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Lab Resource: Multiple Cell Lines

Generation of three iPSC lines (IAIi002, IAIi004, IAIi003) from Rubinstein-Taybi syndrome 1 patients carrying *CREBBP* non sense c.4435G > T, p.(Gly1479*) and c.3474G > A, p.(Trp1158*) and missense c.4627G > T, p.(Asp1543Tyr) mutations

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ABSTRACT

Rubinstein-Taybi syndrome (RSTS) is a neurodevelopmental disorder characterized by growth retardation, skeletal anomalies and intellectual disability, caused by heterozygous mutations in either *CREBBP* (RSTS1) or *EP300* (RSTS2) genes. We characterized 3 iPSC lines generated by Sendai from blood of RSTS1 patients with unique non sense c.4435G > T, p.(Gly1479*), c.3474G > A, p.(Trp1158*) and missense c.4627G > T, p.(Asp1543Tyr) *CREBBP* mutations. All lines displayed iPSC morphology, pluripotency markers, trilineage differentiation potential, stable karyotype and specific mutations. Western-blot using a CREB-Binding Protein N-terminus antibody demonstrated the same amount of full length protein as control in the missense mutation line and reduced amount in lines with stop mutations.

Resource details

Rubinstein-Taybi syndrome (RSTS) is a rare multiple congenital anomaly and intellectual disability syndrome characterized by growth retardation, skeletal deformities and cognitive impairment, mainly caused by *de novo* heterozygous mutation in either *CREBBP* or *EP300* genes, encoding the homologous acetyltransferases and transcriptional coactivators CBP and p300. RSTS1 results from inactivating or missense *CREBBP* mutations leading to CBP protein either in reduced quantity or defective in enzymatic function. RSTS1 accounts for 60% of clinically diagnosed RSTS patients, *versus* 10% accounted for by RSTS2, hence representing the main RSTS entity. The overall clinical phenotype, and particularly cognitive impairment is more severe than that of RSTS2 (Hennekam, 2006). Following institutional ethical committee approval and patient informed consent, peripheral blood was withdrawn from three patients carrying *CREBBP* mutations (Table 1) who have been clinically and molecularly described under the codes 34, 149 (inactivating mutations) and 46 (missense mutation) (Bentivegna et al., 2006; Lopez-Atalaya et al., 2012; Spena et al., 2015). The original patients' codes are included in the alternative names of the iPSC lines (Table 1). Induced pluripotent stem cells (iPSCs) were generated from peripheral blood mononuclear cells (PBMNCs) using integration-free Sendai virus (SeV) particles transducing target cells with replication-competent RNAs encoding the four reprogramming Yamanaka factors. iPSCs were grown on irradiated Mouse Embryonic Fibroblasts (MEF) feeder layers. Twenty days after

Abbreviations: a-CGH, array-Comparative Genome Hybridization; CBP, CREB-Binding Protein; CNVs, Copy Number Variants; CREBBP, CREB-Binding Protein; CTR, Control; EP300, E1A binding protein, 300KDa; FACS, Fluorescence Activated Cell Sorter; HRP, Horseradish Peroxidase; IF, Immunofluorescence; MEF, Mouse Embryonic Fibroblasts; PBMNCs, Peripheral Blood Mononuclear Cells; PCR, Polymerase Chain Reaction; RSTS1, Rubinstein-Taybi syndrome 1; RSTS2, Rubinstein-Taybi syndrome 2; RT, Reverse Transcriptase; SeV, Sendai Virus; WB, Western Blot

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
IAIi002RSTS1-34-A	IAIi002	Male	13	Caucasian	c.4435G > T p.(Gly1479*)	RSTS1
IAIi003RSTS1-46-A	IAIi003	Female	14	Caucasian	c.4627C > T p.(Asp1543Tyr)	RSTS1
IAIi004RSTS1-149-A	IAIi004	Male	7	Caucasian	c.3474G > A p.(Trp1158*)	RSTS1

reprogramming, iPSC colonies were manually selected and culture expanded. The iPSC lines described here, named IAIi002RSTS1-34-A, IAIi003RSTS1-46-A and IAIi004RSTS1-149-A were characterized by evaluating distinctive morphology and expression of the pluripotency markers by immunofluorescence and FACS (SSEA4, OCT3/4, TRA-1-60, Fig. 1A; 99% SSEA4+ cells; 81%,91% and 94% TRA-1-60+ cells, Fig. 1B); potential to differentiate along ectodermal, TRA1-60+ cells, mesodermal and endodermal lineages (NESTIN/PAX6, αSMA and SOX17, Fig. 1C). Cytogenetic analysis, performed on > 30 mitoses, showed that all three iPSC lines were karyotypically normal in the number and structure of chromosomes at passage 6 (P6) (Suppl Fig. 1). Array-Comparative Gnome Hybridization (a-CGH) also ruled out submicroscopic rearrangements and allowed by CNVs analysis to match the identity of the three iPSC lines to that of donor patients peripheral blood cells (Table 2). Sanger sequencing on extracted DNAs revealed the patients germline *CREBBP* exon 27 non sense mutation c.4435G > T (p.(Gly1479*)), exon 28 missense mutation c.4627G > T (p.(Asp1543Tyr)) and exon 18 non sense mutation c.3474G > A (p.(Trp1158*)) (Fig. 1D). Western Blot analysis using an antibody recognizing CREB Binding Protein (CBP) N-terminus showed that full length CBP was present in the same amount than in control cells in IAIi003RSTS1-46-A (carrying a missense mutation), while it was reduced in IAIi002RSTS1-34-A and IAIi4004RSTS1-149-A lines (carrying non sense mutations) (Fig. 1E) by 46% and 68%, respectively, as shown by densitometry (CBP: actin ratio) (Fig. 1F).

Materials and methods

Reprogramming of RSTS2 patients' erythroblasts to iPSCs

PBMCs were collected *via* gradient centrifugation from blood of 3 RSTS1 patients with *CREBBP* mutations (Table 1) and cultured for 9 days in enriched StemSpan™ Medium (Stemcell Technologies) at 37 °C in 5% CO_2. Reprogramming was performed by SeV (Cytotune 2.0, LifeTech) (Soares et al., 2016). Transduced cells were plated on MEF feeders in HESC (human embryonic stem cell) medium (DMEM-F1220% KOSR, 1 mM L-glutamine, 1 × NEAA, 4 ng/ml FGF (all reagents from Life Technologies) and 100 mM β-mercaptoethanol (Sigma)) and fed every other day. Colonies were picked at day 20 and manually passaged weekly by cutting through the single colony in several places with a sterile syringe needle and then removing the colony by scraping it. Passage ratio was 1:5. iPSCs were harvested in 60% HESC medium, 30% FBS and 10% DMSO and stored in liquid nitrogen.

Pluripotency marker immunocytochemistry

Cells were fixed in 4% paraformaldehyde (20 min, 37 °C). Antibodies in gelatin dilution buffer (0.2% gelatin (for blocking), 0.3% Triton-X 100 (for permeabilization), 20 mM Sodium Phosphate Buffer pH 7.4, 0.45 M NaCl, all by Sigma) were incubated at 4 °C overnight (primary) and 2 h at RT (secondary). Images were acquired with a Nikon Eclipse Ti microscope. Nuclei were counterstained with DAPI.

Flow cytometry

iPSCs were dissociated in PBS/0.5 mM EDTA, fixed using BD Cytofix™ buffer (BD Biosciences) and stained with TRA-1-60 or SSEA4 antibody (both 1 h, 4 °C) followed by the specific fluorescently tagged secondary antibody (1 h 4 °C). Cells were analyzed using a Gallios (Beckman Coulter) flow cytometer and Kaluza software. An iPSC line from a healthy donor was used as a characterization control.

In vitro trilineage differentiation potential assay

iPSCs were cultured on vitronectin-coated chamber slides and differentiated using the STEMdiff™ trilineage differentiation kit (Stemcell Technologies) according to the manufacturer's instructions. (primary and secondary antibody details in Table 3.)

Karyotyping

Chromosomes were prepared from the 3 iPSCs at P6. After colcemid (10 μg/ml) overnight at 37 °C (5% CO_2, 95% rH) iPSCs were incubated in hypotonic solution (KCl 0.56%) at RT for 6 min, washed with acetic acid 5% for 3 min, fixed with methanol/acetic acid (3:1). Q-banded metaphases were photographed at 100× (Leica microscope and camera) and karyotyped (> 30 mitoses) using CytoVision software (Leica).

Array-CGH analysis

High-resolution a-CGH was performed on genomic blood and iPSC DNA using the SurePrint G3 Human CGH Microarray Kit 4x180K in accordance with the manufacturer's instructions (Agilent Technologies). Data were then extracted and analyzed for copy number changes using Agilent CytoGenomics v.3.0.

CREBBP mutation analysis by Sanger sequencing

Genomic DNA was extracted from iPSCs using QIAmp DNA Mini kit (Qiagen). *CREBBP* exons 27, 28, 18 were amplified with GoTaq Flexi DNA polymerase (Promega) using exon flanking primers (details in Table 4) and sequenced with Big Dye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems). Electropherograms were analyzed with ChromasPro software 2.1.5 (Technelysium Pty Ltd) using ENSG00000005339 as the *CREBBP* reference.

Western Blot

Cells grown on vitronectin were pelleted and lysed in ice-cold 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Igepal, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.2 mM Na_3VO_4, protease and phosphatase inhibitors cocktail (Sigma/Aldrich). Nuclear proteins were released with DNase I (20 U) in 20 mM Tris-HCl (pH 7.4) 2.5 mM $MgCl_2$, 20 mM

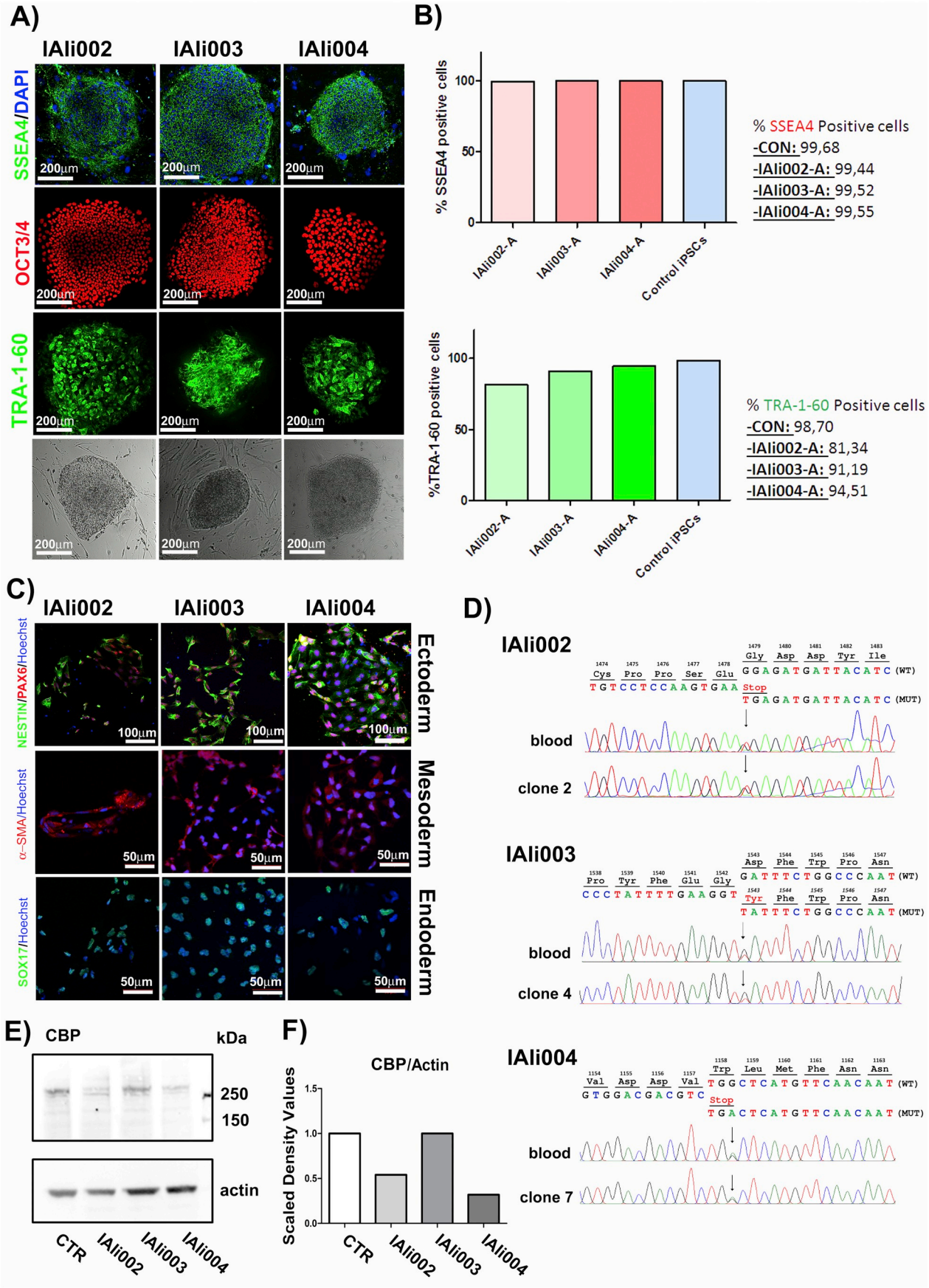

Fig. 1. Characterization of IAli002, IAli004, IAli003.

Table 2
Characterization and validation.

Category	Test	Details and Results	Data
Phenotype	Morphology	Phase contrast microscopy revealed normal stem cell-like morphology	Fig. 1A
Marker expression	Immunofluorescence	Positive for expression of pluripotency markers: SSEA4, OCT3/4, TRA-1-60	Fig. 1A
	Flow cytometry	Determined cell surface expression of SSEA4 and TRA-1-60	Fig. 1B FACS profiles available with Authors
Genotype	Karyotype (G-banding) or alternative	IAIi002: 46XY. Q-banding shows normal karyotype at a resolution of 10 Mb IAIi003: 46XX. Q-banding shows normal karyotype at a resolution of 10 Mb IAIi004:46XY. Q-banding shows normal karyotype at a resolution of 10 Mb	Supplementary Fig. S1
	Reprogramming factors	n/a	n/a
	Blood group genotyping	n/a	n/a
	HLA tissue typing	n/a	n/a
	Sequencing*	Confirmed *CREBBP* mutations in exons 27 (IAIi002-A), 28 (IAIi003-A) and 18 (IAIi004-A)	Fig. 1D
	Western Blot	IAIi002-A: 46% reduction of full length CBP IAIi003-A: full length CBP amount comparable to control IAIi004-A: 68% reduction full length CBP	Fig. 1E-F
	Southern Blot*	n/a	n/a
	WGS	n/a	n/a
Identity*	STR analysis	Substituted by CNV Analysis Number of markers tested: 20 (IAIi002-A); 27 (IAIi003-A); 11 (IAIi004-A) 100% matched against donors' PBMCs CNVs	with author(s)
	Microsatellite PCR	n/a	n/a
Microbiology*	Mycoplasma spp.	Negative Myc test	with author(s)
	Virus screen	SeV genome detected by RT- PCR in iPSCs at first passages, no more detectable at passage 6.	with author(s)
Differentiation	3 germ layer differentiation	Directed differentiation. Determined the expression of markers for each of the three germ layers: NESTIN and PAX6 ectoderm; αSMA, mesoderm; SOX17, endoderm	Fig. 1C

Table 3
Antibody details.

Antibody description	Conjugate	Application	Dilution	Company	Catalog #	RRID
Rabbit anti-OCT3/4		IF Pluripotency Markers	1:200	Santa Cruz Biotechnology	sc-9081	AB_2167703
Mouse anti-TRA-1-60		IF Pluripotency Markers	1:100	Santa Cruz Biotechnology	sc-21,705	AB_628385
Mouse anti-SSEA4		IF Pluripotency Markers	1:100	Thermo Fisher Scientific	14-8843-80	AB_657847
Mouse anti-SSEA4		FACS Pluripotency Markers	1:100	Abcam	ab16287	AB_778073
Mouse anti-TRA-1-60		FACS Pluripotency Markers	1:100	Abcam	ab16288	AB_778563
Mouse anti-NESTIN		IF Differentiation markers	1:150	Abcam	ab22035	AB_446723
Rabbit anti-PAX6		IF Differentiation Markers	1:300	BioLegend	PRB-278P	AB_291612
Rabbit anti-SOX17		IF Differentiation markers	1:200	Cell Signaling Inc.	81,778	AB_2650582
Mouse anti-αSMA		IF Differentiation markers	1:200	Millipore	CBL171	AB_2223166
Rabbit polyclonal anti- CBP (A-22)		WB	1:300	Santa Cruz Biotechnology	sc-369	AB_631006
Mouse monoclonal anti-ACTIN antibody		WB	1:2000	Sigma	A3853	AB_262137
anti-rabbit IgG Secondary Antibody	HRP	WB	1:2000	Millipore	AP307P	AB_92641
anti-mouse IgG Secondary Antibody	HRP	WB	1:2000	Millipore	AP124P	AB_90456
F(ab')2-Goat anti- Rabbit IgG(H + L) Secondary Antibody	Alexa®Fluor 555	IF	1:300	Thermo Fisher Scientific	A-21430	AB_2535851
Anti-Mouse IgG Secondary Antibody	Alexa®Fluor 488	IF	1:500	Thermo Fisher Scientific	A-11001	AB_2534069
Anti-Mouse IgG Secondary Antibody	Alexa®Fluor 488	FACS/IF	1:400 (for SSEA4) 1:300 (for NESTIN)	Thermo Fisher Scientific	A11059	AB_2534106
Anti-Rabbit IgG Secondary Antibody	Alexa®Fluor 488	IF	1:200 (for SOX17)	Thermo Fisher Scientific	A11034	AB_2576217
Anti-Mouse IgM Secondary Antibody	Alexa®Fluor 488	FACS	1:200 (for TRA-1-60)	Thermo Fisher Scientific	A21042	AB_2535711
Anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody	Alexa®Fluor 633	IF	1:200 (for αSMA)	Thermo Fisher Scientific	A-21136	AB_2535775
Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody	Alexa®Fluor 594	IF	1:300 (for PAX6)	Thermo Fisher Scientific	A-11037	AB_2534095

Table 4
Oligo details.

Oligo name	Target	Description	Sequence (5′-3′)	Product Size
*CREBBP_Ex27*_Forward	*CREBBP_ Ex27 c.4435G > T* (IAIi002-A)	Genomic	CTTAAAGGCAGGGCCGATT	301 bp
*CREBBP_Ex27*_Reverse	*CREBBP_ Ex27 c.4435G > T* (IAIi002-A)	Genomic	TGCAAGAAAAAGGCACACAA	301 bp
*CREBBP_Ex28*_Forward	*CREBBP_ Ex28 c.4627G > T* (IAIi003-A)	Genomic	CACACATGCATGGGACTCTG	327 bp
*CREBBP_Ex28*_Reverse	*CREBBP_ Ex28 c.4627G > T* (IAIi003-A)	Genomic	GACACGTGGGCAATGGAG	327 bp
*CREBBP_Ex18*_Forward	*CREBBP_ Ex18 c.3474G > A* (IAIi004-A)	Genomic	GCCAGATGAGACTGGCATTT	445 bp
*CREBBP_Ex18*_Reverse	*CREBBP_ Ex18 c.3474G > A* (IAIi004-A)	Genomic	CAGGCATCAACTGTGTCACC	445 bp

NaCl, and 1 mM PMSF (20 min at 4 °C) mixed with the soluble fraction in SDS-loading buffer and boiled at 70° C for 10 min. Proteins (120 μg) were separated on NuPAGE 4–12% Bis-Tris Gel (Invitrogen), transferred to nitrocellulose and blocked with 5% BSA in PBS-0.2% Tween 20 (PBS-T). The membrane was incubated (1 h, RT) with antibodies to CBP N-terminus and actin, (30 min, RT) in HRP-labeled secondary antibodies (Table 3), then washed in PBS-T, and chemiluminescence signals revealed with a Westar R imager (Hi-Tech Cyanagen). ImageJ was used for densitometry.

Mycoplasma test

We ruled out the presence of Mycoplasma by using EZ-PCR Mycoplasma Test Kit (Biological Industries) according to the manufacturer's instructions. Positive Control was included in the kit.

Resource table

Unique stem cell line identifier	IAIi002-A IAIi003-A IAIi004-A
Link to hPSCreg entry	https://hpscreg.eu/cell-line/IAIi002-A https://hpscreg.eu/cell-line/IAIi003-A https://hpscreg.eu/cell-line/IAIi004-A
Alternative name(s) of stem cell line	IAIi002RSTS1-34-A IAIi003RSTS1-46-A IAIi004RSTS1-149-A
Institution	Istituto Auxologico Italiano (IAI)-IRCCS, Milan, Italy
Contact information of distributor	Lidia Larizza, l.larizza@auxologico.it
Type of cell line	iPSC
Origin	Species: Human Age: 13; 14; 7 Gender: Male; Female; Male Ethnicity: Caucasian
Cell Source	Peripheral blood mononuclear cells (PBMCs)
Clonality	Clonal
Multiline rationale	Same disease non-isogenic cell lines
Reprogramming method	Sendai virus kit
Associated disease	Rubinstein-Taybi syndrome 1 (RSTS1)
Disease associated locus	*CREBBP*, 16p13.3
Known mutations or modification	Spontaneous mutation c.4435G > T, p.(Gly1479*) c.4627G > T, p.(Asp1543Tyr) c.3474G > A, p.(Trp1158*)
Method of modification	n/a
Name of transgene and/or resistance	n/a
Inducible/constitutive system	n/a
STR analysis	Substituted by CNV Analysis Number of CNVs tested: 20 (IAIi002-A); 27 (IAIi003-A); 11 (IAIi004-A) 100% matched against donors' PBMC CNVs
Date archived/stock date	IAIi002-A: March 2016 IAIi003-A: November 2016 IAIi004-A: January 2018
Cell line repository/bank	n/a
Ethical approval	IAI Ethical Committee (CE). CE code: 12_15_2015_02
Have these lines been published before	Yes
If yes, Publication reference/s	Alari V. et al., Stem Cell Res 30, 130–140. https://doi.org/10.1016/j.scr.2018.05.019
Description of the publication	#34: IAIi002-A #46: IAIi003-A #149: IAIi004-A iPSC generation described: Yes QC assays done: Immunofluorescence, RT-PCR, Karyotype, a-CGH, DNA sequencing, transcript sequencing. Novelty of current publication: in-depth characterization (FACS, Trilineage Assay, Myc Test, Western Blot analysis) of three iPSC lines from RSTS1 donors with different mutations and cognitive phenotypes which embrace and validate the disease model.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101553.

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