Supplemental appendix

For the manuscript entitled "Activating PRKACB somatic mutation in cortisol producing adenomas" by Stéphanie Espiard, Matthias J. Knape, Kerstin Bathon, Guillaume Assié, Marthe Rizk-Rabin, Simon Faillot, Windy Luscap-Rondof, Daniel Abid, Laurence Guignat, Davide Calebiro, Friedrich W. Herberg, Constantine A. Stratakis, Jérôme Bertherat

Supplemental Methods

Genetic testing

Exome DNA was captured using the SureSelectXT Human All Exon version 4 Kit (Agilent), following the manufacturer's protocol, and then sequenced on a pair of SOLiD 5500xl flowchips (12 lanes) as pair-end sequencing reads (75 plus 35 bp) on a 5500 SOLiD sequencer (Life Technologies). Colour space reads were mapped to the GRCh37/hg19 reference genome using LifeScope software version 2.5.1 (Life Technologies). Variants were identified using GATK version 2.1 (Genome Analysis Toolkit, Broad Institute) along with Picard tools version 1.77 or LifeScope version 2.5.1 (Life Technologies) and annotated with ANNOVAR version 2012Mar08 (supplemental table 1).

DNA constructs

For PKA activity experiments, the wild-type and mutant DNA constructs were obtained from Blue Heron. The *PRKACB* sequence wild-type (WT) (NM_002731) and mutant were introduced into the pCMV6-AC-DDK expression vector containing a C-terminal DDK-tag (Blue Heron PS100005). The same coding sequence (WT) was cloned into the pGFP-C3 (N-terminal GFP tag, Perkin Elmer) and pET30 vector (Novagen). The S54L mutation was introduced by site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene) with specific primer pairs (5'-GGAACAGGTTTATTTGGAAGAG-3'). Successful subcloning and mutagenesis was verified by Sanger sequencing. For the holoenzyme formation experiment using SPR the sequence encoding the human regulatory (R) subunit RIα of PKA was introduced into the expression vector pGEX-KG (N-terminal GST tag).

Cell culture

Cell lines were obtained from American Type Culture Collection (ATCC). Human embryonic kidney cells HEK293 cells were cultured in DMEM GlutaMax medium containing 10% fetal bovine serum. Mediums were supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL).

Bioluminescence Resonance Energy Transfer (BRET) assay were performed in HEK293 cells (DSMZ, Braunschweig, Germany) co-transfected with plasmids (pGFP-C and pRluc8-N) encoding the human PKA catalytic (C) isoforms GFP-Cββ1, GFP-Cβ1S54L and the human PKA regulatory isoform RIα-Rluc8, RIβ-Rluc8, RIIα-Rluc8, or RIIβ-Rluc8 respectively, allowing the analysis of defined PKA holoenzymes (1). HEK293 cells were seeded in 96-well Nunc Nunclon plates (Thermo Scientific) at a density of $2x10^4$cells/well and cultured in DMEM with high glucose (GE Healthcare) supplemented with 10% fetal calf serum gold (PAA Laboratories) at 37 °C and 5% CO_2. The following day, cells were transfected.

For the *in vitro* PKA activity assay, HEK293 cells were seeded in 6-well plates at a density of $0.25x10^6$cells/well and allowed to grow for 24 h, before transfection with Effectene (Qiagen) according to the manufacturer´s instructions. Since protein expression of S54L-Cβ was lower *in cellulae* than the WT-Cβ, we increased the transfected amount of the catalytic subunit vectors while keeping the regulatory subunit constant to obtain comparable Cβ expression.

Expression, purification of recombinant PKA-Cβ1 and GST-PKA-RIα

Recombinant expression of PKA-C in *E. coli* and purification by IP20 affinity chromatography was performed as described earlier (2, 3). For this, pET30-PKA-Cβ1 (encoding the human PKA catalytic subunit β1) was transformed and expressed in *E. coli* BL21(DE3). For expression of PKA-Cβ1 S54L, pET30-PKA-Cβ1 S54L was co-transformed with the plasmid pGEX-KG-PDK1 coding for the putative activator kinase of PKA, 3-phosphoinositide-dependent protein kinase-1 (PDK1). (Auto)phosphorylation of the activation loop and of the turn motif was verified using site-specific antibodies.

Human PKA-RIα (non-tagged and GST-tagged) was expressed in *E. coli* BL21(DE3) RIL cells and purified using a cAMP resin (Sp-8-AEA-cAMPS agarose, A008, Biolog) to obtain cAMP-free R subunit as described earlier (4).

Immunohistochemistry

Sections were deparaffinized in xylene and rehydrated through 100% ethanol. For antigen unmasking, the slides were incubated in antigen unmasking solution (Vector) a citrate buffer pH6 for 20 min at 99° C in a microwave (Biocare medical,Eurobio). Endogenous peroxidases were inhibited by incubation in 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) in water for 5 min. Slides were incubated with 10% normal goat serum (life technologies) for 30min to block non specific binding sites, than primary antibodies PRKACB (1:500) (sc-904 Santa Cruz®) and PRKACA (610981 BD Biosciences®) (1:50) were incubated overnight at 4° C. Sections were washed than using Vectastain ABC working solutions (Vector) sections were incubated with biotinylated second conjugated antibodies for 1H, and than vectastain ABC reagent for 30min. Slides were washed and antigen were revealed with diaminobenzidin (peroxydase substrate Kit DAB ,Vector) for 5-10min and counterstained with hematoxilin (Sigma®). Slides were scanned by Lamina (Perkin –Elmer) and viewed by Panoramic Viewer software.

References

1. Schwede F et al. Rp-cAMPS Prodrugs Reveal the cAMP Dependence of First-Phase Glucose-Stimulated Insulin Secretion. *Mol. Endocrinol. Baltim. Md* 2015;vol. 29(no. 7):p. 988-1005.

2. Knape MJ et al. Divalent Metal Ions Mg^{2+} and Ca^{2+} Have Distinct Effects on Protein Kinase A Activity and Regulation. *ACS Chem. Biol.* 2015;vol. 10(no. 10):p. 2303-2315.

3. Olsen SR, Uhler MD. Affinity purification of the C alpha and C beta isoforms of the catalytic subunit of cAMP-dependent protein kinase. *J. Biol. Chem.* 1989;vol. 264(no. 31):p. 18662-18666.

4. Bertinetti D et al. Chemical tools selectively target components of the PKA system. *BMC Chem. Biol.* 2009;vol. 9:p. 3.

Supplemental table 1: variants list found after filtering in the 6 CPA

Sample	Gene	Transcript	Function	cDNA level	Protein level	ExAC_Freq	Depth	% Var
Ad1	PRKACB	NM_002731	missense	c.C161T	p.S54L	NA	49	27
Ad1	ARHGAP5	NM_001030055	missense	c.G1439A	p.R480Q	8.237e-06	70	16
Ad1	CABIN1	NM_001201429	missense	c.A1142G	p.D381G	NA	64	33
Ad1	E2F5	NM_001083588	nonsense	c.C323G	p.S108X	NA	13	23
Ad1	HCFC1	NM_005334	missense	c.G98C	p.R33P	NA	13	31
Ad1	ITIH4	NM_001166449	missense	c.C2108G	p.S703C	NA	12	25
Ad1	ITIH4	NM_001166449	missense	c.C2098A	p.Q700K	NA	8	38
Ad1	KIF1A	NM_001244008	missense	c.G2720A	p.G907E	NA	18	28
Ad1	KIR2DL1	NM_014218	missense	c.G16A	p.V6I	4.2e-05	29	10
Ad1	MICAL1	NM_001159291	missense	c.T2254G	p.S752A	NA	13	54
Ad1	MTRNR2L8	NM_001190702	missense	c.C35T	p.S12L	NA	29	38
Ad1	NCOR2	NM_001077261	missense	c.C6712A	p.Q2238K	NA	17	24
Ad1	NCOR2	NM_001077261	missense	c.A6711C	p.E2237D	NA	17	24
Ad1	RFWD2	NM_001286644	missense	c.A329G	p.N110S	NA	61	31
Ad1	USP15	NM_006313	missense	c.G2723C	p.S908T	NA	152	19
Ad2	CPT1B	NM_001145137	missense	c.G49A	p.D17N	NA	21	19
Ad2	CTDSP2	NM_005730	missense	c.C9A	p.H3Q	NA	9	33
Ad2	DCAF16	NM_017741	missense	c.A457T	p.S153C	NA	233	15
Ad2	FAM179B	NM_015091	missense	c.T4596G	p.F1532L	NA	18	28
Ad2	MTRNR2L8	NM_001190702	missense	c.C35T	p.S12L	NA	24	25
Ad2	ORMDL3	NM_139280	missense	c.A157C	p.N53H	NA	35	20
Ad2	PEX26	NM_001127649	missense	c.C32T	p.P11L	9.453e-05	21	43
Ad2	PRDM11	NM_001256695	missense	c.T284G	p.F95C	NA	24	13
Ad2	PRKRIP1	NM_024653	missense	c.A308C	p.Q103P	NA	23	26
Ad2	SLCO5A1	NM_001146008	missense	c.G245C	p.S82T	8.236e-06	34	29
Ad2	SOX13	NM_005686	missense	c.G1094T	p.S365I	NA	112	35
Ad2	TPRX1	NM_198479	missense	c.A643T	p.I215F	NA	20	20
Ad2	TPSAB1	NM_003294	missense	c.C412T	p.H138Y	NA	13	54
Ad2	ZNF333	NM_032433	frameshift	c.1702_1785del	p.568_595del	NA	229	14
Ad3	BSN	NM_003458	missense	c.A7292T	p.Q2431L	NA	29	10
Ad3	CCDC79	NM_001136505	missense	c.G1705T	p.D569Y	NA	12	33
Ad3	CCDC85B	NM_006848	missense	c.G521C	p.G174A	NA	4	75
Ad3	COLGALT1	NM_024656	missense	c.T1214C	p.L405P	NA	109	10
Ad3	DNMT3B	NM_001207056	missense	c.T2077C	p.C693R	NA	155	25
Ad3	DYNLT1	NM_001291602	missense	c.G130A	p.G44R	NA	135	30
Ad3	EGFL7	NM_201446	missense	c.G622A	p.D208N	0.000009	8	50
Ad3	FOLH1		splicing			NA	19	21
Ad3	GNAS	NM_000516	missense	c.C601T	p.R201C	8.236e-06	73	27
Ad3	NFRKB	NM_006165	missense	c.T385C	p.F129L	NA	35	17
Ad3	PKD2L1	NM_001253837	missense	c.G334A	p.D112N	NA	17	47
Ad3	POLR3D	NM_001722	missense	c.A421C	p.K141Q	NA	34	18
Ad3	SAFB	NM_001201340	missense	c.C1985A	p.A662D	NA	154	37
Ad3	SKIDA1	NM_207371	missense	c.G1286A	p.G429E	NA	18	28
Ad3	SORL1	NM_003105	missense	c.A4795G	p.I1599V	NA	207	14
Ad3	SYCP1	NM_001282542	missense	c.C2512A	p.P838T	NA	47	17
Ad3	SYCP1	NM_001282542	missense	c.C2513A	p.P838Q	NA	44	16
Ad3	TMEM132D	NM_133448	missense	c.C2273T	p.A758V	3.295e-05	189	24
Ad3	WDR66	NM_001178003	missense	c.G184A	p.G62R	NA	23	52

Ad3	WDR66	NM_001178003	missense	c.G185A	p.G62E	1.668e-05	24	58
Ad3	ZNF695	NM_020394	missense	c.G1421A	p.G474D	NA	40	13
Ad3	ZNF695	NM_020394	missense	c.G1420A	p.G474S	NA	38	16
Ad3	ZNF709	NM_152601	missense	c.G1395A	p.M465I	NA	39	10
Ad4	CCDC84	NM_198489	missense	c.T386C	p.F129S	NA	34	18
Ad4	CDH9	NM_016279	missense	c.C469G	p.P157A	NA	13	62
Ad4	CDH9	NM_016279	missense	c.G466C	p.E156Q	NA	12	50
Ad4	CGN	NM_020770	missense	c.A2597G	p.K866R	NA	30	23
Ad4	EP300	NM_001429	missense	c.A4391T	p.Q1464L	NA	211	15
Ad4	EP300	NM_001429	missense	c.A4394G	p.E1465G	NA	213	17
Ad4	FOXR1	NM_181721	missense	c.G718C	p.G240R	NA	209	22
Ad4	FOXR1	NM_181721	missense	c.G719C	p.G240A	NA	197	23
Ad4	MPHOSPH10	NM_005791	missense	c.T1889C	p.F630S	NA	16	31
Ad4	MTRNR2L8	NM_001190702	missense	c.C35T	p.S12L	NA	26	35
Ad4	PRKACA	NM_002730	missense	c.T617G	p.L206R	NA	120	23
Ad4	SLC25A11	NM_001165418	missense	c.T81G	p.F27L	NA	16	38
Ad4	TLK1	NM_001136555	missense	c.T1842A	p.F614L	NA	10	30
Ad4	TLK1	NM_001136555	missense	c.T1841A	p.F614Y	NA	11	27
Ad4	TLK1	NM_001136555	missense	c.T1840A	p.F614I	NA	11	27
Ad4	ZBTB21	NM_020727	missense	c.A65C	p.E22A	NA	231	23
Ad5	CDC42EP4	NM_012121	missense	c.C278T	p.T93I	NA	10	30
Ad5	CFAP97		splicing			NA	18	22
Ad5	CLASP2	NM_001207044	missense	c.A2295T	p.R765S	NA	10	30
Ad5	DPF1	NM_004647	missense	c.T856C	p.S286P	NA	20	15
Ad5	GNAS	NM_000516	missense	c.C601T	p.R201C	8.236e-06	61	38
Ad5	IFNGR1	NM_000416	missense	c.T221A	p.I74N	NA	11	27
Ad5	KIAA0754	NM_015038	missense	c.G3841A	p.A1281T	NA	8	38
Ad5	LRRC16B	NM_138360	missense	c.G2939C	p.R980P	NA	7	43
Ad5	LYPD2	NM_205545	frameshift	c.170_178del	p.57_60del	NA	21	24
Ad5	P3H1	NM_001146289	missense	c.T1355C	p.L452P	NA	24	46
Ad5	POLR3D	NM_001722	missense	c.A421C	p.K141Q	NA	20	35
Ad5	PPAPDC3	NM_032728	missense	c.G575C	p.G192A	NA	16	44
Ad5	RTKN2	NM_001282941	nonsense	c.C283T	p.R95X	8.238e-06	76	28
Ad5	SLC25A2	NM_031947	missense	c.C44G	p.A15G	NA	37	24
Ad5	SLC52A2	NM_001253815	missense	c.A116G	p.K39R	3.296e-05	13	23
Ad5	SMARCC2	NM_003075	frameshift	c.3579_3617del	p.1193_1206del	NA	15	33
Ad5	SRP72	NM_001267722	missense	c.A1487G	p.K496R	NA	13	23
Ad5	STRN3	NM_001083893	missense	c.A167G	p.E56G	NA	4	75
Ad5	TNKS1BP1	NM_033396	missense	c.G4337C	p.R1446P	NA	14	21
Ad5	TXNDC2	NM_001098529	missense	c.C895A	p.L299I	1.648e-05	8	38
Ad5	ZNF385D		splicing			NA	40	30
Ad5	ZNF721	NM_133474	missense	c.C1793T	p.T598I	NA	22	32
Ad5	ZNF721	NM_133474	missense	c.A1790C	p.Y597S	NA	24	42
Ad5	ZNF721	NM_133474	missense	c.C1786T	p.R596W	0.0001569	24	50
Ad5	ZSCAN25	NM_145115	missense	c.C1339G	p.Q447E	NA	14	29
Ad6	ATXN7L2	NM_153340	missense	c.C1000T	p.R334C	NA	216	31
Ad6	CCSER1	NM_001145065	missense	c.G2173T	p.G725C	NA	20	35
Ad6	CFAP70	NM_145170	missense	c.A431G	p.Q144R	NA	214	30
Ad6	CPA2	NM_001869	missense	c.T80C	p.V27A	NA	9	33
Ad6	CRY1	NM_004075	missense	c.G58C	p.A20P	NA	18	28
Ad6	CTAGE1	NM_172241	missense	c.A1256C	p.Q419P	NA	34	18

Ad6	CTAGE1	NM_172241	missense	c.C1255A	p.Q419K	NA	33	18
Ad6	CTNNB1	NM_001098209	missense	c.T133C	p.S45P	NA	207	29
Ad6	ELAC2	NM_001165962	missense	c.A1868G	p.Y623C	NA	142	20
Ad6	GHRL	NM_001134944	missense	c.G67A	p.V23I	3.926e-05	218	22
Ad6	GTF3C1	NM_001286242	missense	c.A1232G	p.K411R	4.942e-05	166	29
Ad6	H2AFZ	NM_002106	missense	c.G254A	p.R85H	NA	77	30
Ad6	LTN1	NM_015565	missense	c.A4268C	p.E1423A	NA	228	26
Ad6	MUC12	NM_001164462	missense	c.C12217T	p.L4073F	NA	17	41
Ad6	NPEPL1	NM_024663	missense	c.C852G	p.C284W	NA	32	38
Ad6	PBRM1	NM_018313	frameshift	c.1334delT	p.L445X	NA	25	20
Ad6	PDE8B	NM_001029851	nonsense	c.C2206T	p.Q736X	NA	178	25
Ad6	RAD17		splicing			NA	38	16
Ad6	SGK2	NM_016276	missense	c.A337G	p.K113E	NA	156	28
Ad6	SLC25A46		splicing			NA	20	30
Ad6	SLC9A3	NM_001284351	missense	c.G2461A	p.E821K	NA	13	38
Ad6	SLC9A3	NM_001284351	missense	c.C2459T	p.P820L	NA	17	29
Ad6	SLC9A3	NM_001284351	missense	c.C2458T	p.P820S	NA	16	31
Ad6	SUSD4		splicing			NA	18	50
Ad6	SYN1	NM_006950	missense	c.C1328T	p.P443L	NA	17	41
Ad6	TMF1	NM_007114	missense	c.A3046G	p.I1016V	NA	38	26
Ad6	TNRC6C	NM_001142640	missense	c.G1288T	p.G430W	NA	120	33
Ad6	TPST2	NM_001008566	missense	c.G750T	p.K250N	NA	199	36
Ad6	USP25	NM_001283041	missense	c.T1088G	p.F363C	NA	38	47
Ad6	XIRP1	NM_001198621	missense	c.A893T	p.E298V	NA	107	34
Ad6	ZFR	NM_016107	missense	c.G196T	p.V66F	NA	54	35

Supplemental table 2: *PRKACB* primers

Exons	Primers sequence: forward (above), reverse (below)	PCR product size	annealing temperature
Exon 1	GAAGATACAGTCGGGCCAGG	476	63
	CCTCAGAAGCTGCGACCC		
Exon 2	TCCTTACACCTGAAAACAGCTC	669	58
	AGTATAACTATGGCAAAATTCCC		
Exon 3	AGCATTTATAATTCCCATAGTGTT	226	56
	CCCTGAAGGTAAATAACAACAA		
Exon 4	ACCGCAATTACTTATGCACCA	368	56
	TCTTCAGGCTTATCATCTGGT		
Exon 5	TCCCAGACTAGTTTGTGGCA	350	58
	TCTGGTTAAACAACACAAGCA		
Exon 6	TCCCCATAAATTTATCAGAGCGT	517	60
	AACAAGTTCCTTTCATAACAGGA		
Exon 7	CTTTTCTTTGAGTTGAGGAAGGA	361	60
	AGGGATGCATGTCTCATATTTCT		
Exon 8	TCTGTCCTGAATAGCTGCTCA	481	56
	CAGAAATTGCTTTACTGTTTTGT		
Exon 9	TCATGAGTCTTAGAATGTGTGTTT	290	56
	AAGTTGAAAATATCTCCACCACA		
Exon 10	GCTAACCTACTGCTTGCTG	384	60
	CTGCTTCAACAAGGACGGTC		