Assessment of a split homing based gene drive for efficient knockout of multiple genes

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Abstract
Homing based gene drives (HGD) possess the potential to spread linked cargo genes into natural populations and are poised to revolutionize population control of animals. Given that host-encoded genes have been identified that are important for pathogen transmission, targeting these genes using guide RNAs as cargo genes linked to drives may provide a robust method to prevent transmission. However, effectiveness of the inclusion of additional guide RNAs that target separate host encoded genes has not been thoroughly explored. To test this approach, here we generated a split-HGD in Drosophila melanogaster that encoded a drive linked effector consisting of a second gRNA engineered to target a separate host encoded gene, which we term a gRNA-mediated effector (GME). This design enabled us to assess homing and knockout efficiencies of two target genes simultaneously, and also explore the timing and tissue specificity of Cas9 expression on cleavage/homing rates. We demonstrate that inclusion of a GME can result in high efficiency of disruption of its target gene during super-Mendelian propagation of split-HGD. However, maternal deposition and embryonic expression of Cas9 resulted in the generation of drive resistant alleles which can accumulate and limit the spread of such a drive. Alternative design principles are discussed that could mitigate the accumulation of resistance alleles while incorporating a GME.
Introduction

For standard Mendelian inheritance, any particular allele has a 50% chance in being transmitted to its offspring. While mechanisms of meiosis generally bias selection against violators of Mendel’s rules, there are many examples of naturally occurring selfish genetic elements (SGEs) that succeed in bypassing these rules. These SGEs enhance, or “drive” their transmission into subsequent generations, despite often times being harmful to the harboring individual (i.e. imposing a fitness load). These include, for example, transposable elements (TEs), meiotic drivers, B chromosomes, post segregation killers, heritable microbes, and homing endonuclease genes (Burt and Trivers, 2006; McLaughlin and Malik, 2017; Werren, 2011; Werren et al., 1988). Drawing inspiration from these natural systems, strategies for exploiting drive to alter the genetics of wild pest populations have been proposed (Burt and Trivers, 2006; Champer et al., 2016; Esvelt et al., 2014; McLaughlin and Malik, 2017; Werren, 2011; Werren et al., 1988), and some have even been experimentally tested in the laboratory, however none have been implemented in the field. For those tested in the laboratory, some examples include synthetic Medea elements (Akbari et al., 2014; A. Buchman et al., 2018; Chen et al., 2007), engineered underdominance systems (Akbari et al., 2013; A. B. Buchman et al., 2018), and those whose development was accelerated by the CRISPR revolution (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013) including toxin-antidote based systems (Oberhofer et al., 2019), and homing based gene drive systems (HGDs) (Champer et al., 2016; Esvelt et al., 2014; Gantz and Bier, 2016; Marshall and Akbari, 2018). HGDs are perhaps the furthest along in development, and in fact in addition to model systems these have already been tested in mosquitoes and even mammals (Champer et al., 2018, 2017; DiCarlo et al., 2015; Gantz et al., 2015; Gantz and Bier, 2015; Grunwald et al., 2019; Hammond et al., 2016, 2018; KaramiNejadRanjbar et al., 2018; Kyrou et al., 2018; Li et al., 2019; Windbichler et al., 2011; Yan and Finnigan, 2018). They function by encoding the Cas9 endonuclease and an independently expressed guide RNA (gRNA) responsible for mediating DNA/RNA base pairing and cleavage at a predetermined site (Champer et al., 2016; Esvelt et al., 2014; Gantz and Bier, 2016; Marshall and Akbari, 2018). When the HGD is positioned within its target site in a heterozygote, double stranded DNA breakage of the opposite chromosome can result in the drive allele being used as a template (i.e. donor chromosome) for DNA repair mediated by homologous recombination. This can result in copying, or “homing,” of the HGD into the broken chromosome (i.e. receiver chromosome), thereby converting heterozygotes to homozygotes in the germline, which can bias Mendelian inheritance ratios and result in an increase in HGD frequency in a population.

Given the recent progress toward developing HGDs in pest species such as mosquitoes (Gantz et al., 2015; Hammond et al., 2016, 2018, 2019; Kyrou et al., 2018; Li et al., 2019), there is significant enthusiasm regarding their potential use to control wild populations. For example, release of
HGDs linked with effector genes inhibiting mosquito pathogen transmission (Buchman et al., 2019a, 2019b; Isaacs et al., 2011; Jupatanakul et al., 2017) may lead to replacement of disease-susceptible mosquitoes with disease-resistant counterparts resulting in reduced pathogen transmission (i.e. population modification drive). Alternatively, HGDs targeting genes affecting the fitness of female mosquitoes could also spread, resulting in gradual population declines and potentially even elimination (i.e. population suppression drive) (Kyrou et al., 2018; Windbichler et al., 2011, 2008). Given these features, both modification and suppression drives possess the potential to transform mosquito population control measures (Burt, 2003; Champer et al., 2016; Esvelt et al., 2014), and therefore have excited significant ongoing discussions involving their potential usage, regulation, safety, ethics and governance (Adelman et al., 2017; Akbari et al., 2015; National Academies of Sciences, Engineering, and Medicine et al., 2016; Oye et al., 2014). Despite these exciting developments however, the elephant in the room persists - can a gene drive actually work in the wild? There are a number of open questions looming as to the efficiency of a HGDs. For example, can a drive spread to fixation in the wild? Will it simply breakdown due to resistance? Will the linked anti-pathogen effector work efficiently given the expected diversity of parasites/virus genomes found in the wild? Can the pathogen evolve to become resistant to the anti-pathogen effector and perhaps even become more virulent? These are just a minority of legitimate concerns regarding the potential use of a gene drive that would need to be resolved prior to any release.

While many questions loom, there has been some effort to resolve these concerns safely in the lab. For example, with regard to the HGD breakdown due to resistance, multiple studies have explored design criteria attempting to suppress the effects of resistance alleles on drive propagation. For example, some studies have had some success using germline-restricted promoters to express Cas9 increasing rates of HDR, resulting in increased homing rates, as opposed to error-prone pathways such as non-homologous end joining (NHEJ) which results in the generation of resistant alleles (Champer et al., 2018; Hammond et al., 2018). Other studies have described (Champer et al., 2016; Esvelt et al., 2014; Marshall et al., 2017) and tested (Champer et al., 2018; J. Champer et al., 2019; S. E. Champer et al., 2019; Oberhofer et al., 2018) multiplexed gRNAs in drives resulting in moderate increases in drive efficacy. While others have had some success targeting highly conserved recessive fertility/viability genes whose homozygous mutants are inviable, or cannot reproduce, and therefore are expected to not affect the spread of HGDs (Hammond et al., 2016; KaramiNejadRanjbar et al., 2018; Kyrou et al., 2018; Oberhofer et al., 2018). However, despite these efforts, resistance alleles are still problematic, leaving open the question as to what is the best method to prevent their generation.

Here, to further explore this paramount issue of resistance to HGD we use Drosophila melanogaster as our model. We use a genetic safeguarded split-drive design as a safety feature and also encode a linked effector to the drive. This effector consisted of a second gRNA engineered to target a separate host encoded gene which we term a gRNA-mediated effector (GME) (Fig. S1A–B). Given that there are many host-encoded genes that are important for
pathogen transmission (Cheng et al., 2016; Dong et al., 2018), one potential application of a HGD is to incorporate a cargo GME that targets a host encoded factor that is important for some aspect of pathogen transmission. If the GME is effective, then disruption of its target in the population should in principle occur as the drive spreads, thereby immunizing that population from pathogen transmission. Therefore, encoding a GME in a drive may be useful feature going forward and worth further exploring. As a proof of concept to test the efficiency of a HGD linked GME, we designed both the drive and effector to target phenotypic genes which resulted in easily scorable recessive viable phenotypes. This novel drive architecture enabled us to test many germline Cas9 expressing promoters, while simultaneously measuring homing and cleavage efficiencies in both the germline and soma for both target genes over successive generations. While homing rates were modest, cleavage rates were high. For example, we determined that we can reproducibly achieve complete penetrance of somatic mosaic phenotypes for both target genes with up to 100% efficiency stemming from a combination of Cas9 maternal deposition and embryonic expression. However, despite the robust cleavage efficiencies and impressive efficacy of the HGD linked GME, drive resistance alleles were still generated which would hinder spread. Given these results, alternative design principles are proposed that could potentially mitigate these issues while also incorporating a drive linked GME (Fig. S2).

Results

Design of split-HGD encoding two gRNAs

To assess the feasibility and efficiency of utilizing a HGD to bias transmission while also expressing a GME, we designed a HGD that expressed two gRNAs (Lopez del Amo et al., 2019). The homing component of the split-HGD system, referred hereon as a Gene Drive element (GDe), encodes a gRNA targeting white (gRNA\textsuperscript{w}, driver gRNA), a separate cargo GME targeting yellow (gRNA\textsuperscript{y}, effector), a 3xP3-\textit{eGFP} dominant marker, all together flanked by 1kb homology arms from the white target locus to direct targeted HDR mediated integration (Fig. S1A). The GDe was integrated at the white locus (\textit{wGDe}) in \textit{D. melanogaster} via HDR (Fig. S1B). In the presence of Cas9, the GDe directs cleavage at both white and yellow, both X-linked loci, and is also capable of homing into the white locus (Fig. 1A). Importantly, in \textit{D. melanogaster} homozygous loss-of-function (LOF) mutants of both white and yellow are viable and fertile with scorable recessive phenotypes in the eye and body, respectively, enabling cleavage events to be directly quantified over successive generations. Additionally, males have only one X chromosome, and are therefore hemizygous for white and yellow, restricting the quantification of homing to heterozygous females (y–,\textit{wGDe}/y+,w+, Fig. 1A).

High penetrance of \textit{F}_1 somatic mutations generated by Cas9 through both maternal deposition and embryonic expression

To explore the effects of tissue specificity and timing of Cas9 expression on cleavage and homing in the germline, we used four separate promoters with distinct expression profiles to
express Cas9-T2A-GFP: nanos (nos) (Van Doren et al., 1998) and vasa (vas) promoters known for early germline-limited expression (Hay et al., 1988; Sano et al., 2002; Van Doren et al., 1998); Bicaudal C (BicC) promoter supporting later germline-limited expression (Saffman et al., 1998); and Ubiquitin 63E (Ubi) promoter with strong expression in both somatic and germ cells (Akbari et al., 2009; Preston et al., 2006). To control for position effect variegation (PEV), each Cas9 construct (Fig. S1A) was integrated site specifically on the 3rd chromosome using φC31-mediated integration (Groth, 2004). To confirm germline expression, we imaged the expression of a self-cleaving T2A-eGFP tag attached to the coding sequence of Cas9, and each promoter robustly expressed GFP in the ovaries (Lowest -Nanos-Cas9 < Vasa-Cas9 <Ubi-Cas9 <BicC-Cas9 Highest). (Fig. S3).

To quantify cleavage efficiencies, we performed bi-directional crosses between hemizygous or homozygous GDe lines mated to heterozygous Cas9 lines (Fig. 1B). From these crosses we determined that maternally deposited Cas9 protein is sufficient to induce both yellow and white somatic LOF mutations in F1 females heterozygous for the GDe both in presence (♀# 2; y–,wGDe/y+,w+; Cas9/+; Fig. 1B) and in the absence (♀ # 1; y–,wGDe/y+,w+; Fig. 1B) of Cas9 gene inheritance. To determine whether embryonic expression of Cas9 can also induce somatic mutations, we scored white and yellow LOF somatic mutations in F1 trans-heterozygous females inheriting Cas9 exclusively from their fathers (i.e. paternal Cas9). Unexpectedly, F1 trans-heterozygous female progeny inheriting Cas9 as a gene (♀#4; y–,wGDe/y+,w+; Cas9/+; Fig. 1B) from their fathers had mutations in both white and yellow with varying frequencies depending on which promoter drove Cas9 expression. For example, nos-Cas9 and vas-Cas9 – induced 100% white and yellow LOF somatic mutations in F1 trans-heterozygous females, while Ubi-Cas9 resulted in 100% of white and 91.3% ± 9.7% of yellow LOF somatic mutations, and BicC-Cas9 resulted in only white LOF mutations in 64.3% ± 2.6% of the F1 y–,wGDe/y+,w+; BicC-Cas9/+ progeny (Fig. 1B). Interestingly however, 100% of F1 heterozygous female progeny from the same fathers that did not inherit Cas9 as a gene (♀#3; y–,wGDe/y+,w+; +/+; Fig. 1B) had wild type (wt) phenotypes, for both white (red eyes) and yellow (brown body), presumably resulting from lack of sufficient Cas9 protein deposited paternally to induce mutations in the zygote (Table S1). Taken together these data strongly indicate that the Cas9 promoters tested here are highly active both maternally and embryonically and can promote very high cleavage efficiency.

To determine whether yellow and white alleles were mutated by maternally deposited Cas9 in germ cells of F1 y–,wGDe/y+,w+ females, we mated these females to y+,w– males and scored recessive yellow phenotypes in resulting F2 male progeny (y–) and recessive white phenotypes in resulting F2 male and female progeny (w–/w–). Interestingly, we found that maternally deposited Cas9 protein expressed under nos and vas promoters did not induce yellow LOF mutations in germ cells of F1 females, while expression from Ubi and BicC promoters resulted in 26% ± 15% and 89.4% ± 9.4% of yellow alleles being mutated in germ cells of F1 females, respectively, perhaps due to a stronger maternal deposition of Cas9 protein by these promoters (Fig. S3) combined with possible prefentail gRNA loading by Cas9 (Fig. 1B). Despite the lack of LOF
germline mutations in yellow by nos and vas, every tested Cas9 line provided a sufficient amount of maternally deposited Cas9 protein to knocking out the white allele in 94.9% ± 4.5% to 98.8% ± 1.1% of F1 germ cells (measured in F2 progeny). To molecularly confirm whether the w+ alleles (1.2% – 5.1%) were cut by Cas9 and perhaps repaired into cleavage resistant alleles, we Sanger sequenced PCR amplicons of the white target locus from individual male flies. Each tested F2 male with red eyes (w+) indeed had a wt w+ allele, however we did not find any white in-frame functional resistant alleles in F1 germ cells suggesting that these alleles were likely remained uncut in the germline.

Maternally deposited Cas9 is sufficient to induce homing of GDe in germ cells

The Cas9/gRNA w-induced DSBs at white locus can be repaired either by HDR resulting in homing of the GDe (wGDe/wGDe) or NHEJ incorporating indel mutations that can render the target locus unrecognizable by the Cas9/gRNA w machinery, and when these mutations occur in germ cells they are referred to as resistance alleles (wR): here LOF and in-frame functional resistance alleles are referred as wR2 and wR1, respectively (Fig. S1C). To directly estimate the frequency of wGDe homing and wR generation in the absence of additional somatic mutations resulting from embryonic expression of Cas9, we analyzed white phenotypes in the F2 progeny of the F1 wGDe/w+ females with maternally deposited Cas9 in a w– recessive mutant background (Fig. 1B). Every tested Cas9 promoter provided a sufficient amount of maternally deposited Cas9 in the F1 germ cells to enable the conversion of 59% – 72% of w+ alleles into wGDe (i.e. homing of GDe) in y–,wGDe/y+,w+ females. This conversion which occurs in the presence of Cas9 protein, but absence of inheritance of the Cas9 gene, was previously noted and termed “shadow drive” (Guichard et al., 2019). The remaining DSBs at w+ alleles were repaired by NHEJ and generated around 38% – 23% wR2 alleles (Fig. 1B). To explore molecular changes at white locus, we PCR amplified and Sanger sequenced wR2 alleles from individual F2 male progeny and identified indels localized at the white cut site in each sequenced male (Fig. S4A). The maternally deposited Cas9 by BicC promoter resulted in the lowest homing and the highest resistance allele rates (59.3% ± 12.3% and 38.7% ± 13.7%, respectively), though no significant difference was identified between each pairwise comparison. Nevertheless, each tested promoter supplied Cas9 protein to the progeny that enabled shadow drive, and thus resulted in super-Mendelian propagation of wGDe in their grandchildren.

Maternal deposition of Cas9 protein reduces the homing efficiency

Maternally deposited Cas9 can induce white cleavage and repair mediated by NHEJ as opposed to HDR in mitotically dividing germ cells which can result in a bias toward generating resistance alleles (wR2 and wR1) at the expense of homing wGDe. To explore this effect, we compared homing rates between F1 trans-heterozygous females that inherited Cas9 either maternally (♀#2; y–,wGDe/y+,w+; Cas9/+;  Fig 1B). For nos-Cas9, vas-cas9, and BicC-Cas9, maternal deposition of Cas9 did not result in a significant bias in homing efficiencies. However, for Ubi-Cas9 homing rates were significantly lower (67%) in the trans-
heterozygous females that inherited Cas9 maternally (♀ #2; w<sup>GDe</sup>/w+; Ubi-Cas9/+; Fig. 1B). In addition to the lower homing rates for Ubi-Cas9, the rate of w<sup>R2</sup> alleles was significantly higher with maternally deposited Ubi-Cas9 as compared to paternally deposited Ubi-Cas9: 9.9% ± 5.7% vs 27.3% ± 10.0%, P > 0.025, vs 26.5% ± 4.4%, P > 0.029, respectively (Fig. 1B). Taken together, these results suggest that strong maternal deposition of Cas9 protein into developing oocytes can result in white cleavage in mitotic cells, prior to developmental stages where efficient HDR repair occurs, therefore leading to a higher w<sup>R</sup> frequency.

**Resistance alleles accumulate between F<sub>2</sub> and F<sub>3</sub> generations**

Resistance alleles generated in germ cells are immune to subsequent cleavage by the Cas9/gRNA<sup>w</sup> complex. Drosophila white and yellow LOF homozygotes are viable and fertile, as a result, the frequency of resistance alleles can potentially increase from generation to generation. To explore this possibility, we crossed F<sub>2</sub> trans-heterozygous females (♀ #6, w<sup>GDe</sup>/w+; Ubi-Cas9/+; Fig. 1C) to wt (y+, w+) males, and scored their F<sub>3</sub> progeny for yellow and white phenotypes, as well as for inheritance of the GDe. Indeed, the frequency of white LOF mutations (w<sup>R2</sup>) increased significantly between F<sub>2</sub> and F<sub>3</sub> progenies for each Cas9 promoter: 11.2% ± 6.2% vs 81.7% ± 7.5% for nos-Cas9; 13.2% ± 5.6% vs 82.4% ± 10.4% for vas-Cas9; 18.6% ± 12.0% vs 84.6% ± 9.5% for Ubi-Cas9; and 36.7% ± 7.5% vs 81.6% ± 7.1% for BicC-Cas9, P > 0.0001, respectively. This rise of w<sup>R2</sup> frequency negatively affected the homing rate, as it plummeted between F<sub>2</sub> and F<sub>3</sub> generations: from 80.0% ± 7.7% to 11.3% ± 4.8% for nos-Cas9; from 80.2% ± 7.4% to 10.8% ± 10.4% for vas-Cas9; from 78.0% ± 13.2% to 7.4% ± 8.4% for Ubi-Cas9; and from 53.9% ± 9.8% to 7.6% ± 6.2% for BicC-Cas9 (♀ #6, w<sup>GDe</sup>/w+; Ubi-Cas9/+; Fig. 1C). To avoid any ambiguity caused by somatic expression of Cas9, the same analysis was repeated with the F<sub>2</sub> heterozygous females carrying maternally deposited Cas9 protein but lacking the Cas9 gene resulting in similar conclusions (♀ #5, y–, w<sup>GDe</sup>/y+, w+; Fig. 1C). To assess the accumulation of resistance alleles, we compared mean frequencies of homing and resistance alleles between F<sub>2</sub> and F<sub>3</sub> generations. The frequency of resistance alleles rose from 28.5% ± 12.2% to 92.6% ± 5.0% in heterozygous females or from 19.9% ± 12.8% to 82.6%±8.2% in trans-heterozygous females, and resulted in the decrease of homing rate from 69.0% ± 10.8% to 6.1% ± 4.2% or from 73.0%±14.6% to 9.2% ± 7.5%, respectively (P > 0.0001, Fig. 1D). As expected, the frequency of LOF resistance alleles at white locus (w<sup>R1</sup>) also increased from F<sub>2</sub> to F<sub>3</sub> progenies and restricted further homing of the GDe. The frequency of in-frame functional white and yellow mutations (w<sup>R1</sup> and y<sup>R1</sup>) could also increase in the F<sub>3</sub> progeny, but unfortunately it could not be directly estimated. The white cleavage frequency significantly decreased in the F<sub>3</sub> progeny of F<sub>2</sub> y–, w<sup>GDe</sup>/y+, w+; Ubi-Cas9/+ females, and could be explained by the increase of w<sup>R1</sup> allele rate that were indistinguishable from w+ alleles phenotypically: from 3.4% ± 2.6% in F<sub>2</sub> to 9.7% ± 3.1% in F<sub>3</sub>, P > 0.004 (Fig. 1B–C). To further explore this possibility, we Sanger sequenced F<sub>3</sub> wt males with red eyes and brown bodies, and identified in-frame indels and substitutions in the majority of tested males for each Cas9 promoter (w<sup>R1</sup> and
alleles, Fig. S4). Therefore, many germ cells of F₂ trans-heterozygous and heterozygous with maternally deposited Cas9 females has indel mutations at white and yellow loci (y⁻,w⁻\textsuperscript{GDe} \textsuperscript{R},w⁻\textsuperscript{R}) that were indeed resistant to further cleavage by Cas9/gRNA\textsuperscript{w} and Cas9/gRNA\textsuperscript{y}, respectively.

Discussion

Homing based gene drives require efficient cleavage and copying in the germline in order to bias their transmission and are therefore sensitive to both existing and induced target sequence variation. In fact, the NHEJ-mediated generation of resistance alleles in germ cells was previously identified as the major force opposing the spread of HGD into populations (Champer et al., 2017; Gantz et al., 2015; Hammond et al., 2017; Oberhofer et al., 2018). Here, in a split-drive design we further explored the effect of timing and expression of Cas9 on both homing and cleavage efficiencies. Additionally, we linked a GME to the drive to measure the efficacy of this approach. This drive architecture enabled us to draw several conclusions including: i) expression of a drive mediating gRNA in addition to a linked GME can result in 100% penetrance of both scorable LOF phenotypes; ii) each tested Cas9 expression promoter (nos, vas, BicC, Ubi) also results in significant embryonic expression; iii) Cas9 maternal protein deposition or embryonic expression are both sufficient for homing; iv) maternal deposition of Cas9 protein is not required for homing in females; v) paternal Cas9 protein deposition in the sperm is insufficient for either homing or cleavage; vi) resistant alleles accumulate over subsequent generations which are predicted to impair the spread of the drive. Below we discuss these conclusions further and also propose novel drive architectures to potentially overcome these issues.

Somatic expression of Cas9 results in high mutagenesis rates

The maternal deposition and embryonic expression of Cas9 in the presence of a gRNA transgene were previously shown to induce LOF mutations in F₁ progeny from a cross using nos- or vas-driven Cas9 and U6-gRNA lines (Kandul et al., 2019; Lin and Potter, 2016; Oberhofer et al., 2018; Port et al., 2014); however, the somatic nature of F₁ LOF mutations was not fully explored. This is in part due to the fact that when Cas9 and gRNA are linked together in the single-locus HGD, somatic and germline LOF mutations are not easily distinguishable from heritable mutations occuring in prior generations which can result in overestimation of homing rates. Therefore, unlinking these components enables a better method for carefully distinguishing these events. Here, using a split-drive design, we were able to carefully assess the effects of timing, expression and inheritance of Cas9 on both homing and cleavage efficiencies. As reported previously, we found that maternal Cas9 protein deposition was sufficient to induce shadow drive (Guichard et al., 2019) in addition to high rates of F₁ somatic LOF mutations (Kandul et al., 2019; Lin and Potter, 2016; Oberhofer et al., 2018; Port et al., 2014). However, unexpectedly we found that embryonic expression of Cas9 was also sufficient to induce cleavage and homing. In fact, both nos and vas promoters, which were previously characterized by early germline-limited expression (Sano et al., 2002; Van Doren et al., 1998), do support significant embryonic expression of Cas9. For example, F₁ progeny with either maternally deposited Cas9
protein, or embryonically expressed Cas9 gene inherited from their fathers, both had moderate rates of HDR in addition to white and yellow LOF somatic mutations with up to 100% efficiency (Fig. 1B); consistent observations were generated in a recent work using a trans-complementing Gene Drive (tGD) system (Lopez del Amo et al., 2019). These data conclusively demonstrate that Cas9 is embryonically expressed and that embryonic expression is sufficient to generate high rates of cleavage and moderate rates of HDR. Given this high penetrance of cleavage, this data suggest that effectors that mechanically rely on cleavage (i.e. GME’s) could indeed be quite effective if linked to an efficacious drive.

**Resistant alleles accumulate over subsequent generations**

Consistent with previous studies, we also found that maternal deposition of Cas9 protein into the embryo can result in both cleavage and homing in the germ cells (Guichard et al., 2019). In addition to this observation, we also found that paternal Cas9 protein deposition was not sufficient to induce either cleavage or homing, presumably due to the low quantities of Cas9 carried by the sperm into the egg. Moreover, we determined that maternal deposition is not a requirement for homing, and in fact females that inherit the Cas9 gene paternally can indeed express Cas9 embryonically resulting in both cleavage and homing. Notwithstanding, regardless of whether Cas9 was maternally or paternally inherited, there was no significant difference between the rates of appearance of drive resistance alleles which were generated at moderate frequencies in the F2. Interestingly, the frequency of resistance alleles (w^R) increased dramatically between F2 and F3 generations and correlated with decreases in homing (Fig. 1D). Taken together, these results suggest that homing occurs post embryonically and requires sufficient Cas9 to be present in the egg via either maternal deposition, or embryonic expression, both of which lead to the generation and accumulation of resistant alleles predicted to impede the spread of the drive.

**Novel strategies for disarming resistant alleles in germ cells**

The accumulation of drive resistant alleles reported here was in part due to the fact that white is recessive viable, enabling targeted drive resistant alleles to accumulate. Given this accumulation, perhaps targeting non-essential genes using HGD may not be ideal. To avoid this issue, targeting essential genes would be a more appropriate design to ensure gene drive stability and spread. By targeting essential genes, it is more possible that non-drive alleles could be actively selected against using a phenomenon previously termed lethal mosaicism (Guichard et al., 2019; Kandul et al., 2019) or by natural selection due to increased fitness costs. Lethal mosaicism results in dominant biallelic knockouts of target genes throughout development which could eliminate cleavage resistant alleles as they would be non-viable. We envision two novel drive design architectures that incorporate a GME and rely on lethal mosaicism to limit the generation of resistant alleles. First, haplo-sufficient genes essential for insect viability or fertility can be targeted by HGD designed to express a recoded version of the disrupted gene that is resistant to gRNA-mediated cleavage in addition to a linked GME (HGD+R+GME). This ensures that only the progeny that
inherit the HGD+R+GME survive, while all progeny that inherit a cleaved allele perish due to non-rescued lethal mosaicism (Fig. S2). Second, a cleavage-only gene drive with rescue could be designed that incorporates a GME (CGD+R+GME) which mechanistically relies exclusively on cleavage for biased inheritance and selection against drive resistant alleles (Fig. S2). Both of these strategies would likely be effective to limit the accumulation of drive resistance alleles. However, in-frame functional mutations (RI type) that confer resistance against the Cas9/gRNA and do not cause fitness costs carriers may still be generated which could limit the spread of a drive. To summarize, our results demonstrate that inserting a GME into a HGD efficient knockouts of multiple genes can be achieved while simultaneously biasing GDe transmission rates into subsequent generations. However, resistant alleles were generated, and accumulated, which would limit the efficacy and spread of this system. To overcome these limitations, novel drive architectures are proposed and remain to be tested in future studies.

Materials and methods

Design and assembly of constructs

The genetic assembly of the Gene Drive element with gRNAs and 3xP3-eGFP (GDe) and employment of the $w^{GDe}$/$w^{GDe}$ which was previously used in for a different purpose by (Lopez del Amo et al., 2019) to generate a split trans-complementing Gene Drive system. The assembly of BicC-Cas9 construct followed the same steps previously described for the other three Cas9 lines: nos-Cas9, vas-Cas9, and Ubi-Cas9 (Kandul et al., 2019). The 2831 bases upstream of BicC-RA’s start codon (Bicaudal C, CG4824) was PCR amplified with CGACGGTCACGGCGGGCATGTCGACGCGGCCGCATAATTATATAATAAACTGCATGC (BicC-F) and TCCGTCGTGGTCCTTATAGTCCATGTTTAAACTGTGGAATTCGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT (BicC-R) from Drosophila melanogaster genome, and enzymatically assembled (Gibson et al., 2009) into Ubi-Cas9 plasmid (addgene #112686) (Kandul et al., 2019) digested with NotI and XhoI.

Fly genetics and imaging

Flies were maintained under standard conditions at 25°C. Embryo injections were carried at Rainbow Transgenic Flies, Inc. (http://www.rainbowgene.com). The BicC-Cas9 construct was inserted at the PBac{y+-attP-3B}KV00033 on the 3rd chromosome (Bloomington #9750) with φC31-mediated integration (Groth, 2004). Transgenic flies were balanced with Df(3L)R/TM6C,cu1,Sh1,Tb1 (Bloomington #57) and CxD,ryBM/TM3, Sb1,Ser1 (Bloomington #1704) in the $w^+$ genetic background.
To assess the cleavage rates and homing efficiencies of the split-HGD system, we genetically crossed the GDe line to four different Cas9 lines in both directions. Two types of F1 trans-heterozygous y−,wGDe(eGFP)/y+,w+; Cas9(RFP)/+ females carrying either maternal or paternal Cas9 (F1 ♀ #2 or ♀ #4, respectively) and the F1 heterozygous y−,wGDe(eGFP)/y+,w+ females with maternally deposited Cas9 were generated (F1 ♀ #1, Fig. 1B). Their yellow and white LOF mutations and transgene markers were scored. To explore whether yellow and white loci were also mutated in the germ cells of the F1 trans-heterozygous and heterozygous females, we genetically crossed them to w+,y+ and w−,y+ males, respectively, and examined their F2 progeny. LOF yellow mutations were scored only in male progeny that inherited their single X chromosome from mothers. To explore the behaviour of resistance alleles over multiple generations, the F2 trans-heterozygous and heterozygous virgin female (♀ #6 or ♀ #5, respectively) progeny of F1 ♀ #2 were also collected, and genetic crosses and phenotype scoring were repeated for an additional generation, F3. The above crossing schemes are depicted in Fig. 1B. To generate means and standard deviations for statistical comparisons, each genetic cross was set up in triplicate using 10♂ and 10♀ flies for each replicate cross. Cleavage and homing frequencies are presented as percentages of y+ and w+ alleles in heterozygous females, aka. they normalized to 50% (Table S1).

Flies were examined, scored, and imaged on the Leica M165FC fluorescent stereo microscope equipped with the Leica DMC2900 camera. To analyze Cas9 expression in ovaries of four homozygous Cas9 lines, their ovaries were dissected in PBS buffer, examined, and imaged utilizing the same settings. The eGFP fluorescence was used as a proxy of Cas9 expression, since it was tagged to Cas9 transgene as via a T2A sequence (Fig. S3).

Genotyping loci targeted with gRNAs.

To explore the molecular changes that caused LOF and in-frame functional mutations in yellow and white loci, we PCR amplified the genomic regions containing target sites for gRNAw and gRNAy:

\[
\text{GGCGATACTTGGATGCCCTCGG and GTTTTTGGACACTGGAACCGTGG, respectively.}
\]

Single-fly genomic DNA preps were prepared by homogenizing a fly in 30µl of a freshly prepared squishing buffer (10mM Tris-Cl pH 8.0, 1mM EDTA, 25mM NaCl, 200 µg/mL Proteinase K), incubating at 37°C for 35 minutes, and heating at 95°C for 2 minutes. 2 µl of genomic DNA was used as template in a 40 µL PCR reaction with LongAmp® Taq DNA Polymerase (NEB). The 415bp PCR fragment of white target was amplified with CGTTAGGGAGCCGATAAAAGAGGTCATCC (w.sF) and AAGAACGCTGATTTCTATTCCGAGTGCG (w.sR); and CACTCTGACCTATAAAGATGGACCAGCTTG (y.sF) and CCAATTCTACGGCAAATAGGGATATGCAT (y.sR) primers were used to amplify the 375bp PCR fragment of yellow. PCR aplicons were purified using QIAquick PCR purification kit (QIAGEN), and sequenced in both directions with Sanger method at Source BioScience. To characterize molecular changes at the targeted sites, sequence AB1 files were aligned against the corresponding reference sequences in SnapGene® 4.
Statistical analysis

Statistical analysis was performed in JMP 8.0.2 by SAS Institute Inc. At least three biological replicates were used to generate statistical means for comparisons. To estimate the effect of Cas9 maternal deposition on homing efficiency, rates of cleavage, homing, and resistance alleles in F1 ♀ #4 with paternal Cas9 were compared to the corresponding values in F1 ♀ #1 and ♀ #2 with maternally deposited Cas9 protein (Fig. 1B). To assess the significance of resistance allele accumulation and homing rate decline between F2 and F3 generations, rates of cleavage, homing, and resistance alleles in F2 ♀ #5 and F2 ♀ #6 (Fig. 1C) were compared to the corresponding values in F1 ♀ #1 and F1 ♀ #2, respectively (Fig. 1B). P values were calculated for a two-sample Student’s t-test with equal variance.

Gene Drive safety measures

All crosses using gene drives genetics were performed in accordance to an Institutional Biosafety Committee-approved protocol from UCSD in which full gene-drive experiments are performed in a high-security ACL2 barrier facility and split drive experiments are performed in an ACL1 insectary in plastic vials that are autoclaved prior to being discarded in accord with currently suggested guidelines for laboratory confinement of gene-drive systems (Akbari et al., 2015; National Academies of Sciences, Engineering, and Medicine et al., 2016).

Ethical conduct of research

We have complied with all relevant ethical regulations for animal testing and research and conformed to the UCSD institutionally approved biological use authorization protocol (BUA #R2401).

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Author Contributions

O.S.A, and N.P.K. conceptualized the study. A.B. and V.M.G. designed and assembled constructs for this project. N.P.K. and J.L. performed molecular and genetic experiments. All authors contributed to the writing, analyzed the data, and approved the final manuscript.

Competing Interests

V.M.G., E.B., and O.S.A have an equity interest in Agragene, Inc. and serve on the company’s Scientific Advisory Board; V.M.G. and E.B. have an equity interest in Synbal, Inc. and serve on
the company’s Scientific Advisory Board.; V.M.G. and E.B. also serve on both companies' Board of Directors; these companies may potentially benefit from the research results. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies.

Figures
Fig. 1. Split-HGD system forces super-Mendelian transmission and knocks out multiple genes. (A) Gene Drive element (GDe) with two gRNAs targeting yellow and white genes, and 3xP3-eGFP marker gene was interated via HDR at the white cut site on X sex chromosome (wGDe). SpCas9 (Cas9) endonuclease expressed with one out of four promoters – nanos (nos), vasa (vas), Bicaudal C (BicC), and Ubiquitin 63E (Ubi) – and Opie2-dsRed marker gene (Fig. S1A) were integrated at the same site on the 3rd chromosome. In heterozygous y+,wGDe/y+,w+; Cas9/+ females generated by a genetic cross between the wGDe line and a Cas9 line, GDe
induces DSBs in $\gamma^+$ and $w^+$ alleles resulting in cleavage of both genes and copying itself into the cut $w^+$ allele via HDR (i.e. homing). The $w^{GDe}$ allele cannot home in $Drosophila$ males, because they have only one X chromosome, aka. hemizygous. (B) Each tested Cas9 promoter, including previously characterised germline-limited $nos$, $vas$, and $BicC$ promoters, supported Cas9 expression in $F_1$ somatic tissues and resulted in white and yellow loss-of-function (LOF) mutations in the $F_1$. Furthermore, maternal deposition of Cas9 protein alone was sufficient to generate $F_1$ somatic white and yellow LOF mutations as well as induce both homing ($w^{GDe}$) and formation of resistance alleles ($w^{R2}$) in $w^+$ alleles of their germ cells ($F_1 \ \#1$). Paternal deposition of Cas9 protein did not induce mutations in somatic or germ cells ($F_1 \ \#3$). Notably, while 100% $F_1$ parents had yellow LOF somatic mutations with each tested Cas9 line, only $Ubi$ and $BicC$ promoters deposited Cas9 protein sufficient to induce yellow LOF mutations in some germ cells ($F_1 \ \#1$). Embryonic expression of Cas9 gene under $nos$, $vas$, and $Ubi$ promoters induced white and yellow LOF mutations in 100% trans-heterozygous females, while embryonic expression of $BicC-Cas9$ caused only white LOF mutation in 64.3% ± 2.6% of trans-heterozygous females ($F_1 \ \#4$). Rates of homing and resistance alleles were not significantly different among two types of trans-heterozygous ($F_1 \ \#2$ and $\#4$) and heterozygous ($\#1$) females with maternally deposited Cas9. Only maternal deposition of Cas9 under $Ubi$ promoter negatively affected homing rates (green arrows) in germ cells. (C) Resistance alleles are expected to be immune to the further cleavage by the same Cas9/gRNA system and if their carrier are fertile can propagate at the expense of homing. To explore this idea, $F_2 \ \#5$ and $F_2 \ \#6$ collected among progeny of $F_1 \ \#2$ were genetically crossed with $w^-$ and $w^+$ males, respectively, and their $F_3$ progeny were scored. While the cleavage rate in $F_2$ germ cells decreased only in $F_2 \ \#6$ with $Ubi-Cas9$ (red arrow) likely due to the rise of functional $w^{R1}$ alleles, the homing frequency fell significantly for each tested split-HGD system with and without Cas9 gene (green arrows). The fall of homing rate was accompanied by the accumulation of the $w^{R2}$ alleles. (D) Accumulation of $w^{R2}$ alleles resistant to cleavage by Cas9/gRNA suppressed homing of GDe. Frequencies of homing and resistance alleles were averaged for all tested promoters and presented separately for progeny of heterozygous and trans-heterozygous females, $F_2 \ \#5$ and $F_2 \ \#6$, respectively. Resistance allele frequency increase from 28.5% or 19.9% to 92.6% or 82.6% between $F_2$ and $F_3$ (blue arrows) and caused the dramatic decline in homing from 69.0% or 73.0% to 6.1% or 9.2% (green arrows). Notably, scoring of $w^{R2}$ alleles in $w^-$ recessive background resulted in the higher estimation of white LOF mutations alleles, since $w^{R2}$ alleles were complemented by $w^+$ alleles inherited from wild type males. Bar plots show the average ± SD over at least three biological replicate crosses. Statistical significance was estimated using a $t$ test with equal variance. ($P \geq 0.05^{ns}$, $P < 0.05^*$, $P < 0.01^{**}$, and $P < 0.001^{***}$).
Supplementary Fig. S1. Development of the CRISPR/Cas9-mediated split-HGD system.

The HGD system was split into two components: Gene Drive element (GDe) and Cas9 endonuclease (Cas9). (A) Schematic maps (not to scale) of genetic constructs used to assemble split-HGD systems. The GDe contains two guide RNAs (gRNAs) targeting the DNA cleavage at white and yellow loci, and an eye-specific marker (3xP3-GFP) all surrounded by Left and Right Homology Arms (LHA and RHA) complementary to the white cut site. Four Cas9 constructs expressing SpCas9 (Cas9) in early germline cells with nanos (nos) and vasa (vas) promoters, in late germ cells with Bicaudal C (BicC) promoter, and in both germ and somatic cells with Ubiquitin 63E (Ubi) promoter were integrated at the same appB site on the 3rd chromosome. To track Cas9 expression, its coding sequence was linked to eGFP via a self-cleaving T2A sequence. Cas9 constructs also carry a body specific marker of transgenesis (Opie2-DsRed). (B) GDe was site-specifically inserted at white locus on X chromosome in Drosophila via HDR-mediated integration, w$^{GDe}$. In the presence of Cas9, GDe direct cleavage at both white and yellow loci and can home at white locus from the carrier allele into a naive allele via HDR in heterozygotes (Fig. 1A). (C) Each element is inactive on its own and can be maintained as a homozygous parental line (P). The cross between the homozygous lines results in 100% trans-heterozygous F1 progeny that carry both elements. gRNA$^w$ expressed by GDe directs cleavage at white locus by Cas9, which can be repaired in three different ways: via HDR using GDe as a repair template and result in homing of GDe; and via Non-Homologous End Joining (NHEJ) and lead to white loss-of-function resistance (w$^{R2}$) allele or an in-frame functional resistance (w$^{R1}$) allele (Fig. S4).
Supplementary Fig. S2. gRNA-mediated Effector (GME) incorporated into two novel gene drive designs mechanistically based on lethal biallelic mosaicism. (A) Schematic of Homing Gene Drive targeting an essential gene with a recoded Rescue and GME (HGD+R+GME). The HGD+R+GME expresses Cas9 and two gRNAs targeting an essential gene (EG) and host gene (HG), a marker gene (eGFP), and the cleavage-resistant recorded portion of the essential gene that is being targeted by the gRNA/Cas9 complex (rescue), which can rescue the knockout phenotype, flanked by Left and Right Homology Arms (LHA and RHA). Mechanistically, once HGD+R+GME is integrated precisely inside the EG it will direct cleavage of the EG\textsuperscript{wt} allele on a receiver chromosome, and induce knockout mutations that will either result in lethal biallelic mosaicism, or convert the receiver chromosome into EG\textsuperscript{HGD+R+GME} via homology directed repair (HDR). This ensures that only the progeny that inherit EG\textsuperscript{HGD+R+GME} survive, while all progeny that inherit a cleaved allele perish due to non-rescued lethal mosaicism. In addition, the HGD+R+GME induces knockout of HG located on another (or the same) chromosome, leading to desired phenotype (i.e. pathogen resistance) to its carriers. The Punnett square below depicts the genetics of how HGD+R+GME achieves a 100% transmission rate and refractoriness in F\textsubscript{1} progeny. Female heterozygous for HGD+R+GME maternally deposits Cas9/gRNA complexes into every oocyte knocking out both EG and HG, and only zygotes that inherit the HDR+R+GME would survive as F\textsubscript{1} progeny. Notably, HDR will convert EG\textsuperscript{wt} alleles into EG\textsuperscript{HGD+R+GME} alleles and further increase numbers of surviving F\textsubscript{1} progeny and this non-Mendelian inheritance rate will depend on homing efficiencies. (B) Schematic of Cleavage-only Gene Drive targeting essential gene with recoded Rescue and GME (CGD+R+GME). The CGD+R+GME expresses Cas9 with multiple gRNAs targeting an ES (gRNA.1) and HG (gRNA.2), a marker gene (eGFP), and the cleavage-resistant recorded essential gene (recue) integrated at a separate genomic location from the target gene. Mechanistically, a CGD+R+GME drive relies exclusively on cleavage with no HDR required for biased inheritance. A Punnett
Supplementary Fig. S3. Fluorescent microscopy imaging of relative amount of maternally deposited Cas9-T2A-eGFP protein in four homozygous lines expressing Cas9 under different promoters. Nanos (nos-Cas9), vasa (vas-Cas9), Ubiquitin-63E (Ubi-Cas9), and Bicaudal C (BicC-Cas9) constructs (Fig. S1A) were inserted at the same site on the 3rd chromosome using φC31-mediated integration. A self-cleaving T2A-eGFP sequence, which was attached to the 3’-end of Cas9 coding sequence, provided an indicator of Cas9 expression (Fig. S1A). Expression levels of eGFP in ovaries of a homozygous female from each Cas9 line were compared to that in wild type (wt) ovaries. Both nos-Cas9 and vas-Cas9 supported weak maternal deposition, while Ubi-Cas9 and especially BicC-Cas9 resulted in strong maternal deposition in developing eggs. Out of four tested Cas9 promoters, nanos and Bicaudal C supported the weakest and the strongest, respectively, maternal deposition into developing late eggs. Scale bars correspond to 500 μm.
Fig. S4. Examples of white and yellow resistance alleles generated by Cas9/gRNA-mediated DNA cleavage. Not every DSB induced by Cas9/gRNA in germ cells is repaired by HDR resulting in homing. NHEJ pathway also ligates DSBs and can lead to base insertions or deletions (indels) incorporated at the ligated DSBs. These indels change recognition sequence for gRNA and can result in mutations that are resistant to further cleavages by the same Cas9/gRNA system. We identified both types of resistance alleles – loss-of-function (LOF) (R2) and in-frame functional (R1) mutations – generated at both white and yellow loci. Diversities of \( w^{R2} \) and \( w^{R1} \) (A), and \( y^{R2} \) and \( y^{R1} \) (B) found at white and yellow loci, respectively. Homozygous LOF mutations of both white and yellow genes are viable and fertile in Drosophila, and thus frequencies of resistance alleles increased between F2 and F3 generations (Fig. 1D).
References


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