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Lab Resource: Genetically-Modified Multiple Cell Lines

Generation of a homozygous and a heterozygous *SNCA* gene knockout human-induced pluripotent stem cell line by CRISPR/Cas9 mediated allele-specific tuning of *SNCA* expression

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A B S T R A C T

Aggregation of alpha-synuclein (aSyn) is closely linked to Parkinson's disease, probably due to the loss of physiological functions and/or gain of toxic functions of aggregated aSyn. Significant efforts have been made elucidating the physiological structure and function of aSyn, however, with limited success thus far in human-derived cells, partly because of restricted resources. Here, we developed two human-induced pluripotent stem cell lines using CRISPR/Cas9-mediated allele-specific frame-shift deletion of the aSyn encoding gene *SNCA*, resulting in homo- and heterozygous *SNCA* knockout. The generated cell lines are promising cellular tools for studying aSyn dosage-dependent functions and structural alterations in human neural cells.

1. Resource Table

Unique stem cell lines identifier	UKERi010-A-1 UKERi010-A-2
Alternative name(s) of stem cell lines	UKERi7MN-010-1, *SNCA*(−/−) UKERi7MN-010-2, *SNCA*(+/−)
Institution	Department of Molecular Neurology University Hospital Erlangen Friedrich-Alexander-University Erlangen-Nürnberg
Contact information of the reported cell line distributor	Wei Xiang, Email: wei.xiang@fau.de
Type of cell lines	iPSC
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 52 Sex: female Ethnicity: Caucasian
Cell Source	Original cell type induced for reprogramming: dermal fibroblasts
Method of reprogramming	Invitrogen™ CytoTune™-iPS 2.0 Sendai Reprogramming Kit
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	Sendai Reprogramming Kit is based on a non-integrating Sendai virus remaining in the cytoplasm
Cell culture system used	iPSC line was grown on basement membrane extracted from murine tumors in mTeSR
Type of Genetic Modification	Induced mutation
Associated disease	N/A
Gene/locus	*SNCA*, chromosome 4q22.1
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	RNP
All genetic material introduced into the cells	Recombinant Cas9, sgRNAs
Analysis of the nuclease-targeted allele status	Sanger sequencing of targeted alleles
Method of the off-target nuclease activity surveillance	Targeted PCR/sequencing
Name of transgene	No transgene was inserted
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A

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Abbreviations: aSyn, Alpha-synuclein; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; SNCA, Gene encoding alpha-synuclein; PD, Parkinson's disease; hiPSCs, Human-induced pluripotent stem cells; sgRNA, single guide RNA.

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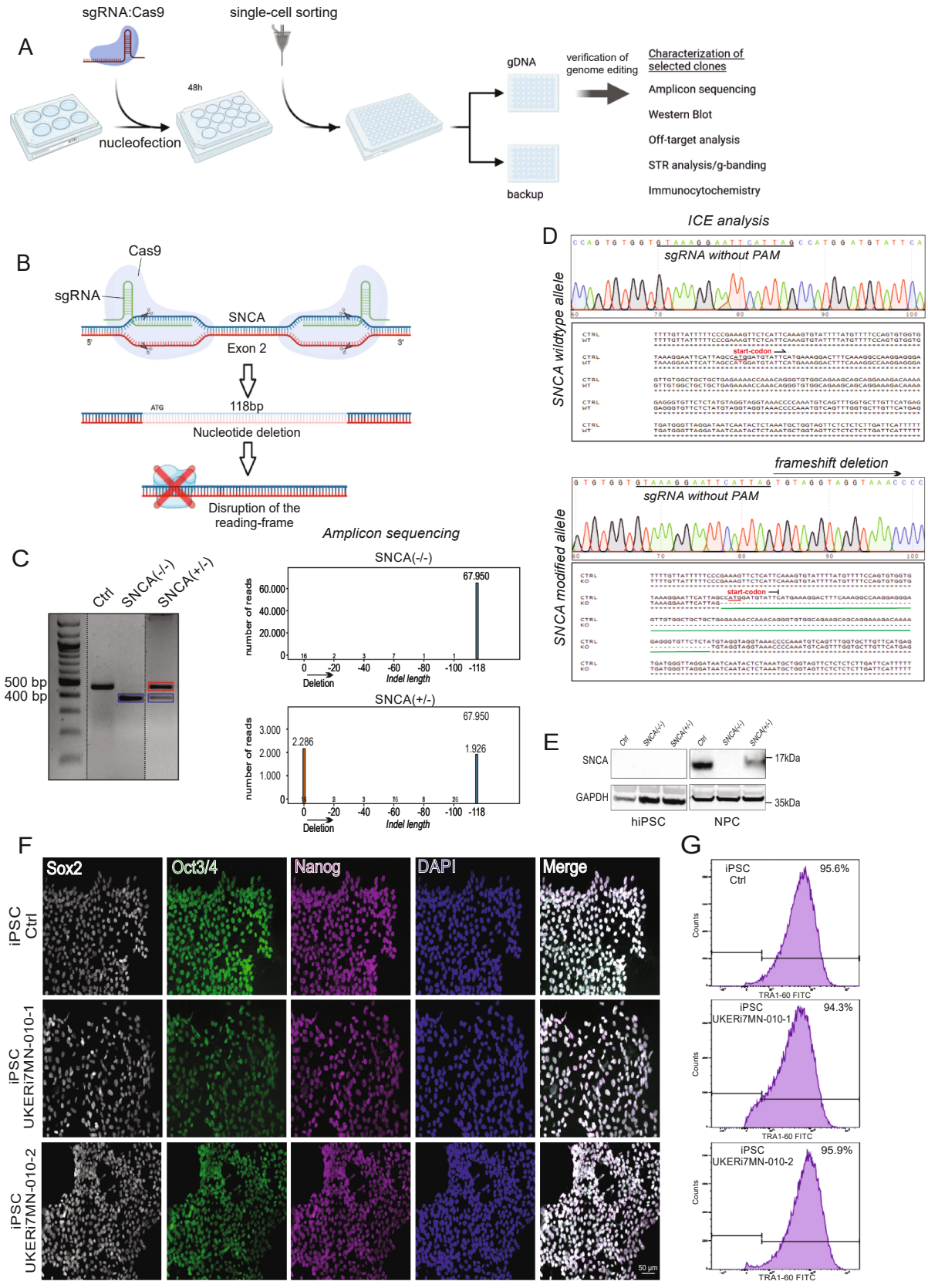

Fig. 1. Workflow and characterization of CRISPR/Cas9-mediated allele-specific gene editing of SNCA.

(*continued*)

Inducible/constitutive system details	N/A
Date archived/stock date	07/2022
Cell line repository/bank	UKERi010-A-1: https://hpscreg.eu/user/celline/edit/UKERi010-A-1 UKERi010-A-2: https://hpscreg.eu/user/celline/edit/UKERi010-A-2
Ethical/GMO work approvals	Institutional Review Board approval number: 259_17B (31/01/2022), Friedrich-Alexander-University Erlangen-Nürnberg
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

2. Manuscript section expected contents clarification

2.1. Resource utility

The reported hiPSC lines were generated from a healthy donor without interference of other known pathogenic mutations. Thus, these hiPSC lines with reduced or fully inactive aSyn allow to study the involvement of aSyn in cellular processes of human-derived cells, in particular in different cellular CNS derivatives.

2.2. Resource details

Alpha-synuclein (aSyn) encoded by the *SNCA* gene is involved in synucleinopathies, including Parkinson's disease (PD). aSyn aggregation-mediated loss of physiological functions and/or gain of toxic functions are believed to induce synucleinopathies (Oliveira et al., 2021). Despite intense efforts, physiological structure and function of aSyn, in particular in human-derived cells, remains elusive. Recent studies on aSyn pathophysiology frequently used neural cells differentiated from human-derived iPSCs (hiPSCs) generated from monogenic PD patients carrying *SNCA* gene multiplications. Isogenic hiPSC lines with corrected *SNCA* dosage have already been generated, which are powerful for investigating specific effects of aSyn in inducing PD-related phenotypes (Zafar et al., 2022). For studying physiological role of aSyn, there is an urgent need of human cells with down-regulated aSyn expression, however, without the multiplication of other genes flanking *SNCA*. Here, we generated a homozygous and a heterozygous *SNCA* knockout cell line (*SNCA*−/−, *SNCA*+/−) from a hiPSC line originally derived from a healthy donor without any history of neurological disease and known pathogenic gene mutations.

SNCA consists of 6 exons, of which exons 2–6 encode full-length aSyn. For precise *SNCA* knockout, we targeted exon 2 which contains the start codon ATG taking advantage of the error-prone non-homologous end joining (NHEJ). The gene editing approach was performed using CRISPR/Cas9 technology (Fig. 1A). For an efficient and straightforward evaluation of *SNCA* knockout, two single guide RNAs (sgRNAs, Table 2) were designed flanking exon 2 (Fig. 1B). Since the Cas9 nuclease cuts a specific sequence 3 base pairs (bp) upstream of the PAM sequence of the sgRNAs, using the designed sgRNAs might result in a 118 bp deletion within exon 2 of *SNCA*. For gene editing, the parental hiPSC line was nucleofected with synthetic sgRNAs and recombinant Cas9 as ribonucleoprotein. Afterwards, single hiPSCs were sorted by flow cytometry into a 96-well plate for expansion. Clonal hiPSCs were next splitted in two 48-well plates for genotype screening (Fig. 1A, gDNA) by PCR evaluation of the deleted 118 bp, followed by further verification approaches (Fig. 1C) and for freezing (Fig. 1A, backup). The modified hiPSC clones were verified for the predicted deletion of 118 bp by amplicon sequencing (Fig. 1C, right) of the PCR products (Fig. 1C, left). In the *SNCA*−/− line there are no remaining wildtype alleles, while

Table 1
Characterization and validation

Classification	Test	Result	Data
Morphology	Photography	Visual records of the hiPSC line's cellular morphology.	Phase-contrast images of the edited hiPSC lines compared to the parental hiPSC line. Fig. S1D
Pluripotency status evidence for the described cell line	Qualitative analysis	Immunocytochemical staining of pluripotency markers Sox2, Oct3/4, and Nanog	Fig. 1F
	Quantitative analysis	Flow cytometry of TRA 1–60 UKERi7MN-010-1 (*SNCA*−/−): 94.3 % positive cells UKERi7MN-010-2 *(SNCA*+/−*)*: 95.9 % positive cells	Fig. 1G
Karyotype	Karyotype (G-banding)	46XX, Resolution 450 bhps	Fig. S2B
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site	PCR and agarose gel electrophoresis with subsequent sequencing of fragments for confirmation of homo-/ and heterozygous *SNCA* knockout status	Fig. 1C and D
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	N/A	N/A	Synthetic guide RNAs – no plasmids were applied
Parental and modified cell line genetic identity evidence	STR analysis	STR analysis of the 9 core STR markers. All the loci of the modified cell lines matched the parental line.	STR analysis file submitted in the archive with journal
Mutagenesis/ genetic modification outcome analysis	Sequencing of genomic DNA	Amplicon-EN NGS-based sequencing	Fig. 1C
	PCR-based analyses	Detection of deletion on agarose gel electrophoresis	Fig. 1C
	Western blotting for *SNCA* knock out	NPCs differentiated from UKERi010-A-1 (*SNCA*−/−, absence of aSyn immunosignals) and from UKERi010-A-2 (*SNCA*+/−, reduced aSyn levels)	Fig. 1E
Off-target nuclease analysis-	PCR across top 6 predicted top likely off-target sites	Lack of NHEJ-caused mutagenesis in the top predicted off-target Cas nuclease activity	Fig. S1A
Specific pathogen-free status	Mycoplasma testing by luminescence	Modified lines are negative	Fig. S1B
Multilineage differentiation potential	Embryoid body formation and directed three germ layer differentiation	Parental and modified cell lines are capable of differentiating in three germ layers as well as forming embryoid bodies	Fig. S1C and S1D
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype – additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Primary antibody – Western blot	Mouse anti-aSyn (Syn1)	1:1,000	BD Biosciences Cat# 610787, RRID:AB_398108
Primary antibody – Immunocytochemistry	Rat anti-aSyn (15G7) antibody	1:200	Enzo Life Sciences Cat# ALX-804–258, RRID: AB_2270759
Pluripotency marker – Immunocytochemistry	Rabbit anti-Sox2 (D6D9)	1:400	Cell Signaling Technology Cat# 3579, RRID: AB_2195767
Pluripotency marker – Immunocytochemistry	Goat anti-Nanog	1:200	R and D Systems Cat# AF1997, RRID:AB_355097
Pluripotency marker – Immunocytochemistry	Mouse anti-Oct-3/4 (C-10)	1:200	Santa Cruz Biotechnology Cat# sc-5279, RRID: AB_628051
Pluripotency marker – flow cytometry	Alexa Fluor(R) 488 anti-human TRA-1–60-R antibody	1:50	BioLegend Cat# 330614, RRID:AB_2119064
Loading control – Western blot	Rabbit anti-GAPDH polyclonal antibody, unconjugated	1:1,000	Abcam Cat# ab9485, RRID:AB_307275
Neuronal differentiation – Immunocytochemistry	Mouse anti-Nestin	1:400	Millipore Cat# MAB5326, RRID:AB_2251134
Secondary antibodies	Donkey anti-mouse IgG (H + L) antibody, Alexa Fluor 488, conjugated	1:1,000	Molecular Probes Cat# A-21202, RRID: AB_141607
	Donkey anti-mouse antibody, Alexa Fluor 647, conjugated	1:500	Jackson ImmunoResearch Labs Cat# 715–605-151, RRID:AB_2340863
	Donkey anti-mouse IgG, (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 568, conjugated	1:1,000	Thermo Fisher Scientific Cat# A10037, RRID: AB_2534013
	Donkey anti-rat IgG (H + L) antibody, Alexa Fluor 488 conjugated	1:1,000	Molecular Probes Cat# A-21208, RRID: AB_141709
	Donkey anti-Goat IgG (H + L) antibody, Alexa Fluor 568, conjugated	1:1,000	Molecular Probes Cat# A-11057, RRID: AB_142581
Nuclear staining – Immunocytochemistry	4′,6-Diamidino-2-phenylindole (DAPI)	1:10,000	Sigma-Aldrich Cat#D8417
Site-specific nuclease			
Nuclease information	spCas9		
Delivery method	Nucleofection		
Selection/enrichment strategy	Single-cell sorting		

Primers and Oligonucleotides used in this study		
	Target	Forward/Reverse primer (5′-3′)
Genotyping (desired allele/transgene presence detection)	PCR specific for the targeted allele	CCTTCTGCCTTTCCACCCT/TTGTCAGGATCCACAGGCAT; Representative PCR gel in Fig. 1C: Product size: 449 bp See supplementary file 1 for PCR parameters and device used
Targeted mutation sequencing	Amplicon PCR Primer without adapter sequence	CCTTCTGCCTTTCCACCCT/TTGTCAGGATCCACAGGCAT
sgRNA oligonucleotide	sgRNA oligonucleotides (synthetic)	sgRNA (5′-end of exon 2): GTAAAGGAATTCATTAGCCA sgRNA (3′-end of exon 2): AAAGAGGGTGTTCTCTATGT
Genomic target sequence	Including PAM and other sequences likely to affect UCN activity	APE file submitted in archive with journal
Top exonal off-target predicted site sequencing	Off-target #1 AC003088.1	GAAACATGGACTTCTGGCCC/TTGGCTGAGTCTGAGAGTGG TCTCCTGCCTCAACCTCTTG/ACTTTCGGAGGCTGAGTCTG
	Off-target #2CLMP	TTCCATGCTTAGAGAGTCAGC/
	Off-target #3 IL1RAPL1	AAGATCTATTGTCTAGGCAAGGG GGCCAAGGTCTGATTGCAAA/ACACAGGAAGGAAGCAGACA
	Off-target #4 RP11-297D21.4	AGGAGAAGTTGAAGGCAGGG/GGTTTCAAGCCCCACACCTA ACTTGAACAGATTGAGAAGAGCT/
	Off-target #5 TTC28	TGCAAGGGACAGTAGATACCA
	Off-target #6 ANAPC13	

the *SNCA*+/−line shows an equal distribution of sequencing reads of the wildtype allele and the modified allele with deletion. To further verify modification-induced frame-shift, hiPSC lines were analysed using sequencing and ICE analysis (Fig. 1D, Fig. S2C), confirming the unchanged sequence of the wildtype allele in the *SNCA*+/− line and the identical frame-shift deletion of the modified allele(s) of both *SNCA* −/− and *SNCA* +/− lines.

Six genomic regions representing the highest homology to the sgRNA target region were analysed for potential off-target effects by PCR amplification (Fig. S1A). Due to very low expression of aSyn in hiPSCs, neural progenitor cells (NPCs) were generated from the reported hiPSC lines (*SCNA*−/− and *SNCA*+/−) to determine aSyn levels. The lack of aSyn expression in *SNCA*−/− NPCs and a reduced aSyn expression in *SNCA*+/− NPCs were confirmed by Western blot analysis (Fig. 1E) and immunocytochemistry (Fig. S2A). Moreover these expression data exclude the persistence of functional transcripts and alternative start codons for *SNCA* expression in the remaining coding sequence. Furthermore, morphology and pluripotency of the reported hiPSC lines were analysed by phase contrast microscopy (Fig. S1D, Table 1), and immunocytochemical or flow cytometric analyses of pluripotency markers (Sox2, Oct3/4, Nanog) (Fig. 1F) or TRA 1-60 (Fig. 1G, Table 1). Differentiation potential of the generated hiPSC lines was also verified by trilineage differentiation assay (Fig. S1C).

3. Materials and methods

3.1. Cell culture

hiPSCs were cultured on a 6-well plate pre-coated with Geltrex™ (Thermo Fisher Scientific) in mTeSR (STEMCELL Technologies) with 1

% Penicillin/Streptomycin at 37 °C and 5 % CO_2. Cells were passaged every 3–5 days using Gentle Cell Dissociation Reagent (STEMCELL Technologies) in a ratio of 1:3–1:8. Gene editing was performed at passage 20 and all subsequent analyses were performed 5–10 passages after gene editing. Mycoplasma testing was performed using MycoAlertTM Mycoplasma Detection Kit (Lonza).

3.2. Genome editing

The gene editing was based on a previous protocol (Turan et al., 2019) (Fig. 1A). sgRNAs flanking exon 2 of *SNCA* (Table 2) were designed using the CRISPOR tool (http://crispor.tefor.net/). Recombinant Cas9 protein (Synthego) was adjusted to 20 μM and synthetic sgRNAs (Synthego) were diluted in nuclease-free, Tris-EDTA buffer to 100 μM. For assembly of the sgRNAs and Cas9, 300 pmol of sgRNAs and 40 pmol Cas9 were mixed in 5 μl and incubated for 10 min at RT. Next, cells were dissociated by accutase and electroporated using the nucleofector 2B (Lonza, program B-16) and the Human Stem Cell Nucleofector™ Kit 2 (Lonza). Single cells were subsequently replated on a 6-well plate. After 48 hrs, cells were sorted as single clones onto 96-well plates in mTeSR containing 10 % CloneR supplement (STEMCELL Technologies). After another 5–10 days, each clone was duplicated on two 48-well plates, respectively for genotyping and cryopreservation (Fig. 1A). Cryopreservation was performed using Bambanker (Nippon). Genomic DNA was extracted using Quickextract (Lucigen) according to manufacturer's protocol and next amplified using Q5®High-Fidelity DNA Polymerase (New England Biolabs) and the primers (Table 2) for the screening purpose. More specific information for PCR is available in supplementary file 1. Final verification of the deletion of exon 2 was carried out by NGS-based Amplicon-EZ (Genewiz).

3.3. Off-target analysis

Genomic loci with a high homology to the target of the designed sgRNAs were identified using CRISPOR (http://crispor.tefor.net/) (Table 2). Six loci with the highest predictability for off-target modification were selected (primers in Table 2), amplified by PCR (supplementary file 1), and sequenced (LGC Genomics, Germany).

3.4. Short tandem repeat (STR) analysis

Genomic DNA was extracted by using Quickextract (Lucigen) according to manufacturer's protocol and analysed for STR by the service provider CLS, Germany.

3.5. Trilineage differentiation

Trilineage analysis was performed using the STEMdiff™ Trilineage Differentiation Kit (STEMCELL Technologies). Immunocytochemical evaluation of germ layer-specific marker expression was performed using Human Three Germ Layer 3-Color Immunohistochemistry Kit (R&D Systems).

3.6. Western blot (WB), immunocytochemistry, and flow cytometry

For protein analysis, generation of cell homogenates as well as WB and immunocytochemistry were performed according to Seebauer et al. 2022 and flow cytometry according to Turan et al 2019 (supplementary file 1 for more specific experimental information).

3.7. Generation of embryoid body (EB) and differentiation into neural progenitor cells (NPCs)

EBs and NPCs were generated from hiPSC using a small molecule-based protocol as described in Seebauer et al., 2022.

3.8. G-banding

Karyotyping was performed by GTG banding at 450 bands resolution using standard protocols for lymphocyte cultures.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2022.102952.

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