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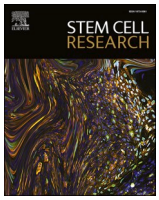

Generation of a homozygous CRYAB p.Arg120Gly mutant (UKEi001-A-1) from a human iPSC line

Niels Pietsch [a,b], Jiancheng Cheng [a], Antonietta Fazio [a,c], Leonie Ewald [a], Erda Alizoti [a], Elisabeth Krämer [a,b], Ellen Orthey [a,b], Lucie Carrier [a,b], Sonia R Singh [a,b,*]

[a] Institute of Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[b] DZHK (German Centre for Cardiovascular Research), Partner Site Hamburg/Kiel/Lübeck, Hamburg, Germany
[c] Department of Biomedical and Neuromotor Sciences (DIBINEM), University of Bologna, Italy

A B S T R A C T

Variants in *CRYAB* can lead to desmin-related (cardio-)myopathy (DRM), a genetic muscle disorder with no curative treatment available. We introduced a homozygous *CRYAB* c.358G > A (p.Arg120Gly) mutation, which is established for the study of DRM in mice, into a donor human induced pluripotent stem cell (hiPSC) line. Control and mutant hiPSCs were tested for karyotype integrity and pluripotency marker expression. HiPSCs could be differentiated into endoderm, ectoderm and cardiomyocytes as a mesodermal derivative *in vitro*. CRYABhom hiPSC-derived cardiomyocytes developed intracellular CRYAB aggregates, which is a hallmark of DRM. This newly created mutant can be utilized to study DRM and cardiac proteinopathy in a human context.

Resource Table:

Unique stem cell line identifier	UKEi001-A-1
Alternative name(s) of stem cell line	CRYABhom
Institution	Institute of Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
Contact information of the reported cell line distributor	Dr. Sonia R Singh; s.singh@uke.de
Type of cell line	iPSC
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 60–64 Female
Cell Source	Dermal fibroblasts
Method of reprogramming	Non-integrating Sendai virus vectors (CytoTune™, expression of OCT-4, SOX2, c-Myc, KLF4)
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-qPCR Sendai Virus, negative at master cell bank stage
The cell culture system used	mTeSR plus during clonal expansion. FTDA for maintenance culture.
Type of the Genetic Modification	Induced mutation (missense)
Associated disease	Desmin-related (cardio-)myopathy
Gene/locus	*CRYAB*/chr11 (q23.1) Homozygous mutant: c.358A > G and c.360G > A (silent mutation); p. Arg120Gly

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(*continued*)

Method of modification / user-customisable nuclease (UCN) used, the resource used for design optimisation	CRISPR/Cas9, IDT
User-customisable nuclease (UCN) delivery method	Nucleofection of RNP complex (crRNA, tracrRNA and Cas9)
All double-stranded DNA genetic material molecules introduced into the cells	None
Analysis of the nuclease-targeted allele status	Sanger sequencing and qPCR
Method of the off-target nuclease activity prediction and surveillance	Targeted PCR and Sanger sequencing of the Top 10 off-target loci
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	26.04.2023
Cell line repository/bank	hPSCreg.eu – UKEi001-A-1
Ethical/GMO work approvals	The donor gave written informed consent for research purposes and the study was approved by the ethical committee of the UKE, Hamburg (PV4798/28.10.2014).
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

* Corresponding author at: Institute of Experimental Pharmacology and Toxicology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.
E-mail address: s.singh@uke.de (S.R. Singh).

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The manuscript section expected contents clarification.

1. Resource utility

Differentiated (cardio-)myocytes of this hiPSC line and its isogenic control can serve as a model system to study DRM. The homozygous mutant allows the exclusive study of the DRM-causing *CRYAB* variant in a human context to investigate disease mechanisms and potential therapies with an ethical and potentially inexhaustible resource (see Table 1 and Table 2).

2. Resource details

The term DRM encompasses several genetic disorders with gene variants in the intermediate filament desmin (*DES*) or different interacting partners, including, among others, filamin C (*FLNC*), titin (*TTN*), myotilin (*MYOT*), or αB-crystallin (*CRYAB*). Depending on the affected gene and mutated locus, variants can be autosomal dominant or recessive and result in different severity of disease symptoms, which affect skeletal and cardiac muscles. The main hallmarks of DRM include mitochondrial dysfunction, excitation–contraction coupling defects and intracellular disarray, which are accompanied by DES- and CRYAB-positive, insoluble aggregates in the majority of cases (reviewed in

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	A visual record of the line's cellular morphology: typical pluripotent human stem cell morphology	Fig. 1 panel A
Pluripotency status evidence for the described cell line	Qualitative analysis - RT-PCR	Expression of *NANOG* and *SOX2*	Fig. 1 panel B
	Quantitative analysis - Flow cytometry	>90% SSEA3-positive cells	Fig. 1 panel C
Karyotype	G-banding for UKEi001-A (not showns), qPCR, in house nCounter NanoString karyotyping panel) for UKEi001-A-1	46, XX	Fig. 1 panel D and 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 F
	Evaluation of the - (homo-/hetero-/hemi-) zygous status of introduced genomic alteration(s)	qPCR, PCR + Sanger sequencing	Fig. 1 D and F
	Transgene-specific PCR (when applicable)	N/A	
Verification of the absence of random plasmid integration events	PCR/Southern	N/A	
Parental and modified cell line genetic identity evidence	STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq	Available for parental line UKEi001-A	Submitted to hpscreg.eu
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	Genomic DNA PCR and sequencing; homozygous, introduction of c.358A > G and c.360G > A (silent)	Fig. 1 F
	Immunofluorescence	Mutant CRYAB aggregates	Fig. 1 I
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	Western blot showing lower CRYAB and desmin protein levels in CRYABhom	Fig. 1 J
Off-target nuclease activity analysis	PCR across top 10 predicted top likely off-target sites, whole genome/exome sequencing	Demonstration of the lack of NHEJ-caused mutagenesis in the top predicted off-target Cas nuclease activity	Supplemental Fig. 1
Specific pathogen-free status	Mycoplasma	PCR-negative	Not shown
Multilineage differentiation potential	Directed differentiation; Expression of marker markers determined by RT-qPCR, flow cytometry or immunofluorescence	STEMdiff Trilineage Differentiation Kit and cardiomyocyte differentiation (mesoderm); RT-qPCR: Ectoderm - *NCAM1* and *PAX6* Endoderm – *FOXA2* and *SOX17* Flow cytometry: Mesodermal lineage, differentiation to cardiomyocytes – cardiac troponin T (TNNT2) Immunofluorescence: Mesodermal lineage, differentiation to cardiomyocytes – α-actinin 2 (ACTN2)	Fig. 1 panel G, H and H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	HIV 1 Taq-PCR QL, HBV-PCR HCV-PCR QL on primary fibroblasts under the detection threshold	Not shown but available with author
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	Not performed	
	HLA tissue typing	Not performed	

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry/Western blot			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency marker	Rat anti-human SSEA3 antibody, PE-conjugated	1:5	BD Biosciences Cat# 560237, RRID: AB_1645542
	rat IgM, k antibody	1:80	BD Biosciences Cat# 553943, RRID:AB_10056839
Differentiated cardiomyocyte marker	Cardiac troponin T antibody, anti-human/mouse/rat, REAfinity™	1:50	Miltenyi Biotec Cat# 130-119-674, RRID: AB_2751795
	REA control antibody (I), human IgG1, REAfinity™		Miltenyi Biotec Cat# 130-120-709, RRID: AB_2784399
Immunofluorescence/Western blot	Anti-ACTN2	1:800 (IF)/	Sigma Aldrich Cat# A7811, RRID: AB_476766
	Anti-CRYAB	1:10000 (WB)	Enzo Life Sciences Cat# ADI-SPA-222-F, RRID: AB_1659585
	Anti-DES	1:100 (IF)/	Millipore Cat# 04-585, RRID: AB_838263
		1:1000 (WB)	
		1:1000 (WB)	
Nuclear stain	DAPI	1 μg/mL	Invitrogen Cat# D1306
Site-specific nuclease			
Nuclease information	Alt-R® S.p. Cas9	Nuclease 3NLS, 2 nmol, Cat# 1,081,058	
Delivery method	Nucleofection of RNP complex (crRNA, tracrRNA and Cas9)	Alt-R® CRISPR-Cas9 crRNA, 2 nmol (IDT®); Alt-R® CRISPR-Cas9 tracrRNA, ATTO™ 550, 5 nmol: Cat# 1,075,927 (IDT®); Alt-R® Cas9 Electroporation Enhancer, 10 nmol: Cat# 1,075,916 (IDT®)	
Selection/enrichment strategy	N/A		
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
Pluripotency marker (RT-(q)PCR)	*NANOG* (stem cell)	F: GATTTGTGGGCCTGAAGAAA	
		R: AAGTGGGTTGTTTGCCTTTG	
	SOX2 (stem cell)	F: AGTCTCCAAGCGACGAAAAA	
		R: TTTCACGTTTGCAACTGTCC	
Differentiation Markers (RT-(q)PCR)	*NCAM1* (ectodermal)	F: ATGGAAACTCTATTAAAGTGAACCTG	
		R: TAGACCTCATACTCAGCATTCCAGT	
	PAX6 (ectodermal)	F: TGGGCAGGTATTACGAGACTG	
		R: ACTCCCGCTTATACTGGGCTA	
	FOXA2 (endodermal)	F: GAGCGGTGAAGATGGAAGG	
		R: TGTACGTGTTCATGCCGTT	
	SOX17 (endodermal)	F: CGCACGGAATTTGAACAGTA	
		R: GGATCAGGGACCTGTCACAC	
Housekeeping gene (RT-(q)PCR)	*GAPDH*	F: CCTCAAGATCATCAGCAATGCC	
		R: ATGTTCTGGAGAGCCCCGC	
Targeted mutation analysis/sequencing	Sequencing data from both alleles		
Allele frequency qPCR	*CRYAB*	F: CAGGATGCCTGAGTTCTGGG	
		R: CCATTCACAGTGAGGACCCC	
	OPA1	F: AGGACATCCTTTCAGCAGTTCT	
		R: CTTTTTGGCTGTGTAGCCACC	
	TTL	F: TGAGTCCTGCACATGGTTCC	
		R: AAACGTTGCCCTCTCCATCC	
	CD248	F: GCCAGCAGATGTGTGTCAAC	
		R: CCAGCAACTCATCTCCGAGG	
crRNA sequence + PAM	TCATCTCCAGGGAGTTCCAC AGG		
Top10 off-target (OT) mutagenesis predicted site sequencing primers	OT1: TCATTTCTAGAGAGTTCCAC CAG	OT1 F: AAATGAGGGTGCCACTGCTT	
		R: GCCTCAGACACTGACCCAAT	
	OT2: ACATCTCCTAGGGAGTTCCAC TAG	OT2 F: GAATGCAAACCCTGGTAGGTG	
		R: GCCCAAGATGGGCACTGAATA	
	OT3: TAATATCCAGGAAGTTCCAC TGG	OT3 F: ACAGAGGTATCATGGGAGTTCAC	
		R: CTTCCAAACTCTCCTGGCTTAGA	

(*continued on next page*)

Table 2 (*continued*)

Antibodies and stains used for immunocytochemistry/flow-cytometry/Western blot			
	Antibody	**Dilution**	**Company Cat # and RRID**
	OT4: CCATATCCAGGAAGTTCCAC CAG	OT4 F: CACTCGCTTCATTCTGCACG R: CATTCTGTCCTGGCTCCCTG	
	OT5: TCAGCACCATGGAGTTCCAC GGG	OT5 F: GAGACTTGCAGAAAGGTCTGGATA R: CAGGCCTGCAAGATGATTGGT	
	OT6: ACAACTCCAGGAAGTTCCAC AGG	OT6 F: GTGGTACATGTACACATGGATG R: TTGAACAGATGGTCCCCAAG	
	OT7: TCATCTCTAGA-AGTTCCAC TGG	OT7 F: CCTTCTGGTACTCCAATTATGC R: GCTGCGGACCTTCATAAGTG	
	OT8: CCAACTCCAGGGAGTTCCAA AGG	OT8 F: GTGCCACTGCTTGAGGACTT R: CAGCCACCAGCAGGATAGAAT	
	OT10: TCAGCTTGAAGGAGTTCCAC TGG	OT9 F: GCCAAGAACACAGCACTTACC R: TCCCAGTTGCTCTGCTTCAG OT10 F: CAGTCCGGCCTTACTGGATT R: AGCCTCAGACACTGACCCA	
103 mer ssODN used as template for HDR-mediated site-directed mutagenesis in antisense direction (5′-3′)		CAGGGATGAAGTAATGGTGAGAGGGTCTACATC AGCTGGGATCCGGTATTTTCCGTGGGAACTCCCT GGAGATGAAACCATGTTCATCCTAACCCAA AAGAATG	

Singh et al, 2020). Clinically, patients present with skeletal muscle weakness, different cardiomyopathy types and conduction defects, with DES filaments highly abundant in both cardiomyocytes and cells of Purkinje fibers. While the onset and progression of the disease vary and no curative treatment is available for DRM patients, symptoms can be ameliorated with physiotherapy and pacemaking/assist devices (reviewed in van Spaendonck-Zwarts et al, 2011). The *CRYAB* p. Arg120Gly variant was first described in 1998 to cause DRM in a French family and, has been established as a model of cardiac proteinopathy in mice (Vicart et al, 1998; Wang et al, 2001). Located in exon 4, the mutation leads to a loss of chaperone function and results in protein aggregates, which stain positive for DES and CRYAB. We introduced a homozygous p.Arg120Gly mutation (c.358A > G) into hiPSCs from a healthy donor to create a human DRM model of cardiac proteotoxicity. The created mutant line (CRYABhom; UKEi001-A-1) was examined for essential characteristics and compared to its isogenic control (ERC001; UKEi001-A). A second homozygous CRYAB p.Arg120Gly mutant line was obtained in a second run and was partly characterized (Supplemental Fig. 2). UKEi001-A-1 showed normal hiPSC morphology (Fig. 1A) and high expression of the pluripotency markers *NANOG* and *SOX2* determined by RT-qPCR (Fig. 1B) and SSEA-3 determined by flow cytometry (Fig. 1C). Normal *CRYAB* allele frequency and karyotype were validated by qPCR and NanoString nCounter profiling respectively (Fig. 1D and E). Homozygous c.358A > G and silent c.360G > A mutations were confirmed in CRYABhom by Sanger sequencing (Fig. 1F). To evaluate specificity in targeting the mutation site with CRISPR-Cas9 we sequenced the top 10 most likely off-targets and did not observe any changes to wild-type sequences, indicating no off-target effects of the CRISPR approach (Supplemental Fig. 1). To confirm pluripotency, hiPSCs were differentiated into ectoderm and endoderm and expression of specific marker genes were evaluated by RT-qPCR (Fig. 1G). Furthermore, we differentiated hiPSCs from both lines into cardiomyocytes (mesodermal lineage) and evaluated differentiation efficiency by staining cardiac troponin T (TNNT2). CRYABhom was differentiated into cardiomyocytes with over 70% TNNT2-positive cells in three independent differentiation runs (Fig. 1H). Immunofluorescence images of CRYABhom cardiomyocytes showed intracellular CRYAB aggregates 30 days after differentiation in a fraction of cells, which were absent in the ERC001 control (Fig. 1I). Western blot analysis revealed overall low protein levels of CRYAB and DES in CRYABhom compared to ERC001 in water-, SDS- and urea-soluble fractions (Fig. 1J).

3. Materials and methods

3.1. Genome editing with CRISPR/Cas9

To introduce c.358A > G into the CRYAB gene, a gRNA was designed with the CRISPOR.org web tool and validated with the IDT CRISPR-Cas9 guide RNA design checker (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM). A 100-nt ssODN template was designed to introduce two specific base edits to the coding sequence and introduce a PAM-silencing mutation to prevent excess cutting of the target. The gRNA was annealed to a tracrRNA and co-nucleofected into passage 26 ERC001sv1162 (UKEi001-A) hiPSCs with the 4D-NucleofectorTM System (Lonza). Nucleofected cells were seeded into a 12-well plate, precoated with Matrigel. After 48 h, cells were detached and seeded at low density in a 6-well plate with mTeSR+ and 10 μM Y-27632 to obtain single-cell clones. When reaching sufficient size, colonies derived from single cells were picked, re-plated in a 48-well plate and passaged once 1:2. Cells from each clone were cryopreserved (1x 48-well) and harvested for DNA extraction (1x 48-well). DNA extraction was performed with DNA Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. PCR amplification of the CRYAB locus was achieved with AmpliTaq DNA Polymerase (Thermo Fisher Scientific) and amplicons sequenced at Eurofins Genomics.

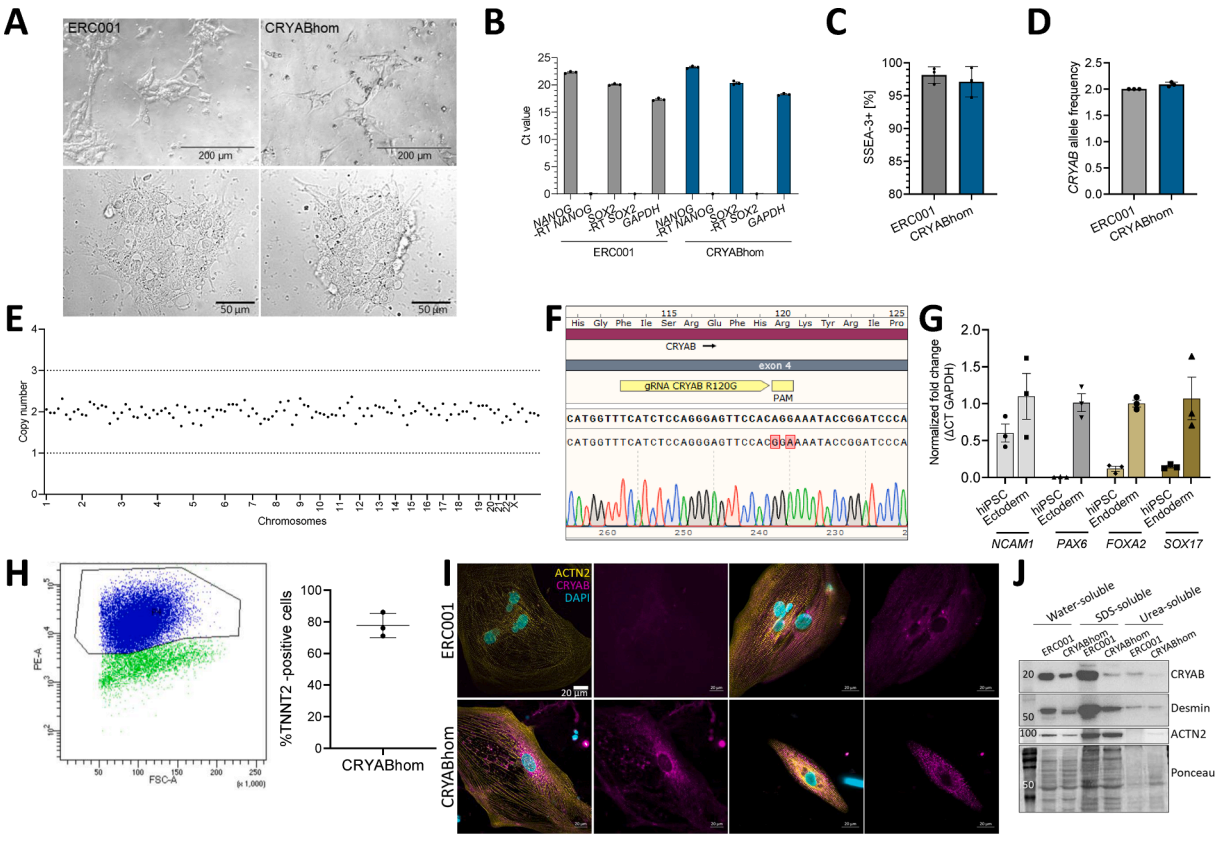

Fig. 1. STR analysis or other genotypic identity evidence types. Available for parental line UKEi001-A at hpscreg.eu.

3.2. Validation of allele frequency by genomic qPCR

To ensure the correct CRYAB allele frequency of the mutant line and exclude a potential hemizygous mutation, a quantitative PCR was performed with genomic DNA. CT-values for CRYAB were normalized to three independent genes (OPA1, TTL and CD248). ΔCT-values were then normalized to the ERC001 control.

3.3. Karyotype analysis

HiPSC lines were tested for karyotypic abnormalities with an in-house NanoString nCounter® Human Karyotype panel according to the manufacturer's protocol.

3.4. Flow cytometry analysis

To determine the pluripotency of hiPSC cultures or cardiomyocyte percentage after cardiac differentiation, cells were stained with an anti-SSEA-3 or an anti-TNNT2 antibody, respectively, and flow cytometry was performed (FACSCanto II Flow Cytometer; BD Biosciences).

3.5. Germ layer differentiation

Pluripotency of hiPSCs was tested with the STEMdiff™ Trilineage Differentiation Kit (Stemcell Technologies, #05230) according to the manufacturer's instructions. The capacity to differentiate into mesoderm was shown by directed monolayer differentiation into cardiomyocytes according to (Mosqueira et al, 2018).

3.6. RNA extraction and RT-qPCR

Total RNA was isolated from hiPSCs and differentiated cells with TRIzol (Life Technologies) according to the manufacturer's instructions. cDNA synthesis with 200 ng RNA was performed using SuperScript III cDNA Synthesis Kit (Thermo Fisher Scientific) with oligo(dT) and random primers according to the manual. Expression levels for different genes of interest were determined with RT-qPCR with GAPDH as housekeeping control.

3.7. Immunofluorescence

For immunofluorescence, cardiomyocytes were fixed in 4% PFA, permeabilized with PBS/0.5% Triton X100, and stained with anti-α-actinin 2 (ACTN2) and anti-CRYAB antibodies in blocking solution (1% BSA, 0.1% Tween20).

3.8. Western blot

For Western blot, cardiomyocytes were lysed in buffer, extracts were run on a 12% gel, transferred to a nitrocellulose membrane and stained with anti-α-actinin 2 (ACTN2), anti-CRYAB and anti-DES antibodies.

3.9. Mycoplasma test

To ensure the absence of mycoplasma contamination of hiPSC cultures, PCR was conducted routinely as previously described (Shibamiya et al. 2020).

Declaration of Competing Interest

LC is member of the DiNAQOR Scientific Advisory Board and has shares in DiNAQOR. The remaining authors declare no 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.2023.103188.

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