



HAL
open science

A novel mutation in the NR3C1 gene associated with reversible glucocorticoid resistance

Margaux Laulhé, Emmanuelle Kuhn, Jérôme Bouligand, Larbi Amazit, Julie Perrot, Elise Lebigot, Peter Kamenický, Marc Lombès, Jérôme Fagart, Say Viengchareun, et al.

► To cite this version:

Margaux Laulhé, Emmanuelle Kuhn, Jérôme Bouligand, Larbi Amazit, Julie Perrot, et al.. A novel mutation in the NR3C1 gene associated with reversible glucocorticoid resistance. *European Journal of Endocrinology*, 2024, 190 (4), pp.284-295. 10.1093/ejendo/lvae031 . hal-04566600

HAL Id: hal-04566600

<https://hal.science/hal-04566600v1>

Submitted on 2 May 2024

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.

A novel mutation in the *NR3C1* gene associated with reversible glucocorticoid resistance

Margaux Laulhe¹, Emmanuelle Kuhn², Jérôme Bouligand^{1,3}, Larbi Amazit⁴, Julie Perrot¹, Elise Lebigot⁵, Peter Kamenicky^{7,6}, Marc Lombès¹, Jérôme Fagart⁷, Say Viengchareun^{1*}, Laetitia Martinerie^{1,8,9*}.

¹ Université Paris-Saclay, Inserm, Physiologie et Physiopathologie Endocriniennes, 94276 Le Kremlin-Bicêtre, France

² Service d'Endocrinologie et Métabolisme, Hôpital Pitié-Salpêtrière GHU APHP Sorbonne Université, 75013 Paris, France

³ Service de Génétique Moléculaire, Pharmacogénétique et Hormonologie, Hôpital Bicêtre GHU APHP Paris Saclay, 94270, Le Kremlin Bicêtre, France

⁴ UMS 44/ Institut Biomédical du Val de Bièvre, Université Paris-Saclay, 94276 Le Kremlin Bicêtre, France

⁵ Service de Biochimie, Hôpital Bicêtre GHU APHP Paris Saclay, 94270, Le Kremlin Bicêtre, France

⁶ Service d'Endocrinologie et des Maladies de la Reproduction, Hôpital Bicêtre GHU APHP Paris Saclay, 94270 Le Kremlin-Bicêtre, France

⁷ CNRS UMR 7654, Ecole Polytechnique, 91120 Palaiseau, France

⁸ Endocrinologie Pédiatrique, Centre de Référence Maladies Endocriniennes Rares de la Croissance et du Développement, Hôpital Universitaire Robert-Debré GHU APHP Nord, 75019 Paris, France

⁹ Université Paris Cité, Faculté de Santé, UFR de Médecine, Paris, France.

*These authors contributed equally

Corresponding authors:

Pr Laetitia Martinerie, MD, PhD
Pediatric Endocrinology Department
CHU Robert Debré
49, boulevard Sérurier
75019, Paris
laetitia.martinerie@aphp.fr

AND

Dr Say Viengchareun, PhD
Inserm U1185
Faculté de Médecine Paris-Saclay
Bâtiment Recherche
63 rue Gabriel Péri,
94276 Le Kremlin-Bicêtre cedex, France
say.viengchareun@universite-paris-saclay.fr

Short Title: Reversible glucocorticoid resistance: case report

Keywords: adrenal adenoma, glucocorticoid resistance syndrome, glucocorticoid receptor, *NR3C1* gene.

Word Count: 4511

45 **Abstract**

46 **Objective** Glucocorticoid resistance is a rare endocrine disease caused by variants of
47 the *NR3C1* gene encoding the Glucocorticoid Receptor (GR). We identified a novel
48 heterozygous variant (GR_{R569Q}) in a patient with uncommon reversible glucocorticoid
49 resistance syndrome.

50 **Methods** We performed *ex vivo* functional characterization of the variant in patient
51 fibroblasts and *in vitro* through transient transfection in undifferentiated HEK 293T cells
52 to assess transcriptional activity, affinity, and nuclear translocation. We studied the
53 impact of the variant on tertiary structure of the Ligand Binding Domain (LBD) through
54 3D modeling.

55 **Results** The patient presented initially with an adrenal adenoma with mild autonomous
56 cortisol secretion and undetectable ACTH levels. Six months after surgery, biological
57 investigations showed elevated cortisol and ACTH (UFC 114 µg/24h, ACTH 10.9
58 pmol/L) without clinical symptoms, evoking glucocorticoid resistance syndrome.
59 Functional characterization of the GR_{R569Q} showed decreased expression of target
60 genes (in response to 100 nM cortisol: *SGK1* control +97% vs. patient +20%, $P <$
61 0.0001) and impaired nuclear translocation in patient fibroblasts compared to control.
62 Similar observations were made in transiently transfected cells, but higher cortisol
63 concentrations overcame glucocorticoid resistance. GR_{R569Q} showed lower ligand
64 affinity (Kd GR_{WT}: 1.73 nM vs. GR_{R569Q}: 4.61 nM). Tertiary structure modeling
65 suggested a loss of hydrogen bonds between H3 and the H1-H3 loop.

66 **Conclusion** This is the first description of a reversible glucocorticoid resistance
67 syndrome with effective negative feedback on corticotroph cells regarding increased
68 plasma cortisol concentrations due to the development of mild autonomous cortisol
69 secretion.

70 **Significance Statement:**

71 We described the original case of a patient with reversible glucocorticoid resistance
72 syndrome, initially masked by adrenal adenoma with mild autonomous cortisol
73 secretion. We characterized a novel heterozygous variant (GR_{R569Q}) of the *NR3C1*
74 gene, located within the Ligand Binding Domain of the Glucocorticoid Receptor (GR),
75 responsible for a decrease in GR affinity and transactivation activity. The negative
76 feedback of GR on the pituitary gland was restored with high cortisol concentrations
77 despite the absence of clinical symptoms of hypercortisolism, reinforcing knowledge of
78 the tissue specificity of glucocorticoid actions. Furthermore, our results suggest that
79 glucocorticoid resistance syndrome should be considered in patients with adrenal
80 incidentalomas, particularly in cases of rapid post-surgical recovery of the HPA axis.

81

82 Introduction

83 Glucocorticoids are steroid hormones secreted according to a circadian rhythm and in
84 response to acute stress factors to maintain homeostasis (1,2). Glucocorticoid
85 secretion is controlled by the Hypothalamic-Pituitary-Adrenal axis (HPA axis). The HPA
86 axis is composed of Corticotropin-Releasing Hormone (CRH)-secreting neurons in the
87 hypothalamus and corticotroph cells in the pituitary gland, secreting the
88 Adrenocorticotropin Hormone (ACTH), which stimulates the synthesis of
89 glucocorticoids in the adrenal glands. Glucocorticoids exert their functions through
90 activation of the Glucocorticoid Receptor (GR). The GR, encoded by the *NR3C1* gene
91 (3,4), is a nuclear receptor composed of three functional domains: the N-terminal
92 domain (NTD), the DNA-Binding Domain (DBD), and the Ligand-Binding Domain
93 (LBD). The glucocorticoid-bound GR is released from a multi-protein complex of heat
94 shock proteins (Hsp90, Hsp70) and immunophilins (FKBP51, FKBP52) and
95 translocated to the nucleus (3,5). Dimeric GR binds DNA to regulate the expression of
96 target genes, such as *SGK1* or *TSC22D3*. The GR also acts as a monomer without
97 binding directly to DNA but through interaction with other transcription factors such as
98 NFκB (6).

99 Glucocorticoid resistance syndrome (7) is characterized by biological hypercortisolism
100 without clinical symptoms of Cushing syndrome and the absence of negative feedback
101 on the HPA axis (8). Chronic ACTH excess increases adrenal steroid secretion,
102 producing androgenic and/or mineralocorticoid effects (9,10). Phenotypes of patients
103 with glucocorticoid resistance may vary from asymptomatic to clinical manifestations
104 of excess mineralocorticoids (hypertension, hypokalemia) or androgens (hirsutism,
105 hypo fertility) (7,11). For example, systolic hypertension and hypokalemia due to

106 mineralocorticoid receptor activation in excess and elevated serum ACTH due to a lack
107 of negative feedback on corticotroph cells were described for the first time in 1976 (10).
108 This syndrome is generally caused by heterozygous loss-of-function variants of the
109 *NR3C1* gene. To date, more than 30 different variants responsible for glucocorticoid
110 resistance have been reported (7,12). The resulting receptors presented a decreased
111 affinity for ligands (13–16) or exerted a dominant negative effect on the wild-type
112 receptor (17). In addition, abnormalities in nuclear translocation or reduced DNA
113 binding have also been described (18,19). Here, we present the case of a man with
114 glucocorticoid resistance syndrome, initially masked by an adrenal incidentaloma with
115 mild autonomous cortisol secretion that efficiently repressed ACTH secretion (20).
116 Genetic explorations revealed a heterozygous variant of the *NR3C1* gene located in
117 the LBD.

118

119 **Materials and Methods**

120 **Ethics**

121 The patient gave written informed consent for all genetic analyses, skin biopsy,
122 fibroblast cultures, characterization of the mutation, and publication of the results.

123 **Clinical and biochemical parameters**

124 Clinical features, including CT-scan, MRI, clinical examination, biochemical
125 parameters, and hormonal explorations, were collected from the patient's medical
126 records during his hospital stays. Hormonal assessments (Cortisol and ACTH) were
127 performed using immunochemiluminescence (Roche Cobas).

128 **DNA analysis**

129 Genomic DNA was extracted from the patient's whole blood using standard methods
130 (QIAAsymphony, QIAGEN, Hilden city, Germany). The coding regions of hGR α and
131 hGR β genes were amplified and sequenced as previously described (18).

132 **Cell Culture**

133 Human HEK 293T cells were cultured at 37°C with 5% CO₂ in DMEM (Life
134 Technologies, Villebon-sur-Yvette, France) with 20 mM HEPES (Life Technologies),
135 100 UI/ml penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies)
136 and 10% of fetal bovine serum (Biowest, Nuaille, France).

137 Fibroblasts were cultured at 37°C with 5% CO₂ in DMEM with high glucose
138 concentration (4.5 g/L), and the addition of 20 mM HEPES, 100 UI/ml penicillin,

139 100 µg/ml streptomycin, and plasmocin (5 mg/L) (InvivoGen, Toulouse, France) and
140 15% of fetal bovine serum.

141 **Site-directed mutagenesis**

142 Plasmids pcDNA3-hGR α -R569Q (c.1706G>A, p.R569Q), pcDNA3-hGR α -R569K
143 (c.1705_1706delCGinsAA, p.R569K) were obtained using the QuickChange Site-
144 Directed Mutagenesis kit (Stratagene, La Jolla, CA) with pcDNA3-hGR α
145 (NM_000176.2) as a template.

146 **Transactivation assay**

147 HEK 293T cells were transfected with plasmids expressing wild-type (WT;
148 pcDNA3hGR α) or mutated GR (pcDNA3hGR α R569Q; 40 ng/well of 96-well plates), a
149 reporter MMTV-luciferase plasmid (40 ng/well) and pMIR β -galactosidase (β -gal)
150 encoding the β -gal used to normalize luciferase activity (35 ng/well), using
151 Lipofectamine 2000 (Life Technologies) as previously described (21). Six hours after
152 transfection, the medium was replaced by a steroid-free medium. Twenty-four hours
153 post-transfection, cells were treated with vehicle or increasing cortisol concentrations
154 (0.1 to 100 nM). As previously described, Luciferase and β -gal activities were
155 measured from cell lysates (18). Transactivation activity is expressed relative to the
156 maximum transactivation activity obtained for GR_{WT} with 100 nM agonist concentration,
157 arbitrarily set at 100%. EC₅₀ is obtained using nonlinear regression from 6 independent
158 experiments performed in 8 replicates. Results are expressed as median [Q1; Q2] of
159 at least 8 replicates from 6 independent experiments. Dominant negative effect is
160 expressed relative to the maximum transactivation activity obtained for GR_{WT} (30 ng)

161 with 100 nM agonist concentration, arbitrarily set at 100%. Results are expressed as
162 median [Q1; Q2] of at least 8 replicates from 2 independent experiments.

163 **RNA extraction and RT-qPCR**

164 Human fibroblasts were treated with vehicle or cortisol (100 nM) for 8 h. Total RNA
165 was extracted with the TRI Reagent® (Euromedex, Souffelweyersheim, France),
166 according to the manufacturer's recommendations. Reverse transcription and real-time
167 quantitative PCR (RT-qPCR) were performed as previously described (18). cDNA
168 samples were amplified by RT-qPCR using the Quant-Studio 6 flex system (Life
169 Technologies, Carlsbad, CA, USA). Relative expression in each sample was
170 normalized to the internal reference *36B4* mRNA values, with control condition values
171 arbitrarily set at 1. Results are expressed as mean±SEM (after confirming normal
172 distribution with the d'Agostino-Pearson normality test) of at least 6 independent
173 samples from 2 independent experiments performed in duplicates. Primers used for
174 RT-qPCR are listed in Supplemental Data 4.

175 **Western blot analysis**

176 As previously described, total protein extracts were prepared from cells lysed at 4°C
177 (22). Immunoblots were incubated overnight at 4°C with an anti-GR antibody (sc-
178 393232, Santa Cruz, Heidelberg, Germany dilution 1/1000) with the loading control (β -
179 actin; #A2066-.2ML, Sigma, St Quentin-Falavier, France, dilution 1/5000). Membranes
180 were incubated at room temperature for 45 min with secondary antibodies (Pierce;
181 Dyelight 680 #35518 or 800 #35571, Life Technologies). Bands were visualized and
182 quantified using Odyssey®Fc, Dual-Mode Western Imager (Li-Cor, Lincoln, NE).
183 Results are expressed as median [Q1; Q3] of 3 to 6 independent samples from 3
184 independent experiments.

185 **Hydrocortisone Binding and Scatchard plot diagram**

186 *In vitro*-translated GR_{WT} and GR_{R569Q} were prepared using the rabbit reticulocyte lysate
187 method with the TnT-T7 Quick Coupled Transcription/Translation kit (Promega,
188 Charbonnieres-les-Bains, France) as previously described (23). Lysates were diluted
189 4-fold in TEGW buffer (20 mM Tris-HCl, 1 mM EDTA, 10% glycerol (v/v), 20 mM
190 sodium tungstate pH 7.4) and incubated with increasing concentrations of [³H]-
191 Hydrocortisone (2590 GBq/mmol; Perkin Elmer, Villebon-sur-Yvette) for 4h at 4°C.
192 Bound and unbound hydrocortisone were separated using the dextran-charcoal
193 method (24). For an accurate calculation of the K_d values, non-specific binding (NS)
194 was calculated using the binding-saturation model of non-linear regression with one
195 specific site and nonspecific binding of Prism (version 10.0.2, GraphPad) and removed
196 from each point using the formula: $B = B_{\max} * X / (Kd + X) + NS * X$, where X is the ligand
197 concentration and B_{max} the number of binding sites. The K_d and B_{max} were then
198 calculated using the Lineweaver-Burk representation of the change in B_{specific}/Unbound
199 as a function of B_{specific}. Results are expressed as individual values representative
200 of 2 independent experiments.

201 **Immunohistochemistry, High-Throughput Microscopy (HTM) and 3D** 202 **deconvolution Microscopy.**

203 Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and
204 permeabilized for 30 min with a 0.5% solution of PBS-Triton X100. Cells were then
205 washed 3 times with PBS and incubated for 1 h at room temperature in PBS-Tween 20
206 0.1% (v/v) buffer containing 5% nonfat dry milk and consecutively incubated with a
207 mouse anti-GR antibody overnight at 4 °C (sc-393232, Santa Cruz), followed by
208 incubation with an Alexa Fluor 555 anti-mouse secondary antibody for 30 min at room

209 temperature (#A32727, Life Technologies). After antibody labeling, cells were
210 postfixed for 10 min with 4% paraformaldehyde, washed 3 times with PBS, and a
211 nuclear counterstaining was performed with 0.5 µg/ml DAPI. HTM Images were
212 acquired (20×/0.4 NA), analyzed, and quantified by the ArrayScan VTI imaging
213 platform (Thermo Fisher Scientific, Asnières-sur-Seine, France) as previously
214 described (30). Briefly, DAPI and GR fluorescence were captured using sequential
215 acquisition to give separate image files for each. The Molecular Translocation V4
216 Bioapplication algorithm (vHCS Scan, version 6.3.1, Build 6586) was used to quantify
217 the average value ratio of the nuclear intensity to the cytoplasmic intensity calculated
218 for each selected cell per well (n > 1000 cells).

219 Fluorescence deconvolution microscopy was performed with Image Pro Plus AMS
220 software (Media Cybernetics Inc, Marlow, UK) using a Mono Q Imaging Retiga 2000R
221 Fast 1394 camera (Q Imaging Inc., Surrey, British Columbia, Canada). Cells were
222 observed and acquired with an automated upright BX61 microscope (Olympus,
223 Rungis, France) at 40X or 60X objective lens (1.4 NA). A z-series of focal planes were
224 digitally imaged and deconvolved with the 3D blind iterative algorithm (Image Pro Plus
225 AMS) to generate high-resolution images. The 3D datasets were flattened in 2D
226 images by applying a Maximum Intensity Projection Method.

227 **Statistical Analyses**

228 Data are expressed as mean ± SEM when normal distribution was validated using the
229 d'Agostino-Pearson test. Data are described as median and interquartile [Q1; Q3] if
230 they do not follow a normal distribution. All experiments have been performed at least
231 two times independently. We used a non-parametric Mann-Whitney test to compare
232 two variables and a Kruskal-Wallis test to compare more than two variables. For

233 grouped analysis, we used multiple Mann-Whitney tests or Two-Way ANOVA test, with
234 a Bonferroni correction, when the distribution of the variable was normal (GraphPad
235 Prism 10.0.1, San Diego, CA). *P* values less than 0.05 were considered statistically
236 significant. When independent experiments were pooled, the *P* value was adjusted
237 using the Bonferroni correction.

238 **Results**

239 **Clinical Presentation**

240 A 45-year-old man was seen in the Endocrinology Department for diagnosis of an
241 adrenal incidentaloma, measuring 4 cm and with low density (<10 HU) on the CT scan,
242 as shown in Figure 1A. The CT scan was performed for the exploration of prolonged
243 cough in the context of chronic tobacco use. The patient had been diagnosed with type
244 1 diabetes for 13 years; he displayed no complications despite a slightly elevated
245 HbA1c of around 8.5%. He had no arterial hypertension, his weight was stable, and he
246 did not display any other clinical features of Cushing syndrome. Hormonal
247 investigations revealed cortisol-secreting adenoma: 24 h urinary free cortisol (UFC)
248 was increased at 111 [106;116] µg/24 h (median of 2 independent assessments, $N <$
249 50 µg/24 h, Figure 1B) and ACTH was < 2.6 pmol/L (equivalent to undetectable by the
250 assay, Figure 1C). Plasma cortisol remained elevated after the 1 mg Dexamethasone
251 Suppression Test (DST) at 96 nmol/L ($N \leq 50$ nmol/L, (25)). We did not find evidence
252 for differential diagnosis (24 h urinary metanephrines were below normal ranges, and
253 plasma potassium concentration was within the normal range). Given the discrepancy
254 between clinical and biochemical parameters, they were controlled after four months.
255 At this point, UFC was further increased (286.5 [265;308] µg/24 h), and cortisol
256 nycthemeral rhythm was altered with elevated midnight plasma cortisol (158 nmol/L,

257 N < 50 nmol/L, Figure 1D); however, the patient still presented no clinical symptoms of
258 Cushing syndrome. He was diagnosed with mild autonomous cortisol secretion
259 (8,20,26) and, due to elevated cardiovascular risk, underwent laparoscopic surgery for
260 unilateral adrenalectomy one month later (27). Pathological analysis was consistent
261 with an adrenocortical adenoma measuring 40 × 27 mm with a Weiss score of 0. On
262 the first day after surgery, plasma cortisol levels were low (49.6 nmol/L). The patient
263 was supplemented with hydrocortisone at 20 mg/day (8). The supplementation was
264 discontinued one month after, following a positive response to the Standard Synacthen
265 Test (plasma cortisol rose from T0: 485 nmol/L to T60: 662 nmol/L). Approximately six
266 months later, the patient described asthenia, anxiety, and sleep disorders. Clinical
267 examination retrieved no symptoms of Cushing syndrome, and the abdominal CT scan
268 found no contralateral adrenal mass. Diabetes equilibrium was stable (HbA1c 8%).
269 New biochemical explorations revealed an elevation in the 24 h UFC (114
270 [101;167] µg/24 h), with no negative feedback on ACTH (10.9 [8.6;15.8] pmol/L). The
271 post-dexamethasone (1 mg) cortisol was unsuppressed (130 nmol/L), evoking an
272 ACTH-dependent hypercortisolism. MRI of the pituitary gland revealed no adenoma,
273 and there was no evidence of ectopic ACTH secretion (positive response to CRH and
274 desmopressin tests). Considering these results, we investigated glucocorticoid
275 resistance syndrome and found a novel *NR3C1* loss-of-function heterozygous variant
276 (Figure 2A). As the patient was asymptomatic, regular monitoring was decided.
277 However, there was no monitoring for two years due to lost follow-up. Four years after
278 surgery, in the context of persistent asthenia, a new hormonal assessment revealed
279 an elevated UFC (57 [38;76] µg/24 h) with unsuppressed ACTH (17.8 [11.6;24]
280 pmol/L). Circadian rhythm secretion of cortisol and ACTH was preserved, although with
281 elevated values (midnight cortisol 88 nmol/L). Interestingly, 100 mg hydrocortisone

282 injection efficiently repressed ACTH secretion (Supplemental Data 1). These results
283 demonstrated effective negative feedback in response to high plasma cortisol levels
284 (2000 nmol/L after 100 mg IV hydrocortisone), explaining the patient's initial
285 presentation of high cortisol levels associated with repressed ACTH levels. Thus, these
286 observations suggested reversible glucocorticoid resistance in corticotroph cells.

287 **Genetic Analysis**

288 Sanger sequencing of the *NR3C1* gene revealed a heterozygous variant c.1706 G>A
289 in exon 5 of *NR3C1* transcript NM_000176.3 (28) (Figure 2A, 2B). This yet unreported
290 variation results in the substitution of Arg569 to glutamine (p.Arg569Gln) in the LBD.
291 Unfortunately, exploration of the propositus family was not possible.

292 **Decreased GR function in patient fibroblasts**

293 We performed a cutaneous biopsy to obtain cultured patient fibroblasts to assess
294 glucocorticoid sensitivity *ex vivo*. In the patient fibroblasts, both GR transcripts were
295 expressed (Figure 2C), and total GR protein expression was not lower than in control
296 fibroblasts (Figure 2D, E), showing that this variant does not undergo proteasomal
297 degradation. We first studied the expression of GR target genes in response to cortisol.
298 As the patient reversible phenotype was observed with endogenous cortisol secretion,
299 we used cortisol in our experiments rather than the potent glucocorticoid agonist
300 dexamethasone. Hormone-induced *SGK1* mRNA expression in response to 100 nM
301 cortisol was lower in the patient fibroblasts compared to controls (Figure 3A, Control
302 1.88 ± 0.06 attomoles vs. Patient 1.08 ± 0.03 attomoles, $P < 0.0001$). Similarly,
303 expression of *TSC22D3* mRNA in response to 100 nM cortisol was lower in the patient
304 fibroblasts compared to control (Figure 3B, Control 15.02 ± 1.11 attomoles vs. Patient

305 11.59 ± 0.85 attomoles, $P = 0.0025$). As negative feedback on corticotroph cells is
306 based on transrepression, we studied *IL6* mRNA expression, which is negatively
307 regulated by GR through NFκB tethering (29). In basal condition, *IL6* mRNA expression
308 is repressed only in control fibroblasts (Figure 3C, Control 1.00 ± 0.06 vs. Patient 3.24
309 ± 0.43, $P < 0.0001$). In response to 100 nM cortisol, *IL6* mRNA expression is decreased
310 similarly in patient and control fibroblasts (Figure 3C, Control 0.20 ± 0.02 vs. Patient
311 0.40 ± 0.02, NS), suggesting a rescue of transrepression activity under high cortisol
312 concentrations. Further, as the GR LBD also contains a Nuclear Localization Signal
313 (NLS), we investigated the nuclear translocation of GR in fibroblasts. It is worth noting
314 that both the wild-type (GR_{WT}) and the variant receptor (GR_{R569Q}) are expressed in the
315 patient fibroblasts and detected by the same antibody (Figure 2C). Cells were
316 stimulated for one hour with increasing cortisol concentrations (from 0 to 100 nM).
317 Nuclear translocation was quantified using automated high-throughput microscopy by
318 measuring the nuclear-to-cytoplasmic ratio as previously described (30). As shown in
319 Figure 3D, GR_{WT} is exclusively nuclear when cells are stimulated with 10 nM cortisol.
320 In contrast, it was both nuclear and cytoplasmic in the patient fibroblasts (Figure 3E, at
321 10 nM Cortisol nuclear/cytoplasmic ratio Control 3.92 ± 0.07 vs. Patient 2.87 ± 0.16, P
322 < 0.0001). These data are consistent with a gene-specific decreased GR activity in
323 patient cells. These data confirmed that GR_{R569Q} activity is reduced in response to
324 cortisol, consecutively to a defect in nuclear translocation.

325 **The GR_{R569Q} is associated with a reduced affinity for cortisol.**

326 To assess the specific transcriptional activity of GR_{R569Q} compared to GR_{WT}, we
327 designed expression vectors comprising either GR_{R569Q} or GR_{WT} that we transiently
328 transfected in HEK 293T cells. We used HEK 293T cells because endogenous GR

329 expression is almost null. We first showed that GR_{R569Q} transactivated the MMTV-Luc
330 reporter gene in a dose-dependent manner, but with a higher half-maximal effective
331 concentration (EC₅₀) compared to the GR_{WT} (Figure 4A, EC₅₀ for GR_{WT}: 0.87
332 [0.30;0.95] nM vs. EC₅₀ for GR_{R569Q}: 1.91 [1.73;5.07], *P* = 0.0011). When we
333 transfected equal amount of GR_{WT} and GR_{R569Q}, we observed a dominant negative
334 effect on transcriptional activity in the presence of 10 nM cortisol (Figure 4B, GR_{WT}
335 70.50 [67.25;84.50] vs GR_{WT/R569Q} 45.50 [40;55.50], *P* < 0.001) that was rescued with
336 100 nM cortisol (Figure 4B, GR_{WT} 104.5 [92.75;109.75] vs GR_{WT/R569Q} 113.5
337 [100.75;131], *P* = 0.196). In transfected HEK 293T cells, GR_{R569Q} is localized in the
338 cytoplasm even at 10 nM cortisol compared to cells transfected with the GR_{WT}, where
339 GR is mainly nuclear. These results were confirmed by automated high-throughput
340 microscopy (Figure 4D, N/C at 10 nM cortisol for GR_{WT} 3.04 ± 0.04 vs N/C at 10 nM
341 cortisol for GR_{R569Q} 1.76 ± 0.04, *P* < 0.0001). At 100 nM cortisol, the
342 nuclear/cytoplasmic ratio appeared to be less different (Figure 4D, N/C at 100 nM
343 cortisol for GR_{WT} 3.98 ± 0.07 vs N/C at 100 nM cortisol for GR_{R569Q} 3.64 ± 0.07,
344 *P* < 0.0001). These results are consistent with reversible glucocorticoid resistance in
345 presence of high concentrations of cortisol. As the genetic variation is located within
346 the LBD, we hypothesized that a decreased affinity for cortisol may be responsible for
347 the phenotype. GR-expressing lysates were incubated with increasing concentrations
348 of [³H]-hydrocortisone, and the dissociation constants were measured at equilibrium.
349 As expected, GR_{R569Q} showed a reduced affinity for cortisol (Figure 4E, GR_{WT} 1.73 nM
350 (IC₉₅ 0.69;0.46) vs. GR_{R569Q} 4.61 nM (IC₉₅ 0.28;0.14), *P* < 0.0001).

351

352 **Three-dimensional model of the GR-LBD**

353 Arg569 is located within the helix H3 of the LBD, which is a determinant that allows
354 ligand binding (Figure 5A) (29). The crystal structure of the GR-LBD bound to
355 dexamethasone and chaperone proteins revealed that Arg569 establishes two strong
356 hydrogen bonds with the main carbonyl chain of Tyr545 and Ala546 located within the
357 loop connecting the Helix H1 to Helix H3 (PDB 7KRJ, Figure 5B) (5). As shown in
358 Figure 5C, substituting Arg569 with a shorter glutamine may result in the loss of these
359 hydrogen bonds that may destabilize the LBD structure even without a direct
360 interaction with the ligand. In the co-chaperones complex of the unliganded structure
361 of the GR-LBD, Arg569 also connects the H1-H3 loop at Ser551 and Val552 through
362 hydrogen bonds, whereas Gln569 is unable to maintain such bonds (Supplemental
363 Data 2). Interestingly, those amino acids are implicated in physiological glucocorticoid
364 resistance in Guinea Pig (32) through decreased GR ligand-binding activity *via*
365 interaction with FKBP51 and FKBP52 co-chaperone proteins. To determine whether
366 these modifications are responsible for the elevated EC_{50} observed with GR_{R569Q} , we
367 mutated Arg569 to a lysine (GR_{R569K}). This basic amino acid is closer to arginine and
368 may reestablish the hydrogen bonds. Our results showed that the reverse variant,
369 GR_{R569K} exhibited an improved transactivation capacity (Supplemental Data 3A and
370 3B, EC_{50} GR_{WT} 0.93 [0.89;1.00], GR_{R569Q} 2.10 [1.76;4.83], GR_{R569K} 1.60 [1.29;1.69],
371 $P=0.015$) but without complete reversion, suggesting a more complex function for
372 Arg569.

373

374

375 **Discussion**

376 We identified a novel missense variant within the GR LBD associated with reversible
377 glucocorticoid resistance. Primary glucocorticoid resistance syndrome is characterized
378 by hypercortisolemia with normal or elevated ACTH without corresponding clinical
379 signs of Cushing syndrome (7). In this case, the patient presented with an adrenal
380 adenoma with mild autonomous cortisol secretion. The association between adrenal
381 hyperplasia or adenoma and glucocorticoid resistance has already been described but
382 is usually associated with normal or elevated ACTH levels (18,22,31). HPA axis
383 stimulation, due to impaired negative feedback, can lead to adrenal hyperplasia, as
384 suggested in mice with GR haploinsufficiency (33). Further, the Muta-GR study
385 estimated *NR3C1* loss-of-function variants in 5 of 100 patients with adrenal hyperplasia
386 associated with either biological hypercortisolism and/or hypertension without clinical
387 symptoms of Cushing syndrome (34). The five patients presented with variants in the
388 DBD (GR_{R469X} and GR_{R477S}) and the LBD (GR_{R491S} , GR_{Q501H} , and GR_{L672P}). Among
389 these, four presented with biological evidence of glucocorticoid resistance. Some of
390 the variants were extensively described in different studies (18,22), showing either the
391 absence of DNA binding (GR_{R469X} and GR_{R477S}) or increased proteasomal degradation
392 (GR_{L672P}). The authors concluded that *NR3C1* variants should be investigated in
393 patients presenting with adrenal hyperplasia associated with low aldosterone and
394 potassium levels and biological hypercortisolism. Although our results reinforce the
395 hypothesis of elevated prevalence of *NR3C1* variants in adrenal hyperplasia, utilization
396 of those criteria would not have permitted to diagnose the patient as aldosterone levels
397 and kaliemia were both normal.

398 Other cases of glucocorticoid resistance have been described in association with
399 adrenal incidentaloma. The first case, published by Zhu *et al.*, was a 56-year-old
400 patient with a right adrenal incidentaloma with elevated ACTH and UFC levels
401 persisting after surgery (35). The patient displayed a heterozygous variant (GR_{T556I})
402 within the fifth exon of the *NR3C1* gene. Nicolaidis *et al.* showed a decreased affinity
403 (50%), transcriptional activity (50%), and nuclear translocation of the variant, but no
404 evidence for a dominant negative effect (31). The variant GR_{T556I} exhibited a defective
405 interaction of its AF-2 domain with GRIP1 and an increased transrepressive activity.
406 The second patient with left adrenal incidentaloma and glucocorticoid resistance was
407 described by our team and displayed a heterozygous variant corresponding to a
408 tyrosine-to-cysteine substitution at amino acid 478 (GR_{Y478C}) within the DBD.
409 Functional characterization of this variant revealed a decreased transactivation,
410 decreased nuclear translocation, and DNA binding without any dominant negative
411 effect or decreased affinity (22). None of these patients presented effective negative
412 feedback, as ACTH levels were within the normal range at diagnosis. In these cases,
413 glucocorticoid resistance-related HPA axis activation may have been responsible for
414 adrenal incidentaloma. In our patient's case, glucocorticoid resistance related to HPA
415 axis activation may have led to autonomous cortisol secretion. Subsequently, high
416 plasma cortisol levels may have led to effective negative feedback on the HPA axis,
417 which might explain why glucocorticoid resistance was not initially diagnosed.
418 Hypothesis of two unrelated events is possible but seemed unlikely as plasma ACTH
419 levels were repressed after 100 mg hydrocortisone injection, showing effective
420 negative feedback on the corticotroph cells (Supplemental Data 1). Symptomatic
421 glucocorticoid resistance syndrome treatment is based on mineralocorticoid-sparing
422 synthetic glucocorticoids, such as dexamethasone. The aim is to suppress the HPA

423 axis activity and reduce ACTH secretion and adrenal stimulation (36), suggesting that
424 a high concentration of glucocorticoid agonists may restore glucocorticoid negative
425 feedback. Decreased sensitivity of the receptor, which can be overcome with high
426 concentrations of agonists, is commonly observed in variations within the LBD of
427 nuclear receptors such as androgen receptor (AR), mineralocorticoid receptor (MR),
428 or vitamin D receptor (37). Interestingly, urinary-free cortisol levels evolved as a mirror
429 image of plasma ACTH levels in the case described herein. Currently, UFC above 150
430 $\mu\text{g}/24\text{h}$ is associated with a decrease in plasma ACTH levels, suggesting that
431 endogenous hypercortisolism is sufficient to achieve effective negative feedback.

432 *In vitro* characterization of the *NR3C1* heterozygous variant GR_{R569Q} showed a lower
433 transactivation activity with an increased EC₅₀. Transactivation activity results from
434 several steps that the variant may have impaired. First, GR_{R569Q}'s affinity for
435 hydrocortisone was 1.5 times lower than GR_{WT} (Figure 4E). The mutated arginine is
436 localized within the LBD on helix H3 (Figure 5A). While helix H3 is a Ligand Binding
437 Pocket (LBP) component, there is no direct interaction between Arg569 and the ligand
438 as it is orientated outside the LBP (34,35). Modeling of the GR-LBD bound to
439 dexamethasone (PDB 7KRJ, Figure 5) showed that Arg569 makes hydrogen bonds
440 with Tyr545 and Ala546 on the H1-H3 loop. The hydrogen bond with Tyr545 is
441 conserved in antagonist and agonistic conformation of the LBD (5,38–40) but not in
442 unliganded conformation. In the latter, Arg569 makes hydrogen bonds with Ser551 and
443 Val552 in H1-H3 loop, suggesting a role for Arg569 in the conformational change of
444 the unliganded-LBD to the liganded-LBD. These amino acids are involved in
445 glucocorticoid resistance observed in guinea pigs (41). Guinea pig exhibits
446 physiological glucocorticoid resistance with elevated cortisol levels due to changes in
447 amino acids within the LBD (42) and, more precisely, within the H1-H3 loop.

448 Modification of the H1-H3 loop results in altered interactions with chaperone proteins
449 FKBP51 and FKBP52 through a particular 3D-conformation of the LBD, conferring
450 glucocorticoid resistance (32). FKBP51 and FKBP52 regulate GR and other steroid
451 receptors affinity for agonists and nuclear translocation (43,44). Consistent with these
452 data, we showed decreased nuclear translocation in patient fibroblasts and transiently
453 transfected cells (Figure 3D, E, Figure 4B, C). Therefore, it would be interesting to
454 study interactions of GR_{R569Q} with FKBP52 and FKBP51.

455 Further, we observed a dominant-negative effect of GR_{R569Q} on GR_{WT} transactivation
456 capacity (Figure 4B). Indeed, transfection of both GRs in equal amounts led to a
457 decreased transcriptional activity compared to GR_{WT} alone, consistent with the results
458 obtained in the patient's fibroblasts. However, glucocorticoid resistance was not
459 reversible with high cortisol levels depending on the gene, suggesting gene-specific
460 cortisol sensitivity associated with this variant. The gene-specific impact of GR variants
461 has been described concerning other well-known variants, such as N363S
462 polymorphism (45) and MR resistance syndrome (46). Furthermore, mice GR^{dim/dim}
463 with a reduced capacity of GR dimerization exhibited gene- and tissue-specific
464 alteration in glucocorticoid signaling pathways (47,48). Tissue-specific GR regulation
465 may explain why the patient did not present clinical symptoms of hypercortisolism,
466 even when HPA axis negative feedback was efficient, and plasma ACTH levels were
467 low, mirroring high cortisol levels. Further this discrepancy may be explained by the
468 different mechanisms of GR signaling. The differential impact of *NR3C1* loss-of-
469 function variants on transactivation and transrepression activity has already been
470 described, but the molecular mechanism remains unclear (31,51). At the pituitary level,
471 glucocorticoid-dependent transrepression of the ACTH precursor, the Pro-
472 opiomelanocortin (*POMC*) gene, depends in part on inhibition of Nur77 transactivation

473 activity through tethering (49,50). Unfortunately, we were not able to study GR_{R569Q}
474 impact in corticotroph cells, nor did we characterize the variant *in vivo* in a mice model.
475 Nevertheless, we showed that transrepression of the NFκB target gene, *IL6*, is rescued
476 in the patient fibroblasts in response to 100 nM cortisol. This suggests that
477 transrepression of GR target-genes through tethering mechanisms may be restored at
478 plasma cortisol concentrations lower than those necessary to regain full GR
479 transactivation through direct DNA-binding.

480 In conclusion, we described a novel heterozygous variant of the *NR3C1* gene
481 associated with glucocorticoid resistance. To our knowledge, this is the first description
482 of a patient with constitutive glucocorticoid resistance initially masked by adrenocortical
483 adenoma with autonomous cortisol secretion. Thus, it emphasizes the question,
484 previously addressed in the Muta-GR study, of the prevalence of GR variants in
485 patients with adrenal incidentaloma and mild autonomous cortisol secretion,
486 particularly in the case of rapid post-surgical recovery of the HPA axis (34). Our
487 functional studies support the critical role of H3 and the H1-H3 loop in the ligand
488 binding. Finally, this is the first demonstration of a reversible glucocorticoid resistance
489 with effective negative feedback on corticotroph cells secondary to mild autonomous
490 cortisol secretion, which brings new insight into tissue-specific glucocorticoid regulation
491 of target genes.

492 **Data Availability**

493 The data supporting this study's findings are available upon request from the
494 corresponding author. The data are not publicly available due to privacy or ethical
495 restrictions.

496 **Acknowledgments**

497 The authors are grateful to A. Blin and P. Tandabany for technical assistance.

498 **Funding Statement**

499 This research program was supported by Inserm (Institut national de la santé et de la
500 recherche) and Université Paris-Saclay. Margaux Laulhé was the recipient of an
501 SFEDP (Société Française d'Endocrinologie et de Diabétologie Pédiatrique) price
502 during her Master 2 Degree. During her PhD program, she benefits from financial
503 support from ITMO Cancer of Aviesan (Institut Thématique Multi-Organisme Cancer
504 de l'Alliance nationale pour les sciences de la vie et de la santé) within the framework
505 of 2021-2030 Cancer Control Strategy, on funds administered by Inserm.

506 **Author contribution**

507 Conceptualization: Margaux Laulhé, Emmanuelle Kuhn, Jérôme Bouligand, Larbi
508 Amazit, Julie Perrot, Peter Kamenicky, Jérôme Fagart, Marc Lombès, Say
509 Viengchareun and Laetitia Martinerie

510 Formal analysis: Margaux Laulhé, Larbi Amazit, Jérôme Fagart, Say Viengchareun
511 and Laetitia Martinerie

512 Funding acquisition: Say Viengchareun and Laetitia Martinerie

513 Investigation: Margaux Laulhé, Emmanuelle Kuhn, Julie Perrot, Jerome Bouligand,
514 Elise Lebigot, Jérôme Fagart, Say Viengchareun and Laetitia Martinerie

515 Methodology: Margaux Laulhé, Emmanuelle Kuhn, Julie Perrot, Jérôme Fagart, Larbi
516 Amazit, Say Viengchareun and Laetitia Martinerie

517 Project administration: Say Viengchareun and Laetitia Martinerie

518 Resources: Elise Lebigot, Jérôme Bouligand; Jérôme Fagart

519 Validation: Margaux Laulhé, Emmanuelle Kuhn, Jérôme Bouligand, Larbi Amazit, Julie
520 Perrot, Peter Kamenicky, Marc Lombès, Jérôme Fagart, Say Viengchareun and
521 Laetitia Martinerie

522 Writing-original draft: Margaux Laulhé, Larbi Amazit, Jérôme Fagart, Say
523 Viengchareun, and Laetitia Martinerie

524 Writing-review & editing: Margaux Laulhé, Emmanuelle Kuhn, Jérôme Bouligand, Larbi
525 Amazit, Julie Perrot, Elise Lebigot, Peter Kamenicky, Marc Lombès, Jérôme Fagart,
526 Say Viengchareun and Laetitia Martinerie

527 **Ethic Declaration**

528 Written consent for genetic testing and additional written consent for the publication
529 were obtained from the patient.

530 **Conflict of Interest**

531 The authors declare no conflict of interest.

532

533 **Figures Legend**

534 **Figure 1. Clinical and biological phenotype**

535A) CT-scan figuring a right adrenal incidentaloma of 44x27 mm with low attenuation (3
536 UH) (White Arrow).

537B) Graphic representation of ACTH (pmol/L, solid line) throughout the years. Data are
538 expressed as median [Q1;Q3] from at least two values whenever possible. The normal
539 range is figured in grey. HC: Hydrocortisone supplementation.

540C) Graphic representation of 24 h Urinary Free-Cortisol ($\mu\text{g}/24\text{h}$, dashed line) throughout
541 the years. Data are expressed as median [Q1;Q3] from at least two values whenever
542 possible. The normal range is figured in grey. HC: Hydrocortisone supplementation.

543D) Graphic representation of midnight plasma cortisol (nmol/L, dashed line) throughout
544 the years. Data are expressed as median [Q1;Q3] from at least two values whenever
545 possible. The normal range is figured in grey. HC: Hydrocortisone supplementation.

546 **Figure 2. A novel heterozygous missense variant located in the LBD**

547A) Schematic representation of the Glucocorticoid Receptor with the localization of the
548 missense variation from gene to protein (NTD: N-Terminal Domain, DBD: DNA Binding
549 Domain, LBD: Ligand Binding Domain).

550B) Chromatograph of the genomic DNA extracted from patient fibroblasts. The
551 heterozygous variation is figured with a black arrow.

552C) Chromatograph of the complementary DNA extracted from patient fibroblasts.
553 Expression of both alleles is figured by the double-peak shown by a black arrow.

554D) Protein expression of GR in patient fibroblasts (90 kDa). The lysate and protein extract
555 of the transfected cells were analyzed by SDS-PAGE. GR proteins were detected by

556 Western Blot analyses using mouse antibody targeting hGR α N-terminal domain. β -
557 actin was used as a loading control. Representative image of 3 independent
558 experiments is shown.

559E) Quantification of protein level. Specific bands were quantified by Odyssey FC, Dual
560 Mode Western Imaging. Results are expressed as median [Q1; Q2] and normalized to
561 β -actin loading, with control condition values arbitrarily set at 1. Data are expressed
562 from individual samples from 3 independent experiments. Statistical analysis was
563 performed using Mann Whitney t-test with Bonferroni correction, **** $P < 0.0001$.

564 **Figure 3. Expression of the GR_{R569Q} is associated with impaired transactivation**
565 **and delayed nuclear translocation but effective transrepression.**

566A) Relative mRNA expression of GR target gene, *SGK1*, measured by RT-qPCR. Control
567 and patient fibroblasts were incubated with 0 or 100 nM concentration of cortisol for
568 8 h. *SGK1* mRNA expression, expressed in attomoles, was normalized with *36B4*
569 mRNA, and expressed relative to the minimum expression obtained for GR_{WT} with no
570 agonist, arbitrarily set at 1. Data are expressed as mean \pm SEM from 6 replicates of 2
571 independent experiments. Statistical significance was calculated with 2-way ANOVA
572 test with Bonferroni correction, **** $P < 0.0001$.

573B) Relative mRNA expression of GR target gene, *TSC22D3*, measured by RT-qPCR.
574 Control and patient fibroblasts were incubated with 0 or 100 nM concentration of
575 cortisol for 8 h. *TSC22D3* mRNA expression, expressed in attomoles, was normalized
576 with *36B4* mRNA, expressed relative to the minimum expression obtained for GR_{WT}
577 with no agonist, arbitrarily set at 1. Data are expressed as mean \pm SEM from 6
578 replicates of 2 independent experiments. Statistical significance was calculated with 2-
579 way ANOVA test with Bonferroni correction, ** $P = 0.0025$.

580C) Relative mRNA expression of GR target gene *IL6* measured by RT-qPCR. Control and
581 patient fibroblasts were incubated with 0 or 100 nM concentration of cortisol for 8 hours.
582 *IL6* mRNA expression expressed in attomoles was normalized with *36B4* mRNA, and
583 expressed relative to the minimum expression obtained for GR_{WT} with no agonist,
584 arbitrarily set at 1. Data expressed as mean±SEM from 6 replicates of 2 independent
585 experiments. Statistical significance was calculated with 2-way ANOVA test with
586 Bonferroni correction, *****P* < 0.0001.

587D) Control and patient fibroblasts were incubated with 0 to 100 nM cortisol for 1 h. Cells
588 were fixed and processed for immunocytochemistry as described in the Materials and
589 Methods section—representative images of the subcellular localization of GR (red). A
590 z-series of focal planes was digitally imaged and deconvolved using
591 ImageProPlusAMS iterative algorithm to generate high-resolution images. DAPI
592 staining delineates the nuclei (blue). GR nuclear localization is figured in the Merged
593 panel (pink); scale bar represents 100 μM.

594 D) Automated high-throughput microscopy quantification of the molecular translocation
595 of GR_{WT} and GR_{R569Q}. Cells were treated as described in C), and fluorescence
596 acquisition was performed with the Array Scan VTI fluorescent microscope. The
597 translocation index (nucleo-cytoplasmic ratio) represents the average ratio of the
598 nuclear fluorescence intensity to the cytoplasmic fluorescence intensity calculated for
599 each transfected cell (n>1000). Data expressed as mean ± SEM from each field (n =
600 50) of 2 independent experiments. Statistical significance was calculated with the 2-
601 way ANOVA test with Bonferroni correction, * *P* = 0.001, *****P* < 0.0001.

602 **Figure 4. Decreased transcription activity of the GR_{R569Q} can be overcome with**
603 **high concentrations of cortisol**

604A) HEK 293T cells were transiently transfected with GR_{WT} or GR_{R569Q} encoding plasmids
605 (40 ng), a reporter luciferase plasmid pMMTV-Luc (40 ng) and pMIR β gal (35 ng). Cells
606 were treated 24 h with increasing cortisol concentrations (0.1 to 100 nM).
607 Transactivation activity is expressed relatively to the maximum transactivational
608 activity obtained for GR_{WT} with 100 nM agonist concentration, arbitrarily set at 100%.
609 EC₅₀ is obtained using nonlinear regression from 6 independent experiments
610 performed in 8 replicates. Statistical significance was calculated with the Mann-
611 Whitney t-test, ** $P = 0,0011$.

612B) HEK 293T cells were transiently transfected with either 30 ng of GR_{WT} or GR_{R569Q}
613 encoding plasmid alone, or 30 ng of both, associated with a reporter luciferase plasmid
614 pMMTV-Luc (40 ng) and pMIR β gal (35 ng). Cells were treated for 24 h with increasing
615 cortisol concentrations (0, 10 or 100 nM). Transactivation activity was expressed
616 relatively to the maximum transactivational activity obtained for 30 ng GR_{WT} at 100 nM
617 cortisol, arbitrarily set at 100%). Data expressed as median[Q1; Q3] from 8 replicates
618 of 2 independent experiments. Statistical significance was calculated with the Multiple
619 Mann-Whitney test with Bonferroni correction, **** $P < 0.0001$.

620C) COS-7 cells were transiently transfected with GR_{WT} or GR_{R569Q} encoding plasmids
621 (400 ng). Transfected cells were incubated with 0 to 100 nM cortisol for 1 h and 24 h
622 after transfection. Cells were fixed and processed for immunocytochemistry as described
623 in the Materials and Methods section—representative images of the subcellular
624 localization of GR (red). A z-series of focal planes was digitally imaged and
625 deconvolved using ImageProPlusAMS iterative algorithm to generate high-resolution
626 images. DAPI staining delineates the nuclei (blue). GR nuclear localization is figured
627 in the Merged panel (pink); scale bar represents 50 μ M.

628D) Automated high-throughput microscopy quantification of the molecular translocation of
629 GR_{WT} and GR_{R569Q}. Cells were treated as described in C), and fluorescence acquisition
630 was performed with the Array Scan VTI fluorescent microscope. The translocation
631 index (nucleo-cytoplasmic ratio) represents the average ratio of the nuclear
632 fluorescence intensity to the cytoplasmic fluorescence intensity calculated for each
633 transfected cell (n>1000). Nuclear cytoplasmic ratio was expressed as fold change
634 from GR_{WT} without cortisol, arbitrarily set at 1. Data expressed as mean ± SEM from
635 each field (n = 50) from 2 independent experiments. Statistical significance was
636 calculated with the 2-way ANOVA test with Bonferroni correction, *****P* < 0.0001.

637E) Scatchard analysis of hydrocortisone binding to GR_{WT} and GR_{R569Q}. *In vitro* expressed
638 GR_{WT} or GR_{R569Q} were incubated with increasing concentrations of [³H]-hydrocortisone
639 (0.5 to 70 nM) for 4h at 4°C. Bound and unbound ligands were separated by the
640 dextran-charcoal method; after removing the nonspecific component, the evolution of
641 the bound/unbound as a function of the amount bound was plotted, and the
642 dissociation constant at equilibrium (K_d) was calculated from the slope obtained with
643 linear regression. Statistical analysis of the slopes showed that the difference between
644 the slopes is significant (*P*<0.0001) representative data of 2 independent experiments.

645 **Figure 5. Three-dimensional model of the ligand-bound GR-LBD.**

646 A) Picture showing the overall organization of the agonist-bound GR-LBD (PDB 7KRJ).
647 The α-helices and beta-sheets are depicted as ribbons and arrows, respectively.
648 Selected residues are shown with their carbon, oxygen, and nitrogen atoms colored in
649 white, red, and blue, respectively. Dexamethasone is shown with its carbon, oxygen,
650 and fluoride atoms colored in violet, red, and pale blue, respectively.

651 B) Focus on the hydrogen-bound network involving Arg569 from helix H3 and Tyr545
652 and Ala546 from Loops 1-3.

653 C) Focus showing the loss of hydrogen bonds after substitution of Arg569 to Gln569.

654 **Supplemental Data 1. Restoration of an effective negative feedback on ACTH**
655 **secretion.**

656 A) Graphical representation of ACTH (pmol/L, solid line) secretion in response to
657 100 mg hydrocortisone injection four years post-adrenalectomy. Normal range
658 is figured in light grey.

659 **Supplemental Data 2. Three-dimensional model of the unliganded GR-LBD.**

660 A) Picture showing the overall organization of the apo GR-LBD (PDB 7KW7). The α -
661 helices and beta-sheets are depicted as ribbons and arrows, respectively. Selected
662 residues are shown with their carbon, oxygen, and nitrogen atoms colored in white,
663 red, and blue, respectively.

664 B) Focus on the hydrogen bound network involving Arg569 from helix H3 and Ser551
665 and Val552 from Loop 1-3.

666 C) Focus showing the loss of hydrogen bonds after substitution of Arg569 to Gln569.

667 **Supplemental Data 3. Transactivation activity of the GR_{R569K}**

668A) HEK 293T cells were transiently transfected with GR_{WT} or GR_{R569Q} or GR_{R569K} encoding
669 plasmids (40 ng), a reporter luciferase plasmid pMMTV-Luc (40 ng) and pMIR β gal (35
670 ng). Cells were treated 24 h with increasing cortisol concentrations (0 to 100 nM).
671 Transactivation activity was expressed relatively to the maximum transactivational

672 activity obtained for GR_{WT} at 100 nM, arbitrarily set at 100%. Data are representative
673 of 3 experiments performed in 8 replicates. Statistical significance was calculated with
674 2-way ANOVA test with Bonferroni correction, **** $P < 0,0001$.

675B) HEK 293T cells were transiently transfected with GR_{WT} or GR_{R569Q} or GR_{R569K}
676 encoding plasmids, a reporter luciferase plasmid pMMTV-Luc and pMIR β gal. Cells
677 were treated 24h with increasing cortisol concentrations (0.1 to 100 nM).
678 Transactivation activity is expressed relatively to the maximum transactivational
679 activity obtained for GR_{WT} with 100 nM agonist concentration, arbitrarily set at 100%.
680 EC₅₀ is obtained using nonlinear regression from 3 independent experiments
681 performed in 8 replicates. Statistical significance was calculated with the Kruskal Wallis
682 test, * $P = 0,015$.

683 **Supplemental Data 4. Target genes primers**

684 All primer sequences are shown from 5' to 3'.

685 **References**

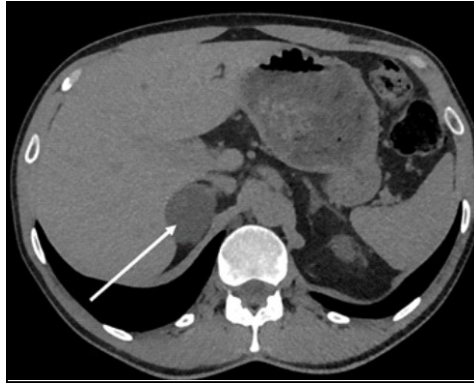
- 686 1. Whirlledge S, DeFranco DB. Glucocorticoid Signaling in Health and Disease: Insights From Tissue-
687 Specific GR Knockout Mice. *Endocrinology*. 2018 Jan 1;159(1):46–64.
- 688 2. Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs.
689 *N Engl J Med*. 2005 Oct 20;353(16):1711–23.
- 690 3. Kadmiel M, Cidlowski JA. Glucocorticoid receptor signaling in health and disease. *Trends*
691 *Pharmacol Sci*. 2013 Sep;34(9):518–30.
- 692 4. Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, et al. Primary structure and
693 expression of a functional human glucocorticoid receptor cDNA. *Nature*. 1985 Dec
694 19;318(6047):635–41.
- 695 5. Wang RYR, Noddings CM, Kirschke E, Myasnikov AG, Johnson JL, Agard DA. Structure of Hsp90-
696 Hsp70-Hop-GR reveals the Hsp90 client-loading mechanism. *Nature*. 2022 Jan;601(7893):460–4.
- 697 6. Ratman D, Vanden Berghe W, Dejager L, Libert C, Tavernier J, Beck IM, et al. How glucocorticoid
698 receptors modulate the activity of other transcription factors: a scope beyond tethering. *Mol Cell*
699 *Endocrinol*. 2013 Nov 5;380(1–2):41–54.
- 700 7. Nicolaidis NC, Charmandari E. Primary Generalized Glucocorticoid Resistance and Hypersensitivity
701 Syndromes: A 2021 Update. *Int J Mol Sci*. 2021 Oct 7;22(19):10839.
- 702 8. Fleseriu M, Auchus R, Bancos I, Ben-Shlomo A, Bertherat J, Biermasz NR, et al. Consensus on
703 diagnosis and management of Cushing’s disease: a guideline update. *Lancet Diabetes Endocrinol*.
704 2021 Dec;9(12):847–75.
- 705 9. Reul JM, Sutanto W, van Eekelen JA, Rothuizen J, de Kloet ER. Central action of adrenal steroids
706 during stress and adaptation. *Adv Exp Med Biol*. 1990;274:243–56.
- 707 10. Vingerhoeds AC, Thijssen JH, Schwarz F. Spontaneous hypercortisolism without Cushing’s
708 syndrome. *J Clin Endocrinol Metab*. 1976 Nov;43(5):1128–33.
- 709 11. Vitellius G, Trabado S, Bouligand J, Delemer B, Lombès M. Pathophysiology of Glucocorticoid
710 Signaling. *Annales d’Endocrinologie*. 2018 Jun;79(3):98–106.
- 711 12. Paragliola RM, Costella A, Corsello A, Urbani A, Concolino P. A Novel Pathogenic Variant in the N-
712 Terminal Domain of the Glucocorticoid Receptor, Causing Glucocorticoid Resistance. *Mol Diagn*
713 *Ther*. 2020 Aug 1;24(4):473–85.
- 714 13. Charmandari E, Raji A, Kino T, Ichijo T, Tiulpakov A, Zachman K, et al. A Novel Point Mutation in
715 the Ligand-Binding Domain (LBD) of the Human Glucocorticoid Receptor (hGR) Causing
716 Generalized Glucocorticoid Resistance: The Importance of the C Terminus of hGR LBD in Conferring
717 Transactivational Activity. *The Journal of Clinical Endocrinology & Metabolism*. 2005
718 Jun;90(6):3696–705.
- 719 14. Chrousos GP, Vingerhoeds A, Brandon D, Eil C, Pugeat M, DeVroede M, et al. Primary cortisol
720 resistance in man. A glucocorticoid receptor-mediated disease. *J Clin Invest*. 1982 Jun;69(6):1261–
721 9.

- 722 15. Nader N, Bachrach BE, Hurt DE, Gajula S, Pittman A, Lescher R, et al. A novel point mutation in
723 helix 10 of the human glucocorticoid receptor causes generalized glucocorticoid resistance by
724 disrupting the structure of the ligand-binding domain. *J Clin Endocrinol Metab.* 2010
725 May;95(5):2281–5.
- 726 16. Nicolaides NC, Charmandari E, Chrousos GP, Kino T. Recent advances in the molecular mechanisms
727 determining tissue sensitivity to glucocorticoids: novel mutations, circadian rhythm and ligand-
728 induced repression of the human glucocorticoid receptor. *BMC Endocr Disord.* 2014 Dec;14(1):71.
- 729 17. Trebble P, Matthews L, Blaikley J, Wayte AWO, Black GCM, Wilton A, et al. Familial glucocorticoid
730 resistance caused by a novel frameshift glucocorticoid receptor mutation. *J Clin Endocrinol Metab.*
731 2010 Dec;95(12):E490-499.
- 732 18. Bouligand J, Delemer B, Hecart AC, Meduri G, Viengchareun S, Amazit L, et al. Familial
733 Glucocorticoid Receptor Haploinsufficiency by Non-Sense Mediated mRNA Decay, Adrenal
734 Hyperplasia and Apparent Mineralocorticoid Excess. Reitsma PH, editor. *PLoS ONE.* 2010 Oct
735 22;5(10):e13563.
- 736 19. Karl M, Lamberts SW, Koper JW, Katz DA, Huizenga NE, Kino T, et al. Cushing’s disease preceded
737 by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative
738 glucocorticoid receptor mutation. *Proc Assoc Am Physicians.* 1996 Jul;108(4):296–307.
- 739 20. Pelsma ICM, Fassnacht M, Tsagarakis S, Terzolo M, Tabarin A, Sahdev A, et al. Comorbidities in
740 mild autonomous cortisol secretion and the effect of treatment: systematic review and meta-
741 analysis. *Eur J Endocrinol.* 2023 Oct 17;189(4):S88–101.
- 742 21. Vitellius G, Delemer B, Caron P, Chabre O, Bouligand J, Pussard E, et al. Impaired 11 β -
743 Hydroxysteroid Dehydrogenase Type 2 in Glucocorticoid-Resistant Patients. *J Clin Endocrinol*
744 *Metab.* 2019 Nov 1;104(11):5205–16.
- 745 22. Vitellius G, Fagart J, Delemer B, Amazit L, Ramos N, Bouligand J, et al. Three Novel Heterozygous
746 Point Mutations of NR3C1 Causing Glucocorticoid Resistance. *Hum Mutat.* 2016 Aug;37(8):794–
747 803.
- 748 23. Fagart J, Hillisch A, Huyet J, Bäracker L, Fay M, Pleiss U, et al. A new mode of mineralocorticoid
749 receptor antagonism by a potent and selective nonsteroidal molecule. *J Biol Chem.* 2010 Sep
750 24;285(39):29932–40.
- 751 24. Fagart J, Seguin C, Pinon GM, Rafestin-Oblin ME. The Met852 residue is a key organizer of the
752 ligand-binding cavity of the human mineralocorticoid receptor. *Mol Pharmacol.* 2005
753 May;67(5):1714–22.
- 754 25. Fassnacht M, Tsagarakis S, Terzolo M, Tabarin A, Sahdev A, Newell-Price J, et al. European Society
755 of Endocrinology clinical practice guidelines on the management of adrenal incidentalomas, in
756 collaboration with the European Network for the Study of Adrenal Tumors. *European Journal of*
757 *Endocrinology.* 2023 Jul 20;189(1):G1–42.
- 758 26. Prete A, Subramanian A, Bancos I, Chortis V, Tsagarakis S, Lang K, et al. Cardiometabolic Disease
759 Burden and Steroid Excretion in Benign Adrenal Tumors : A Cross-Sectional Multicenter Study. *Ann*
760 *Intern Med.* 2022 Mar;175(3):325–34.
- 761 27. Bancos I, Prete A. Approach to the Patient With Adrenal Incidentaloma. *J Clin Endocrinol Metab.*
762 2021 Oct 21;106(11):3331–53.

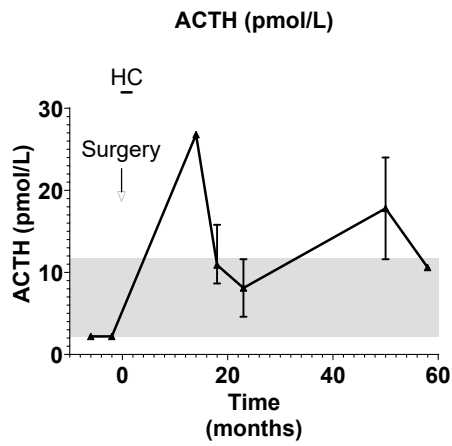
- 763 28. Freeman PJ, Hart RK, Gretton LJ, Brookes AJ, Dalgleish R. VariantValidator: Accurate validation,
764 mapping, and formatting of sequence variation descriptions. *Hum Mutat.* 2018 Jan;39(1):61–8.
- 765 29. Hudson WH, Vera IMS de, Nwachukwu JC, Weikum ER, Herbst AG, Yang Q, et al. Cryptic
766 glucocorticoid receptor-binding sites pervade genomic NF- κ B response elements. *Nat Commun.*
767 2018 Apr 6;9(1):1337.
- 768 30. Amazit L, Le Billan F, Kolkhof P, Lamribet K, Viengchareun S, Fay MR, et al. Finerenone Impedes
769 Aldosterone-dependent Nuclear Import of the Mineralocorticoid Receptor and Prevents Genomic
770 Recruitment of Steroid Receptor Coactivator-1. *J Biol Chem.* 2015 Sep 4;290(36):21876–89.
- 771 31. Nicolaides NC, Skyrla E, Vlachakis D, Psarra AMG, Moutsatsou P, Sertedaki A, et al. Functional
772 characterization of the hGR α T556I causing Crousos syndrome. *Eur J Clin Invest.* 2016
773 Jan;46(1):42–9.
- 774 32. Cluning C, Ward BK, Rea SL, Arulpragasam A, Fuller PJ, Ratajczak T. The helix 1-3 loop in the
775 glucocorticoid receptor LBD is a regulatory element for FKBP cochaperones. *Mol Endocrinol.* 2013
776 Jul;27(7):1020–35.
- 777 33. Michailidou Z, Carter RN, Marshall E, Sutherland HG, Brownstein DG, Owen E, et al. Glucocorticoid
778 receptor haploinsufficiency causes hypertension and attenuates hypothalamic-pituitary-adrenal
779 axis and blood pressure adaptations to high-fat diet. *FASEB J.* 2008 Nov;22(11):3896–907.
- 780 34. Vitellius G, Trabado S, Bouligand J, Hoeffel C, Mantel AG, Delemer B, et al. Résultats du PHRC
781 National muta-GR : prévalence des mutations NR3C1 dans l'hyperplasie bilatérale des surrénales.
782 *Annales d'Endocrinologie.* 2017 Sep 1;78(4):216.
- 783 35. Zhu H Juan, Dai Y fei, Wang O, Li M, Lu L, Zhao W gang, et al. Generalized glucocorticoid resistance
784 accompanied with an adrenocortical adenoma and caused by a novel point mutation of human
785 glucocorticoid receptor gene. *Chin Med J (Engl).* 2011 Feb;124(4):551–5.
- 786 36. Tatsi C, Xekouki P, Nioti O, Bachrach B, Belyavskaya E, Lyssikatos C, et al. A novel mutation in the
787 glucocorticoid receptor gene as a cause of severe glucocorticoid resistance complicated by
788 hypertensive encephalopathy. *J Hypertens.* 2019 Jul;37(7):1475–81.
- 789 37. Achermann JC, Schwabe J, Fairall L, Chatterjee K. Genetic disorders of nuclear receptors. *Journal*
790 *of Clinical Investigation.* 2017 Apr 3;127(4):1181–92.
- 791 38. Bledsoe RK, Montana VG, Stanley TB, Delves CJ, Apolito CJ, McKee DD, et al. Crystal structure of
792 the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization
793 and coactivator recognition. *Cell.* 2002 Jul 12;110(1):93–105.
- 794 39. He Y, Yi W, Suino-Powell K, Zhou XE, Tolbert WD, Tang X, et al. Structures and mechanism for the
795 design of highly potent glucocorticoids. *Cell Res.* 2014 Jun;24(6):713–26.
- 796 40. Kauppi B, Jakob C, Färnegårdh M, Yang J, Ahola H, Alarcon M, et al. The three-dimensional
797 structures of antagonistic and agonistic forms of the glucocorticoid receptor ligand-binding
798 domain: RU-486 induces a transconformation that leads to active antagonism. *J Biol Chem.* 2003
799 Jun 20;278(25):22748–54.
- 800 41. Zuckerman SH, Bendele AM. Regulation of serum tumor necrosis factor in glucocorticoid-sensitive
801 and -resistant rodent endotoxin shock models. *Infect Immun.* 1989 Oct;57(10):3009–13.

- 802 42. Keightley MC, Curtis AJ, Chu S, Fuller PJ. Structural determinants of cortisol resistance in the guinea
803 pig glucocorticoid receptor. *Endocrinology*. 1998 May;139(5):2479–85.
- 804 43. Vandevyver S, Dejager L, Libert C. On the Trail of the Glucocorticoid Receptor: Into the Nucleus
805 and Back: Nuclear Transport of Glucocorticoid Receptor. *Traffic*. 2012 Mar;13(3):364–74.
- 806 44. Kirschke E, Goswami D, Southworth D, Griffin PR, Agard DA. Glucocorticoid receptor function
807 regulated by coordinated action of the Hsp90 and Hsp70 chaperone cycles. *Cell*. 2014 Jun
808 19;157(7):1685–97.
- 809 45. Jewell CM, Cidlowski JA. Molecular Evidence for a Link between the N363S Glucocorticoid
810 Receptor Polymorphism and Altered Gene Expression. *J Clin Endocrinol Metab*. 2007
811 Aug;92(8):3268–77.
- 812 46. Fernandes-Rosa FL, Hubert EL, Fagart J, Tchitchek N, Gomes D, Jouanno E, et al. Mineralocorticoid
813 Receptor Mutations Differentially Affect Individual Gene Expression Profiles in
814 Pseudohypoaldosteronism Type 1. *The Journal of Clinical Endocrinology & Metabolism*. 2011 Mar
815 1;96(3):E519–27.
- 816 47. Timmermans S, Verhoog NJD, Van Looveren K, Dewaele S, Hochepped T, Eggermont M, et al. Point
817 mutation I634A in the glucocorticoid receptor causes embryonic lethality by reduced ligand
818 binding. *J Biol Chem*. 2022 Feb;298(2):101574.
- 819 48. Jewell CM, Scoltock AB, Hamel BL, Yudt MR, Cidlowski JA. Complex human glucocorticoid receptor
820 dim mutations define glucocorticoid induced apoptotic resistance in bone cells. *Mol Endocrinol*.
821 2012 Feb;26(2):244–56.
- 822 49. Martens C, Bilodeau S, Maira M, Gauthier Y, Drouin J. Protein-protein interactions and
823 transcriptional antagonism between the subfamily of NGFI-B/Nur77 orphan nuclear receptors and
824 glucocorticoid receptor. *Molecular Endocrinology (Baltimore, Md)*. 2005 Apr;19(4):885–97.
- 825 50. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, et al. DNA binding of the
826 glucocorticoid receptor is not essential for survival. *Cell*. 1998 May 15;93(4):531–41.
- 827 51. Nicolaidis NC, Roberts ML, Kino T, Braatvedt G, Hurt DE, Katsantoni E, et al. A novel point mutation
828 of the human glucocorticoid receptor gene causes primary generalized glucocorticoid resistance
829 through impaired interaction with the LXXLL motif of the p160 coactivators: dissociation of the
830 transactivating and transrepressive activities. *J Clin Endocrinol Metab*. 2014 May;99(5):E902-907.
- 831

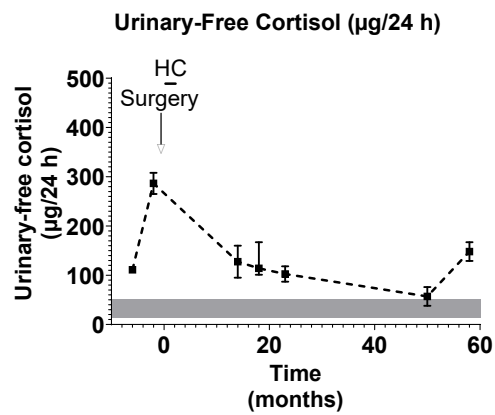
A



B



C



D

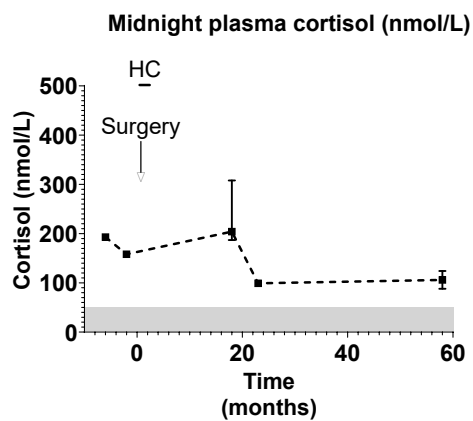


Figure 1. Clinical and biological phenotype

NM_000176.3 c.1706 G>A

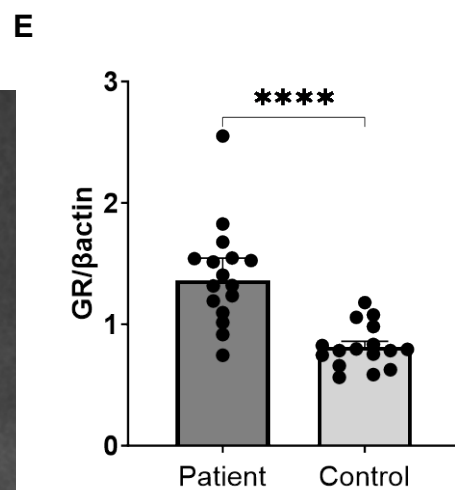
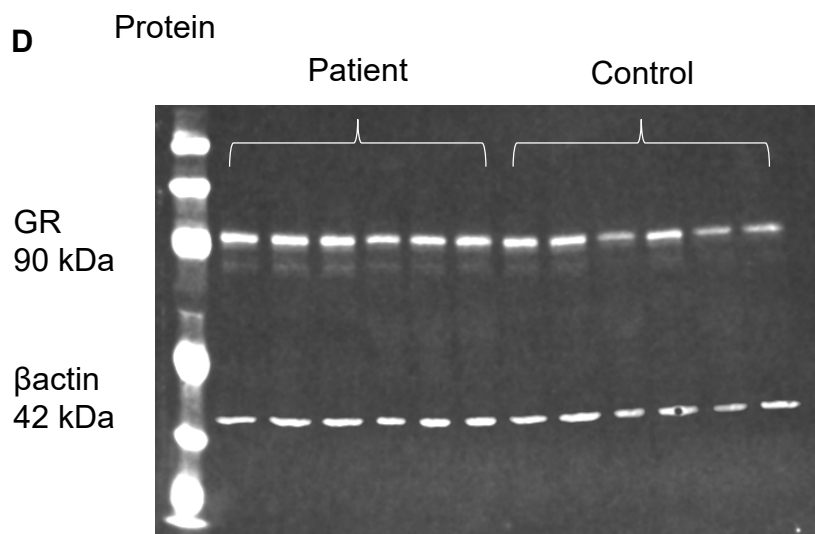
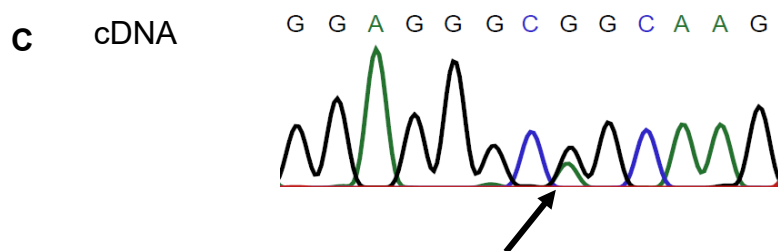
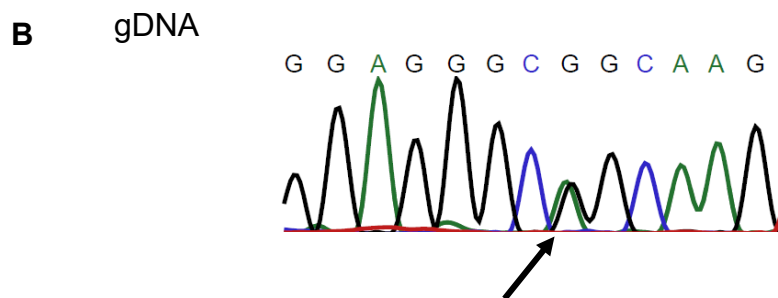
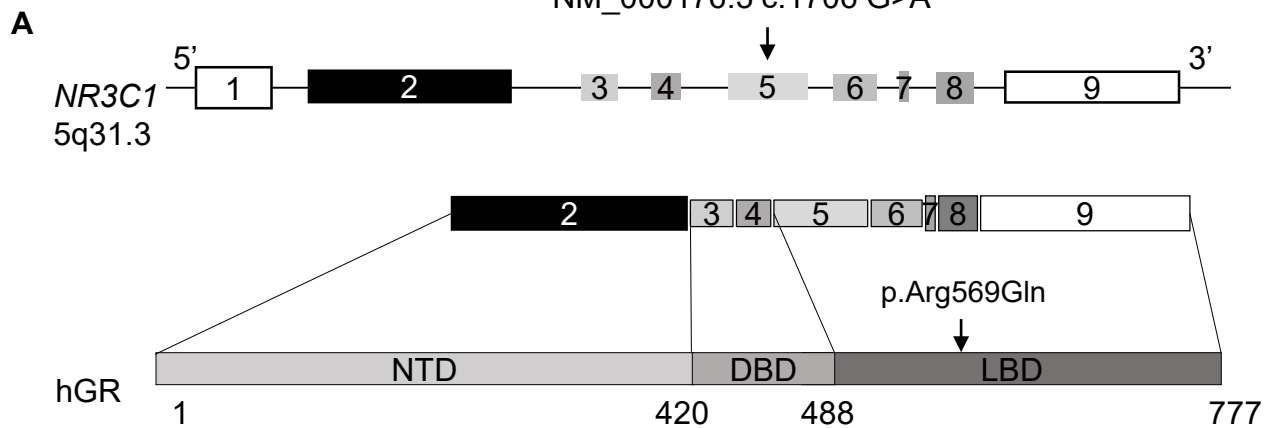


Figure 2. A novel heterozygous missense variant located in the GR LBD

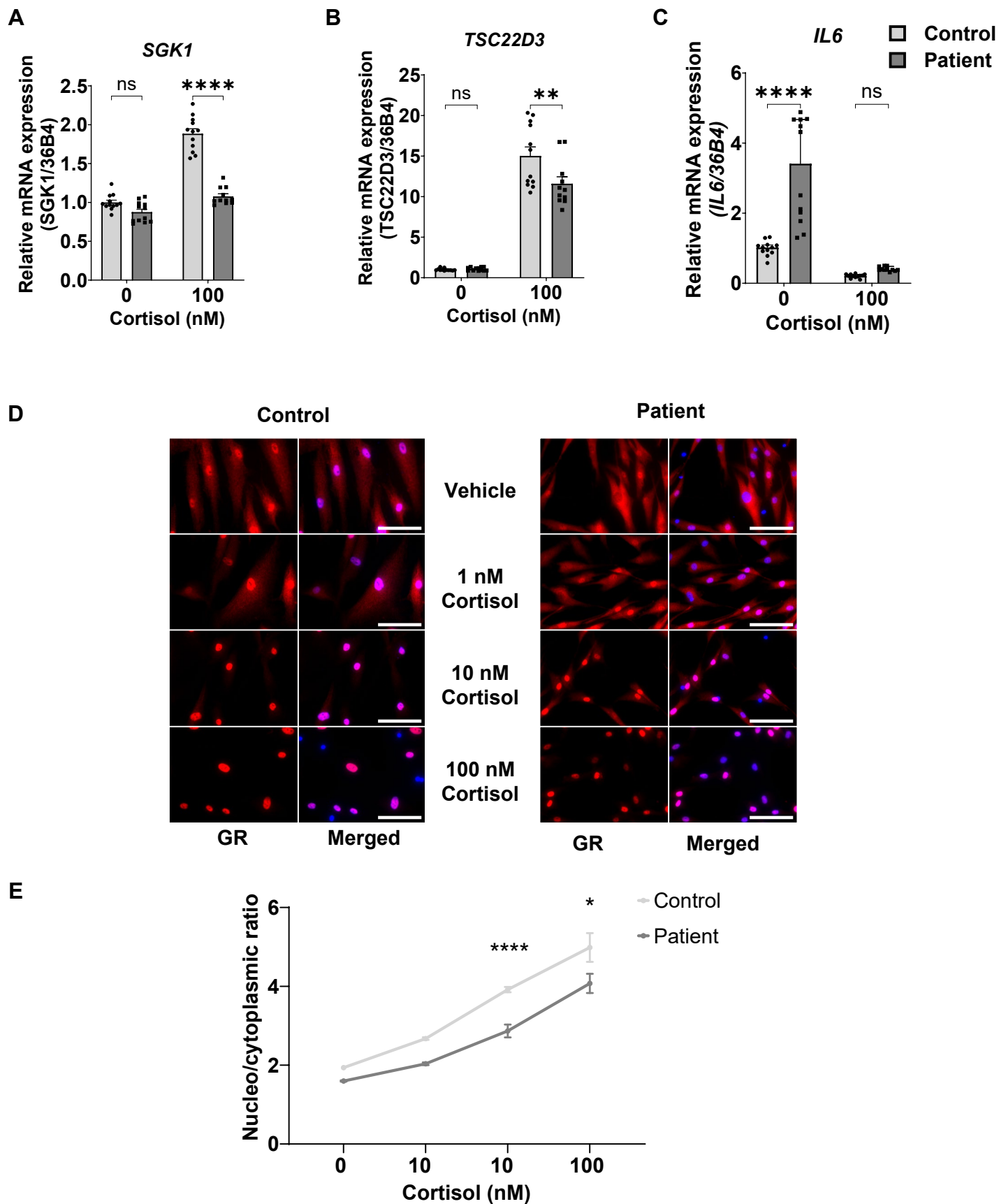


Figure 3. Expression of the GR_{R569Q} is associated with impaired transactivation and delayed nuclear translocation but effective transrepression

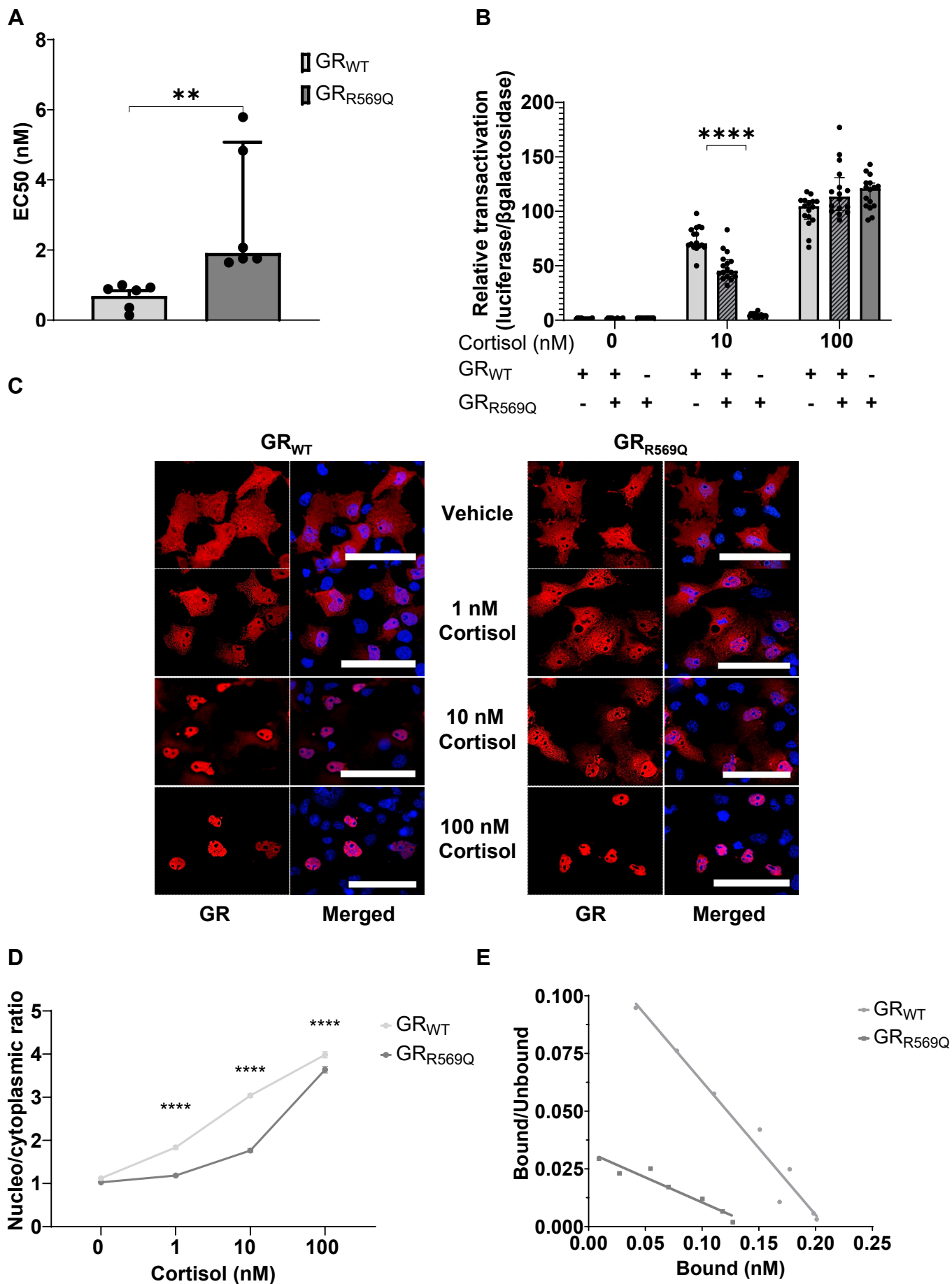


Figure 4. Decreased transcription activity of the GR_{R569Q} can be overcome with high concentrations of cortisol

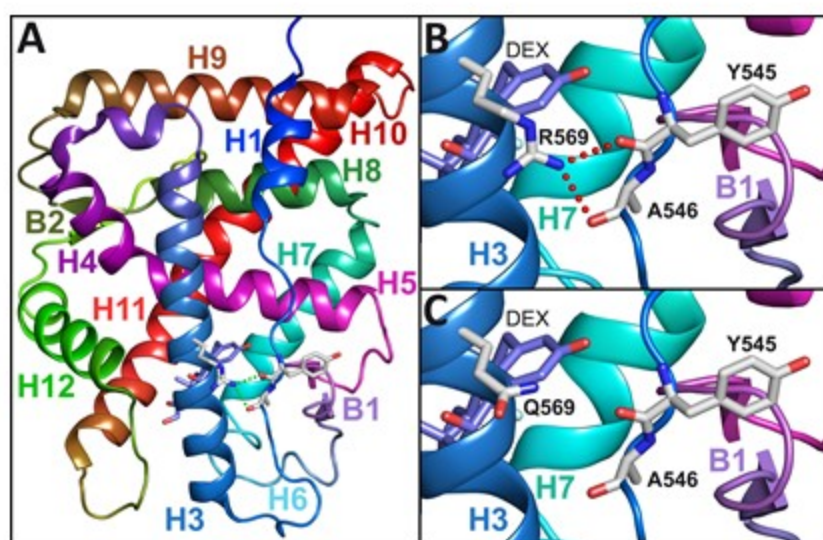
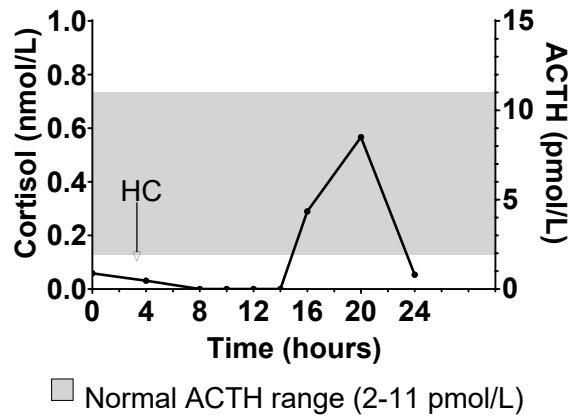
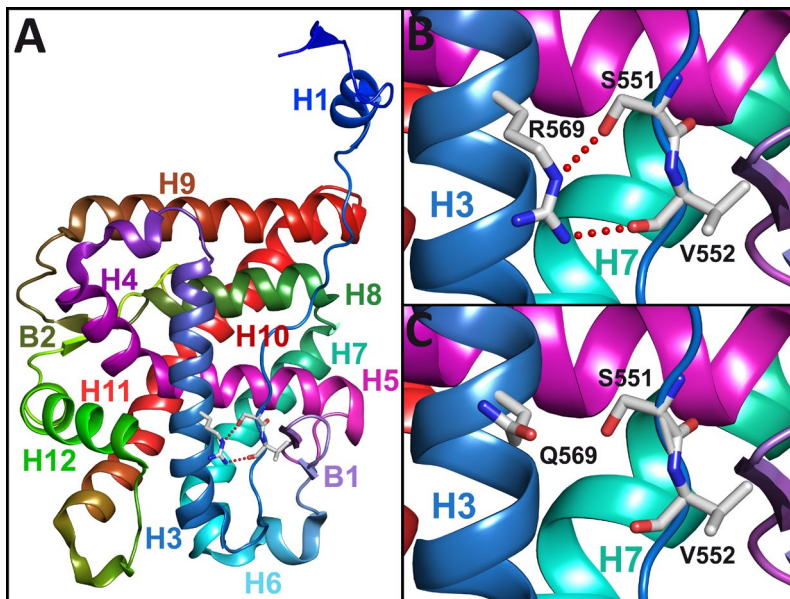


Figure 5. Three-dimensional model of the ligand-bound GR-LBD

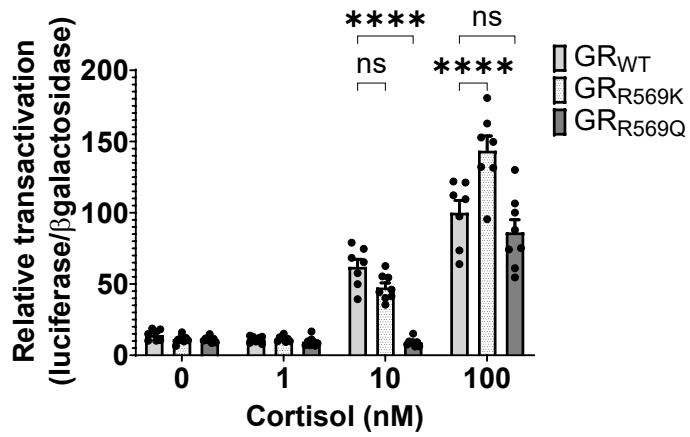


Supplemental Data 1. Restoration of an effective negative feedback on ACTH secretion

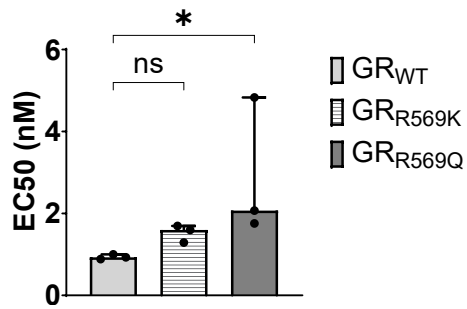


Supplemental Data 2. Three-dimensional model of the unliganded GR-LBD

A



B



Supplemental Data 3. Transactivation activity of the GR_{R569K}

GENE	Primer sequence
<i>h36B4</i> _F	CCC-ATT-CTA-TCA-TCA-ACG-GGT-ACA-A
<i>h36B4</i> _R	CAG-CAA-GTG-GGA-AGG-TGT-AAT-CC
<i>hTSC22D3</i> _F	TCC-TGA-AGG-AGC-AGA-TCC-GA
<i>hTSC22D3</i> _R	TCT-TCA-GGG-CTC-AGA-CAG-GA
<i>hSGK1</i> _F	GTC-GCA-ATT-CTC-ATC-GCT-TTC
<i>hSGK1</i> _R	CTT-CAG-GGT-GTT-TGC-ATG-CAT
<i>hIL6</i> _F	AGG-GCT-CTT-CGG-CAA-ATG-TA
<i>hIL6</i> _R	TGC-CCA-GTG-GAC-AGG-TTT-CT

Supplemental Data 4. Target genes primers