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Biology of Human Tumors

Clinical Cancer Research

# Biochemical, Molecular, and Clinical Characterization of Succinate Dehydrogenase Subunit A Variants of Unknown Significance

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## Abstract

**Purpose:** Patients who inherit a pathogenic loss-of-function genetic variant involving one of the four succinate dehydrogenase (SDH) subunit genes have up to an 86% chance of developing one or more cancers by the age of 50. If tumors are identified and removed early in these high-risk patients, they have a higher potential for cure. Unfortunately, many alterations identified in these genes are variants of unknown significance (VUS), confounding the identification of high-risk patients. If we could identify misclassified SDH VUS as benign or pathogenic SDH mutations, we could better select patients for cancer screening procedures and remove tumors at earlier stages.

**Experimental Design:** In this study, we combine data from clinical observations, a functional yeast model, and a computational model to determine the pathogenicity of 22 *SDHA* VUS. We gathered *SDHA* VUS from two primary sources: The OHSU Knight Diagnostics Laboratory and the literature. We used a yeast model to identify the functional effect of a VUS on mitochondrial function with a variety of biochemical assays. The computational model was used to visualize variants' effect on protein structure.

**Results:** We were able to draw conclusions on functional effects of variants using our three-prong approach to understanding VUS. We determined that 16 (73%) of the alterations are actually pathogenic, causing loss of SDH function, and six (27%) have no effect upon SDH function.

**Conclusions:** We thus report the reclassification of the majority of the VUS tested as pathogenic, and highlight the need for more thorough functional assessment of inherited SDH variants. *Clin Cancer Res; 23(21); 6733–43. ©2017 AACR.*

## Introduction

SDH, succinate dehydrogenase, also known as complex II of the electron transport chain (ETC), is a four-subunit complex encoded by nuclear genes (SDHA, SDHB, SDHC, and SDHD, collectively referred to as SDHx). The assembled SDH complex localizes to the inner membrane of the mitochondria and links the tricarboxylic acid (TCA) cycle to the ETC, making SDH function critical for aerobic respiration (1).

Loss-of-function mutations affecting the SDH complex predispose patients to develop multiple cancers, including gastrointestinal stromal tumor (GIST), paraganglioma, pheochromocytoma, renal cell carcinoma, Hodgkin lymphoma, chronic lymphocytic leukemia, thyroid cancer, pituitary adenomas, and neuroendocrine tumors of the pancreas (2–6). Tumor formation due to SDH-deficiency requires the complete loss of function of at least one SDHx subunit (e.g., A, B, C, or D), causing destabilization and loss of enzymatic function of the entire SDH complex (7). There are several genetic mechanisms that can lead to SDH-deficiency. Typically, loss of function of an SDHx subunit is the result of a combination of an inactivating germline mutation (first hit) with a somatic LOH or other inactivating mutation affecting the other allele (second hit). Less commonly, loss of SDH complex occurs due to somatic inactivation of both alleles of a given complex subunit or SDH assembly factor. Finally, SDH-deficiency can be caused by an *SDHC* epimutation, defined as hypermethylation of the *SDHC* promoter, which leads to repression of SDHC transcription and depletion of SDHC protein levels, without a known underlying heritable cause (8).

Importantly, germline loss-of-function genetic *SDHx* variants are associated with a high lifetime risk of developing the

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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## Translational Relevance

Molecular testing plays an important role in the clinical management of gastrointestinal stromal tumors, including decision making about the most appropriate medical or surgical therapy. As the routine use of multi-gene sequencing panels has expanded, there has also been an increase in reported variants of unknown significance (VUS) of the *SDHA* gene. In many cases, these *SDHA* VUS are present in the germline and are therefore potentially heritable by other family members. In order to understand the functional consequences of these variants, we combined clinical observations, data from a functional yeast model, and computational modeling to classify these *SDHA* VUS as having no effect or causing loss of function. These results will be helpful for appropriate genetic counseling of individuals with these germline variants. In addition, our data highlight the limitations of SDHA immunohistochemistry in clinical testing of tumors with SDHA VUS.

aforementioned malignancies. For example, the chance of a patient with germline loss-of-function *SDHD* variant of developing one or more primary tumors by the age of 50 was reported to be 86% (9, 10). Therefore, if we could identify high-risk patients through genetic testing and follow them serially with specialized screening tests, early tumor detection may lead to curative surgical resection before the tumors are metastatic/incurable. Early detection is crucial because there are no effective medical treatments for patients with advanced SDH-deficient cancers.

Currently, an SDH-deficient tumor is identified by measuring SDHB protein abundance using immunohistochemistry (IHC); absence of SDHB protein is indicative of loss of SDH function. However, it can be challenging to determine the underlying cause of SDH-deficiency in a tumor lacking SDHB expression. Clinical sequencing panels may turn up missense mutations in SDHx genes, but many of these are variants of unknown significance (VUS). In addition, such panels can miss large intragenic deletions and are not designed to identify epigenetic silencing of the *SDHC* promoter. Some SDHB, C, and D VUS have been functionally characterized to determine their effect on function, and thus their pathogenicity in tumors like paraganglioma and pheochromocytoma. However, the study of the functional consequences of SDHA VUS has lagged behind that of other SDHx subunits (7).

GIST is a heterogeneous group of tumors that arise from the interstitial cells of Cajal. However, there are several different driver genes that when mutated give rise to GIST (11). The molecular classification of GIST is especially important because of the treatment implications of the different genetic drivers. The majority of GISTs have an activating receptor tyrosine kinase (RTK) mutation, but about 13% of GISTs lack RTK mutations (RTK-WT). Most RTK-WT GISTs are SDH-deficient as assessed by IHC for SDHB. *SDHA* pathogenic variants are found in 47% of SDH-deficient GIST, and the majority of these *SDHA* mutations are germline, and thus heritable, variants (12). However, some the *SDHA* variants we find in GIST are VUS. A universal problem in the field is that these variants are rarely seen with a complete clinical and pathogenic annotation, making it difficult to draw conclusions on functional effect from clinical data alone. Our study gathered these VUS from two primary sources: OHSU and the literature (12, 13). We then combine data from clinical observations, a functional yeast model, and a computational model to understand the effects of *SDHA* VUS identified in GIST specimens on SDH complex function. Historically, yeasts have been a robust system for identifying the assembly and enzymatic activity of SDH (14–16). In addition, yeasts have proven to be an ideal model to study the functional effect of *SDHB/C/D* variants on SDH complex activity (17–20). Yeasts are able to survive without functional mitochondria (e.g., lacking SDH complex activity) if they are provided a fermentable carbon source; thus, they provide a unique model system to study the biochemical effects of *SDHA* VUS (21).

Based on our findings, we discriminated between *SDHA* VUS that affected or did not affect SDH complex activity, and thus, their potential for pathogenicity. These data will aid clinicians' ability to provide genetic counseling and tumor surveillance to patients with germline inheritance of these specific *SDHA* variants.

## Materials and Methods

### Yeast strains and vectors

All *Saccharomyces cerevisiae* strains used in this study were derivatives of BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). The *SDH1* (yeast homolog to *SDHA*) deletion strain (*sdh1Δ)* was purchased from the ATCC (catalog #4004998). The *sdh1Δ* was constructed as part of the *Saccharomyces* Genome Deletion Project by homologous recombination using the *KanMX4* cassette (22). We verified the deletion of *SDH1* using PCR mapping of the *SDH1* locus with primer pairs recommended by the *Saccharomyces* Genome Deletion Project.

An amplicon containing the wild-type (WT) *SDH1* [including the native *SDH1* promoter and 3′ untranslated region (UTR)] was generated from WT BY4741 and cloned into the pRS416 plasmid (ref. 23; ATCC) and expressed in the *sdh1Δ* strain. The *SDH1* point mutations were introduced by the QuikChange mutagenesis PCR system (Agilent Technology; cat #200521). All mutations were confirmed by Sanger sequencing. Yeast strains were transformed using Frozen EZ Yeast Transformation II (Zymo Research; catalog # T2001). Strains were grown in synthetic complete medium lacking uracil to maintain plasmid selection with either 2% glucose or 3% glycerol as the carbon source.

### Alignment of multiple species' succinate dehydrogenase flavoprotein subunit

Cluster Omega was used to align the protein sequences of SDH flavoproteins including *Escherichia coli* (*E. coli*; P0AC41), yeast (Q00711), human (P31040), and pig (Q0QF01).

### Immunoblotting

Intact mitochondria were isolated using a previously described method (24).Steady-state levels of mitochondrial proteins were resolved on SDS-PAGE, transferred to nitrocellulose membrane, probed using the indicated primary antibodies, and visualized using Amersham-enhanced chemiluminescence Western Blotting Detection Reagent (GE Life Sciences; catalog #RPN2106) with horseradish peroxidase–conjugated secondary antibodies (BioRad; catalog #1662408EDU). We used previously described polyclonal rabbit antibodies for immunodetection of Sdh1 and

Sdh2 (25). Anti-porin was purchased from ThermoFisher Scientific (catalog # 459500).

### Analysis of Sdh1-bound flavin adenine dinucleotide and total mitochondrial flavin adenine dinucleotide

Levels of flavin adenine dinucleotide (FAD) covalently bound to Sdh1 were analyzed as previously described (14, 26). Briefly, mitochondrial proteins were resolved on SDS-PAGE, and the gel was placed in a 10% acetic acid solution for 20 minutes to oxidize flavin. FAD was visualized upon exposure to UV light using a Bio-Rad ChemiDoc MP Imaging System.

### Oxygen consumption assay

Yeast strains were grown to confluency in glucose-based media and then switched to glycerol-based media for 12 hours. Two million cells were plated onto commercially available microplates with oxygen sensors (Oxoplate; PreSens catalog #OP96U; ref. 27). Kinetic reading of oxygen consumption was measured using a spectrofluorometer. A Student *t* test was used to determine statistical significance between a variant of interest and yeast complemented with WT Sdh1.

### Computational modeling of Sdh1 variants

A model of yeast Sdh1 (Q00711) from the Swiss Model repository was refined using Yasara Homology Modelling to include the liganded FAD interactions with the peptide chain, followed by optimization of the loop and side-chains interactions. Side-chain rotamers were fine-tuned considering electrostatic and knowledge-based packing interactions as well as solvation effects. An unrestrained high-resolution refinement with explicit solvent molecules was run, using YAMBER, a second-generation self-parameterizing force field derived from the AMBER force field. Clinically identified variants were introduced in the homology model, and the structures were minimized as described earlier. The variants were compared with the WT model of *E. coli* or yeast Sdh1 using PYMOL.

### FoldX analysis

FoldX was used to measure the effect of point mutations on the stability of the Sdh1 yeast model (28). Except as noted, we used the default software settings. The move neighbors setting was turned off, and the average ΔΔG was calculated after three runs.

### Mutation analysis

We gathered *SDHA* VUS from two primary sources: The OHSU Knight Diagnostics Laboratory (Portland, OR) and the literature (12, 13). The majority of the variants pulled from the literature were found in a review highlighting the need for a functional model to characterize *SDHx* VUS (12). The remaining literature variants are from an article identifying novel causes of GIST that were WT for any known oncogenic driver of GIST (13). A collaboration with the OHSU Knight Diagnostics Laboratory, which offers a targeted exome panel to identify genetic drivers in GIST which includes all four SDHx subunits (https://www.ohsu.edu/custom/knight-diagnostic-labs/home/test-details?id=GeneTrails±GIST±Genotyping±Panel), leads to identification of several novel variants. All of the variants came from tumor samples that lacked any known oncogenic driver of GIST (e.g., no KIT mutations).

## Results

### Clinical analysis

All the known clinical information on the *SDHA* VUS identified in this study, including SDHA/B IHC, tumor-mutant allele fraction, other disease references, population data from the ExAC database (29), and ClinVar clinical significance (30), is listed in Table 1. Unfortunately, there were few variants with complete clinical and pathologic annotation, emphasizing the challenge of trying to understand the functional effects of these variants from available clinical data alone. Currently, SDHx subunit testing remains uncommon for GIST, and only a limited number of reference laboratories offer this testing. However, these laboratories usually do not have access to full clinical annotation and/or prior SDHB IHC testing results. This limitation applies to the published cases as well as the cases from the Knight Diagnostic laboratory. We listed all available additional clinical information for tumors with VUS in Supplementary Table S2.

### Functional studies in yeast

To understand the functional consequences of VUS in human *SDHA*, we used complementation studies in a yeast strain lacking Sdh1 (*sdh1*Δ). The flavoprotein subunit of the SDH complex is highly conserved across all species, including yeast Sdh1 (ySdh1) and human SDHA (hSDHA; Supplementary Fig. S1). For simplicity, we use yeast nomenclature throughout this study and reference the corresponding human variant in all of the tables.

As a positive control, we generated a WT *SDH1* amplicon from BY4741 that included the promoter and the 3′UTR which was cloned into a pRS416 plasmid and expressed in *sdh1*Δ yeast. We used *sdh1*Δ as our negative control. S*dh1*Δ do not have a functional SDH complex and were unable to utilize their mitochondria for canonical TCA or ETC pathways, allowing growth on a fermentable carbon source (glucose), but preventing growth with a nonfermentable carbon source such as glycerol (Fig. 1). Complementation with WT *SDH1* (+Sdh1) restored growth on glycerol (Fig. 1). Using this strategy, we tested the ability of plasmids encoding individual VUS to complement the *sdh1*Δ strain and restore a normal growth phenotype. The two loss-of-function controls (yR19X and yH90S), as well as 16 of the variants shown in Fig. 1 (yG97R, yT134M, yR179W, yG251R, yH287Y, yR303C, yY399C, yG410R, yC431F, yG432E, yR444C, yR444H, yA447E, yR458W, yR582G, yH601Y), were unable to grow on glycerol, indicating that these variants result in loss of oxidative phosphorylation (Fig. 1A). In contrast, six of the variants (yN109S, yR162H, yR186W, yT501I, yW605FF, and yV633I) were able to grow on glycerol, indicating these variants had no effect on the Sdh1 function as their phenotype was the same as WT Sdh1 (Fig. 1B).

As a secondary measure of SDH complex activity, we measured oxygen consumption to assess oxidative phosphorylation, a surrogate for measuring the ability of the SDH complex to transfer electrons to the ETC. In concordance with our previous results, the same 18 variants that were unable to grow on glycerol consumed significantly less ($P < 0.0001$) oxygen than WT Sdh1 (Fig. 1C). Variants that grew on glycerol had similar oxygen consumption to WT Sdh1, confirming that these variants do not disrupt the ETC (Fig. 1D). To ensure oxygen consumption was due to the ETC, azide was added to each cell strain, and this promptly abolished oxygen consumption for all controls and variants.

To better understand how each variant affects Sdh1 protein function, we measured the covalent attachment of FAD to Sdh1, a

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**Table 1.** Clinical variants of human *SDHA* with unknown significance

| hSDHA Mutation | ySdh1 Mutation | Source | SDHB/ SDHA IHC | Tumor-mutant allele fraction | Other disease references | Allele frequency from EXAC database | ClinVar clinical significance | Conclusions drawn |
|---|---|---|---|---|---|---|---|---|
| R31X | R19X | Control | Neg/neg (38) | N/A | GIST (38, 39) Paraganglioma (40) | $1.647 \times 10^{-4}$ | Pathogenic/likely pathogenic | LOF (Control) |
| H99S | H90S | Control (14) | N/A | N/A | None | N/A | N/A (H99Y Likely pathogenic) | LOF (Control) |
| G106R | G97R | OHSU | N/A | 92% | Novel | N/A | N/A | LOF |
| N118S | N109S | OHSU | N/A | 50.7% | Novel | N/A | N/A | No effect |
| T143M | T134M | Literature (13) | N/A | 22% | Novel | N/A (T143R reported $8.31 \times 10^{-6}$) | Uncertain significance | LOF |
| R171H | R162H | OHSU | N/A | 34% | Novel | $8.24 \times 10^{-6}$ | Uncertain significance | No effect |
| R171H | R162H | OHSU | N/A | 40.3% | Novel | $8.24 \times 10^{-6}$ | Uncertain significance | No effect |
| R188W | R179W | Literature (12) | Neg/pos [30] | N/A | None | N/A (R188Q reported $1.65 \times 10^{-5}$) | N/A | LOF |
| R195W | R186W | OHSU | Neg/ND | 66.7% | GIST (37) | N/A (R195Q reported $8.23 \times 10^{-6}$) | N/A | No effect |
| G260R | G251R | Literature (12) | Pos/pos [32] | N/A | None | N/A | Uncertain significance | LOF |
| H296Y | H287Y | Literature (13) | N/A | 87% | Novel | N/A | N/A | LOF |
| R312C | R303C | OHSU | N/A | 34.5% | Novel | N/A | N/A (R312P Uncertain significance) | LOF |
| R408C | Y399C | OHSU | N/A | 52.8% | GIST (41); Late onset neurodegenerative disease (42) | N/A | N/A | LOF |
| G419R | G410R | OHSU | N/A | 77% | GIST (43) | N/A | N/A | LOF |
| C438F | C431F | OHSU | N/A | 48.5% | Novel | N/A | N/A | LOF |
| G439E | G432E | OHSU | N/A | 87.6% | Novel | N/A | N/A | LOF |
| R451C | R444C | Literature (13) | N/A | 18% | Complex II deficiency (42) | N/A (R451H reported $8.23 \times 10^{-6}$) | N/A | LOF |
| R451H | R444H | OHSU | N/A | 47.5% | Novel | $8.23 \times 10^{-6}$ | N/A | LOF |
| R451H | R444H | OHSU | N/A | 39.5% | Novel | $8.23 \times 10^{-6}$ | N/A | LOF |
| A454E | A447E | Literature (12) | Neg/pos [31] | N/A | None | N/A | N/A (A454T Uncertain significance) | LOF |
| R465W | R458W | OHSU | N/A | 50% | Novel | $8.23 \times 10^{-6}$ | Uncertain significance | LOF |
| T508I | T501I | OHSU | N/A | 50.6% | Cardiomyopathy and leukodystrophy (44) | $7.60 \times 10^{-4}$ | Conflicting interpretations of pathogenicity | No effect |
| R589G | R582G | OHSU | N/A | 40.2% | Paraganglioma (45) | $8.29 \times 10^{-6}$ | Uncertain significance | LOF |
| H625W | H601Y | Literature (12) | Neg/neg [39] | N/A | Pituitary adenoma and pheochromocytoma/ paraganglioma | N/A | N/A | LOF |
| Y629F | W605F | OHSU | N/A | 99.6% | | $1.52 \times 10^{-1}$ | Benign/likely benign | No effect |
| V657I | V633I | OHSU | N/A | 95.1% | Not pathogenic (46) | $1.30 \times 10^{-1}$ | Likely benign | No effect |

NOTE: All of the genetic variants were found in GIST, except for hG260R, which was found in a paraganglioma. A summary of available clinical data for each variant is listed, including source of the variant, clinical IHC results for SDHB/SDHA, frequency of the variant in tumor, other SDHA variants found in the same tumor, information on functional effect of other variants in the tumor, other disease references to the variant of interest in the literature, population allele frequency, and results from our yeast model.

Abbreviations: LOF, loss of function; N/A, not available; neg, negative- SDHx absent; ND, not done; pos, positive- SDHx expression.

process known as flavination. We also measured the protein abundance of Sdh1 and Sdh2 (Fig. 2). Sdh1 flavination is critical for the catalytic activity of Sdh1; without covalent attachment of flavin, the Sdh1 protein is unable to oxidize succinate (14). As a positive control for a variant that inhibited insertion of FAD into Sdh1, we used the previously described Sdh1 H90S variant (14). Variants that complemented *sdh1*Δ yeast in our functional assays had similar levels of flavinated Sdh1, total Sdh1, and total Sdh2 as yeast complemented with WT Sdh1 (Fig. 2D). However, variants that were unable to perform oxidative phosphorylation had differential effects on the levels of flavinated Sdh1 and total Sdh1 protein (Fig. 2A–C). Some loss-of-function variants inhibited flavination without affecting the abundance of Sdh1 (H90S, G97R, T134M, R444C, and H601Y), whereas others caused a marked decrease in Sdh1 protein abundance (R179W, G251R, H287Y, R303C, Y399C, G410R, C431F, G432E, R444H, A447E, R458W, and R582G). All of the loss-of-function variants resulted in a decrease in Sdh2 protein abundance, consistent with loss of a functional SDH complex as described previously (25). In a subset of no effect and loss-of-function variants, we confirmed that Sdh1 mRNA expression was not significantly different from WT.

Based on all of the above results, we characterized 16 variants (73%) as causing loss of function and six variants (27%) as having no effect on SDH function (Table 2). All the loss-of-function variants were unable to grow on glycerol, had decreased oxygen consumption, and showed decreased Sdh2 protein abundance. In contrast, the no effect group had similar results to cells complemented with WT Sdh1 protein in these assays.

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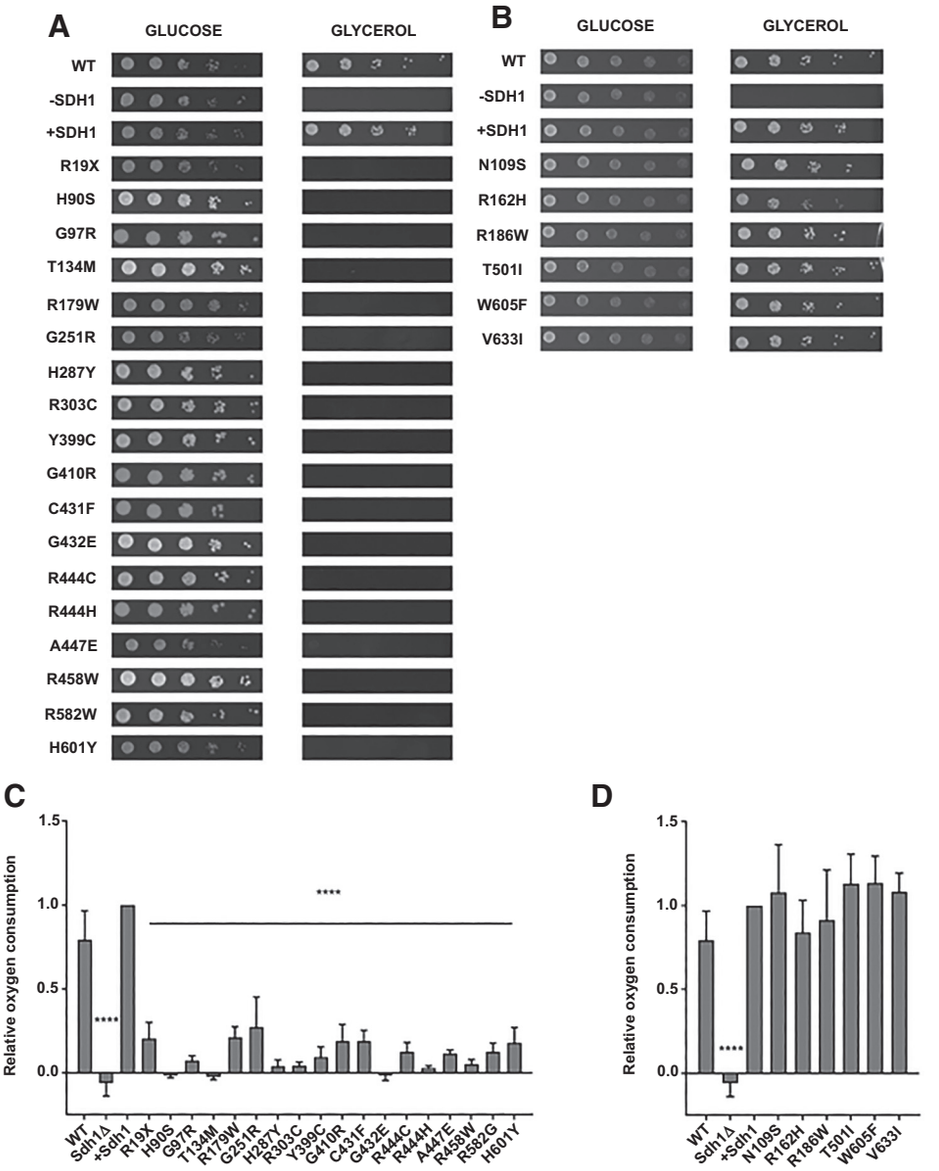


**Figure 1.**

ySdh1 variants causing loss of function are unable to use glycerol as a sole carbon source or consume oxygen. Yeasts were grown to a confluency of $2 \times 10^7$/mL and then serially diluted and plated onto either glucose or glycerol media plates (left to right: highest to lowest). All variants, regardless of SDH complex function, can grow on glucose, but only those variants that complement *sdh1*Δ and restore SDH complex activity are able to grow on glycerol and consume oxygen. **A,** Loss-of-function variants that are unable to grow with glycerol as their sole carbon source. **B,** Variants with no effect are able to grow with glycerol as their sole carbon source. **C,** Sdh1 variants that consume significantly less oxygen than cells complemented with WT Sdh1 (+Sdh1 control). **D,** Variants that consume oxygen at a similar rate to the +Sdh1 control. ****, $P < 0.0001$.

### Computational modeling

We next performed computational modeling to predict the potential structural implications of each variant. Using the two groups (loss-of-function and no effect) identified in our yeast model, we further characterized each variant by visualizing the location in the Sdh1 protein. Both groups, loss-of-function and no effect, were located throughout the four domains of Sdh1, suggesting that there are not "hotspot" areas or domains for pathogenic variants, unlike the situation for some proteins. Within the loss-of-function group, 12 variants [yH90S (loss-of-function control), yG97R, yG251R, yH287Y, yR303C, yY399C, yG432E, yR444C, yR444H, yA447E, yR582G, yH601Y] were identified as being involved in cofactor (FAD) or substrate (succinate) binding in the Sdh1-active site (Fig. 3, Table 2, and Supplementary Video S1). Structural studies from the homologous protein quinol: fumarate reductase in *E. coli* suggests an important catalytic function for most of these amino acids (31). In addition to the amino acids directly interacting with the flavin-binding pocket, it has been previously shown that the C-terminal domain of Sdh1 is crucial for the flavination of Sdh1 (25). Two variants located in the C-terminal domain of Sdh1, yR582G and yH601Y, both inhibited flavination of Sdh1. To visualize the substrate (succinate) and cofactor (FAD), which were not visualized using the yeast structure, these Sdh1 variants were modeled using the *E. Coli* crystal structure of Sdh1.

The other six loss-of-function variants [yR19X (control—not pictured in computational models because it is an early truncation of the protein), yT134M, yR179W, yG410R, yG431F, and yR458W] are not located in the active site of Sdh1. Changes in protein structure based on each of these variants are visualized in Fig. 4A and Supplementary Video S1. Further, FoldX analyses of the variants compared with WT provided insight into how the

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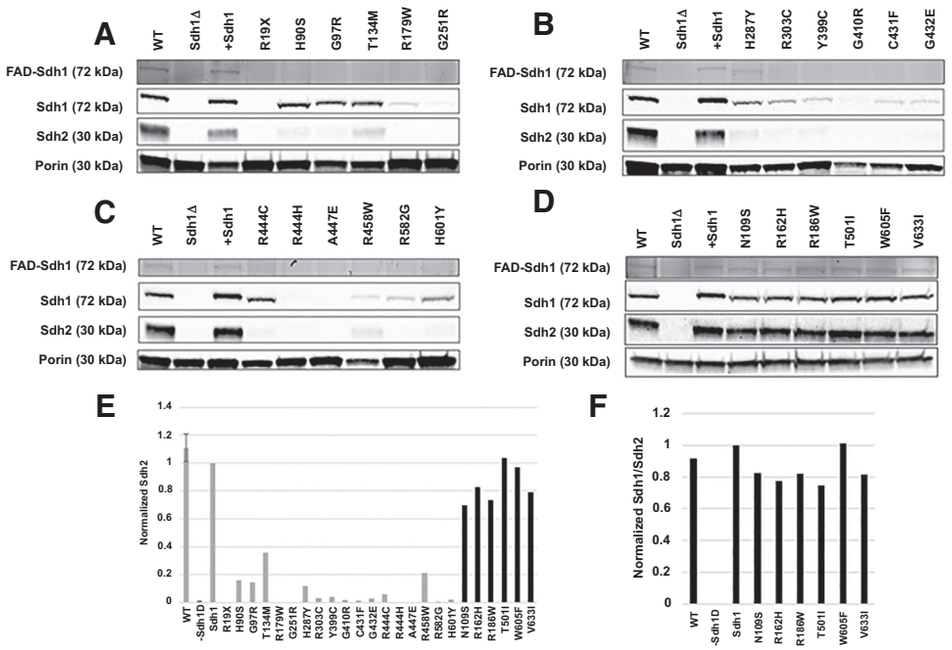


**Figure 2.**

Abundance of flavinated Sdh1, total Sdh1, and total Sdh2 in mitochondrial lysates from yeast expressing each variant. Western blotting of mitochondrial lysates from each variant is shown. Positive and negative control lanes are included on each Western blot (WT, SDH1delta, +SDH1). WT and +Sdh1 show bands at expected size for each protein. Sdh1Δ does not show bands for Sdh1-FAD, Sdh1, or Sdh2, but does have normal expression of the mitochondrial marker, porin. **A–C,** Loss-of-function variants are shown in numerical order by altered amino acid residue. **D,** No effect variants are shown in numerical order by altered amino acid residue and have with no reduction in flavin, Sdh1, or Sdh2 expression compared with WT controls. **E,** Sdh2 protein abundance was calculated relative to Sdh1 and then normalized to porin (control for mitochondrial protein loading). The no-effect variants are labeled in black, whereas the loss-of-function variants are in gray. No-effect variants have Sdh2 protein abundance more similar to complemented Sdh1, and loss-of-function variants have decreased Sdh2 similar to *sdh1*Δ. **F,** Ratio of Sdh1/Sdh2 protein abundance in no effect variants.

variants affect stability (Supplementary Table S2; ref. 28). yT134M, yR179W, yG410R, and yG431F all have positive ΔΔG (change in free energy), indicating these changes are highly destabilizing. Some of these also were associated with decreased Sdh1 protein abundance (Fig. 2).

Interestingly, all the variants with no functional effects were localized to the surface of the protein (Fig. 4B; Supplementary Video S1) where it would be predicted that they would be less likely to affect protein structure and function. Notably, these mutations did not map into any of the known critical domains of Sdh1 or predicted Sdh2 interaction sites.

## Discussion

Molecular testing plays an important role in the clinical management of GIST, including decision making about appropriate medical and surgical therapy. SDH deficiency is a potential cause of GIST in tumors lacking known oncogenic drivers such as gain-of-function KIT mutations. To attempt to identify the cause of SDH deficiency in such GIST, it is necessary to use multi-gene sequencing panels to search for SDHx pathogenic mutations; however, as the routine use of multi-gene sequencing panels has expanded, there has also been an increase in reported VUS. Due to the multiple potential causes of SDH-deficiency, it is difficult to determine if a newly discovered VUS is responsible for the defect, or if a different genetic mechanism is responsible for the loss of SDH complex activity. This problem is exacerbated in GIST as loss of SDHA protein is the most common cause of SDH deficiency in GIST, but SDHA variants have not been extensively characterized at the functional level. Finally, many SDHA VUS are also found in the germline, meaning that they can be inherited. Knowing a patient has a loss-of-function *SDHA* variant in a tumor dramatically changes clinical decision making concerning screening recommendations for tumor syndromes, which affects both the patient and other family members. Because there are no effective medical treatments for advanced SDH-deficient tumors, early detection of tumors allowing curative surgical resection is crucial.

A major problem in the field has been the lack of a validated model system to assess the biochemical function of *SDHA* VUS. Currently, there are no human SDHA-deficient cell lines, and assessing SDH complex activity in tumor samples is difficult. Previously, it has been shown that a yeast model can be used to evaluate the pathogenic significance of *SDHB* mutations (17, 18). Extending these studies, we report the successful use of a yeast model to characterize *SDHA* variants found in GIST tumors as either causing loss of function or having no biochemical consequence. Together with computational modeling, structural homology, and patient data, we have drawn conclusions on how and why the *SDHA* variants we studied affect SDH function, providing clinicians with information to guide genetic counseling of patients and family members who harbor one of these germline VUS.

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**Table 2.** Consistent findings across multiple functional assays for each variant in our yeast model

| hSDHA Mutation | ySdh1 Mutation | Growth phenotype on glycerol | Oxygen consumption (% of complemented) | Sdh2 Protein (% of complemented) | Structural implications of variants | Group name |
|---|---|---|---|---|---|---|
| R31X (control) | R19X (control) | Unable to grow | Decreased (20%) | Decreased (0%) | Truncates mitochondrial targeting sequence (47) | Loss-of-function (control) |
| H99S (control) | H90S (control) | Unable to grow | Decreased (−2%) | Decreased (15%) | Inhibits covalent bond to FAD (14) | Loss-of-function (control) |
| G106R | G97R | Unable to grow | Decreased (7%) | Decreased (14%) | Distorts the active site (31) | Loss-of-function |
| N118S | N109S | Growth | Similar (108%) | Similar (83%) | Surface of protein | No effect |
| T143M | T134M | Unable to grow | Decreased (3%) | Decreased (35%) | No obvious disturbances but causes loss of function | Loss-of-function |
| R171H | R162H | Growth | Similar (84%) | Similar (78%) | Surface of protein | No effect |
| R188W | R179W | Unable to grow | Decreased (21%) | Decreased (0%) | Disrupts salt bridge with D108 | Loss-of-function |
| R195W | R186W | Growth | Similar (91%) | Similar (82%) | Surface of protein | No effect |
| G260R | G251R | Unable to grow | Decreased (27%) | Decreased (0%) | Obstructs flavin binding pocket | Loss-of-function |
| H296Y | H287Y | Unable to grow | Decreased (4%) | Decreased (11%) | Inhibits succinate binding (31) | Loss-of-function |
| R312C | R303C | Unable to grow | Decreased (4%) | Decreased (3%) | Contributes to proper orientation and activation of the flavin isoalloxazine ring to facilitate formation of the covalent FAD bond and disrupts salt bridge (48) | Loss-of-function |
| R408C | Y399C | Unable to grow | Decreased (9%) | Decreased (4%) | In flavin binding site | Loss-of-function |
| G419R | G410R | Unable to grow | Decreased (19%) | Decreased (2%) | Distorts helix | Loss-of-function |
| C438F | C431F | Unable to grow | Decreased (19%) | Decreased (2%) | Bulky change disrupts helix | Loss-of-function |
| G439E | G432E | Unable to grow | Decreased (-2%) | Decreased (3%) | Obstructs flavin binding pocket | Loss-of-function |
| R451C | R444C | Unable to grow | Decreased (12%) | Decreased (6%) | Disrupts flavin binding, succinate binding and proton shuttle necessary for catalytic activity (31) | Loss-of-function |
| R451H | R444H | Unable to grow | Decreased (3%) | Decreased (0%) | Disrupts flavin binding, succinate binding, and proton shuttle necessary for catalytic activity (31) | Loss-of-function |
| A454E | A447E | Unable to grow | Decreased (11%) | Decreased (0%) | Lines succinate binding site (31) | Loss-of-function |
| R465W | R458W | Unable to grow | Decreased (5%) | Decreased (21%) | Disrupts salt bridge with E136 | Loss-of-function |
| T508I | T501I | Growth | Similar (113%) | Similar (75%) | Surface of protein | No effect |
| R589G | R582G | Unable to grow | Decreased (12%) | Decreased (0%) | Inhibits C-terminal flavination (25) | Loss-of-function |
| H625W | H601Y | Unable to grow | Decreased (18%) | Decreased (2%) | Inhibits C-terminal flavination | Loss-of-function |
| Y629F | W605F | Growth | Similar (113%) | Similar (101%) | Surface of protein | No effect |
| V657I | V633I | Growth | Similar (108%) | Similar (82%) | Surface of protein | No effect |

NOTE: A summary of results, including the growth on glycerol, oxygen consumption, Sdh2 protein abundance, consequences of changing the WT amino acid using computational modeling, and literature search for studies in other species' flavoprotein, is tabulated along with our classification of each variant based on our yeast model. Numerical values for oxygen consumption were taken from Fig. 1C and D. Numerical values for Sdh2 protein were taken from Fig. 2E.

Using our yeast model, we characterized variants as either causing loss of protein function or having no effect. All variants in the loss-of-function group were unable to grow on a nonfermentable carbon source (glycerol). In addition, these variants also failed to consume oxygen, indicating defective oxidative phosphorylation. Interestingly, these variants had differential effects on Sdh1 flavination and/or protein abundance. As a group, all of the loss-of-function variants were associated with a dramatic decrease in Sdh2 protein abundance, which is in concordance with current clinical testing to determine SDH-deficiency in tumors—assessment of SDHB (human equivalent to ySdh2) expression using IHC (2, 32–36). Our data provide further evidence supporting the role of SDHB IHC as the most reliable clinical test to identify SDH-deficiency in a tumor. Notably, we

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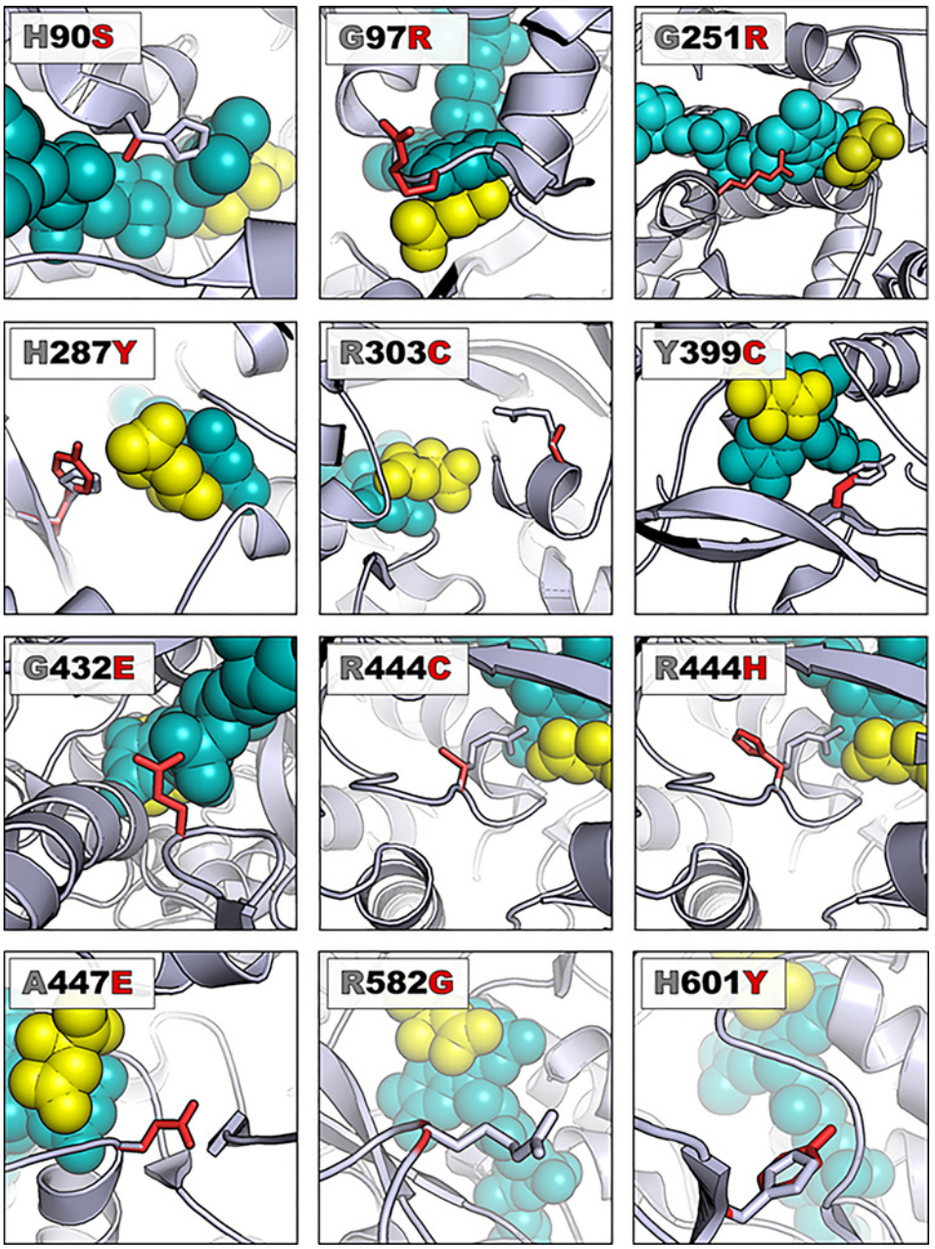


**Figure 3.**
Variants involving the active site cause loss of Sdh1 function. A ribbon representation of Sdh1 (*E. coli* model PBD file 2WP9) is shown with WT protein carbons colored gray and the variant carbons colored red. The FAD is shown as teal-colored spheres, whereas the dicarboxylic acid substrate (succinate analog) is depicted with yellow-colored spheres. In each case, the view has been rotated so that residues of interest are clearly observed. References detailing structural implications of each variant can be found in Table 2.

identified four novel loss-of-function variants that did not affect Sdh1 protein abundance (yG97R, yT134M, yR444C, yH601Y). These variants prevent flavination of Sdh1, making Sdh1 dysfunctional, but still expressed at normal levels. Our findings are consistent with previous reports where SDHA expression was retained in some *SDHA*-mutant tumors that lack SDHB expression (37), confirming that not all dysfunctional SDHA (or Sdh1) variants lead to decreased protein expression of this subunit. There are three clinical samples (R188W, G260R, A454E) that we characterized as loss-of-function variants but are associated with normal sdh1 in our yeast model. Based on these results and coupled with the fact the SDHA IHC is not universally available, we advocate for the use of SDHB IHC to identify tumors that are SDH-deficient.

The conclusions drawn from our yeast model are largely supported by our computational modeling results. For many variants, we could find confirmatory structural modeling evidence that the amino acids affected by these mutations played a role in the catalytic site of Sdh1. The computational model and energy minimization also allowed us to explain why some variants could reduce Sdh1 protein abundance. Furthermore, all the variants that did not affect protein function were on the surface of the protein as visualized by our computational model, suggesting that they would not interact with the catalytic mechanism of Sdh1.

Based on our biochemical data, there are still several tumors that have no known mechanism for loss of SDH activity (Table 1). One of these tumors had the hR195W (yR186W)

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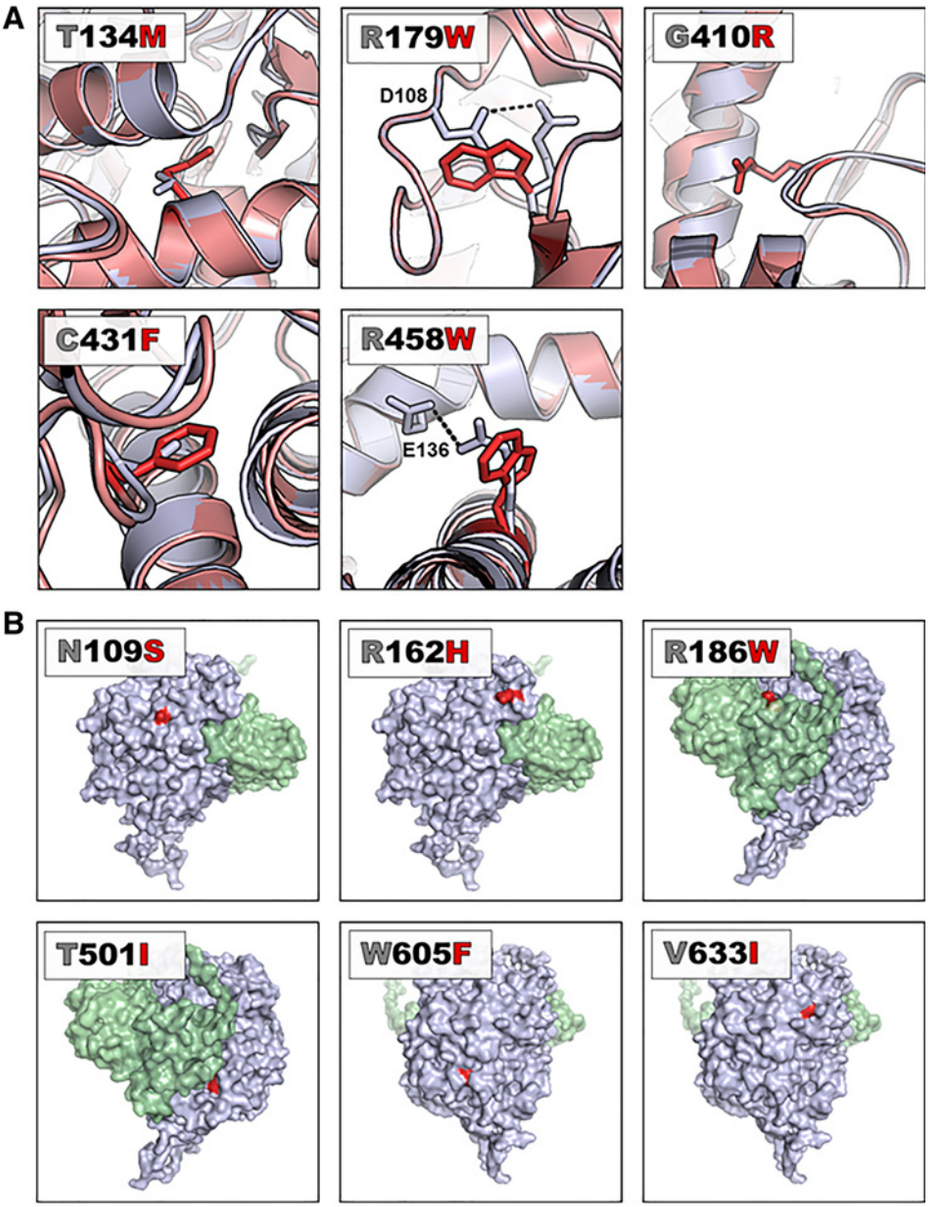


**Figure 4.**

Structural consequences of variants visualized in a yeast model (yeast model PBD file 1ORZ). **A,** Variants not in the active site causing loss of function. WT Sdh1 protein is represented as a gray ribbon with the amino acid of interest labeled as a gray stick and the refolded variant Sdh1 protein as a red ribbon with the amino acid of interest indicated by a red stick. In each case, the view has been rotated such that residues of interest are clearly observed. Potential structural implications of each variant can be found in Table 2. **B,** Variants that do not affect protein function are located on the surface of Sdh1. The surface area of WT Sdh1 is shown in gray, the variant residue is labeled red, and the surface area of Sdh2 is labeled in green. In each case, the view has been rotated such that residues of interest are clearly observed.

variant with an allele frequency of 66.7% and negative SDHB IHC. This tumor is likely driven by a different mechanism of SDH-deficiency. The rest of the unexplained tumors had no IHC data available, so it is possible that these GISTs are not driven by SDH-deficiency and instead belong to a different subtype of GIST (11). Alternatively, these GISTs may be SDH-deficient by a different genetic mechanism than the *SDHA* variant identified using targeted exome sequencing. There are several weaknesses of various targeted exome sequencing panels including potential lack of coverage for regions of certain *SDHx* genes, failure to detect large genomic deletions involving SDHx subunits, as well as the inability to measure hypermethylation of the *SDHC* promoter. In addition, mutations that inactivate newly identified SDH assembly factors (SDHAF3, 4) are typically not included in clinical cancer gene sequencing panels (15, 16). Given the limitations of using deidentified results from clinical testing, we did not have access to residual tumor samples to perform additional IHC or genomic testing.

We used a yeast model to characterize 22 *SDHA* VUS. These data revealed 16 (73%) of SDHA VUS as loss of function (and therefore pathogenic), highlighting the importance of understanding such variants to provide better clinical recommendations for genetic counselors concerning family screening and early detection protocols. However, our approach using a functional yeast model paired with computational modeling can distinguish between *SDHA* VUS that cause loss of function and those that have no biochemical effect, allowing us to discriminate between pathogenic and nonpathogenic variants.

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## Disclosure of Potential Conflicts of Interest

J.K. Sicklick reports receiving commercial research grants from Foundation Medicine, Inc., and Novartis Pharmaceuticals. M.C. Heinrich is an employee of MolecularMD. No potential conflicts of interest were disclosed by the other authors.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** A.E. Bannon, I. Forquer, A. Town, C. Corless, U. Shinde, M.C. Heinrich
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A.E. Bannon, J. Kent, I. Forquer, L.R. Klug, K. McCann, O. Harismendy, U. Shinde, M.C. Heinrich
**Writing, review, and/or revision of the manuscript:** A.E. Bannon, J. Kent, L.R. Klug, K. McCann, O. Harismendy, J.K. Sicklick, C. Corless, U. Shinde, M.C. Heinrich
**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** A.E. Bannon, C. Beadling, M.C. Heinrich
**Study supervision:** A.E. Bannon, I. Forquer, M.C. Heinrich

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Clinical Cancer Research

# Biochemical, Molecular, and Clinical Characterization of Succinate Dehydrogenase Subunit A Variants of Unknown Significance

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