TSPAN12 is a novel negative regulator of aldosterone production in

adrenal physiology and aldosterone-producing adenomas

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Short title: TSPAN12 in aldosterone physiology and pathophysiology

Abstract

1 **Background**: Aldosterone-producing adenomas (APA) are a major cause of primary
2 aldosteronism, a condition of low-renin hypertension, in which aldosterone overproduction is
3 usually driven by a somatic activating mutation in an ion pump or channel. *TSPAN12*
4 (tetraspanin 12) is differentially expressed in different subgroups of APAs suggesting a role in
5 APA pathophysiology. Our objective was to determine the function of TSPAN12 in adrenal
6 physiology and pathophysiology.

7 **Methods**: APA specimens, pig adrenals under dietary sodium modulation, and a human
8 adrenocortical cell line HAC15 were used for functional characterization of TSPAN12 in vivo
9 and in vitro.

10 **Results**: Gene ontology analysis of 21 APA transcriptomes dichotomized according to high
11 versus low *TSPAN12* transcript levels highlighted a function for *TSPAN12* related to the renin-
12 angiotensin system. *TSPAN12* expression levels in a cohort of 30 APAs were inversely
13 correlated with baseline plasma aldosterone concentrations (R=-0.47, P=0.009). In a pig
14 model of renin-angiotensin system activation by dietary salt restriction, *TSPAN12* mRNA levels
15 and TSPAN12 immunostaining were markedly increased in the zona glomerulosa layer of the
16 adrenal cortex. *In vitro* stimulation of human adrenocortical HAC15 cells with 10 nM
17 angiotensin II for 6 h caused a 1.6-fold ± 0.13 increase in *TSPAN12* expression, which was
18 ablated by 10 μM nifedipine (P=0.0097) or 30 μM W-7 (P=0.0022). Gene silencing of *TSPAN12*
19 in HAC15 cells demonstrated its inverse effect on aldosterone secretion under basal and
20 angiotensin II stimulated conditions.

21 **Conclusions**: Our findings show that TSPAN12 is a negative regulator of aldosterone

22 production and could contribute to aldosterone overproduction in primary aldosteronism.

Key words

Adrenal cortex, aldosterone, angiotensin II, hyperaldosteronism, pig model, steroidogenesis

23

Nonstandard Abbreviations and Acronyms

AngII	angiotensin II
APA	aldosterone-producing adenoma
HAC15	human adrenocortical cells
PA	primary aldosteronism
RAS	renin-angiotensin system
zG	zona glomerulosa

Introduction

24 Primary aldosteronism (PA) is the most common cause of endocrine hypertension and is
25 characterized by inappropriately high plasma aldosterone to renin concentrations.[1] A major
26 subtype of PA is caused by a unilateral aldosterone-producing adenoma (APA), which can be
27 cured by adrenalectomy.[2] Somatic APA mutations in some ion pumps and channels can cause
28 a disturbance in intracellular ion homeostasis resulting in activation of Ca^{2+} signaling, which
29 drives an increase in *CYP11B2* (aldosterone synthase) gene expression and aldosterone
30 production.[3] CYP11B2 immunohistochemistry-guided next generation sequencing has helped
31 identify somatic aldosterone-driver mutations in more than 90% APAs.[4,5]

32 Aldosterone is produced by adrenal zona glomerulosa (zG) cells in response to the
33 physiological stimuli angiotensin II (AngII), circulating potassium, and adrenocorticotropic
34 hormone.[6,7] In healthy individuals, suppression of the circulating renin-angiotensin system
35 (RAS) can lower aldosterone levels in response to high salt or volume expansion.[7] Thus, RAS
36 suppression and elevated plasma aldosterone concentrations should be mutually exclusive
37 under normal physiological conditions. Immunohistochemistry of adult adrenals shows that
38 CYP11B2 expression in the zG layer is mostly suppressed and restricted to aldosterone-
39 producing micronodules that carry aldosterone-driver mutations.[8,9] Several genes have been
40 identified in adrenal zG cells that downregulate *CYP11B2* gene transcription.[10-13] Of these,
41 *NEFM* (Neurofilament medium chain),[11,14] and *LGR5* (Leucine rich repeat containing G protein-
42 coupled receptor 5)[13,14] are differentially expressed in APAs and might function under

43 pathological conditions to modulate aldosterone production.

44 TSPAN12 belongs to the transmembrane 4 superfamily (or tetraspanin family) and functions
45 in a cell type-specific and disease-specific manner. Loss of endothelial TSPAN12 in the retina
46 impairs vascular morphogenesis[15], and aberrant TSPAN12 expression in epithelial cells and
47 fibroblasts contributes to cancer progression via FZD4 (frizzled class receptor 4)/LRP5 (low-
48 density-lipoprotein receptor-related protein 5)-mediated β-catenin signaling.[16] Additionally,
49 *Tspan12* transcripts are abundantly expressed in mouse pancreatic α-cells and have been
50 linked to the pathogenesis of diabetes.[17] Recently, we used RNAseq analysis to identify
51 differentially expressed genes in APAs.[18] *TSPAN12* was inversely correlated with APA diameter
52 and was downregulated in APAs with an APA somatic mutation in *KCNJ5* (encoding a G protein-
53 activated inward rectifier potassium channel) compared with APAs with wild type *KCNJ5*.[18,19]
54 Its function in adrenal physiology and pathology are unknown but might feasibly play a role in
55 steroidogenesis because *TSPAN12* gene expression is a component of the most informative
56 transcriptome signature for adrenal zone-specific steroid production.[20]

57 Herein, we investigated the role of *TSPAN12* under conditions of RAS activation and
58 suppression using a combined approach of transcriptome analysis of adrenal tumors and of
59 adrenals from a pig model of activated RAS, and functional *in vitro* studies in human
60 adrenocortical cells.

Materials and Methods

61 The expanded methods are available in the Data Supplement. The authors declare that all
62 supporting data are available within the article and Data Supplement.

Patients

63 APAs were surgically resected from patients diagnosed with unilateral disease at the Klinikum
64 der Ludwig-Maximilians-Universität München, Munich, Germany, and the Division of Internal
65 Medicine and Hypertension, Turin, Italy.[21-23] Blood pressure, hormonal and biochemical
66 measurements were assessed as described. Diagnosis of PA and differentiation of unilateral
67 from bilateral PA by adrenal venous sampling was performed according to recent
68 recommendations.[1,24,25] The presence of an APA in the resected adrenal was evaluated by
69 CYP11B2 immunohistochemistry.[26,27] APA *KCNJ5* mutations were identified as described
70 previously.[18]

Human Biosamples and Transcriptome data

71 RNA-sequencing (RNA-seq) datasets were publicly available from previously published data,
72 either from our group (APAs and pig adrenal zones)[18,28] or from others (comparison of zona
73 glomerulosa adjacent to APA and pheochromocytoma).[14] This study also included a separate
74 cohort of 30 APAs for TaqMan real time gene expression analysis and the corresponding
75 peripheral blood plasma samples were used for steroid measurements. Adrenal steroid
76 measurements of aldosterone, 18-oxocortisol, and 18-hydroxycortisol in peripheral venous
77 plasma were determined by liquid chromatography-tandem mass spectrometry (LC-

78 MS/MS).[29] All patients gave written informed consent for use of clinical data and biomaterial

79 in accordance with local ethics committees

Animal Samples

80 Adrenal samples from 6-week-old male German Landrace DanBred pigs fed a 14-day diet

81 comprising 0.04% sodium (low salt group) or 0.7% sodium (high salt group) were from a

82 previous study.[28]

***In vitro* functional assays**

83 The HAC15 human adrenocortical cell line[30] was used to investigate the functional role of

84 TSPAN12 *in vitro* as detailed in the online data supplement.

Statistical analyses and bioinformatics

85 GraphPad Prism 9.3.1 was used for all statistical analyses. An unpaired Student's t-test was

86 used for normally distributed data or a Mann-Whitney U for non-normally distributed data

87 among two groups. For multiple groups, one-way or two-way analysis of variance (ANOVA)

88 followed by Tukey's post hoc test was performed for normally distributed data or Kruskal-

89 Wallis for non-normally distributed data. Data in time-course experiments were analysed by

90 repeated measures two-way ANOVA. Chi-square and Fisher's exact tests were performed to

91 compare categorical variables. Correlations were performed using Spearman correlations. P-

92 values less than 0.05 were considered to indicate significant differences. Differential gene

93 expression analyses were performed using R package DESeq2 (v1.32.0)[31] and R package

94 clusterProfiler (v4.0.5) was used to analyze Gene Ontology. [32]

Results

Clinical parameters of patients with APA according to *TSPAN12* gene expression levels

95 The RNA-seq transcriptome profiles of 21 APAs were dichotomized into those with high or low
96 *TSPAN12* expression levels (*TSPAN12*^{high} versus *TSPAN12*^{low}) according to the median value.
97 Gene Ontology analysis of the *TSPAN12*^{high} versus *TSPAN12*^{low} groups demonstrated that
98 *TSPAN12* gene expression was closely related to the activity of the RAS and in particular blood
99 pressure regulation by the circulatory RAS and by hormones (**Figure 1A**, **1B**).

100 *TSPAN12* gene expression levels were also determined in a separate sample set of 30 APAs,
101 which were likewise dichotomized into *TSPAN12*^{high} and *TSPAN12*^{low} groups according to the
102 median *TSPAN12* expression level in this sample set. Patients in the *TSPAN12*^{high} group
103 displayed higher systolic blood pressure (BP) compared with the *TSPAN12*^{low} group (160
104 mmHg *versus* 145mmHg, P=0.011), and higher diastolic BP (105.7 mmHg *versus* 90 mmHg, P=
105 0.012) (**Table 1**). A higher proportion of APAs with a *KCNJ5* mutation were identified in the
106 *TSPAN12*^{low} group than in the *TSPAN12*^{high} group (80.0% in *TSPAN12*^{low} *versus* 33.3% in
107 *TSPAN12*^{high} APAs, P = 0.027). Lower APA *TSPAN12* gene expression was associated with higher
108 peripheral plasma concentrations of aldosterone (R=-0.47, P=0.009; **Figure 1C**), and the hybrid
109 steroids 18-oxocortisol (R=-0.53, P=0.0028; **Figure 1D**), and 18-hydroxycortisol (R=-0.63,
110 P=0.00025; **Figure 1E**) measured by LC-MS/MS.

TSPAN12 gene expression in zG cells adjacent to an APA or pheochromocytoma

111 Because Gene Ontology analysis highlighted a link between *TSPAN12* and the RAS, we

112 assessed *TSPAN12* gene expression levels in zG cells adjacent to an APA (a form of low renin
113 hypertension) and adjacent to a pheochromocytoma (a form of high renin hypertension) using
114 publicly available transcriptome data (GSE64957).[14] *TSPAN12* expression levels in zG cells
115 adjacent to APA (n=13) were 3.93-fold lower than in zG cells adjacent to pheochromocytoma
116 (n=7, *P*<0.001) (**Figure S1A**). Indeed, the absence of TSPAN12 immunostaining was observed
117 in zona glomerulosa adjacent to an APA **(Figure S2)** indicating the downregulation of TSPAN12
118 under conditions of a suppressed RAS. In addition, *TSPAN12* gene expression in APAs with a
119 *KCNJ5* mutation (n=17) was downregulated compared with APAs without a *KCNJ5* mutation
120 (n=13) (*P*<0.05) (**Figure S1B and Table 1**) and markedly lower intensity of TSPAN12
121 immunostaining was detected in *KCNJ5* mutated relative to *KCNJ5* wild type APAs (**Figure 2**).

TSPAN12 expression in pig adrenals in response to RAS activation

122 To explore the physiological response of *TSPAN12* to RAS activation, we evaluated *TSPAN12*
123 gene expression and TSPAN12 immunostaining in adrenal glands from pigs after RAS
124 activation by a 14-day low salt diet compared with a high salt diet.[28] RNA-seq analysis of the
125 dissected zG layer demonstrated notably increased *TSPAN12* gene expression in pigs following
126 the low salt diet (*P*<0.001) (**Figure 3A**). In addition, immunohistochemistry established higher
127 TSPAN12 expression localized to the zG of pig adrenals on the low *versus* high sodium diet
128 (**Figure 3B**).

***In vitro* functional analysis of *TSPAN12* expressed in human adrenocortical cells**

129 *CYP11B2* gene expression was quantified in HAC15 cells after overexpression or silencing of

130 *TSPAN12* (**Figure 4A and 4C**). *TSPAN12* overexpression displayed a 33% reduction in *CYP11B2*
131 gene expression (*P*=0.0036, **Figure 4B**). Silencing *TSPAN12* resulted in a 30% increase in
132 *CYP11B2* expression (*P*<0.0001, **Figure 4D**) and a 41% increase in aldosterone production
133 (*P*<0.05, **Figure 4E**). Angiotensin II (10 nM) stimulation of HAC15 cells led to a transient 1.43-
134 fold increase in *TSPAN12* expression at 4h compared with basal levels (*P*=0.0004) and as
135 expected, an increase in *CYP11B2* gene expression over the 16 h time course (**Figure 3C and**
136 **3D**). The stimulatory effect of AngII on aldosterone biosynthesis in zG cells is mediated by the
137 calcium signaling pathway. To investigate if calcium signaling mediates the effect of AngII on
138 *TSPAN12* gene expression, HAC15 cells were treated with 10 nM AngII for 6 h in the presence
139 and absence of either 10 µM of the calcium channel blocker nifedipine or 30 µM of the
140 calmodulin antagonist W-7 (N-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide
141 hydrochloride). Both calcium pathway inhibitors ablated the AngII-induced increase in
142 *TSPAN12* expression levels (*P*=0.0097 and *P*=0.0022) (**Figure 3E**).

143 *TSPAN12* gene silencing in HAC15 cells caused an amplification of *CYP11B2* mRNA levels and
144 aldosterone secretion in response to AngII compared with AngII-stimulated HAC15 cells
145 transfected with a control siRNA (14.6-fold increase in *CYP11B2* mRNA with *TSPAN12* gene
146 silencing *versus* 10.2-fold increase in AngII-stimulated control cells, *P*=0.0004; 2.5-fold
147 increase in aldosterone secretion with *TSPAN12* gene silencing *versus* 2.1-fold increase in
148 AngII-stimulated control cells, *P*<0.05) (**Figure 3F**).

149 We investigated the effect of *TSPAN12* on HAC15 cell death and proliferation. Cell viability and

150 flow cytometry analyses demonstrated the absence of an effect of *TSPAN12* gene silencing or
151 overexpression on HAC15 cell proliferation (**Figure S3A and S3B**), staurosporine-induced
152 apoptosis (**Figure S3C and S3D**) or RSL3-induced cell death by ferroptosis (**Figure S3E and S3F**)
153 under the conditions tested.[18]

Discussion

154 TSPAN12 has a widely studied role in endothelial cells in the promotion of vascular
155 morphogenesis and barrier formation (blood-brain barrier and blood-retina barrier).[15,33-35]
156 Endothelial cell-specific inactivation of *TSPAN12* results in abnormal angiogenesis and barrier
157 dysfunction.[15] The role of TSPAN12 in the adrenal gland is unknown but a potential role in APA
158 pathophysiology is suggested by the inverse correlation of *TSPAN12* gene expression with APA
159 nodule diameter[18] and the downregulation of *TSPAN12* expression in APAs carrying a *KCNJ5*
160 mutation (compared with APAs without this variant).[19]

161 In this study we combined transcriptome data from both human adrenal tissue samples and
162 a pig translational model of human adrenal steroidogenesis, with functional *in vitro* studies in
163 adrenocortical cells to identify TSPAN12 as a negative regulator of basal *CYP11B2* gene
164 expression with a functional role linked to the RAS. *CYP11B2* expression was upregulated by
165 *TSPAN12* gene silencing and downregulated by *TSPAN12* overexpression under basal
166 conditions in human adrenocortical HAC15 cells. *TSPAN12* expression was upregulated in
167 response to AngII stimulation by Ca^{2+}/calmodulin signaling in HAC15 cells and in response to
168 RAS activation in vivo. Conversely, under pathological conditions of RAS suppression, *TSPAN12*

169 expression was downregulated in zG cells adjacent to an APA compared with zG cells adjacent
170 to a pheochromocytoma thereby demonstrating differential expression of *TSPAN12* under
171 conditions of low versus high renin hypertension.

172 A study of 677 individuals without primary aldosteronism demonstrated a progressive decline
173 of plasma renin activity with increasing age associated with age-related autonomous
174 aldosteronism.[9] The same study also demonstrated the change of CYP11B2 expression in the
175 zG layer with age from a continuous to a discontinuous pattern and the accumulation of
176 aldosterone-producing micronodules[8,9] such that most zG cells in the adult human adrenal do
177 not express CYP11B2.[26,36] Zhou J et al.[10] identified 7 highly upregulated genes (more than 10-
178 fold) from a transcriptome analysis of zG versus zona fasciculata. Of these, 4 were negative
179 regulators of aldosterone production (*LGR5*, *ANO4*, *NEFM*, *DACH1*)[10-13] and 2 were positive
180 regulators (*NR4A2* and *VSNL1*).[37,38] These findings, together with the data of the present study,
181 highlight genes that potentially function in the suppression of aldosterone production.

182 Like our findings with *TSPAN12*, a low sodium diet and AngII infusion induced *RGS2* (regulator
183 of G protein signaling 2) and *RGS4* (regulator of G protein signaling 4) gene expression through
184 the calcium/calmodulin-dependent kinase pathway in human adrenocortical cells.[39,40]
185 Overexpression of *RGS2* or *RGS4* resulted in decreased AngII-induced aldosterone secretion,
186 supporting the hypothesis that counterregulatory mechanisms function in the maintenance
187 of appropriate levels of aldosterone production *in vivo*.[41]

188 We observed a positive association between TSPAN12 gene expression and blood pressure in

189 our cohort instead of an expected negative correlation. This apparent paradox can be
190 explained by the multiple factors that influence blood pressure such as glomerular filtration
191 rate, distal tubular sodium delivery, arterial compliance, and the duration and amplitude of
192 aldosterone excess.[42,43] Our transcriptome data from APAs with high versus low *TSPAN12* gene
193 expression indicate that *TSPAN12* function is linked with RAS activity under pathological
194 conditions. In PA, aldosterone production is autonomous of the RAS. However, most PA
195 patients, approximately 40% of patients with an APA and most patients with bilateral PA,
196 display increased aldosterone production in response to RAS activation by upright posture
197 (posture-responsive APAs).[7,44,45] The majority of posture-responsive APAs do not have a *KCNJ5*
198 mutation and comprise a relatively high proportion of compact eosinophilic cells (zG-like
199 cells).[46,47] In contrast, posture-unresponsive APAs show a slight preponderance of *KCNJ5*
200 mutations and are predominantly composed of clear zona fasciculata-like cells.[47-51] Patients
201 with posture-unresponsive APAs display higher plasma concentrations of the hybrid steroids
202 18-oxocortisol and 18-hydroxycortisol, which are reported as a feature of APAs with a *KCNJ5*
203 mutation.[52-54] Therefore, the link between the RAS and *TSPAN12* function underscored by the
204 transcriptomics analyses is conceivably related to the posture-responsive APA phenotype
205 (**Figure 5**).

Perspectives

206 *TSPAN12* gene expression is upregulated by increased RAS activity and may comprise a
207 counterregulatory response to elevated aldosterone through downregulation of *CYP11B2*

208 expression. Further studies are required to elucidate the mechanism by which TSPAN12 exerts

209 its negative regulatory effects on *CYP11B2* gene expression and adrenal aldosterone

210 production. In addition, the potential role of chronic RAS suppression in APA pathophysiology

211 and *TSPAN12* function in posture-responsive and posture-unresponsive APAs remains to be

212 elucidated.

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Conflicts of Interest/Disclosures

None

References

1. Reincke M, Bancos I, Mulatero P, Scholl UI, Stowasser M, Williams TA. Diagnosis and treatment of primary aldosteronism. *Lancet Diabetes Endocrinol*. 2021;9:876-892. doi: 10.1016/S2213-8587(21)00210-2

2. Williams TA, Lenders JWM, Mulatero P, Burrello J, Rottenkolber M, Adolf C, Satoh F, Amar L, Quinkler M, Deinum J, et al. Outcomes after adrenalectomy for unilateral primary aldosteronism: an international consensus on outcome measures and analysis of remission rates in an international cohort. *Lancet Diabetes Endocrinol*. 2017;5:689-699. doi: 10.1016/S2213-8587(17)30135-3

3. Scholl UI. Genetics of Primary Aldosteronism. *Hypertension*. 2022;79:887-897. doi: 10.1161/HYPERTENSIONAHA.121.16498

4. Nanba K, Rainey WE. GENETICS IN ENDOCRINOLOGY: Impact of race and sex on genetic causes of aldosterone-producing adenomas. *Eur J Endocrinol*. 2021;185:R1-R11. doi: 10.1530/EJE-21-0031

5. De Sousa K, Boulkroun S, Baron S, Nanba K, Wack M, Rainey WE, Rocha A, Giscos-Douriez I, Meatchi T, Amar L, et al. Genetic, cellular, and molecular heterogeneity in adrenals with aldosterone-producing adenoma. *Hypertension*. 2020;75:1034-1044. doi: 10.1161/HYPERTENSIONAHA.119.14177.

6. Hattangady NG, Olala LO, Bollag WB, Rainey WE. Acute and chronic regulation of aldosterone production. *Mol Cell Endocrinol*. 2012;350:151-162. doi: 10.1016/j.mce.2011.07.034

7. Stowasser M, Gordon RD. Primary aldosteronism: changing definitions and new concepts of physiology and pathophysiology both inside and outside the kidney. *Physiol Rev*. 2016;96:1327-1384. doi: 10.1152/physrev.00026.2015.

8. Nishimoto K, Tomlins SA, Kuick R, Cani AK, Giordano TJ, Hovelson DH, Liu CJ, Sanjanwala AR, Edwards MA, Gomez-Sanchez CE, et al. Aldosterone-stimulating somatic gene mutations are common in normal adrenal glands. *Proc Natl Acad Sci U S A*. 2015;112:E4591-4599. doi: 10.1073/pnas.1505529112

9. Nanba K, Vaidya A, Williams GH, Zheng I, Else T, Rainey WE. Age-related autonomous aldosteronism. *Circulation*. 2017;136:347-355. doi: 10.1161/CIRCULATIONAHA.117.028201

10. Zhou J, Shaikh LH, Neogi SG, McFarlane I, Zhao W, Figg N, Brighton CA, Maniero C, Teo AE, Azizan EA, et al. DACH1, a zona glomerulosa selective gene in the human adrenal, activates transforming growth factor-beta signaling and suppresses aldosterone secretion. *Hypertension*. 2015;65:1103-1110. doi: 10.1161/HYP.0000000000000025

11. Maniero C, Garg S, Zhao W, Johnson TI, Zhou J, Gurnell M, Brown MJ. NEFM (neurofilament medium) polypeptide, a marker for zona glomerulosa cells in human adrenal, inhibits D1R (dopamine D1 receptor)-mediated secretion of aldosterone. *Hypertension*. 2017;70:357-364. doi: 10.1161/HYPERTENSIONAHA.117.09231

12. Maniero C, Scudieri P, Haris Shaikh L, Zhao W, Gurnell M, Galietta LJV, Brown MJ. ANO4 (anoctamin 4) is a novel marker of zona glomerulosa that regulates stimulated aldosterone secretion. *Hypertension*. 2019;74:1152-1159. doi:

10.1161/HYPERTENSIONAHA.119.13287

13. Shaikh LH, Zhou J, Teo AE, Garg S, Neogi SG, Figg N, Yeo GS, Yu H, Maguire JJ, Zhao W, et al. LGR5 activates noncanonical Wnt signaling and inhibits aldosterone production in the human adrenal. *J Clin Endocrinol Metab*. 2015;100:E836-844. doi: 10.1210/jc.2015-1734

14. Zhou J, Lam B, Neogi SG, Yeo GS, Azizan EA, Brown MJ. Transcriptome pathway analysis of pathological and physiological aldosterone-producing human tissues. *Hypertension*. 2016;68:1424-1431. doi: 10.1161/HYPERTENSIONAHA.116.08033

15. Zhang C, Lai MB, Pedler MG, Johnson V, Adams RH, Petrash JM, Chen Z, Junge HJ. Endothelial cell-specific inactivation of TSPAN12 (tetraspanin 12) reveals pathological consequences of barrier defects in an otherwise intact vasculature. *Arterioscler Thromb Vasc Biol*. 2018;38:2691-2705. doi: 10.1161/ATVBAHA.118.311689

16. Otomo R, Otsubo C, Matsushima-Hibiya Y, Miyazaki M, Tashiro F, Ichikawa H, Kohno T, Ochiya T, Yokota J, Nakagama H, et al. TSPAN12 is a critical factor for cancer-fibroblast cell contact-mediated cancer invasion. *Proc Natl Acad Sci U S A*. 2014;111:18691-18696. doi: 10.1073/pnas.1412062112

17. Yin Q, Ni Q, Wang Y, Zhang H, Li W, Nie A, Wang S, Gu Y, Wang Q, Ning G. Raptor determines beta-cell identity and plasticity independent of hyperglycemia in mice. *Nat Commun*. 2020;11:2538. doi: 10.1038/s41467-020-15935-0

18. Yang Y, Tetti M, Vohra T, Adolf C, Seissler J, Hristov M, Belavgeni A, Bidlingmaier M, Linkermann A, Mulatero P, et al. BEX1 is differentially expressed in aldosterone-

producing adenomas and protects human adrenocortical cells from ferroptosis. *Hypertension*. 2021;77:1647-1658. doi: 10.1161/HYPERTENSIONAHA.120.16774

19. Monticone S, Hattangady NG, Nishimoto K, Mantero F, Rubin B, Cicala MV, Pezzani R, Auchus RJ, Ghayee HK, Shibata H, et al. Effect of KCNJ5 mutations on gene expression in aldosterone-producing adenomas and adrenocortical cells. *J Clin Endocrinol Metab*. 2012;97:E1567-1572. doi: 10.1210/jc.2011-3132

20. Baker JE, Plaska SW, Qin Z, Liu CJ, Rege J, Rainey WE, Udager AM. Targeted RNA sequencing of adrenal zones using immunohistochemistry-guided capture of formalin-fixed paraffin-embedded tissue. *Mol Cell Endocrinol*. 2021;530:111296. doi: 10.1016/j.mce.2021.111296

21. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, Clement DL, Coca A, de Simone G, Dominiczak A, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension. *Eur Heart J*. 2018;39:3021-3104. doi: 10.1093/eurheartj/ehy339

22. Riester A, Fischer E, Degenhart C, Reiser MF, Bidlingmaier M, Beuschlein F, Reincke M, Quinkler M. Age below 40 or a recently proposed clinical prediction score cannot bypass adrenal venous sampling in primary aldosteronism. *J Clin Endocrinol Metab*. 2014;99:E1035-1039. doi: 10.1210/jc.2013-3789

23. Mulatero P, Bertello C, Sukor N, Gordon R, Rossato D, Daunt N, Leggett D, Mengozzi G, Veglio F, Stowasser M. Impact of different diagnostic criteria during adrenal vein sampling on reproducibility of subtype diagnosis in patients with primary aldosteronism. *Hypertension*. 2010;55:667-673. doi:

10.1161/HYPERTENSIONAHA.109.146613

24. Mulatero P, Monticone S, Deinum J, Amar L, Prejbisz A, Zennaro MC, Beuschlein F, Rossi GP, Nishikawa T, Morganti A, et al. Genetics, prevalence, screening and confirmation of primary aldosteronism: a position statement and consensus of the Working Group on Endocrine Hypertension of The European Society of Hypertension. *J Hypertens*. 2020;38:1919-1928. doi: 10.1097/HJH.0000000000002510

25. Mulatero P, Sechi LA, Williams TA, Lenders JWM, Reincke M, Satoh F, Januszewicz A, Naruse M, Doumas M, Veglio F, et al. Subtype diagnosis, treatment, complications and outcomes of primary aldosteronism and future direction of research: a position statement and consensus of the Working Group on Endocrine Hypertension of the European Society of Hypertension. *J Hypertens*. 2020;38:1929-1936. doi: 10.1097/HJH.0000000000002520

26. Gomez-Sanchez CE, Qi X, Velarde-Miranda C, Plonczynski MW, Parker CR, Rainey W, Satoh F, Maekawa T, Nakamura Y, Sasano H, et al. Development of monoclonal antibodies against human CYP11B1 and CYP11B2. *Mol Cell Endocrinol*. 2014;383:111-117. doi: 10.1016/j.mce.2013.11.022

27. Williams TA, Gomez-Sanchez CE, Rainey WE, Giordano TJ, Lam AK, Marker A, Mete O, Yamazaki Y, Zerbini MCN, Beuschlein F, et al. International histopathology consensus for unilateral primary aldosteronism. *j Clin Endocrinol Metab*. 2021;106:42-54. doi: 10.1210/clinem/dgaa484

28. Vohra T, Kemter E, Sun N, Dobenecker B, Hinrichs A, Burrello J, Gomez-Sanchez EP,

Gomez-Sanchez CE, Wang J, Kinker IS, et al. Effect of dietary sodium modulation on pig adrenal steroidogenesis and transcriptome profiles. *Hypertension*. 2020;76:1769-1777. doi: 10.1161/HYPERTENSIONAHA.120.15998

29. Peitzsch M, Dekkers T, Haase M, Sweep FC, Quack I, Antoch G, Siegert G, Lenders JW, Deinum J, Willenberg HS, et al. An LC-MS/MS method for steroid profiling during adrenal venous sampling for investigation of primary aldosteronism. *J Steroid Biochem Mol Biol*. 2015;145:75-84. doi: 10.1016/j.jsbmb.2014.10.006

30. Parmar J, Key RE, Rainey WE. Development of an adrenocorticotropin-responsive human adrenocortical carcinoma cell line. *J Clin Endocrinol Metab*. 2008;93:4542-4546. doi: 10.1210/jc.2008-0903

31. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15:550. doi: 10.1186/s13059-014-0550-8

32. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, Feng T, Zhou L, Tang W, Zhan L, et al. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation (N Y)*. 2021;2:100141. doi: 10.1016/j.xinn.2021.100141

33. Bucher F, Zhang D, Aguilar E, Sakimoto S, Diaz-Aguilar S, Rosenfeld M, Zha Z, Zhang H, Friedlander M, Yea K. Antibody-mediated inhibition of Tspan12 ameliorates vasoproliferative retinopathy through suppression of β-catenin signaling. *Circulation*. 2017;136:180-195. doi: 10.1161/CIRCULATIONAHA.116.025604

34. Junge HJ, Yang S, Burton JB, Paes K, Shu X, French DM, Costa M, Rice DS, Ye W. TSPAN12

regulates retinal vascular development by promoting Norrin- but not Wnt-induced FZD4/beta-catenin signaling. *Cell*. 2009;139:299-311. doi: 10.1016/j.cell.2009.07.048

35. Shoda T, Wen T, Caldwell JM, Ben-Baruch Morgenstern N, Osswald GA, Rochman M, Mack LE, Felton JM, Abonia JP, Arva NC, et al. Loss of endothelial TSPAN12 promotes fibrostenotic eosinophilic esophagitis via endothelial cell-fibroblast crosstalk. *Gastroenterology*. 2022;162:439-453. doi: 10.1053/j.gastro.2021.10.016

36. Gomez-Sanchez CE, Gomez-Sanchez EP, Nishimoto K. Immunohistochemistry of the human adrenal CYP11B2 in normal individuals and in patients with primary aldosteronism. *Horm Metab Res*. 2020;52:421-426. doi: 10.1055/a-1139-2079

37. Bassett MH, Suzuki T, Sasano H, White PC, Rainey WE. The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. *Mol Endocrinol*. 2004;18:279-290. doi: 10.1210/me.2003-0005

38. Williams TA, Monticone S, Crudo V, Warth R, Veglio F, Mulatero P. Visinin-like 1 is upregulated in aldosterone-producing adenomas with KCNJ5 mutations and protects from calcium-induced apoptosis. *Hypertension*. 2012;59:833-839. doi: 10.1161/HYPERTENSIONAHA.111.188532

39. Romero DG, Plonczynski MW, Gomez-Sanchez EP, Yanes LL, Gomez-Sanchez CE. RGS2 is regulated by angiotensin II and functions as a negative feedback of aldosterone production in H295R human adrenocortical cells. *Endocrinology*. 2006;147:3889-3897. doi: 10.1210/en.2005-1532

40. Romero DG, Zhou MY, Yanes LL, Plonczynski MW, Washington TR, Gomez-Sanchez CE,

Gomez-Sanchez EP. Regulators of G-protein signaling 4 in adrenal gland: localization, regulation, and role in aldosterone secretion. *J Endocrinol*. 2007;194:429-440. doi: 10.1677/JOE-07-0153

41. Aptel HB, Johnson EI, Vallotton MB, Rossier MF, Capponi AM. Demonstration of an angiotensin II-induced negative feedback effect on aldosterone synthesis in isolated rat adrenal zona glomerulosa cells. *Mol Cell Endocrinol*. 1996;119:105-111. doi: 10.1016/0303-7207(96)03805-1

42. Safar ME, Asmar R, Benetos A, Blacher J, Boutouyrie P, Lacolley P, Laurent S, London G, Pannier B, Protogerou A, et al. Interaction between hypertension and arterial stiffness. *Hypertension*. 2018;72:796-805. doi: 10.1161/HYPERTENSIONAHA.118.11212

43. Vaidya A, Mulatero P, Baudrand R, Adler GK. The expanding spectrum of primary aldosteronism: implications for diagnosis, pathogenesis, and treatment. *Endocr Rev*. 2018;39:1057-1088. doi: 10.1210/er.2018-00139

44. Ahmed AH, Cowley D, Wolley M, Gordon RD, Xu S, Taylor PJ, Stowasser M. Seated saline suppression testing for the diagnosis of primary aldosteronism: a preliminary study. *J Clin Endocrinol Metab*. 2014;99:2745-2753. doi: 10.1210/jc.2014-1153

45. Wisgerhof M, Carpenter PC, Brown RD. Increased adrenal sensitivity to angiotensin II in idiopathic hyperaldosteronism. *J Clin Endocrinol Metab*. 1978;47:938-943. doi: 10.1210/jcem-47-5-938

46. Tunny TJ, Gordon RD, Klemm SA, Cohn D. Histological and biochemical distinctiveness of atypical aldosterone-producing adenomas responsive to upright posture and

angiotensin. *Clin Endocrinol (Oxf)*. 1991;34:363-369. doi: 10.1111/j.1365-2265.1991.tb00306.x

47. Guo Z, Nanba K, Udager A, McWhinney BC, Ungerer JPJ, Wolley M, Thuzar M, Gordon RD, Rainey WE, Stowasser M. Biochemical, histopathological, and genetic characterization of posture-responsive and unresponsive APAs. *J Clin Endocrinol Metab*. 2020;105. doi: 10.1210/clinem/dgaa367

48. Azizan EA, Murthy M, Stowasser M, Gordon R, Kowalski B, Xu S, Brown MJ, O'Shaughnessy KM. Somatic mutations affecting the selectivity filter of KCNJ5 are frequent in 2 large unselected collections of adrenal aldosteronomas. *Hypertension*. 2012;59:587-591. doi: 10.1161/HYPERTENSIONAHA.111.186239

49. Azizan EA, Lam BY, Newhouse SJ, Zhou J, Kuc RE, Clarke J, Happerfield L, Marker A, Hoffman GJ, Brown MJ. Microarray, qPCR, and KCNJ5 sequencing of aldosterone-producing adenomas reveal differences in genotype and phenotype between zona glomerulosa- and zona fasciculata-like tumors. *J Clin Endocrinol Metab*. 2012;97:E819-829. doi: 10.1210/jc.2011-2965

50. Monticone S, Castellano I, Versace K, Lucatello B, Veglio F, Gomez-Sanchez CE, Williams TA, Mulatero P. Immunohistochemical, genetic and clinical characterization of sporadic aldosterone-producing adenomas. *Mol Cell Endocrinol*. 2015;411:146-154. doi: 10.1016/j.mce.2015.04.022

51. Dekkers T, ter Meer M, Lenders JW, Hermus AR, Schultze Kool L, Langenhuijsen JF, Nishimoto K, Ogishima T, Mukai K, Azizan EA, et al. Adrenal nodularity and somatic

mutations in primary aldosteronism: one node is the culprit? *J Clin Endocrinol Metab*. 2014;99:E1341-1351. doi: 10.1210/jc.2013-4255

52. Williams TA, Peitzsch M, Dietz AS, Dekkers T, Bidlingmaier M, Riester A, Treitl M, Rhayem Y, Beuschlein F, Lenders JW, et al. Genotype-specific steroid profiles associated with aldosterone-producing adenomas. *Hypertension*. 2016;67:139-145. doi: 10.1161/HYPERTENSIONAHA.115.06186

53. Tezuka Y, Yamazaki Y, Kitada M, Morimoto R, Kudo M, Seiji K, Takase K, Kawasaki Y, Mitsuzuka K, Ito A, et al. 18-Oxocortisol synthesis in aldosterone-producing adrenocortical adenoma and significance of KCNJ5 mutation status. *Hypertension*. 2019;73:1283-1290. doi: 10.1161/HYPERTENSIONAHA.118.12064

54. Turcu AF, Wannachalee T, Tsodikov A, Nanba AT, Ren J, Shields JJ, O'Day PJ, Giacherio D, Rainey WE, Auchus RJ. Comprehensive analysis of steroid biomarkers for guiding primary aldosteronism subtyping. *Hypertension*. 2020;75:183-192. doi: 10.1161/HYPERTENSIONAHA.119.13866

55. Burrello J, Burrello A, Stowasser M, Nishikawa T, Quinkler M, Prejbisz A, Lenders JWM, Satoh F, Mulatero P, Reincke M, et al. The primary aldosteronism surgical outcome score for the prediction of clinical outcomes after adrenalectomy for unilateral primary aldosteronism. *Ann Surg.* 2020;272(6):1125-1132. doi: 10.1097/SLA.0000000000003200.

56. Yang Y, Gomez-Sanchez CE, Jaquin D, Aristizabal Prada ET, Meyer LS, Knosel T, Schneider H, Beuschlein F, Reincke M, Williams TA. Primary aldosteronism: KCNJ5 mutations and

adrenocortical cell growth. *Hypertension*. 2019;74:809-816. doi: 10.1161/HYPERTENSIONAHA.119.13476

213

Novelty and Relevance

What Is New?

- Transcriptomics analyses of APAs highlight a link between the renin-angiotensin system and *TSPAN12* gene expression.
- TSPAN12 (tetraspanin 12) is highly expressed in pig adrenal zG cells in response to renin–angiotensin system activation by dietary sodium restriction.
- Angiotensin II stimulation of human adrenocortical cells promotes *TSPAN12* expression by the Ca^{2+}/calmodulin signaling pathway.
- Ablation of *TSPAN12* gene expression enhances angiotensin II stimulated *CYP11B2* (aldosterone synthase) gene expression and aldosterone production.
- *TSPAN12* expression in APAs is inversely associated with preoperative plasma aldosterone concentrations in the corresponding patients.

What Is Relevant?

- TSPAN12 is an endogenous negative regulator of *CYP11B2* gene expression and aldosterone production.
- Downregulation of *TSPAN12* in APAs by suppression of the renin–angiotensin system. might contribute to pathological aldosterone production.

Clinical/Pathophysiological Implications

TSPAN12 is a novel target of the renin-angiotensin system under physiological and pathophysiological conditions.

Figure Legends

Figure 1. Transcriptomics and steroidomics analyses of patients with APA.

Panel A, RNAseq analysis of 21 APAs was used to dichotomize APAs into those with high and low *TSPAN12* mRNA levels according to the median value (APA-$TSPAN12^{high}$ and APA-$TSPAN12^{low}$). **Panel B**, Bar plots showing Gene Ontology (GO) terms significantly enriched in RNA-seq analysis of APAs in the $TSPAN12^{high}$ group (*n*= 10) versus the $TSPAN12^{low}$ group (*n*= 11). **Panel C**, **D**, and **E**, Scatter plots showing the correlation of *TSPAN12* mRNA levels with plasma aldosterone concentrations, 18-oxocortisol, and 18-hydroxycortisol in a cohort of 30 patients with an APA. *P* and r values were calculated by Spearman correlation. ADX, adrenalectomy; APA, aldosterone-producing adenoma; BP, blood pressure; RAS, renin-angiotensin system; SRL, signaling receptor ligand; PAC, plasma aldosterone concentration.

Figure 2. TSPAN12 immunohistochemistry of APAs with or without a *KCNJ5* mutation

Immunohistochemical staining of TSPAN12 in APAs with a *KCNJ5* mutation (Panel A) or in APAs without a *KCNJ5* mutation (Panel B). APA from patient 4 and patient 5 carry *CACNA1D* mutations. The APA from patient 6 carries an *ATP1A1* mutation. Pictures are representative of 10 adrenals per group. Scale bars = 200 µm. *KCNJ5*, gene encoding G-protein–coupled inwardly rectifying potassium channel; *CACNA1D*, gene encoding Cav1.3 Ca^{2+} channel; *ATP1A1*, gene encoding Na^+/K^+ ATPase 1; TSPAN12, tetraspanin 12.

Figure 3. Effect of renin angiotensin system activation and angiotensin II on *TSPAN12* in pig adrenals and in human adrenocortical cells

Panel A, *TSPAN12* gene expression levels using FPKM normalization were assessed in the zG of pig adrenals on low *versus* high sodium diet from RNA-seq data (*n*= 3 per group). Data are represented as box-and-whisker plots with min. to max. values. **Panel B**, Representative immunohistochemistry staining of TSPAN12 in adrenal sections from pigs on low *versus* high sodium diet. Scale bar, 2 mm (whole images) and 200 µm (magnifications). **Panels C** and **D**, time course of *TSPAN12* (**Panel C**) and *CYP11B2* (**Panel D**) mRNA levels in HAC15 cells treated with 10 nM AngII (*n*=8 independent experiments per group). Data represent mean ± SEM. Statistical significance was determined by repeated measures two-way ANOVA. **Panel E**, Levels of *TSPAN12* mRNA in HAC15 cells treated for 6h with vehicle (DMSO) or AngII (10 nmol/L) in the presence or absence of the calcium channel blocker nifedipine (10 µmol/L) or calmodulin antagonist W-7 hydrochloride (30 µmol/L) (6 independent experiments per group). **Panel F**, Levels of *CYP11B2* mRNA or aldosterone production in HAC15 cells transfected with control siRNA (siControl) or *TSPAN12*-specific siRNA (si*TSPAN12*) treated with or without AngII (10 nmol/L) for 6h (*n*=6 independent experiments per group) or 24h (right, n=3 independent experiments per group). Data represent mean ± SEM. Statistical significance was calculated by one-way ANOVA and Tukey's post-hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars (**Panel B, upper**) = 2 µm; scale bar (**Panel B, lower**) = 200 µm. IHC, immunohistochemistry. AngII, angiotensin II; DMSO, dimethyl sulfoxide; FPKM, fragments per kilo base of transcript per million mapped fragments; HAC15 cells, human adrenocortical cell line; zG, zona glomerulosa.

Figure 4. Effect of *TSPAN12* gene expression on *CYP11B2* gene expression and aldosterone production in human adrenocortical cells

Real-time PCR gene expression assays of *TSPAN12* (**Panel A**) and *CYP11B2* (**Panel B**) in HAC15 cells transfected with empty vector (EV) or pcDNA3.1-*TSPAN12* (p*TSPAN12*) (8 independent experiments per group). Real-time PCR analysis of *TSPAN12* (**Panel C**) and *CYP11B2* (**Panel D**) gene expression in HAC15 cells transfected with non-targeting siRNA (siControl) or *TSPAN12*-specific siRNA (si*TSPAN12*). *TSPAN12* silencing in HAC15 cells caused increased aldosterone production compared with controls. Cell medium was collected 48-hour after transfection for aldosterone measurements (n=23) and aldosterone levels were normalized relative to total protein in cell lysates (**Panel E**). Data are presented as mean of 8 independent experiments ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by a two-sided unpaired Student's *t*-test.

Figure 5. TSPAN12 is regulated by the renin-angiotensin-system under physiological and pathophysiological conditions

TSPAN12 gene expression is upregulated by increased RAS activity and may form part of a counterregulatory response to elevated aldosterone production by inhibition of *CYP11B2* expression. Figure produced using https://smart.servier.com/

Variables	Total cohort (n=30)	*TSPAN12* gene expression		P value
		High (n=15)	Low (n=15)	
Age at surgery, y (n=30)	46.30 ± 10.55	48.27 ± 11.42	44.33 ± 9.57	0.316
Sex (ref. women; n=30)	17 (56.7%)	7 (46.7%)	10 (66.7%)	0.461
BMI, kg/m2 (n=30)	27.76 (22.88-30.33)	29.00 (24.90-30.98)	25.60 (22.20-28.82)	0.101
Systolic BP, mmHg (n=30)	150.00 (140.75-163.00)	160.00 (148.85-182.15)	145.00 (136.50-151.50)	0.011
Diastolic BP, mmHg (n=30)	94.15 (86.75-104.28)	105.70 (95.00-110.00)	90.00 (85.50-94.15)	0.012
Duration HTN, mo (n=30)	77.50 (41.25-112.50)	91.00 (59.00-131.50)	67.00 (18.50-101.00)	0.245
Anti-HTN meds (DDD; n=30)	4.00 (3.00-5.62)	5.00 (3.00-6.00)	3.50 (1.25-4.88)	0.080
PAC, pmol/L (n=30)	792.00 (575.60-1301.42)	643.60 (448.00-1004.20)	1090.20 (606.10-1451.35)	0.272
DRC, mU/L (n=15)	3.30 (2.00-9.20)	3.10 (2.00-7.35)	4.50 (1.95-10.25)	0.728
ARR_DRC (n=15)	180.50 (62.15-324.20)	180.50 (79.25-254.50)	191.10 (47.78-358.72)	1
PRA, pmol/L per min (n=15)	2.56 (2.56-3.84)	3.20 (2.56-5.44)	2.56 (1.92-3.20)	0.206
ARR_PRA (n=15)	418.30 (242.70-708.15)	253.55 (116.95-411.75)	609.00 (488.20-829.50)	0.049
Lowest serum K^+, mmol/L (n=30)	2.98 ± 0.57	3.03 ± 0.54	2.94 ± 0.62	0.687
Nodule	15.00	13.00	17.00	0.021

diameter, mm (n=30)	(12.25-17.75)	(10.50-15.50)	(14.00-24.50)	
TSPAN12 expression	0.30 (0.09-1.24)	1.27 (0.80-2.81)	0.09 (0.04-0.16)	<0.001
Genotype				0.027
KCNJ5 mutation	17 (56.7%)	5 (33.3%)	12 (80.0%)	
KCNJ5 wild type	13 (43.3%)	10 (66.7%)	3 (20.0%)	

Table 1. Clinical parameters of patients with an APA stratified by *TSPAN12* gene expression levels

Baseline variables were stratified as APA-*TSPAN12*^{high} (>median *TSPAN12* gene expression level) and APA-*TSPAN12*^{low} (≤median). *TSPAN12* relative expression for each sample was calculated using the $2^{-\Delta\Delta Ct}$ formula. Quantitative normally distributed variables are shown as means ± SD and nonnormally distributed variables are reported as medians (IQR). Categorical variables are shown as absolute numbers and percentage. *P* values were calculated using χ^2 and Fisher exact tests or *t* tests or Mann-Whitney U test as appropriate. *P* values of <0.05 were considered significant. The defined daily dose (DDD) is the assumed average maintenance dose per day for a drug used for its main indication in adults (https://www.who.int/tools/atc-ddd-toolkit/about-ddd) and was calculated using the online tool available at https://github.com/ABurrello/PASOPredictor/raw/master/00—PASO Predictor.xlsm.[55] The 30 APAs used for this analysis were from patients diagnosed at 2 different centres (Munich and Turin) using different methods for the measurement of renin levels (DRC or PRA). APA,

aldosterone-producing adenoma; ARR, aldosterone-to-renin ratio; BMI, body mass index; BP, blood pressure; DDD, defined daily dose; PAC, plasma aldosterone concentration; DRC, direct renin concentration; HTN, hypertension; K^+, potassium ions; *KCNJ5*, gene encoding potassium inwardly rectifying channel subfamily J member 5; meds, medications; N, number; PRA, plasma renin activity; ref, reference.

Figure 1

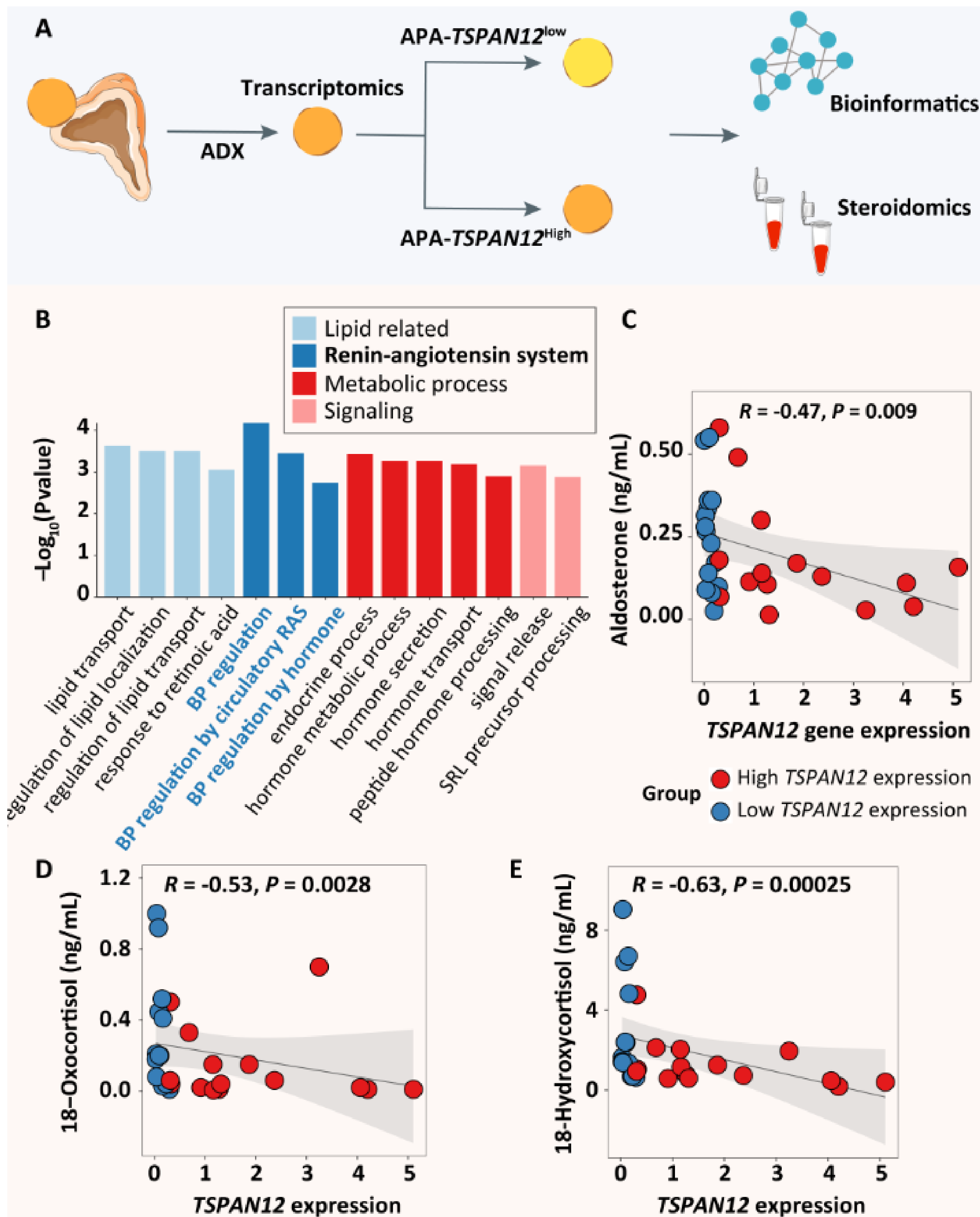


Figure 2

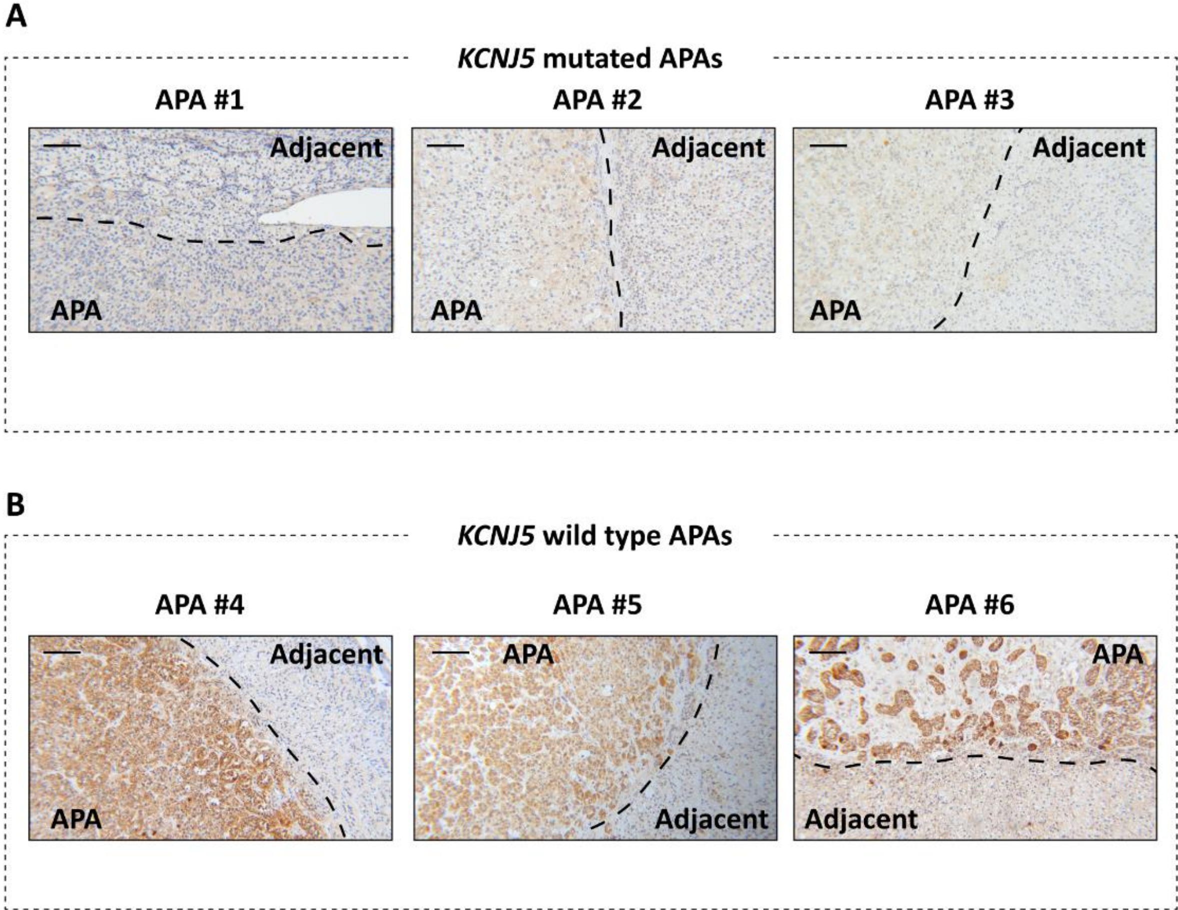


Figure 3

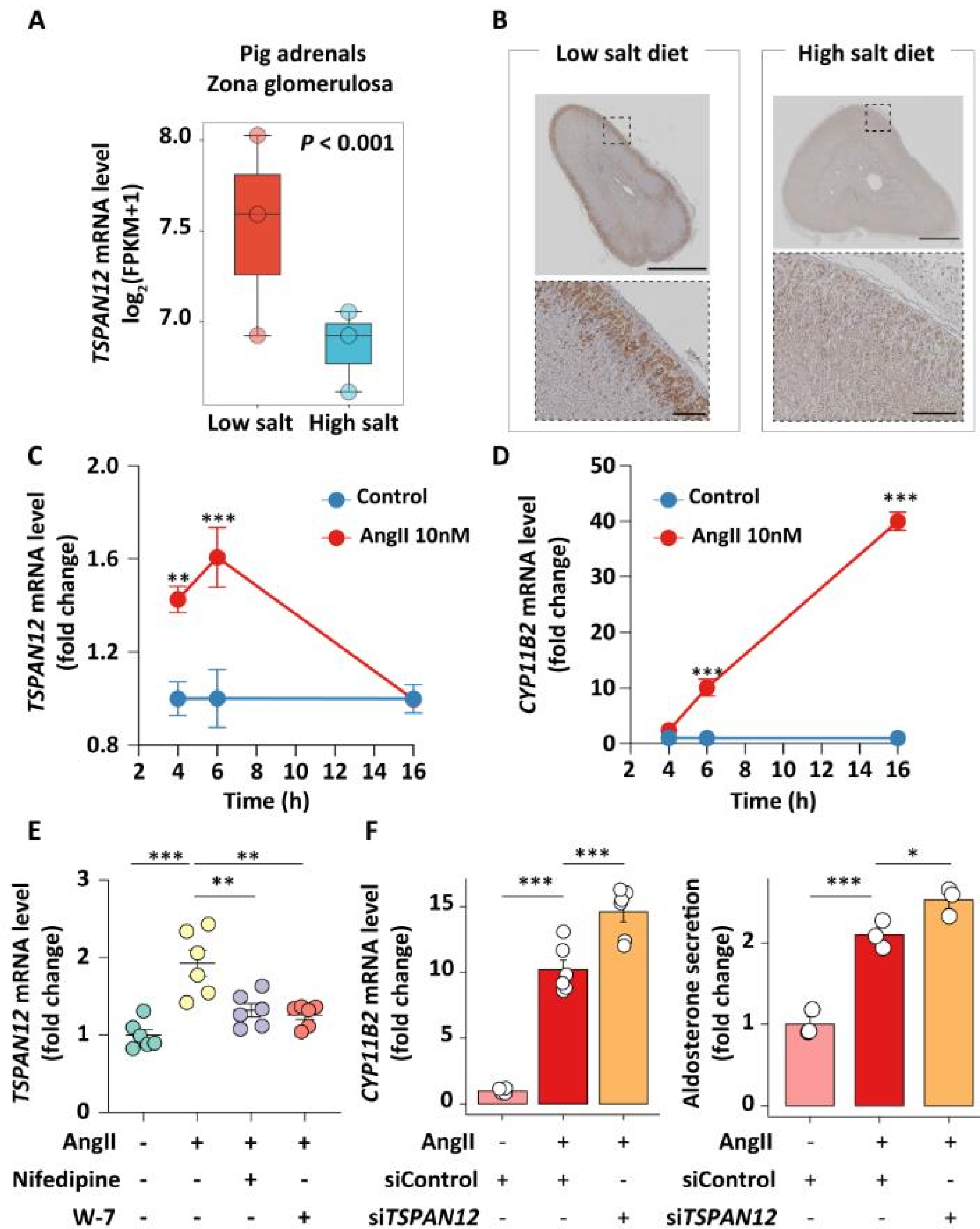


Figure 4

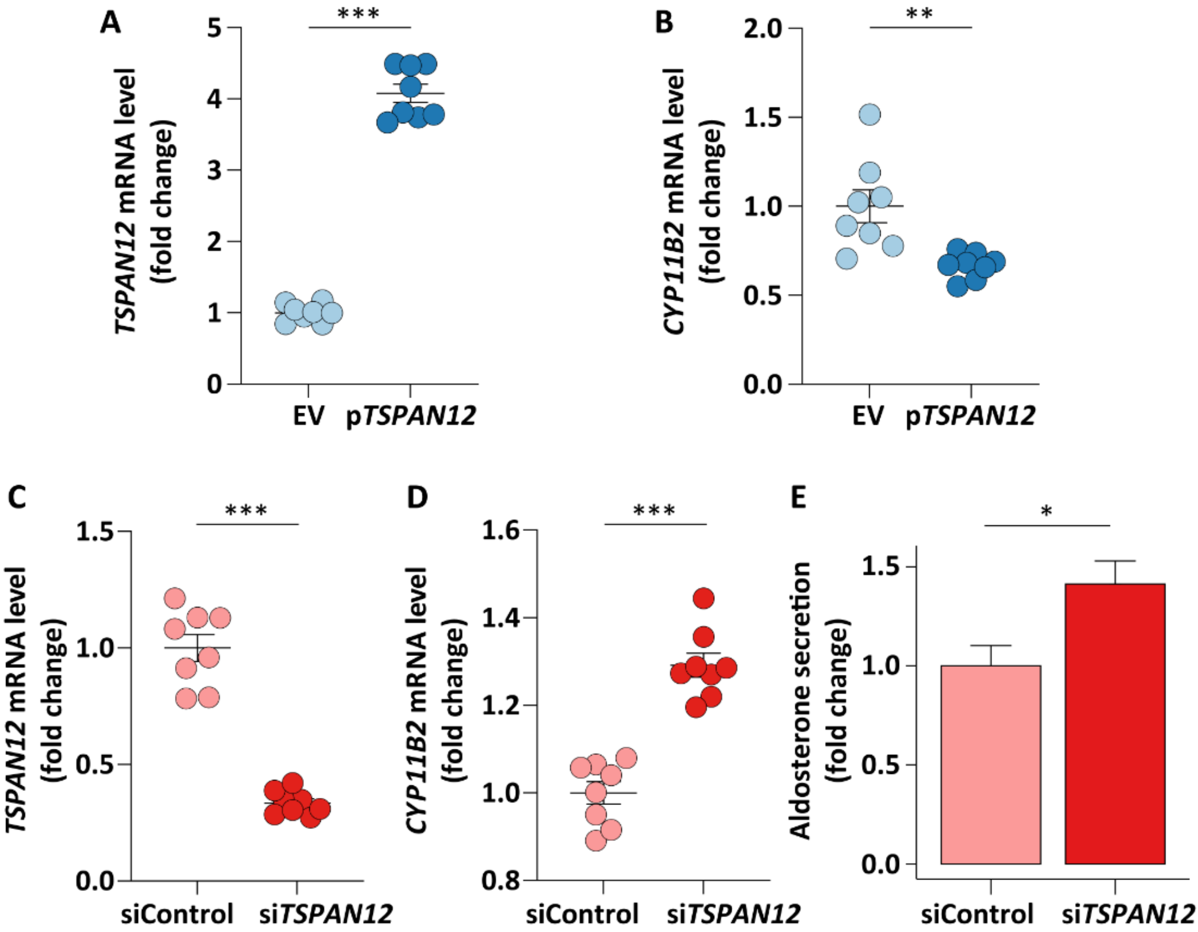
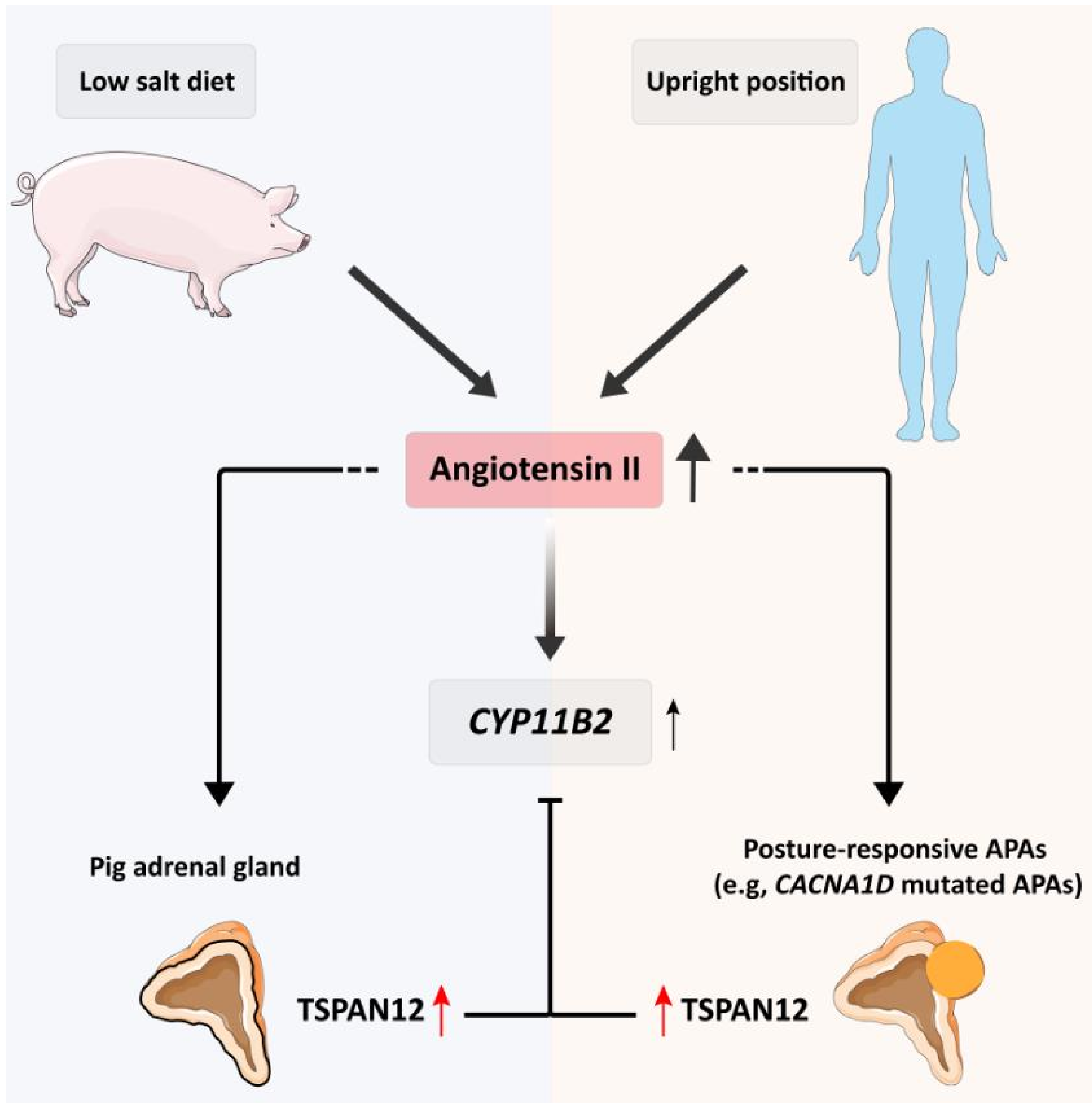


Figure 5



Low salt diet
Upright position
Angiotensin II
CYP11B2
Pig adrenal gland
Posture-responsive APAs
(e.g, CACNA1D mutated APAs)
TSPAN12
TSPAN12

Online-only supplement

TSPAN12 is a novel negative regulator of aldosterone production in adrenal physiology and aldosterone-producing adenomas

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Expanded Materials and Methods

Clinical measurements

Baseline blood pressure (BP) was measured at the first visit under treatment using a mercury sphygmomanometer. BP measurements were initiated after the patients was seated in a quiet room for 3 to 5 minutes. Seated blood pressure measurements were evaluated at least twice, at a 1-to-2-minute interval and the average BP was calculated.[1]

Hormonal measurements were performed following withdrawal of interfering medications and correction of hypokalemia with potassium supplementation. Plasma aldosterone, cortisol, and direct renin concentrations or plasma renin activity were measured according to local protocols.[2,3]

Immunohistochemistry

Formalin-fixed paraffin-embedded adrenals from 6-week-old male German Landrace DanBred pigs fed a 14-day 0.04% sodium diet (low salt group) or 0.7% sodium (high salt group) were from a previous study.[4] Pig and APA adrenal sections were cut (3 µm thick) and used for TSPAN12 immunohistochemistry with an anti-TSPAN12 primary antibody diluted 1:100 (LSBio)[5] and KCNJ5 immunohistochemistry with the KCNJ5 monoclonal antibody (clone No. 36-33-5, dilution 1:2000, a kind gift from Prof. Celso Gomez-Sanchez, University of Mississippi, US). Negative controls were performed on APA and pig adrenal sections by replacing the primary antibody with equivalent dilutions and concentrations of rabbit immunoglobulin (Ig)G fractions (X0903; Dako). TSPAN12 staining in the human placental tissues were used as positive controls (Figure S4).

In vitro functional assays

Human adrenal carcinoma cell line HAC15 cells[6] were cultured at 37°C in 5% CO_2 in DMEM/F12 (1:1) medium supplemented with 10% Cosmic Calf serum, 1x insulin-transferrin-selenium, 1% antibiotic-antimycotic, and 0.01% Gentamicin. Prior to cell treatments, cells were incubated overnight in starvation medium (DMEM/F12 medium containing 0.1% Cosmic Calf serum). Cells were treated with combinations of 10 nM angiotensin II (AngII), 10 µM nifedipine; 30 µM W-7 hydrochloride as indicated. Cells were pre-incubated with inhibitors for 1 hour before AngII stimulation as appropriate.

Silencer Select small interfering RNA (siRNA) for *TSPAN12* (Thermofisher Scientific) and pcDNA3.1+/C-(k)DYK-*TSPAN12* was from GenScript. For gene silencing experiments, HAC15 cells ($1x10^6$) were transfected with 1 µL of a 100 µmol/L solution of silencer select *TSPAN12* siRNA. For TSPAN12 overexpression experiments, HAC15 cells ($3x10^6$) were transfected with 3 µg pcDNA3.1-*TSPAN12* using the Amaxa Cell Line Nucleofector Kit R and the Nucleofector™ 2b (program X-005) (Lonza) according to manufacturers´ instructions. Samples were collected for RT-qPCR analysis at 48h post-transfection. Total RNA was extracted using a Maxwell 16 device and reverse transcription was performed with GoScript™ reverse transcriptase mix, oligo (dT) (Promega) according to the manufacturer's protocol. qPCR reactions were performed on the QuantStudio 5 Real-Time PCR instrument (Applied Biosystems) using TaqMan gene expression assays. *GAPDH* was used for normalization. The following TaqMan

probes were used: *TSPAN12*, Hs01113125_m1; *CYP11B2*, Hs01597732_m1; *GAPDH*, Hs02786624_g1. $2^{-\Delta\Delta Ct}$ relative quantification method was used for calculating gene expression levels.

Aldosterone and protein assays

Transfected HAC15 cells were seeded on 24-well plates. After 24 hours, cells were washed with DPBS, serum deprived overnight and then incubated for another 48 h in starvation medium or 24h in starvation medium with 10 nM AngII. Following this, supernatant and cells were harvested for aldosterone measurements and protein quantification of cell lysates, respectively. Aldosterone was measured in cell culture supernatants using the Aldosterone Parameter Assay Kit (KGE016, R&D systems) following the manufacturer's instructions. Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermofisher Scientific). Aldosterone levels per well were normalized to protein concentrations.

Cell proliferation and viability assay

For cell proliferation experiments, HAC15 cells (4×10^4 cells/well) were plated in 96-well plates and incubated for 72 hours. For apoptosis experiments, transfected HAC15 cells (2.5×10^4 cells/well) were plated in 96-well plates. After 24 hours, cells were treated with 1 µmol/L staurosporine (STS, inducer of apoptosis) for 24 hours. Cell proliferation and cell apoptosis were determined with a water-soluble tetrazolium salt-1 (WST-1) assay (Roche). After incubation with WST-1 at 37°C for 3 hours, absorbance at 450 nm and 690 nm were determined on a FLUOstar Omega plate reader (BMG LABTECH).

Flow Cytometry

For cell death experiments, transfected HAC15 cells were seeded into 6-well plates ($1x10^6$ cells/well) and incubated for 24 hours, followed by a further 24 hours in starvation medium. Cells were then treated for 24 hours with either vehicle alone (0.03% DMSO) or an inducer of ferroptosis (1 µmol/L and 1.5 µmol/L RSL3 ([1S,3R]-RSL3).[7] Viable and floating dead cells were collected and stained with propidium iodide (PI) (eBioscience, catalog no. 00-6990-50). At least 10,000 cells were acquired per sample. Data analysis was conducted using the FlowJo Software (v.10.4).

References

1) Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, Clement DL, Coca A, de Simone G, Dominiczak A, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension. *Eur Heart J*. 2018;39:3021-3104. doi: 10.1093/eurheartj/ehy339

2) Riester A, Fischer E, Degenhart C, Reiser MF, Bidlingmaier M, Beuschlein F, Reincke M, Quinkler M. Age below 40 or a recently proposed clinical prediction score cannot bypass adrenal venous sampling in primary aldosteronism. *J Clin Endocrinol Metab*. 2014;99:E1035-1039. doi: 10.1210/jc.2013-3789

3) Mulatero P, Bertello C, Sukor N, Gordon R, Rossato D, Daunt N, Leggett D, Mengozzi G, Veglio F, Stowasser M. Impact of different diagnostic criteria during adrenal vein sampling on reproducibility of subtype diagnosis in patients with primary aldosteronism. *Hypertension*. 2010;55:667-673. doi: 10.1161/HYPERTENSIONAHA.109.146613

4) Vohra T, Kemter E, Sun N, Dobenecker B, Hinrichs A, Burrello J, Gomez-Sanchez EP, Gomez-Sanchez CE, Wang J, Kinker IS, et al. Effect of dietary sodium modulation on pig adrenal steroidogenesis and transcriptome profiles. *Hypertension*. 2020;76:1769-1777. doi: 10.1161/HYPERTENSIONAHA.120.15998

5) Bucher F, Zhang D, Aguilar E, Sakimoto S, Diaz-Aguilar S, Rosenfeld M, Zha Z, Zhang H, Friedlander M, Yea K. Antibody-mediated inhibition of Tspan12 ameliorates vasoproliferative retinopathy through suppression of β-catenin signaling. *Circulation*. 2017;136:180-195. doi: 10.1161/CIRCULATIONAHA.116.025604

6) Parmar J, Key RE, Rainey WE. Development of an adrenocorticotropin-responsive human adrenocortical carcinoma cell line. *J Clin Endocrinol Metab*. 2008;93:4542-4546. doi: 10.1210/jc.2008-0903

7) Yang Y, Tetti M, Vohra T, Adolf C, Seissler J, Hristov M, Belavgeni A, Bidlingmaier M, Linkermann A, Mulatero P, et al. BEX1 is differentially expressed in aldosterone-producing adenomas and protects human adrenocortical cells from ferroptosis. *Hypertension*. 2021;77:1647-1658. doi: 10.1161/HYPERTENSIONAHA.120.16774

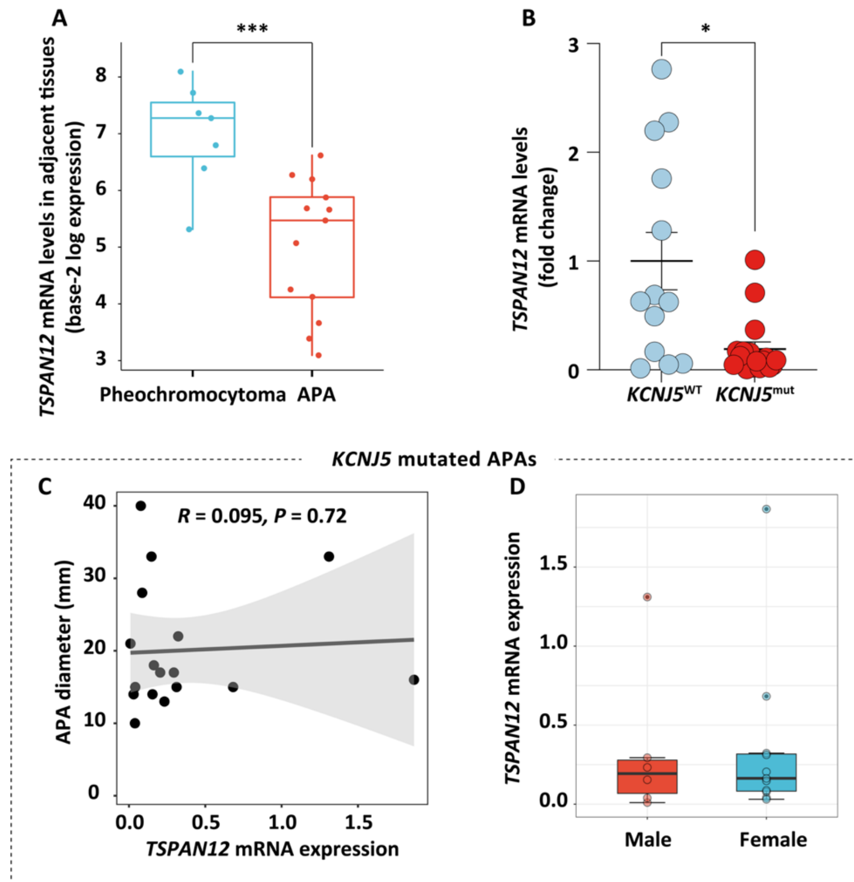

Figure S1. Differential expression of *TSPAN12* in adrenal tissues.

(A) *TSPAN12* gene expression is downregulated in the zG adjacent to APAs (n=13) compared with zG adjacent to pheochromocytomas (n=7) (transcriptome data from GSE64957). **(B)** real-time PCR analysis of *TSPAN12* gene expression in APA without a *KCNJ5* mutation (*KCNJ5*^{WT}, n=13) and with a *KCNJ5* mutation (*KCNJ5*^{mut}, n=17). **(C),** There was no correlation between APA diameter and *TSPAN12* expression in *KCNJ5* mutated APAs (n=17). *P* and r values were calculated by Spearman correlation. **(D),** *TSPAN12* expression was independent of sex within groups of *KCNJ5* mutated APAs. Data represent mean ± SEM. Statistical significance was calculated using a Mann-Whitney test. *$P < 0.05$, ***$P < 0.001$. APA, aldosterone-producing adenoma; zG, zona glomerulosa.

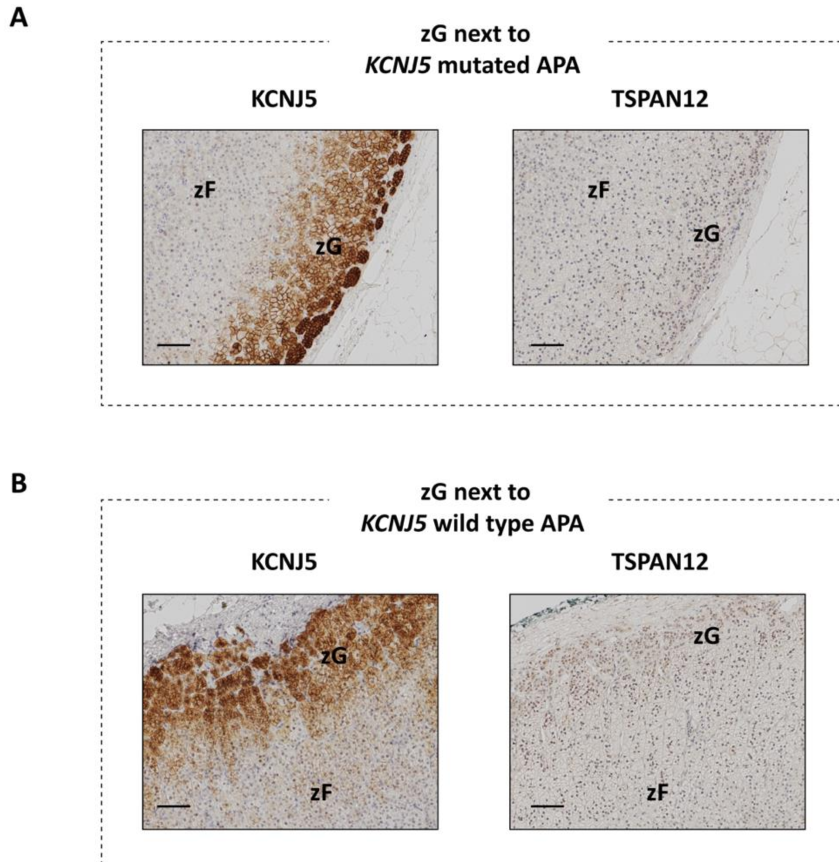

Figure S2. TSPAN12 immunohistochemistry of adrenal zona glomerulosa adjacent to APA.
TSPAN12 immunostaining was absent in the zona glomerulosa (zG) adjacent to APA. Representative immunohistochemistry of KCNJ5 (as a zG marker) and TSPAN12 in zG adjacent to a *KCNJ5* mutated APA **(Panel A)** or adjacent to a *KCNJ5* wild type APA **(Panel B)**. The negative and positive controls for TSPAN12 immunohistochemistry are shown in Figure S4. Scale bars = 100 μm. APA, aldosterone-producing adenoma; zG, zona glomerulosa; zF, zona fasciculata.

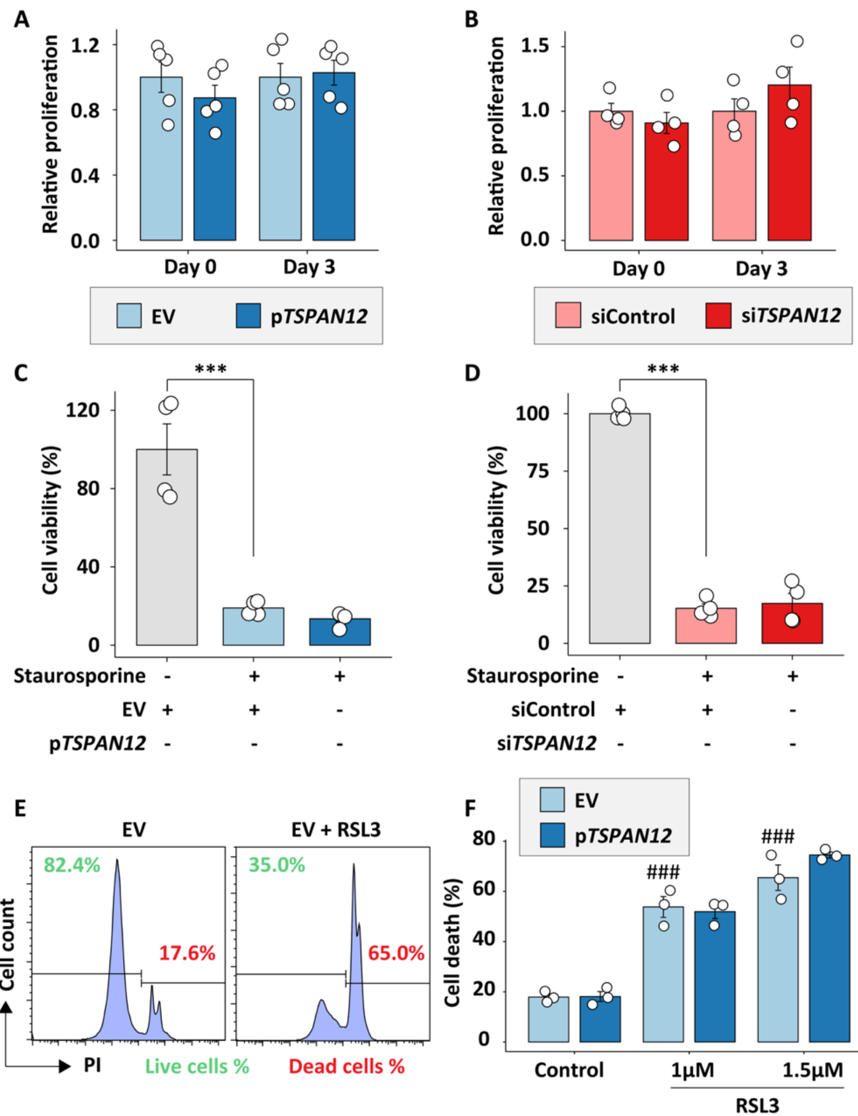

Figure S3. *TSPAN12* has no effect on cell proliferation, and adrenocortical cell death.
A-B, Analysis of cell viability by WST-1 assay in HAC15 cells with *TSPAN12* overexpression **(A)** or in HAC15 cells with *TSPAN12* siRNA knockdown **(B)** compared with control cells transfected with empty vector (EV) or a control siRNA, as appropriate. Data are normalized to negative control. Statistical significance was calculated by unpaired Student's *t*-test. **C-D**, Cell viability of HAC15 cells with *TSPAN12* overexpression **(C)** or in HAC15 cells with *TSPAN12* siRNA knockdown **(D)** compared with control cells transfected with empty vector (EV) or a control siRNA after 24 h treatment with vehicle (0.02% DMSO) or 1 µmol/L STS (staurosporine, inducer of apoptosis). Cell viability was measured using WST-1 assay and STS-treated HAC15 cells were used as positive control for the experiment. Statistical significance was calculated by one-way ANOVA with Tukey's multiple comparisons test. **E**, Representative flow cytometry plots of PI^- (live cells in green) or PI^+ (dead cells in red) HAC15 EV cells untreated (left) or treated (right) with 1.5 µmol/L ferroptosis inducer RSL3 ([1S, 3R]-RSL3) for 24 h. RSL3-treated HAC15 cells serve as positive control for flow cytometry experiments. **F**, Flow cytometry analysis of cell death in *TSPAN12*-overexpressing HAC15 cells compared to negative control (EV) after treatment with vehicle (0.03% DMSO), 1 µmol/L RSL3 and 1.5 µmol/L RSL3 for 24h. Statistical significances were calculated by two-way ANOVA with Tukey's multiple comparisons test. ***$P < 0.001$. ### difference (P<0.001) from HAC15 EV cells treated with vehicle. Data are presented as mean ± SEM.

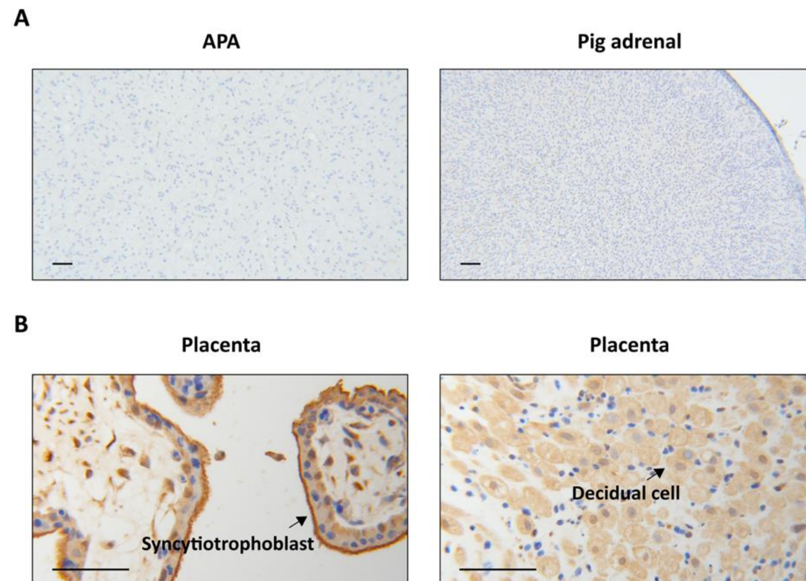

Figure S4. TSPAN12 antibody validation for immunohistochemistry.
A, IHC (immunohistochemistry) analysis of paraffin-embedded APA (left) and pig adrenal (right) sections, using negative control rabbit immunoglobulin (Ig)G fractions (X0903, Dako). **B**, TSPAN12 IHC of paraffin-embedded human placenta sections, used as positive controls. Strong TSPAN12 immunostaining was evident in syncytiotrophoblasts and decidual cells. Scale bars = 100 μm. APA, aldosterone-producing adenoma