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Supplemental Information

Efficient Precision Genome Editing in iPSCs via Genetic Co-targeting with Selection

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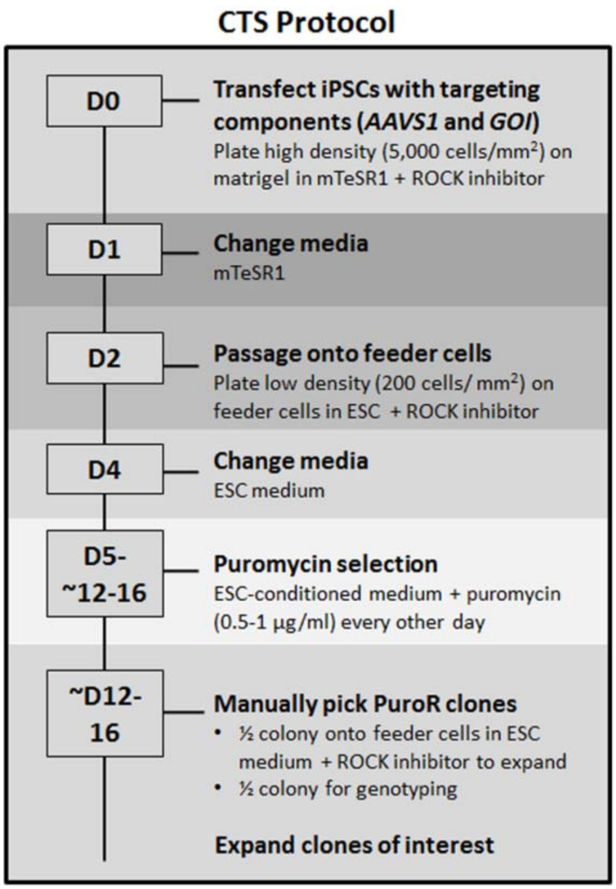
Supplemental Information

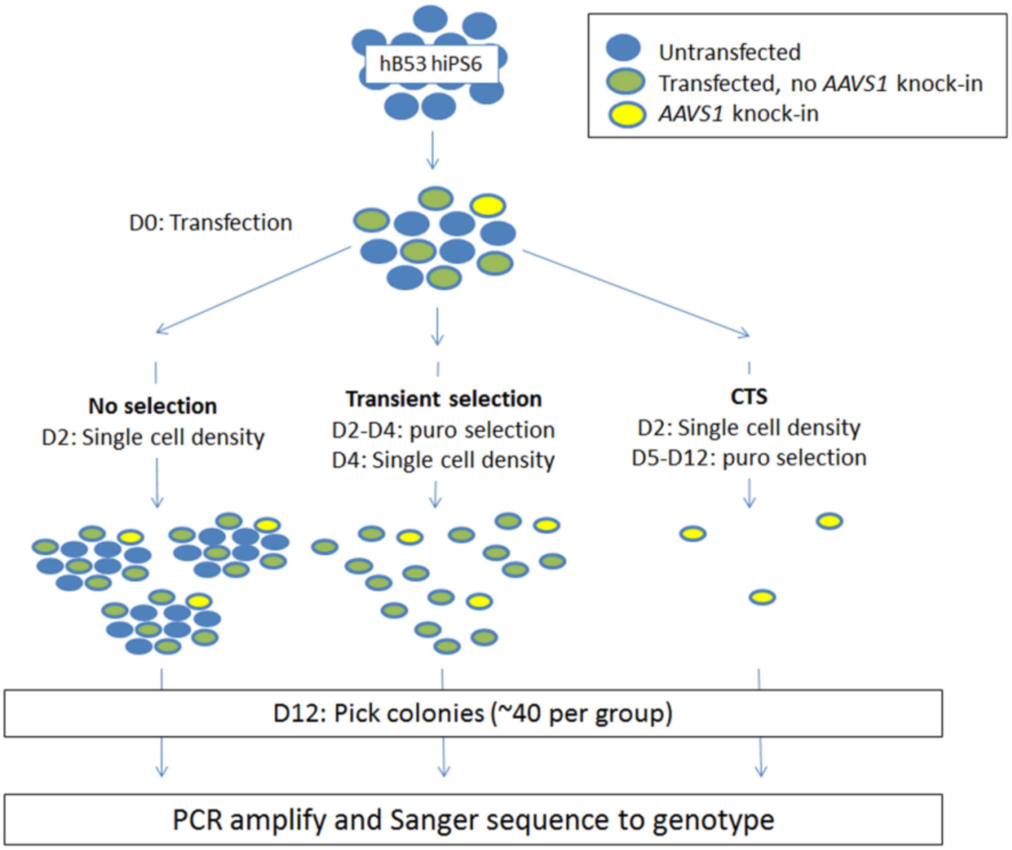
Supplemental Figures

Figure S1: Timeline for CTS protocol. Related to Figure 1.



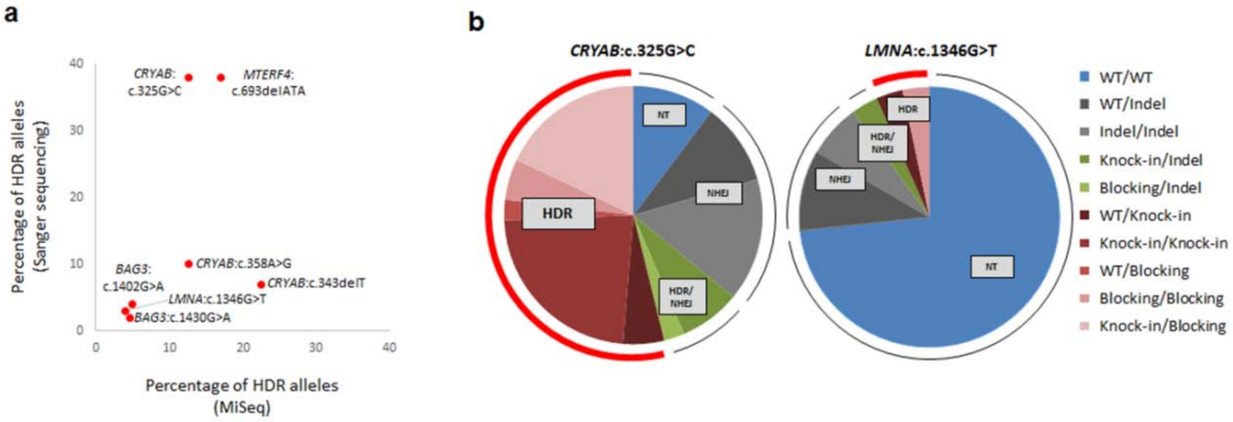
iPSCs are transfected with targeting components shown in Figure 1a on day 0 (D0) and plated at high density on matrigel to promote survival in mTeSR1 medium supplemented with ROCK inhibitor. On D1, media is changed to fresh mTeSR1 and dead cells are washed away. Cells are passaged on D2 to mitomycin-C-treated SNL feeders cells at low density. Puromycin selection (0.5 μg/ml) is performed D5-picking time (~D12-16) in order to limit selection based on transient expression of puromycin resistance and promote selection for incorporation of the puromycin cassette into the *AAVS1* locus. Selection is continued until picking to ensure all cells picked are puromycin resistant. Colonies are manually picked ~D12-16, with half of the colony utilized for genotyping while the other half is re-plated for expansion.

Figure S2: Experimental design comparing selection methods. Related to Figure 1.



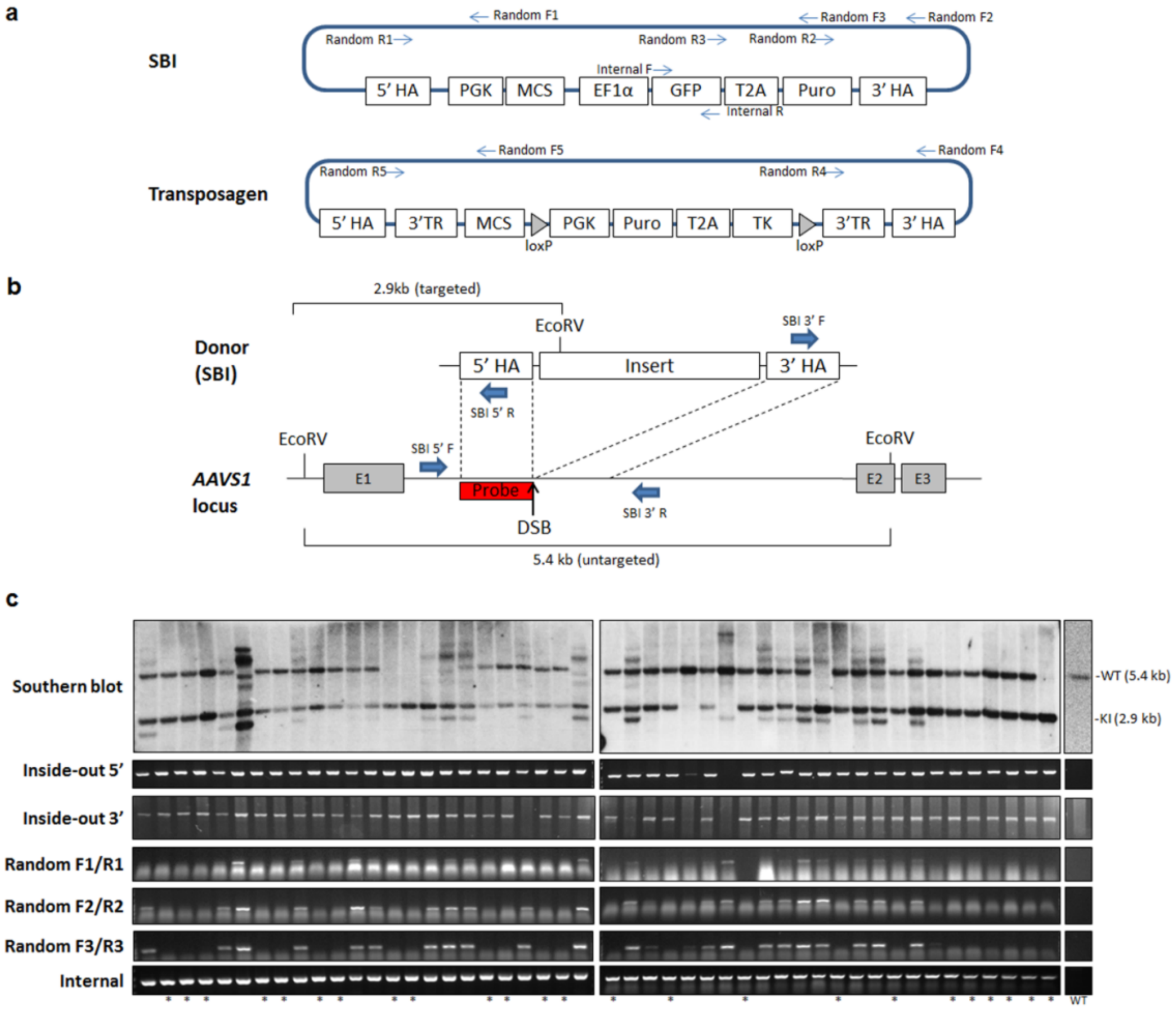
Experimental design employed for comparing selection strategies (no selection, transient selection, and CTS) used for generating data shown in Table S1 and Figure 1c.

Figure S3: Concordance between deep-sequencing pooled samples and direct sequencing clonal samples. Related to Figure 2-3 and Table S2-S3.



(a) Correlation of allelic HDR frequency between deep-sequencing (MiSeq) of pooled populations and Sanger sequencing of clonal populations. (b) Representative examples of high (CRYAB:c.325G>C) and low (LMNA:c.1346G>T) efficiency loci showing genotypes derived from clonally expanded populations. ‘Blocking’ indicates knock-in of only the silent, Cas9-blocking mutation (i.e., without the variant of interest), whereas ‘Knock-in’ indicates incorporation of both the silent mutation and variant of interest. ‘WT’ indicates unmodified alleles. Genotypes are categorized as not-targeted (NT), NHEJ, HDR and NHEJ (HDR/NHEJ), or HDR.

Figure S4: Knock-in and random integration at *AAVS1*. Related to Figures 1 and 3.
(a) Schematics of commercially available targeting vectors used in experiments from System Biosciences (SBI) and Transposagen. Abbreviations: HA, homology arm; PGK, phosphoglycerate kinase promoter; MCS, multiple cloning site; EF1α, elongation factor 1 α promoter; GFP, green fluorescent protein; T2A, self-cleaving peptide sequence; Puro, puromycin resistance gene (*pac*); TR, *piggyBac* terminal repeats; TK, thymidine kinase. Relative locations of PCR Primers used for random integration and internal control primers are shown. (b) Schematic representation of HDR-mediated integration at the *AAVS1* locus. Relative positions of Southern blotting probe (red block), double strand break (DSB), exons (E), EcoRV cut sites, and inside-out PCR primers (SBI 5' F/R and SBI 3' F/R) are shown. Expected sizes for targeted (2.9 kb) and untargeted (5.4 kb) fragments by Southern blot are shown. (c) Forty-eight clones derived from transfection of the AAVS1 donor vector and TALEN constructs following the CTS protocol (Figure S1) were screened by Southern blotting and a PCR-based integration assay for knock-in at *AAVS1*. For Southern blotting, genomic DNA was digested with EcoRV and hybridized with a ^{32}P-labeled probe recognizing the 5' homology arm (shown in b). A 5.4 kb untargeted or wildtype (WT) band and a 2.9 kb targeted or knock-in (KI) band is observed. Additional bands observed are predicted to be random integration events of the donor vector elsewhere in the genome. PCR was performed using inside-out primers (demonstrating targeted integration), random primers (specific for the vector backbone), and internal primers (integration control) (primer sequences and fragment sizes are shown in Supplemental Experimental Procedures) and run on agarose gels. Clones with targeted knock-in (either heterozygous or homozygous) and without additional random integration events are indicated with asterisk (*). Genomic DNA used in the final column was from untargeted (WT) cells as a control.



Supplemental Tables

Table S1: Genotypes of clones analyzed comparing different selection strategies. Related to Figure 1.

Selection	None	Transient	CTS
WT/WT	39	43	4
WT/Indel	3	1	4
Indel/Indel	1	1	6
Knock-in/Indel	1	2	3
Blocking/Indel	0	0	1
WT/Knock-in	0	0	2
Knock-in/Knock-in#	0	1	9
WT/Blocking	1	0	1
Blocking/Blocking#	0	0	2
Knock-in/Blocking	1	0	7
Total	46	48	39

Genotypes of clones analyzed by direct Sanger sequencing of PCR products (presented in Figure 1c). ‘Blocking’ indicates knock-in of only the silent, Cas9-blocking mutation (i.e., without the variant of interest), whereas ‘Knock-in’ indicates incorporation of both the silent mutation and variant of interest. ‘WT’ indicates unmodified alleles. CTS column from this table is the same data shown in Table S3.

#Though these clones appear to be homozygous through Sanger sequencing (i.e. ‘clean’ sequence), a Southern blot would be required prior to phenotyping to ensure there is not a large deletion on one allele that prevents PCR amplification.

Table S2: MiSeq experimental results. Related to Figure 2.

hB53 hiPS6	**Total # reads**	**Total # edits**	**HDR**		**NHEJ**		**WT**	
Sample			**# reads**	**% of total**	**# reads**	**% of total**	**# reads**	**% of total**
CRYAB:c.343delT_CTS-	262144	152720	**2458**	***1***	150262	*57*	109424	*42*
CRYAB:c.343delT_CTS+	134752	134752	**30210**	***22***	86683	*64*	17859	*13*
CRYAB:c.325G>C_CTS-	264167	168170	**2967**	***1***	165437	*63*	95997	*36*
CRYAB:c.325G>C_CTS+	135680	123981	**17227**	***13***	106754	*79*	11699	*9*
CRYAB:c.358A>G_CTS-	233189	129405	**2568**	***1***	126837	*54*	103784	*45*
CRYAB:c.358A>G_CTS+	299329	282460	**37946**	***13***	244515	*82*	16869	*6*
BAG3:c.1430G>A_CTS-	155400	13104	**439**	***0***	12665	*8*	142296	*92*
BAG3:c.1430G>A_CTS+	183997	61279	**8526**	***5***	52753	*29*	122718	*67*
BAG3:c.1402G>A_CTS-	180651	14651	**108**	***0***	14543	*8*	166000	*92*
BAG3:c.1402G>A_CTS+	160393	50311	**8042**	***5***	42269	*26*	110082	*69*
LMNA:c.1346G>T_CTS-	207467	9211	**20**	***0***	9191	*4*	198256	*96*
LMNA:c.1346G>T_CTS+	202658	25455	**2410**	***1***	23045	*11*	177203	*87*
MTERF4:c.693delATA_CTS-	188846	9255	**353**	***0***	8902	*5*	179591	*95*
MTERF4:c.693delATA_CTS+	174497	90735	**29916**	***17***	60819	*35*	83762	*48*
Average_CTS-	**213123**	**70931**	**1273**	**0.4**	**69691**	**28.4**	**142193**	**71.1**
Average_CTS+	**184472**	**109853**	**19182**	**10.9**	**88120**	**46.6**	**77170**	**42.7**

hB119 hiPS9	**Total # reads**	**Total # edits**	**HDR**		**NHEJ**		**WT**	
Sample			**# reads**	**% of total**	**# reads**	**% of total**	**# reads**	**% of total**
CRYAB:c.343delT_CTS-	121572	23748	**3108**	***3***	20640	*17*	97824	*80*
CRYAB:c.343delT_CTS+	110499	108887	**27905**	***25***	80982	*73*	1612	*1*
CRYAB:c.325G>C_CTS-	118811	69039	**774**	***1***	68265	*57*	49772	*42*
CRYAB:c.325G>C_CTS+	109238	75900	**9260**	***8***	66640	*61*	33338	*31*
CRYAB:c.358A>G_CTS-	100932	16806	**2447**	***2***	14359	*14*	84126	*83*
CRYAB:c.358A>G_CTS+	77869	75359	**41505**	***53***	33854	*43*	2510	*3*
BAG3:c.1430G>A_CTS-	91759	7878	**139**	***<1***	7739	*8*	83881	*91*
BAG3:c.1430G>A_CTS+	98513	21496	**10510**	***11***	10986	*11*	77017	*78*
BAG3:c.1402G>A_CTS-	95556	6920	**37**	***<1***	6883	*7*	88636	*93*
BAG3:c.1402G>A_CTS+	207563	45135	**11727**	***6***	33408	*16*	162428	*78*
LMNA:c.1346G>T_CTS-	112816	5463	**48**	***0***	5415	*5*	107623	*95*
LMNA:c.1346G>T_CTS+	220533	42597	8521	***4***	34436	*16*	177576	*81*
MTERF4:c.693delATA_CTS-	130614	9997	**1087**	***1***	8910	*7*	120617	*92*
MTERF4:c.693delATA_CTS+	93752	11555	**8111**	***9***	3444	*4*	82197	*88*
Average_CTS-	**110294**	**19979**	**1091**	**1.4**	**18887**	**16.4**	**90354**	**82.3**
Average_CTS+	**131138**	**54418**	**16791**	**16.6**	**37679**	**32.0**	**76668**	**51.4**

MiSeq experimental results for each cell line (hB53 hiPS6- top and hB119 hiPS9- bottom) including total number of reads, total number of edits, number of reads with HDR, percent of reads with HDR, number of reads with NHEJ, percent of reads with NHEJ, number of WT (unmodified) reads, and percent WT reads. Data for each variant with (CTS+) and without (CTS-) CTS are shown in individual rows with the final two rows showing average values including all variants. Each row indicates a single editing experiment.

Table S3: Information regarding generation of knock-in cell lines. Related to Figure 2-4.

Variant Information	Gene	*Crystallin, Alpha B or HSPB5 (CRYAB)*			*BCL2-Associated Athanogene 3 (BAG3)*		*Lamin A/C (LMNA)*	*Mitochondrial Transcription Termination Factor 4 (MTERF4)*
	Location	11q23.1			10q25.2-q26.2		1q22	2q37.3
	Mutation	c.343delT	c.325G>C	c.358A>G	c.1430G>A	c.1402G>A	c.1346G>T	c.[693delATA];[787C>T] *
	Protein change	p.S115Pfs*14	p.D109H	p.R120G	p.R477H	p.V468M	p.G449V	p.[E231D,Y232del];[Q263*]
	Disease	skeletal myopathy	cardiomyopathy, skeletal myopathy, cataracts	cardiomyopathy, skeletal myopathy, cataracts	dilated cardiomyopathy	dilated cardiomyopathy	muscular atrophy	hypertrophic cardiomyopathy
	Reference	(Forrest et al., 2011)	(Sacconi et al., 2012)	(Vicart et al., 1998)	(Norton et al., 2011)	(Villard et al., 2011)	(Dialynas et al., 2012)	Unpublished data
Targeting Information	AAVS1 Targeting Vector	Transposagen	SBI	Transposagen	SBI	SBI	Transposagen	SBI
	sgRNA/ssODN Orientation	R+	R+	R+	R-	R-	R+	R-
	Cell Line	hB53 hiPS6	hB53 hiPS6	hB53 hiPS6	hB53 hiPS6	hB53 hiPS6	hB119 hiPS9	hB53 hiPS6
Genotype	Unmodified	9	4	13	61	75	22	13
	WT/Indel	0	4	0	2	7	3	0
	Indel/Indel	4	6	5	0	2	2	2
	Knock-in/indel	0	3	0	1	1	1	4
	Blocking/Indel	0	1	0	0	1	0	0
	WT/Knock-in	0	2	0	1	3	1	0
	Knock-in/Knock-in#	1	9	2	0	1	0	11
	WT/Blocking	0	1	0	0	0	0	0
	Blocking/Blocking#	0	2	0	0	0	1	0
	Knock-in/Blocking	0	7	0	0	1	0	0
	Total	14	39	20	65	91	30	30

Variants of interest knocked-in to iPSCs using CTS. Gene name, mutation, corresponding protein change, disease, and references are given for each variant. The *AAVS1* targeting vector utilized in each case is shown (see Experimental Procedures). The sgRNA/ssODN orientation is listed as either R+ or R-, indicating agreement or disagreement with the Richardson et al. model for ssODN strand design (Richardson et al., 2016). The cell line utilized to generate each variant is also listed (hB53 hiPS6 or hB119 hiPS9). The numbers of clones isolated with each genotype are indicated below each variant and are derived from one experiment per variant. *The patient variant *MTERF4*:c.[693delATA];[787C>T] was generated by an ssODN incorporating c.693delATA and screening for an indel on the second allele to mimic c.787C>T, which generates a stop codon. #Though these clones appear to be homozygous through Sanger sequencing (i.e. ‘clean’ sequence), a Southern blot would be required prior to phenotyping to ensure there is not a large deletion on one allele that prevents PCR amplification.

Supplemental Experimental Procedures

CRISPR target site design and plasmid construction

CRISPR target sites proximal (within 35 bps) to the SNP of interest were identified using ZiFiT Targeter Version 4.2. Target sites as unique as possible – based on dissimilarity to other genomic loci - were selected and are shown in the table below. Typically, sites were chosen that had zero or one 'off by 0' or 'off by 1' matches elsewhere in the genome. pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230). Reverse complementary oligonucleotide pairs with BbsI overhangs were purchased from Sigma (or Life Technologies) and hybridized, and cloned into the pX330 vector as described previously (Cong et al., 2013).

Locus	**Guide RNA complementarity regions (5'-3')**	**Cleavage efficiency (%)**
CRYAB	GGGATCCGGTATTTCCTG ()	5.4
BAG3	GGGACGAGCCGATGTGCGTC	8.8
LMNA	CATTGGACTTGTTGCGCAGC	13.3
MTERF4	ACTTGTATTCCAGTTGACCC	4.8

Culture and transfection of HEK293T cells and Cel-1 surveyor assay

HEK293T cells, maintained in Dulbecco's modified Eagle medium (DMEM) with high glucose, sodium pyruvate and L-glutamine (Life Technologies) supplemented with 10% fetal bovine serum (FBS- Life Technologies) and 100 u penicillin/100ug streptomycin/ml media (P/S- Life Technologies), were passaged using 0.05% Trypsin (Life Technologies) for transfection. For validating cleavage efficiencies of designed CRISPR guides, HEK 293T cells were mixed with SF nucleofection solution (Lonza) and various pX330 plasmids and transfected with the 4D Nucleofector™ (Lonza) using program CM-130. Cells were harvested 48 hours later and CRISPR activity was validated using the Cel-1 Surveyor assay (cutting efficiencies displayed in last column of the above table) as previously described (Geurts et al., 2009; Miller et al., 2007).

ssODN design

We typically designed ssODNs to flank the variant and/or cut-site by approximately 60bp on either side. In keeping with previous reports (Chen et al., 2011; Long et al., 2014; Paquet et al., 2016; Ponce de León et al., 2014), silent mutations were incorporated into the ssODNs to prevent re-cutting by Cas9 following HDR. This was achieved either through disruption of the PAM sequence or multiple disruptions within the target sequence. ssODNs utilized in experiments are shown in the table below.

Variant	**ssODN Sequence (5'-3')**
CRYAB: c.343delT	TGTTCTTATCTCTCTGCCTCTTTCCTCATTCTTTTGGGTTAGGATGAACATGGTTTCATCCCAGGGAGTTCAACAGGAAATACCGGATCCCAGCTGATGTAGACCCTCTCACCATTACTTCATCCCTGTCATCTGA
CRYAB: c.325G>C	GCAGGTGATAATAGTTCCTGTTCTTATCTCTCTGCCTCTTTCCTCATTCTTTTGGGTTAGCATGAACATGGTTTCATCTCCAGGGAGTTTCACAGGAAATACCGGATCCCAGCTGATGTAGACCCTCTCACCATTACTTCATCCCTGTCATCTGA
CRYAB: c.358A>G	GCCTCTTTCCTCATTCTTTTGGGTTAGGATGAACATGGTTTCATCTCCAGGGAGTTTCACGGGAAATACCGGATCCCAGCTGATGTAGACCCTCTCACCATTACTTCATCCCTGTCATCTGA
BAG3: c.1430G>A	CCTGATGATCGAAGAGTATTTGACCAAAGAGCTGCTGGCCCTGGATTCAGTGGACCCCGAGGGGCGGGCAGACGTCCATCAGGCCAGGAGAGACGGTGTCAGGAAGGTTCAGACCATCTTGGAAAAACTTGAACAGAAAG
BAG3: c.1402G>A	CCTGATGATCGAAGAGTATTTGACCAAAGAGCTGCTGGCCCTGGATTCAATGGACCCCGAGGGGCGGGCAGACGTCCGGCAGGCCAGGAGAGACGGTGTCAGGAAGGTTCAGACCATCTTGGAAAAACTTGAACAGAAAG
LMNA: c.1346G>T	ACGCACTAGCGGGCGCGTGGCCGTGGAGGAGGTGGATGAGGAGGGCAAGTTTGTACGGCTGCGCAACAAGTCCAATGAGgtaggctcctgctcagggtctaaggggatacagctgcatca
MTERF4: c.693delATA	ccagtcattctcacctgaaactacgctaatcacgctatcagtcattctcacCTGAAACTTGTCTAGCTGCCCCAGGTCCTCTCGAAGAACAGAGGGGCAACTGTGCAAAATCTTGGTGACTTGCTGTACCGTGAAAAGGCA

AAVS1 targeting plasmids

The *AAVS1* Safe Harbor TALE-Nuclease kit was purchased from System Biosciences (SBI), including TALENs previously shown to have minimal off-target cleavage (Hockemeyer et al., 2011). Plasmids include the HDR donor vector (Figure S4a), p*AAVS1* Dual Promoter Donor Vector (GE602A-1) containing GFP-Puromycin resistance cassette driven by an *EF1α* promoter, and the TALE-Nuclease Vectors, pZT-*AAVS1* L1 TALE-N Vector (GE601A-1) and pZT-*AAVS1* R1 TALE-N Vector (GE601A-1). These were used for all Illumina MiSeq experiments (except hB119 hiPS9 *LMNA*:c.1346 G>T), and for clonal knockin of *BAG3*:c.1430G>A, *BAG3:*c.1402G>A, *MTERF4*:c.693delATA, and *CRYAB*:c.325G>C. A second *AAVS1* Safe-harbor kit was purchased from Transposagen (Puro-TK with XTN™ TALEN, Catalog # KSH-004) with the donor vector that includes a puromycin resistance gene and thymidine kinase selection cassette driven by a *PGK* promoter and flanked by *piggyBac* repeats (Figure S4a), which can be sequentially, seamlessly removed with excision by *piggyBac* transposase if desired, as well as the accompanying *AAVS1*-specific XTN Forward and Reverse TALEN nucleases. The Transposagen system was used for generating *CRYAB*:c.358A>G and *CRYAB*:c.343delT clones. Additionally, we designed and validated a sgRNA targeting the *AAVS1* locus (guide RNA complementarity region (5'-3') GTCACCAATCCTGTCCCTAG) cloned into pX330 as described above. This pX330-*AAVS1* was used in concert with the *AAVS1* donor vector from Transposagen for generating *LMNA*:c.1346G>T clones and for Illumina MiSeq analysis of hB119 hiPS9 *LMNA*:c.1346G>T.

iPSC lines

All human subject research was approved by the Medical College of Wisconsin and University of Utah Institutional Review Boards. The human iPSC lines used in this study are hB53 hiPS6 (Riedel et al., 2014), derived from a 25-year-old Caucasian male and hB119 hiPS9, derived from peripheral blood mononuclear cells of a healthy 34-year-old Caucasian male using a polycistronic lentivirus containing *OCT4*, *KLF4*, *SOX2*, and *c-MYC* as previously described (Riedel et al., 2014). Informed consent was obtained for this procedure. hB53 hiPS6 was used for generating cell lines with the following knock-in mutations: *BAG3*:c.1402G>A, *BAG3*:c.1430G>A, *MTERF4*:c.693delATA, *CRYAB*:c.358A>G, *CRYAB*:c.325G>C and *CRYAB*:c.343delT, as well as the dual targeting experiment. hB119 hiPS9 was used for generating the cell line containing *LMNA*:c.1346G>T. We successfully applied our CTS method to two other iPSC lines (data not shown): knocking-in *CRYAB*:c.358A>G and *CRYAB:*c.325G>C mutations into hB119 hips10 (an alternate iPSC line derived from the same individual as hB119 hiPS9 using the same method, unpublished data) and reverting homozygous *CRYAB:*c.343delT to homozygous wildtype in a female iPSC line derived from the patient (Forrest et al., 2011), which was reprogrammed using retrovirus (Mitzelfelt et al., 2016).

iPSC culture

Prior to transfection, iPSCs were cultured as previously described (Mitzelfelt et al., 2016) in feeder-free conditions on Matrigel (Corning)-coated 6-well plates with mTeSR1 (Stem Cell Technologies) or StemMACS iPS-Brew XF (Miltenyi Biotec). Cells were passaged every 3-4 days using Accutase (Life Technologies) and seeded in media containing 10μM Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor (Y-27632, Selleck) for 24 hours following passaging.

iPSC Transfection

<u>CTS for generation of knock-in iPSC lines</u>– iPSCs were pretreated for 3-4 hours with 10μM ROCK inhibitor, washed once with Dulbecco's phosphate buffered saline (DPBS- Life Technologies), and incubated with Accutase (Life Technologies) for 5-8 minutes. Wash medium (Knockout DMEM/F12 supplemented with 10% FBS- both from Life Technologies) was added and cells were pipette vigorously to generate a single cell solution and counted using a Countess Automated Cell Counter (Life Technologies). For each transfection (*day 0*), 1μg of the gene-specific pX330 CRISPR/Cas9 plasmid, 2μl of a 40μM stock solution or 1.5μl of a 20μM stock solution of the relevant ssODN, 1μg of each of the two *AAVS1*-specific TALEN plasmids (or 1μg of the *AAVS1*-specific pX330 CRISPR plasmid) and 1μg *AAVS1* donor plasmid (see Targeting Reagents above) were added to 100μl P4 solution (Lonza) and electroporated using program CB-150 on a 4D Nucleofector™ into iPSCs ($1x10^6$ cells/transfection). Cells from each transfection were then seeded into one well of Matrigel-coated 24-well plate (5,000 cells/mm^2) for recovery in mTeSR1 or StemMACS iPS-Brew XF supplemented with 10μM ROCK inhibitor. The following day (*day 1*), cells were washed once with DPBS to remove dead cells and media was changed to mTeSR1 or StemMACS iPSC-Brew XF. Two days post-transfection (*day 2*), iPSCs were dispersed using Accutase and distributed across a 6-well plate pre-seeded with Mitomycin C (SantaCruz) -treated SNL feeder cells (Cell Biolabs) in ESC medium, composed of Knockout DMEM (Life Technologies) supplemented with 20% Knockout Serum Replacement (Life Technologies), MEM-NEAA (Life Technologies), 2mM L-glutamine (Life Technologies), P/S, 0.1mM β-mercaptoethanol (Sigma), 10ng/ml human basic fibroblast growth factor (bFGF, Cell Signaling), and 50ng/ml L-ascorbic acid (Sigma), supplemented with 10μM ROCK inhibitor. Media was changed two days later

(*day 4*) to ESC medium minus ROCK inhibitor. Three days post-seeding (*day 5*), puromycin (0.5-1µg/ml)-supplemented ESC-conditioned media (ESC media conditioned on SNL feeder cells with bFGF and vitamin C added post-conditioning) was added and replaced thereafter every 2 days until picking time (~7-10 days). Waiting until *day* 5 to begin puromycin selection limits the extent of selection for transient expression of puromycin resistance and selecting until picking ensures that all colonies picked have integration of the *pac* cassette. Following ~7-10 days of maintenance in puromycin-containing media (*day 12-16*), distinct colonies (~1mm diameter) were apparent and manually/mechanically transferred each to a single well of a 24-well plate pre-seeded with feeder cells in ESC media plus ROCK inhibitor. Half of each isolate was retained for expansion and half for DNA isolation to genotype. Following genotyping (see below), desired clones were passaged to single wells of 12-well matrigel-coated dishes in mTeSR1 plus ROCK inhibitor and further expanded for pluripotency immunocytochemistry and karyotyping (see below) and frozen for future culture in freezing medium composed of FBS plus 10% dimethyl sulfoxide (DMSO- Sigma). Isolated knock-in iPSC lines were frequently subcloned to ensure homogeneity of the population.

Transfection and CTS for Illumina MiSeq experiments – With the aim to assess the effect of our CTS regimen on editing outcomes, we carried out next-generation sequencing using the Illumina MiSeq platform via a pooled amplicon strategy, including 7 different variants of interest (Table S3) across 4 different genes (*CRYAB*, *BAG3*, *LMNA* and *MTERF4*,) in two different cell lines (hB53 hiPS6 and hB119 hiPS9). Samples were prepared as described above except, two days post-transfection (*day 2*), iPSCs were dispersed such that 10,000 iPSCs were allocated across 3 wells (*Puro*$^-$) and the remainder across the other 3 wells (*Puro*$^+$) of a 6-well plate with corresponding *Puro*$^-$ and *Puro*$^+$ groups derived from the same transfection. Media was changed every other day for 1 week (*day 5-12*) with or without puromycin, accordingly. Following 1 week maintenance (*day 12*), all three *Puro*$^+$ wells and all three *Puro*$^-$ wells were collected and combined separately. In order to deplete the feeder cell sub-population, cells were reseeded in one Matrigel-coated 6-well plate wells in mTeSR1 plus ROCK inhibitor. At confluence, cells were again dispersed, combined and pelleted for isolation of genomic DNA and library preparation for Illumina MiSeq analysis (see below).

Genotyping PCR and Sanger sequencing for clones

To isolate genomic DNA from clones, 30µl Quick Extract Solution (Epicentre) was added to each cell pellet (half colony) and incubated for 15 minutes at 65°C, followed by 5 minutes at 95°C. PCR was carried out using gene-specific primers (see below table) and the resulting amplicons were PCR-purified using a PureLink Quick PCR Purification Kit (Life Technologies) and Sanger sequencing was performed by Retrogen (San Diego, CA) with the same primers used for amplification. Sequences were analyzed using Sequencher software.

Primer Name	**Primer Sequence (5'-3')**
CRYAB_F	AGACAGTTATCTGTTGCTGAATGATATT
CRYAB_R	GGCAATTTCATCTTAGCTGCAA
BAG3_F	TGAAAGTGGAAGCCATCCTG
BAG3_R	GGCTGATCTGCTTCAAGGTT
LMNA_F	CCCCACTTGGTCTCCCTC
LMNA_R	GGCTCACCCTGGTCCACC
MTERF4_F	TTATATGCTTGCCTTTTTTGAA
MTERF4_R	GTCCGAGGCTTCTTATCCATAT

Illumina MiSeq library preparation

Genomic DNA was isolated from cell pellets using a PureLink Genomic DNA Mini Kit (Life Technologies). Samples were prepared for analysis with Illumina MiSeq as previously described (Kistler et al., 2015). PCR 1 primers are listed in the below table with adapter sequences in red and green text for the forward and reverse primers, respectively. PCR2 was performed using the Nextera XT Index Kit (#15055293) from Illumina according to manufacturer's instructions. Individual amplicons were quantified via qPCR (KAPA Biosystems) and pooled at 3nM concentrations. To ensure high sequencing quality, following pooling, final amplicon pools were quantitated by qPCR to determine the precise molarity of the pool as a whole. Samples were sequenced using Single read sequencing (250bp read) and dual indexing on an Illumina MiSeq following the manufacturer's instructions. The pool was run with a 30% spike-in of Phi-X to avoid issues with low-complexity Amplicon Libraries.

Primer Name	**Primer Sequence (5'-3')**
CRYAB_MiSeq_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTGAGTTCTGGGCAGGTGAT
CRYAB_MiSeq_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAATTTGGGCCTGCCCTTAG
BAG3_MiSeq_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACCAAAGAGCTGCTGGCCCT
BAG3_MiSeq_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCGCTGCTGCTGTGGCTTCT
LMNA_MiSeq_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCAGCAGCTTCTCACAGCAC
LMNA_MiSeq_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGCACACGGATACCTTATCTTT
MTERF4_MiSeq_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGCAGAAAAGTTGAAGAATAGGTTT
MTERF4_MiSeq_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGAAGTGCCTTTTCACGGTACA

Illumina MiSeq analysis methods

In all cases, reads were inspected using the FASTX-Toolkit to assess general quality and then 3'-clipped where the Q score in a 4 nucleotide sliding window fell below 15 and filtered as to retain only those of 100 nucleotides or longer using Trimmomatic (Bolger et al., 2014). Pipeline error rate was estimated by deep-sequencing unedited amplicons derived from each target gene and aligning FATSTQ-derived sorted/indexed BAM file to reference sequences (obtained from Ensembl release 84) with Bowtie2 and assessing sequence divergence in the informative segment (10 nucleotides up/downstream of the nucleotide to be mutated and the CRISPR PAM site) of each target read. Average coverage was ~240,000X and average sequence divergence from the reference at Q30 was 0.1%. For knock-in experiments, we quantified the extent of HDR-mediated donor integration by interrogating pre-processed FASTQ files (as described above) for informative segments of the donor sequence (typically ~50 nucleotides, spanning the targeted nucleotide and CRISPR cut site (not simply the targeted nucleotide, as this may occur in the presence of an indel and lead to overestimation of knock-in) using a Linux grep command (of format : grep -A 2 -B 1 'ssODN sequence' 'INPUT.FQ' | sed '/^--$/d' > 'OUTPUT.FQ') as well as via manual inspection of FASTQ files for confirmation. For comparative value, HDR was also quantified using the Church lab's CRISPR Genome Analyzer (Güell et al., 2014) and we observed good agreement with our estimates (data not shown).

Southern blotting

Southern blotting was performed to analyze zygosity of the *pac* cassette at the *AAVS1* locus and determine the rate of random integration. The probe was designed to the 5' homology arm (Figure S4b) and was digested from the SBI targeting vector using KpnI and ClaI restriction endonucleases (NEB) and radiolabeled. Genomic DNA was isolated from 48 puromycin resistant clones derived from transfection of the SBI targeting construct and TALENs followed by the CTS puromycin selection protocol. Southern blotting was performed based on previously described methods (Haque et al., 2000) with genomic DNA digested by EcoRV (NEB).

PCR-based assay for targeted and untargeted integration of the *AAVS1* donor construct

PCR to confirm HDR at *AAVS1* locus – As an independent confirmation of the Southern blotting data, the same 48 clones were screened with inside-out PCR using the SBI primers in the below table such that one falls inside the homology arm (i.e., in the exogenous sequence) and one falls outside (i.e., in the endogenous locus). Representative cell lines in Figure 3 were also screened in this way.

Name	**Primer Sequence (5'-3')**	**Expected PCR Product Size**
SBI 5' Forward	AGTCCGGACCACTTTGAGCTCTACT	1061bp
SBI 5' Reverse	GAGGAGTAGAAGGTGGCGCGAA	
SBI 3' Forward	AGGTTTAGCCCCGGAATTGACTG	1036bp
SBI 3' Reverse	CCAAAAGGCAGCCTGGTAGACA	
Transposagen 5' Forward	CTCTTTCCGGAGCACTTCC	711bp
Transposagen 5' Reverse	CCGATAAAACACATGCGTCA	

Transposagen 3' Forward	ACTTACCGCATTGACAAGCA	805bp
Transposagen 3' Reverse	CCAGATAGCACTGGGGACTC	

PCR to Screen for random integration of the *AAVS1* donor vector - To screen for random integration of the *AAVS1* donor construct, in addition to the Southern blot, three sets of PCR primers were designed for the SBI donor vector and two sets for the Transposagen vector that amplify the backbone region (i.e., the region of the vector outside of the homology arms) (see below table). PCR was performed using Accuprime Supermix II (Life Technologies) with plasmid DNA as a positive control and DNA from hB53 hiPS6 and hB119 hiPS9 iPSCs as negative controls. The same 48 clones from Southern blotting were screened in this way (Figure S4c). Internal primers were used as a control. Additionally, bands were undetectable in generated knock-in cell lines from Figure 3 (data not shown).

Vector	**Primer Name**	**Primer Sequence (5'-3')**	**Expected PCR Product Size**
SBI	Random F1	GTGCCACCTAAATTGTAAGCGTT	495bp
SBI	Random R1	AACAGTTGCGCAGCCTGAAT	
SBI	Random F2	TGCTGCTGCATTGACGTTGA	408bp
SBI	Random R2	TGCTTCCGGCTCGTATGTTG	
SBI	Random F3	CAACATACGAGCCGGAAGCA	412bp
SBI	Random R3	CCTCTGACTTGAGCGTCGATTT	
SBI	Internal F	ACCCCAGCATCCTGCAGAAC	445bp
SBI	Internal R	ACCCACACCTTGCCGATGTC	
Transposagen	Random F4	GCTTCCTCGCTCACTGACTC	409bp
Transposagen	Random R4	CGACCTACACCGAACTGAGA	
Transposagen	Random F5	CGGTGAAAACCTCTGACACA	380bp
Transposagen	Random R5	ATTCACTGGCCGTCGTTTTA	

Immunocytochemistry

Immunocytochemistry was performed as previously described (Mitzelfelt et al., 2016). Briefly, iPSCs were seeded onto 12mm glass coverslips in 12 well plates coated with Matrigel in mTeSR1 or StemMACS iPS-Brew XF supplemented with 10µM ROCK inhibitor. The following day, media was changed minus ROCK inhibitor and incubated for 4 hours. iPSCs on coverslips were washed with DPBS, fixed with 4% paraformaldehyde at room temperature for 15 minutes, washed two times with DPBS, and stored in DPBS at 4°C until staining. Cells were permeabolized with 0.1% triton-X 100 in DBPS for 10 minutes, washed once in DPBS, and blocked for 1 hour at room temperature with 3% bovine serum albumin (BSA-Sigma) in DPBS. Primary antibody was added in 3% BSA/DPBS and incubated for 2 hours at room temperature. Primary antibodies: Nanog (Cell Signaling 4903p, USA, 1:200) and stage-specific embryonic antigen-4 (SSEA-4) (Stem Cell Technologies 60062AD, USA, 1:40). Cells were washed three times with DPBS. Secondary antibody was added in DBPS and incubated for 1 hour at room temperature. Secondary antibody: Alexa Fluor 555 donkey anti-rabbit IgG (A31572). Cells were washed three times and mounted with Ultracruz Hard Set Mounting Media plus DAPI (Santa Cruz). Representative images were taken using the inverted Nikon Eclipse TE 2000.

Karyotyping

Karyotyping, performed as previously described (Mitzelfelt et al., 2016), was carried out by Wisconsin Diagnostic Laboratories (formerly Dynacare Laboratories), Milwaukee WI. Chromosomes of 20 proliferating cells were counted and fully analyzed using G-banding with representative images shown (Figure 3).

Potential off-target analysis

Clone Analysis: Potential off-target sites were predicted by CRISPR RGEN Tools Cas-OFFinder (Bae et al., 2014) and are shown in the below table (with lowercase text indicating mismatches from the guide sequence). We chose the top 3-5 off-target sites for each CRISPR guide and designed primers that amplify a 300-500bp region around the off-target site. Off-target genomic regions were amplified using Accuprime Supermix II in all isolated knock-in clones. Amplicons were PCR-purified using a PureLink Quick PCR Purification Kit and Sanger sequencing was performed by Retrogen in both the 5’ and 3’ directions with the amplification primers. Sequences were analyzed using Sequencher software. Sequencing results were compared with the originating cell line (either hB53 hiPS6 or hB119 hiPS9). No mutations were noted (data not shown).

CRISPR	Potential Off-Target Site	Chromosome	Position	Primer Name	Primer Sequence (5'-3')
CRYAB	GaGATCCGGTAaTTCCTGAGG	chrY	17785850	OT_1_F	AGCATGCAGTTTAATCATTG
				OT_1_R	CCTATAATGTGTTGGGGAAA
	GGGATCCaGTAaTTCCTGAGG	chrY	18489869	OT_2_F	CAGCATGCAGTTCAATCAT
				OT_2_R	TATAACGTGTTGGGCAAAGT
	GGGATCCaGTAaTTCCTGTGG	chrY	23737276	OT_3_F	AGCCTGCAGTTCAATCAT
				OT_3_R	CATAAGGTGTTGGGCATATT
	GGGAaCaGGTATTTCCTGCGG	chr6	40874614	OT_4_F	TTCCTTGCTTTCTTCCTATG
				OT_4_R	AGGCCAGGGAAGTTAAAGTA
	GGGAaCCGcTATTTCCTGAGG	chrX	153640375	OT_5_F	GGTCTGACAGGAAACAAGAT
				OT_5_R	GGAGAAACATTTGGATTGAA
BAG3	GGGAaGAGCCAGATGgGgGTCAGG	chr12	53068310	OT_1_F	CAGCTCCATGAGCAAAAA
				OT_1_R	AAGTGTGTGGGGGAAGAC
	GGGAaGAGCaGATTGTGCGTaGGG	chr3	62611736	OT_2_F	CACAGAGCTTGGCTTCTAGT
				OT_2_R	CTACAAGGCCTTTGATGAGT
	GGGACGAGCCaATGTGCtTtAGG	chr7	57160206	OT_3_F	TCTGCATGTTGAAATTGTTT
				OT_3_R	TGGGAAAATTTTGGAGATTA
	GGcACGAGCTACcATGTcCGTCTGG	chr4	147603021	OT_4_F	CAATCCTCCCACCTTAGC
				OT_4_R	TCCTTTTATGCACCAAGTTT
LMNA	tATTGGACTTcTTGgGCAGCCGG	chr12	26116974	OT_1_F	CTAATGGTTTTAGCCCACAA
				OT_1_R	TCACATACAGCAAGCAAAAC
	CATgGGACTTGTTGCGtAGaTGG	chr10	6557892	OT_2_F	ATGTTTCAAAGCAAGGAAGA
				OT_2_R	CCCCTGTATTCAACTCCATA
	CAcTGGAaTTtTTGCtCAGCAGG	chr8	4084793	OT_3_F	AACACATTCACTTCCTTTGG
				OT_3_R	TTGGGAGAGAGAAAATGAAA
	CATTGGgCTgGTTGgGCAcCAGG	chr8	41681653	OT_4_F	TCCTATGTGGGAGAAGCA
				OT_4_R	TGTCCAGAATCAGCTTCTTT
	CATTGGgCTTaggGCGCAGCTGG	chr8	69992997	OT_5_F	AAGTCCAAAACGAAGGTGTT
				OT_5_R	CTGAGGCAGGAGAATCACT
MTERF4	ACcTGTATTCCAGTCTGAtCCTGG	chr12	115848234	OT_1_F	TTTTGAAATTGGGAGATGAG
				OT_1_R	TGAAGCTTTGTTCTTCCTGT
	cCTTGTAcTCCATGTTGACCCAGG	chr3	105899012	OT_2_F	CGTGTACTTTATCATTTAACAGC
				OT_2_R	GATCTGTGGACAGAAAGTCC
	ACTTtTATTCCAGcTGACCACAGG	chr5	53828304	OT_3_F	TCTTGTTTTACTGTGGCTGA
				OT_3_R	ATGGCTGTACAAAATTGAGC

Illumina MiSeq Analysis: To ensure CTS does not enrich for off-target effects, we chose our most active CRISPR (targeting *CRYAB*), and analyzed the top 12 potential off-target sites by deep-sequencing using the Illumina MiSeq. CRISPR RGEN Tools Cas-OFFinder (Bae et al., 2014) was used to identify potential off-target sites with sequences identified in the below table (lowercase text indicates mismatches from the guide sequence). Primers were designed flanking these sites and samples were prepared and analyzed using the Illumina MiSeq (as described above). PCR 1 primers are listed in the table below (adapter sequences in red and green text for the forward and reverse primers, respectively). Reads were inspected and 3'-clipped as described above before being aligned to the appropriate off-target reference sequences using Bowtie2 (with the 'local' alignment setting to maximize the chance of finding indels). Resulting SAM files were converted to sorted/indexed BAM files and loaded into IGV for viewing. We found no measurable difference in indel presence between pooled populations following CTS compared to without CTS.

Potential Off-Target Site	**Chr**	**Position**	**Primer Name**	**Primer Sequence (5'-3')**
GaGATCCGGTAaTTCCTGAGG	chrY	17785850	COT_MS_F_1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAGGCATGTTGTTGTTAATC*
			COT_MS_R_1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTAGCTGGAAATTGTGATCC*
GGGATCCaGTAaTTCCTGAGG	chrY	18489869	COT_MS_F_2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTCATCAACCAAGCAGGT*
			COT_MS_R_2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTCAGCATTAGCTGGAAAT*
GGGATCCaGTAaTTCCTGTGG	chrY	23737276	COT_MS_F_3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAAGCAGGTTGTTGTAGTCA*
			COT_MS_R_3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTCAGCATTAGCTGGAAAT*
GGGATCCaGTAaTTCCTGAGG	chrY	23941131	COT_MS_F_4	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTAAGCAGGTTGTTGTTGTCA*
			COT_MS_R_4	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTCAGCATTAGCTGGAAAT*
GGGAaCaGGTATTTCCTGCGG	chr6	40874614	COT_MS_F_5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGACCCTGTGTGAGACAGAAA
			COT_MS_R_5	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTGCCTTCTCATAGGGAAT
GGGAaCCGcTATTTCCTGAGG	chrX	153640375	COT_MS_F_6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCAAGCAAACTTGCACTC
			COT_MS_R_6	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAAAGGCTTGGTGGGTGAG
GGtATCCtGTATTTCCTcTGG	chr8	41832706	COT_MS_F_7	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTATTGCACAGGACAGACTTG
			COT_MS_R_7	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGCAGATGTTAGGTCAGAA
GaGATCaGGTAcTTCCTGGGG	chr8	120828204	COT_MS_F_8	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCTCTTTGTTCTGAAGGA
			COT_MS_R_8	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGTGTTGACAAGCATCTGA
GGGATCaGGTgTTTCtTGTGG	chr12	128299314	COT_MS_F_9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGACCAGCTCATCTTTTCAA
			COT_MS_R_9	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGTATTTAACGCTGAGAGCA
aGaATCtGGTATTTCCTGTGG	chr3	123694853	COT_MS_F_10	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGGCAGCTTTTCCAACCT
			COT_MS_R_10	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTATAGCAGAGCCTCCAG
GaGATtCGGTcTTTCCTGAGG	chr3	179083191	COT_MS_F_11	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATGGACATCTGACCTACT
			COT_MS_R_11	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGGAGATTAGAATGTTGCT
GGGAaCaGGgATTTCCTGCGG	chr7	45889831	COT_MS_F_12	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGAGGAATTAGGACTTCCAT
			COT_MS_R_12	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCAGCTTCTCCATCCTTAAA

*Primers amplified >1 locus with same sequence

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