Structural and functional consequences of succinate dehydrogenase subunit B mutations

E Kim^{1,*}, E M Rath^{3,*}, V H M Tsang^{1,2,*}, A P Duff⁴, B G Robinson^{1,2}, W B Church³, D E Benn¹, T Dwight¹ and R J Clifton-Bligh^{1,2}

¹Cancer Genetics, Kolling Institute of Medical Research, Royal North Shore Hospital, and University of Sydney, Sydney, New South Wales, Australia

²Department of Endocrinology, Royal North Shore Hospital, Sydney, New South Wales, Australia

³Faculty of Pharmacy, University of Sydney, Sydney, New South Wales, Australia

⁴Australian Nuclear Science and Technology Organisation, Lucas Heights, New South Wales, Australia

*E Kim, E M Rath and V H M Tsang contributed equally to this work

Correspondence should be addressed to E Kim

Email
edward.kim@sydney.edu.au

Abstract

Mitochondrial dysfunction, due to mutations of the gene encoding succinate dehydrogenase (SDH), has been implicated in the development of adrenal pheochromocytomas, sympathetic and parasympathetic paragangliomas, renal cell carcinomas, gastrointestinal stromal tumours and more recently pituitary tumours. Underlying mechanisms behind germline SDH subunit B (*SDHB*) mutations and their associated risk of disease are not clear. To investigate genotype–phenotype correlation of SDH subunit B (*SDHB*) variants, a homology model for human *SDH* was developed from a crystallographic structure. *SDHB* mutations were mapped, and biochemical effects of these mutations were predicted *in silico*. Results of structural modelling indicated that many mutations within *SDHB* are predicted to cause either failure of functional *SDHB* expression (p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG), or disruption of the electron path (p.Cys101Tyr, p.Pro197Arg and p.Arg242His). GFP-tagged WT *SDHB* and mutant *SDHB* constructs were transfected (HEK293) to determine biological outcomes of these mutants *in vitro*. According to *in silico* predictions, specific *SDHB* mutations resulted in impaired mitochondrial localisation and/or SDH enzymatic activity. These results indicated strong genotype–functional correlation for *SDHB* variants. This study reveals new insights into the effects of *SDHB* mutations and the power of structural modelling in predicting biological consequences. We predict that our functional assessment of *SDHB* mutations will serve to better define specific consequences for SDH activity as well as to provide a much needed assay to distinguish pathogenic mutations from benign variants.

Key Words

- ▶ succinate dehydrogenase
- ▶ SDHB
- ▶ pheochromocytoma
- ▶ paraganglioma
- ▶ functional consequences

Endocrine-Related Cancer
(2015) 22, 387–397

Introduction

Pheochromocytoma (PC) and paraganglioma (PGL) are rare neuroendocrine tumours that arise from adrenal glands and extra-adrenal sites respectively (Amar *et al.*

2005, Gimenez-Roqueplo *et al.* 2012). These tumours occur in the context of inherited neoplasia syndromes in more than 30% of cases and are linked to germline

mutations in succinate dehydrogenase subunit A (*SDHA*), *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *VHL*, *RET*, *NF1*, *TMEM127*, *MAX*, *KIF1B*, *EPAS1* (Lorenzo *et al.* 2013), *FH* (Castro-Vega *et al.* 2014 and reviewed by Dahia (2014)) and *MDH2* (Poliakov *et al.* 2014). Mutations in any of the subunits of *SDH*, also known as complex II of the mitochondrial respiratory chain (MCII), have been associated with development of PC/PGL and also renal cell cancers, gastrointestinal stromal tumours and pituitary adenomas (Dahia *et al.* 2005, Dwight *et al.* 2013, Gill *et al.* 2010, 2011). Mutations in *SDHB* are the most commonly found gene mutations in PC/PGL and are associated with younger ages at presentation, higher rates of metastases and poorer prognosis (Srirangalingam *et al.* 2008). Screening programmes have been recommended for surveillance of PC/PGL and the associated neoplasms in subjects carrying *SDHx* mutations (Lenders *et al.* 2014); however, penetrance of disease is highly variable, which therefore burdens many asymptomatic individuals with lifetime screening for low-risk events (Benn *et al.* 2006). Genotype–phenotype correlations have been difficult to establish, although the precise mechanism(s) by which these mutations cause defective SDH function has been under-studied to date. SDH is located in the inner mitochondrial membrane and matrix and consists of four subunits (A, B, C and D), which function both in the Krebs cycle and as part of the respiratory chain. In the respiratory chain, MCII functions to transport electrons to the ubiquinone pool, then to cytochrome *c* of complex III. In the Krebs cycle, SDH catalyses the conversion of succinate to fumarate (Imperiale *et al.* 2013). The SDH structure choreographs the transfer of electrons by catalysing a two-electron reduction of succinate to fumarate via a two-electron reduction of its flavin adenine dinucleotide (FAD) cofactor, finally producing two stepwise single-electron transfers to ubiquinone to reduce it to ubiquinol (Van Vranken *et al.* 2014). Two predictable consequences therefore of SDH inactivation and malfunction are accumulation of succinate and increased production of reactive oxygen species. Both outcomes may contribute to cellular accumulation of hypoxia-inducible factors (HIFs; Denko 2008, Jochmanová *et al.* 2014). The results of various yeast model studies (Barrientos 2003, Goffrini *et al.* 2009, Panizza *et al.* 2013) have indicated that selective missense germline mutations in *SDHB* can lead to significantly reduced SDH enzyme activity. Results of another recent study have indicated that *SDHB* mutations are often associated with impaired mRNA expression and reduced protein stability (Yang *et al.* 2012). *In silico* modelling of the tetrameric SDH enzyme complex from crystallographic data has tentatively

predicted a broad spectrum of biochemical consequences of *SDHB* mutations (Sun *et al.* 2005).

The aim of this study was to carefully assess the functional consequences of disease-associated *SDHB* mutations using *in silico* modelling of SDH followed by two biological assays, to assess the effects of *SDHB* mutations on mitochondrial localisation, SDH activity and interaction with SDHA. We sought to address whether different *SDHB* mutations cause distinct alterations in protein function, thereby providing a platform for more informative genotype–phenotype associations in the future.

Materials and methods

Structural modelling

We created a homology model of human SDH by mapping the human protein sequences onto a template structure in such a way as to respect the biophysical properties and interactions of the residues observed in the template. The template structure used was pig heart SDH, PDBID 1ZOY (Sun *et al.* 2005, resolution 2.40 Å) from the Protein Databank (wwPDB, Berman *et al.* 2000), which was the closest structurally determined homologue to human SDH. The homology model was created because a high-resolution experimentally determined structure of human SDH is not currently available. The Entrez protein database sequences from which the human SDH homology model was built were those of the human *SDH* genes NP_004159, NP_002991, NP_002992 and NP_002993, for SDHA, SDHB, SDHC and SDHD, respectively, and had sequence identities of 96, 98, 91 and 94%, respectively, to the pig heart sequences. This WT human SDH model was then used as the template model to build homology models for mutant human SDH proteins. Our choice of *SDHB* mutations for *in silico* structural modelling was principally based upon their association, in our experience, with severe clinical disease (i.e. young age of onset and/or malignancy; Table 1). Several additional mutations were selected from the Leiden Open Variations Database (LOVD; Fokkema *et al.* 2011), together with single-nucleotide polymorphisms in the LOVD catalogued as not associated with disease (Supplementary Tables 1 and 2, see section on supplementary data given at the end of this article). Overall, our panel of *SDHB* mutants was representative of truncating mutations, nonsense mutations and missense mutations spread throughout the exons. Two software modules, Sorting Intolerant From Tolerant (SIFT; <http://sift.jcvi.org/>; Ng & Henikoff 2001) and

Table 1 Predicted consequences of *SDHB* mutations based on structural modelling or *in silico* tools

<i>SDHB</i> ^a mutation	Predicted structural consequence ^b	Earliest age of onset (years)	Phenotype in families	<i>In silico</i>	
				SIFT	Polyphen-2
p.Arg27*	Truncation mutation – missing protein chain abrogates SDH assembly	15 ^{A,C}	EA	–	–
p.Ala43Pro	Mutation having no obvious consequence and located in a disease-associated mutation hot-spot – peripheral residue may compromise assembly factor binding	27 ^{B,C}	EA, M	D (0.03)	B (0.356)
p.Arg46Gly	Mutation having no obvious consequence and located in a disease-associated mutation hot-spot – peripheral residue may compromise assembly factor binding	29 ^{B,C}	EA, HN, M, PC	D (0.00)	D (1.0)
p.Arg46Gln	Mutation having no obvious consequence and located in a disease-associated mutation hot-spot – peripheral residue may compromise assembly factor binding	23 ^B	EA, HN, M, PC	D (0.00)	D (1.0)
p.Arg90*	Truncation mutation – missing protein chain abrogates SDH assembly	13 ^A	EA, HN	–	–
p.Cys101Tyr	Mutation breaking electron pathways – due to loss of iron–sulphur centre	10 ^C	EA	T (0.10)	D (1.0)
p.Ile127Ser	Mutation having no obvious consequence and located in a disease-associated mutation hot-spot – non-peripheral residue may compromise assembly factor binding	18 ^B	EA, M	D (0.00)	D (0.99)
p.Pro197Arg	Mutation affecting electron pathways – may result in loss of electrons and generation of reactive oxygen species	16 ^{B,C}	EA, PC	D (0.01)	D (0.999)
p.Arg242His	Mutation disrupting macromolecular assembly – compromises SDHD assembly	19 ^A	EA	D (0.00)	D (1.0)
c.88delC	Model not created for this frameshift mutation	13 ^A	M, PC, RCC	–	–
c.311delAinsGG	Model not created for this frameshift mutation	27 ^A	EA, HN, PC, RCC	–	–

SDH, succinate dehydrogenase; SDHB, SDH subunit B; PC, adrenal pheochromocytoma; EA, extra-adrenal paraganglioma; HN, head and neck paraganglioma; M, malignancy; RCC, renal cell carcinoma; B, benign; D, damaging; T, tolerated; SIFT, Sorting Intolerant From Tolerant; Polyphen-2, Polymorphism Phenotyping-2. Source of Clinical Information: ^AAustralian SDH Consortium (DE Benn, T Dwight, K Tucker and R Clifton-Bligh unpublished data); ^BInternational SDH Consortium (Benn et al. 2006) and ^CLeiden Database (Bayley et al. 2005).

^a*SDHB* reference sequence: NM_003000 (mRNA) and NP_002991 (protein).

^bPredictions for the human SDH homology model (based on pig heart SDH, PDBID 1ZOY (Sun et al. 2005)).

Polymorphism Phenotyping-2 (Polyphen-2; <http://genetics.bwh.harvard.edu/pph2/>; Adzhubei et al. 2010), were used to predict pathogenicity of *SDHB* mutants. All homology models were built using the Modeller Software (Šali & Blundell 1993, Eswar et al. 2006) version 9.8 downloaded from <http://salilab.org/modeller/>, using default parameters and the molecular dynamics feature for refining residue positions. Hydrogen bonds in the models were calculated by HBPLUS (McDonald & Thornton 1994) with the LIGPLOT Software (Wallace et al. 1995). The PyMOL Software (DeLano 2002) was used to visually inspect homology models. The Pathways Software (Beratan et al. 1991, Balabin et al. 2012) run in the VMD Software (Humphrey et al. 1996) was used to calculate the electronic coupling of segments of the electron path for the WT SDH model and for mutant models having a mutation between redox centres. Peptide bonds and metal bonds were treated as covalent bonds for the purposes of electron tunnelling mediation calculations.

Generation of *SDHB* expression vectors

A primer was designed to generate the WT *SDHB* in pEGFP-N1 (Clontech) vector: AATGGAATTCTAAAAATGGCGGC-GGTGGTCTGCACTC. GFP vector and *SDHB* insert were digested using EcoRI and BamHI (Fermentas, Waltham, MA, USA). JM109 competent cells (Promega) were added to ligation reactions of empty vector and *SDHB* insert that were then incubated on agar plates at 37 °C overnight. Colonies from agar plates were amplified using PCR and then sequenced for confirmation. Primers were prepared (Supplementary Tables 3, 4 and 5, see section on supplementary data given at the end of this article) in order to perform site-directed mutagenesis according to the manufacturer's instructions using the Quickchange Lightning Site-Directed Mutagenesis Kit (Agilent Stratagene, Santa Clara, CA, USA) to generate *SDHB* mutants (p.Arg27*, p.Ala43Pro, p.Arg46Gly, p.Arg46Gln, p.Arg90*, p.Cys101Tyr, p.Ile127Ser, p.Pro197Arg, p.Arg242His,

c.88delC and c.311delAinsGG). Sanger sequencing was performed on the amplified products to confirm the presence of the mutation.

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in DMEM (Life Technologies) with 10% fetal bovine serum (Life Technologies) at 37 °C and 5% CO₂. Rat PC cells (PC12 and ATCC) were cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and 5% horse serum (Life Technologies). Cells (passages 15–23) were plated at a density of 1.0×10^6 cells/25 cm² flasks and allowed to settle overnight. WT *SDHB*, mutant *SDHB* and empty vector DNA (7.5 µg) were mixed with 15 µl Lipofectamine 2000 (Life Technologies) and then at room temperature for 15 min in 650 µl Opti-MEM (Life Technologies). The mixtures were then used to transfect cells with replenished serum-free DMEM for 24 h (incubated at 37 °C and 5% CO₂). The cells were washed with PBS and lysed using either a subcellular fractioning kit or immunoprecipitation (IP) lysis buffer.

Subcellular localisation

Membrane fractions containing mitochondrial proteins were extracted using a Qproteome cell compartment kit according to the manufacturer's instructions (Qiagen). Membrane protein lysate was homogenised by brief sonication, and protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA).

Immunoprecipitation

Dynabeads M-280 sheep anti-mouse IgG (Life Technologies) was incubated with either a mouse IgG antibody (dilution 1:2000, Thermo-Fisher, Waltham, MA, USA) for negative control or a GFP monoclonal mouse antibody (dilution 1:2000, Roche) for 2 h before washing, then it was incubated overnight with cell extracts in IP lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 0.1% Triton X-100) at 4 °C under gentle rotation. Proteins not associated with GFP-tagged SDHB were removed (3 × 10 min gentle agitation washes) from the beads using IP lysis buffer with a higher salt concentration (500 mM NaCl). Fresh IP lysis buffer was then re-introduced to the beads to prevent drying and degradation.

Western immunoblotting

Cell lysates were sonicated, mixed with NuPAGE LDS sample buffer and dithiothreitol and then separated by SDS-PAGE (4–12% NuPAGE Bis-Tris gels, Invitrogen) under reducing conditions. Proteins were transferred to nitrocellulose membranes and the membrane blocked with 5% skim milk (in TBST) for 1 h at room temperature. The membranes were probed with the following antibodies: GFP (dilution 1:2000, Roche (11814460001)), SDHA (dilution 1:1000, Abcam (ab14715), Cambridge, UK), MT-CO2 (dilution 1:2000, Abcam (ab3298)) and GAPDH (dilution 1:5000, Cell Signaling (D16H11), Danvers, MA, USA), and incubated overnight at 4 °C. Immunoblots were washed three times with TBST for 5–10 min and incubated with the relevant secondary antibody conjugated to HRP. Blots were then washed (three times in TBST for 5 min) and protein detected (ECL Plus Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK)) on a LAS-3000 (Fujifilm, Brookvale, NSW, Australia). Quantitation was performed using the Multi-Gauge 3.11 Software (Fujifilm).

SDH activity assay

The decrease in absorbance due to reduction of the artificial electron acceptor dichlorophenolindophenol (DCPIP) linked to succinate was measured as SDH converting succinate to fumarate. An assay agent containing 25 mM potassium phosphate buffer, 5 mM MgCl₂, pH 7.2, 20 mM sodium succinate, 50 µM DCPIP, 2 mM KCN, 2 µg/ml antimycin A, 2 µg/ml rotenone (in ethanol) and water was prepared for 500 µl reaction volume/sample. The reaction mixture was incubated at 30 °C for 10 min to equilibrate, and then pre-incubated with GFP IP beads for 10 min at 30 °C (Kirby *et al.* 2007). The reaction mixture was separated from the magnetic bead and placed into a 48-well plate, with baseline absorbance measured at a wavelength of 600 nm for 3 min before being mixed with beads again. An additional 6 µl of 5 mM DCPIP was then added to the bead/reaction mixture to initiate the enzyme-catalysed reaction, with reduction of DCPIP measured by decrease in absorption at 600 nm (extinction coefficient of 19.1/mM per cm) at 2, the 7 and 12 min time points. Following this, the activity of the SDH complex (pulled down by GFP) was determined by normalising changes in optical density over time ($\Delta OD/5$ min and $\Delta OD/10$ min) with relative densitometry of SDHB-GFP eluted from the magnetic beads relative to the WT.

Statistical analysis

Experiments were performed in triplicate at least three times unless otherwise stated. Statistical analysis of the data was performed using ANOVA or repeated-measures ANOVA where appropriate. Differences between groups were evaluated by Fisher's protected least significant difference test and results were considered significant if $P < 0.05$. The statistical package StatView for Windows version 5.0 (SAS Institute, Inc., Cary, NC, USA) was used. Results are expressed as mean $\pm$ s.e.m. unless otherwise stated.

Results

In silico structural modelling of SDHB mutations

The human WT SDH model is shown in Fig. 1 and has a root mean square deviation of 0.18 Å with the porcine SDH template for all atoms, or 0.12 Å for backbone atoms. *In silico* analysis using only the models (hydrogen bond and electron path calculations, and visual inspection; refer to Table 1, Supplementary Table 1 and depictions in Supplementary Fig. 2, see section on supplementary data given at the end of this article) led to the identification of the following distinct types of mutations.

Truncation mutations As subunit B is structurally the central component of the SDH complex, the complex cannot be assembled when SDHB is significantly truncated or missing.

Mutations disrupting macromolecular assembly Loss of hydrogen bonding, loss of salt bridges or gain of repulsive charges between subunit B and subunits C or D may destabilise assembly of subunits C and D, leading to loss of electron pathways.

Mutations breaking electron pathways Loss of an iron–sulphur centre due to loss of bonding to the iron–sulphur centre will result in lost electrons generating reactive oxygen species and failure to reduce ubiquinone.

Mutations affecting electron pathways The choreography of transferring two electrons from succinate to arrive in a stepwise sequence at ubiquinone may be perturbed in those mutants that have shortened or lengthened electron paths, resulting in generation of reactive oxygen species caused by lost electrons.

Mutations having no obvious consequences and located in a disease-associated mutation ‘hot-spot’ Some disease-associated mutations did not have obvious structurally deleterious effects and are

located in a disease-associated mutation ‘hot-spot’ at the protein surface where a finger of subunit C binds to the surface of subunit B (pictured in Supplementary Fig. 3, see section on supplementary data given at the end of this article). This leads us to speculate that this ‘hot-spot’ could be involved in binding of an assembly factor, such as an SDHB cofactor insertion or maturation factor (Van Vranken et al. 2014), whose binding to the SDH complex is compromised by these mutations.

Figure 1

Human WT SDH homology model showing secondary protein structure (helices denote α -helical secondary structure and ribbons denote β -sheets). Subunit A (green) is sitting on subunit B (cyan) and together they comprise the soluble component, which is sitting on subunits C (magenta) and D (yellow) that are adjacent to each other in the membrane. Inside the complex is a path of redox factors (spheres, carbon atoms in orange) starting at FAD, passing through iron–sulphur centres and ending at ubiquinone. Other views of this model appear in Supplementary Fig. 1 and the locations of mutations studied are shown in Supplementary Figs 2 and 3.

Impaired mitochondrial localisation of mutant SDHB *in vitro* is mutation-specific

In order to validate these *in silico* predictions that *SDHB* mutations could have diverse functional consequences, we then generated GFP-tagged *SDHB* constructs containing disease-associated mutations (Table 1). We chose mutations that, in our experience, have been associated with severe clinical manifestations (young age of onset, and/or malignant disease). The GFP-*SDHB* vectors were introduced into HEK293 cells, and initially studied for mitochondrial localisation. As expected, *SDHB* truncating mutations (p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG) were not detected in whole-cell lysates (Fig. 2A) or in mitochondrial preparations (Fig. 2B). A strikingly different picture was seen with missense *SDHB* mutations. All our missense mutant *SDHB* constructs (p.Ala43Pro, p.Arg46Gly, p.Arg46Gln, p.Ile127Ser, p.Pro197Arg, p.Cys101Tyr and p.Arg242His) were present in whole-cell lysates at levels similar to those for the WT *SDHB* construct (Fig. 2A). When mitochondrial-enriched preparations were analysed using anti-GFP western blots (and corrected for content of mitochondrial protein MT-CO2 as an internal control), some mutant SDHBs were present at concentrations not significantly different from those for the WT *SDHB* (p.Ile127Ser, p.Pro197Arg and p.Arg242His; Fig. 2B), whereas other mutant SDHBs exhibited markedly lower mitochondrial expression (p.Ala43Pro, p.Arg46Gly and p.Arg46Gln) ($n=4$, $*P<0.05$).

SDHB mutations differentially reduce SDH enzymatic activity

The enzymatic function of SDH complexes containing only transfected *SDHB* (WT or mutant) was measured by means of anti-GFP antibody pulldown that specifically precipitated GFP-containing complexes while removing endogenous *SDHB*. Successful IP was confirmed by western blot (Fig. 3A). The rate of conversion from succinate to fumarate was then assessed in IPs by means of a modified Kirby protocol (Kirby *et al.* 2007), corrected for densitometric quantity of GFP-*SDHB* on western blot (relative to WT), and data are shown in Fig. 3B. No SDH activity was detected in IPs from cells transfected with GFP vector alone, nor in IPs from cells transfected with truncating *SDHB* mutations p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG. Enzymatic activities of SDH complexes containing the missense *SDHB* mutants p.Ala43Pro, p.Arg46Gln and p.Arg242His were moderately reduced to approximately 50% relative to WT (Fig. 3B).

Figure 2 HEK293 cells were transiently transfected (24 h) with GFP-tagged *SDHB* (WT or mutants) or empty vector (pEGFP-N1) and the whole-cell fraction (A) and membrane fraction (B) were isolated. Samples were probed for GAPDH (whole-cell endogenous protein), MT-CO2 (mitochondrial membrane protein marker) and GFP on western blots. (A) GFP-tagged *SDHB* mutants p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG were not detected in the whole-cell fraction ($*P<0.05$, compared with WT), indicating nonsense-mediated mRNA decay or protein degradation. (B) GFP-tagged *SDHB* mutants p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG had baseline readings ($*P<0.05$). Additionally, compared with WT *SDHB*, *SDHB* mutants p.Ala43Pro, p.Arg46Gly and p.Arg46Gln, and p.Cys101Tyr showed significantly reduced localisation to mitochondria ($*P<0.05$). Results represent four independent experiments, with error bars indicating s.e.m., and ANOVA was used to test for statistical significance.

The p.Arg46Gly and p.Cys101Tyr mutants were found to be severely disruptive to SDH activity with the rate of metabolite conversion almost at baseline levels (Fig. 3B). Conversely, the reduction in SDH activity associated with either the p.Ile127Ser or the p.Pro197Arg mutation was not significantly different from that associated with the WT (Fig. 3B).

When the results of SDH assays were correlated with those from mitochondrial localisation, we found an excellent agreement for most mutations (Fig. 4). Notably, p.Arg242His was an obvious outlier with significantly reduced SDH activity despite near-normal mitochondrial abundance (Fig. 4).

Figure 3

Activity of SDH isolated through pulldown by GFP-tagged SDHB. SDH complexes with transfected GFP-tagged SDHB in HEK293 cells were isolated through co-immunoprecipitation (IP) using magnetic beads. Enzyme activities were determined by measuring the reduction of the artificial electron acceptor DCPIP during conversion of succinate to fumarate using an absorbance assay (Kirby *et al.* 2007) over time, relative to WT and then normalised by pulled down SDHB-GFP western blotting. (A) Western blot of SDHB-GFP (WT and mutants) or empty vector (pEGFP-N1) after GFP IP. (B) GFP-tagged SDHB mutants, p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG, resulted in the absence of SDHB attached to GFP and SDH activities were not detected. While GFP-tagged SDHB mutants, p.Ala43Pro, p.Arg46Gln and p.Arg242His, significantly reduced SDH activity ($*P < 0.05$), the greatest effect with respect to reduction of SDH activity was observed with p.Arg46Gly and p.Cys101Tyr ($*P < 0.05$). Results represent four independent experiments, with error bars indicating s.e.m., and ANOVA was used to test for statistical significance.

To assess whether tissue-specific factors may be important for determining mutation-specific SDH function, we repeated transfection studies of the same GFP-SDHB vectors in the rat PC cell line PC12. SDH activity in IPs from these cells was strikingly similar to that in HEK293 cells (Supplementary Fig. 4, see section on supplementary data given at the end of this article).

SDHB mutations differentially reduce association with SDHA

SDHA is normally non-covalently associated with the SDHB subunit on the catalytic end of the SDH complex. The effects of SDHB mutations on association between these two subunits were investigated by means of anti-GFP IP from whole-cell lysates, followed by western blotting with anti-SDHA (Fig. 5) and then correcting for densitometric quantity of GFP-SDHB (Fig. 5). As expected, IPs

from cells transfected with truncating SDHB mutations p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG did not express any SDHA (Fig. 5). SDHA was detected in IPs from cells transfected with missense SDHB mutant constructs in amounts similar to those for GFP-WT SDHB IPs, except for p.Cys101Tyr that had significantly lower SDHA association ($n = 4$, $*P < 0.05$).

Discussion

SDH dysfunction, arising as a result of mutations within any of the four SDH subunits, has been correlated with a range of diseases, including hereditary PC and PGL syndromes, renal cell carcinoma, gastrointestinal stromal tumours and Leigh syndrome (Horvath *et al.* 2006, Vicha *et al.* 2014). In general, PC and PGL tumours containing germline SDH gene mutations exhibit markedly diminished SDH function (Gimenez-Roqueplo *et al.* 2002). Two predictable consequences of SDH inactivation are accumulation of succinate and increased production of reactive oxygen species, and both outcomes may contribute to the cellular accumulation of HIFs that is thought to mediate aberrant biological processes leading to tumour formation (Selak *et al.* 2005). However, functional consequences have not been precisely determined for most SDHB mutations and this has fundamentally limited the ability to accurately correlate between genotype and phenotype in hereditary PGL syndrome type 4.

Herein, we used various techniques to investigate the functional outcomes of specific SDHB mutations associated with hereditary PC and PGL. First, structural modelling of SDH was performed using X-ray crystallography data to predict the effect of mutations on protein stability, changes

Figure 4

Correlation between mitochondrial expression of SDHB and SDH activity. Mean values (percentage relative to WT) of mitochondrial expression of GFP-tagged SDHB were plotted against mean values (percentage relative to WT) of SDH activity. A trendline encompassing investigated SDHB mutants (excluding p.Arg242His) was added to indicate the direct relationship between SDH activity and SDHB mitochondrial localisation.

Figure 5

Association of mutant SDHB with WT SDHA. SDH complexes pulled down by GFP-tagged SDHB following 24 h of transfection in HEK293 cells were immunoblotted for SDHA and GFP. The p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG mutants show complete disassociation with SDHA due to absence of SDHB–GFP protein. Densitometry results of SDHA blots were normalised to SDHB–GFP proteins that were bound by the process of co-immunoprecipitation and showed that p.Ala43Pro, p.Arg46Gly, p.Arg46Gln and p.Cys101Tyr *SDHB* mutants significantly reduced the association between SDHB and SDHA (* $P < 0.05$). Results represent four independent experiments, with error bars indicating S.E.M., and ANOVA was used to test for statistical significance.

in spatial dimensions affecting charges and binding sites *in silico*. These predictions were then compared against assessment of intracellular localisation and biochemical assays after transfecting HEK293 cells with various *SDHB* mutants *in vitro*. We developed a novel means for assaying SDH function in human cells transfected with *SDHB* mutant constructs, by specifically immunoprecipitating mutant-containing SDH complexes from whole cell lysates. Our results indicate that *SDHB* mutations can result in three biological consequences: i) loss of protein due to nonsense-mediated mRNA decay and/or accelerated degradation; ii) loss of correct mitochondrial targeting or iii) loss of enzyme function due to changes in specific active sites.

Structural modelling of disease-associated *SDHB* mutations p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG predicted nonsense-mediated mRNA decay or truncation of the protein. Complete loss of SDHB expression in both cytosolic and mitochondrial compartments associated with truncating mutations from our *in vitro* data indicates either the occurrence of nonsense-mediated decay or that the truncated proteins were unstable and degraded. In keeping with the localisation data, no measurable SDH activity was present in IPs containing these truncating mutants.

The second group of disease-associated *SDHB* mutations (p.Ala43Pro, p.Arg46Gly and p.Arg46Gln)

were those shown to exhibit reduced expression of mitochondrial SDHB despite being expressed at normal levels in cytoplasm. Again, in keeping with this localisation data, SDH activity was impaired by these mutations (but not totally abolished, as observed with the truncating mutations). Interestingly, p.Arg46Gly demonstrated severely impaired SDH activity, whereas p.Arg46Gln had a more modest effect on SDH function. These results indicate that these two different mutations have different effects on the SDH catalytic site.

The third group of disease-associated mutations (p.Cys101Tyr, p.Pro197Leu and p.Arg242His) were those associated with apparently normal mitochondrial SDHB abundance but impaired functional activity. Our structural modelling of these mutants led to the prediction of loss of the electron path (p.Cys101Tyr), changes to the electron path (p.Pro197Leu) or destabilisation of SDH structure leading to loss of the electron path (p.Arg242His). *SDHB* mutants p.Cys101Tyr and p.Arg242His were both associated with significantly reduced SDH activity when compared with the WT. Using our *in silico* results we predicted loss of bonding to the 2Fe–2S centre at p.Cys101, which is more tolerant to amino acid substitution than the other two (4Fe–4S), (3Fe–4S) domains of *SDHB* (Werth *et al.* 1990, Iverson *et al.* 2012). Mutation at p.Arg242 was predicted to disrupt hydrogen bonds between SDH subunits, thereby reducing their biochemical efficiency and perhaps could also explain the loss of function in an otherwise fully compartmentalised SDH complex. The effect of p.Pro197Leu on measurable SDH activity in our assay was not significant, although our structural modelling predicts that this mutation has a clear effect on electron transport; it is intriguing to speculate that this mutation may actually discriminate between these two functions of the SDH complex.

In order to confirm that reduced expression of mitochondrial SDHB was in fact associated with loss of SDH assembly, we then examined the interaction of SDHB (WT or mutant) and SDHA. Our results were entirely consistent with the hypothesis that some mutations (p.Ala43Pro, p.Arg46Gly, p.Arg46Gln and p.Cys101Tyr) produce proteins that are normally stable but have impaired trafficking to mitochondria and/or assembly into mature SDH. The mechanism(s) by which this occurs remains speculative. Recent studies (Lane *et al.* 2014, Maio *et al.* 2014, Na *et al.* 2014) have highlighted the importance of iron–sulphur (Fe–S) clusters that are incorporated into *SDHB* after engaging with two LYR motifs, including p.Arg46 and p.Arg242. This indicates

that mutation of these residues may cause failed LYR-mediated assembly of Fe-S clusters.

Reports of two previous studies have described functional impairments associated with disease-associated *SDHB* mutations. Using transfection experiments in HeLa cells, Yang *et al.* (2012) proposed that the major effect of missense mutations was on protein stability, which we also found for some mutations (p.Ala43Pro and p.Arg46Gln). However, in contrast to Yang *et al.*'s (2012) suggestion that subcellular localisation and complex formation are not affected by *SDHB* mutations, our results also convincingly indicated that some mutations either did not affect protein stability and instead selectively impaired SDH function (p.Arg242His), or had an effect on function that was disproportionate to the effect on mitochondrial SDHB expression (p.Cys101Tyr and p.Arg46Gly). In this respect, our findings were in agreement with the results of Panizza *et al.* (2013), who also found that *SDHB* mutants p.Arg46Leu, p.Cys101Tyr and p.Arg242Ser were severely impaired for SDH biochemical function in yeast. While Panizza *et al.* (2013) studied the biochemical function of *SDHB* mutants in *sdh2*null yeast cells, our novel approach of selectively immunoprecipitating mutant-containing SDH complexes allowed us to study the functional impact of these *SDHB* mutations in human cells.

The results of this study have not enabled the identification of the biological effects of p.Ile127Ser. Structural modelling of this mutation did not identify an obvious pathological consequence. There was no clear effect on either mitochondrial SDH assembly or SDH activity, and the interaction between this mutant protein and SDHA appeared to be preserved. SDH activity was similarly preserved in p.Ile127Ser-containing IPs from PC12 cells. Despite this seemingly normal SDH function, in our experience, this mutation is recurrently associated with severe disease in multiple (unrelated) families. We therefore propose the hypothesis that p.Ile127Ser disrupts some aspects of SDHB function other than SDH activity (e.g. electron transport) and that further studies to precisely define pathogenic mechanisms for this mutant will be important.

Many studies (Gimenez-Roqueplo *et al.* 2001, 2002, Lima *et al.* 2007) have emphasised the importance of SDH structural integrity, and that specific mutations in any of the SDH subunits would disrupt assembly, anchorage to the mitochondrial membrane, surface exposure of binding sites and stabilisation of the complex. Our results indicate that particular *SDHB* mutations may have specific effects on SDH structure and/or function as well as indicating a correlation between *SDHB* mitochondrial expression and

SDH activity (Fig. 4). Our results provide a platform upon which future genotype–phenotype correlations might be properly identified, by accurately characterising their mutational consequences. Furthermore, this study reveals new insights into the effects of *SDHB* mutations and the power of structural modelling in predicting their biological consequences. In combination with recent metabolomics profiling (Richter *et al.* 2014), we predict that our functional assessment of *SDHB* mutations will serve to better define specific consequences on SDH activity as well as to provide a much needed assay to distinguish pathogenic mutations from benign variants. In summary, pathogenic mutations of *SDHB* have been shown to be associated with diverse structural and functional consequences broadly categorised into three groups: those that cause loss of SDHB protein, those that impair SDHB mitochondrial localisation and those that specifically disrupt enzyme sites.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0099>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Hillcrest Foundation (Perpetual Trustees) and the Pheo-Para Alliance.

Author contribution statement

E Kim conducted *in vitro* studies, performed data analyses and drafted the manuscript. V H M Tsang carried out selection and generation of *SDHB* expression vectors. E M Rath, A P Duff and W B Church carried out *in silico* studies of *SDHB*, providing predicted biological consequences as well as critically revising the manuscript. B G Robinson provided intellectual input and critically revised the manuscript. D E Benn participated in the design of the study, provided intellectual input and critically revised the manuscript. T Dwight participated in the design of the study, provided intellectual input and critically revised the manuscript. R J Clifton-Bligh conceived, designed and coordinated the study and critically revised the manuscript.

Acknowledgements

The authors are grateful to Professor David Thorburn (Murdoch Childrens Research Institute, Melbourne) for advice on biochemical assays of SDH activity and to Dr Martyn Bullock (Kolling Institute, RNSH) for expert help with immunoprecipitation assays. They also thank our co-investigator Dr Kathy Tucker in the Australian SDH Consortium, and all the patients and families who are part of this ongoing study.

References

- Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS & Sunyaev SR 2010 A method of server for predicting damaging missense mutations. *Nature Methods* **7** 248–249. (doi:10.1038/nmeth0410-248)
- Amar L, Bertherat J, Baudin E, Ajzenberg C, Paillerets B, Chabre O, Chamontin B, Delemer B, Giraud S, Murat A *et al.* 2005 Genetic testing in pheochromocytoma or functional paraganglioma. *Journal of Clinical Oncology* **23** 8812–8818. (doi:10.1200/JCO.2005.03.1484)
- Balabin IA, Hu X & Beratan DN 2012 Exploring biological electron transfer pathway dynamics with the Pathways plugin for VMD. *Journal of Computational Chemistry* **33** 906–910. (doi:10.1002/jcc.22927)
- Barrientos A 2003 Yeast models of human mitochondrial diseases. *International Union of Biochemistry and Molecular Biology* **55** 83–95. (doi:10.1002/tbmb.718540876)
- Bayley J, Devilee P & Taschner PE 2005 The SDH mutation database: an online resource for succinate dehydrogenase sequence variants involved in pheochromocytoma, paraganglioma and mitochondrial complex II deficiency. *BMC Medical Genetics* **6** 39–45. (doi:10.1186/1471-2350-6-39)
- Benn DE, Gimenez-Roqueplo AP, Reilly JR, Bertherat J, Burgess J, Byth K, Croxson M, Dahia PL, Elston M, Gimm O *et al.* 2006 Clinical presentation and penetrance of pheochromocytoma/paraganglioma syndromes. *Journal of Clinical Endocrinology and Metabolism* **91** 827–836. (doi:10.1210/jc.2005-1862)
- Beratan DN, Betts JN & Onuchic JN 1991 Protein electron transfer rates set by the bridging secondary and tertiary structure. *Science* **252** 1285–1288. (doi:10.1126/science.1656523)
- Berman H, Henrick K & Nakamura H 2000 Announcing the worldwide Protein Data Bank. *Nature Structural & Molecular Biology* **10** 980. (doi:10.1038/nsb1203-980)
- Castro-Vega LJ, Buffet A, De Cubas AA, Cascon A, Menara M, Khalifa E, Amar L, Azriel S, Bourdeau I, Chabre O *et al.* 2014 Germline mutations in *FH* confer predisposition to malignant pheochromocytomas and paragangliomas. *Human Molecular Genetics* **23** 2440–2446. (doi:10.1093/hmg/ddt639)
- Dahia PL 2014 Pheochromocytoma and paraganglioma pathogenesis: learning from genetic heterogeneity. *Nature Reviews. Cancer* **14** 108–119. (doi:10.1038/nrc3648)
- Dahia PL, Ross KN, Wright ME, Hayashida CY, Santagata S, Barontini M, Kung AL, Sanso G, Powers JF, Tischler AS *et al.* 2005 A HIF1 α regulatory loop links hypoxia and mitochondrial signals in pheochromocytomas. *PLoS Genetics* **1** 72–80. (doi:10.1371/journal.pgen.0010008)
- DeLano WL 2002 The PyMOL Molecular Graphics System (DeLano Scientific LLC, San Carlos, CA, USA, <http://www.pymol.org>).
- Denko NC 2008 Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nature Reviews. Cancer* **8** 705–713. (doi:10.1038/nrc2468)
- Dwight T, Benn DE, Clarkson A, Vilain R, Lipton L, Robinson BG, Clifton-Bligh RJ & Gill AJ 2013 Loss of *SDHA* expression identifies *SDHA* mutations in succinate dehydrogenase-deficient gastrointestinal stromal tumors. *American Journal of Surgical Pathology* **37** 226–233. (doi:10.1097/PAS.0b013e3182671155)
- Eswar N, Webb B, Marti-Reimon MA, Madhusudhan MS, Eramian D, Shen MY, Pieper U & Sali A 2006 Comparative protein structure modeling using Modeller. *Current Protocols in Bioinformatics* **15** 5.6.1–5.6.30. (doi:10.1002/0471250953.bi0506s15)
- Fokkema IF, Taschner PE, Schaafsma GC, Celli J, Laros JF & den Dunnen JT 2011 LOVD v.2.0: the next generation in gene variant databases. *Human Mutation* **32** 557–563. (doi:10.1002/humu.21438)
- Gill AJ, Chou A, Vilain R, Clarkson A, Lui M, Jin R, Tobias V, Samra J, Goldstein D, Smith RC *et al.* 2010 Immunohistochemistry for SDHB divides gastrointestinal stromal tumors (GISTs) into 2 distinct types. *American Journal of Surgical Pathology* **34** 636–644. (doi:10.1097/PAS.0b013e3181d6150d)
- Gill AJ, Pachter NS, Chou A, Young B, Clarkson A, Tucker KM, Winship IM, Earls P, Benn DE, Robinson BG *et al.* 2011 Renal tumors associated with germline *SDHB* mutation show distinctive morphology. *American Journal of Surgical Pathology* **35** 1578–1585. (doi:10.1097/PAS.0b013e318227e7f4)
- Gimenez-Roqueplo AP, Favier J, Rustin P, Mourad J, Plouin P, Corvol P, Rotig A & Jeunemaitre X 2001 The R22X mutation of the *SDHD* gene in hereditary paraganglioma abolishes the enzymatic activity of complex II in the mitochondrial respiratory chain and activates the hypoxia pathway. *American Journal of Human Genetics* **69** 1186–1197. (doi:10.1086/324413)
- Gimenez-Roqueplo AP, Favier J, Rustin P, Mourad JJ, Plouin PF, Corvol P, Rotig A & Jeunemaitre X 2002 Functional consequences of a *SDHB* gene mutation in an apparently sporadic pheochromocytoma. *Journal of Clinical Endocrinology and Metabolism* **87** 4771–4774. (doi:10.1210/jc.2002-020525)
- Gimenez-Roqueplo AP, Dahia PL & Robledo M 2012 An update on the genetics of paraganglioma, pheochromocytoma, and associated hereditary syndromes. *Hormone and Metabolic Research* **44** 328–333. (doi:10.1055/s-0031-1301302)
- Goffrini P, Ercolino T, Panizza E, Giachè V, Cavone L, Chiarugi A, Dima V, Ferrer I & Mannelli M 2009 Functional study in a yeast model of a novel succinate dehydrogenase subunit B gene germline missense mutation (C191Y) diagnosed in a patient affected by a glomus tumor. *Human Molecular Genetics* **18** 1860–1868. (doi:10.1093/hmg/ddp102)
- Horvath R, Abicht A, Holinski-Feder E, Laner A, Gempel K, Prokisch H & Jaksch M 2006 Leigh syndrome caused by mutations in the flavoprotein (Fp) subunit of succinate dehydrogenase (*SDHA*). *Journal of Neurology, Neurosurgery, and Psychiatry* **77** 74–76. (doi:10.1136/jnnp.2005.067041)
- Humphrey W, Dalke A & Schulten K 1996 VMD: visual molecular dynamics. *Journal of Molecular Graphics* **14** 33–38. (doi:10.1016/0263-7855(96)00018-5)
- Imperiale A, Moussallieh FM, Sebag F, Brunaud L, Barlier A, Elbayed K, Bachellier P, Goichot B, Pacak K, Namer JJ *et al.* 2013 A new specific succinate-glutamate metabolomic hallmark in *Sdhx*-related paragangliomas. *PLoS ONE* **8** e80539. (doi:10.1371/journal.pone.0080539)
- Iverson TM, Maklashina E & Cecchini G 2012 Structural basis for malfunction in complex II. *Journal of Biological Chemistry* **287** 35430–35438. (doi:10.1074/jbc.R112.408419)
- Jochmanová I, Zelinka T, Widimský J & Pacak K 2014 HIF signaling pathway in pheochromocytoma and other neuroendocrine tumors. *Physiological Research* **63** S251–S262.
- Kirby DM, Thorburn DR, Turnbull DM & Taylor RW 2007 Biochemical assays of respiratory chain complex activity. *Methods in Cell Biology* **80** 93–119. (doi:10.1016/S0091-679X(06)80004-X)
- Lane DJ, Merlot AM & Richardson DR 2014 The lure of a LYR: the logistics of iron sulfur cluster delivery. *Cell Metabolism* **19** 348–350. (doi:10.1016/j.cmet.2014.02.011)
- Lenders J, Duh QY, Eisenhofer G, Gimenez-Roqueplo AP, Grebe S, Murad MH, Naruse M, Pacak K & Young W 2014 Pheochromocytoma and paraganglioma: an endocrine clinical practice guideline. *Journal of Clinical Endocrinology and Metabolism* **99** 1915–1945. (doi:10.1210/jc.2014-1498)
- Lima J, Feijao T, Ferreira da Silva A, Pereira-Castro I, Fernandez-Ballester G, Máximo V & Garcia-Rostan G 2007 High frequency of germline succinate dehydrogenase mutations in sporadic cervical paragangliomas in northern Spain: mitochondrial succinate dehydrogenase structure–function relationships and clinical–pathological correlations. *Journal of Clinical Endocrinology and Metabolism* **92** 4853–4864. (doi:10.1210/jc.2007-0640)
- Lorenzo FR, Yang C, Ng Tang Fui M, Vankayalapati H, Zhuang Z, Huynh T, Grossmann M, Pacak K & Prchal JT 2013 A novel *EPAS1/HIF2A* germline mutation in a congenital polycythemia with paraganglioma. *Journal of Molecular Medicine* **91** 507–512. (doi:10.1007/s00109-012-0967-z)
- Maio N, Singh A, Uhrigshardt H, Saxena N, Tong WH & Rouault TA 2014 Cochaperone binding to LYR motifs confers specificity of iron sulfur cluster delivery. *Cell Metabolism* **19** 445–457. (doi:10.1016/j.cmet.2014.01.015)

- McDonald IK & Thornton JM 1994 Satisfying hydrogen bonding potential in proteins. *Journal of Molecular Biology* **238** 777–793. (doi:10.1006/jmbi.1994.1334)
- Na U, Yu W, Cox J, Bricker DK, Brockmann K, Rutter J, Thummel CS & Winge DR 2014 The LYR factors SDHAF1 and SDHAF3 mediate maturation of the iron–sulfur subunit of succinate dehydrogenase. *Cell Metabolism* **20** 253–266. (doi:10.1016/j.cmet.2014.05.014)
- Ng PC & Henikoff S 2001 Predicting deleterious amino acid substitutions. *Genome Research* **11** 863–874. (doi:10.1101/gr.176601)
- Panizza E, Ercolino T, Mori L, Rapizzi E, Castellano M, Opocher G, Ferrero I, Neumann HP, Mannelli M & Goffrini P 2013 Yeast model for evaluating the pathogenic significance of SDHB, SDHC and SDHD mutations in PHEO–PGL syndrome. *Human Molecular Genetics* **22** 804–815. (doi:10.1093/hmg/ddt487)
- Poliakov E, Managadze D & Rogozin I 2014 Generalized portrait of cancer metabolic pathways inferred from a list of genes overexpressed in cancer. *Genetics Research International* **646193** 1–8. (doi:10.1155/2014/646193)
- Richter S, Peitzsch M, Rapizzi E, Lenders J, Qin N, de Cubas A & Eisenhofer G 2014 Krebs cycle metabolite profiling for identification and stratification of pheochromocytomas/paragangliomas due to succinate dehydrogenase deficiency. *Journal of Clinical Endocrinology and Metabolism* **99** 3903–3911. (doi:10.1210/jc.2014-2151)
- Šali A & Blundell TL 1993 Comparative protein modelling by satisfaction of spatial restraints. *Journal of Molecular Biology* **234** 779–815. (doi:10.1006/jmbi.1993.1626)
- Selak MA, Armour SM, Mackenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB & Gottlieb E 2005 Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- α prolyl hydroxylase. *Cancer Cell* **7** 77–85. (doi:10.1016/j.ccr.2004.11.022)
- Srirangalingam U, Walker L, Khoo B, MacDonald F, Gardner D, Wilkin T, Skelly R, George E, Sponner D, Chew SL et al. 2008 Clinical manifestations of familial paraganglioma and pheochromocytomas in succinate dehydrogenase B (SDH-B) gene mutation carriers. *Clinical Endocrinology* **69** 587–596. (doi:10.1111/j.1365-2265.2008.03274.x)
- Sun F, Huo X, Zhai Y, Wang A, Xu J, Su D, Bartlam M & Rao Z 2005 Crystal structure of mitochondrial respiratory membrane protein complex II. *Cell* **121** 1043–1057. (doi:10.1016/j.cell.2005.05.025)
- Van Vranken JG, Na U, Winge DR & Rutter J 2014 Protein-mediated assembly of succinate dehydrogenase and its cofactors. *Critical Reviews in Biochemistry and Molecular Biology* **9** 1–13. (doi:10.3109/10409238.2014.990556)
- Vicha A, Taieb D & Pacak K 2014 Current views on cell metabolism in SDHx-related pheochromocytoma and paraganglioma. *Endocrine-Related Cancer* **21** 261–277. (doi:10.1530/ERC-13-0398)
- Wallace AC, Laskowski RA & Thornton JM 1995 LIGPLOT: a program to generate schematic diagrams of protein–ligand interactions. *Protein Engineering, Design & Selection* **8** 127–134. (doi:10.1093/protein/8.2.127)
- Werth MT, Cecchini G, Manodori A, Ackrell BA, Schröder I, Gunsalus RP & Johnson MK 1990 Site-directed mutagenesis of conserved cysteine residues in *Escherichia coli* fumarate reductase: modification of the spectroscopic and electrochemical properties of the [2Fe–2S] cluster. *PNAS* **87** 8965–8969. (doi:10.1073/pnas.87.22.8965)
- Yang C, Matro JC, Huntoon KM, Donald YY, Huynh YT, Flidner SM, Breza J, Zhuang Z & Pacak K 2012 Missense mutations in the human SDHB gene increase protein degradation without altering intrinsic enzymatic function. *FASEB Journal* **26** 4506–4516. (doi:10.1096/fj.12-210146)

Received in final form 2 March 2015

Accepted 23 March 2015