Nigericin	Calbiochem-Novabiochem, Bad Soden Germany
Nitrocellulose membrane	Schleicher & Schuell, Dassel, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
pEGFP(YFP-CFP)-N1 vector	Clontech, Heidelberg, Germany
Penicillin	Sigma-Aldrich, Steinheim, Germany
Phosphoric acid	Merck, Darmstadt, Germany
Ponceau S	Roche Diagnostics, Mannheim, Germany
Potassium cloride	Sigma-Aldrich, Steinheim, Germany
Kaleidoscope prestained marker	Bio Rad, Munich, Germany
PVDF-filters, Immobilon-P	Millipore Corporation, Bedford, USA
Sodium chloride	Sigma-Aldrich, Steinheim, Germany
Sodium citrate	Merck, Darmstadt, Germany
Sodium hydroxide	Carl Roth, Karlsruhe, Germany
Sodium phosphate	Merck, Darmstadt, Germany
Sodium-dihydrogen phosphate	Merck, Darmstadt, Germany
Sodiumdodecyl sulfate (SDS)	Sigma-Aldrich, Steinheim, Germany
Sodim-hydrogen carbonate	Sigma-Aldrich, Steinheim, Germany
Streptomycine	Sigma-Aldrich, Steinheim, Germany
Tetramethylendiamine (TEMED)	Sigma-Aldrich, Steinheim, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Tween-20	Sigma-Aldrich, Steinheim, Germany
Zeocin	Invitrogen**,** Leek, The Netherlands

Antibodies:

monoclonal c-myc (9E10) antibody	Roche Diagnostics, Mannheim, Germany
monoclonal FLAG (M2) antibody	Sigma-Aldrich, Steinheim, Germany
monoclonal GFP (JL-8) antibody	BD Biosciences
rabbit anti-NT-ET_A antibody	Dr.Jens Furkert, FMP-Berlin, Germany
rabbit anti-CT-ET_A antibody	Dr.Jens Furkert, FMP-Berlin, Germany
rabbit anti-NT-ET_B antibody	Dr.Jens Furkert, FMP-Berlin, Germany
rabbit anti-CT-ET_B antibody	Dr.Jens Furkert, FMP-Berlin, Germany

2. Materials and Experimental procedures

2.1 Material

2.1.1 Chemicals, antibodies, cDNA's and receptor ligands

Acetic acid	Merck, Darmstadt, Germany
Acrylamide, Bisacrylamide	Serva, Heidelberg, Germany
Ammoniumpersulfat (APS)	Sigma-Aldrich, Steinheim, Germany
Aprotinin	Merck, Darmstadt, Germany
Bacitracin	Merck, Darmstadt, Germany
Bovine serum albumine (BSA)	Sigma-Aldrich, Steinheim, Germany
Disodium hydrogenphosphate	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, Steinheim, Germany
Dried milk powder (low-fat)	Néstle AG, Frankfurt, Germany
Dulbecco's modified Eagle's medium	Sigma-Aldrich, Steinheim, Germany
Ethanol	J.T. Baker, Deventin, The Netherlands
Ethylendiamine-tetraacetate (EDTA)	Sigma-Aldrich, Steinheim, Germany
Ethylene glycol bis-tetraacetate (EGTA)	Sigma-Aldrich, Steinheim, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
FuGENE 6 Transfection Reagent	Roche Diagnostics, Mannheim, Germany
Glucose	Sigma-Aldrich, Steinheim, Germany
Glutamine	Sigma-Aldrich, Steinheim, Germany
Glycerin	Sigma-Aldrich, Steinheim, Germany
Glycine	Carl Roth, Karlsruhe, Germany
Hydrochloric acid	Merck, Darmstadt, Germany
IGEPAL CA-630 (NP-40)	Sigma-Aldrich, Steinheim, Germany
Isopropanol	J.T. Baker, Deventin, The Netherlands
LipofectAMINE	Invitrogen
Lumi-Light solution	Roche Diagnostics, Mannheim, Germany
Methanol	J.T. Baker, Deventin, The Netherlands
Monensin	Calbiochem-Novabiochem, Bad Soden, Germany

Peroxidase-conjugated goat anti-rabbit antibodies (F_{ab} fragments)	Dianova, Hamburg, Germany
Peroxidase-conjugated donkey anti-mouse antibodies (F_{ab} fragments)	Dianova, Hamburg, Germany

cDNAs, constructs:

Epidermal growth factor receptor cDNA (HER1)	kindly provided by Dr. Alexander Sorkin, Health Science Center,University of Colorado, Denver, USA
K44A.dynamin I	kindly provided by Dr. S. L. Schmid, The Scripps Research Institute, La Jolla, CA

Endothelin receptor ligands:

BQ123	Alexis, Läufelfingen, Switzerland
BQ788	Calbiochem-Novabiochem, Bad Soden Germany
BQ3020	was synthesized using the solid phase method, Calbiochem-Novabiochem, Bad Soden, Germany
ET-1	was synthesized using the solid phase method, Calbiochem-Novabiochem, Bad Soden, Germany
ET-3	Calbiochem-Novabiochem, Bad Soden Germany
^{125}I-ET-1 (2000 Ci/mmol)	Amersham Pharmacia Biotech, Freiburg, Germany
^{125}I-ET-3 (2000 Ci/mmol)	Amersham Pharmacia Biotech, Freiburg, Germany
^{125}I-PD151242 (2000 Ci/mmol)	Amersham Pharmacia Biotech, Freiburg, Germany

2.1.2 Apparatus and software

AA-gel cast-stand	BIORAD Laboratories
AA-gel electrophoresis chamber	BIORAD Laboratories
Centrifuges	Beckmann TLK 100
	Beckmann Optima L70
	Sorvall RC 285
	Haereus Biofuge pico
Lumi-Imager F1	Roche Diagnostics
Microscopes	Zeiss 510 META inverted confocal laser scanning microscope
	Zeiss Axiovert 100
Photometer	GeneQuantII, Pharmacia Biotech
Pipettes	Eppendorf
Power supplies	Bio-Rad, Amersham Pharmacia Biotech
Rotator	Stuart Scientific, Blood tube rotator SB1
Scintillator	Wallac 1409
Semi-dry Western Blot chamber	Trans Blot SD, Bio-Rad
Sonicator	Sonoplus UW 2040, Bandelin Electronics
Thermomixer	Eppendorf Thermomixer 5436

Software:

Axio Vision	Zeiss
Excel 2000, Excel X	Microsoft
GraphPad Prism 3.02	GraphPad software
Photoshop 5.0, Photoshop 4.0	Adobe
Powerpoint 2000, Powerpoint X	Microsoft
Word 2000, Word X	Microsoft

2.1.3 Cells

HEK293 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

2.2 Commonly used buffers

10 x blot buffer:
112 g glycine
22 g tris-base
ad 1 liter de-ionized H_2O

1 x blot buffer:
100 ml 10x blot buffer
200 ml methanol
ad 1 liter de-ionized H_2O

10 x gel running buffer:
60 g tris-base
280 g glycine
10 g SDS
ad 1 liter de-ionized H_2O

1 x gel running buffer:
100 ml 10x gel running buffer
ad 1 liter de-ionized H_2O

4 x Laemmli sample buffer (Laemmli buffer):
10% β-mercaptoethanol (w/v)
4% SDS (w/v)
2% bromphenol blue (w/v)
20% glycerol (w/v)
250 mM Tris, pH 6.8

Native lysis buffer :

20 mM Tris-HCl, pH 7.5
1% (w/v) IGEPAL CA-630 (NP-40)
1 mM ethylene glycol tetraacetic acid
1 mM ethylenediamine tetraacetic acid
1 mM dithiothreitol
0.5% (w/v) sodium deoxycholate
0.1% (w/v) SDS
1.5 mM $MgCl_2$
150 mM NaCl
0.5 mM phenyl methyl sulforyl fluoride
2 mg/ml soy bean trypsin inhibitor
1.43 mg/ml aprotinin
0.5 mM benzamidine

PBS :
137 mM NaCl
2.7 mM KCl
1.5 mM KH_2PO_4
8.0 mM Na_2HPO_4
pH 7.4

PBST :
PBS
0.1% (w/v) SDS
1% (w/v) IGEPAL CA-630 (NP-40)

Tris-BAME:
50 mM Tris
2 mM EGTA
10 mM $MgCl_2$
0.15 mM Bacitracin
0.0015 % (w/v) Aprotinin

2.1.3 Plasmids/constructs

Plasmid/Vector	Encoded protein	Source
ET_Amyc.CFP (pEGFP-N1, BD Biosciences, Heidelberg, Germany)	ET_A receptor with an N-terminal c-myc epitope and a C-terminal CFP moiety.	Plasmid kindly provided by Dr. Alexander Oksche, FMP Berlin, Germany.
ET_B.CFP/ ET_B.GFP/ ET_B.YFP (pEGFP-N1, BD Biosciences, Heidelberg, Germany)	ET_B receptor with a C-terminal CFP, GFP or YFP moiety.	Plasmids kindly provided by Dr. Alexander Oksche, FMP Berlin, Germany.
ET_Bflag.YFP (pEGFP-N1, BD Biosciences, Heidelberg, Germany)	ET_B receptor with an N-terminal FLAG epitope and a C-terminal YFP moiety.	Plasmid kindly provided by Dr. Alexander Oksche, FMP Berlin, Germany.
ET_Amyc (pcDNA3.1, Invitrogen, Leek, The Netherlands)	ET_A receptor with an N-terminal c-myc epitope.	Plasmid kindly provided by Dr. Alexander Oksche, FMP Berlin, Germany.
ET_Bflag (pcDNA3.1, Invitrogen, Leek, The Netherlands)	ET_B receptor with an N-terminal FLAG epitope.	Plasmid kindly provided by Dr. Alexander Oksche, FMP Berlin, Germany.

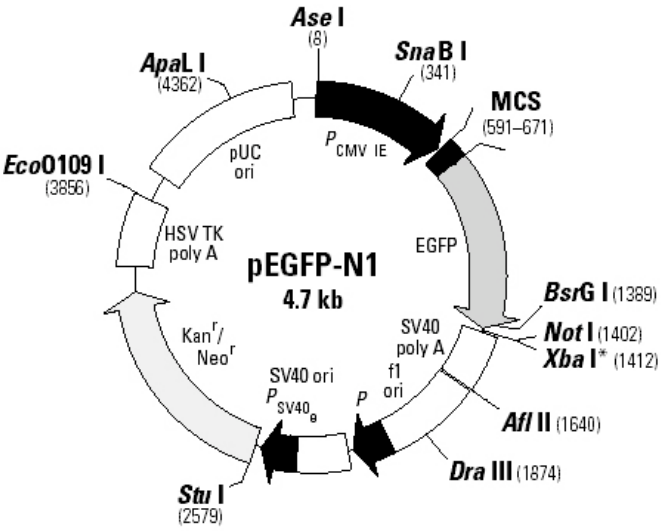

Fig. 4. **Model of the pEGFP-N1 vector.**
The figure above summarizes the features of the pEGFP-N1 vector.

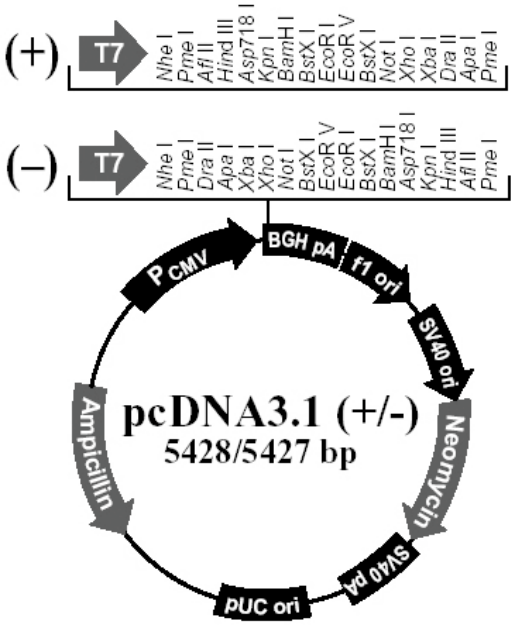

Fig. 5. **Model of the pcDNA3.1 vector.**
The figure above summarizes the features of the pcDNA3.1 (+) vector.

2.2 Experimental procedures

2.2.1 Cell culture

HEK293 cells (obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were maintained in DMEM medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate at 37°C in a humidified atmosphere of 95% air / 5% CO_2. For fluorescence microscopy or laser scanning microscopy, cells were grown on glass cover slips for 48 h. For biochemical analyses, cells were grown for 48-72 h until 80% confluence was achieved.

2.2.2 Peptide synthesis and fluorescence labeling

ET-1 and BQ3020 (N-acetyl-[Ala^{11,15}]-6-21-endothelin-1) were synthesized using the solid phase method (chlorotrityl-resin, 1.05 mmol/g; Calbiochem-Novabiochem) and standard 9-fluorenyl-methoxy-carbonyl chemistry (double couplings with 8 Eq of 9-fluorenyl-methoxy-carbonyl-amino acid derivates). After the final cleavage/deblocking, the crude peptide (50 mg) was dissolved in 500 ml of aqueous 4 mM $NaHCO_3$ solution and kept for two days at room temperature. The final purification was carried out by preparative HPLC (Polyencap A 300, 250 x 20 mm) applying a linear gradient 20 to 60% within 70 min [A, trifluoroacetic acid/water (0.1:99, v/v); B, trifluoroacetic acid/acetonitrile/water (0.1:80:19.9, v/v/v)]. The masses of purified ET-1 and BQ3020 were verified by electrospray mass spectrometry. Fluorescence labeling of ET-1 and BQ3020 was carried out by selective modification of the ε-amino group of Lys-9 of ET-1 and Lys-4 of BQ3020 using Cy3 monoreactive succinimidyl ester (Amersham Biosciences) in 0.1 M $NaHCO_3$ at pH 9.3 followed by preparative HPLC purification.

2.2.3 Transient and stable transfection of HEK293 cells

For transient transfection, FuGENE6 was used according to the instructions of the manufacturer (4 µl FuGENE 6/1 µg DNA). DNA if required was diluted in sterile de-ionized water and pipetted into a reaction tube. FuGENE 6 was diluted in serum- free media (in 100 µl per 25 mm or 700 µl per 92 mm culture dish) and incubated for 5 min at room temperature before plasmid DNA was added, followed by an incubation for 30 min at room temperature. Finally the mix was added to cells at 50–80% confluency.

Stable transfection of HEK293 cells with LipoFECTAMINE:

1st. day: 200 000 cells were seeded in a 60 mm dish

2nd. day: 2 µg DNA was diluted in 100 µl serum-free medium without antibiotics (SFM) and 7.5 µl LipoFECTAMINE was diluted in 100 µl SFM. Both solutions were mixed and incubated for 30 min at room temperature. 1800 µl SFM was added and the mixture was pipetted to cells (which were washed before one time in SFM). Following incubation for 4 to 6 hours at 37°C the medium was replaced by 5 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate.

Cells were grown for 2-3 days before they were split in selection medium containing G418 (400 µg/ml) or Zeocin (17 µg/ml).

The selection medium was changed every 3-4 days and after 10 days resistant cell clones were picked with sterile Q-tips and transferred to 24 well plates containing 1 ml selection medium per well. Cell clones were tested in binding studies and/or fluorescence microscopy.

2.2.4 Generation and affinity-purification of polyclonal antibodies

A polyclonal NT-ET_B serum was raised against a synthetic peptide corresponding to the amino acids 19-37 in the N terminus of the ET_B receptor (P24530; CGLSRIWGEERGFPPDRATP) coupled to the carrier protein keyhole limpet hemocyanin (KLH; Calbiochem-Novabiochem). NT-ET_B antibody was purified with the synthetic N-terminal peptide conjugated to protein Sepharose 6B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturers protocol. The IgG fraction obtained was dialyzed against sodium phosphate buffer (20 mM sodium phosphate buffer, 150 mM NaCl, pH 7.5) and stored in aliquots at –20°C.

Buffers used for the affinity-purification of antibodies:

Binding buffer:	20 mM sodium phosphate buffer, pH 7-7.5
Elution buffer I:	0.2 M glycine 150 mM NaCl pH 2.5
Elution buffer II:	0.2 M glycine 150 mM NaCl pH 2.25
Strip buffer:	0.3 M $NaHCO_3$ 1 mM EDTA pH 8.4

Wash buffer: 0.1 M acetic acid
0.5 M NaCl
1 mM EDTA
50 mM HEPES, pH 8.0
PBS + 0.5% Tween 20
2M Tris-HCl, pH 9.0

Preparation of the sepharose for peptide binding:

250–500 mg thiopropyl sepharose 6B (Pharmacia Biotech AB, Uppsala, Sweden) was mixed with 12 ml H_2O in a 15 ml reaction tube and incubated on ice for 10 min. The sepharose was precipitated by centrifugation for 3 min at 780 x g. Then the following washing steps were performed:
2 x 12 ml H_2O
1 x 12 ml strip buffer.
After centrifugation the sepharose was incubated in 12 ml strip buffer for 40 min at room temperature on a rotator wheel, followed by two additional washing steps with washing buffer. Prior to peptide binding the sepharose was equilibrate with three washes of binding buffer (4°C).

Binding of the peptide:

5 mg of the peptide was dissolved in 400 µl sterile water and the pH was titrated to pH 7–7.5 with 50 mM HEPES, pH 8.0. The sepharose was resuspended in the peptide solution and incubated for 1 hour at 4°C in a rotator wheel. The following wash steps were performed prior to the addition of the antiserum:

1 x 12 ml binding buffer (4°C)
1 x 12 ml elution buffer, pH 2.5 (4°C)
3 x 12 ml binding buffer (4°C)

Binding of the antibodies:

5 to 10 ml of the antiserum were added to the sepharose and incubated in a rotator wheel at 4°C over night.
The following wash steps were performed:

5 x 10 ml PBS/0.5% Tween 20 (4°C)

Then the sepharose was dissolved in 10 ml PBS/0.5% Tween 20 and transfered to a column (BioRad) followed by an another wash with 10 ml PBS/0.5% Tween 20 prior to elution of the antibodies.

Elution of the antibodies:

To elute the antibodies elution buffer I and elution buffer II were applied to the column as follows.

3x 1 ml elution buffer I (pH 2.5)
1x 4ml elution buffer I
3x 1 ml elution buffer II (pH 2.25)
The flow-through was collected in 1.5 ml reaction tubes containing 50 µl 2 M Tris (pH 9) buffer to adjust the pH of the acidic eluate to pH 7–7.5.

Concentration of the eluated antibodies:

To concentrate the antibodies the eluate (in 2 ml aliquots) was pipetted to Centricon-tubes (Centricon YM 30, Millipore, 30 kDa cut-off) and centrifuged at 4500 x g for 30 min at 4°C. Centricon concentrators can concentrate proteins on the basis of molecular weight. The buffer of the antibodies was changed by multiple centrifugation steps with binding buffer (approx. 10 ml). Finally the

concentrated antibodies were recovered by a reverse spin at 1000 x g for 15 min. at 4°C and 0.05% Na-acid was added.

2.2.5 Immunoblots for the detection of ET_B.YFP and ET_Amyc.CFP

HEK293 cell clones stably expressing the fusion proteins were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.4) and harvested with lysis buffer. To remove cell debris the lysates were centrifuged for 15 min at 14, 000 rpm. Lysates (30 µg protein per lane) were separated by SDS-PAGE (10% gels, 20 mA per gel) and transferred (100 mA per gel) to nitrocellulose filters (Schleicher und Schuell, Dassel, Germany). Filters were blocked for 1 h at room temperature in PBS/5% milk/5% FCS/0.05% Tween 20 and then probed for 1 h at room temperature or alternatively for 16 h at 4°C with polyclonal NT-ET_B antibody (diluted 1 : 5000 in PBS/5% milk), monoclonal c-myc or monoclonal GFP antibodies (diluted 1 : 2000 in PBS/5% milk). Filters were then washed for 15 min with PBST (PBS + 0.1% SDS, 1% NP-40) followed by PBS wash and a final PBST wash. Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG or with horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories, USA). Secondary antibodies were diluted 1 : 2000 in PBS + 5% milk, and filters were probed for 20 min at room temperature. Finally the filters were washed alternately for 15 min in PBST, PBS, PBS and antibodies were detected using Lumi-Light Western blotting substrate (Roche Diagnostics).

2.2.6 Immunoprecipitation experiments

HEK293 cell clones were grown in 75 cm^2 cell culture flasks for 48 h to near confluence. Cells were washed twice with PBS, and lysis buffer (1.2 ml) was added. The cells were then harvested with a rubber policeman, homogenized five times by passage through a 27G needle and centrifuged (800 x g) for 10 min

at 4°C. The supernatant was transferred to a new reaction tube and centrifuged (26,000 x g) for 30 min. The new supernatant was mixed with the NT-ET_B antibody (diluted 1 : 2000 in PBS/5% milk) and protein A Sepharose (3.5 mg) in a final volume of 1.0 ml and incubated for 12 h in a shaker at 4°C. After 3 washes with lysis-buffer, the pellet was resuspended in 1 x Laemmli buffer and analyzed in immunoblot experiments.

2.2.7 Fluorescence resonance energy transfer (FRET)

FRET is a quantum mechanical process and is widely used as a biochemical tool to study protein-protein interactions. Excitation of a donor fluorophore can give rise to non-radiative transfer of the absorbed energy to an adjacent acceptor fluorophore provided that the emission spectrum of the donor overlaps sufficiently with the excitation spectrum of the acceptor. Direct energy transfer depends on the coupling of the respective dipole moments. Since the probability of energy transfer is proportional to orientation and inversely proportional to the sixth power of the distance ($1/R^6$) between the donor and the acceptor, FRET can be used to deduce the respective positional or rotational contexts of the two molecules. The positional constraints limit efficient FRET to distances of less than 10 nm and therefore sufficiently close to infer interaction. This makes FRET an ideal tool to study protein-protein interactions.

Cells were grown on glass coverslips for 48 h. Glass coverslips were mounted in a custom-made chamber and covered with incubation buffer (138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose, 2 mg/ml BSA and 10 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.5). FRET analysis was performed as described (Brock et al. 2003), using an inverted microscope Axiovert 100 equipped with a Plan-Apochromat 63x/1.4 objective (both Carl Zeiss, Göttingen, Germany). In brief, CFP and YFP were alternately excited at 410 and 515 nm with a monochromator (Polychrome II; TILL Photonics, Gräfelfing, Germany) in combination with a dual reflectivity dichroic mirror (<460 nm and 500-520 nm; Chroma Technology, Rockingham,

VT, USA). Emitted light was filtered through 475-505 nm (CFP) or 535-565 nm (YFP) band pass filters changed by a motorized filter wheel (Lambda 10/2; Sutter Instruments, Novato, USA), and detected with a cooled CCD camera (Imago; TILL Photonics). For FRET analysis, CFP (40 ms) and YFP (8 ms) emission without acceptor bleach were recorded during 20 cycles with a 10-20 ms exposure per cycle. Then, 60 cycles were recorded with an additional 2 s illumination per cycle at 512 nm to bleach YFP. FRET was assessed as recovery of CFP (donor) fluorescence during YFP (acceptor) bleach. The FRET efficiency E can be expressed as $E = 1 - (F_{DA} / F_D)$, where F_{DA} and F_D are intensities of the donor fluorescence in the presence and in the absence of the FRET acceptor, respectively. The increase in CFP fluorescence during acceptor photobleaching was plotted against the remaining YFP fluorescence, yielding a regression line, which served to extrapolate F_{DA} and F_D.

The influence of ligands on FRET efficiency was investigated by incubation of cells with 50 nM BQ3020, BQ788, BQ123 or ET-1 for 5 or 30 min at 37°C prior to FRET analysis. To clamp the pH at 7.5 or pH 5.0 in control experiments, monensin (10 µM) and nigericin (10 µM) were added. To block ligand-induced receptor sequestration, cells were co-transfected with ET_Amyc.CFP, ET_B.YFP and K44A.dynamin or incubated with 450 mM sucrose.

2.2.8 Receptor sequestration assay

HEK293 cell clones (40,000 cells/well) were seeded into 96-well plates. After 24 h, cells were stimulated with ET-1 (50 nM) or BQ3020 (50 nM) for up to 2 h at 37°C. The cells were then washed twice with PBS and fixed for 15 min at room temperature (2.5% formaldehyde in 100 mM sodium cacodylate, 100 mM sucrose, pH 7.5). After two rinses with PBS, monoclonal peroxidase-conjugated FLAG antibody (diluted 1 : 2000, Sigma) was added for 1 h at 37°C. Cells were washed twice with PBS and stained with 95 µl reaction buffer (200 mM citric acid, 200 mM K_2HPO_4, 0.01% H_2O_2) and 5 µl/well substrate solution (20 mM tetramethylbenzidine in propanol) for 15 min at room temperature. The

reaction was terminated with 100 µl/well H_2SO_4 (1.5 M). The absorbance at 450 nm was determined in an ELISA bio-kinetiks reader (Bio-Tek instruments, Winooski, VT, USA).

2.2.9 Fluorescence microscopy and image analysis

Living HEK293 cells grown on glass coverslips were analyzed with a Zeiss 510 META inverted confocal laser scanning microscope equipped with a Plan-Apochromat 63x/1.4 objective. Excitation and emission wavelengths were: λ_{exc} = 458 nm and λ_{em} = 470-505 nm for CFP, λ_{exc} = 488 nm and λ_{em} = 515-550 nm for YFP, and λ_{exc} = 543 nm, and λ_{em} > 570 nm for Cy3.

2.2.10 ^{125}I-ET-1 displacement binding experiments

Radioligand experiments were performed as described (Oksche et al. 2000). In brief, membranes (0.1-0.5 µg) were incubated in 200 µl Tris/BAME buffer containing 50 pM of ^{125}I-ET-1 without or with increasing concentrations of unlabelled ligand (1 x 10^{-13} to 1 x 10^{-4} M) for 3 h at 25°C in a shaking water bath. The samples were then transferred to GF/C filters (Whatman, Maidstone, UK) pretreated with 0.1% (w/v) polyethylenimine and washed twice with PBS using a Brandel cell harvester. Filters were finally transferred into 5 ml vials and radioactivity was determined in a γ–counter. Data were analyzed with RadLig Software 4.0 (Cambridge, UK), and graphs were generated with Prism Software 2.01 (GraphPad, San Diego, USA).