

Metabolic context and cellular ploidy enable functional classification of SDHB exon3 variants

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Abstract

Loss-of-function variants in *SDHB*, encoding an iron-sulfur subunit of mitochondrial complex II, predispose to hereditary pheochromocytoma and paraganglioma (PPGL), with metastatic disease developing in up to 30% of affected individuals. Yet 47% of germline *SDHB* single-nucleotide variants reported in ClinVar remain variants of uncertain significance (VUS), limiting the clinical utility of genetic testing. We hypothesized that limiting metabolic compensation for impaired mitochondrial respiration would enhance detection of hypomorphic variants, while integrating haploid and hemizygous diploid backgrounds would improve functional discrimination. We developed a media- and ploidy-optimized multiplexed assay of variant effect (MAVE) in HAP1-LIG4KO-Cas9 cells to characterize 318 single-nucleotide variants across *SDHB* exon 3, encompassing the 2Fe-2S cluster, and flanking regions. *SDHB*-deficient cells exhibited pyruvate-dependent proliferation, enabling detection of functionally-abnormal variants. Variant effects across ploidies were integrated using a discordance-aware probabilistic framework. Incorporating MAVE-derived PS3/BS3 evidence into ACMG/ClinGen criteria classified 143 (~45%) variants as likely benign, 135 (~42%) as (likely) pathogenic, and 40 as VUS (~13%). Structurally, intolerant variants clustered around the 2Fe-2S cluster and an adjacent helix. These findings establish assay context as a critical determinant of variant discrimination and provide a framework to reduce the VUS burden and improve personalized medicine for *SDHB* and beyond.

Introduction

Monoallelic loss-of-function germline variants in *SDHB* are attributing 50–55% of hereditary pheochromocytoma and paraganglioma (PPGL) (Else et al., 2018). *SDHB* acts as a tumour suppressor gene, with metastatic disease developing in roughly 30% of individuals with *SDHB*-associated PPGL in proband-enriched cohorts. Since early detection can reduce mortality (Davidoff et al., 2022), accurate risk stratification is essential for patient outcomes (Taïeb et al., 2024).

Genetic testing for PPGL is increasingly implemented in clinical practice, yet the interpretive yield remains substantially limited by variants of uncertain significance (VUS). As of August 2026, 1,429 germline *SDHB* single-nucleotide variants are listed in ClinVar, of which ~47% are classified as VUS, with missense variants comprising ~91%. This substantially limits the clinical utility of genetic testing and hinders accurate risk stratification in the absence of complementary evidence, familial segregation patterns, and functional evaluation (Richards et al., 2015).

The functional evaluation of variants depends strongly on assay design and the phenotypic context in which variants are tested, including the cellular model, media composition, and assay readout (Brnich et al., 2020). *SDHB* encodes the Fe-S protein subunit of succinate dehydrogenase (*SDH*; mitochondrial complex II), housing the 2Fe-2S, 4Fe-4S, and 3Fe-4S clusters within the complex that is composed of *SDHA*, *SDHB*, *SDHC*, and *SDHD* (together SDHx). As a central component of mitochondrial respiration, it links the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) by oxidizing succinate to fumarate. In PPGL tumorigenesis, biallelic loss of *SDHB* function disrupts oxidative phosphorylation,

elevates the succinate:fumarate ratio, and drives oncogenesis through downstream epigenetic reprogramming and pseudohypoxic signaling (Martínez-Reyes and Chandel, 2020). Functional characterization of primary tumours by immunohistochemistry of *SDHB* (van Nederveen et al., 2009; Gill et al., 2010; Pai et al., 2014; Udager et al., 2018) and succinate:fumarate ratio (Lendvai et al., 2014; Kim et al., 2017; Richter et al., 2019; Cascón and Robledo, 2024) can improve variant interpretation. However, patient-derived tumor material is not always accessible, and tissue-based approaches are inherently retrospective and cannot scale to address the growing VUS burden prospectively.

Computational predictors offer scalable, first-pass assessments of variant impact, with recent calibrated predictors providing increasingly robust evidence for missense variant interpretation (Pejaver et al., 2022; Zhuo et al., 2026). However, computational predictions remain distinct from direct experimental evidence of molecular dysfunction, motivating complementary functional approaches for variant interpretation. Plasmid-based functional assays in immortal cell lines like HEK293 have successfully resolved RNA splicing effects and succinate:fumarate ratio change for individual variants (Lee et al., 2025; Köhler et al., 2026), but these approaches are low-throughput, labour-intensive, and typically evaluate variants in isolation rather than at scale. Multiplexed Assays of Variant Effect (MAVE) address this limitation by enabling the parallel functional interrogation of hundreds to thousands of variants within their native genomic context (Starita et al., 2017). MAVEs have been applied to generate functional landscapes for several major tumor suppressor genes, including *BRCA1*, *BRCA2*, *BAP1*, *VHL*, and *TP53*, and have demonstrated superior performance over computational predictions alone (Findlay et al., 2018; Waters et al., 2024; Buckley et al., 2024; Funk et al., 2025; Huang et al., 2025; Sahu et al., 2025).

Cancer cells frequently rely on aerobic glycolysis even with intact mitochondrial respiration, a phenomenon termed the Warburg effect (Vander Heiden et al., 2009). Loss of *SDHB* function impairs complex II activity, driving a Warburg-like metabolic rewiring including increased glycolysis, pyruvate carboxylation, and glutamine-dependent anaplerosis depending on the model system and cell line of study (Cardaci et al., 2015; Tabebi et al., 2022). Metabolic adaptations, including pyruvate transamination and NAD biosynthesis, sustain aerobic glycolysis and biosynthesis processes despite impaired *SDHx* function maintaining proliferation (Ricci et al., 2023). This metabolic plasticity is not unique to loss of *SDHx*, as respiratory-deficient cells have been shown to require exogenous pyruvate as an electron acceptor to sustain NAD⁺ regeneration and glycolytic flux in cancer cells (Yin et al., 2016), suggesting that presence of pyruvate in the culture media could mask the fitness cost of *SDHB* loss. In the context of disease, PPGLs follow the classical two-hit tumor suppressor model (Knudson, 1971), in which somatic loss of the remaining wild-type allele (loss of heterozygosity) drives tumorigenesis in cells with a pathogenic germline variant. MAVE assays commonly employ the chronic myelogenous leukemia derived haploid HAP1 cells, maximize adjusted-sensitivity to loss-of-function by preventing complementation from a second allele (Llargués-Sistac et al., 2023), but do not recapitulate the physiological diploid context in which pathogenic germline variants undergo a second somatic hit through loss of heterozygosity.

We hypothesized that optimizing media conditions to suppress Warburg-like metabolic compensation would improve detection of hypomorphic variants, and that integrating data from both haploid and hemizygous (diploid cells with one *SDHB* copy and deletion of the other) backgrounds would provide a better balance of adjusted-sensitivity and adjusted-specificity than either model alone. In addition, comparing results from both ploidies could potentially identify context-dependent and context-independent variants, with concordant and discordant classifications between the two backgrounds offering mechanistically informative insight into variant behavior. To test this, we developed a media- and ploidy-optimized MAVE in HAP1-LIG4KO-Cas9 cells targeting *SDHB* exon 3 and its flanking splice regions. This region encodes the highly constrained 2Fe-2S iron-sulfur cluster, including the canonical coordinating residue Cys93, making it a biologically and clinically important target for functional interrogation. Using pyruvate-dependent growth as a sensitive readout of *SDHB* function, we quantified the effects of hundreds of coding and splice-region variants across both genetic backgrounds. We then integrated these functional data into ACMG/ClinGen variant interpretation, demonstrating that a media- and ploidy-optimized MAVE can improve germline *SDHB* variant classification and support clinical decision-making in hereditary paraganglioma-pheochromocytoma syndromes.

Result

Pyruvate availability determines proliferative fitness in *SDHB*-deficient cells

SDHB loss is associated with increased glycolytic dependence and pyruvate consumption (Cardaci et al., 2015). We therefore hypothesized that optimizing media culture conditions to limit glycolytic compensation would increase mitochondrial dependency and enhance the phenotypic distinction between *SDHB* knockout (KO) and wild-type (WT) HAP1-LIG4KO-Cas9 cells (from here on referred to as HAP1).

To generate *SDHB* KO in haploid and diploid HAP1 backgrounds, a CRISPR-Cas9 deletion strategy was employed and KOs were confirmed with Western blot and breakpoint PCR (Fig. S1; generation and confirmation in supplementary Material and Methods). Up to four clones per condition and ploidy state were selected for succinate:fumarate ratio validation by liquid chromatography tandem mass spectrometry (LC-MS/MS), with the exception of hemizygous cells for which only one confirmed clone was available. Generation of hemizygous clones (deletion of one *SDHB* allele from exon 1 to 8) in diploid HAP1 required simultaneous independent editing events on a single allele, resulting in low efficiency (1/35 clones). Previous studies have shown an increase in succinate:fumarate ratio in both tumours and in vitro models with *SDHB* loss of function (Richter et al., 2014; Kim et al., 2017; Lee et al., 2025). Similar to these findings, WT cells maintained low succinate:fumarate ratios across haploid (0.02–1.34) and diploid (0.02–0.93) backgrounds. The hemizygous diploid clone showed an equally low ratio (1.38), consistent with retained SDH activity in cells carrying one functional *SDHB* allele. KO cells showed markedly elevated ratios in haploid (79–984) and diploid (64–980) backgrounds (Fig. 1a; Table S1).

Next, we sought to identify media conditions that best distinguished WT and KO clones in both ploidy states. Because haploid HAP1 cells spontaneously diploidize during prolonged culture (Olbrich et al., 2017), the initial media optimization was performed using two independently derived diploid WT and KO HAP1 clones to minimize ploidy-related variability. To define metabolic conditions that either minimized or accentuated fitness differences between *SDHB* WT and KO cells, we systematically varied the carbon source (glucose or galactose) and pyruvate availability. In pyruvate-deficient media, KO cells exhibited minimal to negligible proliferation with either glucose or galactose as the sole carbon source, whereas WT cells continued to proliferate under both conditions, with mean doubling times of 13.8 h in glucose and 72.3 h in galactose. Pyruvate supplementation restored proliferation of KO cells in glucose-containing media, resulting in doubling times of 17.5 h and 17.8 h at 2 and 4 mM pyruvate, respectively (Fig. S2a), although proliferation remained slower than that of WT cells. In contrast, pyruvate supplementation failed to restore proliferation of KO cells in galactose-containing media (Fig. S2b). WT proliferation was only modestly affected by pyruvate supplementation in glucose (doubling times of 10.8 h and 12.3 h at 2 and 4 mM pyruvate, respectively) (Fig. S2a), whereas growth in galactose was substantially improved by 2 mM pyruvate (doubling time, 25.6 h) (Fig. S2b).

These results prompted us to modulate pyruvate availability in low-glucose media, shifting between 'proliferation permissive' and 'selective starvation' conditions to distinguish *SDHB* WT and KO clones. We expanded the study to include diploid, hemizygous, and haploid cells and measured the AUC of $\log_2$ normalized proliferation from day 0 to day 3. Similar to KO diploid cells, KO haploid cells were dependent on pyruvate for proliferation unlike their WT counterparts (Fig. 1b). Under pyruvate starvation, a clear divergence between WT (doubling time 18.8h and 12.8h in haploid and diploid respectively) and KO cells (negligible proliferation) was observed in both haploid and diploid backgrounds (Fig. 1c, 1e), in contrast to pyruvate supplementation (doubling time 32.1h and 11.5h in haploid and diploid respectively) (Fig. 1d, 1f). Unlike KO cells, hemizygous *SDHB* cells were able to maintain proliferation under pyruvate starvation (doubling times of 31.2h and 17.8h at 0 and 2 mM pyruvate) (Fig. 1e, 1f).

Together, these results demonstrate three key findings. First, *SDHB* KO in HAP1 cells causes succinate:fumarate elevation over 100-fold above the levels of WT cells. Second, *SDHB* loss in HAP1 cells increases dependence on exogenous pyruvate for proliferation. Third, hemizygous *SDHB* cells are similar to WT cells in their succinate:fumarate ratio and proliferation pattern, supporting their use alongside haploid cells in the downstream MAVE assay.

Multiplexed functional profiling of 318 *SDHB* exon 3 variants in haploid and hemizygous cells

For our *SDHB* MAVE assay, we generated a saturation mutagenesis library consisting of all possible SNVs across exon 3 and its flanking regions ($n = 318$) and introduced the library into HAP1 haploid and hemizygous cells. To improve the Homology-Directed Repair (HDR) in haploid HAP1 with lower editing efficiency, we combined DNA-PK with Pol θ inhibition (2iHdRi) (Fig. S3) (Wimberger et al., 2023). After four days of growth in pyruvate supplemented media, cells were switched to pyruvate starvation. Functional scores were calculated from changes in variant allele frequency as $\log_2(\text{day8}/\text{day4})$

(Materials and Methods). Scores were normalized and converted to posterior probabilities of pathogenicity, which were benchmarked against annotated variants to calibrate ACMG/AMP PS3 and BS3 evidence strengths for classification (Fig. 2a).

Following normalization, functionally-normal and functionally-abnormal variants localized to distinct peaks of ground-truth variants with highly-stringent criteria for annotation (defined in supplementary Material and Methods), whereas VUS from ClinVar (multiple submitters) were broadly distributed (Fig. 2b, 2c). Functional scores were highly correlated across replicate sets (Fig. S4) and both the haploid and hemizygous assay exhibited consistently bimodal distributions with Gaussian mixture modelling supporting two well-separated components (difference in Bayesian information criterion ΔBIC = 84–187; Ashman's D = 3.49–3.99; Table S2).

To assign PS3/BS3 functional evidence for ACMG/ClinGen variant classification, a two-component GMM was applied to normalized MAVE scores to assign each variant a posterior probability of pathogenicity (Materials and Methods, Table S3). Given the modest sample size, we used pre-specified biologically meaningful thresholds rather than dataset-specific threshold optimization. Variants with $p \geq 0.95$ were classified as functionally-abnormal-like, $p \leq 0.05$ as functionally-normal, and intermediate scores as uncertain, yielding 130 functionally-normal, 29 uncertain, and 158 functionally-abnormal variants and 1 unclassified variant in haploid cells, and 164 functionally-normal, 24 uncertain, and 130 functionally-abnormal in hemizygous cells. PCA supported the classifications, with functionally-normal variants forming compact clusters while functionally-abnormal and uncertain variants showed greater dispersion (Fig. 2d, 2e).

Classification by variant type revealed clear biological stratification of the GMM derived calls. Stop-gain variants were exclusively functionally-abnormal in both backgrounds (15/15). Canonical splice variants in haploid cells (11/12) and hemizygous cells (12/12) were functionally-abnormal. Splice-region (35/44 haploid, 39/44 hemizygous), synonymous (51/54 haploid, 50/54 hemizygous), and intronic (5/6 haploid, 6/6 hemizygous) variants were predominantly functionally-normal. In contrast, missense variants were distributed across all three classes, with 91% receiving a definitive classification in haploid and 93% in hemizygous cells (Fig. 2f, 2g). To externally validate these classifications, we benchmarked both classifiers against a curated annotated variant dataset. The haploid classifier achieved Receiver Operating Characteristic-Area Under the Curve, (ROC-AUC) = 0.998, adjusted-sensitivity = 0.97, and adjusted-specificity = 0.893, whereas the hemizygous classifier achieved ROC-AUC = 1, adjusted-sensitivity = 1, and adjusted-specificity = 0.929 (Table S4). The reported adjusted-sensitivity and adjusted-specificity were calculated using all benchmark variants, including those remaining unclassified (uncertain) (defined in supplementary Materials and Methods).

Together, these findings demonstrate that both ploidy-specific classifiers provide highly accurate functional classification, with the hemizygous model modestly outperforming the haploid model.

Dual-ploidy integration balances sensitivity and specificity for conservative variant classification

To understand the relationship between the haploid and hemizygous assays, we first assessed the concordance and discordance of variant classifications (Table S3). Haploid and hemizygous scores were highly correlated (Pearson's $r = 0.928$; Spearman's $\rho = 0.852$; Fig. 3a, Fig. S5), indicating that both backgrounds capture a shared functional signal. However, a subset of variants showed discordant classifications ($n = 62$), with large number of discordant variants classified as functionally-abnormal in haploid cells ($n = 31$) but functionally-normal ($n = 17$) or uncertain ($n = 14$) in hemizygous cells. In contrast, only one functionally-abnormal variant in hemizygous cells was classified as functionally-normal in haploid cells (Fig. 3b). This discordance was concentrated among missense variants ($n = 45$) but also included synonymous ($n = 7$), non-canonical splice variants ($n = 9$) and intronic ($n = 1$) (Fig. 3c). Together, these findings suggest that the haploid classification is more permissive for functionally abnormal calls, especially with respect to missense variants, whereas the hemizygous classification is more conservative.

Nevertheless, the high correlation between assays and the predominantly one-sided discordance indicated that the haploid assay retained informative functional evidence. We therefore combined haploid and hemizygous posterior probabilities as related but non-equivalent functional measurements using an odds-based probabilistic framework (Materials and Methods, Table S3). To conservatively account for inter-ploidy discordance, definitive integrated classifications were assigned only to variants meeting predefined concordance and confidence thresholds while all others were classified as uncertain (Materials and Methods, Table S3). This discordance-aware integrated classifier achieved improved performance on highly-stringent annotated ground-truth annotations (ROC-AUC = 1, adjusted-sensitivity = 1, adjusted-specificity = 0.964) (Fig. 3d, Table S4). Integration retained concordant classifications across both ploidy models while resolving 14 of 29 haploid-uncertain variants to definitive classifications (13 functionally-normal and 1 functionally-abnormal). Conversely, 1 functionally-normal and 21 functionally-abnormal were reclassified as uncertain. Similarly, 15 of 24 hemizygous-uncertain variants were resolved to definitive classifications (5 functionally-normal and 10 functionally-abnormal), whereas 26 functionally-normal and 2 functionally-abnormal variants were reclassified as uncertain. These reclassifications confirm that the discordance filter conservatively withheld definitive classifications when evidence from the two ploidy models conflicted, yielding a final integrated classification of 138 functionally-abnormal, 143 functionally-normal, and 37 uncertain variants (Fig. 3e).

Among *in-silico* comparators, which were calibrated using medium-stringency ground-truth annotations, the high-stringency ground-truth set contained no benign missense variants. We therefore supplemented this set with computationally supported variant annotations, following a modified version of published recommendations (Pejaver et al., 2022; Rowlands et al. 2025; see Supplementary Materials and Methods for details). Medium-stringency ground-truth consists of 104 variants (64 functionally-normal, 40 functionally-abnormal). The MAVE integrated model outperformed CADD in adjusted-sensitivity and adjusted specificity across all variant types (Fig. S5, Table S4). For missense variants specifically, MAVE integrated model outperformed AlphaMissense and CADD in adjusted-specificity and adjusted-sensitivity (Fig. S6, Table S4). For splice variants, the MAVE integrated model outperformed CADD in adjusted-sensitivity (Fig. S7, Table S4). We did not compare our model with REVEL, BayesDel, and SpliceAI

because these were used in constructing the medium-stringency ground-truth dataset (Pejaver et al., 2022; Rowlands et al., 2025). However, per-consequence-class performance should be interpreted cautiously given the small subset sizes, where ROC-AUC estimates are sensitive to individual variant classifications.

Pairwise correlation analysis showed stronger agreement among the MAVE datasets ($\rho = 0.84\text{--}0.96$) than between MAVE functional scores and *in-silico* predictors. Among missense predictors, AlphaMissense exhibited the strongest correlation with the integrated MAVE score ($\rho = 0.709$), followed by BayesDel ($\rho = 0.65$), REVEL ($\rho = 0.562$), and CADD ($\rho = 0.43$). For splice prediction, both CADD and SpliceAI showed moderate correlations with the integrated MAVE score ($\rho = 0.489$ and 0.516 , respectively). Within the MAVE assay, haploid and hemizygous showed moderate correlation with each other for splice variants ($\rho = 0.632$). These findings indicate that MAVE-derived functional scores capture functional variation that is only partially reflected by computational prediction tools (Fig. S8).

Collectively, these results demonstrate that the integrated ploidy-aware classifier achieves higher adjusted-sensitivity and adjusted-specificity, and identifies context-specific discordant variants compared to ploidy-specific approaches. It also outperformed current computational variant effect predictors across the benchmarked dataset.

Ploidy-integrated functional mapping reveals conserved missense hotspots

Three prominent missense-constrained regions were detected: the region flanking the C93 residue of the 2Fe-2S cluster (17033063–17033083; amino acids 88–95), the adjacent helical scaffold (17033118–17033135; amino acids 71–76), and the C68-G69 motif (17033140–17033144; amino acids 68–69) (Fig. 4a, 4b).

The first and most constrained region (amino acids 88–95) contains C93, a canonical 2Fe-2S cluster-coordinating cysteine essential for electron transfer (Saxena 2016; Du et al., 2023) and previously confirmed to be likely-pathogenic (Lee et al., 2025) (Fig. 4c). In our assay, most substitutions flanking this residue were classified as functionally-abnormal, consistent with the critical structural role of C93. The second region (amino acids 71–76) corresponds to the MVLDAL hydrophobic helical motif, forming part of an α -helical segment adjacent to the 2Fe-2S cluster (Fig. 4c). Substitutions at hydrophobic positions were generally functionally-abnormal, particularly those introducing polar or charged residues, consistent with the strict hydrophobic packing requirements of this helical segment. The third region (amino acids 68–69) corresponds to the C68-G69 motif. Most substitutions at C68 and G69 were functionally-abnormal, although C68S and G69A remained classified as functionally-normal and VUS respectively. The adjacent P70 displayed broader mutational tolerance, with several substitutions remaining functional.

Sequence conservation corroborated the MAVE-identified constrained regions: the MVLDAL motif (residues 71–76) and TFRRSCR region (residues 88–94) showed near-complete conservation across species, while residues 68–69 exhibited more moderate conservation, suggesting MAVE identified

functional constraints beyond evolutionary conservation alone (Fig. 4d, Table S5, supplementary Material and Methods).

MAVE-derived evidence refines ACMG/AMP classification of *SDHB* variants

To evaluate the clinical impact of the assay, we incorporated MAVE integrated model-derived functional evidence into a modified ACMG/ClinGen variant interpretation framework using the ClinGen SVI functional assay validation approach (Brnich et al., 2020). We used the highly-stringent ground-truth annotation for calculating the Odds ratio for PS3 and BS3 evidence. For haploid and hemizygous assay calibration, the intermediate functional scores were excluded prior to OddsPath calculation, consistent with the treatment of indeterminate functional scores in subsequent implementations of this framework (Fayer et al., 2021; O'Neill et al., 2024). The resulting OddsPath values exceeded the thresholds for strong functional evidence proposed by Brnich *et al.*, supporting application of PS3 and BS3 at strong strength of haploid, hemizygous and the integrated model (Table S6). Although haploid and hemizygous functional evidence was independently calibrated, due to modestly improved adjusted-sensitivity and adjusted-specificity, the integrated ploidy-aware classifier was used as the primary model for downstream interpretation.

To conservatively approximate the impact of the MAVE integrated model-derived functional evidence on ACMG/ClinGen variant evaluation, we adjusted the evidence codes and strengths in an automated variant assessment (Material and Methods, Table S7). Without MAVE-derived evidence, 279 variants remain unclassified while 39 variants have a definitive class (LB = 5, LP = 27, P = 7). After incorporating MAVE integrated model-derived functional evidence, 40 variants remain unclassified, resulting in ~ 86% decrease in VUS annotations. A total of 278 of the 318 exon3 variants (~ 87%) get a definitive classification (LB = 143, LP = 101, P = 34) (Fig. 5a; Table S7).

Among ClinVar-annotated variants (n = 127), all variants submitted as (likely) pathogenic (n = 22) received a PS3 code and a classification of likely pathogenic, while ~ 93% of variants submitted as (likely) benign (n = 28/30) were supported by BS3. A total of ~ 79% of ClinVar VUS (n = 52/66) were reclassified as either likely benign (n = 24, ~ 36%) or likely pathogenic (n = 28, 42%). In addition, 9 of 9 variants with conflicting ClinVar interpretations were resolved as likely pathogenic (n = 7) or likely benign (n = 2). Among the 191 unannotated variants on ClinVar, ~ 87% were assigned a classification, including likely benign (n = 89, ~ 47%), likely pathogenic (n = 62, ~ 32%), and pathogenic (n = 16, ~ 8%) variants (Fig. 5b). However, our assay reclassified one B/LB and one LB as VUS.

All stop-gains and canonical splice variants were classified as (likely) pathogenic. All intronic variants were likely benign, while ~ 84% (n = 37) splice region and 96% (n = 52) of synonymous variants were classified as benign. Overall, 57% of missense variants were classified as likely pathogenic (n = 99) or pathogenic (n = 7), whereas 26% (n = 48) were classified as likely benign (Fig. S9). The inclusion of functional evidence derived from the integrated MAVE model substantially improved variant classification, reducing the number of variants of uncertain significance (VUS) from 180 to 33 for

missense variants, from 43 to 5 for splice-region variants, and from 50 to 2 for synonymous variants (Fig. S9).

Together, these findings demonstrate the clinical utility of a media- and ploidy-optimized MAVE framework for resolving *SDHB* VUS at scale, providing a template for functional interrogation of other exons and *SDHx* genes where variant interpretation remains a barrier to patient care.

Material and methods

Saturation genome editing (SGE), Homology-directed DNA repair (HDR) and sgRNA design

All genomic coordinates refer to the hg38/GRCh38 reference genome. Coding positions correspond to the *SDHB* MANE Select transcript (NM_003000.3 / ENST00000375499.8), as defined by the Matched Annotation from NCBI and EMBL-EBI (MANE) project (Morales et al., 2022) and amino acid positions refer to the *SDHB* protein reference NP_002991.2 (*SDHB* gene – Gene ID: 6390, OMIM: 185470) and UniProt P21912 (The UniProt Consortium). Exon3 and 10 bp flanking the exon was extracted from the hg38 reference genome in chromosome 1, spanning position 17033050–17033155. A synonymous variant annotated as benign with no conflict, multiple submitters, in ClinVar <https://www.ncbi.nlm.nih.gov/clinvar/> (Landrum et al., 2014), located in the PAM region at position 17033082G > C was introduced into the sequence. All possible single-nucleotide variants (SNVs) in this sequence with the PAM switch were designed, resulting in a library with 318 SNVs within a 106 bp window. The sgRNA sequence to target exon3 was designed on CHOPCHOP <https://chopchop.cbu.uib.no/> (Labun et al., 2019) based on priority and low off target output.

Template library preparation

Double-stranded Ultramer™ DNA oligonucleotides (Integrated DNA Technologies) were purchased in 384-well plate format, with each well containing oligonucleotides encoding all three possible single-nucleotide substitutions at a specific genomic position. Wells were dissolved in nuclease-free water as instructed from the manufacturer. Three mini-libraries were generated from 35–36 wells each by PCR amplification (KAPA HiFi HotStart ReadyMix, 2 × 25 µL reactions containing X ng or X µL template, 18 cycles; 98°C for 20 s, 60°C for 15 s, 72°C for 20 s, with an initial denaturation of 95°C for 3 min and a final extension of 72°C for 1 min) using flanking primers that anneal to the constant regions of the oligonucleotide templates (Table S8). PCR amplicons were purified using the High Pure PCR Product Purification Kit (Roche, #11732668001) and quantified by Qubit fluorometer (Thermo Fisher Scientific). Mini-libraries were pooled at equal molar concentrations to generate one full variant library. For each experimental replicate, mini-library PCR amplification was repeated from the original oligonucleotide templates and pooled to generate a fresh full-variant library, ensuring independent library preparation across replicates.

Design and cloning of variant library in plasmid

To clone the variant library into the modified PX459 plasmid (px459-Cas9-KO-sgRNA-Exon3HDR, Supplementary material and Methods), the vector was linearized by PCR amplification using primers flanking the SNV insertion site (NEB Q5® High-Fidelity DNA Polymerase with Q5 High GC Enhancer, 25 µl reaction, containing 20 ng template, 30 cycles; 98°C for 10 s, 66°C for 45 s, 72°C for 4 mins, with an initial denaturation of 98°C for 30 s and a final extension of 72°C for 10 mins), thereby removing the wild-type exon 3 sequence and generating an open vector with compatible ends for assembly (Table S8). The linearized vector and insert full-library amplicon were assembled at a 5:1 insert-to-vector molar ratio using the NEBuilder HiFi DNA Assembly Master Mix (NEB, #E2621L, at 50°C for 60 min). The assembled library plasmid was transformed into DH5α competent cells (NEB, #C29871) and extracted by miniprep (Qiagen, #27104) for use in all downstream SGE experiments.

Proliferation assay and analysis

To identify optimal culture conditions for cells of varying ploidy, a medium screening experiment was performed. Cells maintained in medium (Table 1) were washed twice with 1xPBS, trypsinized at 37°C for 3 minutes, and resuspended in the respective screening medium. Each cell type (haploid, diploid, and hemizygous; WT, KO) was suspended at 5,000 cells per 200 µl in 1.5 ml tubes, then transferred in 200 µl aliquots into 96-well plates. Cells were cultured under standard conditions (37°C at 5% CO₂) and the medium was refreshed every 3 days. Proliferation was monitored by live-cell imaging using the Incucyte system (Sartorius) with 10x objective, images acquired every 6 hours per well. Each independent clone was cultured in three technical replicate wells, with 1–5 regions of interest (ROIs) imaged per well, depending on the experiment. Cell areas were segmented using the Sartorius Basic Analyzer plugin with a minimum object area threshold of 200 µm². Technical replicate wells were averaged for each clone, and independent clones were used as the biological replicates for all downstream analyses. Confluency values were normalized to the first timepoint for each well and normalized area under the curve as well as normalized growth curves were plotted. Differences in AUC between wild-type and knockout clones under each condition were assessed using two-sided Mann–Whitney U tests.

Table 1
Media conditions for proliferation assay

Media condition	feature
DMEM, 10% FBS, 10 mM Glucose, 2 mM pyruvate	Low glucose, high pyruvate
DMEM, 10% FBS, 25 mM Glucose, 2 mM pyruvate	High glucose, high pyruvate
DMEM, 10% FBS, 25 mM Glucose, 4 mM pyruvate	High glucose, very high pyruvate
DMEM, 10% FBS, 25 mM Galactose, 2 mM pyruvate	High galactose, high pyruvate
DMEM, 10% FBS, 10 mM Glucose	Low glucose
DMEM, 10% FBS, 25 mM Glucose	High glucose
DMEM, 10% FBS, 25 mM Galactose	High galactose

Gibco™DMEM(Thermo Fisher Scientific, #11966-025), Gibco™FBS (Thermo Fisher Scientific, #A5256701), Glucose (Carl Roth, #HN06.1), Galactose (Carl Roth, #8878.1), pyruvate (Thermo Fisher Scientific,#11360070).

Succinate and fumarate measurements by LC-MS/MS

Cells (500,000 per well) were cultured overnight in a 6-well plate in Gibco™DMEM(Thermo Fisher Scientific, #11966-025) with Gibco™FBS (10%, Thermo Fisher Scientific, #A5256701), Glucose (10 mM, Carl Roth, #HN06.1), pyruvate (2 mM, Thermo Fisher Scientific,#11360070). Media was removed and cells were washed on ice with PBS (4x) followed by lysis with ice-cold methanol and addition of internal standard mix. Cell extracts were cleared by centrifugation and taken to dryness. For analysis, extracts were resuspended in the mobile phase and analysed by ultrahigh pressure liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) as previously described (Wallace et al., 2020).

Saturation Genome Editing pipeline

FACS sorted haploid/hemizygous HAP1-LIG4KO-Cas9 cells (Supplementary Methods) were thawed and expanded for 4 days prior to the experiment. A total of 5×10^6 cells per biological replicate was seeded in 15 cm plates (or equivalent T175). Hemizygous cells were maintained in Gibco™DMEM (Thermo Fisher Scientific, #11966-025) supplemented with (10 mM, Carl Roth, #HN06.1), Gibco™ sodium pyruvate (2 mM, Thermo Fisher Scientific,#11360070), Gibco™FBS (10%, Thermo Fisher Scientific, #A5256701), and DAB (10 μ M, Selleck Chemicals, #S2409) to prevent spontaneous diploidization. Haploid cells were cultured in the same base medium with an additional supplementation for 2iHDRi methodology with AZD7648 (0.5 μ M, Selleck Chemicals, HY-111783) and PolQi2 (1 μ M, MedChemExpress, HY-150279) to improve HDR efficiency (Wimberger et al., 2023), as initial sequencing at equivalent depth revealed lower editing rates in haploid compared to hemizygous cells. The optimal inhibitor concentrations for haploid cells were determined empirically using the multiEDITR Sanger sequencing analysis tool (Fig. S3) (<https://moriaritylab.shinyapps.io/multieditr/>; accessed [19,December, 2025], Kluesner et al., 2021).

When cells had reached approximately 80% confluence the following day, 93 μ g of plasmid DNA was transfected using 27.9 μ l of Xfect polymer (Takara Bio,#631317) in a total reaction volume of 1800 μ l at a 1:0.3 DNA:polymer ratio (w/v) according to the manufacturer's instructions. Puromycin selection (1 μ g/ml, invivoGen #ant-pr-1) was initiated 48 hours post-transfection and maintained for 3 days with media replenishment every 1–2 days.

Following completion of puromycin selection, cells were transferred to proliferation-permissive media Gibco™DMEM (Thermo Fisher Scientific, #11966-025) supplemented with D+(-)Glucose (10 mM, Carl Roth, #HN06.1), Gibco™ sodium pyruvate (2 mM, Thermo Fisher Scientific,#11360070), Gibco™FBS (10%, Thermo Fisher Scientific, #A5256701), and DAB (10 μ M, Selleck Chemicals, cat. #S2409) for 24 hours, after which 2×10^6 cells were harvested for timepoint 1. Cells were then switched to selective starvation media Gibco™DMEM (#11966-025) supplemented with Glucose (10 mM, Carl Roth, #HN06.1), Gibco™FBS (10%, Thermo Fisher Scientific, #A5256701), and DAB (10 μ M, Selleck Chemicals, cat.

#S2409) for the 4-day interval between timepoints, with splitting plates as required to maintain subconfluent growth below 80% throughout. A second harvest of 2×10^6 cells was performed at timepoint 2.

Genomic DNA was extracted using a modified DNeasy Mini Kit protocol (Qiagen, #69504) as described in Radford et al., 2023. Briefly, cell pellets were washed twice in DPBS to remove residual drugs, treated with PureLink™ RNase A (20 mg/ml, 37°C, 15 min, invitrogen, #12091021), and digested with Proteinase K in Buffer AL (56°C, 30 min). DNA was precipitated with 100% ethanol, bound to a silica spin column, washed sequentially with Buffers AW1 and AW2, eluted in 100 µl pre-warmed Buffer AE (60°C), and quantified by NanoDrop (Thermo Fisher Scientific) and Qubit™ dsDNA BR Assaykit (Thermo Fisher Scientific, #Q32854). Each ploidy had three biological repeats for statistical robustness.

Amplicon preparation for NGS

Library allele frequency was assessed in plasmid and gDNA. The plasmid amplicon was prepared for NGS with a 2-step PCR. A 171 bp region in the plasmid containing the variant library and flanking intronic sequence was PCR amplified (Roche KAPA HiFi HotStart Ready Mix #07958927001 with 2 ng of plasmid, 30 cycles; 98°C for 20 s, 67°C for 15 s, 72°C for 15 s, with an initial denaturation of 95°C for 3 min and a final extension of 72°C for 30 s) with partial adaptor primers (Table S8), the resulting amplicon was purified with High Pure PCR purification kit (Merck, #11732668001). Indexing of the PCR product was done using xGen™ UDI Primers (IDT, 10005975) via PCR (Roche KAPA HiFi HotStart Ready Mix #07958927001 with 25 ng amplicon, 7 cycles; 98°C for 10 s, 60°C for 30 s, 72°C for 20 s, with an initial denaturation of 98°C for 30 s and a final extension of 72°C for 10 mins). The final amplicon was purified with High Pure PCR purification kit (Merck, #11732668001). PCR amplicons were sequenced on an Illumina NextSeq 2000 instrument (2×150 bp paired-end) using NextSeq 1000/2000 XLEAP P3 Reagents (300-cycle kit).

gDNA was prepared for NGS with a 3-step PCR adapted from Radford et al., 2023. Briefly, genomic *SDHB* was amplified over plasmid *SDHB* by PCR amplification of *SDHB* sequence containing exon3 and flanking intron sequence absent in plasmid containing the HDR (Roche KAPA HiFi HotStart Ready Mix #07958927001 with 2.5 µg of gDNA split in 10 tubes, 23 cycles; 98°C for 20 s, 65°C for 15 s, 72°C for 20 s, with an initial denaturation of 98°C for 3 min and a final extension of 72°C for 1 min). The resulting 711 bp amplicon was purified High Pure PCR purification kit (Merck, #11732668001) and was used as input for PCR2 (Roche KAPA HiFi HotStart Ready Mix #07958927001 with 150–400 ng amplicon split in 4 tubes, 9 cycles; 98°C for 10 s, 65°C for 15 s, 72°C for 20 s, with an initial denaturation of 98°C for 3 min and a final extension of 72°C for 1 mins) with partial adaptors (Table S8). The resulting 224 bp amplicon was purified with High Pure PCR purification kit (Merck, #11732668001) and used as input for PCR3 (Roche KAPA HiFi HotStart Ready Mix #07958927001 with 25 ng amplicon, 7 cycles; 98°C for 10 s, 60°C for 30 s, 72°C for 20 s, with an initial denaturation of 98°C for 30 s and a final extension of 72°C for 10 mins) using xGen™ UDI Primers (IDT, 10005975). The final amplicon was purified with High Pure PCR

purification kit (Merck, #11732668001). PCR amplicons were sequenced on an Illumina NextSeq 2000 instrument (2 × 150 bp paired-end) using NextSeq 1000/2000 XLEAP P3 Reagents (300-cycle kit).

Calculation of Functional Score and probability

Functional scores were calculated by comparing variant allele frequencies between two time points (day 4 and day 8). Variant allele frequencies were merged across time points based on genomic position and alleles. To avoid division by zero and stabilize low-frequency estimates, missing or zero values were replaced with a small constant ($\epsilon = 6 \times 10^{-5}$, corresponding to approximately 10 reads). Frequencies were then bound to a minimum of this value. The functional score for each variant was defined as the $\log_2$ fold change in allele frequency between day 6 and day 4:

$$Functional\ score = \log_2 \left(\frac{AF_{day8}}{AF_{day4}} \right)$$

Positive values indicate enrichment over time, while negative values indicate depletion.

Functional scores from three biological sets were extracted. To ensure comparability across sets and reduce the influence of outliers, each score was normalized using the transformation $z = (x - \text{median}) / \text{IQR}$, where IQR represents the interquartile range. The normalized data were then modelled using a two-component Gaussian mixture model (GMM). Posterior probabilities from the GMM were used to classify each variant. Variants with a probability ≥ 0.95 were labeled as functionally-abnormal-like, those with probability ≤ 0.05 were labeled as functionally-normal, and all others were classified as uncertain. The custom codes are available on gitlink (https://github.com/NatashaSLewis/SGE_SDHB_Exon3/tree/main).

Integrated ploidy-based probability of pathogenicity score

Functional probabilities were independently estimated in haploid and hemizygous cellular contexts. Although functional scores between ploidies were correlated, categorical discordance was observed for a subset of variants (62/318), suggesting partially overlapping but non-identical information content. Therefore, haploid and hemizygous results were treated as related but non-equivalent functional measurements. The combined probability of pathogenicity $p_{combined}$ was computed from the haploid $p_{haploid}$ and hemizygous probabilities $p_{hemizygous}$ using an odds from Bayes's theorem, as an approximation under an independence assumption (Bishop and Nasrabadi, 2006). This Bayesian formulation is conceptually consistent with probabilistic frameworks for clinical variant interpretation (Tavtigian et al., 2018). Specifically,

$$p_{combined} = \frac{p_{haploid} \cdot p_{hemizygous}}{p_{haploid} \cdot p_{hemizygous} + (1 - p_{haploid})(1 - p_{hemizygous})}$$

To conservatively account for inter-ploidy discordance, variants were assigned a definitive integrated classification only if three criteria were met: (i) at least one ploidy context classified the variant as

functionally-abnormal or functionally-normal; (ii) the two ploidy contexts did not produce conflicting definitive classifications (i.e., one functionally-abnormal and the other functionally-normal); and (iii) the integrated posterior probability met a stringent threshold ($p \geq 0.99$ for functionally-abnormal or $p \leq 0.01$ for functionally-normal). Variants failing any criterion were classified as uncertain (Table S3). The custom code is available on gitlink (https://github.com/NatashaSLewis/SGE_SDHB_Exon3/tree/main).

Integration of MAVE data into ACMG/ClinGen variant evaluation

We have translated our functional score per variant into a PS3/BS3 code according to Brnich *et al.*, with a weight of strong (Brnich *et al.*, 2019). To approximate the impact of the MAVE codes on ACMG/ClinGen variant evaluation (Richards *et al.*, 2015; Rehm *et al.*, 2015) and since *SDHB* specific ACMG/ClinGen criteria are currently being developed by the ClinGen Endocrine Tumour Predisposition Variant Curation Expert Panel (<https://clinicalgenome.org/affiliation/51050/>), we have adjusted an automated variant assessment by Franklin, a clinical-grade genetic variant interpretation platform (Genoox Ltd., n.d.), and GeneBe (Stawiński, P. and Płoski, R., 2024), according to gene specific considerations. The following codes were provided for these variants from one of the resources and considered for variant evaluation: PVS1 (Franklin), PS3 (Franklin), PS4 (Franklin), PM2 (GeneBe), PM5 (Franklin), PP1 (Franklin), PP3 (Franklin), BP4 (Franklin), and BP7 (Franklin). Weights of specific codes were conservatively adjusted according to gene specific (Aim *et al.*, 2022) or general ClinGen SVI recommendations: PM2 (threshold 0.0001) and PM5 were used at a maximum weight of supporting. The following codes were not applied: PM1, as specific functional domains/residues are not well defined (Aim *et al.*, 2022), PP2 as the GnomAD v.4.1.1 derived *SDHB* missense z-score of 0.18 did not suggest a low rate of benign missense variation (Lek *et al.*, 2016) and PP5/BP6 as not suggested by ClinGen (Biesecker, L.G. and Harrison, S.M., 2018). Automated assessments were compared with and without PS3/BS3 of this study using a quantitative point-based framework (Tavtigian *et al.*, 2020) (Table S7).

Discussion

Functional interpretation of *SDHB* germline variants, which may inform both disease predisposition and prognosis, remains a major clinical challenge (Richter and Bechmann, 2024). The growing gap between variant discovery and clinically actionable interpretation highlights the need for robust functional assays to support personalized care and prevention. Here, we adapted MAVE to the biology of *SDHB*-associated PPGL by optimizing culture conditions to preserve loss-of-function phenotypes and evaluating variants in complementary haploid and hemizygous diploid backgrounds. Together, these approaches enabled assessment of how cellular context influences functional classification. The resulting functional scores showed strong concordance with control variants and provided functional evidence that enabled ACMG/ClinGen classification of approximately 42% of variants as (likely) pathogenic and 45% as (likely) benign.

Our findings suggest a general framework for MAVE design in which gene- and disease-specific selective pressures, applied across multiple media conditions or cell lines, can reveal the underlying biology by

identifying both concordant and discordant variant effects. In PPGL tumors, *SDHB* deficiency also drives succinate-mediated epigenetic reprogramming and metabolic rewiring (Letouzé et al., 2013; Lendvai et al., 2014; Cubiella et al., 2026). Tailoring media and cellular context (here with ploidy) conditions in the assay is particularly relevant for *SDHB*, where complete loss of function induces Warburg-like metabolic rewiring including increased glycolysis, pyruvate carboxylation, and glutamine-dependent anaplerosis depending on the model system and cell line of study (Cardaci et al., 2015; Tabebi et al., 2022). Accordingly, complete *SDHB* knockout cells failed to proliferate in the absence of exogenous pyruvate in both haploid and diploid zygosity of HAP1. Exploiting this metabolic dependency created sufficient proliferative separation between functionally-normal and functionally-abnormal variants within the four-day interval between MAVE time points, enabling sensitive functional discrimination. This pyruvate starvation-based readout can be applied directly to patient-specific SNVs, indels and potentially in patient derived cells. It can be further used to study the specific effects of variants on RNA or protein level to investigate mechanistic and possibly therapeutic opportunities. Although our study was limited to exon 3 of *SDHB*, the underlying strategy is scalable. SDH-deficient cells depend on GPT2-mediated pyruvate transamination to sustain reductive glutamine carboxylation, maintain NAD⁺ homeostasis, and support aerobic glycolysis (Ricci et al., 2023), suggesting that pyruvate withdrawal may provide a broadly applicable functional readout for assessing *SDHB* and *SDHx* complex variants. Outside of the SDHx complex, respiratory chain deficiency creates characteristic metabolic dependencies including nonviability in galactose medium when glycolysis is suppressed (Robinson et al., 1992), and reliance on exogenous pyruvate as an electron acceptor to sustain NAD⁺ regeneration (Yin et al., 2016). More broadly, this framework may be applicable to other metabolically constrained genes (Calame and Emrick, 2024), including tumour suppressors that undergo Warburg-associated metabolic rewiring (Vander Heiden et al., 2009) such as *VHL* and *FH* (Gottlieb and Tomlinson; 2005; Gossage et al., 2015), although the optimal selection conditions will likely differ. The concept of using biologically relevant selective pressure to resolve hypomorphic variants has also emerged in other disease settings. For example, cisplatin and *PARP* inhibition revealed hypomorphic *BRCA2* variants that were indistinguishable in conventional proliferation assays (Sahu et al., 2025).

The inclusion of complementary haploid and hemizygous backgrounds revealed variants with context-dependent functional effects that would have been missed using either background alone. The discordant functionally-abnormal variants in haploid cells were functionally-normal (n = 17) or uncertain (n = 14) in hemizygous cells, suggesting that the haploid background acts as a sensitized system capable of revealing hypomorphic variants with partial loss of function, however the sparse data on hypomorphic variants causing a more severe mitochondrial disease (Leigh syndrome) in a biallelic state in this exon limits further correlation. Only one variant 17033073T > C, c.273A > G (p.Arg91=), was classified as functionally-normal in haploid cells but functionally-abnormal in hemizygous cells. The discordant classification may reflect a genuine context-dependent effect, as the variant has a low predicted splice impact (SpliceAI delta score = 0.17). Given this discordance, our framework did not assign PS3 evidence for this variant, underscoring the value of requiring concordant functional evidence across complementary assay backgrounds. The increased adjusted-sensitivity of haploid HAP1 cells is

reflected in 50% of variants showing abnormal function versus 41% in hemizygous HAP1, while functionally-normal classifications were more common in the hemizygous background (52%) than in the haploid background (41%). The high prevalence of functionally-abnormal variants in both ploidies is not a surprise given the missense constraints in the Fe-S domains of *SDHB* (Garrett et al., 2022). The higher functionally-abnormal call preferentially in haploid in particular may reflect their intrinsic susceptibility to mild perturbations, arising from chromosome segregation defects resulting in activation of p53-dependent cytotoxic response (Olbrich et al., 2017), which can shift variants with modest functional impairment across the assay threshold. While the hemizygous background likely more closely recapitulates the physiological context of *SDHB*-associated tumorigenesis and may therefore mask weaker functional defects despite the applied metabolic selection. The use of multiple cellular contexts has also proven valuable for functional variant interpretation in other genes, including humanized mouse embryonic stem cells expressing a single human gene copy in a diploid background (Sahu et al., 2023) and iPSC-based systems with engineered second alleles (Fayer et al., 2025). This strategy is likely to be particularly informative for genes in which variants exhibit both dominant and recessive effects, such as *FH* (Coman et al., 2020; Tomlinson et al., 2002), *BRCA1/2*, and other Fanconi anaemia genes (D'Andrea., 2010).

The integrated classifier outperformed individual assay conditions and existing computational prediction methods. Although functional scores correlated with sequence-based predictors (best performing tool was AlphaMissense with 1 adjusted-sensitivity and 0.875 adjusted-specificity, Spearman coefficient = 0.709 with MAVE, albeit with 18 annotations), they captured experimentally measurable effects that computational algorithms alone cannot resolve (Livesey et al., 2025). Overall, our functional classifications showed strong concordance with independent experimental studies despite methodological differences. Complete agreement was observed for variants previously classified in a recent *SDHB* minigene assay (n = 7). Furthermore, seven of the eight variants that remained unresolved in the minigene assay due to predicted in-frame or missense effects were functionally classified by our assay, demonstrating the added value of MAVE as a complementary approach for variant interpretation (Köhler et al., 2026). Similarly, our results agreed with the two of four variants evaluated using plasmid-based biochemical complementation assays in *SDHB*-deficient HEK293 cells (Lee et al., 2025). However, I79F, I79M were classified as functionally-abnormal in our endogenous editing assay but reported as likely benign using transient plasmid expression in *SDHB*-null HEK293 cells (Lee et al., 2025) and VUS on ClinVar with multiple and single submitter respectively. These discrepancies likely reflect fundamental differences between transient overexpression systems and endogenous CRISPR-edited alleles, where native transcriptional regulation, splicing and stoichiometric assembly of complex II are preserved. These observations highlight the complementary strengths of endogenous genome editing and overexpression-based functional assays, which interrogate variant effects in different biological contexts.

Independent clinical corroboration further supports the biological relevance of our classifications by cross-referencing our results with the A5 Consortium PPGL cohort (n = 94 *SDHB*-mutant tumors, n = 67 variants) (Cerami et al., 2012; Gao et al., 2013; Flynn et al., 2025). As one example, the recurrent

pathogenic truncating variant R90* (identified in seven patients) was classified as functionally-abnormal in our assay (Flynn et al., 2025). As a novel classification, c.205G > T, p.G69C, currently a VUS in ClinVar, was classified as functionally-abnormal in our integrated MAVE within the C68-G69 constrained motif. The classification of this variant as functionally-abnormal is further supported by the observation of this variant in one patient with *SDHB*-associated paraganglioma from the A5 Consortium cohort (Flynn et al., 2025), and a somatic loss-of heterozygosity of the wildtype allele (tumor variant allele frequency 70%) accessed via cBioPortal (Cerami et al., 2012; Gao et al., 2013) which were proposed as ACMG/ClinGen codes (Aim et al., 2022). Although these observations do not constitute formal clinical validation, they provide independent orthogonal support that our functional classifications reflect biologically meaningful constraints.

An important unanswered question is whether functional assays can distinguish variants associated with hereditary PPGL from hypomorphic alleles causing recessive mitochondrial disease. Previous studies have suggested that distinct classes of *SDHB* variants underlie Leigh syndrome and inherited PPGL (Alston et al., 2012), although known Leigh variants and functional datasets remain limited. Functional analysis with succinate:fumarate ratio in *SDHB* KO HEK293 cells with plasmid-derived variant overexpression resulted in BS3_moderate for 2 Leigh variants while one variant received PS3_moderate (Lee et al., 2025). Because exon 3 contains only a single Leigh-associated variant, our study was not powered to define disease-specific functional thresholds. However, previous studies have shown that fibroblasts from patients with Leigh syndrome caused by isolated complex I deficiency exhibit pyruvate dependence in galactose-containing, pyruvate-free medium (Iannetti et al., 2018). In contrast, our experiments using galactose instead of glucose as the primary carbon source showed that *SDHB* knockout HAP1 cells failed to proliferate regardless of pyruvate supplementation, suggesting that distinct mitochondrial defects exhibit different metabolic dependencies and that functional assay media conditions should be tailored to the underlying disease biology.

Although our study was not powered to distinguish PPGL-associated from Leigh-associated alleles, the broad distribution of functionally-abnormal variants in PCA suggests that functionally-abnormal *SDHB* variants comprise a spectrum of residual function rather than a binary loss-of-function class. This is consistent with the variable functional impairment observed for p.Arg90Gln and supports the concept that quantitative differences in *SDHB* activity may contribute to phenotypic variability. Variant c.269G > A p.Arg90Gln, has conflicting ClinVar classifications (3x (likely) pathogenic, 7xVUS), and has been repeatedly observed in apparently healthy individuals (38x heterozygous, 0x homozygous in GnomAd v.4.1.1, > 20x ClinVar Accession: SCV000581189.7, and 3x SCV004832447.2) but also in at least 9 individuals with predominantly head and neck paraganglioma (Garrett et al., 2022; Bernardo-Castineira et al., 2019; Bernardo-Castineira et al., 2019, Currás-Freixes et al., 2015), permitting a very strong likelihood ratio for PS4 (Rowlands et al., 2024). Previous functional studies in yeast and HEK293 overexpression reported only a mild-to-moderate impairment (Panizza et al., 2013; Lee et al., 2025), consistent with the intermediate mean normalized functional scores (haploid: -0.52; hemizygous: -0.68) observed in both ploidy states in our assay, leading to a final PS3_strong and a likely pathogenic assessment in the automated assessment and a final pathogenic assessment considering PS4_very strong. Together,

these findings support a reduced penetrance or a hypomorphic effect rather than complete loss of function (Lee *et al.*, 2025). This model could explain that missense variants are enriched in head and neck paraganglioma (Bayley *et al.*, 2023), and showed lower succinate:fumarate ratios in the assay by (Lee *et al.*, 2025) as these tumors possibly require lower succinate:fumarate build up compared to PPGLs. Moreover, mouse models have shown that succinate accumulation alone is insufficient to initiate pheochromocytoma (Armstrong *et al.*, 2022), suggesting that tumour formation depends on the tissue of origin and additional genetic or cellular factors beyond metabolic dysfunction alone. These observations reinforce the concept that *SDHB* dysfunction exists along a continuum and that quantitative and cumulative functional measurements may ultimately improve genotype to phenotype prediction.

Several limitations should be considered with the present study. As proof-of-principle study, we focused on a single, functionally critical exon, resulting in a smaller dataset than those in larger MAVE studies spanning multiple exons or entire genes, this challenged identification of benign ground truth variants. Due to technical challenges in generating sufficient *SDHB* hemizygous cells the MAVE assay relied on a single cell clone, raising the possibility of clone-specific effects for the hemizygous model. Nevertheless, this clone showed wild-type-like proliferation, and wild-type succinate:fumarate ratios, suggesting minimal evidence of clonal artefacts. A further limitation is the exclusive use of HAP1 cells. Physiologically relevant chromaffin and neuroendocrine cell models, as well as patient-derived fibroblasts, have limited proliferative capacity (Bayley *et al.*, 2022; Maier and Westendorp, 2009), restricting their scalability for multiplex assays. Moreover, biallelic *SDHB* loss is embryonic lethal in zebrafish (Parisien-La Salle *et al.*, 2025) and mice (Lewis *et al.*, 2024), complicating large-scale functional screening *in vivo*. Nevertheless, these models could complement MAVE screening through individual variant validation under tailored media conditions. Finally, although our classifications showed strong concordance with available clinical evidence, the functional boundaries separating complete loss-of-function, hypomorphic, and mitochondrial disease-associated alleles remain incompletely defined and will require integration with larger clinical cohorts and orthogonal functional assays.

In summary, our study demonstrated that functional interpretation of *SDHB* variants is context dependent. By integrating complementary ploidy states with disease-relevant metabolic selection, this framework balances both adjusted-sensitivity, adjusted-specificity and identified cellular context dependent discordant variants that reduce the VUS burden. More broadly, we established a scalable strategy for tailored assays facilitating functional genomics of metabolically constrained disease genes that support the incorporation of multiplex functional evidence into clinical variant interpretation.

Declarations

Data availability:

MAVEDB (will be upload after publishing)

Code availability

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Conflict of interest:

AJ is a member of the ClinGen Endocrine Tumour Predisposition Variant Curation Expert Panel (<https://clinicalgenome.org/affiliation/51050/>). AJ has received honoraria as a member of the speakers' bureau for AstraZeneca.

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Figures

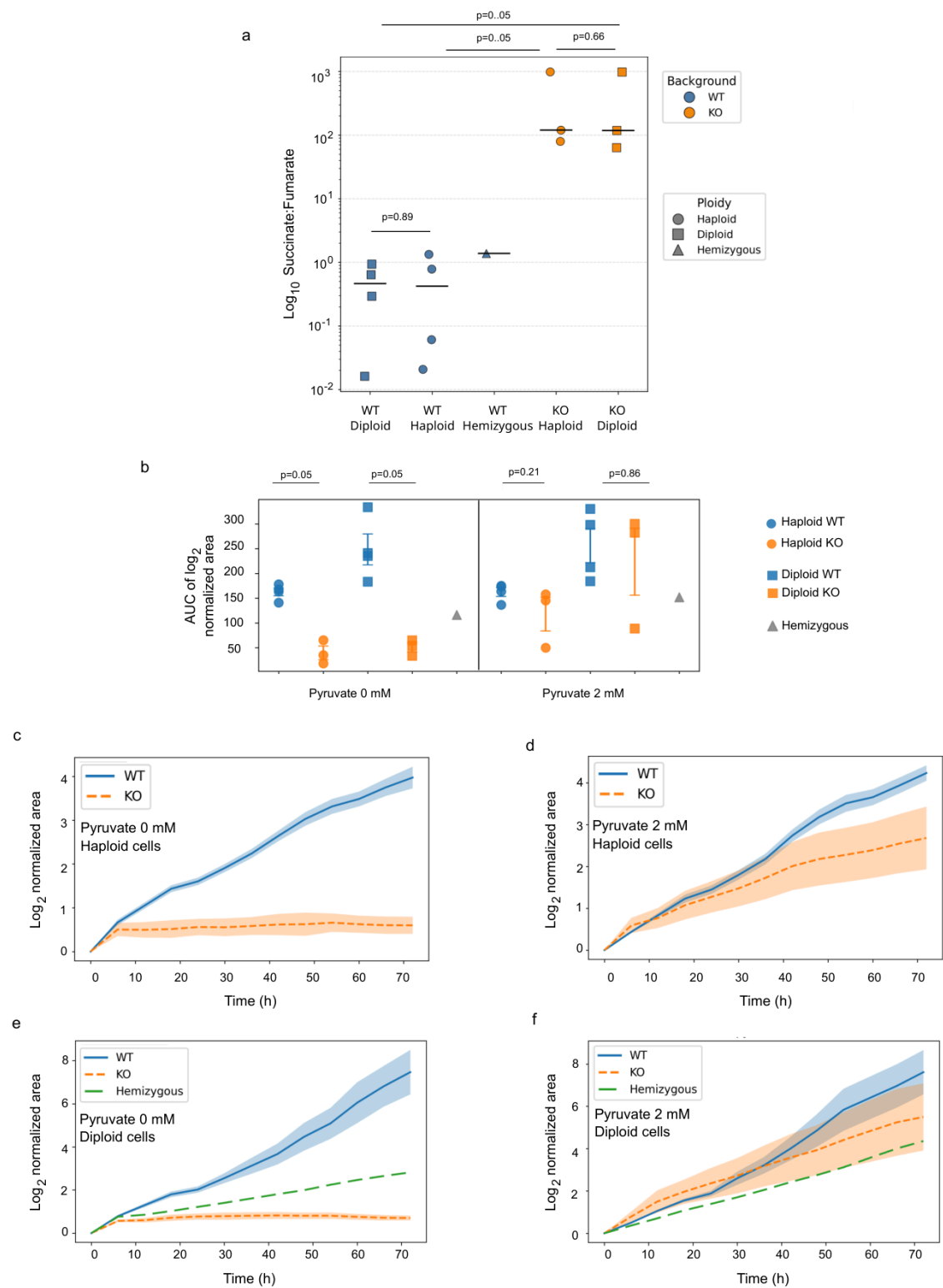


Figure 1

***SDHB* loss induces pyruvate-dependent proliferation and elevated succinate:fumarate ratios across HAP1 ploidy states.**(a) log₁₀ Succinate:fumarate ratios measured by LC-MS/MS in wild-type (WT) and knockout (KO) HAP1-LIG4KO-Cas9 cells across haploid, diploid, and hemizygous (diploid with one *SDHB*

allele deleted) backgrounds. Individual clones (WT diploid n=4 clones, WT haploid n=4 clones, WT hemizygous n=1 clone, KO haploid n=4 clones, KO diploid n=3 clones) are shown as data points with median indicated by a horizontal bar. Statistical comparisons between WT and KO within each ploidy state were performed by Mann-Whitney U test (p-values shown). (b) Area under the curve (AUC) of log₂-normalized proliferation from 0 to 72 hours in haploid, diploid, hemizygous WT and KO cells under pyruvate-free (0 mM, left panel) and pyruvate-supplemented (2 mM, right panel) conditions. Individual clones are shown with mean and standard error with WT diploid n=4 clones, WT haploid n=4 clones, WT hemizygous n=1 clone, KO haploid n=4 clones, KO diploid n=3 clones. Hemizygous cells are shown as a single data point (triangle). Statistical comparisons between WT and KO within each condition were performed by Mann-Whitney U test (p-values shown). (c, d) Mean log₂-normalized proliferation curves (solid line) with standard error (shaded area) for haploid WT (blue, solid) and KO (orange, dashed) cells under 0 mM (c) and 2 mM pyruvate (d) conditions over 72 hours. (e, f) Mean log₂-normalized proliferation curves for diploid WT (blue, solid), KO (orange, dashed), and hemizygous (green, dashed) cells under pyruvate-free (e) and 2 mM pyruvate (f) conditions over 72 hours. Shaded areas represent standard error across clones.

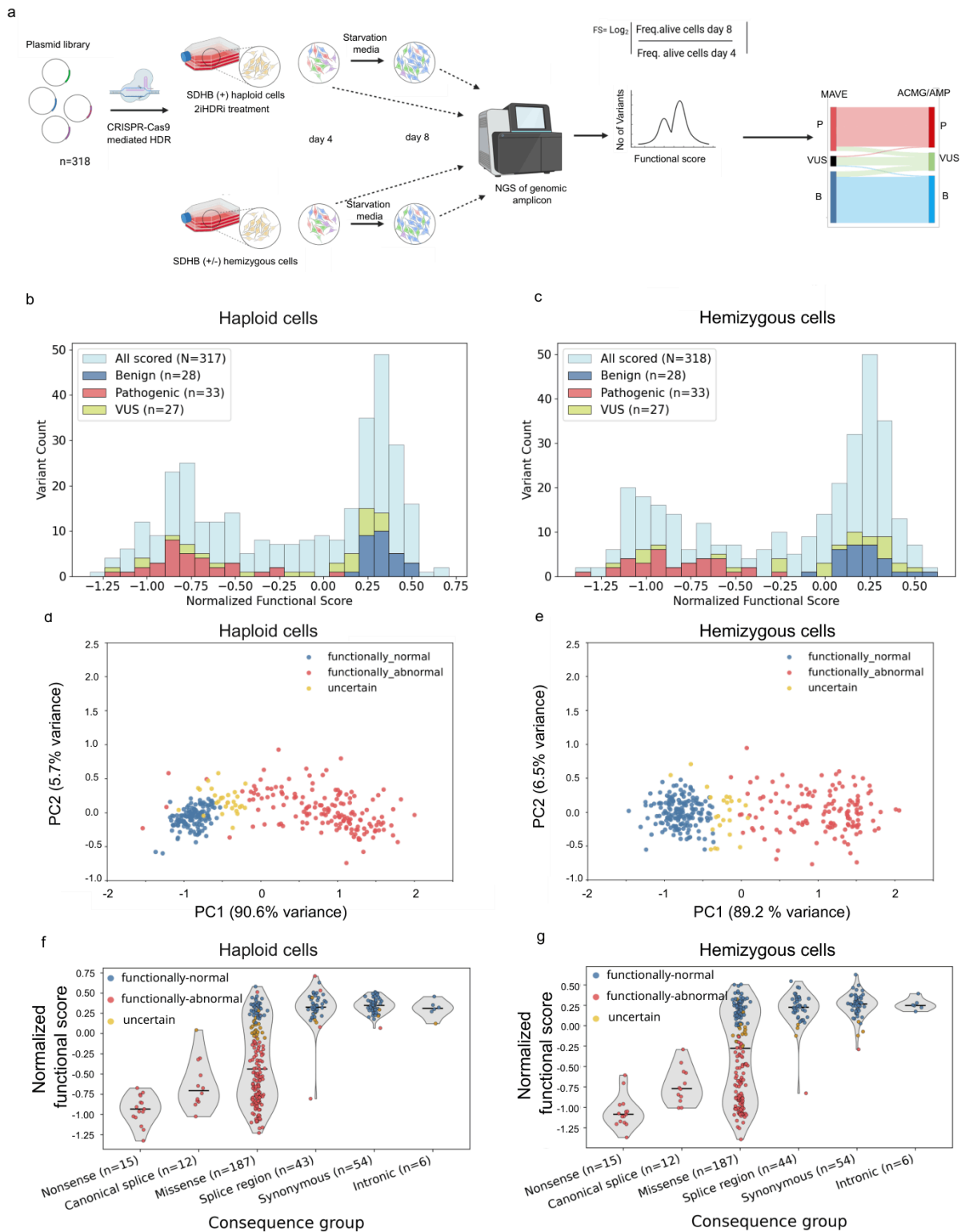


Figure 2

Multiplex functional profiling of 318 *SDHB* exon 3 variants in haploid and hemizygous cells. (a) Schematic overview of the saturation genome editing (SGE) assay. A plasmid library encoding all 318 possible single-nucleotide variants across *SDHB* exon 3 and flanking splice regions was introduced into *SDHB*(+) haploid cells treated with 2iHdRi (AZD7648 and PolQi2) and diploid *SDHB*(+/-) hemizygous HAP1-LIG4KO-Cas9 cells by CRISPR-Cas9-mediated Homology-Directed Repair (HDR). Cells were

cultured under proliferation-permissive conditions until day 4, then switched to pyruvate-free starvation media until day 8. Genomic DNA was harvested at both timepoints and variant allele frequencies quantified by NGS of the target amplicon. Functional scores were calculated as $\log_2(\text{frequency day 8} / \text{frequency day 4})$, normalized, and converted to posterior probabilities of pathogenicity using a two-component Gaussian mixture model for ACMG/AMP variant classification. The assay was performed in three sets per ploidy. (b, c) Distribution of robust-normalized functional scores in haploid (b) and hemizygous (c) cells across all 318 variants (light blue) overlaid with highly-stringent annotated variants: functionally-normal (blue, n=20), functionally-abnormal (red, n=23), and VUS (yellow, n=27). Bimodal score distributions are evident in both ploidy contexts, with high-confidence functionally-normal and functionally-abnormal variants localizing to distinct peaks. (d, e) Principal component analysis of robust-normalized functional scores in haploid (d) and hemizygous (e) cells, colored by GMM classification (functionally-normal blue, functionally-abnormal red, uncertain yellow). PC1 explains 90.6% and 89.2% of variance respectively, confirming functional classification as the dominant source of variation. Functionally-normal variants form compact clusters while functionally-abnormal and uncertain variants show greater dispersion. (f, g) Violin plots showing robust-normalized functional scores stratified by VEP-predicted consequence class in haploid (f) and hemizygous (g) cells. Individual variants are overlaid as points colored by GMM classification. Horizontal bars indicate median scores. Consequence classes shown: nonsense (n=15), canonical splice (n=12), missense (n=187), splice region (n=44), synonymous (n=54), and intronic (n=6).

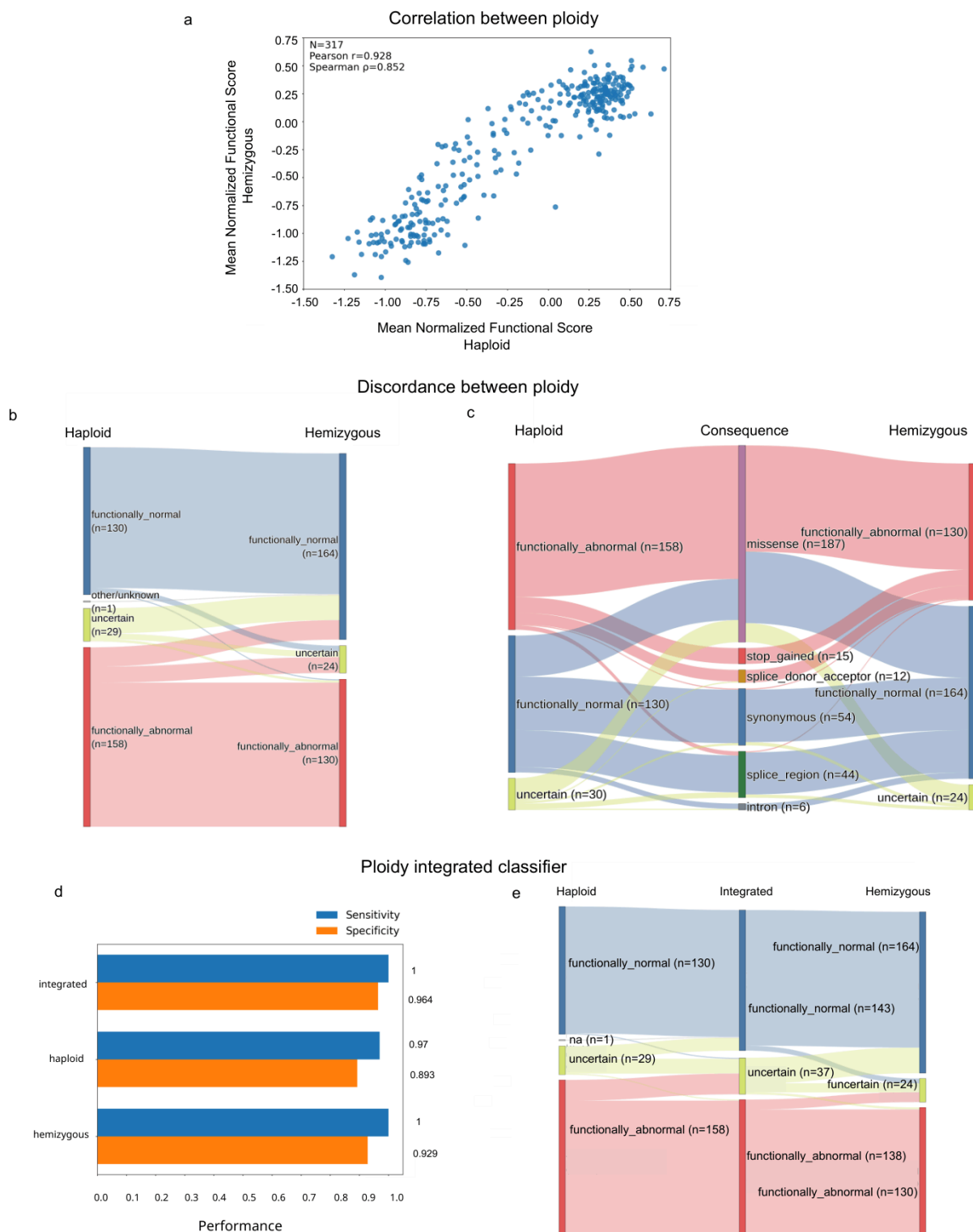


Figure 3

Discordance-aware integration of haploid and hemizygous measurements for conservative variant classification.(a) Scatter plot of mean robust-normalized functional scores for all 317 variants in haploid (x-axis) versus hemizygous (y-axis) cells. Pearson $r=0.928$ and Spearman $\rho=0.852$ indicate strong overall correlation between ploidy states, with a subset of variants showing discordant classifications. (b) Sankey plot showing classification transitions between haploid and hemizygous models across all

318 variants. Most variants were concordantly classified as functionally-normal (n=124), functionally-abnormal (n=127), uncertain (n=5). Discordant classifications are visible as crossing flows (n=62) (c) VEP-consequence Sankey plot showing classification transitions between haploid and hemizygous models. Discordance is concentrated in the missense, synonymous and splice region. (d) Modified adjusted-sensitivity and adjusted-specificity including unclassified ground-truth variants and calibrated on highly-stringent ground-truth annotations, are shown for the haploid, hemizygous, and integrated models. (e) Sankey plot showing classification transitions from flanking haploid and hemizygous to the central integrated model. Integration of haploid and hemizygous posterior probabilities using a discordance-aware odds-based framework yields 138 functionally-abnormal-like, 143 functionally-normal, and 37 uncertain variants. All sankey plots show node labels indicating the number of variants in each category, and ribbon widths are proportional to the number of variants transitioning between categories

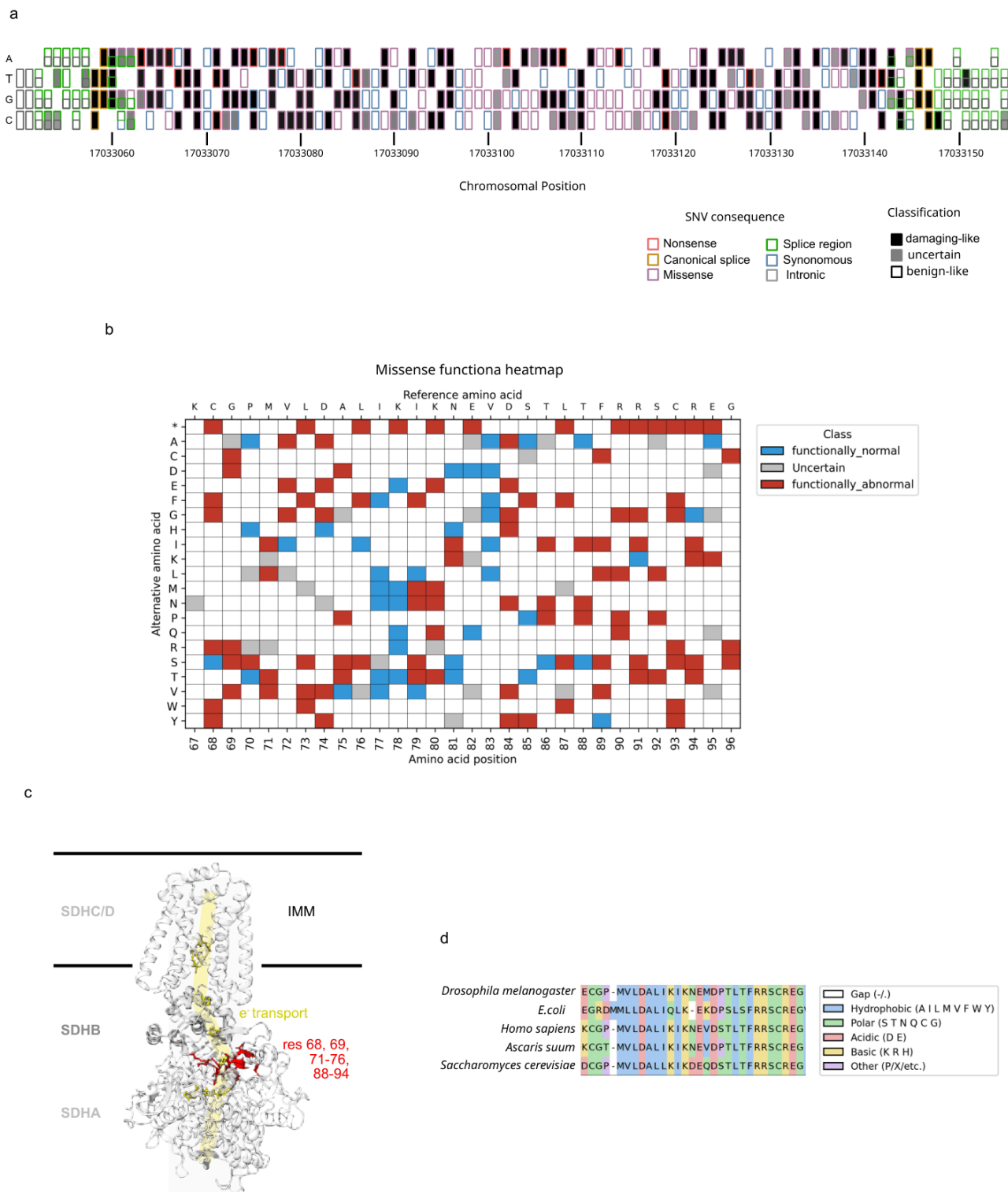


Figure 4

Nucleotide-level functional constraint map of *SDHB* exon 3 reveals structural and evolutionary determinants of variant pathogenicity. (a) Tile plot showing integrated MAVE classifications for all 318 single-nucleotide variants across *SDHB* exon 3 and flanking splice regions (chromosomal positions 17033050-17033155). Rows represent the four possible nucleotide substitutions (A, T, G, C) and columns represent genomic positions. Fill color indicates functional classification (black = functionally-

abnormal, grey = uncertain, white = functionally-normal). Outline color indicates predicted SNV consequence class (red = nonsense, yellow = canonical splice, purple = missense, green = splice region, blue = synonymous, light-grey = intronic). More than one annotation is depicted with half the perimeter of the rectangle. (b) Missense functional heatmap showing integrated MAVE classifications for all missense variants across the 30 amino acid positions encoded by exon 3 (positions 67-96). Rows represent the 19 possible alternative amino acids and columns represent amino acid positions (reference amino acid shown above each column). Fill color indicates classification (blue = functionally-normal, grey = uncertain, red = functionally-abnormal). Empty cells indicate positions where the substitution is not possible by a single nucleotide change. (c) Cryo-EM structure of human mitochondrial complex II (PDB 8GS8) showing the *SDHB* subunit (grey) with the electron transport chain indicated (yellow) was modified using ChimeraX 1.12. Residues corresponding to the three functionally constrained regions identified by MAVE are highlighted in red (residues 68-69, 71-76, and 88-94), all located in proximity to the 2Fe-2S cluster within the *SDHB* iron-sulfur domain. *SDHA* and *SDHC/D* subunits and the inner mitochondrial membrane (IMM) are indicated. (d) Multiple sequence alignment of the *SDHB* exon 3-encoded region across five species (*Drosophila melanogaster*, *Escherichia coli*, *Homo sapiens*, *Ascaris suum*, and *Saccharomyces cerevisiae*). Residues are colored by physicochemical properties (hydrophobic = blue, polar = green, acidic = red, basic = yellow, other = purple, gap = white).

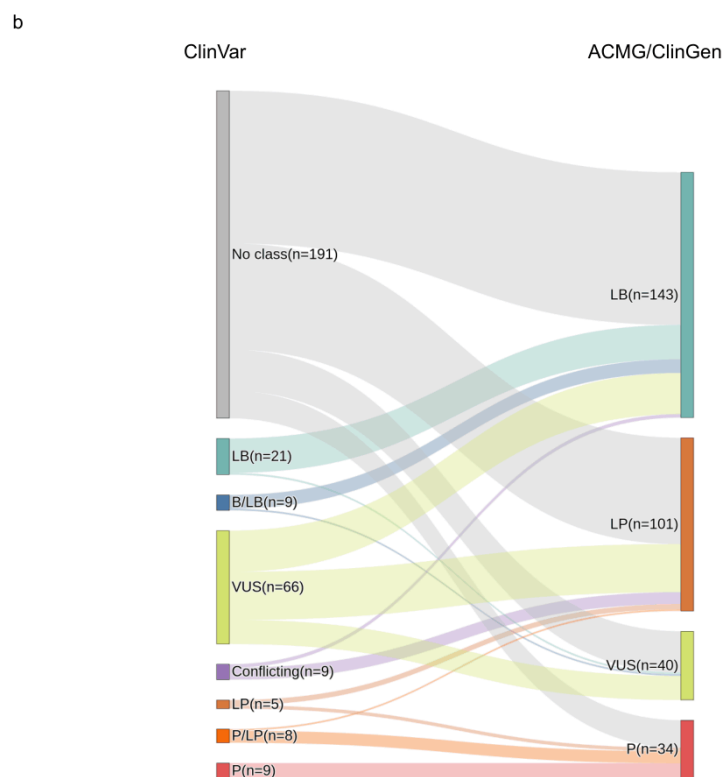
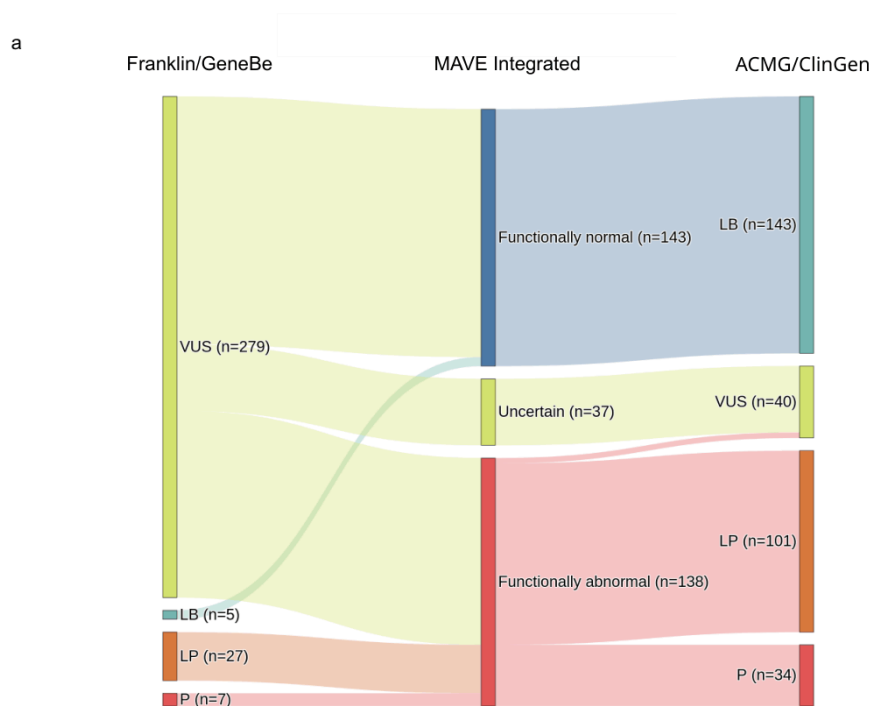


Figure 5

MAVE-derived functional evidence refines ACMG/ClinGen variant classification of *SDHB* exon 3 variants. (a) Sankey plot showing the transition from semi-automated-curated Franklin/GeneBe evidence class to ACMG/ClinGen variant classifications following incorporation of PS3/BS3 from MAVE integrated assay. (b) Sankey plot showing the relationship between prior ClinVar classifications and imputed ACMG/ClinGen classifications derived from semi-automated-curated Franklin/GeneBe evidence class

and PS3/BS3 evidence weights from MAVE integrated assay. Node labels indicate the number of variants in each category, and ribbon widths are proportional to the number of variants transitioning between categories.

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