Germline SUCLG2 Variants in Patients with Pheochromocytoma and Paraganglioma

Author

Vanova, Katerina Hadrava, Pang, Ying, Krobova, Linda, Kraus, Michal, Nahacka, Zuzana, Boukalova, Stepana, Pack, Svetlana D, Zobalova, Renata, Zhu, Jun, Huynh, Thanh-Truc, Jochmanova, Ivana, Uher, Ondrej, Hubackova, Sona, Neuzil, Jiri, et al.

Published

2021

Journal Title

JNCI: Journal of the National Cancer Institute

Version

Accepted Manuscript (AM)

DOI

10.1093/jnci/djab158

Germline *SUCLG2* Variants in Patients with Pheochromocytoma and Paraganglioma

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

† Co-first authors. *These authors contributed equally.

[1] Section of Medical Neuroendocrinology, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA;

[2] Institute of Biotechnology, Czech Academy of Sciences, BIOCEV, Vestec, Prague West, Czech Republic;

[3] Faculty of Science, Charles University, Prague, Czech Republic;

[4] Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA;

[5] Systems Biology Center, National Heart Lung Blood Institute, National Institutes of Health, Bethesda, MD, USA;

[6] 1st Department of Internal Medicine, Pavol Jozef Safarik University in Kosice, Faculty of Medicine and Teaching Hospital of Louis Pasteur, Kosice, Slovakia;

[7] Department of Medical Biology, Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic;

[8] Southeast Center for Integrated Metabolomics, Clinical and Translational Science Institute, University of Florida, Gainesville, FL, USA;

[9] Department of Medicine, Division of Endocrinology, Malcom Randall VA Medical Center, University of Florida, Gainesville, FL, USA;

[10] Cancer Research Technology Program, Frederick National Laboratory for Cancer Research, Frederick, MD, USA;

[11] Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic;

[12] Section of Endocrinology, Boston Medical Center, Boston University, Boston, MA, USA;

[13] 3rd Department of Internal Medicine, University Hospital and Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic;

[14] Department of Urology, University Hospital Olomouc and Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic;

[15] Endocrine Surgery Section, Surgical Oncology Program, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA;

[16] Department of Nuclear Medicine, La Timone University Hospital, CERIMED, Aix-Marseille University, Marseille, France;

[17] School of Pharmacy and Medical Science, Griffith University, Southport, Qld, Australia;

[18] Neuro-Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.

Corresponding author:

Karel Pacak, MD, PhD, DSc, FACE, Chief, Section on Medical Neuroendocrinology, Professor of 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.

Phone: (301) 402-4594

Fax: (301) 402-4712

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 subjects 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 eight germline variants in the GTP-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 CII and faulty assembly of the complex, 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.

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% cases (2-6). Moreover, 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 ADP or GDP to succinate and ATP or GTP, respectively (9). SUCL is a heterodimer comprising α subunit encoded by *SUCLG1* and a β 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 due to 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. noted that the Suclg2 gene could be essential for embryogenesis in mice as *Suclg2*$^{-/-}$ mice were embryonically lethal (11). 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 SDH activity, SQR activity, native blue gel electrophoresis, mtDNA assay, respiration assay, prediction of functional impact, and 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* NICHD IRB. All patients provided written informed consent approved by the NIH 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* variants description come from public databases including www.cbioportal.org, www.varsome.com, cancer.sanger.ac.uk/cosmic databases, and gnomad.broadinstitute.org from 25th of November 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 FISH analysis, BAC DNA probes RP11-146E16 and RP11-927D18 labeled with orange fluorescence were purchased from Empire Genomics (Buffalo, NY, USA). For chromosome quantification, 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 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) were maintained in the RPMI medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum, antibiotics, and 1 mM pyruvate at 37 °C under 5% CO_2. Genomic deletion of *SUCLG2* in hPheo1 cells was performed using the CRISPR/AsCas12a (also known as AsCpf1) system (17), plus 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 PCR are detailed in the **Supplementary Methods** (available online).

For SUCLG2 re-expression, *SUCLG2* DNA constructs were sub-cloned into the pCDH-CMV-MCS-EF1-Puro vector (CD510B-1; System Biosciences, Palo Alto, CA, USA). Recombinant lentiviral particles were obtained from calcium phosphate-transfected HEK 293T cells using the packaging plasmid psPAX2 (12260; Addgene, Watertown, MA, USA) 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 μg/ml; InvivoGen, San Diego,

CA, USA). 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 two-dimensional gas chromatography with mass spectrometric detection (GC×GC-MS; Pegasus 4D; LECO Corporation, St. Joseph, MO, USA) with more specific details in Supplementary Methods. 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 MS on a Thermo Quantiva instrument with Dionex 3000 high-performance liquid chromatography system (Thermo Fisher Scientific) using isotope dilution. Briefly, 40 µl of the tissue homogenate (100 mg/ml) was transferred to a microcentrifuge tube, to which 10 µl of internal standard mixture was added. Derivatization was performed by adding 50 µl of 0.4 M o-benzylhydroxylamine (methanol/200 nM ammonium formate; 1:1) and 10 µl of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride in water to each tube. The samples were incubated for 10 min, followed by the addition of 100 µl water to each tube and 600 µl of ethyl acetate. The tubes were mixed and centrifuged at 18,000g for 5 min, after which 100 µl 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 three independent experiments. Data were evaluated by one-way ANOVA with multiple comparison post-hoc Tukey correction in GraphPad Prism (v.7.03; GraphPad Software, La Jolla, CA, USA) and a Mann–Whitney non-parametric test for patient metabolomic data. All statistical tests were 2-sided. Data were considered statistically significant at $p < 0.05$.

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 1A, B; Table 1**). Seven males and eight females 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. 1A; Supplementary Figure 1**, available online). Nine patients presented with PHEO, five with PGL, and one with primary tumor of unknown origin. Seven patients developed metastatic disease. Most patients presented with the noradrenergic biochemical phenotype. However, four 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 three patients #2 (c.302A>G), #10 (c.302A>G), and #12 (c.158G>A) one of the parents was a carrier of a particular variant. In one case a sample from sibling was collected and presented with the same variant. For patient #11 (c.539_540delTG), only one parental DNA sample was available and was negative for the variant (**Table 2**).

SUCLG2 variant sites were located within highly evolutionarily conserved positions (**Figure 1E**). 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 (gnomAD). 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 COSMIC (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**; **Figure 1C, D; Figure 2A, B, Supplementary Table 2,** available online).

Fluorescence *in situ* hybridization (FISH) detected gene deletion in available tumor tissues (**Figure 1C**), i.e., archival material from 3 patients, ID #2 (c.302A>G), #4 (c.349G>A), and #11 (c.539_540delTG). All three cases demonstrated loss of one 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 (#2), c.349G>A (#4), and c.539_540delTG (#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 two 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 1D**).

Metabolomic analysis of tumor biopsies from six patients with *SUCLG2* variants (patients #1-6 in **Table 1**) showed increased succinate-to-fumarate ratio relative to normal adrenal medulla (NAM) (mean [SD] = 12.25 [5.46] vs 2.07 [1.89], p=0.02), a shift to glycolytic metabolism, and dysregulation of the TCA cycle (**Supplementary Table 2**, available online), pointing to dysfunction of SDH. Western blot analysis of tumor but not NAM mitochondria showed substantially decreased level of the SDHB protein and no effect on the SDHA protein (**Figure 2A**). Consistently, decreased SDH in-gel activity and CII-dependent respiration were detected in tumor mitochondria (**Figure 2B, C**), suggesting that SUCLG2 defects may compromise SDH.

To investigate the impact of *SUCLG2* variants in more detail, we established *SUCLG2*-knockout ($SUCLG2^{KO}$) hPheo1 cells, in which we subsequently re-expressed the protein to prepare $SUCLG2^{rec}$ cells. Western blot revealed that absence of the SUCLG2 protein had little effect on SUCLG1 and no effect on SUCLA2 levels (**Figure 2D**). Assessment of CII subunits indicated unchanged SDHA, but reduced SDHB protein levels in $SUCLG2^{KO}$ cells relative to parental cells,

which was recovered by *SUCLG2* re-expression (**Figure 2E**). While there was some variability in the extent of SDHB downregulation in individual *SUCLG2*^{KO} clones (data not shown), reversal of the phenotype by cDNA confirmed the specificity. Native blue gel electrophoresis revealed impaired CII assembly in *SUCLG2*^{KO} cells, which was present as the CII_{low} subassembly, consistent with decreased SDHB (18), while fully assembled CII was observed in parental and *SUCLG2*^{rec} cells (**Figure 2F**).

These data indicate a disruption of CII in SUCLG2-deficient cells, possibly affecting mitochondrial function. Therefore, we assessed CII activity in *SUCLG2*^{KO} and *SUCLG2*^{rec} cells, comprising SDH-dependent conversion of succinate to fumarate in the TCA cycle and succinate quinone reductase (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*^{KO} cells to 40.3% (SD=16.1, $p<0.001$) and 25.3% (SD=7.8), $p<0.001$), respectively, and recovered in *SUCLG2*^{rec} cells to parental cell values (**Figure 2G, H**). Consistently, CII-dependent respiration was depressed in *SUCLG2*^{KO} cells compared to parental cells (mean [SD] = 20.1 [3.5] pmol/(s*10^6 cells) vs 53.7 [10.2] pmol/(s*10^6 cells), $p<0.001$) and restored in *SUCLG2*^{rec} cells (**Figure 2I**). Moreover, the succinate-to-fumarate ratio was increased by up to 10-fold in *SUCLG2*^{KO} cells relative to that in control (mean [SD] = 9.3 [0.2] vs 1.1 [0.2], $p<0.001$) and *SUCLG2*^{rec} cells (mean [SD] = 0.95 [0.04], $p<0.001$) (**Figure 2J**), 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*^{KO} cells. mtDNA was significantly reduced in both tumor tissues (**Table 1**) and cells where it recovered upon re-expression of SUCLG2 (**Figure 2K**), 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 two major reasons. First, its products ATP or GTP connect the enzyme to mtDNA synthesis and maintenance via mitochondrial nucleoside diphosphate kinase (10, 11), which links SUCL 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 SUCL subunits have not been reported in cancer till now.

We evaluated 352 apparently sporadic PPGL patients and found 15 patients (4.3%) with 8 germline variants located within the GTP-binding domain of SUCLG2 (**Table 1**). Since 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, the tumor was negative for SUCLG2, showed decrease 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/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 IHC 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 IHC staining suggesting damaging impact of the variant. 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 (20, 24-26). 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 (27-29), 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) (30, 31). 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 both 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* (32, 33). We were able to recruit family members of 4 patients; however, for 2 of these patients we had only one parental sample. We found parental carriers of variants c.302A>G and c.158G>A and while 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 limited number of relatives was available, *SUCLG2* variants seem to have incomplete penetrance that was repeatedly recorded for novel PPGL-related TCA genes like fumarate hydratase (34) or malate dehydrogenase (35).

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. described impairment of SDH activity in non-*SDHx* mutated PPGLs, possibly due to germline variants in other known (i.e. *MAX*) or unknown susceptibility genes (36). 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-grade 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 to 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*-defficient cells identified association and mechanism between *SUCLG2* variants and PPGL, and we propose *SUCLG2* as 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.

Funding

This work was supported by the Intramural Research Program of the National Institutes of Health: *Eunice Kennedy Shriver* National Institute of Child Health and Human Development and National Cancer Institute; and in part by the Czech Science Foundation (18-10832S, 19-20553S, 20-11724Y, and 20-05942S), the Czech Health Foundation (17-30138A), and the Australian Research Council. The project was also supported in part by the Program of Ministry of Education Youth and Sports of the Czech Republic (MEYS) grant LM2018130.

Notes

Role of the funders: The funders were not involved in the design, conduct, or reporting of the study as well as the writing of the manuscript.

Disclosure: The authors declare no competing interests.

Author contributions: Conceptualization and design: K.H.V., Y.P., J.R., J.N., C.Y., and K.P. Methodology, investigation, data curation, and formal analysis: K.H.V., Y.P., L.K., M.K., Z.N., S.B., S.D.P., R.Z., J.Z., T-T.H., I.J., O.U., S.H., S.D., T.J.G., H.K.G., J.C., and X.W. Visualization: K.H.V., Y.P., and C.Y. Resources: K.P., P.E.K., Z.F., I.H., B.S., H.K.G., D.T., and N.N. Supervision and project administration: K.H.V., J.N., C.Y., and K.P. Funding acquisition: J.N., C.Y., S.B., J.R., B.S., K.P. Writing – original draft: K.H.V., Y.P., J.R., J.N., C.Y., and K.P. All authors reviewed and edited the original draft.

Acknowledgments: We would like to acknowledge Dr. Lukas Lacina for preparation of NAM (equipment of "Center for Tumor Ecology", reg. no. CZ.02.1.01/0.0/0.0/16_019/0000785), Dr. Yanqin Yang for help to analyze the gene sequencing data, Nidhi Kapoor for help with metabolomics, and Leah Meuter for tireless collection and evaluation of the patient data.

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.

References

1. Lenders JW, Eisenhofer G, Mannelli M, *et al.* Phaeochromocytoma. Lancet 2005;366(9486):665-75.

2. Neumann HP, Bausch B, McWhinney SR, *et al.* Germ-line mutations in nonsyndromic pheochromocytoma. N Engl J Med 2002;346(19):1459-66.

3. Dahia PL. Pheochromocytoma and paraganglioma pathogenesis: learning from genetic heterogeneity. Nat Rev Cancer 2014;14(2):108-19.

4. Favier J, Amar L, Gimenez-Roqueplo AP. Paraganglioma and phaeochromocytoma: from genetics to personalized medicine. Nat Rev Endocrinol 2015;11(2):101-11.

5. Fishbein L, Leshchiner I, Walter V, *et al.* Comprehensive Molecular Characterization of Pheochromocytoma and Paraganglioma. Cancer Cell 2017;31(2):181-193.

6. Crona J, Lamarca A, Ghosal S, *et al.* Genotype-phenotype correlations in pheochromocytoma and paraganglioma: a systematic review and individual patient meta-analysis. Endocr Relat Cancer 2019;26(5):539-550.

7. Letouze E, Martinelli C, Loriot C, *et al.* SDH mutations establish a hypermethylator phenotype in paraganglioma. Cancer Cell 2013;23(6):739-52.

8. de Cubas AA, Korpershoek E, Inglada-Perez L, *et al.* DNA Methylation Profiling in Pheochromocytoma and Paraganglioma Reveals Diagnostic and Prognostic Markers. Clin Cancer Res 2015;21(13):3020-30.

9. Johnson JD, Mehus JG, Tews K, *et al.* Genetic evidence for the expression of ATP- and GTP-specific succinyl-CoA synthetases in multicellular eucaryotes. J Biol Chem 1998;273(42):27580-6.

10. Miller C, Wang L, Ostergaard E, *et al.* The interplay between SUCLA2, SUCLG2, and mitochondrial DNA depletion. Biochim Biophys Acta Mol Basis Dis 2011;1812(5):625-629.

11. Kacso G, Ravasz D, Doczi J, *et al.* Two transgenic mouse models for β-subunit components of succinate-CoA ligase yielding pleiotropic metabolic alterations. Biochem J 2016;473(20):3463-3485.

12. Elpeleg O, Miller C, Hershkovitz E, *et al.* Deficiency of the ADP-forming succinyl-CoA synthase activity is associated with encephalomyopathy and mitochondrial DNA depletion. Am J Hum Genet 2005;76(6):1081-6.

13. Ostergaard E. Disorders caused by deficiency of succinate-CoA ligase. J Inherit Metab Dis 2008;31(2):226-9.

14. Maas RR, Marina AD, de Brouwer AP, *et al.* SUCLA2 Deficiency: A Deafness-Dystonia Syndrome with Distinctive Metabolic Findings (Report of a New Patient and Review of the Literature). JIMD Rep 2016;27:27-32.

15. Pack SD, Zhuang Z. Fluorescence in situ hybridization : application in cancer research and clinical diagnostics. Methods Mol Med 2001;50:35-50.

16. Ghayee HK, Bhagwandin VJ, Stastny V, *et al.* Progenitor cell line (hPheo1) derived from a human pheochromocytoma tumor. PLoS One 2013;8(6):e65624.

17. Zetsche B, Heidenreich M, Mohanraju P, *et al.* Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. Nat Biotechnol 2017;35(1):31-34.

18. Bezawork-Geleta A, Wen H, Dong L, *et al.* Alternative assembly of respiratory complex II connects energy stress to metabolic checkpoints. Nat Commun 2018;9(1):2221.

19. Carrozzo R, Verrigni D, Rasmussen M, *et al.* Succinate-CoA ligase deficiency due to mutations in SUCLA2 and SUCLG1: phenotype and genotype correlations in 71 patients. J Inherit Metab Dis 2016;39(2):243-52.

20. Selak MA, Armour SM, MacKenzie ED, *et al.* Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. Cancer Cell 2005;7(1):77-85.

21. Huang J, Fraser ME. Structural basis for the binding of succinate to succinyl-CoA synthetase. Acta Crystallogr D Struct Biol 2016;72(Pt 8):912-21.

22. Bezawork-Geleta A, Rohlena J, Dong L, *et al.* Mitochondrial Complex II: At the Crossroads. Trends Biochem Sci 2017;42(4):312-325.

23. Amar L, Baudin E, Burnichon N, *et al.* Succinate dehydrogenase B gene mutations predict survival in patients with malignant pheochromocytomas or paragangliomas. J Clin Endocrinol Metab 2007;92(10):3822-8.

24. Favier J, Brière J-J, Burnichon N, *et al.* The Warburg Effect Is Genetically Determined in Inherited Pheochromocytomas. PLoS One 2009;4(9):e7094.

25. Letouzé E, Martinelli C, Loriot C, *et al.* SDH mutations establish a hypermethylator phenotype in paraganglioma. Cancer Cell 2013;23(6):739-52.

26. Hescot S, Curras-Freixes M, Deutschbein T, *et al.* Prognosis of Malignant Pheochromocytoma and Paraganglioma (MAPP-Prono Study): A European Network for the Study of Adrenal Tumors Retrospective Study. J Clin Endocrinol Metab 2019;104(6):2367-2374.

27. Amar L, Bertherat J, Baudin E, *et al.* Genetic Testing in Pheochromocytoma or Functional Paraganglioma. J Clin Oncol 2005;23(34):8812-8818.

28. Benn DE, Gimenez-Roqueplo A-P, Reilly JR, *et al.* Clinical Presentation and Penetrance of Pheochromocytoma/Paraganglioma Syndromes. J Clin Endocrinol Metab 2006;91(3):827-836.

29. Brouwers FM, Eisenhofer G, Tao JJ, *et al.* High Frequency of SDHB Germline Mutations in Patients with Malignant Catecholamine-Producing Paragangliomas: Implications for Genetic Testing. J Clin Endocrinol Metab 2006;91(11):4505-4509.

30. Kantorovich V, Pacak K. Pheochromocytoma and paraganglioma. Prog Brain Res 2010;182:343-373.

31. Fliedner SMJ, Brabant G, Lehnert H. Pheochromocytoma and paraganglioma: genotype versus anatomic location as determinants of tumor phenotype. Cell Tissue Res 2018;372(2):347-365.

32. Maniam P, Zhou K, Lonergan M, *et al.* Pathogenicity and Penetrance of Germline SDHA Variants in Pheochromocytoma and Paraganglioma (PPGL). J Endocr Soc 2018;2(7):806-816.

33. van der Tuin K, Mensenkamp AR, Tops CMJ, *et al.* Clinical Aspects of SDHA-Related Pheochromocytoma and Paraganglioma: A Nationwide Study. J Clin Endocrinol Metab 2018;103(2):438-445.

34. Clark GR, Sciacovelli M, Gaude E, *et al.* Germline FH Mutations Presenting With Pheochromocytoma. J Clin Endocrinol Metab 2014;99(10):E2046-E2050.

35. Cascón A, Comino-Méndez I, Currás-Freixes M, *et al.* Whole-Exome Sequencing Identifies MDH2 as a New Familial Paraganglioma Gene. J Natl Cancer Inst 2015;107(5).

36. Rapizzi E, Ercolino T, Canu L, *et al.* Mitochondrial function and content in pheochromocytoma/paraganglioma of succinate dehydrogenase mutation carriers. Endocr Relat Cancer 2012;19(3):261-9.

Tables

Table 1. **Clinical characteristics of patients**.

ID	Sex	Age at initial diagnosis	Mutation in *SUCLG2*	Protein change	mtDNA depletion	Biochemistry at initial diagnosis[a]	Initial tumor type	Primary tumor size, cm	Metastatic	Recurrent
1[b]	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[b]	male	25	c.349G>A	A117T	Yes	NE[c], NMN[c]	PHEO	7.5	No	Yes
5	male	51	c.295G>T	G99C	Yes	NE[c], NMN[c]	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[b]	female	29	c.349G>A	A117T	NA	CgA[c]	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[b]	male	56	c.349G>A	A117T	NA	MTX, CgA	UNP	NA	Yes	No

15[b]	female	25	c.601G>C	D201H	NA	NE[c], NMN[c], EPI[c], MN[c], DA[c]	PHEO	12.4	Yes	No

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

[b]Indicates death of the patient.

[c]Biochemistry done at the NIH, at the time of initial diagnosis not done or no data available.

.

Table 2. Detection of *SUCLG2* variants in patients' relatives[a]

ID sample	*SUCLG2* variant	Sample	Variant status	Cancer history
2	c.302A>G	Paternal	present	NR
		Maternal	ND	NR
		Sibling	present	pancreatic NET
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

[a]Available saliva samples from first-degree relatives were tested for presence of particular *SUCLG2* variants by Sanger Sequencing.

NA = sample not available; NR = not reported; ND = not detected.

Table 3. *SUCLG2* variants (chr.3, NM_003848.4), *in silico* prediction and summary of functional outcomes.

SUCLG2 variants	ID sample							
	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_540delTG	c.158G>A	c.601G>C
Amino acid change	I235N	K101R	A117T	G99C	Q203P	Val180GlyfsTer10	G53E	D201H
Exon	7	3	4	3	6	5	2	6
Position in Hg19	67559284	67579535	67578624	67579542	67568723	67570936-7	67659947	67568730
gnomAD allele frequency (No. of homozygotes)	0.000004029 = 1/248218 (0)	0.004736 = 1326/279992 (7)	0.007629 = 2141/280634 (57)	0.0001643 = 46/280026 (0)	0.0001465 = 41/279854 (0)	0.00001781 = 5/280762 (0)	not described	0.000004035 = 1/247828 (0)
ClinVar ID	-	257601 benign	774168 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)[b]	5/7	6/5[c]	6/5[c]	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								
IHC SUCLG2	negative	#2;3 negative	#4;6 negative	negative	-	negative	-	-
Western blot[d]	decrease	#2 decrease	#4 decrease	-	-	-	-	-
FISH SUCLG2	-	#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	-	-	-	-

[a]Data comes from www.cbioportal.org, www.varsome.com, and gnomad.broadinstitute.org. Functional studies data are summarized from experiments and results reported in **Figures 1-2** and **Supplementary Table 2** (available online). IHC: immunohistochemistry; FISH: fluorescence in situ hybridization; SDH: succinate dehydrogenase; Succ: succinate; Fum: fumarate.

[b] - includes *in silico* predictive programs available on www.varsome.com used in the ACMG Classification - BayesDel_addAF, DEOGEN2, EIGEN, MVP, MutationAssesor, PrimateAI, SIFT, DANN, FATHMM-MKL, LIST-S2, M-Cap, MutationTaster.

[c]M-Cap not available.

[d]Western blot of SUCLG2 in mitochondria extract from tumor tissue.

Figure Legends

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. PPGL = pheochromocytoma and paraganglioma; NAM = normal adrenal medulla; SP = sporadic PPGL.

Figure 2 Mitochondrial Complex II functions in *SUCLG2*-mutated tumors and *SUCLG2*-silented 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*. Pt1, 2, and 4 refers to patients 1, 2, and 4 in **Table 1**. Data are presented as mean [SD] and evaluated by a two-sided Student *t* test. **D)** *SUCLG2* mRNA (left) and SUCLG2, SUCLA2, and SUCLG1 protein levels (right) in parental (PAR), $SUCLG2^{KO}$, and $SUCLG2^{rec}$ cells. **E)** SDHA and SDHB protein levels in PAR, $SUCLG2^{KO}$, and $SUCLG2^{rec}$ cells. **F)** Native blue gels showing the assembly of mitochondrial CII in PAR, $SUCLG2^{KO}$ and $SUCLG2^{rec}$ cells using antibodies against SDHA and SDHB. **G)** In-gel SDH activity in PAR, $SUCLG2^{KO}$ and $SUCLG2^{rec}$ cells. **H)** SQR activity in PAR, $SUCLG2^{KO}$ and $SUCLG2^{rec}$ cells. **I)** Routine (left) and CII-dependent (right) respiration in PAR, $SUCLG2^{KO}$, and $SUCLG2^{rec}$ cells. **J)**

Succinate-to-fumarate ratio in PAR, *SUCLG2*^{KO}, and *SUCLG2*^{rec} cells. **K)** Levels of mtDNA in PAR, *SUCLG2*^{KO}, and *SUCLG2*^{rec} cells. All *in vitro* experiments were performed at least three times independently. Data are presented as mean (SD) and evaluated by one-way ANOVA with post-hoc Tukey test. All statistical tests were 2-sided. PAR = parental hPheo1 cells; NAM = normal adrenal medulla; CII = mitochondrial Complex II.

Fig. 1

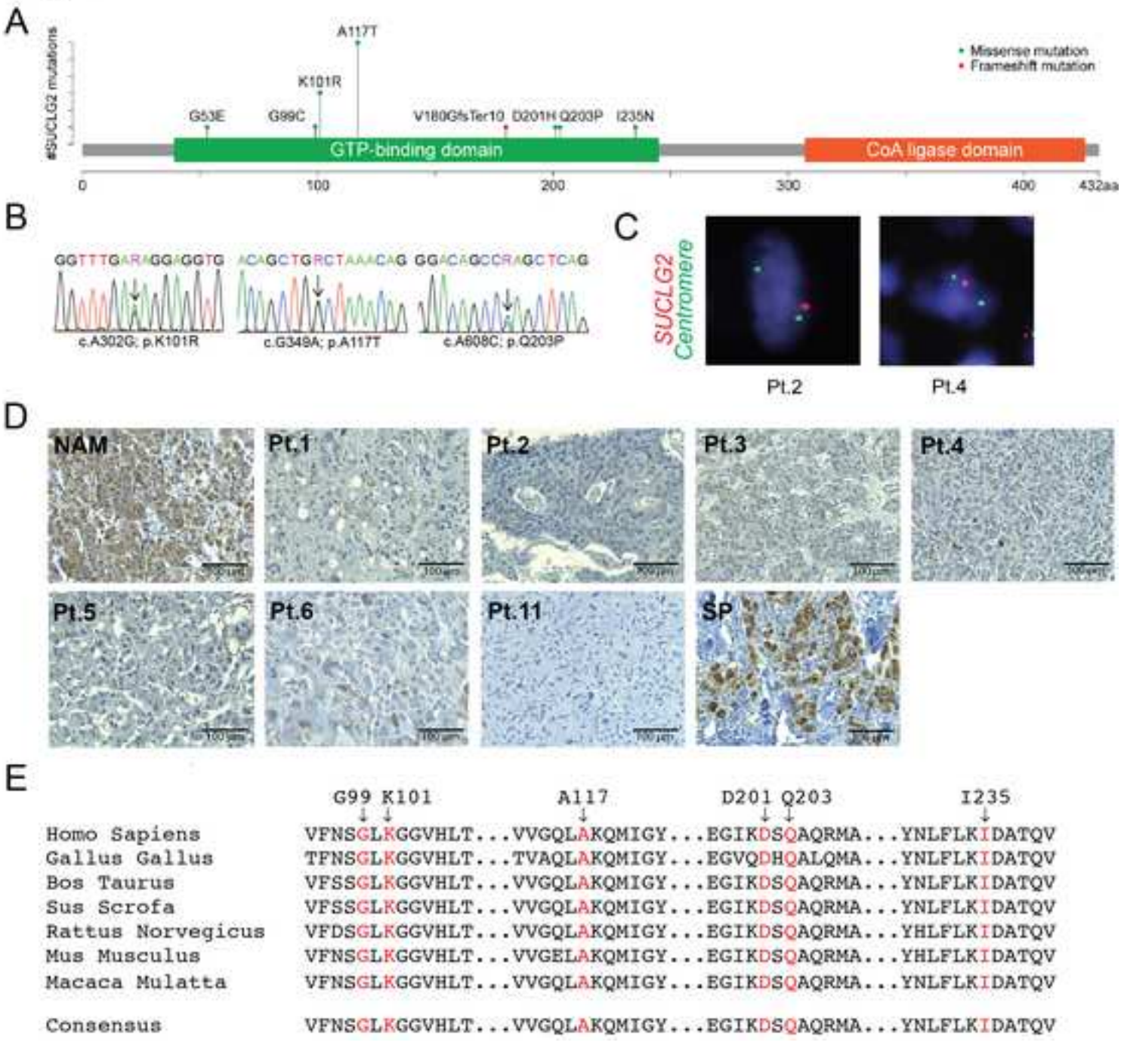
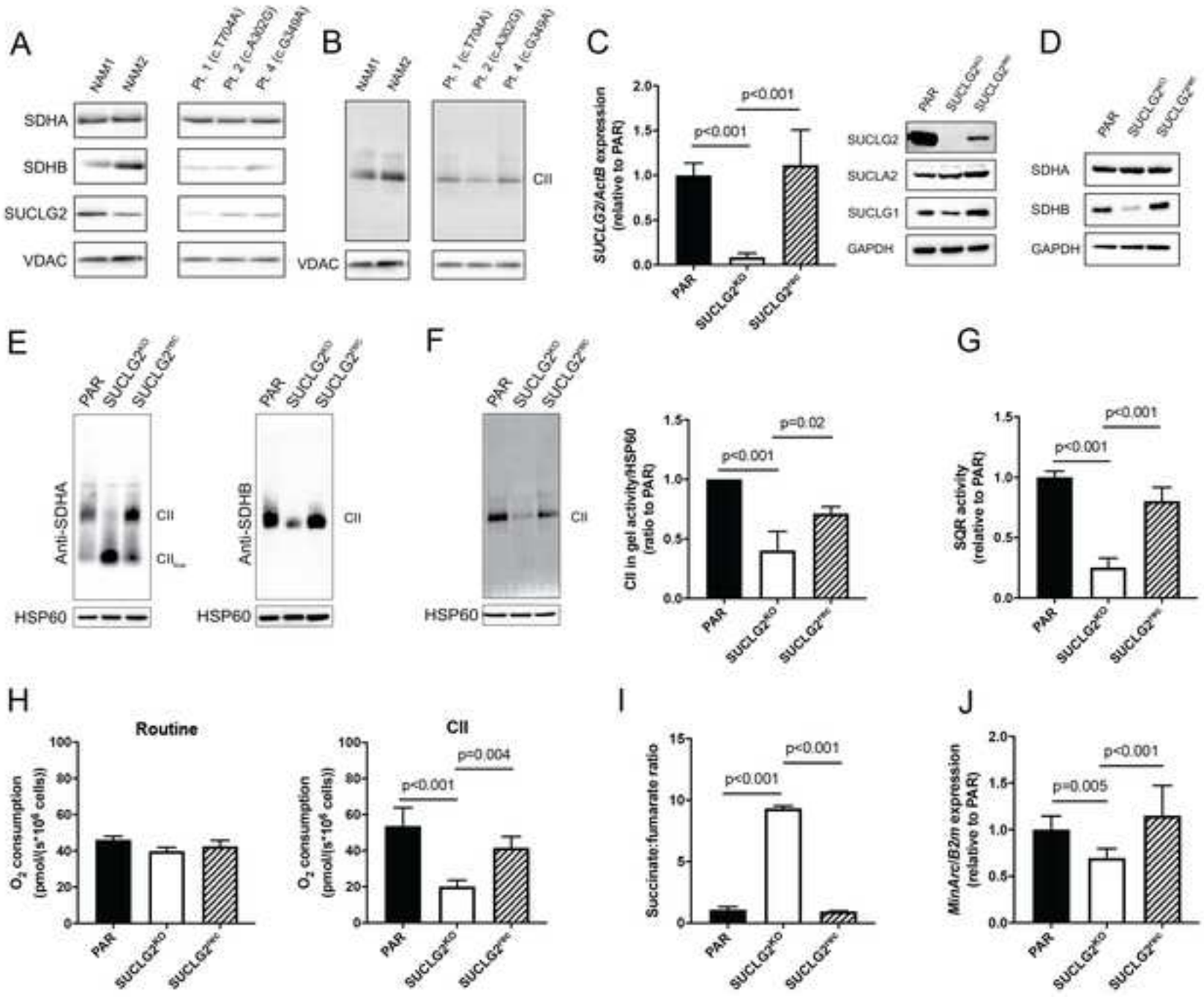


Fig. 2


A
NAM1
NAM2
Pt. 1 (c.T704A)
Pt. 2 (c.A302G)
Pt. 4 (c.G349A)
SDHA
SDHB
SUCLG2
VDAC
B
CII
C
SUCLG2/ActB expression (relative to PAR)
p<0.001
p<0.001
PAR
SUCLG2KO
SUCLG2rec
SUCLA2
SUCLG1
GAPDH
D
E
Anti-SDHA
Anti-SDHB
CII
CIIlow
HSP60
F
CII in gel activity/HSP60 (ratio to PAR)
p=0.02
G
SQR activity (relative to PAR)
H
Routine
CII
O2 consumption (pmol/(s*10^6 cells))
p=0.004
I
Succinate:fumarate ratio
J
MinArc/B2m expression (relative to PAR)
p=0.005