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SUPPLEMENTARY INFORMATION

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A gain-of-function mutation in the *CLCN2* chloride channel gene causes primary aldosteronism

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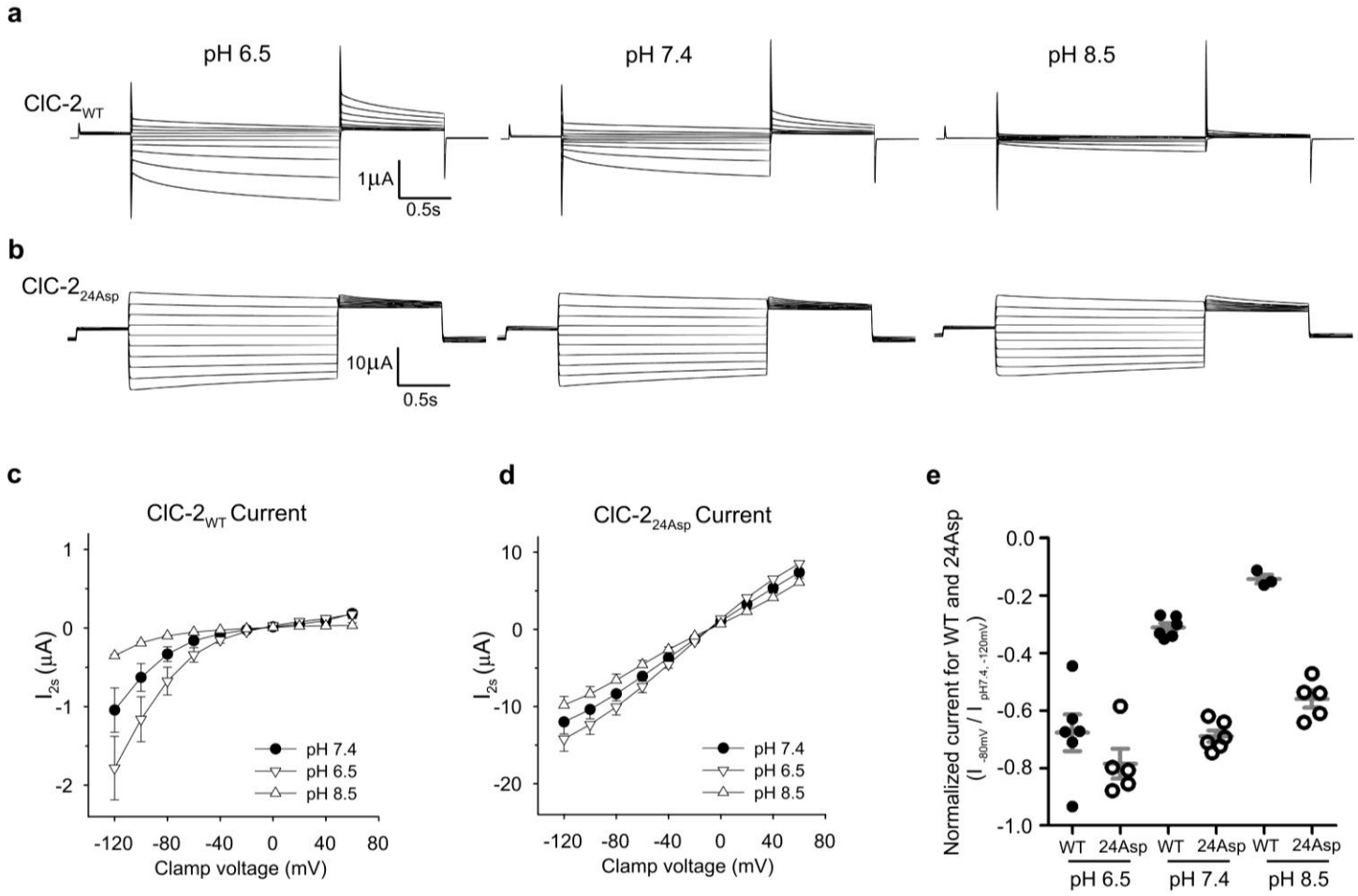
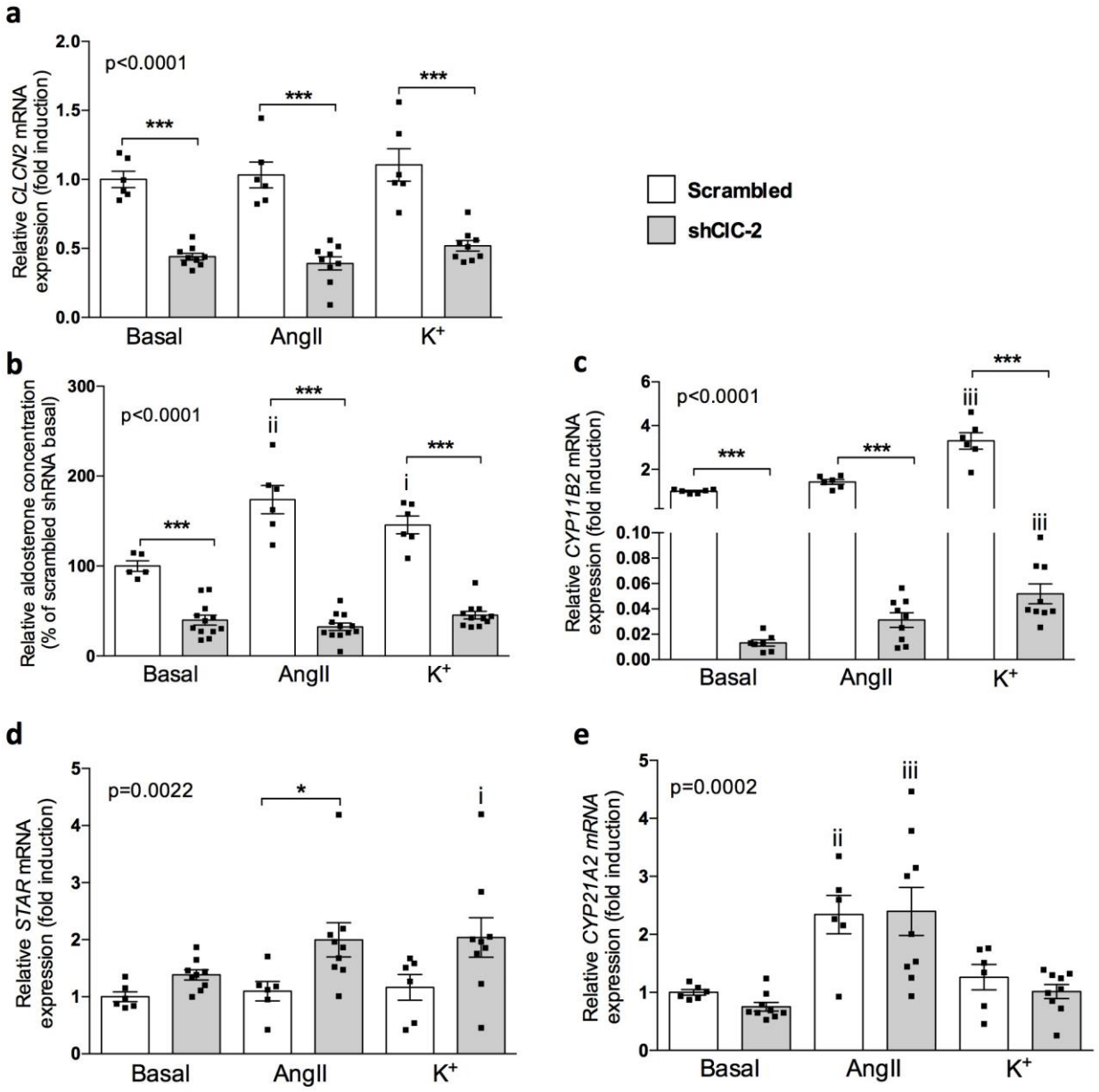


Supplementary Figure 1

Dependence of ClC-2^{WT} and ClC-2^{Asp24} currents on external pH

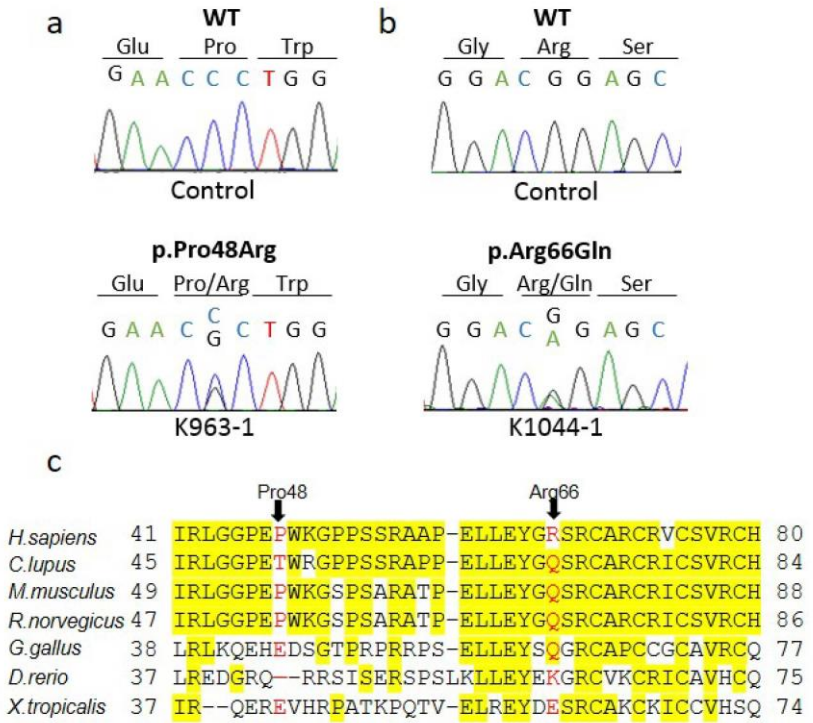
WT and mutant channels were expressed in *Xenopus* oocytes and measured by two-electrode voltage-clamp using a pulse protocol that clamped the oocytes in 2-s-long 20-mV steps from +60 to –120 mV. **a**,**b**, Representative current traces obtained from WT (**a**) and G24D mutant (**b**) ClC-2 at indicated pH values. **c**,**d**, Mean ClC-2^{WT} (**c**) and ClC-2^{Asp24} (**d**) currents measured after 2 s as a function of voltage and pH. *n* = 3–6 oocytes; error bars, s.e.m. (**e**) Currents at –80 mV (approximately the resting voltage of glomerulosa cells) from ClC-2^{WT} (filled circles) and ClC-2^{Asp24} (open circles) normalized to respective currents at –120 mV at pH 7.4. Note the large pH dependence of WT currents, which is strongly reduced but not abolished by the Gly24Asp mutation.



Supplementary Figure 2

Effect of ClC-2 downregulation on aldosterone production and expression of genes involved in aldosterone biosynthesis

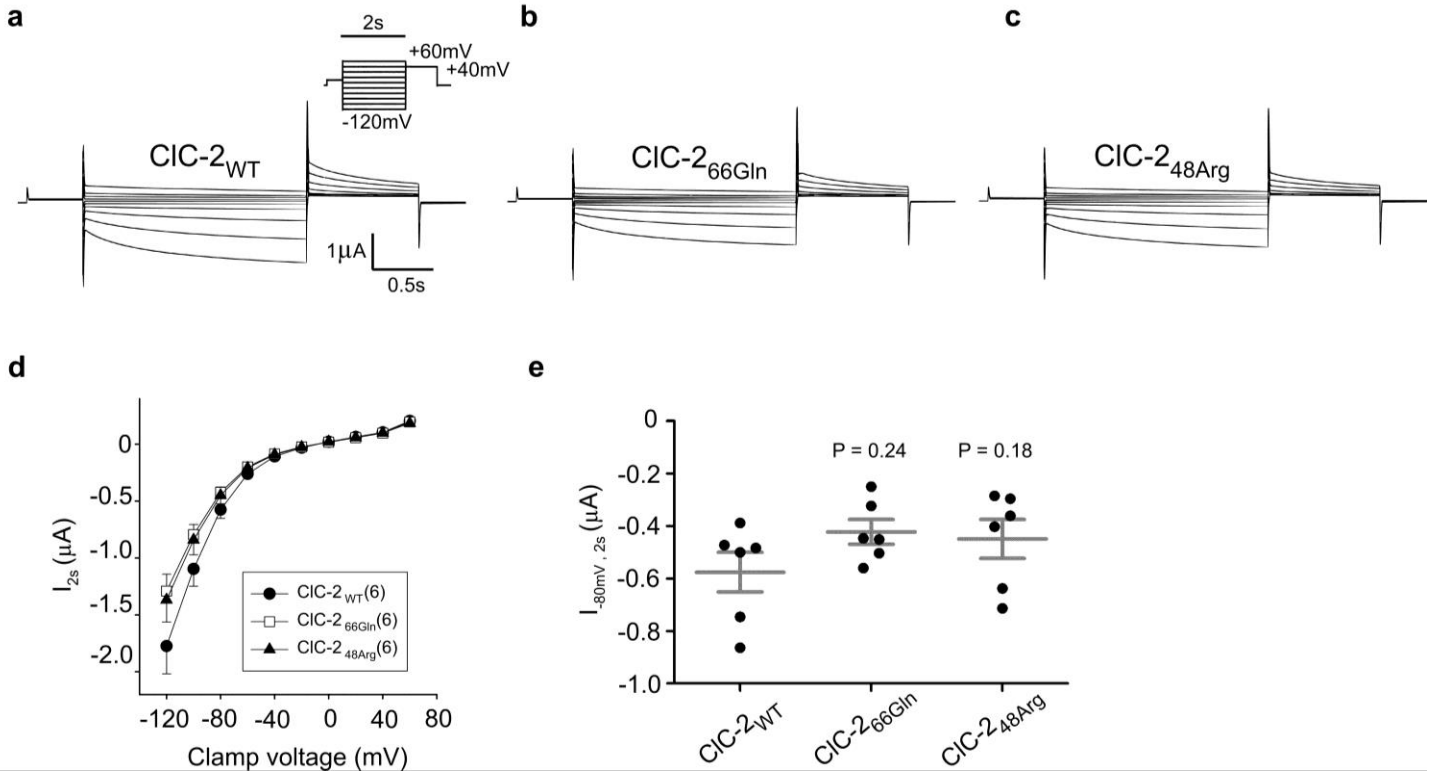
a, Basal and stimulated (Ang II or K^+) mRNA expression of *CLCN2* in H295R-S2 cells infected with scrambled (open bars) or ClC-2 (filled bars) shRNA (one-way ANOVA, $P < 0.0001$, $F = 28.11$). **b**, Basal and stimulated aldosterone production by H295R-S2 cells infected with scrambled or ClC-2 shRNA. **c**–**e**, Basal and stimulated mRNA expression of *CYP11B2* (one-way ANOVA, $P < 0.0001$, $F = 84$) (**c**), *STAR* (Kruskal–Wallis, $P = 0.0022$) (**d**), and *CYP21A2* (Kruskal–Wallis, $P = 0.0002$) (**e**) in H295R-S2 cells transfected with scrambled or ClC-2 shRNA. Results of mRNA expression are represented as fold induction of cells infected with scrambled shRNA in basal conditions. Values of all experiments are represented as means ± s.e.m. of two independent experiments performed in experimental triplicate for each condition ($n = 6$ for scrambled shRNA, $n = 12$ for ClC-2 shRNA). *$P < 0.05$; ***$P < 0.001$; (i) $P < 0.05$ stimulated versus basal condition; (ii) $P < 0.01$ stimulated versus basal condition; (iii) $P < 0.001$ stimulated versus basal condition.



Supplementary Figure 3

***CLCN2* variants identified in subjects with bilateral adrenal hyperplasia**

a, Sanger sequencing chromatograms showing the *CLCN2* wild-type sequence and the *CLCN2* variant c.143C>G (p.Pro48Arg) identified in subject K963-1 with bilateral adrenal hyperplasia. **b**, Sanger sequencing chromatograms showing the *CLCN2* wild-type sequence and the *CLCN2* variant c.197G>A (p.Arg66Gln) identified in subject K1044-1 with bilateral adrenal hyperplasia. **c**, Alignment and conservation of residues encoded by ClC-2 orthologs. Residues that are conserved among more than three sequences are highlighted in yellow.



Supplementary Figure 4

Electrophysiological analyses of ClC-2^{Gln66} and ClC-2^{Arg48} channels

a–c, Representative chloride current traces measured by two-electrode voltage-clamp from *Xenopus* oocytes injected with 9.2 ng of human ClC-2^{WT} (**a**), ClC-2^{Gln66} (**b**), or ClC-2^{Arg48} (**c**) cRNA. **d**, Mean ± s.e.m. currents measured after 2 s from experiments in **a**–**c** plotted as a function of clamp voltage. The number of cells, obtained from two different batches of oocytes is indicated in parentheses. **e**, Summary of Cl^- currents at –80 mV and after 2 s for **a**–**c**. Statistical analyses for ClC-2^{Gln66} and ClC-2^{Arg48} were performed by comparison with ClC-2^{WT}, Mann–Whitney test.

Supplementary material

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Supplementary Table 1: Expression of plasma membrane chloride channels in the human adrenal cortex.

Gene name	protein	mRNA expression*
CLCN2	ClC-2	1.65±0.23/1.16±0.18**
CLCN1	ClC-1	0.51±0.07
CFTR	CFTR	0.46±0.05
LRRC8A [$]	LRRC8A	1.09±0.22
LRRC8B [#]	LRRC8B	0.62±0.14/0.42±0.041**
LRRC8C [#]	LRRC8C	2.11±0.22
LRRC8D [#]	LRRC8D	9.95±0.84
LRRC8E [#]	LRRC8E	0.79±0.11
TMEM16A	Anoctamin-1	0.44±0.065

*mRNA expression was retrieved from a transcriptome study including 123 APA and 11 CA [1]. Values represent median centred, log2-transformed and model-adjusted expression levels respresented as mean±SEM. **Values represent expression levels detected by two different probes. [$]Essential and [#]non-essential components of the volume-regulated anion channel (VRAC) [2].

Supplementary Table 2. Primers used for *CLCN2* sequence

Exon	**Forward primer**	**Reverse primer**
1	CAGGACAGAGCCGGAACC	GGACAGGATTAGGGTAGGCC
2	CATAAGCATGGTCCACTCCC	AGCAGCTCTAATGGCCTCTG
10	AGGCTCCTTTTCACTCAGGT	CCTGTTTTGACTGGGCCATT

Supplementary Table 3. Primers used for real-time RT-qPCR

Gene Symbol	Forward primer	Reverse primer
18S	CCCTGCCTTTGTACACACC	CGATCCGAGGGCCTCACTA
HPRT	CTCAACTTTAACTGGAAAGAATGTC	TCCTTTTCACCAGCAAGCT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
CLCN2	TTGATCCTGCTCCCTTCCAG	CATAAGCATGGTCCACTCCC
StAR	ATGAGTAAAGTGGTCCCAGATG	ACCTTGATCTCCTTGACATTGG
CYP21A2	GAGTAGTCTCCCAAGGACAGGT	GTGGTGCTGAACTCCAAGAGGA
CYP11B2	GTGTGGAAGGAGCACTTTGAGG	GATGCCTGTGTAGTGTTGAGGC

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2. Voss, F.K. *et al.* Identification of LRRC8 heteromers as an essential component of the volume-regulated anion channel VRAC. *Science* **344**, 634-8 (2014).