Ghrelin is a gastric peptide hormone with important physiological functions. The unique feature of ghrelin is its Serine 3 acyl-modification, which is essential for ghrelin’s activity. However, it remains to be elucidated why the acyl-modification of ghrelin is necessary for activity. To address these questions, we solved the crystal structure of the ghrelin receptor bound to antagonist. The ligand-binding pocket of the ghrelin receptor is bifurcated by a salt bridge between E124 and R283. A striking feature of the ligand-binding pocket of the ghrelin receptor is a wide gap (crevasse) between the TM6 and TM7 bundles that is rich in hydrophobic amino acids, including a cluster of phenylalanine residues. Mutagenesis analyses suggest that the interaction between the gap structure and the acyl acid moiety of ghrelin may participate in transforming the ghrelin receptor into an active conformation.
Ghrelin, a peptide hormone consisting of 28 amino acids, was originally discovered in the stomach as an endogenous ligand for GHSR (growth hormone secretagogue receptor, now called the ghrelin receptor), a member of the β-branch in the class A GPCRs. Ghrelin is the only peptide hormone that induces appetite stimulation following peripheral injection, and is thus functionally opposite to leptin. Moreover, ghrelin has a wide range of physiological functions, playing roles in growth hormone secretion, adiposity, energy homeostasis, memory formation, and hippocampal neurogenesis.

A salient feature of ghrelin is the O-acyl-modification at Ser3, which is essential for its activity; des-acyl ghrelin (i.e., ghrelin lacking the acyl-modification) is inactive. No other peptide hormone is known to require such an acyl-modification for its activity. The acyl-modification of ghrelin is catalyzed by ghrelin O-acyltransferase (GOAT). Although GOAT can transfer fatty acids ranging in size from acetic to palmitic acid onto des-acyl ghrelin, GOAT prefers medium-chain fatty acids as its substrates. The main acyl-modified and most potent active form of ghrelin is n-octanoyl ghrelin, but other medium-chain fatty acids are also detected as endogenous forms of ghrelin. For example, n-decanoyl ghrelin has been purified from the stomachs of frog and bird at levels comparable to those of n-octanoyl ghrelin. Moreover, calcium mobilization assays using synthesized ghrelin with fatty acids of various lengths revealed that ghrelin is less active when modified with a shorter-chain fatty acid. In addition, several reports indicated that the ghrelin receptor exhibits strong ligand-independent activity. The structural basis for its high constitutive activity is thought to be an aromatic amino acid cluster on transmembrane helices 6 and 7. However, because of the lack of structural information about the ghrelin receptor, the precise mechanisms of its high constitutive activity and activation by acyl-modified ghrelin remained to be elucidated.

Here, we report the first high-resolution crystal structure of the ghrelin receptor bound to an antagonist. We found that the ghrelin receptor has a wide gap (crevasse) between the TM6 and TM7 bundles, which is rich in hydrophobic amino acids including a cluster of phenylalanine residues. Thus, the interaction between the gap structure and the acyl acid moiety of ghrelin may participate in transforming the ghrelin receptor into an active conformation.

**Results**

**Crystal structure of the ghrelin receptor.** To facilitate the determination of the structure, we designed an engineered ghrelin receptor construct (Supplementary Fig. 1). For crystallization, 28 and 20 residues were removed from the N and C termini of the receptor, respectively, and the thermostabilized apocytochrome b562 (bRIL) protein from *Escherichia coli* was fused to the deleted N terminus. Deletion of the N-terminal 28 amino acids resulted in loss of cell-surface expression and receptor activity, whereas both expression and activity were retained when the C-terminal 20-amino acids were deleted (Supplementary Fig. 2a–d).

In addition, two mutations were introduced: T130K (superscripts indicate residue numbering using the Ballesteros-Weinstein nomenclature) to improve thermostability and expression, and N188Q in extracellular loop 2 (ECL2) to avoid glycosylation. Moreover, both a Fab fragment antibody (Fab 7881) specific for the ghrelin receptor and an antagonist, Compound 21, were added to bRIL-conjugated truncated ghrelin receptor to increase thermostability (Supplementary Fig. 3a, b). Compound 21 was synthesized from YIL-781, another ghrelin receptor antagonist, to improve antagonistic activity and lipophilic efficiency. Compound 21 had no effect on the constitutive activity of the ghrelin receptor, indicating that it is a neutral antagonist (Supplementary Fig. 3c). Binding assays using [3H]-Compound 21 confirmed that Compound 21 binds to the ghrelin receptor construct, although the binding affinity of the construct (dissociation constant, KD = 17.2 ± 7.1 nM) was lower than that of the WT ghrelin receptor (KD = 4.42 ± 0.76 nM) (Supplementary Fig. 3d). Fab 7881 binds to intracellular loop 3 (ICL3); the flexibility of ICL3 prevents the crystallization of the receptor. Thus, Fab 7881 seems to stabilize ICL3 and improve the thermostability of the ghrelin receptor, allowing it to form crystals. The ghrelin receptor was crystallized in lipidic cubic phase and solved at 3.3 Å resolution (Fig. 1, Supplementary Fig. 3e–g, Supplementary Table 1).
The ghrelin receptor structure, as a class A GPCR, shares a canonical seven-transmembrane helical architecture and an intracellular amphipathic helix 8 (Fig. 1)\(^1\). Similar to other peptide hormone receptors, ECL2 forms anti-parallel β-strands with a short hairpin and is stabilized by the highly conserved disulfide bond between C116\(^3.28\) and C198\(^ECL2\). ECL3 between TM6 and TM7 has not been observed, probably due to its unstable structure. The structure superimposed well with that of the inactive OX\(_2\) orexin receptor (PDB ID: 4S0V)\(^19\), with a root-mean-squared deviation of 0.819 Å. Thus, our structure of the TM6 and TM7 interface is occupied by bulky side chains that completely separate the ligand binding cavity from the lipid bilayer; consequently, ligands cannot be observed from outside the receptors (Supplementary Fig. 7b–f). In contrast, within the β-branch of class A GPCRs, with the exception of the ghrelin receptor, the TM6–TM7 interface is occupied by bulky side chains that completely separate the ligand binding cavity from the lipid bilayer; consequently, ligands cannot be observed from outside the receptors (Supplementary Fig. 7b–f). In contrast, the peptide hormone GPCRs, several GPCRs with lipid ligands

### Bifurcated ligand-binding pocket and the gap structure.

The ghrelin receptor has two main differences relative to other peptide hormone GPCRs. It is the bifurcated ligand-binding pocket and the hydrophobic wide gap structure of TM6 and TM7. The binding pocket of ghrelin receptor is bifurcated into two cavities by a salt bridge between E124\(^3.33\) and R283\(^6.55\) (Fig. 2a–c, Supplementary Fig. 4). We call the larger one cavity I, and the smaller one cavity II (Fig. 2b, c). In addition, the top of cavity II is covered with ECL2. Note that the A204E mutation in ECL2 causes loss of constitutive activity of the ghrelin receptor and is associated with familial short stature\(^20\). In fact, we observed loss of basal receptor activity and a decrease in ghrelin-induced receptor function (Supplementary Fig. 5a, b). Thus, mutation of A204 may block ghrelin’s access to the cavities.

No such bifurcated pocket has been observed in the class A peptide hormone GPCRs in the β-branch family whose structures have been determined thus far (e.g., the neurotensin receptor NTSR1\(^21\), the OX\(_1\) and OX\(_2\) orexin receptors\(^19,22,23\), the endothelin type B receptor\(^24\), and the NPY Y\(_1\) receptor\(^25\)) (Supplementary Fig. 6a–e).

Another characteristic feature of ghrelin receptor is the gap structure between TM6 and TM7. The extracellular side of the TM7 bundle curves outwardly to a greater degree than the TM6 bundle (Supplementary Fig. 6a). Furthermore, the G282\(^6.54\) and the bulky side chains of the five phenylalanines (F279\(^6.51\), F286\(^6.58\), F290\(^6.62\), F309\(^7.39\), and F312\(^7.42\)) in TM6 and TM7 are positioned to open the space between the TM6 and TM7 bundles. These structural features enable the ghrelin receptor to form a wide gap between the TM6 and TM7 bundles. We refer to this gap as the crevasse (Fig. 2b, d, Supplementary Fig. 7a). However, within the β-branch of class A GPCRs, with the exception of the ghrelin receptor, the TM6–TM7 interface is occupied by bulky side chains that completely separate the ligand binding cavity from the lipid bilayer; consequently, ligands cannot be observed from outside the receptors (Supplementary Fig. 7b–f). In contrast to the peptide hormone GPCRs, several GPCRs with lipid ligands.

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**Fig. 2** Bifurcated ligand-binding pocket in the ghrelin receptor, and the structure of the gap. a The cutaway surface for the ghrelin receptor in complex with the antagonist Compound 21 reveals a bifurcated ligand-binding pocket. b The ligand-binding pocket of the ghrelin receptor. c Schematic model of Fig. 2b. d The phenylalanine cluster forms a crevasse in the ghrelin receptor. The five phenylalanines are shown as sticks in dark orange. The crevasse is shown as a red dashed line. G282 is shown as spheres with carbon atoms in orange, nitrogen in blue, and oxygen in red.
have gaps between TM1 and TM7 (S1P1, EP₄, and TP)²⁶-²⁸, TM3 and TM4 (GPR40)²⁹, and TM4 and TM5 (PAFR and LPA₆)³⁰,³¹ (Supplementary Fig. 7g–l). These gaps have been proposed to be the access routes of the lipid ligands from outside the lipid bilayers into the pockets where they bind.

**Binding mode of Compound 21.** Compound 21 covers the salt bridge and fills the two cavities (Fig. 3a, b). The crystal structure of ghrelin receptor bound to Compound 21 revealed that the 1-isopropylpiperidine moiety of the Compound 21 sits in cavity I, whereas the 4-bromo-2-fluorobenzene moiety sits in cavity II, and the 5-azaquinazolinone moiety (5-aza core) links between cavities I and II. Moreover, the 5-nitrogen of the 5-aza core forms a hydrogen bond with R283⁶.⁵⁵, which connects with E12⁴.³³ through a salt bridge and forms the boundary between the cavities I and II (Fig. 2b, Supplementary Fig. 4). In addition, the 5-aza core makes a π–cation interaction with R10².⁶³ (Fig. 3a, b). The nitrogen of the 1-isopropylpiperidine moiety of Compound 21 forms hydrophobic interactions with the F2⁷⁹⁶.⁵¹, F3⁰⁹⁷.⁵⁸, and F3¹²⁷.⁴² (Fig. 3a, b). In particular, the F2⁷⁹⁶.⁵¹ and F3¹²⁷.⁴² mutants decreased the specific binding of [³⁵H]-Compound 21 and basal receptor activity (Supplementary Fig. 8f, i, j, Supplementary Fig. 9k), although cell-surface expression of the mutant ghrelin receptors was retained (Supplementary Fig. 9f, i, j). The 4-bromo-2-fluorobenzene moiety forms three hydrophobic contacts with residues I¹⁷⁸⁴.⁶⁰, L¹⁸¹⁴.⁶³, and M₂¹³⁵.³⁹ in cavity II. The fact that Compound 21 covers the two cavities and interacts with eight residues may explain the high antagonistic affinity of Compound 21 for the ghrelin receptor. However, the results of the binding assay using [³⁵H]-Compound 21 and site-directed mutants of the ghrelin receptor suggest that the amino acids in cavity I are more important than those in the cavity II for the interaction between Compound 21 and the receptor (Supplementary Fig. 8a–j, Supplementary Fig. 9, Supplementary Fig. 10).

**Amino acid residues in the ligand-binding pocket.** Mutations in the salt bridge, E₁₂⁴.³³⁻A and R₂⁸³⁶.⁵⁵⁻A, completely abolished ghrelin-induced receptor function, whereas replacement with the cognate amino acids mutants E₁₂⁴.³³⁻D and R₂⁸³⁶.⁵⁵⁻K retained function, although their ghrelin-induced receptor activities were significantly reduced (Fig. 4c, d). Note that the cell-surface expression of E₁₂⁴.³³⁻A mutant was retained at 111.5% of the WT level, while that of R₂⁸³⁶.⁵⁵⁻A mutant was significantly decreased (66.3% of the WT level) (Supplementary Fig. 9a, d, e). Recent NMR data and structural modeling suggest that the N-terminus of ghrelin extends down into the bottom of the ligand-binding pocket of the receptor, where it interacts with E₁₂⁴.³³⁻³². Furthermore, alanine mutation of two other polar amino acids in the ligand-binding pocket, D₉⁹⁶.⁶⁰ and R₁₀².⁶³, abolishes the receptor activity (Fig. 4a, b). Polar amino acids in the ligand-binding pocket are also important in other peptide hormone GPCRs, such as R₃₂⁷⁶.⁵⁴ in NTSR1 (PDB ID: 4GRV)³¹, R₃⁴³⁶.⁵⁵ and R₃⁵⁷⁷.²⁴ in ET₆ (PDB ID: 5GLH)²⁴, and K₂¹⁵⁵.⁴², D₂⁷⁹⁶.⁵⁸, and D₂⁹⁷⁷.³² in AT₉R (PDB ID: 5XJM)³³. These polar amino acids interact with the peptide core and side chains to fix the ligand in the binding pocket.

In addition, our crystal structure of the ghrelin receptor suggests a possible mechanism for receptor activation by acyl-modified ghrelin, and in particular the role of the acyl acid. We propose that the interaction between the acyl acid of ghrelin and the hydrophobic environment in the crevasse induces the transformation of the ghrelin receptor into an active form. Mutagenesis analyses of the phenylalanine cluster in the crevasse suggested that the cluster is important for the receptor activity. When F₂⁷⁹⁶.⁵¹, F₃⁰⁹⁷.⁵⁸, and F₃¹²⁷.⁴² were individually mutated to alanine, basal and ghrelin-induced receptor activities were significantly reduced (Fig. 4e, h, i, Supplementary Fig. 9k), although the cell-surface expression of the mutant receptors was retained at 78.9 – 112.1% of the WT level. By contrast, the basal and ghrelin-induced receptor activities of the F₂⁸⁶⁶.⁵⁸⁻A and F₂⁹⁰⁶.⁶²⁻A mutants were only slightly lower than that of the WT ghrelin receptor (Fig. 4f, g, Supplementary Fig. 9k). Moreover, when the phenylalanines were each mutated to tyrosine, the receptor activities were remained at WT levels, with the exception of F₃¹²⁷.⁴²⁻Y (Fig. 4e–i). It should be noted that both F₂⁸⁶⁶.⁵⁸ and F₂⁹⁰⁶.⁶² are located near the extracellular surface of the receptor, whereas F₂⁷⁹⁶.⁵¹, F₃⁰⁹⁷.⁵⁸, and F₃¹²⁷.⁴² are located in the bottom of the ligand-binding pocket (Fig. 2d). These observations suggest that both F₂⁸⁶⁶.⁵⁸ and F₂⁹⁰⁶.⁶² play a role in the entry of ghrelin into the receptor core, whereas F₂⁷⁹⁶.⁵¹, F₃⁰⁹⁷.⁵⁸, and F₃¹²⁷.⁴² contribute to recognition of the acyl-modification of ghrelin. A recent study combining NMR with molecular modeling supports the concept that the n-octanoyl moiety of ghrelin is essential for access to the ligand-binding pocket.⁴

Meanwhile, we found that I¹₁⁷⁸⁴.⁶⁰, L¹₈¹⁴.⁶³, and M₂¹³⁵.³⁹ in cavity II are not important for the interaction with ghrelin, as their alanine mutations caused no decrease in ghrelin-induced activity, although the basal receptor activities were suppressed (Supplementary Fig. 10a–c). In fact, we found that des-acyl ghrelin not only was unable to activate the ghrelin receptor, but also did not bind to the ghrelin receptor at all (Supplementary Fig. 11), suggesting that the acyl-modification is necessary for ghrelin to
enter the binding pocket of the ghrelin receptor. An in silico docking study of inverse agonists to a homology model of ghrelin receptor also suggested that F2796.51, F3097.39, and F3127.42 are key residues for receptor activation/inactivation. For other lipid receptors, the equivalent cavities contain multiple phenylalanine residues and accommodate hydrophobic moieties of their lipoligands. For example, the acyl tail of ML056, an antagonist of the S1P1 receptor, and both the tricyclic tetrahydrocannabinol ring and alkyl chain of AM11542, an antagonist of the CB1 receptor, are placed in the cavity (Fig. 5a, b). These facts suggest that the acyl moiety of ghrelin could be located in the cavity, where it interacts with phenylalanine residues essential for receptor activation.

Discussion

This study provides insights into the interactions between the ghrelin receptor and ghrelin, and our findings may explain why the acyl-modification of the ghrelin peptide is necessary for ghrelin receptor activation. Several ghrelin mimetics have properties of both peptide hormone GPCRs and lipid GPCRs. As described above, the binding pocket of the ghrelin receptor is bifurcated into two cavities, in which cavity I is more important for the binding of ghrelin, as determined by the results of mutagenesis analyses. In the peptide hormone GPCRs, such as NTSR1, ET3R, and AT2 receptor, the polar amino acids in the ligand binding pockets are important for recognition of the peptide cores of the endogenous ligands. In the case of the ghrelin receptor, the polar amino acids in cavity I is the core for recognition of the peptide part of ghrelin, because the alanine mutations of the polar amino acids in the cavity I abolish receptor activity (Fig. 4a–d).

On the other hand, in many lipid GPCRs, a wide gap between transmembrane bundles is proposed to serve as the access route for lipid ligands. However, in the case of the ghrelin receptor, the gap structure (crevasse) is probably not the access route for ghrelin into the ligand binding pocket, but instead seems to be the recognition site for the acyl-modification of ghrelin. This idea is supported by the results of hydrophobic amino acid mutations in the gap region, in particular mutations of the phenylalanine residues such as F2796.51, F3097.39, and F3127.42. In the S1P1 and CB1 receptor, which are known as typical lipid GPCRs, the position of the ligand-binding pockets is equivalent to the position of cavity I in the ghrelin receptor (Fig. 5a, b). Thus, in the ghrelin receptor, the hydrophobic environment of the ligand-binding pocket seems to accommodate the acyl acid moiety of ghrelin, just as lipid GPCRs accommodate hydrophobic moieties of their lipoligands.

Furthermore, the gap structure of the ghrelin receptor is composed of TM6 and TM7, whose movements are thought to...
change the GPCR conformation into its active form\(^{37}\). Thus, the interaction between the gap structure and the acyl acid moiety of ghrelin may participate in transforming the ghrelin receptor into an active conformation. Further details, obtained by structural analysis of the active form of ghrelin receptor bound to acyl-modified ghrelin, will be required to fully elucidate the mechanism of ghrelin receptor activation.

### Methods

#### Expression and purification of the ghrelin receptor

Human GHSR cDNA, which is based on the nucleotide sequence of the human GHSR gene (encoded by GHSR; UniProt accession Q92847), was cloned into a modified pFastBac vector (Invitrogen) containing an expression cassette with a hemagglutinin signal sequence and a FLAG epitope tag followed by a human rhinovirus (HRV) C3 protease recognition site at the N terminus, and an HRV C3 protease recognition site followed by an enhanced green fluorescent protein (eGFP) and an α-HEX tag at the C terminus. Twenty-eight amino acids of the N terminus (residues 1–28) were replaced with the destabilized apocyclochrome b562RIL (bRIL) from Escherichia coli (M7W, H102I, and R106L) protein\(^{38,39}\), and twenty amino acids were deleted from the C terminus (residues 347–366). Two mutations were introduced. T130K to improve thermostability and N188Q to avoid glycosylation. Recombinant baculovirus was prepared using the Bac-to-Bac baculovirus expression system (Invitrogen). Spodoptera frugiperda (Sf9) insect cells (Thermo Fisher Scientific) were infected with baculovirus at a cell density of 2–3.5 × 10\(^6\) cells ml\(^{-1}\) at multiplicity of infection of 1 in PSFM-J1 medium (Wako) supplemented with 2% (v/v) fetal calf serum, 50 units ml\(^{-1}\) penicillin, 50 µg ml\(^{-1}\) streptomycin, and 0.5 µg ml\(^{-1}\) amphotericin B. Infected cells were harvested by centrifugation 48 h post-infection, and the cell pellets were stored at −80 °C for future use.

The cell pellets were homogenized using a Dounce homogenizer in hypotonic buffer containing 10 mM HEPES-NaOH (pH 7.5), 20 mM KCl, 10 mM MgCl\(_2\), and EDTA-free protease inhibitor cocktail (Nacalai Tesque). The cell membranes were isolated by ultracentrifugation at 100,000 ×g for 30 min at 4 °C. Washing of the membranes was performed by two rounds of Dounce homogenization and centrifugation in high-osmolality buffer containing 10 mM HEPES-NaOH (pH 7.5), 1.0 M NaCl, 20 mM KCl, and 10 mM MgCl\(_2\). Purified membranes were resuspended in a buffer containing 50 mM HEPES-NaOH (pH 7.5), 500 mM NaCl, 2 mg ml\(^{-1}\) iodoacetamide (Wako), and 5 µM YL781 (Tocris), and incubated overnight at 4 °C in the dark. The membranes were then solubilized in a solubilization buffer containing 50 mM HEPES-NaOH (pH 7.5), 500 mM NaCl, 1% (w/v) n-dodecyl-β-D-maltospyranoside (DDM; Anatrace), 0.2% (w/v) cholesteryl hemisuccinate (CHS; sigma), and 0.2% (w/v) sodium cholate (Doyindo) for 2 h at 4 °C, followed by ultracentrifugation at 100,000,000 ×g for 30 min at 4 °C. Solubilized supernatant supplemented with 5 mM imidazole was mixed with 10 ml of Ni-NTA agarose (Qagen) in batch-binding mode for 2 h at 4 °C. After washing, the Ni resin was washed with 10 column volumes of Ni wash buffer containing 30 mM HEPES-NaOH (pH 7.5), 750 mM NaCl, 0.1% DD, 0.02% CHS, 0.02% sodium cholate, and 5 µM YL781. The protein was eluted with three column volumes of Ni wash buffer containing 500 mM imidazole. Elution from the Ni resin was supplemented with 2 mM calcium and loaded onto M1 anti-FLAG affinity resin (Sigma). Detergent was gradually exchanged on the M1 resin from DDM to 0.01%(v/v) lauryl maltose neopentyl glycol (LMNG, Anatrace), and YIL781 was replaced with 5 µM Compound 21, a ghrelin receptor antagonist. The receptor was eluted with 3 column volumes of FLAG elute buffer containing 20 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 0.01% LMNG, 0.001% CHS, 200 µg ml\(^{-1}\) Flag peptide (Peptide Institute Inc.), 5 mM EDTA, and 5 µM Compound 21. The N-terminal FLAG tag and C-terminal eGFP-8xHis tag were cleaved by 3 C protease overnight at 4 °C. The purified receptor was concentrated with a 50-kDa molecular weight cut-off Amicon Ultra concentrator (Millipore) and loaded onto Superdex 200 Increase 10/300 GL (GE Healthcare) in buffer containing 20 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 0.01% LMNG, and 0.001% CHS. The peak fractions were collected and flash-frozen with liquid nitrogen and stored at −80 °C until use.

#### Protein thermostability assay

The thermal stability analysis was performed with the thiol-specific fluorochrome N-[4-(7-diethylamino-4-methyl-3-coumarinyl) phenyl]maleimide (CPM)\(^{40}\). CPM at 4 mg ml\(^{-1}\) in DMSO was diluted 40-fold into buffer containing 20 mM HEPES-NaOH (pH 7.5), 100 mM NaCl, 0.01% LMNG, and 0.001% CHS. Purified protein (20 µg) was mixed with diluted CPM and incubated on ice for 15 min in darkness. The reaction mixture (20 µl) was heated in a controlled manner with a ramp rate of 3 °C min\(^{-1}\) in a MyiQ2 thermal cycler (Bio-Rad). The excitation wavelength was set at 387 nm, and the emission wavelength at 463 nm. Assays were performed over a temperature range starting from 4 °C and ending at 80 °C. The peak in the derivative of the fluorescence signal was used to determine the melting temperature.

#### Antibody generation

All animal experiments described in this study conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals of Japan and were approved by Kyoto University Animal Care Committee (approval No. Med Kyō 16043). For use as antigen, purified human ghrelin receptor [29–34] (T130K/N188Q) was reconstituted into liposomes containing chicken egg yolk phosphatidylcholine (eggPC, Avanti) and monophosphoryl lipid A (Sigma-Aldrich). Briefly, Nrl/lpr mice were immunized three times at 10–14-day intervals with 100 µg purified ghrelin receptor mutant. The immunized mice were sacrificed, and single-cell suspensions were prepared from their spleens. These cells were fused with NS-1 myeloma cells using polyethylene glycol (PEG) according to conventional methods. To screen for antibodies that specifically recognize native receptors, we developed an ELISA method using proteoliposomes\(^{41}\). For liposome ELISA, we used purified ghrelin receptor mutant reconstituted into liposomes containing biotinyl phosphatidylethanolamine (Avanti) to maintain the protein in its native conformation and effectively immobilize liposomes onto Streptavidin-coated plates (Nunc). To eliminate antibodies recognizing the flexible loops, the N and C termini, or unstructured regions of the ghrelin receptor, we performed ELISA using ghrelin receptor denatured with 1% (v/v) SDS. The selected cells were isolated by limiting dilution to establish monoclonal hybridoma cell lines producing antibodies against the ghrelin receptor. The supernatant of the large-scale cell culture was loaded onto HiTrap Protein G HP (GE Health care) to purify mouse IgG. Fab fragments were obtained by proteolytic cleavage of IgG with papain (Worthington) and purified by Superdex200 10/60 (GE Healthcare). Purified Fab monomer (Fab 7881) was concentrated to 10 mg ml\(^{-1}\) using a 10-kDa molecular weight cut-off Amicon Ultra...
concentrator. Sequences of Fab fragments were determined according to the standard 5′-RACE method using total RNA isolated from hybridoma cells. The Fab 7881 obtained in this manner was used for crystallization of the ghrelin receptor–Fab complex.

Crystallization of the ghrelin receptor–Fab complex. Purified ghrelin receptor and the Fab 7881 were mixed in a 1:1.5 molar ratio and incubated for 1 h at 4 °C. The complex was purified by gel filtration using Superdex 200 Increase 10/300 GL. Concentration of the bound antibody to the peak fraction of the gel filtration was 2.8 mg/mL, and the complex was concentrated to 30 mg/mL using an Ultra concentrator. Sequences of Fab fragments were determined according to the standard 5′-RACE method using total RNA isolated from hybridoma cells. The Fab 7881 obtained in this manner was used for crystallization of the ghrelin receptor–Fab complex.

Cell-surface expression levels of ghrelin receptors. HEK293 cells (ATCC) were seeded in 6-well plates at a cell density of 8 × 105 cells/well and cultured for 24 h. The cells were transfected with complex of vector DNA (the FLAG-tagged N188Q ghrelin receptor (control) or the indicated FLAG-tagged ghrelin receptor mutants) and FuGENE HD transfection reagent (Promega). The next day, transfected HEK293 cells were harvested in PBS containing 1 mM EDTA, and then incubated for 30 min on ice with 2.5 μg/mL anti-FLAG antibody (Wako) and 5 μg/mL Alexa Fluor 488–conjugated anti-mouse IgG polyclonal antibody (Thermo Fisher Scientific) in FACS buffers (PBS, 2% FBS, 0.05% NaN3). Cell-surface expression levels were evaluated by flow cytometry on a Guava EasyCyte Plus flow cytometer. FLAG-positive cells were defined as cell populations with signals greater than the top 5% of MOCK cells.

Luciferase assay. To detect constitutive activity of ghrelin receptor, CHO cells were seeded in 6-cm dishes at a density of 2.5 × 105 cells/dish and cotransfected with pcDNA3.1/myc-His6C/Flag/SP7 (0.5 μg, Promega) and the indicated ghrelin receptor plasmid (5 μg) and the indicated FLAG-tagged ghrelin receptor mutants (1 μg) with the reporter plasmid (0.5 μg). Reporter assays were performed using the FuGENE HD transfection reagent (Promega). The next day, the transfected cells were collected and plated into 96-well white microplates (Corning) at a density of 2.5 × 104 cells/well. After a 1-day incubation, the luciferase activities were determined using dual-luciferase Reporter Assay System (Promega). When assessing Compound 21 activity, the transfected cells were incubated with various concentrations of Compound 21 for 1 h just before luciferase activity measurement. All firefly luciferase activities were normalized against Renilla luciferase activity. All experiments were performed in sextuplicate.

Data availability. Data are presented as means ± s.e.m. The outliers on the luciferase activity were detected and removed based on the Smirnov–Grubbs test. The GraphPad Prism 8 software (GraphPad Software Inc.) was used for statistical analysis. Differences between groups were analyzed using the two-tailed Student t test. P < 0.05 was considered statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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