Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Beuschlein F, Fassnacht M, Assié G, et al. Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome. N Engl J Med 2014;370:1019-28. DOI: 10.1056/NEJMoa1310359

Supplementary Appendix

Supplement to:

Beuschlein F, Fassnacht M, Assie G et al. Constitutive activation of PRKACA in adrenal Cushing's syndrome

CONTENT

SUPPLEMENTARY TEXT

Variant detection

Pools of 12 indexed libraries were sequenced on four lanes to an average depth of coverage between 88x and 160x. Image analysis and base calling was performed using Illumina Real Time Analysis. Reads were aligned against the human assembly hg19 (GRCh37) using Burrows-Wheeler Aligner (BWA v 0.5.9). We performed single-nucleotide variant and small insertion and deletion (indel) calling specifically for the regions targeted by the exome enrichment kit, using SAMtools (v 0.1.18) and custom scripts. Subsequently the variant quality was determined using the SAMtools varFilter script. We used default parameters, with the exception of the maximum read depth (-D) and the minimum P-value for base quality bias (-2), which we set to 9999 and 1e-400, respectively. Additionally, we applied a custom script to mark all variants with adjacent bases of low median base quality. All variants were then annotated using custom Perl scripts. Annotation included information about known transcripts (UCSC Known Genes and RefSeq genes), known variants (dbSNP v 135), type of mutation and - if applicable – amino acid change in the corresponding protein. The annotated variants were then inserted into our in-house database. To discover putative somatic variants, we queried our database to show only those variants of a tumor that were not found in the corresponding control tissue. To reduce false positives we filtered out variants that were already present in our database, had a variant quality of less than 40, or failed one of the filters from the filter scripts. We then manually investigated the raw read data of the remaining variants using the Integrative Genomics Viewer (IGV).

PRKACA sequencing

DNA was amplified using intron-spanning primers for the coding region of the catalytic subunit of PKA. Bidirectional Sanger sequencing was performed using the ABI BigDye Terminator v.3.1 Cycle Sequencing Kit. Primer sequences (*PRKACA*_ex6-7_F, 5'-GTTTCTGACGGCTGGACTG-3'; *PRKACA*_ex6-7_R, 5'-AGTCCACGGCCTTGTTGTAG-3') were designed using the ExonPrimer software as described earlier.[1] For the *PRKACA* gene screening for germline mutations of the patients with bilateral ACTH independent adrenal hyperplasia, intron-spanning primers for the coding region of the catalytic subunit of PKA were designed using the software Primer3Web version 4.0.0 (http://bioinfo.ut.ee/primer3/, sequence on request).

Comparative genomic hybridization and fluorescent in situ hybridization

Array-CGH analysis was performed using 4×180K oligonucleotide commercial arrays (Agilent Technologies, Santa Clara, CA) as previously described[2] and a custom designed 8x60K array was used (Agilent Technologies), covering with an increased resolution the coding region (average probe spacing 100 bp) and the flanking 1 Mb (average probe spacing 500 bp) of the *PRKACA* gene and of 6 other genes encoding for PRKA subunits (*PRKAR1A*, *PRKAR2A*, *PRKAR1B*, *PRKAR2B*, *PRKACB* and *PRKACG*) and their 1 Mb flanking regions (average probe spacing of 500 bp). The arrays were scanned in a dual-laser scanner (DNA Microarray Scanner with Sure Scan High-Resolution Technology, Model G2565CA, Agilent Technologies) and the images were extracted and analyzed through the Agilent Feature Extraction software (v10.5.1.1) and the Genomic Workbench v. 5.0.14 software (Agilent, ADM-2 algorithm with a threshold of 5, respectively. DNA copy number changes were observed as the deviation of the log2ratio value from the value of 0 of at least three consecutive probes. The quality of each experiment was assessed by using a parameter

provided by the Agilent software (QC metric) and on the basis of DNA quality. Copy number changes identified in the samples were compared to the Database of Genomic Variants (http://projects.tcag.ca/variation/) and also visualized by using the UCSC Genome Browser website (http://genome.ucsc.edu/). Included in the deletion interval shared by the four duplications are the full open-reading frames of 12 protein-coding genes *C19orf57* (ID 79173), *CC2D1A* (ID 54862), *PODNL1* (ID 79883), *DCAF15* (ID 90379), *RFX1* (ID 5989), *RLN3* (ID 117579), *IL27RA* (ID 605350), *PALM3* (ID 342979), *C19orf67* (ID 646457), *SAMD1* (ID 90378), *PRKACA* (ID 5566), *ASF1B* (ID 55723). The positions of oligomers refer to the Human Genome assembly (hg19). Detected abnormalities were confirmed by fluorescent in situ hybridization which was performed using commercially available BAC clones specific to the *PRKACA* gene locus and performed as described previously.[2]

Protein homology analysis

The protein sequence alignment for the catalytic subunit of protein kinase A in man (accession no. NP_002721), zebrafish (NP_001076309), acorn worm (XP_002740161) and fruitfly (NP_995672) was performed with the CLC Sequence Viewer 6.5.1 software (CLC Bio A/S, Sweden).

FRET assays

HEK293 cells were cultured in DMEM+10% FCS at 37 °C and in the presence of 5% (vol/vol) CO_2. Cells were plated on 24-mm round glass coverslips and transfected with the indicated amount of DNA and Effectene (Qiagen), following the manufacturer's protocol. Cells were analyzed 24h after transfection in a buffer containing 144 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM HEPES, pH 7.3. FRET imaging was performed as previously described.[3] Equimolar concentrations of a cell-permeable pair of synergistic cAMP analogs (N6-mono-tert.-butylcarbamoyladenosine-cAMP and 5,6-dichloro-1-β-D-

ribofuranosylbenzimidazole-3',5'-cyclic monophosphorothioate Sp-isomer) were used to selectively activate PKA II [4]. HEK293 cells were transfected with the following constructs: 0.2 µg AKAR4-NES PKA sensor, 0.1µg PKA Cα subunit (wild-type or mutant) and 0.8 µg of either RIIβ or empty vector. For co-expression of mutant and wild-type PKA Cα subunits, 0.5µg of each corresponding construct were used. Cells were stimulated with increasing concentrations of cAMP analogs, followed by maximal stimulation of cAMP production with 10µM forskolin and PKA inhibition with 20µM H89. PKA activity was continuously monitored during the course of the experiment by FRET microscopy. Data are normalized to the maximal (i.e. after forskolin) and minimal (i.e. after H89) values.

Real-time PCR

RNA from leukocytes and adrenal tissue was extracted and subjected to a SYBR Green PCR master mix and *PRKACA* cDNA primer pairs (Exon 3-4: 5'-AGGAGACCGGGAACCACTAT-3' and 5'-TTCAGGGTGTGTTCGATCTG-3'. Exon 5-6: 5'-CGGGGAGATGTTCTCACAC-3' and 5'-ATCTGGGCCGCGTAGAAAC-3'. Exon 7-8: 5'-CGGCACCCCTGAGTACCT-3' and 5'-AGTCCACGGCCTTGTTGT-3') to determine the relative quantity of *PRKACA* RNA levels. Actin beta (*ACTB*, forward primer: 5'-CCTAGGCACCAGGGTGTG-3' and reverse primer: 5'-CTTCTCCATGTCGTCCCAGT-3') was used as an endogenous control. The reactions were run in an Applied Biosystems ViiA™ 7 Real-time System using standard parameters. Relative quantification was performed using the $2-\Delta\Delta CT$ method.[5]

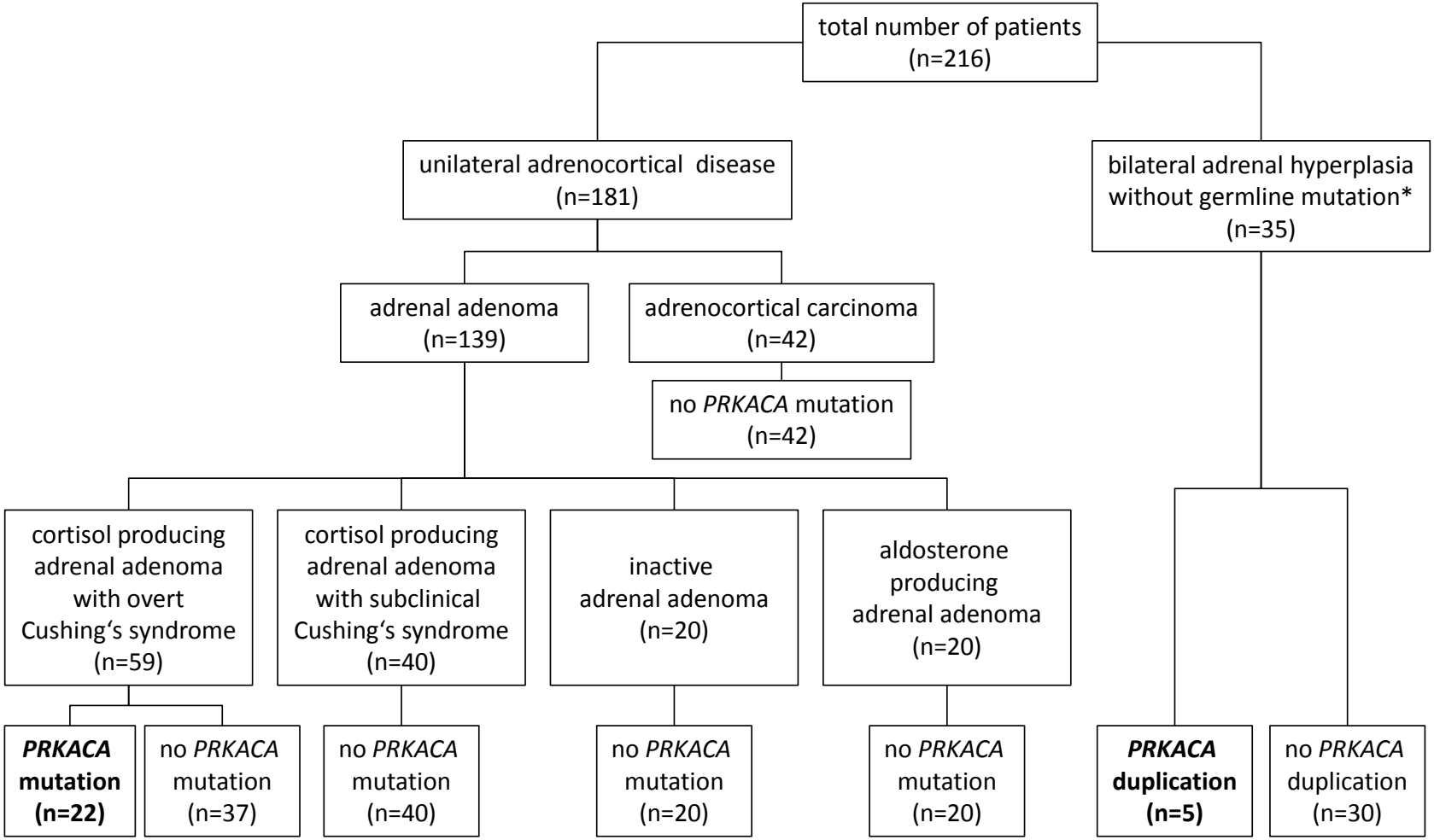
Microarray analysis

Tissue RNA was extracted as described earlier.[6] Microarray experiments were performed using Affymetrix U133 Plus 2.0 chips. The full dataset can be found at Array Express (http://www.ebi.ac.uk/arrayexpress, experiment E-TABM-311). All the transcriptome analyses were carried out using "R" and normalization was performed using the RMA method. For specific expression analysis no correction for multiple testing was implemented. The functional annotation of gene lists was performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7, using Gene Ontology (http://www.geneontology.org) annotations.

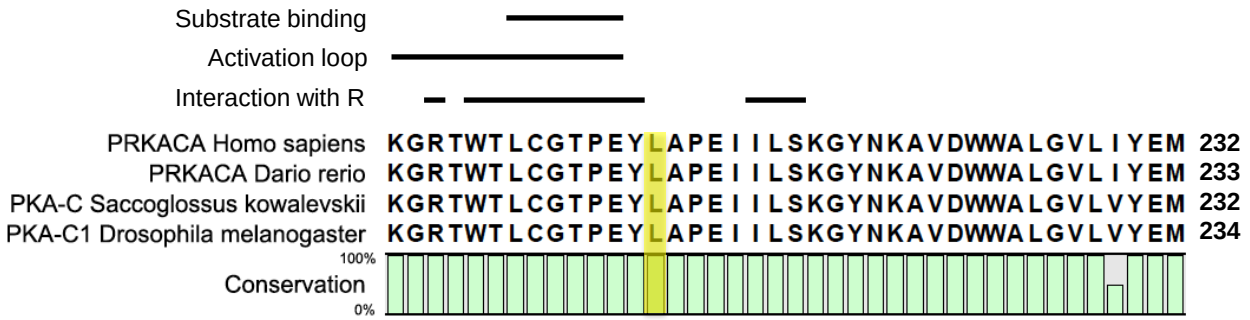
Reference ranges for endocrine parameters

The clinical investigations utilized in this retrospective analysis were performed in different centers and laboratories resulting in some variation between the reference ranges and study protocols. All centers used the same normal range for plasma ACTH (10-50 pg/ml) and for serum cortisol after 1 mg dexamethasone (< 1.8 µg/dl). However, there was significant variation between clinical centers for urinary free cortisol due to assay differences (upper limit of normal for urinary free cortisol, 38-218 µg/24 hours) and on the procedure to assess midnight hypercortisolism (midnight salivary < 1.5ng/ml or midnight serum cortisol < 5µg/dl), respectively. Therefore, for the latter parameters the results were calculated and expressed as fold changes of the upper limit of normal.

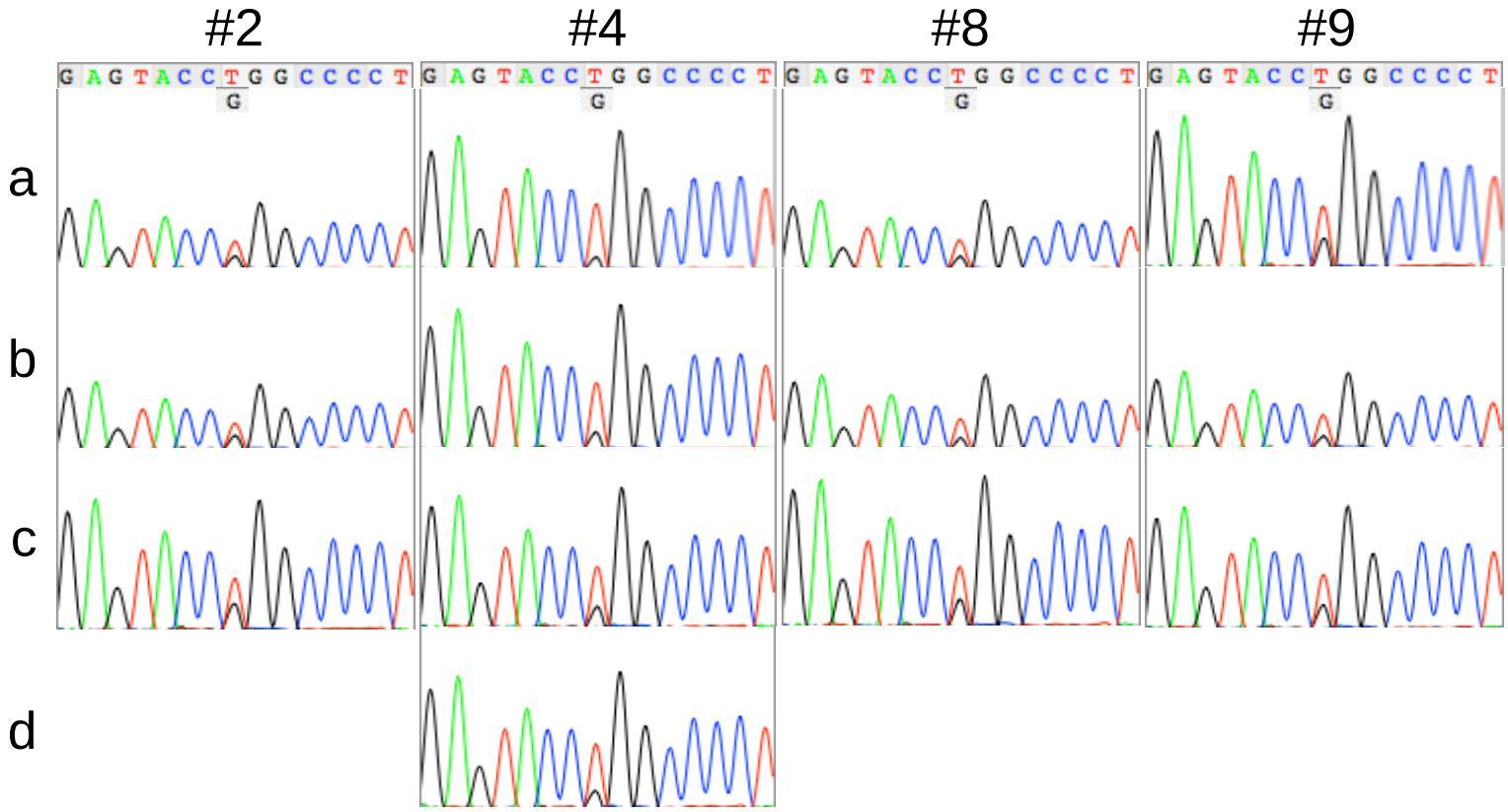
Supplementary Figure S1: Overview on the patient series included in the genetic analysis. (*) Patients with bilateral adrenal hyperplasia and ACTH independent Cushing's syndrome without germline mutations in *PRKAR1A*, *PDE11A* or *PDE8B* or somatic *GNAS* mutations.



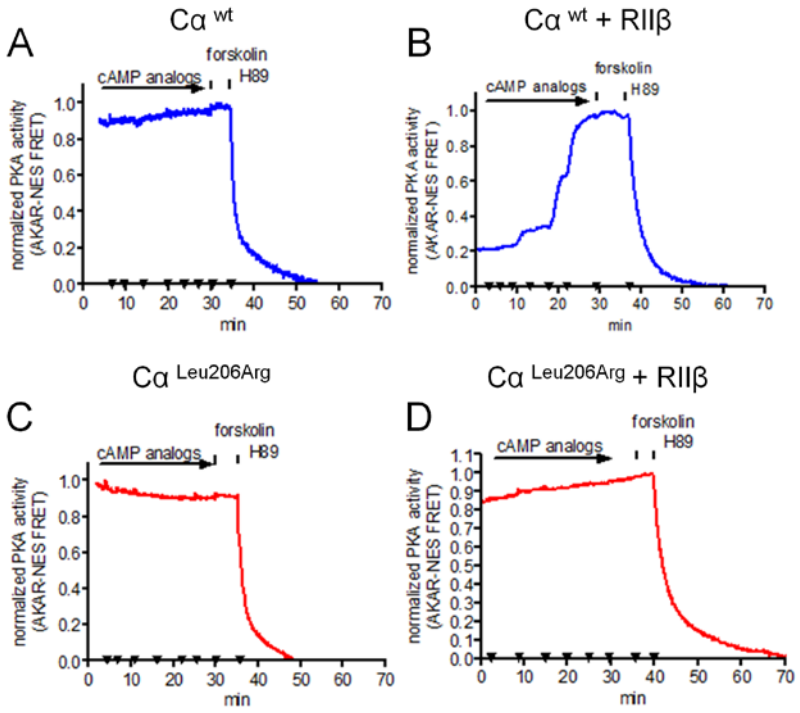
Supplementary Figure S2: Leu206 in PRKACA is directly adjacent to the activation loop (aa. 184-204), a major substrate binding site (aa. 198-204), and a major site of interaction with the regulatory subunits (aa. 197-205), and displays a high level of conservation among species.



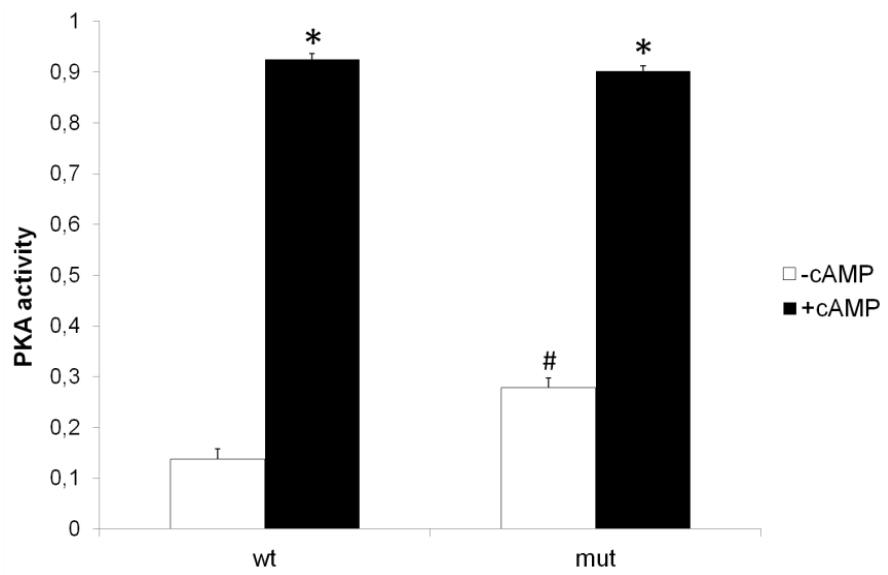
Supplementary Figure S3: To address the question of genetic tumor heterogeneity a subset of adenomas (n=4) carrying the mutation were dissected thereby generating 3-4 fragments per adenoma. As demonstrated by Sanger sequencing in all fragments the mutation was detected at comparable ratios.



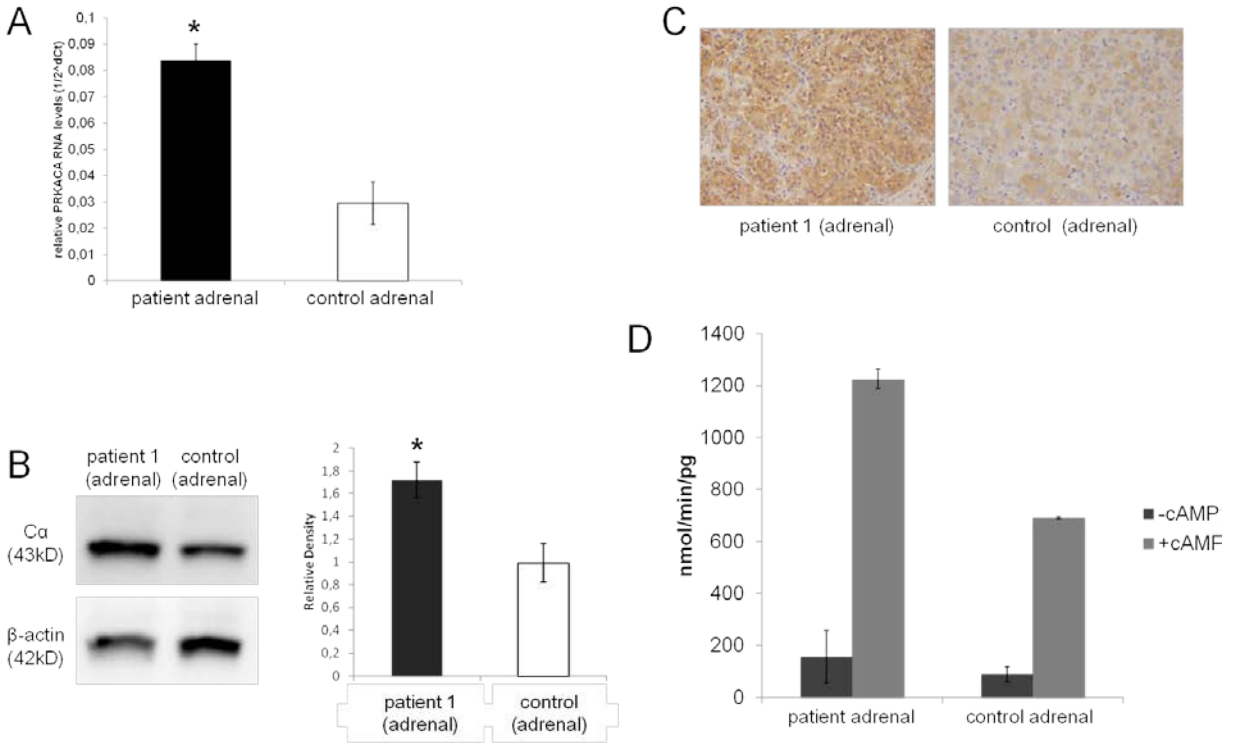
Supplementary Figure S4: HEK293 cells were transfected and stimulated with increasing concentrations of a PKA II-selective pair of cAMP analogs, followed by maximal stimulation of cAMP production with forskolin and PKA inhibition with H89. Each black triangle on the x-axis indicates the addition of cAMP analogs (from 3.175 to 100 μM). PKA activity in cells transfected only with either wild-type *PRKACA* ($C\alpha^{wt}$) or the Leu206Arg mutant ($C\alpha^{Leu206Arg}$) remained high and was not affected by stimulation with cAMP analogs, indicating that both variants were catalytically active. Whereas co-transfection of wild-type RIIβ decreased basal PKA activity and rendered cells transfected with wild-type *PRKACA* responsive to cAMP analogs, this was not observed for the Leu206Arg mutant.



Supplementary Figure S5: (A) PKA enzymatic activity measured in tissue samples from patients with adrenocortical adenomas in dependence of their *PRKACA* mutational status. (*) indicates significant differences between cAMP treated and baseline values while (#) indicates differences of baseline activity between wild type (wt) and *PRKACA* mutated (mut) adenomas.



Supplementary Figure S6: Functional consequences of *PRKACA* gene duplication: Higher adrenal *PRKACA* mRNA expression (A) and PRKACA protein levels (B) as well as more intense immunostaining (C) in an adrenal tumor from a patient with a *PRKACA* duplication (patient 1) in comparison to a cortisol producing tumor from a patient without any known genetic defects. (D) PKA activity quantified in adrenal samples of patients harboring the *PRKACA* duplication in comparison to control adrenals indicating elevated PKA activity under cAMP stimulated conditions.



Supplementary Table S1: Available genetic and molecular investigations for included patient series.

	cortisol producing adrenal adenoma with overt Cushing's syndrome (n=59)*	cortisol producing adrenal adenoma with subclinical Cushing's syndrome (n=40)*	inactive adrenal adenoma (n=20)*	aldosterone producing adrenal adenoma (n=20)*	adrenocortical carcinoma (n=42)*	bilateral adrenal hyperplasia without germline mutation (n=40)*
Exome sequencing (tumor/hyperplasia)	10	-	-	9	-	10
PRKACA sequencing (tumor/hyperplasia)	14	15	4	-	15	-
PRKACA targeted sequencing (tumor/hyperplasia)	37	23	16	11	27	-
SNP analysis (tumor/hyperplasia)[#]	7	9	8	-	22	-
Comparative genomic hybridization (L-DNA)	-	-	-	-	-	35
Micorarray expression analysis[§]	19	11	9	-	-	-

* total number of included patients (as given in Supplementary Figure 1); due to overlapping investigations, numbers per column do not count up to total n. [#] patient series reported in Ronchi et al.[7] [§] patient series partly reported in Wilmot et al. [8]

Supplementary Table S2: Clinical characteristics of the patient group with cortisol producing adrenal adenoma included in exome sequencing

pat. #	sex	age at diagnosis (years)	tumor size (mm)	ACTH (pg/ml)	midnight salivary cortisol (ng/ml)	midnight serum cortisol (µg/dl)	serum cortisol after 1mg Dex (µg/dl)	24h urinary cortisol (µg/d)	*PRKACA* mutation
#1	f	43	25	<5.0	6.1	-	15.9	-	p.Leu206Arg
#2	f	47	30	<5.0	21.0	-	21.0	799	p.Leu206Arg
#3	f	48	21	-	4.0	-	14.8	288	p.Leu199_Cys200insTrp
#4	f	37	24	<5.0	5.8	-	12.6	447	p.Leu206Arg
#5	f	42	21	<5.0	-	-	23.0	-	p.Leu206Arg
#6	m	70	110	5.0	-	9.5	11.4	-	none
#7	f	55	50	8.1	-	13.7	15.7	-	none
#8	f	29	35	5.0	-	-	39.0	1772	p.Leu206Arg
#9	f	41	35	<5.0	-	-	22.4	418	p.Leu206Arg
#10	f	42	30	<5.0	-	22.6	17.2	172	p.Leu206Arg

Dex, dexamethasone. To convert ACTH from pg/ml to pmol/l multiply by 0.22, salivary cortisol from ng/ml to µmol/l by 0.0028, serum cortisol from µg/dl to µmol/l by 0.028 and urinary cortisol from µg/d to nmol/d by 2.76, respectively. For reference ranges of the endocrine parameters see Supplementary Text above (page 6).

Supplementary Table S3: Overview on mutations found in individual tumor samples upon exome sequencing

								# of reads from tumor			# of reads from blood	
Pat.#	**Type**	**Genomic position**	**Transcript**	**cDNA level**	**Protein level**	**Gene symbol**	**Effect on protein**	**Ref. allele**	**Non-ref. allele**	**% of all reads**	**Ref. allele**	**Non-ref. allele**
#1	missense	chr10:g.127967523C>T	NM_003474.4	c.221G>A	p.Arg74Gln	ADAM12	Arg74Gln	29	12	29.27%	57	1
	missense	chr19:g.14208416A>C	NM_002730.3	c.617T>G	p.Leu206Arg	**PRKACA**	Leu206Arg	220	98	30.82%	283	0
	missense	chr2:g.207615789G>C	NM_001039845.1	c.921C>G	p.Asp307Glu	MDH1B	Asp307Glu	72	27	27.27%	158	1
	missense	chr4:g.57182589C>T	NM_020722.1	c.2921C>T	p.Ala974Val	KIAA1211	Ala974Val	156	87	35.80%	235	0
#2	missense	chr1:g.93659282C>A	NM_206886.3	c.679C>A	p.Gln227Lys	CCDC18	Gln227Lys	3	3	50.00%	9	0
	missense	chr1:g.165621232C>A	NM_004528.3	c.209C>A	p.Pro70His	MGST3	Pro70His	99	21	17.50%	114	0
	missense	chr10:g.61413447C>A	NM_194298.2	c.1337G>T	p.Gly446Val	SLC16A9	Gly446Val	61	24	28.24%	136	0
	missense	chr12:g.6708983G>A	NM_001273.2	c.1438C>T	p.Leu480Phe	CHD4	Leu480Phe	156	102	39.53%	263	2
	missense	chr16:g.150437T>C	NM_001039476.1	c.163A>G	p.Lys55Glu	NPRL3	Lys55Glu	31	16	34.04%	32	0
	indel	chr16:g.58030765_58030767delAGA	NM_020807.1	c.1403_1405delTCT	p.Phe468del	ZNF319	Phe468del	133	26	16.35%	149	0
	missense	chr19:g.14043654C>T	NM_001146254.1	c.1397G>A	p.Gly466Asp	PODNL1	Gly466Asp	78	20	20.41%	91	0
	missense	chr19:g.14208416A>C	NM_002730.3	c.617T>G	p.Leu206Arg	**PRKACA**	Leu206Arg	253	136	34.96%	445	2
	missense	chr19:g.17306250C>G	NM_001130065.1	c.4014C>G	p.His1338Gln	MYO9B	His1338Gln	16	8	33.33%	14	0
	indel	chr21:g.43309330_43309332dup	NM_199050.2	c.1527_1529dup	p.Lys510dup	C2CD2	Lys510dup	122	22	15.28%	101	0
	missense	chr22:g.50962431C>T	NM_001169109.1	c.410G>A	p.Cys137Tyr	SCO2	Cys137Tyr	169	53	23.87%	271	1
	nonsense	chrX:g.110654004G>A	NM_000555.3	c.199C>T	p.Gln67*	DCX	Gln67*	70	26	27.08%	109	1
	missense	chr1:g.16069136C>A	NM_001013641.1	c.82C>A	p.Leu28Met	TMEM82	Leu28Met	491	122	19.90%	683	1
	missense	chrX:g.37850357G>A	NM_012274.1	c.265G>A	p.Val89Met	CXorf27	Val89Met	162	31	16.06%	189	0
#3	missense	chr1:g.164768954A>G	NM_001204961.1	c.529A>G	p.Thr177Ala	PBX1	Thr177Ala	108	26	19.40%	108	0
	missense	chr12:g.99060032A>G	NM_001160.2	c.1226A>G	p.Asn409Ser	APAF1	Asn409Ser	78	23	22.77%	143	0
	splice	chr13:g.113750862A>T	NM_001112732.2	c.3189-2A>T		MCF2L		57	20	25.97%	50	1
	missense	chr16:g.57490503C>T	NM_020312.3	c.466C>T	p.Arg156Trp	COQ9	Arg156Trp	93	42	31.11%	137	0
	frameshift	chr19:g.17514614_17514614delT	NM_004335.2	c.433delA	p.Ser145Alafs*2	BST2	Ser145Alafs*2	142	27	15.98%	156	0
	missense	chr20:g.20033207G>T	NM_016652.4	c.263C>A	p.Ser88Tyr	CRNKL1	Ser88Tyr	122	58	32.22%	142	0
	missense	chr20:g.48156124C>A	NM_000961.3	c.656G>T	p.Arg219Leu	PTGIS	Arg219Leu	47	22	31.88%	54	0

	missense	chr21:g.47961713C>T	NM_015151.3	c.2081C>T	p.Ser694Leu	DIP2A	Ser694Leu	48	17	26.15%	67	0
	missense	chr4:g.96044999A>G	NM_001203.2	c.388A>G	p.Ile130Val	BMPR1B	Ile130Val	19	12	38.71%	85	0
	missense	chr6:g.76782198A>G	NM_001563.2	c.8T>C	p.Leu3Ser	IMPG1	Leu3Ser	37	20	35.09%	107	0
	missense	chrX:g.46359311A>T	NM_001039891.2	c.1713T>A	p.His571Gln	ZNF674	His571Gln	91	43	32.09%	228	0
	indel	chr19:g.14208434_14208436dup	NM_002730.3	c.597_599dup	p.Leu199_Cys200insTrp	**PRKACA**	Leu199_Cys200insTrp	368	79	17.67%	315	1
#4	missense	chr1:g.53556345C>T	NM_006671.4	c.1165G>A	p.Ala389Thr	SLC1A7	Ala389Thr	70	15	17.65%	74	1
	missense	chr2:g.120979606G>A	NM_024121.2	c.947C>T	p.Pro316Leu	TMEM185B	Pro316Leu	53	15	22.06%	74	0
	missense	chr19:g.14208416A>C	NM_002730.3	c.617T>G	p.Leu206Arg	**PRKACA**	Leu206Arg	224	59	20.85%	302	0
	indel	chr1:g.52827213_52827215delTCC	NM_032449.2	c.288_290delGGA	p.Glu97del	CC2D1B	Glu97del	63	7	10.00%	78	0
	missense	chr19:g.14208416A>C	NM_002730.3	c.617T>G	p.Leu206Arg	**PRKACA**	Leu206Arg	181	66	26.72%	288	0
#5	indel	chr19:g.33792755_33792757delGGC	NM_004364.3	c.564_566delGCC	p.Pro189del	CEBPA	Pro189del	25	3	10.71%	23	0
	missense	chr8:g.56436103_56436104delTGinsAA	NM_052898.1	c.1270_1271delTGinsAA	p.Cys424Asn	XKR4	Cys424Asn	164	74	31.09%	220	0
	frameshift	chr1:g.114510495_114510505delCCCATGTTGTC	NM_152696.3	c.2489_2499delCCCATGTTGTC	p.Ala830Glufs*14	HIPK1	Ala830Glufs*14	308	35	10.20%	409	0
	nonsense	chr1:g.23395062C>T	NM_001009999.2	c.1210C>T	p.Gln404*	KDM1A	Gln404*	73	13	15.12%	182	0
	missense	chr19:g.994242G>A	NM_024100.3	c.1198G>A	p.Val400Ile	WDR18	Val400Ile	155	56	26.54%	132	0
	missense	chr19:g.8807973C>A	NM_178525.3	c.1079G>T	p.Arg360Leu	ACTL9	Arg360Leu	77	25	24.51%	82	1
#6	nonsense	chr22:g.42422922G>T	NM_152613.2	c.667G>T	p.Gly223*	WBP2NL	Gly223*	172	51	22.87%	212	0
	missense	chr5:g.140237750C>A	NM_018901.2	c.2117C>A	p.Ser706Tyr	PCDHA10	Ser706Tyr	79	91	53.53%	169	0
	missense	chr6:g.74207609G>T	NM_001123226.1	c.2027G>T	p.Arg676Leu	MTO1	Arg676Leu	46	14	23.33%	78	0
	missense	chr6:g.90385235C>T	NM_014611.1	c.12709G>A	p.Val4237Ile	MDN1	Val4237Ile	261	108	29.27%	327	1
	missense	chr8:g.113358347T>C	NM_052900.2	c.6109A>G	p.Ile2037Val	CSMD3	Ile2037Val	43	10	18.87%	50	0
	frameshift	chr12:g.49434924_49434925delGG	NM_003482.3	c.6628_6629delCC	p.Pro2210Aspfs*32	MLL2	Pro2210Aspfs*32	49	11	18.33%	52	0
#7	missense	chr21:g.38309183C>T	NM_000411.6	c.562G>A	p.Asp188Asn	HLCS	Asp188Asn	146	49	25.13%	226	0
	missense	chr4:g.39257509T>G	NM_025132.3	c.3043T>G	p.Leu1015Val	WDR19	Leu1015Val	108	23	17.56%	183	1
	missense	chr11:g.63413986G>A	NM_015459.3	c.611C>T	p.Pro204Leu	ATL3	Pro204Leu	44	9	16.98%	68	0
	missense	chr17:g.7144186A>C	NM_007278.1	c.341T>G	p.Val114Gly	GABARAP	Val114Gly	36	9	20.00%	43	0
#8	missense	chr19:g.14208416A>C	NM_002730.3	c.617T>G	p.Leu206Arg	**PRKACA**	Leu206Arg	261	103	28.30%	207	0
	missense	chr3:g.118865593C>A	NM_152539.2	c.557C>A	p.Ser186Tyr	C3orf30	Ser186Tyr	92	15	14.02%	95	0
	indel	chr4:g.25032262_25032264delCAG	NM_018176.3	c.52_54delCTG	p.Leu18del	LGI2	Leu18del	22	3	12.00%	15	0

#9	frameshift	chr1:g.157068544delG	NM_001004341.2	c.440delC	p.Pro147Leufs*32	ETV3L	Pro147Leufs*32	15	8	34.78%	13	0
	missense	chr11:g.85361319A>C	NM_032273.3	c.20A>C	p.Asn7Thr	TMEM126A	Asn7Thr	19	5	20.83%	27	0
	missense	chr15:g.83936926T>C	NM_001717.3	c.158A>G	p.His53Arg	BNC1	His53Arg	48	21	30.43%	82	1
	missense	chr19:g.14208416A>C	NM_002730.3	c.617T>G	p.Leu206Arg	**PRKACA**	Leu206Arg	172	72	29.51%	245	0
	missense	chr8:g.11301874C>T	NM_053279.2	c.47G>A	p.Gly16Glu	FAM167A	Gly16Glu	145	68	31.92%	219	0
	missense	chrX:g.119070617C>T	NM_024528.3	c.496G>A	p.Glu166Lys	NKAP	Glu166Lys	70	41	36.94%	111	0
	missense	chr19:g.21216347G>T	NM_025189.3	c.182G>T	p.Arg61Ile	ZNF430	Arg61Ile	13	4	23.53%	24	0
#10	missense	chr19:g.14208416A>C	NM_002730.3	c.617T>G	p.Leu206Arg	**PRKACA**	Leu206Arg	285	107	27.30%	233	0

Supplementary Table S4: Clinical characteristics of the patient series with bilateral ACTH independent adrenal hyperplasia included in the genetic analysis

pat. #	sex	disease*	age at diagnosis (years)	catabolic Cushing symptoms (yes/no)	ACTH (pg/ml)	midnight salivary cortisol (ng/ml)	midnight serum cortisol (µg/dl)	serum cortisol after 1mg Dex (µg/dl)	24h urinary cortisol (µg/d)	*PRKACA* mutation	*PRKACA* duplication
1	M	PPNAD	9	yes	4. 7		35. 3	28. 5	740	no	**yes**
2	M	PPNAD	3	yes	<5		29. 4	23. 6	704	no	**yes**
3[%]	M	PPNAD	23	yes	<5			28		no	**yes**
4[%]	F	PPNAD	39	yes	<10			25	206	no	**yes**
5	M	AIMAH	3	yes	7. 2	157		21. 9		no	**yes**
6	F	PPNAD / acromegaly	41	yes	1		27	36	1134	no	no
7	F	PPNAD	35	yes	4. 7	3. 18	19. 8	19. 1	984	no	no
8	F	PPNAD	41	yes						no	no
9	F	PPNAD	9	yes	11		7. 2		975	no	no
10	M	PPNAD	2	yes	<5					no	no
11	M	PPNAD	20	yes	<5.0		23. 5	28. 2	598	no	no
12	F	PPNAD	47	yes	<5					no	no
13	F	PPNAD	46	yes	<5.0				1638	no	no
14	F	PPNAD	1	yes	5. 7		24	28	836	no	no
15	F	PPNAD	5	yes	5. 8		6		775	no	no
16	F	PPNAD	3	yes	6. 9				879	no	no
17	M	PPNAD	7	yes					1072	no	no
18	M	PPNAD	13	yes	<5.0		19. 9	19. 9	350	no	no
19	F	PPNAD	12	yes	13. 7		10. 4		661	no	no
20	F	PPNAD	9	yes	<5.0			21		no	no
21	M	PPNAD	7	yes	<10		24		100	no	no
22	F	PPNAD / thyroid cancer	68	yes						no	no
23	F	PPNAD	15	yes	<5.0			26. 6		no	no

24	F	PPNAD	12	yes	<2	17. 7	18. 3	1450	no	no
25	F	PPNAD	22	yes	<5.0		>5	elevated	no	no
26	F	iMAD	1	yes	<5				no	no
27	F	PPNAD	7	yes	<5	22.3		135	no	no
28	F	PPNAD	19	yes	<5				no	no
29	?	AIMAH	60		<5				no	no
30	F	PPNAD	14	yes	<5				no	no
31	M	iMAD	13	yes	5. 4	17	11	4200	no	no
32	F	PPNAD	10	yes	<5	16.6		391	no	no
33	M	PPNAD	11	yes	<5.0	25. 3	5. 43	4245	no	no
34	F	PPNAD	30	yes	<5.0	26. 6	26. 6	elevated	no	no
35	F	PPNAD	27	yes	4	<1		1136	no	no

*PPNAD, primary pigmented nodular adrenal disease; iMAD, isolated micronodular adrenocortical disease; AIMAH, ACTH independent macronodular adrenal hyperplasia; Dex, dexamethasone

[%] indicates mother and son. For reference ranges of the endocrine parameters see Supplementary Text above (page 6).

Supplementary Table S5: Expression analysis of adrenal adenomas dependent on their mutational status (*PRKACA* mutated vs. non-mutated)

Expression analysis of adenomas independent of functional status (n=39)[#]

gene symbol	probe set ID	no *PRKACA* mutation (n=31)*	*PRKACA* mutation (n=8)*	p†
MC2R	208568	7.03±1.05	8.65±0.52	**<0.001**
CYP11B1	1552493_s	10.94±0.51	10.71±0.20	0.05
StAR	204548	12.33±0.27	12.79±0.14	**<0.001**
CYP21A2	214622	10.94±1.42	12.31±0.34	**<0.001**
HSD3B2	206294	12.21±0.45	12.95±0.15	**<0.001**
CYP11A1	204309	10.97±0.63	11.95±0.26	**<0.001**

Expression analysis of adenomas associated with overt Cushing's syndrome (n=19)

gene symbol	probe set ID	no *PRKACA* mutation (n=11)*	*PRKACA* mutation (n=8)*	p†
MC2R	208568	7.34±1.29	8.65±0.52	**0.009**
CYP11B1	1552493_s	10.71±0.68	10.71±0.20	0.99
StAR	204548	12.34±0.36	12.79±0.14	**0.002**
CYP21A2	214622	10.40±2.26	12.31±0.34	**0.02**
HSD3B2	206294	12.41±0.42	12.95±0.15	**0.001**
CYP11A1	204309	11.29±0.67	11.95±0.26	**0.01**

n indicates the number of subjects within each group; [#] complete group consisting of inactive adenomas (n=9), and adenomas associated with subclinical Cushing's syndrome (n=11) and overt Cushing's syndrome (n=19);* values indicate mean±SD; † values between adenomas of the respective groups were compared using t-test.

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REFERENCES

1. Beuschlein F, Boulkroun S, Osswald A, et al. Somatic mutations in ATP1A1 and ATP2B3 lead to aldosterone-producing adenomas and secondary hypertension. Nat Genet 2013;doi: 10.1038/ng.2550.
2. Vetro A, Manolakos E, Petersen MB, et al. Unexpected results in the constitution of small supernumerary marker chromosomes. European journal of medical genetics 2012;55:185-90.
3. Calebiro D, Nikolaev VO, Gagliani MC, et al. Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. PLoS Biol 2009;7:e1000172.
4. Calebiro D, de Filippis T, Lucchi S, et al. Selective modulation of protein kinase A I and II reveals distinct roles in thyroid cell gene expression and growth. Molecular endocrinology 2006;20:3196-211.
5. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.
6. Vincent-Dejean C, Cazabat L, Groussin L, et al. Identification of a clinically homogenous subgroup of benign cortisol-secreting adrenocortical tumors characterized by alterations of the protein kinase A (PKA) subunits and high PKA activity. Eur J Endocrinol 2008;158:829-39.
7. Ronchi CL, Sbiera S, Leich E, et al. Single nucleotide polymorphism array profiling of adrenocortical tumors - evidence for an adenoma carcinoma sequence? PLoS One 2013;8:e73959.
8. Wilmot Roussel H, Vezzosi D, Rizk-Rabin M, et al. Identification of gene expression profiles associated with cortisol secretion in adrenocortical adenomas. J Clin Endocrinol Metab 2013;98:E1109-21.