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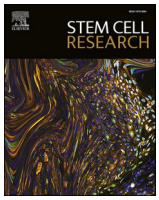

Generation of the human iPSC lines AKOSi011-A carrying the mutation p.Pro65Ser/p.Asp35T and AKOSi012-A, carrying the mutation p.Tyr231His, derived from FAHN patient fibroblasts

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A B S T R A C T

Fatty acid hydroxylase-associated neurodegeneration (FAHN) is a hereditary neurodegenerative disease caused by mutations in the *FA2H* gene. Patients show a wide range of neurological symptoms and an abnormal myelination. Here we describe the generation of the human induced pluripotent stem cell (hiPSC) lines AKOSi011-A and AKOSi012-A, derived from FAHN-patient fibroblasts, carrying the compound heterozygous mutation p.Pro65Ser/p.Asp35Tyr and the homozygous mutation p.Tyr231His, respectively. The hiPSC lines were generated using a non-integrating Sendai virus. The obtained hiPSCs show an unobtrusive karyotype, carry the mutations of the original fibroblasts, express pluripotency markers and can differentiate into cells of the three germ layers.

1. Resource Table

Unique stem cell lines identifier	AKOSi011-A AKOSi012-A
Alternative name(s) of stem cell lines	FAHN13_1 FAHN18_1
Institution	Translational Neurodegeneration Section "Albrecht Kossel", Department of Neurology, University Medical Centre Rostock, 18147 Rostock, Germany
Contact information of distributor	Moritz Frech, PhD, Translational Neurodegeneration Section „Albrecht Kossel", Department of Neurology, University Medical Centre Rostock, 18147 Rostock, Germany
Type of cell lines	*iPSC*
Origin	human
Additional origin info required	*Age/Sex:* AKOSi011-A:12y/female AKOSi012-A:7y/male *Ethnicity if known: N/A*
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Sendai virus
Genetic Modification	N/A
Type of Genetic Modification	N/A
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-/q-PCR
Associated disease	Fatty acid hydroxylase-associated neurodegeneration (FAHN)
Gene/locus	*FA2H*/16q23.1
Date archived/stock date	AKOSi011-A: November 2021 AKOSi012-A: October 2022
Cell line repository/bank	AKOSi011-A: https://hpscreg.eu/cell-line/AKOSi011-A AKOSi012-A: https://hpscreg.eu/cell-line/AKOSi012-A
Ethical approval	Children's Hospital of Eastern Ontario Research Ethics Board (CHEO REB). 401 Smyth Road, Ottawa, ON K1H8L1; CHEOREB# 20/79X

2. Resource utility

Fatty acid hydroxylase-associated neurodegeneration (FAHN) is a rare hereditary neurodegenerative disease which has findings of

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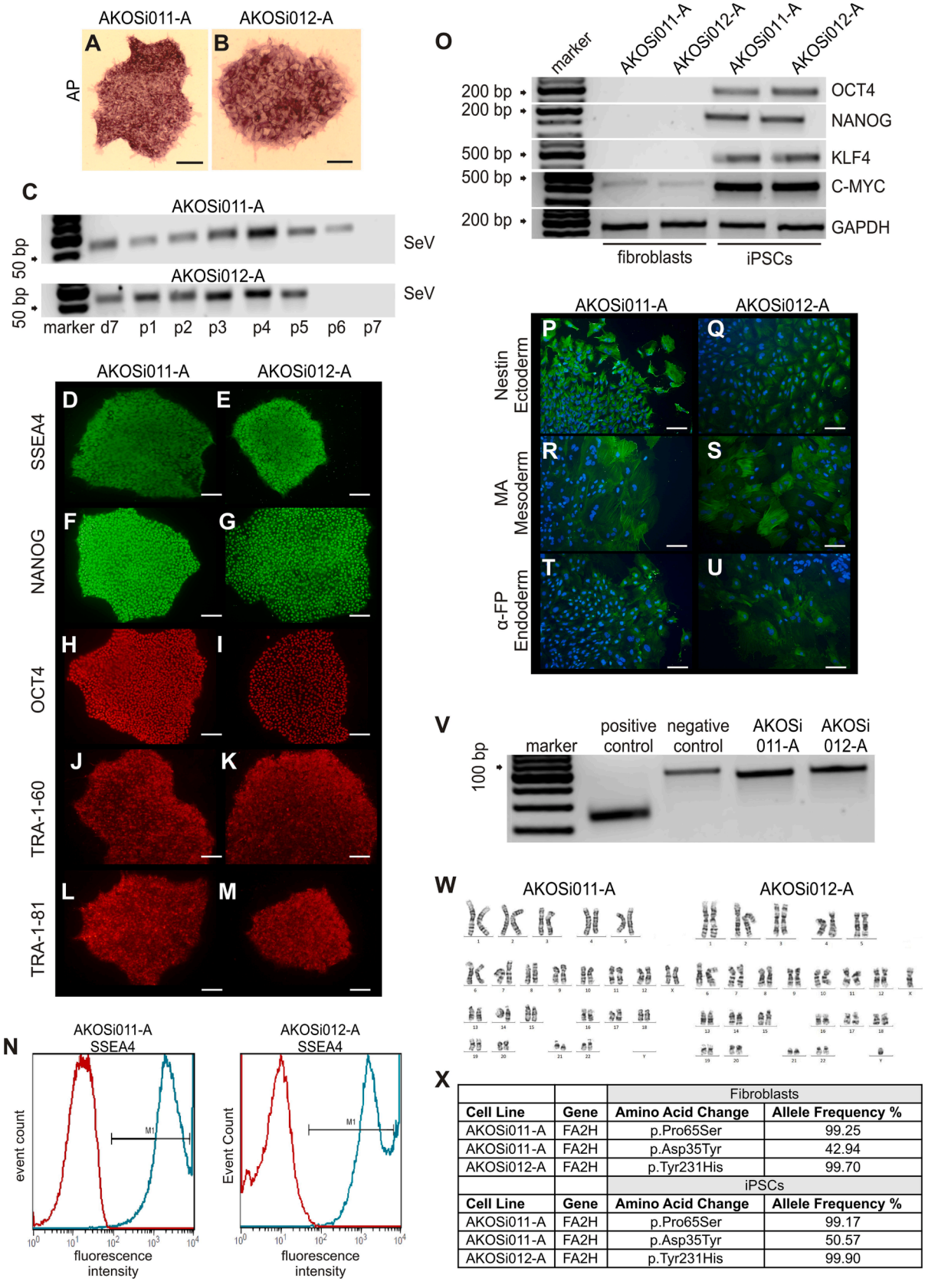

		Fibroblasts	
Cell Line	**Gene**	**Amino Acid Change**	**Allele Frequency %**
AKOSi011-A	FA2H	p.Pro65Ser	99.25
AKOSi011-A	FA2H	p.Asp35Tyr	42.94
AKOSi012-A	FA2H	p.Tyr231His	99.70
		iPSCs	
Cell Line	**Gene**	**Amino Acid Change**	**Allele Frequency %**
AKOSi011-A	FA2H	p.Pro65Ser	99.17
AKOSi011-A	FA2H	p.Asp35Tyr	50.57
AKOSi012-A	FA2H	p.Tyr231His	99.90

Fig. 1. Characterization of AKOSi011-A and AKOSi012-A iPSC lines.

Table 2
Reagents details.

	Antibodies used for immunofluorescence/flow-cytometry			
	Antibody	**Dilution**	**Company Cat #**	**RRID**
Pluripotency Marker (IF)	Rabbit anti-OCT4	1:100	Stemgent Cat# 09-0023	RRID: AB_2167689
Pluripotency Marker (IF)	Rabbit anti-NANOG	1:100	Stemgent Cat# 09-0020	RRID: AB_2298294
Pluripotency Marker (IF)	Mouse anti-SSEA4	1:100	Stemgent Cat# 09-0006	RRID: AB_1512169
Pluripotency Marker (IF)	Mouse anti-TRA-1-60	1:100	Stemgent Cat# 09-0010	RRID: AB_1512170
Pluripotency Marker (IF)	Mouse anti-TRA-1-81	1:100	Stemgent Cat# 09-0011	RRID: AB_1512171
Pluripotency Marker (FC)	Alexa Fluor 488 anti-OCT4, mouse IgG2b	1:20	BioLegend Cat# 653705	RRID: AB_2562250
Pluripotency Marker (FC)	Alexa Fluor 647 anti-NANOG, mouse IgG1	1:50	BioLegend Cat# 674210	RRID: AB_2650619
Pluripotency Marker (FC)	Alexa Fluor 647 anti- SSEA-4, mouse IgG3	1:500	BioLegend Cat# 330407	RRID: AB_1089201
Pluripotency Marker (FC)	PE anti-human TRA-1-60, mouse IgM	1:20	BioLegend Cat# 330609	RRID: AB_1279447
Pluripotency Marker (FC)	Alexa Fluor 488 anti-TRA-1-81, mouse IgM	1:20	BioLegend Cat# 330709	RRID: AB_2561741
Differentiation Marker (IF)	Mouse anti-Muscle actin	1:50	Agilent Dako Cat# M0635	RRID: AB_2242301
Differentiation Marker (IF)	Mouse anti-Nestin	1:100	R and D Systems Cat# MAB1259	RRID: AB_2251304
Differentiation Marker (IF)	Mouse anti-α fetoprotein	1:20	R and D Systems Cat# MAB1368	RRID: AB_357658
Differentiation Marker (IF)	Anti-PAX6 antibody	1:50	Abcam Cat# EPR15858	RRID: AB_195045
Differentiation Marker (IF)	PAX3 Antibody	1:50	Thermo Fisher Scientific Cat# 38-1801	RRID: AB_2533359
Differentiation Marker (IF)	Anti-SOX17 antibody		Abcam Cat# EPR20684	RRID: AB_224637
Secondary antibody	Alexa Fluor 488, Goat anti-mouse IgG	1:500	Thermo Fisher Scientific Cat# A-11029	RRID: AB_2534088
Secondary antibody	Alexa Fluor 568, Goat anti-mouse IgM	1:500	Thermo Fisher Scientific Cat# A-21043	RRID: AB_2535712
Secondary antibody	Alexa Fluor 488, Goat anti-rabbit IgG	1:500	Thermo Fisher Scientific Cat# A-11034	RRID: AB_2576217

	Primers		
	Target	**Size of band**	**Forward/Reverse primer (5′-3′)**
Pluripotency Marker (RT-PCR)	*C-MYC*	325 bp	GCGTCCTGGGAAGGGAGATCCGGAGC/ TTGAGGGGCATCGTCGCGGGAGGCTG
Pluripotency Marker (RT-PCR)	*NANOG*	128 bp	TGTGTTCTCTTCCACCCAGC/ACCAGGTCTTCACCTGTTTGT
Pluripotency Marker (RT-PCR)	*OCT4*	144 bp	GACAGGGGGAGGGGAGGAGCTAGG/ CTTCCCTCCAACCAGTTGCCCCAAAC
Pluripotency Marker (RT-PCR)	*KLF4*	397 bp	ACGATCGTGGCCCCGGAAAAGGACC/ TGATTGTAGTGCTTTCTGGCTGGGCTCC
Sendai reprogramming vector (RT-PCR)	*SeV*	181 bp	GGATCACTAGGTGATATCGAGC/ACCAGACAAGAGTTT AAGAGATATGTATC
House-Keeping Gene (RT-PCR)	*GAPDH*	112 bp	CATGTTCCAATATGATTCCACCC/GGGATCTCGCTCCTGGAAGAT
House-Keeping Gene (RT-PCR)	*G6PD*	76 bp	TGCCCCCGACCGTCTAC/ATGCGGTTCCAGCCTATCTG

neurodegeneration with brain iron accumulation (NBIA), leukodystrophy and hereditary spastic paraplegia. The pathophysiology underlying FAHN is inadequately studied and understood. The iPSC lines described here will contribute to a better understanding of the pathophysiology of FAHN.

3. Resource details

Fatty Acid Hydroxylase-associated Neurodegeneration (FAHN, OMIM #611026), also known as Hereditary Spastic Paraplegia Type 35 (HSP35), is an inherited autosomal recessive disease, caused by mutations in the fatty acid 2-hydroxylase (FA2H) gene ((Gregory et al., 1993; Rattay et al., 2019).

FA2H encodes an enzyme that catalyzes the hydroxylation of 2-hydroxy fatty acids, which are a precursors for the synthesis of ceramides. Ceramides are in turn major components of sphingolipids and myelin. Consequently, FAHN patients display abnormal myelination or demyelination in parts of the CNS. However, the pathophysiology underlying the observed myelin abnormalities are yet not well understood, which might be due to the fact that FAHN is an ultra-rare disease and thus only a low number of patients with FAHN have been described worldwide to date (Kolarova et al., 2022). Therefore, cellular model systems are needed to study the pathophysiological processes of FAHN. Recently, we developed an iPSC line, derived from patient-specific fibroblasts from a symptomatic FAHN patient, carrying the compound heterozygous mutation p.Gly45Arg/p.His319Arg (Efendic et al., 2022). Here, we describe now the generation of two additional iPSC cell lines, namely AKOSi011-A and AKOSi012-A, carrying the compound heterozygous mutation p.Pro65Ser/p.Asp35Tyr and the homozygous mutation p.Tyr231His, respectively.

Patient-derived fibroblasts were reprogrammed into iPSCs using a non-integrating Sendai virus encoding KLF4, OCT 3/4, SOX2, and C-MYC. Obtained iPSC colonies were positive for alkaline phosphatase (AP) and showed stem cell-like morphology (Fig. 1A,B, scale bars = 200 μm). RT-PCR was performed to confirm the absence of the Sendai virus backbone. After passage 7 (p7) the backbone was no longer detectable in both cell lines. (Fig. 1C). Pluripotency was proven by immunofluorescence staining of the pluripotency markers SSEA4, NANOG, OCT4, TRA-1–60, and TRA-1–81 (Fig. 1D-M, scale bars = 200 μm). Quantitative analysis of the pluripotency markers was done by flow cytometry. An example is shown for SSEA4 (Fig. 1N). In addition, the pluripotency related genes OCT4, NANOG, KLF4, and C-MYC were detected by RT-PCR in the iPSCs (Fig. 1O). The expression of specific markers for the ectoderm (Nestin, Pax6), the mesoderm (muscle actin, Pax3/Pax7) and the endoderm (α-feto-protein, Sox17) in embryoid bodies (EB), was proven by immunofluorescence staining. Examples are shown for Nestin (Fig. 1P, Q, scale bars = 200 μm), muscle actin (MA, Fig. 1R,S, scale bars = 200 μm) and α-feto-protein (α-FP, Fig. 1T,U, scale bars = 200 μm). See Table 2 for the antibodies and primers used. These results confirm the differentiation capability of the iPSCs. The iPSC lines were negative for mycoplasma contamination (Fig. 1V). A normal female karyotype (46, XX) and a normal male karyotype were observed in G-banding karyotyping analysis for AKOSi-011A and AKOSi-012A, respectively (Fig. 1W). The match of mutations in the fibroblasts and iPSCs was confirmed by NGS (Fig. 1X). Short tandem repeat analysis of 18 genomic loci confirmed that iPSCs originated from the patient specific fibroblasts (Table 1). Overall, we succeeded in establishing two iPSC lines carrying disease causing mutations of *FA2H*.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Bright field photography	Normal	Fig. 1 A, B
Phenotype	Qualitative analysis	Immunofluorescence: SSEA4, NANOG, OCT4, TRA-1-60, TRA-1-81	Fig. 1 D-M
	Quantitative analysis	FACS: AKOSi011-A: NANOG: 99% OCT4: 97% SSEA4: 97% TRA-1-60: 96% TRA-1-81: 97% AKOSi012-A: NANOG: 98% OCT4: 97% SSEA4: 98% TRA-1-60: 96% TRA-1-81: 97%	Example for SSEA4 Fig. 1 N
Genotype	Karyotype (G-banding) and resolution	AKOSi011-A: 46, XX, normal AKOSi012-A: 46, XY, normal Resolution 400 – 450 bands	Fig. 1 W
Identity	STR analysis	18 STR loci tested, all matched	with author
Mutation analysis	Targeted sequencing	Mutations confirmed	Fig. 1 X
Microbiology and virology	Mycoplasma testing by RT-PCR	negative	Fig. 1 V
Differentiation potential	Embryoid body formation	Ectoderm: Nestin, PAX6	Example of Nestin Fig. 1 P, Q
		Mesoderm: MA, Pax3	MA Figure 1 R, S
		Endoderm: α-FP, SOX17	α-FP Figure 1 T, U
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

4. Materials and methods

4.1. Reprogramming of patient-specific fibroblasts

Fibroblasts were cultured in high glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (GE Healthcare) and 1% penicillin/streptomycin. The CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific) was used for transfection. For each transfection $1x10^4$ fibroblasts were transduced with KLF4, OCT 3/4, SOX2 and C-MYC. The MOIs of 5–5-3 (KOS-cMyc-Klf4), as recommended by the manufacturer, were used for transduction. Manually picked iPSC colonies were maintained on Matrigel coated dishes in E8 medium (Thermo Fisher Scientific). Medium was changed daily. For passaging cells were detached using ReLeSR passaging reagent (Stem Cell Technologies) and further cultivated mTeSR supplemented with ROCK inhibitor (1:1000, Stem Cell Technologies). All cells were cultivated at 37 °C in a saturated humidity atmosphere containing 5 % CO2.

4.2. Alkaline phosphatase staining

Ice-cold methanol was used to fix iPSC colonies from passage 7 (p7) for 10 min. Afterwards cells were incubated for 15 min in the staining solution composed of: 75% distilled water, 10% 1 M sodium chloride, 10% 1 M Tris (pH 9.8), 5% 1 M magnesium chloride, 1:50 NBT/BCIP stock solution (Roche).

4.3. Immunofluorescence staining

For immunofluorescence staining, the iPSC colonies (p10) were fixed in 4% paraformaldehyde for 15 min. Blocking was carried out using PBS containing 10% normal goat serum and cells were permeabilized with 0.1% Triton-X-100 for 45 min. Primary antibodies were incubated overnight at 4 °C and secondary antibodies for 1 h at room temperature (RT). DAPI was added for 5 min at RT. Images were acquired using a Keyence BZ-8000 K microscope (Keyence).

4.4. Flow cytometry

Cells (p10) were collected with Gentle Cell Dissociation Reagent (STEMCELL Technologies). For the analysis of the pluripotency markers NANOG and SSEA4, cells were prepared with the True-NuclearTM Transcription Factor Buffer Set (Biolegend). Antibodies were incubated for 1 h at RT. Per sample $5x10^4$ cells were measured with a FACSCalibur (BD) and analysis was done with the FCSalyzer software (version 0.9.18-alpha).

4.5. Embryoid body (EB) formation

IPSC colonies (p11) were transferred to low attachment plates in differentiation medium (knockout DMEM, 20 % FBS, 1 % MEM NEAA, 2 mM GlutaMAX, and 0.1 mM beta-mercaptoethanol) to induce formation. After seven days EBs were reseeded onto gelatin-coated glass cover slips and allowed to differentiated for 10 days. Expression of nestin, MA, and α-FP was proven by immunofluorescence staining as described above.

4.6. Short tandem repeat (STR)-Analysis

STR-Analysis of 18 STR loci was carried out with the ATCC FTA Sample Collection Kit for Human Cell Authentication Service (ATCC). Samples of fibroblasts (p7) and iPSCs (p11), containing $1x10^6$ cells/ml PBS, were collected on Whatman® FTA® cards according to the manufacturer's protocol.

4.7. Mycoplasma test

Absence of mycoplasma in iPSCs (p10) was proven with the PCR-Mycoplasma-Test-Kit I/C (PromoCell) according to manufacturer's instructions.

4.8. Targeted sequencing

Quick-DNATM Miniprep Kit (Zymo Research) was used to obtain genomic DNA (gDNA) of fibroblasts (p7) and iPSCs (p10). Targeted sequencing library construction was performed using a custom designed Ion AmpliSeq™ FA2H Panel (Thermo Fisher Scientific). 10 ng of gDNA was used for library construction covering the complete coding sequence. Sequencing was carried out on an Ion Torrent™ Personal Genome Machine™ System, using an Ion Torrent 318 V2 chip. Sequence analysis was performed using the hg19 assembly of the human genome using Torrent Suite™ software and the variant caller plugin version 5.12.V2 (Thermo Fisher Scientific).

4.9. RT-PCR

The Quick-RNA Miniprep kit (Zymo Research) was used to extract RNA of iPSCs (p10) and an Eppendorf 5331 Master Cycler was used to perform an One-step RT-PCR (QIAGEN). Per sample 50 ng of RNA was used and PCR products were run on TBE agarose gels.

4.10. Transgene expression silencing

Cells were collected 7 days after reprogramming and subsequently after each passage until passage 7. RNA of iPSCs was isolated with a Quick-RNA Miniprep kit (Zymo Research) and cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen). Absence of transgene was detected by PCR using LightCycler® FastStart DNA MasterPLUS SYBR Green I Kit (Roche) and transgene-specific primers.

4.11. Karyotyping

Karyotype analysis of iPSCs (p11) was performed commercially by the Center for Regenerative Therapies Dresden (CRTD, Technische Universität Dresden). G-banding of 20 metaphases spreads, with a resolution of 400 – 450 bands per haploid set, was performed.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Fatima Efendic reports financial support was provided by NBIA Disorders association. Fatima Efendic reports financial support was provided by Centre for Transdisciplinary Neurosciences Rostock. Andreas Hermann reports financial support was provided by Hermann und Lilly Schilling-Stiftung für medizinische Forschung im Stifterverband.

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