Materials and Methods:

**Reagents-** 17β-estradiol, 17α-estradiol, 4-hydroxytamoxifen, LY294002, Pluronic®, Dulbecco’s modified Eagle’s medium (DMEM) were from Sigma. Pertussis toxin, tyrophostin AG1478 and U73122 were from Calbiochem. ICI 182,780 was from Tocris Chemicals. Mitotracker Red (chloromethyl X-rosamine), indo-1 AM, Alexa Fluor carboxylic acid succinimidyl esters, rhodamine phalloidin, TO-PRO-3 and Alexa568-conjugated secondary antibodies were from Molecular Probes. Anti-KDEL antibody was purchased from Stressgen Biotechnologies. Peptides representing the amino and carboxy termini of GPR30 (MDVTSQARGVGLEMPGAQPAAC and CAVIPDSTEQSDVRFSSAV, respectively) with an added cysteine residue for KLH conjugation, were synthesized and used for antibody production in rabbits by New England Peptide Inc.

**Cell culture and transfection-** Cell lines were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS until 24 to 48 h prior to experimentation, when they were placed in serum-free and phenol red-free DMEM/Ham’s F-12 medium. Transient transfection experiments were performed 24 h after seeding cells using LipofectAMINE 2000 (Invitrogen) according to manufacturer’s instructions.

**Construction of expression vectors-** GPR30, mRFP1 and ERα/β-GFP cDNA were gifts from Drs. Weigel (S1), Tsien (S2), and Matsuda (S3), respectively. pEYFP-ER (an endoplasmic reticulum-targeted YFP) was from Clontech. TGN38-GFP and PH-GFP, from which PH-mRFP1 was generated by PCR, were gifts from Drs. V. Deretic and Balla (S4), respectively. GPR30-GFP/RFP and amino terminally FLAG-tagged and carboxy-terminally truncated GPR30 constructs were generated by PCR and confirmed by dideoxy sequencing. Antisense vectors for GPR30 and the β2-adrenergic receptor were generated by cloning of the entire open reading frame of the receptor cDNA in the reverse orientation in pcDNA3.1 Hygro (−).
Localization of GPR30- COS7 cells were cotransfected with GPR30-mRFP1 and β2-adrenergic receptor-GFP, TGN38-GFP or pEYFP-ER. The cells were reseeded on to 12 mm glass coverslips 24 h after transfection. The cells on coverslips were then fixed with 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and mounted in Vectashield (Vector Laboratories). Laser confocal scanning images were obtained using a Zeiss LSM 510 confocal system. For studying localization of GPR30 with respect to mitochondria, COS7 cells expressing GPR30-GFP were loaded with 25 nM Mitotracker Red for 20 min at 37°C. The cells were then fixed and visualized by confocal microscopy. For F-actin staining, cells on coverslips, transiently expressing GPR30-GFP were fixed with 2% PFA in PBS for 15 min at 37°C. The cells were stained with actin staining solution (100 µg of lysolecithin, 0.05% BSA and 2 units of rhodamine phalloidin (dissolved in methanol) in one ml of PBS) for 10 min at 37°C. BSA (2%) in PBS was added for 2 min at 37°C, and the coverslip was washed three times and mounted in Vectashield (S5).

Immunofluorescence staining- Cells expressing GPR30 were plated on 12 mm coverslips and fixed using 2% PFA in PBS, followed by blocking and permeabilization with 0.25% Triton X-100 in PBS with 3% bovine serum albumin. The primary antibody, diluted in 3% goat serum, was applied for 2 h at room temperature. The cells were subsequently washed three times with PBS and incubated with the appropriate secondary antibody diluted in 3% goat serum. The coverslips were washed three times with PBS and mounted using Vectashield. Confocal images were collected on a Zeiss LSM 510 confocal system. For quantitation of GPR30 expression, cells were stained using the anti-carboxy terminal antibody and analyzed on a FACS Calibur using Cell Quest software.

Estrogen derivative synthesis, labeling and purification- 17α-[4-aminomethylphenylethynyl]-estra-1,3,5(10)-triene-3, 17β-diol was produced as described (S6) . Briefly, a solution of palladium acetate and triphenylphosphine in diethylamine was stirred under argon for 10 min. Copper(I) iodide and N-(tert-butoxycarbonyl)-4-bromobenzylamine were added. After 5 min of stirring, 17α-ethynylestradiol was added
and the solution was stirred for an additional 4 h at 60° C. The volatiles were removed \textit{in vacuo}, and the residue was chromatographed to yield 17α-[N-(tert-butoxycarbonyl)-4-aminomethyl-phenylethynyl]-estra-1,3,5(10)-triene-3,17β-diol, which was subsequently dissolved in ethanol and acidified by dropwise addition of conc. HCl. The reaction was stirred at room temperature for 7 h and the mixture was concentrated in vacuo followed by precipitation upon addition of water to yield the final product 17α-[4-aminomethyl-phenylethynyl]-estra-1,3,5(10)-triene-3,17β-diol hydrochloride. This estrogen derivative was conjugated to Alexa Fluor carboxylic acid succinimidyl esters (Alexa-546 and Alexa-633) in DMSO at room temperature for 16 h in the dark. The Alexa conjugates were purified by reverse phase HPLC using a Symmetry 300 C18 column (Waters).

\textbf{Ligand binding assay} - COS7 cells expressing nuclear estrogen receptor (ER)-GFP or GPR30-GFP were serum starved for 24 h before the experiment. For microscopic assays, the cells were seeded on 12 mm coverslips and treated with 2-5 nM E2-Alexa 633 or E2-Alexa 546 diluted in permeabilization buffer (0.025 % saponin, 5 mM EGTA, 100mM NaCl and 1 mM MgCl$_2$ in 80 mM piperazine-N-N′-bis(2-ethane sulfonic acid)-KOH (pH 6.8)) for 10 min at 37°C. For staining cells without permeabilization E2-Alexa 633 or E2-Alexa-546 was diluted in permeabilization buffer lacking saponin and incubated for 10 min at 37°C. To determine the specific binding of E2-Alexa-546 and E2-Alexa-633, binding was carried out in presence of 1 µM 17β-estradiol. The cells on coverslip were fixed with chilled 2% PFA in PBS with 1 mM CaCl$_2$ and 1 mM MgCl$_2$ for 15 min at 37°C. For flow cytometric analysis of E2-Alexa-633 binding to GPR30-GFP or ER-GFP expressing cells, the cells were washed once with PBS and analyzed on FACS Calibur using Cell Quest. Nonspecific binding was determined in presence of 1 µM 17β-estradiol. Competition binding was carried out in the presence of the indicated concentration of 17β-estradiol.

\textbf{Intracellular calcium mobilization} - Cells (5 X 10$^6$) were incubated at room temperature in HBSS containing 5 µM indo1-AM and 0.05% pluronic acid. After 30 min cells were washed once with HBSS and resuspended in HBSS at a density of 10$^7$ cells/mL. Relative and absolute Ca$^{++}$ measurements were determined ratiometrically using $\lambda_{ex}$ 340 nm and $\lambda_{em}$
\( \lambda_{em} \) 400, 490 nm at 37°C in a spectrofluorometer (PTI QM-2000-2) equipped with a magnetic stirrer and temperature control. The relative 490nm/400nm ratio is plotted as a function of time. Absolute intracellular calcium concentrations were determined according to established methods (S7).

**Activation of PI3kinase and PIP3 accumulation**- The PIP3 binding domain of Akt fused to mRFP1 (PH-mRFP1) was used to assess PIP3 production by PI3K. COS7 cells cotransfected with GPR30-GFP or ERα-GFP and PH-mRFP1 were plated on coverslips and serum starved for 24 h. They were then treated with the indicated inhibitor and stimulated with ligands as indicated in the figure legend. In some merged images, the location of the nucleus is shown by staining with TO-PRO-3. The cells on coverslips were fixed with 2% PFA in PBS, washed and mounted in Vectashield. The cells were analyzed by confocal microscopy.

**Inhibitors**- Inhibitors were used as follows (concentration, preincubation time): pertussis toxin (200ng/mL, overnight), U73122 (10\( \mu \)M, 20 min), AG1478 (25 \( \mu \)M, 60 min), LY294002 (10\( \mu \)M, 20 min). All inhibitors were present during ligand stimulation.

**References and Notes**


