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

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

Namita Ganesh Hattangady[1], Jessica Foster[1], Antonio Marcondes Lerario[1], Daniela Ponce-Balbuena[2], Juilee Rege[3], Silvia Monticone[4], José Jalife[2], William E. Rainey[1,4], Paolo Mulatero[3], and Tobias Else[1]

Department of Internal Medicine, Division of Metabolism, Endocrinology & Diabetes, University of Michigan, Ann Arbor, Michigan, USA, [2]Center for Arrhythmia Research, University of Michigan, Ann Arbor, Michigan, USA, [3]Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan, USA.[4], Department of Medical Sciences, Division of Internal Medicine and Hypertension, University of Torino, Via Genova 3, 10126 Torino, Italy.

Corresponding author: Dr. Tobias Else, M.D.

Address: University of Michigan

Department of Internal Medicine

Division of Metabolism, Endocrinology and Diabetes

1150 West Medical Center Drive

Ann Arbor, MI 48109

Email: telse@umich.edu

USA phone: 734-647-7258

ABSTRACT

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

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

INTRODUCTION

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

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

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

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

RESULTS

Clinical Characteristics

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

ATP2B4 expression in different tissues

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

Effect of *ATP2B4* knock down in HAC15 cells.

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

Effect of *ATP2B4* variants in HAC15 cells.

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

dox-inducible wildtype *ATP2B3* (*ATP2B3^{WT})* and a previously described pathogenic variant containing the deletion Leu425-Val426 (*ATP2B3$^{\Delta 425\text{-}426}$*) (22) were expressed in HAC15 cells, using a similar strategy. This is the first ever study to develop and use a dox-inducible model for *ATP2B3* characterization. In this model system, incubation with dox induced *ATP2B3* transcript levels by ~4-fold *(3, Panel E)*. The *ATP2B3^{WT}* variant decreased CYP11B2 transcript levels, in agreement with previous reports of reduced basal calcium levels in cells over-expressing the gene variant. Furthermore, *ATP2B3$^{\Delta 425\text{-}426}$* variant increased basal *CYP11B2* levels by ~15-fold, a more potent effect than earlier reports using transient transfection (22). The stimulatory effects of agonists were also enhanced in cells expressing *ATP2B3$^{\Delta 425\text{-}426}$* compared to *ATP2B3^{WT}* expressing adrenal cells *(Figure 3, Panels F-H)*.

Effect of *ATP2B4* variants on steroidogenesis.

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

Electrophysiological analyses of *ATP2B4* variants.

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

MATERIALS AND METHODS

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

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

http://n2t.net/addgene:45952; RRID:Addgene_45952)] (24). Lentiviruses (~1 x 10^6 TU/mL) were generated at the University of Michigan Biomedical Vector Core and used for transducing adrenal cells. Similarly, *ATP2B3*^{WT} and the previously described pathogenic variant *ATP2B3*$^{\Delta Leu425\text{-}Val426}$ (kindly donated by Dr. Sasha Bandulik) (22), were also cloned in to the pCLX-pTF- R1-DEST-R2-EBR65 lentiviral system. These served as controls for the effect of the ATP2B family in adrenal cells.

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

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

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

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

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

internal standard of deuterated steroids, was deproteinated using acetonitrite as previously described (Rege J et al, 2015). Following deproteinization, extraction of Δ4 steroids was performed using 1 mL methyl-t-butyl ether (MTBE). The organic phase was dried down and concentrated under nitrogen, reconstituted with 50 µL of 1:1 methanol:deionized water and transferred to a 0.25 mL vial insert . The LC-MS/MS assay was performed as previously described (26).

Electrophysiology: Ion currents were recorded in whole-cell patch-clamp configuration using a MultiClamp 700B amplifier and Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA, USA) as described earlier (25). Patch pipettes had resistances of 5-8 MO when filled with intracellular pipette solution and placed in extracellular solution. External solution containing 137 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, and 5mM HEPES (pH 7.4) was perfused through the system. The internal pipette solution contained 95mM K-gluconate, 30 mM KCl, 4.8 mM Na_2HPO_4, 1.2 mM NaH_2PO_4, 5 mM glucose, 2.38 mM $MgCl_2$, 0.726 mM $CaCl_2$, 1 mM EGTA, and 3 mM ATP (pH 7.2). Data acquisition and analysis were performed using pCLAMP software (Molecular Devices, Sunnyvale, CA, USA). Normalization of current amplitudes to cell size was performed by dividing amplitudes by cell capacitance (Cm) to generate current densities (pA/pF) (25).

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

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

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

DISUSSION

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

Herein, we used exome sequencing of germline DNA obtained from patients with BAH in an attempt to identify potential pathogenic gene variants. We focused on three rare variants in *ATP2B4,* which encodes the Ca^{2+} ATPase Pump PMCA4. The mechanistic characterization of the variants involved knock down of endogenous *ATP2B4* as well as expression of wild type and rare *ATP2B4* variants in basal and agonist-stimulated conditions in the human adrenocortical carcinoma cell line (HAC15). The $ATP2B4^{C3152A}$ did not affect CYP11B2 transcript levels or the production of steroids aldosterone, cortisol, 18-Hydroxycortisol or 18-Oxocortisol in basal conditions, although a minor increase in K^+-stimulated aldosterone observed. The $ATP2B4^{C3158T}$ and $ATP2B4^{G3346A}$ variants increased basal *CYP11B2* mRNA levels, although not more than the $ATP2B4^{WT}$ transgene itself and far less than an *ATP2B3* variant observed in APAs that served as a positive control. No changes in current density was observed using electrophysiological analysis. Parallel analysis of a disease-causing variant in *ATP2B3* displayed dramatic increases of *CYP11B2* expression, making it unlikely that the subtle changes observed with *ATP2B4* variants are of pathophysiological relevance. Thus, overall, an overt pathogenic role with large increases in aldosterone production was excluded for the *ATP2B4* variants.

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

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

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

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

ACKNOWLEDGEMENTS

We would like to acknowledge Dr. Celso Gomez-Sanchez for his advice on sub-cloning strategies and Dr. Sasha Bandulik for generously sharing expression vectors for $ATP2B3^{WT}$ and $ATP2B3^{\Delta 425\text{-}426}$.

FIGURE LEGENDS

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

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

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

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

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

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

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

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

REFERENCES

1. Akerstrom T, Crona J, Delgado Verdugo A, Starker LF, Cupisti K, Willenberg HS, Knoefel WT, Saeger W, Feller A, Ip J, Soon P, Anlauf M, Alesina PF, Schmid KW, Decaussin M, Levillain P, Wangberg B, Peix JL, Robinson B, Zedenius J, Backdahl M, Caramuta S, Iwen KA, Botling J, Stalberg P, Kraimps JL, Dralle H, Hellman P, Sidhu S, Westin G, Lehnert H, Walz MK, Akerstrom G, Carling T, Choi M, Lifton RP, Bjorklund P. Comprehensive re-sequencing of adrenal aldosterone producing lesions reveal three somatic mutations near the KCNJ5 potassium channel selectivity filter. PLoS One 2012; 7:e41926

2. Azizan EA, Murthy M, Stowasser M, Gordon R, Kowalski B, Xu S, Brown MJ, O'Shaughnessy KM. Somatic mutations affecting the selectivity filter of KCNJ5 are frequent in 2 large unselected collections of adrenal aldosteronomas. Hypertension 2012; 59:587-591

3. Azizan EA, Poulsen H, Tuluc P, Zhou J, Clausen MV, Lieb A, Maniero C, Garg S, Bochukova EG, Zhao W, Shaikh LH, Brighton CA, Teo AE, Davenport AP, Dekkers T, Tops B, Kusters B, Ceral J, Yeo GS, Neogi SG, McFarlane I, Rosenfeld N, Marass F, Hadfield J, Margas W, Chaggar K, Solar M, Deinum J, Dolphin AC, Farooqi IS, Striessnig J, Nissen P, Brown MJ. Somatic mutations in ATP1A1 and CACNA1D underlie a common subtype of adrenal hypertension. Nat Genet 2013; 45:1055-1060

4. Beuschlein F, Boulkroun S, Osswald A, Wieland T, Nielsen HN, Lichtenauer UD, Penton D, Schack VR, Amar L, Fischer E, Walther A, Tauber P, Schwarzmayr T, Diener S, Graf E, Allolio B, Samson-Couterie B, Benecke A, Quinkler M, Fallo F, Plouin PF, Mantero F, Meitinger T, Mulatero P, Jeunemaitre X, Warth R, Vilsen B, Zennaro MC, Strom TM, Reincke M. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. Nat Genet 2013; 45:440-444, 444e441-442

5. Charmandari E, Sertedaki A, Kino T, Merakou C, Hoffman DA, Hatch MM, Hurt DE, Lin L, Xekouki P, Stratakis CA, Chrousos GP. A novel point mutation in the KCNJ5 gene causing primary hyperaldosteronism and early-onset autosomal dominant hypertension. J Clin Endocrinol Metab 2012; 97:E1532-1539

6. Choi M, Scholl UI, Yue P, Bjorklund P, Zhao B, Nelson-Williams C, Ji W, Cho Y, Patel A, Men CJ, Lolis E, Wisgerhof MV, Geller DS, Mane S, Hellman P, Westin G, Akerstrom G, Wang W, Carling T, Lifton RP. K+ channel mutations in adrenal aldosterone-producing adenomas and hereditary hypertension. Science 2011; 331:768-772

7. Kuppusamy M, Caroccia B, Stindl J, Bandulik S, Lenzini L, Gioco F, Fishman V, Zanotti G, Gomez-Sanchez C, Bader M, Warth R, Rossi GP. A Novel KCNJ5-insT149 Somatic Mutation Close to, but Outside, the Selectivity Filter Causes Resistant Hypertension by Loss of Selectivity for Potassium. J Clin Endocrinol Metab 2014; 99:E1765-1773

8. Monticone S, Hattangady NG, Penton D, Isales CM, Edwards MA, Williams TA, Sterner C, Warth R, Mulatero P, Rainey WE. a Novel Y152C KCNJ5 mutation responsible for familial hyperaldosteronism type III. J Clin Endocrinol Metab 2013; 98:E1861-1865

9. Mulatero P, Tauber P, Zennaro MC, Monticone S, Lang K, Beuschlein F, Fischer E, Tizzani D, Pallauf A, Viola A, Amar L, Williams TA, Strom TM, Graf E, Bandulik S, Penton D, Plouin PF, Warth R, Allolio B, Jeunemaitre X, Veglio F, Reincke M. KCNJ5 mutations in European families with nonglucocorticoid remediable familial hyperaldosteronism. Hypertension 2012; 59:235-240

10. Murthy M, Azizan EA, Brown MJ, O'Shaughnessy KM. Characterization of a novel somatic KCNJ5 mutation delI157 in an aldosterone-producing adenoma. J Hypertens 2012; 30:1827-1833

11. Scholl UI, Goh G, Stolting G, de Oliveira RC, Choi M, Overton JD, Fonseca AL, Korah R, Starker LF, Kunstman JW, Prasad ML, Hartung EA, Mauras N, Benson MR, Brady T, Shapiro JR, Loring E, Nelson-Williams C, Libutti SK, Mane S, Hellman P, Westin G, Akerstrom G, Bjorklund P, Carling T, Fahlke C, Hidalgo P, Lifton RP. Somatic and germline CACNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. Nat Genet 2013; 45:1050-1054

12. Scholl UI, Stolting G, Nelson-Williams C, Vichot AA, Choi M, Loring E, Prasad ML, Goh G, Carling T, Juhlin CC, Quack I, Rump LC, Thiel A, Lande M, Frazier BG, Rasoulpour M, Bowlin DL, Sethna CB, Trachtman H, Fahlke C, Lifton RP. Recurrent gain of function mutation in calcium channel CACNA1H causes early-onset hypertension with primary aldosteronism. Elife 2015; 4:e06315

13. Daniil G, Fernandes-Rosa FL, Chemin J, Blesneac I, Beltrand J, Polak M, Jeunemaitre X, Boulkroun S, Amar L, Strom TM, Lory P, Zennaro MC. CACNA1H Mutations Are Associated With Different Forms of Primary Aldosteronism. EBioMedicine 2016; 13:225-236

14. Scholl UI, Nelson-Williams C, Yue P, Grekin R, Wyatt RJ, Dillon MJ, Couch R, Hammer LK, Harley FL, Farhi A, Wang WH, Lifton RP. Hypertension with or without adrenal hyperplasia due to different inherited mutations in the potassium channel KCNJ5. Proc Natl Acad Sci U S A 2012; 109:2533-2538

15. Nanba K, Omata K, Else T, Beck PCC, Nanba AT, Turcu AF, Miller BS, Giordano TJ, Tomlins SA, Rainey WE. Targeted Molecular Characterization of Aldosterone-Producing Adenomas in White Americans. J Clin Endocrinol Metab 2018; 103:3869-3876

16. Nanba K, Omata K, Gomez-Sanchez CE, Stratakis CA, Demidowich AP, Suzuki M, Thompson LDR, Cohen DL, Luther JM, Gellert L, Vaidya A, Barletta JA, Else T, Giordano TJ, Tomlins SA, Rainey WE. Genetic Characteristics of Aldosterone-Producing Adenomas in Blacks. Hypertension 2019; 73:885-892

17. Fernandes-Rosa FL, Williams TA, Riester A, Steichen O, Beuschlein F, Boulkroun S, Strom TM, Monticone S, Amar L, Meatchi T, Mantero F, Cicala MV, Quinkler M, Fallo F, Allolio B, Bernini G, Maccario M, Giacchetti G, Jeunemaitre X, Mulatero P, Reincke M, Zennaro MC. Genetic

spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. Hypertension 2014; 64:354-361

18. Monticone S, Hattangady NG, Nishimoto K, Mantero F, Rubin B, Cicala MV, Pezzani R, Auchus RJ, Ghayee HK, Shibata H, Kurihara I, Williams TA, Giri JG, Bollag RJ, Edwards MA, Isales CM, Rainey WE. Effect of KCNJ5 mutations on gene expression in aldosterone-producing adenomas and adrenocortical cells. J Clin Endocrinol Metab 2012; 97:E1567-1572

19. Fernandes-Rosa FL, Daniil G, Orozco IJ, Goppner C, El Zein R, Jain V, Boulkroun S, Jeunemaitre X, Amar L, Lefebvre H, Schwarzmayr T, Strom TM, Jentsch TJ, Zennaro MC. A gain-of-function mutation in the CLCN2 chloride channel gene causes primary aldosteronism. Nat Genet 2018; 50:355-361

20. Scholl UI, Stolting G, Schewe J, Thiel A, Tan H, Nelson-Williams C, Vichot AA, Jin SC, Loring E, Untiet V, Yoo T, Choi J, Xu S, Wu A, Kirchner M, Mertins P, Rump LC, Onder AM, Gamble C, McKenney D, Lash RW, Jones DP, Chune G, Gagliardi P, Choi M, Gordon R, Stowasser M, Fahlke C, Lifton RP. CLCN2 chloride channel mutations in familial hyperaldosteronism type II. Nat Genet 2018; 50:349-354

21. Oki K, Plonczynski MW, Luis Lam M, Gomez-Sanchez EP, Gomez-Sanchez CE. Potassium channel mutant KCNJ5 T158A expression in HAC-15 cells increases aldosterone synthesis. Endocrinology 2012; 153:1774-1782

22. Tauber P, Aichinger B, Christ C, Stindl J, Rhayem Y, Beuschlein F, Warth R, Bandulik S. Cellular Pathophysiology of an Adrenal Adenoma-Associated Mutant of the Plasma Membrane Ca(2+)-ATPase ATP2B3. Endocrinology 2016; 157:2489-2499

23. Campeau E, Ruhl VE, Rodier F, Smith CL, Rahmberg BL, Fuss JO, Campisi J, Yaswen P, Cooper PK, Kaufman PD. A versatile viral system for expression and depletion of proteins in mammalian cells. PLoS One 2009; 4:e6529

24. Giry-Laterriere M, Cherpin O, Kim YS, Jensen J, Salmon P. Polyswitch lentivectors: "all-in-one" lentiviral vectors for drug-inducible gene expression, live selection, and recombination cloning. Hum Gene Ther 2011; 22:1255-1267

25. Hattangady NG, Karashima S, Yuan L, Ponce-Balbuena D, Jalife J, Gomez-Sanchez CE, Auchus RJ, Rainey WE, Else T. Mutated KCNJ5 activates the acute and chronic regulatory steps in aldosterone production. Journal of molecular endocrinology 2016; 57:1-11

26. Rege J, Nishimoto HK, Nishimoto K, Rodgers RJ, Auchus RJ, Rainey WE. Bone Morphogenetic Protein-4 (BMP4): A Paracrine Regulator of Human Adrenal C19 Steroid Synthesis. Endocrinology 2015; 156:2530-2540

27. Omata K, Satoh F, Morimoto R, Ito S, Yamazaki Y, Nakamura Y, Anand SK, Guo Z, Stowasser M, Sasano H, Tomlins SA, Rainey WE. Cellular and Genetic Causes of Idiopathic Hyperaldosteronism. Hypertension 2018; 72:874-880

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
	cC3158T	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

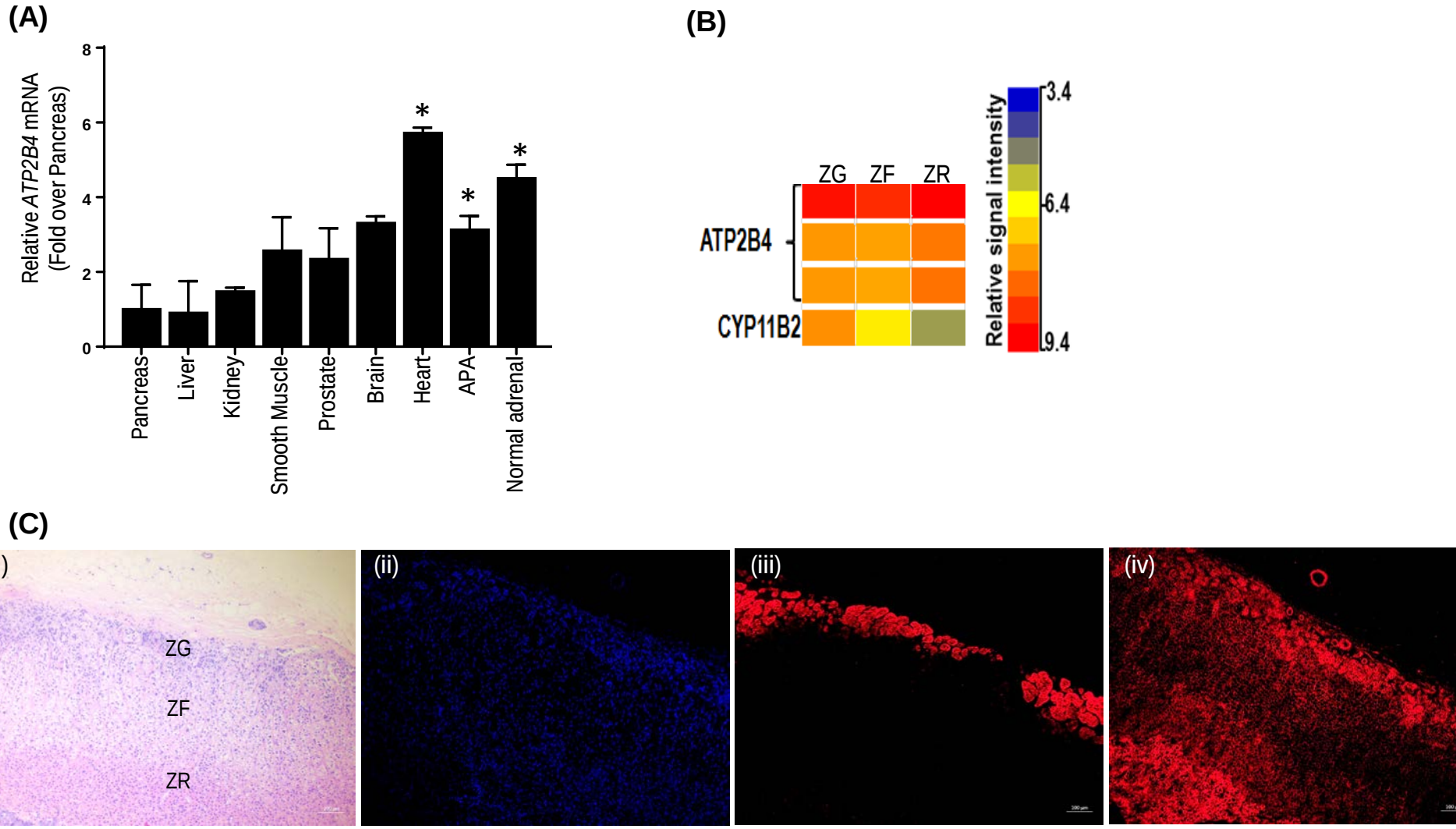
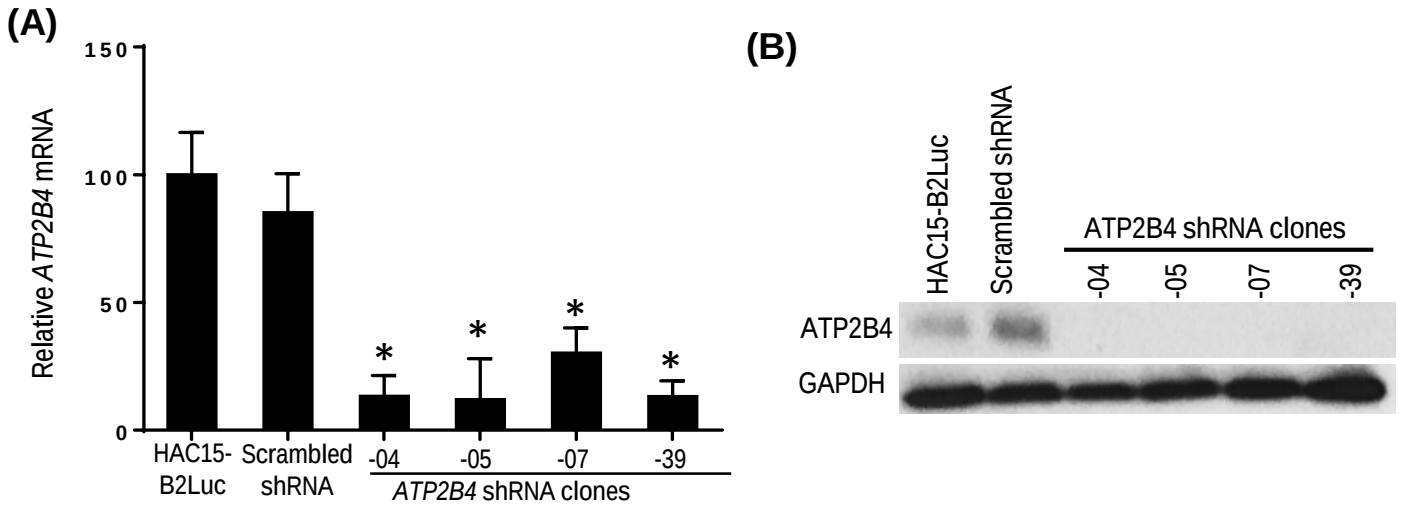


Figure 2. Effect of ATP2B4 knock down in adrenal cells



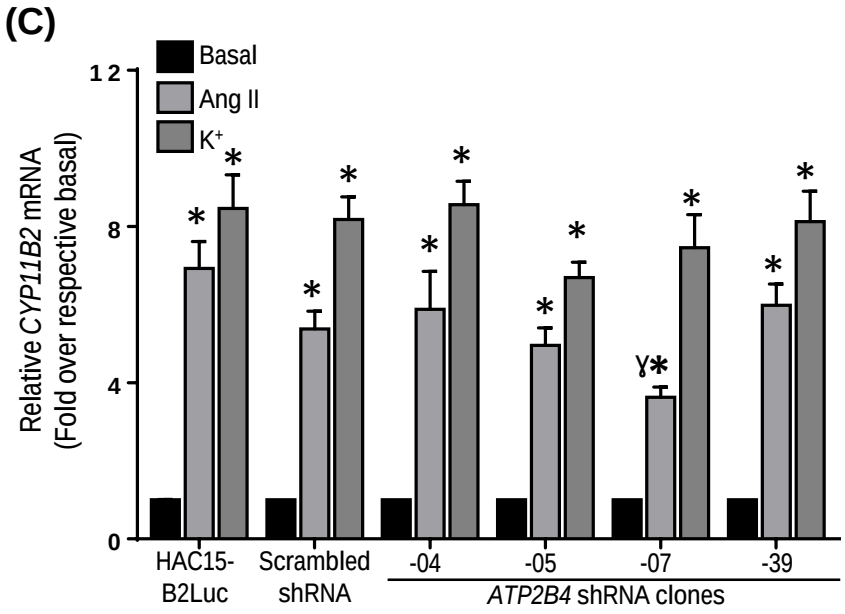


Figure 3. Characterization of HAC15 cells with dox-inducible ATP2B4 variants

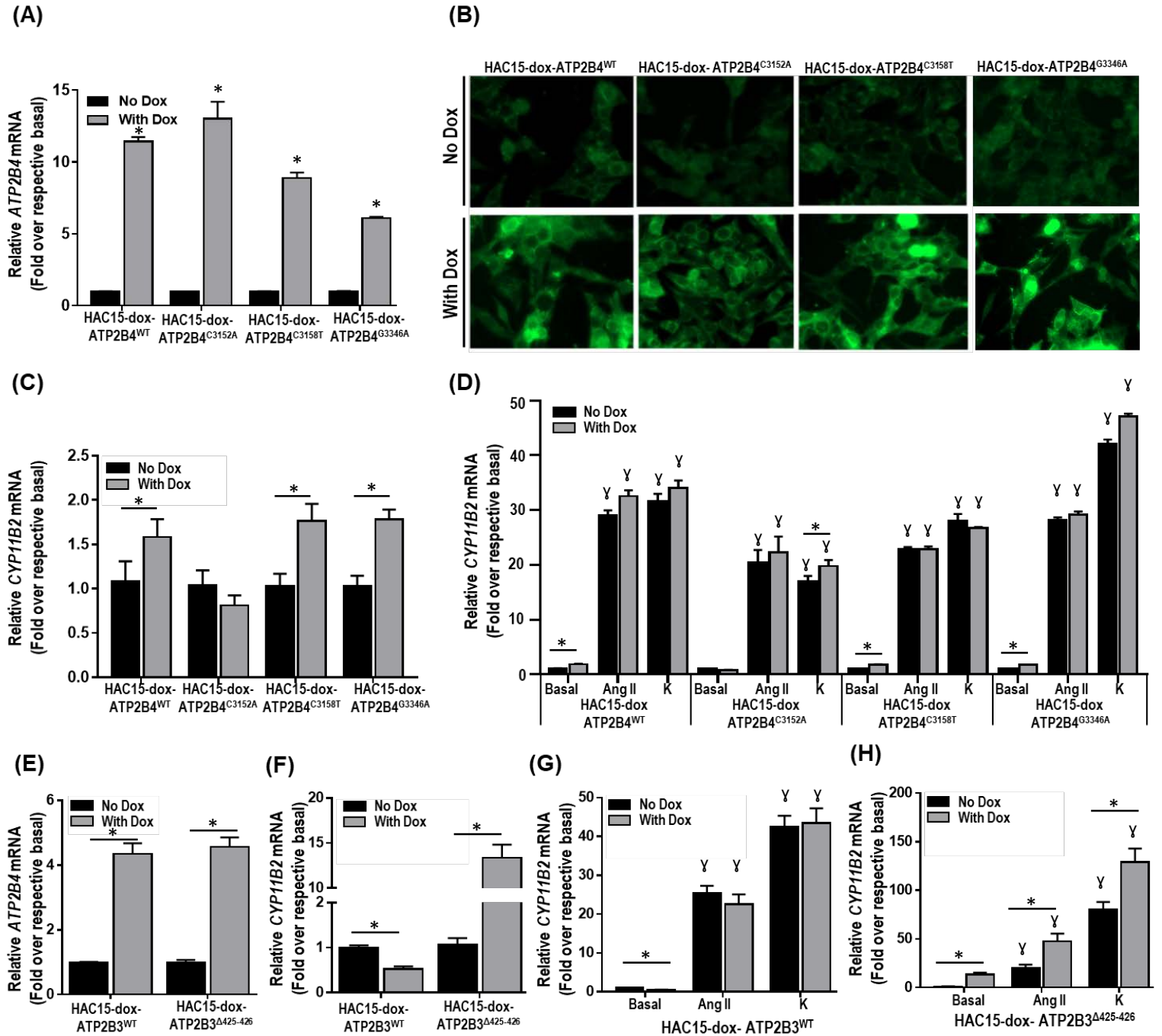


Figure 4. Effect of PMCA4b variants on steroid production

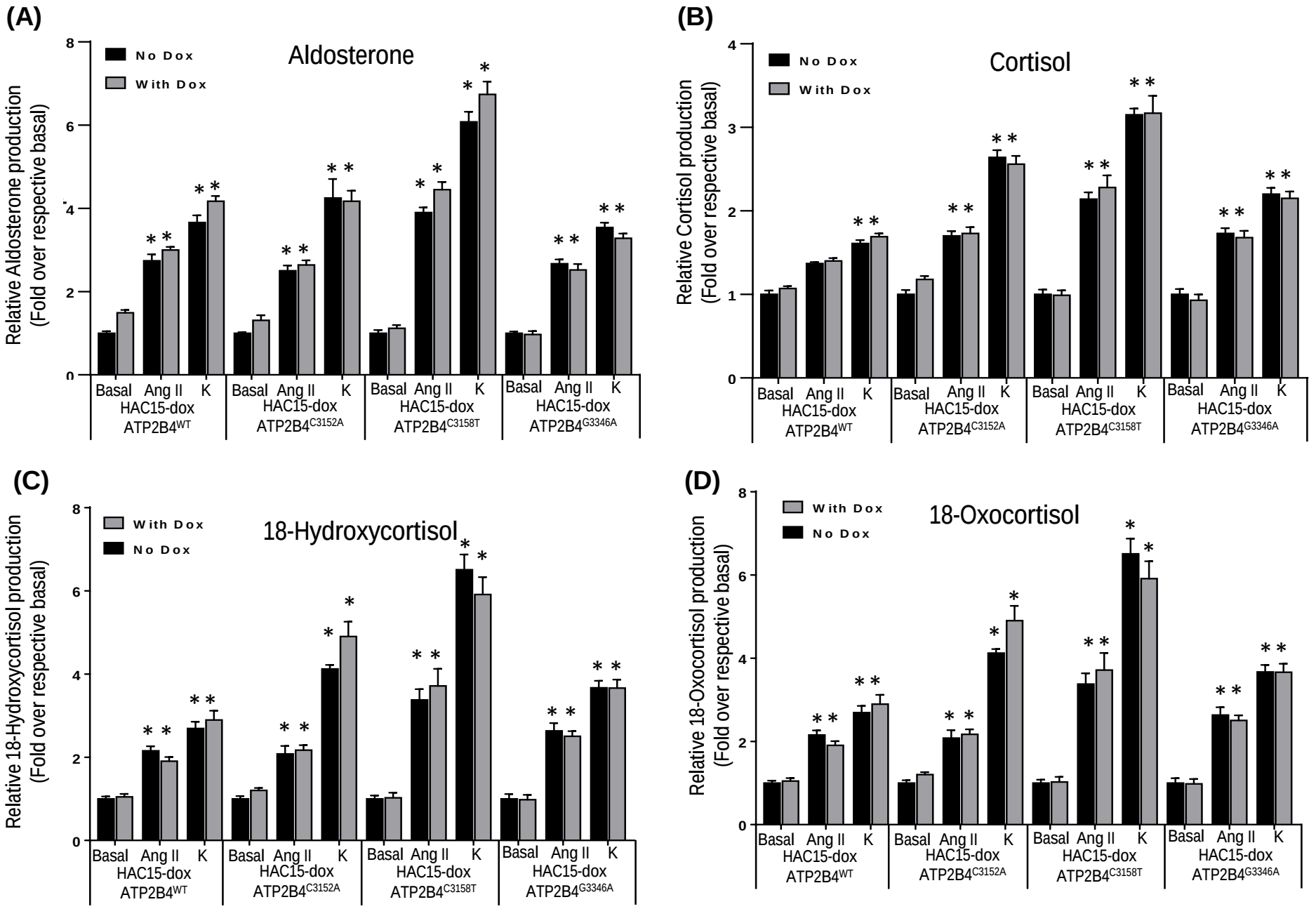


Figure 5. Electrophysiological analyses of ATP2B4 variants