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## Generation of a CLTA reporter human induced pluripotent stem cell line, CRMi001-A-1, using the CRISPR/Cas9 system to monitor endogenous clathrin trafficking

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### Abstract

The most highly studied endocytic pathway, clathrin-dependent endocytosis, mediates a wide range of fundamental processes including nutrient internalization, receptor recycling, and signal transduction. In order to model tissue specific and developmental aspects of this process, CRISPR/Cas9 genomic editing was utilized to fluorescently label the C-terminus of clathrin light chain A (*CLTA*) within the phenotypically normal, parental CRMi001-A human induced pluripotent stem cell line. Successfully edited cells were isolated by fluorescently activated cell sorting, remained karyotypically normal, and maintained their differentiation potential. This cell line facilitates imaging of endogenous clathrin trafficking within varied cell types.

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Declarations of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2018.10.001>.

**Resource Table.**

Unique stem cell line identifier	CRMi001-A-1
Alternative name(s) of stem cell line	SANFi002-A-1 CRMi001-A-1-CLTA-TQ2 NCRM-5-CLTA-TQ2
Institution	Sanford Research
Contact information of distributor	Kevin Francis; Kevin.Francis@SanfordHealth.org
Type of cell line	iPSC
Origin	Human
Additional origin info	Age: Fetal Sex: Male
Cell Source	Ethnicity if known: Unknown Umbilical cord blood derived CD34 + cells
Clonality	Mixed
Method of reprogramming	Episomal vectors: Oct4, Sox2, c-Myc, KLF4, Lin28 SV40 Large T antigen
Genetic Modification	Yes
Type of Modification	Knock-in
Associated disease	N/A
Gene/locus	CLTA/9p13.3
Method of modification	CRISPR/Cas9
Name of transgene or resistance	Tq2 and puromycin resistance gene
Inducible/constitutive system	N/A
Date archived/stock date	05/30/17
Cell line repository/bank	N/A
Ethical approval	The CRMi001-A hiPSC line was obtained from the National Heart, Lung, and Blood Institute iPSC Core in Bethesda, MD. All research involving hiPSCs was approved by the Institutional Biosafety Committee at Sanford Research (approval no. 2015101).

## Resource utility

Generation of CRMi001-A-1 human induced pluripotent stem cells enable monitoring and manipulation of endogenous clathrin trafficking dynamics in pluripotent or differentiated cell types.

## Resource details

A CRISPR/Cas9 mediated knock-in system was utilized to generate human induced pluripotent stem cells (hiPSCs) expressing *CLTA* tagged with the fluorescent protein Turquoise2 (Tq2), followed by a self-cleavable P2A peptide sequence and the puromycin *N*-acetyltransferase gene. 1kB homology arms targeted a Tq2-P2A-puro cassette to the C-terminal end of the *CLTA* locus (Fig. 1A). A single guide RNA (sgRNA) was designed to target the stop codon in Exon 7 and demonstrated efficient cleavage in a T7 endonuclease assay (Fig. S1). CRMi001-A hiPSCs were transfected with the knock-in vector and sgRNA/Cas9 expression plasmid by nucleofection. Selection for Tq2 positive cells by fluorescence-activated cell sorting (FACS) demonstrated a knock-in efficiency of approximately 1% (Fig. S2A) and co-localized expression with clathrin heavy chain protein CLTC (Fig. S2B). As a control for random integration, CRMi001-A hiPSCs were transfected with the knock-in vector alone by nucleofection. Tq2 positive cells were collected and pooled to minimize potential off-targeting effects. Knock-in to the *CLTA* locus was validated by PCR and Sanger sequencing (Fig. 1B). Targeted integration to the C-terminus of *CLTA* was confirmed by PCR analysis of edited versus unedited cells using a primer pair specific to the donor cassette and the genomic region outside of the homology arms (Fig. 1F-1, F-2). PCR amplification with primers designed to span the homology arms suggested predominately heterozygous insert integration (Fig. 1F-3).

CRMi001-A-1 hiPSCs maintained human pluripotent stem cell-like morphology (Fig. 1C, Table 1). Pluripotency of the CRMi001-A-1 line was confirmed by immunofluorescent expression of transcription factors Oct4A, Nanog, and Sox2, as well as surface markers SSEA4, TRA-1-60 and TRA-1-81 (Fig. 1E), and FACS quantitation of Oct4A-Alexa Fluor 488 expression (Fig. 1D). Pluripotency was further confirmed by embryoid body (EB) formation, loss of pluripotent transcription expression (*POU5F1*) and spontaneous germ layer differentiation exhibited by expression of endodermal (*AFP*), mesodermal (*TBX1*), and ectodermal (*MSI1*) markers (Fig. 1H) assessed by qRT-PCR. Transcript levels were normalized to GAPDH expression and plotted relative to expression levels in respective hiPSC lines. Directed neural differentiation confirmed ectodermal potential through expression of human-specific Nestin (hNestin), expressed by human neural progenitors, and the neuronal-specific intermediate filament, Neurofilament, medium-chain polypeptide (NF-M) (Fig. 1I). Karyotypic analysis detected no chromosomal abnormalities (Fig. 1G) and hiPSCs were mycoplasma free (Fig. S3). Authentication of CRMi001-A-1 hiPSCs was verified by short tandem repeat analysis in comparison to parental CRMi001-A hiPSCs at 17 distinct loci (submitted in archive with journal).

## Materials and methods

### Cell culture

hiPSCs were cultured in StemMACS iPS-Brew XF medium (Miltenyi Biotec) on Corning™ Matrigel™ hESC-qualified-coated plates. Cells were maintained at 37 °C in humidified 5% CO<sub>2</sub> and 21% O<sub>2</sub>. hiPSCs were passaged every 5 days with 0.5 mM EDTA.

### Targeting of the *CLTA* locus

sgRNAs targeting the *CLTA* locus were designed at [crispr.mit.edu](http://crispr.mit.edu).

An oligo pair, 5'-CACCGCAGATGTAGTGTTCACACA-3' and 3'-CGTCTACA TCACAAAGGTGTCAAA-5', was annealed and subcloned into the pX330-U6-Chimeric\_BB-CBh-hSpCas9 vector (kind gift from Feng Zheng). sgRNA activity was validated by T7 endonuclease assay.

1kB homology arms flanking the Turquoise2-P2A-puromycin insert (Fig. 1A) were amplified from genomic DNA and inserted into the pDONOR3 vector by Golden gate assembly (Scott et al., 2018). To prevent Cas9 recognition after integration, the insert's PAM sequence was mutated (Table 1).

1 × 10<sup>6</sup> CRMi001-A hiPSCs were transfected using the Nucleofector 2b system, Nucleofector Kit V, 4 μg donor vector and 2.5 μg sgRNA/Cas9 vector. hiPSCs were plated onto Matrigel-coated plates in iPSC-Brew XF. Tq2 positive cells were isolated with a BD FACSJazz Cell Sorter.

### Targeted integration analysis

Homology-directed repair was validated by PCR utilizing primers listed in Table 2. CRMi001-A hiPSCs transfected with donor vector alone were used to control for non-selective integration. The knock-in was sequenced entirely (3' and 5' targeting in Fig. 1B).

### Karyotyping, STR analysis, and mycoplasma detection

G-banding karyotype analysis (450 band resolution) of eight cells was performed by the Sanford Cytogenetics Laboratory. STR analysis was performed using the ATCC Cell Line Authentication Service and compared to the CRMi001-A line. Mycoplasma assessment was performed using a PCR Detection Kit (Applied Biological Materials).

### Immunocytochemistry

hiPSCs were grown on Matrigel-coated chamberslides, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated in blocking buffer (0.1% Triton X-100, 5% donkey serum). Primary antibodies were incubated overnight at 4 °C in blocking buffer. Alexa Fluor conjugated secondary antibodies and Hoechst counterstain were incubated for 1 hr at room temperature. Images were taken using a Nikon NiE fluorescent microscope and processed using NIS Elements software and Adobe Photoshop.

### Quantitative analysis of pluripotency

hiPSCs were dissociated with accutase and fixed in 2% paraformaldehyde overnight at 4 °C. Cells were incubated with Alexa Fluor 488-conjugated Oct4A and analyzed on a BD LSRFortessa.

### Germ layer differentiation

hiPSCs were incubated with 10  $\mu$ M Y27632 prior to accutase dissociation.  $1 \times 10^6$  cells were plated into the AggreWell™800 system (Stem Cell Technologies). After 5 days in differentiation media (DMEM/F12, 20% Knockout Serum Replacement, 2 mM L-glutamine, 1% NEAA, 1000 units/mL Penicillin-streptomycin, 100  $\mu$ M  $\beta$ -mercaptoethanol), aggregates were transferred to gelatinized plates and spontaneously differentiated for 10 days.

### Directed neural differentiation

Neural rosettes were isolated from EBs, plated onto laminin/PLO coated coverslips and differentiated for 7 days toward neural lineages.

### Real-time PCR analysis

RNA was isolated using the E.Z.N.A RNA Kit (Omega Biotek). 1  $\mu$ g of RNA was reverse transcribed using the Superscript III First-Strand Synthesis System (Invitrogen). Quantitative PCR was performed with Absolute Blue qPCR Master Mix on an Applied Biosystems 7500 instrument. Samples were analyzed in triplicate from independent experiments. Transcript abundance was normalized to GAPDH and quantified using the  $2^{-C_t}$  method. Primers used are listed in Table 2.

### Supplementary Material

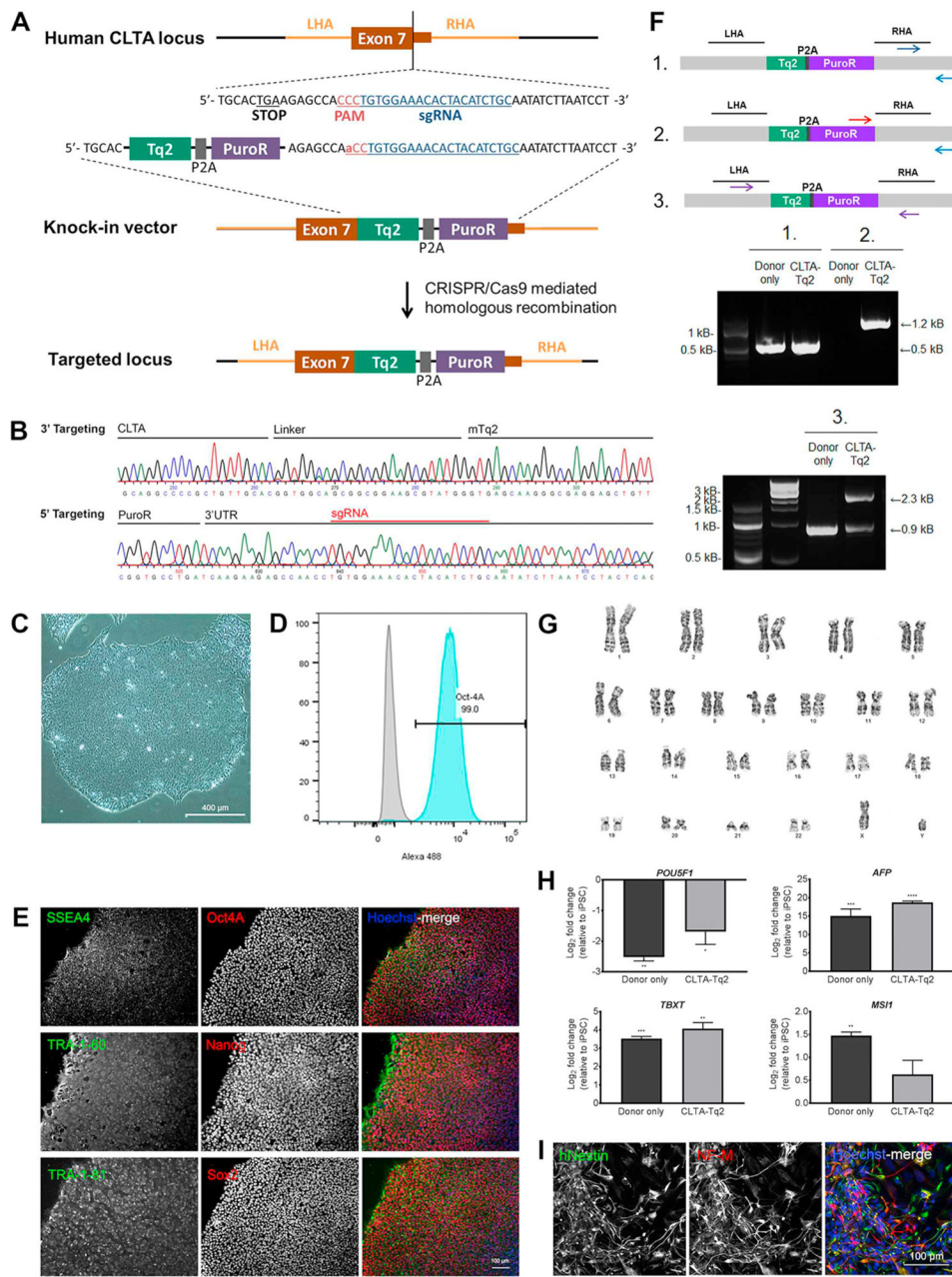
Refer to Web version on PubMed Central for supplementary material.

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### References

Scott BL, et al. , 2018. Membrane bending occurs at all stages of clathrin-coat assembly and defines endocytic dynamics. Nat. Commun 9, 419. 10.1038/s41467-018-02818-8. [PubMed: 29379015]



**Fig. 1.** Targeting of the *CLTA* locus for genomic insertion and CRMi001-A-1 validation.

Table 1

Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal hiPSC morphology	Fig. 1 Panel C
Phenotype	Qualitative analysis	Immunohistochemistry of pluripotency markers: Oct4A, Nanog, Sox2, TRA-1-60, TRA-1-81	Fig. 1 Panel E
Genotype	Quantitative analysis	Expression of pluripotency marker Oct4 by flow cytometry	Fig. 1 Panel D
Identity	Karyotype (G-banding) and resolution	Normal Karyotype: 46,XY, resolution: 480 band level	Fig. 1 Panel G
Mutation analysis (IF APPLICABLE)	STR analysis	17 loci tested, 100% match	Submitted in archive with journal
	PCR and sequencing	Heterozygous targeted integration	Fig. 1 Panel F
	Southern Blot OR WGS	Not performed. Top 10 predicted coding and non-coding off-targets were sequenced, detecting no indel formation.	Available by request
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Supplementary Fig. 4
Differentiation potential	Embryoid body formation and Directed neural differentiation	Expression of genes from three germ layers following spontaneous differentiation: <i>AFP</i> , <i>TBX7</i> , <i>MSI1</i>	Fig. 1 Panel H and I
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Directed neural differentiation immunohistochemistry: Human Nestin, Medium-length neurofilament polypeptide	
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A
		Not performed	N/A

Table 2

## Reagents details

Antibodies used for immunocytochemistry/flow-cytometry			
Antibody	Dilution	Company Cat # and RRID	
Pluripotency markers	1:400	Cell Signaling Technology Cat# 2840, RRID:AB_2167691	
Rabbit anti-OCT4	1:400	Cell Signaling Technology Cat# 4755, RRID:AB_1264259	
Mouse anti-SSEA4	1:200	Cell Signaling Technology Cat# 4903, RRID:AB_10559205	
Rabbit anti-NANOG	1:400	Cell Signaling Technology Cat# 4746, RRID:AB_2119059	
Mouse anti-TRA-1-60	1:200	Cell Signaling Technology Cat# 3579, RRID:AB_2195767	
Rabbit anti-SOX2	1:400	Cell Signaling Technology Cat# 4745P, RRID:AB_10829904	
Mouse anti-TRA-1-81	1:1000	Molecular Probes Cat# A-31572, RRID:AB_162543	
Differentiation markers	1:1000	Millipore Cat# MAB5326, RRID:AB_11211837	
Mouse anti-human Nestin	1:1000	Molecular Probes Cat# A-31572, RRID:AB_162543	
Rabbit anti-Neurofilament M, CT	1:1000	Thermo Fisher Scientific Cat# A-11001, RRID:AB_2534069	
Secondary antibodies	1:1000	Thermo Fisher Scientific Cat# A-21244, RRID:AB_2535812	
Alexa Fluor 555 donkey anti-rabbit IgG (H + L)	1:50	Cell Signaling Technology Cat# 5177S, RRID:AB_10693303	
Alexa Fluor 488 goat anti-mouse IgG (H + L)	1:50	Cell Signaling Technology Cat# 2975S, RRID:AB_10699151	
Alexa Fluor 647 goat anti-rabbit IgG (H + L)			
Flow cytometry			
Oct-4A (C30A3) Rabbit mAb			
Rabbit mAb Alexa Fluor 488 conjugate			
Rabbit (DAIE) mAb IgG XP Isotype Control			
Alexa Fluor 488 conjugate			

Primers		
Target	Forward/Reverse primer (5'-3')	
Pluripotency Markers (qPCR)	<i>POU5F1</i>	CCAAGGAATAGTCTGTAGAAAGTGC/ TGCATGAGTCAGTGAACAGG
Differentiation Markers (qPCR)	<i>AFP</i>	TCTGCATGAATTATACATTGACCAC/ AGGAGATGTGCTGGATTGTC
	<i>MSI1</i>	TCGTTCCGAGTCACCATCTTG/ GGCTTCGTCACTTTCATGGA
	<i>TBX1</i>	CTATGTGGATTCCAGGGCTCATAC/ CGTCTCCTTCAGCAAAGTCAA
House-Keeping Genes (qPCR)	<i>GAPDH</i>	GGCCCAATACGACCAA/ CTCTGTGCTCCTCCTGTTTC
Targeted integration analysis/sequencing	<i>PCR analysis</i>	
	1. Template control	TTGCTTCCAGTGTCCCTCAGTTTA/ GCTTTAATAATTGCTTGGAAACATCACCT
	2. T.q2-Puro Integration	TACGAGCGGCTCCGGCTTCA GCTTTAATAATTGCTTGGAAACATCACCT



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## Primers

Target	Forward/Reverse primer (5'-3')
3. Mono-allelic integration	ATCTTGGGAAAAGCCAGAAATGTCATT / CCTAAACTGAGGGACACTGGCAA
<i>Sequencing</i> 5' targeting	ATCTTGGGAAAAGCCAGAAATGTCATT TACCAGCAGAACACCCCCAT
3' targeting	TCACCGAGCTGCCAAGAACTCTT CCTAAACTGAGGGACACTGGCAA
T7 endonuclease assay 1 kB amplicon	ATCTTGGGAAAAGCCAGAAATGTCATT CCTAAACTGAGGGACACTGGCAA