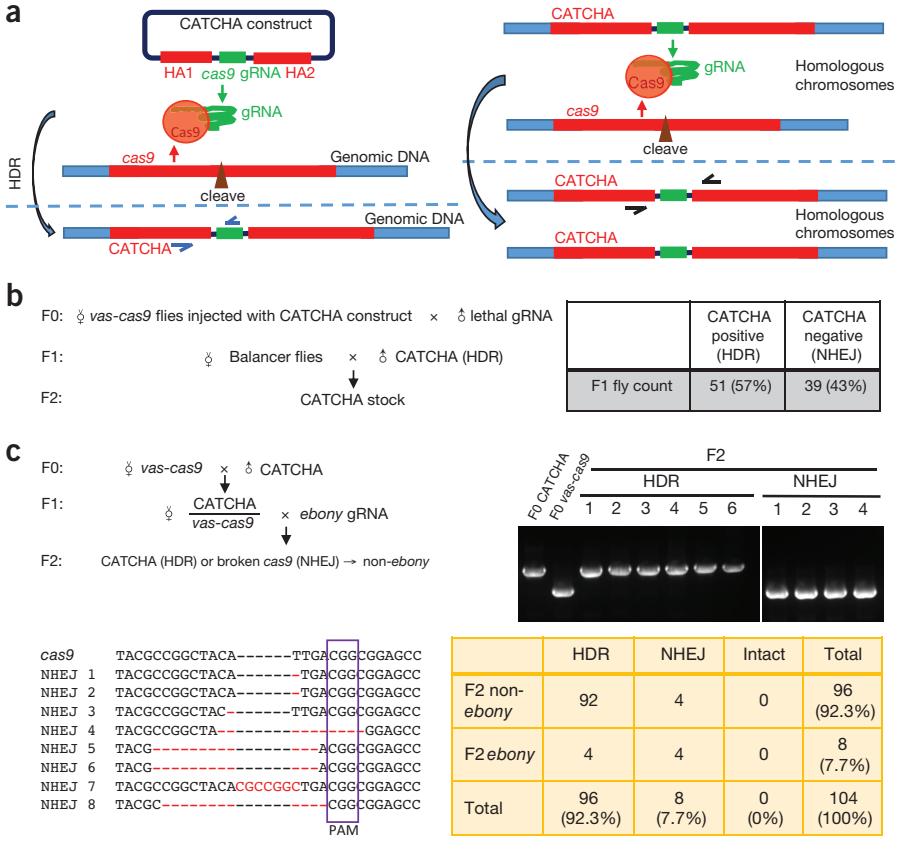
Cas9-triggered chain ablation of *cas9* as a gene drive brake

To the Editor:

With the advent of clustered, regularly interspaced, short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) technology[1,2], researchers can construct gene drives that can bias the inheritance of edited alleles to alter entire populations[3,4]. As demonstrated with the mutagenic chain reaction in *Drosophila*[4], the CRISPR-Cas9 system can propagate genomic modification together with the genome-editing machinery itself. Although gene drives might have the potential to control insect-borne diseases and agricultural pests[5], substantial concerns have been raised over unanticipated ecological consequences as a result of drive use[6]. Here we report the development of a potential Cas9-based gene drive 'brake' that remains inert in a wild-type genome but is activated by Cas9 to both cleave the genomic *cas9* sequence and to convert an incoming *cas9* allele into a brake. This means that the propagation of the brake is favored in a *cas9*-carrying population.

We designed and synthesized a transgene system that we named Cas9-triggered chain ablation (CATCHA). The CATCHA transgene encodes a guide RNA (gRNA) that is expressed ubiquitously from a U6:2 promoter. The gRNA targets a site within the DNA sequence of *cas9*. The guide RNA is flanked by homology arms (of 1,042 bp and 1,003 bp) that match the *cas9* sequences next to the gRNA-specified cleavage site (**Fig. 1a**). In the presence of both CATCHA and *cas9*, Cas9 proteins will be guided to cleave the *cas9* genomic locus from which Cas9 proteins are expressed. Upon repair of the cleaved *cas9* by homology-directed repair (HDR), the *cas9* locus will be converted to CATCHA. Such conversion in heterozygous offspring favors amplification of CATCHA in the *cas9*-carrying population (**Fig. 1a**).

In a proof-of-principle experiment, we used CATCHA in *vas-cas9* transgenic flies, which are commonly used for CRISPR-based transgenesis[7], and in which Cas9 likely follows endogenous *vas* expression in



	CATCHA positive (HDR)	CATCHA negative (NHEJ)
F1 fly count	51 (57%)	39 (43%)

	HDR	NHEJ	Intact	Total
F2 non-*ebony*	92	4	0	96 (92.3%)
F2 *ebony*	4	4	0	8 (7.7%)
Total	96 (92.3%)	8 (7.7%)	0 (0%)	104 (100%)

Figure 1 Schematics and proof of principle of CATCHA. (**a**) Left, the CATCHA construct encodes a gRNA targeting *cas9*, flanked by homology arms (*HA1* and *HA2*) corresponding to *cas9* sequences next to the cleavage site specified by the gRNA (see **Supplementary Methods** for details). In the presence of genomically encoded Cas9, the CATCHA construct is integrated into the target locus via HDR. Successful HDR events can be identified by PCR with suitable primers (blue). Right, the genomically integrated CATCHA can further convert allelic *cas9* sequences to CATCHA via HDR. Indels and converted alleles can be distinguished with PCR primers (black arrows). Primer information is available in **Supplementary Methods**. (**b**) Integrating CATCHA into the genome. *vas-cas9* donor females injected with the CATCHA construct were crossed to males expressing gRNA targeting *wg* to select for ablated *cas9* alleles. PCR was performed for each F1 survivor to identify CATCHA-positive progenies and establish stable stocks. NHEJ is presumed on the basis of PCR results. Balancers are fly chromosomes with multiple inversions that impede recombination and thereby maintain transgenes and mutations in stable stocks. (**c**) Converting genomic *cas9* using genomic CATCHA. All 104 F2 offspring from one CATCHA male crossed to *vas-cas9* females were genotyped using PCR. Representative DNA gel lanes are shown (top right). F0 CATCHA and F0 *vas-cas9* are controls indicating the respective sizes of CATCHA (~1,200 bp) and NHEJ (~650 bp) bands. In sum, 96 F2 flies carried CATCHA, suggesting a conversion rate of 85%, estimated by the formula (CATCHA – total × 50%) / (total × 50%). All eight F2 flies with short bands (non-HDR) were sequenced (bottom left) and confirmed to carry NHEJ-mediated indels (nucleotides differing from *cas9* sequence are shown in red). The four indels that maintain the reading frame (NHEJ 5–8) correspond to the four *ebony* flies. The purple box highlights the protospacer adjacent motif (PAM) sequence. See **Supplementary Table 1** for the summary of all F2 phenotypes.

the germline throughout development and is maternally deposited into embryos[8,9]. To introduce the CATCHA transgene into the genome, we injected *vas-cas9* embryos with a plasmid carrying CATCHA (**Fig. 1a**, **Supplementary Fig. 1** and **Supplementary Methods**). To enrich for converted alleles, the resultant F0 females were crossed to transgenic males that stably express gRNA that targets the essential gene *wg*[10]. This way, all surviving F1 progeny would carry ablated *cas9* (**Fig. 1b**). Flies that still express functional Cas9 cannot survive owing to the engagement of the lethal gRNA. We found that the *cas9* locus was converted to CATCHA in 57% (51 out of 90) of F1 progeny (**Fig. 1b**) as assayed by PCR (**Fig. 1a**), demonstrating that CATCHA can convert *cas9*, even with extragenomic delivery. The rest of the surviving F1 population (39 flies) are likely to carry *cas9* loci with deletions or insertions (indels) resulted from nonhomologous end joining (NHEJ).

Next, we tested whether genomically encoded CATCHA can efficiently convert allelic *vas-cas9* to CATCHA (**Fig. 1a**). We crossed five independent F0 CATCHA males to *vas-cas9* females (**Fig. 1c**). To estimate the rate of *cas9* ablation in one generation, we crossed F1 females to transgenic males stably expressing gRNA against the *e* (*ebony*) gene[10], the disruption of which will result in a markedly darkened cuticle (**Supplementary Fig. 2**). The emergence of F2 flies with wild-type cuticle color (and thus, failed disruption of the *ebony* gene) is an indicator for the efficiency of CATCHA-mediated *cas9* ablation. Overall, 93.4% of F2 cuticles had wild-type color, demonstrating successful impairment of *cas9* function (**Supplementary Table 1**). To determine the nature of ablated *cas9* alleles, we genotyped all F2 descendants of the first CATCHA male using PCR followed by sequencing (**Fig. 1c**). Of the 104 F2 flies, 96 carried either direct replication of the F0 CATCHA allele or HDR-mediated conversion of *vas-cas9*, indicating that 85% of *vas-cas9* alleles were successfully converted to CATCHA (**Fig. 1c**). In addition, all eight non-CATCHA F2 flies carried NHEJ-mediated indels (**Fig. 1c**). Thus, genomic CATCHA is highly efficient in inactivating *cas9* and converting most alleles to CATCHA.

In summary, we propose here a chain reaction named CATCHA that could potentially be used to inactivate genomic *cas9* in insect populations and demonstrate that CATCHA can inactivate *cas9* to near completion in a laboratory population of flies. However, it is important to note that further improvements and analyses will be required to establish whether CATCHA, or a similar approach, might be suitable for eventual use in either the laboratory or the field. First, it is worth testing whether *cas9* can be inactivated using a nonallelic CATCHA. Given that gRNAs function *in trans*, we anticipate highly efficient cleavage regardless of CATCHA's locus, as suggested by our own data (0 out of 104 F2 carried intact cas9 sequences, **Fig. 1c**) and previous work[4,10]. Although such a strategy would not provide an allelic template to convert the drive, it is much more convenient, as one premade CATCHA released at high frequency could be used to target different gene drives. Second, a small percentage of in-frame indels (**Supplementary Table 1**) might produce CATCHA-resistant alleles that encode functional Cas9 proteins; in that case, it might be possible to modify CATCHA to include two gRNAs to mediate double cleavage, deleting a longer segment of the *cas9* coding sequence. Third, the interactions between a Cas9-based gene drive, CATCHA and wild-type alleles could be affected by the frequency of each of them in the population and the carrier insects' fitness. To understand the efficacy of CATCHA under different scenarios, it will be necessary to estimate population dynamics using mathematical modeling. Lastly, field experiments would clearly need to be performed before deployment of CATCHA in any ecosystem is contemplated.

We envision that an improved version of CATCHA might be useful in selected situations. In a facility that is used to house *cas9*-based gene drive animals, it might be beneficial to maintain CATCHA animals in the same space; the latter might be able to neutralize the former in the event of mass escape, for example, that is caused by a catastrophe, such as an earthquake. Alternatively, if a *cas9*-based gene drive is already present in a wild population, CATCHA animals might be released into the wild to potentially function as a 'cordon' to contain *cas9* propagation, followed by other sophisticated gene drives[5] that could be added to remedy the damage. Scientists must be alert to the ethical implications of research[6], but policies and regulations are not immune to human malice, negligence or natural disasters. Technical advancements based on this set of proof-of-principle experiments might offer a mechanism to reduce the spread of a Cas9-based gene drive through a population and thereby function as a potential safeguard against the unwanted consequences of such drives.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/nbt.3444).

Accession codes. GenBank: The plasmid sequence of CATCHA has been deposited under accession number KU212289. The sequencing results of eight NHEJ alleles have been deposited under accession numbers KU212290, KU212291, KU221292, KU221293, KU221294, KU221295, KU221296 and KU212297.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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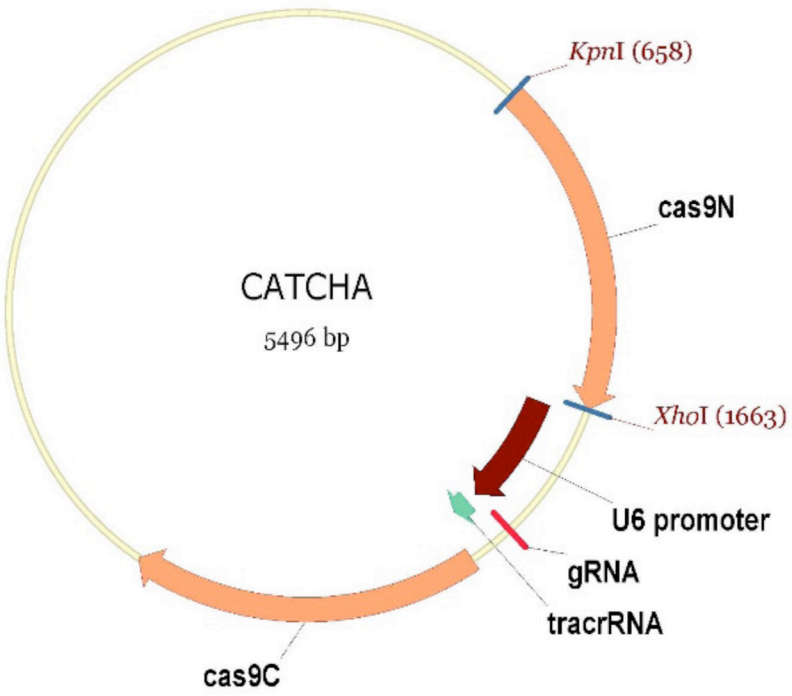
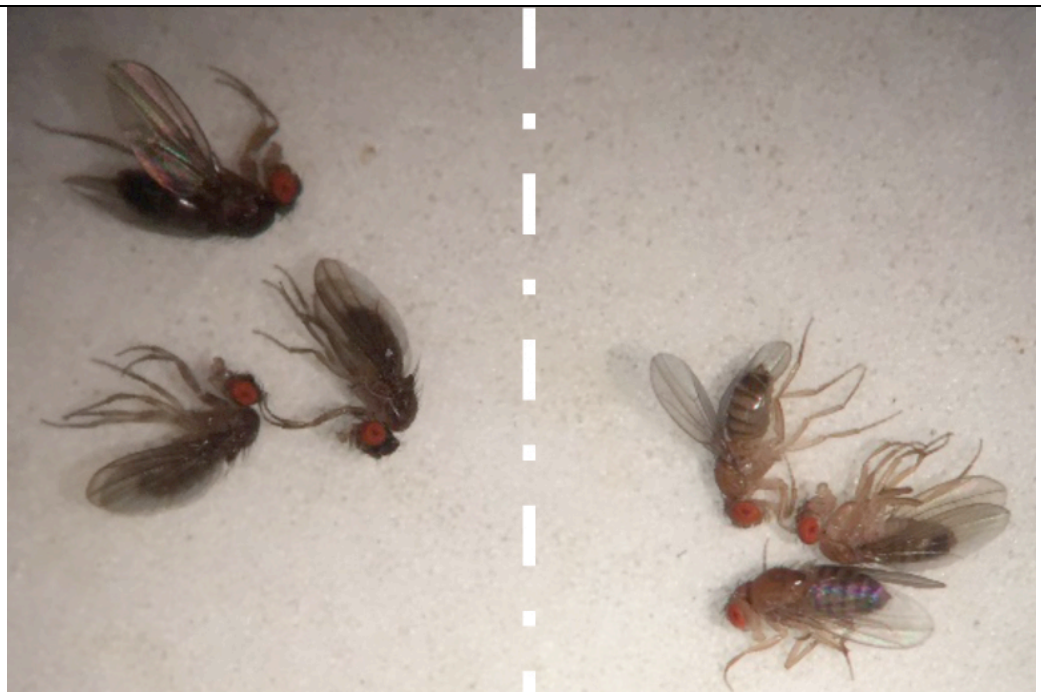


Supplementary Figure 1

Diagram for CATCHA construct.



Supplementary Figure 2

Representative view of ebony (left) and non-ebony (right) F2 flies from experiments described in Fig. 1c.

	F0 #1	F0 #2	F0 #3	F0 #4	F0 #5	Total (#1 to #5)	*vas-cas9* /+ control
F2 *ebony* fly count	8	6	5	11	7	37	121
F2 non-*ebony* fly count	96	103	89	139	97	524	0
Fraction of non-*ebony*	92.3%	94.5%	94.7%	92.7%	93.3%	93.4%	0.0%

Supplementary Table 1. *ebony* phenotypic analysis for assessing CATCHA-induced *cas9* ablation.
Columns from F0 #1 to F0 #5 were from five experiments in parallel using five CATCHA stocks. Each CATCHA stock (F2 in **Fig. 1b**) originated from a single F1 male that is positive for CATCHA. In *vas-cas9*/+ control group, *vas-cas9*/+ flies were used as F1 females in experiment described in **Fig. 1c**, demonstrating the efficiency of *ebony* gRNA. All flies in the first column (highlighted in yellow) were molecularly genotyped in **Fig. 1c**, which revealed that the 8 *ebony* flies also carry either CATCHA or NHEJ-mediated indels. Thus, the fraction of non-*ebony* in F2 is likely an underestimate of ablation efficiency. The disruption of the *ebony* gene in these 8 flies may be caused by the following two factors. First, the maternal contribution of *cas9* mRNA and/or Cas9 protein can disrupt *ebony* in the zygote. Since *vas-cas9* is expressed in nurse cells, *cas9* mRNA and Cas9 protein can be deposited into fertilized eggs regardless of the latter's genotype. If CATCHA-mediated ablation occurs late in the germline, sufficient numbers of nurse cells may still carry functional *vas-cas9* and deposit maternal Cas9 to cleave *ebony* in the zygote. That 100% (rather than 50%) *vas-cas9*/+ progeny are *ebony* supports this interpretation.

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Materials and Methods

Construction of CATCHA plasmid and verification

gRNA sequence targeting *cas9* was generated by annealing oligos cas9_gRNA_F (CTTCGGCTACGCCGGCTACATTGA) and cas9_gRNA_R (AAACTCAATGTAGCCGGCGTAGCC). The resulting product was ligated into BbsI-digested plasmid pU6-BbsI-chiRNA[1] (Addgene ID #45946), followed by transformation into TOP10 Chemically Competent *E. coli* (Life Technologies). Positive pU6-cas9-chiRNA colonies were confirmed by sequencing.

The homology arm (1042 bp) for the downstream of the *cas9* cleaved site were amplified by PCR using primers cas9C_infus_F (GGGGATCCACTAGTTGACGGCGGAGCCAGC) and cas9C_infus_R (TGGCGGCCGCTCTAGCTTTCTGGATGTCCTCT), from template plasmid pHsp70-Cas9[1] (Addgene ID #45945), followed by PCR purification (Qiagen). In-Fusion (Clontech) was performed to insert the product into vector pU6-cas9-chiRNA at the Xba1 site. Transformation and colony confirmation were conducted as described above, yielding a pU6-cas9-chiRNA-cas9C plasmid.

The homology arm (1003 bp) for the upstream of the *cas9* cleaved site was amplified by PCR using primers cas9_N_Kpn1F (ATGGTACCGCAAGAAATTCAAGGTGCTG) and cas9_N_Xho1R (ATCTCGAGATGTAGCCGGCGTAGCCGTTCT). Products were purified and treated with Kpn1 and Xho1 restriction enzymes (New England Biolabs). Purified products were then ligated with pU6-cas9-chiRNA-cas9C vector digested by Kpn1 and Xho1. The final cas9N-pU6-cas9-chiRNA-cas9C plasmid (CATCHA construct, **Supplementary Fig. 1**) was verified by sequencing. Plasmid sequence is available at Harvard Dataverse (https://dataverse.harvard.edu/), with the title "Cas9-Triggered Chain Ablation (CATCHA) sequence materials".

Drosophila stocks

w[1118]; PBac{y[+mDint2]=vas-Cas9}VK00027[2] (Bloomington stock # 51324) was injected with the CATCHA construct. This strain also served as the allelic *vas-cas9* to test conversion efficiency of integrated CATCHA. Transgenic gRNAs against *wingless* and *ebony* were gifts from Fillip Port[3].

CATCHA stocks were confirmed by PCR with primers cas9_F_seq (CTGAGCGCCTCTATGATC) and pU6_R_seq (AACTAGTGGATCCCCCG) (blue primers in Fig. 1a, left). A 705-bp band was yielded from CATCHA positive stock. cas9_F_seq (CTGAGCGCCTCTATGATC) and cas9_R_seq (TCTCATTCCCTCGGTCACGT) (black primers in Fig. 1a, right) were used to obtain a 1221-bp band from CATCHA positive (HDR) flies, and a 654-bp band from flies carrying NHEJ alleles. The NHEJ alleles were sequenced using nest_cas9_R_seq primer (TGGTCAGCTCGTTATACACGGTG). The sequencing results are deposited at Harvard Dataverse.

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Supplementary References

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