A novel mutation in SPART gene causes a severe neurodevelopmental delay due to mitochondrial dysfunction with Complex I impairments and altered pyruvate metabolism

Running title: SPART and mitochondria in Troyer syndrome

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Abbreviation
AcCoA Acetyl-coA
ALS Amyotrophic Lateral Sclerosis
BMP Bone Morphogenic Protein
Ca^{2+} Calcium
CAT Catalase
Complex I NADH dehydrogenase
Complex I+III NADH-cytochrome c reductase activity
Complex II+III succinate-cytochrome c reductase activity
CS Citrate Synthase
DAPI 4',6-diamidino-2-phenylindole
DB Decylbenzoquinone
DCFDA 2',7'-dichlorofluorescin diacetate
DDS Deciphering Consortium
DMEM Dulbecco’s modified Eagle’s medium
DTNB 5,5'-dithiobis-2-nitrobenzoic acid
ECM Extra-Cellular Matrix
ExAc Exome Aggregation database
FCCP carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
GH Growth Hormone
gnomAD Genome Aggregation database
gRNA guide RNA
GRP75 Glucose-Regulated Protein 75
hESC human embryonic stem cell
hNSC human neural stem cell

hpf hours post fertilization

HPLC High Performance Liquid Chromatography

HSP Hereditary Spastic Paraplegia

IMS Inter-Membrane Space

IUGR Intra-Uterine Growth Restriction

MIM Mitochondrial Inner Membrane

MIT Microtubule Interacting and Trafficking

MOM Mitochondrial Outer Membrane

MPC Mitochondrial Pyruvate Carrier

MTG Mitotracker Green

mΔψ Mitochondrial transmembrane potential

OXPHOS oxidative phosphorylation

PDH Pyruvate Dehydrogenase

pSpCas9n(BB)-2A-GFP Cas9 from *S. pyogenes* with 2A-EGFP

pSpgRNA *S. pyogenes* Cas9 guide RNA

RIPA Radioimmunoprecipitation assay

ROH Runs Of Homozygosity

ROS reactive oxygen species

SD standard deviations

SDS-PAGE sodium dodecyl sulfate polyacrylamide electrophoresis

SEM Standard Error of Mean

SNPs Single Nucleotide Polymorphisms

SOD1 Superoxide Dismutase

SOD2 Manganese Superoxide Dismutase

ssODN single stranded oligonucleotide

TBS Tris Buffered Saline
TMRM Tetramethylrhodamine methyl ester
UPD7 Uniparental disomy 7
WES Whole Exome Sequencing
Abstract

Loss-of-function mutations in *SPART* gene cause Troyer syndrome, a recessive form of spastic paraplegia resulting in muscle weakness, short stature and cognitive defects. *SPART* encodes for Spartin, a protein linked to endosomal trafficking and mitochondrial membrane potential maintenance. Here, we identified with whole exome sequencing (WES) a novel frameshift mutation in the *SPART* gene in two brothers presenting an uncharacterized developmental delay and short stature. Functional characterization in a SH-SY5Y cell model shows that this mutation is associated with increased neurite outgrowth. These cells also show a marked decrease in mitochondrial Complex I activity, coupled to decreased ATP synthesis and defective mitochondrial membrane potential. The cells also presented an increase in reactive oxygen species, extracellular pyruvate and NADH levels, consistent with impaired Complex I activity. In concordance with a severe mitochondrial failure, Spartin loss also led to an altered intracellular Ca\(^{2+}\) homeostasis that was restored after transient expression of wild-type Spartin.

Our data provide for the first time a thorough assessment of Spartin loss effects, including impaired Complex I activity coupled to increased extracellular pyruvate. In summary, through a WES study we assign a diagnosis of Troyer syndrome to otherwise undiagnosed patients, and by functional characterization we show that the novel mutation in *SPART* leads to a profound bioenergetic imbalance.
Introduction

Neurodevelopmental disorders affect 2–5% of individuals and are genetically heterogeneous (1). They constitute a large proportion of the life-long global health burden in terms of medical care, hospitalizations, and mortality (2). An example of a rare developmental disorder is Troyer syndrome (OMIM #275900), which is an autosomal-recessive form of hereditary spastic paraplegia (HSP) characterized by lower extremity spasticity and weakness, short stature, cognitive defects, distal amyotrophy and degeneration of corticospinal tract axons (3, 4, 5, 6). In Troyer syndrome loss-of-function mutations occur in the SPART gene, which codes for Spartin, a multifunctional protein consisting of a N-terminal Microtubule Interacting and Trafficking (MIT) domain and a C-terminal senescence domain (7, 8) (Supplementary Fig. 1A). Spartin is expressed in a wide range of tissues at embryonic and adult stages. In the Eurexpress mouse database (http://www.eurexpress.org) expression of the homologous murine Spg20 was identified in the nervous and olfactory systems of the developing mouse at embryonic day 14.5 (9). In the Human Protein Atlas SPART is ubiquitously expressed, with a high expression level in the central nervous system, gastrointestinal tract and reproductive system (https://www.proteinatlas.org/ENSG00000133104-SPG20). Spartin functions in a range of cellular processes including epidermal growth factor receptor trafficking, lipid droplets turnovers, bone morphogenetic protein (BMP) signalling and cytokinesis (8-14). Specifically, the impaired cytokinesis in Spg20/- mice leads to a prominent number of binucleated chondrocytes in epiphyseal growth plates of bones, accounting for the short stature and skeletal defects observed in Troyer syndrome (15).

Interestingly, a few studies suggested that Spartin loss might impair mitochondrial function, documenting alterations in the mitochondrial network and decreases in the mitochondrial membrane potential (16-19).

In this study we used whole exome sequencing analysis, to identify a novel mutation in SPART gene in two brothers born from healthy consanguineous parents (first degree cousins). The brothers
had been referred for pre- and post-natal growth retardation, syndromic short stature and
developmental delay with severe speech impairment, and both carried a homozygous mutation,
c.892dupA, which confers a premature stop codon. Although Troyer syndrome had not been
considered in these two particular cases, a careful re-evaluation identified common features and we
therefore investigated effects of this SPART loss-of-function mutation, which specific focus on
mitochondria. We first evaluated the effects of Spartin loss using gene silencing in human neural
stem cells (hNSCs), documenting altered neuronal growth and exhibited significantly longer
neurites, compared to cells transfected with siRNA. We next generated a neuroblastoma-derived
SH-SY5Y cell line carrying the mutation via CRISPR/Cas9-genome editing. Compared to control
SH-SY5Y cells, the mutant cells exhibited increased neurite outgrowth and altered distribution and
structure of the mitochondrial network. Importantly these cells also showed metabolic changes with
severe decrease in Complex I activity, increased production of mitochondrial reactive oxygen
species (ROS) and elevated extracellular pyruvate, which reflects defective mitochondrial oxidation
of this molecule. Interestingly, in a recent study heterologous expression of human or Drosophila
Spartin extended yeast lifespan, reduced age-associated ROS production and cell death (19). Spartin
localized to the proximity of mitochondria, physically interacting with proteins related to
mitochondrial and respiratory metabolism in yeast (19). Nevertheless, a thorough analysis of human
SPART mutation effects in a human genome-edited neuronal cell model, including a quantitative
mitochondrial respiration and OXPHOS activity assessment, has not been carried out yet. Our novel
findings related to Spartin loss might provide clues to the neurological impairments in Troyer
syndrome. Our data suggest that the mitochondrial impairment could affect neurons by inducing an
energetic failure that could be coupled to excessive ROS production and extended axonal
morphology.

In summary, through a WES study we were able to assign a diagnosis of Troyer syndrome to
otherwise undiagnosed patients, and by functional characterization we elucidated that the novel
mutation in SPART led to a profound bioenergetic imbalance.
Patients and methods

Subjects

Two brothers born from consanguineous healthy parents, first-degree cousins of Moroccan origin, were first evaluated at the Clinical Genetics Unit when they were 42-months and 12-months-old, respectively. Family history was unremarkable. They both presented with IUGR (intrauterine growth restriction), stature and weight below -2 SD (standard deviations), relative macrocrania, dysmorphic features (very long eyelashes, dolichocephaly, prominent maxilla, pectus excavatum) and mild psychomotor retardation, with severe language delay. They started walking independently at 18 months. Other shared anomalies included delayed bone age, pes planus, euphoric behavior, and joint hyperlaxity. The first evaluation did not disclose signs of neuromuscular involvement. Genetic analyses included: karyotype and analysis of subtelomeric regions, UPD7 and H19 methylation analysis, and mutation screening of PNPLA6 (OMIM#603197), all of which were negative.

After a 5 years follow-up, the eldest brother (age 8 years and 9 months) gradually developed muscular hypotrophy in upper and lower limbs, increased muscle tone in the lower limbs (distal>proximal) and brisk deep tendon reflexes. Sporadic aggressive behavior and inappropriate crying was reported by parents. The younger sib (6 years and 3 months) developed muscular hypotrophy as well, mild hyper-reflexia and difficulty to walk on toes or heels. No cerebellar signs were reported, nor dysarthria/tongue dyspraxia but language impairment remained severe. Stature was constantly around the 3rd percentile in both sibs. The eldest brother showed a partial Growth Hormone (GH) deficiency, treated with a GH analogue.

High-throughput SNP genotyping and Whole Exome Sequencing (WES)

High-Throughput SNP genotyping
High-throughput SNP (Single Nucleotide Polymorphisms) genotyping was performed on Illumina Infinium HD Assay Gemini platform (Illumina, San Diego, CA, USA), according to manufacturer’s protocol, starting from 400 ng of genomic DNA from peripheral blood. Genotypes were converted into PLINK format with custom scripts. PLINK v1.07 (http://ngu.mgh.harvard.edu/~purcell/plink/) was used to isolate individual Runs Of Homozygosity (ROH) that showed > 1 Mb overlap between the three affected siblings (20).

Whole Exome Sequencing (WES)

WES was performed on genomic DNA extracted from peripheral blood (QIAGEN, Hilden, Germany) from the two affected brothers. Genomic DNA libraries, starting from 100 ng genomic DNA, were prepared using the Illumina Pair-End Nextera Kit (Illumina) and library was enriched for exomic sequences using the Nextera coding exome kit. The captured regions were sequenced on the Illumina HiScanSQ platform for 200 cycles (100 cycles paired-ends, Illumina). The read files were aligned to hg19 version of the human genome sequencing, annotation and variant prioritization was performed according to our internal pipeline for exome annotation as previously reported (21). The identified variants were confirmed by Sanger sequencing.

Cell lines

SH-SY5Y cells (ATCC, Middlesex, UK) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Euroclone, Milan, Italy) supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

Human neural stem cells (hNSC), derived from the NIH approved H9 (WA09; WiCell Research Institute, Madison, WI, USA) human embryonic stem cells (hESCs), were grown in 6-well plates coated with CTSTM CELLstart™ Substrate (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and maintained in KnockOut D-MEM/F-12 with 2mM of GlutaMAX-I supplement, 20 ng/ml of bFGF, 20 ng/ml of EFG and 2% of StemPro® Neural Supplement (Thermo Fisher Scientific). All cells were grown in a humidified incubator with 95% air and 5% CO₂ at 37°C.
Silencing SPART in Neural Stem Cells (hNSC)

To transiently knock-down SPART, hNSC were transfected every 36 hours with a combination of 3 siRNAs (Thermo Fisher Scientific; see Supplementary Table 1 for the sequences), using Lipofectamine3000 (Thermo Fisher Scientific) according to the manufacturer’s instruction. At day 0, 4 and 8 cells were collected and processed for western blot and imaging analysis.

Generation of SPART c.892dupA knock-in SH-SY5Y cell line

The SPART mutation was generated in SH-SY5Y genome using guide RNAs (gRNAs) designed with the CRISPR Design Tool (mit.edu.crispr) (22). Annealed oligos containing the target sequence for Cas9 were cloned into pSpgRNA that expresses gRNA driven by a U6 promoter (S. pyogenes Cas9 guide RNA #47108; Addgene, Cambridge, MA, USA, 23) and sequenced. Sequences of gRNAs and single stranded-oligonucleotide (ssODN) carrying the variant c.892dupA are reported in Supplementary Table 1. Cells were plated at 80% confluence and transfected with 4.7 µg of pSpCas9n(BB)-2A-GFP (PX458, #48140; Addgene; 24), 0.8 µg of each gRNA expression plasmid and 10 µM of ssODN with Lipofectamine®3000 (Thermo Fisher Scientific) according to the manufacturer’s instruction. After 28 hours, cells were sorted with an automated Fluorescence-Activated Cell Sorting (FACS) system (Influx, Becton Dickinson, Franklin Lakes, NJ, USA) and single cells were plated in 96-well plates coated with Poly-D-Lysine (Sigma-Aldrich). Clones were amplified and screened by PCR and direct sequencing of the target region. A clone carrying the specific change and with no off-target mutations was selected for the analysis (hence defined SPART<sup>c.892dupA</sup>). The SH-SY5Y clone that underwent the same CRISPR/Cas9 genome editing approach but did not carry any change was used as control cell line (hence defined SPART<sup>wt</sup> throughout the text).
**Western blotting**

Cells were lysed in ice-cold RIPA buffer: 50 mM HEPES (EuroClone), 1 mM EDTA (Sigma-Aldrich), 10% glycerol (Thermo Fisher Scientific), 1% Triton X-100 (Sigma-Aldrich), 150 mM NaCl in the presence of proteases and phosphatases inhibitors (Sigma-Aldrich). Total protein was measured using the Lowry protein assay kit (Bio-Rad DC Protein Assay; Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instruction. Protein samples (70 μg) were subsequently separated on 10% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gels or on 4-20% pre-cast SDS-PAGE gels (Bio-Rad). Gels were then electro-transferred onto nitrocellulose membranes (Trans-Blot Turbo Transfer System, Bio-Rad). Membranes were blocked in Tris Buffered Saline (TBS) with 1% Casein (Bio-Rad) for 1 hour at room temperature and incubated with primary antibodies at 4°C for 16 hours. Membranes were washed three times in Tris-buffered saline containing 0.1% Tween and incubated with peroxidase-conjugated secondary antibodies for 45 minutes at room temperature. Bands were visualized using WESTAR Supernova (Cyanagen, Bologna, Italy) and detected with the ChemiDoc™ XRS+ system (Bio-Rad). Densitometric analysis was performed with ImageLab software (Bio-Rad). Primary antibodies used were: GAPDH (mouse, 1:10,000; Abcam, Cambridge, UK), γ-tubulin (mouse, 1:10,000; Sigma-Aldrich), T-STAT3 (mouse, 1:500; OriGene, Rockville, MD, USA), P-STAT3 (rabbit, 1:500; Cell Signaling, Leiden, Netherlands), Spartin (rabbit, 1:1,000; 13791-1-AP, N-terminal; ProteinTech, Rosemont, IL, USA) and GRP75 (goat, 1:500; Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibodies used were anti-mouse IgG (1:5,000), anti-rabbit IgG (1:5,000; Sigma-Aldrich), anti-goat IgG (1:5,000; Dako, Glostrup, Denmark).

**Immunofluorescence microscopy**

Cells were plated in ibiTreat µ-Slide 8 Well (Ibidi, Martinsried, Germany). When 80% confluent, they were fixed in 4% paraformaldehyde in PBS for 10 minutes at 4°C. Samples were blocked and permeabilized in 10% newborn calf serum (Sigma-Aldrich), 0.3% Triton X-100 (Sigma-Aldrich) in
PBS for 1 hour at room temperature. Samples were incubated 16 hours at 4°C in primary antibody
diluted in 5% newborn calf serum, 0.15% Triton X-100 in PBS. After three 30-minutes washes in
PBS, sample were incubated 16 hours at 4°C in secondary antibodies diluted in 5% newborn calf
serum, 0.15% Triton X-100 in PBS. After three 30-minutes washes in PBS, samples were mounted
in Fluoroshield containing DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich). Microscopies
used for imaging were Leica DM5500B equipped with a Leica DCF3000 G camera (Leica
Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) and Axiovert 200 inverted microscope (Carl
Zeiss, Oberkochen, Germany). Primary antibodies were as follows: α-III tubulin (mouse, 1:500;
Abcam), Spartin (rabbit, 1:1,000; ProteinTech), Nestin (mouse, 1:300; Abcam) and PGP9.5 (rabbit,
1:300; Thermo Fisher Scientific), and secondary antibodies: Alexa Fluor 488 goat anti-rabbit IgG,
Alexa Fluor 488 donkey anti-mouse, Alexa Fluor 555 goat anti-rabbit and Alexa Fluor 555 donkey
anti-mouse (all diluted 1:800; Abcam). Quantitative evaluation of α- tubulin, Spartin and Nestin
fluorescence intensity have been performed using ImageJ.

**RNA isolation and quantitative PCR (qPCR) in cell lines**

Total RNA was isolated from SH-SY5Y cultures using the RNeasy Mini Kit (QIAGEN). cDNA
from 1 µg of \(SPART^{\text{wt}}\) and \(SPART^{892\text{dupA}}\) RNA was synthesized using the SuperScript™ VILO™
cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR was performed using SYBR®
Green master mix (Bio-Rad). All samples were run in triplicate on the ABI7500 Fast PCR machine
(Thermo Fisher Scientific). Melting curve analysis for each primer pair was carried out to ensure
specific amplification. Relative mRNA expression levels of \(CAT, SOD1, SOD2\) were normalized to
the house-keeping gene β-actin using the \(\Delta\DeltaCt\) method. Primers are reported in Supplementary
Table 1.

**Quantification of neurite outgrowth and elongation**
Cells were seeded on Poly-L-Lysine (Sigma-Aldrich) coated glasses and immunofluorescence for
the neuronal marker PGP9.5 was performed as described above. Mean neurite number and length
was measured using the NeuronGrowth plugin (ImageJ, National Institute of Health, Bethesda) by
tracing the individual neurites of cells according to Fanti et al. (25). For each experiment twenty
cells were examined.

**Spheroid formation assay**

We generated matrix-free SH-SY5Y spheroid cultures by seeding 2x10^4 SPART^wt and SPART^892dupA
SH-SY5Y cells in ultra-low attachment µ-slide 8-wells (Ibidi). Cells were grown to allow the
spontaneous formation of spheroids. After 4 days, spheroids were examined and photographed
using an Axiovert 200 inverted microscope (Carl Zeiss), then RNA was extracted. Spheroids
morphology was measured using ImageJ software.

**ROS quantification**

*Intracellular ROS measurement using DCFDA*

Control and SPART^892dupA SH-SY5Y cell lines were seeded at 5 x 10^3 cells/well and incubated
overnight. Cells were treated with 10 µM DCFDA (2′,7′-dichlorofluorescin diacetate, Sigma-
Aldrich) dissolved in medium for 1 hour. Cells were washed with PBS and the fluorescence
emission from each well was measured (λ Excitation = 485 nm; λ Emission = 535 nm) using a
multi-plate reader (Enspire, Perkin Elmer, Waltham, MA, USA) and normalized for protein content
using a Lowry assay. Data are reported as the mean ± standard deviation of at least three
independent experiments.

*Mitochondrial ROS measurement using MitoSOX*

Mitochondrial superoxide production was measured using MitoSOX™ Red (Molecular Probes,
Thermo Fisher Scientific) following manufacturer instructions with minor modifications. Briefly,
cells were seeded in 96-well plates (OptiPlate black, Perkin Elmer) at 5 x 10^3 cells/well and
incubated for 16 hours to allow adhesion. Cells were then treated with 5 μM MitoSOX Red dissolved in medium for 30 minutes. Cells were washed twice times with warm PBS and the fluorescence emission from each well was measured (λ. Excitation = 510 nm; λ. Emission = 580 nm) using a multi-plate reader (Enspire, Perkin Elmer) and normalized for protein content using a Lowry assay. Data are reported as the mean ± standard deviation of at least six independent experiments.

*Mitochondrial network and morphology assessment via live cell imaging*

To visualize the mitochondrial network in live cells, 3×10^4 cells were plated in ibiTreat μ-Slide 15 Well (Ibidi, Germany) in 50 μl of complete medium and incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂. After 24 hours, cells were transfected with a plasmid carrying the GFP protein targeted to mitochondria, following the manufacturer’s instructions (CellLight™ Mitochondria-GFP, BacMam 2.0, Thermo Fisher Scientific). Mitochondrial network morphology was assessed by live cell imaging, using a Nikon C1si confocal microscope (Nikon, Tokio, Japan) following the procedure of Dagda et al. (26) with minor modification using ImageJ software. Briefly, the green channel was subjected to both a background subtraction with a radius of 10 pixels and a median filter to reduce noise. An automatic threshold value was then applied to delineate the particles. The mean area/perimeter ratio was employed as an index of mitochondrial interconnectivity according to the previously published method reported in Dagda et al. (26).

*Mitochondrial three-dimensional (3D) network analysis*

In order to visualize the 3D structure of the mitochondrial network, cells were plated in ibiTreat μ-Slide 8 Well (Ibidi, Martinsried, Germany). After 24 hours, cells were transfected with a plasmid carrying the GFP protein targeted to mitochondria as described above. The mitochondrial 3D network analysis was performed according to Giuliani et al. (27). Briefly, cell preparations were scanned with a Nikon Ti-E fluorescence microscope coupled to an A1R confocal system and the
NIS-Elements AR 3.2 software. An air-cooled argon-ion laser system with 488 wavelength output was used. Images were acquired with oil immersion (60x) with an optical resolution of 0.18 micron, 3 x scanner zoom, and 1024 x 1024 pixel resolution. All the stacks were collected with optical section separation (interval) values suggested by the NIS-Elements AR 3.2 software (0.5µm step). Five randomly selected fields per sample were acquired. 3D images were analyzed by the IMARIS software (Bitplane, Concord, MA, USA). The software analyzes the volumes of all detected isosurfaces and calculates the average value, corresponding to the average volume of interconnected mitochondria per cell. This analysis allows to measure the average volume of single interconnected mitochondrial isosurfaces, directly linked to mitochondrial fragmentation.

**Mitochondrial oxygen consumption**

To measure mitochondrial oxygen consumption in *SPART*<sup>wt</sup> and *SPART*<sup>892dupA</sup> cells, 1.5x10<sup>6</sup> cells for each cell line were harvested at 70-80% confluence, washed in PBS, re-suspended in complete medium and assayed for oxygen consumption at 37°C using a thermostatically regulated oxygraph chamber (Instech Mod.203, Plymouth Meeting, PA, USA) in 1.5 ml of culture medium. Basal respiration was compared with the one obtained after injection of oligomycin (1 µM). FCCP (1–6 µM). Antimycin A (5 µM) was added at the end of experiments to completely block mitochondrial respiration. The respiratory rates were expressed in µmol O₂/min/mg of protein referring to 250 nmol O₂/ml of buffer as 100 % at 30°C (28). Data were normalized to protein content determined using the Lowry assay.

**ATP and ADP determination**

Nucleotides were extracted and detected following Jones DP, 1981 (29), using a Kinetex C18 column (250 × 4.6 mm, 100 Å, 5 µm; Phenomenex, CA, USA). Absorbance (260 nm) was monitored with a photodiode array detector (Agilent 1100 series system). Nucleotide peaks were identified by comparison and coelution with standards, and quantification by peak area
measurement compared with standard curves. The ATP level was also measured in presence or absence of rotenone (a specific Complex I inhibitor, Sigma-Aldrich). $4 \times 10^5 \text{SPART}^{\text{wt}}$ and $\text{SPART}^{892\text{dupA}}$ cells were seeded and treated with 200 nM of rotenone for 72 hours, then ATP level was measured as describes above.

**Respiratory chain complex activities**

Cell lysates were resuspended in a 20 mM hypotonic potassium phosphate buffer (pH 7.5) followed by spectrophotometric analysis of mitochondrial complexes activity at 37°C using a Jasco-V550 spectrophotometer (Jasco, Easton, MD, USA) equipped with a stirring device. Complex I activity was measured in 50 mM phosphate buffer at 340 nm ($\varepsilon=6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) after the addition of 150 µg of cell lysate, 1 mM KCN, 10 µM antimycin a, 2.5 mg fatty acid-free BSA, 100 µM NADH, 60 µM decylbenzoquinone (DB, Sigma-Aldrich). Complex I (NADH dehydrogenase) specific activity was obtained by inhibiting complex I with 10 µM rotenone. The succinate-cytochrome c reductase activity (II+III activity) was measured in 50 mM phosphate buffer at 550 nm ($\varepsilon=18.5 \text{ mM}^{-1} \text{ cm}^{-1}$) after the addition of 100 µg of cell lysate, 1 mM KCN, 20 mM succinate and 50 µM oxidized cytochrome c. The specific complex II+III activity was obtained by inhibiting complex II with 500 µM TTFA. The NADH-cytochrome c reductase activity (I+III activity) was measured in 50 mM phosphate buffer at 550 nm ($\varepsilon=18.5 \text{ mM}^{-1} \text{ cm}^{-1}$) after the addition of 100 µg of cell lysate, 1 mg/ml of fatty acid-free BSA, 1 mM KCN, 50 µM cytochrome c and 200 µM NADH. The specific complex I+III activity was obtained by inhibiting complex I with 10 µM rotenone. Citrate synthase activity was measured in 100 mM TRIS buffer with 0.1% Triton X-100 at 412 nm ($\varepsilon=13,600 \text{ M}^{-1} \text{cm}^{-1}$) after the addition of 30 µg of cell lysate, 0.1 mM acetyl-CoA, 0.5 mM oxaloacetate, and 0.1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB; Sigma-Aldrich).

**Mitochondrial transmembrane potential ($m\Delta\psi$)**

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Mitochondrial transmembrane potential and mass were measured following Kirk et al. (30) with minor modifications. Briefly, cells were seeded at the density of $10^4$ cells/well in 96-well culture plates (OptiPlate Black, Perkin Elmer). After 24 hours, cells were loaded with 50 nM tetramethylrhodamine methyl ester (TMRM, 544 Ex; 590 Em, Thermo Fisher Scientific) and 25 nM MitoTracker Green (MTG, 490ex; 516em, Thermo Fisher Scientific) for 30 minutes and washed twice with PBS. The fluorescence emission from each well was measured with a multi-plate reader (Enspire, Perkin Elmer). TMRM fluorescence emission intensity was normalized by comparing to MitoTracker Green fluorescence.

**NADH quantification**

NADH autofluorescence measurements were performed as described by Frezza et al., 2011 (31) with minor modifications. Briefly, cells were seeded at a density of $3\times10^3$ cells/ well in 15-well µ-Slides (Ibidi) following manufacturer’s instructions and incubated for 16 hours to allow adhesion. Images were collected using a Nikon C1si confocal microscope equipped with UV laser. NADH quantification was performed using ImageJ software after background subtraction.

**Lactate and pyruvate quantification**

Extracellular lactate was determined by HPLC (High Performance Liquid Chromatography). Briefly, cells were seeded in 6-well dishes and after 72 hours the culture medium was collected for HPLC analysis. Prior to injection, the culture medium was diluted 1:10 in mobile phase and centrifuged at 14,000 g for 5 minutes at 4°C. The supernatant was then injected manually into the HPLC system. Metabolites were separated on a C18 column (Agilent ZORBAX SB-Phenyl, 5 µm, 250×4.6 mm, Santa Clara, CA, USA), using a mobile phase consisting of 50 mM KH$_2$PO$_4$, pH 2.9, at a flow rate of 0.8 ml/min. Lactate and pyruvate were detected using an Agilent UV detector set to 210 nm and quantified using Agilent ChemStation software. The retention time was determined by
injecting standard solution. All injections were performed in triplicate. The peak area was normalized for protein content as measured using a Bradford assay.

**Measurement of intracellular Ca\(^{2+}\)**

Intracellular calcium level was assessed in live cells using Fura-2 AM probe (Thermo Fisher Scientific) following manufacturer’s instruction. The emission of the calcium-free probe was measured using a Nikon C1si confocal microscope (Nikon, Tokio, Japan) at \(\lambda = 380\) nm Excitation and \(\lambda = 515\) nm Emission. The quantification of fluorescence intensity was carried out using the ImageJ software, with an automated acquisition of Fura-2 AM probe fluorescence (at least 50 cells per condition were acquired).

**Rescue of the phenotype with Spartin wild type**

Human \(SPART\) coding sequence was PCR-amplified from SH-SY5Y-derived cDNA using the KAPA HiFi HotStart Taq Polymerase (Kapa Biosystems, Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. Primers are reported in Supplementary Table 1. The amplified fragment was digested with \(XhoI\) and \(HindIII\) (New England Biolabs, Hitchin, UK), cloned in pcDNA3.1 vector and sequenced to verify the correct insertion. For rescue experiments, \(4 \times 10^5\) \(SPART^{wt}\) and \(SPART^{892dupA}\) cells were plated. The plasmids expressing wild-type \(SPART\) (3 \(\mu\)g/experiment) was transfected into \(SPART^{892dupA}\) cells using Lipofectamine 3000 (Life Technologies) following the manufacturer’s instructions. In parallel, the pcDNA3.1 empty vector (3 \(\mu\)g/experiment) was transfected into \(SPART^{wt}\) and \(SPART^{892dupA}\) cells. Forty-eight hours after transfection, cells were pelleted and washed twice with PBS. Western blot analysis was used verify Spartin overexpression, determination of ATP and ADP synthesis and assessment of intracellular \(Ca^{2+}\) were performed as previously described.
Gene expression of spg20b in zebrafish developmental stages and zebrafish adult tissues

Total RNA from developmental stages between 16–32 cells, up to 120 hours post-fertilization (hpf) was extracted using the RNeasy Mini kit according to the manufacturer’s instructions (QIAGEN) using at least 50 embryos at each stage. Heart, liver and brain were dissected from 5 adult fish, flash-frozen on dry ice and stored at -80°C until the RNA was extracted. Animals were handled following the guidelines from European Directive 2010/63/EU and euthanised with Schedule 1 procedures of the Home Office Animals (Scientific Procedures) Act 1986. RNA was synthesized using the SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific) following the manufacturer’s protocol. Gene expression level of spg20b was assessed using quantitative PCR (qPCR) conducted with the SYBR Green master mix (Bio-Rad). All samples were run in triplicate on the ABI7500 Fast PCR machine (Thermo Fisher Scientific). Relative mRNA expression level of spg20b was normalized to the eukaryotic translation eef1a1l2 as endogenous control gene. Primers are reported in Supplementary Table 1. All zebrafish studies were approved by the Animal Welfare and Ethics Committee at the University of St. Andrews.

Image analysis

Images were analyzed using ImageJ (public software distributed by the National Institutes of Health), Chemidoc (Bio-Rad) and IMARIS (Bitplane).

Statistical analysis

Statistical analysis was conducted with Prism 7 (GraphPad, San Diego, CA, USA). All experiments were carried out at least in triplicates. Results are expressed as the mean ± SEM. Unpaired Student’s t-test with Welch’s correction, Fisher’s exact test or ANOVA test with Tukey-multiple comparison test were used to determine the differences between groups, when appropriate. A p-value <0.05 (two-tailed) was considered statistically significant.


**Results**

**Whole exome sequencing identified a novel loss-of-function mutation in SPART gene**

We performed a combined analysis of high-density SNPs genotyping and WES in the two affected brothers (Fig. 1A: II-1, II-2) born to consanguineous partners. The two siblings presented with a history of intrauterine growth restriction, stature and weight below -2 SD, relative macrocrania, dysmorphic features (very long eyelashes, dolichocephaly, prominent maxilla, pectus excavatum, Fig. 1B) and psychomotor retardation, with severe language delay (for an exhaustive description of cases see the Materials and Methods section). In consideration of the degree of inbreeding, a search for runs of homozygosity (ROH) using SNP data from Illumina 350K array identified a region of homozygosity on chromosome 13 (5 Mb). WES analysis identified a novel insertion on chr13:g3690561insT (hg19), leading to a c.892dupA (NM_001142294) in the SPART gene (OMIM *607111). The mutation was homozygous in the two affected sibs and caused a frameshift with the insertion of a premature stop codon (p.Thr298Asnfs*17) in the protein Spartin. The variant was carried by the parents (Fig. 1A, I-1 and I-2) and was not present in the Exome Aggregation database (ExAc) and Genome Aggregation database (gnomAD) (as accessed on 20/11/2018), nor in an in-house whole exome database consisting of 650 exomes.

**Generation of biological models**

To understand the effect of SPART ablation we generated two biological models. First, we transiently silenced SPART with siRNA in human Neural Stem cells (hNSCs), using siRNA specific to SPART transcripts vs cell treated with scramble siRNA. Silencing efficiency was evaluated using western blot analysis (Supplementary Fig. 1B). Second, we generated a stable SH-SY5Y cell line with a knock-in of the c.892dupA mutation using the CRISPR/Cas9 technology. Cells were transfected with paired gRNAs and Cas9-nickase plasmids and the oligo DNA carrying the
c.892dupA variant to insert the specific modification into the SH-SY5Y genome (Fig. 1C). The variant is predicted to generate a shorter protein of 33KDa, however western blot analysis showed that Spartin in \( \text{SPART}^{892\text{dupA}} \) cells was completely absent (Fig. 1D).

**Spartin depletion affects neuronal morphology leading to neuronal differentiation**

To understand the effect of \( \text{SPART} \) loss on neuronal morphology, we transiently silenced the gene in hNSCs up to 8 days of treatment, using siRNA specific to \( \text{SPART} \) transcripts vs scrambled siRNA (Fig 2A, B). In \( \text{SPART} \)-silenced hNSCs, we observed an increased neurite outgrowth, visualized via nestin staining (Fig. 2A, panel f and Fig. 2B, panel a) compared to scramble-treated hNSCs (Fig. 2A, panel e and Fig. 2B, panel b). After 8 days of \( \text{SPART} \) silencing, we found no significant differences in \( \alpha \)-tubulin and nestin expression between scramble-transfected and \( \text{SPART} \)-silenced hNSCs (Student’s t-test with Welch’s correction, \( p=0.5394 \) and \( p=0.9883 \) respectively, Supplementary Fig. 1C-D). At day 8, in agreement with western blot data (Supplementary Fig. 1B), Spartin staining was not detectable in \( \text{SPART} \)-silenced hNSCs, as compared with those treated with scrambled siRNA (Student’s t-test with Welch’s correction, \( p<0.0001 \), Supplementary Fig. 1E).

In the stable knock-in cell line carrying the c.892dupA mutation, immunostaining for PGP9.5 (a specific neuronal marker localized to cell bodies and neurites) revealed an altered neuronal morphology compared to control \( \text{SPART}^{\text{wt}} \) cells (Fig. 2C). \( \text{SPART}^{892\text{dupA}} \) cells showed extensive and branched neurite-like formations (Fig. 2C, panels g, h) compared to \( \text{SPART}^{\text{wt}} \) (Fig. 2C, panels c, d). Neuritogenesis was measured using a quantitative evaluation of the length and number of neurites using the NeuronGrowth software. \( \text{SPART}^{892\text{dupA}} \) cells showed significantly longer neuronal processes, compared to \( \text{SPART}^{\text{wt}} \) cells (Student’s t-test with Welch’s correction, \( p=0.0001 \); Supplementary Fig. 1F) and an increase in average number of neurites per cell extending from the cell body (Student’s t-test with Welch’s correction, \( p=0.0337 \); Supplementary Fig. 1G).

Moreover, Spartin-depleted cell models (both hNSC and SH-SY5Y) presented an extensive cell loss compared to controls (Fig. 2D, 2E).
Spartin loss alters cell morphology in 3D cultures

Since the data on $SPART^{892dupA}$ cell extended neurites suggested an altered neuronal differentiation, we evaluated cell morphology in a matrix-free environment. When cells were shifted from a 2D to a 3D micro-environment (spheroids), $SPART^{wt}$ and $SPART^{892dupA}$ cultures showed a different morphology. Twenty-four hours after seeding both cell lines started to aggregate, and they grew into spheroids-like structures at 72 hours (Fig. 3A). $SPART^{wt}$ cells formed condensed and disorganized aggregates (Fig. 3A, panels b, c), whereas $SPART^{892dupA}$ cells formed rounder and more compact aggregated more similar to spheroids (Fig. 3A, panels e, f). These differences, already detectable at 24 hours, were confirmed by the quantitative measurement of spheroid circularity at 24 and 72 hours, using ImageJ software. We found increased circularity for $SPART^{892dupA}$-derived spheroids compared to $SPART^{wt}$, both at 24 and 72 hours (Student’s t-test with Welch’s correction, p<0.0001 and p=0.0001 respectively, Fig. 3B). According to the classification of Kenny et al. (32), $SPART^{892dupA}$-derived spheroids could be classified as “round group”, characteristic of non-malignant and more differentiated cells, since there was a prevalence of rounded spheroids. $SPART^{wt}$-derived spheroids could be classified in the “grape-like” group, characterized by cancer stem cell-like properties, with high frequency of disorganized formations (88 round spheroids and 9 grape-like spheroids for $SPART^{892dupA}$ cells vs 11 round spheroids and 55 grape-like spheroids for $SPART^{wt}$ cells; Fisher’s exact test, p<0.0001, Fig. 3C, D).

Spartin loss alters the mitochondrial network

Previous studies showed that Spartin co-localized with mitochondria and contributed to mitochondrial stability (16, 18, 33). Therefore, we evaluated the effects of the c.892dupA mutation on different mitochondrial characteristics. Assessment of mitochondrial network was performed using live-cell microscopy in mitoGFP-transfected cells (Fig. 4A). The total mitochondrial mass per cell was similar between $SPART^{wt}$ and $SPART^{892dupA}$ cells (Supplementary Fig. 1H) but decrease in
the total number of mitochondria was observed in neurite-like extensions in mutants compared to controls (Fig. 4A, arrows). \textit{SPART}^{892dupA} cells displayed a fragmented and disorganized mitochondrial network morphology (Fig. 4A, right panel) compared to control cells (Fig. 4A, left panel). In particular, we found a decrease of the mean perimeters (Student’s t-test with Welch’s correction, \(p=0.0242\)), mean areas (\(p=0.0117\)), and an increase of roundness value (\(p<0.0001\)) of mitochondria in mutant cells compared to controls (Fig. 4B; mitochondria in \(n=35\) cells for each cell lines were measured). Moreover, mitochondria in \textit{SPART}^{892dupA} cells exhibited a decreased interconnectivity, indicated by a decreased area-to-perimeter ratio, compared to the \textit{SPART}^{\text{wt}} control cells (Student’s t-test with Welch’s correction, \(p<0.0001\); Fig. 4C).

In order to further analyze the altered connectivity of the mitochondrial network due to Spartan loss, we performed a three-dimensional acquisition of mitochondrial-GFP transfected cells with confocal microscopy. Z-stacks were analyzed using IMARIS software, in order to quantify the mean volume of the isosurfaces reconstructed from GFP fluorescence and representing the mitochondrial network. The 3D view of the mitochondrial network emphasized the fragmented organization in cells lacking Spartan (Fig. 4D). The analysis showed a strong reduction in the mean volume per cell of the elaborated network, in \textit{SPART}^{892dupA} compared to \textit{SPART}^{\text{wt}} (Student’s t-test with Welch’s correction, \(p=0.0237\); Fig. 4E). These data support the hypothesis that Spartan loss might cause mitochondrial network fragmentation.

**Spartin loss determines a specific OXPHOS Complex I impairment**

Mitochondrial morphology reflects its functionality (34), therefore we measured the oxygen consumption rates in \textit{SPART}^{\text{wt}} and \textit{SPART}^{892dupA} intact cells, in absence and in presence of the specific ATPase inhibitor oligomycin A and of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) as uncoupling agent. No difference in endogenous respiration (basal) was observed, but the uncoupled oxygen consumption (FCCP) was significantly decreased in \textit{SPART}^{892dupA} mutant cells (\(n=3\) independent experiments, one-way ANOVA with Tukey’s multiple
comparisons test, p=0.0173; Fig. 5A). We found that cells lacking Spartin exhibited a significantly lower ATP/ADP ratio in comparison to controls, due to the concomitant decrease of ATP and increase of ADP levels (n=3 independent experiments, Student’s t-test with Welch’s correction, p=0.0065; Fig. 5B). We investigated the OXPHOS enzyme activities by measuring: NADH-cytochrome c oxidoreductase activity (Complex I+III); succinate dehydrogenase-cytochrome c oxidoreductase activity (Complex II+III) and NADH-DB oxidoreductase activity (Complex I).

**SPART**892dupA mutant cells showed a 50% decrease of Complex I+III and Complex I activities (n=3 independent experiments, Student’s t-test with Welch’s correction, p<0.0001 and p=0.0268 respectively; Fig. 5C, D), whereas no difference compared to controls was found for Complex II+III activity (p=0.6485; Fig. 5E). These data show that cells lacking Spartin presented a Complex I impairment. To further investigate the effect of Spartin on Complex I activity, we measured the ATP level in **SPART**wt and **SPART**892dupA grown for 72 hours in the presence and absence of 200 nM of the specific Complex I inhibitor rotenone. **SPART**wt cells were significantly impaired in ATP synthesis in presence of rotenone (n=4 independent experiments, one-way ANOVA with Tukey’s multiple comparisons test, p=0.0077; Fig. 5F), whereas **SPART**892dupA were less sensitive to rotenone, since their Complex I activity was already severely impaired by the mutation in **SPART** (p=0.5496, Fig. 5F).

Consistently with an impaired OXPHOS Complex I activity, we observed a significant reduction (20%) in mitochondrial membrane potential in **SPART**892dupA cells, compared to control cells (n=12 independent experiments, Student’s t-test with Welch’s correction, p<0.0001; Fig. 5G).

**Altered pyruvate metabolism in **SPART**892dupA mutant cells**

Since OXPHOS respiration was impaired, we investigated a possible metabolic switch to glycolysis. We measured intracellular NADH levels and extracellular lactate. In **SPART**892dupA mutated cells, the intracellular NADH level was increased in comparison to control cells (13 fields for each cell type, Student’s t-test with Welch’s correction, p=0.0026, Fig. 6A). HPLC analysis of
extracellular culture medium did not detect any change in extracellular lactate levels (Fig. 6B). Nevertheless, in the extracellular culture medium of \textit{SPART}^{892dupA} cells, we identified a 2.5 folds increase in pyruvate levels in comparison to controls (n=3 independent experiments, Student’s t-test with Welch’s correction, \textit{SPART}^{892dupA} = 231.4±27.65 vs \textit{SPART}^{wt} = 77.56±7.639; p=0.0241, Fig. 6C-D).

**STAT3 activation in \textit{SPART}^{892dupA} mutant cells**

Decreased oxidative phosphorylation has been correlated to STAT3 activation, and constitutive activation of STAT3 in several cell models indicated a major role for this transcription factor in promoting increased glycolysis (35). Thus, we investigated the phosphorylation/activation status of STAT3 in mutant and control cell lines using western blot analysis of phosphorylated (p-STAT3) and total (T-STAT3) STAT3 protein. In \textit{SPART}^{892dupA} cells STAT3 was phosphorylated, whereas no activation was observed in \textit{SPART}^{wt} cells. Comparable levels of total STAT3 were present in both cell types (Fig. 6E).

**Spartin loss increased mitochondrial Reactive Oxygen Species (ROS) and altered intracellular Ca$^{2+}$ homeostasis**

Increases in cellular superoxide production are implicated in a variety of pathologies, including neurodegeneration (36). Mitochondrial superoxide is generated as a by-product of oxidative phosphorylation and in healthy cells occurs at a controlled rate. Given the specific NADH-dehydrogenase activity (Complex I) impairment detected in mutant cells, we investigated whether ROS production was altered by Spartin loss. Intracellular ROS levels, measured with the fluorescent probe 2',7'--dichlorofluorescin diacetate (DCFDA), were significantly increased in \textit{SPART}^{892dupA} cells (n=3 independent experiments, Student’s t-test with Welch’s correction, p<0.0001; Fig. 7A). Moreover, by staining live cells with MitoSOX™ Red, a highly selective probe
for mitochondria-specific superoxide, we found a significant increase in superoxide production in

\( SPART^{892\text{dupA}} \) cells compared to the control (\( n=3 \) independent experiments, Student’s t-test with

Welch’s correction, \( p<0.0001; \) Fig. 7B). To further investigate the oxidative stress status of mutated
cells, we tested the expression of the major ROS-detoxifying enzymes. RT-qPCR revealed a
significant reduction in expression of \( CAT \) (Catalase), \( SOD1 \) (Superoxide Dismutase) and \( SOD2 \)
(mitochondrial Manganese Superoxide Dismutase) in cells lacking Spartin, compared to controls
(\( n=3 \) independent experiments, Student’s t-test with Welch’s correction, \( p=0.0027 \) for \( CAT; \)
\( p=0.0280 \) for \( SOD1; \) \( p=0.0172 \) for \( SOD2 \); Fig. 7C-E, respectively).

Glucose-Regulated Protein 75 (GRP75) has a major role in neuronal cells for mitochondrial
function regulation and protection from stress-induced ROS and physically interacts with Spartin (36, 37). Therefore, we evaluated its protein levels, and found a higher expression of GRP75 in

\( SPART^{892\text{dupA}} \) compared to \( SPART^{\text{wt}} \) cells (\( n=3 \) independent experiments, Student’s t-test with
Welch’s correction, \( p=0.0327; \) Fig. 7F).

GRP75 also coordinates the exchange and transfer of \( \text{Ca}^{2+} \), thereby affecting mitochondrial function
and intracellular \( \text{Ca}^{2+} \) homeostasis (37). Accordingly, we assessed intracellular free \( \text{Ca}^{2+} \) in

\( SPART^{\text{wt}} \) and \( SPART^{892\text{dupA}} \) cells by quantifying \( \text{Ca}^{2+} \) probe Fura-2 AM-relative fluorescence. We
found a significant increase of intracellular \( \text{Ca}^{2+} \) in \( SPART^{892\text{dupA}} \) cells, compared to \( SPART^{\text{wt}} \) cells
(\( n=3 \) independent experiments, Student’s t-test with Welch’s correction, \( p<0.0001; \) Fig. 7G). These
results are consistent with previous data showing that the decreased expression of Spartin via
transient silencing led to a dysregulation of intracellular \( \text{Ca}^{2+} \) levels (18).

In order to provide additional evidence that these observed defects were specifically due to Spartin
absence, we re-expressed Spartin in \( SPART^{892\text{dupA}} \) cells. Spartin re-expression in \( SPART^{892\text{dupA}} \) cells
rescued altered intracellular \( \text{Ca}^{2+} \), restoring \( \text{Ca}^{2+} \) levels as in control cells (\( n=3 \) independent
experiments, one-way ANOVA Tukey’s multiple comparisons test, \( SPART^{\text{wt}} \) vs. \( SPART^{892\text{dupA}} \)
\( p<0.0001; \) \( SPART^{\text{wt}} \) vs. \( SPART^{892\text{dupA}}+ SPART^{\text{wt}} \) \( p=0.5690; \) \( SPART^{892\text{dupA}} \) vs. \( SPART^{892\text{dupA}}+ SPART^{\text{wt}} \)
\( p<0.0001; \) Fig. 7H).
Discussion

Next generation sequencing (NGS) technologies are powerful tools for the identification of rare mutations that cause neurodevelopmental disorders (38-44). The identification of a causative mutation can support diagnosis, prognosis, and available treatment (45). Using WES technology, we identified a novel homozygous insertion (c.892dupA) in the SPART gene in two male sibs with syndromic short stature, developmental delay and severe speech impairment, with consanguineous healthy parents. This novel SPART mutation generated a frameshift and a premature stop codon in the Spartin protein. Loss-of-function mutations in SPART cause Troyer syndrome (OMIM 275900), a very rare recessive form of HSP (3-6). Although Troyer syndrome was not firstly diagnosed, the WES data prompted a thorough clinical reassessment, that identified muscular hypotrophy in upper and lower limbs, increased muscle tone in lower limbs (distal>proximal) and brisk deep tendon reflexes, all symptoms characteristic of this specific HSP form due to mutations in the SPART gene (46).

Nonetheless, so far very few SPART loss-of-function mutations have been reported (3, 4, 6, 47-51, Supplementary Table 2). Animal (null mice) and cellular models (Spartin silencing/overexpression) showed a role for Spartin in different processes, including neuronal survival/sprouting, cell division and mitochondrial stability (7, 8, 10, 12, 13, 19).

Based on this evidence, we focused our functional studies to investigate the effect of SPART c.892dupA mutation on mitochondrial network integrity and mitochondrial functionality. We used two different cell models: hNSCs (silenced for SPART) and SH-SY5Y cell line genome-edited via CRISPR/Cas9 technology to introduce the mutation c.892dupA.

We found that both hNSCs silenced for SPART and SH-SY5Y cells carrying the SPART loss-of-function mutation presented significant neurite outgrowth/length coupled to extensive cell loss (Fig. 2), in line with the data observed in animal models (14). Gene expression analysis in zebrafish embryos identified high expression of the SPART homologous (spg20b) through the initial phases, during cleavage and blastula periods - 0.75-5 hours post fertilization (Supplementary Fig. 2A).
adult zebrafish brain, spg20b expression was 18 times higher than in heart and liver tissues (Supplementary Fig. 2B).

We observed significant changes in the mitochondrial network and significant mitochondrial fragmentation in absence of Spartin (Fig. 4D-E) with possible detrimental effects on dendrites and axons during synaptic transmission (52, 53).

In concordance, we observed that SPARTdup892A cells presented a mitochondrial impaired respiration with decreased ATP synthesis specifically due to a decreased Complex I activity (Fig. 5), with a reduced mitochondrial membrane potential and increased oxidative stress (with concomitant decreased expression of ROS detoxifying enzymes; Fig. 7). These data demonstrated that Spartin depletion led to mitochondrial Complex I deficiency. We propose that the mitochondrial impairments found in SPARTdup892A cell possibly contribute to neurodegeneration (Fig. 8A, B).

Perturbations of mitochondrial dynamics or energetic imbalances underpin many neurodegenerative disorders, often with overlapping clinical features such as HSP, Alzheimer’s disease, Parkinson’s disease, Amyotrophic Lateral Sclerosis and Huntington’s disease (54). All neuronal cellular processes are energy demanding and require significantly active mitochondria. Moreover, neurons with long axons, such as peripheral sensory neurons and motor neurons, are more susceptible to neurodegeneration, since they are more sensitive to mitochondrial defects (55).

It is intriguing to note that another form of HSP is due to mutations in spastic-paraplegia-7 gene (SPG7), encoding for Paraplegin, a protein forming large complexes in the inner membrane of mitochondria (56, 57). Loss-of-function mutations in SPG7 lead to a defective Complex I assembly and consequent defective respiratory chain activity (58). Like Paraplegin, Spartin also interacts with GRP75 (Fig. 8A), a member of mitochondrial complex for the import of nuclear-encoded proteins into the mitochondria (37). We hypothesize a role for Spartin in assembly or stability of Complex I, similar to what described for Paraplegin (58). In line with this hypothesis, the increased levels of GRP75 in SPART mutant cells might indicate a compensatory effect for Spartin absence (Figure 7F).
In addition to mitochondrial defects, mutant cells presented also specific metabolite imbalances. We identified for the first time an excess of pyruvate in the context of Troyer syndrome (Fig. 6C). Pyruvate is directed into mitochondria through the mitochondrial pyruvate carrier (MPC) located in the mitochondrial inner membrane (MIM). Here, pyruvate functions as fuel input for the citric acid cycle and for mitochondrial ATP generation. Disruption in pyruvate metabolism affects tissues with high demand for ATP. The nervous system is particularly vulnerable because of its high demand of carbohydrate metabolism for ATP generation (59). A previous study in yeast indicated a protective effect of Spartin overexpression on PDH activity, a key enzyme of glucose metabolism that converts pyruvate into acetylCoenzyme A (AcCoA) (19). Our study in a human neuronal cell model identified an increased pyruvate excretion, possibly underlying an impaired pyruvate metabolism, in absence of Spartin, due to the novel human SPART mutation (Fig. 8A, B).

Furthermore, Spartin loss was associated with a constitutive phosphorylation (hence activation) of STAT3 (Fig. 6E). STAT3 activation promoted a faster neurite outgrowth (60). Nevertheless, constitutive STAT3 activation was shown to increase glycolysis and decrease oxidative phosphorylation, counteracting PDH activity (35).

PDH inhibition has been also reported in presence of oxidative stress (61-63) and decreased mitochondrial Ca^{2+} levels (64). It is worth noting that the GRP75/VDAC (Voltage-dependent anion channel) complex regulates mitochondrial Ca^{2+} intake from the endoplasmic reticulum, thereby affecting mitochondrial function and intracellular Ca^{2+} homeostasis (37, 65). Consistently, we observed an increase in intracellular Ca^{2+} in SPART^{892dupA} cells compared to SPART^{wt} cells (Fig. 7G), which was restored to normal levels with the re-expression of Spartin (Fig. 7H). In accordance to Joshi and Bakowska (18), these results suggest that Spartin loss itself affects trafficking and buffering cytosolic and mitochondrial Ca^{2+} via GRP75 interaction.

In summary, through a WES study we were able to assign a diagnosis of Troyer syndrome to otherwise undiagnosed patients and we provided for the first time a thorough assessment of Spartin mutations in a human neuronal cell model. Functional characterization elucidated that the novel
mutation in \textit{SPART} led to a profound bioenergetic imbalance, including impaired Complex I activity coupled to increased extracellular pyruvate. Our data support the hypothesis that Spartin coupled with GRP75 might modulate mitochondrial protein import and Ca$^{2+}$ levels, maintaining low ROS levels and a normal ATP production (Fig. 8A). Spartin loss determines an energetic failure, due to altered Complex I function, with increased ROS production and altered intracellular Ca$^{2+}$ possibly leading to reduced PDH activity (59). Therefore, pyruvate is not efficiently converted into acetyl-coA (AcCoA) and is accumulated and excreted from \textit{SPART} mutant cells (Fig. 8B).

Hence, we propose that Troyer syndrome due to \textit{SPART} mutations might be considered a mitochondrial disease. We propose that the observed neuronal phenotypes result from defective protein assembly within mitochondria or defective mitochondrial Complex I function, coupled to an excess of pyruvate and ROS production, generating energetic imbalances that have been already connected to several neurodegenerative disorders, as mentioned previously (54). As the expansion of personalized medicine proceeds, with increasing potential for active analyses of genomic data, the early identification of molecular and genetic defects can lead to a better clinical refinement and the possibility of applying timely targeted therapies. Most importantly, it is crucial to carry out the functional characterization of proteins and mutations, to move forward translational work from gene identification.

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\textbf{Author contributions}
CD, CB, RD, IL, FB, LM, VAB, NR, FB performed the experiments (including NGS analysis, cell model generation and characterization and mitochondrial assessments); CD, RF, TP, AT, EB performed data analysis; AW, EM, DMC, MS carried out patient assessment; CD, MS, SP, EB wrote the manuscript and supervised the study.

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**Competing interests**

The authors report no competing interests.

**URL**

Exome Aggregation database (ExAc): http://exac.broadinstitute.org/

Genome Aggregation database (gnomAD): http://gnomad.broadinstitute.org/

Eurexpress: http://www.eurexpress.org

Human Protein Atlas: https://www.proteinatlas.org/ENSG00000133104-SPG20

CRISPR design: crispr.mit.edu
References


**Figure Legends**

**Figure 1. Identification of SPART c.892dupA variant.** (A) Pedigree of the consanguineous family and electropherograms of the sequences in family members showing the co-segregation of the change with the spastic paraplegia phenotype. The two sibs are homozygous for the mutation, whereas both parents are heterozygous carriers. (B) Representative images of patient’s skeletal defects. Hand X-ray showed a delayed bone age of 1 year at 2 years of chronological age and of 2.5 years at 5.5 years of chronological age. (C) Generation of SPART c.892dupA knock-in SH-SY5Y cell line. Electropherogram of the SH-SY5Y clone sequence carrying the mutation c.892dupA in SPART gene and alignment between reference sequence and clone sequence are reported. (D) Representative western blot of Spartin protein from SPART<sup>wt</sup> and SPART<sup>892dupA</sup>-SH-SY5Y clone using a specific anti-Spartin antibody) Western confirmed that in SPART<sup>892dupA</sup> cells, the predicted 33KDa mutant Spartin is not produced, whereas in control cells Spartin is normally synthesized with molecular weight of 75-84 KDa. Gamma tubulin was used as endogenous control.

**Figure 2. Spartin depletion affects neuronal morphology and cells growth.** (A) Representative immunofluorescence images showing SPART-silencing in hNSCs. Images showed hNSCs day 0 (not silenced, panels a, d), hNSC scramble day 8 (panels b, e) and hNSC SPART-silenced (panels c, f). Panel a-c showed immunostaining for Spartin (red), α-tubulin (green) and DAPI (blue). Panels d-f showed immunostaining for Nestin (green) and DAPI (blue). Images showed an increased neuronal outgrowth in Spartin-depleted hNSCs compared to controls. Scale bars 10 µm. (B) Photograms in panel a and b are magnifications showing respectively hNSC SPART-silenced and hNSC scramble cells at day 8, immunostained for Nestin (green) and DAPI (blue). Arrows indicate neurite extensions that are absent in scramble-treated cells (arrowheads). Scale bars 50 µm. (C) Representative immunofluorescence images of SPART<sup>wt</sup> and SPART<sup>892dupA</sup> SH-SY5H cell lines stained against the neuronal marker PGP9.5. SPART<sup>892dupA</sup> cells showed an increased neuronal morphology compared to SPART<sup>wt</sup> cells. Scale bars 50µm. Merged images (panels d and h) show at
higher magnification the insets of the regions in panels c and g, respectively. Arrowheads in panel d showed the absence of neurites formation in $SPART^{wt}$ cells. Arrows in panel h indicate the neurite length generation in $SPART^{892dupA}$ cell line. Scale bar 50 µm. (D) Representative images of hNSCs after 8 days of $SPART$ depletion (panel c, hNSCs si$SPART$ Day 8), showing a strong cell loss compared to control cells (panel a and b, hNSCs at day 0 and hNSC scramble-transfected at day 8). Scale bars 10 µm. (E) Representative images showing $SPART^{wt}$ (control) and $SPART^{892dupA}$ cell lines, showing a strong cell loss in $SPART^{892dupA}$ cells (panel b) compared to control cells (panel a). Scale bars 50 µm.

Figure 3. Morphological characterization of $SPART^{892dupA}$ spheroids. (A) Morphological evaluation of $SPART^{wt}$ and $SPART^{892dupA}$-derived spheroids at 24 and 72 hours (panels a, b representing $SPART^{wt}$ spheres and panels d, e representing $SPART^{892dupA}$ spheres). Scale bar 20 µm. Both wild-type and mutant cell lines aggregate at 24 hours and formed spheroids after 72 hours. Panels c and f showed magnification of the indicated regions in panels b and e. $SPART^{892dupA}$ spheroids were more rounded (panel f) than control $SPART^{wt}$ cells, whereas (panel e) formed more condensed and disorganized aggregates. (B) Circularity analysis of spheroids performed with ImageJ at 24 and at 72 hours post seeding. An increased circularity value was observed in $SPART^{892dupA}$-derived spheroids compared to $SPART^{wt}$ cells at 24 and 72 hours. Unpaired t-test with Welch’s correction was performed. ****p<0.0001, ***p<0.0001, mean ± SEM. (C) According to the classification of morphological groups of 3D cell cultures (Kenny et al., 2007). $SPART^{wt}$-derived spheroids meet the criteria for “grape-like”, whereas $SPART^{892dupA}$-derived spheroids could be classified as “round”, characteristic of more differentiated cells. (D) Frequency of classification type for $SPART^{wt}$ and $SPART^{892dupA}$ 3D cell cultures derived from N=46 images for $SPART^{wt}$ and n=38 for $SPART^{892dupA}$.
Figure 4. Spartin loss alters mitochondrial morphology and network. (A) Representative z-stack image of mitochondrial network evaluated using mito-GFP probe by live cell imaging. Control cells showed a more diffused distribution of mitochondria within the cytoplasm of cells, also in neurites (left panel, arrows). However, SPART<sup>892dupA</sup> cells showed a notable perinuclear distribution and absence of mitochondria in neurites (right panel, arrowheads). Scale bars 50 µm. (B) Quantitative assessment of mitochondrial interconnectivity measured via live cell imaging (n=35 live cells measured for each cell line) using ImageJ Mitochondrial Morphology plugin. ****p<0.0001, mean ± SEM. (C) Mitochondrial morphology assessment showed decreased mean perimeter and mean area and increased roundness value in SPART<sup>892dupA</sup> vs control cells (*p<0.05, ****p<0.0001, mean ± SEM). (D) Representative images of 3D z-stacks and relative IMARIS reconstruction of the mitochondrial network visualized via mito-GFP transfection in SPART<sup>wt</sup> and SPART<sup>892dupA</sup> cells. Scale bars: 10 µm. (E) Quantification of mitochondrial network volume represented as the mean GFP-isosurface volume per cell (n=5 pictures per group). A significant decrease in the mitochondrial isosurface volume was observed in SPART<sup>892dupA</sup> cells (*p<0.05, Student’s t-test).

Figure 5. Spartin loss alters mitochondrial activity. (A) Oxygen consumption rates analysis in intact cells in SPART<sup>wt</sup> (n=3 independent experiments) and SPART<sup>892dupA</sup> cells (n=3 independent experiments). Respiration was measured in DMEM (basal respiration), in presence of oligomycin A (non-phosphorylating respiration) and in presence of FCCP (uncoupled respiration). Data were normalized on citrate synthase activity. Colour legend: white box= SPART<sup>wt</sup> cells; black box= SPART<sup>892dupA</sup> cells; white dotted box= SPART<sup>wt</sup> cells oligomycin A-treated; black dotted box=SPART<sup>892dupA</sup> cells oligomycin A-treated; white striped box= SPART<sup>wt</sup> cells FCCP-treated cells; black striped box= SPART<sup>892dupA</sup> cells FCCP-treated cells. **p<0.01, mean ± SEM. (B) ATP/ADP ratio in cellular extracts from SPART<sup>wt</sup> and SPART<sup>892dupA</sup> cells showing a decreased ATP/ADP ratio in mutant cells. Bars indicate standard errors. **p<0.01, mean ± SEM. (C-E)
OXPHOS complex activity measurements. (C) Complex I+III activity (NADH-cytochrome c oxidoreductase activity) in cell homogenate from $SPART^{wt}$ (n=3 independent experiments) and $SPART^{892\text{dup}A}$ cells (n=3 independent experiments). ****p<0.0001, mean ± SEM. (D) Complex I activity (NADH-dehydrogenase) in cell homogenate from wild type (n=3 independent experiments) and mutant SHSY-5Y cells (n=3 independent experiments). Data were normalized on citrate synthase activity (CS). *p<0.05, mean ± SEM. (E) Complex II+III activity (succinate-cytochrome c oxidoreductase activity) in cell homogenate from $SPART^{wt}$ (n=3 independent experiments) and $SPART^{892\text{dup}A}$ cells (n=3 independent experiments). (F) ATP level in cellular extracts from $SPART^{wt}$ and $SPART^{892\text{dup}A}$ cells grown for 72 hours in the presence and absence of 200 nM rotenone (n=4 independent experiments) **p<0.01, mean ± SEM. (G) Mitochondrial membrane potential measurement assessed with Tetramethylrhodamine (TMRM) probe. The TMRM fluorescence emission was normalized on MitoTracker Green emission, (n=12 independent experiments). ****p<0.0001, mean ± SEM.

**Figure 6. Mutant $SPART^{892\text{dup}A}$ cells showed an increased NADH level and pyruvate excretion.**

(A) NADH autofluorescence measurement showing an increased level in mutant cells compared to controls. **p<0.01, mean ± SEM. (B) Extracellular lactate content determination by HPLC in $SPART^{wt}$ and $SPART^{892\text{dup}A}$ cells. The extracellular lactate content in culture cell medium was quantified after 24 hours of cell growth by HPLC analysis. The peak area corresponding to lactate was normalized on cell number. (C) Extracellular pyruvate production $SPART^{wt}$ and $SPART^{892\text{dup}A}$ cells measured by HPLC analysis after 72 hours of cell growth. The peak area corresponding to pyruvate was normalized on protein content by Bradford assay. *p<0.05, mean ± SEM. (D) Representative HPLC chromatograms of extracellular media from $SPART^{wt}$ and $SPART^{892\text{dup}A}$ cells. Red line indicates medium from $SPART^{892\text{dup}A}$ cells, green line indicates medium only (DMEM high glucose) and blue line indicates medium from $SPART^{wt}$ cells. Vertical red line indicates pyruvate. (E) Representative western blot analysis showing the expression of phosphorylated and total
STAT3 in the two cell lines. Gamma tubulin was used as endogenous control. \textit{SPART}^{892\text{dupA}} showed an increase of P-STAT3 compared to \textit{SPART}^{\text{wt}} cells, indicating an over-activation of STAT3 in mutant cells. Graph showed the relative quantification of western blot.

**Figure 7.** Spartin loss increases oxidative stress and alters the homeostasis of calcium. (A) Assessment of reactive oxygen species (ROS) production in \textit{SPART}^{\text{wt}} (n=35) and \textit{SPART}^{892\text{dupA}} (n=35) SH-SY5Y live cells using dichlorofluorescein diacetate (DCFDA) as fluorescent probe. (B) Assessment of mitochondrial superoxide production in \textit{SPART}^{\text{wt}} (n=35) and \textit{SPART}^{892\text{dupA}} (n=35) SHSY-5Y live cells using MitoSOX Red as specific fluorescent probe. Data were normalized on protein content using the Lowry assay. ****p<0.0001, mean ± SEM. (C) \textit{CAT}, (D) \textit{SOD1} and (E) \textit{SOD2} mRNA relative expression in \textit{SPART}^{\text{wt}} and \textit{SPART}^{892\text{dupA}} cell lines. *p<0.05, **p<0.01, mean ± SEM. (F) Representative western blot analysis showing the expression of GRP75 protein and the relative quantification in the two cell lines. Gamma tubulin was used as endogenous control. \textit{SPART}^{892\text{dupA}} showed a higher expression of GRP75 compared to \textit{SPART}^{\text{wt}} cells. *p<0.05, mean ± SEM. (G) Representative confocal microscopy images showing \textit{SPART}^{\text{wt}} and \textit{SPART}^{892\text{dupA}} cells stained with Fura-2-AM (right panel). Left panel showed quantification of the Ca^{2+}-free Fura-2 AM relative fluorescence. \textit{SPART}^{892\text{dupA}} (n=50) cells showed an increase of intracellular Ca^{2+} compared to control cells (n=50). ****p<0.0001, mean ± SEM. (H) Representative confocal microscopy images of Fura-2 AM showing \textit{SPART}^{\text{wt}} and \textit{SPART}^{892\text{dupA}} cells and re-expression of Spartin in mutant cells (right panel). Left panel showed Spartin re-expression evaluated by western blot and the quantification of the intracellular-free Ca^{2+} measuring the Fura-2 AM relative fluorescence in the three samples. Re-expression of Spartin rescued the intracellular Ca^{2+} concentration. ****p<0.0001, Data are reported as the mean ± SEM of at least three independent experiments.

**Figure 8.** Model of Spartin functions in mitochondrial metabolism. (A) Spartin interacts with GRP75, modulating the import of mitochondrial proteins encoded by the nucleus via the TIM-TOM
complexes into the mitochondria (Milewska et al., 2009), allowing a normal ATP production through Krebs’s cycle and OXPHOS activity (Complex I-V). (B) Spartin loss, as observed in our experimental settings, determines an energetic failure due to an altered Complex I activity, with a decreased ATP production, and a halt in mitochondrial oxidative phosphorylation leading to decreased mitochondrial membrane potential, and increased oxidative stress, due to enhanced production of mitochondrial ROS. Increased oxidative stress/increased ROS are known inhibitors of PDH activity (Gray et al., 2014). Moreover, Spartin loss results in a reduction of mitochondrial Ca\(^{2+}\) levels (Joshi and Bakowska, 2011) which could alter the activity of PDH. Accordingly, we observed that pyruvate, which enters mitochondria through the mitochondrial pyruvate carrier (MPC), was not efficiently converted into acetyl-coA (AcCoA), Krebs’s cycle substrate, thus accumulating and excreted from \(\textit{SPART}^{892\text{dupA}}\) mutant cells. Therefore, we hypothesize that the energetic failure observed in absence of Spartin, probably related to an impaired mitochondrial protein import of the nuclear-encoded subunits, is pivotal in generating the neurodegenerative defects observed in neurons in Troyer syndrome.

Abbreviations: MPC, mitochondrial pyruvate carrier; PDH, pyruvate dehydrogenase; \(\Delta\psi\), mitochondrial membrane potential; MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; IMS, inter-membrane space; ECM, extracellular matrix. I-V: mitochondrial OXPHOS complexes; TOM, translocase of the outer membrane; IP3R, Inositol trisphosphate receptor, Phosphatidylinositol 3-phosphate; VDAC, Voltage-dependent anion-selective channel.
Figure 3

(A) 24 h vs. 72 h

- SPART wt
  - a: Organized nuclei, Robust cell-cell adhesion
  - b: Disorganized nuclei, Poor cell-cell adhesion
  - c: Invasive processes
- SPART^892dupA
  - d: Organized nuclei, Robust cell-cell adhesion
  - e: Disorganized nuclei, Poor cell-cell adhesion
  - f: Invasive processes

(B) Circularity values of spheroids at 24 h and 72 h

- Average circularity value of spheroids at 24 h
  - SPART wt
  - SPART^892dupA

- Average circularity value of spheroids at 72 h
  - SPART wt
  - SPART^892dupA

(C) Cell line: SPART wt vs. SPART^892dupA

- SPART wt: Round: 11, Grape-like: 55
- SPART^892dupA: Round: 88, Grape-like: 9

(D) Fisher's exact test

- SPART wt: P<0.0001
Figure 3 track changes

A

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B

Circularity values of spheroids at 24 h:

- SPART wt: [Bar graph]
- SPART 892dupA: [Bar graph]

Circularity values of spheroids at 72 h:

- SPART wt: [Bar graph]
- SPART 892dupA: [Bar graph]

C

- Round
- Mass
- Grape-like
- Stellate

Organized nuclei: Robust cell-cell adhesion
Disorganized nuclei: Poor cell-cell adhesion
Invasive processes

D

<table>
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<tr>
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Figure 4

(A) Images showing mitochondrial distribution in cells expressing wild-type (SPART<sup>wt</sup>) and 882dupA variants of SPART.

(B) Graphs comparing interconnectivity, mean perimeter, mean area, and roundness of mitochondria for wild-type (SPART<sup>wt</sup>) and 882dupA variants.

(D) Images from confocal 3D acquisition and IMARIS elaboration showing mitochondrial structures in wild-type and 882dupA variants.

(E) Bar graph showing mean mitochondrial isosurface volume for wild-type (SPART<sup>wt</sup>) and 882dupA variants.
Figure 5: Track changes

A

B

C

D

E

F

G

Figure 5: Track changes

A

B

C

D

E

F

G
Supplementary Figure 1

A

B

MW (Kda)  

hNSC Day 0  
UNT Day 4  
scramble Day 4  
siSPART Day 4  
UNT Day 8  
scramble Day 8  
siSPART Day 8

100

Spartin

50

γ-Tubulin

C

D

E

F

G

H

Mean neurite length (pixel)

Mean number of neurite per cell

Average number of mitochondria per cell
Supplementary Figure 2

A

spg20b mRNA relative expression

16-32 cells 3-5 hpf 6 hpf 12 hpf 24 hpf 36 hpf 48 hpf 72 hpf 96 hpf 120 hpf

B

spg20b mRNA relative expression

Liver Brain Heart

ns **** ****
Supplementary Tables

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