Repository of the Max Delbrück Center for Molecular Medicine (MDC) in the Helmholtz Association

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

Fernandes-Rosa F.L., Daniil G., Orozco I.J., Göppner C., El Zein R., Jain V., Boulkroun S., Jeunemaitre X., Amar L., Lefebvre H., Schwarzmayr T., Strom T.M., Jentsch T.J., Zennaro M.C.

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

Fabio L Fernandes-Rosa[1,2,3*#], Georgios Daniil[1,2*], Ian J Orozco[4,5*], Corinna Göppner[4,5$], Rami El Zein[1,2$], Vandana Jain[6^], Sheerazed Boulkroun[1,2^], Xavier Jeunemaitre[1,2,3], Laurence Amar[1,2,7], Hervé Lefebvre[8,9,10], Thomas Schwarzmayr[11], Tim M Strom[11,12], Thomas J Jentsch[4,5#], Maria-Christina Zennaro[1,2,3#]

[1]INSERM, UMRS_970, Paris Cardiovascular Research Center, Paris, France
[2]Université Paris Descartes, Sorbonne Paris Cité, Paris, France
[3]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service de Génétique, Paris, France
[4]Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Berlin, Germany
[5]Max-Delbrück-Centrum für Molekulare Medizin (MDC), Berlin, Germany
[6]Division of Pediatric Endocrinology, Department of Pediatrics, All India Institute of Medical Sciences, New Delhi, India
[7]Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Unité Hypertension artérielle, Paris, France
[8]Normandie Univ, UNIROUEN, Rouen, France
[9]INSERM, DC2N, Rouen, France
[10]Department of Endocrinology, Diabetes and Metabolic Diseases, University Hospital of Rouen, Rouen, France
[11]Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg, Germany
[12]Institute of Human Genetics, Technische Universität München, Munich, Germany
*,$,^equal contribution
#Corresponding authors

Address correspondence to:
Maria-Christina Zennaro, MD, PhD
INSERM, U970
Paris Cardiovascular Research Center – PARCC
56, rue Leblanc,
75015 Paris – France
Tel : +33 (0)1 53 98 80 42
Fax : + 33 (0)1 53 98 79 52
e-mail : maria-christina.zennaro@inserm.fr

Fabio Fernandes Rosa, MD, PhD
INSERM, U970
Paris Cardiovascular Research Center – PARCC
56, rue Leblanc,
75015 Paris – France
Tel : +33 (0)1 53 98 80 43
Fax : + 33 (0)1 53 98 79 52
e-mail : fabio.fernandes-rosa@inserm.fr

Thomas J. Jentsch, MD, PhD
FMP / MDC
Robert-Rössle-Strasse 10
13125 Berlin – Germany

Tel: +49 30 9406 2961
Fax: +49 30 9406 2960
e-mail: Jentsch@fmp-berlin.de

INTRODUCTORY PARAGRAPH

Primary aldosteronism is the most common and curable form of secondary arterial hypertension. We performed whole exome sequencing in patients with early-onset primary aldosteronism and identifed a *de novo* heterozygous c.71G>A/p.Gly24Asp mutation in the *CLCN2* gene, coding for the voltage-gated ClC-2 chloride channel [1], in a patient diagnosed at age 9 ys. Patch-clamp analysis of glomerulosa cells of mouse adrenal gland slices revealed hyperpolarization-activated Cl^- currents that were abolished in *Clcn2*$^{-/-}$ mice. The p.Gly24Asp mutation, located in a well conserved 'inactivation domain' [2,3], abolished the voltage- and time-dependent gating of ClC-2 and strongly increased Cl^- conductance at resting potentials. Expression of ClC-2$_{24Asp}$ in adrenocortical cells increased expression of aldosterone synthase and aldosterone production.

Our data indicate that *CLCN2* mutations cause primary aldosteronism. They highlight for the first time the important role of chloride in aldosterone biosynthesis and identify ClC-2 as the foremost chloride conductor of resting glomerulosa cells.

1   **MAIN TEXT**

2   Arterial hypertension is a major cardiovascular risk factor [4]. Primary aldosteronism is

3   the most common and curable form of secondary arterial hypertension, with an estimated

4   prevalence of ~10% in referred patients and 4% in primary care[5], and up to 20% of patients

5   with resistant hypertension [6]. Primary aldosteronism results from autonomous aldosterone

6   production in the adrenal cortex [7], caused in most cases by a unilateral aldosterone-producing

7   adenoma or bilateral adrenal hyperplasia (BAH). It is diagnosed on the basis of hypertension

8   associated with increased aldosterone to renin ratio and often hypokalemia [8]. Compared to

9   essential hypertension, increased aldosterone levels in primary aldosteronism are associated

10   with increased cardiovascular risk in particular coronary artery disease, heart failure, renal

11   damage and stroke [9,10].

12   Gain-of-function mutations in different genes, coding for cation channels (*KCNJ5* [11],

13   *CACNA1D* [12,13], *CACNA1H* [14,15]) and ATPases (*ATP1A1* and *ATP2B3*, [12,16]), regulating

14   intracellular ion homeostasis and plasma membrane potential, have been described in

15   aldosterone producing adenoma and familial forms of primary aldosteronism, but the

16   pathophysiology of many cases is still unknown.

17 We performed whole exome sequencing on germline DNA from 12 patients with young onset

18 hypertension and hyperaldosteronism diagnosed before age 25 ys. Two index cases were

19 investigated together with their parents and unaffected sibling to search for *de novo* variants.

20 A *de novo* germline *CLCN2* variant c.71G>A (NM_004366; p.Gly24Asp) was identified in

21 subject K1011-1, but not in her asymptomatic parents (K1011-3 and K1011-4) and sibling

22 (K1011-2, Fig. 1a and 1b, Table 1). The variant was absent from more than 120.000 alleles in

23 the Exome Aggregation Consortium (ExAC) and in our in-house database. We did not find

24 additional *CLCN2* variants among the other 11 investigated individuals. *CLCN2* encodes the

25 chloride channel ClC-2. The variant *CLCN2* p.Gly24Asp localizes to its N-terminal

26 cytoplasmic domain (Fig. 1c, d). Gly24 is highly conserved in ClC-2 orthologs of species as

27 distant as zebrafish and *Xenopus* (Fig. 1c).

28   The patient carrying the *CLCN2* p.Gly24Asp variant is a 9 year old girl, who presented

29 with severe headache and vomiting lasting for 1 year (Table 1). The child was

30 developmentally normal, first born to a non-consanguineous couple. There was history of

31 mild hypertension in maternal grandmother and granduncle. Blood pressure (BP) was 172/100

32 mm Hg, heart rate was 120/minute. The rest of the examination was normal. Her work-up

33 revealed persistent hypokalemia (serum K^+ ranging from 1.8 to 2.4 meq/L), elevated serum

34 aldosterone (868.1 pg/ml, reference range 12-340 pg/ml) and suppressed plasma renin activity

35 (0.11 ng/ml/hr, reference range 1.9 to 6.0 ng/ml/hr in upright posture), suggestive of primary

36 aldosteronism. Abdominal CT scan showed no adrenal abnormalities. Other parameters

37 including 24 hours urinary vanillylmandelic acid and serum cortisol were normal.

38 Hypertension was initially managed with amlodipin, enalapril and atenolol. Once the

39 diagnosis of primary aldosteronism was made, spironolactone was added, enalapril was

40 stopped and doses of amlodipin and atenolol were reduced. Serum K^+ normalized. A positive

41 glucocorticoid suppression test (aldosterone 949.3 pg/ml at baseline and 56.9 pg/ml after

42   administration of oral dexamethasone 0.5 mg every 6 hours for 48 hours) suggested the

43   possibility of glucocorticoid remediable aldosteronism (GRA), a rare familial form of

44   hyperaldosteronism [17]. However, genetic analysis for a chimeric *CYP11B1*/*CYP11B2* gene

45   was negative. The child's hypertension has been well controlled over the last 18 months with

46   prednisolone 5mg/m^2/day, spironolactone and amlodipin. On treatment, her serum aldosterone

47   and plasma renin are 421 pg/ml (reference range 25 to 392) and 8.22 µU/mL (4.4 to 46.1).

48   After exclusion of GRA by genetic testing, prednisolone treatment was stopped.

49   Cl$^-$ conductances can regulate the excitability of neuronal, muscle, and endocrine cells

50   [18-21]. In zona glomerulosa cells, ACTH-activated Cl$^-$ currents have been described [22], but their

51   outward rectification sets them apart from hyperpolarization-activated ClC-2 currents. ClC-2

52   is expressed in almost all tissues [1] and may have roles in ion homeostasis and transepithelial

53   transport [23]. *Clcn2*$^{-/-}$ mice display early postnatal retinal and testicular degeneration [24] as well

54   as leukodystrophy [25,26]; in humans, *CLCN2* loss-of-function mutations result in

55   leukodystrophy[27] that may be associated with azoospermia[28]. These phenotypes have been

56   ascribed to a role of ClC-2 in extracellular ion homeostasis [24,25].

57   Data retrieved from a transcriptome analysis including eleven human adrenals [29]

58   showed high expression of *CLCN2* in human adrenal cortex (Supplementary Table 1). In

59   mice, Western blots revealed similar expression of ClC-2 in whole adrenal gland as in brain

60   (Fig. 2a), which expresses substantial, physiologically important amounts of ClC-2 [25]. Patch-

61   clamp analysis of glomerulosa cells *in situ* revealed typical hyperpolarization-activated

62   currents in WT, but not in *Clcn2*$^{-/-}$ mice (Fig. 2 b,c). Their magnitude was similar to those

63   observed in Bergmann glia which prominently express ClC-2 [26]. The almost complete

64   absence of Cl$^-$ currents in *Clcn2*$^{-/-}$ cells demonstrates that under resting conditions ClC-2

65   mediates the bulk of glomerulosa cell Cl$^-$ currents.

66   The *CLCN2* p.Gly24Asp mutation is located in a highly conserved 'inactivation

67   domain' [2,3] of the channel. Deletions and point mutations in this region and an intracellular

68   loop [2] (highlighted in Fig. 1c, d) lead to 'open' ClC-2 channels that have lost their sensitivity

69   to voltage, cell swelling, or external pH [2,3]. Likewise, insertion of the p.Gly24Asp mutation

70   drastically changed voltage-dependent gating of ClC-2 (Fig. 2d,e,h) and dramatically

71   increased current amplitudes when expressed in *Xenopus* oocytes (Fig. 2d-g). When measured

72   at -80 mV, the approximate resting potential of glomerulosa cells [30], current amplitudes from

73   the mutant channel were much larger compared to WT (Fig. 2d-f). Linear, ohmic currents like

74   those of the mutant channel might be due to unspecific electrical leaks; however, currents of

75   both WT and mutant ClC-2 were markedly reduced when extracellular chloride was replaced

76   by iodide (Fig. 2d-f), agreeing with the $Cl^->I^-$ selectivity of CLC channels in general [23] and

77   ClC-2 in particular [1]. The activation of ClC-2 by acid extracellular pH can be almost

78   abolished by mutations in the 'inactivation domain'[2]. Likewise, the ClC-2$_{24Asp}$ mutant had

79   largely reduced pH sensitivity (Fig. 2i-j, Supplementary Fig.1). In conclusion, the

80   p.Gly24Asp mutation results in a strong gain of function, explaining the dominant disease

81   phenotype of the mutation that is present at the heterozygote state. It also suggests a

82   pathophysiological mechanism in which a strong increase in Cl^- currents may depolarize

83   glomerulosa cells, thereby opening voltage-gated Ca^{2+} channels and activating transcriptional

84   programs via an increase of cytosolic Ca^{2+}.

85   Expression of the mutant ClC-2$_{24Asp}$ channel in adrenocortical H295R-S2 cells, and

86   conversely, knock-down of ClC-2 by shRNA significantly affected aldosterone production

87   and expression of steroidogenic enzymes. Despite similar expression of ClC-2 in H295R-S2

88   cells stably transfected with ClC-2$_{24Asp}$ or ClC-2$_{WT}$ (Fig. 3a and 3b), aldosterone synthase

89   expression (Fig. 3a and 3c) and aldosterone production (Fig. 3d, e) were significantly

90   increased in ClC-2$_{24Asp}$ expressing cells. Stimulation with angiotensin II (AngII 10nM) or K^+

91   (12mM) increased aldosterone production in cells expressing WT ClC-2 (Fig. 3e). A further

92   increase was observed in cells expressing ClC-2$_{24Asp}$ after AngII stimulation, but not after K$^+$

93   stimulation (Fig. 3e). Nevertheless, also after stimulation, aldosterone production in cells

94   expressing ClC-2$_{24Asp}$ was significantly higher compared to cells expressing ClC-2$_{WT}$ (Fig.

95   3e). Infection of H295R-S2 cells with ClC-2 shRNA reduced *CLCN2* expression by ~50%

96   (Supplementary Fig. 2a) compared with a scrambled shRNA, and significantly reduced

97   aldosterone production, both at baseline and after stimulation (Supplementary Fig. 2b),

98   suggesting that even WT ClC-2 currents, although much smaller than currents from the

99   Gly24Asp mutant, significantly increase the excitability of H295R-S2 adrenocortical cells.

100   These changes were paralleled in both models by concomitant modifications of the expression

101   of steroidogenic genes. A significant increase of mRNA expression of *CYP11B2* (encoding

102   aldosterone synthase, Fig. 3f), *StAR* (encoding the Steroidogenic acute regulatory protein, Fig.

103   3g) and *CYP21A2* (encoding Steroid 21-hydroxylase, Fig. 3h) was observed in ClC-2$_{24Asp}$

104   compared with ClC-2$_{WT}$ overexpressing cells in basal conditions. AngII increased expression

105   of *StAR* and *CYP11B2*, while K$^+$ stimulation increased mRNA expression of *CYP11B2*.

106   Conversely, knock-down of ClC-2 led to a significant decrease in *CYP11B2* expression in all

107   conditions (Supplementary Fig. 2c). These data further support the notion that a gain-of-

108   function *CLCN2* mutation may depolarize the cell, activate the steroidogenic pathway, and

109   increase aldosterone production. While knock-down of ClC-2 influences aldosterone

110   production in H295R-S2 cells which have a resting potential of about − 65 mV (Fig. 4a, b),

111   this may not be the case in native glomerulosa cells. Because their V$_m$ is close to the K$^+$

112   equilibrium potential [30], they are unlikely to markedly hyperpolarize upon loss of ClC-2. No

113   changes of blood pressure have been reported for mice or patients lacking ClC-2 [24,25,27], but

114   this issue has not been investigated in detail.

115   We next explored the effect of the ClC-2 Gly24Asp mutation on the membrane

116   potential and on calcium influx through voltage-gated calcium channels. These studies were

117   performed with the perforated patch clamp technique which does not disturb the intracellular

118   chloride concentration and is required to see the full effect of 'inactivation domain'[2,3]

119   mutations[31,32]. In the stably transfected H295R-S2 cells used to investigate steroidogenesis

120   (Fig. 3), there was a trend of V_m to be depolarized in ClC-2_{24Asp} transfected compared to ClC-

121   2_{WT} transfected cells (mean values of roughly -52 and -67 mV, respectively) (Fig. 4a).

122   However, because these cell lines are not clonal, the variability was large and the difference

123   was not statistically significant.

124   We therefore resorted to transient transfection of H295R-S2 cells which allowed us to

125   select ClC-2 expressing cells by fluorescence of co-transfected GFP (Fig. 4b-g). Although

126   these cells express ClC-2 less efficiently than *Xenopus* oocytes (compare Fig. 4f and 2d) and

127   HEK cells [31,32], ClC-2_{24Asp} expressing cells displayed robust chloride currents that lacked

128   strong voltage-dependence (Fig. 4c-g). The observed increase in currents may reflect both an

129   increase in currents per channel and in the number of channels; both must be considered when

130   analyzing pathogenic effects of ion channel mutants. This increase in curents correlated with a

131   strong depolarization from $V_m = -65 \pm 4$ (ClC-2_{WT}) to -46 ± 4 mV in ClC-2_{24Asp} transfected

132   cells (Fig. 4b), indicating that chloride concentration in H295R-S2 cells is higher than

133   predicted by the electrochemical equilibrium. This depolarization may open voltage-

134   dependent calcium channels. Indeed, nifedipine (an L-type calcium channel blocker) and/or

135   mibefradil (a T-type calcium channel blocker) strongly reduced aldosterone production in

136   cells expressing the ClC-2_{24Asp} mutant (Fig. 4i). The involvement of L-type calcium channels

137   appeared to be larger in ClC-2_{24Asp} expressing cells (Fig. 4h), possibly because of their

138   depolarized plasma membrane potential which is required to open these channels [33]. However,

139   we cannot exclude that nifedipine acted partially through T-type calcium channels, which are

140   also blocked by this compound at depolarized voltages[34].

141   To investigate whether the *CLCN2* p.Gly24Asp mutation could be involved in other

142   forms of primary aldosteronism, we sequenced exon 2 of *CLCN2* in 100 patients with BAH.

143   While *CLCN2* p.Gly24Asp was not identified among these subjects, we identified two rare

144   *CLCN2* variants, c.197G>A (p.Arg66Gln, rs755883734) and c.143C>G (p.Pro48Arg,

145   rs115661422) in two subjects (Supplementary Fig. 3). Minor allele frequencies of these

146   variants are very low in the ExAC database (*CLCN2* p.Arg66Gln 0.00003; *CLCN2*

147   p.Pro48Arg 0.0017). Both variants failed to significantly change ClC-2 currents in

148   heterologous expression (Supplementary Fig. 4), in spite of a previously described[35] moderate

149   reduction of ClC-2$_{Pro48Arg}$ current amplitudes. Nonetheless, it is noteworthy that the two

150   patients were diagnosed with hypertension at young age, 29 and 19 ys respectively (Table 1)

151   and in both cases during pregnancy. Finally, sequencing the *CLCN2* exons encoding the N-

152   terminal domain (exon 1 and 2) and the loop between helices J and K (exon 10),

153   corresponding to the ClC-2 'inactivation domains'[2,3], in 20 additional patients with

154   hypertension before age 20 did not identify additional mutations. Among these patients, nine

155   had a history of hypertension before the age of 15 years (one before age 10 ys), indicating that

156   *CLCN2* mutations might underlie very young onset forms of primary aldosteronism.

157   In conclusion, we show that a gain-of-function mutation in the ClC-2 chloride channel

158   underlies a genetic form of secondary arterial hypertension and identify ClC-2 as the foremost

159   chloride conductor of resting glomerulosa cells. We suggest that increased Cl⁻ currents

160   induced by the ClC-2 p.Gly24Asp mutation could depolarize the zona glomerulosa cell

161   membrane, thereby opening voltage-gated calcium channels which trigger autonomous

162   aldosterone production by increasing intracellular Ca^{2+} concentrations (Fig. 5b, red arrows).

163   We hypothesize that the increased Cl⁻ currents may overcome the hyperpolarizing currents of

164   K$^+$ channels that normally determine the glomerulosa cell resting potential. The inhibition of

165   these potassium channels e.g. upon AngII stimulation, or the depolarizing currents mediated

166   by these channels upon increases in extracellular K$^+$, are the main mechanisms triggering

167   aldosterone production under physiological conditions (Fig. 5a, dashed black arrows).

168   Not only mutations in the amino-terminal ClC-2 'inactivation domain' [2,3], like the

169   Gly24Asp mutation found here, but also in the cytoplasmic linker between transmembrane

170   helices J and K may cause primary aldosteronism (Fig. 1d). Several point mutations in that

171   linker result in constitutively open ClC-2 channels [2]. We propose both regions as potential

172   hotspots for mutations causing primary aldosteronism. The discovery that a chloride channel

173   is involved in primary aldosteronism opens new and unexpected perspectives for the

174   pathogenesis and treatment of arterial hypertension.

175

Conflict of interest

The authors have nothing to disclose.

Author Contributions

MCZ, FFR, GD, IJO and TJJ designed experiments and wrote the manuscript. TMS, MCZ, TS and FFR performed and analyzed whole exome sequencing. MCZ, FFR, GD, REZ and SB performed and analyzed *in vitro* studies on H295R-S2 cells. IJO performed electrophysiological studies that were analyzed by IJO and TJJ. CG characterized adrenal glands from WT and *Clcn2*$^{-/-}$ mice and performed Western blots. VJ, XJ, LA, and HL were responsible for patients' recruitment, medical care and clinical data acquisition. All authors revised the manuscript draft.

200 **References**

201   1.   Thiemann, A., Grunder, S., Pusch, M. & Jentsch, T.J. A chloride channel widely

202   expressed in epithelial and non-epithelial cells. *Nature* **356**, 57-60 (1992).

203   2.   Jordt, S.E. & Jentsch, T.J. Molecular dissection of gating in the ClC-2 chloride

204   channel. *EMBO J.* **16**, 1582-92 (1997).

205   3.   Grunder, S., Thiemann, A., Pusch, M. & Jentsch, T.J. Regions involved in the opening

206   of CIC-2 chloride channel by voltage and cell volume. *Nature* **360**, 759-62 (1992).

207   4.   Worldwide trends in blood pressure from 1975 to 2015: a pooled analysis of 1479

208   population-based measurement studies with 19.1 million participants. *Lancet* **389**, 37-

209   55 (2017).

210   5.   Hannemann, A. & Wallaschofski, H. Prevalence of primary aldosteronism in patient's

211   cohorts and in population-based studies--a review of the current literature. *Horm.*

212   *Metab. Res.* **44**, 157-62 (2012).

213   6.   Calhoun, D.A. Hyperaldosteronism as a common cause of resistant hypertension.

214   *Annu. Rev. Med.* **64**, 233-47 (2013).

215   7.   Zennaro, M.C., Boulkroun, S. & Fernandes-Rosa, F. Genetic Causes of Functional

216   Adrenocortical Adenomas. *Endocr. Rev.* **38**, 516-537 (2017).

217   8.   Funder, J.W. *et al.* The Management of Primary Aldosteronism: Case Detection,

218   Diagnosis, and Treatment: An Endocrine Society Clinical Practice Guideline. *J. Clin.*

219   *Endocrinol. Metab.* **101**, 1889-916 (2016).

220   9.   Savard, S., Amar, L., Plouin, P.F. & Steichen, O. Cardiovascular complications

221   associated with primary aldosteronism: a controlled cross-sectional study.

222   *Hypertension* **62**, 331-6 (2013).

223   10.   Rossi, G.P. *et al.* A prospective study of the prevalence of primary aldosteronism in

224   1,125 hypertensive patients. *J. Am. Coll. Cardiol.* **48**, 2293-300 (2006).

225   11.   Choi, M. *et al.* K+ channel mutations in adrenal aldosterone-producing adenomas and
226   hereditary hypertension. *Science* **331**, 768-72 (2011).

227   12.   Azizan, E.A. *et al.* Somatic mutations in ATP1A1 and CACNA1D underlie a common
228   subtype of adrenal hypertension. *Nat. Genet.* **45**, 1055-60 (2013).

229   13.   Scholl, U.I. *et al.* Somatic and germline CACNA1D calcium channel mutations in
230   aldosterone-producing adenomas and primary aldosteronism. *Nat. Genet.* **45**, 1050-4
231   (2013).

232   14.   Scholl, U.I. *et al.* Recurrent gain of function mutation in calcium channel CACNA1H
233   causes early-onset hypertension with primary aldosteronism. *Elife* **4**, e06315 (2015).

234   15.   Daniil, G. *et al.* CACNA1H Mutations Are Associated With Different Forms of
235   Primary Aldosteronism. *EBioMedicine* **13**, 225-236 (2016).

236   16.   Beuschlein, F. *et al.* Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-
237   producing adenomas and secondary hypertension. *Nat. Genet.* **45**, 440-4, 444e1-2
238   (2013).

239   17.   Lifton, R.P. *et al.* A chimaeric 11beta-hydroxylase aldosterone synthase gene causes
240   glucocorticoid-remediable aldosteronism and human hypertension. *Nature* **355**, 262-
241   265 (1992).

242   18.   Moss, S.J. & Smart, T.G. Constructing inhibitory synapses. *Nat. Rev. Neurosci.* **2**,
243   240-50 (2001).

244   19.   Rinke, I., Artmann, J. & Stein, V. ClC-2 voltage-gated channels constitute part of the
245   background conductance and assist chloride extrusion. *J. Neurosci.* **30**, 4776-86
246   (2010).

247   20.   Koch, M.C. *et al.* The skeletal muscle chloride channel in dominant and recessive
248   human myotonia. *Science* **257**, 797-800 (1992).

249 21. Guo, J.H. *et al.* Glucose-induced electrical activities and insulin secretion in

250   pancreatic islet beta-cells are modulated by CFTR. *Nat. Commun.* **5**, 4420 (2014).

251 22. Chorvatova, A., Gendron, L., Bilodeau, L., Gallo-Payet, N. & Payet, M.D. A Ras-

252   dependent chloride current activated by adrenocorticotropin in rat adrenal zona

253   glomerulosa cells. *Endocrinology* **141**, 684-92 (2000).

254 23. Jentsch, T.J. Discovery of CLC transport proteins: cloning, structure, function and

255   pathophysiology. *J. Physiol.* **593**, 4091-109 (2015).

256 24. Bosl, M.R. *et al.* Male germ cells and photoreceptors, both dependent on close cell-

257   cell interactions, degenerate upon ClC-2 Cl(-) channel disruption. *EMBO J.* **20**, 1289-

258   99 (2001).

259 25. Blanz, J. *et al.* Leukoencephalopathy upon disruption of the chloride channel ClC-2. *J.*

260   *Neurosci.* **27**, 6581-9 (2007).

261 26. Hoegg-Beiler, M.B. *et al.* Disrupting MLC1 and GlialCAM and ClC-2 interactions in

262   leukodystrophy entails glial chloride channel dysfunction. *Nat. Commun.* **5**, 3475

263   (2014).

264 27. Depienne, C. *et al.* Brain white matter oedema due to ClC-2 chloride channel

265   deficiency: an observational analytical study. *Lancet Neurol.* **12**, 659-68 (2013).

266 28. Di Bella, D. *et al.* Subclinical leukodystrophy and infertility in a man with a novel

267   homozygous CLCN2 mutation. *Neurology* **83**, 1217-8 (2014).

268 29. Boulkroun, S. *et al.* Prevalence, Clinical, and Molecular Correlates of KCNJ5

269   Mutations in Primary Aldosteronism. *Hypertension* **59**, 592-8 (2012).

270 30. Spat, A. & Hunyady, L. Control of aldosterone secretion: a model for convergence in

271   cellular signaling pathways. *Physiol. Rev.* **84**, 489-539 (2004).

272 31. Varela, D., Niemeyer, M.I., Cid, L.P. & Sepulveda, F.V. Effect of an N-terminus

273   deletion on voltage-dependent gating of the ClC-2 chloride channel. *J. Physiol.* **544**,

274   363-72 (2002).

275 32. Pusch, M., Jordt, S.E., Stein, V. & Jentsch, T.J. Chloride dependence of

276   hyperpolarization-activated chloride channel gates. *J. Physiol.* **515 (Pt 2)**, 341-53

277   (1999).

278 33. Barrett, P.Q. *et al.* Role of voltage-gated calcium channels in the regulation of

279   aldosterone production from zona glomerulosa cells of the adrenal cortex. *J. Physiol.*

280   **594**, 5851-5860 (2016).

281 34. Perez-Reyes, E., Van Deusen, A.L. & Vitko, I. Molecular pharmacology of human

282   Cav3.2 T-type Ca2+ channels: block by antihypertensives, antiarrhythmics, and their

283   analogs. *J. Pharmacol. Exp. Ther.* **328**, 621-7 (2009).

284 35. Paul, J. *et al.* Alterations in the cytoplasmic domain of CLCN2 result in altered gating

285   kinetics. *Cell. Physiol. Biochem.* **20**, 441-54 (2007).

286 36. Dutzler, R., Campbell, E.B., Cadene, M., Chait, B.T. & MacKinnon, R. X-ray

287   structure of a ClC chloride channel at 3.0 A reveals the molecular basis of anion

288   selectivity. *Nature* **415**, 287-94 (2002).

289
290

291

FIGURE LEGENDS

293 **Figure 1. A *CLCN2* variant identified in a patient with early onset primary**

294 **aldosteronism**. (a) Pedigree of kindred K1011. The subject with PA is shown with a black

295 symbol and non-affected subjects are shown with white symbols. (b) Sanger sequencing

296 chromatograms showing the *CLCN2* wild-type sequence of the unaffected parents (K1011-3

297 and K1011-4) and brother (K1011-2) and the *CLCN2* variant c.71G>A (p.Gly24Asp)

298 identified in the patient with early onset primary aldosteronism (K1011-1). (c) Alignment and

299 conservation of residues encoded by *CLCN2* orthologs. The red box indicates the amino-

300 terminal 'inactivation domain' of ClC-2. Several deletions and mutations in this region of rat

301 ClC-2 led to constitutive open ClC-2 channels (uninterrupted line) or partially opened

302 channels (dashed line) [3]. Residues that are conserved among more than 3 sequences are

303 highlighted in yellow. (d) Position of the disease-causing Gly24Asp mutation in the ClC-2

304 protein (schematic transmembrane topology drawing modified from [36]). 'Inactivation

305 domains' previously identified by structure-function analysis in the amino-terminus [3] and an

306 intracellular loop [2] of ClC-2 are shown in red. Several point mutations and deletions in these

307 domains open the ClC-2 channel [2,3] similar to the Gly24Asp substitution described here.

308 CBS1 and CBS2, cystathionine-β-synthase domains which can affect gating of CLC channels

309 [23].

310 **Figure 2 ClC-2 expression in mouse adrenals and electrophysiological analyses of WT**

311 **and mutant channels.** (a) ClC-2 immunoblot of brain and adrenals from *Clcn2* [+/+] and *Clcn2*

312 [-/-] mice. All lanes are from the same blot, which has been cut as indicated. Similar amounts of

313 protein were loaded with actin serving as loading control. This blot is representative for three

314 independent experiments. (b) Representative whole-cell chloride current traces of mouse zona

315 glomerulosa cells from *Clcn2*^{+/+} (top) and *Clcn2*^{-/-} (bottom) adrenal slices using voltage steps

316 as indicated above. (c) Mean ± SEM currents measured after 1.5 s from experiments in (b)

317   plotted as a function of clamp voltage. (d, e) Representative chloride current traces measured

318   by two-electrode voltage-clamp from *Xenopus* oocytes injected with either human ClC-2$_{WT}$

319   (d) or ClC-2$_{24Asp}$ (e) cRNA, using the protocol shown in (d). For some measurements (below)

320   extracellular chloride was replaced with iodide. (f) Mean ± SEM currents measured in (d,e)

321   plotted as a function of voltage. Number of cells indicated in parenthesis. (g, h) Summary of

322   Cl$^-$ currents at -80 mV (I$_{-80mV}$) (g) and current ratios (I$_{-120mV}$ / I$_{+60mV}$) as measure of

323   rectification (h) (always measured at 2s) for panels (d-f). ****p < 0.0001, Mann-Whitney

324   two-tailed test. (i, j) Effect of external pH on currents mediated by ClC-2$_{WT}$ (i) or ClC-2$_{24Asp}$

325   (j) in *Xenopus* oocytes. Currents were normalized to mean currents from respective construct

326   measured after 2s at -120 mV and pH 7.4. Number of oocytes indicated in parenthesis, error

327   bars indicate SEM.

328   **Figure 3. Effect of ClC-2$_{WT}$ and mutant ClC-2$_{24Asp}$ channels on aldosterone production**

329   **and expression of genes and proteins involved in aldosterone biosynthesis.** (a) Western

330   blots for ClC-2 and aldosterone synthase of H295R-S2 cells stably transfected with ClC-2$_{WT}$

331   or mutant ClC-2$_{24Asp}$. These blots are representative of three independent experiments, with

332   actin serving as loading control. (b) Quantification of ClC-2 protein expression in ClC-2$_{WT}$

333   and ClC-2$_{24Asp}$ H295R-S2 cells (T test p=0.10, F=3.19). (c) Quantification of aldosterone

334   synthase expression in ClC-2$_{WT}$ or ClC-2$_{24Asp}$ H295R-S2 cells (T test p=0.0025, F=136). (d)

335   Basal aldosterone production by H295R-S2 cells transfected with ClC-2$_{WT}$ or mutant ClC-

336   2$_{24Asp}$ (T test, p=0.0008, F=142). (e) Basal and stimulated aldosterone production by H295R-

337   S2 cells transfected with ClC-2$_{WT}$ (open bars) or mutant ClC-2$_{24Asp}$ (filled bars) (1way

338   ANOVA p<0.0001, F=23.46). (f-h) Basal and stimulated mRNA expression of (f) *CYP11B2*

339   (1way ANOVA p<0.001, F=18.39), (g) *STAR* (Kruskal-Wallys p=0.0033)*,* and (h) *CYP21A2*

340   (1way ANOVA p<0.0001, F23.27) in H295R-S2 cells transfected with ClC-2$_{WT}$ (open bars) or

341 mutant ClC-2$_{24Asp}$ (filled bars). Quantification of protein expression (using actin as loading

342 control) and aldosterone production are represented as percentage of ClC-2$_{WT}$ in basal

343 conditions and results of mRNA expression are represented as fold induction of ClC-2$_{WT}$ in

344 basal conditions. Values of all experiments are represented as mean ± SEM of three

345 independent experiments performed in experimental triplicates (n=9) for each condition. *

346 p<0.05; ** p<0.01; *** p<0.001; i) p<0.05 stimulated vs basal condition, ii) p<0.01

347 stimulated vs basal condition; iii) p<0.001 stimulated vs basal condition.

348 **Figure 4 Functional impact of the ClC-2$_{24Asp}$ mutation.** (a-g) Effect on membrane

349 potential V$_m$ and plasma membrane anion currents in H295R-S2 cells. (a) Resting membrane

350 potential of ClC-2$_{WT}$ and ClC-2$_{24Asp}$ stably transfected H295R-S2 cells which were used to

351 determine aldosterone secretion. Note strong mean depolarization (ClC-2$_{WT,}$ -67 ± 2 mV

352 (n=8); ClC-2$_{24Asp}$, -52 ± 6 mV (n=12)), which, however, was not significant (Two-tailed

353 Mann-Whitney p = 0.14) owing to large variability of V$_m$ of ClC-2$_{24Asp}$ transfected cells that

354 were not cloned. (b) Similarly determined values of V$_m$ for non-transfected, and transiently

355 transfected H295R-S2 cells (non-transfected, -65 ±4 mV (n=8); ClC-2$_{WT}$, -69 ± 7 mV (n=4);

356 ClC-2$_{24Asp}$, -46 ± 4 mV (n=6); **, Two-tailed Mann-Whitney p = 0.0095)) (c,d)

357 Corresponding plasma membrane currents measured after 1.5 s under conditions eliminating

358 cation inward currents as function of voltage (c) or as plot of individual values at

359 physiological V$_m$ of glomerulosa cells (d) *, Two-tailed Mann-Whitney p = 0.019. (e-g)

360 corresponding averaged current traces with 20 mV voltage steps between 0 and -120 mV. (h-i)

361 Effect of calcium channel blockers on aldosterone production in H295R-S2 cells expressing

362 (h) ClC-2$_{WT}$ (Kruskall Wallis p=0.0005) and (i) ClC-2$_{24Asp}$ (Kruskal Wallis, p=0.0032). Values

363 represent mean ± SEM of two independent experiments performed in experimental triplicates

364 (n=6) for each condition. * p<0.05; ** p<0.01.

365

Figure 5. Proposed model for autonomous aldosterone secretion in adrenal zona glomerulosa cells with the ClC-2$_{24Asp}$ mutation. (a) In unstimulated conditions, the ZG cell membrane potential closely follows the potassium resting potential at approximately -80 mV. Increasing extracellular K$^+$ concentration, or inhibition of K$^+$ channels by AngII through its receptor (AT1R), leads to cell membrane depolarization, opening of voltage-gated Ca^{2+} channels, and increased intracellular calcium concentrations, the major trigger for aldosterone biosynthesis. Binding of AngII to AT1R also leads to Gαq-mediated signaling and IP3-mediated release of Ca^{2+} from the endoplasmic reticulum. (b) The ClC-2 p.Gly24Asn mutation abolishes the voltage-dependent gating of ClC-2. The resulting pronounced increase of Cl$^-$ currents at resting potentials is proposed to result in cell depolarization, opening of voltage gated Ca^{2+} channel, stimulation of Ca^{2+} signaling, and finally increased expression of steroidogenic genes and aldosterone production.

378

379 **Table 1. Clinical and biological characteristics of patients carriers of *CLCN2* variants.**

	K1011-1	K963-1	K1044-1
Sex	F	F	F
Age at HTN dg (ys)	9	19	29
Age at primary aldosteronism dg (ys)	9	27	48
SBP at primary aldosteronism dg (mmHg)	172	139	173
DBP at primary aldosteronism dg (mmHg)	100	90	114
Lowest plasma K^+ (mmol/L)	1.8	2.9	2.5
Urinary aldosterone (nmol/24h)	ND	60	ND
Plasma aldosterone (pmol/L)[a]	2406	927	1061
Plasma renin (mU/L)[a]	0.9	1.9	<1
ARR (pmol/mU)[b]	481.2	185.4	212.2
Adrenal abnormalities on imaging	No	No	No
Lateralization at AVS	ND	No	No

380 dg, diagnosis; m, months; y, years ; HTN, hypertension; SBP, systolic blood pressure; DBP, diastolic
381 blood pressure; K, potassium; ARR, aldosterone to renin ratio; Hormonal data are at diagnosis of
382 primary aldosteronism. ND, not determined. For comparison within this table, plasma aldosterone
383 levels for patient K1011-1 have been converted to pmol/L and plasma renin activity to plasma renin
384 concentration. [b]for ARR calculation, renin values <5 have been transformed to 5. Conversion factor
385 used for plasma aldosterone: 1 ng/l = 2.77 pmol/L; conversion factor used for plasma renin: 1 ng/ml/h
386 = 8.2 mU/L.

387

388 **Online Methods**

389 *Patients*

390 Patients with primary aldosteronism were recruited within the COMETE (COrtico- et

391 MEdullo-surrénale, les Tumeurs Endocrines) network (COMETE-HEGP protocol,

392 authorization CPP 2012-A00508-35) or in the context of genetic screening for familial

393 hyperaldosteronism at the Genetics department of the HEGP. Methods for screening and

394 subtype identification of primary aldosteronism were performed according to the Endocrine

395 Society guidelines[8]. In patients diagnosed with primary aldosteronism, a thin slice CT scan or

396 MRI of the adrenal and/or an adrenal venous sampling (AVS) were performed to differentiate

397 between unilateral and bilateral aldosterone hypersecretion. All patients gave written

398 informed consent for genetic and clinical investigation. Procedures were in accordance with

399 institutional guidelines.

400

401 *DNA isolation*

402 DNA from peripheral blood leukocytes was extracted using QIAamp DNA midi kit (Qiagen,

403 Courtaboeuf Cedex, France) or salt-extraction.

404

405 *Whole exome sequencing and variant detection*

406 Exomes were enriched in solution and indexed with SureSelect XT Human All Exon 50 Mb

407 kits (Agilent). Sequencing was performed as 100 bp paired-end runs on HiSeq2000 systems

408 (Illumina). Pools of 12 indexed libraries were sequenced on four lanes. Image analysis and

409 base calling was performed using Illumina Real Time Analysis. CASAVA 1·8 was used for

410 demultiplexing. BWA (v 0.5.9) with standard parameters was used for read alignment against

411 the human genome assembly hg19 (GRCh37). We performed single-nucleotide variant and

412 small insertion and deletion (indel) calling specifically for the regions targeted by the exome

413   enrichment kit, using SAMtools (v 0.1.18). Subsequently the variant quality was determined

414   using the SAMtools varFilter script. We used default parameters, with the exception of the

415   maximum read depth (-D) and the minimum P-value for base quality bias (-2), which we set

416   to 9999 and 1e-400, respectively. Additionally, we applied a custom script to mark all

417   variants with adjacent bases of low median base quality. All variants were then annotated

418   using custom Perl scripts. Software is available on request (https://ihg4.helmholtz-

419   muenchen.de/cgi-bin/mysql/snv-vcf/login.pl). Annotation included information about known

420   transcripts (UCSC Known Genes and RefSeq genes), known variants (dbSNP v 135), type of

421   mutation, and - if applicable – amino acid change in the corresponding protein. The annotated

422   variants were then inserted into our in-house database. To reduce false positives we filtered

423   out variants that were already present in our database, had variant quality less than 40, or

424   failed one of the filters from the filter scripts. We then manually investigated the raw read

425   data of the remaining variants using the Integrative Genomics Viewer (IGV).

426

Sanger sequencing

CLCN2 DNA was amplified using the intron-spanning primers described in supplementary

table S2. PCR were performed on 100 ng of DNA in a final volume of 25 µl containing 0.75

mM $MgCl_2$, 400 nM of each primer, 200 µM deoxynucleotide triphosphate, and 1.25 U Taq

DNA Polymerase (Sigma). Cycling conditions for *CLCN2* were as previously described [37]

with an annealing temperature of $60^{o}C$. Direct sequencing of PCR products was performed

using the ABI Prism Big Dye Terminator® v3.1 Cycle Sequencing Kit (Applied Biosystems,

Foster City, CA) on an ABI Prism 3700 DNA Analyzer (Applied Biosystems).

Site directed mutagenesis

The ClC-2_{24Asp} construct was generated by site-directed mutagenesis using the QuikChange II

XL site-directed mutagenesis kit (Agilent). The mutation was introduced into the human ClC-

2 cDNA fragment inserted into the pFROG expression vector [38] and their presence confirmed

by Sanger sequencing.

Western blot

The membrane fractions of tissue homogenate from brain and adrenal gland of adult *Clcn2* [+/+]

and *Clcn2* [-/-] mice were isolated and lysed in 50mM Tris pH 6.8, 140mM NaCl, 0.5mM

EDTA and 2% SDS with protease inhibitors (4 mM Pefabloc and Complete® EDTA-free

protease inhibitor cocktail, Roche). Equal amounts of protein were separated by SDS–PAGE

(10 % polyacrylamide) and blotted onto nitrocellulose. Rabbit polyclonal antibodies against a

modified carboxy-terminal ClC-2 peptide have been described previously [26]. Blots were

reprobed with mouse anti-β-actin (Clone AC-74, Sigma A2228, 1:1000) as a loading control.

H295R-S2 cells were lysed using RIPA buffer (Bio Basic Canada Inc.) with protease and

phosphatase inhibitors mini tablets, EDTA free (Thermo Scientific). Proteins were solubilized

452 for 30 min at 4°C, under end-over-end rotation, and then centrifuged at 13000 rpm for 15 min

453 at 4°C. Equal amounts of proteins were submitted to 10% SDS-PAGE and transferred onto

454 nitrocellulose membrane. Membranes were blotted with rabbit anti-ClC-2 antibody (1:500),

455 mouse anti-aldosterone synthase antibody (1:500, clone CYP11B2-41-13, kindly provided by

456 Dr C Gomez Sanchez[39]) and mouse anti-β-actin (A2228, 1:10000, Sigma).

457

458

459 *Electrophysiological recordings*

460 Patch clamp analysis were performed in adrenal sections from wild type and *Clcn2*^{-/-} mice [24],

461 similarly to previously described [40]. Bicarbonate based buffers were used which were

462 continuously bubbled with 95% O_2 and 5% CO_2. Briefly, adrenal glands were removed and

463 placed into cold low-Ca^{2+} solution composed of (in mM): 140 NaCl, 2 KCl, 26 $NaHCO_3$, 10

464 glucose, 5 $MgCl_2$, 0.1 $CaCl_2$, pH 7.4. The adrenals, after removal of surrounding fat tissue,

465 were embedded in 3% low-melting agarose, sectioned at 70 µm (Leica VT1200S), and

466 incubated at room temperature in solution containing: 140 NaCl, 2 KCl, 26 $NaHCO_3$, 10

467 glucose, 2 $MgCl_2$, 2 $CaCl_2$, and adjusted to pH 7.4. After at least 1 hour, slices were then

468 transferred to a recording chamber and imaged with a 60x objective and DIC optics (Olympus

469 BX51WI). Cells of the zona glomerulosa were identified by their rosette organization.

470 Electrical signals were acquired at room temperature using a microelectrode amplifier

471 (Multiclamp 700B) and software (Clampex 10.3, Molecular Devices, USA). As expected,

472 cells when patched with a K^+-based solution displayed spontaneous spiking which could be

473 stimulated with angiotensin II. For measuring chloride currents, patch pipettes were filled

474 with solution containing: 117.5 $CsMeSO_3$, 17.5 CsCl, 4 NaCl, 10 Hepes, 1 EGTA, 1 $MgCl_2$,

475 adjusted to pH 7.3 while the bath solution contained: 117 NMDG-Cl, 23 NMDG-HCO_3, 5

476 CsCl, 1.3 $MgCl_2$, 9 glucose, 2 $CaCl_2$, adjusted to pH 7.3. Voltage steps from +40 to −120 mV

477 from a holding potential of $-10\,mV$ were used, with a final 1 s step at $+40\,mV$. Signals were

478 digitized at $10\,kHz$, filtered at $2\,kHz$ and stored off-line for analysis with Clampfit software

479 10.4.

480 For two electrode voltage clamp in *Xenopus laevis* oocytes, human wildtype and Gly24Asp

481 ClC-2 cRNAs were prepared from pFROG vectors (Ambion mMESSAGE mMACHINE T7

482 kit) and injected into *X. laevis* oocytes, 13.8 ng and 9.2 ng per cell, respectively. Following

483 two days of expression at $17°C$, two electrode voltage clamp was performed at room

484 temperature using a TurboTEC amplifier (npi electronic GmbH, Tamm, Germany) and

485 pClamp Software (Molecular Devices) to elicit ClC-2 currents (2s steps from $+60mV$ to -

486 $120mV$ with a final 1s step at $+40mV$) in ND109 solution containing (in mM): 109 NaCl, 2

487 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 2 HEPES and adjusted to pH 7.4. To test for the typical $Cl^->I^-$

488 selectivity of CLC channels, currents were sequentially measured in solutions containing 109

489 mM Cl^- or 109 mM I^-. To determine the pH sensitivity of currents, ND109 was buffered with

490 5 mM MES for pH 6.5 and with 5 mM Tris for pH 8.5. Off-line analysis was performed with

491 Clampfit software 10.4. Statistical significance was assessed using the Mann–Whitney test

492 (Prism, GraphPad Software, USA).

493 For patch clamp of transiently transfected H295R-S2 cells, cells were seeded at 30%

494 confluency onto poly-L-lysine (Sigma) coated glass coverslips. Once adhered they were

495 transfected (X-fect) with bicistronic plasmids encoding emGFP (for identification of

496 transfected cells) and, after an an IRES sequence, $ClC-2_{WT}$ or $ClC-2_{G24D}$. Cells were

497 measured 12-24 hours later. Both transiently and stably transfected cells were measured

498 using a Multiclamp 700B (Axon Instruments) amplifier Gramicidin-perforated patch clamp

499 was performed to retain the intracellular chloride concentration. The tips of patch pipettes

500 were first filled with gramicidin-free internal pipette solution (in mM): 100 $KMeSO_3$, 30 KCl,

501 4 NaCl, 10 Hepes, 1 $MgCl_2$, 1 EGTA, 3 MgATP (pH 7.3, 280 mOsm/L) and then back-filled

502   with the same solution containing 25µg/mL gramicidin. GFP expressing cells were selected

503   for analysis. Approximately 20 minutes following tight giga-seal formation, stable membrane

504   potential measurements (I=0 configuration) could be acquired with access resistances of <100

505   MΩ in bath solution containing 140 NaCl, 5 KCl, 10 Hepes, 1.8 $MgCl_2$, 1.8 $CaCl_2$ (pH 7.4,

506   300 mOsm/L). When adequate access resistance was attained (<35 MΩ), a Na^+- and K^+-free

507   bath solution containing 140 CsCl, 10 Hepes, 1.8 $MgCl_2$, 1.8 $CaCl_2$, 20 sucrose (pH 7.3, 300

508   mOsm/L) was perfused to measure anion membrane currents in the voltage clamp

509   configuration (1s steps from +60 mV to -120 mV from a holding clamp of -10 mV).

510   Measurements were performed at room temperature (22-24°C). Data are presented as mean

511   ± SEM.

512

513   *Functional studies in H295R-S2 cells*

514   The human adrenocortical carcinoma cell line H295R strain 2 (H295R-S2), kindly

515   provided by W. E. Rainey [41] was cultured in DMEM/Eagle's F12 medium (GIBCO, Life

516   technologies, Carlsbad, CA) supplemented with 2% Ultroser G (PALL life sciences, France),

517   1% insulin/transferrin/selenium Premix (GIBCO, Life technologies, Carlsbad, CA), 10mM

518   HEPES (GIBCO, Life technologies, Carlsbad, CA), 1% penicillin, and streptomycin (GIBCO,

519   Life technologies, Carlsbad, CA) and maintained in a 37°C humidified atmosphere (5%

520   CO2).

521   For overexpression experiments, H295R-S2 cells were seeded into tissue culture dishes 100 in

522   groups of 5.000·000 cells per dish, and maintained in the conditions described. After 24h,

523   cells were resuspended in 100 µl Nucleofector R solution (AMAXA kit, Lonza) and

524   transfected with 3 µg of the ClC-2$_{WT}$ or ClC-2$_{24Asp}$ pFROG construct or a GFP construct,

525   using the electroporation program P-20. To select only stably transfected cells, 48h post

526   transfection cells were changed to medium containing 500 µg/mL G418-Genetycin (Gibco)

27

527 and used after all GFP transfected cells were dead. G418 selection was kept during all

528 functional studies. For aldosterone measurements and RNA extraction, cells were serum

529 deprived in DMEM/F12 containing 0.1% Ultroser G for 24h and then incubated for another

530 24h with fresh medium containing 0.1% Ultroser G with no secretagogue or vehicle (basal),

531 or secretagogues AngII (10nM) or K^+ (12mM), or calcium channel bloquers Nifedipine (L-

532 Type calcium channel blocker, 10μM, Sigma) or Mibefradil (T-type calcium channel

533 blocker, 10μM, Sigma). At the end of the incubation time, supernatant and cells from each

534 well were harvested for aldosterone measurement and RNA extraction. Three experiments

535 using aldosterone secretagogues (n=9) and two experiments using calcium channel blockers

536 (n=6) were independently conducted in triplicate.

537 Human ClC2-targeting (TRCN0000427876) and non-mammalian control (SHC002V)

538 MISSION shRNA lentiviral transduction particles were obtained from Sigma-Aldrich. The

539 shRNA sequences were inserted into TRC2 pLKO-puro plasmid backbone. For the lentiviral

540 infections, manufacturer's protocol was followed with slight modifications. 1 x 10^4 H295R-S2

541 cells were seeded in a 96-well plate. After 24 hours, medium was changed and supplemented

542 with 2 μg/ml polybrene (Sigma). Lentiviral particles were then added at a multiplicity of

543 infection of 10 and medium changed after overnight incubation. For selection, 2 μg/ml

544 puromycin (Gibco) was added to the medium. After two passages cells were characterized in

545 terms of mRNA expression and aldosterone production after incubation with or without

546 secretagogue as mentioned above.

547

548 ***RNA extraction and RT-qPCR***

549 Total RNA was extracted in Trizol reagent (Ambion Life technologies, Carlsbad CA)

550 according to the manufacturer's recommendations. After deoxyribonuclease I treatment (Life

551 Technologies, Carlsbad, CA), 500 ng of total RNA were retrotranscribed (iScript reverse

552   transcriptase, Biorad, Hercules, CA). Primers used for qPCR are described in supplementary

553   table 3. The quantitative PCR was performed using SYBRgreen (Sso advanced universal

554   SyBr Green supermix, Biorad, Hercules, CA) on a C1000 touch thermal cycler of Biorad

555   (CFX96 Real Time System), according to the manufacturer's instructions. Controls without

556   template were included to verify that fluorescence was not overestimated from primer dimer

557   formation or PCR contaminations. RT-qPCR products were analyzed in a post amplification

558   fusion curve to ensure that a single amplicon was obtained. Normalization for RNA quantity,

559   and reverse transcriptase efficiency was performed against three reference genes (geometric

560   mean of the expression of Ribosomal *18S* RNA, *HPRT*, and *GAPDH*), in accordance with the

561   MIQE guidelines [42]. Quantification was performed using the standard curve method. Standard

562   curves were generated using serial dilutions from a cDNA pool of all samples of each

563   experiment, yielding a correlation coefficient of at least 0.98 in all experiments.

564

565   ***Aldosterone and protein assays***

566   Aldosterone levels were measured in cell culture supernatants by ELISA. Aldosterone

567   antibody and aldosterone-3-CMO-biotin were kindly provided by Dr Gomez-Sanchez [43].

568   Aldosterone concentrations were normalized to cell protein concentrations (determined using

569   Bradford protein assay).

570

571   ***Statistical analyses***

572   Quantitative variables are reported as means ± standard error of the mean (SEM) when

573   Gaussian distribution or medians and interquartile range when no Gaussian distribution.

574   Pairwise comparisons were done with unpaired t-test or Mann-Whitney test respectively;

575   multiple comparisons were done with the ANOVA test followed by a test for pairwise

576   comparison of subgroups according to Bonferroni when Gaussian distribution, or Kruskal-

577   Wallis followed by Dunn's test when no Gaussian distribution. Comparisons between two

578   groups were performed with two-tailed T test or two-tailed Mann-Whitney test. A p value <

579   0.05 was considered significant. For functional experiments, all results were expressed as

580   mean ± SEM of three separate experiments performed in triplicate for ClC-2 overexpression

581   studies with secretagogues, two separate experiments performed in triplicate for ClC-2

582   expression studies with calcium channel blockers, and two to four separate experiments

583   performed in triplicate for ClC-2 knockdown studies. Analyses were performed using Prism5

584   (GraphPad software Inc, San Diego, CA).

585

586   **Data availability.**

587   The data that support the findings of this study are available from the authors on reasonable

588   request, see author contributions for specific data sets. Disease-causing variants have been

589   submitted to ClinVar. Exome data are available on request within a scientific cooperation.

590   **Online Methods References**

591   37.   Hubert, E.L. *et al.* Mineralocorticoid receptor mutations and a severe recessive

592   pseudohypoaldosteronism type 1. *J. Am. Soc. Nephrol.* **22**, 1997-2003 (2011).

593   38.   Gunther, W., Luchow, A., Cluzeaud, F., Vandewalle, A. & Jentsch, T.J. ClC-5, the

594   chloride channel mutated in Dent's disease, colocalizes with the proton pump in

595   endocytotically active kidney cells. *Proc. Natl. Acad. Sci. U S A* **95**, 8075-80 (1998).

596   39.   Gomez-Sanchez, C.E. *et al.* Development of monoclonal antibodies against human

597   CYP11B1 and CYP11B2. *Mol. Cell. Endocrinol.* **383**, 111-7 (2014).

598   40.   Hu, C., Rusin, C.G., Tan, Z., Guagliardo, N.A. & Barrett, P.Q. Zona glomerulosa cells

599   of the mouse adrenal cortex are intrinsic electrical oscillators. *J. Clin. Invest.* **122**,

600   2046-53 (2012).

601   41.   Wang, T. *et al.* Comparison of aldosterone production among human adrenocortical

602   cell lines. *Horm. Metab. Res.* **44**, 245-50 (2012).

603   42.   Bustin, S.A. *et al.* The MIQE guidelines: minimum information for publication of

604   quantitative real-time PCR experiments. *Clin. Chem.* **55**, 611-22 (2009).

605   43.   Gomez-Sanchez, C.E. *et al.* The production of monoclonal antibodies against
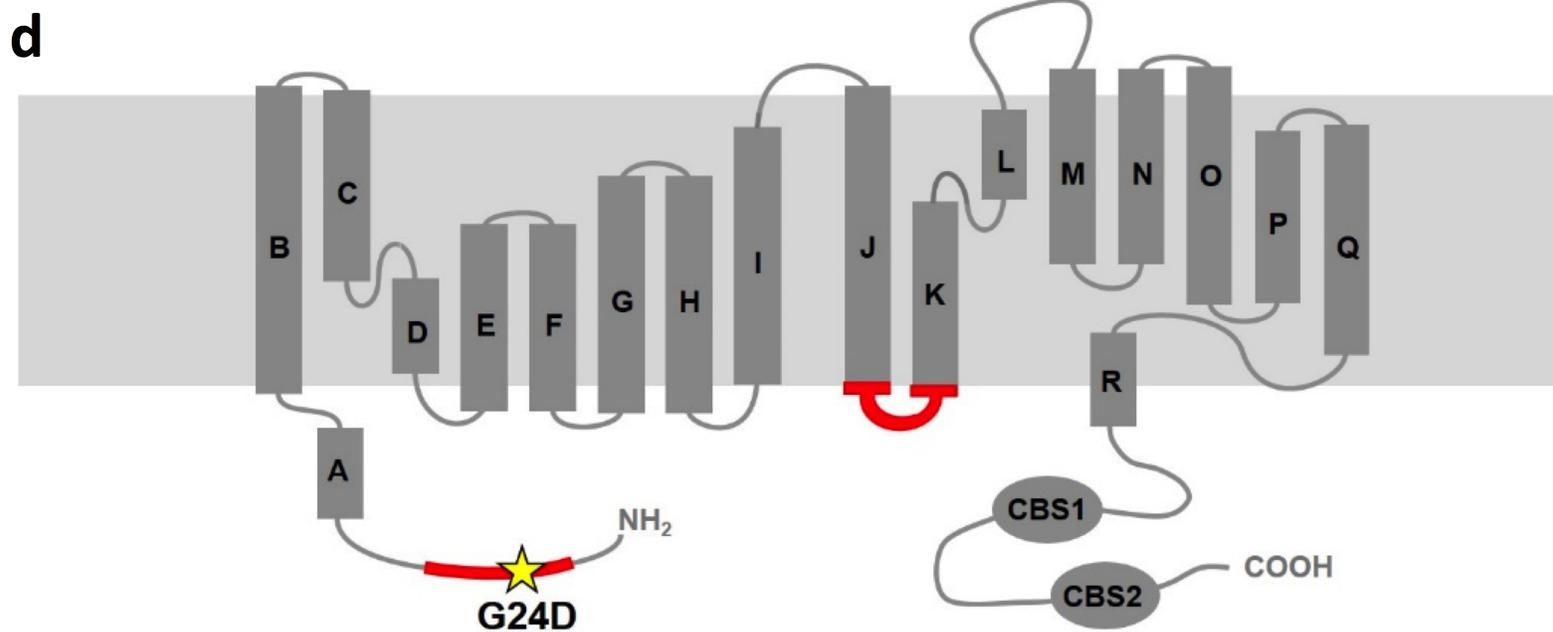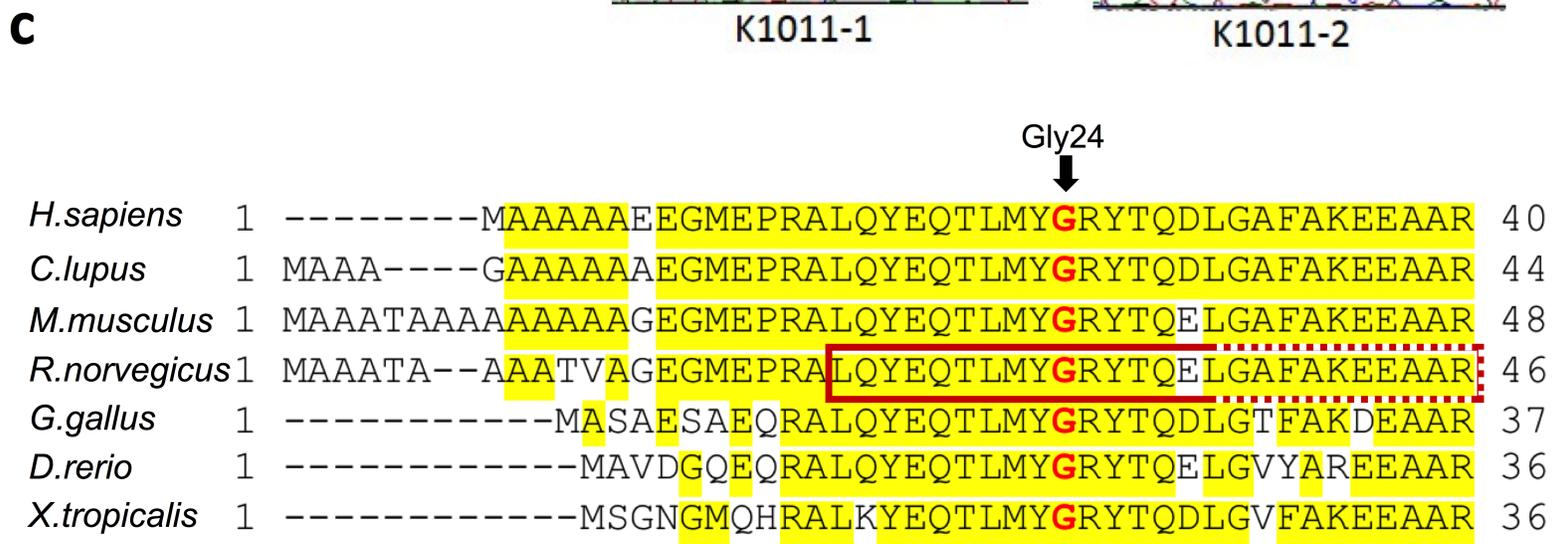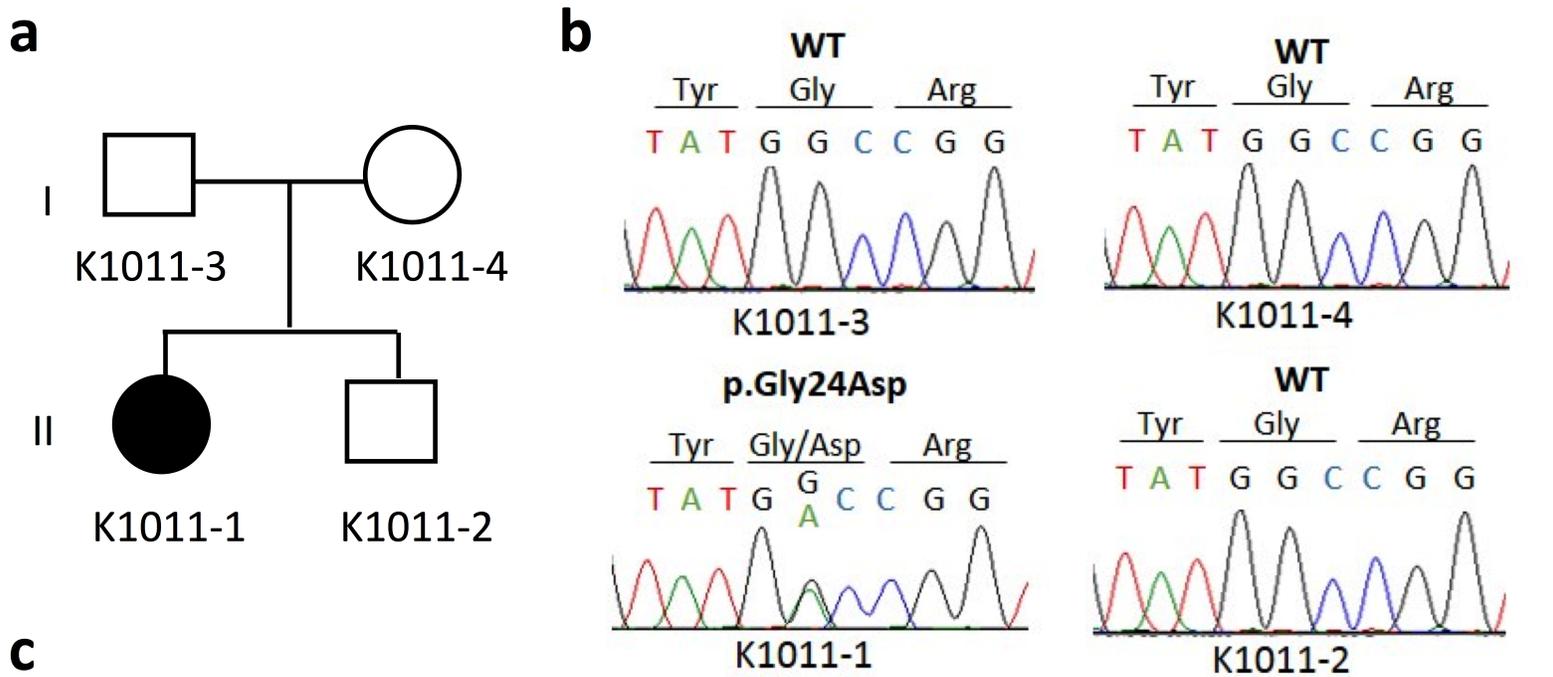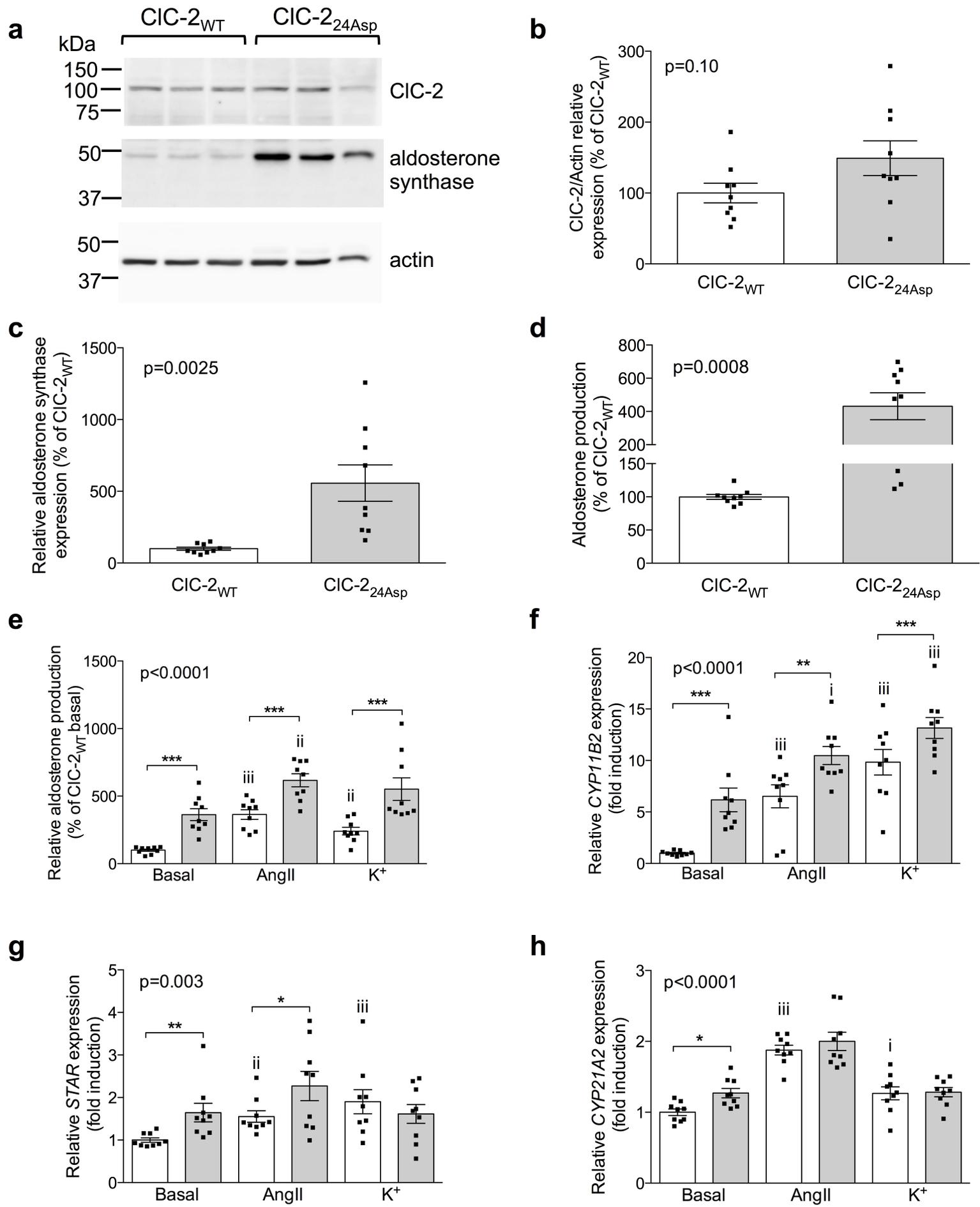
606   aldosterone. *Steroids* **49**, 581-7 (1987).

607

Figure 1
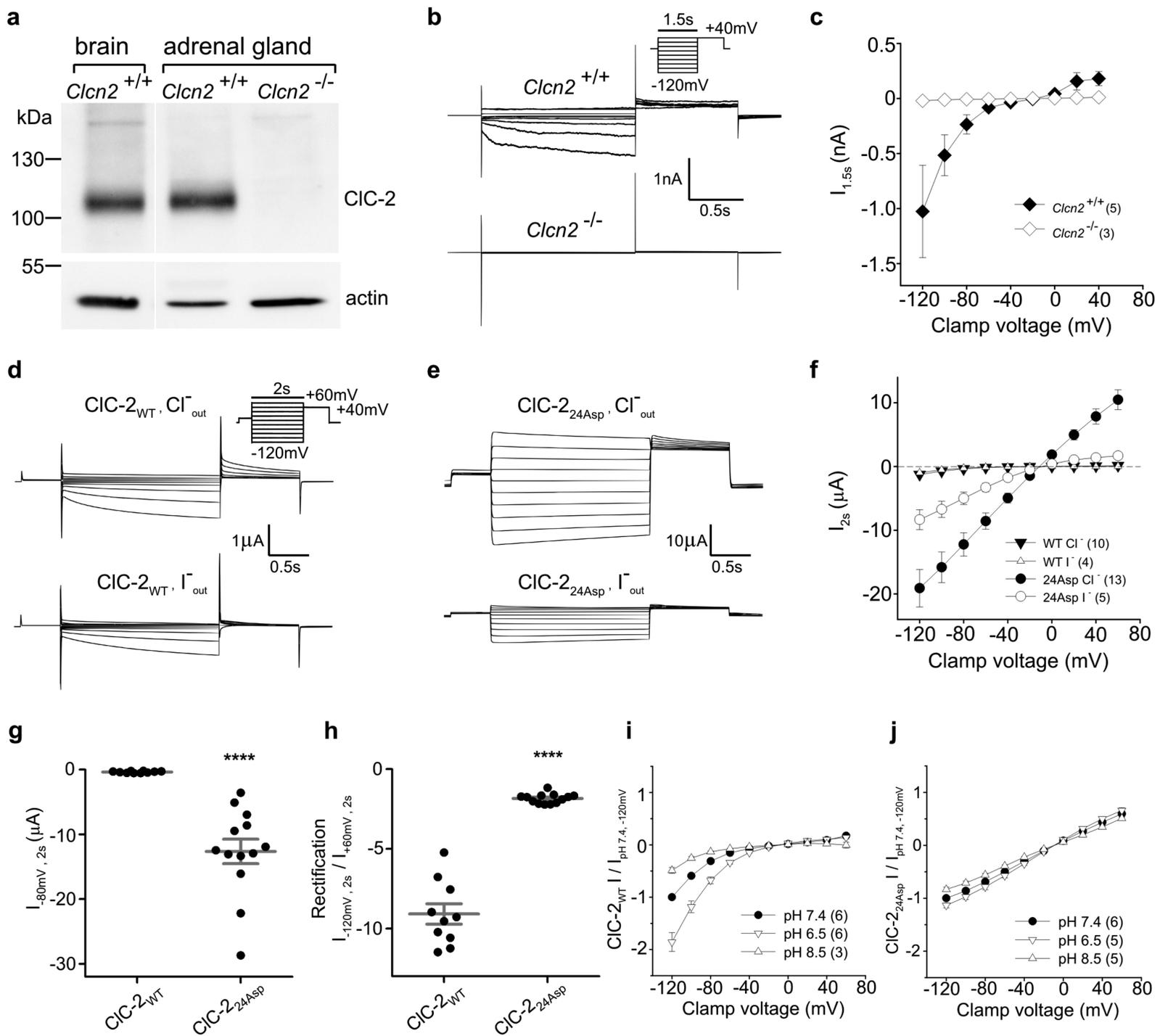
Figure 2

Figure 3

Figure 4

Figure 5