Activating mutations in *CTNNB1* in aldosterone producing adenomas

Tobias Åkerström[1], Rajani Maharjan[1], Holger Sven Willenberg[2], Kenko Cupisti[3], Julian Ip[4], Ana Moser[5], Peter Stålberg[1], Bruce Robinson[4], K. Alexander Iwen[5], Henning Dralle[6], Martin K. Walz[7], Hendrik Lehnert[5], Stan Sidhu[4], Celso Gomez-Sanchez[8], Per Hellman[1], and Peyman Björklund[1*].

[1]Department of Surgical Sciences, Uppsala University, Uppsala, Sweden.

[2]Department of Endocrinology and Metabolism, Rostock University Medical Center, Germany.

[3]General, Visceral and Pediatric Surgery University Hospital Düsseldorf, Düsseldorf, Germany.

[4]University of Sydney, Endocrine Surgical Unit and Cancer Genetics, Hormones and Cancer Group , Kolling Institute of Medical Research, Royal North Shore Hospital, Sydney, Australia .

[5]Department of Medicine I, University of Lübeck, University Hospital Schleswig-Holstein, Lübeck, Germany

[6]Department of General, Visceral and Vascular Surgery, University Hospital, University of Halle-Wittenberg, Halle/Saale, Germany. Sweden.

[7]Klinik für Chirurgie und Zentrum für Minimal Invasive Chirurgie, Kliniken Essen-Mitte, Essen, Germany.

[8]Endocrine Section, Department of Medicine, G.V. (Sonny) Montgomery VA Medical Center and University of Mississippi Medical Center, Jackson, USA.

*Corresponding author: Peyman Björklund, Experimental Surgery Group, Uppsala University, Akademiska sjukhuset, ing 70 FOA2, 75185 Uppsala, Sweden. Phone: +46186114911 Fax: +46186114911 Email: peyman.bjorklund@surgsci.uu.se
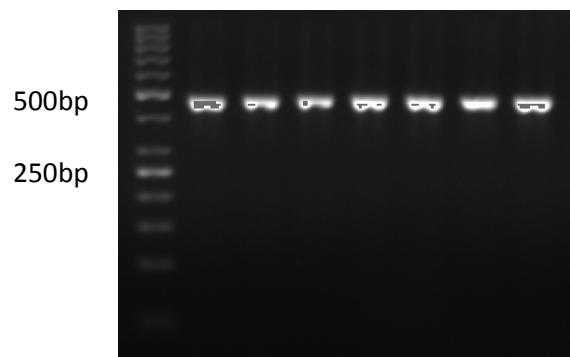
Supplementary Fig. S1. Investigation of large exon 3 *CTNNB1*deletions.

RT-PCR on cDNA from non-mutated APA samples using mRNA specific primers. Expected size of amplicon 464bp.
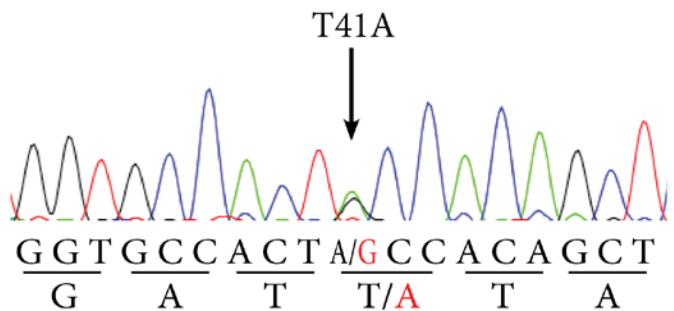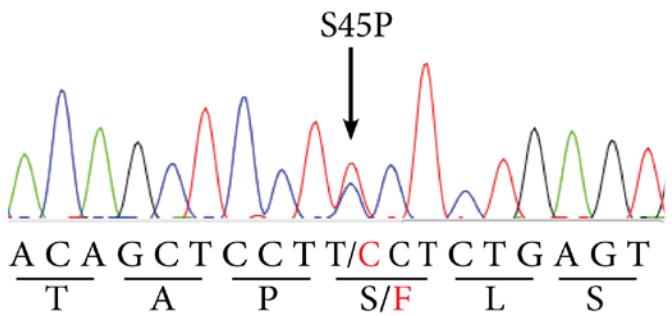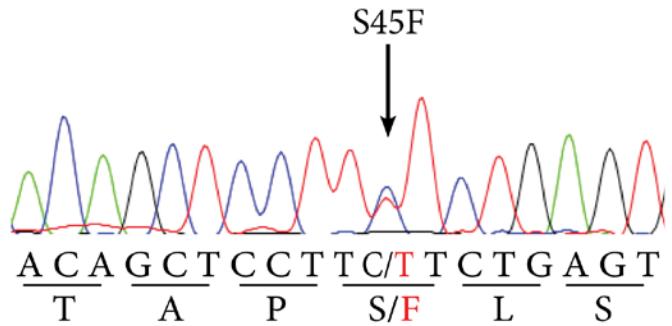
Supplementary Figure S2. Observed missense mutations in *CTNNB1*.

Chromatograms showing the different somatic *CTNNB1* mutations in APAs.
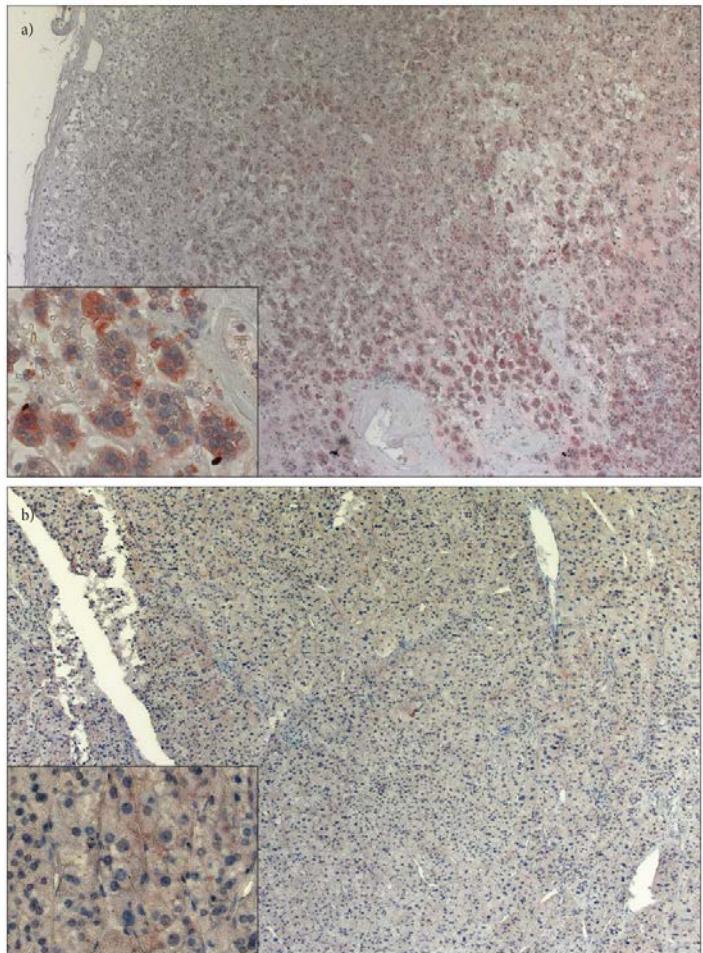
Supplementary Fig. S3. **Immunohistochemical analysis of β-catenin.**

APA with a *KCNJ5* mutation showing an area of ZG-like cells with strong cytoplasmic staining (a). APA with a *KCNJ5* mutation with mostly ZF-like cells and membranous expression (b).
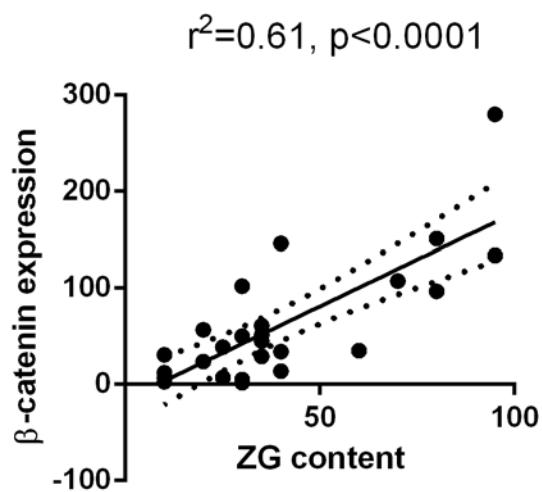
Supplementary Fig. S4. β-catenin expression and ZG-like cell content.

Correlation of ZG-like cell content and β-catenin expression. ZG=Zona Glomerulsosa
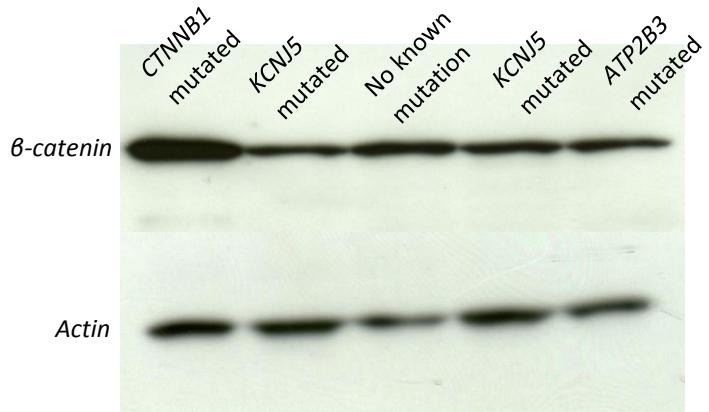
Supplementary Figure S5. **Western blot analysis of antibody used for immunohistochemistry.**

Western blot using a goat polyclonal anti β-catenin in one tumor with *CTNNB1* mutation (p.Ser45Pro) and four without *CTNNB1* mutation. Anti-actin as loading control.

Cohort	No. of APAs	CTNNB1
Sweden	41	3 (7.3%)
Germany	123	5 (4.1%)
Australia	34	2 (5.9%)
Total:	198	10 (5.1%)

Supplementary Table S1. *CTNNB1* **mutations found in different cohorts.**

	T41																S45															
NP_001091679.1 (H. Sapiens)	26-	Q	Q	S	Y	L	-	D	S	G	I	H	S	G	A	T	T	T	A	P	S	L	S	G	K	G	N	P	E	E	E	-55
XP_001138023.1 (P.Troglodytes)	26-	Q	Q	S	Y	L	-	D	S	G	I	H	S	G	A	T	T	T	A	P	S	L	S	G	K	G	N	P	E	E	E	-55
NP_001131124.1 (C. Lupus)	26-	Q	Q	S	Y	L	-	D	S	G	I	H	S	G	A	T	T	T	A	P	S	L	S	G	K	G	N	P	E	E	E	-55
NP_445809.2 (R. Norvegicus)	26-	Q	Q	S	Y	L	-	D	S	G	I	H	S	G	A	T	T	T	A	P	S	L	S	G	K	G	N	P	E	E	E	-55
NP_001159374.1 (M. Musculus)	26-	Q	Q	S	Y	L	-	D	S	G	I	H	S	G	A	T	T	T	A	P	S	L	S	G	K	G	N	P	E	E	E	-55
NP_001069609.1 (B. Taurus)	26-	Q	Q	S	Y	L	-	D	S	G	I	H	S	G	A	T	T	T	A	P	S	L	S	G	K	G	N	P	E	E	E	-55
NP_990412.1 (G. Gallus)	26-	Q	Q	S	Y	L	-	D	S	G	I	H	S	G	A	T	T	T	A	P	S	L	S	G	K	G	N	P	E	E	E	-55
NP_571134.2 (D.Rerio)	26-	Q	Q	S	Y	L	-	D	S	G	I	H	S	G	A	T	T	T	A	P	S	L	S	G	K	G	N	P	E	D	D	-55
XP_309245.4 (A. Gambiae)	36-	Q	N	S	Y	L	G	D	S	G	I	H	S	G	A	V	T	Q	V	P	S	L	S	G	K	-	-	-	D	D	D	-62
NP_996328.1 (D. Melanogaster)	26-	Q	N	S	Y	L	G	D	S	G	I	H	S	G	A	V	T	Q	V	P	S	L	S	G	K	-	-	-	E	D	E	-52

Supplementary Table S2. Conservation of the mutated amino acid residues in *CTNNB1*.

No.	CTNNB1 mutation	β-catenin IHC	CYP11B2 IHC (SCORE)	CYP11B1 IHC (SCORE)	Cell morphology	Gender 1=Male, 0=female	Age at op.	Adenoma size (mm)	Aldosterone pmol/L	Renin	Hypokalemia 1=yes, 0=no	Subtype classification	Postoperative outcome
1	S45P	Cytoplasmic	375	0.67	ZF	0	39	40	1168	<1	1	CT	Normotensive, eukalemic
2	S45P	Cytoplasmic/nuclear	277	0.22	ZG/ZF	0	51	13	790	7.0	1	AVS	na
3	S45P	Cytoplasmic	300	101	ZF	0	51	23	1567	1.0	1	na	na
4	S45F	Cytoplasmic/heterogenous	28.7	291	ZF>ZG	0	26	39	477	2.3	1	na	na
5	T41A	na	na	na	na	1	35	25	2441	4.0	na	CT	na
6	S45P	na	na	na	na	1	66	45	na	na	na	MRI	na
7	S45P	Cytoplasmic/nuclear	30.0	213	ZG>ZF	1	30	10	2011	1.5	1	CT/AVS	Normotensive, eukalemic
8	S45P	Membranous/nuclear	18.3	202	ZG/ZF	1	34	20	2808	2.1	1	CT/AVS	Normotensive, eukalemic
9	S45P	Cytoplasmic/heterogenous	0.97	326	ZF>ZG	0	76	30	2300	1.6	1	CT/AVS	Normotensive, eukalemic
10	S45P	Cytoplasmic/nuclear	244	0.58	ZG>ZF	0	32	15	2520	<1	1	CT/AVS	Normotensive, eukalemic

Supplementary Table S3. Characteristics of patients with *CTNNB1* mutated tumors.

Tumor size was measured either at surgery or by the pathologist. Workup describes the lateralization diagnostics made before surgery. *Plasma renin concentration measured in mU/L. Numbers in parenthesis shows postoperative values. CYP11B2 are graded from 0-400 using a modified H-score. ARR= Aldosterone to renin ratio, AVS=Adrenal vein sampling, CT=Computer tomography. na=not available. ZG=Zona Glomerulosa. ZF=Zona Fasciculata.

Patient no.	Mutation	H-SCORE	ZF %	ZG %
1	Non-mutated	61,11	65	35
2	KCNJ5	23,96	80	20
3	KCNJ5	13,75	60	40
4	Non-mutated	51,39	65	35
5	KCNJ5	50,00	70	30
6	Non-mutated	35,00	40	60
7	KCNJ5	107,43	30	70
8	KCNJ5	4,38	90	10
9	Non-mutated	29,03	65	35
10	KCNJ5	2,67	90	10
11	Non-mutated	45,00	65	35
12	Non-mutated	12,00	90	10
13	KCNJ5	1,94	70	30
14	Non-mutated	30,69	90	10
15	Non-mutated	56,88	80	20
16	Non-mutated	280,00	5	95
17	Non-mutated	7,08	75	25
18	Non-mutated	96,67	20	80
19	Non-mutated	133,68	5	95
20	CTNNB1	38,96	75	25
21	KCNJ5	34,31	60	40
22	KCNJ5	6,67	90	10
23	KCNJ5	102,22	70	30
24	KCNJ5	5,15	70	30
25	CTNNB1	151,04	20	80
26	KCNJ5	146,25	60	40

Supplementary Table S4. **Immunohistochemical analysis of β-catenin in APAs.**

Scoring of expression and amount of ZF-, and ZG like cells. ZF=Zona Fasciculata, ZG=Zona Glomerulosa

	(A) WT (n=84)	(B) *KCNJ5* (n=92)	(C) *CTNNB1* (n=10)	Between groups comparison (A-C) (*P*)	A vs C (*P*)	B vs C (*P*)
Age at operation	53.5 (±7.8)	45.6 (±11.0)	37.0 (26-76)	0.000018	ns	ns
Gender, Female in %	40	83	60	$4.4e^{-8}$	ns*	ns*
Tumor size (mm)	14.7 (±7.9)	18.4 (±6.8)	24 (10-45)	0.000024	0.01	ns
Aldosterone (pmol/L)	1137 (±789)	1377 (±1694)	1567 (477-2520)	ns	ns	ns

Supplementary Table S5. Clinical data analysis.

Values are presented as mean ±SD for parametric data, and median and range for non-parametric data. Statistical analysis was performed by between groups tests (A, B, C) using a Kruskal-Wallis test. Post hoc comparison (A vs C and B vs C) using a multiple comparison corrected Mann-Whitney U-test with p<0.05/4 as cutoff point. *Chi squared test. WT= No mutation in *KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D* or *CTNNB1*.