SI appendix for

Classification: BIOLOGICAL SCIENCES - GENETICS

Title: Efficient non-meiotic allele introgression in livestock using custom endonucleases

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Keywords: TALEN, CRISPR/Cas9, Livestock, allele introgression

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Materials and Methods

TALEN designing and production. Candidate TALEN target DNA sequences and RVD sequences were identified using the online tool "TAL Effector Nucleotide Targeter" (https://tale-nt.cac.cornell.edu/about). Plasmids for TALEN DNA transfection or in vitro TALEN mRNA transcription were then constructed by following the Golden Gate Assembly protocol(1) using pC-GoldyTALEN (Addgene ID 38143) and RCIscript-GoldyTALEN (Addgene ID 38143) as final destination vectors(2). The final pC-GoldyTALEN vectors were prepared by using PureLink® HiPure Plasmid Midiprep Kit (Life Technologies) and sequenced before usage. Assembled RCIscript vectors prepared using the QIAprep Spin Miniprep kit (Qiagen) were linearized by SacI to be used as templates for in vitro TALEN mRNA transcription using the mMESSAGE mMACHINE® T3 Kit (Ambion) as indicated previously(2). Refer to **Supplementary Table 3** for the RVD sequences of all the TALENs used in this paper. Modified mRNA was synthesized from RCIScript-GoldyTALEN vectors as previously described(2) substituting a ribonucleotide cocktail consisting of 3'-0-Me-m7G(5')ppp(5')G RNA cap analog (New England Biolabs), 5-methylcytidine triphosphate pseudouridine triphosphate (TriLink Biotechnologies, San Diego, CA) and adenosine triphosphate and guanosine triphosphate. Final nucleotide reaction concentrations are 6 mM for the cap analog, 1.5 mM for guanosine triphosphate, and 7.5 mM for the other nucleotides. Resulting mRNA was DNAse treated prior to purification using the MEGAclear Reaction Cleanup kit (Applied Biosciences).

CRISPR/Cas9 design and production. Gene specific gRNA sequences were cloned into the Church lab gRNA vector (Addgene ID: 41824) according their methods(3). The Cas9 nuclease was provided either by co-transfection of the hCas9 plasmid (Addgene ID: 41815) or mRNA synthesized from RCIScript-hCas9. This RCIScript-hCas9 was constructed by sub-cloning the XbaI-AgeI fragment from the hCas9 plasmid (encompassing the hCas9 cDNA) into the RCIScript plasmid. Synthesis of mRNA was conducted as above except that linearization was performed using KpnI.

Donor repair template preparation

BB-HDR (1,623bp) plasmid. A 1,695bp fragment encompassing the Belgian Blue allele was PCR amplified (btGDF8 BB 5-1: **5'-**CAAAGTTGGTGACGTGACAGAGGTC; btGDF8 BB 3-1: 5'-GTGTGCCATCCCTACTTTGTGGAA) from Belgian Blue genomic DNA and TOPO cloned into the PCR 2.1 vector (Life Technologies). This plasmid was used as positive control template for analytical primer sets and for derivation of the 1,623bp BB-HDR template by PCR with following primers (BB del HR 1623 5-1: 5'-GATGTATTCCTCAGACTTTTCC; BB del HR 1623 3-1: 5'- GTGGAATCTCATCTTACCAA) and TOPO cloned as before. Each plasmid was sequence verified prior to use. Transfection grade plasmid was prepared using the Fast-Ion MIDI Plasmid Endo-Free kit (IBI Scientific)**.**

rAAV packaging. BB-HDR was cloned into pAAV-MCS and packaged into using the Adeno-Associated Virus Helper-Free system (Agilent). Briefly, a 10cm dish AAV-293 cells was transfected with 5 µg each: pAAV-Helper, pAAV-RC and the AAV-BB-HDR plasmid. Two days post transfection, the cells were removed from the plate by scraping into 1 ml of growth media. Viral particles were released by 3 freeze-thaw cycles prior to centrifugation at maximum speed in a microcentrifuge for 5 minutes. The supernatant was aspirated and used directly for infection of target cells.

Pc *HDR template.* A 1,784bp fragment encompassing the Celtic *POLLED* allele was PCR amplified (F1: 5'-GGGCAAGTTGCTCAGCTGTTTTTG; R1- 5'-TCCGCATGGTTTAGCAGGATTCA) from angus genomic DNA and TOPO cloned into the PCR 2.1 vector (Life Technologies). This plasmid was used as positive the control template for analytical primer sets and for derivation of the 1,592bp HDR template by PCR with following primers (1594 F: 5'-ATCGAACCTGGGTCTTCTGCATTG; R1: 5'-TCCGCATGGTTTAGCAGGATTCA) and TOPO cloned as before. Each plasmid was sequence verified prior to use. Transfection grade plasmid was prepared using the Fast-Ion MIDI Plasmid Endo-Free kit (IBI Scientific) and 5 µg or 10 µg was transfected along with 2 µg HP1.3 TALEN mRNA.

Oligonucleotide templates. All oligonucleotide templates were synthesized by Integrated DNA Technologies, 100 nmole synthesis purified by standard desalting, and resuspended to 400 µM in TE. See **Supplementary Table 4** for the complete list of oligo templates.

Tissue culture and transfection. Pig, cattle or goat fibroblasts were maintained at 37 or 30 °C (as indicated) at 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 I.U./ml penicillin and streptomycin, and 2mM L-Glutamine. For transfection, all TALENs, CRISPR/Cas9 and HDR templates were delivered through transfection using the Neon Transfection system (Life Technologies) unless otherwise stated. Briefly, low passage Ossabaw, Landrace, Wagyu, Holstein or goat fibroblasts reaching 100% confluence were split 1:2 and harvested the next day at 70-80% confluence. The goat cells used in this study derived from a 35-40 day male Nubian x Boer fetus. Each transfection was comprised of 500,000-600,000 cells resuspended in buffer "R" mixed with plasmid DNA or mRNA and oligos and electroporated using the 100ul tips by the following parameters: input Voltage; 1800V; Pulse Width; 20ms; and Pulse Number; 1. Typically, 2-4 µg of TALEN expression plasmid or 1-2 µg of TALEN mRNA and 2-3 µM of oligos specific for the gene of interest were included in each transfection. Deviation from those amounts is indicated in the figure legends for both TALENs and CRISPR/Cas9 experiments. After transfection, cells were divided 60:40 into two separate wells of a 6-well dish for three days' culture at either 30 or 37°C respectively. After three days, cell populations were expanded and at 37°C until at least day 10 to assess stability of edits.

Plasmid and rAAV HDR in Wagyu Fibroblasts. Low passage Wagyu fibroblasts were cultured to 70-90% confluence and transfected by Nucleofection (Lonza) with 2 µg each TALEN expression plasmid (btGDF83.1L+NR, **Supplementary Table 3**) along with 750 ng of *Sleeping Beauty* transposon components as previously described(2). For conditions where plasmid HDR template was used, 2 µg of BB-HDR plasmid was also included in the transfection. Transfected cells were split between two wells of a 6-well plate for culture at 30 or 37°C. For conditions using rAAV HDR template, 150 µl of viral lysate was added to each well 2 hours post transfection. After incubation for three days, cells were harvested by trypsinization, a portion of which were lysed for analysis of HDR at day 3, and the remainder were plated for colony isolation as previously described(2).

Dilution cloning: Three days post transfection, 50 to 250 cells were seeded onto 10 cm dishes and cultured until individual colonies reached circa 5mm in diameter. At this point, 6 ml of TrypLE (Life Technologies) 1:5 (vol/vol) diluted in PBS was added and colonies were aspirated, transferred into wells of a 24-well dish well and cultured under the same conditions. Colonies reaching confluence were collected and divided for cryopreservation and genotyping.

Sample preparation: Transfected cells populations at day 3 and 10 were collected from a well of a 6-well dish and 10-30% were resuspended in 50 µl of 1X PCR compatible lysis buffer:10 mM Tris-Cl pH 8.0, 2 mM EDTA, 0.45% Tryton X-100(vol/vol), 0.45% Tween-20(vol/vol) freshly supplemented with 200 µg/ml Proteinase K. The lysates were processed in a thermal cycler using the following program: 55°C for 60 minutes, 95°C for 15minutes. Colony samples from dilution cloning were treated as above using 20-30 µl of lysis buffer.

Surveyor mutation detection and RFLP analysis. PCR flanking the intended sites was conducted using Platinum Taq DNA polymerase HiFi (Life Technologies) with 1 µl of the cell lysate according to the manufacturer's recommendations. Primers for each site are listed in **Supplementary Table 5**. The frequency of mutation in a population was analysed with the Surveyor Mutation Detection Kit (Transgenomic) according to the manufacturer's recommendations using 10 ul of the PCR product as described above. RFLP analysis was performed on 10 µl of the above PCR reaction using the indicated restriction enzyme. Surveyor and RFLP reactions were resolved on a 10% TBE polyacrylamide gels and visualized by ethidium bromide staining. Densitometry measurements of the bands were performed using ImageJ; and mutation rate of Surveyor reactions was calculated as described in Guschin et al. 2010(4). Percent HDR was calculated via dividing the sum intensity of RFLP fragments by the sum intensity of the parental band + RFLP fragments. For analysis of *mloxP* insertion, small PCR products spanning the insertion site were resolved on 10% polyacrylamide gels and the insert versus wild type alleles could be distinguished by size and quantified. RFLP analysis of colonies

was treated similarly except that the PCR products were amplified by 1X MyTaq Red Mix (Bioline) and resolved on 2.5% agarose gels.

For analysis of clones for introgression of the *GDF8* G938A-only (oligos lacked a novel RFLP), colonies were initially screened by a three primer assay that could distinguish between heterozygous and homozygous introgression. Briefly, lysates from pig or cattle colonies were analysed by PCR using 1X MyTaq Red Mix (Bioline) using the following primers and programs. Cattle *GDF8* (Outside F1: 5'-CCTTGAGGTAGGAGAGTGTTTTGGG, Outside R1: 5'-TTCACCAGAAGACAAGGAGAATTGC, Inside F1: 5'-TAAGGCCAATTACTGCTCTGGAGACTA; and 35 cycles of (95°C, 20 s; 62°C, 20 s; 72°C, 60 s). Pig *GDF8*: Outside F1: 5'-CCTTTTTAGAAGTCAAGGTAACAGACAC, Outside R1: 5'-TTGATTGGAGACATCTTTGTGGGAG, Inside F1: 5'-TAAGGCCAATTACTGCTCTGGAGATTA; and 35 cycles of (95°C, 20 s; 58°C, 20 s; 72°C, 60 s). Amplicons from candidates were sequenced directly and/or TOPO cloned (Life Technologies) and sequenced by Sanger sequencing.

To detect TALEN-mediated HDR at with the BB-HDR template, either 1 µl or 1 µl of a 1:10 dilution of PCR-lysate (1,000 cells/ul) was added to a PCR reaction with PCR primers bt GDF8 BB 5-1 (primer "c") and primer "c' " (BB-Detect 3-1- 5'-GCATCGAGATTCTGTCACAATCAA) and subjected to PCR with using 1X MyTaq Red mix (Bioline) for 40 cycles (95°C, 20 s; 66°C, 20 s; 72°C, 60 s). To confirm HDR in colonies identified by the above PCR, amplification of the entire locus was performed with primers bt GDF8 BB 5-1 and bt GDF8 BB 3-1 followed by TOPO cloning (Life Technologies) and sequencing.

Detection of Pc introgression was performed by PCR using the F1 primer (see above) and the "P" primer (5'-ACGTACTCTTCATTTCACAGCCTAC) using 1X MyTaq Red mix (Bioline) for 38 cycles (95°C, 25 s; 62°C, 25 s; 72°C, 60 s). A second PCR assay was performed using (F2: 5'-GTCTGGGGTGAGATAGTTTTCTTGG; R2- 5'-GGCAGAGATGTTGGTCTTGGGTGT). Candidates passing both tests were analysed by PCR using the flanking F1 and R1 primers followed by TOPO cloning and sequencing.

Detection of *FecB* introgression was performed as previously described for sheep(5). *Callipyge* introgression was detected by an AvaII RFLP assay using primers indicated in **Supplementary Table 5**.

Amplicon sequencing and analysis. DNA was isolated from transfected populations and 100-250 ng was added to a 50 µl Platinum *Taq* DNA Polymerase High Fidelity (Life Technologies) assembled per the manufacturer's recommendations. Each sample was assigned a primer set with a unique barcode to enable multiplex sequencing (**Supplementary Table 6**). A portion of the PCR product was

resolved on a 2.5% agarose gel to confirm size prior to PCR cleanup using the MinElute PCR Purification Kit (Qiagen). Samples were submitted to the University of Minnesota Genomics Center where they were quantified and pooled into a single sample for sequencing. The single combined sample was spiked with 25% PhiX (for sequence diversity) and sequenced on an Illumina MiSeq sequencer generating 150 base-pair paired-end reads. Read quality was assessed using FastQC (www.bioinformatics.bbsrc.ac.uk/projects/fastqc). Read-pairs with overlapping ends were joined using fastq-join from the ea-utils package(6). A custom perl script was used to demultiplex the joined reads and count insert types. Exact matches to the forward and reverse primers were required in the demultiplexing step.

Cloned animals were genotyped by RFLP assay and sequencing.

Supplementary Table 1: Sequence analysis of clones introgressed with the Pc allele					
Clone	**Haplotype predicted by PCR**	**Allele**	**Homology Template**	**% Identity** (From primer pair HP1748F to HP1748R)	**Number of INDELs**
HP8 P6 A2	Heterozygous	*HORNED*	5 µg plasmid HP1594bp	86.8%	2
		POLLED		100%	0
HP8 P3 B5	Heterozygous	*HORNED*	5 µg plasmid HP1594bp	99.4%	1
		POLLED		100%	0
HP7 P4 A1	Homozygous	*POLLED*	10 µg plasmid HP1594bp	100%	0
HP14-30 P2 B4	Homozygous	*POLLED*	5 µg plasmid HP1594bp	100%	0
HP14-30 P3 B6	Homozygous	*POLLED*	5 µg plasmid HP1594bp	100%	0

Supplementary Table 2: Sequence analysis of SNP-introgressed cellular clones		
Colony ID	**Allele 1**	**Allele 2**
btGDF8 G938A only		
C8	iSNP	iSNP
E3	iSNP	iSNP + Indel
A12	iSNP	WT
E7	iSNP	Indel
H5	iSNP	Indel
G8	iSNP + indel	WT
H3	iSNP +indel	Indel
D3	WT	WT
H10	WT	WT
ssP65		
B4	iSNP	iSNP
D4	iSNP	iSNP
D8	Homozygous iSNP + indel	
E7	Homozygous iSNP + indel	
B6	Homozygous iSNP + indel	
ssP65 Rep		
A8	iSNP	iSNP
E9	iSNP	WT
D2	iSNP	WT
C7	Homozygous iSNP + indel	
A4	Homozygous iSNP + indel	
D1	Homozygous iSNP + indel	
D7	iSNP + indel	indel
A3	iSNP + indel	WT
B7	iSNP + indel	indel
A10	Indel	WT
ssGDF8 G938A only		
4C6	iSNP	iSNP
3A1	iSNP + indel	WT
2D2	iSNP + indel	WT
3D2	ND	ND
iSNP = Intended SNP		

Supplementary Table 3. TALEN sequences		
TALEN pair	**TALEN RVD sequence**	**DNA Target sequence (Sense strand)**
ssLDLR2.1	HD NG HD HD NG NI HD NI NI NN NG NN NN NI NG NG NG HD NN NN NI HD HD HD NN NG HD HD NG NG NN HD NI HD NG	CTCCTACAAGTGGATTTGTGATGGGAACACCGAGTGCAAGGACGGGTCCG
btGDF83.1L+NR	NN NG NN NI NG NN NI NI HD NI HD NG HD HD NI HD NI NN NI NI NG HD NG NG HD NI NI NI NI NG HD HD NI HD NI NN NG NG NI NN NI NN	GTGATGAACACTCCACAGAATCTCGATGCTGTCGTTACCCTCTAACTGTGGATTTTGA
ssDAZL3.1	NN NN NI NG NN NI NI NI HD HD NN NI NI NI NG NG HD NG NG NG NG NI HD NG NN NI NI HD HD NI NG NI NG	GGATGAAACCGAAATTAGAAGTTTCTTTGCTAGATATGGTTCAGTAAAAG
ssAPC14.2	NN NN NI NI NN NI NI NN NG NI NG HD NI NN HD HD NI NG NN NI HD HD HD NI NN NI NI NG NG NG HD NG NN NG	GGAAGAAGTATCAGCCATTCATCCCTCCCAGGAAGACAGAAATTCTGGGTC
ssTp53	NN NN HD NI HD HD HD NN NG NN NG HD HD NN HD NN HD HD NI NG NN NG NI HD NG HD NG NN NI HD NG NG	GGCACCCGTGTCCGCGCCATGGCCATCTACAAGAAGTCAGAGTACATG
ssKissR3.2	NN HD NG HD NG NI HD NG HD NG NI HD HD HD HD NN HD NI HD NI NG NN NI NI NN NG HD NN HD HD HD NI	GCTCTACTCTACCCCCTACCAGCCTGGGTGCTGGGCGACTTCATGTGC
ssEIF4GI14.1	HD HD NN NG HD HD NG NG NG NN HD HD NI NI HD HD NG NG NG NN NN NN NN NN HD HD HD NI HD NN NN NG NG NN HD NG	CCGTCCTTTGCCAACCTTGGCCGACCAGCCCTTAGCAACCGTGGGCCCCCA
btGGTA9.1	HD NG NN HD NN HD NG HD HD NG NG HD NI NI NI NN NG NN NG HD HD NG NN HD HD NI HD HD NG HD NG NG HD NG	CTGCGCTCCTTCAAAGTGTTTAAGATCAAGCCTGAGAAGAGGTGGCAGGAC
ssRAG2.1	NI HD HD NG NG HD HD NG HD HD NG HD NG HD HD NN HD NG HD NG NI NI NN HD NG NN HD NG NG NG NG NN NI NI NG	ACCTTCCTCCTCTCCGCTACCCAGCCACTTGCACATTCAAAAGCAGCTTAG
ssIL2Rg2.1	HD HD HD NI NI NI NN NN NG NG HD NI NN NG NN NG NG NG HD HD NI NI NN NG NN HD NI NI NG NG HD NI NG NN NG NI HD NG	CCCAAAGGTTCAGTGTTTTGTGTTCAATGTTGAGTACATGAATTGCACTTGG
btGDF83.6-A	NN HD NG HD NG NN NN NI NN NI NI NG NI NG NI NG NN NI NN NN NI NG NI HD NG NG NG NG	GCTCTGGAGAATATGAATTTGTATTTTTGCAAAAGTATCCTCAT
btGDF83.6-G	NN HD NG HD NG NN NN NI NN NI NI NG NNNG NI NG NN NI NN NN NI NG NI HD NG NG NG NG	GCTCTGGAGAATGTGAATTTGTATTTTTGCAAAAGTATCCTCAT
ssGDF83.6	NI HD NG NN HD NG HD NG NN NN NI NN NI NN NG NN NG NN NI NN NN NN NG NI NG NG NG NG NG NN NG	ACTGCTCTGGAGAGTGTGAATTTGTATTTTTACAAAAATACCCTCAC
btRosa1.2	HD NG HD NN HD NI NG NG NN HD HD HD NI HD NG HD NG HD NG HD NG HD HD NI HD HD HD NG NI HD HD NG	CTCGCATTGCCCACTGGGTGGGTGCTTAGGTAGGTAGGGTGGAGAGAG

ssSRY3.2	NI NG NI HD NI NG NG NG NG NI HD NI HD NI HD NI NG NI NG NI NN NN NG NG HD NI NN NN HD HD NI NG NG NI NI NG	ATACATTTTACACACATATATATGAAACTGACAGTATTAATGGCCTGAACCT
caFecB6.1	NI HD NI NN NI NN NN NI NN NN HD HD NI NN HD NG NN NN NG NG HD NI NG HD NI NI HD NI HD HD NN NG HD NG NN NI NG NI NG	ACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAATATATCAGACGGTGTT GATG
caCLPG1.1	NN NI NN NI NN HD NN HD NI NN NN NI NI NG HD HD NI NN NN HD NG NN NI HD NI NN NN NG NN NN NG HD HD HD NI NN HD	GAGAGCGCAGGAATCCAGGCGCAGGGGCCCGAGGGCTGGGACCACCTGTC AG
btHP1.3	NG NG NG HD NG NG NN NN NG NI NN NN HD NG NN NN NI NI NI NI NN NI NN NI NN NG NG NG NG NN NI NG	TTTCTTGGTAGGCTGGTATTCTTGCTCTTTAGATCAAAACTCTCTTTTC
ssP65_11.1	NN HD HD HD HD HD HD NI HD NI HD NI NN HD NG NI NG NI NN HD HD NG HD NI NN NN NN NG NI HD NG	GCCCCCCCACACAGCTGAGCCCATGCTGATGGAGTACCCTGAGGCTAT
ssP65.8	HD NG HD HD NG HD HD NI NG NG NN HD NN NN NI NN NI NG HD NG NN NI HD NG HD NI NN NI NI NN	CTCCTCCATTGCGGACATGGACTTCTCAGCCCTTCTGAGTCAGATC

Abbreviations: ss= *Sus scrofa*; bt= *Bos taurus*; ca= *Capra aegagrus.*

Note: RVD sequences for left and right TALEN monomers are shown top and bottom respectively oriented from the N to C terminus. Red text indicates TALEN binding sites.

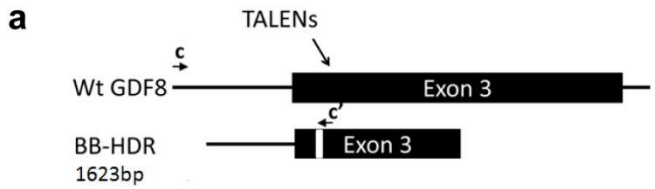
Supplementary Table 4. Oligonucleotide HDR templates		
TALEN pair	**ssODN design**	**Sequence**
ssLDLR2.1	46_SNPs BamHI	CCTACAAGTGGATTTGTGGGATCCACACCGAGTGCAAGGACGGGTC
ssLDLR2.1	90_SNPs BamHI	TGCCGAGACGGGAAATGCATCTCCTACAAGTGGATTTGTGGGATCCACACCGAGTGCAAGGACGGGTCCGATGAGTCCCTGGAGACGTGC
ssLDLR2.1	90_ins4_BM BamHI	CCGAGACGGGAAATGCACCTCCTACAAGTGGATTTGTGATGGATCCGAACACCGAGTGCAAGGACGGGTCCGCTGAGTCCCTGGAGACGT
ssLDLR2.1	90_SNPs_BM BamH1	TGCCGAGACGGGAAATGCACCTCCTACAAGTGGATTTGTGGGATCCACACCGAGTGCAAGGACGGGTCCGCTGAGTCCCTGGAGACGTGC
ssLDLR2.1	60_SNPs_BM BamH1	TGCACCTCCTACAAGTGGATTTGTGGGATCCACACCGAGTGCAAGGACGGGTCCGCTGAG
ssLDLR2.1	86_del4_BM BamH1	TGCCGAGACGGGAAATGCACCTCCTACAAGTGGATTTGGGATCCACCGAGTGCAAGGACGGGTCCGCTGAGTCCCTGGAGACGTGC
ssDAZL3.1	90_ins4_BM BamHI	AATTCTTCTCCATAGACGGATGAAACCGAAATTAGAAGTTGGATCCTTTGCTAGATATGGTTCAGTAAAAGGAGTGAAGATATTCACAGA
ssAPC14.2	90_ins4_BM HindIII	CCAGATCGCCAAAGTCACGGAAGAAGTATCAGCCATTCATCCCTCCCAGTGAAGCTTACAGAAATTCTGGGTCGACCACGGAGTTGCACT
ssTp53	90_ins5_BM HindIII	AGCTCGCCACCCCCGCCGGGCACCCGTGTCCGCGCCATGGCCATCTAAGCTTAAAGAAGTCAGAGTACATGCCCGAGGTGGTGAGGCGCT
ssKissR3.2	90_ins4 HindIII	GTGCTGCGTGCCCTTTACTGCTCTACTCTACCCCCTACCAGCCTAAGCTTGTGCTGGGCGACTTCATGTGCAAGTTCCTCAACTACATCC
ssEIF4GI14.1	90_SNP-NL-DF EagI	CCCAGACTTCACTCCGTCCTTTGCCGACTTCGGCCGACCAGCCCTTAGCAACCGTGGGCCCCCAAGGGGTGGGCCAGGTGGGGAGCTGCC
btGGTA9.1	90_del4_BM HindIII	GCCTTTGATAGAGTTGGGTCCCCTGCGCTCCTTCAAAGTGTTTAAGCTTCTGAGAAGAGGTGGCAGGACCTCAGCATGATGCGCATGAAG
ssRAG2.1	90_ins4_BM HindIII	CTCTAAGGATTCCTGCCACCTTCCTCCTCTCCGCTACCCAGACTAAGCTTGCACATTCAAAAGCAGCTTAGGGTCTGAAAAACATCAGT
ssIL2Rg2.1	90_ins4_BM	TTCCACTCTACCCCCCCCAAAGGTTCAGTGTTTTGTGTAAGCTTCAATGTTGAGTACATGAATTGCACTTGGGACAGCAGCTCTGAGCTC

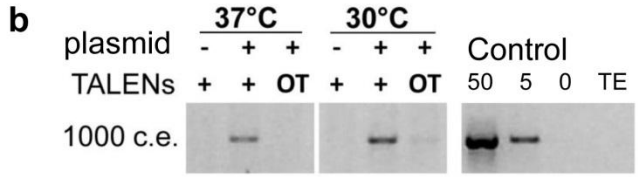
	HindIII	
btRosa1.2	41_mLoxP 75bp total	CTCGCATTGCCCACTGGGTGATAACTTCGTATAGCATACATTATAGCAATTTATGGTGCTTAGGTAGGTAGGGTG
btRosa1.2	60_LoxP 94bp total	GGGACTCTCGCATTGCCCACTGGGTGGGTATAACTTCGTATAATGTATGCTATACGAAGTTATGCTTAGGTAGGTAGGGTGGAGAGAGACTTGG
ssSRY3.2	DS 3.2 mLoxP 86bp total	ATACATTTTACACACATATATATGAAAATAACTTCGTATAGCATACATTATAGCAATTTATCTGACAGTATTAATGGCCTGAACCT
caFecB6.1	FecB-A-G	AAAGTGTTCTTCACTACAGAGGAGGCCAGCTGGTTCCGAGAGACAGAAATATATCGGACGGTGTTGATGAGGCATGAAAACATCTTGGGC
caCLPG1.1	CLPG A- G AvaII (loss of)	TGCTGAGAGCGCAGGAATCCAGGCGCAGGGGCCCGAGGGCTGGGGCCACCTGTCAGATCCTTTCCCCAGCTGAAGGCAGGGTGTGGGTGA
btGDF83.1	71_del11	GGAGAGATTTTGGGCTTGATTGTGACAGAATCTCGATGCTGTCGTTACCCTCTAACTGTGGATTTTGAAGC
btGDF83.6-G	90_SNPs_BM EcoRI	CTAAAAGATATAAGGCCAATTACCGCTCTGGAGAATATGAATTCGTATTTTTGCAAAAGTATCCTCATCCCCATCTTGTGCACCAAGCAA
btGDF83.6-G	90_SNPs EcoRI	CTAAAAGATATAAGGCCAATTACTGCTCTGGAGAATATGAATTCGTATTTTTGCAAAAGTATCCTCATACCCATCTTGTGCACCAAGCAA
btGDF83.6-G	90_SNP	CTAAAAGATATAAGGCCAATTACTGCTCTGGAGAATATGAATTTGTATTTTTGCAAAAGTATCCTCATACCCATCTTGTGCACCAAGCAA
ssP65.8	90_SNP XmaI	GGGCCTCTGGGCTCACCAACGGTCTCCTCCCGGGGGACGAAGACTTCTCCTCCATTGCGGACATGGACTTCTCAGCCCTTCTGAGTCAGA
ssP65-S-P (CRISPR)	S-P-HDR XmaI	GCTCCCACTCCCCTGGGGGCCTCTGGGCTCACCAACGGTCTCCTCCGGGGGACGAAGACTTCTCCTCCATTGCGGACATGGACTTCTCA
ssGDF83.6	90_SNPs_BM EcoRI	CCCAAAAGATATAAGGCCAGTCACTGCTCTGGAGAGTATGAATTCGTATTTTTACAAAAATACCCTCACCCTCATCTTGTGCACCAAGCA
ssGDF83.6	90_SNPs EcoRI	CCCAAAAGATATAAGGCCAGTTACTGCTCTGGAGAGTATGAATTCGTATTTTTACAAAAATACCCTCACACTCATCTTGTGCACCAAGCA
ssGDF83.6	90_SNP	CCCAAAAGATATAAGGCCAGTTACTGCTCTGGAGAGTATGAATTTGTATTTTTACAAAAATACCCTCACACTCATCTTGTGCACCAAGCA

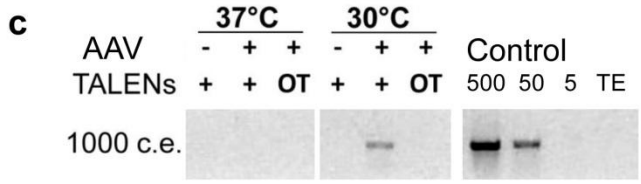
Oversized red text represents intended SNPs; regular size red text stands indicates BMs or nucleotide changes to generate restriction sites for RFLP screening; orange texts indicates insertions; blue text indicates TALEN or CRISPR binding sites; novel restriction sites are underlined.

Supplementary Table 5. Primers used for Surveyor or RFLP analysis.		
TALEN pair	**Primer Forward 5' to 3'**	**Primer Reverse 5' to 3'**
ssLDLR2.1	CACAGCCGTAATAATGCCAGCTCC	CCTTCTCCGCCCACATCCTAATTC
btGDF83.1	CCTTGAGGTAGGAGAGTGTTTTGGG	CTCATGAACACCCACAGCGATCTAC
ssDAZL3.1	ATTTGGGCCCTGTTGAAAAC	ACTCACCCTTTGGACACACC
ssAPC14.2	CAGTGTTGCCCAGCTCCTCTTCA	GCGTGTGAGTGGGCAGTAGAGCTT
ssTp53	TATAGCGATGGTGAGTGGGCGG	AAGGCCACGGACAAACCCCTCT
ssKissR3.2	AAGGATGTCAGCACCTCTCTGGGG	ACCCACCCGGACTCTACTCCTACCA
ssEIF4GI14.1	GGAGCCAGAGGTCCTGAAAGAGTTG	TGAGTCAGCCAACCTGTGACACCA
ssIL2Rg2.1	CTCCCCCACTTCATTTTCTCCCC	GATTCCACAGTCCAGCCTCAGCTC
ssRAG2.1	CCCAGCTGCCTGGATTTTTGC	CCGTCCTCCAAAGAGAACACCCA
btGGTA9.1	AAGCCTGCAGAAATCCCAGAGGTT	TTCGCCGAAGGGAATGTATGCTG
btRosa1.2	CGCCTGTCAGTTACAGCCTCG	CAGCCCTACCTCCCCGTGG
ssSRY3.2	GCTCCTGGCCATCTCTTTGGTCA	TGCCTGCCTGCTTGCATCTCTCA
caCLPG1.1	CTGCTCAGAGAGGCCAGATGCT	TGCTGGCAGGAGAGACGGTTA
btGDF83.6-G **btGDF83.6-A**	CCTTGAGGTAGGAGAGTGTTTTGGG	CTCATGAACACCCACAGCGATCTAC
ssP65_11.1 **ssP65.8**	GCAATAACACTGACCCGACCGTG	GCAGGTGTCAGCCCTTTAGGAGCT
ssGDF83.6	AGGCGAAGACCTCAGGGAAATTTA	TTGATTGGAGACATCTTTGTGGGAG

Supplementary Table 6. Amplification primers for Illumina sequencing		
btGDF8	btGDF8-DS F1	TTTGGGCTTGATTGTGATGA
	btGDF8-DS F1_A	ATCACGTTTGGGCTTGATTGTGATGA
	btGDF8-DS F1_B	CGATGTTTTGGGCTTGATTGTGATGA
	btGDF8-DS F1_C	TTAGGCTTTGGGCTTGATTGTGATGA
	btGDF8-DS R1	AACCTCTGGGGTTTGCTTG
ssLDLR	ssLDLR2-DS F1	GGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_A	ATCACGGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_B	CGATGTGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_C	TTAGGCGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_D	TGACCAGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS F1_E	ACAGTGGGGAGTATGGTCACTTGCTGA
	ssLDLR2-DS R1	TTCCCACCGAGTCTATCACC
ssAPC	ssAPC14 DS F1	TAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_A	ATCACGTAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_B	CGATGTTAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_C	TTAGGCTAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_D	TGACCATAGGCAACTACCATCCAGCAACAG
	ssAPC14 DS F1_E	ACAGTGTAGGCAACTACCATCCAGCAACAG
	ssAPC NJ 14 R1	GCGTGTGAGTGGGCAGTAGAGCTT
ssP53	ss tp53 E6 NJ F1	CTCCCCTGCCCTCAATAAGCTGTT
	ss tp53 E6 NJ F1_A	ATCACGCTCCCCTGCCCTCAATAAGCTGTT
	ss tp53 E6 NJ R1	TGGGAATGAGGGGTTTGGCAG
P65	ssP65-DS-F1	TGAGGCTATAACTCGCTTGG
	ssP65-DS-F1_A	ATCACGTGAGGCTATAACTCGCTTGG
	ssP65-DS-R1	ATCCGTAAGTGCTGGCTCTG
Barcode for multiplexing is underlined.		



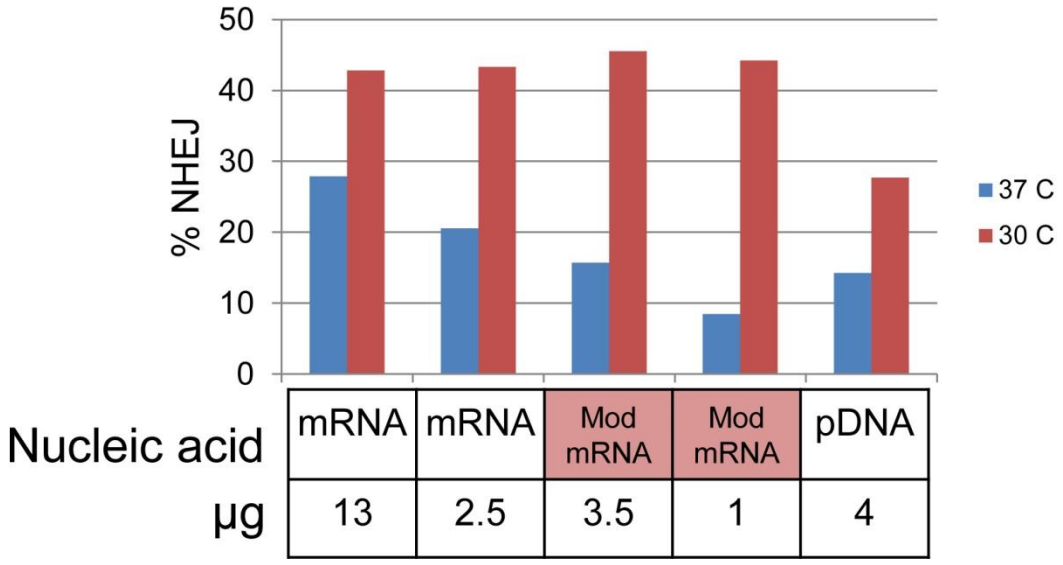




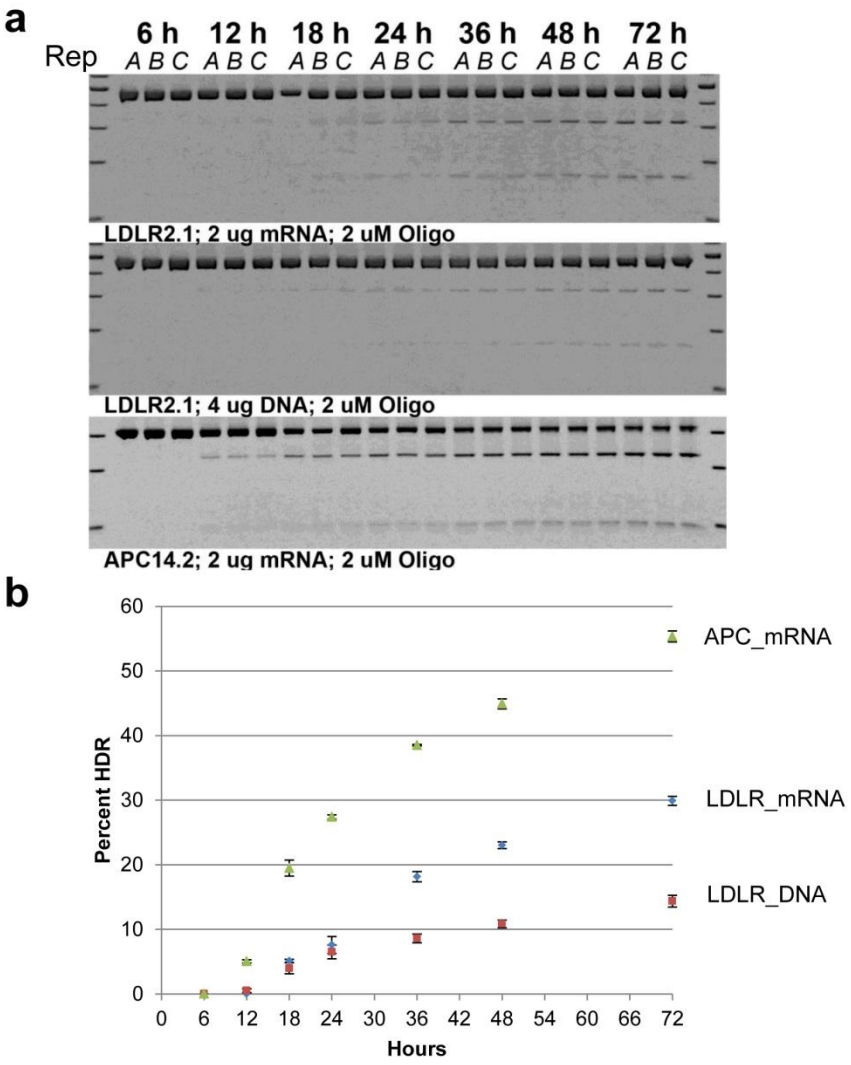
d

Template	HR+ /Puro^{R} colonies(%)
Plasmid	8/1007 (1)
AAV	36/280(13)

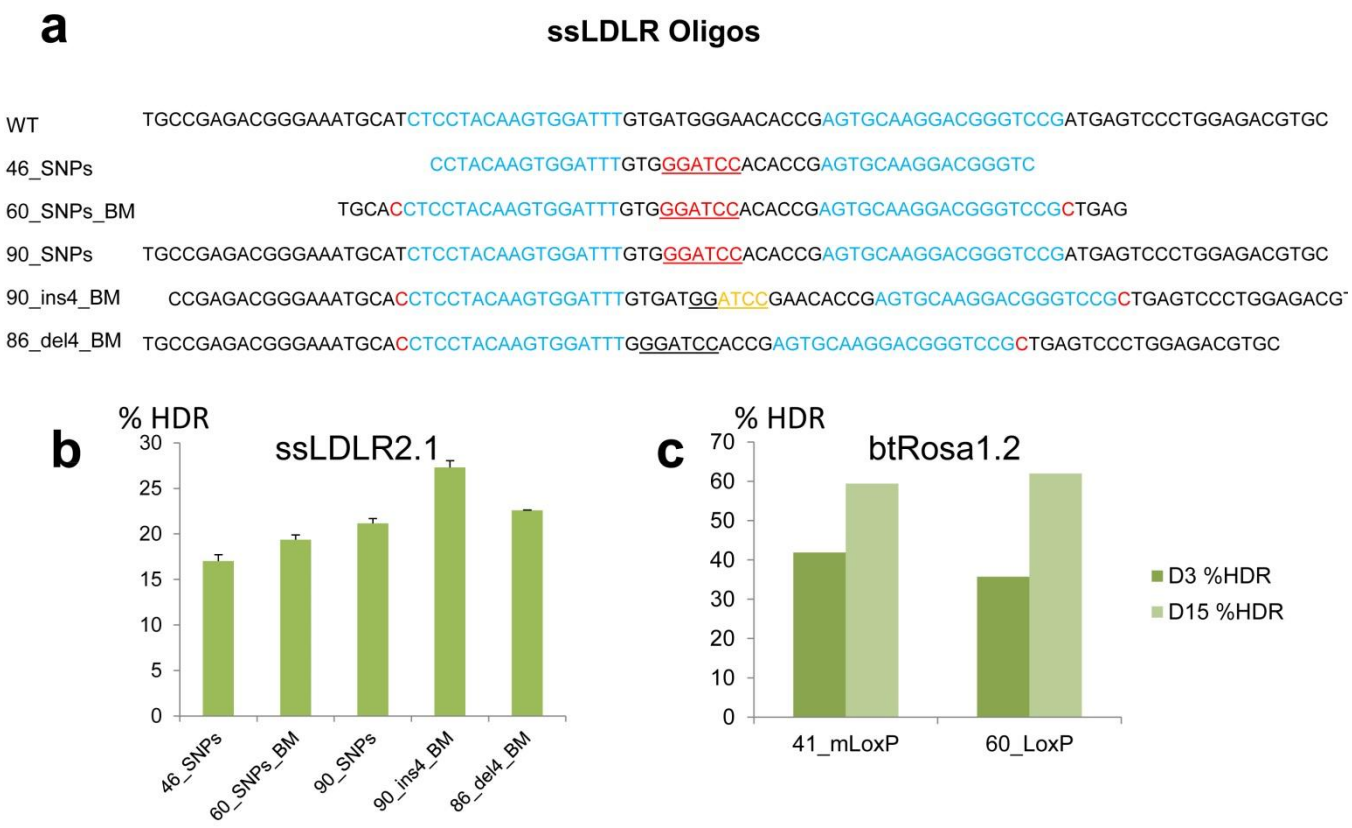
Supplementary Figure 1. TALEN stimulated allele transfer into Wagyu fibroblasts using plasmids or rAAV as repair templates. a) TALENs, btGDF83.1, and a homologous template (BB-HDR) were designed to introduce an 11bp deletion to exon 3 of bovine *GDF8* (Belgian Blue mutation) by TALEN stimulated homologous recombination. The homology template was prepared either as supercoiled plasmid DNA or packaged as a recombinant adeno-associated virus (rAAV) genome. Half of the binding site for the left TALEN is missing in the BB-HDR template due to the 11bp deletion, thus should be resistant to TALEN cleavage. **b, c**) Allele specific PCR demonstrates that HDR induction is dependent on co-transfection of TALENs and the BB-HDR template. The PCR assay was developed to specifically detect HDR modified *GDF8* alleles using primers c and c' (panel a). The 3' end of primer c' spans the 11 base pair deletion, and cannot amplify the wild type allele. Five hundred cell equivalents were included in each PCR reaction and positive controls consisted of 5-500 copies of a synthetic DNA corresponding to the outcome of homologous recombination. **d)** The allele specific PCR was conducted on individual puromycin resistant colonies derived from transposon co-transfected populations (30°C conditions only) as described previously(2). Candidate clones were confirmed by sequencing. Use of rAAV template resulted in a 16-fold enrichment in homologous recombination frequency in comparison to plasmid template.



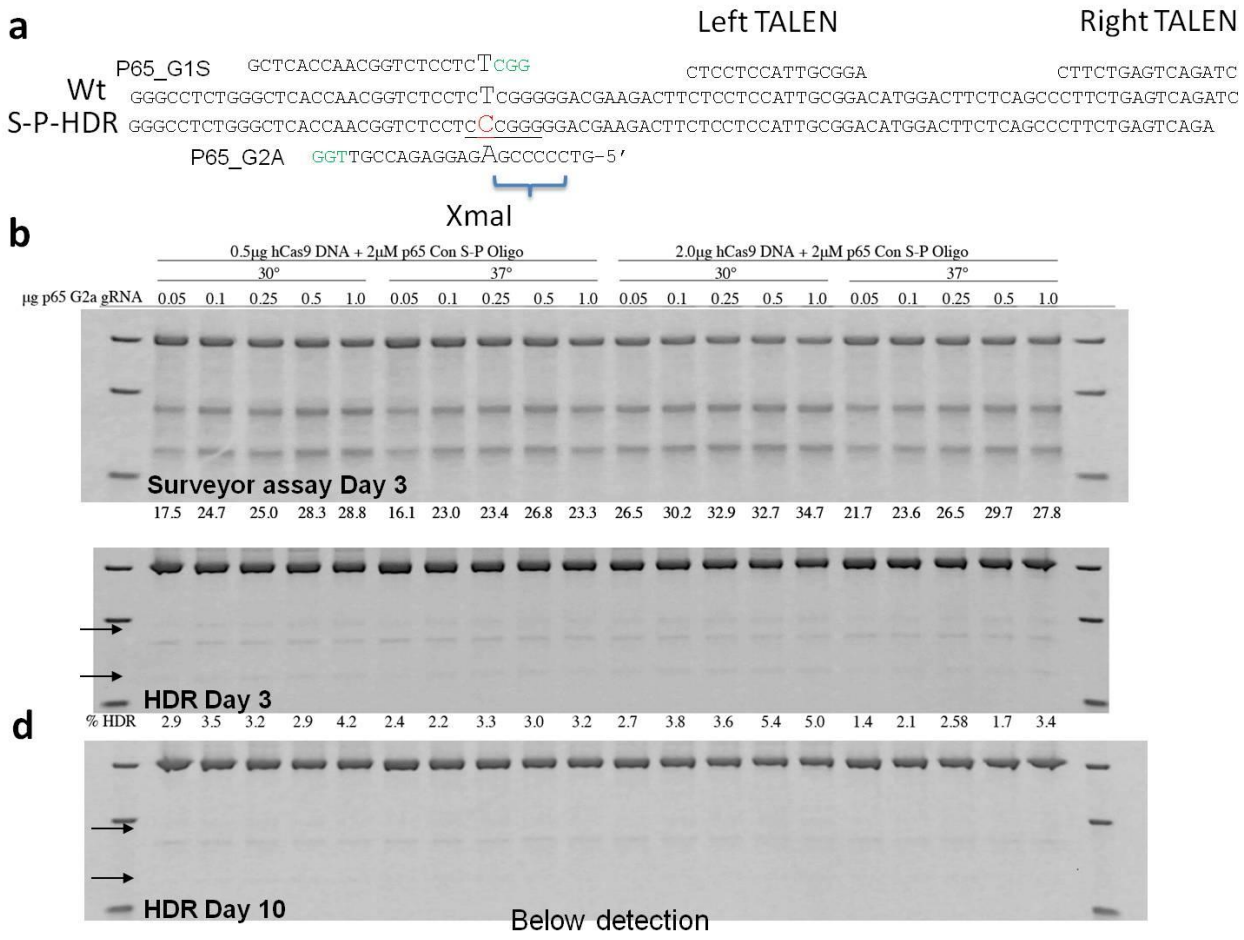
Supplementary Figure 2. Evaluation of transfected mRNA as a source of TALENs. The p65_11.1 TALENs were introduced into pig fibroblasts encoded by either unmodified mRNA, modified mRNA (mod mRNA) or plasmid DNA (pDNA). Two quantities of each TALEN preparation were transfected into cells by nucleofection (Lonza), cultured 3 days at 30°C or 37°C prior to analysis of indels. Percent NHEJ was similar for all mRNA transfections incubated at 30°C, while a dosage response could be observed for transfected cells incubated at 37°C. Notably, mRNA transfection in all groups incubated at 30°C significantly outperformed the TALENs transfected as plasmid DNA under the same conditions. Sample size was not sufficient to distinguish a benefit of modified versus unmodified mRNA in this test.



Supplementary Figure 3. Kinetics of TALEN induced HDR with oligonucleotide templates. Porcine fibroblasts were transfected with either TALEN-encoding mRNA or plasmid DNA and oligos with 4 base pair insertions targeting *LDLR* or *APC* genes. Cells from each transfection were then evenly split into seven 24-well plate wells, cultured at 30°C and assayed by RFLP at the indicated time points. **a)** RFLP analysis on cell populations at indicated time points. **b)** Results from panel ***a*** were quantified by densitometry and the averages were plotted as a function of time with SEM (n = 3). HDR signal first appears 12 hours post-transfection and accumulates over time. The onset of HDR at *LDLR* was independent of TALEN source, but the rate of HDR between 24 and 72 hours was much higher when mRNA was used compared to plasmid DNA.



Supplementary Figure 4. Influence of mutation type on the frequency of HDR. a) The sequence of five oligos used to target ss*LDLR*. Oligos vary in length and the type of mutation they are intended to introduce. TALEN binding sites are indicated in blue text and the novel BamHI site is underlined. SNPs including BMs are in red while insertions are marked in orange. **b)** Cells were transfected with LDLR2.1 TALEN mRNA (1 µg) and oligos (2µM final). HDR at day 3 was determined by RFLP analysis and the average with SEM (n=3) was plotted. The results suggest that insertion alleles are more efficiently incorporated than SNPs or deletions, but that homology length from 46-90 bp has negligible influence on HDR efficiency. **c)** Cattle cells were transfected with btRosa1.2 TALEN mRNA and either 41_mloxP or 60_loxP oligos (2µM final). The numbers 41 and 60 refer to the number of homologous bases. Each oligo contains a 34bp *loxP* site, either a modified (*mloxP)* or wild type *(loxP)* version, in the center of the spacer. Densitometry at day 3 and 15 show that insertion of *loxP* sites is both efficient and stable.



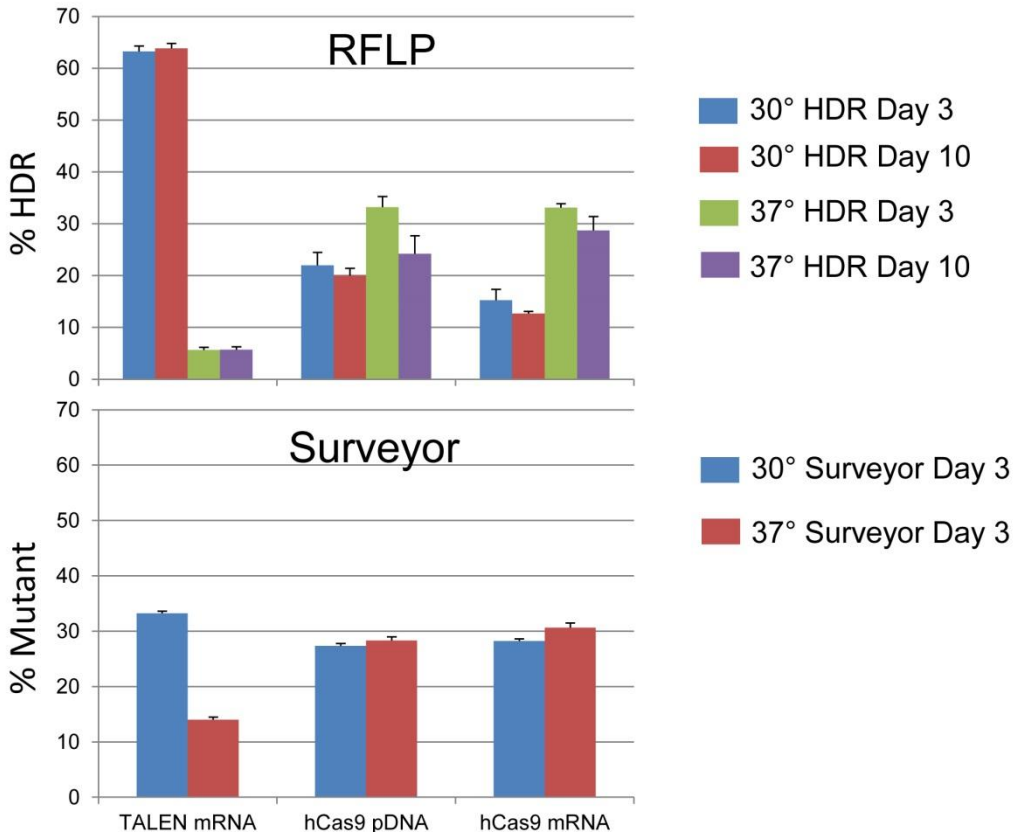
Supplementary Figure 5. CRISPR/Cas9 mediated HDR to introgress the *p65* S531P mutation from warthogs into conventional swine. a) The S531P missense mutation is caused by a T-C transition at nucleotide 1591 of porcine *p65* (*RELA*)(7). The S-P HDR template includes the causative T-C transition mutation (oversized text) which introduces a novel XmaI site and enables RFLP screening. Two gRNA sequences (P65_G1S and P65_G2A) are shown along with the p65.8 TALENs used in previous experiments. **b)** Landrace fibroblasts were transfected with S-P-HDR oligos (2µM), two quantities of plasmid encoding hCas9 (0.5 µg v.s. 2.0 µg); and five quantities of the G2A transcription plasmid (0.05 to 1.0 µg). Cells from each transfection were split 60:40 for culture at 30 and 37°C respectively for 3 days before prolonged culture at 37°C until day 10. Surveyor assay revealed activity ranging from 16-30%. **c and d)** RFLP analysis of cells sampled at days 3 and 10. Expected cleavage products of 191 and 118bp are indicated by black arrows. Despite close proximity of the DSB to the target SNP, CRISPR/Cas9 mediated HDR was less efficient than TALENs for introgression of S531P. Individual colonies were also analyzed using each gRNA sequence and are reported in Table 1.

a

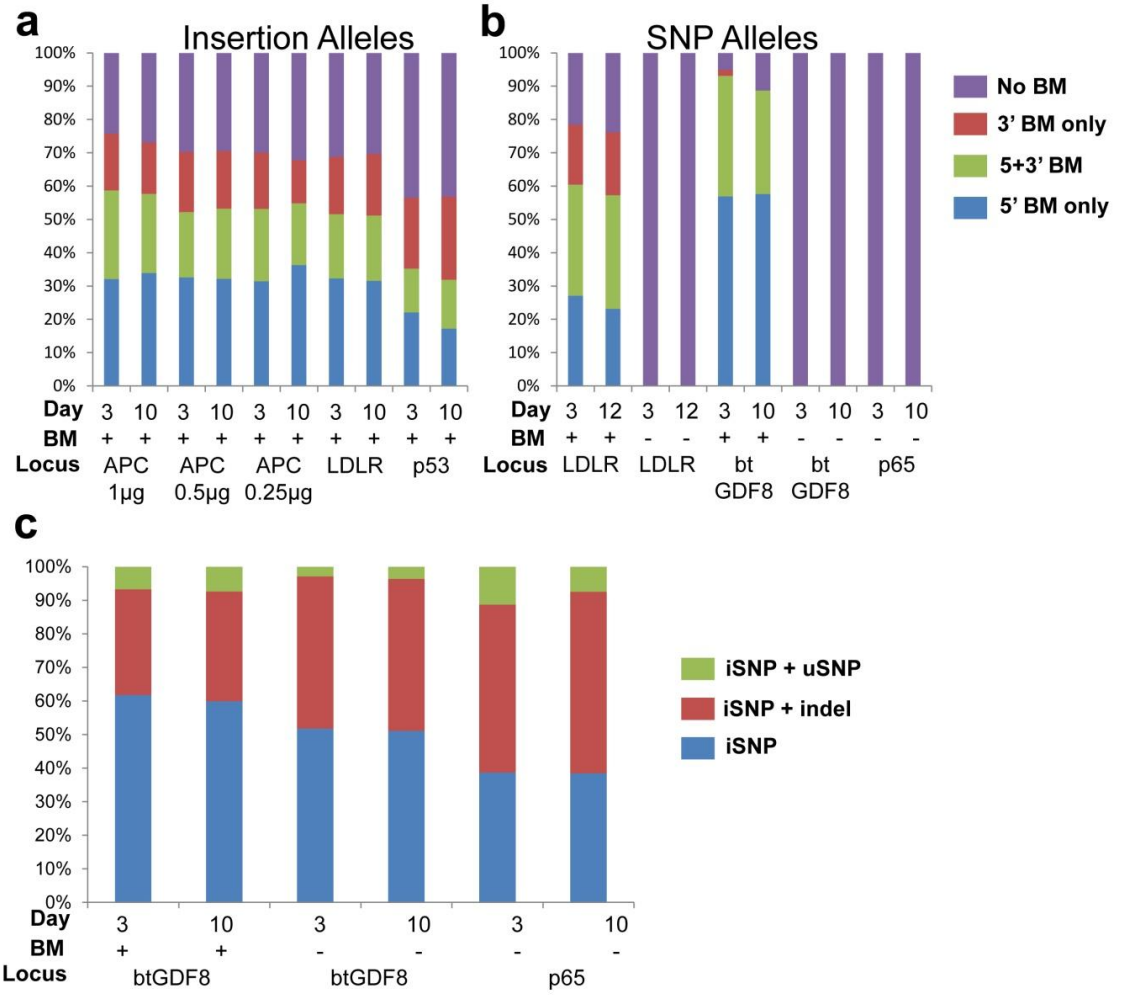
```
          APC14.2 TALENs GGAAGAAGTATCAGCCAT                    ACAGAAATTCTGGGTC-ANTISENSE STRAND
Wt  CCAGATCGCCAAAGTCATGGAAGAAGTATCAGCCATTCATCCCTCCCAGGAAGACAGAAATTCTGGGTCAACCACGGAGTTGCACT
                          APC14.2 G1a GGGAGGGTCCTTCTGTCTTTAAG-5'

HDR CCAGATCGCCAAAGTCACGGAAGAAGTATCAGCCATTCATCCCTCCCAGTGAAGCTTACAGAAATTCTGGGTCGACCACGGAGTTGCACT
```

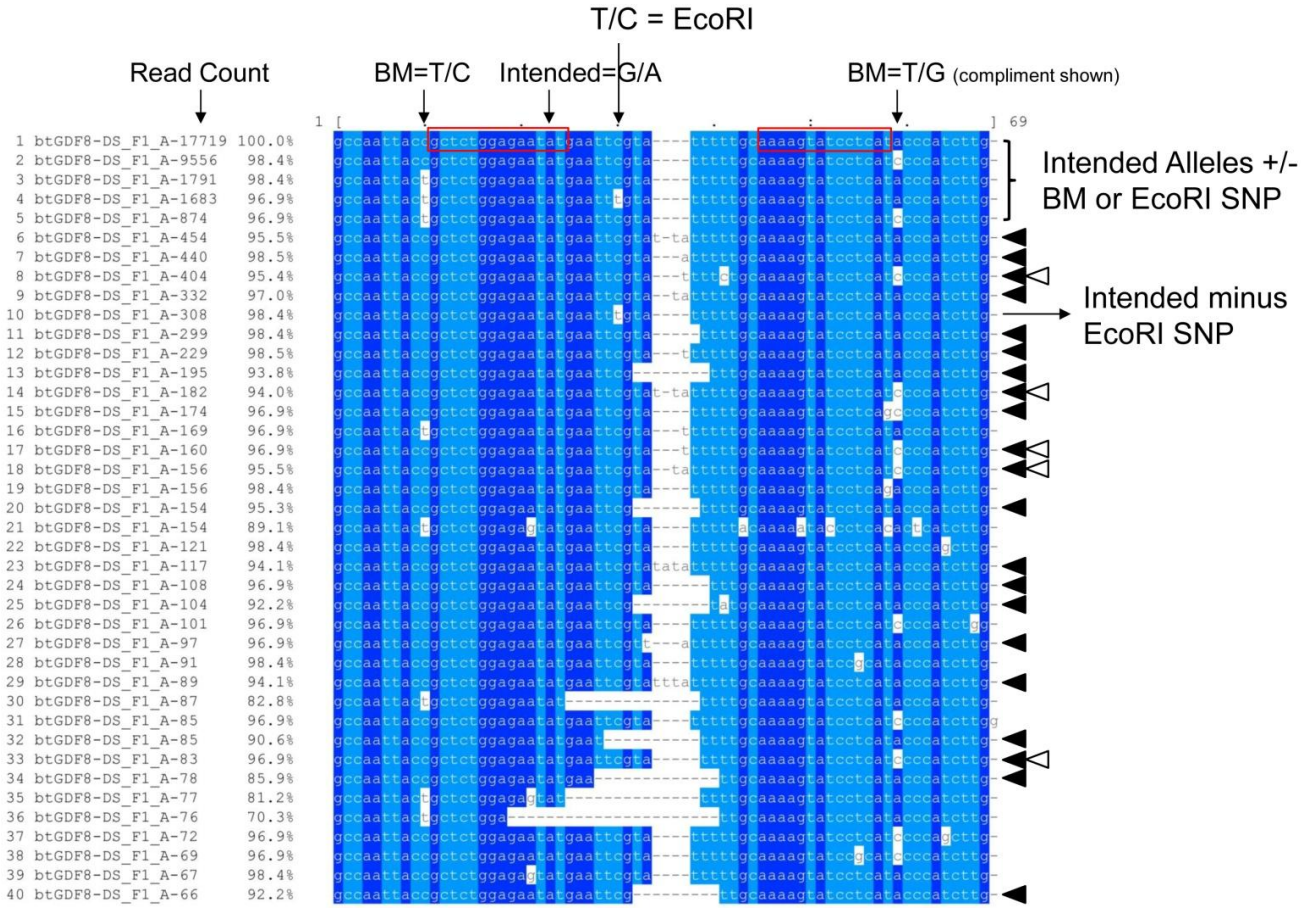
b



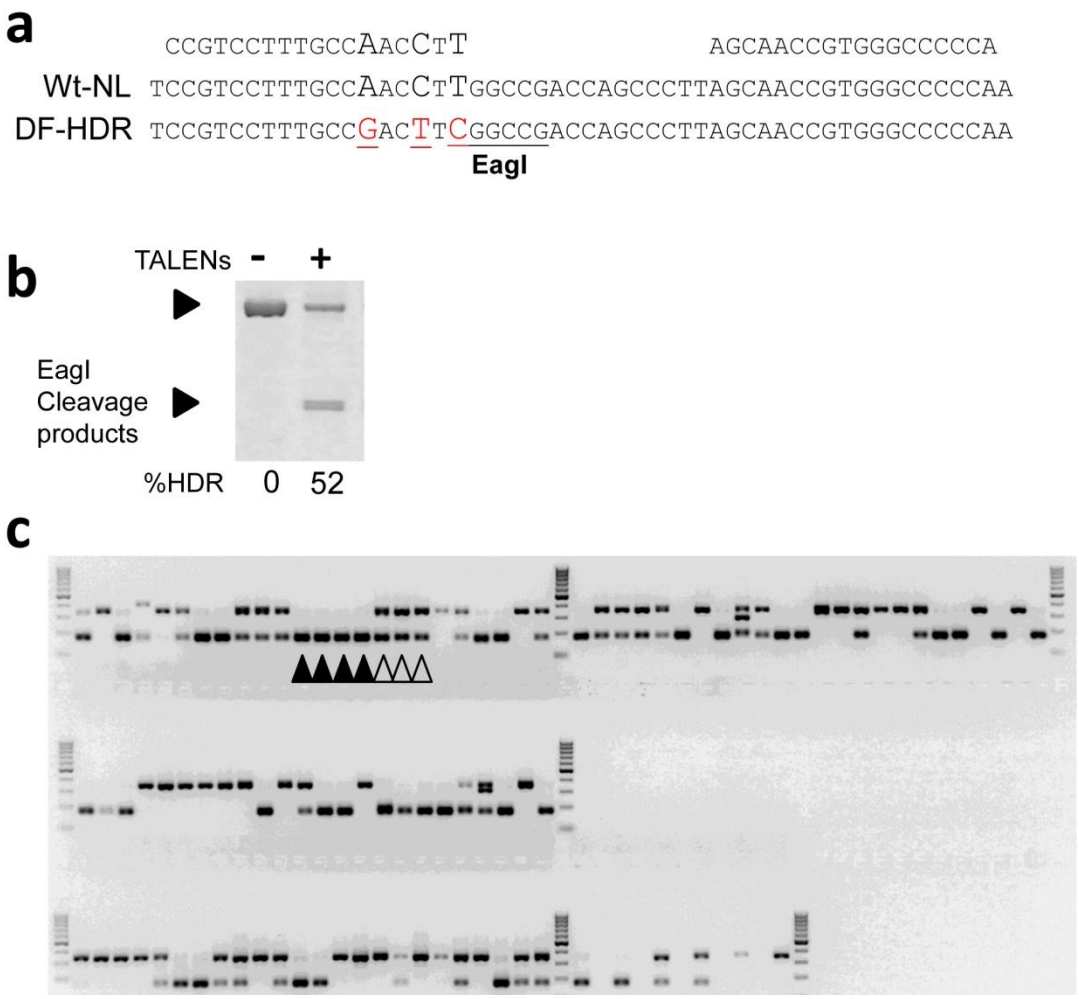
Supplementary Figure 6. Comparison of TALENs and CRISPR/Cas9 mediated HDR at porcine APC. a) APC14.2 TALENs and the gRNA sequence APC14.2 G1a are shown relative to the wild type *APC* sequence. Below, the HDR oligo is shown which delivers a 4bp insertion (orange text) resulting in a novel HindIII site. Pig fibroblasts transfected with 2μM of oligo HDR template, and either 1μg TALEN mRNA, 1 μg each plasmid DNA encoding hCas9 and the gRNA expression plasmid; or 1 μg mRNA encoding hCas9 and 0.5 μg of gRNA expression plasmid, were then split and cultured at either 30 or 37°C for 3 days before expansion at 37°C until day 10. **b)** Charts displaying RFLP and Surveyor assay results. As previously determined TALEN stimulated HDR was most efficient at 30°C, while CRISPR/Cas9 mediated HDR was most effective at 37°C. For this locus, TALENs were twice as effective as the CRISPR/Cas9 system for stimulation of HDR as measured by RFLP. At 30°C, the HDR was so efficient with TALENs that it exceeded the sensitivity of the Surveyor assay, which depends on allelic heterogeneity. In contrast to TALENs, there was little difference in HDR when hCas9 was delivered as mRNA versus plasmid.



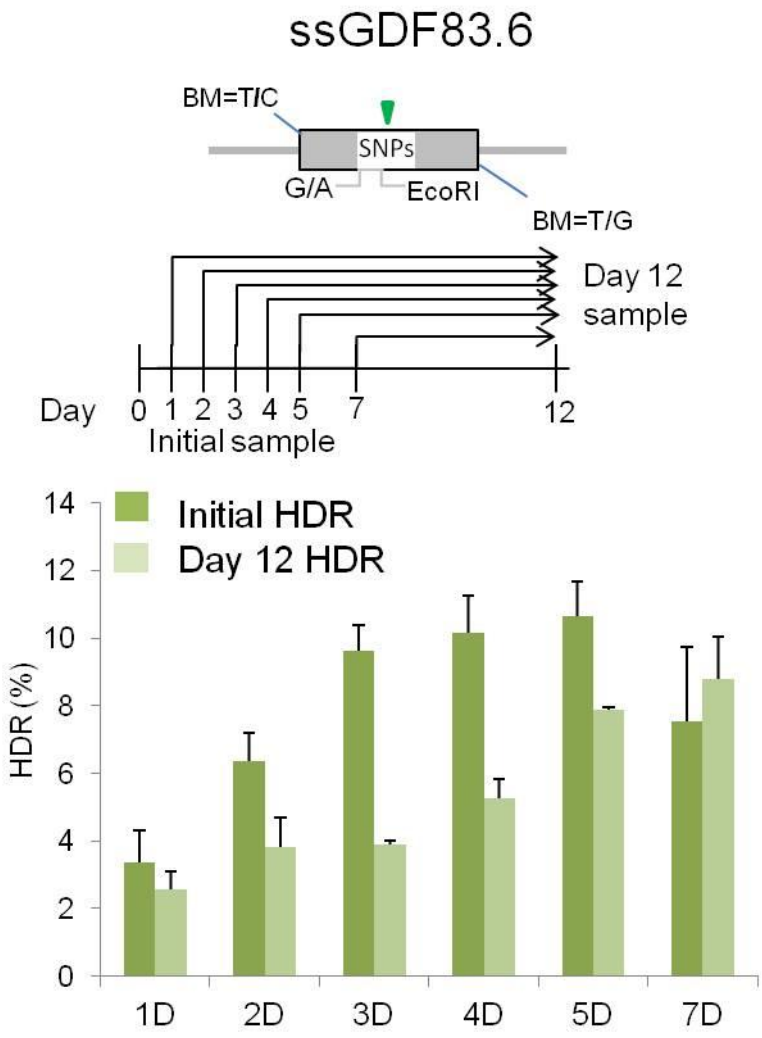
Supplementary Figure 7. Sequence analysis of HDR alleles. Sequencing reads containing the correct insertion (**a**) or SNP allele (**b**) were analyzed for incorporation of BM. The target locus, time point and whether or not BMs were included in the oligo are indicated below each graph. In general, the 5' BM was incorporated most frequently into the HDR conversion tract, followed by inclusion of both BMs, or the 3' BM only. The distribution of BM is somewhat skewed towards incorporation of both BM when the intended mutation to *LDLR* is a SNP versus a 4 bp insertion allele. It is also interesting to note that the majority of intended reads for bt*GDF8* have incorporated at least one BM, but seldom have the 3' BM alone. Thus, while BM did not have a significant impact on the frequency of maintaining the intended SNP (iSNP) allele in culture, their enrichment relative to *APC*, *p53* and *LDLR,* suggests that they might have offered some protection from TALEN re-cleavage. **c**). The data of **Fig. 4c** was further classified by mutation type and compared. Some reads contained only the iSNP, others had a concomitant indel (iSNP + indel), or a concomitant unintended SNP (iSNP + uSNP). There appears to be some elevation in the frequency of iSNP + indel when BMs were not included in the template, and the majority of indels were located in the spacer region so are likely to be the result of re-cutting of already converted alleles.



Supplementary Figure 8. Sequencing reveals indels in HDR alleles with incorporated BM. An alignment of the top 40 reads sorted positive for the iSNP (G938A) are shown. These data are derived from day 10 btGDF83.6-G treated cells transfected with an oligo template that included BMs, an EcoRI site and the G938A SNP. Each of these sites and the TALEN binding sites (red boxes) are indicated. The number of reads counted for each line is indicated in the left margin. Reads displaying an intended outcome are shown; the 4bp gap is introduced by the alignment software to accommodate reads containing 4bp insertions. Several reads having the 5' BM (filled triangle), the 3' BM (open triangle), or both BMs (open and filled triangles) also have indels in the spacer region proving the TALENs can bind and cleave without the conserved 5' thymidine nucleotide. Several reads differ by only a single base mismatch with the intended allele; we suspect a small proportion of these are due to errors in oligo synthesis or DNA sequencing. To aid visualization, purines (A and G) are colored dark blue while pyrimidines (T and C) are light blue.



Supplementary Figure 9. Multiple SNPs in the TALEN DNA-binding site stabilize HDR alleles in the *EIF4GI* gene. a) A portion of wild type *EIF4GI* Wt-NL is shown. One pair of TALENs was designed to cut the wild type *EIF4GI* to stimulate homologous recombination. Also aligned to the Wt sequence is the core sequence of the donor oligo, DF-HDR, used to introduce three SNPs (red oversized letters) into the genome. The third SNP creates a novel EagI restriction site that was used for RFLP analysis. Pig fibroblasts were transfected with EIF4GI14.1 TALEN mRNA (2µg) and DF-HDR (2µM) and then cultured at 30°C for 3 days prior to analysis and colony propagation. **b)** RFLP analysis on population three days post transfection. Expected product sizes of 344, 177 and 167bp are indicated by filled triangles. **c)** RFLP assay on isolated cellular clones. Day 3 cells were used to derive monoclonal colonies through dilution cloning. An example of colonies with heterozygous (open triangles) or homozygous (filled triangles) HDR alleles are indicated.



Supplementary Figure 10. Extension of hypothermic treatment enhances maintenance of SNP HDR alleles. Pig fibroblasts were transfected with TALEN mRNA (1µg) and oligos (3µM). Cells from two independent transfections were pooled for each replicate and evenly distributed into six wells of a 6-well plate and cultured at 30°C. Samples were collected from these populations for RFLP analysis on days 1-7 (minus day 6, 1D to 7D along X-axis) post-transfection and the remaining cells were transferred to 37°C. Samples for each condition were collected again at day 12 for RFLP analysis. The average HDR and SEM (n = 3) is shown at the initial collection and once again at day 12. The frequency of HDR plateaus after 3 days of hypothermic treatment, however, longer hypothermic treatment enhances the stability of HDR alleles in culture.

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