

Efficient, footprint-free human iPSC genome editing by consolidation of Cas9/CRISPR and piggyBac technologies

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Genome editing of human induced pluripotent stem cells (hiPSCs) offers unprecedented opportunities for *in vitro* disease modeling and personalized cell replacement therapy. The introduction of Cas9-directed genome editing has expanded adoption of this approach. However, marker-free genome editing using standard protocols remains inefficient, yielding desired targeted alleles at a rate of ~1–5%. We developed a protocol based on a doxycycline-inducible *Cas9* transgene carried on a piggyBac transposon to enable robust and highly efficient Cas9-directed genome editing, so that a parental line can be expeditiously engineered to harbor many separate mutations. Treatment with doxycycline and transfection with guide RNA (gRNA), donor DNA and piggyBac transposase resulted in efficient, targeted genome editing and concurrent scarless transgene excision. Using this approach, in 7 weeks it is possible to efficiently obtain genome-edited clones with minimal off-target mutagenesis and with indel mutation frequencies of 40–50% and homology-directed repair (HDR) frequencies of 10–20%.

INTRODUCTION

hiPSCs offer an unprecedented opportunity for *in vitro* disease modeling and for personalized cell replacement therapy¹. Applications of iPSCs have been greatly expanded by the advent of genome editing, in which the genomic sequence at a target site is altered by insertion or deletion ('indel') mutations, or by introduction of precisely programmed ('knockin') modifications². Here we present a highly efficient and reproducible protocol to edit the genome of hiPSCs through the combined use of the CRISPR/Cas9 RNA-guided nuclease and piggyBac transposase^{3–5}. This protocol is best suited to applications in which a common starting cell line is edited many different times to yield isogenic daughter cell lines that differ by the introduced mutations.

Genome editing relies on the introduction of a double-strand break at a target locus using 'designer nucleases' that selectively target one site in the genome. The cell repairs the double-strand break through either nonhomologous end joining (NHEJ), creating indel mutations, or HDR, resulting in knock-in modification near the nuclease cutting site. Potential nuclease platforms include zinc-finger nucleases and transcription-activator-like effector nucleases⁶. However, designing these nucleases is labor-intensive and not readily multiplexed. More recently, the CRISPR/Cas9 nuclease has emerged as a powerful and malleable tool to introduce targeted double-strand breaks⁶. Unlike zinc-finger nucleases and transcription-activator-like effector nucleases, Cas9 specificity is determined by Watson–Crick base pairing between an engineered gRNA and the target site^{7,8}. As a result, Cas9 targeting is easily achieved by synthesizing the desired gRNA.

Although Cas9 efficiently directs target-site cleavage, the efficiency of targeted genome modification was initially reported to be ~1–2% in hiPSCs^{7,9}. At this efficiency, recovery of properly targeted clones without positive selection is labor-intensive and inconsistent. We and others previously showed that low transfection efficiency of the relatively large Cas9 expression construct limits the

yield of targeted clones in pluripotent stem cells¹⁰. Procedures that select for Cas9-transfected cells, such as cell sorting for a fluorescent protein expressed from a co-transfected plasmid, increase the recovery of modified clones¹¹. However, cell sorting is stressful for stem cells, exposes them to contamination risks and can be cumbersome when performing modifications on multiple cell lines in parallel. Gonzalez *et al.*¹² showed that knock-in of inducible Cas9 into a safe harbor locus enhances the genome-editing efficiency. However, this strategy consumed the adeno-associated virus integration site 1 (AAVS1) safe harbor locus, and the *Cas9* transgene was not excisable. We have refined this strategy and present here an optimized protocol to permit footprint-free, highly efficient and consistent genome modification in hiPSCs. This procedure can be used to develop isogenic cell lines that differ from each other by sequence variations introduced by genome editing, as we described for an iPSC-based model of Barth syndrome (BTHS)³. The high efficiency of the procedure can also be used to simultaneously disrupt multiple genes or sequences that are present multiple times in the genome. For example, we used this strategy to simultaneously disrupt 62 copies of porcine endogenous retrovirus in a porcine cell line⁵. The inducibility of Cas9 in our system might also be exploited to permit temporally controlled gene inactivation in cells differentiated from iPSCs, thereby potentially circumventing the need to establish stable mutant cell lines.

Development of the protocol: Dox-inducible *Cas9* transgene encapsulated on a piggyBac transposon

We reasoned that Cas9 genome-editing efficiency could be enhanced by generating a stable cell line that harbors an inducible *Cas9* transgene encapsulated on a piggyBac transposon (**Fig. 1a**). Genome editing is performed by Cas9 induction accompanied by transfection of gRNA and a HDR DNA donor template. The *Cas9* transgene can be removed by transient transfection with piggyBac transposase.

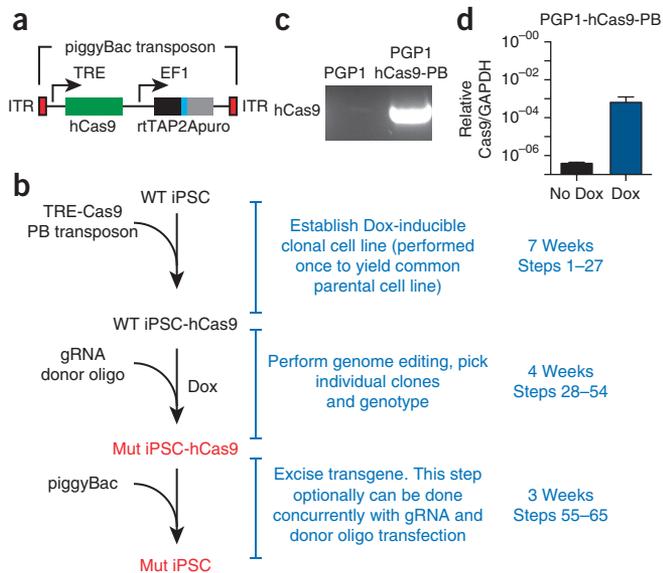


Figure 1 | Enhanced genome editing with Dox-inducible Cas9. (a) Schematic of the piggyBac transposon, which contains Dox-inducible Cas9. (b) Overview of the genome-editing strategy. A piggyBac transposon containing the reverse tet activator, a tet-activator-responsive promoter driving humanized Cas9, and a puromycin resistance cassette are integrated into the genome of wild-type hiPSCs. Treatment with Dox and co-transfection with gRNA and donor DNA oligonucleotide efficiently yield mutant iPSC clones, indicated in red. The transposon is efficiently removed by transfection with an excision-only piggyBac transposase, either as a separate step or concurrently with the transfection of gRNA and the donor oligo. (c) PCR genotyping of PGP1 cells, confirming stable integration of hCas9-PB. (d) Induction of hCas9 by the addition of Dox (2 $\mu\text{g}/\text{ml}$). hCas9 expression was measured by qRT-PCR and normalized to GAPDH. Chart shows mean \pm s.e.m. $n = 3$. EF1, elongation factor 1 promoter; hCas9, human codon-optimized Cas9; Mut, mutant; ITR, piggyBac transposon inverted terminal repeats; rtTAP2puro, reverse tet-activator that followed the self-cleaving P2A peptide and the puromycin resistance marker; TRE, tet-activator-response element; WT, wild-type.

We generated a doxycycline-inducible, human-codon-optimized Cas9 (hCas9) construct contained within a piggyBac transposon⁷. The piggyBac transposon construct was stably introduced into the male PGP1-iPS cell line by co-transfecting it with a plasmid encoding the piggyBac transposase (Fig. 1b,c). The resulting PGP1-hCas9-PB stable cell line showed >1,000-fold induction of Cas9 by doxycycline (Dox) treatment (Fig. 1d).

Stable expression of hCas9 allowed us to efficiently target a human disease gene in iPSCs. We targeted Tafazzin (*TAZ*), a gene on the X chromosome that is mutated in BTHS, a mitochondrial cardiomyopathy¹³. We designed a gRNA and an HDR template to introduce a known BTHS mutation (c.517delG)¹⁴ into *TAZ* exon 6 and co-transfected them into PGP1-hCas9-PB with Dox treatment. The surveyor mutation detection assay suggested efficient *TAZ* gene modification with Dox treatment, and no detectable modification in the absence of Dox (Fig. 2a). High-throughput sequencing of the targeted locus from pooled genomic DNA⁹ showed that 30% of cells had an indel near the engineered double-strand break, whereas 50% had undergone HDR and harbored the sequence variant programmed by the HDR donor (Fig. 2b).

We evaluated the recovery of individual *TAZ*-modified clones. After transfection with gRNA and the HDR donor, cells were plated at low density and treated with Dox. Colonies were then picked

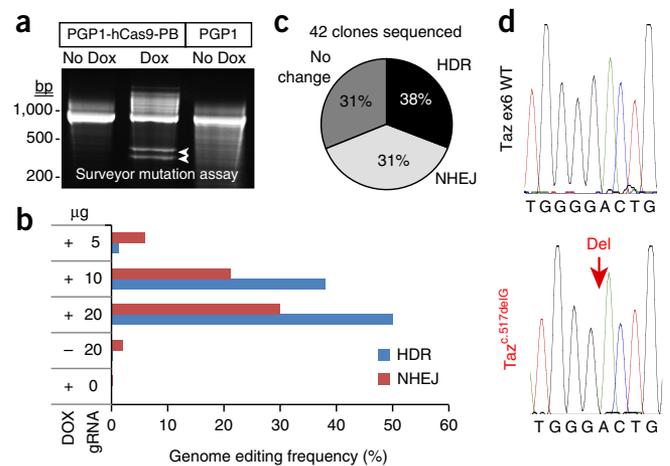


Figure 2 | Efficiency of genome editing with Dox-inducible Cas9. (a) Surveyor mutation assay to detect genome modification. The indicated hiPSC lines were treated or not treated with Dox. Modification efficiency in genomic DNA at the *TAZ* locus was assessed using Surveyor nuclease followed by native gel separation of reaction products. Arrowheads indicate nuclease cleavage products. (b) Deep sequencing analysis of the frequency of HDR or NHEJ genome modification at the *TAZ* locus. A PCR amplicon encompassing the *TAZ* gRNA target site was sequenced using a MiSeq Illumina sequencer at a minimum depth of 100,000 reads per amplicon. The amount of gRNA expression construct is shown in micrograms. (c) After Dox-induced genome editing at the *TAZ* locus on the X chromosome of a male iPSC line, individual clones were picked and genotyped by Sanger sequencing. The pie chart displays the frequency of *TAZ* modification by HDR or NHEJ. (d) Representative Sanger sequencing chromatograms, showing a clone that underwent HDR-mediated genomic modification (red arrow indicating a one-base HDR-programmed deletion) compared with a control.

and genotyped by DNA sequencing. Of 42 clones sequenced, 13 (31%) contained an indel and 16 (38%) contained the donor-programmed sequence variant (Fig. 2c,d). The efficiency of our strategy and protocol has been further tested in a different human embryonic stem cell line and at different loci, with HDR rates of ~20–35% and NHEJ rates of ~50% (Supplementary Fig. 1).

Development of the protocol: excision of the Dox-inducible Cas9 transgene by piggyBac transposase

Encapsulating the *hCas9* transgene on a piggyBac transposon enabled its efficient excision. To illustrate this, we transiently transfected PGP1-hCas9-PB-*TAZ*^{c.517delG} with an excision-competent, integration-defective piggyBac transposase expression plasmid¹⁵ and assessed *hCas9* transgene excision by loss of puromycin resistance, encoded on the piggyBac transposon. PiggyBac transposase reduced the frequency of puromycin-resistant clones, as assessed by crystal violet visualization of puromycin-resistant clones, demonstrating efficient transposon excision (Fig. 3a). Most individual clones recovered after transient piggyBac transposase expression were negative for the *hCas9* transgene, as determined by PCR genotyping. For establishment of the PGP1-*TAZ*^{c.517delG} line lacking the *hCas9* transgene, we genotyped 34 clones, and 22 (64%) had undergone successful transgene removal (Fig. 3b). We have further streamlined the protocol by introducing piggyBac transposase into Dox-induced cells in the same transfection as gRNA and donor DNA. We found that co-transfection of the excision-only piggyBac mutant did not substantially reduce the yield of genome-edited clones, yet most of the recovered clones had still successfully

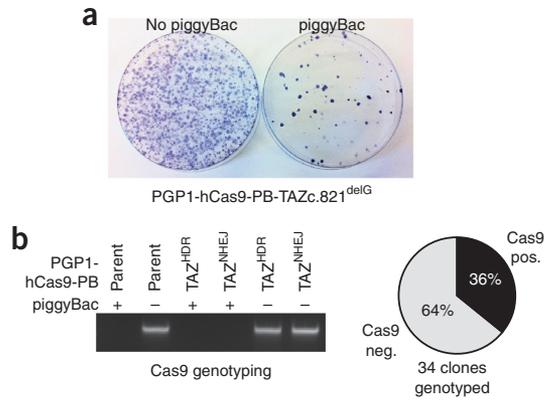


Figure 3 | Excision of Cas9-bearing transposon using piggyBac transposase. (a) PGP1-hCas9-PB-TAZc.821delG cells were transfected with piggyBac transposase expression vector. Puromycin-resistant clones, the clones that failed to undergo transposon excision, were visualized by crystal violet staining. (b) PCR genotyping of individual clones obtained after transfection with piggyBac expression vector. Representative examples of genotyping results of cas9-positive and cas9-negative clones are shown. Pie chart summarizes the genotyping results for 34 clones.

undergone piggyBac transgene excision (Supplementary Fig. 2). Thus, including the excision-only piggyBac mutant in the transfection mix with gRNA and donor DNA permits efficient, single-step genome editing and transgene excision.

Development of the protocol: quality control of recovered clones
We performed quality control on the genome-edited cell lines. PGP1e-TAZc.517delG cells had a normal karyotype (Supplementary Fig. 3a), expressed the pluripotency genes *OCT4* and *NANOG* at levels comparable to the human embryonic stem (ES) cell line H7 (Supplementary Fig. 3b,c) and differentiated into all three germ layers in teratoma assays (Supplementary Fig. 3d–g). The cell lines differentiated efficiently into cardiomyocytes using a common directed differentiation protocol (Supplementary Fig. 3h)¹⁶. Indeed, we showed that the genome-edited PGP1e-TAZc.517delG iPSC line effectively recapitulates hallmarks of BTHS (Supplementary Fig. 4).

A concern of Cas9-based genome-editing strategies has been off-target mutagenesis. Recently, several studies used whole-genome sequencing to demonstrate that Cas9 genome editing does not substantially affect the mutation burden of iPSCs^{4,17,18}. We confirmed that our strategy is not substantially mutagenic by deep sequencing of 31 potential off-target sites (Fig. 4a). At each site, we sequenced a minimum of 100,000 amplicons, which we have previously shown to yield a detection sensitivity of 0.07%⁴. At the 31 computationally predicted potential off-target sites, 30 sites had three nucleotide mismatches to the reference genome. Significant off-target activity was not detected at these sites. The final site, site 28, was designed to also have three nucleotide mismatches to the reference sequence, but as we reported previously⁴, a single-nucleotide polymorphism (SNP) in the PGP1 genome sequence eliminated one mismatch. This site with two mismatches was frequently mutated, highlighting the potential for SNPs to affect off-target mutagenesis at specific sites. Whole-genome sequencing of six individual clones isolated using this protocol from separate genome-editing experiments at three loci (TAZ, DNAJC19 and JUP) showed that Cas9 does not induce frequent

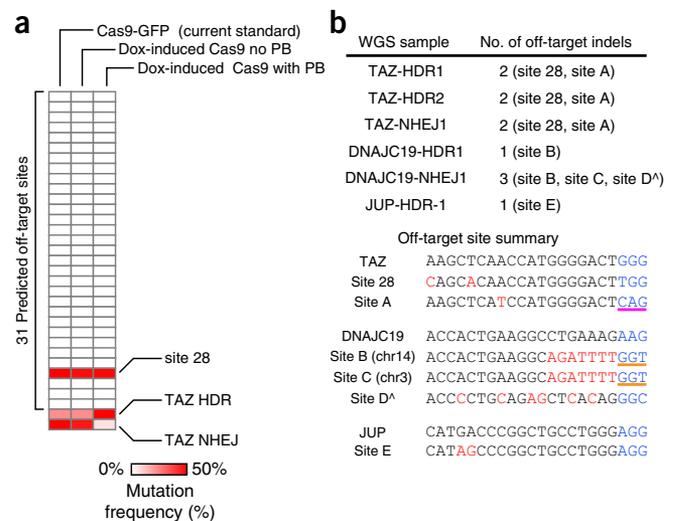


Figure 4 | Analysis of Cas9 off-target activity. (a) Off-target activity at 31 computationally predicted potential off-target sites for TAZ gRNA. Cas9-PB iPSCs were treated with DOX+TAZ gRNA+donor. Off-target activity at 31 sites was analyzed by PCR amplification of the candidate sites from pooled genomic DNA, followed by deep sequencing (>100,000 sequences per site). The candidate sites had three mismatches from the gRNA, with the exception of site 28, which had a SNP that created a 2-bp mismatch, as we previously reported⁴. ‘Cas9 no PB’ and ‘Cas9 with PB’ refer to omission or inclusion of piggyBac transposase expression plasmid in the transfection of gRNA and DNA donor. ‘TAZ HDR’ and ‘TAZ NHEJ’ indicate the frequency of on-target HDR and NHEJ at the TAZ locus. (b) Whole-genome sequencing of six independently isolated clones derived from PGP1-Cas9-PB (listed under WGS sample) after targeting at three loci (TAZ, DNAJC19 and JUP). HDR or NHEJ indicates the type of mutation found at the target site. In each whole-genome sequence, we identified 10–15 indels. These were analyzed for homology to the gRNA, presence of protospacer-adjacent motif (PAM) sequences (blue) and recurrence in multiple clones or genomic locations. On the basis of this analysis, most of the indels were unrelated to the gRNA sequences and may have arisen spontaneously during clonal expansion from a single cell. Those that were related to the gRNA sequence are listed as ‘off-target indels’ and named with a number if they were among the 31 predicted potential off-target sites (described in panel a; e.g., site 28) or a letter if they were not among these top 31 predicted off-target sites. Red letters indicate differences from the gRNA sequence, which is shown next to the locus name. The three separate TAZ clones sequenced all shared indels at the same two sites: site 28 and site A. Interestingly, site A differs from the gRNA target by only one nucleotide but was not computationally predicted because of its atypical PAM (CAG, pink underline). The two DNAJC19 clones had different indels at the same site, site B. An identical genomic sequence on a different chromosome (site C) also contained an indel in one of these clones. However, sites B and C had neither a functional PAM (orange underline) nor close homology to the gRNA within the Cas9 seed sequence. [^] indicates a site that is listed for completeness but may not have arisen from Cas9 activity (single occurrence; lack of PAM; multiple gRNA mismatches). Copy-number variation analysis of the whole-genome sequencing data found no significant copy-number variation in these clones.

off-target mutagenesis. However, between 1 and 3 mutations that were probably linked to Cas9 were identified in each of the clones (Fig. 4b). With only one exception, we did not detect significant off-target activity at sites with three or more gRNA mismatches. The exception (site B, Fig. 4b) consisted of an indel mutation at a genomic site on chromosome 14 that matched the gRNA at bases 8–19 (where position 1 is adjacent to the protospacer-adjacent motif (PAM)). However, ‘seed’ nucleotides 1–7 did not match, and the PAM sequence, GGT, has no predicted activity¹⁹. An identical

sequence exists on chromosome 3, and this site also contained an indel mutation in one of the two clones (site C, **Fig. 4b**), suggesting that mutation at these sites was related to Cas9 activity. In addition, we detected clear off-target activity at a site (site A, **Fig. 4b**) with a 1-bp gRNA mismatch but a variant PAM sequence, CAG, with known partial activity¹⁹. Together these results suggest that Cas9 does not induce widespread genomic instability or rearrangements but does induce off-target mutagenesis at rare sites that cannot be fully anticipated by current prediction rules.

We also investigated the potential for piggyBac excision to leave residual genomic scars. Our analysis (**Supplementary Data 1**) confirmed scarless piggyBac excision in all clones that we examined.

Limitations

Off-target mutagenesis is one potential consequence of genome editing. Whole-genome sequencing of several mutant iPSC lines generated through application of this protocol did not reveal a

Box 1 | Design of gRNAs, HDR donor DNA oligonucleotide and genotyping primers.

Several excellent resources are available that provide detailed instructions on designing genome-editing reagents. Some of these are as follows:

- Graham and Root, "Resources for the design of CRISPR gene editing experiments"²³.
- The Addgene e-book, *CRISPR 101: A Desktop Resource* (<http://www.addgene.org>).
- Yang *et al.*, "CRISPR/Cas9-directed genome editing of cultured cells"²⁴.

gRNA expression construct

This procedure is described in greater detail in Yang *et al.*²⁴. Although computational prediction of effective gRNAs has improved, there is still substantial variation in gRNA efficiencies. Therefore, it can be more efficient to generate several different gRNA constructs in parallel and then use them in Steps 28–47. Subsequent steps could then focus on the most efficient gRNA.

1. Find genomic sites of the form 5'-N19NGG-3' (**Fig. 5a**) within ± 50 bp of your intended target site (optimally within ±10 bp). The sequence can be on either strand. Publicly available gRNA design tools such as <http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design> are useful for selecting the most active gRNAs with the lowest chance of off-target activity. Additional design tools are described in Graham and Root²³. For HDR, give priority to gRNAs located closest to the intended target site.
2. Replace the bold, red 'X' in the following sequence with the best N19 target sequence to yield the complete 455-bp sequence, which contains a U6 promoter and the gRNA. The U6 promoter prefers a G as the transcribed base, and we have incorporated this into the sequence as indicated by the green 'G':

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TGTACAAAAA AGCAGGCTTT AAAGGAACCA ATTCAGTCGA CTGGATCCGG TACCAAGGTC GGCAGGAAG AGGGCCATTT
TCCCATGATT CCTTCATATT TGCATATACG ATACAAGGCT GTTAGAGAGA TAATTAGAAT TAATTTGACT GTAAACACAA
AGATATTAGT ACAAATACG TGACGTAGAA AGTAATAATT TCTTGGGTAG TTTGCAGTTT TAAAATTATG TTTTAAATG
GACTATCATA TGCTTACCGT AACTTGAAAG TATTTTCGATT TCTTGGCTTT ATATATCTTG TGAAAGGAC GAAACACCX
GTTTTAGAGC TAGAAATAGC AAGTTAAAAT AAGGCTAGTC CGTTATCAAC TTGAAAAAGT GGCACCGAGT CGGTGC'TTTT
TTTCTAGACC CAGCTTTCCT GTACAAAGTT GGCATTA
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3. Synthesize the gRNA as a double-stranded DNA. For example, use gBlocks from Integrated DNA Technologies.
4. Clone the synthesized dsDNA into an empty backbone vector such as pCR-Blunt II-TOPO from Life Technologies. Validate the resulting clone by sequencing and prepare a plasmid prep. Resuspend the DNA in 10 mM Tris, pH 8.0, and 1 mM EDTA at a concentration of >0.5 mg/ml.

HDR donor oligonucleotide

1. To generate donor DNA for oligo-mediated HDR, identify the positions targeted by the gRNA for cleavage and the desired modification site (**Fig. 5b**). Include the 45 nt on either side of this modification region as the homology arms of the donor DNA. 45-nt homology arms are sufficient for base changes or small insertions (<40 nt). Larger insertions require larger homology arms¹⁰. If possible within the constraints of the desired experiment, introduce additional modifications in the HDR oligo that will alter the gRNA recognition sequence in the HDR donor oligo so that the oligo and the corrected genomic DNA will be refractory to gRNA-Cas9 cleavage. For instance, place silent mutations in the gRNA seed sequence, or disrupt the PAM sequence. It is also desirable to introduce or eliminate a restriction enzyme cleavage site to facilitate genotyping of candidate clones.
2. Synthesize the sequence as a single-stranded oligonucleotide at a 25-nmole scale. Desalted oligonucleotide is acceptable quality. Resuspend the oligonucleotide in ddH₂O at a stock concentration of 10 µg/µl.

Genotyping primers

1. Surveyor assay primers: forward and reverse primers should be designed to produce an amplicon of ~700 bp, positioned so that predicted cleavage products will be easily resolved (e.g., 200 and 500 bp) (**Fig. 5b**). Each primer should be at least 70 bp from the target modification site. Resuspend at a 100 µM stock concentration and a 10 µM working concentration. PCR using the primers should yield a robust single band.
2. Sanger sequencing/PCR primers: forward and reverse PCR primers should be designed to be ~150 bp from the target modification site. These will be used to PCR-amplify the target region and to perform Sanger sequencing of the PCR amplicon. Resuspend at a 100 µM stock concentration and a 10 µM working concentration.

substantial burden of off-target mutation, although each of the six cell lines that underwent whole-genome sequencing did acquire 10–15 off-target mutations each, of which 1–3 were attributable to gRNA-related Cas9 activity. Interestingly, the mutation sites were in some cases poorly predicted by current algorithms.

At present, whole-genome sequencing of each genome-edited clone is not feasible. Rather, we suggest that at least two independent clones be recovered for each genome-editing experiment. This will help to control for potential confounding effects of off-target mutations, although our whole-genome sequencing data show that some off-target sites are recurrently mutated in independent clones. Therefore, it is optimal to use two independent gRNAs. Rescue of mutant cells by transient cDNA expression is an alternative strategy to control for off-target mutation³.

This protocol requires establishing a parental cell line with the stably integrated transgene, which represents an extra step as compared with methods based on transient transfection coupled with cell-sorting-based enrichment of transfected cells. However, the benefit of our strategy is increased consistency and higher efficiency for recovering genome-edited clones once the parental line is established. Therefore, we use this strategy in situations in which it is desirable to make many different genome-edited cell lines from a common parent, such as when generating a series of cell lines that are isogenic, except for introduced mutations. On the other hand, this strategy would be cumbersome for editing many different cell lines, such as for correction of mutations in patient-derived iPSC lines.

Our method involves stable, random integration of Cas9, followed by scarless excision. It may be desirable in future iterations of this protocol to target a piggyBac-excisable *Cas9* transgene to a safe harbor locus, such as *AAVS1*. This would eliminate problems with transgene copy number and reduce the chance of deleterious integration sites. Although our data suggest that piggyBac excision is robust and that the frequency of leaving a scar at the

excision sites was below our detection limits (0/75 excision sites evaluated; **Supplementary Data 1**), a targeted integration strategy would nevertheless reduce the risk and facilitate validation of scar-free excision.

Experimental design

gRNA and donor template design. Critical parameters that should be optimized for genome editing are the design of the gRNA and the HDR donor (**Box 1**). As we noted previously, SNPs not represented in the reference genome may lead to off-target sites not predicted by such design algorithms⁴.

Genotyping strategy. It is critical to design the genome-editing strategy to facilitate genotyping of clones, as this step can otherwise be costly and rate-limiting. We have written this protocol using the most generic case, in which the genome-editing strategy does not incorporate specific features that facilitate identification of the desired modification. As a result, Steps 37–47 use the Surveyor nuclease to determine whether efficient modification has occurred in pooled genomic DNA. Sanger sequencing is used in Steps 51–54 to genotype individual clones.

However, it is preferable to design features into the genome-editing strategy that facilitate identification of clones with the desired genotype without relying on Sanger sequencing. In cases in which a simple deletion is desired, using two gRNAs spaced ~100 bp apart may slightly increase mutation efficiency¹⁰ and will facilitate genotyping of individual clones. For HDR, the DNA donor could be designed to insert or remove a restriction endonuclease site (**Box 1**). If these features are incorporated into the genome-editing strategy, then they can be substituted for Steps 37–47 and 51–54.

Controls. A negative control (omission of gRNA) should be included in the workflow. A useful positive control for newcomers to the protocol is to target the *TAZ* locus using the sequences outlined in **Supplementary Data 2**. *TAZ*, located on the X chromosome, is present in a single copy in XY iPSCs.

MATERIALS

REAGENTS

- Pluripotent stem cell line, e.g., the PGP1 iPSC line (Coriell Institute) or the CHB10 hESC line (Daley laboratory at Boston Children's Hospital)
- **CAUTION** The cell lines used should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.
- Human Stem Cell Nucleofector Kit 1 (Lonza, cat. no. VPH-5012)
- mTeSR1 complete kit for hES maintenance (StemCell Technologies, cat. no. 05850) **▲ CRITICAL** Avoid warming the mTeSR1 stock to 37 °C to prevent degradation of basic fibroblast growth factor (bFGF) contained in the medium.
- mFreSR medium (Stemcell Technologies, cat. no. 05854)
- Knockout DMEM (Thermo Fisher Scientific, cat. no. 11330-032)
- Dulbecco's PBS (DPBS; Life Technologies, cat. no. 21600)
- Corning Matrigel hESC-Qualified Matrix (Corning, cat. no. 354277)
- Versene (Thermo Fisher Scientific, cat. no. 15040-066)
- Penicillin–streptomycin, 100× (Life Technologies, cat. no. 15140-163) **▲ CRITICAL** All cell culture media used in this protocol contain 1× penicillin–streptomycin.
- Puromycin dihydrochloride (Life Technologies, cat. no. A11138-03)
- Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Millipore, cat. no. SCM075)
- Doxycycline (Dox; Sigma-Aldrich, cat. no. D3072-1ML) **▲ CRITICAL** Dox is light sensitive. The sterile solution should be stored in a light-shielded container.
- DNA Loading Dye, 6× (Fermentas/Thermo Fisher Scientific, cat. no. R0611)

- Ethidium bromide (TianGen, cat. no. RT203) **! CAUTION** Ethidium bromide is a mutagen. Handle it with care and use personal protective equipment.
- Proteinase K (Roche, cat. no. 03115887001)
- Tris, pH 8.0 (Boston BioProducts, cat. no. BBT-423)
- EDTA (Boston BioProducts, cat. no. BM-150)
- Tris-EDTA buffer (TE; 10 mM Tris, pH 8.0, and 1 mM EDTA)
- NaCl (Boston BioProducts, cat. no. BM-244)
- 10× Taq PCR Buffer (GenScript, cat. no. B0005)
- gBlocks (Integrated DNA Technologies)
- Isopropanol (Sigma, cat. no. 19516)
- Sarcosyl (Sigma-Aldrich, cat. no. L7414)
- GlycoBlue (Life Technologies, cat. no. AM9515)
- Plasmid Mini Kit (Life Technologies, cat. no. K2100-10)
- Plasmid Midi Kit (Life Technologies, cat. no. K2100-04)
- TBE Gels, 4–20%, 1.0 mm, 15-well (Life Technologies, cat. no. EC62255BOX)
- Agarose for gel electrophoresis (BioExpress GeneMate LE agarose, cat. no. E-3120-125)
- QIAquick PCR Purification Kit (50) (Qiagen, cat. no. 28104)
- KAPA HiFi Hot Start ReadyMix PCR Kit (KAPA, cat. no. KK2601)
- Quantitative PCR master mix (Affymetrix Veriquest probe and Sybr qPCR 2× master mix, cat. nos. 75650 and 75600)
- Surveyor Mutation Detection Kit, S25 (IDT, cat. no. 706025)
- TA Cloning Kit (Life Technologies, cat. no. K202020)

- pPB-rtTA-hCas9-puro-PB plasmid (Dox-inducible hCas9 on a piggyBac transposon with a puromycin resistance marker). This plasmid is available from the authors upon request
- Excision only piggyBac transposase expression vector (System Biosciences, cat. no. PB220PA-1)
- Super piggyBac transposase expression vector (System Biosciences, cat. no. PB210PA-1)
- pCR-Blunt II-TOPO (Life Technologies, cat. no. K2800-02)
- gRNA expression constructs and HDR donor oligonucleotide (see **Box 1** and **Figure 5**)
- PCR primers for SURVEYOR analysis or sequencing (see **Box 1** and **Figure 5**)

hCas9 genotyping primers

- hCas9-F: aggtggcgtaccatgaaag (amplicon size = 571 bp; Integrated DNA Technologies)
 - hCas9-R: gctttggtgatctcgtgtt (amplicon size = 571 bp; Integrated DNA Technologies)
- 20× hCas9 qPCR primers/probe assay**
- hCas9-qPCR-F: aagaacggcctgtttgtaa (premixed in TE at a final concentration of 5 μM; Bio-Rad, custom-made)
 - hCas9-qPCR-R: gttgaagctggcattctcg (premixed in TE at a final concentration of 5 μM; Bio-Rad, custom-made)
 - hCas9-probe (FAM): cgccctgctcaactgggctgacc (premixed in TE at a final concentration of 5 μM; Bio-Rad, custom-made)
 - 20× EIF2C1 qPCR probe assay, HEX-labeled (Bio-Rad, assay ID dHsaCP2500349)

EQUIPMENT

- Tissue culture plates (6- and 24-well; Nunc, cat. nos. 140675 and 142475)
- Sterilized Pasteur pipettes (Fisher, cat. no. 13-678-20D)
- Microcentrifuge tube (1.7 ml; Fisher Scientific, cat. no. 05-408-129)
- Conical centrifuge tube, polypropylene, 15 ml (BD Falcon, cat. no. 352097)
- Conical centrifuge tube, polypropylene, 50 ml (BD Falcon, cat. no. 352070)
- Serological pipettes (5, 10 and 25 ml; Fisher Scientific, cat. nos. 13-678-11D, 13-678-11E and 13-678-11)
- Stericup filtration system (500 ml; Millipore, cat. no. SCGPU05RE)
- Steriflip-HV filter unit (0.45 μm (50 ml); Millipore, cat. no. SE1M003M00)
- Filter-tip pipette tips (20 μl, 200 μl, 1 ml; ISC-BioExpress)
- Humidified tissue culture incubator (37 °C, 5% CO₂; Forma Steri-Cycle)
- Tissue culture hood
- Hemocytometer (Hausser Scientific, cat. no. 02-671-52)
- Inverted phase-contrast microscope (Nikon, model no. Eclipse TS100)
- Nucleofector 2b Device (Lonza, cat. no. AAB-1001)
- Bench-top cell culture centrifuge (Thermo Scientific, cat. no. 004260F)
- Microfuge (Eppendorf, model no. 5424)
- Gel electrophoresis tanks, vertical (Lifetech XCell SureLock Mini-Cell)
- Gel electrophoresis tanks, horizontal (Owl EasyCast B2)
- Gel electrophoresis power supply (Bio-Rad PowerPac300)
- Gel documentation system (UVP GelDoc-It)
- PCR thermocycler (Bio-Rad C1000 Touch)

PROCEDURE

Establishment of a Dox-inducible iP/ES line ● TIMING 33 d to obtain candidate lines

1| Grow low-passage iPSCs (between passages 10 and 40) to 70% confluence in a six-well plate. iPSCs should be grown in dedicated tissue culture incubators (37 °C, 5% CO₂, humidified).

▲ **CRITICAL STEP** It is crucial that cell culture environments be free of mold, bacteria and mycoplasma.

2| Prepare a Matrigel-coated six-well dish (**Box 2**) and place it in the tissue culture incubator.

3| Lift cells as directed in **Box 3**.

▲ **CRITICAL STEP** To achieve highly efficient transfection, the cells must be dissociated into single cells.

4| Count the cells with a hemocytometer. Adjust the cell density with PBS to 1 million cells per ml.

5| Prepare nucleofection solution: combine 82 μl of human stem cell nucleofector solution and 18 μl of supplement 1 from the Human Stem Cell Nucleofector Kit 1 in a sterile 1.5-ml microfuge tube. Mix well.

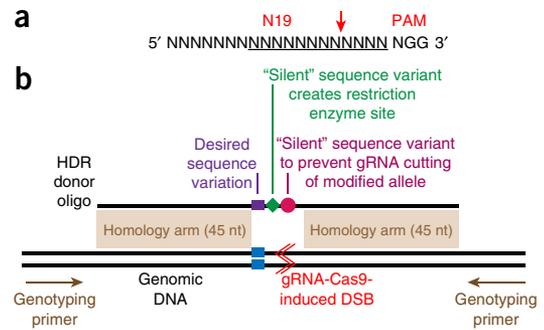


Figure 5 | Design of genome-editing reagents. (a) gRNA recognition site. Red arrow indicates the gRNA cleavage site. Underline indicates the 12-nucleotide ‘seed’ sequence that is generally considered to be the most important for Cas9 targeting. The PAM (protospacer-adjacent motif) sequence is required for Cas9 targeting. **(b)** A gRNA is designed to introduce a double-strand break (DSB, red lines) close to the desired modification site (blue). The HDR donor oligo is designed to have homology arms that are at least 45-nt long on either side of the desired sequence modification (purple). Two additional sequence modifications are desirable if they can be achieved without impacting the ultimate experimental goal. First, introduce ‘silent’ sequence variants (magenta) that eliminate the gRNA recognition sequence after HDR. Second, introduce or remove a restriction enzyme site so that HDR genomes can be identified by restriction digestion of PCR products (green). Genotyping PCR primers are designed flanking the targeted region. These genotyping primers should not overlap the homology arms.

- Pipettors, 20 μl, 200 μl, 1 ml (Eppendorf Research plus)
- 37 °C bacterial incubator and shaker (ATR Multitron)
- Coolcell LX cell freezing box (Biocision, cat. no. BCS-405)
- Nucleofector device (Lonza, model no. AAB-1001 Nucleofector 2b)

REAGENT SETUP

Matrigel-coated dishes Prepare the dishes as described in **Box 2**.

Y-27632 stock solution (5 mM) Add 1 mg of Y-27632 to 624 μl of sterile dH₂O. Divide the solution into aliquots to avoid repeated freeze–thaw cycles. Store it at –20 °C. This solution is stable for 6 months.

Y-27632 should be freshly added to the culture media with each use.

Per 10 ml of mTeSR1, add 20 μl of Y-27632 stock solution.

Culture media Add penicillin–streptomycin to all cell culture media to a final concentration of 1×. Media containing penicillin–streptomycin are stable for several months at 4 °C.

Lysis buffer Lysis buffer is 10 mM Tris, pH 8.0, 10 mM EDTA, 10 mM NaCl, 0.5% (wt/vol) sarcosyl and 40 μg/ml proteinase K. Buffer without proteinase K can be stored at 4 °C for up to a year. Add proteinase K immediately before use.

Box 2 | Matrigel coating of dishes

Preparing Matrigel aliquots

1. Thaw a 5-ml vial of Matrigel overnight at 4 °C.
2. Prechill the pipette tips and sterile 50-ml tubes to 4 °C.
3. Add into each 50-ml tube the volume that will be diluted to 25 ml. This volume, typically between 300 and 500 µl, is lot-specific and specified by the manufacturer.
4. Freeze the mixture at -20 °C.

■ **PAUSE POINT** Frozen aliquots are stable for >1 year.

Coating dishes with Matrigel

1. Thaw sufficient aliquots of Matrigel in 50-ml tubes at 4 °C overnight. Each will yield 25 ml of diluted Matrigel.
2. Prechill the pipette tips.
3. Add the 25 ml of cold knockout DMEM to the Matrigel in the 50-ml tubes. Gently mix. Do not vortex the mixture.
4. Add sufficient volume of diluted Matrigel to cover the bottom of the culture dishes, as follows:

Tissue culture dish format	Volume of diluted Matrigel
10-cm dish	8 ml/dish
6-cm dish	2 ml/dish
six-well dish	1 ml per well
24-well dish	0.25 ml per well

5. To use the dish immediately, keep the dish at 37 °C for at least 20 min and not >2 h. Alternatively, the plate can be stored at 4 °C for up to 1 week.

Prepare coated dish for use

1. To prepare a plate for use, put the plate into the tissue culture incubator for at least 20 min but no longer than 2 h. Just before adding cells suspended in culture medium, aspirate the diluted Matrigel without scratching the surface coating.

- 6| Incubate the solution at 37 °C for 5 min.
- 7| Add 1 ml of cell suspension to a sterile 1.5-ml Eppendorf tube and centrifuge at room temperature (~20–23 °C) at 300g for 5 min in a bench-top centrifuge.
- 8| Discard the supernatant and resuspend the cells in 100 µl of human stem cell nucleofactor solution from Step 6.
- 9| Add 1 µg of Super piggyBac transposase expression vector plasmid and 5 µg of pPB-rtTA-hCas9-puro-PB plasmid to the cell suspension.
- 10| Mix cells and DNA by gentle swirling. Transfer the cells to a nucleofactor cuvette from the Nucleofactor Kit using a 1-ml pipette tip. Put the cuvette into the nucleofactor. Select Program B-016 and nucleofect the cells by pressing button X.

Box 3 | Lifting of cells

The procedure below is written for one well of a six-well dish. For different-sized dishes, scale by the number of wells. For example, for a 24-well dish, use 1/4 of the indicated volumes for each well.

1. Pretreat the culture by adding ROCK inhibitor Y-27632 to mTeSR1 medium to a concentration of 10 µM. Incubate the mixture overnight.
2. Aspirate the cell culture medium. Gently rinse the cells with 2 ml of DPBS for each well of the six-well plate.
3. Aspirate the DPBS, add 2 ml per well of Versene and put the culture back into the 37 °C incubator until the cells become rounded up and loosely adherent, but not detached.

▲ **CRITICAL STEP** This requires 3–7 min and should be determined by observing cells under the microscope every 2–3 min. Sufficient time is needed for complete dissociation to single cells, but excessive time will lead to cell death.

4. Gently aspirate the Versene. Add 1 ml of mTeSR1 with Y-27632 (10 µM) and dislodge the cells by gently flowing mTeSR1 over them with a P1000 pipette.

▲ **CRITICAL STEP** The cells should be dislodged easily by the flow of medium over the cells. The cells should not require scraping to dislodge; if they do, then rinse with DPBS, add more Versene and increase the time of incubation.

▲ **CRITICAL STEP** Total Versene incubation time should not exceed 15 min.

5. Collect the dislodged cells, and gently triturate them into a single-cell suspension.

11| Add 500 µl of mTeSR1 medium with 10 µM Y-27632 to the nucleofection cuvette and aspirate the nucleofected cells from the cuvette using the provided plastic pipette.

12| Transfer the cells drop-wise into one well of the Matrigel-coated six-well dish from Step 2, and incubate the cells at 37 °C overnight.

13| The next day (day 1 after nucleofection) change the medium to mTeSR1 and add puromycin at a final concentration of 1 µg/ml. Thereafter, change the medium with puromycin daily. Nonresistant cells will die, peaking at day 4. When distinct colonies are visible to the naked eye (usually about day 7), proceed to the next step.

? TROUBLESHOOTING

14| Prepare a 10-cm Matrigel-coated dish (**Box 2**) and place it in the tissue culture incubator.

15| Lift the cells as described in **Box 3**.

16| Count the cells with a hemocytometer. Plate 250 cells in the Matrigel-coated 10-cm dish from Step 14 with 5 ml of mTeSR1 medium with 10 µM Y-27632.

? TROUBLESHOOTING

17| Culture cells until individual clonal outgrowths are visible (~12 d), changing the medium daily.

18| Pick 5–10 colonies and expand them as directed in **Box 4**. The procedure described in **Box 4** will yield iPSC clones in 24-well dishes, to be used in Step 19, and matching genomic DNA samples, to be analyzed in Steps 20–25 below.

19| When the iPSC clones growing in 24-well dishes reach 70% confluence, lift the cells as described in **Box 3**. Freeze a sample of each iPSC-hCas9-PB clone as directed in **Box 5** pending genotyping results, which are obtained by analyzing each clone's genomic DNA sample as directed in Steps 20–25.

■ PAUSE POINT Cells can remain frozen for several weeks while awaiting genotyping results.

Quality control of Dox-inducible iPSC/ESC clones: genotyping ● TIMING 2 d

20| To confirm transgene integration, prepare PCR genotyping reaction to analyze the genomic DNA samples from Step 18 as follows:

Component	Amount
Genomic DNA from Step 18	50 ng
hCas9F primer, 10 µM	1 µl
hCas9R primer, 10 µM	1 µl
ddH ₂ O	to 12.5 µl
2× KAPA HIFI Hot Start ReadyMix	12.5 µl
Final volume	25 µl

21| Run the PCR program as follows:

Step	Temperature	Time	Comment
1	95 °C	3 min	
2	98 °C	20 s	
3	60 °C	15 s	
4	72 °C	15 s	Go to 2, 30 times
5	72 °C	1 min	

Box 4 | Picking and expanding individual clonal outgrowths

1. Treat clones that will be picked by changing the medium to mTeSR1 containing ROCK inhibitor Y-27632 at 10 μ M. Incubate the mixture overnight.
2. Prepare a Matrigel-coated 24-well plate (**Box 2**) by putting it in the tissue culture incubator for 20 min–2 h. Then, aspirate the solution and replace it with mTeSR1 containing 10 μ M Y-27632, at a volume of 500 μ l per well.
3. Put the dish containing the individual clones into the culture hood and pick colonies with a P10 pipette with filter tips and set at 10 μ l. Pick up a clone by scratching it into small pieces. Transfer each clone to a separate well of the Matrigel-coated 24-well plate from the previous step. Use a different filter tip for each clone.
4. Change the medium daily with mTeSR1 without Y-27632.
5. After 4–5 d, the cells within each well should reach ~40% confluence and will be ready to split. Culture cells overnight in mTeSR1 with 10 μ M Y-27632, at a volume of 500 μ l per well.
6. Prepare a fresh 24-well plate coated with Matrigel (**Box 2**) by placing it in the incubator for 20 min–2 h. Aspirate the solution and replace it with 125 μ l of mTeSR1.
7. Using the dish containing the cells from step 5 of this box, repeat steps 2–5 of **Box 3** (using 1/4 volumes).
8. Transfer 125 μ l of cell suspension into a well of the Matrigel-coated 24-well plate from step 6 of this box. Add 125 μ l of mTeSR1 containing Y-27632 (10 μ M).
9. Transfer 125 μ l of cell suspension from step 7 of this box to a 1.5-ml Eppendorf tube and use to prepare genomic DNA, as described in **Box 6**.

22| Add 4 μ l of DNA Loading Dye to the 25 μ l of PCR reaction mix and load on a 2% (wt/vol) agarose gel. Run the gel at 7 V/cm for 30 min and visualize by staining with ethidium bromide (0.5 μ g/ml). A 571-bp product in the puromycin-selected cells that is absent from the untransfected cells indicates successful stable transgene integration (see example in **Fig. 3b**).

23| Determine the transgene copy number using quantitative PCR. The hCas9 amplicon is detected with FAM-labeled probe, and the genomic reference amplicon (EIF2C1) is detected with the HEX-labeled probe. Assemble the following reaction in triplicate technical replicates:

Component	Amount
Genomic DNA from Step 18	10 ng
20 \times hCas9 qPCR probe assay	1 μ l
20 \times EIF2C1 qPCR probe assay	1 μ l
ddH ₂ O	to 10 μ l
2 \times Affymetrix Veriquest quantitative PCR master mix for probe assays	10 μ l
Final volume	20 μ l

24| Perform quantitative PCR, detecting both FAM (carboxyfluorescein) and HEX (hexachlorofluorescein), using the following PCR program:

Step	Temperature	Time	Comment
1	95 $^{\circ}$ C	10 min	
2	95 $^{\circ}$ C	30 s	
3	60 $^{\circ}$ C	60 s	Go to 2, 40 times

25| Calculate the copy number per genome using the following formula: $2 \times 2^{(Ct_{EIF2C1} - Ct_{Cas9})}$.

▲ CRITICAL STEP When interpreting the data, keep in mind that in our experience this assay underestimated by about three fold the actual transgene copy number, determined by whole-genome sequencing and confirmed by targeted PCR amplification of each transgene. If digital droplet PCR is available, it may estimate transgene copy number slightly more accurately using the same qPCR probe assays (**Supplementary Fig. 3**).

Additional quality control of selected iPSC/ESC lines ● **TIMING 3–4 weeks**

26| Using the genotyping results from Step 22 and the transgene copy number from Step 25, select those cell lines that are positive for the transgene and that contain a low number of transgene copies (e.g., 1–5 copies) for further characterization. Thaw the chosen cell lines from Step 19 as directed in **Box 5**, and perform quality control for expression of pluripotency

Box 5 | Freezing and thawing of iPSCs

These procedures for freezing and thawing iPSCs are written for a well of a 24-well plate. For plates of different sizes, adjust the volumes accordingly.

Freezing

1. When the culture reaches roughly 60–70% confluence, harvest the cells following steps 2–5 of **Box 3**, using 1/4 volumes to adjust for use of a 24-well plate, and using mFreSR medium to dislodge and resuspend cells.
2. Quantify the cell density using a hemocytometer and adjust to 0.5 million cells per ml.
3. Using a 1-ml pipette, transfer the 0.25-ml of cell-containing mFreSR medium into a labeled cryovial.
4. Put the vials into a Coolcell LX cell freezing box and place them at $-80\text{ }^{\circ}\text{C}$ overnight. For long-term storage, transfer the vials to a liquid nitrogen tank. Cells can be stored in this state for many years.

Thawing

One vial of cells cryopreserved as described above should be thawed into one to two wells of a six-well plate. Cells should be thawed and plated as quickly as possible.

1. Prepare a six-well dish coated with Matrigel (**Box 2**).
2. Thaw the vial of cryopreserved cells in a $37\text{ }^{\circ}\text{C}$ water bath. Gently and continuously agitate the cryovial. The cells should thaw within 1–2 min.
3. Transfer the contents of the cryovial to a 15-ml tube with a 1-ml pipette.
4. Put 6 ml of warm mTeSR1 into the 15-ml tube.
5. Centrifuge the cells at 300g for 5 min at room temperature.
6. Remove the supernatant and resuspend the cell pellet in 4 ml of mTeSR1 containing $10\text{ }\mu\text{M}$ Y-27632.
7. Aspirate the medium from the Matrigel-coated dish from step 1.
- ▲ **CRITICAL STEP** Do not scratch the surface coating.
8. Transfer 2 ml of the cell suspension to each of two wells of the precoated six-well plate.
9. Put the plate in a $37\text{ }^{\circ}\text{C}$ incubator. Distribute the cells evenly by gently rocking the plate front-to-back and side-to-side. The plate should not be disturbed for 24 h.
10. Change the mTeSR1 medium daily until the cells are ready for splitting (typically in 4–5 d).

markers (qRT-PCR and immunostaining for Oct4, Nanog and Sox2)²⁰. Confirm normal karyotype by performing G-banded karyotyping through a cytogenetics testing service. Using qRT-PCR²¹ and the hCas9 qPCR probe assay, confirm robust Cas9 upregulation by DOX ($2\text{ }\mu\text{g/ml}$). Confirm Cas9 excisability as detailed in Steps 55–62.

27| Expand and freeze (**Box 5**) the best 2–3 lines for future use.

Genome editing using the stable iPSC-hCas9-PB cell line ● TIMING 5 d

28| Prepare one of the iPSC-hCas9-PB stable cell lines from Step 27 for nucleofection, as described in Steps 1–8.

29| To the cell suspension, add the following DNAs in a maximum volume of $<20\text{ }\mu\text{l}$:

Component	Amount
gRNA expression construct	4–20 μg
Donor DNA	4 μg
Excision-only piggyBac transposase plasmid (optional; see CRITICAL STEP note below)	2 μg

▲ **CRITICAL STEP** We found that 4 μg of gRNA expression construct is the minimal amount required for high (70–80%) transfection efficiency and $>20\text{ }\mu\text{g}$ of DNA will cause excessive cell death. Generally, 10 μg works best, but increasing or decreasing the amount can improve efficiency.

▲ **CRITICAL STEP** Addition of excision-only PB transposase plasmid is optional; for one-step genome editing with concurrent removal of the *hCas9-PB* transgene, add 2 μg of excision-only PB expression plasmid to the transfection mix. If further genome editing is planned, omit this step.

30| Repeat Steps 10–12, adding Dox at a final concentration of $2\text{ }\mu\text{g/ml}$ to the cells in the six-well plate before overnight incubation.

31| The next day, change the medium to mTeSR1 with Dox ($2\text{ }\mu\text{g/ml}$) but without Y-27632. Change the medium daily with mTeSR1 containing Dox ($2\text{ }\mu\text{g/ml}$). Cells should not exceed 70% confluence; if they do, then lift the cells (**Box 3**), replat them and continue Dox treatment.

Box 6 | Preparation of genomic DNA

The protocol below is for preparing genomic DNA from a six-well dish. The volumes can be scaled proportionately for other dish sizes. For example, use 1/4 of the volumes for a well of a 24-well dish.

1. Add 250 µl of lysis buffer to a cell pellet or to a well of a 6-well dish.
2. Incubate the cells at 55 °C overnight.
3. Precipitate DNA by adding 250 µl of isopropanol.
4. Centrifuge for 30 min in a microcentrifuge at maximum speed (20,000g, room temperature). Aspirate the supernatant. Wash with 1 ml of 70% (vol/vol) ethanol and repellet by centrifuging at 20,000g at room temperature for 5 min. Aspirate the supernatant and air-dry for 5 min.
- ▲ **CRITICAL STEP** The DNA pellet may be small and translucent. Be careful not to disturb the pellet when aspirating the supernatant. If DNA recovery is problematic, a coprecipitant such as GlycoBlue may increase yield and help to visualize the pellet.
5. Resuspend the DNA with 100–200 µl of dH₂O. Vortex the mixture vigorously. Allow several hours for the DNA to be resolubilized.

- 32| On the fourth day after nucleofection, change the medium to mTeSR1 containing 10 µM Y-27632 but without Dox.
▲ **CRITICAL STEP** In subsequent culture steps, use media without Dox.

- 33| On the fifth day after nucleofection, prepare three Matrigel-coated 10-cm dishes (**Box 2**).

- 34| Lift cells from Step 32 (as described in **Box 3**). Adjust the cell density with DPBS to 100,000 cells/ml.

? TROUBLESHOOTING

- 35| Seed the cells onto the Matrigel-coated 10-cm dishes from Step 33, each containing 5 ml of mTeSR1 medium with 10 µM Y-27632. Seed the cells at densities of 5 K, 10 K and 40 K per dish. For culture of these cells, continue to Step 48.

- ▲ **CRITICAL STEP** Be sure to distribute the cells evenly to avoid overlap of clones.

- 36| Extract genomic DNA from the remaining cells from Step 34, as directed in **Box 6**, and proceed to the Surveyor mutation detection assay (Step 37).

Surveyor mutation detection ● TIMING 1 d

- 37| To prepare PCR amplicons of the target area using Surveyor mutation genotyping primers (see Reagent Setup), assemble the following reaction mix:

Component	Amount
Genomic DNA from Step 36	50–100 ng
Forward primer, 10 µM	1 µl
Reverse primer, 10 µM	1 µl
ddH ₂ O	to 10 µl
2× Kapa Hifi Hotstart ReadyMix	10 µl
Final volume	20 µl

- 38| Run the following PCR program:

Step	Temperature	Time	Comment
1	95 °C	3 min	
2	98 °C	20 s	
3	60 °C	15 s	
4	72 °C	15 s	Go to 2, 30 times
5	72 °C	1 min	

39| Purify the PCR products using the QiaQuick PCR Purification Kit, according to the manufacturer's instructions.

40| Make 400 ng of purified PCR product to 40 µl in 1× Taq PCR Buffer.

41| Melt and anneal PCR products by placing them in a thermocycler running the following program:

Step	Temperature	Time
1	95 °C	10 min
2	95–85 °C at –2 °C/s	
3	85–75 °C at –0.3 °C/s	
4	75 °C	1 min
5	75–65 °C at –0.3 °C/s	
6	65 °C	1 min
7	65–55 °C at –0.3 C/s	
8	55 °C	1 min
9	55–45 °C at –0.3 C/s	
10	45 °C	1 min
11	45–35 °C at –0.3 C/s	
12	35 °C	1 min
13	35–25 °C at –0.3 C/s	
14	25 °C	1 min

42| Treat with Surveyor nuclease by adding the following:

Component	Amount
0.15 M MgCl ₂	4 µl
Surveyor enhancer S	1 µl
Surveyor nuclease S	2 µl

43| Mix well and incubate the mixture at 42 °C for 60 min.

44| Add 1/10 volume stop solution from the Surveyor Mutation Detection Kit to terminate the reaction and 1/6 volume DNA Loading Dye.

45| Analyze 10 µl of Surveyor nuclease digestion product by electrophoresis through the 4–20% TBE Gel at 200 V for ~60 min.

46| Stain the gel with 0.5 µg/ml ethidium bromide in 1× TBE for 10 min. Wash the gel in water for 10 min.

47| Image the gel with a UV transilluminator. Detectable Surveyor nuclease cleavage fragments typically indicate a Cas9 cutting efficiency that is sufficient to proceed with screening of individual clones. See **Figure 2a** and **Supplementary Fig. 1c** for examples. **Figure 6** includes an example of inefficient gRNA cleavage. If detectable Surveyor nuclease cleavage fragments are visible, then one can proceed to analyzing single clonal outgrowths (see Step 48). If gRNA cleavage was inefficient, terminate the experiment and see Troubleshooting section.

? TROUBLESHOOTING

PROTOCOL

Picking and analyzing individual clonal outgrowths

● TIMING 20 d

48| For the cells plated in Step 35, on the day after plating, change the medium to mTeSR1 without Y-27632. Change the medium daily until individual clonal outgrowths are visible to the naked eye (~12 d). Do not allow clones to become too big or to adhere to one another.

49| Pick and expand individual clones as directed in **Box 4**. The procedure described in **Box 4** will yield iPSC clones in 24-well dishes, to be used in Step 50, and matching genomic DNA samples, to be analyzed in Steps 51–54 below.

50| When the iPSC clones growing in 24-well dishes reach 70% confluence, lift the cells as described in **Box 3**. Freeze a sample of each clone, as directed in **Box 5**, pending genotyping results, which are obtained by analyzing each clone's genomic DNA sample as directed in Steps 51–54.

■ **PAUSE POINT** The cells can remain frozen for several weeks while awaiting genotyping results.

Genotyping of clones by Sanger sequencing ● TIMING 3 d

51| To evaluate individual clones for Cas9 genome editing, PCR-amplify genomic DNA for each clone obtained in Step 49, as described in Steps 37 and 38. Design the primers so that putative changes are in the center of a 200- to 300-bp amplicon. The PCR primers should efficiently amplify genomic DNA to yield a single band.

52| Submit the PCR product for Sanger sequencing with appropriate sample processing for crude PCR products.

53| Analyze the Sanger sequencing chromatograms to detect desired sequence changes. Software such as PolyPeakParser²² may help to deconvolute overlapping, out-of-phase chromatograms, which frequently result from indel mutation on one or both alleles. It may be necessary to clone PCR amplicons (e.g., using a TA Cloning Kit) and then sequence individual bacterial colonies to definitively determine the sequence of each individual allele.

? TROUBLESHOOTING

54| If piggyBac transposase was included in the transfection mix in Step 28, then also perform genotyping for the *hCas9* transgene as described in Steps 20–22.

PiggyBac transposon removal ● TIMING 20 d

▲ **CRITICAL** If multiple genome-editing steps are anticipated, it may be desirable to leave the piggyBac transposon in place, and then remove it at a final independent step.

55| Expand iPSC clones (from Step 50) that have the desired genotype and contain the *hCas9* transgene, as described in **Box 4**.

56| On the day before transfection, prepare a Matrigel-coated 10-cm dish (**Box 2**).

57| On the day of transfection, remove the solution from the Matrigel-coated 10-cm dish. Replace it with 6 ml of mTeSR1 medium with 10 μ M Y-27632.

58| Prepare the iPSC clone from Step 55 for nucleofection, following Steps 1–8.

59| To the cell suspension, add 2 μ g of excision-only piggyBac transposase plasmid.

60| Repeat Steps 10 and 11.

61| Of the 500 μ l of the suspension of nucleofected cells from Step 60, plate 30 μ l onto the Matrigel-coated 10-cm dish from Step 56. Distribute cells evenly over the dish. Incubate the cells at 37 °C overnight.

? TROUBLESHOOTING

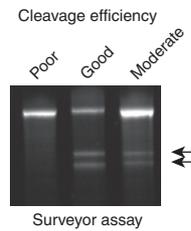


Figure 6 | Surveyor nuclease assay. Examples of good, moderate and poor gRNA-directed Cas9 cleavage followed by NHEJ, as determined by the surveyor assay. Arrows indicate surveyor nuclease cleavage products.

62 | The next day, change the medium to mTeSR1 without Y-27632 and change the medium daily.

63 | Once the clones are big enough to see with the naked eye (~12 d), pick 10 and expand (**Box 4**). The procedure in **Box 4** will yield iPSC clones in a 24-well dish, to be used in Step 64, and matching genomic DNA samples, to be analyzed in Step 65.

64 | When the iPSC clones growing in 24-well dishes reach 70% confluence, lift the cells as described in **Box 3**. Freeze a sample of each clone as directed in **Box 5**, pending genotyping results, which are obtained by analyzing genomic DNA from each clone from Step 63, as directed in Step 65.

65 | Genotype the genomic DNA of the clones from Step 63 for the *hCas9* transgene, as described in Steps 20–22.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Step 13	No puromycin-resistant iPSC clones	Problem with plasmids	Check the piggyBac expression plasmid and pPB-rtTA-hCas9-puro-PB plasmids by PCR and restriction digestion
		Poor transfection efficiency	Check transfection efficiency by transfecting a GFP expression plasmid. At least 50% of cells should be transfected, as ascertained by FACS analysis Use a GFP expression plasmid to optimize nucleofection efficiency Make sure that iPSCs are dissociated to a single-cell suspension
Steps 16, 34, and 61	Low iPSC number after nucleofection	Problem with puromycin solution	Check that the puromycin concentration is correct
		Low cell number	Grow cells to ~70% confluence before lifting for nucleofection
		ROCK inhibitor not working	Change to a new aliquot of Y-26732
		Incomplete cell dissociation	Incubate iPSCs with Versene until they become round and loosely adherent. Cells should easily separate from the dish and from other cells when gently sprayed with medium
Step 47	No surveyor nuclease cleavage product (see Fig. 5 for example)	Excessive cell treatment with Versene	Excessive Versene treatment causes cell death. Observe cells under a microscope so that they are collected without excessive incubation time
		Problem with Matrigel coating. Cells will appear round and will not attach well	Coat dishes with Matrigel as directed in Box 2
		Problem with surveyor nuclease assay	Perform positive control to make sure that the nuclease is working properly Use alternative genotyping strategy, e.g., creation of significant deletion or alteration of a restriction site
Step 47	No surveyor nuclease cleavage product (see Fig. 5 for example)	Poor transfection efficiency	Check transfection efficiency by transfecting a GFP expression plasmid. At least 50% of cells should be transfected, as ascertained by FACS analysis Use a GFP expression plasmid to optimize nucleofection efficiency Make sure that iPSCs are dissociated to a single-cell suspension
		Inefficient gRNA	Design alternative gRNAs
		Dox is degraded	Test procedure using a gRNA with known robust activity Use a new Dox aliquot. Protect Dox from light

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
Step 53	No HDR mutants obtained	Poor DNA donor design	Efficient yield of NHEJ, as ascertained by sequencing of clones and by heteroduplex detection with the Surveyor assay, but inefficient recovery of HDR clones suggests an issue with the DNA donor template. Increase the size of the DNA homology arms ¹⁰ and make sure that the donor is refractory to gRNA cleavage
Step 65	Low efficiency of piggyBac transposon excision	Problem with plasmid Low transfection efficiency	Make sure that you are using the excision-only piggyBac variant Check plasmid integrity by sequencing and restriction nuclease digestion Check transfection efficiency by transfecting a GFP expression plasmid. At least 50% of cells should be transfected, as ascertained by FACS analysis Use a GFP expression plasmid to optimize nucleofection efficiency Make sure that iPSCs are dissociated to a single-cell suspension

● **TIMING**

Obtaining Dox-inducible iPSC/ES clones (33 days)

Steps 1 and 2, growth of cells to 70% confluence in one well of a six-well dish: ~4 d, depending on starting point

Steps 3–12, nucleofection with Dox-inducible hCas9 plasmid and piggyBac: 2 h

Step 13, outgrowth of puromycin-resistant cells: 7 d

Steps 14–17, clonal expansion of puromycin-resistant cells: 12 d

Step 18, picking of individual puromycin-resistant clones: 2 h; outgrowth of puromycin-resistant clones, expansion, splitting and preparation of genomic DNA: 5 d

Step 19, growing of clones to 70% confluence and freezing: 3 d

Steps 20–22, confirmation of the presence of the *hCas9* transgene: 1 d

Steps 23–25, determination of *hCas9* transgene copy number: 1 d

Additional QC assays (can be done in parallel with each other)

Step 26, cDNA preparation from cells: 4 h; qRT-PCR for pluripotency markers: 4 h; qRT-PCR to determine basal and induced hCas9 levels: 4 h; immunostaining and microscopy to visualize pluripotency proteins: 1 d; G-banded karyotyping by cytogenetics testing service: 2 weeks; confirmation of Cas9 excisability: 16 d (can be done in parallel with karyotyping)

Step 27, Expand and freeze the best 2–3 lines for future use: 4 d

Genome editing using a stable iPSC-hCas9-PB line

Steps 28–30, nucleofection with gRNA and HDR donor DNAs: 2 h

Steps 31–33, outgrowth of nucleofected cells: 5 d

Step 34–35, lifting and replating of cells for clonal outgrowth: 1 h

Steps 36–47, confirmation of efficient genomic DNA modification: 1 d

Step 48, growth of clonal outgrowths: 12 d

Step 49, picking of individual clones into 24-well dishes, expansion, splitting and preparation of genomic DNA: 5 d

Step 50, growth of clones to 70% confluence and freezing of individual clones: 3 d

Steps 51 and 52, PCR-amplification of genomic DNA and submission of amplicons for Sanger sequencing: 2 d

Step 53, analysis of sequencing results for identification of candidate clones: 1 d

Step 54, PCR analysis of hCas9 excision, if performing one-step editing with concurrent hCas9 excision: 4 h, in parallel with Steps 51–53

Steps 55–60, nucleofection with excision-only piggyBac transposase plasmid: 2 h

Steps 61 and 62, outgrowth of single-cell-derived clones: 12 d

Step 63, picking of individual clones into 24-well dishes, expansion, splitting and preparation of genomic DNA: 5 d

Step 64, growth of clones to 70% confluence and freezing of individual clones: 3 d

Step 65, PCR analysis of hCas9 excision: 4 h

ANTICIPATED RESULTS

The first stage of this protocol is to integrate the piggyBac transposon containing the Dox-inducible Cas9 expression cassette into iPSCs, to yield iPSC-Cas9-PB. This step needs to be done only once to obtain a common parental line that is conducive to genome editing.

Genome editing using iPSC-Cas9-PB cells is highly efficient. As shown in **Figure 2** and **Supplementary Fig. 1**, we routinely obtain clones in which 19% have undergone HDR and 54% have undergone NHEJ. The Surveyor nuclease assay is a useful predictor of efficient genome modification: if a robust nuclease cleavage product is observed, then analysis of 48–96 clones will probably yield the desired genome-edited product. On the other hand, a weak or absent nuclease cleavage product (**Fig. 6**) suggests that the experiment should be halted and steps should be taken to troubleshoot the genome-editing efficiency (**Table 1**).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

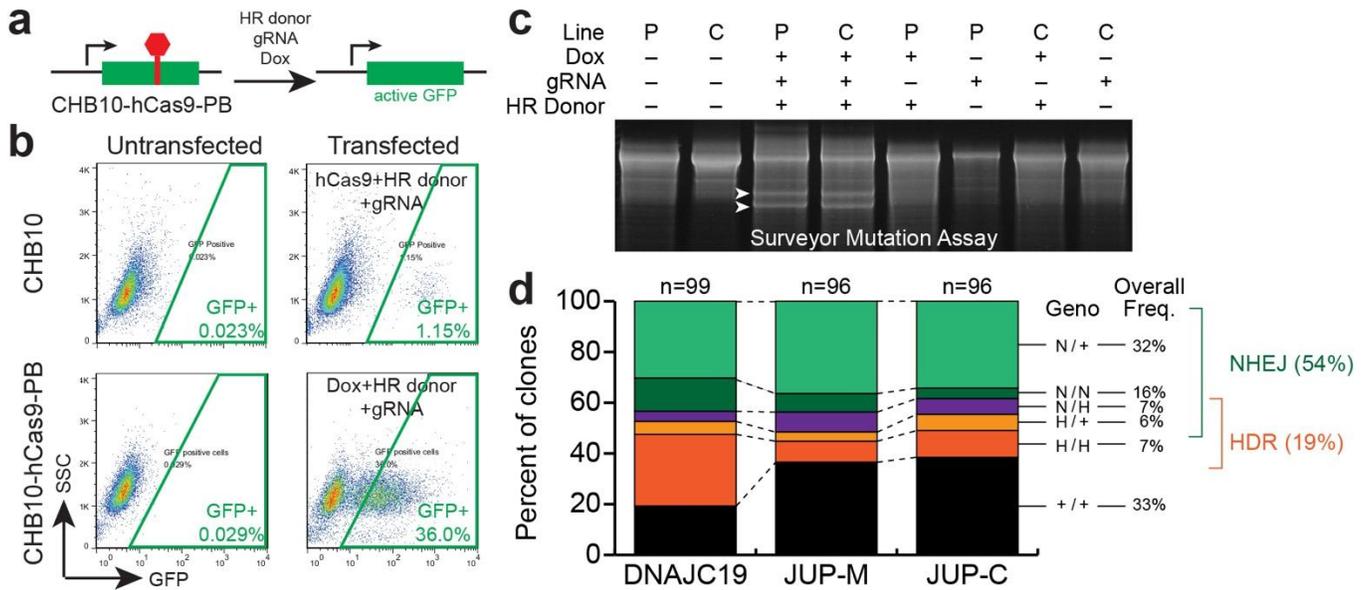
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AUTHOR CONTRIBUTIONS G.W. and L.Y. developed the protocol. L.Y. and D.G. analyzed sequencing data. G.W. and W.T.P. wrote the manuscript with input from the other authors. W.T.P. and G.M.C. supervised the project. L.Y.Y. performed experiments. K.L. contributed to the gRNA design, molecular cloning, and nucleofection. D.Z. contributed to iPSC culture and differentiation. Y.H. contributed genome-editing data. X.R. contributed to development of the Dox-inducible Cas9 expression plasmid.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details are available in the [online version of the paper](#).

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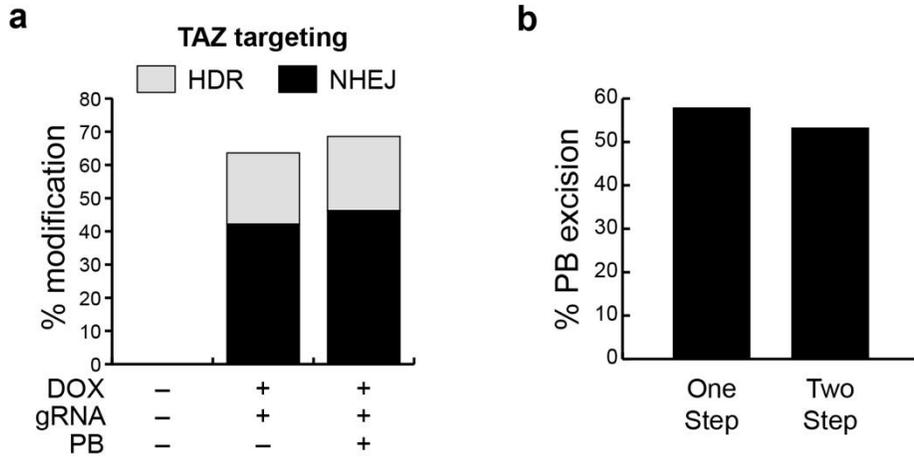
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Supplementary Figure 1

Efficient genome editing in human pluripotent stem cells.

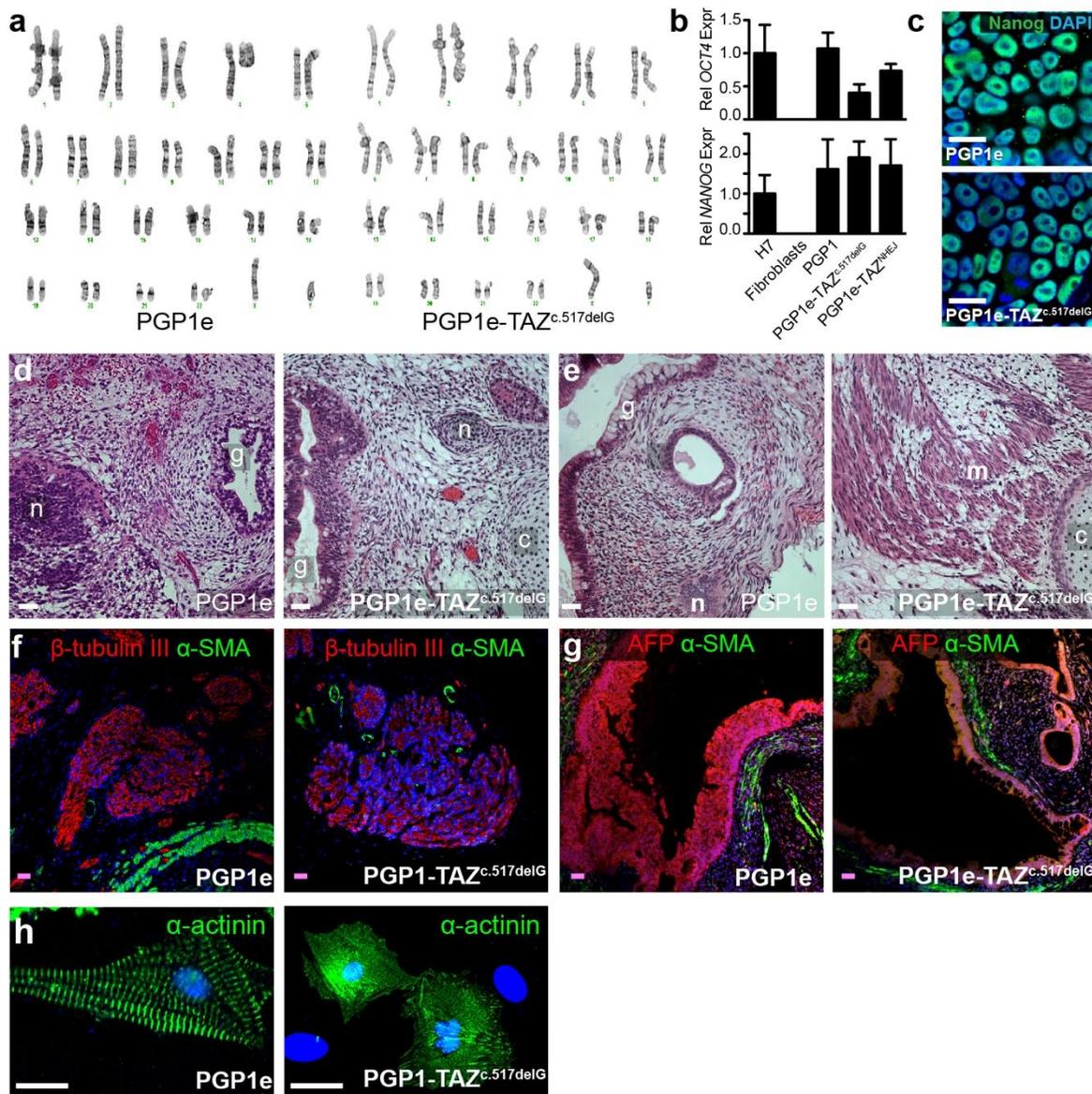
Our genome editing protocol was highly efficient in an additional pluripotent human cell line (CHB10) and at several different loci. **a-b.** We tested the efficiency of our protocol in a different pluripotent cell line, the human embryonic stem cell line CHB10, and a different locus, an integrated GFP reporter that is inactive due to a stop codon in the open reading frame (red symbol, panel a). Efficiency of HDR-mediated correction of the frameshift mutation was measured by FACS detection of GFP expression (panel b). **c.** Genome editing at the autosomal DNAJC19 locus of PGP1-hCas9-PB (P) or CHB10-hCas9-PB (C) pluripotent cell lines. Arrowheads indicate bands diagnostic of targeted genome modification. **d.** Genome editing efficiency at three autosomal sites, one in DNAJC19, and two within JUP (named JUP-M and JUP-C). Graph summarizes the results of Sanger sequencing of individual clones. Both homozygous and heterozygous mutations were efficiently recovered. Genotypes at each of the cell's two alleles is indicated as N, indel mutation from NHEJ; H, point mutation from HDR; +, wild-type. Overall frequency of each genotype class across the 3 experiments is summarized to the right.



Supplementary Figure 2

Efficient genome editing and piggyBac excision in a single step.

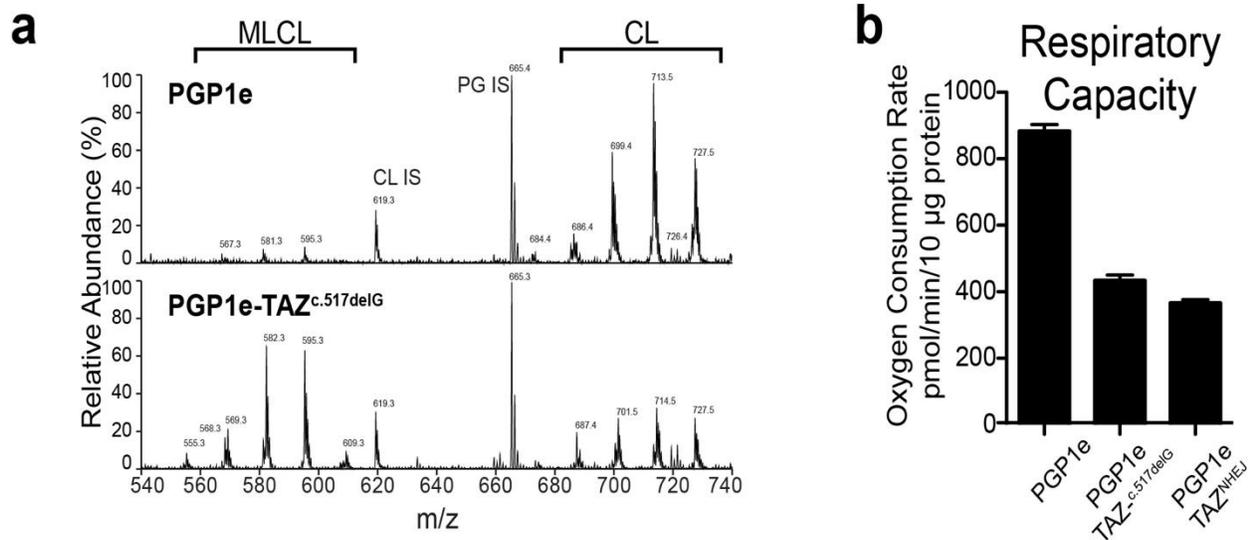
a. The co-transfection of the excision-only piggyBac mutant (PB) at the same as gRNA and HDR donor ("one-step" genome editing) did not substantially reduce the yield of genome-edited clones compared to sequential editing followed by excision ("two-step" genome editing). TAZ targeting frequencies were determined by next generation sequencing of genomic DNA from pooled cells. **b.** The frequency of transgene excision was not substantially different between one-step and two-step protocols. With either protocol, the majority of the recovered clones had successfully undergone excision of the piggyBac transgene, as determined by PCR genotyping of at least 79 independent clones per group over three separate experiments. These results show that including the excision-only piggyBac mutant into the transfection mix with gRNA and donor DNA permits efficient, single step genome editing and transgene excision.



Supplementary Figure 3

Efficient transposon removal by piggyBac transposase transient transfection yielded high quality iPSCs.

a. iPSC lines before after Cas9 genome editing had normal 46-XY karyotype. PGP1-hCas9-PB after transposon removal was designated PGP1e, and PGP1-hCas9-PB-TAZc.517delG was designated PGP1-TAZc.517delG. bar = 20 μm. **b-c.** Expression of pluripotency markers by control and mutant lines, as determined by qRT-PCR (b) or immunostaining (c). **d-g.** Hematoxylin and eosin staining of teratomas indicated formation of structures from all three germ layers. n, neural. g, glandular. c, cartilagenous. m, muscular. white bar = 100 μm; pink bar = 200 μm. **h.** Cardiac differentiation of genome-edited, piggyBac excised iPSCs. bar = 20 μm.



Supplementary Figure 4

iPSCs with induced mutation at the TAZ locus recapitulate features of Barth Syndrome patients.

TAZ mutation causes mitochondrial dysfunction and cardiomyopathy by blocking maturation of cardiolipin, the major phospholipid of the inner mitochondrial membrane. (Wang, G., McCain, M. L., Yang, L., He, A., et al. Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies. *Nat Med* 20, 616-623 (2014); Houtkooper, R. H., Turkenburg, M., Poll-The, B. T., Karall, D., et al. The enigmatic role of tafazzin in cardiolipin metabolism. *Biochim Biophys Acta* 1788, 2003-2014 (2009)). **a**. Cardiolipin phospholipid mass spectroscopy analysis control (PGP1e) and TAZ mutant (PGP1e-TAZ^{c.517delG}) iPSC-derived cardiomyocytes. As expected, PGP1e-TAZ^{c.517delG} iPSC-derived cardiomyocytes (iPSC-CMs) showed abnormal cardiolipin maturation, a signature of Barth syndrome **b**. Normal CL content is required for optimal function of the mitochondrial electron transport chain (Pfeiffer, K., Gohil, V., Stuart, R. A., Hunte, C., et al. Cardiolipin stabilizes respiratory chain supercomplexes. *J Biol Chem* 278, 52873-52880 (2003)). Respiratory capacity, the rate of oxygen consumption in the presence of the mitochondrial uncoupler trifluorocarbonyl cyanide phenylhydrazine (FCCP), is a measure of maximal electron transport chain activity. Oxygen consumption rate of control and mutant iPSC-CMs. Cells were treated with FCCP and analyzed using a Seahorse Biosciences Extracellular Flux Analyzer. Respiratory capacity, the rate of oxygen consumption in the presence of the mitochondrial uncoupler trifluorocarbonyl cyanide phenylhydrazine (FCCP), is a measure of maximal electron transport chain activity. We confirmed that respiratory capacity was markedly impaired in iPSC-CMs containing introduced TAZ mutations.

Supplementary Data 1

Scarless piggyBac excision of hCas9 transgene.

We examined the sites of transgene integration to verify that piggyBac excision was indeed scarless. Using the software tool ITIS (Jiang, C., Chen, C., Huang, Z., Liu, R. & Verdier, J. ITIS, a bioinformatics tool for accurate identification of transposon insertion sites using next-generation sequencing data. *BMC Bioinformatics* 16, 72 (2015)) we examined whole genome sequencing data from six independent genome-edited clones (listed in Fig. 4b) derived from the same parental line, PGP1-hCas9-PB. We identified 15 integration sites. These 15 integration sites were each confirmed by site-specific PCR of genomic DNA from PGP1-hCas9-PB using a primer within the transgene and a primer in the flanking genomic DNA. Interestingly, quantitative PCR and digital droplet PCR both slightly underestimated the transgene copy number (each estimated a transgene copy number of 4 or 8, respectively). In 5 separate piggyBac-treated clones that lacked the hCas9 transgene by PCR genotyping, we individually sequenced each piggyBac insertion site. At all 15 sites in all 5 clones (75 sites total), the wild-type sequence was restored. These data indicate that piggyBac excision is accurate and did not leave a detectable scar within the limits of detection of this assay.

Supplementary Data 2. Sequences used to target *TAZ*. These are useful as a positive control.

gBlock encoding gRNA (target sequence in red)

```
TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGC
AGGAAGAGGGCCTATTTCCCATGATTCTTCATATTTGCATATACGATACAAGGCTGTTAGAGA
GATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAG
TAATAATTTCTTGGGTAGTTTGCAGTTTTAAATTTATGTTTTAAATGGACTATCATATGCTTA
CCGTAACCTGAAAAGTATTTGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGA
AGCTCAACCATGGGACTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTAT
CAACTTGAAAAGTGGCACCGAGTCGGTGCTTTTTTTCTAGACCCAGCTTTCTTGTACAAAGTT
GGCATT
```

HDR donor oligonucleotide (generates BTHH *TAZ* mutation)

```
CTACCAGAAGGGGATGGACTTCATTTTGGAGAAGCTCAACCATGGGACTGGGTGCATATCTTCC
CAGAAGGTCAGCAGGGCTGACTGGGT
```

Sequencing primers

Forward

```
TAAGCTAACCTGTCACCCCA
```

Reverse

```
AGAGCACAGAGGCGAGGCTT
```