BMC Research Notes

RESEARCH NOTE

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Identification of a novel transcript of mouse Sdha

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Abstract

Objective This study aimed to identify novel isoforms of mouse succinate dehydrogenase complex flavoprotein subunit A (Sdha) arising from internal exon skipping, analogous to the process observed in human ortholog SDHA.

Results We identified a novel isoform, designated Δ3-10, which lacked the final 104 nucleotides of exon 3 and all of exons 4 through 10, yet did not alter the reading frame. The Δ3-10 Sdha cDNA was cloned into expression vectors, and overexpression resulted in a protein localized to the mitochondria. However, the endogenous Δ3-10 Sdha protein was not detected with the available antibodies.

Keywords Sdha, Isoforms, Mitochondria, Mouse

Introduction

Succinate dehydrogenase (SDH) has a critical role in cellular energy metabolism through its dual role in the Krebs cycle and as part of the electron transport chain (mitochondrial complex 2). It is a hetero-tetrameric protein located on the inner mitochondrial membrane, and consists of four subunits: A, B, C and D. The catalytic subunit SDHA is the largest subunit and generates FADH2 by oxidizing succinate to fumarate as part of the tricarboxylic acid cycle. SDHB contains three iron-sulfur clusters that accept the electrons from FADH2 and transfer them to SDHC and SDHD, which are embedded in the inner mitochondrial membrane and constitute the CII function within the electron transport chain serving as the site for ubiquinone binding and reduction to ubiquinol [1–3]. Pathogenic variants affecting the SDH/CII genes result in mitochondrial dysfunction. This can manifest either as primary mitochondrial diseases, or as susceptibility to tumorigenesis [4].

The human SDHA gene is located on chromosome 5p15.33 and consists of 15 exons. Besides the primary transcript (variant 1), there are two other in-frame transcript variants: one missing exon 4 (variant 2) and another missing exons 12 and 13 (variant 3). The existence of these three isoforms has not been experimentally identified at the protein level. The mouse Sdha gene is located on chromosome 13 C1 and, like its human ortholog, consists of 15 exons. No alternative transcripts have been described in mice, so we aimed to explore their expression on both transcript and protein levels.

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Main text

Methods

Cell lines

The human osteosarcoma cell line U2OS (catalog number HTB-96) and the mouse macrophage cell line RAW 264.7 (catalog number TIB-71) were obtained from ATCC. The U2OS SDHA knockout (KO) cells were previously generated in our laboratory. The mouse pancreatic alpha cell line αTC1.9, the mouse pancreatic beta cell line Min6, and the mouse hepatocyte cell line AML12 were generously provided by Dr. Germán Perdomo (IBGM, Valladolid). The U2OS, RAW 264.7 and MIN6 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (Pen/Strep). The αTC1.9 cell line was maintained in DMDM containing 16 mM glucose, 10% FBS and 1% Pen/Strep. The AML12 cell line was maintained in DMEM/F-12 medium containing 10% FBS, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 40 ng/mL dexamethasone and 1% Pen/Strep.

Mice

This study was conducted with 6- to 8-week-old C57BL/6 J mice (Jackson Laboratory #000664) and BALB/cJ (Jackson Laboratory #000651) of both sexes. Mice were euthanized in a CO2 chamber, followed by the collection of various tissues. Bone marrow-derived macrophages (BMDMs) were generated as previously described [5].

Conventional and quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). For conventional RT-PCR, RT of RNA was primed with oligo(dT) and the resultant cDNA was used as the template for PCR amplification using primers P1 and P2. The PCR products were extracted from an agarose gel before being subjected to Sanger sequencing. For quantitative RT-PCR (RT-qPCR), RT of RNA was primed with a mixture of the following gene specific primers: P5 and P6 for Sdha, P8 and P9 for the housekeeping gene Hprt1, and P11 and P12 for the housekeeping gene Ef-2. The cDNA is then used as the template for the SYBR Green quantitative PCR using the following primers: P4 and P5 for the canonical Sdha transcript and P3 and P5 for the ΔE3-10 Sdha transcript, P7 and P8 for Hprt1, and P10 and P11 for Ef-2. The sequences of the primers used in this study are provided in Table S1.

Plasmids construction

Canonical and ΔE3-10 Sdha transcripts, amplified using primers P1 and P2, were cloned into the pJET1.2/blunt vector (Thermo Fisher Scientific) for sequencing and subsequent use as templates for expression vectors. To generate C-terminally EGFP-tagged constructs, Sdha isoforms were amplified using primers P13 and P14 and cloned into the pEGFP-N1 vector (Clontech #6085-1). To create C-terminally FLAG-tagged constructs, Sdha isoforms were amplified using primers P15 and P16 and cloned into an expression vector designated pFLAG-PURO. This vector, previously developed in our laboratory from pEGFP-N1, was created by replacing EGFP with a puromycin cassette (SA-T2A-Puro-pA) and introducing a 3xFLAG sequence containing an EcoRV site at the N-terminus of this cassette. To generate the mitoCherry plasmid, the mitochondrial targeting sequence of human COX8A was amplified using primers P17 and P18 and cloned into the mCherry vector (Clontech #632523). All final constructs were verified by DNA sequencing.

Immunofluorescence

U2OS cells seeded on 12-mm coverslips in 24-well plates were cotransfected with Sdha-EGFP plasmids and mitoCherry plasmid. Forty-eight hours later, cells were fixed in 4% paraformaldehyde and nuclei were stained with 5 µg/ml Hoechst 33342. Coverslips were mounted and confocal images were obtained with a TCS SP5 confocal system (Leica).

Western blotting

Protein was extracted by RIPA buffer plus protease inhibitors. Twenty-microgram protein samples separated by SDS-PAGE and transferred to PVDF membranes. The membranes were used for the immunodetection of β-actin (AC-40, Sigma-Aldrich), Sdha (B-1, Santa Cruz Biotechnology) and Flag (M2, Sigma-Aldrich).

Protein tertiary structure prediction

Homology modelling was carried out with the SWISS-MODEL [6] server using the PDB identification code 8GS8 for the Cryo-EM structure of the human respiratory complex II [7] as template. UCSF ChimeraX (1.8) [8] was used for visualization. The obtained models were scored using the GMQE (global model quality estimation) and QMEAN scoring functions [9]. The GMQE

has values between 0 and 1, reflecting the accuracy of the model built with that specific alignment and template. The higher the number, the higher the reliability of the model is. The QMEAN score indicates the degree of nativeness of the structure in the model. Values around 0 mean good quality agreement between the modeled structure and experimental structures of similar size. Values less than -4 indicate models of low quality.

Results

To investigate the presence of exon-skipping isoforms of mouse Sdha, we conducted conventional RT-PCR using primers that flank the coding region in various tissues, as well as in BMDMs. As depicted in Fig. 1A, amplification of cDNA from BMDMs showed a band of ~ 2000 bp, corresponding to the expected size of the canonical isoform, and a smaller band of ~ 800 bp. The DNA fragments were extracted from the gel, and Sanger sequencing revealed that the upper band corresponded to the canonical isoform, while the lower band represented a newly identified isoform. This novel isoform, which we named Δ3-10, lacked the final 104 nucleotides of exon 3 and all of exons 4 through 10, but this did not result in a change in the reading frame (Figure S1). Next, cDNAs from various tissues of two different mouse strains, C57BL/6 J and BALB/cJ, were amplified using PCR conditions optimized to favor the appearance of the band of ~ 800 bp (Figure S2). The electropherograms displayed sharp, regularly spaced, single peaks, indicating the presence of a single template, which was identified as Δ3-10 in all cases (Figure S2). Conventional RT-PCR is qualitative, allowing for the detection of target mRNAs but not providing precise quantification, merely offering pseudo-quantification through normalization to an external control standard. To more accurately assess Δ3-10 isoform mRNA expression, we conducted a RT-qPCR. RT was performed with two reverse primers specific for exon 11, common to both Sdha isoforms, and the housekeeping genes Hprt1 and Ef-2, instead of conventional oligo(dT) or random hexamer primers. For each transcript to be quantified, quantitative PCR with SYBRGREEN was performed using the more upstream reverse primer from those used in RNA priming and

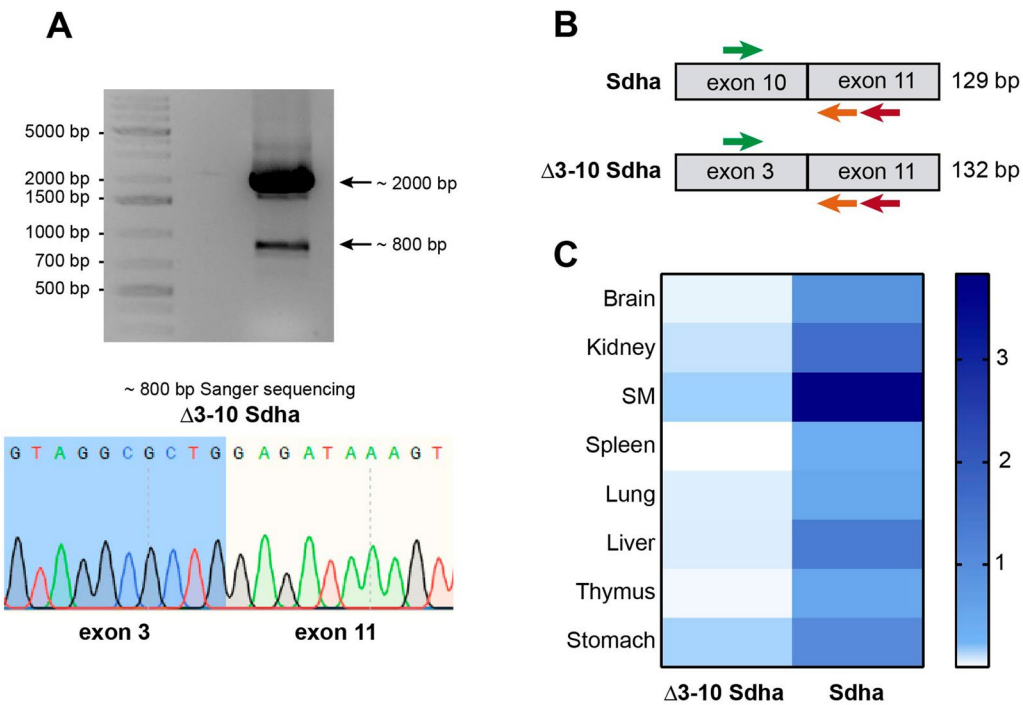

Fig. 1 Sequence analysis and relative expression of ΔE3-10 Sdha transcript. **A** Gel electrophoresis image showing DNA fragments generated by conventional RT-PCR using primers flanking the Sdha coding region, with RNA extracted from BMDMs. Below, a representative Sanger sequencing electropherogram depicts the exon 3 (blue shaded)/11 junction region within the ~ 800 bp DNA fragment. **B** Schematic representation of the RT-qPCR method used to determine the relative expression of canonical and ΔE3-10 Sdha transcripts. The green arrows indicate the forward primers, and the orange and red arrows indicate the reverse primers (listed in Table S1). RNA was primed with two specific reverse primers located in exon 11. Subsequent quantitative PCR with SYBRGREEN was performed using the upstream reverse primer (orange arrow) and forward primers on exon 3 for Δ3-10 Sdha and on exon 10 for canonical Sdha. The sizes of the corresponding amplicons are depicted. **C** A heat map illustrates the relative log expression levels of Sdha isoforms in the specified mouse tissues. SM: skeletal muscle

forward primers designed to amplify similarly sized amplicons: 132 bp for Δ3-10 (forward primer on exon 3), 129 bp for canonical Sdha (forward primer on exon 10), 147 bp for Hprt1, and 120 bp for Ef-2 (Fig. 1B). The heat map in Fig. 1C illustrates that the canonical isoform is significantly more abundant than the Δ3-10 isoform across all tested tissues, with both isoforms being most abundant in skeletal muscle. Notably, despite the relatively low expression of the Δ3-10 isoform, its Ct values ranged between 25 and 30 in all tissues tested, indicating reliable quantification within the acceptable Ct value range (Table S2).

Next, we generated vectors expressing C-terminal FLAG-tagged Sdha isoforms and transfected them into U2OS cells. The overexpression of the FLAG-tagged proteins was analyzed using Western blotting. The canonical full-length Sdha-FLAG construct encoded a protein of approximately 75 kDa, while the Δ3-10 Sdha construct encoded a protein of about 30 kDa (Fig. 2A and Figure S1). We then investigated the endogenous expression of the Δ3-10 Sdha isoform using a commercially available antibody specifically chosen for its suitability in detecting the Δ3-10 Sdha isoform. Typically, anti-SDHA antibodies are produced using an immunogen that is either proprietary or a complete recombinant protein. The antibody we used is a monoclonal antibody specific to an epitope located between amino acids 461–664 at the C-terminus of canonical Sdha. Of the 207 amino acids in this epitope, 190 are present in the C-terminus of the Δ3-10 Sdha isoform. As expected, the antibody recognized the overexpressed Δ3-10 Sdha-FLAG isoform (Fig. 2A). However, we were unable to detect the endogenous form (Fig. 2B). The successful translation of the overexpressed Δ3-10 Sdha transcript suggests that the lack of detection is likely due to antibody sensitivity. Consequently, we believe that this isoform is expressed, albeit at low levels. To validate the antibody's specificity, we used cell lysates from SDHA KO U2OS cells (Fig. 2A).

To determine the intracellular localization of Δ3-10 Sdha, we generated constructs encoding the Sdha isoforms fused to GFP at their C-terminus. These GFP vectors were co-transfected into U2OS cells along with the mitoCherry plasmid, which encodes the mitochondrial targeting sequence of human COX8A fused with

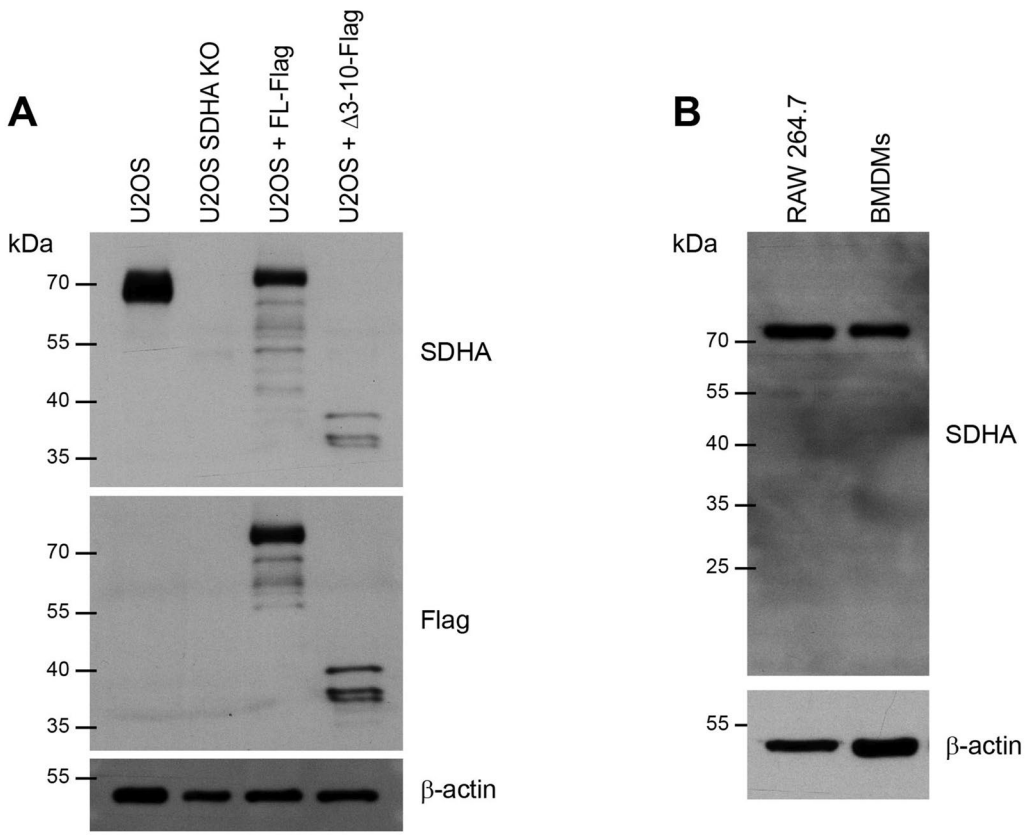

Fig. 2 Western blot analysis of endogenous Sdha protein and recombinant canonical and ΔE3-10 Sdha proteins. **A** Western blotting of whole cell lysates from the indicated transfected and untransfected U2OS cells using antibodies against Flag, SDHA, or β-actin. FL: canonical full-length isoform; KO: knockout. **B** Western blotting of whole cell lysates from RAW 264.7 cells and mouse BMDMs using antibodies against Sdha or β-actin

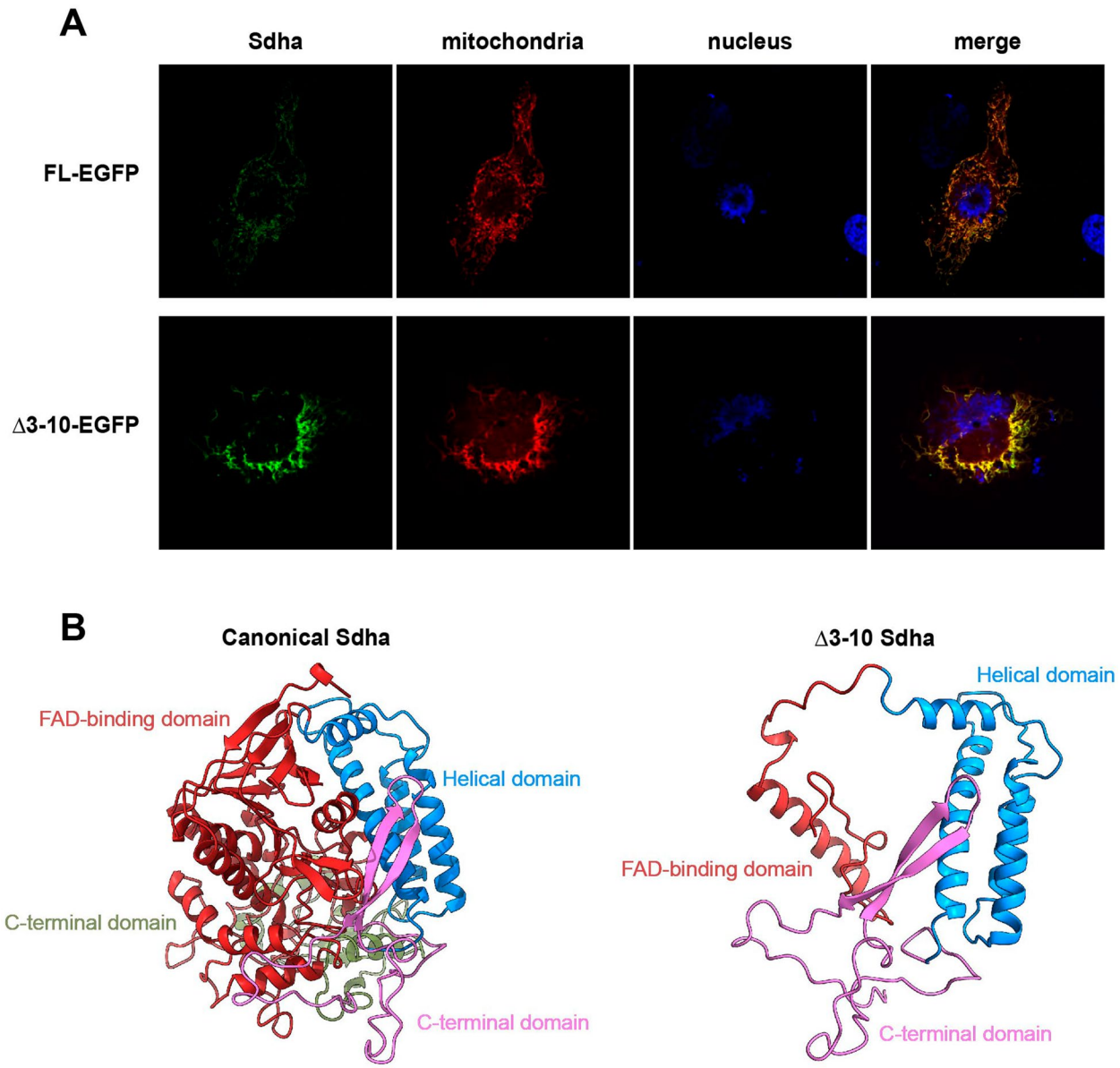

Fig. 3 Cell localization of ΔE3-10 Sdha protein and prediction of its 3D structure. **A**. Confocal images obtained in fixed U2OS cells co-transfected with the mitochondrial targeting signal of the cytochrome c oxidase subunit 8A (COXA) fused to a mCherry protein as a mitochondria marker (mitoCherry) and constructs containing the Sdha isoforms fused to an EGFP protein. Green, EGFP; Red, mCherry; Blue, nucleus. Individual and merge images are shown. **B** Prediction of the 3D structure of the canonical and ΔE3-10 Sdha proteins using the SWISS model server. The FAD-binding domain, the capping domain, the helical domain, and the C-terminal domain are depicted in different colors. The Δ3-10 Sdha isoform contains the helical domain and the C-terminal domain

mCherry. As shown in Fig. 3A, the Δ3-10 Sdha localizes to the mitochondria, similar to the canonical Sdha isoform. In an effort to deduce the function of Δ3-10 Sdha, we examined the domains it contains. The Sdha subunit comprises four domains: the FAD-binding domain (residues A51 to A315 and A403 to A487), the capping domain (residues A316 to A402), the helical domain (residues A488 to A585), and the C-terminal domain (residues A596 to A664) [7, 10]. The active site for succinate-fumarate interconversion is located at the interface between the FAD-binding domain and the capping domain. The Δ3-10 Sdha isoform contains only the helical domain and the C-terminal domain, thus lacking enzymatic activity. The predicted 3D structure of the Δ3-10 Sdha isoform and its overlap with the canonical form are shown in Fig. 3B. It

is important to note that the canonical Sdha model had a GMQE score of 0.90 and a QMEAN Z-score of -1.58, indicating a good overall model quality. In contrast, the Sdha Δ3-10 isoform model showed a GMQE score of 0.76 and a QMEAN Z-score of − 3.68, indicating reduced structural quality, likely attributable to its shorter sequence relative to the canonical isoform used for modeling.

Limitations

In this study, we provide evidence for the presence of a novel Sdha transcript in various tissues of two different mouse species. As previously described, this isoform lacks most of exon 3 and exons 4 through 10. Further investigation is necessary to understand the molecular mechanisms behind the generation of this new isoform. Our analysis did not identify a cryptic splice donor site within exon 3 that could explain the partial deletion of exon 3 and the complete deletion of exons 4–10. Interestingly, the exon 3/11 junction sequence (CTGGAG) in Δ3-10 Sdha appears twice in the canonical Sdha mRNA: once in exon 3, where the skipping event initiates, and again at the exon 10/11 junction. Subsequently, we performed an exhaustive search of the complete mouse genome sequence databases for processed Sdha pseudogenes and did not detect any.

Experiments must be conducted to detect the endogenous form of the Δ3-10 Sdha isoform by generating antibodies against the small isoform. Additionally, it is crucial to determine whether the Δ3-10 Sdha isoform has any function. It is highly unlikely that it binds to the SDHAF2 and SDHAF4 chaperones and forms a complex with the other subunits [11, 12]. This possibility, although unlikely, should be explored by co-immunoprecipitation assays and tracking of alternative SDH/CII complex species by BN-PAGE. Instead, the Δ3-10 Sdha isoform might function in a free form, inhibiting the interaction between SDHA and SDHB. This hypothesis arises from the observation that the Δ3-10 isoform of Sdha retains the helical domain, which in the human SDHA ortholog is positioned at the interface with SDHB [7]. This hypothesis can be tested through in vitro competition assays with recombinant Sdha, Δ3-10 Sdha, and Sdhb proteins, under conditions with and without the chaperones SDHAF2 and SDHAF4. Complementary experiments in murine cells overexpressing the Δ3-10 isoform of Sdha would allow assessment of its impact on the Sdha-Sdhb interaction via immunoprecipitation analysis.

Abbreviations

BMDMs	Bone marrow-derived macrophages
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
FL	Full-length canonical isoform
KO	Knockout
Pen/Strep	Penicillin/Streptomycin
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SDH	Succinate dehydrogenase
SM	Skeletal muscle

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-025-07149-8.

Supplementary material 1.

Acknowledgements

None.

Author contributions

M.J.E.-A., A.T., D.G., P.S.-L, J.J.T., M.T.P.-G., and P.K. performed all procedures and analyzed the data. M.A.d.l.F. and M.S. conceived the study and wrote the manuscript. All authors confirmed the final version of the manuscript.

Funding

Agencia Estatal de Investigación, Grant Number PID2023-150506OB-I00; Consejería de Educación, Junta de Castilla y León, Grant Number VA172P20; Consejería de Sanidad, Junta de Castilla y León, Grant Number GRS 2201/A/2020; Programa Estratégico Instituto de Biomedicina y Genética Molecular (IBGM), Junta de Castilla y León, Award Number CCVC8485.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The sequence of ΔE3-10 Sdha transcript has also been deposited on GenBank under accession number PQ237693 (https://www.ncbi.nlm.nih.gov/nuccore/PQ237693.1/).

Declarations

Ethics approval and consent to participate

The Animal Care and Use Committee of the University of Valladolid approved all experiments (protocol #9409958). All mice procedures conformed to European Directive 2010/63/EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 53/2013.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 16 July 2024 Accepted: 12 February 2025
Published online: 25 February 2025

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