Primary aldosteronism, a common cause of severe hypertension, features constitutive production of the adrenal steroid aldosterone. We analyzed a multiplex family with familial hyperaldosteronism type II (FH-II) and 80 additional probands with unsolved early-onset primary aldosteronism. Eight probands had novel heterozygous variants in CLCN2, including two de novo mutations and four independent occurrences of a mutation encoding an identical p.Arg172Gln substitution; all relatives with early-onset primary aldosteronism carried the CLCN2 variant found in the proband. CLCN2 encodes a voltage-gated chloride channel expressed in adrenal glomerulosa that opens at hyperpolarized membrane potentials. Channel opening depolarizes glomerulosa cells and induces expression of aldosterone synthase, the rate-limiting enzyme for aldosterone biosynthesis. Mutant channels show gain of function, with higher open probabilities at the glomerulosa resting potential. These findings for the first time demonstrate a role of anion channels in glomerulosa membrane potential determination, aldosterone production and hypertension. They establish the cause of a substantial fraction of early-onset primary aldosteronism.

More than 1.1 billion people worldwide have hypertension, the single largest cause of premature mortality. About 6% of hypertensive patients in primary care have primary aldosteronism, with higher frequencies among patients with severe hypertension. The plasma aldosterone level in primary aldosteronism is constitutively elevated despite low levels of the normal upstream regulator renin; hypokalemia is variable. Aldosterone-producing adrenal adenomas (APAs) and idiopathic hyperaldosteronism are common causes of primary aldosteronism. Somatic mutations in KCNJ5, CACNA1D, ATP1A1 or ATP2B3 that cause increased glomerulosa cell Ca²⁺ are sufficient for producing APAs; germline mutations alter CYP11B2 in glucocorticoid-remediable aldosteronism (GRA, also known as FH-I), KCNJ5 in FH-III, CACNA1H in FH-IV and CACNA1D in PASNA syndrome. These mutations define a common pathway for induction of aldosterone biosynthesis—glomerulosa cell membrane depolarization activates voltage-gated Ca²⁺ channels, which induces the rate-limiting enzyme for aldosterone biosynthesis, aldosterone synthase (encoded by CYP11B2), along with other enzymes in the biosynthetic pathway; increased mitochondrial Ca²⁺ may also contribute.

In 1992, Stowasser et al. described a multiplex kindred featuring autosomal dominant primary aldosteronism that was clinically distinct from GRA, the only dominant syndrome then known, and hence called it FH-II. The responsible gene in this kindred has not been identified. We recruited an additional affected individual of this kindred (family 3; Fig. 1, Table 1 and Supplementary Note) and performed exome sequencing of three affected subjects, identifying two shared novel protein-changing heterozygous variants in CLCN2 (chr. 3: g.184075850C>T (hg19), p.Arg172Gln, NP_004357) and LINGO1 (chr. 15: g.77906476G>C, p.His591Gln, NP_116197) (Supplementary Table 1). CLCN2 was considered the more likely candidate gene on the basis of conservation (Fig. 1), expression levels in human adrenal cortex (8.14 for CLCN2, 5.91 for LINGO1, log scale, mean expression of all genes = 7.20) and segregation analysis in the pedigree (Fig. 1 and Supplementary Table 2).

We next searched for rare (allele frequency <10⁻³ in public databases) damaging variants in CLCN2 and LINGO1 among the exomes of 35 unrelated individuals diagnosed with unsolved primary aldosteronism by age 10 years—an extreme phenotype. Only CLCN2 showed such variants. All four were heterozygous and were absent in public databases (Supplementary Table 1). Notably, one
proband had the identical p.Arg172Gln variant found in family 3. In the three additional subjects, chr. 3: g.184076907A>T (p.Met22Lys) and chr. 3: g.184076907A>T (p.Tyr26Asn) occurred at positions conserved from invertebrates to humans and one variant produced a new splice donor site resulting in an in-frame deletion (chr. 3: g.184074782T>G (p.Ser865Arg)). Sanger sequencing identified eight carriers of the p.Arg172Gln variant in family 3 (Fig. 1, Table 1, Supplementary Fig. 1 and Supplementary Table 2). In addition to having later onset, she was distinct in having increased aldosterone with upright posture, typical of sporadic idiopathic hyperaldosteronism5. In kindred 1786, the proband’s affected mother and brother carried the p.Arg172Gln variant. The brother had borderline ARR with suppressed renin and non-lateralizing aldosterone production. One subject tested had positive confirmatory fludrocortisone suppression tests (FSTs) and non-lateralizing aldosterone production. One subject had repeatedly normal ARR, suggesting incomplete penetrance. Subject 3-1, diagnosed with hypertension in her thirties and with borderline elevated ARR, tested had positive confirmatory fludrocortisone suppression tests (FSTs) and non-lateralizing aldosterone production. One subject had repeatedly normal ARR, suggesting incomplete penetrance. Subject 3-1, diagnosed with hypertension in her thirties and with borderline elevated ARR, had repeatedly normal ARR, suggesting incomplete penetrance. Subject 3-1, diagnosed with hypertension in her thirties and with borderline elevated ARR, had repeatedly normal ARR, suggesting incomplete penetrance.

Among the four kindreds with p.Arg172Gln, the mutation was de novo in one kindred (kindred 318; absent in the proband’s biological parents; Fig. 1 and Supplementary Table 4). Among the other three kindreds, the maximum lengths of shared mutation-linked haplotypes between pairs of individuals ranged from 4,894 to 357,885 bp (Supplementary Table 5), with the putative last shared ancestor occurring ~651 (95% confidence interval (CI), 203–2,615) to ~50,000 (95% CI, 10,000–infinity) generations ago14. Although extremely remote common ancestry is a possibility, independent occurrence is overwhelmingly likely. After finding this mutation in the first family, the probability of finding, by chance, three additional independent instances of the mutation encoding p.Arg172Gln (one de novo) among 80 probands is 6.5 × 10−12 (Methods). The probability of any one of these mutations being identical by descent from a remote common ancestor is even lower. Lastly, the burden of rare protein-altering CLCN2 variants in primary aldosteronism kindreds is significantly higher than in controls (8/81 versus 6/3,578, P = 1.3 × 10−16, relative risk = 58.9; Supplementary Table 6).

Sanger sequencing identified eight carriers of the p.Arg172Gln variant in family 3 (Fig. 1, Table 1, Supplementary Fig. 1 and Supplementary Note). Seven carriers had an elevated aldosterone/renin ratio (ARR; a screening test for primary aldosteronism); those tested had positive confirmatory fludrocortisone suppression tests (FSTs) and non-lateralizing aldosterone production. One subject had repeatedly normal ARR, suggesting incomplete penetrance. Subject 3-1, diagnosed with hypertension in her thirties and with primary aldosteronism at age 66 years, was wild type for CLCN2 (Supplementary Table 2). In addition to having later onset, she was distinct in having increased aldosterone with upright posture, typical of sporadic idiopathic hyperaldosteronism5. In kindred 1786, the proband’s affected mother and brother carried the p.Arg172Gln variant. The brother had borderline ARR with suppressed renin and prehypertension at age 13 years. Subject 1492-1 was diagnosed with hypertension at age 13 years. Subject 1492-1 was diagnosed with hypertension at age 13 years.
hypertension and primary aldosteronism in infancy but became normotensive by age 2, suggesting variable expressivity with age. Among other probands, the mutation encoding the p.Met22Lys variant was de novo (Supplementary Table 4). Thus, in two of the four kindreds with parental data, the rare CLCN2 mutations were de novo.

In silico splice-site analysis of the variant in kindred 1492 predicted the creation of a new splice donor site at the end of exon 10, 3 bp upstream of the normal splice donor site. In a splicing assay in HEK cells (Supplementary Note), the wild-type exon was normally spliced but the mutation resulted exclusively in splicing at the predicted upstream site, producing an in-frame deletion of codon 362 (Supplementary Fig. 1).

CLC-2, the chloride channel encoded by CLCN2, is found in many tissues, including brain, kidney, lung and intestine. Additionally, CLCN2 RNA is found in the adrenal gland. Immunohistochemistry with an antibody specific for CLC-2 showed intense staining of the human adrenal zona glomerulosa (Fig. 2 and Supplementary Fig. 2), with an antibody specific for ClC-2 showing partial colocalization with YFP-tagged ClC-2WT and ClC-2MUT with a membrane potential of about –80 mV. This depolarization is predicted to activate voltage-gated Ca2+ channels, inducing aldosterone biosynthesis.

A facilitative subunit of CIC-2 in glia, GlialCAM, also known as HEPACAM,23 is not expressed in human adrenal gland (Genotype-Tissue Expression (GTEx) portal; see URLs). We therefore expressed wild-type CIC-2 (CIC-2WT) and each of the mutant CIC-2 proteins (CIC-2MUT) alone in HEK293T cells and performed whole-cell patch-clamp electrophysiology at [Cl–]i = 75 mM. CIC-2WT channels are closed at depolarized voltages and activate slowly at voltages negative to the chloride reversal potential.31,24,26 All mutants shifted the activation curve to more positive voltages (Fig. 3, Supplementary Fig. 2 and Supplementary Table 8). CLC channels are homodimers, with each subunit forming a separate pore. Each of these conduction pathways can be individually opened and closed by a fast protopore gate, while a common slow gating mechanism acts on both pores.24,26 Where p.Ser865Arg slowed down closing its minimum open probability and accelerating its activation deactivation of both gates and altered the protopore open probability,24,26. Whereas p.Ser865Arg slowed down deactivation of both gates and altered the protopore open probability, all other variants modified the common gate by increasing its minimum open probability and accelerating its activation (Fig. 3 and Supplementary Fig. 3). Mass spectrometry demonstrated that Ser865 was phosphorylated, suggesting a regulatory mechanism (Supplementary Fig. 4). The observed gating alterations with the CIC-2MUT channels predict significantly larger chloride efflux versus CIC-2WT channels in glomerulosa cells at physiological membrane potentials.

To characterize the impact of the CIC-2WT and CIC-2MUT channels in human adrenal glomerulosa cells, we expressed these channels in human H295R adrenocortical cancer cells, an established model of aldosterone production.22,27 Confocal microscopy showed partial colocalization of YFP-tagged CIC-2WT and CIC-2MUT with a cell surface membrane marker; the Met22Lys variant showed less colocalization than CIC-2WT (Supplementary Fig. 2). RNA-seq (Fig. 4a and Supplementary Table 9) demonstrated that transfection...
of cells to express untagged wild-type or Arg172Gln CIC-2 in both cases significantly increased expression of CYP11B2 and its upstream regulator NR4A2 (NURR1)30; RG54, which provides feedback inhibition of angiotensin II (Ang II)-triggered signaling30, was also upregulated. Quantitative real-time PCR of CYP11B2 showed that transfection with CLCN2WT produced significantly greater increases in CYP11B2 expression than were observed with CLCN2mut (Fig. 4b). In contrast, transfection with CLCN2 encoding loss-of-function mutations49 did not change CYP11B2 expression (Supplementary Fig. 5). H295R cells and their subclone HAC15 expressing loss-of-function mutations30 did not change expression of cells to express untagged wild-type or Arg172Gln CIC-2 in both cases significantly increased expression of CYP11B2 and its upstream regulator NR4A2 (NURR1)30; RG54, which provides feedback inhibition of angiotensin II (Ang II)-triggered signaling30, was also upregulated. Quantitative real-time PCR of CYP11B2 showed that transfection with CLCN2WT produced significantly greater increases in CYP11B2 expression than were observed with CLCN2mut (Fig. 4b). In contrast, transfection with CLCN2 encoding loss-of-function mutations49 did not change CYP11B2 expression (Supplementary Fig. 5). H295R cells and their subclone HAC15 expressing loss-of-function mutations30 did not change expression of...
Fig. 4 | CIC-2 increases aldosterone synthase expression in H295R cells. a, RNA sequencing of H295R cells transfected with CLCN2 (WT or Arg172Gln) or vector control. CLCN2 and CYP11B2 show the largest increase in expression versus control. Genes involved in adrenal function or calcium pathways are highlighted. FPKM, fragments per kilobase of transcript per million fragments mapped. b, Relative expression levels of CYP11B2 (box, interquartile range; whiskers, 1.5 times the interquartile range: line, median) in the H295R cell line after transfection with empty vector (control), CLCN2WT (blue) or CLCN2MUT (orange). Parallel transfections and real-time PCR reactions were performed in each group. CYP11B2 expression significantly increases after transfection with CLCN2MUT (see Supplementary Table 8 for statistical analysis). c, Resting membrane potential (plots as in b) of HAC15 cells stably expressing CLCN2 (WT or Arg172Gln) and untransfected controls. WT and Arg172Gln channels cause significant depolarization versus the control, and Arg172Gln causes significant depolarization versus WT channels (see Supplementary Table 8 for statistical analysis and tests used). d, Model of CIC-2 function in human adrenal glomerulosa. Resting cells are hyperpolarized. Ang II and hyperkalemia cause depolarization, activation of voltage-dependent calcium channels, calcium influx and increased CYP11B2 expression via the transcription factor NR4A2 (NURR1). CIC-2MUT causes increased CYP11B2 expression by membrane depolarization via increased chloride efflux. **P < 0.01; ***P < 0.001; ****P < 0.0001.

Proband with FH-II showed early-onset primary aldosteronism and hypertension, often with hypokalemia. Hypertension was controlled with mineralocorticoid receptor antagonists or other antihypertensives (Supplementary Note). The phenotype appeared indistinguishable from that of patients with CACNA1H mutations. Hybrid steroid production and/or response to glucocorticoids, historically used to diagnose GRA, was absent, as were massive adrenal hyperplasia (present in many subjects with KCNJ5 variants) and neurodevelopmental abnormalities (characteristic of subjects with CACNA1D mutation). Despite widespread CLCN2 expression, subjects with gain-of-function CLCN2 variants shared no apparent pathology other than primary aldosteronism, whereas loss-of-function CLCN2 variants cause leukoencephalopathy with ataxia, with a similar phenotype in mice. Incomplete penetrance or phenotypic amelioration with age, as sometimes occurs with germline mutations in CYP11B2, KCNJ5, CACNA1D and CACNA1H, occurred in some subjects with CLCN2 mutations.

Our findings implicate the activity of an anion channel in the regulation of aldosterone biosynthesis, primary aldosteronism and hypertension. Whether previously described slowly activating tiny chloride currents at strongly negative voltages in rat glomerulosa cells or Ras-dependent chloride currents represent CIC-2 activity is unclear. In vivo, CIC-2 may contribute to hyperpolarization-induced depolarization of adrenal glomerulosa cells, cyclic membrane potential oscillations and aldosterone production. Variants implicated in primary aldosteronism would likely amplify these effects. Mouse models may prove useful to study such effects.

Methods
Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41588-018-0048-5.
23. Jeworutzki, E. et al. GlialCAM, a protein defective in a leukodystrophy, serves as a common substrate for multiple signaling pathways. *Clin. Exp. Pharmacol. Physiol.* 19, 319–322 (1992).

22. Hu, C., Rusin, C. G., Tan, Z., Guagliardo, N. A. & Barrett, P. Q. Zona glomerulosa cells of the mouse adrenal cortex are intrinsic electrical oscillators. *J. Physiol.* 568, 2851–2862 (2006).

21. Lifton, R. P. et al. A chimaeric zona glomerulosa cell transgenic mouse model for the study of primary aldosteronism. *Nat. Genet.* 45, 1050–1054 (2013).

20. Fernandes-Rosa, F. L. et al. Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension* 64, 354–361 (2014).

19. Dogan, R. I., Getoor, L., Wilbur, W. J. & Mount, S. M. SplicePort—an interactive splice-site analysis tool. *Nucleic Acids Res.* 33, W253–W258 (2005).

18. Genin, E., Tullio-Pelet, A., Begeot, F., Lyonnet, S. & Abel, L. Estimating the frequency of frameshift mutations underlying childhood benign cerebral lymphangiomyomatosis (CBL). *J. Med. Genet.* 45, 440–444 (2012).

17. Scholl, U. I. et al. Somatic and germline CAGNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat. Genet.* 45, 445–449 (2013).

16. Carss, K. J., Stowasser, M., Gordon, R. D. & O'Shaughnessy, K. M. Further evidence that the calcium-sensitive potassium channel KCNB1 is a new gene for familial hyperaldosteronism type 1. *Hum. Mol. Genet.* 13, 489–500 (2004).

15. Korah, H. E. & Scholl, U. I. An update on familial hyperaldosteronism. *J. Hum. Hypertens.* 25, 1610–1613 (2009).

14. Gilissen, C., van der Knaap, M. S. & Vissers, L. E. L. Mutations in the *SHOC2* gene cause X-linked dominant nephropathy with advanced renal disease and intellectual disability. *Hum. Mol. Genet.* 23, 2224–2230 (2014).

13. Chorvátová, A., Gendron, L., Bilodeau, L., Gallo-Payet, N. & Payet, M. D. A Ras-dependent chloride current activated by adrenocorticotropin in rat adrenal zona glomerulosa cells. *Endocrinology* 141, 684–692 (2000).

12. Romero, R. M. et al. Mapping of the transcription start site for the human *KCNJ5* gene in adrenal zona glomerulosa cells. *J. Endocrinol.* 207, 249–256 (2008).

11. Lifton, R. P. et al. A genetic corepressor network regulates adrenal aldosterone production. *Science* 331, 678–772 (2011).

10. Fernandes-Rosa, F. L. et al. Genetic spectrum and clinical correlates of somatic mutations in aldosterone-producing adenoma. *Hypertension* 64, 354–361 (2014).

9. Beuschlein, F. et al. Somatic mutations in *ATP1A1* and *ATPB3* lead to aldosterone-producing adenomas and secondary hypertension. *Nat. Genet.* 45, 440–444 (2013).

8. Azizan, E. A. et al. Somatic mutations in adrenal zona glomerulosa cells of the mouse adrenal cortex are intrinsic electrical oscillators. *J. Physiol.* 568, 2851–2862 (2006).

7. Scholl, U. I. et al. Somatic and germline CAGNA1D calcium channel mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat. Genet.* 45, 1050–1054 (2013).

6. Choi, M. et al. K+ channel dysfunction in a leukodystrophy. *Nature* 355, 262–265 (1992).

5. Lim, S. S. et al. A comparative risk assessment of burden of disease and injury attributable to 69 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380, 2243–2249 (2012).

4. Monticone, S. et al. Prevalence and clinical manifestations of primary aldosteronism encountered in primary care practice. *J. Am. Coll. Cardiol.* 69, 1813–1820 (2017).

3. NCD Risk Factor Collaboration (NCD-RisC). Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479 population-based measurement studies with 19.1 million participants. *Lancet* 389, 37–57 (2017).

2. Stölling, G. et al. Regulation of ClC-2 gating by intracellular ATP. *J. Physiol.* 586, 388–400 (2017).

1. Monticone, S. et al. Prevalence and clinical manifestations of primary aldosteronism encountered in primary care practice. *J. Am. Coll. Cardiol.* 69, 1813–1820 (2017).
DNA preparation and exome sequencing. DNA was prepared from venous blood or saliva samples using standard procedures. Exome capture was performed using the 2.1 M NimbleGen Exome reagent (Roche NimbleGen), and 75-bp paired-end sequencing was performed on the Illumina platform with analysis performed as described\(^1\).

Sanger sequencing of genomic DNA and genotyping of parent–offspring trios. Direct bidirectional Sanger sequencing of candidate variants from genomic DNA of the indicated subjects was performed at Beckman Coulter Genomics or the Keck DNA sequencing facility at Yale University following PCR amplification. Rare variants identified in index cases through exome sequencing were confirmed by Sanger sequencing of genomic DNA and genotyping of parent–offspring trios.

Immunohistochemistry and immunofluorescence. Formalin-fixed, paraffin-embedded 5-µm human adrenal gland sections were obtained from US Biomax and Panomics. Immunohistochemistry was performed as described, with the exception that 10% donkey serum was used for blocking. The concentration of the antigenic peptide was 0.4 mg/ml, and 1 µg of peptide per microgram of antibody was used. Images were recorded on a Zeiss Axioplan 2 Imaging microscopy (10x and 40x objectives) with a Zeiss AxioCam MRc5 camera. Image cropping was performed in Adobe Illustrator CS4. The primary antibody against ClC-2 was HPA014454 (Sigma-Aldrich Prestige Antibodies; 1:100 dilution, incubation overnight at 4°C), and the antibody against Dab2 was sc-13982 (Santa Cruz Biotechnology; 1:100 dilution). The secondary antibody was donkey anti-rabbit antibody (Jackson, 035-152; 1:200 dilution, incubation for 2 h at room temperature) for human samples. To confirm selection of the zona glomerulosa in mouse adrenal slices for FLIM, slices were stained with the antibody to Dab2 (1:100 dilution, incubation overnight at 4°C). The secondary antibody was donkey anti-rabbit antibody conjugated to Alexa Fluor 647 (A-31573, Thermo Fisher Scientific, 1:1,000 dilution, incubation for 2 h at room temperature).

Immunofluorescence images were recorded on a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems).

Molecular cloning. Site-directed mutagenesis (QuikChange, Agilent Technologies) was performed to introduce mutations into pcDNAs/FRT/TO CIC-2\(^2\) according to the manufacturer’s instructions. Primer sequences (M2ZK_F/R, Y26N_F/R, R172Q_F/R, K362 del new_F/R) are given in Supplementary Table 10. Each construct was validated by sequencing of the entire coding region. Mutant CIC-2s were subcloned in frame into the pRcCMV vector containing YFP cDNA with NotI and PmlI for use in confocal microscopy only. Two independent clones were assayed in all experiments (Supplementary Fig. 3).

Generation of stable cell lines. Stable HEK293 cell lines were generated using the Flp-In T-REx system (Invitrogen, Life Technologies) according to the manufacturer’s instructions. HEK293 cells do not express HEPACAM (Human Protein Atlas; see URLs). Flp-In T-REX 293 cells (authenticated, Line Authentication Service) and cultured in DMEM/F12 (GlutaMAX, Gibco) at 37 °C and 5% CO\(_2\) in a humidified atmosphere. Stable cell lines were prepared using the Piggybac transposon system (System Biosciences). CDNAs of CLCN2 (wild type and encoding p.Arg172Gln) were subcloned into pENTR-2B-Dual using NotI and Xhol. Gateway LR recombination (Invitrogen) was performed with pPiggybac-EF1 Neo and pTRE-LTA (a kind gift of C. Gomez-Sanchez, University of Mississippi). Inserts were verified by Sanger sequencing. HAC15 cells were transfected using an Amaxa Nucleofector 1 (Lonza; 2 million cells, 2 µg of plasmid DNA, 0.8 µg of Super Piggybac transposase; program X-005). After 48 h, selection was initiated by addition of 5 µg/ml g bicarbonate-buffered saline (BBS) (125 mM NaCl, 2 mM KCl, 26 mM NaHCO\(_3\), 0.1 mM CaCl\(_2\), 5 mM MgCl\(_2\), 10 mM glucose, constantly oxygenated with 5% CO\(_2\) in O\(_2\)) for the removal of surrounding fat. The adrenal glands were embedded in 4% agarose in BBS, mounted, cut at 4°C (150–200 µm thick) with a Microm HM 650V (Thermo Scientific; frequency 60 Hz, amplitude 1 mm, drive 10) and held at 35°C for 30 min in BBS. Slices were subsequently stored in BBS at 37 °C for further experiments. During each experiment, slices were constantly perfused with solution at 37°C, and all measurements were completed within 8 h of organ removal.

Fluorescence lifetime imaging microscopy. Prior to chloride imaging experiments, adrenal slices were incubated in BBS containing 10 mM MQAE (Sigma-Aldrich)\(^3\) for 45–60 min at room temperature. Slices were transferred to an imaging chamber and perfused with BBS solution containing 2 mM indocyanine green instead of 0.1 mM Ca\(^{2+}\); FLIM was performed as described\(^4\). The solution was perfused through a heating coil, resulting in a temperature of 37°C in the perfusion chamber. Fluorescence was stimulated by two-photon excitation (\(\lambda_{\text{exc}}=750\) nm). MQAE fluorescence was filtered (short-pass filter 500 nm, \(\lambda_{\text{em}}\leq510\) nm; Omega Optical), and mean fluorescence lifetimes were measured using multidimensional time-correlated single-photon counting (TCSPC). TCSPC electronics (SPC-152, Becker & H Hickl) and acquisition software were used for FLIM as described\(^5\). We recorded data for 12 slices from five different C57BL/6 mice (two male, three female) of 3 months or older.

The chloride concentration calibration, MQAE fluorescence lifetimes of preset \([\text{Cl}^-]\) values were measured. Adrenal slices were incubated in HEPS-buffered solution (140 mM K\(^+\), 10 mM Na\(^+\), 10 mM HEPES, 10–80 mM Cl\(^-\), 70–140 mM glucose, adjusted to 310 mM osmol/L, with potassium glutamate and to pH 7.4 with KOH, 37°C) containing 10 µM nigericin (sodium salt; Sigma-Aldrich) and 10 mM tributyltin (chloride salt; Sigma-Aldrich)\(^6\). The inverse fluorescence lifetime (1/\(\tau\)) was plotted, and the calibration curve was fitted as described before\(^7\). The Stern–Volmer constant (\(K_{SV}=3.96\)) was determined as the product of \(\tau_0\) and the slope of the calibration curve. Because the fluorescence lifetime of MQAE is reduced by chloride via collisional quenching, MQAE fluorescence lifetimes and \([\text{Cl}^-]\) show a linear relationship.

Zona glomerulosa \([\text{Cl}^-]_{\text{int}}\) could then be calculated according to this relationship.

The three outermost cell layers were assumed to form the zona glomerulosa based on their characteristic nucleus-to-cytoplasm ratio and the corresponding staining with antibody to Dab2 (sc-13982, Santa Cruz Biotechnology) performed separately. Each cell was defined as a region of interest (ROI) with the exclusion of the nucleus, and fluorescence lifetimes were determined as mean values of the average fluorescence lifetimes of all pixels in a given ROI. Fluorescence lifetimes were calculated using SPCImage 5.6 (Becker & Hickl) and exported for further extraction in Fiji. Statistical analysis was performed using SigmaPlot 12 (Systat) and Python 3.5.2 + scipy 0.18.1 + pandas 0.19.2 + seaborn 0.7.1 using built-in functions. The FLIM datasets generated or analyzed during the current study are available on request. Python scripts for analysis are based on built-in functions of the above-mentioned packages but are available on request.

Electrophysiological recordings. Flp-In T-REx stable cell lines were used for electrophysiological recordings. For each construct, at least two clones from at least two separate preparations were included in the analysis. To avoid chloride depletion at large current amplitudes\(^8\), Flp-In T-REx cells were used without induction. Whole-cell patch–clamp currents were recorded on an EPC10 amplifier using PatchMaster software (both HEKA). Borosilicate glass pipettes with open resistances between 0.9 and 2.5 M\(\Omega\) were used. The extracellular solution for whole-cell recordings contained in mM 140 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\) and 10 HEPEs at pH 7.4. The intracellular solution contained (in mM) 73 NaCl, 1 MgCl\(_2\), 42 sodium gluconate, 5 EGTA, 10 HEPEs and 1 Mg-ATP at pH 7.4. The liquid

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Instantaneous current amplitudes at the fixed tail step to +60 mV were normalized and plotted against the preceding voltage step to show relative open probability curves. The durations of the voltage steps were 5 s for CIC-2 WT and CIC-2<sup>hewish</sup> and 1 s for all other mutants, so that steady-state open probabilities were determined. Open probabilities (P<sub>open</sub>) were fitted using a modified Boltzmann equation

\[ P_{\text{open}}(V) = \frac{1}{1 + e^{\frac{V - V_0}{\tau}}}. \]

\( V_0 \) is the half-maximal activation voltage, and \( \tau \) is the time constant.

The pipette solution contained (in mM) 140 KCl, 10 HEPES, 2 CaCl<sub>2</sub>, and 1 MgCl<sub>2</sub>, adjusted to a pH of 7.4. The pipette solution (in mM) 130 KCl, 10 HEPES and 5 NaCl with the pH adjusted to 7.4. To maintain the native intracellular [Cl<sup>-</sup>], we included the pore-forming, monovalent-cation-selective antibiotic gramicidin D (Sigma-Aldrich) in the pipette solution to obtain access to the inside of the cell<sup>45</sup>. Gramicidin stock solution (50 mg/ml in DMSO) was prepared daily, and the diluted solution (final concentration of 100 µg/ml) was prepared every 2 h. The tip of the pipette (open resistance of 1.3–3 MΩ) was filled with solution lacking gramicidin to facilitate gigaseal formation. Break-in was typically observed after 15–45 min. Resting potentials were determined using a HEKA EPC-10 patch-clamp amplifier and PatchMaster software (HEKA Elektronik) from the mean of 10- to 60-s voltage recording segments with the current clamped to 0 pA. Only cells that exhibited ClC-2-like currents (visible slow activation upon hyperpolarization and a current clamp step pulse, the ClC-2 fast gate is maximally opened (P<sub>open</sub> fast) 44, and the common gate open probability can be determined. Fast protopore open probabilities were calculated by dividing the open probability by the common gate open probability. Resting potentials in untransfected or induced (1 µg tetracycline/ml medium for 24 h) stably transfected HEK293 cells were measured using the above-mentioned patch technique<sup>2</sup> in the current-clamp mode. The extracellular solution contained (in mM) 140 NaCl, 10 HEPES, 2 CaCl<sub>2</sub> and 1 MgCl<sub>2</sub> adjusted to a pH of 7.4.

## Shared haplotypes and estimation of the age of the mutation encoding p.Arg172Gln in CLCN2.

Genotypes of SNPs flanking the CLCN2<sup>hewish</sup> mutation were extracted from exome data. To estimate the age of the mutation in CLCN2 encoding p.Arg172Gln testing the assumption that the mutation is identical by descent among all possible pairs of kindreds with the variant (except the documented de novo mutation), we used the ESTIAGE algorithm to estimate the pairwise time of coalescence for the three pairs of kindreds as previously described<sup>13</sup>. ESTIAGE uses a maximum-likelihood approach to estimate the mutation age, which takes into account the frequencies of the shared allele at each marker and the recombination fractions between the mutation of interest and polymorphic markers located within or at the boundaries of the shared haplotype.

Seventeen polymorphic markers spanning the shared haplotype were used for input (Supplementary Table S5). The marker allele frequencies were estimated from the Finnish and non-Finnish European populations in the ExAC database<sup>8</sup>, and the mutation rate was set to 2 × 10<sup>−8</sup>.

## Statistics

The statistical analyses used throughout the manuscript are described in the corresponding results and methods paragraphs, figure legends or supplementary tables.

## UIRLS

ClinVar, [https://www.ncbi.nlm.nih.gov/clinvar/](https://www.ncbi.nlm.nih.gov/clinvar/); HEPACAM localization in Human Protein Atlas (retrieved 27 June 2017), [http://www.proteinatlas.org/ENSG0000016478-HEPACAM/cell/HKE+&929;](http://www.proteinatlas.org/ENSG0000016478-HEPACAM/cell/HKE+&929;); FastQC, [https://www.bioinformatics.babraham.ac.uk/projects/fastqc/](https://www.bioinformatics.babraham.ac.uk/projects/fastqc/); GTEx Portal HEPACAM expression, [http://www.gtexexportal.org/home/gene/HEPACAM](http://www.gtexexportal.org/home/gene/HEPACAM).

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

## Data availability

CLCN2 variants have been deposited in ClinVar (accessions 441164, 441165, 441166, 441167 and 441168), and RNA-seq data have been deposited at GEO (accession GSE107030).
References
37. Torpy, D. J. et al. Familial hyperaldosteronism type II: description of a large kindred and exclusion of the aldosterone synthase (CYP11B2) gene. J. Clin. Endocrinol. Metab. 83, 3214–3218 (1998).
38. Krumn, N. et al. Excess of rare, inherited truncating mutations in autism. Nat. Genet. 47, 582–588 (2015).
39. Verkman, A. S. Development and biological applications of chloride-sensitive fluorescent indicators. Am. J. Physiol. 259, C375–C388 (1990).
40. Kaneko, H., Putzier, I., Frings, S., Kaupp, U. B. & Gensch, T. Chloride accumulation in mammalian olfactory sensory neurons. J. Neurosci. 24, 7931–7938 (2004).
41. Bevensee, M. O., Apkon, M. & Boron, W. F. Intracellular pH regulation in cultured astrocytes from rat hippocampus. II. Electrogenic Na/HCO3 cotransport. J. Gen. Physiol. 110, 467–483 (1997).
42. Chao, A. C., Dix, J. A., Sellers, M. C. & Verkman, A. S. Fluorescence measurement of chloride transport in monolayer cultured cells. Mechanisms of chloride transport in fibroblasts. Biophys. J. 56, 1071–1081 (1989).
43. Accardi, A. & Pusch, M. Fast and slow gating relaxations in the muscle chloride channel CLC-1. J. Gen. Physiol. 116, 433–444 (2000).
44. de Santiago, J. A., Nehrke, K. & Arreola, J. Quantitative analysis of the voltage-dependent gating of mouse parotid CIC-2 chloride channel. J. Gen. Physiol. 126, 591–603 (2005).
45. Rhee, J. S., Ebihara, S. & Akaike, N. Gramicidin perforated patch-clamp technique reveals glycine-gated outward chloride current in dissociated nucleus solitarii neurons of the rat. J. Neurophysiol. 72, 1103–1108 (1994).
46. Kim, D. et al. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36 (2013).
47. Trapnell, C. et al. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat. Biotechnol. 31, 46–53 (2013).
48. Samocha, K. E. et al. A framework for the interpretation of de novo mutation in human disease. Nat. Genet. 46, 944–950 (2014).
49. Zou, J. et al. Quantifying unobserved protein-coding variants in human populations provides a roadmap for large-scale sequencing projects. Nat. Commun. 7, 13293 (2016).
50. Lek, M. et al. Analysis of protein-coding genetic variation in 60,706 humans. Nature 536, 285–291 (2016).
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

1. Sample size
   Describe how sample size was determined.
   Sample sizes for electrophysiological recordings and FLIM experiments were estimated according to previous experience with similar proteins and experimental setups; sample size for real-time PCRs was estimated based on electrophysiological results and results of RNAseq.

2. Data exclusions
   Describe any data exclusions.
   For electrophysiological recordings (technically acceptable recording, presence of CIC-2-like currents) and FLIM (outer layers of the adrenal gland as described in Methods) experiments, only inclusion criteria were defined with no data being excluded afterwards.
   For CYP11B2 qPCR (Figure 4C), the first transfection was excluded because the experimentator performed this protocol and transfection (via electroporation) for the first time and used it for practice.
   For confocal microscopy, cells without sufficient detection of CFP and/or YFP were excluded.
   For membrane potential measurements, cells without CIC-2-like currents were excluded.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   Electrophysiological experiments, FLIM data, real-time PCRs, and confocal microscopy were performed using multiple separate preparations and at least two DNA or stable cell line clones and were reliably reproduced. IHC was reliably reproduced (2 technical replicates each of two biological replicates). Splicing assay was performed using two independent clones with indistinguishable results. Mass spectrometry was performed on two independent preparations, with reproducible results. Similar results were obtained from two preparations using a different solvent.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Samples for electrophysiological, FLIM experiments, confocal microscopy, IHC and real-time PCRs were not randomized.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   For electrophysiological, FLIM and RNA sequencing experiments, investigators were not blinded to group allocation. For CYP11B2 qPCR (Figure 4C) the investigator was blinded to group allocation of two replicates by replacing the plasmid names with numbers.
   The investigator was not blinded to group allocation for confocal microscopy and immunohistochemistry.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
### 6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☑   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
| ☑   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | A statement indicating how many times each experiment was replicated |
| ☑   | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
| ☑   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
| ☑   | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
| ☑   | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

### Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Analysis of all electrophysiological experiments were performed using FitMaster software (HEKA Elektronik), SigmaPlot 12 (Systat) and Python 3.5.2+numpy 1.12.1+scipy 0.18.1+pandas 0.19.2 using built-in functions. Fluorescence lifetimes were calculated using SPCImage 5.6 (Becker&Hickl) and exported for further extraction in Fiji. Statistics were performed using SigmaPlot 12 (Systat) and Python 3.5.2+numpy 1.12.1+scipy 0.18.1+pandas 0.19.2+seaborn 0.7.1 using built-in functions. Real-time PCR results were analyzed with Microsoft Excel 2010 and Graph Pad Prism 6. Confocal microscopy was analyzed in ZEN 2012 (black edition, Carl Zeiss) and Graphpad Prism 6. Mass spectrometry was analyzed using MaxQuant version 1.5.1.2.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

### Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There are restrictions on availability of subject DNAs and exome sequencing data because of a lack of consent for data sharing and potential identification of subjects from exome data.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Anti-CLCN2 (HPA014545): Sigma-Aldrich; Lot # R06751; polyclonal; IgG; validation is described in human protein atlas (http://www.proteinatlas.org/ENSG00000114859-CLCN2/antibody)  
Dab2 (H-110): Santa Cruz sc-13982; Lot # E3013; polyclonal; validation is described in the following citations: PMID: # 24889971, PMID: # 23293299, PMID: # 23840954
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used.  
      H295R: gift of Dr. Matthias Haase (HHU Düsseldorf); HAC15: gift of Dr. William Rainey (University of Michigan); Flp-In-T-REx 293 cells were obtained from Invitrogen.
   b. Describe the method of cell line authentication used.  
      H295R/HAC15: short tandem repeat (STR) analysis by ATCC (03/30/2016)  
      HEK293T: short tandem repeat (STR) analysis by Eurofins Genomics (10/13/2016)
   c. Report whether the cell lines were tested for mycoplasma contamination.  
      H295R, HAC15 and HEK293 tested negative for mycoplasma contamination.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.  
      H295R and HAC15 (as a subclone of H295R) are the most established human aldosterone-producing adrenal cell lines. We used HAC15 for stable cell line generation because H295R is not a clonal cell line.  
      HEK293 cells are an established system for patch clamp electrophysiology.

> Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study.  
      Five C57BL/6 mice were used for FLIM experiments (2 males, 3 females, age > 3 mo)

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants.  
      This information is provided in Table 1 and Supplementary Table 3 of the manuscript. Additional probands are described in Ref. 13.