

Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome.

Felix Beuschlein, Martin Fassnacht, Guillaume Assié, Davide Calebiro, Constantine Stratakis, Andrea Osswald, Cristina Ronchi, Thomas Wieland, Silviu Sbiera, Fabio Faucz, et al.

► **To cite this version:**

Felix Beuschlein, Martin Fassnacht, Guillaume Assié, Davide Calebiro, Constantine Stratakis, et al.. Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome.. New England Journal of Medicine, Massachusetts Medical Society, 2014, 370 (11), pp.1019-28. <10.1056/NEJ-Moa1310359>. <inserm-01068048>

HAL Id: inserm-01068048

<http://www.hal.inserm.fr/inserm-01068048>

Submitted on 24 Sep 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

ORIGINAL ARTICLE

Constitutive Activation of PKA Catalytic Subunit in Adrenal Cushing's Syndrome

Felix Beuschlein, M.D., Martin Fassnacht, M.D., Guillaume Assié, M.D., Ph.D., Davide Calebiro, M.D., Ph.D., Constantine A. Stratakis, M.D., D.Sc., Andrea Osswald, M.D., Cristina L. Ronchi, M.D., Ph.D., Thomas Wieland, M.Sc., Silviu Sbiera, Ph.D., Fabio R. Faucz, Ph.D., Katrin Schaak, Ph.D., Anett Schmittfull, M.S., Thomas Schwarzmayr, M.Sc., Olivia Barreau, M.D., Ph.D., Delphine Vezzosi, M.D., Ph.D., Marthe Rizk-Rabin, Ph.D., Ulrike Zabel, Ph.D., Eva Szarek, Ph.D., Paraskevi Salpea, Ph.D., Antonella Forlino, Ph.D., Annalisa Vetro, Ph.D., Orsetta Zuffardi, Ph.D., Caroline Kisker, Ph.D., Susanne Diener, M.Sc., Thomas Meitinger, M.D., Martin J. Lohse, M.D., Martin Reincke, M.D., Jérôme Bertherat, M.D., Ph.D., Tim M. Strom, M.D., and Bruno Allolio, M.D.

ABSTRACT

BACKGROUND

Corticotropin-independent Cushing's syndrome is caused by tumors or hyperplasia of the adrenal cortex. The molecular pathogenesis of cortisol-producing adrenal adenomas is not well understood.

METHODS

We performed exome sequencing of tumor-tissue specimens from 10 patients with cortisol-producing adrenal adenomas and evaluated recurrent mutations in candidate genes in an additional 171 patients with adrenocortical tumors. We also performed genomewide copy-number analysis in 35 patients with cortisol-secreting bilateral adrenal hyperplasias. We studied the effects of these genetic defects both clinically and in vitro.

RESULTS

Exome sequencing revealed somatic mutations in *PRKACA*, which encodes the catalytic subunit of cyclic AMP-dependent protein kinase (protein kinase A [PKA]), in 8 of 10 adenomas (c.617A→C in 7 and c.595_596insCAC in 1). Overall, *PRKACA* somatic mutations were identified in 22 of 59 unilateral adenomas (37%) from patients with overt Cushing's syndrome; these mutations were not detectable in 40 patients with subclinical hypercortisolism or in 82 patients with other adrenal tumors. Among 35 patients with cortisol-producing hyperplasias, 5 (including 2 first-degree relatives) carried a germline copy-number gain (duplication) of the genomic region on chromosome 19 that includes *PRKACA*. In vitro studies showed impaired inhibition of both PKA catalytic subunit mutants by the PKA regulatory subunit, whereas cells from patients with germline chromosomal gains showed increased protein levels of the PKA catalytic subunit; in both instances, basal PKA activity was increased.

CONCLUSIONS

Genetic alterations of the catalytic subunit of PKA were found to be associated with human disease. Germline duplications of this gene resulted in bilateral adrenal hyperplasias, whereas somatic *PRKACA* mutations resulted in unilateral cortisol-producing adrenal adenomas. (Funded by the European Commission Seventh Framework Program and others.)

The authors' affiliations are listed in the Appendix. Address reprint requests to Dr. Fassnacht at the Department of Medicine I, University Hospital Würzburg, Oberdürrbacherstr. 6, 97080 Würzburg, Germany, or at fassnacht_m@ukw.de.

Drs. Beuschlein, Fassnacht, Assié, Calebiro, and Stratakis contributed equally to this article.

This article was published on February 26, 2014, at NEJM.org.

N Engl J Med 2014;370:1019-28.

DOI: 10.1056/NEJMoa1310359

Copyright © 2014 Massachusetts Medical Society.

ENDOGENOUS HYPERCORTISOLISM, REFERRED to as Cushing's syndrome, is associated with substantial morbidity and mortality.¹ When Cushing's syndrome is severe, patients have catabolic symptoms such as muscle weakness, skin fragility, osteoporosis, and severe metabolic sequelae.² Hypersecretion of cortisol can be driven by an excess of pituitary or ectopic corticotropin or can be due to adrenocortical tumors or hyperplasias with corticotropin-independent cortisol production. Adrenal adenomas are common, with a prevalence of at least 3% among persons older than 50 years of age.³ Whereas only a subset of these tumors is associated with overt Cushing's syndrome, some degree of cortisol excess is present, depending on the diagnostic criteria applied,⁴ in up to 47% of patients with adrenal adenomas and is associated with a range of phenotypes, from hypertension to the metabolic syndrome and osteoporosis.⁵

The molecular pathogenesis of cortisol-producing adrenal adenomas is not well understood. Whereas somatic mutations in the gene encoding beta-catenin (*CTNNB1*) have been found primarily in nonsecreting adrenocortical adenomas,⁶ there is some evidence that increased endocrine activity may be linked to aberrant cyclic AMP (cAMP) signaling.^{7,8} For instance, ectopic expression of G-protein-coupled receptors for neuroendocrine hormones or neurotransmitters that mediate their effects through cAMP has been implicated in syndromes such as food-dependent hypercortisolism and related conditions^{9,10} that are caused by cortisol-producing adenomas or bilateral hyperplasias of the adrenal cortex. Moreover, somatic mutations in the gene encoding the α subunit of the stimulatory G protein (*GNAS1*) cause adenomas or hyperplasias leading to Cushing's syndrome in patients with McCune-Albright syndrome¹¹ or macronodular hyperplasia.^{12,13} Finally, mutations in the genes encoding the cAMP-degrading phosphodiesterase 11A (*PDE11A*)¹⁴ and phosphodiesterase 8B (*PDE8B*)¹⁵ and in the gene encoding the regulatory subunit of the cAMP-dependent protein kinase (protein kinase A [PKA]) (*PRKARIA*)^{16,17} have been identified in patients with Cushing's syndrome due to primary pigmented nodular adrenocortical disease and in a small number of cortisol-producing adrenal adenomas. These genetic alterations, however, explain only a small fraction of cases. The observation that a subset of adrenal adenomas is characterized by abnormal PKA activity, despite the absence of muta-

tions in these candidate genes,¹⁸ suggests yet unknown alterations in the cAMP-PKA signaling cascade in these tumors.

METHODS

STUDY PATIENTS AND DNA EXTRACTION

Patients were recruited at three centers that participate in the European Network for the Study of Adrenal Tumors and at the U.S. National Institutes of Health. We evaluated 139 patients with adrenal adenoma, 42 patients with adrenocortical carcinoma, and 35 patients with corticotropin-independent bilateral adrenal hyperplasia who did not have germline mutations in *PRKARIA*, *PDE11A*, or *PDE8B* or somatic *GNAS* mutations (33 with micronodular hyperplasia [31 with primary pigmented nodular adrenocortical disease and 2 with isolated micronodular adrenocortical disease] and 2 with macronodular hyperplasia) (Fig. S1 and Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). In all cases, the diagnosis was histologically confirmed after surgical resection. All the patients gave written informed consent, and the study was approved by the ethics committee at each participating institution.

The diagnosis of corticotropin-independent Cushing's syndrome was based on a combination of biochemical hallmarks of hypercortisolism — elevated urinary excretion of free cortisol, increased late-night salivary or serum cortisol levels, suppressed plasma corticotropin levels (<10 pg per milliliter [<2.2 pmol per liter]), and nonsuppressible serum cortisol levels (>5 μ g per deciliter [>138 nmol per liter]) after the administration of 1 mg of dexamethasone — as well as on the presence of catabolic signs of hypercortisolism. Patients were classified as having overt Cushing's syndrome if they had at least three abnormal biochemical test results or if they had typical catabolic features (i.e., muscle weakness, skin fragility, and osteoporosis) plus at least two abnormal biochemical test results. Patients were considered to have endocrine-inactive adrenal lesions if they had normal biochemical test results and no catabolic signs. All patients who had no catabolic signs but had at least one abnormal result in the abovementioned tests were classified as having subclinical Cushing's syndrome.

DNA was extracted as described previously¹⁹⁻²¹ from unilateral adrenocortical tumors in 181 patients and from corresponding normal tissue

in 26 of these patients. Furthermore, germline DNA was available for all 35 patients with bilateral hyperplasia, and DNA from adrenal-tissue samples obtained during surgery was available for 10 of these 35 patients.

EXOME AND *PRKACA* SEQUENCING

Exomes were enriched in solution and indexed with the use of the SureSelect XT Human All Exon 50Mb kit, version 4 (Agilent Technologies). Sequencing was performed as paired-end reads of 100 bp on HiSeq2000 systems (Illumina). Pools of 12 indexed libraries were sequenced on four lanes. Image analysis and base calling were performed with the use of Real-Time Analysis software (Illumina). Methods of variant detection and *PRKACA* sequencing are described in the Supplementary Appendix.

COMPARATIVE GENOMIC HYBRIDIZATION

Array-based comparative genomic hybridization analysis was performed with the use of commercial arrays (Agilent Technologies), according to the manufacturer's instructions and as described previously.²² Technical details are provided in the Supplementary Appendix.

IN SILICO ANALYSIS OF HUMAN MUTATIONS

Structural images were prepared with the use of PyMOL software (www.pymol.org). The structure of the mouse full-length tetrameric RII β (2):C α (2) holoenzyme²³ (Protein Data Bank entry 3TNP) was used to display the structures of the PKA catalytic subunit (C α) and regulatory subunit (RII β).

DNA CONSTRUCTS AND SITE-DIRECTED MUTAGENESIS

Plasmids encoding nonmutant human RII β or C α subunits were purchased from OriGene Technologies. The *PRKACA*-containing plasmid was used for site-directed mutagenesis, with the c.617A→C and the c.595_596insCAC mutation introduced with the use of the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies), according to the manufacturer's protocol. The mutation was confirmed by means of sequencing.

QUANTIFICATION OF PKA ACTIVITY IN INTACT CELLS

Human embryonic kidney 293 cells were transfected with the AKAR4-NES (a protein activity reporter 4 with a nuclear export signal) sensor²⁴ so that PKA activity could be monitored by means of fluorescence resonance energy transfer (FRET) imaging. Transfection and FRET imaging were

performed as described previously.²⁵ Equimolar concentrations of a cell-permeable pair of synergistic cAMP analogues were used to activate PKA II.²⁶

QUANTIFICATION OF *PRKACA* PROTEIN AND PKA ENZYMATIC ACTIVITY

Whole-cell or tissue lysates were studied for PKA C α subunit expression by means of Western blotting with the use of a specific antibody (sc-903, Santa Cruz Biotechnology). COS-7 cells were transfected with the use of the X-tremeGENE HP DNA Transfection Reagent (Roche) and 500 ng of plasmid DNA per well for 24 hours. For transfections including both PKA C α (nonmutant or Leu206Arg variant) and RII β subunits, a molar ratio of 1:8 was used. In lysed cells from the transfection experiments or patient-derived cells, PKA activity was determined by means of an enzymatic assay (Enzo Life Sciences).

GENE-EXPRESSION MICROARRAY ANALYSIS AND REAL-TIME POLYMERASE-CHAIN-REACTION (PCR) ANALYSIS

An earlier microarray analysis of 22 adenomas²⁷ was expanded to include 39 adenomas in the current study (see Table S1 in the Supplementary Appendix). For quantification of *PRKACA* expression, real-time quantitative PCR analysis was used. Details of the microarray experiments and real-time PCR analysis are provided in the Supplementary Appendix.

STATISTICAL ANALYSIS

Data were compared between two groups with the use of the Mann–Whitney U test and among three groups with the use of the Kruskal–Wallis test. All comparisons were two-sided, and P values of less than 0.05 were considered to indicate statistical significance. The analyses were performed with the use of SPSS software, version 20 (IBM).

RESULTS

SOMATIC *PRKACA* MUTATIONS AND GERMLINE DUPLICATIONS IN CORTISOL-PRODUCING LESIONS

Exome sequencing was performed in samples from 10 patients with unilateral cortisol-producing adenomas and overt Cushing's syndrome (Table S2 in the Supplementary Appendix) and revealed a low number of somatic mutations per adenoma (median, 5; range, 1 to 14) (Table S3 in the Supplementary Appendix). Within this small set of genetic alterations, somatic variants in *PRKACA*, encoding the PKA C α subunit, were found in

8 of 10 tumors (c.617A→C, p.Leu206Arg in 7 and c.595_596insCAC, Leu199_Cys200insTrp in 1). The affected amino acids were highly conserved across a variety of species (Fig. S2 in the Supplementary Appendix).

On the basis of these initial results, *PRKACA* was sequenced in 129 additional adenomas (including 89 cortisol-producing, 20 aldosterone-producing, and 20 inactive adenomas). In 33 of these samples, the entire coding sequence was investigated, and in the remaining 96 samples, sequencing of hot-spot regions was performed. The Leu206Arg variant was identified in 14 of these 129 adenomas, and all 14 patients with this variant also had overt Cushing's syndrome, according to the predefined criteria. Whole-exome and targeted sequencing indicated that both the nonmutated and mutated alleles were present in tumor tissue, consistent with a heterozygous state of *PRKACA* mutations (Fig. 1A and 1B, and Fig. S3 in the Supplementary Appendix). In the affected patients, there were no *PRKACA* mutations in DNA derived from leukocytes (19 patients) or fat tissue (1 patient) or in adjacent normal adrenal tissue (6 patients).

Comparative genomic hybridization of samples from 35 patients with cortisol-secreting bilateral adrenal hyperplasias and overt Cushing's syndrome identified 5 patients (4 kindreds) with copy-number gains (duplications) of the genomic region on chromosome 19p that includes *PRKACA* (Fig. 1C, and Table S4 in the Supplementary Appendix). In one case, the defect was inherited: a mother and son, both carriers of the same *PRKACA* duplication, were affected by bilateral macronodular hyperplasia. In another case, that of a 3-year-old boy with Cushing's syndrome due to bilateral micronodular hyperplasia, the defect was *de novo*, because neither parent carried the *PRKACA* duplication. No amplification of *PRKACA* was found in 24 cortisol-producing adrenal adenomas analyzed by means of single-nucleotide polymorphism array profiling.²⁸

No *PRKACA* mutations were detected in 1600 in-house exomes or in the 1000 Genomes Project data set (version 0.0.14). Although *PRKACA* duplications are reported in public databases of copy-number variants in at least six instances (occurring in patients referred for genetic testing because of developmental delay), no *PRKACA* whole-gene duplications are included in the Database of Genomic

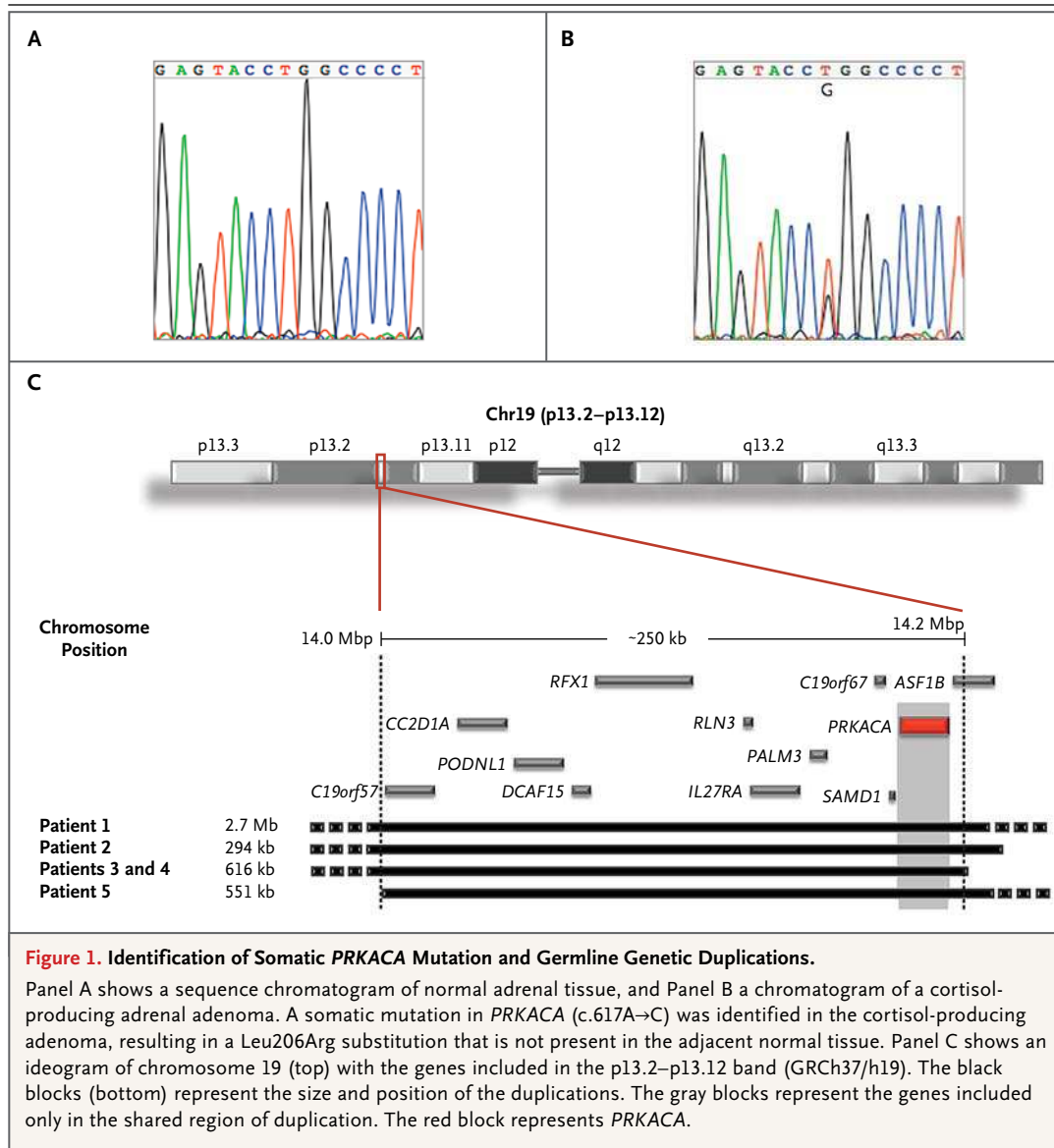
Variants, which is based on the general population. Moreover, no *PRKACA* duplications were found in 2000 persons with intellectual disability, congenital malformations, or both in an internal database.

PRKACA MUTATIONS AND REGULATION OF CATALYTIC SUBUNITS BY REGULATORY SUBUNITS

Analysis of the mouse full-length tetrameric RIIβ(2):Cα(2) holoenzyme structure²³ revealed that this mutation is located in the highly conserved core of the interaction between the regulatory (RIIβ) and catalytic (Cα) subunits of PKA — a finding that supports a functional relevance of the Leu206Arg variant. Leu206 is part of the active-site cleft of the catalytic subunit to which the inhibitory sequence of the regulatory subunit binds, mimicking a substrate for the catalytic subunit. This interaction keeps the catalytic subunit inactive in the absence of cAMP. Exchanging Leu206 with the bulky and positively charged amino acid Arg *in silico* yields steric hindrance between the side chain of the mutated Arg206 in the Cα subunit and Val115 and Tyr228 in the RIIβ subunit (Fig. 2A and 2B).²⁹

The functional consequences of the two detected variants (Leu206Arg and Leu199_Cys200insTrp) were investigated in intact cells by means of FRET microscopy with the use of a sensor for PKA activity (AKAR4-NES).²⁴ The PKA activity in cells transfected only with either nonmutant Cα or the variants was high and was not further stimulated by cAMP analogues, indicating preservation of the catalytic activity in the mutants (Fig. 2C, and Fig. S4A and S4C in the Supplementary Appendix). However, after cotransfection with excess nonmutant RIIβ, basal PKA activity was decreased in cells transfected with nonmutant Cα and became responsive to cAMP analogues, whereas PKA activity in the cells transfected with the mutants remained high and was not responsive to cAMP analogues (Fig. 2C, and Fig. S4B and S4D in the Supplementary Appendix). This finding indicates that the mutations made the catalytic subunit resistant to the physiologic suppression by the regulatory subunit. The lack of suppression remained when an equal amount of nonmutant Cα was cotransfected, indicating a dominant effect of the mutations (Fig. 2D).

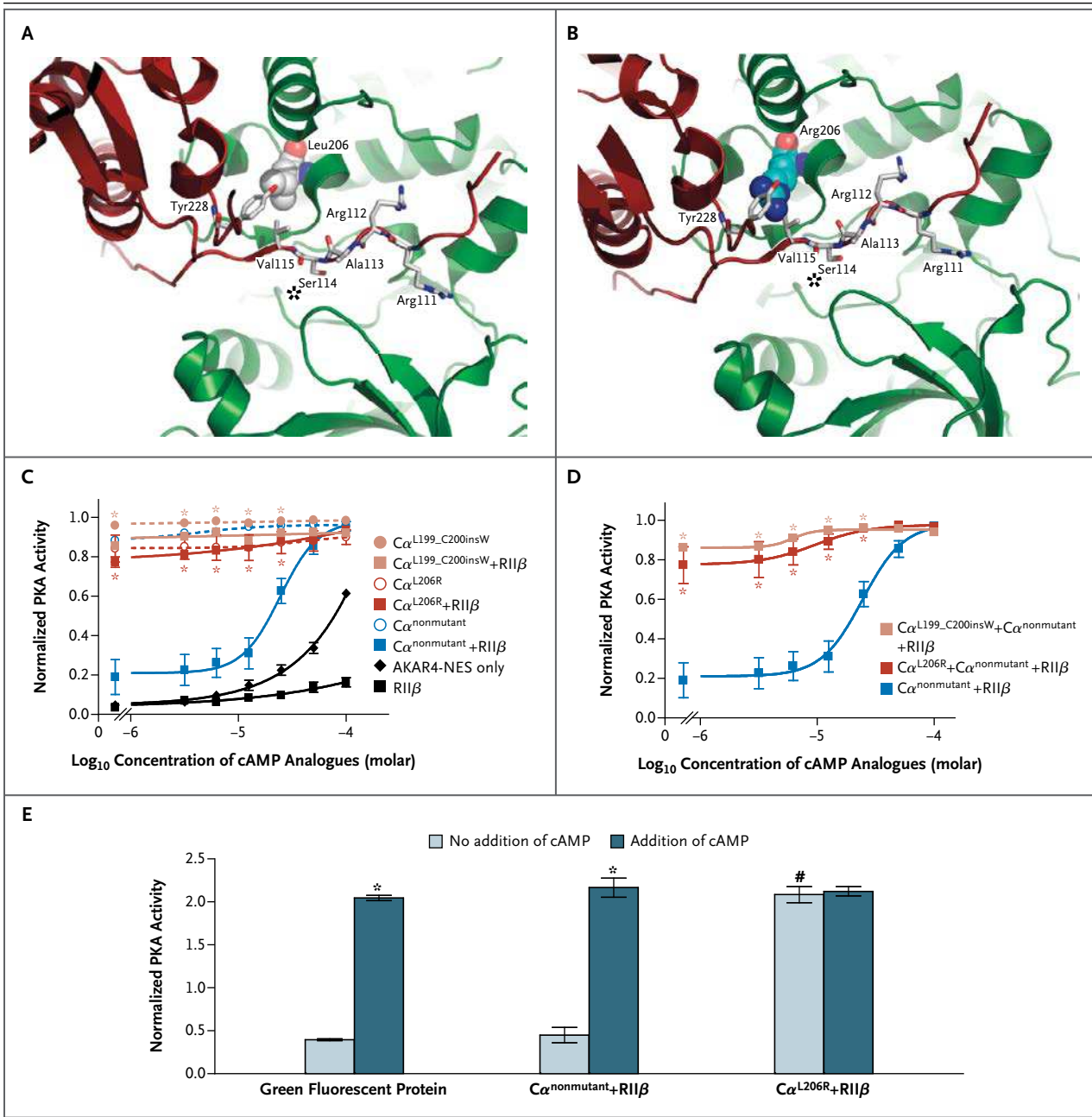
Similarly, transfection of the mutant Cα Leu206Arg variant caused a profound increase in PKA activity under basal conditions so that PKA activity was in the same range as that in cells



transfected with the nonmutant $C\alpha$ on cAMP stimulation, and this activity was not suppressed by cotransfection with $R11\beta$, indicating again a lack of suppression of the activity of the mutant catalytic subunit by the regulatory subunit (Fig. 2E). Consistent with these findings, basal PKA activity in tumor tissue was found to be higher in adenoma samples with *PRKACA* mutations than in those without such mutations (Fig. S5 in the Supplementary Appendix). Taken together, these data indicate that the Leu206Arg mutant protein is constitutively active and is not suppressed by the regulatory subunit.

ASSOCIATION OF GERMLINE DUPLICATION OF *PRKACA* WITH INCREASED PROTEIN LEVELS AND PKA ACTIVITY

As compared with tumor-tissue samples from patients without any known genetic defects, tumor-tissue samples from patients with duplications of *PRKACA* had higher PKA $C\alpha$ messenger RNA and protein levels (Fig. S6A and S6B, respectively, in the Supplementary Appendix). Immunohistochemical experiments confirmed higher expression of the PKA $C\alpha$ subunit in adrenal tissue from these patients (Fig. S6C in the Supplementary Appendix). The higher expression of the PKA $C\alpha$ subunit was associated with higher basal and cAMP-



stimulated PKA activity (Fig. S6D in the Supplementary Appendix).

CLINICAL PHENOTYPES AND PRKACA MUTATION STATUS
 Whereas 22 of 59 patients (37%) with overt Cushing's syndrome due to a unilateral adenoma harbored a PRKACA mutation, this alteration was not present in any adenoma associated with subclinical Cushing's syndrome (40 patients), nor was it present in inactive adrenal adenomas (20 patients),

aldosterone-producing adrenal adenomas (20 patients), or adrenocortical carcinomas (42 patients) or in adrenal tissue (10 patients) or lymphocytic DNA (35 patients) from patients with corticotropin-independent adrenal hyperplasia (35 patients). Furthermore, in the group of patients with overt Cushing's syndrome, the presence of PRKACA mutations was associated with a more severe phenotype (Table 1). Accordingly, expression levels of a variety of steroidogenic enzymes in mutant ade-

Figure 2 (facing page). Functional Characterization of PRKACA Variants.

Panel A shows the structure derived from the protein kinase A (PKA) tetramer, with the nonmutant catalytic subunit ($C\alpha$) depicted in green and the regulatory subunit (RII β) depicted in red. A zoomed view into the region of Leu206 in the $C\alpha$ subunit is shown. Leu206 is depicted as a space-filling representation; the two residues in close proximity (Val115 and Tyr228) and additional residues from the inhibitory site (Arg111–Ser114, marked with an asterisk) of the regulatory subunit are depicted as sticks. Panel B shows the same region of the PKA tetramer, with Leu206 in the $C\alpha$ subunit replaced by Arg206, also depicted as a space-filling representation. Panel C shows PKA activity of nonmutant and mutant PKA $C\alpha$ subunits transfected in human embryonic kidney 293 cells, as determined by means of fluorescence resonance energy transfer (FRET) assay with a PKA reporter (for details, see Fig. S4 in the Supplementary Appendix). The results indicate that the mutant variants are constitutively active. Asterisks indicate $P < 0.05$ for the comparison with $C\alpha^{\text{nonmutant}} + \text{RII}\beta$. AKAR4-NES denotes a protein activity reporter 4 with a nuclear export signal. Panel D shows that high constitutive PKA activity was maintained when either mutant was cotransfected with an equal amount of nonmutant $C\alpha$ subunit. Asterisks indicate $P < 0.05$ for the comparison with $C\alpha^{\text{nonmutant}} + \text{RII}\beta$. The data in Panels C and D were compared by means of a two-way analysis of variance followed by Bonferroni's test. Panel E shows the quantification of enzymatic PKA activity; COS-7 cells were transfected with $C\alpha$ (nonmutant or mutant) and RII β , with or without the addition of cyclic AMP (cAMP). Asterisks indicate $P < 0.05$ for the comparison between samples with and those without the addition of cAMP. The hatch mark indicates $P < 0.05$ for the comparison between samples transfected with nonmutant $C\alpha$ subunit and those transfected with mutant $C\alpha$ subunit without the addition of cAMP. In Panels C, D, and E, the I bars represent the standard error.

noma tissues were higher in the presence of a *PRKACA* mutation (Table S5 in the Supplementary Appendix).

There were no obvious phenotypic differences between patients with germline *PRKACA* duplications and those without duplications, although the number of patients with duplications was small. The affected mother and son had mild Cushing's syndrome of insidious onset, caused by bilateral macronodular hyperplasia manifesting in the third and fourth decade of life. The three young boys presenting with Cushing's syndrome due to bilateral hyperplasia (two with micronodular disease and one with macronodular disease) (Table S4 in the Supplementary Appendix) had severe disease similar to that of patients

with somatic *PRKACA* mutations. One patient with *PRKACA* duplication had a paradoxical increase in cortisol secretion after administration of dexamethasone,³⁰ whereas the other four patients did not undergo a long-term dexamethasone suppression test (Liddle's test) before surgery.

DISCUSSION

Despite evidence that enhanced cAMP signaling is the culprit in many benign adrenal lesions leading to Cushing's syndrome,^{8,18} the search for tumorigenic mutations in adrenal adenomas with the use of a candidate-gene approach has revealed only very rare mutations in a distinct subgroup of patients.^{12,13,17,31} The current study suggests that more than one third of cortisol-producing adenomas associated with overt Cushing's syndrome have unique somatic mutations in *PRKACA* (which encodes the main catalytic subunit of PKA), resulting in constitutive PKA activation. Although the mutation is present only in tumor cells in these patients, germline duplication of *PRKACA* was identified in a group of patients with bilateral adrenal hyperplasias. Consistent with the hypothesis that the resulting increased PKA activity is responsible for adrenocortical tumor formation, patients with somatic defects had single adenomas, whereas patients with germline duplications presented with bilateral adrenal hyperplasias. All the patients with *PRKACA* defects, whether germline or somatic, had overt Cushing's syndrome, and none of the patients with subclinical Cushing's syndrome or other adrenal tumors had genetic *PRKACA* alterations.

Thus, our findings provide evidence that *PRKACA* activation leads to marked excess of cortisol, as one would expect from constitutive activation of the enzymes that mediate corticotropin-dependent effects on adrenal steroidogenesis. The findings also indicate that subclinical Cushing's syndrome is not an early form of overt disease but a pathophysiologically distinct entity. Because *PRKACA* mediates most of the effects of inactivating *PRKARIA* mutations³² and because mutations of *PRKARIA* are associated with a variety of tumors in humans and mice,^{33,34} we would speculate that somatic *PRKACA* defects might also play a role in other forms of endocrine and nonendocrine tumors.

PKA, a cAMP-dependent serine–threonine kinase, is perhaps the best characterized protein

Table 1. Clinical Characteristics of Patients with Adrenal Adenomas in Relation to *PRKACA* Mutational Status.*

Characteristic	Endocrine-Inactive Adenoma (N=20)	Subclinical Cushing's Syndrome (N=40)	Overt Cushing's Syndrome (N=59)		P Value		
			Total	No <i>PRKACA</i> Mutation	<i>PRKACA</i> Mutation	Comparison of Endocrine-Inactive Adenoma, Subclinical Cushing's Syndrome, and Overt Cushing's Syndrome†	Comparison of Overt Cushing's Syndrome with and without <i>PRKACA</i> Mutation‡
Age at diagnosis (yr)							
Median	49.5	54.0	41.0	41.0	41.0	<0.001	0.37
Interquartile range	39.3–59.0	48.3–66.0	34.0–49.0	35.0–50.0	33.0–47.3		
Adenoma size (mm)§							
Median	43.0	40.0	30.0	30.0	30.0	<0.001	0.86
Interquartile range	33.0–50.0	30.0–50.0	30.0–36.0	29.5–40.0	30.0–35.5		
Corticotropin (pg/ml)¶							
Median	14.0	6.0	2.5	2.3	2.5	<0.001	0.69
Interquartile range	11.0–18.0	5.0–12.9	1.0–5.0	1.0–5.0	1.0–6.0		
Serum cortisol after 1 mg of dexamethasone (µg/dl)							
Median	1.2	2.9	15.9	15.0	22.0	<0.001	0.005
Interquartile range	1.0–1.7	2.3–7.5	13.3–23.0	9.4–19.5	16.5–27.7		
Urinary cortisol ÷ ULN**							
Median	0.62	0.46	3.67	3.14	5.33	<0.001	0.03
Interquartile range	0.38–0.72	0.26–0.99	1.88–6.01	1.53–5.23	2.46–9.08		
Midnight cortisol ÷ ULN††							
Median	0.6	1.6	3.9	3.6	4.3	<0.001	0.02
Interquartile range	0.4–1.0	0.8–2.6	2.8–4.6	2.3–4.5	3.9–5.9		

* For reference ranges of the endocrine characteristics, see the Methods section in the Supplementary Appendix. ULN denotes upper limit of the normal range.

† Values were compared with the use of the Kruskal–Wallis test.

‡ Values were compared with the use of the Mann–Whitney U test.

§ Data were not available for 1 patient with endocrine-inactive adenoma, 2 patients with subclinical Cushing's syndrome, and 4 patients with overt Cushing's syndrome (3 with no *PRKACA* mutation and 1 with *PRKACA* mutation).

¶ Data were not available for 9 patients with endocrine-inactive adenoma, 9 patients with subclinical Cushing's syndrome, and 12 patients with overt Cushing's syndrome (9 with no *PRKACA* mutation and 3 with *PRKACA* mutation). To convert values for corticotropin to picomoles per liter, multiply by 0.22.

|| Data were not available for 12 patients with endocrine-inactive adenoma, 11 patients with subclinical Cushing's syndrome, and 22 patients with overt Cushing's syndrome (14 with no *PRKACA* mutation and 8 with *PRKACA* mutation). To convert values for serum cortisol to nanomoles per liter, multiply by 27.6.

** Values are based on 24-hour urine samples. Data were not available for 3 patients with endocrine-inactive adenoma, 11 patients with subclinical Cushing's syndrome, and 6 patients with overt Cushing's syndrome (3 with no *PRKACA* mutation and 3 with *PRKACA* mutation).

†† Values are based on midnight salivary or serum cortisol levels. Data were not available for 12 patients with endocrine-inactive adenoma, 21 patients with subclinical Cushing's syndrome, and 23 patients with overt Cushing's syndrome (15 with no *PRKACA* mutation and 8 with *PRKACA* mutation).

kinase and provides a clear example of allosteric regulation.³⁵ In its inactive state, it is a tetrameric holoenzyme consisting of a dimer of two regulatory and two catalytic subunits; under physiologic conditions, PKA activity is induced by G-protein-coupled receptors through increased levels of cAMP.^{23,35} On binding of cAMP, the regulatory subunits dissociate from the catalytic subunits, allowing the enzyme to become active.³⁵ Although some randomly introduced mutations in *PRKACA* have been shown to result in unopposed catalytic activation *in vitro*,³⁶ such alterations have not been linked to human disease. The only naturally found gain-of-function *PRKACA* mutations are those described in the *Cos1(A1) drosophila* mutant.³⁷ The two *PRKACA* mutants identified in the current study alter the structure of the catalytic subunit at a site that is essential for interaction with the regulatory subunit, thus maintaining high activity of the catalytic subunit in the absence of cAMP.^{23,35} The critical position of the Leu206Arg mutation at the core of the interaction between the catalytic subunit and the inhibitory site of the regulatory subunit, combined with the steric hindrance involving Val115 and Tyr228 in the regulatory subunit, may explain the high grade of specificity of this particular mutation.

In conclusion, the current study links genetic variants of the main catalytic subunit of PKA with both hyperplasias and adenomas of the adrenal cortex leading to corticotropin-independent Cushing's syndrome. These observations

are consistent with the known role of the cAMP signaling pathway in adrenal lesions that have been associated with Cushing's syndrome.

Supported by a grant (FP7/2007-2013) from the European Commission Seventh Framework Program under grant agreement 259735 (to Drs. Beuschlein, Fassnacht, Bertherat, and Allolio) and, in part, by grants from the Wilhelm Sander-Stiftung (2012.095.1, to Dr. Allolio), the Else Kröner-Fresenius-Stiftung (2012_A103, to Dr. Reincke), Bundesministerium für Bildung und Forschung (BMBF 01EO1004-D2, to Drs. Fassnacht and Allolio), the COMETE Network (Programme Hospitalier de Recherche Clinique grant AOM95201), the Institut National du Cancer Recherche Translationnelle 2009-RT-02, INSERM (with Dr. Assié a recipient of a Contrat d'Interface), the Conny-Maeva Charitable Foundation, the European Research Council (Advanced Grant TOPAS, to Dr. Lohse), the Deutsche Forschungsgemeinschaft (DFG) (Rudolf Virchow Center and DFG Research Center for Experimental Biomedicine FZT82, to Drs. Kisker and Lohse, and DFG grant CA1014/1-1, to Dr. Calebiro), the Fondazione Telethon 2010 (GGP10121, to Dr. Zuffardi), and the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD).

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank the staff at the tumor bank of Cochin Hospital (in particular, Prof. Benoît Terris) for help with sample collection; Drs. Anelia Horvath (now at George Washington University), Isaac Levy (now at Toronto Children's Hospital, Toronto), Kiran Nadela (now at Otsuka America Pharmaceutical), and Maria Nesterova (NICHD) for work on earlier phases of this project; Jin Zhang (Johns Hopkins University School of Medicine) for providing the AKAR4-NES sensor; JoAnn C. Kelly, Zunyan Dai, Eric D. Smith, Marc A. Sanidad, and Philip Mowrey (Quest Diagnostics Nichols Institute) for technical assistance with the fluorescence *in situ* hybridization analysis; Marco Fichera for help with the Database of Genomic Variants; Drs. Glenn Braunstein (Cedars-Sinai Medical Center, Los Angeles), Louise Izatt (Guy's Hospital, London), and James Gardner (Pediatric Endocrinology Clinic, Our Lady of the Lake Physician Group, Baton Rouge, LA) for referral of patients who were then found to have *PRKACA* defects; and Prof. Wiebke Arlt (University of Birmingham) for helpful comments on an earlier version of the manuscript.

APPENDIX

The authors' affiliations are as follows: Medizinische Klinik und Poliklinik IV, Ludwig-Maximilians-Universität München (F.B., M.F., A.O., S.S., K.S., M.R.), Institute of Human Genetics, Technische Universität München (T.M., T.M.S.), and Deutsches Zentrum für Herz-Kreislauf-Forschung partner site, Munich Heart Alliance (T.M., T.M.S.), Munich, the Department of Medicine I, Endocrine and Diabetes Unit, University Hospital (M.F., S.S., B.A.), Comprehensive Heart Failure Center (M.F., M.J.L., B.A.), Institute of Pharmacology and Toxicology (D.C., U.Z., M.J.L.), Rudolf Virchow Center and Deutsche Forschungsgemeinschaft Research Center for Experimental Biomedicine (D.C., C.K., M.J.L.), and Comprehensive Cancer Center Mainfranken (C.L.R.), University of Würzburg, Würzburg, and the Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg (T.W., A.S., T.S., S.D., T.M., T.M.S.) — all in Germany; INSERM Unité 1016, Centre National de la Recherche Scientifique Unité Mixte de Recherche 8104, Institut Cochin, Faculté de Médecine Paris Descartes, Université Paris Descartes, Sorbonne Paris Cité (G.A., O.B., D.V., M.R.-R., J.B.), and the Department of Endocrinology, Referral Center for Rare Adrenal Diseases, Assistance Publique-Hôpitaux de Paris, Hôpital Cochin (G.A., O.B., J.B.), Paris; Section on Endocrinology and Genetics, Program on Developmental Endocrinology and Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD (C.A.S., F.R.F., E.S., P.S.); and Dipartimento di Medicina Molecolare, University of Pavia (A.F., O.Z.), and Biotechnology Research Laboratory, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico, Policlinico San Matteo (A.V.), Pavia, Italy.

REFERENCES

1. Lindholm J, Juul S, Jørgensen JO, et al. Incidence and late prognosis of Cushing's syndrome: a population-based study. *J Clin Endocrinol Metab* 2001;86:117-23.
2. Newell-Price J, Bertagna X, Grossman AB, Nieman LK. Cushing's syndrome. *Lancet* 2006;367:1605-17.
3. Grumbach MM, Biller BM, Braunstein GD, et al. Management of the clinically inapparent adrenal mass ("incidentaloma"). *Ann Intern Med* 2003;138:424-9.
4. Mansmann G, Lau J, Balk E, Rothberg

- M, Miyachi Y, Bornstein SR. The clinically inapparent adrenal mass: update in diagnosis and management. *Endocr Rev* 2004; 25:309-40.
5. Terzolo M, Pia A, Reimondo G. Subclinical Cushing's syndrome: definition and management. *Clin Endocrinol (Oxf)* 2012;76:12-8.
 6. Bonnet S, Gaujoux S, Launay P, et al. Wnt/ β -catenin pathway activation in adrenocortical adenomas is frequently due to somatic CTNNB1-activating mutations, which are associated with larger and non-secreting tumors: a study in cortisol-secreting and -nonsecreting tumors. *J Clin Endocrinol Metab* 2011;96(2):E419-E426.
 7. Lacroix A, Bourdeau I, Lampron A, Mazzuco TL, Tremblay J, Hamet P. Aberrant G-protein coupled receptor expression in relation to adrenocortical overfunction. *Clin Endocrinol (Oxf)* 2010;73:1-15.
 8. Stratakis CA. cAMP/PKA signaling defects in tumors: genetics and tissue-specific pluripotential cell-derived lesions in human and mouse. *Mol Cell Endocrinol* 2013;371: 208-20.
 9. Lacroix A, Bolté E, Tremblay J, et al. Gastric inhibitory polypeptide-dependent cortisol hypersecretion — a new cause of Cushing's syndrome. *N Engl J Med* 1992; 327:974-80.
 10. Lacroix A, Hamet P, Boutin JM. Leuprolide acetate therapy in luteinizing hormone-dependent Cushing's syndrome. *N Engl J Med* 1999;341:1577-81.
 11. Carney JA, Young WF, Stratakis CA. Primary bimorphic adrenocortical disease: cause of hypercortisolism in McCune-Albright syndrome. *Am J Surg Pathol* 2011;35:1311-26.
 12. Fragoso MC, Domenice S, Latronico AC, et al. Cushing's syndrome secondary to adrenocorticotropin-independent macronodular adrenocortical hyperplasia due to activating mutations of GNAS1 gene. *J Clin Endocrinol Metab* 2003;88:2147-51.
 13. Hsiao HP, Kirschner LS, Bourdeau I, et al. Clinical and genetic heterogeneity, overlap with other tumor syndromes, and atypical glucocorticoid hormone secretion in adrenocorticotropin-independent macronodular adrenal hyperplasia compared with other adrenocortical tumors. *J Clin Endocrinol Metab* 2009;94:2930-7.
 14. Horvath A, Boikos S, Giatzakis C, et al. A genome-wide scan identifies mutations in the gene encoding phosphodiesterase 11A4 (PDE11A) in individuals with adrenocortical hyperplasia. *Nat Genet* 2006; 38:794-800.
 15. Horvath A, Mericq V, Stratakis CA. Mutation in PDE8B, a cyclic AMP-specific phosphodiesterase in adrenal hyperplasia. *N Engl J Med* 2008;358:750-2.
 16. Kirschner LS, Carney JA, Pack SD, et al. Mutations of the gene encoding the protein kinase A type I-alpha regulatory subunit in patients with the Carney complex. *Nat Genet* 2000;26:89-92.
 17. Bertherat J, Groussin L, Sandrini F, et al. Molecular and functional analysis of PRKAR1A and its locus (17q22-24) in sporadic adrenocortical tumors: 17q losses, somatic mutations, and protein kinase A expression and activity. *Cancer Res* 2003; 63:5308-19.
 18. Bimpaki EI, Nesterova M, Stratakis CA. Abnormalities of cAMP signaling are present in adrenocortical lesions associated with ACTH-independent Cushing syndrome despite the absence of mutations in known genes. *Eur J Endocrinol* 2009; 161:153-61.
 19. 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;45:440-4.
 20. Ronchi CL, Leich E, Sbierra S, et al. Single nucleotide polymorphism microarray analysis in cortisol-secreting adrenocortical adenomas identifies new candidate genes and pathways. *Neoplasia* 2012;14:206-18.
 21. Vincent-Dejean C, Cazabat L, Groussin L, et al. Identification of a clinically homogeneous 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.
 22. Bonaglia MC, Giorda R, Beri S, et al. Molecular mechanisms generating and stabilizing terminal 22q13 deletions in 44 subjects with Phelan/McDermid syndrome. *PLoS Genet* 2011;7(7):e1002173.
 23. Zhang P, Smith-Nguyen EV, Keshwani MM, Deal MS, Kornev AP, Taylor SS. Structure and allostery of the PKA RII β tetrameric holoenzyme. *Science* 2012;335: 712-6.
 24. Herbst KJ, Allen MD, Zhang J. Spatiotemporally regulated protein kinase A activity is a critical regulator of growth factor-stimulated extracellular signal-regulated kinase signaling in PC12 cells. *Mol Cell Biol* 2011;31:4063-75.
 25. Calebiro D, Nikolaev VO, Gagliani MC, et al. Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biol* 2009;7(8):e1000172.
 26. 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. *Mol Endocrinol* 2006;20:3196-211.
 27. 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(6): E1109-E1121.
 28. Ronchi CL, Sbierra S, Leich E, et al. Single nucleotide polymorphism array profiling of adrenocortical tumors — evidence for an adenoma carcinoma sequence? *PLoS One* 2013;8(9):e73959.
 29. Taylor SS, Ilouz R, Zhang P, Kornev AP. Assembly of allosteric macromolecular switches: lessons from PKA. *Nat Rev Mol Cell Biol* 2012;13:646-58.
 30. Louiset E, Stratakis CA, Perraudin V, et al. The paradoxical increase in cortisol secretion induced by dexamethasone in primary pigmented nodular adrenocortical disease involves a glucocorticoid receptor-mediated effect of dexamethasone on protein kinase A catalytic subunits. *J Clin Endocrinol Metab* 2009;94:2406-13.
 31. Kobayashi H, Usui T, Fukata J, Yoshimasa T, Oki Y, Nakao K. Mutation analysis of Gsalpha, adrenocorticotropin receptor and p53 genes in Japanese patients with adrenocortical neoplasms: including a case of Gsalpha mutation. *Endocr J* 2000; 47:461-6.
 32. Meoli E, Bossi I, Cazabat L, et al. Protein kinase A effects of an expressed PRKAR1A mutation associated with aggressive tumors. *Cancer Res* 2008;68: 3133-41.
 33. Horvath A, Bertherat J, Groussin L, et al. Mutations and polymorphisms in the gene encoding regulatory subunit type I-alpha of protein kinase A (PRKAR1A): an update. *Hum Mutat* 2010;31:369-79.
 34. Kirschner LS, Kusewitt DF, Matyakhina L, et al. A mouse model for the Carney complex tumor syndrome develops neoplasia in cyclic AMP-responsive tissues. *Cancer Res* 2005;65:4506-14.
 35. Das R, Esposito V, Abu-Abed M, Anand GS, Taylor SS, Melacini G. cAMP activation of PKA defines an ancient signaling mechanism. *Proc Natl Acad Sci U S A* 2007;104:93-8.
 36. Orellana SA, McKnight GS. Mutations in the catalytic subunit of cAMP-dependent protein kinase result in unregulated biological activity. *Proc Natl Acad Sci U S A* 1992;89:4726-30.
 37. Collier LS, Suyama K, Anderson JH, Scott MP. Drosophila Costal1 mutations are alleles of protein kinase A that modulate hedgehog signaling. *Genetics* 2004; 167:783-96.

Copyright © 2014 Massachusetts Medical Society.