

# Supplementary Materials for Engineered Reproductively Isolated Species Drive Reversible Population Replacement

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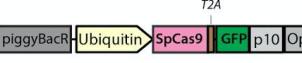
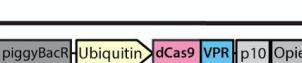
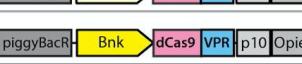
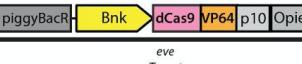
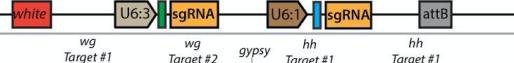
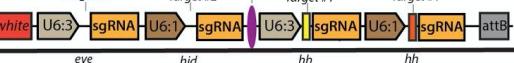
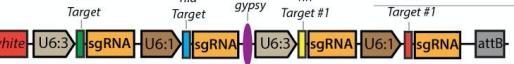
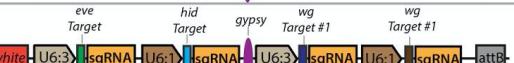
**Supplementary Table 5** Primers used in this study.

**Supplementary Table 6.** Population studies with associated fitness costs of SPECIES strains relative to WT

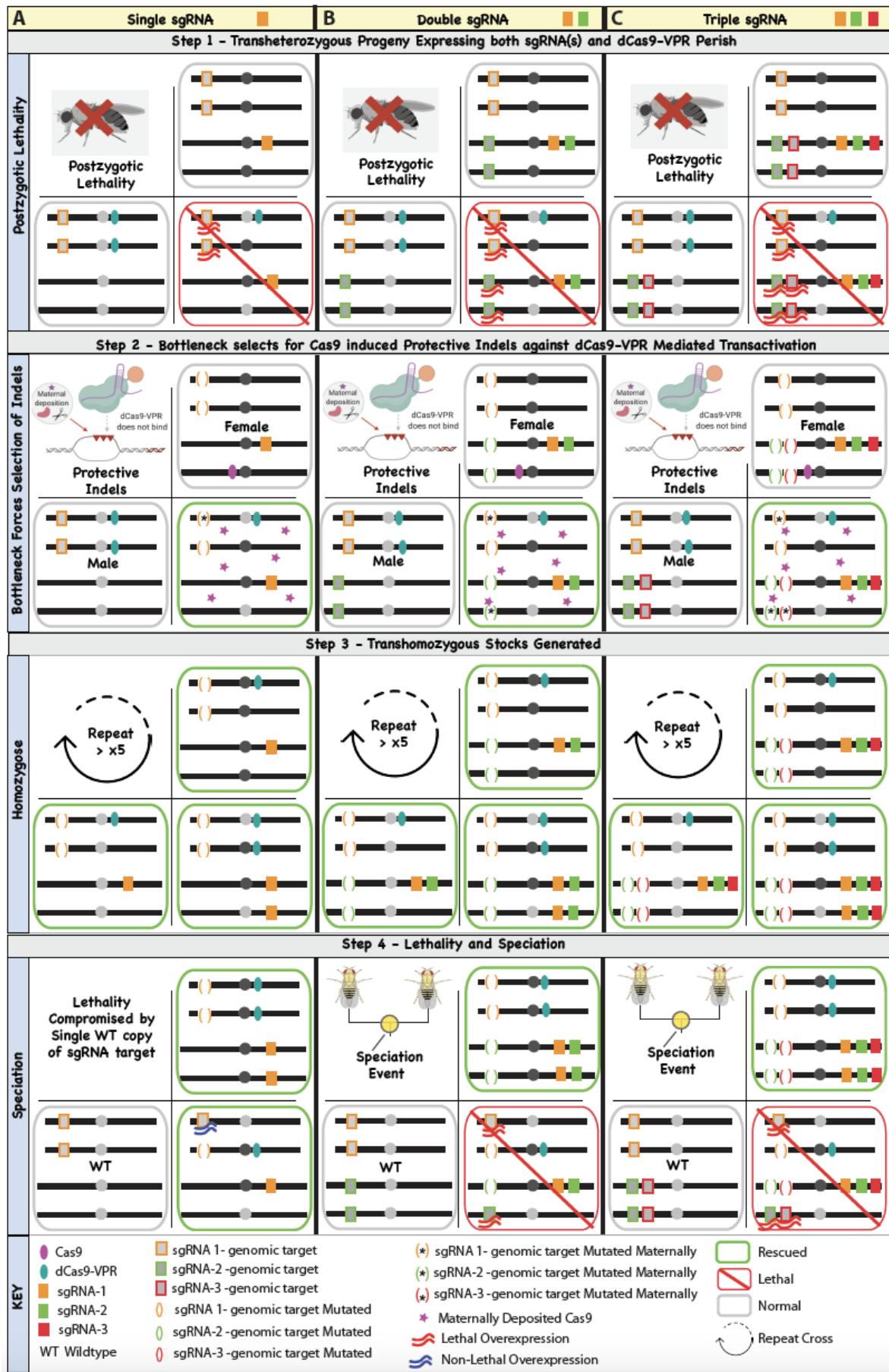
**Supplementary Table 7.** Correlation between all RNA-seq samples.

**Supplementary Table 8.** Outcrosses to Global Diversity Lines.

## SUPPLEMENTARY FIGURES

	ID	Schematic Construct Map	attP Site (Chrom)	AddGene ID	BSC#	Citation	
SpCas9	OA-874W		9750 (III)	112686	79005	(Kandul et al., 2019)	
	OA-986B		36304 (II)	124999			
	OA-986C		36304 (II)	125000	91792		
	OA-986D		36304 (II)	125001			
	OA-986E		36304 (II)	125002			
sgRNA Constructs	dCas9 -AD		9732 (III)	125003	91791	This study	
			8622 (III)				
	2 Targets		8622 (III)	125006			
			86fa (III)				
	3 Targets		9732 (III)	125004			
			8622 (III)				
	3 Targets		9732 (III)	125005			
			8622 (III)				
	3 Targets		8622 (III)	125007			
			86fa (III)				

**Supplementary Figure 1. Constructs used in this study.** A list of constructs used in this study, providing the construct ID, construct schematics, chromosomal insertion sites, Addgene ID number, Bloomington Stock number and citation.



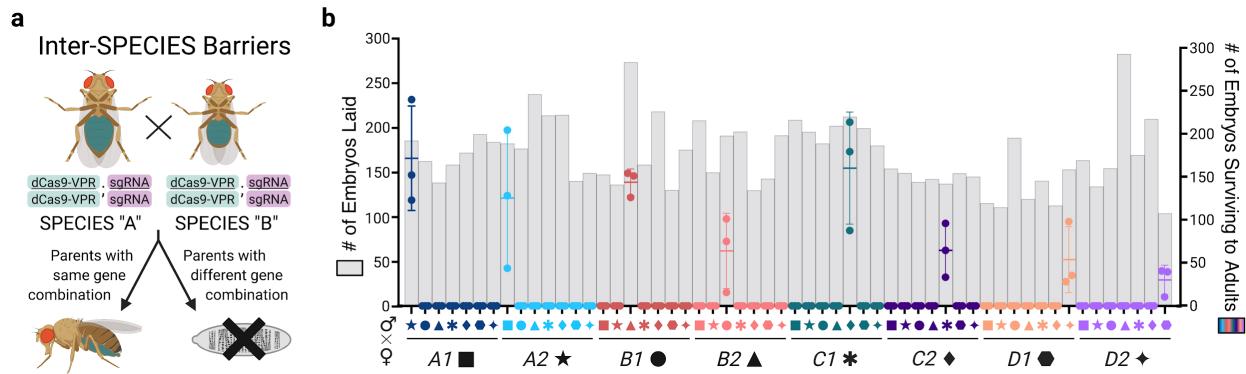
**Supplementary Figure 2. Schematic of the genetic crossing scheme used to engineer SPECIES.** (A) Complete lethality (100%) was observed in transheterozygotes (dCas9+/; sgRNA+) when an sgRNA was crossed to dCas9-VPR due to lethal overexpression (Step 1). To generate protective indels, the sgRNA was first crossed to Cas9, then transheterozygous (Cas9+/; sgRNA+) females were crossed to dCas9-VPR males generating a bottleneck by which a small proportion of transheterozygotes (dCas9+/; sgRNA+) survived due to protective indels generated by Cas9/sgRNA (Step 2). Surviving individuals (inheriting Cas9 protein maternally but lacking Cas9 as a gene) were inbred for many generations (>5) to generate homozygous stocks (Step 3). To assess lethality and speciation, homozygous stocks were bidirectionally outcrossed to WT. For a single sgRNA system, complete synthetic lethality and speciation was not observed due to the fact that one WT copy of the target promoter was not sufficient to induce lethal overexpression (A, Step 4). To overcome this issue, we multiplexed using either two sgRNAs (**B**), or three sgRNAs (**C**), and repeated steps 1-4 to engineer reproductively isolated synthetic species.

Transgenes Used					
Synthetic Species	Cas9	dCas9-AD	gRNAs	attP Site (Chrom)	Citation
A1	874W attP Site 9750 Chrom III	OA-986C attP Site 36304 Chrom III	OA-1045A	9732 (III)	This study
A2				8622 (III)	
B1			OA-1045B	9732 (III)	
B2				8622 (III)	
C1			OA-1045C	9732 (III)	
C2				8622 (III)	
D1			OA-1045D	8622 (III)	
D2				86Fa (III)	

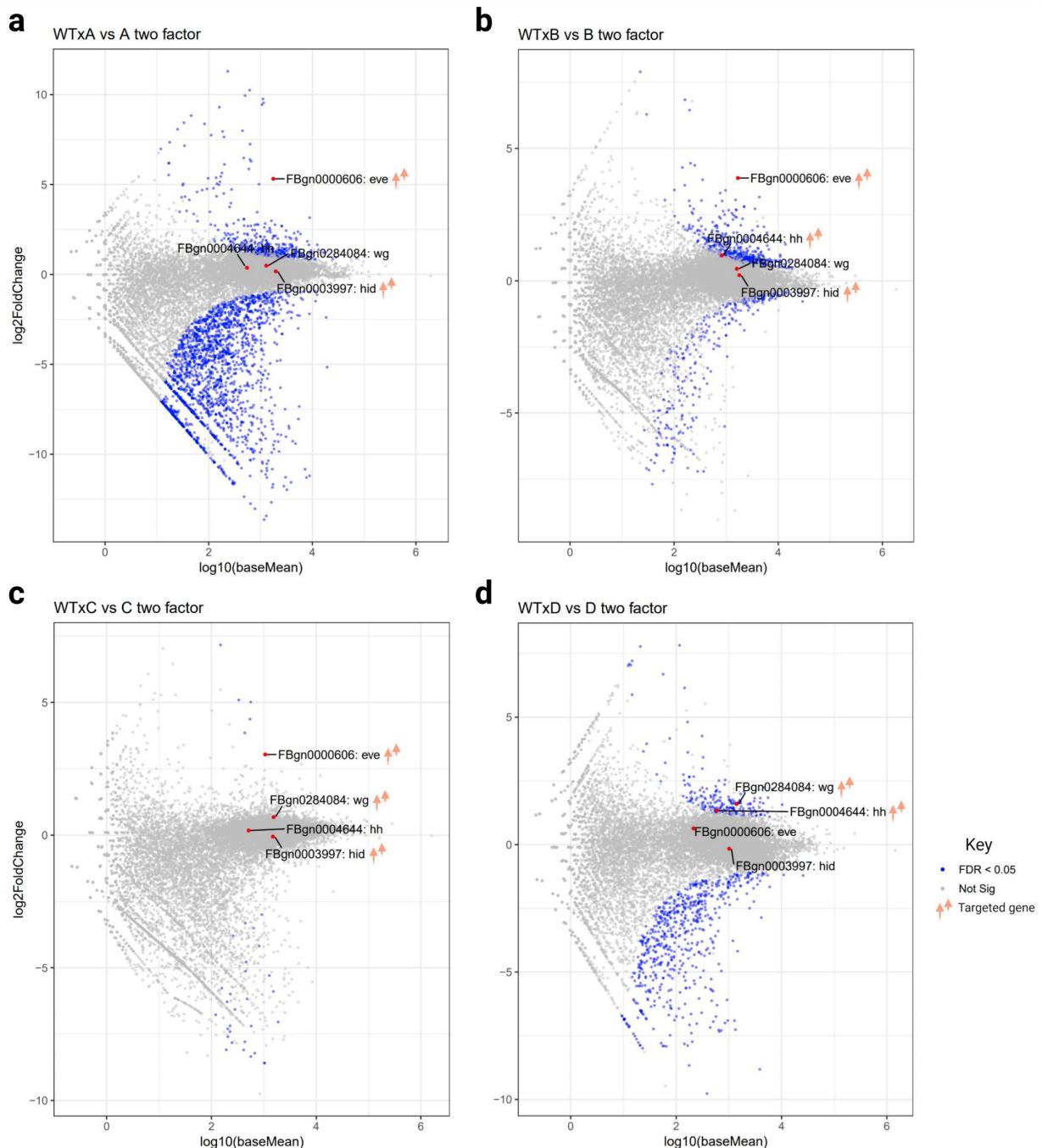
**Supplementary Figure 3. Generation of eight SPECIES.** For each synthetic species (A1,A2,B1,B2,C1,C2,D1,D2) the transgene ID, and chromosomal insertion site are listed.

9732 1045A (eve+hid) - A1	
WT   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
1   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
2   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
3   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
4   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
1   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
hid WT   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
18   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
PAM	sgRNA
8622 1045A (eve+hid) - A2	
WT   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
eve 11   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
1   CAGCACCGCACGATTAGCACCGTTCCGCTCAG	
WT   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
2   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
1   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
3   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
hid 2   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
1   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
3   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
1   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
PAM	sgRNA
9732 1045B (eve+hid+hh) - B1	
WT   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCT	
eve 3   CAGCACCGCACGATTAGCACCGTTCCGCT	
6   CAGCACCGCACGATTAGCACCGTTCCGCT	
1   CAGCACCGCACGATTAGCACCGTTCCGCT	
WT   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
2   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
hid 12   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
2   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
WT   TCTGGTTGTCCTCCACTTCTGGCATATAAGCAGCATAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCACACAAT	
13   TCTGGTTGTCCTCCACTTCTGGCATATAAGCAGCATAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCACACAAT	
3   CCTGAGCTTGGCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
4   CCTGAGCTTGGCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
hh 1   CCTGAGCTTGGCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
3   CCTGAGCTTGGCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
1   CCTGAGCTTGGCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
1   CCTGAGCTTGGCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
PAM	sgRNA
8622 1045B (eve+hid+hh) - B2	
WT   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
eve 10   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
hid 10   CAAGTGTGCCCTCTTCACGATGCACTGTCAGGGAGGGAGCGAGA	
WT   TCTGGTTGTCCTCCACTTCTGGCATATAAGCAGCATAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCACACAAT	
4   TCTGGTTGTCCTCCACTTCTGGCATATAAGCAGCATAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCACACAAT	
hh 8   TCTGGTTGTCCTCCACTTCTGGCATATAAGCAGCATAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCACACAAT	
PAM	sgRNA
9732 1045C (eve+hid+wg) - C1	
WT   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
eve 6   CAGCACCGCACGATTAGCACCGTTCCGCTCAG	
5   CAGCACCGCACGATTAGCACCGTTCCGCTCAG	
hid 20   CAAGTGTGCCCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
WT   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCCATT	
wg 1   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCCATT	
2   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCCATT	
9   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCCATT	
PAM	sgRNA
8622 1045C (eve+hid+wg) - C2	
WT   CAGCAGCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
eve 4   CAGCACCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
8   CAGCACCGCATCCACTCTCAGCACCGCACGATTAGCACCGTTCCGCTCAG	
hid 10   CAAGTGTGCCCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
WT   CAAGTGTGCCCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
wg 14   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCCATT	
PAM	sgRNA
8622 1045D (hh+wg) - D1	
WT   TCTGGTTGTCCTCCACTTCTGGCATAAAGCAGCATAAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCACACAAT	
hh 13   TCTGGTTGTCCTCCACTTCTGGCATAAAGCAGCATAAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCACACAAT	
5   CCTGAGCTTGGCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
8   CCTGAGCTTGGCTCTTCATGCACTGTCAGGGAGGGAGCGAGA	
WT   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCC	
4   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCC	
1   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCC	
PAM	sgRNA
8622 1045D (hh+wg) - D2	
WT   TCTGGTTGTCCTCCACTTCTGGCATAAAGCAGCATAAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCACACAAT	
hh 5   TCTGGTTGTCCTCCACTTCTGGCATAAAGCAGCATAAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCATACAAAT	
5   TCTGGTTGTCCTCCACTTCTGGCATAAAGCAGCATAAAAATGAACACCACAGCCGATCGAACGTCAGTGCGATACGCATACAAAT	
WT   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCCATT	
wg 12   CTATGACGAAATTTCATGAGGGTTCGCAAATAATCGGGCAATACAACTCGATTACACCGAAAATGCGGGCAGAGTTTTCCATTCCGCCATT	
PAM	sgRNA

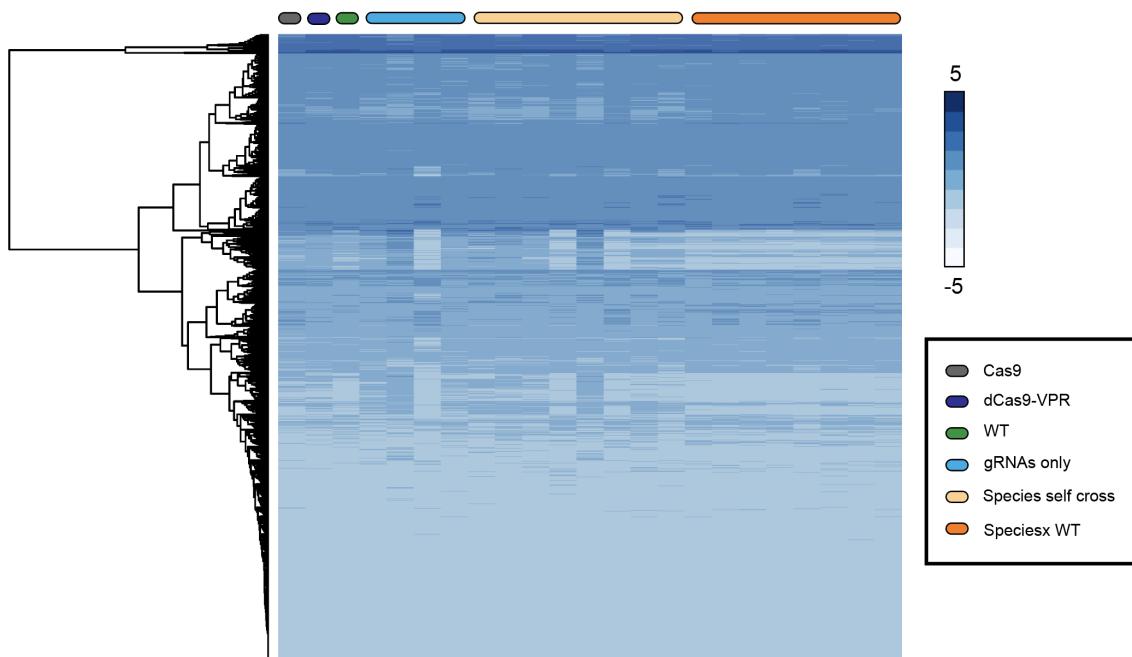
**Supplementary Figure 4. Molecular characterization of protective indel mutations.** For the generation of each independent synthetic species (*A1,A2,B1,B2,C1,C2,D1,D2*) the gRNA target site was sanger sequenced and the indels were confirmed. Number to the left of each sequence indicates the number of individuals sequenced with this mutation.



**Supplementary Figure 5. Reproductive isolation between double-homozygous speciated lines.** (a) Individuals from each SPECIES were crossed to one another to determine the extent of reproductive isolation between SPECIES. (b) The total # of embryos laid is plotted in grey as bars on the left y-axis, while the total # of embryos surviving to adults is plotted on the right y-axis as points. N = 3 biologically independent replicates of all eight SPECIES bidirectionally crossed to the remaining seven species. For embryos laid, each bar represents the mean. For embryos surviving to adults, middle lines indicate mean, while error bars represent standard deviation. In the graph, SPECIES "A" is listed by color and there is a symbol indicating SPECIES "B" in the cross (i.e., the star above the A1 group indicates the cross between an A1 female and an A2 male). Source data are provided as a Source Data file.



**Supplementary Figure 6. Two-Factor RNAseq comparisons.** (a) Deseq comparisons between RNAseq samples WTxA1 (sample 8), WTxA2 (sample 9), A1xA1 (sample 10), A2xA2 (sample 11) observing target/non-target gene misexpression. (b) Deseq comparisons between RNAseq samples WTxB1 (sample 10), WTxB2 (sample 20), B1xB1 (sample 16) and B2xB2 (sample 22). (c) Deseq comparisons between RNAseq samples WTxC1 (sample 11), WTxC2 (sample 21), C1xC1 (sample 17), and C2xC2 (sample 23). (d) Deseq comparisons between RNAseq samples WTxD1 (sample 12), WTxD2 (sample 13), D1xD1 (sample 18), and D2xD2 (sample 19). A full list of RNAseq sample IDs are listed in Supplementary Table 1 and RNAseq data can be found in Supplementary Tables 1-3, 7. Genes that are significantly misexpressed (FDR < 0.05) are colored blue. Source data are provided as a Source Data file.



**Supplementary Figure 7. Heatmap of the RNAseq Data.** Hierarchical clustering heat map of the RNAseq data (Supplementary Table 2).

**Supplementary Table 5. Primers used in this study.**

Primer	Primer Sequence, 5' to 3'	Source
<i>attP</i> sequence 986.C1 986.C2	CCCACAATGGTTAATTGAGCTGCCCGGGCCTAGGTCGACGATG TAGGTACCGTCTC  GTTATTTAAAAACGATTCAATTCTAGTTAATTAAGTCGACATGCCG CCGTGACCGTCGA	plasmid M{3xP3-RFP <i>attP</i> }
p10 3'UTR 986.C3 986.C4	TCGACGGTCACGGCGGGCATGTCGACTTAATTAACAGAAATGAATC GTTTTAAAATAAC  AAAAGTTGGTGGTGGGGAGGCCACCGAGTATGGGCGCGCCCCGGC CGTTAACTCGAACATCG	Addgene plasmid #100580
opie2 promoter fragment 986.C5 986.C6	GCTGGCTTGGATAGCGATTGAGTTAACGGCCGGGGCGCGCCATA CTCGGTGGCCTCCC  CCCCGGTGAACAGCTCCTGCCCTTGCTCACCATCTGAGCACCAAG AGACAGGTTGCAGC	Translocation plasmid B
eCFP 986.C7 986.C8	CGCCATCCAACCGCCGCCGCAACCTGTCTCTGGTGCTCGAGATGGT GAGCAAGGGCGAGG  GTGGTATGGCTGATTATGATCTAGAGTCGCGGCCGCTTACTTGTAC AGCTCGTCCATGCC	pJFRC81- 10XUAS-IVS- Syn21-GFP- p10
<i>Ubiquitin-63E</i> promoter fragment (for 986B) 986.C9 986.C10	AGCGGGTTCTCGACGGTCACGGCGGGCATGTCGACGCCGCCGC GCAGATCGCCGATG  CAATGGAGTACTTCTTGTCCATGGTGGCAGTTAAACTCTGCGGGT CAAATAGAGATGT	<i>D.</i> <i>melanogaster</i> genomic DNA

dCas9-VPR (for 986B)		Addgene plasmid #78898
986.C11	ATTTCCACATCTCTATTTGACCCGCAGAGTTAAACTGCCACCATTGGACAAGAAGTAC	
986.C12	ATTGATTGTTATTTAAAAACGATTCAATTCTAGTTAATTAAATCAAAACAGAGATGTGTC	
<i>bottleneck</i> promoter fragment (for 986C)		<i>D. melanogaster</i> genomic DNA
986.C13	GCGGGTTCTCGACGGTCACGGCGGGCATGTCGACGCCGCATTAGATGAACCCCATGG	
986.C14	CCCAATGGAGTACTTCTTGTCCATGGTGGCAGTTAACAGCCGAA TTCGTTGACGGTTG	
dCas9-VPR (for 986C)		Addgene plasmid #78898
986.C15	TTCGTACTTCAACCGTCAACGAATTGGCTGTTAACTGCCACCATTGGACAAGAAGTAC	
986.C12	ATTGATTGTTATTTAAAAACGATTCAATTCTAGTTAATTAAATCAAAACAGAGATGTGTC	
<i>Ubiquitin-63E</i> promoter fragment (for 986D)		<i>D. melanogaster</i> genomic DNA
986.C9	AGCGGGTTCTCGACGGTCACGGCGGGCATGTCGACGCCGCAGATCGCCGATG	
986.C16	TCGTGGCCGCCGGCTTTCATGGTGGCAGTTAAACTCTGCGGGTC AAAATAGAGATGT	
dCas9-VP64 (for 986D)		Addgene plasmid #78897
986.C17	TTCCACATCTCTATTTGACCCGCAGAGTTAAACTGCCACCATTGGAA AAGGCCGGCGGCC	
986.C18	ATTGATTGTTATTTAAAAACGATTCAATTCTAGTTAATTAAATTAGC CCTCCCCACACATA	

<i>bottleneck</i> promoter fragment (for 986E)		<i>D.</i> <i>melanogaster</i> genomic DNA
986.C13	GC GG GTTCTCGACGGTCACGGCGGCATGTCGACGCCGCATTAGATGAACCCCATGG	
986.C19	TTT CGTGGCCGCCGGCCTTTCATGGTGGCAGTTAACAGCCGAA TTCGTTGACGGTTG	
dCas9-VP64 (for 986E)		Addgene plasmid #78897
986.C20	GTACTTCAACCGTCAACGAATTGGCTGTTAAACTGCCACCATGA AAAGGCCGGCGGCC	
986.C18	ATTGATTGTTATTTAAAAACGATTCTAGTTAATTAAATCAA ACAGAGATGTGTC	
U6:3 promoter fragment		Addgene plasmid #49411
1045.C1	TTGGGAATTGGCAATATTAAATGGCGCGCGCCGAATTCTTTTGCTCACCTGTGAT	
1045.C2	CTTATTAACTGCTATTCTAGCTCTAAAACCCTAGGCCGACGTT AAATTGAAAATAG	
sgRNA scaffold		Addgene plasmid #49411
1045.C3	ATATATAGACCTATTTCAATTAAACGTCGGCCTAGGGTTTAGAGC TAGAAATAGCAAG	
1045.C4	AGTGGATCTCTAGAGGTACCGTTGGCGCGCTTAATTAAAAAA GCACCGACTCGGTG	
<i>eve</i> -sgRNA-U6:1- promoter- <i>hid</i> - sgRNA fragment		Custom gBlocks® Gene Fragment
1045.C5	GTTCGTATATAGACCTATTTCAATTAAACGTCGGATCGTGGT GCTGAGAG	
1045.C6	CGGACTAGCCTATTAACTTGCTATTCTAGCTCTAAACTCATG CACGTGCATGTGC	

<i>Gypsy</i> -U6:1-promoter- <i>hh1</i> -sgRNA-U6:3-promoter- <i>hh2</i> -sgRNA fragment (for OA-1045B)		pCFD- <i>hh</i>
1045.C7	GGTGCTTTTAATTAAAACGCGGCCGCAACGGTACCTGCAGCCAC GTAATAAGTGTGCG	
1045.C8	ACACTAGTGGATCTCTAGAACAACTCTCAGGCTCCAGGTAGGCAA AAAGCACCGACTCG	
<i>Gypsy</i> -U6:1-promoter- <i>wg1</i> -sgRNA-U6:3-promoter- <i>wg2</i> -sgRNA fragment (for OA-1045C)		pCFD- <i>wg</i>
1045.C7	GGTGCTTTTAATTAAAACGCGGCCGCAACGGTACCTGCAGCCAC GTAATAAGTGTGCG	
1045.C8	ACACTAGTGGATCTCTAGAACAACTCTCAGGCTCCAGGTAGGCAA AAAGCACCGACTCG	
U6:1-promoter- <i>wg1</i> -sgRNA-U6:3-promoter- <i>wg2</i> -sgRNA fragment (for OA-1045D)		pCFD- <i>wg</i>
1045.C9	CCGGGAATTGGATTGGCAATTAAATGGCGGCCAGCC GATCAATTGAGATC	
1045.C10	TGTTTTGCGAATAAATTCAACGCACACTTATTACGTGCATATGAAC AACTCTCAGGCTC	
<i>Gypsy</i> -U6:1-promoter- <i>hh1</i> -sgRNA-U6:3-promoter- <i>hh2</i> -sgRNA fragment (for OA-1045D)		pCFD- <i>hh</i>
	CTGGAGCCTGAGAGTTGTCATATGCACGTAATAAGTGTGCGTTG	

1045.C11	TTTATTGAACAACTCTCAGGCTCCAGGTAGTCTAGAGCAAAAAGC ACCGACTCGGTGCC	
1045.C12		
tRNA- <i>eve</i> - sgRNA-tRNA- <i>hid</i> -sgRNA- tRNA- <i>hh1</i> - sgRNA-tRNA- <i>hh2</i> -sgRNA-U6:3 UTR fragment (for OA-1045E)		Gene synthesized vector
1045.C13	TCGTATATATAGACCTATTTCAATTAACGTCGGTTAATTAAAGGGC TTTGAGTGTGTGT	
1045.C14	TCGTCGACACTAGTGGATCTCTAGAGGTACCGTTGC GGCCGCATGC ATACGCATTAAGCG	