Supplemental Material

SUPPLEMENTARY MATERIALS AND METHODS

Materials

Cell culture media, supplements and antibiotics were obtained from Invitrogen (Darmstadt, Germany and San Diego, CA) and Sigma (Taufkirchen, Germany and St Louis, MO). Chemicals were purchased from Sigma (Taufkirchen, Germany), Roth (Karlsruhe, Germany) or Applichem (Darmstadt, Germany), unless otherwise noted. All enzymes and competent bacteria were purchased from New England Biolabs (Frankfurt, Germany). Primers and pUC19 vector were obtained from Invitrogen (Darmstadt, Germany), while remaining plasmids were purchased from Clontech (Heidelberg, Germany).

Cell Culture

COS7s were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin G and 100 µg/ml streptomycin. Melanocytes were grown in a mixture of 80% RPMI-20% DMEM medium supplemented with 10% FCS, 1% sodium pyruvate, 1% glutamate, 5 U/ml penicillin, 5 µg/ml streptomycin, 1% non-essential amino acids and 200 nM 12-*O*-tetradecanoyl phorbol 13-acetate. COS7s were transfected with jetPRIME transfection kit (Polyplus Transfection, Darmstadt, Germany) according to the manufacturer's recommendations. DNA electroporation of melanocytes was performed using Nucleofector device following manufacturer's instructions of Amaxa kit (Lonza, Walkersville, MD).

Plasmids

The human GPR143 gene was firstly cloned into the pUC19 vector using SalI and KpnI restriction sites and then two point mutations corresponding to double amino acid exchange (L223A-L224A and W329A-E330A) were inserted with site-directed mutagenesis using polymerase chain reaction (PCR). The designed mutagenesis primers contain the corresponding mismatch flanked by 14-15 nucleotides at

the 3'- and 5'-end. The obtained DNA was digested by DpnI, a methylation sensitive restriction enzyme, and then transformed into *E.coli* Top10 bacteria. Single clones were isolated and the plasmid DNA was sequenced by GATC Biotech (Konstanz, Germany). Wildtype (wt) and double mutated GPR143 were then subcloned into a DiscoverX vector containing a ProLink tag (PK2 vector, DiscoverX, Birmingham, UK) using NheI and BglII restriction sites and into pEYFP-N1 vector using KpnI and AgeI. The human tyrosinase (TYR) gene was cloned into pEGFP-N3 vector using HindIII and BamHI restriction sites.

Immunofluorescence Microscopy

Transfected cells were cultured on coverslips in a 12 well plate (Sarstedt) and fixed with a cold 1:1 methanol/acetone solution for 20 min at -20 °C. Cells were then washed with phosphate-buffered saline (PBS) and blocked 1 h with 1% bovine serum albumin (BSA)/PBS solution. Cells were incubated in the dark 60 min with the first antibody and then 30 min with the secondary antibody, both diluted in 1% BSA/PBS. In between and after antibody incubations, cells were washed with PBS. Nuclei staining was carried out using DAPI. At last coverslips were mounted using ProLong Gold antifade reagent (Invitrogen, Darmstadt, Germany) and stored in the dark at 4 °C. Mouse monoclonal anti-PK/PL antibody (PathHunter DiscoverX, Birmingham, UK), rabbit polyclonal anti-pan Cadherin antibody (Abcam, Cambridge, UK), rabbit antiserum αPEP-7 (Dr. V. J. Hearing, National Cancer Institute), donkey anti-mouse-AlexaFluor594 (Jackson Immuno Research, Hamburg, Germany) and donkey anti-rabbit-DyLight649 (BioLegend, San Diego, CA) were used to stain TYR and GPR143. A Nikon A1 Spectral confocal microscope (Pharmaceutical Institute, University of Bonn, Germany) and Carl Zeiss LSM 700 (Department of Cell Biology, New York University, USA) were used for imaging. Each transfection and staining was repeated three times and at least ten squares (60x objective) containing 3-15 cells each were imaged for each sample. The cell that was most representative of the majority of each condition was shown.

Glycosylation Studies

The glycosylation studies were performed using two enzymes: endoglycosidase H (EndoH) and Peptide N-Glycosidase (PNGaseF). Cells were transiently transfected and after 48 h of incubation they were detached and sonicated (3 times for 10 sec). 50 μg of total lysates were mixed with buffers corresponding to each endoglycosidase, following the manufacturer´s instruction. The reaction mixture was incubated 1 h at 37 °C. Thereafter, each reaction was mixed with loading buffer, the proteins were separated and transferred to a membrane (see Western Blot).

Immunoprecipitation and Western Blot

Two days after DNA transfection, COS7 and melanocytes were scraped off the dishes and lysed on ice with lysis buffer (20 mM HEPES pH 7.2, 1% NP-40, 10% glycerol and protease inhibitor cocktail (Sigma, Taufkirchen, Germany). The lysate was centrifuged 15 min at 21000 g and the protein concentration was determined with BCA Protein assay reagent kit (Thermo Fisher Scientific, Grand Island, NY) or with Bradford reagent. The protein samples were mixed with loading buffer containing 2% SDS (or 14% SDS for GPR143) and boiled for 2 min (or warmed at 37 °C for 30 min for GPR143). For immunoprecipitation samples, 1 mg of lysate was mixed to 2 μg of antibody and incubated overnight at 4 °C on a rotating wheel. Protein A-agarose beads (50 μl of suspension for each sample - Thermo Fisher Scientific, Grand Island, NY) were washed twice with lysis buffer and then pre-incubated 1 h in 0.1% BSA/lysis buffer. Then, beads were washed in lysis buffer twice and incubated with the lysate-antibody for 2 hours at 4 °C on a rotating wheel. After the beads were washed four times, the proteins were eluted in loading buffer containing 2% SDS (or 14% SDS for GPR143) and boiled for 2 min (or warmed at 37 °C for 30 min for GPR143). Immunoprecipitated proteins were separated in 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (PROTRAN – Nitrocellulose Transfer Membrane – Whatman, Sigma, Taufkirchen, Germany) or polyvinylidene difluoride membrane (Immobilon, Millipore, Waltham, MA). The Precision Plus Protein Kaleidoscope

Prestained Protein Standard and the *Novex Sharp Prestained Protein Standard* were used as protein markers. The membrane was blocked 1 hour in a 5% powdered milk/PBS-Tween solution (for COS7 lysates) or StartingBlock (TBS) Blocking buffer (for melanocyte lysates, Thermo Fisher Scientific, Grand Island, NY). Afterward, the membrane was incubated with the primary antibody overnight at 4 °C or 1 hour at RT, washed 1 h with PBS-Tween, incubated with secondary antibody and washed again. The detection was performed with ECL kit (GE Healthcare, Amersham, Arlington, IL – Dassel, Germany) according to the manufacturer's instructions. Each IP was repeated at least twice in both cell lines, COS7s and melanocytes. The following antibodies were used: mouse monoclonal anti-PK/PL (PathHunter DiscoverX, Birmingham, UK), mouse monoclonal anti-GPF (for COS7s, Covance, Freiburg, Germany), rabbit polyclonal anti-eGFP (Thermo Scientific, Darmstadt, Germany), rabbit antiserum αPEP-7 (Jiménez, Tsukamoto, and Hearing, 1991), mouse monoclonal anti-GFP (for melanocytes, Covance, Emeryville, CA), mouse monoclonal anti-GAPDH (Santa Cruz, Dallas, TX), anti-mouse-HRP and anti-rabbit-HRP (Jackson Immuno Research, Hamburg, Germany). Western blots of melanocyte lysates were detected with Odyssey (LI-COR, Lincoln, NE) using the following secondary antibodies: ODYSSEY goat anti-rabbit IRDye 680 and ODYSSEY goat anti-mouse IRDye 800CW.

Fluorescence Resonance Energy Transfer (FRET)

The GPR143 coding sequence was cloned into the pEYFP-N1 vector using KpnI and AgeI restriction enzymes, while the TYR was cloned into the pECFP-N1 plasmid using HindIII and BamHI. The fusion protein ECFP-EYFP was created by cloning ECFP into the pEYFP-N1 plasmid using KpnI and AgeI restriction enzymes. COS7s (10^5 cells $well^{-1}$) were seeded on sterile glass coverslips in 12 well-plates and transfected with different combinations of DNA plasmids. After 48 h cells were fixed with an ice-cold 1:1 methanol/acetone solution for 20 min and mounted on slides using Mowiol 4-88 medium (Roth, Karlsruhe, Germany). A Nikon A1 Spectral confocal microscope operating with an argon laser

was used for imaging. Cells were examined with a 60X oil immersion objective. NIS Element Advanced Research software 4.0 was used for image analysis.

For the sensitized emission method, different optical configurations were set up: “Dd channel” for excitation and emission of the donor chromophore (ECFP, excitation filter: 457 nm, emission filter: 482/35 nm), “Aa channel” for excitation and emission of the acceptor chromophore (EYFP, excitation filter: 514 nm, emission filter: 540/30 nm) and “FRET channel” for the excitation of the donor and emission of the acceptor chromophore (excitation filter: 457 nm, emission filter: 540/30 nm). For each image, parameters (high voltage, offset and laser intensity) were adjusted in order to limit the spectral bleed through and to avoid the pixel over-saturation. The laser intensity was equalized in both FRET and donor channel, while the high voltage was equalized in both FRET and acceptor channel. The FRET calibration was performed with single transfected cells expressing either GPR143-YFP or TYR-CFP. Correction parameters (CoA and CoB) were calculated by the software using the following formulas: $CoA = D_{a\ ACCEPTOR}/A_{a\ ACCEPTOR}$ and $CoB = D_{a\ DONOR}/D_{d\ DONOR}$, where A_a corresponds to the channel where the excitation and the emission of acceptor is measured, D_d corresponds to the channel of excitation and emission of the donor and D_a corresponds to the channel of the excitation of the donor and emission of the acceptor. $Xx_{\ DONOR/ACCEPTOR}$ coefficients are average intensities of the donor/acceptor-only images. The corrected FRET signal and the FRET efficiency were calculated for each image using the following formulas: $FRET_{CORR} = D_{a\ FRET} - (D_{d\ FRET} \times CoB) - (A_{a\ FRET} \times CoA)$ and $FRET_{\ EFFICIENCY}\ [\%] = (FRET_{\ CORR} / D_{d\ FRET}) \times 100$, where $Xx_{\ FRET}$ members are average intensities of assigned FRET image components.

For the acceptor photobleaching method, images were captured before and after the photobleaching of acceptor molecules in a specific region of the cell. If any interactions leading to energy transfer were present, photobleaching of the acceptor will lead to an increase of donor fluorescence, as it is no longer quenched by the acceptor. Acceptor photobleaching was performed with a high-intensity laser pulse at

514 nm. Images in the A_a and D_d channels were captured simultaneously before and after the photobleaching. FRET efficiency was calculated using the following formula: $FRET_{EFFICIENCY}$ [%]= $(I_A-I_B) \times 100/ I_A$, where I_A is the CFP intensity emission after bleaching and I_B is the CFP intensity emission before bleaching.

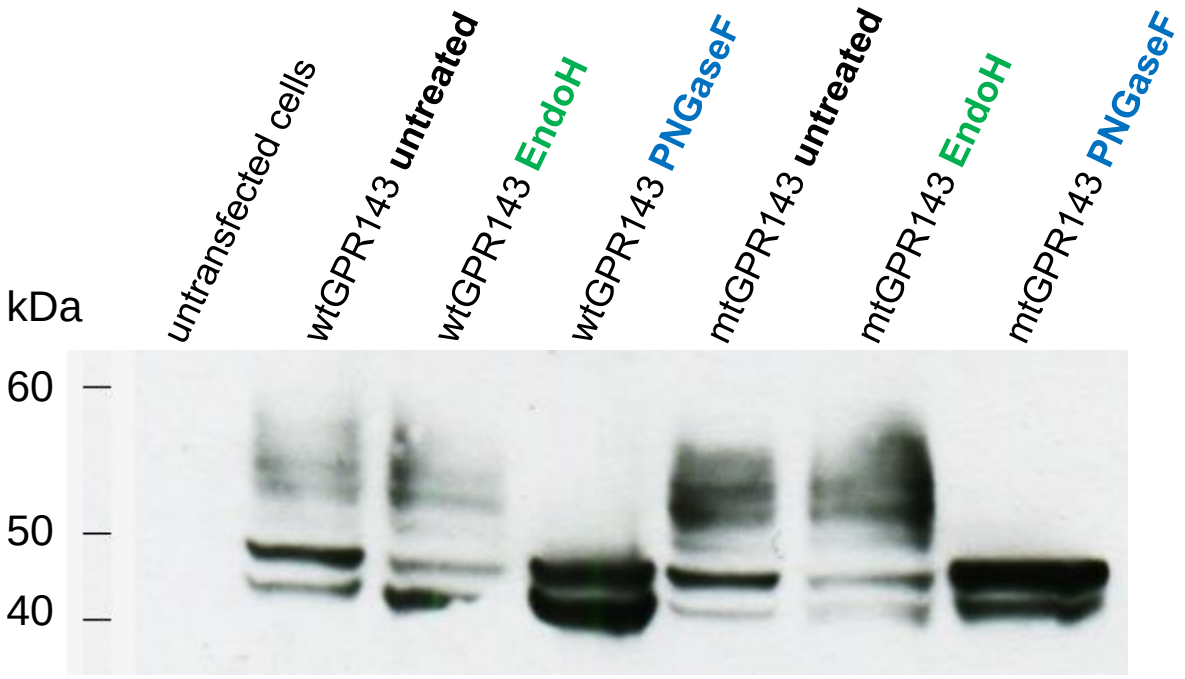


Figure S1. Glycosylation study of GPR143 expressed in COS7 cells. The wildtype and mtGPR143 were treated with different endoglycosidases (EndoH and PNGaseF) reacting at different levels of the glycosylation pattern. GPR143 was subjected to complex glycosylation in the Golgi apparatus and to different post-translational modifications apart from N-glycosylation. Each lane of the SDS-PAGE was loaded with 50 μg of total protein amount from the lysate, after treatment with the corresponding enzyme.

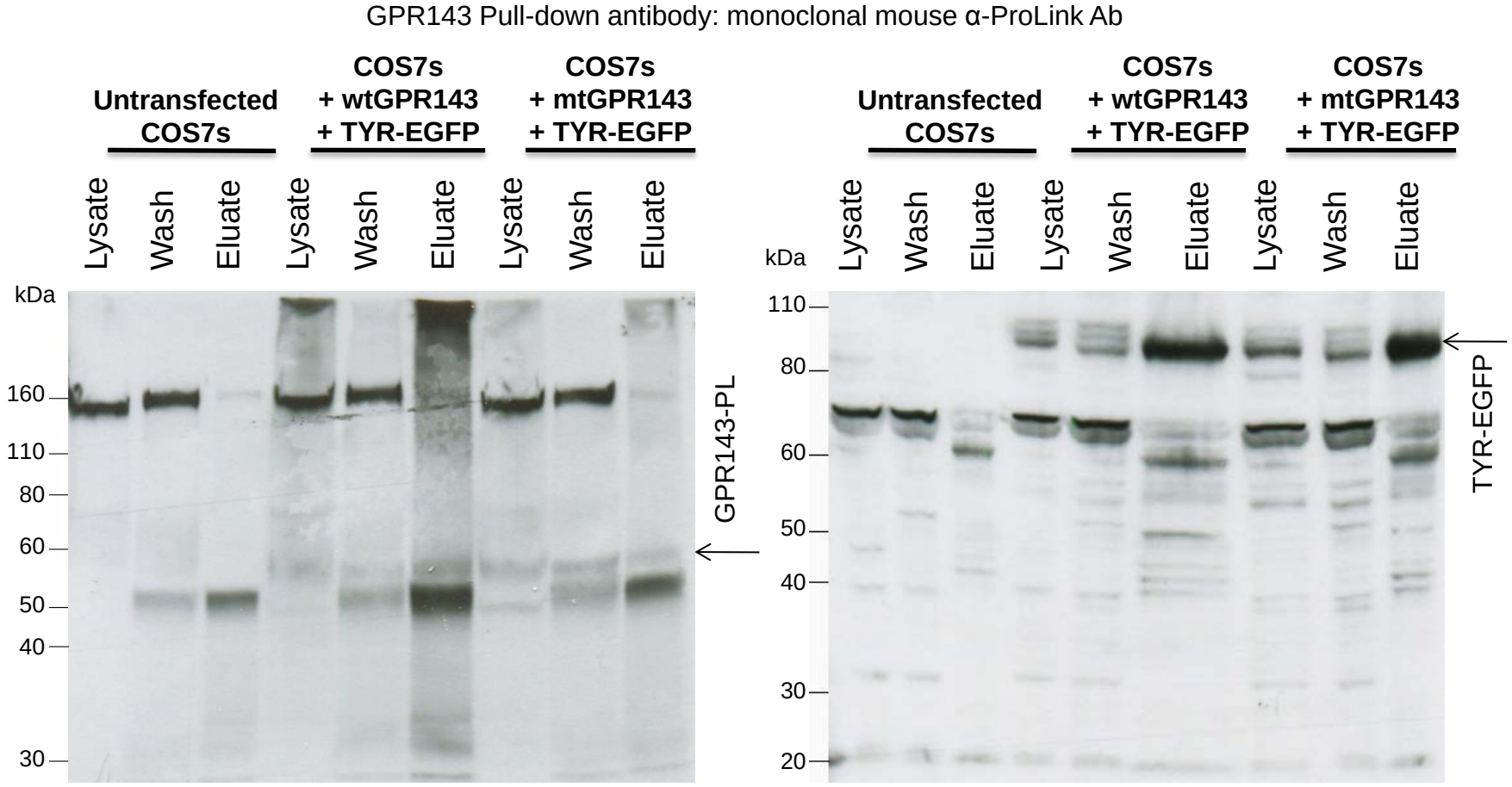


Figure S2. Complete blots prior to cropping for Figure 2a- Immunoprecipitation (IP) of GPR143 in COS7 cells. COS7s were co-transfected with wildtype or mtGPR143-PL and tyrosinase-EGFP. The IP was performed through the monoclonal anti-ProLink antibody. The blots were stained with the monoclonal antibody anti-ProLink (for PL tagged-GPR143, left image) and a polyclonal anti-GFP antibody (for EGFP tagged-TYR, right image). Untransfected COS7s were used as control. PL, ProLink tag; mt, mutant; TYR, tyrosinase; wt, wildtype.

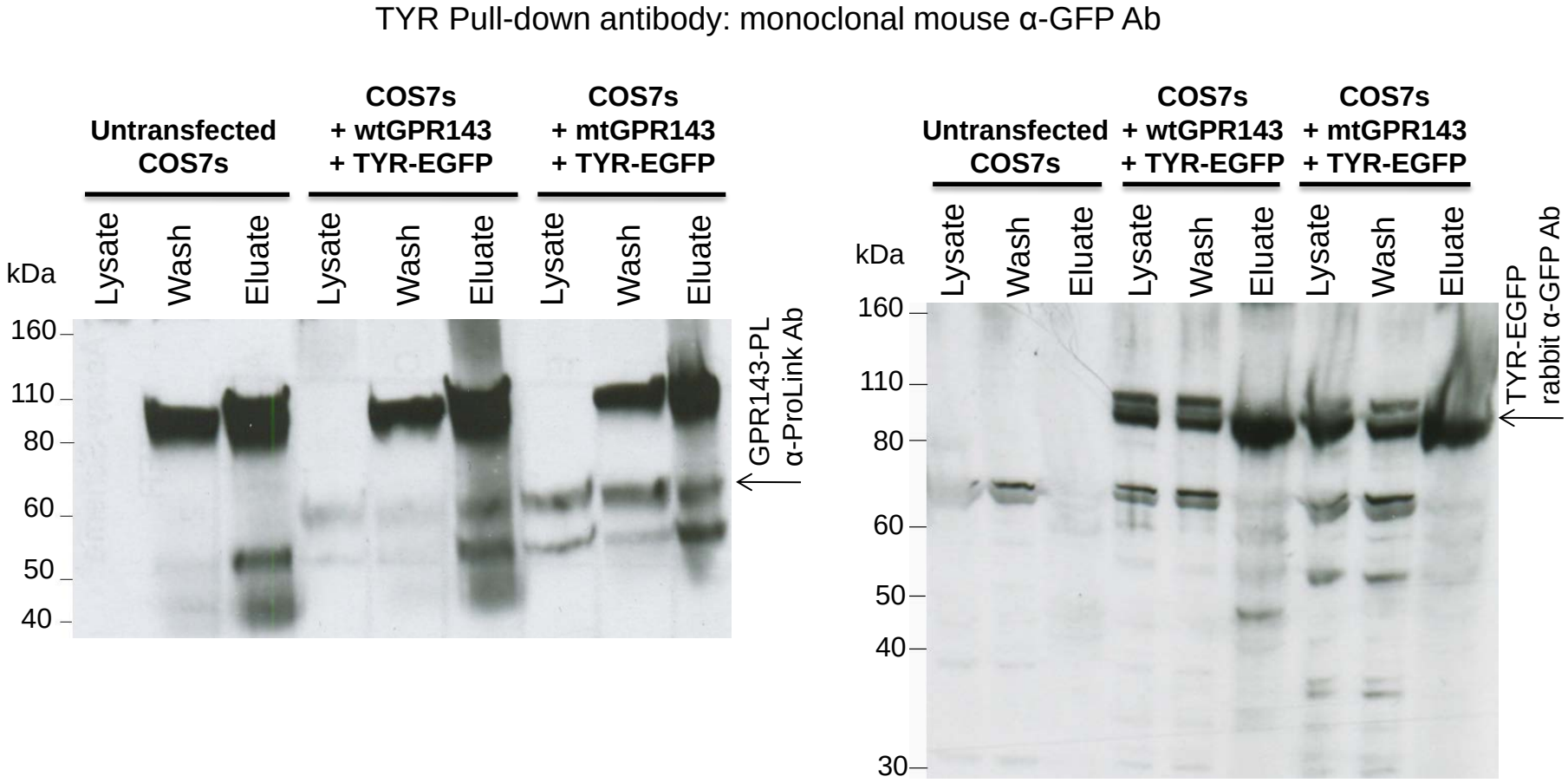


Figure S3. Immunoprecipitation (IP) of tyrosinase in COS7 cells. COS7s were co-transfected with wildtype or mtGPR143-PL and tyrosinase-EGFP. The IP was performed through a monoclonal anti-GFP antibody. The blots were stained with a monoclonal antibody anti-ProLink (for PL tagged-GPR143, left image) and a polyclonal anti-GFP antibody (for EGFP tagged-TYR, right image). Untransfected COS7s were used as controls. mt, mutant; TYR, tyrosinase; wt, wildtype.

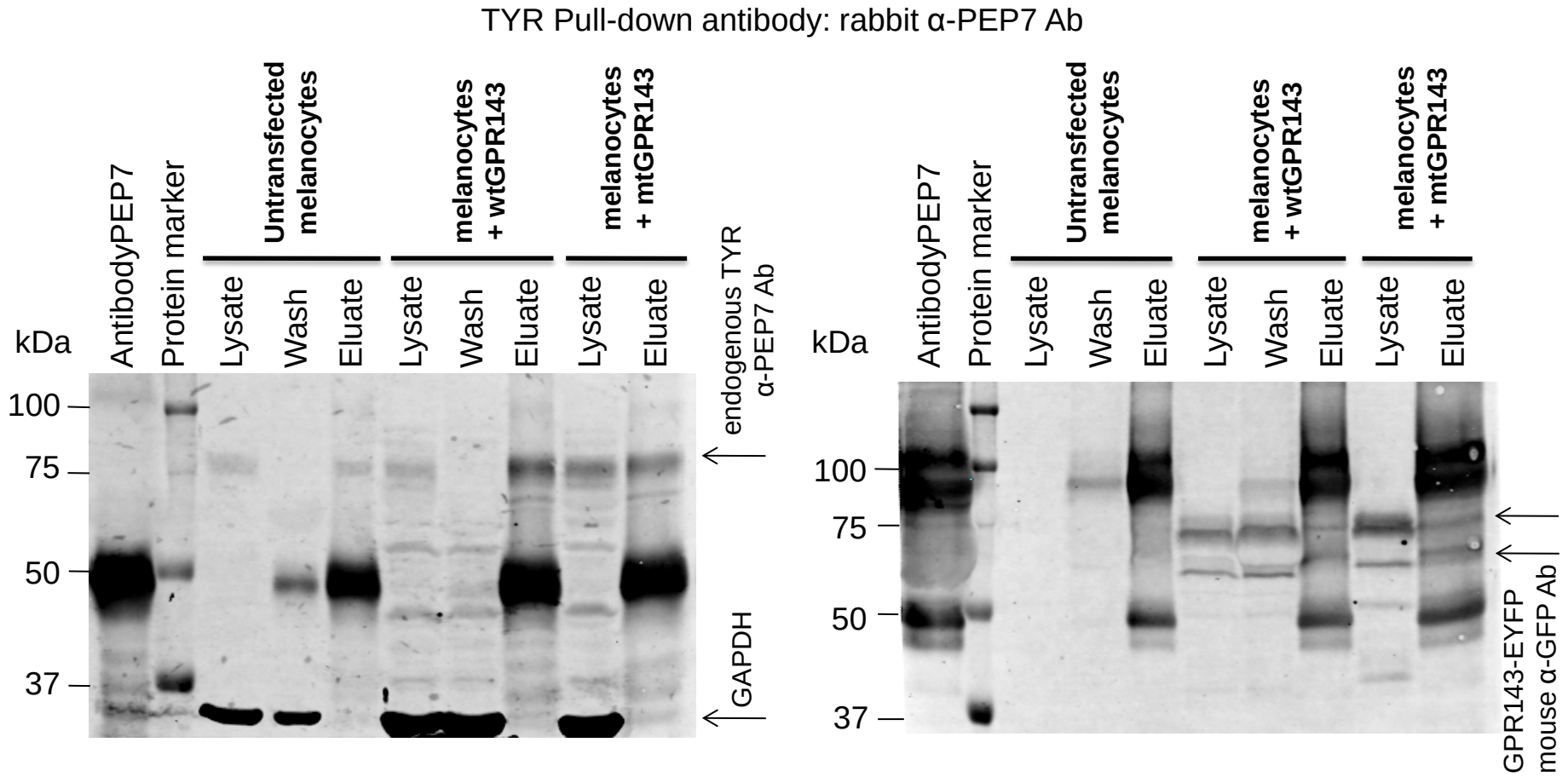


Figure S4. Complete blots prior to cropping for Figure 2b. Immunoprecipitation (IP) of endogenous tyrosinase in melanocytes. Melanocytes were transfected with GPR143-EYFP and the IP was performed through αPEP7 antibody. The blots were stained with a monoclonal antibody anti-GFP (for EYFP tagged-GPR143, right image), an anti-GAPDH (as control, left image) and the αPEP7 (for the endogenous tyrosinase, left image). Untransfected melanocytes were used as control. In the first lane only αPEP7 was loaded, after adding loading buffer and warming it up as the samples in the relative blot. mt, mutant; TYR, tyrosinase; wt, wildtype.

Figure S5

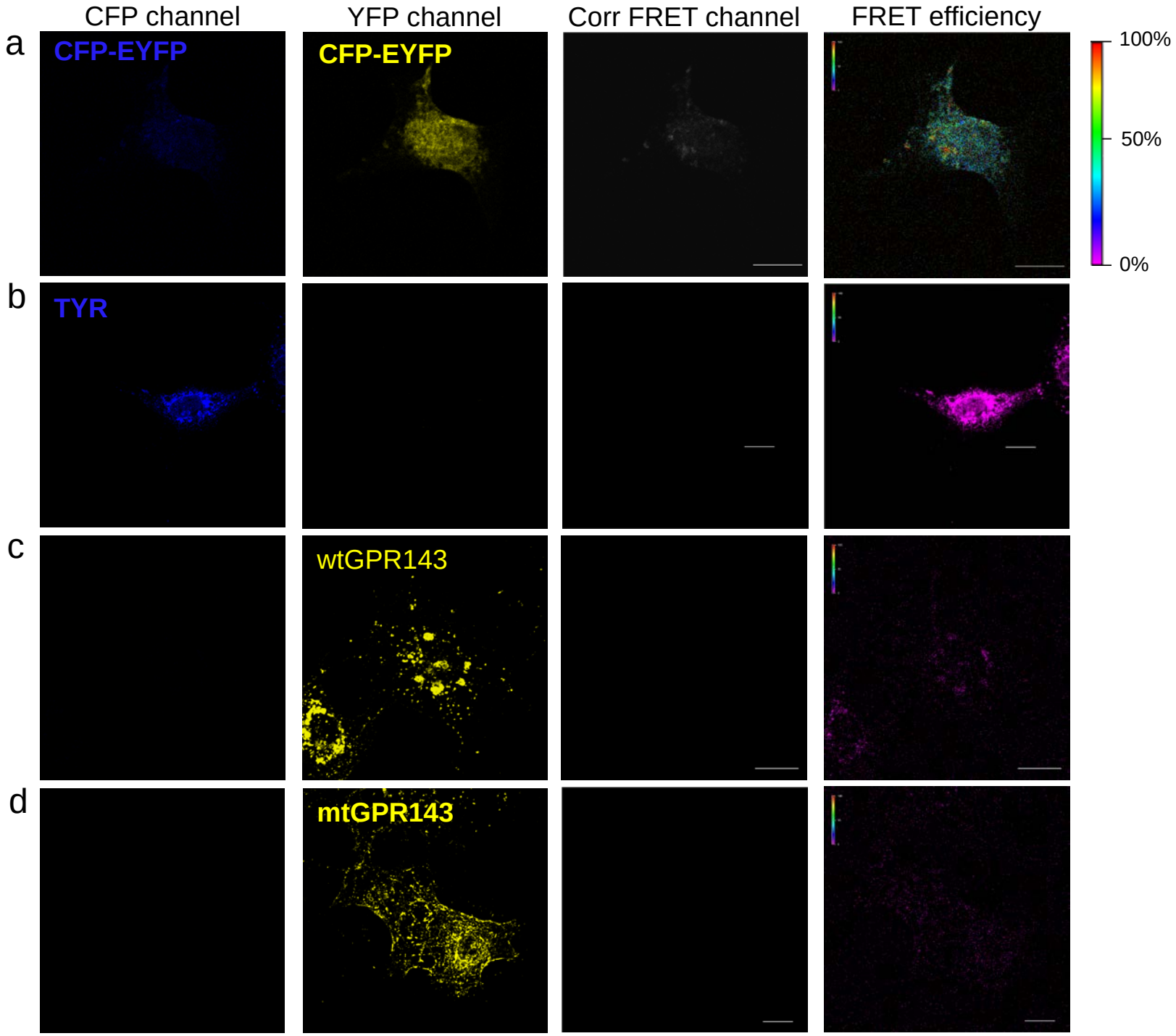

CFP channel
YFP channel
Corr FRET channel
FRET efficiency
a
CFP-EYFP
CFP-EYFP
100%
50%
0%
b
TYR
c
wtGPR143
d
mtGPR143

Figure S5. Control images of sensitized emission FRET. Sensitized emission method was used to detect interaction of GPR143 (YFP channel) and tyrosinase (CFP channel) in COS7s transfected either with (**a**) ECFP-EYFP fusion protein or (**b**) TYR-ECFP or (**c**) wtGPR143-EYFP or (**d**) mtGPR143-EYFP. FRET signal, corrected by CoA and CoB parameters, and FRET efficiency (color scale on the far right) are shown. Scale bar = 20 μm. FRET, fluorescence resonance energy transfer; mt, mutant; TYR, tyrosinase; wt, wildtype.

Figure S6

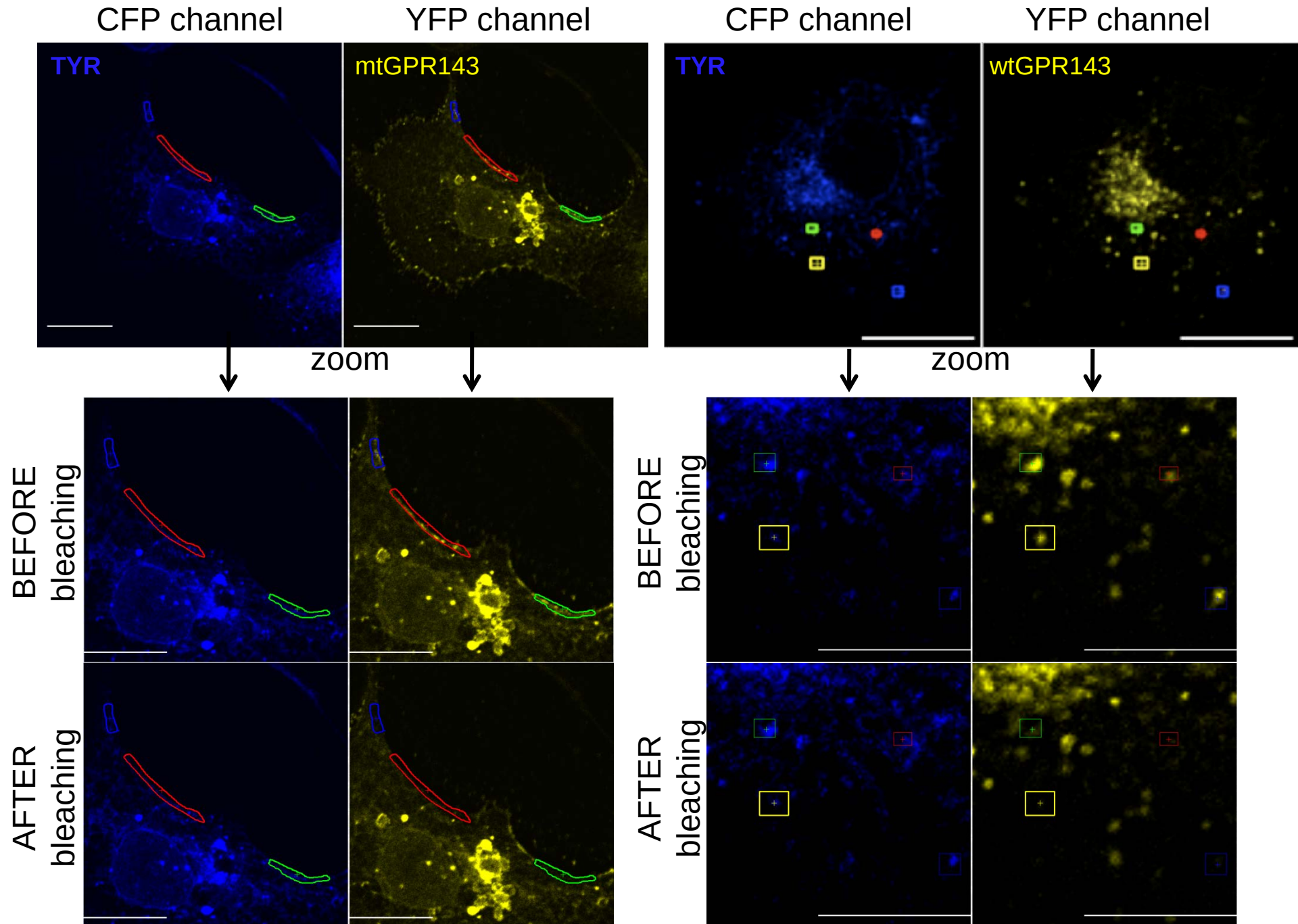
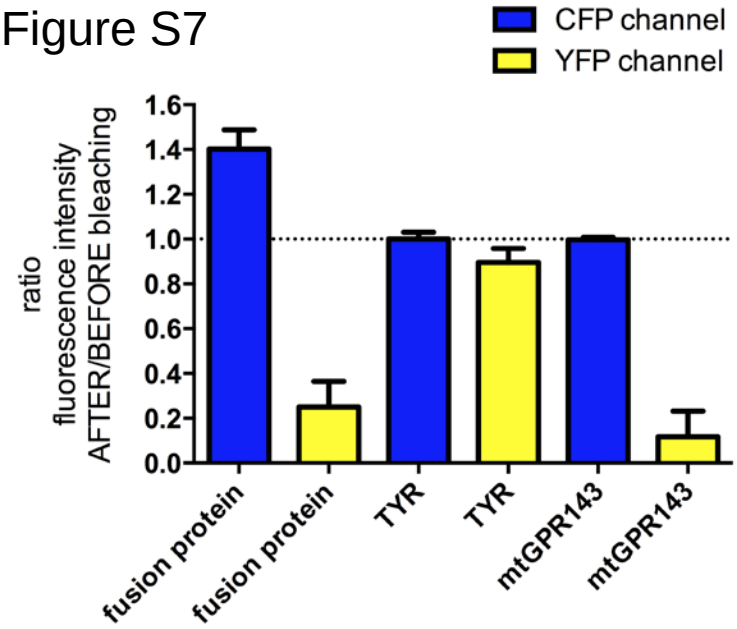
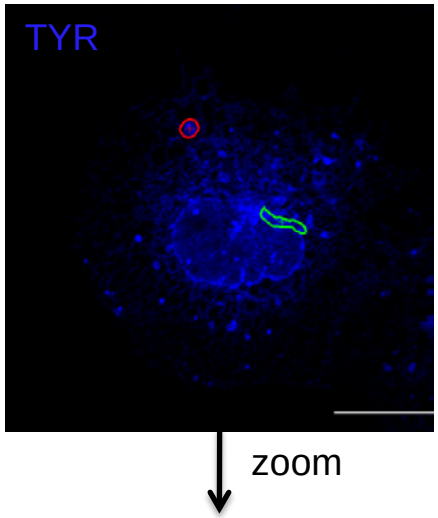
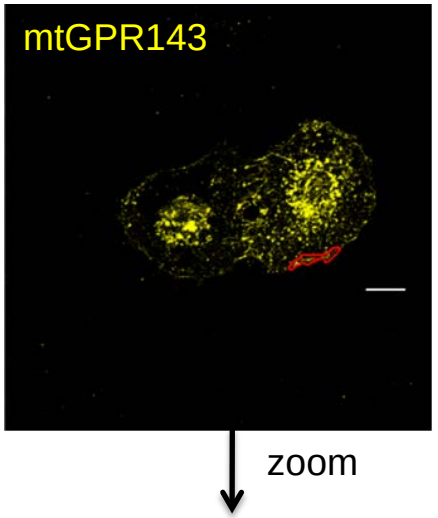

CFP channel
YFP channel
CFP channel
YFP channel
TYR
mtGPR143
TYR
wtGPR143
zoom
zoom
BEFORE bleaching
AFTER bleaching
BEFORE bleaching
AFTER bleaching

Figure S6. **Acceptor photobleaching FRET in COS7 cells.** COS7s were co-transfected with wildtype or mtGPR143-EYFP and tyrosinase-ECFP. The EYFP photobleaching was performed and detected in delimited regions (highlighted and zoomed in the pictures). The fluorescence of GPR143 (YFP channel) and tyrosinase (TYR; CFP channel) were detected before and immediately after the acceptor photobleaching. Controls are shown in Figure S7. Scale bar = 20 μm. FRET, fluorescence resonance energy transfer; mt, mutant; TYR, tyrosinase; wt, wildtype.

Figure S7


CFP channel
YFP channel
ratio
fluorescence intensity
AFTER/BEFORE bleaching
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
fusion protein
fusion protein
TYR
TYR
mtGPR143
mtGPR143


TYR
zoom


mtGPR143
zoom

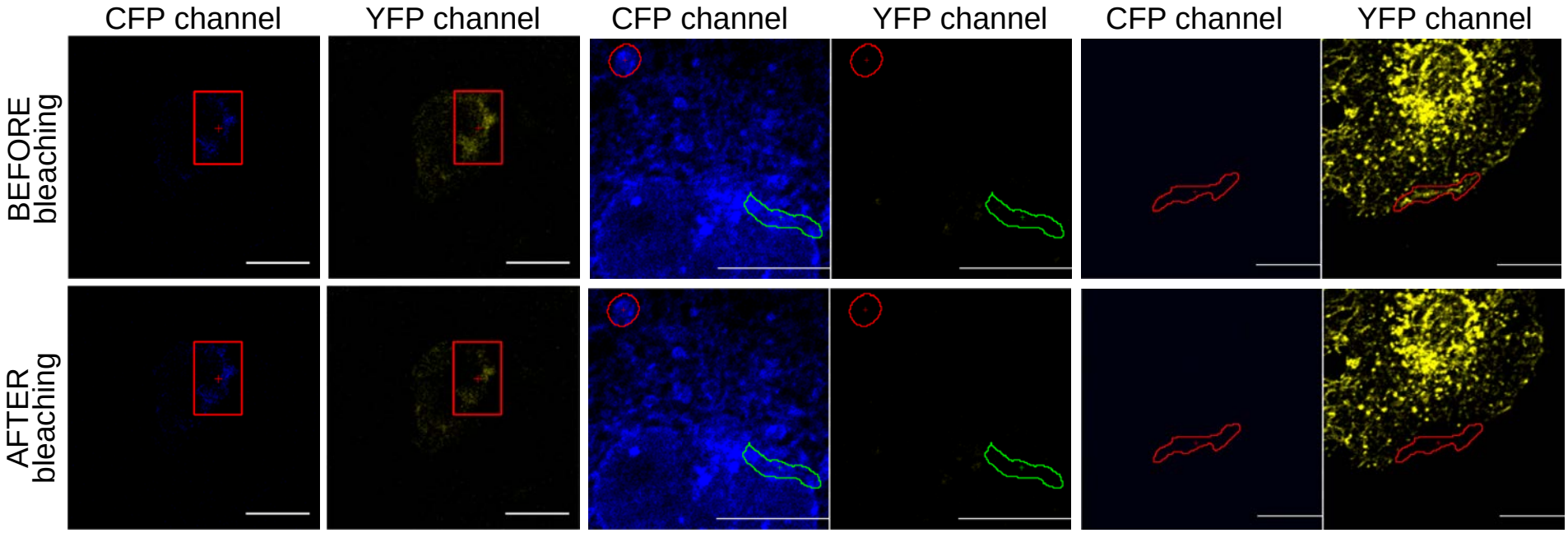

CFP channel
YFP channel
CFP channel
YFP channel
CFP channel
YFP channel
BEFORE bleaching
AFTER bleaching
CFP-YFP fusionprotein
TYR-CFP
Single transfection
mtGPR143-YFP
Single transfection

Figure S7. Control images of FRET acceptor photobleaching. The EYFP photobleaching was performed and detected in delimited regions (highlighted and zoomed in the pictures) of COS7s transfected with either ECFP-EYFP fusion protein (left panel) or tyrosinase-ECFP (middle panel) or mtGPR143-EYFP (right panel). Images of GPR143 (YFP channel) and TYR (CFP channel) fluorescence are shown before and immediately after the acceptor photobleaching. Data analysis of the controls is plotted in the inset graph. For corresponding FRET efficacies see Figure 5b. Scale bar = 20 μm. FRET, fluorescence resonance energy transfer; mt, mutant; TYR, tyrosinase; wt, wildtype.

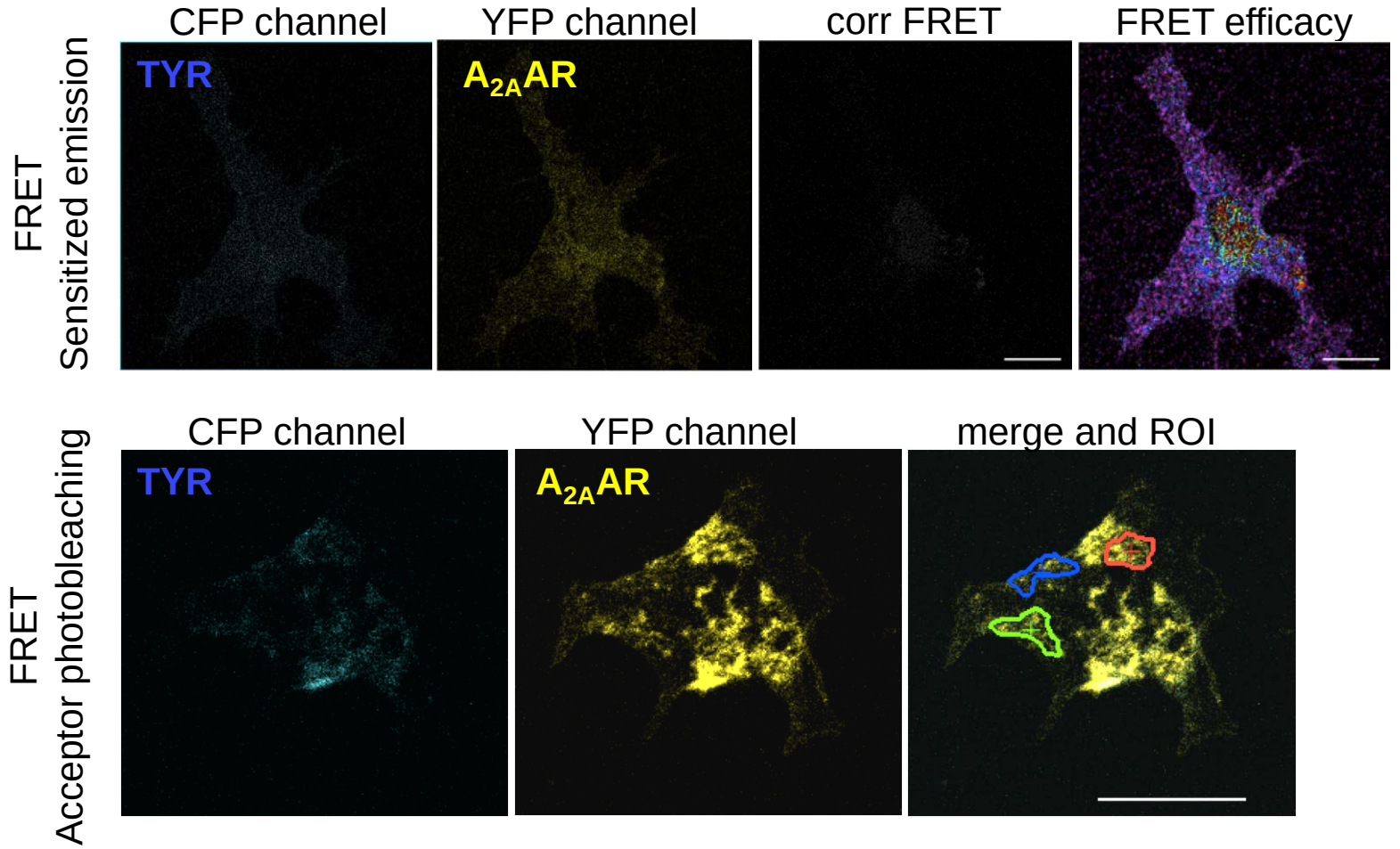


Figure S8. Negative control images of FRET. Sensitized emission method (upper panel) was used to detect interaction of adenosine A_{2A} receptor (YFP channel) and tyrosinase (CFP channel) in COS7s transfected. FRET signal, corrected by CoA and CoB parameters, and FRET efficiency (color scale on the far right) are shown. The EYFP photobleaching (lower panel) was performed and detected in delimited regions (highlighted in the pictures) of COS7s transfected with TYR-ECFP + A_{2A}AR-EYFP. Images of YFP channel and CFP channel fluorescence are shown before the acceptor photobleaching. Data analysis of the controls (fluorescence ratio and FRET efficacy) is plotted in Figure 5. Scale bar = 20 μm. A_{2A}AR, adenosine receptor A_{2A}, FRET, fluorescence resonance energy transfer; mt, mutant; TYR, tyrosinase; wt, wildtype.