Structural and functional consequences of succinate dehydrogenase subunit B mutations

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Abstract

Mitochondrial dysfunction, due to mutations of the gene encoding succinate dehydrogenase (SDH), has been implicated in the development of adrenal phaeochromocytomas, 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
- phaeochromocytoma
- paraganglioma
- functional consequences

Introduction

Phaeochromocytoma (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...
Transfers to ubiquinone to reduce it to ubiquinol (Vancofactor, finally producing two stepwise single-electron reduction of its flavin adenine dinucleotide (FAD) choreographs the transfer of electrons by catalysing a two-translation of hypoxia-inducible factors (HIFs; Denko 2008, species. Both outcomes may contribute to cellular accumu-
of succinate and increased production of reactive oxygen fore of SDH inactivation and malfunction are accumulation 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 there-
fore 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 represen-tative 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
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 (Sali & 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.

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

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

SDH, succinate dehydrogenase; SDHB, SDH subunit B; PC, adrenal phaeochromocytoma; 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: Australian SDH Consortium (DE Benn, T Dwight, K Tucker and R Clifton-Bligh unpublished data); International SDH Consortium (Benn et al. 2006) and Leiden Database (Bayley et al. 2005). SDHB reference sequence: NM_003000 (mRNA) and NP_002991 (protein).

**Generation of SDHB expression vectors**

A primer was designed to generate the WT SDHB in pEGFP-N1 (Clontech) vector: AATGGAATTCTAAAAATGGCGGGTGGTCGCACTC. GFP vector and SDHB insert were digested using EcoR1 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 Lightening 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) and 5% horse serum (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 \times 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 10 min gentle agitation washes) from 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 (1181446001)), 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 (ΔOD/5 min and ΔOD/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 ± 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 mutations 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.
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 introduced into HEK293 cells, and initially studied for mitochondrial localisation. As expected, SDHB truncating mutations (p.Arg27*, p.Arg90*, c.88delC and c.311del-AinsGG) 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.311del-AinsGG. Enzymatic activities of SDH complexes containing the missense SDHB mutants p.Ala43Pro, p.Arg46Gly and p.Arg242His were moderately reduced to approximately 50% relative to 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).
HEK293 cells (Supplementary Fig. 4, see section on in IPｓ from these cells was strikingly similar to that in SDHB vectors in the rat PC cell line PC12. SDH activity

...from 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 SDH–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 in HEK293 cells were isolated through pulldown by GFP-tagged SDHB. SDH activity (percentage relative to WT) of SDH activity. A trendline encompassing investigated SDHB 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

**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, IP’s 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 IP’s from cells transfected with missense SDHB mutant constructs in amounts similar to those for GFP-WT SDHB IP’s, 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.

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 IP’s 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).
in spatial dimensions affecting charges and binding sites \textit{in silico}. These predictions were then compared against assessment of intracellular localisation and biochemical assays after transfecting HEK293 cells with various \textit{SDHB} mutants \textit{in vitro}. We developed a novel means for assaying \textit{SDH} function in human cells transfected with \textit{SDHB} mutant constructs, by specifically immunoprecipitating mutant-containing \textit{SDH} complexes from whole cell lysates. Our results indicate that \textit{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 \textit{SDHB} mutations p.Arg27*, p.Arg90*, c.88delC and c.311delAinsGG predicted nonsense-mediated mRNA decay or truncation of the protein. Complete loss of \textit{SDH} expression in both cytosolic and mitochondrial compartments associated with truncating mutations from our \textit{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 \textit{SDH} activity was present in IPs containing these truncating mutants.

The second group of disease-associated \textit{SDHB} mutations (p.Ala43Pro, p.Arg46Gly and p.Arg46Gln) were those shown to exhibit reduced expression of mitochondrial \textit{SDHB} despite being expressed at normal levels in cytoplasm. Again, in keeping with this localisation data, \textit{SDH} activity was impaired by these mutations (but not totally abolished, as observed with the truncating mutations). Interestingly, p.Arg46Gly demonstrated severely impaired \textit{SDH} activity, whereas p.Arg46Gln had a more modest effect on \textit{SDH} function. These results indicate that these two different mutations have different effects on the \textit{SDH} catalytic site.

The third group of disease-associated mutations (p.Cys101Tyr, p.Pro197Leu and p.Arg242His) were those associated with apparently normal mitochondrial \textit{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 \textit{SDH} structure leading to loss of the electron path (p.Arg242His). \textit{SDHB} mutants p.Cys101Tyr and p.Arg242His were both associated with significantly reduced \textit{SDH} activity when compared with the WT. Using our \textit{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 \textit{SDHB} (Werth et al. 1990, Iverson et al. 2012). Mutation at p.Arg242 was predicted to disrupt hydrogen bonds between \textit{SDH} subunits, thereby reducing their biochemical efficiency and perhaps could also explain the loss of function in an otherwise fully compartmentalised \textit{SDH} complex. The effect of p.Pro197Leu on measurable \textit{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 \textit{SDH} complex.

In order to confirm that reduced expression of mitochondrial \textit{SDHB} was in fact associated with loss of \textit{SDH} assembly, we then examined the interaction of \textit{SDHB} (WT or mutant) and \textit{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 \textit{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 \textit{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 sdh2null 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.
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