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Lab Resource: Genetically-Modified Multiple Cell Lines

Generation of iPSC lines with SLC16A2:G401R or SLC16A2 knock out

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ABSTRACT

The X-linked Allan-Herndon-Dudley syndrome (AHDS) is characterized by severely impaired psychomotor development and is caused by mutations in the *SLC16A2* gene encoding the thyroid hormone transporter MCT8 (monocarboxylate transporter 8). By targeting exon 3 of *SLC16A2* using CRISPR/Cas9 with single-stranded oligodeoxynucleotides as homology-directed repair templates, we introduced the AHDS patient missense variant G401R and a novel knock-out deletion variant (F400Sfs*17) into the male healthy donor hiPSC line BIHi001-B. We successfully generated cerebral organoids from these genome-edited lines, demonstrating the utility of the novel lines for modelling the effects of MCT8-deficency on human neurodevelopment.

1. Resource table

Unique stem cell line identifier	BIHi001-B-1 BIHi001-B-7 BIHi001-B-8
Alternative name(s) of stem cell line	NA
Institution	Berlin Institute of Health at Charité and Charité – Universitätsmedizin Berlin
Contact information of the reported cell line distributor	Harald Stachelscheid, PhD cusco@bih-charite.de
Type of cell line	iPSC
Origin	human
Additional origin info	1., 2., 3.: Age: neonate; Sex: male; ethnicity: Caucasian
Cell Source	1., 2., 3.: dermal fibroblasts
Method of reprogramming	1., 2., 3.: Non-intergating single-stranded RNA replicon vector
Clonality	Clonal; single cell seeding using IsoCell Iota sciences
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	1., 2., 3.: yes, RT-PCR
The cell culture system used	1., 2., 3.: Feeder-free culture in Essential 8 media with enzyme free dissociation (EDTA)
Type of the Genetic Modification	1. SLC16A2 disease associated mutation introduction

(*continued on next column*)

(*continued*)

Unique stem cell line identifier	BIHi001-B-1 BIHi001-B-7 BIHi001-B-8
	2. SLC16A2 gene knock-out 3. SLC16A2 gene knock-out
Associated disease	Allan-Herndon-Dudley syndrome (AHDS): OMIM: #300523
Gene/locus	1., 2., 3.: SLC16A2 (NCBI Gene ID: 6567), Xq13.2
Method of modification / user-customisable nuclease (UCN) used, the resource used for design optimisation	CRISPR/Cas9
User-customisable nuclease (UCN) delivery method	Ribonucleoprotein complex (RNP)
All double-stranded DNA genetic material molecules introduced into the cells	NA
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele
Method of the off-target nuclease activity prediction and surveillance	NA
Descriptive name of the transgene	NA
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	NA

(*continued on next page*)

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(*continued*)

Unique stem cell line identifier	BIHi001-B-1 BIHi001-B-7 BIHi001-B-8
Inducible/constitutive expression system details	NA
Date archived/stock creation date	1. 31.05.2021 2. 23.01.2023 3. 23.01.2023
Cell line repository/bank	Berlin Institute of Health, Core Unit pluripotent Stem Cells & Organoids: https://www.bihealth.org/en/research/core-facilities/stem-cells/ and https://hpscreg.eu/
Ethical/GMO work approvals	Ethical approval can be found at: https://hpscreg.eu/cell-line/BIHi001-B
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	NA

2. Resource utility

The p.G401R mutation in SLC16A2, a thyroid hormone transporter, results in Alan-Herndon-Dudley (AHD) syndrome, a neuro-developmental disorder causing moderate to severe mental disability and movement disorder (Friesema et al., 2010, p. 8; van Geest et al., 2022, p. 8). SLC16A2 knock-out, and G401R mutant iPSC lines will be a valuable tool for understanding molecular mechanisms underlying the condition using *in vitro* models e.g. cerebral organoids (Table 1).

3. Resource details

SLC16A2-G401R and knock-out (KO) cell lines were generated by transfection of recombinant Cas9 protein and synthetic gRNA with (G401 mut) or without ssODN into a hiPSC line dervided from a healthy donor (parental, BIHi001-B). Both modifications were generated using single gRNA targeting exon 3 (Fig. 1**A,** Table 2). SLC16A2-G401R mutation was introduced through homology directed repair (HDR) using ssODN template (Fig. 1**A,** Table 2). The ssODN template included the mutation found in the patients with AHDS: c.1201G > A, silent mutation to disrupt PAM sequence: c.1200C > T, and 4 silent mutations to disrupt seed sequence (c.1185C > T, c.1186C > A, c.1188C > A, c.1191C > T) (Fig. 1**A and F**). For SLC16A2 KO generation the same gRNA guide was used and clones with frame shift resulting in premature STOP codon were chosen. In both reported KO cell lines (BIHi001-B-7 and −8)

Table 1
Characterization and validation.

Classification (optional *italicized*)	**Test**	**Result**	**Data**
Morphology	Photography	Typical iPSC morphology	Fig. 1 panel B
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	Cells are positive for Nanog, SSEA4, Tra-1-60 and Oct3/4 (1.p7; 2.p6; 3.p6)	Fig. 1 panel C
	Quantitative analysis (Flow cytometry)	Percentage of cells positive for each marker: 1. Oct3/4: 98.2 %; Nanog: 99.8 %; TRA1-60: 83 %; SSEA4: 90.4 % 2. Oct3/4: 97.5 %; Nanog: 99.5 %; TRA1-60: 87.7 %; SSEA4: 95.7 % 3. Oct3/4: 98.6 %; Nanog: 99.3 %; TRA1-60: 84.7 %; SSEA4: 95 % (1.p7; 2.p5; 3.p5)	Fig. 1 panel D
Karyotype	Karyotype (G-banding) and higher-resolution, array-based assays (SNP)	Normal karyotype: no reportable instabilities detected in engineered cell lines in 20 metaphases assessed (1.p5; 2.p4; 3.p4) or in SNP analysis as compared to parental line (1.p4; 2.p11; 3.p4).	G-banding: Fig. 1 panel C and Supplementary Files 3 and 4; SNP: Supplementary File 5
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site	1. Hemizygous HDR incorporation 2. Hemizygous knock-out 3. Hemizygous knock-out	Fig. 1 panel F
	Evaluation of the - (homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s)	NA	NA
	Transgene-specific PCR (when applicable)	NA	NA
Verification of the absence of random plasmid integration events	PCR/Southern	NA	NA
Parental and modified cell line genetic identity evidence	STR analysis	Parental and modified cell lines are of the same origin Loci tested: H01, TPOX, vWA, Amelogenin, CSF1PO, D16S539, D7S820, D13S317 and D5S818	Supplementary file 2, submitted in the archive with journal
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR)	1. Hemizygous HDR incorporation 2. Hemizygous knock-out 3. Hemizygous knock-out	Fig. 1 panel F
	PCR-based analyses	NA	NA
	RNAseq and western blotting (for knock-outs, KOs)	2. and 3. mRNA levels decreased; protein not detected for the targeted gene	Fig. 1 panels G and H
Off-target nuclease activity analysis	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	Not performed	NA
Specific pathogen-free status	Mycoplasma	Mycoplasma testing with RT-PCR, cell lines are mycoplasma free. (1.p5; 2.p4; 3.p4)	Supplementary Fig. S1 panel A
Multilineage differentiation potential	Directed differentiation	Positive for CD140b and CD144 (Mesoderm), Sox2 and Pax6 (Ectoderm), Sox17 and CD184 (Endoderm)	Supplementary Fig. S1 panel B
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	NA	NA
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	NA	NA
	HLA tissue typing	NA	NA

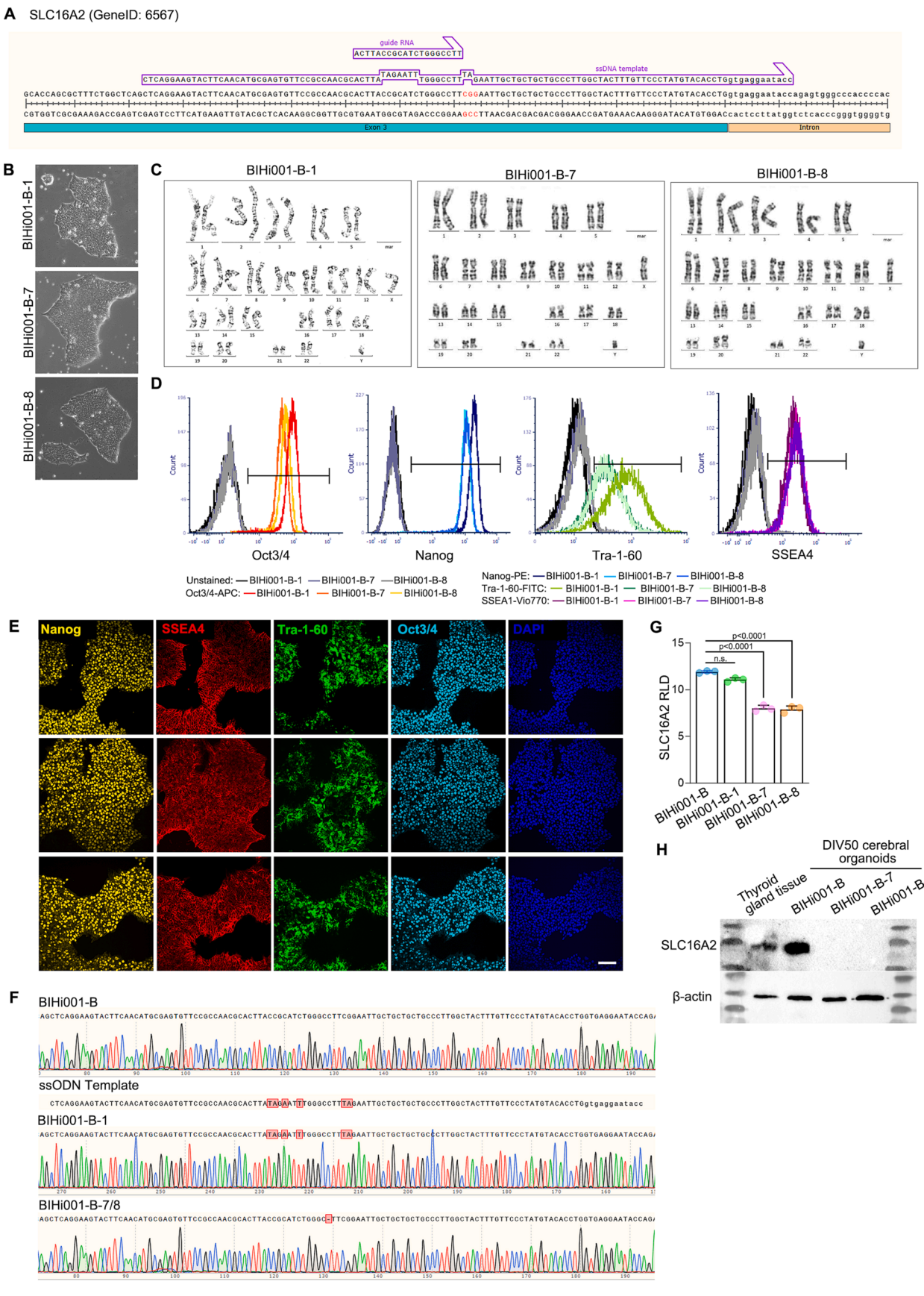

Fig. 1. Figure 1. Characterization and validation of SLC16A2:G401R or SLC16A2 knock out

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers (FACS)	recombinant anti-SSEA-4 VioBlue	FACS 1:20	Miltenyi Biotec, 130-098-366 AB_2653521
Pluripotency Markers (FACS/IF)	recombinant anti-Oct3/4 APC	FACS 1:100 IF 1:10	Miltenyi Biotec, 130-123-257 AB_2819457
Pluripotency Markers (FACS/IF)	recombinant anti-TRA-1-60 Vio488	FACS 1:700 IF 1:100	Miltenyi Biotec, 130-106-872 AB_2654228
Pluripotency Markers (FACS/IF)	rabbit anti-Nanog (D73G4) PE	FACS 1:100 IF 1:50	Cell Signaling, 14955S AB_2798659
Pluripotency Markers (IF)	recombinant anti-SSEA4 PerCpVio700	IF 1:10	Miltenyi Biotec, 130-105-053 AB_2653527
Trilineage differentiation (FACS)	Sox2 Antibody, anti-human/mouse, FITC, REAfinity™	FACS: 1:250	Miltenyi Biotec, 130-120-721 AB_2784458
Trilineage differentiation (FACS)	CD140b Antibody, anti-human, APC, REAfinity™	FACS: 1:50	Miltenyi Biotec, 130–105-280 AB_2655085
Trilineage differentiation (FACS)	CD144 (VE-Cadherin) Antibody, anti-human, FITC, REAfinity™	FACS: 1:50	Miltenyi Biotec,130-100-742 AB_2655151
Trilineage differentiation (FACS)	CD184 (CXCR4) Antibody, anti-human, APC, REAfinity™	FACS: 1:250	Miltenyi Biotec, 130–120-708 AB_2752173
Trilineage differentiation (FACS)	Sox17 Antibody, anti-human, Vio B515, REAfinity™	FACS: 1:250	Miltenyi Biotec, 130-111-031 AB_2653497
Trilineage differentiation (FACS)	PAX-6 Antibody, anti-human, APC, REAfinity™	FACS: 1:50	Miltenyi Biotec, 130-107-776 AB_2653169
Confirmation of gene editing (WB)	rabbit polyclonal anti-SLC16A2	WB 1:750	Atlas, HPA003353 AB_1079343
Confirmation of gene editing (WB)	mouse Anti-β-Actin	WB 1:1000	Sigma, A1978 AB_476692
Confirmation of gene editing (WB)	goat Anti-Rabbit Immunoglobulins/HRP antibody	WB 1:2000	Agilent, P0448 AB_2617138
Confirmation of gene editing (WB)	goat Anti-Mouse Immunoglobulins/HRP antibody	WB 1:2000	Agilent, P0447 AB_2617137
Site-specific nuclease			
Nuclease information	recombinant HiFi Cas9	Alt-R® S.p. HiFi Cas9 Nuclease V3 (IDT)	
Delivery method	nucleofection	4D-Nucleofector™ system (Lonza); program CM150 P3 Primary Cell 4D-Nucleofector® X Kit L (Lonza)	
Selection/enrichment strategy	single cell clonal plating	IsoCell single cell plating into grid chambers (IotaSciences)	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
Genotyping (desired allele/transgene presence detection)	SLC16A2	Fwd: TCTTATGCTGCTTTCACTCACC Rev: TTCCACTAAGGTCTTCCCTCAA	
Potential random integration-detecting PCRs	NA	NA	
gRNA oligonucleotide/crRNA sequence	sgRNA (DNA binding region)	ACUUACCGCAUCUGGGCCUU	
Genomic target sequence(s)	SLC16A2 exon 3	ACTTACCGCATCTGGGCCTTCGG	
ssODN	SLC16A2	IDT-HDR C*T* CAG GAA GTA CTT CAA CAT GCG AGT GTT CCG CCA ACG CAC TTA TAG AAT TTG GGC CTT TAG AAT TGC TGC TGC TGC CCT TGG CTA CTT TGT TCC CTA TGT ACA CCT GGT GAG GAA TA*C* C	

Table 2 (*continued*)

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**

double strand break repair resulted in single base (C) deletion: c.1197_1197delC, F400Sfs*17. Genetic modifications of the SLC16A2 locus were confirmed by Sanger sequencing (Fig. 1**F**). The karyotype of the described cell lines is 46XY. Male donor line was selected on purpose, as this X-linked disease manifests most often in males. Therefore, all the described modifications are hemizygous. iPSCs do not express high levels of SLC16A2 gene. Therefore, described cell lines and the parental line (BIHi001-B) were differentiated into cerebral organoids to assess *SLC16A2* mRNA levels. G401 mutation of SLC16A2 did not affect RNA levels, but both KO cell lines showed a significant decrease in SLC16A2 mRNA levels (Fig. 1**G**). As the mRNA depletion was not total, likely due to exons 1–3 still being expressed, protein levels of SLC16A2 were assessed in cerebral organoids. No protein was detected in organoids derived from either of the SLC16A2-KO lines as compared to organoids from parental (BIHi001-B) cell line or primary human thyroid tissue (positive control) (Fig. 1**H**). All resulting cell lines had normal morphology (Fig. 1**B**), unmodified karyotypes (Fig. 1**C**), and expressed markers of undifferentiated cells as visualized by FACS (Fig. 1**D**) and immunofluorescence (Fig. 1**E**).

4. Materials and methods

4.1. Cell culture

iPSCs were maintained in Essential 8 medium on plates coated with Geltrex (both Thermo) at 37 °C, 5 % CO2, 5 % O2. For regular maintenance, the media was changed daily, and the cells were passaged as clumps (EDTA) at 70 % confluence. Prior to transfection, the media was changed to StemFlex (Thermo) and cells were passaged using Accutase (Thermo). During single-cell handling the media was supplemented with CloneR (StemCell Technologies).

4.2. CRISPR-Cas9 editing

TrRNA:crRNA duplexes (200 pmol) were annealed at 95°C for 5 min, cooled down to RT, and incubated with Cas9 nuclease (123 pmol) at RT for 1 h (all IDT). 0.5x10^6 cells were nucleofected with RNPs using a 4D-Nucleofector™ system and a P3 Primary Cell 4D-Nucleofector® X Kit L (program CM150, Lonza), then plated on Geltrex coated plates in StemFlex supplemented with CloneR for 48 h. Cells were replated for single cell cloning using IsoCell (IotaSciences). Single colonies were transferred to 96-well plates and expanded for sequencing (Ludwik et al., 2023).

4.3. PCR and sequencing

Genomic DNA was extracted using Phire Tissue Direct PCR Kit (Thermo). PCR was performed using Kappa2GRobust (Sigma) according to manufacturer instructions with primers listed (Table 2). PCR products (SLC16A2: 402 bp) were purified using Qiaquick PCR-Purification Kit (Qiagen) and sequenced (Mycrosynth AG).

4.4. FACS staining

Cells were harvested using TrypLE™ Select (Life Technologies). 2 ×

10ˆ5 live cells were incubated with TRA-1-60 and SSEA-4 antibodies for 10 min at 4 °C in 100 µL FACS buffer (DPBS, 2 mM EDTA, 0.5 % BSA). Cells were fixed and permeabilised using FoxP3 Staining Buffer Set (Miltenyi). Cells were incubated with Nanog and Oct3/4 antibodies for 30 min at 4 °C in 100 µL of permeabilization buffer. Cells were analysed by MACS-Quant® VYB (Miltenyi).

4.5. IF staining

15x10ˆ4 cells were plated in a CellCarrier-96 Black Imaging Plate (Perkin Elmer) and after 48 h fixed for 10 min at RT with ROTI®Histofix 4 % (Roth), followed by 30 min incubation with Perm/Wash buffer (BD) with 5 % FBS. Cells were incubated for 1 h at RT with antibodies diluted in Perm/Wash. Nuclei were stained with Hoechst 33,342 (10 µg/mL; Thermo Fisher). Microscopy was performed with an OperaPhenix (Perkin Elmer), brightness and contrast were modified for image presentation using Harmony 4.9 (PerkinElmer). Scale bar in Fig. 1D: 100 µm.

4.6. Trilineage differentiation

Directed differentiation was performed using StemMACS™ Trilineage Differentiation Kit (Miltenyi) according to manufacturer instructions.

4.7. Karyotyping

For SNP assessment, gDNA was analysed on Infinium Global Screening Array-24 BeadChip (Illumina); results analysed for CNV using GenomeStudio V2.0.5. For G-banding, cells were harvested (Howe et al., 2014) and the cell pellet was sent to Praxis für Humangenetik Dr. med. Eun Kyung Suk (Berlin) for karyotype assessment.

4.8. Generation of cerebral organoids

Cerebral organoids were generated using STEMdiff™ Cerebral Organoid Kit (Stemcell Technologies) following the manufactureŕs instructions.

4.9. Bulk RNA sequencing

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) following the manufactureŕs instructions. RNA quality was assessed by TapeStation. The mRNA was sequenced using Novaseq 6000 (single-end, 75 bp read-length, 30mln reads/sample).

4.10. Bioinformatic processing of bulk RNA sequencing data

The analysis was performed using an in-house pipeline (github.com/bihealth/seasnap-pipeline). Adapters were trimmed using trimadap (v.r11, https://github.com/lh3/trimadap). Reads were mapped to GRCh38, using STAR aligner (v.2.7.3a). Downstream analysis was performed in R (v.4.2.0). Differential expression analysis was performed using DESeq2 (v.1.38.0). P-values were corrected for multiple testing using the Benjamini-Hochberg method.

4.11. Western blot

Snap frozen DIV50 cerebral organoids were incubated on ice for 30 min in Tris Buffered Saline (TBS) supplemented with 0,1 % Triton-X-100 and Protease inhibitor (Roche). Samples were centrifuged at 16000g/4°C, protein concentration was determined in the supernatant. Protein was resolved on 10 % SDS polyacrylamide gel in a Mini PROTEAN 3Cell chamber (Biorad) (150 V, 75 min) and transferred to a nitrocellulose membrane using the Transblot Turbo Transfer System (Biorad) (1,5A, 25 V, 15 min). The membrane was blocked in TBS 0,01 % Tween 20 (TBS-T) + 4 % milk powder (1 h, RT), incubated with the primary antibody (Table 2, TBS-T + 2 % BSA; O/N, 4 °C) washed 3x5min (TBS-T), incubated with the secondary antibody (Table 2, TBS-T + 4 % milk; 75 min, RT), and washed 3x (TBS-T). Protein was detected using Lumi-LightPLUS Western-Blot-Substrat (Roche, 12015196001) and Chem-iDocXRS (Biorad).

CRediT authorship contribution statement

Katarzyna Anna Ludwik: . **Robert Opitz:** Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Writing – original draft. **Sabine Jyrch:** Investigation. **Matthias Megges:** Investigation, Methodology, Validation, Writing – original draft. **January Weiner:** Data curation, Formal analysis, Visualization. **Dieter Beule:** Software, Supervision. **Peter Kühnen:** Conceptualization, Funding acquisition, Project administration, Supervision. **Harald Stachelscheid:** Conceptualization, Funding acquisition, Project administration, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Harald Stachelscheid reports financial support was provided by German Research Foundation.

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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.2023.103256.

References

Friesema, E.C.H., Visser, W.E., Visser, T.J., 2010. Genetics and phenomics of thyroid hormone transport by MCT8. Mol. Cell. Endocrinol. Spec. Issue: Genet. Thyroid Dis. 322, 107–113. https://doi.org/10.1016/j.mce.2010.01.016.

Howe, B., Umrigar, A., Tsien, F., 2014. Chromosome Preparation From Cultured Cells. J. vis. Exp. 50203 https://doi.org/10.3791/50203.

Ludwik, K.A., Telugu, N., Schommer, S., Stachelscheid, H., Diecke, S., 2023. ASSURED-optimized CRISPR protocol for knockout/SNP knockin in hiPSCs. STAR Protocols 4, 102406. https://doi.org/10.1016/j.xpro.2023.102406.

van Geest, F.S., Groeneweg, S., van den Akker, E.L.T., Bacos, I., Barca, D., van den Berg, S.A.A., Bertini, E., Brunner, D., Brunetti-Pierri, N., Cappa, M., Cappuccio, G., Chatterjee, K., Chesover, A.D., Christian, P., Coutant, R., Craiu, D., Crock, P., Dewey, C., Dica, A., Dimitri, P., Dubey, R., Enderli, A., Fairchild, J., Gallichan, J., Garibaldi, L.R., George, B., Hackenberg, A., Heinrich, B., Huynh, T., Kłosowska, A., Lawson-Yuen, A., Linder-Lucht, M., Lyons, G., Monti Lora, F., Moran, C., Müller, K. E., Paone, L., Paul, P.G., Polak, M., Porta, F., Reinauer, C., de Rijke, Y.B., Seckold, R., Menevşe, T.S., Simm, P., Simon, A., Spada, M., Stoupa, A., Szeifert, L., Tonduti, D., van Toor, H., Turan, S., Vanderniet, J., de Waart, M., van der Wal, R., van der Walt, A., van Wermeskerken, A.-M., Wierzba, J., Zibordi, F., Zung, A., Peeters, R.P., Visser, W.E., 2022. Long-Term Efficacy of T3 Analogue Triac in Children and Adults With MCT8 Deficiency: A Real-Life Retrospective Cohort Study. J. Clin. Endocrinol. Metab. 107, e1136–e1147. https://doi.org/10.1210/clinem/dgab750.