APPLICATION NOTE

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Generating functional protein knockout in iPSCs using Edit-R™ reagents

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Introduction

Whether a researcher is studying normal human cell biology or a disease state, primary human cell models are not always available and can be challenging to work within the lab. Induced pluripotent stem cells (iPSCs) are a useful research tool because they are a renewable model for karyotypically normal cells. Additionally, they can be manipulated in the pluripotent state and differentiated into various isogenic cell types.

Here we demonstrate the ability to modify the genome and create functional knockout models within a human iPSC line with Horizon Discovery Edit-R CRISPR reagents.

Results

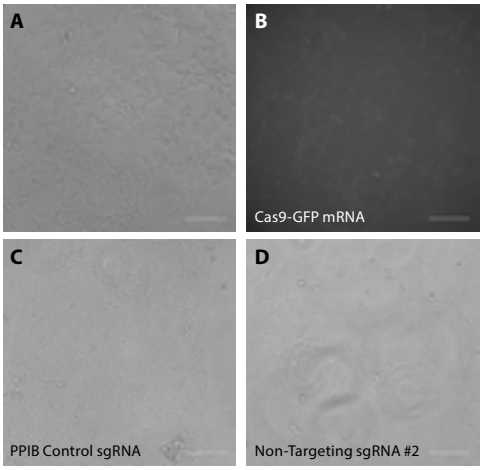
Electroporation of Edit-R Synthetic sgRNAs and Edit-R Cas9 Nuclease mRNA in human iPSCs

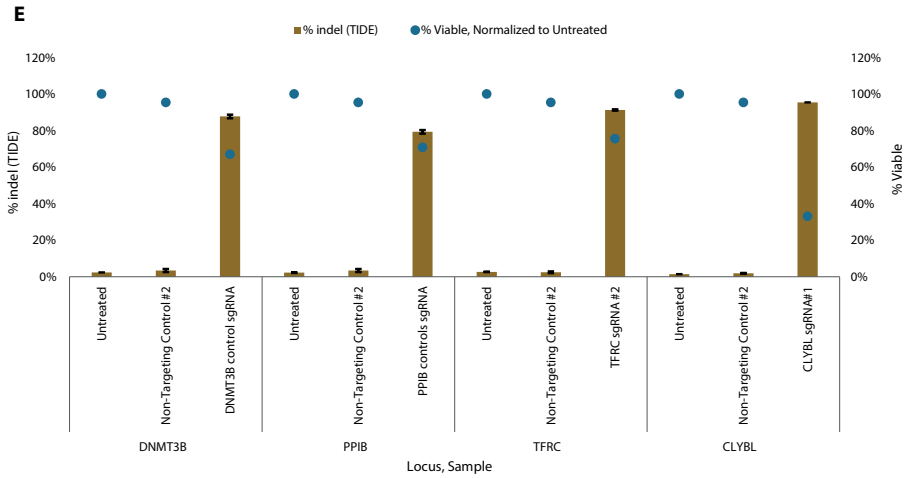
To demonstrate the use of Edit-R Cas9 Nuclease mRNA with GFP fluorescence in iPSCs, Cas9 Nuclease mRNA co-expressing a GFP reporter was electroporated with synthetic sgRNAs targeting four genes using previously optimized conditions (see Methods). Six hours post-transfection, GFP fluorescence was observed in a high percentage of iPSCs, suggesting a highly efficient transfection (Figure 1A–B). Cells were harvested 5 days post-transfection when they exhibited expected iPSC morphology (smooth rounded colonies with tightly packed cells) and a confluence of approximately 70% (Figure 1C–D). Cells were lysed, and PCR primers were used to amplify approximately 300 bp on each side of the sgRNA binding site. Sanger sequencing was used to perform a TIDE analysis (see Methods), indicating that the percentage of indel formation at each target locus was greater than 75% (Figure 1E). Cells were also fixed and stained with antibodies against four iPSC pluripotency markers. Visual analysis confirmed no loss of pluripotency marker expression in any of the treated samples compared to the untreated control cells, which is expected since none of the four proteins targeted had been reported to affect the pluripotency of iPSCs (Figure 1F).

Edit-R Cas9 Nuclease mRNA and Edit-R Synthetic sgRNA targeting *SOX2* induce loss-of-pluripotency phenotype in iPSCs

Indel formation in a gene of interest may not always lead to a functional protein knockout. To demonstrate a functional knockout phenotype, we targeted *SOX2*, a known controller of iPSC pluripotency (Rizzino 2013). Here, we use Edit-R Cas9 Nuclease mRNA and three pre-designed Edit-R Synthetic sgRNAs targeting the SOX2 locus to identify the best target for functional protein knockout. Individual synthetic sgRNAs were electroporated into healthy iPSCs with Cas9 Nuclease mRNA, and five days later, replicate wells were lysed for PCR and Sanger sequencing required for TIDE analysis. Cells were also fixed and stained with antibodies against four iPSC pluripotency markers, including the target gene of interest, *SOX2*.

Each sgRNA used caused greater than 80% indels at the target locus, and visible changes in iPSC morphology were seen on the day of harvest (large, flat cells not growing in tightly packed colonies, Figure 2A–B). Pluripotency marker staining showed that the non-targeting control sample expressed all four pluripotency markers (SSEA4, OCT4, SOX2, and TRA-1-60) as expected for healthy iPSCs (Figure 2C, left series). Cells treated with sgRNAs against *SOX2* show varying levels of diminished *SOX2* expression (Figure 2C, third row highlighted in green). In *SOX2* sgRNA treated samples, cells also show loss of *OCT4* and TRA-1-60 expression (Figure 2C). This result suggests a functional loss of pluripotency is also achieved with the knockout of *SOX2* and agrees with previously published work describing the highly interdependent network of factors that control pluripotency (Rizzino 2013).





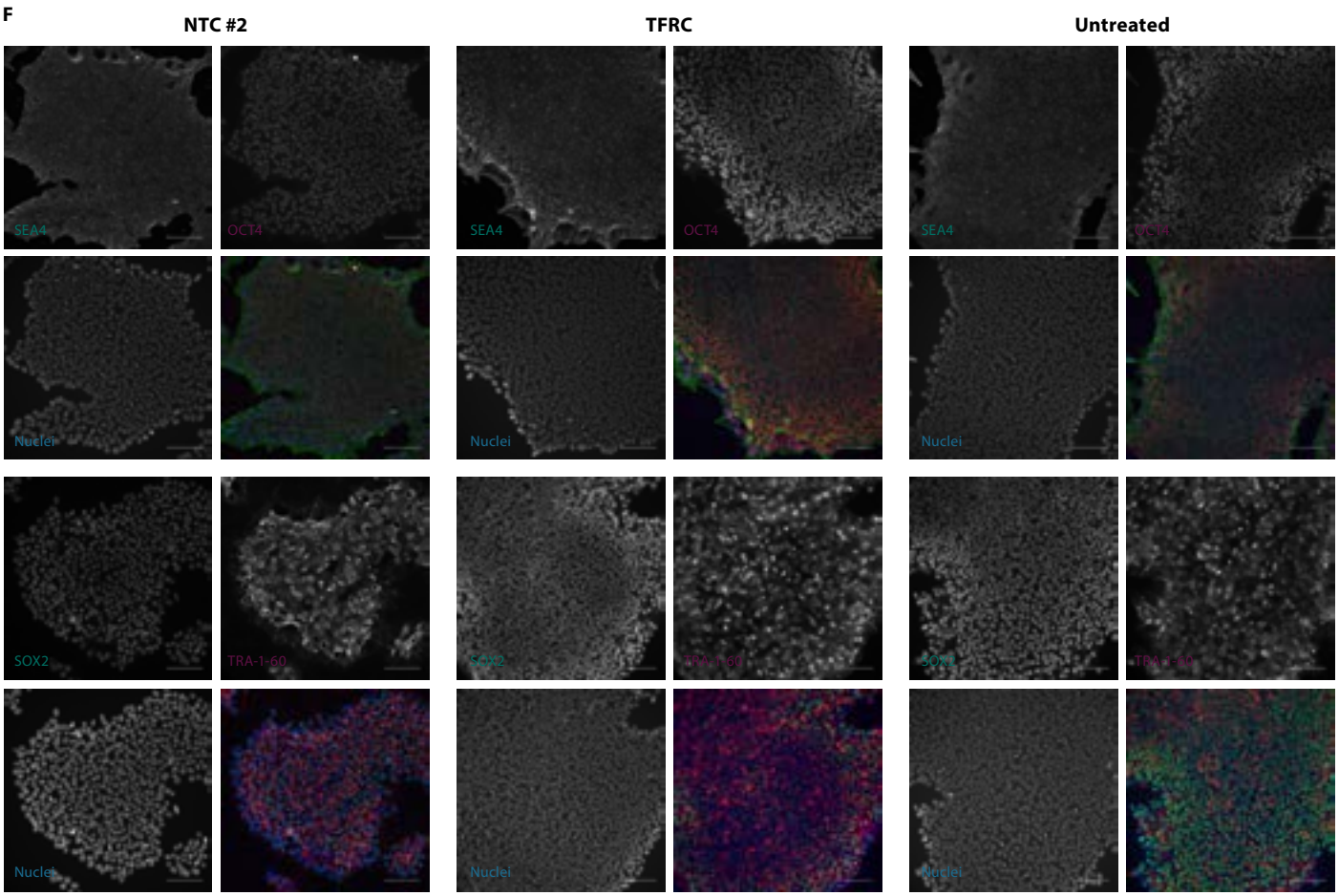


Figure 1. Gene Editing in iPSCs with Cas9-mRNA Nuclease and synthetic sgRNAs delivered by nucleofection is an effective editing method that is non-perturbing to iPSC morphology and pluripotency. A–B) PPIB Control sgRNA sample imaged 6 hours post-transfection where B) shows GFP signal from the Cas9-GFP mRNA and indicates successful delivery. C–D) Phase images post-transfection after 5 days (passaged 1 time). Cells show expected iPSC morphology. A–D) Scale bars 100 µm. E) Percent indel formation by TIDE analysis. F) Staining for iPSC pluripotency markers SSEA4, OCT4, SOX2, and TRA-1-60 5 days after nucleofection of Non-Targeting Control #2 Synthetic sgRNA (NTC#2, left series), TFRC Synthetic sgRNA (middle series), and Untreated cells (right series).

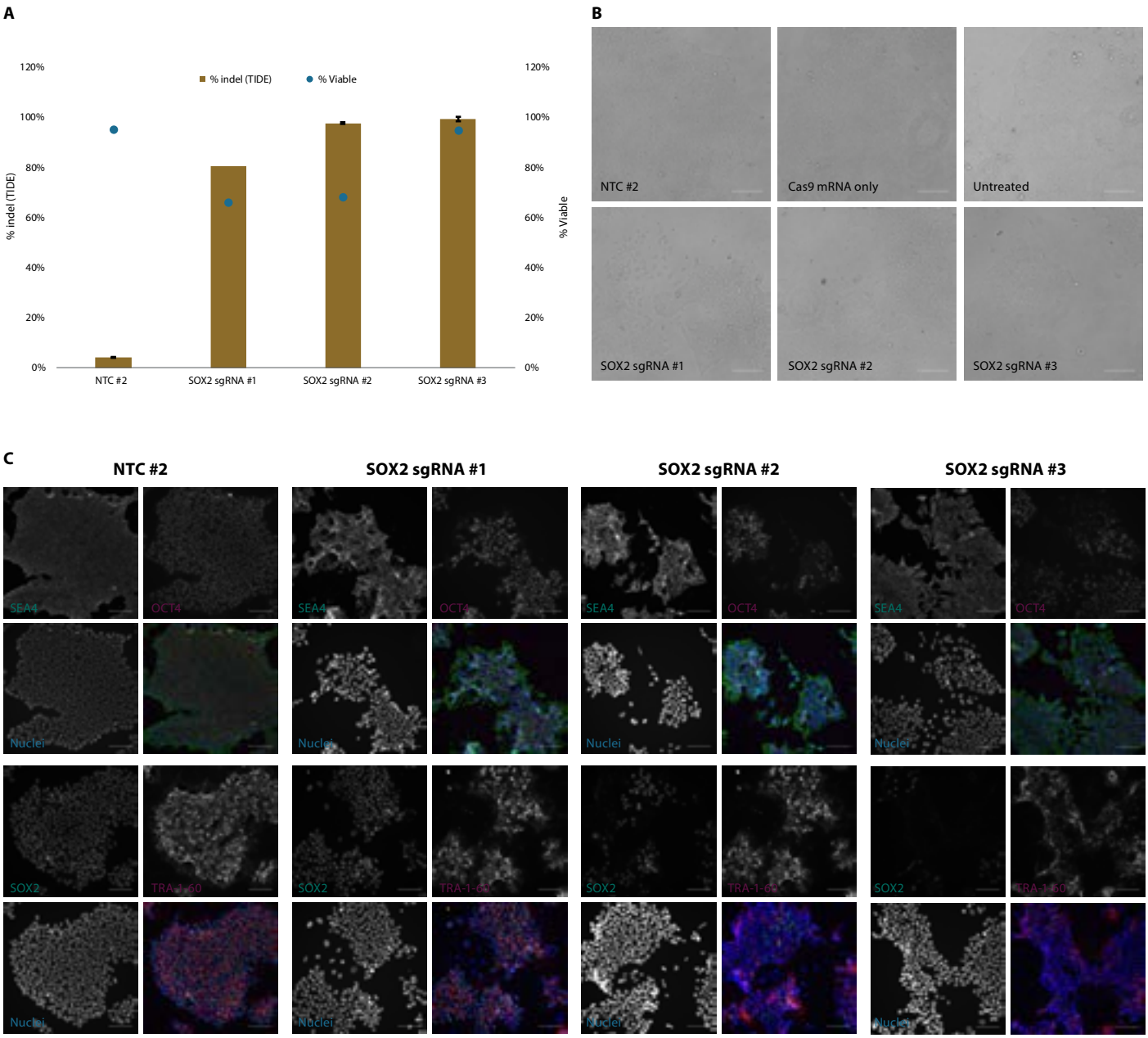


Figure 2. Gene Editing in iPSCs with Cas9-mRNA Nuclease and synthetic sgRNAs delivered by nucleofection to target pluripotency transcription factor SOX2 generates a functional knockout with high efficiency. A) Percent indel formation by TIDE. B) Knockout of SOX2 induces changes in iPSC morphology. No changes to morphology are seen with Non-targeting control, or Cas9 mRNA only compared to untreated iPSCs. Images were taken 5 days after nucleofection, including one passage. Scale bars are 100 µm. C) Pluripotency marker staining of fixed cells 5 days after electroporation. Scale bars are 100 µm.

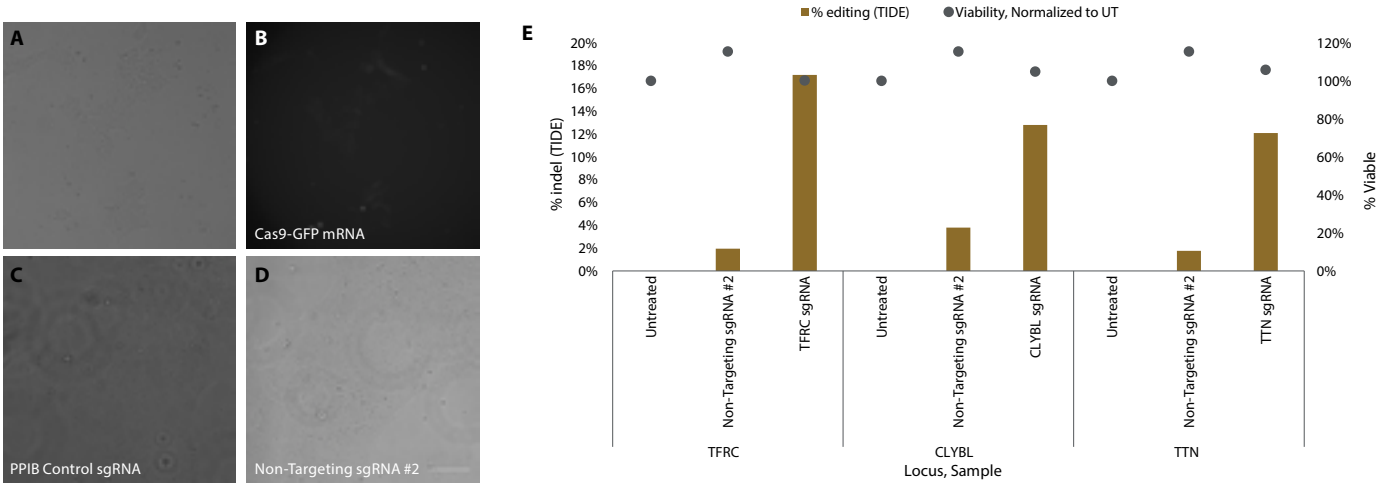


Figure 3. Gene Editing in iPSCs with Cas9-mRNA Nuclease and synthetic sgRNAs delivered by DharmaFECT Duo Transfection Reagent is an alternative delivery method that is non-perturbing to iPSC morphology. A–B) TFRC sgRNA sample imaged 20 hours post-transfection where A) is the phase image and B) shows GFP signal from the Cas9-GFP mRNA and indicates successful delivery. C–D) Phase images post-transfection after 72 hours. Cells show expected iPSC morphology. A–D) Scale bars 100 µm. E) Percent indel formation by TIDE analysis.

Lipid transfection of Edit-R Synthetic sgRNAs and Edit-R Cas9 Nuclease mRNA in iPSCs

While electroporation is the optimal method for delivery of Edit-R Cas9 Nuclease mRNA and synthetic sgRNAs for iPSCs, the optimal methods for each cell line may require specific equipment that a lab may not have access to such as a nucleofection instrument. As an alternative, lipid transfection can also be used to deliver Edit-R gene editing reagents to iPSCs.

Here, optimized transfection conditions using DharmaFECT Duo Transfection Reagent (see Methods) were used to deliver Edit-R Cas9 Nuclease mRNA and synthetic sgRNAs targeting three genes that are either expected to not affect iPSC viability or morphology (*TFRC, CLYBL*) or are not expressed in iPSCs (*TTN*). After transfection, GFP signal from the Cas9-GFP mRNA is observed, indicating successful delivery (Figure 3A–B). On the day of harvest, cells exhibit expected iPSC morphology (Figure 3C–D) and high viability compared to untreated controls (Figure 3E). Cells were lysed, and PCR products were Sanger sequenced and subjected to TIDE analysis, which resulted in indel formation between 12–18% for samples treated with sgRNAs targeted to each of the three genes analyzed (Figure 3E).

Discussion

Here we have demonstrated the effectiveness of Edit-R Cas9 Nuclease mRNA and Synthetic sgRNAs in editing human iPSCs. Optimized electroporation protocols are ideal for the delivery of these reagents into this cell type, though exact conditions may vary by cell line. Alternatively, reagents can also be delivered by lipid transfection. To study the impact of a specific protein of interest on cellular phenotype or function populations of edited iPSCs exhibiting a high percentage of indel formation and functional protein knockout can be used directly in comparison with unedited isogenic controls, or alternatively, clonal cell lines can be generated.

Using Horizon's DNA-free reagents prevents unwanted random genomic integration, preserving the integrity of the iPSC models' genetic composition. Additionally, synthetic sgRNA and Cas9 Nuclease mRNA are active in the cell for less time than plasmid-based reagents, which reduces the risk for off-target editing activity. Using Cas9 Nuclease mRNA also precludes the need for using a reagent with a cell-line specific optimized promoter. This application note demonstrates the utility of synthetic sgRNA and Cas9 Nuclease mRNA as a convenient source of CRISPR reagents for effective genome editing in human iPSCs.

Materials and methods

Cell culture

A Human Episomal human iPSC Line (Cat # A18945, Thermo Fisher Scientific) found to be karyotypically normal was cultured on non-tissue culture treated plastics (recommended for use with Vitronectin coating) coated with truncated Vitronectin (VTN-N) Recombinant Human Protein (Cat # A14700, Thermo Fisher Scientific) according to manufacturer's recommendations. Cells were cultured in antibiotic-free mTeSR™ Plus media (Cat # 05825 StemCell Technologies). Cultures were routinely maintained by clump passaging two times a week with Gibco™ Versene Solution (Cat #15040066, Thermo Fisher Scientific) per manufacturer's instructions. Cells were incubated at 37 °C and 5% CO_2.

Electroporation

Prior to electroporation, cells were treated with 10 µM ROCK inhibitor molecule Y-27632 (Cat #72302, StemCell Technologies) for 1–4 hours to promote viability post-electroporation. Cells were passaged into a single cell suspension using StemPro Accutase Cell Dissociation Reagent (Cat # A1110501 Thermo Fisher Scientific) and resuspended in mTeSR™ Plus media supplemented with 10 µM ROCK inhibitor molecule Y-27632. Cell line-specific (see Cell Culture section for specific lineage) electroporation conditions, previously optimized with pMax-GFP expression plasmid, were as follows: Lonza™ P3 Primary Cell 4D-Nucleofector™Kit (Cat # V4SP-3096) was used to transfect 3.4×10^5 cells per reaction in an Amaxa™ 96-well Shuttle™ Device (Cat # AAM-1001S) using setting CM-113. Each reaction contained 1 µg Edit-R EGFP Cas9 Nuclease mRNA (Cat # CAS11860) and 2 µM Edit-R synthetic sgRNA in 20 µL reactions.

The specific guide RNA sequences used were:

- Edit-R Human TFRC synthetic sgRNA, Cat# SG-003941-02-0003
- Edit-R Human SOX2 synthetic sgRNA, Cat # SG-011778-01-0003, SG-011778-02-0003, and SG-011778-03-0003
- Edit-R Human CLYBL synthetic sgRNA, Cat # SG-018275-01-0003
- Edit-R Human TTN synthetic sgRNA, Cat # SG-005395-01-0003

After transfection was performed, 80 µL mTeSR™1 Without Phenol Red media (Cat # 05876 StemCell Technologies) supplemented with 10 µM ROCK inhibitor molecule Y-27632 was added to each reaction well, and 25 µL of each reaction was plated into a prepared Vitronectin-coated 96-well plate containing 0.2 mL pre-equilibrated mTeSR™1 Without Phenol Red media supplemented with 10 µM ROCK inhibitor molecule Y-27632. Cells were incubated at 37 °C and 5% CO_2. After 6–20 hours, cells were imaged for GFP fluorescence from the Edit-R EGFP Cas9 mRNA as an indication of transfection efficiency. Twenty-four hours post-transfection, media was changed to mTeSR™ Plus, and cells were fed daily until the time of harvest or passaged one time before harvest.

Lipid transfection

One day before transfection, healthy iPSC cultures with 65-75% confluence were passaged into a single cell suspension using StemPro Accutase Cell Dissociation Reagent and resuspended in mTeSR™ Plus media supplemented with 10 µM ROCK inhibitor molecule Y-27632. Cells were replated into non-tissue culture treated 96-well plates coated with Vitronectin at a density of 6×10^3 cells/well in 0.1 mL mTeSR™ Plus media supplemented with 10 µM ROCK inhibitor molecule Y-27632 and incubated at 37 °C and 5% CO_2. This seeding density was optimized for this iPSC line to give 60-75% confluence on the day of harvest. The day after plating, the transfection mix was prepared so that each reaction contained 200 ng Edit-R EGFP Cas9 Nuclease mRNA, 50 nM Edit-R synthetic sgRNA, and 0.3 µg/mL DharmaFECT Duo Transfection Reagent (Cat #T-2010-01) (the amount of transfection reagent needed should be empirically determined through optimization experiments for each iPSC line). Reagents were pre-complexed for 20 minutes at room temperature in HyClone MEM-RS (Cat #SH30564, Fisher Scientific) before mTeSR™1 without Phenol Red media was added to the transfection mix for a final volume of 0.1 mL/reaction. Expired growth media was replaced with 0.1 mL transfection mix, and cells were returned to the incubator. After 6–20 hours, cells were imaged for GFP fluorescence from the Edit-R EGFP Cas9 mRNA as an indication of transfection efficiency. Cells were fed every 24 hours with mTeSR™ Plus media until the day of harvest (typically 72 hours post-transfection).

Viability assay

Replicate wells were harvested for viability analysis by CellTiter-Glo® Luminescent Cell Viability Assay (Cat #G7572, Promega) per the manufacturer's instructions. Luminescence was read on an Envision 2 Plate Reader.

TIDE analysis

On the day of harvest, replicate wells were washed 1X in DPBS (Cat #SH30028, Fisher Scientific) and lysed in 0.1 mL/well Thermo Scientific Phusion HF Buffer (Cat #F-518L, Thermo Fisher Scientific) with 5 µL each of Proteinase K (Cat# E00492, Thermo Fisher Scientific) and RNase A (Cat #EN0531, Thermo Fisher Scientific), and incubated for 1 h at 56 °C then heat-inactivated at 98 °C for 10 min. Fifty microliter PCR reactions were carried out in 1× Phusion HF buffer (Cat #F-518, Thermo Fisher Scientific), using 0.5 µL Phusion Hot Start II DNA Polymerase (Cat #F-549S, Thermo Fisher Scientific), 200 µM each dNTP (Cat #F-549S, Thermo Fisher Scientific), 0.5 µM forward and reverse primers, and 5 µL direct cell lysis template. Touchdown PCR cycling was run for each sample: denature/enzyme activation at 98 °C for 3 min followed by 10 cycles of 98 °C for 10 s, 72 °C for 15 s –1 °C/cycle, and 72 °C for 30 s, then 25 cycles of 98 °C for 3 min followed by 10 cycles of 98 °C for 10 s, 62 °C for 15 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. Each PCR product was verified to be single amplicons by running 5 µL of the reaction on 1.2% agarose gels before proceeding with Sanger sequencing. The resulting AB1 files were analyzed by an in-house program that utilizes an adapted version of the TIDER algorithm described in Brinkman *et al.* (2018) to estimate the likely indel frequencies present within a Sanger sequence trace. Samples are first assessed and filtered on the basis of sequence trace quality, and the analysis parameters determined for each individual input. Treated samples are then aligned to and compared against untreated controls to calculate the sample editing efficiency, the frequency of indels, the rate of indel occurrence, and corresponding p-values. Each possible indel within the specified window of detection was individually tested to determine the likelihood of such an indel being explained by the observed Sanger trace data, and a confidence estimate was then assigned to this indel.

Immunocytochemistry

Replicate wells were washed once with DPBS and then fixed and stained with the Invitrogen™ Pluripotent Stem Cell 4-Marker Immunocytochemistry Kit (Cat# A24881, Thermo Fisher Scientific) per the manufacturer's instructions.

References

1. A. Rizzino. The Sox2-Oct4 Connection: Critical players in a much larger interdependent network integrated at multiple levels. *Stem Cells*. **31**(6): 1033–1039 (2013).

2. E. Brinkman *et al.* Easy Quantification of template-directed CRISPR/Cas9 editing. *Nucleic Acids Research*. **46**(10): e58 (2018).

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