Supplemental Data

Thyrotropin Receptor: Allosteric Modulators Illuminate Intramolecular Signaling Mechanisms at the Interface of Ecto- and Transmembrane Domain

Journal: Molecular Pharmacology

Patrick Marcinkowski[1], Annika Kreuchwig[1], Sandro Mendieta[1], Inna Hoyer[1], Franziska Witte[1,3], Jens Furkert[1], Claudia Rutz[1], Dieter Lentz[2], Gerd Krause[1], Ralf Schülein[1]

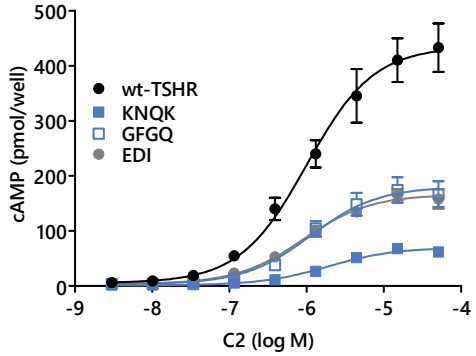
[1] Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), 13125 Berlin, Germany

[2] Institut für Chemie und Biochemie - Anorganische Chemie, Freie Universität Berlin, 14195 Berlin, Germany

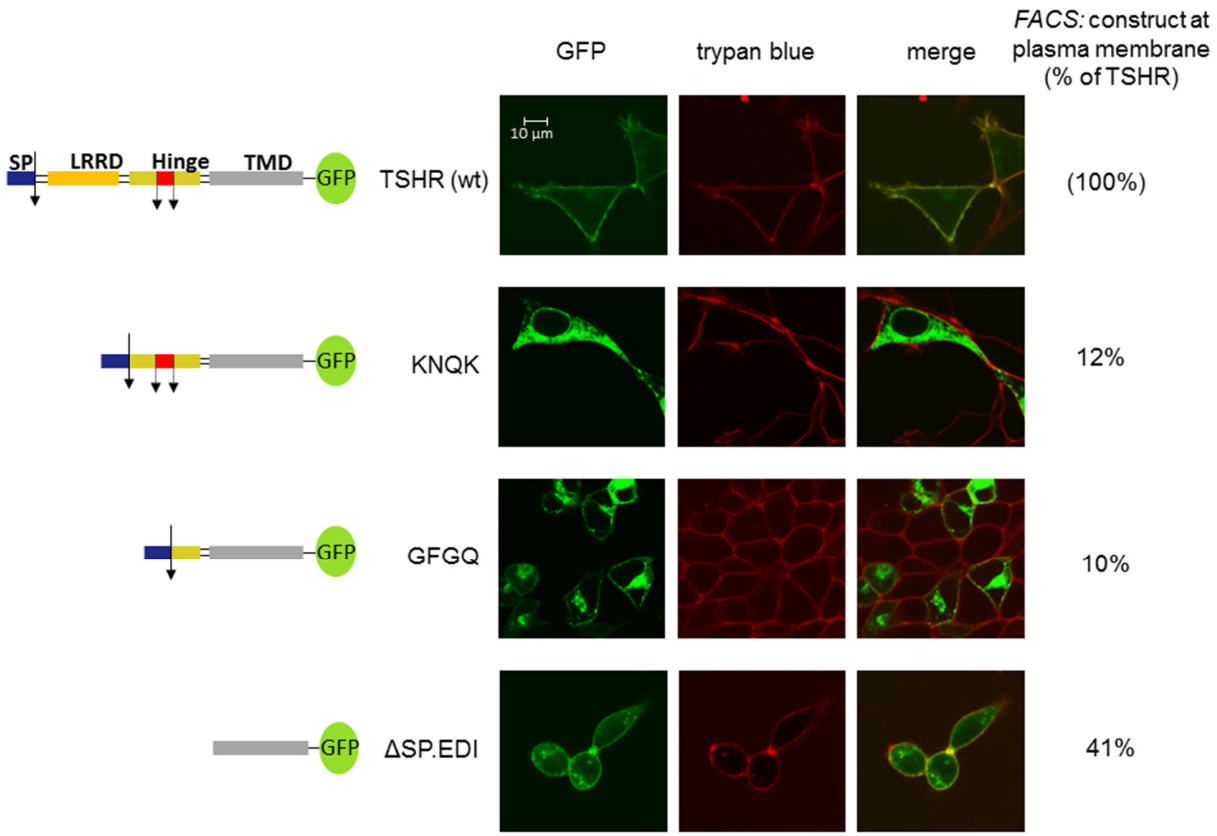
[3] current affiliation: Max Delbrück Center for Molecular Medicine (MDC), 13125 Berlin, Germany

Supplemental Data

Supplemental Figures

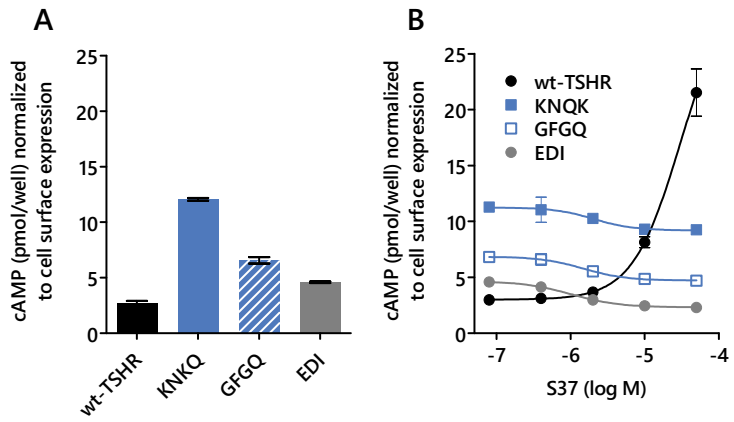


Supplemental Figure 1: Concentration-dependent activation of truncated TSHR by small allosteric agonist C2. The graph shows cAMP accumulation in transiently transfected HEK293 cells.

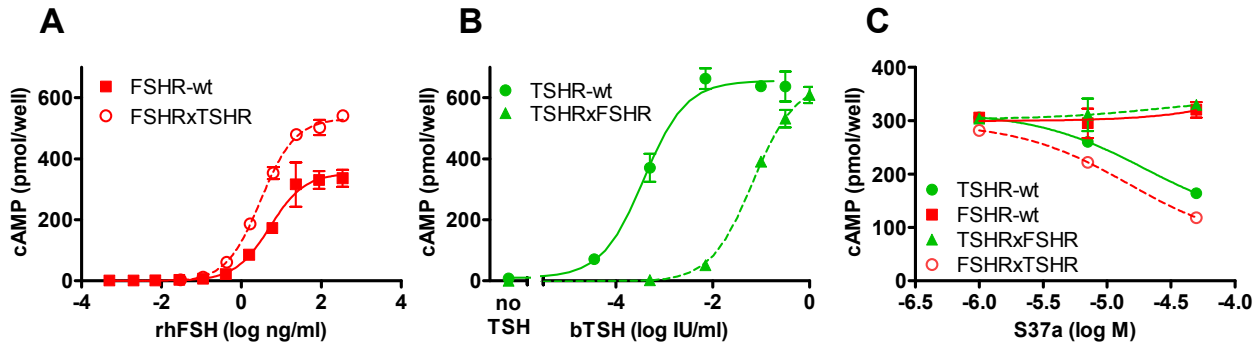


Supplemental Figure 2: Expression of truncated TSHR constructs (scheme, left panel) in transiently transfected HEK293 cells. Expression data by confocal laser scanning microscopy (LSM, 3 central panels) and flow cytometry data (FACS, right panel) of truncated TSHR constructs indicate that the EDI constructs is well expressed on the cell surface in HEK 293T cells although it is lacking the entire ectodomain. Confocal laser scanning microscopy images show the GFP fluorescence signals of the constructs (green) and the trypan blue stained plasma membrane (red). The overlay of green and red channels show yellow color, thus demonstrating the plasma membrane expression of the constructs. $2x10^5$ cells per well were seeded on glass cover slips in 6 well plates. After 24 h of incubation they were transfected

with plasmids containing the TSHR constructs using 1.6 µg polyethylenemimine and 0.8 µg DNA per well. Another 48 h later medium was replaced by PBS and the cells were examined with the microscope ConfoCor2 (Carl Zeiss) using the objective Plan-Apochromat 63x/1.40 Oil DIC (Carl Zeiss) at room temperature. Plasma membranes were stained with a drop of a 0.02 % trypan blue solution put directly into the PBS on top of the cells. GFP signals were visualized using an argon laser (λ_{exc} = 488 nm) and a 505-550 nm bandpass filter, trypan blue using a helium-neon laser (λ_{exc} = 543 nm) and a 560 nm long pass filter. The overlay images were generated using Zeiss LSM Image Browser software version 4.2.0.121. Brightness and contrast was adjusted for all images equally. Flow cytometry (FACS) measurements of the plasma membrane signals of the constructs confirm these results (right panels in % of wt-TSHR). PE fluorescence was measured after staining with mouse-anti-FLAG and PE-conjugated anti-mouse antibodies. Plasma membrane expression of the truncated constructs was substantially lower than that of the wt TSHR and was accompanied by their intracellular accumulation. Most likely, this intracellular retention results from increased misfolding of the truncation mutants which is detected by the quality control system of the early secretory pathway.

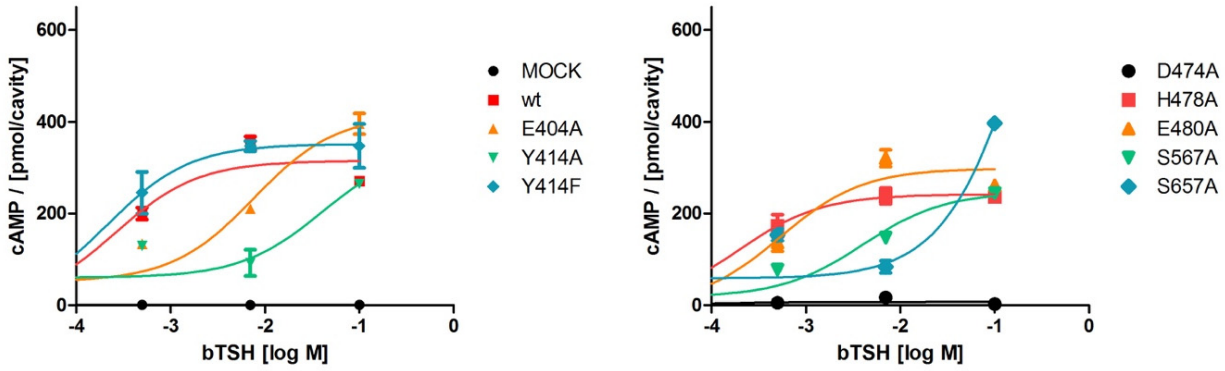


Supplemental Figure 3: Basal activity (A) and effect of S37 alone (B) in truncated TSHR constructs normalized to plasma membrane expression. Basal activity is elevated in truncated TSHR. S37 has a low intrinsic efficacy (E_{max}) for cAMP signaling which means that it activates the wt-TSHR without any other ligand by about 10% of bTSH E_{max}. This effect was completely abolished in the truncated TSHR. On the contrary, it even acts as an inverse agonist by reducing their constitutive activity.



Supplemental Figure 4: cAMP formation mediated by the TSHR-FSHR chimeras. A, B) TSHR/FSHR chimeras which are activated by the respective LRRD-binding hormone (rhFSH→FSHR-LRRD, bTSH→TSHR-LRRD) (also see Schaarschmidt *et al.*, 2014). **C)** Chimera activated with respective EC_{80} of rhFSH (red) and bTSH (green) were inhibited using S37a. S37a is TSHR-selective and does not inhibit FSHR activation, the chimera containing the S37a binding region of TSHR should be inhibited. S37a only inhibits activation of the FSHR-LRRD/TSHR chimera but not that of the TSHR-LRRD/FSHR chimera. These data prove that S37a does not bind at the LRRD of TSHR but instead at the hinge region and/or the TMD.

Supplemental Data



Supplemental Figure 5: cAMP formation mediated by the TSHR variants. Mutant receptor constructs were transiently transfected in HEK 293T cells and activated by bTSH concentration-dependently. cAMP accumulation was induced in all constructs except D474A. The EC_{50} values were elevated in all variants except of Y414F, H478A and E480A.