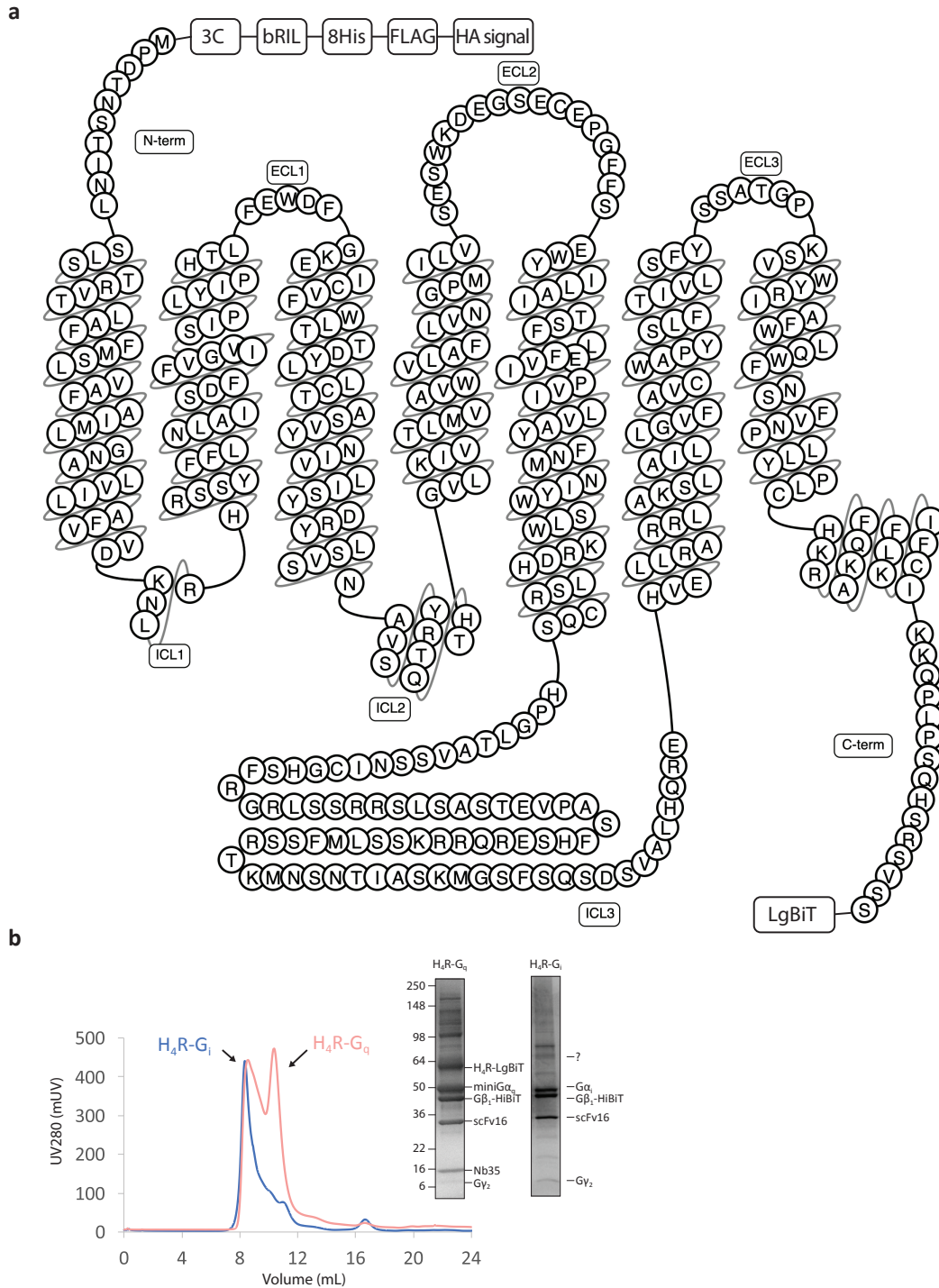


Supplementary Information

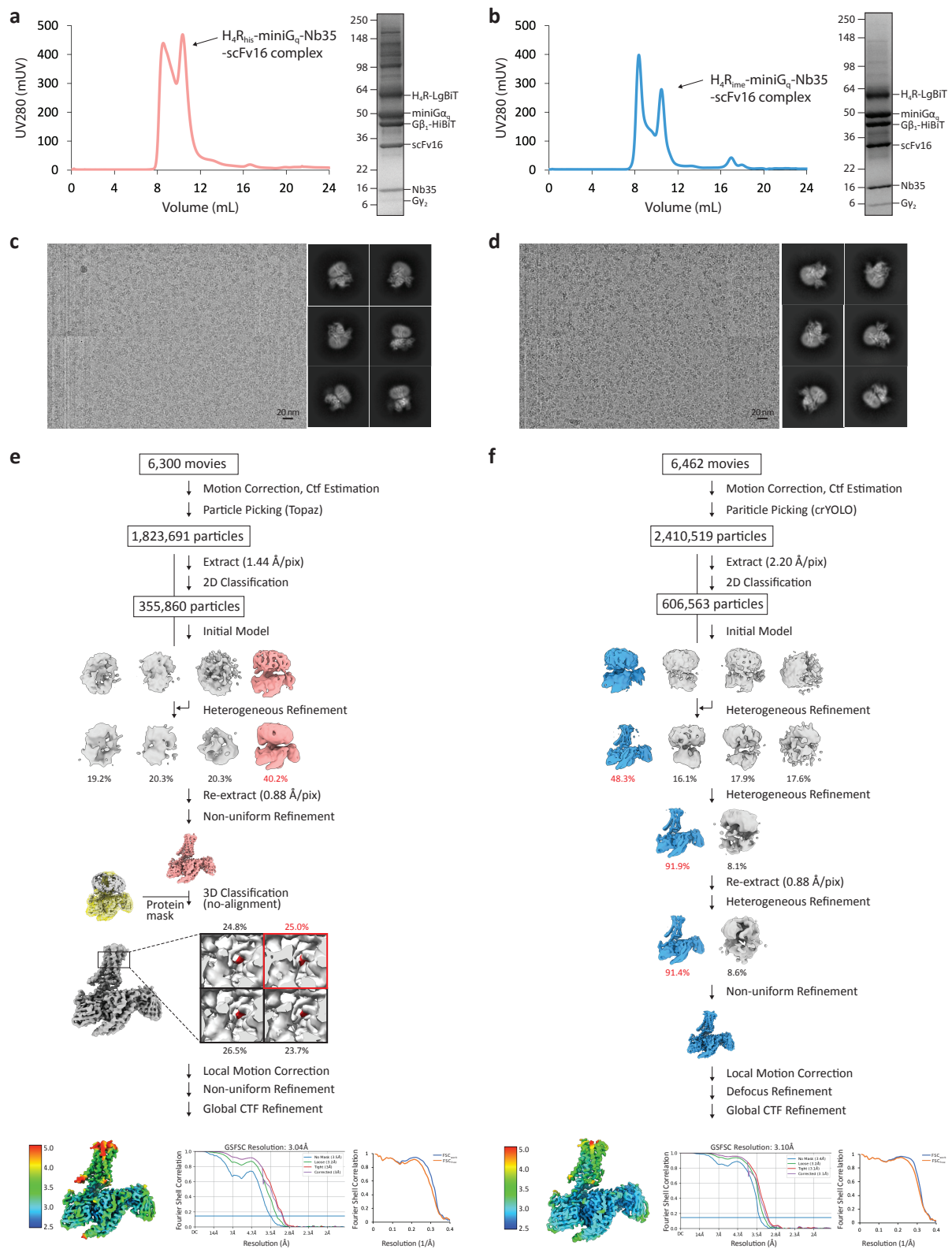
**Structural Insights into the Agonists Binding and Receptor Selectivity of Human Histamine  
H<sub>4</sub> Receptor**

D. Im *et al.*



### Supplementary Figure 1 | Sample preparation of $H_4R-G_q$ complex.

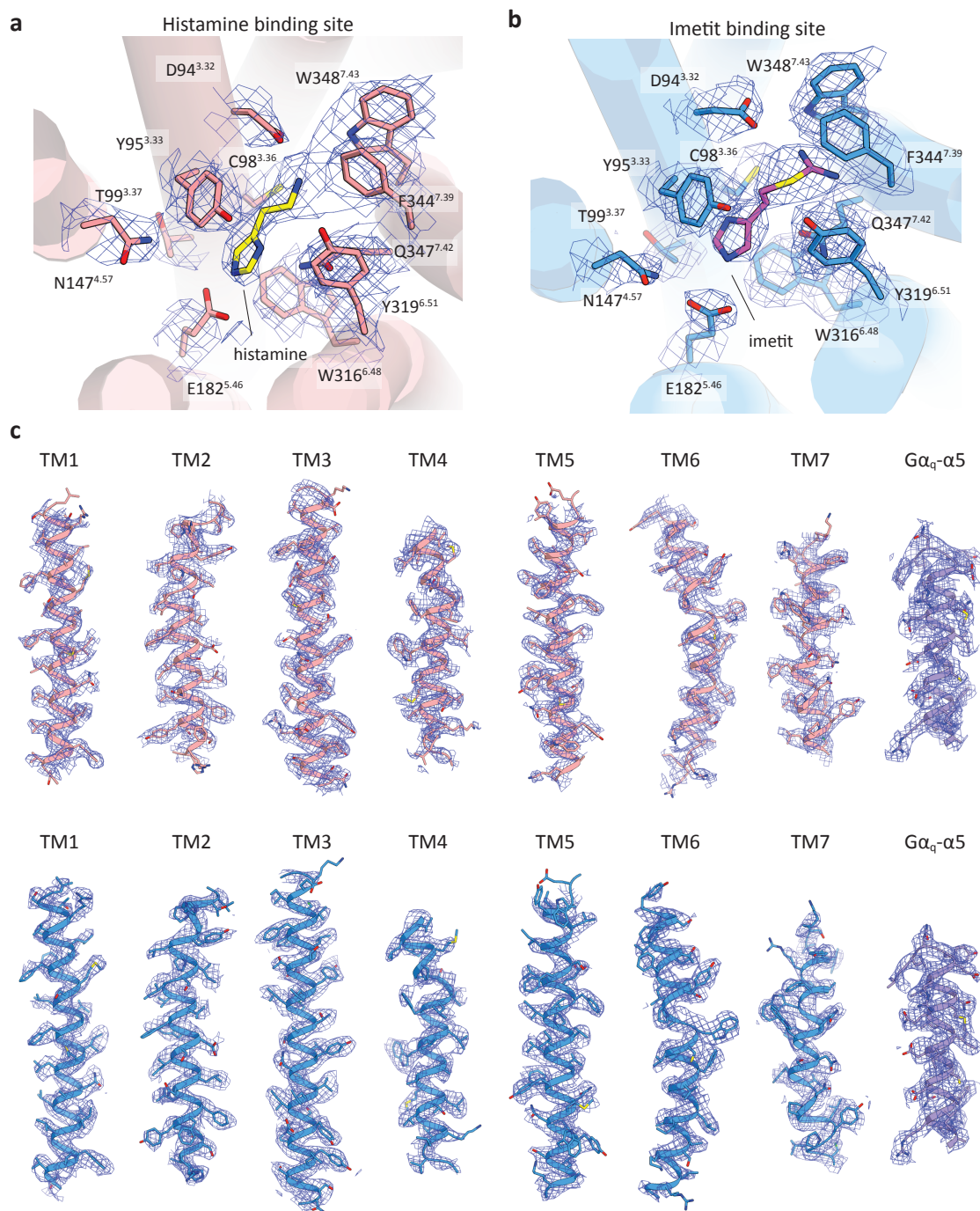
**a**, Snake-shape diagram of the  $H_4R-LgBiT$  construct used in complex assembling. The snake-diagram of  $H_4R$  was adopted and modified from the GPCRdb (<https://www.gpcrdb.org>). **b**, Size exclusion chromatography profiles of  $H_4R/G_q$  and  $H_4R/G_i$  complexes.



**Supplementary Figure 2 | Single-particle cryo-EM analysis procedure.**

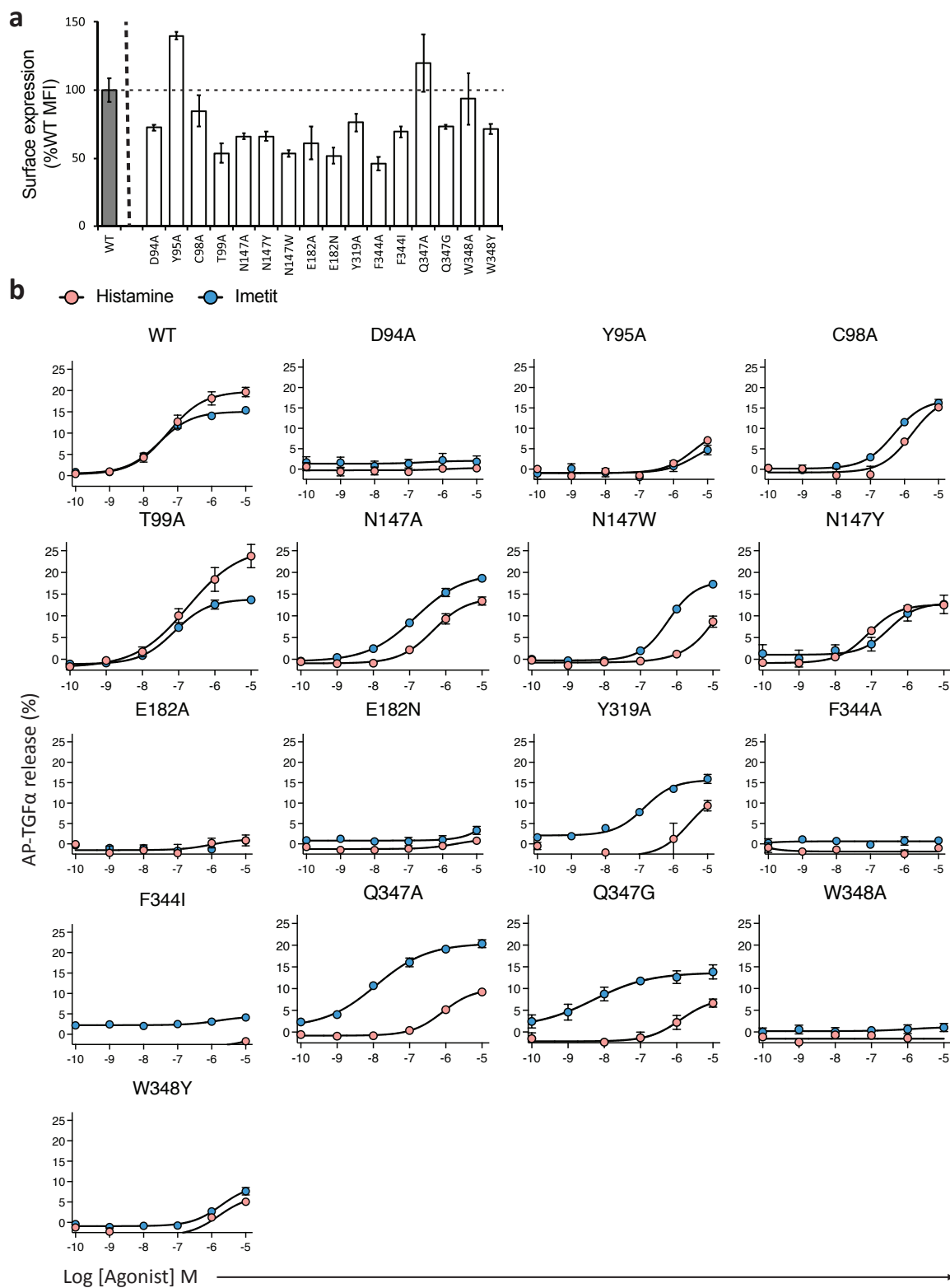
**a, b**, Size exclusion chromatography profiles and SDS-PAGE analysis of the histamine-bound H<sub>4</sub>R-G<sub>q</sub> (**a**) and imetit-bound H<sub>4</sub>R-G<sub>q</sub> complexes (**b**). **c, d**, Representative cryo-EM micrographs and two-dimensional classification averages of the histamine-bound H<sub>4</sub>R-G<sub>q</sub> (**c**) and imetit-bound H<sub>4</sub>R-G<sub>q</sub> complexes (**d**). **e, f**, Cryo-EM data processing workflows of the histamine-bound H<sub>4</sub>R-G<sub>q</sub> (**e**) and imetit-bound H<sub>4</sub>R-G<sub>q</sub> complexes (**f**).





### Supplementary Figure 3 | Cryo-EM density map.

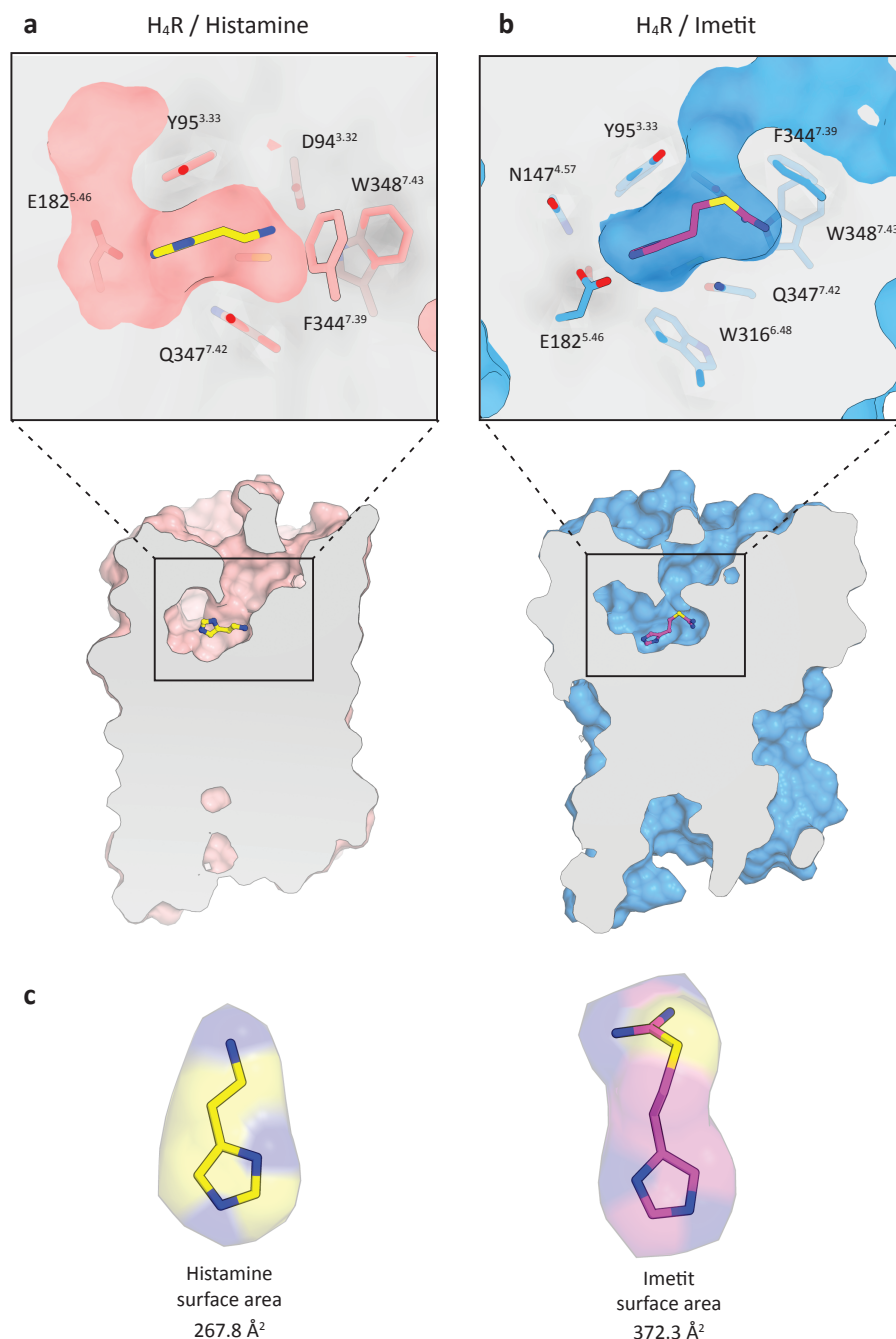
**a, b,** The agonist-binding site of the histamine-bound H<sub>4</sub>R-G<sub>q</sub> (**a**) and imetit-bound H<sub>4</sub>R-G<sub>q</sub> complexes (**b**). **c,** The transmembrane helices 1–7, helix 8, and the α5 helix of the G<sub>q</sub> of the histamine-bound H<sub>4</sub>R-G<sub>q</sub> (top panel) and imetit-bound H<sub>4</sub>R-G<sub>q</sub> complexes (lower panel). The density map was drawn by CueMol at contour level 3.0.



**Supplementary Figure 4 | Agonist activities against the WT and mutant H<sub>4</sub>R.**

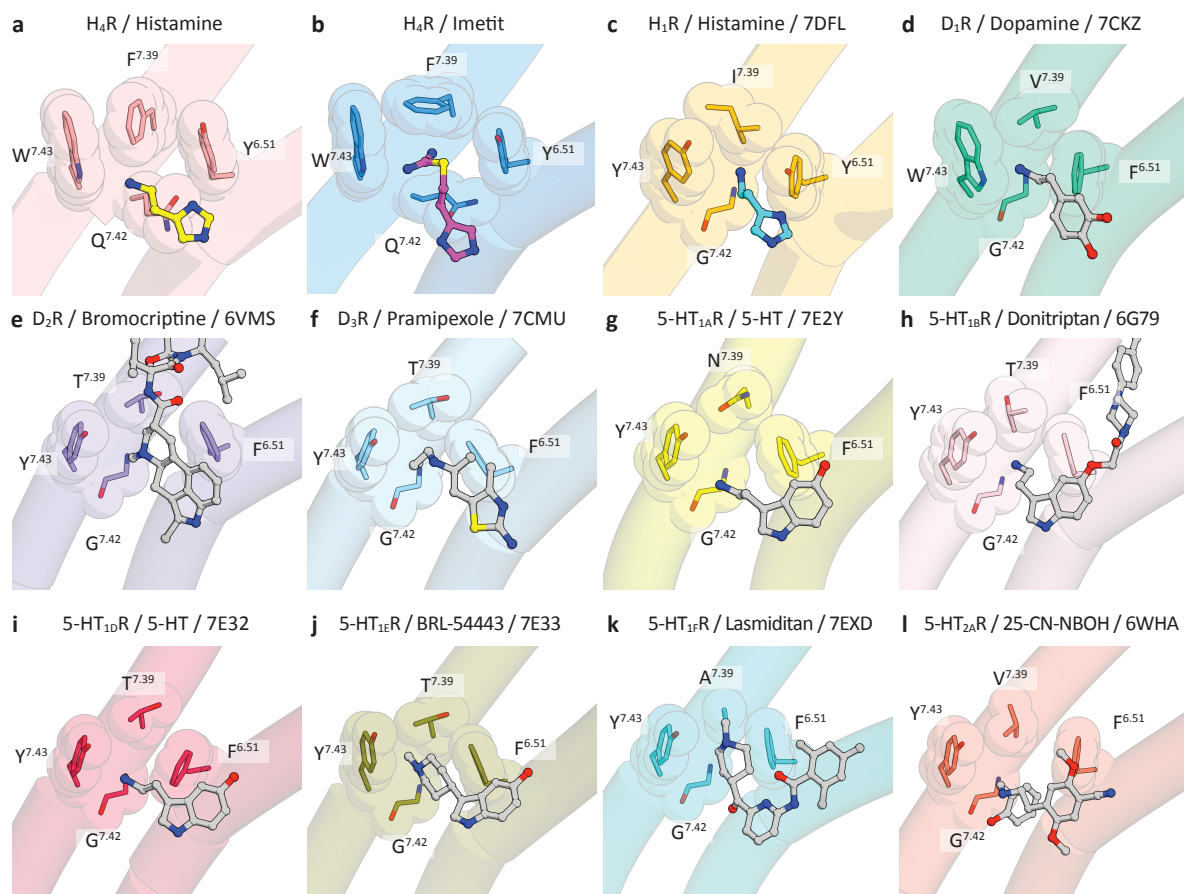
**a**, Surface expression of the H<sub>4</sub>R mutants analyzed by flow cytometry. **b**, Graphical representation

of the TGF $\alpha$  shedding assay data for the identified H<sub>4</sub>R WT and mutants. X-axis values are displayed as the logarithmic (Log<sub>10</sub>) concentration of the respective agonist. Y-axis values are displayed as the percentage of AP-TGF $\alpha$  release. Dose response curves were further analyzed using the “log(agonist) vs. response-variable slope (three or four parameters)” function in GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA) (f). All data are presented as mean  $\pm$  standard error of the mean of three independent experiments.



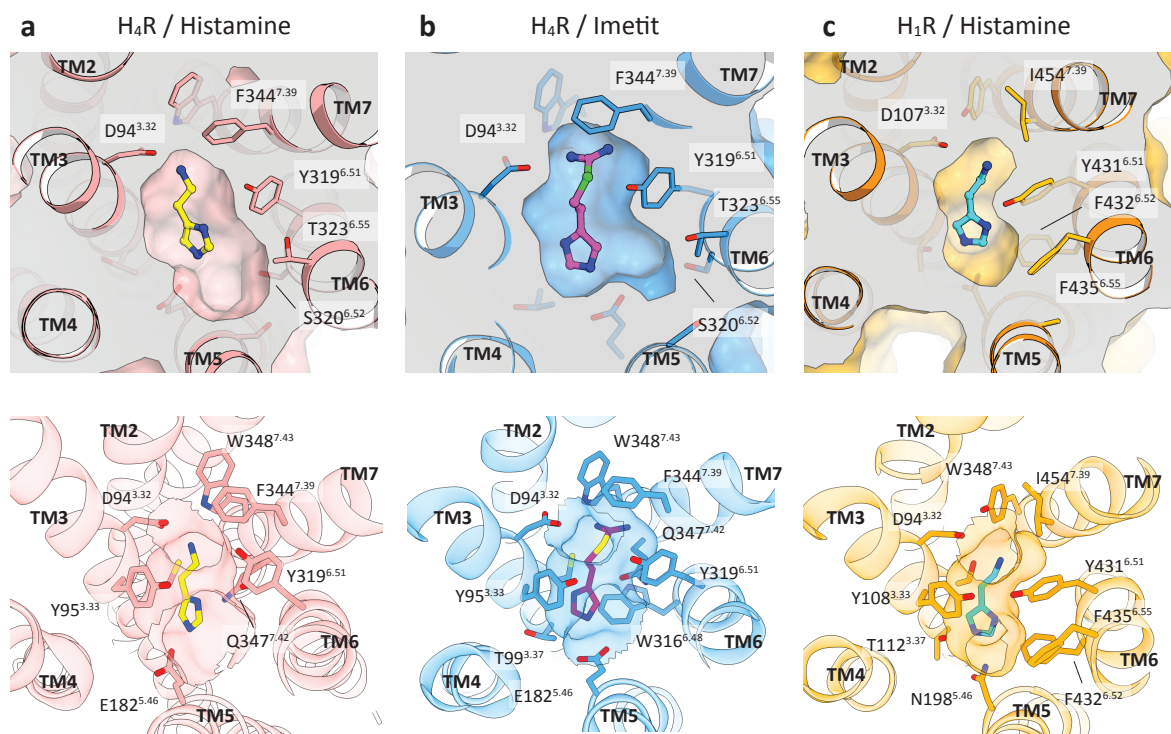
### Supplementary Figure 5 | The agonist-binding pocket of H<sub>4</sub>R<sub>his</sub> and H<sub>4</sub>R<sub>ime</sub>.

**a, b**, Vertical cross-section of H<sub>4</sub>R<sub>his</sub> (**a**, pink) and H<sub>4</sub>R<sub>ime</sub> (**b**, blue). The top panels show a close-up view of the ligand-binding pocket. Structures are shown as surfaces. Ligands (histamine: yellow, imetit: magenta) and residues are shown as stick models. **c**, Comparison of the surface area of histamine (left panel) and imetit (right panel) in H<sub>4</sub>R<sub>his</sub> and H<sub>4</sub>R<sub>ime</sub>.



### Supplementary Figure 6 | Comparison of the aromatic slot residues on the agonist-bound aminergic receptors.

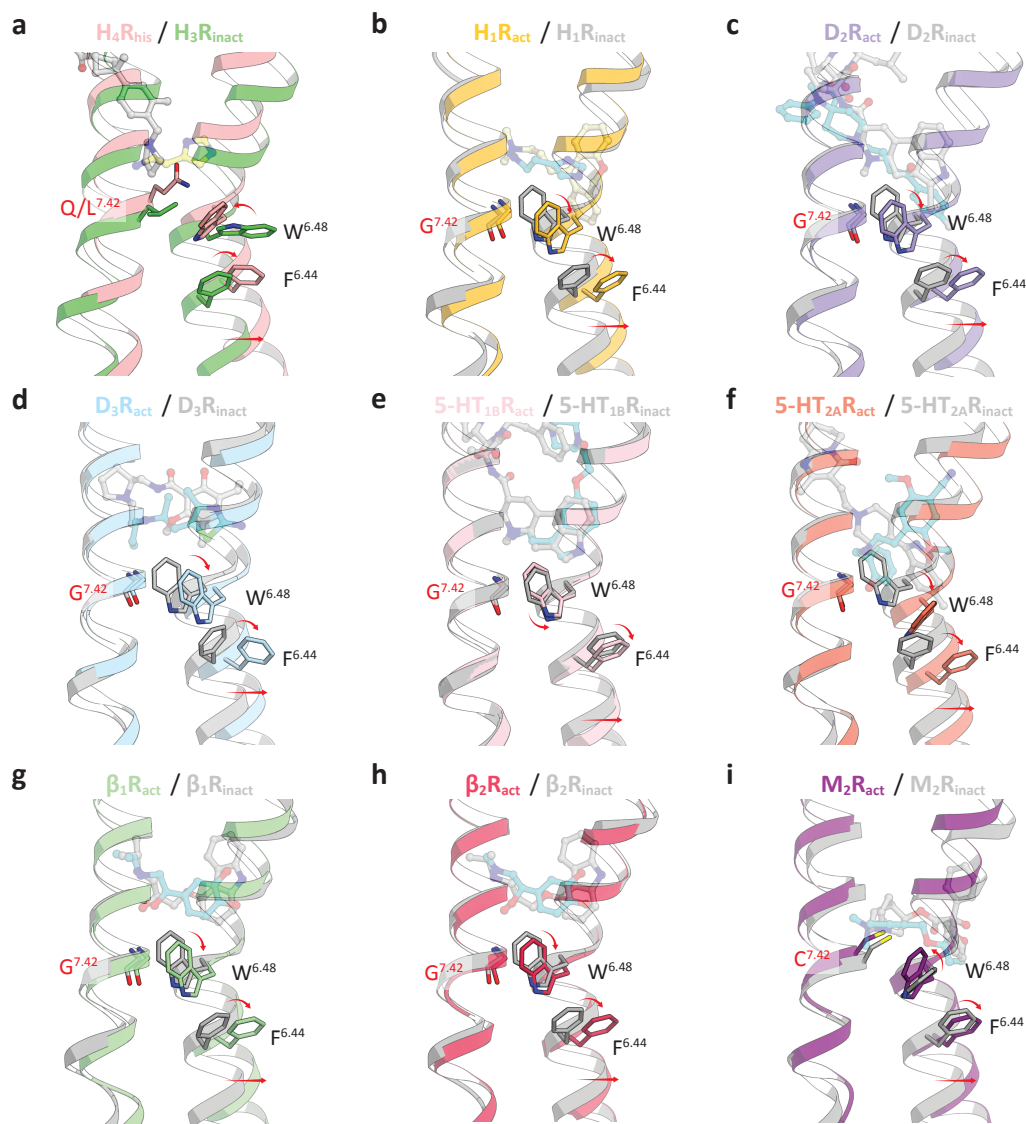
Receptors and agonists are shown as cylinders and sticks, respectively. Side chains are indicated by sticks and transparent spheres. The aromatic slot on H<sub>4</sub>R<sub>ime</sub> (**b**; blue) and corresponding region for H<sub>4</sub>R<sub>his</sub> (**a**; pink), H<sub>1</sub>R<sub>his</sub> (**c**; orange), D<sub>1</sub>R (**d**; green, PDB 7CKZ), D<sub>2</sub>R (**e**; purple, PDB 6VMS), D<sub>3</sub>R (**f**; light blue, PDB 7CMU), 5-HT<sub>1A</sub>R (**g**; yellow, PDB 7E2Y), 5-HT<sub>1B</sub>R (**h**; light pink, PDB 6G79), 5-HT<sub>1D</sub>R (**i**; red, PDB 7E32), 5-HT<sub>1E</sub>R (**j**; olive, PDB 7E33), 5-HT<sub>1F</sub>R (**k**; cyan, PDB 7EXD), and 5-HT<sub>2A</sub>R (**l**; tomato, PDB 6WHA).



**Supplementary Figure 7 | H<sub>4</sub>R formed a specific sub-pocket between TM5 and 6.**

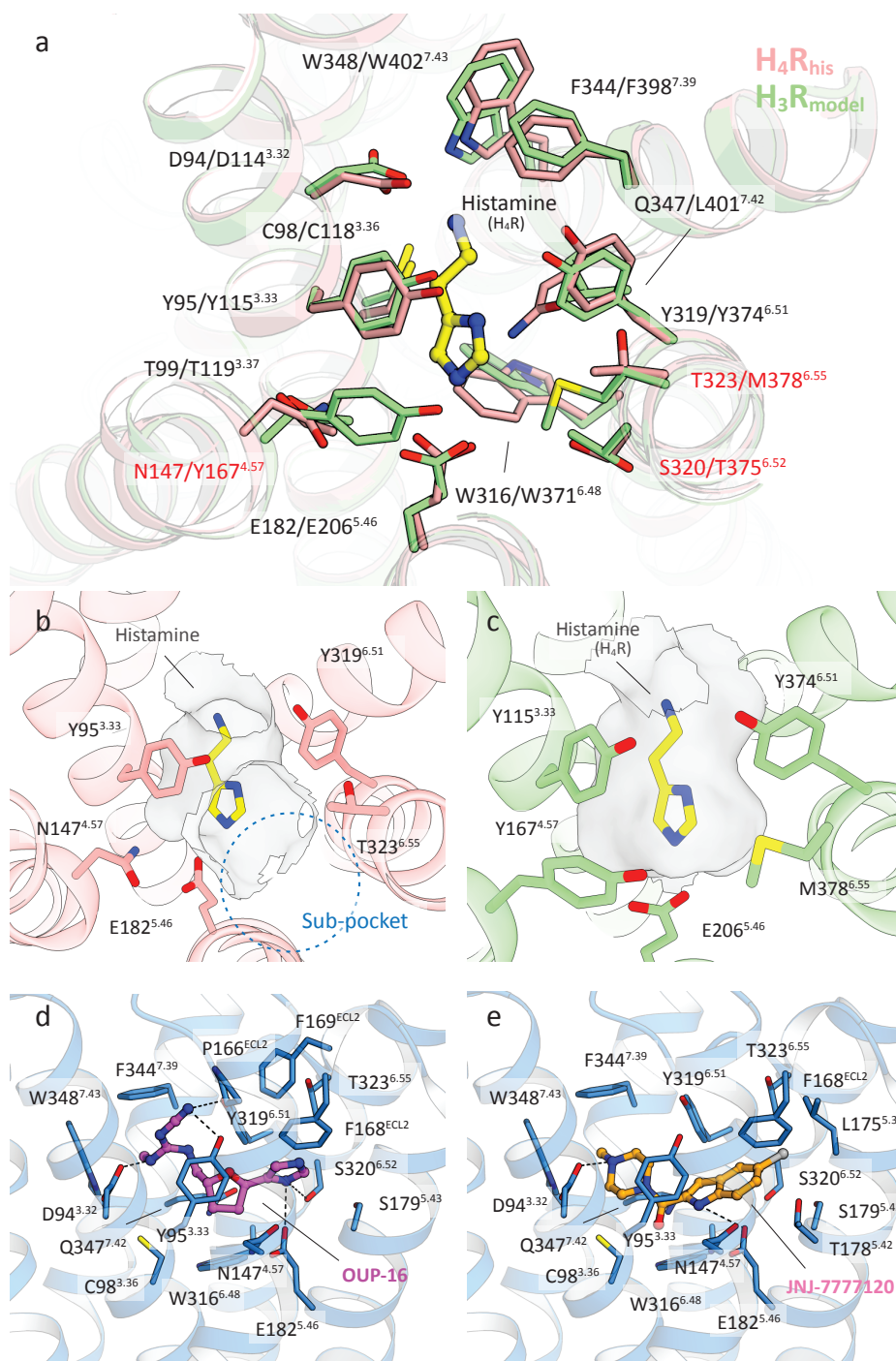
**a-c**, Extracellular view of the ligand-binding pocket in H<sub>4</sub>R<sub>his</sub> (**a**, pink), H<sub>4</sub>R<sub>ime</sub> (**b**, blue), and H<sub>1</sub>R<sub>his</sub> (**c**, orange). The top panels show horizontal cross-sections of each structure. The surface of the ligand-binding pocket is shown in the lower panels.





**Supplementary Figure 8 | Coordination of Gln347<sup>7.42</sup> and the basal activity of H<sub>4</sub>R.**

**a–i**, Comparison of the peripheral structure of the residue at position 7.42. Superimposition of the inactive (gray) and active structure (each color) of aminergic receptors. The PDB IDs are shown in parentheses. **(a)** H<sub>4</sub>R<sub>his</sub>/H<sub>3</sub>R<sub>inact</sub> (7YFC, pink/7F61, green) **(b)** H<sub>1</sub>R<sub>act</sub>/H<sub>1</sub>R<sub>inact</sub> (7DFL/3RZE), **(c)** D<sub>2</sub>R<sub>act</sub>/D<sub>2</sub>R<sub>inact</sub> (6VMS/7DFP), **(d)** D<sub>3</sub>R<sub>act</sub>/D<sub>3</sub>R<sub>inact</sub> (7CMU/3PBL), **(e)** 5-HT<sub>1B</sub>R<sub>act</sub>/5-HT<sub>1B</sub>R<sub>inact</sub> (6G79/4IAR), **(f)** 5-HT<sub>2A</sub>R<sub>act</sub>/5-HT<sub>2A</sub>R<sub>inact</sub> (6WHA/6A93), **(g)** β<sub>1</sub>R<sub>act</sub>/β<sub>1</sub>R<sub>inact</sub> (7JJO/2YCW), **(h)** β<sub>2</sub>R<sub>act</sub>/β<sub>2</sub>R<sub>inact</sub> (4LDO/2RH1), **(i)** M<sub>2</sub>R<sub>act</sub>/M<sub>2</sub>R<sub>inact</sub> (6OIK/3UON). Histamine, each antagonist, and agonist are shown as yellow, gray, and cyan transparent sticks.

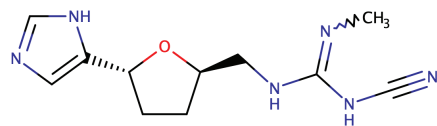


**Supplementary Figure 9 | Comparison of H<sub>4</sub>R and H<sub>3</sub>R homology models demonstrated a specific sub-pocket of H<sub>4</sub>R.**

**a**, Structural alignment of the ligand-binding pocket of H<sub>4</sub>R<sub>his</sub> (pink) and putative pocket of a model of H<sub>3</sub>R (green). The homology model of H<sub>3</sub>R was generated using Maestro (Schrodinger, Inc.) based on the H<sub>4</sub>R<sub>his</sub> structure. Histamine (yellow) and the side chains are shown as sticks. **b**, **c**,

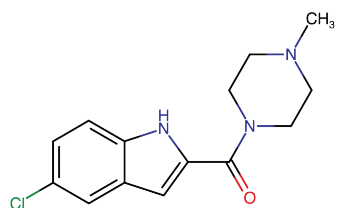


Sub-pocket formed between TM5 and 6 in H<sub>4</sub>R<sub>his</sub> (**b**) and the corresponding region in the H<sub>3</sub>R model (**c**). **d**, **e**, Docking simulation of H<sub>4</sub>R specific ligands. Docking pose of the H<sub>4</sub>R agonist OUP-16 (**d**), and the antagonist JNJ-7777120 (**e**).



OUP-16

(H<sub>4</sub>R-selective agonist)



JNJ-7777120

(H<sub>4</sub>R-selective antagonist)

**Supplementary Figure 10 | Chemical structures of histamine receptor agonists and antagonists.**

**Supplementary Table 1 | Pharmacological parameters of the H<sub>4</sub>R mutants against the agonists.**

|       | Histamine                       |                                    |                       |                   | Imetit                          |                                    |                          |                   |
|-------|---------------------------------|------------------------------------|-----------------------|-------------------|---------------------------------|------------------------------------|--------------------------|-------------------|
|       | pEC <sub>50</sub><br>mean ± SEM | E <sub>max</sub> (%)<br>mean ± SEM | EC <sub>50</sub> (nM) | fold of<br>change | pEC <sub>50</sub><br>mean ± SEM | E <sub>max</sub> (%)<br>mean ± SEM | EC <sub>50</sub><br>(nM) | fold of<br>change |
| WT    | 7.12 ± 0.08                     | 19.42 ± 1.15                       | 55.4                  | 1                 | 7.53 ± 0.06                     | 14.93 ± 1.19                       | 29.9                     | 1                 |
| D94A  | ND                              | ND                                 | ND                    | +++               | ND                              | ND                                 | ND                       | +++               |
| Y95A  | ND                              | ND                                 | ND                    | +++               | ND                              | ND                                 | ND                       | +++               |
| C98A  | ND                              | ND                                 | ND                    | +++               | 6.32 ± 0.03*                    | 17.06 ± 1.72                       | 486.2                    | 16.3              |
| T99A  | 6.93 ± 0.05                     | 22.78 ± 4.74                       | 134.9                 | 2.4               | 7.12 ± 0.01                     | 13.72 ± 1.67                       | 76.0                     | 2.5               |
| N147A | 6.15 ± 0.13                     | 14.11 ± 1.55                       | 510.3                 | 9.2               | 6.94 ± 0.04*                    | 17.18 ± 0.52                       | 116.1                    | 3.9               |
| N147W | ND                              | ND                                 | ND                    | +++               | 6.22 ± 0.01*                    | 18.45 ± 1.29                       | 602.8                    | 20.2              |
| N147Y | 7.09 ± 0.05                     | 12.71 ± 0.59                       | 82.9                  | 1.5               | 6.94 ± 0.04                     | 11.10 ± 1.47                       | 319.4                    | 10.7              |
| E182A | ND                              | ND                                 | ND                    | +++               | ND                              | ND                                 | ND                       | +++               |
| E182N | ND                              | ND                                 | ND                    | +++               | ND                              | ND                                 | ND                       | +++               |
| Y319A | ND                              | ND                                 | ND                    | +++               | 6.85 ± 0.16                     | 15.81 ± 1.58                       | 159.4                    | 5.3               |
| F344A | ND                              | ND                                 | ND                    | +++               | ND                              | ND                                 | ND                       | +++               |
| F344I | ND                              | ND                                 | ND                    | +++               | ND                              | ND                                 | ND                       | +++               |
| Q347A | 6.06 ± 0.04*                    | 10.06 ± 0.39                       | 826.9                 | 14.9              | 7.91 ± 0.07*                    | 19.34 ± 1.10                       | 12.7                     | 0.4               |
| Q347G | 6.47 ± 0.38                     | 8.80 ± 2.03                        | 2570.2                | 46.4              | 8.33 ± 0.24                     | 12.90 ± 1.22                       | 6.2                      | 0.2               |
| W348A | ND                              | ND                                 | ND                    | +++               | ND                              | ND                                 | ND                       | +++               |
| W348Y | ND                              | ND                                 | ND                    | +++               | ND                              | ND                                 | ND                       | +++               |

ND, EC<sub>50</sub> or E<sub>max</sub> values cannot be detected due to their low signal; +++, Exceedingly high fold of change due to undetectable or very low agonist activity; All data are presented as mean ± SEM from three independent experiments performed in triplicate. \*p < 0.05, one-way ANOVA, followed by Dunnett's post-test.

4 **Supplementary Table 2 | List of primers sequences for site-direct mutagenesis studies.**

| Oligonucleotides primer | Forward                         | Reverse                        |
|-------------------------|---------------------------------|--------------------------------|
| D94A                    | ACCACCGCTTACCTGCTGTGCACCGCT     | CAGGTAAGCGGTGGTCAGCCAGAAAAC    |
| Y95A                    | ACCGACGCTCTGCTGTGCACCGCTTCC     | CAGCAGAGCGTCGGTGGTCAGCCAGAA    |
| C98A                    | CTGCTGGCTACCGCTTCCGTGTACAAC     | AGCGGTAGCCAGCAGGTAGTCGGTGGT    |
| T99A                    | CTGTGCGCTGCTTCCGTGTACAACATC     | GGAAGCAGCGCACAGCAGGTAGTCGGT    |
| N147A                   | TTCCTGGTCGCTGGTCCCATGATCCTGGTG  | CATGGGACCAGCGACCAGGAAAGCCAGCAC |
| N147W                   | CTGGTCTGGGGTCCCATGATCCTGGTG     | GGGACCCCAGACCAGGAAAGCCAGCAC    |
| N147Y                   | TTCCTGGTCTATGGTCCCATGATCCTGGTG  | CATGGGACCATAGACCAGGAAAGCCAGCAC |
| E182A                   | TTCTTGGCTTTCGTGATCCCCGTGATC     | CACGAAAGCCAAGAACGATGTGATAGC    |
| E182N                   | TCGTTCTTGAATTTTCGTGATCCCCGTGATC | GATCACGAAATTCAAGAACGATGTGATAGC |
| Y319A                   | TGGGCTCCCGCTTCCCTGTTCACCATCGTC  | GAACAGGGAAGCGGGAGCCCAGCAAACGGC |
| F344A                   | AGGATCGCCGCTTGGCTGCAGTGGTTCAAC  | CTGCAGCCAAGCGGCGATCCTGTACCACAC |
| F344I                   | ATCGCCATTTGGCTGCAGTGGTTCAAC     | CAGCCAAATGGCGATCCTGTACCACAC    |
| Q347A                   | TGGCTGGCTTGGTTCAACTCCTTCGTG     | GAACCAAGCCAGCCAGAAGGCGATCCT    |
| Q347G                   | TTCTGGCTGGGTTGGTTCAACTCCTTCGTG  | GTTGAACCAACCCAGCCAGAAGGCGATCCT |
| W348A                   | CTGCAGGCTTTCAACTCCTTCGTGAAC     | GTTGAAAGCCTGCAGCCAGAAGGCGAT    |
| W348Y                   | CTGCAGTATTTCAACTCCTTCGTGAAC     | GTTGAAATACTGCAGCCAGAAGGCGAT    |