Supplemental file: Annotated donor plasmid sequences and primer list

Supplemental text A*:* Annotated pGEM-WingGFP-*tan* sequence (7290bp):

Underlined sequences represent primers used for plasmid construction and screening.
Double underlines represent areas where two primers overlap

5’ homology arm
5’ target PAM
Unique portion of 5’ target site for reporter excision (restriction sites: BglII, BsiWI, Acc65I)
Pupal wing enhancer from *D. melanogaster yellow* gene
EGFP with Hsp70 promoter and SV40 polyadenylation signal
Unique portion of 3’ target site for reporter excision (restriction sites: NarI, Bsu36I, ClaI)
3’ homology arm
3’ target PAM
Restriction sites within pGEM backbone for homology arm removal: 5’ end – ApaI, SphI; 3’ end – SbfI, MluI

ATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGT
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GGGAAGGGCGATCGGTGCGGGCCTCTTCGCT

Supplemental text B: Annotated pGEM-3XP3.RFP-*tan* sequence (6653bp):

5’ homology arm
5’ target PAM
Unique portion of 5’ target site for reporter excision (restriction sites: BglII, BsiWI, Acc65I)
3XP3 eye-expression Promoter
RFP with α-tubulin 3’UTR
Unique portion of 3’ target site for reporter excision (restriction sites: NarI, Bsu36I, ClaI)
3’ homology arm
3’ target PAM
Restriction sites for homology arm removal: 5’ end – ApaI, NcoI; 3’ end – SexAI, MluI

ATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGT
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CGATCGGTGCGGGCCTCTTCGCT

Table S1: Oligonucleotides and primers used in reagent construction and molecular screening: All base-pairing nucleotides are in capital letters. Tailing sequences added to primers are in lowercase letters.

Pair #	Purpose	Forward (description)	Reverse (description)
1	Screen for plasmid backbone integration on the 5' end of the donor. (282bp band if backbone is present)	GATGTGCTGCAAGGCGATTAA (In pGEM-WingGFP-tan plasmid backbone, 5' of homology arm)	GAGCAGGCAACGCATATAGAAAC (Within the pGEM-WingGFP-tan 5' homology arm)
2	Screen for plasmid backbone integration on the 3' end of the donor. (481bp band if backbone is present)	GCGACCTATCGTTCCTATGC (Within the pGEM-WingGFP-tan 3' homology arm)	CAGCTGGCACGACAGGTTTC (In pGEM-WingGFP-tan plasmid backbone, 3' of homology arm)
3	Confirm wing-GFP reporter insertion at correct locus on the 5' side. (1541bp band if correctly integrated)	AAACGAACCGCAACTGATATTGAAC (In *D. americana tan* transgene DNA outside the 5' homology arm. Represented as primer X in Figure 2.)	GTCAATTTCCACTGTCCCGATTG (In the pGEM-WingGFP-tan reporter sequence, specifically the pupal wing enhancer from the *D. melanogaster yellow* gene)
4	Confirm wing-GFP reporter insertion at correct locus on the 3' side. (1163bp band if correctly integrated)	TGGTTTGTCCAAACTCATCAA (In the pGEM-WingGFP-tan reporter sequence, specifically in GFP)	AATATAGAGCGCAGCGGCTGTT (In *D. americana tan* transgene DNA outside the 3' homology arm. Represented as primer Y in Figure 2.)
5	Amplify 5' homology arm for pGEM-WingGFP-tan tailed with Gibson assembly overlaps	catggcggccgcgggaattcgatCTCTGGATAAGCGTCAGCCTC (Represented as primer A in Figure 2.)	gatgagtatggatggtaccgtacgagatctTGCCGGAGGACATGCTGGAC
6	Amplify 3' homology arm	ttataaccctggcgcctaaggatcgatACTTGGGGATACCCTGAACC	gccgcgaattcactagtgatGCTTGTGCGGGGTATTATGAG

	for pGEM-WingGFP-tan tailed with Gibson assembly overlaps		(Represented as primer B in Figure 2)
7	Amplify wing-expressing GFP reporter tailed with Gibson assembly overlaps	agatctcgtacggtaccATCCATACTCATCAATGTATCTTATAGCTTATCGTTACGTGTA	agtatcgatccttaggcgccAGGGTTATAATTACCACAGGTCG
8	Amplify *tan* sequence to replace wing-GFP reporter in second stage of allele replacement	catggcggccgcgggaattcgatCTCTGGATAAGCGTCAGCCTC (Same forward primer as in Pair #5 from this table. Represented as primer A in Figure 2.)	gccgcgaattcactagtgatGCTTGTGCGGGGTATTATGAG (Same reverse primer as in Pair #6 from this table. Represented as primer B in Figure 2.)
9	Spanning PCR across edited *tan* locus after second replacement step (~2.5kb band if repaired correctly)	AAACGAACCGCAACTGATATTGAAC (Same forward primer as in Pair #3 from this table. Represented as primer X in Figure 2.)	AATATAGAGCGCAGCGGCTGTT (Same reverse primer as in pair #4 from this table. Represented as primer Y in Figure 2.)
10	Amplify new 3' homology arm which in the modified pGEM-WingGFP-tan before the construction of pGEM-3XP3.RFP-tan (cloned into pGEM-WingGFP-tan using Bsu36I and MluI)	atcctgcctaaggatcGATGCTAGGTCTGACTTTTATATAGAATAATC (Tailed with Bsu36I and ClaI restriction sites.)	gatacaacgcgtatctGAGAAGCAACCTGGTC (Tailed with MluI restriction sites.)
11	Amplify 3XP3-RFP reporter out of M{3XP3-RFP.attP}ZH-51C landing site to assemble	ttatggcgcgCCAACGTGTCGGTACCAATTG (Contains Acc65I restriction site in the primer.)	atcgatccttaggcgccAGAGAGCTTCGCATGGTTTTGC (Tailed with NarI and Bsu36I restriction sites.)

	pGEM-3XP3.RFP-tan		
12	Oligo-nucleotide pair for cloning sgRNA expression plasmid pCFD3 targeting t5 site (see Figure 2)	gtcGCGACGGGGCAATATCTTGC (Sense oligo. which matches t5 target site. Note that the sgRNA does not include the PAM nucleotides. "gtc" tail is for cloning into the BbsI restriction site in pCFD3. Instructions for pCFD3 cloning at: http://www.crisprflydesign.org/wp-content/uploads/2014/05/Cloning-with-pCFD3.pdf)	aaacGCAAGATATTGCCCCGTCG (Antisense oligo. to anneal with sense oligo to form insert for cloning. "aaac" tail is for cloning into the BbsI restriction site in pCFD3.)
13	Oligo-nucleotide pair for cloning sgRNA expression plasmid pCFD3 targeting t3 site (see Figure 2)	gtcGTTCAGGGTATCCCCAAGTC (See description for Pair #12)	aaacGACTTGGGGATACCCTGAA (See description for Pair #12)
14	Oligo-nucleotide pair for cloning sgRNA expression plasmid pCFD3 targeting t5re site (see Figure 2)	gtcGGTACCGTACGAGATCTTGC (See description for Pair #12)	aaacGCAAGATCTCGTACGGTAC (See description for Pair #12)
15	Oligo-nucleotide pair for cloning sgRNA expression plasmid pCFD3 targeting t3re site (see Figure 2)	gtcGGCGCCTAAGGATCGATACT (See description for Pair #12)	aaacAGTATCGATCCTTAGGCGC (See description for Pair #12)
Seq1	Sanger sequencing 5' PolyT	CTCTGGATAAGCGTCAGCCTCG	
Seq2	Sanger sequencing 5' PolyT	CGCCCATTGCTGTTATTGTT	
Seq3	Sanger sequencing 5' target sites	ATTACTGCTCATCCTGCTCG	
Seq4	Sanger	TCACTATGAGGTCGGCTTCG	

	sequencing 3' PolyT		
Seq5	Sanger sequencing 3' PolyT and 3' target sites		TTGACTAGTGTCTACGCAGATCG

Table S2: Genome editing rates in the first stage of the allele swap

Embryos injected	Injected adults crossed	Founders[1]	Homozygous edited lines established from progeny of founders	Proportion of founders' progeny with wing GFP expression	Homozygous lines with carrying correct[2] repair events
1220	150	6	43	2.5%-25.4%	13

1: In this experiment, founders are defined as injected adults who produced progeny with pupal wing GFP expression.
2: Correct repair events were identified first by the PCR screens described in the results section. All 13 lines that passed the PCR screen were later verified to carry correctly repaired sequence by Sanger sequencing.

Table S3: Genome editing rates in the second stage of the allele swap

Embryos injected	Injected adults crossed	Founders[1]	Homozygous edited lines established from progeny of founders	Proportion of founders' progeny that lost wing GFP expression	Homozygous lines with carrying correct[2] repair events
1361	179	5	10	0.68%-1.1%	1

1: In this experiment, founders are defined as injected adults who produced progeny that did not express GFP in the pupal wings.
2: Correct repair events were identified first by the PCR screen described in the results section. The single line that passed the PCR screen was later verified to have a correctly repaired sequence by Sanger sequencing.

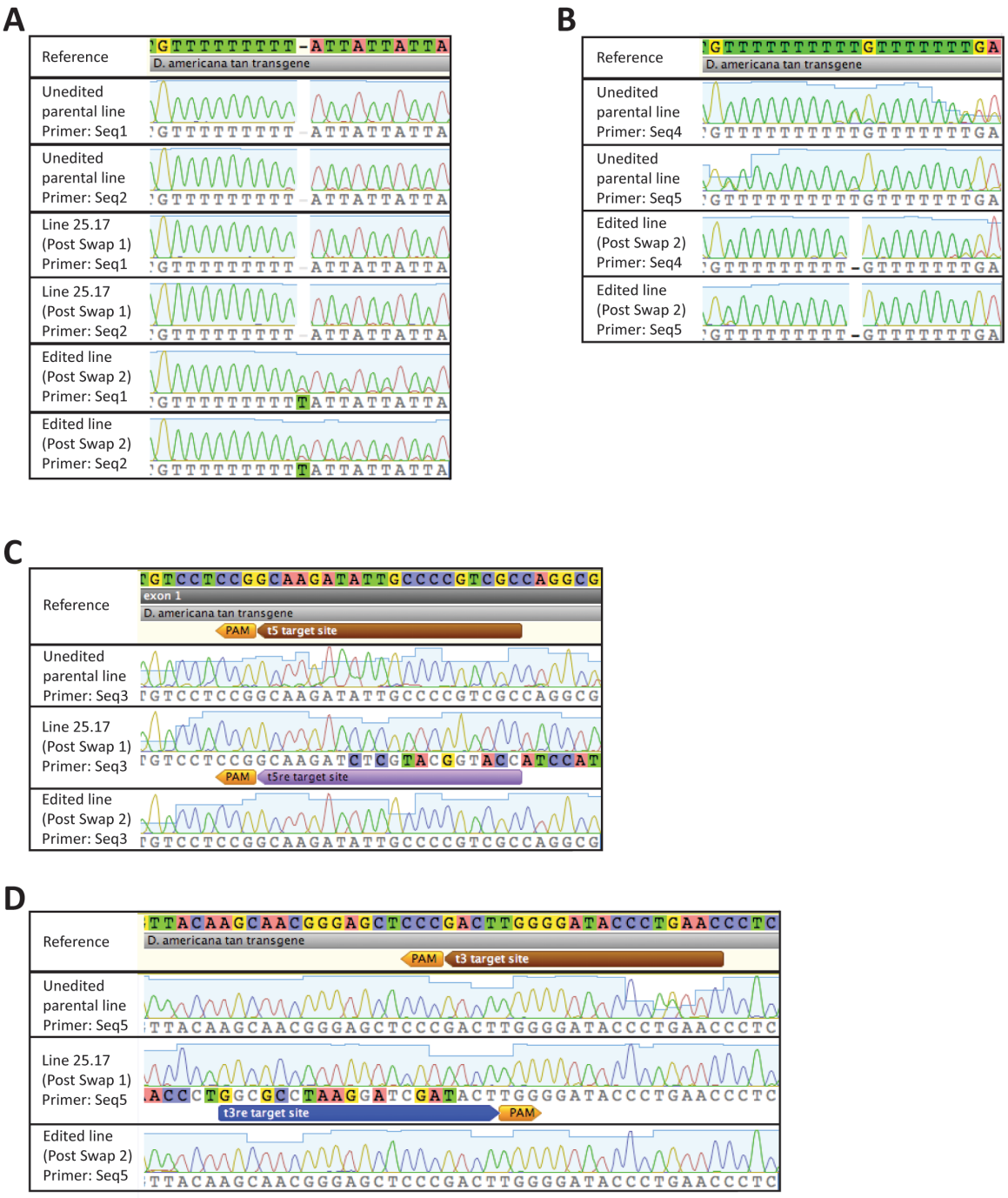

Figure S1: Chromatograms showing the sequence of the edited sites before and after each stage of the editing process. In all panels, the reference sequence in the top row represents the unedited *D. americana tan* transgene that was targeted for genome editing in this study. The sequencing primers are listed to the left of the chromatograms, and all primer sequences are provided in Table S1. Panels (A) and (B) show chromatograms of the edited homopolymer runs in the 5' homology arm (A) and in first intron (B) of the *D. americana tan* transgene throughout the allele swap process. Sequencing data for homopolymer runs are shown for two different primers per DNA template. Panels (C) and (D) show chromatograms of the 5' target sites and 3' target sites, respectively, across the stages of genome editing. The PAM sequences and sgRNA target sites are labeled.