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Creation of CD63-deficient HEK293 cell lines using a polycistronic CAS9/EGFP/HSVtk/PuroR expression vector

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Disciplines
Cell Biology
Cell Biology
Genetics

Keywords
CD63
CRISPR
Endosome
Lysosome

Type of Observation
Standalone

Type of Link
Standard Data

Submitted Mar 23, 2016
Published Apr 21, 2016

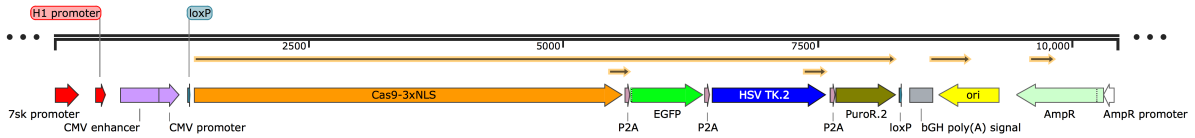
Triple Blind Peer Review
The handling editor, the reviewers, and the authors are all blinded during the review process.

Full Open Access
Supported by the Velux Foundation, the University of Zurich, and the EPFL School of Life Sciences.

Abstract
CD63 is an integral membrane protein that is present at the plasma membrane but highly enriched in endosomes and lysosomes. CD63 has been implicated in several cell biological processes and is a common constituent of small, secreted vesicles (diameter of ~30–300 nm) known as exosomes. Here, we report a polycistronic Cas9/EGFP/HSVtk/PuroR expression vector that is designed to allow for (i) guide RNA (gRNA)-mediated mutation of a specific gene, (ii) puromycin selection of Cas9-expressing cells, (iii) Cre-mediated excision of the Cas9-encoding ORF, and (iv) ganciclovir killing of Cas9-expressing cells. It was further modified to express gRNAs that target the human CD63 gene. This plasmid was transfected into HEK293 cells followed by the expansion of single cell clones (SCCs). Immunoblot analysis revealed that multiple SCCs lacked detectable levels of CD63 expression. Four such CD63-deficient SCCs were chosen for further analysis. In each of three or more trials, these SCCs were found to lack CD63 protein expression. Furthermore, these four CD63-deficient cell lines all had DNA insertions or deletions in exon 2 of this gene that shifted the reading frame, precluded the expression more than the first 44–46 amino acids of CD63, and are predicted to induce nonsense-mediated decay of the CD63 mRNA.

Introduction
Like all tetraspanins [1], CD63 is co-translationally translocated into the endoplasmic reticulum as a multipass, four transmembrane-domain protein with its N- and C-terminus oriented in the cytoplasm. CD63 is subsequently trafficked to the plasma membrane, endosomes, multivesicular bodies (MVBs), and the lysosomes that they form, but is particularly enriched in endosomes, MVBs, and lysosomes [2]. CD63 has been implicated in a number of cell biological processes, including endo/lysosomal sorting of its partner proteins [3] and the biogenesis of multivesicular bodies [4]. CD63 is also a common constituent of small secreted vesicles known as exosomes [5].
To better understand the role(s) of CD63 in human cell biology, we created a Cas9-expressing plasmid designed to introduce mutations in the CD63 gene due to its simultaneous expression of CD63-directed guide RNAs [6] [7]. This Cas9/CRISPR vector co-expresses Cas9-3xNLS in a polycistronic ORF together with EGFP, Herpes Simplex Virus thymidine kinase, and the puromycin-resistance enzyme, each separated by viral 2a peptides. This plasmid is designed for the Cas9/gRNA-mediated mutation of the CD63 gene, but also for the positive selection of Cas9 ORF-expressing cells, Cre recombinase-mediated deletion of the Cas9 ORF, and the positive ganciclovir selection of cells that no longer express this ORF. This plasmid successfully used to induce mutations in the human CD63 gene in HEK293 cells, and we report the nucleotide sequences of 4 CD63-deficient HEK293 cell lines.

Objective
The objectives were to (1) generate a Cas9/gRNA expression plasmid that can be used to generate CD63-deficient human cells and (2) use this plasmid to generate CD63-deficient HEK293 cell lines.



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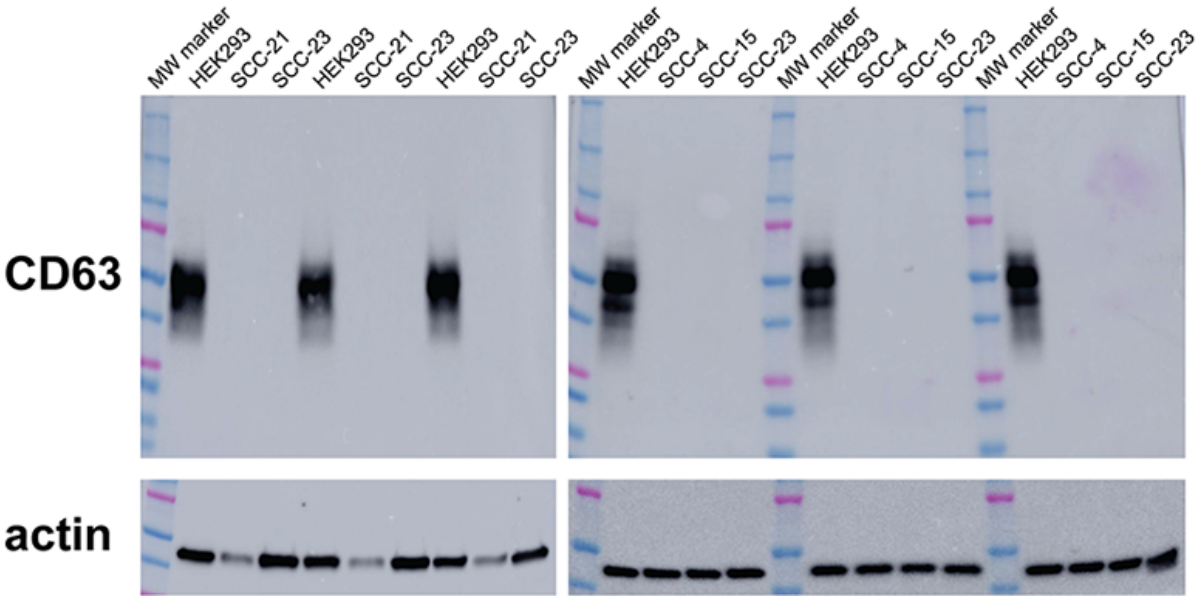

b

Database sequence: 5′-CAGACCATAATCCAGGGGGCTACCCCTGGCTCTCTGTTG-3′
3′-GTCTGGTATTAGGTCCCCCGATGGGGACCGAGAGACAAC-5′

gDNA source		DNA sequence	Resulting ORF
HEK293		5′-CAGACCATAATCCAGGGGGCTACCCCTGGCTCTCTGTTG-3′	CD63(aa1-238)
SCC_{CD63ko}-4			
allele 1	1174.2	5′-CAGACCATAATCCAGGGG[113bp]CTACCCCTGGCTCTCTGTTG-3′	CD63(aa1-46) + 16aa junk
allele 2	1174.3	5′-CAGACCATAATCCAGGGG-CTACCCCTGGCTCTCTGTTG-3′	CD63(aa1-46) + 36aa junk
SCC_{CD63ko}-15			
allele 1	1175.2	5′-CAGACCATAATCCAGGG--CTACCCCTGGCTCTCTGTTG-3′	CD63(aa1-46) + 60aa junk
allele 2	1175.3	5′-CAGACCATAATCCAGGG--CTACCCCTGGCTCTCTGTTG-3′	CD63(aa1-46) + 60aa junk
SCC_{CD63ko}-21			
allele 1	1121.1	5′-CAGACCATAATCCAGGGAGGCTACCCCTGGCTCTCTGTTG-3′	CD63(aa1-46) + 61aa junk
allele 2	1139.1	5′-CAGACCATAATCC----GGCTACCCCTGGCTCTCTGTTG-3′	CD63(aa1-44) + 37aa junk
SCC_{CD63ko}-23			
allele 1	1122.1	5′-CAGACCATAATCCAGG----------CTCTCTGTTG-3′	CD63(aa1-45) + 33aa junk
allele 2	1122.8	5′-CAGACCATAAT----------ACCCCTGGCTCTCTGTTG-3′	CD63(aa1-44) + 35aa junk

c

Figure Legend

Figure 1. Inducing CD63 mutations in HEK293 cells.

(A) Plasmid map of pFF4. The two CD63-targeting guide RNAs are designed to be expressed from the PolIII promoters of the human 7sk gene and H1 gene, marked in thick red arrows. The CMV enhancer/promoter (thick purple arrow) drives expression of a single long ORF (thin yellow/black arrow) that encodes Cas9-3xNLS-p2a(18) (thick orange arrow), EGFP-p2a(18) (thick bright green arrow), HSVtk-p2a (thick blue arrow) and Puro (thick olive green arrow). This ORF is flanked by loxP sites and followed by

the polyadenylylation signal from bovine growth hormone gene (thick gray box). The sequence of pFF4 is provided as supplementary data.
(B) Immunoblot analysis of whole-cell protein lysates isolated from multiple independently grown and lysed cultures (3 each) of HEK293 cells and of SCCs CD63ko-21, and -23 cells (left panels), and of HEK293 cells and of SCCs CD63ko-4, -15, and -23 cells (right panels) blotted with antibodies specific for (upper panels) CD63 and (lower panels) beta-actin. Protein size markers are 250 kDa, 150 kDa, 100 kDa, 75 kDa (red), 50 kDa, 37 kDa, 25 kDa (red), 20 kDa, 15 kDa, and 10 kDa. CD63 has a predicted molecular mass of 26 kDa but runs on non-reducing SDS-PAGE gels as a mixture of products of ~35–55 kDa due to heterogeneous glycosylation. In contrast, actin migrates at its predicted molecular mass of 42 kDa.
(C) Summary of CD63 exon 2 sequences in HEK293 and CD63-deficient SCCs. Both strands of the human CD63 gene sequence in the vicinity of the gRNA target site are shown at the top, with the gRNA target sequence on the non-coding strand underlined and the PAM domain in bold. Sequences obtained by amplifying exon 2 from HEK293 cells and 4 SCCs are shown below the database sequence, along with the possible encoded protein.

Plasmids

All plasmids were introduced into DH10B cells by electroporation, maintained in this strain, amplified by culturing the cognate strain, and purified from bacterial lysates using Qiagen mini-prep and midi-prep plasmid purification kits. Automated Sanger DNA sequencing was performed using custom primers and dye-linked dideoxynucleotides (Applied Biosystems). DNA sequences were maintained as SnapGene files, and this software was used to create plasmid maps and to perform DNA sequence alignments. The plasmid pFF4 was assembled from a combination of sources, including in vitro synthesized EGFP, HSV TK.2, and Puro.2 genes, in vitro synthesized 7sk promoter, H1 promoter, and guide RNA sequence, on a backbone derived from pcDNA3. Its structure was confirmed by restriction enzyme mapping. In addition, most of the pFF4 structure was confirmed by DNA sequence analysis, with the exception of the SpCas9 ORF [6] and the PciI-SgrDI fragment from pcDNA3 (Invitrogen) containing its bacterial origin of replication and the ampicillin resistance gene. The sequence of the pFF4 plasmid is submitted as raw data. Modification of pFF4 to express different guide RNAs can be accomplished by synthesizing ~350 bp long fragments containing a guide RNA, the H1 promoter, and a second guide RNA and inserting the fragment downstream of the Acc65i site (located near the 3' end of the 7sk promoter) and the MluI site (located upstream of the CMV promoter). 2 sequence files for this plasmid are provided in the supplementary data, a TXT file and a Snapgene file. We also created a large number of plasmids by first amplifying exon 2 containing and exon 4 containing DNA fragments from various gDNA preparations, and then cloning them between the SgrD1 and NotI sites of pCJM by Gibson cloning (New England Biolabs). Selected subclones from each cloning reaction were subjected to DNA extraction, restriction enzyme mapping, and DNA sequence analysis as outlined above.

HEK293 cell culture

HEK293 cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM (high glucose; Gibco/BRL) supplemented with 10% fetal calf serum (FCS; ThermoFisher) in a tissue culture incubator equilibrated to 5% CO_2 and maintained at 37°C. Selection of puromycin-resistant cells was accomplished by adding puromycin to growth medium to a final concentration of 3 µg/ml and maintaining cells in this medium for the next 7 days. Transfection was by lipofection using Lipofectamine2000 reagent (ThermoFisher). In brief, 5 µg plasmid DNA was diluted into 0.3 ml of Opti-Mem medium (Gibco/BRL), while 15 µl of Lipofectoamine2000 was diluted into a separate 0.3 ml of Opti-Mem medium, and both mixtures were incubated separately for 5 min. The two mixtures were mixed together and incubated for a further 15 min. Next, growth medium was removed from a T-25 flask (Sarstedt) containing HEK293 cells (at ~70% confluency), the cells were washed with 5 ml of 37°C Opti-Mem medium, all liquid was removed, and the 0.6 ml mixture of DNA, Lipofectamine2000 and Opti-Mem was added to the flask. After gentle rocking to distribute the mixture across the entire flask surface, the flask was placed in a tissue culture incubator. 15 min later the flask was removed, the

DNA/Lipofectamine2000 solution was removed by aspiration, the cells were fed with 5 ml DMEM/FCS and returned to the tissue culture incubator. To obtain single cell clones (SCCs), puromycin-selected cells were trypsinized 5 days after transfection and suspended at a concentration of 1 cell per each 200 µl of DMEM/10% FCS. 200 µl of this solution was added to each well of three 96 well tissue culture plates. Each well was scanned visually under a low power light microscope within 1 h, and then every 2–3 days thereafter. Any wells that contained evidence of receiving more than a single cell, or of having more than a single growing clone, were eliminated from further analysis. The remaining single cell clones were expanded continually for the next 3–4 weeks to obtain ~107 cells, which were then used to generate frozen stocks of each SCC, as well as to create protein lysates and genomic DNA preparations.

Immunofluorescence

For immunofluorescence microscopy, cells were released from the tissue culture flask by trypsinization followed by re-plating on tissue culture dishes to which sterile (autoclaved) borosilicate coverglasses had been added. Cells were grown an additional 8 h (or more) on the coverglasses before processing for fluorescence microscopy. For examination of the cells, cells were first fixed by placing cell containing coverglasses in a solution of 3.7% formaldehyde (in Dulbecco's modified phosphate-buffered saline (DPBS), pH 7.4) for 15 min. This was followed by permeabilization of the cells in 1% Triton X-100/DPBS for 5 min, and then washed twice in DPBS. To label CD63 in these cells we diluted a mouse monoclonal antibody directed specifically against human CD63 (clone E-12, #sc-365604, Santa Cruz Biotechnology) into DPBS containing 1% bovine serum albumin (BSA), and placed 40 µl of this antibody mixture on a small sheet of parafilm (Bemis). Next, a coverglass was placed cell-side down on this drop of antibody and incubated for 15 min. The coverglasses were then washed 5 times with 1 ml DPBS, and then incubated with a solution containing TexasRed-labeled goat anti-mouse IgG antibody, again diluted into DPBS/1% BSA. After 15 min incubation, the coverglasses were washed 5 times in DPBS, and then mounted on glass slides on a drop (8 µl) of mounting solution (90% glycerol, 100 mM Tris pH 8.5, 0.01% *para*-phenylenediamine). After removal of excess mounting solution, the cells were examined using an Olympus BH-2 fluorescence microscope equipped with a 60x oil lens and the percentage of CD63-positive cells was determined by visual examination of the samples.

Immunoblots

To obtain whole-cell lysates, a confluent 10 cm dish of cells (either HEK293 cells or an SCC) was lysed in 2 mls 2x SDS-PAGE sample buffer lacking reducing agent (the antigen recognized by our anti-CD63 antibody is dependent upon its disulfide bridges, and is destroyed by reducing agent). The 2 ml was then boiled for 15 min, and then spun at 13,000 x g to generate a clarified lysate. 15 µl of this lysate was then loaded in a lane of an acrylamide gel (BioRad) alongside molecular weight markers and other samples. The protein lysates were then separated by SDS-PAGE at 150 V for 90 min. Proteins were then transferred from the gels to immobilon-P membrane, PVDF, 0.45 µm (Millipore), and these were processed for immunoblot as described [19] using a mouse monoclonal antibody specific for human CD63 (clone E-12, #sc-365604, Santa Cruz Biotechnology) or a rabbit polyclonal anti-actin antibody (ab8227, Abcam), followed by HRP-linked secondary antibodies directed against either mouse IgG or rabbit IgG, respectively. HRP was then detected using ECL reagents and visualized using an Amersham Imager 600 (GE Healthcare Life Sciences). Figures were assembled using Adobe Illustrator.

Genomic DNA analysis

To prepare genomic DNAs (gDNAs), ~1×106 cells were lysed and processed using the TRIzol reagent (Life Technologies). Gene-specific primers flanking exon 2 and exon 4 of the CD63 gene were synthesized (IDT) and used to amplify the corresponding of genomic DNA from HEK293 gDNA and from each SCC gDNA preparation. Each PCR product was analyzed by agarose gel electrophoresis with molecular weight markers loaded in parallel, to determine the relative sizes of PCR products. To determine the sequence of each PCR product in each sample, the PCR products were cloned into pCJM using the Gibson cloning kit and used to transform DH10 cells to ampicillin resistance. Between 4 and 10 clones from each cloning reaction were grown and plasmids were

extracted for subsequent restriction enzyme mapping and DNA sequence analysis. The resulting DNA sequences were aligned to the CD63 genomic DNA sequence using SnapGene software, revealing the precise nature of the mutations in each cell line.

Results & Discussion

Creating a system for positive and negative selection of Cas9

Cas9-mediated mutation of human genes is well established [6] [7], and improvements in Cas9-mediated mutagenesis continue to be developed [8] [9]. One route to improved creation of mutant cell lines is to use an expression system that allows one to enrich for cells that express high levels of Cas9, yet subsequently delete the introduced Cas9 gene and select for cells that lack its expression.

Toward this end, we generated a new Cas9 expression vector, pFF4 (Fig. 1A). This plasmid contains a single PolII transcription unit controlled by the cytomegalovirus (CMV) early enhancer/promoter. The CMV promoter is followed by a pair of loxP sites that flank a single yet polycistronic open reading frame (ORF). This polycistronic ORF encodes, in succession, (1) Cas9-3xNLS, a form of the SpCas9 protein [6], containing three copies of the SV40 nuclear localization signal, (2) the 19 amino acid-long porcine teschovirus 2a peptide (p2a; ATNFSLLKQAGDVEENPGP [10]), (3) enhanced green fluorescent protein (EGFP [11]), (4) another copy of the p2a peptide, (5) the herpes simplex virus thymidine kinase (HSVtk [12]), (6) a third copy of the p2a peptide, and (7) Puro, a puromycin-resistance protein [13]. Downstream of the 3' loxP site is a polyadenylylation signal from the bovine growth hormone gene. As was done previously for SpCas9 and EGFP, the HSV TK.2 and PuroR.2 ORFs are distinct from the original HSV TK and PuroR gene sequences, respectively, in that the HSV TK.2 and PuroR.2 sequences were designed for optimal human codon usage and synthesized *in vitro*.

Translation of the encoded polycistronic mRNA is predicted to yield 4 separate polypeptides due to the action of the three copies of p2a peptide sequence, which inhibits peptide bond formation between the penultimate glycine and terminal proline of the peptide. The four predicted protein products are, in succession: (i) Cas9-3xNLS-p2a(18), which should carry out Cas9 functions, be more efficiently imported into the nucleus due to its inclusion of three copies of the nuclear localization signal from SV40T antigen (data not shown), carry the first 18 amino acids of the p2a peptide (ATNFSLLKQAGDVEENPG-COOH) at its C-terminus; (ii) EGFP-p2a(18), which should confer green fluorescence on expressing cells and consist of EGFP with an additional proline at its N-terminus and the p2a remnant peptide (ATNFSLLKQAGDVEENPGCOOH) at its C-terminus; (iii) HSVtk-p2a(18), which should confer ganciclovir sensitivity on puromycin-resistant cells due to its (relatively) non-specific kinase activity, carry an extra proline at its N-terminus, and carry the p2a remnant peptide (ATNFSLLKQAGDVEENPGCOOH) at its C-terminus; and (iv) Puro, which should confer resistance to the antibiotic puromycin due to its puromycin N-acetyltransferase activity and have an extra proline at its N-terminus.

The pFF4 plasmid also contains two PolIII-transcribed genes. One consists of the human 7sk promoter [14] driving expression of a Cas9 guide RNA (gRNA) [6] [7] that targets exon 2 of the human CD63 gene, and one consists of the human H1 promoter [15] driving expression of a Cas9 guide RNA (gRNA) that targets exon 4 of the human CD63 gene.

Creation of CD63-deficient human cells

HEK293 cells were transfected with pFF4. 12 h later the cells were examined by immunofluorescence microscopy, which revealed that more than half the cells expressed detectable levels of EGFP fluorescence and that nearly all cells still expressed detectable levels of CD63. The cells were then diluted into normal growth medium containing puromycin and grown for an additional 5 days. An aliquot of the mixed pool of transfected cells were again examined by immunofluorescence microscopy and we now observed a smaller percentage of EGFP-positive cells but a higher percentage of cells that no longer expressed detectable levels of CD63. These results are consistent with the fact that transfected cells express higher transgene levels shortly after transfection, and that introduction of null mutations in a gene will not be reflected until previously synthesized mRNA and protein have to turn over before loss of the protein can be observed. On day 5 after transfection the cells were also diluted into growth medium lacking puromycin

and diluted at an average of 1 cell per well in 96 well plates to generate single cell clones (SCCs).

Protein lysates were prepared from 40 different SCCs and from the parental HEK293 cell line. Immunoblot analysis using antibodies specific for CD63 indicated that 9/40 SCCs lacked detectable levels of CD63 protein. However, this initial screen was uncontrolled, and we therefore performed a more detailed analysis of 4 of the candidate CD63-deficient SCCs. Specifically, we grew 3 or more separate cultures of each of the 4 SCCs and the parental HEK293 cell line (actually, 6 separate cultures in the cases of HEK293 and SCCCD63ko-23 cells), lysed the cells, and processed them for immunoblot using antibodies specific for (Fig. 1B, upper panels) CD63 and (Fig. 1B, lower panels) beta-actin. We failed to detect CD63 protein in the lysates from the SCCs SCCCD63ko-4, SCCCD63ko-15, and SCCCD63ko-21 in each of three trials, and for SCCCD63ko-23 in each of 6 trials. In contrast, we detected actin in all samples, even in the lysates that had been generated from the SCCCD63ko-21 cell line, which grows far more slowly than the parental HEK293 cells or the other SCCs.

To determine whether these four SCCs had mutations in the expected positions of either exon 2 or exon 4 of the CD63 gene, we performed exon-specific PCR on each genomic DNA (gDNA) preparation and (i) determined the size of the PCR products by agarose gel electrophoresis, and (ii) cloned the PCR products and determined the DNA sequence of the PCR products by sequencing the inserts in multiple plasmid subclones. We hoped to identify no more than two mutant alleles in each SCC, as CD63 is located on chromosome 12, whereas HEK293 cells are triploid for the X, tetraploid for chromosomes 17 and 22, yet diploid for much of the remaining genome [16]. Amplification of exon 2 from the four CD63-deficient SCCs yielded PCR products of varying size, some of which were of the same size as the fragment that we amplified from HEK293 gDNA, and some that were larger or smaller. All PCR products amplified from HEK293 cells had the WT sequence.

Amplification of the exon 2 containing fragment from SCCCD63ko-4 gDNA yielded two products, one that was close to WT size and another that was ~100 bp longer. Sequence analysis showed that the longer product had a 113 bp insertion at the position noted in the figure, whereas the normal-sized PCR product had a bp deletion at the noted position. PCR products amplified from SCCCD63ko-15 gDNA all had the same sequence, indicating that this mutation may have been generated on both alleles. The SCCCD63ko-21 exon 2 PCR products also appeared to be of WT size, but sequence analysis of subclones identified two classes of PCR products, one with a 1 bp insertion and another containing a 4 bp deletion. The SCCCD63ko-21 exon 2 PCR products appeared to be slightly smaller than WT, as did the inserts in the subclones that were generated using the PCR products. Sequence analysis was consistent with our gel electrophoresis results, as one set of subclones had a 13 bp deletion and the other had a 10 bp deletion. All of the mutations detected in these CD63-deficient cell lines are predicted to shift the CD63 reading frame, resulting in the potential expression of proteins that encode only the first 44–46 amino acids of CD63 followed by varying but short stretches of "junk" amino acids. Given that these mutations all introduce premature stop codons in exon 2 of this 7 exon-containing gene, they are also predicted to induce nonsense-mediated RNA decay of the CD63 transcript [17].

In contrast to what we observed at exon 2, the exon 4 PCR products amplified from control or SCC gDNAs displayed only the WT size and sequence, indicating that no mutations were introduced at this site. Control experiments in which we amplified and sequenced the exon 2 and exon 4 amplicons from HEK293 genomic DNA revealed that the sequences of these regions of the CD63 gene in HEK293 cells were identical to that reported in the human genome sequence and had the target sequences complementary to the gRNAs encoded by pFF4.

Conclusions

We conclude that transfection of human cells with pFF4 can induce mutations in exon 2 of the CD63 gene due to its expression of an exon 2-targeted gRNA from the human 7sk promoter. In addition, we conclude that we have successfully created HEK293 cell lines that lack expression of human CD63 due to the presence of inactivating mutations

in exon 2 of the CD63 gene. Thus, loss of CD63 does not have a gross defect in cell growth or viability, consistent with the prior observation that CD63-deficient mice and mouse cells are viable [18]. TheCD63-deficient HEK293 cell lines reported here should be useful for assessing the role(s) of CD63 in various cell biological processes, from MVB biogenesis to exosome secretion, and from signal transduction to endo/lysosomal protein trafficking.

Limitations

One limitation is that we were unable to identify a second mutation in SCCCD63ko-15, and therefore do not know for certain whether this mutation is present on one or both CD63 alleles in this particular clone. However, we did not detect any evidence for other PCR products when we amplified exon 2 from SCCCD63ko-15 gDNA, and we obtained the same sequence in each of five separate subclones of this PCR product. Another limitation is that we did not sequence the entire genome of the SCCs, and we can therefore not exclude the possibility that there may be additional Cas9/gRNA-induced mutations in these CD63-deficient HEK293 SCCs. In addition, we did not test whether Cre-mediated excision of the Cas9/EGFP/HSVtk/PuroR ORF and subsequent ganciclovir selection of deleted clones worked as predicted.

We think that these CD63-deficient cell lines will be useful for future studies of how CD63 contributes to human cell biology. We also hypothesize that expression of Cre in these cell lines will convert them from Cas9-expressing, ganciclovir-sensitive cells, to Cas9-deleted, ganciclovir-resistant cells.

Additional Information

Methods

Plasmids

All plasmids were introduced into DH10B cells by electroporation, maintained in this strain, amplified by culturing the cognate strain, and purified from bacterial lysates using Qiagen mini-prep and midi-prep plasmid purification kits. Automated Sanger DNA sequencing was performed using custom primers and dye-linked dideoxynucleotides (Applied Biosystems). DNA sequences were maintained as SnapGene files, and this software was used to create plasmid maps and to perform DNA sequence alignments. The plasmid pFF4 was assembled from a combination of sources, including in vitro synthesized EGFP, HSV TK.2, and Puro.2 genes, in vitro synthesized 7sk promoter, H1 promoter, and guide RNA sequence, on a backbone derived from pcDNA3. Its structure was confirmed by restriction enzyme mapping. In addition, most of the pFF4 structure was confirmed by DNA sequence analysis, with the exception of the SpCas9 ORF [6] and the PciI-SgrDI fragment from pcDNA3 (Invitrogen) containing its bacterial origin of replication and the ampicillin resistance gene. The sequence of the pFF4 plasmid is submitted as raw data. Modification of pFF4 to express different guide RNAs can be accomplished by synthesizing ~350 bp long fragments containing a guide RNA, the H1 promoter, and a second guide RNA and inserting the fragment downstream of the Acc65i site (located near the 3' end of the 7sk promoter) and the MluI site (located upstream of the CMV promoter). 2 sequence files for this plasmid are provided in the supplementary data, a TXT file and a Snapgene file. We also created a large number of plasmids by first amplifying exon 2 containing and exon 4 containing DNA fragments from various gDNA preparations, and then cloning them between the SgrD1 and NotI sites of pCJM by Gibson cloning (New England Biolabs). Selected subclones from each cloning reaction were subjected to DNA extraction, restriction enzyme mapping, and DNA sequence analysis as outlined above.

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Immunoblots

To obtain whole-cell lysates, a confluent 10 cm dish of cells (either HEK293 cells or an SCC) was lysed in 2 mls 2x SDS-PAGE sample buffer lacking reducing agent (the antigen recognized by our anti-CD63 antibody is dependent upon its disulfide bridges, and is destroyed by reducing agent). The 2 ml was then boiled for 15 min, and then spun at 13,000 x g to generate a clarified lysate. 15 µl of this lysate was then loaded in a lane of an acrylamide gel (BioRad) alongside molecular weight markers and other samples. The protein lysates were then separated by SDS-PAGE at 150 V for 90 min. Proteins were then transferred from the gels to immobilon-P membrane, PVDF, 0.45 µm (Millipore), and these were processed for immunoblot as described [19] using a mouse monoclonal antibody specific for human CD63 (clone E-12, #sc-365604, Santa Cruz Biotechnology) or a rabbit polyclonal anti-actin antibody (ab8227, Abcam), followed by HRP-linked secondary antibodies directed against either mouse IgG or rabbit IgG, respectively. HRP was then detected using ECL reagents and visualized using an Amersham Imager 600 (GE Healthcare Life Sciences). Figures were assembled using Adobe Illustrator.

Genomic DNA analysis

To prepare genomic DNAs (gDNAs), ~1×106 cells were lysed and processed using the TRIzol reagent (Life Technologies). Gene-specific primers flanking exon 2 and exon 4 of the CD63 gene were synthesized (IDT) and used to amplify the corresponding of genomic DNA from HEK293 gDNA and from each SCC gDNA preparation. Each PCR product was analyzed by agarose gel electrophoresis with molecular weight markers loaded in parallel, to determine the relative sizes of PCR products. To determine the sequence of each PCR product in each sample, the PCR products were cloned into pCJM using the Gibson cloning kit and used to transform DH10 cells to ampicillin resistance. Between 4 and 10 clones from each cloning reaction were grown and plasmids were extracted for subsequent restriction enzyme mapping and DNA sequence analysis. The resulting DNA sequences were aligned to the CD63 genomic DNA sequence using SnapGene software, revealing the precise nature of the mutations in each cell line.

Supplementary Material

Please see https://sciencematters.io/articles/201604000004.

Funding Statement

This work was funded by support from the NIH (1U19CA179563).

Acknowledgements

We thank Michael Wolfgang for generous gift of the SpCas9 gene and for advice and discussion throughout the course of our studies. We also thank Michael Caterina for his suggestion to incorporate HSVtk and negative ganciclovir selection into our strategy.

Ethics Statement

All work was performed according to Johns Hopkins University regulations regarding use of recombinant DNA and human cell lines.

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