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Thibaud Tranchant, Guillaume Durand, Christophe Gauthier, Pascale Crépieux, Alfredo Ulloa-Aguirre, et al.. Preferential β -arrestin signalling at low receptor density revealed by functional characterization of the human FSH receptor A189V mutation. *Molecular and Cellular Endocrinology*, 2010, 331 (1), pp.109. 10.1016/j.mce.2010.08.016 . hal-00639767

HAL Id: hal-00639767

<https://hal.science/hal-00639767v1>

Submitted on 10 Nov 2011

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Accepted Manuscript

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PII: S0303-7207(10)00430-2
DOI: doi:10.1016/j.mce.2010.08.016
Reference: MCE 7628

To appear in: *Molecular and Cellular Endocrinology*

Received date: 29-4-2010
Revised date: 26-7-2010
Accepted date: 19-8-2010

Please cite this article as: Tranchant, T., Durand, G., Gauthier, C., Crépieux, P., Ulloa-Aguirre, A., Royère, D., Reiter, E., Preferential β -arrestin signalling at low receptor density revealed by functional characterization of the human FSH receptor A189 V mutation, *Molecular and Cellular Endocrinology* (2010), doi:10.1016/j.mce.2010.08.016

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

3

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

5

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21 **Keywords:** FSH, FSH receptor, A189V mutation, β -arrestin, selective signalling.

22 T.T. was granted by The “Fondation d’Aide à la Recherche d’Organon” (FARO). G.D. was
23 funded by a fellowship from the French MRT. A.U.-A is Le STUDIUM Invited Professor at
24 the INRA.

25 **Disclosure statement:** The authors have nothing to disclose.

26

27 **ABSTRACT**

28
29
30 The A189V inactivating mutation of the human FSH receptor (FSHR) leads to subfertility in
31 men and primary ovarian failure in women. This mutation has previously been associated
32 with intracellular retention of the FSHR and impaired cAMP production. Here, we show that
33 the A189V FSHR stably expressed in HEK293N cells provoked ERK MAP kinases
34 phosphorylation through β -arrestins, independently of the canonical cAMP/PKA pathway.
35 Interestingly, both the A189V and wild-type (Wt) FSHRs selectively activated cAMP-
36 independent ERK phosphorylation when expressed at low plasma membrane densities. These
37 data indicate that the selective intracellular signalling triggered by the A189V FSHR resulted
38 from reduced membrane expression rather than by switching receptor coupling. Hence,
39 receptor density at the plasma membrane might control the balance between distinct signal
40 transduction mechanisms. Furthermore, our results help to clarify why mutations of FSH β are
41 more deleterious to human fertility than the FSHR A189V mutation which preserves parts of
42 receptor signalling repertoire.

43

44 1 INTRODUCTION

45
46

47 Follicle-stimulating hormone (FSH) is a glycoprotein synthesized by the pituitary
48 gland. This hormone binds to its cognate receptor (FSHR) in the gonads. The FSHR is
49 expressed by the granulosa cells of the ovarian follicle and by the Sertoli cells of the
50 seminiferous tubules. This gonadotropin promotes folliculogenesis and supports
51 spermatogenesis thereby playing a key role in reproduction.

52 Knock-out of either the FSH β -subunit or the FSHR gene in mice suggests that FSH action is
53 fundamental for female reproduction whereas in males, although necessary to achieve normal
54 testis size and sperm production, it is not essential for fertility (Kumar *et al.* 1997; Dierich *et*
55 *al.* 1998; Krishnamurthy *et al.* 2000). In women, inactivating mutations of either the FSH β -
56 subunit or the FSHR lead to primary amenorrhea and infertility, again suggesting that FSH
57 action is crucial for female reproduction (Matthews *et al.* 1993; Aittomaki *et al.* 1995;
58 Layman *et al.* 1997; Layman *et al.* 2002; Huhtaniemi 2006). In men, the A189V mutation is
59 the only FSHR inactivating mutation reported so far. In agreement with the FSHR knock-out
60 male phenotype, the five Finnish men presenting the A189V mutation displayed variable
61 degrees of oligozoospermia and subfertility (Tapanainen *et al.* 1997). Surprisingly however,
62 men with inactivating mutations of the FSH β -subunit were completely infertile due to
63 azoospermia (Lindstedt *et al.* 1998; Phillip *et al.* 1998; Layman *et al.* 2002). The reason(s) for
64 this apparent discrepancy between the severity of the phenotypes associated with inactivating
65 mutations in the FSH β -subunit *versus* the FSHR has previously been pointed out but still
66 remains to be clarified (Huhtaniemi 2006).

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

70 by activation of enzyme effectors, production of second messengers, and transduction of
71 intracellular signals (Neer 1995). Until recently, second messengers were thought to be the
72 only mediators for GPCRs to trigger signalling pathways following agonist stimulation.
73 Therefore, mutations at GPCRs inhibiting second messenger production were classified as
74 inactivating or loss-of-function mutations. Accordingly, the A189V mutation, which
75 dramatically decreases expression of the FSHR at the plasma membrane (Rannikko *et al.*
76 2002) and suppresses FSH-stimulated cAMP production has been classified as an inactivating
77 mutation (Aittomaki *et al.* 1995).

78 However, it has been recently described for many GPCR (Luttrell *et al.* 1999; DeFea *et al.*
79 2000a; DeFea *et al.* 2000b; Luttrell *et al.* 2001; Reiter and Lefkowitz 2006; Shenoy *et al.*
80 2006), including the FSHR (Kara *et al.* 2006), that β -arrestins -molecules known for their key
81 role in desensitization and internalisation of GPCRs- are able to activate signalling pathways
82 in response to agonist stimulation. Moreover, it has been demonstrated that G protein- and β -
83 arrestin-dependent signalling pathways are independent. A number of GPCR mutants have
84 been reported to selectively trigger β -arrestin-dependent signalling pathways without inducing
85 G protein coupling (Wei *et al.* 2003; Reiter and Lefkowitz 2006; Shenoy *et al.* 2006). In
86 addition, some GPCR ligands have recently been reported to selectively activate β -arrestin-
87 mediated signalling (Azzi *et al.* 2003; Wei *et al.* 2003; Gesty-Palmer *et al.* 2006; Wisler *et al.*
88 2007; Drake *et al.* 2008). Consistent with this concept, we recently demonstrated that a
89 partially deglycosylated equine LH was able to selectively activate β -arrestin-dependent
90 signalling pathways in the absence of cAMP production at the FSHR (Wehbi *et al.* 2010a)

91 In light of this emerging paradigm for GPCR signalling, one hypothesis that may explain the
92 discrepancy between subfertility of men carrying the A189V FSHR mutation *versus* infertility
93 caused by the FSH β -subunit inactivating mutations would be that the A189V receptor might
94 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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98 **2 Materials and methods**

99

100 *2.1 Reagents*

101 3-isobutyl-1-methylxanthine (IBMX) and forskolin were purchased from Sigma-Aldrich (St
102 Louis, MO, USA). H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-iso-quinolinesulfonamide)
103 was purchased from Calbiochem (San Diego, CA, USA). G418 was from Invivogen
104 (Invivogen, San Diego, CA, USA). Minimum essential medium, fetal bovine serum,
105 penicillin, streptomycin, trypsin-EDTA and bovine serum albumin were obtained from PAA
106 Laboratories GmbH (Pasching, Austria). Rabbit polyclonal anti-ERK2 antibody was from
107 Santa Cruz Biotechnology (Santa Cruz, CA, USA), while rabbit polyclonal anti-phospho-
108 p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody was from Cell Signaling Technology Inc.
109 (Danvers, MA, USA). Rabbit polyclonal anti- β -arrestin 1 and 2 antibody (A1CT) was a kind
110 gift from Dr. R.J. Lefkowitz (Duke University, Durham, NC, USA). The Fugene 6
111 transfection reagent was purchased from Roche Diagnostics (Basel, Switzerland). The
112 SuperSignal West Pico Chemiluminescent substrate was obtained from Pierce (Pierce
113 Biotechnologies, Rockford, IL, USA). Highly purified porcine FSH (pFSH) was kindly
114 provided by Dr. J. Closset (Université de Liège, Belgium) and Dr. G.R. Bousfield
115 (Department of Biological Sciences, Wichita State University, Wichita, KA, USA).

116

117 *2.2 Plasmids and cells*

118 The plasmid encoding the full-length human FSHR-FLAG fusion protein was generated
119 employing the procedure previously described to construct the rat FSHR-FLAG (Reiter *et al.*
120 2001). The original full-length human FSHR cDNA was obtained from the Missouri S&T
121 cDNA Ressource Center (Rolla, MO, USA; www.cdna.org). HEK293N cells were grown in
122 minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 100

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

132

133 *2.3 Binding assays*

134 pFSH was labeled following the IODO-GEN (Pierce, Rockford, IL, USA) method. Total
135 binding of [¹²⁵I]-labelled pFSH to the Wt or A189V FSHR were measured after 1 hour
136 exposure to intact cells plated in 96-well plates at 37°C. For ¹²⁵I-FSH displacement
137 experiments, cells were incubated 1 hour at 37°C with a mix containing 25,000 cpm of ¹²⁵I-
138 FSH and increasing doses (0 to 33 nM) of unlabelled pFSH. Binding reactions were carried
139 out in 41.5 µl of binding buffer (minimum essential medium, 10 U/ml penicillin, 10 µg/ml
140 streptomycin, 250 mM Hepes and 0.1 % BSA) with 100,000 cpm of ¹²⁵I-pFSH. At the end of
141 the incubation period, cell monolayers were washed thrice with 200 µl PBS and then lysed in
142 200 µl of 0.5 M NaOH. The amount of radioactivity in each lysate was quantitated by
143 spectroscopy using a Wallac Wizard scintillation counter (Amersham Biosciences, Pittsburgh,
144 PA, USA). Each condition was assayed by triplicate.

145

146 *2.4 cAMP assay*

147 HEK293N cells stably expressing either the human Wt or A189V FSHR (cultured on 24-well
148 plates and grown to 50% confluency) were serum-starved in the presence of 100 μ M IBMX
149 for 30 min prior to stimulation. Cells were incubated for 1 hour with pFSH in the presence of
150 100 μ M IBMX at 37°C in a humidified 5% CO₂ incubator. Total cAMP was measured using a
151 commercially available cAMP ELISA kit (Biomedical Technologies, Inc., Stoughton, MA,
152 USA) according to the manufacturer's instructions.

153

154 2.5 FRET assays

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

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

178

179 *2.6 Reporter gene assay*

180 HEK293N cells stably expressing either the human Wt or A189V FSHR were transiently
181 transfected with the pSOMLuc reporter gene as previously described (Lazari *et al.* 1999;
182 Troispoux *et al.* 1999). Cells were split into 12-well plates 24 hours after transfection.
183 Forty-eight hours after transfection, cells were serum-starved for 24 hours, then stimulated for
184 6 hours with pFSH. Cell were then washed twice with cold PBS and lysed with passive lysis
185 buffer. The luciferase activity was measured using the luciferase assay system supplied by
186 Promega Corp (Madison, WI, USA). Each sample (20 μ l) was mixed with 50 μ l of luciferase
187 assay reagent, containing the substrate. The light produced was measured in a luminescence
188 counter (Luminoskan Ascent, Thermo Labsystems, Waltham, MA, USA) and expressed as
189 fold increase over basal.

190

191 *2.7 Small interfering RNA transfection*

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

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

202

203 *2.8 Western blotting*

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

215

216 *2.9 Statistical analysis*

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

221

222 **3 Results**

223

224 **3.1 The A189V mutation leads to a dramatic reduction of FSHR expression at the** 225 **plasma membrane in stably transfected HEK293N cells**

226

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

237

238 **3.2 The A189V mutation suppresses FSHR coupling to the cAMP/PKA pathway.**

239

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

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

262

263 **3.3 The A189V FSHR mutant induces ERK MAPK phosphorylation in response to FSH** 264 **stimulation**

265

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

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

282

283

284

285 **3.4 FSH-induced ERK MAPK phosphorylation by the A189V FSHR mutant is PKA-** 286 **independent**

287

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

292

293 **3.5 FSH-induced ERK MAPK phosphorylation by the A189V FSHR mutant is primarily** 294 **β -arrestin dependent**

295

296 Previous studies from our group have demonstrated that FSH-induced ERK phosphorylation
297 is triggered by both cAMP/PKA- and β -arrestin-dependent pathways (Kara *et al.* 2006; Wehbi
298 *et al.* 2010a; Wehbi *et al.* 2010b). To investigate the contribution of β -arrestin-dependent
299 transduction mechanisms to ERK phosphorylation, HEK293N cells stably expressing the Wt
300 or the A189V FSHR were transfected with siRNA targeting both β -arrestin 1 and 2. As
301 expected, in cells transfected with β -arrestin siRNA endogenous β -arrestin expression was
302 dramatically reduced compared to cells transfected with control siRNA (Fig. 6A). Dose-
303 response curves demonstrated that ERK phosphorylation induced by the activated Wt receptor
304 was only partially sensitive to β -arrestins depletion (Figs. 6B and 6C), whereas ERK
305 phosphorylation induced by the A189V mutant was abrogated by knocking down β -arrestins
306 1/2 (Figs. 6B and 6D). Side-by-side comparison confirmed that the A189V mutant was
307 significantly more sensitive to β -arrestins depletion than the Wt FSHR (Fig. 6E). These results
308 demonstrated that β -arrestins-dependent signalling accounts for most of ERK phosphorylation
309 mediated by the A189V mutant but not by the Wt FSHR, which exhibited a bimodal
310 regulation of ERK phosphorylation.

311

312 **3.6 β -arrestins-dependent ERK phosphorylation predominates at low levels of Wt FSHR** 313 **plasma membrane expression**

314

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

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

333

334 **4 DISCUSSION**

335
336 It has been shown that the A→V substitution at position 189 of the FSHR leads to
337 gonadal dysgenesis and hypergonadotrophic hypogonadism in women (Aittomaki *et al.*
338 1995). It has also been demonstrated that this mutation provokes intracellular retention of the
339 receptor [due to misfolding of the receptor protein (Rannikko *et al.* 2002)] as well as defective
340 function as disclosed by a marginal or null ability to transduce signals *via* G proteins
341 (Aittomaki *et al.* 1995; Tena-Sempere *et al.* 1999; Rannikko *et al.* 2002). In fact, this so-
342 called inactivating mutation is well known to prevent agonist-stimulated cAMP production
343 and phosphoinositide turnover (Aittomaki *et al.* 1995; Tena-Sempere *et al.* 1999). In the
344 present study, we provide experimental evidence supporting the notion that the A189V
345 mutation in the FSHR retains its ability to promote G protein-independent signalling. Our
346 results show that this mutant can phosphorylate ERK MAPK despite being essentially
347 uncoupled from the canonical cAMP/PKA pathway. This finding offers a plausible
348 explanation for the mild abnormalities detected in men bearing the A189V mutation (e.g.
349 oligozoospermia) when compared to those carrying FSH β inactivating mutations (Matthews
350 *et al.* 1993; Aittomaki *et al.* 1995; Layman *et al.* 1997; Layman *et al.* 2002). Thus, whereas
351 FSH β inactivating mutations lead to complete suppression of all potential FSH-dependent
352 signalling pathways due to inability of the mutant FSH to bind and activate the FSHR, the
353 A189V mutation only impairs G α s- (cAMP/PKA-dependent) and G α q/11-mediated
354 pathways. The overall data suggest that G protein-dependent pathways are essential for
355 female reproduction whereas in men reproduction could still be feasible, though with lower
356 efficiency, independently of G protein-coupling.

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

359 siRNA-mediated depletion of endogenous β -arrestins 1 and 2 primarily abrogated FSH-
360 promoted ERK phosphorylation by the A189V FSHR but not by the Wt receptor. These data
361 are consistent with our previous studies showing that β -arrestins can induce G protein-
362 independent signalling at the FSHR (Kara *et al.* 2006; Wehbi *et al.* 2010a; Wehbi *et al.*
363 2010b). Interesting, in contrast to what has been reported for the rat FSHR, which shows a
364 slow and sustained ERK phosphorylation upon agonist exposure (Kara *et al.* 2006), we
365 detected that agonist-provoked activation of the A189V mutant was followed by a rapid and
366 transient β -arrestin-dependent ERK phosphorylation. This apparent discrepancy could be due
367 to the lack of G protein coupling of the mutant receptor, which may potentially abrogate the
368 competition between G proteins and β -arrestins for receptor recruitment. In this scenario, β -
369 arrestin coupling to the mutant receptor and subsequent signalling would proceed faster than
370 that presented by Wt receptor. Although physiological roles for β -arrestin-dependent
371 signalling have recently been described for other GPCR (Premont and Gainetdinov 2007;
372 Gesty-Palmer *et al.* 2009), it is the first time that β -arrestins are suspected to play a role in
373 reproductive function.

374 It has been previously reported that the A189V mutation leads to intracellular retention
375 of the FSHR associated with a dramatic decrease in plasma membrane expression level and
376 attenuation of G protein coupling (Rannikko *et al.* 2002). In the present study, we showed that
377 the preferential β -arrestin coupling exhibited by the A189V FSHR is paradoxically due to the
378 low level of plasma membrane expression rather than to the mutation itself. In fact, our data
379 demonstrate that in cells expressing the Wt FSHR at a plasma membrane density comparable
380 to that of the A189V FSHR, FSH exposure promoted ERK phosphorylation without
381 triggering detectable intracellular signalling mediated by the cAMP/PKA pathway. In this
382 vein, Rannikko *et al.* (2002) showed that overexpression of the A189V FSHR mutant in COS-
383 7 cells resulted in agonist-stimulated cAMP accumulation detectable in the presence of

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

391 The results of the present study, suggest that the A189V FSHR can be phosphorylated
392 by GRKs and recruit β -arrestins as revealed by the receptor's ability to signal through β -
393 arrestin scaffolding. A growing body of evidence supports the notion that signalling at GPCR
394 can be selectively triggered or dampened by using either biased ligands or modified receptors
395 (Wehbi *et al.*; Wei *et al.* 2003; Shenoy *et al.* 2006; Violin and Lefkowitz 2007). In this vein,
396 our data establish for the first time that selective signalling may also be achieved by
397 modulating cell surface plasma membrane levels of the receptor, underlining the importance
398 of receptor density as a regulatory mechanism to control cellular signalling. In the present
399 study, induction of β -arrestins-dependent biased signalling was achieved by decreasing the
400 number of functional receptors in HEK293N cells to levels found in primary granulosa cells
401 (1000 receptors per cell; McNatty *et al.* 1989) or primary Sertoli cells from 19 days old rat
402 [6400 receptors per cell as calculated from our previously published data (Musnier *et al.*
403 2009)]. Since plasma membrane expression of the FSHR varies depending on the
404 developmental and differentiation stages of the gonads (Simoni *et al.* 1997), it is tempting to
405 speculate that varying FSHR levels might lead to differential coupling to effectors and,
406 consequently, to distinct biological responses known to be associated with the gonadotropic
407 signal. Undoubtedly, the cellular context should be considered when trying to understand Gs
408 protein/ β -arrestin balance in a given experimental paradigm. In fact, it has been shown that rat

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

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

422

423 **Acknowledgments**

424 The authors thank the members of the BIOS group for their support throughout this project.

425 This work was supported by the Action d'Envergure (AE) Institut National de la Recherche
426 en Automatique et en Informatique (INRIA)/INRA Regate, and Region Centre project. BIOS
427 group is a young research team from INRA.

428

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430 **Figure Legends**

431

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

433 **(A)** Schematic representation of the structure of the FSHR with the A189V substitution (EC:
434 Extracellular domain; TM: Transmembrane Domain; IC: Intracellular Domain). The
435 endogenous signal peptide was replaced by the FLAG and the hemagglutinin signal peptide
436 (HA-SP) sequences in both the Wt and A189V FSHR. **(B)** HEK293N cells stably expressing
437 the Wt or A189V human FSH receptor were serum-starved for 1 hour and then incubated for
438 1 hour at 37°C with 100,000 cpm of ^{125}I -FSH. Specific binding values were normalized
439 according to protein concentration. Results are expressed as mean \pm sem of at least 3
440 independent experiments.

441

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

443

444 **(A)** HEK293N cells stably expressing the Wt or A189V FSHR were serum-starved for 30 min
445 in the presence of IBMX and then stimulated with increasing concentrations of pFSH (zero to
446 4 nM) for 1 hour. After stimulation, cell extracts were lysed as described in *Materials and*
447 *Methods*. Intra- and extra-cellular cAMP produced was assayed by ELISA. Results are
448 normalized according to protein concentration and expressed as mean \pm sem of four
449 independent experiments. **(B)** Cells were transiently transfected with the cAMP FRET sensor
450 ICUE. Forty-eight hours after transfection, cells were serum-starved for 1 hour at 37°C before
451 ICUE FRET ratio measurement (CFP emission/YFP emission). After 5 min of baseline
452 measurement, cells were stimulated with 3.3 nM pFSH at 37°C. Forskolin (20 μM) was added
453 40 minutes after FSH stimulation, as a positive control. Results are expressed as mean \pm sem
454 of 20 individuals cells responses measured in 3 different experiments. **(C)** Cells were

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

466

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

469

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

480

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

483

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

498

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

501

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

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

511

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

514

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

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

531

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

534

535 **(A)** HEK293N cells transiently transfected with either the Wt or the A189V human FSHR
536 were serum-starved for 1 hour and then incubated with 100,000 cpm of ^{125}I -FSH for 1 hour at
537 37°C . Low plasmid amounts of Wt FSHR were transfected and compensated with pcDNA3.1
538 empty vector. Results are expressed as total binding normalized according to protein
539 concentration and compared to the results obtained in HEK293N cells stably expressing the
540 Wt human FSH receptor from at least 2 individual experiments. **(B)** HEK293N cells were
541 transiently co-transfected with the ICUE cAMP FRET sensor and either the Wt or the A189V
542 FSHR. Forty-eight hours after transfection, cells were serum-starved for 1 hour before ICUE
543 FRET *ratio* measurement (CFP emission/YFP emission) at 37°C . After 5 min of baseline
544 measurements, cells were stimulated with pFSH (3.3 nM). Forskolin (20 μM) was added 40
545 minutes after FSH stimulation as a positive control. Results are expressed as mean \pm sem of at
546 least 17 individual cells measured in 3 different experiments. **(C, D)** HEK293N cells
547 transiently transfected with either the Wt or the A189V human FSHR were serum-starved for
548 4 hours and then stimulated with 3.3 nM pFSH for increasing time period. After stimulation,
549 cell extracts were prepared as described in Materials and Methods. Endogenous
550 phosphorylated ERK were revealed using anti-pERK antibody. Membranes were stripped as
551 described in Materials and Methods and revealed with anti-ERK2 antibody for normalisation.
552 **(C)** Representative immunoblots for FSH-induced ERK phosphorylation. **(D)** ERK
553 phosphorylation signals were quantified by densitometry and expressed as a ratio of

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

566

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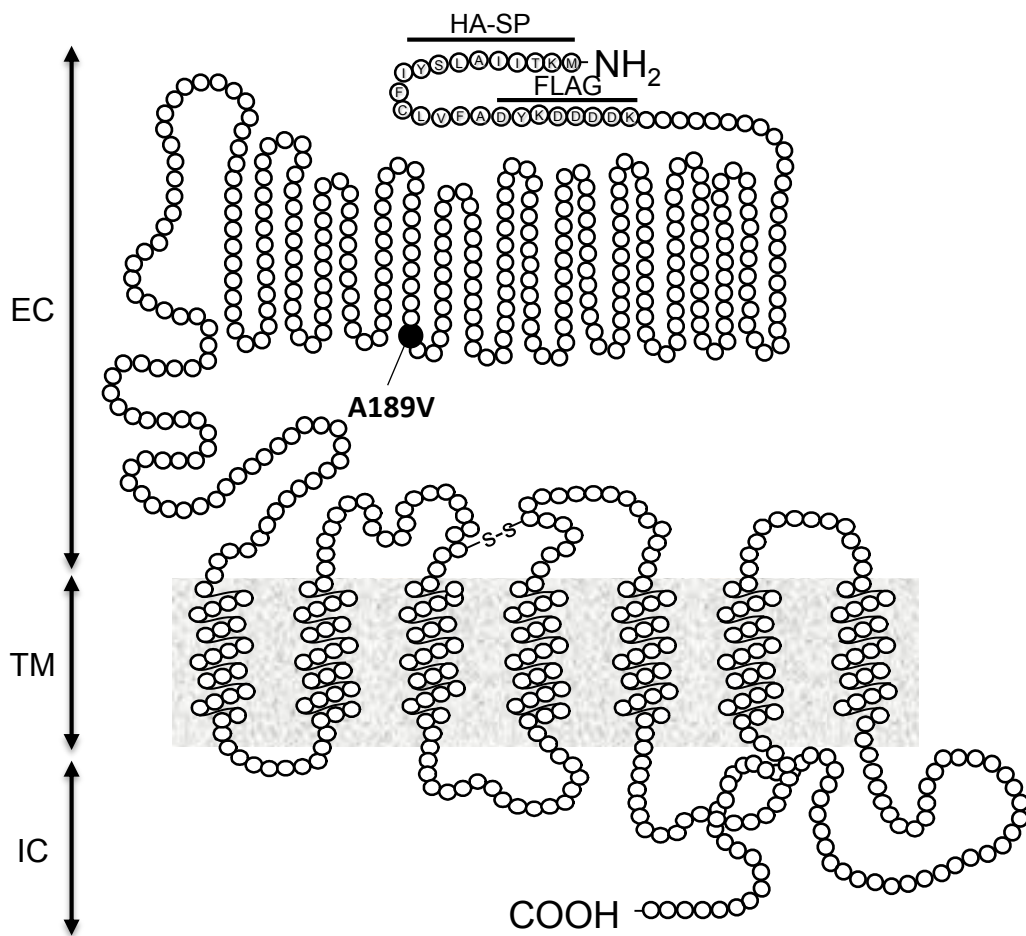
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A.



B.

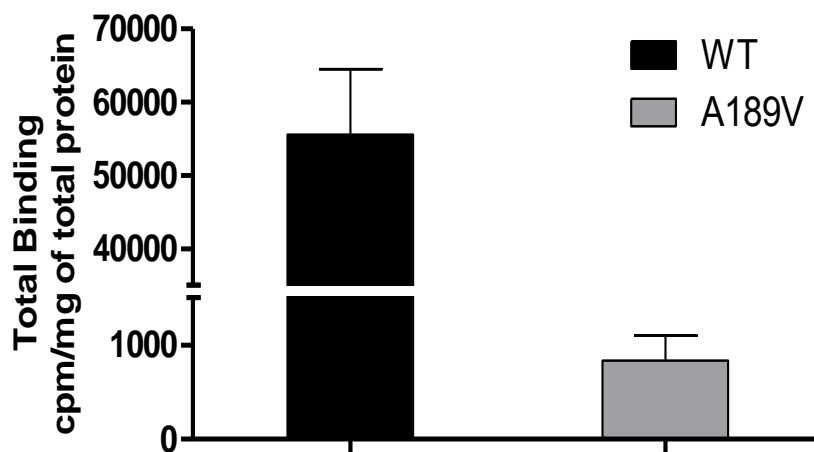


Figure 1

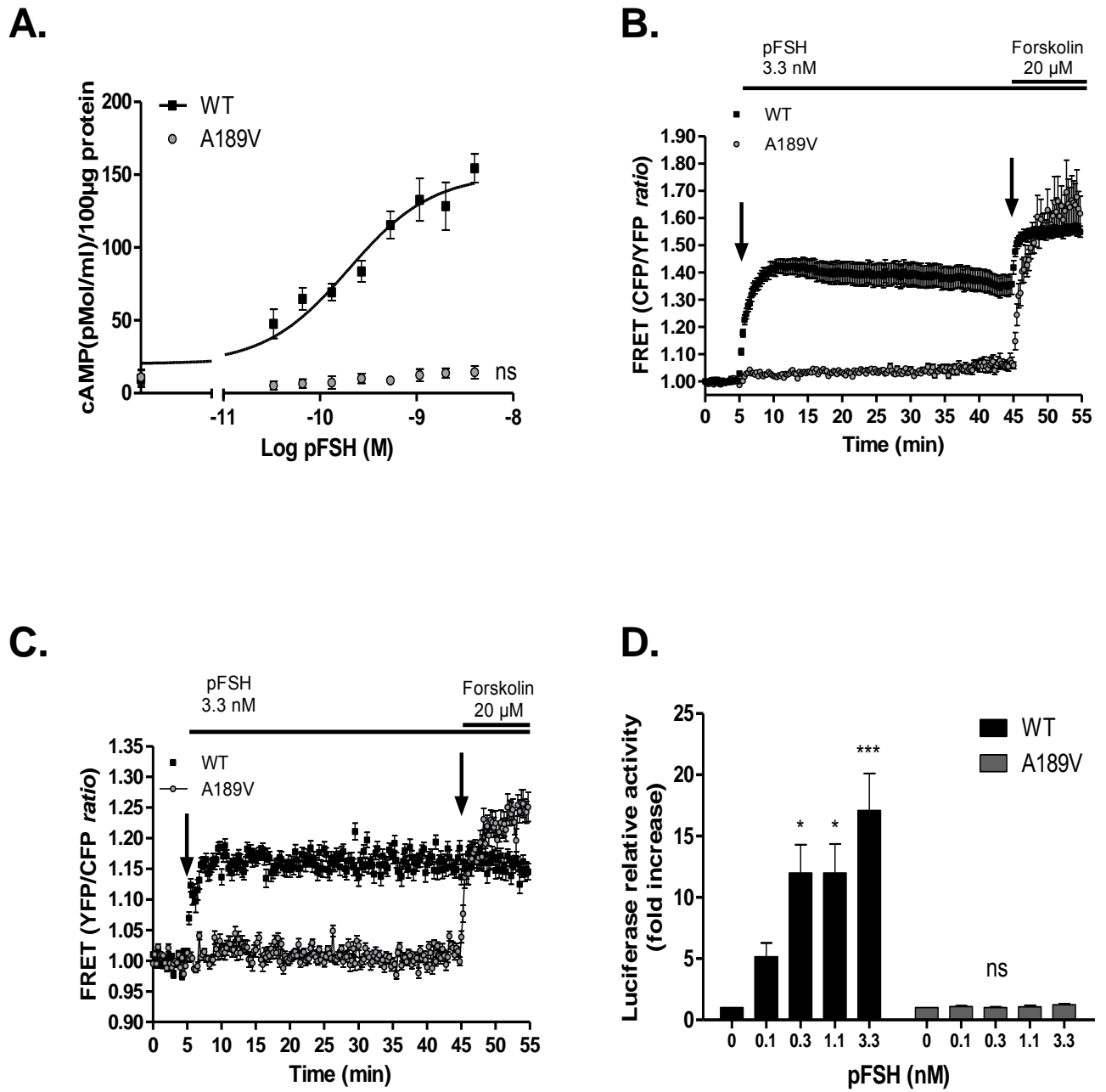
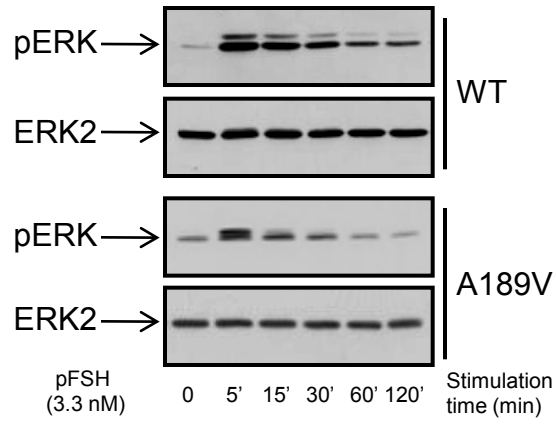


Figure 2

A.



B.

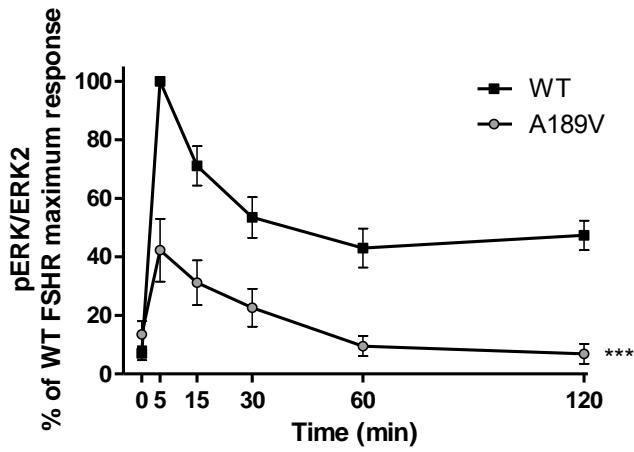
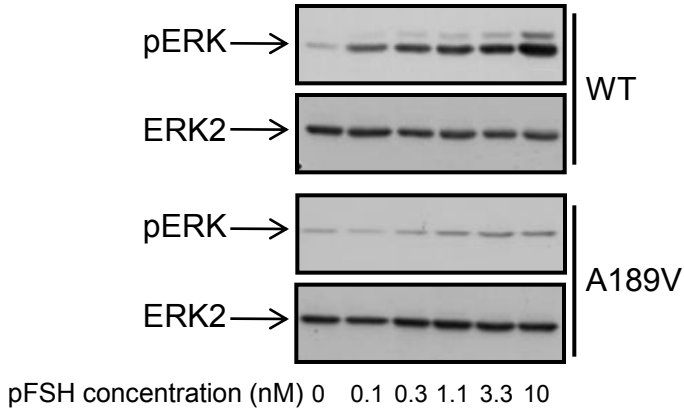
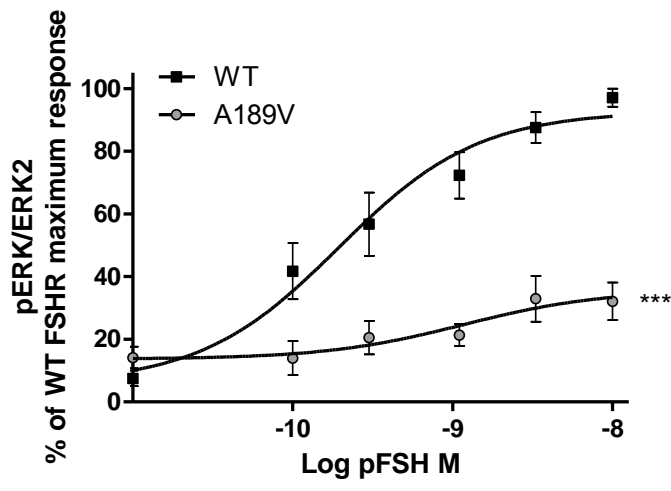


Figure 3

A.



B.



C.

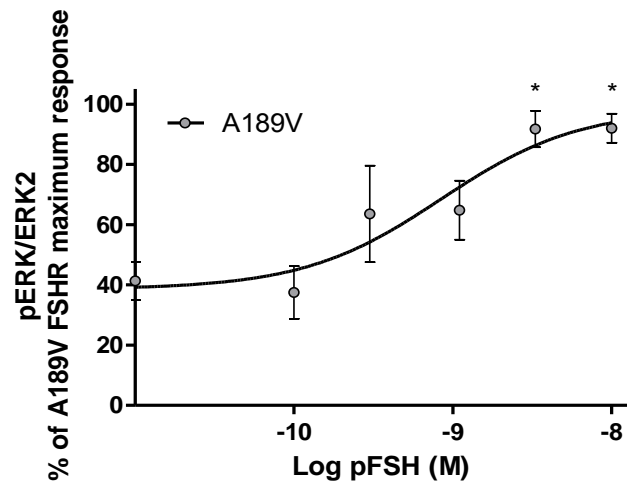


Figure 4

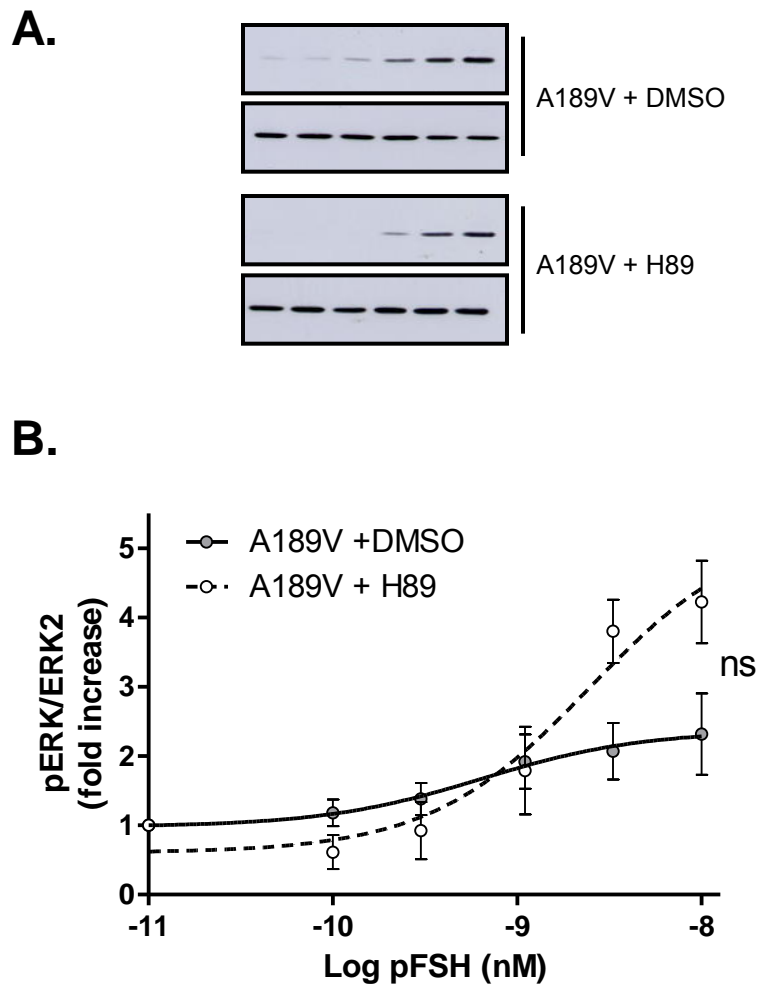


Figure 5

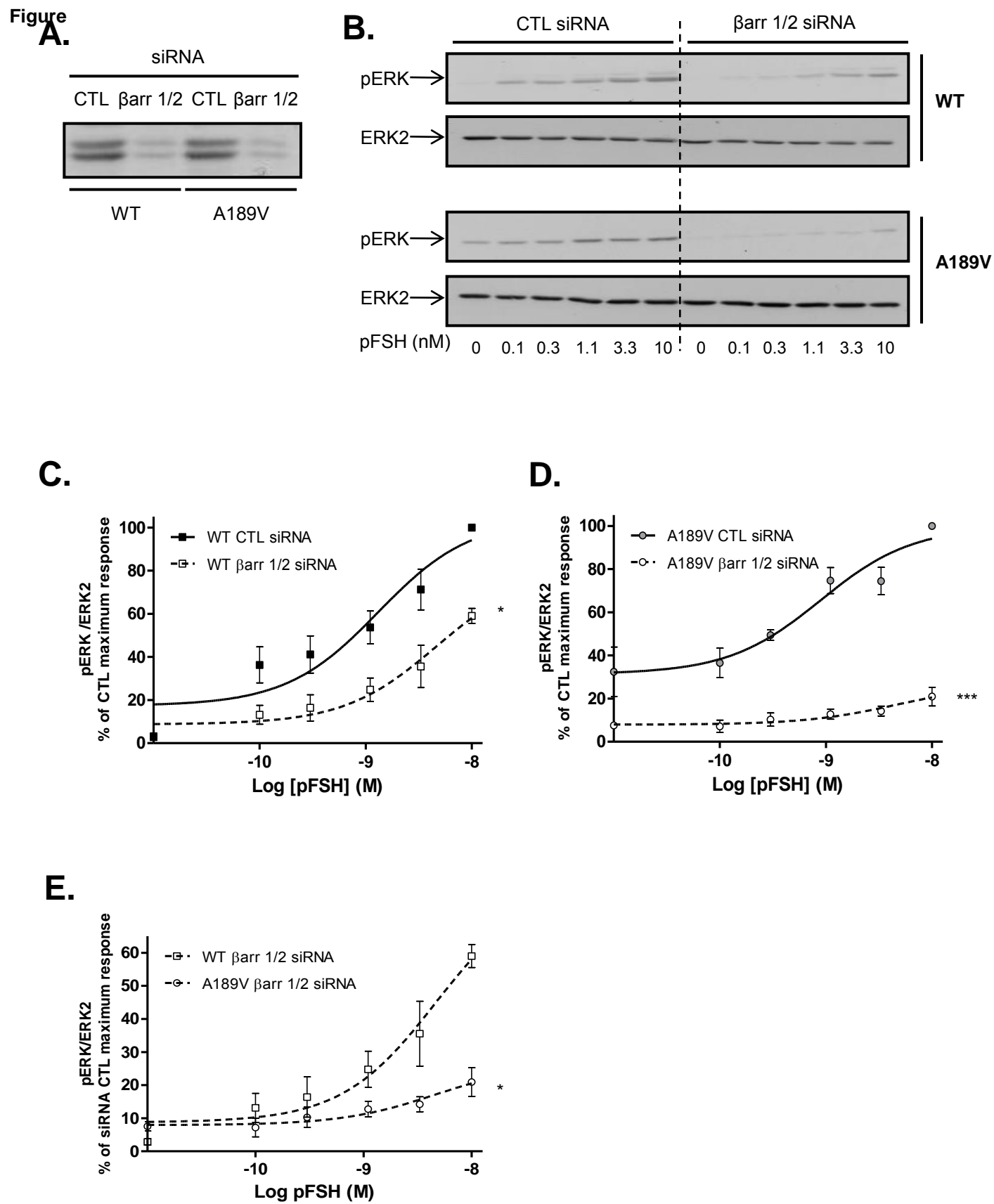


Figure 6

Figure

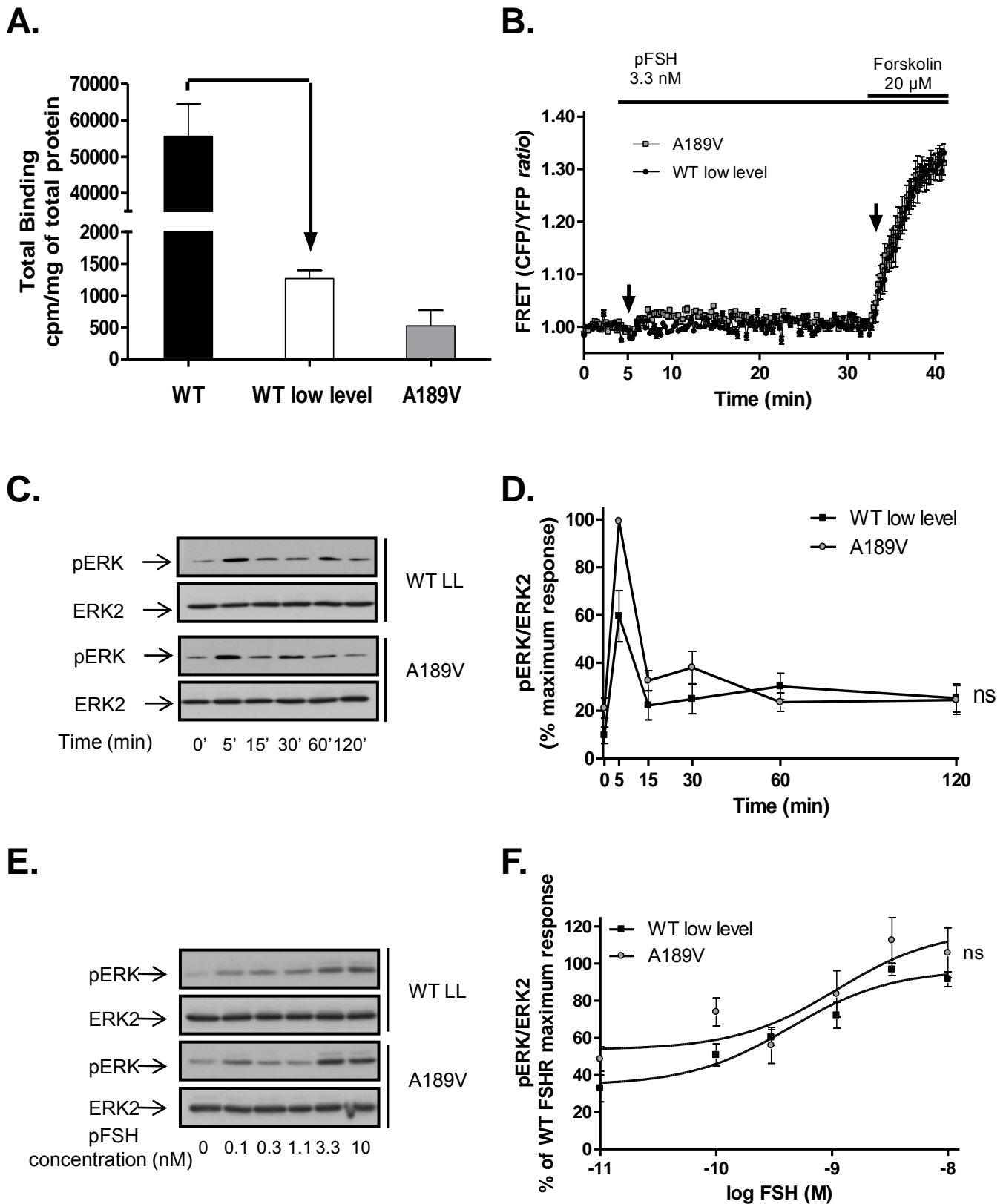


Figure 7