Rare germline mutations identified by targeted next-generation sequencing of susceptibility genes in pheochromocytoma and paraganglioma

Supplemental Information

Supplementary Materials and Methods

Samples from PCC and PGL patients

Tumor samples (72 PCCs and 14 PGLs) were obtained from 86 unselected patients operated at the Karolinska University Hospital, Stockholm, Sweden, during the period 1986-2011. Clinical data for subsets of the cases have been previously published (1-3) and are detailed here in Table S1. Eighteen of the tumors were from patients with a known syndromic form of the disease (nine MEN2A, two VHL, three NF1) or a known mutation (four with *SDHB* mutation (1)). The remaining tumors (58 PCCs and 10 PGLs) were from apparently sporadic cases, i.e. non-familial and without syndromic features.

Tumors were histopathologically diagnosed as PCC or PGL in the routine setting according to the WHO classification (4). All cases were retrospectively characterized concerning gender, age at surgery, plasma- or urine hormone levels prior to surgery, tumor location, weight and size, associated syndrome, other diseases, follow-up time and outcome (Table S1). Tumor malignancy was assessed based on the presence of metastasis according to the WHO criteria (4) as well as by incorporating extensive local invasion from the AFIP criteria (5). Frozen tissue samples were collected through an established biobank system where an experienced endocrine pathologist identified the tumor and normal tissue macroscopically. Included samples had been snap-frozen in liquid nitrogen after surgery and stored in -80°C without thawing. For tumors with identified mutations, corresponding blood samples or normal frozen or paraffin-embedded tissue was obtained whenever available.

As additional sensitivity controls, three PCC samples with known *NF1* alterations from a previous study (6) were included in the NGS analysis: one with a germline mutation, one with a somatic mutation and one with a whole-gene deletion of *NF1* but no detected point mutation.
All samples were collected and studied with informed consent and approval from the local ethic committees.

DNA and RNA preparation

DNA was extracted from frozen tissue samples using a DNeasy Blood and tissue kit (Qiagen) and from blood samples using a Blood DNA Midi kit (Qiagen). Tumor DNA samples were quantified using a Quant-iT PicoGreen dsDNA assay (Invitrogen) with fluorescence measured in a VICTOR^3 plate reader (PerkinElmer) and a standard curve prepared from lambda DNA (Invitrogen). Samples were diluted to 50 ng/µl prior to enrichment and sequencing.

Total RNA was extracted from frozen tumor samples using a *mir*Vana miRNA Isolation Kit (Ambion, Life technologies). RNA samples were quantified using NanoDrop spectrophotometer (ND-1000).

RNA quality was measured using a Bioanalyzer 2100 (Agilent) which showed RNA Integrity Numbers (RIN) >7 for samples included in the study (RIN values for subsets of cases have been previously published (7)). One micro gram of total RNA was transcribed into cDNA with the Maxima First Strand cDNA synthesis kit (Thermo Scientific).

Library preparation and next-generation sequencing

Probes for targeted sequencing with the TruSeq Custom Amplicon kit (Illumina) were designed to cover the genes *EGLN1*, *KIF1Bβ*, *MAX*, *MEN1*, *NF1*, *RET*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127* and *VHL* (Table S2 and S3) including exon-intron boundaries, using the online DesignStudio software (Illumina). For *RET*, the hot-spot exons 8, 10, 11, 13, 14, 15 and 16 (8) were targeted. In all, the targeted regions included 176 exons and specific probes could be designed for 99% of the target sequence, but design was not possible for exons 9 and 10 of *MEN1* which therefore had to be excluded. A built-in function was used to avoid known SNPs in the probe sites. In total, the custom-designed oligo pool contained probes for 272 amplicons with an average length of 250 bp (longer exons were covered by overlapping amplicons). For each sample, 250 ng of DNA was used for library preparation. The DNA library was prepared according to the manufacturer´s protocol which included oligo probe hybridization to genomic DNA, extension-ligation of the bound oligos, PCR-amplification of the extension-ligation products with addition of barcodes for multiplexing, and finally normalization and pooling of samples. Two library pools of 48 samples, each including one Illumina TruSeq control sample, were prepared and sequenced in two separate runs on a MiSeq sequencer (Illumina), using 2x150 bp paired end reads. In total, 96 samples were sequenced (86 from the study cohort, three *NF1*-mutated sensitivity controls, two Illumina controls and five patient samples for clinical genetic testing, not included in this research study). Obtained raw sequencing data is available upon request.

Bioinformatical analysis

Alignment of NGS data to the human reference genome, UCSC hg19, and variant calling, including annotation of known SNPs, were performed in the MiSeq Reporter Software v. 2.1.43 (Illumina), using the Smith-Waterman algorithm and the Somatic Variant Caller algorithm, respectively. The threshold for insertions and deletions allowed during the alignment was increased from the default value of 25 bp to 100 bp. This was done to increase the capacity to detect larger insertions and deletions, as we noted that these were otherwise missed or incorrectly aligned and annotated. However, it should be noted that the length of detectable insertions and deletions is still limited by the read length of the technology. Quality and variant evaluation was performed in the Amplicon Viewer software v. 1.1.0 (Illumina). Sequence variants that were detected in more than 10% of the reads of one sample were checked against all samples and then against Ensembl to investigate potential mutation effect (e.g. amino acid substitution) and population frequencies. Common polymorphisms, silent sequence variants and missense variants that were reported in the HapMap or 1000 Genomes projects were excluded, except for very rare (allele frequency ≤ 0.02) missense variants which are listed separately (Table S4). Remaining variants were classified as mutations. Their effects were predicted with two different algorithms: PolyPhen-2 (9) and MutationTaster (10). Deletions or insertions were double-checked by exporting alignment (.bam) files for inspection of sequence reads in Integrative Genomics Viewer v. 2.3.8 (11).

Statistical analysis

Statistical analyses were carried out in GraphPad Prism v. 6.02. Student's t-test was performed to compare the age of patients with and without germline mutations. For further analysis of age and tumor weight, analysis of variance (ANOVA) was used to test for differences in means between genotype groups. If significant, a post-hoc test with Tukey correction for multiple comparisons was performed to compare pairs of groups. For categorical data (malignancy, tumor type and hormone levels), Fisher's exact test was performed to compare each group that showed a trend (e.g. malignancy in *SDHB*-mutated cases) against the rest of the cohort. Hormone levels were also compared after grouping tumors into two groups (Cluster 1: *VHL*/*SDHx*/*EPAS1* and Cluster 2: *RET*/*NF1*/*TMEM127*/*KIF1Bβ*) based on their genotype, according to the previous knowledge of gene expression patterns (12-16). Significance was accepted at P-values <0.05.

Sanger sequencing

Direct Sanger sequencing was applied to validate mutations in tumor DNA and to investigate mutational status in corresponding constitutional DNA from blood or normal tissue samples. Primer design was aided by the Primer3 software (17), and care was taken to avoid SNPs (reported in Ensembl) in the primer sites. Amplification of known pseudogenes was avoided by specificity-screening in the BiSearch software (18). Approximately 30 ng of DNA was PCR-amplified for 35 cycles using HotStar Taq Polymerase (Qiagen) and PCR products were purified with ExoSAP-IT (GE Healthcare). Dideoxynucleotide chain termination was performed using BigDye Terminator 3.1 (Applied Biosystems) and capillary electrophoresis was performed on a 3500 Genetic Analyzer (Applied Biosystems). Sequences were analyzed by visual inspection in Sequence Scanner v. 1.0 (Applied Biosystems) and by alignment to the Ensembl sequence using the NCBI BLAST tool. In cases of probable or potential splice-site mutations, *NF1* cDNA was sequenced as previously described (6). Sanger sequencing was also applied for exon 1 and exon 8 of *NF1* for which the MiSeq sequencing did not yield results (Table S5). As the involvement of the *EPAS1* gene was not known when we designed our NGS assay, the mutation-prone exons 9 and 12 of *EPAS1* were analyzed separately with Sanger sequencing, using previously described methodology (unpublished data, revised manuscript under review). In one case with two different *EPAS1* mutations, cloning and sequencing of *EPAS1* cDNA was performed to determine if the mutations occurred in *cis* or *trans*, using previously described methodology (6).

Mutations detected in constitutional DNA are termed "germline mutations" throughout the paper, though it should be noted that it is unknown whether they were inherited or *de novo* and whether they are present in the germ cells.

Table S1. Details of cases included in the study.

Case ID	Mutation(s)	Gender	Age at surgery	Syndrome	Tumor weight [g]	Tumor size [cm]	EPI[a]	NE[a]	DA[a]	Tumor type	Malignancy AFIP	Malignancy WHO	Metastasis (M) or relapse	Multi-focal	Other cancers or poly-cythemia	Follow up time [months]	Outcome	CIMP
1		F	58		1350	16x10x4	E	E	-	PCC	Benign	Benign			Not found	>200+	AWOD	No[c]
2		F	22		133	5x5x6	N	N	N	PCC	Benign	Benign			Not found	>200+	AWOD	No[c]
3		F	46		640	10x10x9	E	E	-	PCC	Benign	Benign			DCIS	>200+	AWOD	No[c]
4		F	69		22	3x3x4	N	E	-	PCC	Benign	Benign			Not found	192	DOC	No[c]
5	*RET*	M	53	MEN2A	32	3x3x4	N	E	-	PCC	Benign	Benign		Bilateral	MTC	>200+	AWOD	No[b]
6		F	44		-	2x2x1	N	E	-	PCC	Benign	Benign			IDC	>200+	AWOD	No[c]
7	*KIF1Bβ*	F	54		16	3x3x2.5	N	E	-	PCC	Benign	Benign			Endometrial carcinoma	>200+	AWOD	No[c]
8	*NF1*	M	59	NF1	12	1.5x2x2	E	N	-	PCC	Benign	Benign			Fibromas	144	DOC	No[c]
9		F	56		11	1.5x2x2	N	N	-	PCC	Malignant	Benign			Mesotelioma	1	DOC	No[b]
10		F	75		19	2x3x3	N	E	-	PCC	Benign	Benign			Melanoma	132	DOC	No[c]
11		M	45		22	3x3.5x3	E	E	-	PCC	Benign	Benign			Not found	>200+	AWOD	No[c]
12		M	48		1993	15x13x9	E	E	E	PCC	Benign	Benign			Not found	>200+	AWOD	No[c]
13	*RET*	F	45	MEN2A	21	3x2x2	-	-	-	PCC	Benign	Benign		Bilateral	MTC	>200+	AWOD	No[c]
14		F	52		74	5.5x5.5x3.5	-	-	-	PCC	Benign	Benign			Leiomyoma	>200+	AWOD	No[c]
15		F	37		59	-	E	E	N	PCC	Benign	Benign			Not found	>200+	AWOD	No[c]
16		F	30		34	6x3.5x3.5	N	E	N	PCC	Benign	Benign			Not found	228	AWOD	No[c]
17		M	58		59	7.5x7.5x3.5	N	E	-	PCC	Benign	Benign			Not found	>200+	AWOD	No[c]
18	*NF1, KIF1Bβ*	F	46	NF1	54	4x4.5x4.5	E	E	-	PCC	Benign	Benign			Fibromas	240	DOC	No[b]
19		M	37		135	10x5x5.5	E	E	N	PCC	Benign	Benign			Seminoma	>200+	AWOD	Not run
20	*NF1*	F	68		23	3.5x3x2	-	-	-	PCC	Benign	Benign			Not found	72	DOC	No[c]
21	*VHL*	M	31	VHL	25	4x3x2.5	N	N	N	PCC	Benign	Benign			Not found	1	AWOD	No[c]
22		M	35		7	2x2x2	E	N	-	PCC	Benign	Benign			Basal-cell carcinoma	>200+	AWOD	Not run
23	*RET*	F	41	MEN2A	4	4x2x1	E	N	-	PCC	Benign	Benign			MTC	>200+	AWOD	No[b]
24	*NF1*	F	74		-	5x3x2	-	-	-	PCC	Benign	Benign			PHPT, CLL, IDC	132	DOC	No[c]
25	*VHL*	M	13	VHL	42	5x5x3	N	N	-	PCC	Malignant	Benign			Not found	>200+	AWOD	No[c]
26	*RET*	F	65		-	6x5x5.5	-	-	-	PCC	Benign	Benign			Not found	>200+	AWOD	No[c]
28		F	77		314	10x10x7	-	-	-	PCC	Malignant	Benign			Not found	84	DOC	No[c]

29	*RET*	F	47		32	4.5x4x3	N	N	N	PCC	Benign	Benign			MTC, mucoepi-dermoid carcinoma (salivary gl)	>200+	AWOD	No[c]
30	*RET*	F	44	MEN2A	3	2.5x2.5x2	N	E	N	PCC	Benign	Benign			MTC	>200+	AWOD	No[b]
31	*RET*	F	34	MEN2A	29	5x0.5x2	-	-	-	PCC	Benign	Benign			MTC	>200+	AWOD	No[b]
32		F	44		7	6x5.5x6.5	N	E	E	PCC	Malignant	Malignant	M: 5 years later		Not found	>200+	AWOD	No[c]
34	*RET*	M	41	MEN2A	183	5x5x5	E	E	N	PCC	Benign	Benign		Bilateral	MTC, PHPT, PCC x3	192+	AWOD	No[c]
36		M	40		434	11x10x9	E	E	E	PCC	Malignant	Malignant	M: Simultaneous		Not found	0	DOD	No[c]
37		F	52		38	4x3.5x3	E	E	N	PCC	Benign	Benign			Not found	192+	AWOD	No[c]
38	*NF1*	M	53		33	3x3x3	E	E	N	PCC	Benign	Benign			Not found	190+	AWOD	No[c]
39	*NF1*	F	69		22	3x3x0.5	E	N	N	PCC	Malignant	Benign			Not found	168+	AWOD	No[c]
43	*RET*	M	69		85	4.5x6.5x4	N	-	N	PCC	Benign	Benign			Not found	168+	AWOD	No[c]
44	*NF1*	F	50		17	2x1.5x2	N	N	N	PCC	Benign	Benign			Not found	156+	AWOD	No[c]
46		F	73		66	-	N	N	N	PCC	Benign	Benign			Melanoma	144+	AWOD	No[c]
47	*NF1*	M	63		17	2x2x2.5	N	E	N	PCC	Benign	Benign			Not found	144+	AWOD	Not run
48	*NF1*	M	52	NF1	165	8x8x6	N	N	E	PCC	Benign	Benign			Fibromas, PHPT	132+	AWOD	No[c]
49		M	76		78	4.5x4.5x4.5	N	N	N	PCC	Benign	Benign			Basal-cell carcinoma	132	DOC	No[c]
50		M	66		26	4x3x2.5	E	E	N	PCC	Benign	Benign			Prostate carcinoma	132+	AWOD	No[c]
51		F	65		10	2x2x2	N	E	-	PCC	Malignant	Benign			Not found	132+	AWOD	No[b]
52	*NF1*	M	45		16	2.5x1.5x1	N	E	-	PCC	Benign	Benign			Not found	132+	AWOD	Not run
58		M	27		172	9x5x4.5	E	E	-	PCC	Benign	Benign			Not found	108+	AWOD	No[c]
61	*NF1*	F	74		90	6x7x5	N	E	-	PCC	Benign	Benign			Basal-cell carcinoma	108+	AWOD	Not run
62		F	64		15	4.2x3x1.8	N	E	-	PCC	Benign	Benign			IDC, melanoma	108+	AWOD	Not run
63	*RET*	F	45	MEN2A	19	7x3.5x2.5	N	E	-	PCC	Benign	Benign			MTC	96+	AWOD	Not run
64	*RET*	M	74		11	1.8x2x1.6	N	E	-	PCC	Benign	Benign			Not found	96+	AWOD	Not run
65		M	39		33	2.5x3x2.5	N	N	-	PCC	Benign	Benign			Not found	96+	AWOD	Not run
71		M	65		23	2x2x2	N	E	-	PCC	Benign	Benign			Not found	84+	AWOD	Not run
72	*RET, NF1*	F	30	MEN2A	7	1.6x3x1	E	E	-	PCC	Benign	Benign	Relapse		MTC, PCC relapse	84+	AWOD	Not run

74		M	76		14	2x2x2	N	E	-	PCC	Malignant	Benign			Not found	72+	AWOD	No[b]
77	*NF1*	M	66		29	2x2x2	E	E	-	PCC	Benign	Benign			Not found	60+	AWOD	Not run
81	*RET*	M	33	MEN2A	10	2x2x2	E	E	-	PCC	Benign	Benign			MTC	48+	AWOD	Not run
82	*EPAS1*	F	64		11	1x1x1	N	E	-	PCC	Benign	Benign			Colon adenoma	48+	AWOD	Not run
83	*NF1*	F	41		245	12x8x8	N	N	-	PCC	Benign	Benign			Not found	48+	AWOD	Not run
84		M	71		263	11x7x5	N	E	-	PCC	Benign	Benign			Renal-cell carcinoma	40+	AWOD	Not run
85		F	62		31	5.5x4x2.5	N	E	-	PCC	Malignant	Benign			Endometrial carcinoma	40+	AWOD	No[b]
86	*NF1, MAX*	F	57		22	6.5x3.5x2	E	E	-	PCC	Benign	Benign			Not found	36+	AWOD	Not run
87	*TMEM127*	F	55		48	5.5x4x2.5	N	E	-	PCC	Benign	Benign			Not found	36+	AWOD	Not run
88		M	73		151	7x6x5	E	E	-	PCC	Benign	Benign			Not found	36+	AWOD	Not run
89	*NF1*	F	36		28	6x4x3	E	E	-	PCC	Benign	Benign			Not found	36+	AWOD	Not run
90		M	51		17	7x3.5x1.5	N	E	-	PCC	Benign	Benign			Not found	36+	AWOD	Not run
91		M	46		40	4x3x3	N	E	-	PCC	Benign	Benign			Not found	30+	AWOD	Not run
92		F	57		31	4.5x3.7x2.3	N	E	-	PCC	Benign	Benign			Not found	30+	AWOD	Not run
93	*NF1*	F	57		-	6x4.5x3.5	E	E	-	PCC	Benign	Benign			Not found	30+	AWOD	Not run
94	*EPAS1*	M	67		-	4.2x3.8x2.8	N	E	-	PCC	Benign	Benign			PCV, meningioma, duodenal carcinoma	30+	AWOD	Not run
95	*SDHA*	M	64		66	6x5x3	N	E	-	PCC	Benign	Benign			Not found	24+	AWOD	Not run
96	*SDHA, VHL, EGLN1*	M	47		23	3.6x2.5x1.6	-	-	-	PCC	Benign	Benign			Not found	24+	AWOD	Not run
97		F	53		51	6x5x4	E	E	-	PCC	Benign	Benign			Not found	18+	AWOD	Not run
100		M	26		50	3x3x3	-	-	-	PGL	Malignant	Malignant	M: Simultaneous		Not found	0	DOC	No[c]
101	*SDHB*	M	14		26	4.5x3.5x2	N	E	-	PGL	Benign	Benign			Not found	>200	AWOD	CIMP[c]
102	*SDHB*	F	41		103	7x7.5x3.5	N	E	E	PGL	Malignant	Malignant	M: 4 years later		Not found	156	DOC	CIMP[c]
103	*SDHB*	M	25		16	4.5x2x2x	N	E	N	PGL	Malignant	Benign	Relapse		PGL relapse	>200+	AWOD	CIMP[c]
104		F	57		75	7x6x4.5	E	E	N	PGL	Malignant	Benign			Not found	>200+	AWOD	No[c]
105	*SDHB*	F	35		-	3x2x3	N	N	N	PGL	Malignant	Malignant	M: 12 years later		Cholesteatomas x 3	>200+	AWOD	No[b]
106	*EPAS1*	M	24		23	5x3.5x3	N	N	N	PGL	Benign	Benign		Multiple	PCV	>200+	AWOD	No[c]
107	*EPAS1*	M	73		13	2.5x4.5x2	N	E	N	PGL	Benign	Benign			Rectal carcinoma	192	DOC	No[c]

108	*VHL*	F	65		47	5.5x3x3	N	E	N	PGL	Malignant	Benign			Basal-cell carcinoma	144	AWOD	No[c]
109	*EPAS1*	M	46		8	3x2x2	E	E	N	PGL	Benign	Benign			Not found	72	AWOD	No[c]
110		F	27		15	2x3x3	-	-	-	PGL	Malignant	Malignant	M: Simultaneous		Not found	50	DOD	No[b]
111		M	61		36	4.5x4x3	E	E	E	PGL	Malignant	Benign			Not found	144+	AWOD	No[b]
113	*SDHB*	M	26		11	4x3x3	N	E	E	PGL	Malignant	Malignant	M: Simultaneous	Multiple	Not found	24	DOD	CIMP[c]
112		M	42		173	5x4.5x2.5	-	-	-	PGL	Malignant	Malignant	M: 4 years later		Not found	168	DOC	CIMP[c]

AFIP, malignancy according to AFIP (5); AWOD, alive with no evidence of disease; CIMP, CpG island methylator phenotype; CLL, chronic lymphoid leukemia; DA, dopamine levels in urine before surgery; DCIS, ductal carcinoma in situ ; DOC, dead of other cause; DOD, dead of disease; E, elevated; EPI, epinephrine levels in plasma or urine before surgery; F, female; IDC, invasive ductal carcinoma; M, male; MEN 2A, multiple endocrine neoplasia type 2A; MTC, medullary thyroid carcinoma; NE, norepinephrine levels in plasma or urine before surgery; NF1, neurofibromatosis type 1 (von Recklinghausen syndrome); N, normal; PCC, pheochromocytoma; PCV, polycythemia vera; PGL, paraganglioma; PHPT, primary hyperparathyroidism; VHL, von Hippel-Lindau syndrome; WHO, malignancy according to WHO (4); +, persisted follow-up; -, no value.

[a]Since different methods had been used for measurements of hormone levels during the period, each case was classified as having a "normal" or "elevated" level according to the appropriate reference value for each method. [b]Published in reference (1). [c]Published in reference (19).

Table S2. Details of genes included in the study.

Gene	Full official gene name (according to NCBI)	Chromosomal location	Number of exons	Coding sequence [nt]	Protein name (according to NCBI)
EGLN1 (*PHD2*)	Egl-9 family hypoxia-inducible factor 1	1q42.1	5	1281	Egl nine homolog 1 (also known as HIF prolyl hydroxylase 2)
EPAS1 (*HIF2A*)	Endothelial PAS domain protein 1	2p.21	16	2613	Endothelial PAS domain-containing protein 1 (also known as Hypoxia-inducible factor 2 alpha)
KIF1Bβ	Kinesin family member 1B (β)	1p36.22	47	5313	Kinesin-like protein KIF1B, Beta
MAX	MYC associated factor X	14q23.3	5	483	Protein MAX
MEN1	Multiple endocrine neoplasia I	11q13.1	10	1833	Menin
NF1	Neurofibromin 1	17q11.2	58	8520	Neurofibromin
RET	RET proto-oncogene	10q11.21	20	3345	Proto-oncogene tyrosine-protein kinase receptor Ret
SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein	5p15.33	15	1995	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial
SDHB	Succinate dehydrogenase complex, subunit B, iron sulfur	1p36.13	8	843	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial
SDHC	Succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa	1q23.3	6	510	Succinate dehydrogenase cytochrome b560 subunit, mitochondrial
SDHD	Succinate dehydrogenase complex, subunit D, integral membrane protein	11q23.1	4	480	Succinate dehydrogenase [ubiquinone] cytochrome b small subunit, mitochondrial
SDHAF2 (*SDH5*)	Succinate dehydrogenase complex assembly factor 2	11q12.2	4	501	Succinate dehydrogenase assembly factor 2, mitochondrial
TMEM127	Transmembrane protein 127	2q11.2	4	717	Transmembrane protein 127
VHL	von Hippel-Lindau tumor supresssor, E3 ubiquitin protein ligase	3p25.3	3	642	von Hippel-Lindau disease tumor suppressor

nt, nucleotides.

Table S3. Summary of probe design for targeted regions from DesignStudio (Illumina).

Target	Chr	Start [bp]	Stop [bp]	Length [bp]	Padding[a] [bp]	Amplicons	Avoid SNPs	Coverage [%]	DS score	Comment
KIF1B_Exon_2063862	1	10270764	10270936	173	15	2	Yes	100	60	
KIF1B_Exon_2063781	1	10292308	10292492	185	15	2	Yes	100	87	
KIF1B_Exon_2063698	1	10316305	10316381	77	15	1	Yes	100	80	
KIF1B_Exon_2064531	1	10318551	10318730	180	15	2	Yes	100	98	
KIF1B_Exon_2061430	1	10321963	10322028	66	15	1	Yes	100	96	
KIF1B_Exon_2063952	1	10327438	10327616	179	15	2	Yes	100	88	
KIF1B_Exon_2061933	1	10328210	10328321	112	15	1	Yes	100	96	
KIF1B_Exon_2061861	1	10331560	10331637	78	15	1	Yes	100	96	
KIF1B_Exon_2064043	1	10332299	10332364	66	15	1	Yes	100	95	
KIF1B_Exon_2062103	1	10335486	10335561	76	15	1	Yes	100	96	
KIF1B_Exon_2061688	1	10336379	10336457	79	15	1	Yes	100	96	
KIF1B_Exon_2062104	1	10338044	10338186	143	15	1	Yes	100	95	
KIF1B_Exon_2061862	1	10342458	10342591	134	15	1	Yes	100	96	
KIF1B_Exon_2062105	1	10351140	10351219	80	15	1	Yes	100	96	
KIF1B_Exon_2063782	1	10352105	10352180	76	10	1	Yes	100	96	
KIF1B_Exon_2063953	1	10355144	10355223	80	15	1	Yes	100	96	
KIF1B_Exon_2064044	1	10355718	10355824	107	15	1	Yes	100	80	
KIF1B_custom-merged	1	10356630	10357314	685		5	Yes	100	94	
KIF1B_Exon_2207886	1	10380101	10380194	94	15	1	Yes	100	98	
KIF1B_Exon_2208477	1	10381767	10381915	149	10	1	Yes	100	96	
KIF1B_Exon_2209899	1	10383942	10384120	179	20	2	Yes	100	96	
KIF1B_Exon_2209740	1	10384816	10384953	138	15	2	Yes	100	98	
KIF1B_Exon_2209900	1	10386169	10386417	249	10	2	Yes	100	80	
KIF1B_Exon_2208478	1	10394578	10394696	119	15	1	Yes	100	96	
KIF1B_Exon_2208055	1	10396715	10396800	86	15	1	Yes	100	96	
KIF1B_custom-merged	1	10397112	10397611	500		4	Yes	100	83	
KIF1B_Exon_2209741	1	10399827	10399917	91	15	1	Yes	100	96	
KIF1B_Exon_2207465	1	10402108	10402226	119	15	1	Yes	100	95	
KIF1B_Exon_2207716	1	10403290	10403345	56	15	1	Yes	100	96	
KIF1B_Exon_2207386	1	10405903	10406011	109	15	1	Yes	100	96	
KIF1B_Exon_2209990	1	10407819	10407885	67	15	1	Yes	100	96	
KIF1B_Exon_2208143	1	10408707	10408791	85	15	1	No	100	80	SNPs allowed[b]
KIF1B_Exon_2207387	1	10412689	10412794	106	15	1	Yes	100	80	
KIF1B_Exon_2207717	1	10420987	10421101	115	15	1	Yes	100	96	
KIF1B_Exon_2207388	1	10421750	10421883	134	15	1	Yes	100	96	
KIF1B_Exon_2207887	1	10423291	10423452	162	0	1	Yes	70	96	
KIF1B_custom-merged	1	10425148	10425716	569		3	Yes	91	96	
KIF1B_Exon_2207888	1	10428525	10428596	72	15	1	Yes	100	96	
KIF1B_Exon_2209991	1	10431199	10431320	122	15	1	Yes	100	96	
KIF1B_Exon_2207889	1	10434374	10434523	150	15	1	Yes	100	96	
KIF1B_custom-merged	1	10434897	10435446	550		3	Yes	90	90	
KIF1B_3'_Exon_2207389	1	10436603	10436720	118	15	1	Yes	100	96	
SDHB_Exon_1959892	1	17345225	17345453	229	15	2	Yes	100	79	
SDHB_Exon_1959132	1	17349103	17349225	123	15	1	Yes	100	96	
SDHB_Exon_1959133	1	17350468	17350569	102	15	1	Yes	100	96	
SDHB_Exon_1959893	1	17354244	17354360	117	15	1	Yes	100	95	
SDHB_Exon_1957709	1	17355095	17355231	137	15	1	Yes	100	96	
SDHB_Exon_1959973	1	17359555	17359640	86	15	1	Yes	100	96	
SDHB_Exon_1957299	1	17371256	17371383	128	15	1	Yes	100	95	
SDHB_Exon_1957375	1	17380443	17380665	223	15	2	Yes	100	60	
SDHC_Exon_1841603	1	161284166	161284215	50	15	1	Yes	100	60	
SDHC_Exon1843529	1	161293218	161293466	249	0	1	Yes	84	60	
SDHC_Exon_1841262	1	161298186	161298287	102	10	1	Yes	100	96	
SDHC_Exon_1841957	1	161310384	161310445	62	15	1	Yes	100	96	

SDHC_Exon_1841263	1	161326467	161326630	164	10	1	Yes	100	96	
SDHC_3'_Exon_1841958	1	161332119	161332300	182	15	2	Yes	100	78	
EGLN1_3'_Exon_2158355	1	231502110	231502221	112	15	1	Yes	100	95	
EGLN1_Exon_2156091	1	231503315	231503382	68	15	1	Yes	100	95	
EGLN1_Exon_2158356	1	231506308	231506444	137	15	1	Yes	100	96	
EGLN1_Exon_2156335	1	231509726	231509845	120	15	1	Yes	100	96	
EGLN1_ex1_Exon_2155751	1	231556744	231557743	1000	15	7	Yes	100	63	
TMEM127_3prim_Exon_2091697	2	96919349	96919853	505	17	4	Yes	100	88	
TMEM127_Exon_2088569	2	96920571	96920735	165	15	2	Yes	100	78	
TMEM127_Exon_2088570	2	96930876	96931250	375	15	3	Yes	100	66	
TMEM127_Exon_2088836	2	96931607	96931751	145	15	1	Yes	100	60	
VHL_Exon_2011217	3	10183320	10183871	552	15	3	Yes	100	60	
VHL_Exon_2267839	3	10188198	10188320	123	15	1	Yes	100	96	
VHL_3'_Exon_2011555	3	10191471	10191710	240	15	2	Yes	100	89	
SDHA_Exon_2025897	5	218356	218533	178	15	2	Yes	100	60	
SDHA_Exon_2025898	5	223597	223683	87	15	1	Yes	100	96	
SDHA_Exon_2024462	5	224475	224636	162	15	2	Yes	100	60	
SDHA_Exon_2026918	5	225534	225677	144	15	1	Yes	100	96	
SDHA_Exon_2024320	5	225998	226162	165	15	1	Yes	100	80	
SDHA_Exon_2024321	5	228300	228448	149	10	2	Yes	100	98	
SDHA_Exon_2024896	5	230991	231115	125	10	1	Yes	100	96	
SDHA_Exon_2025899	5	233592	233760	169	20	2	Yes	100	79	
SDHA_Exon_2024572	5	235259	235454	196	15	2	Yes	100	80	
SDHA_Exon_2027785	5	236543	236714	172	10	1	Yes	100	96	
SDHA_Exon_2027199	5	240473	240591	119	10	1	Yes	100	96	
SDHA_custom-merged	5	251092	251598	507		4	Yes	100	74	
SDHA_Exon_2024897	5	254508	254621	114	10	1	Yes	100	96	
SDHA_Exon_2024322	5	256449	256814	366	15	3	Yes	100	79	
RET_Exon_1929860	10	43607547	43607672	126	9	1	Yes	100	60	
RET_Exon_1929009	10	43609004	43609123	120	9	1	Yes	100	80	
RET_Exon_1931470	10	43609928	43610184	257	9	2	Yes	100	60	
RET_Exon_1927266	10	43613821	43613928	108	9	1	Yes	100	96	
RET_Exon_1927505	10	43614979	43615193	215	6	2	Yes	100	80	
RET_Exon_1927181	10	43615529	43615651	123	12	1	Yes	100	80	
RET_Exon_1929268	10	43617394	43617464	71	9	1	Yes	100	60	
SDHAF2_Exon_1833445	11	61197597	61197654	58	15	1	Yes	100	80	
SDHAF2_custom-merged	11	61205082	61205600	519		3	Yes	92	91	
SDHAF2_3prim_Exon_1840775	11	61213413	61213694	282	20	3	Yes	100	66	
MEN1_3prim_ex-merged *Excluded (see main text)*	11	64571674	64572759	1086	0	0	No	0	0	Excluded[c]
MEN1_Exon_1851016	11	64573107	64573242	136	15	1	Yes	100	60	
MEN1_Exon_1850844	11	64573704	64573840	137	15	1	Yes	100	80	
MEN1_custom-merged	11	64574473	64574701	229		2	Yes	100	78	
MEN1_custom-merged	11	64575014	64575581	568		4	Yes	100	75	
MEN1_Exon_1850913	11	64577122	64577604	483	15	4	Yes	100	65	
SDHD_Exon_1922582	11	111957571	111957683	113	15	1	Yes	100	96	
SDHD_Exon_1919386	11	111958581	111958697	117	15	1	Yes	100	96	
SDHD_Exon_1920069	11	111959591	111959735	145	15	1	Yes	100	96	
SDHD_3'_Exon_1920070	11	111965529	111965800	272	15	3	Yes	100	92	
MAX_3prim_Exon_1838797	14	65542958	65543381	424	10	3	Yes	100	79	
MAX_Exon_1838095	14	65544631	65544754	124	15	1	Yes	100	80	
MAX_Exon_1833728	14	65560426	65560533	108	15	1	Yes	100	80	
MAX_Exon_1936492	14	65568264	65568290	27	10	1	Yes	100	80	
MAX_Exon_1834823	14	65569022	65569227	206	20	2	Yes	100	70	
NF1_Exon_1842979	17	29421945	29422387	443	25	4	Yes	100	60	
NF1_Exon_1847907	17	29483001	29483144	144	25	2	Yes	100	96	
NF1_Exon_1843057	17	29486028	29486111	84	25	1	Yes	100	96	
NF1_Exon_1845671	17	29490204	29490394	191	15	2	Yes	100	98	
NF1_Exon_1848458	17	29496909	29497015	107	25	1	Yes	100	96	

NF1_custom-merged	17	29508415	29508828	414		3	Yes	100	96	
NF1_Exon_1844672	17	29509526	29509683	158	15	2	Yes	100	95	
NF1_Exon_1842980	17	29527440	29527613	174	25	2	Yes	100	79	
NF1_custom-merged	17	29528030	29528528	499		4	Yes	100	95	
NF1_Exon_1847748	17	29533258	29533389	132	25	1	Yes	100	96	
NF1_Exon_1847749	17	29541469	29541603	135	25	1	Yes	100	96	
NF1_Exon_1843198	17	29546023	29546136	114	25	1	Yes	100	96	
NF1_Exon_1844673	17	29548868	29548947	80	25	1	Yes	100	96	
NF1_Exon_1846784	17	29550462	29550585	124	25	1	Yes	100	96	
NF1_Exon_1847750	17	29552113	29552268	156	25	2	Yes	100	96	
NF1_Exon_1845915	17	29553453	29553702	250	20	3	Yes	100	91	
NF1_custom-merged	17	29554211	29554649	439		3	Yes	100	91	
NF1_Exon_1843370	17	29556043	29556483	441	25	3	Yes	100	96	
NF1_Exon_1845672	17	29556853	29556992	140	15	1	Yes	100	96	
NF1_Exon_1849263	17	29557278	29557400	123	15	1	Yes	100	96	
NF1_Exon_1842981	17	29557860	29557943	84	25	1	Yes	100	98	
NF1_Exon_1847908	17	29559091	29559207	117	25	1	Yes	100	96	
NF1_custom-merged	17	29559693	29560256	564		4	Yes	100	82	
NF1_custom-merged	17	29562604	29563064	461		4	Yes	100	96	
NF1_Exon_1843059	17	29576002	29576137	136	25	2	Yes	100	95	
NF1_Exon_2166252	17	29579956	29580018	63	25	1	Yes	100	96	
NF1_Exon_1848460	17	29585362	29585520	159	25	2	Yes	100	78	
NF1_Exon_1845041	17	29586050	29586147	98	20	2	Yes	100	77	
NF1_Exon_1843141	17	29587387	29587533	147	25	2	Yes	100	98	
NF1_Exon_1849265	17	29588729	29588875	147	25	1	Yes	100	96	
NF1_Exon_1845674	17	29592247	29592357	111	25	1	Yes	100	96	
NF1_Exon_1849266	17	29652838	29653270	433	20	3	Yes	95	85	
NF1_Exon_1847752	17	29654517	29654857	341	25	3	Yes	100	91	
NF1_Exon_1843475	17	29657314	29657516	203	25	2	Yes	100	78	
NF1_Exon_1843142	17	29661856	29662049	194	20	2	Yes	100	78	
NF1_custom-merged	17	29663326	29663957	632		4	Yes	100	98	
NF1_custom-merged	17	29664366	29665177	812		6	Yes	100	90	
NF1_Exon_1846508	17	29665722	29665823	102	25	1	Yes	100	96	
NF1_Exon_1847279	17	29667523	29667663	141	25	2	Yes	100	96	
NF1_Exon_1849267	17	29670027	29670153	127	25	1	Yes	100	98	
NF1_Exon_1843200	17	29676138	29676269	132	25	1	Yes	100	95	
NF1_Exon_1847753	17	29677201	29677336	136	25	1	Yes	100	95	
NF1_Exon_1843371	17	29679275	29679432	158	25	2	Yes	100	79	
NF1_Exon_1844675	17	29683478	29683600	123	25	1	Yes	100	96	
NF1_custom-merged	17	29683953	29684412	460		4	Yes	100	87	
NF1_Exon_1849268	17	29685498	29685640	143	25	2	Yes	100	97	
NF1_Exon_1844676	17	29685987	29686033	47	25	1	Yes	100	95	
NF1_Exon_1843201	17	29687505	29687721	217	20	2	Yes	100	77	
NF1_3'_Exon_1843143	17	29701031	29701440	410	25	4	Yes	100	78	

Chr, Chromosome; bp, base pairs; DS score, DesignStudio score estimating the relative enrichment performance (0-100) of each amplicon compared to all other amplicons in the pool.

[a]Padding refers to the number of flanking nucleotides that are included in the design for each exon to cover the exon-intron boundary. Between 10 and 25 nucleotides upstream and downstream of each exon were generally included, with the exception of long 5'- and 3'-UTRs, where the translation start site and stop site flanks were covered instead. Shorter flanking regions were allowed for the proto-oncogene *RET* and for two additional exons (in *KIF1B* and *SDHC*) where design was otherwise not possible. Exon positions were automatically derived from hg19 in DesignStudio (Illumina) except for regions termed "custom-merged" which were defined manually in the software to allow merging of adjacent exons and their flanking regions.

[b]SNPs allowed due to difficult target.

[c]Excluded after several designs had been attempted.

Table S4. Missense variants of unknown pathogenic significance identified by next-generation sequencing in 72 pheochromocytomas and 14 paragangliomas.

Sample(s)	Gene	Variant Nucleotide, protein	Predicted mutation impact PolyPhen-2	 MutationTaster	Allele frequency in cohort	Population allele frequency[a]
49	*VHL*	rs35460768 c.74C>T, p.Pro25Leu	Probably benign (0.000)	Polymorphism	0.006	0.004
3, 47, 61, 65	*SDHB*	rs33927012 c.487T>C, p.Ser163Pro	Probably benign (0.000)	Disease causing	0.023	0.013
18	*SDHD*	rs34677591 c.34G>A, p.Gly12Ser	Probably benign (0.005)	Disease causing	0.006	0.011
20, 85, 89, 93, 96	*KIF1Bβ*	rs77172218 c.4660G>A, p.Val1554Met	Probably benign (0.005)	Disease causing	0.029	0.017
17, 85, 100	*MEN1*	rs607969 c.512G>A, p.Arg171Gln	Probably damaging (0.995)	Polymorphism	0.017	0.020

Nucleotide and protein nomenclature were based on the following Ensembl transcript identifiers: *KIF1Bβ*: ENST00000263934, *MEN1*: ENST00000312049, *SDHB*: ENST00000375499, *SDHD*: ENST00000375549, *VHL*: ENST00000256474. The two prediction algorithms gave contradictory results for four of the five variants. The pathogenicity of the variant rs607969 (Arg171Gln) in *MEN1* has previously been disputed (20, 21).

[a]Population frequencies refer to a summary of all reported individuals of European ancestry in Ensembl. Two-tailed Fisher's exact tests were performed to compare allele frequencies between the cohort and the reported population, but no significant differences were found.

Table S5. Sanger sequencing of *NF1* exons that did not yield results with next-generation sequencing.

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature	Taq polymerase	Number of samples analyzed
1	CTTGCCTCTTCCCTC ACCTCAGCCTC [a]	ACCCCTCACCTCCC GCCTTGG [a]	67.5°C	MyTaq (Bioline)	78/86[b]
8	TTGCTTAAATGAAG TTCCATGTTT	TGCCTGGTATTATT TTCCCTCT	56°C	HotStar (Qiagen)	86/86

[a]Primers for exon 1 were designed according to a previous study (22).
[b]The *NF1* exon 1 proved difficult to analyze, probably due to the high GC-content of the promoter region overlapping with exon 1. Three different primer pairs were tested with two different enzymes and a temperature gradient (with and without DMSO) before obtaining specific amplification of the correct PCR product. The optimized conditions were: use of the above previously published primers (22), with MyTag polymerase (Bioline), an annealing temperature of 67.5°C and with 5% DMSO in the reaction mixture. For unknown reasons, amplification was not possible for eight samples.

Table S6. Mutations identified by next-generation sequencing in pheochromocytomas (PCCs) and paragangliomas (PGLs) from cases with syndromic disease or known germline mutations.

Sample	Tumor type	Clinical syndrome	Gene	Mutation	Protein alteration
5	PCC	MEN2A	*RET*	c.1900T>G	p.Cys634Gly
13	PCC	MEN2A	*RET*	c.1900T>G	p.Cys634Gly
23	PCC	MEN2A	*RET*	c.1900T>G	p.Cys634Gly
30	PCC	MEN2A	*RET*	c.1900T>G	p.Cys634Gly
31	PCC	MEN2A	*RET*	c.1900T>C	p.Cys634Arg
34	PCC	MEN2A	*RET*	c.1900T>C	p.Cys634Arg
63	PCC	MEN2A	*RET*	c.1900T>C	p.Cys634Arg
72	PCC	MEN2A	*RET* *NF1*	c.1858T>C c.7891A>G	p.Cys620Arg p.Thr2631Ala[a]
81	PCC	MEN2A	*RET*	c.1900T>G	p.Cys634Gly
21	PCC	VHL	*VHL*	c.217C>T	p.Gln73X
25	PCC	VHL	*VHL*	c.193T>G	p.Ser65Ala
8	PCC	NF1	*NF1*	c.2446C>T	p.Arg816X
18	PCC	NF1	*NF1* *KIF1Bβ*	c.586+5G>A c.1633G>A	p? (splice site mutation) p.Gly545Arg[b]
48	PCC	NF1	*NF1*	c.1971dupT	p.Leu658*fs*
101	PGL	(PGL4)	*SDHB*	c.190delG	Asp64*fs*
102	PGL	(PGL4)	*SDHB*	c.423+1G>A	p? (splice site mutation)
103	PGL	(PGL4)	*SDHB*	c.148_151dupGACA	p.Lys51*fs*
113	PGL	(PGL4)	*SDHB*	c.148_151dupGACA	p.Lys51*fs*

Nucleotide and protein nomenclature were based on the following Ensembl transcript identifiers: *KIF1Bβ*: ENST00000263934, *NF1:* ENST00000358273, *RET*: ENST00000355710, *SDHB*: ENST00000375499, *VHL*: ENST00000256474. All mutations were verified with Sanger sequencing and were, as expected, also detected in constitutional DNA (Figure S1), with the exception of case 25 where only tumor DNA was available. Nine cases carried *RET* missense mutations involving codon 634 in eight cases and codon 620 in one case, in agreement with the common alterations of *RET* exon 11 in MEN2. Two cases were identified with *VHL* mutations, three with *NF1* mutations and four with *SDHB* mutations. In addition, one of the NF1-associated tumors harboured a somatic missense *KIF1Bβ* mutation predicted to be damaging. One of the MEN2A cases also harboured a probably benign *NF1* variant in addition to the *RET* mutation.

[a]The *NF1* mutation was germline and was predicted as benign by PolyPhen (score 0.000), but as disease causing by MutationTaster. It has previously been reported as the only detected mutation in a patient with Neurofibromatosis type 1 (23).
[b]The *KIF1Bβ* mutation was somatic (not present in constitutional DNA) and was predicted as probably damaging by PolyPhen (score 1.000) and as disease causing by MutationTaster.

Table S7. Sensitivity control samples with previously defined *NF1* alterations[a] (not included in the study cohort).

Sample	Tumor type	Clinical syndrome	Gene	Mutation
PH12	PCC	NF1	*NF1*	c.5609G>A
PH3	PCC	Sporadic	*NF1*	c.1721+3A>T
PH4	PCC	Sporadic	*NF1*	No mutation[b]

[a]Samples were analyzed for *NF1* in a previous study, with the same results (6).
[b]In agreement with previous copy number results (6), allelic imbalance for SNPs may indicate LOH at the *NF1* locus in this sample. For example, the three first (in base pair position) heterozygous SNPs had the following read frequencies of the two alleles: rs2269855: 69.1% A, 30.9% G; rs2952976: 64.7% A, 35.3% G; rs1801052: 82.7 % A, 17.3% G.

Table S8. Number of sequencing reads for mutations detected with next-generation sequencing of tumor DNA.

Sample	Gene	Mutation	Position[a] [Chr:bp]	Total number of reads covering the locus (depth)	Proportion of reads with mutated allele
96	*EGLN1*	c.799G>A	1:231556836	1178	0.462
7	*KIF1Bβ*	c.2504A>G	1:10384920	1068	0.494
18	*KIF1Bβ*	c.1633G>A	1:10355818	1393	0.234
86	*MAX*	c.97C>T	14:65560500	1249	0.364
8	*NF1*	c.2446C>T	17:29556079	2632	0.870
18	*NF1*	c.586+5G>A	17:29497020	1091	0.786
20	*NF1*	c.1413_1440del	17:29541484	1664	0.722
24	*NF1*	c.935delG	17:29527484	2305	0.294
38	*NF1*	c.879delC	17:29509673	967	0.855
39	*NF1*	c.3638_3700delinsA	17:29560177	956	0.675
44	*NF1*	c.1340T>C	17:29533337	776	0.376
47	*NF1*	c.7901_7924del	17:29684316	1605	0.596
48	*NF1*	c.1971dupT	17:29552237	614	0.932
52	*NF1*	c.5327_5375dup	17:29654574	627	0.349
61	*NF1*	c.2806A>T	17:29556439	1450	0.694
72	*NF1*	c.7891A>G	17:29684308	1700	0.490
77	*NF1*	c.5123delT	17:29653121	1339	0.815
83	*NF1*	c.3354_3375del	17:29559743	1708	0.548
86	*NF1*	c.4174-2A>T	17:29585360	1264	0.573
89	*NF1*	c.3232delT	17:29559122	436	0.374
93	*NF1*	c.5991G>A	17:29662034	1266	0.476
5	*RET*	c.1900C>G	10:43609948	1106	0.582
13	*RET*	c.1900C>G	10:43609948	756	0.519
23	*RET*	c.1900C>G	10:43609948	811	0.527
26	*RET*	c.2753T>C	10:43617416	1094	0.334
29	*RET*	c.2753T>C	10:43617416	1082	0.401
30	*RET*	c.1900C>G	10:43609948	932	0.589
31	*RET*	c.1900T>C	10:43609948	743	0.510
34	*RET*	c.1900T>C	10:43609948	776	0.537
43	*RET*	c.2372A>T	10:43613908	946	0.451
63	*RET*	c.1900T>C	10:43609948	806	0.455
64	*RET*	c.2694_2705del	10:43615611	888	0.322
72	*RET*	c.1858T>C	10:43609102	364	0.659
81	*RET*	c.1900C>G	10:43609948	754	0.647
95	*SDHA*	c.629G>A	5:228307	2374	0.502
96	*SDHA*	c.223C>T	5:224547	990	0.479
101	*SDHB*	c.190delG	1:17371265	881	0.645
102	*SDHB*	c.423+1G>A	1:17355094	808	0.869
103	*SDHB*	c.148_151dupGACA	1:17371304	789	0.559
105	*SDHB*	c.683_684delAG	1:17349183	848	0.460
113	*SDHB*	c.148_151dupGACA	1:17371304	666	0.732
87	*TMEM127*	c.665C>T	2:96919598	610	0.361
21	*VHL*	c.217C>T	3:10183748	141	0.440
25	*VHL*	c.193T>G	3:10183724	103	0.418
96	*VHL*	c.386T>C	3: 10188243	457	0.578
108	*VHL*	c.593T>G	3:10191600	1010	0.283

[a]Base pair positions are according to hg19. For alterations affecting several base pairs, the first affected position is listed.

Chr, chromosome; bp, base pair

Figure S1. Sanger sequencing results for validation of mutations and investigation in corresponding constitutional DNA. *Continued on page 16.*

Figure S1. Sanger sequencing results for validation of mutations and investigation in corresponding constitutional DNA. *Continued from page 15*.

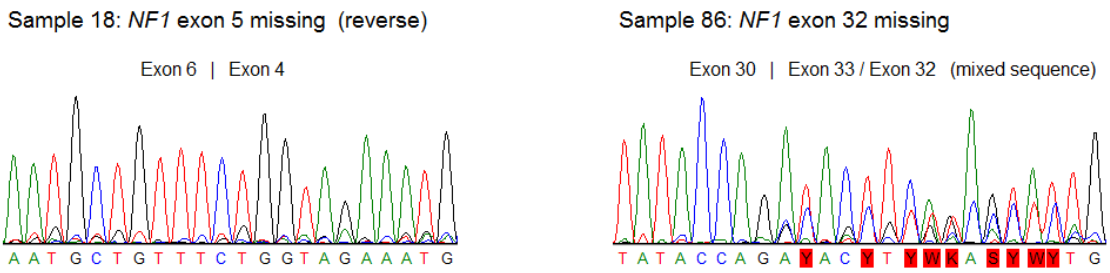

Figure S2. Validation of splice-site mutations by sequencing of *NF1* cDNA.

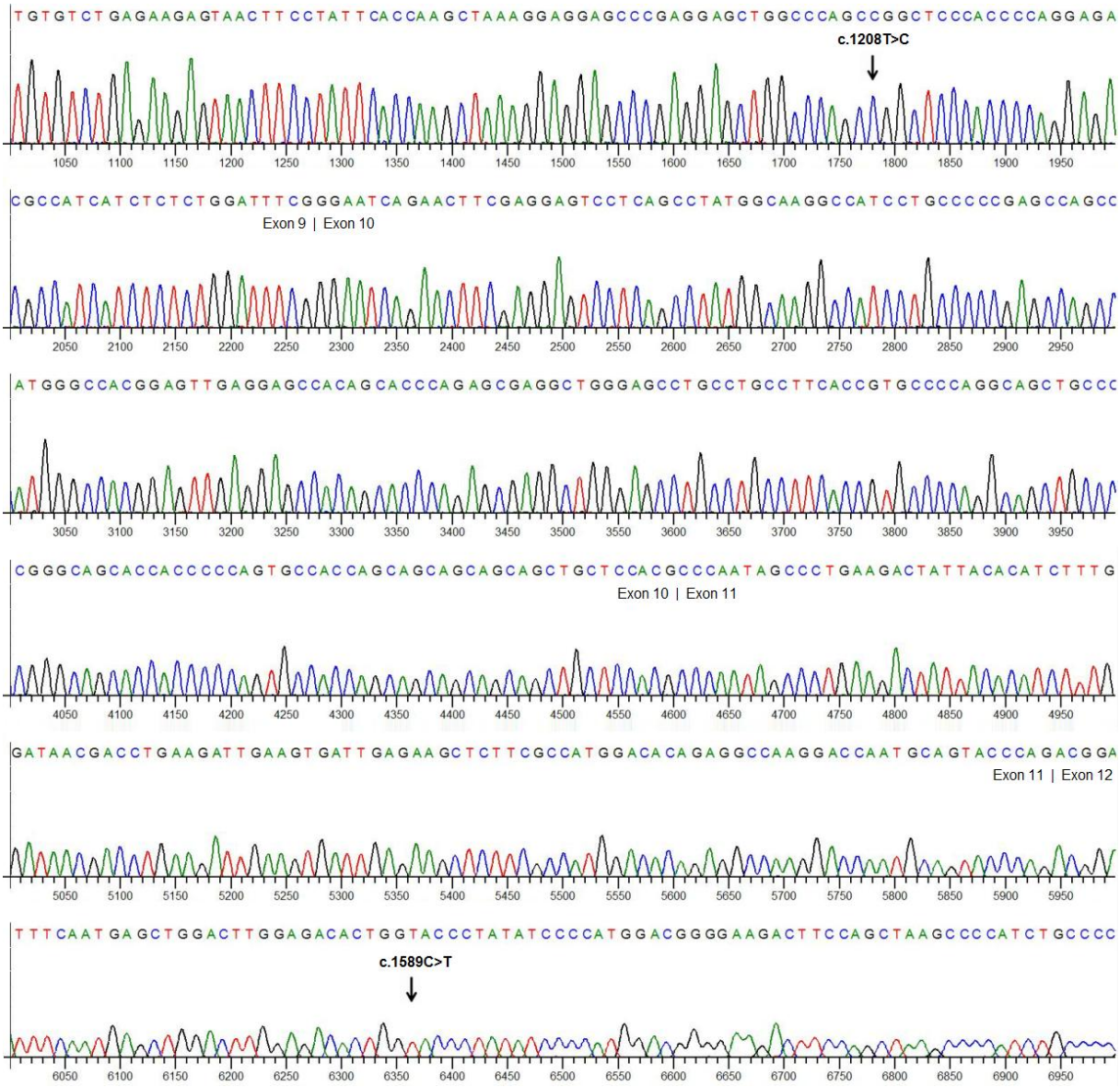

Figure S3. Sequencing results after molecular cloning of *EPAS1* cDNA from a tumor carrying two different somatic mutations (sample 106). The two mutations, c.1208T>C (Leu403Pro) and c.1589C>T (Ala530Val) were present in the same clone, implying that they occur in *cis*.

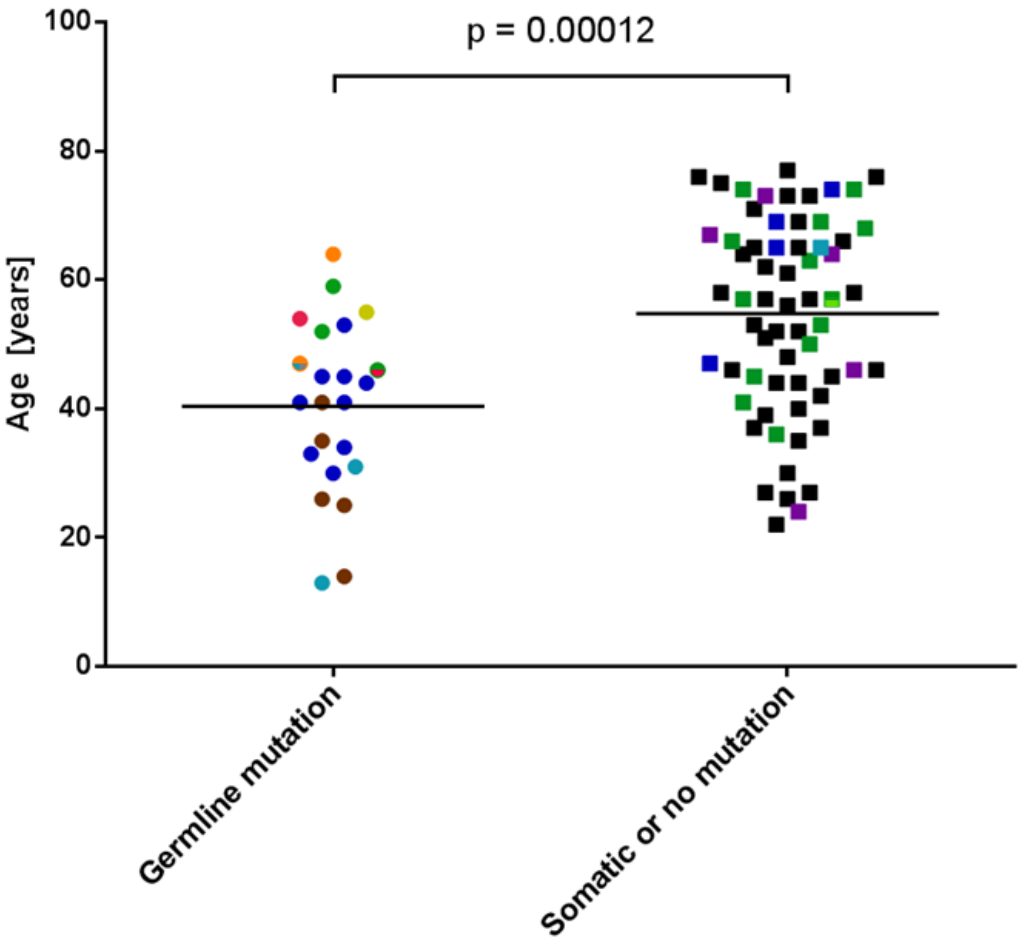

Figure S4. Age (at surgery) of patients with pheochromocytoma and paraganglioma. The difference between those with and without germline mutations was accessed with a two-tailed t-test. Cases with germline or somatic mutations are color-coded as follows (in conformity with Figure 1 in the main article): dark blue=*RET*, light blue=*VHL*, dark green=*NF1*, light green=*MAX*, orange=*SDHA*, brown=*SDHB*, yellow=*TMEM127*, gray=*EGLN1*, pink=*KIF1Bβ*, violet=*EPAS1*, black=no detected mutation (samples with multiple colors have more than one mutation). Horizontal bars indicate mean values for each group.

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