

## G Protein-Coupled Estrogen Receptor 1 (GPER1)/GPR30 Localizes in the Plasma Membrane and Trafficks Intracellularly on Cytokeratin Intermediate Filaments

Caroline Sandén<sup>1</sup>, Stefan Broselid<sup>1</sup>, Louise Cornmark, Krister Andersson, Joanna Daszkiewicz-Nilsson, Ulrika E.A. Mårtensson, Björn Olde, and L.M. Fredrik Leeb-Lundberg

Department of Experimental Medical Science, Lund University, SE-22184 Lund, Sweden

**Running title:** GPER1/GPR30 trafficking

**Address correspondence to:** L.M. Fredrik Leeb-Lundberg, Department of Experimental Medical Science, Lund University, BMC A12, SE-22184 Lund, Sweden. Tel: +46-46-2223944; Fax: +46-46-2220568; E-mail: [fredrik.leeblundberg@med.lu.se](mailto:fredrik.leeblundberg@med.lu.se)

**Document information:**

Number of text pages (Introduction through Discussion) – 17

Figures – 8

Tables – 0

References – 47

Number of words in:

Abstract – 226

Introduction – 534

Discussion – 1534

**Nonstandard abbreviations:** GPER1, G protein-coupled estrogen receptor 1; B2 bradykinin receptor, B2R; CK, cytokeratin; E2, 17 $\beta$ -estradiol; ER, endoplasmatic reticulum; PM, plasma membrane; HEK293 cells, human embryonic kidney cells; MDCK cells, Madine Darby canine kidney cells; GPCR, G protein-coupled receptor; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecylsulphate; TBS, tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PNGase F, peptide:N-glycanase.

## Abstract

GPR30, or G protein-coupled estrogen receptor 1 (GPER1), was recently introduced as a membrane estrogen receptor and a candidate cancer biomarker and therapeutic target. However, several questions surround the subcellular localization and signaling of this receptor. In native cells, including mouse myoblast C<sub>2</sub>C<sub>12</sub> cells, Madine-Darby canine kidney (MDCK) epithelial cells, and human ductal breast epithelial tumor T47-D cells, G-1, a GPER1 agonist, and 17 $\beta$ -estradiol (E2) stimulated GPER1-dependent cAMP production, a defined plasma membrane (PM) event, and recruitment of  $\beta$ -arrestin2 to the PM. Staining of fixed and live cells showed that GPER1 was localized both in the PM and on intracellular structures. One such intracellular structure was identified as cytokeratin (CK) intermediate filaments, including those composed of CK7 and CK8, but apparently not endoplasmatic reticulum (ER), Golgi, or microtubules. Reciprocal co-immunoprecipitation of GPER1 and CKs confirmed an association of these proteins. Live staining also showed that the PM receptors constitutively internalize apparently to reach CK filaments. Receptor localization was supported using FLAG- and HA-tagged GPER1. We conclude that GPER1-mediated stimulation of cAMP production and  $\beta$ -arrestin2 recruitment occur in the PM. Furthermore, the PM receptors constitutively internalize and localize intracellularly on CK. This is the first observation that a G protein-coupled receptor (GPCR) is capable of associating with intermediate filaments, which may be important for GPER1 regulation in epithelial cells and the relationship of this receptor to cancer.

## Introduction

GPR30, or GPER1, is a GPCR that was recently proposed to be an estrogen receptor responsible for at least some non-genomic estrogen signaling (Filardo and Thomas, 2005; Prossnitz et al., 2008). Estrogens are important sex hormones in both genders that have long been recognized to act through both genomic and non-genomic mechanisms. The genomic mechanisms are the best described and involves the binding of estrogens to two nuclear estrogen receptors, ER $\alpha$  and ER $\beta$ , which function as nuclear transcription factors regulating gene expression (Heldring et al. 2007). Membrane-associated full-length ER $\alpha$  also exists that is at least in part responsible for non-genomic estrogen signaling (Razandi et al., 2004).

GPER1 is ubiquitously expressed in both human and rodents, and GPER1-deficient mice show that this receptor may participate in metabolic, cardiovascular, bone, and immune regulation, at least in part in an estrogen-dependent manner (Olde and Leeb-Lundberg, 2009; Mårtensson et al., 2009, Windahl et al., 2009). In addition, GPER1 was found to be associated with the growth of both breast and endometrial cancers (Filardo et al., 2006; Smith et al., 2007). An agonist, G-1, with selectivity for GPER1 over ER $\alpha$  and ER $\beta$ , was reported (Bologa et al., 2006) and is now being used extensively to study this receptor. Furthermore, some anti-estrogens, e.g. hydroxytamoxifen, act as agonists at this receptor (Maggiolini et al., 2004; Thomas et al., 2005).

At the cellular level, GPER1 was reported to bind E2 with high affinity (Thomas et al., 2005; Revankar et al., 2005), to influence growth factor signaling pathways including trans-activation of the EGF receptor, intracellular Ca<sup>2+</sup> mobilization, PI-3 kinase translocation, Src activation, ERK activation, and cAMP production (Filardo and Thomas, 2005; Prossnitz et al., 2008), and to modulate downstream transcription factor networks (Pandey et al., 2009). GPER1 was anti-proliferative in ER $\alpha$ - and ER $\beta$ -positive MCF-7 breast cancer cells but proliferative in ER $\alpha$ - and ER $\beta$ -negative SkBr3 breast cancer cells (Ariazi et al., 2010) suggesting that GPER1 function depends on the genetic environment of the cell.

Limited detailed studies have been done on the subcellular localization and membrane trafficking of GPER1, and then mostly in recombinant cells. Based on available studies, the localization of the receptor and receptor signaling is in debate with some groups stating that the receptor is present and functions exclusively intracellularly in the ER either with (Revankar et al., 2005) or without estrogen receptor functions (Otto et al., 2008), whereas others state that GPER1 is present and acts as an estrogen receptor in the PM (Funakoshi et al., 2005; Filardo et al., 2007), as would be expected of a typical GPCR.

Here, we used several native cell lines to show that GPER1 is functional and localizes in the PM and intracellularly on CK intermediate filaments. CKs are proteins important for the structural integrity primarily of epithelial cells. The human genome contains a total of 54 functional CK genes of which 37 are epithelial (Schweizer et al., 2006; Moll et al., 2008). Filamentous CK structures form by heteromeric pairing of acidic type I and basic or neutral type II CKs. Little is still known about CK beyond structural roles, but evidence is accumulating that CK may also serve additional roles as signaling platforms in cell adhesion, apoptosis/survival, and proliferation (Eriksson et al., 2009).

## Materials and Methods

*Cell culture and DNA constructs* – C<sub>2</sub>C<sub>12</sub> cells, MDCK cells, and HeLa cells (ATCC) were grown in phenol-free Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (HyClone) in 5% CO<sub>2</sub> at 37°C. T47-D cells (ATCC) were grown in RPMI 1640 media supplemented with 10% FBS and 10 µg/ml insulin in 5% CO<sub>2</sub> at 37°C. HEK293 cells (ATCC) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS in 10% CO<sub>2</sub> at 37°C. The human GPER1 cDNA was subcloned into the pIRESpuro vector (Clontech) containing a puromycin selection marker. The mouse GPER1 cDNA and human B2 bradykinin receptor (B2R) were subcloned into a pcDNA3.1 vector containing a zeosin selection marker. An N-terminal artificial signal sequence, as previously described (Whistler et al., 2002; Enquist et al., 2007), and the FLAG sequence tag were added in series to make the GPER1 construct FGPER1 and B2R construct FB2R. To make mouse GPER1 antisense cDNA, the mouse GPER1 sequence was amplified from a plasmid by PCR using primers: upper 5'-CAAGCGGCCGCTATGGATGCGACTACTCCAGC-3' and lower 5'-CAGAAGCTTAGCACTGCTGAACCTGACCT-3' containing a *NotI* and a *HindIII* site, respectively. The insert was then cloned in reverse orientation into the *NotI/HindIII* site of the pEAK12 vector. Clones containing mouse GPER1, in reverse orientation, were identified by *HindIII/NotI* digestion and sequencing using the BigDye terminator sequencing kit (PerkinElmer Life and Analytical Sciences). A cDNA construct of GPER1 tagged in the N terminus with three HA epitopes in series (HGPER1) was obtained from Missouri S&T cDNA Resource Center (Rolla, MO). A β-arrestin2-GFP cDNA construct was kindly provided by Dr. Marc Caron (Duke University Medical Center, Durham, NC) (Barak et al., 1997).

HEK293 cells were transfected with FGPER1 and FB2R cDNA using the calcium phosphate precipitate method and HeLa cells with human GPER1 by electroporation as previously described (Kotarsky et al., 2001). Single colonies were then chosen and propagated in the presence of selection-

containing media to generate clonal stable cell lines. C<sub>2</sub>C<sub>12</sub> cells, MDCK cells, and/or T47-D cells were transiently transfected with antisense GPER1 cDNA,  $\beta$ -arrestin2-GFP cDNA, FGPER1 cDNA, HGPER1 cDNA, and/or GPER1 siRNA using lipofectamine and lipofectamine PLUS (Invitrogen) or Fugene-6 (Roche) according to the manufacturer's instructions.

*RNA isolation and PCR* – Isolation of RNA was performed using a previously described method (Chomczynski and Sacchi, 1987). cDNA synthesis was performed using the Superscript first-strand synthesis system for PCR (Life Technologies). The GPER1 cDNA was amplified using PCR with GPER1 primers: upper 5'-TGGCTTTGTGGGCAACATCC-3' and lower 5'-GGTGCTTGGTGCGGAAGAGGC-3' (mouse), upper 5'-TCTACACCATCTTCCTCTTCC-3' and lower 5'-GTAGCGATCAAAGCTCATCC-3' (canine and human), and the products visualized on a 0.8% agarose gel.

*Immunoprecipitation and immunoblotting* – Confluent cells grown on 10-cm dishes were washed twice with ice-cold PBS and lysed in 0.5-1 ml lysis buffer (0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 25 mM KCl) with complete protease inhibitor cocktail (Roche). Lysates were cleared by centrifugation at 10,000 x g for 10 min at 4°C. Receptors were immunoprecipitated by incubating the cleared lysates overnight at 4°C with goat anti-GPER1 antibody (R&D Systems) coupled to protein G-Sepharose (GE Healthcare) or mouse anti-M2 FLAG agarose (Sigma-Aldrich) overnight at 4°C, and CKs were immunoprecipitated with mouse anti-pan CK (clone C-11, Sigma-Aldrich), mouse anti-CK7 (Sigma-Aldrich), or mouse anti-CK8 antibody coupled to protein G-Sepharose (Sigma-Aldrich). The precipitate was washed extensively and sequentially in the lysis buffer and in 10 mM Tris-HCl, pH 7.4. For immunoblotting, proteins were denatured in sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer including 6%  $\beta$ -mercaptoethanol for 30 min at 37°C, fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and the membrane blocked for at least 45 min in Tris-buffered saline (TBS) and 10% nonfat milk. The proteins were stained by incubating with goat anti-GPER1 antibody (1:200), mouse anti-M2 FLAG antibody (1:1000; Sigma-Aldrich), mouse HA.11 antibody (1:1000, Biosite Inc.), mouse anti-pan CK antibody (1:1000), mouse anti-CK7 antibody

(1:200), or mouse anti-CK8 antibody (1:200) for 1 h at 22°C. Immunoreactive bands were visualized with a chemiluminescence immunodetection kit using peroxidase-labeled secondary antibody (Invitrogen) according to the procedure described by the supplier (PerkinElmer Life and Analytical Sciences).

*Enzymatic deglycosylation* - To determine the presence of N-glycosylation in FGPER1, immunoprecipitates were treated with 500 units PNGase F (New England Biolabs) in 10 mM Tris-HCl, pH 7.4, for 2 h at 37°C.

*Immunofluorescence microscopy* – Cells were propagated to about 50% confluency in growth media on glass coverslips, coated with poly-D-lysine (Sigma-Aldrich) or 0.1% gelatin (Sigma-Aldrich), and then incubated in serum- and phenol-free media for at least 1 h at 37°C before treatment. For live cell staining, live cells were incubated in serum- and phenol-free media containing goat anti-GPER1 antibody (1:100) or mouse anti-M1 FLAG antibody (Sigma-Aldrich; 1:500) for 30 min at 37°C. In some experiments, live cells were treated with 0.4 M sucrose for 60 min at 37°C prior to incubation with antibody to disrupt clathrin-mediated endocytosis (Heuser and Anderson, 1989). Cells were then fixed with 3.7% formaldehyde in PBS and permeabilized with blotto (3% dry milk, 0.1% Triton X-100, 1 mM CaCl<sub>2</sub>, 50 mM Tris-HCl pH 7.4). For fixed cell staining, cells were incubated in serum- and phenol-free medium and then fixed and permeabilized. The cells were then incubated in blotto containing goat anti-GPER1 antibody (1:100), mouse anti-M1 FLAG antibody (1:500), and/or mouse HA.11 antibody (1:1000) for 1 h at 22°C. In all experiments, cells were then washed with PBS and receptors visualized by incubation with secondary Alexa488-labeled anti-goat antibody, anti-mouse IgG2b antibody (Invitrogen), or anti-mouse IgG1 antibody (Invitrogen). For colocalization studies, fixed and permeabilized cells were also incubated for 1 h at 22°C with rabbit anti-calnexin antibody (Sigma-Aldrich; 1:200), mouse anti-GM130 antibody (BD Biosciences; 1:250), mouse anti- $\alpha$ -tubulin antibody (Sigma-Aldrich; 1:4000), mouse pan-CK antibody (1:1000), or mouse anti-CK7 antibody (1:200). Alexa568-labeled anti-mouse IgG1 or anti-rabbit antibody (Invitrogen) were then used as secondary antibodies. For  $\beta$ -arrestin2-GFP imaging, cells were incubated in serum- and phenol-free with or without 1  $\mu$ M E2 (Sigma-Aldrich), 1  $\mu$ M G-1 (Calbiochem),



1  $\mu$ M isoproterenol (Sigma-Aldrich), or DMSO vehicle for 30 min at 37°C and then fixed with 3.7% formaldehyde in PBS and washed with PBS. Images were collected using a Nikon Eclipse confocal fluorescence microscope. Some fluorescence images were analyzed using NIS Elements software (Nikon) and then graphed.

*cAMP production* – Cells were grown to near confluence in 6-well plates (Sarstedt). The cells were washed one time with serum- and phenol-free medium followed by incubation in the same medium for 1 h at 37°C. This was followed by 20 min incubation at 37°C in medium containing 25  $\mu$ M rolipram (Sigma-Aldrich). Different concentrations of E2 and G-1 were added and the cells further incubated for 30 min, after which the cells were lysed in 0.20 ml ice-cold 0.1 M HCl for 30 min at 4°C, scraped, and centrifuged at 13,000 x g for 10 min. The amount of cAMP in the supernatant was assayed using an EIA kit (Cayman Chemical) according to the manufacturer's instructions.

*Data analysis* – Data are presented as means  $\pm$  SEM. Student's two-tailed t-test for unpaired data was done to evaluate statistical significance. *P*-values less than 0.05 were regarded as statistically significant. Data analysis was performed using the Prism program (GraphPad).

## Results

*GPER1 expression* – Several native cell systems relevant to GPER1 physiology from several species were used to study GPER1 including canine kidney epithelial MDCK cells, mouse myoblast C<sub>2</sub>C<sub>12</sub> cells, and human ductal breast epithelial tumor T47-D cells. All the cells expressed GPER1 as determined at the mRNA level (Fig. 1A). Receptor expression at the protein level was monitored with a goat anti-GPER1 antibody raised against the human receptor N-terminal domain, which was used previously to detect GPER1 (Kolkova et al., 2010). To verify that this antibody reacts specifically with GPER1, we immunoblotted control HeLa cells (HeLa-Ctr.) (Fig. 1B, lane 1) and cells stably expressing human GPER1 (HeLa-GPER1) (lane 2). No bands were observed in HeLa-Ctr. cells, whereas major bands were observed in HeLa-GPER1 cells. The 40-45 kDa band corresponds most closely with the theoretical mass of the receptor, whereas the lower bands (20 kDa and 30 kDa) are probably degradation products thereof. To further evaluate receptor expression, control HEK293 cells (HEK-Ctr.) (Fig. 1C, lane 1) and cells stably expressing mouse GPER1 with the FLAG epitope inserted at the N-terminal end (FGPER1) (HEK-FGPER1) (lane 2) were immunoprecipitated with M2 FLAG antibody covalently coupled to agarose and immunoblotted with FLAG antibody. A receptor band at 40-45 kDa was present also in HEK-FGPER1 cells as well as a band at 20 kDa. In addition, these cells contained receptor bands at 65 kDa, 100 kDa, and above that may be receptor complexes.

Immunoblotting of T47-D cell (Fig. 1D, lane 1) and MDCK cell lysates (lane 2) with GPER1 antibody revealed bands common to these cells at about 40-45 kDa as well as at 30 kDa and 50-55 kDa albeit with slightly different relative intensities. The GPER1 antibody did not recognize the mouse receptor as determined with both mouse C<sub>2</sub>C<sub>12</sub> cells and HEK-FGPER1 cells. Thus, to further evaluate the GPER1 antibody specificity, T47-D cells and MDCK cells were transfected with or without a cDNA of human GPER1 containing the HA epitope at the N-terminal end (HGP<sub>ER1</sub>) and then immunoprecipitated with protein G-Sepharose with and without pre-coupled GPER1 antibody. As shown in Fig. 1E (lanes 2 and 6), the GPER1 antibody recognized HGP<sub>ER1</sub> bands at 40-45 kDa and 50-55 kDa as well as a weak

band at about 100 kDa in both T47-D-HGPER1 cells and MDCK-HGPER1 cells that were absent in mock-transfected T47-D-Ctr. and MDCK-Ctr. cells (lanes 1 and 5) and immunoprecipitates with only protein G-Sepharose (lanes 3 and 7). In MDCK cells transfected with FGPER1 cDNA (MDCK-FGPER1), FGPER1-specific bands were present at 50-55 kDa, in part overlapping with the IgG heavy chain, and at about 65 kDa and 90-100 kDa (Fig. 1F, lane 2) that were absent in mock-transfected MDCK- Ctr. cells (Fig. 1F, lane 1). Thus, FGPER1 migrates in part differently in MDCK and HEK293 cells with a band at 40-45 kDa in the former cells and a band at 50-55 kDa band in the latter cells. However, the presence of a 50-55 kDa band also in HEK293 cells was indicated following PNGase deglycosylation (see below). On the other hand, HGPER1 clearly migrates at both masses. Thus, GPER1 appears to reside on proteins of 40-45 kDa and 50-55 kDa as determined with GPER1 antibody, which reacts with the native receptor, FLAG antibody, which reacts with FGPER1, and HA antibody, which reacts with HGPER1. Receptor immunoprecipitates also enriched for a higher mass receptor form(s) at about 100 kDa, which may be a detergent-resistant receptor complex(es). Together, these results show that the GPER1 antibody is specific for GPER1.

*GPER1 N-glycosylation* – The presence of multiple immunoreactive GPCR species is typical and often caused by variations in receptor N-glycosylation. To address this modification as a basis for GPER1 heterogeneity, FLAG immunoprecipitates from HEK-FGPER1 cell lysates were N-deglycosylated with PNGase F and then immunoblotted with FLAG antibodies. Surprisingly, PNGase F treatment resulted in a dramatic decrease in the intensities of the 20-kDa, 40-45-kDa, and 65-kDa bands and an increase in the intensity of the 100-kDa band (Fig. 2A), which is contrary to the typical decrease in apparent receptor mass that occurs upon deglycosylation. A decrease did occur in the mass of a FLAG-specific protein corresponding to that of the IgG heavy chain (50-55 kDa) supporting the presence of a receptor band here also in these cells. Similar changes occurred upon PNGase F treatment of FGPER1 immunoprecipitates from lysates of MDCK-FGPER1 cells and T47-D-FGPER1 cells (Fig. 2B), again with the notable decrease in mass at 50-55 kDa. These results show that the receptor is N-glycosylation and that this modification has additional effects on the physical properties of on the receptor, at least FGPER1.

*GPER1-mediated cAMP production* – G-1, a substance previously reported to be an agonist on GPER1-mediated intracellular  $\text{Ca}^{2+}$  signaling (Bologa et al., 2006), dose-dependently increased cAMP production in a saturable manner in mouse C<sub>2</sub>C<sub>12</sub> cells with an EC<sub>50</sub> value of  $282 \pm 52$  nM (Fig. 3A). E2 also potently stimulated cAMP production in these cells with an EC<sub>50</sub> value of  $1.8 \pm 0.3$  nM (Fig. 3A). However, the maximal response, or efficacy, of E2 was only about 10% of that of G-1. To address the dependency of these responses on GPER1, we transiently transfected C<sub>2</sub>C<sub>12</sub> cells with a mouse anti-sense GPER1 cDNA construct previously used and validated in detail (Ahola et al., 2002; Revankar et al., 2005). The G-1- (Fig. 3B) and E2-promoted responses (Fig. 3C) were both inhibited by this anti-sense construct. G-1 and E2 also stimulated cAMP production in MDCK cells with relative efficacies and potencies similar to those in C<sub>2</sub>C<sub>12</sub> cells (Fig. 3D). A canine GPER1-specific siRNA, but not a scrambled non-specific siRNA, inhibited the G-1 response in MDCK cells (Fig. 3E) indicating that this response was also dependent on GPER1. G-1 also stimulated cAMP production in human T47-D cells (Fig. 3F), which express GPER1 (Fig. 1A). Thus, G-1- and E2-stimulated cAMP production in these native cells is mediated at least in part by GPER1.

*GPER1-mediated  $\beta$ -arrestin2 recruitment* – Receptor-mediated cAMP production is a PM-dependent event. To further address the subcellular localization of GPER1 signaling, we analyzed the distribution of  $\beta$ -arrestin2, a regulatory and signaling effector protein that physically associates with many GPCR at their site of function. Consistent with cAMP signaling, G-1 and E2 increased  $\beta$ -arrestin2-GFP in the PM in MDCK cells (Fig. 4). Isoproterenol was used as a positive control presumably by acting through a small but significant number of  $\beta_2$ -adrenergic receptors expressed on these cells (Meier et al., 1983). PM recruitment of  $\beta$ -arrestin2-GFP by these agents was also observed in T47-D cells (data not shown). These results provide additional evidence that GPER1 functions at least in part in the PM. Even though only semi-quantitative, this assay further suggests that G-1 and E2 exhibit similar efficacies on this response.

*Subcellular GPER1 trafficking* – Limited detailed studies have been done to localize GPER1 subcellularly in native cells. To do so, we performed confocal immunofluorescence microscopy using the

GPHER1 antibody. The dependence of antibody reactivity on GPHER1 expression was again confirmed by positive staining in HeLa-GPHER1 cells but not in HeLa-Control cells (Fig. 5A). A calnexin antibody was used as a control to show ER staining in both cells types. Some overlap in receptor and ER staining occurred, which is common in over-expressed recombinant GPCR cell systems and often due to saturation of maturation mechanisms (Fig. 5A). The specificity of the antibody for GPHER1 was further underlined by colocalization of GPHER1 antibody and FLAG antibody staining in T47-D cells transfected with human GPHER1 and FGPER1 (Fig. 5B). Furthermore, FLAG antibody and HA antibody staining colocalized in cells transfected with FGPER1 and HGPHER1 (Fig. 5B). Co-staining of GPHER1 and HGPHER1 with the GPHER1 antibody could not be done because both receptor construct are of human origin and thus recognized by this antibody. Interestingly, the intracellular co-staining had a web-like appearance in all cells suggesting receptor localization on a cytoskeletal structure.

Staining of endogenous GPHER1 with the GPHER1 antibody in fixed MDCK cells was observed both at the cell periphery and intracellularly, the latter also exhibiting a web-like cytoskeletal appearance (Fig. 5B; Supplemental Figure 1). Staining also occurred under live conditions at 37°C providing direct evidence for PM receptors because the antibody is directed against the extracellular N-terminal receptor domain (Fig. 5C). A significant amount of the live staining was intracellular showing that the PM receptors undergo constitutive endocytosis (Fig. 5C). The live intracellular staining exhibited the same web-like pattern as the fixed intracellular staining (Fig. 5C), suggesting that this pattern is at least in part caused by constitutive receptor internalization.

To confirm constitutive GPHER1 endocytosis, we used HEK-FGPER1 cells. Fixed staining with M1 FLAG antibody showed that FGPER1 was present primarily intracellularly (Fig. 5C) similar to HeLa-GPHER1 cells (Fig. 5A). Live staining was again observed indicating the presence of PM receptors also in these cells. Similar to MDCK cells, the live staining was almost exclusively intracellular again showing constitutive receptor internalization (Fig. 5D). The live staining in HEK-FGPER1 cells was punctate rather than web-like, which again may be due to the heterologous nature of this over-expressed recombinant cell system. Incubating cells in the absence of serum for 24 hr did not change the live

staining pattern showing that it was not caused by a serum-derived factor. The relative amount of cell surface receptor staining increased dramatically by treating the cells prior to antibody incubation with hyperosmotic sucrose for 30 min, which blocks endocytosis by yielding abnormal clathrin polymerization resulting in empty microcages in the membrane (Heuser and Anderson, 1989) (Fig. 5D). The live intracellular staining was not caused by non-specific uptake of the antibody or antibody-promoted receptor internalization because GPCR vary in their ability to generate such staining as previously described by us (Enquist et al., 2007). Indeed, FLAG-tagged B2 bradykinin receptors (FB2R) stably expressed in HEK293 (HEK-FB2R) remained in the PM during live staining until exposed to the agonist bradykinin, upon which the receptor-antibody complex internalized (Fig. 5D). Thus, two different cell systems (MDCK cells and HEK-FGPER1) using two different receptor-specific antibodies (GPER1 antibody and M1 FLAG antibody) show that at least a fraction of the cellular GPER1 is localized in the PM and undergoes constitutive endocytosis to populate intracellular structures.

*Subcellular GPER1 localization* – Antibodies against various subcellular marker proteins were then used to determine the intracellular localization of GPER1 in native cells. Intracellular GPER1 staining in MDCK cells was not associated with ER as determined by the lack of overlap with calnexin staining (Fig. 6A). Also, no overlap occurred with GM130 staining of Golgi (data not shown). The web-like intracellular staining pattern suggested receptor association with a cytoskeletal structure. Lack of costaining with  $\alpha$ -tubulin staining showed that this structure is not microtubules (Fig. 6B). Also, receptor staining appeared too disorganized to be associated directly with actin filaments. On the other hand, a significant overlap occurred in receptor and CK staining (Fig. 6C). The same overlap was observed in T47-D cells (Fig. 6C). The pan-CK antibody that was used recognizes several CKs including the simple epithelial CK8, but not CK7. Using a specific CK7 antibody, overlap was also found with this CK subtype (Fig. 6D). Thus, in MDCK cells and T47-D cells GPER1 is localized in the PM and intracellularly at least in part on CK intermediate filaments but not in the ER, Golgi, or on microtubules.

*GPER1-CK association* – GPER1-CK association was further addressed by co-immunoprecipitation. Immunoblotting of MDCK and T47-D cells with anti-pan-CK antibody revealed that both cells express

CKs at masses of 50-55 kDa, which is typical of epithelial CKs (Fig. 7A, lanes 1 and 2). The T47-D cell line was the richest source of CK, which is expected of a breast cancer epithelial cell line. Typical of a simple epithelial cell, T47-D cells expressed the basic type-II CKs CK8 (Fig. 7A, lane 3) and CK7 (lane 4), often as doublets, and as previously reported (Ferrero et al., 1989). Even though MDCK cells have been reported to express CK8 (Pollack et al., 1997), we were unable to effectively detect CK7 or CK8 in MDCK cell lysates, which is probably due to the relatively low CK expression in this cell line (Fig. 7A, lane 1).

Consistent with GPER1-CK association, GPER1 immunoprecipitates from both MDCK and T47-D cells contained CK (Fig. 7B, lanes 1 and 2) including both CK7 (lanes 3 and 4) and CK8 (lanes 5 and 6). CKs detected by the pan-CK antibody in both cells (Fig. 7B, lanes 1 and 2) and CK7 antibody in MDCK cells (lane 3) migrated as monomers, whereas higher mass forms were detected of CK7 in T47-D cells (lane 4) and CK8 in both cells (lanes 5 and 6). The reason for this is unknown but may be due in part to detergent-resistant CK complexes with e.g. GPER1. Indeed, pan-CK immunoprecipitates from T47-D cells contained a GPER1 antibody-reactive band at about 100 kDa (Fig. 7C, lane 2) that was not present in protein G-Sepharose precipitates (lane 1). The same specific band was present in CK8 and/or CK7 immunoprecipitates of MDCK cells (Fig. 7D, lanes 2 and 3) and T47-D cells (lane 4). Occasionally, weaker bands at about 50 kDa appeared in protein G-Sepharose precipitates (Fig. 7D, lane 1). PNGase F treatment of the pan-CK immunoprecipitate from T47-D cells resulted in a small downward shift in the 100 kDa GPER1 band (Fig. 7E, lanes 1 and 2), whereas this treatment did not influence the CK band in the GPER1 immunoprecipitate (Fig. 7E, lanes 3 and 4).

FLAG immunoprecipitates of MDCK-FGPER1 cells also contained pan-CK and CK8 immunoreactivities of higher masses (Fig. 8, lanes 2 and 4) that were not present in mock-transfected MDCK-Ctr. cells (lane 1). Consistent with native cells, pan-CK immunoprecipitates from MDCK-FGPER1 cells contained a 100 kDa FLAG-receptor-specific band (Fig. 8, lane 5). Thus, the 100 kDa receptor species observed in both FGPER1 and HGPER1 immunoprecipitates seems to be a major CK-interacting partner as determined by immunoblotting with both GPER1 antibody for the native receptor

MOL # 69500

(Fig. 7C-E) and FLAG antibody for FGPER1 (Fig. 8, lane 5). In all, these results confirm those obtained by immunofluorescence microscopy that at least some GPER1 in MDCK cells and T47-D cells associates with CK intermediate filaments.



## Discussion

Here, we investigated the subcellular distribution and signaling of GPER1 expressed endogenously in a series of pathophysiologically relevant cell lines from various species including MDCK cells, T47-D cells, and C<sub>2</sub>C<sub>12</sub> cells. GPER1 was localized both in the PM and on intracellular cytoskeletal structures. The PM receptors were subject to relatively rapid constitutive endocytosis to reach such structures. These structures were identified at least in part as CK intermediate filaments, including those composed of CK7 and CK8, but not ER, Golgi, or microtubules. The GPER1 agonist G-1 and E2 stimulated GPER1-dependent cAMP production and  $\beta$ -arrestin2 recruitment to the PM. We conclude from this study that GPER1 couples to cAMP and  $\beta$ -arrestin2 signaling in the PM and associates intracellularly with CK, the latter which may be a mechanism for subcellular redistribution of the receptor following endocytosis.

The subcellular localization of GPER1 has been a point of debate ever since this receptor was introduced as a putative estrogen receptor in 2005. Four studies were published early that addressed this issue using epitope-tagged receptors expressed in recombinant cell systems. Three of these studies used receptors tagged at the N terminus with either the HA epitope (Thomas et al., 2005; Filardo et al., 2007) or the FLAG epitope (Funakoshi et al., 2006) to claim that the receptor localized in the PM but reached intracellular compartments via E2-promoted endocytosis. Two other studies used receptors tagged in the C terminus with GFP, in the N terminus with the FLAG epitope, or non-tagged receptor to claim that the receptor is more or less exclusively localized intracellularly in the ER (Revankar et al., 2005; Otto et al., 2008). Another study found that when GPER1 was tagged at the N terminus with the FLAG epitope it localized in the PM, whereas when tagged at the C terminus with GFP it localized in the ER (Funakoshi et al., 2006), which agrees with some other epitope-tagged GPCR (Brothers et al., 2003) and indicates that tagging can influence GPCR trafficking. Since then, several studies have addressed endogenous receptor localization using various receptor antibodies and reported immunoreactivity at the cell periphery and/or intracellularly in the ER and/or Golgi (Funakoshi et al., 2006; Sakamoto et al., 2007; Brailoiu et al., 2007; Otto et al., 2008; Matsuda et al., 2008; Lin et al., 2009). Even though not emphasized, many of

these reports however also show significant intracellular immunoreactivity not associated with either of these structures. Only in one study was PM localization of endogenous GPER1 directly addressed. In this case, specific radiolabeled E2 binding to a PM fraction from SkBr3 breast cancer cells was detected that was sensitive to prior treatment of the cells with a GPER1 siRNA (Thomas et al., 2005).

In this study, using an antibody that we confirmed is specific for GPER1, immunostaining of fixed cells showed that endogenous GPER1 is localized both at the cell periphery and intracellularly in MDCK cells and T47-D cells. Furthermore, human GPER1, mouse FGPER1, and human HGPER1 colocalized on the same cytoskeletal-like cellular structures. MDCK cells stained positive under live non-fixed conditions indicating that GPER1 is also present at the PM in these cells. No significant overlap occurred between receptor staining and either ER or Golgi staining, which suggests that the receptor matures normally in these cells. The ability of GPER1 to reach the PM was confirmed by live staining of HEK-FGPER1 cells with FLAG antibody. Live staining of MDCK cells and HEK-FGPER1 cells also showed that the PM receptors can reach intracellular structures via constitutive endocytosis. In MDCK cells, live and fixed staining had the same cytoskeletal-like patterns suggesting that it is at least in part the constitutively internalized receptors that give rise to such staining.

Three experimental observations led us to conclude that at least a portion of the cytoskeletal-like receptor staining represents receptors associated with CK intermediate filaments including 1) co-localization of GPER1 and CK staining as determined by confocal immunofluorescence microscopy, 2) identification GPER1 staining on filamentous structures projecting between the nuclear membrane and the PM, and 3) reciprocal co-immunoprecipitation of GPER1 and CK including CK7 and CK8. To our knowledge, this is the first observation that a GPCR is capable of reaching and interacting with intermediate filaments. CK7 and CK8 are simple-epithelial ductal-type CKs that are widely distributed and often co-expressed. Little is known about CK7, which usually pairs with CK19, but the related CK8, which pairs with CK18, has been shown to associate with the external leaflet of the PM in cancer cells (Gires et al., 2005). Also, CKs are necessary for membrane incorporation of GLUT1 and GLUT3 (Vijayaraj et al., 2009). One suggestion is that endocytic vesicles use CK filaments to redistribute GPER1

to unique functional locations in the cell such as cell-cell or cell-basement membrane contacts (Toivola et al., 2005) via interaction with the adapter complex AP3 involved in clathrin-mediated endocytosis in a way similar to the CK-related proteins vimentin, peripherin, and  $\alpha$ -internexin (Styers et al., 2004).

GPER1 expression at the protein level is heterogeneous both in native and recombinant cells with products observed both below and above the theoretical receptor mass, which may be consequences of detergent-resistant protein complexes, glycosylation, and degradation. The receptor monomer appears to be a 40-45 kDa protein as observed in both native and recombinant cells. The relationship of this protein to the 50-55-kDa receptor protein is not clear, but the latter may in part be a glycosylated form of the former because it decreased in mass upon PNGase F treatment. The 100-kDa receptor band identified primarily in receptor immunoprecipitates may be a detergent-resistant receptor dimer or a monomer interacting with another protein. Interestingly, the 100 kDa band was the primary receptor band enriched in CK immunoprecipitates suggesting that it is this receptor form that interacts with CK. Alternatively, CK may serve as a scaffold for receptor interactions with other proteins. Some evidence was obtained that N-glycosylation plays a role in receptor interactions, at least for FGPER1, because PNGase treatment increased the amount of the 100 kDa FGPER1 band apparently at the expense of the lower mass bands. One explanation is that N-deglycosylation makes the proteins in the immunoprecipitate more hydrophobic thus promoting complex formation. Alternatively, N-deglycosylation of the two asparagines in the receptor N-terminal domain may influence the immunoreactivity of the nearby N-terminal FLAG epitope. Apparent receptor degradation products were also observed, which should be cellular because all preparations contained a complete protease inhibitor cocktail. It is interesting to note that CK intermediate filaments associate with proteasomes (Olink-Coux et al., 1994), and ER $\alpha$  is degraded via a ubiquitin-proteasome pathway involving receptor association with CK8 and CK18 (Long and Nephew, 2006).

The localization of GPER1 signaling is also a matter of debate. Using recombinant cells systems and membrane-permeable and -impermeable E2 analogs, GPER1 was proposed to function either in the PM or intracellularly in the ER. PM signaling was based on GPER1 localization in this compartment, stimulation of cAMP production by membrane-impermeable E2 analogs, E2-stimulated PM GTP $\gamma$ S

binding, and GPER1-dependent PM E2 binding (Filardo et al., 2002; Thomas et al., 2005; Filardo et al., 2007). On the other hand, ER-associated signaling was based on the identification of GPER1 in this compartment in some cells and stimulation of intracellular  $\text{Ca}^{2+}$  signaling and PI3-kinase translocation only by membrane-permeable E2 analogs (Revankar et al., 2005, 2007).

Here, we show that G-1 and E2 both stimulated GPER1-dependent cAMP production in several native cell lines from several species, which is a PM-defined event. G-1 and E2 also recruited  $\beta$ -arrestin2-GFP to the PM, which is consistent with the receptor for these agonists being localized in this compartment and also a possible mechanism of GPER1-mediated ERK signaling as previously shown for other receptors (Galandrin and Bouvier, 2006). Thus, we conclude that it is in the PM that GPER1 couples to  $\text{G}_s$ /adenylate cyclase and  $\beta$ -arrestin2 in native cells. Whether or not GPER1 is able to couple to other signals in other subcellular compartments such as intermediate filaments remains an open question. Interestingly, the efficacy of E2 on GPER1-mediated cAMP production in these cells was only a fraction of that of G-1, which suggests that E2 may act as a partial agonist or G-1 as a superagonist on this response. While our assay of  $\beta$ -arrestin2 recruitment is only semi-quantitative, the efficacies of G-1 and E2 on this response appeared to be approximately equal. Thus, E2 may exhibit biased signaling at GPER1, i.e. full agonist on  $\beta$ -arrestin2 recruitment and partial agonist on cAMP production, a behavior described for agonists at several other GPCR (Kenakin, 2007) including receptors coupled to both cAMP and  $\beta$ -arrestin signaling (Galandrin and Bouvier, 2006).

In summary, we show that GPER1 is localized and signals via cAMP production and  $\beta$ -arrestin2 recruitment in the PM. Receptors also reach CK intermediate filaments. CK is highly expressed in cancer epithelial cells and has long been used to classify cancer subtypes (Moll et al., 2008). Considering that GPER1 influences growth factor signaling pathways (Filardo and Thomas, 2005; Prossnitz et al., 2008) and cancer cell proliferation (Pandey et al., 2009; Ariazi et al., 2010), and that receptor expression is associated with cancer growth (Filardo et al., 2006; Smith et al., 2007), it is tempting to propose that GPER1-CK association in epithelial cells provides an important functional link in this disease.

## Acknowledgements

We thank J. Enquist for assistance in constructing HEK293 cells stably expressing FGPER1 and FB2R, M.G. Caron for providing  $\beta$ -arrestin2-GFP, and M. Sjöström for experimental assistance.

## Authorship Contribution

Participated in research design: Sandén, Broselid, Olde, Leeb-Lundberg.

Conducted experiments: Sandén, Broselid, Cornmark, Andersson, Mårtensson, Daszkiewicz-Nilsson.

Performed data analysis: Sandén, Broselid, Olde, Leeb-Lundberg.

Wrote or contributed to the writing of the manuscript: Sandén, Broselid, Leeb-Lundberg.

Other: Leeb-Lundberg acquired funding for the research.

## References

- Ahola TM, Manninen T, Alkio N, and Ylikomi T (2002) G protein-coupled receptor 30 is critical for a progesterin-induced growth inhibition in MCF-7 breast cancer cells. *Endocrinology* **143**:3376-3384.
- Ariazi EA, Brailoiu E, Yerrum S, Shupp HA, Slifker MJ, Cunliffe HE, Black MA, Donato AL, Arterburn JB, Oprea TI, Prossnitz ER, Dun NJ, and Jordan VC (2010) The G protein-coupled receptor GPR30 inhibits proliferation of estrogen receptor-positive breast cancer cells. *Cancer Res* **70**:1184-1194.
- Barak LS, Ferguson SSG, Zhang J, and Caron MG (1997) A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J Biol Chem* **272**:27497-27500.
- Bologa CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, Parker MA, Tkachenko SE, Savchuck NP, Sklar LA, Oprea TI, and Prossnitz ER (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* **2**:207-212.
- Brailoiu E, Dun SL, Brailoiu GC, Mizuo K, Sklar LA, Oprea TI, Prossnitz ER, and Dun NJ (2007) Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. *J Endocrinol* **193**:311-321
- Brothers SP, Janovick JA, and Conn PM (2003) Unexpected effects of epitope and chimeric tags on gonadotropin-releasing hormone receptors: implications for understanding the molecular etiology of hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* **88**:6107-6112.
- Chomczynski P and Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**:156-159.
- Eriksson JE, Dechat T, Grin B, Helfand B, Mendez M, Pallari HM, and Goldman RD (2009) Introducing intermediate filaments: from discovery to disease. *J Clin Invest* **119**:1763-1771.
- Enquist J, Skräder C, Whistler JL, and Leeb-Lundberg LMF (2007) Kinins Promote B<sub>2</sub> Receptor Endocytosis and Delay Constitutive B<sub>1</sub> Receptor Endocytosis. *Mol Pharmacol* **71**:494-507.
- Ferrero M, Spyrtos F, Desplaces A, Andrieu C, Phillips E, and Rouëssé J (1989) Cytokeratin analysis of breast and leukemia tumor cell lines by flow cytometry. *Biol Cell* **65**:305-308.

- Filardo EJ, Quinn JA, Frackelton AR Jr, and Bland KI (2002) Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. *Mol Endocrinol* **16**:70-84.
- Filardo EJ and Thomas P (2005) GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. *Trends Endocrinol Metab* **16**:362-367.
- Filardo EJ, Graeber CT, Quinn JA, Resnick MB, Giri D, DeLellis RA, Steinhoff MM, and Sabo E (2006) Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression. *Clin Cancer Res* **12**:6359-6366.
- Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, and Thomas P (2007) Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. *Endocrinology* **148**:3236-3245.
- Funakoshi T, Yanai A, Shinoda K, Kawano MM, and Mizukami Y (2006) G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane. *Biochem Biophys Res Commun* **346**:904-910.
- Galandrin S and Bouvier M. (2006) Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* **70**:1575-1584.
- Gires O, Andratschke M, Schmitt B, Mack B, Schaffrik M. (2005) Cytokeratin 8 associates with the external leaflet of plasma membranes in tumour cells. *Biochem Biophys Res Commun* **328**:1154-1162.
- Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Ström A, Treuter E, Warner M, and Gustafsson JA (2007) Estrogen receptors: how do they signal and what are their targets. *Physiol Rev* **87**:905-931.
- Heuser JE and Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol* **108**:389-400.



- Kenakin T (2007) Functional selectivity through protean and biased agonism: who steers the ship? *Mol Pharmacol* **72**:1393-1401.
- Kolkova Z, Noskova V, Ehinger A, Hansson S, and Casslén B (2010) G protein-coupled estrogen receptor 1 (GPER, GPR 30) in normal human endometrium and early pregnancy decidua. *Mol Hum Reprod*, **in press**.
- Kotarsky K, Owman C, and Olde B (2001) A chimeric reporter gene allowing for clone selection and high-throughput screening of reporter cell lines expressing G-protein-coupled receptors. *Anal Biochem* **288**:209-215.
- Lin BC, Suzawa M, Blind RD, Tobias SC, Bulun SE, Scanlan TS, and Ingraham HA (2009) Stimulating the GPR30 estrogen receptor with a novel tamoxifen analogue activates SF-1 and promotes endometrial cell proliferation. *Cancer Res* **69**:5415-5423.
- Long X and Nephew KP (2006) Fulvestrant (ICI 182,780)-dependent interacting proteins mediate immobilization and degradation of estrogen receptor- $\alpha$ . *J Biol Chem* **281**:9607-9615.
- Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, Montanaro D, Musti AM, Picard D, and Andò S (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17 $\beta$ -estradiol and phytoestrogens in breast cancer cells. *J Biol Chem* **279**:27008-27016.
- Matsuda K, Sakamoto H, Mori H, Hosokawa K, Kawamura A, Itose M, Nishi M, Prossnitz ER, and Kawata M (2008) Expression and intracellular distribution of the G protein-coupled receptor 30 in rat hippocampal formation. *Neurosci Lett* **441**:94-99.
- Meier KE, Snively MD, Brown SL, Brown JH, and Insel PA (1983) Alpha 1- and beta 2-adrenergic receptor expression in the Madin-Darby canine kidney epithelial cell line. *J Cell Biol* **97**:405-415.
- Moll R, Divo M, and Langbein L (2008) The human keratins: biology and pathology. *Histochem Cell Biol* **129**:705-733.
- Mårtensson UEA, Salehi SA, Windahl S, Gomez MF, Swärd K, Wendt A, Daszkiewicz-Nilsson J, Andersson N, Hellstrand P, Grände P-O, Owman C, Rosen CJ, Adamo ML, Lundquist I, Nilsson B-O, Rorsman P, Ohlsson C, Olde B, and Leeb-Lundberg LMF (2009) Deletion of the G protein-

- coupled receptor GPR30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. *Endocrinology* **150**:687-698.
- Olde B and Leeb-Lundberg LMF (2009) GPR30/GPER1: searching for a role in estrogen physiology. *Trends Endocrinol Metab* **20**:409-416.
- Olink-Coux M, Arcangeletti C, Pinardi F, Minisini R, Huesca M, Chezzi C, and Scherrer K (1994) Cytolocation of prosome antigens on intermediate filament subnetworks of cytokeratin, vimentin and desmin type. *J Cell Sci* **107**:353-366.
- Otto C, Rohde-Schulz B, Schwarz G, Fuchs I, Klewer M, Brittain D, Langer G, Bader B, Prella K, Nubbemeyer R, and Fritzemeier KH (2008) G protein-coupled receptor 30 localizes to the endoplasmic reticulum and is not activated by estradiol. *Endocrinology* **149**:4846-4856.
- Pandey DP, Lappano R, Albanito L, Madeo A, Maggiolini M, and Picard D (2009) Estrogenic GPR30 signalling induces proliferation and migration of breast cancer cells through CTGF. *EMBO J* **28**:523-532.
- Pollack V, Scheiber K, Pfaller W, and Schramek H (1997) Loss of cytokeratin expression and formation of actin stress fibers in dedifferentiated MDCK-C7 cell lines. *Biochem Biophys Res Commun* **241**:541-547.
- Prossnitz ER, Arterburn JB, Smith HO, Opream TI, Sklar LA, and Hathaway HJ (2008) Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol* **70**:165-190.
- Razandi M, Razandi M, Pedram A, Merchenthaler I, Greene GL, and Levin ER (2004) Plasma membrane estrogen receptors exist and functions as dimers. *Mol Endocrinol* **18**:2854-2865.
- Revankar CM, Cimino DF, Sklar LA, Arterburn JB, and Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307**:1625-1630.
- Revankar CM, Mitchell HD, Field AS, Burai R, Corona C, Ramesh C, Sklar LA, Arterburn JB, and Prossnitz ER (2007) Synthetic estrogen derivatives demonstrate the functionality of intracellular GPR30. *ACS Chem Biol* **2**:536-544.

- Sakamoto H, Matsuda K, Hosokawa K, Nishi M, Morris JF, Prossnitz ER, and Kawata M. (2007) Expression of G protein-coupled receptor-30, a G protein-coupled membrane estrogen receptor, in oxytocin neurons of the rat paraventricular and supraoptic nuclei. *Endocrinology* **148**:5842-5850.
- Schweizer J, Bowden PE, Coulombe PA, Langbein L, Lane EB, Magin TM, Maltais L, Omary MB, Parry DA, Rogers MA, and Wright MW (2006) New consensus nomenclature for mammalian keratins. *J Cell Biol* **174**:169-174.
- Smith HO, Leslie KK, Singh M, Qualls CR, Revankar CM, Joste NE, and Prossnitz ER (2007) GPR30: a novel indicator of poor survival for endometrial carcinoma. *Am J Obstet Gynecol* **196**:386.e1-9.
- Styers ML, Salazar G, Love R, Peden AA, Kowalczyk AP, and Faundez V (2004) The endo-lysosomal sorting machinery interacts with the intermediate filament cytoskeleton. *Mol Biol Cell* **15**:5369-5382.
- Thomas P, Pang Y, Filardo EJ, and Dong J (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* **146**:624-632.
- Toivola DM, Tao GZ, Habtezion A, Liao J, and Omary MB (2005) Cellular integrity plus: organelle-related and protein-targeting functions of intermediate filaments. *Trends Cell Biol* **15**:608-617.
- Vijayaraj P, Kröger C, Reuter U, Windoffer R, Leube RE, and Magin TM (2009) Keratins regulate protein biosynthesis through localization of GLUT1 and -3 upstream of AMP kinase and Raptor. *J Cell Biol* **187**:175-184.
- Whistler JL, Enquist J, Marley A, Fong J, Gladher F, Tsuruda P, Murray SR, and von Zastrow M (2002) Modulation of postendocytic sorting of G protein-coupled receptors. *Science* **297**:615-620.
- Windahl SH, Andersson N, Chagin AS, Mårtensson UEA, Carlsten H, Olde B, Swanson C, Moverare-Skrtic S, Sävendahl L, Lagerquist MK, Leeb-Lundberg LMF, and Ohlsson C (2009) The role of the G protein-coupled receptor GPR30 in the effects of estrogen in ovariectomized mice. *Am J Physiol* **296**:E490-496.

## Footnotes

This work was supported in part by funds from European Foundation for the Study of Diabetes/Servier, Swedish Research Council, Alfred Österlund Foundation, The Swedish Diabetes Association, Konsul Thure Carlssons Minne Foundation, Magnus Bergvalls Foundation, Diabetes Association in Malmö, Syskonen Svenssons Foundation, Anders Otto Swärds Foundation, Royal Physiographic Society in Lund, and the Faculty of Medicine at Lund University through the Vascular Wall prioritized program.

To whom reprint requests should be addressed: L.M. Fredrik Leeb-Lundberg, Ph.D., BMC A12, SE-22184 Lund, Sweden, Tel.: 46-46-2223944, Fax: 46-46-2220568, E-mail: [fredrik.leeblundberg@med.lu.se](mailto:fredrik.leeblundberg@med.lu.se)

<sup>1</sup> Both authors contributed equally to this work.

## Figure Legends

Figure 1. GPER1 expression. (A), RNA from MDCK cells, T47-D cells, and C<sub>2</sub>C<sub>12</sub> cells was isolated and cDNA synthesized and analyzed with (*cDNA+RT*, lane 1) and without reverse transcriptase (*cDNA-RT*, lane 2). (B), HeLa cells without (*HeLa-Ctr.*, lane 1) and with stable expression of human GPER1 (*HeLa-GPER1*, lane 2) were lysed and immunoblotted with goat GPER1 antibody (*GP*). (C), HEK293 cells without (*HEK-Ctr.*, lane 1) and with stable expression of mouse FGPER1 (*HEK-FGPER1*, lane 2) were lysed, immunoprecipitated with mouse M2 FLAG antibody-agarose, and immunoblotted with M2 FLAG antibody (*FLAG*). (D), T47-D cells (*T47-D*, lane 1) and MDCK cells (*MDCK*, lane 2) were lysed and immunoblotted with GPER1 antibody (*GP*). (E), Mock-transfected MDCK cells (*MDCK-Ctr.*, lane 1) and T47-D cells (*T47-D-Ctr.*, lane 5) and MDCK cells (*MDCK-HGPER1*, lanes 2 and 3) and T47-D cells (*T47-D-HGPER1*, lanes 6 and 7) transiently transfected with HGPER1 cDNA were lysed, immunoprecipitated with protein G-Sepharose without (-, lanes 3 and 7) and with GPER1 antibody (*GP*) (+, lanes 2 and 3), and immunoblotted with HA antibody. (F), Mock-transfected MDCK cells (*MDCK-Ctr.*, lane 1) and MDCK cells transiently transfected with FGPER1 cDNA (*MDCK-FGPER1*, lane 2) were lysed, immunoprecipitated with M2 FLAG antibody beads, and immunoblotted with M2 FLAG antibody. Molecular mass (*M<sub>r</sub>*) standards (kDa, kilo Daltons) or base pairs (*bp*) (*left side arrows*), and position of IgG heavy chain (*h.c.*) and light chain (*l.c.*) (*right side arrows*) are indicated. The results are representative of experiments performed at least three times.

Figure 2. GPER1 N-glycosylation. (A), HEK293 cells stably expressing mouse FGPER1 (*HEK-FGPER1*) were lysed, immunoprecipitated with mouse M2 FLAG antibody-agarose, treated without (lane 1) and with PNGase F (lane 2), and immunoblotted with M2 FLAG antibody. (B), MDCK cells (*MDCK-FGPER1*, lanes 1 and 2) and T47-D cells (*T47-D-FGPER1*, lanes 3 and 4) transiently transfected with FGPER1 cDNA were lysed, immunoprecipitated with M2 FLAG antibody beads, treated without (lanes 1 and 3) and with PNGase F (lanes 2 and 4), and immunoblotted with M2 FLAG antibody. Molecular mass

( $M_r$ ) standards (kDa, kilo Daltons) (*left side arrows*) are indicated. The results are representative of experiments performed at least three times.

Figure 3. GPER1-mediated cAMP production. (A), C<sub>2</sub>C<sub>12</sub> cells were stimulated with increasing concentrations of G-1 ( ) or E2 ( ) for 30 min at 37°C and then assayed for cAMP production. (B, C), C<sub>2</sub>C<sub>12</sub> cells were transfected with a mouse GPER1 anti-sense construct (+AS) or empty vector (-AS), stimulated without (*Control*) or with (B) 1  $\mu$ M G-1 (*G-1*) or (C) 1  $\mu$ M E2 (*E2*) for 30 min at 37°C and then assayed for cAMP production. (D), MDCK cells were stimulated with increasing concentrations of G-1 (*G-1*) or E2 (*E2*) for 30 min at 37°C and then assayed for cAMP production. (E), MDCK cells were transfected with a canine GPER1 siRNA (*GPER1 siRNA*) or scrambled siRNA (*Scr siRNA*) construct, stimulated without (*Control*) or with 1  $\mu$ M G-1 (*G-1*) for 30 min at 37°C and then assayed for cAMP production. (F), T47-D cells were stimulated with increasing concentrations of G-1 and then assayed for cAMP production. Data are presented as % of Control where control corresponds to 15-20 pmol cAMP/well. Values are means  $\pm$  SEM with each data point performed in quadruplicates. NS, not significant; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

Figure 4. GPER1-mediated  $\beta$ -arrestin2 recruitment. MDCK cells transiently transfected with  $\beta$ -arrestin2-GFP cDNA were stimulated without (*Control*) or with 1  $\mu$ M G-1 (*G-1*), 1  $\mu$ M E2 (*E2*), or 1  $\mu$ M isoproterenol (*ISO*) for 30 min at 37°C. Images of fixed cells were collected using a Nikon Eclipse confocal microscope, 60x objective, 50  $\mu$ m zoom. *Arrows* indicate cellular distance that was analyzed for changes in  $\beta$ -arrestin2-GFP fluorescence intensity upon agonist stimulation using the NIS Elements software program (Nikon), and the results are graphed. Distance (*arrow*) is 2  $\mu$ m. The results are representative of experiments performed at least three times.

Figure 5. Subcellular GPER1 trafficking. (A), HeLa cells without (*HeLa-Control*) and with stable expression of human GPER1 (*HeLa-GPER1*) were fixed and permeabilized prior to incubation with goat

GP1R antibody (*GP1R*) and rabbit calnexin antibody (*calnexin*). (B), T47-D cells transfected with human GP1R and FG1R (*T47-D-GP1R/FG1R*) or FG1R and HGP1R (*T47-D-FG1R/HGP1R*) were fixed and permeabilized prior to incubation with goat GP1R antibody (*GP1R*), mouse FLAG antibody (*FLAG*), and/or mouse HA antibody (*HA*). (C), MDCK cells (*MDCK*) were fixed and permeabilized prior to incubation with GP1R antibody (*GP1R, fixed*) or preincubated live with GP1R antibody for 30 min at 37°C prior to fixation and permeabilization (*GP1R, live*). (D), HEK293 cells stably expressing mouse FG1R (*HEK-FG1R*) and human FB2R (*HEK-FB2R*) were fixed and permeabilized prior to incubation with mouse M1 FLAG antibody (*FG1R, fixed; FB2R, fixed*) or preincubated live without (*FG1R, live; FB2R, live*) or with 0.4 M sucrose for 60 min (*FG1R, live+sucr*) or 1 µM bradykinin for 30 min (*FB2R, live+agonist*) at 37°C prior to incubation with M1 FLAG antibody for an additional 30 min at 37°C. The cells were then fixed and permeabilized. In (A-D), cells were subsequently incubated with secondary donkey anti-goat, rabbit anti-mouse, or mouse anti-rabbit ALEXA488- or ALEXA568-labeled antibody. The individual and merged (*Merge*) images were collected using a Nikon Eclipse confocal microscope, 60x objective, 50 µm zoom. The results are representative of experiments performed at least three times.

Figure 6. Subcellular GP1R localization. (A-D), MDCK cells (*MDCK*) or T47-D cells (*T47-D*) were fixed and permeabilized prior to incubation with goat GP1R antibody (*GP1R*), rabbit calnexin antibody (*calnexin*), mouse  $\alpha$ -tubulin antibody ( *$\alpha$ -tubulin*), mouse pan-CK antibody (*pan-CK*) or mouse CK7 antibody (*CK7*). Cells were subsequently incubated with secondary mouse anti-rabbit, rabbit anti-mouse, or donkey anti-goat ALEXA488- or ALEXA568-labeled antibody. The individual and merged (*Merge*) images were collected using a Nikon Eclipse confocal microscope, 60x objective, 50 µm zoom. The results are representative of experiments performed at least three times.

Figure 7. Native GPER1-CK association. (A), MDCK cells (*MDCK*, lane 1) and T47-D cells (*T47-D*, lanes 2-4) were lysed and immunoblotted with mouse pan-CK antibody (lanes 1 and 2), mouse CK8 antibody (lane 3), or mouse CK7 antibody (lane 4). (B), MDCK cells (*MDCK*, lanes 1, 3, and 5) and T47-D cells (*T47-D*, lanes 2, 4, and 6) were lysed, immunoprecipitated with goat GPER1 antibody (*GP*) precoupled to protein G-Sepharose, and then immunoblotted with pan-CK antibody (lanes 1 and 2), CK7 antibody (lanes 3 and 4) or CK8 antibody (lanes 5 and 6). (C), T47-D cells (*T47-D*) were lysed, immunoprecipitated with protein G-Sepharose without (-, lane) and with precoupled pan-CK antibody (+, lane), and then immunoblotted with GPER1 antibody (*GP*). (D), MDCK cells (*MDCK*, lanes 1-3) and T47-D cells (*T47-D*, lane 4) were lysed, immunoprecipitated with CK8 antibody (lanes 1 and 2) or CK7 antibody (lane 3) precoupled to protein G-Sepharose, and then immunoblotted with GPER1 antibody. (E), T47-D cells (*T47-D*) were lysed, immunoprecipitated with pan-CK antibody (lanes 1 and 2) or GPER1 antibody (lanes 3 and 4) precoupled to protein G-Sepharose, respectively, treated without (lanes 1 and 3) or with PNGase F (lanes 2 and 4) and then immunoblotted with GPER1 antibody. Molecular mass ( $M_r$ ) standards (kDa, kilo Daltons) (*left side arrows*) are indicated. The results are representative of experiments performed at least three times.

Figure 8. Recombinant GPER1-CK association. MDCK cells without (*MDCK-Ctr.*) and with transient transfection of FGPER1 cDNA (*MDCK-FGPER1*) were lysed, immunoprecipitated with M2 FLAG antibody (lanes 1-3) or pan-CK antibody (lane 4), and immunoblotted with pan-CK antibody (lanes 1 and 2), CK8 antibody (lane 3), or M2 FLAG antibody (lane 4). Molecular mass ( $M_r$ ) standards (kDa, kilo Daltons) (*left side arrows*) are indicated. The results are representative of experiments performed at least three times.



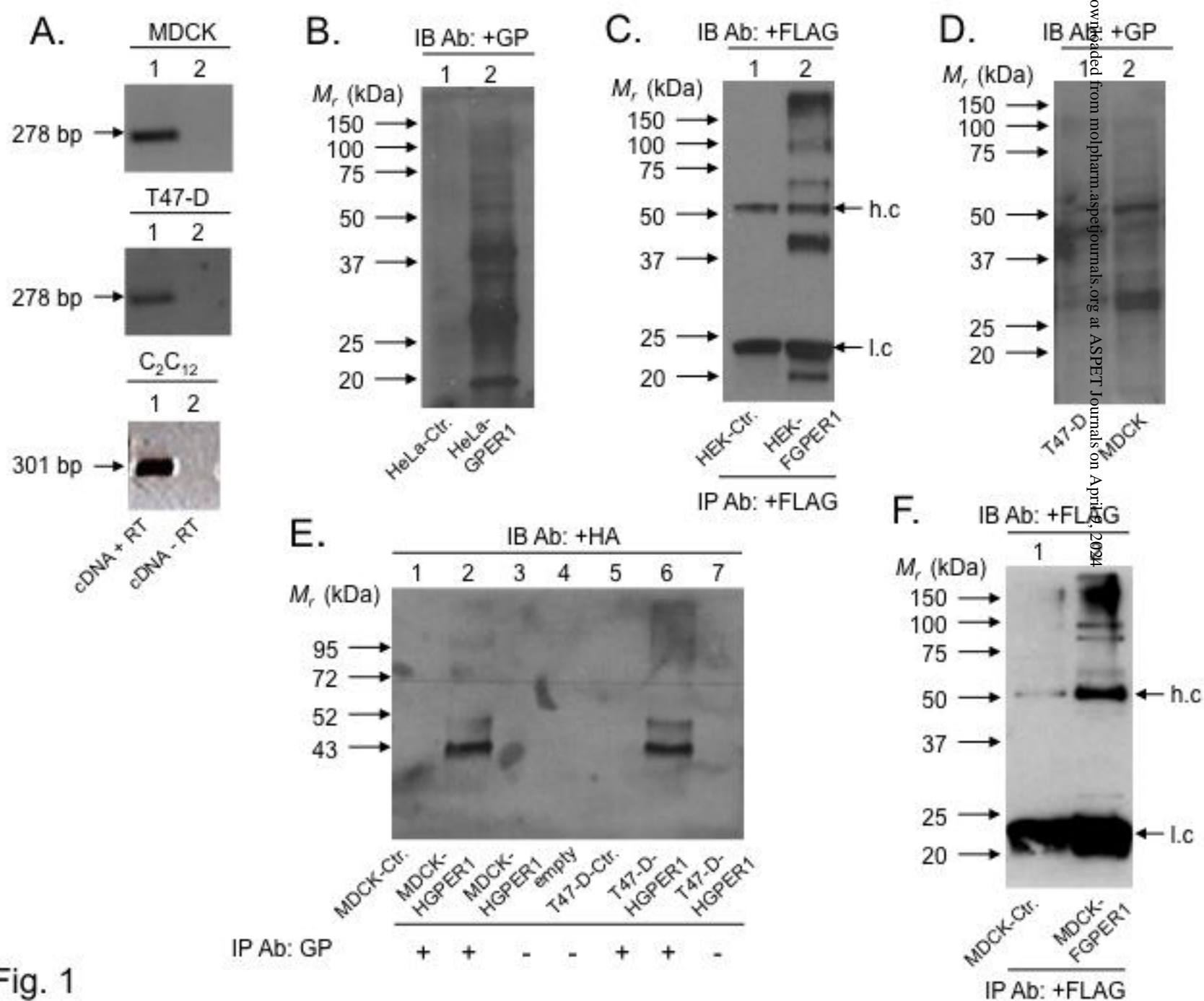


Fig. 1

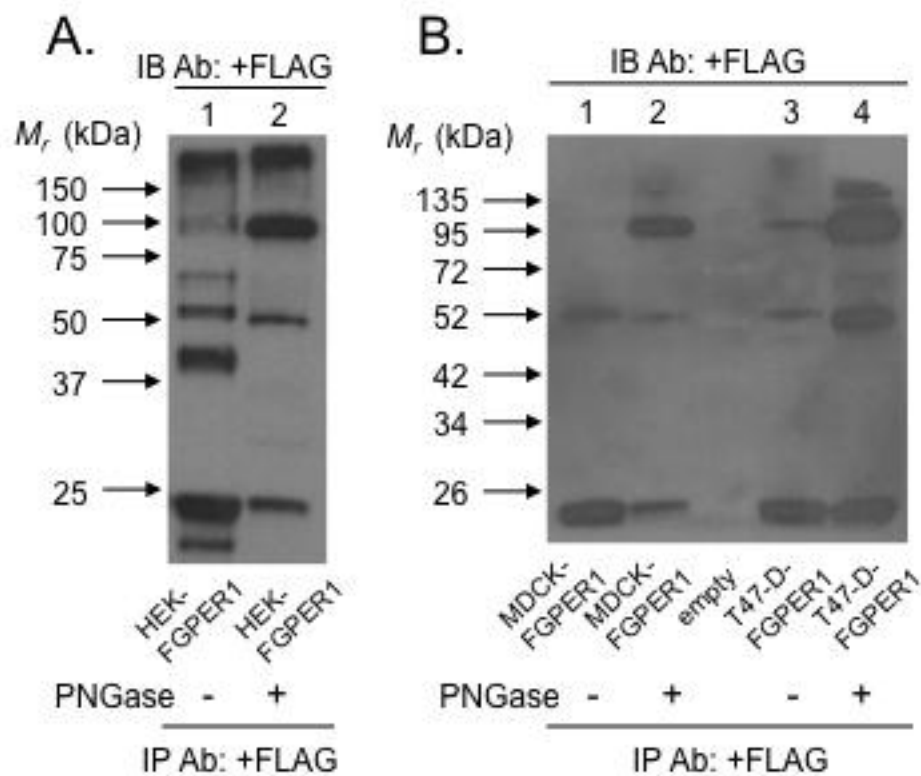
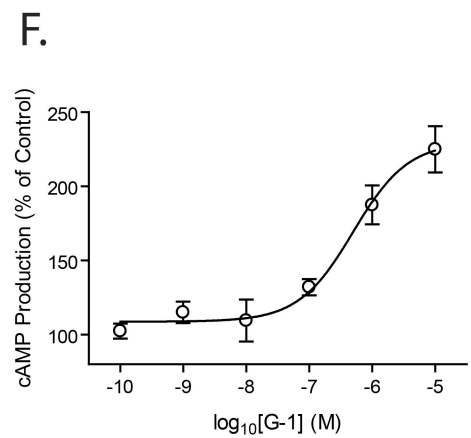
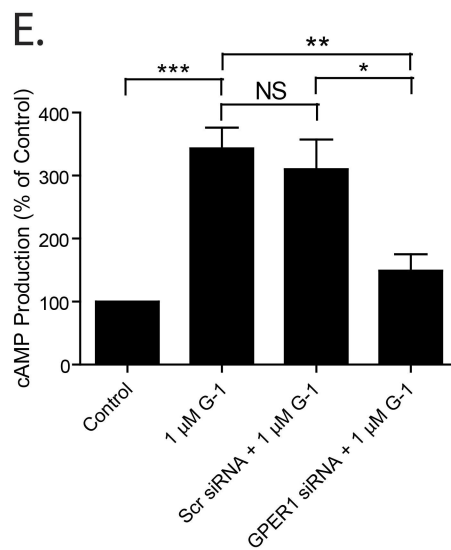
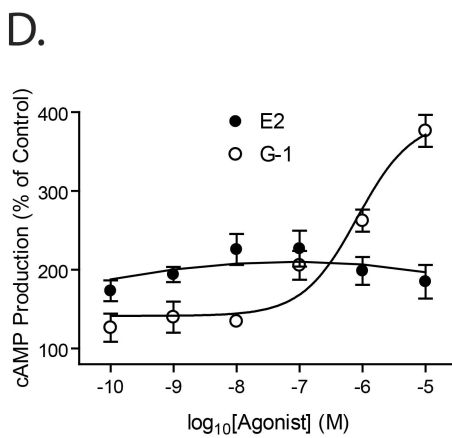
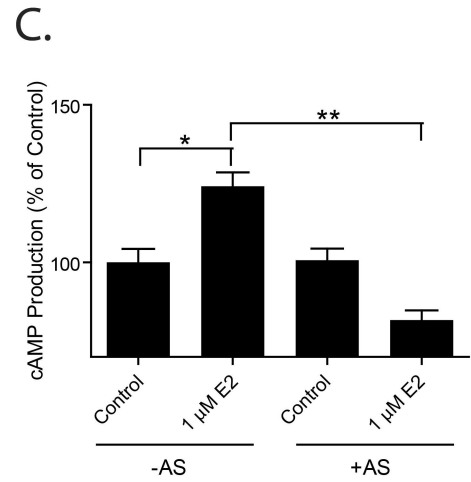
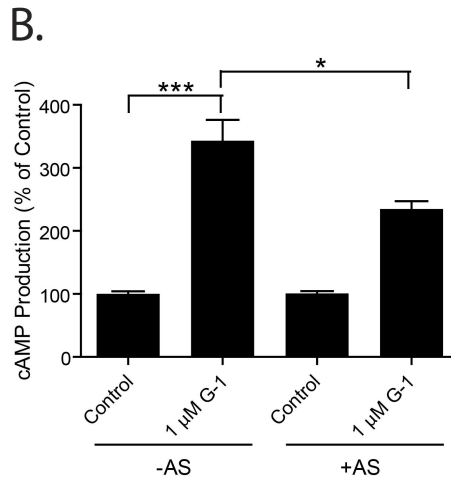
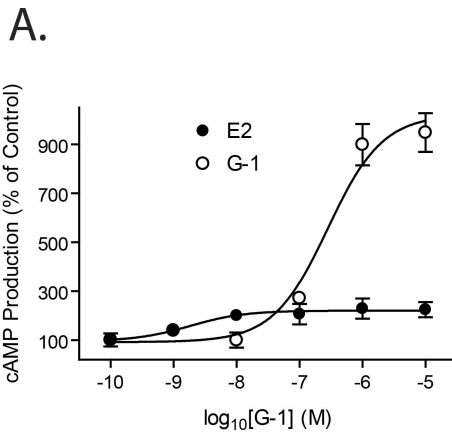


Fig. 2

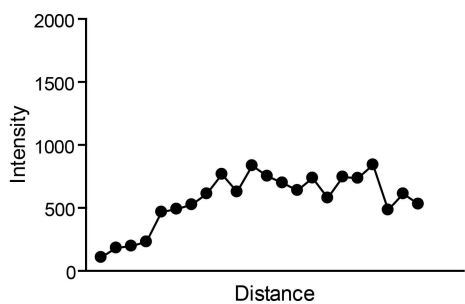
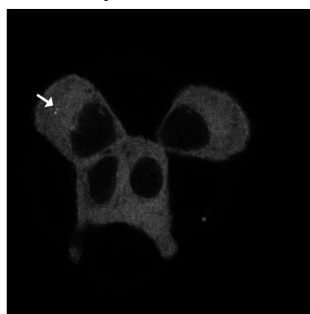


**Fig. 3**

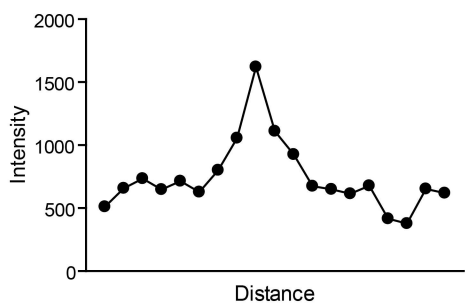
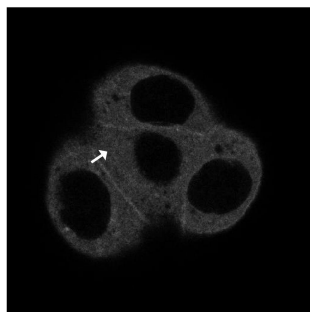
$\beta$ -Arr2

$\beta$ -Arr2 intensity distr.

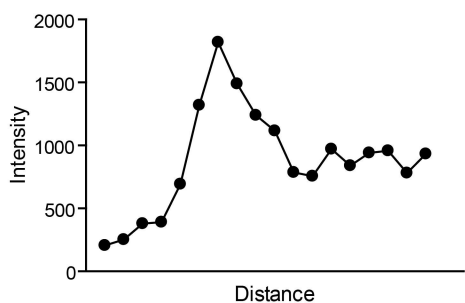
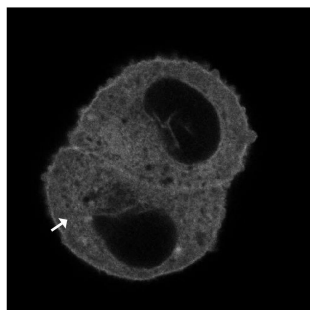
Control



G-1



E2



ISO

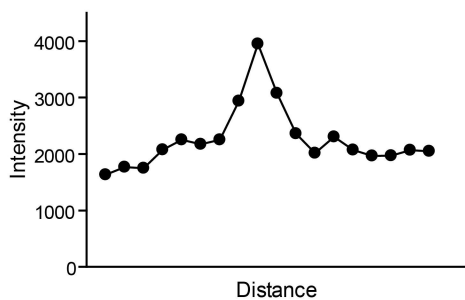
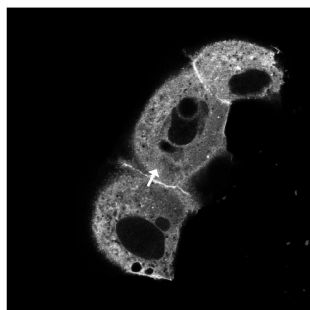
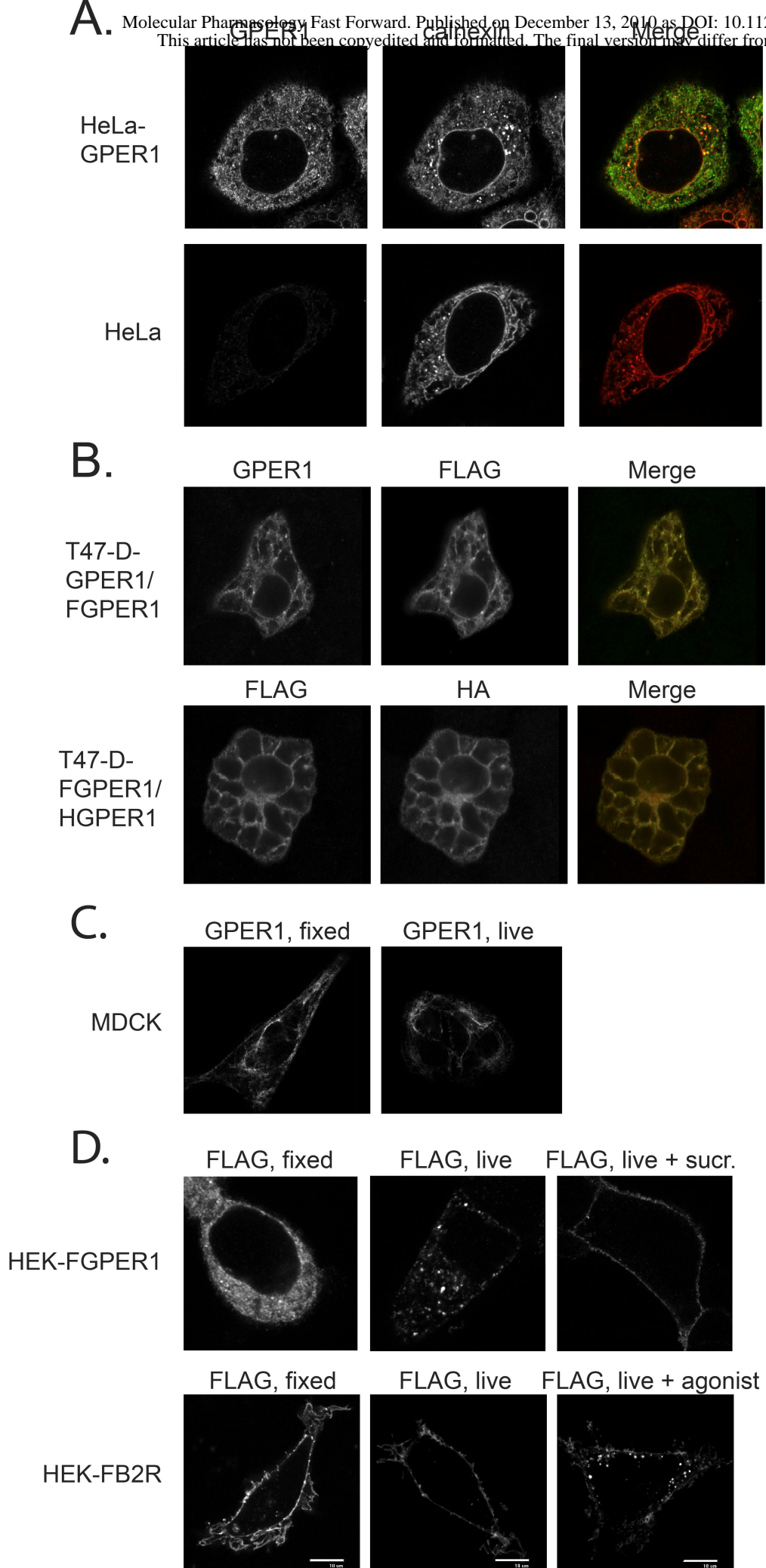


Fig.4



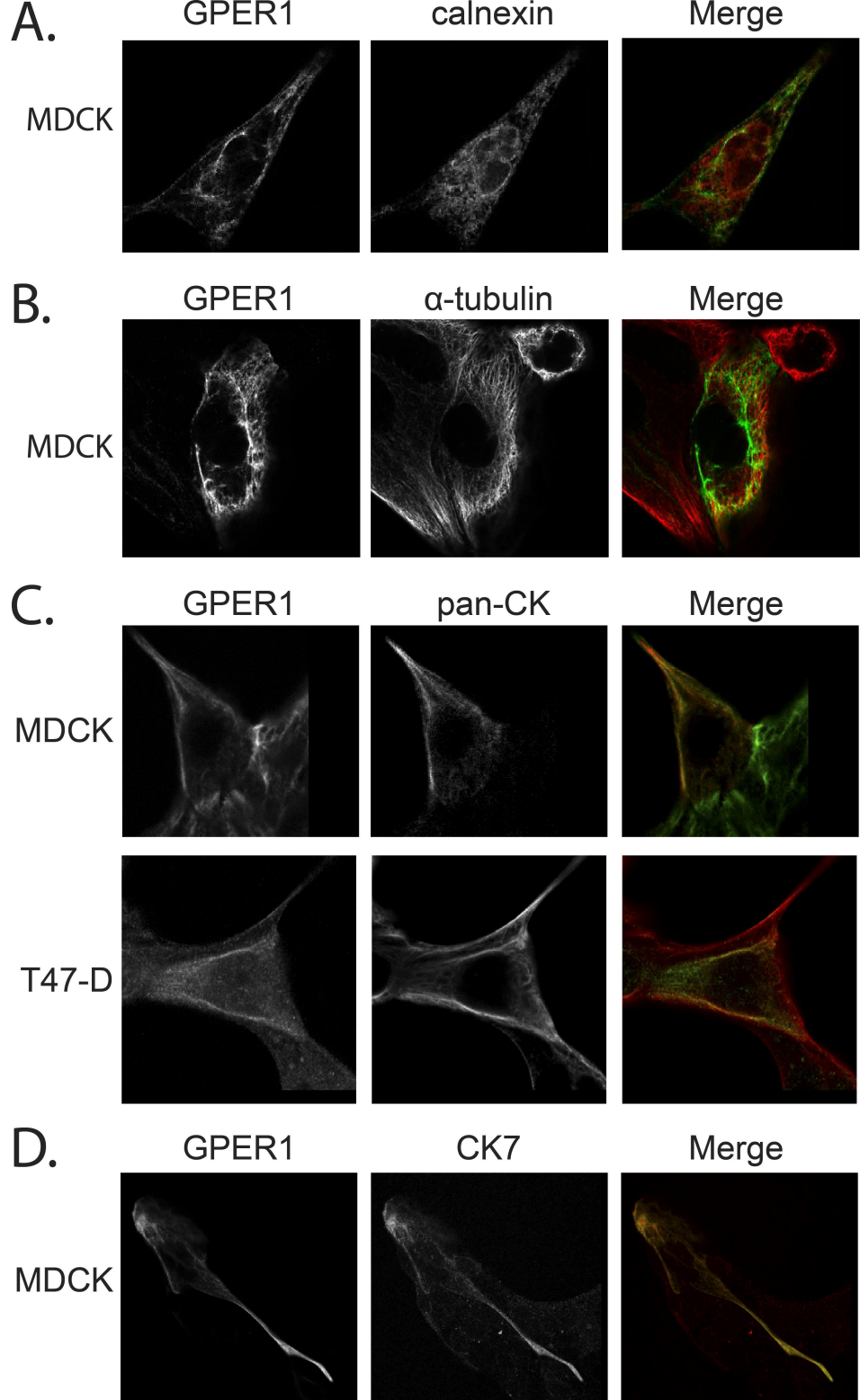


Fig.6

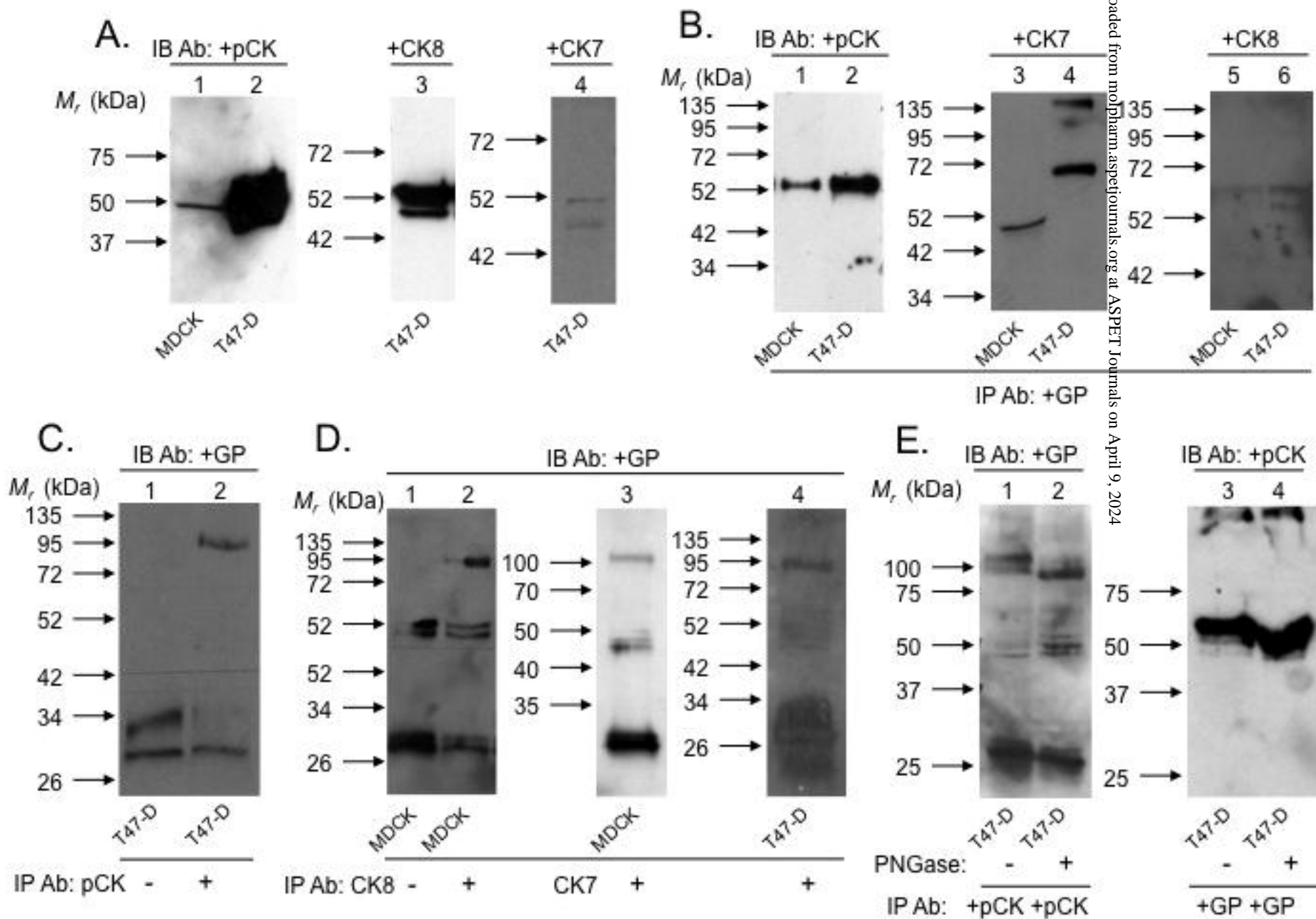


Fig. 7

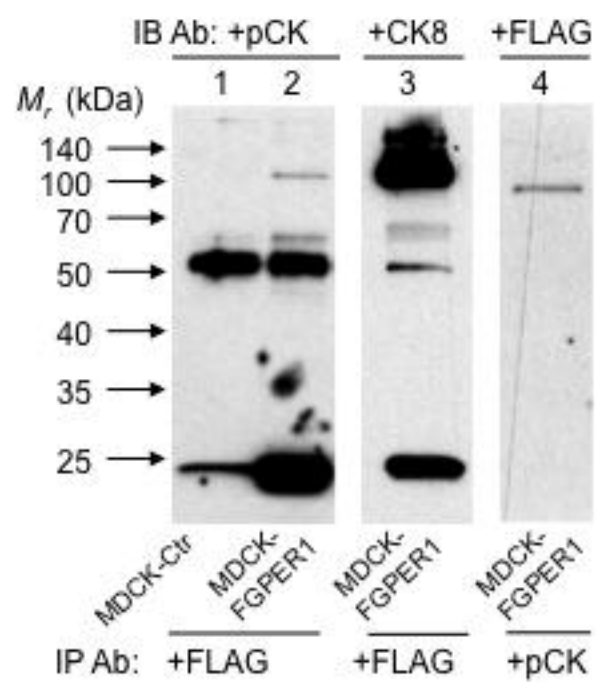


Fig. 8