

# Adrenal GIPR expression and chromosome 19q13 microduplications in GIP-dependent Cushing's syndrome

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GIP-dependent Cushing's syndrome is caused by ectopic expression of glucose-dependent insulinotropic polypeptide receptor (GIPR) in cortisol-producing adrenal adenomas or in bilateral macronodular adrenal hyperplasias. Molecular mechanisms leading to ectopic GIPR expression in adrenal tissue are not known. Here we performed molecular analyses on adrenocortical adenomas and bilateral macronodular adrenal hyperplasias obtained from 14 patients with GIP-dependent adrenal Cushing's syndrome and one patient with GIP-dependent aldosteronism. *GIPR* expression in all adenoma and hyperplasia samples occurred through transcriptional activation of a single allele of the *GIPR* gene. While no abnormality was detected in proximal *GIPR* promoter methylation, we identified somatic duplications in chromosome region 19q13.32 containing the *GIPR* locus in the adrenocortical lesions derived from 3 patients. In 2 adenoma samples, the duplicated 19q13.32 region was rearranged with other chromosome regions, whereas a single tissue sample with hyperplasia had a 19q duplication only. We demonstrated that juxtaposition with *cis*-acting regulatory sequences such as glucocorticoid response elements in the newly identified genomic environment drives abnormal expression of the translocated *GIPR* allele in adenoma cells. Altogether, our results provide insight into the molecular pathogenesis of GIP-dependent Cushing's syndrome, occurring through monoallelic transcriptional activation of *GIPR* driven in some adrenal lesions by structural variations.

**Authorship note:** A.L. Lecoq, C.A. Stratakis, S. Viengchareun, and R. Chaligné contributed equally to this work

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## Introduction

Adrenal Cushing's syndrome results from excessive pituitary adrenocorticotropin-independent (ACTH-independent) cortisol production by benign or malignant adrenocortical tumors or from bilateral adrenal hyperplasia. The recent identification of activating somatic mutations of the *PRKACA* gene in one-third of

cortisol-secreting adrenal adenomas (1–3) and of germline and secondary somatic inactivating mutations of *ARMC5* in approximately 50% of operated patients with apparently sporadic primary bilateral macronodular adrenal hyperplasias (4) has provided important new insights into the molecular pathogenesis of adrenal Cushing's syndrome. Regulatory mechanisms of cortisol secretion in patients with bilateral macronodular hyperplasia, such as ectopic adrenal ACTH production, have also been recently discovered (5).

In a subset of patients with adrenal Cushing's syndrome, the cortisol excess is driven by aberrant (ectopic or excessive) expression of certain G protein-coupled receptors in adrenal lesions (6, 7). Although the molecular pathogenesis of food-dependent Cushing's syndrome is unknown, the condition is associated with ectopic glucose-dependent insulinotropic polypeptide receptor (*GIPR*) expression in adrenocortical adenomas or bilateral macronodular adrenal hyperplasia (8–10). Adrenal *GIPR* exposes the gland to inappropriate postprandial stimulation by glucose-dependent insulinotropic polypeptide (*GIP*). Activation of the ectopic receptor, functionally coupled to cAMP signaling, triggers adrenal cell proliferation and excessive hormone production (6). Here, we report molecular events leading to adrenal *GIPR* expression in a large international collection of adrenocortical adenomas and bilateral macronodular adrenal hyperplasias derived from patients with *GIP*-dependent adrenal Cushing's syndrome or with *GIP*-dependent primary aldosteronism.

## Results

**Clinical presentation.** Detailed clinical phenotypes are described in Table 1 and Supplemental Figure 1; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.92184DS1>. Twelve patients had overt Cushing's syndrome, two patients had subclinical Cushing's syndrome and one patient had primary aldosteronism. One patient (#13) presented with familial nonsyndromic Cushing's syndrome, and one patient (#15) presented with multiple endocrine neoplasia type 1 (*MEN 1*), caused by the frameshift 219–220delCG mutation in the *MEN1* gene; other patients had nonsyndromic and nonfamilial presentations.

**Adrenal expression of *GIPR* occurs from a single allele of the *GIPR* gene.** We detected a somatic *CTNNB1* p.Ser45Cys mutation in the tumor DNA of patient #1. No abnormalities in other genes known to be associated with adrenocortical adenomas and bilateral macronodular adrenal hyperplasias were detected in the tumor DNA of all 15 patients. The *GIPR* expression profile in the adrenal samples is shown in the Figure 1A. The relative abundance of *GIPR* transcripts in the normal adrenal tissue samples was very low, estimated at 26 molecules/ $\mu$ g RNA. All 16 adrenal samples selected for the study showed abundant *GIPR* expression (more than 10-fold of the mean expression in the unaffected adrenal tissue samples). The relative abundance of *GIPR* transcripts was not different between the adenoma and hyperplasia samples. Peritumoral adrenal tissue from patient #1 did not express *GIPR*. Interestingly, as reported previously (11), concomitant aberrant expression of one or more G protein-coupled hormone receptors was detected in one adenoma (from patient #4) and in 3 hyperplasia samples (from patient #6, #10, and #11) in addition to *GIPR* (Supplemental Figure 2).

To determine how many *GIPR* alleles are actively transcribed in patients' adrenal cells, we applied nascent RNA FISH, allowing detection of nascent RNA molecules at their transcription sites. This method enables the study of *GIPR* expression at the single-cell level and takes into account the heterogeneity of the adrenal lesions. The method enables the quantification of the number of *GIPR* alleles transcribed in the patients' adrenal cells. Consistent with quantitative PCR (RT-qPCR) results, normal adrenal tissue did not show *GIPR* expression. All adenoma and hyperplasia samples expressed *GIPR* from a single allele in a different proportion of nuclei (Figure 1B, representative samples showing monoallelic *GIPR* expression). Furthermore, samples showing the highest *GIPR* transcript levels measured by RT-qPCR (Figure 1A) also exhibited the highest proportion of nuclei expressing *GIPR* (Figure 1C).

**Identification of 19q13.32 duplications.** We studied the molecular events leading to transcriptional activation of a single allele of the *GIPR* gene in the adrenal lesions. We performed pyrosequencing of the *GIPR* promoter region (chr19: 46,171,165–46,171,452), containing 10 CpG islands. Of note, 3 CpGs were located close to functional Sp1/Sp3 binding motifs as described previously (12). Pyrosequencing showed absence of methylation in the proximal *GIPR* promoter in normal adrenal tissue, adenoma, and hyperplasia samples (Supplemental Figure 3).

Alternatively, transcriptional activation of a single allele of *GIPR* could be related to structural chromosomal imbalances. Oligonucleotide-based array comparative genomic hybridization analysis (array-CGH) was used to map somatic DNA copy number alterations (CNAs) in all adenoma and hyperplasia samples.

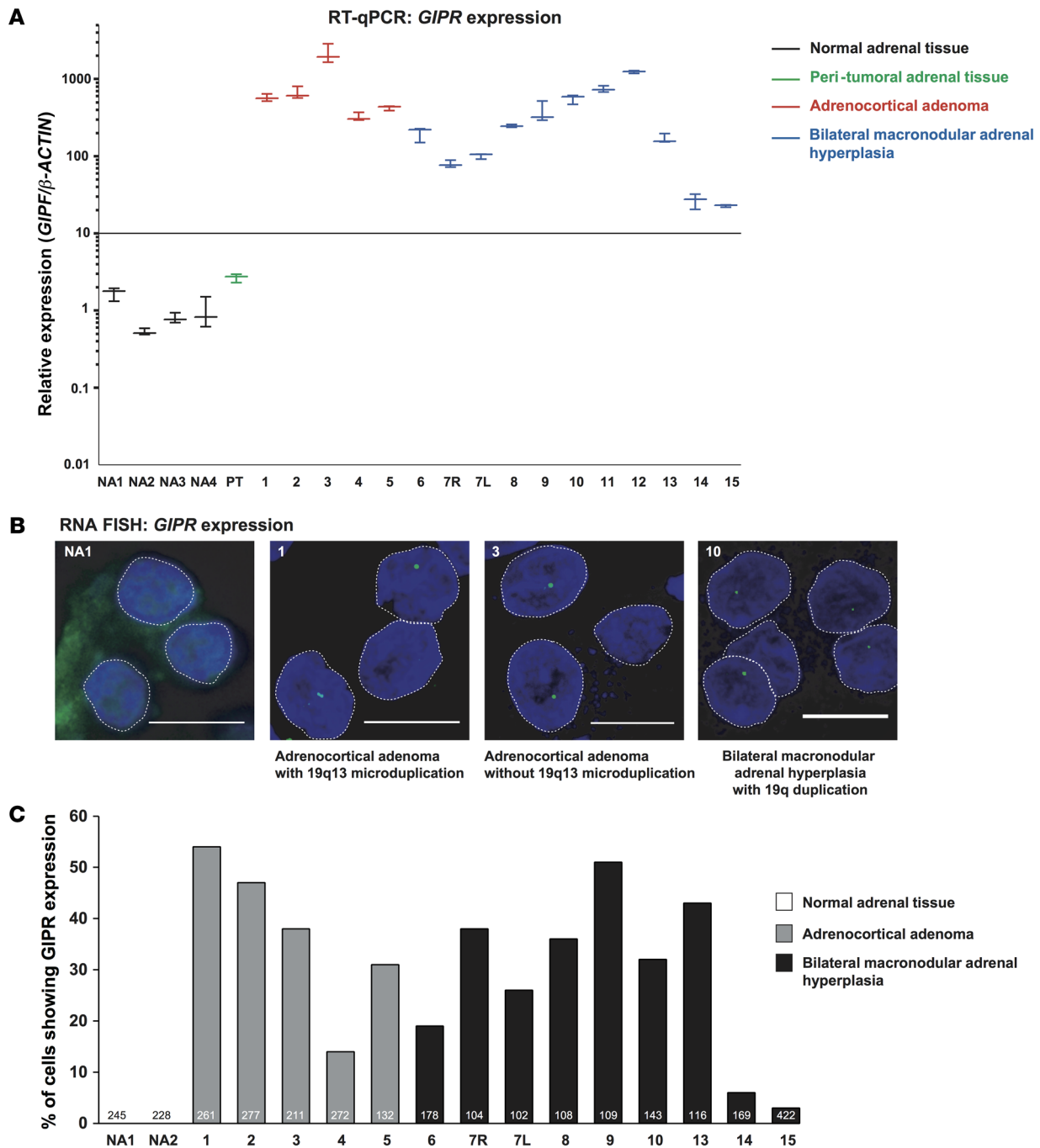
Table 1. Characteristics of the Patients

Patient no.	Sex	Age at surgery (yr)	Clinical presentation	Urinary free cortisol ( $\mu\text{g}/24\text{ h}$ )	Morning plasma corticotropin ( $\text{pg}/\text{ml}$ )	Morning plasma cortisol ( $\mu\text{g}/\text{dl}$ )	Postprandial plasma cortisol ( $\mu\text{g}/\text{dl}$ )	Adrenal histology	Adrenal lesion size (mm)	Refs.
1	F	35	Cushing's syndrome	692	<5	5.2	42.5	Adenoma	44	Unpublished, cf. Supplemental Fig 1
2	F	45	Cushing's syndrome	205	<2	1.7	43.9	Adenoma	30	31, 32
3	F	33	Cushing's syndrome	605	<5	2.4	18.0	Adenoma	20	33, 34 (patient #1)
4	M	56	Primary aldosteronism	17	11	10.0	15.7	Adenoma	20	35
5	F	58	Cushing's syndrome	304	<5	2.3	26.2	Adenoma	30	Unpublished
6	F	33	Cushing's syndrome	390	1.4	10.1	35.9	Hyperplasia	R, 2 nodules, 25 and 30	24, 25
7	F	55	Cushing's syndrome	43	<15	3.1	28.3	Hyperplasia	R 50, L 50	(36)
8	M	35	Cushing's syndrome	372	15	5.0	27.0	Hyperplasia	R 35, L 35	Unpublished
9	F	43	Cushing's syndrome	230	5	15.0	39.8	Hyperplasia	R 104, L 65	37 (patient 12)
10	F	45	Cushing's syndrome	525	<5	6.0	35.2	Hyperplasia	R 35, L 40	26 (patient 2), 27 (patient 2)
11	F	54	Cushing's syndrome	707	<5	15.9	76.5	Hyperplasia	R 50, L 54	26 (patient 4), 27 (patient 3)
12	F	43	Cushing's syndrome	213	<1	5.9	25.9	Hyperplasia	R 23, L 23	Unpublished
13	F	42	Cushing's syndrome, familial presentation	463	<1	5.2	34.7	Hyperplasia	R 60, L 32	Unpublished
14	F	46	Subclinical Cushing's syndrome	34	7	12.8	NA	Hyperplasia	R 10, L 28	Unpublished
15	F	25	Subclinical Cushing's syndrome, MEN 1	63	9	11.2	NA	Hyperplasia	R 38, L 12	Unpublished

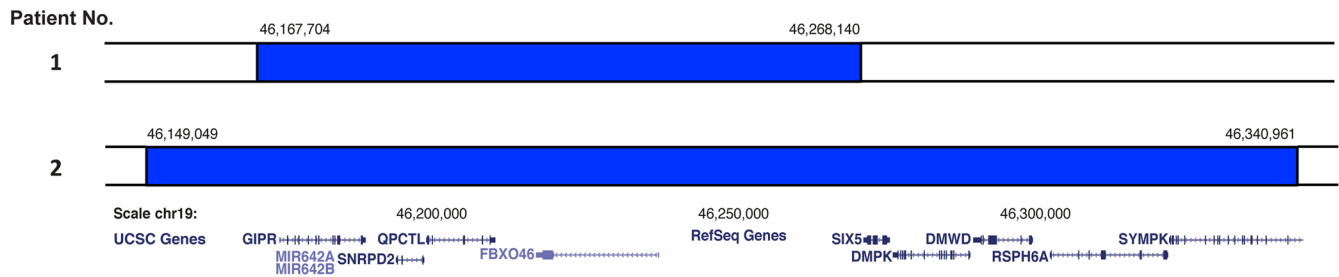
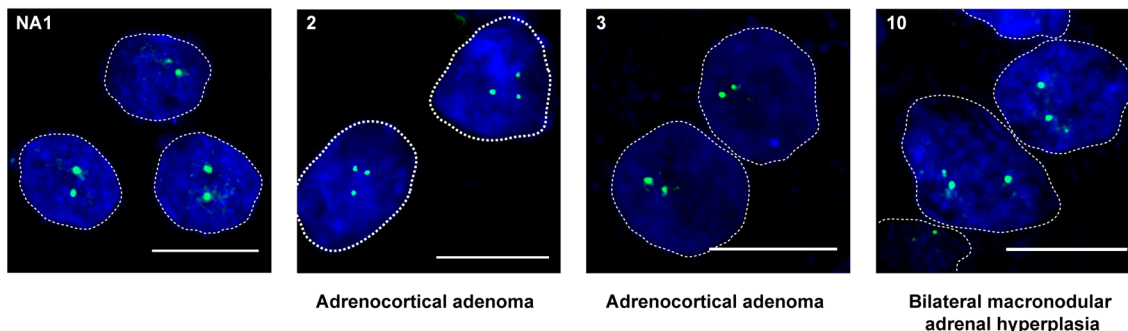
R, right; L, left; MEN 1, multiple endocrine neoplasia type 1.

The global CGH array profile is presented in Supplemental Figure 4, and identified CNAs are described in detail in Supplemental Tables 1–3. Very few CNAs were detected in adenoma samples, suggesting that GIPR-expressing adenomas belong to the “CNA-negative” subgroup of adrenocortical tumors (2). In contrast, as previously shown (13), hyperplasia samples had a higher number of extensive chromosomal imbalances.

In 3 samples, we identified somatic duplication in 19q13.32. In patient #1, array-CGH revealed a microduplication of 19q13.32 encompassing a 100.4-kb region (hg19 assembly, chr19:46,167,704–46,268,140) in the adrenal adenoma (Figure 2A). The duplication was not found in adjacent non-tumorous adrenal tissue and in the germline DNA. In patient #2, array-CGH also revealed a microduplication of 19q13.32 encompassing a 191.9-kb region (hg19 chr19:46,149,049–46,340,961) in the adrenal adenoma (Figure 2A). In patient #10, the entire long arm of chromosome 19 was duplicated in hyperplasia tissue, with ratios suggesting mosaicism. Again, this duplication was absent in the germline DNA. No duplications in 19q13.32 were identified in 4 GIP nonresponsive adenoma samples and 11 GIP nonresponsive hyperplasia samples.



**Figure 1. Adrenal glucose-dependent insulinotropic polypeptide receptor (*GIPR*) expression in patients with *GIP*-dependent Cushing's syndrome or aldosteronoma.** (A) *GIPR* expression profile in adrenal tissue samples obtained on reverse transcription quantitative PCR (RT-qPCR) and normalized to the  $\beta$ -actin housekeeping gene. Similar results were obtained when *GIPR* expression was normalized to *18S*. Four samples of normal adrenal tissue (NA1-NA4); non-tumorous adrenal tissue (PT) adjacent to adenoma of patient #1; 5 adrenocortical adenomas; and 10 bilateral adrenal hyperplasias from patients with aberrant adrenal sensitivity to *GIP* were studied. In one patient with bilateral adrenal hyperplasia (#7), samples from both adrenal lesions (R, right; L, left) were analyzed. Results are expressed as relative *GIPR* expression compared with mean expression level in the unaffected adrenal tissue samples, arbitrary set at 1, representing  $1.60 \times 10^{-4} \pm 0.54 \times 10^{-4}$  amol/amol of  $\beta$ -actin. The expression of *GIPR* in adrenal lesions of patients with *GIP*-dependent Cushing's syndrome or aldosteronoma was increased ( $570 \pm 159$ -fold, ranging from 23- to 2,152-fold) compared with the mean expression level in the unaffected adrenal tissue ( $P < 0.0001$ , Mann-Whitney *U* test). Experiments were performed twice; one representative experiment is shown here. Three independent determinations were done for each sample. The lines represent the median values; the whiskers represent the minimal and maximal values. (B) Representative examples of adrenal *GIPR* expression as assessed by RNA FISH in adrenal lesions with or without 19q13 duplication. Monoallelic (a maximum of one RNA FISH signal is visible per nucleus) *GIPR* expression in interphase nuclei is visible as one green pinpoint in adrenal lesions of patients with *GIP*-dependent Cushing's syndrome or aldosteronoma. RNA FISH analysis was performed on all adrenal lesions with aberrant sensitivity to *GIP*, except for lesions of patient #11 and #12, due to the lack of good quality frozen tissue in these two samples. Normal adrenal tissue did not show any *GIPR* expression. (C) Percentage of nuclei with monoallelic *GIPR* expression determined by RNA FISH. At the bottom of the histogram, the number of analyzed nuclei is indicated. Scale bars: 5  $\mu$ m.

**A** Array-CGH**B** DNA FISH : *GIPR* locus

**Figure 2. Genomic 19q13 duplications in adrenal lesions of 3 patients with glucose-dependent insulinotropic polypeptide-dependent (GIP-dependent) Cushing's syndrome.** (A) 19q13 microduplications, as seen on oligonucleotide-based array comparative genomic hybridization analysis (array-CGH), in the adenomas of patients #1 and #2. Duplicated genomic segments are shown in blue and adjacent nonduplicated segments in white. The genomic coordinates of the duplicated segments are shown above the blue boxes. The magnification scale of chromosome 19 and the genomic content in the duplicated regions are shown below the boxes (adapted from the UCSC Genome Browser, <http://genome.ucsc.edu/>). (B) Representative examples of *GIPR* (*GIPR*) loci visualized by DNA FISH analysis. Three *GIPR* loci (green) are visible in the majority of the interphase nuclei in the adrenal tissue of 3 patients with genomic 19q13 duplication on array-CGH (Supplemental Figure 4), represented here by samples of patients #2 and #10. DNA FISH analysis of the adrenal sample of patient #1 is shown in Figure 3C. Two *GIPR* loci are present in the interphase nuclei of the normal adrenal tissue and in adrenal lesions without 19q13 duplication on array-CGH, represented by adenoma of patient #3. Scale bars = 5  $\mu$ m.

The smallest regions of overlap (hg19 chr19: 46,167,704–46,268,140) encompassed 5 genes, namely *GIPR*, *SNRPD2*, *QPCTL*, *FBXO46*, and *SIX5*. RT-qPCR of RNA of these adrenal samples versus RNA from unaffected adrenal tissue showed that *GIPR* was the only overexpressed gene. Expression of the other 4 duplicated genes was not significantly different (Supplemental Figure 5).

DNA FISH confirmed duplication of the *GIPR* locus in adenoma samples from patients #1 and #2 and a hyperplasia sample from patient #10. As expected, two *GIPR* loci were observed in normal adrenal tissue. Other adenoma and hyperplasia samples also had two detectable *GIPR* alleles as resolved by DNA FISH (Figure 2B).

*Characterization of the chromosomal rearrangements.* To further characterize the molecular events leading to transcriptional activation of a single allele of *GIPR*, we performed mate-pair sequencing on DNA samples from 6 selected adrenal lesions. We analyzed DNA from the 3 adrenal lesions harboring the 19q13.32 duplication and from 3 lesions without CNAs on the *GIPR* locus. Sequencing of mate-pair libraries yielded  $121,226,487 \pm 18,679,783$  mapped pairs with insert sizes of  $3.4 \pm 0.8$  kb. This provided effective coverage of  $68 \pm 14\times$  relative to the six billion base pair diploid genome in adrenal cells. BAM preprocessing with SVDetect yielded  $12,752,546 \pm 2,772,124$  abnormally mapped pairs.

In the adenoma of patient #1, mate-pair SVDetect analysis identified an interchromosomal rearrangement between the duplicated segments 19q13.32 and 20q13.12. Abnormal pairs indicated two breakpoints (hg19 chr19:46,166,265–chr20:45,950,228 and chr19:46,271,027–chr20:46,030,117) compatible with duplication of one of these chromosome segments, separated by insertion of the other nonduplicated segment in inverted orientation. Similarly, in adenoma from patient #2, mate-pair analysis identified an intrachromosomal rearrangement between the duplicated segments 19q13.32 and 19q13.43. Abnormal pairs revealed two breakpoints (hg19 chr19:46,152,434–57,454,276 and chr19:46,344,667–57,820,369), indicating



a similar configuration of the rearrangement (Figure 3A). In both adenoma samples, breakpoints close to the *GIPR* gene were characterized at base pair resolution by conventional Sanger sequencing of the PCR fragments (Figure 3B), showing a junction of canonical nonhomologous end-joining typical of chromosomal translocations in human cells (14). In the hyperplasia sample of patient #10 with 19q duplication, mate-pair analysis did not identify breakpoints at the pericentromeric region. In the other 4 samples, mate-pair SVDetect analysis did not reveal rearrangements, including at the *GIPR* locus.

*The rearranged genomic environment drives aberrant monoallelic expression of GIPR.* To further explore the functional impact of the *GIPR* genomic rearrangements as identified in adenoma of the patient #1, we performed DNA and RNA FISH on this sample. We confirmed the insertion of the duplicated 19q13.32 region in close proximity to the *ZMYND8* gene (zinc finger and MYND [myeloid, Nervy, and DEAF-1] domain containing 8) on chromosome 20. RNA FISH revealed that *GIPR* was only expressed from the allele translocated on chromosome 20, as indicated by *ZMYND8* expression (Figure 3C).

We investigated the functional impact of the newly observed genomic environment in the tumor of patient #1. In this tumor, the chromosome rearrangement juxtaposed the *GIPR* gene to the second intron of the *ZMYND8* gene, which contains an alternative start site for RNA polymerase II and several *cis*-acting regulatory elements, including a glucocorticoid response element (GRE) (15) (Supplemental Figure 6A) and Sp1 binding motifs. Transient transfections of HEK 293T cells with a *ZMYND8* intron 2-pGL4.26-luciferase vector showed transcriptional activation of *ZMYND8* intron 2 by glucocorticoids, confirming the functional status of the GRE (Figure 4A). Expression of a chimeric *ZMYND8* intron 2-pGL4.26-*GIPR* vector (Supplemental Figure 6B) in HEK 293T cells and in adrenocortical H295R cells resulted in increased cAMP signaling in the presence of  $10^{-7}$  M GIP, which was abolished by the protein-kinase A inhibitor H-89. Co-treatment with dexamethasone resulted in a significant increase in cAMP production by HEK 293T cells (Figure 4B) and by H295R cells (Figure 4C). Thus, this GRE-containing fragment of intron 2 of *ZMYND8* was sufficient to drive *GIPR* expression.

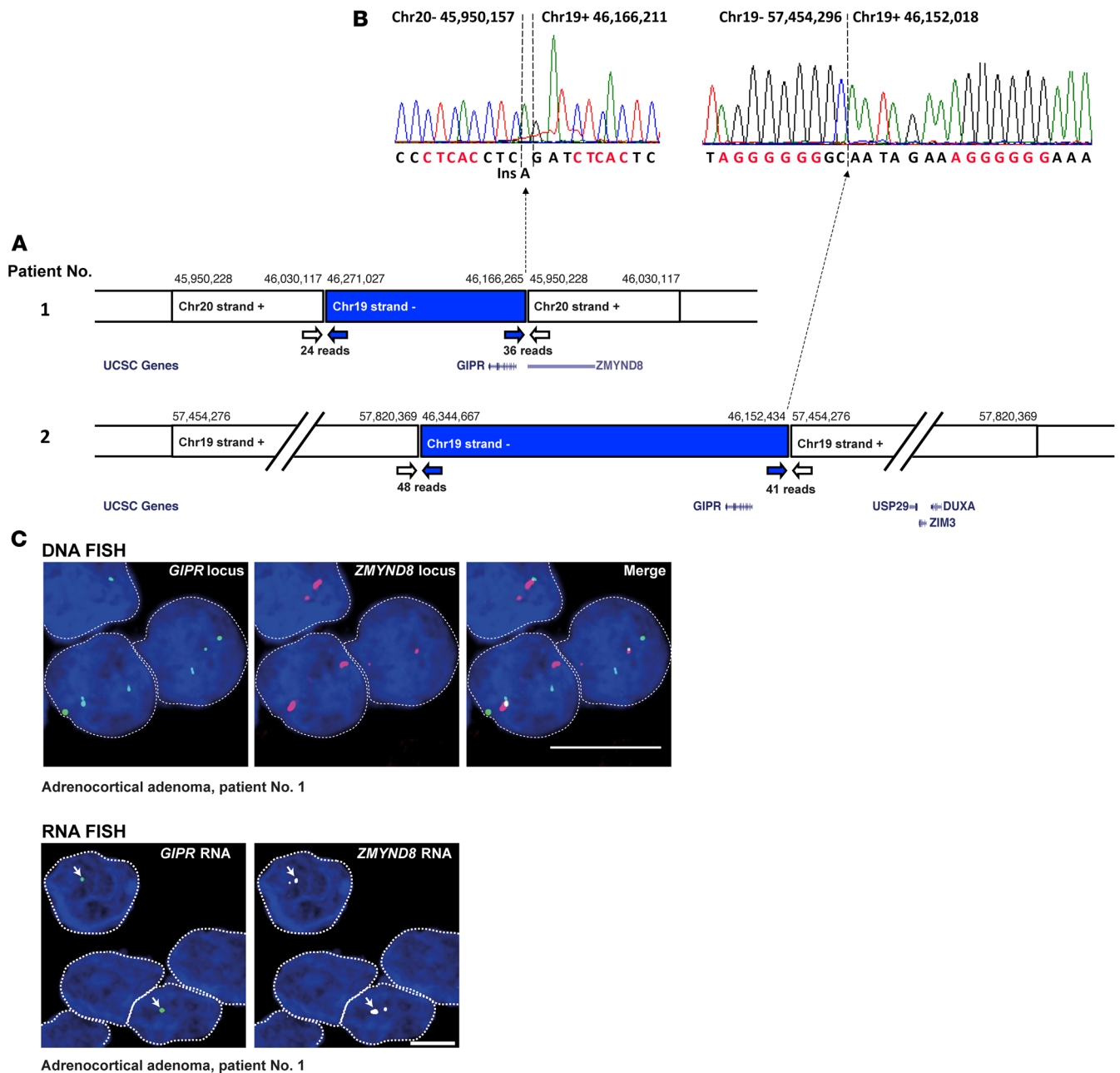
## Discussion

Since the initial description of GIP-dependent Cushing's syndrome (9, 10), molecular mechanisms responsible for ectopic *GIPR* expression in adrenal tissue have remained elusive (6). Here, we describe the results of combined molecular analyses in the largest reported collection of adrenocortical adenomas and bilateral macronodular adrenal hyperplasias from patients with GIP-dependent Cushing's syndrome or aldosteronism. *GIPR* expression analysis by RNA FISH consistently demonstrated *GIPR* expression from a single allele in all adenoma and hyperplasia samples with aberrant sensitivity to GIP, whereas *GIPR* was not expressed in normal adrenal cells. Monoallelic *GIPR* expression observed in all adrenal lesions suggested a common disease mechanism leading to transcriptional activation of a single allele of this gene. Either chromosome rearrangements and point mutations and/or epigenetic abnormal modifications of the *GIPR* regulatory region (promoter or enhancer) could underlie inappropriate adrenal lesion *GIPR* expression.

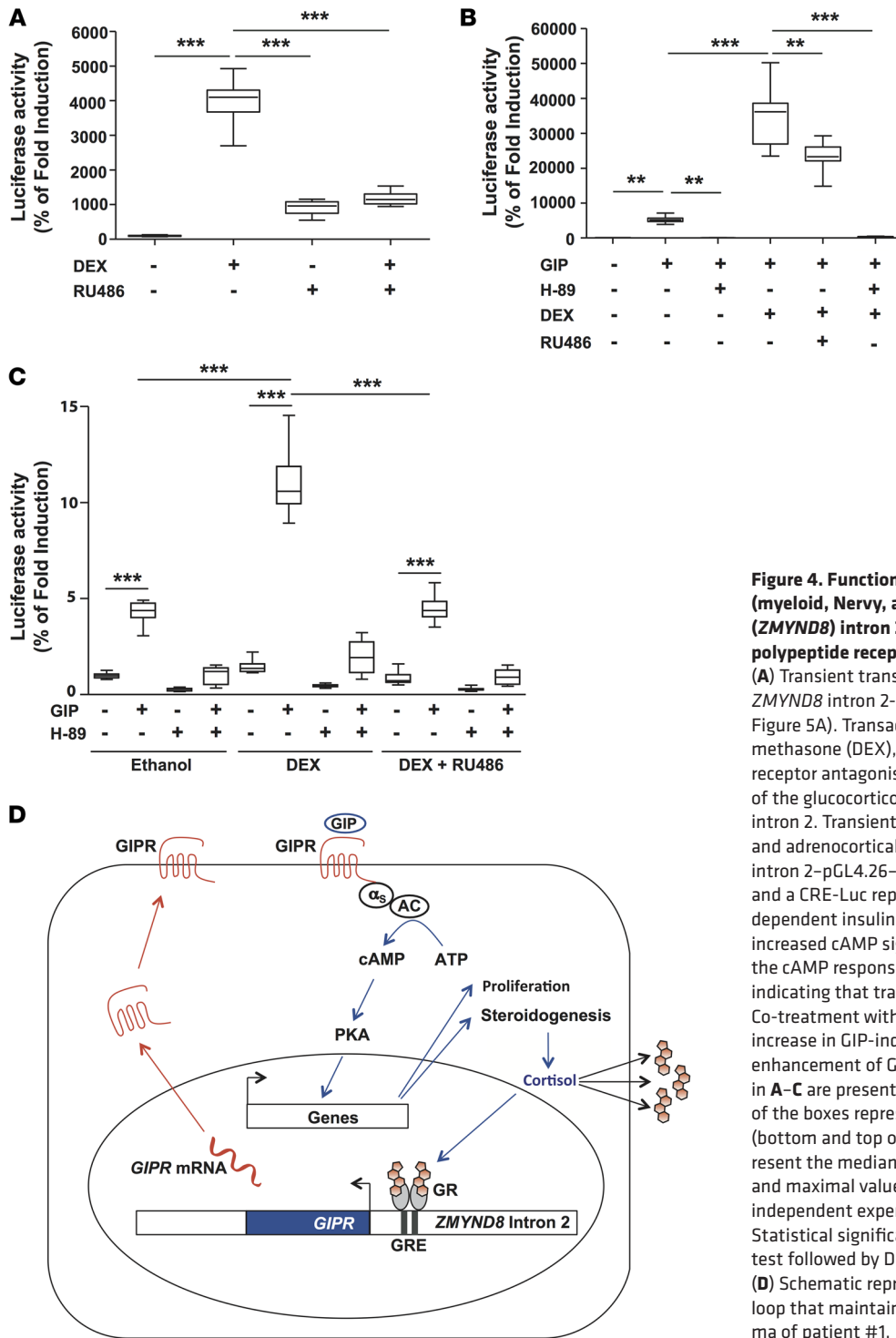
Indeed, molecular and cytogenetic assessments showed that 3 adrenal lesions harbored somatic duplications in chromosome region 19q13.32. The duplicated region that was common to all 3 samples contained 5 protein-encoding genes, of which only *GIPR*, located at the extremity of the duplicated region, was markedly overexpressed in these adrenocortical lesions. Recently, we described Xq26.3 microduplications in patients with early-onset acrogigantism (16), showing that genomic gains may promote inappropriate hormonal hypersecretion.

In two adenoma samples, the duplicated 19q13.32 region was rearranged with other chromosome regions, whereas in one hyperplasia sample, 19q duplication without chromosome rearrangements was detected. Examples of genomic rearrangements in endocrine tumors include gene fusion involving the *RET* oncogene in papillary thyroid carcinomas (17), a chimeric *11 $\beta$ -hydroxylase/aldosterone synthase* gene in glucocorticoid-remediable aldosteronism (18), and juxtaposition of the parathyroid hormone promoter with the *cyclin D* gene in some parathyroid adenomas (19). In the two tumors described here, chromosome rearrangements did not result in fusion of the coding sequences of two genes but placed *GIPR* in a chromatin environment favoring transcriptional activation in the vicinity of *cis*-acting regulatory regions. As shown by RNA FISH experiments, this genomic environment drove abnormal expression of the translocated *GIPR* allele in the cells in both adenomas.

In the adenoma of patient #1, *GIPR* was fused with an intronic regulatory region of the *ZMYND8* gene located in chromosome region 20q13.12. *ZMYND8* is ubiquitously expressed and plays a role in chromatin



**Figure 3. Chromosome rearrangements in adrenocortical adenoma of two patients with glucose-dependent insulinotropic polypeptide-dependent (GIP-dependent) Cushing's syndrome drive ectopic GIP receptor (*GIPR*) expression in adenoma cells.** (A) Genomic rearrangements in adenoma samples from patient #1 and #2, as revealed by mate-pair analysis. Next-generation sequencing (NGS) data identified abnormally mapped reads corresponding in tumor from patient #1 to interchromosomal rearrangement between chromosome segments 19q13.32 and 20q13.12, and in tumor from patient #2 to intrachromosomal rearrangement between chromosome segments 19q13.32 and 19q13.43. The genomic coordinates of the rearranged genomic segments are shown above the boxes. White and blue arrows indicate abnormal reads corresponding to break points. Selected genes in the rearranged chromosome segments are shown below the boxes (adapted from the UCSC Genome Browser). The break point at position hg19 chr20:45,950,228 in adenoma from patient #1 corresponds to the second intron of the zinc finger and the MYND (myeloid, Nervy, and DEAF-1) domain containing 8 (*ZMYND8*) gene, which is consequently truncated. In the adenoma of patient #2, the break point at position hg19 chr19:57,454,276 falls within a noncoding region of chromosome 19. (B) The breakpoints in the proximity of the *GIPR* gene at base-pair resolution, characterized by conventional Sanger sequencing of the PCR fragments, showing one base-pair insertion (Ins) typical of canonical nonhomologous end-joining. Microhomologies are indicated in red. (C) DNA (top) and RNA FISH (bottom) analyses in adenoma of patient #1. On DNA FISH, 3 *GIPR* loci (green) and two *ZMYND8* loci (red) are visible in each interphase nucleus. Thus, DNA FISH confirms duplication of the 19q13.32 region and its insertion in close proximity to *ZMYND8* in this sample. Representative examples of *GIPR* expression (green) and of *ZMYND8* expression (white) in adenoma of patient #1, as analyzed by RNA FISH, are also shown. Arrows indicate *GIPR* expression from a single allele in each nucleus. The RNA FISH signal corresponds to the translocated *GIPR* allele fused with intron 2 of the *ZMYND8* gene located in chromosome region 20q13.12. Scale bars: 5  $\mu$ m.



**Figure 4. Functional analysis of zinc finger and MYND (myeloid, Nervy, and DEAF-1) domain containing 8 (ZMYND8) intron 2-glucose-dependent insulinotropic polypeptide receptor (GIPR) chromosome rearrangement.** (A) Transient transfection assays of HEK 293T cells with a ZMYND8 intron 2-pGL4.26-Luciferase vector (Supplemental Figure 5A). Transactivation of ZMYND8 intron 2 by dexamethasone (DEX), and its prevention by the glucocorticoid receptor antagonist RU486, confirms the functional status of the glucocorticoid response element (GRE) in ZMYND8 intron 2. Transient transfection assays of HEK 293T cells (B) and adrenocortical H295R cells (C) with a chimeric ZMYND8 intron 2-pGL4.26-GIPR plasmid (Supplemental Figure 5B) and a CRE-Luc reporter vector. Treatment with glucose-dependent insulinotropic polypeptide (GIP) resulted in increased cAMP signaling, as shown by transactivation of the cAMP response elements (CRE) in the CRE-Luc vector, indicating that transfected cells expressed functional GIPR. Co-treatment with dexamethasone resulted in a significant increase in GIP-induced cAMP production, demonstrating enhancement of GIPR expression by glucocorticoids. Data in A-C are presented as box-and-whisker plots. The bounds of the boxes represent the first and the third quartiles (bottom and top of the box); the lines within the boxes represent the median values; the whiskers depict the minimal and maximal values. Presented data are the means of 2 independent experiments, with  $n = 8$  for each experiment. Statistical significance was evaluated by Kruskal-Wallis test followed by Dunn's post-test.  $**P < 0.01$ ,  $***P < 0.001$ . (D) Schematic representation of the positive regulatory loop that maintains ectopic GIPR expression in the adenoma of patient #1.

recognition of transcription-associated DNA damage (20, 21). We show that GIPR expression in this tumor was positively regulated by glucocorticoids via a functional GRE located in the second intron of ZMYND8 (15). Consequently, GIPR stimulation by GIP led to postprandial cortisol production, which maintained inappropriate GIPR expression, thus forming an intracrine and/or paracrine positive regulatory loop responsible for excess cortisol (Figure 4D). In the adenoma of patient #2, GIPR was placed in a vast intergenic region, with the closest protein-coding gene USP29 situated 176 kb from the break point. Recently, the Encyclopedia of DNA Elements (ENCODE) enabled us to assign biochemical functions for 80% of the genome, in particular outside of the well-studied protein-coding regions, pointing out the importance of



such apparent “desert” intergenic regions in transcriptional regulation (15).

The observation of structural variations on the 19q13.32 locus does not apply to other adenoma and bilateral macronodular adrenal hyperplasia cases analyzed. Genetic or epigenetic alterations in the *GIPR* promoter region could be the cause of transcriptional activation of *GIPR* in these adrenal lesions without structural variations on the 19q13.32 locus. However, no changes in methylation of the proximal *GIPR* promoter were detected in this study (Supplemental Figure 3). Considering the possible link between chronic pituitary ACTH stimulation in Cushing’s disease and adrenal GIPR expression (22), in some cases of primary bilateral macronodular hyperplasia adrenal GIPR expression could be amplified by ectopic ACTH produced within the adrenocortical tissue (5).

Several adrenal lesions expressed more than one aberrant receptor (Supplemental Figure 2). No genomic duplications in the corresponding loci were detected by array-CGH analysis in these 4 patients. Of note, in patients #7 (left adrenal lesion) and #10, the entire long arm of the chromosome 1 was duplicated. Chromosome 1q contains the gene encoding vasopressin receptor type 1b receptor, *AVPR1b*, but *AVPR1b* was not expressed in these lesions. In contrast to microduplications with chromosome rearrangements, which create a novel genomic environment favorable for transcription, arm-level duplications alone are probably not sufficient to drive aberrant gene expression. How several receptors encoded on different chromosomes can be expressed in the same tissues remains enigmatic. Recently, a link between activating *CTNNB1* p.Ser33Cys, p.Gly34Arg, and p.Ser45Phe mutations and overexpression of gonadotropin-releasing hormone receptor and luteinizing hormone/chorionic gonadotropin receptor in 3 aldosterone-producing adenomas has been suggested (23). We did not identify these *CTNNB1* mutations, in particular in 3 bilateral macronodular adrenal hyperplasia samples (24–27) that also overexpressed luteinizing hormone/chorionic gonadotropin receptor (Supplemental Figure 2). As reported by others (28), the relation between these *CTNNB1* mutations and gonadal receptor expression thus seems not to be a recurrent finding.

However, one GIPR-expressing adrenal adenoma harbored a somatic *CTNNB1* p.Ser45Cys mutation, which could contribute to tumor development. By analogy, the *MEN1* mutation in patient #15 may have presented a genetic hit leading to bilateral adrenal hyperplasia development. No mutations in genes known to be involved in adrenal tumorigenesis were identified in the remaining 14 adrenal lesions. In particular, consistent with previous reports (4, 29), no *ARMC5* mutations were detected in any of the patients with GIP-dependent Cushing’s syndrome studied. Thus, *ARMC5*-related bilateral macronodular adrenal hyperplasia and GIPR-dependent adrenal proliferation seem to have different pathogeneses. Activation of the ectopic GIP receptor, functionally coupled to cAMP signaling, not only triggers excessive hormone production, but very likely contributes to adrenal cell proliferation and tumor development as demonstrated in mice (30).

In conclusion, we demonstrated that ectopic GIPR expression in adrenocortical adenomas and in bilateral macronodular adrenal hyperplasias of patients with GIP-dependent Cushing’s syndrome or GIP-dependent aldosteronism is consistently associated with transcriptional activation of a single allele of the *GIPR* gene. Three adrenal lesions harbored somatic duplications in 19q13.32 containing the *GIPR*; in two, genomic gain was associated with chromosomal rearrangements, creating a chromatin environment favoring *GIPR* transcription.

## Methods

**Patients.** We analyzed 16 adrenal samples derived from 15 patients who had undergone adrenalectomy for adrenocortical adenoma or ACTH-independent bilateral macronodular adrenal hyperplasia. In a patient with primary bilateral macronodular adrenal hyperplasia (#7, Table 1), samples from both adrenal lesions were available for analysis. Samples were collected at 8 endocrine centers in Europe, the USA, and Canada. All adrenal lesions with proven GIPR expression available in these participating centers were selected for the study. Fourteen specimens were obtained from 12 patients with overt GIP-dependent Cushing’s syndrome and from one patient with GIP-dependent aldosteronism. Eight of these cases were previously reported (24–27, 31–37). GIP-dependent Cushing’s syndrome was diagnosed according to standard criteria showing hypercortisolism (38) in combination with low morning (fasting) plasma cortisol and ACTH concentrations and an increase in plasma cortisol concentration in response to a meal or to other tests, such as GIP infusion. GIP-dependent aldosteronism was diagnosed in a patient presenting with classical clinical and biological criteria of hyperaldosteronism (39) and a significant postprandial and post-GIP infusion renin-independent increase in plasma aldosterone concentration. Two additional primary bilateral macronodular adrenal hyperplasia samples from patients with subclinical Cushing’s syndrome without presurgical biochemical

demonstration of aberrant adrenal sensitivity to food were included (patients #14 and #15), based on *GIPR* expression screening among 78 adrenal lesion samples derived from patients with overt or subclinical Cushing's syndrome collected at the NIH. We further included 4 samples of adrenocortical adenomas and 11 samples of bilateral adrenal hyperplasia with no *GIPR* expression as controls. Eleven of these control samples were obtained from patients with adrenal Cushing's syndrome, two samples were from patients with primary hyperaldosteronism, and one sample was from a patient with nonfunctioning adrenal adenoma. We also studied samples of macroscopically and histologically unaffected adrenals obtained from 4 subjects who underwent total nephrectomy with adrenalectomy for renal cell carcinoma and peritumoral adrenal tissue from patient #1 (Table 1). We analyzed leukocyte DNA from patients #1 and #10.

*Molecular analysis of the adrenal lesions and in vitro studies.* We sequenced the *ARMC5*, *PRKACA*, and *PRKAR1A* genes, exon 3 of the *CTNNB1* gene, and exons 7–9 of the *GNAS* gene in DNA extracted from the adrenal lesions. We measured gene expression levels in adrenal samples using reverse transcription qPCR (RT-qPCR). We analyzed expression of *ADRB1*, *AGTR1*, *AVPR1a*, *GIPR*, *GnRHR*, *HTR4*, *LHCGR*, *MC2R*, *QPCTL*, *SIX5*, *SNRPD2*, *FBOX46*, and *ZMYND8* (Supplemental Table 4). We applied DNA and RNA FISH to frozen samples. We performed array-CGH on DNA samples from the adrenal lesions. We performed mate-pair analyses on the tumor DNA of 6 patients. We transfected human HEK 293T cells and adrenocortical H295R cells with the *ZMYND8* intron 2-pGL4.26-Luc and *ZMYND8* intron 2-pGL4.26-GIPR plasmid constructs for in vitro studies. More details are provided in Supplemental Methods, available online with this article.

*Study approval.* Patients with adrenocortical adenomas and bilateral macronodular adrenal hyperplasias expressing *GIPR* and patients from whom unaffected adrenal tissue was obtained gave written informed consent for genetic analyses; all studies were approved by the local ethics committee at Bicêtre hospital, Le Kremlin-Bicêtre, France.

*Statistics.* Data are expressed as mean  $\pm$  SEM. Differences between groups were analyzed by nonparametric Mann-Whitney *U* test or Kruskal-Wallis test, followed by Dunn's post-test (Prism, GraphPad Software). *P* values less than 0.05 were considered statistically significant.

## Author contributions

ALL, CAS, SV, RC, VD, NS, HL, EL, AL, PC, J. Bouligand, and PK contributed to the conception and design of the research; ALL, SV, RC, LT, VD, MH, PH, HGB, GT, VB, EC, EL, J. Bouligand, and PK conducted experiments; ALL, CAS, SV, RC, LT, VD, AB, FRF, PH, HGB, SS, GT, AGM, JY, ML, and EL acquired data; ALL, SV, RC, LT, VD, AGB, FRF, PH, EL, J. Bouligand, and PK analyzed data; CAS, CA, IB, DM, AT, J. Bertherat, HL, WdH, and AL provided tissue samples; CAS, SV, RC, J. Bouligand, and PK wrote the manuscript.

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1. Beuschlein F, et al. Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome. *N Engl J Med*. 2014;370(11):1019–1028.
2. Goh G, et al. Recurrent activating mutation in PRKACA in cortisol-producing adrenal tumors. *Nat Genet*. 2014;46(6):613–617.
3. Cao Y, et al. Activating hotspot L205R mutation in PRKACA and adrenal Cushing's syndrome. *Science*. 2014;344(6186):913–917.
4. Assié G, et al. ARMC5 mutations in macronodular adrenal hyperplasia with Cushing's syndrome. *N Engl J Med*. 2013;369(22):2105–2114.
5. Louiset E, et al. Intraadrenal corticotropin in bilateral macronodular adrenal hyperplasia. *N Engl J Med*. 2013;369(22):2115–2125.
6. Lacroix A, Ndiaye N, Tremblay J, Hamet P. Ectopic and abnormal hormone receptors in adrenal Cushing's syndrome. *Endocr Rev*. 2001;22(1):75–110.
7. Lacroix A. Heredity and cortisol regulation in bilateral macronodular adrenal hyperplasia. *N Engl J Med*. 2013;369(22):2147–2149.
8. Hamet P, Larochelle P, Franks DJ, Cartier P, Bolte E. Cushing syndrome with food-dependent periodic hormonogenesis. *Clin Invest Med*. 1987;10(6):530–533.
9. Lacroix A, et al. Gastric inhibitory polypeptide-dependent cortisol hypersecretion — a new cause of Cushing's syndrome. *N Engl J Med*. 1992;327(14):974–980.
10. Reznik Y, et al. Food-dependent Cushing's syndrome mediated by aberrant adrenal sensitivity to gastric inhibitory polypeptide. *N Engl J Med*. 1992;327(14):981–986.
11. El Ghorayeb N, Bourdeau I, Lacroix A. Multiple aberrant hormone receptors in Cushing's syndrome. *Eur J Endocrinol*. 2015;173(4):M45–M60.
12. Boylan MO, Jepeal LI, Wolfe MM. Sp1/Sp3 binding is associated with cell-specific expression of the glucose-dependent insulinotropic polypeptide receptor gene. *Am J Physiol Endocrinol Metab*. 2006;290(6):E1287–E1295.
13. Almeida MQ, et al. Integrated genomic analysis of nodular tissue in macronodular adrenocortical hyperplasia: progression of tumorigenesis in a disorder associated with multiple benign lesions. *J Clin Endocrinol Metab*. 2011;96(4):E728–E738.
14. Ghezraoui H, et al. Chromosomal translocations in human cells are generated by canonical nonhomologous end-joining. *Mol Cell*. 2014;55(6):829–842.
15. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. *Nature*. 2012;489(7414):57–74.
16. Trivellini G, et al. Gigantism and acromegaly due to Xq26 microduplications and GPR101 mutation. *N Engl J Med*. 2014;371(25):2363–2374.
17. Grieco M, et al. PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. *Cell*. 1990;60(4):557–563.
18. Lifton RP, et al. A chimaeric 11 beta-hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. *Nature*. 1992;355(6357):262–265.
19. Motokura T, et al. A novel cyclin encoded by a bcl1-linked candidate oncogene. *Nature*. 1991;350(6318):512–515.
20. Malovannaya A, et al. Analysis of the human endogenous coregulator complexome. *Cell*. 2011;145(5):787–799.
21. Gong F, et al. Screen identifies bromodomain protein ZMYND8 in chromatin recognition of transcription-associated DNA damage that promotes homologous recombination. *Genes Dev*. 2015;29(2):197–211.
22. Swords FM, et al. The aberrant expression of the gastric inhibitory polypeptide (GIP) receptor in adrenal hyperplasia: does chronic adrenocorticotropin exposure stimulate up-regulation of GIP receptors in Cushing's disease? *J Clin Endocrinol Metab*. 2005;90(5):3009–3016.
23. Teo AE, et al. Pregnancy, primary aldosteronism, and adrenal CTNNB1 mutations. *N Engl J Med*. 2015;373(15):1429–1436.
24. N'Diaye N, Hamet P, Tremblay J, Boutin JM, Gaboury L, Lacroix A. Asynchronous development of bilateral nodular adrenal hyperplasia in gastric inhibitory polypeptide-dependent cushing's syndrome. *J Clin Endocrinol Metab*. 1999;84(8):2616–2622.
25. Lampron A, Bourdeau I, Hamet P, Tremblay J, Lacroix A. Whole genome expression profiling of glucose-dependent insulinotropic peptide (GIP)- and adrenocorticotropin-dependent adrenal hyperplasias reveals novel targets for the study of GIP-dependent Cushing's syndrome. *J Clin Endocrinol Metab*. 2006;91(9):3611–3618.
26. Bertherat J, et al. In vivo and in vitro screening for illegitimate receptors in adrenocorticotropin-independent macronodular adrenal hyperplasia causing Cushing's syndrome: identification of two cases of gonadotropin/gastric inhibitory polypeptide-dependent hypercortisolism. *J Clin Endocrinol Metab*. 2005;90(3):1302–1310.
27. Louiset E, et al. Expression of serotonin7 receptor and coupling of ectopic receptors to protein kinase A and ionic currents in adrenocorticotropin-independent macronodular adrenal hyperplasia causing Cushing's syndrome. *J Clin Endocrinol Metab*. 2006;91(11):4578–4586.
28. Berthon A, Drelon C, Val P. Pregnancy, primary aldosteronism, and somatic CTNNB1 mutations. *N Engl J Med*. 2016;374(15):1493–1494.
29. Espiard S, et al. ARMC5 mutations in a large cohort of primary macronodular adrenal hyperplasia: clinical and functional consequences. *J Clin Endocrinol Metab*. 2015;100(6):E926–E935.
30. Mazzucco TL, Chabre O, Sturm N, Feige JJ, Thomas M. Ectopic expression of the gastric inhibitory polypeptide receptor gene is a sufficient genetic event to induce benign adrenocortical tumor in a xenotransplantation model. *Endocrinology*. 2006;147(2):782–790.
31. de Herder WW, et al. Food-dependent Cushing's syndrome resulting from abundant expression of gastric inhibitory polypeptide receptors in adrenal adenoma cells. *J Clin Endocrinol Metab*. 1996;81(9):3168–3172.
32. N'Diaye N, Tremblay J, Hamet P, De Herder WW, Lacroix A. Adrenocortical overexpression of gastric inhibitory polypeptide receptor underlies food-dependent Cushing's syndrome. *J Clin Endocrinol Metab*. 1998;83(8):2781–2785.
33. Luton JP, Bertherat J, Kuhn JM, Bertagna X. [Aberrant expression of the GIP (gastric inhibitory polypeptide) receptor in an adrenal cortical adenoma responsible for a case of food-dependent Cushing's syndrome]. *Bull Acad Natl Med*. 1998;182(9):1839–1849.
34. Groussin L, et al. The ectopic expression of the gastric inhibitory polypeptide receptor is frequent in adrenocorticotropin-

- independent bilateral macronodular adrenal hyperplasia, but rare in unilateral tumors. *J Clin Endocrinol Metab.* 2002;87(5):1980–1985.
35. Lampron A, et al. Regulation of aldosterone secretion by several aberrant receptors including for glucose-dependent insulinotropic peptide in a patient with an aldosteronoma. *J Clin Endocrinol Metab.* 2009;94(3):750–756.
  36. Preumont V, Mermejo LM, Damoiseaux P, Lacroix A, Maiter D. Transient efficacy of octreotide and pasireotide (SOM230) treatment in GIP-dependent Cushing's syndrome. *Horm Metab Res.* 2011;43(4):287–291.
  37. Doppman JL, Chrousos GP, Papanicolaou DA, Stratakis CA, Alexander HR, Nieman LK. Adrenocorticotropin-independent macronodular adrenal hyperplasia: an uncommon cause of primary adrenal hypercortisolism. *Radiology.* 2000;216(3):797–802.
  38. Lacroix A, Feelders RA, Stratakis CA, Nieman LK. Cushing's syndrome. *Lancet.* 2015;386(9996):913–927.
  39. Funder JW, et al. The management of primary aldosteronism: case detection, diagnosis, and treatment: an Endocrine Society Clinical practice guideline. *J Clin Endocrinol Metab.* 2016;101(5):1889–1916.