SUPPLEMENTARY MATERIAL

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Germline *SUCLG2* variants in patients with pheochromocytoma and paraganglioma

SUPPLEMENTARY METHODS

Adrenomedullary tissue separation

Normal human adrenal glands from anonymous donors without adrenal dysfunction or tumor were obtained from patients undergoing radical nephrectomy or within 2-5 hours after confirmed brain. Whole adrenal glands were snap-frozen upon removal and stored at −80 °C. Separation of cortex and medulla was performed as previously described (1). Frozen adrenal tissue was transferred in Petri dish and placed on wet ice to warm slightly to prevent tissue from cracking during processing. The adrenals were cut centrally, perpendicular to the longest axis and the cut surface was visually inspected for the presence of adrenomedullary tissue. The border between the cortex and medulla was identified by the red/brown color of the cortical zona reticularis. If sufficient gray/pink medulla for separation was visible, sections of 2-5 mm width were taken, if not, the tissue was again cut perpendicular to the longest axis about 1 cm from previous cut, until an area with wider section of adrenal medulla was found. Afterward, the tissue cuts were examined under the dissection microscope, and the medulla was carefully separated from the surrounding cortex with size 11 scalpel blades and microsurgical scissors with 4 mm flat or curved blades. Medulla, cortex, and inseparable mixtures of medullary and cortical tissue were collected in separate containers. The tissue was kept frozen on dry ice at all times.

FISH analysis

For interphase FISH analysis, BAC DNA probes RP11-146E16 and RP11-927D18 labeled with orange fluorescence were purchased from Empire Genomics (Buffalo, NY, USA). For chromosome enumeration, we used Vysis CEP 3 (alpha satellite) Spectrum Green-labeled probes

(Abbott Molecular, Abbott Park, Il, USA). FISH assays were performed on 5-µm formalin-fixed-paraffin-embedded (FFPE) tumor sections using laboratory standardized protocol with slight modifications. Briefly, 5-µm thick sections of FFPE tissue blocks were de-paraffinized and rehydrated. Antigen retrieval was performed with IHC-Tek epitope retrieval solution (IHC World, Ellicott City, MD, USA) by steaming for 25 min. After cooling, slides were subjected to 50 mg/ml pepsin treatment at 37 °C and rinsed in phosphate-buffered saline (PBS), followed by dehydration in an ethanol series. Co-denaturation of the probe and target DNA at 73 °C in HYBrite (Abbott Molecular) for 5 min was followed by overnight hybridization at 37 °C. The next day, slides were washed at 72 °C in 0.4× SSC/0.3% Tween-20 for 2 min and then in 2× SSC/0.1% Tween-20 at room temperature for 1 min. The slides were then counterstained, mounted with DAPI/Antifade (Vector Laboratories, Burlingame, CA, USA), and analyzed on the BioView Duet-3 fluorescent scanning station (BioView, Billerica, MA, USA) using a 63×-oil objective and DAPI/FITC/Rhodamine single band-pass filters (Semrock, Rochester, NY, USA). At least 100 tumor cell nuclei were scored for each specimen. The number of red signals from <2 copies per cell in >20% of cells (cut-off) was defined as deletion.

Immunohistochemistry

Paraffin-embedded tumor biopsies were cut into 10-µm sections, and tissue slides were deparaffinized with Histo-Clear (National Diagnostics, Atlanta, GA, USA) and rehydrated by sequential incubation in ethanol at different concentrations. Antigen retrieval was performed by incubation of the slides in boiling citrate buffer for 20 min, followed by incubation with 3% H_2O_2 and blocking buffer (Sigma-Aldrich, St. Louis, MO, USA). The slides were probed with rabbit anti-SUCLG2 IgG (ab187996; 1:5000; Abcam, Cambridge, UK) overnight at 4 °C. The signal was

amplified using Anti-Rabbit EnVision+ System, HRP (Dako, Carpinteria, CA, USA) for 1h at room temperature and visualized using the liquid DAB+ Substrate Chromogen System (Dako). Counterstaining was performed with hematoxylin.

Cell lines

For *in vitro* experiments, human pheochromocytoma cells (hPheo1) were cultured in RPMI medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, antibiotics, and 1 mM pyruvate at 37 °C and 5% CO_2. Genomic deletion of *SUCLG2* in hPheo1 cell lines was performed using the CRISPR/AsCas12a (also known as AsCpf1) system (2) and our chimeric Cas12a and guide RNA-expression plasmid pX AsCpf1-Venus-NLS. Suitable crRNAs were identified using Crispor software (http://crispor.tefor.net/). An oligonucleotide was designed to provide the overhangs 5'-AGAT and 3'-TTTT (AAAA reverse complement) for cloning and contain an array of three crRNAs targeting 5'-CTT CTC TCA ACT CTG GGT TGC AG within TGT AGG CAG TTC AAT TAA CCT CC and 3'-GGC AAG TGT ATT TCA CCC ATT AT of the critical exon 2 of human *SUCLG2* and separated by the AsCas12a direct-repeat sequence: AGA TCT TCT CTC AAC TCT GGG TTG CAG AAT TTC TAC TCT TGT AGA TTG TAG GCA GTT CAA TTA ACC TCC AAT TTC TAC TCT TGT AGA TGG CAA GTG TAT TTC ACC CAT TAT TTT T. The oligonucleotides were cloned into a plasmid cleaved by FastDigest *Bpi*I (Thermo Fisher Scientific, Waltham, MA, USA), and the correct insertion was confirmed by colony PCR and DNA sequencing. hPheo1 cells were transfected with the verified AsCas12a constructs using Lipofectamine 3000 (Thermo Fisher Scientific), followed by single-cell sorting for Venus-positive cells into a 96-well culture plate. Clones were collected, and deletion of the targeted locus was confirmed by genomic PCR using primers GGG GAG CCA TAT CCA TGT AAT AGA G and

TCT CCT CCC AGG TCA GCA GG. The selected clone was further tested by Sanger sequencing and confirmed for SUCLG2 absence by WB.

For SUCLG2 re-expression, *SUCLG2* DNA constructs were subcloned into the pCDH-CMV-MCS-EF1-Puro vector (CD510B-1; System Biosciences, Palo Alto, CA, USA). For PCR, we used iProof high-fidelity DNA polymerase (Bio-Rad, Hercules, CA, USA). Digestion of the target vector and PCR products was performed using FastDigest *BamH*I and FastDigest *EcoR*I (Thermo Fisher Scientific), and the subsequent ligation reaction was performed using T4 DNA ligase (Thermo Fisher Scientific) according to manufacturer instructions. DNA constructs were analyzed using DNA electrophoresis and purified with Nucleospin Gel and PCR clean-up (Macherey-Nagel, Düren, Germany). DNA concentration was detected using a NanoDrop ND-2000 (Thermo Fisher Scientific).

Constructs were used to transform OneShot Stbl3™ chemically competent *Escherichia coli* cells (C737303; Invitrogen, Carlsbad, CA, USA). Plasmids with SUCLG2 constructs were isolated using NucleoSpin Plasmid (NoLid) and NucleoBond Xtra midi kits (both Macherey-Nagel). Recombinant lentiviral particles were obtained from calcium-phosphate-transfected HEK 293T cells using packaging plasmids psPAX2 (12260; Addgene, Watertown, MA, USA) and pMD2.G (12259; Addgene) together with SUCLG2 pCDH constructs. The medium containing lentiviral particles was harvested 36- to 48-h post-transfection, and the viral particles were precipitated using PEG-it (System Biosciences). Target cells were transduced with viruses at a multiplicity of infection of 5 to 10 and selected for puromycin resistance (2 µg/ml; InvivoGen, San Diego, CA, USA).

Isolation of mitochondria

Mitochondria for *in vitro* experiments were isolated as previously described (3). Briefly, cells were homogenized in isolation buffer containing 250 mM sucrose, 10 mM Tris, and 1 mM EDTA using a Balch homogenizer (Isobiotec, Heidelberg, Germany). Cell suspension containing 40×10^6 to 50×10^6 cells/5 ml was passed three times through an 8-μm wide opening using a hand-driven 1-ml syringe. The homogenate was centrifuged at 800*g* for 5 min at 4 °C followed by centrifugation of the supernatant at 3,000*g* for 5 min. The supernatant containing mitochondria was centrifuged for 15 min at 10,000*g*. Pelleted mitochondria were either stored at -80 °C or re-suspended in 100 μl to 200 μl of the isolation buffer for further experiments.

For tissue samples, 30-100 mg of tissue was homogenized in buffer containing 20 mM HEPES-KOH (pH 7.4), 220 mM D-mannitol, 70 mM sucrose, and 1 mM EDTA using Dounce Tissue Grinder (tube 885303-0007, pestle 885302-0007, both Kimble Chase, DWK Life Sciences, Milville, NJ, USA) with 60 strokes. The homogenate was centrifuged at 1,000*g* for 10 min at 4 °C. The supernatant was collected, and the pellet was once more homogenized using 60 strokes, centrifuged at 1,000*g* 10 min, and the supernatant mixed with the previous one. Pooled supernatant was centrifuged for 15 min at 10,000*g*, the mitochondrial pellet was re-suspended in the isolation buffer and used for analysis.

Western blot analysis

For *in vitro* experiments, samples were harvested from cell lines using the radioimmunoprecipitation assay lysis buffer supplemented with a protease- and phosphatase-inhibitor cocktail (Thermo Fisher Scientific). Tissues were homogenized using a rotor-stator homogenizer (Omni International, Kennesaw, GA, USA) on ice and incubated on ice for 1 h. Cell

lysates were incubated on ice for 30 min. Samples were then centrifuged at 13,000*g* for 20 min at 4 ºC, and supernatants were used for the analysis. For tissue samples, solubilized mitochondria lysate was used for the analysis. Protein content was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific). Samples were boiled for 5 min in reducing loading buffer before separation by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Wet blotting was used to transfer the separated proteins to nitrocellulose membranes (Millipore, Billerica, MA, USA), and immunoblotting was performed in Tris-buffered saline/Tween-20 (TBS-T) supplemented with 5% non-fat dried milk overnight at 4 ºC. The following primary antibodies were used: anti-SUCLG2 (ab187996; Abcam), anti-SUCLA2 (ab183513; Abcam), anti-SUCLG1 (PA5-57766; Thermo Fisher Scientific), anti-GAPDH (#5174; Cell Signaling Technology, Danvers, MA, USA), anti-SDHA (ab14715; Abcam), anti-SDHB (ab14714; Abcam), anti-VDAC1 (ab15895; Abcam). Horseradish peroxidase-conjugated secondary antibodies were used in TBS-T with 5% non-fat dried milk for 1 h at room temperature. Protein bands were quantified using AzureSpot 2.0 software (Azure Biosystems, Dublin, CA, USA).

In-gel SDH activity

Digitonin-solubilized mitochondria (30 µg) were mixed with sample buffer containing 50% glycerol and 0.1% Ponceau dye and run on a precast 4% to 16% NativePAGE Novex Bis-Tris gel (Life Technologies, Carlsbad, CA, USA) at a constant voltage of 30 V for 1 h, followed by 2 h at 150 V. Deoxycholate (0.05%) and lauryl maltoside (0.01%) were added to the cathode buffer for higher resolution, as previously described (4). Gels with separated protein complexes were incubated for 30 min in assay buffer containing 20 mM sodium succinate, 0.2 mM phenazine methosulfate, and 0.25% Nitrotetrazolium Blue in 5 mM Tris-HCl (pH 7.4). The reaction was

stopped using a solution of 50% methanol and 10% acetic acid, and gels were immediately photographed and evaluated by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

SQR activity

Protein lysates (20 µg) were incubated in 200 µl of 25 mM phosphate potassium buffer (pH 7.4) containing 20 mM succinate, 2 µM antimycin A, 5 µM rotenone, 10 mM sodium cyanide, bovine serum albumin (BSA; 50 mg/ml), and 2,6-dichlorphenol indophenol (0.015% w/v) for 5 min in a 96-well plate. After 30 s of recording of the signal intensity at 600 nm, 100 µM decylubiquinone was added, and the reaction was recorded for another 10 min. Identical measurements were performed in the presence of 20 mM malonate, and net SQR activity was obtained by subtracting the malonate-insensitive rate.

Native blue gel electrophoresis (NBGE)

NBGE was performed as previously described (5). Briefly, mitochondria were isolated from hPheo1 cells using the described protocol. Digitonin-solubilized mitochondria (15 µg) were separated on 4% to 16% NativePAGE Novex BisTris gradient gels (Life Technologies). After electrophoresis, the gels were incubated in the transfer buffer for 10 min with SDS, and proteins were transferred to polyvinylidene difluoride membranes (Millipore) that were probed with specific antibodies against SDHA or SDHB, using anti-HSP60 IgG (#12165; Cell Signaling Technology) as the loading control.

mtDNA assay

Total DNA was extracted using the Wizard SW Genomic DNA purification system (Promega, Madison, WI, USA) for *in vitro* experiments and the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) for tumor samples. DNA (100 ng) was mixed with 10 μM forward and reverse primers for mtMinArc (6) and 5× HOT FIREPol EvaGreen qPCR Mix Plus (ROX; Solis Biodyne, Tartu, Estonia), and the reaction was performed according to manufacturer instructions. Data were expressed as ratios of levels of β-2 microglobulin representing nuclear DNA (nDNA) (6). The mtDNA:nDNA was compared with that in parental hPheo1 cells for *in vitro* experiments and with NAM for tumor samples.

Respiration assays

Respiration was evaluated as previously described (7). Briefly, cells were trypsinized, washed with PBS, resuspended in Mir05 medium [0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 110 mM sucrose, 1 g/l essentially fatty acid-free BSA, and 20 mM HEPES (pH 7.1)] at 30 °C and transferred to the chamber of an Oxygraph-2k instrument (Oroboros, Innsbruck, Austria). Respiration measurements were performed at 37 °C. After closing the chamber, routine respiration was recorded. To allow access of exogenously added substrates, cells were permeabilized with 4 μg digitonin per 10^6 cells. CII-mediated respiration was determined in the presence of 10 mM succinate, 3 mM ADP, and 10 μM cytochrome c. CII-specific oxygen-consumption rates were verified by the addition of malonate. Antimycin A (2.5 μM) was added at the end of each assay to inhibit the electron-transport chain, and residual oxygen consumption after antimycin A addition was subtracted from all results in order to obtain mitochondria-specific rates. For respiration in NAMs and SUCLG2 tumors from frozen tissue, we

based our protocol on a recently published methodology for respiration using frozen tissue and the Oxygraph-2k instrument (8). In brief, 10-20 mg of snap-frozen tissue was homogenized by Schredder homogenizer (8 s on level 1, 6 s on level 2) (Oroboros) in 2.2 ml of Mir05 medium. Homogenate was transferred into Oxygraph chamber and analyzed for uncoupled CII-mediated respiration in presence of 1 μM rotenone and 10 mM succinate. Antimycin A (2.5 μM) was added at the end of the assay to inhibit the electron-transport chain, and residual oxygen consumption after antimycin A addition was subtracted from results in order to obtain mitochondria-specific rates.

Succinate-to-fumarate ratio in hPheo1 cells

Cells were grown in 12-well plates for 24 h, followed by washing twice with ice-cold 0.9% NaCl solution and metabolite extraction with 500 μl of 80% methanol in water. After centrifugation for 5 min at 16,000*g* at 2 ºC, 400 μl of the extract was transferred to a 2-ml glass vial with a screwcap and dried *in vacuo*. The content was then re-dissolved in 100 μl of anhydrous pyridine (Sigma-Aldrich), and 30 μl of N-tert-butyldimethylsilyl-N-methyl trifluoroacetamide (Sigma-Aldrich) was added. The content was then vortexed and incubated at 70 ºC for 30 min under constant shaking, after which 300 μl of hexane (VWR Chemical, Radnor, PA, USA) was added. The silylated extract was analyzed using two-dimensional gas chromatography with mass spectrometric detection (GC×GC-MS; Pegasus 4D; LECO Corporation, St. Joseph, MO, USA). The first dimension was run on an Rxi-5Sil MS column (30 m × 0.25 mm ID × 0.25 μm dF; Restek, Bellefonte, PA, USA), and the second dimension was run using a BPX50 column (1-1.5 m × 0.1 mm ID × 0.1 μm dF; SGE). Operating conditions were as follows: primary oven temperature gradient, 50 ºC (1 min) with increases of 10 ºC/min to 240 ºC and then 20 ºC/min to 320 ºC (4

min); the secondary oven was set at 5 ºC above the primary oven temperature; modulation period, 3 s to 5 s; injection temperature, 30 ºC; split-less injection mode was applied; injection volume, 1 µl; carrier gas, helium; and corrected constant flow, 1 ml/min. MS-detection parameters were as follows: electron ionization, −70 eV; transfer line temperature, 280 ºC; and ion source temperature, 280 ºC. We used ChromaTOF software (v.4.51; LECO Corporation) for instrument control, data acquisition, and data processing.

Succinic and fumaric acids were analyzed as tert-butyl silyl derivatives, with their identities confirmed by co-elution with standards. Analytes were quantified using masses of m/z 289 (succinic acid) and m/z 287 (fumaric acid).

Prediction of functional impact

The frequency of *SUCLG2* variants was investigated in public databases: gnomAD (gnomad.broadinstitute.org), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), dbSNP (https://www.ncbi.nlm.nih.gov/snp/), COSMIC (cancer.sanger.ac.uk/cosmic) and VarSome (www.varsome.com) databases. Variants identified below 0.001 allele frequency of the population and without homozygotes described were considered very rare. We used the GERP++ method as *in silico* approach of evolutionary conservation in the genome. The cut-off for high deleterious variants is over 4. For protein function prediction, t Mutation Assessor, SIFT, Polyphen-2 programs, and all functional impact tools included in VarSome database were applied.

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Supplementary Table 1. Analysis from custom panel of 54 genes[a,b]

ID	Other mutations	Cancer Hotspot	Onco KB status	OncoKB variant	ClinVar
#3	*RET*(c.C166A:p.L56M)	-	Oncogene	no	benign/likely benign
#5	*RET* (c.C2944T; p.R982C)	-	Oncogene	no	benign/likely benign
#6	*EPAS1* (c.A2296C:p.T766P)	-	?	no	benign
#9	*IDH2* (c.G67A:p.V23M)	-	Oncogene	no	not reported
#10	*ATRX* (c.A5465G: p.N1822S)	-	TSG	no	benign/likely benign
#13	*GOT2* (c.G460A: p.D154N)	-	-	no	benign
#14	*SDHA* (c.G563A: p.R140Q). Validation failed	-	TSG	no	uncertain significance

[a]List of genes: *ACLY; ACO1; ACO2; AKT1; ALK; APC; ARID2; ARNT; ATM; ATRX; BAP1; CREB1; CS; EGLN1; EGLN2; EPOR; FGFR2; FGFR3; FH; GLS2; HIF1AN; HIF2A; HMOX1; HMOX2; IDH1; IDH2; IREB2; JAG1; JAK2; JUN; K-RAS; KIF1Bbeta; KIT; MAX; MDH2; mTOR; NF1; NOTCH1; OGDH; PIK3CA; PRKAR1A; PTPN5; RET; SART1; SDHA; SDHAF2; SDHB; SDHC; SDHD; SIRT1; SUCLG2; TMEM127; TP53; VHL*

[b]List of additional mutations in known PPGL susceptibility genes in 7 patients (# as listed in **Table 1**) and analysis of relevance from Cancer Hotspot (www.cancerhotspots.org), OncoKB (Chakravarty et al., 2017), and ClinVar did not indicate any pathogenic mutations with oncogenic potential.

Supplementary Table 2. Metabolomic analysis of tumor and NAM tissues[a]

Group and Sample/ Patient ID[b]	Succinate	Fumarate	Succinate/ fumarate	Malate	Citrate	alpha-Ketoglutarate	Pyruvate	Lactate	3-Hydroxybutyric acid
	nmoles/mg dry powder Wt.	nmoles/mg dry powder Wt.	ratio	nmoles/mg dry powder Wt.	nmoles/mg dry powder Wt.	nmoles/mg dry powder Wt.	nmoles/mg dry powder Wt.	nmoles/mg dry powder Wt.	nmoles/mg dry powder Wt.
NAM									
1	0.43	0.54	0.80	2.86	0.93	0.02	0.62	88.99	1.53
2	0.53	0.45	1.17	2.15	0.87	0.03	0.74	97.58	0.60
3	0.38	0.09	4.25	0.63	0.14	0.00	0.15	28.68	2.12
No.	3	3	3	3	3	3	3	3	3
Mean (SD)	0.44 (0.08)	0.36 (0.24)	2.07 (1.89)	1.88 (1.14)	0.64 (0.44)	0.02 (0.01)	0.51 (0.31)	71.75 (37.54)	1.42 (0.77)
Tumor tissue									
1	0.98	0.20	4.98	0.90	2.13	0.02	0.36	108.40	BLOQ
2	1.92	0.16	11.90	1.16	2.93	0.04	1.28	256.62	0.72
3	3.45	0.45	7.68	2.39	4.61	0.04	0.74	239.08	1.37
4	3.28	0.21	15.51	1.44	1.72	0.05	1.13	175.84	3.71
5	4.32	0.33	13.19	1.80	2.53	0.03	0.45	188.47	0.75
6	4.33	0.21	20.23	1.45	2.29	0.03	0.87	297.96	0.42
No.	6	6	6	6	6	6	6	6	6
Mean (SD)	3.05 (1.35)	0.26 (0.11)	12.25 (5.46)	1.52 (0.52)	2.70 (1.02)	0.04 (0.01)	0.81 (0.36)	211.06 (67.38)	1.39 (1.34)
P^c	0.02	0.58	0.02	0.71	0.02	0.17	0.29	0.02	0.79

[a]Normal adrenal medulla (NAM; $n = 3$) and patient tissues ($n = 6$) were analyzed for metabolites by LC-MS/MS. BLOQ: below the limit of quantification; LC-MS/MS: liquid chromatography tandem mass spectrometry; NAM: normal adrenal medulla; Wt: weight.
[b]Patient ID refers to patients 1 through 6 in **Table 1**.
[c]Statistical significance between NAM and patient samples was assessed by a 2-tailed Mann–Whitney non-parametric test.

Supplementary Figure 1. Putative interaction between the SUCLG1/SUCLG2 complex and GTP/GDP binding sites. Mutant amino acids of SUCLG2 were identified around the GTP/GDP binding pocket.