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Lab Resource: Genetically-Modified Multiple Cell Lines

Generation of four gene-edited human induced pluripotent stem cell lines with mutations in the *ATM* gene to model Ataxia-Telangiectasia

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ABSTRACT

Ataxia-Telangiectasia (A-T) is an autosomal recessive multi-system disorder caused by mutations in the ataxia-telangiectasia mutated (*ATM*) gene, resulting, among other symptoms, in neurological dysfunction. ATM is known to be a master controller of signal transduction for DNA damage response, with additional functions that are poorly understood. CRISPR/Cas9 technology was used to introduce biallelic mutations at selected sites of the *ATM* gene in human induced pluripotent stem cells (hiPSCs). This panel of hiPSCs with nonsense and missense mutations in ATM can help understand the molecular basis of A-T.

Resource Table:

Unique stem cell line identifier	Cell line 1: PEIi019-A-13 https://hpscreg.eu/cell-line/PEIi019-A-13 Cell line 2: PEIi019-A-14 https://hpscreg.eu/cell-line/PEIi019-A-14 Cell line 3: PEIi019-A-15 https://hpscreg.eu/cell-line/PEIi019-A-15 Cell line 4: PEIi019-A-16 https://hpscreg.eu/cell-line/PEIi019-A-16
Alternative name(s) of stem cell line	Cell line 1: KOLF2-C1 ATM R35* Cell line 2: KOLF2-C1 ATM K3016Q Cell line 3: KOLF2-C1 ATM L1465P Cell line 4: KOLF2-C1 ATM A3054V
Institution	Paul-Ehrlich-Institut, Langen
Contact information of the reported cell line distributor	Wasifa Nurieva: wasifa.nurieva@pei.de Zoltán Ivics: zoltan.ivics@pei.de
Type of cell line	iPSC
Origin	human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 55–59 Sex: male Ethnicity: European - White British
Cell Source	Fibroblasts
Method of reprogramming	Non-integrating Sendai virus reprogramming system
Clonality	Clonal

(*continued on next column*)

(*continued*)

Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
The cell culture system used	Feeder-free when banked and since then also kept in feeder-free culture StemFlex™ Medium
Type of the Genetic Modification	Cell line 1_PEIi019-A-13: Homozygous point mutation C->T in exon 3 at R35 in the *ATM* gene. Introduction of R35* stop codon Cell line 2_PEIi019-A-14: Homozygous point mutation A->C in exon 63 at K3016 in the *ATM* gene. Introduction of K3016Q Cell line 3_PEIi019-A-15: Homozygous point mutation T->C in exon 29 at L1465 in the *ATM* gene. Introduction of L1465P Cell line 4_PEIi019-A-16: Homozygous point mutation C->T in exon 63 at A3054 in the *ATM* gene. Introduction of A3054V
Associated disease	Ataxia-Telangiectasia (A-T) OMIM code: 208900
Gene/locus	*ATM* gene/11q22.3

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography bright field, 10x magnification	Normal	Fig. 1 Panel B
Pluripotency status evidence for the described cell line	Qualitative analysis (i.e. immunocytochemistry, Western blotting)	Positive staining of pluripotency markers: NANOG & TRA-1–60	Fig. 1 Panel F
	Quantitative analysis flow cytometry	SSEA3 & TRA 1–60 positive cells: >99 %	Fig. 1 Panel H
Karyotype	Karyotype (G-banding)	46XY, Resolution 450–500 bhps	Fig. 1 Panel E
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	PCR + Sanger sequencing	Fig. 1 Panel C
	Evaluation of the - (homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s)	SNP Sanger sequencing	Supplementary Fig. S1
	Transgene-specific PCR (when applicable)	N/A	N/A
Verification of the absence of random plasmid integration events	PCR/Southern	N/A	N/A
Parental and modified cell line genetic identity evidence	STR analysis	Short tandem repeat (STR) analysis of 16 allele loci showed identical results in parental cell line and newly generated hiPS cells. 16 independent PCR systems D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338,AMEL, D5S818, FGA, D19S433, vWA, TPOX and D18S51 were investigated (Thermo Fisher, AmpFlSTR® Identifiler® Plus PCR Amplification Kit). In parallel, positive and negative controls were carried out yielding correct results.	Supplementary Files
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	Homozygous mutations	Fig. 1, Panel C
	PCR-based analyses	N/A	N/A
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	N/A	N/A
Specific pathogen-free status	Mycoplasma	Negative	Supplementary Fig. S2
Off-target nuclease activity analysis	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing [Optional but highly-recommended if Cas editing is used]	N/A	N/A
Multilineage differentiation potential	Directed trilineage differentiation	Trilineage differentiated cells showed expression of lineage-specific markers in all four cell lines.	Fig. 1, Panel D
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

(*continued*)

Method of modification / user-customisable nuclease (UCN) used, the resource used for design optimisation	CRISPR/Cas9 Alt-R® S.p. HiFi Cas9 Nuclease V3, (IDT) Integrated DNA Technologies #1081061
User-customisable nuclease (UCN) delivery method	RNP, nucleofection with Amaxa 4D Nucleofector (Lonza)
All double-stranded DNA genetic material molecules introduced into the cells	Single-stranded HDR donor template
Analysis of the nuclease-targeted allele status	Sanger sequencing
Method of the off-target nuclease activity prediction and surveillance	N/A
Descriptive name of the transgene	N/A
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	N/A
Inducible/constitutive expression system details	N/A
Date archived/stock creation date	Cell line 1: PEIi019-A-13 – 25.07.2021 Cell line 2: PEIi019-A-14 – 07.02.2022 Cell line 3: PEIi019-A-15 – 31.01.2022 Cell line 4: PEIi019-A-16 – 13.04.2022
Cell line repository/bank	Cell line 1: PEIi019-A-13 https://hpscreg.eu/cell-line/PEIi019-A-13 Cell line 2: PEIi019-A-14 https://hpscreg.eu/cell-line/PEIi019-A-14 Cell line 3: PEIi019-A-15 https://hpscreg.eu/cell-line/PEIi019-A-15 Cell line 4: PEIi019-A-16 https://hpscreg.eu/cell-line/PEIi019-A-16
Ethical/GMO work approvals	Paternal cell line WTSIi018-B obtained from HipSci (HUMAN INDUCED PLURIPOTENT STEM CELL INITIATIVE) https://hpscreg.eu/cell-line/WTSIi018-B-1
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

1. Resource utility

Understanding the molecular basis of A-T has been hampered by the lack of appropriate animal and cell culture models that accurately reflect the disease phenotypes. *In vitro* generation of neurons can provide valuable insights into mechanisms of A-T-associated neurodegeneration, which may provide novel targets for future therapies.

2. Resource details

The *ATM* gene encodes a serine-threonine protein kinase that is responsible for detecting and signaling the presence of genomic DNA double-strand breaks (DSBs). When DNA damage occurs, the ATM

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry/western blot	**Antibody**	**Dilution**	**Company Cat # and RRID**
Pluripotency markers	Mouse anti-Tra1-60	1:100	Thermo Fisher Scientific Cat# 41-1000, RRID: AB_2533494
	Rat SSEA3	1:100	Developmental Studies Hybridoma Bank Cat# MC-631, RRID: AB_528476
	Rabbit anti-NANOG	1:800	Cell Signaling Technology Cat# 3580, RRID:AB_2150399
Western blot	Rabbit anti-ATM	1:1000	Cell Signaling Technology Cat# 2873, RRID:AB_2062659
	Anti-GAPDH Mouse	1:1000	Millipore Cat# CB1001, RRID:AB_2107426
Differentiation markers	Rabbit anti-Nestin	1:200	Abcam Cat# ab105389, RRID:AB_10859398
	Rabbit anti-Brachyury	1:1000	Abcam Cat# ab209665, RRID:AB_2750925
	Rabbit anti-Sox17	1:1000	Abcam Cat# ab92494, RRID:AB_10585428
Secondary antibodies	Goat anti-Rat IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	4 μg/mL	Thermo Fisher Scientific Cat# A-11006, RRID: AB_2534074
	F(ab')2-Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	4 μg/mL	Thermo Fisher Scientific Cat# A-11020, RRID: AB_2534087
	Mouse anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, HRP	1:10.000	Thermo Fisher Scientific Cat# 31464, RRID: AB_228378
	Goat anti-Mouse IgG F(ab')2 Secondary Antibody, HRP	1:10.000	Thermo Fisher Scientific Cat# 31436, RRID:AB_228313
Nuclear stain	DAPI	2 μg/mL	Invitrogen Cat #D1306
Site-specific nuclease			
Nuclease information	Alt-R® S.p. HiFi Cas9 Nuclease V3	IDT SpCas9 Hi-Fi v.3, 1081061	
Delivery method	Nucleofection	Lonza; 4D-NucleofectorTM core Unit + X Unit P3 Primary Cell 4D-Nucleofector™ X Kit L Pulse code: CA-137	
Selection/enrichment strategy	Single cell-derived colonies	Analyzed by Sanger sequencing	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
	Mycoplasma detection	GPO-3 Primer: 5́' - GGGAGCAAACAGGATTAGATACCCT - 3́' MGSO Primer: 5́' - TGCACCATCTGTCACTCTGTTAACCTC – 3'	
Genotyping (desired allele/transgene presence detection)	PCR specific for the targeted R35 locus	Forward primer: 5′ – TCCTGCTACTACTGCAAGCAA – 3′ Reverse primer: 5′ – GCATCAACCCTGTTTGCCAC – 3′ Annealing temperature 67 °C	
	PCR specific for the targeted K3016 locus	Forward primer: 5′ – GAGGAAGAATTGGGCCCTACA– 3′ Reverse primer: 5′ – GAAAGCAGAGATGTTCCTTAAGAC– 3′ Annealing temperature 64 °C	
	PCR specific for the targeted L1465 locus	Forward primer: 5′ – ATGAGAACAGAATCTTGTGACACT– 3′ Reverse primer: 5′ – TGTTTCCCAGGCAAGTAGCG– 3′ Annealing temperature 64 °C	
	PCR specific for the targeted A3054 locus	Forward primer: 5′ – GAGGAAGAATTGGGCCCTACA– 3′ Reverse primer: 5′ – GAAAGCAGAGATGTTCCTTAAGAC– 3′ Annealing temperature 64 °C	
	PCR specific for the targeted SNP rs186556743 locus	Forward primer: 5′ – ACTACAAGAGCACACTACCAC – 3′ Reverse primer: 5′ – TGGGAGGATCACAAGGTCAG – 3′ Annealing temperature 65 °C	
	PCR specific for the targeted SNP rs559077054 locus	Forward primer: 5′ – AGCGAGACCCTATCTCATAAAC – 3′ Reverse primer: 5′ – AAAATAGAGCAGATGCCATCC – 3′ Annealing temperature 63 °C	
sgRNA oligonucleotide	R35 locus	TTGTTTCAGGATCTCGAATCAGG (reversed)	
	K3016 locus	AGACTACAAGAGAAACTGAAAGG	
	L1465 locus	AATAACGTCTCGAAGAACAAAGG (reversed)	
	A3054 locus	CACACCCAAGCTTTCCATCCTGG (reversed)	

(*continued on next page*)

Table 2 (*continued*)

Antibodies and stains used for immunocytochemistry/flow-cytometry/western blot	Antibody	Dilution	Company Cat # and RRID
Genomic target sequence(s)	Including PAM and other sequences likely to affect UCN activity	R35 locus, exon 3: Homo sapiens chromosome 11q22.3, GRCH38: 108.227.806 K3016 locus, exon 63: Homo sapiens chromosome 11q22.3, GRCH38: 108.365.383 L1465 locus, exon 29: Homo sapiens chromosome 11q22.3, GRCH38: 108.289.759 A3054 locus, exon 63: Homo sapiens chromosome 11q22.3, GRCH38: 108.365.498	
Bioinformatic gRNA on– and -off-target binding prediction tool used, specific sequence/outputs link(s)	HTGT WGE	R35 locus: https://wge.stemcell.sanger.ac.uk/crispr/1088290180 K3016 locus: https://wge.stemcell.sanger.ac.uk/crispr/1088301744 L1465 locus: https://wge.stemcell.sanger.ac.uk/crispr/1088295510 A3054 locus: https://wge.stemcell.sanger.ac.uk/crispr/1088301760	
ODNs/plasmids/RNA molecules used as templates for HDR-mediated site-directed mutagenesis.	Ultramer® DNA oligo, Alt-R® HDR modified for R35 locus	ATTAGTAACCCATTATTATTTCCTTTTTATTTTCAGAAAGAAGTTGAGAAATTTAAGCGCCTGATTTGAGATCCTGAAACAATTAAACATCTAGATCGGC	
	Ultramer® DNA oligo, Alt-R® HDR modified for K3016 locus	CCTGCTGTATGAGCAAATTCACTTGTCCACCAACACTGAGCACAGTGCCTTCTTCCACTCCTTTCAGTTGCTCTTGTAGTCTCATTAAGACACGTTCAGC	
	Ultramer® DNA oligo, Alt-R® HDR modified for L1465 locus	TCACCTGTTTGTTAGTTTATTACTGAAAGATATAAAAAGTGGCTTAGGAGGAGCTTGGGCCTTTGTTCCTCGAGACGTTATTTATACTTTGATTCACTAT	
	Ultramer® DNA oligo, Alt-R® HDR modified for A3054 locus	GACAAGTGAATTTGCTCATACAGCAGGCCATAGACCCCAAAAATCTCAGCCGACTTTTCCCAGGATGGAAAGTTTGGGTGTGATCTTCAGTATATGAATT	

protein is activated, recruited to the DSBs, and initiates a cascade of phosphorylation events, ultimately leading to the activation of various downstream effector proteins that coordinate DNA repair processes, cell cycle checkpoints and apoptosis, thereby playing a crucial role in maintaining genomic stability (McKinnon, 2004; Shiloh, 2003). The parental cell line used for the generation of human iPSCs (hiPSCs) was the subclone KOLF2-C1, isolated in the Skarnes Lab from the cell line WTSIi018-B (Skarnes et al., 2019). Characterizations of the cell lines are summarized in Table 1.

CRISPR/Cas9 technology was used to introduce mutations at selected sites by designing specific single guide RNAs (sgRNAs) (see Table 2) targeting the respective sequences in the *ATM* gene. The four designed mutations are spanning across the entire *ATM* gene (Fig. 1, Panel A). Due to DSBs that are introduced at the desired sites, repair pathways are triggered. By providing exogenous template DNA (see Table 2), DSB repair pathway choice can be shifted to provoke cellular machineries to repair the gap predominantly by homology-directed repair (HDR), which can copy the desired mutations into the *ATM* locus from synthetic, single-stranded oligonucleotides. Since A-T only manifests in a homozygous mutant background, biallelic mutations needed to be established (Fig. 1, Panel B). To exclude deleterious on-target effects in the edited cell lines, Sanger sequencing was performed to ensure that no loss of heterozygosity or larger deletions occurred by detecting single nucleotide polymorphisms (SNPs). All hiPSCs lines showed the presence of selected SNPs rs186556743 and rs559077054 (Supplementary Fig. S1).

The hiPSC lines exhibit typical stem cell-like morphology which display a high nuclear-to-cytoplasmic ratio (Fig. 1, Panel C).

The multilineage differentiation potential was confirmed by a trilineage differentiation assay and demonstrated positive signals in the immunofluorescence analysis for the ectodermal marker Nestin, mesodermal marker Brachyury and endodermal marker Sox17 (Fig. 1, Panel D).

Karyotyping analysis (Szuhai and Tanke, 2006) at passage 6 (PEIi019-A-14) and passage 8 (PEIi019-A-13, PEIi019-A-15, PEIi019-A-16) showed normal numbers and structures of chromosomes (46, XY) (Fig. 1, Panel E). Immunofluorescence analysis showed, that the cell lines express pluripotency markers such as TRA-1–60 and NANOG (Fig. 1, Panel F).

Western blot analysis revealed a full knock out for ATM in PEIi019-A-13 cells and reduced protein amounts in PEIi019-A-14, PEIi019-A-15 and PEIi019-A-16 cells (Fig. 1, Panel G) compared to the parental cell line.

Expression of stem cell surface markers TRA-1–60 and SSEA3 was tested with flow cytometry analysis, and showed positive signals for > 99 % cells (Fig. 1, Panel H).

Short tandem repeats (STRs) analysis confirmed that the reported new cell lines were identical to the original parental cell line KOLF2-C1 (Skarnes et al., 2019). The hiPSC cell lines were healthy during culture and no mycoplasma contamination was observed (Supplementary Fig. S2).

Hence, the PEIi019-A-13, PEIi019-A-14, PEIi019-A-15 and PEIi019-A-16 lines, carrying *ATM*-specific genetic modifications, provide valuable model cell lines for the further analysis of A-T pathology on a cellular level.

3. Materials and methods

3.1. hiPSC culture

hiPSCs were cultured on Synthemax-coated (Corning® Synthemax® II-SC Substrate, 3535) 6-well plates in StemFlex™ Medium (Thermo Fisher Scientific, A3349401) at 37 °C and 5 % CO_2. Cell passage was conducted at a ratio of 1:10 every 5–6 days with ReLeSR™ (Stemcell, 100–0484).

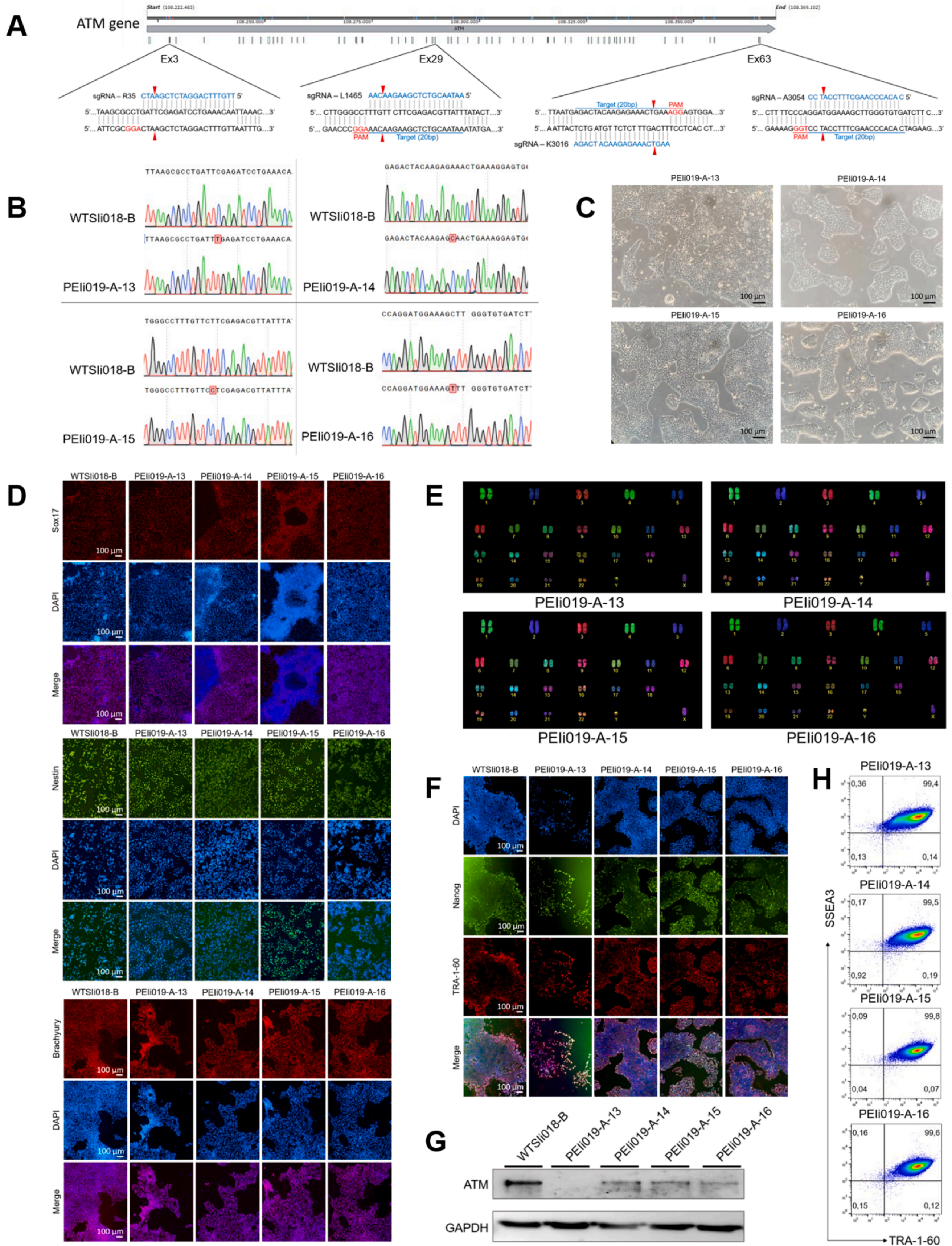

Fig. 1. Characterization of hiPSC lines with ATM mutations.

3.2. 4D nucleofection of hiPSCs

Nucleofection was performed on 10^6 cells following the Skarnes et al., 2019 protocol for delivery of Cas9 RNP and oligo using the Amaxa 4D Nucleofector. hiPSCs were nucleofected for each clones with a mix of 16 µg of sgRNA and 20 µg of recombinant Cas9 protein (20 µg HiFi Cas9 nuclease v3; IDT), then reseeded on Synthemax-coated 6-well plates in StemFlex™ containing Revitacell (100X stock solution, Life Technologies, A26445-01) with addition of HDR enhancer (IDT, 1081073) to a 30 µM final concentration. The medium was replaced the next day without Revitacell and HDR enhancer. After cells reached ~ 80 % confluency, they were dissociated by using Accutase (Stemcell, 07920) and replated for single clone expansion.

3.3. Immunofluorescence

Immunofluorescence was performed using standard protocols (Reprocell) at passage 5 with the antibodies listed in Table 2.

3.4. Western blotting

Cell lysates of each hiPSC line and the parental wild type line were prepared with RIPA buffer containing a protease inhibitor cocktail (Roche, 11697498001). Western blotting and RIPA buffer preparation were performed using the standard protocol for Western blots by Abcam. Antibodies are listed in Table 2.

3.5. Flow cytometry

For flow cytometry analysis, 10^6 hiPS cells (passage 10) were dissociated into single cells with Accutase, washed with 2 % FCS in PBS and resuspended and stained in the primary antibody solution (SSEA3-AF488 and TRA-1–60-AF647, Table 2) at 4 °C for 2 h. They were then washed twice and treated with secondary antibodies at room temperature for 2 h. After staining, the cells were analyzed with the MACSQuant® and the FlowJo software.

3.6. Mycoplasma detection

Mycoplasma detection was performed with PCR testing. Primers are listed below in Table 2.

3.7. Genotyping

Isolation of genomic DNA and PCR followed by Sanger sequencing (Eurofins Genomics) was used to determine the genotype of the clones as described in Skarnes et al., 2019. Additionaly, SNP sequencing was performed targeting heterozygous SNPs previously identified in the WTSIi018-B cell line. Primers are listed in Table 2. PCR was done by using Q5® High-Fidelity DNA polymerase (New England Biolabs) with the following cycle parameters: 98 °C 30 sec, followed by 35 cycles of 98 °C 10 sec, primer annealing 30 sec, 72 °C 30 sec, and completed by 72 °C 2 min.

3.8. Karyotyping

Karyotyping was performed at the Leiden University Medical Center as previously described by Szuhai and Tanke, 2006.

3.9. Trilineage differentiation

Each cell line was differentiated into the three germ layers by using the STEMdiff™ Trilineage Differentiation Kit (Stemcell Technologies, 05230) according to manufacturer's instructions.

3.10. STR analysis

STR analysis was performed by Multiplexion GmbH.

Declaration of Competing Interest

The authors declare no financial interests/personal relationships which may be considered as potential competing interests.

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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.2023.103247.

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