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Safeguarding CRISPR-Cas9 gene drives in yeast

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RNA-guided gene drives capable of spreading genomic alterations made in laboratory organisms through wild populations could be used to address environmental and public health problems. However, the possibility of unintended genome editing occurring through the escape of strains from laboratories, coupled with the prospect of unanticipated ecological change, demands caution. We report the efficacy of CRISPR-Cas9 gene drive systems in wild and laboratory strains of the yeast *Saccharomyces cerevisiae*. Furthermore, we address concerns surrounding accidental genome editing by developing and validating methods of molecular confinement that minimize the risk of unwanted genome editing. We also present a drive system capable of overwriting the changes introduced by an earlier gene drive. These molecular safeguards should enable the development of safe CRISPR gene drives for diverse organisms.

Synthetic gene drive systems have the potential to address diverse ecological problems by altering the traits of wild populations. These genetic elements spread not by improving the reproductive fitness of the organism, but by increasing the odds that they themselves will be inherited. Because this inheritance advantage can overcome the fitness costs associated with the drive itself or adjacent genes carried with the drive, such genetic elements are theoretically capable of 'driving' unrelated traits through populations over many generations[1].

Inheritance-biasing is a common strategy in nature[2]. One elegant class of inheritance-biasing genes spreads by cutting homologous chromosomes that do not contain them, thereby inducing the cellular repair process to copy them onto the damaged chromosome by homologous recombination (**Fig. 1a**). This process is known as 'homing'[3]. The best-characterized homing endonuclease gene is *I-SceI*, whose product cuts the gene encoding the large rRNA subunit of *S. cerevisiae* mitochondria. Most homing endonucleases are extremely efficient, for example, *I-SceI* is correctly copied 99% of the time[4].

Austin Burt suggested in 2003 that homing endonucleases might form the basis of synthetic gene drives that could alter wild populations of sexually reproducing organisms (**Fig. 1b**)[5]. The *I-Sce*I endonuclease gene was subsequently demonstrated to exhibit homing in transgenic laboratory populations of mosquitoes[6] and fruit flies[7,8] in which an I-*Sce*I recognition site was inserted into the recipient strain at the same locus as the *I-SceI* gene in the donor strain. However, the difficulty of retargeting homing endonucleases to cleave specific sequences in wild-type genomes has limited their utility for synthetic gene drive elements[9].

CRISPR-Cas9, which cleaves target sequences specified by single guide RNA (sgRNA) molecules, has facilitated attempts to edit the genomes of diverse species[10–17]. We previously outlined the potential for CRISPR-Cas9 RNA-guided gene drives to alter wild populations. We also described molecular confinement methods robust to human error[1] that could be used with such systems and called for public discussions and regulatory reform[18]. Here we report the validation of our hypotheses by constructing multiple CRISPR-Cas9 RNA-guided gene drive systems for use in *S. cerevisiae*. The ability of these gene drives to bias inheritance, and the effectiveness of confinement measures, are quantified, and we also present a method to overwrite and restore edited traits.

RESULTS

Before starting our experiments, we established an experimental set-up to minimize the risk of synthetic gene drive elements escaping from the laboratory and altering wild yeast populations. All of our studies used a barrier protocol to reduce contact with wild yeast. The rarity of sexual reproduction in wild *S. cerevisiae* provides an additional, natural obstacle to synthetic gene drives in the wild (**Supplementary Note**). Most importantly, all experiments used one of two forms of molecular confinement, allowing us to test the efficacy of this form of safeguard.

Molecularly confined sgRNA-only gene drives

For our initial experiments, we used a form of molecular confinement[1] in which the Cas9-based gene drive system was split into two physically separate parts: an episomal plasmid encoding Cas9 and a gene drive element encoding a guide RNA inserted into the targeted genomic locus. This allowed us to avoid creating a self-sufficient inheritance-biasing cassette while enabling homing in wild-type yeast strains. This simple form of molecular confinement is not vulnerable to human error because even if drive-containing yeast were to escape into the wild, the required and relatively unstable Cas9 episomal plasmid would rapidly be segregated from the sgRNA-only drive element (**Supplementary Fig. 1**), thereby preventing the drive from spreading exponentially.

To measure the efficiency of RNA-guided gene drives in yeast, we used the *ADE2* gene encoding phosphoribosylaminoimidazole

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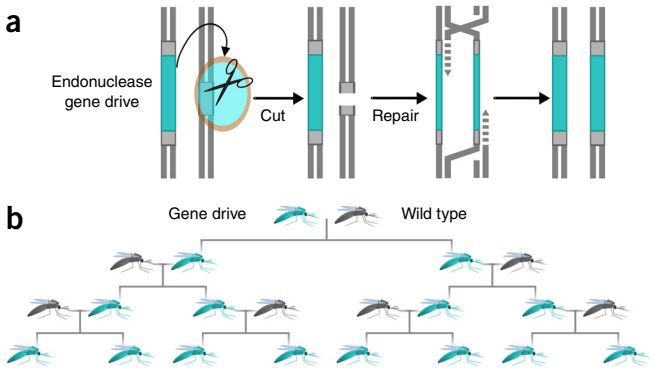

Figure 1 Mechanism and population-level effect of endonuclease gene drives. (**a**) Homing endonucleases cut competing alleles, inducing the cell to repair the damage by copying the endonuclease gene. (**b**) By converting heterozygous germline cells into homozygotes containing two copies (teal), gene drives increase the odds that they will be inherited and consequently spread themselves and associated changes through wild populations (gray). Reproduced from reference 1.

carboxylase as a visual marker[19]. Cells with wild-type *ADE2* are cream colored whereas *ade2* mutants are red. If red *ade2* haploids are mated with cream-colored wild-type haploids, the resulting heterozygous diploids inherit one functional copy of *ADE2* and are cream-colored. When these diploids undergo meiosis and reproduce by sporulation, half the resulting haploids inherit the mutated copy and are red; the other half inherit the intact, unmutated copy and are cream-colored (**Fig. 2a**).

If the red haploids encode a functional gene drive system knocked into the *ADE2* locus and designed to target the wild-type *ADE2* sequence are mated to wild-type haploids, the drive will cut and replace the intact *ADE2* locus that is inherited from the wild-type parent, yielding red diploids. Following meiosis, all haploid progeny will inherit one of the two gene drive alleles and will also be red (**Fig. 2b**). Thus, the cutting efficiency of a gene drive system that replaces *ADE2* can be assessed by mating drive-containing haploids with wild-type haploids, and quantifying the fraction of diploid cells that are red.

We built a split CRISPR-Cas9 gene drive system as described above by inserting a guide RNA targeting wild-type *ADE2* into the wild-type *ADE2* locus such that *ADE2* function was disrupted and the target site removed. We mated these red *ade2*::sgRNA haploids to wild-type yeast of the opposite mating type in the presence or absence of the episomal Cas9 plasmid and examined the color of the resulting diploid colonies to check for gene drive (**Fig. 2c**). More than 99% of diploid colonies were red when the Cas9 plasmid was present, indicating highly efficient cutting of the *ADE2* copy inherited from the wild-type parent. As expected, we did not observe any red diploid colonies in the absence of a Cas9-encoding plasmid, validating the effectiveness of sgRNA-only drive confinement.

Once sporulation of mated diploids took place, we isolated and examined haploid progeny to verify that the *ADE2* alleles from drive-containing diploids were disrupted. Upon dissecting 18 *cas9*$^+$ diploids, we observed a perfect Mendelian 4:0 ratio of red/cream haploids, confirming that all WT copies of the *ADE2* locus were disrupted. In contrast, 18 cream-colored *cas9*$^-$ diploids yielded a 2:2 red/cream ratio, indicating normal Mendelian inheritance of the inactivated drive and the wild-type *ADE2* allele (**Fig. 2d**).

To determine whether *ADE2* disruptions in red diploids were the result of successful copying of the drive element, we sequenced all 72 red haploids that we derived from dissected *cas9*$^+$ diploids. All sequenced haploid colonies contained intact sgRNA-only drive elements without additional mutations. These results indicate that the *ade2::sgRNA* drive element efficiently cut and replace the native ADE2 locus upon mating with wild-type haploids. Homing took place only in the presence of the unlinked episomal Cas9 plasmid, demonstrating that split gene drive elements cannot spread in the absence of the non-driving element.

We next tested whether CRISPR-Cas9 gene drive systems could be designed to bias the inheritance of not only a minimal drive ele-

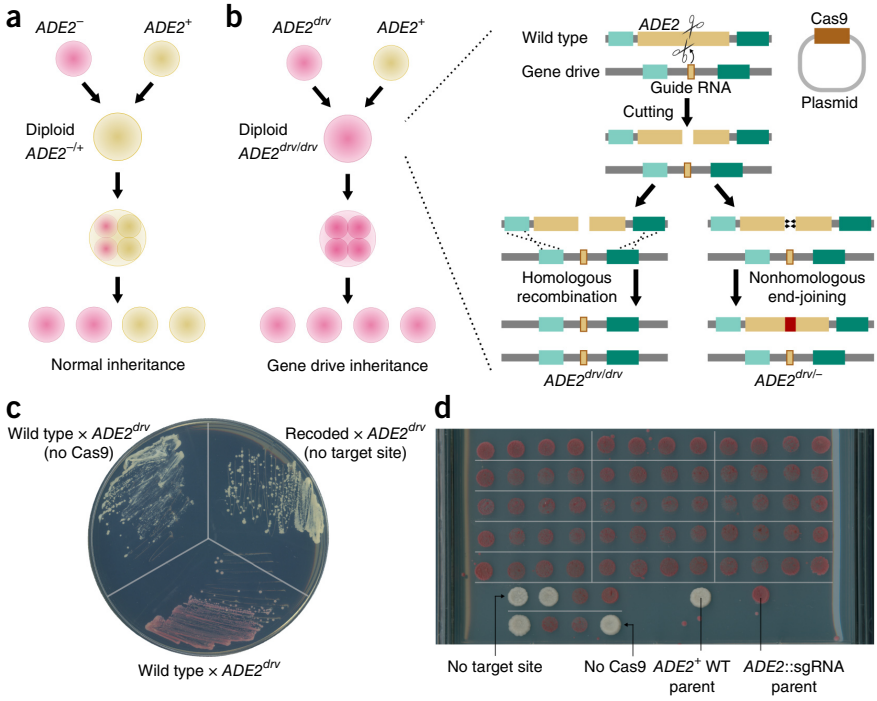

Figure 2 Biased inheritance of an *ADE2* gene drive element in *S. cerevisiae*. (**a**) Mutations in *ADE2* generate a red phenotype on adenine-limiting media due to the buildup of red pigments. Mating a red mutant haploid to a wild-type haploid produces cream-colored diploids, which yield 50% red and 50% cream-colored progeny upon sporulation. (**b**) When haploids with a gene drive element targeting *ADE2* mate with wild-type haploids in the presence of Cas9, cutting and subsequent replacement or disruption of *ADE2* produces red diploids that upon meiosis yield exclusively red progeny. (**c**) Diploids produced by mating wild-type and *ade2*::sgRNA gene drive haploids yield cream-colored colonies in the absence of Cas9 or when the target site is removed by recoding but uniformly red colonies when both are present, demonstrating Cas9-dependent disruption of the wild-type *ADE2* copy. (**d**) Spores from 15 dissected tetrads produce uniformly red colonies on adenine-limited plates, confirming disruption of the *ADE2* gene inherited from the wild-type parent. In the absence of the target site or Cas9, normal 2:2 segregation is observed.

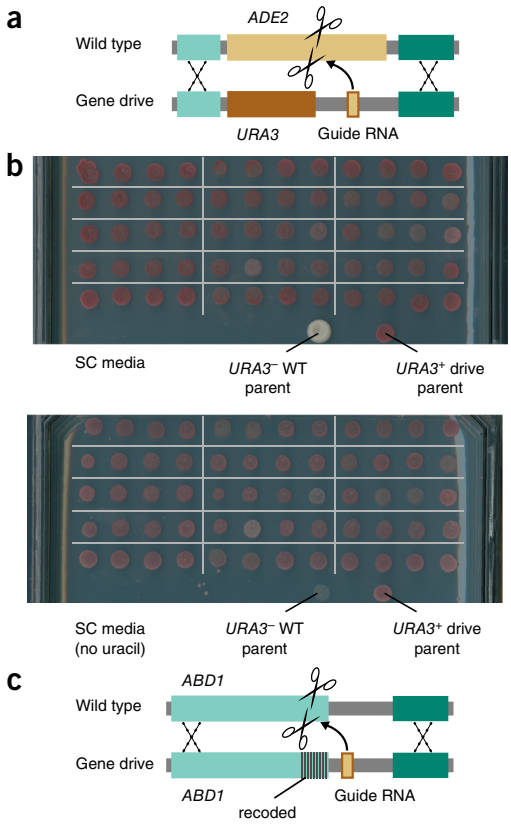

Figure 3 Gene drives and cargo genes remain intact upon copying and can spread by targeting both nonessential and essential genes. (**a**) The *ADE2*-targeting gene drive was modified to carry *URA3* as a cargo gene. (**b**) Diploids produced by mating wild-type *URA3*⁻ haploid yeast with haploids encoding the gene drive carrying *URA3* were allowed to sporulate and tetrads dissected to isolate colonies arising from individual spores. Pictures are spores from 15 of these tetrads. All grew on replica plates lacking uracil, demonstrating that the drive successfully copied *URA3* in all diploids. (**c**) A gene drive designed to cut and recode the 3′ end of the essential *ABD1* gene.

ment, but also any closely associated 'cargo' gene(s) whose spread through an existing population might be desirable. As a proof-of-principle experiment, we inserted *URA3 in cis* with the *ade2*::sgRNA drive element. *URA3* allows laboratory-modified yeast strains to grow in the absence of uracil supplementation (**Fig. 3a**). We mated *URA3*-containing drive haploids with wild-type haploids in the presence of an episomal Cas9 plasmid and allowed the resulting diploids (all of which were red) to sporulate; we dissected the resulting tetrads and examined the phenotype of the resulting haploid colonies. As was the case for the *ADE2* gene drive, all of the dissected haploid cells formed red colonies, indicative of biased inheritance. Crucially, all haploids grew without uracil in the growth medium, which demonstrates that *URA3* was efficiently copied along with the *ADE2* drive (**Fig. 3b**).

We next sought to determine whether CRISPR-Cas9 gene drive systems can recode an essential gene but leave its function intact. Especially when targeting multiple sites, such a drive element should be more evolutionarily stable as its target locus cannot readily acquire mutations that prevent homing without disrupting the function of the essential gene and thus compromising viability[1]. We designed and synthesized a split-drive element in which a guide RNA targeting the essential gene *ABD1* was encoded adjacent to a recoded version of the same gene (**Fig. 3c**)[20] and mated this haploid strain with wild-type cells containing the Cas9-expressing episomal plasmid. Isolated diploid cells were allowed to sporulate and 18 of the resulting tetrads were dissected. The 72 resulting segregants were allowed to form colonies before being sequenced at the *ABD1* locus. All 72 haploids contained the drive element and recoded *ABD1* locus. The strains with the drive element and recoded *ABD1* locus did not exhibit any obvious fitness defects as compared to wild-type strains, supporting the use of essential gene recoding as a potential strategy to build evolutionarily stable CRISPR-Cas9 gene drives.

Gene drive efficacy in diverse genetic backgrounds

Gene drive efficacy may differ for many reasons, including but not limited to the types of repair machinery available to the cell at the time of the cut, the chromatin status of the locus, and the degree of homology between the sequences flanking the gene drive and the targeted locus. To test gene drive efficacy in different genetic backgrounds, we mated *ADE2* drive-containing haploids with six phylogenetically and phenotypically diverse wild-type strains of *S. cerevisiae*. Rather than relying on visual markers of genome editing, we used quantitative PCR on populations of the resulting diploids using one set of primers specific to the drive element and another set designed to amplify either wild-type alleles or those disrupted by nonhomologous end-joining.

The mean fraction of diploid chromosomes containing the *ADE2* gene drive element was more than 99% regardless of wild-type parent strain used (**Fig. 4**), attesting to the robustness of the drive in diverse backgrounds. The copying efficiency was identical for the *URA3*-carrying gene drive element and the drive element that targeted and recoded the essential *ABD1* gene, suggesting that CRISPR-Cas9 gene drives are robust to variance in genetic background, cargo size and target site selection. In addition, the drive-containing haploid progeny of an *ADE2* gene drive exhibited equivalent copying effi-

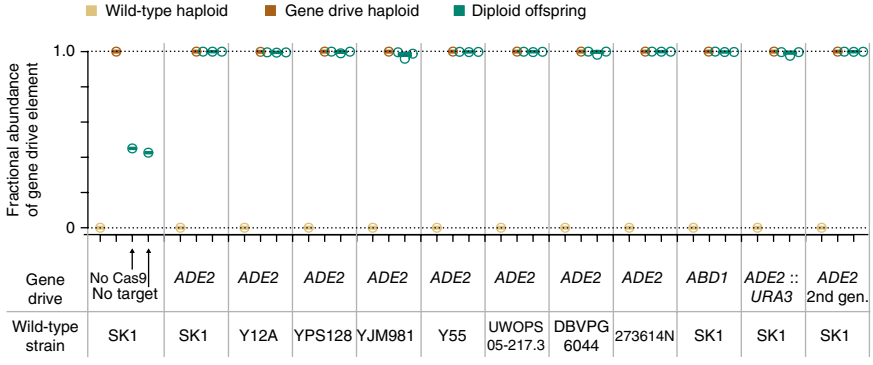

Figure 4 Quantitative PCR shows relative abundance of wild-type and drive-containing alleles in diploids. Highly efficient inheritance biasing by split drives across diverse yeast strains in the presence of Cas9 resulted from matings between SK1 haploids bearing gene drives and diverse wild-type haploid strains. "No Cas9" and "No target" refer to haploid cells containing the *ADE2* drive mated to wild-type haploids in the absence of Cas9 or to an otherwise wild-type strain with Cas9 that has a mutation in the targeted sequence that blocks cutting. "2nd gen." refers to the haploid progeny of an earlier mating. Data points are from independent cultures or mating events and represent the mean of three technical replicates.

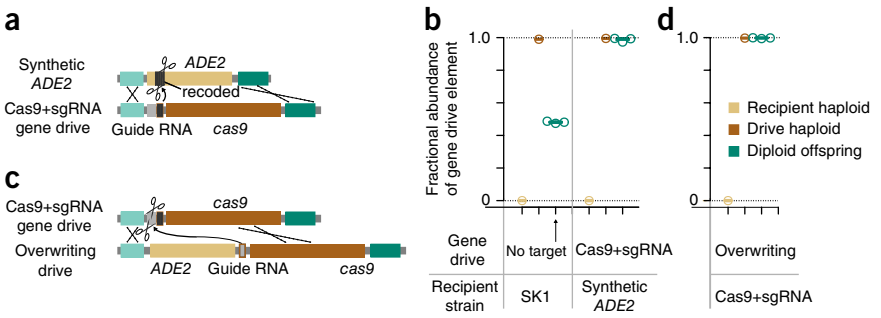

Figure 5 Available safeguards include targeting synthetic sequences and reversing drive-spread phenotypic changes with subsequent drives. (**a**) An autonomous Cas9+sgRNA gene drive that cuts and replaces the recoded *ADE2* gene. (**b**) Quantitative PCR results depicting the relative abundance of wild-type and drive-containing alleles in diploids arising from matings between SK1 haploids bearing the above gene drive and wild-type SK1 yeast. (**c**) A drive that cuts the autonomous drive and restores *ADE2*. (**d**) Quantitative PCR results for diploids arising from matings between SK1 haploids bearing the *ADE2*-disrupting and *ADE2*-restoring gene drives. Data points are from independent cultures (n = 3 technical replicates).

ciency in a subsequent cross to the original parental wild-type yeast strain, demonstrating that the drive construct is stable during homing (**Fig. 4**).

Molecularly confined autonomous gene drives

Our first experiments used a split-drive molecular confinement strategy in which Cas9 was encoded on an unlinked episomal plasmid and the gene drive element contained only the sgRNA. Because the gene encoding Cas9 is required and is unlinked from the drive, and wild yeast populations do not encode Cas9, the sgRNA-only drive is unable to spread in wild organisms lacking Cas9. In contrast, an autonomous drive element capable of propagating in wild populations would encode *cas9* and sgRNA *in cis*. This ~5-kb genetic construct is much larger than the ~500-bp sgRNA-only system that we had observed driving at a rate >99%. We hypothesized that the larger autonomous drive cassette might be copied less efficiently than the smaller sgRNA-only split-drive cassette. To safely test an autonomous Cas9+sgRNA drive under molecular confinement, we created a recipient yeast strain with a recoded *ADE2* gene containing a synthetic DNA sequence that is not present in wild populations (**Fig. 5a**). We subsequently built a Cas9+sgRNA gene drive construct targeting the synthetic sequence such that homing will disrupt *ADE2*. Diploids produced by crossing autonomous drive-carrying haploids and otherwise wild-type haploids encoding the synthetic target site were universally red. Quantitative PCR analysis of populations of diploid colonies confirmed that the Cas9+sgRNA drive system was copied at an average efficiency of more than 99%, demonstrating that inclusion of the large *cas9* gene *in cis* did not impede gene drive function in yeast (**Fig. 5b**). Crucially, crossing drive-carrying haploids to wild-type cells lacking the synthetic target sequence did not yield detectable homing, demonstrating the efficacy of synthetic site targeting as a molecular confinement strategy.

Reversibility of driven genome edits but not transgenesis

We next sought to determine whether the loss of *ADE2* function induced by this Cas9+sgRNA drive might be corrected using another gene drive to overwrite the earlier change[1]. In principle, such an overwriting drive can restore the original phenotype—in this case the loss of *ADE2*—but the resulting organisms will still be transgenic due to the presence of residual *cas9* and guide RNA. We designed a Cas9+sgRNA gene drive element (~7 kb) to cleave a synthetic sequence contained within the first *ADE2*-disrupting drive and restore an intact copy of the *ADE2* gene upon homing (**Fig. 5c**). Crosses between *ADE2*-disrupting drive haploids and *ADE2*-restoring drive haploids yielded all-white diploid colonies. Quantitative PCR analysis of diploids (**Fig. 5d**) showed that the *ADE2*-restoring drive overwrote the earlier drive at >99% efficiency. Finally, when diploid colonies were sporulated and the phenotype of their haploid progeny examined, *ADE2* function had been restored in all cells (**Supplementary Fig. 2**).

DISCUSSION

We have shown that CRISPR-Cas9 gene drives can bias inheritance in diverse wild yeast strains over successive generations at efficiencies over 99%. A previous report describing a method to produce biallelic loss-of-function mutations using a CRISPR-Cas9 gene drive construct in the fruit fly *Drosophila melanogaster* exhibited 97% copying efficiency, with noteworthy implications for potential applications in mosquitoes[21]. Although it is impossible to generalize from two studies, that neither study employed any optimization suggests that RNA-guided gene drives may function in many of the sexually reproducing organisms that have to date been engineered with Cas9.

Our potential ability to construct synthetic gene drive elements in multiple species demands caution. Scientists share a fundamental obligation to ensure that the products of laboratory experiments are confined to the laboratory. Because a single escaped organism carrying a synthetic CRISPR-Cas9 gene drive system could alter a substantial fraction of the wild population with unpredictable ecological consequences, the decision to deploy a gene drive must be made collectively by society. Any accidental release could severely damage public trust in scientists, transgenics and the field of gene drive research. It is therefore imperative for scientists performing experiments with gene drive constructs to use stringent confinement measures to minimize the risk of accidentally altering wild populations.

Barrier protocols are the traditional confinement measure used in laboratories working with dangerous organisms. However, human pathogen research has conclusively demonstrated that barrier protocols are vulnerable to human error and will eventually fail[22]. The CRISPR-Cas9 gene drive construct tested in *D. melanogaster* relied exclusively on a barrier protocol, causing concerns that led to recommendations that future experiments employ additional confinement strategies[23].

We report two easy-to-use methods of molecular confinement that are robust to human error. For example, studies requiring cutting or replacement of wild-type genes could use an sgRNA-only split-drive element in a Cas9-expressing strain. Although the episomal Cas9 plasmid used in this study cannot be used in all eukaryotic species, there is no known reason why a chromosomally encoded copy of *cas9* could not be used for split-drive molecular confinement. Strains in which chromosomally integrated Cas9 is expressed under one of several different housekeeping and germline promoters are available in multiple species[24–26]. Similarly, experiments intended to study intact Cas9+sgRNA gene drives in model systems could exclusively target synthetic sequences that are only present in laboratory populations.

We recommend that future gene drive experiments use at least one form of molecular confinement unless they aim to create a candidate for eventual release. A combination of molecular and barrier confinement may suffice in yeast, the nematode worm *Caenorhabditis elegans*, and other organisms that rarely reproduce sexually, because gene drives in such organisms can impose only a tiny fitness cost if they are to spread (**Supplementary Note**). That said, natural endonuclease gene drives such as *I-SceI* do exist in yeast. Whether CRISPR-Cas9 gene drives will constitute this level of burden is as yet unknown. We openly acknowledge that the barrier protocol used for the experiments in this study should have been stronger; we now perform such experiments using a more stringent protocol involving biological containment hoods.

Gene drive experiments in species that always reproduce sexually pose a greater risk of spread if any organisms should escape from the laboratory. We therefore suggest that gene drive experiments in such organisms use multiple confinement measures that are not vulnerable to human error. For example, laboratories might use both forms of molecular confinement (an sgRNA-only drive element that targets a synthetic sequence), molecular as well as ecological confinement (in which experiments take place in geographic areas where the organism in question cannot survive or find mates)[1], or molecular as well as reproductive confinement (the use of genetic backgrounds that are entirely unable to reproduce with wild-type organisms, such as *Drosophila* strains with compound autosomes[27]). These precautions are most important in flying insects and other species that easily escape physical confinement.

Even scientists not intending to work with gene drives should consider taking precautions, as any unintended insertion of *cas9* and guide RNAs near a targeted site could produce a gene drive element. Fortunately, a simple and free precaution is available and already used for different reasons by many laboratories, namely, researchers avoid delivering *cas9* on a DNA cassette that also encodes a guide RNA. As we have shown, guide RNAs alone cannot bias inheritance in the absence of Cas9 and therefore cannot spread through wild populations (**Fig. 4**).

Our results lay the groundwork for future efforts to design highly efficient and stable gene drives intended for real-world applications. These experiments could initially create and test sgRNA-only gene drive systems, but must eventually create a gene drive capable of spreading in the wild, at which point they are likely limited to ecological and barrier confinement methods. Our demonstration that a subsequently released gene drive can correct changes made by an earlier gene drive constitutes an additional safeguard: in the event of a premature release or unanticipated ecological effect, the override drive could mitigate the damage. We recommend that in ongoing gene drive efforts researchers consider constructing an appropriate corrective drive element in parallel with the candidate gene drive construct. In addition, the success of our *ABD1* gene drive shows that it is feasible to target and recode genes important for fitness, a strategy that may improve the evolutionary stability of gene drive systems[1].

More generally, our findings suggest that yeast could be a useful platform for rapid testing of CRISPR gene drives before moving them into multicellular organisms. The power of yeast genetics and the ease of genome manipulation could facilitate combinatorial investigations into gene drive optimization. For example, studies might explore how biasing repair pathway choice[28] affects the efficiency of copying for gene drives of various sizes. Because the factors involved in these pathways are broadly conserved, these experiments could guide gene drive optimization in other organisms[29].

Lastly, the confinement strategies we report will enable researchers to exploit the power of gene drives for facile genome engineering. For example, mating libraries of organisms in which sgRNA-only cassettes have been used to replace nonessential target genes could quickly generate all viable combinations of knockouts to better understand interaction networks and potentially identify relevant therapeutic pathways in pathogenic organisms. Furthermore, the successive combinatorial knockouts created by our outlined gene drive approach could eventually enable the identification of candidate minimal genomes.

In conclusion, we hope that reversible and molecularly confined gene drives in yeast will inform, safeguard and accelerate efforts to build CRISPR-Cas9 gene drives in other organisms. Given the considerable potential for this technology to address global problems in health, agriculture and conservation, these results underscore the urgent need for formal guidelines specifying requisite safeguards, inclusive public discussions and regulatory reform[18] to build a reliable foundation for future humanitarian applications.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GenBank: KT876200 (*ADE2* KO) and KT876201 (*ABD1* recoding). NCBI Trace Archive: 2342918196-2342918339.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.L.D. initiated the study; J.E.D., A.C., S.L.D. and K.M.E. designed the experiments; J.E.D. performed the experiments with assistance from A.C.; J.E.D., A.C., S.L.D. and K.M.E. analyzed the data; and K.M.E. wrote the paper with A.C. and contributing input from J.E.D., S.L.D. and G.M.C.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Physical confinement of gene drives. All experiments were carried out using a barrier protocol to supplement the molecular confinement strategies. Plates and tubes containing gene drive strains were kept in separate areas of bench surfaces, cold room surfaces and freezer space. All plates were secured with Parafilm and bench surfaces were cleaned daily using a 70% ethanol solution. Exposed glassware was first soaked for 24 h in Wescodyne solution before cleaning. All waste was bagged in biohazard-specific bags, sealed and incinerated. Future experiments will use a stricter barrier protocol in which all work is carried out in a biological hood as a further precaution.

Plasmids and genomic cassettes. Gene drive cassettes were synthesized from gBlocks (Integrated DNA Technologies, Coralville, IA) and inserted into SK1 cells by Cas9-mediated genome modification as follows. Guide RNAs for each drive were cloned into p416-Cas9 containing plasmids with expression driven by the SNR52 promoter[16]. 60 base pair homology arms to the target locus were added on both ends of the gene drive cassette via PCR and 5 µg of PCR product was co-transformed with the p416-Cas9-gRNA plasmids. Correctly integrated gene drives were verified by sequencing and p416-Cas9-gRNA plasmids were cured using 5-Fluoroorotic Acid (5-FOA) selection.

To create the *URA3*-containing *ADE2* gene drive, the *ADE2* gene drive was cloned next to the *Candida albicans URA3* gene in the pAG60 plasmid. The entire URA3 cassette and gene drive were PCR amplified and inserted using Cas9-mediated genome modification into the *ADE2* locus of haploid SK1 cells.

The recoded C terminus of the *ABD1* gene and corresponding gene drive were synthesized as a gBlock to remove homology and generate mutations in the seed sequence by means of synonymous changes. The TEF1 terminator was inserted at the 3′ end of the recoded *ABD1* gene between the gene and the gRNA as *ABD1* shares a terminator with the *VHC1* gene. The entire cassette was integrated into the haploid SK1 genome using Cas9-mediated genome modification.

The *ADE2* gene was recoded by cotransforming a double-stranded oligonucleotide and a p416 plasmid containing Cas9 and a gRNA targeting the ADE2 region to recode. The oligonucleotide silently recoded the *ADE2* gene and included an orthogonal target and PAM sequence. The complete gene drive (Cas9 and gRNA, targeting the recoded ADE2 gene) was generated by cloning a gRNA into the p416-Cas9 plasmid. An orthogonal genomic target was also included in the complete gene drive to later be targeted by the reversal drive. The Cas9 and gRNA linear construct was amplified by PCR using the same homology arms as the sole gRNA gene drive construct. The construct was co-transformed into By4723 cells with the plasmid it was amplified from and the cells were selected for uracil prototrophy. Correct integrations were screened via colony PCR. This plasmid was later removed using 5-FOA.

The overwriting drive (Cas9 and gRNA integrated upstream of the ADE2 gene) was generated by cloning an alternately encoded gRNA into a p414 plasmid containing Cas9. This alternatively encoded gRNA contains less homology to previously used gRNAs and would reduce the chance of unwanted recombination when used to replace the complete gene drive. This gRNA targets a 20-bp region inserted with the complete gene drive. The *TRP1* gene with the Cas9 and gRNA were PCR amplified with homology arms to the 5′ region of the *ADE2* and the product was transformed into SK1 A cells. Cells were selected for tryptophan prototrophy and screened by PCR for correct integrations.

The p416-Cas9-gRNA plasmid (conferring uracil prototrophy) is a variant of the previously described p414-Cas9-gRNA plasmid (conferring tryptophan prototrophy)[16] (Addgene #43802). One or the other was used in each mating experiment. The pRS413 vector was transformed into select cell types to confer histidine prototrophy as a marker to select for diploid cells.

Strain genotypes:

SK1A: MATa ho::LYS2 lys2 ura3 leu2::hisG his3::hisG trp1::hisG;
SK1 α: MATα ho::LYS2 lys2 ura3 leu2::hisG his3::hisG trp1::hisG;
Y12A: MATa ho::HygMX ura3::KanMX;
YPS128: MATa ho::HygMX ura3::KanMX;
YJM981: MATa ho::HygMX ura3::KanMX; Y55: MATa ho::HygMX ura3::KanMX;
UWOPS05-217-3: MATa ho::HygMX ura3::KanMX;
DBVPG6044: MATa ho::HygMX ura3::KanMX;
273614N: MATa ho::HygMX ura3::KanMX.

Yeast mating experiments. Haploid drive-containing SK1 yeast and haploid wild-type strains of the opposite mating type were mixed in equal amounts in YPAD liquid media and incubated overnight. The resulting diploids were washed in sterile water and plated on selective media for both parental genotypes. Specific crosses and selection conditions are detailed in **Supplementary Table 1**.

Sporulation and tetrad dissection. After mating in liquid YPAD and selection for diploids on selection plates, the selection plates were scraped into 10 ml selective media and grown overnight at 30°C. A fresh 5 ml YPAD culture was then inoculated to an OD = 0.1 and grown 4–5 h at 30 °C. The entire culture was then washed twice in 10 ml water, inoculated into 2 ml of sporulation media (1% potassium acetate), and incubated at room temperature for 3 days or until spores were visible. Sporulated cells were suspended in 50 µl of a stock solution of zymolyase (50 µg/ml in 1 M sorbitol) and incubated at 30 C for 5 min, transferred to ice, diluted with 150 µl cold H_2O, microdissected using a Zeiss tetrad dissection microscope, and isolated spores grown on YPAD plates.

Selection for URA3 function. Dissected spores were grown in synthetic complete (SC) media and then spotted onto SC medium as well as SC medium without uracil. To enhance red color, all SC solid media used for plate images contained 0.5× adenine hemisulfate (final concentration of 0.08 mM).

Verification of chromosomal segregation. Three genes on chromosome 15 flanking the *ADE2* gene were sequenced in two dissected tetrads of the complete gene drive cross. VAM3, TRS33 and DPP1 were amplified using PCR from colonies and sequenced using Sanger sequencing to verify that homologous recombination copied only the gene drive cassette rather than the entire chromosome.

Measuring mitotic plasmid loss. BY4722 cells containing p416-Cas9 plasmids were grown overnight in SC-uracil media. 50 µl of saturated cultured was then used to inoculate 10 ml cultures of YPAD. The YPAD cultures were grown overnight and then plated. Overnight dilutions were plated on YPAD and 5-FOA plates and the difference in colony counts between the two medias were used to determine plasmid loss rate. To determine the total number of population doublings after overnight growth cell counts before overnight growth and after overnight growth were measured.

Measuring meiotic plasmid loss. A *URA3* gene placed under a haploid specific promoter (STE5) was used to select for diploids and haploids. SK1α ho::pSTE5-CaURA3 ade2::gRNA containing p413-Cas9 plasmid was mated with SK1 A ho::pSTE5-CaURA3. After mating in YPAD, cells were passaged an additional day in YPAD media. Diploids were then selected for in SC-histidine+5-FOA. After diploid selection, cells were grown to log phase in YPAD and then placed in sporulation media (2% potassium acetate) and allowed to sporulated for 3 days. After visual confirmation of asci formation, asci were disrupted using 0.5 mg/ml zymolyase (20-T) for 20 min and then vortexing for 5 min. Disrupted sporulated cultures were then plated to either SC-uracil (haploid selection) or SC-uracil-histidine (haploid+Cas9 plasmid selection), and the difference between colony numbers was calculated.

Quantitative PCR. Candidate primer pairs were designed to amplify short regions specific to each drive or the wild-type sequence replaced by the drive, as well as the *ACT1* gene as a control. All sequences are included in **Supplementary Table 2**. Genomic DNA was extracted using Method A as described in Lõoke *et al.*[30].

KAPA SYBR FAST qPCR Master Mix (2×) was used to perform the qPCR reaction along with 25 ng of genomic DNA. The amplification efficiency and relative specificity of each primer pair were measured by amplifying dilutions of genomic DNA from wild-type and drive haploids, respectively, and the best-performing and well-matched pairs selected for use (see below for all primers used). Quantitative PCR reactions were performed on genomic DNA isolated from each parental haploid as well as from diploids arising from three independent mating events. Three reactions (technical replicates) were performed per sample on a LightCycler 96 machine by Roche.

Calculations. Results from three technical replicates were averaged for calculations. In order to directly calculate the ratio of alleles before PCR amplification, we first determined the efficiencies of the different primer pairs. Efficiencies were calculated from qPCR runs of serial dilutions (6 orders of magnitude) as:

$$\text{Efficiency} = 10^{-1/\text{slope}}$$

R^2 values were higher than 0.99 in all cases except for one pair (ade2::URA3+sgRNA).

The allelic ratios were calculated as:

$$x_a . E_a^{Ct,a} = x_b . E_b^{Ct,b}$$
$$x_a / x_b = E_b^{Ct,b} / E_a^{Ct,a}$$

with x_a and x_b being the initial concentration of drive and WT DNA, E_a and E_b the efficiency of the respective primer pairs and Ct,a and Ct,b the Ct values for each sample.

Figures 4 and **5** were generated using BoxPlotR[31].

Raw data and calculations are available in the Source Data.

Sequencing traces. Trace files for sequences corresponding to the spores of the 18 tetrads dissected for the *ADE2* knockout gene drive and the *ABD1* recoding gene drive were uploaded to the NCBI Trace Archive with TI numbers 2342918196-2342918339. Templates corresponding to expected sequences used for alignments are available as GenBank Accession KT876200 (*ADE2* KO) and KT876201 (*ABD1* recoding).

Oligonucleotides. Sequences of oligonucleotides used in the study (Integrated DNA Technologies, Coralville, IA) are listed in **Supplementary Table 2**.

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