



Compound heterozygous *SPATA5* variants in four families and functional studies of *SPATA5* deficiency

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Abstract

Variants in the *SPATA5* gene were recently described in a cohort of patients with global developmental delay, sensorineural hearing loss, seizures, cortical visual impairment and microcephaly. *SPATA5* protein localizes predominantly in the mitochondria and is proposed to be involved in mitochondrial function and brain developmental processes. However no functional studies have been performed. This study describes five patients with psychomotor developmental delay, microcephaly, epilepsy and hearing impairment, who were thought clinically to have a mitochondrial disease with subsequent whole-exome sequencing analysis detecting compound heterozygous variants in the *SPATA5* gene. A summary of clinical data of all the *SPATA5* patients reported in the literature confirms the characteristic phenotype. To assess *SPATA5*'s role in mitochondrial dynamics, functional studies were performed on rat cortical neurons. *SPATA5*-deficient neurons had a significant imbalance in the mitochondrial fusion-fission rate, impaired energy production and short axons. In conclusion, *SPATA5* protein has an important role in mitochondrial dynamics and axonal growth. Biallelic variants in the *SPATA5* gene can affect mitochondria in cortical neurons and should be considered in patients with a neurodegenerative disorder and/or with clinical presentation resembling a mitochondrial disorder.

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Introduction

Autosomal recessive variants in the *SPATA5* (spermatogenesis-associated protein 5, MIM: 613940) gene were recently associated with a specific disease phenotype. Tanaka et al. [1], Kurata et al. [2] and Buchert et al. [3] reported 25 individuals with intellectual disability,

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Table 1 Detailed clinical data of our five patients

Phenotype	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Nucleotide and amino-acid changes—RefSeq NM_145207.2(SPATA5)	c.250C>T, p.(Arg84*) and c.989_991del, p.(Thr330del)	c.250C>T, p.(Arg84*) and c.989_991del, p.(Thr330del)	c.554G>A, p.(Gly185Glu) and c.989_991del, p.(Thr330del)	c.394C>T, p.(Gln132*) and c.989_991del, p.(Thr330del)	c.700C>T, p.(Gln234*) and c.2384C>G, p.(Pro795Arg)
Sex	Female	Male	Female	Male	Female
Current age	Died at 4 y 11 m	Died at 3 y	9 y	3 y	5 y
Family history	Younger sister of patient 2	Older brother of patient 1	Single case	Single case	Single case
Consanguinity	No	No	No	No	No
Age of onset	Early infancy	Early infancy	Early infancy	Early infancy	Early infancy
Pregnancy/delivery	Uneventful	Uneventful	Uneventful	Uneventful	Uneventful pregnancy, emergency caesarean section—meconium present
Growth parameters					
Birth weight (g)	3150	3230	2850	2910	NI
Birth length (cm)	50	55	50	48	NI
OFC at birth (cm)	No data	No data	33	34	NI
Height at later age (cm) 1 y:	73 (−1.5 SD)	2 y5 m: 88 (−1 SD)	9 y: 140 (P50)	1 y3 m: 83 (P74, SD 0.81)	5 y: P10
Weight at later age (kg) 1 y:	7.35 (−3 SD)	2 y5 m: 11.5 (−2.5 SD)	9 y: 29 (P25)	1 y3 m: 8.3 (P2, SD −2.11)	5 y: P10
OFC at later age (cm) 2 y3 m:	44 (−3.5 SD)	2 y5 m: 47 (−2.5 SD)	9 y: 50.5 (<P3)	1 y3 m: 43 (<P1, SD −4.67)	5 y: P3
Microcephaly	Yes	Yes	Yes	Yes	Yes
Neurological findings					
Developmental delay	Regression at 7 m	Regression at 10 m	Regression at 3–4 m	Yes, from the birth	Yes
Motor milestones	4 m: head control; 5 m: rolling over; 7 m: all motor abilities were lost; 2y3m: no head control	2 y5 m: no head control	2 y6 m: sitting; 4 y6 m: walking with support; 8 y: few steps alone	3 y: cannot sit unsupported	4 y: cannot sit unsupported
Speech	No words	No words	No words	No words	No words
Epilepsy	Tonic-clonic	Myoclonic	Clonic, tonic-clonic	Tonic, myoclonic	Myoclonic
Muscle tone	Spasticity, dystonia, opisthotonus	Spasticity, dystonia	Spasticity, dystonia	Spasticity, dystonia, axial hypotonia, orofacial dyskinesia	Spasticity, dystonia, axial hypotonia, peripheral hypertonia
Hyperreflexia	Yes	Yes	Yes	Yes	Yes
Hearing	Sensorineural hearing loss	Sensorineural hearing loss	Sensorineural hearing loss	Sensorineural hearing loss	Sensorineural hearing loss
Ocular symptoms					
Vision	Cannot be evaluated	Cannot be evaluated	Visual impairment	Cortical visual impairment	Visual impairment
Strabismus	Yes	Yes	Yes	No	No
Nystagmus	No	No	No	Pendel nystagmus	No

Table 1 (continued)

Phenotype	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Eye contact	None	None	Yes	None	NI
Skeletal findings	Normal	Normal	Hip dysplasia	Normal	Bilateral hip dislocation
Dysphagia	No	No	No	Yes	Yes
Gastrointestinal problems	2y3m puree feeding	Mild hepatosplenomegaly, 2y5m: Recurrent gastroenteritis puree feeding	2y5m: Recurrent gastroenteritis puree feeding	Puree feeding	Severe gastro-oesophageal reflux and dysmotility, puree feeding
Disorder of the circadian rhythm	No	No	No	Yes	No
Metabolic investigations					
Serum lactate (mmol/l) (normal <2.2)	3.6	2.0	NI	2.55	Normal
Serum	AA, VLCFA normal	AA, acylcarnitines normal	AA, acylcarnitine, CDG, AA, VLCFA normal	AA, acylcarnitine, CDG, AA, VLCFA normal	AA, acylcarnitine, TIEF, VLCFA normal
Urine	AA, OA, MPS, oligosaccharides, sialic acid, imidazoles, creatinine/GUAA normal	AA, OA normal	AA, OA normal	AA, OA normal	AA, OA, MPS, oligosaccharides, sulfatides normal
CSF	Lactate 2.1 mmol/l, AA, NT, folic acid normal		Homocysteine, AA, NT, lactate normal	Homocysteine, AA, NT, normal	Lactate normal
Lysosomal enzymes	Normal				Normal
Muscle biopsy	Not done	Not done	Normal	Not done	Normal
Respiratory chain enzyme analysis	Normal enzyme activities (from cultured skin fibroblasts)	Not tested	Normal	Not tested	Combined respiratory chain defects of complex I and IV
Instrumental investigations					
EEG	Pathologic (multifocal epileptiform discharges)	Pathologic (multifocal sharp waves)	Pathologic (bilateral and multifokal spikes)	Pathologic (hypsarrhythmia)	Pathologic (epileptiform activity with polyspikes)
Brain MRI	1 y and 2y3m Progressive brain atrophy and atrophic caudate nucleus. Slightly elevated signal intensity on T2-weighted images in white matter	10 m and 2y5m Brain atrophy. Delayed myelination	2 y and 3 y Mild delayed myelination with improvement	6 m, 14 m and 16 m Development of hypoplasia of corpus callosum. Delayed myelination appeared at 14 m. Progressive white matter atrophy.	4 m and 2 y Marked cerebral atrophy. Global leukodystrophy with relative preservation of the grey matter and of basal ganglia
Mt DNA	Not tested	Not tested	Depletion-deletion screening, sequencing normal	Sequencing normal	Sequencing normal
Dysmorphic features	Relatively big ears, up-slanted palpebral fissures, high narrow palate, rethrognathia	Relatively big ears, up-slanted palpebral fissures, high narrow palate, rethrognathia	Mild rethrognathia	None	None

AA amino acids, CDG congenital disorder of glycosylation, GUAA guanidinoacetic acids, MPS mucopolysaccharides, NI no information, NT neurotransmitters, OA organic acids, TIEF transferrin isoelectric focusing, VLCFA very long chain fatty acids

microcephaly, hypotonia, spasticity, seizures, sensorineural hearing loss and cortical visual impairment.

The *SPATA5* gene and the protein encoded by it were first described by Liu et al. [4] as a spermatogenesis-associated factor (SPAF). The SPAF protein is a member of the AAA (ATPase Associated with diverse Activities) protein subfamily having two highly conserved ATPase modules and a putative mitochondrial matrix-targeting sequence. Liu et al. [4] concluded that *SPATA5* gene may be important in spermatogenesis, through remodelling of mitochondria from an orthodox form to a condensed form, as SPAF's predominant expression was in the spermatogonia and spermatocytes, but not in spermatids, and subcellularly in the inner membrane and matrix of mitochondria. However, no functional studies to link variants in the *SPATA5* gene to mitochondrial dysfunction have been done. In addition, the recent association of *SPATA5* variants with a neurodegenerative disease [1–3], suggests a role of the *SPATA5* gene not only in spermatogenesis, but also in neuronal development.

This paper reports five new patients from four families with compound heterozygous variants in the *SPATA5* gene and reviews the clinical features and reported mutations of all the patients described so far.

To analyse the pathomechanisms of *SPATA5* gene deficiency on a cellular level, functional studies on rat cortical neurons were undertaken. An association with neuronal development and mitochondrial dysfunction is suggested.

Patients

Five patients from four different families were enrolled in this study. The first family with two affected individuals (Patients 1 and 2) was identified at the Department of Clinical Genetics, Tartu University Hospital, Tartu, Estonia. Three other families with single affected cases (Patients 3, 4 and 5) were detected at the Institute of Human Genetics, Technical University Munich, Germany. The detailed information about the patients is given in Table 1 and in the Supplementary File. Common features for all the patients were global developmental delay, moderate microcephaly, myoclonic/tonic–clonic epilepsy, abnormal EEG, brain atrophy and/or delayed myelination (Supplementary Fig. S1A–D), sensorineural hearing loss, hyperreflexia, spasticity and dystonia. The facial phenotype of Patient 3 is illustrated on Supplementary Fig. S1E, F. The patients were suspected clinically to have a mitochondrial disorder. However, screening analyses for metabolic disorders including mitochondrial diseases from blood, urine and cerebrospinal fluid were mostly unremarkable. Mitochondrial respiratory chain enzyme analyses from muscle showed combined defects of complex I and IV in Patient 5,

but were normal in Patient 1 (from cultured skin fibroblasts) and Patient 3 (from muscle). Whole-exome sequencing (WES) was performed in Patients 1, 3, 4 and 5 in the context of two separate research projects.

Methods

The study of the Estonian family was approved by Research Ethics Committee of the University of Tartu (approval date 17/11/2014 and number 242/M-10). The ethics committee of the Technical University Munich approved the study of Patients 3, 4 and 5.

Whole-exome sequencing

WES analysis of the Patients 1, 3, 4 and 5 was carried out using methods described earlier [5, 6] and detected compound heterozygous variants in the *SPATA5* gene. All the variants were submitted to the LOVD (<http://databases.lovd.nl/shared/genes/SPATA5>) with individual IDs 00103212-00103215.

Functional studies

To analyse the functional consequences of *SPATA5* gene deficiency on the central nervous system, the effects of *SPATA5* shRNA silencing on rat cortical neurons were investigated using the following methods.

Plasmids

Plasmids expressing scrambled shRNA or shRNA targeted against rat *SPATA5* were obtained from SABiosciences and were previously validated by PCR. Plasmids expressing GW1-PercevalHR (49082) and neuron-specific pAAV-hSyn-DsRedExpress (22907) were obtained from Addgene. Mito-KikGR1 was constructed as described earlier [7].

Neuronal cultures

Primary cultures of rat cortical cells were prepared from neonatal Wistar rats as described [8]. Neurons were grown in Neurobasal A medium supplemented with B27 with or without Phenol Red on poly-L-lysine-coated 96-well white plates, 35-mm plastic or glass bottom dishes. All culture media and supplements were obtained from Life Sciences. For transfection of cells growing on glass bottom dishes, the conditioned medium was replaced with 100 μ l Opti-MEM I medium containing 2% Lipofectamine 2000 and 1–2 μ g total DNA containing an equal amount of each plasmid. The

dishes were incubated for 3–4 h, after which fresh medium was added.

Mitochondrial fusion and fission rate analysis

For mitochondrial fusion and fission rate analysis, cortical neuronal cultures were transfected with mito-KikGR1 plasmid and plasmids of interest as described earlier [7] and examined at DIV 7–8 by a laser scanning confocal microscope (LSM 780, Carl Zeiss Microscopy GmbH). For fusion acquisition, mito-KikGR1 was illuminated with a 488-nm argon laser line to visualize the intense green mitochondrial staining. Selected mitochondria were then photoconverted to red using a 405-nm diode laser and illuminated using a 561 nm DPSS laser. The images were taken at 10-s intervals for 10 min, the fate of all activated mitochondria was followed throughout the time-lapse, and the fusion and fission events were recorded.

ATP/ADP ratio measurement

Neurons expressing the ATP/ADP ratio sensor PercevalHR at neuronal endings were excited using a 405 nm diode laser and a 488 nm line of an Argon laser and collected using a 494–553 nm emission window. The ratio of fluorescence intensities, when exciting at 488 nm divided by 405 nm (F488 nm/F405 nm), was calculated from the collected signal from 50 axonal endings from five dishes per group.

Axonal growth

For neuronal maturation experiments, cortical neurons were transfected at day 1 in vitro (DIV1) with a plasmid expressing neuron-specific pAAVhSyn-DsRed1 and plasmids of interest. For the analysis of axonal growth, images of cultured cortical neurons at DIV4 were captured using an Olympus IX70 inverted microscope with a 20x objective and traced manually using NeuroLucida software (MBF Bioscience) and Fiji [9].

Immunohistochemistry

Neurons, HeLa, COS7 and SHSY5Y cells were fixed using 4% paraformaldehyde solution in Neurobasal™ A containing 5% sucrose for 10 min at 37 °C. Fixed cells were permeabilised using 0.1% Triton X-100 in PBS for 7 min and then blocked using 10% normal goat serum and 3% BSA for 60 min at room temperature. The cells were then incubated with the primary antibodies rabbit anti-myc (1:300, ab 9106, Abcam, USA) and mouse anti-TOM20 (1:200, ab 56783, Abcam, USA) in the presence of 10% normal goat serum and 3% BSA at 4 °C for 24 h. After washing, the cells were further incubated with respective

Alexa-Fluor-488- or Alexa-Fluor-594-conjugated secondary antibodies at room temperature for 1 h and subsequently examined using confocal microscope.

Statistics

Data are presented as the mean \pm SEM. *T*-test or one-way ANOVAs followed by Bonferroni post hoc test were used to compare differences between experimental samples and control groups. *P* values of <0.05 were considered statistically significant.

Results

Whole-exome sequencing

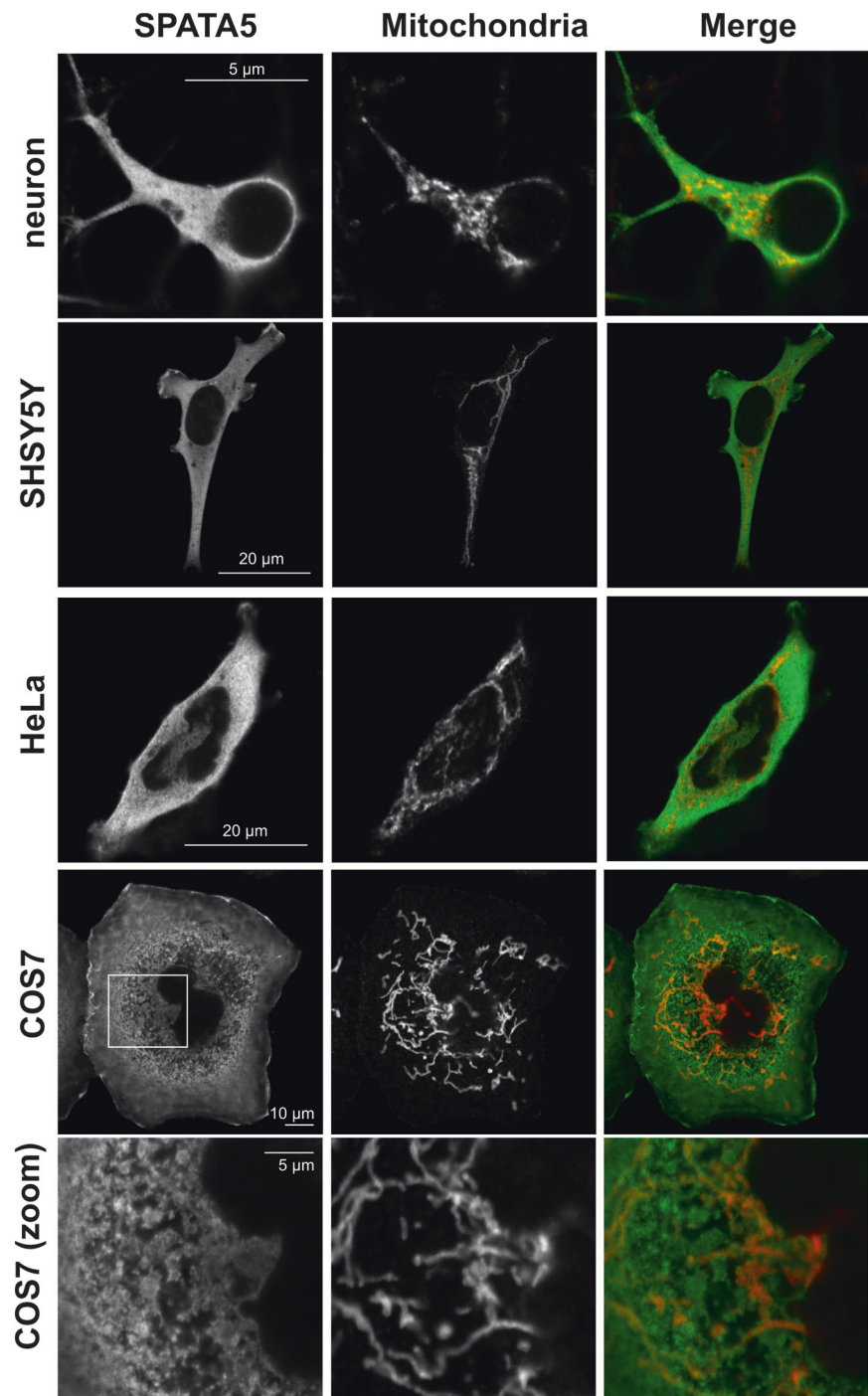
In Patient 1, after filtering for rare protein altering possibly biallelic variants two heterozygous variants in the *SPATA5* gene were identified by WES: c.250C>T, p.(Arg84*) and c.989_991del, p.(Thr330del) (RefSeq NM_145207.2). The c.250C>T is a nonsense variant presumably causing loss-of-function, which appears only in 1 out of 121,318 alleles (i.e. one heterozygous carrier) in the Exome Aggregation Consortium (ExAC) database [10]. The c.989_991del in-frame deletion has been previously reported in multiple patients with *SPATA5*-related disorder [1–3]. Both variants were confirmed by Sanger sequencing in both, Patients 1 and 2. Their mother was confirmed to carry only the c.250C>T variant, DNA from the father was not available for testing.

In Patient 3, after filtering the results of WES with the frequency of online databases (MAF < 0.1% in 1000 genomes, ExAC), pathogenicity prediction programs (PolyPhen-2, Sift) and ClinVar, *SPATA5* was the only gene with both variants predicted to be probably damaging (c.554G>A, p.(Gly185Glu)) or having a likely pathogenic rating in ClinVar (c.989_991del, p.(Thr330del)). The compound heterozygous state of the two variants was confirmed by Sanger sequencing, the mother carried the missense variation, the father the deletion.

WES analysis of Patient 4, using the above-described filtering, identified only *SPATA5* gene containing a nonsense loss-of-function heterozygous variant, namely c.394C>T, p.(Gln132*). The patient also carried the c.989_991del, p.(Thr330del) in-frame deletion in heterozygous state in the *SPATA5* gene. The mother was the carrier of the nonsense variant and the deletion came from the father.

The same filtering method of Patient 5 resulted in only one nonsense loss-of-function variant (c.700C>T, p.(Gln234*)) in the *SPATA5* gene. This variant was detected in a heterozygous state predicted to be compound

Fig. 1 SPATA5 has a dominantly cytosolic localization and does not co-localize with mitochondria. Overexpressed SPATA5-myc (detected using an anti-myc antibody, green) is showing relatively homogenous localization in cortical neurons, SH-SY5Y and HeLa cells. In COS7 cells, part of the SPATA5 was in filamentous network-like structures but as demonstrated by the zoom panels it was nevertheless not co-localizing with mitochondria. Mitochondrial outer membrane was stained using TOM20 antibody



heterozygous with the c.2384C>G, p.(Pro795Arg) missense variant. This assumption was confirmed by Sanger sequencing, where the healthy mother was the carrier of the c.700C>T nonsense variant and the healthy father carried the c.2384C>G missense variant, each in heterozygous state.

Patient 1 was identified in the Estonian research project, which included 21 patients with a clinical suspicion of a

mitochondrial disorder, but no genetic diagnosis. These 21 cases were selected from a database of 181 patients whose fibroblast cell cultures have been stored from 2003-2014. Patients 3–5 were identified in the German research project, which included 353 patients with a suspicion of a mitochondrial disorder to whom WES analysis was performed and the three patients with *SPATA5* variants were identified.

The diagnostic yield was 0.6% and 0.8% in those two cohorts, respectively.

The results of the functional studies on rat cortical neurons

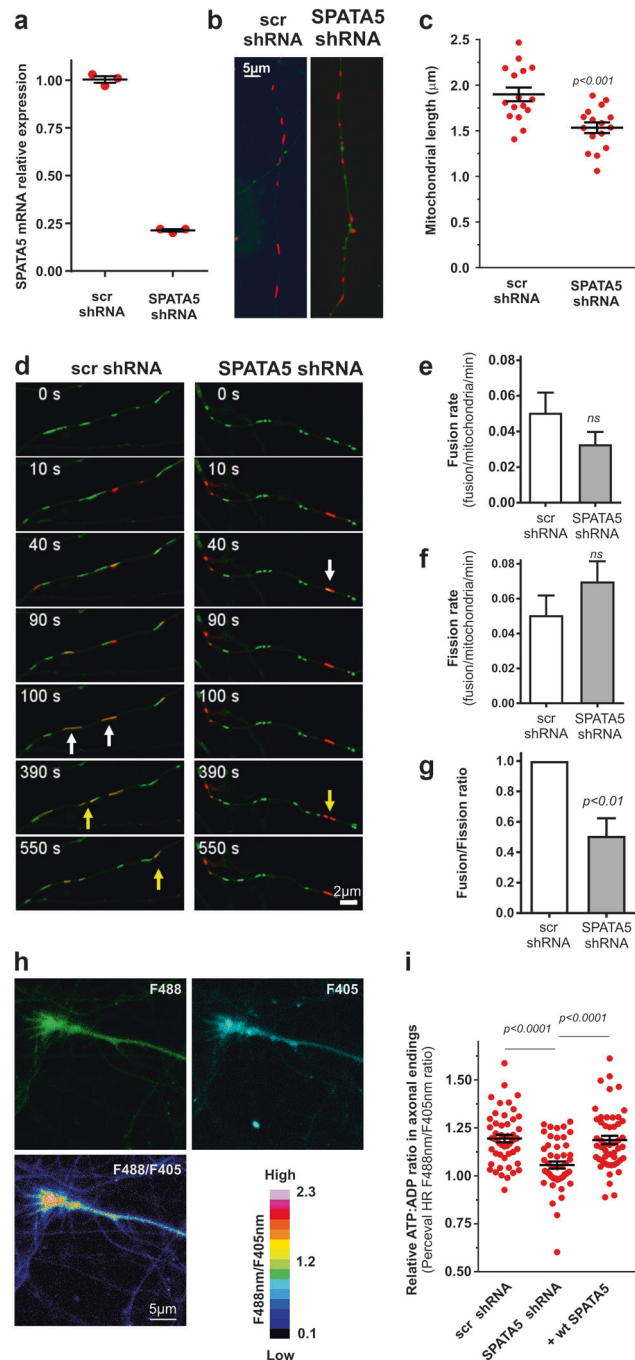
SPATA5 is cytosolic and not co-localizing with the mitochondrial marker

SPATA5 protein has a putative mitochondrial matrix-targeting sequence and has been shown to localize in

mitochondria in mouse testis [4]. However, subcellular localization data of SPATA5 in neurons and other cell types has not been reported. To our surprise the localization of overexpressed SPATA5 in cultured primary cortical neurons was dominantly cytosolic and clearly not co-localizing with the mitochondrial marker (Fig. 1). Similar relatively homogenous cytosolic localization was observed in neuronal SH-SY5Y cells and HeLa cells. The signal was less homogenous in COS7 cells, but also clearly not co-localizing with the mitochondrial marker. Unfortunately, we were not able to visualize endogenous SPATA5 as the antibody staining was too weak for co-localization study. It should also be noted that despite having the mitochondrial targeting sequence, several subcellular localization prediction tools like PSORT II [11], Yloc [12] and Cello [13] suggested cytosolic localization of SPATA5.

SPATA5 deficiency affects mitochondrial morphology and inhibits mitochondrial dynamics

Previously, it has been suggested that SPATA5 has a role in mitochondrial morphogenesis during spermatogenesis [4]. Therefore, we first tested whether the SPATA5 deficiency affects mitochondrial morphology in neurons. Indeed, there was a significant, 20% decrease in mitochondrial length in SPATA5 shRNA-treated neurons (efficiency of SPATA5 shRNA is demonstrated in Fig. 2a) compared with scrambled shRNA-treated controls (Fig. 2b, c). The



◀ **Fig. 2** SPATA5 deficiency impairs mitochondrial dynamics and ATP production. **a** Relative mRNA expression levels in neuronal PC cells transfected with scrambled or SPATA5 shRNA. The expression level of SPATA5 was normalized to CYC. **b, c** Neurons were transfected with mito-DsRed, GFP and scrambled shRNA or SPATA5 shRNA. SPATA5 shRNA neurons demonstrate significantly reduced mitochondrial length ($n = 16$ neurons in each group). **d–g** Primary cortical neurons were transfected with the photoconvertible mitochondrially targeted construct mito-Kikume—Green and scrambled shRNA or SPATA5 shRNA. Selected mitochondria were irradiated using a 405-nm laser line, thereby converting mito-Kikume—Green into mito-Kikume—Red. Fusion events between mito-Kikume—Green and photoactivated mito-Kikume—Red mitochondria are visible when mitochondria become yellow after mixing of the contents of the red and green mitochondria (white arrows) and fission events when these mitochondria split (yellow arrows) (**d**). In primary cortical neurons, SPATA5 shRNA tends to decrease the fusion rate (**e**) and increase the fission rate (**f**) leading to significant decrease in fusion–fission ratio (**g**) (four fields per dish were imaged and at least four dishes per condition were used). **h–i** Neurons were transfected with the ATP sensor Perceval and scrambled SPATA5 shRNA or SPATA5 shRNA plus shRNA insensitive human SPATA5 encoding plasmid. FRET (F488), CFP (F405) and pseudocolour ratiometric (F488/F405) images of an axonal ending of control neuron expressing PercevalHR are depicted (**h**). SPATA5-deficient neurons show a lower cytosolic ATP level as compared to the control (**i**). Note that the lower ATP levels in SPATA5 deficient neurons is restored by wt SPATA5 overexpression ($n = 50$ neurons)

mitochondrial length is controlled by mitochondrial fusion–fission balance [7]. Therefore, we then measured mitochondrial fusion and fission using photoconvertible mitochondria targeted Kikume Green-Red, which enables the quantification of fusion events between green- and red-emitting mitochondria (Fig. 2d). There was a slight statistically non-significant decrease in the number of fusion events in SPATA5 shRNA-treated neurons and slight statistically non-significant increase in the number of fission events in SPATA5 shRNA-treated neurons (Fig. 2e, f). However, together these changes led to two fold statistically significant decrease in fusion–fission ratio suggesting strong imbalance between the fusion and fission events in neuronal axons (Fig. 2g).

SPATA5 deficiency is associated with decreased cellular ATP

To test whether the changes in mitochondrial morphology and dynamics could affect the energetic status of the neurons a quantitative analysis of cell ATP/ADP ratio was performed using the genetically encoded fluorescent ratio-metric probe PercevalHR, which senses ATP/ADP ratio (Fig. 2h) [14]. Neurons were first transfected with SPATA5 shRNA encoding plasmid and compared with scrambled shRNA-treated controls. Further experiments performed at axonal endings of scrambled or SPATA5 shRNA expressing neurons showed a statistically significant 12% decrease in signal in the SPATA5 shRNA group, suggesting a decrease in ATP levels (Fig. 2i). Overexpression of human shRNA-insensitive SPATA5 restored the ATP/ADP ratio in the SPATA5 shRNA-treated group demonstrating the specificity of shRNA.

SPATA5 deficiency delays neuronal development

The results demonstrate that SPATA5 deficiency delayed the development of cortical neurons markedly. The longest axon was significantly shorter in developing DIV4 SPATA5 shRNA-transfected neurons (Fig. 3). Similarly, overexpression of human shRNA-insensitive SPATA5 restored the axonal growth in the SPATA5 shRNA-treated group.

Discussion

Including these 5 subjects, 30 patients with homozygous or compound heterozygous variants in the *SPATA5* gene have been reported in the literature [1–3]. All patients share similar features and thus a characteristic phenotype can be described.

Symptoms start in early infancy with all of the patients developing global developmental delay with severely limited speech or no words, hearing impairment (most

frequently sensorineural hearing loss, 77%), and a pathological EEG with symptomatic epilepsy being present in 73% of patients. Other prominent features are microcephaly (90%), gastrointestinal problems (73%), abnormal brain MRI (67%, predominantly brain atrophy and delayed myelination), visual impairment (most frequently cortical visual impairment, 41%) and no or reduced eye contact (48%). The patients tend to have a combination of axial hypotonia and peripheral hypertonia with spasticity. Detailed clinical data of all reported patients is summarized in Table 2.

Together, 25 different variants have been found across the gene: 14 missense, 5 nonsense, 3 frameshift variants, 2 in-frame deletions and 1 intronic splice-site variant. These are illustrated in Fig. 4. The most common reported variant is an in-frame deletion (c.989_991del, p.(Thr330del)) that was seen in 6 out of 18 different families (33%) and in 8 of the 30 patients. Three other variants (p.(Arg84Gln); p.(Arg186*); p.(Asp628Gly)) have occurred in two different families each. Interestingly, there is a homozygous individual with the p.(Thr330del) variant in the ExAC database [10], but also one patient described by Tanaka et al. [1] has a homozygous p.(Thr330del) variant. There are no mutation hot spots in the gene and there is no clear association between the location of the variants and severity of the phenotype. The siblings in Tanaka et al. [1] underlie this fact, as the younger sister has a milder phenotype than her elder brother, although they have the same genotype (NM_145207.2: c.2351G>A and c.269G>T). The precise investigation of the 3D structure of the protein could help for better understanding of the effects of the variants.

Previous researchers have suggested that SPATA5 is associated with mitochondrial morphology and function [4]. This study shows that SPATA5 deficient neurons have a significant imbalance of mitochondrial fusion and fission events leading to mitochondrial shortening that in turn result in decreased ATP production at axonal endings. This confirms that *SPATA5* deficiency alters the morphology and function of mitochondria in neurons and therefore can be indirectly categorized under mitochondrial disorders. This also supports the patients' phenotype and the clinicians' initial suspicion of a mitochondriopathy.

In contrast to Liu et al. [4] suggesting that SPATA5 is localized in the inner membrane or matrix of mitochondria in mouse testis, our experiments in cortical neurons, SH-SY5Y, HeLa and COS7 cells showed that it was dominantly cytosolic. We observed no co-localization with mitochondria suggesting that the putative mitochondrial targeting sequence might not be sufficient to drive SPATA5 to the mitochondria in these cell types. It should be noted here that Liu et al. [4] did also demonstrate that SPATA5 and the mitochondrial marker signals do not overlap. There are many examples where non-mitochondrial proteins play an

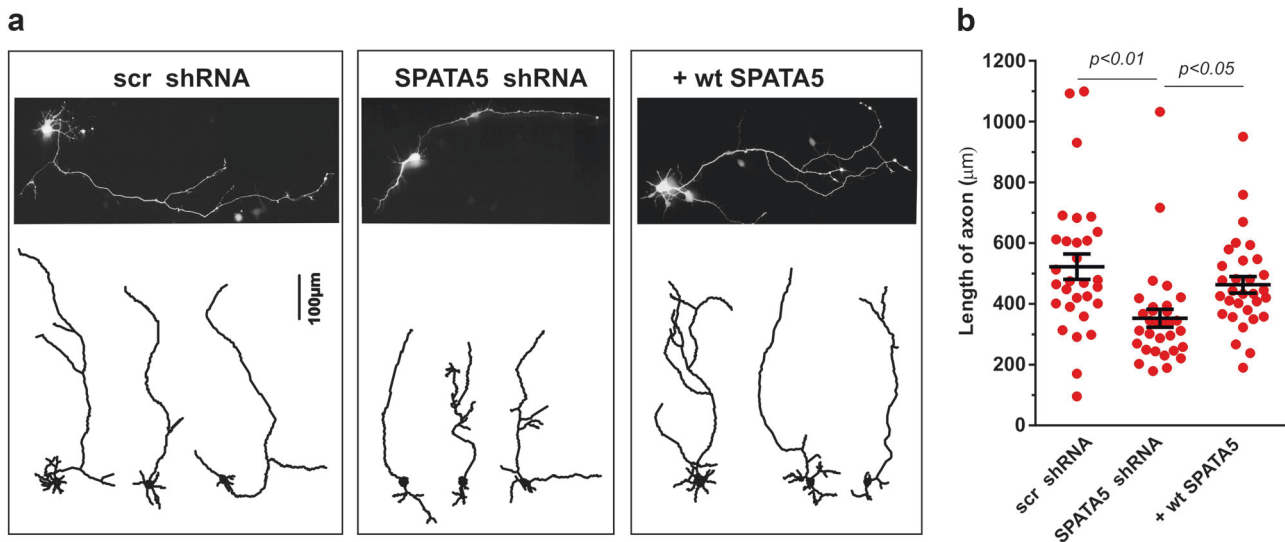


Fig. 3 SPATA5 deficiency impairs neuronal growth. Primary cortical neurons were transfected with the neuronal marker pAAV-hSyn-DsRed1 and scrambled shRNA or SPATA5 shRNA at DIV 1, and neuronal morphology was assessed at DIV 4. **a** Microscopy images

(above) and examples of NeuroLucida reconstructions (below) are depicted. **b** SPATA5 deficiency retards growth of the longest axon that is restored by wt SPATA5 overexpression ($n = 30$ neurons in each group)

active role in mitochondrial function. These proteins control mitochondrial biogenesis, mitochondrial trafficking and anchoring, as well as mitochondrial quality control mechanisms (e.g. mitophagy).

For example, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is caused by variants in the *TYMP* gene encoding cytosolic thymidine phosphorylase. These variants cause loss of thymidine phosphorylase activity and lead to accumulation of thymidine and deoxyuridine. This impairs mtDNA replication leading to accumulation of point mutations, deletions, and depletion of mtDNA [15–17]. Variants in the gene encoding KIF5A (Kinesin Family Member 5A), a protein that interacts with mitochondrial adaptor proteins and is responsible for mitochondrial transport in axons, are leading to hereditary spastic paraplegia [18, 19]. Variants in Parkin, a cytosolic ubiquitin E3 ligase known to be a crucial component of mitochondrial quality control mechanism, are associated with hereditary Parkinson's disease [20, 21]. Another Parkinson's disease related protein LRRK2, known to be involved in the regulation of mitochondrial dynamics and function, is also primarily localized to the cytoplasm [22]. Variants in the *GBA* gene encoding lysosomal glucocerebrosidase cause Gaucher disease and increase susceptibility to Parkinson's disease. Elegant paper by Osellame et al. [23] shows that this is related to downregulation of mitophagy and the ubiquitin–proteasome system resulting in accumulation of dysfunctional and fragmented mitochondria leading in turn to impaired energy and free radical homeostasis.

The majority of Wolfram syndrome cases are caused by variants in the *WFS1* (Wolfram syndrome 1) gene encoding

an endoplasmic reticulum (ER) protein. However, the clinical features of the syndrome resemble mitochondrial disease symptoms [24]. We have recently shown that downregulation of *WFS1* in neurons leads to dramatic changes in mitochondrial dynamics (inhibited mitochondrial fusion, altered mitochondrial trafficking and augmented mitophagy), which results in lower levels of ATP and inhibits neuronal development [25]. Another example of the ER protein variant responsible for mitochondrial damage is *RYR1* (ryanodine receptor type 1) variant in central core disease [26]. One of the characteristics of this type of myopathy is the lack of mitochondria within the centres of muscle fibres. Therefore, our results allow to suggest that similarly to these proteins, SPATA5 is indirectly or directly interacting with mitochondria and required for their proper dynamics. Nevertheless, we cannot completely exclude a possibility that a small fraction of SPATA5 is still localized to the mitochondria and controls the mitochondrial function from there.

This study also shows that the *SPATA5* gene plays an important role in the development of cortical neurons. Local ATP supply is critical for axonal growth. In neurons, most of the ATP supply is produced in the mitochondria and decline in ATP production could impair the axonal growth. Depletion of mitochondria at or before axogenesis prevents axon formation [27]. Similarly, a lack of synaptic or terminal axonal mitochondria results in aberrant organelle transport and dysfunctional synapses [28, 29]. Vaarmann et al. [30] and Cagalinec et al. [25] have recently shown that increased mitochondrial ATP production in axonal endings supports axonal growth and suggests that mitochondrial

Table 2 Phenotype overview of all the patients with *SPATA5* variants reported in the literature so far, including our five patients

Phenotype	5 Patients from this study	Tanaka et al. [1]	Kurata et al. [2]	Buchert et al. [3]	All 30 patients	% of a feature
	5 Patients in 4 families	14 Patients in 10 families	3 Patients in 2 families	8 Patients in 2 families	30 Patients in 18 families	
Sex	Female (3/5) Male (2/5)	Female (8/14) Male (6/14)	Female (1/3) Male (2/3)	Female (3/8) Male (5/8)	Female (15/30) Male (15/30)	50% 50%
Family history	Familial case (2/5) Single case (3/5)	Familial case (8/14) Single case (6/14)	Familial case (2/3) Single case (1/3)	Familial case (7/8) Single case (1/8)	Familial case (19/30) Single case (11/30)	63% 37%
Consanguinity	—	—	—	Yes (7/8)	Yes (7/30)	23%
Pregnancy/delivery	Uneventful (4/5)	Not mentioned, but probably uneventful (14/14)	Uneventful (2/3)	Uneventful (4/8)	Uneventful (10/30); not mentioned, but probably uneventful (14/30)	80%
	Emergency caesarean section (CS) (1/5)	—	IUGR (1/3)	Complicated (maternal diabetes, neonatal hypotonia, born with cyanosis) (4/8)	Complicated (6/30)	20%
Age of onset	Early infancy (5/5)	Not mentioned	Early infancy (3/3)	Early infancy (8/8)	Early infancy (16/16)	100%
Microcephaly	Yes (5/5)	Yes (12/13)	Yes (2/3)	Yes (7/8)	Yes (26/29)	90%
Global developmental delay	Yes (5/5)	Yes (14/14)	Yes (3/3)	Yes (8/8)	Yes (30/30)	100%
Developmental regression	Yes (3/5)	Yes (1/14)	—	—	Yes (4/30)	13%
Speech	No words (5/5)	No words/one word (13/13)	No words (3/3)	Severely limited (8/8)	No words/severely limited (29/29)	100%
Hearing	—	—	Hearing impairment (3/3)	Hearing impairment (3/4)	Hearing impairment, incl SNHL (26/26)	100%
Ocular symptoms	SNHL (5/5) Visual impairment (2/3)	SNHL (14/14) Visual impairment (2/13)	—	SNHL (1/4) Visual impairment (2/8)	SNHL (20/26) Visual impairment, incl CVI (17/27)	77% 63%
	CVI (1/3) Strabismus (2/5)	CVI (9/13) Strabismus (2/13)	—	Cortical blindness (1/8) Strabismus (1/8)	CVI (11/27) Strabismus (5/29)	41% 17%
	—	Astigmatismus (1/13)	Astigmatismus (1/3)	—	Astigmatismus (2/29)	7%
	Pendelnystagmus (1/5) No eye contact (3/5)	Nystagmus (3/13) Visual disinterest (1/13)	—	—	Nystagmus (4/29)	14%
	Hypotonia (axial) (2/5)	Hypotonia (primarily axial) (11/14)	Hypotonia (1/3)	Reduced eye contact (8/8)	No/ reduced eye contact (14/29)	48%
Muscle tonicity	Hypertonia (5/5) Spasticity (5/5) Dystonia (5/5)	Hypertonia (primarily peripheral) (8/14) Spasticity (7/14) Dystonia (2/14)	Hypertonia (2/3)	—	Hypotonia (18/30) Hypertonia (15/30) Spasticity (14/30) Dystonia (9/30)	60% 50% 47% 30%

Table 2 (continued)

Phenotype	5 Patients from this study	Tanaka et al. [1]	Kurata et al. [2]	Buchert et al. [3]	All 30 patients	% of a feature
Hyperreflexia	5 Patients in 4 families	14 Patients in 10 families	3 Patients in 2 families	8 Patients in 2 families	30 Patients in 18 families	
Epilepsy	Dyskinesia (1/5) Yes (5/5) Yes (5/5) — Myoclonic (3) Tonic (3) Abnormal (5/5) Abnormal (5/5) Brain atrophy (4) Hypoplasia of corpus callosum (1) Delayed myelination (3)	— Not mentioned Yes (13/14) Infantile spasms (5) Myoclonic (2) Tonic (4) Abnormal (14/14) Abnormal (7/12) Brain atrophy (4) Hypoplasia of corpus callosum (2) Delayed myelination/ hypomyelination (3)	Dyskinesia (1/3) Yes (1/3) Yes (3/3) — — Tonic (2) Abnormal (3/3) Abnormal (3/3) Brain atrophy (3) Thin corpus callosum (3) Delayed myelination (3)	— Not mentioned Yes (1/8) Infantile spasms (1) — — Abnormal (2/2) Abnormal (1/4) Brain atrophy (1) — —	Dyskinesia (2/30) Yes (6/8) Yes (22/30) Infantile spasms (6) Myoclonic (5) Tonic (9) Abnormal (24/24) Abnormal (16/24) Brain atrophy (12) Hypoplasia of corpus callosum (6) Delayed myelination/ hypomyelination (9) Yes (22/30)	7% NA 73% — — — 100% 67% — —
EEG						
Brain MRI						
Gastrointestinal problems	Yes (pure feeding, GERD, HSM, gastroenteritis) (5/5)	Yes (pure feeding, constipation, vomiting, G-tube, FTT) (13/14)	Yes (vomiting, GERD) (1/3)	Yes (constipation, FTT) (3/8)	Yes (22/30)	73%
Skeletal findings	— Hip dysplasia/ dislocation (2/5)	Scoliosis (4/14) Hip dysplasia/ dislocation (4/14)	— —	Scoliosis (1/8) —	Scoliosis (5/30) Hip dysplasia/ dislocation (6/30)	17% 20%
Cardiac problems	—	—	—	—	Yes (2/30)	7%
Immunodeficiency	—	Yes (4/14)	—	—	Yes (4/30)	13%
Thrombocytopenia	—	Yes (3/14)	—	—	Yes (3/30)	10%
Blood copper level	Not tested	Not mentioned	Elevated (2/3)	Not mentioned	Elevated (2/3)	NA
Muscle biopsy	Normal (2/2)	Abnormal mitochondria (2/2)	Not done	Not done	Abnormal mitochondria (2/4)	NA
Respiratory chain enzyme analysis	Complex I, IV defects (1/3)	Reduced activity (1/2)	Not tested	Not tested	Abnormal (2/5)	NA
Microanomalies	Rethrognathia (3/5) Large ears (2/5)	— —	Rethrognathia (3/3) Large ears (1/3) Low-set ears (2/3)	— — —	Rethrognathia (6/30) Large ears (3/30) Low-set ears (2/30) Long nose (7/30) Depressed nasal ridge/ bridge (4/30) Broad eyebrows (4/30)	20% 10% 7% 23% 13% 13%

CVI cortical visual impairment, FTT failure to thrive, GERD gastroesophageal reflux disease, HSM hepatosplenomegaly, IUGR intrauterine growth retardation, NA not applicable, SNHL sensorineural hearing loss

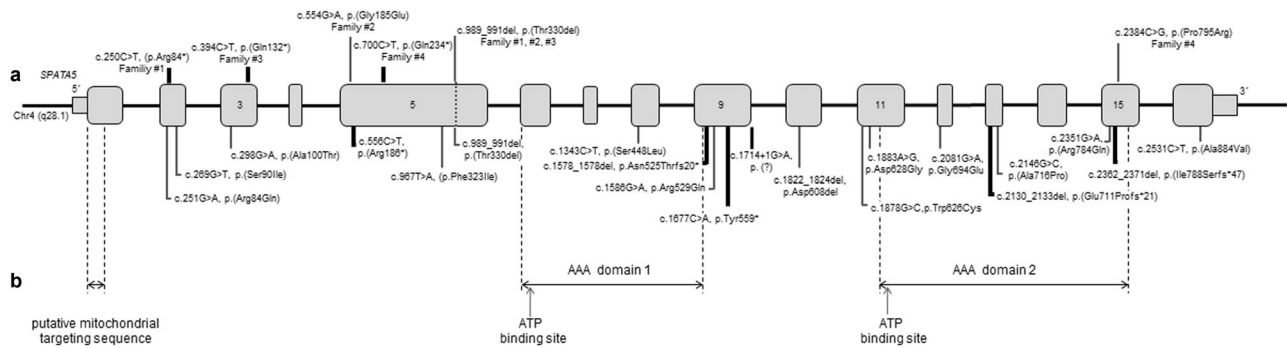


Fig. 4 The schematic figure of the *SPATA5* gene with the variants reported so far. **a** Exons are shown as grey boxes. All six variants from our study are shown above, and all published are shown below the

gene. The loss-of-function variants are tagged with bold black lines. **b** The putative mitochondrial targeting sequence and the two AAA domains are marked with dashed lines

ATP production is required for proper neuronal development.

In conclusion, *SPATA5* protein is required to sustain mitochondrial morphology, dynamics and ATP production in neurons and its deficiency leads to impaired axogenesis in vitro, in primary cortical neurons. *SPATA5* deficiency results in a syndrome with severe global developmental delay, severe speech impairment, hearing loss, abnormal electroencephalogram and microcephaly. Biallelic variants in the *SPATA5* gene can affect mitochondria in cortical neurons and should be considered in patients with a neurodegenerative disorder and/or with clinical presentation resembling a mitochondrial disorder.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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