

1 **Supplementary Methods**

2 *Genetic examination*

3 One hundred eighty-one cases were tested for germline or somatic pathogenic variants
4 in established susceptibility genes to detect tumor mutation status by the local centers or
5 by the Spanish National Cancer Research Centre (CNIO) in Madrid. For this, Sanger
6 sequencing and/or NGS, and multiplex ligation-dependent probe amplification or
7 custom array comparative genomic hybridization (CGH) for detection of deletions were
8 applied. Tumors with unknown genetic backgrounds were additionally analyzed by
9 customized panel sequencing (Illumina, San Diego, CA) including coding exons and
10 20bp of intronic flanking regions of known PPGL-associated genes *RET*, *VHL*, *NF1*,
11 *MAX*, *TMEM127*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *MDH2*, *FH*, *EPAS1*, *HRAS*,
12 *KIF1B*, *EGLN1*, *EGNL2*, *IDH1*, and *IDH2*. Library preparation was performed using
13 Illumina's TruSeq Nano DNA Library Prep Kit according to the manufacturer's
14 instructions. One hundred fifty nucleotide paired-end sequencing was carried out with a
15 minimum median coverage of 1000-fold either on an Illumina MiSeq or NextSeq
16 sequencer. Reads were aligned to the reference genome (GRCh37/hg19) and variant
17 calling was performed using in-house workflows, as described previously (1). Variants
18 of interest were validated by Sanger sequencing.

19 *Measurement of urine samples*

20 Urine samples of 65 patients were measured as part of the prospective monoamine
21 producing tumor (PMT study) by LC-MS/MS according to a protocol and
22 standard-operating procedures available online (<https://pmt-study.pressor.org>). Data
23 from the individual referral centers were used in the remainder of samples.

24

25 *Tissue microarrays, immunohistochemistry, and digital image analysis.*

26 Tissue microarrays (TMAs) were constructed by sampling of two or three tumor tissue
27 cores (each 1.0 mm in diameter) as previously described (2). Paraffin-embedded tissue
28 blocks were utilized and representative areas selected on the basis of hematoxylin and
29 eosin (H&E) stained tissue sections by the experienced pathologists (S.K., A.W. and
30 A.K.W.).

31 The technical construction of the tissue microarrays was performed on a
32 manual tissue arrayer (AlphaMetrix Biotech, Rödermark, Germany). A semi-automated
33 rotation microtome (Microm HM 355S, Thermo Fisher Scientific, MA) was used to cut
34 3- μ m tissue microarray sections that were transferred to glass slides for
35 immunohistochemistry (IHC) and H&E staining. IHC was performed with
36 commercially available antibodies: mouse monoclonal Ab14715 antibody (Mitosciences,

37 Abcam, Cambridge, UK; 1:500 dilution) against SDHA and rabbit polyclonal
38 HPA002868 antibody (Sigma-Aldrich, St Louis, MO, USA; 1:400 dilution) against
39 SDHB on an automatic Ventana Benchmark Ultra System (Ventana Medical Systems,
40 Tuscon, AZ, USA) using Ultraview DAB detection system preceded by heat-induced
41 epitope retrieval with Ventana Cell Conditioning 1 (pH 8.4) at 97°C for 52 and 92min,
42 respectively.

43 Following IHC, stained tissue microarray slides were scanned at 20× lens using
44 a digital Mirax Desk slide scanner (Carl Zeiss Microscopy GmbH, Jena, Germany) prior
45 to import into the image analysis software Definiens Developer XD2 (Definiens AG,
46 Munich, Germany), as previously described (3). Manual annotation was performed to
47 exclude stromal tissue parts and blood vessels from the analysis, and a rule set was
48 defined to detect and quantify staining intensities from IHC in the annotated tumor area,
49 with operators blinded with regard to corresponding clinical annotations.

50

51 *Sample preparation and MALDI-MSI*

52 TMA samples were sectioned with a thickness of 4 μm, and water-bath mounted onto
53 indium-tin oxide (ITO)-coated glass slides (Bruker Daltonik GmbH, Bremen, Germany).
54 FFPE sections were incubated for 1 h at 70°C, deparaffinised in xylene (2 × 8 min), and

55 air-dried. For MALDI-MSI of endogenous metabolites, the matrix solution was 10
56 mg/ml 9-aminoacridine hydrochloride monohydrate matrix (9-AA) (Sigma-Aldrich
57 Chemie GmbH, Munich, Germany) in water/methanol 30:70 (v/v). 9-AA was chosen as
58 a matrix since it is known to exhibit very few matrix-derived interferences in the
59 low-mass range, achieve high sensitivity and high linearity in negative ion mode for a
60 wide range of low molecular-weight metabolites (4-8). The matrix solution was sprayed
61 on the tissue sections using a SunCollect automatic sprayer (SunChrom, Friedrichsdorf,
62 Germany) at room temperature. The matrix application was performed at flow rates of
63 10, 20, 30 and 40 $\mu\text{L}/\text{min}$, respectively, for the first four layers. The other four layers
64 were performed at 40 $\mu\text{L}/\text{min}$.

65

66 *Bioinformatics and statistical analysis*

67 MATLAB R2014b (v.7.10.0, Mathworks, Inc., Natick, MA) was used for MALDI
68 spectral preprocessing. Peak picking was performed as described in detail previously (9).
69 Briefly, the parameters of peak picking included m/z 0.0005 minimal peak width, signal
70 to noise threshold of 2, and intensity threshold of 0.01%. Isotopes were automatically
71 identified and excluded. List of m/z species with respective intensities for ROIs were
72 uploaded to MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca>) (10), processed with a

73 mass tolerance of m/z 0.0001. Heatmap-based clustering and orthogonal partial least
74 squares discriminant analysis (ortho-PLSDA) were created with MetaboAnalyst and
75 data were exported as figures.

76 Statistical comparisons between metabolome and clinical characteristics including
77 genotype were performed with Mann-Whitney U test, Kruskal-Wallis test or
78 Spearman's rank correlation coefficient (r_s). Multivariate cox models were built to find
79 covariates related to survival. Survival curves were calculated with the Kaplan-Meier
80 method and differences between curves were determined using the log-rank test. A P
81 value of <0.05 was considered significant. All calculations were performed using R

82 3.4.3.

83

84 *Cell culture and siRNA transfection*

85 Rat pheochromocytoma PC12 cells (ATCC; LGC Promochem) were kind gifts from
86 Natalia Pellegata, Helmholtz Zentrum München, Munich, Germany (11). PC12 cells
87 were cultured in Ham's F-12K (Kaighn's) Medium (Thermo Fisher Scientific, Gibco)
88 supplemented with 0.15 v/v horse serum (Thermo Fisher Scientific, Gibco), 0.025 v/v
89 fetal bovine serum (Thermo Fisher Scientific, Gibco) and 0.01 v/v,
90 penicillin/streptomycin (Thermo Fisher Scientific, Gibco).

91 RNA interference was performed as follow: cells were grown in culture
 92 medium and, 24 h later, the cells were transfected with Silencer Select siRNA (Ambion)
 93 using RNAiMAX (Invitrogen) according to the manufacturer's instructions. At the same
 94 time, non-targeting Silencer Select siRNA (Ambion) was transfected as a negative
 95 control (scrambled siRNA).

96

97 *Quantitative real-time PCR*

98 Total RNA was isolated from PC12 cells transiently transfected with siRNA, using
 99 Maxwell[®] 16 LEV simplyRNA Tissue kit (Promega). Total RNA (one µg) was reverse
 100 transcribed in a 20 µl reaction volume, with GoScript[™] Reverse Transcription Mix,
 101 Oligo(dT), according to the manufacturer's instructions. Quantitative real-time PCR
 102 was carried out using Mx3000PTM real-time PCR system (Stratagene) and SsoFast
 103 EvaGreen[®] Supermix (Bio-Rad Laboratories, Inc), to quantify the expression level of
 104 *Sdhb* and *Vhl* mRNA normalized to *Rpl13a*. Primer probes are described as follow.

105 Primers for quantitative real-time PCR

Gene		
<i>Sdhb</i>	forward	5'-GCTGGAGATAAACCTCGAATGC-3'
	reverse	5'-TCTCTGCATGATCTTCGGAAGG-3'
<i>Vhl</i>	forward	5'-CCCTCTCAGGTCATCTTCTGC-3'
	reverse	5'-CATCCCTGAAGAGCCAAAGGT-3'
<i>Rpl13a</i>	forward	5'-GGATCCCTCCACCCTATGACA-3'

106

107 *Immunoblotting*

108 Immunoblotting was carried out as previously described (12), using mouse monoclonal
109 anti-Sdhb (ab14714, 1:500, Abcam), anti-Vhl (sc-135657, 1:500, Santacruz), and
110 anti- α -Tubulin (T6074, 1:1000, Sigma-Aldrich).

111

112 *Metabolome analysis of in vitro models*

113 PC12 cells (2.0×10^5 cells/sample) were grown in culture medium for 24 h and
114 transfected with siRNA (see supplementary methods). After 48 h, cells were washed
115 with cooled H₂O (4 °C) and quenched and extracted with precooled 80% MeOH
116 (-80 °C). After incubation at -80 °C for 20 min, cells were scraped and centrifuged for 5
117 min. After supernatant was collected and centrifuged, samples were dried under reduced
118 pressure in Concentrator 5301 (Eppendorf). Fifteen biological replicates were prepared
119 for each siRNA transfection.

120 All samples were dissolved in 50 μ L buffer consisting of 15% mobile phase A
121 (20 mM ammonium acetate in 95% H₂O, 5% acetonitrile with pH adjusted to 9.1 using
122 ammonium hydroxide) and 85% mobile phase B (acetonitrile). A pool consisting of 10
123 μ L from each sample was prepared and analyzed randomly throughout the run to check

124 for reproducibility. Ten μl from each sample was injected twice and analyzed by liquid
125 chromatography high resolution tandem mass spectrometry (LC-HR-MS/MS) using
126 electrospray ionization in positive and negative mode with a system setup containing an
127 ultra performance liquid chromatography system (Aquity I-class, Waters) coupled to a
128 quadrupole – time-of-flight (QToF) instrument further equipped with an ion mobility
129 spectrometer (IMS) (Vion IMS-QToF, Waters). Chromatographic separation was
130 performed using an XBridge Amide HILIC column (4.6 x 100 mm, 3.5 μm , Waters) at
131 40°C. The gradient was as follows: 0-10 min gradient from 15% A to 50% A, 10-15 min
132 gradient from 50% A to 95% A, 15-16min gradient from 95 to 15% A, 16-23 min
133 equilibration with 15% A. The LC-HR-MS/MS was set to High Definition MS^e mode in
134 the mass to charge ratio (m/z) range of 50-1000 with a scan rate of 0.375 s. The capillary
135 voltage was set to 3.0kV, source and desolvation temperatures were set to 120°C and
136 500°C, respectively, while cone and desolvation gas flows were set to 50 L/h and 900
137 L/h. The collision cell was set to 6 eV (low energy) for 25 % of the scan time before it
138 was ramped from 28-56 eV (high energy) for the remaining 75% of the time. Repeated
139 injections of a Leucine-Enkephalin solution served as reference allowing for mass
140 correction.

141 Metabolites were identified by comparing the measured features to either an

142 internal library using the UNIFI software package (Waters) built with pure compounds,
143 or against the two publically available databases, HMDB and KEGG, using the
144 Chemspider plugin in Progenesis QI (Nonlinear). Precursor mass deviation of 5ppm,
145 retention time shift of 0.2 min and CCS deviation of 3% was allowed. Obtained data
146 were corrected with cell numbers and analyzed by MetaboAnalyst 3.0 as described
147 above. Statistical comparisons were performed with Mann-Whitney *U* test or
148 Kruskal-Wallis test. A *P* value of <0.05 was considered significant. All calculations
149 were performed using R 3.4.3.

150

151 **References of supplementary methods**

- 152 1. Richter S, Geldon L, Pang Y, Peitzsch M, Huynh T, Leton R, Viana B,
153 Ercolino T, Mangelis A, Rapizzi E, et al. Metabolome-guided genomics to identify
154 pathogenic variants in isocitrate dehydrogenase, fumarate hydratase, and succinate
155 dehydrogenase genes in pheochromocytoma and paraganglioma. *Genet Med.*
156 2019;21(3):705-17.
- 157 2. Rauser S, Weis R, Braselmann H, Feith M, Stein HJ, Langer R, Hutzler P,
158 Hausmann M, Lassmann S, Siewert JR, et al. Significance of HER2 low-level copy gain
159 in Barrett's cancer: implications for fluorescence in situ hybridization testing in tissues.
160 *Clin Cancer Res.* 2007;13(17):5115-23.
- 161 3. Feuchtinger A, Stiehler T, Jutting U, Marjanovic G, Lubert B, Langer R, Walch
162 A. Image analysis of immunohistochemistry is superior to visual scoring as shown for
163 patient outcome of esophageal adenocarcinoma. *Histochem Cell Biol.* 2015;143(1):1-9.
- 164 4. Edwards JL, Kennedy RT. Metabolomic analysis of eukaryotic tissue and
165 prokaryotes using negative mode MALDI time-of-flight mass spectrometry. *Anal Chem.*
166 2005;77(7):2201-9.
- 167 5. Shroff R, Muck A, Svatos A. Analysis of low molecular weight acids by
168 negative mode matrix-assisted laser desorption/ionization time-of-flight mass

- 169 spectrometry. *Rapid Commun Mass Spectrom.* 2007;21(20):3295-300.
- 170 6. Miura D, Fujimura Y, Tachibana H, Wariishi H. Highly sensitive
171 matrix-assisted laser desorption ionization-mass spectrometry for high-throughput
172 metabolic profiling. *Anal Chem.* 2010;82(2):498-504.
- 173 7. Miura D, Fujimura Y, Yamato M, Hyodo F, Utsumi H, Tachibana H, Wariishi H.
174 Ultrahighly sensitive in situ metabolomic imaging for visualizing spatiotemporal
175 metabolic behaviors. *Anal Chem.* 2010;82(23):9789-96.
- 176 8. Sun N, Ly A, Meding S, Witting M, Hauck SM, Ueffing M, Schmitt-Kopplin P,
177 Aichler M, Walch A. High-resolution metabolite imaging of light and dark treated retina
178 using MALDI-FTICR mass spectrometry. *Proteomics.* 2014;14(7-8):913-23.
- 179 9. Buck A, Ly A, Balluff B, Sun N, Gorzolka K, Feuchtinger A, Janssen KP,
180 Kuppen PJ, van de Velde CJ, Weirich G, et al. High-resolution MALDI-FT-ICR MS
181 imaging for the analysis of metabolites from formalin-fixed, paraffin-embedded clinical
182 tissue samples. *J Pathol.* 2015;237(1):123-32.
- 183 10. Xia J, Wishart DS. MetPA: a web-based metabolomics tool for pathway
184 analysis and visualization. *Bioinformatics.* 2010;26(18):2342-4.
- 185 11. Lee M, Waser B, Reubi JC, Pellegata NS. Secretin receptor promotes the
186 proliferation of endocrine tumor cells via the PI3K/AKT pathway. *Mol Endocrinol.*

187 2012;26(8):1394-405.

188 12. Siebert C, Ciato D, Murakami M, Frei-Stuber L, Perez-Rivas LG,

189 Monteserin-Garcia JL, Nolting S, Maurer J, Feuchtinger A, Walch AK, et al. Heat Shock

190 Protein 90 as a Prognostic Marker and Therapeutic Target for Adrenocortical Carcinoma.

191 Front Endocrinol (Lausanne). 2019;10:487.

192

193 **Supplementary Figure Legends**

194 **Supplementary Figure 1:** MSI images of distinctive metabolites across the entire
195 TMA.

196 Zoomed images of patient 165 with triplicated tissue cores were represented as an
197 example.

198

199 **Supplementary Figure 2:** Clinical and immunohistochemistry markers of metastatic
200 PPGLs.

201 Kaplan-Meier plots for metastasis-free survival in relation to clinical parameters
202 including maximal tumor size, tumor location, 24h urinary metanephrine and mutation
203 status (A). Boxplots of staining intensities (SI) of SDHB and SDHA protein with
204 respect to mutation status (B). Kruskal-Wallis test were used for statistical analysis. P
205 <0.05 , compared to **SDHB* mutation, †*SDHC* mutation, ‡*SDHD* mutation, and § *VHL*
206 mutation. Boxplots of staining intensities of SDHB and SDHA protein with respect to
207 *SDHx* mutation status (C). Mann-Whitney U test was used for statistical analysis. ** P
208 <0.001 . A ROC curve for prediction of *SDHx* mutation by SI of SDHB and area under
209 curves (AUC) were also shown (D). Kaplan-Meier plots for metastasis-free survival in
210 relation to staining intensities of SDHB protein (E). Cut off were shown as red and
211 black lines. Log rank test was used to statistically compare the curves and P -values are

212 provided.

213

214 **Supplementary Figure 3:** Metabolomic features of PPGLs.

215 Unsupervised hierarchical clustering analysis of metabolome profiles of 184 PPGLs

216 including cluster 1, cluster 2 and wildtype (A). Groups based on metabolites pattern,

217 mutation status, tumor location and metastatic behavior are shown below heat map.

218 Distribution of patient numbers with regard to mutation status is shown. Ortho-PLSDA

219 analysis of metabolome profiles of 141 PPGLs in which sample age and institution were

220 annotated (B). Comparisons between *SDHx* and *VHL* mutation status in the purine

221 metabolism (C); adenosine monophosphate (AMP), adenosine diphosphate (ADP) and

222 adenosine triphosphate (ATP). Mann-Whitney U test was used for statistical analysis

223 and *P*-values are shown.

224

225 **Supplementary Figure 4:** *In vitro* models using PC12 cells.

226 Knockdown of *Sdhb* (si*Sdhb*) and *Vhl* gene (si*Vhl*) in PC12 cells, confirmed by

227 quantitative real time PCR ($P = 1.63E-07$ and $1.29E-05$, respectively, A) and

228 immunoblot with Sdhb and Vhl protein and corresponding control (B). Succinate

229 concentration and succinate/fumarate ratio in scrambled siRNA and knockdown of Sdhb

230 gene in PC12 cells (C). Significant differences of tryptophan in PC12 cells ($P = 0.005$,

231 D). Student's t test or Kruskal-Wallis test were used for statistical analysis. * $P < 0.05$,
232 ** $P < 0.001$.

233

234 **Supplementary Figure 5:** Scoring system using maximal tumor size, staining intensity

235 of SDHB and xanthurenic acid for prediction of metastatic behavior of PPGLs.

236 Patients profiles of a scoring system for metastasis, using maximal tumor size, SDHB

237 mutation status and xanthurenic acid (A). Kaplan-Meier plots for metastasis-free

238 survival in relation to scoring system and the examination of scoring system by cox

239 proportional hazards model were shown (B). Log rank test was used to statistically

240 compare the curves and P -values are provided.