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Targeted mutagenesis in human iPSCs using CRISPR genome-editing tools

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ABSTRACT

Mutagenesis studies have rapidly evolved in the era of CRISPR genome editing. Precise manipulation of genes in human induced pluripotent stem cells (iPSCs) allows biomedical researchers to study the physiological functions of individual genes during development. Furthermore, such genetic manipulation applied to patient-specific iPSCs allows disease modeling, drug screening and development of therapeutics. Although various genome-editing methods have been developed to introduce or remove mutations in human iPSCs, comprehensive strategic designs taking account of the potential side effects of CRISPR editing are needed. Here we present several novel and highly efficient strategies to introduce point mutations, insertions and deletions in human iPSCs, including step-by-step experimental protocols. These approaches involve the application of drug selection for effortless clone screening and the generation of a wild type control strain along with the mutant. We also present several examples of application of these strategies in human iPSCs and show that they are highly efficient and could be applied to other cell types.

1. Introduction

Genome-editing tools have greatly evolved in the past decade, ever since the groundbreaking investigations on clustered regularly interspaced short palindromic repeats (CRISPR) [1–4]. Discoveries on CRISPR drove the rapid development of convenient, flexible and efficient genome-editing tools, and CRISPR tools quickly took over the territories that were first innovated by Zinc finger nucleases (ZFN) and Transcription activator-like effector nucleases (TALENs) [5–9].

The Class 2 CRISPR system consists of a single nuclease protein to cleave target sequences, in contrast to the requirement of a complex of multiple proteins for the Class 1 system. One Class 2 system, type II-A CRISPR-Cas9, provided the first CRISPR tools for genome editing in cultured human cells [3,4] and is still the most commonly used genome-editing tool in a variety of organisms. The engineered CRISPR-Cas9 system consists of a single Cas9 nuclease (most commonly from *Streptococcus pyogenes*, SpCas9) and a single guide RNA (sgRNA). The Cas9-sgRNA ribonucleoprotein complex cleaves the target DNA segment complementary to the sgRNA, and the recognition also requires a 3–5 base pair protospacer adjacent DNA motif (PAM). SpCas9 utilizes a PAM with a very short sequence of NGG, which is relatively abundant in mammalian genomes. Therefore, SpCas9 has been widely applied in mammalian genome editing. In contrast, the type V Cas12a system recognizes a T-rich PAM and can be used for editing A/T rich regions [10].

Stem cell research plays a key role in understanding cell differentiation and tissue development. Induced pluripotent stem cells (iPSCs) are derived from somatic cells through reprogramming [11]. iPSCs are extremely valuable in human disease study, because human iPSCs (hiPSCs) bypass the ethical concerns of using human embryonic stem cells (hESCs) and can be derived in a patient-matched manner. It is conceivable that genetic mutations in patients could be corrected using CRISPR genome-editing tools, and then the edited hiPSCs could be differentiated into healthy tissues for disease treatment. Besides treatment purposes, iPSCs have been widely used in biomedical research to study consequences of mutations, importance of individual exon/introns and functions of specific genes. All these applications require targeted mutagenesis in iPSCs using genome editing.

Here we provide comprehensive guides to three main schemes for precise and efficient genome editing in iPSCs: base substitutions or point mutations, insertion of additional sequences, and deletions (Fig. 1). We explain that drug selection facilitates successful and efficient editing [8,12]. In addition, making wild type control cell lines by the same procedure is essential for downstream functional and phenotypical investigations, as the CRISPR-edited locus differs from the parental gene in several ways, and the editing can also cause off-target alterations

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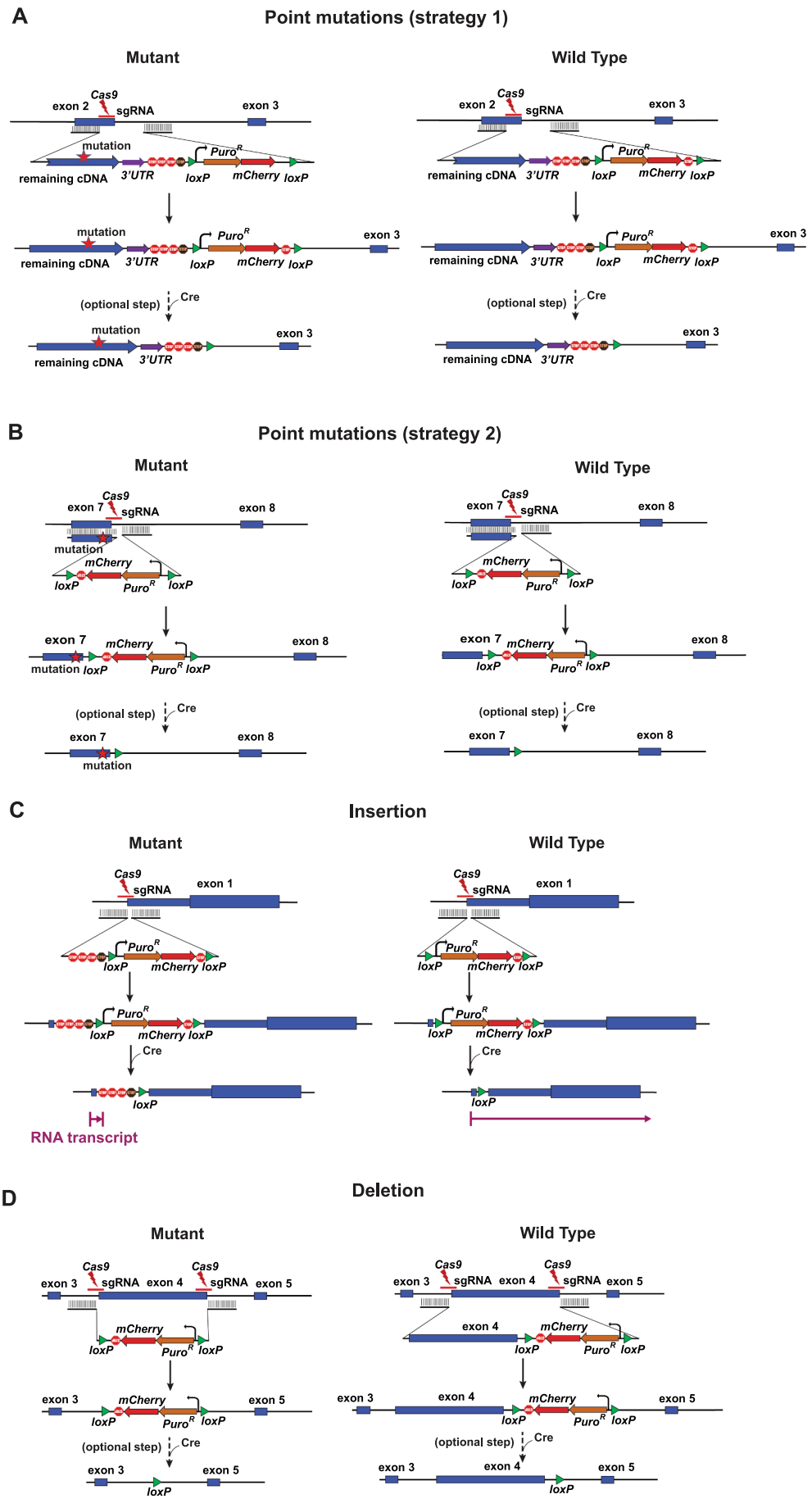


Fig. 1. Schematic design for genome editing of point mutations, insertions and deletions using CRISPR-Cas9. A. Strategy 1 to introduce point mutations. SpCas9 is used to generate a double-stranded break at an early exon-intron junction (e.g., exon 2 and intron 2), and homologous recombination fuses the remaining cDNA with the mutations to the end of this exon along with a *LoxP*-flanked selection cassette. Removal of the selection cassette using Cre is optional. A wild type control is made exactly the same way but without the mutations. B. Strategy 2 to introduce point mutations. SpCas9 cleaves near the exon-intron junction of the sequence targeted for mutation, and the point mutation present in the homology arms is introduced by homologous recombination. The selection cassette is inserted in the antisense direction to minimize interference with target gene expression. C. Strategy to insert sequences at specific genomic loci. In the example here, polyadenylation (poly(A)) signals (indicated by the STOP signs) are inserted immediately after the transcription start site of a gene. This insertion results in a truncated RNA transcript compared to the wild type control. D. Strategy to delete sequences at specific genomic loci. The example here represents deletion of Exon 4 of a gene. Two Cas9 cleavage sites flank the targeted exon, and the exon is replaced with a selection cassette by homologous recombination. The wild type control differs in that the targeted exon is reintroduced along with the selection cassette. *Puro*^{R}: puromycin-resistant gene.

including large sequence deletions and complex rearrangements [13].

2. Materials and methods

Here we present an optimized protocol for introducing mutations in human iPSCs (Fig. 2), and this protocol could also be easily adapted for other cell types.

2.1. iPSC feeder-free culture

Most iPSC strains have been adapted to feeder-free culture nowadays. We routinely use a well-characterized human iPSC strain WTC-11 made by Dr. Bruce R. Conklin's group (Gladstone Institute of Cardiovascular Disease, UCSF, cell line distributed by the Coriell Institute #GM25256). A variety of iPSC culture media and coating matrix formulations have been developed in the past two decades, and many of them are available commercially: (a) medium – Essential 8 medium (Thermo Fisher A1517001) and mTeSR1 (StemCell Technologies 85850); (b) coating matrix – Vitronectin (Thermo Fisher A14700), Matrigel (Corning 356234) and Geltrex (Thermo Fisher A1413201). These media and coating materials can be used in any combination. We regularly maintain our iPSC strains in Essential 8 Flex medium (E8 Flex, Thermo Fisher A2858501) combined with Vitronectin as the coating matrix. This combination maintains the cell pluripotency very well, and E8 Flex medium offers every-other-day medium change instead of the regular daily medium change with most other media.

For preparing the culture medium

- E8 Flex Medium Preparation
  - For a 500 ml E8 Flex Medium Bottle
    - Thaw E8 Flex supplement (stored in –20 °C freezer) at room temp (takes ~ 2–3 h on bench, or ~ 30 min in a water bath or metal block)
    - The remaining steps are done in a sterile hood
      - Mix E8 Flex supplement by shaking the bottle several times
      - Transfer entire E8 Flex supplement bottle (10 ml) to the 500 ml E8 Medium bottle
    - Add 2.5 ml of 100x Penicillin-Streptomycin (Sigma-Aldrich P4333) to the medium. Aliquot in 50-ml conical tubes and store for up to two weeks at 4 °C

For passaging the cells

- Coating plates with Vitronectin (VTN-N)
  - Recombinant VTN-N (Gibco A14700) is aliquoted into 30 or 60 μl that can coat 3 or 6 wells of a 6-well plate. These aliquots are then stored in a −80 °C freezer.
  - To coat a 6-well plate, remove an aliquot of VTN-N from the −80 °C Freezer. Thaw at room temp (takes ~ 1–2 min)
  - Add VTN-N to sterile dPBS (Dulbecco's Phosphate-Buffered Saline)
    - 6 ml for 60 μl aliquot
    - 3 ml for 30 μl aliquot
      - Note: Use a 200 μl pipette and rinse the tube with dPBS to collect all the VTN-N solution
  - Mix the VTN-N solution by capping the tube and inverting several times
  - Add 1 ml of VTN-N solution to each well that will be coated
  - Leave plate in a sterile hood for at least 1 h
    - Note: Take the E8 medium out of the fridge at this point and place in the hood to warm up
- Passaging
  - Let E8 medium and VTN-N coated plates equilibrate to room temperature (if the 6-well plates were stored at 4 °C)
  - Aspirate old E8 medium from the 6-well plate
  - Rinse each well with 2 ml of dPBS
  - Aspirate dPBS and add 1 ml of 0.5 mM EDTA in dPBS
  - Place in incubator for 5–8 min
  - Aliquot E8 Flex Medium into a separate tube
    - For most passages use a 1:6 or 1:8 dilution –> for each well passaging, dilute cells into 6 ml of new medium
  - After 5 min, look at cells under microscope – cells will separate and colonies will appear to round up. Colonies will also have holes in them.
  - Aspirate out EDTA solution
  - To recover cells, add E8 Flex medium into the well (~2 ml per well of the 6-well plate) from the aliquoted medium
    - Gently squirt medium to remove cells from the entire well. Try to avoid making bubbles and pipetting too much because mechanical stress is deleterious.
    - Use a 5 or 10 ml pipette tip to avoid creating too much mechanical stress
  - Collect and add the recovered cells to the aliquoted E8 Flex medium
  - Gently mix the cell solution by capping the tube and inverting several times
  - Remove the VTN-N solution from the new plate by aspiration
  - Add 2 ml of the cell solution to each well (recommended: add 5 μM inhibitor of ROCK (Rho-associated, coiled-coil containing protein kinase), Tocris 1254)
  - Place the new 6-well plate into the incubator and shake back and forth several times to spread the cells evenly across the surface
  - Change medium on the next day
  - After the first medium change, E8 Flex medium can be changed every other day until confluency

For freezing of iPSCs

- Prepare Freezing Medium
  - Combine 0.9 ml of E8 Flex Medium with 0.1 ml of DMSO for each well of a 6-well plate to be frozen down
  - Place freezing medium into the 4 °C fridge until ready to use
- Aspirate old E8 medium from the 6-well plate
- Rinse each well with 2 ml of dPBS
- Aspirate out dPBS and add 1 ml of 0.5 mM EDTA (in dPBS)
- Place in incubator for 5–8 min. Meanwhile, take the E8 Flex medium out of fridge.
- After 4 min, look at cells under microscope – cells will separate and colonies will appear to round up. Colonies will also have holes in them.
- Aspirate out EDTA solution
- To recover cells, add freezing medium into the well (1 ml per well) from the aliquoted medium
  - Gently squirt medium to remove cells from the entire well. Try to avoid making bubbles and over-pipetting to avoid mechanical stress.
  - Use a 5–10 ml pipette again to avoid creating too much mechanical stress
- Combine the contents of all identical wells into a tube and then aliquot 1 ml of cell suspension into each cryotube
- Place into the cell cryofreezing container (Thermo Scientific 5100–0001) and move to −80 °C overnight
- After overnight storage at −80 °C, transfer the cells to the large liquid nitrogen tank.

For thawing iPSCs

- Coat 6-well plates with Vitronectin as described above
- Thaw iPSCs
  - Add 10 ml of room temperature E8 medium into a 15 ml tube
  - Remove iPSCs from the liquid nitrogen storage tank
  - Immerse vial in 37 °C water bath and swirl it gently

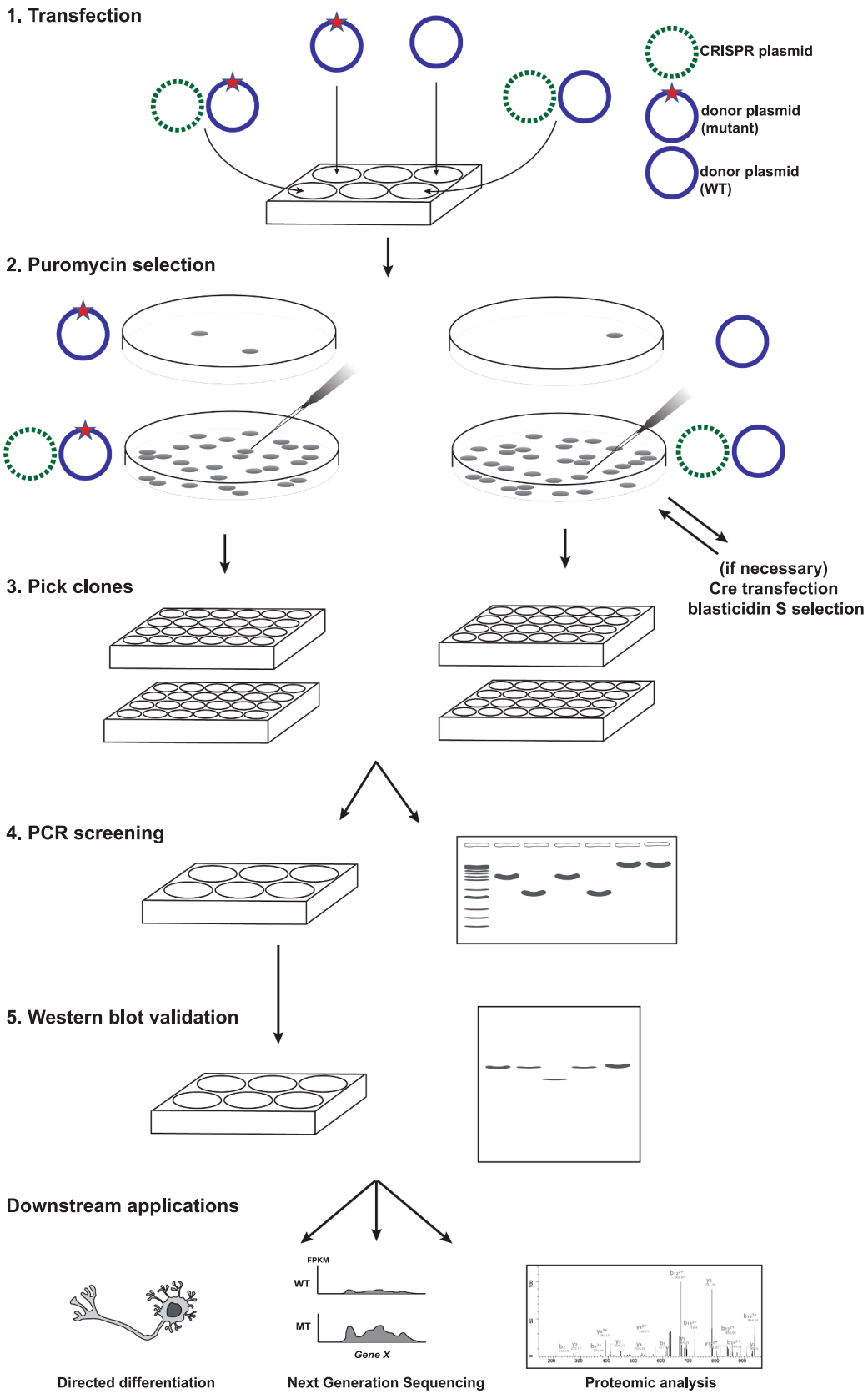


Fig. 2. Genome editing procedure to introduce gene mutations in human iPSCs. 1. The workflow starts with four parallel transfection experiments in a 6-well plate culturing human iPSCs: a CRISPR plasmid expressing the SpCas9 and sgRNA and a mutant donor DNA, CRISPR plasmid and a WT donor, and two donor DNA-only controls. 2. The transfected cells are passaged to a larger 10-cm petri dish, and drug (puromycin) selection is applied at confluency. While the two donor-only controls generally have very few or no surviving colonies, the CRISPR + donor transfections generally have many more surviving clones. 3. After two weeks of selection, undifferentiated colonies are hand-picked under a stereomicroscope into 24-well plates. 4. These clones will be cryo-preserved or cultured continuously, and their genotype (successful integration, homozygous or heterozygous) will be screened using genomic PCR usually followed by Sanger sequencing of the PCR products. 5. PCR-validated clones will be further characterized by western blot to examine the expression of the edited genes. The one-step edited clones are now ready for downstream analysis such as directed differentiation, next-generation sequencing, and proteomic analysis. If desired, the *LoxP*-flanked selection cassette can be removed by transfection of Cre. The Cre transfection could be done after validation of single clones or could be applied immediately in the polyclonal population after the drug selection. Cre-expression plasmid contains a blasticidin S resistant gene for drug selection after transfection, and clonal screening is performed as in the earlier steps.

- When only a small ice crystal is left, move to the hood (use caution as the label might come off)
- Collect the cells with a 5 ml pipette and add dropwise into 10 ml of E8 Flex medium
    - Drop-wise prevents osmotic damage to the cells
- Rinse the vial with 1 ml of E8 Flex medium to collect any remaining cells
- Spin down the cell suspension in a 15 ml tube at 200 g for 5 min
- Aspirate the supernatant
- Resuspend cells in 2 ml (or appropriate volume based on the cell number and dish to be used) of E8 medium (per vial thawed) by pipetting up and down several times. Add 5 μM ROCK inhibitor (important for robust cell survival after thawing).
- Aspirate the VTN-N solution from the plate
- Add 2 ml of cell solution per well
- Place in the incubator and shake plate back and forth several times to spread the cells evenly
- Change medium on the next day to E8 Flex medium without ROCK inhibitor
- After the first medium change, E8 Flex medium can be changed every other day until confluency

2.2. Transfection of CRISPR and donor plasmids

Two plasmids are used for the delivery of the editing machinery: a CRISPR plasmid containing the SpCas9 and sgRNA and a donor plasmid containing the homologous recombination cassette with homology arms and antibiotic resistance cassette (Fig. 2).

It is generally more difficult to transfect iPSCs than other commonly used immortalized cell lines (e.g., HEK293T), but several relatively effective methods have been developed including electroporation and lipid-mediated transfection [14,15]. As electroporation-based transfection may have low cell survival rate, we routinely use Lipofectamine 3000 (Thermo Fisher L3000015) or Stem Reagent (Thermo Fisher STEM00015) to deliver the CRISPR and donor plasmids.

- Seeding iPSCs for transfection
    - Passage the cells on the day before the transfection, aiming to obtain ~ 40–50% confluency at transfection in a 6-well plate.
- Transfection using Lipofectamine 3000
    - Prepare an appropriate volume (2 ml per well for 6-well plates) of regular E8 medium (Thermo Fisher A1517001) without antibiotics, warm up to room temperature. Note: we have found that transfection works better in regular E8 medium compared to the E8 flex medium, and removal of the antibiotics is essential for cell survival after transfection.
    - For each well of a 6-well plate, dilute 3 μg DNA (1.5 μg of the CRISPR plasmid and 1.5 μg of the donor plasmid) in 125 μl Opti-MEM medium (Thermo Fisher 11058021). Add 6 μl P3000 reagent (2 μl/μg DNA), mix well. It is important to include a "donor DNA only" control to differentiate stable genomic integration from transient expression. For this control, omit the CRISPR plasmid and only include 1.5 μg donor plasmid.
    - For each well of a 6-well plate, dilute 7.5 μl Lipofectamine 3000 reagent in 125 μl Opti-MEM medium. Mix well.
    - Combine the diluted DNA and the diluted lipofectamine reagent. Mix it well and incubate at room temperature for 15 min.
    - During the 15 min, take out the cultured iPSCs (in 6-well plates) from the incubator. Aspirate the medium and replace it with the freshly prepared E8 medium without antibiotics.
    - Add 250 μl of the DNA-lipid complex to each well. Gently stir the plate to mix well.
    - After 6 h or on the next day, replace the medium with E8 flex medium. mCherry fluorescence can be examined to determine transfection efficiency. mCherry intensity and transfection efficiency are expected to be similar between the experiment and the "donor DNA only" control.

As an alternative to our plasmid-based CRISPR delivery system, pre-assembled ribonucleoprotein preparations of Cas9 and sgRNA may be able to achieve high efficiency [16,17], but we have not tested this approach in iPSCs.

2.3. Drug selection

The purpose of the drug selection is to select not only the transfected cells, but also the successful genomic integration. In general, iPSCs lose the transient transfected plasmids gradually and completely at around two weeks post-transfection. This means that the selection needs to be performed at least for two weeks. A key to successful selection is to use Matrigel or Geltrex, rather than vitronectin, as the coating matrix.

- Passage cells on to Matrigel or Geltrex coated 10-cm plate
    - Maintain the growth of transfected iPSCs in the 6-well plate by changing E8 flex medium every other day.
    - When the wells reach 80% confluency, passage the cells in each well to a new 10-cm plate coated with Geltrex (Thermo Fisher A1413301) or Matrigel (Corning 354277).
    - To coat a 10-cm plate with Geltrex or Matrigel, take out a 70 μl aliquot of Geltrex or Matrigel that was stored in −80 °C. Place it immediately on ice and bring the ice bucket in a sterile hood.
    - In the hood, add 1 ml of cold DMEM (Dulbecco's Modified Eagle's Medium) basal medium without any supplement (Gibco 12800-082) to the Geltrex/Matrigel. Pipette up and down to thaw and dilute the Geltrex/Matrigel.
    - Add 6 ml of the cold DMEM medium to a new 10-cm plate, and then add the diluted Geltrex/Matrigel. Rock back and forth to mix the solutions.
    - Place the plate in 37 °C incubator for at least 30 min, and then the plate is ready to be used for iPSC culture. If the coated plate won't be used on the same day, wrap it in parafilm and store it at 4 °C.
    - Cell passaging is done similar to what is described above. In detail, cells grown in each well of the 6-well plate are detached using 0.5 mM EDTA in dPBS (note: try breaking cell clusters when pelleting) and transferred to 11 ml of E8 flex medium in a 10-cm plate. Importantly, 5 μM Rock inhibitor is added to the medium to prevent cell death during matrix change. Place the 10-cm plate in 37 °C 5% CO_2 incubator for overnight.
    - On the next day, replace medium with 11 ml fresh E8 flex medium to remove the Rock inhibitor. Check cell survival and attachment under a microscope.
- Drug selection
    - Perform medium change every other day until the confluency reaches 70–80%.
    - Thaw a 1 mg/ml puromycin aliquot (diluted from Gibco A1113802). Add 5.5 μl to 11 ml fresh E8 flex medium (final concentration of puromycin is 0.5 μg/mL) and mix it well.
    - Replace the old medium in the 10-cm plate with the fresh medium supplemented with 0.5 μg/ml puromycin.
    - On the next day, a large fraction of cells should have lifted from the plate, while some cell clusters remain attached. Change medium to E8 flex supplemented with 0.25 μg/ml puromycin. This medium change also helps to remove the lifted cells.
        - Note: we use half concentration of puromycin starting at the second day of the selection, because we observed that a lower concentration of puromycin is required at lower cell density. In other words, on the first day of selection a higher concentration is needed when the cell density is high, while a reduced concentration is required for survival of the positive clones. We have confirmed that the

optimal concentration of puromycin positively correlates with the cell density by performing drug killing curves.
  - o Replace medium every other day with E8 flex supplemented with 0.25 μg/ml puromycin for around two weeks until the survival colonies are almost touching each other.
  - o During the two-week selection process, the "donor DNA only" control gradually loses its viable colonies, and successful genomic integration is indicated by a large difference in the number of surviving colonies between the experiment and the "donor DNA only" control.

2.4. Colony isolation and clonal screening

How many colonies need to be screened may depend on several factors: (a) the efficiency of the homologous recombination using the transfected DNA donor, (b) the accessibility of the specific genomic locus, (c) whether homozygous or heterozygous mutants are desired. Factors 1 and 2 can be optimized in experimental design. Regarding factor 3, 48–72 colonies would be ideal to obtain a homozygous mutant, while 24 colonies are often sufficient to screen for a heterozygous mutant.

- Pick colonies using a stereo microscope
  - o Prepare Matrigel/Geltrex-coated 24-well plates similar to what was described above for 10-cm plates. The volume of DMEM-diluted Matrigel/Geltrex to be add in each well is 300 μl. Incubate the plates at 37 °C for at least 30 min.
  - o Prepare the culture medium by supplementing E8 flex medium (already contains 0.5 × Penicillin-Streptomycin as described earlier) with 5 μM Rock inhibitor and 0.5 μg/ml Amphotericin B (Gibco 15290018). Amphotericin B prevents fungal contamination and can be omitted if the colony picking environment is sterile (e.g., the stereo microscope is placed in a sterile hood).
  - o Remove the Matrigel/Geltrex solution from the 24-well plates. Distribute 500 μl of prepared E8 flex medium in each well of the 24-well plates.
  - o Under a stereo microscope (Nikon SMZ1500), examine the surviving colonies and select the ones with circular shape, smooth and well-defined edge, single layer and uniformed organization of cells. Using an ethanol-wiped pipetman with a 10 μl sterile filtered tip (USA Scientific 1180-3810), scrap the selected colony in stripes carefully under the microscope without touching other colonies.
  - o Aspirate the stripes of the picked colony carefully under the microscope and place them in a well of the coated 24-well plates. Label the well with a check mark on the lid, to avoid cross-contamination.
  - o Repeat the picking process for other undifferentiated colonies until the number of desired colonies is reached (48–72 colonies for homozygous and 24 for heterozygous).
  - o Rock the plates back and forth several times to evenly distribute the scraped cells.
  - o (optional) Spin down the plates in a swinging-bucket tabletop centrifuge at 200g for 4 min.
  - o Place the 24-well plates in a 37 °C 5% CO_2 incubator.
- Maintaining the picked colonies
  - o Change medium on the next day with regular E8 flex medium without Rock inhibitor and Amphotericin B.
  - o Perform medium change every other day until the cells are confluent enough to be passaged. Please note that different wells will reach confluency at different days.
  - o Examine the wells that are ready to be passaged under the microscope. Mark the wells with cells that have good stem cell morphology (smooth colony edge, tight cell-to-cell contact in each colony and so on). Omit the wells that have a significant fraction of undifferentiated cells. Only proceed with the marked wells for passaging.
  - o Aspirate the medium, wash cells once with dPBS, and add 300 μl 0.5 mM EDTA in dPBS. Incubate at 37 °C for 7 min, aspirate the EDTA solution, and resuspend the detached cells in 1 ml E8 flex medium.
  - o Split the 1 ml resuspended cells into two fractions: 400 μl for cryo-preservation and 600 μl for genomic DNA extraction.
  - o For cryo-preservation, mix the 400 μl resuspended cells with 500 μl E8 flex medium and 100 μl DMSO. Perform slow freezing as described earlier.
  - o Spin down the remaining 600 μl at 800 g for 5 min. Discard the supernatant and store the pellet in −20 °C until ready for genomic DNA extraction.

2.5. Validation of successful integration using PCR and western blot

PCR validation is key to characterization of the guided repair at the Cas9 cleavage site, and it can help to determine the homozygous or heterozygous status of the mutation. Besides the conventional PCR approaches, droplet digital PCR (ddPCR) can be used to decipher homozygous from heterozygous mutations with precision and sensitivity [18]. Sanger sequencing of the PCR products is recommended to further validate the sequence alteration. We have adapted a previously developed quick genomic DNA extraction procedure [19], and here we describe a comprehensive protocol for genomic DNA PCR.

While correct repair at the DNA level is primary, correct expression of the edited gene also needs to be examined. This is important because mRNA processing, translation and protein stability may be perturbed as a result of a DNA sequence alteration.

- Genomic DNA PCR
  - o Supplement the lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, stored at room temperature) with 100 μg/mL Proteinase K (Invitrogen AM2544)
  - o Resuspend the cell pellet in 200 μl supplemented lysis buffer. Incubate at 55 °C for 30 min.
  - o Add 200 μl isopropanol to each tube at room temperature. Invert several times to mix. A white precipitate may be visible.
  - o Spin down at 13,000 g for 15 min.
  - o Aspirate the supernatant and air-dry the pellet for 3–5 min.
  - o Resuspend the pellet in 20 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8). Incubate the solution at 55 °C for 30 min to dissolve the nucleic acids.
  - o Set up two PCR reactions (e.g., primer pairs P1 + P2 and P1 + P3 in Fig. 3A) for each clone and a parental genomic DNA control: 0.02 U/μl Phusion DNA polymerase (Thermo Scientific F530), 1X Phusion GC buffer, 200 μM each dNTPs, 3% DMSO, 0.5 μM each primer, 0.5 μl genomic DNA in a 20 μl reaction.
  - o Proceed with the following thermal cycles: 98 °C for 30 s; 30 cycles of {98 °C for 10 s, X(calculated using the ThermoFisher Tm calculator)°C for 30 s and 72 °C for 30 s per kilobase of the amplicon}; 72 °C for 30 s; 4 °C hold.
  - o Add 4 μl 6X loading dye (New England Biolabs B7024S), and load 12 μl on a 1% agarose 1XTAE gel with 0.5 μg/mL ethidium bromide (Sigma-Aldrich E1510). Also load a lane with 1 μg of the 1 kb plus DNA ladder (Invitrogen 10787018). Run gel at 120 V for 35 min (adjust the time according to amplicon sizes). Visualize the gel using an Alpha Innotech FluorChem HD2 chemiluminescent imager (ProteinSimple, Inc).
  - o PCR fragments for validated clones are gel-extracted using a gel extraction kit (Omega Bio-Tek D2500-02) and the DNA is sequenced by the Sanger method.
- Western blot to validate expression
  - o PCR-validated clones are thawed and grown in E8 flex medium. For well-behaved (undifferentiated and proliferating normally) clones, cells are passaged to two wells in 6-well plates. One well is used for cryo-preservation or downstream applications, while the

other one is used for western blot analysis of the protein product of the edited gene.
- o At cell confluency, aspirate the medium and add 20 μl lysis buffer (1X NuPAGE LDS Sample Buffer (Invitrogen NP0008) supplemented with 1.25 unit of Benzonase (Millipore E1014, to degrade DNA and RNA)) directly to the adherent cells.
- o Incubate the 6-well plate in a 37 °C incubator for 10 min. Rock the plate every 3 min.
- o Transfer the lysate to a 1.7 ml Eppendorf tube and resume 37 °C incubation for another 20 min.
- o The lysate can be immediately used for western blot analysis or stored at −20 °C.
- o For western blot analysis, load 10 μl of each lysate in a NuPage 4–12% Bis-Tris gel (Invitrogen # NP0322BOX, 12 well) and include a protein ladder (SeeBlue Plus2 Pre-stained Protein Standard, Invitrogen LC5925).
- o Run gel(s) at 140–150 V for 1 h in 1X MES running buffer (Invitrogen NP000202).
- o Transfer the proteins from the gel to a nitrocellulose membrane (Amersham Protran 0.45 NC, GE Healthcare 10600002) in cold 1X Western buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS). Run at 0.5 Amp constant for 1 h.
- o Disassemble and examine that the pre-stained protein ladder has been transferred to the nitrocellulose membrane.
- o Place the membrane in a square petri dish, and block the membrane with 10 ml blocking buffer for 15 min (StartingBlock T20 PBS Blocking, Thermo Scientific 37539).
- o Discard the blocking buffer and add 10 ml of diluted primary antibody in the blocking buffer. Incubate overnight with rotation.
- o Remove the primary antibody solution and store it at 4 °C with a supplement of 0.02% sodium azide (can be used for at least 5 more times). Wash the membrane with PBST (1X PBS pH 7.4, 0.05% Tween) for three times.
- o Add secondary antibody diluted in the blocking buffer. Incubate for one hour with rotation.
- o Wash the membrane with PBST for three times, and 1XPBS once.
- o Develop western with SuperSignal West Pico Chemiluminescent Substrate kit (Termo Scientific 34080). Mix 5 ml of SuperSignal West Pico Stable Peroxide Solution (#1856135) and 5 ml of SuperSignal West Pico Lumino/Enhancer Solution (#1856136) first, and then add the 10 ml solution to the membrane.
- o Wait for one min, and image with an Alpha Innotech FluorChem HD2 chemiluminescent imager.

- Verified iPSC clones can be sent to WiCell (https://www.wicell.org) for karyotyping to confirm that there is no genome abnormity. Pluripotency of the clones can be examined by immunofluorescence using SOX2, OCT4 and SSEA4 antibodies as described previously [20]. Moreover, p53 loss of function and other associated effects have been seen in CRISPR-manipulated stem cells [21]. To help guard against genome sequence mutations acquired during the CRISPR process, which could be misleading, multiple independent clones should be obtained and tested.
- The validated clones are now ready for downstream analysis, which can include directed differentiation, next-generation sequencing, and proteomic analysis.

2.6. Optional removal of the LoxP cassette using Cre

The *LoxP*-flanked cassette can be removed by transfection of the Cre recombinase. This transfection can be performed after validation of the single clones from the last step, but more efficiently it can be performed immediately in the polyclonal population after the puromycin selection. The latter avoids repeating the clonal screening steps and significantly saves time and effort.

- o [continued from the end of 2.3] All colonies in the 10-cm petri dish are detached using 0.5 mM EDTA in dPBS. Half of cells are split into a well of a 6-well plate for Cre transfection, while the other half are cryo-preserved in E8 flex medium with 10% DMSO.
- o On the next day, transfect the cells with a Cre expression plasmid with a Blasticidin S resistance gene (e.g., addgene 140284). Transfection protocols are same as described earlier.
- o Blasticidin S selection is performed similar to what was described earlier for puromycin, except for the concentration: 2 μg/mL blasticidin is applied on the first day of selection, 1 μg/mL on the second and third day (drug concentration is reduced for the same reason as mentioned above in the puromycin selection), and 0 μg/mL afterwards.
- o Clonal screening and western validation are performed exactly as described earlier.

3. Results

Here we describe comprehensive guides and examples of three main schemes of genome editing to introduce mutations (Fig. 1). Two important features of the design are:

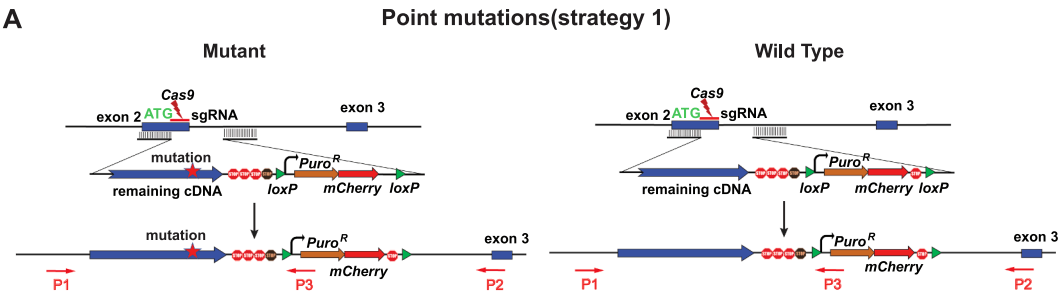
(a) Construction of a WT control alongside the mutant is essential for downstream analysis. Other sequence changes beyond the desired mutations have been made to the genome. Introns have been removed, and they can contain regulatory sequences. An additional DNA sequence (*LoxP*) has been added as well. Moreover, recent studies have shown that CRISPR editing generates unexpected changes to the genome [13,22,23], and therefore for mutational study it is extremely important to obtain a WT control that is edited exactly the same way except for the mutated sequences. In addition, the Cre transfection could lead to significant alteration to the cell [24], and a WT control that has undergone the same Cre transfection step is much more justified as the control than the parental cell lines.

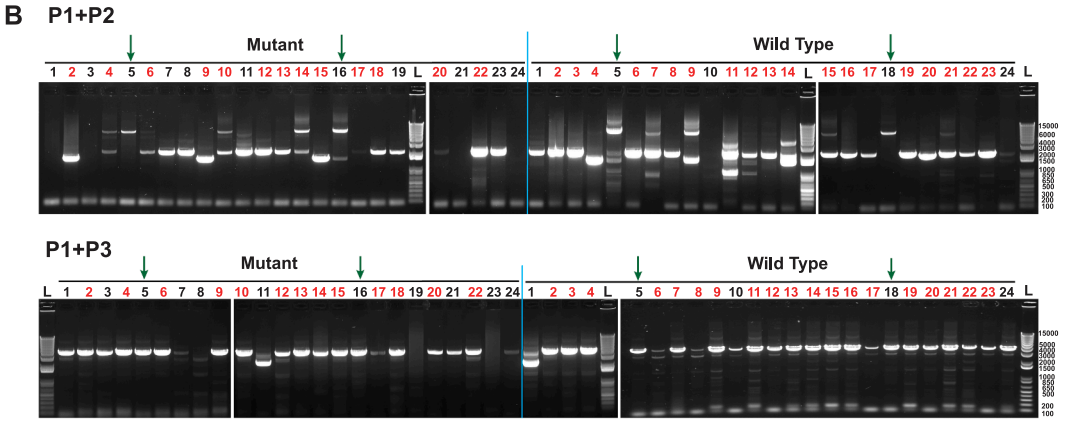
(b) Application of drug selection is usually more efficient than protocols relying on screening. We found that drug selection often eliminates the vast majority of the cells after transfection of the CRISPR and donor plasmids, indicating that most cells do not have the donor DNA integrated as desired. Clonal screening using PCR in this unselected population would cost significantly more effort, and many clones would need to be screened to find the rare successful integration events.

(c) Regarding the CRISPR system, we have chosen the conventional Cas9 from *Streptococcus pyogenes* (SpCas9), which has been widely used in editing of mammalian genomes due to the commonly found PAM sequence (*NGG*). The conventional SpCas9 generates a double-stranded break using one sgRNA. In contrast, SpCas9 D10A nickase has been engineered to increase specificity of Cas9 cleavage, due to the requirement of two adjacent single-stranded nicks to generate a double-stranded break [4,25,26]. Both regular and nickase version of the SpCas9 can be used in the strategies described in this study. We used the regular Cas9 for examples described in Figs. 3 and 5 and the Cas9 nickase in Fig. 4. The Sanger Institute CRISPR finder (https://wge.stemcell.sanger.ac.uk//find_crisprs) offers location-oriented guide RNA selection for both the regular and nickase versions of SpCas9 on a genome browser with customized specificity filters [27], and thus it is extremely suitable for selecting guide RNAs in our approach.

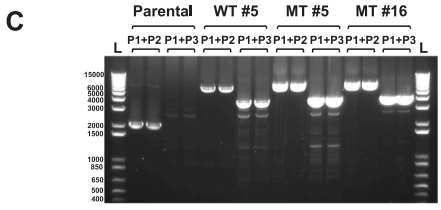
3.1. Manipulation of point mutations in individual genes

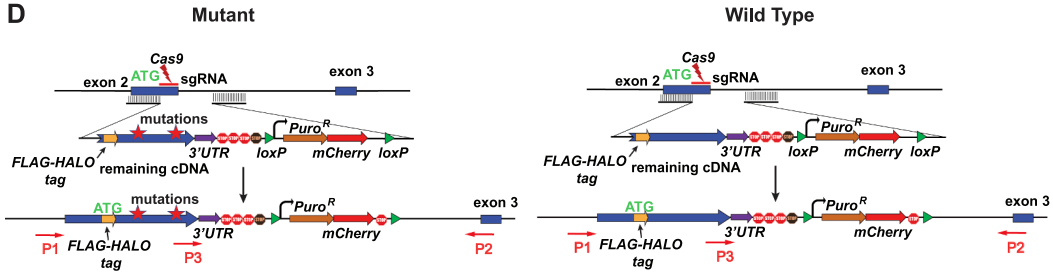
Manipulation (removal or introduction) of point mutations has been vital to modern biomedical research. These mutations include disease mutations, loss-of-function mutations and gain-of-function mutations,

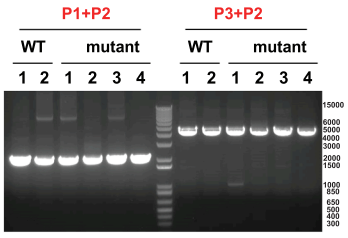




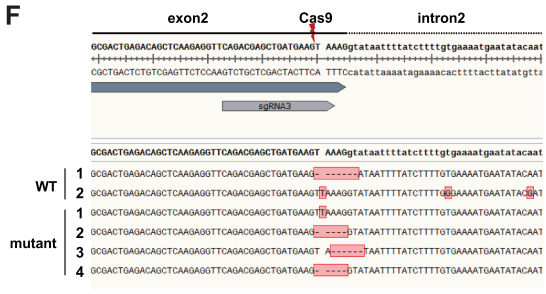
Amplicon	Edited	Parental
P1+P2	6797 bp	1925 bp
P1+P3	4066 bp	N/A

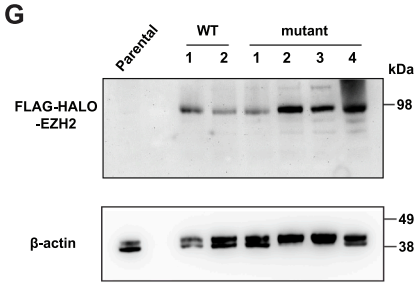






Amplicon	Edited	Parental
P3+P2	4491 bp	N/A
P1+P2	8605 bp	2154 bp





(caption on next page)

Fig. 3. Genome editing to introduce point mutations in *EZH2* gene in human iPSCs. A. Schematic design of the editing strategy. Red arrows (P1, P2 and P3) indicate the binding sites of the primers used in the PCR validation. B. PCR screening of 24 mutant and 24 wild type clones using P1 + P2 or P1 + P3 as the primer pair. PCR products were resolved on a 1% agarose TAE gel, and a 1 kb plus DNA ladder (Invitrogen) was used as the marker. Green arrows indicate potential homozygous clones, and red clone numbers indicate heterozygous clones. Size (base pairs) of representative bands in the ladder is labeled on the side the gels. Expected sizes of the PCR products in either edited or parental cells are shown in the table below the gels. C. PCR validation of selected clones (WT#5, MT#5&16) with parental cells as the negative control. D. Schematic design for introducing mutations at two distant sites of the *EZH2* gene. At the same time, 3XFLAG- and Halo-tags are added at the N-terminus of the *EZH2* ORF. E. PCR validation of six clones (2 wt and 4 MT) picked after the puromycin selection. Expected sizes (base pairs) of PCR products are listed in the table beside the gel. F. Sanger sequencing of the major P1 + P2 PCR product from all six clones indicated indels (shaded pink) at the Cas9 cleavage site for all six clones. Parental sequence is shown on the top and red arrow indicates the Cas9 cleavage site. Alignment was generated by the SnapGene software. G. Western blot analysis of the lysates from the six clones using anti-FLAG antibody. β-actin is used as the loading control. The purpose here was to assess successful genome editing at the protein level; differential expression of the edited gene may exist among clones.

and introduction of these mutations in vivo plays a key role in understanding their functional consequences. Previous studies have successfully introduced point mutations using single-stranded donor oligodeoxynucleotides (ssODN) as the donor DNA for guided repair [28–30]. However, the fraction of successfully mutated cells is generally extremely low due to the lack of selection, and clonal screening can be very labor and time consuming [30,31].

Here we describe two strategies for introducing point mutations (Fig. 1A and B), both of which utilize drug selection to achieve high efficiency of guided repair. Strategy 1 includes a single CRISPR-Cas9 cleavage at an early exon (usually exon 2 to preserve at least one intron for appropriate mRNA processing) followed by insertion of all remaining cDNA sequences at the cleavage site using homologous recombination. This strategy does not require a second step to remove the selection cassette, which simplifies the experimental procedure. In addition, the cDNA fusion conveniently enables mutations of several distant locations at the same time and the addition of epitope tags for downstream applications (e.g., live-cell imaging). This strategy has been used in two of our recent studies [20,32]. Although Strategy 1 serves as our primary method for introducing point mutations, it could be difficult when editing genes with extremely large size (for example, *TTN*, the gene coded for titin) due to the overwhelming size of the donor plasmid. For editing genes with large cDNA, Strategy 2 is very advantageous. This strategy similarly employs a single Cas9 cleavage site and introduces the mutation at one of two homology arms of the donor DNA (Fig. 1B). The selection cassette is inserted in the intron adjacent to the mutated site. Removal of the selection cassette with Cre may not be required if the cassette is inserted in an antisense direction of the edited gene, but it could still be optimal to leave only a *LoxP* scar in the intron because strong transcription from the *LoxP* cassette may cause abnormal gene expression in nearby genomic areas [33].

The optimal Cas9 cleavage site is at the exon-intron junction at the 3′-end of the edited exon (exon 2 for Strategy 1 and the exon to be mutated for Strategy 2). We choose to cleave the exon-intron junction, rather than regions inside the exon, to facilitate the insertion of the complete donor DNA sequence. If the cleavage occurred in the middle of an exon, homologous recombination could occur directly using the cDNA sequence (for Strategy 1) or only the left homology arm (for Strategy 2), leaving out most of the donor sequences including the entire selection cassette. As shown in previous studies, this misguided repair happens because HDR repair can efficiently occur using as short as 35 bp sequences that match the two sides of the dsDNA break created by Cas9 cleavage [34–36]. This cleavage site design strategy applies to all protein-coding genes with the purpose of avoiding misguided HDR repair.

To test Strategy 1, we aimed to mutate three residues in the *EZH2* gene in human iPSC strain WTC-11, generating the homozygous mutant to study the physiological importance of these three residues. A CRISPR plasmid encoding the SpCas9 and the guide RNA was made by inserting the sgRNA sequence (CAGACGAGCTGATGAAGTAA) targeting the exon 2 – intron 2 junction of the *EZH2* gene in pX330 (Addgene 42230 [3]). Two donor plasmids carrying either the WT or mutant *EZH2* cDNA were made by assembling the following fragments into a previously described donor plasmid [12]: left homology arm (-951 to −14, relative to the ATG start codon), remaining *EZH2* cDNA, *EZH2* 3′UTR (872 bp immediately after the stop codon), 3X SV40 polyadenylation sites, 1X bGH polyadenylation site, SV40 promoter, puromycin resistance ORF, T2A self-cleavage site, mCherry ORF, SV40 polyadenylation site and right homology arm (+25 to +830, relative to the ATG start codon).

We co-transfected the CRISPR and donor plasmids into the WTC-11 strain, and we also included a donor plasmid only control to differentiate permanent integration from the transiently present drug resistant gene. After two weeks of drug selection, distinct colonies appeared on both WT and mutant plates, while the control transfection with only the donor plasmids usually had no or many fewer colonies. 24 colonies were picked from each WT and mutant plate, and PCR was performed with their genomic DNA to characterize the integration. Three primers were used in the PCR characterization: P1 and P2 binding sites are outside of the two homology arms, and P3 matches sequence in the promoter of the selection cassette (Fig. 3A). The original screening identified two homozygous clones of WT and mutant, with most of the remaining clones being heterozygous (Fig. 3B). While both homozygous and heterozygous clones have a P1 + P3 PCR product of 4066 bp, they could be differentiated by the P1 + P2 PCR product based on the absence (homozygous) or presence (heterozygous) of the 1925 bp band. Homozygous clones are characterized by the presence of a 6797 bp band and the absence of the 1925 bp band (for example, mutant #5, 16, and wild type #5, 18). However, the 6797 bp band could be missing in the P1 + P2 PCR product of some homozygous clones, due to the difficulty in amplifying large genomic fragments. Therefore, MT #1, 3, 21 and WT #10, 24 may be homozygous clones as well, since they are all missing the 1925 bp P1 + P2 product but contain the 4066 bp P1 + P3 product. In summary, out of the 48 clones that we picked for WT and MT strains, 4 (or 9) of them (8 or 19%) are likely to be homozygous clones, while 31 of them (65%) are heterozygous clones. These heterozygous clones only have one allele correctly integrated, while the other allele is either unedited or has undergone non-homologous end joining (NHEJ) at the Cas9 cleavage site. We continued the culture of two MT and one WT clones for further studies, and PCR validation was performed again to confirm the homozygous status of these clones at this later stage (Fig. 3C). These clones exhibit a convincing homozygous genotype as illustrated by the clean single 6797 bp band in the P1 + P2 PCR product. Because a PCR product of the correct length could still contain indels or other mutations, we always sequence the PCR product before using the clone for downstream analysis [20,37].

We also tested our strategy for mutating multiple sites and adding epitope tags. We aimed to mutate two distant sites in the *EZH2* gene and add two epitope tags (3XFLAG- and Halo-Tags) at the beginning of the open reading frame. Conventional approaches would require at least three steps of genome editing to make the final product, but here we used only a single step to achieve the goal. The CRISPR plasmid including the guide RNA was exactly the same as used in Fig. 3A-C. The WT donor DNA encoded N-terminal Halo- and 3XFLAG-tags similar to what was used previously [38,39], and the MT donor DNA also included the mutations at two distant sites of the *EZH2* gene (site 1: F32A R34A D36A K39A; site 2: PRKKKR489-494NAAIRS) in addition to the epitope tags (Fig. 3D). Because heterozygous clones were sufficient for the downstream imaging and immunoprecipitation applications, we only

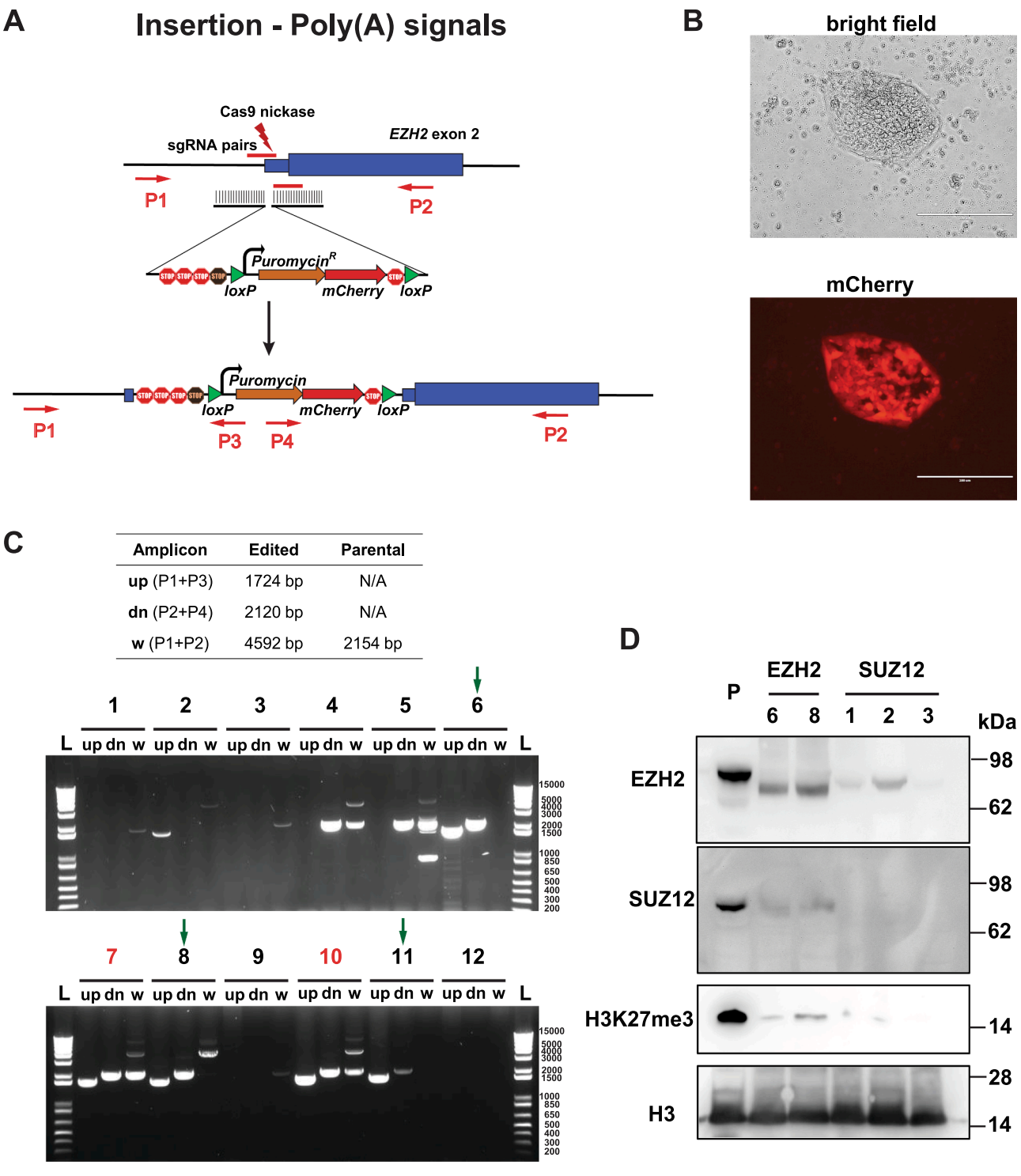


Fig. 4. Insertion of Polyadenylation signals at the start of *EZH2* and *SUZ12* genes in human iPSCs. Schematic design of the poly(A) signal insertion in *EZH2* gene for transcription perturbation. Poly(A) signals are indicated with the STOP signs, and PCR validation primers (P1-4) are marked with red arrows. B. bright field and mCherry fluorescence images of a representative iPSC colony after a week of puromycin selection. Scale bar represents 200 μm. C. PCR screening of 12 clones in the EZH2 poly(A) signal insertion experiment. Three primer combinations (up: P1 + P3; dn: P2 + P4; w: P1 + P2) were used in the PCR. Expected PCR product size for edited or unedited (parental) cells are shown in the table. Green arrows indicate potential homozygous clones, and red clone numbers indicate heterozygous clones. D. Western blot analysis of two *EZH2* insertion and three *SUZ12* insertion clones using anti-EZH2, SUZ12, H3K27me3 and H3 antibodies. H3 level is used as a loading control. In the EZH2 blot, the proteins that run faster than EZH2 in lanes 6, 8 and 2 have not been identified, and could be truncated EZH2 polypeptides initiated from a downstream alternative transcription start site or potential contaminants.

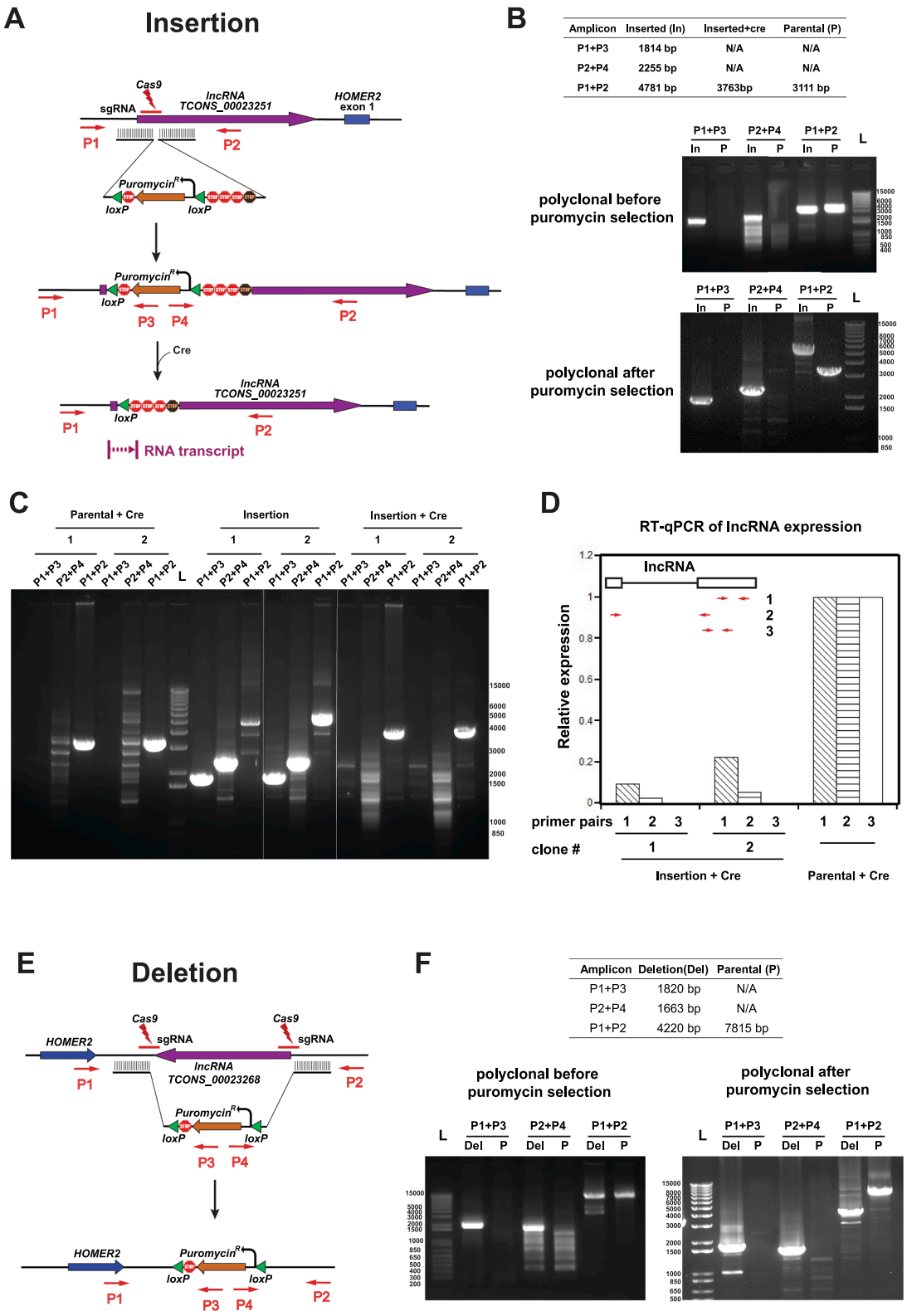


Fig. 5. Application of insertion and deletion editing in HEK293T cells. A. Schematic design of poly(A) signal insertion near the TSS of a lncRNA gene (*TCONS_00023251*). PCR primer binding sites are labeled with red arrows. Dashed arrow at bottom shows approximate truncated transcript (which could truncate at any of the poly(A) sites). B. Genomic PCR analysis of the polyclonal population before and after the puromycin selection. Three primer pairs (see table) were used. C. PCR validation of two clones from each experiment (Parental + Cre control, Insertion, Insertion + Cre). D. RT-PCR analysis of the lncRNA gene in the two clones of final edited product (Insertion + Cre) and a negative control (Parental + Cre). Three primer pairs targeting different regions of the lncRNA were used, and lncRNA levels were normalized to GAPDH mRNA and the negative control. E. Schematic design of the deletion of a lncRNA gene (*TCONS_00023268*). Cas9 cleavage sites flank the gene, which is replaced by a puromycin-resistance gene expression cassette using homologous recombination. F. Genomic PCR analysis of the polyclonal population before and after the puromycin selection with the expected PCR product sizes listed in the table.

needed to characterize the genotypes of two WT and four MT clones using PCR (Fig. 3E). P1 + P2 are same as the ones used in the previous experiment, and P3 is a new forward primer matching the puromycin-resistant gene sequence. The P3 + P2 PCR products of all six clones exhibited a 4491 bp band, indicating successful integration at one allele at least of the *EZH2* gene. The P1 + P2 PCR products of all clones included the parental size 2154 bp band, suggesting that the other allele of all clones did not contain the insertion of the donor DNA sequence. P1 + P2 PCR on three of clones (WT #2, MT#1 and 3) successfully picked up the edited 8605 bp band, amplified from the inserted allele. However, PCR reactions are usually strongly biased towards amplifying the smaller amplicons, making detection of the 8605 bp band difficult. When using CRISPR to target a gene locus, it is always essential to understand what is occurring on both alleles of an autosomal gene. In addition to correct targeting one allele, more often than not, the other allele is mutated with an indel mutation. To test this "indel" hypothesis, we further gel-extracted the 2154 bp P1 + P2 product band, and Sanger sequencing indicated that all the "parental" alleles contained indels that would cause frame shift in the parental allele (Fig. 3F). WT #2 and MT #1 contained a single T insertion in exon 2, WT #1 and MT #2&4 contained a 5-nucleotide deletion at the end of exon 2, and MT #3 had a 6-nucleotide deletion at the exon-intron junction (3 in exon and 3 in intron). These sequencing results indicated that the uninserted allele would not express a functional WT EZH2 protein, making these heterozygous clones suitable for phenotypic and functional downstream studies. Lastly, the western blot using anti-FLAG antibody successfully detected the expression of the inserted allele in all clones (Fig. 3G).

3.2. Insertion of sequences at a specific genomic location

Mutagenesis via sequence insertion is an important tool for biological studies. For example, gain-of-function studies rely on the de-novo insertion of sequence elements in genes of interest; insertion of expression-control elements (e.g., doxycycline switches) at the promoter region allows regulation of gene expression; and loss-of-function can be achieved by insertion of polyadenylation (poly(A)) signals immediately downstream of the transcription start site of genes. Furthermore, insertion of sequences encoding epitope tags allows the protein product to be tracked or recovered by immunoprecipitation. Finally, insertion of sequences encoding fluorescent proteins or the HaloTag allows cyto-localization and live-cell imaging.

Our insertion strategy (Fig. 1C) again involves the incorporation of a *LoxP*-flanked selection cassette using homologous recombination (e.g., a puromycin-resistance gene for drug selection and a mCherry gene for fluorescence-activated cell sorting). Removal of the *LoxP* cassette using Cre recombinase may be unnecessary if the cassette has been inserted in intronic or intergenic regions, but is absolutely essential if the cassette is in exonic regions even if it is oriented in the antisense direction of the gene. The WT control is made exactly the same way without the inserted sequences (for the example of poly(A) insertion in Fig. 1C, the poly(A) sequences are missing in the WT).

Here we applied our CRISPR editing scheme to insertion of poly(A) signals just downstream from the promoters of *EZH2* and *SUZ12* genes, in order to curtail their expression in human iPSCs [40–46]. In this case we utilized the engineered SpCas9 D10A nickase (in pX335, addgene 42335) to achieve the best specificity and least off-target effects. Guide RNA pairs were designed using the Sanger Institute CRISPR finder, aiming at creating the double-stranded break adjacent to the ATG start codon. Donor plasmids contained left and right homology arms flanking the two DNA nicks. Four polyadenylation signals (3X SV40 and 1X bGH poly(A) signals) were inserted before the *LoxP*-flanked puromycin-resistant gene and mCherry expression cassette (Fig. 4A). The usage of multiple poly(A) signals is a much underappreciated factor of successful transcription termination, and previous studies have shown than multiple tandem poly(A) signals are often necessary for eliminating transcription [47,48]. Post-transfection puromycin selection eliminated most cells, and surviving cells grew into individual colonies with distinct boundaries. Most of these colonies expressed the mCherry signal (Fig. 4B), which could be used for flow cytometry cell sorting in place of the puromycin drug selection or for enriching the cells that lose the *LoxP* cassette after Cre transfection (if necessary). For *EZH2* poly(A) insertion, PCR characterization of 12 picked clones identified one homozygous (#8, and two potentially homozygous, #6 and 11) and two heterozygous (#7 and 10) clones (Fig. 4C). Correct integration was characterized by the 1724 bp band in the up (P1 + P3) PCR reaction and 2120 bp band in the dn (P2 + P4) reaction. Heterozygous clones showed two bands in the w (P1 + P2) PCR reaction, while homozygous clones were missing the smaller parental band (2154 bp). Several other clones may have had correct integration as well, but failure to amplify up or dn PCR reaction was concerning. Similar results were obtained for the *SUZ12* insertion experiment, and we proceeded with five homozygous clones from *EZH2* and *SUZ12* poly(A) insertion experiments. Western blot analysis indicated the poly(A) signal insertion abolished expression for all edited genes (Fig. 4D). The residual band of smaller size in the EZH2 blot is clearly non-functional (unable to maintain H3K27me3 level) and is potentially a truncated isoform that is translated from an mRNA product initiated from a downstream transcription start site. Since EZH2 and SUZ12 are inter-dependent for formation of a protein complex (Polycomb Repressive Complex 2, or PRC2), both EZH2 and SUZ12 proteins were greatly diminished in all five clones even though only one of the two genes was inactivated. PRC2′s catalytic product – trimethylation of lysine 27 in histone H3 (H3K27me3) – was also much diminished in all five clones compared to the parental iPSCs (Fig. 4D).

Conveniently, the same mutagenesis strategy including this insertion approach can also be applied in other cell types. For example, we used a very similar approach to insert poly(A) signals in a long noncoding RNA (lncRNA) gene in human HEK293T cells. We also included the *Cre* step to show the convenience of performing the *LoxP* cassette removal. Regular SpCas9 was used in this experiment, and the guide RNA targeted the sequence immediately preceding the transcription start site (TSS) of the lncRNA *TCONS_00023251*. Donor DNA was designed similarly except that: [a] the poly(A) signals were inserted after the *LoxP* cassette, [b] the mCherry gene was omitted, and [c] the puromycin expression cassette was oriented in the reverse direction (Fig. 5A). PCR characterization was performed before and after the puromycin selection, to evaluate the efficiency and specificity of the selection. Surprisingly, the selection resulted in an almost completely homozygous population for all cells in the polyclonal pool, indicated by the shift of the P1 + P2 PCR band from 3111 bp to 4781 bp characteristic of the insertion (Fig. 5B). This suggests that the guided DNA repair via homologous recombination is extremely efficient for this genomic locus and strategic editing design in HEK293T cells.

To obtain single clones, we used flow cytometry and sorted the single cells in the polyclonal population after puromycin selection. Without much difficulty, two homozygous clones were verified in the PCR screening (Fig. 5C, insertion #1 and 2). For removal of the *LoxP* cassette, we transfected the post-selection polyclonal population with a Cre-GFP expression plasmid, and sorted single cells expressing GFP on the day after the transfection. Two homozygous clones were also obtained with the complete loss of the *LoxP* cassette on all alleles, which was indicated by the absence of any PCR product for P1 + P3 or P2 + P4 and the presence of a 3763 bp band in the P1 + P2 PCR reaction (Fig. 5C, Insertion + Cre #1 and 2). Acknowledging that Cre transfection could cause inevitable changes to cells [24,49–51], we also included a parental + Cre control, and two single clones were obtained using the same experimental flow starting from Cre transfection. To validate the loss of lncRNA expression by insertion of the poly(A) signals, we extracted the total RNA and performed RT-qPCR on the four clones with Cre transfection using three different primer pairs targeting this lncRNA. The results indicated that lncRNA expression was largely abolished (and certainly reduced to below 20% of the parental cell level) (Fig. 5D).

3.3. Deletion of a segment, exon/intron or the complete CDS of individual genes

Mutagenesis by deletion is common in loss-of-function studies. For example, to study the physiological effect of exon skipping, one would benefit from a homozygous mutant that deletes this skipped exon. Deletion genome editing is also important in studies of the function of specific genomic elements including sequence motifs, enhancers and insulators. Our deletion strategy (Fig. 1D) involves two Cas9 cleavage events flanking the region to be deleted, which is replaced by a *LoxP*-flanked selection cassette using homologous recombination. Designing the WT control for the deletion editing can be less straightforward: the best strategy is to use the identical Cas9 cleavage sites but include the deletion sequence in the donor DNA (right panel of Fig. 1D); however, if the region to be deleted is too large (>5 kb), the strategy to make the WT control can be adjusted to only use one of the two Cas9 cleavage sites and insert the *LoxP* cassette at this site. Removal of the *LoxP* cassette using Cre is often optional if the cassette is left in an antisense direction inside an intron.

Here we tested our deletion strategy to delete a lncRNA gene in HEK293T cells and demonstrated that it is very efficient. Two guide RNAs were designed to create two double-stranded breaks flanking the lncRNA gene *TCONS_00023268* using the regular SpCas9 (Fig. 5E). The donor plasmid contained two homology arms flanking the lncRNA genes and a puromycin-resistance gene expression cassette. We collected the polyclonal population before and after the puromycin selection and used three PCR pairs to characterize the genotype of the populations. Similar to the results in Fig. 5B, puromycin selection efficiently converted most parental alleles to deleted alleles, indicated by the downshift from a 7815 bp band before selection to a 4220 bp band after selection in the P1 + P2 PCR reaction of the Del sample (Fig. 5F).

4. Discussion

As genome-editing tools evolve rapidly, more and more biomedical investigations and applications require precise targeted mutagenesis of genomic loci. Human iPSCs have been one of the most popular and versatile platforms for these genome-editing experiments, due to their self-renewal ability, differentiation potential and ability to be customized to disease models or patients. In this study we describe a comprehensive guide for introducing precise mutations in human iPSCs, mutations which include point mutations, insertion and deletions. These experimental strategies are not only efficient in human iPSCs but can also be applied in other mammalian cell types including the HEK293T cells used in our study.

Here we used Cas9 as the effector nuclease to create double-stranded breaks. SpCas9 recognizes an NGG PAM sequence and generates blunt ends at the break. The guided repair in our study utilizes homologous recombination, which requires proliferating cells. For editing non-dividing cells such as primary cells, homology-independent integration methods using Cas9 have been developed [52–54], and an approach using either zinc finger nucleases (ZFNs) or Tale nucleases (TALENs) has been reported as well [55]. Alternatively, Cpf1 could be a very useful option. Cpf1 creates a staggered cut with a 5′-overhang of four nucleotides, enabling the precise insertion of sequences with a matching sticky end [10,56]. In addition, Cpf1 recognizes a T-rich PAM sequence (TTTN), which is more widespread in some organisms including *Dictyostelium discoideum* [57] and *Plasmodium falciparum* [10].

Application of drug selection significantly enhances the efficiency of obtaining clones with successful integration [8,12,58,59]. The puromycin selection we used in human iPSCs generally eliminates more than 95% of cells that don't have the correct integration, and this makes clone screening hassle-free since most of the surviving cells are either homozygous or heterozygous clones. The selection is extremely important for difficult-to-transfect cells as the low delivery rate further reduces the editing efficiency. Puromycin serves as our top choice for drug selection due to its high specificity, and alternatively blasticidin S or hygromycin B could be used if multiple mutation experiments need to be performed in tandem. G418/Geneticin is not recommended because of its high false positive rate in mammalian cell selections. Drug selection may also increase the risk of unexpected insertions of the donor vector containing the resistance cassette [60]. Besides obtaining multiple clones for downstream applications, techniques including vector backbone PCR screening and Southern blot analysis can be performed to screen against such unexpected insertions.

Other selection-free strategies have also been developed and used to make the editing fully scarless and avoid the potential side effects of antibiotic selection. For example, a method has been developed by the Conklin lab to efficiently capture rare mutational events and enable isolation of mutant lines with single-base substitutions [61]. The editing efficiency in the selection-free strategies can also be enhanced using the pre-assembled ribonucleoprotein (RNP) delivery method, short ssDNA oligos or overexpression of other protein factors [62–64]. Thus, both selection-based and selection-free strategies are of great value to the research community, and which to use may depend on the specific needs and concerns of each experiment.

In summary, our study presents genome-editing strategies to introduce point mutations, insertions and deletions in human iPSCs, and provides a step-by-step guide to application of these strategies. Most of these editing strategies offer the convenience of one-step editing and effortless clone screening thanks to the selection cassette. We emphasize the importance of generating a WT control strain along with the mutant, which helps to filter-out any side-effects of CRISPR editing and the potential complexity of perturbing genome integrity and cellular homeostasis via the procedure.

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.

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Author contributions

Y.L. and T.R.C. conceived the study and designed experiments. Y.L. performed the experiments and analyzed the data. Y.L. and T.R.C. wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2021.01.002.

References

[1] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337 (6096) (2012) 816–821.

[2] G. Gasiunas, R. Barrangou, P. Horvath, V. Siksnys, Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria, Proc. Natl. Acad. Sci. U.S.A. 109 (39) (2012) E2579–E2586.

[3] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, W. Jiang, L.A. Marraffini, F. Zhang, Multiplex genome engineering using CRISPR/Cas systems, Science 339 (6121) (2013) 819–823.
[4] P. Mali, L. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. DiCarlo, J.E. Norville, G. M. Church, RNA-guided human genome engineering via Cas9, Science 339 (6121) (2013) 823–826.
[5] D. Carroll, Genome engineering with targetable nucleases, Annu. Rev. Biochem. 83 (1) (2014) 409–439.
[6] D. Carroll, K.J. Beumer, Genome engineering with TALENs and ZFNs: repair pathways and donor design, Methods 69 (2) (2014) 137–141.
[7] F. Zhang, L.e. Cong, S. Lodato, S. Kosuri, G.M. Church, P. Arlotta, Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription, Nat. Biotechnol. 29 (2) (2011) 149–153.
[8] D. Hockemeyer, R. Jaenisch, Gene targeting in human pluripotent cells, Cold Spring Harb. Symp. Quant. Biol. 75 (0) (2010) 201–209.
[9] J. Boch, TALEs of genome targeting, Nat. Biotechnol. 29 (2) (2011) 135–136.
[10] B. Zetsche, J. Gootenberg, O. Abudayyeh, I. Slaymaker, K. Makarova, P. Essletzbichler, S. Volz, J. Joung, J. van, der Oost, A. Regev, E. Koonin, F. Zhang, Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, Cell 163 (3) (2015) 759–771.
[11] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (4) (2006) 663–676.
[12] L.H. Xi, J.C. Schmidt, A.J. Zaug, D.R. Ascarrunz, T.R. Cech, A novel two-step genome editing strategy with CRISPR-Cas9 provides new insights into telomerase action and TERT gene expression, Genome Biol. 16 (2015) 231.
[13] M. Kosicki, K. Tomberg, A. Bradley, Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements, Nat. Biotechnol. 36 (8) (2018) 765–771.
[14] P. Chatterjee, Y. Cheung, C. Liew, Transfecting and nucleofecting human induced pluripotent stem cells, J Vis Exp. (56) (2011), https://doi.org/10.3791/3110.
[15] S.M. Byrne, P. Mali, G.M. Church, Genome editing in human stem cells, Methods Enzymol. 546 (2014) 119–138.
[16] S. Kim, D. Kim, S.W. Cho, J. Kim, J.-S. Kim, Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins, Genome Res. 24 (6) (2014) 1012–1019.
[17] P. Gee, M.S.Y. Lung, Y. Okuzaki, N. Sasakawa, T. Iguchi, Y. Makita, H. Hozumi, Y. Miura, L.F. Yang, M. Iwasaki, X.H. Wang, M.A. Waller, N. Shirai, Y.O. Abe, Y. Fujita, K. Watanabe, A. Kagita, K.A. Iwabuchi, M. Yasuda, H. Xu, T. Noda, J. Komano, H. Sakurai, N. Inukai, A. Hotta, Extracellular nanovesicles for packaging of CRISPR-Cas9 protein and sgRNA to induce therapeutic exon skipping, Nat. Commun. 11 (1) (2020), https://doi.org/10.1038/s41467-020-14957-y.
[18] S.D. Findlay, K.M. Vincent, J.R. Berman, L.-M. Postovit, T.T. Wang, A digital PCR-based method for efficient and highly specific screening of genome edited cells, PLoS ONE 11 (4) (2016) e0153901.
[19] P.W. Laird, A. Zijderveld, K. Linders, M.A. Rudnicki, R. Jaenisch, A. Berns, Simplified mammalian DNA isolation procedure, Nucl. Acids Res. 19 (15) (1991), 4293.
[20] Y. Long, T. Hwang, A.R. Gooding, K.J. Goodrich, J.L. Rinn, T.R. Cech, RNA is essential for PRC2 chromatin occupancy and function in human pluripotent stem cells, Nat. Genet. 52 (9) (2020) 931–938.
[21] F.T. Merkle, S. Ghosh, N. Kamitaki, J. Mitchell, Y. Avior, C. Mello, S. Kashin, S. Mekhoubad, D. Ilic, M. Charlton, G. Saphier, R.E. Handsaker, G. Genovese, S. Bar, N. Benvenisty, S.A. McCarroll, K. Eggan, Human pluripotent stem cells recurrently acquire and expand dominant negative P53 mutations, Nature 545 (7653) (2017) 229–233.
[22] H.Y. Shin, C. Wang, H.K. Lee, K.H. Yoo, X. Zeng, T. Kuhns, C.M. Yang, T. Mohr, C. Liu, L. Hennighausen, CRISPR/Cas9 targeting events cause complex deletions and insertions at 17 sites in the mouse genome, Nat. Commun. 8 (2017) 15464.
[23] R. Tuladhar, Y. Yeu, J. Tyler Piazza, Z. Tan, J. Rene Clemenceau, X. Wu, Q. Barrett, J. Herbert, D.H. Mathews, J. Kim, T. Hyun Hwang, L. Lum, CRISPR-Cas9-based mutagenesis frequently provokes on-target mRNA misregulation, Nat. Commun. 10 (1) (2019), https://doi.org/10.1038/s41467-019-12028-5.
[24] E.K. Pugach, P.A. Richmond, J.G. Azofeifa, R.D. Dowell, L.A. Leinwand, Prolonged Cre expression driven by the alpha-myosin heavy chain promoter can be cardiotoxic, J. Mol. Cell. Cardiol. 86 (2015) 54–61.
[25] B. Shen, W. Zhang, J. Zhang, J. Zhou, J. Wang, L.i. Chen, L.u. Wang, A. Hodgkins, V. Iyer, X. Huang, W.C. Skarnes, Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects, Nat. Methods 11 (4) (2014) 399–402.
[26] P. Mali, J. Aach, P.B. Stranges, K.M. Esvelt, M. Moosburner, S. Kosuri, L. Yang, G. M. Church, CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering, Nat. Biotechnol. 31 (9) (2013) 833–838.
[27] A. Hodgkins, A. Farne, S. Perera, T. Grego, D.J. Parry-Smith, W.C. Skarnes, V. Iyer, WGE: a CRISPR database for genome engineering, Bioinformatics 31 (2015) 3078–3080.
[28] F. Chen, S.M. Pruett-Miller, Y. Huang, M. Gjoka, K. Duda, J. Taunton, T. N. Collingwood, M. Frodin, G.D. Davis, High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases, Nat. Methods 8 (9) (2011) 753–755.
[29] L. Yang, M. Guell, S. Byrne, J.L. Yang, A. De Los Angeles, P. Mali, J. Aach, C. Kim-Kiselak, A.W. Briggs, X. Rios, P.-Y. Huang, G. Daley, G. Church, Optimization of scarless human stem cell genome editing, Nucl. Acids Res. 41 (2013) 9049–9061.
[30] J. Lin, K. Musunuru, Genome engineering tools for building cellular models of disease, FEBS J. 283 (17) (2016) 3222–3231.
[31] Q. Ding, S. Regan, Y. Xia, Leoníe A. Oostrom, C. Cowan, K. Musunuru, Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs, Cell Stem Cell 12 (4) (2013) 393–394.
[32] X. Wang, Y. Long, R.D. Paucek, A.R. Gooding, T. Lee, R.M. Burdorf, T.R. Cech, Regulation of histone methylation by automethylation of PRC2, Genes Dev. 33 (19-20) (2019) 1416–1427.
[33] J.M. Engreitz, J.E. Haines, E.M. Perez, G. Munson, J. Chen, M. Kane, P.E. McDonel, M. Guttman, E.S. Lander, Local regulation of gene expression by lncRNA promoters, transcription and splicing, Nature 539 (7629) (2016) 452–455.
[34] A. Paix, A. Folkmann, D.H. Goldman, H. Kulaga, M.J. Grzelak, D. Rasoloson, S. Paidemarry, R. Green, R.R. Reed, G. Seydoux, Precision genome editing using synthesis-dependent repair of Cas9-induced DNA breaks, Proc. Natl. Acad. Sci. U.S. A. 114 (50) (2017) E10745–E10754.
[35] A. Hayashi, K. Tanaka, Short-homology-mediated CRISPR/Cas9-based method for genome editing in fission yeast, G3 (Bethesda) 9 (2019) 1153–1163.
[36] O. Kanca, J. Zirin, J. Garcia-Marques, S.M. Knight, D. Yang-Zhou, G. Amador, H. Chung, Z. Zuo, L. Ma, Y. He, et al., An efficient CRISPR-based strategy to insert small and large fragments of DNA using short homology arms, eLife 8 (2019).
[37] X. Wang, Y. Long, R.D. Paucek, A.R. Gooding, T. Lee, T.R. Cech, Regulation of histone methylation by automethylation of PRC2, bioRxiv 343020 (2018).
[38] J.C. Schmidt, A.J. Zaug, T.R. Cech, Live cell imaging reveals the dynamics of telomerase recruitment to telomeres, Cell 166 (5) (2016) 1188–1197.e9.
[39] D.T. Youmans, J.C. Schmidt, T.R. Cech, Live-cell imaging reveals the dynamics of PRC2 and recruitment to chromatin by SUZ12-associated subunits, Genes Dev. 32 (11-12) (2018) 794–805.
[40] N.J. Proudfoot, G.G. Brownlee, 3' non-coding region sequences in eukaryotic messenger RNA, Nature 263 (5574) (1976) 211–214.
[41] N.J. Proudfoot, J.I. Longley, The 3' terminal sequences of human alpha and beta globin messenger RNAs: comparison with rabbit globin messenger RNA, Cell 9 (1976) 733–746.
[42] F. Zhang, R.M. Denome, C.N. Cole, Fine-structure analysis of the processing and polyadenylation region of the herpes simplex virus type 1 thymidine kinase gene by using linker scanning, internal deletion, and insertion mutations, Mol. Cell. Biol. 6 (12) (1986) 4611–4623.
[43] M.D. Sheets, S.C. Ogg, M.P. Wickens, Point mutations in AAUAAA and the poly (A) addition site: effects on the accuracy and efficiency of cleavage and polyadenylation in vitro, Nucl. Acids Res. 18 (19) (1990) 5799–5805.
[44] B. Tian, J. Hu, H. Zhang, C.S. Lutz, A large-scale analysis of mRNA polyadenylation of human and mouse genes, Nucl. Acids Res. 33 (2005) 201–212.
[45] T. Gutschner, M. Baas, S. Diederichs, Noncoding RNA gene silencing through genomic integration of RNA destabilizing elements using zinc finger nucleases, Genome Res. 21 (11) (2011) 1944–1954.
[46] Y. Yin, P. Yan, J. Lu, G. Song, Y. Zhu, Z. Li, Y.i. Zhao, B. Shen, X. Huang, H. Zhu, S. Orkin, X. Shen, Opposing roles for the lncRNA Haunt and its genomic locus in regulating HOXA gene activation during embryonic stem cell differentiation, Cell Stem Cell 16 (5) (2015) 504–516.
[47] D.B. Batt, Y. Luo, G.G. Carmichael, Polyadenylation and transcription termination in gene constructs containing multiple tandem polyadenylation signals, Nucl. Acids Res. 22 (14) (1994) 2811–2816.
[48] Y. Liu, X. Han, J. Yuan, T. Geng, S. Chen, X. Hu, I.H. Cui, H. Cui, Biallelic insertion of a transcriptional terminator via the CRISPR/Cas9 system efficiently silences expression of protein-coding and non-coding RNA genes, J. Biol. Chem. 292 (14) (2017) 5624–5633.
[49] V. Janbandhu, D. Moik, R. Fässler, Cre recombinase induces DNA damage and tetraploidy in the absence of loxP sites, Cell Cycle 13 (3) (2014) 462–470.
[50] P.T. Lam, S.L. Padula, T.V. Hoang, J.E. Poth, L. Liu, C. Liang, A.S. LeFever, L. M. Wallace, R. Ashery-Padan, P.K. Riggs, J.E. Shields, O. Shaham, S. Rowan, N. L. Brown, T. Glaser, M.L. Robinson, Considerations for the use of Cre recombinase for conditional gene deletion in the mouse lens, Hum. Genomics 13 (1) (2019), https://doi.org/10.1186/s40246-019-0192-8.
[51] K. Bersell, S. Choudhury, M. Mollova, B.D. Polizzotti, B. Ganapathy, S. Walsh, B. Wadugu, S. Arab, B. Kuhn, Moderate and high amounts of tamoxifen in alphaMHC-MerCreMer mice induce a DNA damage response, leading to heart failure and death, Dis. Model Mech. 6 (2013) 1459–1469.
[52] J.M. Geisinger, S. Turan, S. Hernandez, L.P. Spector, M.P. Calos, In vivo blunt-end cloning through CRISPR/Cas9-facilitated non-homologous end-joining, Nucl. Acids Res. 44 (2016), e76.
[53] K. Suzuki, Y. Tsunekawa, R. Hernandez-Benitez, J. Wu, J. Zhu, E.J. Kim, F. Hatanaka, M. Yamamoto, T. Araoka, Z. Li, M. Kurita, T. Hishida, M.o. Li, E. Aizawa, S. Guo, S. Chen, A. Goebl, R.D. Soligalla, J. Qu, T. Jiang, X. Fu, M. Jafari, C.R. Esteban, W.T. Berggren, J. Lajara, E. Nuñez-Delicado, P. Guillen, J. M. Campistol, F. Matsuzaki, G.-H. Liu, P. Magistretti, K. Zhang, E.M. Callaway, K. Zhang, J.C.I. Belmonte, In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration, Nature 540 (7631) (2016) 144–149.
[54] T.O. Auer, K. Duroure, A. De Cian, J.-P. Concordet, F. Del Bene, Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair, Genome Res. 24 (1) (2014) 142–153.
[55] M. Maresca, V.G. Lin, N. Guo, Y. Yang, Obligate ligation-gated recombination (ObLiGaRe): custom-designed nuclease-mediated targeted integration through nonhomologous end joining, Genome Res. 23 (3) (2013) 539–546.
[56] T. Yamano, H. Nishimasu, B. Zetsche, H. Hirano, I. Slaymaker, Y. Li, I. Fedorova, T. Nakane, K. Makarova, E. Koonin, R. Ishitani, F. Zhang, O. Nureki, Crystal structure of Cpf1 in complex with guide RNA and target DNA, Cell 165 (4) (2016) 949–962.
[57] R. Sekine, T. Kawata, T. Muramoto, CRISPR/Cas9 mediated targeting of multiple genes in Dictyostelium, Sci. Rep. 8 (2018) 8471.

[58] W. Supharattanasitthi, E. Carlsson, U. Sharif, L. Paraoan, CRISPR/Cas9-mediated one step bi-allelic change of genomic DNA in iPSCs and human RPE cells in vitro with dual antibiotic selection, Sci. Rep. 9 (2019) 174.

[59] M. Kaulich, Y.J. Lee, P. Lönn, A.D. Springer, B.R. Meade, S.F. Dowdy, Efficient CRISPR-rAAV engineering of endogenous genes to study protein function by allele-specific RNAi, Nucl. Acids Res. 43 (2015) e45.

[60] B.V. Skryabin, D.-M. Kummerfeld, L. Gubar, B. Seeger, H. Kaiser, A. Stegemann, J. Roth, S.G. Meuth, H. Pavenstädt, J. Sherwood, T. Pap, R. Wedlich-Söldner, C. Sunderkötter, Y.B. Schwartz, J. Brosius, T.S. Rozhdestvensky, Pervasive head-to-tail insertions of DNA templates mask desired CRISPR-Cas9-mediated genome editing events, Sci. Adv. 6 (7) (2020) eaax2941, https://doi.org/10.1126/sciadv.aax2941.

[61] Y. Miyaoka, A.H. Chan, L.M. Judge, J. Yoo, M. Huang, T.D. Nguyen, P.P. Lizarraga, P.-L. So, B.R. Conklin, Isolation of single-base genome-edited human iPS cells without antibiotic selection, Nat. Methods 11 (3) (2014) 291–293.

[62] J. Jacków, Z. Guo, C. Hansen, H.E. Abaci, Y.S. Doucet, J.U. Shin, R. Hayashi, D. DeLorenzo, Y. Kabata, S. Shinkuma, J.C. Salas-Alanis, A.M. Christiano, CRISPR/Cas9-based targeted genome editing for correction of recessive dystrophic epidermolysis bullosa using iPS cells, Proc. Natl. Acad. Sci. U.S.A. 116 (52) (2019) 26846–26852.

[63] X.-L. Li, G.-H. Li, J. Fu, Y.-W. Fu, L.u. Zhang, W. Chen, C. Arakaki, J.-P. Zhang, W. Wen, M. Zhao, Highly efficient genome editing via CRISPR-Cas9 in human pluripotent stem cells is achieved by transient BCL-XL overexpression, Nucl. Acids Res. 46 (2018) 10195–10215.

[64] R.M. Martin, K. Ikeda, M.K. Cromer, N. Uchida, T. Nishimura, R. Romano, A. J. Tong, V.T. Lemgart, J. Camarena, M. Pavel-Dinu, C. Sindhu, V. Wiebking, S. Vaidyanathan, D.P. Dever, R.O. Bak, A. Laustsen, B.J. Lesch, M.R. Jakobsen, V. Sebastiano, H. Nakauchi, M.H. Porteus, Highly efficient and marker-free genome editing of human pluripotent stem cells by CRISPR-Cas9 RNP and AAV6 donor-mediated homologous recombination, Cell Stem Cell 24 (5) (2019) 821–828.e5.