Dear Editor,

The ten mammalian Frizzleds (FZD1–10) belong to the class F of G protein-coupled receptors (GPCRs) and mediate WNT signaling through interaction with transducer proteins including Dishevelled (DVL) or heterotrimeric G proteins.1 Their involvement in human disease has put FZDs at the forefront of drug targets, especially anti-cancer therapy.2 However, no drugs have been developed for efficient pharmacological modulation of FZDs, partially owing to the limited understanding of FZD structure and activation mechanisms.1,3 Among class F, FZD7 is intensively pursued due to its relevance in various tumor models, particularly in intestinal cancers.4 Detailed structures of the receptor complexes would allow for structure-guided discovery of new drug candidates. FZD1–10 share structural similarity with the related class F member Smoothered (SMO), which mediates Hedgehog signaling and is a validated target for cancer therapy.2 In an effort to understand the structural basis of FZD activation and transducer interaction, we solved the structure of human FZD7 in complex with heterotrimeric mini Gs (mGs).5

Based on the evidence that FZD7 interacts with Gs to mediate muscle hypertrophy,4,6 we assessed its ability to activate heterotrimeric Gs independently of WNT stimulation. Co-expression of FZD7 with a bioluminescence resonance energy transfer (BRET)-based Gs biosensor,8 reporting the rearrangement or dissociation of Ga and Gβγ following receptor engagement and G protein activation, revealed that FZD7 exhibits constitutive activity similar to the class A β2-adrenoceptor (Fig. 1a; Supplementary information, Fig. S1a, b). Using an analogous assay that measures activity-dependent Ga translocation (Supplementary information, Fig. S1c), we found that the constitutive activity of FZD7 correlates with increased receptor expression (Supplementary information, Fig. S1d, e). Given the robust constitutive activity of FZD7 towards Gaβγ, we reconstituted purified, full-length human FZD7, heterotrimeric mGs, and Nanobody35 (Nb35), which stabilizes the nucleotide-free Gaα and Gβ subunits,9 in the absence of ligand and obtained pure complexes following size exclusion chromatography (Supplementary information, Fig. S2). The final complex was composed of FZD7, mGsα, Gβγ, Ny and Nb35, which could be clearly identified by 2D classification (Fig. 1b; Supplementary information, Fig. S2d). We used single-particle cryo-EM analysis to determine the 3D structure of this complex. After several rounds of classification and auto-refinement, the resolution of the final structure reached 3.2 Å allowing us to build an atomic model based on the density map (Fig. 1c; Supplementary information, Figs. S3–S5, Table S1).

In accordance with the functional evidence for constitutive activity, the FZD7–mGs complex structure provides the structural basis for ligand-independent G protein coupling (Fig. 1c). The interface between FZD7 and mGs is dominated by the distal C-terminal segment of the α5-helix in mGsα (Fig. 1d, e). The C-terminal leucine residues (L393H5.25, L394H5.26; superscripts refer to the residue position in the common Ga numbering scheme for G proteins/GPCRdb) are inserted into the helical bundle of the receptor. L393H5.25 and L394H5.26 establish extensive interactions with FZD7 residues yielding a locally converged network that stabilizes the complex (Fig. 1e). The terminal carboxyl group of L394H5.26 in mGsα forms an ionic bond with K4666.28, and residues R281ICL1, K552 8.49 and R470β5.32 of FZD7 are located in close proximity (superscript numbers refer to the Ballesteros and Weinstein numbering system). Y391H5.23 forms a hydrogen bond with the backbone of W369ICL2. Residues I4505.72, I4535.75 and M4545.76 of FZD7 form a hydrophobic cleft accommodating L388H5.20. Furthermore, R385H5.17 forms an ionic bond with D457 in ICL3, further strengthening the interaction between the α5-helix and FZD7. In summary, the recognition of Gaα by FZD7 is primarily governed by a network of hydrogen bonding and electrostatic interactions contributed from the C-terminal segment of the α5-helix (D383H5.13, L394H5.26), among which, interactions with L394H5.26 lock the α5-helix tail in an uncoiled, elongated conformation (Fig. 1e).

The placement of the α5-helix of mGsα in the core of FZD7 stabilizes an open FZD7 conformation. We compared the FZD7–mGs structure with the available inactive-state FZD4 crystal structure (PDB: 6BD4) and the inactive-state FZD4 cryo-EM structure (PDB: 6WW2) and observed a clear outward bending of TM6 and an inward shift of TM5 at the cytoplasmic side (Fig. 1f–h)—a conformational change characteristic of active-state class A and B GPCRs. This helical rearrangement is achieved through interaction of TM6 and TM5 with mGsα and opening of the molecular switch between TM6 and TM7 (R5.32/W7.55; Fig. 1i).7 Comparing inactive FZD4 with FZD7–mGs reveals that the extracellular portion of TM6 of FZD7 extends above the surface of the lipid bilayer at an angle of 45° (Supplementary information, Figs. S6, S7), similar to what we have predicted in previous models10 and in contrast to the almost 90° bending in the FZD4 structure.11 Moreover, conserved cysteines within the hinge domain form disulfide bonds to both stabilize its structure and to link it with ECL1 (C210–C230; C234–C315ECL1) (Supplementary information, Fig. S6).12

To better understand the activation mechanism of FZD7 and G protein coupling to class F receptors, we compared the FZD7–mGs structure with the agonist (24(S), 25-epoxycholesterol)-bound structure of SMO–Gα13 (PDB: 6O70). The helical arrangement at the upper portion of the FZD7 transmembrane core is more compact, presumably due to the absence of ligand (Supplementary information, Fig. S7). At the lower portion of TM6, substantially distinct conformations are observed between the SMO–Gα and FZD7–mGs structures. Most strikingly, TM6 in SMO–Gα undergoes a parallel outward movement compared to inactive SMO, whereas TM6 in the FZD7–mGs complex accomplishes a
The ionic interactions between TM6, ICL3 and the α5-helix of mGαs (K4666.28–L394H5.26 and D457–R385H5.17) are likely to be the main contributors in maintaining this kink. In addition, Y4786.40 forms π–π interaction with W3543.43 to further maintain the bent TM6 conformation (Supplementary information, Fig. S7).

While the most evident structural rearrangements relate to TM6, additional positional shifts of TM2, TM3, TM4 and TM5 in the FZD7–mGα complex are observed when compared to the SMO–Gαi5

** Similar displacement of the cytoplasmic portion through a kink in the helix (Fig. 1h). The ionic interactions between TM6, ICL3 and the α5-helix of mGαs (K4666.28–L394H5.26 and D457–R385H5.17) are likely to be the main contributors in maintaining this kink. In addition, Y4786.40 forms π–π interaction with W3543.43 to further maintain the bent TM6 conformation (Supplementary information, Fig. S7).
Fig. 1 Structure of constitutively active FZD7 in complex with heterotrimeric mGα. a Normalized BRET values of ΔFZD7ΔCRD–HEK293 cells transiently co-transfected with the Gαi BRET sensor along with either negative control (mock), the β1-adrenergic receptor (β1AR) or FZD7. Data are represented as the means ± SEM of raw BRETs that were obtained from simple linear regression of five independent experiments measured in quadruplicates shown in Supplementary information, Fig. S1A and normalized to the negative control. *P < 0.05; **P < 0.01 (one-way ANOVA followed by Sidak’s multiple comparison). b An annotated 2D class average of FZD7–mGα–Nb35 complex. c Overall density map and atomic model of overall FZD7–mGα–Nb35 complex (complex omitted due to linker flexibility). FZD7, blue; mGα, orange; Nb35, green; Gαi, yellow; Nb35, gray. d Insertion of the α5-helix (mGα, orange) into FZD7 helical bundle represented as surface (ICL1, blue; ICL2, pink; ICL3, yellow; TM7/H8, green). e Schematics of interactions between FZD7 and α5-helix. Hydrogen bonds are shown as red dashed lines. The red circle represents the hydrophobic interaction network. Yellow shades indicate residues that reside in TM5/6/ICL3; pink, TM3/4/ICL2; blue, TM1/2/ICL1; green, TM7/H8. f Superposition of FZD7 (blue) and FZD4 (yellow) structures, viewed from the intracellular side (bottom view). g Superposition of the active FZD7 structure (blue) with the inactive FZD5 (PDB: 6DBD, yellow), inactive FZD3 (PDB: 6WW2, light pink), active SMO (PDB: 6O7O, gray) and inactive SMO (PDB: 5VS7, green) structures. h Comparison of the cytoplasmic portion of TM6 (from ΔFZD5 to 6O7O in FZD5; ΔFZD7 in FZD4, active SMO and inactive SMO structures. i R6.32–W5.35 and W6.36–E6.32 in FZD7. Gray dashed lines indicate the distance of R6.32–W5.35 and W6.36–E6.32 in active SMO structure. j Normalized BRET values of ΔFZD7ΔCRD–HEK293 cells transiently co-transfected with rGFP-CAAX and Gαi7, Δ-Rflu, along with either negative control (mock), wild-type FZD7, ΔCRD–FZD7 or the indicated FZD7 mutants. Data are represented as the means ± SEM of raw BRETs that were obtained from simple linear regression of four independent experiments measured in quadruplicates shown in Supplementary information, Fig. S11b and normalized to the negative control. *P < 0.01; **P < 0.001 (one-way ANOVA followed by Tukey’s multiple comparison). k Normalized FRET values of ΔFZD7ΔCRD–HEK293 cells transiently co-transfected with the FRET-based cAMP biosensor along with either negative control (mock), wild-type FZD7, ΔCRD–FZD7 or the indicated FZD7 mutants. Data are represented as the means ± SEM of raw FRETs that were obtained from simple linear regression of five independent experiments measured in quadruplicates shown in Supplementary information, Fig. S11c and normalized to the negative control. **P < 0.01; ****P < 0.0001 (one-way ANOVA followed by Sidak’s multiple comparison). complex. These four helices constitute a more compact bundle in the FZD7–mGα structure, partially stabilized by a network of interactions (Supplementary information, Fig. S7e). In a cooperative manner, these interactions promote the cytoplasmic portion of TM4 shifting inward by ~2 Å (comparing the Ca of L3834.47 in FZD7–mGα with corresponding L3624.47 in SMO–Gα complex structures) (Supplementary information, Fig. S7f, black arrow).

A conserved molecular switch between TM6 and TM7 was previously identified for all class F GPCRs, maintaining the receptor in an inactive conformation (observed as a hydrogen-bonding distance between R4706.32 and the backbone of W5477.55 in all inactive class F receptor structures). The polar interactions between R4706.32 and W5477.55. The overall hallmark of FZD7 activation due to its small size while minimizing the effect on receptor dynamics. These MD simulations allowed us to monitor general receptor integrity and the status of the molecular switch by assessing the angle of the kinked TM6 and the distance between R4706.32 and W5477.55. The overall hallmark of FZD7 activation—its role in maintaining the constitutive activity of FZD7 towards Gαi, we employed a mutagenesis-based approach in combination with assessment of Gαi translocation and cAMP production as functional readouts of Gαi-dependent signaling. We focused on D457 (in ICL3) and K4662.39, which interact with the α5-helix of mGα. Mutating either D457 or K4662.39 to alanine alone did not affect the constitutive activity of FZD7 on Gαi translocation or cAMP production (Fig. 1j, k). Supplementary information, Figs. S11, S12). However, the double mutant D457A/K4662.39A abrogated FZD7 constitutive activity towards Gαi, suggesting that these mutations collectively interfere with G protein coupling. In contrast, the double mutant did not affect the ability of FZD7 to mediate WNT-induced activation of the WNT/β-catenin pathway as assessed by the TOPFlash reporter assay (Supplementary information, Fig. S13), underlining the concept of conformational selection for DVL-dependent signaling over G protein coupling as has been suggested previously.7,15 Although the CRD could not be resolved in the present structure, we observed that removal of the CRD (ΔCRD-FZD7) resulted in the inability to reconstitute the receptor–mGα complex in vitro to the same extent as that of full-length FZD7 (Supplementary information, Fig. S14). Thus, we surmised that the CRD is required for FZD–mGα complex stability and that removal of the CRD could decrease constitutive activity. Therefore, we assessed the ability of the ΔCRD-FZD7 construct to functionally couple to Gαi by assessing Gαi translocation and cAMP production (Fig. 1j, k). Removal of the CRD blunted the constitutive activity towards Gαi signaling as evidenced by the lack of Gαi translocation and cAMP production. These data underline the requirement for the CRD to maintain constitutive activity of FZD7 towards heterotrimERIC G proteins through intramolecular allosteric.

In conclusion, we report the cryo-EM structure of FZD7–mGα, demonstrating how constitutive activity feeds into downstream signaling via heterotrimERIC G proteins. With respect to the overall diversity among GPCRs, FZD7 has evolved a unique way to maintain certain homologous movements consistent with class A and B GPCR activation, while adapting its class-specific architecture to mediate G protein activation. While the classical hallmarks of G protein engagement are present in our structure, several differences can be found at the interface between the receptor and the G protein suggesting that FZD7s harbor their own
selectivity determinants for heterotrimeric G proteins. In short, the present structure of constitutively active FZD7-mGn, alongside previously published inactive structures of FZD4 and FZD5, opens the door to more accurate modeling of other FZDs and a platform for in silico drug discovery, which will aid in the discovery of new treatments to help those afflicted with diseases of WNT-FZD signaling.

Lu Xu1,2,3,4, Bo Chen1,9, Hannes Schihada5,9, Shane C. Wright5,6,9, Ainoleena Turku5,9, Yiran Wu6, Gye-Won Han7, Maria Kowalski-Jahn1, Pawel Kozieliewicz1, Carl-Fredrik Bowin2, Xianjun Zhang1,2,3,4,7, Chao Li1,2,3,4,7 and Fei Xu5,1,2,3,4,5

1Human Pharmaco R&D, ShanghaiTech University, Shanghai, China. 2School of Life Science and Technology, ShanghaiTech University, Shanghai, China. 3Center for Excellence in Molecular Cell Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. 4University of Chinese Academy of Sciences, Beijing, China. 5Section of Receptor Biology & Signaling, Department of Physiology & Pharmacology, Karolinska Institutet, Stockholm, Sweden. 6Institute for Research in Immunology and Cancer, Department of Biochemistry & Molecular Medicine, Université de Montréal, Montréal, QC, Canada. 7Departments of Biological Sciences and Chemistry, Bridge Institute, University of Southern California, Los Angeles, CA, USA. 8Present address: Orion Pharma R&D, Espoo, Finland. 9These authors contributed equally: Lu Xu, Bo Chen, Hannes Schihada, Shane C. Wright

Email: gunnar.schulte@ki.se; xufei@shanghaitech.edu.cn

DATA AVAILABILITY
The cryo-EM 3D map of the FZD7-mGn-Nb35 complex has been deposited in EMD database with accession code EMD-31340; the coordinates have been deposited in PDB database with accession code 7EVW. The MD simulation data is available at www.gpcrmd.org with ID 245.

REFERENCES
1. Schulte, G. & Wright, SC. Trends Pharmacol. Sci. 39, 828–42 (2018).
2. Taipale, J. & Beachy, PA. Nature 411, 349–54 (2001).
3. Kozieliewicz, P., Turku, A. & Schulte, G. Mol. Pharmacol. 97, 62–71 (2020).
4. Phesse, T., Flanagan, D. & Vincan, E. Cancers 8, https://doi.org/10.3390/cancers8050050 (2016).
5. Nehmé, R., Carpenter, B., Singhal, A., Strege, A., Edwards, PC & White, CF. PLoS ONE 12, e0175642 (2017).
6. von Maltzahn, J., Bentzinger, CF. & Rudnicki, MA. Nat. Cell. Biol. 14, 86–91 (2012).
7. Wright, SC, Kozieliewicz, P., Kowalski-Jahn, M., Petersen, J., Bowin, CF & Slodkowicz, G. et al. Nat. Commun. 10, 667 (2019).
8. Schihada, H., Shekhanli, R. & Schulte, G. bioRxiv https://doi.org/10.1011/2021.02.05.429900 (2021).
9. Rasmussen, SG. et al. Nature 477, 549–55 (2011).
10. Kozieliewicz, P. et al. Nat. Commun. 11, 414 (2020).
11. Yang, S. et al. Nature 560, 666–70 (2018).
12. Valshoova, J., Kowalski-Jahn, M., Sunahara, RK. & Schulte, G. J. Biol. Chem. 293, 17875–87 (2018).
13. Qi, X. et al. Nature 571, 279–83 (2019).
14. Turku, A., Schihada, H., Kozieliewicz, P., Bowin, CF. & Schulte, G. Nat. Commun. https://doi.org/10.1038/s41467-021-24004-z (2021).
15. Bowin, CF., Inoue, A. & Schulte, G. J. Biol. Chem. 294, 11677–84 (2019).

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AUTHOR CONTRIBUTIONS
L.X. performed cloning, protein purification, cryo-EM sample preparation, data collection and structure analysis; B.C. performed cryo-EM data processing, model building and refinement; Y.W. assisted with the structure analysis and some calculations; G.W.H. was responsible for structure quality control; X.Z. and C.L. characterized the protein expression at early phase of the project; H.S. and S.C.W. performed functional biosensor experiments; M.K.J. and P.K. performed FZD7 construct mutagenesis for functional analysis; C.F.B. validated FZD7 surface expression; A.T. performed the MD simulation and analysis and contributed to model interpretation and visualization. F.X. conceived the project. F.X. and G.S. designed, coordinated and supervised the experiments. L.X., B.C., S.C.W., H.S., M.B., G.S. and F.X. wrote the manuscript.

COMPETING INTERESTS
M.B. is the president of the scientific advisory board for Domain Therapeutics. M.B. has filed patent applications related to some of the biosensors used in this work and the technology has been licensed to Domain Therapeutics.

ADDITIONAL INFORMATION
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Correspondence and requests for materials should be addressed to G.S. or F.X.

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