LGR5 activates non-canonical Wnt-signaling and inhibits aldosterone production in the human adrenal

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44

Abstract:

Context. Aldosterone synthesis and cellularity in the human adrenal zona glomerulosa (ZG) is sparse and patchy, presumed due to salt excess. The frequency of somatic mutations causing aldosterone-producing adenomas (APA) may be a consequence of protection from cell loss by constitutive aldosterone production.

Objective. To delineate a process in human ZG which may regulate both aldosterone production and cell turnover.

Design. Comparison of 20 pairs of ZG and zona fasciculata (ZF) transcriptomes from adrenals adjacent to an APA (n=13) or a pheochromocytoma (n=7).

Interventions. Over-expression of top ZG gene (*LGR5*), or stimulation by its ligand (R-spondin-3).

Main Outcome Measures. 1) Transcriptome profile of ZG and ZF; 2) Aldosterone production, cell kinetic measurements, and Wnt signaling activity of *LGR5* transfected or R-spondin-3-stimulated cells.

Results. LGR5 was the top gene up-regulated in ZG (25-fold). The gene for its cognate ligand R-spondin-3, *RSPO3*, was 5-fold up-regulated. In total, 18 genes associated with the Wnt pathway were >2-fold up-regulated. ZG selectivity of *LGR5,* and its absence in most APAs, were confirmed by qPCR and immunohistochemistry. Both R-spondin-3 stimulation and *LGR5* transfection of human adrenal cells suppressed aldosterone production. There was reduced proliferation and increased apoptosis of transfected cells, and the non-canonical AP-1/Jun pathway was stimulated more than the canonical Wnt pathway (3-fold vs. 1.3-fold). ZG of adrenal sections stained positive for apoptosis markers.

Conclusion. LGR5 is the most selectively expressed gene in human ZG, and reduces aldosterone production and cell number. Such conditions may favour cells whose somatic mutation reverses aldosterone inhibition and cell loss.

Introduction

Since their discovery by Conn nearly 60 years ago, APAs have been regarded as infrequent – less than 1% of all hypertension; recent estimates of prevalence, however, have risen to several times this figure (1). Most APAs, though, are diagnosed too late for complete cure of hypertension (2), and there is a need for better-tolerated drugs that block the increased aldosterone production. Current treatments either compete with aldosterone for its receptor, or inhibit the Na^+ channel downstream. In addition to limitations of efficacy or selectivity, such drugs increase aldosterone production (3). Novel drug targets can be identified by the discovery of either a gene whose gain-of-function mutation increases aldosterone production in APAs, or pathways coupled to inhibition of aldosterone in the normal adrenal.

APAs are often a heterogeneous mixture of cells; paradoxically, the cells of classical Conn's tumours appear more like cortisol-producing ZF cells than the supposedly aldosterone-producing cells of normal ZG (4,5). This paradox may be resolved by the frequent finding of small APAs (often missed on conventional adrenal imaging) that consist mainly of compact cells, and express genes, such as *NPNT* (encoding for nephronectin), not expressed in the ZF of human adrenals (6,7). Exome-sequencing of ten such 'ZG-like' APAs led us to identify somatic mutations in three genes, encoding $Ca_V1.3$, Na^+, K^+-ATPase, and β-catenin (7); no ZG-like APAs have yet been found to have one of the *KCNJ5* mutations common in larger, more ZF-like, APAs (8-11). The small size of many of these ZG-like APAs, sometimes documented as unchanged in serial CT scans over many years, inferred limited growth before diagnosis. This observation suggested to us that cells in a ZG-like APA may derive a survival advantage from constitutive aldosterone production, rather than cell division, thus explaining the frequency of somatic mutations – 19 different mutations in $Ca_v1.3$ alone – which switch on constitutive aldosterone production (7,11,12).

In most species, ZG is unusually proliferative for an endocrine tissue, stimulated by the renin-angiotensin II response to salt depletion; but in typically salt-loaded humans, the reverse appears true (13,14). We

94   hypothesized the existence of a local process, in addition to withdrawal of renin, which might suppress ZG

95   cell steroidogenesis or proliferation when not required. Previous comparisons of human adrenal ZG and

96   ZF have depended on techniques, such as immunohistochemistry (IHC) and in situ hybridisation that

97   require investigation of specific candidate molecules (15,16). The striking difference in appearance,

98   following cresyl violet staining, between lipid-poor and lipid-rich cells of ZG and ZF (6), enabled us to

99   carry out laser capture microdissection of snap-frozen fresh adrenal sections; and thus whole transcript

100 expression analysis and profiling of ZG and ZF. The resulting top ZG gene identified, *LGR5*, is postulated

101 to be key to this local suppression process of ZG cell activity, highlighting both a possible novel drug

102 target that can inhibit aldosterone production, and a possible link between the frequency of APAs and

103 aldosterone secretion.

104

105 **Materials and Methods**

106 *Study design*

107 To determine genes and pathways regulating aldosterone production, transcriptome profiles of supposedly

108 aldosterone-producing ZG was compared with its paired cortisol-producing ZF from 20 human adrenals.

109 The detail method of the microarray assay and clinical demographics of the patients involved is as

110 described previously (17), and briefly described in **Supplementary Methods**. Adrenals were collected

111 within 30 minutes of adrenalectomy (due to an APA or pheochromocytoma) with local ethical approval

112 and informed consent from the patients. Adrenals immediately underwent collagenase dispersion for

113 primary cell culture, or were stored at -70ºC for RNA analyses, histology and IHC.

114

115 Comparison of ZG and ZF transcriptome profile led us to hypothesise an important role for *LGR5* (the top

116 ZG gene identified) and the Wnt signaling pathway in the regulation of aldosterone production in the

117 human adrenal. The hypothesis was tested in the human adrenocortical carcinoma cell line, H295R, and in

118 primary human adrenal cell cultures. The primary outputs of cell culture experiments were aldosterone

119 secretion into the supernatant of cultures, and qPCR for *CYP11B2* expression (encoding for the enzyme

120 aldosterone synthase). These were measured in response to key proteins modulating the Wnt signaling

121 pathway, either administered externally, or by transfection of cDNA constructs.

122

123 *Laser capture microdissection (LCM)*

124 LCM was used to obtain samples of ZF and ZG cells from adrenal tissue adjacent to APAs or

125 pheochromocytomas as previously described (5). For differentiation of ZG from ZF, sections were stained

126 with cresyl violet using the LCM Staining Kit (AM1935, Ambion).

127

128 *Quantification of mRNA expression*

129 Cells were kept in RNAlater and TRIzol (both from Ambion) until extracted for RNA. Total DNA-free

130 RNA was isolated using the PureLink® RNA Mini Kit with the PureLink® DNase Set (Life

131 Technologies) according to manufacturer's instructions. Reverse transcription was performed using the

132 Reverse Transcriptase System (Promega) with a 1:1 mixture of random hexamer and oligo-dT primers

133 according to manufacturer's instructions. mRNA expression of genes of interest was quantified using

134 TaqMan probes (Applied Biosystems), and *CYP11B2* expression was quantified using custom-made

135 TaqMan probes that had been validated for specificity (20). The housekeeping 18S rRNA (Applied

136 Biosystems) was used for normalization.

137

138 *Immunohistochemistry (IHC)*

139 IHC was performed on formalin-fixed, paraffin-embedded human adrenal sections (4 μm) using a

140 chromogen based detection system, 3,3′-diaminobenzidine (DAB). Commercial antibodies to LGR5

141 (#TA503316, Origene; 1:300 dilution), β-catenin (#610154, BD Transduction Laboratories; 1:300

142 dilution), LEF1 (#ab53293, Abcam plc; 1:100), c-JUN (#ab32137, Abcam; 1:250 dilution) and phospho-c-

JUN (#SC-822, Santa Cruz Biotechnology; 1:100) were used on slides selected from patient adrenals included in the microarray. Negative controls, in which primary and then secondary antibodies were omitted were used, and resulted in absence of staining. Slides were counter-stained with haematoxylin solution (Harris modified; Sigma-Aldrich). To negate cross reactivity of LGR5 antibody with LGR5's close homologs, LGR4 and LGR6, additional anti-LGR5 antibodies (Novus Biologicals NBP128904, Abcam ab75732) that binds to epitopes which through Blast analysis (NCBI) aligned specifically to *LGR5* but differs in sequence to *LGR4* and *LGR6,* were also tested in serial human adrenal sections.

Apoptosis was examined using TUNEL staining visualized by a rhodamine-labeled anti-digoxigenin antibody (#11093088910, Roche Diagnostics Corp) and proliferation was captured using an anti-Ki67 antigen (#M7240, Dako; 1: 400 dilution) according to manufacturer's instructions. Images were captured using a standard bright-field microscope, a U-TV1-X digital camera and CellD software (Olympus).

Cell culture experiments

H295R human adrenocortical carcinoma cells and primary adrenocortical cells were cultured in DMEM/Nutrient F-12 Ham supplemented with 10% FCS, 100 U of penicillin, 0.1 mg/ml streptomycin, 0.4 mM L-glutamine and insulin-transferrin–sodium selenite medium (ITS) at 37 °C in 5% CO_2 as previously described (17). Cells were transfected with vector controls pCMV6-AC-GFP (Origene) or pcDNA3.1/His A (Invitrogen) and GFP tagged *LGR5* (Origene; 600ng/ul). Transfections were carried out using Cell Line Nucleofector Kit R (VACA-1001; Lonza) according to the manufacturers' guidelines. Cell fate assays were performed in *LGR5* transfected cells as described in **Supplementary Methods**. Cells were silenced using either ON-TARGETplus Non-Targeting siRNA as control or SMART pool: ON-TARGET plus *LGR5* siRNA (Dharmacon; 50nM). Transfected cells were plated into 24-well plates (100,000 cells per well) in 0.25 ml of growth medium. After 24h of transfection, H295R cells were serum deprived in unsupplemented medium for 24h, further drug treatments were carried out with 8hr incubation

168   from this point. Recombinant hR-spondin-3 (Sino Biological Inc and R&D Systems) was used where

169   specified at 100 nM. Supernatants for aldosterone concentration measurement were collected after

170   respective 8h treatments, and cells were harvested for analysis of mRNA. Aldosterone concentrations

171   were determined by a commercially available ^{125}I radioimmunoassay (Diagnostic Products Corp) as

172   detailed in **Supplementary Methods**.

173

174   *Wnt signaling pathway activity assays*

175   TCF/LEF response was measured using Cignal TCF/LEF Reporter (luc) Kit (SABiosciences). AP1/JUN

176   pathway response was measured using a construct created by cloning an oligo containing 7 copies of the

177   AP1 binding element (TGACTAA) into a luciferase construct pGL4.10 (luc2) (Promega) at its KpnI and

178   XhoI sites. pRL-TK was used for normalisation (Promega). NFAT response was also measured using a

179   luciferase reporter construct (plasmid #10959, Addgene).

180

181   Plasmids modulating Wnt signaling pathway were used as comparator to show effect on aldosterone of

182   activation or suppression of the canonical pathway. To activate the pathway, pcDNA3 ΔN47 β-catenin

183   was used; and to suppress the pathway, pcDNA/Myc ΔN TCF4 (both from Addgene). Where indicated the

184   vector control, pcDNA3.1-V5His (Invitrogen) was used. To measure luciferase (and renilla for

185   normalization) Dual-Glo® Luciferase Assay System (Promega) was used according to the manufacturer's

186   protocol. The selective porcine inhibitor, LGK-974 (1μM, Selleck Chemicals) (21), and the selective c-

187   Jun N-terminal kinase (JNK) inhibitor, JNK-IN-8 (1μM, Selleck Chemicals)(22), were used to analyse the

188   effect of blocking respectively, all Wnts secretion or just the non-canonical Wnt signaling pathway.

189

190   *Statistical analysis*

191   Unless otherwise stated, results are expressed as mean values ± SEM. Statistical analysis was performed

192   using the Student's unpaired *t* test or analysis of variance on standard statistical software.

193

Results

LGR5 and several other genes associated with the Wnt signaling pathway are selectively and abundantly expressed in human adrenal ZG

Transcriptome analysis by Affymetrix microarray identified 213 genes, which were between 2- and 25-fold more abundant in human adrenal ZG than their paired ZF (**Supplemental Fig. 1**). The top 'hit' was *LGR5* (P=1.6x10^{-23}; **Fig. 1a and Table 1**)(17), which in several tissues is recognized as a stem-cell marker (23,24). The possibility of *LGR5* playing a key role in ZG was supported by finding that the expression of the gene for its cognate ligand R-spondin-3, *RSPO3,* was also up-regulated in ZG by 5-fold (P=3x10^{-11}). Overall, 18 of the 213 'ZG-genes' encode previously identified proteins associated with the Wnt signaling pathway (**Table 1**). By contrast, only 1 of 117 'ZF-genes' (those >2-fold more abundant in ZF than ZG) encodes for a known protein from this Wnt signaling pathway.

The expression of *LGR5* in ZG was confirmed by qPCR as 306-fold up-regulated compared to ZF (n=18, P=3.01x10^{-9}). By contrast, in 12 APAs qPCR found expression of *LGR5* to be only 1.7% of that in the adjacent ZG. mRNA results were confirmed at protein level through IHC using three commercial antisera raised against differing epitopes (**Supplemental Fig. 2a**). IHC of key Wnt genes (β-catenin, the central signaling molecule of the canonical Wnt pathway; LEF1, the nuclear transcription factor activated by β-catenin; c-Jun, the target of the polarity non-canonical Wnt pathways; and phospho-c-Jun) in serial human adrenal sections showed ZG-selective distribution with co-localization of LGR5 (**Fig. 1b and Supplemental Fig. 2b**). In addition to the ZG-selective staining, some adrenals showed radial streaking into ZF for LGR5 in serial sections, reminiscent of the pattern previously associated with centripetal migration of ZG cells (**Supplemental Fig. 2a**) (25,26). Staining of the positive control, small intestine supported the specificity of LGR5 staining in the adrenals (**Supplemental Fig. 2c**). Most β-catenin staining was membranous or cytoplasmic, with a few cells showing the nuclear staining induced by

218 canonical Wnt activation **(Fig. 1b).** Microarray analysis detected neither of the canonical Wnt ligands,

219 Wnt1 or Wnt3a, as up-regulated in ZG, and their absolute expression in both adrenal zones were low.

220

221 *Transfection and treatment of LGR5 and R-spondin-3 show reduction of aldosterone secretion in H295R*

222 *cells and primary adrenals*

223 Functional studies, over-expression and silencing of *LGR5* and treatment with its cognate ligand R-

224 spondin-3, were performed on either the immortalised human adrenocortical H295R cell line or normal

225 primary adrenal cells cultured from adrenalectomized adrenals of patients diagnosed with either primary

226 hyperaldosteronism or pheochromocytoma.

227

228 In both cell line and primary cultures, 100 nM of R-spondin-3 treatment caused a reduction in aldosterone

229 secretion and *CYP11B2* expression **(Fig. 2a-d)**. *LGR5* transfection similarly reduced aldosterone

230 production **(Fig. 2e, f)**. Conversely, silencing of *LGR5* caused an increase in aldosterone production and

231 *CYP11B2* transcription **(Fig. 2g, h)**. Some of the inhibition by R-spondin-3 and *LGR5* appeared due to

232 reduction in cell number as seen on protein assay. However, even after protein correction, there was a

233 reduction in aldosterone secretion, which was most marked for *LGR5*-transfected cells exposed to R-

234 Spondin-3 (56±5%, *P*=0.002 **Fig. 2i)**.

235

236 *LGR5 transfection in H295R cells shows reduction of cells*

237 We consistently noted a qualitative reduction in cell number and adhesion following *LGR5* transfection or

238 R-spondin-3 stimulation. Kinetic live-cell imaging of *LGR5* transfected cells directly quantified a 43±11%

239 reduction in the proliferation of H295R cells (P=1.5x10^{-5}) **(Fig. 3a)**, seen also in a cell viability assay

240 **(Supplemental Fig. 3).** These observations led us to investigate indices of proliferation and apoptosis in

241 sections of human adrenal cortex, and in *LGR5* transfected H295R cells. In other species, the accepted

242 model of adrenocortical cell fate has been of a unitary progenitor cell in the capsule that migrates

243 centripetally through ZG and ZF to zona reticularis (ZR), where it undergoes apoptosis (27,28). In the

244 absence of aldosterone production – e.g. in the *CYP11B2$^{-/-}$* mouse – the processes of migration and

245 apoptosis are accelerated (29). By contrast, in human adrenal we found that the main site of apoptosis is

246 ZG, as seen on both TUNEL and cleaved caspase 3 staining (**Fig. 3b and Supplemental Fig. 4**). Staining

247 of H295R cells with Annexin V and separation by flow cytometry quantified higher apoptosis in *LGR5*-

248 transfected cells compared to vector controls (54±3 % *vs*. 34±3 %; **Fig. 3c**).

249

250 *LGR5 activates AP1/Jun non-canonical Wnt pathway*

251 Previous studies have shown that canonical Wnt activation increases proliferation and aldosterone

252 production (15,30). We hypothesized that the inhibition of aldosterone and net cell loss is due to *LGR5*

253 inhibition of the canonical Wnt activation (31), though there have been previously postulated non-

254 canonical Wnt effects in human adrenal (32). In order to determine which is the more likely mechanism,

255 we co-transfected H295R cells with *LGR5* or a luciferase reporter with a promoter recognition site for

256 either TCF/LEF – the canonical Wnt transcription factors, or one of the non-canonical Wnt targets, AP-

257 1/Jun or NFAT (**Fig. 4 and Supplemental Fig. 5**). In addition, we also investigated the effect on

258 aldosterone production of a constitutively active or dominant negative canonical Wnt pathway through

259 transfection of a constitutively active β-catenin (ΔN47 β-catenin) or dominant negative TCF (ΔN TCF4);

260 and using small molecule inhibitors, we interrogated non-selective inhibition of Wnt signaling pathways

261 (through inhibition of the Wnt chaperone porcupine) and selective inhibition of the non-canonical Wnt

262 signaling pathway (through inhibition of JNK).

263

264 H295R cells, whose S45P mutant β-catenin is constitutively active, had the expected high basal levels of

265 TCF/LEF activity, with only slight further activation on the addition of the ΔN47 β-catenin construct (**Fig.**

266 **4b**). On the other hand, the ΔN TCF4 construct markedly reduced both TCF/LEF activity and aldosterone

267 production (**Fig. 4b, c**). In contrast, transfection of *LGR5* caused a slight increase in TCF/LEF activity

268 while decreasing aldosterone secretion (**Fig. 4b, c**). Supporting a predominant action of *LGR5* on a non-

269 canonical Wnt pathway (**Fig. 4d**), *LGR5* transfection increased AP-1/Jun activity by almost 3-fold and the

270 non-canonical selective JNK inhibitor, JNK-IN-8, ameliorated this increase (**Fig. 4e**). JNK-IN-8

271 inhibition, however, was insufficient to reverse *LGR5*'s mediated suppression of aldosterone secretion

272 **(Fig. 4f)**, suggesting that AP1/Jun is not the only non-canonical pathway involved. The consequences of

273 blocking Wnt signaling with the non-selective porcupine inhibitor, LGK974, had a similar inhibitory

274 effect on aldosterone production as was seen with the TCF ΔN construct **(Fig. 4e, f)**. *LGR5* had no

275 significant influence on the NFAT reporter construct (Fig. S7)

276

277 **Discussion**

278 We found the most-up-regulated gene in normal human ZG, compared to its adjacent ZF, to be *LGR5*, and

279 that its transfection into human adrenocortical cells results in inhibition of aldosterone production,

280 reduction in cell number, and activation of a non-canonical Wnt signaling pathway postulated as the

281 mediator of the *LGR5* response.

282

283 Wnt signaling has well-documented roles in normal adrenal development, adrenocortical tumours, and

284 H295R cells (30,32,33). However, no definite physiological role has been ascribed to Wnt in the

285 regulation of aldosterone, especially in humans (34). The dominant inhibitory role of Wnt that we have

286 found in human ZG helps to explain the lack of similarity between our microarray findings from the

287 current study and those reported from adrenal tissues in which aldosterone production is stimulated. For

288 example, none of the genes associated with the Wnt signaling pathway that were up-regulated in normal

289 human ZG were up-regulated in our previous microarray analyses of APAs – one comparing APAs with

290 adjacent adrenal, and the other comparing ZG-like APAs with more ZF-like APAs (6,7). Further, *LGR5*

291 was almost absent from adjacent APAs in the present study. Finally, only one of our 18 Wnt related genes

292 (*GPC3*) was similarly up-regulated in a microarray comparison of CYP11B2 positive cells in rat ZG with

293 adjacent ZF (35). We believe that the key difference between human ZG on the one hand, and either

294 adjacent APAs or rat ZG on the other, is that the dominant signaling pathway activated in the latter are

295 those which stimulate aldosterone production, whilst the reverse appears true of human ZG.

296

297 We found that, unlike its reported effects in other tissues (23,24), LGR5 in human adrenal cells stimulates

298 predominantly non-canonical pathways. The AP-1/Jun pathway, which was markedly stimulated by LGR5

299 transfection of H295R cells, influences cell migration (36). Its activation is also a measure for Rho/ROCK

300 regulation of both migration and polar cell polarity (see **Fig 3d)**. The latter has not been studied in human

301 ZG cells, but could be key to the formation of glomerular structure and tight junctions, as known genes

302 associated with these structures (*TJP1* and *CLDN1*) were among our ZG-selective genes (==**Supplemental**

303 **Fig. S1**==). Our models of migration or adhesion did not detect an effect of *LGR5* (==**Supplemental Fig. S6**==),

304 but probably do not exclude their occurrence in the intact human adrenal. Intact adrenocortical structure

305 was recently shown to be essential for generation of the voltage oscillations that regulate aldosterone

306 secretion (37,38). We have not yet established which non-canonical Wnt pathway may be responsible for

307 inhibition by *LGR5* of aldosterone production, since the AP1/Jun inhibitor, JNK-IN-8, had little impact on

308 the inhibition.

309

310 Our study of ZG genes was prompted by the frequency of somatic mutations leading to common, small

311 APAs. If aldosterone is required for ZG cell survival, as implied by $CYP11B2^{-/-}$ mice (29), cells that

312 mutate to protect aldosterone production will have a selective advantage. Most cells in human ZG do not

313 express *CYP11B2* (39). ==The sparseness of *CYP11B2* in ZG is a general phenomenon whether adjacent to==

314 ==an APA or to a pheochromocytoma (39,40).== The most likely explanation of this down-regulation of

315 *CYP11B2* is a physiological response to high salt intake. If physiological inhibition of aldosterone

316 production has the same consequences as genetic deletion, as is suggested by our apoptosis data (**Fig. 3**),

317 then constitutive activation of aldosterone production following somatic mutations would confer a local

318 selection advantage to mutated cells. Moreover, since the fate of ZG cells on centripetal migration is to

319 proliferate and then disappear (26), the survival advantage to ZG cells may derive from entering synthetic

320 rather than proliferative mode, providing an explanation as to why ZG-like APAs are often small.

321

322 Few completely normal adrenals are removed at surgery. We therefore restricted our choice to those

323 adjacent to either an APA or pheochromocytoma, the commonest indications for adrenalectomy in our

324 hospital. These tumours have opposite effects on sodium balance, and future analyses will consider

325 differences in expression between the adrenal adjacent to the two tumour types. While, however, a

326 limitation of our study design is the use of adrenals adjacent to a tumour, which may differ from

327 completely normal adrenals, there was no difference in expression of LGR5 between these two groups of

328 patients. It seems unlikely therefore that its abundance is due to the tumours. The immortalised adrenal

329 cell line, H295R, is also not a perfect model for native ZG cells (41). Although primary adrenal cells are

330 less easy to transfect in culture than H295R cells, we have supported our findings in the latter where

331 possible using primary adrenal cells. The assumption taken is that whilst the ZG cells are a minority of the

332 mixture of ZG, ZF, and ZR cells, only ZG cells contribute to the measurements of aldosterone production.

333

334 Our discovery suggests a hypothetical translational consequence, arising from evidence that the survival

335 of ZG cells is dependent upon production of aldosterone (29). Current drug treatments for

336 hyperaldosteronism antagonise aldosterone response, but increase plasma aldosterone levels (3). Selective

337 inhibition of aldosterone production has been problematic because of the 95% homology of *CYP11B1*

338 (cortisol synthase enzyme) with *CYP11B2*. But the discovery of adrenally expressed genes whose

339 mutation activates aldosterone production, and of adrenal pathways linked to reduced aldosterone

340 secretion, provides novel targets, with potential for clinical development.

341

342 Chronic human diseases are often attributed to maladaptation when scarcity is replaced by plenty. We

343 speculate that inhibition of aldosterone production by a R-spondin-3-*LGR5*-non-canonical Wnt signaling

344 pathway creates a mechanism which is an appropriate response to excess salt, but could paradoxically

345 create a survival advantage for cells with somatic mutations causing autonomous aldosterone production.

346 Identification of this pathway may now provide novel targets for prevention and treatment of

347 hyperaldosteronism.

348

360

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482

483 **Table 1: Wnt genes with > 2-fold <mark>up-regulation in ZG compared to ZF</mark>, ranked in order of fold-**

484 **change (ZG vs. ZF)**

Rank	Gene name	Symbol	P-Value	Fold-Change
1	Leucine-rich repeat-containing G protein-coupled receptor 5	LGR5	1.6×10^{-23}	25.0
9	Secreted frizzled-related protein 4	SFRP4	2.3×10^{-10}	8.7
17	Lymphoid enhancer-binding factor 1	LEF1	4.3×10^{-15}	5.9
19	Cadherin 12, type 2 (N-cadherin 2)	CDH12	4.4×10^{-10}	5.8
27	R-spondin 3 homolog (Xenopus laevis)	RSPO3	3.3×10^{-11}	5.3
44	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	5.7×10^{-6}	3.8
53	Frizzled-related protein	FRZB	2.4×10^{-8}	3.5
76	Glypican 3	GPC3	1.7×10^{-6}	3.1
89	Transcription factor 21	TCF21	2.4×10^{-5}	2.9
90	Jun B proto-oncogene	JUNB	5.6×10^{-6}	2.9
92	Claudin 1	CLDN1	2.3×10^{-10}	2.8
98	FBJ murine osteosarcoma viral oncogene homolog	FOS	9.6×10^{-5}	2.8
105	Frizzled homolog 6 (Drosophila)	FZD6	9.4×10^{-11}	2.7
157	Wingless-type MMTV integration site family, member 4	WNT4	3.2×10^{-8}	2.2
179	Matrix metallopeptidase 2	MMP2	3.5×10^{-6}	2.1
183	Dickkopf homolog 3 (Xenopus laevis)	DKK3	4.7×10^{-10}	2.1
206	Low density lipoprotein receptor-related protein 1B	LRP1B	3.5×10^{-6}	2.0
209	CD44 molecule (Indian blood group)	CD44	6.6×10^{-8}	2.0

485 [*]Wnt genes were identified using the list provided at http://www.stanford.edu/group/nusselab/cgi-bin/wnt/

486 or http://www.rndsystems.com/molecule_group.aspx?g=484&r=2, or due to the attachment to the GO

487 term GO:0016055.

488

Fig. 1. Wnt related genes show selective expression in the ZG of human adrenal

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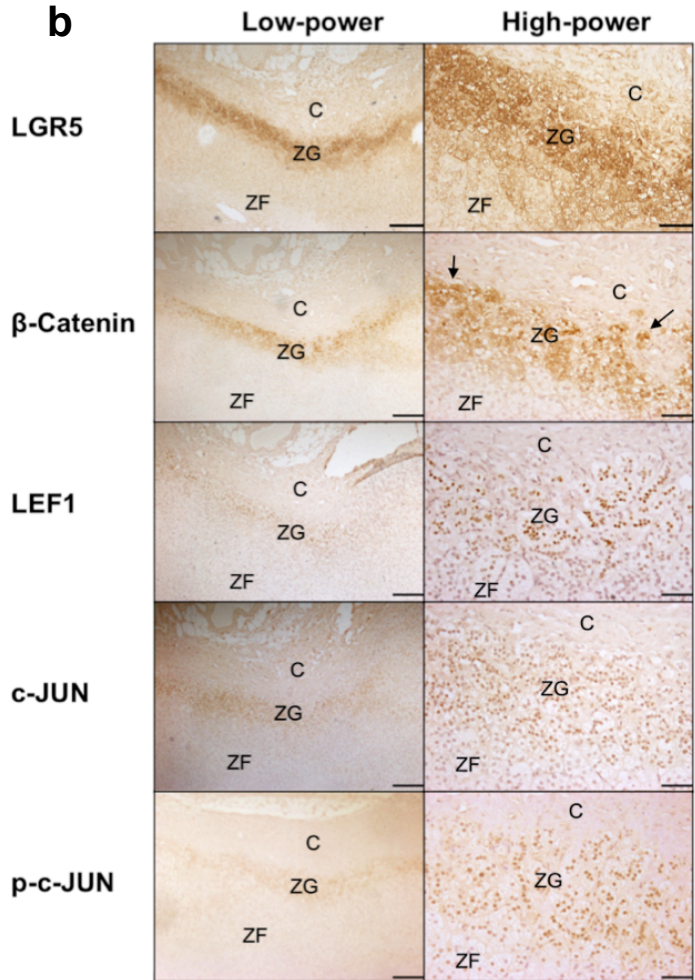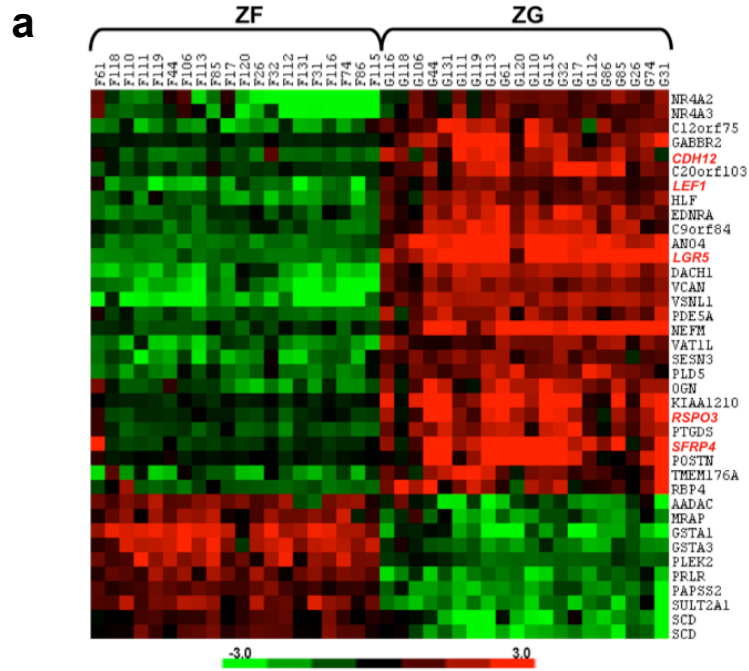
492   **(a)** Heatmap of 37 genes (shown on the vertical axis) which are $\geq$ 5-fold differentially expressed in ZG

493   and ZF. Genes previously associated with the Wnt signaling pathway are highlighted in red. On the

494   horizontal axis, each pairs of ZG and ZF from the 20 adrenals are anonymized and numbered. Probe set

495   and detailed microarray information are available from the NCBI Gene Expression Omnibus (GEO) under

496   accession GSE64957.

497   **(b)** IHC localisation of LGR5 and downstream Wnt signaling proteins in the ZG; from the canonical β-

498   catenin pathway– β-catenin and LEF1, and from the non-canonical AP1/JUN pathway– c-JUN and p-c-

499   JUN. IHC was performed on formalin-fixed, paraffin-embedded human adrenal sections (4 μm) using a

500   chromogen based detection system (DAB) which results in a positive brown staining. Interestingly, β-

501   catenin staining was mostly membranous or cytoplasmic (rather than nuclear staining as highlighted by

502   the arrows) indicating limited canonical Wnt activation. Pictures are representative of 6 normal adrenal

503   sections that were used in our microarray study; 4 from primary hyperaldosteronism patients and 2 from

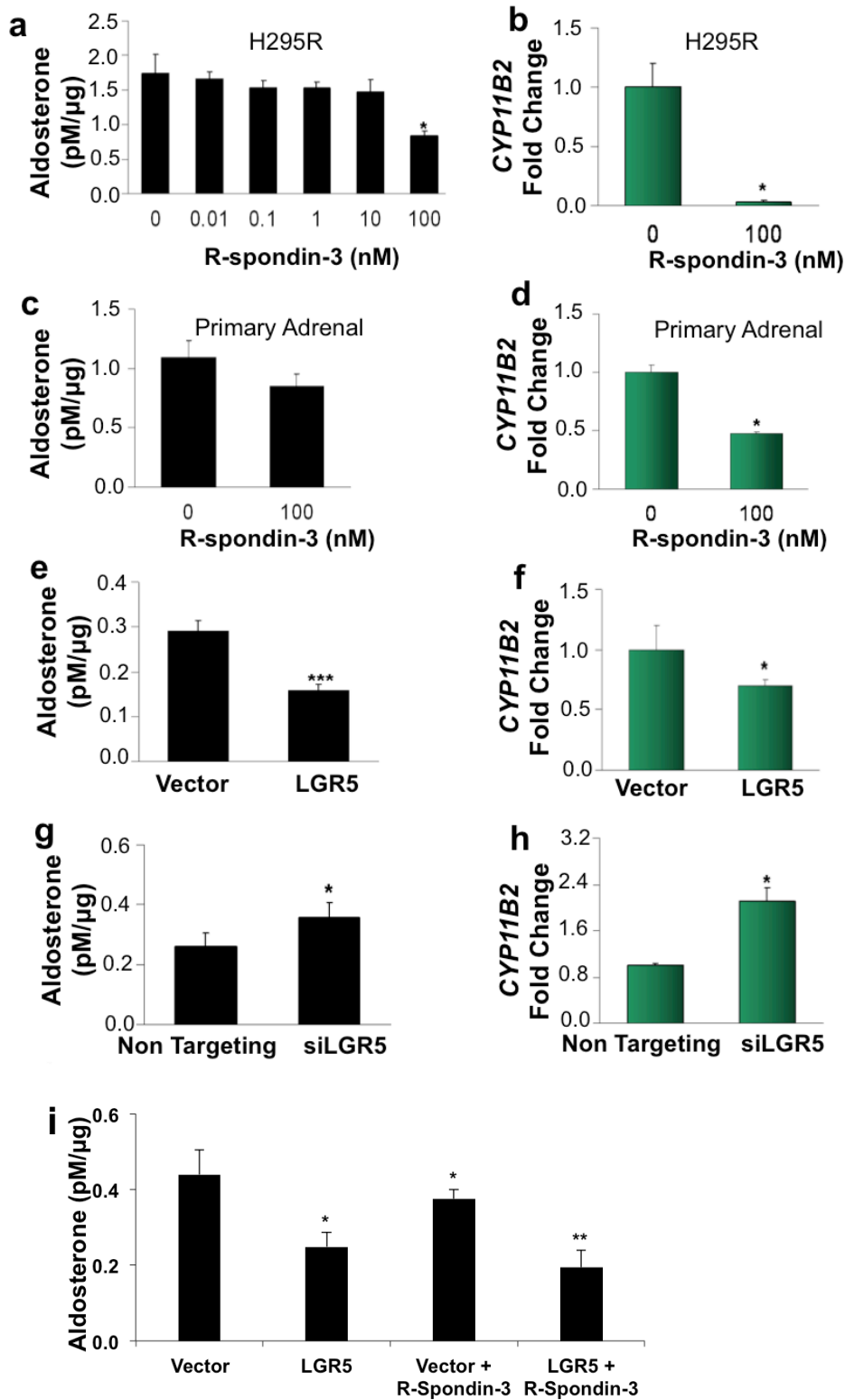504   pheochromocytoma patients. Scale bar = 500μm (low power) and 100μm (high power).

505   C: Capsule. ZF, F: Zona fasciculata. ZG, G: Zona glomerulosa.

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Fig. 2. LGR5 and its cognate ligand R-spondin-3 inhibit aldosterone production

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515   R-spondin-3 inhibition of **(a)** aldosterone secretion (n=12) and **(b)** *CYP11B2* transcription (n=3) by

516   H295R cells; and **(c)** aldosterone secretion (n=7) and **(d)** *CYP11B2* transcription (n=3) by normal human

517   primary adrenal cells from patients with primary hyperaldosteronism. H295R and human primary adrenal

518   cells were serum deprived in unsupplemented medium for 24h before exposed to vehicle control or R-

519   spondin-3 as specified. Supernatants for aldosterone concentration measurement were collected after 8h

520   incubation with drug, and cells were harvested for analysis of *CYP11B2* mRNA.

521   *LGR5* transfection of H295R cells, showing **(e)** inhibition of aldosterone secretion (n=17) after 48h of

522   transfection and **(f)** inhibition of *CYP11B2* transcription (n=3). pCMV6-AC-GFP was used as vector

523   control (Vector). Silencing of *LGR5* in H295R cells (siLGR5) after 48h, showing **(g)** stimulation of

524   aldosterone (n=7), and **(h)** increased CYP11B2 transcription (n=3). ON-TARGET plus Non-Targeting

525   siRNA (Non Targeting) transfected cells were used as experimental control.

526   **(i)** Additive effect of R-spondin-3 treatment on aldosterone production of *LGR5* transfected H295R cells

527   (n=8). H295R cells were transfected with vector controls pCMV6-AC-GFP or GFP-tagged *LGR5*

528   (Origene; 600ng/ul). After 24h of transfection, H295R cells were serum deprived in unsupplemented

529   medium for 24h, and further R-spondin-3 treatments were carried out with 8h incubation from this point.

530   Results are expressed in mean ±S.E.M. *P*-values show significance between treatments and baseline

531   control using student *t* test. *, $P<0.05$. **, $P<0.01$. ***, $P<0.001$
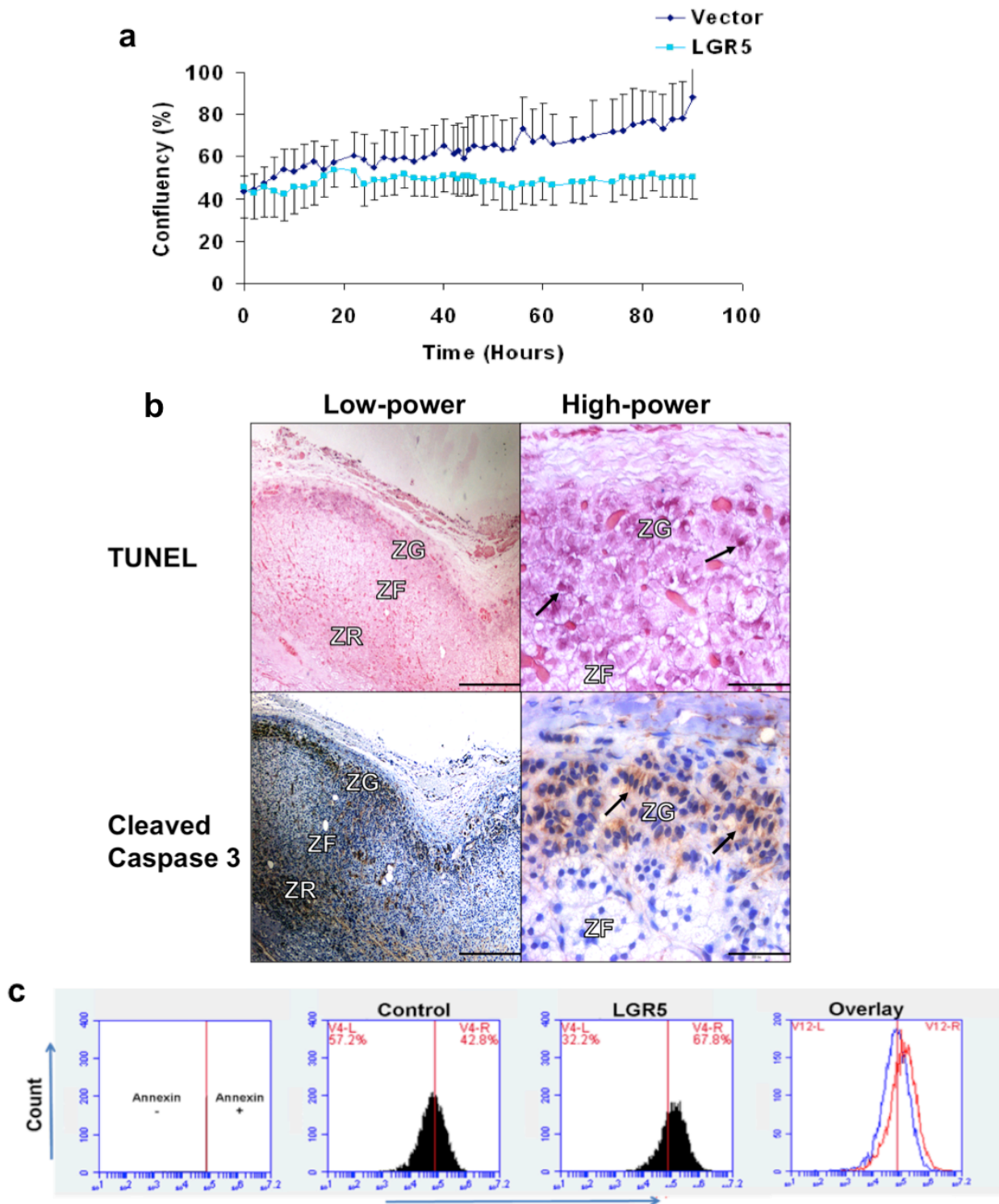
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535 **Fig. 3. LGR5 reduces human adrenocortical cell proliferation and increases apoptosis**

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538   **(a)** Kinetic live-cell imaging compared *LGR5* and vector transfected H295R cells. n=6, $P=1.5 \times 10^{-5}$ using

539   student *t* test.

540   **(b)** Human adrenal section showing staining for TUNEL (apoptosis marker, dark purple) and cleaved

541   caspase 3 (apoptosis marker, brown) in the ZG (as arrowed). Pictures are representative of 6 normal

542   adrenal sections that were used in our microarray study; from 4 primary hyperaldosteronism patients and 2

543   pheochromocytoma patients. ZG: zona glomerulosa. ZF: zona fasciculata. ZR: zona reticularis. Scale bar=

544   500um (low power) and 50um (high power).

545   **(c)** GFP-tagged *LGR5* transfected H295R cells undergoing apoptosis were Annexin V stained for flow

546   cytometry quantification. In the overlay, the blue line denotes the number of H295R cells stained with

547   Annexin V that were transfected with vector control while the red line denotes the same for LGR5

548   transfected cells. n=3, *P*=0.009 using student *t* test.

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Fig. 4. Dominant action of LGR5 on the non-canonical (AP1 /Jun) pathway
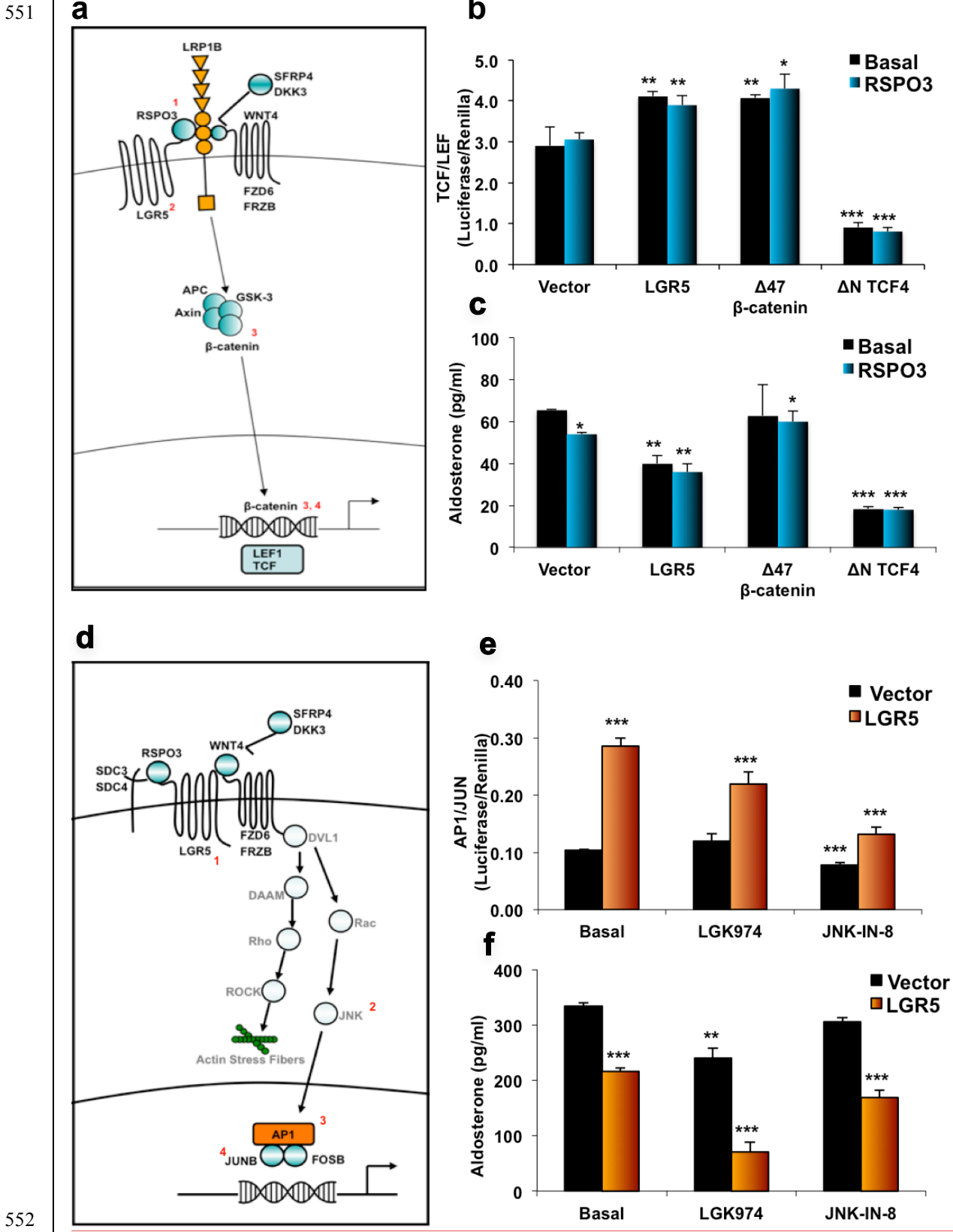
554   **(a)** Schema showing genes expressed in the <mark>human adrenal from the canonical Wnt signaling pathway.</mark>

555   Numbers (in red) show site of action of Wnt related proteins, which were either used or measured in the

556   experiment (1:R-spondin-3, 2:LGR5, 3:β-catenin, 4:TCF). **(b)** Canonical <mark>Wnt signaling activity of H295R</mark>

557   <mark>cells transfected with control vectors (Vector), *LGR5*, ΔN47 β-catenin (encoding a constitutively active β-</mark>

558   <mark>catenin) or ΔN TCF4 (encoding a dominant negative TCF) were measured through co-transfection with a</mark>

559   <mark>luciferase construct that had a TCF/LEF-responsive promoter (Cignal TCF/LEF Reporter (luc) Kit,</mark>

560   <mark>SABiosciences).</mark> **(c)** Aldosterone secretion during the same experiments (n=5).

561   **(d)** Schema showing Wnt genes expressed in the adrenal from the non-Canonical Wnt signaling pathway.

562   Numbers (in red) show site of action of Wnt related proteins, which were either used or measured in the

563   experiment (1:LGR5, 2:JNK, 3:AP1, 4: JUN). **(e)** AP-1/Jun non-canonical Wnt activity measured in the

564   presence of LGK974 (porcupine inhibitor) and JNK-IN-8 (JNK inhibitor) (both 1µM). **(f)** Aldosterone

565   secretion during the same experiment (n=4).

566   <mark>Results are expressed in mean ±S.E.M and *P*-values show significance between treatments and baseline</mark>

567   <mark>control.*, $P<0.05$. **, $P<0.01$. ***, $P<0.001$ using student *t* test.</mark>

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