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Generation of a homozygous knock-in human embryonic stem cell line expressing mEos4b-tagged CTR1

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Abstract

Copper transporter 1 (CTR1) is the major membrane protein responsible for cellular copper (Cu) uptake and mediates cellular copper homeostasis. To elucidate CTR1′s behavior using imaging approaches, we generated a homozygous knock-in human embryonic stem cell (hESC) clone expressing photoconvertible fluorescence protein mEos4b-tagged endogenous CTR1 using CRISPR-Cas9 mediated homologous recombination. The engineered cells express functional CTR1-mEos4b fusion and have normal stem cell morphology. They remain pluripotent and can be differentiated into all three germ layers *in vitro*. This resource allows the study of CTR1 at an endogenous level in different cellular contexts using microscopy.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2022.102845.

1. Resource table

Unique stem cell line identifier	WAe001-A-79
Alternative name(s) of stem cell line	H1_CTR1-mEos4b / UHMCe002-A-79
Institution	Department of Chemistry, University of Houston, Houston
Contact information of the reported cell line distributor	Tai-Yen Chen (tchen37@central.uh.edu)
Type of cell line	Human embryonic stem cells
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: N/A Sex: Male
	Ethnicity if known: N/A
Cell Source	H1 hESCs (NIH Registration Number: 043, WiCell, passage 28–30)
Method of reprogramming	N/A
Clonality	Clonal by manual single-cell colony picking
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Cell culture system used	mTeSR™plus/Matrigel™ (1%)
Type of Genetic Modification	Transgene generation (fluorescent protein tagging)
Associated disease	N/A
Gene/locus	9q32; *SLC31A1* (ENSG00000136868)
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Electroporation
All genetic material introduced into the cells	Linearized HDR donor vector (carried by pMiniT 2.0)
	Cas plasmid (pSpCas9(BB)-2A-Puro (PX459))
Analysis of the nuclease-targeted allele status	Genotyping PCR, sequencing of the targeted allele, and Southern blotting
Method of the off-target nuclease activity surveillance	N/A
Name of transgene	mEos4b followed by a Flag-tag
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A
Inducible/constitutive system details	N/A
Date archived/stock date	2022-05-12
Cell line repository/bank	Human Pluripotent Stem Cell Registry: https://hpscreg.eu/cell-line/WAe001-A-79
Ethical/GMO work approvals	Cell lines were used according to institutional guidelines. UTHealth approval number: SCRO-16–01
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	pSpCas9(BB)-2A-Puro (PX459) was a gift from Feng Zhang (Addgene plasmid # 48139; http://n2t.net/addgene:48139; RRID: Addgene_48139)

2. Resource utility

The generation of this homozygous knock-in hESC line expressing mEos4b-tagged copper transporter 1 (H1_CTR1-mEos4b) enables the study of CTR1 using single-molecule imaging approaches in various human cells at an endogenous level. The differentiated

engineered hESCs will be used to dissect the molecular behaviors of CTR1 and understand cellular Cu homeostasis.

3. Resource details

Copper transporter CTR1, encoded by *SLC31A1*, is a homotrimeric and the major membrane protein responsible for cellular Cu uptake. Heterozygous deficiency of CTR1 in mammals shows cerebral Cu accumulation; homozygous mutation is embryonic lethal because defective cuproenzymes lead to poor connective tissue development (Lee et al., 2001). Despite its substantial role in cellular Cu supply and systematic Cu homeostasis, the molecular regulations of CTR1′s cell-type-specific distributions, intracellular trafficking, and oligomerization dynamics are still unclear. To elucidate the behaviors of CTR1 at an endogenous level in different cellular contexts using single-molecule super-resolution microscopy (Xie et al., 2018), we genetically modified H1 hESCs by CRISPR-Cas9 mediated homologous recombination to insert a photoconvertible fluorescence protein, mEos4b with a Flag-tag, intramolecularly at the location four amino acids before the HCH domain of the endogenous CTR1 cytoplasmic tail to avoid perturbing Cu uptake function (Fig. 1A). A heterozygous clone was first obtained and subjected to a repeat of the CRISPR-Cas9 induced mEos4b knock-in to generate the homozygous line, H1_CTR1-mEos4b. The targeted homozygous mEos4b insertion is confirmed by genotyping PCR and Southern blot analysis using CTR1 specific probes (Fig. 1B and Suppl. Fig. 1, respectively). The sequence of the genomic DNA PCR product matches the reference engineered genome, showing that the mEos4b insertion is precise and in-framed (Fig. 1C and Suppl. Fig. 2). The expression of CTR1-mEos4b fusion is validated by detecting mRNA spanning across the CTR1-mEos4b junction using RT-qPCR (Fig. 1D). mEos4b mRNA level was also measured to investigate any off-target mEos4b expression. Compared to the heterozygous line, the H1_CTR1-mEos4b line shows a doubled mEos4b and CTR1-mEos4b transcripts, indicating that there is no extra copy of mEos4b expressed other than the CTR1-mEos4b fusion (Fig. 1D). The fluorescence measurements also show the expected elevated mEos4b fluorescence signal compared to the heterozygous, supporting the conclusion of the absence of off-target mEos4b expression and, at the same time, suggesting the presence of CTR1-mEos4b fusion at the protein level (Fig. 1E). To assess the functionality of H1_CTR1-mEos4b, we conducted ICP-MS measurements to compare the intracellular Cu content with the parental H1 under Cu treatment. H1_CTR1-mEos4b line shows a comparable Cu uptake capability, indicating that the engineered protein is functional (Fig. 1F). H1_CTR1-mEos4b has a matched short tandem repeat (STR) identity to its H1 parental (data available upon request), and 16 out of 22 metaphases show normal karyotype (Suppl. Fig. 3). The cells exhibit normal hESC morphology (Fig. 1G). The expressions of pluripotency transcription factors NANOG, OCT3/4, and SOX2 are demonstrated by both RT-qPCR and immunostaining (Fig. 1H and I, respectively). *In vitro* differentiation shows that the line can be differentiated into ectoderm, mesoderm, and endoderm, demonstrated by positive immunostaining of the differentiated cells using germ-layer-specific markers (Fig. 1J). The characterization and validation of H1_CTR1-mEos4b are summarized in Table 1. In short, H1_CTR1-mEos4b is a homozygous pluripotent hESC line expressing mEos4b tagged endogenous CTR1. Its

ability to differentiate into different tissue types provides a versatile platform for imaging-based studies of Cu uptake mechanisms. Table 2.

4. Materials and methods

4.1. Cell culture

H1 hESCs were cultured as previously described (Huang et al., 2021; Wen et al., 2019). Accutase was used for cell detachment, and ROCK-inhibitor was added on the day of cell attachment.

4.2. CRISPR-Cas9

We adopted the protocol from Koch et al. (Koch et al., 2018) and designed sgRNAs targeting near the stop codon of CTR1 using Benchling. H1 hESCs were co-electrotransfected with a sgRNA-containing plasmid (PX459, Addgene) and the linearized (EcoRI digested) recombination donor sequence carried by pMiniT 2.0 (NEB). Transfected cells were plated on feeder cells (Gibco), followed by puromycin selection for 3 days. Clones were then manually picked and expanded under a feeder-free system again.

4.3. PCR and sequencing

Genomic DNA was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre). PCR for screening was performed using AccuStart II GelTrack SuperMix (QuantaBio) with the annealing temperature (T_a) of 60 °C. Genomic fragments for Sanger sequencing were amplified using Q5 Hot Start High-Fidelity DNA Polymerase (NEB) with the same primer set at T_a = 66 °C.

4.4. Southern blotting

Genomic DNA was digested with HindIII-HF, resolved via electrophoresis, and transferred to blots. The probe was synthesized using PCR DIG Probe Synthesis Kit (Roche) at T_a = 63 °C. Southern blot was performed according to the Roche DIG Manual for Filter Hybridization with the hybridization temperature of 37 °C. The expected fragment sizes are 4617 bp for CTR1 wild type and 3694 bp for CTR1-mEos4b.

4.5. RT-qPCR

Total RNA was extracted the same way as DNA and converted to cDNA using iScript R.T. Supermix (Bio-Rad). Quantitative PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) with the following program: 50 °C for 10 min, 95 °C for 5 min, 40 cycles of 95 °C for 10 sec and 60 °C for 30 sec (1 min 45 sec for CTR1mEos4b junctional primers), 95 °C for 10 sec, and Melt Curve 65 to 95 °C, increment 0.5 °C for 5 sec. Data were analyzed in triplicate and normalized to GAPDH expression.

4.6. Inductively coupled plasma–mass spectrometry (ICP-MS)

Cells with Cu-treatment (50 μM $CuCl_2$, 1 h) were dissociated from dishes and washed two times with PBS. The cell pellets were digested in ultrapure 70% HNO_3, heated at 70 °C for 30 min followed by 95 °C for additional 2 h, cooled down to room temperature, and further

diluted in ultrapure water. Copper and sulfur contents were detected by Agilent 8800 triple quadrupole ICP-MS. Copper contents of each sample were normalized to sulfur.

4.7. Fluorescence measurement

Cells were dissociated and washed. Around one million cells were resuspended in PBS. Fluorescent intensity of mEos4b was detected by Spark® multimode microplate reader (Tecan) with Ex 480 nm (width 10 nm)/Em 516 nm (width 20 nm).

4.8. Karyotyping and STR analysis

The G-band karyotyping and STR analysis was carried out by the University of Texas MD Anderson Cancer Center Cytogenetics and Cell Authentication Core Facility.

4.9. In vitro differentiation into three germ layers

In vitro differentiation was performed using STEMdiff Trilineage Differentiation Kit (STEMCELL Technologies).

4.10. Mycoplasma testing

Mycoplasma testing was performed using Mycoplasma PCR Detection Kit (Applied Biological Materials).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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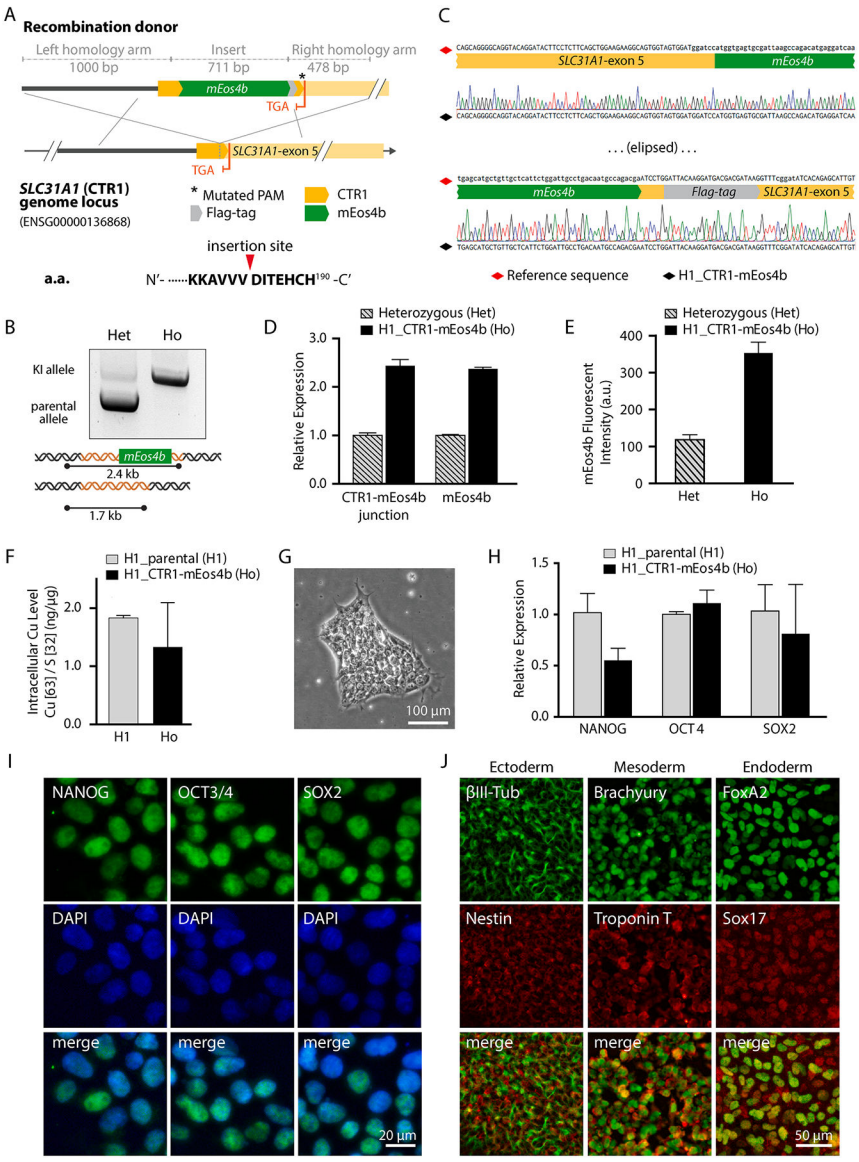

Fig. 1.

Characterization of H1_CTR1-mEos4b.

Table 1

Characterization and validation.

Classification (optional *italicized)*	**Test**	**Result**	**Data**
Morphology	Photography	Normal	Fig. 1G
Pluripotency status evidence for the described cell line	Qualitative analysis: *Immunocytochemistry*	Positive nuclear staining for pluripotency markers NANOG, OCT3/4, and SOX2	Fig. 1I
	Quantitative analysis: *RT-qPCR*	*NANOG*: 0.55 ± 0.119%; *SOX2*: 0.81 ± 0.482%; *OCT4*: 1.11 ± 0.129% (Mean ± SEM)	Fig. 1H
Karyotype	Karyotype (G-banding)	Band resolution: 450	Supplementary Fig. 3
		A total of 22 metaphases were karyotyped by G-banding: 16 showed normal karyotype 46, XY.	
Genotyping for the desired genomic alteration/allelic status of the gene of interest	Targeted allele-specific PCR	Genomic DNA PCR aiming at the targeted site indicates homozygous knock-in	Fig. 1B
	Transgene-specific PCR	See “Sequencing (genomic DNA PCR)”	See “Sequencing (genomic DNA PCR)”
Verification of the absence of random plasmid integration events	PCR	pSpCas9(BB)-2A-Puro (PX459): no random integration; pMiniT 2.0: random backbone integration (does not contain the transgene)	Supplementary Fig. 4
Parental and modified cell line genetic identity evidence	STR analysis	14 sites were tested; all match parental H1	Data available with authors and submitted in an archive with the journal
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR)	Genomic DNA PCR sequence across the targeted site matches the reference engineered genome	Fig. 1C and Supplementary Fig. 2
	PCR-based analyses	See “Targeted allele-specific PCR” and “Sequencing (genomic DNA PCR)	See “Targeted allele-specific PCR” and “Sequencing (genomic DNA PCR)”
	Southern Blot	Homozygous transgene insertion at the targeted allele	Supplementary Fig. 1
Off-target nuclease analysis-	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	N/A	N/A
Specific pathogen-free status	Mycoplasma: *PCR*	Negative	Supplementary Fig. 5
Multilineage differentiation potential	*In vitro* differentiation: *Immunocytochemistry*	Differentiated cells show positive staining for ectoderm markers: Nestin and β3-Tubulin; mesoderm markers: Brachyury and Troponin T; endoderm markers: FoxA2 and Sox17	Fig. 1J
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

Table 2

Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Marker	Goat anti-NANOG	10 μg/ml (ICC)	R&D Systems Cat# AF1997, RRID: AB_355097
	Mouse anti-OCT3/4	1:250 (ICC)	Santa Cruz Biotechnology Cat# sc-5279, RRID: AB_628051
	Mouse anti-SOX2	1:250 (ICC)	Santa Cruz Biotechnology Cat# sc-365823, RRID: AB_10842165
Differentiation Marker	Mouse anti-Nestin	1:100 (ICC)	R&D Systems Cat# MAB1259, RRID: AB_2251304
	Rabbit anti-β3-Tubulin	1:300 (ICC)	Cell Signaling Technology Cat# 5568, RRID: AB_10694505
	Goat anti-Brachyury	10 μg/ml (ICC)	R&D Systems Cat# AF2085, RRID: AB_2200235
	Mouse anti-Troponin T	10 μg/ml (ICC)	R&D Systems Cat# MAB1874, RRID: AB_2206731
	Rabbit anti-FOXA2	1:400 (ICC)	Cell Signaling Technology Cat# 8186, RRID: AB_10891055
	Mouse anti-SOX17	1: 50 (ICC)	R&D Systems Cat# MAB1924, RRID: AB_2195646
Secondary Antibody	Donkey anti-Mouse IgG (Alexa Fluor 488 conjugated)	1:1000 (ICC)	Thermo Fisher Scientific Cat# A-21202, RRID: AB_141607
	Donkey anti-Goat IgG (Alexa Fluor 488 conjugated)	1:1000 (ICC)	Thermo Fisher Scientific Cat# A-11055, RRID: AB_2534102
	Donkey anti-Rabbit IgG (Alexa Fluor 488 conjugated)	1:1000 (ICC)	Thermo Fisher Scientific Cat# A-32790TR, RRID: AB_2866495
	Donkey anti-Mouse IgG (Alexa Fluor 594 conjugated)	1:1000 (ICC)	Thermo Fisher Scientific Cat# A-21203, RRID: AB_141633
Nuclear stain	DAPI	600nM	Sigma Aldrich Cat# D9542
Site-specific nuclease			
Nuclease information	CRISPR/Cas9		
Delivery method	Electroporation		300 V / 500 μF
Selection/enrichment strategy	Puromycin selection for gRNA-containing PX459 transfection followed by manual single-cell colony picking		Puromycin 0.5 μg/ml

Primers and Oligonucleotides used in this study		
	Target	**Forward/Reverse primer (5’ −3’)**
Pluripotency Markers (qPCR)	*NANOG* (116bp)	TTTGTGGGCCTGAAGAAAAC T/AGGGCTGTCCTGAATAAGCAG
	OCT4 (123bp)	AACCTGGAGTTTGTGCCAG GGTTT/TGAACTTCACCTTCCCTCCAACCA
	SOX2 (150bp)	AGAAGAGGAGAGAGAAA GAAAGGGAGAGA/GAGAGAG GCAAACTGGAATCAGGATCAAA
House-Keeping Genes (qPCR)	*GAPDH* (102bp)	CCACTCCTCCACCTTTGAC/ACCCTGTTGCTGTAGCCA

Primers and Oligonucleotides used in this study

	Target	Forward/Reverse primer (5’ −3’)
Target Genes (qPCR)	*mEos4b* (119bp)	TTGACTTTCGAAGACGGG GG/CCCGCCAATGGTCCAGTTAT
	CTR1-mEos4b junction (769bp)	TCAATACAGCTGGAGAAAT GGC/CCCGCCAATGGTCCAGTTAT
Genotyping	*SLC31A1* intron 4 to exon 5 (Wild type: 1718bp; transgene insertion: 2444bp)	AGGTCATGAGCAGGC CAAAGGA /ATCTCAT GGGTGGCTTCTCCTTA
Southern Blot Probe Synthesis	*SLC31A1* intron 4 (227bp)	AGGTCATGAGCAGGCCAAAGGA /G AGGACAGGGCTGACTAGGTG
Potential random integration-detecting PCRs	pSpCas9(BB)-2A-Puro (PX459) (1883bp)	CGGACAGGTATCCGGTAAGC / A GAAGCTGTCGTCCACCTTG
	pMiniT 2.0 backbone (1868bp)	GGGAACCGGAGCTGAAT GAA / CTTCTATAGTGTC ACCTAAATGCGT
gRNA oligonucleotide	*SLC31A1* exon 5	GCCATAGAGTTTGATGTCAA
Genomic target sequence(s)	*SLC31A1* exon 5	Hg38 reference genome: chr9, 113260272– 113264492 PAM: TGG Cut locus: chr9: −113260473

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Primers and Oligonucleotides used in this study

	Target	Forward/Reverse primer (5' −3')
DNA fragment used as the template for HDR-mediated site-directed mutagenesis	*SLC31A1* intron 4 to exon 5-mEos4b-FLAG tag-*SLC31A1* exon 5	GGGCCCAGAAGGATTGTGAC CTACCTAAGGTGGCACAGGGGATGAGTAGCCAGGTCTCCT TATCCCTAATTAAGTAATTTCTTCTATACTACCCAACCCTCA ATTCTATTTTTTTTTCTTTTTTTCTTTCTTTTTTTTTTTTTTTC TGGAGACAGGGTCTCCCTCTATTGCCCAGGCTGAAGTGCAG TGGCACTATCTTGGCTCACTGCAATCTCTGCCTTCCAGACTCAAG TGATCCTCCCACCTCAGCCTCCTAAGTAGCTGGAACTACAGGCATG AGCCACCACACCCAGCTAATTTTTTTTATTTTTTGTAGAGACAAG GTTTCGCCATGTTGCCCAGGCTGGTCTGAAACTCCTGAGCTCAA GCAATCAGCCCACCTCGGCCTCCCAAAGTGCTGGGATTAAAG GCATTAGCCACCACCCCCAGCCTCTGTTTCTGTTTTTGTAGA ATGTAAACTTTGCAAAGGTGGGAAGCCTAAAAATATTCGGGC AGATGAGCGTGAGAGGGTTTACGGCTTCCCTTTCAGGATCTA AGGTGGAGGTAAGTCGGGATTACTCCTCGTCCTTTTCTCCTC CTCCGGAAAAGGGTTCCTTCTACTGGAGGAACCACAGCCATA TCTGGCCAATGTTTATAAGCCTGACAGCCCTGTGCTGTTTCAT TTCCATGGACCAATCAAAGAACATTCAAGTACCCATGAGTT GCCAGAGTGGCTGTCCAGTTCCAGCTGAGCTCATGGCAAG AACTCTTCCCTCTTTCTCCTGATCTGCAGAACTCCTAGGAGT CCCTGATTGTTGTGTCCCTGTTTACTCTCCAGGCAACAGATG CTGAGCTTTCCTCACCTCCTGCAAACAGTGCTGCACATCATC CAGGTGGTCATAAGCTACTTCCTCATGCTCATCTTCATGACC TACAACGGGTACCTCTGCATTGCAGTAGCAGCAGGGGCAGG TACAGGATACTTCCTCTTCAGCTGGAAGAAGGCAGTGGTAG TGGATGGATCCATGGTGAGTGCGATTAAGCCAGACATGAGG ATCAAACTCCGTATGGAAGGCAACGTAAACGGGCACCACTTT GTGATCGACGGAGATGGTACAGGCAAGCCTTATGAGGGAAAA CAGACCATGGATCTTGAAGTCAAAGAGGGCGGACCTCTGCC TTTTGCCTTTGATATCCTGACCACTGCATTCCATTACGGCAA CAGGGTATTCGTGAAATATCCAGACAACATACAAGACTAT TTTAAGCAGTCGTTTCCTAAGGGGTATTCGTGGGAACGAAGC TTGACTTTCGAAGACGGGGGCATTTGCAATGCCAGAAACGAC ATAACAATGGAAGGGGACACTTTCTATAATAAAGTTCGATTTT ATGGTACCAACTTTCCCGCCAATGGTCCAGTTATGCAGAAGA AGACGCTGAAATGGGAGCCCTCCACTGAGAAAATGTATGTGC GTGATGGAGTGCTGACGGGTGATATTGAGATGGCTTTGTT GCTTGAAGGAAATGCCCATTACCGATGTGACTTCAGAACT ACTTACAAAGCTAAGGAGAAGGGTGTCAAGTTACCAGGCG CCCACTTTGTGGACCACGCCATTGAGATTTTAAGCCATGACA AAGATTACAACAAGGTTAAGCTGTATGAGCATGCTGTTGCTC ATTCTGGATTGCCTGACAATGCCAGACGAATCCTGGATTA CAAGGATGACGACGATAAGGTTTCGGATATCACAGAGCA TTGTCATTGACATCAAACTCTATGGCGTGACCTTATCGAT TGCAGTGGGAAGTTGTTGAAGACTTGAAGACGTGATTCCTG CTCCAATCATCCCTTCTTGCTCCTCTTTGTGCACGTACAC ACACACACACACACACACACACACACACACCCCTGCTCAACA GAGGTTTAGTTTACAGTCTCTGAACTAAAGTAGTAACCTC CCAAATTGTTTTTTCTAATAAGCTGAGATTCCCATTTCTC TTAAGGAGAAGCCACCCATGAGATGTCTTTTCCTTCTCC ATCATCTTAGAGCCAAGTTATATGTTCTTGTCTAATCCA TGTAGCTTTTTGTTCAATGACTTGATCATCTGCTTCCTTT TTGAATTTTTAACAGATAGTAAGTAAATTTGGTGGTTTTTT CCCCTGGGTCAGTGATGGAAAGGGGTTAACTTCAGCCAGGA TTGATGGCAGCTGAGGGAAATTCTT