Active Genetic Neutralizing Elements for Halting or Deleting Gene Drives

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In Brief
Xu et al. describe two genetic systems for neutralizing an active gene drive that efficiently attenuate drive frequency in both pair crosses and cage-population experiments. These neutralization systems either delete the gene drive from the genome (ERACRs) or inactivate the Cas9 protein (e-CHACRs) to halt the gene drive.

Highlights
- e-CHACRs can efficiently copy and inactivate Cas9 activity (~99%)
- e-CHACRs spread to 100% prevalence in cage trials and eliminate Cas9 activity
- ERACRs often copy but can also damage the target chromosome
- ERACRs can efficiently delete and completely replace a gene drive in population cages
Active Genetic Neutralizing Elements for Halting or Deleting Gene Drives

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SUMMARY

CRISPR-Cas9-based gene drive systems possess the inherent capacity to spread progressively throughout target populations. Here we describe two self-copying (or active) guide RNA-only genetic elements, called e-CHACRs and ERACRs. These elements use Cas9 produced in trans by a gene drive either to inactivate the cas9 transgene (e-CHACRs) or to delete and replace the gene drive (ERACRs). e-CHACRs can be inserted at various genomic locations and carry two or more gRNAs, the first copying the e-CHACR and the second mutating and inactivating the cas9 transgene. Alternatively, ERACRs are inserted at the same genomic location as a gene drive, carrying two gRNAs that cut on either side of the gene drive to excise it. e-CHACRs efficiently inactivate Cas9 and can drive to completion in cage experiments. Similarly, ERACRs, particularly those carrying a recoded cDNA-restoring endogenous gene activity, can drive reliably to fully replace a gene drive. We compare the strengths of these two systems.

INTRODUCTION

Harnessing natural or synthetic gene drives to bias inheritance of beneficial traits in populations was proposed more than 60 years ago (Curtis, 1968), and variations on efficient “low-threshold” systems, such as homing endonucleases (Chevalier and Stoddard, 2001; Macreadie et al., 1985), have been modeled extensively over the past two decades (Burt, 2003; Deredec et al., 2008; Eckhoff et al., 2017). Recently, CRISPR-Cas9 genome editing tools have enabled the development of several highly efficient gene drive (or active genetic) systems in insects (Gantz and Bier, 2015; Gantz et al., 2015; Hammond et al., 2016; Kyrour et al., 2018; Li et al., 2019), yeast (DiCarlo et al., 2015), and bacteria (Valderrama et al., 2019). A mammalian guide RNA (gRNA)-only “split-drive” prototype has also shown significant promise in the mouse (Grunwald et al., 2019).

CRISPR-Cas9-based gene drives propagate by creating double-stranded DNA breaks at the precise site on the homologous chromosome where they are inserted into the genome. In the germline, the homology-directed repair (HDR) pathway copies the gene drive element into the break on the homologous chromosome, resulting in super-Mendelian transmission of that element to progeny. Mathematical modeling predicts that such efficient gene drive systems should spread rapidly throughout a population following logistic growth dynamics, even when released at low seeding levels (Burt, 2003; Eckhoff et al., 2017; Gantz and Bier, 2016).

Discussion in the scientific literature, workshops, and the media has raised several potential concerns, including scenarios in which a low-threshold system spreads beyond its intended zone of application (Adelman et al., 2017; James et al., 2018; National Academies of Sciences, Engineering, and Medicine, 2016; Warmbrod et al., 2020). One mitigation strategy for limiting gene drive systems to a chosen region is to develop neutralizing genetic systems that eliminate or prevent further dissemination of the gene drive.

We previously proposed two designs for self-copying (or “active”) neutralizing genetic elements that either inactivate Cas9 carried by a gene drive (e-CHACR [erasing construct hitchhiking on the autocatalytic chain reaction]) or delete and replace the gene drive (ERACR [element reversing the autocatalytic chain reaction]) (Gantz and Bier, 2016; Figure 1A). A key design feature of both elements is that they encode gRNAs but not Cas9. e-CHACRs can be inserted into the genome at any desired location and encode two or more gRNAs. One gRNA cuts at the
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genomic site of e-CHACR insertion, enabling self-copying in the presence of a trans-acting source of Cas9. The additional gRNA(s) target cleavage and inactivation of the cas9 transgene component of a gene drive element. ERACRs are inserted at the same genomic site as a gene drive and encode two gRNAs that combine with Cas9 produced by the drive, to cut on either side of the drive element to delete and replace it.

When seeded into a gene drive population, such gRNA-only neutralizing systems should follow the same logistic growth trajectory as a gene drive released into a native population (Gantz and Bier, 2016). In the absence of a Cas9 source, however, these neutralizing elements are inherited in a standard Mendelian fashion, as when they have either inactivated (e-CHACR) or eliminated (ERACR) a gene drive element.

In this study, we test and analyze the activities of several e-CHACR and ERACR elements in Drosophila melanogaster (D. mel.). We find that although the e-CHACRs vary in their copying efficiency, all mutate and inactivate Cas9 efficiently, and the one tested in population cages drives to completion. Similarly, ERACRs often copy and delete a gene drive element as intended but can damage target chromosomes and generate various rare recombinant outcomes. Despite these imperfections, ERACRs, particularly those carrying functional recoded sequences that restore endogenous gene activity, can fully replace a gene drive element in cage experiments. We discuss these results with regard to the potential utility of e-CHACRs and ERACRs to neutralize gene drives.

RESULTS

Active genetic elements such as gene drives and gRNA-only neutralizing elements (Figure 1A) bypass standard rules of inheritance imposed by independent chromosome assortment and linkage of nearby loci. Tracking such fluidly copying elements requires careful genetic bookkeeping by following each genetic element with a different fluorescent transgene and using genetic markers to carefully record the fate of each element. Careful genetic bookkeeping is needed to record the fate of each element.

Super-Mendelian Transmission of the MCR-GFP Gene-Drive Element

As a first step in our analysis, we generated an eGFP-marked MCR-GFP gene drive element carrying a cas9 transgene expressed under control of the vasa promoter (vasaCas9) and a gRNA (gRNA-y1) directing its copying at the y locus (Gantz and Bier, 2015) using a genetic “tagging” method (STAR Methods; Figure 1B). We assessed the drive performance of the MCR-GFP element in numerous parallel single-pair mating crosses in which so-called F1 “master females” (females carrying both gRNA and cas9 transgenes) were crossed to y− w+ males (Figure 1C). A substantial fraction (~20%–35%) of crosses resulted in 100% transmission of the MCR-GFP element to all F2 progeny (Figure 1D). Overall, the MCR-GFP element exhibited an average super-Mendelian transmission rate of ~85%.

Experiments with w+ marked receiver chromosomes (Figure S2A) revealed that the two chromosome homologs were inherited following standard Mendelian segregation (Figure S2B). We conclude that the great majority of the super-Mendelian inheritance displayed by the MCR-GFP element in such crosses can be attributed to gene drive copying events rather than to biased transmission of donor versus target receiver chromosomes.

e-CHACRs Efficiently Inactivate Cas9 but Copy with a Range of Frequencies

We inserted e-CHACRs into loci on different chromosomes: the X-linked white locus (e-CHACR-wG, eGFP marked; e-CHACR-wR, white apricot eyes) and the one tested in population cages drives to completion. We discuss these results with regard to the potential utility of e-CHACRs and ERACRs to neutralize gene drives.

Figure 1. Gene-Drive and Neutralizing Drive Elements

(A) Scheme depicting gene drives and neutralizing elements. Left: MCR (mutagenic chain reaction) gene drive element carrying Cas9, an eGFP fluorescent marker, and a gRNA for copying. Center: e-CHACR carrying two or more gRNAs: one gRNA (blue) for copying at its genomic insertion site, a second gRNA (purple) targeting Cas9, and a DsRed (∗ or eGFP) fluorescent eye marker. e-CHACRs are typically inserted at a different chromosomal site (locus A) than the gene drive (locus B). Right: ERACR carrying gRNAs that target sequences flanking the MCR-GFP element and a DsRed marker.

(B) Two MCR elements inserted at the same site in the y locus: (1) MCR lacking a fluorescent marker (third row) and (2) MCR-GFP, an eGFP-marked version (bottom row) carrying the same core components (vasa-Cas9 and gRNA-y1).

(C) Cross scheme for generating MCR-GFP F1, “master females” and their F2 progeny. Phenotypes of F0, F1, and F2 progeny are depicted schematically.

(D) Percentage of F2 progeny (carrying MCR-GFP element) recovered per cross. Error bars indicate standard deviation; asterisks indicate p values: *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001.

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DsRed marked) and the third chromosome *ebony* (e-CHACR-e) and *knirps* (e-CHACR-k) loci (see Data S1 for further details on e-CHACR analysis). Strains carrying these e-CHACRs were crossed to those harboring a standard “static” (non-copying) vasaCas9 source (Figures S3–S5 and S10) or the active MCR-GFP gene drive inserted at *y* (Figure 2; Figures S7–S9 and S11) to generate F1-generation master females bearing both elements. The phenotypes of F2 progeny from many parallel pair-mating crosses between F1 master females and males of informative genotypes (i.e., *y*±, *w*±, *ac*±) were tabulated, as discussed below. We evaluated two key e-CHACR performance parameters: (1) the efficiency of anti-Cas9 gRNAs (gRNA-C1, carried by the *w* and *kni* e-CHACRs, or gRNA-C3, carried by the ebony e-CHACR) in mutating and inactivating Cas9 and (2) the rate of e-CHACR copying to the target receiver chromosome.

**Figure 2. e-CHACR-wR versus the MCR-GFP Element**
(A) Schematic of DsRed+ e-CHACR-wR element linked to the *y* ac*^±* allele (e-wR*e*) and the *y* ac*^* marked MCR-GFP allele.
(B) Crossing scheme for testing the e-CHACR-wR (e-wR) against the MCR-GFP element. e-CHACR-wR females were mated to MCR-GFP males to generate F1 master females that were then pair-mated to ac*^±* males. F2 progeny were screened and analyzed for MCR-GFP presence and presence (DsRed*) or absence (w*NHEJ*) of e-CHACR-wR on ac*^±* or ac*^* marked chromosomes.
(C and C0) Percentage of fluorescence phenotypes in total female (C) or male (C0) F2 progeny per cross.
(D and D0) Prevalence of GFP* and GFP* alleles in MCR-GFP donor (left) and receiver (right) F2 (D) females or (D0) males.
(E and E0) Prevalence of body color in total F2 (E) females or (E0) males.
(F) Percentage of MCR-GFP donor (ac*, black dots) and receiver (ac*, pink dots) alleles in F2 males. Error bars indicate standard deviation; asterisks indicate p values: *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001.

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Cas9 inactivation by e-CHACRs was assessed in several ways. For e-CHACRs inserted at w (Figure 2; Figures S3, S4, and S7), we monitored a highly penetrant Cas9-dependent somatic mosaic eye phenotype (eyes consist of red and white sectors). By this mosaic eye metric, we estimate that Cas9 was inactivated in ~99% of F2 progeny by either gRNA-C1 or gRNA-C3, both of which target sites in the cas9 transgene encoding catalytically essential amino acids. Furthermore, non-mosaic F2 progeny carrying e-CHACR-wG and the presumably mutated Cas9 source transmitted the GFP+ element to F2 progeny at standard Mendelian ratios (Figure S3E), in contrast to the high super-Mendelian frequencies observed in F2 progeny (Figures S3C and S3C). In experiments involving the MCR-GFP drive (Figure 2; Figures S7,S9, and S11), we also assessed the full-body y+ pigmentation phenotype, which, as mentioned above, is fully penetrant in F2 progeny of master females carrying an active MCR-GFP element. 100% of F2 progeny had y+ phenotypes, revealing that Cas9 activity was eliminated by all e-CHACRs. We also sequenced genomic DNA isolated from individual F2 progeny, confirming that the gRNA target sites had been mutated in all samples analyzed from crosses with e-CHACR-wG (carrying gRNA-C1) and e-CHACR-e (carrying gRNA-C3) (Figure S6).

We observed a range of e-CHACR transmission frequencies. Copying was highly efficient (95%–99% transmission to F2 progeny) for both GFP andDsRed-marked e-CHACRs inserted at w (e-CHACR-wG and e-CHACR-wR, respectively) (Figure 2; Figures S3–S5). e-CHACR-k, inserted in the knirps (kn) locus, exhibited intermediate levels of copying (87%–90% transmission; Figure S11), while e-CHACR-e, inserted at ebony (e), was transmitted at Mendelian frequencies (Figure S9). The absence of drive for e-CHACR-e was puzzling, as previous experiments with other split-drive elements indicated that the same gRNA-e1 carried by e-CHACR-e sustained modest super-Mendelian copying (López Del Amo et al., 2019). We speculated that efficient mutagenesis of the cas9 transgene by gRNA-C3 might prevent e-CHACR-e from copying. Indeed, a cleavage-resistant form of the cas9 transgene (cas9) that could not be targeted by gRNA-C3 sustained modest copying of e-CHACR-e (~60% transmission) (Figure S10), similar to that previously reported for the split system driven by the same gRNA-e1 (López Del Amo et al., 2019). We conclude that all three e-CHACRs are highly efficient at eliminating Cas9 activity with either of two Cas9 targeting gRNAs and that their differing transmission most likely reflects the relative activities of the gRNAs that sustain their copying (see Data S1 and Discussion for further analysis).

**e-CHACRs Efficiently Inactivate the MCR-GFP Drive**

We tested the ability of the three different e-CHACRs to inactivate the MCR-GFP full-drive element, and all functioned efficiently to mutate Cas9, as judged by the virtual absence of F2 individuals displaying mosaic eye (>99%) or full-body y+ (100%) phenotypes (Figures 2E and 2F; Figures S9E, S9E, S11E, and S11E). All three e-CHACRs also markedly reduced the frequency of MCR-GFP copying, albeit to varying extents. e-CHACR-wR reduced MCR-GFP copying by 5- to 10-fold (i.e., from ~70% [Figure 1D] to 7%–13% [Figures 2D and 2D]; Figures S7D and S7D), e-CHACR-e by ~5-fold (Figures S9D and S9D), and e-CHACR-k by ~2-fold (Figures S11D and S11D). We conclude that e-CHACRs efficiently inactivate Cas9, copy themselves in the presence of a full-drive element or static sources of Cas9, and significantly reduce copying of the MCR-GFP gene drive element (see Discussion). These various reinforcing activities likely contribute to the efficient performance of e-CHACR-wR in population cage experiments described below.

**e-CHACRs Can Preferentially Bias Inheritance of Donor Chromosomes**

Visible markers (e.g., w+, ac+) closely linked to the e-CHACR and MCR-GFP elements permitted us to determine whether the donor and receiver chromosome homologs segregated in a biased fashion. We observed two types of significant bias in chromosomal inheritance. First, when the X-linked e-CHACR-wG targets a static Cas9 source inserted at the closely linked y locus, we observed a nearly 2:1 bias in the inheritance of the donor e-CHACR chromosome over the receiver chromosome in male (Figure S3C; Figure S4C) but not female (Figure S3C; Figure S4C). F2 progeny, which was accompanied by a corresponding excess of females to males (Figures S8B, S8D, and S8F). In contrast, when autosomal e-CHACR-e targeted a nearby Cas9-GFP source at w, the single-cut target chromosome was inherited at Mendelian frequencies in F2 progeny of both sexes (Figure S10A–S10C). Also, e-CHACR-wR driven by an autosomal source of Cas9 did not bias the sex of recovered F2 progeny (Figure S9). The handicap against receiver chromosomes in the former crosses, however, did not permanently damage target chromosomes, as F2 females carrying mutated Cas9 chromosomes transmitted them to ~50% of their F3 progeny (Figure S3E).

The second type of biased chromosome inheritance occurred when any of the three e-CHACRs targeted the MCR-GFP element. In these scenarios, the same locus on both chromosome homologs is targeted for cleavage (the donor MCR-GFP allele with anti-Cas9 gRNAs, and the receiver target allele with gRNA-y1 expressed by the MCR-GFP element). We observed excess transmission of y+ac- receiver chromosomes in F2 males, but not females (Figure 2F; Figures S7F, S9F, and S11F). We also recovered an unexpected class of GFP+ donor chromosomes (Figures 2D and 2D; Figures S7D, S7D, S9D, and S9D). Of 17 such isogenized GFP− target chromosomes tested, 10 (59%) carried male-lethal alleles, and all could be rescued by a duplication covering the y locus (Figure S12A; Table S1). As analyzed in greater detail in Data S1, we conclude that at least two different types of chromosome bias can be induced by e-CHACR elements: one, seen when cutting chromosomes carrying a static Cas9 source twice, does not irreparably damage the receiver chromosome. The second, associated with e-CHACRs targeting the MCR-GFP drive element, can induce local damage to the donor chromosome resulting in male lethality (or homozygous lethality in females).

**ERACR versus MCR-GFP Crossing Schemes**

We tested three DsRed-marked ERACR constructs designed to delete and replace the MCR-GFP element inserted at y (see Data S2 for in-depth analysis of ERACRs). All three ERACRs carry two gRNAs (gRNA-y2 and gRNA-y3) that direct Cas9 cleavage to either side of the MCR-GFP element (Figure 3A). ERACR-min carries just the aforementioned minimal elements (DsRed and...
two gRNAs), while ERACR-1 and ERACR-2 also carry recoded y cDNA coding sequences at their 5' junction that seamlessly restore function of the y locus. ERACR-2 shares fewer homologous sequences with the MCR-GFP element than ERACR-1. y cDNA sequences are fully recoded for ERACR-2, whereas only the 5' junction is recoded in ERACR-1. Also, U6-promoter sequences driving expression of gRNAs carried by ERACR-2 derive from a distantly related Drosophilid (D. grimshawi), which have little sequence homology and are oriented in the opposite direction to those carried by the MCR-GFP element to minimize potential spurious recombination events between the two elements.

Fly strains carrying the different DsRed+ ERACR constructs were crossed to a w+ marked MCR-GFP strain (Figure 3B; see also Figure S13 and Data S2 for additional crossing schemes and analysis). F1 master females carrying the w+ MCR-GFP chromosome and w- ERACR elements were crossed to w- males and their F2 progeny scored for fluorescence, eye color, and whole-body pigmentation phenotypes. This scheme permits w- ERACR-bearing donor chromosomes to be distinguished from w+ MCR-GFP target chromosomes.

**ERACRs Frequently Delete and Often Replace a Gene-Drive Element**

Three prominent outcomes were observed among progeny inheriting the w+ receiver chromosome: (1) deletion and replacement of the MCR-GFP element with the ERACR (phenotype: w-), while ERACR-1 and ERACR-2 also carry recoded y cDNA coding sequences at their 5' junction that seamlessly restore function of the y locus. ERACR-2 shares fewer homologous sequences with the MCR-GFP element than ERACR-1. y cDNA sequences are fully recoded for ERACR-2, whereas only the 5' junction is recoded in ERACR-1. Also, U6-promoter sequences driving expression of gRNAs carried by ERACR-2 derive from a distantly related Drosophilid (D. grimshawi), which have little sequence homology and are oriented in the opposite direction to those carried by the MCR-GFP element to minimize potential spurious recombination events between the two elements.

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Figure 4. ERACRs Delete and Replace the MCR-GFP Drive
Phenotypic frequencies and deduced gene conversion events in F₂ progeny from crosses depicted in Figure 3A and additional crossing schemes in Figures S13A–S13E. Black type, mean of percentages across all vials; orange type, estimated receiver conversion frequencies (e.g., DsRed⁺ w⁺ males/total w⁺ males) (see Data S1 for details).
(A) DsRed⁺ inheritance is a proxy for scoring ERACR prevalence (DsRed⁺,GFP⁺ and DsRed⁺,GFP⁺ progeny included).
(A') The subset of data plotted in (A) with traceable donor and receiver chromosomes re-plotted by donor (w⁺; left graph) versus receiver (w⁺; right graph) chromosomes.
(B) Proportion of F₂ males or females inheriting the donor (w⁺) versus receiver (w⁺) chromosome.
(C and E) Schematics illustrating predicted gene conversion events responsible for specific phenotypes: sequences of relevant junctions shown in (Figure S14).
(D) GFP inheritance is a proxy for MCR-GFP prevalence (DsRed⁺,GFP⁺ plus DsRed⁺,GFP⁺ F₂ progeny). Error bars indicate standard deviation; asterisks indicate p values: *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001.

DsRed⁺,GFP⁺: Figures 4A–4C); (2) retention of the MCR-GFP element (phenotype: DsRed⁻,GFP⁺; Figures 4D and 4E) with nonhomologous end joining (NHEJ)-induced indels at both the gRNA-y2 and gRNA-y3 cut sites (i.e., both ERACR gRNAs direct efficient target cleavage); and (3) deletion of the MCR-GFP element without copying the ERACR (phenotype: DsRed⁺,GFP⁺; Figures 5A–5E), a category abundantly observed only in females. Comprehensive analysis of the various ERACR
MCR Deletion Without ERACR Replacement

A. Prevalence of Non-Fluorescence in Total Progeny (Proxy for Deletion)

<table>
<thead>
<tr>
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<th>eGFP- and DsRed- (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERACR-min</td>
<td>16.6%</td>
</tr>
<tr>
<td>ERACR-1</td>
<td>49.6%</td>
</tr>
<tr>
<td>ERACR-2</td>
<td>22.7%</td>
</tr>
</tbody>
</table>

A'. Proportion of MCR Excision Events

|     | MCR Deletion (y-) | MCR Recombination (y+)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ERACR-1</td>
<td>24.6%</td>
<td>1.5%</td>
</tr>
<tr>
<td>ERACR-2</td>
<td>23.6%</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

B. Target Deletion

- X Chromosome - Truncated
- X Chromosome - Complete
- Female Viable
- Male Lethal

C. MCR Deleted (y2 Recombination)

- Inferred Gene Editing
- ERACR
- y2
- y3

- Resulting Sequence
- y2+y3

D. MCR Deleted (NHEJ)

- Inferred Gene Editing
- ERACR
- y2
- y3

- Resulting Sequence
- y2+y3

E. MCR Deleted (y2 Recombination)

- Inferred Gene Editing
- ERACR
- y2
- y3

- Resulting Sequence
- y2+y3

F. Prevalence of DsRed,GFP in Total Progeny (Proxy for ERACR+MCR Hybrid)

<table>
<thead>
<tr>
<th></th>
<th>eGFP- and DsRed+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERACR-min</td>
<td>0.2%</td>
</tr>
<tr>
<td>ERACR-1</td>
<td>1.6%</td>
</tr>
<tr>
<td>ERACR-2</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

G. ERACR/MCR Hybridization

- Inferred Gene Editing
- ERACR
- y2
- y3

- Resulting Sequences
- Yellow
- cas9
- eGFP
- gRNA-y1
- yellow
- DsRed
- gRNA Cassette

(legend on next page)
versus MCR-GFP outcomes is presented in Figure 4, Figure 5, and Figures S14–S17A, and in-depth analysis of these events is presented in Data S2).

Several salient features emerged from these experiments. First, the three ERACRs performed similarly overall (Figures 4 and S5; Figure S15), deleting and replacing the MCR-GFP element (~31%), leaving the MCR-GFP in place (~24%), and, in females, deleting the MCR-GFP without copying (~43%). There were also various rare outcomes (~1%) based on illegitimate recombination events, which differed on the basis of ERACR design (discussed further in Data S2). Second, the DsRed+·GFP+ phenotype was recovered >10-fold more often in females than males (Figures 5A and 5A). The great majority of such alleles were male lethal, resulting in a 2:1 excess of female over male F2 progeny (Figure S17A). In all tested cases, lethality of these DsRed+·GFP+ alleles could be rescued by a duplication of the tip of the X chromosome covering the y locus (Figure S12B). These findings indicate that ERACRs often damaged the target chromosome, disrupting essential functions in the vicinity of the target locus. Finally, although illegitimate recombinant events were rare, they were recovered (Figures 5C and 5E–5G). A few events lead to the generation of DsRed+·GFP+ mosaic elements (Figures 5F and 5G) that retained some capacity to copy all (Figures S20A–S20C) or portions (Figures S20D and S20D) of the elements. Also noteworthy, the frequency of such outlier events was significantly reduced for ERACR-2 relative to ERACR-1 (Figures 5A, 5A, and 5F; Figures S14 and S15), indicating that elimination of homologous sequences between ERACR and gene drive targets is a favorable design strategy.

One-Sided Homology Mismatch Underlies ERACR-Induced Chromosome Damage

Like e-CHACRs targeting receiver chromosomes for dual cleavage, ERACRs favor inheritance of donor chromosomes, suggesting that ERACR-induced chromosome damage might result from cutting the receiver chromosome twice at neighboring sites. We tested this hypothesis by constructing single-cut versions of ERACR-2, which should not generate damage according to the double-cut model. Single-cut ERACRs carried either only gRNA-y2 (ERACR2-y2, cutting 5’ and centromere distal to the MCR-GFP element), or only gRNA-y3 (ERACR2-y3, cutting 3’ and centromere proximal to the MCR-GFP element) (Figure 6A). We tested these elements for super-Mendelian transmission as well as induction of damage to the target chromosome.

Copying of single-cut ERACRs was assessed by pair-mating them to w0 MCR-GFP individuals according to the scheme depicted in Figure 6B, which is analogous to that used for the double-cut ERACRs (Figure 3B). Analysis of fluorescence associated with w0 receiver chromosomes in F2 progeny (Figures 6C and 6D) revealed that the frequency of deleting and replacing the MCR-GFP element was significantly reduced for both ERACR2-y2 (average = 4.9%; Figure 6D) and ERACR2-y3 (average = 1.7%; Figure 6D) relative to the dual-cutting ERACR-2 (average = 16%; Figure 4A, right panels), even when combined.

Crosses of ERACR2-y3 to the MCR-GFP element also reveal whether these single-cut elements might damage the target chromosome. We observed a prominent category (17%) of DsRed+·GFP+ · F2 female progeny that was absent in male siblings (Figure 6C), which is indicative of deleting the MCR-GFP element without copying the ERACR. This rate of DsRed+·GFP+ progeny is similar to that observed for the double-cut ERACR-2 (22.7% in F2 females versus 1.1% in males; Figure 5A), suggesting that single-cut ERACR2-y3 damages the target chromosome at a frequency comparable with that of the double-cut elements. Although crosses of ERACR2-y2 to the MCR-GFP element did not produce a prominent class of DsRed+·GFP+ · F2 female progeny, we did observe a similar ~10% disparity in the fraction of GFP+ female versus male progeny. This finding could be explained by chromosome damage being induced distal to the gRNA-y2 cut site, leaving the centromere-proximal GFP transgene intact. Both single-cut-ERACRs also lead to an excess of female over male F2 progeny inheriting the receiver chromosome (Figure S17B), as did all double-cut ERACRs (Figure S17A). These results suggest that both single-cut ERACRs damage the target chromosome at appreciable frequencies and that damage is localized centromere-distal to the gRNA-directed cleavage sites.

We also compared the frequencies with which single- versus double-cut ERACRs copied and induced chromosome damage when confronting a wild-type y0 allele in a so-called copy-cat configuration with a static source of Cas9 provided in trans (Figures S18 and S19). Here, the single-cut ERACR2-y2 (Figure S19B) copied nearly as well as the full double-cut ERACR-2 (Figure S19A), as judged by the proportion of DsRed+·GFP+ progeny (~20%). ERACR2-y3 copied less well, albeit notably better (~8%) than when challenged with the MCR-GFP (~1.5%). Both single- and double-cut ERACRs again induced comparable reductions in transmission of the GFP-marked

Figure 5. Alternative ERACR versus MCR-GFP Outcomes

Partial copying or fusion of sequences on the receiver chromosome.

(A) Non-fluorescent DsRed+·GFP+ · F2 progeny as a proxy for MCR-GFP deletion events (crosses as in Figure 4A).

(A1) Data in (A) re-plotted by inheritance of y0 (lethal deletion) versus y+ (recombination) alleles. Body color permits inference of the type of excision event that occurred, as depicted in (C–E) (data from y+ F2 fathers excluded).

(B) Loss of essential sequences distal to the ERACR gRNA cut sites result in male-lethal alleles. Viability of several such alleles can be rescued in males by duplications covering the tip of the X chromosome (Figure S12B).

(C) The MCR-GFP element is deleted, but not replaced, by ERACR, producing three distinct observed outcomes. (C) Hypothesized repair mediated by partial pairing of un-recorded y sequences carried by ERACR-1 and endogenous y sequences 3’ to the MCR-GFP element result in expression of the recorded y cassette and a wild-type body color. (D) NHEJ events joining adjacent sequences at the gRNA-y2 and gRNA-y3 cut sites. (E) Likely pairing between 17 bp of the gRNA-y2 genomic target sequences 5’ to the MCR-GFP with correspondingly oriented sequences in the gRNA-y2 transgene carried by either ERACR-min or ERACR-1.

(F) Prevalence of DsRed+·GFP+ · F2 progeny in which MCR-GFP and ERACR sequences are both present on the receiver chromosome.

(G) Two examples of MCR-GFP/ERACR fusion events (see Figure S14). Error bars indicate standard deviation; asterisks indicate p values: *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001.

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target chromosome in F2 males, but not females, consistent with damage to receiver chromosomes. We conclude that the likely basis for the majority of ERACR-induced chromosomal damage is lack of DNA homology on one side of the ERACR (the side missing the second gRNA), rather than cutting the target chromosome twice. We also note that single-cut ERACRs copy better with less distance between flanking homology sequences (e.g., 2.2 kb in a copy-cat versus 11.3 kb in the MCR-GFP mode).

**Figure 6. Drive Performance of Single-Cut ERACRs versus MCR-GFP Drive**

(A) Schemes illustrating single- and double-cut ERACR designs.  
(B) Crossing scheme for generating and testing transmission by single-cut w^A ERACR/MCR-GFP F1 master females.  
(C–E) Single-cut versions of ERACR-2 are placed in trans to the MCR-GFP element, where the gRNA-y2 and gRNA-y3 are separated by a distance of 11.3 kb (see also Figure S19 for Copy-Cat analysis).  
(C) Fluorescent phenotypes for single-cut DsRed+ ERACR2-y2 and ERACR2-y3 and MCR-GFP.  
(D) Percent DsRed+ females or males inheriting either the donor (w^A) ERACR-2 single-cut chromosome or the receiver (w^y) chromosome.  
(E) Donor (w^-) vs. receiver (w^y) chromosome transmission for single-cut ERACR2-y2 and ERACR2-y3. Error bars indicate standard deviation; asterisks indicate p values: *p < 0.05; **p < 0.01; ***p < 0.001; and ****p < 0.0001.

**ERACRs and e-CHACRs Do Not Sustain Shadow Drive**

We previously documented a maternal “shadow drive” phenomenon in individuals descended from Cas9-bearing mothers that...
inherit a gRNA-based drive element but not the cas9 transgene (Guichard et al., 2019). As both e-CHACRs and ERACRs eliminate Cas9 activity, we wondered whether this early action might preclude accumulation of sufficient Cas9/gRNA stores to sustain shadow drive in the subsequent generation. We examined the drive potential of e-CHACR or ERACR elements in F2 progeny of F1 master females lacking the cas9 transgene and thus carrying only maternal stores of the endonuclease provided by their F1 mothers (Figures S21A and S21C). We tested e-CHACR or ERACR transmission to F3 progeny from such F2 females and observed Mendelian frequencies of GFP or DsRed transmission, respectively (Figures S21 B and S21D–S21F), revealing that neither neutralization element sustained significant shadow drive.

**Figure 7. Cage Experiments: MCR-GFP versus e-CHACR-wR and ERACRs**

(A–F) Modeling of MCR-GFP versus e-CHACR-wR (A) and ERACR (C and E) dynamics (solid lines; same as B, D, and F) and model fits (dotted lines) plotted for the frequencies of MCR-GFP (green) and e-CHACR-wR or ERACRs (red). (B) Plot of fraction of individuals with different phenotypes over 12 generations. Green, MCR-GFP; red, e-CHACR-wR prevalence in the total population (e.g., both males and females). Orange, females carrying both elements. Yellow, females with mosaic eyes indicative of Cas9 activity. Dark traces represent separate cage replicates, and pale lines denote model simulations (also in D and F). (C and E) Modeling of MCR-GFP versus ERACR dynamics over 26 generations (C) and ERACR-2 (E). (D and F) ERACR-min (D) and ERACR-2 (F) versus MCR-GFP cage experiments. Red, DsRed+ ERACRs; green, MCR-GFP+; yellow, both markers.

**e-CHACR-wR Inactivates the MCR-GFP Gene-Drive in Population Cages**

Results from pair-mating crosses revealed that e-CHACR-wR efficiently eliminated Cas9 activity and copied efficiently when combined with either static (Figures S3–S5) or gene drive (Figure 2; Figure S7) borne sources of Cas9. We generated mathematical models on the basis of these single-generation data (Data S1) and fitted parameters on the basis of observed time-series data from the cage trials (Figure S22; Figure 7A, solid lines). These initial values were then used to run simulations, shown as arrays of pale-colored lines, matching the schemes for the different displayed phenotypes (Figure 7B). Simulations with the set of fitted parameters (Data S1, Table S1) were largely consistent with experimental values for the frequencies of
different phenotypic classes observed in the pair-mating crosses. We were also able to infer parameters revealing informative deviations from assumptions of random mating between all genotypes. In particular, the modeling indicated a relatively high degree of assortative mating within groups having shared eye pigmentation (i.e., w males preferentially mating with w females over w males).

In conjunction with the modeling above, we competed e-CHACR-wR against the MCR-GFP element in population cages. We seeded triplicate cages with equal numbers of homozygous e-CHACR-wR and MCR-GFP individuals. At each generation (n), we scored half of the individuals per cage (approximately 150 random flies) for their fluorescent and y0 phenotypes, while the other half was used to seed the next generation (n + 1) of cages. In all three cages, e-CHACR (red curves) drove to completion in nine generations (Figures 7A and 7B). In addition, we observed a transient appearance and then disappearance (after eight generations S22B and S22B)

gonous females but lethal in homozygous or hemizygous condi-
posing elements should also behave as designed to eliminate the
the MCR-GFP element, are fully viable in heterozygous CHACR-wR had achieved 100% homozygous introgression (Figures 7C and 7E).
We conducted three or four separate cage replicates per ERACR composed of 25% homozygous ERACR and 75% homozygous MCR-GFP virgin male and female individuals in each cage. The frequency of the DsRed-expression ERACR cassette for the ERACR-min experiment tripled in about four generations and then gradually approached fixation over the next five generations, with a modest degree of variation (~10%–15%) exhibited among the four cages tested (Figure 7D). Reciprocally, the frequency of the MCR-GFP marker decreased until it was nearly eliminated by generation 9. Consistent with random mating among y-ERACR-min and MCR-GFP individuals, 30%–35% of progeny from the first generation were trans-heterozygotes carrying both fluorescent markers. This double-positive (GFP+,DsRed+) population peaked in generation 3 and then steadily diminished. In contrast, the proportion of DsRed-individuals in the ERACR-2 cages (Figure 7F) remained approximately constant for one generation and then increased steeply and in tight synchrony, reaching fixation in five or six generations, with concomitant reduction and then complete elimination of the MCR-GFP element. Notably, ERACR-2 replicates displayed little variation in their drive trajectories. These experimental results match well with simulations (Figures 7D and 7F, pale lines) on the basis of parameters identified by model fitting (Figures 7C and 7E, dotted lines), capturing key features of the overall kinematics and inter-trial variations.

Although there was an excellent overall fit between the observed data and modeling (Figures 7C and 7E; S23F–S23G), two ERACR-min replicates (1 and 4) pursued moderately different trajectories from the other two (2 and 3), with the latter two matching the predicted modeling (Figures 7C and 7E). As no such events were observed in any of the y-ERACR-2 replicates, these outlier trajectories may result from formation of y- resistance alleles that would have been selected against in the ERACR-2 replicates because of assortative mating but that could persist and potentially spread in the y-ERACR-2 replicates, where they were on even footing. In addition, modeling and cage studies (Figure S22) suggest that a fitness cost is associated with the MCR-GFP element, which might be aggravated by cryptic ERACR-induced damage by gRNA-y2.

We also competed the y-ERACR-2 against a y-NHEJ-induced point allele generated at the gRNA-y1 cleavage site (i.e., the same gRNA driving the MCR-GFP element) in the absence of Cas9 to isolate the effect of assortative mating (Figure S23E). Over about

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ten generations, chromosomes carrying the \( y^+ \) ERACR-2 successfully overtook the \( y^- \) NHEJ population with typical inter-cage variation (Figure S23E). These observations confirm that the \( y^+ \) genotype has an appreciable competitive advantage over \( y^- \) in a cage setting. Also consistent with \( y^+ \) ERACR-2 females mating preferentially with \( y^+ \) ERACR-2 males, the frequency of DsRed\(^+\),GFP\(^+\) (ERACR-2 + MCR-GFP) flies in first-generation progeny (Figure 7F, yellow curves) was considerably lower (15%–20%) than that predicted by random mating (37.5%) or observed for ERACR-min (Figure 7D). We conclude that the modeling predictions conform closely to the experimental outcomes and that ERACRs, particularly those carrying a recoded transgene restoring function of a functionally important gene, have the potential to eliminate and replace a gene drive element even once it has attained fixation in a population.

**DISCUSSION**

Overall, both e-CHACRs (Figure 8A) and ERACRs (Figure 8B) offer promise for fulfilling their purpose of countering a gene drive element even once it has attained fixation in a population.
system (Figures 8C and 8D), but it is also important to take into account some of their unintended actions.

**Drive Performance of Drive and Neutralization Systems**

Several factors contribute to levels of drive we observed, which ranged in transcription frequencies from >95% (e-CHACR-wG/R inserted into the w locus) to only ~50% Mendelian inheritance (e-CHACR-e, but see below). e-CHACR-k and the MCR-GFP drive element copied at intermediate rates (~85%), which for the MCR-GFP is somewhat lower than estimated in prior experiments in which transmission of an unmarked MCR element was estimated on the basis of full yellow body phenotypes (Gantz and Bier, 2015). As a significant fraction of crossovers using the marked MCR-GFP element transmitted it to 100% of progeny, this may also have been the case in those original experiments, which were based on only a small number of crossovers. Alternatively, it is possible that we underestimated gene conversion (e.g., the mosaic group might not have inherited the MCR). The size of the drive cassette could also affect copying efficiency, as we have noted higher inheritance rates for a smaller drive element using the same gRNA-y1 (e.g., Lopez Del Amo et al., 2019), which was in the 90% range.

In pair-mating crosses, all three ERACRs deleted the target MCR-GFP gene drive element from 74% of target chromosomes and copied themselves in its place as in 31% of those cases. The combined effect of these two outcomes is pronounced super-Mendelian inheritance of ERACR alleles in F2 males (85%–90%). In females, only copying events contributed to biased inheritance of the ERACR (e.g., ~65%), as male-lethal MCR-deleted chromosomes can survive in the heterozygous state (~20% of total females). In multi-generational contexts, however, damaged alleles carried by females are rapidly purged from the population (see below).

As noted above, e-CHACRs copied at different rates, which most likely reflect relative gRNA efficiencies. Two cases are informative in this regard. First, e-CHACR-e did not copy when combined with Cas9, although in a split-drive configuration, without the Cas9-targeting gRNA, it exhibited modest drive (~60%) (Lopez Del Amo et al., 2019). When combined with a cleavage-resistant Cas9* form, however, e-CHACR-e drove at levels similar to those in the split-drive experiments. We hypothesize that a “weak” gRNA, such as that carried by e-CHACR-e, can fail entirely if the source of Cas9 is eliminated prematurely. Similarly, the intermediate level of MCR-GFP drive (~85%) was reduced 2- to 10-fold when combined with various e-CHACRs, again suggesting that early elimination of Cas9 activity can reduce the performance of suboptimal gRNAs. In contrast, when the e-CHACR-wG/Rs carrying an efficient gRNA were combined with static or MCR-GFP sources of Cas9, they copied at rates equal to those observed in split-drive experiments (Lopez Del Amo et al., 2019). Thus, choosing an efficient gRNA seems to be an important design feature for e-CHACR elements.

ERACRs carry two gRNAs targeting the receiver chromosome at two neighboring sites. The two gRNAs increase the rate of ERACR copying relative to single-cut ERACRs that carry only one or the other gRNA, suggesting some form of synergy even though each gRNA was expected to act independently. This disparity was significantly more pronounced when the two gRNA cut sites were spaced further apart (e.g., when the MCR-GFP element is inserted between them).

**Neutralizing Elements Can Bias Chromosomal Inheritance**

Marking donor versus receiver chromosomes permitted us to discern three different types of biased inheritance of these chromosome holomologs. First, e-CHACRs cutting the same chromosome twice generated a 2:1 bias in favor of transmitting the donor X chromosome in males, but not females. This bias did not lead to any obvious permanent damage, however, because females segregated both donor and receiver chromosomes to their progeny with equal frequency (Figure S5E). Second, e-CHACRs generated a class of donor MCR-GFP alleles lacking the internal GFP marker, more than half of which were male-lethal. In this scenario, gRNAs from e-CHACRs (anti-Cas9 gRNAs) and the MCR-GFP element (driving gRNA) target both chromosome holomologs in almost exactly same location, a situation most often favoring the receiver allele, which, unlike the cas9 transgene, is flanked by perfect homology on both sides. Finally, ERACRs with two gRNAs targeting neighboring sites on the same chromosome homolog generated an abundant class of damaged chromosomes. This effect was not the result of dual cutting per se, as single-cut ERACRs generated comparable frequencies of damage. Thus, one-sided homology mismatch rather than dual cutting of the target most likely underlies ERACR-induced chromosome damage.

**e-CHACRs and ERACRs Perform Effectively as Designed in Cage Experiments**

Despite imperfections revealed in pair matings, e-CHACRs and ERACRs performed largely as intended in multi-generational cage studies. In these experiments, e-CHACR-wR completely eliminated Cas9 activity and drove itself to 100% homozygous introgression in nine or ten generations. Similarly, ERACR-2 entirely replaced the MCR-GFP element over six generations. Several factors contributed to these successful outcomes (Figure 8B): (1) efficient Cas9 mutagenesis by the targeting gRNA, (2) efficient e-CHACR-wR copying, and (3) absence of somatic mosaicism or shadow drive caused by accumulated Cas9/gRNA complexes (this factor, which greatly reduces formation of drive-resistant NHEJ alleles, likewise pertains to ERACR performance). Also, a fitness cost may be associated with the MCR-GFP element, inferred from modeling and competition between the MCR-GFP element and a y− allele in cages (Figure S22A). In addition, single-generation crosses revealed that e-CHACRs can damage MCR-GFP chromosomes, generating a class of GFP− male-lethal alleles. These alleles would be invisible in the cage experiments, as they do not survive either in males or homozygous females but would deplete the MCR-GFP allelic pool.

Three notable features distinguished the drive trajectories of y− ERACR-min and y− ERACR-2, all of which derive from their y− versus y+ phenotypes. First, ERACR-2 rapidly drove to 100% replacement, while ERACR-min plateaued and then increased more slowly without entirely eliminating the MCR-GFP element. Second, inter-cage variation was much reduced for the ERACR-2 trials than for ERACR-min, for which the observed variation was more typical (~5%–10%). Finally,
ERACR-2 experienced a one-generation lag in producing progeny carrying both the DsRed+ ERACR and MCR-GFP elements (yellow curves in Figure 7D versus Figure 7F).

The significant assortative mating advantage conferred upon y+ ERACR-2 males relative to y- MCR-GFP males is well documented (Barker, 1962; Bastock, 1956; Diederich, 1941; Merrell, 1949; Sturtevant, 1915) and was confirmed in our own experiments (Figures S23A–S23E). Indeed, the y+ ERACR-2 allele overtook a point mutant y- allele in competitive cage experiments (Figure S23E), albeit more gradually than when driving in a Cas9-dependent fashion against the MCR-GFP element (Figure 7F). The more rapid and complete drive of ERACR-2 compared with ERACR-min can be attributed to dual (Cas9-mediated + assortative mating) versus single (Cas9-mediated) drive mechanisms being operative. Similarly, the initial delay in generating ERACR-2/ MCR-GFP heterozygotes is expected if y+ ERACR-2 females preferentially mated with their own kind. The much reduced inter-cage variation observed for the ERACR-2 trajectories may reflect two independently acting processes: drive produced by Cas9 delivered in trans and a selective pressure resulting from assortative mating favoring y+ males. These dual factors provide fewer opportunities for stochastic variation to accumulate.

### Strengths of ERACR and e-CHACR Neutralizing Elements

These studies provide encouraging support for neutralizing a gene drive with e-CHACRs or ERACRs. The major strength of e-CHACRs is that they act generically and can neutralize SpCas9 gene drives inserted at various locations in the genome. e-CHACRs can efficiently inactivate Cas9 and copy themselves (e-CHACR-wG/R elements). The primary advantage of ERACRs is that they can delete and replace the gene drive, thereby eliminating any undesired effect associated with that element. ERACRs perform as intended less often, replacing the gene drive as designed only about a third of the time (Figures 4A and 4B). Nonetheless, ERACRs, particularly ERACR-2 carrying recoded sequences to restore target locus function, performed very effectively in population cages.

In the future, these two neutralizing strategies could be combined to exploit the strengths of each system. These systems also could be implemented with other proposed mitigation measures such as elements carrying anti-Cas9 proteins or inductive releases of strains carrying functional cleavage-resistant alleles of the targeted locus. Such passive elements could spread rapidly if insertion of a gene drive disrupted locus function. Also a CATCHA construct has been described that is inserted at the site of the gene drive (similar to ERACRs) and is designed to copy into the cas9 transgene (Wu et al., 2016). Although CATCHA elements could target Cas9 sources located elsewhere in the genome, they would not copy in those contexts. Also, because the cassette must be flanked with cas9 homology arms, it does not readily deliver an in-frame recoded target gene.

Another potential strategy is to incorporate a “weakened” gRNA* targeting the same essential Cas9 amino acid residues as gRNA-C1 or gRNA-C3 into the drive element itself. gRNA’s carrying one or two base pair mismatches to the target sequence should reduce Cas9 cleavage by 1–2 logs. Although not expected to appreciably interfere with the spread of a gene drive carrying such a gRNA*, the cas9 transgene should be mutated at rates ~10^6 greater than by spontaneous mutation once the drive attains full introgression into a population, thereby accelerating elimination of Cas9 from the population if it imposed a fitness cost.

### Implications for Potential Implementations of Neutralizing Systems

Although e-CHACR and ERACR elements behave largely as expected to curtail the spread of gene drives, they also produce unexpected outcomes (e.g., generation of drive-competent chimeric ERACR/MCR-GFP elements) and can falter in copying (e.g., e-CHACRs with non-optimal gRNAs). Thus, although these experiments provide optimism for strategies to retard wayward gene drive systems if necessary, we agree with the recommendations of the National Academies of Sciences, Engineering, and Medicine (2016) report on gene drives regarding the cautious use of such neutralization systems. We concur with their appraisal that the decision to go forward with potential releases of gene drive systems should not be predicated on constructing neutralizing elements and that such systems should be developed only for precautionary purposes. If one has concerns about a potential gene drive system somehow going awry, surely such concerns would only be amplified by release of a second element that could generate yet more complex genetic outcomes. Nonetheless, the present in-depth studies provide encouraging support for the use of these types of mitigating strategies should there be an unanticipated need for them.

### STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- **QUANTIFICATION AND STATISTICAL ANALYSIS**
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### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.molcel.2020.09.003.
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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

E.B., V.M.G., and O.S.A. have equity interest in Symbal Inc. (E.B. and V.M.G.) and Agragene (E.B., V.G., and O.S.A.). These companies may potentially benefit from the research results. E.B. and V.M.G. also serve on the Board of Directors and Scientific Advisory Board for Symbal Inc., E.B., V.M.G., and O.S.A. serve on the Scientific Advisory Board for Agragene Inc. The terms of these arrangements have been reviewed and approved by the University of California, San Diego, in accordance with its conflict-of-interest policies. All other authors declare no competing interests.

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SUPPORTING CITATIONS

The following references appear in the Supplemental Information: Bozas et al. (2009); Brunner et al. (2019); Delattre et al. (1995); Do et al. (2014); Drury et al. (2017); Joyce et al. (2012); Lindsley et al. (2016); López Del Amo et al. (2020); Pham et al. (2019); Vella et al. (2017); Wei and Rong, (2007); Xu et al. (2017).

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## STAR METHODS

### KEY RESOURCES TABLE

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### Experimental Models: Organisms/Strains

| Oregon-R | Bloomington Drosophila Stock Center | BDSC #5905 |
| W1118 | Bloomington Drosophila Stock Center | BDSC #2376 |
| FM7a | Bloomington Drosophila Stock Center | BDSC #785 |
| Basc | Bloomington Drosophila Stock Center | BDSC #806 |
| w[a] | Bloomington Drosophila Stock Center | BDSC #148 |
| ac[4] w[a] | Bloomington Drosophila Stock Center | BDSC #8 |
| y-: w- (used in MCR cage trial) | Gift from the Gantz Lab | N/A |
| y-: w- (F5 line used in ERACR cage trial) | Gantz et al., 2015 | N/A |
| Vasa Cas9 in yellow (y1) w[1118A] | Gift from the Gantz Lab | N/A |
| Vasa Cas9 in yellow (y1) w+ | Gift from the Gantz Lab | N/A |
| Vasa Cas9 in white (w2) | Gift from the Gantz Lab | N/A |
| w- MCR GFP | This study | N/A |
| w[a] MCR GFP | This study | N/A |
| w+ MCR GFP | This study | N/A |
| e-CHACR-AG; see Table S3 | This study | N/A |
| e-CHACR-AR; see Table S3 | This study | N/A |
| eCHACR-e; see Table S3 | This study | N/A |
| e-CHACR-k; see Table S3 | This study | N/A |
| vCas9-R; see Table S3 | This study | N/A |
| ERACR-min; see Table S3 | This study | N/A |
| ERACR-1; see Table S3 | This study | N/A |
| ERACR-2; see Table S3 | This study | N/A |
| ERACR-2 y2 Single-cut; see Table S3 | This study | N/A |
| ERACR-2 y3 Single-cut; see Table S3 | This study | N/A |
| X-duplication lines; see Table S5 | Bloomington Drosophila Stock Center | Various; See Table S5 |

### Oligonucleotides

| Cloning/construct design primers; see Table S2 | N/A | N/A |
| PCR primers; see Table S5 | N/A | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ethan Bier (ebier@ucsd.edu).

Materials Availability
All unique plasmids or Drosophila lines generated by this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability
Original data have been deposited on Mendeley Data (https://doi.org/10.17632/hjcpd6j8rn.1). Modeling code is available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila rearing and Genetic Experiments
All genetic experiments were conducted in a high-security Arthropod Containment Level 2 (ACL2) barrier facility, in accordance with protocols approved by the Institutional Biosafety Committee from University of California San Diego. All materials and waste are frozen for 48 hours prior to removal from the facility followed by autoclaving. Flies were kept triple-contained in shatter-proof polypropylene plastic vials. Stocks were maintained at 18°C while crosses were carried out at 25°C on a 12 hour day/night cycle on cornmeal media.

Multi-Generational Population cage studies
All population cage experiments were conducted at 25°C with a 12 hour day-night cycle, in 250 mL bottles containing standard cornmeal medium. Seeding populations for all drive experiments included equal numbers of unmated females and males for each genotype. 5-7 days following introduction into the cage and successful mating, all flies (generation n) are removed. Subsequent progeny (generation n+1) were collected, then randomly separated into two pools, one group is further analyzed and screened while the other pool is used for further seeding of the next cage (generation n+2).

METHOD DETAILS

Plasmid Construction
All constructs were cloned using standard recombinant DNA techniques, including PCR amplification with Q5 Hotstart master mix (NEB #M0494S) and Gibson assembly with NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Cat. # E2621).
Following successful cloning, the plasmids were transformed into NEB 5-alpha Electrocompetent Competent E. coli (New England Biolabs, Cat. # C2989). We list starting plasmids, oligos, and restriction enzymes used to generate each construct. Refer to supplementary materials (Table S2) for full sequences of plasmids and oligos.

**MCR-GFP construct by “tagging”**

The MCR-GFP construct used for these studies was generated by genetic “tagging” of the unmarked prototype yMCR construct (Gantz and Bier, 2015). Briefly, a plasmid construct carrying the eGFP gene expressed under control of the 3XP3 eye promoter, followed by 3’ SV40 polyadenylation sequences, flanked by homology sequences present in the unmarked yMCR construct (cas9 transgene and U6 gRNA homology arms), and carrying a construct expressing a gRNA directing cleavage between the cas9 transgene and U6-gRNA of the yMCR was inserted into the genome at the same site as the yMCR using gRNA-y1. Transgenic fly strains carrying this tagging construct (eGFP-Tagger) were recovered and crossed to the yMCR line to generate MCR/eGFP-Tagger females. Single crosses were performed by mating with Basc balancer males and the daughters in the resulting offspring were screened for a y-body color, indicative of Cas9 activity. Vials with Cas9 activity indicated a successful tagging of the yMCR construct and individual males were crossed with cut resistant gRNA-y1 site females (contain static Cas9 at gRNAy1 site) to generate isogenic homozygous body color, indicative of Cas9 activity. Vials with Cas9 activity indicated a successful tagging of the yMCR construct and individual males were crossed with cut resistant gRNA-y1 site females (contain static Cas9 at gRNAy1 site) to generate isogenic homozygous stocks for use in this study.

**Microinjection of ERACR and e-CHACR Constructs**

Plasmids were purified using the QIAGEN Plasmid Midi kit (#12191). ERACR and e-CHACR constructs were co-injected with a transient source of pAct-Cas9 (pAct-Cas9 was a gift from Fillip Port (Addgene plasmid # 62209) by Best Gene Inc. Injection mixes were assembled with either ERACR or e-CHACR donor plasmids (final concentration: 700 ng/μl) and pAct-Cas9 (final concentration: 300 ng/μl) in a volume of 50 μl. ERACR mixes were injected into a w1118 stock (BDSC #5905) while e-CHACR mixes were injected into an Oregon-R stock (BDSC #2376). All constructs were fully sequenced prior to injection and subsequently generated transgenic stocks were confirmed through sanger sequencing as well.

**Recovery of Transformants and Genomic DNA Isolation**

Injected flies were crossed the w1118 stock and progeny were screened for DsRed or GFP positive individuals, which were then subsequently crossed to an FM7 (first chromosome) or TM3 (third chromosome) balancer. Homozygous lines were established based on the absence of balancer alleles. For analysis of individuals containing gene drive elements, flies were frozen in an ACL-2 facility for 48 hours prior to removal from the facility and DNA isolation. Genomic DNA was prepared from individual male flies according to protocols by Gloer, 1993.

**Drosophila PCR and Sequence Analysis**

Sequencing PCR reactions were assembled with Q5 Hotstart master mix (NEB #M0494S), KOD Xtreme Hot Start DNA Polymerase (Millipore #71975), Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo-Fisher #F531S), or Platinum SuperFi DNA Polymerase (ThermoFisher #12351010). Primers used to amplify PCR products are listed in Table S4. PCR samples were purified using the QIAquick PCR purification kit (QiAGEN #28104) prior to sanger sequencing.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Mathematical Modeling**

Model fitting was carried out for the ERACR-min and ERACR-2 cage experiments using Markov chain Monte Carlo (MCMC) methods in which genotype-specific fitness parameters were estimated, including 95% credible intervals (CrIs). We considered discrete generations and Mendelian inheritance rules at the gene drive locus, with the exception that, for females heterozygous for the ERACR (E) and drive (H) allele, a proportion of the drive alleles are cleaved, while a proportion remain H alleles (Figure S1). Of those that are cleaved, a proportion are subject to accurate homology-directed repair (HDR) and become E alleles, while a proportion become resistant alleles. Of those that become resistant alleles, a proportion become in-frame, cost-free resistant (R) alleles, while the remainder become out-of-frame or otherwise costly resistant (broken, B) alleles. Parameter values were fixed for and based on the experimental results depicted in Figure S12. These considerations allowed us to calculate the expected genotype frequencies in the next generation, and to explore the fitness and assortative mating parameters that maximize the likelihood of the experimental data. Estimated parameters include the fitness cost associated with males having the drive allele, females homozygous for the drive allele, and having one copy of the drive or broken allele in females or respectively. BB females and BY males were known to be unviable. Populations were assumed to be randomly mixing, with the exception of the ERACR-2 experiment, in which females having the y+ phenotype preferentially mated with males having the y− phenotype. For ERACR-2, we estimated an additional parameter representing the fraction by which y+ females reduce their mating with y− males. The modeling framework is described in full in the Supporting Information.
Statistical Methods

Statistical analysis was performed using GraphPad Prism. One-way independent measures ANOVA followed by Sidak’s Multiple Comparisons test were performed for graphs in Figures 1D and 6 (all panels). All other graphs use one-way independent measures ANOVA followed by Tukey’s post hoc test. Significance was determined as follows: * denotes p < 0.05, ** denotes p < 0.01, *** denotes p < 0.001, and **** denotes p < 0.0001.
Supplemental Information

Active Genetic Neutralizing Elements for Halting or Deleting Gene Drives

Xiang-Ru Shannon Xu, Emily A. Bulger, Valentino M. Gantz, Carissa Klanseck, Stephanie R. Heimler, Ankush Auradkar, Jared B. Bennett, Lauren Ashley Miller, Sarah Leahy, Sara Sanz Juste, Anna Buchman, Omar S. Akbari, John M. Marshall, and Ethan Bier
Figure S1

A

F₀

MCR

MCR

F₁

MCR

y+

y+

F₂ Progeny Analysis

MCR

y+

y+

MCR

y+

MCR

y+

F₂ Male Progeny from MCR-GFP Male

F₂ Female Progeny from MCR-GFP Male

F₂ Progeny from MCR-GFP Female

F₂ Progeny from MCR-GFP Male

F₂ Progeny from MCR-GFP Female

Total F₂ Progeny from MCR-GFP Male

Total F₂ Progeny from MCR-GFP Female

C

F₂ Female Progeny from MCR-GFP Male

C'

F₂ Male Progeny from MCR-GFP Male

C''

Total F₂ Progeny from MCR-GFP Male

D

F₂ Female Progeny from MCR-GFP Female

D'

F₂ Male Progeny from MCR-GFP Female

D''

Total F₂ Progeny from MCR-GFP Female

Body Color

Eye Fluorescence

Eye Color

Phenotype

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<th>eGFP-</th>
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</tr>
<tr>
<td>22.0%</td>
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<td></td>
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<tr>
<td>0.0%</td>
<td>yellow-</td>
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</tr>
<tr>
<td>0.0%</td>
<td>yellow-</td>
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Phenotype

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<td>yellow-</td>
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Fig. S1: Performance of the MCR-GFP drive element when inherited maternally, related to Figure 1

A) Crossing scheme using MCR-GFP element inserted at the yellow locus when inherited from an F0 female (corresponds with Fig. 1C, in which the MCR-GFP was derived from an F0 male). Crossing scheme includes flies observed in the F0 and F1 generations and outlines the observed F2 female (top) and male (bottom) progeny. Fly heads represent phenotypic characterizations of each individual. B) Graph showing percentage of eGFP+ progeny per total males or total females, as a proxy to determine prevalence of the w^a MCR-GFP element. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. C-C’’,D-D’’) Histograms of MCR GFP transmission of F2 female, male, and total progeny. Conversion rates are binned in intervals of 5% on the X-axis and the number of vials with each conversion rate are represented on the Y-axis. The MCR crosses exhibited a profound dip in the 90-95% inheritance bins, then another peak around 80-85% transmission, followed by another dip and then a final peak between 65-70%. Each of these peaks (100%, 80-85%, 65-70%) are denoted with a black arrow. C-C’’) Analysis of conversion rate of F2 progeny when derived from an MCR-GFP male. D-D’’) Analysis of conversion rate of F2 progeny when derived from MCR-GFP female.
Figure S2

A

Chromosomes

F0  x  MCR-

y+, w-

F1  X

y+, w-

"Master Females"

Assay MCR Copying Frequency in F2 Progeny

B

w+ Marked MCR-GFP F2 Progeny

C

e-CHACR-wG Female F2 GFP Transmission

C'
e-CHACR-wG Male F2 GFP Transmission

C'' e-CHACR-wG Combined F2 GFP Transmission

Assay MCR Copying Frequency in F2 Progeny
Fig. S2: Performance of w^a MCR-GFP element and histograms of eCHACR-wG transmission, related to Figures 1 and 2

A) MCR-GFP w^a males were crossed to homozygous y^+w^- females to generate F_1 master females of the genotype MCR-GFP/w^-, which were crossed y^+w^- males to generate the scored F_2 progeny generation. B) Graph showing percentage of eGFP^+ progeny per total males or total females, as a proxy to determine prevalence of the w^a MCR-GFP element. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. C-C’”: Histograms of e-CHACR-wG GFP transmission of F_2 female, male, and total progeny. Conversion rates are binned in intervals of 5% on the X-axis and the number of vials with each conversion rate are represented on the Y-axis. The e-CHACR crosses exhibited a profound dip in the 90-95% inheritance bins, then another peak around 80-85% transmission, followed by another dip and then a final peak between 65-70%. Each of these peaks (100%, 80-85%, 65-70%) are denoted with a black arrow. C) Histogram of e-CHACR-wG GFP transmission of F_2 female progeny. C’) Histogram of e-CHACR-wG GFP transmission of F_2 male progeny. C”’) Histogram of e-CHACR-wG GFP transmission of F_2 total progeny.
Figure S3

A

Donor X Chromosome

yellow Locus

Homology Arm

Dmel U6:1 P

Dmel U6:3 P

Dmel U6:3 P

SV40 P-A eGFP 3xP3

Homology Arm

white Locus

Receiver

3' UTR cas9 Vasa P SV40 DsRed P-A

B

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F0 y+ e-wG x y+ e-wG

Master Females

F1 y+ e-wG x y+ w+

Analyze F2 Progeny

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<table>
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<tr>
<td>y+ e-wG</td>
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</tbody>
</table>

F2 ♂ y+ e-wG x y+ w+

F2 ♀ y+ e-wG x y+ w+

Assess Cas9 Function by presence of Mosaic F2 Females

C

e-CHACR-wG Female F2 Progeny

% Phenotype

47.6% 42.2% 52.2% 94.4%

Total DsRed+ Both eGFP+ only Total eGFP+

C'
e-CHACR-wG Male F2 Progeny

% Phenotype

37.1% 32.1% 62.6% 94.7%

Total DsRed+ Both eGFP+ only Total eGFP+

D

F2♀ Cas9 e-wG x y+ w+

Analyze F3 Progeny

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<th>Eye Color</th>
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</thead>
<tbody>
<tr>
<td>Eye Fluorescence</td>
</tr>
</tbody>
</table>

E

Cas9, e-CHACR-wG F3 Progeny

% Phenotype

52.7% 47.3% 47.8% 52.3%

Both None Both None
Figure S3: e-CHACR-wG versus a static source of Cas9 inserted at y, related to Figures 1 and 2

A) Schematic depicting the action of e-CHACR-wG, inserted at the w locus, on a static source of Cas9 inserted into the y locus, both loci residing on the X chromosome. e-CHACR-wG contains two gRNAs: gRNA-w2 and gRNA-C1. e-CHACR-wG targets insertion and copying of e-CHACR-wG into the w locus. gRNA-C1 targets sequences encoding catalytic residues of the Cas9 carried on the MCR-GFP full gene-drive element. e-CHACR-wG was marked with eGFP expressed under the control of a 3xP3 promoter to follow the transmission of the element through fluorescence in the eyes. This element was tested against a static (non-driving) source of Vasa-Cas9 inserted into the yellow locus and marked with DsRed in the eye. B) Crossing scheme for e-CHACR-wG (e-wG) experiments where marked alleles are denoted with corresponding colors (i.e. red and green) used for phenotypic screening and the angled chromosome represents the Y chromosome in male flies. Homozygous e-CHACR-wG females were crossed to males carrying Cas9 to generate heterozygous F1 “master females” that exhibited mosaic eye phenotypes. The master females were subsequently placed into single-pair mating crosses with wild type males, followed by scoring of the F2 progeny. The mosaic eye phenotype was utilized to assess the presence of functional Cas9 in F2 females. C-C') Each dot represents the percentage of the indicated fluorescence per cross. For each fluorescence group depicted, the corresponding mean value is also shown underneath the X-axis. The mean of each fluorescence group was calculated per cross by dividing the number of individuals in a phenotypic class by the total number of females (panel C) or males (panel C') in that vial. Additionally, because a static Cas9 transgene was utilized in these crosses, DsRed fluorescence also served as a reliable marker for the receiver chromosome on which the e-CHACR-wG was acting. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001. C) Percentage of fluorescence in female F2 progeny. The total DsRed category represents the amount of receiver chromosomes able to be targeted by the e-CHACR-wG. The group exhibiting both fluorescent markers indicates highly efficient copying of e-CHACR-wG, as 89.3% of the receiver (DsRed alleles) were successfully converted. The total GFP+ group also indicates the efficient transmission of the element. C') Percentage of fluorescence in male F2 progeny. Note the percentage of total DsRed was significantly lower than that expected 50% (37.1%), suggesting a substantial amount
of target chromosome damage. This transmission bias was not observed in F$_2$ females, most likely due to rescue by a wild-type copy of the X chromosome. D) Cross scheme to phenotypically assess Cas9 function by crossing individual F$_2$ females positive for both DsRed and eGFP expression (indicating copying of the e-CHACR-wG element and targeting of Cas9) to wild type males and scoring F$_3$ progeny. E) Percentage of fluorescence in total F$_3$ females or males per cross. We observed Mendelian inheritance of the element (yellow dots) and wild type chromosomes (grey dots), indicating proper disruption of Cas9 function. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
**Figure S4**

A

Donor X Chromosome

yellow Locus

Homology Arm

Receiver

3' UTR cas9 Vasa P SV40 DsRed 3xP3 white Locus

Homology Arm

B

Donor y+ e-wG Cas9 w+ Analyze F2 Progeny

Receivers y+ e-wG Cas9 w+

C

e-CHACR-wG Female F2 Progeny

% Phenotype

50.3% 47.3% 49.7% 96.9%

C'
e-CHACR-wG Male F2 Progeny

% Phenotype

33.7% 31.5% 65.6% 97.2%
Fig. S4: Reverse e-CHACR-wG versus a static source of Cas9, related to Figures 1 and 2

A) e-CHACR-wG construct and schematic.  B) Cross scheme used to test performance of e-CHACR-wG against a static form of Cas9 when the e-CHACR-wG comes from a male instead of a virgin female. Males with the e-CHACR-wG element (marked with GFP) were crossed to virgin females with the static Cas-9 element (marked with DsRed) to generate the F1 master females containing both elements that are then crossed to wild-type males in single-pair mating crosses to obtain F2 progeny that are screened and scored for eye color, body color, and fluorescence. The mosaic eye phenotype was utilized to assess the presence of functional Cas9 in F2 females.  

C-C’) Percent fluorescence in female F2 progeny from the cross scheme depicted in panel B. Fluorescent categories are depicted on the X-axis, and each dot represents the number of females counted of that fluorescent category per vial. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. 

C) e-CHACR-wG transmission in F2 female progeny, determined from the total GFP category, is 96.9%.  

C’) e-CHACR-wG transmission in F2 male progeny, determined from the total GFP category, is 97.1%. Note an overall reduction of receiver chromosomes (total DsRed) measured in males when compared with females.
Figure S5

A

X Chromosome

y-, e-CHACR-wR x Vasa-Cas9 (III)

Female F2 Progeny

Total GFP+

Both

DsRed+ only

Total DsRed+

% Phenotype

54.4% 51.4% 44.3% 95.6%

6.0% 93.9%

% Phenotype

C

y-, e-CHACR-wR x Vasa-Cas9 (III)

Female F2 Progeny

% Phenotype

54.4% 51.4% 44.3% 95.6%

3.0% 97.0%

% Phenotype

C'

y-, e-CHACR-wR x Vasa-Cas9 (III)

Male F2 Progeny

% Phenotype

50.7% 48.7% 48.6% 95.4%

2.5% 97.5%

% Phenotype

D

Prevalence in

Donor F2 Females

% Phenotype

75.0% 50.0% 25.0% 0.0%

Non-Deleted + Deleted

Prevalence in

Receiver F2 Females

% Phenotype

75.0% 50.0% 25.0% 0.0%

Non-Deleted + Deleted

D'

Prevalence in

Donor F2 Males

% Phenotype

75.0% 50.0% 25.0% 0.0%

Non-Deleted + Deleted

Prevalence in

Receiver F2 Males

% Phenotype

75.0% 50.0% 25.0% 0.0%

Non-Deleted + Deleted

E

Total Female vs Male

e-CHACR-wR F2 Progeny

% Phenotype

0% 25% 50% 75% 100%

0% 25% 50% 75% 100%

0% 25% 50% 75% 100%

0% 25% 50% 75% 100%

0% 25% 50% 75% 100%
Fig. S5: e-CHACR-wR versus a static autosomal Cas9 source, related to Figures 1 and 2

A) e-CHACR-wR construct and schematic depicting the e-CHACR-wR element inserted into the X-linked w locus and associated with a y allele in order to distinguish between the original donor chromosome and e-CHACR-wR copying events. This strain was tested against a static Cas9 source marked with GFP on the third chromosome and wild type for both y and w.  

B) Schematic illustrating the crossing scheme for combining in the F₀ generation the y-, e-CHACR-wR (y-, e-wR) element from mothers with a paternal static Cas9 source to generate F₁ master females, which were then crossed to y⁻, w⁺ males.

C, C') Percentage of female (C) and male (C’) F₂ progeny from the crossing scheme depicted in panel B carrying the DsRed and/or eGFP fluorescence markers. Note that e-CHACR-wR copies efficiently (~95% transmission) and that the Cas9 bearing third chromosome is inherited at approximately Mendelian frequencies in both males and females. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001.

D) Prevalence of DsRed⁺ and DsRed⁻ alleles in e-CHACR-wR donor (left) and receiver F₂ females (right). Mean values were calculated by dividing the number of DsRed⁺/⁻ females per cross by the total number of y⁻ or y⁺ females in each vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001.  

D’) Prevalence of DsRed⁺ and DsRed⁻ alleles in e-CHACR-wR donor (left) and receiver F₂ males (right). Mean values were calculated for males in a similar fashion as for panel D, in which DsRed⁺/⁻ males were divided by the total number of y⁻ or y⁺ males.  Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001. 

E) Prevalence of male and female F₂ progeny out of total F₂ progeny indicating an even distribution for both sexes within the flies screened. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001.
Fig. S6: e-CHACR sequencing data, related to Figures 1 and 2
Sequence analysis of NHEJ mutations generated at the catalytic site of the coding sequence of Cas9 by the e-CHACR-wG C1 gRNA or by the e-CHACR-e C3 gRNA. The wild-type sequences of the gRNA-C1 (for panel A) or gRNA-C3 (for panel B) cleavage sites are referenced at the top with sequencing data below. Wild-type sequences of each gRNA are written in blue text and sequences of the PAM site are written in red text. Inserted bases are written in green text and deleted bases are denoted with dots. Sequences with in-frame indels are highlighted in red, and the number of bases changed are noted on the right of the sequence read. A) Male F₂ flies with yellow body color and both DsRed and GFP fluorescence, as depicted in the cross scheme in Fig. S3B, are collected and sequenced on the receiver allele to determine the indel sequence surrounding the cut site of the gRNA-C1. B) Male F₂ flies with yellow body color and both DsRed and GFP fluorescence, as depicted in the cross scheme in Fig. S9B, were collected and sequenced on either the donor or the receiver MCR-GFP allele to determine the indel sequence surrounding the cut site of the gRNA-C3.
**Figure S7**

**A**

- **X Chromosome**
- **yellow Locus**
- **Homology Arm**
- **Dmel U6:1 P**
- **Dmel U6:3 P**
- **5V40 P-A**
- **DsRed**
- **3xP3**
- **Homology Arm**

**B**

- **Chromosomes**
- **F0**
  - **Y+** e-wR
  - **y+** e-AR
- **F1**
  - **MCR+** W+
  - **ac-** MCR
- **F2**
  - **MCR+** W+=
  - **ac-** MCR
  - **MCR+** e-wR=
  - **ac-** MCR
  - **MCR+** e-wR
  - **ac-** MCR

**C**

- **e-CHACR-wR Female F2 Progeny**
- **% Phenotype**
- **Total eGFP+**
- **Both DsRed+ Only**

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<td>93.6%</td>
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**C’**

- **e-CHACR-wR Male F2 Progeny**
- **% Phenotype**
- **Total eGFP+**
- **Both DsRed+ Only**

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**D**

- **MCR-GFP Donor F2 Female Progeny**
- **MCR-GFP Receiver F2 Female Progeny**

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**E**

- **Body Color of F2 Females**
- **(None Observed)**
- **Total eGFP+**
- **Both DsRed+ Only**

- **ac-**
  - **Y+** e-wR
  - **y+** e-AR

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**E’**

- **Body Color of F2 Males**
- **Donor vs. Receiver in F2 Males**

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**F**

- **Body Color of F2 Females**
- **(None Observed)**
- **Total eGFP+**
- **Both DsRed+ Only**

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Fig. S7: Reverse eCHACR-wR crosses: ac<sup>4</sup>-marked MCR-GFP donor chromosome, related to Figures 1 and 2

**A)** e-CHACR-wR construct and schematic. This schematic is similar to the schematic depicted in Fig. 2A except that the element associated with the *achaete*<sup>4</sup> (ac<sup>4</sup>) viable loss-of-function allele has been crossed onto the MCR-GFP chromosome instead of being associated with the e-CHACR-wR chromosome. In this case, the original MCR-GFP allele will be ac<sup>−</sup> (ac<sup>4</sup> = ac<sup>−</sup>, and eGFP<sup>+</sup>) whereas the converted allele (on the y locus of the original e-CHACR-wR chromosome) will be ac<sup>+</sup> (wild type *achaete*<sup>+</sup> = ac<sup>+</sup>, and eGFP<sup>+</sup>).

**B)** Cross scheme used to test performance of eCHACR-AR against the full gene-drive (MCR-GFP). e-CHACR-wR virgins were crossed to *achaete*<sup>−</sup> (ac<sup>4</sup>) marked MCR males to generate the F<sub>1</sub> master females carrying both elements, which were then crossed individually to ac<sup>4</sup> males to obtain F<sub>2</sub> progeny, which were screened and scored for eye color, body color, and fluorescence.

**C)** Percent fluorescence in female F<sub>2</sub> progeny from the cross scheme depicted in panel B. Fluorescent categories are depicted on the X-axis, and each dot represents the number of females counted of that fluorescent category per vial (tabulation also applies for D). Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

**C’)** Percent fluorescence in male F<sub>2</sub> progeny from the cross scheme depicted in panel B. Fluorescent categories are depicted on the X-axis, and each dot represents the total number of males counted of that fluorescent category per vial (tabulation also applies for D’). Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

**D)** Percentages shown below graph represent the mean of each category. Left panel: Percentage of flies with or without GFP fluorescence on the donor MCR (ac<sup>−</sup> = ac<sup>4</sup>) allele in the F<sub>2</sub> female progeny. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Right panel: Percentage of flies with or without GFP fluorescence on the receiver MCR (ac<sup>4</sup>) allele in the F<sub>2</sub> female progeny. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

**D’)** Percentages shown below graph represent the mean of each category. Left panel: Percentage of flies with or...
without GFP fluorescence on the donor MCR (ac^d) allele in the F2 male progeny. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Right panel: Percentage of flies with or without GFP fluorescence on the receiver MCR (ac^+) allele in the F2 male progeny. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

E) Percentage of flies with brown (y^+) or yellow (y^-) body color on both the donor (ac^d) and receiver (ac^+) chromosomes in F2 female progeny. Phenotypic categories (ac^d, y^+; ac^+, y^+, ac^d, y^-; and ac^+, y^-) are depicted on the X-axis, and each dot represents the total number of females counted of that phenotypic category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

E') Percentage of flies with brown (y^+) or yellow (y^-) body color on both the donor (ac^d) and receiver (ac^+) chromosomes in F2 male progeny. Phenotypic categories (ac^d, y^+; ac^+, y^+, ac^d, y^-; and ac^+, y^-) are depicted on the X-axis, and each dot represents the total number of females counted of that phenotypic category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

F) Percent of F2 males that have the MCR donor (ac^d) allele or the MCR receiver (ac^+) allele. Phenotypic categories (ac^d or ac^+) are depicted on the X-axis, and each dot represents the total number of males counted of that phenotypic category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
Figure S8

A. Female vs. Male Prevalence in Total e-CHACR-wG F2 Progeny

B. Total Female vs. Male e-CHACR-wG F2 Progeny

C. Female vs. Male Prevalence in Total e-CHACR-e F2 Progeny

D. Total Female vs. Male e-CHACR-e F2 Progeny

E. Female vs. Male Prevalence in Total e-CHACR-wR F2 Progeny

F. Total Female vs. Male e-CHACR-wR F2 Progeny

G. Fitness Cost of ac[4] in Female F2 Progeny

H. Fitness Cost of ac[4] in Male F2 Progeny

I. Total Female vs. Male ac[4] control F2 Progeny
Fig. S8: Breakdown of eCHACR-wG events, related to Figures 1 and 2

Because we observed a bias in the inheritance of the donor versus receiver chromosome in our eCHACR experiments, we analyzed this bias further by comparing the percentages for the various fluorescence categories in both males and females for each of the different eCHACR experiments shown in Fig. S3 (panels A,B), Fig. S9 (panels C,D), and Fig. 2 (panels E,F).

A) Male versus female prevalence of the following fluorescence categories for eCHACR-wG: DsRed only, “Both” markers, GFP only, and none. Note that the Both and GFP only fluorescence categories seem significantly lower in males than females while the DsRed only and none categories have no significant difference. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

B) Female versus male prevalence for total e-CHACR-wG F2 progeny. Note that there is 8-9% more females and 8-9% less males in the total F2 progeny. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

C) Male versus female prevalence of the following fluorescence categories for e-CHACR-e: Total DsRed, Both, Total GFP, and none. Here total DsRed is plotted instead of DsRed only because we were unable to distinguish donor from receiver chromosome for the DsRed copying element (e-CHACR-e). Note that all fluorescence categories seem significantly lower in males with the exception of the none category. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

D) Female versus male prevalence for total e-CHACR-e F2 progeny. Note that there is 9-10% more females and 9-10% less males in the total F2 progeny. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

E) Male versus female prevalence of the following fluorescence categories for e-CHACR-wR: DsRed only, Both, GFP only, and none. Note that both the DsRed only and Both fluorescence categories seem significantly lower in males. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

F) Female versus male prevalence for total e-CHACR-wR F2 progeny. Note that there is 10-11% more females and 10-11% less males in the total
F₂ progeny. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. G,H) Estimates of fitness cost associated with the ac⁴ allele which was used to mark either the MCR-GFP or e-CHACR bearing chromosome in experiments with e-CHACR-wR shown in Fig. 2 and Fig. S7. ac⁴/ac⁴ virgin females were crossed to wild-type males (+/Y males). F₁ virgin females (ac⁴/+ ) were then crossed to achaete males (ac⁴/Y) to generate F₂ progeny that were scored for the presence (ac⁻) or absence (ac⁺) of the achaete phenotype. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. G) Percent of F₂ female or male progeny that are either ac⁺ or ac⁻. We observed a 62:38 bias in females and a 56:44 bias in males for preferred inheritance of the wild-type (ac⁺) allele over the achaete (ac⁻) allele. H) Percent of male and female F₂ progeny out of total F₂ progeny. Since we see a normal 50:50 distribution for the percentage of males and females from the same cross as depicted in panel G, we conclude that the skew present among the ac⁺ and ac⁻ F₂ progeny results from unequal inheritance of the two alleles among males and females, and not from a sex-specific inheritance bias.
Figure S9

(A) X Chromosome
yellow Locus
W-
Homology Arm
Dmel U6-1 P
3' UTR
cas9 U6a P
Dmel U6-3 P
Dmel U6-3 Y
gRNA
e1 gRNA
3' UTR
ebony Locus
Homology Arm
3' Chromosome

(B) Chromosomes

F₀

1st 1st
MCRway+
X
1st 1st
MCRway+
X

F₁

Master Females

F₂

Analyse MCR containing F₂ Progeny

F₂♂

Assess MCR Function by presence of yellow- F₂ Female Progeny
(None Observed)

F₂♀

(C) e-CHACR-e Female F₂ Progeny

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(D) MCR-GFP Donor F₂ Female Progeny

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(E) Body Color of F₂ Females

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(F) Donor vs. Receiver in F₂ Females

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(G) MCR-GFP Receiver F₂ Female Progeny

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(H) MCR-GFP Donor F₂ Male Progeny

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(K) MCR-GFP Receiver F₂ Male Progeny

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(L) Body Color of F₂ Males

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(M) Body Color of F₂ Males

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(N) Body Color of F₂ Males

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Fig. S9: e-CHACR-e versus the MCR-GFP drive element, related to Figures 1 and 2

A) Schematic depicting the action of the autosomal e-CHACR-e on the X-linked MCR-GFP drive element inserted at the y locus. e-CHACR-e contains two gRNAs: gRNA-e1 and gRNA-C3. gRNA-e1 targets insertion and copying of e-CHACR-e into the ebony locus on the third chromosome. gRNA-C3 targets the Cas9 coding sequence 34 amino acids downstream of gRNA-C1 (carried by e-CHACR-w). e-CHACR-e was marked with 3xP3-DsRed and combined with a w– allele on the X chromosome. We tested e-CHACR-e against the MCR-GFP element, which was linked to the w^3 marker. 

B) For the e-CHACR-e (e-e) crossing scheme, females homozygous for the e-CHACR-e were crossed to males carrying the MCR-GFP (full gene-drive element) inserted into the y locus. The MCR-GFP donor chromosome was marked with w^a in order to distinguish it (MCR^wa) from copying to the w– receiver chromosome (MCR). Heterozygous F1 master females were then crossed individually to wild-type males followed by screening of the F2 progeny. Residual Cas9 function was assessed from the generation of y– F2 females (none observed). 

C) Percentage of fluorescence per cross in total female F2 progeny. Females exhibited Mendelian inheritance of the element, as indicated by the total DsRed+ group. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001. 

C') Percentage of fluorescence in total male F2 progeny. Note that males also show decreased numbers of MCR-GFP alleles. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. 

D) Prevalence of GFP+ and GFP– alleles in MCR-GFP donor (left) and receiver F2 females (right). Mean values were calculated by dividing the number of GFP+/– females per cross by the total number of w^3 or w– females in each vial. The MCR-GFP element was found to still be able to copy onto its w– receiver chromosome, but at significantly reduced rates. We also observed a significant number of flies that are w^a but not GFP+, indicating possible NHEJ events following targeting by the e-CHACR-e. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001. 

D') Prevalence of GFP+ and GFP– alleles in MCR-GFP donor (left) and receiver F2 males (right). Mean values were calculated for males in a similar fashion as
for panel D, in which GFP+/– males were divided by the total number of w+a or w– males. Mirroring the trend in female F2 progeny, we observed reduced rates of MCR-GFP copying, but also an increased number of GFP– w+a individuals (*Note: 4 single-pair mating crosses did not generate any w+a males and thus were excluded from the graph). Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. E) Scoring of body color in total F2 females. We observed equal percentages of w+a and w– chromosomes, as well as no y– F2 female progeny, indicating return to Mendelian inheritance of the alleles and proper inactivation of Cas9 function in the gene-drive. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. E') Scoring of body color in total F2 males. We observed a decreased number of the MCR w+a chromosomes (as shown in the y– w+a group), as well as an increased fraction of y+ w+a progeny suggesting an ~2-fold increase in recombination. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. F) Percentage of MCR-GFP donor (w+a, orange dots) and receiver (w–, black dots) alleles in F2 males. We observed a significant bias against w+a alleles, possibly due to cuts being generated on all four strands of the y locus and no option for homology-dependent repair of the cleaved Cas9 transgene carried by the MCR-GFP element. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
Figure S10

A. Chromosomes

F₀

F₁

Master Females

W- e-e

Cas9²

W- e-e

Cas9²

Analyze F2 Progeny

B. e-CHACR-e x Vasa-Cas9 (X)
Female F₂ Progeny

C. e-CHACR-e x Vasa-Cas9 (X)
Male F₂ Progeny

D. Chromosomes

F₀

F₁

Master Females

W- e-e

Cas9²

W- e-e

Analyze F2 Progeny

E. e-CHACR-e x Resistant Vasa-Cas9 (X)
Female F₂ Progeny

F. e-CHACR-e x Resistant Vasa-Cas9 (X)
Male F₂ Progeny
Fig. S10: e-CHACR-e crossed to cleavage sensitive versus resistant Cas9 sources, related to Figures 1 and 2

A) Crossing scheme used to test performance of eCHACR-e (e-e) inserted into the ebony locus on the third chromosome against a static form of Cas9 on the third chromosome. F₀ eCHACR-e females were crossed to males carrying a static form of Cas9 to generate F₁ master females carrying both elements. w⁻/Cas9; e-e/e⁺ master females were then crossed individually to w⁻ males to obtain F₂ progeny, which were screened and scored for body color and fluorescence. B) Percent fluorescence in female F₂ progeny from the cross scheme depicted in panel A. Fluorescent categories are depicted on the X-axis, and each dot represents the number of females counted of that fluorescent category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. C) Percent fluorescence in male F₂ progeny from the cross scheme depicted in panel A. Fluorescent categories are depicted on the X-axis, and each dot represents the number of males counted of that fluorescent category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. D) Cross scheme used to test performance of eCHACR-e (e-e) inserted into the white locus on the X chromosome against a static form of Cas9 on the third chromosome. eCHACR-AR virgins were crossed to males carrying a static form of Cas9 to generate master females carrying both elements, which were then crossed to wild type males to obtain F₂ progeny that were screened and scored for body color and fluorescence. E,F) The e-CHACR-e element drives when crossed to a cleavage resistant Cas9 source (Cas9*). E) Percent fluorescence in female F₂ progeny from the cross scheme depicted in panel D. Fluorescent categories are depicted on the X-axis, and each dot represents the number of females counted of that fluorescent category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. F) Percent fluorescence in male F₂ progeny from the cross scheme depicted in panel D. Fluorescent categories are depicted on the X-axis, and each dot represents the number of males counted of that fluorescent category per vial. Percentages shown below graph represent the mean of each category. Error bars
indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Note that e-CHACR-e is transmitted 60.7% of female F2 progeny and 62.4% of male progeny in contrast to its approximately Mendelian transmission when crossed to a cleavage sensitive source of Cas9 (panel B).
B Chromosomes

F0

1st Chromosome 3rd Chromosome

X Chromosome yellow Locus Homology Arm Homology Arm

MCRway+ MCRway+

kni+ kni+

MCR-GFP Donor MCR-GFP Receiver

F2 Female Progeny

MCR-GFP Donor MCR-GFP Receiver

F2 Male Progeny

Assess MCR Function by presence of yellow- F2 Female Progeny (None Observed)

E Body Color of F2 Females

F Body Color of F2 Males

Donor vs. Receiver in F2 Males
Fig S11: e-CHACR-k in the autosomal knirps locus drives and inactivates Cas9, related to Figures 1 and 2

A) e-CHACR-k construct and schematic. The e-CHACR-k element is inserted into the cis-regulatory element required for driving knirps expression in a narrow stripe required for formation of the second longitudinal wing vein (L2). Although deletions of this region are associated with viable L2 vein loss phenotypes, surprisingly the L2 vein developed normally in adults homozygotes for the e-CHACR-k insertion. B) Cross scheme used to test performance of e-CHACR-k against the MCR-GFP element. e-CHACR-k virgins were crossed to w^a marked MCR-GFP males to generate the F1 master females carrying both elements. Master females were individually crossed w^- males to obtain F2 progeny, which were screened and scored for eye color, body pigmentation, and fluorescence phenotypes. C) Percent fluorescence in female F2 progeny from the cross scheme depicted in panel B. Fluorescent categories are depicted on the X-axis, and each dot represents the number of females counted of that fluorescent category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. C') Percent fluorescence in male F2 progeny from the cross scheme depicted in panel B. Fluorescent categories are depicted on the X-axis, and each dot represents the total number of males counted of that fluorescent category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. D) Left panel: Percentage of flies with or without GFP fluorescence on the donor w^a allele in the F2 female progeny. Phenotypic categories are depicted on the X-axis, and each dot represents the total number of females counted of that phenotypic category for per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Right panel: Percentage of flies with or without GFP fluorescence on the donor (w^a) allele in the F2 female progeny. Phenotypic categories are depicted on the X-axis, and each dot represents the total number of females counted of that phenotypic category for per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
D') Left panel: Percentage of flies with or without GFP fluorescence on the donor (wa) allele in the F2 male progeny. Phenotypic categories are depicted on the X-axis, and each dot represents the total number of males counted of that phenotypic category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Right panel: Percentage of flies with or without GFP fluorescence on the MCR receiver (w−) allele in the F2 male progeny. Phenotypic categories are depicted on the X-axis, and each dot represents the total number of males counted of that phenotypic category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

E) Percentage of flies with brown (y+) or yellow (y−) body color on both the donor (wa) and receiver (ac+) chromosomes in F2 female progeny. Phenotypic categories (wa, y+; w−, y+, wa, y−; and w−, y−) are depicted on the X-axis, and each dot represents the total number of females counted of that phenotypic category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

E') Percentage of flies with brown (y+) or yellow (y−) body color on both the MCR donor (wa) and MCR receiver (w−) chromosomes in F2 male progeny. Phenotypic categories (wa, y+; w−, y+, wa, y−; and w−, y−) are depicted on the X-axis, and each dot represents the total number of females counted of that phenotypic category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

F) Percent of F2 males that have the MCR donor (wa) allele or the MCR receiver (w−) allele. Phenotypic categories (wa or w−) are depicted on the X-axis, and each dot represents the total number of males counted of that phenotypic category per vial. Percentages shown below graph represent the mean of each category. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
Fig. S12. Rescue of e-CHACR and ERACR induced male-lethal alleles by a duplication covering the tip of the X chromosome, related to Figures 2 and 5

A) In Fig. 2D,D’, Fig. 5A,A’ and Figs. S7D,D';S9D,D';S11D,D’ we identified a category of GFP– w a progeny inheriting the MCR-GFP donor chromosome. We hypothesized that some of these alleles may have sustained chromosome damage resulting in loss of the GFP marker and perhaps extending to deletion of other potentially essential genes. Indeed, a fraction of such balanced GFP– w a alleles are viable in heterozygous females but are lethal in males (and females in the homozygous condition), consistent with potential local damage to the X chromosome. We tested whether a duplication covering the tip of the X chromosome which includes the y locus might rescue such lethality using the depicted crossing scheme. In the F0 generation, DsRed+ e-CHACR-e (e-e) flies were crossed to MCR-GFP males. In the F1 generation, heterozygous MCR-GFP/+; e-CHACR-e/+ “master female” individuals were collected and crossed to FM7 males. Females lacking either fluorescent marker were recovered in the F2 generation and were assessed for the viability of the non-FM7 chromosome. Of 17 such crosses, 10 lines proved to carry e-CHACR-induced male-lethal (eml) alleles. Females trans-heterozygous for an eml/FM7 were crossed to males carrying an X Duplication covering the tip of the X chromosome (including the y locus - Bloomington X Duplication Kit, stock #30220) (Cook et al., 2010). All 10 eml lines were fully rescued in these crosses as judged by the abundant recovery of y+ non-FM7 male progeny (Table S1, page 1). Control crosses of eml/FM7 females to males carrying the viable achaete allele ac4, however, resulted in no rescue of male lethality, nor did any non-FM7 females display any bristle-loss phenotype indicative of potential DNA deletions extending from the MCR-GFP element into the neighboring centromere proximal achaete locus (Table S1, page 2).

B) In Figs. 5A,A’ we identified a category of DsRed– and GFP– progeny, greatly enriched in female F2 progeny, that we hypothesized carried damage to the tip of the X chromosome (Fig. 5B), resulting in alleles that were viable when heterozygous in females, but lethal when hemizygous in males (or homozygous in females). Characterization of the X-lethal phenotype observed was done according to the cross scheme depicted. In the F0 generation, DsRed+ ERACR-2 flies were crossed to MCR-GFP males. In the F1 generation, heterozygous ERACR-2/MCR-GFP “master female” individuals were collected and crossed to FM7 males. Females lacking either fluorescent marker were recovered in the F2 generation and were crossed
to males of various X Duplication stocks from the Bloomington X Duplication Kit (Cook et al., 2010). Crossing these non-fluorescent F2 females to X Duplication males with irrelevant duplications (or wild-type males) resulted in the male lethality pattern shown on the left (gray). However, vials that gave rise to both female and male F3 progeny revealed that the X Duplication carried by the parental F2 male rescued the lethal phenotype (right schematic - green). Three ERACR-2 lines from the cross scheme presented in Fig. 3, in addition to two ERACR-min lines and one ERACR-1 line from the cross scheme presented in Supplemental Fig. 13E were rescued by the X Duplication stock #30220.
**Figure S13**

**Variant ERACR Crossing Schemes**

<table>
<thead>
<tr>
<th>Donor/Receiver Allele Unmarked</th>
<th>Receiver Allele Marked with ( W^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong> Experiment 1: ERACR-1 and ERACR-2</td>
<td></td>
</tr>
<tr>
<td><strong>B</strong> Experiment 2: ERACR-1 and ERACR-2</td>
<td></td>
</tr>
<tr>
<td><strong>C</strong> Experiment 3: ERACR-min and ERACR-2</td>
<td></td>
</tr>
<tr>
<td><strong>D</strong> Experiment 4: ERACR-min, ERACR-1, and ERACR-2</td>
<td></td>
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<tr>
<td><strong>E</strong> Experiment 5: ERACR-1 and ERACR-2</td>
<td></td>
</tr>
</tbody>
</table>

**Reversed \( F_0 \) Cross (GFP-tagged MCR: Cas9 inherited from Female Parent)**

**E'** F2 Phenotypic Distribution

- **ERACR-1**
- **ERACR-2**

---

*Note: The diagram includes detailed representations of the crosses and phenotypic distributions for each experiment.*
Fig. S13: Additional ERACR crosses and experiments, related to Figures 4 and 5

Alternative configurations in which an ERACR was challenged with the MCR-GFP gene-drive: F₁ “master females” carrying a MCR-GFP element, marked or not with w⁰, in-trans to an ERACR were crossed to F₁ males of differing genotypes, including Basc, FM7, and w⁻. **A)** ERACR-1 or ERACR-2 female crossed to an MCR-GFP male. The F₁ “master female” was then crossed to a Basc male and F₂ progeny were analyzed. **ERACR-1:** total flies = 581; females = 368; males = 213; 10 vials. **ERACR-2:** total flies = 728, females = 470; males = 258; 13 vials. **B)** Individual ERACR-1 or ERACR-2 females were crossed to single MCR-GFP males. F₁ “master females” were then pair-mated to FM7 males and F₂ progeny were analyzed. **ERACR-1:** total flies = 1460; females = 897; males = 563; 17 vials. **ERACR-2:** total flies = 1259; females = 774; males = 485; 17 vials. **C)** ERACR-min or ERACR-2 females were pair-mated to MCR-GFP w⁰ males. F₁ “master females” were pair-mated to FM7 males and F₂ progeny were analyzed. **ERACR-min:** total flies = 713; females = 422; males = 291; 12 vials. **ERACR-2:** total flies = 1366; females = 840, males = 526; 21 vials. **D)** ERACR-min, ERACR-1, or ERACR-2 females were pair-mated to MCR-GFP w⁰ males. F₁ “master females” were crossed to w⁻ males and F₂ progeny were analyzed. **ERACR-min:** total flies = 863; females = 478; males = 385; 22 vials. **ERACR-1:** total flies = 757, females = 448; males = 309; 18 vials. **ERACR-2:** total flies = 1092, females = 651, males = 441, 22 vials. **E)** ERACR-1 or ERACR-2 male crossed to a MCR-GFP female. F₁ master females crossed to FM7 males and the F₂ progeny were analyzed. **ERACR-1:** total flies = 1885; females = 1212; males = 673; 17 vials. **ERACR-2:** total flies = 1684, females = 1015, males = 669; 16 vials. **E’)** Proportion of F₂ males or females displaying a given phenotype. Supplying the Cas9 allele either paternally or maternally supported comparable levels of ERACR copying efficiency. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
ERACR Allele Inherited

Resulting Sequence

Ref: ATGAGCTGTCGCC TCTGGGTCCTGATA
E1: ATGAGCTGTCGCC TCTGGGTCCTGATA (x5)
E2: ATGAGCTGTCGCC TCTGGGTCCTGATA (x5)

Ref: AGCTTTTGTGACACATTATAGATAC
E1: AGCTTTTGTGACACATTATAGATAC (x5)
E2: AGCTTTTGTGACACATTATAGATAC (x5)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CCGGGTTAAGACCCTGCTTT (x2)

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Resulting Sequence

Inferred Gene Editing

- E1: 73.7% 49.8%
- E2: 64.7% 53.7%
- MCR/ERACR region of homology

MCR and ERACR-2 Recombination at SV40 Region

Resulting Sequence

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.7% 0.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.2% 1.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.7% 0.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.2% 1.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.7% 0.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.2% 1.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.7% 0.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.2% 1.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.7% 0.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)

Inferred Gene Editing

- E2: 0.7% 0.4%
- MCR/E2 Jct

Ref: CTACATAAGACGGCCCCTAC
E2: CTACATAAGACGGCCCCTAC (x1)

Ref: GATTCCTTCTACATTATAGAT
E2: GATTCCTTCTACATTATAGAT (x2)

Ref: CGGGTTAAGACCCTGCTTT
E2: CGGGTTAAGACCCTGCTTT (x2)
Fig. S14: Additional observed gene editing events, related to Figures 4 and 5

A-F) Sequencing results of the predicted junction fragments (resulting from cleavage by gRNA-y1, gRNA-y2, or gRNA-y3) of the phenotypes illustrated in Fig. 4 and Fig. 5. For each, the top schematic depicts the predicted gene editing event with green reference sequences indicating an MCR-GFP receiver chromosome origin and red reference sequences a donor ERACR chromosome origin. Unknown junction sequences between the ERACR and MCR-GFP are depicted in brown. The sequences below are derived from F2 males from the cross shown in Fig. 3B, with the relevant genotype with expected copying events depicted in black text, insertions in blue text, or deletions as dots. The ERACR construct giving rise to particular male sequenced is noted to the left of the sequence. If multiple males from independent vials share a sequence, the number of replicates is shown in parentheses to the right (e.g., “x6”). The number of available sequences is heavily dependent on the scarcity of each event. G-K). In addition to the predominant phenotypes and genotypes described in Fig. 4 and Fig. 5, we have also observed a subset of five additional rare events. Consistent with schematics in Fig. 4 and Fig. 5, we hypothesize that these events were also mediated by directional gene conversion, resulting in the following D-loop schematics. Each of the genotypes described in this figure are validated by sequence analysis at gRNA-y1, gRNA-y2, and/or gRNA-y3 junction points, as shown. Sequencing data is derived from male F2 progeny from the cross scheme in Fig. 3B. The tables in each panel display the average frequency of the phenotype observed in F2 progeny per cross according to the scheme shown in Fig. 3B.

G) The ERACR donor allele, associated with the closely linked w− chromosomal marker, is inherited in a Mendelian fashion. H) ERACR-2 copied onto the MCR-GFP imperfectly. Sequence analysis shows a NHEJ-deletion at the gRNA-y2 cut site and addition of part of the MCR-GFP construct (the vasa promoter, the Cas9, and the 3’ vasa UTR) after the gRNA-y2 cut site, possibly due to recombination between the SV40 region of the ERACR and MCR-GFP alleles. Sequence at the MCR-GFP/ERACR-2 junction is depicted with green = MCR-GFP (left), red = ERACR (right), and gray (middle) = SV40 sequences. The recombination event that created this MCR-GFP/ERACR-2 hybrid is likely to have occurred within a region of SV40 homology. I) An ERACR-2/MCR-GFP hybrid recombined at the SV40 promoter, which is shared between the MCR-GFP and ERACR elements. J) An MCR-GFP/ERACR-min hybrid with recombination near gRNA-y2 fused ERACR-min to MCR-GFP sequences. The MCR-ERACR junction sequence is currently
unknown as well as the sequence at the gRNA-y3 cut site. The sequence at the MCR-ERACR junction is depicted with red = ERACR (left), green = MCR-GFP (right), and gray (middle) = SV40 sequences. Recombination leading to this MCR-ERACR hybrid is likely to have occurred somewhere in the SV40 region. K) MCR-GFP and ERACR-1 recombined at y, resulting in a yellow+ hybrid that contains both the ERACR-1 and MCR-GFP cassette. Genomic sequence including the gRNA-y1 cut site is present at the ERACR-MCR junction, indicating that recombination occurred between the y region of the MCR-GFP and some sequence present in the ERACR construct (currently unknown). There is also a mutation at the gRNA-y2 cut site that results in a base change but no reading frame alteration, allowing this fly to remain y+. The sequence from the eGFP transgene to the gRNA-y3 cut site is currently unknown.
Fig. S15: Prevalence of various ERACR-induced events on receiver chromosomes, related to Figures 4 and 5

A) Graph summarizing all experimental data from this study relating to the prevalence of different fluorescent categories specifically on the receiving (w^a) allele. Each dot represents the percentage of w^a progeny displaying a given phenotype with respect to all w^a progeny in a given vial, divided by sex. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Line 1: “Average of w^a Vial Averages” refers to the mean of all individual vials of a given category. Line 2: “Average of w^a Vial Averages SD” refers to the standard deviations of the above averages. Line 3: “Average from Pooled w^a Raw Fly Counts” refers to averages calculated using pooled raw fly counts, irrespective of the vial the progeny were derived from. Line 4: “Estimated Average from All Vial Averages” refers to the estimated percentages of each phenotype by multiplying the phenotypic prevalence from both marked and unmarked w^a experiments by the percent of w^a receiver chromosomes inherited. These values are equivalent to the orange values displayed in red in Fig. 4A',D; Fig. 5A,F. Note that these estimated values (line 4) and the calculated values for events that occurred on the receiving allele (lines 1,3) are similar, indicating that the values in Figs. 4,5 are a plausible estimates of recombination events on the receiving chromosome. “w^a Data” bracket: ERACR-min: total flies = 1576; females = 900; males = 676; 34 vials (Fig. S13C,D). ERACR-1: total flies = 757; females = 448, males = 309; 18 vials (Fig. S13D). ERACR-2: total flies = 2458; females = 1491; males = 967, 43 vials (Fig. S13C,D). “All Data” Bracket: ERACR-min: total flies = 1576; females = 900; males = 676; 34 vials. ERACR-1: total flies = 2798; females = 1713; males = 1085; 45 vials. ERACR-2: total flies = 4445; females = 1710; males = 2735; 72 vials (data collected according to crossing schemes depicted in Fig. 3B and Fig. S13A-D).

B) Data tabulated from Figs. 4,5 by gender for all crosses (genetically marked with w^a or not). Compare to performance of single-cut elements shown in Fig. 6D. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
Figure S16

A

Phenotypic Classes of F₄ Progeny:
White<sup>apricot</sup> MCR-GFP F₂ Male with NHEJ
Derived from ERACR-1 F₁ Male

Phenotypic Classes of F₄ Progeny:
White<sup>apricot</sup> MCR-GFP F₂ Male, No NHEJ
(Control)

Phenotypic Classes of F₄ Progeny:
White<sup>apricot</sup> ERACR-2 F₂ Male with NHEJ

Validation of MCR Efficiency
Validation of ERACR Efficiency
Fig. S16: ERACR and MCR-GFP drive efficiency with imperfect homology, related to Figure 4

In certain cases, some recombination events between an ERACR and MCR-GFP produced flies that were eGFP\(^+\) (indicative of an active MCR-GFP element) or DsRed\(^+\) (indicative of an active ERACR). For the eGFP\(^+\) elements DNA sequencing revealed indels at both the gRNA-y1 and gRNA-y2 cut sites (Fig. 4, Fig. S14B,H). GFP\(^+\) F\(_2\) individuals, derived from the cross scheme depicted in Fig. 3B, were used to generate isogenized lines, and individuals from these lines were tested for drive efficiency. A similar cross scheme was used to test the activity of DsRed\(^+\) for a line carrying a mutated gRNA-y2 cut site adjacent to an ERACR-2 element. A) Crossing scheme for assessing the copying efficiency of indel-bearing MCR-GFP and ERACR alleles. Left: In the F\(_2\) generation, homozygous w\(^–\) females were crossed to MCR-GFP w\(^a\) males that have indels at the gRNA-y1 cut site. In the F\(_3\) generation, flies heterozygous for the MCR-GFP w\(^a\) and w\(^–\) were collected and crossed to w\(^–\) males. F\(_4\) progeny were then assayed for body color, eye color, and eye fluorescence, all indicators of drive element copying. Right: In the F\(_2\) generation, y\(^–\), DsRed\(^+\) ERACR males were crossed to homozygous MCR-GFP w\(^a\) females. In the F\(_3\) generation, female flies heterozygous for the MCR-GFP w\(^a\) and the DsRed\(^+\) ERACR were collected and crossed to y\(^–\), w\(^–\) males. F\(_4\) progeny were then assayed for body color, eye color, and eye fluorescence, all indicators of drive element copying. B-D) Observed F\(_4\) progeny derived from the left cross scheme in A. The four categories of observed progeny include phenotypes that indicate that the MCR-GFP was inherited in a Mendelian-like fashion (w\(^a\), y\(^–\), GFP\(^+\), DsRed\(^–\)), the MCR-GFP element recombined at y, resulting in y\(^+\) individuals (w\(^–\), y\(^+\), GFP\(^+\), DsRed\(^–\)), the MCR-GFP was copied (w\(^–\), y\(^–\), GFP\(^+\), DsRed\(^–\)), or neither element was copied (w\(^–\), y\(^–\), GFP\(^–\), DsRed\(^–\)). Total MCR-GFP inheritance = the sum of MCR-GFP inheritance and MCR-GFP copying events. Grey shading on the graphs indicate the inheritance of MCR-GFP elements. Numbers shown below each graph represent the mean of each category, as a percentage of total females or total males. Error bars indicate standard deviation and stars indicate p-values: \(\ast = p < 0.05\), \(\ast\ast = p < 0.01\), \(\ast\ast\ast = p < 0.001\), and \(\ast\ast\ast\ast = p < 0.0001\). B) F\(_0\) male is ERACR-1. C) F\(_0\) male is ERACR-2. D) Control: MCR-GFP w\(^a\) males were crossed to homozygous w\(^–\) females to generate F\(_1\) master females of the genotype MCR-GFP/w\(^–\),
which were crossed $w^-$ males to generate the scored F2 progeny generation. E) Observed F4 progeny derived from the crossing scheme on the right in panel in A for a hybrid ERACR-2/MCR-GFP element shown in Fig. S14H. Categories observed include ERACR-2 inherited ($w^a, y^-, GFP^-, DsRed^+$), MCR-GFP inherited ($w^a, y^-, GFP^+, DsRed^-$), and neither element inherited ($w^a, y^-, GFP^-, DsRed^-$). Results from this experiment indicate that ERACR-2/MCR-GFP element retains efficient drive capability, as these percentages are comparable to those observed in Fig. 5A-A’ for the cleanly inserted parental ERACR-2 element.
Figure S17

A  Double-cut ERACRs
Sex-Based Inheritance Bias on Receiving Allele

\[
\begin{array}{ccc}
\text{ERACR-min} & \text{ERACR-1} & \text{ERACR-2} \\
\text{\( w^\circ \) (Donors)} & \text{\( w^\circ \) (Receivers)} & \text{\( w^\circ \) (Receivers)} \\
\text{\( w^\bullet \) (Donors)} & \text{\( w^\bullet \) (Receivers)} & \text{\( w^\bullet \) (Receivers)} \\
\end{array}
\]

\[
\begin{array}{ccc}
49.9\% & 50.1\% & 65.6\% 34.4\% \\
50.7\% & 50.3\% & 72.9\% 27.1\% \\
52.1\% & 47.9\% & 72.6\% 27.4\% \\
\end{array}
\]

B  Single-cut ERACRs
Sex-Based Inheritance Bias on Receiving Allele

\[
\begin{array}{ccc}
\text{ERACR-2} & \text{ERACR-2} & \text{ERACR-2} \\
\text{Single Cut at \( y_2 \)} & \text{Single Cut at \( y_3 \)} & \\
\text{\( w^\circ \) (Donors)} & \text{\( w^\circ \) (Receivers)} & \text{\( w^\circ \) (Receivers)} \\
\text{\( w^\bullet \) (Donors)} & \text{\( w^\bullet \) (Receivers)} & \text{\( w^\bullet \) (Receivers)} \\
\end{array}
\]

\[
\begin{array}{ccc}
51.4\% & 48.6\% & 59.3\% 40.7\% \\
48.5\% & 51.5\% & 64.4\% 35.6\% \\
40.7\% & 48.5\% & 51.5\% \\
\end{array}
\]

**** **** **** ****
**Fig. S17: Sex ratios observed for ERACR donor versus receiver chromosomes, related to Figures 4 and 5**

**A)** Female sex bias for inheriting the receiver (w<sup>a</sup>) allele. Each dot represents the percent of the w<sup>a</sup> or w<sup>-</sup> progeny of a given phenotype and sex with respect to all progeny in a given vial. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. For each ERACR, there is a roughly equal proportion of males and females (~50%) who inherit the donor (w<sup>-</sup>) allele, but there is a large skew towards females inheriting the receiving allele (w<sup>a</sup>). This trend is observed for all three ERACR constructs and may explain the inheritance bias shown in **Fig. 4** for the different fluorescent categories. For each ERACR, the grey dots represent the w<sup>-</sup> allele, while the orange dots represent the w<sup>a</sup> allele. ERACR-min: n=1576, female n=900, male n=676, 34 vials (**Fig. S13C-D**). ERACR-1: n=757, female n=448, male n=309, 18 vials (**Fig. S13D**). ERACR-2: n=2458, female n=1491, male n=967, 43 vials (**Fig. S13C-D**).

**B)** Sex bias ratios for single-cut ERACR2-y2 and ERACR2-y3 tabulated by donor (w<sup>-</sup>) versus receiver (w<sup>a</sup>) chromosomes. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Note the excess of females inheriting receiver chromosomes, but no gender bias among donor chromosomes.
Figure S18

B  Control Cross: Static Cas9

Chromosomes

$1^{st}$ (X) $y^2$ $w^-$ $y^1$ $w^+$ $y^3$ $w^-$

"Master Females" $y^+$ $w^-$ $y^+$ $w^-$ $y^+$ $w^-$

F1 Progeny Analysis

F2 Control Females

% of total females

Yellow eGFP DsRed

C  F2 Control Females

% of total females

0.0% 48.0% 51.1% 0.0% 0.0% 0.0% 0.0% 0.0%

D  Experimental Cross: ERACR-min and ERACR-2

F2 Progeny Analysis

F0

Cas9 $E$ $w^-$ $y^+$ $w^-$

E  F2 ERACR-min Females

% of total females

Yellow eGFP DsRed

E'  F2 ERACR-2 Females

% of total females

Yellow eGFP DsRed

C'  F2 Control Males

% of total females

0.0% 48.0% 51.1% 0.0% 0.0% 0.0% 0.0% 0.0%

E'  F2 ERACR-min Males

% of total males

Yellow eGFP DsRed

F'  F2 ERACR-2 Males

% of total males

Yellow eGFP DsRed

Y3 Y2 Y1 yellow gene 2.2 Kb white gene

A  Chromosomes

y2 y1 y3 yellow gene white gene

y2 y1 y3 yellow gene white gene
Fig. S18: ERACR-min and ERACR-2 drive as CopyCat elements, related to Figures 3, 4, and 5

ERACR-min and ERACR-2 were tested for their ability to copy themselves onto wild-type chromosomes in the presence of static Cas9. **A** The gRNA-y2 and gRNA-y3 cut sites are located approximately 2.2 kb apart from each other. The w locus is approximately 1.5 cM centromere-proximal to the y locus. **B** Control crosses: Females homozygous for DsRed Cas9 in yellow are crossed to males hemizygous for GFP Cas9 in w. Both the GFP-Cas9 and DsRed-Cas9 elements are “static” sources of Cas9 inherited in a standard Mendelian fashion in the F2 generation. **C,C’** Percentage of each phenotype observed in F2 progeny from the control cross scheme in **panel B**. Results show an overall Mendelian inheritance of each element, as ~50% of the progeny inherited eGFP and ~50% of the progeny inherited DsRed, as expected. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Total flies = 390; females = 225; males = 165, 8 vials. **D** Genetic crossing scheme for so-called CopyCat configuration. In this case, the receiver chromosome is marked with GFP. In the F0 generation, ERACR-min (y−) and ERACR 2 (y+) were crossed to static Cas9 source inserted at the w locus. Since this static form of Cas9 does not contain any gRNAs, it is unable to copy itself. F1 “master females” that are heterozygous for either ERACR-min or ERACR-2 (DsRed+) and a static source of Cas9 (GFP+) were crossed in bulk to w− males. The F2 progeny of these crosses were scored for percentage of eGFP, DsRed, and yellow body color. **E-E’** F2 progeny with both DsRed and GFP fluorescence indicate successful ERACR transmission. **E-E’** Percentage of each phenotype for ERACR-min observed in F2 progeny from the cross scheme in **panel D**. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. Total flies = 661; females = 343; males = 318; 11 vials. **F-F’** Percentage of each phenotype for ERACR-2 observed in F2 progeny from the cross scheme in **panel D**. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001. E=ERACR. Red shading = ERACR inherited. Green shading = MCR inherited. Total flies = 589; females = 311; males = 278, 11 vials.
Figure S19

A  Double-Cut ERACRs as CopyCat Elements

B  Single-Cut ERACRs as CopyCat Elements

ERACR-2  
Single-cut at y2

ERACR-2  
Single-cut at y3

ERACR-min  

% Phenotype

0  25  50  75  100

74.9% 91.3% 50.8% 31.2% 0.0% 0.0% 25.7% 29.5%

68.5% 73.8% 44.9% 0.5% 2.5% 19.5% 21.1% 57.7% 67.0% 47.5% 38.7% 0.7% 0.3% 5.9% 6.0%

DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+  DsRed+  eGFP+

0  25  50  75  100

46.5% 38.8% 0.0% 0.3% 20.5% 31.0%

57.7% 67.0% 47.5% 38.7% 0.7% 0.3% 5.9% 6.0%

47
Fig. S19: Double-cut and single-cut ERACRs versus wild-type y locus (CopyCats), related to Figure 6

A,B) Distributions of different phenotypic classes for double-cut (A) and single-cut (B) ERACRs are indicated for males and females separately. Note the similar performance profiles for the double-cut ERACR-2 and the single-cut ERACR2-y2 (see also Fig. S18 for comparison of single- versus double-cut ERACRs challenging the MCR-GFP element). As when challenged with the MCR-GFP bearing chromosome, ERACR2-y2 copied more efficiently than ERACR2-y3 (yellow symbols). Also, note that for all three elements, the percentage of GFP⁺ males was less than that for females, consistent with a significant class of receiver chromosomes being male lethal. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.
**Figure S20**

A. Chromosomes

- **F₀**
  - MCR⁺E
  - W⁻
  - "Master Females"

- **F₁**
  - MCR⁺E
  - W⁻

- **F₂** Progeny Analysis

A'. Diagrams of Phenotypes

- **B** Female F₂ Progeny of yellow- MCR-ERACR-1 Hybrid
- **B'** Male F₂ Progeny of yellow- MCR-ERACR-1 Hybrid

- **C** Female F₂ Progeny of yellow- MCR-ERACR-2 Hybrid
- **C'** Male F₂ Progeny of yellow- MCR-ERACR-2 Hybrid

- **D** Female F₂ Progeny of yellow- MCR-ERACR-min Hybrid
- **D'** Male F₂ Progeny of yellow- MCR-ERACR-min Hybrid

- **E** Female F₂ Progeny of yellow+ MCR-ERACR-1 Hybrid*
**Fig. S20: MCR-ERACR Hybrid drive efficiency, related to Figure 5**

An extremely rare subset of individuals identified in crosses shown in Fig. 5F,G and Fig. S14F,J-K, displayed a double-positive DsRed*, GFP* phenotype, which genotypically and phenotypically appear to be ERACR/MCR-GFP hybrids. As these hybrids are an unintended result of the mating between ERACR and MCR-GFP flies, we show in this experiment how these hybrid elements copy themselves when crossed to wild-type (w−) males. For each phenotypic category depicted along the X-axis, each graphed dot represents the percentage of females or males from one pair-mated vial with that particular recovered phenotype. Receiver chromosome phenotypes (w−) are highlighted in different colors: yellow = w−, GFP*, DsRed*, green = w−, GFP*, red = w−, DsRed*, and gray = w− (no fluorescence). A) Crossing scheme used to assess copying frequency of ERACR/MCR-GFP hybrids (denoted MCR+E). Homozygous w− F0 females were crossed with w− hybrid DsRed* GFP* F0 males (y+ or y−). Hybrid/w− F1 “master females” were then crossed to w− males to assay copying efficiency. A') Rows 1 and 2: Reproduced schematics from the y− ERACR-1/MCR-GFP and ERACR-2/MCR-GFP hybrids (Fig. 5F). Row 3: y− ERACR-min/MCR-GFP hybrid (Fig. S14J). Row 4: y+ ERACR-1/MCR-GFP hybrid (Fig. S14K). B,B'). Phenotypic prevalence of female and male F2 progeny from a y− ERACR 1/MCR-GFP hybrid copying onto a wild-type allele. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001. ERACR-1 (y−): total flies = 338, females = 156; males = 182; 4 F1 vials derived from one F0 mating. C-C') Phenotypic prevalence of female and male F2 progeny from a y− ERACR-2/MCR-GFP hybrid copying onto a wild-type allele. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001. ERACR-2: total flies = 62; females = 32; males = 30, 1 vial. D-D') Phenotypic prevalence of female and male F2 progeny from a y− ERACR-min/MCR-GFP hybrid copying onto a wild-type allele. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001. ERACR-min: total flies = 330; females = 171; males = 159; 3 F1 vials derived from one F0 mating. E-E') Phenotypic prevalence of female and male F2 progeny from a y+ ERACR-1/MCR-GFP hybrid copying onto a wild-type allele. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001,
and **** = p < 0.0001. ERACR-1 (y^+): total flies = 296; females = 135; males = 161, 3 F_1 vials derived from one F_0 mating.
Indication of Shadow Drive in F3 Progeny

A

Chromosomes

$F_0$

$y^+ e^{-wG} \ y^+ e^{-wG}$

Cas9 $w^+$

$\times$

$F_1$

$y^+ e^{-wG}$

Cas9 $w^+$

$y^+ w^+$

$\times$

$F_2$

$y^+ e^{-wG}$

$y^+ w^+$

$\times$

Analyze $F_3$ Progeny

$F_3$ e-CHACR-wG

B

% eGFP+ per vial

$\frac{52}{52}$

$\frac{52}{52}$

$\frac{52}{52}$

$\frac{52}{52}$

C

Chromosomes

$F_0$

$E MCR$

$\times$

"Master Females"

$F_1$

MCR $w^-$

$\times$

$F_2$

$E w^-$

$\times$

Analyze $F_3$ Progeny

$F_3$ ERACR-min

D

Indication of Shadow Drive in $F_3$ Progeny

$>50\% \ E$ $E$ $<50\% \ y^- w^- y^+ w^-$

E

% DsRed per vial

$\frac{52}{52}$

$\frac{52}{52}$

$\frac{52}{52}$

$\frac{52}{52}$

F

% DsRed per vial

$\frac{52}{52}$

$\frac{52}{52}$

$\frac{52}{52}$

$\frac{52}{52}$
Fig. S21: Absence of shadow-drive in e-CHACR-wG and ERACR F3 progeny, related to Figures 2, 3, 4, and 5

A) Cross scheme to determine shadow drive in F3 progeny in eCHACR-wG. B) No shadow drive was observed as revealed by the recovery of Mendelian (~50%) male and female GFP+ proportions of F3 progeny. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001. C) Cross scheme to determine shadow drive in F3 progeny for ERACR-2. F1 DsRed+, GFP+ “master females” were crossed to y+ w– males. DsRed+ female F2 progeny were then crossed to y– w– males. Equivalent crosses were conducted with ERACR-min, which is y– (not depicted). D) Shadow drive is evident if greater than 50% of F3 progeny exhibit a DsRed+ phenotype. E,F) The Mendelian distribution of DsRed+ alleles suggests a lack of shadow drive in ERACR-min (E) or ERACR-2 (F) F3 progeny. Each dot represents the percentage of DsRed+ females or males per vial for either ERACR-min or ERACR-2. Error bars indicate standard deviation and stars indicate p-values: * = p < 0.05, ** = p <0.01, *** = p < 0.001, and **** = p < 0.0001. ERACR-min: n=1082, female n=613, male n=469, 17 vials. ERACR-2: n=1078, female n=594, male n=484, 14 vials.
Figure S22

A

MCR-GFP vs yellow-

Generation 10 Crosses

MCR-GFP Inactivation within Female Progeny

MCR-GFP Inactivation within Female Progeny

B

Generation 9 and 10 ♀

♀ MCR e-wR x y+ w+

♀ MCR e-wR x ? ?

Analyze Female Progeny

♀ y+ w+ MCR

♀ y- w+ MCR

Inactive MCR Phenotype

Active MCR Phenotype

(B')

MCR-GFP Inactivation within Female Progeny

Generation 9 Crosses

Generation 10 Crosses

% Phenotype

0 25 50 75 100

0% 100% 0% 100% 0%

♀ y+ w+ mosaic eyes

♀ y- w+ mosaic eyes

C

Generation 9 and 10 ♀

♀ MCR e-wR x y+ w-

♀ MCR e-wR x ? ?

Analyze Progeny

♀ y+ w- MCR

♀ y- w- MCR

♀ e-wR yNHEJ MCR

♀ e-wR wNHEJ MCR

♀ e-wR MCR

♀ e-wR MCR

♀ e-wR MCR

C'

Inheritance of Inactivated MCR-GFP

Generation 9 Crosses

Generation 10 Crosses

% Phenotype

0 25 50 75 100

73.8% 26.2% 0% 70.7% 29.4% 0% 65.1% 34.8% 0% 64.4% 35.6% 0%

♀ Male Crosses

♀ Female Crosses

54
Fig. S22: Supplemental e-CHACR cage experiments, related to Figure 7

A) Control MCR-GFP versus \(y^-\) cage experiments. Multi-generation plot showing the kinetics of MCR-GFP copying in a \(y^-\) genetic background. The \(y^-\) point mutation used for this experiment is near the 5' end of the gene and does not interfere with cutting by gRNA-y1 carried by the MCR-GFP gene-drive element. Note that the frequency of the MCR-GFP peaks at generation 4 in the 80-85% range and then gradually decreases in subsequent generations suggesting that the MCR-GFP element incurs some fitness cost.

B,B') DsRed\(^+\), GFP\(^+\) \(F_0\) males from generations 9 and 10 were tested for Cas9 activity in crosses to wild-type Oregon-R females. \(F_1\) female progeny were examined for the presence of mosaic eyes, which is a reliable indicator of somatic Cas9 activity. B) Crossing scheme used for assessing residual Cas9 activity of the MCR-GFP gene-drive element in males and females from cage experiments. \(F_0\) male progeny (n=25) and \(F_0\) female progeny (n=15) expressing both the DsRed and eGFP fluorescence markers, indicating the presence of e-CHACR-wR and MCR-GFP and elements respectively, procured from each cage replicate of generations 9 and 10, were crossed with wild-type flies (\(y^+w^+\)). Female \(F_1\) progeny were then screened and analyzed for Cas9 activity. B') Scoring of body color and mosaic eyes phenotype in female progeny from the cross scheme depicted in panel B. Phenotypic categories are depicted on the X-axis, and each dot represents the total number of females counted of that phenotypic category per vial. We observed 100% of female \(F_1\) progeny had a \(y^+w^+\) phenotype. Since \(y^-\) or mosaic eyed female progeny were entirely absent from \(F_1\) progeny of generation 9 or 10 males, we conclude that no residual Cas9 activity remained in these populations. Similarly, none of the \(F_1\) female progeny of \(F_0\) females exhibited mosaic eyes and all of their \(F_1\) progeny (both males and females) carried the DsRed marker, indicative of the e-CHACR-wR element having being homozygous in their \(F_0\) mothers. These results are consistent with the hypothesis based on single-generation crosses (e.g., Fig. 2; Figs. S3;S4;S7;S9;S11) that Cas9 activity is nearly always eliminated when combined with an e-CHACR element.

C) Crossing scheme for assessing e-CHACR-wR and MCR-GFP transmission from generation 9 and 10 females. C') Prevalence of MCR and e-CHACR-wR element were scored in male progeny. We observed a significant number of male progeny with both fluorescence and the rest with DsRed only. Note that no GFP-only males were recovered from females from generations 9 and 10.
Figure S23

A) 

- **F₀**
  - 50% **y⁺**
  - 25% **y⁻** or 25% **y⁺**

- **F₁**
  - **y⁻**

- **F₂**
  - Analyze Male

- **F₂** Progeny

B) Male F₂ Progeny

- % of total males
  - 100%
  - 75%
  - 50%
  - 25%
  - 0%

- **y⁺**
  - 80.8%
  - 19.2%

- **y⁻**

C) 

- **F₀**
  - 50% **y⁺**
  - 25% **y⁻** or 25% **y⁺**

- **F₁**
  - **y⁻**

- **F₁** Progeny

- **F₂**

- **F₂** Progeny

D) Female F₂ Progeny

- % of total females
  - 100%
  - 75%
  - 50%
  - 25%
  - 0%

- **y⁺**
  - 44.3%
  - 55.7%

- **y⁻**

E) ERACR-2 vs yellow Point Mutant

- Population Ratio vs Generation

- Chart with lines indicating % of total males and females across generations.

F) Model Fitting for ERACR-min Females

- eGFP+, DsRed-
- eGFP-, DsRed-
- eGFP+, DsRed+
- eGFP-, DsRed+

G) Model Fitting for ERACR-2 Females

- **y⁻**, eGFP+, DsRed-
- **y⁺**, eGFP+, DsRed-
- **y⁻**, eGFP+, DsRed+
- **y⁺**, eGFP+, DsRed+

F’) Model Fitting for ERACR-min Males

- eGFP+, DsRed-
- eGFP-, DsRed-
- eGFP+, DsRed+
- eGFP-, DsRed+

G’) Model Fitting for ERACR-2 Males

- **y⁻**, eGFP+, DsRed-
- **y⁺**, eGFP+, DsRed-
- **y⁻**, eGFP+, DsRed+
- **y⁺**, eGFP+, DsRed+
Fig. S23. Additional ERACR cage trial data, related to Figure 7

A,C) Crossing schemes for testing sexual selection of preferences of $y^+$ virgin females (A), or $y^-$ virgin females (C), for $y^+$ or $y^-$ males. In the crossing scheme shown in panel A, $F_0$ $y^+$ virgin females were crossed to both $y^+$ and $y^-$ males. $F_1$ virgin females were then crossed to wild-type ($y^+$) males, male $F_2$ progeny of which were analyzed to determine the sexual preference of the $F_0$ $y^+$ virgin females. For the crossing scheme depicted in panel C, $F_0$ $y^-$ virgin females are crossed to both $y^+$ and $y^-$ males to generate $F_1$ progeny, of which females are analyzed to determine the sexual preference of the $F_0$ $y^-$ virgin females. B,D) Percentage of each phenotype ($y^+$ or $y^-$) for either male $F_2$ progeny (B) or for female $F_1$ progeny (D) for the respective cross schemes shown in panels A and C. B shows a 80:20 skew for mating preference of $y^+$ virgin females for $y^+$ males over $y^-$ males. D shows no obvious skew (50:50) or mating preference of $y^-$ virgin females for $y^+$ males over $y^-$ males. Error bars indicate standard deviation and stars indicate p-values: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$. E) ERACR-2 x $y^-$ point mutant. Virgin ERACR and MCR-GFP flies were seeded at a ratio of 25%:75%. Note that at generation 1, the frequency of $y^-$ individuals (avg. = 0.41) approximates that predicted by random mating (~37%). Over 4-5 generations the prevalence of the ERACR increases sharply in concert with a corresponding reduction in $y^-$ individuals. By generation 10, ERACR-2 is nearly fixed and the yellow point mutant eliminated in all cages. Red traces indicate DsRed$^+$ progeny (proxy for ERACR-2), and yellow traces indicate progeny that are yellow in body color (proxy for $y^-$ mutant). Each line represents a separate cage replicate. F and F') Observed and predicted dynamics of the ERACR-min gene-drive remediation system for each marker phenotype. Population cage experiments were set up with 19 females and 19 males homozygous (EE) or hemizygous (EY) for the X-linked ERACR-min construct (E) and 57 females and 57 males homozygous (HH) or hemizygous (HY) for the X-linked drive system (H) that the ERACR-min construct targets. Population counts were monitored over 21 or 26 generations. Results from these experiments are shown as solid lines, with fitted model predictions shown as dashed lines. Each marker phenotype is known to be produced by certain genotypes. For females, EH and ER ("R" represents an in-frame, cost-free resistant allele) are GFP$^+$/DsRed$^+$, EE and EB ("B" represents and out-of-frame or otherwise costly resistant
“broken” allele) are GFP–/DsRed+, HH, HR, HB, RR and RB are GFP+/DsRed–, and BB are GFP–/DsRed–. For males, EY are GFP–/DsRed+, HY and RY are GFP+/DsRed–, and BY are GFP–/DsRed–. Observed data are consistent with the conversion efficiency results shown in Fig. S15A, namely an accurate copying efficiency of 28.2%, with 47.6% of resistant alleles being in-frame, cost-free (R), with the remainder being out-of-frame or otherwise costly (B). Given these rates, the data are consistent with the following fitness costs: females and males homozygous (BB) and hemizygous (BY) for broken allele are inviable, females homozygous for the driving allele (HH) have a fitness cost of 99.7% (95% CrI: 98.8-100%), males hemizygous for the driving allele (HY) have a fitness cost of 21% (95% CrI: 18-24%), females having one copy of the drive allele have a fitness cost of 0.5% (95% CrI: 0-2.0%), and females having one copy of the broken allele have a fitness cost of 92% (95% CrI: 62-100%). The ERACR-min system spreads in all repetitions, although not always to fixation. G and G’) Observed and predicted dynamics of the ERACR-2 gene-drive remediation system for each marker phenotype. Population cage experiments were set up with 19 females and 19 males homozygous (EE) or hemizygous (EY) for the X-linked ERACR-2 construct (E), and 57 females and 57 males homozygous (HH) or hemizygous (HY) for the X-linked drive system (H) that the ERACR-2 construct targets. Population counts were monitored over 10 generations. Results from these experiments are shown as solid lines, with fitted model predictions shown as dashed lines. Each marker phenotype is known to be produced by certain genotypes. For females, EH and ER (“R” represents an in-frame, cost-free resistant allele) are y+/GFP+/DsRed+, EE and EB (“B” represents an out-of-frame or otherwise costly resistant “broken” allele) are y+/GFP–/DsRed+, HH, HR, HB, RR and RB are y–/GFP+/DsRed–, and BB are y–/GFP–/DsRed–. For males, EY are y+/GFP+/DsRed+, HY and RY are y–/GFP+/DsRed–, and BY are y–/GFP–/DsRed–. Observed data are consistent with the conversion efficiency results shown in Fig. S15A, namely an accurate conversion efficiency of 32.4%, with 57.2% of resistant alleles being in-frame, cost-free (R), with the remainder being out-of-frame or otherwise costly (B). Given these rates, the data are consistent with the following fitness costs: females and males homozygous (BB) or hemizygous (BY) for the broken allele are inviable, females homozygous for the drive allele (HH) have a fitness cost of 77% (95% CrI: 69-84%), males hemizygous for the drive allele (HY) have a fitness cost of 66% (95% CrI: 33-80%), females having one copy of the
drive allele have a fitness cost of 73% (95% CrI: 57-89%), and females having one copy of the broken allele have a fitness cost of 48% (95% CrI: 4-97%). Assortative mating is seen for $y^+$ females, who are estimated to reduce their mating with $y^-$ males by 63% (95% CrI: 49-77%), with a corresponding increase in mating with $y^+$ males. The ERACR-2 system reaches fixation in all three repetitions.
Table S1: Data from individual e-CHACR-induced 10 male-lethal (eml) alleles, related to Figure 1 and 2

Lethal line (GFP-, w-) X 30220 on Y (w-)

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<td>y+</td>
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Category is highlighted (in blue) which indicates rescue of lethality by X-duplication stock
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Category is highlighted (in blue) which indicates Akit phenotype (which you should see if lethal line contains an Akit deletion)
Table S1: Data from individual e-CHACR-induced 10 male-lethal (eml) alleles, related to Figures 1 and 2

Data from individual e-CHACR-induced 10 male-lethal (eml) alleles crosses diagrammed in Fig. S21C-F which were performed in triplicate (crosses yielding no progeny are boxed in red). Page 1 of the Excel spreadsheet tabulates the results for crosses to males carrying an X Duplication covering the tip of the X Chromosome (including the y locus - Bloomington X Duplication Kit, stock #30220) (Cook et al., 2010) and page 2 tabulates the results for crosses to males carrying the viable ac^4 allele. Note also that none of the non-FM7 females recovered (eml/ac^4) displayed any bristle-loss phenotypes that would be indicative of DNA lesions disrupting the function of the achaete locus located ~9 kb away (centromere proximal) from the MCR-GFP insertion into the y locus.
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<tr>
<th>Vector Name</th>
<th>Template</th>
<th>Primer sequences</th>
<th>Restriction Enzymes</th>
<th>Method</th>
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<td>ERACR- min (pVG155)</td>
<td>pVG138</td>
<td>422: CTTAaccggGAAAGTGAGGATAGTAGCA GGTTGGATCTAGCTTGC</td>
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<td>279: TTAAGATACATTGATGAGGTGACAAA CCACAAGTACGAT</td>
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| e-CHACR-wG (pVG315) | pVG315 | 1079: AACGTGGGACATCGGCACCAACTCTGGTTTAGAGCTAGAATAGCAAGTTAAAAT AAGG  
| | | 1080: CGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGC |
| | | 1081: GTGGCGACCGGATGCCGCCACCATGGTGAGCAAGGGC |
| | | GTTGGTGCCGATGTCCGACGTTAAATTGAAAATAGCTATATACGAAACTGAGTCTGG |
| e-CHACR-wR (pVG429) | pVG315 | 359: CGATTTGTTTAGCTTGTTCAGCTGCG  
| | | 1522: CTCCCCCTGAACCTGAAACATAAAATG |
| | | 931: CAAGCGCAGCTGAACAAGCTAAACAATC |
| | | 1633: CATTGTATGCCGCTTACCAAGG |
| e-CHACR-e (pVG359) | pVG304 | 559: CGATTTGTTTAGCTTGTTCAGCTGCG  
| | | 1285: CTGGCTATTTTCTAGCTAAAAACGATCGGAGCCCTGCTGTTCGAGCTGACGTTGAATAG |
| | | 931: CAAGCGCAGCTGAACAAGCTAAACAATC |
| | | 656: gttttagctagaaatagcaagttaaaataaggc |
| e-CHACR-k (pVG467) | Kni-HA-A_TOPO | 1754: TTGAATTAGATCCCCGGGCGAGCTCGcatATAGAAGGTAAGGCCAAATATGGACG |
| | | 1755: TATACTATCGAGGACGTTGAAAATGacgGTTGGGCTACCACAGG |
| eCHACR-E-gRNA_pCFD4 | pVG429 | 1732: CTATTTCACGTCCCTGATAGTATAGTG |
| | | 1733: ctagcACAAAAAGCTGGAGCTGCAGT |
| | | 1735: AGCTCGGCCCGGAGATCTAATTCAATAG |
| | | 1734: CAGGAGCTCCAGCTTTTGTgctag |
| Vasa Cas9 Resistant to eCHACR-e (pVG470) | pVG316 | 1798: CTTGATGCGcTGTGTTTGACACGCGGC |
| | | 885: GAACTCTGACATCATCCATACACTGAGC |
| eCHACR-E-gRNA_pCFD4 | pVG133 | 366: CTCCAGGGATCCTTTTTTGCTCACCTGTGATTGCTCC |
| MCR-Tagger- | | | Gibson Assembly |
| | | | Gibson Assembly |
| | | | Gibson Assembly |
| | | | Gibson Assembly |
Table S3: Transgenic *Drosophila melanogaster* flies generated in this study, related to STAR methods

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<td>ERACR-2 y2 Single-cut</td>
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<td>ERACR-2 y3 Single-cut</td>
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<td>Vasa Cas9 resistant to e-CHACR-e</td>
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Table S5: List of *Drosophila melanogaster* X-duplication Stocks, related to STAR methods

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Methods S1

Methods, Figure 2: e-CHACR-wR versus the MCR-GFP element

B) Crossing scheme used for testing the e-CHACR-wR (e-wR) against the eGFP tagged MCR. Homozygous e-CHACR-wR females were mated to MCR-eGFP males to generate heterozygous master females that were subsequently singly mated to ac− males. The F2 progeny were then screened and analyzed for presence of the MCR-GFP element, presence (DsRed+) or absence (w^NHEJ) of e-CHACR-wR on ac+ marked chromosome. As observed for e-CHACR-e, no y− females were recovered from the F2 generation, indicating the absence of any Cas9 activity produced by the gene-drive element. However, individuals with mosaic eye phenotypes were recovered at the low frequency of 0.83% in F2 females.

C) Percentage of fluorescence in total F2 female progeny per cross. Overall DsRed transmission reveals efficient copying of the e-CHACR-wR element, comparable to that of e-CHACR-wG. C’) Percentage of fluorescence in total F2 male progeny per cross. Similar to F2 females, overall DsRed transmission was very high in males. However, unlike the e-CHACR-wG and e-CHACR-e experiments, we did not observe a reduced fraction of Cas9-bearing chromosomes targeted by the element.

D) Prevalence of GFP+ and GFP− alleles in MCR donor (left) and receiver F2 females (right). D’) Prevalence of GFP+ and GFP− alleles in MCR donor (left) and receiver F2 males (right). Paralleling results from e-CHACR-e, we observed similar reduced rates of MCR copying onto target chromosomes (*Note 2 single-pair mating crosses did not generate any ac+ males and thus were excluded from the graph).

E) Scoring of body color in total F2 females. We observed equal percentages of ac+ and ac− chromosomes, of which none were y−, suggesting that the gene-drive element has been efficiently targeted and inactivated. E’) Scoring of body color in total F2 males. F) Percentage of MCR donor (ac+, black dots) and receiver (ac−, pink dots) alleles in F2 males. We observed only a slight reduction of ac+ alleles, in contrast to significant decreases of e-CHACR target alleles found in e-CHACR-wG and e-CHACR-e experiments.
Methods, Figure 3: ERACR construct designs and crossing schemes.

A) Design features of MCR and ERACR elements. The top line illustrates the location of the \(y\) and \(w\) loci, which are located 1.5 centimorgans (cM) apart at the end of the X-chromosome. Just below, is a magnified view of the \(y\) locus into which the MCR and ERACR elements are inserted. The third line from the top depicts the eGFP-labeled MCR, which carries the gRNA-y1 guide RNA that cuts at the genomic site of MCR-GFP insertion. The MCR-GFP bearing chromosome is also marked with the \(w\textsuperscript{a}\) allele (orange-colored eyes). gRNA cut sites marked with asterisks indicate sites that have been rendered uncleavable by the gRNA in the process of inserting an active genetic element (e.g., \(y\textsuperscript{1*}\) indicates the site disrupted by MCR insertion via gRNA-y1 cleavage, and \(y\textsuperscript{2*}\) and \(y\textsuperscript{3*}\) denote sites eliminated by ERACR integration mediated by gRNA-y2 and gRNA-y3 respectively). The lower three lines illustrate the structures of the ERACR-min, ERACR-1, and ERACR-2 constructs respectively, which all carry both gRNA-y2 and gRNA-y3 directing genome cleavage on either side of the MCR-GFP element. ERACR-1 and ERACR-2 also carry recoded versions of a rescuing \(y\)-cDNA (extent of recoded sequences indicated by hatched brown boxes), and ERACR-2 features an inverted gRNA cassette and \textit{D. grimshawi} regulatory regions. Fly heads on the left indicate the phenotype of each strain. The cassette size of each type of drive element is shown to the right in kilobases (kb). Discrepancies between the sum of element length and the total construct length can be attributed to rounding differences. Schematic is not drawn to scale.

B) Crossing schemes depict single pair mating cross in the F\(_0\), generation between an ERACR bearing female (indicated by white eyes with emanating red fluorescence) and an MCR-GFP bearing male (indicated with orange eyes with emanating green fluorescence) to generate F\(_1\) “master females” carrying the ERACR \textit{in-trans} to the MCR-GFP element. ERACR copying events indicated by red arrow heads occur in the germline of F\(_1\) females. The cross scheme for the \(y\textsuperscript{−}\) ERACR-min element is shown on the left and the genetic scheme for the \(y\textsuperscript{+}\) ERACR-1 and ERACR-2 elements is shown on the right. Dotted lines around fly heads indicate expected phenotypes and all other fly heads indicate observed phenotypes. As indicated in the key in the bottom right, fly heads depict eye color (wild-type dominant \(w\textsuperscript{+}\) (red), recessive loss-of-function \(w\textsuperscript{−}\) (white), or partial loss-of-function \(w\textsuperscript{a}\)(orange)), eye fluorescence (radial emanating lines of eGFP (green),
DsRed (red), both eGFP and DsRed (alternating green and red), or neither fluorescence (white), and body color: dominant \( y^+ \) (brown) or recessive \( y^- \) (yellow). In certain experiments, the MCR-GFP strain was not marked with \( w^a \). Also, in some instances, FM7 or Basc F1 males were used in place of F1 \( w^- \) males to permit scoring of other markers (Supplemental Fig. 1). E = ERACR. P = Promoter; P-A = poly-A tail. 3’ = 3’ UTR; \( D. \) gri = Drosophila grimshawi; \( D. \) mel = Drosophila melanogaster.

**Methods, Figure 4: Analysis of ERACR deletion and replacement of MCR drive**

Phenotypic frequencies and deduced gene conversion events in F2 progeny when a hemizygous MCR individual was confronted with a homozygous ERACR individual (Detailed cross schemes provided in Figs. S13A-D). Dot plots represent the phenotypic distribution of either male or female F2 progeny with respect to the total flies of each sex. Each dot represents the percentage of F2 individuals of a given phenotype emerging from a single pair mating event (e.g. one vial). Percentages below each plot (in black type) tabulate the mean of these percentages across all vials. Each graph is based on pooled data from a combination of experiments that include the \( w^a \)-marked cross scheme shown in Fig. 3B and Fig. S13A-D. All vials contained >20 flies and crosses (vials) with fewer than 20 flies were not included. **ERACR-min:** total flies = 1576; females = 900; males = 676, 34 vials. **ERACR-1:** total flies = 2798, females = 1713; males = 1085; 45 vials. **ERACR-2:** total flies = 4445; females =1710; males = 2735; 72 vials. **A)** DsRed\(^+\) inheritance is a proxy for scoring ERACR prevalence (DsRed\(^+\), GFP\(^-\) and DsRed\(^+\), GFP\(^+\) progeny included). The mean of each fluorescence group was calculated per cross by dividing the number of individuals in a phenotypic class by the total number of females or males in that vial. **A’)** The subset of data plotted in Fig. 4A with traceable donor and receiver chromosomes re-plotted by donor (\( w^- \); left graph) versus receiver (\( w^a \); right graph) chromosomes. **ERACR-min:** total flies = 1576; females = 900; males = 676; 34 vials (Fig. S13C-D). **ERACR-1:** total flies = 757; females = 448; males = 309; 18 vials (Fig. S13D). **ERACR-2:** total flies = 2458; females = 1491; males = 967; 43 vials (Fig. S13C-D). Percentages written below the graph in orange type represent the ERACR prevalence as a portion of the receiver chromosomes of F2 progeny (e.g., DsRed \( w^a \)
males/total w³ males). B) The proportion of F₂ males or females inheriting the donor (w⁻) versus receiver (w³) chromosome, which reveals approximately Mendelian inheritance of the two chromosomes in females but a substantial bias towards inheriting the receiver allele in males. C,E) Schematics illustrating the predicted gene conversion event responsible for a given phenotypic observation. Phenotypic data (y w, ±GFP, ±DsRed) and an illustrative fly head are shown to the left. The upper schematic, labeled “Inferred Gene Editing”, uses a black arrow to illustrate the gene conversion event between the two parental alleles in F₁ “Master Females”. The final product of this editing event, evident in the F₂ progeny, is illustrated below and labeled “Resulting Sequence.” MCR cassettes are highlighted in green, and ERACR cassettes are highlighted in red. Unless specified, the ERACR schematic depicted is based off the ERACR-1 cassette and is not intended to represent any one ERACR in particular. All schematics shown are validated by sequence analysis at junction points (Fig. S14). C) Top: schematic representation of the likely HDR-mediated repair event that led to the copying of ERACR allele to the w³ marked donor chromosome. A D-loop intermediate forming as a step in SDSA branch of HDR repair is shown, resulting in directional gene conversion wherein the genomic segment of the ERACR between gRNA-y2 and gRNA-y3 cleavage sites is copied onto the MCR. Panel D and Figs. 5A,F) Because not all of these experiments have clearly marked donor and receiver alleles, we estimated the proportion of the receiver chromosome made up by the progeny of a given phenotype (“% Rec” in orange type) from all experimental crosses by multiplying the average frequency of that phenotype across all vials by the frequency of receiver alleles inherited (panel 2B). (Example: mean percent of phenotype per vial derived from panel 2A multiplied by the percent receiver alleles inherited derived from panel 2B (See Fig. S15 for full details). Note that panel 2A’ does not display estimated data because calculations are confounded by ERACR donor allele inheritance. D) eGFP inheritance is a proxy for scoring the MCR prevalence. Data includes DsRed⁺, GFP⁺ and DsRed⁻, GFP⁺ progeny. E) Top: schematic representation of a likely HDR-mediated repair event leading to retention of an MCR-GFP element. The MCR-GFP although intact, is acted on by the ERACR at the gRNA-y2 and gRNA-y3 cleavage sites...
to create NHEJ-induced mutations as indicated by insertions and deletions (indels) at y2 and y3 cut sites, but the MCR element is not replaced by the ERACR element.

**Methods, Figure 5: Analysis of alternative ERACR outcomes**

A) GFP\(^{-}\), DsRed\(^{-}\) inheritance is a proxy for scoring MCR deletion events. Non-fluorescent or double-negative (DsRed\(^{-}\), GFP\(^{-}\)) F\(_2\) progeny, which lack markers for both the ERACR and MCR elements, are much more frequently recovered in female than male F\(_2\) progeny, suggesting that double-negative events may be male lethal. A’) Data in panel A re-plotted by inheritance of y\(^{-}\) (lethal deletion) versus y\(^{+}\) (recombination) alleles. Body color permits inference of the type of excision event that occurred. Female data only includes experiments with y\(^{-}\) F\(_1\) males. All available males are included as they do not inherit a confounding paternal y\(^{+}\) allele. ERACR-min data were excluded from panel A’ due to y\(^{-}\) body color. ERACR-1: total flies = 1982; females = 897, 18 vials (Fig. S13B), male n=1085; 45 vials (Fig. S13A,B,D). ERACR-2: total flies = 3324; females = 1614; 39 vials (Fig. S13B-C), males = 1710; 72 vials (Fig. S13A-D). B) Hypothesized deletion of essential sequences distal to the ERACR gRNA cut sites resulting in a male lethal allele (e.g., tip of the X-chromosome truncated). The viability of several such alleles can be rescued in males by duplications covering the tip of the X-chromosome (see Fig. S12B). C) Scheme depicting hypothesized repair event mediated by partial pairing of un-recoded y sequences carried by ERACR-1 and endogenous y sequences 3’ to the MCR-GFP element, resulting in expression of the full recoded y cassette and a wild-type body color. D,E) The MCR-GFP element is deleted, but not replaced with the ERACR, producing two distinct observed outcomes: D) NHEJ events joining adjacent sequences at the gRNA-y2 and gRNA-y3 cut sites. E) Likely pairing between 17 bp of the gRNA-y2 genomic target sequences 5’ to the MCR with corresponding sequences in the gRNA-y2 transgene carried by either ERACR-min or ERACR-1. The y coding region is interrupted in this allele, resulting in a yellow body color. Note that this class of events was not recovered for ERACR-2 in which the gRNAs are oriented in the opposite direction. F) Prevalence of DsRed\(^{+}\), GFP\(^{+}\) F\(_2\) progeny in which MCR-GFP and ERACR sequences are both present on the receiver chromosome. G) Two examples of MCR-GFP/ERACR fusion events. In the first case, mis-aligned pairing between endogenous y sequences 3’ to the MCR-GFP...
insertion and un-recoded y sequences carried by ERACR-1 likely resulted in the observed MCR-GFP/ERACR-1 fusion element. In the second instance, an illegitimate pairing or non-homology based repair event led to the fusion of endogenous y sequences 3’ to the MCR with recoded y sequences carried by ERACR-2. We note that an octomer denoted by an asterisk (see box) which lies immediately 3’ to the junction site also occurs 1.2 kb upstream within endogenous y sequences.

Methods, Figure 6: Drive performance of single-cut ERACRs versus MCR drive
A) Schemes illustrating single-cut and double-cut ERACR designs. Note that the receiver chromosome is marked with \( w^a \). Double-cut ERACRs carry both gRNA\(_y2\) and gRNA-\(y_3\) directing Cas9 cleavage to both sides of the MCR-GFP gene-drive element. ERACR2-\(y_2\) carries only gRNA-\(y_2\), while ERACR2-\(y_3\) harbors gRNA-\(y_3\). (Note: gRNA-\(y_1\) from the MCR element is unable to target any of the ERACR constructs due to lack the cleavage site for this gRNA). B) Crossing scheme for generating and testing transmission by single-cut ERACR/MCR F\(_1\) master females. C-E) Single-cut versions of ERACR-2 are placed in-trans to the MCR-GFP element where the gRNA-\(y_2\) and gRNA-\(y_3\) are separated by a distance of 11.3 kb, while in Fig. S19 these elements are confronted in CopyCat mode with a wild-type y locus where the two gRNA cut sites are only 2.2 kb apart. C) Tabulation of fluorescent phenotypes for single-cut DsRed\(^+\) ERACR2-\(y_2\) and ERACR2-\(y_3\) and MCR-GFP as a percentage of total female (F) or male (M) progeny. Orange type indicates percentages restricted to the receiving allele only. Note that for ERACR2-\(y_2\), a greater fraction of males than females are DsRed\(^+\) and the proportion of GFP\(^+\) progeny is less for males than females. These two skews are consistent with a male lethal class (~22% = 43.5%-33.7%/43.5%) of MCR-GFP target alleles that retain the GFP marker. In the case of ERACR2-\(y_3\), note the abundant class (17%) of double-negative female progeny (gray symbols), which are entirely absent in males. Similarly, for ERACR2-\(y_2\), there is a prominent reduction in the fraction of GFP\(^+\) males relative to females (~10% difference). D) Percent DsRed\(^+\) (ERACR) females or males inheriting either the donor (\(w\)) ERACR-2 single-cut chromosome or the receiver (\(w^a\)) chromosome. Note the prominent skew in favor of donor chromosomes in males but not in females and that the rate of copying for
ERACR2-y2 (y2 cut) is greater than for ERACR2-y3 (y3 cut). Percentages along the X-axis in standard type indicate percentages relative to the total population. E) Donor versus receiver chromosome transmission for single-cut ERACR2-y2 and ERACR2-y3. While both chromosomes are inherited in approximately Mendelian frequencies in females, a pronounced bias for the donor chromosome is observed in males, paralleling results from the double-cut ERACRs (Fig. 4B).

**Methods, Figure 7. ERACR versus MCR population cage trials and modeling**  
A) Graph showing the fraction of individuals with different phenotypes over 12 generations. Equal numbers of homozygous MCR-GFP and e-CHACR-wR (DsRed*) males and females (25% of each) were seeded into bottles. Following each generation, half of the population was anesthetized and scored for fluorescence and eye color phenotypes (including mosaic eyes indicative of first generation contact between the MCR and e-CHACR elements), while the remaining flies (100-150) were used to start the next generation. Green = MCR and Red = e-CHACR-wR prevalence in the total population (e.g., both males and females). Orange = females carrying both elements. Yellow = females with mosaic eyes indicative of Cas9 activity. B) Mathematical modeling of MCR-GFP versus e-CHACR-wR single-pair. Data presented in panel A as solid lines are shown here as corresponding dotted lines. The dark solid lines (Fits) indicate best fits based on the model and parameters used, and the array of lighter lines (Stoch.) indicate individual stochastic simulations based on the model. C,D) Modeling of MCR-GFP versus ERACR dynamics. D,F) ERACR versus MCR cage experiments. Virgin ERACR and MCR-GFP flies were seeded at a ratio of 25%:75%. At each generation 50% of the flies in a cage were counted and scored for eye fluorescence and body color phenotypes and 100 flies randomly selected from the remainder were added to fresh cages. Red traces indicate DsRed* progeny, green traces denote GFP* progeny, and yellow traces signify the presence of both markers. Female MCR-GFP individuals were homozygous for the MCR element. Each line represents a separate cage replicate. C) Modeling of ERACR-min versus MCR-GFP dynamics over 26 generations with separate data (solid curves) and model fits (dotted curves) plotted for the frequencies of MCR-GFP (top) and DsRed* ERACR-min (bottom). D) Virgin ERACR-min (y-, DsRed*) males and females were added
to virgin MCR-GFP (y– GFP+) males and females in generation zero to initiate the drive experiments. Note that at generation 2, the frequency of double-positive (DsRed+, GFP+) individuals (avg. = 32.9%) approximates that predicted by random mating (37.5%). Over 4-5 generations the prevalence of the ERACR increases sharply in concert with a corresponding reduction in MCR-GFP (and double positive) individuals. By generation 10, ERACR-min is nearly fixed and the MCR-GFP eliminated in all cages, with a modest degree of frequency variation between cages at each generation. E) Modeling of ERACR-2 versus MCR-GFP dynamics over 10 generations with separate data (solid curves) and model fits (dotted curves) plotted for frequencies of the MCR-GFP and DsRed+ ERACR-2 phenotypes. F) Virgin ERACR-2 (y+, DsRed+) males and females were added to virgin MCR-GFP (y– GFP+) males and females in generation zero to initiate the drive experiments. Note that at generation 2, the frequency of double-positive (DsRed+, GFP+) individuals (avg. = 15.1%) was significantly lower than predicted by random mating (37.5%), consistent with a strong assortative mating behavior associated with y– mutants wherein wild-type (y+) females exhibit a strong bias to mate with y+ males over y– males. The ERACR-2 element drives to near completion by 5 generations with little variation between cages.
Data S1

In-depth analysis of e-CHACR Performance

An X-linked e-CHACR efficiently copies and inactivates Cas9

Based on the design rationale illustrated in Fig. 1A, we first tested an e-CHACR element (e-CHACR-wG, Fig. S3A) carrying an eGFP marker and two gRNAs. One guide (gRNA-w2) targets the white locus to enable its own copying in the presence of Cas9 (e-CHACR-wG integration or NHEJ events at this site disrupt w gene function), and a second (gRNA-C1) targets sequences essential for Cas9 catalytic activity. In initial experiments, we assessed the ability of e-CHACR-wG to inactivate a static (non-driving) DsRed-marked source of Cas9 inserted at the same site in the y locus as the MCR-GFP element. Since the y and w loci are closely linked on the X chromosome (1.5 cM apart), and because the Cas9 element is inherited in a standard Mendelian fashion, the presence of DsRed also serves as a marker of the receiver chromosome (98.5% of the time) providing a reliable indicator for e-CHACR-wG copying events and other potential Cas9-dependent outcomes.

Following the genetic crossing scheme depicted in Fig. S3B, we crossed w− F0 females homozygous for the e-CHACR-wG element to w+ males carrying a DsRed-marked Cas9 transgene inserted at y. 100% of resulting F1 master females bearing these two elements in-trans displayed mosaic eye phenotypes comprised of w− (white-colored) and w+ (red-colored) sectors generated by somatic activity of Cas9/gRNA complexes targeting the w locus. This mosaicism, therefore, served as a fully penetrant and readily scorable indicator of Cas9 activity. F1 master females were crossed in parallel single-pair matings to wild-type y+w+ males and their F2 progeny were tabulated according to their fluorescence phenotypes (Fig. S3C). We analyzed two genetic outcomes in these F2 male and female progeny: 1) transmission frequencies of e-CHACR-wG (GFP, donor) versus Cas9 (DsRed, receiver) chromosomes (Fig. S3C), and 2) the fraction of mosaic DsRed+, GFP+ progeny (i.e., individuals carrying both the targeted Cas9 transgene and
gRNA-w2, targeting \( w \)) since, as mentioned above, this mosaic phenotype served as a faithful indicator of Cas9 activity.

In these experiments, the e-CHACR-wG element was efficiently transmitted to both female (avg. = 94%) and male (avg. = 95%) \( F_1 \) progeny (Fig. S3C,C'). Nearly three-quarters of these crosses (73%), exhibited 100% transmission of the e-CHACR-wG element to both male and female offspring (Fig. S2C,C',C''). This non-Gaussian distribution of inheritance rates displayed three discrete peaks, mirroring the pattern we observed for the MCR-GFP gene-drive experiments (Fig. S1C-D''). In females (Fig. S3C), we observed approximately equal proportions of donor (GFP only) versus receiver (DsRed\(^+\), GFP\(^\pm\)) chromosomes in accord with expected Mendelian inheritance. Based on these ratios, we can estimate the frequency of e-CHACR-wG copying to the target chromosome as \(~92\%\) (i.e., DsRed\(^+\), GFP\(^+\)/total DsRed). In males (Fig. S3C'), however, we observed significantly biased transmission of GFP-only donor chromosomes (63%) relative to total DsRed\(^+\) receiver chromosomes (37.1%). This nearly 2:1 skewing of inheritance suggests that target receiver chromosomes incur a significant transmission handicap in males. Since the fraction of copying events observed in \( F_2 \) males (\(~86\%) was comparable to that in \( F_2 \) females (\(~89\%), we conclude that e-CHACR-wG copying events occurred at nearly equal frequencies regardless of whether they suffered reduced transmission to males. The deficit in receiver chromosome transmission to males versus females also results in a corresponding bias in the percentages of total male versus female progeny (Fig. S8B).

In addition to being transmitted to progeny at high frequencies, e-CHACR-wG was also very efficient in eliminating Cas9 activity as judged by the virtual absence of DsRed\(^+\), GFP\(^+\) \( F_2 \) female progeny displaying mosaic eye phenotypes (e.g., only 60/4355 = 1.37\% of total progeny). We further confirmed this phenotypic assessment of Cas9 inactivation by extending the single-pair mating crossing scheme to the \( F_3 \) generation in which non-mosaic \( F_2 \) females carrying both the e-CHACR-wG and Cas9 (presumably inactivated) were crossed to wild-type \( y^+\), \( w^+ \) males (Fig. S3D). The resulting \( F_3 \) generation (Fig. S3E)
confirmed full Cas9 inactivation by two criteria: the continued absence of any mosaic eye phenotypes, and the reversion to standard Mendelian inheritance of both elements. We also note that approximately equivalent percentages of male and female F3 progeny were recovered, suggesting that the significant majority of DsRed⁺, GFP⁺ receiver chromosomes were viable in both sexes (this point is also relevant to e-CHACR induced damage to the MCR-GFP allele, as discussed below). DNA sequencing of a panel of DsRed⁺, GFP⁺ chromosomes transmitted to male F₂ offspring revealed that 100% of targeted chromosomes carried NHEJ-induced mutations at the target site, with ~1/3 of them being in-frame mutations, consistent with there being a wide array of quasi-random NHEJ mutations induced by this particular gRNA (Fig. S6). The lack of Cas9 activity despite the abundant class of in-frame mutations suggests that gRNA-C1, which targets a site encoding critical enzymatic residues, generates almost exclusively non-functional NHEJ alleles that disrupt Cas9 transgene function. We also generated F₁ master females by providing the e-CHACR-wG element from F₀ fathers and found little difference as compared to providing the element from F₀ mothers (Fig. S4).

As mentioned above, maternally provided Cas9/gRNA complexes can act on the paternal chromosome to create somatic mutations even in the absence of genetically encoded CRISPR components. We have also shown that this trans-generational effect includes the germline where it produces a significant, albeit reduced, level of gene-drive - a phenomenon we refer to as "shadow drive" (Guichard et al., 2019). Because e-CHACR-wG is very efficient at mutating and inactivating Cas9, and thereby abolishing mosaic eye phenotypes, we tested whether shadow drive might also be impacted in F₂ females carrying the e-CHACR-wG element, but lacking genetically encoded Cas9 (i.e., DsRed⁻, GFP⁺ females). We crossed such DsRed⁻, GFP⁺ F₂ females to wild-type males (Fig. S21A) and observed only the expected Mendelian frequency of e-CHACR-wG transmission in both male and female F₃ progeny (Fig. S21B). Thus, as in somatic cells, germline Cas9 activity was greatly attenuated or eliminated in F₂ females resulting in little, if any, shadow drive in the subsequent generation.
An autosomal e-CHACR inactivates and substantially reduces transmission of a full gene-drive element

Given that e-CHACR-wG efficiently copied itself and inactivated a static source of Cas9, we next tested three e-CHACRs located on different chromosomes for their ability to target Cas9 carried on the full gene-drive MCR-GFP element inserted at the y locus. We scored three genetic events: 1) frequency of e-CHACR transmission, 2) efficiency of Cas9 inactivation by e-CHACR, and 3) copying efficiency of the MCR-GFP element.

We first tested several independently derived lines of a DsRed marked e-CHACR-e element inserted into the coding region of the ebony gene on the third chromosome. e-CHACR-e carries gRNA-e1 to drive its own copying in ebony and gRNA-C3 for targeting Cas9 (at a different catalytically essential site from that of gRNA-C1 carried by e-CHACR-wG). Homozygous (y^+ w^-; e-CHACR-e) F0 females were crossed to hemizygous y^- w^a MCR-GFP males to generate trans-heterozygous F1 y^- w^a MCR-GFP/y^+ w^-; e-CHACR-e/+ master females (Fig. S9B). F1 master females were then crossed to y^+; e^+ males, and transmission frequencies for GFP, DsRed, as well as eye and body color phenotypes were scored in F2 progeny to assess e-CHACR-e copying on the third chromosome as well as MCR-GFP outcomes on both MCR-donor (w^a) and MCR-receiver (w^-) X chromosomes.

With regard to Cas9 targeting, gRNA-C3 carried by the e-CHACR-e element was fully effective at inactivating Cas9 as substantiated by 100% of F2 female progeny exhibiting a yellow^+ phenotype (Fig. S9E). This observation indicated that the y^+ allele inherited from the wild-type F1 male parent always remained intact (i.e., unmutated by Cas9) in female F2 progeny when present in-trans to the presumably mutated Cas9 transgene carried on the MCR-GFP element. In contrast, nearly all F2 female progeny exhibited a y^- phenotype in control experiments in which F1 master females carrying an intact MCR element were crossed to y^+ males (Fig. 1D).
Surprisingly, while e-CHACR-e demonstrated high efficiency in targeting Cas9, in five independently-isolated lines, this active genetic element was only transmitted at Mendelian frequencies. The same gRNA-e1, however, sustained moderate copying in split-drive experiments in which one element carried two gRNAs, one for copying itself and another for propagating a separate Mendelian element that expresses Cas9 (López Del Amo et al., 2019). Similarly, overall inheritance of the MCR-GFP element (i.e., GFP⁺ progeny) was markedly reduced (Fig. S9C,C’) relative to control MCR-GFP experiments (Fig. 1D; Fig. S2B). In F₂ females, the overall inheritance of the MCR-GFP element was ~50% (compared to 85% in controls - Fig 1C). This transmission rate was not just the result of simple Mendelian segregation, however, since copying to the receiver chromosome did occur (Fig. S9D), albeit at a substantially reduced rate (15.2%) in comparison to that observed in control MCR-GFP crosses (Fig. 1D; Fig. S2B). The MCR-GFP element was similarly copied onto the receiver chromosome at modest frequencies in F₂ males (11.2%) (Fig. S9D’), although this copying accounted for only ~1/3 of the y⁻ mutants recovered on the receiver chromosome (Figs. S9D’,E’). The remainder of y⁻ mutants (~25% of total receiver events) presumably carry NHEJ alleles, which arise at frequencies comparable to those recovered in baseline MCR-GFP copying experiments (Fig. 1D).

The significantly higher frequency of GFP transmission in females compared to males (Fig. S9C,C’) was largely the result of preferential inheritance in males of the w⁻ target chromosome over that of the MCR-GFP wᵃ chromosome, which occurred at a ratio of more than 2:1 (Fig. S9F). In females, however, w⁻ and wᵃ chromosomes were inherited in expected equal Mendelian proportions (Fig. S9E). The pronounced skew in male chromosomal inheritance suggests that nearly half of the MCR-bearing chromosomes carry some appreciable handicap as a result of cleavage by the e-CHACR-e element. Consistent with this hypothesis, a significant fraction of wᵃ donor chromosomes (14-15%) lacked the GFP marker associated with the intact MCR element (Fig. S9D,D’), which greatly exceeded the expected percentage due to recombination between the y and w loci (separated by only 1.5 cM). We established 17 independent balanced lines from
individual F\textsubscript{2} females carrying GFP\textsuperscript{−} MCR donor chromosomes and 10 of these chromosomes (59\%) were indeed male lethal. Consistent with the Cas9/gRNA-C1 induced damage being localized to the chromosomal region carrying the MCR-GFP element, a duplication covering the tip of the X chromosome (Fig. S12A), which includes the \textit{y} locus, rescued male lethality in all 10 lines. These DNA lesions did not extend to the neighboring centromere proximal \textit{achaete} locus, however, as control crosses to the viable \textit{ac\textsuperscript{d}} allele revealed (Table S1). Thus, e-CHACR-e can induce more than simple NHEJ-mediated mutagenesis of the Cas9 target site, potentially generating larger lesions such as deletions encompassing the GFP marker and at least one essential gene located distal to the MCR insertion site at the \textit{y} locus (see Discussion for more in depth consideration of these results).

When e-CHACR-e targeted Cas9 carried by the MCR-GFP element, we again observed Mendelian inheritance of the e-CHACR-e element, reflecting the inability of gRNA-e1 to sustain e-CHACR-e copying in the context of a full MCR-GFP drive, as we observed above for a static Cas9 source. In addition, e-CHACR-e did not generate NHEJ induced mutations in any of the F\textsubscript{2} progeny we sequenced. However, copying could be restored to previously reported levels (López Del Amo et al., 2019) by crossing e-CHACR-e to a recoded, cleavage-resistant version of Cas9 (Fig. S10D-F). This Cas9\textsuperscript{*} transgene, which is immune to cleavage by gRNA-C3, was inserted at the same site in the \textit{w} locus as the cleavage sensitive Cas9 form (Fig. S10B,C). These data confirm that e-CHACR-e is competent to copy, but that premature inactivation of Cas9 greatly reduces drive mediated by inefficient gRNA-e1.

As further evidence of autosomal sites being competent to sustain both copying and Cas9 inactivation, e-CHACR-k, inserted into wing-specific cis-regulatory sequences of the essential \textit{kni} locus (Fig. S11A), copied at intermediate levels (89.5\% in females and 87.0\% in males; Fig. S11C,C'), comparable to that achieved by the MCR-GFP full drive (~85\%; Fig 1D). e-CHACR-k was also highly efficient in inactivating Cas9 carried by the MCR-GFP element (Fig. S11B,E).
The X-linked e-CHACR-wR copies efficiently, inactivating and greatly reducing transmission of a full gene-drive element

The next neutralizing element we pitted against the MCR-GFP drive is identical to e-CHACR-wG (Fig. S3A) except that it carries DsRed (e-CHACR-wR, Fig. 2A) in place of eGFP to distinguish it from the GFP-marked MCR. As a control, we first tested several independently derived e-CHACR-wR lines against a static GFP-marked Cas9 transgene carried on the third chromosome (Fig. S5A,B) paralleling experiments described in Fig. S3 for e-CHACR-wG. We associated the donor eCHACR-wR element with a y− allele in order to distinguish between copying events versus biased inheritance of the donor chromosome. In these experiments, the e-CHACR-wR efficiently inactivated Cas9 (e.g., eliminated eye color mosaicism due to somatic mutagenesis of the w locus in F2 females) and copied itself to the y+ target chromosome (Fig. S5C-D’) at similar frequencies to the GFP-marked version of this element (Fig. S3C,C’). One notable difference between targeting the Cas9 at the X-linked y locus versus the third chromosome Cas9 source, however, was that the latter did not result in a sex-specific bias in F2 progeny (Fig. S5E, compare to Fig. S8B). As considered further in the discussion, this difference in the frequency of handicapping receiver chromosomes may reflect the number of distinct gRNA-mediated cleavage events sustained by the same chromatid.

Having established that e-CHACR-wR copied itself and inactivated Cas9 comparably to e-CHACR-wG, we next assessed its ability to forestall a fully active MCR-GFP element. In the first round of these experiments (Fig. 2B), the e-CHACR-wR chromosome was marked with a viable recessive allele of the immediately adjacent achaete-scute locus (ac4, ~9 kb away from the y gene) which results in a highly penetrant loss of innervated bristles. The opposing MCR-GFP bearing chromosome was associated with a wild-type ac+ allele. As observed for e-CHACR-wG (Fig. S3), F1 master females of the genotype MCR-GFP, ac+/ac4, e-CHACR-wR displayed mosaic eye pigmentation with large white patches. Similarly, paralleling the efficient copying of its GFP-marked counterpart (Fig. S3), e-CHACR-wR was transmitted to nearly all progeny (Fig. 2C,C’), including those carrying the wild-type ac+ allele linked to the targeted MCR-GFP element. Recapitulating
the Cas9 neutralizing effect of e-CHACR-e (Fig. S9), e-CHACR-wR reduced MCR-GFP copying to the $ac^4$ marked chromosome by >5-fold (12.5% of receivers in males, Fig. 2D,D’) while having little effect on the rate of generating NHEJ alleles (21% of receivers) in F$_2$ progeny (Fig. 2E’). Consistent with this profound reduction in Cas9 activity, no F$_2$ female progeny displayed a $y^-$ phenotype (Fig. 2E), and fewer than 1% of F$_2$ progeny carrying the GFP marker displayed $w^-$ mosaicism - a more readily detected measure of residual Cas9 activity. We also observed a frequency bias in favor of the $ac^4$ marked chromosome over the $ac^+$ chromosome in F$_2$ male progeny (~1.2:1 - Fig. 2F), but not in F$_2$ females (Fig. 2E), despite the fact that the mutant $ac^4$ allele incurs a significant fitness cost relative to the wild-type allele (Fig. S8G). Since the $ac^4$ allele carries a fitness cost, we also generated a reverse configuration of this experiment in which the F$_1$ master females carried an $ac^4$ marked MCR-GFP chromosome while the e-CHACR-wR chromosome carried the wild-type $ac^+$ allele. This marker inversion produced similar results (Fig. S7), albeit with a slightly increased bias for the $ac^+$ chromosome (~1:4:1), as might be expected given the fitness advantage associate with the $ac^+$ allele. In these crosses we also observed an over-abundance of the GFP negative MCR-GFP chromosome class (GFP$^-$, $ac^+$) (8.6-9.8%), which was somewhat less than that noted above for the e-CHACR-e experiments (13.8-14.7%).

e-CHACR-wR spreads to completion in competition with the MCR-GFP gene-drive in population cages

Results from single generation pair-mating crosses revealed that e-CHACRs carrying either of two gRNAs targeting Cas9 were highly effective in eliminating Cas9 activity. In addition, e-CHACR-wR inserted into the $w$ locus demonstrated highly efficient drive when confronting either a stationary Cas9 transgene or Cas9 carried by the MCR-GFP gene-drive element inserted at $y$ (Fig. 2; Figs. S3,S4,S5,S7). Since these performance characteristics suggested that the DsRed marked e-CHACR-wR should drive well in population cages when challenging the MCR-GFP element, we seeded cages in triplicate
with homozygous e-CHACR-wR and MCR elements each at 50%. We carried out these drive trials for 12 generations (Fig. 7A) and observed in all three cages that the e-CHACR drove to completion in 9 generations (red curve in Fig. 7A). In addition, we observed a transient appearance and then disappearance (after 8 generations) of a population of DsRed⁺, GFP⁺ females that exhibited mosaic eye phenotypes (yellow curve in Fig. 7A), indicative of Cas9 activity in the generation when the two elements first come into contact. Crosses sampling DsRed⁺, GFP⁺ males and females from generations 9 and 10 revealed that all tested MCR-GFP alleles had lost Cas9 activity, as judged by the absence of female progeny with mosaic eyes (Fig. S22B,C). These results are consistent with the single-pair mating experiments between e-CHACR-wR and the MCR-GFP element, which demonstrated efficient Cas9 inactivation and e-CHACR copying (Fig. 2). In addition, we crossed DsRed⁺, GFP⁺ females from generations 9 and 10 to y⁺ w⁻ males (Fig. S22C) and all progeny carried the DsRed marker indicating that the e-CHACR-wR had achieved 100% homozygous introgression (Fig. S22C’).

Another multi-generational trend of the cage experiments was that following an initial expected increase, the fraction of individuals carrying the MCR-GFP element peaked at generation 7, and then subsequently displayed a precipitous decline (green curve in Fig. 7A). Although a similar trend of initial surge followed by waning was also observed in control crosses of the MCR-GFP element to y⁻ mutants (which have an intact gRNA-y1 cleavage site) (Fig. S22A), the kinetics differed significantly with an earlier peak time (generation 5) followed by a more gradual loss of the MCR-GFP element in those experiments. These later observations suggest that the MCR-GFP element imposes a fitness cost on its own (hence its gradual loss in control experiments). However, when the MCR-GFP is confronted with an e-CHACR element, potentially ensuing DNA damage associated with incomplete repair of the Cas9 transgene on the MCR-GFP donor chromosome may accelerate clearance of the MCR-GFP element.

We generated a mathematical model based on the data collected from the parallel single-generation pair-mating crosses and optimized parameters based on observed time-series data from the cage trials (Fig. Smod1 in Data S1, Fig. 7B - solid lines). These initial
values were then used to run simulations, shown as arrays of pale-colored lines, matching the schemes for the different displayed phenotypes (Fig. 7A - translucent lines). Overall, the set of optimized parameters (Table Smod4 in Data S1) is consistent with experimental values for the frequencies of different phenotypic classes observed in the pair-mating crosses. We were also able to infer parameters revealing informative deviations from assumptions of random mating between all genotypes. In particular, the modeling indicated a relatively high degree of assortative mating within groups having shared eye pigmentation (i.e., \(w^+\) females preferentially mating with \(w^+\) males over \(w^-\) males). Overall, modeling simulations derived from these assumptions matched well with the observed efficient experimental performance of the e-CHACR (Fig. 7B).
Distilled assessment of e-CHACR action

The four e-CHACRs, tested in combination with different Cas9 sources, provided insights into factors influencing their efficacy and activities. Most importantly, all e-CHACRs efficiently inactivated Cas9 (99-100%), deploying either of two gRNAs that target sites encoding amino acids essential to Cas9 catalytic activity. The ability of e-CHACRs to copy themselves varied. e-CHACR-e, which copies with the least efficient gRNA-e1 at the autosomal ebony locus was only inherited at Mendelian frequencies, but regained its modest drive capacity when paired with a cleavage-resistant Cas9 target source. Another autosomal element, e-CHACR-k, copied at intermediate frequencies comparable to the MCR-GFP gene-drive. At the other end of the spectrum, e-CHACR-w (G or R), which carry the same highly-efficient gRNA-w2 targeting the w locus, displayed strong super-Mendelian inheritance. e-CHACR-wR also copied nearly perfectly when combined with the MCR-GFP element, however, drive of the MCR-GFP element was markedly reduced (~5-fold), consistent with gRNA-y1 being less effective at sustaining drive than gRNA-w2. Several factors may contribute to these differences in copying efficiency including: gRNA cleavage efficiency, genomic location of drive insertion, overall size of the drive element, developmental timing of Cas9 expression, epigenetic influences, or differences in the off-rates of gRNAs bound to Cas9. These results suggest that by mutating and inactivating Cas9, e-CHACRs can reduce MCR copying in the first generation and efficiently prevent any further spread of that gene-drive element in subsequent generations.

e-CHACRs can induce two different forms of damage to the target chromosome:
When e-CHACR-wR and the Cas9 source were located on different chromosomes, the Cas9 chromosome was inherited at approximately Mendelian frequencies in both male and female progeny. However, when e-CHACR-wG, which is inserted at exactly the same genomic location and is copied using the same gRNA, was combined with a Cas9 source located on the same chromosome, a nearly 2:1 bias was observed in favor of donor versus receiver chromosome transmission to male progeny. Nearly equal transmission of the two chromosomes, however, was observed in females. This pronounced male-specific deficit of target chromosome transmission suggests that receiver chromosomes sustain some type of cleavage associated damage that selectively handicaps their
inheritance in males. Also, targeting of a full-drive element from an e-CHACR located on a different chromosome can lead to significant biases in transmission of donor versus receiver chromosomes and, in the case of e-CHACR-e, to an abundant class (~15%) of GFP− MCR donor chromosomes, more than half of which were male-lethal. The elevated frequencies of generating damaged target chromosomes when they are cut twice but not once by same e-CHACR-wG element suggests that a contributing factor may be whether the receiver chromosome is cut only once (resulting in Mendelian inheritance of both donor and receiver chromosomes) or twice (resulting in biased inheritance of the donor chromosome).

At least two different forms of e-CHACR induced male-biased inheritance can be distinguished, the first being when the e-CHACR targets the same chromosome for both its copying and for Cas9 inactivation (e.g., e-CHACR-wG/R). Double-cutting ERACR elements also create a pronounced male bias favoring donor chromosome transmission, which reflects the generation of an abundant class of male-lethal alleles. In the case of e-CHACR-wG/R, however, we found no evidence of it creating male lethal alleles when neutralizing a static X-linked source of Cas9 (i.e., in a double-cut scenario). We recovered individual females containing receiver Cas9 alleles targeted by e-CHACR-wG and indeed, all tested examples proved fully viable in subsequent male progeny (Fig. S3D,E). The basis for this form of biased inheritance, which appears to involve some form of transient, but resolvable chromosome handicap, remains to be determined. One hypothesis to pursue in future experiments is that early post-fertilization repair events may benefit from the presence of an intact chromosome homolog, perhaps serving a scaffolding function, to achieve optimal efficiency.

The second type of male bias includes the generation of receiver alleles that are male lethal, as can occur in the context of autosomal e-CHACR-e acting on Cas9 carried by the MCR-GFP element inserted at the X-linked y locus. Cleavage of both copies of a chromosome at nearly the same site (e.g., gRNA-y1 of the MCR targeting the y locus and gRNA-C3 of the e-CHACR targeting the MCR-GFP element) may result in preferential repair of the MCR receiver chromosome carrying the native y allele mediated by flanking homology sequences present on the MCR-GFP allele (resulting in MCR-GFP copying).
In contrast, the donor MCR-GFP allele has no equivalent neighboring homology template from which to repair cleavage in the Cas9 transgene. The simultaneous cleavage of both donor and receiver chromosomes may result in interrupted copying events on the MCR-GFP donor allele, which are then resolved as deletions, as can occur in aborted transposon copying (Delattre et al., 1995). In support of the biased allele repair hypothesis, when e-CHACR-e targeted a static Cas9 source inserted at the w locus in F1 master females, the Cas9 element was inherited at approximately Mendelian frequencies in both F2 males and females (Fig. S10B,C). In this scenario, the Cas9 transgene is inserted at w on the X chromosome (i.e., 1.5 cM from the y insertion site of the MCR-GFP gene-drive construct, Fig. S10A), but is in-trans to an intact y homolog.

Another potential event, leading to viable GFP− MCR outcomes may result from partial pairing between a gRNA and its genomic target sequence that leads to copying of a portion of wild-type sequences matching the gRNA transgene onto the MCR donor chromosome. This relatively low frequency phenomenon was also observed in the ERACR studies (Data S2) and by others (Pham et al., 2019). It is also possible that basal meiotic recombination rates between y and w are augmented by cutting both homologs leading to production of a greater fraction of GFP− wα recombinants in the experiment presented in Fig. S9D,D’, perhaps mediated by non-homology based joining of chromosome homologs (Brunner et al., 2019). The order of the Cas9 and eGFP transgenes relative to the chromosomal orientation (Fig. S9A), however, does not readily explain the generation of this sizeable proportion of GFP+ w− progeny by such a simple mechanism of illicit recombination, and no significant recombination distortion was observed with the MCR-GFP element alone. Further analysis of individual molecular events will be required to resolve the genesis of these different classes of potential outcomes.

Non-Gaussian distribution of copying events: A defining element of our experimental approach was conducting many parallel pair-wise matings rather than fewer mass matings. Sampling of germline events from single individuals can reveal patterns of inheritance that would be obscured by bulk crosses. Indeed, frequency binned histograms
from the gene-drive and e-CHACR/MCR-GFP crosses revealed discrete patterns of transmission rather than a normal Gaussian distribution centered around the mean (Fig. S1C-D”; Fig. S2C-C”). The most abundant class of events in these crosses was inheritance of the active genetic element by 100% of the progeny. When binned in 5% intervals, both the MCR and e-CHACR crosses exhibited a profound dip in the 90-95% inheritance bins, then another peak around 80-85% transmission, followed by another dip and then a final peak between 65-70%. These discrete three peaks of super-Mendelian inheritance, reminiscent of a Poisson-like distribution, could reflect potential drive outcomes in a small number of germline precursor cells, which has been estimated to be ~3-4 cells at the 128-cell blastoderm stage embryo (Lindsley et al., 2016). One model (gauntlet model) to account for such a pattern of inheritance would be that NHEJ mutagenesis of the gRNA target has a low random probability of happening at each cell division in the pre-blastoderm embryo. If such so-called resistant alleles are not generated before germline cells are specified (e.g., by the end of the blastoderm stage), then the likelihood of gene-drive copying is very high in all descendent germline precursor cells that have not undergone NHEJ. Consistent with the gauntlet model, when a full gene-drive element was challenged by two different e-CHACR elements, the frequency of copying events was greatly reduced (e.g., by ~7-fold) while the frequency of generating NHEJ induced mutations at the y locus remained virtually unaltered. These divergent outcomes are consistent with NHEJ events being generated at an early time point (e.g., in the blastoderm embryo) when Cas9 protein is still abundant, with copying events taking place at a later stage, by which time Cas9 protein has become limiting. Alternatively, a competition model could apply wherein the decision to undergo NHEJ or copying takes place stochastically at approximately the 128-cell stage. Future experiments including single cell sequencing from developing gonads should help resolve the basis for the observed non-Gaussian inheritance patterns.

Hierarchies of gRNA copying efficiency: One hypothesis emerging from these studies is that different gRNAs have a range of drive potential. Although all e-CHACRs tested in this study are highly efficient in mutating and inactivating Cas9 (e.g., ~99%), they copy with varying efficiencies (Fig. 8C). e-CHACRs driven by gRNA-w1 copied with the highest
efficiencies (95-99%), e-CHACR-k (gRNA-k1) drives at intermediate frequencies similar to that of the MCR-GFP (driven by gRNA-y1). The weakest copying element, e-CHACR-e, failed to demonstrate any detectable drive activity when combined with a cleavable Cas9 target, despite displaying modest drive when combined with a cleavage resistant form of Cas9, similar to that observed in split-drive experiments (López Del Amo et al., 2019). Although the basis for gRNA efficacy remains to be determined, it is possible that if the copying gRNA (e.g., gRNA-e1) is inefficient in directing cleavage, the source of Cas9 becomes mutated (e.g., by gRNAs-C1/C3) before copying can occur. Consistent with this hypothesis of gRNA drive hierarchies, e-CHACR-wR copied with very high efficiency in the same crosses in which the MCR-GFP element copied at greatly reduced frequencies. Since several studies have suggested that the cleavage/copying activities of drive elements can vary based on genetic background (Champer et al., 2017; Drury et al., 2017), this question deserves further scrutiny as an e-CHACR driving through a genetically non-homogeneous population might encounter genotypes that could render its copying less effective.

**e-CHACRs can drive efficiently in population cages:** The strong performance characteristics of e-CHACR-w(G/R) elements summarized above suggested that they would effectively compete with the MCR-GFP gene-drive in population cages. Indeed, the e-CHACR-wR element rapidly spread to full introgression after ~ 9 generations and, as expected, inactivated Cas9 in the process. The ability of e-CHACR-wR to achieve 100% homozygosity in all cages is consistent with its high rate of drive in pair-mating experiments, and also with the lack of maternal transmission of Cas9/gRNA complexes manifested as shadow drive. Since the same gRNA-w1 carried by e-CHACR-wR was observed to produce a high frequency of drive-resistant NHEJ alleles when transmitted together with Cas9 maternally for two generations as a split-drive system (Lopez Del Amo et al., 2020), the ability of e-CHACRs to abrogate this maternal activity is likely to be a primary factor in attaining complete introgression.

Another notable feature of the population experiments is that in all three cages, the prevalence of the MCR-GFP element initially increased, but then fell precipitously. A
similar, although more gradual decline in MCR-GFP frequency was also observed in control experiments where the MCR-GFP element was crossed to a \( y^- \) point mutant strain. Modeling of these MCR-GFP decay dynamics suggests that two processes may contribute to its elimination. First, since a gradual decrease is also observed in control crosses, the MCR-GFP element may carry a fitness cost, perhaps associated with Cas9 activity. Second, given that a fraction of the \( w^a \) marked MCR-GFP donor chromosomes lack GFP and are male lethal or experience other handicaps, a proportion of the GFP\(^+\) chromosomes may experience strong negative selection. This possibility is consistent with the observed deficit in male receiver chromosome transmission and the pronounced female over male gender bias.

In summary, the combined performance of the e-CHACR to copy itself and inactivate Cas9, together with potential transient elimination of functional Cas9-bearing drive elements and the generation of damaged MCR-GFP alleles, result in a robust system for neutralizing the gene-drive. If one associated beneficial factors (e.g., anti-malarial effector molecules) with an e-CHACR, such elements should drive these beneficial traits to a high level of introgression in the process of neutralizing the drive element. A drive element could carry one set of effectors and the e-CHACR a second complimentary or reinforcing set. In such a scenario, the sequential release of a full gene-drive followed by neutralization with an e-CHACR offers a promising approach to take advantage of low-threshold CRISPR-based drive systems to deliver multiple beneficial traits and halt the further spread of those elements beyond the intended target region.
We modeled the population dynamics of the e-CHACR double-locus gene drive remediation system, under laboratory cage conditions, assuming discrete generations and randomly mixing populations, with the exception of assortative mating preferences of $y^+$ females for $y^+$ males. Model fitting was carried out using a likelihood-based Markov chain Monte Carlo (MCMC) algorithm. Here, we describe the likelihood calculation for the e-CHACR system, both loci of which are located on the X chromosome, one of which is being driven into a locus occupied by a X-linked homing system initially fixed in the laboratory population.

We modeled the X-linked e-CHACR system by considering two unlinked loci on the X chromosome. At both loci, the default wild-type allele is denoted by “W,” with its position in the genotype string indicating either locus one or locus two. At the first locus, the homing allele is denoted by “H”. At the second locus, we have the e-CHACR element, denoted by “E.” At both loci are the possibility of in-frame resistance alleles, “R,” and out-of-frame resistance alleles, “B.” For males, we denote the Y chromosome as “YY”, representing the two loci that we follow on the X chromosome. Consequently, there are 20 unique male genotypes and 210 unique female genotypes. We denote the proportion of organisms having each genotype at the $k$th generation by $p_k^x$, where $x$ denotes one of the 230 genotypes.

Given the large number of possible mating pairs, it is not feasible to show the complete equations for the next generation genotype frequencies. Instead, we will define a set of simple rules that dictate how offspring are determined in a complete set of distinct classes of mating. First, if the mother does not have the H allele, there is no Cas9 available, and any allele present in the parental genotypes is inherited by the offspring in a Mendelian fashion.

Second, if the mother has the H allele but there is no E allele present, then Mendelian inheritance rules apply at the H locus, with the exception that, for HW heterozygotes,
proportion, \( c^H \), of the H alleles are cleaved, while a proportion, \( 1 - c^H \), remain as H alleles. Of those that are cleaved, a proportion, \( p_{HDR}^H \), are subject to accurate homology-directed repair (HDR) and and become H alleles, while a proportion \( 1 - p_{HDR}^H \), become resistant alleles. Of those that become resistant alleles, a proportion, \( p_{RES}^H \), of these become in-frame, cost-free resistant (R) alleles, while the remainder, \( 1 - p_{RES}^H \), become out-of-frame or otherwise costly resistant (broken, B) alleles. The resulting alleles segregate in a Mendelian fashion.

Third, if the mother has both the H and E alleles present, then homing of both elements is a possibility. If the H allele is present at the first locus, and the E allele at the second locus, then Mendelian inheritance rules apply at the E locus, with the exception that, for EW heterozygotes, W alleles are cleaved and a proportion, \( p_{HDR}^E \), are subject to accurate HDR and become E alleles, while a proportion \( 1 - p_{HDR}^E \), become resistant alleles. Of those that become resistant alleles, a proportion, \( p_{RES}^E \), of these become in-frame, cost-free resistant (R) alleles, while the remainder, \( 1 - p_{RES}^E \), become out-of-frame or otherwise costly resistant (broken, B) alleles at the second locus. The resulting alleles segregate in a Mendelian fashion.

Considering the H allele at the first locus (in the presence of the E allele), Mendelian inheritance rules again apply, with the exception that, for HW heterozygotes, a proportion, \( c^{HE} \), of the H alleles are cleaved, while a proportion, \( 1 - c^{HE} \), remain as H alleles. Of those that are cleaved, a proportion, \( p_{HDR}^{HE} \), of cleaved H alleles are subject to accurate HDR and become E alleles, while a proportion \( 1 - p_{HDR}^{HE} \), become resistant alleles. Of those that become resistant alleles, a proportion, \( p_{RES}^{HE} \), of these become R alleles, while the remainder, \( 1 - p_{RES}^{HE} \), become B at the first locus. Following this, the e-CHACR element (E) targets the homing allele (H) for cleavage. As this is implemented after the H element homes, new H alleles are also available for cleavage by the e-CHACR element. A proportion, \( p_{HDR}^{EH} \), of H alleles are cleaved by the e-CHACR element, with a proportion, \( p_{RES}^{EH} \), of the cleaved H alleles becoming R alleles and the remainder, \( 1 - p_{RES}^{EH} \), becoming B alleles at the first locus.
Dynamics are significantly simpler in males due to the fact that both loci are X-linked, and in males, there is only one X chromosome. The only departure from X-linked Mendelian inheritance rules are that, for males that are hemizygous for the H and E alleles (i.e., genotype HYEY), a proportion, \( p^{EH}_{HDR} \), of H alleles are cleaved by the e-CHACR element, with a proportion, \( p^{EH}_{RES} \), of the cleaved H alleles becoming R alleles and the remainder, \( 1 - p^{EH}_{RES} \), becoming B alleles at the first locus, as per the case for cleavage of the homing locus by the e-CHACR element in females.

In addition to the parameters describing inheritance biases due to the action of the homing and e-CHACR constructs, we need to account for fitness costs associated with having certain alleles and allele-induced phenotypes: i) possession of a functional Cas9 element is associated with a fitness reduction denoted by \( s_{Cas9} \) for each Cas9 element, ii) out-of-frame resistant alleles are associated with a fitness cost of \( s_B \) per B allele, and iii) the white- eye phenotype, displayed by any organism lacking both copies of the wild-type (W) allele at the second (e-CHACR) locus, is associated with a fitness cost of \( s_{white,f} \) in females and \( s_{white,m} \) in males. Given the discrete generation nature of the laboratory gene drive experiments, we applied these fitness costs to reductions in female fecundity and male mating competitiveness in our model, as these variables reduce the contribution of a given genotype to next generation offspring proportionately to the fitness cost. Fitness costs are combined here in an additive fashion, with a maximum value of 1 - e.g. the fitness of an organism having the genotype HWBW is \( 1 - max((s_{Cas9} + s_B),1) \).

The final component of our model accounts for assortative mating related to the white eye color phenotype. The e-CHACR element is carried at the white locus, resulting in individuals with white- eyes when there are no wild-type alleles at the e-CHACR locus in an organism’s genotype, and white+ eyes otherwise (i.e. when at least one wild-type allele is present at the e-CHACR locus). Females having the white+ eye color phenotype are more likely to mate with males having the white+ phenotype; however, females having the white- phenotype do not have this mating preference. To account for this, we upweight crosses between white+ females and white+ males by a factor, \( a_W/m_W \), where \( a_W \) represents the fraction by which white+ females increase their mating with white- males.
and $m_w$ represents a normalizing term given by $a_w \left( p_k^{WYY} \right) + \left( p_k^{xYY} + p_k^{xRYY} + p_k^{xBY} \right)$, where $x \in \{H, R, B, W\}$. We also downweight crosses between white+ females and white- males by a factor, $1/m_w$.

These considerations (inheritance biases, fitness costs and assortative mating) allow us to calculate the expected genotype frequencies in the next generation. The next generation genotype frequencies are normalized to ensure they sum to 1.

The likelihood of the population cage data was calculated by assuming a multinomial distribution of individuals having each sex and marker phenotype, and by using the model predictions to generate expected proportions for each set of parameter values. I.e., by calculating the log likelihood,

$$\log L(\theta) \propto \sum_{i=1}^{3} \sum_{k=1}^{n_i} \left( \sum_{P \in \{P_F\}} N_{f,i,k}^P \cdot \log(p_{f,k}^{P}(\theta)) + \sum_{P \in \{P_M\}} N_{m,i,k}^P \cdot \log(p_{m,k}^{P}(\theta)) \right).$$

Here, $N_{f,i,k}^P$ is the number of females at generation $k$ in experiment $i$ having phenotype $P \in \{P_F\}$, and $N_{m,i,k}^P$ is the number of males at generation $k$ in experiment $i$ having phenotype $P \in \{P_M\}$. Possible phenotypes are listed in Table Smod1, genotype-to-phenotype mappings for females are provided in Table Smod2, and genotype-to-phenotype mappings for males are provided in Table Smod3. The $i$th experiment is run for $n_i$ generations, and expected phenotype frequencies in females, $p_{f,k}^{P}$, and males, $p_{m,k}^{P}$, at generation $k$ are dependent on the model parameters, $\theta = \{c^H, p_{HDR}^H, p_{RES}^H, p_{HDR}^E, p_{RES}^E, c^{HE}, p_{HDR}^{HE}, p_{RES}^{HE}, s_{Cas9}, s_B, s_{white,f}, s_{white,m}, a_w\}$, as defined earlier.

Experiments were seeded with 160 individuals – 40 HHW females, 40 WEWE females, 40 HWYY males, and 40 WEYY males. We used a Differential Evolution Makov chain Monte Carlo (MCMC) sampling procedure from the R package “BayesianTools” to estimate each of the model parameters, including 95% credible intervals. The fitting procedure was run for two million iterations, with fitted parameters listed in Table Smod4 and resulting model fits depicted in Fig. Smod1.
A stochastic version of the model was implemented by assuming a population size equal to the mean experimental population size at each generation, and by sampling the number of individuals having each genotype at the next generation according to a multinomial distribution with model-predicted genotype frequencies. This was used to generate the stochastic trajectories depicted in Fig. 7.
Figure Smod1. Cage trials and model fit for e-CHACR females and males. Observed and predicted dynamics of the e-CHACR gene drive remediation system for the four predominant marker phenotypes. Population cage experiments were set up with 160 individuals – 40 HWHW females, 40 WEWE females, 40 HWYY males, and 40 WEYY males – i.e. with half of the females and males having the e-CHACR construct only, and half of the females and males having the MCR construct only. Population counts were monitored over 14 generations. Results from these experiments are shown as solid blue lines, with fitted model predictions shown as dashed magenta lines. Each marker phenotype is known to be produced by certain genotypes, which are listed in Table Smod2 for females and Table Smod3 for males. Observed data are consistent with the parameter values listed in Table Smod4. The initial decline in W+/DsRed-/GFP+ and W-/DsRed+/GFP- individuals reflects decline in the number of individuals having the e-CHACR or MCR construct, but not both. Coincidently, there is an increase in DsRed+/GFP+ individuals that have both constructs. The mosaic phenotype (W?) also increases in females as the e-CHACR and MCR constructs begin to co-occur. Mosaics are not possible in males because they have only one X chromosome. Once both the e-CHACR and MCR constructs are well established in the population (around generations 8-10), the MCR element begins to drop out, signaled by a decline in the GFP+ marker phenotype, due to the action of the e-CHACR construct and the inherent fitness cost of the MCR construct. At the same time, there is an increase in individuals having only the e-CHACR construct, signaled by an increase in the DsRed+/GFP- marker phenotype.
<table>
<thead>
<tr>
<th>Triplet signature</th>
<th>White</th>
<th>DsRed</th>
<th>GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-++</td>
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<tr>
<td>?--</td>
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</tr>
</tbody>
</table>

**Table Smod1. Phenotype code designation.** This table lists the possible phenotypes, and their triplet short-form, for the e-CHACR experiments. The MCR element, at the first locus, carries a GFP within the construct. The e-CHACR element, at the second locus, carries a DsRed within the construct. Additionally, the e-CHACR element targets the white locus, so homology-directed repair (HDR) and non-homologous end-joining (NHEJ) instances at that locus will cause the individual to be white.
<table>
<thead>
<tr>
<th>Phenotype observed</th>
<th>Possible female genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>+++</td>
<td>BEWW, BERW, BWWE, BWRE, BEBW</td>
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<td>-++</td>
<td>HEWE, BEWE, HRWE, BRWE, HBWE, BBWE, HEHE, HERE, BEHE, HEWR, HEHR, HERR, BRHE, HEWB, HBHE, HERB, BBHE, BERE, HRRE, BRRE, HBRE, BBRE, BEBE, BEWR, BEHR, BERR, BEBR, BEWB, BEHB, BERB, BBBE</td>
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<td>WRWR, RRWR, WBWR, RBWR, RRWR, RRWB, RBRR, WBWB, RBWB, RBBB</td>
</tr>
</tbody>
</table>

**Table Smod2. Phenotype-to-genotype mappings for females.** Possible female phenotypes are depicted on the left, with corresponding genotypes that give rise to those phenotypes on the right. Genotypes are written as four letter strings, with the first two letters representing the alleles on one X chromosome, and the last two letters representing the alleles on the other. For each X chromosome, the first letter represents the first locus, which hosts the MCR allele, and the second letter represents the second locus, which hosts the e-CHACR allele (e.g., an individual having the genotype BEWW, has one B and one W allele at the first locus, and one E and one W allele at the second locus). The haplotype phasing of this individual is BE/WW. The phenotypes are designated as per Table Smod1, with the inclusion of a “?” in the white signifier that indicates a mosaic female.
<table>
<thead>
<tr>
<th>Phenotype observed</th>
<th>Possible male genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>-++</td>
<td>HEYY, BEYY, HBYY, BBYY</td>
</tr>
<tr>
<td>+--</td>
<td>HWYY, BWYY</td>
</tr>
<tr>
<td>--+</td>
<td>HRYY, BRYY</td>
</tr>
<tr>
<td>-+-</td>
<td>WEYY, REYY, WBYY, RBYY</td>
</tr>
<tr>
<td>+--</td>
<td>WWYY, RWYY</td>
</tr>
<tr>
<td>---</td>
<td>WRYY, RRYY</td>
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</tbody>
</table>

**Table Smod3. Phenotype-to-genotype mappings for males.** Possible male phenotypes are depicted on the left, with corresponding genotypes that give rise to those phenotypes on the right. Genotypes are written as four letter strings, with the first two letters representing the alleles on the X chromosome, and the last two letters representing the Y chromosome. For each X chromosome, the first letter represents the first locus, which hosts the MCR allele, and the second letter represents the second locus, which hosts the e-CHACR allele. E.g. an individual having the genotype HEYY, has an H allele at the first locus of the X chromosome, and an E allele at the second locus of the X chromosome. The phenotypes are designated as per Table Smod1.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Differential Evolution MCMC parameter estimates</th>
</tr>
</thead>
<tbody>
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<td>$c^H$</td>
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<td>$p^H_{HDR}$</td>
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<td>$p^H_{RES}$</td>
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<td>$p^E_{HDR}$</td>
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<td>$p^E_{RES}$</td>
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<tr>
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<td>$p^H_{HDR}$</td>
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<td>$p^E_{HDR}$</td>
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<tr>
<td>$p^E_{RES}$</td>
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<td>$s_{Cas9}$</td>
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<tr>
<td>$s_{white,f}$</td>
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<td>$s_{white,m}$</td>
<td>0.57</td>
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<tr>
<td>$a_w$</td>
<td>0.12</td>
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Table Smod4. Parameter estimates from e-CHACR cage experiments. Parameters were estimated using the model described in the “Model Fitting” section of the manuscript and supporting information. A Differential Evolution MCMC algorithm was used to calculate the maximum a posteriori estimate for each parameter (the value that appears most often in the MCMC chain after burn-in), as well as the 2.5%, 50% and 97.5% quantiles of the posterior distribution for each parameter.
ERACRs efficiently delete and frequently replace a gene-drive element

Categories of genotypes recovered in F_2 male progeny of F_1 master females are summarized schematically in Fig. 3, and also represent the genotypes present in females. We note that in the case of ERACR-min, all male progeny were \( y^- \), while for the ERACR-1 and ERACR-2, which carry rescuing cDNA sequences restoring function of the \( y \) gene, inheritance of the donor allele or accurate copying of ERACR elements to the \( w^a \) chromosome resulted in a dominant \( y^+ \) phenotype in both males and females (Fig. 3B, F_2 male phenotype schematics). The most prominent male genotypes/phenotypes are illustrated from left to right in Fig. 3B: 1) inheritance of the original ERACR element on the \( w^- \) donor chromosome (DsRed\(^+\), \( w^- \); Fig. S14G); 2) inheritance of the ERACR copied onto the \( w^a \) receiver chromosome accomplished by deleting and replacing the MCR-GFP element (DsRed\(^+\), \( w^a \); Fig. 4A-C; Fig. S14A); and 3) retention of the MCR-GFP element on the \( w^a \) chromosome (GFP\(^+\), \( w^a \); Fig. 4D,E; Fig. S14B). A fourth prominent phenotypic class, which was recovered only at significant frequencies in female progeny, lacked both the DsRed and GFP markers (GFP\(^-\), DsRed\(^-\), \( w^a \)). These "double-negative" progeny comprised several different genotypes in which the MCR-GFP element was deleted but the ERACR did not copy, including a predominant category of events that were inviable in males (Fig. 5A-E; Fig. S14C-E). There also were several less frequent outcomes: non-homologous end-joining (NHEJ) events fusing sequences flanking the two ERACR gRNA cut sites (Fig. 5D), incomplete copying of ERACR sequences (Fig. 5C,E), or rare apparently homology-independent fusion of ERACR and MCR-GFP sequences (Fig. 5F,G; Fig. S14F,H-K), which are discussed in more detail below.

A more in-depth analysis of the F_2 phenotypes, genotypes, and associated molecular events in progeny of F_1 ERACR/MCR-GFP master females is presented in Fig. 4, Fig. 5, and Fig. S14. The two metrics bearing most palpably on ERACR performance are the
frequencies of ERACR copying (Fig. 4A-C) versus retention of the MCR-GFP element (Fig. 4D,E). With regard to ERACR inheritance (copying and direct transmission), all three ERACRs performed similarly, with prevalence ranging between 85-89% in males, but notably lower frequencies in females (~ 65%) (Fig. 4A). In crosses where the MCR-GFP drive was linked to the \( w^a \) marker, ERACR elements could be traced to donor \( (w^-) \) versus receiver \( (w^a) \) chromosomes (Fig. 4A'). These crosses revealed that the sex-based differences in ERACR prevalence could be ascribed primarily to preferential transmission of the donor chromosome in males (Fig. 4A', left graph; Fig. 4B), rather than to differences in rates of copying to the receiver chromosome (Fig. 4A', right graph).

In this, and in subsequent panels of Fig. 4, we quantified copying frequencies in two ways. The mean DsRed prevalence per vial among total progeny, separated by gender, is presented in black type on the top row beneath each panel. We also estimated the percentage of converted receiver chromosomes in which the ERACR replaced the MCR-GFP element across all experiments (some of which did not include the \( w^a \) marker; Fig. S13A,B) by multiplying the phenotypic prevalence (Fig. 4A') by receiver allele frequency in Fig. 4B. This estimate of the conversion rate is shown on the bottom row in dark orange type (see orange type estimates also in Fig. 4D, Fig. 5A,F and full data is presented for marked donor crosses in Fig. S15A and for all crosses in Fig. S15B), which ranged from 29.6 – 38.5% in females (avg. = 33.5%, Fig. 4A', right panel).

Sequence analysis of individual ERACR copying events on \( w^a \) marked receiver chromosomes \( (y^+, DsRed^+, GFP^-, w^a) \), revealed perfect junctions at both the 5' gRNA-y2 and 3' gRNA-y3 cleavage sites in all molecularly characterized F2 individuals (Fig. S14A). Similarly, sequences of ERACR donor chromosomes revealed unaltered 5' and 3' junction fragments (Fig. S14G). As discussed further below, relatively rare instances of imperfect copying were also identified for ERACR-1 and ERACR-2 by virtue of individuals exhibiting a \( y^- \) phenotype \( (y^-, DsRed^+, GFP^- w^a) \), indicative of the rescuing \( y \) sequences being thrown out of frame.
Prevalence of the MCR-GFP elements among total F2 progeny (GFP+, w^a) was markedly below the 50% rate expected from random chromosome segregation (range 9.6% - 18.3%; Fig. 4D), and did not display any pronounced sex bias. MCR-GFP retention estimated on female target chromosomes ranged from 25.1% - 37.5% (avg. = 29.2%, Fig. 4D, dark orange type; Fig. S15A). MCR-GFP retaining receiver chromosomes analyzed by sequencing all revealed NHEJ-induced indels at both the gRNA-y2 and gRNA-y3 cleavage site, indicating these two gRNAs were highly efficient in cutting their respective target sites (Fig. 4E; Fig. S14B). Two tested MCR-GFP NHEJ mutants retained approximately full drive activity when challenged with a pristine y^+ w^- test chromosome, as expected from the substantial distance between gRNA-y2 and gRNA-y3 induced NHEJ mutations and the MCR-GFP’s gRNA-y1 cleavage site. (Fig. S16A-C), which would not be expected to disrupt HDR. We conclude that confrontations between the MCR-GFP element and all three ERACRs result in substantial reduction in MCR-GFP transmission and that this effect depends on a combination of ERACR copying, which is not sex-dependent, and an excess of donor ERACR-bearing chromosomes being transmitted in males. The combination of these two effects results in efficient super-Mendelian transmission of ERACRs in male F2 progeny, and moderate enhanced transmission in female progeny.

**ERACRs can damage the target receiver chromosome**

The strong sex-bias observed for ERACR transmission in F2 progeny suggested that a category of receiver X chromosomes might be viable in females but not males. This hypothesis is further supported by the approximately equal transmission of donor and receiver chromosomes in F2 female progeny (Fig. 4B), but a ~2:1 overabundance of donor chromosomes in F2 male progeny (Fig. 4A',B). Additionally, equivalent numbers of male and female progeny inherited the w^- ERACR-bearing donor chromosome (Fig. S17A, gray symbols), indicating that there is no sex-bias *per se* in ERACR transmission. A candidate class of female F2 progeny carrying such male-lethal chromosomes are those with the double-negative fluorescence phenotype (DsRed^-, GFP^-), which was much more abundant among total females (16.6-24.6%) than total males (0.3-5.3%) (Fig. 5A). This
16-34 fold excess of females presumably results from deletion of the MCR-GFP in the absence of ERACR copying. Among receiver chromosomes recovered in F2 females for the different ERACRs, this double-negative phenotype ranged from 34.0 - 49.6% (avg. = 43.1%) (dark orange type in Fig. 5A; Fig. S15A).

**Rescue of damaged double-negative chromosomes in males**

In accord with the hypothesis that the great majority of $y^{-}$, DsRed$^{-}$, GFP$^{-}$, $w^{a}$ receiver chromosome alleles were inviable in a hemizygous condition in males, nearly all balanced stocks derived from such individual F2 double-negative females were shown to carry male-inviable X chromosomes. Since a variety of attempts to PCR amplify sequences spanning the gRNA-y2 and gRNA-y3 cleavage sites in these stocks were unsuccessful and because flies carrying a deletion of the entire $y$ locus are viable, we asked whether these male-lethal chromosomes might have sustained larger scale chromosomal damage as schematized in Fig. 5B. We tested this hypothesis by crossing heterozygous females harboring a specific DsRed$^{-}$, GFP$^{-}$ lethal allele to males carrying Y-linked duplications covering various regions of the X chromosome (Fig. S12B). In several such crosses involving independently isolated male lethal alleles, we observed rescue of male progeny viability by a duplication covering just the tip of the X chromosome. Because the $y$ gene is located very close to the telomere, this rescue suggests that some portion of the distal region of the X chromosome was deleted or damaged in each of these male-lethal lines as a consequence of its cleavage by gRNA-y2 and/or gRNA-y3 at the $y$ locus.

**ERACRs do not sustain shadow-drive**

As described in the main text and Data S1, e-CHACRs can copy efficiently without generating an associated maternal drive phenomenon referred to as “shadow drive” (Guichard et al., 2019). Shadow-drive can occur in F2 individuals descended from F1 Cas9-bearing mothers that inherit a gRNA-based drive element, but not the Cas9 transgene. The maternal perdurance of Cas9/gRNA complexes in these individuals results in substantial super-Mendelian transmission of the drive element in the following F3 generation (Guichard et al., 2019). We tested whether ERACRs that had confronted
an MCR-GFP element in F1 master females and replaced it (i.e., had copied to the \( w^a \) receiver chromosome), could then be transmitted in a super-Mendelian fashion from F2 females (via generate shadow-drive) to the subsequent F3 generation (Fig. S21C,D). Such tests conducted with DsRed\(^+\) F2 females resulted in only standard Mendelian transmission (~50%) of the DsRed\(^+\) marker to F3 progeny (Fig. S21E,F). When Cas9 is expressed by the somewhat leaky germline vasa promoter, under conditions where shadow-drive has been observed (Guichard et al., 2019), there appear to be two phases of Cas9 expression. One is maternally provided and the other depends on zygotic expression. We propose the following hypothesis to explain the absence of shadow drive in both the e-CHACR and ERACR experiments: maternal Cas9 stores from an F1 generation female alone are sufficient for providing germline drive as well as inducing somatic phenotypes (e.g., mosaic eyes, full-body pigmentation phenotypes) for highly efficient gRNAs such as gRNA-w2 that directs cleavage at the white locus. If the second (zygotic) phase of expression is precluded in an F2 generation female (e.g. by the action of an ERACR or e-CHACR), then she will not be able to load her eggs with Cas9 and therefore will exhibit neither shadow-drive nor mosaic somatic phenotypes.

These findings reinforce conclusions from e-CHACR experiments indicating that distinct Cas9 activities mediate first generation gene-drive (i.e., germline-restricted) versus trans-generational shadow-drive (i.e., mediated by maternal Cas9/gRNA stores). These concordant findings suggest that it should be possible, in principle, to develop highly-efficient drive systems that only produce Cas9 at specific stages of development thereby enabling exclusive homology-directed copying of the element.

**Size of the target region does not significantly impact ERACR performance**

The gRNA-y2 and gRNA-y3 cleavage sites are separated by 2.2 kb in the endogenous y locus, which is increased to 11.3 kb by insertion of the 9.1 kb MCR-GFP element at the gRNA-y1 target between these two ERACR gRNA sites (Fig. 3A, Fig. S18A). Also, the MCR-GFP element shares some limited sequence homology with all ERACRs tested. We therefore tested whether the distance between the two gRNA cleavage sites or possible interactions between ERACR and MCR-GFP sequences contributed to the
frequent generation of damaged MCR-GFP deleted (DsRed−, GFP−) chromosomes. We placed ERACR-min or ERACR-2 in-trans to wild-type y target sequences in the presence of a separate source of Cas9 (Fig. S18D). ERACR + Cas9 bearing females were crossed to y+ males carry a closely linked GFP-marked static source of Cas9 inserted at the w locus (Fig. S18D). F1 master females were crossed to y+, w− males, and the F2 progeny were scored for: 1) ERACR prevalence on donor (DsRed+ only) versus receiver (DsRed+, GFP+) chromosomes, 2) failure to copy the ERACR (GFP+ only), and 3) a ±yellow phenotype indicative of potential damage to the receiver chromosome (abundant y−, GFP+ female class) (Fig. S18C-F′, Fig. S19A). We observed that both ERACR-min (Fig. S18E,E′) and ERACR-2 (Fig. S18F,F′) copied at comparable frequencies to ERACRs placed in-trans to the MCR-GFP element (Fig. S19A, compare to Fig. S15B). Also, both ERACRs produced comparable increases in the proportion of inherited donor (DsRed+ only) versus receiver (GFP+ only) chromosomes in males (Fig. S18E′,F′), but not females (Fig. S18E,F). In control experiments (Fig. S18B), F1 females carrying a trans-configuration of static Cas9 elements in y (DsRed-marked) and w (GFP-marked), which were out-crossed to y+, w− males, produced expected Mendelian segregation ratios (Fig. S18C,C′). We conclude that the ability of ERACRs to copy and damage receiver chromosomes is similar for both MCR-GFP and wild-type y alleles and is largely insensitive to target size (11.3 kb versus 2.2 kb) or the presence of limited homology between the target (i.e., MCR-GFP) and the ERACR.

ERACR-induced damage to receiver chromosomes can be attributed primarily to one-sided homology mismatch

As described above, ERACRs induce male-lethal alleles on the receiver chromosome at a substantial frequency (~40%). One possible mechanism underlying this outcome could be that dual cleavage of the receiver chromosome directed by gRNA-y2 and gRNA-y3 destabilizes DNA repair intermediates, leading to loss or damage of local chromosomal sequences. We tested this hypothesis by constructing two single-cut versions of ERACR-2 (Fig. 6A), each carrying only a single gRNA (both gRNAs required for insertion of these elements into the genome were transiently supplied during transgenesis). We also
examined whether these single-cut ERACRs would be capable of copying and if so, how frequently they perform relative to the corresponding double-cut ERACR-2 element.

We constructed single-cut versions of ERACR-2 either carrying only gRNA-y2 (ERACR2-y2), which cleaves between the MCR-GFP element and the tip of the X chromosome, or only gRNA-y3 (ERACR2-y3), which cuts toward the centromere (Fig. 6A). Paralleling experiments in which double-cut (complete) ERACRs were placed in-trans to the MCR-GFP drive element (Fig. 4A,B; Figs. S17A,B), F2 progeny of the single-cut ERACR/MCR-GFP master females (Fig. 6B) displayed similar classes of primary outcomes, albeit with differing proportions of these events (Fig. 6C-E). For example, the frequency of properly deleting and replacing the MCR-GFP element was significantly reduced for both ERACR2-y2 (3.5% in females, 6.3% in males - Fig. 6D) and, most prominently, for ERACR2-y3 (1.6% in females, 1.8% in males - Fig. 6D) relative to the double-cut ERACR-2 rate (16.0% in females, 15.7% in males% - Fig. 4A', right side). Notably, the attenuated rates of single-cut ERACR copying, even when summed, fell short of that achieved by the full ERACR-2 element, suggesting some form of cooperative action between the two gRNAs underlies the higher efficiency of double-cut ERACRs in replacing the MCR-GFP element.

A second prominent F2 phenotypic group generated from single-cut ERACR2-y3/MCR-GFP F1 master females was a double-negative (DsRed\(^–\), GFP\(^–\)) class recovered only in females (Fig. 6C). This non-fluorescent category was observed at similar frequencies in F2 female progeny of single-cut and double-cut crosses: 17% for ERACR2-y3 (Fig. 6C) versus 22.7% for ERACR-2 (Fig. 5A, see also Fig. S15B for proportional display of all phenotypes similar to that shown in Fig. 6C). This double-negative F2 category was entirely absent among single-cut ERACR2-y3 male progeny (Fig. 6C) mirroring its rarity in double-cut ERACR-2 male progeny (1.1%, Fig. 5A). In the case of ERACR2-y2, GFP expression would not be expected to be eliminated following cleavage at y2 distal to the MCR-GFP element. However, for this single-cut element, a similar reduction in the fraction of GFP\(^+\) males relative to females (\(~10\%\) difference) was observed instead, revealing a comparable candidate class of alleles that were viable in females, but not in
males (Fig. 6C). Consistent with the inferred generation of a significant fraction of male lethal alleles by both the single-cut and double-cut ERACRs, we observed a nearly 2-fold excess in the recovery of donor versus receiver chromosomes for all three elements in males, while nearly Mendelian ratios of the chromosomes prevailed among females (Fig. 6E; Fig. 4B). Also, as expected, a substantial excess of female over male progeny was recovered for both single-cut ERACRs (Fig. S17B), comparable to that noted for the double-cut ERACR-2 (Fig. S17A).

We also tested the performance of the single-cut ERACRs when placed in-trans to a wild-type y allele in what we refer to as a "CopyCat" configuration (Xu et al., 2017) (Figs. S18D; S19B), where the gRNA-y2 and gRNA-y3 cut sites are only 2.2. kb apart (compared to 11.3 kb on the MCR-GFP chromosome). In this context, the copying efficiency of the single-cut ERACR2-y2 (19.5-21%; Fig. S19B) more nearly approximated that of the double-cut ERACRs (20.5 - 31%; Fig. S19A). ERACR2-y3 again displayed lower copying rates (5.9-6.0%), which nonetheless exceeded the copying rates observed when tested against the MCR-GFP chromosome by three-fold. Once again, comparable rates of target chromosome damage can be inferred for all three elements based on the greater proportion of receiver chromosomes (GFP+ progeny) recovered in females versus males. We conclude that lethal damage to the target chromosome is not primarily attributable to the target being cut twice at nearby sites, but rather to homology being limited to only a single side of a gRNA-directed cleavage site (the other side being within the single-cut ERACR construct).

ERACRs can serve as partial DNA repair templates

Although the great majority of ERACR-induced DsRed−, GFP− F2 events were recovered in females, rare double-negative male-viable events were also recovered. Molecular analysis of this infrequent category of y−, DsRed−, GFP− male progeny (Fig. 5A′, left graph) revealed two primary outcomes. The first category was deletion of the MCR-GFP element following Cas9 directed cleavage at both the gRNA-y2 and gRNA-y3 cut sites leading to a composite indel at the deletion site (Fig. 5D). Alternatively, partial gene conversion mediated by likely pairing between 17 bp of the recognition portion of gRNA-
y2 and the full target site present in the same 5’->3’ orientation upstream of the MCR-GFP element on the receiver chromosome resulted in retention of a fragment of the gRNA-2 an intact gRNA-3 transgene (Fig. 5E). A similar phenomenon has also been noted in mosquito gene-drive experiments (Pham et al., 2019). The former clean deletion of the MCR-GFP element was observed with all three double-cut ERACRs. The latter partial homology-based copying event, however, was only observed for ERACR-min and ERACR-1. Similarly, we recovered significantly fewer progeny of the double-negative fluorescent class for ERACR-2 relative to ERACR-1 (Fig. 5A,A’), consistent with ERACR-2 carrying both gRNA sequences in reverse orientation to avoid such illegitimate recombination events (Fig. 3A). Finally, the infrequent class of partial copying events depicted in Fig. 5C most likely resulted from misaligned pairing between the non-recoded portion of the y transgene carried by ERACR-1 and 3’ endogenous y sequences downstream of the MCR-GFP element. As would be expected, the prevalence of this phenotypic class was also significantly reduced for ERACR-2 for which the entire rescuing y coding sequences were recoded (Fig. 5A and 5A’, right graph).

Novel ERACR/MCR-GFP recombinant events

Another rare category of events recovered in F2 progeny had a double-positive fluorescence phenotype (y–, DsRed+, GFP+, wA; Fig. 5F,G), indicative of a possible fusion of MCR-GFP and ERACR sequences on the receiver chromosome. Molecular analysis of one such homology-based double-positive MCR-GFP/ERACR-1 fusion event reveals that it is likely to have been the product of misaligned pairing between endogenous y sequences 3’ to the MCR-GFP element and corresponding 5’ non-recoded rescuing y sequences in ERACR-1 (Fig. 5G; Fig. S14F; Fig. S20A’). This illegitimate conversion product relies on offset pairing of the same sequences mediating MCR-GFP deletion in Fig. 5C, albeit in reverse order. As expected, the frequency of double-positive homology-based MCR-GFP/ERACR fusion events is considerably lower for ERACR-min and ERACR-2 constructs, which either lack (ERACR-min) or carry fully-recoded (ERACR-2) rescuing y sequences (Fig. 5F).
Despite their inability to pair erroneously with endogenous 3' y sequences, ERACR-min and ERACR-2 also generated occasional double-positive MCR-GFP fusions (Fig. 5F,G). While no obvious pairing between homologous sequences can account for the junction sequence observed in the case of the MCR-GFP/ERACR-2 fusion event illustrated in Fig. 5G, we note that the eight bases immediately 3' to the junction, which derive from recoded yellow sequences in ERACR-2, match an octomer (asterisk) that lies approximately 1.2 kb upstream within endogenous y sequences. Although this may only be a fortuitous sequence match, it is also possible that a transient pairing between these short sequences occurred, which was then resolved by a sequence-independent ligation event. In the case of ERACR-min, we characterized an illegitimate conversion event between gRNA-y2 carried by the ERACR and MCR-GFP encoded Cas9 3' UTR sequences leading to an ERACR/MCR-GFP fusion with the ERACR upstream of the MCR-GFP element (Fig. S14J). Additional partial ERACR/MCR-GFP repair events suggest disjointed pairing mediated by shared SV40 3' sequences (Figs. S14H,I) as well as a combination of pairing-dependent and independent junctions (Fig. S14K), resulting in rare y⁺, GFP⁺, wᵃ phenotypes. In the case shown in Fig. S14H, an indel was observed at the gRNA-y2 cut site followed by an intact copy of the Cas9 transgene fused to ERACR components including DsRed, and gRNA-y2, gRNAy3, and wild-type sequences retained at the gRNA-y3 site. This autonomously powered hybrid element with a mutated gRNA-y2 site copied at a rate comparable to that of the parent ERACR-2 element when tested against an intact MCR-GFP element (Fig. S16A,C).

Double-positive ERACR/MCR-GFP recombinants exhibit varying drive activity
Molecular analysis of double-positive ERACR/MCR-GFP fusion events summarized above revealed that several of these chimeric elements carried Cas9 as well as all three gRNAs collectively encoded by the two elements (Fig. 5G, Fig. S14F,J), suggesting that these chimeras might possess potential drive activity. We tested four different fusion chimeras (Fig. S20A’) to determine whether they might be able to copy all or part of themselves efficiently to a wild-type chromosome. Master F₁ females carrying the double-positive (y⁺, DsRed⁺, GFP⁺, wᵃ) donor chromosome in-trans to a y⁺, w⁻ receiver chromosome (Fig. S20A) were crossed to y⁺, w⁻ males, and the F₂ progeny were
assessed for transmission frequencies of various phenotypes. For three fusion chimeras (\(y^{-}\) ERACR-min/MCR-GFP, \(y^{-}\) MCR-GFP/ERACR-2, and \(y^{-}\) MCR-GFP/ERACR-1), we observed biased inheritance of the \(w^a\) donor chromosome (~2:1) in males, but not females, and a prominent class (~25%) of non-fluorescent (\(y^{-}\), DsRed\(^-\), GFP\(^-\), \(w^{-}\)) receiver progeny in females, but not in males (Fig. S20B-D). Both of these phenotypes are consistent with male lethal alleles being generated on the target receiver chromosome. In the case of the \(y^{-}\) MCR-GFP/ERACR-1 (Fig. S20B,B') and \(y^{-}\) MCR-GFP/ERACR-2 (Fig. S20C,C') fusions, we also observed moderate copying frequencies of the full hybrid fusion element (\(y^{-}\), DsRed\(^+\), GFP\(^+\), \(w^{-}\)) recovered in both male and female progeny (11-17%), as well as an approximately equal fraction (9-17%) inheriting just the GFP\(^+\) transgene (\(y^{-}\), GFP\(^+\), \(w^{-}\)). Copying of the complete double-positive fusion element most likely involved the action of the ERACR encoded gRNA-y2 and gRNA-y3, while copying of the GFP\(^+\) element alone presumably was mediated by the MCR-GFP encoded gRNA-y1. Consistent with chimeric GFP\(^+\) elements being able to copy by themselves, the ERACR-1/MCR-GFP and ERACR-2/MCR-GFP fusions analyzed at the molecular level (Fig. 5G; Fig. S14F; Fig. S20A') retained at least 1 kb of homologous sequences flanking the gRNA-y1 cleavage site on both sides of the MCR-GFP element. The \(y^{-}\), DsRed\(^+\), GFP\(^+\), \(w^a\) element derived from ERACR-min (Fig. S14J; Fig. S20A') copied itself very poorly (2%), but copied just the DsRed marker at a modest frequency (25%) (Figure S20D,D'). Finally, we identified a \(y^+\), DsRed\(^+\), GFP\(^+\), \(w^a\) chimera from an ERACR-1 cross (Fig. S14K; Fig. S20A'), which appeared to exhibit little, if any, copying ability or mutagenic activity (Figure S20E,E'), suggesting the Cas9 or gRNAs had compromised function. This hybrid also has an in-frame indel at the gRNA-y2 cut site, consistent with its \(y^+\) phenotype (Fig. S14K). We have yet to identify the exact molecular natures of these latter two chimeric elements.

In summary, several different behaviors of double-positive (DsRed\(^+\), GFP\(^+\)) chimeric elements were observed including: modest copying of 1) the full chimeric element (i.e., both the DsRed\(^+\), GFP\(^+\) markers), 2) GFP\(^+\) only (\(y^{-}\) hybrids with ERACR-1,2), 3) DsRed\(^+\)
only (hybrids with ERACR-min), or 4) minimal/no copying ($y^+$ hybrids with ERACR-1). Further characterization of these and additional tertiary events will be required to determine the full range of possible outcomes from unintended rare recombination events, but several of these chimeras clearly comprise active or partially active genetic elements.

**ERACRs efficiently replace a gene-drive in population cages**

Results described above revealed that ERACR elements counteract a gene-drive element by two primary mechanisms: 1) deletion and replacement of the drive by the ERACR, or 2) deletion of the drive without ERACR copying. The latter outcome most often results in damaged receiver chromosomes that are inviable unless in-trans to a wild-type chromosome in females. Together these two ERACR activities lead to efficient super-Mendelian inheritance of the ERACR-bearing donor allele in males and to a more moderate transmission bias in females. Females also harbor a substantial fraction of drive-deleted male lethal alleles.

We performed mathematical modeling to determine whether the various outcomes of ERACR/MCR-GFP confrontation would result in ERACRs fulfilling their designed function of eliminating the gene-drive element over multiple generations (Fig. 7C,E, Fig. S23F,F',G,G'). In these simulations we made the following simplified core assumptions based on the predominant classes and frequencies of various genotypes we have described above: 1) ERACRs carried either on the donor chromosome, or that copied and replaced an MCR-GFP element, retain their initial activities in subsequent generations; 2) MCR-GFP elements that have been retained following a confrontation with an ERACR are immune to further action by ERACRs (due to generation of cleavage resistant NHEJ mutations at both cut sites); and 3) double-negative (DsRed\textsuperscript{−}, GFP\textsuperscript{−}) alleles generated by ERACRs deleting but not replacing the MCR-GFP element are fully viable in heterozygous females, but lethal in homozygous or hemizygous individuals. In addition, consistent with prior classic studies (Barker, 1962; Bastock, 1956; Diederich, 1941; Merrell, 1949; Sturtevant, 1915) we modeled a range of potential fitness costs for $y^-$ versus ERACR-2 ($y^+$) males in gaining mating access to $y^+$ females as well as an
apparent fitness cost associated with the MCR-GFP gene-drive relative to the ERACR element. We confirmed this assortative mating preference of wild-type females for wild-type versus \( y^- \) males in single generation control crosses performed with our own stocks (Fig. S23A-D), and in competitive multi-generational cage experiments (Fig. S23E). Sampling over a broad range of parameters suggests that ERACR-min (\( y^- \)) and, particularly ERACR-2 (\( y^+ \)), should produce sufficient drive in mixed populations carrying the MCR-GFP and ERACR elements (but no wild-type alleles) to replace and ultimately eliminate the gene-drive over several generations (Fig. 7C,E).

Based on these encouraging modeling results, two ERACRs were tested in cage experiments: ERACR-min, which like the MCR-GFP drive element causes insertional inactivation of the \( y \) locus, and ERACR-2, which carries fully recoded \( y \) sequences to restore yellow activity. Because of the strong sexual selection bias wherein wild-type (\( y^+ \)) females show a strong predilection for mating with wild-type versus \( y^- \) males, we expected that this effect might result in differing drive trajectories for ERACR-min versus ERACR-2 in cage populations.

We initiated cage experiments (3-4 separate replicates per ERACR) by combining unmated "virgin" male and female flies composed of 25% homozygous ERACR and 75% homozygous MCR-GFP virgin male and female individuals in each cage. At each generation (\( n \)) we scored half of the individuals per cage (approximately 150 random flies) for their fluorescent and \( y^\pm \) phenotypes, while the other half was used to seed the next generation (\( n+1 \)) of cages. In the ERACR-min experiment, the frequency of the DsRed marker, and therefore the ERACR cassette, tripled in ~4 generations and then gradually approached fixation over the next 5 generations, with a modest degree of variation (~10-15%) exhibited between the four cages tested (Fig. 7D). Reciprocally, the frequency of the MCR-GFP marker decreased until it was almost eliminated by generation 9. Consistent with random mating among \( y^- \) ERACR-min and MCR-GFP individuals, 30-35% of progeny from the first generation were trans-heterozygotes carrying both fluorescent markers. The frequency of these double-positive (GFP\(^+\), DsRed\(^+\)) flies peaked
in generation 3 and then steadily declined. In contrast, the frequency of the DsRed marker in the ERACR-2 cages (Fig. 7F) remained approximately constant for one generation and then increased steeply and in tight synchrony, reaching fixation in 5-6 generations with concomitant reduction and then complete elimination of the MCR-GFP element. These experimental results match well with simulations (pale lines in Figs. 7D, 7F) based on parameters identified by the modeling (dotted lines in Figs. 7C, E), capturing key features of both the overall trajectories as well as inter-trial variations.

Although there was an excellent overall fit between the observed data and modeling based on key drive outcomes (i.e., relative frequencies of ERACR copying, MCR retention, and MCR-GFP deletion with associated chromosome damage) (Fig. 7C-F; Fig. S23F-G') we note that two ERACR-min replicates (#1 and #4) pursued moderately different trajectories from the other two (#2, #3), with the latter two matching the predicted modeling (Fig. 7C, D). Further analysis revealed that the deviation in replicate #4 was associated with a late expansion of an abundant GFP+ DsRed− category (i.e., retained MCR-GFP elements: ~30% of receivers), which fluctuated at modest frequencies beginning after generation 15 (Fig. S23F'), while in replicate (#1) a rare GFP− DsRed− category (~4-5%) that was not observed in the other replicates (#2-4) began an irregular pattern of increase after generation 10. These varied trajectories may result from stochastic drift of y− alleles that have equivalent fitness to the y− ERACR-min since no such events were observed in any of the ERACR-2 replicates.

In addition to their differing drive dynamics, another marked difference in the behaviors of the two ERACR elements was that unlike ERACR-min cages, which showed modest levels of variation between cage replicates, ERACR-2 trajectories displayed very little variance between replicates. As for ERACR-min, there was a close fit between experimental and simulated trials. Consistent with our prediction that assortative mating might contribute to the ERACR-2 drive trajectory, the frequency of double-positive (GFP+, DsRed+) flies in first generation progeny was considerably less (15-20%) than that predicted by random mating (37.5%), suggesting that y+ females initially may have preferentially mated with y+ males as reported in previous studies (Barker, 1962; Bastock,
1956; Diederich, 1941; Merrell, 1949; Sturtevant, 1915) and confirmed in our control crosses (Fig. S23A,B). According to this scenario, $y^+$ males presumably also mated successfully with $y^-$ females (Fig. S23C,D), leading to subsequent positive feedback cycles in which the combination of ERACR-2-biased assortative mating and potent CRISPR-based drive lead to rapid population replacement of the MCR-GFP element with ERACR-2 (Fig. 7E,F). We hypothesize that, in addition to resulting in faster population replacement for ERACR-2, this additional selective pressure against $y^-$ individuals imposed fitness costs on some mutational variants (i.e. the $GFP^-$, $DsRed^-$, $y^-$ male variant in replicate 1 for ERACR-min and the $GFP^+$, $DsRed^-$, $y^-$ male variant in replicate 4 for ERACR-min), shus reducing the variance in marker phenotype frequencies for ERACR-2 replicates.

Cage experiments were also conducted setting the $y^+$ ERACR-2 against a strain carrying a $y^-$ NHEJ-induced point mutation at the gRNA-y1 cleavage site (i.e., the same gRNA driving the MCR-GFP element) in the absence of Cas9 to assess the potential role of assortative mating in these experiments (Fig. S23E). Over ~10 generations, chromosomes carrying the $y^+$ ERACR-2 element were able to successfully overtake the $y^-$ NHEJ population, although this process was more gradual than that observed in the ERACR-2/MCR-GFP cages and also showed typical inter-cage variation (Fig. S23E). These observations support the hypothesis that the $y^+$ genotype has a strong competitive advantage over $y^-$ in a cage setting. Another factor that could contribute to the strong drive displayed by ERACR-2, and perhaps also for ERACR-min, is a fitness cost that may be associated with the MCR-GFP element. This apparent contribution to the drive, which was extracted from the modeling, might reflect a fraction of MCR-GFP chromosomes that have been damaged by ERACR action, such as those produced in the ERACR2-y2 single-cut experiments. We conclude that the modeling predictions conform closely to the experimental outcomes and that ERACRs, particularly those carrying a recoded transgene restoring function of a functionally important gene, have the potential to eliminate a gene-drive element even once it has attained fixation in a population.
Distilled assessment of ERACR action

Data from this study support the following hypothesis to explain the major inheritance patterns we observed in male and female F2 progeny of F1 ERACR/MCR-GFP master females. The two ERACR gRNAs efficiently cut both target sites leading to frequent deletion of the MCR-GFP construct on the target chromosome. Deletion of the MCR-GFP element is typically repaired either by copying the ERACR into the gap (its intended action), or by damaging the receiver chromosome such that it is rendered lethal when hemizygous in males (or homozygous in females).

An advantage of the ERACR design paradigm is that they can also carry a functional recoded version of a gene that has been inactivated by insertion of the gene-drive element (in this study the y locus). Such recoded genes thereby come under the control of endogenous cis-regulatory sequences, restoring native activity of the target gene. If a gene-drive element disrupts a target gene under strong selection for viability, fertility, or sexual selection (e.g., the y locus), rescue of target gene activity can provide a potent selective advantage to ERACR-bearing individuals.

In addition to the primary ERACR-mediated events resulting in deletion of the gene-drive target gene, we also observed a variety of infrequent partial gene conversion events mediated by shared DNA sequences in the same orientation between ERACR and MCR-GFP elements. These homology-based repair events were presumably the result of synthesis-dependent strand annealing (SDSA) D-loop repair resolved by non-crossover directional gene conversion events (Bozas et al., 2009; Do et al., 2014; Joyce et al., 2012; Wei and Rong, 2007).

An informative example of offset D-loop conversion was presumably mediated by pairing between partially recoded y sequences carried by the ERACR-1 element and endogenous y 3' sequences flanking the MCR element. These recombination events could take place in two different configurations, one generating y+ deletions of the MCR (GFP−, DsRed−) and the other generating concatenated y− MCR-ERACR hybrid elements (GFP+, DsRed+). The frequency of both of these events was greatly reduced for ERACRs
with minimal homology to the MCR-GFP drive: ERACR-2, which carries fully recoded \( y \) sequences, and ERACR-min, which entirely lacks rescuing \( y \) sequences. Despite these measures to reduce recombination between ERACR and MCR-GFP sequences, \( y^+ \) recombinants and hybrid elements were still generated at a very low frequency for both ERACR-2 and ERACR-min. Sequence analysis of these rare events suggests that they may result from various spurious repair outcomes. For example, very short stretches of sequence homology may guide transient alignments which then resolve in illegitimate end-joining reactions, micro-homology mediated end-joining (MMEJ) repair, or other unpredictable end-joining events. Further analysis of the sequence requirements underlying these repair outcomes may provide insights to minimize such illicit recombination events.

**Cas9-mediated copying versus damage of target chromosome:** One important design feature shared by the ERACR and e-CHACR experiments was to mark donor versus receiver chromosomes, thereby making it possible to differentiate the fates of the two chromosomes independently from the distinct fluorescently-labeled active genetic elements. Such genetic bookkeeping revealed that super-Mendelian inheritance of the single-cut MCR-GFP gene-drive resulted almost exclusively from its copying to the receiver chromosome, since the receiver and donor chromosomes were inherited at equal Mendelian rates (50%).

In contrast to the single-cutting MCR-GFP element, ERACRs, which cut the receiver chromosome twice were estimated to damage \(~40\%\) of target X chromosomes, and did so whether the receiver chromosome carried an MCR-GFP insertion or wild-type chromosomal sequences at the target site. Since the sum of ERACR copying and damage rates (70\%) approximates that of the MCR-GFP element, many of the damaged chromosome outcomes may reflect derailed copying events.

As mentioned above, single-cut versions of ERACRs induced target chromosome damage, and surprisingly, at rates comparable to the double-cut elements. One possibility is that the two gRNAs carried by full ERACRs do not cut simultaneously, thereby resulting in two tandem single-cut events. Interestingly, while one of the single
gRNAs (gRNA-y2) sustains a higher rate of copying than the other (gRNA-y3), when summed, their copying rates still fell short of that attained by the double-cut element when challenged with larger gRNA separation distances. Little difference in performance was observed between the single-cut (gRNA-y2) versus double-cut ERACRs, however, when the two gRNA cleavage sites spaced closer together. These findings suggest that for larger cargo sizes, double-cut elements benefit from some form of cooperative interaction between the two gRNAs in promoting HDR.

Another informative feature of the single-cut ERACR experiments is that ERACR2-y3 which cuts on the centromere side of the MCR-GFP element generated an abundant class of GFP⁻, DsRed⁻ progeny, as did the double-cut ERACR. A corresponding prominent GFP⁻, DsRed⁻ class was not observed for ERACR2-y2, where the cut is distal to the MCR-GFP element. However, ERACR2-y2 generated significantly fewer male than female GFP⁺ progeny, suggesting that a similar lethal class was generated, but that these damaged alleles retained sequences centromere-proximal to the gRNA-y2 cut site. These results suggest that the chromosome damage induced by ERACRs begins precisely where the gRNAs cut the chromosome and extend distally to delete or inactivate at least one essential gene.

It is important to emphasize that the types of damage generated by ERACRs and e-CHACRs in various configurations reflect distinct outcomes in different contexts and may be mediated by alternative repair mechanisms. While over 90% of the GFP⁻, DsRed⁻ receiver chromosomes recovered in females exposed to ERACRs were male-lethal, all tested individual female target chromosomes that had been subject to double-cleavage by e-CHACR-wG (at y and w) were found to be viable in males, and moreover, displayed Mendelian transmission (Fig. S3E). The precise nature of the damage incurred by receiver chromosomes exposed to the double-cutting e-CHACR remains to be determined, however, the Mendelian transmission of these chromosomes to both sexes suggests that the handicap in males is not associated with unrepairable damage. Furthermore, it is possible to rule out mechanisms involving either meiotic segregation distortion or significant fitness costs associated with receiver chromosomes recovered in females. Future studies should shed light on the repair-based mechanisms underlying
the differing forms of chromosomal damage produced by double cut e-CHACR configurations and ERACRs. It will also be important to analyze the types of damage resulting from e-CHACRs targeting a full-drive element (e.g., Fig. S9), which produces both viable and male lethal events. These latter male lethal events, which were recovered on the MCR-donor chromosome, were rescuable by duplications covering the tip of the X chromosome. Such lesions may represent the outcome of yet another distinct category of imperfect DNA repair in which the gRNA cut site (in Cas9) is far from any homology providing sequences, and thus is unable to cleanly define a border of the damaged region.

**Unexpected activities of ERACRs:** Although the majority of events induced by ERACRs matched our expectations, we also observed unintended outcomes. As discussed above, the most frequent of such events was the deletion and damage of the target chromosomes. We also observed the generation of novel categories of hybrid elements that could be accounted for either by partial pairing taking place between shared ERACR and MCR-GFP sequences or by non-homology based end-joining events. Although most of these partial or chimeric elements resulted in non-driving outcomes, a subset of them with a double-positive (GFP⁺, DsRed⁺) phenotype encoded Cas9 and carried all three gRNAs from the ERACR and MCR-GFP element. These fusion elements were able copy themselves to a wild-type chromosome and also induced damage to those receiver chromosomes, consistent with multiple gRNAs acting locally. In addition to these full-copying events, different chimeric elements exhibited various classes of progeny, including those with either "GFP-only = MCR-GFP" or "DsRed-only = ERACR" phenotypes. The performance of these various tertiary elements will be interesting to examine in future experiments. While eliminating large stretches of sequence homology between ERACR and MCR-GFP elements reduced the products of such illegitimate pairing events to very low frequencies, such recombinant phenotypic classes were nonetheless occasionally recovered when either only short stretches of sequence complementarity were present nearby or even when no significant homology was evident. As mentioned above, such rare events can become more common if favored by some type of selective advantage or benefiting from a stochastic effect, as was observed in one of the ERACR-min cage replicates. These and other imperfect outcomes potentially
generated in subsequent generations are factors to evaluate when considering the deployment of such neutralizing systems.

**ERACRs can drive efficiently in populations cages:** ERACRs were conceived to eliminate gene-drive elements from populations (Gantz and Bier, 2016). Initial modeling of randomly mixed infinite populations consisting of gene-drive, ERACR, and wild-type alleles predicts that oscillatory dynamics can emerge, which eventually reach a stable equilibrium wherein ERACR and wild-type alleles are most prevalent, and the gene-drive allele (with active Cas9) is greatly reduced in frequency (Vella et al., 2017).

Despite the experimental results presented in this study indicating that ERACRs are imperfect in deleting and replacing the target MCR-GFP element, both ERACR-min and ERACR-2 proved effective in replacing the MCR-GFP drive in cage experiments. ERACR-min, like the MCR-GFP, disrupts the function of the y locus, while ERACR-2 carries a recoded y transgene that restores yellow activity in a single copy. Both ERACRs achieved ~75% frequencies in 5-6 generations. ERACR-min then took another 6-7 generations to near fixation, while ERACR-2 drove rapidly to completion by generation 7. In addition, instead of the typical degree of cage-to-cage variation observed for ERACR-min, the ERACR-2 replicates displayed very little variation. The difference in performance of the two ERACRs most likely results from the potent assortative mating selection imposed by the y− mutation resulting from wild-type y+ females strongly preferring to mate with y+ versus y− males (Barker, 1962; Bastock, 1956; Diederich, 1941; Merrell, 1949; Sturtevant, 1915). In addition to resulting in faster spread of the ERACR-2 allele, and hence less opportunities for stochastic variation to accumulate, its y+ advantage likely placed an additional selective pressure against y− variants, which were observed in ERACR-min experimental replicates (i.e., the GFP−, DsRed−, y− male variant in replicate 1 and the GFP+, DsRed−, y− male variant in replicate 4). Evidence of assortative mating in the ERACR versus MCR-GFP cage experiments can be inferred from the first-generation results for ERACR-2 versus ERACR-min wherein the frequency of progeny carrying both fluorescent markers was consistent with random mating for ERACR-min,
but was half of that expected value (37.5%) for ERACR-2. In addition, we observed that ERACR-2 rapidly outcompeted a $y^-$ point mutant allele in separate cage experiments, providing direct evidence for a fitness advantage associated with the $y^+$ ERACR-2 genotype, consistent with the preference of $y^+$ females for mating with $y^+$ versus $y^-$ males in control pair-mating crosses. The much-reduced inter-cage variation observed for the ERACR-2 trajectories may reflect two independently acting processes: drive produced by Cas9 delivered \textit{in-trans}, which is approximately equal to that observed for ERACR-min; and a selective pressure resulting from assortative mating favoring $y^+$ males. These two factors provide fewer opportunities for stochastic variation to accumulate. The likelihood of failure to transmit the ERACR-2 element can be estimated by taking the product of the independent failure probabilities: 1) the simultaneous failure to inherit the ERACR based on its drive performance ($f_1 = 1 - \text{transmission frequency} \sim 0.2$) and 2) exceptions to assortative mating ($f_2 = 1 - \text{mating bias} \sim 0.3$). A composite “inheritance bias” for the ERACR-2 element can then be computed (i.e., $1 - f_1 f_2 \sim 0.94$). By reducing the frequency of transmission failures, the stochastic variation between cage replicates for the dual ERACR-2 drive should also be reduced, as is observed. Another perspective to account for the reduced variance between of ERACR-2 cage replicates relative to ERACR-min is that the rare phenotypic categories that displayed late increases in two of the ERACR-min replicates had a $y^-$ phenotype, which would have been strongly selected against in the ERACR-2 cages, thus reducing variance associated with such stochastic or weakly selected events. Both ERACRs may also gain an advantage from a fitness cost incurred by the MCR-GFP. In addition, based on the results of the single cut ERACR2-y2, which retains GFP expression, but likely generates $\sim$20-30% lethal target alleles, a similar fraction of GFP$^+$ MCR-GFP alleles presumably sustain similar damage, and would thus be eliminated in males or homozygous females. Whether expression of the Cas9 transgene, other design features of the MCR-GFP element, or potential ERACR-inflicted damage to the MCR-GFP bearing chromosome (e.g., double-negative male lethal alleles) contribute to this latter fitness cost remains to be determined.
Although the drive trajectories we observed for both ERACRs agreed well with model simulations based on primary drive outcomes, as noted above, two replicates of ERACR-min deviated modestly from the others. These outliers presumably reflected random drift or selection of a rare event that conferred an advantage to a retained MCR-GFP allele (a significant class of ERACR-induced receiver chromosome events ~30%) or a male viable GFP−, DsRed− allele (~4% of target chromosome outcomes). This result highlights the need to consider rare events in assessing drive outcomes in populations. In aggregate, this study suggests that despite imperfections in the ERACR performance observed in single pair-matings, these neutralizing elements perform effectively as intended to replace a gene-drive element under competitive conditions, particularly if one provides the ERACR with an additional fitness advantage. Such dual-drive may be a key design feature for optimizing the chances of successfully neutralizing a gene-drive system.
We modeled the population dynamics of the two single-locus gene drive remediation systems, ERACR-min and ERACR-2, under laboratory cage conditions assuming discrete generations and randomly mixing populations, with the exception of assortative mating preferences of y+ females for y+ males for ERACR-2. Model fitting was carried out using a likelihood-based Markov chain Monte Carlo (MCMC) algorithm. Here, we first describe the likelihood calculation for the ERACR-min construct being driven into a locus occupied by a X-linked homing system initially fixed in the laboratory population. We then describe minor modifications for the likelihood calculation for the ERACR-2 system, including the incorporation of assortative mating, followed by the details of the MCMC algorithm.

To model the X-linked ERACR-min system, we consider an X-linked ERACR allele, “E,” a previously fixed homing allele, “H,” an in-frame, cost-free resistant allele (i.e. a homing allele that is resistant to cleavage by the ERACR system), “R,” and an out-of-frame or otherwise costly resistant (broken) allele, “B.” As male *D. melanogaster* have only one X chromosome, we denote the Y chromosome by “Y.” Consequently, there are 10 possible female genotypes (EE, ER, EB, EH, RR, RB, HR, BB, HB, and HH), and four possible male genotypes (EY, RY, BY, and HY). We denote the proportion of organisms having each genotype at the kth generation by $p^x_k$, where $x$ denotes one of the 14 genotypes.

Given the large number of possible mating pairs (40), it is not feasible to show the complete equations for the next generation genotype frequencies here, so we instead depict them in Fig. Smod2, and describe them here in brief. We consider Mendelian inheritance rules at the gene drive locus, with the exception that, for EH heterozygotes, a proportion, $c$, of the H alleles are cleaved, while a proportion, $1 - c$, remain as H alleles. Of those that are cleaved, a proportion, $p_{HDR}$, are subject to accurate homology-directed repair (HDR) and become E alleles, while a proportion, $1 - p_{HDR}$, become resistant alleles. Of those that become resistant alleles, a proportion, $p_{RES}$, of these become in-
frame, cost-free resistant (R) alleles, while the remainder, \(1 - p_{RES}\), become out-of-frame or otherwise costly resistant (broken, B) alleles. The resulting alleles segregate in a Mendelian fashion.

These considerations allow us to calculate the expected genotype frequencies in the next generation before accounting for fitness costs. Let us denote these frequencies by \(\hat{p}_{k+1}^x\), where \(x\) denotes the genotype and \(k + 1\) denotes the next generation. Normalizing these ratios to account for fitness costs – \(s_{HY}\) and \(s_{BY}\) associated with males having the homing and broken alleles, respectively, \(s_{HH}\) and \(s_{BB}\) associated with females homozygous for the homing and broken alleles, respectively, and \(s_H\) and \(s_B\) associated with having one copy of the homing or broken allele in females – the genotype frequencies in the next generation are given by,

\[
\begin{align*}
(p_{k+1}^{HH}, p_{k+1}^{RR}, p_{k+1}^{HR}) &= (\hat{p}_{k+1}^{HH}, \hat{p}_{k+1}^{RR}, \hat{p}_{k+1}^{HR})(1 - s_{HH})/W_{k+1} , \\
(p_{k+1}^{HB}, p_{k+1}^{RB}) &= (\hat{p}_{k+1}^{HB}, \hat{p}_{k+1}^{RB})(1 - s_H)(1 - s_B)/W_{k+1} , \\
(p_{k+1}^{EH}, p_{k+1}^{ER}) &= (\hat{p}_{k+1}^{EH}, \hat{p}_{k+1}^{ER})(1 - s_H)/W_{k+1} , \\
p_{k+1}^{BB} &= \hat{p}_{k+1}^{BB}(1 - s_{BB})/W_{k+1} , \\
p_{k+1}^{EB} &= \hat{p}_{k+1}^{EB}(1 - s_B)/W_{k+1} , \\
(p_{k+1}^{EE}, p_{k+1}^{EY}) &= (\hat{p}_{k+1}^{EE}, \hat{p}_{k+1}^{EY})/W_{k+1} , \\
(p_{k+1}^{HY}, p_{k+1}^{RY}) &= (\hat{p}_{k+1}^{HY}, \hat{p}_{k+1}^{RY})(1 - s_{HY})/W_{k+1} , \\
p_{k+1}^{BY} &= \hat{p}_{k+1}^{BY}(1 - s_{BY})/W_{k+1} .
\end{align*}
\]

Here, \(W_{k+1}\) is a normalizing term given by,

\[
W_{k+1} = (\hat{p}_{k+1}^{HH} + \hat{p}_{k+1}^{RR} + \hat{p}_{k+1}^{HR})(1 - s_{HH}) + (\hat{p}_{k+1}^{HB} + \hat{p}_{k+1}^{RB})(1 - s_H)(1 - s_B) + (\hat{p}_{k+1}^{EH} + \hat{p}_{k+1}^{ER})(1 - s_H) + \hat{p}_{k+1}^{BB}(1 - s_{BB}) + \hat{p}_{k+1}^{EB}(1 - s_B) + \hat{p}_{k+1}^{EE} + \hat{p}_{k+1}^{HY} + (\hat{p}_{k+1}^{HY} + \hat{p}_{k+1}^{RY})(1 - s_{HY}) + \hat{p}_{k+1}^{BY}(1 - s_{BY}) .
\]

Note that fitness costs are measured relative to the EE and EY genotypes, we have assumed multiplicative fitness costs due to the H and B alleles, and the R allele is assumed to have the same fitness cost as the H allele due to its close resemblance.
The likelihood of the population cage data was calculated by assuming a multinomial distribution of individuals having each sex and marker phenotype, and by using the model predictions to generate expected proportions for each set of parameter values. I.e., by calculating the log likelihood,

$$\log L(\theta) \propto \sum_{i=1}^{3} \sum_{k=1}^{n_i} A_{f,i,k} \log \left( p_k^{EH}(\theta) + p_k^{ER}(\theta) \right) + B_{f,i,k} \log \left( p_k^{HH}(\theta) + p_k^{HR}(\theta) + p_k^{HB}(\theta) + p_k^{RR}(\theta) + p_k^{RB}(\theta) \right) + B_{m,i,k} \log \left( p_k^{HY}(\theta) + p_k^{RY}(\theta) \right) + C_{f,i,k} \log \left( p_k^{EE}(\theta) + p_k^{EB}(\theta) \right) + C_{m,i,k} \log \left( p_k^{EY}(\theta) \right) + D_{f,i,k} \log \left( p_k^{BB}(\theta) \right) + D_{m,i,k} \log \left( p_k^{BY}(\theta) \right).$$

Here, $A_{f,i,k}$ is the number of GFP+, DsRed+ females (genotypes EH and ER) at generation $k$ in experiment $i$, $B_{f,i,k}$ and $B_{m,i,k}$ are the corresponding numbers of GFP+, DsRed- females (genotypes HH, HR, HB, RR and RB) and males (genotypes HY and RY), respectively, $C_{f,i,k}$ and $C_{m,i,k}$ are the corresponding numbers of GFP-, DsRed+ females (genotypes EE and EB) and males (genotype EY), respectively, and $D_{f,i,k}$ and $D_{m,i,k}$ are the corresponding numbers of GFP-, DsRed- females (genotype BB) and males (genotype BY), respectively. The $i$th experiment is run for $n_i$ generations, and the expected genotype frequencies are dependent on the model parameters, $\theta = \{c, p_{HDR}, p_{RES}, s_{HY}, s_{BY}, s_{HH}, s_{BB}, s_H, s_B\}$, as defined earlier.

Given prior knowledge that the BY and BB genotypes confer lethality, we assumed that $s_{BY} = s_{BB} = 1$, and applied these fitness costs prior to phenotype scoring. Other fitness costs were manifest after phenotyping, i.e. in the contribution of gametes to the next generation. The initial condition for all ERACR-min and ERACR-2 experiments was 19 EE females, 19 EY males, 57 HH females, and 57 HY males (i.e. 25% of both males and females had the ERACR allele, with females being homozygous). Finally, we considered only three of the four drive experiments conducted for the ERACR-min system in the model fitting process, as in one of them (experiment 1), an allele emerged with a GFP-, DsRed- marker phenotype that displayed different properties to the corresponding allele in the other three experiments. Accommodating this allele would have required a new
model specific to that experiment. We intend to explore this in subsequent modeling studies.

A slight modification was made to our model for the ERACR-2 experiment to allow for assortative mating. Here, individuals having the ERACR allele, in addition to having the DsRed+ marker phenotype, also have the y+ eye color phenotype. Females having the y+ eye color phenotype (i.e. EE, ER, EB and EH females) are more likely to mate with males having the y+ phenotype (i.e. EY males) than with males having the y- phenotype (i.e. RY, BY and HY males); however, females having the y- phenotype do not have this mating preference. To account for this, we downweighted crosses between y+ females and y- males by a factor, \((1 - s_y)/m_y\), where \(s_y\) represents the fraction by which y+ females reduce their mating with y- males, and \(m_y\) is a normalizing term given by: \(1 - s_y)(p^{RY}_k(\theta) + p^{BY}_k(\theta) + p^{HY}_k(\theta)) + (1 + s_y)p^{EY}_k(\theta)\). We also upweighted crosses between y+ females and y+ males by a factor, \((1 + s_y)/m_y\). Other than that, the model and likelihood calculation for the ERACR-2 experiment was the same as that for the ERACR-min experiment, with the addition of one more fitted model parameter, \(s_y\).

As identifiability was an issue with estimating parameter values, we fixed the values of \(c\), \(p_{HDR}\) and \(p_{RES}\), based on the experimental results depicted in Fig. S3, and fixed the values of \(s_{BY}\) and \(s_{BB}\), as mentioned previously. We used a Markov chain Monte Carlo (MCMC) sampling procedure to estimate the remaining fitness parameters, \(s_H\), \(s_B\), \(s_{HH}\) and \(s_{HY}\), including 95% credible intervals. The resulting model fits are depicted in Fig. 7 and Figs. Smod3,4.

A stochastic version of the model was implemented by assuming a population size of 150 individuals at each generation, for general consistency with the cage experiments, and by sampling the number of individuals having each genotype at the next generation according to a multinomial distribution with model-predicted genotype frequencies. This was used to generate the stochastic trajectories depicted in Fig. 7.
Figure Smod2. Crosses representing the inheritance pattern of an X-linked ERACR construct. “E” denotes the X-linked ERACR system, “H” denotes the X-linked homing construct targeted by the ERACR system, “R” denotes an in-frame, cost-free homing-resistant allele, “B” denotes an out-of-frame or otherwise costly “broken” homing-resistant allele, and “Y” denotes the Y chromosome. Alleles segregate in a Mendelian fashion, with the exception of the H allele of EH females. A proportion, \( p_E = cP_{HDR} \), of these H alleles are converted into E alleles, where \( c \) is the proportion of H alleles that are cleaved, and \( P_{HDR} \) is the proportion of those cleaved that are subject to accurate homology-directed repair (HDR). Of those that are cleaved but not converted into H alleles, a proportion, \( p_{RES} \), become R alleles, while the remainder, \( 1 - p_{RES} \), become B alleles. I.e., a proportion, \( p_R = c(1 - P_{HDR})p_{RES} \), of the H alleles of EH females become R alleles, while a proportion, \( p_B = c(1 - P_{HDR})(1 - p_{RES}) \), become B alleles. Finally, a proportion, \( p_H = 1 - c \), are not cleaved and remain as H alleles. The inheritance pattern depicted here is incorporated into the model fitting process described in the Materials and Methods section of the manuscript, and in the Model Fitting section of the Supporting Information.
Figure Smod3. Cage trials and modeling of ERACR-2 system

Observed and predicted dynamics of the ERACR-2 gene-drive remediation system for each marker phenotype. Population cage experiments were set up with 19 females and 19 males homozygous (EE) or hemizygous (EY) for the X-linked ERACR-2 construct (E) and 57 females and 57 males homozygous (HH) or hemizygous (HY) for the X-linked homing system (H) that the ERACR-2 construct targets. Population counts were monitored over 10 generations. Results from these experiments are shown as solid lines, with fitted model predictions shown as dashed lines. Each marker phenotype is known to be produced by certain genotypes. For females, EH and ER (“R” represents an in-frame, cost-free resistant allele) are y+/GFP+/DsRed+, EE and EB (“B” represents an out-of-frame or otherwise costly resistant “broken” allele) are y+/GFP-/DsRed+, HH, HR, HB, RR and RB are y-/GFP+/DsRed+, and BB are y-/GFP-/DsRed-. For males, EY are y+/GFP-/DsRed+, HY and RY are y-/GFP+/DsRed+, and BY are y-/GFP-/DsRed-. Observed data are consistent with the homing efficiency results shown in Fig. S3, namely an accurate homing efficiency of 32.4%, with 57.2% of resistant alleles being in-frame, cost-free (R), with the remainder being out-of-frame or otherwise costly (B). Given these rates, the data are consistent with the following fitness costs: females and males homozygous (BB) or hemizygous (BY) for the broken allele are unviable, females homozygous for the homing allele (HH) have a fitness cost of 48% (95% CrI: 38-56%), males hemizygous for the homing allele (HY) have a fitness cost of 49% (95% CrI: 28-63%), females having one copy of the homing allele have a fitness cost of 98% (95% CrI: 89-100%), and females having one copy of the broken allele have a fitness cost of 65% (95% CrI: 7-98%). Assortative mating is seen for y+ females, who are estimated to reduce their mating with y- males by 71% (95% CrI: 57-87%), with a corresponding increase in mating with y+ males. The ERACR-2 system reaches fixation in all three repetitions.
Figure Smod4. Cage trials and modeling of ERACR-min system

Observed and predicted dynamics of the ERACR-min gene-drive remediation system for each marker phenotype. Population cage experiments were set up with 19 females and 19 males homozygous (EE) or hemizygous (EY) for the X-linked ERACR-min construct (E) and 57 females and 57 males homozygous (HH) or hemizygous (HY) for the X-linked homing system (H) that the ERACR-min construct targets. Population counts were monitored over 21 or 26 generations. Results from these experiments are shown as solid lines, with fitted model predictions shown as dashed lines. Each marker phenotype is known to be produced by certain genotypes. For females, EH and ER (“R” represents an in-frame, cost-free resistant allele) are GFP+/DsRed+, EE and EB (“B” represents and out-of-frame or otherwise costly resistant “broken” allele) are GFP-/DsRed+, HH, HR, HB, RR and RB are GFP+/DsRed-, and BB are GFP-/DsRed-. For males, EY are GFP-/DsRed+, HY and RY are GFP+/DsRed-, and BY are GFP-/DsRed-. Observed data are consistent with the homing efficiency results shown in Fig. S3, namely an accurate homing efficiency of 28.2%, with 47.6% of resistant alleles being in-frame, cost-free (R), with the remainder being out-of-frame or otherwise costly (B). Given these rates, the data are consistent with the following fitness costs: females and males homozygous (BB) and hemizygous (BY) for broken allele are unviable, females homozygous for the homing allele (HH) have a fitness cost of 79.4% (95% CrI: 76.8-81.8%), males hemizygous for the homing allele (HY) have a fitness cost of 19% (95% CrI: 15-22%), females having one copy of the homing allele have a fitness cost of 0.6% (95% CrI: 0.2-2.9%), and females having one copy of the broken allele have a fitness cost of 96% (95% CrI: 82-100%). The ERACR-min system spreads in all repetitions, although not always to fixation.