Supplementary Information for

Gene drive, and resilience through renewal with next generation *Cleave and Rescue* selfish genetic elements

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This PDF file includes:

Fig. S1 to S9

Table S1 to S12

Other Supplementary Materials for this manuscript include the following:

Dataset S1. **DataS1.xlsx**; primer sequences, gRNA sequences, *ClvR* constructs GenBank files
Dataset S2. **DataS2.xlsx**; genotype count table for drive experiments.

Supplementary Figures

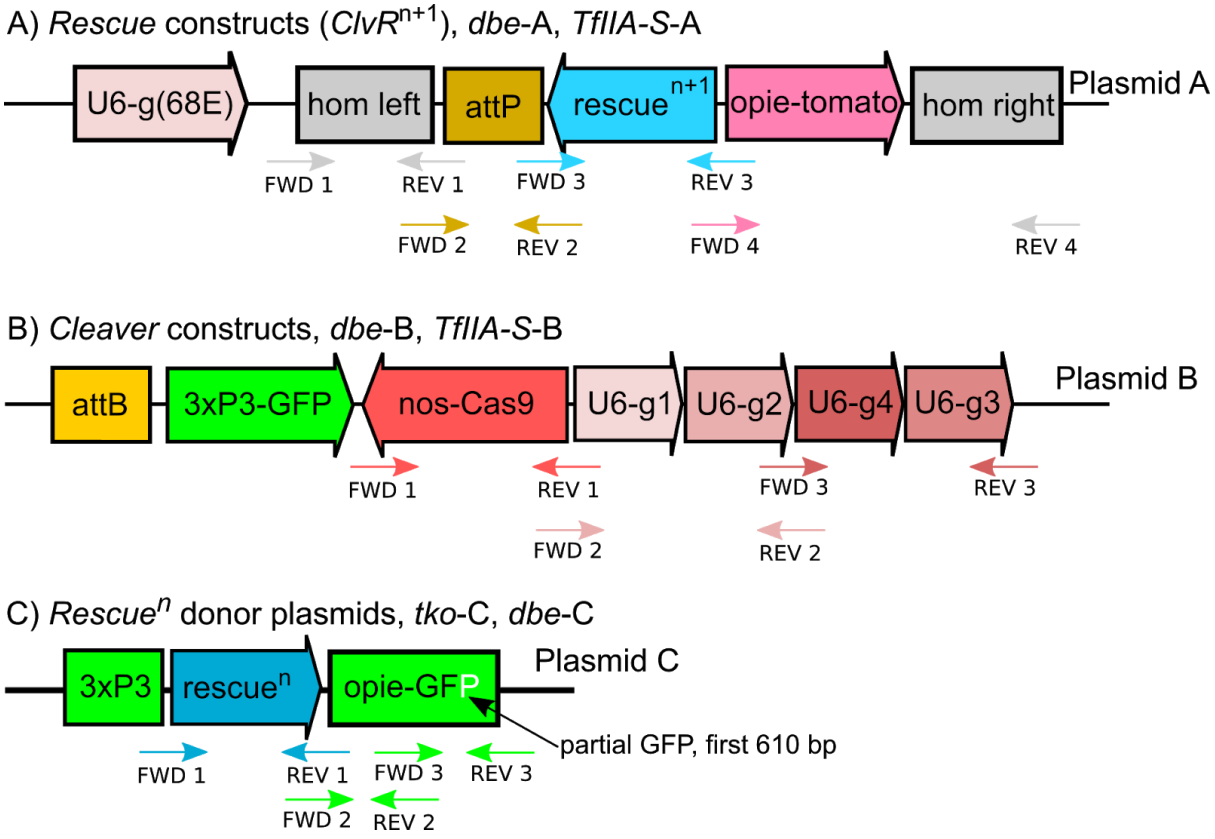

Fig. S1. Constructs used to create 1st and 2nd generation *ClvR* flies. Schematic representation of *ClvR* constructs including primers used for cloning. Primer sequences are in Dataset S1. **(A)** *Rescue* constructs including the *Rescue* gene, a *td-tomato* marker and an *attP* site, flanked by homology arms to facilitate insertion into the fly genome at 68E on the third chromosome. The gRNA to target 68E was driven from a U6 promoter located outside of the homology arms. **(B)** *Cleaver* constructs having an *attB* site, a *3xP3*-GFP marker gene, germline Cas9 under the control of the *nanos* promoter and UTRs, as well as a set of 4 gRNAs each driven by a U6 promoter. **(C)** *Rescue*^{n} donor plasmid having a *3xP3* promoter serving as homology arm, the *Rescue*^{n} gene, a ubiquitous *opie* promoter and a partial GFP ORF (110 bp +UTR (SV40) missing the C-terminus of GFP served as the other homology arm.

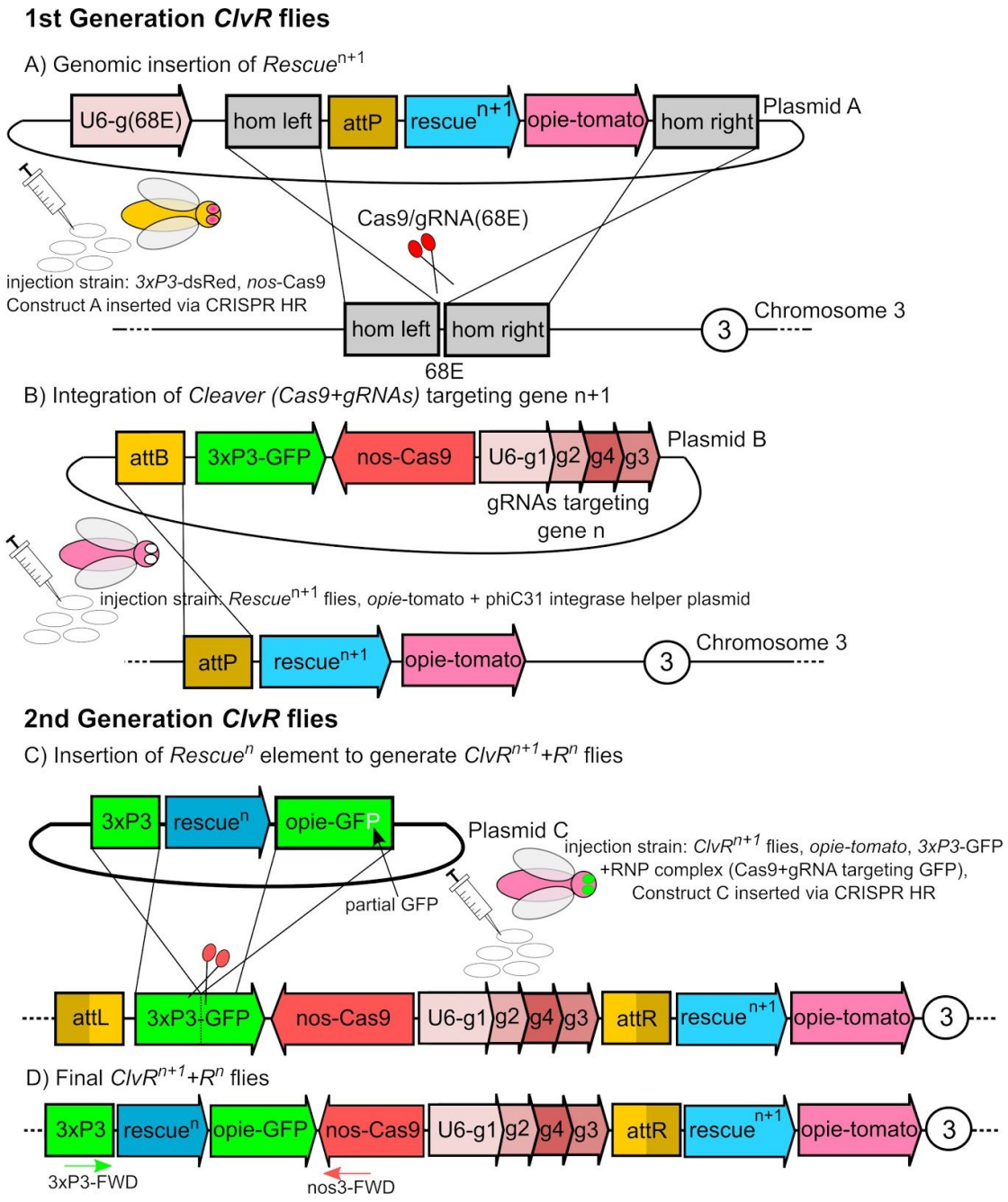

Fig. S2. Synthesis strategy to create 1st and 2nd generation *ClvR* flies. (A) CRISPR HR mediated insertion of *Rescue*$^{n+1}$. Plasmid A having the *Rescue*$^{n+1}$ and a marker was injected into a strain expressing Cas9 in the germline (*nos*-Cas9). A gRNA targeting a genomic region at 68E was expressed from the plasmid outside the homology arms. **(B) *PhiC31* mediated integration of *Cleaver*$^{n+1}$ (Cas9 and gRNAs).** Plasmid B having Cas9 and gRNAs targeting essential gene (n+1) was injected into flies from step A with a helper plasmid as the source for *phiC31* integrase. **(C) CRISPR HR mediated insertion of *Rescue*^{n} into flies that will become *ClvR*$^{n+1}$+*R*^{n} flies.** Cas9/gRNA RNP complexes were injected to induce a DSB between the *3xP3* promoter and GFP alongside a donor template that had the *Rescue*^{n}. The homology arms were designed in a way so that successful insertion will switch GFP expression from eye-specific to ubiquitous. **(D) Final *ClvR*$^{n+1}$+*R*^{n} flies.** These flies were used in the gene drive experiments to replace populations carrying *ClvR*^{n} elements. Red and green arrows indicate primers that were used to confirm correct insertion of the new *Rescue*^{n}.

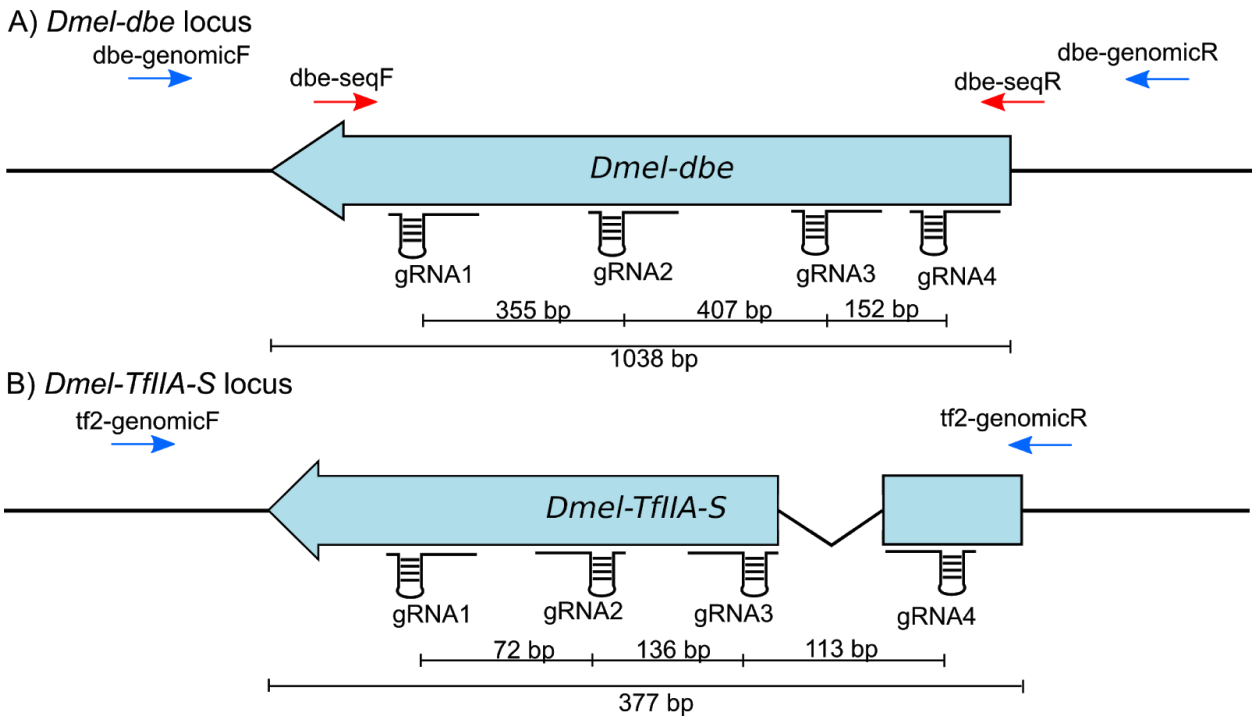

Fig. S3. Schematic of target genes genomic loci. Shown are exons (blue) and introns of the two *ClvR* target genes with gRNA binding sites and directions, primers used for sequencing, and scale bars giving distances in bp. **(A)** *Dmel-dbe* locus **(B)** *Dmel-TfIIA-S* locus

```
                    Start
Dsuz-dbe        cga--aataATGagcgacagcgaagcggaagacactaaagttagcacggagccggtggac
Dmel-dbe        cgagtaacgatgagcgaaagtgaagcggaggagaccaaaatcagcaccgagccggtggac
                ***  **..******** **.********.** **.***.*.***** ***********

Dsuz-dbe        aatgcgtgggccatgaagatcccggctttcaagcaggaggacaacccgcacggcatggtg
Dmel-dbe        aatgcgtggtccatgaagatccctgccttcaggcaggaggacaacccgcatGGGatggtg
                ********* ************* **.****.******************.** ******
                                                      gRNA4
Dsuz-dbe        gaagagagctccttcgccacgctgttccccaaataccgggagcgatatctcaaggaggtc
Dmel-dbe        gaggagagctccttcgccacgctgtttcccaaatatcgggagcgctatcttaaggaggtt
                **.**********************.********.******** *****.********.

Dsuz-dbe        tggcccctggtggagcagtgcctggaggagcaccacctaaaggcggaactggacctgatg
Dmel-dbe        tggcctctggtggagcagtgcttggcggagcaccacctgaaggcggagctagatttgatg
                *****.***************.*** ************.********.**.**..*****
                                                        gRNA3
Dsuz-dbe        gagggcagcatggtggtgaagaccagtcgtaagacctgggacccctacatcattatcaag
Dmel-dbe        gagGGGagcatggtggtgaagaccagtcgcaagacctgggatccctacatcatcatcaag
                ***** **********************.***********.***********.******

Dsuz-dbe        gcgagggacatgatcaagctgatggccaggagtgtgcccttgagcaggccaagcgggtc
Dmel-dbe        gcgcgggatatgatcaagctgatggccagaagtgttcccttcgagcaggccaagcgggtc
                *** ****.********************.***** *****.******************

Dsuz-dbe        ctgcaggatgacattgggtgcgacatcatcaaaatcggcaatctagtccacaagaaggag
Dmel-dbe        ctgcaggacgacattgggtgtgacatcatcaaaatcggaaaccttgtgcacaagaaggag
                ********.***********.***************** **.** ** ***********

Dsuz-dbe        aagttcgtgaagcggcgccagcgtttgatagggcccaacggagccaccctgaagtccatc
Dmel-dbe        aagttcgtgaagcggcgacagcgtttgatcggacctaacggcgccacccttaagtccatt
                ***************** *********** **.**.***** ******** ********.

Dsuz-dbe        gaactgcttaccgactgctatgttctggtacaaggaaacaccgtctccgccttgggtcca
Dmel-dbe        gaactgctcaccgattgctacgttttggttcaaggaaacacagtctccgccttgggtcct
                ********.*****.*****.***.**** *********** *****************

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Dmel-dbe        tacaagggccttcagcaggtgcgggatatagtcctggagaccatgaacaatgtgcacccc
                ***********.***************** ********.** **************.***
                                gRNA2
Dsuz-dbe        atatacaacatcaaggccctgatgatcaagcgcgagctgatgaaggatccccgcctggcc
Dmel-dbe        atatacaacattaaggctctgatgatcaagCGGgagctgatgaaggatccgcgtctggcc
                ***********.*****.************** ***************** **.******

Dsuz-dbe        aacgaggattggtctaggttcctgccgaagttcaagaacaagaacatcagcaaacgcaag
Dmel-dbe        aacgaggactggtcccgattcctgcccaagttcaagaacaaaaacattagcaaacgcaag
                ********.*****. *.******** ***************.*****.************

Dsuz-dbe        cagccgaaggtgaagaagcagaagaaggagtacactccattcccgcccagccagccggag
Dmel-dbe        cagccgaaggtcaagaagcaaaagaaggagtacaccccattcccgcccagccagccggag
                *********** ********.**************.************************

Dsuz-dbe        agcaaggtggacaagcagttggccagcggagagtacttcctcaaccaggagcagaagcag
Dmel-dbe        agcaaggtggacaagcagctggccagcggagagtacttcctcaaccaggagcagaagcaa
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Dsuz-dbe        gccaagcggaaccaggagcgcaccgaaaagcagaaggaggcggccaagcgccagggcgag
Dmel-dbe        gcgaagcgcaaccaggagcgcaccgaaaagcagaaggaggcggccaagcgccaggacgaa
                ** ***** ********************************************.***.
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Fig. S4. Alignment of *dbe* target region with the *Rescue* fragment. Shown is the DNA sequence alignment of the *dbe* locus gRNA target sites in *D. melanogaster* with the *Rescue* fragment form *D. suzukii*. Note how the gRNAs can only target the *D. melanogaster* locus. CDS in green, Start/Stop in uppercase, gRNA target sites including PAM in yellow, PAM in uppercase.

Fig. S5. Alignment of *TfIIA-S* target region with the *Rescue* fragment. Shown is the DNA sequence alignment of the *TfIIA-S* locus gRNA target sites in *D. melanogaster* with the *Rescue* fragment from *D. suzukii*. Note how the gRNAs can only target the *D. melanogaster* locus. CDS in green, Start/Stop in uppercase, gRNA target sites including PAM in yellow, PAM in uppercase.

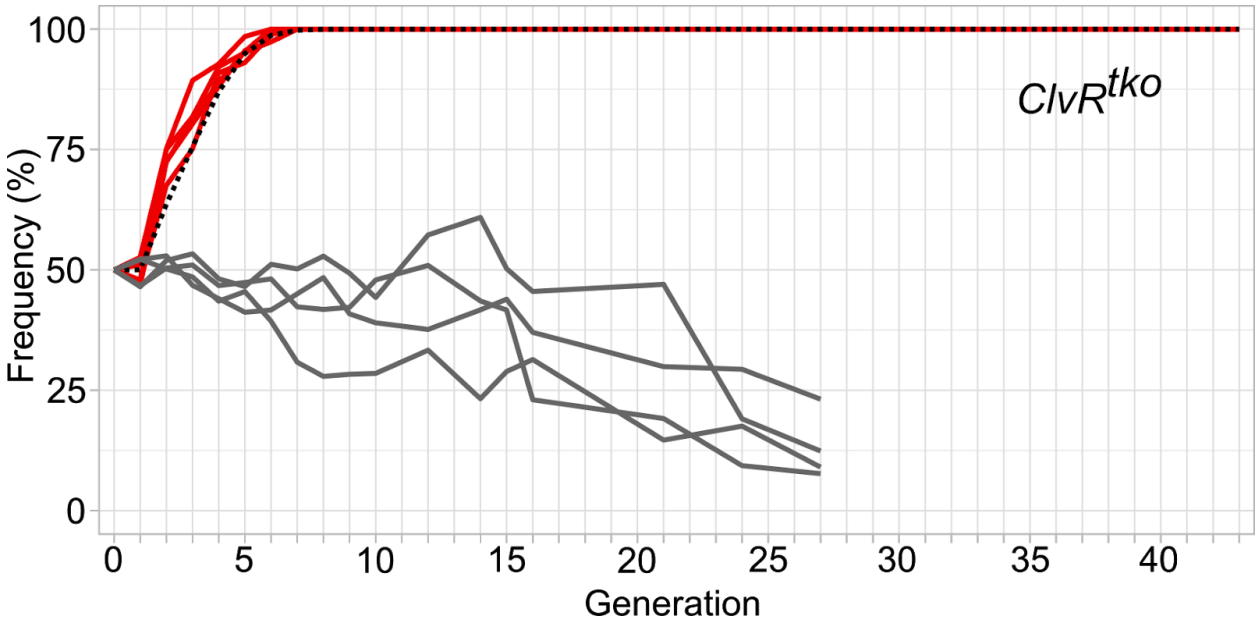

Fig. S6. Continuation of a *ClvR^{tko}* gene drive experiment reported in (25)**.** Red lines represent drive data from five replicates. Grey lines show data from control experiments in which a construct carrying only the *tko Rescue* and a dominant marker was introduced into a w^{1118} population. Dotted black line shows model behavior for an element with no fitness cost. Data from ref. (25) (generation 0-18).

>*Dsuz-TfIIA-S* rescue fragment

tgttccataatgcattgcgctgctttccagaagatggattgaccaagatgtcggtgaagaagtccatggagatcttcgatggtaggctgctcaagtacagggaactggctgccactgccaagg
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Fig S7. *Dsuz-TfIIA-S Rescue* fragment fasta file. Exons in yellow, intron in green, up- and downstream annotated gene neighbors in blue, total length=3802 bp. The *Rescue* fragment was chosen to include sequence from neighboring genes to maximize the likelihood that all regulatory sequences needed for normal expression of the *Rescue* transgene are present.

>*Dsuz-dbe* rescue fragment

gagcaaaaaagaattcatcagacgctgacctaagtgcttaaccttgtatattttagaaagatatgtacatatatagctcagtgtaaaaataaatctgttagccgatcggagtatt
aggctataggctttaaaaatgtaattatatttagggtattccttaaacaattgaatataaacttaataggtacattttatctattgggatggcataaagtaagaatgtgtataagaaa
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gaatcgctttttttggtaagctacaaaaatattttaaattgcggggaaattataaggacc

Fig S8. *Dsuz-dbe Rescue* fragment fasta file. Exon in yellow, up- and downstream annotated gene neighbors in blue, total length=1961 bp. The *Rescue* fragment was chosen to include sequence from neighboring genes to maximize the likelihood that all regulatory sequences needed for normal expression of the *Rescue* transgene are present.

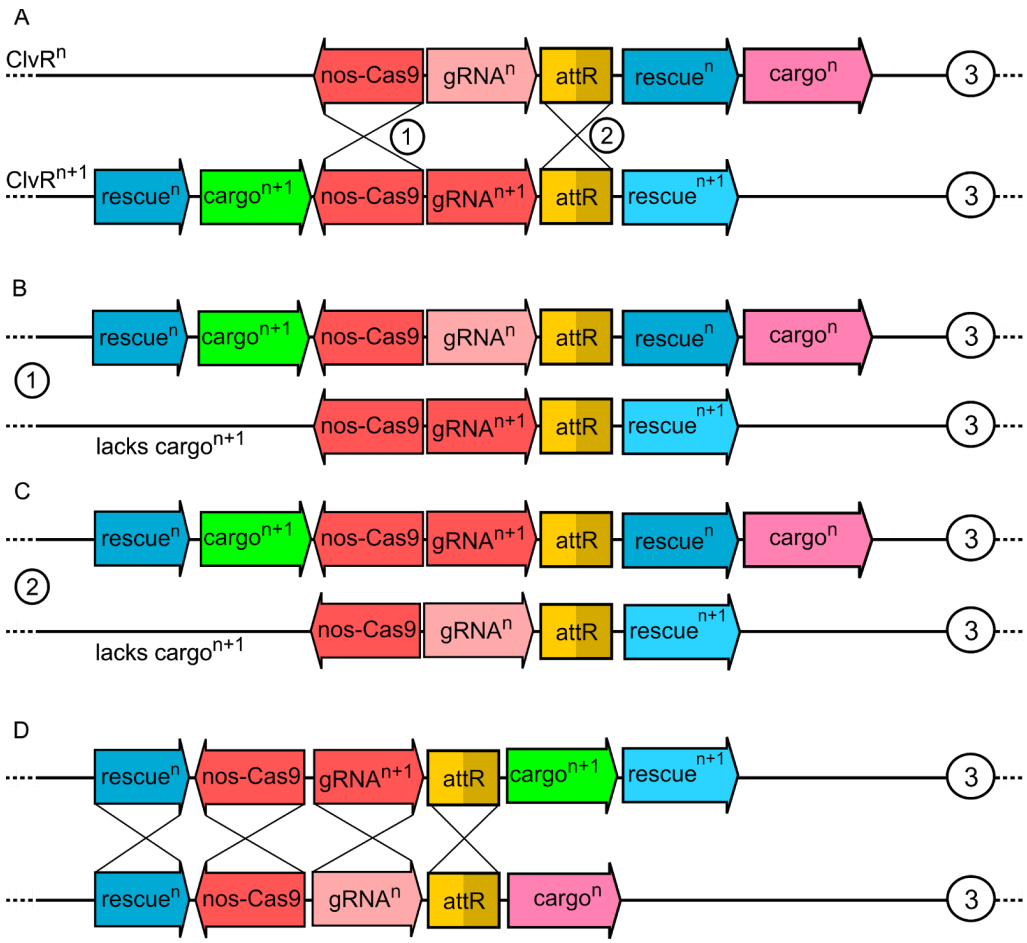


Fig. S9. Recombination products of *ClvR*^{n} and *ClvR*$^{n+1}$. (A) Shown are the actual arrangements of components used in this study. Recombination (crossed lines) is to be expected between sequences with high homology. Two important cases are highlighted (circled numbers) with the recombination products drawn out below. The products of most concern are those that carry *Rescue*$^{n+1}$ and lack cargo^{n+1}. While such elements lack gRNAs that can work with Cas9 to drive *Rescue*$^{n+1}$ through the population, this activity would be provided by the majority of other elements in the population, which are intact. If the empty *Rescue*$^{n+1}$ element had a higher fitness than that of an intact cargo^{n+1}-bearing element the former would spread at the expense of the latter, resulting in some drive element-bearing individuals that lack the cargo. **(B)** Recombination event 1 creates a chromosome that lacks cargo^{n+1}. This chromosome is expected to spread because it carries Cas9 targeting gene n+1 along with a Rescue copy of n+1. However, it lacks *Rescue*^{n} and is therefore at risk of ending up in a fly without functional copies of gene n. This may slow its spread, depending on when during the process of drive it occurs **(C)** Recombination event 2 creates another chromosome without cargo^{n+1}. In addition it carries Cas9 targeting gene n but has no *Rescue* for gene n and is thus expected to be at risk for loss, as in B. **(D)** The components of the second generation element are configured such that recombination between regions of homology shared between *ClvR*^{n} abd *ClvR*$^{n+1}$ has no effect. Recombinant products are either *ClvR*^{n} or *ClvR*$^{n+1}$. Most importantly, cargo^{n+1} remains tightly linked to rescue^{n+1}.

Supplementary Tables

Table S1: Rates of LOF allele creation in the combined maternal germline and zygote of *ClvR^{dbe}* and *ClvR$^{TfIIA\text{-}S}$*. We scored the genotype of offspring from a cross of *ClvR*/+ heterozygous mothers to wildtype *w^{1118}* males. The genotype frequencies of the offspring of these crosses were used to calculate a combined LOF allele creation rate coming from cleavage in the germline and cleavage of the paternal target gene allele due to maternal carry over. Crosses were set up in bottles with ~40 *ClvR*/+ virgins each. The 4 presumably wildtype flies (escapers) were analyzed further (see Table S4)

A) Parental cross: ♀*ClvR^{dbe}* XX ♂*w^{1118}*

fly bottle	*ClvR^{dbe}*	wildtype	sum	*ClvR* frequency (%)	Cleavage to LOF
1	851	0	851	100	100
2	974	1	975	99.9	99.79
3	894	0	894	100	100
4	878	1	879	99.9	99.77
5	668	0	668	100	100
6	529	0	529	100	100
7	716	2	718	99.7	99.44
8	462	0	462	100	100
total	**5972**	**4**	**5976**	**99.93**	**99.87**

B) Parental cross: ♀C*ClvR$^{TfIIA\text{-}S}$* XX ♂*w^{1118}*

fly bottle	*ClvR$^{TfIIA\text{-}S}$*	wildtype	sum	*ClvR* frequency (%)	Cleavage to LOF
1	679	0	679	100	100
2	632	0	632	100	100
3	708	0	708	100	100
4	588	0	588	100	100
5	705	0	705	100	100
total	**3312**	**0**	**3312**	**>99.97**	**>99.94**

Table S2: Rates of LOF allele creation in the paternal germline. The target genes of our *ClvR* lines are recessive lethal. To determine rates of cleavage and LOF allele creation in progeny from heterozygous *ClvR*/+ males we crossed them to deficiency stocks that completely lacked the target gene locus. The deficiency-bearing chromosome was maintained in trans to a balancer chromosome that is dominantly marked and that is wildtype for the target essential gene. By focusing on the offspring that carry the deficiency we can calculate the rate at which LOF alleles are created in the male germline by dividing the number of flies that carry the deficiency and *ClvR* by half the number of flies that carry the deficiency: male germline LOF allele rate= [*Df(dbe),ClvR*^{dbe}] / sum[*Df(dbe)/2*]. The 21 flies that carried the deficiency but not *ClvR* (escapers) were analyzed further below, Table S5)

A) Parental cross: ♂*ClvR*^{dbe}XX ♀ *Df(dbe)/CyO*

vial	*Df(dbe),ClvR*^{dbe}	*Df(dbe)*	*CyO,ClvR*^{dbe}	*CyO*	sum *Df(dbe)*	*ClvR* frequency (%)	Cleavage to LOF
1	15	0	21	9	15	100	100
2	29	0	25	29	29	100	100
3	9	0	3	5	9	100	100
4	43	4	50	30	47	91.5	82.98
5	16	0	15	16	16	100	100
6	5	0	7	2	5	100	100
7	18	4	25	25	22	81.8	63.64
8	23	0	26	25	23	100	100
9	31	0	35	28	31	100	100
10	17	0	28	26	17	100	100
11	14	0	19	29	14	100	100
12	34	2	38	33	36	94.4	88.89
13	15	0	17	14	15	100	100
14	40	1	44	31	41	97.6	95.12
15	22	2	24	23	24	91.7	83.33
16	33	0	28	28	33	100	100
17	33	0	25	15	33	100	100
18	21	0	21	27	21	100	100
19	43	4	36	42	47	91.5	82.98
20	24	0	18	26	24	100	100
21	18	0	27	23	18	100	100
22	23	0	22	23	23	100	100
23	26	1	27	35	27	96.3	92.60
24	31	0	35	40	31	100	100

25	19	0	13	25	19	100	100
26	19	0	19	18	19	100	100
27	27	0	20	26	27	100	100
28	20	2	32	33	22	90.9	81.82
29	12	0	18	18	12	100	100
30	29	0	22	35	29	100	100
31	29	1	38	39	30	96.7	93.33
32	20	0	20	21	20	100	100
33	18	0	20	19	18	100	100
total	**776**	**21**	**818**	**818**	**797**	**97.4**	**94.73**

B) Parental cross: ♂*ClvR$^{TfIIA-S}$* XX ♀ *Df(TfIIA-S)/TM6B,Tb*

vial	***Df(TfIIA-S)/ClvR$^{TfIIA-S}$***	***Df(TfIIA-S)***	*ClvR$^{TfIIA-S}$/TM6B, Tb*	*TM6B,Tb*	sum *Df(TfIIA-S)*	*ClvR* frequency (%)	Cleavage to LOF
1	32	0	27	26	32	100	100
2	20	0	28	21	20	100	100
3	26	1	28	32	27	96.3	92.60
4	34	0	36	23	34	100	100
5	27	0	28	26	27	100	100
6	34	0	29	24	34	100	100
7	29	0	35	35	29	100	100
8	13	0	36	27	13	100	100
9	19	0	16	15	19	100	100
10	23	0	10	13	23	100	100
11	28	0	27	24	28	100	100
12	3	0	2	3	3	100	100
13	32	0	42	37	32	100	100
14	26	0	20	18	26	100	100
15	36	0	41	31	36	100	100
16	19	0	17	19	19	100	100
17	26	0	32	31	26	100	100
18	11	0	13	10	11	100	100
19	24	0	31	30	24	100	100

20	35	0	42	31	35	100	100
21	30	0	31	34	30	100	100
22	11	1	21	18	12	91.7	83.33
23	28	0	24	27	28	100	100
24	34	0	32	28	34	100	100
25	23	0	23	24	23	100	100
26	31	0	27	22	31	100	100
27	18	0	25	24	18	100	100
total	**672**	**2**	**723**	**653**	**674**	**99.7**	**99.41**

Table S3: Rates of LOF allele creation in the combined maternal germline and zygote of the second generation *ClvR* elements *ClvR^{dbe}+R^{tko}* and *ClvR^{tko}+R^{dbe}*. We scored the genotype of offspring from a cross of (*ClvR^{n+1}+R^{n}*)/+ heterozygous mothers to wildtype *w^{1118}* males. The genotype frequencies of the offspring of these crosses were used to calculate a combined LOF allele rate coming from cleavage in the germline and cleavage of the paternal target gene allele due to maternal carry over. Crosses were set up in bottles with ~40 (*ClvR^{n+1}+R^{n}*)/+ virgins each.

A) Parental cross: ♀(*ClvR^{dbe}+R^{tko}*)/+ XX ♂*w^{1118}*

fly bottle	*ClvR^{dbe}+R^{tko}*	wildtype	sum	*ClvR* frequency (%)	Cleavage to LOF
1	1138	0	1138	100	100
2	1053	0	1053	100	100
3	812	0	812	100	100
total	**3003**	**0**	**3003**	**>99.97**	**>99.87**

B) Parental cross: ♀(*ClvR^{tko}+R^{dbe}*)/+ XX ♂*w^{1118}*

fly bottle	*ClvR^{tko}+R^{dbe}*	wildtype	sum	*ClvR* frequency (%)	Cleavage to LOF
1	1106	0	1106	100	100
2	1329	0	1329	100	100
3	1164	0	1164	100	100
total	**3599**	**0**	**3599**	**>99.97**	**>99.89%**

Table S4: Analysis of escapers from crosses of heterozygous *ClvR^{dbe}*/+ females to *w^{1118}* in Table S1.

Out of 5972 flies scored we found 4 flies that did not carry the *ClvR* marker. We crossed all 4 of them again to heterozygous *ClvR*/+ females to test whether the escaper chromosomes remained sensitive to *ClvR*. Results of this cross are in the last 3 columns of the table below. After allowing the escaper flies to mate we extracted genomic DNA and sequenced over the gRNA target sites. Sequencing results are shown in the table below. '+' indicates an unaltered target site, del indicates a deletion followed by the number of deleted bp. One fly died for which we couldn't obtain a sequencing signal. The other three had a common 3bp in frame deletion at the target site for gRNA4. Two of these flies had an additional 3bp in frame deletion at target site for gRNA3 . Target sites for gRNA1 and gRNA2 were unaltered. Backcrosses of these flies to *ClvR*/+ females showed that the escaper chromosome could still be cleaved/mutated (Cleavage to LOF of 93.75 and 100%, 1 fly was sterile).

Fly ID	gRNA1	gRNA2	gRNA3	gRNA4	tomato+	tomato-	*ClvR* frequency (%)	Cleavage to LOF
2.1	+	+	3bp del	3bp del	62	2	96.88	93.75
4.1	no signal	+	3bp del	3bp del	sterile	sterile	NA	NA
6.1	+	+	+	3bp del	51	0	100	100

Table S5: Analysis of escapers from crosses of heterozygous *ClvR^{dbe}*/+ and *ClvR$^{TfIIA-S}$*/+ males to the deficiency strains from Table S2. For the cross of ♂*ClvR^{dbe}* XX *Df(dbe)/CyO* we found 21 escapers among 2433 flies scored. For the cross of ♂*ClvR$^{TfIIA-S}$* XX *Df(TfIIA-S)/TM3,Tb* we found 2 escapers among 2050 flies scored. We extracted genomic DNA from all of the escapers and sequenced over the target region. In addition, male escapers were backcrossed to heterozygous *ClvR*-bearing females to check whether the escaper chromosome could still be cleaved and mutated to LOF. Results are summarized in the table below. '+' stands for unaltered target site, SNP is likely a pre-existing polymorphism in the target site, 'del' stands for deletion followed by the number of deleted bases, 'sub' stands for substitution of bases. The last four columns show the results of the backcrosses of male escapers to heterozygous *ClvR*/+ females to check whether the escaped target locus could still be cleaved. For the 21 escapers coming from male *ClvR^{dbe}*/+ fathers, we found a 3bp in frame deletion at the target site for gRNA4 in five flies. One fly had a polymorphism at the target site of gRNA3 and one fly had a 2bp substitution at target site of gRNA3. All other target sites were unaltered. 12 of the 21 flies were males, which were backcrossed to *ClvR*/+ females. In one of theses crosses the cleavage rate to LOF was 85%, for all the others it was 100% (see Table S5A), again showing that the escaper chromosomes were not resistant to Cas9 cleavage. The 2 escapers coming from *ClvR$^{TfIIA-S}$* fathers had a polymorphism at the target site of gRNA2, while all the other target sites remained unaltered. When the 2 escapers were backcrossed to *ClvR*/+ females, the cleavage rate to LOF in the progeny was 100% (Table S5B).

A) ClvR^{dbe}

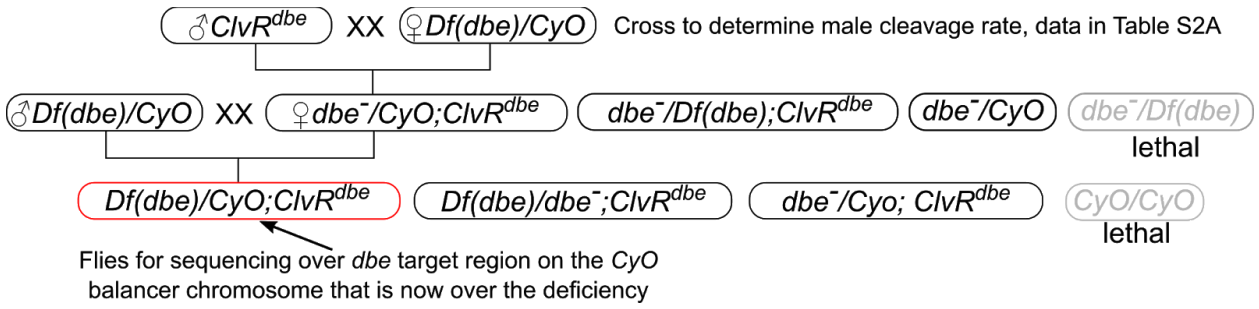
Fly ID	gRNA1	gRNA2	gRNA3	gRNA4	tomato+	tomato-	*ClvR* frequency(%)	Cleavage to LOF	notes
4.1	+	+	+	+	74	0	100	100	male
4.2	+	+	+	+	64	0	100	100	male
4.3	+	+	+	3bp del	NA	NA	NA	NA	female
4.4	+	+	+	+	71	0	100	100	male
7.1	+	+	SNP	3bp del	NA	NA	NA	NA	female
7.2	+	+	+	+	76	0	100	100	male
7.3	+	+	+	+	53	4	93.0	84.9	male
7.4	+	+	+	+	NA	NA	NA	NA	female
12.1	+	+	+	+	NA	NA	NA	NA	female
12.2	+	+	+	+	66	0	100	100	male
14.1	+	+	+	3bp del	NA	NA	NA	NA	female
15.1	+	+	+	+	sterile	sterile	NA	NA	male

15.2	+	+	+	+	54	0	100	100	male
19.1	+	+	2bp sub	+	NA	NA	NA	NA	female
19.2	+	+	+	+	NA	NA	NA	NA	female
19.3	+	+	+	3bp del	77	0	100	100	male
19.4	+	+	+	3bp del	71	0	100	100	male
23.1	+	+	+	+	NA	NA	NA	NA	female
28.1	+	+	+	+	68	0	100	100	male
28.2	+	+	+	+	61	0	100	100	male
31.1	+	+	+	+	66	0	100	100	male

B) ClvR$^{TfIIA\text{-}S}$

Fly ID	gRNA 1	gRNA2	gRNA3	gRNA4	tomato+	tomato-	*ClvR* frequency (%)	Cleavage to LOF	notes
3.1	+	SNP	+	+	71	0	100	100	male
22.1	+	SNP	+	+	89	0	100	100	male

Table S6: Cleavage events in chromosomes exposed to *ClvR^{dbe}* for the first time. We sequenced the target locus of *ClvR^{dbe}* flies in a chromosome that was exposed to *ClvR^{dbe}* for the first time. The crossing scheme to obtain these flies is provided below. We started with the cross to determine the male germline cleavage rate to LOF by crossing ♂*ClvR^{dbe}* XX ♀*Df(dbe)/CyO* (Data in Table S2). This cross produced ♀*dbe^{-}/CyO;ClvR^{dbe}*. The *CyO* balancer chromosome came from the deficiency stock and is now exposed to the *ClvR* element for the first time. Next we backcrossed these females again to males of the deficiency stock: ♀*dbe^{-}/CyO;ClvR^{dbe}* XX ♂*Df^{dbe}/CyO*. Among the progeny of this cross were flies that had the *CyO* balancer chromosome of the mother in trans to the deficiency from the father. Flies with that genotype were used to sequence over the target region on the *CyO* balancer chromosome. To simplify the scheme below it is assumed that *ClvR^{dbe}* always creates mutations at the wildtype *dbe* locus, indicated as *dbe^{-}*.



Sequencing results of *Df(dbe)/CyO;ClvR^{dbe}* flies:

Fly ID	gRNA1	gRNA2	gRNA3	gRNA4
27	+	SNP	del between gRNA3 and 4	
28	+	SNP	2bp indel	37bp del
29	+	SNP	9bp del	8bp del
30	+	SNP	11bp insertion	6bp del
31	+	SNP	+	+
32	del between gRNA1 and 2-3		9bp del	3bp del
33	17bp del	6bp del	4bp del	4bp indel
34	+	SNP	del between gRNA3 and 4	
35	3bp del	5bp indel	11bp insertion	3bp del
36	+	SNP	del between gRNA3 and 4	
37	+	SNP	+	+
38	+	SNP	11bp insertion	3bp del
39	+	SNP	11bp indel	38 bp del
40	+	SNP	3 bp indel	7bp del
41	+	SNP	13 bp deletion	5bp insertion
42	+	SNP	4 bp deletion	2bp deletion

Table S7: Cleavage events in chromosomes of *ClvR^{dbe}* drive populations after 22 Generations. We took 4 flies of each drive replicate bottle, crossed them individually to the deficiency stock, and sequenced progeny of this cross that had a presumably cleaved/mutated *dbe* locus in trans to the deficiency. Sequencing results are summarized in the table below.

Fly ID	gRNA1	gRNA2	gRNA3	gRNA4	notes
D1	***	1bp del	39bp del	***	inversion of locus between gRNA1 and gRNA4
D2	***	***	***	***	whole locus deleted
D3	3bp del	4bp del	21bp del	2bp del	
D4	3bp del	21bp indel	11bp insertion	2bp del	
D5	3bp del	6bp indel	4bp del	3bp del	
D6	10bp del	6bp del	11bp insertion	3bp del	
D7	11bp insertion	3bp insertion	11bp insertion	3bp del	
D8	11bp indel	3bp del	del between gRNA3 and 4		
D9	3bp del	10bp indel	2bp del	44bp del	
D10	24bp del	8bp indel	del between gRNA3 and 4		
D11	3bp del	2bp del	del between gRNA3 and 4		
D12	7bp del	28bp indel	9bp indel	4bp del	
D13	25bp del	3bp indel	9bp indel	3bp del	
D14	NS	NS	NS	NS	no signal
D15	48bp del	4bp del	9bp indel	3bp del	
D16	***	1bp del	39bp del	***	inversion of locus between gRNA1 and gRNA4

Table S8: Cleavage events in chromosomes exposed to *ClvR$^{TfIIA\text{-}S}$*. For *ClvR$^{TfIIA\text{-}S}$*, the *ClvR* selfish element as well as the target gene are located on the same chromosome. Therefore, we could not set up crosses easily where it would be clear that the cleaved target locus was only generated in that generation. However, as an attempt to generate what were likely new mutations at the *TfIIA-S* locus we took heterozygous *ClvR/+* females and outcrossed them to *w^{1118}* males for 5 generations. We then crossed *ClvR/+* females to a deficiency strain for the *TfIIA-S* locus and sequenced the target locus in progeny that were ClvR$^{TfIIA\text{-}S}$/*Df*. Results are summarized in the following table.

Fly ID	gRNA1	gRNA2	gRNA3	gRNA4	notes
1	3bp del	2bp ins, 3 bp sub	19bp del	8bp del	
2	del between gRNA1 and gRNA2		19 bp del	26bp del	
3	9bp del	2bp del	11bp del	8bp del	
4	deletion between gRNA1 and gRNA4				
5	3bp del	2bp ins, 3 bp sub	19bp del	8bp del	
6	del between gRNA1 and gRNA2		19 bp del	26bp del	
7	del between gRNA1 and gRNA2		19 bp del	26bp del	
8	del between gRNA1 and gRNA2		19 bp del	26bp del	
9	del between gRNA1 and gRNA2		19 bp del	26bp del	
10	del between gRNA1 and gRNA2		19 bp del	26bp del	
11	del between gRNA1 and gRNA2		19 bp del	26bp del	
12	9bp del	2bp del	11bp del	8bp del	
13	NS	NS	NS	NS	no signal
14	del between gRNA1 and gRNA2		19 bp del	26bp del	
15	del between gRNA1 and gRNA2		19 bp del	26bp del	
16	del between gRNA1 and gRNA2		19 bp del	26bp del	

Table S9: Cleavage events in chromosomes of *ClvR$^{TfIIA\text{-}S}$* drive populations after 22 Generations. We took 4 flies from each drive replicate bottle, crossed them individually to the *Df* stock for the *TfIIA-S* region, and sequenced progeny of this cross that were *ClvR$^{TfIIA\text{-}S}$* and carried the *Df* chromosome. These flies presumably had a cleaved *TfIIA-S* locus in trans to the deficiency. Sequencing results are summarized in the table below.

Fly ID	gRNA1	gRNA2	gRNA3	gRNA4	notes
T1	3bp del	6bp del	11bp del	30bp del	
T2	del between gRNA1 and 2		19bp del	26 bp del	
T3	del between gRNA1 and 2		19bp del	26 bp del	
T4	del between gRNA1 and 2		19bp del	26 bp del	
T5	del between gRNA1 and 2		19bp del	26 bp del	
T6	del between gRNA1 and 2		19bp del	26 bp del	
T7	del between gRNA1 and 2		19bp del	26 bp del	
T8	del between gRNA1 and 2		19bp del	26 bp del	
T9	NS	NS	NS	NS	no signal
T10	del between gRNA1 and 2		19bp del	26 bp del	
T11	del between gRNA1 and 2		19bp del	26 bp del	
T12	del between gRNA1 and 2		19bp del	26 bp del	
T13	del between 1.1kb upstream of gRNA1 and 3			3bp del	
T14	del between gRNA1 and 2		19bp del	26 bp del	
T15	del between gRNA1 and 2		19bp del	4bp del	
T16	2bp del	1bp del	del between gRNA3 and 4		

Table S10: Allele frequency of *ClvR* elements in gene drive experiments. The number of *ClvR* homozygotes and heterozygotes in different gene drive populations was determined by outcrossing 100 males from each gene drive bottle to w^{1118} females. If 100% of the progeny of these crosses carried the $ClvR^n$ marker, the male was homozygous for $ClvR^n$. If half carried the $ClvR^n$ marker the father was heterozygous. In addition, virgins were collected from the bottles at the assayed generations and used to seed the $ClvR^n$+R^{n-1} drive experiments presented in Fig. 4.

ClvR target	Replicate	Generation	$ClvR^n$ allele frequency [%]
dbe	A	22	90.43
dbe	B	22	82.76
dbe	C	22	87.36
dbe	D	22	89.20
TfIIA-S	A	22	91.07
TfIIA-S	B	22	93.75
TfIIA-S	C	22	94.71
TfIIA-S	D	22	95.70
tko	A	32	93.96
tko	B	32	95.60
tko	C	32	97.65
tko	D	32	100.00
tko	E	32	100.00

Table S11. Allele frequencies of *ClvR^{n+1}+R^{n}*, *ClvR^{n}*, and wildtype in 2nd generation *ClvR* gene drive experiments at generation 12. The allele frequencies in these gene drive populations was determined by outcrossing 100 males from each gene drive bottle to w^{1118} females. If 100% of the progeny of these crosses carried the *ClvR^{n+1}+R^{n}* marker, the male was homozygous for *ClvR^{n+1}+R^{n}*. If half carried the *ClvR^{n}* marker but not *ClvR^{n+1}+R^{n}* the father was transheterozygous for the 2nd and 1st generation *ClvR* element. If half carried no marker the father was heterozygous. Starting allele frequency for *ClvR^{n+1}+R^{n}* at generation 0 in all gene drive experiments was 25% (starting allele frequencies of *ClvR^{n}* are in Table S10). Note how *ClvR^{n+1}+R^{n}* increases in frequency at the cost of *ClvR^{n}*. Phenotype frequencies are plotted in Fig. 4 (green lines).

A) Allele frequencies of *ClvR^{dbe}+R^{tko}* driving into a population of *ClvR^{tko}* at generation 12 in Fig.4A (green lines)

	Allele frequencies (%)		
Replicate	***ClvR^{dbe}+R^{tko}***	***ClvR^{tko}***	**wildtype**
A	91.18	8.24	0.59
B	87.18	12.82	0.00
C	90.24	9.15	0.61
D	87.36	12.64	0.00

B) Allele frequencies of *ClvR^{tko}+R^{dbe}* driving into a population of *ClvR^{dbe}* at generation 12 in Fig. 4B (green lines)

	Allele frequencies (%)		
Replicate	***ClvR^{tko}+R^{dbe}***	***ClvR^{dbe}***	**wildtype**
A	84.88	14.53	0.58
B	88.46	10.44	1.10
C	88.61	10.76	0.63
D	85.00	13.33	1.67

Table S12. Allele frequencies of *ClvR^{n+1}+R^{n}* driving into populations of *w^{1118}* at generation 12. The allele frequencies in these gene drive populations was determined by outcrossing 100 males from each gene drive bottle to *w^{1118}* females. If 100% of the progeny carried the *ClvR^{n+1}+R^{n}* marker, the male was homozygous for *ClvR^{n+1}+R^{n}*. If half carried no marker the father was heterozygous. Starting allele frequency for *ClvR^{n+1}+R^{n}* at generation 0 in all gene drive experiments was 25%. Note how *ClvR^{n+1}+R^{n}* increases in frequency at the cost wildtype (*w^{1118}*). Phenotype frequencies are plotted in Fig. 4 (red lines)

A) Allele frequencies of *ClvR^{dbe}+R^{tko}* driving into a population of *w^{1118}* at generation 12 in Fig.4A (red lines)

	Allele frequencies (%)	
Replicate	***ClvR^{dbe}+R^{tko}***	**wildtype**
A	84.76	15.24
B	81.33	18.67
C	84.83	15.17
D	87.34	12.66

B) Allele frequencies of *ClvR^{tko}+R^{dbe}* driving into a population of *w^{1118}* at generation 12 in Fig. 4B (red lines)

	Allele frequencies (%)	
Replicate	***ClvR^{tko}+R^{dbe}***	**wildtype**
A	83.73	16.27
B	87.67	12.33
C	82.72	17.28
D	86.14	13.86