

# Generation and genetic repair of 2 iPSC clones from a patient bearing a heterozygous c.1120del18 mutation in the ACVRL1 gene leading to Hereditary Hemorrhagic Telangiectasia (HHT) type 2

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# Citation

Bouma, M. J., Orlova, V., Hil, F. E. van den, Mager, H. J., Baas, F., Knijff, P. de, ... Freund, C. (2020). Generation and genetic repair of 2 iPSC clones from a patient bearing a heterozygous c.1120del18 mutation in the ACVRL1 gene leading to Hereditary Hemorrhagic Telangiectasia (HHT) type 2. *Stem Cell Research*, 46. doi:10.1016/j.scr.2020.101786

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**Note:** To cite this publication please use the final published version (if applicable).

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Lab Resource: Multiple Stem Cell Lines

# Generation and genetic repair of 2 iPSC clones from a patient bearing a heterozygous c.1120del18 mutation in the *ACVRL1* gene leading to Hereditary Hemorrhagic Telangiectasia (HHT) type 2



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### ABSTRACT

Fibroblasts from a patient carrying a heterozygous 18bp deletion in exon 8 of the *ACVRL1* gene (c.1120del18) were reprogrammed using episomal vectors. The inframe deletion in ACVRL1 causes the loss of 6 amino acids of the protein, which is associated with Hereditary Hemorrhagic Telangiectasia (HHT) type 2 (Letteboer et al., 2005). CRISPR-Cas9 editing was used to genetically correct the mutation in the induced pluripotent stem cells (iPSCs). The top5-predicted off-target sites were not altered. Patient and isogenic iPSCs showed high pluripotent marker expression, *in vitro* differentiation capacity into all three germ layers and displayed a normal karyotype. The obtained isogenic pairs will enable proper *in vitro* disease modelling of HHT (Roman and Hinck, 2017).

#### **Resource Table:**

Unique stem cell lines	LUMCi030-A
identifier	LUMCi030-B
	LUMCi030-A-1
	LUMCi030-B-1
Alternative names of st-	LUMC0110iALK04
em cell lines	LUMC0110iALK10
	iso03LUMC0110iALK04
	iso01LUMC0110iALK10
Institution	Leiden University Medical Center, Leiden, the Netherlands
Contact information of	V. Orlova, PhD, HYPERLINK "mailto: V.Orlova@lumc.nl
distributor	
Type of cell lines	iPSC
Origin	Human
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogram-	Episomal vectors: OCT3/4, SOX2, KLF4, L-MYC, LIN28
ming	-
Multiline rationale	Gene corrected clones
Gene modification	Yes
Type of modification	Correction of heterozygous 18bp mutation (c.1120del18)
Associated disease	Hereditary Hemorrhagic Telangiectasia type 2
Gene/locus	ACVRL1
Method of modification	CRISPR-Cas9
	Not applicable

Name of transgene or r- esistance	
Inducible/constitutive system	Not applicable
Date archived/stock da-	18-03-2020
te	
Cell line repository/ba-	https://hpscreg.eu/cell-line/LUMCi030-A
nk	https://hpscreg.eu/cell-line/LUMCi030-A-1
	https://hpscreg.eu/cell-line/LUMCi030-B
	https://hpscreg.eu/cell-line/LUMCi030-B-1
Ethical approval	Biopsies were taken with an informed consent at the St.
	Antonius Hospital, Nieuwegein, The Netherlands. The generation of the lines was approved by the Leiden University ethics committee under the P13.080 "Parapluprotocol: hiPSC"

# 1. Resource utility

Patients with a heterozygous c.1120del18 mutation in *ACVRL1* display Hereditary Hemorrhagic Telangiectasia (HHT) type 2, which is associated with regular bleedings, telangiectasia and arteriovenous malformations (Letteboer et al., 2005). iPSCs were generated from a patient carrying this mutation and CRISPR-Cas9 mediated repair was used to correct the mutation, hereby enabling in vitro disease modelling

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https://doi.org/10.1016/j.scr.2020.101786

Received 7 February 2020; Received in revised form 19 March 2020; Accepted 30 March 2020 Available online 28 May 2020 1873-5061/ © 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/BY/4.0/).

## Summary of lines.

iPSC line names	Abbreviation in Fig.s	Gender	Age	Ethnicity	Genotype of locus	Disease
LUMCi030-A LUMCi030-B LUMC030-A-1	LUMC0110iALK04 LUMC0110iALK10 iso03LUMC0110iALK04	Male Male Male	42 42 42	Caucasian Caucasian Caucasian	ACVRL1:c.1120del18 ACVRL1:c.1120del18 ACVRL1:no mutation	Hereditary Hemorrhagic Telangiectasia Hereditary Hemorrhagic Telangiectasia None (isogenic)
LUMC030-B-1	iso01LUMC0110iALK10	Male	42	Caucasian	ACVRL1:no mutation	None(isogenic)

#### Table 2

#### Characterization and validation.

Classification	Test	Result	Data
Morphology	Transmission light microscopy	Normal	Fig. 1B
Phenotype	Pluripotency markers, qualitative analysis: Immunofluorescent staining	OCT3/4, SSEA4 & Nanog expressed	Fig. 1A
	Pluripotency markers, quantitative	% of cells double positive for Nanog & SSEA4:	Fig. 1C
	analysis:	LUMC0110iALK04: 85%	
	Flow cytometry	iso03LUMC0110iALK04: 87%	
		LUMC0110iALK10: 97%	
		iso01LUMC0110iALK10: 96%	
Genotype	Karyotype	LUMC0110iALK04: 46XY	1) Fig. 1G
	KaryoStat	iso03LUMC0110iALK04: 46XY	2) Fig. 1H
	Resolution >1-2 Mb	LUMC0110iALK10: 46XY	
	2) G-banding	iso01LUMC0110iALK10: 46XY	
	Resolution 5-10 Mb		
Identity	STR analysis	22 loci tested, 100% matching identity	Available with the authors
Mutation analysis	TOPO TA cloning, Sanger sequencing	Heterozygous 18bp deletion	Fig. 1D
-	Southern Blot OR WGS	Not performed	Not performed
Microbiology and virology	Mycoplasma	Mycoplasma tested by luminescence: Negative	Supplementary Table 1
Differentiation potential	Spontaneous differentiation or directed	LUMC0110iALK04, LUMC0110iALK10:	Fig. 1F
	differentiation	areas with b3-tubulin + - (ectoderm), AFP + - (endoderm) and CD31 +	C C
		(mesoderm) expressing cells	
		iso03LUMC0110iALK04/iso01LUMC0110iALK10: Expression of PAX6,	
		b3-tubulin (ectoderm), FOXA2, SOX17 (endoderm), Vimentin and NCAM	
		(mesoderm)	
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	Not performed
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	Not performed
	HLA tissue typing	Not performed	Not performed

of HHT type 2 (Roman and Hinck, 2017).

#### 2. Resource Details

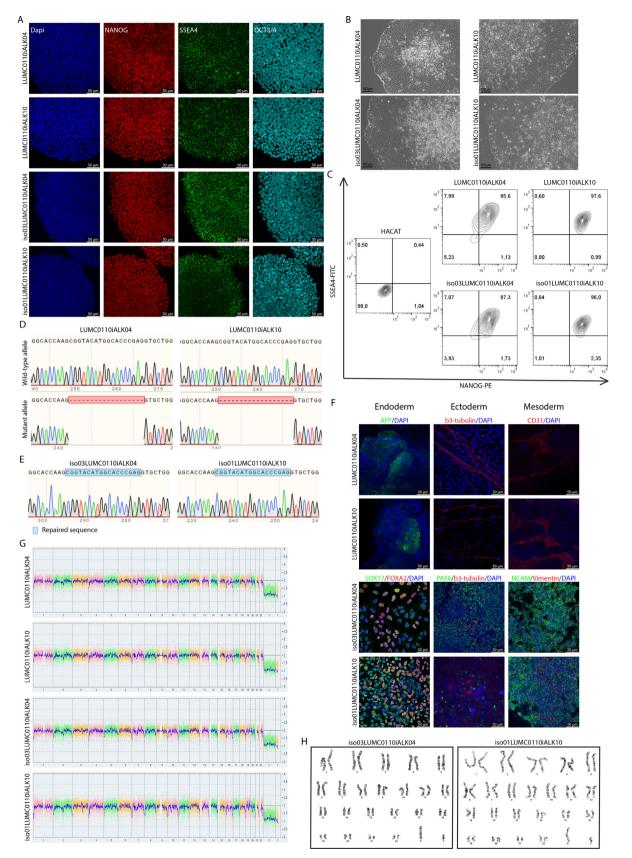
Fibroblasts were isolated from a skin biopsy of a 42 year old male Hereditary Hemorrhagic Telangiectasia (HHT) type 2 patient with a heterozygous c.1120del18 mutation in ACVRL1 and reprogrammed at three using oriP/EBNA1-based episomal passage vectors (Letteboer et al., 2005, Okita et al., 2011). The iPSC clones LUM-C0110iALK04 and LUMC0110iALK10, displayed a typical morphology and a normal karyotype (passage 17 and 15 respectively) as assessed by the KaryoStat assay (Table 1Table 2, Fig 1B/G). Immunofluorescence staining showed homogenous expression of the pluripotency markers OCT3/4, NANOG and SSEA4 and flow cytometry analysis illustrated high percentages of pluripotency marker expression: 85% of NANOG<sup>+</sup>/ SSEA4<sup>+</sup> cells for LUMC0110iALK04 and 97% for LUMC0110iALK10 (Fig 1A/C, Table 2). Moreover, the iPSCs were able to differentiate into derivatives of all three germ layers upon spontaneous differentiation in vitro (Fig 1F). The heterozygous 18bp deletion in exon 8 of ACVRL1 was confirmed by Sanger sequencing (Fig 1D) and the mutated allele was repaired by transfection with Cas9-ribonucleoprotein (RNP) complex, with a mutation-specific single guide RNA (sgRNA) and a singlestranded oligodeoxynucleotide (ssODN) containing the wild-type sequence as a donor template (Table 3). Single-cell derived colonies were screened for repair using the XCMI restriction enzyme, which only recognizes the wild-type sequence, and corrected clones were confirmed by Sanger sequencing (Fig 1E). The repaired iPSCs showed a typical morphology and a normal karyotype using KaryoStat at passage 6 after gene editing (Table 2, Fig 1B/G). As balanced translocations cannot be detected by KaryoStat, additionally G-banding was performed for the repaired iPSCs. Using this method, again both clones appeared to have a normal karyotype (Fig 1H). Moreover, the pluripotency markers OCT3/ 4, NANOG and SSEA4 were expressed at high levels (87% NANOG<sup>+</sup>/ SSEA4<sup>+</sup> cells for iso03LUMC0110iALK04 and 96% for iso01-LUMC0110iALK10) (Fig 1A/C) and the clones were able to differentiate into ectoderm, mesoderm and endoderm after directed differentiation (Fig 1F). All lines were mycoplasma negative (Supplementary Table 1). The origin of the isogenic pairs was confirmed by short tandem repeat (STR) analysis which fully matched the profile of the patient's somatic cells. Finally, the absence of off-target mutations was confirmed by Sanger sequencing of the top5-predicted sites by CRISPOR (crispor.tefor.net; data not shown) (Haeussler et al., 2016).

# 3. Materials and Methods

# 3.1. Reprogramming

A 4 mm skin biopsy was dissociated and fibroblasts were cultured in DMEM/F12 supplemented with 10% Fetal Bovine Serum, 1% Non-Essential Amino Acids, 1% Pen/Strep and 0.18%  $\beta$ -mercaptoethanol (all Gibco). At passage three, 5\*10^5 cells were electroporated with 1  $\mu$ g of episomal plasmids pCXLE-hOCT3/4, pCXLE-hSK and pCXLE-hUL (Addgene, #ID27076, #ID27078, #ID27080,) respectively, using the NHDF nucleofector kit with program U-023 of the NucleofectorII device

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**Fig. 1.** Characterization of iPSC lines. A. Immunofluorescent staining for pluripotency markers NANOG, SSEA4 and OCT3/4. B. Morphology of the iPSC-colonies. C. Flow cytometry analysis of SSEA4 and NANOG. D. Sanger sequencing data of LUMC0110iALK04 and LUMC0110iALK10. E. Sanger sequencing data of iso03LUMC0110iALK04 and iso01LUMC0110iALK10. F. Immunofluorescent staining for endo-, ecto- and mesoderm markers after spontaneous (LUMC0110iALK04 and LUMC0110iALK10) or directed differentiation (iso03LUMC0110iALK04 and iso01LUMC0110iALK10). G. Results of KaryoStat assay. H. G-banding analysis.

#### Table 3 Reagents details.

Pluripotency Marker         mouse IgG1 anti-NANOG         1:150         Santa Cruz, Sc-293121, RRID: AB_2665475           Pluripotency Marker         mouse IgG3 anti-SSEA4         1:30         Biolegend, #330402, RRID: AB_1089208           Pluripotency Marker         human IgG1 anti-SSEA4-FITC         1:25         Miltenyi, #130-098-371, RRID: AB_2653517           Pluripotency Marker         mouse IgG1 anti-NANOG-PE         1:5         BDBioscience, #560433, RRID: AB_1645522           Differentiation Marker,         mouse IgG2 anti-b3-tubulin         1:4000         Covance, #MMS-435P, RRID: AB_2716839           Erctoderm           Cuartett, #2011200530, RRID: AB_2716839           Endodern           Santa Cruz, Sc-293121, RRID: AB_2665475           Differentiation Marker,         Rabbit IgG anti-AFP         1:5         DBIB           Differentiation Marker,         mouse IgG1 anti-FPCAM-1         1:100         DAKO, #M0823, RRID: AB_2114471           Mesoderm          1:200         Cell Signaling Technology, #60433, RRID: AB_2797599           Erctoderm           Standa Cruz, Sc-293121, RRID: AB_390153           Endoderm           Standa Cruz, Sc-293121, RRID: AB_390153           Endoderm           Standa Cruz, Sc-293, RRID: AB_390153							
Multipotentry Marker         mouse [gG2 battl-OCT3/4         1:100         Sunt Cruz, 5<2392, RRID: AB, 2663475           Planpotenty Marker         mouse [gG1 anti-SRA40C         1:180         Santa Cruz, 5<2392, RRID: AB, 2663475	Antibodies used for immu		Dilution	Company Cat # and PPID			
Plurijonersy Marker mouse jed anit-ANOC 1:50 Source ACCACCCCCACACCC ACCCCCCCCCCCCCCCCCCCC		Antibody	Dilution	Company Cat # and KKiD			
Plamponery Markermouse leg3 ant-SSLA41:30Biolegend, #3304, RRID: AB, 1089208Plamponery Markernuose lgG1 anti-NANOG-PE1:50DDRisocience, #0408-437, RRID: AB, 2013773Plamponery Markernuose lgG1 anti-NANOG-PE1:4000Corace, #MMS-4359, RRID: AB, 2013773EctoderEctoderEctoderMifferentiation Marker,Rabbi IgG anti-SSLA41:00DAKO, #M0823, RRID: AB, 2716839Mifferentiation Marker,Rabbi IgG anti-PACA1:00DAKO, #M0823, RRID: AB, 2716839EctoderMifferentiation Marker,Rabbi IgG anti-SCN171:100Cell Signaling Technology, #60433, RRID: AB, 2797599EctoderEctoderEctoderBiolegen # 230400Nitrogen Gar# 2124, RRID: AB, 390153EctoderDifferentiation Marker,Nabit IwentinScondary antibodyAleca 47 Gor Anti-Mouse IgG11:250Invitrogen Gar# 21216, RRID: AB, 253576Scondary antibodyAleca 48 Goa Anti-Mouse IgG11:250Invitrogen Gar# 2115, RRID: AB, 253576Scondary antibodyAleca 48 Goa Anti-Mouse IgG11:250Invitrogen Gar# 2115, RRID: AB, 253576Scondary antibodyAleca 48 Goa Anti-Mouse IgG11:250Invitrogen Gar# 2115, RRID: AB, 253576Scondary antibodyAleca 4	Pluripotency Marker	mouse IgG2b anti-OCT3/4	1:100	Santa Cruz, Sc-5279, RRID: AB_628051			
Plaripotency Marker human igG1 anti-SRA0FITC 1.25 MILEony, #13-009371, RBL7. A2 353517 Plaripotency Marker nouse igG2a anti-NA7 bit 15 0000 Covance, #MINE-4359, RBL7. A2 313773 Ecodern Ecodern Ecodern Hiderentiation Marker nouse igG2 anti-NA7 Eadedern Holferentiation Marker nouse igG1 anti-PRCM-1 100 DAG0, #M0823, RBL7 A2 2114471 Mesodern Holferentiation Marker nouse igG1 anti-PRCM-1 100 Cell Signaling Technology, #60433, RBL7 A2 2197599 Ecodern Hiderentiation Marker nouse igG1 anti-PRCM-1 100 Cell Signaling Technology, #60433, RBL7 A2 397599 Ecodern Hiderentiation Marker nouse igG1 anti-SAT7 100 Cell Signaling Technology, #60433, RBL7 A2 390153 Ecodern Hiderentiation Marker nouse igG2 not-SAT7 100 Cell Signaling Technology, #60433, RBL7 A2 390153 Ecodern Hiderentiation Marker nouse igG2 Cell 2.20 Ecodern Hiderentiation Marker nouse igG3 Cell 2.20 Ecodern Hiderentiation Marker nouse igG3 Ecodern Hiderentiation Marker nouse igG3 Ecodern Hide	Pluripotency Marker	mouse IgG1 anti-NANOG	1:150	Santa Cruz, Sc-293121, RRID: AB_2665475			
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Differentiation Marke moue igGa = mt b3 tubuli p 14000 Covance, #MSI-435P, RRID: AB_2313773 Endodern	Pluripotency Marker	human IgG1 anti-SSEA4-FITC	1:25	Miltenyi, #130-098-371, RRID: AB_2653517			
Ecodem         Signalise	Pluripotency Marker	mouse IgG1 anti-NANOG-PE	1:5	BDBioscience, #560483, RRID: AB_1645522			
Endodem       DAKO, #M0823, RRD: AB_2114471         Mesodern       Differentiation Marker,       Roubit IgG anti-PCAM-1       1:100       DAKO, #M0823, RRD: AB_2797599         Differentiation Marker,       Goat IgG anti-SOX17       1:100       R&D, #AP1924, RRD: AB_355060         Endodern       Differentiation Marker,       Rabbit IgG anti-SOX17       1:100       R&D, #AP1924, RRD: AB_355060         Endodern       Differentiation Marker,       Rabbit IgG anti-SOX17       1:100       Cell Signaling Technology, #5741, RRD: AB_290153         Endodern       Differentiation Marker,       Rouse anti-NCAM       1:400       Cell Signaling Technology, #5741, RRD: AB_2149540         Mesodern       Alexa 496 Goat Anti-Mouse IgG2b       1:250       Invitrogen Cat# 21242, RRD: AB_2535764         Secondary antibody       Alexa 496 Goat Anti-Mouse IgG2b       1:250       Invitrogen Cat# 21151, RRD: AB_2535764         Secondary antibody       Alexa 589 Goat Anti-Mouse IgG2       1:250       Invitrogen Cat# 21147, RRD: AB_2535764         Secondary antibody       Alexa 589 Goat Anti-Mouse IgG2       1:250       Invitrogen Cat# 21147, RRD: AB_2535764         Secondary antibody       Alexa 589 Goat Anti-Mouse IgG2       1:250       Invitrogen Cat# 21147, RRD: AB_2535764         Secondary antibody       Alexa 549 Goat Anti-Mouse IgG2       1:250       Invitrogen Cat# 21147, RRD: AB_253	Differentiation Marker, Ectoderm	mouse IgG2a anti-b3-tubulin	1:4000	Covance, #MMS-435P, RRID: AB_2313773			
Mesodern     Normaline     Abbit VgG anti-PAG     1:200     Cell Signaling Theology, #0433, RRID: AB_2797599       Bit forematiation Marker,     Goarl tgG anti-SOX17     1:100     RR.D, #AF1924, RRID: AB_355600       Bit forematiation Marker,     Goarl tgG anti-SOX17     1:100     RR.D, #AF1924, RRID: AB_355600       Bit forematiation Marker,     Rabbit tgG anti-FOXA2     1:100     Cell Signaling Technology, #5741, RRD: AB_10695459       Bit forematiation Marker,     Mabbit tgG anti-FOXA2     1:400     Cell Signaling Technology, #3576, RRD: AB_2149540       Bit forematiation Marker,     Mouse anti-NCAM     1:400     Cell Signaling Technology, #3576, RRD: AB_2149540       Mesodern     Alexa 485 Goat Anti-Mouse IgG3     1:250     Invitrogen Cat# 21151, RRD: AB_2358764       Secondary antibody     Alexa 485 Goat Anti-Mouse IgG3     1:250     Invitrogen Cat# 21124, RD: AB_2353764       Secondary antibody     Alexa 485 Goat Anti-Mouse IgG3     1:250     Invitrogen Cat# 21124, RD: AB_2353764       Secondary antibody     Alexa 480 Donky Anti-Gai IgG     1:500     Invitrogen Cat# 21246, RRD: AB_2535864       Primer     Target     For: TGCCTGCTTGCCACCACACACA     Invitrogen Cat# 21247, RRD: AB_2535864       Primer     Target     For: TGCCTGCTTGCCACCACACACACACACACACACACACACA	Differentiation Marker, Endoderm	Rabbit IgG anti-AFP	1:25	Quartett, #2011200530, RRID: AB_2716839			
Ecodem     Ecodem     Ecodem     Ecodem       Differentiation Marker, Endoderm     Goat IgG anti-SOX17     1:100     R&D, #AF1924, RID: AB_35060       Differentiation Marker, Mesoderm     Rabbit IgG anti-FOXA2     1:100     Millipore, #07-633, RID: AB_300153       Differentiation Marker, Mesoderm     Rabbit anti-Vimentin     1:200     Cell Signaling Technology, #5741, RRD: AB_2149540       Mesodern     Hesodern     Cell Signaling Technology, #3776, RRD: AB_2149540     Millipore, 4375, RRD: AB_2149540       Mesodern     Hesodern     Invitrogen Cat# 21242, RRD: AB_2535784       Secondary antibody     Alexa 488 Goat Anti-Mouse IgG 1     1:250     Invitrogen Cat# 21243, RRD: AB_2535766       Secondary antibody     Alexa 586 Goat Anti-Mouse IgG 1     1:250     Invitrogen Cat# 21124, RRD: AB_2535766       Secondary antibody     Alexa 586 Goat Anti-Mouse IgG 1     1:250     Invitrogen Cat# 21124, RRD: AB_2535766       Secondary antibody     Alexa 647 Donkey Anti-Goat IgG 1     1:500     Invitrogen Cat# 21124, RRD: AB_2535766       Secondary antibody     Alexa 647 Donkey Anti-Goat IgG 1     1:500     Invitrogen Cat# 21124, RRD: AB_2535766       Secondary antibody     Alexa 647 Donkey Anti-Goat IgG 1     1:500     Invitrogen Cat# 21124, RRD: AB_2535766       Secondary antibody     Alexa 647 Donkey Anti-Goat IgG 1     1:500     Invitrogen Cat# 21124, RRD: AB_2535766       Secondary antibo	Differentiation Marker, Mesoderm	mouse IgG1 anti-PECAM-1	1:100	DAKO, #M0823, RRID: AB_2114471			
Endodem       File       1:100       Millipore, #07-633, RND: AB_390153         Endodem       Differentiation Marker,       Rabbit anti-Vimentin       1:200       Cell Signaling Technology, #5741, RRD: AB_10695459         Mesodem       Mesodem       Transpace       Transpace       Transpace       Transpace         Mesodem       Hesodem       Transpace	Differentiation Marker, Ectoderm	Rabbit IgG anti-PAX6	1:200	Cell Signaling Technology, #60433, RRID: AB_2797599			
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Mesodern     Cell Signaling Technology, #3576, RRD: AB_2149540       Mesodern	Differentiation Marker, Endoderm	Rabbit IgG anti-FOXA2	1:100	Millipore, #07-633, RRID: AB_390153			
<table-container>      Differentiation Marker, Mesodary     Nouse arti-NCM     1:400     Cell Signaling Technology, #3576, RRID: AB, 214950       Mesodary antiboly     Alexa 485 Got Anti-Mouse IgG2     1:250     Invitrogen Cat# 211242, RRID: AB, 2535784       Secondary antiboly     Alexa 485 Got Anti-Mouse IgG3     1:250     Invitrogen Cat# 21124, RRID: AB, 2535784       Secondary antiboly     Alexa 568 Got Anti-Mouse IgG3     1:500     Invitrogen Cat# 21124, RRID: AB, 25357864       Secondary antiboly     Alexa 568 Got Anti-Mouse IgG3     1:500     Invitrogen Cat# 21124, RRID: AB, 25357864       Secondary antiboly     Alexa 568 Got Anti-Mouse IgG3     1:500     Invitrogen Cat# 21124, RRID: AB, 25357864       Secondary antiboly     Alexa 568 Got Anti-Mouse IgG3     1:500     Invitrogen Cat# 21124, RRID: AB, 25357864       Secondary antiboly     Alexa 647 United Mathitica Igg3     1:500     Invitrogen Cat# 21149, RRID: AB, 25357864       Secondary antiboly     Alexa 647 United Mathitica Igg3     1:500     Invitrogen Cat# 21149, RRID: AB, 25357864       Secondary antiboly     Alexa 647 United Mathitica Igg3     1:500     Invitrogen Cat# 21149, RRID: AB, 25357864       Secondary antiboly     Alexa 647 United Mathitica Igg3     1:500     Invitrogen Cat# 21149, RRID: AB, 25357864       Secondary antiboly     Alexa 647 United Mathitica Igg3     Invitrogen Cat# 21149, RRID: AB, 25357864       Secondary antiboly     Secondary antiboly     <t< td=""><td>Differentiation Marker,</td><td>Rabbit anti-Vimentin</td><td>1:200</td><td>Cell Signaling Technology, #5741, RRID: AB_10695459</td></t<></table-container>	Differentiation Marker,	Rabbit anti-Vimentin	1:200	Cell Signaling Technology, #5741, RRID: AB_10695459			
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Secondary antibody     Alexa 648 Donkey Anti-abbit IgG     1:50     Invitrogen Cat# A21206, RRD:: AB_2535792       Secondary antibody     Alexa 647 Donkey Anti-Goat IgG     1:250     Invitrogen Cat# A21206, RRD:: AB_2535792       Secondary antibody     Alexa 647 Donkey Anti-Goat IgG     1:250     Invitrogen Cat# A21206, RRD:: AB_2535792       Primers     Target     Forward Reverse primer (5'-37)     Invitrogen Cat# A21447, RRD: AB_2535792       PCR mutation site     FW: TOCGGCGCTGCGCCACCACACACT     Invitrogen Cat# A21447, RRD: AB_2535792       Sanger sequencing     exon 8: ACRVL1     FW: TOCGGCGCGCGCACCACACACACACACACACACACACACAC							
<table-container>      Secondary antibody     Alexa 647 Donkey Anti-Goat Ig0     1:250     Invitrogen Cat# 201447, RRD: AB,2535864       Frie     Fage     Forward/Reverse primer (5'-3')       PCR mutation site     Exon 8: ACRVLI     FW: TOGCAGCCTGCACGCACCACACTCA       mutation site     W: ACCCAAGCTAGAGAGACCTGCACGGAGACTACA       mutation site     W: TOGGAACCTAGAGAGACCTCACACTCACACTCACACTCACTC</table-container>							
Primers     Target     Forward/Reverse primer (5'-3')       PCR mutation site     exon 8: ACRVL1     FW: TGCCTGCCTTGCCCACATCT       RV: AGGCAGATGGAGAGCGTGCAGGAGAT     RV: AGGCAGATGGAGCGTGCAGGAGAT       sanger sequencing     exon 8: ACRVL1     FW: TGGGAACCTAGAGAGCGTGCAGGAGAT       mutation site     RV: AGGCAAGATGGAGAGCGTGCAGGAGAAT     RV: AGGCAAGTGGAGCAGCAGCAGCAGA       Offlarget site 1,     intergenic: RNU7-187P-NCAM1     FW: AGCAGCCCAGGGCGCTCTTCATTTTCTTT       PCR & Sanger     sequencing     RV: AACAGCCCAGGGCCCCATCAGCGGCTGCTGCTGCTGCTGTGTGTG				-			
<table-container>      Image:     Forward/Reverse prime (5'-3)       PCR watching in the server in the</table-container>		mena o II Donney mile cour 180	11200				
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Offtarget site 4     intergenic: RP11-347D21.3-SNORD36     FW: CTGGATTTAGCCTGAGGAGATG       PCR & Sanger     RV: GAAGGCAGAGGTGGCTATTT       sequencing     intergenic: RP11-195B3.1-RP11-       PCR & Sanger     482E14.1       PCR & Sanger     482E14.1       PCR & Sanger     640CCCTCGGCTTACCCTCGTCTT       sequencing     rRV: GCAGAGCCCTTCGGCTTACCCTGATGGT       PCR & Sanger     482E14.1       PCR & Sanger     640CCCAGAGCCCTTCGGCTTACCCTGATGT       sequencing     rRV: GCAGAGCCCTTCGGCTTACCCTGATGT       crRNA     crRNA sequence -PAM       crRNAsense     c.1120del18 specific       crRNA     CAACCCGAGAGTGGGCACCA -AGG       ssoDN     sequence       ssoDN for repair c.1120del18     CCTGGCTGTGATGCACTCACAGGGCAGCGATTACCTGGACATCGGCAACACACCCGAGAG- GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGAGCAGATCCGCCAGGAC-	-						
PCR & Sanger     RV: GAAGGCAGAGGTGGCTATTT       sequencing     FW: AGCTGACTGATGGCATTGTGCTTCCGTCTT       PCR & Sanger     482E14.1     FW: AGCTGACTGATGGCATTGTGCTTCCGTCTT       PCR & Sanger     482E14.1     RV: GCAGAGCCCTTCGGCTTACCCTGATGTT       sequencing     rcrRNA     rcrRNA       crrRNA     .1120del18 specific     CAACCCGAGAGTGGCACCA - AGG       ssoDN     sequence     sequence       ssoDN for repair c.1120del18     CCTGGCTGTGATGCACTCACAGGGCAGCGATTACCTGGACATCGGCAACAACCCGAGA-		intergenic: RP11-347D21.3-SNORD36	FW: CTGGATTTAGCCTC	AGGAGATG			
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PCR & Sanger     482E14.1     RV: GCAGAGCCCTTCGGCTTACCCTGTATGTT       sequencing     removed     removed       crRNA     removed     removed       crRNAsense     CAACCCGAGAGTGGGCACCA - AGG       cssODN     sequence     sequence       ssoDN for repair c.1120del18     CCTGGCTGTGATGCACTCACAGGGCAGCGAGTGACGAGCGAG		intergenic: RP11-195B3 1-RP11-	FW: AGCTGACTGATCC	CATTGTGCTTCCGTCTT			
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crRNA     Target     crRNA sequence -PAM       crRNAsense     c.1120del18 specific     CAACCCGAGAGTGGGCACCA -AGG       ssoDN     Sequence       csSODN for repair c.1120del18     CCTGGCTGTGATGCACTCACAGGGCAGCGAGTACCTGGACATCGGCAACAACCCGAGA-       GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGCAGACCACAGCGGAC-       GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGCAGACCAGGAC-	°						
Target     crRNA sequence -PAM       crRNAsense     c.1120del18 specific     CAACCCGAGAGTGGGCACCA -AGG       ssODN     Sequence       crSGGCGCGCACCAGGGGCAGCGACTACCCGGAGCAGCGACTACCCGGAGAGAGCAGCAGAGAGAG							
crRNAsense c.1120del18 specific CAACCCGAGAGTGGGCACCA -AGG ssODN  Sequence CCTGGCTGTGATGCACTCACAGGGCAGCGATTACCTGGACATCGGCAACAACCCGAGA- GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGCAGCACCAGGAC-		Target		crRNA sequence -PAM			
ssODN Sequence CCTGGCTGTGATGCACTCACAGGGCAGCGATTACCTGGACATCGGCAACAACCCGAGA- GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGCAGCAGCAGCAGAACAACCCGAGGAC-	crRNAsense	•		•			
Sequence           ssODN for repair c.1120del18         CCTGGCTGTGATGCACTCACAGGGCAGCGATTACCTGGACATCGGCAACAACCCGAGA-           GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGCAGCAGCAGCAGCACAACAACCCGAGAC-         GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGCAGCAGCACCAGGAC-		c.1120de110 specific					
ssODN for repair c.1120del18 CCTGGCTGTGATGCACTCACAGGGCAGCGATTACCTGGACATCGGCAACAACCCGAGA- GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGCAGCAGCAGCACGAGC-	55021	Sequence					
GTGGGCACCAAGCGGTACATGGCACCCGAGGTGCTGGACGAGCAGATCCGCACGGAC-				GGACATCGGCAACAACCCGAGA_			
	350Div 101 Tepati 0.11200						
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(both Lonza) and subsequently cultured in ReproTeSR (Stem Cell Technologies) on Matrigel (Corning)-coated plates. iPSC colonies were picked manually and expanded in TeSR-E8 medium (Stem Cell Technologies) on VitronectinXF-coated plates (Stem Cell Technologies).

# 3.2. Cell culture

iPSCs were maintained at  $37^{\circ}$ C and 5% CO<sub>2</sub> in TeSR-E8 medium (Stem Cell Technologies) on VitronectinXF-coated plates (Stem Cell Technologies) with daily media changes. Once a week, iPSCs were passaged as small aggregates using Gentle Cell Dissociation Reagent (Stem Cell Technologies) according to manufacturer's protocol. Splitting ratio's ranged from 1:10 till 1:40 depending on cell confluency

at the day of passaging.

# 3.3. Gene editing

LUMC0110iALK04 was cultured until passage 22 and LUMC0110iALK10 until passage 19 prior to gene editing. 1\*10°5 cells were transfected with the Alt-R Cas9-RNP complex and the ssODN (both IDT) using the NEON Transfection System (Invitrogen) at 1200V/ 30ms/1pulse. Cells were plated in a Synthemax II-SC (Corning)-coated plate using TeSR-E8 with CloneR (Stem Cell Technologies). After recovery, 1000 cells were plated in a Synthemax II-SC-coated 10cm dish in TeSR-E8 with CloneR. Half of a single cell-derived colony was used for DNA isolation with QuickExtract solution (Lucigen). The region of interest was PCR-amplified using Terra PCR Direct Polymerase Mix (TaKaRa) and a Bio-Rad S1000 Thermal Cycler with the following parameters: 1: 2min at 98°C, 2: 10sec at 98°C, 3: 1min/kb at 68°C, 35 cycles. Positive clones were identified by successful cutting of the PCR amplified *ACVRL1* fragment by restriction enzyme XCMI according to manufacturer's instructions.(New England Biolabs). The repaired allele in the corrected clones was confirmed by Sanger sequencing, performed by the Leiden Genome Technology Centre (LGTC) using the ABI3730xl system.

# 3.4. Flow cytometry

Single cells were prepared with Gentle Cell Dissociation Reagent (Stem Cell Technologies) and fixed and permeabilized using the FIX & PERM Cell Fixation & Permeabilization Kit (Invitrogen) according to manufacturer's protocol. Cells were stained with the directly conjugated antibodies against SSEA4 and NANOG and subsequently measured using the MACSQuant VYB (Miltenyi Biotech). Data was analyzed with FlowJo version 10.6.1.

# 3.5. Trilineage differentiation

iPSCs were either subjected to spontaneous differentiation (LUMC0110iALK04 & LUMC0110iALK10) in DMEM/F12 + 20% FBS (Gibco) for 21 days or directed differentiation (iso03LUMC0110iALK04, iso01LUMC0110iALK10) using STEMdiff<sup>TM</sup> Trilineage ectoderm-, endoderm- and mesoderm media according to manufacturer's protocol (Stem Cell Technologies).

# 3.6. Phase contrast imaging and immunofluorescent (IF) staining

Phase contrast pictures were taken of live cells 6 days after passage using a Nikon eclipse T1 microscope with a 10x objective. For IF staining, cells were fixed with 2% PFA for 30 min at RT. Subsequently, cells were blocked and permeabilized using 0.1% TritonX-100 + 4% normal swine serum (Jackson) in PBS for 1h at RT. Primary antibodies were diluted in PBS with 4% normal swine serum (Jackson) and applied O/N (4°C), followed by the secondary antibodies in PBS for 1 hour (RT) (Table 3). DNA counterstaining was performed with DAPI (1:1000, Invitrogen). Images were taken with a 40x objective using a Leica TCS SP8 confocal microscope.

## 3.7. Evaluation of off-target effects

Top5 off-target sites were predicted using the CRISPOR website (crispor.tefor.net) (Haeussler et al., 2016). PCR products ranging from 230 up to 908bp covering the predicted sites were screened by Sanger sequencing, performed by the Leiden Genome Technology Centre (LGTC) using the ABI3730xl system.

## 3.8. Mycoplasma detection

The mycoplasma status was assessed using the MycoAlert<sup>™</sup> mycoplasma detection kit (Lonza, #LT07-418) following the manufacturers protocol.

#### 3.9. Short Tandem Repeat (STR) analysis

STR-analysis was performed by the LUMC Forensic Laboratory for DNA-research (FLDO), using the PowerPlex Fusion System 5C autosomal STR kit (Promega) as previously described (Westen et al., 2014).

#### 3.10. Karyotyping

G-banding analysis was conducted at the Laboratory of Clinical Genetics Leiden (LDGA), according to standard procedures. A total of 20 metaphases was analyzed for each line, for iso03LUMC0110iALK04 at passage 5 and for iso01LUMC0110iALK10 at passage 7 after gene editing. The KaryoStat assay was performed for LUMC0110iALK04 at passage 17, LUMC0110iALK10 at passage 15 and for iso03LUMC0110iALK04 and iso01LUMC0110iALK10 at passage 6 after gene editing according to manufacturer's instructions (ThermoFisher Scientific).

#### **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.

# Acknowledgments

This work was supported by ZonMW grant 446002501: "Treating Hereditary Hemorrhagic Telangiectasia through drug repurposing".

# Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101786.

#### References

- Letteboer, T.G., Zewald, R.A., Kamping, E.J., de Haas, G., Mager, J.J., Snijder, R.J., Lindhout, D., Hennekam, F.A., Westermann, C.J., Ploos van Amstel, J.K., 2005. Hereditary hemorrhagic telangiectasia: ENG and ALK-1 mutations in Dutch patients. Hum. Genet. 116, 8–16.
- Roman, B.L., Hinck, A.P., 2017. ALK1 signaling in development and disease: new paradigms. Cell. Mol. Life Sci. 74, 4539–4560.
- Okita, K., Matsumura, Y., Sato, Y., Okada, A., Morizane, A., Okamoto, S., Hong, H., Nakagawa, M., Tanabe, K., Tezuka, K., Shibata, T., Kunisada, T., Takahashi, M., Takahashi, J., Saji, H., Yamanaka, S., 2011. A more efficient method to generate integration-free human iPS cells. Nat. Methods 8, 409–412.
- Haeussler, M., Schonig, K., Eckert, H., Eschstruth, A., Mianne, J., Renaud, J.B., Schneider-Maunoury, S., Shkumatava, A., Teboul, L., Kent, J., Joly, J.S., Concordet, J.P., 2016. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol. 17, 148.
- Westen, A.A., Kraaijenbrink, T., Robles de Medina, E.A., Harteveld, J., Willemse, P., Zuniga, S.B., van der Gaag, K.J., Weiler, N.E., Warnaar, J., Kayser, M., Sijen, T., de Knijff, P., 2014. Comparing six commercial autosomal STR kits in a large Dutch population sample. Forensic Sci. Int. Genet. 10, 55–63.