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Title: Preferential β -arrestin signalling at low receptor density revealed by functional characterization of the human FSH receptor A189V mutation.

Abbreviated title: Preferential β -arrestin signalling at low FSH receptor density.

Thibaud Tranchant^{1#}, Guillaume Durand^{2#}, Christophe Gauthier², Pascale Crépieux², Alfredo Ulloa-Aguirre^{2,3}, Dominique Royère¹ and Eric Reiter^{2*}

From ¹ BINGO group, INRA, UMR85, Unité Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France; CNRS, UMR6175, F-37380 Nouzilly, France; Université François Rabelais, F-37041 Tours, France; Haras Nationaux, F-37380 Nouzilly, France, ² BIOS group, INRA, UMR85, Unité Physiologie de la Reproduction et des Comportements, F-37380 Nouzilly, France; CNRS, UMR6175, F-37380 Nouzilly, France; Université François Rabelais, F-37041 Tours, France ; Haras Nationaux, F-37380 Nouzilly, France; ³ LE STUDIUM Institute for Advanced Studies, Orléans, France.

[#] These authors contributed equally to this work

*To whom correspondence should be addressed: Eric Reiter, UMR 6175, 37380, Nouzilly, France.

Telephone: +33 2 47 42 77 83; Fax: +33 2 47 42 77 43;

E-mail address: eric.reiter@tours.inra.fr

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ABSTRACT

The A189V inactivating mutation of the human FSH receptor (FSHR) leads to subfertility in men and primary ovarian failure in women. This mutation has previously been associated with intracellular retention of the FSHR and impaired cAMP production. Here, we show that the A189V FSHR stably expressed in HEK293N cells provoked ERK MAP kinases phosphorylation through β -arrestins, independently of the canonical cAMP/PKA pathway. Interesting, both the A189V and wild-type (Wt) FSHRs selectively activated cAMP-independent ERK phosphorylation when expressed at low plasma membrane densities. These data indicate that the selective intracellular signalling triggered by the A189V FSHR resulted from reduced membrane expression rather than by switching receptor coupling. Hence, receptor density at the plasma membrane might control the balance between distinct signal transduction mechanisms. Furthermore, our results help to clarify why mutations of FSH β are more deleterious to human fertility than the FSHR A189V mutation which preserves parts of receptor signalling repertoire.

1 INTRODUCTION

Follicle-stimulating hormone (FSH) is a glycoprotein synthesized by the pituitary gland. This hormone binds to its cognate receptor (FSHR) in the gonads. The FSHR is expressed by the granulosa cells of the ovarian follicle and by the Sertoli cells of the seminiferous tubules. This gonadotropin promotes folliculogenesis and supports spermatogenesis thereby playing a key role in reproduction. Knock-out of either the FSH β -subunit or the FSHR gene in mice suggests that FSH action is fundamental for female reproduction whereas in males, although necessary to achieve normal testis size and sperm production, it is not essential for fertility (Kumar *et al.* 1997; Dierich *et al.* 1998; Krishnamurthy *et al.* 2000). In women, inactivating mutations of either the FSH β -subunit or the FSHR lead to primary amenorrhea and infertility, again suggesting that FSH action is crucial for female reproduction (Matthews *et al.* 1993; Aittomaki *et al.* 1995; Layman *et al.* 1997; Layman *et al.* 2002; Huhtaniemi 2006). In men, the A189V mutation is the only FSHR inactivating mutation reported so far. In agreement with the FSHR knock-out male phenotype, the five Finnish men presenting the A189V mutation displayed variable degrees of oligozoospermia and subfertility (Tapanainen *et al.* 1997). Surprisingly however, men with inactivating mutations of the FSH β -subunit were completely infertile due to azoospermia (Lindstedt *et al.* 1998; Phillip *et al.* 1998; Layman *et al.* 2002). The reason(s) for this apparent discrepancy between the severity of the phenotypes associated with inactivating mutations in the FSH β -subunit *versus* the FSHR has previously been pointed out but still remains to be clarified (Huhtaniemi 2006).

The FSHR belongs to the superfamily of G protein-coupled receptors (GPCR). It is widely accepted that upon ligand stimulation GPCRs adopt an active conformation which allows the recruitment of one or more G α subunits of the heterotrimeric G proteins followed

by activation of enzyme effectors, production of second messengers, and transduction of intracellular signals (Neer 1995). Until recently, second messengers were thought to be the only mediators for GPCRs to trigger signalling pathways following agonist stimulation. Therefore, mutations at GPCRs inhibiting second messenger production were classified as inactivating or loss-of-function mutations. Accordingly, the A189V mutation, which dramatically decreases expression of the FSHR at the plasma membrane (Rannikko *et al.* 2002) and suppresses FSH-stimulated cAMP production has been classified as an inactivating mutation (Aittomaki *et al.* 1995).

However, it has been recently described for many GPCR (Luttrell *et al.* 1999; DeFea *et al.* 2000a; DeFea *et al.* 2000b; Luttrell *et al.* 2001; Reiter and Lefkowitz 2006; Shenoy *et al.* 2006), including the FSHR (Kara *et al.* 2006), that β -arrestins -molecules known for their key role in desensitization and internalisation of GPCRs- are able to activate signalling pathways in response to agonist stimulation. Moreover, it has been demonstrated that G protein- and β -arrestin-dependent signalling pathways are independent. A number of GPCR mutants have been reported to selectively trigger β -arrestin-dependent signalling pathways without inducing G protein coupling (Wei *et al.* 2003; Reiter and Lefkowitz 2006; Shenoy *et al.* 2006). In addition, some GPCR ligands have recently been reported to selectively activate β -arrestin-mediated signalling (Azzi *et al.* 2003; Wei *et al.* 2003; Gesty-Palmer *et al.* 2006; Wisler *et al.* 2007; Drake *et al.* 2008). Consistent with this concept, we recently demonstrated that a partially deglycosylated equine LH was able to selectively activate β -arrestin-dependent signalling pathways in the absence of cAMP production at the FSHR (Wehbi *et al.* 2010a).

In light of this emerging paradigm for GPCR signalling, one hypothesis that may explain the discrepancy between subfertility of men carrying the A189V FSHR mutation *versus* infertility caused by the FSH β -subunit inactivating mutations would be that the A189V receptor might actually retain some G protein-independent signalling properties. In the present study, we

95 addressed this hypothesis using HEK293N cells expressing either the wild-type (Wt) or the
96 A189V human FSHR.

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2 Materials and methods

2.1 Reagents

3-isobutyl-1-methylxanthine (IBMX) and forskolin were purchased from Sigma-Aldrich (St Louis, MO, USA). H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-iso-quinolinesulfonamide) was purchased from Calbiochem (San Diego, CA, USA). G418 was from Invivogen (Invivogen, San Diego, CA, USA). Minimum essential medium, fetal bovine serum, penicillin, streptomycin, trypsin-EDTA and bovine serum albumin were obtained from PAA Laboratories GmbH (Pasching, Austria). Rabbit polyclonal anti-ERK2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while rabbit polyclonal anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody was from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit polyclonal anti- β -arrestin 1 and 2 antibody (A1CT) was a kind gift from Dr. R.J. Lefkowitz (Duke University, Durham, NC, USA). The Eugene 6 transfection reagent was purchased from Roche Diagnostics (Basel, Switzerland). The SuperSignal West Pico Chemiluminescent substrate was obtained from Pierce (Pierce Biotechnologies, Rockford, IL, USA). Highly purified porcine FSH (pFSH) was kindly provided by Dr. J. Closset (Université de Liège, Belgium) and Dr. G.R. Bousfield (Department of Biological Sciences, Wichita State University, Wichita, KA, USA).

2.2 Plasmids and cells

The plasmid encoding the full-length human FSHR-FLAG fusion protein was generated employing the procedure previously described to construct the rat FSHR-FLAG (Reiter *et al.* 2001). The original full-length human FSHR cDNA was obtained from the Missouri S&T cDNA Ressource Center (Rolla, MO, USA; www.cdna.org). HEK293N cells were grown in minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 100

U/ml penicillin, and 100 µg/ml streptomycin, at 37°C in a humidified 5% CO₂ incubator. Cells were transfected at 60-70% confluency with 0.018 µg/cm² of the plasmid encoding the full-length human FSHR-FLAG using the Fugene 6 transfection reagent. HEK293N cells stably expressing either the Wt or the A189V FSHR were obtained following G418 selection. To obtain similar levels of plasma membrane expression of the Wt and the mutant A189V FSHRs, HEK293N cells were transiently transfected with 1.05 ng/cm² of the full-length human Wt FSHR or 42 ng/cm² of full length A189V FSHR. The total amount of DNA transfected was kept constant by adding appropriate amounts of pcDNA 3.1 empty vector to wells transfected with the Wt FSHR.

2.3 Binding assays

pFSH was labeled following the IODO-GEN (Pierce, Rockford, IL, USA) method. Total binding of [¹²⁵I]-labelled pFSH to the Wt or A189V FSHR were measured after 1 hour exposure to intact cells plated in 96-well plates at 37°C. For ¹²⁵I-FSH displacement experiments, cells were incubated 1 hour at 37°C with a mix containing 25,000 cpm of ¹²⁵I-FSH and increasing doses (0 to 33 nM) of unlabelled pFSH. Binding reactions were carried out in 41.5 µl of binding buffer (minimum essential medium, 10 U/ml penicillin, 10 µg/ml streptomycin, 250 mM Hepes and 0.1 % BSA) with 100,000 cpm of ¹²⁵I-pFSH. At the end of the incubation period, cell monolayers were washed thrice with 200 µl PBS and then lysed in 200 µl of 0.5 M NaOH. The amount of radioactivity in each lysate was quantitated by spectroscopy using a Wallac Wizard scintillation counter (Amersham Biosciences, Pittsburgh, PA, USA). Each condition was assayed by triplicate.

2.4 cAMP assay

HEK293N cells stably expressing either the human Wt or A189V FSHR (cultured on 24-well plates and grown to 50% confluency) were serum-starved in the presence of 100 μ M IBMX for 30 min prior to stimulation. Cells were incubated for 1 hour with pFSH in the presence of 100 μ M IBMX at 37°C in a humidified 5% CO₂ incubator. Total cAMP was measured using a commercially available cAMP ELISA kit (Biomedical Technologies, Inc., Stoughton, MA, USA) according to the manufacturer's instructions.

2.5 FRET assays

HEK293N cells stably or transiently expressing the human Wt or A189V FSHR were transiently transfected with either the cAMP (ICUE) or PKA (AKAR) plasmid-encoded FRET sensor. Both sensors were kindly provided by Dr. Jin Zhang (Johns Hopkins University, Baltimore, MD, USA) (Zhang *et al.* 2001; DiPilato *et al.* 2004). Twenty-four hours after transfection, cells were splitted and seeded on imaging dishes (BD Biocoat, BD Biosciences, San Jose, CA, USA). Forty-eight hours after transfection, cells were serum-starved for 90 min and imaged in the dark on a 37°C temperature-controlled stage. Cells were imaged on a Leica DM IRBE (Leica Microsystems, Wetzlar, Germany) microscope with a CoolSnap fx cooled charge-coupled device camera (Roper Scientific, Ottobrunn, Germany) controlled by METAFLUOR 7.5 (Universal Imaging Corporation, Downingtown, PA, USA). Dual emission *ratio* imaging used a 436DF10 excitation filter, a 436-510 DBDR dichroic mirror, and 480AF30 and 550AF30 emission filters for CFP and YFP, respectively. The filters were alternated by a Lambda 10-2 filter changer (Sutter Instruments, Novato, CA, USA). Exposure time was 100 ms, and images were taken every 15 sec. Typically, 10 sensor-positive cells and 4 non-specific areas were chosen in the field of the microscope. Base line signals at the wavelength corresponding to YFP and CFP were recorded for 5 min and then, the hormone was added to the dish. Signals were recorded for the next 40 min before

forskolin (20 μ M) was added to the cells as a positive control. The areas that did not respond to forskolin were removed from the analysis. Fluorescent intensity of non-transfected cells was subtracted to the intensity of fluorescent cells expressing the sensor in order to quantify the specific signal. The FRET *ratio* (CFP/YFP for the cAMP sensor and YFP/CFP for the PKA sensor) was calculated for each individual cell. The mean $\pm$ SEM from at least 20 individual cell responses measured in 3 different experiments were plotted.

2.6 Reporter gene assay

HEK293N cells stably expressing either the human Wt or A189V FSHR were transiently transfected with the pSOMLuc reporter gene as previously described (Lazari *et al.* 1999; Troispoux *et al.* 1999). Cells were split into 12-well plates 24 hours after transfection. Forty-eight hours after transfection, cells were serum-starved for 24 hours, then stimulated for 6 hours with pFSH. Cells were then washed twice with cold PBS and lysed with passive lysis buffer. The luciferase activity was measured using the luciferase assay system supplied by Promega Corp (Madison, WI, USA). Each sample (20 μ l) was mixed with 50 μ l of luciferase assay reagent, containing the substrate. The light produced was measured in a luminescence counter (Luminoskan Ascent, Thermo Labsystems, Waltham, MA, USA) and expressed as fold increase over basal.

2.7 Small interfering RNA transfection

The siRNA sequence 5'-AAACCUGCGCCUUCCGCUAUG-3' was used to simultaneously target human β -arrestin 1 (positions 172-190 relative to the start codon) and β -arrestin 2 (position 175-193). One small RNA duplex with no silencing effect was used as a control (5'-UUCUCCGAACGUGUCACGU-3'). These chemically synthesized siRNAs (Eurogentec SA, Liège, Belgium) have been previously described and validated (Gesty-Palmer *et al.* 2006;

Kara *et al.* 2006). Early passage HEK293N cells stably expressing human Wt or A189V FSHR at 10-20% confluency in 100 mm dishes were transfected with siRNA using the GeneSilencer transfection reagent following the manufacturer's recommendations (Genlantis, San Diego, CA, USA). Forty-eight hours after transfection, cells were seeded into 12-well plates. All assays were performed 3 days after transfection.

2.8 Western blotting

Cells were stimulated with pFSH for various time periods (kinetics) or for 5 minutes with increasing doses of hormone in the presence of vehicle or H89 and the supernatants were removed. Cells were then lysed in 2X Laemmli buffer and equivalent amounts of protein were analysed by Western blot. The membranes were incubated overnight at 4°C with anti-phospho-ERK 1/2 (1:3000) and then with secondary anti-mouse IgG horseradish peroxidase conjugate (Biosource International, Armadillo, CA, USA). Signal was developed using the Pierce ECL Western Blotting detection kit (Rockford, IL, USA). Equal protein loading was confirmed in a stripped, washed and reprobed membrane with primary polyclonal antibody against total ERK2 (1:10,000). To monitor β -arrestin protein silencing, endogenous β -arrestins 1 and 2 were detected with A1CT antibody (1:3000), as previously described (Kara *et al.* 2006).

2.9 Statistical analysis

Statistical analysis was performed with one-way analysis of variance (followed by Bonferroni's multiple comparison tests) to compare results from several samples or with two-way analysis of variance to compare entire curves (GraphPad PRISM Software, San Diego, CA, USA).

3 Results

3.1 The A189V mutation leads to a dramatic reduction of FSHR expression at the plasma membrane in stably transfected HEK293N cells

HEK293N cells were stably transfected using the pcDNA 3.1(+) encoding the full length human FSHR sequence bearing the N-terminus FLAG (DYKDDDDK) and hemagglutinin signal peptide (MKTIIALSYIFCLVFA) sequences (Fig. 1A). Total binding experiments displayed that A189V FSHR is expressed at 1.5% of Wt levels (Fig. 1B). These results are consistent with previous studies showing a dramatic decrease in plasma membrane expression of the A189V FSHR when transiently expressed in MSC-1 or COS7 cell lines. Displacement curves and subsequent Scatchard analysis showed that the calculated K_d for the A189V FSHR (1.3 nM) is similar to that previously reported for the Wt FSHR (0.780 nM) and the A189V FSHR (0.820 nM) (Rannikko *et al.* 2002) (Figs. 1A and B of supplementary data); the number of cell surface A189V FSHRs per cell was 1500 as disclosed by this analysis.

3.2 The A189V mutation suppresses FSHR coupling to the cAMP/PKA pathway.

It has been found that FSH-stimulated cAMP production by MSC-1 or COS7 cell lines transiently expressing the A189V mutation is significantly decreased when compared to the Wt FSHR (Aittomaki *et al.* 1995; Rannikko *et al.* 2002). Here, we investigated whether this mutation impaired cAMP response to FSH exposure in HEK293N cells stably expressing the FSHR. Dose-response curves of cAMP accumulation in the presence of the phosphodiesterase inhibitor IBMX clearly showed that the A189V mutation completely suppressed the ability of the FSHR to elicit cAMP production after 1 hour of FSH exposure (Fig. 2A). As expected,

this response was in sharp contrast with that observed in cells expressing the Wt FSHR. FRET assays using the cAMP sensor ICUE (DiPilato *et al.* 2004) were then carried out in living cells in order to rule out the possibility of transient intracellular cAMP production that may pass undetected by the cAMP assay. As shown in Fig. 2B, FRET experiments revealed that the robust and rapid agonist-stimulated cAMP production presented by cells expressing the Wt receptor was completely abrogated in cells expressing the A189V mutant. In both conditions, however, adenylyl-cyclase activity remained inducible as revealed by the addition of forskolin (Fig. 2B). To further corroborate this observation, PKA activation was measured in living cells using the FRET sensor AKAR (Allen and Zhang 2006). Consistent with the results from the cAMP assays, we found that the A189V mutation repressed FSH-induced PKA activation without affecting PKA activity inducible by forskolin (Fig. 2C). Finally, in order to compare the cAMP/PKA transcriptional-associated response elicited by the Wt and mutant FSHRs, cells were transfected with the cAMP-sensitive reporter plasmid pSOMLuc. The results showed that the Wt FSHR induced a robust dose-dependent response in luciferase activity whereas the A189V mutant FSHR failed to trigger any significant response (Fig. 2D).

3.3 The A189V FSHR mutant induces ERK MAPK phosphorylation in response to FSH stimulation

We next examined whether the A189V FSHR, which is unable to activate the cAMP/PKA transduction pathway, retained its ability to trigger ERK 1/2 MAPK phosphorylation, a well known FSHR-mediated signalling event previously reported to be partially cAMP/PKA-independent (Kara *et al.* 2006). A time-course of ERK phosphorylation provoked by FSH stimulation (3.3 nM) was performed in HEK293N cells stably expressing the Wt or A189V FSHR. When pERK 1 and 2 were analysed by Western blot in whole cell lysates a robust

FSH-induced ERK phosphorylation response was detected not only for the Wt FSHR but also for the A189V mutant. Although the mutant FSHR-mediated ERK phosphorylation was clearly lower than that triggered by the Wt FSHR, the response was consistently detected in all experiments despite the low expression levels of the mutant receptor species (Figs. 3A and B). In both FSHRs the increase in pERK signal was rapid, reaching its maximum after 5 minutes of FSH exposure. However, in the case of the Wt receptor FSH-stimulated ERK phosphorylation was detected for over 2 hours whereas for the mutant receptor it only lasted one hour. Dose-response curves at 5 minutes were next carried out (Figs. 4 A and B). The results displayed an unequivocally dose-dependent increase in ERK phosphorylation for both receptors (Figs. 4B and 4C), with a lower efficacy for the A189V FSHR (Fig. 4B).

3.4 FSH-induced ERK MAPK phosphorylation by the A189V FSHR mutant is PKA-independent

To confirm that the PKA-dependent pathway was not involved in FSH-stimulated ERK MAPK phosphorylation by the A189V FSHR, dose-response experiments in the presence of the PKA inhibitor H89 were performed. The results showed that FSH-induced ERK phosphorylation by the A189V FSHR was recalcitrant to H89 treatment (Fig. 5A and 5B).

3.5 FSH-induced ERK MAPK phosphorylation by the A189V FSHR mutant is primarily β -arrestin dependent

Previous studies from our group have demonstrated that FSH-induced ERK phosphorylation is triggered by both cAMP/PKA- and β -arrestin-dependent pathways (Kara *et al.* 2006; Wehbi *et al.* 2010a; Wehbi *et al.* 2010b). To investigate the contribution of β -arrestin-dependent transduction mechanisms to ERK phosphorylation, HEK293N cells stably expressing the Wt or the A189V FSHR were transfected with siRNA targeting both β -arrestin 1 and 2. As expected, in cells transfected with β -arrestin siRNA endogenous β -arrestin expression was dramatically reduced compared to cells transfected with control siRNA (Fig. 6A). Dose-response curves demonstrated that ERK phosphorylation induced by the activated Wt receptor was only partially sensitive to β -arrestins depletion (Figs. 6B and 6C), whereas ERK phosphorylation induced by the A189V mutant was abrogated by knocking down β -arrestins 1/2 (Figs. 6B and 6D). Side-by-side comparison confirmed that the A189V mutant was significantly more sensitive to β -arrestins depletion than the Wt FSHR (Fig. 6E). These results demonstrated that β -arrestins-dependent signalling accounts for most of ERK phosphorylation mediated by the A189V mutant but not by the Wt FSHR, which exhibited a bimodal regulation of ERK phosphorylation.

3.6 β -arrestins-dependent ERK phosphorylation predominates at low levels of Wt FSHR plasma membrane expression

We next sought to investigate whether the selective β -arrestin-dependent ERK phosphorylation observed with the A189V FSHR resulted from the impact of the mutation on receptor function, switching its coupling or alternatively from the very low levels of receptor expression at the plasma membrane. To attenuate the expression levels of the Wt FSHR to a minimum, we transiently transfected low amounts of Wt FSHR cDNA (bearing the sequence encoding its natural signal peptide) in HEK293N cells. This procedure dramatically reduced

Wt receptor plasma membrane expression at levels comparable with those of the A189V FSHR mutant (Fig. 7A). Under this condition, FSH-stimulated cAMP production was monitored by FRET (Fig. 7B). The results showed that when expressed at low levels, the Wt FSHR is unable to mediate detectable cAMP production in response to a high dose (3.3 nM) FSH challenge. As previously observed, no significant agonist-stimulated cAMP production was detected in cells transfected with the A189V FSHR, and in both conditions adenylyl-cyclase remained inducible by forskolin. Time-course experiments to monitor ERK phosphorylation following FSH exposure revealed that activation of both Wt and mutant receptors by agonist provoked a comparable transient increase in ERK phosphorylation, with a maximum at 5 minutes (Figs. 7C and 7D). Dose-response experiments to monitor ERK phosphorylation at 5 minutes following FSH exposure revealed that stimulation of both receptors increased the pERK signal with comparable efficacies (Figs. 7E and 7F).

4 DISCUSSION

It has been shown that the A→V substitution at position 189 of the FSHR leads to gonadal dysgenesis and hypergonadotrophic hypogonadism in women (Aittomaki *et al.* 1995). It has also been demonstrated that this mutation provokes intracellular retention of the receptor [due to misfolding of the receptor protein (Rannikko *et al.* 2002)] as well as defective function as disclosed by a marginal or null ability to transduce signals *via* G proteins (Aittomaki *et al.* 1995; Tena-Sempere *et al.* 1999; Rannikko *et al.* 2002). In fact, this so-called inactivating mutation is well known to prevent agonist-stimulated cAMP production and phosphoinositide turnover (Aittomaki *et al.* 1995; Tena-Sempere *et al.* 1999). In the present study, we provide experimental evidence supporting the notion that the A189V mutation in the FSHR retains its ability to promote G protein-independent signalling. Our results show that this mutant can phosphorylate ERK MAPK despite being essentially uncoupled from the canonical cAMP/PKA pathway. This finding offers a plausible explanation for the mild abnormalities detected in men bearing the A189V mutation (e.g. oligozoospermia) when compared to those carrying FSHβ inactivating mutations (Matthews *et al.* 1993; Aittomaki *et al.* 1995; Layman *et al.* 1997; Layman *et al.* 2002). Thus, whereas FSHβ inactivating mutations lead to complete suppression of all potential FSH-dependent signalling pathways due to inability of the mutant FSH to bind and activate the FSHR, the A189V mutation only impairs Gαs- (cAMP/PKA-dependent) and Gαq/11-mediated pathways. The overall data suggest that G protein-dependent pathways are essential for female reproduction whereas in men reproduction could still be feasible, though with lower efficiency, independently of G protein-coupling.

The results presented herein additionally demonstrate that β-arrestin-dependent signalling is responsible for ERK phosphorylation triggered by the A189V FSHR. In fact,

siRNA-mediated depletion of endogenous β -arrestins 1 and 2 primarily abrogated FSH-promoted ERK phosphorylation by the A189V FSHR but not by the Wt receptor. These data are consistent with our previous studies showing that β -arrestins can induce G protein-independent signalling at the FSHR (Kara *et al.* 2006; Wehbi *et al.* 2010a; Wehbi *et al.* 2010b). Interesting, in contrast to what has been reported for the rat FSHR, which shows a slow and sustained ERK phosphorylation upon agonist exposure (Kara *et al.* 2006), we detected that agonist-provoked activation of the A189V mutant was followed by a rapid and transient β -arrestin-dependent ERK phosphorylation. This apparent discrepancy could be due to the lack of G protein coupling of the mutant receptor, which may potentially abrogate the competition between G proteins and β -arrestins for receptor recruitment. In this scenario, β -arrestin coupling to the mutant receptor and subsequent signalling would proceed faster than that presented by Wt receptor. Although physiological roles for β -arrestin-dependent signalling have recently been described for other GPCR (Premont and Gainetdinov 2007; Gesty-Palmer *et al.* 2009), it is the first time that β -arrestins are suspected to play a role in reproductive function.

It has been previously reported that the A189V mutation leads to intracellular retention of the FSHR associated with a dramatic decrease in plasma membrane expression level and attenuation of G protein coupling (Rannikko *et al.* 2002). In the present study, we showed that the preferential β -arrestin coupling exhibited by the A189V FSHR is paradoxically due to the low level of plasma membrane expression rather than to the mutation itself. In fact, our data demonstrate that in cells expressing the Wt FSHR at a plasma membrane density comparable to that of the A189V FSHR, FSH exposure promoted ERK phosphorylation without triggering detectable intracellular signalling mediated by the cAMP/PKA pathway. In this vein, Rannikko *et al.* (2002) showed that overexpression of the A189V FSHR mutant in COS-7 cells resulted in agonist-stimulated cAMP accumulation detectable in the presence of

phosphodiesterase inhibitors. Under these conditions, cAMP production per receptor unit was comparable between the Wt and the A189V FSHRs (Rannikko *et al.* 2002). Based on these observations and depending on the cellular context, it is reasonable to consider that the A189V FSHR can still couple to G α s but that the G protein-independent signalling observed may result from the low cell surface plasma membrane levels of the mutant, rather than from the functional impairment imposed by the A189V mutation. In this setting, FSH-stimulated cAMP production may still be insufficient to promote PKA activation.

The results of the present study, suggest that the A189V FSHR can be phosphorylated by GRKs and recruit β -arrestins as revealed by the receptor's ability to signal through β -arrestin scaffolding. A growing body of evidence supports the notion that signalling at GPCR can be selectively triggered or dampened by using either biased ligands or modified receptors (Wehbi *et al.*; Wei *et al.* 2003; Shenoy *et al.* 2006; Violin and Lefkowitz 2007). In this vein, our data establish for the first time that selective signalling may also be achieved by modulating cell surface plasma membrane levels of the receptor, underlining the importance of receptor density as a regulatory mechanism to control cellular signalling. In the present study, induction of β -arrestins-dependent biased signalling was achieved by decreasing the number of functional receptors in HEK293N cells to levels found in primary granulosa cells (1000 receptors per cell; McNatty *et al.* 1989) or primary Sertoli cells from 19 days old rat [6400 receptors per cell as calculated from our previously published data (Musnier *et al.* 2009)]. Since plasma membrane expression of the FSHR varies depending on the developmental and differentiation stages of the gonads (Simoni *et al.* 1997), it is tempting to speculate that varying FSHR levels might lead to differential coupling to effectors and, consequently, to distinct biological responses known to be associated with the gonadotropic signal. Undoubtedly, the cellular context should be considered when trying to understand Gs protein/ β -arrestin balance in a given experimental paradigm. In fact, it has been shown that rat

Sertoli cells from animals at 2 different developmental stages (5 and 19 days of age) exhibit similar cell surface membrane levels of FSHR yet differ in coupling to G proteins (Musnier *et al.* 2009, Crepieux *et al.*, 2001).

The A189V mutation of the FSHR has been associated with primary ovarian failure in the Finnish population (Aittomaki *et al.* 1995). Recently, a gene therapy approach using adenoviruses expressing the human FSHR has been proposed as a potential intervention to restore a normal phenotype in women affected by this mutation (Ghadami *et al.* 2008). Since when expressed at high levels the A189V FSHR remains functional a therapeutic approach addressed to improve the expression levels of the A189V FSHR could also be envisioned. In this regard, Janovick and colleagues (2009) recently showed that a membrane permeable allosteric modulator of the FSHR was able to partially rescue plasma membrane expression of the A189V mutant, suggesting that such molecules also represent good candidates for treating patients bearing particular inactivating mutations of the FSHR (Janovick *et al.* 2009).

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Figure Legends

Fig. 1: FSH binding to the Wt or A189V human FSH receptor

(A) Schematic representation of the structure of the FSHR with the A189V substitution (EC: Extracellular domain; TM: Transmembrane Domain; IC: Intracellular Domain). The endogenous signal peptide was replaced by the FLAG and the hemagglutinin signal peptide (HA-SP) sequences in both the Wt and A189V FSHR. (B) HEK293N cells stably expressing the Wt or A189V human FSH receptor were serum-starved for 1 hour and then incubated for 1 hour at 37°C with 100,000 cpm of 125 I-FSH. Specific binding values were normalized according to protein concentration. Results are expressed as mean $\pm$ sem of at least 3 independent experiments.

Fig. 2: FSH-induced cAMP/PKA pathway activation by Wt vs A189V FSHR.

(A) HEK293N cells stably expressing the Wt or A189V FSHR were serum-starved for 30 min in the presence of IBMX and then stimulated with increasing concentrations of pFSH (zero to 4 nM) for 1 hour. After stimulation, cell extracts were lysed as described in *Materials and Methods*. Intra- and extra-cellular cAMP produced was assayed by ELISA. Results are normalized according to protein concentration and expressed as mean $\pm$ sem of four independent experiments. (B) Cells were transiently transfected with the cAMP FRET sensor ICUE. Forty-eight hours after transfection, cells were serum-starved for 1 hour at 37°C before ICUE FRET ratio measurement (CFP emission/YFP emission). After 5 min of baseline measurement, cells were stimulated with 3.3 nM pFSH at 37°C. Forskolin (20 μ M) was added 40 minutes after FSH stimulation, as a positive control. Results are expressed as mean $\pm$ sem of 20 individuals cells responses measured in 3 different experiments. (C) Cells were

transiently transfected with the PKA FRET sensor AKAR. Forty-eight hours after transfection, cells were serum-starved for 1 hour at 37°C before AKAR FRET ratio measurement (YFP emission/CFP emission). After 5 min of baseline measurement, cells were stimulated with 3.3 nM pFSH at 37°C. Forskolin (20 µM) was added 40 minutes after FSH stimulation, as a positive control. Results are expressed as mean ± sem of at least 20 individual cell responses measured in 3 different experiments. **(D)** Cells were transiently transfected with the cAMP-sensitive reporter plasmid pSOMLuc. Forty-eight hours after transfection, cells were serum-starved for 24 hours and then stimulated for 6 hours with increasing doses of pFSH (zero to 3.3 nM). Luciferase activity was then measured in total cell extracts. Results are expressed as mean ± sem of 5 independent experiments. *, $p < 0.05$, ***, $p < 0.001$, compared to the basal response.

Fig. 3: Kinetics of FSH-induced ERK phosphorylation by the Wt and A189V human FSHR

HEK293N cells stably expressing the Wt or A189V FSHR were serum-starved for 4 hours and then stimulated with 3.3 nM pFSH for increasing time periods. After stimulation, cell extracts were prepared as described in *Materials and Methods*. Endogenous phosphorylated ERKs were revealed using an anti-pERK antibody. Membranes were stripped as described in *Materials and Methods* and revealed with anti-ERK2 antibody for normalisation. **(A)** Representative immunoblot for FSH-induced ERK phosphorylation. **(B)** ERK phosphorylation signals were quantified by densitometry on the same film and expressed as the ratio of phosphorylated ERK over total ERK2. The maximal Wt-induced ERK phosphorylation was arbitrarily chosen as 100%. Data correspond to the mean ± sem of at least 5 independent experiments. ***, $p < 0.001$ compared with the entire control curve.

Fig. 4. Doses-response curves of FSH-induced ERK phosphorylation by the Wt and A189V human FSHR.

HEK293N cells stably expressing the Wt or A189V FSHR were serum-starved for 4 hours and then exposed to increasing doses of pFSH (from zero to 10 nM) for 5 minutes. After stimulation, cell extracts were prepared as described in *Materials and Methods*. Endogenous phosphorylated ERK were revealed using an anti-pERK antibody. Membranes were stripped as described in *Materials and Methods* and revealed with anti-ERK2 antibody for normalisation. **(A)** Representative immunoblots of FSH-induced ERK 1/2 phosphorylation. **(B)** ERK phosphorylation signals were quantified by densitometry on the same film and expressed as a ratio of phosphorylated ERK over total ERK2. The maximal Wt FSHR-induced ERK phosphorylation was arbitrarily chosen as 100%. Data correspond to the mean $\pm$ sem of at least 4 independent experiments. ***, $p < 0.001$ vs the entire control curve. **(C)** A189V FSHR-induced ERK phosphorylation signals were quantified by densitometry and expressed as a ratio of phosphorylated ERK over total ERK2. The maximal A189V FSHR-induced ERK phosphorylation was arbitrarily chosen as 100%. Data represent the mean $\pm$ sem of 4 individual experiments. *, $p < 0.05$ when compared to the basal level.

Fig. 5. Contribution of PKA-dependent transduction mechanism to FSH-induced ERK activation by the A189V FSHR

HEK293N cells stably expressing the A189V FSHR were serum-starved for four hours and pre-incubated with either 20 μ M H89 or DMSO (negative control) prior to five minutes stimulation with increasing doses of pFSH (from 0 to 10 nM). Endogenous phosphorylated

ERK were revealed using anti-pERK antibody. Membranes were stripped as described in Materials and Methods and revealed with anti-ERK2 antibody for normalisation. **(A)** Representative immunoblots of FSH-induced ERK phosphorylation in cells incubated with H89 or DMSO. **(B)** ERK phosphorylation signals were quantified by densitometry and expressed as a ratio of phosphorylated ERK over total ERK2. Each data point represents the mean $\pm$ sem of three individual experiments. ns = non significant vs the entire control curve.

Fig. 6. Contribution of β -arrestin-dependent transduction mechanism to FSH-induced ERK phosphorylation by the Wt and the A189V human FSHR

HEK293N cells stably expressing either the Wt or the A189V FSHR were transfected either with siRNA targeting both β -arrestins 1 and 2 or with control siRNA. Forty-eight hours after transfection, cells were serum-starved for 4 hours and then stimulated for 5 minutes with increasing doses of pFSH (from 0 to 10 nM). After stimulation, cell extracts were prepared as described in the *Materials and Methods* section. Endogenous phosphorylated ERK were revealed using anti-pERK antibody. Membranes were stripped as described in *Materials and Methods* and revealed with anti-ERK2 antibody for normalisation. **(A)** Representative immunoblot of endogenous β -arrestins 1 and 2 (β arr 1/2) siRNA-mediated knock-down compared to control siRNA (CTL). **(B)** Representative immunoblots of FSH-induced ERK phosphorylation in cells transfected with either CTL or β -arr1/2 siRNA. **(C, D)** ERK phosphorylation signals were quantified by densitometry and expressed as a ratio of phosphorylated ERK over total ERK2. The maximal phosphorylation of ERK obtained in control (CTL siRNA) was arbitrarily chosen as 100%. Each data point represents the mean $\pm$ sem of at least four individual experiments. *, $p < 0.05$, ***, $p < 0.001$ vs. the entire control

curve. **(E)** Sensitivity of ERK phosphorylation to β -arrestins depletion compared side-by-side for the Wt and the A189V FSHR. *, $p < 0.05$

Fig. 7. Effect of low-level expression of the Wt FSHR on FSH-induced cAMP/PKA-dependent and β -arrestin-dependent pathways.

(A) HEK293N cells transiently transfected with either the Wt or the A189V human FSHR were serum-starved for 1 hour and then incubated with 100,000 cpm of ^{125}I -FSH for 1 hour at 37°C. Low plasmid amounts of Wt FSHR were transfected and compensated with pcDNA3.1 empty vector. Results are expressed as total binding normalized according to protein concentration and compared to the results obtained in HEK293N cells stably expressing the Wt human FSH receptor from at least 2 individual experiments. **(B)** HEK293N cells were transiently co-transfected with the ICUE cAMP FRET sensor and either the Wt or the A189V FSHR. Forty-eight hours after transfection, cells were serum-starved for 1 hour before ICUE FRET *ratio* measurement (CFP emission/YFP emission) at 37°C. After 5 min of baseline measurements, cells were stimulated with pFSH (3.3 nM). Forskolin (20 μM) was added 40 minutes after FSH stimulation as a positive control. Results are expressed as mean $\pm$ sem of at least 17 individual cells measured in 3 different experiments. **(C, D)** HEK293N cells transiently transfected with either the Wt or the A189V human FSHR were serum-starved for 4 hours and then stimulated with 3.3 nM pFSH for increasing time period. After stimulation, cell extracts were prepared as described in Materials and Methods. Endogenous phosphorylated ERK were revealed using anti-pERK antibody. Membranes were stripped as described in Materials and Methods and revealed with anti-ERK2 antibody for normalisation. **(C)** Representative immunoblots for FSH-induced ERK phosphorylation. **(D)** ERK phosphorylation signals were quantified by densitometry and expressed as a ratio of

phosphorylated ERK over total ERK2. Each data point represents the mean $\pm$ sem of seven individual experiments. **(E, F)** HEK293N cells transiently transfected with either the Wt or A189V human FSH receptor were serum-starved for 4 hours and then stimulated for 5 minutes with increasing (zero to 10 nM) doses of pFSH. After stimulation, cell extracts were prepared as described in *Materials and Methods*. Endogenous phosphorylated ERK were revealed using an anti-pERK antibody. Membranes were stripped as described in *Materials and Methods* and revealed with anti-ERK2 antibody for normalisation. **(E)** Representative immunoblots of FSH-induced ERK phosphorylation. **(F)** ERK phosphorylation signals were quantified by densitometry and expressed as the ratio of phosphorylated ERK over total ERK2. The maximal phosphorylation signal of ERK obtained with the Wt receptor was arbitrarily chosen as 100%. Each data point represents the mean $\pm$ sem from 5 individual experiments.

References

- Aittomaki, K., J. L. Lucena, P. Pakarinen, P. Sistonen, J. Tapanainen, J. Gromoll, R. Kaskikari, E. M. Sankila, H. Lehvaslaiho, A. R. Engel, E. Nieschlag, I. Huhtaniemi and A. de la Chapelle (1995). "Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure." *Cell* **82**(6): 959-68.
- Allen, M. D. and J. Zhang (2006). "Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters." *Biochem Biophys Res Commun* **348**(2): 716-21.
- Azzi, M., P. G. Charest, S. Angers, G. Rousseau, T. Kohout, M. Bouvier and G. Pineyro (2003). "Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors." *Proc Natl Acad Sci U S A* **100**(20): 11406-11.
- Crepieux P., S. Marion, N. Martinat, V. Fafeur, Y. L. Vern, D. Kerboeuf, F. Guillou and E. Reiter (2001). "The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation." *Oncogene* **20**(34): 4696-709.
- DeFea, K. A., J. Zalevsky, M. S. Thoma, O. Dery, R. D. Mullins and N. W. Bunnett (2000a). "beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2." *J Cell Biol* **148**(6): 1267-81.
- DeFea, K. A., Z. D. Vaughn, E. M. O'Bryan, D. Nishijima, O. Dery and N. W. Bunnett (2000b). "The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta-arrestin-dependent scaffolding complex." *Proc Natl Acad Sci U S A* **97**(20): 11086-91.
- Dierich, A., M. R. Sairam, L. Monaco, G. M. Fimia, A. Gansmuller, M. LeMeur and P. Sassone-Corsi (1998). "Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance." *Proc Natl Acad Sci U S A* **95**(23): 13612-7.
- DiPilato, L. M., X. Cheng and J. Zhang (2004). "Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments." *Proc Natl Acad Sci U S A* **101**(47): 16513-8.
- Drake, M. T., J. D. Violin, E. J. Whalen, J. W. Wisler, S. K. Shenoy and R. J. Lefkowitz (2008). "beta-arrestin-biased agonism at the beta2-adrenergic receptor." *J Biol Chem* **283**(9): 5669-76.
- Gesty-Palmer, D., M. Chen, E. Reiter, S. Ahn, C. D. Nelson, S. Wang, A. E. Eckhardt, C. L. Cowan, R. F. Spurney, L. M. Luttrell and R. J. Lefkowitz (2006). "Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation." *J Biol Chem* **281**(16): 10856-64.
- Gesty-Palmer, D., P. Flannery, L. Yuan, L. Corsino, R. Spurney, R. J. Lefkowitz and L. M. Luttrell (2009). "A beta-arrestin-biased agonist of the parathyroid hormone receptor (PTH1R) promotes bone formation independent of G protein activation." *Sci Transl Med* **1**(1): 1ra1.
- Ghadami, M., S. A. Salama, N. Khatoon, R. Chilvers, M. Nagamani, P. J. Chedrese and A. Al-Hendy (2008). "Toward gene therapy of primary ovarian failure: adenovirus expressing human FSH receptor corrects the Finnish C566T mutation." *Mol Hum Reprod* **14**(1): 9-15.
- Huhtaniemi, I. (2006). "Mutations along the pituitary-gonadal axis affecting sexual maturation: novel information from transgenic and knockout mice." *Mol Cell Endocrinol* **254-255**: 84-90.
- Janovick, J. A., G. Maya-Nunez, A. Ulloa-Aguirre, I. T. Huhtaniemi, J. A. Dias, P. Verboost and P. M. Conn (2009). "Increased plasma membrane expression of human follicle-

- stimulating hormone receptor by a small molecule thienopyr(im)idine." Mol Cell Endocrinol **298**(1-2): 84-8.
- Kara, E., P. Crepieux, C. Gauthier, N. Martinat, V. Piketty, F. Guillou and E. Reiter (2006). "A phosphorylation cluster of five serine and threonine residues in the C-terminus of the follicle-stimulating hormone receptor is important for desensitization but not for beta-arrestin-mediated ERK activation." Mol Endocrinol **20**(11): 3014-26.
- Krishnamurthy, H., N. Danilovich, C. R. Morales and M. R. Sairam (2000). "Qualitative and quantitative decline in spermatogenesis of the follicle-stimulating hormone receptor knockout (FORKO) mouse." Biol Reprod **62**(5): 1146-59.
- Kumar, T. R., Y. Wang, N. Lu and M. M. Matzuk (1997). "Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility." Nat Genet **15**(2): 201-4.
- Layman, L. C., E. J. Lee, D. B. Peak, A. B. Namnoum, K. V. Vu, B. L. van Lingen, M. R. Gray, P. G. McDonough, R. H. Reindollar and J. L. Jameson (1997). "Delayed puberty and hypogonadism caused by mutations in the follicle-stimulating hormone beta-subunit gene." N Engl J Med **337**(9): 607-11.
- Layman, L. C., A. L. Porto, J. Xie, L. A. da Motta, L. D. da Motta, W. Weiser and P. M. Sluss (2002). "FSH beta gene mutations in a female with partial breast development and a male sibling with normal puberty and azoospermia." J Clin Endocrinol Metab **87**(8): 3702-7.
- Lazari, M. F., X. Liu, K. Nakamura, J. L. Benovic and M. Ascoli (1999). "Role of G protein-coupled receptor kinases on the agonist-induced phosphorylation and internalization of the follitropin receptor." Mol Endocrinol **13**(6): 866-78.
- Lindstedt, G., E. Nystrom, C. Matthews, I. Ernest, P. O. Janson and K. Chatterjee (1998). "Follitropin (FSH) deficiency in an infertile male due to FSHbeta gene mutation. A syndrome of normal puberty and virilization but underdeveloped testicles with azoospermia, low FSH but high lutropin and normal serum testosterone concentrations." Clin Chem Lab Med **36**(8): 663-5.
- Luttrell, L. M., S. S. Ferguson, Y. Daaka, W. E. Miller, S. Maudsley, G. J. Della Rocca, F. Lin, H. Kawakatsu, K. Owada, D. K. Luttrell, M. G. Caron and R. J. Lefkowitz (1999). "Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes." Science **283**(5402): 655-61.
- Luttrell, L. M., F. L. Roudabush, E. W. Choy, W. E. Miller, M. E. Field, K. L. Pierce and R. J. Lefkowitz (2001). "Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds." Proc Natl Acad Sci U S A **98**(5): 2449-54.
- Matthews, C. H., S. Borgato, P. Beck-Peccoz, M. Adams, Y. Tone, G. Gambino, S. Casagrande, G. Tedeschini, A. Benedetti and V. K. Chatterjee (1993). "Primary amenorrhoea and infertility due to a mutation in the beta-subunit of follicle-stimulating hormone." Nat Genet **5**(1): 83-6.
- McNatty K. P., S. Lun, D. A. Heath, N. L. Hudson, L. E. O'Keeffe and K. M. Henderson (1989). "Binding characteristics of 125I-labelled human FSH to granulosa cells from Booroola ewes which were homozygous, heterozygous or non-carriers of a major gene(s) influencing their ovulation rate." J Reprod Fertil **86**(1): 27-38.
- Musnier A., D. Heitzler, T. Boulo, S. Tesseraud, G. Durand, C. Lecureuil, H. Guillou, A. Poupon, E. Reiter and P. Crepieux (2009). "Developmental regulation of p70 S6 kinase by a G protein-coupled receptor dynamically modeled in primary cells." Cell Mol Life Sci **66**(21): 3487-503.
- Neer, E. J. (1995). "Heterotrimeric G proteins: organizers of transmembrane signals." Cell **80**(2): 249-57.

- Phillip, M., J. E. Arbelle, Y. Segev and R. Parvari (1998). "Male hypogonadism due to a mutation in the gene for the beta-subunit of follicle-stimulating hormone." N Engl J Med **338**(24): 1729-32.
- Premont, R. T. and R. R. Gainetdinov (2007). "Physiological roles of G protein-coupled receptor kinases and arrestins." Annu Rev Physiol **69**: 511-34.
- Rannikko, A., P. Pakarinen, P. R. Manna, I. Beau, M. Misrahi, K. Aittomaki and I. Huhtaniemi (2002). "Functional characterization of the human FSH receptor with an inactivating Ala189Val mutation." Mol Hum Reprod **8**(4): 311-7.
- Reiter, E. and R. J. Lefkowitz (2006). "GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling." Trends Endocrinol Metab **17**(4): 159-65.
- Reiter, E., S. Marion, F. Robert, C. Troispoux, F. Boulay, F. Guillou and P. Crepieux (2001). "Kinase-inactive G-protein-coupled receptor kinases are able to attenuate follicle-stimulating hormone-induced signaling." Biochem Biophys Res Commun **282**(1): 71-8.
- Shenoy, S. K., M. T. Drake, C. D. Nelson, D. A. Houtz, K. Xiao, S. Madabushi, E. Reiter, R. T. Premont, O. Lichtarge and R. J. Lefkowitz (2006). "beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor." J Biol Chem **281**(2): 1261-73.
- Simoni, M., J. Gromoll and E. Nieschlag (1997). "The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology." Endocr Rev **18**(6): 739-73.
- Tapanainen, J. S., K. Aittomaki, J. Min, T. Vaskivuo and I. T. Huhtaniemi (1997). "Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility." Nat Genet **15**(2): 205-6.
- Tena-Sempere, M., P. R. Manna and I. Huhtaniemi (1999). "Molecular cloning of the mouse follicle-stimulating hormone receptor complementary deoxyribonucleic acid: functional expression of alternatively spliced variants and receptor inactivation by a C566T transition in exon 7 of the coding sequence." Biol Reprod **60**(6): 1515-27.
- Troispoux, C., F. Guillou, J. M. Elalouf, D. Firsov, L. Iacovelli, A. De Blasi, Y. Combarnous and E. Reiter (1999). "Involvement of G protein-coupled receptor kinases and arrestins in desensitization to follicle-stimulating hormone action." Mol Endocrinol **13**(9): 1599-614.
- Violin, J. D. and R. J. Lefkowitz (2007). "Beta-arrestin-biased ligands at seven-transmembrane receptors." Trends Pharmacol Sci **28**(8): 416-22.
- Wehbi, V., T. Tranchant, G. Durand, A. Musnier, J. Decourtye, V. Piketty, V. Y. Butnev, G. R. Bousfield, P. Crepieux, M. C. Maurel and E. Reiter (2010a). "Partially deglycosylated equine LH preferentially activates beta-arrestin-dependent signaling at the follicle-stimulating hormone receptor." Mol Endocrinol **24**(3): 561-73.
- Wehbi, V., J. Decourtye, V. Piketty, G. Durand, E. Reiter and M. C. Maurel (2010b). "Selective Modulation of Follicle-Stimulating Hormone Signaling Pathways with Enhancing Equine Chorionic Gonadotropin/Antibody Immune Complexes." Endocrinology.
- Wei, H., S. Ahn, S. K. Shenoy, S. S. Karnik, L. Hunyady, L. M. Luttrell and R. J. Lefkowitz (2003). "Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2." Proc Natl Acad Sci U S A **100**(19): 10782-7.
- Wisler, J. W., S. M. DeWire, E. J. Whalen, J. D. Violin, M. T. Drake, S. Ahn, S. K. Shenoy and R. J. Lefkowitz (2007). "A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling." Proc Natl Acad Sci U S A **104**(42): 16657-62.

713 Zhang, J., Y. Ma, S. S. Taylor and R. Y. Tsien (2001). "Genetically encoded reporters of
714 protein kinase A activity reveal impact of substrate tethering." Proc Natl Acad Sci U S
715 A **98**(26): 14997-5002.

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