Efficient delivery of nuclease proteins for genome editing in human stem cells and primary cells

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Targeted nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like (TAL) effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9), have provided researchers with the ability to manipulate nearly any genomic sequence in human cells and model organisms. However, realizing the full potential of these genome-modifying technologies requires their safe and efficient delivery into relevant cell types. Unlike methods that rely on expression from nucleic acids, the direct delivery of nuclease proteins to cells provides rapid action and fast turnover, leading to fewer off-target effects while maintaining high rates of targeted modification. These features make nuclease protein delivery particularly well suited for precision genome engineering. Here we describe procedures for implementing protein-based genome editing in human embryonic stem cells and primary cells. Protocols for the expression, purification and delivery of ZFN proteins, which are intrinsically cell-permeable; TALEN proteins, which can be internalized via conjugation with cell-penetrating peptide moieties; and Cas9 ribonucleoprotein, whose nucleofection into cells facilitates rapid induction of multiplexed modifications, are described, along with procedures for evaluating nuclease protein activity. Once they are constructed, nuclease proteins can be expressed and purified within 6 d, and they can be used to induce genomic modifications in human cells within 2 d.

INTRODUCTION

Site-specific DNA endonucleases are highly flexible tools that are capable of manipulating virtually any genomic sequence across a broad range of cell types and model organisms[1–3]. These systems are configured to induce targeted DNA double-strand breaks (DSBs) that stimulate the cellular DNA repair machinery[4], which typically leads to one of two outcomes: (i) gene knockout by error-prone nonhomologous end joining (NHEJ)[5,6] or (ii) targeted integration or correction via homology-directed repair (HDR)[7–9]. The ease with which these technologies can be customized for genome engineering has revolutionized basic biological research, and it has the potential to transform medicine by providing a means to correct the underlying genetic causes of many diseases. The three technologies that are most commonly used to facilitate genome editing are ZFNs, TALENs and CRISPR/Cas9 (also known as RNA-guided endonucleases (RGENs)).

ZFNs[10] and TALENs[11] are fusions between the cleavage domain of the FokI restriction endonuclease and customizable zinc-finger and TAL effector DNA-binding domains, respectively. In particular, zinc-fingers are among the most frequently occurring proteins in the human genome, whereas TALE proteins are derived from plant pathogenic bacteria, in which they regulate host virulence factors that promote infection. Because the FokI cleavage domain functions as a dimer, two ZFN and TALEN monomers are required for DNA cleavage. Individual zinc-finger domains consist of ~30 amino acid residues in a ββα configuration, with the amino acids that mediate DNA recognition situated within the α-helical domain[12]. Each zinc-finger typically binds three bases, with occasional target site overlap from an adjacent zinc-finger domain. Synthetic zinc-finger proteins[13] can be generated to recognize a wide range of sequences by selection or by using a library of preselected zinc-finger domains, each with predefined specificity (**Box 1**). TALE proteins contain a DNA-binding domain that is composed of a series of 33- to 35-aa repeats that each recognizes a single base pair of DNA. The specificity of each TAL effector repeat is determined by two hypervariable amino acid residues present within each domain, which are termed the repeat variable di-residues (RVDs). Elucidation of the TAL effector protein DNA recognition code[14,15] has facilitated construction of synthetic TALE arrays capable of recognizing nearly any contiguous DNA sequence[16] (**Box 1**).

In contrast to ZFNs and TALENs, which rely on protein engineering methods for their assembly, the CRISPR/Cas9 system enables highly efficient RNA-guided genome editing with minimal design. Originally derived from bacteria, the CRISPR system provides acquired immunity against invading foreign DNA via RNA-guided DNA cleavage by the Cas9 protein[17]. This is achieved through expression of sequence-variable short CRISPR RNA (crRNA) and invariant *trans*-activating crRNA (tracrRNA) that direct sequence-specific cleavage of pathogenic DNA by Cas proteins. For target recognition, the Cas9 nuclease relies primarily on a 20-nt sequence within the crRNA and a conserved protospacer adjacent motif sequence downstream of the target site[17]. This system has been simplified for genome engineering[18–21], and it now consists of only the Cas9 nuclease and a single guide RNA (sgRNA) that contains both the crRNA and tracrRNA elements[17]. Together, with ZFNs and TALENs, these three foundational technologies provide a powerful toolbox capable of achieving sequence-specific genomic modification.

Box 1 | Methods for constructing targeted nucleases ● TIMING ~4–14 d

Before nuclease proteins can be introduced into cells, the genes encoding these proteins must be constructed. Depending on the nuclease platform used, a variety of approaches can be implemented for their assembly. Detailed protocols for many of these methods have been described elsewhere, but they are reviewed below.

Synthetic zinc-fingers with unique DNA-binding specificity can be readily constructed using the 'modular assembly' approach[91], which is based on the use of a preselected library of zinc-finger modules that are capable of recognizing individual DNA triplets generated by selection or rational design. Naturally occurring zinc-finger domains can also be incorporated into this method[92]. Selection-based procedures, including oligomerized pool engineering (OPEN), can facilitate isolation of zinc-finger arrays that consider context-dependent effects[93]. Approaches for building extended arrays around two- or three-finger zinc-finger building blocks have also been described[94,95], including those preselected for context-dependent interactions[96,97].

Numerous methods have also been developed that enable assembly of synthetic TAL effector arrays. Perhaps the simplest approach for assembling these is the Golden Gate cloning method[16]. PCR-based methods have also been described[98]; however, the repetitive nature of the TAL effector repeats pose problems for their amplification. High-throughput approaches based on solid-phase assembly[99] and ligation-independent cloning techniques[100] have also been reported, enabling rapid assembly of TALENs for genome-scale applications[50,99,100].

Unlike zinc-fingers and TALEs, no protein engineering is necessary for redirecting the CRISPR system to new target sites. We recommend starting by searching for potential Cas9 cleavage sites using an online CRISPR design tool or DNA sequencing viewing software. For the SpCas9 protein, search the selected gene sequence for the motif 5′-GNNNNNNNNNNNNNNNNNNN-NGG-3′, where 5′-NGG-3′ is the protospacer adjacent motif (PAM) recognized by SpCas9. Sense and antisense oligonucleotides encoding the selected sgRNA protospacer sequences can be custom-synthesized and cloned directly into a number of previously described CRISPR plasmid backbones[18,101].

However, realizing the full potential of these platforms requires the development of methods that facilitate their safe and efficient delivery into various cell types for basic research and therapeutic applications[22,23].

Methods for delivering nucleases into cells

Nuclease-encoding plasmid DNA can be delivered into cells by a number of standard techniques, such as electroporation[24] or nucleofection[25], as well as chemical methods, including calcium phosphate[26], cationic liposomes[27], polymers[28] or peptides[29,30]. These methods provide a cost-effective and straightforward approach for introducing nucleases into cells alone or in the presence of donor DNA for HDR. However, all of these techniques are associated with drawbacks that limit their effectiveness and practicality, including toxicity from the electroporation process[31] or chemical transfection reagents[32], low transfection efficiency (particularly for primary cells that divide slowly or exhibit quiescence) and, notably, the potential for insertional mutagenesis of foreign DNA into the host genome. Although transfection of nuclease-encoding mRNA offers a route for alleviating the risk of chromosomal insertion of the DNA backbone, this approach remains subject to many of the constraints that accompany electroporation and chemical transfection technologies, and it imposes the additional burdens and challenges associated with handling labile RNA.

In contrast to electroporation or chemical transfection, viral vectors provide a means for delivering nucleases into a broad range of primary cell types *in vitro* and *in vivo*. In particular, adeno-associated virus (AAV) vectors have facilitated the delivery of ZFNs and CRISPR/Cas9 into both mouse liver[33–35] and brain[36], resulting in efficient *in vivo* genome editing. AAV vectors have also facilitated nuclease-mediated genome engineering *in vitro*[37–39]. AAV vectors, however, are constrained by their limited carrying capacity (<5 kb), which hinders or prevents the use of a single AAV particle for the delivery of two TALEN monomers, *Streptococcus pyogenes* Cas9 (SpCas9) with its sgRNA[40] and, in some cases, a nuclease with a donor template. Integrase-defective lentiviral vectors (IDLVs), in contrast, provide an effective means for delivering nucleases into cells with donor DNA for HDR[41,42]. Conventional lentivirus also facilitates genome editing both *in vitro*[43] and *in vivo*[44], and it provides a means for performing genome-wide functional screens[45–47] using CRISPR/Cas9-based technologies. However, lentivirus integrates randomly within the host genome, and IDLVs are prone to silencing effects. In addition, TALEN-encoding genes are prone to rearrangements and truncations within both vector platforms because of the repetitive nature of the DNA-binding domain[48], although strategies have developed to help overcome this[49,50]. Specifically, amino acid degeneracy and alternative codon usage can be used to generate highly variable, preassembled tripartite RVD arrays that are less susceptible to recombination in cells[50]. Alternatively, adenovirus vectors (AdVs) provide another means for delivering nucleases[51,52], including TALENs[48], into primary cells and *in vivo*[53,54]. Unlike IDLVs, which exist as free-ended donor DNA, protein-capped AdVs are capable of mediating scarless HDR[55]. These vectors, however, are particularly difficult and time-consuming to produce, and they are associated with low rates of genomic modification. Nonintegrating gamma-retroviral[56] and baculoviral vector systems[57–59] have also been configured for nuclease delivery, although the expression of TALEN genes from baculovirus could lead to rearrangements within the TAL effector DNA-binding domain[60].

Regardless of the viral or nonviral vector system used, high levels of nuclease expression for an extended period of time from DNA could lead to increased off-target effects[61], a drawback that has serious consequences for myriad genome engineering applications. One approach for improving nuclease specificity is by limiting the amount of time that the nuclease is present within cells[61,62]. This can be achieved in a number of ways[63], including by introducing nucleases into cells as purified protein rather than expressing the nuclease within the cell from nucleic acids[62]. This approach offers the distinct advantage of rapid action

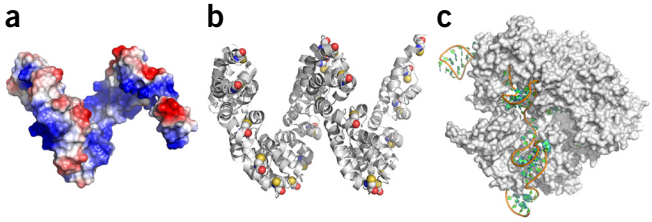

Figure 1 | Modes of nuclease protein delivery. (**a**) Cell-permeable ZFN protein. Cartoon of the electrostatic surface potential of a zinc-finger protein colored from dark red (negatively charged residues) to white (neutral residues) to dark blue (positively charged residues; PDB ID: 2I13)[85]. Intrinsic cell-penetrating activity of the ZFN protein because of the positive charge of the zinc-finger protein. (**b**) Cell-penetrating TALEN protein. Cartoon of the PthXo1 TAL effector DNA-binding domain (PDB ID: 3UGM)[86]. Cell permeability endowed via Cys-conjugation with cell-penetrating peptides. Surface-exposed Cys residues used for conjugation shown as spheres, with oxygen, nitrogen and sulfur atoms colored red, blue and yellow, respectively. (**c**) Cas9 ribonucleoprotein. Cartoon of the Cas9 ribonucleoprotein (PDB ID: 4008)[87]. RNP can be introduced directly into cells via nucleofection or transfection of RNP. Cas9 protein is shown in gray. sgRNA is indicated by green sticks.

and fast turnover, resulting in fewer off-target effects. Notably, this shortened window of activity does not adversely affect the rates of targeted genome modification. To date, all three major types of nucleases have been introduced into cells as purified protein through a number of different strategies (**Fig. 1**). In particular, ZFN proteins are inherently cell-permeable, and they are capable of achieving gene knockout after their direct application to cells in culture[62,64,65]. This property is due to the intrinsic cell-penetrating activity of zinc-finger proteins (**Fig. 1a**), which has recently been leveraged for intracellular protein delivery at rates that exceed many conventional protein transduction systems[66,67]. ZFN proteins can also be engineered for direct cellular internalization using receptor-mediated endocytosis[68] and retrovirus-[56] or lentivirus-mediated[69] protein transfer, the last of which allows for co-delivery of donor DNA into cells for targeted integration or gene replacement. TALENs, which are incompatible with several viral vectors because of their size and repetitive structure, can be also delivered into cells as proteins using lentivirus[69]. Alternatively, cell-penetrating peptides (CPPs) that mediate cellular uptake can be conjugated to the surface of individual TAL effector repeats (**Fig. 1b**)[70] or genetically fused to their termini[71]. Finally, a preformed Cas9-sgRNA ribonucleoprotein (RNP) complex can be delivered into cells using nucleofection[72,73], Lipofectamine-mediated transfection[74], CPP-mediated internalization[75] and small-molecule–induced osmocytosis (**Fig. 1c**)[76].

We and others have shown that nuclease proteins can be delivered into a wide variety of primary cell types, including induced pluripotent[64] and human embryonic stem cells[72,75], primary CD4$^+$ T cells[62,64], hematopoietic stem or progenitor cells[64,68] and mouse hair cells *in vivo*[74], indicating the broad potential of protein delivery for mediating genomic engineering both *ex vivo* and *in vivo*. Strategies for combining nuclease protein delivery with single-stranded DNA oligonucleotides (ssODNs) have also been recently described[72–74], further expanding the scope of modifications that can be achieved using these procedures.

Limitations of nuclease protein delivery

Target cell type, nuclease platform and administration route all contribute to variable levels of uptake and genome editing efficiency. Although this broad variability represents a limitation to the approach, a handful of more specific restrictions also exist. Specifically, methods for delivering nuclease proteins with donor DNA containing transgenes are lacking. In addition, although methods for delivering RNP *in vivo* have recently been described[74], few approaches have been established that enable tissue-specific *in vivo* delivery of nuclease proteins.

Experimental design

Here we describe three step-by-step protocols for introducing ZFN, TALEN and Cas9 protein directly into cells. We focus on primary CD4$^+$ T cells and human embryonic stem cells; however, our procedures have the potential to be applied to a diverse range of cell types.

Successful nuclease protein delivery first requires choosing the most suitable platform for the target application. In our experience, nucleofection of RNP provides the highest degree of flexibility and efficiency: once recombinant Cas9 protein is generated, modifying user-specific genomic loci only requires the production of new sgRNA.

Once a platform is selected, the nuclease must be constructed (see **Box 1** for an overview of nuclease assembly methods) and validated. We recommend using previously described transient transfection methods to rapidly evaluate the activity of nuclease-encoding gene(s) in transformed human cell lines. After these initial proof-of-principal experiments, nuclease-encoding gene(s) are cloned into the pET-28 bacterial expression vector for overexpression and subsequent administration into cells. For each nuclease platform, we previously determined the optimal amount of protein for maximum activity[62,64,70,72]. These conditions are the basis for the procedures described here.

Controls. Finally, for any protein delivery experiment, it is crucial to perform relevant controls, including mock treatments. For experiments using ZFN and TALEN proteins, we recommend treating cells with serum-containing medium containing either no nucleases or only one nuclease monomer. For RNP, we recommend transfecting cells with nucleofection solution containing no additives, Cas9 protein only or sgRNA only. When quantifying genome editing using the Surveyor nuclease assay, background cleavage from mock-treated cells must be subtracted from nuclease-treated samples in order to accurately measure activity.

ZFN protein delivery. This protocol describes gene knockout in primary CD4$^+$ T cells isolated from human peripheral blood mononuclear cells (PBMCs) using ZFN proteins[62,64]. We previously showed that zinc-finger DNA-binding domains enter cells primarily through macropinocytosis and caveolin-dependent endocytosis[66]. Despite the simplicity of this technique, first-generation ZFN protein delivery strategies required consecutive protein treatments for high levels of mutagenesis in cells. To improve on this methodology, we demonstrated that incorporation of tandem repeats of the simian virus (SV40) nuclear localization signal (NLS) sequence improves ZFN protein uptake and gene editing activity[64].

To ensure activity, ZFN proteins must be maintained with two supplements: $ZnCl_2$, which coordinates with Cys and His residues to stabilize the zinc-finger scaffold, and L-Arg, which acts as a protein-stabilizing agent. Once internalized, ZFN proteins must also be exposed to transient 'cold shock' culture conditions in order to enhance their activity in cell culture[77].

TALEN protein delivery. This protocol describes the CPP-mediated delivery of TALEN proteins into mammalian cells[70]. We previously showed that conjugation of individual TAL effector repeats with thiol-reactive poly-Arg_9 moieties imparts enough positive charge to endow TALEN proteins with cell-penetrating activity[70]. These disulfide linkages are reversible under reducing conditions, and poly-Arg_9 is released from the TALEN proteins after their entry into the cytosol. Once purified and concentrated, TALEN proteins are allowed to react with Cys-(Npys)-$(D\text{-}Arg)_9$ under conditions that were previously determined to yield maximum TALEN-mediated gene-editing activity. Before their direct application to cells, the reaction solution is neutralized to physiological pH using 1 M sodium hydroxide and mixed thoroughly with serum-free medium (SFM) in the presence of protease inhibitor. Because of differences in CPP-mediated uptake between certain cell types, optimization of the peptide-to-protein ratio may be necessary to achieve maximum TALEN protein delivery.

Cas9 RNP delivery. This protocol describes the nucleofection of human embryonic stem cells (hESCs) with RNPs, and it represents an optimized procedure that was originally described by Kim *et al.*[72]. This approach is highly flexible and capable of simultaneously delivering multiple RNPs in tandem for inducing targeted chromosomal deletions[72] and multiplexed gene modifications. In addition, RNPs can be delivered into cells with highly recombinogenic ssODN[72–74] for the introduction of single-base substitutions via HDR[78]. Compared with both ZFN and TALEN protein delivery, this approach facilitates the highest levels of modification in hESCs, and thus it could be used to modify cells for regenerative medicine applications.

MATERIALS

REAGENTS

- DNA ladder, 100 bp (New England Biolabs, cat. no. N3231S)
- TAE running buffer, 10× (Life Technologies, cat. no. AM9869)
- Laemmli protein loading dye, 2× (Bio-Rad, cat. no. 161-0737)
- TBE running buffer, 5× (Life Technologies, cat. no. LC6675)
- DNA loading dye, 6× (New England Biolabs, cat. no. B7021S)
- AccuRuler RGB protein marker (BioPioneer, cat. no. PM-001)
- Accutase (Stem Cell Technologies, cat. no. 07920)
- Agarose (Bio-Rad, cat. no. 161-3100)
- Kanamycin (Sigma-Aldrich, cat. no. 70560-51-9)
- Bacto agar (BD Biosciences, cat. no. 214050)
- Bacto tryptone (BD Biosciences, cat. no. 211705)
- Bacto yeast extract (BD Biosciences, cat. no. 212750)
- CD3/CD28 human T-cell activation beads (Life Technologies, cat. no. 11131D)
- Chloroform (Sigma-Aldrich, cat. no. C7559-5VL)
- Collagenase type IV (Life Technologies, cat. no. 17104-019)
  ▲ **CRITICAL** The quality of collagenase type IV can vary between batches and suppliers. Optimal cell dissociation conditions should be tested for each batch.
- CutSmart buffer (New England Biolabs, cat. no. B7204S)
- Cys (Npys)-$(D\text{-}Arg)_9$ peptide (AnaSpec, cat. no. AS-61206)
- D,L-DTT (Sigma-Aldrich, cat. no. D0632)
- DMEM/F12 medium (Life Technologies, cat. no. 11330)
- DMSO (Sigma-Aldrich, cat. no. D2650)
- dNTPs (New England Biolabs, cat. no. N0447S)
- DMEM (Life Technologies, cat. no. 10566-016)
- Dulbecco's PBS, no calcium, no magnesium (DPBS; Life Technologies, cat. no. 14190-144)
- EasySep human $CD4^+$ T-cell enrichment cocktail (Stem Cell Technologies, cat. no. 19052)
- EDTA-free complete protease inhibitor cocktail tablet (Roche, cat. no. 05892791001)
- Ethanol (Sigma-Aldrich, cat. no. E7023) ! **CAUTION** Ethanol is flammable, and it should be stored and handled under appropriate conditions.
- Expand high-fidelity PCR system (Roche, cat. no. 11732641001)
- FBS (Life Technologies, cat. no. 16000-044)
- Ficoll-Paque Plus (GE Healthcare Life Sciences, cat. no. 17-1440-02)
- Glucose (Sigma-Aldrich, cat. no. G7021-100G)
- GlutaMAX (Life Technologies, cat. no. 35050-061)
- Glycerol (Sigma-Aldrich, cat. no. G5516-100ML)
- HBSS with calcium and magnesium (Life Technologies, cat. no. 14025-092)
- HEPES sodium salt (Sigma-Aldrich, cat. no. H3784-100G)
- HiScribe T7 transcription kit (New England Biolabs, E2040S)
- Human blood mixed with anticoagulant (The Scripps Research Institute's Normal Blood Donor Program) ! **CAUTION** Informed consent should be obtained for all human samples. Always use gloves when manipulating human blood. Being previously vaccinated for hepatitis A and B is highly recommended.
- Imidazole (Sigma-Aldrich, cat. no. I5513-100G)
- Isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, cat. no. I5502-1G)
- Knockout serum replacement (SR; Life Technologies; cat. no. 10828-028)
- L-Arginine (L-Arg; Sigma-Aldrich, cat. no. A5006-100G)
- Magnesium chloride hexahydrate (Sigma-Aldrich, cat. no. M2393-100G)
- Matrigel, growth factor reduced (BD Biosciences, cat. no. 354277)
- MEM non-essential amino acid solution, 100× (Life Technologies, cat. no. 11140-050)
- Mouse anti-human CD4 antibody, FITC-labeled (Abcam, cat. no. ab59474)
- mTeSR1 (STEMCELL Technologies, cat. no. 05850)
- mTeSR1 5× supplement (STEMCELL Technologies, cat. no. 05852)
- Nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, cat. no. 30210)
- Nuclease-free water (Promega, cat. no. P1193)
- Penicillin-streptomycin (Life Technologies, cat. no. 15140-148)
- Phenol:chloroform:isoamyl alcohol (25:24:1 (vol/vol); Life Technologies, cat. no. 15593-031) ! **CAUTION** It is toxic if swallowed or inhaled. Handle it according to the manufacturer's instructions.
- Plasmid: pET-28b (EMD Biosciences, cat. no. 69865-3)
- Poly-L-lysine solution (Sigma-Aldrich, cat. no. P4707-50ML)
- Polypropylene gravity-flow purification column (Bio-Rad, cat. no. 732-1010)
- QIAquick gel extraction kit (Qiagen, cat. no. 28704)
- QIAquick PCR purification kit (Qiagen, cat. no. 28104)
- QIAprep spin miniprep kit (Qiagen, cat. no. 27104)
- QuickExtract DNA extraction solution (Epicentre, cat. no. QE0905T)
- Recombinant human basic fibroblast growth factor (bFGF; Life Technologies, cat. no. 13256-029)
- Recombinant interleukin-2 (rIL-2; R&D Systems, cat. no. 202-IL-010)
- RPMI 1640 medium (Life Technologies, cat. no. 11875-119)
- SepMate-50 column (STEMCELL Technologies, cat. no. 15460)
- Sodium acetate (Sigma-Aldrich, W302406-1KG-K)
- Sodium chloride (Sigma-Aldrich, cat. no. S3014-500G)
- Sodium hydroxide (Sigma-Aldrich, cat. no. S8045-500G)
- Sodium phosphate dibasic (Sigma-Aldrich, cat. no. S7907-100G)
- Sodium phosphate monobasic (Sigma-Aldrich, cat. no. S8282-500G)
- SURVEYOR mutation detection kit (Transgenomic, cat. no. 706021)
- SYBR Safe DNA gel stain (Life Technologies, cat. no. S33102)
- T4 DNA ligase (New England Biolabs, cat. no. M0202S)

TABLE 1 | Primer sequences for nuclease cloning.

Primer name	Sequence (5′–3′)	Purpose
NcoI ZFN fwd	aaaCCATGGatgggtcatcatcatcatcatcacggtggcagcccgaaaaaaaaacgcaaa	Forward primer used to PCR-amplify ZFN-encoding genes
XhoI ZFN rev	aaaCTCGAGttaaaagtttatctcgccgtt	Reverse primer used to PCR-amplify ZFN-encoding genes
NcoI TALEN fwd	aaaCCATGGatgatgggtcatcatcatcatcatcacggtggcagcgactacaaagaccatgacggt	Forward primer used to PCR-amplify TALEN-encoding genes
XhoI TALEN rev	aaaCTCGAGttaaaagtttatctcgccgttatt	Reverse primer used to PCR-amplify TALEN-encoding genes
NcoI SpCas9 fwd	aaaCCATGGatgggcagcagccccaagaagaagaggaaggtgggcggctccatggataagaaatactca	Forward primer used to PCR-amplify the SpCas9-encoding gene
XhoI SpCas9 rev	aaaCTCGAGttaatgatgatgatgatgggagccgcccactttgcgtttcttttcggggagccgcc	Reverse primer used to PCR-amplify the SpCas9-encoding gene

Uppercase bases indicate restriction sites.

- T4 DNA ligase reaction buffer (New England Biolabs, cat. no. B0202S)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787-50ML) **! CAUTION** Triton X-100 is hazardous if it is swallowed or inhaled.
- Trypsin-EDTA, 0.05% (wt/vol; Life Technologies, cat. no. 25300-062)
- Zero Blunt TOPO PCR cloning kit (Life Technologies, cat. no. K2800-40)
- Zinc chloride (Sigma-Aldrich, cat. no. 229997-10G)
- β-Mercaptoethanol, 55 mM (β-ME; Life Technologies, cat. no. 21985-023) **! CAUTION** (β-ME is hazardous if it is contacted or ingested.

Vectors and primers

- Primers (**Table 1**; ThermoFisher Scientific, Invitrogen custom DNA oligos)
- M13 forward primer, 20 μM (5′-GTAAAACGACGGCCAGT-3′; Life Technologies)
- Bacterial and mammalian expression vectors encoding the ZFNs, TALENs and RNPs used here can be obtained upon request by contacting J.L.

Cell lines

- HeLa (American Type Culture Collection, cat. no. CCL-2)
- K562 (American Type Culture Collection, cat. no. CCL-243)
- Jurkat E6-1 (American Type Culture Collection, cat. no. TIB-152)
- Chemically competent BL21 (DE3) cells (Life Technologies, cat. no. C6010-03)
- Chemically competent TOP10 cells (Life Technologies, cat. no. C4040-06)
- H9 human embryonic stem cells (hESCs) **! CAUTION** Handle H9 cells in accordance with institutional regulations.

Restriction enzymes

- NcoI (New England Biolabs, cat. no. R0193S)
- XhoI (New England Biolabs, cat. no. R0146S)
- XbaI (New England Biolabs, cat. no. R0145S)

EQUIPMENT

- TBE gel, 10%, wt/vol (Life Technologies, cat. no. EC6275BOX)
- Petri dish, 10 cm (Corning, cat. no. 25373-041)
- Polystyrene round-bottom tubes, 5 ml (Corning, cat. no. 352058)
- Amicon Ultra-15 centrifugal filter units (Millipore, 10-kDa MWCO, cat. no. UFC901024; 30-kDa MWCO, cat. no. UFC903024)
- Avanti J-E centrifuge (Beckman, cat. no. 369001)
- EasySep magnet (Stem Cell Technologies, cat. no. 18000)
- Gel Doc XR system (Bio-Rad, cat. no. 170-8195)
- Hemocytometer (Hausser Scientific, cat. no. 3200)
- Leica microscope (Leica, DFC340FX)
- NanoDrop 2000c spectrophotometer (Thermo Scientific)
- P3 primary cell 4D-nucleofector kit (Lonza, cat. no. V4XP-3032)
- PCR thermocycler (MJ Research PTC-2000)
- Refrigerated microcentrifuge (5415R; Eppendorf, cat. no. 022621408)
- S-450 sonicator (Branson Sonifier, cat. no. 15-338-553)
- Sterile 15-ml conical tubes (Corning, cat. no. 14-959-49D)
- Sterile 50-ml conical tubes (BD Biosciences, cat. no. 352070)
- Sterile serological pipettes (VWR International; 5 ml, cat. no. 89130-896; 10 ml, cat. no. 89130-898; 25 ml, cat. no. 89130-900)
- Tissue culture hood (Labconco, cat. no. 332391120)
- Tissue culture incubator (Thermo Scientific, cat. no. 50116050)
- 4D-Nucleofector system (Lonza; core unit, cat. no. AAF-1002B; X unit, cat. no. AAF-1002X)
- Tris-glycine gel, 4–20%, wt/vol (Life Technologies, cat. no. EC6028BOX)
- Flat-cap PCR tubes, 0.2 ml (Axygen, cat. no. PCR-02-C)
- Low-protein-binding filter (Millipore; 0.22 μm, cat. no. SLGV013SL; 0.45 μm, cat. no. SLHP033RS)
- Sterile 1.5-ml microcentrifuge tubes (Eppendorf, cat. no. 022431021)
- Tissue culture plate (Sigma-Aldrich; six well, cat. no. CLS3516-10EA; 24 well, cat. no. CLS3527-100EA; 96 well, cat. no. CLS3596)

REAGENT SETUP

LB medium (1 liter) Dissolve 10 g of Bacto tryptone, 5 g of yeast extract and 10 g of $NaCl_2$ in dH_2O. Adjust the volume to 1 liter and the pH to 7.5, and then autoclave the solution. Store it at 4 °C for up to 1 month.

LB agar plates (1 liter) Add 15 g of Bacto agar to 1 liter of LB medium. Sterilize it by autoclaving, and pipette 22 ml of LB agar to a sterile Petri dish. Store it at 4 °C for up to 1 month.

HEPES (200 mM stock solution, 1 liter) Dissolve 52.1 g of HEPES sodium salt in dH_2O. Adjust the volume to 1 liter and the pH to 8.0, and then autoclave the solution. Store it at 4 °C for up to 6 months.

Collagenase type IV (1,000× stock solution) Dissolve 1 g of collagenase type IV in 2 ml of HBSS with calcium and magnesium. Store the solution at 4 °C for up to 1 month

Ethanol (75%, vol/vol, 100 ml) Mix 25 ml of dH_2O with 75 ml of ethanol. Store it at room temperature (25 °C) for up to 1 month.

$MgCl_2$ (1 M stock solution, 100 ml) Dissolve 20.3 g of $MgCl_2{\cdot}6H_2O$ in 1 liter of dH_2O and autoclave the solution. Store the solution at room temperature for up to 1 year.

$ZnCl_2$ (9 mM stock solution, 1 liter) Dissolve 1.23 g of $ZnCl_2$ in 1 liter of dH_2O and autoclave the solution. Store it at room temperature for up to 1 year.

Imidazole (2 M stock solution, 100 ml) Dissolve 13.6 g of imidazole in 100 ml of dH_2O and autoclave the solution. Store the solution at room temperature for up to 1 year.

L-Arg (1 M stock solution, 100 ml) Dissolve 17.4 g in 100 ml of dH_2O. Adjust the pH to 7.4 and sterilize it using a 0.22-μm filter. Store the solution at room temperature for up to 6 months.

DTT (1 M stock solution, 10 ml) Dissolve 1.54 g of DTT in 10 ml of dH_2O. Prepare 1-ml aliquots; sterilize the solution using a 0.22-μm filter, and store it at −20 °C for up to 6 months.

Sodium phosphate buffer (200 mM stock solution, 1 liter) Dissolve 23.4 g of sodium phosphate monobasic in 1 liter of dH_2O. Adjust the pH to 5.5 and autoclave the solution. Store the buffer at 4 °C for up to 6 months.

Sodium acetate (3 M stock solution, 100 ml) Dissolve 24.6 g of sodium acetate anhydous powder in 100 ml of dH_2O. Adjust the pH to 5.2 and autoclave the solution. Store it at room temperature for up to 1 year.
Cys (Npys)-(D-Arg)$_9$ peptide solution (2.3 mM stock solution) Dissolve 1 mg of Cys (Npys)-(D-Arg)$_9$ peptide in 250 µl of dH_2O. Sterilize the solution using a 0.22-µm filter, and store it at −20 °C for up to 6 months.
Protease inhibitor cocktail (25× stock solution) Dissolve one Roche protease inhibitor cocktail tablet in 2 ml of dH_2O, and sterilize it using a 0.22-µm filter. Store it at −20 °C for up to 1 week.
Binding buffer (1 liter) Add 100 ml of 200 mM HEPES stock solution, 1 ml of 1 M $MgCl_2$ stock solution, 29.2 g of NaCl and 100 ml of glycerol into 799 ml of dH_2O. For ZFN binding buffer, add 10 ml of 9 mM $ZnCl_2$ stock solution and an additional 87.7 g of NaCl. Adjust the volume to 1 liter and autoclave the solution. Store it at 4 °C for up to 6 months.
Lysis buffer (1 liter) Add 1 mM β-mercaptoethanol (β-ME) and 1× protease inhibitor cocktail to the binding buffer before use. Store the solution at 4 °C for up to 24 h.
Wash buffer A (1 liter) Add 2.5 ml of 2 M imidazole to 1 liter of binding buffer. Store the buffer at 4 °C for up to 6 months.
Wash buffer B (1 liter) Add 17.5 ml of 2 M imidazole to 1 liter of binding buffer. Store the buffer at 4 °C for up to 6 months.
Elution buffer (1 liter) Dissolve 20.4 g of imidazole into 1 liter of binding buffer. Store the buffer at 4 °C for up to 6 months.
Storage buffer (1 liter) Add 100 ml of 200 mM HEPES stock solution, 1 ml of 1 M $MgCl_2$ stock solution, 29.2 g of NaCl and 100 ml of glycerol into 799 ml of dH_2O. For ZFN storage buffer, add 100 ml of 1 M L-Arg. For Cas9 protein storage buffer, add 1 ml of 1 M DTT stock solution. Store the solution at 4 °C for up to 2 weeks.
Complete RMPI medium Add 50 ml of FBS and 5 ml of 100 U/ml of penicillin-streptomycin to 500 ml of RPMI 1640 medium. This solution can be stored at 4 °C for 3 months. Prewarm the medium to 37 °C before use.
Complete DMEM medium Add 50 ml of FBS and 5 ml of 100 U/ml of penicillin-streptomycin to 500 ml of DMEM medium. This solution can be stored at 4 °C for 3 months. Prewarm the medium to 37 °C before use.
Complete DMEM/F12 medium Add 120 ml of KnockOut serum replacement, 6 ml of 100× non-essential amino acids solution, 6 ml of GlutaMAX, 1.5 ml of 55 mM β-ME and 2.4 µg of bFGF into 466 ml of DMEM/F12 medium. Store the medium at 4 °C for 1 month. Prewarm the medium to 37 °C before use.
Complete mTeSR1 medium Add 100 ml of mTeSR1 supplement (5×) into 400 ml of mTeSR1 medium. Store the medium at 4 °C for up to 2 weeks or at −20 °C for up to 6 months.

EQUIPMENT SETUP

Poly-lysine coating Add 250 ml of poly-lysine solution to each well of a 24-well culture plate, and incubate the plate at 37 °C for 1 h. Remove the solution by aspiration and wash it twice with 0.5 ml of DPBS. Dry poly-lysine–treated plates at room temperature for 2 h. Coated plates can be stored at 4 °C for up to 1 month. ▲ **CRITICAL** Allow the plates to dry for 2 h before use to ensure efficient cell attachment.
Matrigel-coated T25 flask One or 2 d before H9 hESC seeding, dilute Matrigel to 5% (vol/vol) with DMEM/F12 medium and add 3 ml of solution into a T25 flask. Store the flask at 4 °C for up to 1 week. Incubate the flask at room temperature for 2 h immediately before seeding.

PROCEDURE

Expression vector construction ● TIMING ~5 d

1| PCR-amplify the gene(s) encoding the 'left' and 'right' ZFN and TALEN monomers or SpCas9 using primers[62,70,72] that encode 5′-NcoI and 3′-XhoI restriction sites (**Table 1**). Carry out a 50-µl reaction using 5 ng of template DNA, 5 µl of 10× Expand high-fidelity buffer with $MgCl_2$, 0.2 µM of each primer, 0.2 mM dNTPs and 0.5 µl of high-fidelity DNA polymerase with water up to 50 µl. Use the following cycling program: initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min for ZFNs, 4 min for TALENs or 6 min for Cas9, and final extension at 72 °C for 10 min.

2| Mix the PCR products from Step 1 with 10 µl of 6× DNA loading dye, and resolve the PCR by gel electrophoresis using a 1% (wt/vol) agarose gel. Run the gel at 100 volts (V) for 45 min. Cut the desired band from the agarose gel, and dissolve it in 500 µl of gel solubilization buffer QG (provided in the QIAquick gel extraction kit) for 20 min at 50 °C. Run the solubilized gel through a Qiagen spin column according to the manufacturer's instructions, and elute DNA using 30 µl of water. Determine DNA concentration by NanoDrop spectrophotometry measuring absorbance at 260 nm (A_{260}) × 50 µg/ml.
▲ **CRITICAL STEP** To ensure that the gel slice is completely dissolved, invert the tube every 5 min. If the solution becomes pink or red after gel slice is dissolved, add 3 M sodium acetate dropwise until the solution becomes yellow.
? TROUBLESHOOTING

3| Digest pET-28 and nuclease-encoding gene(s) from Step 2 in separate 50-µl reactions containing 2 µg of DNA, 5 µl of 10× CutSmart buffer and 20 U each of the restriction enzymes NcoI and XhoI. Incubate the reaction at 37 °C for 3 h. Visualize DNA by agarose gel electrophoresis using SYBR Safe, as described in Step 2.

4| Purify the digested DNA by gel extraction, and then determine the DNA concentration using NanoDrop spectrophotometry, as described in Step 2.

5| Ligate the purified nuclease-encoding gene(s) from Step 4 with the digested pET-28 vector from Step 4 using an insert-to-vector molar ratio of 6:1 in a 10-µl reaction containing ~50 ng of digested DNA, 400 U of T4 DNA ligase and 1 µl of 10× T4 ligase buffer. Incubate the reaction at room temperature for 1 h.

6| Thaw 100 µl of chemically competent TOP10 cells on ice, and mix them gently with 10 µl of the ligation reaction from Step 5.

7| Keep the cells on ice for 30 min. Heat-shock the mixture at 42 °C for 60 s using a water bath, and then incubate the cells on ice for 30 s, and transfer the cell suspension to a culture tube containing 1 ml of LB medium. Incubate the cells with shaking at 250 r.p.m. for 1 h at 37 °C.

8| Spread 100 µl of the bacterial cell culture from Step 7 on an LB agar plate with 50 µg/ml kanamycin and incubate it overnight at 37 °C.

9| The following day, inoculate 4 ml of LB medium containing 50 µg/ml kanamycin with one colony from the LB agar plate, and culture it overnight at 37 °C with shaking.

10| Purify the nuclease expression vectors using the Qiagen plasmid miniprep kit according to the manufacturer's instructions, and confirm plasmid identity by DNA sequencing.
▲ **CRITICAL STEP** Verify the sequence identity of each plasmid by DNA sequencing. Mutations can reduce nuclease activity.
? TROUBLESHOOTING

Expression and purification of nuclease proteins ● TIMING ~6 d
11| After sequence verification, thaw 50 µl of chemically competent BL21 (DE3) cells on ice and mix them gently with 100 ng of sequence-verified nuclease expression vector from Step 10. Transform the cells as described in Steps 7 and 8.

12| The following day, inoculate 20 ml of LB medium containing 50 µg/ml kanamycin with one colony from the LB agar plate, and culture it overnight at 37 °C.

13| The following day, transfer 20 ml of the overnight starter culture from Step 12 into 1 liter of LB medium containing 50 µg/ml kanamycin, 200 mM NaCl and 0.2% (wt/vol) glucose.
▲ **CRITICAL STEP** For ZFN protein expression only, supplement the medium with 90 µM $ZnCl_2$.
▲ **CRITICAL STEP** Induction conditions are highly variable, and they depend on the stability of the protein being expressed. We recommend monitoring the optical density at 600 nm (OD_{600}) every 30 min until an OD_{600} of 0.8 is reached.

14| Induce protein expression once the culture reaches an OD_{600} of 0.8 with 0.1 mM IPTG. After induction, culture ZFN- and TALEN-expressing cultures at room temperature for 4 h, and Cas9-expressing culture at 18 °C for 12 h.
▲ **CRITICAL STEP** Protein yield is improved markedly by culturing cells at reduced temperatures after induction, probably as a result of reduced toxicity from nuclease proteins and improved folding.
? TROUBLESHOOTING

15| After induction, collect the cells by centrifugation at 5,000*g* for 10 min at 4 °C. Decant and discard the supernatant. Do not dry the cell pellets.
■ **PAUSE POINT** Cell pellets can be stored for up to 1 week at –20 °C, or for up to 1 month at –80 °C with no loss in nuclease protein activity.

16| Resuspend the cell pellet from Step 15 with 20 ml of lysis buffer. Transfer the suspension to a fresh collection tube.
▲ **CRITICAL STEP** For ZFN protein expression only, ensure that the lysis buffer contains 90 µM $ZnCl_2$.
▲ **CRITICAL STEP** Insufficient resuspension of the cell pellet will negatively affect the efficiency of sonication. β-ME and protease inhibitor cocktail should be added to the lysis buffer immediately before use.

17| Lyse the cells by sonication using the following settings: 50% power output, and 2 min process time with 5 s on/10 s off intervals.
▲ **CRITICAL STEP** Avoid overheating the solution by keeping the cells on ice during sonication. Multiple rounds of sonication may be necessary for complete lysis.
? TROUBLESHOOTING

18| Centrifuge the cell lysate at 25,000*g* for 30 min at 4 °C and transfer the supernatant into a fresh collection tube. Filter the supernatant through a 0.45-µm low-protein-binding filter.

19| Add to the filtered lysate 1 ml of nickel–nitrilotriacetic acid (Ni-NTA) agarose (50% slurry in 30% ethanol) and incubate it on a rotisserie at 4 °C for 30 min.

20| Rinse an empty polypropylene gravity-flow purification column with 5 ml of lysis buffer, and then transfer the protein-bound slurry into the column with the bottom cap attached.

21| Remove the bottom cap and discard the flow-through. Wash the column with 20 ml of wash buffer A and 5 ml of wash buffer B.

22| Elute the nuclease protein ten times into separate fractions with 0.5 ml of elution buffer.
▲ **CRITICAL STEP** For ZFN proteins only, add 50 µl of 1 M L-Arg to each fraction immediately after elution to enhance protein stability. Failure to add L-Arg can lead to ZFN protein precipitation.

23| Analyze the fractions by SDS-PAGE. Mix 5 µl of eluted protein with 5 µl of 2× Laemmli protein loading dye. Boil at 95 °C for 10 min and resolve on a 4–20% (wt/vol) Tris-glycine gel. Combine the fractions with the highest purity.

24| Buffer-exchange the eluted fractions with storage buffer, and concentrate the proteins to 400–800 µl using an Amicon ultra-15 centrifugal filter unit (use a 10-kDa MWCO for ZFN proteins and a 30-kDa MWCO for TALEN proteins and RNPs). Filter the protein using a 0.22-µm low-protein-binding filter.
▲ **CRITICAL STEP** The protein concentration should be assessed using a BSA standard curve by SDS-PAGE[79], or bicinchoninic acid assay (BCA)[80] or Bradford[81] assay. To ensure maximum dosage into cells, ZFN and TALEN proteins should be concentrated to ~40 µM and Cas9 protein to ~90 µM (15 µg/µl).
? TROUBLESHOOTING

TABLE 2 | *In vitro* cleavage assay reaction.

Component	Amount per sample	Final
CutSmart buffer	2 µl	1×
Target site–encoding PCR amplicon	200 ng	10 ng/µl
BSA, 1 mg/ml	2 µl	0.1 mg/ml
$ZnCl_2$, 900 µM	2 µl	90 µM
L-Arg, 1 M	2 µl	100 mM
ZFN, TALEN or RNP	2 µl	Titrate from 100 to 1 nM
Nuclease-free water	Up to 20 µl	

TALEN and RNP do not require $ZnCl_2$ and L-Arg.

25| Determine nuclease protein activity by *in vitro* cleavage. Prepare the reaction as described in **Table 2** and incubate it at room temperature for 1 h. For *in vitro* cleavage assays using RNP, sgRNA must be *in vitro*–transcribed and purified (see Step 27C(i–xii)).
? TROUBLESHOOTING

26| Add 250 µl of each concentrated protein from Step 24 into 1.5-ml microcentrifuge tubes. Flash-freeze the samples using liquid nitrogen and store them at –80 °C.
▲ **CRITICAL STEP** Avoid repeating freeze-thaw cycles to prevent damaging proteins. Although flash-freezing is recommended to help maintain protein stability, standard freezing at –80 °C is also acceptable.
■ **PAUSE POINT** Nuclease proteins can be stored at –80 °C for at least 1 year without loss of activity.

Protein delivery
27| Introduce ZFNs, TALENs and Cas9 RNPs into cells via (option A) direct application, (option B) CPP-mediated internalization and (option C) nucleofection, respectively.
(A) ZFN protein delivery into primary CD4⁺ T cells ● TIMING ~5 d
(i) Obtain 100 ml of blood, mixed with the anticoagulant heparin, from a qualified supplier.
(ii) Dilute the blood with an equal volume of complete RPMI 1640 medium using a serological pipette. Mix it well.
(iii) Add 15 ml of Ficoll-Paque into a SepMate-50 column, and then carefully pipette 34 ml of diluted blood sample onto the Ficoll-Paque layer in the SepMate column.
(iv) Centrifuge the sample at 1,200*g* for 10 min at room temperature with no brake.
(v) By using a pipette, transfer the mononuclear cell layer to a new 50-ml canonical tube, and wash it with 40 ml of complete RPMI 1640 medium. Centrifuge the cells at 300*g* for 10 min at room temperature.
? TROUBLESHOOTING
(vi) Discard the supernatant and repeat the wash step until the supernatant becomes transparent.
(vii) Resuspend the mononuclear cells in 1.2 ml of DPBS with 2% (vol/vol) FBS and 1 mM EDTA, and transfer the cells to a 5 ml round-bottom conical tube.
(viii) Add 60 µl of EasySep human CD4⁺ T cell enrichment cocktail to 1.2 ml of mononuclear cells. Mix well and incubate the cells at room temperature for 10 min.
▲ **CRITICAL STEP** The human CD4⁺ T cell enrichment cocktail isolates CD4⁺ T cells by negative selection using a tetrameric antibody complex that recognizes CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ and glycophorin A.

(ix) Mix the magnetic beads thoroughly and then add 120 µl of the beads to the cells. Mix well and incubate the tube at room temperature for 5 min.
▲ **CRITICAL STEP** Magnetic beads can precipitate, and they should be vortexed before use.
(x) Insert the 5-ml conical tube containing the magnetic bead mix into the EasySep magnet, and allow it to stand for 5 min.
(xi) Gently invert the tube and transfer the unbound cells into a new 5-ml conical tube. Count the cells using a hemocytometer. Stain the cells using FITC-conjugated anti-human CD4 antibody according to the manufacturer's instructions. Evaluate the purity of isolated $CD4^+$ T cells by flow cytometry, as described by Gaj and Liu[67].
? TROUBLESHOOTING
(xii) Add 1×10^6 $CD4^+$ T cells into one well of a 24-well plate containing 1 ml of complete RPMI 1640 medium with 25 µl of CD3/CD28 human T cell activation beads and 50 U of rIL-2 for stimulation and expansion. Transfer the remaining $CD4^+$ T cells to a 10-cm dish containing 10 ml of complete RPMI 1640 medium, and maintain them at 37 °C with 5% CO_2. Cells can be cultured under these conditions for up to 1 week.
▲ **CRITICAL STEP** Activation of $CD4^+$ T cells is required for achieving maximum ZFN-mediated gene modification rates. Proper cell-to-bead ratio is crucial for cell activation.
(xiii) At 48 h after activation, collect the cells by centrifugation at 300*g* for 10 min at room temperature, discard the supernatant and wash the cells once with serum-free DMEM medium.
▲ **CRITICAL STEP** Activate T cells for 24–72 h. Prolonged activation can decrease genome editing efficiency. Activated cells expand in size.
(xiv) Resuspend the cells in 250 µl of serum-free DMEM containing 2 µM each of 'left' and 'right' ZFN proteins supplemented with 90 µM $ZnCl_2$ and 100 mM L-Arg.
▲ **CRITICAL STEP** Lack of L-Arg will markedly decrease the genome editing efficiency. Maximum modification is observed using ZFN protein concentrations between 0.5 µM and 4 µM. Depending on the purity of ZFN proteins, low levels of cytoxicity could be observed at high protein concentrations.
(xv) Incubate the cells at 37 °C for 1 h.
(xvi) Collect the cells by centrifugation at 300*g* for 10 min at room temperature and discard the superntant. Resuspend the cells with 500 µl of complete RPMI 1640 medium containing 50 U/ml rIL-2.
? TROUBLESHOOTING
(xvii) Incubate the cells at 30 °C for 24 h.
▲ **CRITICAL STEP** Transient incubation at 30 °C enhances the activity of internalized ZFN proteins, and it is recommended for achieving optimal rates of mutagenesis.
(xviii) At 24 h after incubation, transfer the cells to 37 °C and incubate them for an additional 24 h.
(xix) Cetrifuge the cells at 300*g* for 10 min at room temperature, discard the supernatant and resuspend the cells in 100 µl of QuickExtract DNA extraction solution.
(xx) Incubate the samples at 65 °C for 15 min, followed by incubation at 98 °C for 15 min.
■ **PAUSE POINT** Samples can be held at 4 °C for up to 1 week or stored indefinitely at –80 °C until the Surveyor nuclease assay (Step 28) is ready to be performed.

(B) CPP-mediated delivery of TALEN proteins ● TIMING ~4 d

(i) Seed HeLa cells onto a 24-well plate at a density of 1×10^5 cells per well.
(ii) At 24 h after seeding, incubate 3.3 µM each 'left' and 'right' TALEN proteins with 100 µM Cys-(Npys)-$(D\text{-Arg})_9$ peptide and 1× complete protease inhibitor cocktail in 100 mM sodium phosphate buffer, pH 5.5, in a 75-µl reaction. Incubate the cells for 2 h at room temperature.
▲ **CRITICAL STEP** The molar ratio of Arg_9-CPP to TALEN protein must be between 8:1 and 15:1 for efficient cellular internalization and genome-editing activity.
? TROUBLESHOOTING
(iii) Adjust the pH of the reaction by adding 7.5 µl of 1 M sodium hydroxide, and mix the solution well with 175 µl of serum-free DMEM medium.
▲ **CRITICAL STEP** Depending on the purity of the TALEN proteins and the quality of the peptide, precipitation may be observed after the conjugation reaction. Precipitates can be removed by centrifuging the reaction solution at 5,000*g* for 2 min at room temperature.
(iv) Before protein treatment, remove the medium from each well, and wash the cells once by adding 500 µl of SFM. Gently remove SFM and apply the entire TALEN protein solution onto the cells. Incubate the cells at 37 °C for 2 h.
▲ **CRITICAL STEP** In contrast to ZFN proteins, TALEN proteins should be incubated with cells for ~2 h for efficient internalization.
(v) Replace the medium with complete DMEM and incubate the cells at 30 °C for 24 h.
(vi) At 24 h after incubation, transfer the cells to 37 °C and incubate them for an additional 24 h.
(vii) Wash the cells once with DPBS and isolate the cells for the QuickExtract procecdure, as described in Step 27A(xix).

(C) Electroporation of Cas9 RNP into human stem cells ● TIMING ~5 d

(i) PCR-amplify sgRNA from a mammalian expression vector (**Box 1**). Carry out a 50-µl PCR using 5 ng of template DNA, 5 µl of 10× Expand high-fidelity buffer with $MgCl_2$, 0.2 µM of each primer, 0.2 mM dNTPs and 0.5 µl of high-fidelity DNA polymerase with water to 50 µl. Use the following cycling program: initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s, and final extension at 72 °C for 10 min.

▲ **CRITICAL STEP** The PCR product must contain a T7 promoter sequence for downstream *in vitro* transcription reactions. This is achieved by designing a forward primer to include a T7 promoter sequence immediately upstream of the sgRNA.

(ii) Purify the PCR products by gel extraction, as described in Step 2, and determine the DNA concentration using a NanoDrop measuring the A_{260} × 50 µg/ml.

▲ **CRITICAL STEP** Isolation of high-quality DNA template for RNA transcription reaction is essential for subsequent *in vitro* transcription steps.

(iii) Transcribe the sgRNA by 'run-off' reaction using the HiScribe T7 transcription kit. Carry out a 20-µl reaction containing 1× reaction buffer, 1 µg of purified PCR product, 7.5 mM of each dNTP and 1.5 µl of T7 RNA polymerase mix. Incubate the reaction at 37 °C for 16 h.

▲ **CRITICAL STEP** Short RNA requires a 16-h reaction time for maximum transcription.

(iv) After 16 h, add 70 µl of nuclease-free water, 10 µl of 10× DNase I buffer and 2 µl of DNase I (RNase-free) to the transcription reaction. Mix well and incubate the reaction for 15 min at 37 °C.

(v) Add 80 µl of nuclease-free water and 20 µl of 3 M sodium acetate to the DNase I–treated transcription reaction followed by 200 µl of phenol-chloroform-isoamyl alcohol solution, and vortex.

(vi) Centrifuge the tube at 12,000*g* for 5 min at 4 °C, and transfer the supernatant to a new microcentrifuge tube. Add 200 µl of chloroform to extract residual phenol.

(vii) Centrifuge the tube at 12,000*g* for 5 min at 4 °C. Transfer the supernatant to a new microcentrifuge tube.

(viii) Repeat the chloroform extraction procedure once more, as described in Step 27C(v–vii).

(ix) Add 2.5 volumes of 100% ethanol to the chloroform-extracted sgRNA. Store the mixture at –20 °C for 12 h.

(x) Centrifuge at 12,000*g* for 30 min at 4 °C. Remove the supernatant and discard it, and then add 500 µl of ice-cold ethanol (75% (vol/vol)) to remove residual salt.

(xi) Centrifuge the tube at 12,000*g* for 5 min at 4 °C, remove the supernatant and air-dry the pellet.

(xii) Dissolve the pellet in 20 µl of nuclease-free water. Determine the sgRNA concentration using NanoDrop spectrophotometry measuring the A_{260} × 40 µg/ml. Store the sgRNA at –80 °C.

▲ **CRITICAL STEP** Resuspending the pellet in nuclease-free water and storing it at –80 °C is crucial for preventing sgRNA degredation.

? TROUBLESHOOTING

■ **PAUSE POINT** Purified sgRNA can be stored at –80 °C for at least 1 year without loss of activity.

(xiii) (Optional) Perform this step only if single-base genome editing is desired. Prepare a donor plasmid for HDR containing homology arms on each side of the intended modification that are at least 500 bp in length as described by Pruett-Miller and Davis[82], or ssODNs containing at least 40 nt of homology on each side of the intended modification, as described previously[73,78].

(xiv) Acquire H9 hESCs from a qualified supplier. Maintain the cells in a six-well plate on a mouse embryo fibroblast (MEF) feeder layer in 2 ml of complete DMEM/F12 medium.

(xv) After one passage on MEF feeder cells (5–7 d), treat H9 hESCs with 0.5 ml of DMEM/F12 medium supplemented with 0.5 mg/ml collagenase type IV at 37 °C for 30 min. Add 2 ml of complete DMEM/F12 medium to the well to stop the digestion.

▲ **CRITICAL STEP** Cell detachment can be monitored under a microscope.

(xvi) Gently pipette the cells and transfer them to a sterile 15-ml conical tube. Once the cells settle at the bottom of the conical tube, carefully remove the medium by pipetting.

(xvii) Wash the cells twice with complete mTeSR1 medium. Allow the cells to settle at room temperature, and carefully remove the supernatant medium.

▲ **CRITICAL STEP** Two wash steps are neccessary to remove the MEFs, which may interfere with the nucleofection of H9 cells in the following steps.

(xviii) Resuspend the H9 cells with complete mTeSR1 medium and transfer them to a six-well plate precoated with Matrigel.

(xix) After 2–3 d of incubation under feeder-free conditions, remove the medium and detach the H9 cells from Matrigel by incubation with 1 ml of Accutase for 5 min at 37 °C.

(xx) Dissociate the cells into a single-cell suspension by carefully pipetting the cells up and down 4–6 times. Add 5 ml of complete mTeSR1 medium to stop the digestion.

▲ **CRITICAL STEP** Single-cell suspension is crucial for achieving maximum nucleofection efficiency.

? TROUBLESHOOTING

(xxi) Mix 15 µg of Cas9 protein with 7.5–15 µg of sgRNA with a maximum volume of 2 µl, and incubate the mixture at room temeprature for 10 min to form the RNP. Centrifuge 2 × 10^5 H9 cells per transfection at 115*g* for 3 min at room temperature, and resuspend the cell pellet in 22 µl of nucleofection solution containing 16.4 µl of P3 primary cell solution, 3.6 µl of supplement S1 and 2 µl of RNP (15 µg of Cas9 and 7.5–15 µg of sgRNA from Step 27C(xxi)). Optionally, for co-delivery with donor plasmid or ssODN, use 2 µl of RNP (7.5–15 µg Cas9 and 5–10 µg sgRNA from Step 27C(xxi)) mixed with 2–4 µg of donor plasmid or 50–200 pmol ssODN (from Step 27C(xiii)). Note that the full range of RNP amounts indicated here support efficient gene editing. Ranges are given to provide flexibility for experimental planning.

▲ **CRITICAL STEP** The total volume of the RNP should not exceed 2 µl, as a protein solution that exceeds 10% of the nucleofection volume (20 µl) can markedly reduce transfection efficiency.

(xxii) Transfer the cells to 16-well Nucleocuvette strips (included in the nucleofector kit) and electroporate them using a 4D-nucleofector system using the program CB-150.

(xxiii) Immediately after nucleofection, add 100 µl of complete mTeSR1 medium to each well and transfer the cells into a six-well plate containing 2 ml of mTeSR1 medium.

(xxiv) At 48 h after incubation, wash the cells once with DPBS and isolate the cells by incubation with 1 ml of Accutase for 5 min at 37 °C. Quench the reaction with 5 ml of complete mTeSR1 medium. Cetrifuge the cells at 115*g* for 3 min at room temperature and resuspend them in 50 µl of QuickExtract DNA extraction solution and proceed as described in Step 27A(xx).

? TROUBLESHOOTING

Quantification of gene modification

28| Determine the gene editing efficiency of the nuclease proteins via (option A) Surveyor nuclease assay, (option B) restriction fragment length polymorphism (RFLP) or (option C) sequence verification.

(A) Surveyor nuclease assay ● TIMING ~2 d

(i) PCR-amplify the genomic target site by nested PCR, as described previously[62]. Prepare the reaction mixture according to **Table 3**, and use the PCR cycling conditions described in **Table 4**. Use 2 µl of the external PCR as template for the internal PCR.

? TROUBLESHOOTING

(ii) Verify amplification by running 10 µl of the PCR on a 1% (wt/vol) agarose gel, as described in Step 2.

▲ **CRITICAL STEP** Ensuring the absence of PCR side products is crucial for preventing the formation of off-target heteroduplexes.

■ **PAUSE POINT** Samples can be stored at –20 °C for up to 1 month.

(iii) Denature and re-anneal 30 µl of the PCR amplicon using the cycle in **Table 5** to generate mismatched duplex DNA for the Surveyor nuclease assay.

(iv) Mix 10 µl of heteroduplex DNA with 1 µl of 0.15 M $MgCl_2$, 1 µl of Surveyor enhancer S and 1 µl of Surveyor nuclease S. Incubate the reaction at 42 °C for 1 h.

(v) Quench the reaction with 1.5 µl of stop solution and add 2 µl of 10× loading dye to each sample.

TABLE 3 | Genomic PCR reaction components.

	Concentrations	
	External PCR	**Internal PCR**
dNTPs (10 mM)	0.2 mM	0.2 mM
Expand high-fidelity buffer (10×)	1×	1×
Expand high-fidelity polymerase (µl)	0.5	0.5
Forward primer (µM)	0.4	0.2
Reverse primer (µM)	0.4	0.2
Template DNA	2 µl of DNA extract	2 µl of external PCR product
DMSO (%)	5	–
dH_2O	Up to 50 µl	Up to 50 µl

TABLE 4 | Thermocycler settings for Surveyor nuclease assay (Step 28A).

	Conditions	
	External	**Internal**
Initial	95 °C, 5 min	95 °C, 5 min
Cycle	20 cycles	30 cycles
	95 °C, 30 s	95 °C, 30 s
	55 °C, 30 s	55 °C, 30 s
	72 °C, 90 s	72 °C, 40 s
Final	72 °C, 5 min	72 °C, 5 min
Hold	4 °C, ∞	4 °C, ∞

TABLE 5 | Heteroduplex formation conditions.

Temperature (°C)	Time (min)
95	10
95–85	–2.0 °C/s
85	1
85–75	–0.3 °C/s
75	1
75–65	–0.3 °C/s
65	1
65–55	–0.3 °C/s
55	1
55–45	–0.3 °C/s
45	1
45–35	–0.3 °C/s
35	1
35–25	–0.3 °C/s
25	1
4	∞

TABLE 6 | Thermocycler settings for RFLP analysis (Step 28B).

	Conditions	
	External	**Internal**
Initial	95 °C, 5 min	95 °C, 5 min
Cycle	20 cycles	30 cycles
	95 °C, 30 s	95 °C, 30 s
	55 °C, 30 s	55 °C, 30 s
	72 °C, 90 s	72 °C, 90 s
Final	72 °C, 5 min	72 °C, 5 min
Hold	4 °C, ∞	4 °C, ∞

TABLE 7 | RFLP digestion reaction components.

Component	Amount per sample	Final
CutSmart buffer	2 µl	1×
PCR amplicon	300 ng	15 ng/µl
XbaI (or other enzyme)	1 µl	1 U/µl
Nuclease-free water	Up to 20 µl	

XbaI site was included in donor DNA.

(vi) Load the samples on a 10% (wt/vol) TBE gel and run the gel at 140–180 V for ~1 h.
(vii) Remove the gel and stain it with 10 µl of SYBR Safe in 30 ml of 1× TBE for 10 min. Wash the gel three times for 10 s with dH_2O.
(viii) Visualize the gel using the Gel Doc XR system, and measure the density or intensity of each band. The percent gene modification can be determined by measuring the fraction of parental band cleaved at the anticipated location.
? TROUBLESHOOTING

(B) RFLP analysis ● TIMING ~2 d

(i) Carry out the nested PCR as described in Step 28A(i), but use the PCR cycling conditions in **Table 6**. Forward and reverse primers are complementary to the region outside the homology arm sequences.
(ii) Verify the amplification by running 5 µl of the PCR on an agarose gel, as described in Step 2.
(iii) Purify the PCR products using the QIAquick PCR purification kit according to the manufacturer's instructions.
(iv) Digest the PCR amplicons with the restriction enzyme whose recognition site was included within the donor DNA sequence[72], by preparing the reaction as described in **Table 7**. Incubate the digestion reaction at 37 °C for 2 h.
(v) Add 2.5 µl of 10× loading dye to the digested sample, and run the sample on a 2% (wt/vol) agarose gel as described in Step 2.
(vi) Stain the gel with SYBR Safe. Visualize the gel and measure the density or intensity of the cleaved bands. HDR efficiency can be calculated by the following formula $(b + c/a + b + c) \times 100$, where a is the intensity of uncleaved band, and b and c are the intensities for the cleaved bands.

(C) Sequence verification ● TIMING ~5 d

(i) Mix 4 µl of the PCR product from Step 28A(i) or Step 28B(i) with 1 µl of pCRII-Blunt-TOPO reaction mix and 1 µl of salt solution from the Zero Blunt TOPO PCR cloning kit.
(ii) Incubate the reaction for 30 min at room temperature.
(iii) Transform the ligation into TOP10 cells, as described in Steps 6–8.
(iv) Pick individual clones for overnight culture, miniprep and DNA sequencing, or submit the plate to a qualified sequencing service for direct colony sequencing. Use the primer M13F to sequence amplicons.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 8**.

TABLE 8 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Nonspecific amplification	Zinc-finger or TAL effector repeats are contributing to side product amplification	Include ~2.5 M betaine to help prevent nonspecific amplification, or repeat the PCR using <0.2 μM concentration of each primer and >100 ng of template. Alternatively, subclone the nuclease gene from a secondary expression vector
	Random mutations	Polymerase-induced errors	Use a high-fidelity DNA polymerase, such as Phusion DNA polymerase. Synthesize the nuclease gene if problems persist. If synthesized, codon usage optimization for expression in *Escherichia coli* is recommended for maximum yield
10	High background	Insufficient vector digestion	Treat the vector with calf intestinal alkaline phosphatase (CIP) to prevent recircularization If problems persist, use colony PCR[90] to screen for positive clones before DNA sequencing using the cycling conditions described in Step 1
14	Slow bacteria growth	Nuclease toxicity	Culture cells at room temperature, and ensure that NaCl is present in the culture medium to prevent nonspecific nuclease binding and cleavage. Induce protein expression when the culture reaches an OD_{600} of 0.2–0.5
17	Insufficient cell lysis	High density of bacterial cells or incorrect sonication settings	Dilute the cell suspension. Alternatively, lyse cells using lysozyme
24	Slow filtration	Precipitated protein is blocking the filter	Gently mix or pipette the protein solution every 20 min during centrifugation to prevent clogging
	Low purity	Protein contamination	Perform additional purification steps (e.g., size-exclusion or ion exchange chromatography) as described previously[100]
25	Poor nuclease activity	Contamination or protein misfolding	Purify proteins at 4 °C to promote native conditions, reduce the salt concentration in the lysis and elution buffers, or perform additional buffer exchange steps. If trouble continues, purchase purified nuclease protein from a qualified manufacturer (e.g., Cas9 protein can be obtained from ToolGen, Life Technologies or PNA BIO)
27A(v)	Cell pellet is colored red	Red blood cell contamination	Avoid contacting the red blood cells when transferring the mononuclear cell layer
27A(xi)	Low $CD4^+$ T-cell purity	The antibody cocktail mix was oversaturated with PBMCs	Reduce the amount of blood used during purification. Increase the incubation time of PBMCs with the antibody cocktail mix
27A(xvi)	Difficulty recovering cells from centrifugation	Cells are attached to the side of the microcentrifuge tube	Use a swing-bucket centrifuge rotor
27B(ii)	Precipitation during the TALEN conjugation reaction	Low TALEN protein purity	Perform conjugation reaction using high-quality TALEN proteins
27C(xii)	Low sgRNA yield	*In vitro* transcription reaction failed or phenol chloroform extraction did not work. Degradation of sgRNA	Increase the amount of DNA template used for *in vitro* transcription. Consider using an RNA minikit to purify sgRNA. Use RNase inhibitors to prevent potential sgRNA degradation. If trouble continues, purchase synthesized sgRNA from a qualified manufacturer
27C(xx)	Difficulty generating a single-cell suspension	Incorrect protease treatment	Treat H9 cells with fresh Accutase. Mix cells well using a P1000 micropipette

(continued)

TABLE 8 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
27C(xxiv)	No H9 cells attached to the plate after nucleofection	Toxicity from nucleofection or stem cell dissociation	Use the correct nucleofection solution and program. Increase the number of cells or decrease the amount of RNP used in the nucleofection. Use rho-associated kinase (ROCK) inhibitor to enhance the survival of dissociated stem cells
28A(i)	Nonspecific amplification	Improper PCR conditions	Ensure that the annealing temperature is no more than 5 °C below the melting temperature of the primers. Identify the optimal annealing temperature by gradient PCR. Alternatively, design and order new genomic PCR primers
28A(viii)	Low genome-modification efficiency	Inactivate nuclease or poor cell-penetrating activity	Test nuclease activity by transient transfection. If there is no activity, construct new nucleases. Confirm the activity of purified nuclease protein by *in vitro* cleavage assay. Visualize internalized nuclease protein by western blotting. If trouble continues, construct new nucleases

● TIMING

Steps 1–10, construction of nuclease bacterial expression vectors: ~5 d
Steps 11–26, expression and purification of nuclease proteins from bacterial cells: ~6 d
Step 27A, delivery of ZFN proteins into primary cells: ~5 d
Step 27B, conjugation of CPPs onto the surface of TALEN proteins and their delivery into human cells: ~4 d
Step 27C, nucleofection of Cas9 RNP into human embryonic stem cells: ~5 d
Step 28A, surveyor nuclease assay to measure modification frequencies: ~2 d
Step 28B, RFLP assay to measure HDR efficiencies: ~2 d
Step 28C, sequence confirming the presence of mutations: ~5 d
Box 1, construction of nucleases: ~4–14 d

ANTICIPATED RESULTS

Modification of the *AAVS1* locus by direct delivery of ZFN proteins

We expressed and purified ZFN proteins (~1 mg per liter culture) that were designed to target the human *AAVS1* locus[83] and contained one to three tandem repeats of the SV40 NLS (**Fig. 2a**). *In vitro* cleavage analysis revealed that NLS length had a negligable effect on ZFN protein activity (**Fig. 2b**). HeLa, K562 and Jurkat cells treated with 4 μM of three-NLS ZFN proteins demonstrated >30% modification of the *AAVS1* locus after one protein treatment (**Fig. 2c**). CD4+ T cells treated with three-NLS ZFN proteins also showed ~25% modification after a single treatment (**Fig. 2c**). Consistent with a previous study,

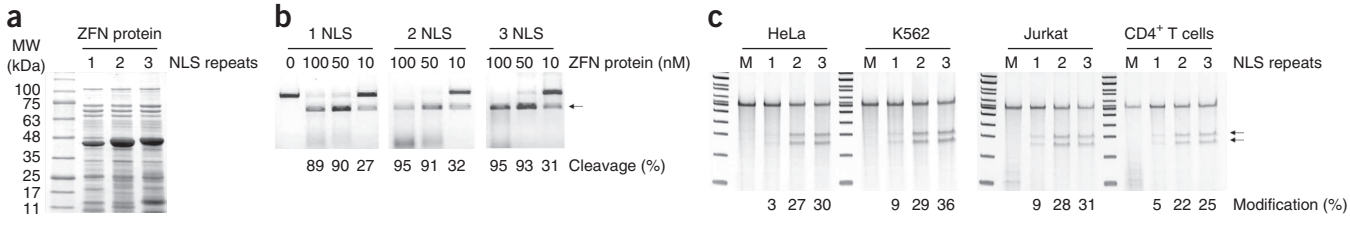

Figure 2 | Modification of the *AAVS1* locus by direct delivery of zinc-finger nuclease (ZFN) proteins. (**a**) SDS-PAGE of one-, two- and three-NLS 'right' AAVS1 ZFN proteins purified from the soluble fraction of bacterial lysate[88]. Molecular-weight (MW) standards are indicated. The number of NLS repeats within each ZFN protein is indicated by '1 NLS', '2 NLS' and '3 NLS.' (**b**) *In vitro* cleavage assay of one-, two- and three-NLS 'right' *AAVS1* ZFN proteins at different concentrations with 100 ng of substrate DNA. Arrow indicates cut substrate. Active ZFN proteins cleave DNA at concentrations as low as 10 nM. The percentage of cleaved DNA is calculated by dividing the density of the cleavage product by the density of the total DNA within the same lane. (**c**) Frequency of *AAVS1* modification in HeLa, K562, Jurkat and CD4+ T cells treated once with 4 μM of one-, two- and three-NLS ZFN proteins, as determined by the Surveyor nuclease assay. 'M' indicates cells treated with serum-free medium only. Arrows indicate expected cleavage products. The modification efficiency is calculated as described previously[89]. (**d**) Sequence analysis of the *AAVS1* locus from CD4+ T cells treated once with 2 μM each of left and right two-NLS ZFN proteins. Deletions (dashes) and insertions (lowercase) induced by ZFN proteins are shown in nine sequences aligned to the wild-type (WT) cleavage site. Mutations were found in 10/45 samples (22.2%) treated with two-NLS ZFN proteins.

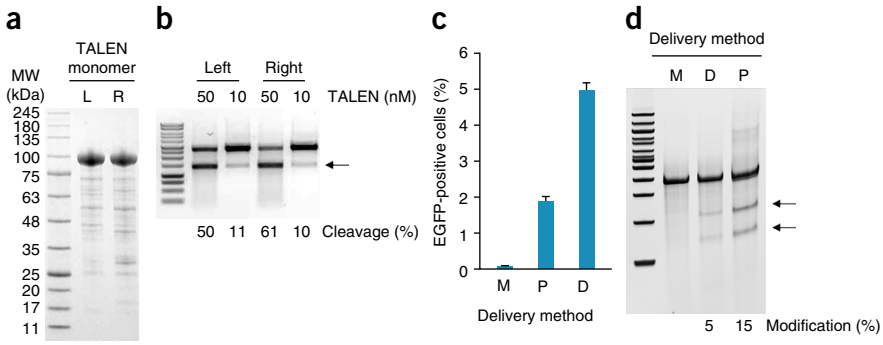

Figure 3 | Modification of the human *CCR5* gene by cell-penetrating TALEN proteins. (**a**) SDS-PAGE of left and right TALEN monomers designed to target human *CCR5*. Molecular-weight (MW) standards are indicated. (**b**) *In vitro* cleavage assay of left and right TALEN proteins at different concentrations incubated with 100 ng of substrate DNA containing symmetrical left and right target sites. Active TALEN proteins cleave DNA at concentrations as low as 10 nM. The percentage of cleaved DNA is calculated by dividing the density of the cleavage product over the density of total DNA within the same lane. Arrow indicates expected cleavage products. (**c**) The percentage of EGFP-positive cells, as determined by flow cytometry in reporter cells treated with 2 μM TALEN proteins (P) or transfected with 200 ng of each TALEN expression vector (D). Error bars indicate s.d. ($n = 3$). (**d**) Modification of the endogenous *CCR5* gene in HeLa cells treated with 2 μM TALEN proteins (P) or transfected with 200 ng of each TALEN expression vector (D), as determined by the Surveyor nuclease assay. 'M' indicates cells treated with serum-free medium. Arrows indicate expected cleavage products. The modification efficiency was calculated as described previously[89].

multiple NLS repeats improved the cellular gene-editing efficiency of ZFN proteins. Sequence analysis of *AAVS1* loci amplified from CD4+ T cells treated with 4 μM of two-NLS ZFN protein confirmed the frequency of ZFN-induced mutations (**Fig. 2d**).

Modification of the human *CCR5* gene by cell-penetrating TALEN proteins

TALEN proteins designed to recognize the human *CCR5* gene were purified from bacteria (~0.5–1.0 mg/l culture) and conjugated to the CPP Cys-(Npys)-(D-Arg)$_9$ (**Fig. 3a**,**b**). We tested the ability of the resulting TALEN proteins to enter the cells and to stimulate mutagenesis using a previously described[84] human embryonic kidney (HEK) 293T reporter cell line containing an integrated EGFP gene whose expression is restored via TALEN-mediated cleavage. Treatment of reporter cells with 2 μM TALEN protein led to ~2% EGFP-positive cells (**Fig. 3c**). In addition, analysis of DNA isolated from HeLa cells treated with 2 μM TALEN proteins revealed ~15% modification of the CCR5 gene, compared with ~5% modification observed after transient transfection of TALEN-encoded plasmid DNA (**Fig. 3d**).

Multiplex gene modification by direct delivery of Cas9 RNP

The SpCas9 nuclease was expressed at high yield (~1 mg/l culture) and >90% purity from the soluble fraction of bacterial lysates and incubated with purified sgRNA targeting the human *CCR5*, *EMX1* and *AAVS1* loci (**Fig. 4a**,**b**). *In vitro* cleavage analysis confirmed that RNP displayed robust cleavage activity (**Fig. 4c**). Analysis of DNA isolated from K562 cells nucleofected with either 7.5 or 15 μg of RNP revealed >20% modification of the CCR5 gene, and >50% modification of the *EMX1* gene and two different *AAVS1* sites (*AAVS1*-1 and *AAVS1*-2; **Fig. 4d**,**e**). K562 cells nucleofected with combinations of RNPs also showed efficient multiplexed gene modifications, with no appreciacable decrease in activity compared with cells modified by single RNPs (**Fig. 4e**,**f**). H9 hESCs nucleofected with single RNP demonstrated >30% modification of the

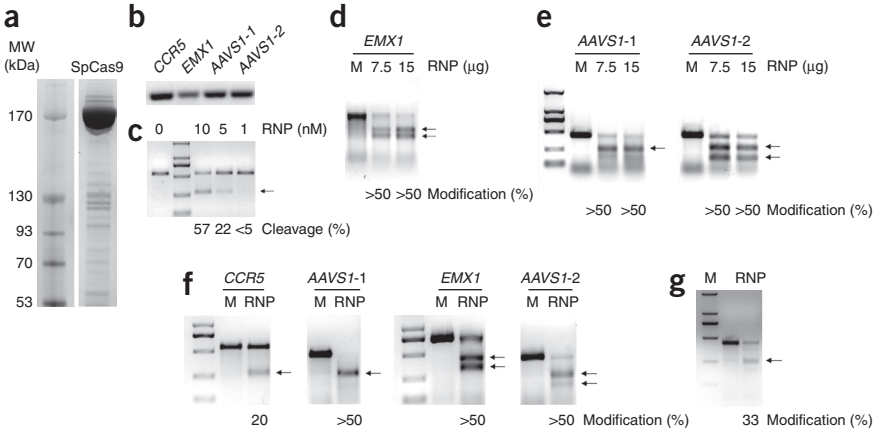

Figure 4 | Modification of multiple genomic loci by direct delivery of Cas9 RNP. (**a**) SDS-PAGE of purified SpCas9 protein. Molecular-weight (MW) standards are indicated. (**b**) Agarose gel of purified sgRNA. All RNA transcripts show specific bands of the anticipated size for sgRNA. (**c**) *In vitro* cleavage assay of *CCR5*-targeting RNP at different concentrations incubated with 100 ng of substrate DNA. Active RNP cleaves DNA at concentrations as low as 5 nM. The percentage of cleaved DNA is calculated by dividing the density of the cleavage product over the density of total DNA within the same lane. (**d**,**e**) Frequency of *EMX1* (**d**) and *AAVS1*-1 and *AAVS1*-2 (**e**) modification in K562 cells nucleofected with RNP complexes composed of 15 μg of Cas9 protein and 7.5 or 15 μg of sgRNA, as determined by the Surveyor nuclease assay. 'M' indicates cells treated with serum-free medium only. Arrows indicate expected cleavage products. The modification efficiency was calculated as described previously[88]. (**f**) Frequency of multiplexed modification of the *CCR5* and *AAVS1*-1, or *EMX1* and *AAVS1*-2 loci in K562 cells nucleofected with 15 μg of Cas9 protein precomplexed with 7.5 μg of each sgRNA, as determined by the Surveyor nuclease assay. (**g**) Modification of the *AAVS1*-1 locus in H9 human embryonic stem cells nucleofected with 30 μg of RNP complex (15 μg of Cas9 protein and 15 μg of sgRNA), as determined by the Surveyor nuclease assay. 'M' indicates cells treated with serum-free medium only. Arrows indicate expected cleavage products. The modification efficiency was calculated as described previously[89].

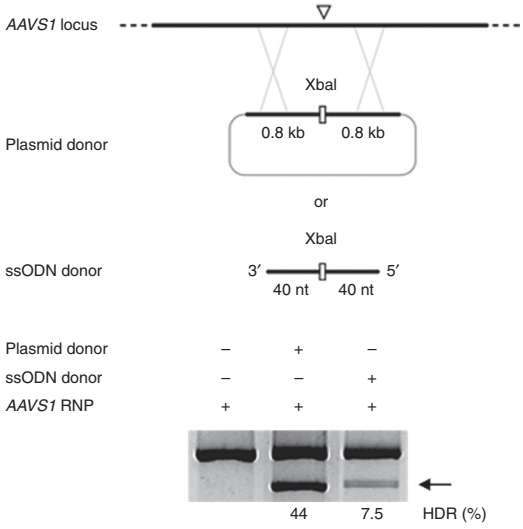

Figure 5 | Modification of the AAVS1 locus by direct delivery of RNP with donor plasmid or ssODN. HDR of the *AAVS1*-1 locus in K562 cells nucleofected with 15 µg of Cas9 protein precomplexed with 15 µg of sgRNA and 2 µg of donor plasmid or 200 pmol of ssODN containing an XbaI restriction site (white bar). Target locus was amplified with primers that are complementary to the outside of the homology arm sequences. PCR amplicons were digested with XbaI to measure the frequency of HDR. Arrow indicates the expected cleavage product. HDR efficiency was calculated as described in Step 28B(vi).

AAVS1-1 locus (**Fig. 4g**). Maximum modification efficiencies are achieved using two- to six-fold molar excess of sgRNA to Cas9 protein. Finally, co-delivery of 15 µg of *AAVS1*-1-specific RNP and 2 µg of HDR-donor plasmid or 200 pmol of ssODNs gave rise to HDR-mediated genome modification at frequencies of 44% or 7.5%, respectively, in K562 cells (**Fig. 5**).

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