# Figure S1

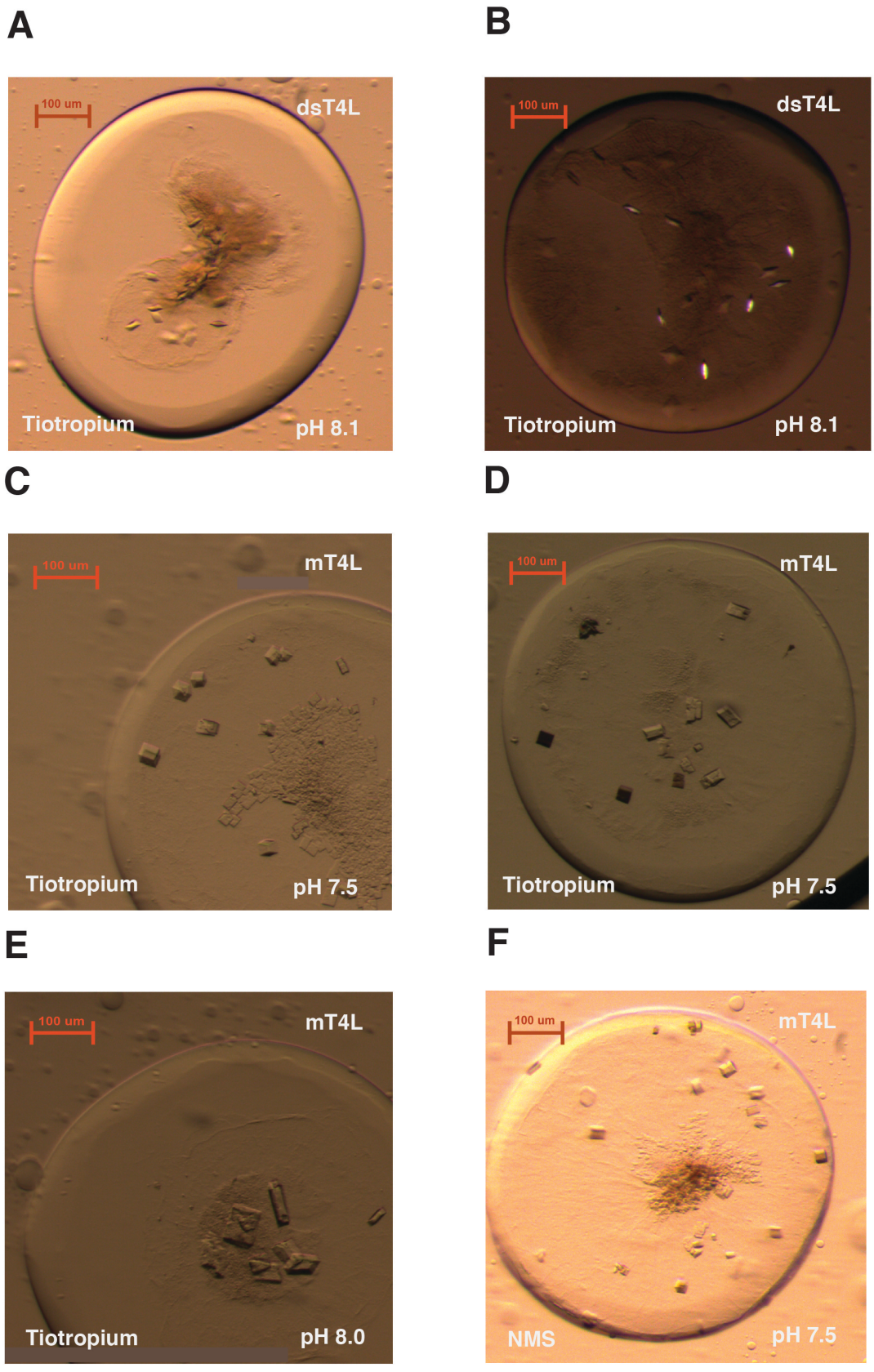
A
100 um
dsT4L
Tiotropium
pH 8.1
B
100 um
dsT4L
Tiotropium
pH 8.1
C
100 um
mT4L
Tiotropium
pH 7.5
D
100 um
mT4L
Tiotropium
pH 7.5
E
100 um
mT4L
Tiotropium
pH 8.0
F
100 um
mT4L
NMS
pH 7.5

**Figure S1, related to Tables 1-3. M3-dsT4L and M3-mT4L crystalized in lipidic cubic phase.**

(A) Image of a 20 nl lipid drop containing plate like M3-dsT4L crystals. (B) Similar crystals as shown in panel a viewed thorough a polarizing filter. (C) 20 nl lipid drop containing block-shaped M3-mT4L crystals. (D) M3-mT4L crystals viewed thorough a polarizing filter. (E) Larger M3-mT4L crystals could be grown at pH 8.0. Though more irregular in shape, they diffracted equally well. (F) The M3 mT4L was also crystalized using NMS as the ligand giving similar appearing crystals as observed with tiotropium.

# Figure S2

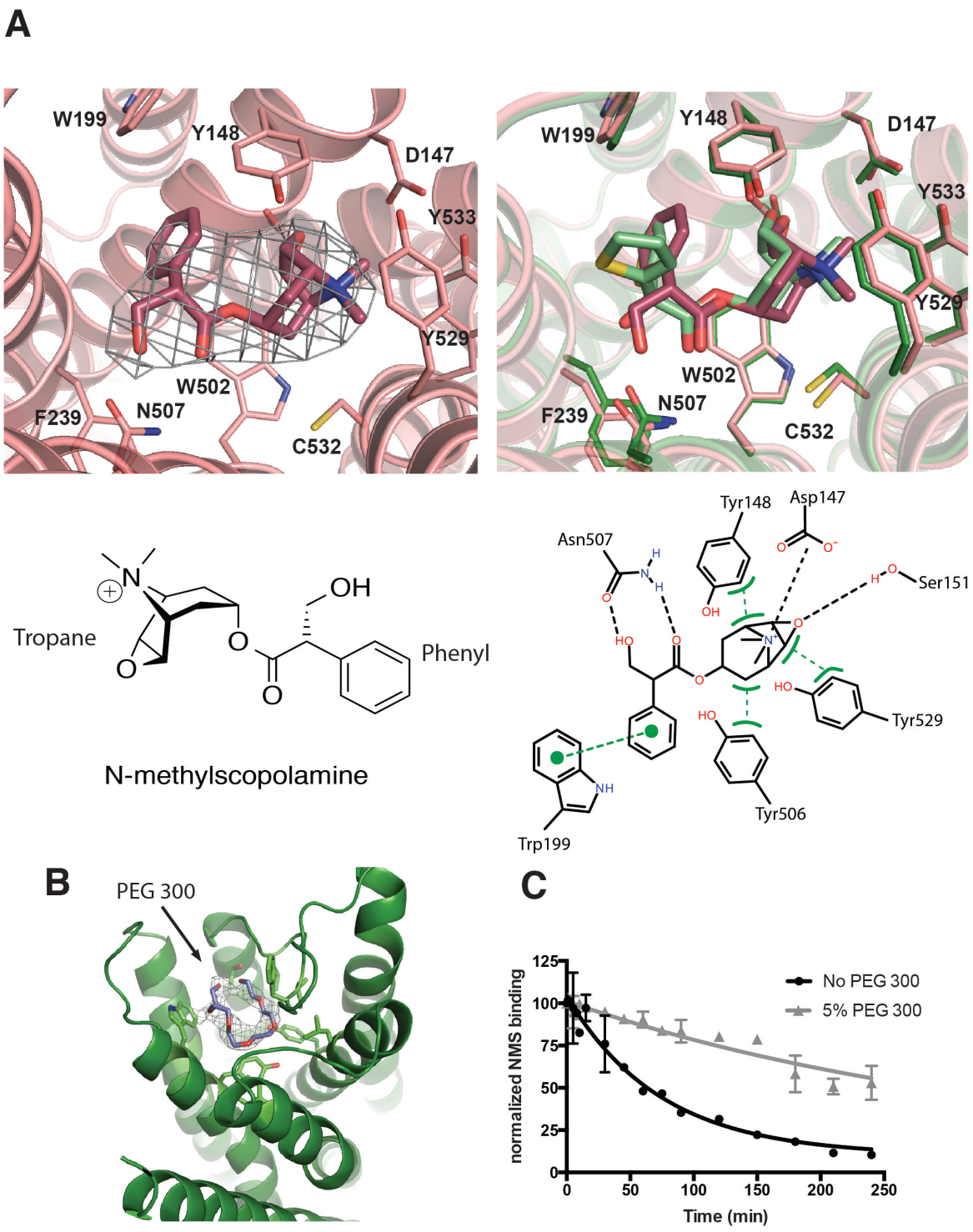


**Figure S2, related to figure 4. Methylscopolamine binding to M3-mT4L**

(A) N-methylscopolamine (NMS) (raspberry) was crystallized bound in M3 mT4L (brown) (PDB 4U16). The $2F_o$-$F_c$ Map around the NMS contoured at 2.0 σ is shown in grey (left panel).

Although the low resolution does not allow for a very exact position of the molecule, it can be seen that NMS binds in the same manner as tiotropium (green) (right panel). The structure of NMS and a lig plot of its interaction with the receptor are also shown.
(B) In the proposed allosteric site of our receptor structures in vicinity of tryptophan 525 a clear density was always present. In the tiotropium bound mT4L M3 structure chain B, the most well resolved chain, a PEG 300 molecule could be fit. The M3 receptor is shown in green and the $2F_o$-$F_c$ Map around the PEG 300 (blue) contoured at 1.0 $\sigma$ is shown in grey. (C) Dissociation experiments show that PEG 300 slows $^3$H-NMS dissociation from the M3 receptor to a $t_{1/2}$ of approx. 240 min compared to a $t_{1/2}$ of approx. 50 min for untreated membranes, supporting a likely binding of PEG in the allosteric site. The data represent the mean +/- s.e.m. of three independent experiments performed in duplicate.

# Figure S3

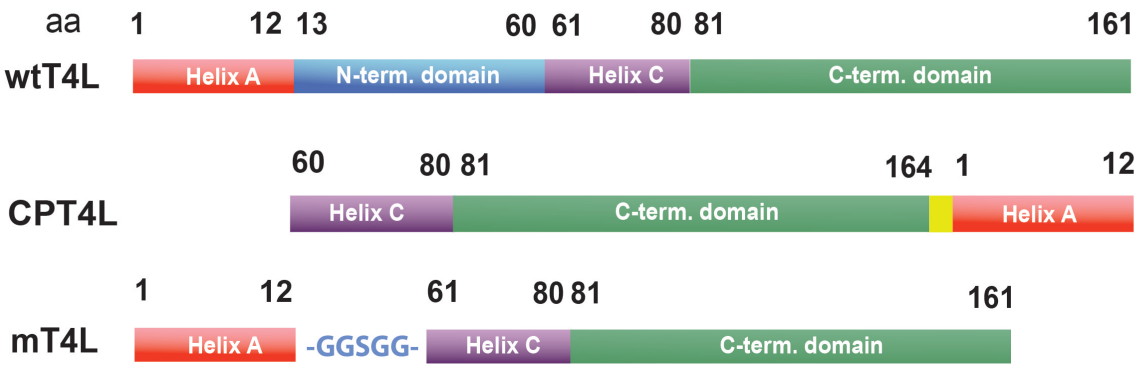


**Figure S3, related to figure 1. Comparison of the wt and mT4L with the previously reported circular permutated CP T4L.**

A circular permutated version of wtT4L (CPT4L) (PDB: 2O7A) was made by moving the N-teminal helix A of wtT4L to the end of the C-terminal domain, and deleting the N-terminal domain. A short SerGly$_4$Ala linker inserted between the C-terminal domain and N-terminal helix is shown in yellow (Chun et al., 2012). A β2AR fusion protein in which this CPT4L was inserted between TMs 5 and 6 expressed poorly and failed to yield crystals (Chun et al., 2012).

# Supplementary methods

Binding experiments

Membrane preparation; 447 mg Sf9 cell pellet expressing M3-mT4L equivalent to 17 ml culture was washed in lysis buffer (10 mM Tris pH 7.5 supplemented with leupeptin and

benzamidine protease inhibitors) and dounce homogenized 50x in 1.3 ml lysis buffer. Pellet was washed 4x in lysis buffer and 1 ul/20 ml benzonase DNase (sigma cat. nr. E1014) and 2 mM $MgCl_2$. Pellet was resuspended in 14 ml lysis buffer and membrane aliquots were frozen. Dissociation experiments were performed using binding buffer (BB) containing 25 mM Tris pH 7.5 and 100 mM NaCl supplemented with 0.1% BSA. Membranes were diluted 50x in BB and supplemented with 10 nM $^{3}H$ NMS tracer (PerkinElmer). The mix was shaken at RT for 90 min to allow NMS binding. 250 ul of the binding reaction was mixed with 250 ul 10 % PEG 300 in BB. Reactions were shaken for an additional 30 min to allow PEG 300 to bind. To begin the dissociation experiments, 10 μM atropine was added to prevent rebinding of NMS. Samples were removed at different time points and were filtered through BB blocked filters in a Brandel harvester and binding was determined using scintillation counting.

# Reference list

Chun, E., Thompson, A.A., Liu, W., Roth, C.B., Griffith, M.T., Katritch, V., Kunken, J., Xu, F., Cherezov, V., Hanson, M.A.*, et al.* (2012). Fusion partner toolchest for the stabilization and crystallization of G protein-coupled receptors. Structure *20*, 967-976.