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Molecular and Electrophysiological Analyses of ATP2B4 Gene Variants in Bilateral Adrenal Hyperaldosteronism

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1 **MOLECULAR AND ELECTROPHYSIOLOGICAL ANALYSES OF ATP2B4 GENE**
2 **VARIANTS IN BILATERAL ADRENAL HYPERALDOSTERONISM**

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21

22 **ABSTRACT**

23 Primary aldosteronism (PA) is the most common cause of secondary hypertension with a
24 high prevalence amongst patients with resistant hypertension. Despite the recent
25 discovery of somatic variants in aldosterone-producing adenoma (APA)-associated PA,
26 causes for PA due to bilateral aldosterone production remain unknown. In this study we
27 identified several rare variants in the *ATP2B4* [coding for a Ca-ATPase Pump, PMCA4],
28 in a cohort of patients with bilateral PA. *ATP2B4* belongs to the same family of Ca-
29 ATPases as *ATP2B3*, which is involved in the pathogenesis of APA. The *ATP2B4* variants
30 were functionally analyzed for effects on aldosterone synthase (*CYP11B2*) expression
31 and steroid production in basal and agonist-stimulated conditions, and for changes in
32 biophysical properties of channel properties. Several *in vitro* molecular analyses were
33 conducted: 1) Real time quantitative PCR (qPCR) confirmed *ATP2B4* mRNA in the
34 various tissue including the human adult adrenal gland, APAs, and the human
35 adrenocortical cell line HAC15. 2) Immunohistochemistry indicated PMCA4 expression
36 throughout the adrenal cortex. 3) Knock down of *ATP2B4* in HAC15 exhibited reduced
37 Angiotensin II-stimulation in one of four shRNA clones. 4) Stable HAC15 cell lines for
38 doxycycline (dox) – inducible wildtype and variant forms of *ATP2B4*, were generated. Q-
39 PCR and immunostaining confirmed dox-induced upregulation of *ATP2B4* mRNA and
40 protein. However, increased expression of the PMCA4b variants did not have a significant
41 effect on basal or agonist-stimulated *CYP11B2* expression. Whole cell recordings in
42 HAC15 cells indicated robust PMCA4b conductance in native cells, but reduced
43 conductance in the WT and variant PMCA4b. The previously defined PA-causing
44 mutation in *ATP2B3*, also a Calcium ATPase family member, served as a positive control

45 for the same dox-inducible system and exhibited elevated *CYP11B2* mRNA compared
46 with the wildtype variant. In conclusion, while this study did not define a pathogenic role
47 for *ATP2B4* variants in Bilateral Adrenal Hyperaldosteronism (BHA), we describe an
48 approach of WES analysis for familial and sporadic BHA and outline a template for the
49 thorough *in vitro* characterization of gene variants in the pathogenesis of BHA.

50

51

52 INTRODUCTION

53 Primary aldosteronism (PA) is the most common form of endocrine hypertension and
54 characterized by renin-independent aldosterone excess. PA can be categorized into two
55 main subtypes: Bilateral and unilateral disease. PA is most often sporadic, but familial
56 forms have been described. Over the past decade, next generation sequencing has
57 identified somatic variants in aldosterone producing adenoma (APA)-related PA. Thus
58 far, NGS has identified several genetic variants in APAs, including *KCNJ5*, *CACNA1H*,
59 *CACNA1D*, *ATP2B3* and *ATP1A1* (1-14), as the underlying mechanism of aldosterone
60 excess in APA. While somatic variants in these genes are found in up to 90% of all APAs,
61 and therefore the majority of cases of unilateral aldosterone excess, germline variants in
62 these or other genes are rare, explaining likely less than 5% of all cases with bilateral
63 aldosterone excess (15,16).

64 The currently known germline variants cause a phenotype of bilateral disease, yet with
65 significant variability in terms of severity, penetrance, age of onset and associated
66 symptoms. For instance, mutations in *KCNJ5* account for up to 40% of APA-related PA
67 (17,18). However, while the germline variant *KCNJ5*^{G151E} was associated with a
68 phenotype of hypertension and negligible hyperplasia, a different germline variant at the
69 same locus, *KCNJ5*^{G151R} exhibited a severe phenotype with massive hyperplasia,
70 requiring bilateral adrenalectomy (9,14). Familial PA due to germline variants in
71 *CACNA1D* cause a novel phenotype of PA associated with seizures and other
72 neurological abnormalities (11). Finally, most recently, whole exome sequencing,
73 revealed germline variants in the inward rectifying chloride channel *CLCN2* in a subgroup
74 of patients with PA (19,20). These patients display bilateral adrenal hyperplasia-related

75 aldosterone excess, and it was classified as Familial Hyperaldosteronism Type II (FH-II).
76 However, the *CLCN2* variants have a low prevalence and the cause for aldosterone
77 excess in the majority Bilateral Adrenal Hyperaldosteronism (BHA) cases remains
78 unexplained.

79 The vast majority of patients with BHA present as apparently sporadic disease. The
80 bilateral nature of the disease, however, suggests a germline genetic predisposition as
81 one putative causative mechanism. Unfortunately, the availability of tissue for detailed
82 analyses is limited as these patients are usually treated medically. Therefore, analysis is
83 largely restricted to germline variants in families with multiple affected members with PA
84 or patients with apparently sporadic PA, assuming pathogenic variants in genes are
85 shared in this population. Using this approach, we identified rare variants in *ATP2B4*,
86 which encodes the Calcium ATPase, transcript variant 4b (PMCA4b). We analyzed these
87 variants through an *in vitro* pipeline involving characterization of gene expression across
88 tissues, analysis of effects of gene knock-down as well as inducible over-expression of
89 the gene variants on cellular mechanisms, LC-MS/MS measurement of steroids and
90 electrophysiological characterization.

91

92

93 RESULTS

94 Clinical Characteristics

95 Patient 1 presented with hypertension (*Table 1*), a serum K of 3.2 mEq/L, elevated
96 aldosterone with suppressed renin (20.4 ng/dL aldosterone, PRA 0.35 ng/mL/h) and an
97 aldosterone to renin ratio of 120.5. Diagnosis of PA was confirmed by the captopril test
98 and bilateral aldosterone excess by adrenal vein sampling. Patient 2, the brother to
99 Patient 1 was also diagnosed with BHA. Whole exome sequencing identified a
100 heterozygous germline variant c. 3152C>A (p.T1051N) (*ATP2B4*^{C3152A}) in both patients,
101 but not in unaffected family members. Additional rare variants (*ATP2B4*^{C3158T} and
102 *ATP2B4*^{G3346A}) in *ATP2B4* were identified in two patients with sporadic BAH (clinical
103 details in *Table 1*. All variants occurred in the calmodulin (CaM) binding domain of
104 PMCA4.

105

106 *ATP2B4* expression in different tissues

107 In order to evaluate differential expression of *ATP2B4* we surveyed different human
108 tissues. Real time quantitative PCR (RT-qPCR) analyses identified *ATP2B4* expression
109 to be highest in the brain, heart and healthy and pathological adrenal tissues (normal
110 adrenals and APA) (*Figure 1, Panel A*). Within the adrenal gland, the *ATP2B4* transcript
111 was expressed throughout the cortex, without zone specificity (*Figure 1, Panel B*). A
112 similar pattern was observed for the PMCA4b peptide, using immunofluorescence (*Figure*
113 *1, Panel C*).

114

115 **Effect of *ATP2B4* knock down in HAC15 cells.**

116 Four clones of short hairpin RNA (shRNA) lentiviruses targeting *ATP2B4* were used to
117 transduce the HAC15 cell line containing a CYP11B2 promoter driven secreted gaussian
118 luciferase (HAC15-B2Luc) (21). Non-targeted scrambled shRNA was used as a control.
119 Real time qPCR analyses confirmed significant, albeit not complete, knock down of
120 endogenous *ATP2B4* transcript and protein levels (*Figure 2, Panels A and B*). Knock
121 down of PMCA4b protein levels was also confirmed using western analysis. Amongst the
122 four clones, only a single clone exhibited reduced angiotensin II (Ang II) stimulation of the
123 *CYP11B2* promoter. *ATP2B4* knock down did not affect basal or agonist (angiotensin II
124 or K⁺-stimulated *CYP11B2* promoter activity in the other clones (*Figure 2, Panel C*).

125

126 **Effect of *ATP2B4* variants in HAC15 cells.**

127 HAC15 cells with doxycycline (dox)-inducible expression of the wildtype (WT) and rare
128 variants of *ATP2B4* (PMCA4b) were generated. Dox treatment increased *ATP2B4*
129 transcript levels by over 5-fold (*Figure 3, Panel A*). Immunocytochemistry confirmed
130 plasma membrane localization of the expressed protein (*Figure 4, Panel B*). Expression
131 of the variant *ATP2B4*^{C3152A} did not increase basal CYP11B2 transcript levels. However,
132 both WT and two of the rare variants (*ATP2B4*^{C3158T} and *ATP2B4*^{G3346A}) did increase
133 basal *CYP11B2* levels (*Figure 3, Panel C*). Further detailed analyses including stimulation
134 by agonists Ang II and K suggested a lack of effect of any of the two of the rare *ATP2B4*
135 variants (*Figure 3, Panel D*). On the other hand, cells expressing the *ATP2B4*^{C3152A} variant
136 displayed elevated CYP11B2 expression in K-stimulated conditions. As a positive control,

137 dox-inducible wildtype *ATP2B3* (*ATP2B3^{WT}*) and a previously described pathogenic
138 variant containing the deletion Leu425-Val426 (*ATP2B3^{Δ425-426}*) (22) were expressed in
139 HAC15 cells, using a similar strategy. This is the first ever study to develop and use a
140 dox-inducible model for *ATP2B3* characterization. In this model system, incubation with
141 dox induced *ATP2B3* transcript levels by ~4-fold (3, Panel E). The *ATP2B3^{WT}* variant
142 decreased *CYP11B2* transcript levels, in agreement with previous reports of reduced
143 basal calcium levels in cells over-expressing the gene variant. Furthermore, *ATP2B3^{Δ425-}*
144 *426* variant increased basal *CYP11B2* levels by ~15-fold, a more potent effect than earlier
145 reports using transient transfection (22). The stimulatory effects of agonists were also
146 enhanced in cells expressing *ATP2B3^{Δ425-426}* compared to *ATP2B3^{WT}* expressing adrenal
147 cells (Figure 3, Panels F-H).

148

149 **Effect of *ATP2B4* variants on steroidogenesis.**

150 Following the observations of the effect of the *ATP2B4* variants on *CYP11B2* expression,
151 we evaluated aldosterone production in these cell lines. Basal aldosterone levels were
152 slightly elevated (1.5 ± 0.12 Fold, Mean \pm S.E.M) in cells expressing *ATP2B4^{WT}*, while
153 they were unchanged for the variants *ATP2B4^{C3158T}* and *ATP2B4^{G3346A}* (Figure 4, Panel
154 A). Agonists Ang II and K stimulated production of steroids (aldosterone, cortisol, 18-
155 Hydroxycortisol and 18-oxocortisol), when compared to basal, remained unchanged in
156 *ATP2B4^{WT}* expressing cells, or any of the cell lines expressing the rare *ATP2B4* variants
157 (Figure 5, Panels B-D).

158

159 **Electrophysiological analyses of *ATP2B4* variants.**

160 A voltage step protocol was used to analyze differences in the biophysical properties of
161 the *ATP2B4* pump variants (*Figure 5, Panels A and B*). The endogenous *ATP2B4* current
162 in HAC15 cells was very robust. HAC15 cells expressing increased *ATP2B4*^{WT} had lower
163 current density. Similarly, the density of current of the rare variants were identical to that
164 of *ATP2B4*^{WT}. The resting membrane potential of adrenal cells expressing the variant
165 forms of *ATP2B4* was unchanged.

166 **MATERIALS AND METHODS**

167 **Next generation sequencing:** NGS was conducted in the UM sequencing core using
168 the Illumina 2500. Paired-end reads (FASTQ file) were aligned to the hg19 assembly with
169 Burrows-Wheeler Alignment (BWA) 92. Then, picard-tools were used for sorting and
170 marking the duplicated reads in the resulting BAM files. Variant calling was performed
171 with FreeBayes 93 and low-quality variants (QUAL<20) was discarded. Common variants
172 (MAF>20) were filtered out in our in-house database. The resulting VCF files were then
173 annotated with ANNOVAR 94 and KGGseq 95. The final list of candidate genes was
174 generated after excluding variants that are common in the population (MAF>1), non-
175 coding and synonymous. From the resulting list, genes variants were selected if present
176 only in the affected family members in each family.

177

178 **Generation of vectors:** The pMM2-hPMCA4b was a gift from John Penniston & Emanuel
179 Strehler (Addgene plasmid # 47759; <http://n2t.net/addgene:47759>;
180 RRID:Addgene_47759). Wildtype PMCA4b was PCR amplified to include using
181 restriction sites DraIII and XbaI at the 5' and 3' ends, respectively. The resultant amplicon
182 was sub-cloned into the shuttle vector pENTR1A-GFP-N2 (FR1) [a gift from Eric
183 Campeau & Paul Kaufman (Addgene plasmid # 19364; <http://n2t.net/addgene:19364>;
184 RRID:Addgene_19364)] (23). The variants of PMCA4b were generated by site directed
185 mutagenesis using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs,
186 Ipswich, MA). The resultant PMCA4b variants were inserted into the lentivector pCLX-
187 pTF- R1-DEST-R2-EBR65 [a gift from Patrick Salmon (Addgene plasmid # 45952;

188 <http://n2t.net/addgene:45952>; RRID:Addgene_45952)] (24). Lentiviruses (~1 x 10⁶
189 TU/mL) were generated at the University of Michigan Biomedical Vector Core and used
190 for transducing adrenal cells. Similarly, *ATP2B3^{WT}* and the previously described
191 pathogenic variant *ATP2B3^{ΔLeu425-Val426}* (kindly donated by Dr. Sasha Bandulik) (22), were
192 also cloned in to the pCLX-pTF- R1-DEST-R2-EBR65 lentiviral system. These served as
193 controls for the effect of the ATP2B family in adrenal cells.

194

195 **Cell culture and viral transduction of cell lines:** The human adrenocortical carcinoma
196 cell line, HAC15, were used as parental cells grown in DMEM:F12 containing 10%CCS,
197 1% ITS and antibiotics (PenStrep and gentamicin), as previously described.
198 Transductions were performed as previously described (25). HAC15 cells were
199 transduced with lentiviruses at a multiplicity of infection of 3, as follows: 2 million cells
200 were plated in a T-75 flask for 24 h, after which they were incubated with fresh GM-Ab
201 containing 10 µg/mL polybrene for 2 hours. Cells were then spinnoculated (centrifuged)
202 at 1000 rpm with lentiviruses and GM-Ab with 8 µg/mL polybrene. After overnight
203 incubation, the cells were recovered by the addition of twice the volume of GM-Ab and
204 incubated for additional 24 h. At the end of 48 h from transduction, the cells were
205 incubated in regular GM containing 5 µg/mL blasticidin (mammalian selection marker). A
206 mixed population of blasticidin-selected HAC15 cells were used to analyze gene
207 mutations. The generated cells carried doxycycline-inducible gene expression systems
208 for *ATP2B4^{WT}*, *ATP2B4^{C3152A}*, *ATP2B4^{C31528T}* and *ATP2B4^{G3346A}*. For experiments,
209 HAC15-dox-ATP2B4 (wildtype or variant cells) were plated in 24-well plates at a density
210 of 200,000 cells/well until 60% confluence. After 48h, cells were starved in 0.1% CCS low

211 serum media (LS) for 24 h and then treated with LS with/without doxycycline (1 µg/mL)
212 for 72 h for maximal induction of the transgene. Agonists (10 nM Ang II and 18 mM K⁺)
213 were introduced for the last 12 h. For LC-MS/MS measurement of steroids, cells were
214 incubated with LS with/without doxycycline and agonists for 72 h. Ang II was replenished
215 every 24 h due.

216 For knock-down studies, HAC15-B2Luc cells (kindly donated by Dr. Celso Gomez-
217 Sanchez) expressing a CYP11B2-driven secreted luciferase were cultured and treated
218 with dox and agonists as described for gene expression studies. These cells have been
219 successfully used for functional analysis of the *KCNJ5^{T158A}* variant in adrenal cells (21).
220 HAC15-B2Luc cells were transduced with four different clones of short-hairpin RNA for
221 ATP2B4 knock-down and selected with puromycin (10 µg/mL). Twenty-five microliters of
222 the media were analyzed for luciferase activity using colentrazine, as described
223 elsewhere (21).

224 For electrophysiological experiments, circular coverslips were coated with 50 µg/mL Poly-
225 D-Lysine for 2 h at room temperature, followed by coating with 1 µg/mL laminin (overnight
226 at 4°C). Coverslips were washed at least three times with 1X PBS and transferred to 24-
227 well plates. At the time of patching, HAC15-dox-ATP2B4 cells incubated with doxycycline
228 for 72 h were trypsinized and plated on the coated coverslips at a density of 100,000
229 cells/well in GM with doxycycline and incubated for 1 h prior to patching.

230

231 **Δ4 Steroid quantitation by liquid chromatography/tandem mass spectrometry (LC-**
232 **MS/MS):** A 150 µL aliquot of media from HAC15-dox-ATP2B4 cells, along with 50 µL

233 internal standard of deuterated steroids, was deproteinated using acetonitrile as
234 previously described (Rege J et al, 2015). Following deproteinization, extraction of $\Delta 4$
235 steroids was performed using 1 mL methyl-t-butyl ether (MTBE). The organic phase was
236 dried down and concentrated under nitrogen, reconstituted with 50 μ L of 1:1
237 methanol:deionized water and transferred to a 0.25 mL vial insert . The LC-MS/MS assay
238 was performed as previously described (26).

239

240 **Electrophysiology:** Ion currents were recorded in whole-cell patch-clamp configuration
241 using a MultiClamp 700B amplifier and Digidata 1440A digitizer (Molecular Devices,
242 Sunnyvale, CA, USA) as described earlier (25). Patch pipettes had resistances of 5-8
243 MO when filled with intracellular pipette solution and placed in extracellular solution.
244 External solution containing 137 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl₂, 1.2
245 mM CaCl₂, and 5mM HEPES (pH 7.4) was perfused through the system. The internal
246 pipette solution contained 95mM K-gluconate, 30 mM KCl, 4.8 mM Na₂HPO₄, 1.2 mM
247 NaH₂PO₄, 5 mM glucose, 2.38 mM MgCl₂, 0.726 mM CaCl₂, 1 mM EGTA, and 3 mM ATP
248 (pH 7.2). Data acquisition and analysis were performed using pCLAMP software
249 (Molecular Devices, Sunnyvale, CA, USA). Normalization of current amplitudes to cell
250 size was performed by dividing amplitudes by cell capacitance (Cm) to generate current
251 densities (pA/pF) (25).

252

253 **Immunofluorescence:** Slides with 5 μ serial sections of the human adrenal cortex were
254 deparaffinized. After antigen retrieval in pH 6 citrate buffer and pH 9 Tris buffer

255 respectively, the sections were incubated for 1 h in primary antibodies for CYP11B2
256 (mouse monoclonal clone 41-17B; 1:100; kindly donated by Dr. Celso Gomez-Sanchez)
257 and PMCA4b (mouse monoclonal clone JA9; Catalog# MA1-914, Millipore; 1:100). After
258 three successive washes in 1X PBS-0.5% Tween (PBS-T), the sections were incubated
259 Alexa Fluor® 647 goat anti-mouse antibody (1:100) for 1 h and with 100 nM DAPI for 5
260 mins. Following washes in 1X PBS-T, coverslips were mounted using the ProLong™ Gold
261 Antifade Mountant without DAPI (Thermo Fisher Scientific). Sectioned treated with only
262 secondary antibody served as negative controls. An H and E stained section of the
263 adrenal was used to identify the zones.

264

265 **RNA isolation and gene expression assays:** RNA was extracted from tissue or cell
266 lines using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), following manufacturer's
267 recommendations for on-column DNase treatment (RNase-free DNase Kit, Qiagen). Two
268 hundred nanograms of RNA were reverse transcribed using the High-Capacity cDNA
269 Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). Real-time
270 quantitative PCR was performed for *ATP2B4* (Hs00608066_m1, Thermo Fisher
271 Scientific), CYP11B2 (as previously described in Hattagady et al 2016) and normalized
272 to PPIA (Hs99999904_m1, Thermo Fisher Scientific). Relative quantification of the data
273 was performed using the $2^{-\Delta\Delta C_t}$ method.

274 **DISUSSION**

275 Over the last decade our understanding of the contribution of somatic genetic changes to
276 APA-related PA has significantly increased. Research on the causes of bilateral disease,
277 however, has been stymied by the very limited tissue availability due to mainly
278 pharmacologic treatment of bilateral disease.

279 Herein, we used exome sequencing of germline DNA obtained from patients with BAH in
280 an attempt to identify potential pathogenic gene variants. We focused on three rare
281 variants in *ATP2B4*, which encodes the Ca²⁺ ATPase Pump PMCA4. The mechanistic
282 characterization of the variants involved knock down of endogenous *ATP2B4* as well as
283 expression of wild type and rare *ATP2B4* variants in basal and agonist-stimulated
284 conditions in the human adrenocortical carcinoma cell line (HAC15). The *ATP2B4*^{C3152A}
285 did not affect *CYP11B2* transcript levels or the production of steroids aldosterone, cortisol,
286 18-Hydroxycortisol or 18-Oxocortisol in basal conditions, although a minor increase in K⁺-
287 stimulated aldosterone observed. The *ATP2B4*^{C3158T} and *ATP2B4*^{G3346A} variants
288 increased basal *CYP11B2* mRNA levels, although not more than the *ATP2B4*^{WT}
289 transgene itself and far less than an *ATP2B3* variant observed in APAs that served as a
290 positive control. No changes in current density was observed using electrophysiological
291 analysis. Parallel analysis of a disease-causing variant in *ATP2B3* displayed dramatic
292 increases of *CYP11B2* expression, making it unlikely that the subtle changes observed
293 with *ATP2B4* variants are of pathophysiological relevance. Thus, overall, an overt
294 pathogenic role with large increases in aldosterone production was excluded for the
295 *ATP2B4* variants.

296 We cannot, however, exclude the possibility that the *ATP2B4* variants act in conjunction
297 with other genes or environmental factors that affect aldosterone production. Indeed, the
298 challenge in finding new genetic causes of PA is that any new gene harboring potential
299 pathogenic variants might be associated with low disease penetrance. An example of this
300 phenomenon can be seen in the recent study investigating the role of several unique
301 hereditary *CLCN2* gene variants, where at least one large kindred (*CLCN2* ^{Arg172Gln})
302 showed incomplete penetrance (20). Similarly, *in vitro* expression of several *CLCN2*
303 variants (p.Gly24Asp, p.Tyr26Asn, p.Met22Lys, p.Ser865Arg, p.Arg172Gln) showed
304 great variation of their effects on *CYP11B2* expression (19,20). Certainly, the incomplete
305 penetrance could be attributed, at least partially, to genomic and epigenomic changes
306 acting as modifiers.

307 The hypothesis of a germline genetic predisposition to PA in BHA is based on the
308 observation of bilateral renin-independent aldosterone production. However, there is also
309 the possibility that a yet to be identified environmental or other pathogenic mechanism
310 contributes to the phenomenon of BHA. To this extent a recent study showed different
311 rare variants in *CACNA1D* in different aldosterone-producing cell clusters found in the
312 same adrenal gland from BAH patients (27). It remains to be shown, whether these
313 multiple somatic mutations could occur by chance or due to an underlying genetic
314 predisposition.

315 In summary, while our study did not identify a pathogenic role of the *ATP2B4* variants, we
316 focused on developing a pipeline for the detailed analyses of gene variants. We
317 successfully demonstrated an efficient lentiviral approach for tight dox-inducible gene
318 expression and the assessment of rare gene variant. Certainly, this system exhibited a

319 remarkable improvement in the effects of the PA-causing *ATP2B3* mutation as compared
320 to previous reports (22). Further, we describe a thorough investigation of these variants
321 including 1) characterization of gene expression across tissues, 2) knock-down analyses
322 of an inactivating gene variant 3) expression of the wildtype and rare gene variant to
323 define their effects on *CYP11B2* transcript levels, steroidogenesis by LC-MS/MS, and 4)
324 electrophysiological analyses of the biophysical properties of the variants in relation to
325 the wildtype protein. Finally, we would like to emphasize the need to publish not only
326 proven pathogenic genetic causes of BHA but also analysis of rare genetic variants, for
327 which experimental data argues against an involvement in the pathogenesis of the
328 disease, even if it is simply to avoid a double effort in the scientific community.

329 ACKNOWLEDGEMENTS

330 We would like to acknowledge Dr. Celso Gomez-Sanchez for his advice on sub-cloning
331 strategies and Dr. Sasha Bandulik for generously sharing expression vectors for
332 *ATP2B3*^{WT} and *ATP2B3*^{Δ425-426}.

333 FIGURE LEGENDS

334 **Table 1.** A) Rare gene variants in *ATP2B4*; B) Clinical characteristics of patients with
335 hereditary or sporadic germline variants in *ATP2B4*.

336

337 **Figure 1.** Expression of *ATP2B4* in tissues. (A) Real time analyses of *ATP2B4* transcript
338 variant 2 in adrenal tissues in comparison with various tissues. *p<0.05 versus pancreas
339 (B) Zonal expression of *ATP2B4* mRNA across the human adrenal using laser capture
340 microscopy. (C) Immunofluorescence for PMCA4b protein on serial sections of the human

341 adrenal cortex: (i) H&E (ii) negative control incubated only with secondary antibody and
342 DAPI (iii, iv) expression of CYP11B2 and PMCA4b, respectively.

343

344 **Figure 2.** Analysis of knock down of *ATP2B4* in adrenal cells. (A,B) Real time analysis
345 demonstrating knock down of *ATP2B4* mRNA and protein in four shRNA clones (-04, -
346 05, -07 and -39) and controls (HAC15-B2Luc and scrambled shRNA). (C) Effect of
347 *ATP2B4* shRNA on agonist stimulation. * $p < 0.05$ versus basal, $\gamma p < 0.05$ versus HAC15
348 Ang II stimulation.

349

350 **Figure 3.** Analysis of conditional expression of WT or variant *ATP2B4* in adrenal cells.
351 (A, B) Real time analysis demonstrating doxycycline-inducible expression of WT and
352 variant *ATP2B4* forms (*ATP2B4*^{WT/C3152A/C3158T/G3346A}) mRNA and protein in the HAC15
353 adrenal cell line. (C,D) Comparison of the effect of *ATP2B4* variants (72 h incubation with
354 1 $\mu\text{g/mL}$ dox) in basal and agonist stimulated (12 h) conditions in HAC15 cells. (E) Real
355 time analysis demonstrating doxycycline-inducible expression of WT and mutant
356 *ATP2B3*, mRNA as positive controls. (F-H) Comparison of the effect of *ATP2B3*^{WT} and
357 *ATP2B3* ^{$\Delta 425-426$} (72 h incubation with 1 $\mu\text{g/mL}$ dox) in basal and agonist stimulated (12 h)
358 conditions in HAC15 cells. * $p < 0.05$ versus respective treatment without dox, $\gamma p < 0.05$
359 versus respective basal.

360

361 **Figure 4.** Effect of of WT and variant *ATP2B4* forms on steroid production on HAC15
362 cells. LC-MS/MS was used to determine the effect of elevated *ATP2B4* variants
363 (*ATP2B4*^{WT/C3152A/C3158T/G3346A}) on steroid production in HAC15 adrenal cells. Amount of

364 steroid was calculated in pg/mL and normalized to amount of protein per well. Means \pm
365 S.E.M were calculated as fold over respective basals. * $p < 0.05$ versus basals in respective
366 cell lines.

367
368 **Figure 5.** Electrophysiological analyses of WT or variant *ATP2B4* in HAC15 cells. IV plots
369 (A) and traces (B) for HAC15 cells expressing endogenous *ATP2B4*, *ATP2B4^{WT}*,
370 *ATP2B4^{C3152A}*, *ATP2B4^{C3158T}* and *ATP2B4^{G3346A}* (n=7-10 for all variants). Internal and
371 external solutions, and protocols followed were as described in the Materials and Methods
372 section.

373

374

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482

Table 1.

A) Rare gene variants in *ATP2B4*

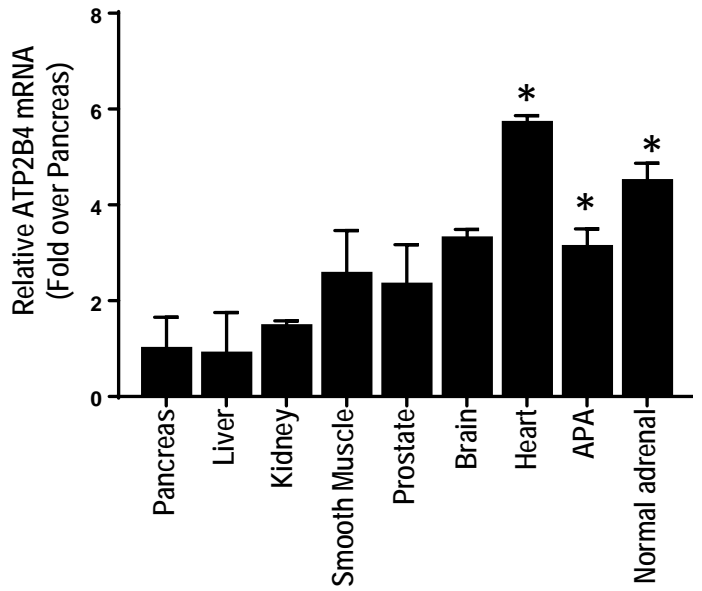
Gene Name	Nucleotide change	protein change	Frequency (EXAC)	Exon number	Transcript variant affected
ATP2B4	c.C3152A	p.T1051N	0.0015	Exon 20	PMCA4a, PMCA4b
	c.C3158T	p.S1052F	0.0081	Exon 20	PMCA4a, PMCA4b
	c.G3346A	p.E1116K	0.0004	Exon 21	PMCA4b only

B) Clinical characteristics of BHA patients with rare *ATP2B4* variants

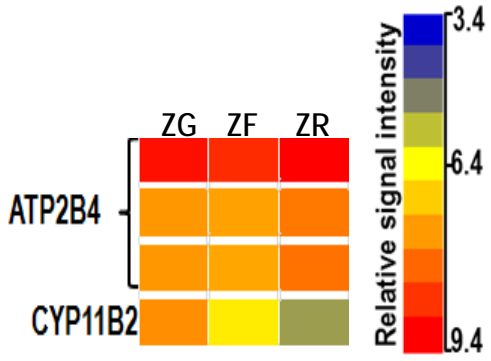
Parameters	Patient 1	Patient 2	Patient 3	Patient 4
ATP2B4 variant	c.C3152A	c.C3152A	c.G3346A	c.C3158T
Serum K ⁺ (mEq/L)	3.2	4	3.4	3.4
Aldosterone (ng/dL)	42.2	20.4	47	23.7
PRA (ng/mL/h)	0.35	0.3	0.5	0.4
ARR	120.5	68	94	59.3
Confirmatory test	37 ARR post captopril	10.9 ng/dL Aldosterone post saline load	11.2 ng/dL Aldosterone post saline load	12.1ng/dL Aldosterone post saline load
AVS	BHA	n/a	BHA	BAH
Imaging				
Diagnosis	BHA	BHA	BHA	Micronodule 7 mm left adrenal

Figure 1. Characterization of ATP2B4 expression across tissues

(A)



(B)



(C)

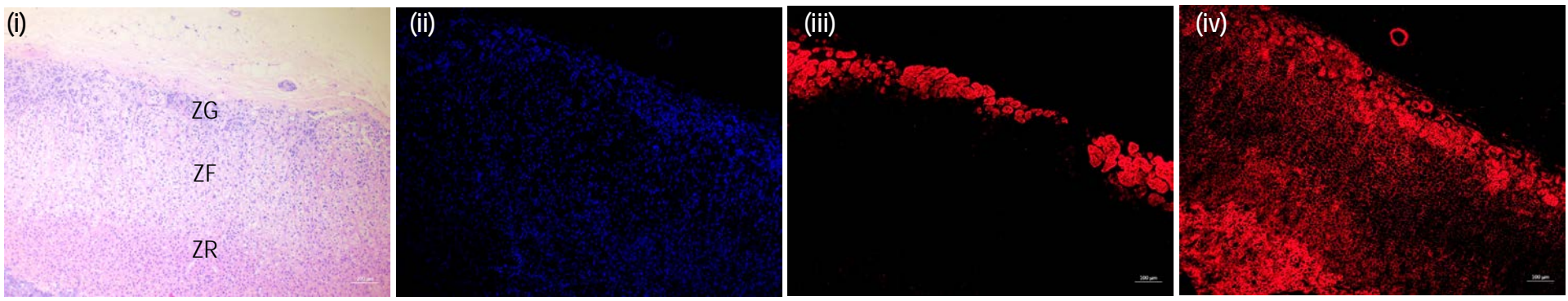


Figure 2. Effect of ATP2B4 knock down in adrenal cells

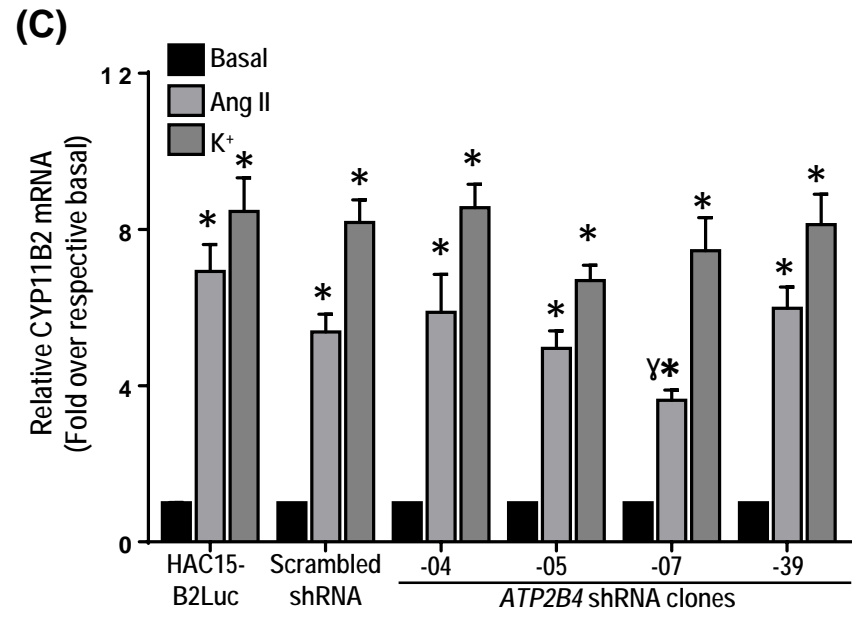
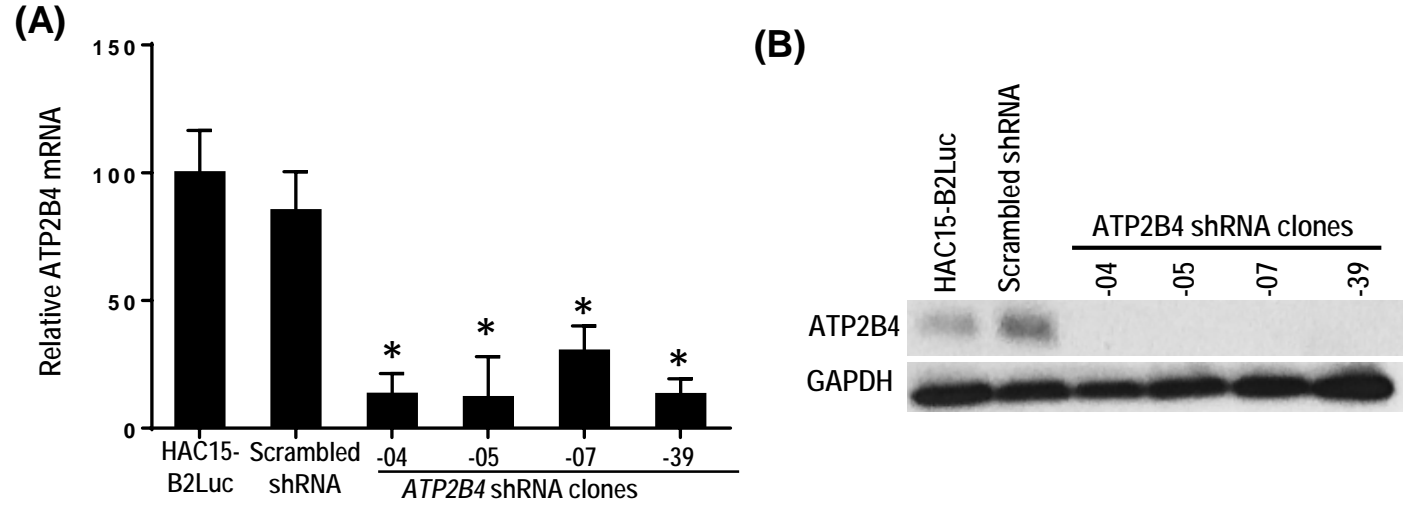


Figure 3. Characterization of HAC15 cells with dox-inducible ATP2B4 variants

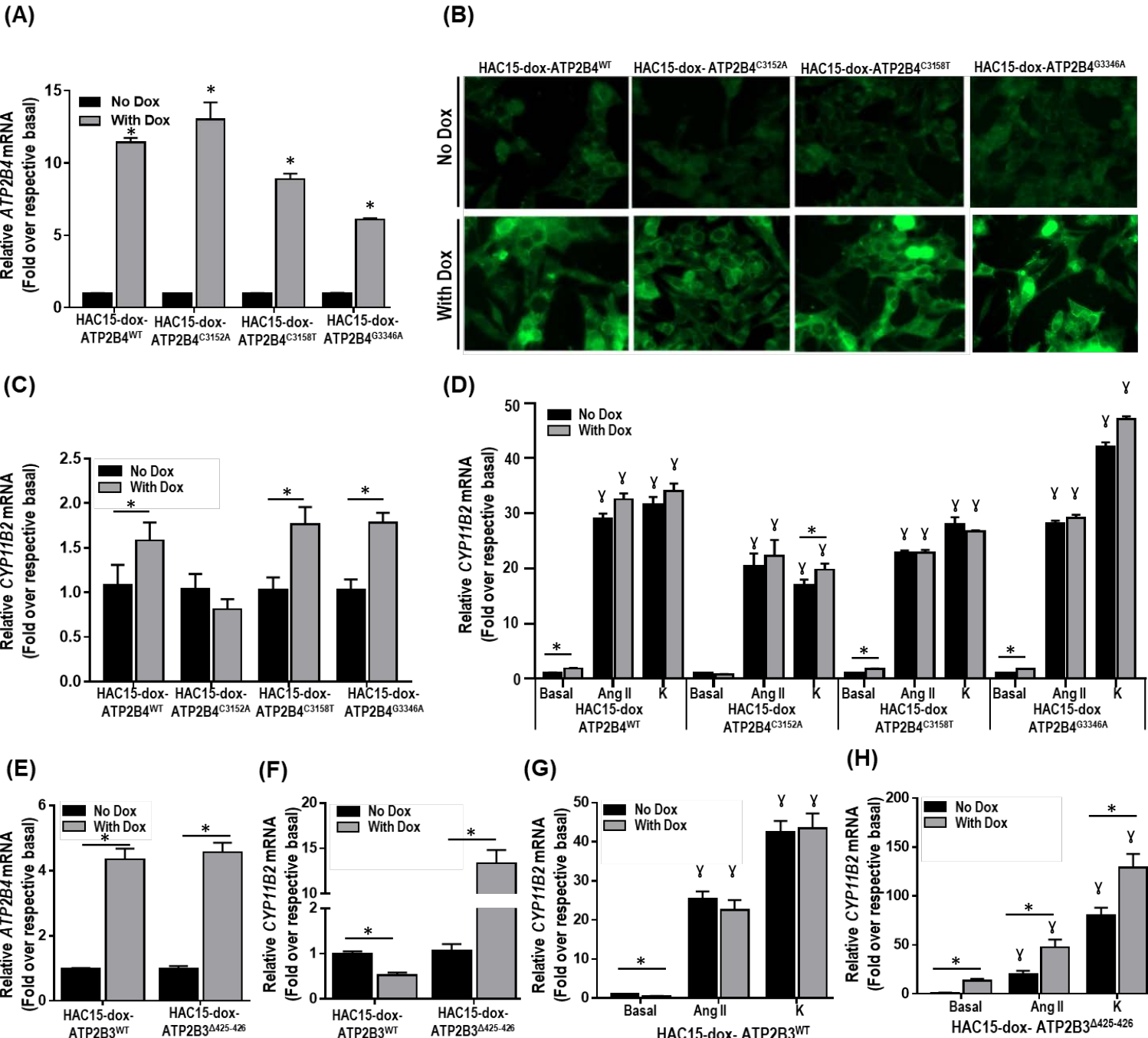


Figure 4. Effect of PMCA4b variants on steroid production

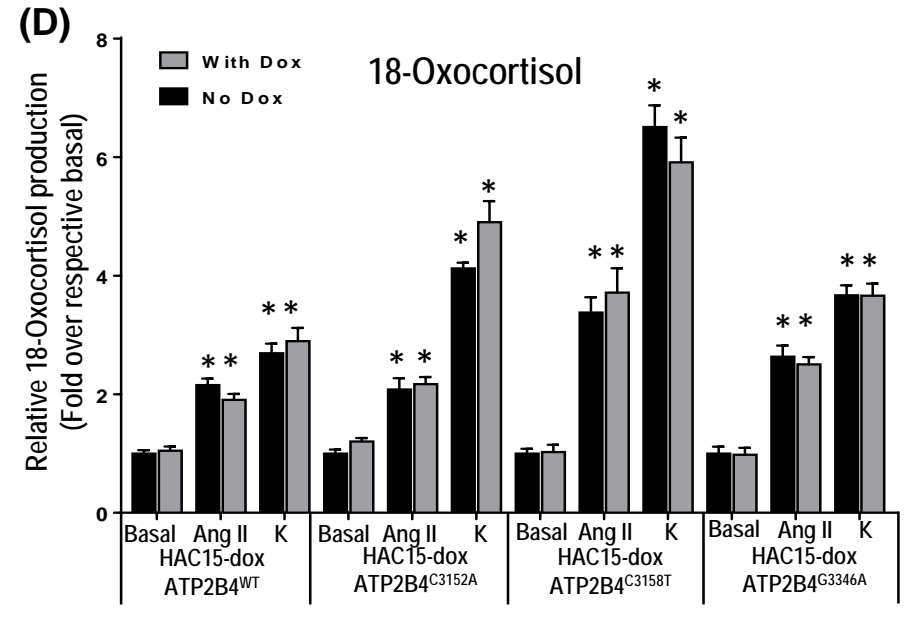
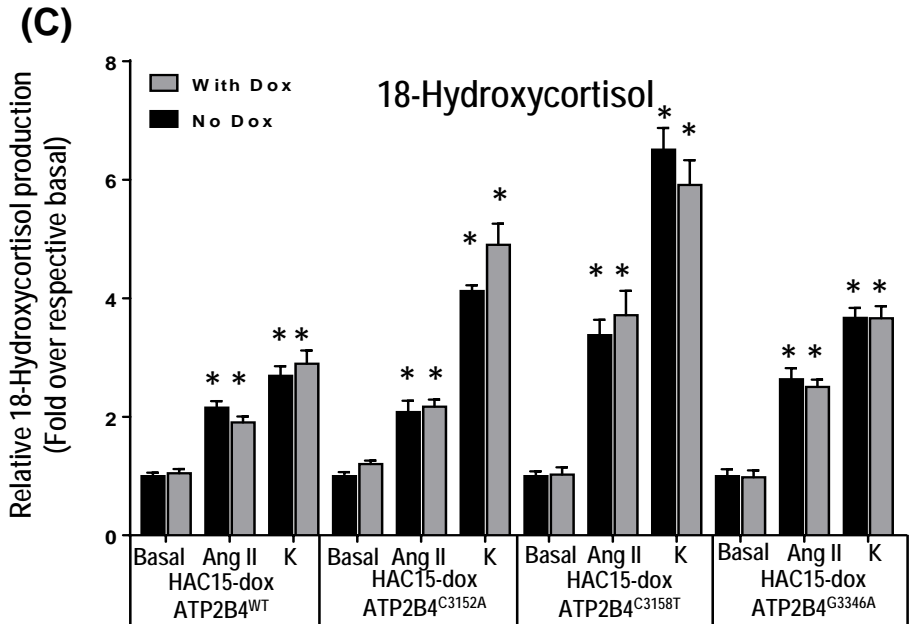
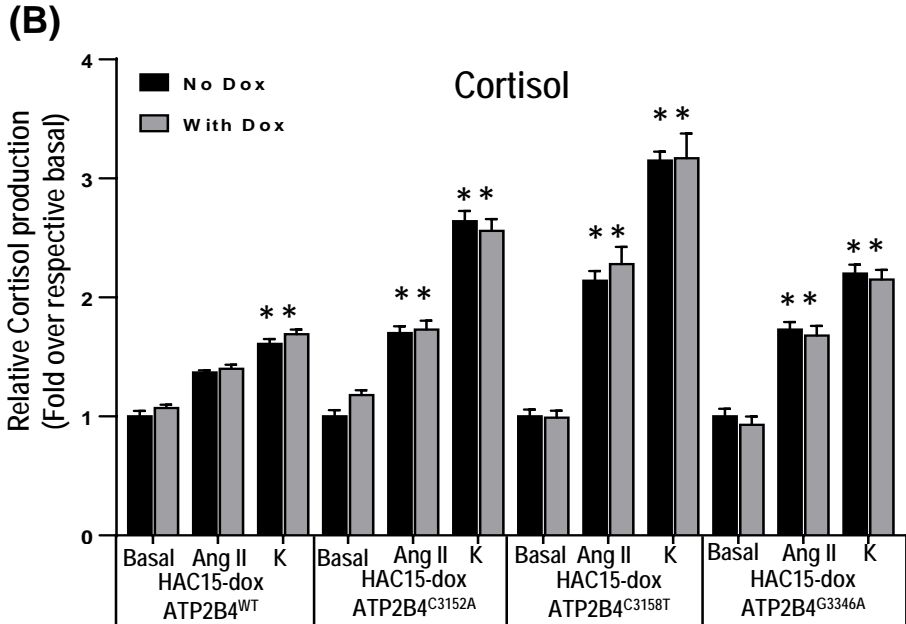
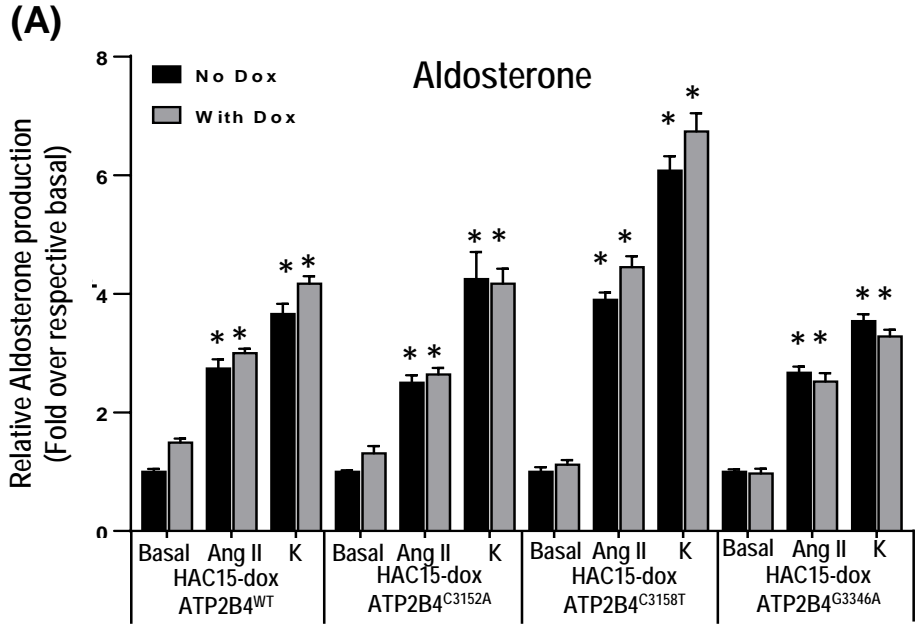
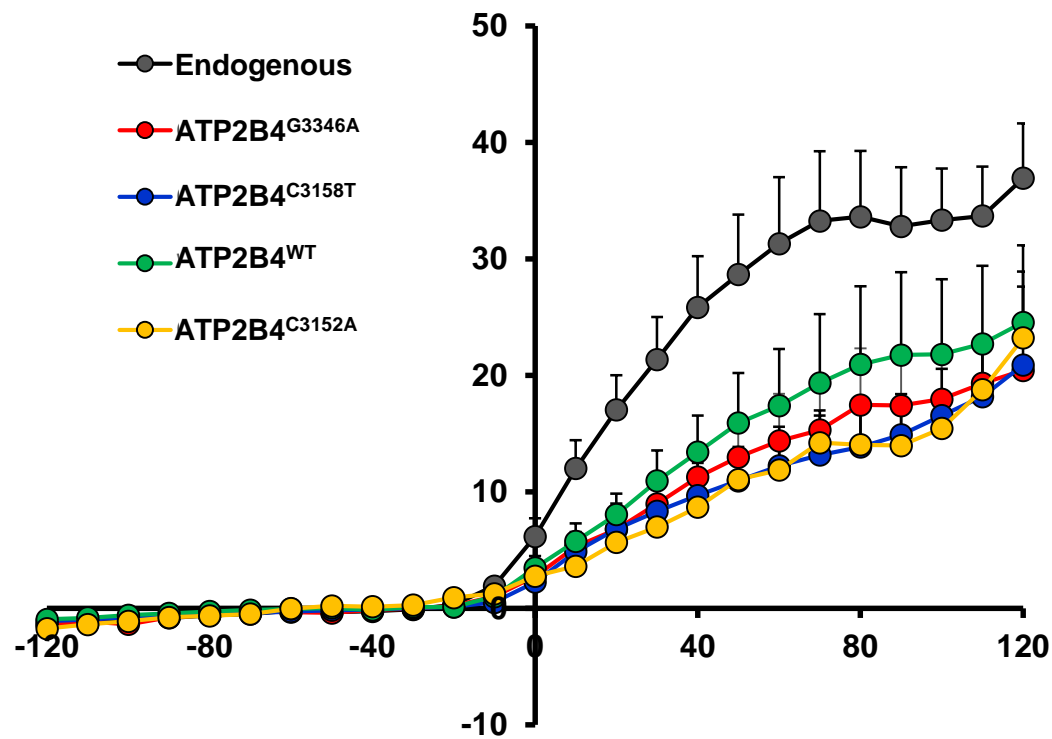


Figure 5. Electrophysiological analyses of ATP2B4 variants

(A)



(B)

