Supporting Information Appendix

Table S1. Larval and adult phenotypes of G_2 progeny of lines 10.1 and 10.2 G_1 outcrosses to wild-type mosquitoes.

G_1 Founder	Larval phenotypes*				Adult phenotypes*							
					Males				Females			
	$DsRed^{+}/kh^{w+}$	$DsRed^{+}/kh^{w-}$	$DsRed/kh^{w+}$	$DsRed^{-}/kh^{w-}$	$DsRed^{+}/kh^{w+}$	$DsRed^{+}/kh^{w-}$	$DsRed^{-}/kh^{w+}$	$DsRed^{-}/kh^{w-}$	$DsRed^{+}/kh^{w+}$	$DsRed^{+}/kh^{w-}$	$DsRed^{-}/kh^{w+}$	$DsRed/kh^{w-}$
10.1 ♂	16				6				8			
10.2 ♂	64		76		32		38		25		34	

*Shaded cells are G_2 progeny positive for DsRed ($DsRed^{+}$).

Table S2. List of oligonucleotide primers.*

Primer	Sequence (5'-3')	Reference
Vg451	GTACGCGTATCGATAAGCTTtaaGATACATTGATGAGTTTGG	This study
Vg452	TAGGCCGGCCGATCTCGGATCTGACAATG	This study
Vg453	GCTTATCGATACGCGTACGCATCTGCATCCTGGTACCACAGTCTTATTGG	This study
Vg454	GTACTTCTTATCCATCTTCCTAGATCGATTTTAAGCACGCCGC	This study
Vg455	CTAGGAAGATGGATAAGAAGTACTCGATCGGTCTGGATATCGG	This study
Vg456	CTCTCGTTACACCTTGCGCTTCTTCTTCGGCG	This study
Vg457	GCAAGGTGTAACGAGAGTTCGGTGCGAATCTCTCTCTTGATTTTTCCC	This study
Vg458	AGATCGGCCGGCCTAACCAAGGCCAGCCTGTTGAGC	This study
Vg464	AAGCTTATCGATACGCGTACCTCAACTGCCGACGAGTTGCTCG	This study
Vg465	ATCCGAGATCGGCCGGCCTACGTTCTGCGTGGCTGTTGTAAGGTTCC	This study
Vg494	GGTATCAGCTCACTCAAAGGCGGTAATACGG	This study
Vg495	GAAAGGGCCTCGTGATACGCCTATTTTTATAGG	This study
Vg498	CCTTTGAGTGAGCTGATACCCCCAAAAATTGACCTCAGATCTCTCAAGAAGCG	This study
Vg499	GCGTATCACGAGGCCCTTTCGCATCTGAACGAGGTGCTGCTAAATGG	This study
Vg500	GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCAC CGAGTCGGTGCTTTTTTTGTGGAAATTTGATTCACTTGTTTTAGG	This study
Vg501	AGGTCTTCTCGAAGACCCCAAGGGAGGGGGCAAATGGGTTGG	This study
Vg505	GTCCACTAACGAAAGAGGTCAAGAGC	This study
Vg507	CGATCGTTTAGTGACGAGATCACGC	This study
Vg537	CTTGATGGTTCCGTTCTACGGGC	This study
Vg538	AAACGCCCGTAGAACGGAACCAT	This study
Vg543	CATGTACATTTCCTTTTACGttaattaaCGTAGAACGGAACCATCGCGTGC	This study
Vg544	ATGGTTCCGTTCTACGttaattaaCGTAAAAGGAAATGTACATGGTAAAGCAATCTGACG	This study
Vg546	CCGGGCGAGCTCGactcgagcCTGGTACCACAGTCTTATTGGCGTGATGG	This study
Vg551	CAATAAGACTGTGGTACCAGgctcgagTTGTTTGATCGCACGGTTCCCACAATGG	This study
Vg552	CCTTACAACAGCCACGCAGAACGggatccGGCAGGGCATGAACGCGGGCTTTGAAGAC	This study
Vg553	GCCCGCGTTCATGCCCTGCCggatccCGTTCTGCGTGGCTGTTGTAAGGTTTCG	This study
Vg554	GCTCAACAGGCTGGCCTTGGTActcgagCTCAACTGCCGACGAGTTGCTCG	This study
Vg555	CAACTCGTCGGCAGTTGAGctcgagTACCAAGGCCAGCCTGTTGAGCAGCTTGC	This study
Vg557	CGATCGTTTAGTGACGAGATCACGC	This study
dsCas9 1 F	TAATACGACTCACTATAGTGCCATCCTGCTGTCGGATA	This study
dsCas9 1 R	TAATACGACTCACTATAGCTTCGGCAGCACCTTCTCGT	This study
dsKu70 F	TAATACGACTCACTATAGCTACGGCATTGGGTTTGTCT	This study
dsKu70 R	TAATACGACTCACTATAGGCTTAGCTTGTAGCCGGATG	This study
G1F2	CGTATGCTGCACGACGTTAAC	This study

G2R1	CTGGACATCTGCTTCTGAG	This study
G2R2	GCAATAGCTGACTCCTG	This study
Km1F1	CGATGCGATCGAGCTAATTG	This study
Km2R1	GTGACGAGATCACGCATCTG	This study
Vg5'R1	CGTTTGGTGCTCAGCTCAGAC	This study
Vg5'R2	GTCGTAGGTGGTGGTATGCTAAC	This study
U6F1	CTACCTGTACGATGGCTTAAG	This study
m2A10 FOR	GAGACGGTGAAGATCTCGTGCAAGG	1
m2A10 REV	GCTTCGTACCACCACCGAACACGTA	1
m1C3 FOR	AAGGGCTCGCTGAAACTGT	1
m1C3 REV	ACAGAAGTACACCGCCAGGT	1
AsVg1 for	CAACATCATGTCCAAGTCGGAGGTGA	2
AsVg1 rev	CTTGAAGCTTTCGTGCTCTTCCTCCG	2
AsCPAFOR	CTTTACGGAAACGCTCGAAG	3
AsCPAREV	CCATAGCAGAATGGACCGTAA-3′	3
Asrib26Sfor	AATCCTTCCCGAAGGACATGAACCG	4
Asrib26Srev	TACGAAACAAATCCCATCCTAATCGAAGC	4

*Lowercase letters are introduced restriction endonuclease cleavage sites.

Table S3. G_3 larval phenotypes of line 10.1 G_2 outcrosses to wild-type mosquitoes.			
Founder[†]	**Cross**	**Larval phenotypes**[*]	
		DsRed$^+$	**DsRed**$^-$
♂	**10.1.1**	346	2
	10.1.3	136	
	10.1.5	129	1
	10.1.6	218	
	Subtotal[‡]	**829**	**3**
♀	**10.1.1**	61	1
	10.1.2	109	2
	10.1.3	48	1
	10.1.4	85	
	10.1.5	85	
	10.1.7	41	
	10.1.8	63	
	Subtotal[‡]	**492**	**4**
	Total	**1321**	**7**

[*]Shaded cells are G_3 progeny positive for DsRed (DsRed$^+$).
[†]Progeny of single G_2 male or female out-crossed to wild-types of the opposite sex.
[‡]X^2 analyses show significant differences (p<<0.0001) in DsRed$^+$:DsRed$^-$ ratios from random segregation.

Table S4. G_3 larval phenotypes of line 10.2 G_2 outcrosses to wild-type mosquitoes.				
Founder	**Cross**	**Larval phenotypes**[*]		
		DsRed$^+$	**DsRed**$^-$	**DsRed**$^-$**/kh**$^{w-}$
♀[†]	**10.2.3**	109		1
	10.2.4	141		
	10.2.5	82		
	10.2.6	100		
	10.2.8	163		1
	10.2.10	146		
	10.2.11	90		
	10.2.12	117	1	
	10.2.13	119		
	10.2.14	70		
	10.2.15	9	1	
	10.2.16	89	7	
	10.2.17	131	1	
	10.2.18	112		1
	10.2.19	93		
	Subtotal[§]	**1571**	**10**	**3**
♂[‡]	**Batch**[§]	**3060**	**25**	
	Total[§]	**4631**	**35**	**3**

[*]Shaded cells are G_3 progeny positive for DsRed (DsRed$^+$).
[†]Progeny of single G_2 female out-crossed to wild-type males.
[‡]27 G_2 males out-crossed to 270 wild-type females.
[§]X^2 analyses show significant differences (p<<0.0001) in DsRed$^+$:DsRed$^-$ ratios from random segregation.

Table S5. G_3 adult phenotypes of line 10.1 G_2 outcrosses to wild-type mosquitoes.

Founder[†]	Cross	Adult phenotypes[*]									
		Males					Females				
		DsRed$^+$/kh^{w+}	DsRed$^+$/kh^{w-}	DsRed$^+$/ mosaic	DsRed$^-$/kh^{w+}	DsRed$^-$/kh^{w-}	DsRed$^+$/kh^{w+}	DsRed$^+$/kh^{w-}	DsRed$^+$/ mosaic	DsRed$^-$/kh^{w+}	DsRed$^-$/kh^{w-}
♂	**10.1.1**	202			2		173				
	10.1.3	54					91				
	10.1.5	58			1		53				
	10.1.6	100					102				
	Total[‡]	**414**			**3**		**419**				
♀	**10.1.1**		20	7	1			18	7		
	10.1.2		26	4				36	4	2	
	10.1.3		9	3				15	3		
	10.1.4		26					32	4		
	10.1.5		38	7				37	7		
	10.1.7		13	2				19			
	10.1.8		23	2				23	2		
	Total[‡]		**155**	**25**	**1**			**180**	**27**	**2**	

[*]Shaded cells are G_3 progeny positive for DsRed (DsRed$^+$).
[†]Progeny of single G_2 male or female out-crossed to wild-types of the opposite sex.
[‡]X^2 analyses show significant differences (p<<0.0001) in DsRed$^+$:DsRed$^-$ ratios from random segregation.

Table S6. G_3 adult phenotypes of line 10.2 G_2 outcrosses to wild-type mosquitoes.

Founder	Cross	Adult phenotypes*									
		Males					Females				
		DsRed^{+}/kh^{w+}	DsRed^{+}/kh^{w-}	DsRed^{+}/ mosaic§		DsRed$^{-/}$kh^{w+}	DsRed^{-}/kh^{w-}	DsRed^{+}/kh^{w+}	DsRed^{+}/kh^{w-}	DsRed^{+}/ mosaic§		DsRed$^{-/}$kh^{w+}	DsRed^{-}/kh^{w-}
♀†	**10.2.3**		66	5	ND				44		ND		
	10.2.4		64		ND				61		ND		
	10.2.5		36	7	ND				32	3	ND		
	10.2.6		36	2	ND				33		ND		
	10.2.8		60		ND				51		ND		
	10.2.10		48	2	20				49	3	14		
	10.2.11		30	1	8				41	2	4		
	10.2.12		36	3	10				38	3	7		
	10.2.13		38	2	7				42	2	1		
	10.2.14		22	1	7				29	2	3		
	10.2.15		4		2				2				
	10.2.16		42	1	1				37		3		
	10.2.17		38	6	20				44	1	19		
	10.2.18		40	4	7				48	3	1		
	10.2.19		37	5	9				34	4	4		
	Total¶		**597**	**130**					**585**	**79**			
♂‡	**Batch¶**	**663**				**8**		**595**				**8**	

*Shaded cells are G_3 progeny positive for DsRed (DsRed^{+}).
†Progeny of single G_2 female out-crossed to wild-type males.
‡27 G_2 males out-crossed to 270 wild-type females.
§The first column numbers are those mosquitoes with mosaicism in the eye. The second column numbers are mosquitoes with the 'colored' eye phenotypes consistent with mosaicism in the rest of the body. 'ND', not determined.
¶X^2 analyses show significant differences (p<<0.0001) in DsRed^{+}:DsRed^{-} ratios from random segregation.

Table S7. G_4 larval phenotypes of line 10.1 G_3 outcrosses to wild-type mosquitoes.		
(Cross)Transgenic parent (n) *	**Larval phenotypes**†	
	DsRed$^+$	**DsRed$^-$**
(1) DsRed$^+$/*kh*$^{w-}$ ♀ (26)	28	20
(2) DsRed$^+$/*kh*$^{w-}$ ♂ (32)	332	303
(5) DsRed$^+$/*kh*$^{w+}$ ♀ (40)‡	645	7
(6) DsRed$^+$/*kh*$^{w+}$ ♂ (40)‡	949	6

*Progeny of batch-mated G_3 males or females out-crossed to wild-types of the opposite sex. (n) is the number of transgenic founder parents.
†Shaded cells are G_4 progeny positive for DsRed (DsRed$^+$).
‡X^2 analyses show significant differences (p<<0.0001) in DsRed$^+$:DsRed$^-$ ratios from random segregation.

Table S8. G_4 larval phenotypes of line 10.2 G_3 outcrosses to wild-type mosquitoes.		
(Cross)Transgenic parent (n)*	**Larval phenotypes**†	
	DsRed$^+$	**DsRed$^-$**
(3) DsRed$^+$/*kh*$^{w-}$ ♀ (43)	204	120
(4) DsRed$^+$/*kh*$^{w-}$ ♂ (25)	372	260
(7) DsRed$^+$/*kh*$^{w+}$ ♀ (50)‡	1134	20
(8) DsRed$^+$/*kh*$^{w+}$ ♂ (50)‡	609	19

*Progeny of batch-mated G_3 males or females out-crossed to wild-types of the opposite sex. (n) is the number of transgenic founder parents.
†Shaded cells are G_4 progeny positive for DsRed (DsRed$^+$).
‡X^2 analyses show significant differences (p<<0.0001) in DsRed$^+$:DsRed$^-$ ratios from random segregation .

Table S9. Table S9. G_4 larval phenotypes of lines 10.1 and 10.2 batch G_3 intercrosses.[1]		
	Larval phenotypes†	
Cross	**DsRed$^+$/*kh*$^{w-}$**	**DsRed$^-$/*kh*$^{w-}$**
Line 10.1	428	71
Line 10.2	1851	361

[1]G_3 DsRed-positive (DsRed$^+$) males and females were intercrossed as a single batch for each line.
†Shaded cells are G_4 progeny positive for DsRed (DsRed$^+$).

Table S10. G_4 adult phenotypes of line 10.1 G_3 outcrosses to wild-type mosquitoes.

Line	(Cross) Founder	Adult phenotypes[1]									
		G_4 males					G_4 females				
		DsRed$^+$/ kh^{w+}	DsRed$^+$/ kh^{w-}	DsRed$^+$/ mosaic †		DsRed$^-$/ kh^{w+}	DsRed$^-$/ kh^{w-}	DsRed$^+$/ kh^{w+}	DsRed$^+$/ kh^{w-}	DsRed$^+$/ mosaic †		DsRed$^-$/ kh^{w+}	DsRed$^-$/ kh^{w-}
10.1	(1) DsRed$^+$/*kh*$^{w-}$ ♀		6		5	4	3		1	3	4	7	2
	(2) DsRed$^+$/*kh*$^{w-}$ ♂‡	153				111		153				137	
	(5) DsRed$^+$/*kh*$^{w+}$ ♀§	1	155	13	85	5	1		223	19	71	4	
	(6) DsRed$^+$/*kh*$^{w+}$ ♂§	403				3		479				3	

*Shaded cells are G_4 progeny positive for DsRed (DsRed$^+$).
†The first column numbers are those mosquitoes with mosaicism in the eye. The second column numbers are mosquitoes with the 'colored' eye phenotypes consistent with mosaicism in the rest of the body.
‡X^2 analyses show significant differences (p=0.0137) in DsRed$^+$:DsRed$^-$ ratios from random segregation .
§ X^2 analyses show significant differences (p<<0.0001) in DsRed$^+$:DsRed$^-$ ratios from random segregation.

Table S11. G_4 adult phenotypes of line 10.2 G_3 outcrosses to wild-type mosquitoes.

Line	(Cross) Founder	Adult phenotypes*											
		G_4 males						G_4 females					
		DsRed$^+$/ kh^{w+}	DsRed$^+$/ kh^{w-}	DsRed$^+$/ mosaic †		DsRed$^{-/}$ kh^{w+}	DsRed$^-$/ kh^{w-}	DsRed$^+$/ kh^{w+}	DsRed$^+$/ kh^{w-}	DsRed$^+$/ mosaic †		DsRed$^{-/}$ kh^{w+}	DsRed$^-$/ kh^{w-}
10.2	(3) DsRed$^+$/*kh*$^{w-}$ ♀‡		40	1	34	36	41		41		53	23	3
	(4) DsRed$^+$/*kh*$^{w-}$ ♂§	157				101		160				139	
	(7) DsRed$^+$/*kh*$^{w+}$ ♀¶	3	288	8	120	5		1	357	16	145		3
	(8) DsRed$^+$/*kh*$^{w+}$ ♂¶	258				10		307				8	

*Shaded cells are G_4 progeny positive for DsRed (DsRed$^+$).

†The first column numbers are those mosquitoes with mosaicism in the eye. The second column numbers are mosquitoes with the 'colored' eye phenotypes consistent with mosaicism in the rest of the body.

‡X^2 analyses show significant differences ($p<0.0001$) in DsRed$^+$:DsRed$^-$ ratios from random segregation.

§X^2 analyses show significant differences ($p=0.0011$) in DsRed$^+$:DsRed$^-$ ratios from random segregation.

¶X^2 analyses show significant differences ($p<<0.0001$) in DsRed$^+$:DsRed$^-$ ratios from random segregation.

Figure S1. Molecular confirmation of the precise insertions of the AsMCRkh2 cargo into the *kh*^{w} locus. Sequencing of the 5'- and 3'-end G1F2-vg5'1 and U6F1-G2R2 junction amplification fragments (left and right, respectively) amplified from the G_1 founder male mosquitoes 10.1 and 10.2 (lower sequencing traces) revealed a perfect match to the sequences from Cas9-mediated cleavage at the kh2 target site and precise integration of the ASMCRkh2 cargo (Figure 1) into that site. Positions of the kh2 homology arms (maroon boxes), guide RNA targeting sequences (blue boxes), PAM site (red arrowhead) and the PCR primers Vg-5prime (orange box) and U6A3'Seq (red box) are shown. Genomic nucleotides depicted in lower case letters indicate restriction endonuclease enzyme recognition sites used in the construction of the AsMCRkh2 cargo.

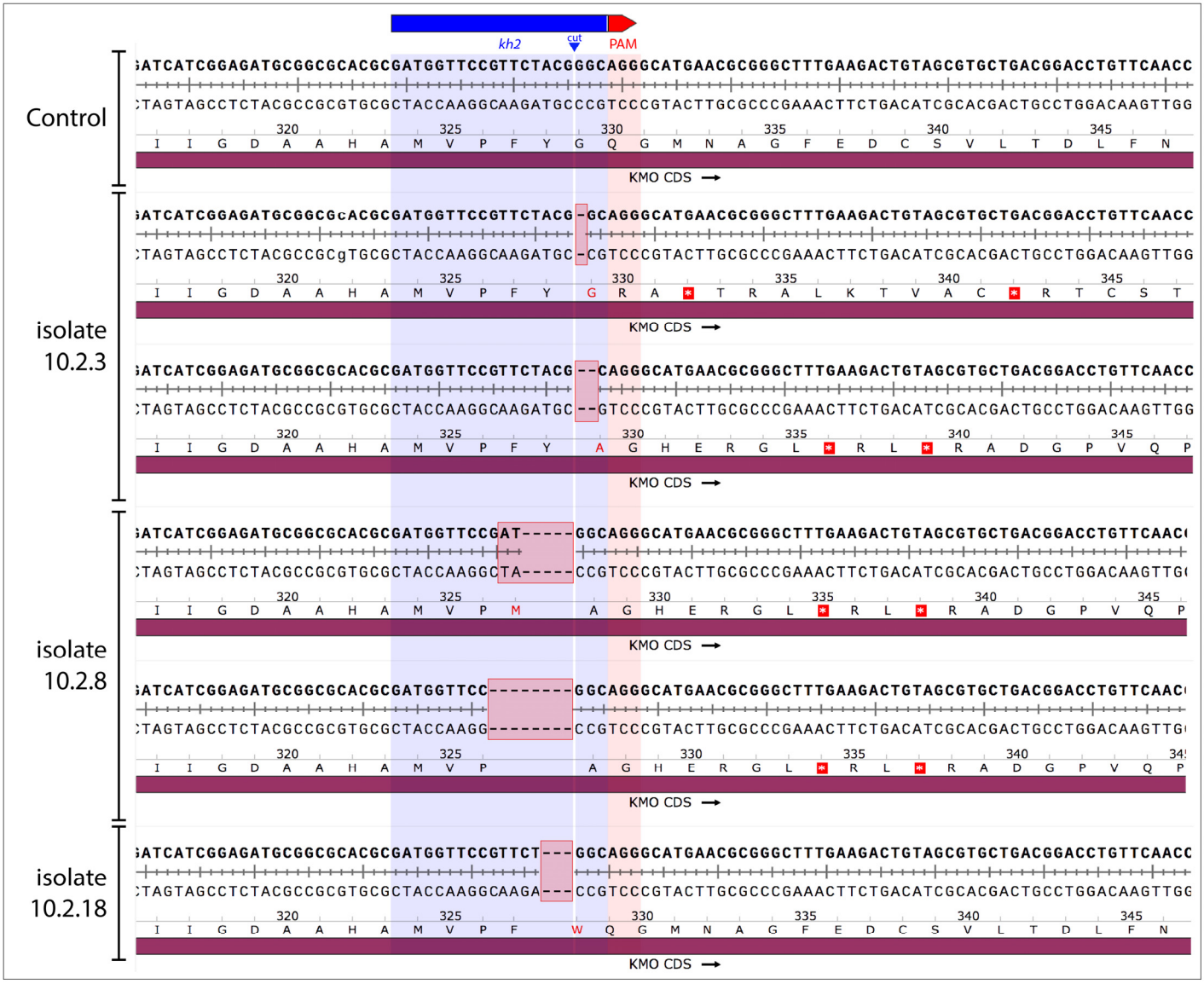

Figure S2. Mutated sequences at the kh2 target site in selected G_2 transgenic mosquitoes. The top sequence is the wild-type reference sequence at the kh2 target site from a control mosquito. The PAM sequence is shaded in peach, the 20 nt targeting sequence is shaded in purple, indels are shaded in pink, and the gRNA directed cleavage site by a thin vertical white line. NHEJ events were detected in three different white-eyed DsRed$^-$ mosquitoes. Different sequences were isolated by cloning PCR products generated from the *kh*^{w} locus in lines 10.2.3 and 10.2.8, supporting the conclusion that these mosquitoes are mosaics carrying at least two distinct NHEJ-directed mutations. Isolate 10.2.18 is derived from a direct sequencing reaction of an amplification product confirming that this transgenic mosquito had only a single NHEJ event. In contrast to the other two cases, this mutation maintains the translation frame but nonetheless results in a white-eye phenotype supporting the conclusion that the deletion of a single amino acid (Y328) and substitution of its neighbor (G329W) eliminates or greatly reduces kynurenine hydroxylase enzymatic activity.

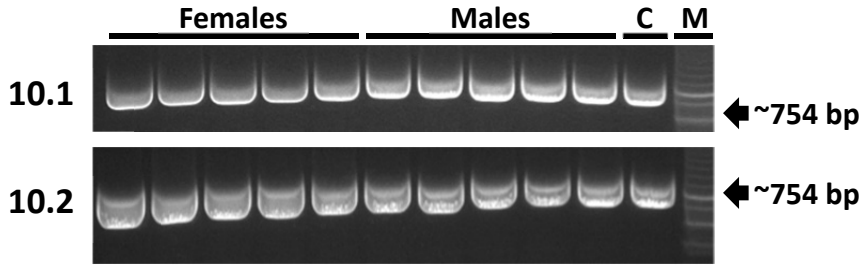

Figure S3. Gene amplification of the kh2 target sites in selected G_3 founder mosquitoes. Genomic DNA prepared from individual male and female G_3 founder mosquitoes positive for DsRed and with white eyes ($DsRed^{+}/kh^{w-}$; crosses 1 - 4, Tables S10 and S11) was used with the gene-specific primers, vg505 and vg557 (Table S2) to amplify a portion of the kh^{w} gene. The diagnostic fragment at 754 base-pairs indicates at least one of the chromosomes had a kh^{w} gene without the large ~17 kb transgene cargo insert. These alleles must be mutant because the eye phenotype of each mosquito from which the DNA was derived is white (kh^{w-}). 'C' is a control sample of DNA from a wild-type mosquito. 'M' are the molecular-weight markers.

Supporting Materials and Methods.

Construction of the *vasa*-Cas9 cassette.
The *Drosophila melanogaster vasa* gene and its *Anopheles gambiae* ortholog AGAP008578 (5) were used as BLAST search queries to identify the ASTE003241 gene as the putative unique *Anopheles stephensi* ortholog. A 4009 bp genomic fragment comprising the *Anopheles stephensi vasa* promoter and regulatory sequences preceding the predicted translational start site was amplified using oligonucleotides vg453/vg454. Four DNA fragments were then joined in the following order: 1) the *An. stephensi vasa* regulatory fragment; 2) a recoded Cas9 gene codon-optimized for translation in *Anopheles stephensi* (pUC57Kan-T7-AsCas9 (amplified with the oligonucleotides vg455/vg456); 3) a 1014bp fragment following the translational stop codon (TAA) of the ASTE003241 (amplified using the oligonucleotides vg457/458), 4) the backbone of the pBacDsRed plasmid (amplified using the oligonucleotides vg451/vg452). These four amplicons were purified, treated with the restriction enzyme *Dpn*I to remove methylated DNA and joined in a Gibson assembly (New England Biolabs, #E5510S) reaction to obtain the plasmid pVG160 (pVG160_pBacDsRed-attB_Aste-Vasa-Cas9).

Construction of the U6A-kh-gRNA cassette. A 923bp fragment spanning the annotated *Anopheles stephensi* U6-snRNA gene ASTE015697 was amplified using the oligonucleotides vg464/vg465, and the amplification product was cloned into pCR2.1-TOPO using a TA Topoisomerase Cloning Kit (Invitrogen, #450641). The resulting plasmid was used as a template for site-directed mutagenesis using the oligonucleotides vg500/vg501 thereby substituting the U6 transcript with a 2x*Bbs*I restriction site linker followed by the gRNA core sequence. The resulting plasmid (pVG145) serves as vector for one-step insertion of 20 bp gRNA guide sequences to generate complete gRNAs under the control of the *An. stephensi* U6 RNA polymerase-III promoter. pVG145 was cut with the restriction enzyme *Bbs*I and a linker generated by annealing the oligonucleotides vg537/vg538 was ligated to close the gap, the resulting plasmid (pVG163) expresses a gRNA targeting cleavage of the *kynurenine hydroxylase-white* (*kh*^{w}) coding sequence at the sequence "GATGGTTCCGTTCTAC**G/GG**CAGG"; the **bolded "GGG"** corresponds to the encoded glycine 329 of the *kh*^{w} gene product.

Construction of the homology arms backbone (pVG159). To minimize the final vector size, a minimal 1943 bp backbone fragment of pUC19 was amplified using vg494/vg495 and fused to a 2.4kb genomic fragment from the *An. stephensi kh*^{w} locus (amplified with vg498/vg499), using Gibson assembly, to yield the plasmid pVG159.

Construction of the pAsMCRkh construct. The final construct, pAsMCRkh, was assembled using restriction cloning in two steps. First, a three-way fusion was generated comprising: 1) a fragment containing the pUC19 plasmid backbone and the *kh*^{w} homology arms was amplified from pVG159 using vg543/vg552, which added *Pac*I and *Bam*HI restriction enzyme cut sites at the ends; 2) a 6.6kb DNA fragment including the 3xP3-DsRed eye marker and a dual-single chain (scFv) antibody cassette expressing m1C3 and m2A10 (1,3) and amplified with vg544/vg551, which added *Pac*I and P*sp*XI sites at the ends); and 3) the U6A-kh2-gRNA cassette (amplified from pVG163 using vg553/vg554, which added *Psp*XI and *Bam*HI sites at the ends). Each of the three fragments was cut with the respective restriction enzymes followed by treatment with *Dpn*I to remove the methylated templates, and ligated together to obtain the intermediate plasmid pVG165. In the second step, the two above fragments were joined. The vasa-Cas9 cassette was amplified from pVG160 (using vg546/vg555, which added *Psp*XI sites at both ends) and the obtained amplicon was cut with *Psp*XI and *Dpn*I, and then ligated to pVG165 linearized with *Psp*XI (and treated with calf intestinal alkaline phosphatase to obtain the final construct.

Reagents: all amplification steps were performed using Phusion High-Fidelity DNA Polymerase (for fragments <6kb, New England Biolabs, Cat.#M0530S) or Q5 High-Fidelity DNA Polymerase (for fragments >6kb, New England Biolabs, Cat.#M0491S). The enzymes mentioned were purchased from New England Biolabs: *Bam*HI-

HF (R3136S), *Bbs*I (R0539S), *Dpn*I (R0176S), *Pac*I (R0547S), *Psp*XI (R0656S), calf intestinal alkaline phosphatase (M0290S).

References

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