



HAL
open science

GnRH Transactivates Human AMH Receptor Gene via Egr1 and FOXO1 in Gonadotrope Cells

Ghislaine Garrel, Chantal Denoyelle, David L'Hote, Jean-Yves Picard, Jose Teixeira, Ursula B. Kaiser, Jean-Noel Laverriere, Joelle Cohen-Tannoudji

► **To cite this version:**

Ghislaine Garrel, Chantal Denoyelle, David L'Hote, Jean-Yves Picard, Jose Teixeira, et al.. GnRH Transactivates Human AMH Receptor Gene via Egr1 and FOXO1 in Gonadotrope Cells. *Neuroendocrinology*, 2019, 108 (2), pp.65-83. 10.1159/000494890 . hal-03201550

HAL Id: hal-03201550

<https://cnrs.hal.science/hal-03201550>

Submitted on 16 Nov 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **GnRH transactivates human AMH receptor gene *via* Egr1 and**
2 **FOXO1 in gonadotrope cells**

3
4 Ghislaine Garrel¹, Chantal Denoyelle¹, David L'Hôte¹, Jean-Yves Picard^{1,2}, Jose Teixeira³,
5 Ursula B. Kaiser⁴, Jean-Noël Laverrière¹ and Joëlle Cohen-Tannoudji^{1, *}.

6 ¹ Sorbonne Paris Cité, Université Paris-Diderot, Centre National de la Recherche Scientifique,
7 Biologie Fonctionnelle et Adaptative UMR 8251, Institut National de la Santé et de la
8 Recherche Médicale, Physiologie de l'axe gonadotrope U1133, Paris, France.

9 ² Present address: Institut National de la Santé et de la Recherche Médicale UMRS 938, Centre
10 de Recherche Saint-Antoine, Sorbonne Université, Paris, France.

11 ³ Michigan State University, Department of Obstetrics, Gynecology, and Reproductive
12 Biology, Grand Rapids, MI 49503, USA.

13 ⁴ Division of Endocrinology, Diabetes and Hypertension, Brigham and Women's Hospital,
14 Harvard Medical School, Boston, MA, USA.

15
16 **Short title:** GnRH enhances hAMHR2 promoter activity

17
18 **Corresponding Author:**

19 Joëlle Cohen-Tannoudji

20 Sorbonne Paris Cité, Université Paris-Diderot

21 Unité de Biologie Fonctionnelle et Adaptative CNRS UMR 8251, Equipe de Physiologie de
22 l'axe gonadotrope INSERM U1133

23 75013 Paris, France

24 joelle.cohen-tannoudji@univ-paris-diderot.fr

25
26 **Key words:** GnRH, AMHR2, Gonadotrope cells, Egr1, FOXO1, β -catenin.

27

28

29 **Abstract**

30 **Background/objectives:** Anti-Müllerian hormone (AMH) signaling is critical for sexual
31 differentiation and gonadal function. AMH receptor type 2 (AMHR2) is expressed in
32 extragonadal sites such as brain and pituitary and emerging evidence indicates that AMH
33 biological action is much broader than initially thought. We recently reported that AMH
34 signaling enhances FSH synthesis in pituitary gonadotrope cells. However, mechanisms
35 regulating AMHR2 expression in these extragonadal sites remain to be explored. **Method/
36 Results:** Here, we demonstrated in perfused murine L β T2 gonadotrope cells that *Amhr2*
37 expression is differentially regulated by GnRH pulse frequency with an induction under high
38 GnRH pulsatility. Furthermore, we showed that GnRH transactivates the human *AMHR2*
39 promoter in L β T2 cells. Successive deletions of the promoter revealed the importance of a
40 short proximal region (-53/-37 bp) containing an Egr1 binding site. Using site-directed
41 mutagenesis of *Egr1* motif and siRNA mediated-knock down of Egr1, we demonstrated that
42 Egr1 mediates basal and GnRH-dependent activity of the promoter, identifying Egr1 as a new
43 transcription factor controlling h*AMHR2* expression. We also showed that SF1 and β -catenin
44 are required for basal promoter activity and demonstrated that both factors contribute to the
45 GnRH stimulatory effect, independently of their respective binding sites. Furthermore, using a
46 constitutively active mutant of FOXO1, we identified FOXO1 as a negative regulator of basal
47 and GnRH-dependent *AMHR2* expression in gonadotrope cells. **Conclusions:** This study
48 identifies GnRH as a regulator of human *AMHR2* expression, further highlighting the
49 importance of AMH signaling in the regulation of gonadotrope function.

50

51 Introduction

52 The neurohormone Gonadotropin-releasing hormone (GnRH) is the central regulator of the
53 reproductive system. GnRH is secreted by hypothalamic neurons in a pulsatile manner and
54 reaches the anterior pituitary where it controls synthesis and secretion of both gonadotropins,
55 Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH), which are dimeric
56 glycoproteins composed of a common α -subunit and rate limiting β -subunits (LH β and FSH β).
57 GnRH activates a G-protein coupled receptor expressed in gonadotrope cells, which activates
58 a wide array of signaling pathways but predominantly the protein kinase C (PKC)/MAPK
59 signaling pathways [1, 2]. This results in rapid activation of genes belonging to the immediate
60 early gene family and culminates with the increased synthesis of genes coding for gonadotropin
61 subunits *i.e.* *Cga*, *Lhb* and *Fshb* [3, 4]. Expression of these subunit genes is differentially
62 regulated by GnRH according to the pulse frequency of its stimulation. *Fshb* expression is
63 optimally induced at low GnRH pulse frequency whereas *Lhb* expression is favored at high
64 pulse frequency, the common α subunit being considered poorly regulated [5]. GnRH induces
65 *Lhb* expression through rapid induction of the immediate early gene *Egr1* [6, 7]. The important
66 role of *Egr1* is emphasized by the lack of *Lhb* in pituitary gonadotrope cells of *Egr1* knockout
67 mice [8, 9]. Upon induction by GnRH, *Egr1* interacts with other transcription factors such as
68 SF1, Pitx1 or Sp1 to enhance *Lhb* transcription [10, 11]. GnRH regulation of *Fshb* occurs
69 notably through the induction of immediate early genes belonging to the activator protein-1
70 family [12]. Recently, GnRH was shown to regulate gonadotropin subunit gene transcription
71 by abrogating the repressive effect of FOXO1 [13-15]. GnRH-induced posttranscriptional
72 modifications of FOXO1 result in FOXO1 export from the nucleus, alleviating FOXO1
73 repression on the *Fshb* promoter [14, 15]. In addition to GnRH, the coordinated transcription
74 of gonadotropins relies on several signals, including locally produced activin, follistatin or
75 bone morphogenetic proteins, as well as feedback signals originating from the gonads, such as
76 steroids and inhibin [12]. We recently demonstrated, by a combination of *in vitro* and *in vivo*
77 approaches, that anti-müllerian hormone (AMH) directly and specifically stimulates *Fshb* and
78 amplifies activin regulation of *Fshb*, identifying AMH as a new actor regulating gonadotrope
79 activity [16].

80 AMH also called Müllerian inhibiting substance is a homodimeric glycoprotein that belongs to
81 the transforming growth factor- β superfamily. AMH is secreted by Sertoli cells of the fetal
82 testis and plays a crucial role during early male sex differentiation by inducing the regression

83 of Müllerian ducts [17]. The expression of AMH is however not restricted to embryonic
84 development as AMH can be found in the Sertoli and granulosa cells of postnatal gonads and
85 AMH produced by ovarian granulosa cells is now recognized as an important regulator of
86 folliculogenesis [18, 19]. In addition, AMH expression was reported in adult mouse brain [20-
87 22] and in pituitaries of immature male and female rats [16]. AMH exerts its function by
88 binding to a specific type 2 receptor, AMHR2, that heterodimerizes with a type 1 receptor [23].
89 Both receptors are transmembrane serine/threonine receptors that recruit and phosphorylate
90 SMAD proteins that will then translocate into the nucleus to regulate the transcription of
91 several target genes [18, 24]. *Amhr2* expression was initially found in mesenchymal cells
92 surrounding the Müllerian epithelium in the bipotential urogenital ridge [17]. *Amhr2* mRNA
93 was then localized in Sertoli and granulosa cells of both fetal and adult gonads as well as in
94 testicular Leydig cells [17, 19]. In addition, more recent studies highlighted *Amhr2* expression
95 in extragonadal tissues, notably in the brain. AMH receptivity has been reported in motor and
96 cortical neurons of rodent brain, in which AMH exerts survival and neuroprotective roles,
97 respectively [20, 21]. Furthermore, accumulating evidence shows that AMH contributes to the
98 central control of gonadal functions. The AMH receptor was indeed identified in a significant
99 subset of hypothalamic GnRH neurons in mice and humans and AMH was found to potently
100 activate the GnRH neuron firing [25, 26]. *Amhr2* transcripts and proteins are also present in rat
101 pituitary gland as well as in the gonadotrope cell line L β T2 [16, 27]. We previously reported a
102 sexually dimorphic expression of *Amhr2* transcripts in postnatal rat pituitary with higher
103 steady-state transcript levels in immature female rats than in males [16]. In line with this
104 difference, AMH treatment was shown to increase the secretion of the gonadotropin FSH in
105 immature females while being ineffective in age-matched males. Furthermore, AMH was
106 reported to up regulate FSH secretion and pituitary *Fshb* without affecting LH [16].

107 The mechanisms regulating AMHR2 gene expression have mostly been identified in the
108 context of urogenital ridge or gonadal cell expression. Cloning of *AMHR2* genes in humans
109 [28, 29] and rodents [30] and studies of their promoters [31-35] have led to the identification
110 of a limited number of regulators and transcription factors responsible for constitutive human
111 or murine AMHR2 gene expression. Among them is the Wnt signaling pathway. *Wnt7-a*
112 deficient mice are indeed sterile with retained Müllerian ducts and exhibit no *Amhr2* mRNA
113 expression [36]. The canonical Wnt signaling pathway is mediated by stabilization of β -catenin
114 and its association with nuclear T-cell factor (TCF)/Lymphoid enhancer binding factor (LEF)
115 transcription factors leading to activation of target gene transcription. Four TCF binding sites

116 have been identified in the h*AMHR2* promoter and β -catenin as well as TCF4 (also designated
117 as TCF7L2) have been reported to transactivate the h*AMHR2* promoter [35]. The steroidogenic
118 factor 1 (SF1/NR5A1), which belongs to the nuclear receptor superfamily, was found to
119 transactivate murine and human *AMHR2* promoters through its binding to SF1-motifs [30, 34].
120 SF1 has also been described to synergistically cooperate with the β -catenin signaling pathway
121 to activate the h*AMHR2* promoter [35]. A handful of hormones regulating *AMHR2* expression
122 have been identified so far, mostly from studies on ovarian tissues. FSH and estradiol were
123 reported to down regulate *Amhr2* transcripts in rat ovarian follicles [37] and an inhibitory effect
124 of LH and estradiol on *AMHR2* mRNA levels was reported in human luteinized granulosa cells
125 [38, 39]. Contrasting with gonadotropins, bone morphogenetic protein 4 (BMP4) and BMP15
126 were recently demonstrated to up regulate *AMHR2* mRNA levels in human granulosa cells
127 through transcriptional activation of the *AMHR2* promoter [40]. However, the signaling
128 pathways and mechanisms by which these hormones regulate the transcriptional activity of the
129 *AMHR2* gene are still unknown. We recently reported that *AMHR2* expression is regulated by
130 GnRH in pituitary gonadotrope cells, adding GnRH to the known regulators of *AMHR2* [16].

131 The present study was undertaken to gain further insight into the mechanisms underlying
132 GnRH regulation of *AMHR2* gene expression. We demonstrate in perfused L β T2 gonadotrope
133 cells that endogenous *Amhr2* expression is differentially regulated by GnRH pulse frequency.
134 We also show by studying human *AMHR2* promoter activity in L β T2 cells that GnRH enhances
135 *AMHR2* transcriptional activity through the combined stimulatory action of Egr1 together with
136 SF1 and β -catenin and the removal of FOXO1 repression.

137

138 **Materials & Methods**

139 **Materials**

140 GF109203X (Bisindolylmaleimide I), SP600125, LY294002, GnRH, GnRH agonist
141 Triptorelin ([D-Trp⁶]GnRH; GnRHa) and GnRH antagonist antide were from Sigma (Saint-
142 Quentin Fallavier, France). JW74 were from Tocris (R&D Systems, Lille, France). Pituitary
143 adenylate cyclase-activating polypeptide (PACAP₃₈) was from Calbiochem (VWR
144 International, Strasbourg, France).

145

146 **Cell cultures**

147 The mouse gonadotrope L β T2 and α T3-1 cell lines and the mouse hypothalamic GnRH
148 neuronal cell line GT1-7, generously provided by Dr P Mellon (University of California, La
149 Jolla, CA) [41, 42], were maintained in monolayer cultures in DMEM-Glutamax (Gibco, Life
150 Technologies) supplemented with 10% fetal calf serum (FBS) and 0.5%
151 penicillin/streptomycin (P/S) (Sigma-Aldrich). Treatments of cells are indicated below, in the
152 corresponding paragraphs. For *Sp1* and *Egr1* mRNA quantification, L β T2 cells were plated in
153 12-well plates (0.8 to 1x10⁶ per well) in duplicate and starved overnight in serum-free medium
154 before incubation for 4 h with 10 nM GnRHa.

155

156 **Pulsatile stimulation of L β T2 cells**

157 The perfusion system was designed according to [43] with modifications. Briefly, L β T2 cells
158 were plated in perfusion chambers mounted on glass slides coated with poly L-Lysine (Sigma-
159 Aldrich). Cells were incubated in the chambers overnight in a static culture system in DMEM
160 containing 10% FBS and 0.5% P/S and then serum starved for 24 h in DMEM 1% FBS and
161 0.1% P/S. The chambers were then mounted in the perfusion system at a constant flow rate of
162 0.3 ml/min and cells were perfused or not with 20 nM GnRH for 21 h in continuous or pulsatile
163 (one pulse every 30 min or one pulse every 120 min) stimulation mode.

164

165 **Plasmid constructs**

166 A 2252 (-2199/+53)-bp human *AMHR2* promoter (Ref-SeqGene NG-015981) [31, 40] was
167 prepared by cloning successively a 428 (-375/+53)-bp fragment and a 1825-bp distal promoter
168 fragment (-2199/-374) into the *Bgl*III and *Hind*III sites of the pGL2-basic vector. The short
169 construct contains only the 428-bp fragment (428-h*AMHR2*-Luc). The expression vector
170 pcDNA3-SF1 was provided by Dr. Bon-chu Chung (Institute of Molecular Biology, Taipei,

171 Taiwan) [44]. Plasmids expressing native and mutant Forkhead Box O1 (FOXO1) were a gift
172 from Domenico Accili [45]. In the constitutively active, non-phosphorylatable mutant (FOXO1-
173 CAM), threonine (T24) and serines (S253, S316) were replaced by alanine (FLAG-
174 FOXO1ADA, Addgene plasmid #12149; FLAG-FOXO1, Addgene plasmid #12148). The 5'
175 deletion constructs from -53 to -5 were obtained by DNA amplification of the native 428-
176 hAMHR2-Luc plasmid using forward primers containing a BglIII restriction site as indicated in
177 table 1 and the reverse primer R3_AMHRm (table 1) that hybridized with luciferase sequence
178 containing a unique XbaI restriction site. The resulting DNA fragments were digested with
179 BglIII/XbaI and then inserted into the corresponding restriction sites of the 428-hAMHR2-luc
180 plasmid. The -247, -174, and -105 deletion constructs were obtained as described below.

181

182 ***Targeted mutagenesis studies***

183 Mutagenesis of SF1 response elements on the 2252-hAMHR2-luc construct was conducted
184 using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), replacing 2-4
185 bp to introduce a KpnI restriction site. Site-directed mutagenesis of TCF, FOXO, SF1 or Egr1
186 motifs was performed in the context of the 428-hAMHR2-luc plasmid by PCR fusion-based
187 approach using the primers listed in Table 1. This generated mutant constructs containing NdeI
188 restriction sites within the TCF, FOXO, SF1 or Egr1 response elements. The SF1, TCF, and
189 FOXO mutants were subsequently used to obtain the -247, -174, and -105 deletion constructs,
190 respectively. These constructs were digested by BglIII and NdeI restriction enzymes, treated by
191 T4 DNA polymerase to fill-in 5' overhangs and purified by agarose gel electrophoresis. The
192 linearized plasmid fragments were then circularized using T4 DNA ligase. All mutant
193 constructs were validated by DNA sequencing.

194

195 ***Transient transfections and luciferase assays***

196 For transient transfections, the human AMHR2 promoter luciferase fusion construct (100
197 ng/well) together with pRL-SV40 as a control for transfection efficiency (5 ng/well) were
198 mixed with 0.5 µl LipofectAMINE2000 reagent (Life Technologies, Gaithersburg, MD) in 10
199 µl OptiMEM-1 for 30 min. The transfection mix was then added to 0.9×10^5 cells in 30 µl
200 OptiMEM-1 before plating in 96-well plates (each point in triplicate). Cells were incubated for
201 5 h before adding 50 µl DMEM supplemented with 5% FBS and 0.25% P/S (Sigma-Aldrich).
202 The next morning, medium was replaced by 100 µl/well DMEM-1% FBS-0.01% P/S and cells
203 were grown an additional 24 h. Then, cells were treated for 4 h with GnRHa (1, 10 nM), GnRH

204 (10, 100 nM) or PACAP₃₈ (20, 50 nM) before luciferase assay (Luciferase Assay System;
205 Promega). When tested, pharmacological inhibitors (5 μM antide, 2 μM GF109203X, 20 μM
206 SP600125, 10 μM JW74 or 20 μM LY294002) were added 1 h before GnRHa stimulation.
207 Relative luciferase activity of the promoter constructs was determined by the ratio of firefly to
208 *Renilla* luciferase activities and then normalized to basic vector (PGL2) activity in the same
209 experiment.

210 For siRNA experiments, LβT2 cells were transiently cotransfected with the 2252-bp hAMHR2
211 promoter and with 75 nM SMARTpool siRNA targeting mouse *Ctnnb1* (siRNA β-catenin),
212 mouse *Nr5a1* (siRNA SF1), mouse *Egr1* (siRNA Egr1) or 75 nM nontargeting SMARTpool
213 siRNA (siRNA NT) as negative control (Dharmacon, Horizon Discovery LTD, Cambridge,
214 UK). Cells were then, 48 h after transfection, stimulated or not with 10 nM GnRHa for 4 h.

215

216 **Real-time PCR**

217 Total RNA from LβT2 were isolated with a RNeasy-kit (Qiagen, France) and first-strand
218 cDNA was obtained from 1-2 μg RNA with Superscript II reverse transcriptase (Invitrogen)
219 using random primers according to the manufacturer's instructions. Quantification of *Amhr2*,
220 *Lhb*, *Fshb*, *Sp1* and *Egr1* mRNA was performed by real-time qPCR using Takyon No ROX
221 SYBR master mix (Eurogentec). Real-time qPCR was carried out in LightCycler 480 (Roche
222 Diagnostics) using 5 μl of the mix containing 0.5 μM of each primer and 5 μl of a 1:20 cDNA
223 dilution. Cycling conditions included an initial heat-denaturing step at 95°C for 3 min, followed
224 by 40 cycles at 95°C for 10 sec, 60°C for 10 sec and 72°C for 10 sec. Oligonucleotide primer
225 sequences are indicated in Table 2 and *Cyclophilin* was used to normalize RNA expression
226 levels. Primer pairs were designed to target cDNA fragments encompassing at least one intron
227 in the gene sequence to prevent amplification of genomic DNA. The specificity of
228 amplification was checked by gel electrophoresis and melting curve analysis. Data were
229 analyzed using the advanced-E-method with standard-curve derived efficiencies obtained from
230 LightCycler 480 software.

231

232 **Statistical analyses**

233 Experiments were performed in triplicate and all given values are the mean ± SEM of at least
234 4 independent experiments. Data were analyzed using the Prism 6 Software (GraphPad
235 Software, Inc). When distribution was normal, statistical differences were determined using
236 one-way or two-way ANOVA followed by a Dunnett's *t* test for multiple comparisons and

237 significance of individual fold changes from control were made using Student's *t* test. A non
238 parametric Mann-Whitney test was used when distribution was not normal. $P \leq 0.05$ was
239 considered as significant.
240

Accepted manuscript

241 **Results**

242

243 **GnRH pulse frequency differentially regulates *Amhr2* transcript levels in L β T2**
244 **gonadotrope cells.**

245 The GnRH pulsatile pattern is critical for appropriate regulation of gonadotrope activity [5].
246 To determine whether endogenous *Amhr2* expression could be differentially regulated
247 depending on GnRH pulse frequency, L β T2 cells were perfused for 21 h and exposed to
248 vehicle alone, to continuous GnRH stimulation or to pulsatile stimulation at either high (every
249 30 min) or low (every 2 h) pulse frequency. The pulse frequencies chosen are reported to favor
250 *Lhb* expression and *Fshb* expression respectively [5, 46]. Accordingly, maximum *Lhb* and
251 *Fshb* transcript levels were found at high and low GnRH pulse frequencies, respectively (Fig
252 1, inset). As shown in Fig 1, a high GnRH pulse frequency significantly increased *Amhr2*
253 transcript levels. In contrast, low pulse frequency of GnRH was ineffective. Continuous
254 exposure of cells with GnRH decreased *Amhr2* transcript levels by about $33 \pm 4.9\%$ as
255 previously observed with the GnRH agonist triptorelin (GnRH α) in static cultures [16]. Under
256 continuous GnRH, *Fshb* transcript levels were also significantly decreased and *Lhb* transcript
257 levels were no longer increased (Fig 1, inset), suggesting that cells are desensitized to GnRH
258 stimulation. Altogether, these data indicate that endogenous *Amhr2* expression is regulated by
259 GnRH in L β T2 gonadotrope cells and is sensitive to GnRH pulse frequency.

260 **GnRH enhances human *AMHR2* promoter activity in gonadotrope cells.**

261 To determine whether GnRH can regulate transcription of the *AMHR2* gene in gonadotrope
262 cells, we took advantage of the human *AMHR2* (*hAMHR2*) promoter that we previously cloned
263 [31] and which shares 70% identity in its proximal region (-375/+78 bp) with the murine
264 promoter. A *hAMHR2* promoter/firefly luciferase reporter gene construct was transiently
265 transfected into α T3-1 and L β T2 cell lines that were derived from immature and mature
266 pituitary cells, respectively [47, 48]. Because a significant subset of GnRH neurons, both in
267 mice and human, express *AMHR2* [25], we also expressed this construct in the hypothalamic
268 GnRH neuronal cell line, GT1-7. GnRH α stimulated *hAMHR2* promoter activity by ~ 2.5 fold
269 in L β T2 gonadotrope cells (Fig. 2A). This effect was not found in α T3-1 or GT1-7 cells. A
270 significant increase in *hAMHR2* promoter activity was also found after a more prolonged
271 treatment (24 h) of L β T2 cells with GnRH α ($170 \pm 6\%$, data not shown) and no noticeable

272 increase could still be observed in α T3-1 or GT1-7 cells. Further analyses revealed that
273 luciferase activity can be significantly stimulated by a 10-fold lower concentration of GnRHa
274 and that GnRHa stimulation was completely blocked by the GnRH antagonist antide (Fig. 2B),
275 demonstrating the specificity of the observed effect. The native hormone, GnRH, also
276 enhanced hAMHR2 promoter activity although to a lesser extent than GnRHa, as expected
277 given the higher potency of the agonist triptorelin. In contrast, the neuropeptide PACAP,
278 known to regulate pituitary gonadotrope function, was unable to activate hAMHR2 promoter
279 activity (Fig. 2B). Altogether, these data indicate that GnRH stimulates the 2252-bp hAMHR2
280 promoter in a cell specific manner, with a significant stimulatory effect in L β T2 gonadotrope
281 cells. We next compared the 2252-bp promoter activity with a shorter 428-bp promoter used
282 for successive cloning of the promoter. As illustrated in Fig. 2C, both promoters exhibited
283 similar basal luciferase activities. Furthermore, GnRHa stimulatory effect did not significantly
284 differ between the two constructs indicating that the cis-acting elements required for GnRH
285 regulation are located within the proximal 428-bp fragment of the hAMHR2 promoter.

286 **A proximal 37-bp promoter region is required for GnRH transactivation of human**
287 **AMHR2.**

288 Sequence analysis of the proximal -375/+53 bases of the hAMHR2 promoter using the CIS-BP
289 database (<http://cisbp.cabr.utoronto.ca/index.php>) [49] revealed consensus binding sites for
290 several regulatory elements which are indicated in the schematic representation shown in Fig.
291 3A. Some of them have already been described, including two SF1 binding sites, two TCF
292 binding sites, and one Sp1 binding site ([34, 35], Fig 3B). Furthermore, we also identified two
293 additional motifs that have not yet been reported: a Egr1 motif at -52 bp, identified with a high
294 confidence, which partially overlaps with the Sp1 motif and a non-canonical FOXO binding
295 site in the reverse orientation (CIS-BP Motif ID M5467-1.02, [50]) at nucleotides -113 to -103
296 (Fig. 3B).

297 In order to locate the region required for GnRH activation, we performed a panel of gradual
298 deletions of the hAMHR2 promoter. As illustrated in Fig. 3C, the loss of distal SF1 and TCF
299 motifs significantly decreased basal promoter activity. In contrast, the extent of GnRH
300 stimulation was not affected (1.78 ± 0.11 and $2.5 \pm 0.39\%$ stimulation for the -375/+53 bp and
301 the -247/+53 bp promoter, respectively). GnRHa-dependent luciferase activity remained
302 unaltered following successive deletions leading to the -53 bp promoter (Fig. 3C). Deletion of
303 the fragment located between -53 and -37 bp of the promoter, which contains the Egr1/Sp1

304 binding site, markedly decreased GnRH-dependent luciferase activity. Further deletions did
305 not induce any additional changes in luciferase activity. Altogether, these experiments indicate
306 that the sequence located between -53 and -37 bp of hAMHR2 promoter is required for GnRH
307 regulation.

308

309 **Egr1 mediates GnRH transactivation of human AMHR2 in gonadotrope cells**

310 To confirm the role of the Egr1/Sp1 binding site in GnRH stimulation of hAMHR2 promoter
311 activity, we performed site-directed mutagenesis. Mutation resulted in a significant decrease
312 in basal as well as in GnRH-dependent luciferase activity as compared to the native promoter
313 fragment (Fig. 4A). Egr1 is highly induced by GnRH in gonadotrope cells and mediates GnRH-
314 stimulated expression of *Lhb* [6, 7]. To examine the potential role of Egr1 in GnRH stimulation
315 of AMHR2 expression, we next used a small interfering RNA (siRNA) strategy to decrease
316 Egr1 expression. LβT2 cells were transfected with a pool of siRNAs specific to mouse *Egr1*
317 (siRNA Egr1) or with a pool of nontargeting siRNAs (siRNA NT) before GnRH_a stimulation.
318 In siRNA NT-transfected cells, GnRH_a stimulated hAMHR2 promoter activity by 218 ± 26%
319 (Fig. 4B). Transfection of cells with siRNA Egr1 reduced basal promoter activity. Furthermore,
320 siRNA Egr1 abrogated the GnRH stimulatory effect indicating that Egr1 is required for GnRH
321 regulation of hAMHR2 expression (Fig. 4B).

322 **FOXO1 represses basal and GnRH-dependent activity of human AMHR2 promoter**

323 To examine the potential involvement of putative FOXO binding site and FOXO transcription
324 factors, we transiently cotransfected LβT2 cells with plasmids expressing wild-type FOXO1
325 (FOXO1-Wt) or a constitutively active, phosphorylation-defective form of FOXO1 (FOXO1-
326 CAM) [51]. The constitutively active form of FOXO1 was previously reported to localize
327 exclusively into the nucleus of LβT2 cells [14, 15]. As illustrated in Fig. 5A, we observed that
328 overexpression of FOXO1-CAM decreased basal hAMHR2 transcriptional activity. Notably,
329 FOXO1-CAM also markedly reduced the GnRH_a stimulatory effect on the promoter (Fig. 5A).
330 Because the phosphoinositide 3-kinase (PI3K) pathway mediates GnRH regulation of FOXO1
331 export in LβT2 cells [14], we next examined whether blocking the PI3K pathway would
332 decrease GnRH-induced hAMHR2 promoter activity (Fig. 5B). Pretreatment of cells with the
333 PI3K signaling pathway inhibitor LY294002 (20 μM) significantly reduced by 48 ± 8%, (Fig.
334 5B *inset*) the GnRH stimulatory effect on hAMHR2 promoter activity. To examine the
335 requirement of the putative FOXO binding site, we next transiently transfected LβT2 cells with
336 a hAMHR2 promoter with a mutated FOXO motif. As shown in Fig. 5C, GnRH_a treatment still

337 increased mutated *hAMHR2* promoter to a similar extent as that observed with the wild-type
338 promoter. This suggests that although FOXO1 decreases GnRH induction of *hAMHR2* activity,
339 it may not act through direct binding to *hAMHR2* promoter as already reported for murine *Fshb*
340 and *Lhb* promoters [13, 14, 52].

341

342 **SF1 and β -catenin are required for basal and GnRH-stimulated human *AMHR2***
343 **promoter activities.**

344 SF1 and TCF4 have been reported to be necessary for basal *hAMHR2* expression [34, 35].
345 Furthermore, both SF1 and β -catenin signaling can be activated by GnRH [53, 54] and are
346 involved in GnRH regulation of *Lhb* expression [10, 11, 55]. To better delineate the role of
347 these two transcription factors, we performed site directed mutagenesis and siRNA-mediated
348 knock down experiments. As shown in Fig. 6A, basal luciferase activity of the *hAMHR2*
349 promoter carrying mutations of both SF1 binding sites [33, 34] was significantly reduced
350 compared to the control promoter in L β T2 cells. In contrast, the GnRH stimulatory effect was
351 still observed, suggesting that intact SF1-binding sites are not required for GnRH stimulation.
352 siRNA-mediated decrease in SF1, leading to a $50 \pm 7\%$ reduction in SF1 transcripts levels after
353 24h transfection (RT-qPCR experiments, data not shown), reduced basal luciferase activity
354 (Fig. 6B) in agreement with results from mutation experiments. Decreasing SF1 expression
355 completely abrogated GnRH stimulation of *hAMHR2* promoter activity (Fig. 6B). This
356 indicates that SF1 contributes to GnRH regulation although it may not bind directly to SF1
357 motifs and suggests an indirect mechanism of control.

358 To examine the potential role of the β -catenin/TCF pathway in basal and GnRH dependent
359 *hAMHR2* activity, we mutated one or both TCF binding sites in the 428-bp *hAMHR2* promoter
360 (Fig. 7A). Mutation of the proximal TCF binding site did not affect basal or GnRH stimulated
361 promoter activity. In contrast, basal luciferase activity was reduced by mutation of the distal
362 TCF motif, which was also observed when both TCF sites were mutated (Fig. 7A). The fold
363 increase in GnRH stimulation was unaffected as both basal and GnRH dependent luciferase
364 activities were reduced under these conditions (Fig. 7A). Significant levels of TCF4 transcripts
365 were found in L β T2 cells (data not shown) suggesting that TCF4 transcription factor
366 contributes to basal promoter activity. To further examine the role of β -catenin, we specifically
367 targeted β -catenin expression using siRNAs. L β T2 cells were transfected with a pool of
368 siRNAs specific to mouse β -catenin (siRNA β -catenin) or with a pool of nontargeting siRNAs

369 (siRNA NT) before GnRHa stimulation. This led to a significant reduction in β -catenin
370 transcript levels in L β T2 cells as assessed by qRT-PCR ($50 \pm 3.5\%$ of siRNA NT-transfected
371 cells, data not shown). As expected in NT-transfected cells, GnRHa stimulated the 2252-bp
372 *hAMHR2* promoter activity by $187 \pm 4\%$ (Fig. 7B). Transfection of cells with siRNA β -catenin
373 reduced basal promoter activity. It also reduced the extent of GnRH stimulation by $48 \pm 17\%$
374 (Fig. 7B, *inset*). In a complementary approach, we inhibited activity of the β -catenin pathway
375 using the pharmacological inhibitor, JW74. As shown in Fig. 7C, we observed that JW74
376 significantly reduced the GnRH stimulatory effect by $44 \pm 7\%$. Activation of β -catenin by
377 GnRH relies on PKC-dependent signaling pathways, including notably the recruitment of c-
378 Jun N-terminal kinases (JNK) [54, 56]. Treatment of cells with the PKC inhibitor GF109203X
379 significantly reduced the GnRH-induced increase of *hAMHR2* promoter activity by $60 \pm 13\%$.
380 Furthermore, GnRH-induced activation was fully prevented by the JNK inhibitor, SP600125.
381 Altogether, these experiments indicate that both SF1 and β -catenin mediate GnRH stimulatory
382 effect on *hAMHR2* transcription in L β T2 gonadotrope cells, although they may not bind to
383 their known response elements.

384

385 Discussion

386 First identified in the mesenchyme of Müllerian ducts and in gonads, AMHR2 was
387 subsequently localized in several other organs, including the brain and the pituitary, suggesting
388 that AMH biological effects are far more diverse than initially thought. However, the
389 mechanisms regulating AMHR2 gene expression, notably in extragonadal sites still remain
390 elusive. Here, we demonstrate that gene expression of both human and murine AMHR2 is
391 regulated by the neuroendocrine hormone, GnRH. Indeed, GnRH was shown to increase
392 endogenous AMHR2 transcripts in the gonadotrope cell line L β T2 and to enhance the activity
393 of a human *AMHR2* promoter. Transactivation of the promoter was not observed in the
394 immature α T3-1 gonadotrope cell line nor in neuronal GT1-7 cell line, although the latter is
395 receptive to both GnRH and AMH.

396 We identified several transcription factors required for GnRH regulation of *AMHR2* gene
397 expression. We notably uncovered the functional importance of the Egr1 transcription factor.
398 We localized an Egr1 binding site in the proximal region of the *hAMHR2* promoter and
399 provided evidence, by deletion and mutation, that this site is needed for GnRH-mediated
400 induction. Furthermore, because siRNA-inhibited expression of Egr1 mRNA in L β T2 cells

401 was shown to prevent GnRH effect, our study strongly suggests that GnRH induces Egr1
402 binding to transactivate the *hAMHR2* promoter. We demonstrated that two factors, *i.e.* SF1 and
403 β -catenin, known to be important for constitutive *hAMHR2* promoter activity [34, 35] also
404 contribute to the GnRH stimulatory effect. Gonadotrope cells, including L β T2 cells, express
405 significant amounts of SF1 and β -catenin [55, 57] and down-regulation of each factor was able
406 to reduce the GnRH-dependent increase in *hAMHR2* promoter activity. The contribution of β -
407 catenin in GnRH regulation of *hAMHR2* promoter was further supported by the fact that
408 pharmacological inhibition of PKC and JNK, known to mediate GnRH activation of β -catenin,
409 prevents GnRH activation of *hAMHR2* promoter. However, because PKC and JNK signaling
410 pathways may act through alternative mediators, additional experiments are needed to establish
411 whether the signaling PKC/JNK/ β -catenin cascade is required for GnRH regulation of
412 *hAMHR2* promoter. Interestingly, GnRH effect was not affected by mutation of either SF1 or
413 TCF motifs suggesting that SF1 and β -catenin/TCF, although contributing to GnRH regulation,
414 do not bind to these sites of the promoter. In line with this result, it was reported in HeLa cells,
415 that SF1 alone does not activate the *hAMHR2* promoter but instead binds to β -catenin and
416 synergistically cooperates with β -catenin to activate the promoter [35]. SF1 is known to
417 physically interact with β -catenin in L β T2 cells [55]. Furthermore, SF1 has been reported to
418 synergize with Egr1 and Sp1 for transactivation of the *Lhb* promoter [11]. Interestingly, it was
419 demonstrated in L β T2 cells that SF1 interacts with Egr1 through β -catenin and that β -catenin
420 colocalization with SF1 and Egr1 on the mouse *Lhb* promoter is increased by GnRH [55].
421 Based on these studies, we can hypothesize from our present findings that, by increasing Egr1
422 binding to the proximal region of the *AMHR2* promoter, GnRH enables the formation of a
423 complex, containing β -catenin and SF1, that cooperates with Egr1 to fully activate the *AMHR2*
424 promoter (see the proposed hypothetical model in Fig. 8). In this study, we also uncovered the
425 functional importance of the transcription factor FOXO1 in the regulation of the *hAMHR2*
426 promoter. FOXO1 was recently detected in native gonadotrope cells as well as in the L β T2 cell
427 line and identified as a negative regulator of FSH β and LH β gene expression [13-15, 52]. Here,
428 we demonstrate using a constitutively active FOXO1 mutant that FOXO1 is a negative
429 regulator of basal and GnRH-dependent *hAMHR2* promoter activity. The PI3K pathway is the
430 major regulator of FOXO1 activity, and GnRH was recently shown to enhance FOXO1 export
431 out of the nucleus through this pathway in L β T2 cells, abrogating the FOXO1 repressive effect
432 on the *Fshb* promoter [14, 15]. Supporting the involvement of FOXO1, we showed in the
433 present study that pharmacological inhibition of the PI3K pathway significantly reduced GnRH

434 stimulation of *hAMHR2* promoter activity. Further experiments are however needed to
435 determine whether the signaling cascade PI3K/FOXO1 is required for GnRH regulation of
436 *hAMHR2* promoter. Altogether these experiments strongly suggest that blockade of FOXO1
437 repressive effect contributes to GnRH stimulation of *hAMHR2* promoter activity (Fig. 8).
438 Mutations of the identified FOXO binding site did not however affect GnRH-stimulated
439 promoter activity. Although this may indicate the use of alternative yet unidentified motifs
440 within the promoter, several studies reported that the FOXO1 suppressive effect on *Fshb* and
441 *Lhb* expression in L β T2 cells occurs without direct binding of FOXO1 to their respective
442 promoters [13, 14, 52]. The authors of those studies postulated that FOXO1 acts through
443 protein-protein interactions with other transcription factors/cofactors. As for gonadotropin
444 genes, factors interacting with FOXO1 to regulate *hAMHR2* still remain to be identified. It is
445 tempting to speculate that FOXO1 may counteract the action of stimulatory factors identified
446 in the present study. By reducing FOXO1 nuclear localization, GnRH could thus enhance the
447 transactivation capacity of SF1, β -catenin, and Egr1. The ability of FOXO1 to inhibit Wnt/ β -
448 catenin signaling has recently been reported in osteoblast precursors as well as in sarcoma cell
449 lines [58, 59], which supports this hypothesis. The underlying molecular mechanisms of this
450 phenomenon are still to be determined.

451 Our study provides new information on the regulation of constitutive *AMHR2* expression in
452 gonadotrope cells. Using a combination of site-directed mutagenesis and siRNA-mediated
453 down regulation experiments, we provide evidence that SF1 and β -catenin contribute to basal
454 *AMHR2* gene expression in gonadotrope cells through their respective motifs in the promoter.
455 We found significant amounts of TCF4 transcript (qRT-PCR experiments, data not shown) in
456 L β T2 cells suggesting that TCF4 transcription factor can be recruited by β -catenin to
457 transactivate *AMHR2* promoter. It was previously reported using mutation experiments that the
458 four TCF binding sites identified in *hAMHR2* promoter are necessary for full activity in HeLa
459 cells [35]. In gonadotrope cells, both deletion and mutation experiments indicate that only one
460 TCF site is needed for constitutive activity. Indeed, the shorter *hAMHR2* promoter, devoid of
461 the two first TCF sites, retained full basal activity and mutation of the most proximal site,
462 reported to be critical in HeLa cells, was ineffective. This difference may reflect cell-specific
463 mechanisms. Interestingly, we found that the TCF motif involved in basal activity in
464 gonadotrope cells partially overlaps with a GATA motif suggesting that GATA transcription
465 factors contribute together with TCF to basal *hAMHR2* expression in gonadotrope cells. Our
466 present study has uncovered the involvement of two new transcription factors in basal

467 h*AMHR2* expression *i.e.* Egr1 and FOXO1. FOXO1 was shown to act as a repressor of *AMHR2*
468 expression as previously reported for gonadotropin β -subunit genes [13-15, 52]. In murine
469 granulosa cells, FOXO1 represses the expression of the luteinizing
470 hormone/choriogonadotropin receptor gene [60], as well as of several genes involved in the
471 steroidogenic biosynthesis pathways [61]. Given the importance of AMH signaling in
472 granulosa cell proliferation and steroidogenesis, it would be of interest to determine whether
473 FOXO1 also represses *AMHR2* gene expression in these cells.

474 It has long been reported that the frequency of GnRH pulses is critical for the appropriate ratio
475 of FSH vs LH synthesis and secretion [5]. This has been proposed as one of the mechanisms
476 allowing gonadotrope cells to interpret the varying pattern of GnRH secretion [4].
477 Gonadotropin genes are not the only genes differentially regulated by GnRH pulse frequency
478 and several other target genes have been identified [62], including follistatin and nitric oxide
479 synthase 1 [63, 64]. Our present study identifies *AMHR2* as one of these genes. Indeed, using
480 L β T2 cells in a perfusion system, we showed that GnRH at high pulse frequency increases
481 *Amhr2* expression whereas lower frequencies were ineffective. This result as well as the
482 positive regulation of *AMHR2* promoter by GnRH is not in agreement with our previous report
483 indicating that GnRHa treatment of L β T2 cells in static cultures decreased *Amhr2* transcript
484 levels by ~ 30%, a decrease which was also observed in rat pituitary, 24 h after GnRHa
485 injection [16]. Our present data using perfused L β T2 cells strongly suggest that this decrease
486 reflected desensitization of cells to GnRH stimulation. Indeed, we observed that *Amhr2*
487 expression is similarly decreased in response to a continuous GnRH stimulation which was
488 associated with a desensitization of the gonadotropin response as reflected by decreased levels
489 of gonadotropin β -subunit transcripts. Furthermore, reevaluation of *Lhb* and *Fshb* transcript
490 levels in the pituitaries of GnRHa-treated rats (unpublished data) were also indicative of
491 gonadotrope cell desensitization to GnRH. Altogether, our data thus indicate that GnRH finely
492 regulates *Amhr2* expression in gonadotrope cells with a selective increase observed with a high
493 GnRH pulse frequency.

494 Accumulating evidence highlights that AMH may act at every level of the gonadotrope axis.
495 Indeed, in addition to gonads, *AMHR2* mRNA has recently been localized in rodent and human
496 GnRH neurons, and intracerebroventricular or peripheral injections of AMH in mice was able
497 to increase LH secretion [25, 26]. Furthermore, *AMHR2* was identified in rodent pituitary as
498 well as in gonadotrope cells and AMH was shown to positively regulate FSH [16, 27, 65]. Very

499 recently, AMH regulation on FSH secretion was also reported in a non-rodent species *i.e.* in
500 heifers [65]. Because we characterized *Amh* transcripts in immature rat pituitary and in L β T2
501 gonadotrope cells [16], this suggests that locally produced AMH could contribute to the
502 regulation of FSH secretion as described for pituitary activin or follistatin [66]. It would be of
503 interest to determine whether AMH is coexpressed with its receptor in the pituitary of other
504 mammalian species. Supporting the functional importance of AMH signaling in the pituitary,
505 AMHR2 gene expression has also been identified in the pituitary of non-mammalian species
506 *i.e.* the teleost Nile Tilapia [67, 68]. The present work reinforces the intriguing hypothesis that
507 AMH contributes to the pituitary control of gonadal functions by demonstrating that
508 gonadotrope AMH receptivity is regulated by the neuroendocrine hormone GnRH. The
509 physiological implication of *Amhr2* up-regulation by high pulse frequency of GnRH remains
510 to be understood. It has been clearly demonstrated that high frequency of GnRH pulses only
511 poorly increases rodent *Fshb* transcript levels [5]. There are, however, physiological situations
512 where high GnRH pulsatility is associated with elevation of both LH and FSH secretion.
513 Among them are the cyclic preovulatory period in adults and the so-called “minipuberty” which
514 is characterized by a transient activation of the Hypothalamic-Pituitary-Gonadal axis in
515 immature animals [69]. We recently detected significant levels of pituitary *Amhr2* transcripts
516 in immature but not adult rats suggesting that AMH could play a crucial role in regulating FSH
517 secretion during the immature period. During this period, a marked increase in FSH secretion
518 and pituitary *Fshb* mRNA levels has been reported in several mammalian species [69]. Hence,
519 increasing AMH receptivity in pituitary gonadotropes may be an indirect mechanism allowing
520 elevated FSH synthesis despite an increase in GnRH tone that would not favor FSH. In rat as
521 in other mammals, this period is characterized by a sexual dimorphism of FSH secretion with
522 higher circulating FSH concentrations and pituitary *Fshb* mRNA levels in females than in
523 males [16, 69]. Interestingly, we reported that pituitary *Amhr2* expression is also higher in
524 female rats during this developmental period [16]. A higher GnRH neurons activity has
525 recently been reported in infantile 2-3-week-female mice as compared to males [70]. Based on
526 our present study, we can hypothesize that this contributes to dimorphism of pituitary *Amhr2*
527 expression and hence to the sexually dimorphic secretion of FSH during infancy.

528 In conclusion, our study shed new light on the mechanisms regulating AMHR2 expression.
529 Moreover, the identification of AMHR2 as a target of GnRH further underlines the role of
530 AMH in the control of pituitary gonadotrope function.

531

532 **Acknowledgements**

533 We are grateful to Dr. Pamela Mellon, (University of California, San Diego, CA) for providing
534 us with the L β T2, α T3-1 and GT1-7 cell lines. We thank A Fernandez-Vega for technical
535 assistance in RT-qPCR experiments. We thank Dr N. di Clemente for helpful discussion of the
536 data.

537 **Disclosure statement:**

538 A preliminary report of this work was presented in a communication at the 42nd Annual
539 Meeting of Société de Neuroendocrinologie (SNE), Dijon, France, September 18-21 2017.

540 The authors have no conflicts of interest to declare.

541

542 **Funding Sources**

543 This study was supported by grants from Paris 7 University, the Centre National de la
544 Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale and, in
545 part, by National Institute of Health HD019938 (U. B. K).

546

547 **Author contributions**

548 GG designed and performed the study, interpreted the results and contribute to the writing of
549 the manuscript, CD performed transfection experiments, DL designed and performed
550 perfusion culture experiments and contributed to the interpretation of promoter studies, JYP
551 generated wild type and SF1-mutated hAMHR2 promoter constructs, JT helped with
552 manuscript preparation, UK helped with the design of perfusion culture experiments and
553 manuscript preparation, JNL designed and performed deletion and site-directed mutagenesis
554 experiments, analyzed the data and contributed to the writing of the manuscript, JCT designed
555 the study, interpreted the results and wrote the manuscript.

556

557 **Abbreviations**

558 bp, base pairs; Egr1, Early growth response protein 1; FOXO, Forkhead Box O; GnRHa, GnRH
559 agonist; JNK, c-Jun N-terminal kinase; PACAP, Pituitary adenylate cyclase-activating
560 polypeptide; PI3K, Phosphatidylinositol 3-kinase; PKC, Protein kinase C; P/S,
561 penicillin/streptomycin; siNT, nontargeting siRNA; TCF/LEF, T-cell factor/Lymphoid
562 enhancer binding factor; SF1, Steroidogenic factor 1; siRNA, small interfering RNA; SMAD,
563 Sma/Mothers against decapentaplegic homolog.

564

565 References

- 566 1 Stojilkovic SS, Reinhart J, Catt KJ: Gonadotropin-releasing hormone receptors: Structure and
567 signal transduction pathways. *Endocr Rev* 1994;15:462-499.
- 568 2 Naor Z, Huhtaniemi I: Interactions of the gnrh receptor with heterotrimeric g proteins. *Front*
569 *Neuroendocrinol* 2013;34:88-94.
- 570 3 Counis R, Laverriere JN, Garrel G, Bleux C, Cohen-Tannoudji J, Lerrant Y, Kottler ML, Magre
571 S: Gonadotropin-releasing hormone and the control of gonadotrope function. *Reprod Nutr Dev*
572 2005;45:243-254.
- 573 4 Burger LL, Haisenleder DJ, Dalkin AC, Marshall JC: Regulation of gonadotropin subunit gene
574 transcription. *J Mol Endocrinol* 2004;33:559-584.
- 575 5 Dalkin AC, Haisenleder DJ, Ortolano GA, Ellis TR, Marshall JC: The frequency of
576 gonadotropin-releasing-hormone stimulation differentially regulates gonadotropin subunit messenger
577 ribonucleic acid expression. *Endocrinology* 1989;125:917-924.
- 578 6 Halvorson LM, Kaiser UB, Chin WW: The protein kinase c system acts through the early
579 growth response protein 1 to increase lhbeta gene expression in synergy with steroidogenic factor-1.
580 *Mol Endocrinol* 1999;13:106-116.
- 581 7 Wolfe MW, Call GB: Early growth response protein 1 binds to the luteinizing hormone-beta
582 promoter and mediates gonadotropin-releasing hormone-stimulated gene expression. *Mol Endocrinol*
583 1999;13:752-763.
- 584 8 Lee SL, Sadovsky Y, Swirnoff AH, Polish JA, Goda P, Gavrilina G, Milbrandt J: Luteinizing
585 hormone deficiency and female infertility in mice lacking the transcription factor ngfi-a (egr-1). *Science*
586 1996;273:1219-1221.
- 587 9 Topilko P, Schneider-Maunoury S, Levi G, Trembleau A, Gourdj D, Driancourt MA, Rao CV,
588 Charnay P: Multiple pituitary and ovarian defects in krox-24 (ngfi-a, egr-1)-targeted mice. *Mol*
589 *Endocrinol* 1998;12:107-122.
- 590 10 Tremblay JJ, Drouin J: Egr-1 is a downstream effector of gnrh and synergizes by direct
591 interaction with ptx1 and sf-1 to enhance luteinizing hormone beta gene transcription. *Mol Cell Biol*
592 1999;19:2567-2576.
- 593 11 Kaiser UB, Halvorson LM, Chen MT: Sp1, steroidogenic factor 1 (sf-1), and early growth
594 response protein 1 (egr-1) binding sites form a tripartite gonadotropin-releasing hormone response
595 element in the rat luteinizing hormone-beta gene promoter: An integral role for sf-1. *Mol Endocrinol*
596 2000;14:1235-1245.
- 597 12 Thackray VG, Mellon PL, Coss D: Hormones in synergy: Regulation of the pituitary
598 gonadotropin genes. *Mol Cell Endocrinol* 2010;314:192-203.
- 599 13 Arriola DJ, Mayo SL, Skarra DV, Benson CA, Thackray VG: Foxo1 transcription factor
600 inhibits luteinizing hormone beta gene expression in pituitary gonadotrope cells. *J Biol Chem*
601 2012;287:33424-33435.
- 602 14 Choi YS, Lee HJ, Ku CR, Cho YH, Seo MR, Lee YJ, Lee EJ: Foxo1 is a negative regulator of
603 fshbeta gene expression in basal and gnrh-stimulated conditions in female. *Endocrinology*
604 2014;155:2277-2286.

605 15 Lannes J, L'Hote D, Garrel G, Laverriere JN, Cohen-Tannoudji J, Querat B: Rapid
606 communication: A microrna-132/212 pathway mediates gnRH activation of fsh expression. *Mol*
607 *Endocrinol* 2015;29:364-372.

608 16 Garrel G, Racine C, L'Hote D, Denoyelle C, Guigon CJ, di Clemente N, Cohen-Tannoudji J:
609 Anti-mullerian hormone: A new actor of sexual dimorphism in pituitary gonadotrope activity before
610 puberty. *Sci Rep* 2016;6:23790.

611 17 Josso N, Picard JY, Rey R, di Clemente N: Testicular anti-mullerian hormone: History,
612 genetics, regulation and clinical applications. *Pediatr Endocrinol Rev* 2006;3:347-358.

613 18 Teixeira J, Maheswaran S, Donahoe PK: Mullerian inhibiting substance: An instructive
614 developmental hormone with diagnostic and possible therapeutic applications. *Endocr Rev*
615 2001;22:657-674.

616 19 Visser JA, de Jong FH, Laven JS, Themmen AP: Anti-mullerian hormone: A new marker for
617 ovarian function. *Reproduction* 2006;131:1-9.

618 20 Wang PY, Koishi K, McGeachie AB, Kimber M, Maclaughlin DT, Donahoe PK, McLennan
619 IS: Mullerian inhibiting substance acts as a motor neuron survival factor in vitro. *Proc Natl Acad Sci U*
620 *S A* 2005;102:16421-16425.

621 21 Lebourrier N, Launay S, Macrez R, Maubert E, Legros H, Leclerc A, Jamin SP, Picard JY,
622 Marret S, Laudénbach V, Berger P, Sonderegger P, Ali C, di Clemente N, Vivien D: Anti-mullerian-
623 hormone-dependent regulation of the brain serine-protease inhibitor neuroserpin. *J Cell Sci*
624 2008;121:3357-3365.

625 22 Kow LM, Florea C, Schwanzel-Fukuda M, Devidze N, Kami Kia H, Lee A, Zhou J,
626 Maclaughlin D, Donahoe P, Pfaff D: Development of a sexually differentiated behavior and its
627 underlying CNS arousal functions. *Curr Top Dev Biol* 2007;79:37-59.

628 23 di Clemente N, Josso N, Gouedard L, Belville C: Components of the anti-mullerian hormone
629 signaling pathway in gonads. *Mol Cell Endocrinol* 2003;211:9-14.

630 24 Josso N, Clemente N: Transduction pathway of anti-mullerian hormone, a sex-specific member
631 of the TGF-beta family. *Trends Endocrinol Metab* 2003;14:91-97.

632 25 Cimino I, Casoni F, Liu X, Messina A, Parkash J, Jamin SP, Catteau-Jonard S, Collier F,
633 Baroncini M, Dewailly D, Pigny P, Prescott M, Campbell R, Herbison AE, Prevot V, Giacobini P:
634 Novel role for anti-mullerian hormone in the regulation of gnRH neuron excitability and hormone
635 secretion. *Nat Commun* 2016;7:10055.

636 26 Tata B, Mimouni NEH, Barbotin AL, Malone SA, Loyens A, Pigny P, Dewailly D, Catteau-
637 Jonard S, Sundstrom-Poromaa I, Piltonen TT, Dal Bello F, Medana C, Prevot V, Clasadonte J,
638 Giacobini P: Elevated prenatal anti-mullerian hormone reprograms the fetus and induces polycystic
639 ovary syndrome in adulthood. *Nat Med* 2018;24:834-846.

640 27 Bedecarrats GY, O'Neill FH, Norwitz ER, Kaiser UB, Teixeira J: Regulation of gonadotropin
641 gene expression by mullerian inhibiting substance. *Proc Natl Acad Sci U S A* 2003;100:9348-9353.

642 28 di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, Tizard R, Picard JY, Vigier B, Josso
643 N, Cate R: Cloning, expression, and alternative splicing of the receptor for anti-mullerian hormone.
644 *Mol Endocrinol* 1994;8:1006-1020.

645 29 Baarends WM, van Helmond MJ, Post M, van der Schoot PJ, Hoogerbrugge JW, de Winter JP,
646 Uilenbroek JT, Karels B, Wilming LG, Meijers JH, et al.: A novel member of the transmembrane
647 serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal
648 cells adjacent to the mullerian duct. *Development* 1994;120:189-197.

649 30 Teixeira J, He WW, Shah PC, Morikawa N, Lee MM, Catlin EA, Hudson PL, Wing J,
650 Maclaughlin DT, Donahoe PK: Developmental expression of a candidate mullerian inhibiting substance
651 type II receptor. *Endocrinology* 1996;137:160-165.

652 31 Imbeaud S, Faure E, Lamarre I, Mattei MG, di Clemente N, Tizard R, Carre-Eusebe D, Belville
653 C, Tragethon L, Tonkin C, Nelson J, McAuliffe M, Bidart JM, Lababidi A, Josso N, Cate RL, Picard
654 JY: Insensitivity to anti-mullerian hormone due to a mutation in the human anti-mullerian hormone
655 receptor. *Nat Genet* 1995;11:382-388.

656 32 Teixeira J, Kehas DJ, Antun R, Donahoe PK: Transcriptional regulation of the rat mullerian
657 inhibiting substance type II receptor in rodent leydig cells. *Proc Natl Acad Sci U S A* 1999;96:13831-
658 13838.

659 33 Klattig J, Sierig R, Kruspe D, Besenbeck B, Englert C: Wilms' tumor protein wt1 is an activator
660 of the anti-mullerian hormone receptor gene amhr2. *Mol Cell Biol* 2007;27:4355-4364.

661 34 de Santa Barbara P, Moniot B, Poulat F, Boizet B, Berta P: Steroidogenic factor-1 regulates
662 transcription of the human anti-mullerian hormone receptor. *J Biol Chem* 1998;273:29654-29660.

663 35 Hossain A, Saunders GF: Synergistic cooperation between the beta-catenin signaling pathway
664 and steroidogenic factor 1 in the activation of the mullerian inhibiting substance type ii receptor. *J Biol*
665 *Chem* 2003;278:26511-26516.

666 36 Parr BA, McMahon AP: Sexually dimorphic development of the mammalian reproductive tract
667 requires wnt-7a. *Nature* 1998;395:707-710.

668 37 Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP,
669 Grootegoed JA: Anti-mullerian hormone and anti-mullerian hormone type ii receptor messenger
670 ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and
671 gonadotropin-induced follicle growth. *Endocrinology* 1995;136:4951-4962.

672 38 Pierre A, Peigne M, Grynberg M, Arouche N, Taieb J, Hesters L, Gonzales J, Picard JY,
673 Dewailly D, Fanchin R, Catteau-Jonard S, di Clemente N: Loss of lh-induced down-regulation of anti-
674 mullerian hormone receptor expression may contribute to anovulation in women with polycystic ovary
675 syndrome. *Hum Reprod* 2013;28:762-769.

676 39 Pierre A, Taieb J, Giton F, Grynberg M, Touleimat S, El Hachem H, Fanchin R, Monniaux D,
677 Cohen-Tannoudji J, di Clemente N, Racine C: Dysregulation of the anti-mullerian hormone system by
678 steroids in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2017;102:3970-3978.

679 40 Pierre A, Estienne A, Racine C, Picard JY, Fanchin R, Lahoz B, Alabart JL, Folch J, Jarrier P,
680 Fabre S, Monniaux D, di Clemente N: The bone morphogenetic protein 15 up-regulates the anti-
681 mullerian hormone receptor expression in granulosa cells. *J Clin Endocrinol Metab* 2016;101:2602-
682 2611.

683 41 Thomas P, Mellon PL, Turgeon J, Waring DW: The l beta t2 clonal gonadotrope: A model for
684 single cell studies of endocrine cell secretion. *Endocrinology* 1996;137:2979-2989.

685 42 Windle JJ, Weiner RI, Mellon PL: Cell lines of the pituitary gonadotrope lineage derived by
686 targeted oncogenesis in transgenic mice. *Mol Endocrinol* 1990;4:597-603.

687 43 Bedecarrats GY, Kaiser UB: Differential regulation of gonadotropin subunit gene promoter
688 activity by pulsatile gonadotropin-releasing hormone (gnrh) in perfused l beta t2 cells: Role of gnrh
689 receptor concentration. *Endocrinology* 2003;144:1802-1811.

690 44 Pincas H, Amoyel K, Counis R, Laverriere JN: Proximal cis-acting elements, including
691 steroidogenic factor 1, mediate the efficiency of a distal enhancer in the promoter of the rat
692 gonadotropin-releasing hormone receptor gene. *Mol Endocrinol* 2001;15:319-337.

693 45 Kitamura YI, Kitamura T, Kruse JP, Raum JC, Stein R, Gu W, Accili D: Foxo1 protects against
694 pancreatic beta cell failure through neurod and mafa induction. *Cell Metab* 2005;2:153-163.

695 46 Thompson IR, Ciccone NA, Xu S, Zaytseva S, Carroll RS, Kaiser UB: GnRH pulse frequency-
696 dependent stimulation of fshbeta transcription is mediated via activation of pka and creb. *Mol*
697 *Endocrinol* 2013;27:606-618.

698 47 Alarid ET, Windle JJ, Whyte DB, Mellon PL: Immortalization of pituitary cells at discrete
699 stages of development by directed oncogenesis in transgenic mice. *Development* 1996;122:3319-3329.

700 48 Pernasetti F, Vasilyev VV, Rosenberg SB, Bailey JS, Huang HJ, Miller WL, Mellon PL: Cell-
701 specific transcriptional regulation of follicle-stimulating hormone-beta by activin and gonadotropin-
702 releasing hormone in the lbeta2 pituitary gonadotrope cell model. *Endocrinology* 2001;142:2284-2295.

703 49 Weirauch MT, Yang A, Albu M, Cote AG, Montenegro-Montero A, Drewe P, Najafabadi HS,
704 Lambert SA, Mann I, Cook K, Zheng H, Goity A, van Bakel H, Lozano JC, Galli M, Lewsey MG,
705 Huang E, Mukherjee T, Chen X, Reece-Hoyes JS, Govindarajan S, Shaulsky G, Walhout AJM, Bouget
706 FY, Ratsch G, Larrondo LF, Ecker JR, Hughes TR: Determination and inference of eukaryotic
707 transcription factor sequence specificity. *Cell* 2014;158:1431-1443.

708 50 Jolma A, Yan J, Whittington T, Toivonen J, Nitta KR, Rastas P, Morgunova E, Enge M, Taipale
709 M, Wei G, Palin K, Vaquerizas JM, Vincentelli R, Luscombe NM, Hughes TR, Lemaire P, Ukkonen
710 E, Kivioja T, Taipale J: DNA-binding specificities of human transcription factors. *Cell* 2013;152:327-
711 339.

712 51 Qiang L, Banks AS, Accili D: Uncoupling of acetylation from phosphorylation regulates foxo1
713 function independent of its subcellular localization. *J Biol Chem* 2010;285:27396-27401.

714 52 Skarra DV, Arriola DJ, Benson CA, Thackray VG: Forkhead box o1 is a repressor of basal and
715 gnrh-induced fshb transcription in gonadotropes. *Mol Endocrinol* 2013;27:1825-1839.

716 53 Haisenleder DJ, Yasin M, Dalkin AC, Gilrain J, Marshall JC: Gnrh regulates steroidogenic
717 factor-1 (sf-1) gene expression in the rat pituitary. *Endocrinology* 1996;137:5719-5722.

718 54 Gardner S, Maudsley S, Millar RP, Pawson AJ: Nuclear stabilization of beta-catenin and
719 inactivation of glycogen synthase kinase-3beta by gonadotropin-releasing hormone: Targeting wnt
720 signaling in the pituitary gonadotrope. *Mol Endocrinol* 2007;21:3028-3038.

721 55 Salisbury TB, Binder AK, Grammer JC, Nilson JH: Maximal activity of the luteinizing
722 hormone beta-subunit gene requires beta-catenin. *Mol Endocrinol* 2007;21:963-971.

723 56 Wang Q, Chikina M, Zaslavsky E, Pincas H, Sealfon SC: Beta-catenin regulates gnrh-induced
724 fshbeta gene expression. *Mol Endocrinol* 2013;27:224-237.

725 57 Laverriere JN, L'Hote D, Tabouy L, Schang AL, Querat B, Cohen-Tannoudji J: Epigenetic
726 regulation of alternative promoters and enhancers in progenitor, immature, and mature gonadotrope cell
727 lines. *Mol Cell Endocrinol* 2016;434:250-265.

728 58 Iyer S, Ambrogini E, Bartell SM, Han L, Roberson PK, de Cabo R, Jilka RL, Weinstein RS,
729 O'Brien CA, Manolagas SC, Almeida M: Foxos attenuate bone formation by suppressing wnt signaling.
730 *J Clin Invest* 2013;123:3409-3419.

731 59 Guan H, Tan P, Xie L, Mi B, Fang Z, Li J, Yue J, Liao H, Li F: Foxo1 inhibits osteosarcoma
732 oncogenesis via wnt/beta-catenin pathway suppression. *Oncogenesis* 2015;4:e166.

733 60 Law NC, Weck J, Kyriakos B, Nilson JH, Hunzicker-Dunn M: Lhcgr expression in granulosa
734 cells: Roles for pka-phosphorylated beta-catenin, tcf3, and foxo1. *Mol Endocrinol* 2013;27:1295-1310.

735 61 Liu Z, Rudd MD, Hernandez-Gonzalez I, Gonzalez-Robayna I, Fan HY, Zeleznik AJ, Richards
736 JS: Fsh and foxo1 regulate genes in the sterol/steroid and lipid biosynthetic pathways in granulosa cells.
737 *Mol Endocrinol* 2009;23:649-661.

738 62 Lawson MA, Tsutsumi R, Zhang H, Talukdar I, Butler BK, Santos SJ, Mellon PL, Webster NJ:
739 Pulse sensitivity of the luteinizing hormone beta promoter is determined by a negative feedback loop
740 involving early growth response-1 and ngfi-a binding protein 1 and 2. *Mol Endocrinol* 2007;21:1175-
741 1191.

742 63 Besecke LM, Guendner MJ, Schneyer AL, Bauer-Dantoin AC, Jameson JL, Weiss J:
743 Gonadotropin-releasing hormone regulates follicle-stimulating hormone-beta gene expression through
744 an activin/follistatin autocrine or paracrine loop. *Endocrinology* 1996;137:3667-3673.

745 64 Garrel G, Simon V, Thieuland ML, Cayla X, Garcia A, Counis R, Cohen-Tannoudji J: Sustained
746 gonadotropin-releasing hormone stimulation mobilizes the camp/pka pathway to induce nitric oxide
747 synthase type 1 expression in rat pituitary cells in vitro and in vivo at proestrus. *Biol Reprod*
748 2010;82:1170-1179.

749 65 Kereilwe O, Pandey K, Borromeo V, Kadokawa H: Anti-mullerian hormone receptor type 2 is
750 expressed in gonadotrophs of postpubertal heifers to control gonadotrophin secretion. *Reprod Fertil*
751 *Dev* 2018

752 66 Winters SJ, Moore JP: Intra-pituitary regulation of gonadotrophs in male rodents and primates.
753 *Reproduction* 2004;128:13-23.

754 67 Poonlaphdecha S, Pepey E, Huang SH, Canonne M, Soler L, Mortaji S, Morand S, Pfennig F,
755 Melard C, Baroiller JF, D'Cotta H: Elevated amh gene expression in the brain of male tilapia
756 (*Oreochromis niloticus*) during testis differentiation. *Sex Dev* 2011;5:33-47.

757 68 Pfennig F, Standke A, Gutzeit HO: The role of amh signaling in teleost fish - multiple functions
758 not restricted to the gonads. *Gen Comp Endocrinol* 2015;223:87-107.

759 69 Ojeda SR, Andrews WW, Advis JP, White SS: Recent advances in the endocrinology of
760 puberty. *Endocr Rev* 1980;1:228-257.

761 70 Dulka EA, Moenter SM: Prepubertal development of gonadotropin-releasing hormone neuron
762 activity is altered by sex, age, and prenatal androgen exposure. *Endocrinology* 2017;158:3943-3953.

763

764

765 **Figure Legends**

766

767 **Figure 1: Pulsatile GnRH regulates *Amhr2* transcript levels in a frequency-dependent**
768 **manner in perfused L β T2 cells.**

769 The effect of different GnRH pulse frequencies on *Amhr2* expression was assayed in L β T2
770 cells in control medium or medium containing GnRH for 21 h in a perfusion chamber under
771 continuous or pulsatile (one pulse every 30 min or every 2 h) stimulation mode. *Amhr2* mRNA
772 levels were analyzed by real-time PCR. Levels of *Fshb* and *Lhb* transcripts were also measured
773 (inset). Results are the mean \pm SEM of four independent experiments and expressed as percent
774 of *Amhr2* mRNA level in control cells. *, $P \leq 0.05$; **, $P \leq 0.01$ compared with control cells,
775 (one-way ANOVA test).

776

777 **Figure 2: GnRH stimulates the activity of human *AMHR2* promoter in L β T2 gonadotrope**
778 **cells.**

779 GnRHa effects on human *AMHR2* promoter activity were assayed in gonadotrope L β T2 and
780 α T3-1 and in hypothalamic neuronal GT1-7 cell lines. (A) A promoter/luciferase fusion
781 construct, containing the 2252 (-2199/+53)-bp human *AMHR2* promoter, was transiently
782 transfected into L β T2, α T3-1 or GT1-7 cells for 48 h. Cells were then stimulated or not by
783 GnRHa for 4 h. Relative luciferase activity was measured as indicated in *Materials and*
784 *Methods* and results are the mean \pm SEM of at least four independent experiments performed
785 in triplicate. ***, $P \leq 0.001$ compared with respective unstimulated cells (control, unpaired
786 Student's *t* test). (B) The effects of GnRHa, GnRH and PACAP₃₈ on 2252-human *AMHR2*
787 promoter activity in L β T2 cells were compared. L β T2 cells were stimulated with GnRHa,
788 GnRH or with PACAP₃₈ for 4 h. Some cells were pretreated with the GnRH antagonist antide
789 (5 μ M) before stimulation with 10 nM of GnRHa. Luciferase activity was expressed as percent
790 of unstimulated promoter activity (control). Data are the mean \pm SEM of at least five
791 independent experiments performed in triplicate. *, $P \leq 0.05$; **, $P \leq 0.01$ compared with
792 control (Mann-Whitney test). (C) The effect of GnRH on a shorter 428-bp h*AMHR2* promoter
793 was analyzed. The 2252-h*AMHR2* promoter or a 428 (-375/+53)-bp h*AMHR2* were transiently
794 transfected in L β T2 cells for 48 h. Cells were then stimulated or not with GnRHa for 4 h and
795 luciferase activities were measured. Results are the mean \pm SEM of six independent

796 experiments performed in triplicate. ***, $P \leq 0.001$ compared with respective control (unpaired
797 Student's *t* test)

798 **Figure 3: Mapping of the 428-human *AMHR2* promoter and delineation of sequence**
799 **required for GnRH effect.**

800 (A) A schematic representation of the proximal -375/+53 bases of the human *AMHR2* promoter
801 (428-h*AMHR2*) illustrating the position of binding sites for SF1 (SF1), TCF7, TCF7L1 or
802 TCF7L2 (TCF), FOXO1, 3 or 4 (FOXO), all members of Egr1 family (Egr1) and Sp1 (Sp1) is
803 shown. The arrow indicates the transcription initiation site (TSS). (B) Detailed nucleotide
804 sequence of the 428-h*AMHR2* with binding sites for SF1, TCF, FOXO, Egr1 and Sp1, shown
805 boxed, except for Egr1 which is highlighted in grey. (C) Delineation of the h*AMHR2* promoter
806 fragment required for GnRH stimulation was analyzed using transient transfection assay in
807 L β T2 cells. Progressive 5' deletions of the human *AMHR2* promoter extending from -375 to -
808 5 were fused to the luciferase reporter gene and tested for their ability to respond to GnRH
809 treatment. Transient transfection assays were performed as described in *Materials and*
810 *Methods*. Results are the mean \pm SEM of six independent experiments performed in triplicate
811 and expressed as fold stimulation over the PGL2 empty vector. ***, $P < 0.001$; **, $P < 0.01$; ns,
812 $P > 0.05$ (two-way ANOVA test).

813

814 **Figure 4: Egr1 mediates GnRH stimulatory effect on h*AMHR2* promoter.**

815 (A) Mutation of the Egr1/Sp1 binding site in the 428-bp h*AMHR2* promoter was performed as
816 described in *Materials and Methods*. Wild type and mutated Egr1/Sp1 sequences are shown.
817 Luciferase activities of wild type and mutated 428-h*AMHR2* promoters were measured in L β T2
818 cells stimulated or not with GnRH α for 4 h. Data are the mean \pm SEM of four independent
819 experiments performed in triplicate and expressed as percent of unstimulated wild type
820 promoter activity (control). **, $P \leq 0.01$ compared with control (two-way ANOVA test). (B)
821 Knockdown of Egr1. L β T2 cells were transiently transfected for 48 h with the 2252-bp
822 h*AMHR2* promoter, together with 75 nM siRNA targeting mouse *Egr1* (siRNA Egr1) or 75
823 nM nontargeting siRNA (siRNA NT) and then stimulated or not with 10 nM GnRH α for 4 h.
824 qRT-PCR analysis confirmed that Egr1 siRNAs significantly reduced Egr1 transcript levels in
825 L β T2 cells ($55 \pm 5\%$ of siRNA NT-transfected cells after 24 h transfection, data not shown).
826 This decrease is probably underestimated as Egr1 mRNAs were measured from the whole
827 population of cells while only a subpopulation was transfected with the siRNA. Luciferase
828 activities are shown as percent of unstimulated siRNA NT-transfected cells (control). Data are

829 the mean \pm SEM of four independent experiments performed in triplicate. *, $P \leq 0.05$; **, $P \leq$
830 0.01 compared with control cells (two-way ANOVA test); α , $P \leq 0.001$ compared with GnRH
831 stimulated, siRNA NT-transfected cells (unpaired Student's t test).

832

833 **Figure 5: FOXO1 represses basal and GnRH-dependent activities of hAMHR2 promoter.**

834 (A) Effect of a constitutively active mutant of FOXO1 on GnRH stimulatory effect. The 2252-
835 hAMHR2 was transiently transfected in L β T2 cells together with FOXO1 wild type (FOXO1-
836 Wt), constitutively active mutant of FOXO1 (FOXO1-CAM) or with empty pcDNA3
837 (pcDNA3) vectors. Cells were then stimulated or not with GnRH α for 4 h. Luciferase activities
838 were measured 48 h later and expressed as percent of unstimulated pcDNA3 transfected cells
839 (control). Data are the mean \pm SEM of five independent experiments performed in triplicate.
840 **, $P \leq 0.01$ compared with control (two-way ANOVA test). (B) Effect of PI3K
841 pharmacological inhibition on GnRH effect. L β T2 cells expressing the 2252-hAMHR2
842 promoter were stimulated or not with GnRH α for 4 h in presence or absence of a PI3K specific
843 inhibitor (LY294002, 20 μ M). Luciferase activities were expressed as percent of unstimulated
844 promoter activity (control). Data are the mean \pm SEM at five independent experiments
845 performed in triplicate. **, $P \leq 0.01$ compared with control. GnRH induction of hAMHR2
846 activity is significantly reduced by 48 \pm 8% in presence of LY294002 (*inset*, unpaired Student's
847 t test). (C) Mutation of the FOXO response element in hAMHR2 promoter. A 428-hAMHR2
848 promoter with mutation of the FOXO motif was designed as described in *Materials and*
849 *Methods*. Wild type (FOXO-Wt) and mutated FOXO sequences extending from -120/-96 bp
850 are shown. Luciferase activities of wild type and mutated hAMHR2 promoters were measured
851 in L β T2 cells stimulated or not with GnRH α for 4 h. Data are the mean \pm SEM of six
852 independent experiments performed in triplicate and shown as percent of unstimulated FOXO-
853 Wt transfected cells (control). **, $P \leq 0.01$ compared with control (two-way ANOVA test).

854

855 **Figure 6: SF1 contributes to basal and GnRH-stimulated hAMHR2 promoter activities**
856 **in L β T2 cells.**

857 (A) Effect of mutation of the two SF1 binding sites in hAMHR2 promoter. A mutant of the
858 2252-hAMHR2 promoter with mutation of both SF1-binding sites was designed as described
859 in *Materials and Methods*. Wild type and mutated SF1 sequences extending from -276/-256
860 and -216/-196 bp are shown. Luciferase activities of wild type and SF1-mutated hAMHR2
861 promoters were measured in L β T2 cells stimulated or not with GnRH α for 4 h and expressed

862 as percent of unstimulated wild type promoter activity (control). Data are the mean \pm SEM of
863 six independent experiments performed in triplicate. *, $P \leq 0.05$; ***, $P \leq 0.001$ compared with
864 control (two-way ANOVA test). (B) Knockdown of SF1. L β T2 cells were transiently
865 transfected during 48 h with the 2252-bp hAMHR2 promoter together with 75 nM siRNA
866 targeting mouse *Nr5a1* (siRNA SF1) or 75 nM nontargeting siRNA (siRNA NT) and then
867 stimulated or not with GnRH α for 4 h. Luciferase activities are shown as percent of
868 unstimulated siRNA NT-transfected cells (control). Data are the mean \pm SEM of four
869 independent experiments performed in triplicate. *, $P \leq 0.05$; **, $P \leq 0.01$ compared with
870 control cells; a, $P \leq 0.001$ compared with GnRH stimulated, siRNA NT-transfected cells (two-
871 way ANOVA test).

872

873 **Figure 7: β -catenin is involved in GnRH stimulation of hAMHR2.**

874 (A) Mutation of the TCF binding sites in the hAMHR2 promoter. Mutants of the 428-hAMHR2
875 promoter with mutations of one or both TCF motifs were designed as described in *Materials*
876 *and Methods*. Wild type and mutated TCF sequences extending from -259/-242 bp and -187/-
877 169 bp are shown. Luciferase activities of wild type and mutated hAMHR2 promoters were
878 measured in L β T2 cells stimulated or not with GnRH α for 4 h. Data are the mean \pm SEM of
879 five independent experiments performed in triplicate and expressed as percent of unstimulated
880 wild type promoter activity (control). **, $P \leq 0.01$ compared with control (two-way ANOVA
881 test). (B) Knockdown of β -Catenin. L β T2 cells were transiently transfected for 48 h with the
882 2252-bp hAMHR2 promoter together with 75 nM siRNA targeting mouse *Ctnnb1* (siRNA β -
883 catenin) or 75 nM nontargeting siRNA (siRNA NT) and then stimulated or not with GnRH α
884 for 4 h. Luciferase activities are shown as percent of unstimulated siRNA NT-transfected cells
885 (control). Data are the mean \pm SEM of four independent experiments performed in triplicate.
886 *, $P \leq 0.05$ compared with control cells (two-way ANOVA test). GnRH induction of hAMHR2
887 is significantly reduced by $48 \pm 17\%$ in presence of siRNA β -catenin (*inset*, unpaired Student's
888 *t* test). (C) Effects of pharmacological inhibition of β -catenin, PKC and JNK signaling
889 pathways on GnRH effect. L β T2 cells were transiently transfected with the 2252-bp hAMHR2
890 promoter and then stimulated or not with GnRH α for 4 h in absence or presence of 10 μ M
891 JW74 (β -catenin pathway inhibitor), 2 μ M GF109203X (GF109, PKC inhibitor) or 20 μ M
892 SP600125 (SP6, JNK inhibitor). Luciferase activities are shown as percent of promoter activity
893 in absence of any treatment (control). Data are the mean \pm SEM of at least five independent

894 experiments performed in triplicate. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ compared with
895 control cells (two-way ANOVA test).

896

897 **Figure 8: Schematic hypothetical model for GnRH induction of human *AMHR2* promoter**
898 **activity.**

899 This schematic drawing illustrates the location of binding sites (in red) and related transcription
900 factors involved in the regulation of *AMHR2* promoter in gonadotrope cells. In the insets are
901 illustrated the levels of Egr1 and Sp1 transcripts (expressed relative to basal Sp1 level) in L β T2
902 cells stimulated or not by GnRH. **(A)** The constitutive activity of h*AMHR2* requires intact TCF,
903 SF1 and overlapping Sp1/Egr1 binding sites. Egr1 is only poorly expressed as compared to
904 Sp1 in basal condition (inset), the transcription factor occupying the Sp1/Egr1 binding site is
905 thus probably Sp1. Significant levels of TCF4 transcripts in L β T2 cells suggest that TCF4
906 transcription factor contributes to basal promoter activity through binding to TCF site. In
907 addition, FOXO1 exerts a repressive action on the promoter, possibly by counteracting
908 stimulatory transcription factors. **(B)** GnRH induces high levels of Egr1 while it does not affect
909 Sp1 transcripts (inset). Egr1 binding to the promoter thus probably leads to the competitive
910 displacement of Sp1. The induction of Egr1 may enable the formation of a transcriptional
911 complex containing β -catenin and SF1 that cooperate with Egr1 to fully activate the h*AMHR2*
912 promoter. The export of FOXO1 from nucleus induced by GnRH also contributes to the
913 transactivation of the promoter.

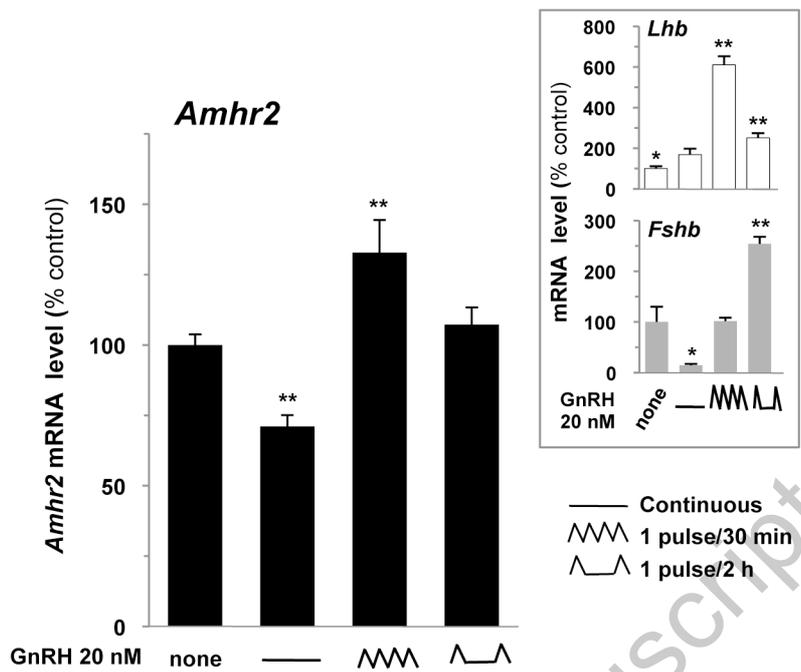


Figure 1

Accepted manuscript

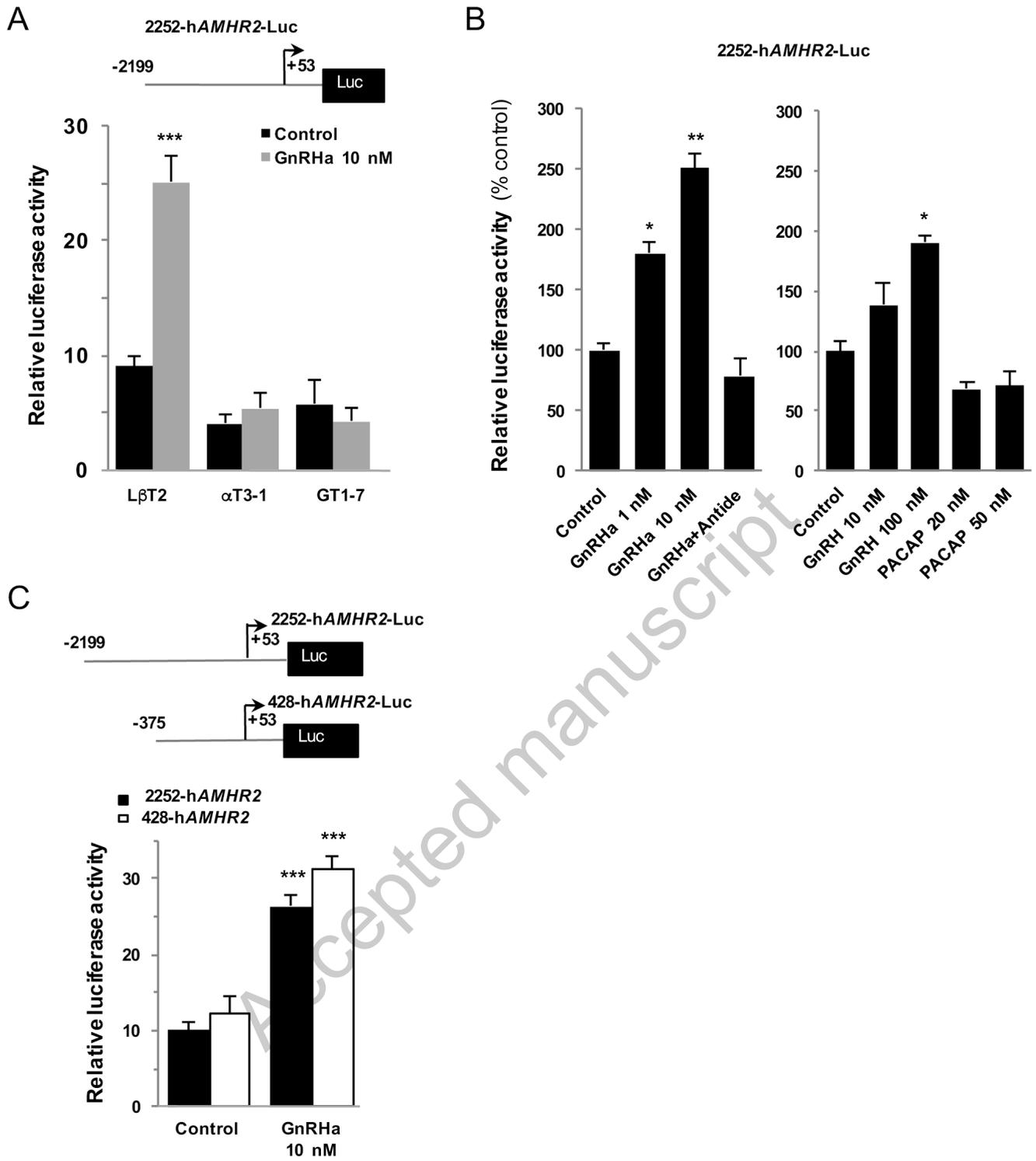
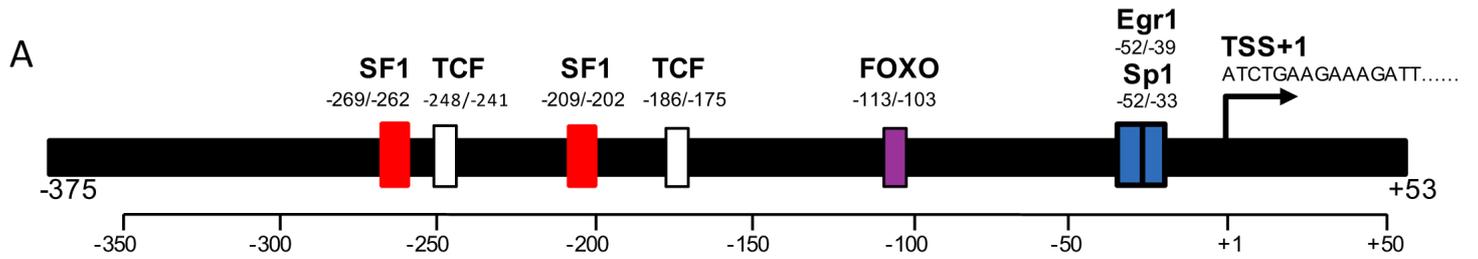


Figure 2



B

-375 AGATCTTAGGCTAGAAAGGATCTTGGGCAGAGCTGAATGGCTCTAAGCATTGACCTCACATTGGTTTCCTCCTG -301

-269 **SF1** -262 -248 **TCF** -241

-300 AGAAGAGTCAACAGAGTCCAGCATCTTCTTC CAAGGTCA GGAAGGGCAAAG ATTTGAAA TTAGGCATCACTGGG -226

-209 **SF1** -202 -186 **TCF** -175

-225 TTCTCAGCTGGGCTC CAAGTTCC CCTCCTCTCACTATG CAGAGCAAAGAG GAGGTTGCAGAAGAGAAGAGGATA -151

-113 **FOXO** -103

-150 TGAGATTGGTTCTCTGCTCCTCCCTTTCTTTCCCTGC TTTCCCCACAC CCACCCTCTCCCCACAGCAGCCTTCT -76

-52 **Egr1** -39 / -52 **Sp1** -33

-75 CCCCCACAGAGGCTGGGATAGGA TGAGGGGGCGGAGTTGGGG ACTGAGGGATCAGAAGCCCCAGGATGCCCTGT -1

+1 ATCTGAAGAAAGATTTGGCCAGGGCAGCTGTGCTGGCTTATGCTCTTCTCCT +53

C

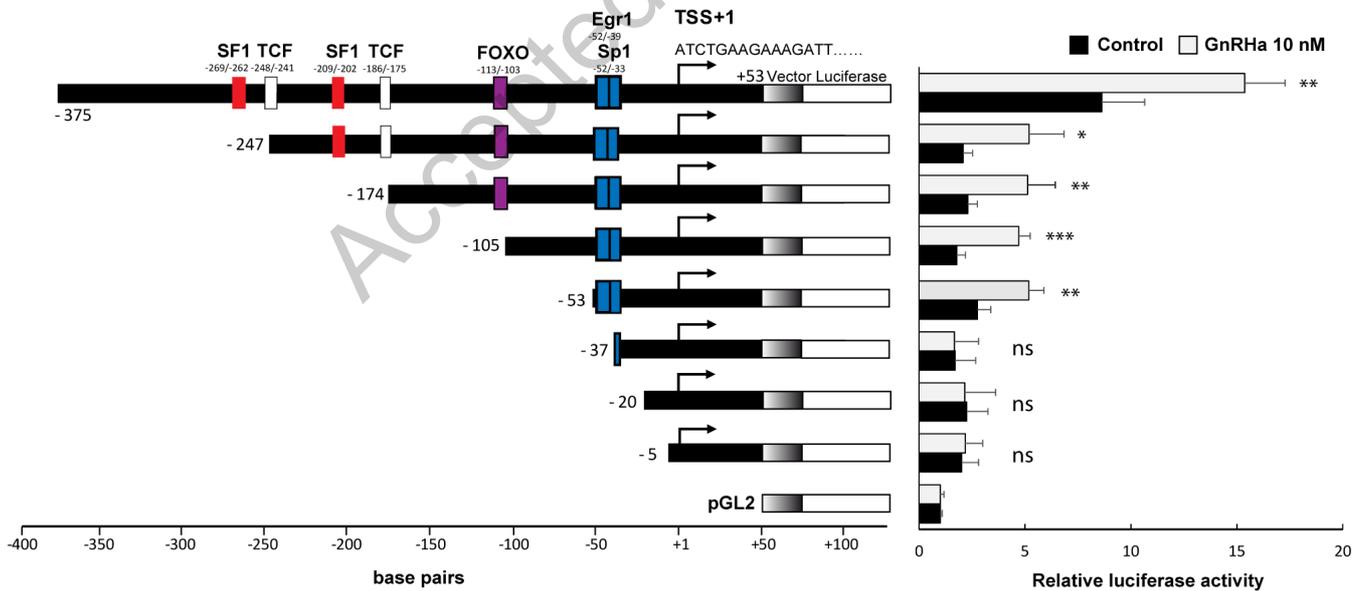
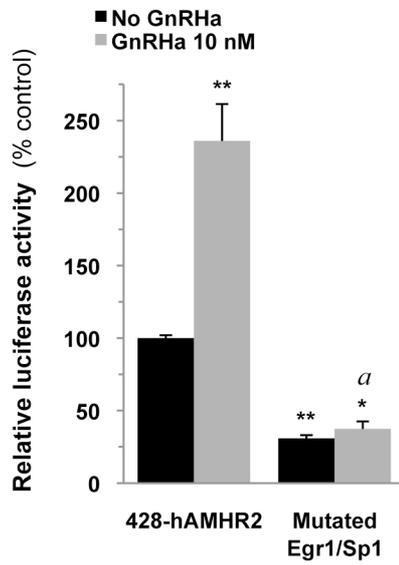


Figure 3

A

Egr1/Sp1-binding site

Wild type	-57 tagga TGAGGGGGCGGAG ttgggg -33
Mutated Egr1/Sp1	tagga TGAGGCATATGGAG ttgggg



B

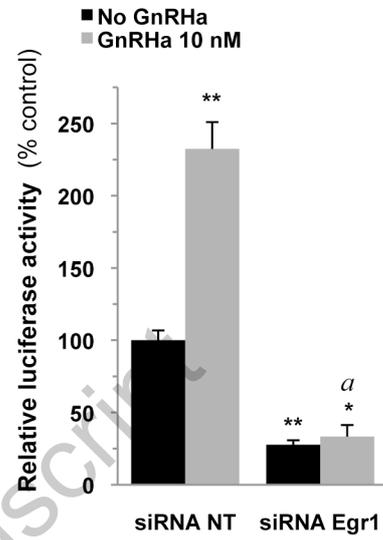
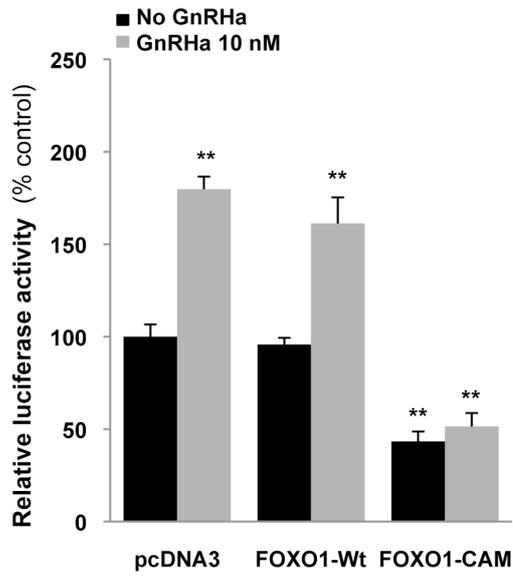
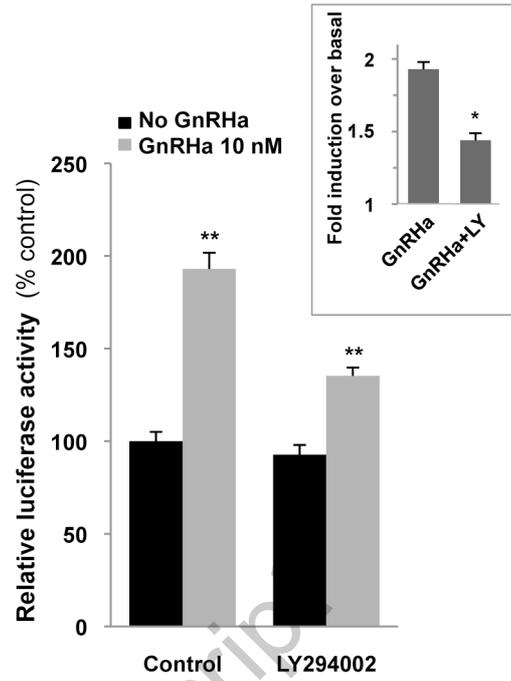


Figure 4

A



B



C

FOXO motif

Wild type	-120	tcctgc	TTTCCCCACAC	Ccaccct	-96
Mutated FOXO		tcctgc	TTCATATGCAC	Ccaccct	

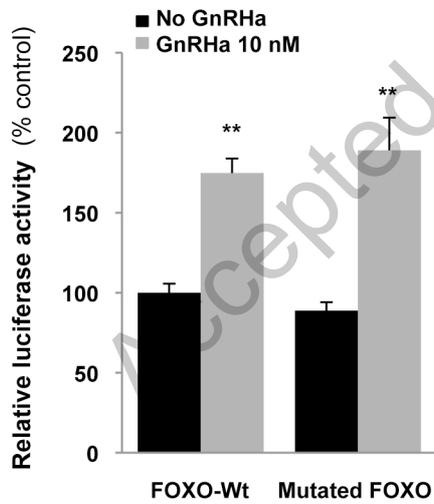
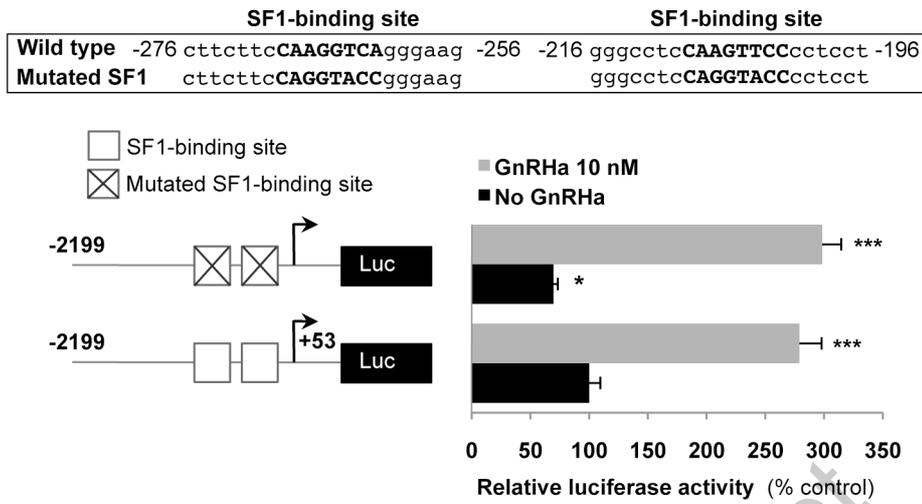


Figure 5

A



B

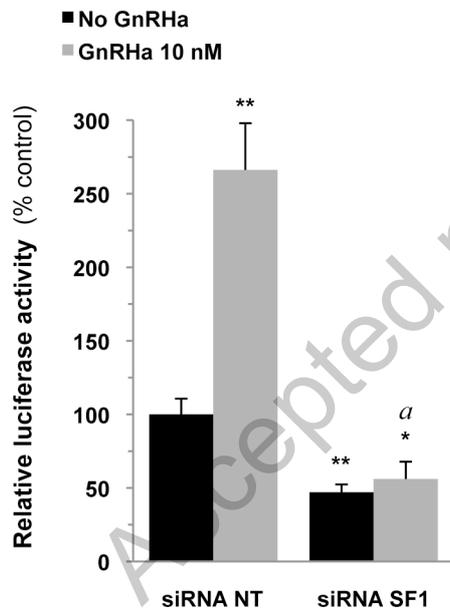
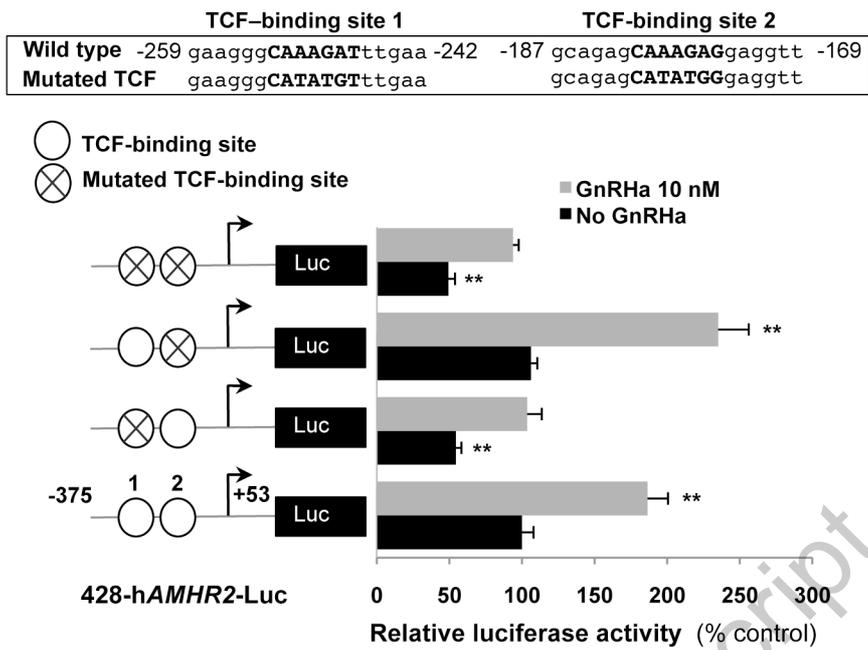
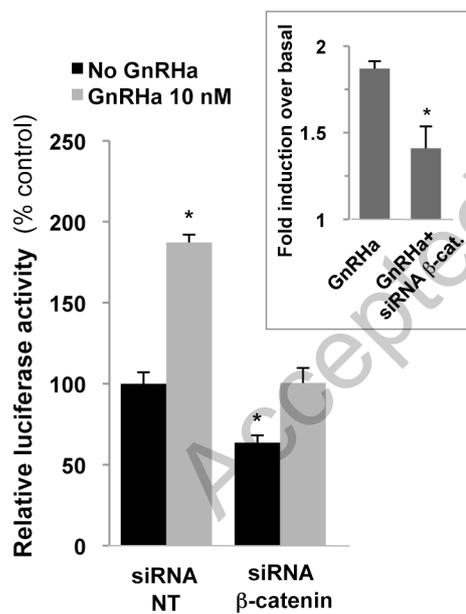


Figure 6

A



B



C

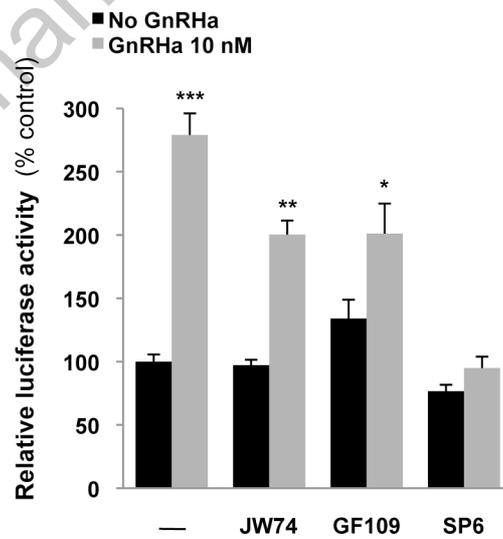


Figure 7

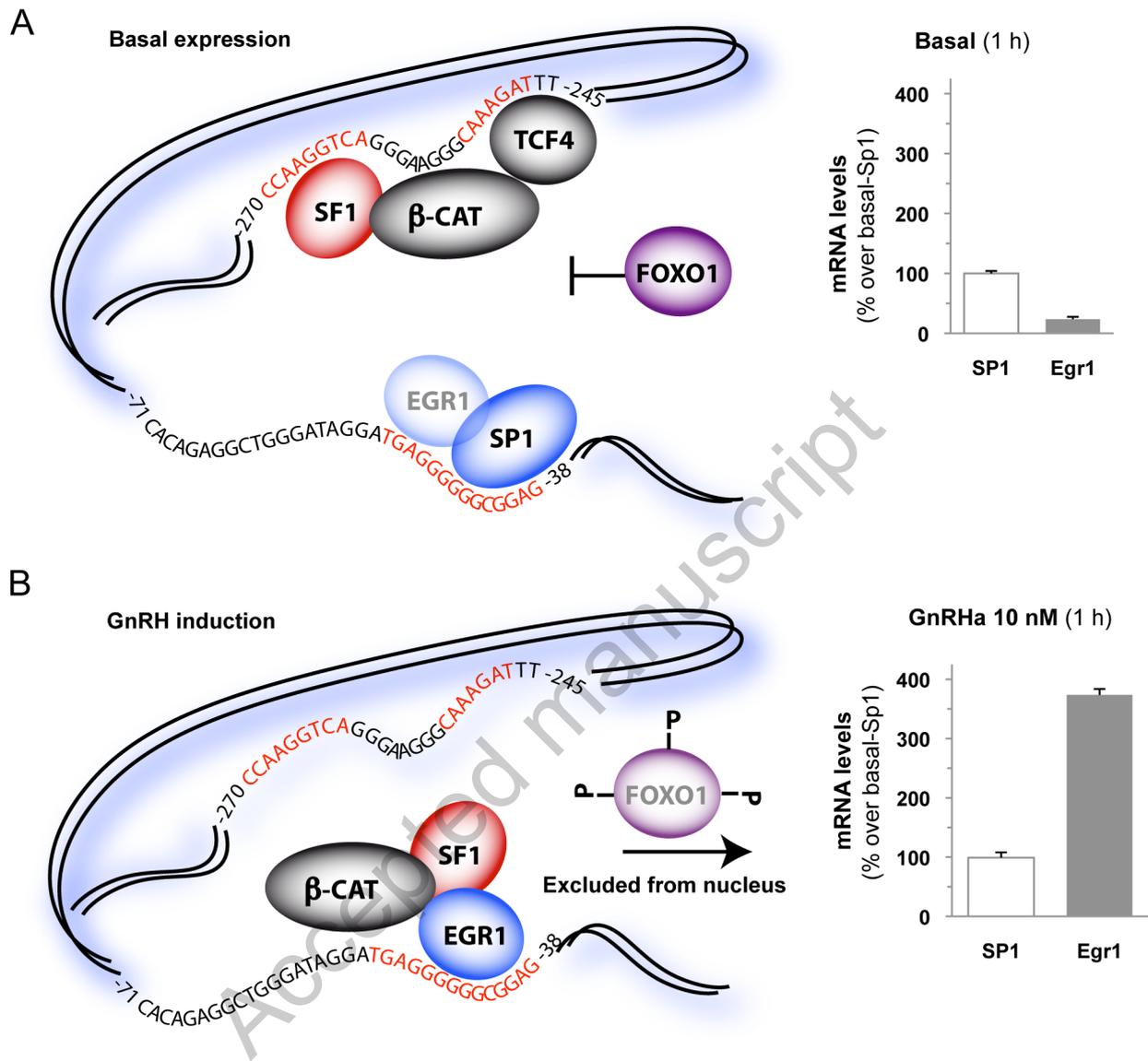


Figure 8

Table 1:**Sequences of the primers used for site-directed mutagenesis of 428-hAMHR2-luc**

Id primer	Sequence (5' > 3')	Restriction site
Common primers		
F1-AMH05	AGCTCG <u>AGATCT</u> TAGGCTAGA	BglII
R3-AMHRm	CCATCC <u>TCTAGA</u> GGATAGAA	XbaI
Primers used for 5' deletions		
AmhF-5	GCCCCA <u>AGATCT</u> CCTGTATCTGAAGAAAGATT	BglII
AmhF-20	ACTGAG <u>AGATCT</u> GAAGCCCCAGGATGCCCTGT	BglII
AmhF-37	GGGGGC <u>AGATCT</u> GGGGACTGAGGGATCAGA	BglII
AmhF-53	GGCTGG <u>AGATCT</u> ATGAGGGGGCGGAGTTG	BglII
AmhF-68	CTTCTC <u>AGATCT</u> AGAGGCTGGGATAGGATGA	BglII
AmhF-85	CTCTCC <u>AGATCT</u> GCAGCCTTCTCCCCACAGA	BglII
Primers used for targeted mutagenesis		
SF1 response element (-271/-262)		
Amhr07_R2	CTTCCCT <u>CATATG</u> GGGAAGAAGATGCTGGACTCT	NdeI
Amhr07_F2	TTCTTCC <u>CATATG</u> AGGGAAGGGCAAAGATTTGA	NdeI
TCF response elements (253/-247 ; -181/-175)		
R1-AMHR05	ATTTCAAA <u>CATATG</u> CCCTTCCCTGACCTTGGA	NdeI
F2-AMHR05	GAAGGG <u>CATATG</u> TTTGAATTAGGCATCACTG	NdeI
R2-AMHRm2	GCAACCTC <u>CATATG</u> GCTCTGCATAGTGAGA	NdeI
F3-AMHRm2	CAGAGC <u>CATATG</u> GAGGTTGCAGAAGAGA	NdeI
FOXO response element (-113/-103)		
R1- AMHR07	GTGGGTG <u>CATATG</u> AAGCAGGGAAAGAAAGGGA	NdeI
F1- AMHR07	CCTGCTT <u>CATATG</u> CACCCACCCTCTCCCCAC	NdeI
Egr1/Sp1 response element (-52/-39)		
AmEgr_F	GGATGAGG <u>CATATG</u> GAGTTGGGGACTGAGGGAT	NdeI
AmEgr_R	CCCAACTC <u>CATATG</u> CCTCATCCTATCCCAGCCT	NdeI

Table 2:**Sequences of the primers used for quantification of transcript levels in L β T2 cells.**

Cyclophilin is used as reference gene for real-time PCR. Unspecified species mean that the primers are functional for both rat and mouse.

Target cDNA	Forward primer (5'>3')	Reverse primer (5'>3')
Mouse <i>Amhr2</i>	CTGAGGTAGCTGTGCTGGTTTTTGC	GTGTCTTTGTGCTTCCCCGCACT
<i>Lhb</i>	ATCACCTTCACCACCAGCAT	GACCCCCACAGTCAGAGCTA
Mouse <i>Fshb</i>	GACAGCTGACTGCACAGGAC	CAATCTTACGGTCTCGTATACC
Mouse <i>Ctnnb1</i> (β -catenin)	CCCAGTCCTTCACGCAAGAG	CATCTAGCGTCTCAGGGAACA
Cyclophilin	CAAAGTTCCAAAGACAGCAG	CTGGCACATGAATCCTGGAA
<i>Nr5a1</i> (SF1)	TTACACGTGCACCGAGAGTC	TGCTTTCCTTGCTGCTTCA
<i>Egr1</i>	AGCAGCGCTTTCAATCCTCA	GTCTCCACCAGCGCCTTCTC
Mouse <i>Sp1</i>	CTCCAGACCATTAACCTCAGTG	ACCAGATCCATGAAGACCAAG

Accepted manuscript