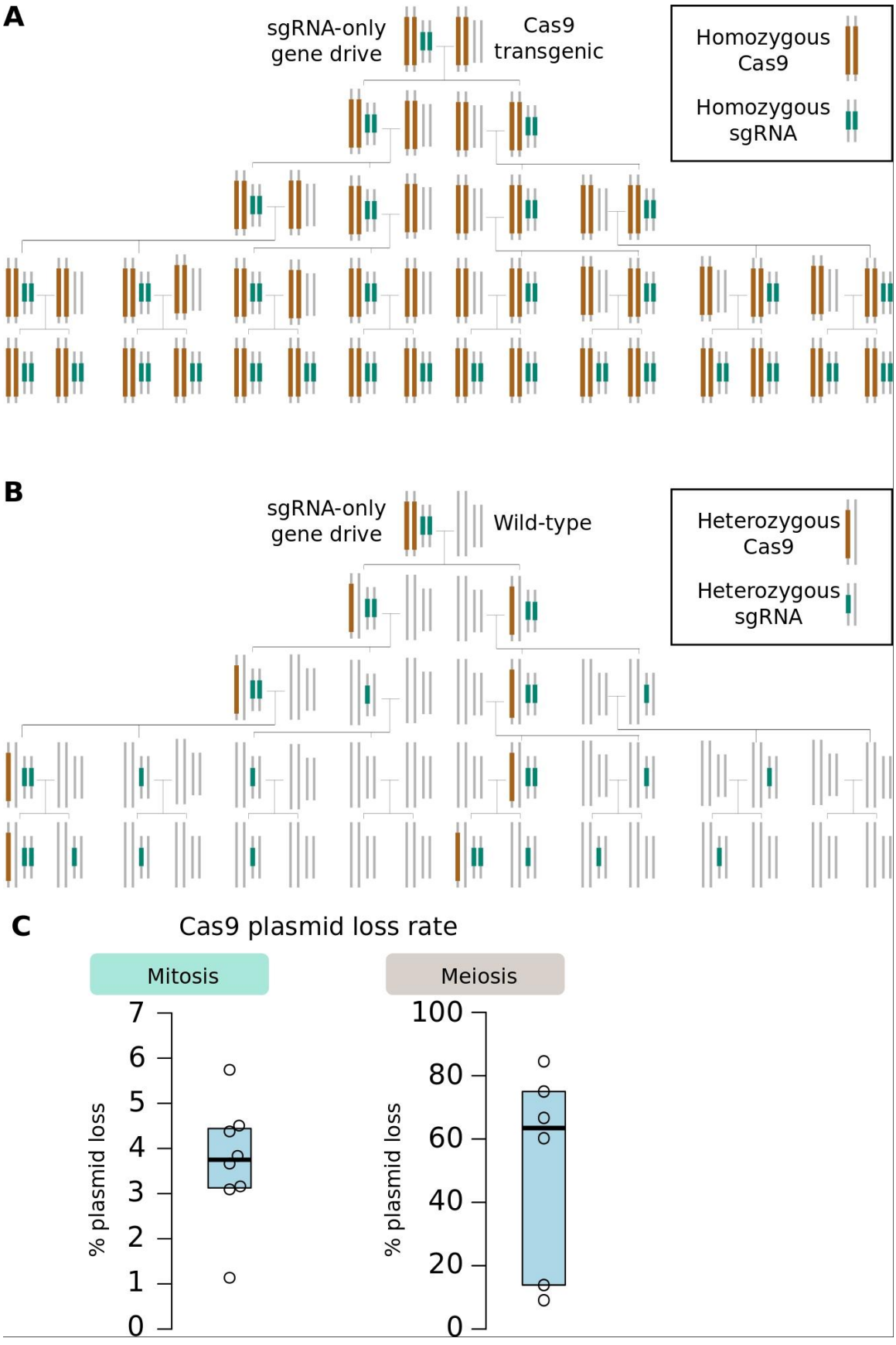

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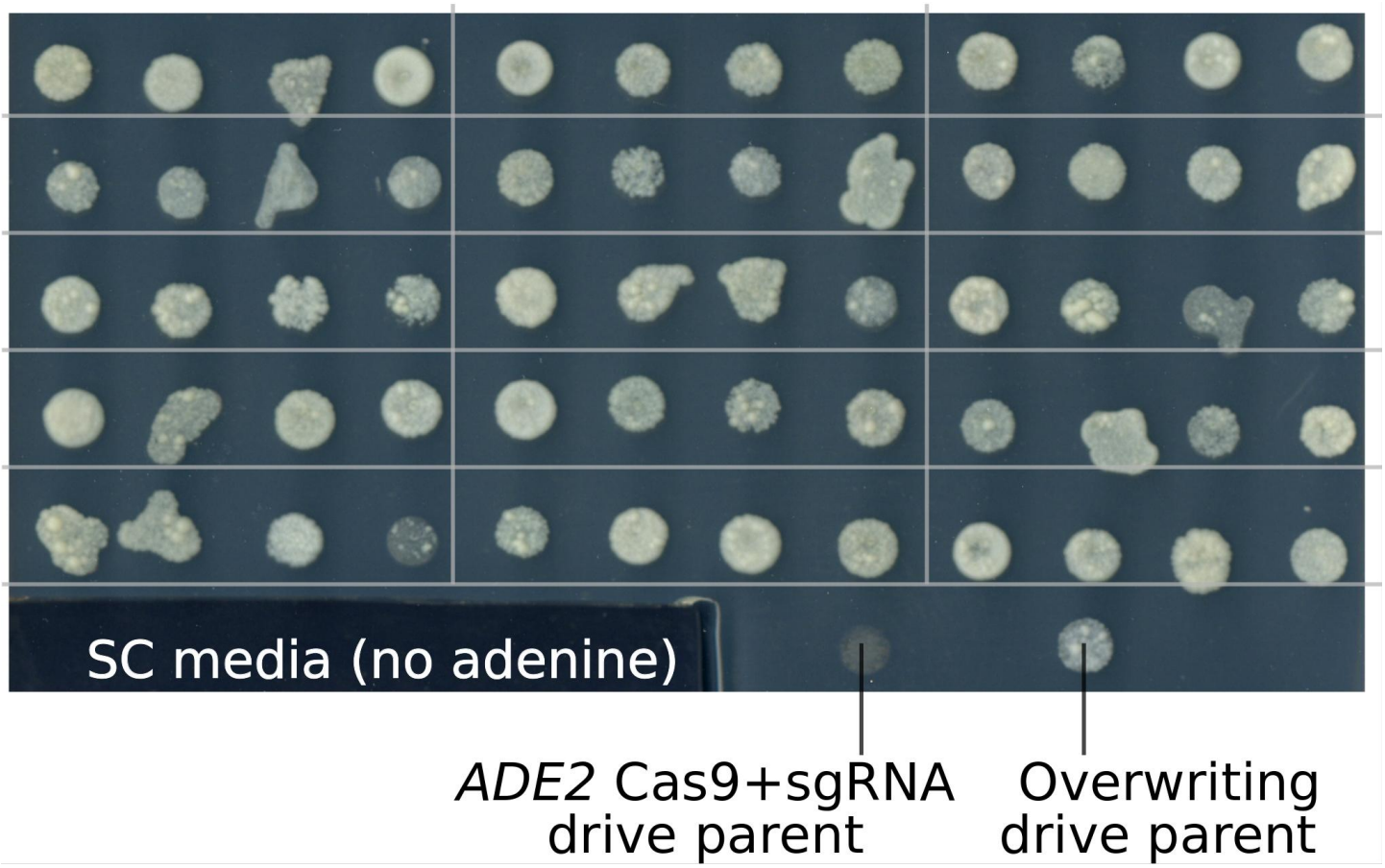
Supplementary Figure 1

Molecular confinement via 'split drive' sgRNA-only cassettes with chromosomal or episomal Cas9.

A) In transgenic laboratory populations expressing Cas9 (brown) from an unlinked locus such as another chromosome, the sgRNA-only drive (green) will be copied in every generation. For clarity, copying is assumed to occur when haploid cells combine to form a diploid. In our S. cerevisiae experiments, Cas9 was encoded on an episomal plasmid with imperfect inheritance that should produce a similar pattern. (B) If escaped organisms encoding an sgRNA-only drive mate with wild-type organisms, the cas9 gene quickly segregates away from the sgRNA-only drive, precluding exponential spread. Any organisms that do encode Cas9 will still exhibit drive, but the total number of copies is limited by the number of escaped organisms and therefore is dwarfed by the wild-type population. If one organism is released from the laboratory for every million wild-type organisms in the population, a perfectly efficient drive with no fitness cost will linearly increase in relative abundance by 2E-6 per generation. This tiny inheritance advantage is exceedingly unlikely to counterbalance the fitness cost of an actual split gene drive. (C) The episomal Cas9-expressing plasmid is unstable in the absence of active selection. With an average loss rate of ~3.8% per generation, more than 2/3 of yeast have lost the plasmid after a single round of asexual overnight growth (10 generations). While variable across independent mating experiments, the plasmid is typically lost at a rate of ~50% during meiotic sporulation, approximately equivalent to a chromosomal transgene. These high loss rates suggest there is minimal risk of Cas9 remaining available to bias the inheritance of the sgRNA-only cassette over generations. Indeed, mitotic loss suggests that the plasmid-encoded gene would likely be eliminated from the population more quickly than a chromosomally-integrated equivalent in the event of an accidental release.



Supplementary Figure 2

Reversal of drive-induced *ADE2* loss by an overwriting drive.

Haploid yeast containing a complete autonomous *ADE2*-disrupting gene drive were mated with haploids containing an overwriting drive that restores *ADE2* function. 15 diploid offspring were sporulated, dissected, and plated on adenine-limited plates. The resulting cream-colored colonies indicate that an intact *ADE2* gene is present in all progeny, indicative of the *ADE2*-restoring drive successfully cutting and replacing the *ADE2*-disrupting gene drive.

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Supplementary Table 1. Haploid matings, genotypes, and selection conditions.

MATa Genotype	**MATα** Genotype	Selection
SK1 pRS414 – Cas9	SK1 ade2::gRNA (gene drive), pRS413	SC-histidine - tryptophan
SK1 ade2::gRNA + URA3 (gene drive), p414-Cas9	SK1 pRS413	SC-histidine - tryptophan
SK1 p414-Cas9	SK1 abd1::ABD1 recoded +gRNA (gene drive), pRS413	SC-histidine - tryptophan
Y12A Hygromycin B resistance (HygR)	SK1 ade2::gRNA (gene drive), p416-Cas9	SC-uracil+300 ug/mL Hygromycin B
YPS128 Hygromycin B resistance (HygR)	SK1 ade2::gRNA (gene drive), p416-Cas9	SC-uracil+300 ug/mL Hygromycin B
YJM981 Hygromycin B resistance (HygR)	SK1 ade2::gRNA (gene drive), p416-Cas9	SC-uracil+300 ug/mL Hygromycin B
Y55 Hygromycin B resistance (HygR)	SK1 ade2::gRNA (gene drive), p416-Cas9	SC-uracil+300 ug/mL Hygromycin B
UWOPS05-217.3 Hygromycin B resistance (HygR)	SK1 ade2::gRNA (gene drive), p416-Cas9	SC-uracil+300 ug/mL Hygromycin B
DBVPG 6044 Hygromycin B resistance (HygR)	SK1 ade2::gRNA (gene drive), p416-Cas9	SC-uracil+300 ug/mL Hygromycin B
273614N Hygromycin B resistance (HygR)	SK1 ade2::gRNA (gene drive), p416-Cas9	SC-uracil+300 ug/mL Hygromycin B
SK1 ADE2:: ADE2 silently recoded genomic target seed sequence, p414-Cas9	SK1 ade2::gRNA (gene drive), pRS413	SC-histidine - tryptophan
SK1, p414-empty	SK1 ade2::gRNA (gene drive), pRS413	SC-histidine - tryptophan
BY4723 ADE2 recoded	SK1 ade2::complete gene drive (Cas9+gRNA,with recoded ADE2 target)	SC-histidine - tryptophan
BY4723	SK1 ade2::complete gene drive (Cas9+gRNA,with recoded ADE2 target)	SC-histidine - tryptophan
Complete Gene drive Haploid from dissected tetrad, p413-empty	SK1 ADE2 +reversal gene drive (Cas9+gRNA, targeting inserted orthogonal target), p416-empty	SC-histidine -uracil

Supplementary Table 2. Oligonucleotide primers and dsDNA fragments used for genome editing and analysis.

Genome modification primers and gBlocks	**Sequence**
ADE2.sgRNA.ade2.1 insert.F	TACGAACCGGGTAATACTAAGTGATTGACTCTTGCTGACCTTTTATTAAGAACTAAATGGtctttgaaaagataatgtatgat tatgctttc
ADE2.sgRNA.ade2.1 insert.R	TAATAAGTGATCTTATGTATGAAATTCTTAAAAAAGGACACCTGTAAGCGTTGATTTCTAagacataaaaaacaaaaaaagca ccac
gRNA+CaURA3.ade2.F	TACGAACCGGGTAATACTAAGTGATTGACTCTTGCTGACCTTTTATTAAGAACTAAATGGagacataaaaaacaaaaaaagca ccaccg
gRNA+CaURA3.ade2.R	TAATAAGTGATCTTATGTATGAAATTCTTAAAAAAGGACACCTGTAAGCGTTGATTTCTAtcgacactggatggcggcgttag tatc
ABD1.recode+gRNA	AGCCAGATGCCATTCAACAAGTTCTTCGTGCAGGAGATACCAAAGTGGATAGAACGTTTCAGCCCAAAGATGCGTGAGGGGCT TCAGCGTAGCGACGGGCGTTACGGGGTGGAGGGTGACGAGAAAGAGGCTGCTAGCTACTTTTACACGATGTTCGCTTTTAGAA AAGTTAAGCAATACATAGAGCCTGAGTCAGTTAAACCAAATTGAACGGCTCCTCGCTGCAGACCTGCGAGCAGGGAAACGCTC CCCTCACAGACGCGTTGAATTGTCCCCACGCCGCGCCCCTGTAGAGAAATATAAAAGGTTAGGATTTGCCACTGAGGTTCTTC TTTCATATACTTCCTTTTAAAATCTTGCTAGGATACAGTTCTCACATCACATCCGAACATAAACAACCATGGGTATGACCGAC CAAGCGACGCCCAACCTGCCATCACGAGATTTCGATCCCACCGCCGCCTTCTATGAAAGGtctttgaaaagataatgtatgat tatgctttcactcatatttatacagaaacttgatgttttctttcgagtatatacaaggtgattacatgtacgtttgaagtaca actctagattttgtagtgccctcttgggctagcggtaaaggtgcgcatttttcacaccctacaatgttctgttcaaaagatt ttggtcaaacgctgtagaagtgaaagttggtgcgcatgtttcggcgttcgaaacttctccgcagtgaaagataaatgatcTGA AGGGGATGAAAAGGAAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACC GAGTCGGTGGTGCTTTTTTTGTTTTTTATGTCT
ABD1.recode+gRNA.int.F	tatgttgtgccattcgaaaccttaagaagtttggctgatgaatacggtttggaactagtaAGCCAGATGCCATTCAACAAGTT C
ABD1.recode+gRNA.int.R	gtaatacggccgaaatacagatgctttatagtagggttattgtttctattcatttttattAGACATAAAAAACAAAAAAAGCA CCACC
ADE2.gRNA	tctttgaaaagataatgtatgattatgctttcactcatatttatacagaaacttgatgttttctttcgagtatatacaaggtg attacatgtacgtttgaagtacaactctagattttgtagtgccctcttgggctagcggtaaaggtgcgcatttttcacaccc tacaatgttctgttcaaaagattttggtcaaacgctgtagaagtgaaagttggtgcgcatgtttcggcgttcgaaacttctcc gcagtgaaagataaatgatcACTTGAAGATTCTTTAGTGTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGT TATCAACTTGAAAAAGTGGCACCGAGTCGGTGGTGCTTTTTTTGTTTTTTATGTCT
ADE2.1.silent.seed.90mer.F	TGATGTGCTAACGATTGAGATTGAGCATGTTGATGTTCCTACCCTGAAAAACCTGCAAGTAAAACATCCCAAATTAAAAATTT ACCCTTC
ADE2.1.silent.seed.90mer.F	GAAGGGTAAATTTTTAATTTGGGATGTTTTACTTGCAGGTTTTTCAGGGTAGGAACATCAACATGCTCAATCTCAATCGTTAG CACATCA
ABD1.ver.F	ATAGATAATGTTCCTGAATATGTTGTGCCA
ABD1.ver.R	TTACTACATATAGAAGTCTTGTAATACGGCCG
ADE2.ver.F	GCTACGAACCGGGTAATACTAAGTGATTG
ADE2.ver.R	CAGGTAATTATTCCTTGCTTCTTGTTACTGG
ade2_recode_MMEJ_90mer.F	tcaaaaatggtatagcagttacccaatccgtaccagttgaacaagcatctgagacgtccctattgaatgttggaaga-gatttgggttttc

ade2_recode_MMEJ_90mer.R	gaaaacccaaatctcttccaacattcaatagggacgtctcagatgcttgttcaactggtacggattgggtaactgctatccatttttga
mali.int.marker.F	ACAGATAAAATTTAAGAGATATTAAATATTAGTGAGAAGCCGAGAATTTTGTAACACCAaattaaccctcactaaaggg
mali.int.marker.R	CCATTTAGTTCTTAATAAAAGGTCAGCAAGAGTCAATCACTTAGTATTACCCGGTTCGTACCTCTGACACATGCAGCTCCCGG
ade.complete.GD.insert.F	AGACAGATAAAATTTAAGAGATATTAAATATTAGTGAGAAGCCGAGAATTTTGTAACACCACATAGCTTCAAAATGTTTCTACTCCTTTT
ade.complete.GD.insert.R	ATGTATGTATAATAAGTGATCTTATGTATGAAATTCTTAAAAAAGGACACCTGTAAGCGTTGATTTCTACCCCACTGTGGGGTGGAGGGG
sgRNAmali.sgRNA2	TATAGGGCGAATTGGtctttgaaaagataatgtatgattatgctttcactcatatttatacagaaacttgatgttttctttcgagtatatacaaggtgattacatgtacgtttgaagtacaactctagattttgtagtgccctcttgggctagcggtaaaggtgcgcatttttttcacaccctacaatgttctgttcaaaagattttggtcaaacgctgtagaagtgaaagttggtgcgcatgtttcggcgttcgaaacttctccgcagtgaaagataaatgatcgtcccctccaccccacagtggttttagagctatgctgaaaagcatagcaagttaaaataaggcagtgatttttaatccagtccgtacacaacttgaaaaagtgcgcaccgattcggtgcTTTTTTTGTTTTTTATGTCTGTACCGGCCGCAAAT
ade2_wt_insert_PAM_gRNA_gBLOCK	TATAGGGCGAATTGGtctttgaaaagataatgtatgattatgctttcactcatatttatacagaaacttgatgttttctttcgagtatatacaaggtgattacatgtacgtttgaagtacaactctagattttgtagtgccctcttgggctagcggtaaaggtgcgcatttttttcacaccctacaatgttctgttcaaaagattttggtcaaacgctgtagaagtgaaagttggtgcgcatgtttcggcgttcgaaacttctccgcagtgaaagataaatgatcattcaatagggacgtctcacGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGGTGCTTTTTTTGTTTTTTATGTCTgtaccggccgcaaat
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TRS33.F	ATGTCCTCTACACATAGTAATAATGTAGGACATCCC
TRS33.R	TTACTGCGGCATTGTGACTTGAACATGG
DPP1.F	ATGGGCAAAACCGCGGATAATCATG
DPP1.R	CTATTTGTCGTCTTTAATGATAGCAGACCTATTAAGG
VAM3.F	ATGTCCTTTTTCGACATCGAAGCACA
VAM3.R	CTAACTTAATACAGCAAGCAATACCACCATG
qPCR Primers	**Sequence**
ade2.WT.qPCR.F	TACGAACCGGGTAATACTAAGTGATTGACTC
ade2.gRNA.qPCR.R	CGCTAGCCCAAGAGGGCACTACA
ade2.WT.qPCR.R	TACCAACTGTTCTAGAATCCATACTTGATTGTTT
URA3.genedrive.ade2.WT.qPCR.F	TACGAACCGGGTAATACTAAGTGATTGACTC
URA3.genedrive.ade2.WT.qPCR.R	CCTCCTAATATACCAACTGTTCTAGAATCCAT
URA3.genedrive.ade2.gRNA.qPCR.R	AAACTTCTCCGCAGTGAAAGATAAATGATC
ABD1_rec_qPCR.R	CGAGGAGCCGTTCAATTTGGTTTAACTGAC
ABD1_rec_qPCR.F	AGATGCGTGAGGGGCTTCAGC
ABD1_WT_qPCR (JDwt1.4).F	**GAAGGGGATGAAAAGGAAGC**

ABD1_WT_qPCR (JDwt1.3).R	**CGCTTTCCGGTTCGATATAC**
ACT1.qPCR.F	**CGAAAGATTCAGAGCCCCAGAAGCT**
ACT1.qPCR.R	**CGGTGATTTCCTTTTGCATTCTTTCG**
mali.qPCR.F	**GTGATTGACTCTTGCTGACCTTTTATTAAGAAC**
mal-i.qPCR.R.reversal	**CAATCATACGTCCCAATTGTCCCCCTC**
ADE2.MME-J.qPCR.F	**AGATAAATGATCAGATGCTTGTTCAACTGG**
ADE2.MME-J.qPCR.R	**AAGGACACCTGTAAGCGTTGATTTCTA**
complete.-gene.-drive.qPCR.GD.F	**GTCCCCTCCACCCCACAGTGG**
complete.-gene.-drive.qPCR.WT.R	**TCCTCGGTTCTGCATTGAGCCG**
complete.-gene.-drive.qPCR.WT.F	**AATGTGGACTTCATACATAGAAATCAACGC**

Supplementary Note

In order to spread through a population, the inheritance advantage provided by a gene drive must outweigh the fitness cost imposed. For example, an RNA-guided gene drive that imposes no fitness cost to a sexually reproducing organism and is perfectly copied upon fertilization will be inherited twice as often as a non-driving gene when rare. But if the drive imposes a fitness penalty of 50%, it will be fitness neutral under the same circumstances: although inherited twice as often, carriers will on average produce only half as many offspring.

For drives spreading through populations that frequently reproduce asexually, the fitness cost must be considerably lower because the inheritance advantage is only realized during sexual reproduction, while the cost also applies to asexual generations. For example, if *S. cerevisiae* reproduces sexually at a frequency comparable to the related *S. paradoxus* in the wild, e.g. only once for every 1000 asexual generations, the fitness cost can presumably be no more than 0.1%[31]. The ability of a synthetic endonucleased-based gene drive element to spread through wild populations of organisms that frequently reproduce asexually is consequently much reduced. Counterintuitively, natural homing endonucleases are most often found in organisms that frequently reproduce asexually. Following Burt and Koufopanou, this may be because rapid fixation of such an element is an evolutionary disadvantage over the long term due to the loss of selective pressure for continued function and subsequent degradation[3].

Supplementary References

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