

# Targeted gene knockout by direct delivery of zinc-finger nuclease proteins

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**Zinc-finger nucleases (ZFNs) are versatile reagents that have redefined genome engineering. Realizing the full potential of this technology requires the development of safe and effective methods for delivering ZFNs into cells. We demonstrate the intrinsic cell-penetrating capabilities of the standard ZFN architecture and show that direct delivery of ZFNs as proteins leads to efficient endogenous gene disruption in various mammalian cell types with minimal off-target effects.**

ZFNs are fusions of the nonspecific cleavage domain from the FokI restriction endonuclease with custom-designed Cys<sub>2</sub>-His<sub>2</sub> zinc-finger proteins (ZFPs)<sup>1</sup>. These chimeric nucleases induce sequence-specific DNA double-strand breaks (DSBs) that can be repaired by error-prone nonhomologous end joining (NHEJ) to yield small alterations at targeted genomic loci. This strategy has enabled highly efficient gene disruption in numerous cell types<sup>2,3</sup> and model organisms<sup>4,5</sup> and has facilitated the progress of targeted gene therapy in humans<sup>6,7</sup>. Despite these advances and more recent methodological improvements<sup>8-10</sup>, there remains a need for new methods that can improve the utility of these enzymes. The development of safe and effective ZFN delivery methods is of particular importance, as the deficiencies of ZFN gene-delivery systems may hinder the continued advancement of this technology. In particular, viral vectors<sup>11</sup> are time consuming to produce and can be associated with undesirable side effects, such as insertional mutagenesis, whereas nonviral DNA and mRNA delivery systems are restricted to certain cell types and have been reported to show toxicity<sup>12,13</sup> and low efficiency<sup>14</sup>. To address this problem, we set out to develop a simple alternative to conventional ZFN delivery systems by investigating the direct delivery of purified ZFN proteins to cells.

We began by introducing protein transduction domains into the established ZFN architecture. For this, we genetically fused either the cell-penetrating peptide sequence from the HIV-1 TAT protein or a polyarginine motif to the N termini of ZFNs designed to target the human chemokine (C-C motif) receptor 5 (*CCR5*) gene<sup>6</sup>. These ZFNs, however, were consistently difficult to express or

purify in quantities sufficient for analysis in cell culture (data not shown). Following these results, and based on the observation that ZFP DNA-binding domains carry a net positive charge (Fig. 1a), we hypothesized that ZFNs might penetrate the cell in the absence of additional modification. We expressed in *Escherichia coli* ZFNs designed to target the *CCR5* gene and lacking any transduction domain and then purified them to homogeneity from either the soluble or the insoluble fractions (Supplementary Fig. 1). *In vitro* analysis confirmed that functional ZFN proteins with similar DNA cleavage profiles could be obtained by either method (Supplementary Fig. 2 and Supplementary Note).

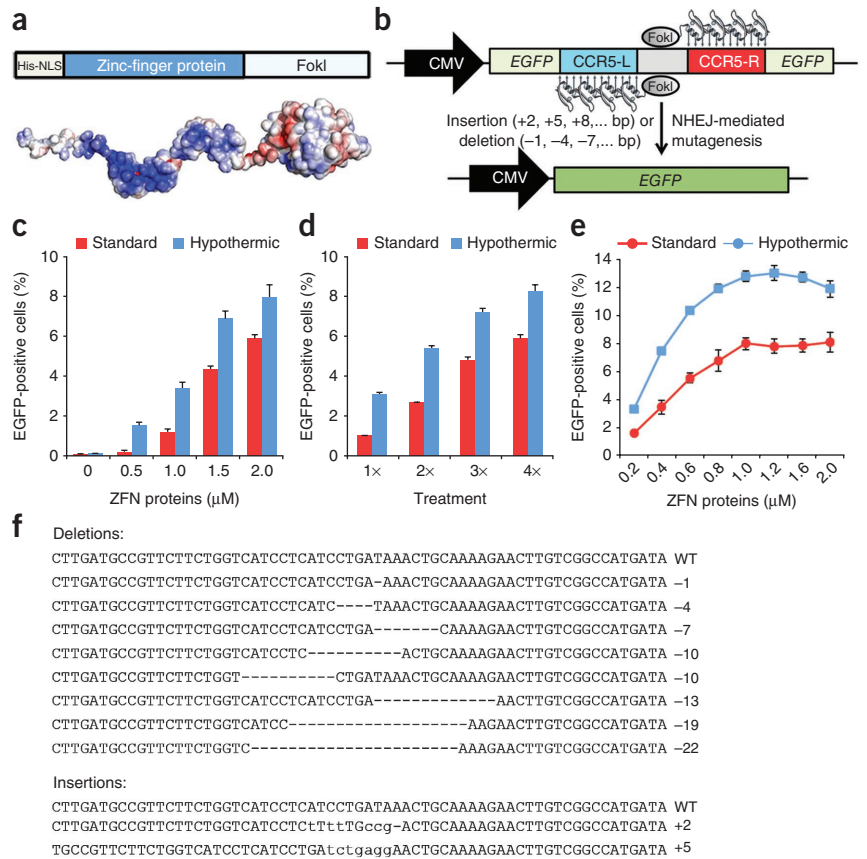
To determine the ability of ZFN proteins to penetrate cells and stimulate mutagenesis, we generated a fluorescence-based reporter system to measure ZFN-induced DSBs (Fig. 1b). This system uses an integrated EGFP gene whose expression has been interrupted by a frameshift mutation introduced by a strategically placed ZFN cleavage site. ZFN proteins that penetrate reporter cells can induce DSBs at this target site and drive the introduction of small insertions and deletions in the EGFP locus by NHEJ. Because NHEJ is a stochastic process, approximately one-third of these mutational events (+2, +5, +8, ... bp or -1, -4, -7, ... bp) will restore the frame and EGFP function.

Direct application of ZFN proteins to reporter cells resulted in a dose-dependent increase in EGFP fluorescence, with maximum activity (6% EGFP-positive cells) achieved after treatment with 2 μM ZFN proteins (Fig. 1c). By comparison, transient transfection of ZFN expression plasmids under saturating conditions resulted in ~7% EGFP-positive cells (Supplementary Fig. 3). We observed no difference in activity between ZFN proteins purified from the soluble fraction or inclusion bodies (Supplementary Fig. 4). At all ZFN concentrations evaluated, the use of transient hypothermic culture conditions<sup>9</sup> enhanced the efficiency of mutagenesis nearly twofold (Fig. 1c,d). Extended periods of incubation (>60 min) did not increase the frequency of genome editing (Supplementary Fig. 5). Consecutive protein treatments, however, did increase the percentage of EGFP-positive cells (Fig. 1d,e). Notably, repeated treatment with ZFN proteins over 3 d using transient hypothermic conditions yielded ~12% EGFP-positive cells (Fig. 1e). Sequence analysis of isolated EGFP-positive cells verified targeted mutagenesis, confirming the presence of the anticipated ZFN-induced insertions and deletions in the EGFP locus (Fig. 1f).

To determine the contribution of each ZFN component to cellular penetration, we incubated cells with fluorescently labeled ZFN or FokI cleavage domain proteins (Supplementary Fig. 6). We observed fluorescence in cell lysate following treatment with ZFN—in the presence or absence of a nuclear localization

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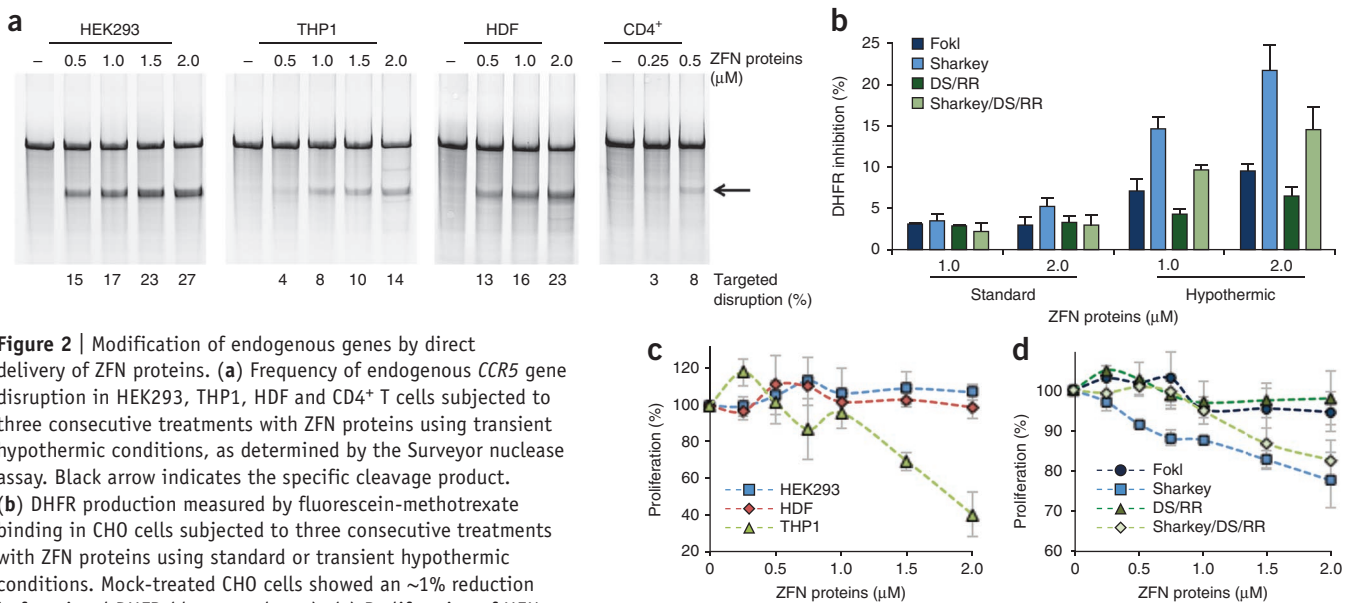
**Figure 1** | ZFN proteins are cell permeable and induce targeted mutagenesis in human cells. **(a)** Diagram of ZFN protein and electrostatic potential of the ZFN protein surface colored from dark red ( $-kT/e$ ) to white ( $0 kT/e$ ) to dark blue ( $+5 kT/e$ ). CMV, cytomegalovirus promoter; NLS, nuclear localization signal. **(b)** Schematic representation of the fluorescence reporter system used to evaluate cellular penetration of ZFN proteins. **(c–e)** Percentage of EGFP-positive cells as determined by flow cytometry in reporter cells treated with increasing amounts of ZFN proteins **(c)** or subjected to consecutive treatments with  $0.2 \mu\text{M}$  ZFN proteins **(d)** or to three consecutive treatments with increasing amounts of ZFN proteins **(e)**. Either standard or transient hypothermic conditions were used in all cases. Error bars, s.d. ( $n = 3$ ). **(f)** Representative sequence analysis of the EGFP locus from isolated EGFP-positive cells. Multiple deletions (dashes) and insertions (lowercase) induced by NHEJ repair are aligned to the cleavage site (wild type (WT)).



sequence—but not with the FokI cleavage domain, suggesting that zinc-finger domains facilitate cellular internalization.

We evaluated the efficacy of this approach for the disruption of endogenous genes by treating human embryonic kidney (HEK) 293 and human acute monocytic leukemia (THP1) cell lines, as well as primary adult human dermal fibroblast (HDF) and primary CD4<sup>+</sup> T cells, with ZFN proteins targeting the *CCR5* gene. These ZFNs used the high-activity *Sharkey* cleavage domain<sup>10</sup>. Analysis of DNA isolated from each cell type with the Surveyor nuclease assay showed efficient and dose-dependent disruption of the endogenous

*CCR5* gene (Fig. 2a). HEK293 and HDF cells subjected to three consecutive treatments with  $2 \mu\text{M}$  ZFN proteins showed gene-disruption frequencies >24%, whereas CD4<sup>+</sup> cells subjected to three consecutive treatments with  $0.5 \mu\text{M}$  ZFN proteins had gene-disruption frequencies >8%. As observed in the reporter system, the frequency of gene disruption increased with repeated protein



**Figure 2** | Modification of endogenous genes by direct delivery of ZFN proteins. **(a)** Frequency of endogenous *CCR5* gene disruption in HEK293, THP1, HDF and CD4<sup>+</sup> T cells subjected to three consecutive treatments with ZFN proteins using transient hypothermic conditions, as determined by the Surveyor nuclease assay. Black arrow indicates the specific cleavage product. **(b)** DHFR production measured by fluorescein-methotrexate binding in CHO cells subjected to three consecutive treatments with ZFN proteins using standard or transient hypothermic conditions. Mock-treated CHO cells showed an ~1% reduction in functional DHFR (data not shown). **(c)** Proliferation of HEK293, HDF and THP1 cells treated with ZFN proteins targeting the *CCR5* gene and containing *Sharkey* mutations. **(d)** Proliferation of CHO cells treated with ZFN proteins targeting the *DHFR* gene and using a variety of specialized cleavage domains. Values normalized to those for mock-treated cells. Error bars, s.d. ( $n = 3$ ).



treatments (Supplementary Fig. 7). Sequence analysis of cloned *CCR5* alleles amplified from each treated cell type confirmed the presence of ZFN-induced insertions and deletions in the *CCR5* gene (Supplementary Fig. 8).

To investigate the cleavage specificity of ZFNs using this approach, we evaluated the activity of the *CCR5* ZFN proteins against nine previously described<sup>6,15</sup> off-target cleavage sites in HEK293 cells (Supplementary Fig. 9). In direct comparison to Lipofectamine-mediated transient transfection of ZFN expression plasmids, we found that cells subjected to consecutive protein treatments showed a marked decrease in ZFN activity at every off-target site, including the *CCR2* locus. Notably, there was no detectable ZFN activity at three of these loci. Western blot analysis showed complete degradation of delivered ZFN proteins <4 h after application, whereas cells transfected with ZFN expression plasmids produced high-levels of protein continuously from 16 h to 72 h after transfection (Supplementary Fig. 10). These results indicate that the differences in cleavage specificity could be attributable to the short half-lives of transduced ZFN proteins and that limiting the duration of ZFN exposure inside cells is a viable method for minimizing toxicity<sup>16</sup>. Consistent with these degradation kinetics, cells treated with ZFN proteins showed maximum activity at 8 h, whereas cells expressing ZFNs from plasmid DNA showed maximum activity at 48 h (Supplementary Fig. 10).

To examine the breadth of this technique, we treated Chinese hamster ovary (CHO) cells with ZFN proteins designed to target the dihydrofolate reductase (*DHFR*) gene<sup>2</sup>. These ZFNs used various specialized cleavage domains, including *Sharkey* and the evolutionarily optimized DS/RR obligate heterodimeric architecture<sup>10</sup>. We observed reduced levels of functional DHFR protein, as determined by fluorescein-labeled methotrexate-based flow cytometry analysis, in CHO cells following three consecutive treatments with DHFR ZFN proteins (Fig. 2b). Notably, CHO cells incubated with ZFNs containing *Sharkey* mutations showed a >12% reduction in functional DHFR. Sequence analysis of cloned *DHFR* alleles amplified from cells treated with ZFN proteins validated these percentages and confirmed the presence of ZFN-induced insertions and deletions in the *DHFR* gene (Supplementary Fig. 11). Examination of DHFR protein levels in expanded clonal populations indicated biallelic *DHFR* gene-disruption frequencies >7% (Supplementary Fig. 12), showing that constitutive ZFN expression from plasmid DNA is not required for high-frequency biallelic modifications and can instead be achieved using directly applied ZFN proteins.

We observed no appreciable toxicity in HEK293 or HDF cells treated with ZFN proteins (Fig. 2c) or in CHO cells incubated with ZFN proteins containing either the wild-type cleavage domain or the DS/RR architecture (Fig. 2d). However, we measured decreased proliferation in CHO and THP1 suspension cells incubated with >1  $\mu$ M ZFN proteins containing *Sharkey* mutations (Fig. 2c,d). We also observed, qualitatively, toxicity in CD4<sup>+</sup> cells subjected to consecutive treatments with >1  $\mu$ M ZFN proteins, suggesting that sensitive cell types may require protein to be administered in consecutive low doses to minimize potential toxic effects.

We have demonstrated the intrinsic cell-penetrating capabilities of the standard ZFN architecture. Furthermore, we have shown that direct delivery of ZFNs as proteins can be used to disrupt the expression of endogenous genes in a variety of mammalian

cell types, including primary CD4<sup>+</sup> T cells and primary adult human dermal fibroblasts, which are frequently used to generate induced pluripotent stem cells. In contrast to methods that require ZFN expression from DNA, ZFN protein delivery leads to comparatively fewer off-target cleavage events and does not carry the risk of insertional mutagenesis. Thus, this method is suitable for genome-editing applications in which minimizing cellular toxicity or maintaining genetic integrity is of particular importance, such as the *in vitro* modeling of human diseases and the *ex vivo* modification of nontransformed human cell types. We show that this method can also be used to modify difficult-to-transfect cell types, including patient-derived leukemia cell lines and primary human lymphocytes, supporting the use of this technique in place of viral-mediated gene delivery for inducing gene knockouts in cultured cells for reverse genetics and drug discovery. As methods for engineering cell permeability into proteins improve, we anticipate that protein delivery and the benefits afforded therein will be extended to other designer nucleases, including TALENs.

## METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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## AUTHOR CONTRIBUTIONS

T.G., J.G., Y.K., S.J.S. and C.F.B. designed the research; J.G. and Y.K. purified ZFN proteins; T.G., J.G., Y.K. and S.J.S. performed experiments; T.G., J.G., Y.K., S.J.S. and C.F.B. analyzed data; T.G., S.J.S. and C.F.B. wrote the manuscript.

## COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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- Kim, Y.G. & Chandrasegaran, S. *Proc. Natl. Acad. Sci. USA* **91**, 883–887 (1994).
- Santiago, Y. *et al. Proc. Natl. Acad. Sci. USA* **105**, 5809–5814 (2008).
- Hockemeyer, D. *et al. Nat. Biotechnol.* **27**, 851–857 (2009).
- Doyon, Y. *et al. Nat. Biotechnol.* **26**, 702–708 (2008).
- Geurts, A.M. *et al. Science* **325**, 433 (2009).
- Perez, E.E. *et al. Nat. Biotechnol.* **26**, 808–816 (2008).
- Holt, N. *et al. Nat. Biotechnol.* **28**, 839–847 (2010).
- Sander, J.D. *et al. Nat. Methods* **8**, 67–69 (2011).
- Doyon, Y. *et al. Nat. Methods* **7**, 459–460 (2010).
- Guo, J., Gaj, T. & Barbas, C.F. III. *J. Mol. Biol.* **400**, 96–107 (2010).
- Thomas, C.E., Ehrhardt, A. & Kay, M.A. *Nat. Rev. Genet.* **4**, 346–358 (2003).
- Warren, L. *et al. Cell Stem Cell* **7**, 618–630 (2010).
- Diebold, S.S. *et al. Science* **303**, 1529–1531 (2004).
- Van Tendeloo, V.F. *et al. Gene Ther.* **7**, 1431–1437 (2000).
- Gabriel, R. *et al. Nat. Biotechnol.* **29**, 816–823 (2011).
- Pruett-Miller, S.M., Reading, D.W., Porter, S.N. & Porteus, M.H. *PLoS Genet.* **5**, e1000376 (2009).

## ONLINE METHODS

**Plasmid construction.** The left (L) and right (R) zinc-finger nuclease (ZFN) proteins designed to target human *CCR5* (ref. 6) and Chinese hamster *DHFR*<sup>2</sup> genes were PCR-amplified from their respective mammalian expression vectors (previously constructed in our laboratory) pVAX1-NH.CCR5.L/R.FN and pVAX1-NH.DHFR.L/R.FN with the primers 5' NdeI-NLS and 3' FN-SacI-Sall. All primer sequences are provided in **Supplementary Table 1**. PCR products were digested with NdeI and Sall and ligated into the NdeI and XhoI restriction sites of the pET-28 (+) expression vector (Novagen) to generate the plasmids pET.CCR5.L/R.FN and pET.DHFR.L/R.FN. Specialized FokI cleavage domain variants, including the high-activity *Sharkey* cleavage domain and the evolutionarily optimized obligate heterodimer architecture DS and RR, were isolated from previously described pDAZ templates<sup>10</sup> and cloned into pET.CCR5.L/R.FN and pET.DHFR.L/R.FN with the restriction sites BamHI and SacI to generate pET.CCR5.L.R/Sh, pET.DHFR.L/R.Sh, pET.DHFR.L/R.DS/RR and pET.DHFR.L/R.Sh.DS/RR (L, left ZFN; R, Right ZFN; FN, FokI cleavage domain; Sh, *Sharkey* cleavage domain; DS/RR, DS or RR architecture; Sh.DS/RR, *Sharkey* cleavage domain with DS or RR architecture). Correct construction of each ZFN expression cassette was verified by sequence analysis (**Supplementary Table 2**).

**ZFN protein expression and purification.** ZFN expression plasmids were transformed into chemically competent *E. coli* BL21 (DE3) pGro7 cells (Stratagene). A single colony was added to 10 ml of LB medium in the presence of 50  $\mu\text{g ml}^{-1}$  kanamycin, 100  $\mu\text{g ml}^{-1}$  chloramphenicol and 1% glucose. Bacteria were grown overnight at 37 °C with shaking. The following day, 500 ml of LB medium supplemented with 100  $\mu\text{M ZnCl}_2$ , 50  $\mu\text{g ml}^{-1}$  kanamycin, 100  $\mu\text{g ml}^{-1}$  chloramphenicol and 0.2% glucose was inoculated with 5 ml of the overnight culture and incubated at 30 °C with shaking. The culture was grown to an optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.4 and then incubated at room temperature with shaking until the  $\text{OD}_{600}$  reached 0.8. Protein synthesis was then induced with 0.1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). After 4 h, cells were harvested by centrifugation at 2,000 RCF for 20 min at 4 °C, and the pellet was resuspended in native lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 100  $\mu\text{M ZnCl}_2$ , 1 mM PMSF, 1 mM  $\text{MgCl}_2$ , 1 mM  $\beta$ -mercaptoethanol, 10 mM imidazole, 0.2% Triton X-100 and 10% glycerol, pH 8.0). Cells were lysed using standard sonication conditions, and the soluble fraction was isolated by centrifugation at 18,000 RCF for 30 min at 4 °C. ZFN proteins were purified using Ni-NTA agarose resin (QIAGEN) and eluted with 500 mM imidazole in the presence of 50 mM Tris-HCl, 500 mM NaCl, 100  $\mu\text{M ZnCl}_2$  and 10% glycerol at pH 8.0. Each protein was concentrated and dialyzed against storage buffer (20 mM HEPES, 90  $\mu\text{M ZnCl}_2$ , 150 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , pH 7.4) and assessed for purity by SDS-PAGE. The protein yield after purification was determined to be between 0.5  $\text{mg l}^{-1}$  and 1.0  $\text{mg l}^{-1}$ .

**Inclusion-body purification.** ZFN proteins were also purified from inclusion bodies using an alternative method. Following the inoculation of 500 ml of LB medium with 5 ml of the overnight culture, cells were incubated at 37 °C with shaking until the  $\text{OD}_{600}$  reached 0.4, and then at room temperature with shaking

until the  $\text{OD}_{600}$  reached 0.9. Protein synthesis was induced by addition of 0.4 mM IPTG and 4 mM arabinose. After 16 h, cells were harvested by centrifugation at 2,000 RCF for 20 min at 4 °C, and the pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, pH 8.0). Cells were lysed using standard sonication conditions and centrifuged at 10,000 RCF for 30 min at 4 °C. Inclusion bodies were then washed with lysis buffer and centrifuged at 10,000 RCF for 30 min at 4 °C. This process was repeated twice and was followed by a procedure designed to enhance protein yield and purity: inclusion bodies were (i) washed with cold water, (ii) centrifuged at 10,000 RCF for 30 min at 4 °C, (iii) resuspended in PBS, (iv) centrifuged as in step (ii), (v) incubated with solubilization buffer (PBS, 500 mM L-arginine, 90  $\mu\text{M ZnCl}_2$ , 1 mM  $\text{MgCl}_2$ , pH 7.4) for 3 h at 4 °C and (vi) centrifuged as in step (ii). L-Arginine was included to facilitate refolding of recombinant proteins from inclusion bodies<sup>17</sup>. Following this procedure, the inclusion-body supernatant was isolated and protein purity was assessed by SDS-PAGE. The protein yield after purification was determined to be between 3  $\text{mg l}^{-1}$  and 5  $\text{mg l}^{-1}$ .

**In vitro cleavage analysis.** Cleavage analysis was performed as described<sup>10</sup>.

**Reporter system construction and analysis.** The EGFP reporter systems used in this study were generated using the Flp-In System (Invitrogen) and constructed as described<sup>10</sup> with the following exception: the CCR5 ZFN cleavage site was amplified with the overlapping oligonucleotides 5' midEGFP-CCR5 and 3' midEGFP-CCR5. Reporter cells were analyzed by flow cytometry as described<sup>10</sup>.

**Cell culture.** HEK293, THP1, HDF (Invitrogen) and CHO cells were seeded onto 24-well plates at a density of  $1 \times 10^5$  cells per well and established in a humidified 5%  $\text{CO}_2$  atmosphere at 37 °C. Peripheral blood mononuclear cells (PBMCs) were obtained from anonymous healthy blood donors through the Scripps Research Institute Normal Blood Donor Program. Primary  $\text{CD4}^+$  T cells were purified from PBMCs using the RosetteSep Human  $\text{CD4}^+$  T Cell Enrichment Cocktail (Stem Cell Technologies) according to the manufacturer's instructions.  $\text{CD4}^+$  cells were seeded onto 96-well plates at a density of  $1 \times 10^5$  cells per well and established in a humidified 5%  $\text{CO}_2$  atmosphere at 37 °C. HEK293 and HDF cells were maintained in DMEM containing 10% (vol/vol) FBS and 1% (vol/vol) Antibiotic-Antimycotic (Anti-Anti; Gibco). THP1, CHO and  $\text{CD4}^+$  cells were maintained in RPMI containing 10% (vol/vol) FBS and 1% (vol/vol) Anti-Anti. CHO cells were maintained with HT supplement (100  $\mu\text{M}$  sodium hypoxanthine and 160  $\mu\text{M}$  thymidine; (Invitrogen)) after treatment with ZFN proteins.

**ZFN protein treatments.** ZFN proteins were prepared for treatment as follows: ZFNs purified from the soluble fraction were diluted into serum-free medium containing 90  $\mu\text{M ZnCl}_2$  at pH 7.4. ZFNs purified from inclusion bodies were diluted into serum-free medium containing 100 mM L-arginine and 90  $\mu\text{M ZnCl}_2$  at pH 7.4. At 24 h after seeding, cells were washed with serum-free medium and treated with ZFN proteins for 1 h at 37 °C. After treatment, cells were washed and maintained at 37 °C with

serum-containing medium for 24 h before the next treatment, or up to 3 d before cells were harvested for analysis. This process was repeated up to four times over four consecutive days.

Transient hypothermic conditions were achieved as follows: 24 h after seeding, cells were washed with serum-free medium and treated with ZFN proteins for 1 h at 37 °C. After treatment, cells were washed and incubated at 30 °C with serum-containing medium for 6–24 h. Cells were then returned to and maintained at 37 °C for the remaining 24 h before the next treatment or up to 3 d before cells were harvested for analysis. This process was repeated up to four times over four consecutive days.

**Internalization of fluorescently labeled proteins.** Purified ZFN and FokI cleavage domain proteins were labeled with fluorescein-5-maleimide (Invitrogen) under conditions specified by the manufacturer. Following conjugation, 2 μM ZFN or FokI cleavage domain proteins were dialyzed against 20 mM HEPES and 90 μM ZnCl<sub>2</sub> at pH 7.4 and then incubated with HEK293 cells for 1 h. Cells were then washed three times with 20 mM HEPES, 90 μM ZnCl<sub>2</sub> and 1 mg ml<sup>-1</sup> heparin at pH 7.4 to remove surface-bound ZFN proteins. Cells were lysed in SDS-PAGE loading buffer (0.2 M Tris-HCl, 10% (vol/vol) SDS, 10 mM β-mercaptoethanol, 0.05% (wt/vol) bromophenol blue, 20% (vol/vol) glycerol, pH 6.8). Relative quantities of internalized protein were determined by fluorescent illumination of SDS-PAGE gels using the GelDoc XR Imaging System (Bio-Rad).

**Surveyor nuclease assay.** Genomic DNA was extracted with Quick Extract DNA Extraction Solution (Epicentre). The *CCR5* gene was amplified by nested PCR with the following primer pairs: 5' hCCR5 External with 3' hCCR5 External, and 5' hCCR5 Internal with 3' hCCR5 Internal. Following PCR amplification of the *CCR5* locus using the Expand High Fidelity Taq System (Roche), the Surveyor mutation detection kit was used according to the manufacturer's instructions (Transgenomics). Cleavage products were visualized by PAGE and the frequency of gene disruption was determined by measuring the ratio of cleaved to uncleaved substrate, as described<sup>18</sup>.

**Western blot analysis.** Western blot analysis was performed as described<sup>19</sup> with the following exception: the internal loading control was β-actin detected with peroxidase-conjugated anti-β-actin antibody.

**Cellular proliferation assay.** HEK293, HDF, THP and CHO cells were seeded onto 96-well plates at a density of  $1 \times 10^5$  cells per well. At 24 h after seeding, cells were treated with ZFN proteins as described above. Decreased cellular proliferation in response to ZFN protein was measured using the Cell Proliferation Kit II (XTT; Roche Applied Science) according to the manufacturer's instructions.

**Fluorescein methotrexate assay.** The fluorescein methotrexate assay was performed as described<sup>2,20</sup>.

**Clonal analysis of CHO cells.** At 16 d after ZFN treatment, CHO cells were seeded onto 96-well plates by limiting dilution with 10% FBS-RPMI, 1% Anti-Anti and HT supplement. Each well was visually inspected to ensure single colony formation. The fluorescein methotrexate flow cytometry assay was used to determine the level of functional DHFR protein. Genomic DNA from clones determined to be *DHFR*<sup>-/-</sup> was isolated using the QIAmp DNA MiniKit (QIAGEN). The *DHFR* alleles were PCR amplified with the primers 5' DHFR BssHII and 3' DHFR EcoRI. PCR products were ligated into the MluI and EcoRI restriction sites of the plasmid pCDNA 3.1 (Invitrogen). Sequence analysis of transformants was used to confirm biallelic gene modification.

**Sequence analysis.** Genomic DNA from EGFP-positive reporter cells (isolated by FACS; FACScan Dual Laser Flow Cytometer, BD Biosciences) was purified with the QIAmp DNA MiniKit (QIAGEN) and the EGFP locus was PCR amplified with the primers 5' EGFP 443–466 BamH1 and 3' EGFP 497–520 EcoRI. Genomic DNA from ZFN-treated HEK293, HDF, THP1 and CD4<sup>+</sup> cells was isolated with Quick Extract DNA Extraction Solution (Epicentre). The *CCR5* locus was PCR amplified with the primers 5' hCCR5 BamH1 and 3' hCCR5 EcoRI. EGFP and *CCR5* PCR products were cloned into the plasmid pUC19 with the restriction sites EcoRI and BamH1. Sequence analysis was performed on individual cloned transformants.

17. Golovanov, A.P., Hautbergue, G.M., Wilson, S.A. & Lian, L.Y. *J. Am. Chem. Soc.* **126**, 8933–8939 (2004).
18. Guschin, D.Y. *et al. Methods Mol. Biol.* **649**, 247–256 (2010).
19. Gordley, R.M., Gersbach, C.A. & Barbas, C.F. III. *Proc. Natl. Acad. Sci. USA* **106**, 5053–5058 (2009).
20. Gaudray, P., Trotter, J. & Wahl, G.M. *J. Biol. Chem.* **261**, 6285–6292 (1986).