




















## Germline *SUCLG2* Variants in Patients With Pheochromocytoma and Paraganglioma

Katerina Hadrava Vanova, PhD,<sup>1,2,†</sup> Ying Pang, MD, PhD,<sup>1,†</sup> Linda Krobova, MS,<sup>2</sup> Michal Kraus, BA,<sup>2,3</sup> Zuzana Nahacka , PhD,<sup>2</sup> Stepana Boukalova , PhD,<sup>2</sup> Svetlana D. Pack , PhD,<sup>4</sup> Renata Zobalova , PhD,<sup>2</sup> Jun Zhu, PhD,<sup>5</sup> Thanh-Truc Huynh, MS,<sup>1</sup> Ivana Jochmanova , MD, PhD,<sup>1,6</sup> Ondrej Uher , MS,<sup>1,7</sup> Sona Hubackova , PhD,<sup>2</sup> Sarka Dvorakova , PhD,<sup>2</sup> Timothy J. Garrett , PhD,<sup>8</sup> Hans K. Ghayee , DO,<sup>9</sup> Xiaolin Wu , PhD,<sup>10</sup> Bjoern Schuster , PhD,<sup>11</sup> Philip E. Knapp , MD,<sup>12</sup> Zdenek Frysak, MD, PhD,<sup>13</sup> Igor Hartmann , MD,<sup>14</sup> Naris Nilubol , MD,<sup>15</sup> Jiri Cerny, PhD,<sup>2</sup> David Taieb, MD, PhD,<sup>16</sup> Jakub Rohlena , PhD,<sup>2</sup> Jiri Neuzil , PhD,<sup>2,17</sup> Chunzhang Yang , PhD,<sup>18,†</sup> Karel Pacak , MD, PhD<sup>1,\* ,†</sup>

<sup>1</sup>Section of Medical Neuroendocrinology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA; <sup>2</sup>Institute of Biotechnology, Czech Academy of Sciences, BIOCEV, Vestec, Prague West, Czech Republic; <sup>3</sup>Department of Cell Biology, Faculty of Science, Charles University, Prague, Czech Republic; <sup>4</sup>Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; <sup>5</sup>Systems Biology Center, National Heart Lung Blood Institute, National Institutes of Health, Bethesda, MD, USA; <sup>6</sup>1st Department of Internal Medicine, Pavol Jozef Safarik University in Kosice, Faculty of Medicine and Teaching Hospital of Louis Pasteur, Kosice, Slovakia; <sup>7</sup>Department of Medical Biology, Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic; <sup>8</sup>Southeast Center for Integrated Metabolomics, Clinical and Translational Science Institute, University of Florida, Gainesville, FL, USA; <sup>9</sup>Department of Medicine, Division of Endocrinology, Malcom Randall VA Medical Center, University of Florida, Gainesville, FL, USA; <sup>10</sup>Cancer Research Technology Program, Frederick National Laboratory for Cancer Research, Frederick, MD, USA; <sup>11</sup>Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic; <sup>12</sup>Section of Endocrinology, Boston Medical Center, Boston University, Boston, MA, USA; <sup>13</sup>3rd Department of Internal Medicine, University Hospital and Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic; <sup>14</sup>Department of Urology, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic; <sup>15</sup>Endocrine Surgery Section, Surgical Oncology Program, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; <sup>16</sup>Department of Nuclear Medicine, La Timone University Hospital, CERIMED, Aix-Marseille University, Marseille, France; <sup>17</sup>School of Pharmacy and Medical Science, Griffith University, Southport, QLD, Australia; and <sup>18</sup>Neuro-Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

<sup>†</sup>Co-first authors.

<sup>‡</sup>Authors contributed equally to this work.

\*Correspondence to: Karel Pacak, MD, PhD, DSc, FACE, Section on Medical Neuroendocrinology, Medicine, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Building 10, CRC, Room 1E-3140, 10 Center Drive MSC-1109, Bethesda, MD 20892-1109, USA (e-mail: karel@mail.nih.gov).

### Abstract

**Background:** Pheochromocytoma and paraganglioma (PPGL) are neuroendocrine tumors with frequent mutations in genes linked to the tricarboxylic acid cycle. However, no pathogenic variant has been found to date in succinyl-CoA ligase (SUCL), an enzyme that provides substrate for succinate dehydrogenase (SDH; mitochondrial complex II [CII]), a known tumor suppressor in PPGL. **Methods:** A cohort of 352 patients with apparently sporadic PPGL underwent genetic testing using a panel of 54 genes developed at the National Institutes of Health, including the *SUCLG2* subunit of SUCL. Gene deletion, succinate levels, and protein levels were assessed in tumors where possible. To confirm the possible mechanism, we used a progenitor cell line, hPheo1, derived from a human pheochromocytoma, and ablated and re-expressed *SUCLG2*. **Results:** We describe 8 germline variants in the guanosine triphosphate-binding domain of *SUCLG2* in 15 patients (15 of 352, 4.3%) with apparently sporadic PPGL. Analysis of *SUCLG2*-mutated tumors and *SUCLG2*-deficient hPheo1 cells revealed absence of *SUCLG2* protein, decrease in the level of the SDHB subunit of SDH, and faulty assembly of the complex II, resulting in aberrant respiration and elevated succinate accumulation. **Conclusions:** Our study suggests *SUCLG2* as a novel candidate gene in the genetic landscape of PPGL. Large-scale sequencing may uncover additional cases harboring *SUCLG2* variants and provide more detailed information about their prevalence and penetrance.

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Pheochromocytoma and paraganglioma (PPGL) are rare neuroendocrine tumors derived from neural crest cells (1). A pioneering study of Neumann and colleagues and recent comprehensive molecular analysis of these tumors identified, to this date, germline variants in more than 20 PPGL-susceptibility genes present in up to 40% of cases (2-6). Moreover, an additional 25%-30% of PPGL patients carry a somatic mutation (3), with up to 39% in 13 genes in some cohorts (5). These and other genetic, epigenetic, and relevant mechanistic studies on PPGL have increased our understanding of their pathogenesis and clinical behavior and uncovered potential new therapeutic targets in these hard-to-treat tumors (3,7,8).

Succinyl-CoA ligase (SUCL), a tricarboxylic acid (TCA) cycle enzyme, catalyzes reversible conversion of succinyl-CoA and adenosine diphosphate (ADP) or guanosine diphosphate (GDP) to succinate and ATP or guanosine triphosphate (GTP), respectively (9). SUCL is a heterodimer comprising  $\alpha$  subunit encoded by *SUCLG1* and a  $\beta$  subunit encoded by either the ATP-forming *SUCLA2* or the GTP-forming *SUCLG2*. SUCL is the only TCA cycle enzyme that can produce ATP- or GTP-linking the enzyme to mitochondrial DNA synthesis (10,11). Besides its important function in the TCA cycle, SUCL also plays a role in a number of anabolic processes, such as heme or lipid synthesis. Alterations in *SUCLG1* and *SUCLA2* activity because of their mutations cause encephalomyopathy presenting as hypotonia, dystonia and Leigh-like syndrome, seizure, psychomotor retardation, deafness, methylmalonic aciduria, and lactic acidosis (12-14). Kacso et al. (11) noted that the *Suclg2* gene could be essential for embryogenesis in mice as *Suclg2*<sup>-/-</sup> mice were embryonically lethal. Yet, the role of *SUCLG2* mutations in pathogenesis of human diseases has not been established.

## Methods

Adrenomedullary tissue separation, isolation of mitochondria, Western blot analysis, in-gel succinate dehydrogenase (SDH) activity, succinate quinone reductase (SQR) activity, native blue gel electrophoresis, mitochondrial DNA (mtDNA) assay, respiration assay, prediction of functional impact, and a detailed description of listed methods are included in the [Supplementary Methods](#) (available online).

## Patient Characteristics and Genetic Analysis

Patients were evaluated using a protocol approved by the Eunice Kennedy Shriver National Institute of Child Health and Human Development Institutional Review Board. All patients provided written informed consent approved by the National Institutes of Health ethics committee. In total, 352 patients with apparently sporadic PPGL underwent genetic testing of blood leukocyte DNA using a panel of 54 genes developed at the National Institutes of Health. Germline *SUCLG2* variants were validated by Sanger sequencing. Additional data for *SUCLG2* variant descriptions come from public databases including [www.ncbi.nlm.nih.gov/bioproject/](http://www.ncbi.nlm.nih.gov/bioproject/), [www.varsome.com](http://www.varsome.com/), [cancer.sanger.ac.uk/cosmic](http://cancer.sanger.ac.uk/cosmic) databases, and [gnomad.broadinstitute.org](http://gnomad.broadinstitute.org) from November 25, 2020. Seven family members of 4 patients were recruited and gave consent to analyze their saliva sample for presence of *SUCLG2* variants. Personal and family history were obtained by clinicians, and the available data were evaluated by clinical staff to record each patient's description, including evaluation of family incidence of PPGL.

## Immunohistochemistry

Immunohistochemistry of *SUCLG2* was performed as described in the [Supplementary Methods](#) (available online).

## FISH Analysis

For interphase fluorescence in situ hybridization (FISH) analysis, bacterial artificial chromosome DNA probes RP11-146E16 and RP11-927D18 labeled with orange fluorescence were purchased from Empire Genomics (Buffalo, NY). For chromosome quantification, we used Vysis CEP 3 (alpha satellite) Spectrum Green-labeled probes (Abbott Molecular, Abbott Park, IL). FISH assays were performed on 5  $\mu$ m formalin-fixed-paraffin-embedded tumor sections using standardized protocol with slight modifications (15), provided in the [Supplementary Methods](#) (available online).

## Cell Lines and In Vitro Gene Manipulation

For in vitro experiments, a progenitor cell line derived from a human pheochromocytoma (hPheo1) (16) was maintained in the RPMI medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, antibiotics, and 1 mM pyruvate at 37°C under 5% CO<sub>2</sub>. Genomic deletion of *SUCLG2* in hPheo1 cells was performed using the CRISPR/AsCas12a (also known as AsCpf1) system (17), as well as the chimeric Cas12a and the guide RNA-expression plasmid pX AsCpf1-Venus-NLS. crRNA sequences were selected using the Crispor software (<http://crispor.tefor.net/>). Oligonucleotide design, transfection, and primers design for genomic DNA polymerase chain reaction are detailed in the [Supplementary Methods](#) (available online).

For *SUCLG2* re-expression, *SUCLG2* DNA constructs were subcloned into the pCDH-CMV-MCS-EF1-Puro vector (CD510B-1; System Biosciences, Palo Alto, CA). Recombinant lentiviral particles were obtained from calcium phosphate-transfected HEK 293T cells using the packaging plasmid psPAX2 (12260; Addgene, Watertown, MA) and pMD2.G (12259; Addgene) and the *SUCLG2* pCDH construct. Target cells were transduced at the multiplicity of infection of 5 to 10 and selected by puromycin (2  $\mu$ g/mL; InvivoGen, San Diego, CA). Detailed protocol and information can be found in the [Supplementary Methods](#) (available online).

## Succinate-to-Fumarate Ratio Evaluation

Cell extracts were prepared and processed as detailed in the [Supplementary Methods](#) (available online). Silylated extracts were analyzed using 2-dimensional gas chromatography with mass spectrometric detection (GC  $\times$  GC-MS; Pegasus 4D; LECO Corp, St Joseph, MO) with more specific details in [Supplementary Methods](#) (available online). We used ChromaTOF software (v.4.51; LECO Corporation) for instrument control, data acquisition, and data processing. Succinic and fumaric acids were detected 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).

## Metabolomics of Patient Tissues

Quantification of organic acids was performed by liquid chromatography tandem mass spectrometry on a Thermo Quantiva instrument with Dionex 3000 high-performance liquid

chromatography system (Thermo Fisher Scientific, Waltham, MA) using isotope dilution. Briefly, 40  $\mu$ l of the tissue homogenate (100 mg/mL) was transferred to a microcentrifuge tube to which 10  $\mu$ l of internal standard mixture was added. Derivatization was performed by adding 50  $\mu$ l of 0.4 M o-benzylhydroxylamine (methanol/200 nM ammonium formate; 1:1) and 10  $\mu$ l of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride in water to each tube. The samples were incubated for 10 minutes, followed by the addition of 100  $\mu$ l water to each tube and 600  $\mu$ l of ethyl acetate. The tubes were mixed and centrifuged at 18 000 g for 5 minutes, after which 100  $\mu$ l of the ethyl acetate layer was transferred and dried under nitrogen. Each sample was reconstituted in 1 mL of a 1:1 methanol: water mixture for analysis. The standard operating procedure is available from the metabolomics workbench (www.metabolomicsworkbench.org).

### Statistical Analysis

Data are expressed as the mean (SD) of at least 3 independent experiments. Data were evaluated by 1-way analysis of variance with multiple comparison post hoc Tukey correction in GraphPad Prism (v.7.03; GraphPad Software, La Jolla, CA) and a Mann-Whitney nonparametric test for patient metabolomic data. All statistical tests were 2-sided. Data were considered statistically significant at a P value less than .05.

### Results

We identified 15 individuals with germline *SUCLG2* variants in our cohort of 352 patients (15 of 352, 4.3%) with apparently sporadic PPGL (Figure 1, A and B; Table 1). Seven men and 8 women were found to carry 7 missense *SUCLG2* variants (c.704T>A in 1 patient; c.302A>G in 3 patients; c.349G>A in 6 patients; c.296G>T in 1 patient; c.608A>C in 1 patient; c.158G>A in 1 patient; c.601G>C in 1 patient) and 1 deletion (c.539\_540delTG) within the GTP-binding pocket of the protein (Figure 1, A; Supplementary Figure 1, available online). Nine patients presented with pheochromocytoma (PHEO), 5 with paraganglioma (PGL), and 1 with primary tumor of unknown origin. Seven patients developed metastatic disease. Most patients presented with the noradrenergic biochemical phenotype. However, 4 patients with PHEO presented with both elevated plasma metanephrine and normetanephrine levels (Table 1).

In PPGL-related gene panel testing, we did not identify any known pathogenic variants in *SUCLG2*-mutated patients (Supplementary Table 1, available online). No patient reported any family history of PPGL. We collected saliva samples from 7 first-degree relatives of 4 patients (Table 2). For 3 patients—patients 2 (c.302A>G), 10 (c.302A>G), and 12 (c.158G>A)—1 of the parents was a carrier of a particular variant. In one case, a sample from a sibling was collected and presented with the same variant. For patient 11 (c.539\_540delTG), only 1 parental DNA sample was available and was negative for the variant (Table 2).

*SUCLG2* variant sites were located within highly evolutionarily conserved positions (Figure 1, E). Consistently, GERP++ (Genomic Evolutionary Rate Profiling) returned scores above 5 (Table 3). Five missense variants (c.704T>A, c.295G>T, c.608A>C, c.158G>A, c.601G>C) were not listed in ClinVar and occurred at a very low frequency ( $<10^{-3}$ ) with no homozygotes in the general population (Genome Aggregation Database). The combination of protein function prediction tools led to

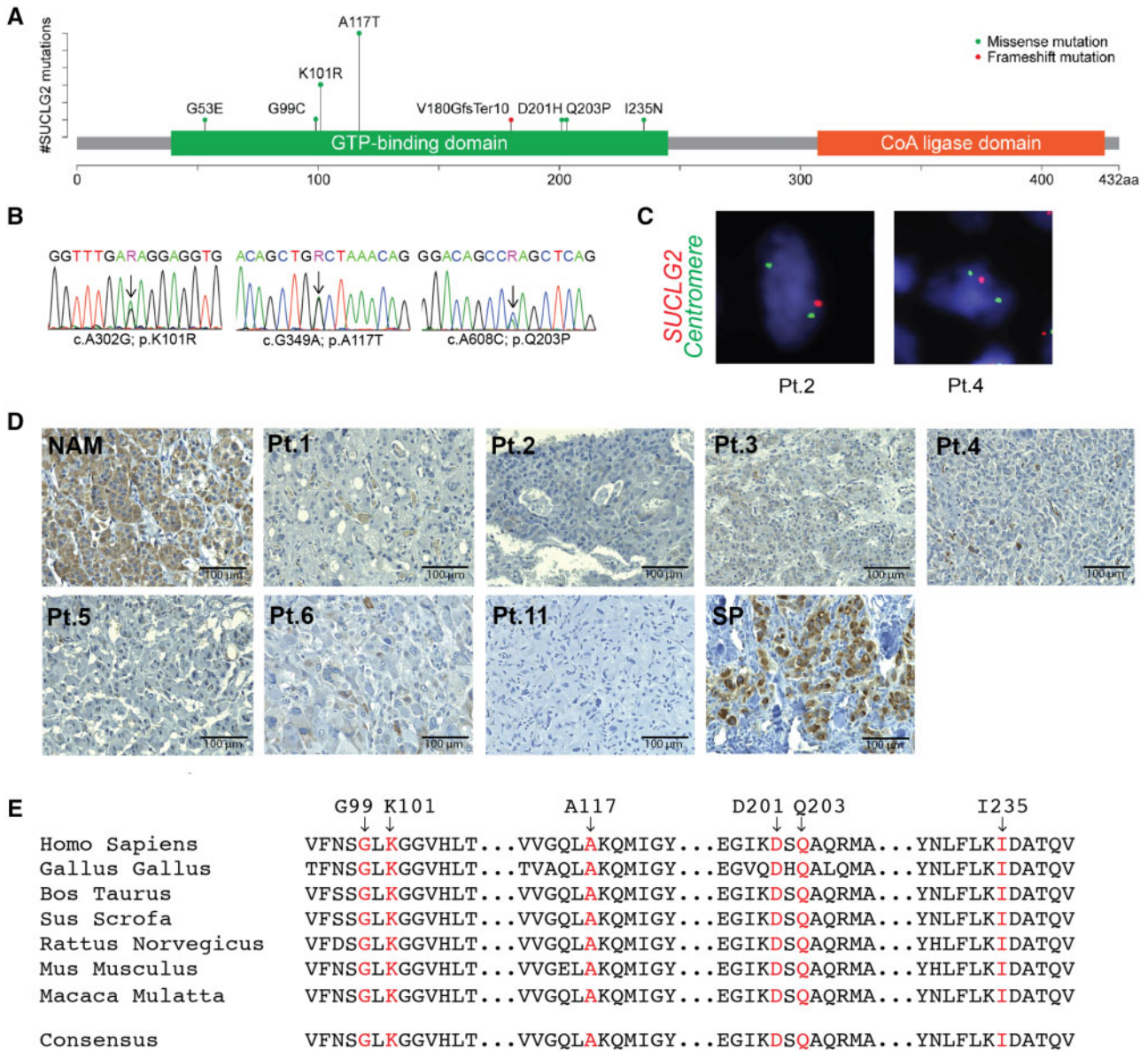
inconclusive results for functional outcomes of variant c.704T>A and c.601G>C and damaging functional outcomes for variants c.295G>T, c.608A>C, and c.158G>A (Table 3). The deletion variant c.539\_540delTG could not be classified by the used prediction tools. Variants c.302A>G and c.349G>A were listed in ClinVar database for germline variants, both with benign and likely benign prediction, with frequency in the general population of 0.0047 and 0.0076, respectively. Evaluation from protein function prediction tools led to inconclusive outcomes (Table 3). The variant c.349G>A was recorded in the Catalogue Of Somatic Mutations In Cancer database in 3 cases of lung cancer and 2 cases of upper aerodigestive tract cancer. Overall, prediction of functional impact was rather inconclusive (Table 3). Thus, we performed functional studies in the available material (Table 3; Figures 1, C and D, and 2, A and B; Supplementary Table 2, available online).

FISH detected gene deletion in available tumor tissues (Figure 1, C), that is, archival material from 3 patients: patients 2 (c.302A>G), 4 (c.349G>A), and 11 (c.539\_540delTG). All 3 patients demonstrated loss of 1 copy of the *SUCLG2* gene. The *SUCLG2* gene deletion was detected in 77.2%, 47.8%, and 44.2% of cells in c.302A>G (patient 2), c.349G>A (patient 4), and c.539\_540delTG (patient 11) tumors, respectively. Loss of the entire chromosome 3 copy was the most common pattern in the tumor cell population, although a hemizygous deletion of the *SUCLG2* gene with the retention of 2 chromosome 3 centromeres was observed in a subpopulation of the tumor cells as well. Furthermore, immunohistochemistry revealed a substantial decrease in the *SUCLG2* protein level in tumor tissue from patients 1-6 and 11 listed in Table 1 (Figure 1, D).

Metabolomic analysis of tumor biopsies from 6 patients with *SUCLG2* variants (patients 1-6 in Table 1) showed increased succinate-to-fumarate ratio relative to normal adrenal medulla (mean [SD] = 12.25 [5.46] vs 2.07 [1.89];  $P = .02$ ), a shift to glycolytic metabolism, and dysregulation of the TCA cycle (Supplementary Table 2, available online), pointing to dysfunction of succinate dehydrogenase (SDH). Western blot analysis of tumor but not normal adrenal medulla mitochondria showed a substantially decreased level of the SDH subunit B (SDHB) protein and no effect on the SDH subunit A (SDHA) protein (Figure 2, A). Consistently, decreased SDH in-gel activity and mitochondrial complex II (CII)-dependent respiration were detected in tumor mitochondria (Figure 2, B and C), suggesting that *SUCLG2* defects may compromise SDH.

To investigate the impact of *SUCLG2* variants in more detail, we established *SUCLG2*-knockout (*SUCLG2*<sup>KO</sup>) hPheo1 cells in which we subsequently re-expressed the protein to prepare *SUCLG2*<sup>rec</sup> cells. Western blot revealed that absence of the *SUCLG2* protein had little effect on *SUCLG1* and no effect on *SUCLA2* levels (Figure 2, D). Assessment of CII subunits indicated unchanged SDHA but reduced SDHB protein levels in *SUCLG2*<sup>KO</sup> cells relative to parental cells, which was recovered by *SUCLG2* re-expression (Figure 2, E). Although there was some variability in the extent of SDHB downregulation in individual *SUCLG2*<sup>KO</sup> clones (data not shown), reversal of the phenotype by cDNA confirmed the specificity. Native blue gel electrophoresis revealed impaired CII assembly in *SUCLG2*<sup>KO</sup> cells, which was present as the CII<sub>low</sub> subassembly, consistent with decreased SDHB (18), and fully assembled CII was observed in parental and *SUCLG2*<sup>rec</sup> cells (Figure 2, F).

These data indicate a disruption of CII in *SUCLG2*-deficient cells, possibly affecting mitochondrial function. Therefore, we assessed CII activity in *SUCLG2*<sup>KO</sup> and *SUCLG2*<sup>rec</sup> cells, comprising SDH-dependent conversion of succinate to fumarate in the



**Figure 1.** Detection of *SUCLG2* variants in PPGL patients. **A)** Schematic illustration of *SUCLG2* germline variants in the GTP-binding site. **B)** Sanger sequencing showing *SUCLG2* germline variants in patient leukocyte DNA. **C)** FISH assay showing apparent heterozygous deletion of *SUCLG2* in tumor specimens. **D)** Immunohistochemistry analysis revealing a substantial decrease in *SUCLG2* level in PPGL with *SUCLG2* germline variants (patient number refers to Table 1) relative to that in normal adrenal medulla (NAM) and sporadic PPGL (SP). **E)** Sequence alignment of *SUCLG2* residues 85 to 240 (human *SUCLG2* nomenclature) showing conservation of the mutated residues. FISH = fluorescence in situ hybridization; GTP = guanosine triphosphate; PPGL = pheochromocytoma and paraganglioma.

TCA cycle and SQR-mediated transfer of electrons from succinate to ubiquinone as part of OXPHOS. We found that both SDH and SQR activities were suppressed in *SUCLG2*<sup>KO</sup> cells to 40.3% (SD = 16.1,  $P < .001$ ) and 25.3% (SD = 7.8,  $P < .001$ ), respectively, and recovered in *SUCLG2*<sup>rec</sup> cells to parental cell values (Figure 2, G and H). Consistently, CII-dependent respiration was depressed in *SUCLG2*<sup>KO</sup> cells compared with parental cells (mean [SD] = 20.1 [3.5] pmol/(s\*10<sup>6</sup> cells) vs 53.7 [10.2] pmol/(s\*10<sup>6</sup> cells);  $P < .001$ ) and restored in *SUCLG2*<sup>rec</sup> cells (Figure 2, I). Moreover, the succinate-to-fumarate ratio was increased by up to 10-fold in *SUCLG2*<sup>KO</sup> cells relative to that in control (mean [SD] = 9.3 [0.2] vs 1.1 [0.2];  $P < .001$ ) and *SUCLG2*<sup>rec</sup> cells (mean [SD] = 0.95 [0.04];  $P < .001$ ) (Figure 2, J), recapitulating the increase succinate-to-fumarate ratio in patient tumors (Supplementary Table 2, available online).

Because *SUCLG1* and *SUCLA2* germline variants are linked to the mtDNA-depletion syndrome (19), we evaluated mtDNA levels in patients with *SUCLG2* germline variants and in *SUCLG2*<sup>KO</sup> cells. mtDNA was significantly reduced in tumor tissues (Table 1) and cells where it recovered on re-expression of *SUCLG2* (Figure 2, K), suggesting that the integrity of SUCL protein plays a role in mtDNA maintenance. However, no patient from our cohort had distinctive symptoms of mitochondrial disorders seen in *SUCLG1* and *SUCLA2* variants.

## Discussion

With respect to metabolic diseases, SUCL has a unique position in the TCA cycle for 2 major reasons. First, its products ATP or

**Table 1.** Clinical characteristics of patients

ID	Sex	Age at initial diagnosis, y	Mutation in <i>SUCLG2</i>	Protein change	mtDNA depletion	Biochemistry at initial diagnosis <sup>a</sup>	Initial tumor type	Primary tumor size, cm	Metastatic	Recurrent
1 <sup>b</sup>	Male	22	c.704T>A	I235N	Yes	NE, NMN, CgA	sPGL	3.5	No	No
2	Male	43	c.302A>G	K101R	Yes	NE, NMN, MN, CgA	PHEO	3.8	No	No
3	Female	45	c.302A>G	K101R	Yes	NE, NMN	PHEO	4.3	No	No
4 <sup>b</sup>	Male	25	c.349G>A	A117T	Yes	NE <sup>c</sup> , NMN <sup>c</sup>	PHEO	7.5	No	Yes
5	Male	51	c.295G>T	G99C	Yes	NE <sup>c</sup> , NMN <sup>c</sup>	PHEO	6.0	Yes	Yes
6	Female	41	c.349G>A	A117T	Yes	NE, NMN, EPI, MN	PHEO	5.8	No	No
7	Female	10	c.349G>A	A117T	NA	NMN	PHEO	4.1	No	No
8 <sup>b</sup>	Female	29	c.349G>A	A117T	NA	CgA <sup>c</sup>	pPGL	NA	Yes	No
9	Male	32	c.608A>C	Q203P	NA	EPI, NE	PHEO	NA	No	No
10	Female	39	c.302A>G	K101R	NA	NE, DA	PGL	4.6	Yes	No
11	Female	38	c.539_540delTG	Val180fs	Yes	NMN, MN	PHEO	6.5	Yes	Yes
12	Male	32	c.158G>A	G53E	NA	NE, NMN, CgA	PGL	7.0	Yes	No
13	Female	70	c.349G>A	A117T	NA	NE, NMN	PGL	4.5	No	No
14 <sup>b</sup>	Male	56	c.349G>A	A117T	NA	MTX, CgA	UNP	NA	Yes	No
15 <sup>b</sup>	Female	25	c.601G>C	D201H	NA	NE <sup>c</sup> , NMN <sup>c</sup> , EPI <sup>c</sup> , MN <sup>c</sup> , DA <sup>c</sup>	PHEO	12.4	Yes	No

<sup>a</sup>Increased analytes displayed. For primary tumor size, only the largest tumor diameter is shown. CgA = chromogranin A; DA = dopamine; EPI = epinephrine; MN = metanephrine measured either in plasma or urine; MTX = methoxytyramine; NA = not available; NE = norepinephrine; NMN = normetanephrine; PHEO = pheochromocytoma; PGL = paraganglioma; pPGL = parasympathetic PGL; sPGL = sympathetic paraganglioma; UNP = unknown primary.

<sup>b</sup>Indicates death of the patient.

<sup>c</sup>Biochemistry done at the National Institutes of Health, at the time of initial diagnosis not done or no data available.

**Table 2.** Detection of *SUCLG2* variants in patients' relatives<sup>a</sup>

ID sample	<i>SUCLG2</i> variant	Sample	Variant status	Cancer history
2	c.302A>G	Paternal	Present	NR
		Maternal	ND	NR
		Sibling	Present	Pancreatic neuroendocrine tumor
10	c.302A>G	Paternal	ND	Lung
		Maternal	Present	Breast
11	c.539_540delTG	Paternal	NA	Prostate, colon, lung
		Maternal	Not detected	NR
12	c.158G>A	Paternal	NA	Suspected liver
		Maternal	Present	NR

<sup>a</sup>Available saliva samples from first-degree relatives were tested for presence of particular *SUCLG2* variants by Sanger Sequencing. NA = sample not available; ND = not detected; NR = not reported.

GTP connect the enzyme to mtDNA synthesis and maintenance via mitochondrial nucleoside diphosphate kinase (10,11), which links *SUCLG2* to inborn mitochondrial pathologies. Second, the other product of the enzyme, succinate, is an oncometabolite (20) and a substrate for CII that is linked to PPGL. Despite such close proximity, mutations in *SUCLG2* subunits have not been reported in cancer until now.

We evaluated 352 apparently sporadic PPGL patients and found 15 (4.3%) patients with 8 germline variants located within the GTP-binding domain of *SUCLG2* (Table 1). Because GTP activity was proposed to be important for phosphorylation of active-site histidine residues to trigger the movement of the carboxylate of succinate into position to be phosphorylated in porcine *SUCLG2* (21), we further analyzed these variants. Albeit, functional impact prediction for variant c.704T>A was inconclusive, and the tumor was negative for *SUCLG2*, showed decreased protein level in the mitochondrial extract, functional changes in

SDH activity, and succinate accumulation, suggesting a damaging effect of the variant. Two most represented variants c.302A>G and c.349G>A in our cohort were evaluated in ClinVar as benign or likely benign; however, detailed information on the cohort and its specificity was not available to us, and the functional impact on protein function was inconclusive. Yet, tumors with these variants presented with negative immunohistochemistry staining for *SUCLG2*, protein level decrease in mitochondrial extract, gene deletion, altered SDH function, and TCA cycle metabolite disbalance, suggesting a damaging effect of the variants. Damaging functional impact from predictions was suggested for the variant c.295G>T that was reflected by negative *SUCLG2* staining and succinate accumulation in available tumor sample. The deletion variant c.539\_540delTG could not be classified by the used prediction tools, but we reported the gene deletion by the FISH assay and negative immunohistochemistry staining suggesting damaging impact of the variant.

Table 3. SUC1G2 variants (chr.3, NM\_003848.4), in silico prediction and summary of functional outcomes<sup>a</sup>

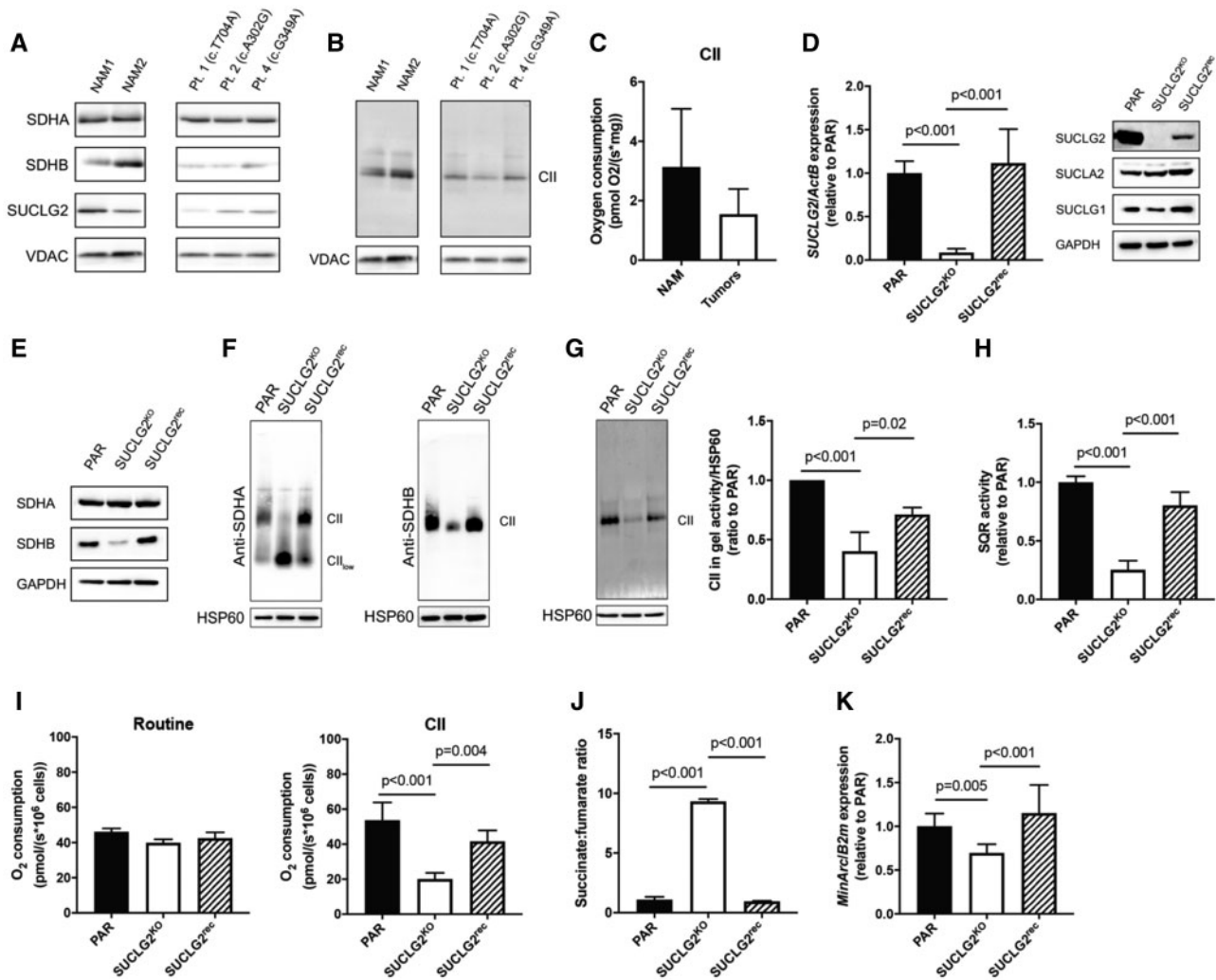
	ID sample														
SUC1G2 variants	1	2, 3, 10	4, 6-8, 13, 14	5	9	11	12	15							
Nucleotide change	c.704T>A	c.302A>G	c.349G>A	c.295G>T	c.608A>C	c.539_540delITG	c.158G>A	c.601G>C							
Amino acid change	I235N	K101R	A117T	G99C	Q203P	Val180ClyfsTer10	G53E	D201H							
Exon	7	3	4	3	6	5	2	6							
Position in Hg19	67 559 284	67 579 535	67 578 624	67 579 542	67 568 723	67 570 936–7	67 659 947	67 568 730							
gnomAD allele frequency (No. of homozygotes)	0.000004029 = 1/248 218 (0)	0.004736 = 1326/279 992 (7)	0.007629 = 2141/280 634 (57)	0.0001643 = 46/280 026 (0)	0.0001465 = 41/279 854 (0)	0.00001781 = 5/280 762 (0)	not described	0.000004035 = 1/247 828 (0)							
ClinVar ID	—	257 601 benign	774 168 benign/ likely benign	—	—	—	—	—							
dbSNP	rs1433609554	rs74675534	rs13318110	rs200916187	rs201652728	—	—	rs902797961							
Protein function prediction	—	—	—	—	—	—	—	—							
Mutation assessor (score)	Neutral (–0.66)	Medium (2.36)	Medium (2.04)	High (3.75)	High (3.91)	—	Medium (2.97)	Medium (2.13)							
SIFT	Tolerated	Damaging	Tolerated	Damaging	Tolerated/damaging	—	Damaging	Tolerated/damaging							
Polyphen-2 (score)	Probably damaging (0.51)	Benign (0.19)	Benign (0.03)	Probably damaging (1)	Probably damaging (0.98)	—	Probably damaging (0.94)	Benign (0.06)							
Functional impact (damaging/benign) <sup>b</sup>	5/7	6/5 <sup>c</sup>	6/5 <sup>c</sup>	11/1	9/3	—	11/1	8/4							
Evolutionary conservation	—	—	—	—	—	—	—	—							
GERP+++ (score)	Conserved (5.8)	Conserved (5.01)	Conserved (5.95)	Conserved (5.01)	Conserved (5.87)	Conserved (5.7; 5.09)	Conserved (5.78)	Conserved (5.87)							
Functional outcomes	Conserved (5.8)	Conserved (5.01)	Conserved (5.95)	Conserved (5.01)	Conserved (5.87)	Conserved (5.7; 5.09)	Conserved (5.78)	Conserved (5.87)							
IHC SUC1G2	Negative	#2; 3 negative	#4; 6 negative	Negative	—	Negative	—	—							
Western blot <sup>d</sup>	Decrease	#2 decrease	#4 decrease	—	—	—	—	—							
FISH SUC1G2	—	#2 deletion (78% cells)	#4 deletion (44% cells)	—	—	Deletion (48% cells)	—	—							
SDH activity	Decrease	#2 decrease	#4 decrease	—	—	—	—	—							
Succ/Fum ratio	Increase	#2; 3 increase	#4; 6 increase	Increase	—	—	—	—							

<sup>a</sup>Data come from [www.ncbi.nlm.nih.gov/variation/portal/](http://www.ncbi.nlm.nih.gov/variation/portal/), [www.ensembl.org](http://www.ensembl.org/), and [gnomad.broadinstitute.org](http://gnomad.broadinstitute.org/). Functional studies data are summarized from experiments and results reported in [Figures 1 and 2](#) and [Supplementary Table 2](#) (available online). FISH = fluorescence in situ hybridization; Fum = fumarate; gnomAD = Genome Aggregation Database; IHC = immunohistochemistry; SDH = succinate dehydrogenase; Succ = succinate.

<sup>b</sup>Includes in silico predictive programs available on [www.varsome.com](http://www.varsome.com) used in the American College of Medical Genetics and Genomics (ACMG) classification—BayesDel\_addAF, DEOGEN2, EIGEN, MVP, MutationAssessor, PrimateAI, SIFT, DANN, FATHMM-MKL, LIST-S2, M-Cap, MutationTaster.

<sup>c</sup>M-Cap not available.

<sup>d</sup>Western blot of SUC1G2 in mitochondria extract from tumor tissue.



**Figure 2.** Mitochondrial complex II functions in *SUCLG2*-mutated tumors and *SUCLG2*-silenced hPheo1 cell line. **A)** Western blot analysis of SDHB, SDHA, and *SUCLG2*; **B)** SDH in-gel activity; and **C)** CII-dependent respiration in mitochondrial lysate of normal adrenal medulla (NAM) and PPGL patients with mutant *SUCLG2*. Pt 1, 2, and 4 refers to patients 1, 2, and 4 in Table 1. Data are presented as mean [SD] and evaluated by a 2-sided Student t test. **D)** *SUCLG2* mRNA (left) and *SUCLG2*, *SUCLA2*, and *SUCLG1* protein levels (right) in parental (PAR), *SUCLG2*<sup>KO</sup>, and *SUCLG2*<sup>rec</sup> cells. **E)** SDHA and SDHB protein levels in PAR, *SUCLG2*<sup>KO</sup>, and *SUCLG2*<sup>rec</sup> cells. **F)** Native blue gels showing the assembly of mitochondrial CII in PAR, *SUCLG2*<sup>KO</sup>, and *SUCLG2*<sup>rec</sup> cells using antibodies against SDHA and SDHB. **G)** In-gel SDH activity in PAR, *SUCLG2*<sup>KO</sup>, and *SUCLG2*<sup>rec</sup> cells. **H)** SQR activity in PAR, *SUCLG2*<sup>KO</sup>, and *SUCLG2*<sup>rec</sup> cells. **I)** Routine (left) and CII-dependent (right) respiration in PAR, *SUCLG2*<sup>KO</sup>, and *SUCLG2*<sup>rec</sup> cells. **J)** Succinate-to-fumarate ratio in PAR, *SUCLG2*<sup>KO</sup>, and *SUCLG2*<sup>rec</sup> cells. **K)** Levels of mtDNA in PAR, *SUCLG2*<sup>KO</sup>, and *SUCLG2*<sup>rec</sup> cells. All in vitro experiments were performed at least 3 times independently. Data are presented as mean (SD) and evaluated by 1-way analysis of variance with post hoc Tukey test. All statistical tests were 2-sided. PAR = parental hPheo1 cells; NAM = normal adrenal medulla; CII = mitochondrial complex II.

Variants c.608A>C, c.158G>A, and c.601G>C were predicted to have damaging impact on protein function, but no tissues for analysis were available to confirm this notion.

Our study revealed that *SUCLG2* germline variants impacted TCA cycle function, resulting in increased succinate levels associated with decreased SDH activity in PPGL, suggesting a possible mechanism for tumor development. SDH links the TCA cycle directly to OXPHOS (22), and genes encoding SDH subunits are frequently mutated in PPGL (23). *SDHx* mutations result in accumulation of succinate, which acts as an oncometabolite that contributes to prolyl hydroxylases suppression associated with stabilization of hypoxia-inducible factors, DNA hypermethylation, and histone modifications, all presumably favoring tumorigenicity (7,20,24,25). Interestingly, in our cohort, the malignancy rate for *SUCLG2*-mutated PPGL (7 of 15 patients, 46.7%,

in time of analysis) was similar to *SDHx*-mutated PPGLs (26-28), and the patients presented mostly with the noradrenergic biochemical phenotype that is generally connected to pseudohypoxia in PPGLs (TCA cycle enzymes and pseudohypoxia signaling mutations) (29,30). Importantly, *SUCLG2* manipulation in hPheo1 cells confirmed a link between the *SUCLG2* mutations and SDH function. Ablation of *SUCLG2* affected the level of the SDHB protein and CII assembly, and consequently SDH functions, resulting in succinate accumulation. These changes mostly recovered by re-expression of *SUCLG2* in the knock-out cells, pointing to specific effects of *SUCLG2* deletion. We did not observe mtDNA-related disease symptoms often reported in patients with *SUCLG1* and *SUCLA2* variants in our cohort of *SUCLG2* mutant patients even though mtDNA levels were decreased in the experimental cell model and patient tumor

tissue, consistent with previous research on the link between SUCL and mtDNA maintenance (11).

Patients described here presented without any family history of PPGL, which is supportive of the low disease penetrance that was previously reported for another TCA cycle gene *SDHA* (31,32). We were able to recruit family members of 4 patients; however, for 2 of these patients, we had only 1 parental sample. We found parental carriers of variants c.302A>G and c.158G>A, and although various cancers were reported in these families, none of them were of PPGL etiology. Interestingly, we found that the sibling of patient 2, who is also a carrier of c.302A>G variant presented with pancreatic neuroendocrine tumor. Even though only a limited number of relatives were available, *SUCLG2* variants seem to have incomplete penetrance that was repeatedly recorded for novel PPGL-related TCA genes like fumarate hydratase (33) or malate dehydrogenase (34).

From a pathophysiological viewpoint, it is possible that patients with *SUCLG2* germline variants consequently develop CII dysfunction leading to PPGL formation. Previously, Rapizzi et al. (35) described impairment of SDH activity in non-SDHx-mutated PPGLs, possibly because of germline variants in other known (ie, *MAX*) or unknown susceptibility genes. It remains to be shown whether *SUCLG2* mutations consistently lead to CII dysfunction and what determines the severity of the effect. Therefore, further studies to reveal the molecular mechanism associated with the link between *SUCLG2* germline variants and CII function are needed and are subjects of our ongoing research.

There are several limitations of our study. First, we did not have a chance to collect and sequence DNA of all first-degree family members to fully confirm the prevalence and penetrance of the reported *SUCLG2* germline variants. Second, we did not have access to or availability of all patient tumor samples to validate all parameters in each sample. We believe that information from future PPGL validation cohorts will help complete the picture and establish the clinical significance of *SUCLG2* variants in PPGLs.

In summary, our analysis of *SUCLG2*-mutated tumors and in vitro experiments in *SUCLG2*-deficient cells identified association and mechanism between *SUCLG2* variants and PPGL, and we propose *SUCLG2* as a PPGL-associated candidate gene, which should be further evaluated to establish clinical significance in the genetic landscape of apparently sporadic PPGL cases. Future studies involving large-scale sequencing may discover additional cases harboring *SUCLG2* variants and provide more detailed information about their prevalence and penetrance. These findings will further clarify the relationship between *SUCLG2* and SDHx proteins, particularly *SDHB*, as well as their role in the disease etiology.

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## Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its [Supplementary Material](#) (available online). Data from consented patients are available in dbGaP with accession number phs002405.v1.p1.

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