CRISPR Stable Knockout Cell Line Generation (Cat. No. C208)

Case Study: Using CRISPR to develop a biallelic LIF Knockout in Mouse Colon Carcinoma Cells

Reference

Kandarian SC, Nosacka RL, Delitto AE, Judge AR, Judge SM, Ganey JD, Moreira JD, Jackman RW. Tumour-derived leukaemia inhibitory factor is a major driver of cancer cachexia and morbidity in C26 tumour-bearing mice. J Cachexia Sarcopenia Muscle. 2018 Sep 30. doi: 10.1002/jcsm.12346.

Summary

- LIF locus in a Mouse Colon Carcinoma Cell Line, was knocked out using CRISPR targeted genome editing.
- Surveyor assay and sequencing results showed genome editing.
- After monoclonal selection biallelic knockout was confirmed by sequencing.

Phase 1: Cas9 and sgRNA Delivery

- Three sgRNA were designed against mouse LIF locus (Mus musculus, NM_008501). Software analysis was performed to ensure the sgRNA had no predicted off targets binding sites. The selected sgRNA design was then cloned into the pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro All-in-One lentivector **(Figure 1)**.
- Recombinant Lentiviruses were packaged using **abm**'s second generation Lentiviral packaging system. A multiplicity of infection (MOI) of 5 was used to transduce the cells.

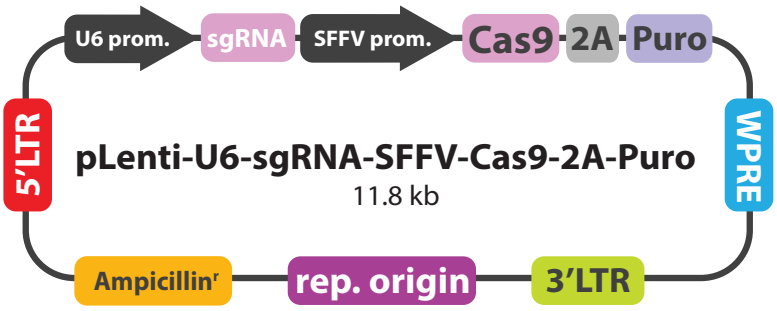

Figure 1 *pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro lentivector is an all-in-one vector for co-expressing sgRNA and Cas9 in mammalian cells. Expression of sgRNA is driven by the U6 promoter, a strong constitutive Pol III promoter. An SFFV promoter drives expression of the Cas9-2A-Puro cassette. By using the Cas9-2A-Puro cassette, cells can be directly screened for expression of Cas9, as they will be resistant to Puromycin.*

Phase 2: First Round of Colony Screening for Edited Clones

- Cell colonies are isolated after puromycin selection. Genomic DNA was extracted and the surveyor assay was performed to confirm genomic editing of the LIF locus.
- A single band in a surveyor assay at the wild-type (WT) size indicates no editing has occurred; two smaller bands (that sum to the length of the WT) indicate editing has taken place.
- The surveyor assay **(Figure 2)** indicated that Colony 3 and 6 were edited; colony 2 was not edited; and colony 1 was inconclusive.

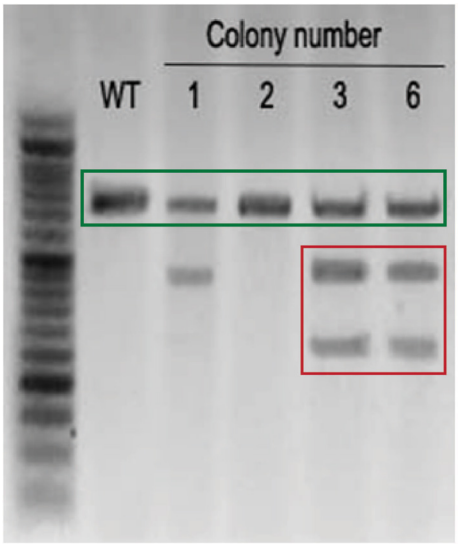

Figure 2 *The surveyor assay indicated that Colony 3 and 6 were edited; colony 2 was not edited; and colony 1 was inconclusive.*

Phase 3: Sequence Analysis of the Edited Colonies

- PCR products from Colonies 3 and 6 were further analyzed via Sanger Sequencing to determine the nature of the knockout **(Figure 3)**.

- For colony 3 only one mutant sequence was detected, indicating that these cells are likely only heterozyotic knockouts. In colony 6 two different mutant sequences were detected.

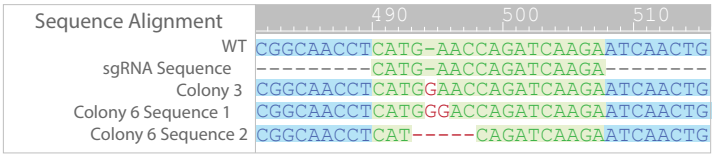

Figure 3 *For colony 3 only one mutant sequence was detected, indicating that these cells are likely only heterozyotic knockouts. In colony 6 two different mutant sequences were detected.*

Phase 4: Second Round of Selection for Monoclonal Biallelic Knockout Clones

- Colony 6 was serial diluted into 96 well plates for monoclonal selection. Genomic DNA was extracted from these clones (i.e. 6a, 6b..), PCR amplified, cloned and sequenced.

- Of the colony 6 clones, sequencing showed that only clone 6a had a frameshift mutation in both alleles **(Figure 4)**. A frameshift mutation disrupts the open reading frame, resulting in nonsense mediated decay of mRNA transcript.

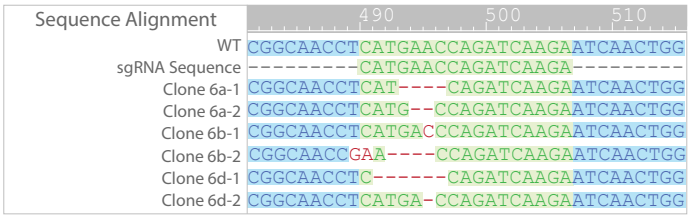

Figure 4 *Clones 6a, 6b and 6d all showed biallelic editing. Only clone 6a had frame shift mutations in both alleles. No WT sequences were detected in all subclones.*

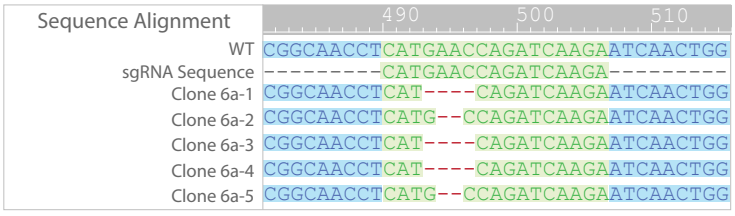

Figure 5 *Further sequencing of 6a confirmed biallelic knock-out. No WT sequences were detected.*

- Further sequencing of clone 6a confirmed that only two mutant alleles were present, the 2 bp and 4 bp deletions, and that no WT or other mutations were detected **(Figure 5)**.

Phase 4: Confirmation of Knockout by Next Generation Amplicon Sequencing

- With next generation sequencing hundreds of thousands of alleles can be sequenced at once, resulting in a more robust dataset. By contrast Sanger sequencing is only feasible for 1-100 clones and therefore it can miss a large proportion of the population.

- Next generation sequencing was performed at each stage of selection to evaluate knockout **(Figure 6)**. Before editing, only WT sequences were observed. After the first round of selection colony 6 showed a mixture of edited (70%) and WT (30%) sequences. Finally after monoclonal selection, clone 6a showed only edited sequences with no WT alleles present.

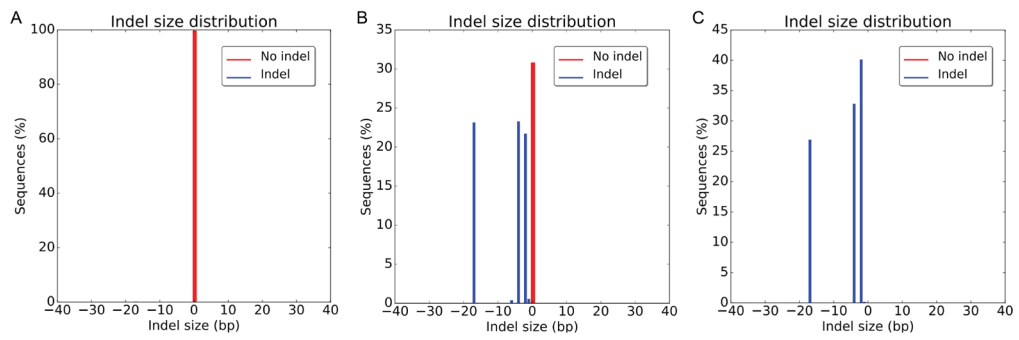

Figure 5 *Next Generation Sequencing for CRISPR Knock-out screening. A) Before knock-out only WT sequences are detected. B) After Cas9 and sgRNA delivery, the first round of selection shows a mixed distribution of indel and WT sequences. C) After the second round of selection only knock-outs remain.*

CRISPR Stable Knock-in Cell Line Generation (Cat. No. C408)

Case Study: Using CRISPR to Knock-in Red Fluorescent Protein (RFP) gene into Human Embryonic Kidney Cells at the AAVS1 Safe Harbour Site

Summary

- An expression cassette containing RFP and puromycin resistance genes (pAAVS1-RFP-DNR) was knocked into the AAVS1 Safe-harbor site in HEK293 cells using CRISPR targeted genome editing via the HDR pathway. Gene insertion at a Safe-harbour site allows stable gene expression without any adverse effects on the fitness of the engineered cells.
- Genomic PCR confirmed Knocked–in RFP integration at AAVS1 Safe-harbor locus.
- RFP expression was confirmed in cells by fluorescence microscopy.

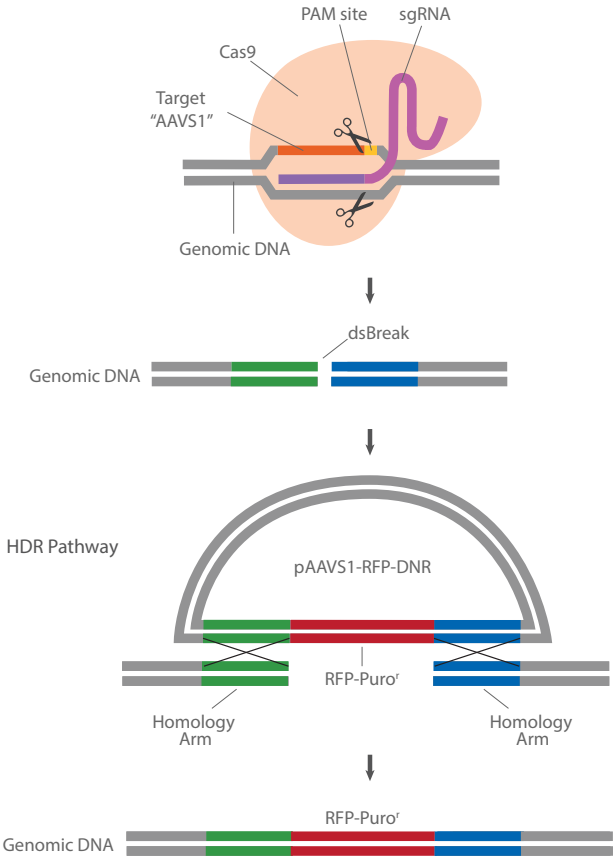

Figure 1 *CRISPR Knock-in requires expression of Cas9 and sgRNA to produce a double-stranded break. The repair template, shown here as pAAVS1-RFP-DNR, is used by the cell to repair the break using homologous recombination. The desired gene and selection marker (RFP and puromycin) included between the homology arms on the repair template will be integrated into the genome.*

Phase 1: Construction and Delivery of sgRNA, Cas9 and Repair Template

- An sgRNA was designed against the human AAVS1 Safe-harbor locus
- Software analysis was performed to ensure the sgRNA had no predicted off target binding sites. The selected sgRNA design, along with the CMV-promoter driven Cas9 gene, was cloned into pCas-Guide to make pCas-Guide-AAVS1 **(Figure 2)**.
- The pAAVS1-RFP-DNR donor plasmid was designed to contain the RFP-puromycin expression cassette, flanked on either side by homology arms of 600 bp **(Figure 2)**.
- HEK293 cells were co-transfected with both plasmids using DNAfectin transfection reagent.

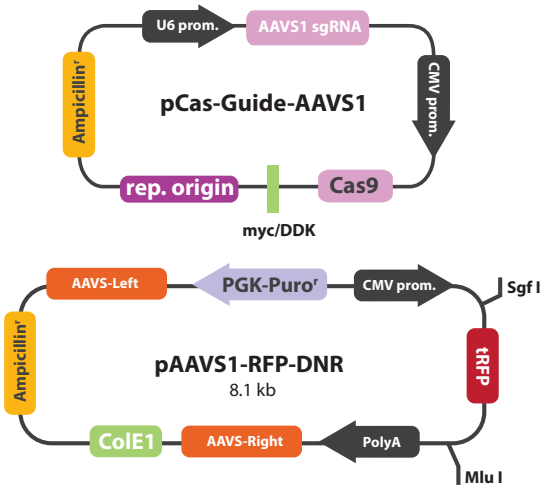

Figure 2 *Vector maps of pCas-Guide-AAVS1 and pAAVS1-RFP-DNR. pCas-Guide-AAVS1 is an all-in-one vector for co-expression of sgRNA and Cas9 in mammalian cells. Expression of sgRNA is driven by the U6 promoter, a strong constitutive Pol III promoter; while a CMV promoter drives the expression of the Cas9 enzyme. pAAVS1-RFP-DNR expresses puromycin resistance marker under the PGK promoter and RFP gene under the CMV promoter. The 5′ and 3′ AAVS1 homology arms ('AAVS-Right' and 'AAVS-Left') provide the cells with a template for Homology Directed Repair.*

Phase 2: Dilution of the Donor Plasmid and Resistance Marker Selection

- Transfected HEK293 cells were passaged ten times to dilute out the episomal donor vector.
- After these passages puromycin was added to the media to select for cells with successful knock-in of the RFP-puromycin resistance cassette.
- After 3-4 weeks of selection, >95% of HEK293 cells were expressing RFP.

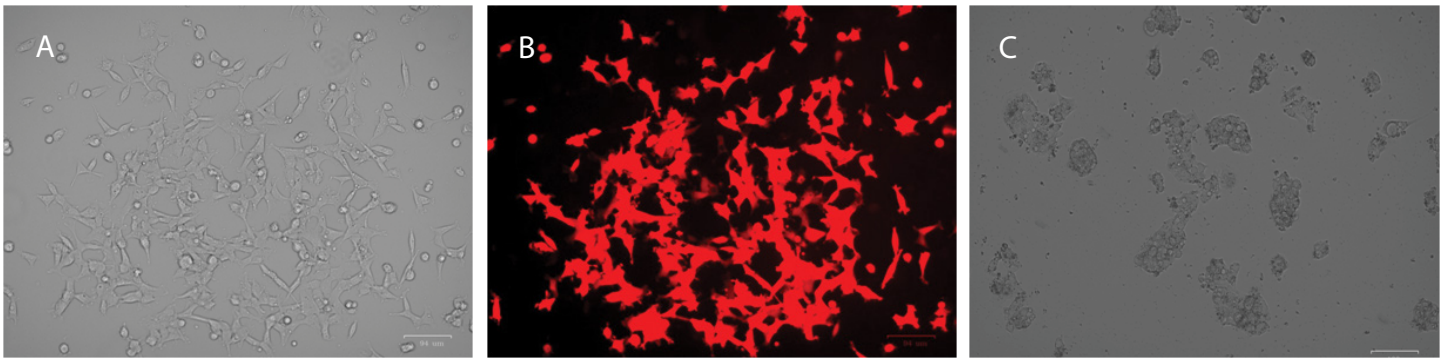

Figure 3 *After transfection, HEK293 cells were passaged ten times to dilute out the episomal vector, then grown in the presence of puromycin for 4 weeks. A) Cells transfected with both pCas-Guide-AAVS1 and pAAVS1-RFP-DNR were healthy after 4 weeks. B) Over 95% of these cells imaged in Figure 3 (A) expressed RFP. C) Control cells not transfected with the vectors died after puromycin treatment.*

Phase 3: Confirmation of Knock-in by Genomic PCR

- To confirm knock-in of RFP in the genomic DNA, a primer pair was designed with Primer 1 targeting the 5' homology arm upstream of RFP and Primer 2 targeting within the RFP-Puromycin resistance cassette.
- PCR product of 1.1 kb indicates successful knock-in at AAVS1 site; absence of PCR amplification indicates unsuccessful cassette insertion **(Figure 4)**.
- No PCR amplification was seen in the control cells ('WT cell') since Primer 2 could not anneal to the genomic DNA.

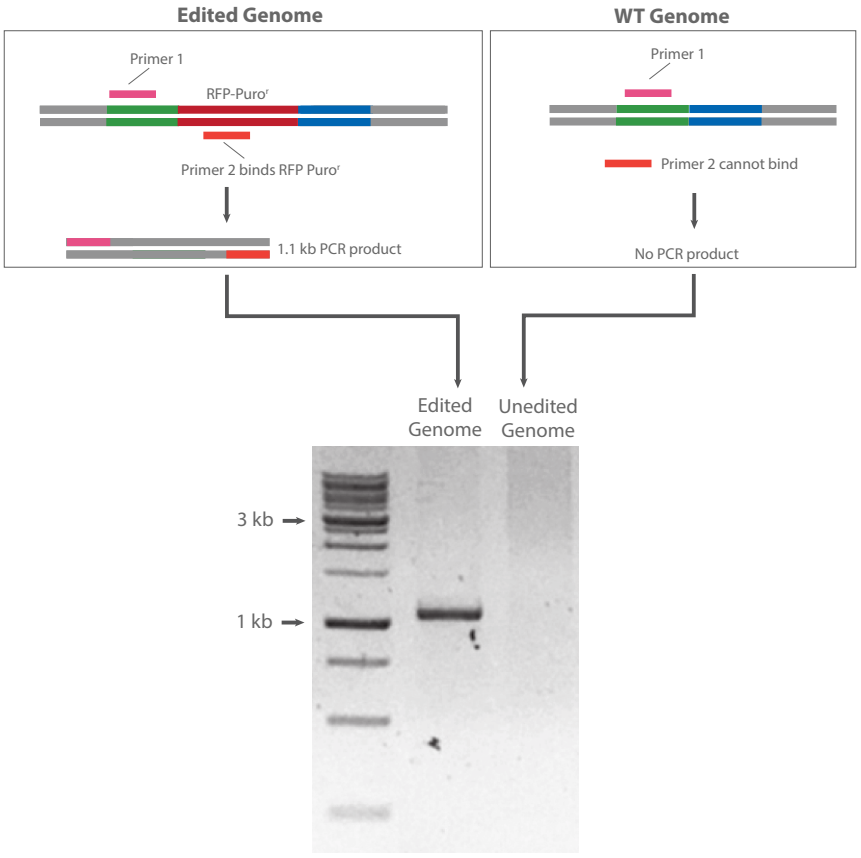

Figure 4 *Genomic PCR was used to confirm the knock-in of RFP. In edited cells, both primer 1 and primer 2 can bind, resulting in a 1.1 kb PCR product. No PCR product is formed in WT cells as primer 2 cannot anneal to the genomic DNA.*