Published in final edited form as:
Nat Methods. ; 8(9): 753–755. doi:10.1038/nmeth.1653.

High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases

Fuqiang Chen[1], **Shondra M Pruett-Miller**[1], **Yuping Huang**[1], **Monika Gjoka**[1], **Katarzyna Duda**[2], **Jack Taunton**[3], **Trevor N Collingwood**[1], **Morten Frodin**[2], and **Gregory D Davis**[1]

[1]Sigma-Aldrich Biotechnology, St. Louis, Missouri, USA.

[2]Biotech Research and Innovation Centre and Centre for Epigenetics, University of Copenhagen, Copenhagen, Denmark.

[3]Howard Hughes Medical Institute, Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, California, USA.

Abstract

Zinc-finger nucleases (ZFNs) have enabled highly efficient gene targeting in multiple cell types and organisms. Here we describe methods for using simple ssDNA oligonucleotides in tandem with ZFNs to efficiently produce human cell lines with three distinct genetic outcomes: (i) targeted point mutation, (ii) targeted genomic deletion of up to 100 kb and (iii) targeted insertion of small genetic elements concomitant with large genomic deletions.

Predefined genomic modifications using zinc-finger nucleases (ZFNs) are typically achieved using donor plasmids that contain homology arms of 200–800 base pairs (bp) flanking the genomic site of modification[1,2]. These donor plasmids are smaller and simpler to build than traditional gene-targeting vectors, yet they still require several weeks to construct. In contrast, single-stranded oligodeoxynucleotides (ssODNs) can be designed and synthesized in just a few days. Substituting plasmid donors with ssODNs would greatly expedite ZFN-based gene targeting studies. Homologous recombination–mediated targeted genome editing in eukaryotic cells is greatly stimulated by targeted double-strand breaks[1–5]. Recent genome-editing work using ssODNs with I-SceI endonuclease and ZFNs has achieved correction of reporter genes at frequencies of up to 0.7% (refs. 6,7). Here we report routine, efficient and experimentally flexible targeted genome editing using ZFNs and ssODNs at endogenous loci.

Double-strand break–facilitated gene conversion is most efficient near the break site[8], so we chose first to insert a restriction site directly into a ZFN cleavage site in the *AAVS1* locus[9,10] using the ssODN AAVS1-95 (numbers indicate total oligo length; here 95 nucleotides), which contained a HindIII site flanked by ~40 bp of homology on each side of the ZFN cut site (**Supplementary Fig. 1** and **Supplementary Note 1**). We readily detected

Correspondence should be addressed to G.D.D. (greg.davis@sial.com)..

AUTHOR CONTRIBUTIONS

F.C., S.M.P.-M., Y.H., M.G. and K.D. performed experiments. G.D.D., F.C., S.M.P.-M. and M.F. designed experiments. G.D.D., T.N.C., M.F., F.C., S.M.P.-M. and J.T. wrote the paper.

Note: Supplementary information is available on the Nature Methods website.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemethods/.

targeted insertion of the HindIII site in pooled cells in seven different transformed cell types. Efficiency of insertion ranged from 7% to 57% and was cell-type–dependent (**Supplementary Fig. 2**). Sequencing of modified A549 cell clones (using ssODN AAVS1-115, **Supplementary Note 1**) confirmed that the insertion was correctly targeted and stable (**Supplementary Fig. 3**). To determine the minimal homology requirements in the ssODN, we systematically reduced the length of total homology of *AAVS1* ssODNs over the 100 base to 30 base range (**Supplementary Note 1**). We observed a drop in efficiency on transition from 40 to 30 total bases of homology (**Supplementary Fig. 4**). We did not detect HindIII site insertion using ssODNs distal to the cut site (for example, 0.1–1 kb away; data not shown). The extent of modification achieved using an ssODN donor was approximately twofold higher than that obtained using a plasmid donor (**Supplementary Fig. 5**). The single-stranded format of oligodeoxynucleotides resulted in fewer (if any) nonfaithful integrations at the double-strand break than a double-stranded oligodeoxynucleotide composed of the same ssODN and its complement (**Supplementary Fig. 6**). Expressing ZFNs in either plasmid DNA or mRNA format enabled efficient ssODN-mediated targeting, with mRNA giving ~1.4-fold higher frequency of targeting under these experimental conditions (**Supplementary Fig. 7**). With the exception of data shown in **Supplementary Figures 4** and **7**, all data reported in this paper were collected using capped poly(A)-tailed mRNA for ZFN expression.

The preceding studies at the *AAVS1* locus demonstrated ssODN-mediated modification directly at the cut site. In practice, however, it is rarely possible to generate ZFNs that cut precisely at the desired point of mutation. We attempted to create a targeted codon modification in the *RPS6KA3* gene encoding the kinase RSK2, implicated in mental retardation, psychomotor and skeletal disorders, and in cancer. Previous data with exogenous RSK2 predicted that a cysteine to valine conversion in the active site will render RSK2 insensitive to the pharmacological RSK kinase inhibitor fmk[11,12]. We sought to recreate this 3-bp mutation at the endogenous *RPS6KA3* locus (here we refer to it by the synonym *RSK2*) using the ssODN-based approach. We engineered a ZFN that cuts 27 bp away from the desired mutation site in *RSK2*. We designed an ssODN donor (ssODN RSK2-125) to span both the mutation site and the ZFN cleavage site as well as flanking sequence (**Fig. 1a** and **Supplementary Note 1**). To enable restriction fragment length polymorphism (RFLP)-based detection of targeted clones, we introduced a silent cytosine to adenine mutation to create a BamHI restriction site 15 bp from the Cys436 codon location, but on the opposite side of Cys436 codon to the ZFN binding site and 42 bp away from the cleavage site. Thus, if the integration of the ssODN occurs via the directional homologous recombination mechanisms previously characterized for dsDNA donors[8], the majority of BamHI site–containing alleles would be expected to contain the mutation encoding C436V. Finally, we included two silent ZFN-blocking mutations in the ZFN binding site. When incorporated into the target locus, the ZFN-blocking mutations disrupt the ZFN target site, thereby protecting the modified locus from additional cutting by the ZFNs after gene conversion, which could otherwise result in unwanted small deletions or insertions via nonhomologous end-joining repair. Although not absolutely required, ZFN-blocking mutations are advantageous in analogous plasmid-based codon conversion experiments (data not shown). Upon transfection of ssODN RSK2-125 and mRNA encoding the *RSK2* ZFN into K562 cells, we detected the BamHI site conversion at rates of 22–32% as estimated by RFLP assay (**Fig. 1b**). Single-cell cloning yielded at least 20 clones from ~750 (3%) with biallelic BamHI conversion and the desired biallelic C436V conversion (**Supplementary Fig. 8**). Thus, creation of mutations at substantial distances (42 bp in this instance) from the ZFN cut site using ssODNs is feasible and efficient. Sequence-based analysis of the fidelity of these modifications showed this approach enables isolation of correctly targeted mutant

clones (**Supplementary Fig. 9**). Three randomly selected clones encoding the C436V mutant demonstrated the predicted insensitivity of RSK2 to fmk (**Fig. 1c**).

Targeted deletion of exons or entire genes is also a frequently desired outcome in genome editing. To date, most ZFN-mediated genomic deletions have resulted from capturing misrepair events during nonhomologous end joining[13] of targeted double-strand breaks. Deletions arising from the use of a single ZFN pair are typically <50 bp. Larger deletions can be obtained by using two ZFN pairs that cleave at a prescribed distance from each other on the same chromosome and excise the intervening sequence[14,15], but this approach may be offset by the need to generate additional ZFNs that target at each boundary of the deletion. A simple and efficient process whereby a single ZFN can be used to generate large deletions in a defined manner is needed. Similar to previous work in yeast[16], we investigated the use of ssODNs to facilitate deletion between a mammalian double-strand break and a distal locus in the same chromosome that is uniquely specified by the design of the ssODN. In **Figure 2a** we illustrate the general design used for deletion ssODNs (nucleotide resolution designs for various loci are shown in **Fig. 2b** and **Supplementary Notes 2** and **3**). We synthesized ssODNs to operate in concert with the ZFNs targeting the *AAVS1* locus. We chose distal deletion points to systematically investigate the effect of deletion size without regard to genetic or sequence specific genomic context. We generated chromosomal deletions 0.1–100 kilobases (kb) in size extending from the ZFN cut site (**Fig. 2c** and **Supplementary Note 2**). First we screened clones from transfected K562 cells by PCR with primers flanking the respective deletion boundaries to identify those in which the deletion had occurred. Then, to detect remaining alleles where deletion had not occurred, we screened clones containing the deletion by junction PCR using one primer that bound inside the deleted region and another that bound outside the deleted region. In K562 cells the *AAVS1* locus is triploid. Nevertheless, the PCR data from the clones indicate that we isolated complete triallelic deletions of 1 kb, 5 kb and 10 kb at frequencies of 2.9%, 0.6% and 0.2%, respectively, after a single ZFN treatment (**Supplementary Table 1** and **Supplementary Figs. 10**–**12**). We verified PCR data indicating a triallelic 10 kb deletion by Southern blotting (**Supplementary Figs. 13** and **14**). In K562 cells, smaller deletions at the *AAVS1* locus of 100 bp and 500 bp produced higher triallelic deletion rates of 21% and 10.8%, respectively, as assessed by analysis of single-cell clones (**Supplementary Table 1** and **Supplementary Fig. 15**). PCR screening >1,000 clones identified two isolates heterozygous for 100-kb deletions (**Supplementary Fig. 16** and **Supplementary Table 1**). We tested this deletion method in seven different cell lines and on four different genes (**Supplementary Fig. 17**). Also we attempted simultaneous deletion in both the 5′ and 3′ directions from the *AAVS1* ZFN cut site by transfecting K562 cells with *AAVS1* ZFNs and two ssODNs designed to achieve 100-bp deletions in each direction. PCR sequencing of the target locus identified deletion events that were exclusively in one direction or the other but never in both directions (data not shown). Also we generated large deletions concomitant with insertion of short sequences such as a *loxP* recombinase site (**Supplementary Fig. 18**).

Earlier studies using ssODNs for targeted gene modification had documented adverse cellular responses, raising concerns as to the viability and safety of ssODN-mediated approaches[6,17,18]. A common aspect of these studies was the use of ssODNs containing phosphorothioate linkages. A recent study has demonstrated that the observed toxicity is due to the presence of the phosphorothioate modification, and that ssODNs themselves have little impact on cell integrity[18], consistent with other work in mammalian cells[7] and in yeast[16]. We conducted our experiments with unmodified ssODNs and did not observe adverse cellular responses. Moreover, we isolated clones even from pools that had undergone relatively low frequency modifications. We advise that ssODNs be free of phosphorothioate modification and that the impact of any other type of modification be assessed. We encourage others to be aware that although ZFN-induced double-strand breaks

greatly increase genome-editing frequencies, cell types can vary greatly in their response to various nucleic acid delivery methods and DNA repair responses. For a given cell type, initial RFLP-based assessment (**Fig. 1b** and **Supplementary Figs. 1** and **2**) can be used to rapidly determine whether delivery efficiencies, ZFN expression and DNA repair rates are sufficiently high to merit expansion and screening of clones in the absence of selection.

In conclusion, we found that ssODNs can be used with precisely localized ZFNs to achieve three distinct genetic modification events: (i) targeted point mutation, (ii) targeted deletion of small and large sequences and (iii) simultaneous targeted deletion of large sequences and insertion of defined small genetic elements. These events can be achieved at frequencies of 1–30% without antibiotic selection, and we tested the approach for multiple loci and transformed cell types. ssODNs and ZFNs have also recently been reported to be effective at genome editing in human induced pluripotent stem cells[19]. The trivial task of synthesizing ssODN donors provides the researcher with greater freedom and speed to conduct sophisticated genetic modification in mammalian cells and likely in other eukaryotic systems.

Methods

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

M.F. acknowledges support from the Danish Cancer Society (R2-A132-02-S2), the Danish National Research Foundation and the Danish Cancer Research Foundation. J.T. acknowledges support from the US National Institutes of Health (GM071434). We thank our colleagues at Sigma-Aldrich and Sangamo Biosciences for helpful advice and discussion; H. Holemon, P. Sullivan and D. Smoller for support; and D. Carroll for helpful comments and discussion.

ONLINE METHODS

Cell culture and transfection

The human cell lines K562, HCT116, U2OS, A549, HEK293, HepG2 and MCF7 were obtained from American Type Culture Collection (ATCC). K562 was grown in Iscove's Modified Dulbecco's Medium, supplemented with 10% FBS and 2 mM L-glutamine; HCT116 and U2OS were grown in McCoy's 5A medium, supplemented with 10% FBS and 1.5 mM L-glutamine; A549 was grown in Ham's F-12 medium, supplemented with 10% FBS and 2 mM L-glutamine; HEK293 and HepG2 were grown in Dulbecco's modified Eagle's medium, supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM nonessential amino acids; and MCF7 was grown in RPMI 1640 medium, supplemented with 10% FBS, 2 mM L-glutamine and 10 μg ml^{-1} of bovine insulin. All media and supplements were obtained from Sigma-Aldrich. Cultures were split 1 d (K562) or 2 d (all the adherent cell types) before transfection and were at ~0.5 million cells ml^{-1} for K562 and ~80% confluency for all attached cell lines at the time of transfection. K562, HCT116, U2OS, HEK293, HepG2 and MCF7 were each nucleofected with Nucleofector Solution V (Lonza) on a Nucleofector (Lonza) with the following programs: T-016 (K562), D-032 (HCT116), X-001 (U2OS), Q-001 (HEK293), T-028 (HepG2) and P-020 (MCF7). A549 was nucleofected with Solution T and program X-001. Each nucleofection contained 0.5

million cells for K562, or 0.6 million cells for HCT116, U2OS, A549, HEK293, HepG2, or 1.2 million cells for MCF7, suspended in 100 μl of Nucleofector solution. The ssODN was dissolved in 10 mM Tris (pH 7.6) at 100 μM, and 1 or 3 μl of the stock solution was mixed with 4 or 8 μg of *ZFN* mRNA (2 or 4 μg of each *ZFN* mRNA) or 5 μg of *ZFN* DNA (2.5 μg of each *ZFN* DNA) before nucleofection. Cells were grown at 37 °C and 5% CO_2 immediately after nucleofection.

Oligonucleotide and ZFN engineering

All oligonucleotides were manufactured by Sigma-Genosys. ZFNs for the *AAVS1* and *RPS6KA6* (synonym *RSK4*) loci were engineered by Sangamo Biosciences. ZFNs for the *RPS6KA3* (synonym *RSK2*) and *IRAK4* loci were engineered by the CompoZr ZFN Operations Group at Sigma-Aldrich Biotechnology and are available from Sigma-Aldrich. Sigma product identifiers for ZFN reagents are CTI1 for *AAVS1*, CKOZFN2400 for *RPS6KA6* (synonym *RSK4*), CKOZFN2232 for *RPS6KA3* (synonym *RSK2*) and CKOZFN1270 for *IRAK4*.

Restriction fragment length polymorphism assay

Genomic DNA was extracted from transfected cells with GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) 2 d after nucleofection. Genomic DNA was then PCR amplified with primers flanking the ssODN target region (see **Supplementary Table 2** for all PCR primer sequences). For the *AAVS1* locus, three pairs of primers were used in PCR amplification. The first pair of primers, AAVS1-F1 and AAVS1-R1, was used to generate a 303-bp fragment for acrylamide gel analysis after HindIII digestion. The amplification was carried out with JumpStart Taq ReadyMix (Sigma), using the following cycling condition: 98 °C for 2 min for initial denaturation; 32 cycles of 98 °C for 15 s, 62 °C for 30 s and 72 °C for 40 s; and a final extension at 72 °C for 5 min. The second pair of primers, AAVS1-F2 and AAVS1-R2, was used to generate a 1.8-kb fragment for agarose gel analysis after HindIII digestion. The amplification was carried out with Expand High Fidelity PCR System (Roche), using the following cycling condition: 95 °C for 5 min for initial denaturation; 15 cycles of touch-down amplification, consisting of 95 °C for 30 s, 68 °C for 1 min and 30 s with 0.5 °C reduction every cycle; 20 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min and 30 s; and a final extension at 72 °C for 5 min. The third pair of primers used to amplify the *AAVS1* loci, AAVS1-F3 and AAVS1-R3, generated a 469-bp fragment for acrylamide gel analysis after HindIII digestion. The amplification was carried out with JumpStart Taq ReadyMix using the following cycling condition: 98 °C for 5 min for initial denaturation; 30 cycles of 98 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min. For the *RSK2* locus, a 428-bp fragment was amplified with primers RSK2-F1 and RSK2-R1 using the same PCR conditions as described for the *AAVS1* locus short fragment. For the *RSK4* locus, a 1,179-bp fragment was amplified with primers RSK4-F1 and RSK4-R1. The amplification was carried out with Taq polymerase (AmpliconIII), using the following cycling condition: 95 °C for 5 min for initial denaturation; 15 cycles of touch-down amplification, consisting of 95 °C for 30 s, 68 °C for 1 min and 30 s with 0.5 °C reduction every cycle; 24 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min and 10 s; and a final extension at 72 °C for 5 min. All PCR products digested with 20 U of HindIII (*AAVS1* locus) or BamHI (*RSK2* and *RSK4* loci) at 37 °C for 2 h or overnight and resolved on acrylamide or agarose gel.

Deletion PCR assay

Genomic DNA was extracted from transfected cells with GenElute Mammalian Genomic DNA Miniprep kit 2 d after nucleofection. Genomic DNA was then PCR-amplified for each

targeted deletion event with a pair of primers positioned outside of the corresponding deleted sequence (see **Supplementary Table 2** for all PCR primer sequences). For deletions 5′ to the ZFN cut site at the *AAVS1* locus, a common reverse primer, AAVS1-d5R-com, was paired with each of the following 5′ deletion-specific forward primers: AAVS1-dF-0.1 (100-bp deletion), AAVS1-d5F-0.5 (500-bp deletion), AAVS1-d5F-1 (1-kb deletion), AAVS1-d5F-1.5 (1.5-kb deletion), AAVS1-d5F-2 (2-kb deletion), AAVS1-d5F-2.5 (2.5-kb deletion), AAVS1-d5F-3 (3-kb deletion), AAVS1-d5F-3.5 (3.5-kb deletion), AAVS1-d5F-4 (4-kb deletion), AAVS1-d5F-4.5 (4.5-kb deletion), AAVS1-d5F-5 (5-kb deletion), AAVS1-d5F-10 (10-kb and 10.1-kb deletions), AAVS1-d5F-20 (19.9-kb and 20-kb deletions), AAVS1-d5F-50 (50-kb deletion) and AAVS1-d5F-100 (100-kb deletion). For the concerted 5′ 5-kb deletion and *loxP* insertion at the *AAVS1* locus, primers AAVS1-dR-com and AAVS1-dF-5 were used. For the junction-specific PCR, AAVS1-dR-com was used with the forward junction–specific primer, AAVS1-dloxP. For deletions 3′ to the ZFN cut site at the *AAVS1* locus, a common forward primer, AAVS1-d3F-com, was paired with each of the two 3′ deletion-specific reverse primers: AAVS1-d3R-0.1 (100-bp deletion) and AAVS1-d3R-2 (2-kb deletion). The primer pairs used to analyze the deletion events at other loci were IRAK4-d5F-5.9 and IRAK4-d5R-5.9 (5′ 5.9-kb deletion), RSK2-d3F-5.2 and RSK2-d3R-5.2 (3′ 5.2-kb deletion), and RSK4-d3F-5 and RSK4-d3R-5 (3′ 5-kb deletion). The amplification was carried out with JumpStart Taq ReadyMix using the following cycling condition: 98 °C for 2 min for initial denaturation; 35 cycles of 98 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min. PCR products were resolved on 3% agarose gel. Deletion PCR fragments were verified by DNA sequencing.

Cell cloning

Single-cell cloning was performed by limiting dilution (targeted insertion and codon conversion) or flow cytometry cell sorting (targeted deletions). Cells were lysed and screened for targeted mutations by real-time PCR using JumpStart SYBR Green ReadyMix (see **Supplementary Table 2** for all PCR primer sequences). For the targeted insertion at the *AAVS1* locus, clones were first screened with an insertion-specific forward primer AAVS1-C-F1 and a common reverse primer AAVS1-C-R1. Candidate clones were then screened for absence of the wild-type allele with wild-type–specific primers AAVS1-C-F2 and AAVS1-C-R1. Real-time PCR was carried out on a Mx3000P Real-Time PCR system (Stratagene) with the following cycling condition: 98 °C for 2 min for initial denaturation; 40 cycles of 98 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s followed by a dissociation curve segment (95 °C, 1 min; 55 °C, 30 s; 95 °C, 30 s). For the codon conversion at the *RSK2* locus, clones were screened with a BamHI site–specific forward primer, RSK2-Bam-F1, and a wild-type–specific forward primer RSK2-wt-F1, in combination with a common reverse primer, RSK2-Bam-R1. Candidate clones were then screened for biallelic cysteine to valine mutation with a mutation-specific forward primer, RSK2-Val-F, and a wild-type–specific forward primer, RSK2-Cys-F, in combination with the common reverse primer, RSK2-Bam-R1. Real-time PCR was carried out essentially as described above, only with a change of the annealing temperature from 62 °C to 60 °C. For the targeted deletions at the *AAVS1* locus, clones were screened with deletion-specific forward primers AAVS1-d5F-5, AAVS1-d5F-10 or AAVS1-d5F-100 in combination with a common reverse primer, AAVS1-d5R-C. Real-time PCR was carried out as described above for the targeted insertion at the *AAVS1* locus. Candidate clones were then analyzed by the deletion PCR assay as described above. For deletions of 100 bp to 1 kb, clones were screened directly by the deletion PCR assay, using their corresponding primer pairs. Undeleted alleles for clones carrying greater than 100-bp deletions were detected by PCR amplification around their corresponding distal deletion sites and around the common ZFN cut site. The primer pairs for detecting undeleted alleles at the distal deletion sites were: AAVS1-d5F-0.5wt and AAVS1-d5R-0.5wt for 500 bp deletion clones; AAVS1-d5F-1wt and AAVS1-d5R-1wt for

1-kb deletion clones; AAVS1-d5F-5wt and AAVS1-d5R-5wt for 5 kb deletion clones; AAVS1-d5F-10wt and AAVS1-d5R-10wt for 10 kb deletion clones; and AAVS1-d5F-100wt and AAVS1-d5R-100wt for 100 kb deletion clones. Amplification of undeleted alleles around the ZFN cut site was carried out with a common forward primer, AAVS1-Z-CF, and a common reverse primer, AAVS1-ZFN-CR. PCR amplification was conducted using the same condition as for the deletion PCR assay.

Southern blot assay

Genomic DNA was isolated from cell culture with Qiagen Blood & Cell Culture DNA kit and digested (15 μg each) with PstI, EcoRI or EcoRV for 16 h. Digested DNA was resolved on 0.7% agarose gel and transferred to nylon membrane with 20× SSC. Digoxin (DIG)-labeled DNA probes were synthesized by nested PCR amplification of genomic DNA isolated from control K562 cells (see **Supplementary Table 2** for all PCR primer sequences). The primer pairs for amplifying a probe corresponding to an undeleted sequence immediately adjacent to the ZFN cut site were AAVS1-S-F1 and AAVS1-S-R1 for primary PCR, and AAVS1-S-F2 and AAVS1-S-R2 for nested PCR. The primer pairs for amplifying a probe corresponding to a deleted sequence at the ZFN cut site were: AAVS1-PZ-F1 and AAVS1-PZ-R1 for primary PCR, and AAVS1-PZ-F2 and AAVS1-PZ-R2 for nested PCR. The primer pairs for amplifying a probe corresponding to a deleted sequence 5 kb from the ZFN cut site were: AAVS1-Pd5-F1 and AAVS1-Pd5-R1 for primary PCR, and AAVS1-Pd5-F2 and AAVS1-Pd5-R2 for nested PCR. The primer pairs for amplifying a probe corresponding to a deleted sequence at the 10-kb deletion site were AAVS1-Pd10-F1 and AAVS1-Pd10-R1 for primary PCR, and AAVS1-Pd10-F2 and AAVS1-Pd10-R2 for nested PCR. The primer pairs for amplifying a probe corresponding to an undeleted sequence at the 10-kb deletion site were AAVS1-Pwt10-F1 and AAVS1-Pwt10-R1 for primary PCR, and AAVS1-Pwt10-F2 and AAVS1-Pwt10-R2 for nested PCR. Both 10-kb deletion site probes were repetitive sequences and not suitable for Southern blot hybridization. Primary PCR products were purified by gel extraction and the amplicons were then used for DIG-dUTP PCR labeling with nested primers, using Roche PCR DIG Probe Synthesis kit. DNA hybridization and signal detection was carried out with Roche DIG Easy Hyb and DIG Chemiluminescent Detection System according to manufacturer's instructions.

RSK and ERK activity assays

K562 cells at 2 million cells per well were serum-starved for 4 h. Then cells were preincubated for 1 h with 3 μM fmk, followed by 20 min stimulation with 100 nM phorbol 12-myristate 13-acetate (PMA), when indicated. Then, cells were solubilized for 15 min in 500 μl lysis buffer (0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM Na3VO4, 5 mM EDTA, 50 mM NaF, 10 nM calyculin A, 10 μM leupeptin, 5 μM pepstatin and 1 μg ml^{-1} aprotinin) on ice and manipulated at <4 °C thereafter. Cell extracts were clarified by centrifugation for 5 min at 18,000*g*, and the supernatant was incubated for 90 min with 4 μg of anti-RSK2 (MCA3429Z, ABD Serotec), with the addition of 20 μl protein G–agarose beads (Upstate) during the final 30 min. The beads were then precipitated by centrifugation, washed 5 times with lysis buffer, drained and dissolved in SDS-PAGE sample buffer. Aliquots of the precipitates and pre-immunoprecipitation extracts were subjected to SDS-PAGE and immunoblotting with antibodies detecting the phosphorylated hydrophobic motif (HM) of active RSK2 (9341, Cell Signaling Technology), total RSK2 (MCA3429Z, ABD Serotec), phosphorylated active ERK1/2 (V803A, Promega) or total ERK1 and ERK2 (sc-093G and sc-154G, respectively, Santa Cruz Biotechnologies).

References

1. Urnov FD, et al. Nature. 2005; 435:646–651. [PubMed: 15806097]
2. Moehle EA, et al. Proc. Natl. Acad. Sci. USA. 2007; 104:3055–3060. [PubMed: 17360608]
3. Rouet P, Smih F, Jasin M. Mol. Cell. Biol. 1994; 14:8096–8106. [PubMed: 7969147]
4. Bibikova M, et al. Science. 2003; 300:764. [PubMed: 12730594]
5. Porteus MH, Baltimore D. Science. 2003; 300:763. [PubMed: 12730593]
6. Wang Z, et al. Oligonucleotides. 2008; 18:21–32. [PubMed: 18321160]
7. Radecke S, et al. Mol. Ther. 2010; 18:743–753. [PubMed: 20068556]
8. Elliott B, et al. Mol. Cell. Biol. 1998; 18:93–101. [PubMed: 9418857]
9. Hockemeyer D, et al. Nat. Biotechnol. 2009; 27:851–857. [PubMed: 19680244]
10. DeKelver RC, et al. Genome Res. 2010; 20:1133–1142. [PubMed: 20508142]
11. Cohen MS, et al. Science. 2005; 308:1318–1321. [PubMed: 15919995]
12. Doehn U, et al. Mol. Cell. 2009; 35:511–522. [PubMed: 19716794]
13. Santiago Y, et al. Proc. Natl. Acad. Sci. USA. 2008; 105:5809–5814. [PubMed: 18359850]
14. Lee HJ, Kim E, Kim JS. Genome Res. 2010; 20:81–89. [PubMed: 19952142]
15. Liu PQ, et al. Biotechnol. Bioeng. 2010; 106:97–105. [PubMed: 20047187]
16. Storici F, et al. Proc. Natl. Acad. Sci. USA. 2003; 100:14994–14999. [PubMed: 14630945]
17. Olsen PA, et al. DNA Repair (Amst.). 2009; 8:298–308. [PubMed: 19071233]
18. Aarts M, te Riele H. Nucleic Acids Res. 2010; 38:6956–6967. [PubMed: 20601408]
19. Soldner F, et al. Cell. Jul 14.2011 published online. (doi:10.1016/j.cell.2011.06.019).

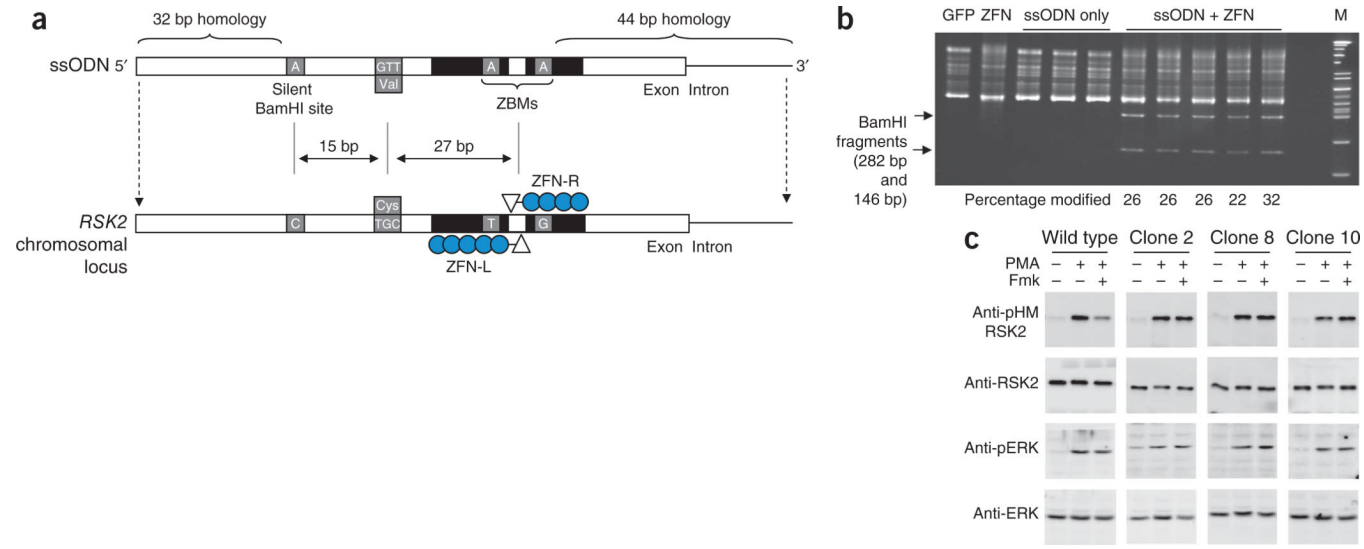

Figure 1.

ssODN design and genome editing at the human *RSK2* locus. (**a**) The schematic shows a 125-mer ssODN (RSK2-125) donor DNA used to incorporate three mutation types into the *RSK2* locus: a silent cytosine to adenine (C to A) mutation to create a silent BamHI site, a codon conversion (TGC to GTT) to create the desired cysteine to valine change in the resulting protein and a ZFN-blocking mutation (ZBM) for each ZFN arm (ZFN-L and ZFN-R). (**b**) Acrylamide gel separation of amplified and BamHI-digested genomic DNA from pooled K562 cells transfected with the indicated constructs or encoding the indicated proteins and collected 2 d after nucleofection. The frequency of BamHI cleavage was quantified by densitometry. Each lane represents pooled cells from an independent transfection event. M, DNA marker (Sigma). (**c**) Immunoblots probing the kinase activity of the RSK2 C436V mutant in K562 cells with wild-type or ssODN-mutated RSK2 C436V (three clones) treated with fmk and phorbol 12-myristate 13-acetate (PMA) to stimulate ERK that activates RSK2 as indicated. Cell extracts were immunoprecipitated with antibodies to RSK2 and the precipitates immunoblotted with antibodies to active phosphorylated hydrophobic motif of RSK2 (anti-pHM RSK2) that detect active RSK2 or to total RSK2 (anti-RSK2). Pre-precipitation cell extracts were immunoblotted with antibodies to phosphoERK (anti-pERK) that detect active ERK or to total ERK (anti-ERK).

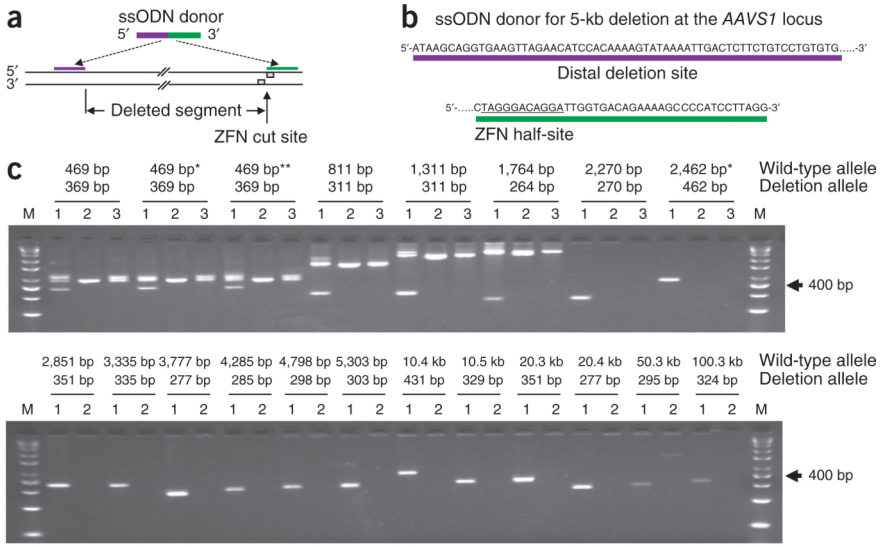

Figure 2.

Deletion of chromosomal segments using ssODNs and ZFNs at the human *AAVS1* locus. (**a**) General ssODN design rules for deletion of chromosomal segments relative to the ZFN cut site. Sequence distal to the ZFN cleavage site (purple) and DNA sequence containing the ZFN half-site farthest from the distal deletion sequence (green) are shown. (**b**) ssODN sequence used to delete 5 kb upstream of the *AAVS1* ZFN cut site. The ZFN binding half-site is underlined. (**c**) Agarose gel separation of amplified genomic DNA from K562 cells transfected with the following constructs 2 d after nucleofection (1, ssODN plus construct encoding ZFN; 2, ssODN only; and 3, construct encoding ZFN only; see **supplementary note 2** for ssODN sequence). The expected fragment sizes of the wild-type and deletion alleles are indicated. PCR fragments greater than 1.5 kb were not detected under the experimental conditions. M, DNA marker (Sigma); *3′ deletion from the ZFN cut site; **5′ and 3′ deletion off the ZFN cut site by transfecting both d5-AAVS1-0.1kb and d3-AAVS1-0.1kb deletion ssODNs; **supplementary note 2**); all other lanes are 5′ deletions from the cut site.