$, $M260^{\{6\}^x}\{36\}$, $I261^{\{6\}^x}\{37\}$; (2)*polar* hydrogen bond and N392 . ^{5}.^{24} interacting with T330$^{8\ 49}$ in helix 8 and the backbone of S325$^{7\ 54}$ (Fig. 6B-C).

Significant ICL2-Gαq interactions are also observed, including the insertion of M163$^{34\ 51}$ into a hydrophobic cavity on Gαq. This interaction is reminiscent of those seen in other Gq-GPCRs[48],[54],[57] (Fig. 6B and 6D). Additionally, R169$^{34\ 57}$ forms a polar interaction with E390 . ^{5}.^{22}, with its backbone carboxyl group interacting with R38 on αN of Gαq. R164$^{34\ 52}$ establishes a hydrogen bond with the D215 backbone of Gαq.

A unique aspect of the GPR30-Gq structure is the increased interaction between TM6-ICL3 of GPR30 and Gαq, facilitated by a smaller outward movement of TM6 in GPR30 (Fig. 6A). Notably, R254$^{6\ 30}$ on GPR30 forms a stabilizing interaction with Q385 . ^{5}.^{17} and D381 . ^{5}.^{13} on Gαq αH5. Furthermore, R248 ^{3} forms a salt bridge with D378 . ^{5}.^{10} on αH5 (Fig. 6E). These additional contacts, unique to the GPR30-Gq complex, highlight the distinct conformational adaptations of GPR30 in its coupling with Gαq.

Discussion

The study of estrogen signaling is pivotal for understanding a wide range of physiological processes, from reproductive health to neurological functions[1],[3]. GPR30, previously conceptualized as a membrane estrogen receptor (GPER)[10], has been central to discussions about rapid, non-genomic estrogen responses[20],[21]. However, our comprehensive structural, biochemical, and functional analyses necessitate a fundamental shift in understanding GPR30' s role. We have clearly demonstrated that GPR30 does not directly bind or become activated by estrogen, thereby challenging its classification as a direct membrane estrogen receptor.

The cryo-EM structures of GPR30 revealed no density for E2, G-1, or the ER-degrader fulvestrant. Instead, the orthosteric pocket of GPR30 exhibits a hydrophilic nature unsuitable for binding hydrophobic steroid hormones like estrogen. This finding aligns with our radioligand binding assays, which also indicate no specific estrogen binding to GPR30. Consequently, we must reinterpret GPR30' s role in estrogen signaling, considering indirect mechanisms or completely estrogen-independent functions of this receptor.

The definitive exclusion of GPR30 as a membrane estrogen receptor redirects our focus to other potential mediators of rapid estrogen responses. Candidates like ERα-36[40], ERα-46[41], and membrane-associated ERs (mERs) warrant further investigation to understand their roles and interactions in membrane-initiated signaling[29],[31],[33]. Clarifying the crosstalk or cooperation between these receptors and GPR30 could provide deep insights into estrogen' s complex signaling pathways.

The nature of GPR30' s native ligand remains an open question, especially since estrogen is now excluded. The hydrophilic pocket architecture of GPR30 and the unique arrangement of its 7TM bundle suggest an affinity for ligands differing from traditional hydrophobic steroids. We have successfully characterized Lys05, a synthetic small molecule that activates GPR30 and the downstream Gq signaling pathway. The extensive interactions between Lys05 and GPR30, as revealed by our structural analysis, advance our understanding of GPR30' s activation and signaling mechanisms.

Our findings have significant implications for the field of hormone signaling and GPCR research. The discovery that GPR30 does not act as a direct estrogen receptor calls for reconsideration of its nomenclature. Referring to GPR30 as GPER may no longer be appropriate, considering its distinct ligand-binding and activation properties. This study lays the groundwork for developing new tools to probe GPR30' s function, potentially leading to a better understanding of its physiological roles and its connections to estrogen biology. Unraveling these mechanisms will offer profound insights into hormone signaling complexities and pave the way for innovative therapeutic approaches targeting GPR30.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (82121005 to X.X., H.E.X. and D.Y., 32130022 to H.E.X., 82330113 to X.X., 82273985 to D.Y., 82304579 to S.G.); CAS Strategic Priority Research Program (XDB37030103 to H.E.X.); Shanghai Municipal Science and Technology Major Project (2019SHZDZX02 to H.E.X.); Shanghai Municipal Science and Technology Major Project (H.E.X.); the Lingang Laboratory, Grant No. LG-GG-202204-01 (H.E.X.); the National Key R&D Program of China (2022YFC2703105 to H.E.X.); Shanghai Municipality Science and Technology Development Fund 21JC1401600 (D.Y.) and Program of Shanghai Academic/Technology Research Leader 23XD1400900 (D.Y.). Cryo-EM data were collected at the Shanghai Advanced Electron Microscope Center, Shanghai Institute of Material Medica and the electron microscopy facility of Shanghai Institute of Materia Medica (SIMM), Chinese Academy of Sciences. We thank Q. Yuan, K. Wu, W. Hu, S. Li, and S. Zhang for providing technical support and assistance during data collection at the Shanghai Advanced Electron Microscope Center, Shanghai Institute of Material Medica. We thank all staff from the electron microscopy facility of SIMM for their support.

Author Contributions

H.E.X. and P.X. initiated the project. H.L. designed and screened expression constructs of GPR30 and prepared protein samples of apo-GPR30-Gq and Lys05-GPR30-Gq complexes for cryo-EM data collection, performed cryo-EM grid preparation, data acquisition, structure determination and model building, and prepared the draft manuscript and figures. S.G. performed cell-based functional assays and participated in figure preparation. A.D. performed the radioligand binding assay. P.X. designed and screened expression constructs of GPR30 and prepared protein samples of GPR30-Gq complexes in the presence of E2, G1, and fulvestrant for cryo-EM data collection, performed cryo-EM grid preparation and data acquisition, and participated in structure determination. K.W. participated in sample screening and data collection. S.H. and X.Z. participated in protein sample preparation and structure determination. Y.L. participated in cell-based functional assays. X.H. participated in figure preparation. D.Y. supervised the radioligand binding assay. X.X. supervised the cell-based functional assays. H.E.X., X.X., and D.Y. conceived and supervised the overall project and participated in manuscript editing. H.E.X. wrote the manuscript with inputs from all authors.

Data Availability

Density maps and structure coordinates have been deposited in the Electron Microscopy Data Bank (EMDB) and the Protein Data Bank (PDB) with accession codes EMD-38527 and 8XOF for the Lys05-GPR30-Gq complex; EMD-38528 and 8XOG for the apo-GPR30-Gq complex; EMD-38529 and 8XOH for the

GPR30-Gq complex in the presence of E2; 8XOI for the GPR30-Gq complex in the presence of fulvestrant; and EMD-38531 and 8XOJ for the GPR30-Gq complex in the presence of G1. Source data is provided with this paper.

Competing Interests

The authors declare no competing interests.

References

1. Cui, J., Shen, Y. & Li, R. (2013). Estrogen synthesis and signaling pathways during aging: *Trends Mol Med* 19, 197-209.

2. Fuentes, N. & Silveyra, P. (2019). Estrogen receptor signaling mechanisms. *Adv Protein Chem Struct Biol* 116, 135-170.

3. Chen, P., Li, B. & Ou-Yang, L. (2022). Role of estrogen receptors in health and disease. *Front Endocrinol (Lausanne)* 13, 839005.

4. Walter, P., Green, S., Greene, G., Krust, A., Bornert, J. M., Jeltsch, J. M., et al. (1985). Cloning of the human estrogen receptor cDNA. *Proc Natl Acad Sci U S A* 82, 7889-7893.

5. Mosselman, S., Polman, J. & Dijkema, R. (1996). ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett* 392, 49-53.

6. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S. & Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A* 93, 5925-5930.

7. Szego, C. M. & Davis, J. S. (1967). Adenosine 3' ,5' -monophosphate in rat uterus: acute elevation by estrogen. *Proc Natl Acad Sci U S A* 58, 1711-1718.

8. Pietras, R. J. & Szego, C. M. (1975). Endometrial cell calcium and oestrogen action. *Nature* 253, 357-359.

9. Pietras, R. J. & Szego, C. M. (1979). Estrogen receptors in uterine plasma membrane. *Journal of Steroid Biochemistry* 11, 1471-1483.

10. Alexander, S. P. H., Mathie, A. & Peters, J. A. (2008). Guide to Receptors and Channels (GRAC), 3rd edition. *British Journal of Pharmacology* 153, S1-S1.

11. Feng, Y. & Gregor, P. (1997). Cloning of a novel member of the G

protein-coupled receptor family related to peptide receptors. *Biochem Biophys Res Commun* 231, 651-654.

12. Owman, C., Blay, P., Nilsson, C. & Lolait, S. J. (1996). Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt' s lymphoma and widely distributed in brain and peripheral tissues. *Biochem Biophys Res Commun* 228, 285-292.

13. Takada, Y., Kato, C., Kondo, S., Korenaga, R. & Ando, J. (1997). Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress. *Biochem Biophys Res Commun* 240, 737-741.

14. Kvingedal, A. M. & Smeland, E. B. (1997). A novel putative G-protein-coupled receptor expressed in lung, heart and lymphoid tissue. *FEBS Lett* 407, 59-62.

15. Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B. & Prossnitz, E. R. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307, 1625-1630.

16. Thomas, P., Pang, Y., Filardo, E. J. & Dong, J. (2005). Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* 146, 624-632.

17. Filardo, E. J., Quinn, J. A., Bland, K. I. & Frackelton, A. R., Jr. (2000). Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Mol Endocrinol* 14, 1649-1660.

18. Filardo, E. J., Quinn, J. A., Frackelton, A. R., Jr. & Bland, K. I. (2002). Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol* 16, 70-84.

19. Nicole, U., Marion, L. & Michael, S. (2023). Multiplex G Protein-Coupled Receptor Screen Reveals Reliably Acting Agonists and a Gq-Phospholipase C Coupling Mode of GPR30/GPER1. *Molecular Pharmacology* 103, 48.

20. Prossnitz, E. R. & Barton, M. (2023). The G protein-coupled oestrogen receptor GPER in health and disease: an update. *Nature Reviews Endocrinology* 19, 407-424.

21. Barton, M., Filardo, E. J., Lolait, S. J., Thomas, P., Maggiolini, M. &

Prossnitz, E. R. (2018). Twenty years of the G protein-coupled estrogen receptor GPER: Historical and personal perspectives. *J Steroid Biochem Mol Biol* 176, 4-15.

22. Vivacqua, A., Bonofiglio, D., Albanito, L., Madeo, A., Rago, V., Carpino, A., Maggiolini, M. (2006). 17beta-estradiol, genistein, and 4-hydroxytamoxifen induce the proliferation of thyroid cancer cells through the g protein-coupled receptor GPR30. *Mol Pharmacol* 70, 1414-1423.

23. Vivacqua, A., Bonofiglio, D., Recchia, A. G., Musti, A. M., Picard, D., Andò, S. & Maggiolini, M. (2006). The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17beta-estradiol and hydroxytamoxifen in endometrial cancer cells. *Mol Endocrinol* 20, 631-646.

24. Petrie, W. K., Dennis, M. K., Hu, C., Dai, D., Arterburn, J. B., Smith, H. O., Prossnitz, E. R. (2013). G protein-coupled estrogen receptor-selective ligands modulate endometrial tumor growth. *Obstet Gynecol Int* 2013, 472720.

25. Bologa, C. G., Revankar, C. M., Young, S. M., Edwards, B. S., Arterburn, J. B., Kiselyov, A. S., Prossnitz, E. R. (2006). Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* 2, 207-212.

26. Pedram, A., Razandi, M. & Levin, E. R. (2006). Nature of functional estrogen receptors at the plasma membrane. *Mol Endocrinol* 20, 1996-2009.

27. Otto, C., Rohde-Schulz, B., Schwarz, G., Fuchs, I., Klewer, M., Brittain, D., Fritzemeier, K. H. (2008). G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol. *Endocrinology* 149, 4846-4856.

28. Langer, G., Bader, B., Meoli, L., Isensee, J., Delbeck, M., Noppinger, P. R. & Otto, C. (2010). A critical review of fundamental controversies in the field of GPR30 research. *Steroids* 75, 603-610.

29. Levin, E. R. (2009). G Protein-Coupled Receptor 30: Estrogen Receptor or Collaborator? *Endocrinology* 150, 1563-1565.

30. Otto, C., Fuchs, I., Kauselmann, G., Kern, H., Zevnik, B., Andreasen, P., Fritzemeier, K. H. (2009). GPR30 does not mediate estrogenic responses in reproductive organs in mice. *Biol Reprod* 80, 34-41.

31. Kang, L., Zhang, X., Xie, Y., Tu, Y., Wang, D., Liu, Z. & Wang, Z. Y.

(2010). Involvement of estrogen receptor variant ER-alpha36, not GPR30, in nongenomic estrogen signaling. *Mol Endocrinol* 24, 709-721.

32. Tutzauer, J., Gonzalez de Valdivia, E., Swärd, K., Alexandrakis Eilard, I., Broselid, S., Kahn, R., Leeb-Lundberg, L. M. F. (2021). Ligand-Independent G Protein-Coupled Estrogen Receptor/G Protein-Coupled Receptor 30 Activity: Lack of Receptor-Dependent Effects of G-1 and 17β-Estradiol. *Mol Pharmacol* 100, 271-282.

33. Levin, E. R. & Hammes, S. R. (2016). Nuclear receptors outside the nucleus: extranuclear signalling by steroid receptors. *Nature Reviews Molecular Cell Biology* 17, 783-797.

34. Luo, J. & Liu, D. (2020). Does GPER Really Function as a G Protein-Coupled Estrogen Receptor in vivo? *Frontiers in Endocrinology* 11.

35. Seok, Y. M., Jang, E. J., Reiser, O., Hager, M. & Kim, I. K. (2012). 17β-Estradiol induces vasorelaxation in a G-protein-coupled receptor 30-independent manner. *Naunyn Schmiedebergs Arch Pharmacol* 385, 945-948.

36. Kim, J., Szinte, J. S., Boulware, M. I. & Frick, K. M. (2016). 17β-Estradiol and Agonism of G-protein-Coupled Estrogen Receptor Enhance Hippocampal Memory via Different Cell-Signaling Mechanisms. *J Neurosci* 36, 3309-3321.

37. Soltysik, K. & Czekaj, P. (2013). Membrane estrogen receptors—is it an alternative way of estrogen action? *J Physiol Pharmacol* 64, 129-142.

38. Razandi, M., Pedram, A., Merchenthaler, I., Greene, G. L. & Levin, E. R. (2004). Plasma membrane estrogen receptors exist and function as dimers. *Mol Endocrinol* 18, 2854-2865.

39. Gingerich, S., Kim, G. L., Chalmers, J. A., Koletar, M. M., Wang, X., Wang, Y. & Belsham, D. D. (2010). Estrogen receptor alpha and G-protein coupled receptor 30 mediate the neuroprotective effects of 17β-estradiol in novel murine hippocampal cell models. *Neuroscience* 170, 54-66.

40. Chaudhri, R. A., Schwartz, N., Elbaradie, K., Schwartz, Z. & Boyan, B. D. (2014). Role of ER$ $36 in membrane-associated signaling by estrogen. *Steroids* 81, 74-80.

41. Li, L., Haynes, M. P. & Bender, J. R. (2003). Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. *Proc Natl Acad Sci U S A* 100, 4807-4812.

42. Liu, H., Zheng, Y., Wang, Y., Wang, Y., He, X., Xu, P., Xu, F. (2023). Recognition of methamphetamine and other amines by trace amine receptor TAAR1. *Nature* 624, 663-671.

43. Zhao, L. H., Ma, S., Sutkeviciute, I., Shen, D. D., Zhou, X. E., de Waal, P. W., Zhang, Y. (2019). Structure and dynamics of the active human parathyroid hormone receptor-1. *Science* 364, 148-153.

44. Liu, H., Zhang, Q., He, X., Jiang, M., Wang, S., Yan, X., Yin, W. (2023). Structural insights into ligand recognition and activation of the medium-chain fatty acid-sensing receptor GPR84. *Nat Commun* 14, 3271.

45. Duan, J., Xu, P., Zhang, H., Luan, X., Yang, J., He, X., Xu, H. E. (2023). Mechanism of hormone and allosteric agonist mediated activation of follicle stimulating hormone receptor. *Nat Commun* 14, 519.

46. Maeda, S., Qu, Q., Robertson, M. J., Skiniotis, G. & Kobilka, B. K. (2019). Structures of the M1 and M2 muscarinic acetylcholine receptor/G-protein complexes. *Science* 364, 552-557.

47. Xu, P., Huang, S., Zhang, H., Mao, C., Zhou, X. E., Cheng, X., Xu, H. E. (2021). Structural insights into the lipid and ligand regulation of serotonin receptors. *Nature* 592, 469-473.

48. Wang, Y., Guo, S., Zhuang, Y., Yun, Y., Xu, P., He, X., Jiang, Y. (2021). Molecular recognition of an acyl-peptide hormone and activation of ghrelin receptor. *Nature Communications* 12, 5064.

49. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Hassabis, D. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature* 596, 583-589.

50. He, X.-h., You, C.-z., Jiang, H.-l., Jiang, Y., Xu, H. E. & Cheng, X. (2023). AlphaFold2 versus experimental structures: evaluation on G protein-coupled receptors. *Acta Pharmacologica Sinica* 44, 1-7.

51. Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S. & Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 138, 863-871.

52. Xu, P., Huang, S., Zhang, H., Mao, C., Zhou, X. E., Cheng, X., Xu, H. E. (2021). Structural insights into the lipid and ligand regulation of serotonin receptors. *Nature* 592, 469-473.

53. Yang, F., Mao, C., Guo, L., Lin, J., Ming, Q., Xiao, P., Zhang, Y. (2020). Structural basis of GPBAR activation and bile acid recognition. *Nature* 587, 152-157.

54. You, C., Zhang, Y., Xu, P., Huang, S., Yin, W., Eric Xu, H. & Jiang, Y. (2022). Structural insights into the peptide selectivity and activation of human neuromedin U receptors. *Nature Communications* 13, 2045.

55. Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G. W., Vardy, E., Stevens, R. C. (2012). Structure of the human -opioid receptor in complex with JDTic. *Nature* 485, 327-332.

56. Wang, Y., Zhuang, Y., DiBerto, J. F., Zhou, X. E., Schmitz, G. P., Yuan, Q., Xu, H. E. (2023). Structures of the entire human opioid receptor family. *Cell* 186, 413-427.e417.

57. Liu, Q., Yang, D., Zhuang, Y., Croll, T. I., Cai, X., Dai, A., Jiang, Y. (2021). Ligand recognition and G-protein coupling selectivity of cholecystokinin A receptor. *Nature Chemical Biology* 17, 1238-1244.

58. Chun, E., Thompson, A. A., Liu, W., Roth, C. B., Griffith, M. T., Katritch, V., Stevens, R. C. (2012). Fusion partner toolchest for the stabilization and crystallization of G protein-coupled receptors. *Structure* 20, 967-978.

59. Liu, P., Jia, M. Z., Zhou, X. E., De Waal, P. W., Dickson, B. M., Liu, B., Jiang, Y. (2016). The structural basis of the dominant negative phenotype of the Gαi1$ 1 $2 G203A/A326S heterotrimer. *Acta Pharmacol Sin* 37, 1259-1269.

60. Maeda, S., Koehl, A., Matile, H., Hu, H., Hilger, D., Schertler, G. F. X., Kobilka, B. K. (2018). Development of an antibody fragment that stabilizes GPCR/G-protein complexes. *Nat Commun* 9, 3712.

61. Mastronarde, D. N. (2005). Automated electron microscope tomography using rapid unsupervised cryo-EM robust prediction of specimen movements. *J Struct Biol* 152, 36-51.

62. Zheng, S. Q., Palovcak, E., Armache, J. P., Verba, K. A., Cheng, Y. & Agard, D. A. (2017). MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat Methods* 14, 331-332.

63. Zhang, K. (2016). Gctf: Real-time CTF determination and correction. *J Struct Biol* 193, 1-12.

64. Scheres, S. H. (2012). RELION: implementation of a Bayesian approach

to cryo-EM structure determination. *J Struct Biol* 180, 519-530.

65. Punjani, A., Rubinstein, J. L., Fleet, D. J. & Brubaker, M. A. (2017). cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nat Methods* 14, 290-296.

66. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C. & Ferrin, T. E. (2004). UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* 25, 1605-1612.

67. Emsley, P. & Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* 60, 2126-2132.

68. Croll, T. I. (2018). ISOLDE: a physically realistic environment for model building into low-resolution electron-density maps. *Acta Crystallogr D Struct Biol* 74, 519-530.

69. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Zwart, P. H. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* 66, 213-221.

70. Pettersen, E. F., Goddard, T. D., Huang, C. C., Meng, E. C., Couch, G. S., Croll, T. I., Ferrin, T. E. (2021). UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Sci* 30, 70-82.

Methods

Cell Lines

Spodoptera frugiperda (Sf9, Expression Systems) and Trichoplusia ni (High Five, Thermo Fisher) cells were grown in ESF 921 medium at 27°C and 120 rpm. HEK293T cells were grown in a humidified 37°C incubator with 5% CO_2 using media supplemented with 100 I.U./mL penicillin and 100 mg/mL streptomycin (Invitrogen). The human cell lines HEK293T were maintained in DMEM (VWR) containing 10% fetal bovine serum (FBS, VWR).

Plasmid Construction

For structure determination of the E2, G1, fulvestrant, and apo GPR30-Gq complexes, the full-length gene sequence of wild-type human GPR30 was cloned into a pFastBac vector with an N-terminal HA signaling peptide sequence followed by a FLAG tag, a 10×His tag, and a BRIL to facilitate expression and purification[58]. The engineered Gαq construct was generated based on mini-Gs/q7[13], which carries two dominant-negative mutations (corresponding to G203A and A326S) to decrease nucleotide-binding affinity

and facilitate stability of the G$\alpha\beta\gamma$ complex[59]. The N-terminal 1–18 amino acids and the α-helical domain of mini-Gs/q71 were substituted with the corresponding sequences of human Gαi1 to contribute to binding to the antibody fragments scFv16[60]. All three G protein complex components—Gαq, rat G$ 1, *andbovineG* 2—*wereclonedintopFastBacvectorsseparately.Forstructuredeterminationofthe*Lys05—*stimulatedGPR*30 — *Gqcomplex, theengineeredG qwasfusedtotheC* — *terminusofGPR*30*withaflexiblelinkerbetweenthem, whileG* 1*andG* $2 were cloned into a pFastBac Dual vector.

Complex Expression and Purification

All complexes were expressed in Hi5 insect cells (Invitrogen). For expression of E2, G1, and fulvestrant-stimulated GPR30-Gq complexes and the apo-GPR30-Gq complex, cell cultures were grown in ESF 921 medium (Expression Systems) to a density of 3$\times$10^{6}$ per mL with virus preparations for GPR30, Gαq, G$ 1, *G* 2, *andscFv*16*ataratioof*1 : 1 : 1 : 1 : 1.*Forexpressionofthe*Lys05 — *activatedGPR*30 — *Gqcomplex, cellculturesweregrowntoadensityof*3$\times$10^{6}$ per mL with two separate virus preparations for GPR30-Gαq and G$ 1 $2 at a ratio of 1:1.2. Infected cells were cultured at 27°C for 48 h before collection by centrifugation, and cell pellets were stored at -80°C.

For purification of the E2, G1, and fulvestrant-stimulated GPR30-Gq complexes, cell pellets from 1 L culture were thawed at room temperature and resuspended in low-salt buffer containing 20 mM HEPES pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 10% glycerol, and protease inhibitor cocktail (Thermo Fisher Scientific). The GPR30-Gq complexes were formed on membrane in the presence of 1 M E2, 1 M G1 (Targetmol, USA), or 1 M fulvestrant (Sigma) and treated with apyrase (20 mU/mL, NEB), followed by incubation for 1 hour at 4°C.

Subsequently, 0.5% (w/v) n-dodecyl-β-d-maltoside (DDM, Anatrace) and 0.1% (w/v) cholesteryl hemisuccinate (CHS, Anatrace) were added to solubilize complexes for 2-3 h at 4°C. Insoluble material was removed by centrifugation at 30,000 g for 30 min, and the supernatant was immobilized by batch binding to Talon affinity resin. After batch binding, the TALON IMAC resin with immobilized protein complex was manually loaded onto a gravity flow column. The TALON IMAC resin was washed with 10 column volumes of 20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 30 mM imidazole, 10% glycerol, 0.1% LMNG (w/v), 0.02% CHS (w/v), plus 1 M E2, G1, or fulvestrant, and eluted with the same buffer plus 250 mM imidazole and the respective ligand. The mixture was then purified by SEC using a Superdex 200 10/300 GL column (GE Healthcare) in 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.00075% (w/v) LMNG, 0.00025% (w/v) GDN, and 1 M of the respective ligand.

For purification of the apo-GPR30-Gq complex, all steps were identical to those described above but without ligand addition. For purification of the Lys05-stimulated GPR30-Gq complex, 500 M Lys05 (Targetmol, USA) was added

throughout the purification, and the eluted protein was incubated with 20 g/mL Nb35 at 4°C for an additional 1 h. The mixture was then purified by SEC using a Superdex 200 10/300 GL column (GE Healthcare) in 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.00075% (w/v) LMNG, 0.00025% (w/v) GDN, and 500 M Lys05. All samples were finally concentrated to ~8 mg/mL for cryo-EM grid preparation.

Cryo-EM Grid Preparation and Data Collection

For cryo-EM grid preparation of the GPR30-Gq complexes, 3 L of protein at ~8 mg/mL was loaded onto a glow-discharged holey carbon grid (Quantifoil Au 300 mesh R1.2/1.3) and subsequently plunge-frozen in liquid ethane using a Vitrobot Mark IV (Thermo Fisher Scientific). Data for the E2, G1, and fulvestrant-stimulated GPR30-Gq complexes were collected at the electron microscopy facility of Shanghai Institute of Materia Medica (SIMM) on a Titan Krios at 300 kV using a Gatan K3 Summit detector with a pixel size of 1.071 Å. Images were taken at a dose rate of about 8.0 e/Å^2/s with a defocus ranging from -1.0 to -2.0 m using Serial-EM software[61]. The total exposure time was 8 s, and 36 frames were recorded per micrograph. A total of 4,773, 4,840, and 7,980 movies were collected for the E2, G1, and fulvestrant-stimulated GPR30-Gq complexes, respectively.

For the apo GPR30-Gq complex, cryo-EM imaging was collected on a Titan Krios at 300 kV using a Gatan K3 Summit detector with a pixel size of 0.824 Å at the Shanghai Advanced Center for Electron Microscopy, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Images were taken at a dose rate of about 8.0 e/Å^2/s with a defocus ranging from -1.0 to -2.0 m using EPU software (FEI Eindhoven, Netherlands). The total exposure time was 8 s, and 36 frames were recorded per micrograph. A total of 6,691 movies were collected.

For the Lys05-activated GPR30-Gq complex, cryo-EM imaging was collected on a Titan Krios equipped with a Falcon 4 direct electron detection device at 300 kV. Images were taken with a pixel size of 0.73 Å, a defocus ranging from -1.0 to -2.0 m using EPU software (FEI Eindhoven, Netherlands). We collected a total of 9,417 movies with a total dose of 50 e/Å^2/s over 2.5 s exposure on each EER format movie. Each movie was divided into 36 frames during motion correction.

Image Processing and Map Construction

For the E2-stimulated GPR30-Gq complex dataset, image stacks were subjected to beam-induced motion correction using MotionCor2[62]. Contrast transfer function (CTF) parameters for each non-dose-weighted micrograph were determined by Gctf[63]. Automated particle selection and data processing were performed using RELION-3.0 beta2[64]. Automatically picked particles were extracted from a binned dataset with a pixel size of 2.142 Å and subjected to reference-free 2D classification, producing 1,199,129 particles with well-defined averages. Further

3D classification produced two good subsets showing clear structural features accounting for 527,631 particles. These particles were re-extracted at a pixel size of 1.071 Å and subsequently subjected to CTF refinement and Bayesian polishing. Additional 3D classifications with no alignment produced high-quality subsets accounting for 177,591 particles. 3D refinement and post-processing generated a map with an indicated global resolution of 3.2 Å at a Fourier shell correlation (FSC) of 0.143.

For the fulvestrant-stimulated GPR30-Gq complex dataset, image stacks were subjected to beam-induced motion correction using MotionCor2[62]. CTF parameters for each non-dose-weighted micrograph were determined by Gctf[63]. Automatically picked particles were extracted from a binned dataset with a pixel size of 2.142 Å and subjected to reference-free 2D classification, producing 3,410,733 particles with well-defined averages. Further 3D classification produced a good subset showing clear structural features accounting for 826,321 particles. These particles were re-extracted at a pixel size of 1.071 Å and subsequently subjected to 3D classifications with no alignment, producing high-quality subsets accounting for 536,691 particles. 3D refinement and post-processing generated a map with an indicated global resolution of 3.2 Å at an FSC of 0.143.

For the G1-stimulated GPR30-Gq complex dataset, image stacks were subjected to beam-induced motion correction using MotionCor2[62]. CTF parameters for each non-dose-weighted micrograph were determined by Gctf[63]. Automated particle selection and data processing were performed using RELION-3.0 beta2[64]. Automatically picked particles were extracted and subsequent reference-free 2D classification produced 1,065,934 well-defined particles that were imported into cryoSPARC.v4[65]. Further ab-initio model and heterogeneous refinement followed by a resolution ladder hetero-refinement produced a high-quality subset accounting for 411,141 particles. Non-uniform refinement generated a map with an indicated global resolution of 3.1 Å at an FSC of 0.143.

For the apo-GPR30-Gq complex dataset, single-particle analysis was performed with cryoSPARC.v4[65]. Dose-fractionated image stacks were subjected to motion correction by MotionCor2[62]. CTF parameters for micrographs were estimated by patch CTF estimation. Auto-picked particles were extracted and subjected to reference-free 2D classification. The selected 1,072,840 particles were subjected to ab-initio model and heterogeneous refinement, producing a high-quality subset accounting for 345,373 particles. Further ab-initio model and heterogeneous refinement produced a subset of 300,561 particles for non-uniform refinement, generating a map with an indicated global resolution of 2.9 Å at an FSC of 0.143.

For the Lys05-activated GPR30-Gq complex dataset, single-particle analysis was performed with cryoSPARC.v4[65]. Dose-fractionated image stacks were subjected to motion correction by MotionCor2[62]. CTF parameters for micrographs were estimated by patch CTF estimation. Auto-picked particles were extracted and subjected to reference-free 2D classification. The selected 1,457,109 particles were subjected to ab-initio model and heterogeneous refinement, producing

a high-quality subset accounting for 274,405 particles. Further ab-initio model and heterogeneous refinement produced a subset of 209,371 particles for non-uniform refinement, generating a map with an indicated global resolution of 2.6 Å at an FSC of 0.143.

Model Building and Refinement

Predicted models of the active-state GPR30 receptor from AlphaFold[49] were used as initial models for rebuilding and refinement against the electron microscopy density map. UCSF Chimera-1.14[66] was used to dock the model into the electron microscopy density map, followed by iterative manual adjustment and rebuilding in COOT-0.9.6[67] and ISOLDE-1.2[68]. Models were further refined and validated in Phenix-1.20 programs[69] (Table S1). Structural figures were generated using UCSF Chimera-1.14[66], ChimeraX-1.2[70], and PyMOL-2.0 (https://pymol.org/2/).

Cell Transfection

The wild-type GPR30 gene was subcloned into a pcDNA3.0 vector with the addition of an N-terminal HA tag. All mutations used for functional studies were generated by QuickChange PCR and verified by DNA sequencing. HEK293 cells were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM supplemented with 10% (v/v) FBS, 100 mg/L penicillin, and 100 mg/L streptomycin in 5% CO_2 at 37°C. For transient transfection, approximately 2$×10^{6}$ cells were mixed with plasmids in 200 µL transfection buffer, and electroporation was carried out with a Scientz-2C electroporation apparatus (Scientz Biotech, Ningbo, China). Experiments were carried out 24 h after transfection.

Surface Expression Analysis

Twenty-four hours after transfection, cells were washed with PBS, fixed with 4% PFA for 15 min, and blocked with 2% BSA for 1 h. Cells were then incubated with polyclonal anti-HA (Sigma, H6908) overnight at 4°C and subsequently with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (CST, 7074S) for 1 h at room temperature. After washing, cells were incubated with 50 L tetramethylbenzidine (Sigma, T0440) for 30 min before the reaction was stopped with 25 L TMB Substrate stop solution (Beyotime, P0215). Absorbance at 450 nm was quantified using a FlexStation III microplate reader (Molecular Devices).

IP1 Accumulation Assay

IP1 accumulation was measured using the IP-ONE Gq HTRF kit (Cisbio, 621PAPEJ). Briefly, 24 hours after transfection, cells were harvested and resuspended in DMEM containing 0.1% BSA at a density of 4 million per milliliter. Five milliliters of cells were aliquoted into a 384-well plate and stimulated with 5 mL of compounds in DMEM (0.1% BSA) supplemented with

50 mM LiCl. After incubation at 37°C for 30 minutes, 5 mL IP1 d2 reagent and 5 mL IP1 Tb cryptate antibody were added. Following incubation at room temperature for another 1 h, HTRF was read on an EnVision multiplate reader (PerkinElmer). EC_{50} values for each curve were calculated by Prism 8.0 software (GraphPad Software).

Calcium Assay

Cells were seeded in 96-well plates at a density of 3$×10^{4}$ cells/well and cultured overnight. Cells were then incubated with 2 mol/L Fluo-4 AM in HBSS (5.4 mmol/L KCl, 0.3 mmol/L Na_2HPO_4, 0.4 mmol/L KH_2PO_4, 4.2 mmol/L $NaHCO_3$, 1.3 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 0.6 mmol/L $MgSO_4$, 137 mmol/L NaCl, 5.6 mmol/L D-glucose, and 250 mol/L sulfinpyrazone, pH 7.4) at 37°C for 40 min. After thorough washing, 50 L of HBSS was added. Following incubation at room temperature for 10 min, 25 L of agonist was dispensed into the well using a FlexStation III microplate reader (Molecular Devices), and intracellular calcium change was recorded at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. EC_{50} and E values for each curve were calculated by Prism 8.0 software (GraphPad Software).

cAMP Accumulation Assay

cAMP accumulation was measured using the cAMP kit (PerkinElmer, TRF0264). Briefly, 24 hours after transfection, cells were harvested and resuspended in DMEM containing 0.1% BSA at a density of 2$×10^{5}$ cells/mL. Cells were then plated onto 384-well assay plates at 1000 cells/5 L/well. To test Gs activity of compounds on GPR30, another 5 L buffer containing compounds with 500 M IBMX was added to the cells. To test Gi activity, 5 L buffer containing compounds with 1 M forskolin and 500 M IBMX was added. After incubation at 37°C for 30 minutes, intracellular cAMP level was measured by a LANCE Ultra cAMP kit and EnVision multiplate reader according to the manufacturer' s instructions. EC_{50} values for each curve were calculated by Prism 8.0 software (GraphPad Software).

Ligand Binding Assay

CV1 cells were cultured in DMEM medium with 10% FBS and seeded at a density of 30,000 cells/well in Isoplate-96 plates (PerkinElmer). Twenty-four hours after transfection with GPR30 or ERα, CV1 cells were washed twice and incubated with blocking buffer (DMEM supplemented with 25 mM HEPES and 0.1% (w/v) BSA, pH 7.4) for 2 h at 37°C. For homogeneous competition binding, radiolabeled [^{3}H]-E2 (PerkinElmer, 3 nM) and unlabeled compound at seven decreasing concentrations (estradiol, G-1, tamoxifen, raloxifene, 10 M to 1 pM) were added and competitively reacted with the cells in blocking buffer at room temperature for 3 h. Following incubation, cells were washed three times with ice-cold PBS and lysed by 50 L lysis buffer (PBS supplemented with 20 mM Tris-HCl, 1% Triton X-100, pH 7.4). Radioactivity was subsequently counted

(counts per minute, CPM) in a scintillation counter (MicroBeta^2 Plate Counter, PerkinElmer) using a scintillation cocktail (OptiPhase SuperMix, PerkinElmer). Data were analyzed by nonlinear regression using GraphPad Prism 8.0 software.

Hi5 cell binding assays were carried out in filter plates. Hi5 membrane homogenates expressing ERα or GPR30 (10 µg protein per well) were incubated in membrane binding buffer (20 mM HEPES-NaOH and 10 mM EDTA, pH 7.4) with 50 nM [^{3}H]-E2 at room temperature for 3 h. Following incubation, samples were rapidly filtered in vacuum through glass fiber filter plates (PerkinElmer). After soaking and rinsing four times with ice-cold PBS, filters were dried before addition of 50 µL scintillation cocktail (PerkinElmer) and counted for radioactivity in a scintillation counter (PerkinElmer). Data were analyzed by GraphPad Prism 8.0 software.

Figure Legends

Fig. 1 Cryo-EM structures contradict GPR30 as a direct estrogen receptor. (A) Cryo-EM maps of GPR30-Gq structures in the presence of E2, G-1, and fulvestrant. (B) No ligand density observed in the GPR30 binding pocket. (C) Cryo-EM density map and cartoon presentation of the apo-GPR30-Gq complex. (D) Comparison of the EM GPR30 structure with the AlphaFold-predicted GPR30 structure. (E-F) Negatively charged (E) and hydrophilic (F) nature of GPR30' s binding pocket.

Fig. 2 Functional assays challenge GPR30 as a direct estrogen receptor. (A) Chemical formula of E2, tamoxifen, raloxifene, and Lys05. (B-D) Radioligand competitive binding results of E2 (B), tamoxifen (C), or raloxifene (D) to GPR30, ERα, and empty cell. Data are mean ± S.E.M. from 3 independent experiments (n = 3). (E-G) Cell-based functional assays evaluating stimulation by E2, Lys05, or E2+Lys05 for Gq (E), Gs (F), or Gi (G) pathways. Data are mean ± S.E.M. from 3 independent experiments (n = 3).

Fig. 3 Structure of GPR30 bound to Lys05. (A) Cryo-EM density map and cartoon presentation of the Lys05-GPR30-Gq complex. The complex density map is shown at a contour level of 0.15. (B) Positively charged Lys05 accommodated in the negatively charged ligand-binding pocket of GPR30.

Fig. 4 Molecular Recognition of Lys05 by GPR30. (A) Diagram of Lys05-interacting residues, with background indicating two hydrophobic (orange) and one hydrophilic (light blue) moieties of Lys05. Residues and dotted lines in red represent polar nature or interactions, while those in black represent hydrophobic nature or interactions. (B, D, E) Detailed interactions of the tri-layered interface between Lys05 and GPR30: bottom hydrophobic interactions (B), center hydrophilic interactions (D), and top hydrophobic interactions (E). (C) Effects of mutating GPR30-Lys05 interacting residues within the ligand-binding pocket. The pEC_{50} is shown in the bar graph. Data are mean ± S.E.M. from 3 independent experiments (n = 3).

Fig. 5 Basal activity and ligand-induced activation of GPR30. (A-B) Side view (A) and top view (B) of water molecules and surrounding residues in the apo-GPR30-Gq structure pocket. (C) Superposition of Lys05 and water molecules in the structures of the GPR30-Gq complexes. (D) Superposition of activated GPR30 (cornflower blue) with active KOR (gray; PDB code: 8F7W) and inactive KOR (orange; PDB code: 4DJH). Notable conformational changes occur at intracellular ends of TM6 and TM7 upon receptor activation, shown in side view (left) and bottom view (right). (E) The "switch" $W272^{6\ 48}$ of GPR30 displays relative rotameric change when sensing Lys05 and further induces conformational change of $F268^{6\ 44}$. (F-H) The key D-$R155^{3\ 50}$-Y motif (F), P-$V145^{3\ 40}$-$F268^{6\ 44}$ (G), and N-$P^{7\ 50}$-xx-$Y324^{7\ 53}$ (H) motifs display conformational rearrangement in activated GPR30.

Fig. 6 Gαq coupling of GPR30. (A) Compared to GSHR (PDB code: 7F9Z), CCKAR (PDB code: 7EZM), and NMUR2 (PDB code: 7W55), the N-terminus of αH5 is positioned closer to the junction of TM5 and TM6 in GPR30, diverging by approximately 15°. (B) Diagram of GPR30-Gαq contacts. (C) Key interactions between the TMs that form the core cavity for Gq coupling and αH5 of Gαq. (D-E) Key interactions between ICL2 (D) and ICL3 (E) from GPR30 and αH5 of Gαq.

Fig. S1 Purification and structure determination of GPR30-Gq complexes in the presence of E2 and fulvestrant. (A) Representative size exclusion chromatography (SEC) profiles and SDS-PAGE analysis of GPR30-Gq complex. (B-C) Representative cryo-EM image (B) and 2D classification averages (C) of GPR30-Gq complex in the presence of E2. (D-E) Cryo-EM data processing flowcharts (D) and Fourier shell correlation (FSC) curves (E) of GPR30-Gq complex in the presence of E2. The global resolution of the final processed density map estimated at FSC = 0.143 is 3.2 Å. (F) Density maps of TM1–TM7 and helix 8 (H8) of GPR30 in GPR30-Gq complex in the presence of E2. (G-H) Representative cryo-EM image (G) and 2D classification averages (H) of GPR30-Gq complex in the presence of fulvestrant. (I-J) Cryo-EM data processing flowcharts (I) and FSC curves (J) of GPR30-Gq complex in the presence of fulvestrant. The global resolution of the final processed density map estimated at FSC = 0.143 is 3.2 Å. (K) Density maps of TM1–TM7 and helix 8 (H8) of GPR30 in GPR30-Gq complex in the presence of fulvestrant.

Fig. S2 Purification and structure determination of GPR30-Gq complexes in the presence of G1 and apo-GPR30-Gq complexes. (A-B) Representative cryo-EM image (A) and 2D classification averages (B) of GPR30-Gq complex in the presence of G1. (C-D) Cryo-EM data processing flowcharts (C) and FSC curves (D) of GPR30-Gq complex in the presence of G1. The global resolution of the final processed density map estimated at FSC = 0.143 is 3.1 Å. (E) Density maps of TM1–TM7 and helix 8 (H8) of GPR30 in GPR30-Gq complex in the presence of G1. (F-G) Representative cryo-EM image (F) and 2D classification averages (G) of apo-GPR30-Gq complex. (H-I) Cryo-EM data processing flowcharts (H) and FSC curves (I) of apo-GPR30-Gq complex. The global resolution of the final

processed density map estimated at FSC = 0.143 is 2.9 Å. (J) Density maps of TM1–TM7 and helix 8 (H8) of GPR30 in apo-GPR30-Gq complex. (K) Local resolution for the density of water molecules (W1–W2) in the ligand-binding pocket of the apo-GPR30-Gq structure.

Fig. S3 Comparisons of GPR30 structures and differences between GPR30' s binding pocket and those of related steroid hormone receptors. (A) Comparisons of GPR30 structures. (B) Chemical formula of hydrophobic ligands E2, G1, and fulvestrant. (C-D) The hydrophilic nature and larger volume of the GPR30 pocket contrast with those of related steroid hormone receptors.

Fig. S4 Binding assay results of E2 to GPR30 and validation of Lys05-induced GPR30 activation and Gq signaling. (A) Binding assay results of [^{3}H]-E2 to GPR30 in Hi5 cell membrane, with ERα as positive control. Data are mean ± S.E.M. from 3 independent experiments performed in duplicate (n = 3). (B-C) Calcium assay and IP1 assay validating that Lys05, but not estrogen-related compounds, induces GPR30 activation and Gq signaling. Data are mean ± S.E.M. from 3 independent experiments (n = 3).

Fig. S5 Purification and structure determination of Lys05-GPR30-Gq complexes. (A) Representative size exclusion chromatography (SEC) profiles and SDS-PAGE analysis of Lys05-activated GPR30-Gq complex. (B-C) Representative cryo-EM image (B) and 2D classification averages (C) of Lys05-GPR30-Gq complexes. (D-E) Cryo-EM data processing flowcharts (D) and FSC curves (E) of Lys05-GPR30-Gq complexes. The global resolution of the final processed density map estimated at FSC = 0.143 is 2.6 Å. (F) Density maps of TM1–TM7 and helix 8 (H8) of GPR30 in Lys05-GPR30-Gq complexes. (G) Comparison of Lys05-bound and apo-GPR30 structures.

Fig. S6 Structural analysis reveals a unique distribution of the transmembrane region of GPR30. (A) Structure comparison of GPR30' s 7TM bundle with that of 5HT1A (PDB code: 7E2Y), GPBAR (PDB code: 7CFM), and GPR183 (PDB code: 7TUZ). (B) Structure comparison of GPR30' s 7TM bundle with that of GHSR (PDB code: 7F9Z), NMUR2 (PDB code: 7W55), and OXR2 (PDB code: 7L1U).

Fig. S7 Mutational effects of Lys05-GPR30 interacting residues on GPR30 activation. (A) Structure comparison of apo-GPR30 and Lys05-GPR30 indicating conformational changes of related residues during Lys05 insertion. (B-E) Dose-response curves of Lys05 activating mutated GPR30; mutations are located in TM2 (B), TM3 (C), TM6 (D), and TM7 (E). Data are mean ± S.E.M. from 3 independent experiments (n = 3).

Table S1 Cryo-EM data collection, refinement, and validation statistics.

Table S2 Effects of Lys05 on activation of GPR30 and its mutants. Receptor mutations include L108$^{2\ 53}$A, E115$^{2\ 60}$A, L137$^{3\ 32}$A, Q138$^{3\ 33}$A, M141$^{3\ 36}$A, W272$^{6\ 48}$A, E275$^{6\ 51}$A, H282$^{6\ 58}$A, H300$^{7\ 29}$A, P303$^{7\ 32}$A, N310$^{7\ 39}$A, and F314$^{7\ 43}$A. pEC_{50} values and E (%) are shown with data as means ± S.E.M.

from at least three independent experiments. Surface expression data were normalized to WT GPR30 expression level in HEK293 cells (means ± S.E.M. from four independent experiments). UD = undetectable, indicating activation level too low to determine EC_{50} values.

Note: Figure translations are in progress. See original paper for figures.