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1 **HIF1 α is an essential regulator of steroidogenesis in the adrenal gland**

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17

18 **Running title: Hypoxia response steers steroidogenesis**

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21

22 **Abstract**

23 Endogenous steroid hormones, especially glucocorticoids and mineralocorticoids, are essential for
24 life regulating numerous physiological and pathological processes. These hormones derive from
25 the adrenal cortex, and drastic or sustained changes in their circulatory levels affect multiple organ
26 systems. Although a role for hypoxia pathway proteins (HPP) in steroidogenesis has been
27 suggested, knowledge on the true impact of the HIFs (Hypoxia Inducible Factors) and oxygen
28 sensors (HIF-prolyl hydroxylase domain-containing enzymes; PHDs) in the adrenocortical cells
29 of vertebrates is scant. By creating a unique set of transgenic mouse lines, we reveal a prominent
30 role for HIF1 α in the synthesis of virtually all steroids under steady state conditions. Specifically,
31 mice deficient in HIF1 α in a part of the adrenocortical cells displayed enhanced levels of enzymes
32 responsible for steroidogenesis and a cognate increase in circulatory steroid levels. These changes
33 resulted in cytokine alterations and changes in the profile of circulatory mature hematopoietic
34 cells. Conversely, HIF1 α overexpression due to combined PHD2 and PHD3 deficiency in the
35 adrenal cortex resulted in the opposite phenotype of insufficient steroid production due to impaired
36 transcription of necessary enzymes. Based on these results, we propose HIF1 α to be a central and
37 vital regulator of steroidogenesis as its modulation in adrenocortical cells dramatically impacts
38 hormone synthesis with systemic consequences. Additionally, these mice can have potential
39 clinical significances as they may serve as essential tools to understand the pathophysiology of
40 hormone modulations in a number of diseases associated with metabolic syndrome, auto-immunity
41 or even cancer.

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50 **Keywords:** Hypoxia, adrenocortical steroids, cytokines, HIF, PHD

51 **Introduction**

52 Steroidogenesis in the adrenal gland is a complex process of sequential enzymatic reactions that
53 convert cholesterol into steroids, including mineralocorticoids and glucocorticoids (1). While
54 glucocorticoids are regulated by the hypothalamic-pituitary-adrenal axis (HPA axis) and are
55 essential for stress management and immune regulation (2, 3), aldosterone, the primary
56 mineralocorticoid, regulates the balance of water and electrolytes in the body (4). As
57 steroidogenesis is a tightly regulated process, proper control of adrenal cortex function relies on
58 appropriate endocrine signaling, tissue integrity, and homeostasis (5). Accordingly, it has been
59 suggested that inappropriately low pO₂, or hypoxia, can lead to both structural changes in the
60 adrenal cortex and interfere with hormone production (6-10).

61 Hypoxia inducible factors (HIFs) are the main transcription factors that are central to cellular
62 adaptation to hypoxia in virtually all cells of our body. The machinery that directly controls HIF
63 activity consists of the HIF-prolyl hydroxylase domain-containing enzymes (PHDs 1-3), which
64 are oxygen sensors that hydroxylate two prolyl residues in the HIF α subunit under normoxic
65 conditions, thereby marking the HIFs for proteasomal degradation. Conversely, oxygen
66 insufficiency renders these PHDs inactive, leading to the binding of the HIF-complex to hypoxia
67 responsive elements (HRE) in the promotor of multiple genes that ensure oxygen delivery and
68 promote adaptive responses to hypoxia such as hematopoiesis, blood pressure regulation, and
69 energy metabolism (reviewed in (11, 12)). Apart from directly activating hypoxia-responsive
70 genes (13, 14), HIFs also indirectly influence gene expression by interfering with the activity of
71 other transcription factors or systems. Of the most intensively studied HIF α genes, HIF1 α has a
72 ubiquitous pattern of expression in all tissues, whereas expression of the paralogue HIF2 α is
73 restricted to a selection of cell types (15, 16).

74 Recent *in vitro* and zebrafish studies have revealed a continuous cross talk between HIF and
75 steroidogenesis pathways, along with potential interference in the production of aldosterone and
76 glucocorticoids (17-20). There is also evidence suggesting a role for the hypoxia pathway in
77 modulating glucocorticoid/glucocorticoid receptor (GR) signaling (21, 22). Importantly, these
78 observations indicate a possible interplay of HIFs and PHDs in modulating the immune-regulatory
79 actions of the HPA axis. Currently, there is huge interest in the development of HIF inhibitors and
80 HIF stabilizers, and their influence on medicine is expected to become significant in the near future

81 (23). However, as the role of HIFs/PHDs is both central and manifold with respect to maintaining
82 oxygen homeostasis, a better understanding of the true impact of Hypoxia Pathway Proteins
83 (HPPs) in the complex interplay of different essential physiological and pathological conditions,
84 including in the adrenal cortex, assumes great importance.

85 We describe the creation and use of a unique collection of transgenic mouse lines that enabled an
86 investigation of the role of HIF α subunits and PHDs in adrenocortical cells. Our results point
87 towards a central role for HIF1 α in the direct regulation of steroidogenesis in the adrenal gland
88 and consequent changes in circulatory hormone levels. Importantly, chronic exposure of mice to
89 such altered hormone levels eventually led to a dramatic decrease in essential inflammatory
90 cytokines and profound dysregulation of circulatory immune cell profiles.

91

92 **Materials and Methods**

93 **Mice**

94 All mouse strains were housed under specific pathogen-free conditions at the Experimental Centre
95 of the Medical Theoretical Center (MTZ, Technical University of Dresden - University Hospital
96 Carl-Gustav Carus, Dresden, Germany). Experiments were performed with male and female mice
97 aged between 8-16 weeks. No significant differences between the genders were observed.
98 Akr1b7:cre-PHD2/HIF1^{ff/ff} (P2H1) or Akr1b7:cre-PHD2/PHD3^{ff/ff} (P2P3) lines were generated by
99 crossing Akr1b7:cre mice (24) to PHD2^{f/f}, HIF1 α ^{f/f} or PHD2^{f/f}; PHD3^{f/f} as previously reported by
100 us (25), and/or the reporter strain mTmG (26). All mice described in this report were born in
101 normal Mendelian ratios. Mice were genotyped using primers described in supplementary Table
102 1. Histological analysis of the adrenal gland of Akr1b7:cre-mTmG^{f/f} reporter mice revealed zonal
103 variation in the penetrance of cre-recombinase activity in the adrenal cortex of all individual mice
104 (GFP⁺ staining). Peripheral blood was drawn from mice by retro-orbital sinus puncture using
105 heparinized micro hematocrit capillaries (VWR, Darmstadt, Germany) and plasma separated and
106 stored at -80 °C until further analysis. Mice were sacrificed by cervical dislocation and adrenals
107 were isolated, snap frozen in liquid nitrogen, and stored at -80°C for hormone analysis or gene
108 expression analysis. All mice were bred and maintained in accordance with facility guidelines on
109 animal welfare and with protocols approved by the Landesdirektion Sachsen, Germany.

110

111 **Blood analysis**

112 White blood cell counts were measured using a Sysmex automated blood cell counter (Sysmex
113 XE-5000) (27).

114

115 **ACTH measurements**

116 Plasma ACTH was determined using a radioimmunoassay, as per manufacturer's instructions
117 (ImmuChem Double Antibody hACTH 125 I RIA kit; MP Biomedicals Germany GmbH,
118 Eschwege, Germany) (28).

119

120 **Hormone detection**

121 Adrenal glands were incubated in disruption buffer (component of Invitrogen™ Paris™ Kit, AM
122 1921, ThermoFisher Scientific, Dreieich, Germany) for 15min at 4°C, homogenized in a tissue
123 grinder, followed by incubation for 15 min on ice and further preparation. *Adrenal steroid*
124 *hormones* were determined by LC-MS/MS as described elsewhere (29). *Catecholamines*,
125 norepinephrine, epinephrine, and dopamine were measured by high pressure liquid
126 chromatography (HPLC) coupled with electrochemical detection, as previously described (30).

127

128 **RNA extraction and qPCRs**

129 RNA from adrenal glands and sorted cells was isolated using the RNA Easy Plus micro kit
130 (Qiagen) (Cat. # 74034Qiagen). cDNA synthesis was performed using the iScript cDNA Synthesis
131 Kit (BIO-RAD, Feldkirchen, Germany). Gene expression levels were determined by performing
132 quantitative real-time PCR using the 'Ssofast Evagreen Supermix' (BIO-RAD, Feldkirchen,
133 Germany). Sequences of primers used are provided in supplemental Table 2. Expression levels of
134 genes were determined using the Real-Time PCR Detection System-CFX384 (BIO-RAD,
135 Feldkirchen, Germany). All mRNA expression levels were calculated relative to β 2M or EF2
136 housekeeping genes and were normalized using the ddCt method. Relative gene expression was
137 calculated using the $2(-ddCt)$ method, where ddCT was calculated by subtracting the average WT
138 dCT from dCT of all samples individually.

139

140 **Immunohistochemistry and immunofluorescence**

141 For preparation of paraffin sections, adrenal glands were isolated, incubated in 4% formaldehyde
142 at 4°C overnight, dehydrated, embedded in paraffin and cut into 5µm sections using a
143 microtome. Sections were rehydrated and subjected to hematoxylin and eosin staining (H&E).
144 For frozen sections, adrenal glands were embedded in O.C.T Tissue-Tek (A. Hartenstein GmbH,
145 Würzburg, Germany) and stored at -20°C. For H&E staining of frozen sections (7µm), samples
146 were first fixed in cold acetone before staining. For immunofluorescence, sections were fixed in
147 cold acetone, air-dried, washed with phosphate-buffered saline containing 0.1% Tween-20,
148 blocked with 5% normal goat serum followed by primary antibody staining (CD31/PECAM –
149 1:500 (31)) or GFP Polyclonal (Antibody ThermoFischer Scientific – 1:200) overnight at 4°C
150 and subsequent secondary antibody staining. After counterstaining with DAPI, slides were
151 mounted in fluorescent mounting medium and stored at 4 °C until analysis.

152

153 **Microscopy**

154 Both brightfield and fluorescent images were acquired on an ApoTome II Colibri (Carl Zeiss, Jena,
155 Germany). Images were analyzed using either Zen software (Carl Zeiss, Jena, Germany) or Fiji
156 (ImageJ distribution 1.52K). Fiji was used to quantify lipid droplet sizes and CD31 staining.

157

158 **Meso Scale Discovery**

159 Meso Scale Discovery (MSD, Rockville, Maryland) was used to measure cytokines in plasma
160 samples using the MSD plate reader (QuickPlex SQ 120). Cytokine concentrations were calculated
161 by converting the measured MSD signal to pg/ml using a standard curve. All values below that of
162 blank (control) were considered as zero. Finally, all cytokine concentrations in individual P2H1
163 mice were normalized to the average value of WT's for every independent experiment; and the
164 average WT value was set as 1.

165

166 **Next generation sequencing**

167 For RNAseq analysis, adrenal glands from Akr1b7:cre-PHD2/HIF1/mTmG^{fff/fff} and Akr1b7:cre-
168 mTmG^{ff/f} (control) mice were isolated directly into the lysis buffer of the RNeasy Plus Micro Kit,
169 RNA was isolated according to manufacturer's instructions, and SmartSeq2 sequencing was
170 performed (SmartSeq2 and data analysis in Supplemental Data). Flow cytometry and cell sorting
171 were performed as described previously (32).

172

173 **Read Quantification**

174 Kallisto v0.43 was first used to generate an index file from the transcript file, which can be
175 downloaded from
176 [:ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_mouse/release_M12/gencode.vM12.transcri](ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_mouse/release_M12/gencode.vM12.transcripts.fa.gz)
177 [pts.fa.gz](ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_mouse/release_M12/gencode.vM12.transcripts.fa.gz). Kallisto v0.43 was then run on all the fastq files using parameters “quant --single -l 75 -
178 s 5 -b 100” to quantify reads for the genes.

179

180 **Differential Gene Expression Quantification**

181 Complete cDNA sleuth v0.30.0 (an R package) was used to evaluate differential expression. The
182 command “sleuth_prep” was run with parameter “gene_mode=TRUE”. Two separate error models
183 were fit using “sleuth_fit” wherein the first was a “full” model with gender and experimental
184 condition as covariates, while the second was a “reduced” model with only gender as the covariate.
185 “sleuth_lrt” (Likelihood Ratio Test) was used to evaluate differential gene expression by
186 comparing the full model and the reduced model.

187

188 **Statistical analyses**

189 All data are presented as mean \pm SEM. Data (WT control versus transgenic line) were analyzed
190 using the Mann–Whitney U-test, unpaired t-test with Welch’s correction as appropriate (after
191 testing for normality with the F test) or as indicated in the text. All statistical analyses were
192 performed using GraphPad Prism v7.02 for Windows (GraphPad Software, La Jolla California
193 USA, www.graphpad.com). Significance was set at $p < 0.05$; “n” in the figure legends denotes
194 individual samples.

195

196 **Results**

197 **A new mouse model to study the effects of alterations in hypoxia pathway proteins (HPPs)** 198 **in the adrenal cortex**

199 We took advantage of the adrenal cortex-specific Akr1b7:cre recombinase mouse line (25) to
200 investigate the effects of adrenocortical HPPs on the structure and functions of the adrenal gland.
201 When combined with the mTmG reporter strain (26), we show up to 40% targeting among all

202 cortical cells (Figure 1A). Next, we generated the *Akr1b7:cre-PHD2/HIF1^{ff/ff}* mouse line
203 (henceforth designated P2H1) by combining *Akr1b7:cre* mice with *PHD2* and *HIF1 α* floxed mice
204 (24). Genomic PCRs on DNA and qPCR analysis using mRNA from whole adrenal glands
205 revealed targeting of *PHD2* and *HIF1 α* , when compared to WT littermates (Figure 1B-C).
206 Importantly, in P2H1 mice, we even detected a significant increase in *HIF2 α* mRNA but not of
207 *PHD3*, which is in line with our earlier report of enhanced HIF2 α -activity in *PHD2/HIF1 α* -
208 deficient cells (24). Therefore, we explored the expression profile of a number of downstream
209 genes known to be transactivated by HIF2 α (33-35) and found a significant increase in *Vegfa*,
210 *Hmox1*, and to a lesser extent *Bnip3* levels, underscoring the functionality of the P2H1 mouse line
211 (Figure 1E).

212

213 **Morphological changes in the adrenal cortex of P2H1 mice**

214 To evaluate the impact of changes in HIF1 α and/or HIF2 α activity in adrenocortical cells, we
215 analyzed adrenal gland morphology using H&E staining on paraffin sections but found no
216 differences between P2H1 mice and WT littermates in the structure of the adrenal gland,
217 especially, at the side of the cortex of P2H1 mice in comparison to WT littermates (Figure 1F). As
218 we detected a significant increase in *Vegfa* in the adrenal glands of P2H1 mice, we used CD31
219 staining to quantify endothelial cells but detected no significant differences between P2H1 and
220 WT mice (Figure 1G). Remarkably, H&E staining on cryosections of P2H1 adrenal glands
221 revealed significantly smaller lipid droplets in the adrenocortical cells (Figure 1H), an effect that
222 is reported to be correlated with greater conversion of cholesterol into pregnenolone (10).

223

224 **Modulation of HPPs in the adrenal cortex enhances synthesis and circulatory levels of steroid 225 hormones**

226 Next, to verify if the observed changes in lipid droplets indeed led to changes in steroidogenesis,
227 we quantified steroid hormones and their precursor levels by LC-MS/MS in the adrenal gland and
228 in plasma. Quantification revealed a significant increase in virtually all of the hormones tested in
229 P2H1 adrenal glands compared to WT littermates (Figure 2A), and importantly, a corresponding
230 increase of progesterone, corticosterone, and aldosterone was found in the plasma (Figure 2B).
231 These observations clearly indicate that central HPPs have an impact on steroidogenesis in the
232 murine adrenal gland and on circulatory levels of steroid hormones.

233

234 **Downstream effects of the chronic increase in the steroidogenesis**

235 Previous reports have stated that glucocorticoids can regulate catecholamine production in the
236 adrenal medulla (36, 37); therefore, we also measured dopamine, norepinephrine, and epinephrine
237 levels in the samples used to quantify steroid levels (as above). However, we found no difference
238 between P2H1 and WT littermates in any of the catecholamines quantified ([Supplementary Figure](#)
239 [1A](#)). Further, although increased steroid levels often result in a negative feedback loop affecting
240 ACTH secretion from the pituitary (38), P2H1 mice displayed no such differences compared to
241 WT littermates ([Supplementary Figure 1B](#)), nor did they have any difference in serum potassium
242 levels or blood glucose levels ([Supplementary Figure 1C-D](#)). Taken together, in contrast to the
243 systemic effects induced by acute and high levels of circulatory cortical hormones (e.g.
244 corticosterone, aldosterone) (3, 4), the P2H1 mice display moderate but chronically enhanced
245 levels of cortical hormones at the described time points.

246

247 **Loss of PHD2/HIF1 α in adrenocortical cells impacts gene expression related to** 248 **steroidogenesis**

249 Previous *in vitro* studies and reports on HIF1 α alterations in zebrafish larvae have suggested
250 negative regulation of StAR, the mitochondrial cholesterol transporter (7, 17, 20). However, data
251 on the effects of HPP alterations in adrenal cortex of mice is scant at best. Therefore, to assess the
252 impact of HIF1 α -deletion and/or HIF2 α -upregulation in adrenal cortical cells, we performed broad
253 transcription analysis of proteins/enzymes involved in steroidogenesis using mRNA from whole
254 adrenals. Our results reveal that almost all of the gene products tested showed either a significant
255 increase or a tendency to do so, including key enzymes like *StAR*, *Cyp11a1*, *Cyp21a1* and *Cyp11b1*
256 ([Figure 3A](#)).

257 To further characterize this phenotype driven by the HPPs, we performed *next generation*
258 *sequencing* (NGS) and compared the steady state transcriptomes of P2H1 and WT littermate mice
259 ([Figure 3B](#)). For this, we specifically created the *Akr1b7:cre-PHD2/HIF1/mTmG^{fff/fff}* mouse line
260 (P2H1 reporter mice) to study only targeted adrenal cortex cells, with *Akr1b7:cre-mTmG^{f/f}*
261 animals used as controls. Bulk RNAseq was performed on GFP⁺-sorted adrenal gland cells as
262 described previously (39) and gene signatures of the various lineages were evaluated using Enrichr

263 or gene set enrichment analyses (GSEA). Concurring with the previous results, we found a number
264 of significant signatures related to the process of steroid synthesis in adrenocortical cells or their
265 response to it (Figure 3C-D). Notably, GSEA also revealed known HIF-dependent associations
266 including, actin cytoskeleton (40, 41), adipogenesis (42) and oxidative phosphorylation (43)
267 (Figure 3E). Furthermore, P2H1 cortical cells also displayed a positive signature related to the
268 regulation of nuclear β -catenin signaling, which is known to be primarily activated in the zona
269 glomerulosa with potential hyperplastic effects (44) (Figure 3F).

270

271 **Modulated adrenocortical HPPs skew cytokine production and leukocyte numbers.**

272 As several studies have reiterated a crucial role for glucocorticoids in immunomodulation (3, 45),
273 and Cushing's syndrome has been described to be accompanied by immune deficiency (3, 38, 46),
274 we measured circulatory cytokine levels. We report a substantial overall decrease in the levels of
275 both pro- and anti-inflammatory cytokines, with the exception of the chemokine and neutrophil
276 attractant CXCL1, which increased almost 2-fold (Figure 4A). Glucocorticoids have been
277 repeatedly shown to promote apoptosis-mediated reduction of lymphocytes (47) and eosinophil
278 reduction (48), along with neutrophilia due to enhanced recruitment from the bone marrow (49).
279 Therefore, we enumerated the various white blood cell (WBC) fractions in P2H1 mice and
280 compared it with that of their WT littermates, which revealed a significant reduction in both
281 lymphocyte and eosinophil fractions (Figure 4B) accompanied by marked elevation in neutrophils
282 (>70% compared to WT) (Figure 4C). Taken together, our data reveal a critical role for HPPs in
283 steady-state cytokine levels and leukocyte numbers, probably through alterations in
284 steroidogenesis pathways.

285

286 **HIF1 α inversely regulates steroidogenesis**

287 To extend our understanding of the role of HIF1 α and/or HIF2 α in adrenocortical cells, we created
288 the Akr1b7:cre-PHD2/PHD3^{ff/ff} mouse line (designated as P2P3), which showed adequate
289 activation efficiency upon genomic PCRs of whole adrenal tissue (supplementary Figure 2).
290 Intriguingly and in contrast to hormone levels in the adrenal glands of the P2H1 mice, P2P3 adrenal
291 glands displayed a marked decrease in corticosterone and aldosterone levels, along with a cognate
292 reduction in their precursors, both in the adrenal gland (Figure 5A) and in circulation (Figure 5B).
293 These results clearly suggest that steroidogenesis is dependent on HIF1 α but not HIF2 α . To further

294 confirm this observation, we performed mRNA expression analyses to identify the levels of central
295 enzymes, similar to that performed in P2H1 mice, and demonstrate an overall decrease in these
296 enzymes (Figure 6A). This observation is contrary to that seen in the P2H1 mice but fits neatly
297 with the observed reduction in steroid levels in the P2P3 mice, thereby underscoring the central
298 role of HIF1 α (Figure 6B).

299

300 Discussion

301 Here, by using a unique collection of adrenocortical-specific transgenic mouse lines, we identify
302 HIF1 α as a central transcription factor that regulates the steroidogenesis pathway by regulating
303 key enzymes. Notably, this directly modifies the entire spectrum of steroid hormones, both in the
304 adrenal gland and in circulation, which eventually impacts the availability of a variety of cytokines.

305 Studies on the role of HIFs in the regulation of steroidogenesis *in vitro* are few, apart from those
306 in zebra fish larvae that describe differential regulation of the enzymes involved in the steroid
307 pathway (7, 18, 20). However, to the best of our knowledge, there are no mouse models to study
308 the role of HPPs in adrenal cortical cells. Undoubtedly, such models would help us to better
309 understand the crosstalk between HPPs and adrenal steroid metabolism, while simultaneously
310 serving as an essential tool to study the pathophysiology of multiple conditions associated with
311 dramatically altered steroid hormone levels (2). Ablation of HIF1 α revealed an important role for
312 this transcription factor in steroidogenesis, which concurs with results from previous studies (20,
313 50). However, our findings that HIF1 α deletion results in the upregulation of mRNA of a vast
314 majority of steroid-related enzymes is counterintuitive to the nature of this transcription factor (12,
315 51), and therefore we believe this effect is most likely indirect with potential involvement of one
316 or more transcriptional repressors (13, 52, 53). This type of transcriptional regulation of adrenal
317 steroidogenesis has already been suggested with miRNAs, which are endogenous noncoding
318 single-stranded small RNAs that suppress the expression of various target genes (54). Hu and
319 colleagues have demonstrated that a HIF1 α -dependent miRNA, miRNA-132, attenuates
320 steroidogenesis by reducing StAR protein levels (55), and similar mechanisms have reported for
321 *Cyp11B2* via miR-193a-3p (56, 57), and *Cyp11B1* and *Cyp11B2* via miR-10b (8). Thus, these new
322 mouse lines will be of great value for in-depth studies on the complex background of HIF1 α
323 involvement in the expression patterns of steroidogenesis-related miRs.

324 Our RNAseq analysis of Akr1b7⁺ P2H1 adrenocortical cells not only unearthed several genetic
325 signatures directly associated with steroidogenesis, but a number of GSEAs revealed prominent
326 HIF-dependent phenotypes previously identified in a variety of other cell types. Recently, we have
327 described a significant role for HIF2 α in the regulation of the actin cytoskeleton, especially in
328 facilitating enhanced neutrophil migration through very confined environments (41), HIF1 α has
329 also been associated with cytoskeleton structure and functionality in a number of cell lineages
330 (reviewed in (40)); this is apart from its role in energy metabolism wherein enhanced oxidative
331 phosphorylation has been demonstrated in various HIF1 α -deficient cell lineages (43). Therefore,
332 it will be of interest to further explore changes in multiple metabolites that are directly or
333 indirectly-associated with the TCA cycle to find a potential link with the overall changes described
334 here.

335 Glucocorticoids and aldosterone are both essential for homeostasis and their substantial increase
336 in P2H1 mice was intriguing, given their pivotal role in immune suppression (3, 58) and blood
337 pressure regulation, respectively. Previous studies have shown that aldosterone not only increases
338 the expression of the potassium channels that secrete potassium but also stimulates K-absorptive
339 pumps in the renal cortex and medulla, thereby stabilizing and maintaining renal potassium
340 excretion (59), a situation we also observed in the P2H1 mice. The significant increase in
341 glucocorticoids upon HIF1 α deletion was clearly associated with immunosuppression, as
342 demonstrated by an overall decrease in both pro- and anti-inflammatory cytokines in circulation,
343 and these observations mirror other reports of immune modulation due to enhanced glucocorticoid
344 levels. Such glucocorticoid elevation can eventually even result in dramatic immune deficiency,
345 for example, as seen in Cushing's disease (3, 38, 45, 58).

346 Intriguingly, we found serum CXCL1 to be significantly enhanced in P2H1 mice, probably
347 because as a central neutrophil attractant it was associated with the massive increase in circulatory
348 neutrophils seen in these mice. It is known that enhanced neutrophil recruitment from the bone
349 marrow is directly associated with glucocorticoids (49), as is their overall survival (60, 61).

350 An essential role of HIF1 α , but not HIF2 α , in the modulation of enzymes and adrenocortical
351 hormones could be further corroborated by the contrasting results seen in the P2P3 mice.
352 Specifically, compared to P2H1 mice, the expression profile of virtually all steroidogenesis
353 regulating enzymes was dramatically inverted in the P2P3 mice, which resulted in an overall

354 impairment of the steroidogenesis pathway. Therefore, these mouse lines will also be helpful to
355 study the potential impact of dramatically modulated steroid levels in a variety of clinically
356 relevant diseases including metabolic and auto-immune disorders.

357 In summary, we reveal a prominent role for HIF1 α as a central regulator of steroidogenesis in mice
358 as two distinct transgenic mouse lines showed persistent but contrasting changes in corticosterone
359 and aldosterone concentrations at levels sufficient to modulate systemic cytokine levels and
360 leukocyte numbers. These P2H1 and P2P3 mouse strains are of significant importance in further
361 exploring the impact of HIF1 α in adrenocortical cells and as an essential component in regulation
362 of steroidogenesis-mediated systemic effects.

363

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370

371 **Conflict-of-interest**

372 The authors have declared that no conflict of interest exists.

373

374 **Author contributions**

375 D.W. designed and performed the majority of experiments, analysed data, and contributed in
376 writing the manuscript. J.S., D.K., A.K., performed experiments and analysed data. A.Me.
377 designed several mouse lines and contributed to the discussion. N.B., A.N., A.E.A. and T.C.
378 provided tools and contributed to the discussion. G.E. and M.P. provided tools, analyzed data and
379 contributed to the discussions. V.I.A. contributed to the discussions. A.Ma. provided essential
380 tools. A.S. performed deep sequencing analysis. L.G.P-R. and M.T. performed ACTH

381 measurements and contributed to the discussion. B.W. designed and supervised the overall study,
382 analysed data, and wrote the manuscript.

383

384 **References**

- 385 1. Miller, W. L., and Auchus, R. J. (2011) The Molecular Biology, Biochemistry, and
386 Physiology of Human Steroidogenesis and Its Disorders. *Endocrine Reviews* **32**, 81-151
- 387 2. Cain, D. W., and Cidlowski, J. A. (2017) Immune regulation by glucocorticoids. *Nat Rev*
388 *Immunol* **17**, 233-247
- 389 3. Straub, R. H., and Cutolo, M. (2016) Glucocorticoids and chronic inflammation.
390 *Rheumatology* **55**, ii6-ii14
- 391 4. Faught, E., and Vijayan, M. M. (2018) The mineralocorticoid receptor is essential for stress
392 axis regulation in zebrafish larvae. *Sci Rep* **8**, 18081
- 393 5. Gallo-Payet, N., and Battista, M. C. (2014) Steroidogenesis-adrenal cell signal
394 transduction. *Compr Physiol* **4**, 889-964
- 395 6. Raff, H., Kohandarvish, S., and Jankowski, A. (1990) The Effect of Oxygen on
396 Aldosterone Release from Bovine Adrenocortical Cells in Vitro:PO2 versus
397 Steroidogenesis*. *Endocrinology* **127**, 682-687
- 398 7. Tan, T., Yu, R. M. K., Wu, R. S. S., and Kong, R. Y. C. (2017) Overexpression and
399 Knockdown of Hypoxia-Inducible Factor 1 Disrupt the Expression of Steroidogenic
400 Enzyme Genes and Early Embryonic Development in Zebrafish. *Gene Regul Syst Bio* **11**,
401 1177625017713193
- 402 8. Nusrin, S., Tong, S. K., Chaturvedi, G., Wu, R. S., Giesy, J. P., and Kong, R. Y. (2014)
403 Regulation of CYP11B1 and CYP11B2 steroidogenic genes by hypoxia-inducible miR-
404 10b in H295R cells. *Mar Pollut Bull* **85**, 344-351
- 405 9. Bruder, E. D., Nagler, A. K., and Raff, H. (2002) Oxygen-dependence of ACTH-stimulated
406 aldosterone and corticosterone synthesis in the rat adrenal cortex: developmental aspects.
407 *Journal of Endocrinology* **172**, 595-604
- 408 10. Lorente, M., Mirapeix, R. M., Miguel, M., Longmei, W., Volk, D., and Cervos-Navarro, J.
409 (2002) Chronic hypoxia induced ultrastructural changes in the rat adrenal zona
410 glomerulosa. *Histol Histopathol* **17**, 185-190

- 411 11. Sormendi, S., and Wielockx, B. (2018) Hypoxia Pathway Proteins As Central Mediators
412 of Metabolism in the Tumor Cells and Their Microenvironment. *Front Immunol* **9**, 40
- 413 12. Wielockx, B., Grinenko, T., Mirtschink, P., and Chavakis, T. (2019) Hypoxia Pathway
414 Proteins in Normal and Malignant Hematopoiesis. *Cells* **8**
- 415 13. Schodel, J., Oikonomopoulos, S., Ragoussis, J., Pugh, C. W., Ratcliffe, P. J., and Mole, D.
416 R. (2011) High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. *Blood*
417 **117**, e207-217
- 418 14. Smythies, J. A., Sun, M., Masson, N., Salama, R., Simpson, P. D., Murray, E., Neumann,
419 V., Cockman, M. E., Choudhry, H., Ratcliffe, P. J., and Mole, D. R. (2019) Inherent DNA-
420 binding specificities of the HIF-1alpha and HIF-2alpha transcription factors in chromatin.
421 *EMBO reports* **20**
- 422 15. Stroka, D. M., Burkhardt, T., Desbaillets, I., Wenger, R. H., Neil, D. A., Bauer, C.,
423 Gassmann, M., and Candinas, D. (2001) HIF-1 is expressed in normoxic tissue and displays
424 an organ-specific regulation under systemic hypoxia. *FASEB J* **15**, 2445-2453
- 425 16. Wiesener, M. S., Jürgensen, J. S., Rosenberger, C., Scholze, C. K., Hörstrup, J. H.,
426 Warnecke, C., Mandriota, S., Bechmann, I., Frei, U. A., Pugh, C. W., Ratcliffe, P. J.,
427 Bachmann, S., Maxwell, P. H., and Eckardt, K.-U. (2003) Widespread hypoxia-inducible
428 expression of HIF-2alpha in distinct cell populations of different organs. *FASEB J* **17**, 271-
429 273
- 430 17. Wang, X., Zou, Z., Yang, Z., Jiang, S., Lu, Y., Wang, D., Dong, Z., Xu, S., and Zhu, L.
431 (2018) HIF 1 inhibits StAR transcription and testosterone synthesis in murine Leydig cells.
432 *J Mol Endocrinol*
- 433 18. Kowalewski, M. P., Gram, A., and Boos, A. (2015) The role of hypoxia and HIF1alpha in
434 the regulation of STAR-mediated steroidogenesis in granulosa cells. *Mol Cell Endocrinol*
435 **401**, 35-44
- 436 19. Yamashita, K., Ito, K., Endo, J., Matsushashi, T., Katsumata, Y., Yamamoto, T., Shirakawa,
437 K., Isobe, S., Kataoka, M., Yoshida, N., Goto, S., Moriyama, H., Kitakata, H., Mitani, F.,
438 Fukuda, K., Goda, N., Ichihara, A., and Sano, M. (2020) Adrenal cortex hypoxia modulates
439 aldosterone production in heart failure. *Biochemical and Biophysical Research*
440 *Communications* **524**, 184-189

- 441 20. Marchi, D., Santhakumar, K., Markham, E., Li, N., Storbeck, K.-H., Krone, N., Cunliffe,
442 V. T., and van Eeden, F. J. M. (2020) Bidirectional crosstalk between HIF and
443 Glucocorticoid signalling in zebrafish larvae. *bioRxiv*, 748566
- 444 21. Kodama, T., Shimizu, N., Yoshikawa, N., Makino, Y., Ouchida, R., Okamoto, K., Hisada,
445 T., Nakamura, H., Morimoto, C., and Tanaka, H. (2003) Role of the glucocorticoid receptor
446 for regulation of hypoxia-dependent gene expression. *J Biol Chem* **278**, 33384-33391
- 447 22. Zhang, C., Qiang, Q., Jiang, Y., Hu, L., Ding, X., Lu, Y., and Hu, G. (2015) Effects of
448 hypoxia inducible factor-1alpha on apoptotic inhibition and glucocorticoid receptor
449 downregulation by dexamethasone in AtT-20 cells. *BMC Endocr Disord* **15**, 24
- 450 23. Semenza, G. L. (2019) Pharmacologic Targeting of Hypoxia-Inducible Factors. *Annu Rev*
451 *Pharmacol Toxicol* **59**, 379-403
- 452 24. Franke, K., Kalucka, J., Mamlouk, S., Singh, R. P., Muschter, A., Weidemann, A., Iyengar,
453 V., Jahn, S., Wiczorek, K., Geiger, K., Muders, M., Sykes, A. M., Poitz, D. M., Ripich,
454 T., Otto, T., Bergmann, S., Breier, G., Baretton, G., Fong, G. H., Greaves, D. R., Bornstein,
455 S., Chavakis, T., Fandrey, J., Gassmann, M., and Wielockx, B. (2013) HIF-1alpha is a
456 protective factor in conditional PHD2-deficient mice suffering from severe HIF-2alpha-
457 induced excessive erythropoiesis. *Blood* **121**, 1436-1445
- 458 25. Lambert-Langlais, S., Val, P., Guyot, S., Ragazzon, B., Sahut-Barnola, I., De Haze, A.,
459 Lefrancois-Martinez, A. M., and Martinez, A. (2009) A transgenic mouse line with specific
460 Cre recombinase expression in the adrenal cortex. *Mol Cell Endocrinol* **300**, 197-204
- 461 26. Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007) A global double-
462 fluorescent Cre reporter mouse. *genesis* **45**, 593-605
- 463 27. Mitroulis, I., Chen, L. S., Singh, R. P., Kourtzelis, I., Economopoulou, M., Kajikawa, T.,
464 Troullinaki, M., Ziogas, A., Ruppova, K., Hosur, K., Maekawa, T., Wang, B.,
465 Subramanian, P., Tonn, T., Verginis, P., von Bonin, M., Wobus, M., Bornhauser, M.,
466 Grinenko, T., Di Scala, M., Hidalgo, A., Wielockx, B., Hajishengallis, G., and Chavakis,
467 T. (2017) Secreted protein Del-1 regulates myelopoiesis in the hematopoietic stem cell
468 niche. *J Clin Invest* **127**, 3624-3639
- 469 28. Castillo, V., Theodoropoulou, M., Stalla, J., Gallelli, M. F., Cabrera-Blatter, M. F., Haedo,
470 M. R., Labeur, M., Schmid, H. A., Stalla, G. K., and Arzt, E. (2011) Effect of SOM230

- 471 (pasireotide) on corticotropic cells: action in dogs with Cushing's disease.
472 *Neuroendocrinology* **94**, 124-136
- 473 29. Peitzsch, M., Dekkers, T., Haase, M., Sweep, F. C., Quack, I., Antoch, G., Siegert, G.,
474 Lenders, J. W., Deinum, J., and Willenberg, H. S. (2015) An LC–MS/MS method for
475 steroid profiling during adrenal venous sampling for investigation of primary
476 aldosteronism. *The Journal of steroid biochemistry and molecular biology* **145**, 75-84
- 477 30. Eisenhofer, G., Goldstein, D. S., Stull, R., Keiser, H. R., Sunderland, T., Murphy, D. L.,
478 and Kopin, I. J. (1986) Simultaneous liquid-chromatographic determination of 3,4-
479 dihydroxyphenylglycol, catecholamines, and 3,4-dihydroxyphenylalanine in plasma, and
480 their responses to inhibition of monoamine oxidase. *Clinical chemistry* **32**, 2030-2033
- 481 31. Klotzsche-von Ameln, A., Muschter, A., Mamlouk, S., Kalucka, J., Prade, I., Franke, K.,
482 Rezaei, M., Poitz, D. M., Breier, G., and Wielockx, B. (2011) Inhibition of HIF prolyl
483 hydroxylase-2 blocks tumor growth in mice through the antiproliferative activity of
484 TGFbeta. *Cancer Res* **71**, 3306-3316
- 485 32. Singh, R. P., Franke, K., Kalucka, J., Mamlouk, S., Muschter, A., Gembarska, A.,
486 Grinenko, T., Willam, C., Naumann, R., Anastassiadis, K., Stewart, A. F., Bornstein, S.,
487 Chavakis, T., Breier, G., Waskow, C., and Wielockx, B. (2013) HIF prolyl hydroxylase 2
488 (PHD2) is a critical regulator of hematopoietic stem cell maintenance during steady-state
489 and stress. *Blood* **121**, 5158-5166
- 490 33. Wiesener, M. S., Turley, H., Allen, W. E., Willam, C., Eckardt, K. U., Talks, K. L., Wood,
491 S. M., Gatter, K. C., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Maxwell, P. H. (1998)
492 Induction of endothelial PAS domain protein-1 by hypoxia: characterization and
493 comparison with hypoxia-inducible factor-1alpha. *Blood* **92**, 2260-2268
- 494 34. Bertout, J. A., Majmundar, A. J., Gordan, J. D., Lam, J. C., Ditsworth, D., Keith, B., Brown,
495 E. J., Nathanson, K. L., and Simon, M. C. (2009) HIF2alpha inhibition promotes p53
496 pathway activity, tumor cell death, and radiation responses. *Proc Natl Acad Sci U S A* **106**,
497 14391-14396
- 498 35. Rankin, E. B., Biju, M. P., Liu, Q., Unger, T. L., Rha, J., Johnson, R. S., Simon, M. C.,
499 Keith, B., and Haase, V. H. (2007) Hypoxia-inducible factor-2 (HIF-2) regulates hepatic
500 erythropoietin in vivo. *J Clin Invest* **117**, 1068-1077

- 501 36. Busceti, L. C., Ferese, R., Bucci, D., Ryskalin, L., Gambardella, S., Madonna, M.,
502 Nicoletti, F., and Fornai, F. (2019) Corticosterone Upregulates Gene and Protein
503 Expression of Catecholamine Markers in Organotypic Brainstem Cultures. *International*
504 *Journal of Molecular Sciences* **20**
- 505 37. Nguyen, P., Peltsch, H., de Wit, J., Crispo, J., Ubriaco, G., Eibl, J., and Tai, T. C. (2009)
506 Regulation of the phenylethanolamine N-methyltransferase gene in the adrenal gland of
507 the spontaneous hypertensive rat. *Neurosci Lett* **461**, 280-284
- 508 38. Newell-Price, J., Bertagna, X., Grossman, A. B., and Nieman, L. K. (2006) Cushing's
509 syndrome. *The Lancet* **367**, 1605-1617
- 510 39. Ramasz, B., Kruger, A., Reinhardt, J., Sinha, A., Gerlach, M., Gerbaulet, A., Reinhardt, S.,
511 Dahl, A., Chavakis, T., Wielockx, B., and Grinenko, T. (2019) Hematopoietic stem cell
512 response to acute thrombocytopenia requires signaling through distinct receptor tyrosine
513 kinases. *Blood* **134**, 1046-1058
- 514 40. Zieseniss, A. (2014) Hypoxia and the modulation of the actin cytoskeleton - emerging
515 interrelations. *Hypoxia (Auckl)* **2**, 11-21
- 516 41. Sormendi, S., Deygas, M., Sinha, A., Krüger, A., Kourtzelis, I., Le Lay, G., Bernard, M.,
517 Sáez, P. J., Gerlach, M., Franke, K., Meneses, A., Kräter, M., Palladini, A., Guck, J.,
518 Coskun, Ü., Chavakis, T., Vargas, P., and Wielockx, B. (2020) HIF2 α is a Direct Regulator
519 of Neutrophil Motility. *bioRxiv*
- 520 42. Wagegg, M., Gaber, T., Lohanatha, F. L., Hahne, M., Strehl, C., Fangradt, M., Tran, C. L.,
521 Schönbeck, K., Hoff, P., Ode, A., Perka, C., Duda, G. N., and Buttgerit, F. (2012) Hypoxia
522 Promotes Osteogenesis but Suppresses Adipogenesis of Human Mesenchymal Stromal
523 Cells in a Hypoxia-Inducible Factor-1 Dependent Manner. *PLOS ONE* **7**, e46483
- 524 43. Thomas, L. W., and Ashcroft, M. (2019) Exploring the molecular interface between
525 hypoxia-inducible factor signalling and mitochondria. *Cell Mol Life Sci* **76**, 1759-1777
- 526 44. Pignatti, E., Leng, S., Yuchi, Y., Borges, K. S., Guagliardo, N. A., Shah, M. S., Ruiz-Babot,
527 G., Kariyawasam, D., Taketo, M. M., Miao, J., Barrett, P. Q., Carlone, D. L., and Breault,
528 D. T. (2020) Beta-Catenin Causes Adrenal Hyperplasia by Blocking Zonal
529 Transdifferentiation. *Cell Rep* **31**, 107524

- 530 45. Brattsand, R., and Linden, M. (1996) Cytokine modulation by glucocorticoids:
531 mechanisms and actions in cellular studies. *Alimentary Pharmacology & Therapeutics* **10**,
532 81-90
- 533 46. Wurzburger, M. I., Prelevic, G. M., Brkic, S. D., Vuckovic, S., and Pendic, B. (1986)
534 Cushing's syndrome--transitory immune deficiency state? *Postgrad Med J* **62**, 657-659
- 535 47. Smith, L. K., and Cidlowski, J. A. (2010) Glucocorticoid-Induced Apoptosis of Healthy
536 and Malignant Lymphocytes. In *Neuroendocrinology - Pathological Situations and*
537 *Diseases* pp. 1-30
- 538 48. Lee, Y., Yi, H. S., Kim, H. R., Joung, K. H., Kang, Y. E., Lee, J. H., Kim, K. S., Kim, H.
539 J., Ku, B. J., and Shong, M. (2017) The Eosinophil Count Tends to Be Negatively
540 Associated with Levels of Serum Glucose in Patients with Adrenal Cushing Syndrome.
541 *Endocrinol Metab (Seoul)* **32**, 353-359
- 542 49. Ronchetti, S., Ricci, E., Migliorati, G., Gentili, M., and Riccardi, C. (2018) How
543 Glucocorticoids Affect the Neutrophil Life. *Int J Mol Sci* **19**
- 544 50. Lai, K. P., Li, J. W., Tse, A. C., Chan, T. F., and Wu, R. S. (2016) Hypoxia alters
545 steroidogenesis in female marine medaka through miRNAs regulation. *Aquat Toxicol* **172**,
546 1-8
- 547 51. Meneses, A. M., and Wielockx, B. (2016) PHD2: from hypoxia regulation to disease
548 progression. *Hypoxia (Auckl)* **4**, 53-67
- 549 52. Yun, Z., Maecker, H. L., Johnson, R. S., and Giaccia, A. J. (2002) Inhibition of PPAR
550 gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for
551 regulation of adipogenesis by hypoxia. *Dev Cell* **2**, 331-341
- 552 53. Fecher, R. A., Horwath, M. C., Friedrich, D., Rupp, J., and Deepe, G. S., Jr. (2016) Inverse
553 Correlation between IL-10 and HIF-1alpha in Macrophages Infected with *Histoplasma*
554 *capsulatum*. *J Immunol* **197**, 565-579
- 555 54. Azhar, S., Dong, D., Shen, W. J., Hu, Z., and Kraemer, F. B. (2020) The role of miRNAs
556 in regulating adrenal and gonadal steroidogenesis. *J Mol Endocrinol* **64**, R21-R43
- 557 55. Hu, Z., Shen, W. J., Kraemer, F. B., and Azhar, S. (2017) Regulation of adrenal and ovarian
558 steroidogenesis by miR-132. *J Mol Endocrinol* **59**, 269-283

- 559 56. Zhang, G., Zou, X., Liu, Q., Xie, T., Huang, R., Kang, H., Lai, C., and Zhu, J. (2018) MiR-
560 193a-3p functions as a tumour suppressor in human aldosterone-producing adrenocortical
561 adenoma by down-regulating CYP11B2. *Int J Exp Pathol* **99**, 77-86
- 562 57. Agrawal, R., Pandey, P., Jha, P., Dwivedi, V., Sarkar, C., and Kulshreshtha, R. (2014)
563 Hypoxic signature of microRNAs in glioblastoma: insights from small RNA deep
564 sequencing. *BMC Genomics* **15**, 686
- 565 58. Coutinho, A. E., and Chapman, K. E. (2011) The anti-inflammatory and
566 immunosuppressive effects of glucocorticoids, recent developments and mechanistic
567 insights. *Mol Cell Endocrinol* **335**, 2-13
- 568 59. Weiner, I. D. (2013) Endocrine and hypertensive disorders of potassium regulation:
569 primary aldosteronism. *Semin Nephrol* **33**, 265-276
- 570 60. Saffar, A. S., Ashdown, H., and Gounni, A. S. (2011) The molecular mechanisms of
571 glucocorticoids-mediated neutrophil survival. *Curr Drug Targets* **12**, 556-562
- 572 61. Kato, T., Takeda, Y., Nakada, T., and Sendo, F. (1995) Inhibition by dexamethasone of
573 human neutrophil apoptosis in vitro. *Nat Immun* **14**, 198-208

574

575

576 **Figure Legends**

577

578 **Figure 1. Characterization of the Akr1b7:cre-P2H1^{ff/ff} mouse line with cortex-specific**
579 **targeting of hypoxia pathway proteins.** A: Representative immunofluorescent image of anti-
580 GFP stained (GFP+) area in the adrenal cortex of the Akr1b7:cre-mTmG mouse line. Region
581 enclosed within the white dotted line represents the medulla and it demarcates the medulla from
582 the cortex (scale bar, 100 μ m). B: qPCR-based mRNA expression analysis of PHD2 and HIF1 α in
583 entire adrenal tissue from P2H1 mice and WT littermates (n=10-13). Relative gene expression was
584 calculated using the 2^{-ddCt} method. The graphs represent data from 2 independent experiments.
585 C: Genomic PCRs for Akr1b7:cre (650bp), PHD2 LoxP (400bp), and PHD2 KO (350bp) in DNA
586 derived from whole adrenal glands of WT and P2H1 mice. D-E: Relative gene expression analysis
587 using mRNA from the entire adrenal tissue in P2H1 mice and their WT counterparts (n=10-13).
588 All graphs represent data from 2 independent experiments. F: Representative images
589 (magnification 20x) of paraffin sections of adrenal glands (H&E) from 8-week old WT and P2H1
590 mice (scale bars represent 100 μ m). G: Representative immunofluorescent images of CD31⁺
591 endothelial cell staining in adrenal gland sections from WT and P2H1 mice (scale bars represent
592 50 μ m). Graph in the right-side panel represents quantification of CD31⁺ area as a fraction of total
593 tissue area. Each data point represents a single measurement of the cortical area in the adrenal
594 gland (collection of n=6 vs 11 individual mice). H: Representative images of cryo-sections of WT
595 and P2H1 adrenal glands (H&E) (scale bars represent 50 μ m). Graph in the right-side panel
596 represents the normalized average size of an individual lipid droplet per section of adrenal gland
597 tissue in WT versus P2H1 mice. Measurements were made from 6 sections per mouse. (n=8
598 individual adrenals per genotype). The graphs in panels G and H are representative of 2
599 independent experiments. Statistical significance was defined using the Mann-Whitney U test
600 (*p<0.05; **p<0.005; ***p<0.001; ****p<0.0001).

601

602 **Figure 2: Adrenal cortex-specific loss of PHD2 and HIF1 leads to enhanced steroidogenesis**
603 **in P2H1 mice.** A: Box and whisker plots showing steroid hormone measurements in adrenal
604 glands from WT mice and compared to littermate P2H1 mice (n=20-31 individual adrenal glands).
605 B: Box and whisker plots showing steroid hormone measurements in the plasma of individual mice
606 (n=5-17). All data were normalized to average measurements in WT mice. The graphs are a

607 representative result of at least 3 independent experiments. Statistical significance was defined
608 using the Mann-Whitney U test (* $p < 0.05$; ** $p < 0.005$).

609 **Figure 3: Gene expression analysis of P2H1 adrenocortical cells.** A: Gene expression analysis
610 of enzymes involved in the steroidogenesis pathway using mRNA from whole adrenals from P2H1
611 mice and WT counterparts (n=10-13). All graphs are the result of 2 independent experiments.
612 Statistical significance was defined using the Mann-Whitney U test (* $p < 0.05$; ** $p < 0.005$). B:
613 Schematic overview of the RNAseq approach which compared sorted GFP⁺ cells from WT
614 controls and P2H1 mice (n=3). C: Gene signature analysis using Enrichr. D. Gene set enrichment
615 analyses (GSEA) showed positive signatures for steroidogenesis related pathways. E: prominent
616 HIF-related pathways. F: the β -catenin nuclear pathway.

617 **Figure 4: Immune system changes in P2H1 mice.** A: Box and whisker plots representing levels
618 of pro/anti-inflammatory cytokines measured in the plasma of P2H1 mice and WT littermate
619 controls (n=7-12). All data were normalized to the average value seen in WT mice. Each dot
620 represents data from one animal. B: Box and whisker plots showing percentage lymphocytes and
621 eosinophils in circulation which revealed reduced fractions in P2H1 mice compared to WT
622 controls. C: Greater numbers of circulating neutrophils in P2H1 mice compared to WT littermates.
623 All graphs represent pooled results of 2 independent experiments. Statistical significance for
624 cytokines in panels A and B was defined using the Mann-Whitney U test, except for TNF α , where
625 the Unpaired t test with Welch's correction was used after verifying data normality. (* $p < 0.05$;
626 ** $p < 0.005$; *** $p < 0.001$).

627 **Figure 5: Adrenal cortex-specific loss of PHD2 and PHD3 leads to reduced steroidogenesis**
628 **in mice.** A: Box and whisker plots showing steroid hormone levels in the adrenal glands of WT
629 mice and compared to that of littermate P2H1 mice (n=14-16 individual adrenal glands). B: Box
630 and whisker plots showing steroid hormone measurements in the plasma of individual mice (n=10-
631 12). All data were normalized to the average value of WT mice and graphs are representative of at
632 least 3 independent experiments. Statistical significance was defined using the Mann-Whitney U
633 test for progesterone, 11-deoxycorticosterone, and 18-OH corticosterone. Unpaired t test with
634 Welch's correction was used for corticosterone and aldosterone after verification of data normality
635 (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$).

636 **Figure 6: Inverse regulation of steroidogenesis in P2P3 mice compared to P2H1 mice** A: Gene
637 expression analysis of enzymes involved in the steroidogenesis pathway in P2P3 mice and their
638 WT counterparts (n=12-13) was performed in mRNA from entire adrenal glands. All graphs
639 represent pooled data from at least 3 independent experiments. Statistical significance was defined
640 using the Mann-Whitney U test (*p<0.05). B: Relative expression profile of all genes analyzed
641 from the adrenal glands of P2H1 and P2P3 mice and compared to their respective WT littermates.
642 Statistical significance was defined using an unpaired multiple t-test (n=13; Benjamini, Krieger
643 and Yekutieli method; *p<0.0001 for all individual genes). C: schematic overview of all changes
644 in adrenocortical enzymes and their corresponding hormones and intermediates reported here in
645 P2H1 (red) and P2P3 (yellow) mice.

FIGURE 1

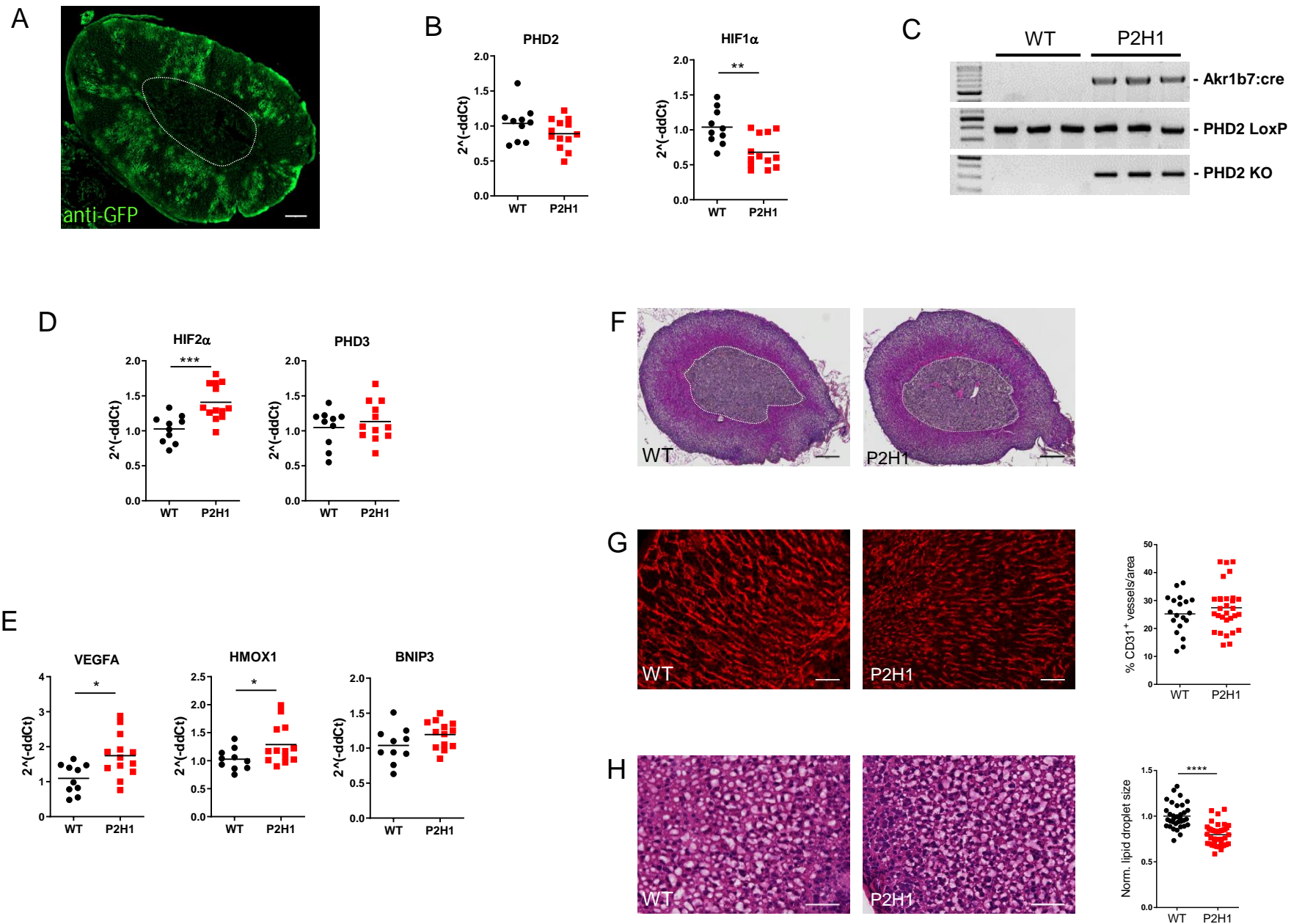
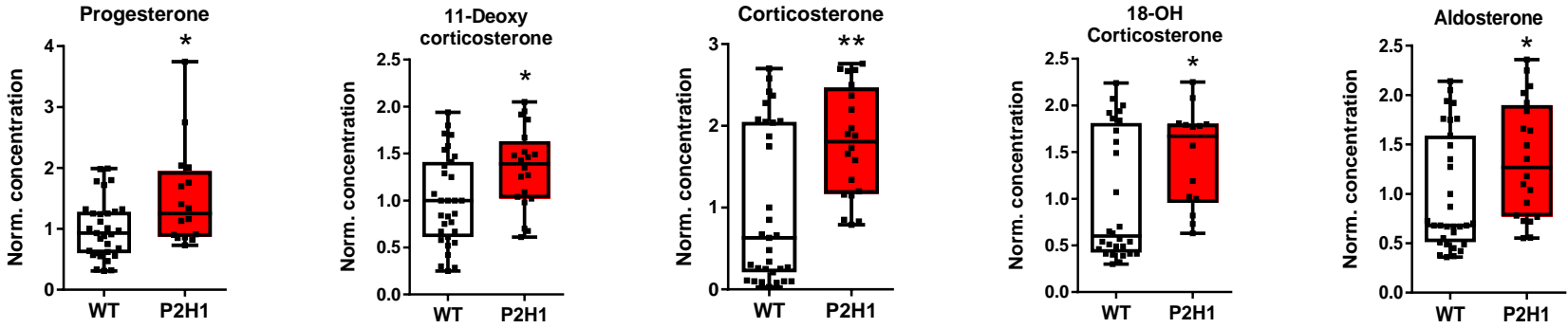


FIGURE 2

A

Adrenal gland



B

Plasma

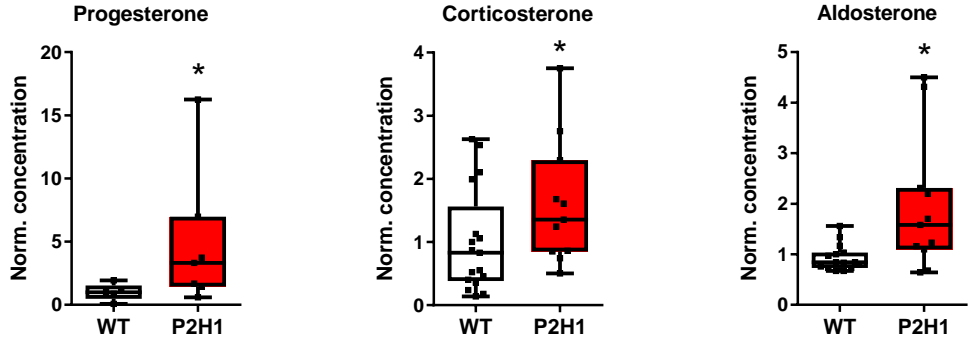
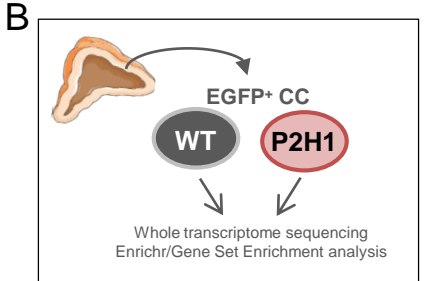
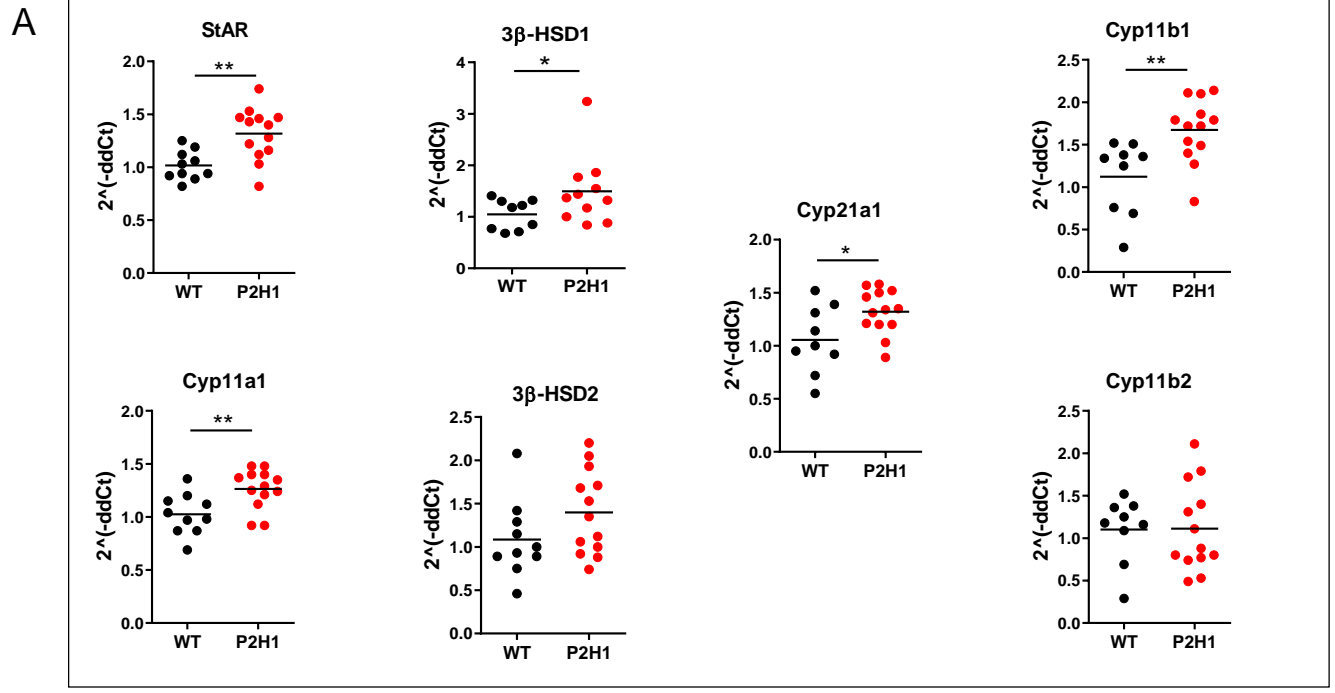


FIGURE 3



C

Gene signatures (Enrichr)	Genes	P-value
Glucocorticoid metabolic process (GO:0008211)	<i>HSD3B1, HSD3B2, CYP11b1, YWAH</i>	0.0008
Cholesterol Biosynthesis (WP103)	<i>FDPS, HMGCR, LSS</i>	0.03
Steroid hormone receptor binding (GO:0035258)	<i>SUMO1, TAF10, ARID5A, EP300, TRIP4, CTNNB1, WIPI1, MMS19, PARK7, RAN, YWAH</i>	0.0002
Corticotropin-releasing hormone signaling pathway (WP2355)	<i>ARRB1, CYP11b1, CTNNB1, ERN1, HSD3B1, HSD3B2, MAPK9, MAPK3</i>	0.004

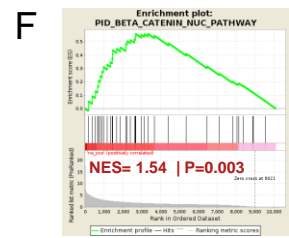
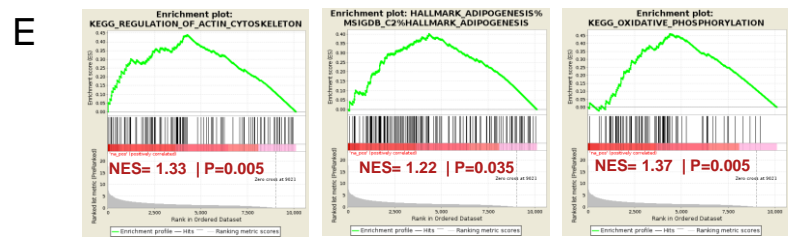
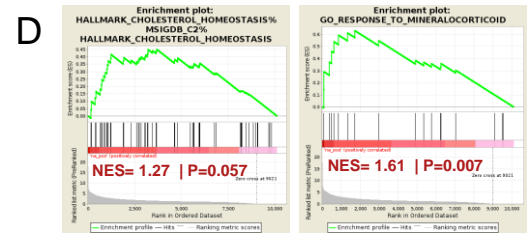


FIGURE 4

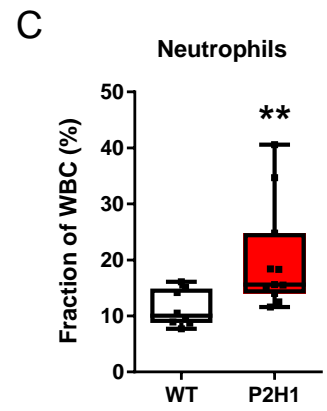
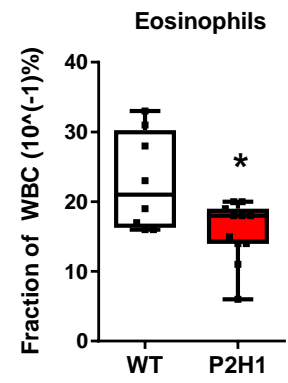
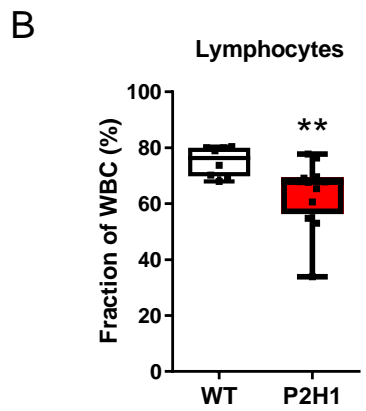
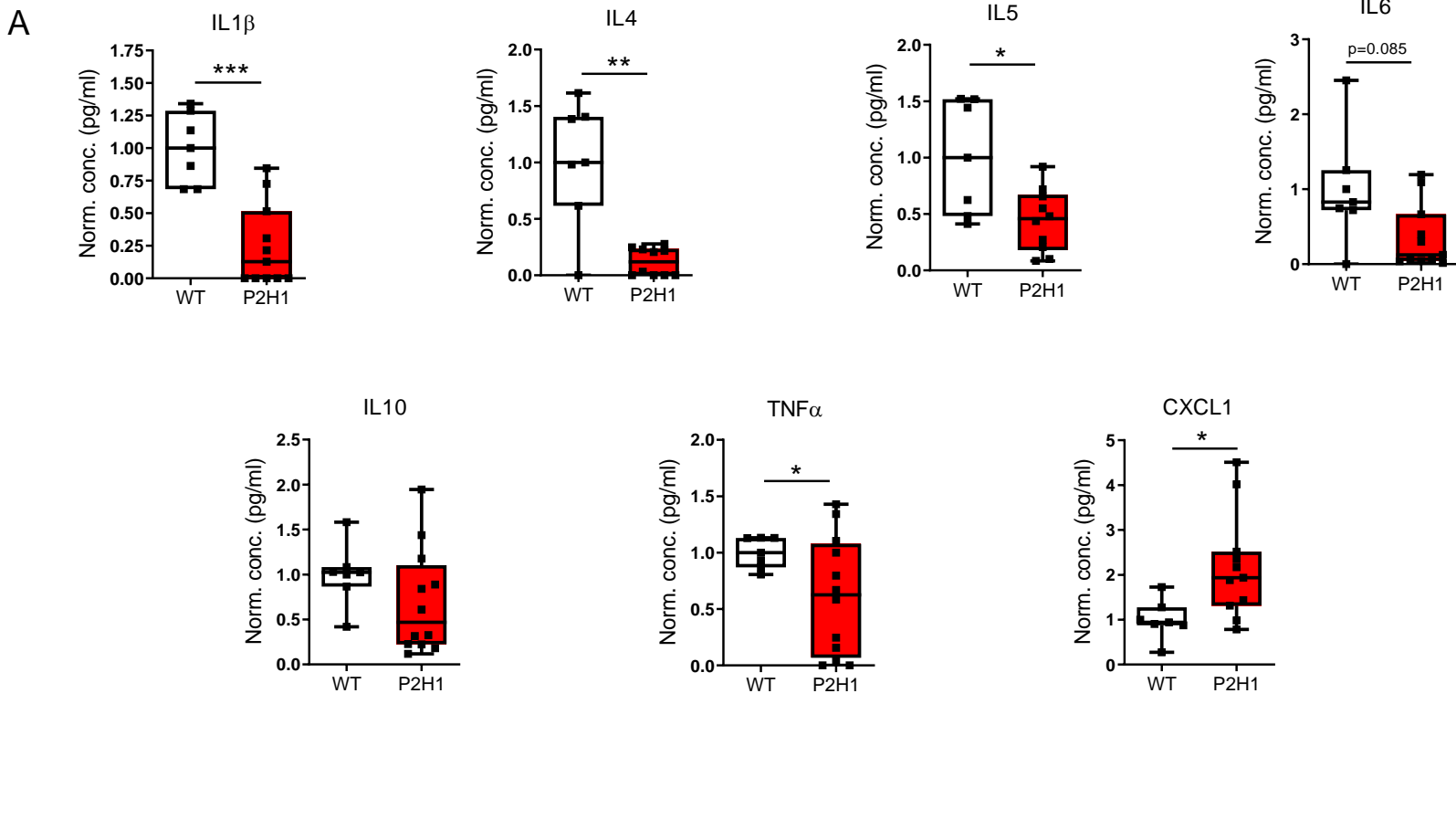
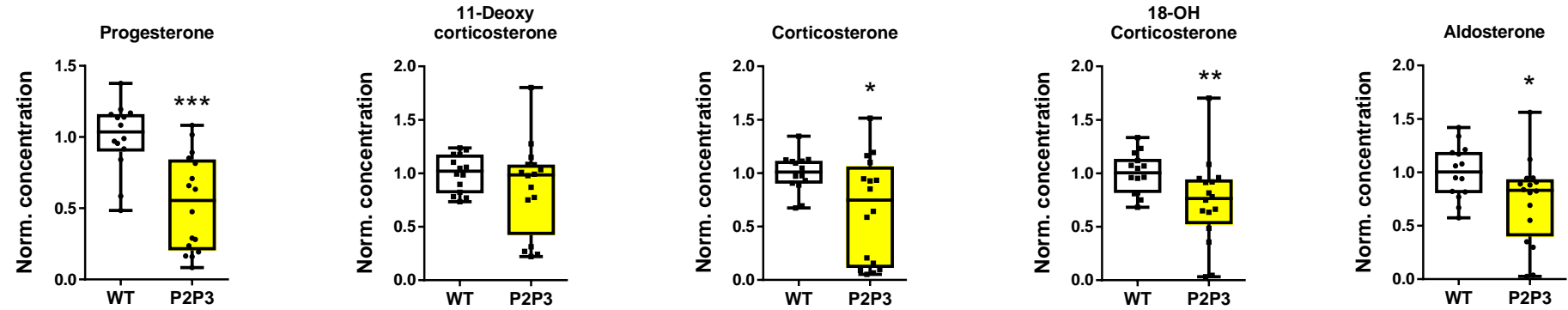


FIGURE 5

Adrenal gland

A



B

Plasma

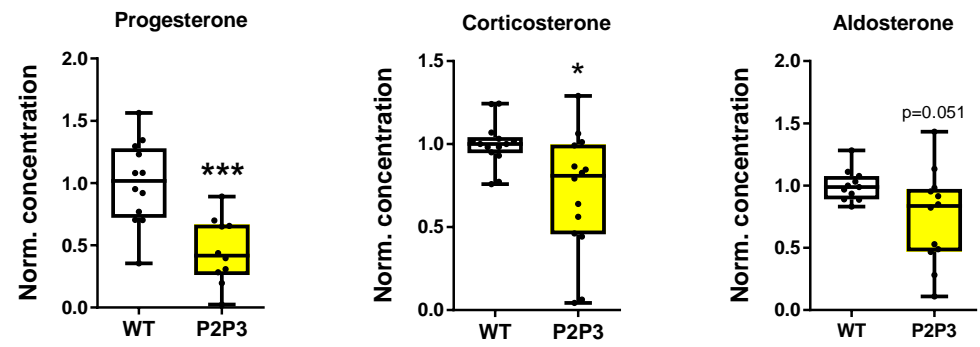
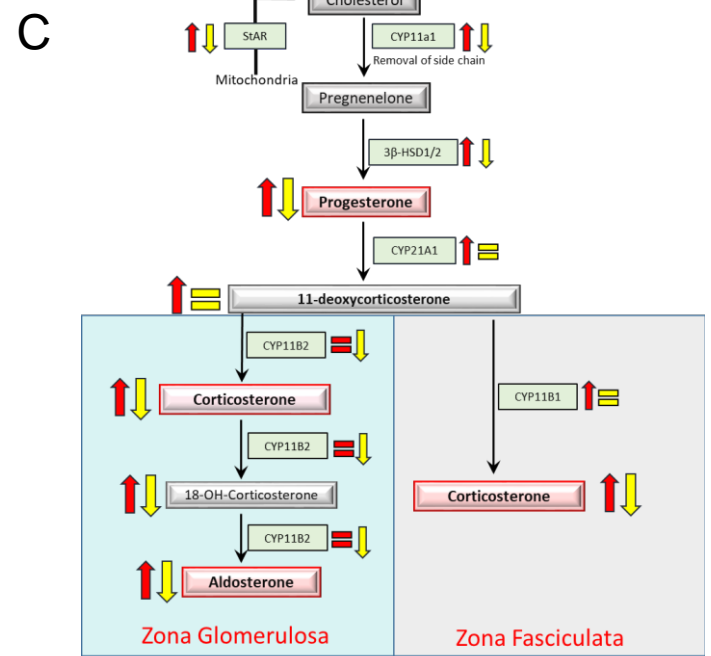
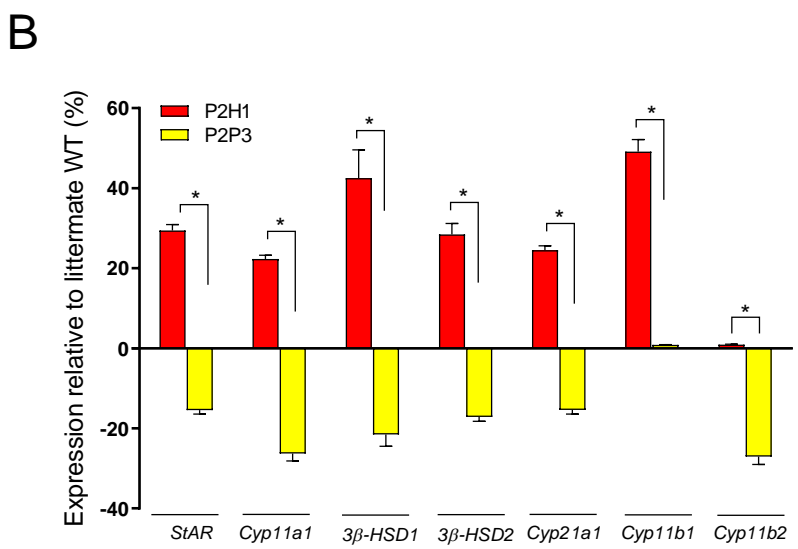
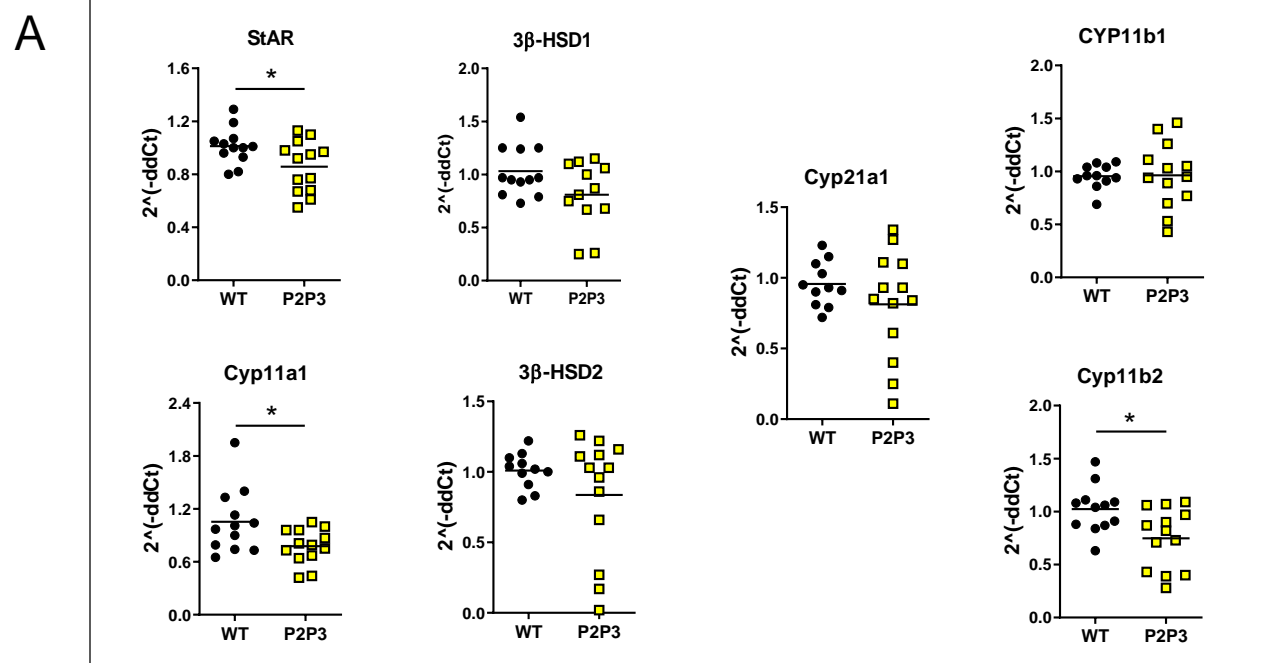
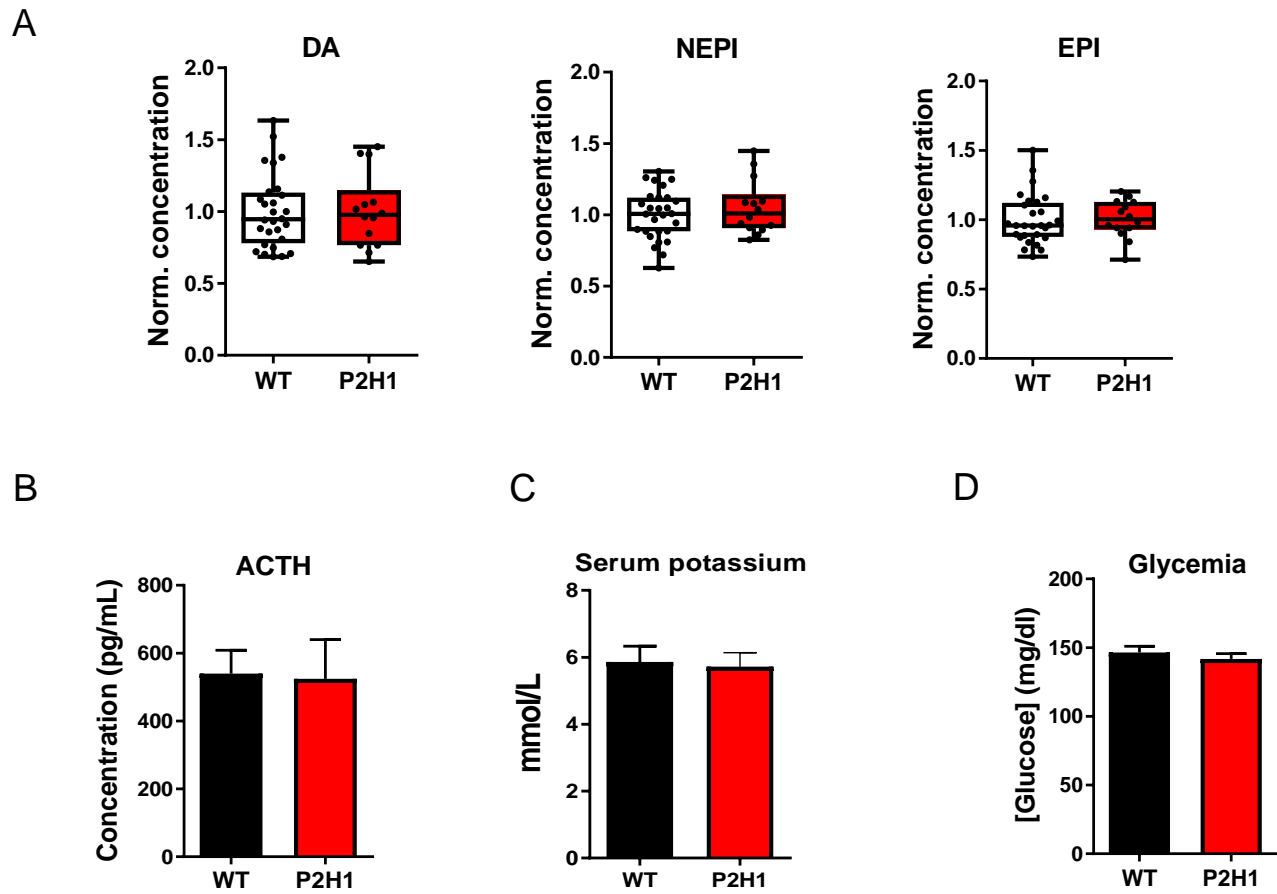


FIGURE 6

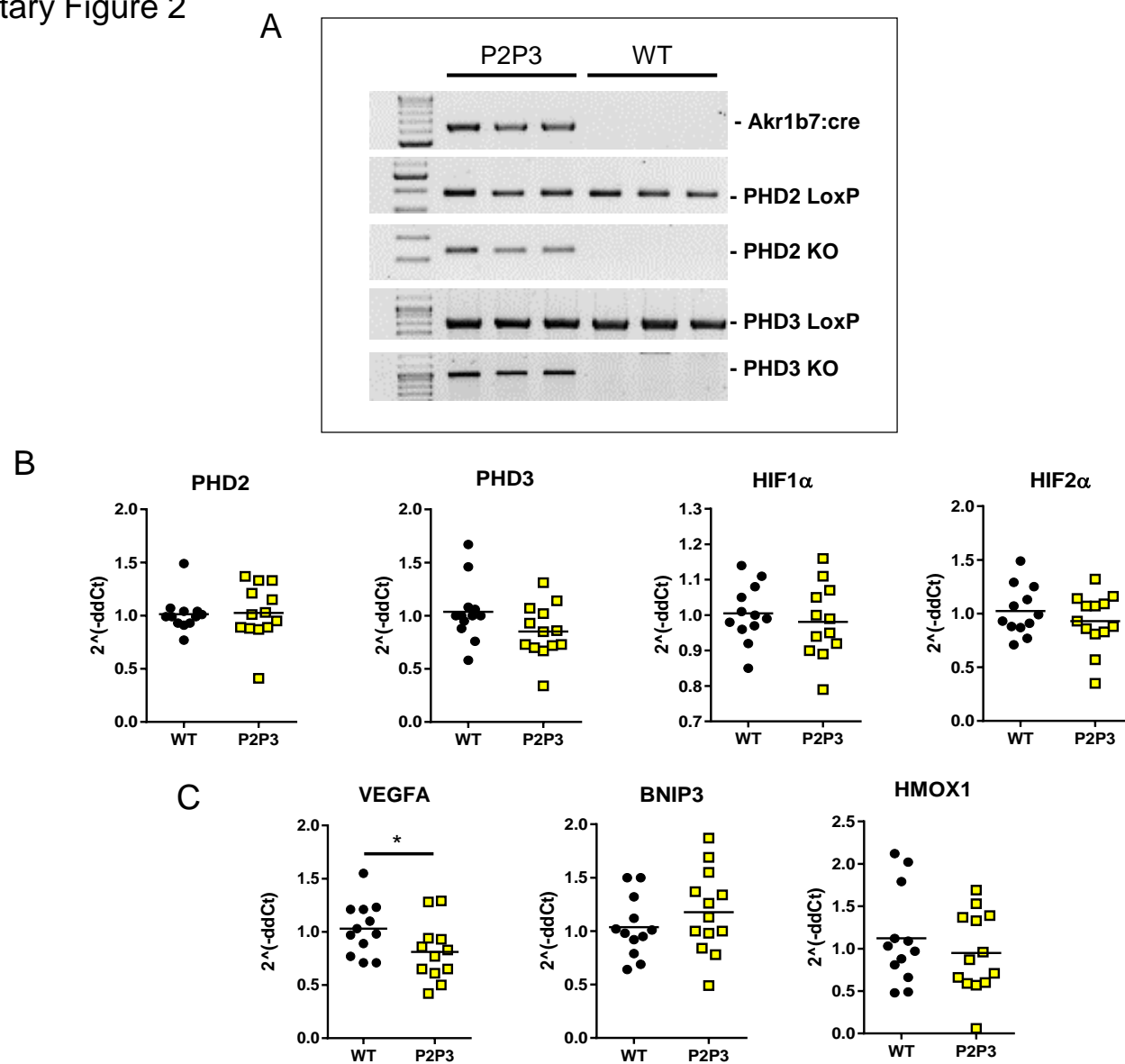


Supplementary Figure 1



Supplementary figure 1: Downstream effects of increased steroidogenesis.

A. Box and whisker plots showing normalized concentrations of all catecholamines (dopamine, norepinephrine (NEPI), and epinephrine (EPI) measured in entire adrenal glands of P2H1 mice and their WT counterparts (n=14-28). Bar graphs represent, respectively, B. Plasma ACTH concentration (n=7-14), C. potassium levels in the serum of WT vs P2H1 mice (n=9-11), D. Blood glucose levels in P2H1 mice vs WT littermate controls (n=5-8). Statistical significance was defined using the Mann-Whitney U test.



Supplementary figure 2: Genetic identification of the *Akr1b7:cre*-P2P3 strain. A. Genomic PCRs for *Akr1b7:cre*, *PHD2 LoxP* (400bp), *PHD2 KO* (350 bp), *PHD3 LoxP* (840bp) and *PHD2 KO* (1000bp) in entire adrenal gland tissue from P2P3 mice and their WT counterparts. B. Relative gene expression analysis by qPCR for *PHD2*, *PHD3*, *HIF1 α* and *HIF2 α* in mRNA from entire adrenal glands of P2P3 and WT counterparts (n=12-13). C. qPCR as in panel B, but for *VEGFA*, *HMOX1*, *BNIP3* (E). Relative gene expression was calculated using the $2^{-(ddCt)}$ method. The graphs are a representative result of 3 independent experiments. Statistical significance was defined using the Mann-Whitney U test – one tailed (*p<0.05).

Table I : Primers for genotyping of mouse strains

Primer name	Primer sequence (5' – 3')
Akr1b7_Fw	GAAAGCAGGCATTTTCATCTGC
Akr1b7_Rev	CAGGGTGTTATAAGCAATCCC
mPHD2_exo2	CGCATCTTCCATCTCCATTT
mPHD2_Intron1	CTCACTGACCTACGCCGTGT
mPHD2_Intron1	CTCACTGACCTACGCCGTGT
mPHD2_Intron3.3	GGCAGTGATAACAGGTGCAA
PHD3mFw	ATGGCCGCTGTATCACCTGTAT
PHD3mRev	CCACGTAACTCTAGAGCCACTGA
PHD3Rec55	CTCAGACCCCCTAAGTATGT
PHD3mouseRev	CCACGTAACTCTAGAGCCACTGA
HIF1a.For	GCAGTTAAGAGCACTAGTTG
HIF1a.Rev	GGAGCTATCTCTCTAGACC

Table II: Primers for qPCR analysis

Primer name	Primer sequence (5' – 3')
StAR_Fwd	TCGCTACGTTCAAGCTGTGT
StAR_Rev	GCTTCCAGTTGAGAACCAAGC
Cyb11a1_Fwd	AGGTCCTTCAATGAGATCCCTT
Cyb11a1_Rev	TCCCTGTAAATGGGGCCATAC
3 β _HSD1_Fwd	TGGACAAAGTATTCCGACCAGA
3 β _HSD1_Rev	GGCACACTTGCTTGAACACAG
3 β _HSD2_Fwd	GGTTTTTGGGGCAGAGGATCA
3 β _HSD2_Rev	GGTACTGGGTGTCAAGAATGTCT
mCyp21a1_Fwd	AACAGAACCATTGAGGAGGCCTTGA
mCyp21a1_Rev	TCTCCAAAAGTGAGGCAGGAGATGA
Cyp11b1_Fwd	CAGATTGTGTTTGTGACGTTGC
Cyp11b1_Rev	CGGTTGAAGTACCATTCTGGC
mCYP11b2_Fwd	CAGTGGCATTGTGGCGGA ACTAATA
mCYP11b2_Rev	GGTCTGACATGGCCTTCTGAGGATT
HIF1 α _Fwd	GGCGAGAACGAGAAGAAAAA
HIF1 α _Rev	AAGTGGCAACTGATGAGCAA
mPHD2_Fwd	AAGCCCAGTTTGCTGACATT
mPHD2_Rev	CTCGCTCATCTGCATCAAAA
mPHD3_Fwd	GGCCGCTGTATCACCTGTAT
mPHD3_Rev	TTCTGCCCTTTCTTCAGCAT
HIF2 α _Fwd	CTGAGGAAGGAGAAATCCCGT
HIF2 α _Rev	TGTGTCCGAAGGAAGCTGATG
HMOX1_Fwd	AAGCCGAGAATGCTGAGTTCA
HMOX1_Rev	GCCGTGTAGATATGGTACAAGGA
BNIP3_Fwd	TCCTGGGTAGAACTGCACTTC
BNIP3_Rev	GCTGGGCATCCAACAGTATTT
VEGFA_Fwd	GCACTGGACCCTGGCTTTAC
VEGFA_Rev	AACTTGATCACTTCATGGGACTTCT