Supplementary information for DeKelver et al., “Functional Genomics, Proteomics, and Regulatory DNA Analysis in Isogenic Settings Using Zinc Finger Nuclease-Driven Transgenesis Into a Safe Harbor Locus in the Human Genome”

Calculation of transgenic haplotype frequency in cell pools enriched for genome-edited chromatids

Even at low (eg, 20-22) PCR cycle numbers, chromatids carrying transgenes at AAVSI will amplify at a reduced efficiency relative to wild-type chromatids. As a result, their frequency will be underestimated. The extent of this amplification bias will differ between loci, and for the same locus, between transgenes. To allow the accurate measurement of such a bias for the experiment where FACS was used to enrich for cells carrying added transgenes (supp. fig. 7), we did the following: a single-cell derived clone biallelic for the transgene of interest was isolated, expanded, and genomic DNA purified. Equal masses of that genomic DNA and that from wild-type K562 cells were mixed, and amplified using body-labelled PCR (see Materials and Methods). The transgenic band signal in this experiment was 17% that of wild-type (EM and RD, data not shown). This provided the “normalization factor” to transition from the observed ratio of transgenic to wild-type chromatids in the FACS-enriched pool to the actual one.

Evaluating Genome-Wide Consequences of ZFN-Driven AAVSI Editing

The goal of our effort is was to establish an approach for transgenesis in isogenic settings for human somatic cell genetics. While the ZFN recognition site is unique in the human genome, it was important to investigate whether ZFN-driven transgene addition to the AAVSI locus is associated with an unacceptably high frequency of undesired effects on the genotype of the target cell.
Three different assays were used to investigate this issue: (i) nucleus-wide measurement of DSB induction in the cell (Miller et al. 2007); (ii) global analysis of donor DNA random integration frequency with and without ZFNs in transformed cells (Moehle et al. 2007); (iii) Southern blotting of single-cell-derived clones. Data from all three assays, shown below, argue that undesired effects, if they do occur, do so at a frequency acceptably low for somatic cell genetic experiments both in transformed and in hES cells.

DSB induction was measured genome-wide using via a hallmark of DSB repair: the assembly of a focus of phosphorylated histone variant H2A.X at the repair site (Paull et al. 2000). Cells were transiently transfected with ZFNs or treated with the DSB-inducing drug etoposide. H2A.X foci that accumulate in these cells were quantitated by immunostaining and FACS-based measurement (Miller et al. 2007). This assay does not measure the absolute number of DSBs per nucleus; instead, it allows a comparison of the AAVS1 ZFNs to those that target the IL2Rγ locus, and characterized earlier for genome-wide editing specificity (Miller et al. 2007; Urnov et al. 2005). The two ZFN pairs showed essentially identical levels of H2A.X staining above the ZFN-untreated samples (supp fig. 3b); in this assay, the AAVS1 ZFNs were 2.5x more active in target locus editing than the IL2Rγ ZFNs (supp fig. 3a, compare lanes 2 and 4). Treatment with etoposide resulted in an increase in H2A.X signal (supp. fig. 3b, right sample).

Next, to determine whether expression of the AAVS1-specific ZFNs would increase the rate of random integration of the donor DNA into the genome, a plasmid donor DNA was used that carries an autonomous expression cassette for a cell surface marker (ΔNGFR) outside the donor homology arms (supp fig 3c). Random integration of this donor plasmid yields ΔNGFR-positive cells; in fact, addition of etoposide – which induces random double-
strand breaks – led to a dose-dependent increase in the number of ΔNGFR-positive cells (supp fig. 3d). No increase in the random donor plasmid integration rate in ZFN- and donor-treated was observed as compared to the level seen in control cells treated with the donor DNA only (supp fig. 3d).

In a separate study (Orlando et al. 2010), we show that gene addition to AAVSI locus can occur using linear DNA fragments carrying short (50-100 bp) homology arms. Of relevance to the issue of potential ZFN-driven donor misintegration, in that study we fail to observe any detectable misintegration of such linear fragments into potential ZFN off-target sites in the genome (Orlando et al. 2010). Here, to address this issue in a different cell system, we made use of donor constructs that carry promotorless selectable markers. These were electroporated into hES cells along with ZFNs, drug-resistant clones selected, and the AAVSI locus genotyped using a probe that would also reveal a donor construct that integrated elsewhere in the genome as well as the AAVSI locus. No misintegration was observed in over 90% of the clones that carried the donor-specified transgene at the AAVSI locus (supp. fig. 8). It is formally possible that some random donor integrations are, in fact, directed by off-target cleavage by the ZFNs, but if that is the case, this is an infrequent event (supp. fig. 8).
Supplementary movie M1

See text and Fig 3 for details. The metaphase-anaphase transition shown in Fig 3 occurs ~22 seconds into the movie.

Supplementary figure 1

“Donor only” sample, Fig 1c, lane 1

Phosphorimager traces of lane 1 (top panel – donor plasmid only) and lane 2 (bottom panel – ZFN expression construct and donor plasmid) from Fig. 1c. The bottom of the autoradiograph is on the right of each image; the major peak is the wild-type chromatid. The edited chromatid is visualized as an additional peak in the lower panel.

“ZFN + donor” sample, Fig. 1c, lane 2
Supplementary figure 2

Upstream chromosome/donor homology arm boundary:

chromosome    donor

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CCCAGGCAGGTCCTGTCTTTCTCTGACCTGC
GGTCCGTCAGGACGAAAGAGACTGGACG
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Chromatograms from single-cell-derived clones:
Supplementary figure 2 (ctd)

Downstream chromosome/donor homology arm boundary:

donor chromosome

TGGCTCTGCTCTTCAGACTGAGCCCCGTTC
ACCGAGACGAGAAGTCTGACTCGGGCAAG

Chromatograms from single-cell-derived clones:

Gene addition to AAVS1 occurs via a homology-directed process. K562 cells were transfected with ZFNs directed against AAVS1 and GFP-carrying donor constructs. GFP-positive single-cell-derived clones were isolated and genotyped at the AAVS1 locus. Three clones that lacked a wild-type-size chromatid (KJP, data not shown) – ie, presumably diallelic for a gene addition event – were chosen at random. The PCR product was cloned without gel purification (ie, to ensure against bias for a product of a particular size) and sequenced with primers that anneal to the vector backbone. In all three cases (see chromatograms above), both the left and right chromosome/donor boundary and the sequence
of the chromosome-resident transgene corresponded to one generated via a homology-based, SDSA-type (Nassif et al. 1994) gene addition event (Moehle et al. 2007).
Experimental measurements of ZFN action specificity (see supplementary discussion for details). A. Surveyor endonuclease measurement of efficiency with which ZFNs that target IL2Rgamma (lane 2) and AAVS1 (lane 4 – here labelled by its RD, data not shown) drive genome editing at their target loci. B. Nucleus-wide measurement of DSB induction, performed using FACS staining for H2AX exactly as described (Miller et al. 2007), in the samples shown in panel “a.” C. Schematic of experiment for determining whether ZFNs increase donor plasmid random integration rates. D. Cells were transfected with indicated plasmids (the donor used is shown in panel C), or transfected with the donor plasmid and treated with etoposide (last three samples), followed by PCR-based measurement showing the ZFNs to be active in editing the AAVS1 locus (RD, data not shown) and FACS-based measurement of percentage of ΔNGFR-positive cells exactly as described (Moehle et al. 2007).
ZFN-driven editing at *AAVS1* in a broad range of transformed cell types (1. A549; 2. DU145; 3. HCT116; 4. HEK293; 5. HeLa; 6. HepG2 7. IMR90; 8. K562; 9. LNCap; 10. MCF7; 11. U-2OS). Homology-directed delivery of a donor DNA specified “patch” (Moehle et al., 2007) was measured using a RFLP knockin assay (Urnov et al. 2005). Positions of wild-type (WT) and edited (TI) chromatids is indicated. Differences in editing frequency may result from those in ZFN expression level between cell types, the epigenetic state of the *AAVS1* locus, as well as in the propensity for resolving a DSB via homology-based pathways (eg refs (Bunz et al. 1998; Mekeel et al. 1997)).
Use of integration-defective lentivirus (IDLV) delivery allows gene addition in hTERT-immortalized human diploid fibroblasts. A. The *AAVS1* locus was genotyped in the indicated samples following transduction with IDLV encoding a donor carrying the GFP ORF (lane 2), or both the ZFNs and the GFP-encoding donor. Positions of transgenic and wild-type (wt) chromatids are indicated. B. Percentage of GFP-positive cells was measured by FACS in the cells genotyped in panel A.
Recruitment of the GR to a chromosomal reporter resident at the PPP1R12C locus is dependent on a functional GRE. Data for a chromatin immunoprecipitation assay performed on isogenic clones (see Fig 4) carrying the indicated reporters at the PPP1R12C locus are shown as “fold increase binding in the presence of dexamethasone relative to cells treated with vehicle only.” Chip experiments were performed as described (Meijsing et al. 2009) using primers targeting the integrated GILZ reporter (GILZjunctionFW: GGGAGGATTTGGGAAGACAATAG and GILZjunctionrev: GGTCATCAAGAACATTCACTGG).
Addition of an shRNA expression cassette to AAVS1 in K562 cells allows its long-term function.

a. Schematic of donor construct used for experiment shown in panel “C” below. The PPP1R12C gene is referred to here by its abridged name, p84.

b. Phenotype (left panel) and genotype at the AAVS1 locus of control cells (lane 1), GFP-positive pool used in Fig 2 (lane 2), and GFP-positive pool used in this experiment (lane 3). PCR of the transgenic chromatid is significantly less efficient than that of the wild-type, and we ran control experiments with defined ratios of wild-type and transgenic DNA to measure the normalization factor in this assay (RD, data not shown). Adjusted for that difference in amplification efficiency, the frequency of transgenic chromatids in both pools is ~80%.

c. Fraction of CD58-positive cells in each indicated sample was measured by FACS.
Supplementary figure 8

[Diagram showing the AAVS1 locus and modified AAVS1 locus with EcoRV, SphI, and Puro markers.]

3' probe external cut

5' probe internal cut

[continued on next page]
Supplementary figure 8 (ctd)

Southern blotting of single-cell-derived hESC clones carrying shRNA expression cassettes at the AAVS1 locus. This dataset complements that shown in Fig 5, panels d-f. The probes used in Southern blotting are shown in the top half of each panel.
Supplementary figure 9

“All-in-one,” single-plasmid system yields comparable ORF addition frequency to that observed with two separate plasmids. The schematic on the left shows the arrangement of DNA constructs, and the data on the right represent a comparison of the efficiency with which gene addition to AAWSI occurs with a two-plasmid or a one-plasmid (lane 3) system in K562 cells. PhosphorImager traces of lanes 1-3 are shown to the right of the autoradiograph, and the targeted integration frequencies are shown below each lane.
References for supplementary information


