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Lab Resource: Multiple Cell Lines

Generation of two *NAGLU*-mutated homozygous cell lines from healthy induced pluripotent stem cells using CRISPR/Cas9 to model Sanfilippo B syndrome

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Mutations in the *NAGLU* gene cause Sanfilippo B syndrome (mucopolysaccharidosis IIIB), a rare lysosomal storage disorder whose main symptom is a severe and progressive neurodegeneration for which no treatment is still available. Here, we generated two homozygous *NAGLU*-mutated cell lines using CRISPR/Cas9 editing in a healthy human induced pluripotent stem cell (hiPSC) line. These novel cell lines express pluripotency specific markers and maintain their capability to differentiate into all three germ layers *in vitro* while exhibit a normal karyotype. These mutated lines in combination with the isogenic control line will be useful to model *in vitro* Sanfilippo B syndrome.

Resource Table

Unique stem cell lines identifier	UBi001-A-3 and UBi001-A-4
Alternative names of stem cell lines	NAGLU3 (UBi001-A-3) and NAGLU4 (UBi001-A-4)
Institution	University of Barcelona (Barcelona, Spain) and Lund Stem Cell Center (Lund, Sweden)
Contact information of distributor	Isaac Canals - isaac.canals@med.lu.se, Daniel Grinberg - dgrinberg@ub.edu
Type of cell lines	Induced pluripotent stem cell line
Origin	Human
Cell Source	Healthy human induced pluripotent stem cell line. Male, 46XY (UBi001-A)
Clonality	Clonal
Method of reprogramming	Retrovirus (the original hiPSC line) (Canals et al., 2015)
Multiline rationale	Isogenic clones
Gene modification	YES
Type of modification	CRISPR/Cas9-mediated indels
Associated disease	Sanfilippo B syndrome
Gene/locus	*NAGLU*/17q21.2
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	31st July 2019
Cell line repository/bank	UBi001-A: https://hpscreg.eu/cell-line/UBi001-A, UBi001-A-3: https://hpscreg.eu/cell-line/UBi001-A-3, UBi001-A-4: https://hpscreg.eu/cell-line/UBi001-A-4
Ethical approval	Institutional Review Board (IRB00003099) of the Bioethical Commission of the University of Barcelona (October 20, 2016)

1. Resource utility

Sanfilippo B syndrome is caused by mutations in the N-acetyl-alpha-glucosaminidase (*NAGLU*) gene. The *NAGLU*-mutated hiPSCs generated here, UBi001-A-3 and UBi001-A-4 together with the isogenic control cell line UBi001-A, can be useful for disease modeling as well as to identify potential therapeutic approaches for Sanfilippo B syndrome.

2. Resource details

NAGLU is a gene coding for a protein involved in the degradation of heparan sulfate (HS), a glycosaminoglycan present in the extracellular matrix. Mutations in *NAGLU* lead to lysosomal accumulation of undegraded HS molecules, resulting in dysfunction of the endolysosomal system and causing Sanfilippo B syndrome. Patients present early-onset progressive and severe neurodegeneration for which there is no treatment available yet.

We targeted exon 5 of the *NAGLU* gene in a previously reported healthy hiPSC line (UBi001-A) (Canals et al., 2015) to generate mutated hiPSC lines using a CRISPR/Cas9-mediated editing system (Fig. 1A). A sgRNA targeting *NAGLU* exon 5 together with the ribonucleoprotein Cas9 were used to disrupt the gene by non-homologous end joining (NHEJ). After 48–72 h of transfecting UBi001-A cell line with Cas9-sgRNA complex, sorted single cells were plated into laminin-coated 96-well plates for single cell culture and expansion. To analyze colony genotypes, genomic DNA was isolated and the regions flanking the

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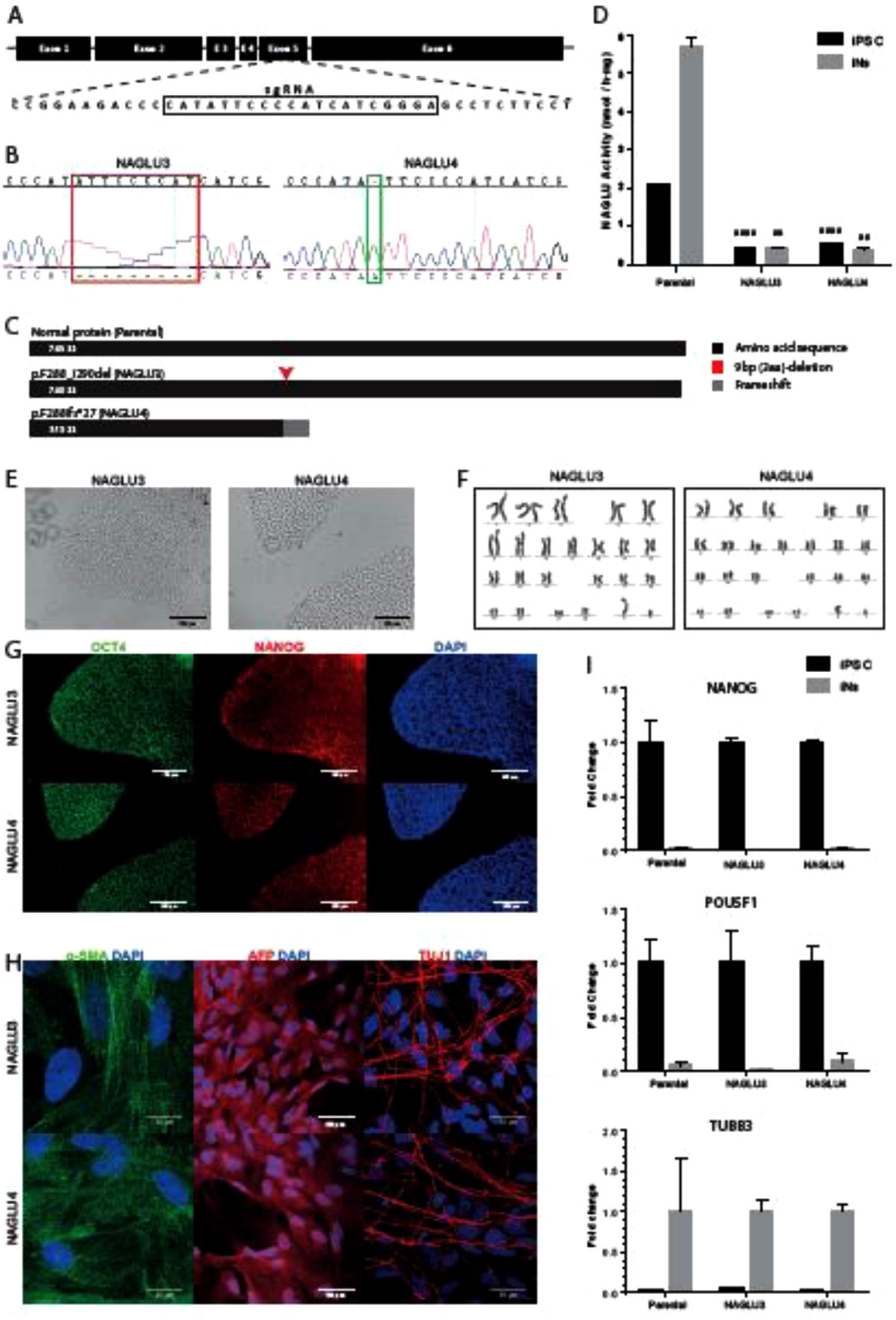

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targeted exon 5 were amplified by PCR, followed by Sanger sequencing, which allowed us to find two homozygous mutated hiPSC clones (Fig. 1B) that were further characterised.

The UBi001-A-3 presented a homozygous 9-bp deletion, which results in the loss of 3 amino acids (p.F288_I290del), from the 743-aminoacid NAGLU protein. UBi001-A-4 had a 1-bp insertion in

Fig. 1. Characterization of *NAGLU*-mutated hiPSC lines UBi001-A-3 (NAGLU3) and UBi001-A-4 (NAGLU4). (A) Illustration showing CRISPR/Cas9 strategy for the *NAGLU* gene, with the sgRNA targeting exon 5. (B) Sanger-sequencing and alignment of both mutated cell lines, showing deletions (in red) and insertions (in green). (C) Scheme of the different resultant proteins, illustrating amino acids in frame (black), amino acid deletions (red) and those after the frameshift (gray). (D) NAGLU activity levels in the different hiPSCs and iNs lines, expressed in nmol/h • mg protein (**** *P* value <0.0001, ** *P* value <0.01, unpaired *t*-test with Welch's correction for 3 independent experiments with 2 technical replicates). (E) Representative bright-field images showing typical stem cell morphology. Scale bar = 100 μm. (F) Results of the karyotype analysis for both cell lines. (G) Representative images of the immunofluorescence staining for pluripotency markers OCT4 (green) and NANOG (red), and DAPI (blue) for nuclei. Scale bar = 100 μm. (H) *In vitro* differentiation assay to the three germ layers: α-SMA (for mesoderm, green, scale bar = 20 μm), AFP (for endoderm, red, scale bar = 50 μm) and TUJ1 (for ectoderm, red, scale bar = 20 μm). (I) Real-time quantitative PCR analysis showing expression of pluripotent genes POU5F1 (OCT4) and NANOG, and neural marker TUBB3, normalized to GAPDH expression both in hiPSC lines (iPSCs) and induced neurons (iNs). Results are represented as the fold change in gene expression of iNs compared to hiPSC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
UBi001-A-3	NAGLU3	Male	N/A	Caucasian	*NAGLU* p.(Phe288_Ile290del)	Sanfilippo B syndrome
UBi001-A-4	NAGLU4	Male	N/A	Caucasian	*NAGLU* p.(F288Ifs*27)	Sanfilippo B syndrome

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 Panel E
Phenotype	Qualitative analysis (Immunocytochemistry)	Expression of pluripotency markers: OCT4 and NANOG	Fig. 1 Panel G
	Quantitative analysis (RT-qPCR)	Relative expression of pluripotency markers: positive for OCT4 and NANOG	Fig. 1 Panel I
Genotype	Karyotype (G-banding) and resolution	46 XY, Resolution 550–650	Fig. 1 Panel F
Identity	Microsatellite PCR (mPCR) OR STR analysis	Not performed	
		16 loci tested, 100% matched	Available with the authors
Mutation analysis (IF APPLICABLE)	Sequencing	Compound heterozygous mutation in both cases	Fig. 1 Panel B
	Off-target analysis	Top 5 predicted off-target analysed and all sequence were correct	Supplementary Figure 1 Panel C
Microbiology and virology	Mycoplasma	Negative	Supplementary Figure 1 Panel B
Differentiation potential	Embryoid body formation and differentiation	Endoderm: a-feto protein (AFP), mesoderm: muscle actin (a-SMA), and ectoderm: P-tubulin (TUJ1).	Fig. 1 Panel H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	
	HLA tissue typing	Not performed	

homozygosis, causing a frameshift that changed the open reading frame (p.F288Ifs*27) and lead to a shorter protein product of only 315 aa (Fig. 1B and C, and Table 1). Both mutations caused a clear reduction in NAGLU enzyme activity when compared to the parental cell line levels, in hiPSCs and induced neurons (iNs) derived from the same hiPSC lines (Fig. 1D). Interestingly hiPSCs lysosomal enzymes have low activity levels, as we have previously reported for HGSNAT (Canals et al., 2015; Benetó et al., 2019).

Both cell lines presented typical stem cell-like morphology (Fig. 1E) normal karyotype (Fig. 1F), and were mycoplasma free (Fig. S1B). Immunocytochemistry assays demonstrated the expression of pluripotency markers OCT4 (aka POU5F1) and NANOG in our mutant hiPSC lines (Fig. 1G), as well as their potential to differentiate into the three germ layers (Fig. 1H). We also confirmed in a RT-qPCR that both mutated hiPSC lines expressed high levels of pluripotency markers *NANOG* and *OCT4* and low levels of the neuronal marker *TUJ1* (aka *TUBB3*) when compared to induced neurons (iNs) (Fig. 1I). Short tandem repeat (STR) analysis confirmed that both cell lines, UBi001-A-3 and UBi001-A-4, had their origin from the hiPSC parental cell line (UBi001-A) (loci listed on Fig. S1A). Finally, the top-five predicted off-target sites of the sgRNA used were sequenced to confirm that no undesired editing was present in these newly established cell lines (Fig. S1C).

3. Materials and methods

3.1. Cell culture

hiPSC were cultured as in (Benetó et al., 2019).

3.2. CRISPR/Cas9-mediated mutation

5 x 10^4 healthy hiPSC per well were plated in Biolaminin521-coated 24-well plates with 10 μM Y-27632 (#72302, STEMCELL Technologies) in StemFlex medium with P/S for 24 h. The day after, cells were transfected with a complex formed by the TrueCut Cas9 Protein v2 (#A36497, Invitrogen) and a pre-designed TrueGuide sgRNA Modified (AssayID: CRISPR1108362_SGM, #A35511, Invitrogen) using Lipofectamine Stem Transfection Reagent (#STEM00001, Invitrogen). 48–72 h after transfection, cells were individually replated after cell sorting onto Biolamina521-coated 96-well plates in the presence of 10 μM Y-27632. Cells were allowed to expand before being analysed by PCR and Sanger sequencing (Table 3).

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-OCT4	1:100	Santa Cruz Biotechnology #sc- 5279, RRID: AB_628051
	Rabbit anti-NANOG	1:100	Abcam #ab21624, RRID: AB_446437
Differentiation Markers	Rabbit anti-AFP	1:100	DakoCytomation (now part of Agilent) #A0008, RRID: AB_2650473
	Mouse anti-SMA	1:100	Sigma-Aldrich #A5228, RRID: AB_262054
	Rabbit anti-TUJ1	1:500	Covance #MRB-435P, RRID: AB_663339
Secondary antibodies	Donkey anti-mouse Cy2	1:200	Jackson Immunoresearch #715225-150, RRID: AB_2340826
	Donkey anti-rabbit Cy3	1:200	Jackson Immunoresearch #711165-152, RRID: AB_2307443

Primers		
	Target	Forward/Reverse primer (5′ – 3′)
Targeted mutation	*NAGLU* Exon 5	GGCAAGAGAAACCAGGAGC/ GTGTGTTATGGCGAGGCATT
Potential off-target	POT1: TTCAGATAAAGGGGAATATG	CCGATTTTACAACTCATTGCC / CAAAT GTTGTCGCT CTTAGT C
	POT2: TATATTCCCCATCATCTAGA	GACAAGAGGTAGGTAGCAAG/ GAGAATGTGGGAGAAAGGAG
	POT3: CAT ATT CT CCATT CTT GGGA	TGCCTGTTCACTCTGATGGT / GGCTCAGAGTAGGTGGTGTT
	POT4: CAT ATT CCCCAT CACT GTGG	GGGT AAAT GAGGTGTCTATCGC / ATCTCAGCACTTTGGAAGGC
	POT5: CAT ATT CT CCAT CGTCTGGG	TGAGACTCCTACTTGAAGCCA / IIIIGTGCTGCTGTCCCATG

Probes for RT-qPCR		
	Target	Assay ID (#4331182, TaqMan)
Pluripotency markers	*NANOG*	Hs02387400_g1
	OCT4	Hs01654807_s1
Neural marker	*TUJ1*	Hs00801390_s1
House Keeping	*GAPDH*	Hs99999905_m1

3.3. Karyotyping, STR analysis and mycoplasma detection

For the karyotype analysis, hiPSC were prepared using 2 ng/ml colcemid (#15212-046, Invitrogen) during 45 min and harvested with StemPro Accutase, then AMBAR (Anàlisis Mèdiques Barcelona) analysed 20 metaphases of each cell line. For STR analysis, AMBAR used gDNA (DNeasy Blood & Tissue Kit #69504, Qiagen) to compare 16 different loci (listed on Fig. S1B) between each mutated cell line and the parental hiPSC. For mycoplasma analysis, Mycoplasma Detection Kit (#4542, Biotools) was used following the manufacturer's instructions.

3.4. NAGLU enzyme activity

Enzyme activity was measured as described in Marsh and Fensom (1985).

3.5. Embryonic body (EB) formation and in vitro differentiation

EBs formation differentiation was performed as in Canals et al. (2015) with minor modifications. hiPSC were maintained in StemFlex medium with P/S, and thiazovivin was added from distribution of hiPSC in 96-well plates until EBs were put in suspension.

3.6. Immunofluorescence staining

Cells were fixed in 4% PFA for 15 min, blocked and permeabilised with TBS containing 0.1% Triton-X 100 (#28817.295, VWR) and 5% normal donkey serum (#S30-100 M, Merck Millipore) (TBS + +) for 2 h at room temperature. Primary antibodies (Table 3) were incubated overnight at 4 °C. Secondary antibodies (Table 3) were incubated 2 h at room temperature. Nuclei were stained with 0.5 μg/ml DAPI (#D1306, Invitrogen). Both antibodies and DAPI were diluted in TBS + +. Slides were mounted with MOWIOL (#475904, Millipore). hiPSC images were acquired with ZOE Fluorescence Cell Imager (Bio Rad), images from EBs differentiated into ectoderm (TUJ1) and mesoderm (α-SMA) were acquired using Zeiss confocal microscope LSM 880, and images from EBs differentiated into endoderm (AFP) were acquired using Leica confocal TCS-SP2 microscope. All images were analysed with the Fiji software (Schindelin et al., 2012).

3.7. Direct neural differentiation and RT-qPCR analysis

Ngn2-induced neurons (iNs) were generated as described in Zhang et al. (2013). Seven days after induction, RNA from iNs was extracted using High Pure RNA Isolation Kit (#11828665001, Roche) and 2 μg of total RNA were used to synthesize cDNA, using the High Capacity cDNA Reverse Transcription kit (#4368814, Applied Biosystems) and the RNase Inhibitor (#N8080119, Applied Biosystems) following manufacturer's instructions. Real-time qPCR was performed in a LightCycler 480 II (Roche) system with the LightCycler 480 Probes Master (#04887301001, Roche). *GAPDH* was used as normaliser. TaqMan probes are listed in Table 3.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2019.101668.

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