Supplemental Methods

Supplemental Figure 1.

AAVS1 donors were constructed, transfected, and TI assayed as described in the main text.

IL2Rg donors were constructed by cloning of fusion PCR products bounded by the following oligonucleotides: For 750 bp donors, 5'-gcc caa gct cct gtt ctc tgc c-3' and 5'-gcc ctt aaa gac cct gca aaa cc-3'; for 500 bp donors, 5'-acc atg cct ggc taa ttt ttg tat ttt tag-3' and 5'-ttt aat cct cac aac agc tcc tgt gag g-3'; for 250 bp donors, 5'-aga ttc aag tca gtg aag gga gca gtg-3' and 5'-aca aca cgc taa ccc aac cct aca c-3'; for 100 bp donors, cct ctt tct cct caa gga aca atc agt g-3' and gat tgg gtc atg tgg gcc cat-3'. Transfection and assay of TI was done analogously as for *AAVS1* but using XhoI.

Rosa donors were constructed by cloning of PCR products made using the following oligonucleotides: For 527 bp left arms, the oligonucleotides used for PCR were 5'-ggc tcg agt gag tca tca gac ttc taa gat cag g-3'; for 413 bp left-arm donors, 5'-ggc tcg agt ttt gat aag gct gca gaa g-3' in conjunction with the reverse primer 5'-ctg aat tcg aat ggg cgg gag tct tct ggg ca-3'. For 640 bp right arms, the oligonucleotides used for PCR were 5'-cca agc ttg gag gta ggt ggg gtg agg-3'; for 200 bp arms, 5'-cca agc tta gtc gct ctg agt tgt tat c-3'; for 100 bp arms, 5'-cca agc ttt ctg gga gtt ctc tgc tgc c-3' in conjunction with the reverse primer 5'-cat tcg aat tca gaa aga ctg gag ttg cag atc-3'. Individual arm amplicons were joined via fusion PCR and cloned to produce donors with varying homology arms. Rosa ZFNs had the following helices and recognition sequences: SBS 18473, DRSARTR QSGHLSR RSDDLSK RNDHRKN (N to C terminus), recognizing 5'-tgg gcg gga gtc-3'; SBS 18477, QSGDLTR TSGSLTR QSGHLAR QSSDLTR RSDNLSE QNAHRKT (N to C terminus), recognizing 5'-aga aag act gga gtt gca-3'. Neuro2a cells (200,000) were co-transfected with 400 ng each of SBS 18473 and 18477 along with 2 μg of the indicated donor in solution SF using the Amaxa-Shuttle Neuro2a high efficiency protocol. Genomic DNA was harvested 72 hours after transfection and 100 ng used for PCR with 5'-cccagctacagcctcgattt-3', 5'-cacaaatggcgtgttttggt-3' and 5 μCi of both ^{32}P-dATP and ^{32}P-dCTP per sample at an annealing temperature of 68ºC with a two minute extension at 72ºC for 28 cycles. Following G-50 column purification, 10

uL of each 50 uL reaction was digested with EcoRI at 37ºC for two hours and loaded onto a 10% polyacrylamide gel.

Supplemental Figure 2.

Analysis of ZFN activity. For all loci, ZFN activity was analyzed via the CEL-I assay (Supplemental Figure 2). K562 cells contain a polymorphism in the 3' region of the *POU5F1* gene resulting in a strong CEL-I signal in addition to that from the ZFNs.

Locus	Forward CEL-I Primer	Reverse CEL-I Primer	Digestion, bp
AAVS1	ccccttacctctctagtctgtgc	ctcaggttctgggagagggtag	293, 243
GS	gggtggcccgtttcatct	cgtgacaactttcccatatcaca	261, 84
POU5F1, 5'	gatagaacgagattccgtcttggtgg	gaaggagattatggaggagggtgacac	115, 110
POU5F1, 3'	ccaaagtgctggaattataggcgtg	gcagagctttgatgtcctgggact	225, 170
BAK, 5'	catctcacatctggaccacagccg	atgaaagtcccacttgcctg	198, 71
BAK, 3'	ttttctctctgctgtccctc	ctgcgggcaaatagatcac	171, 163

Supplemental Figure 3.

Analysis of integration at off-target sites. Off-target sites for the *AAVS1* ZFNs were screened for integration of the pGK-GFP-pA and oligo donors by PCR. Forward PCR primers are identical to those in Supplementary Figure 8 of Hockemeyer *et al.*, paired with the reverse primer 5'-tcc tcg ccc ttg ctc acc at-3' for the pGK-GFP-pA donor and the reverse primer 5'-atc cgc cga att ctc acc ta-3' for the oligonucleotide donor. It was not possible to assay for off-target insertion of the oligonucleotide donors by Southern blot as their small size precluded efficient hybridization.

For the analysis of off-target integration based on GFP expression (Supplemental Figure 3C), cells were transfected as indicated and GFP expression was assayed 21 days post-transfection. The background fluorescence level from *IL2R*γ ZFN treated cells (0.14%) was subtracted from the data. For the indicated samples, etoposide was added to 1μM post-transfection.

Supplemental Figure Legends

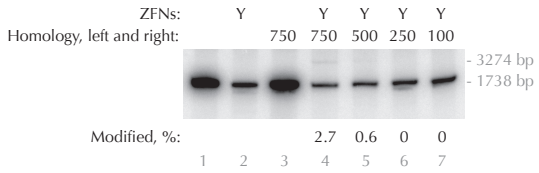
Supplemental Figure 1. The effect of homology arm length on targeted integration of large and small inserts. A) Insertion of the same transgene into the *IL2Rγ* locus. The length of the homology arms was varied as indicated and targeted integration measured by PCR. PCR amplification of the wild-type locus produces a 1738 bp product; integration of the transgene results in a 3274 bp product. B) Insertion of a small HindIII-containing patch into the *AAVS1* locus in 293 cells. The length of the homology arms was varied as indicated and targeted integration measured by PCR and HindIII digestion. Targeted integration produces 1041 and 918 bp HindIII digestion products. C) Insertion of a small XhoI-containing patch into the *IL2Rγ* locus in HeLa cells. The length of the homology arms was varied as indicated and targeted integration measured by PCR and XhoI digestion. Targeted integration produces 873 and 868 bp XhoI digestion products. D) Insertion of a small EcoRI-containing patch into the *Rosa* locus in mouse Neuro2a cells. The length of the homology arms was varied as indicated and targeted integration measured by PCR and EcoRI digestion. Targeted integration produces 890 and 575 bp EcoRI digestion products. For all panels, the percentage of modified chromosomes is shown below the gel in black text.

Supplemental Figure 2. ZFN cleavage during insertion of oligonucleotide donors. A) ZFN action at the *AAVS1* locus was measured for samples in Figure 4b using the CEL-I assay. ZFN action results in 293 and 243 bp CEL-I digestion products. B) ZFN action at the *GS* locus was measured for samples in Figure 4c using the CEL-I assay. ZFN action results in 261 and 84 bp CEL-I digestion products. C) ZFN action of the 5' pairs at the *POU5F1* and *BAK* loci was measured for samples in Figure 5b using the CEL-I assay. ZFN action results in 115 and 110 bp CEL-I digestion products for *POU5F1* and 198 and 71 bp CEL-I digestion products for *BAK*. D) ZFN action of the 3' pairs at the *POU5F1* and *BAK* loci was measured for samples in Figure 5b using the CEL-I assay. ZFN action results in 225 and 170 bp CEL-I digestion products for *POU5F1* and 171 and 163 bp CEL-I digestion products for *BAK*. For all panels, the percentage of modified chromosomes is shown below the gel in black text.

Supplemental Figure 3. Assay of off-target ZFN action. A) Samples treated with ZFNs only, donor only, and ZFNs plus donor were assayed for off-target transgene integration at the top ten potential *AAVS1* off-target sites by PCR specific for the junction of the transgene and each off-target locus. The size of the PCR product expected from off-target integration is shown below each lane. The ZFN plus donor-specific band for locus 7 (lane 21) was sequenced and found to be unrelated to locus 7. B) Samples treated with ZFNs only, donor only, and ZFNs plus donor were assayed for off-target oligonucleotide integration at the top ten potential *AAVS1* off-target sites by PCR specific for the junction of the transgene and each off-target locus. The size of the PCR product expected from off-target integration is shown below each lane. C) Forced mis-integration of a pGK-GFP-pA donor. Cells were transfected with the indicated combinations of ZFNs and donors and GFP expression measured by Guava cell counting.

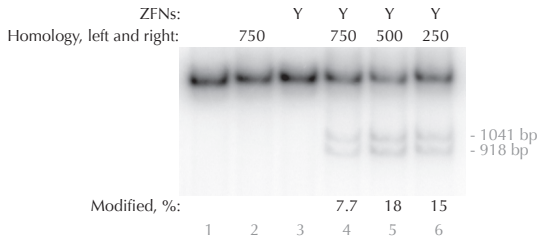
A

Insertion of pGK-GFP-pA into the *IL2Rγ* gene in K562 cells



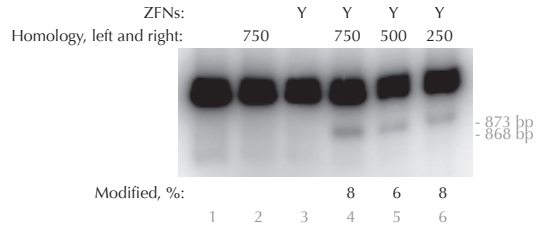
B

Insertion of a HindIII site into *AAVS1* in 293 cells



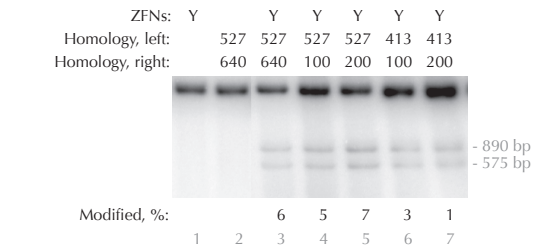
C

Insertion of a XhoI site into *IL2Rγ* in HeLa cells

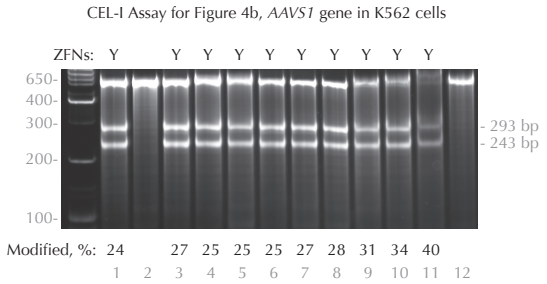


D

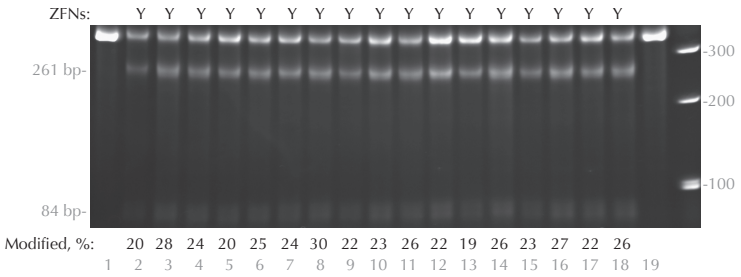
Insertion of a EcoRI site into *Rosa* in Neuro2a cells



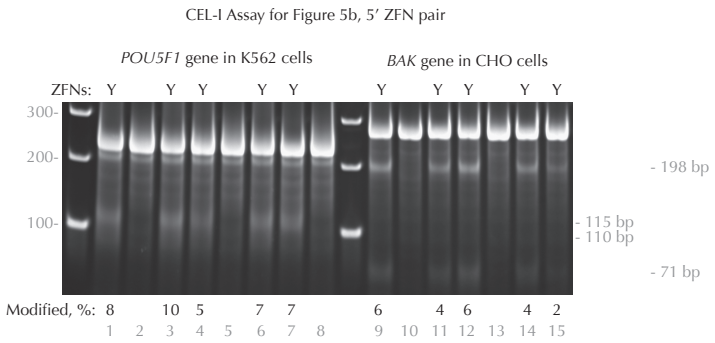
A


CEL-I Assay for Figure 4b, AAVS1 gene in K562 cells
ZFNs: Y Y Y Y Y Y Y Y Y Y
650-
400-
300-
200-
100-
- 293 bp
- 243 bp
Modified, %: 24 27 25 25 25 27 28 31 34 40
1 2 3 4 5 6 7 8 9 10 11 12

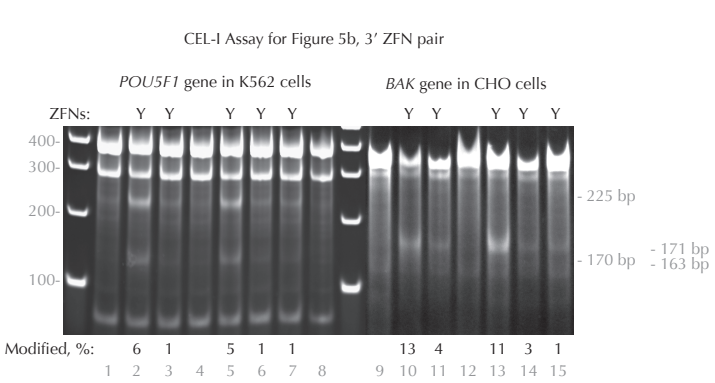
B


CEL-I Assay for Figure 4c, GS gene in CHO cells
ZFNs: Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y
261 bp-
84 bp-
-300
-200
-100
Modified, %: 20 28 24 20 25 24 30 22 23 26 22 19 26 23 27 22 26
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

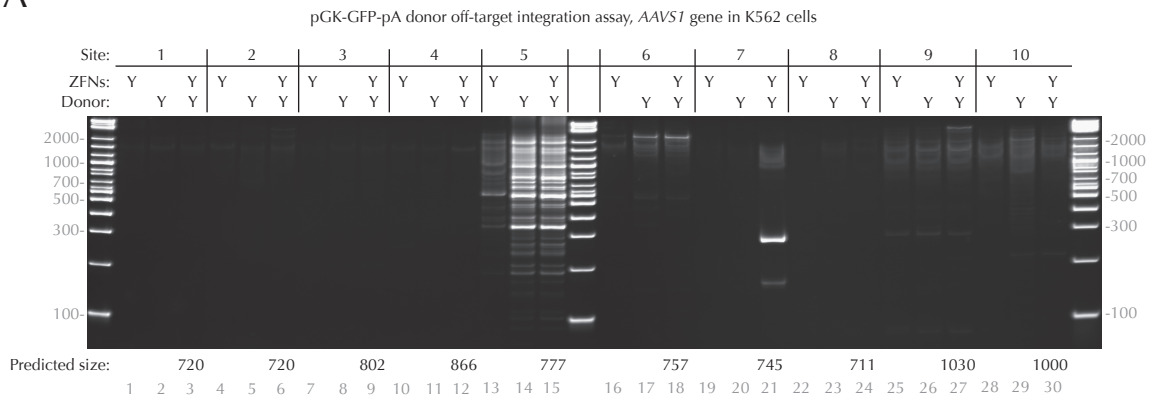
C


CEL-I Assay for Figure 5b, 5′ ZFN pair
POU5F1 gene in K562 cells
BAK gene in CHO cells
ZFNs: Y Y Y Y Y Y Y Y Y Y
300-
200-
100-
- 198 bp
- 115 bp
- 110 bp
- 71 bp
Modified, %: 8 10 5 7 7 6 4 6 4 2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

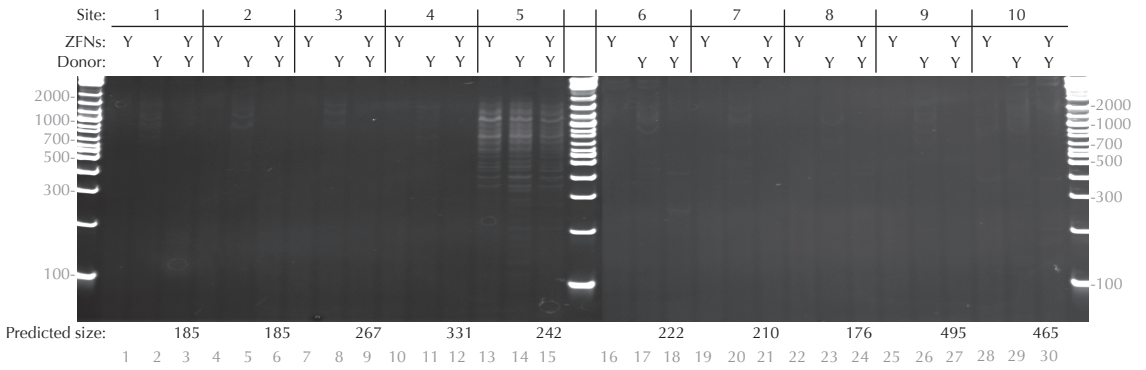
D


CEL-I Assay for Figure 5b, 3′ ZFN pair
POU5F1 gene in K562 cells
BAK gene in CHO cells
ZFNs: Y Y Y Y Y Y Y Y Y Y
400-
300-
200-
100-
- 225 bp
- 170 bp
- 171 bp
- 163 bp
Modified, %: 6 1 5 1 1 13 4 11 3 1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

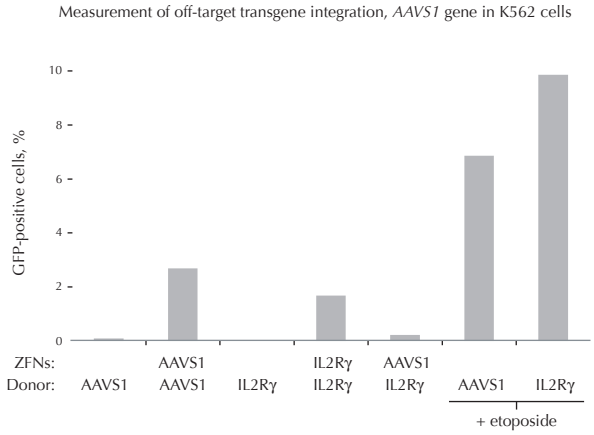
A


pGK-GFP-pA donor off-target integration assay, AAVS1 gene in K562 cells
Site: 1 2 3 4 5 6 7 8 9 10
ZFNs:
Donor:
2000
1000
700
500
300
100
Predicted size: 720 720 802 866 777 757 745 711 1030 1000
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

B


Oligonucleotide donor off-target integration assay, AAVS1 gene in K562 cells
Site: 1 2 3 4 5 6 7 8 9 10
ZFNs:
Donor:
2000
1000
700
500
300
100
Predicted size: 185 185 267 331 242 222 210 176 495 465
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

C


Measurement of off-target transgene integration, AAVS1 gene in K562 cells
GFP-positive cells, %
10
8
6
4
2
0
ZFNs: AAVS1 IL2Rγ AAVS1
Donor: AAVS1 AAVS1 IL2Rγ IL2Rγ IL2Rγ AAVS1 IL2Rγ
+ etoposide