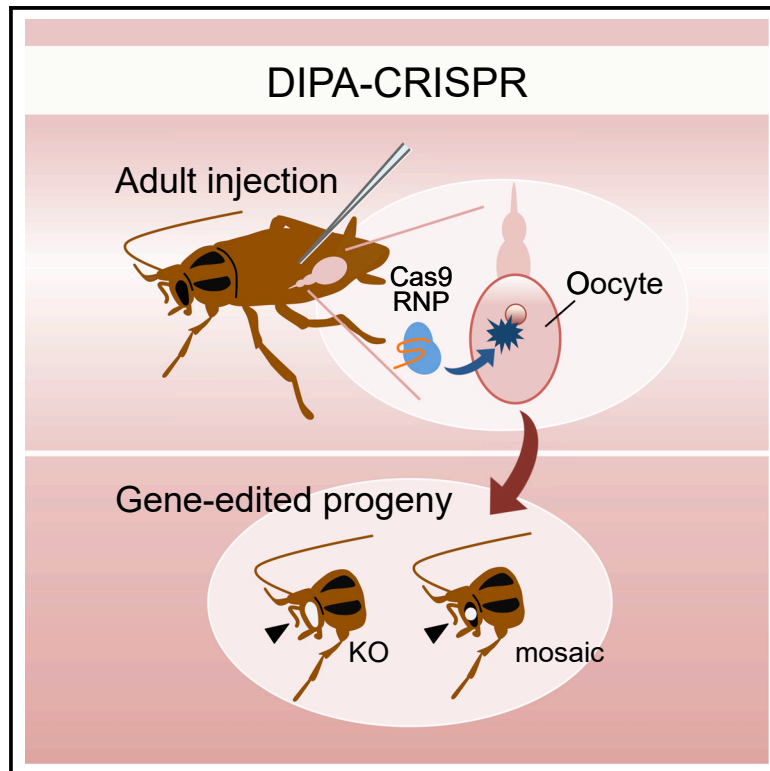**Report**

DIPA-CRISPR is a simple and accessible method for insect gene editing

Graphical abstract

Authors

Yu Shirai, Maria-Dolors Piulachs, Xavier Belles, Takaaki Daimon

Correspondence

daimon.takaaki.7a@kyoto-u.ac.jp

In brief

Shirai et al. develop a simple and accessible method, DIPA-CRISPR, for insect gene editing by direct adult injection of Cas9 ribonucleoproteins. Using it, they successfully establish gene knockouts in cockroaches, in which conventional embryo microinjection cannot be applied. Given its simplicity and versatility, DIPA-CRISPR has the potential to greatly extend the application of gene editing technology to a wide diversity of insects.

Highlights

- A simple and efficient method for insect gene editing

- Based on direct adult injection of Cas9 ribonucleoproteins

- Readily implementable in non-specialist laboratories

- Applicable to a wide diversity of non-model insects

CellPress

Report

DIPA-CRISPR is a simple and accessible method for insect gene editing

Yu Shirai,[1] Maria-Dolors Piulachs,[2] Xavier Belles,[2] and Takaaki Daimon[1,3,*]

[1]Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
[2]Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta 37, Barcelona 08003, Spain
[3]Lead contact
*Correspondence: daimon.takaaki.7a@kyoto-u.ac.jp
https://doi.org/10.1016/j.crmeth.2022.100215

> **MOTIVATION** With over a million species described, insects are a treasure trove of diversity and represent boundless possibilities as research tools for answering fundamental questions in biology. Current approaches for insect gene editing require microinjection of materials into early embryos, which is highly challenging in most species. In this work, we established and optimized a simple and efficient method for insect gene editing by adult injection, which can be readily implemented in any laboratory and directly applied to a great diversity of non-model insect species.

SUMMARY

Current approaches for insect gene editing require microinjection of materials into early embryos. This severely limits the application of gene editing to a great number of insect species, especially to those whose reproduction systems preclude access to early embryos for injection. To overcome these limitations, we report a simple and accessible method for insect gene editing, termed "direct parental" CRISPR (DIPA-CRISPR). We show that injection of Cas9 ribonucleoproteins (RNPs) into the haemocoel of adult females efficiently introduces heritable mutations in developing oocytes. Importantly, commercially available standard Cas9 protein can be directly used for DIPA-CRISPR, which makes this approach highly practical and feasible. DIPA-CRISPR enables highly efficient gene editing in the cockroaches, on which conventional approaches cannot be applied, and in the model beetle *Tribolium castaneum*. Due to its simplicity and accessibility, DIPA-CRISPR will greatly extend the application of gene editing technology to a wide variety of insects.

INTRODUCTION

Recent advances of genome editing tools have enabled sophisticated engineering of insect genomes (Doudna and Charpentier, 2014; Gantz and Akbari, 2018; Matthews and Vosshall, 2020). However, current approaches rely on embryo injection, which requires expensive equipment, a specific experimental setup for each species, and highly skilled laboratory personnel (Matthews and Vosshall, 2020; Tamura et al., 2000). Furthermore, embryo injection must be completed in a small time window, from oviposition to preblastoderm stage, which is not applicable to species that give live birth rather than lay eggs (e.g., viviparous aphids and flies) or species in which an access to very early embryos is highly challenging (e.g., cockroaches, which encapsulate the eggs into a hard egg case or ootheca).

Recently, an alternative method that can bypass the requirement of embryo injection has been developed in the mosquito *Aedes aegypti* (Chaverra-Rodriguez et al., 2018). In this method, called Receptor-Mediated Ovary Transduction of Cargo (ReMOT), peptide ligands derived from yolk protein precursors are fused to Cas9 protein, and the complex of the engineered Cas9 and single-guide RNAs (sgRNAs) is injected into female adults to introduce mutations in developing oocytes. ReMOT-mediated targeted mutagenesis has been successfully used in a few other species, such as the mosquito *Anopheles stephensi* (Macias et al., 2020), the jewel wasp *Nasonia vitripennis* (Chaverra-Rodriguez et al., 2020), the red flour beetle *Tribolium castaneum* (Shirai and Daimon, 2020), and the silverleaf whitefly *Bemisia tabaci* (Heu et al., 2020). Although these examples are encouraging, the ReMOT approach appears to have a number of limitations. For example, the results reported so far indicate that the peptide ligands that are fused to Cas9 should be specifically tuned to a target species for efficient gene editing (Chaverra-Rodriguez et al., 2018; Heu et al., 2020; Shirai and Daimon, 2020), which may be a barrier for non-specialist laboratories.

Interestingly, however, the above studies, together with the study in the spider mite *Tetranychus urticae* (Dermauw et al., 2020), also showed that a very small number of gene-edited
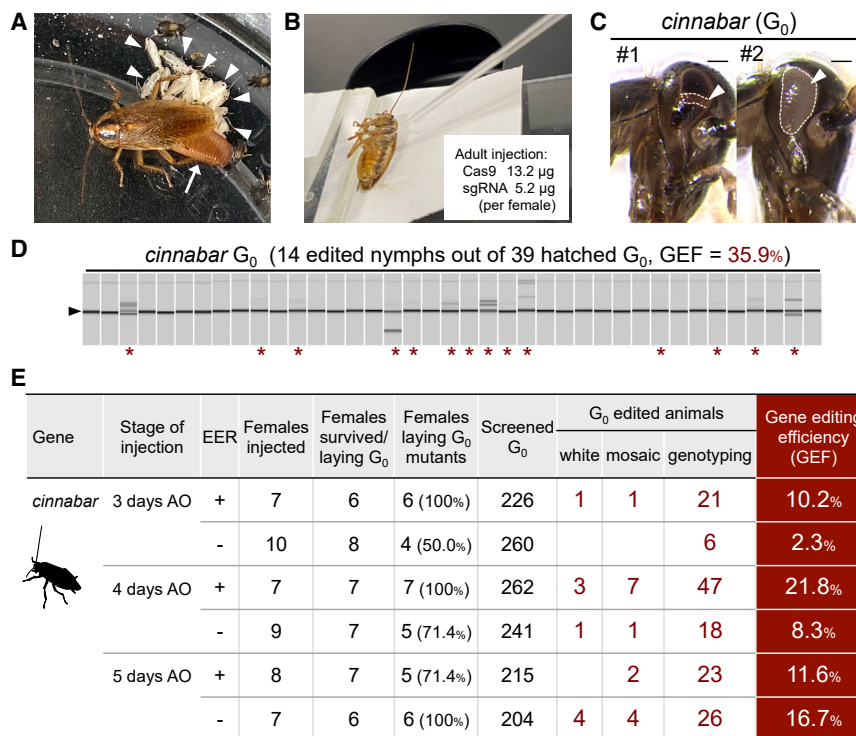
Figure 1. Cockroach gene editing with DIPA-CRISPR

(A) Newly hatched nymphs (arrowheads) from the ootheca (arrow) of *Blattella germanica*.

(B) Adult injection in *B. germanica*.

(C) The eyes of *cinnabar* mosaic G_0 nymphs with loss of black eye pigments. Scale bars, 100 μm.

(D) A representative result of *cinnabar* G_0 genotyping. G_0 nymphs with normal eyes hatched from a single ootheca were analyzed by heteroduplex mobility assay (HMA). Individuals having edited alleles (asterisks) and the homoduplex bands (156 bp, arrowhead) are indicated. See also Figure S1 for the detailed results.

(E) Gene editing efficiency (GEF) of DIPA-CRISPR. G_0 mutants were first screened by phenotypes (white or mosaic), and then those without phenotypes were analyzed by HMA (genotyping). White, having phenotypes in entire regions of both eyes; AO, after ootheca drop; EER, presence or absence of chloroquine (2 mM) in injection solution.

Figure panel captions:
cinnabar (G_0)
#1   #2

cinnabar G_0 (14 edited nymphs out of 39 hatched G_0, GEF = 35.9%)

Adult injection:
Cas9 13.2 μg
sgRNA 5.2 μg
(per female)

Gene	Stage of injection	EER	Females injected	Females survived/ laying G_0	Females laying G_0 mutants	Screened G_0	G_0 edited animals			Gene editing efficiency (GEF)
							white	mosaic	genotyping	
cinnabar	3 days AO	+	7	6	6 (100%)	226	1	1	21	10.2%
		-	10	8	4 (50.0%)	260			6	2.3%
	4 days AO	+	7	7	7 (100%)	262	3	7	47	21.8%
		-	9	7	5 (71.4%)	241	1	1	18	8.3%
	5 days AO	+	8	7	5 (71.4%)	215		2	23	11.6%
		-	7	6	6 (100%)	204	4	4	26	16.7%

able to generate gene knockin beetles by co-injecting single-stranded oligonucleotides (ssODNs) and Cas9 RNPs. The successful application of DIPA-CRISPR in the two evolutionarily distant insect species gives an idea of its generalizability. Without the need of custom-engineering of Cas9 or the use of special reagents that have been considered to facilitate the ovary uptake of injected Cas9 ribonucleoproteins (RNPs) (Chaverra-Rodriguez et al., 2018, 2020), DIPA-CRISPR could be readily implemented in any laboratory, so that it would greatly extend the application of gene editing to a wide diversity of insect species.

RESULTS AND DISCUSSION

Cockroach gene editing with DIPA-CRISPR

In general, cockroach females ovulate the oocytes into the genital atrium, where they are fertilized and then encapsulated into a hard egg case, or ootheca, where they will remain for days or weeks until egg hatching (Figure 1A) (Cornwell, 1968). Because of this unique reproduction system, it is impracticable to inject materials into very early embryos, thus genetic manipulation of cockroaches (i.e., transgenesis or gene editing) has not been achieved so far. To investigate whether adult injection of Cas9 RNPs enables cockroach gene editing, we tested non-tagged Cas9 in *B. germanica*, a global urban pest whose genome has been sequenced (Harrison et al., 2018), by targeting the autosomal eye color gene *cinnabar* (Figure S1), which is involved in the biosynthesis of ommochrome pigments (Lorenzen et al., 2002; Quan et al., 2002).

We first injected commercial Cas9 RNPs into 16 fully matured females not carrying oothecae (Figure 1B). We presumed that they were undergoing a vitellogenic cycle, thus the injected Cas9 RNPs might be non-selectively incorporated into the growing oocytes with vitellogenins by receptor-mediated endocytosis (Ciudad et al., 2006; Cooper and Hausman, 2007;

individuals could be recovered by adult injection of non-tagged Cas9 (i.e., Cas9 without a ligand sequence). Thus, these studies suggest that, although the addition of an appropriate ligand tag to Cas9 could increase gene editing efficiency, its addition is not essential for gene editing by adult injection.

During the course of our work aiming at developing a novel peptide tag for Cas9 that can cover a broad range of insect groups (i.e., a fragment of yolk protein precursor vitellogenin [Li et al., 2003; Murakami et al., 2019; discussed in Shirai and Daimon, 2020]), our group found that female adult injection of non-tagged Cas9 can efficiently introduce heritable mutations in developing oocytes of the German cockroach *Blattella germanica*, to which conventional approaches (i.e., embryo injection) are not feasible. As we used commercially available standard Cas9 protein (i.e., the one sold for general genome editing experiments in animals and cultured cells), such "non-tagged Cas9 approach" would become a more generalized method for insect gene editing by adult injection. To further explore this possibility, we here optimized this method and established it as an accessible technology for insect gene editing, which we named "direct parental" CRISPR (DIPA-CRISPR).

After exploring different optimizing conditions of DIPA-CRISPR in *B. germanica*, we demonstrated that gene editing efficiency (GEF; the proportion of edited individuals out of the total number of individuals hatched) could reach as high as 21.8%, which easily enabled the first establishment of knockout cockroach lines. Furthermore, we tested DIPA-CRISPR in the red flour beetle *T. castaneum*, in which GEF reached over 50%, a percentage comparable with the efficiency reached in conventional approaches (Gilles et al., 2015). Furthermore, we were
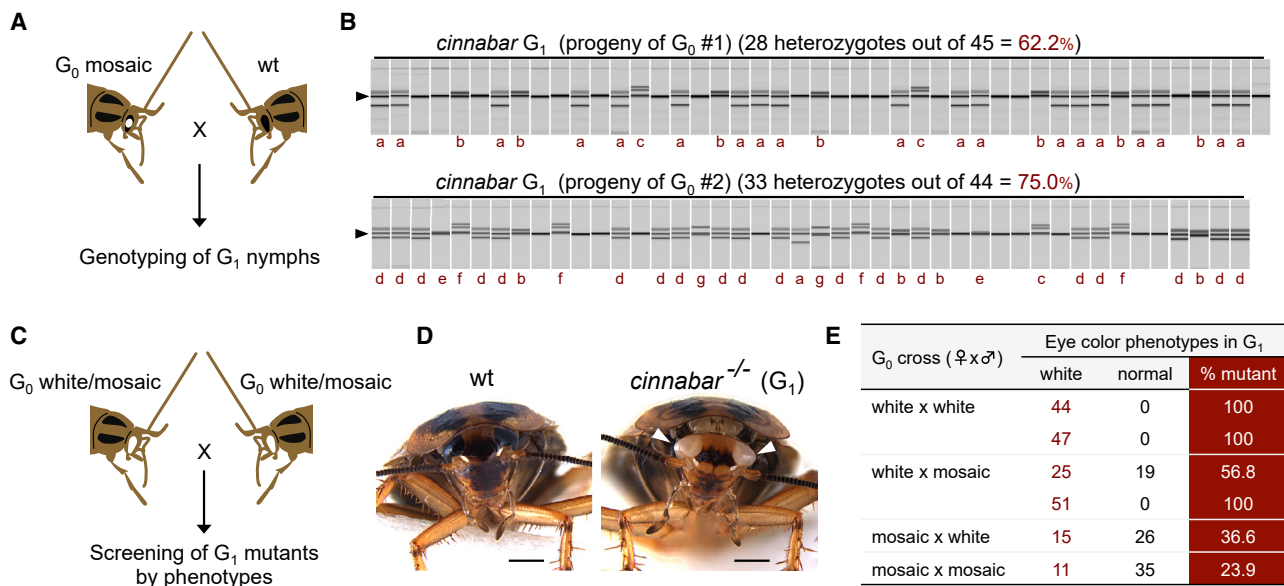
Figure 2. Inheritance of edited alleles in cockroaches

(A and B) A mating scheme (A) and the results of genotyping (B) of G_1 individuals. The two *cinnabar* mosaics shown in Figure 1C (G_0 #1 and #2) were crossed to wild type, and all the G_1 progeny were individually analyzed by heteroduplex mobility assay. The mutant alleles are indicated below the panels (a–g, seven alleles in total). See Figure S1D for their nucleotide sequences.

(C) A mating scheme for screening of G_1 knockout insects. G_0 insects with eye color phenotypes were crossed to obtain G_1 progenies.

(D) A *cinnabar* knockout G_1 adult (right). Arrowheads indicate the white eyes. Scale bars, 1 mm.

(E) Phenotypes in G_1 insects. Each row indicates the result of a single-pair mating of G_0.

Dermauw et al., 2020; Raikhel and Dhadialla, 1992). Notably, 31% (5 out of 16) of the injected females that produced an ootheca after the injection yielded gene-edited G_0 (generation zero) progeny. Of the nine edited G_0 nymphs recovered from 385 hatchlings (GEF = 2.3%), two were eye color mosaics (Figure 1C), and the other seven carried edited alleles as judged by genotyping experiments (see Figure 1D for representative results). As *cinnabar* is an autosomal gene, the mosaic phenotypes observed in the G_0 insects indicate that the incorporated Cas9 RNPs might persist in the oocyte for several days (i.e., from injection to fertilization) and then disrupted paternal alleles after fertilization, yielding cells having biallelic mutations.

The results obtained in this first experiment encouraged us to explore optimal conditions of our method, DIPA-CRISPR, by using carefully staged adults. We tested females at selected days of their reproductive cycle (Cornwell, 1968; Pascual et al., 1992; Treiblmayr et al., 2006) and found that injecting 4 days after ootheca drop and eggs hatching resulted in the highest efficiency (Figures 1E and S1C). In this condition, all the seven injected females produced gene-edited G_0 nymphs, and they yielded 57 edited nymphs from 262 hatchlings in total (GEF = 21.8%) (Figure 1E). Notably, some G_0 nymphs had "white" eyes (i.e., the entire surface of both eyes was white), suggesting that biallelic mutations were introduced at a very early stage of embryogenesis. When the GEF values of injected females were individually measured, some females had values exceeding 40% (Figure S1C). Collectively, our results show that highly efficient cockroach gene editing can be achieved by simple adult injection.

Inheritance of edited alleles in cockroaches

To test if edited alleles in G_0 cockroaches are inherited to the next generation, we performed crossing experiments (Figure 2). When the two mosaic nymphs obtained in the first experiment (Figure 1C) were reared to adults and crossed to wild type (Figure 2A), 62.2% and 75.0% of their respective progenies (G_1) were heterozygous mutants (Figure 2B), showing very high germline mutation rates in these mosaics. To further confirm this result, G_0 adults with eye color phenotypes (white or mosaic) were crossed and their G_1 progenies were screened for knockout phenotypes (Figure 2C). Notably, when G_0 adults with both eyes white were crossed, all of their G_1 progeny became white-eyed knockout insects (Figures 2D and 2E). This suggests that *cinnabar* was disrupted in all (or practically all) cells in the white-eyed G_0 individuals. Similarly, very high germline mutation rates were also exhibited in mosaic-eyed G_0 adults, which were roughly estimated to be >50% (Figure 2E). Together, our results demonstrate that DIPA-CRISPR is a powerful method that easily enabled the first establishment of knockout cockroaches.

DIPA-CRISPR in beetles

To demonstrate potential for broad use, we applied DIPA-CRISPR to much more evolutionarily modified species, the red flour beetle *T. castaneum* (Figure 3). For these experiments, we targeted *cardinal*, an eye color gene on the X chromosome, as their mutant phenotypes are easily visible in hemizygous G_0 males without genotyping (females = XX and males = XY in *T. castaneum*) (Grubbs et al., 2015; Shirai and Daimon, 2020).
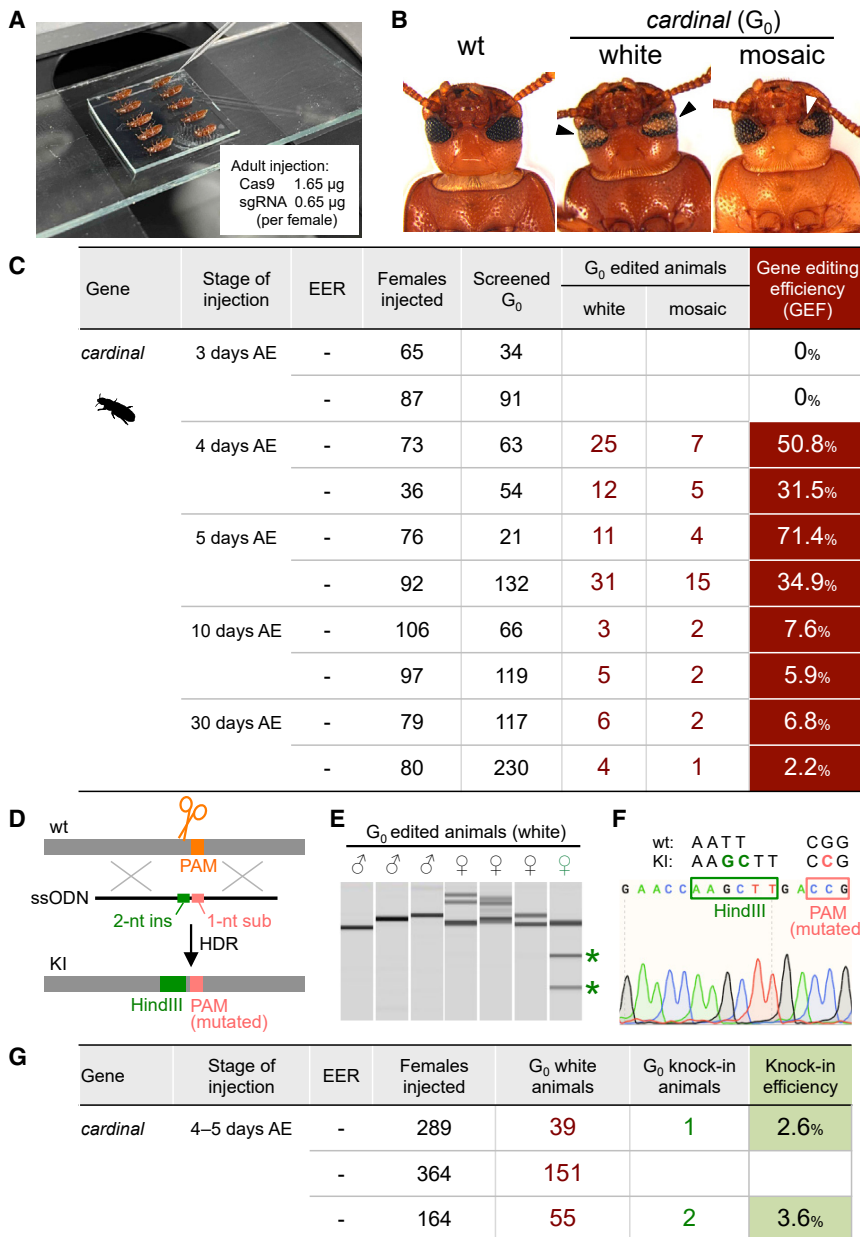
Figure 3. DIPA-CRISPR in beetles

(A) Adult injection in *Tribolium castaneum*.

(B) G_0 adults generated by DIPA-CRISPR targeting *cardinal*. Arrowheads indicate the loss of black eye pigments.

(C) The gene editing efficiency (GEF) in *T. castaneum*. See Figure S2 for the detailed results.

(D) A scheme of knockin using ssODNs as a template for homology-directed repair (HDR). ssODNs that contain 5′- and -3′-homology arms (96 nt each), a 2-nt insertion (producing a HindIII site) and a 1-nt substitution (mutating the PAM sequence) are knocked into the *cardinal* gene.

(E) A representative result of G_0 genotyping. G_0 adults with both eyes white were individually genotyped. The products of genomic PCR were digested with HindIII and analyzed by microchip electrophoresis. Asterisks indicate the HindIII-digested products.

(F) The nucleotide sequence of the recovered knockin (KI) allele.

(G) The efficiency of knockin through DIPA-CRISPR in three independent experiments. All the three knockin beetles carried the precise knockin allele shown in (F).

Panel A label:
Adult injection:
Cas9   1.65 μg
sgRNA 0.65 μg
(per female)

Panel B: wt   *cardinal* (G_0)   white   mosaic

Panel C table:

Gene	Stage of injection	EER	Females injected	Screened G_0	G_0 edited animals white	G_0 edited animals mosaic	Gene editing efficiency (GEF)
cardinal	3 days AE	-	65	34			0%
		-	87	91			0%
	4 days AE	-	73	63	25	7	50.8%
		-	36	54	12	5	31.5%
	5 days AE	-	76	21	11	4	71.4%
		-	92	132	31	15	34.9%
	10 days AE	-	106	66	3	2	7.6%
		-	97	119	5	2	5.9%
	30 days AE	-	79	117	6	2	6.8%
		-	80	230	4	1	2.2%

Panel D: wt / PAM / ssODN / 2-nt ins / 1-nt sub / HDR / KI / HindIII / PAM (mutated)

Panel E: G_0 edited animals (white)

Panel F:
wt: A A T T   C G G
KI: A A **G C** T T   C **C** G
G A A C C A A G C T T G A C C G
HindIII   PAM (mutated)

Panel G table:

Gene	Stage of injection	EER	Females injected	G_0 white animals	G_0 knock-in animals	Knock-in efficiency
cardinal	4–5 days AE	-	289	39	1	2.6%
		-	364	151		
		-	164	55	2	3.6%

We injected Cas9 RNPs into females at selected days after adult emergence (Figures 3A and S2). We found that injection into 4- or 5-day-old adult females exhibited very high gene editing efficiency, with the GEF values being as high as 50.8% for 4-day-old females (32 out of 63 hatchlings) and 71.4% for 5-day-old females (15 out of 21) (Figures 3B and 3C), which is comparable with the efficiency in conventional embryo injection approaches (Gilles et al., 2015).

Cockroaches and beetles used in this study are evolutionarily very distant (Polyneopteran versus Endopterygote) (Harrison et al., 2018; Misof et al., 2014), show radically different modes of metamorphosis (hemimetabolan versus holometabolan) (Belles, 2020), and have different types of ovaries (panoistic in *B. germanica* versus telotrophic in *T. castaneum*) (McLaughlin and Bratu, 2015). Furthermore, previous ReMOT studies have shown that gene editing can be achieved by adult injection in wasps or mosquitos (Chaverra-Rodriguez et al., 2018, 2020; Macias et al., 2020), which have the most derived types of ovaries (i.e., polytrophic). Thus, these results point to DIPA-CRISPR as a generalizable approach for insect gene editing.

Gene knockin by DIPA-CRISPR in beetles

We next tested whether DIPA-CRISPR could be used to generate gene knockin insects, using *T. castaneum* as the experimental subject. We designed an ssODN having homology arms (96 nt each for 5′- and -3′-homology arms), a 2-nt insertion that introduces a novel HindIII restriction site, and a 1-nt substitution that mutates PAM, and used it as a template for homology-directed repair (HDR) (Figures 3D–3G). After injecting a mixture of Cas9 RNPs targeting *cardinal* and the ssODNs into female adults at optimized stages, we screened for white-eyed G_0 adults. Genotyping of these adults showed that three adults (1.2%, 3 out of 245 in total) carried precise knockin alleles generated by HDR (Figures 3E and 3F). Although the efficiency is still low and should be further improved, our results indicate that the application of DIPA-CRISPR can be extended to knockin experiments.

Cas9 for DIPA-CRISPR

The direct use of commercial Cas9 protein for adult injection in the DIPA-CRISPR can eliminate time-consuming processes required in a similar adult injection approach (ReMOT) (Chaverra-Rodriguez et al., 2018), such as the development of a novel ligand that is tuned to target species, engineering of Cas9, and the expression and purification of recombinant Cas9 protein. Thus, the use of commercial Cas9 can enable gene editing in any non-specialist laboratory that cannot implement the above elaborated methods.

We also investigated and compared the performance of commercial Cas9 products from additional three companies in the market (Figure S3A). Although details were not disclosed to the authors, these Cas9 products should be engineered differently by manufacturers in many ways (e.g., the type, number, and location of nuclear localization signals [NLSs] or other epitope/purification tags). Nevertheless, their gene editing efficiencies were comparable and very high when tested in *T. castaneum* (GEF = 24%–32%; Figure S3A). This indicates that there is little requirement to use a particular Cas9 product, although the presence of NLSs should be essential for the delivery of Cas9 RNPs to the nucleus.

It is of note that, unlike this study, very little gene editing was observed with non-tagged Cas9 in previous ReMOT studies. We speculate that the large differences in gene editing efficiency between this study and previous attempts are probably due to the difference in the preparation of Cas9 and doses of Cas9 injected. In previous studies, Cas9 was purified only by a single step of affinity chromatography, which might have led to a product contaminated with undesired materials. Although commercial Cas9 was tested in the jewel wasp *N. vitripennis* (Chaverra-Rodriguez et al., 2020), the dose used was much lower than that used in this study (0.36 versus 3.3 μg/μL in injection solution). To examine this view, we tested a serial dilution of Cas9 RNPs in *T. castaneum* and found a clear trend showing that decreased doses of Cas9 RNPs result in the decreased gene editing efficiency (Figures S3B and S3C). We thus propose using relatively high doses of commercial Cas9 when implementing our method to other species.

The use of endosomal escape reagents

It has been reported that the efficiency of ReMOT-mediated gene editing can be improved with the use of endosomal escape reagents (EERs) that facilitate the release of Cas9 RNPs from the endosome to the cytosol (Chaverra-Rodriguez et al., 2018, 2020; Heu et al., 2020; Macias et al., 2020). Similarly, in our experiments, there were cases where the use of EER increased the efficiency (see Figures 1E and S1C). However, the effect of EER was not always clear, especially at some time points (see Figures S1C and S2B). Furthermore, the number of the eggs laid and/or hatched decreased in some cases (Figure S2B). As EERs often reduce survival rates and/or fecundity of the injected females (Chaverra-Rodriguez et al., 2018, 2020; Heu et al., 2020; Macias et al., 2020), their use in new target species needs empirical optimization through multiple rounds of experiments. Thus, we propose to use only two components, Cas9 and sgRNA, in our DIPA-CRISPR approach, which greatly simplifies the procedures for gene editing experiments.

A key parameter for DIPA-CRISPR

Our experiments demonstrated that the most critical parameter for successful gene editing by DIPA-CRISPR is the stage of the adult females injected (Figure 4), which is also shown in the previous ReMOT studies. In the species tested in this study, the highest GEF was achieved with females actively undergoing vitellogenesis (Cornwell, 1968; Parthasarathy et al., 2010; Pascual et al., 1992; Treiblmayr et al., 2006). This finding, together with the fact that endocytosis results in the non-selective uptake of extracellular materials (Cooper and Hausman, 2007), and that cultured insect ovaries can uptake and accumulate non-vitellogenin proteins (e.g., mouse IgG and bovine γ-globulin) (Kindle et al., 1988; Koller et al., 1989), suggests that the incorporation of Cas9 RNPs into vitellogenic oocytes occurs concomitantly with the massive uptake of vitellogenins from the hemolymph (Raikhel and Dhadialla, 1992) (Figure 4A). Thus, a good knowledge of the vitellogenesis process in the target species can be an important prerequisite for using DIPA-CRISPR.

Like most insects, females of *T. castaneum* produce eggs continuously (i.e., they lay small number of eggs every day), whereas some insects such as cockroaches produce eggs in discrete batches (i.e., discontinuous reproductive cycle) (Figures 4B and 4C). Thus, vitellogenesis occurs almost throughout the adult stage in the former group, whereas at certain times in the latter group. Interestingly, we found a clear peak of GEF values in the former group. In *T. castaneum*, GEF values peaked on days 4 and 5 after emergence, after which the values decreased to a basal level (Figure 4C). Notably, this peak corresponds to the time of the onset of vitellogenesis that begins on day 4 after adult eclosion (Parthasarathy et al., 2010). The reason why these early stages give a very high efficiency is not clear, but our results would be helpful to design DIPA-CRISPR experiments in new target species.

DIPA-CRISPR as an accessible method for insect gene editing

Our method requires only minimal equipment for adult injection, such as a stereomicroscope and a micromanipulator commonly used for larval/nymphal RNAi (Linz et al., 2014; Posnien et al., 2009). Thus, it could be readily implemented in any laboratory. Furthermore, the minimal requirement of reagents (i.e., Cas9 protein and sgRNA) makes this method highly practical and feasible.

As adult injection requires a much larger amount of injection solution compared with embryo microinjection (μL versus nL scale per injection), the cost of reagents required for DIPA-CRISPR is expected to be higher than that for the conventional method. Thus, we calculated the cost of commercial Cas9 per recovered G_0 edited insects (Figure S4). When high doses of Cas9 RNPs (3.3 μg/μL Cas9 in injection solution) were injected at optimized stages, the cost was calculated to be 2.0–7.4 USD for *B. germanica* (Figure S4A) and 4.1–10.5 USD for *T. castaneum* (Figure S4B). We consider that this cost is at an accessible level given the minimal requirement of equipment and no need to produce in-house Cas9. On the other hand, our results in *T. castaneum* suggested that the cost could not be reduced by decreasing the dose of Cas9 RNPs (Figure S4C), because this decreases the total number of recovered mutants (Figure S3C). Thus, injection of relatively high doses of Cas9
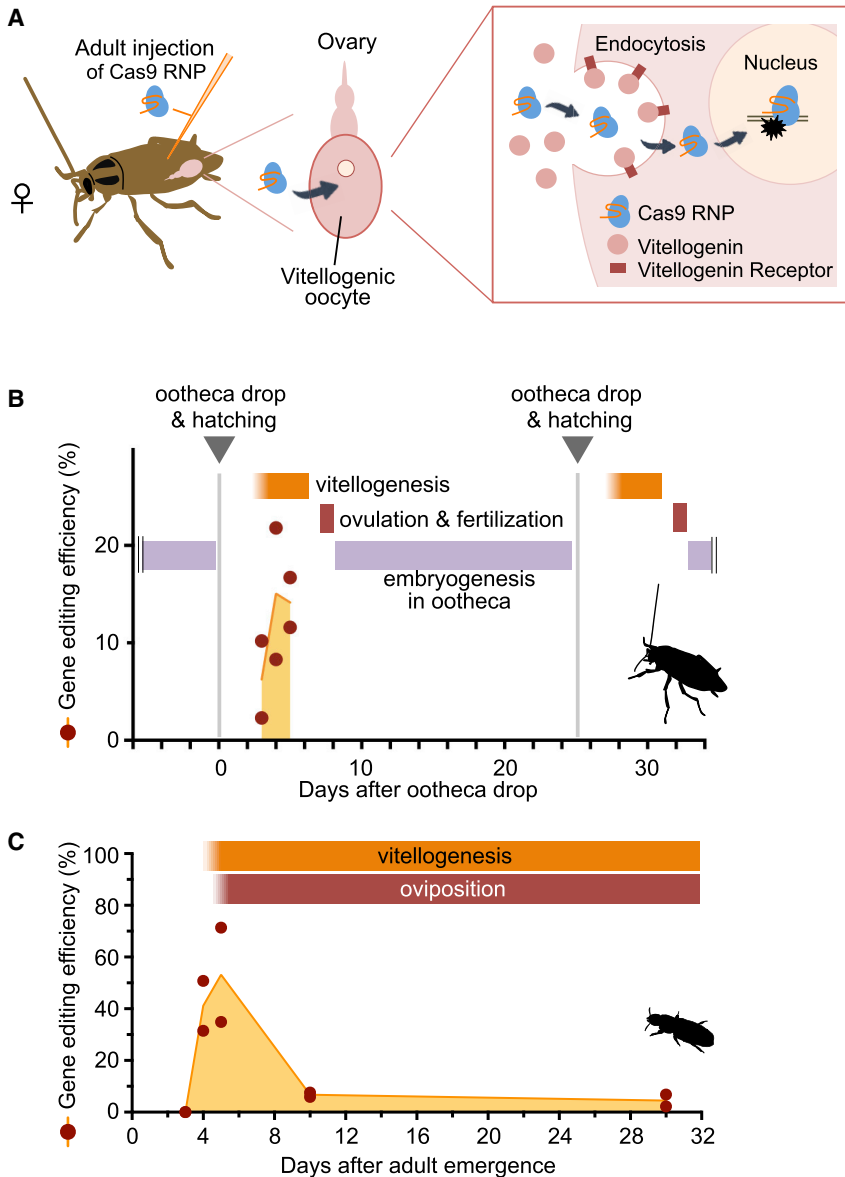
Figure 4. A schematic model and description of DIPA-CRISPR

(A) A schematic model of the uptake of Cas9 ribonucleoproteins (RNPs) by the oocyte. Vitellogenic oocytes massively uptake vitellogenins circulating in the hemolymph via receptor-mediated endocytosis (Raikhel and Dhadialla, 1992). Along with vitellogenin, injected Cas9 RNPs are likely non-selectively incorporated into the endosome of oocytes. Although the details of the process of endosomal escape are not clear, Cas9 RNPs then disrupt target genes in the nuclei of developing oocytes and/or fertilized embryos, producing gene-edited G_0 insects.

(B and C) Relationship of gene editing efficiency and ovary development. Gene editing efficiencies are plotted against the stage of females of *B. germanica* (B) and *T. castaneum* (C). Each point represents the GEF value of each biological replication. Our results suggest that it is critical to set the right timing of adult injection, depending on the reproductive physiology of target species (i.e., continuous in *T. castaneum* versus discontinuous in *B. germanica*). The timetable of ovary development is inferred from Parthasarathy et al. (2010), Pascual et al. (1992), and Treiblmayr et al. (2006).

not be applicable to species in which oogenesis proceeds without apparent vitellogenesis (e.g., aphids undergoing parthenogenetic reproduction) (Miura et al., 2003), or species in which vitellogenesis is mainly an ovarian autosynthetic process. For example, in the fruit fly *Drosophila melanogaster* and some higher Diptera, the major source of yolk proteins is the follicle cells surrounding the oocytes, but not the extraovarian fat body (Brennan et al., 1982; Houseman and Morrison, 1986). Furthermore, the temporal and spatial range of "patency," the opening of intercellular spaces between follicle cells that allows hemolymph-borne materials to reach the surface of oocytes (Raikhel and Dhadialla, 1992), is more limited in *D. melanogaster* than in most other species (Isasti-Sanchez et al., 2021; Row et al., 2021). To empirically establish the limits of our method in ovarian autosynthetic species, we tested it in *D. melanogaster* (Table S1), verifying that it does not work in them. Thus, our DIPA-CRISPR approach would not be directly applicable to some peculiar species that undergo a highly derived mode of vitellogenesis.

RNPs would be a reasonable option, as this optimizes the chances of success without largely increasing the cost of reagents.

Due to its simplicity and accessibility, DIPA-CRISPR will greatly extend the application of gene editing technology to a wide variety of model and non-model insects, including global/local agricultural and medical pests whose genomes have not been manipulated in any way.

Limitations of the study

DIPA-CRISPR requires a good knowledge of ovary development in target species, since injected Cas9 RNPs utilize the process of vitellogenesis to be internalized into the oocyte. Thus, a precise staging of vitellogenic females may be crucial. However, this can be challenging in some species, given the diverse life histories and reproductive strategies in insects. Also, DIPA-CRISPR would

STAR★METHODS

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

- ● KEY RESOURCES TABLE
- ● RESOURCE AVAILABILITY

AUTHOR CONTRIBUTIONS

Conceptualization, Y.S. and T.D.; methodology, all authors; investigation, Y.S.; writing – original draft, Y.S. and T.D.; writing – review & editing, all authors; resources, Y.S. and T.D.; visualization, Y.S. and T.D.; funding acquisition, all authors; supervision, T.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
T7 RiboMAX Express Large Scale RNA Production System	Promega	Cat#P1320
Phenol:chloroform:isoamyl alcohol mixture	Sigma-Aldrich	Cat#77619
Alt-R® S.p. Cas9 Nuclease V3	Integrated DNA Technologies	Cat#1081059
Cas9 Protein	Sigma-Aldrich	Cat#CAS9PROT
Cas9 Nuclease protein NLS	FUJIFILM Wako	Cat#316-08651
GenomeCraft Cas9	Fasmac	Cat#GE-005-S
Chloroquine Diphosphate	FUJIFILM Wako	Cat#038-17971
Saponin Quilaja sp.	Sigma-Aldrich	Cat#S4521
KOD FX Neo	TOYOBO	Cat#KFX-201
Experimental models: Organisms/strains		
B. germanica wildtype Japanese strain	Sumika Technoservice	N/A
T. castaneum wildtype Okinawa strain	Shirai and Daimon, 2020	N/A
D. melanogaster wildtype Canton S strain	Gift from Christen Mirth	N/A
Oligonucleotides		
PCR primers used in this study, see Method Details	This paper	N/A
ssODN (5′- to -3′) used as a HDR template: ACCCTTTATCCGAATTTAATGTCACTTGTA TGGAATTTGTGCGGTCGGCAAATGCCGC CACTTGTTGTCTGGGGCCCAGGGAACAG ATGAACCAAGCTTGACCGCGTTTATAGAC GGGTCGGTTATTTACGGGGTGGAGGAAA AGACAGTTGGGGCGCTCCGGACGATGTC AGGGGGTGAACTCGAAATGTTTG	Integrated DNA Technologies	N/A (Ultramer® DNA Oligonucleotides)
Recombinant DNA		
pDR274	Hwang et al., 2013	Addgene Plasmid #42250
Software and algorithms		
GraphPad Prism (v.6.0h)	GraphPad	RRID:SCR_002798
Leica Application Suite X (LASX)	Leica Microsystems	https://www.leica-microsystems.com/
Adobe Photoshop	Adobe	RRID:SCR_014199
Other		
Femtojet 4i Microinjector	Eppendorf	Cat#5252000021
MultiNA Microchip Electrophoresis System	SHIMADZU	MCE-202
Needle puller	Narishige	PC-100
Borosilicate glass with filament	Sutter Instrument	BF-100-50-10
Stereomicroscope	Leica Microsystems	M165FC
Digital microscope camera	Leica Microsystems	DFC7000T

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Takaaki Daimon (daimon.takaaki.7a@kyoto-u.ac.jp).

Materials availability
The knockout lines generated in this study is available on reasonable request to the lead contact.

Data and code availability
- All data reported in this paper will be shared by the lead contact upon request.
- This study does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Insects
A *Blattella germanica* colony derived from a Japanese population was maintained at $25 \pm 1.5°C$ under a 16 h:8 h light:dark cycle with a constant supply of solid feed (MF, Oriental Yeast) and water. A *Tribolium castaneum* (Okinawa strain) colony was maintained on wheat flour containing 5% (w/w) brewer's dry yeast at $30 \pm 1°C$ and 50%–70% relative humidity as described previously (Shirai and Daimon, 2020). The wildtype *Drosophila melanogaster* strain (Canton S) was reared using a commercial Drosophila diet (Formula 4-24 Instant Drosophila Food, Carolina Biological, Cat#173210).

METHOD DETAILS

Preparation of Cas9-sgRNA RNPs
Single-guide RNAs (sgRNAs) targeting *B. germanica cinnabar* (GenBank: PSN36199), *T. castaneum cardinal* (GenPept: XP_008200769), and *D. melanogaster white* (GenBank: NM_057439) were synthesized as described previously (Shirai and Daimon, 2020). Briefly, annealed oligo DNA was cloned into the BsaI site of the pDR274 vector (Hwang et al., 2013). After linearization with DraI, the vector was used as a template for *in vitro* transcription using the T7 RiboMAX Express Large Scale RNA Production System (Promega, Cat#P1320). The synthesized sgRNAs were extracted with phenol (pH4–5):chloroform:isoamyl alcohol (125:24:1) (Sigma, Cat#77619), and then precipitated with isopropanol and dissolved in RNase-free water. For *D. melanogaster white*, we also purchased and used chemically synthesized sgRNAs from the Integrated DNA Technologies (IDT) (Alt-R® CRISPR-Cas9 sgRNA). Otherwise stated, commercial Cas9 protein purchased from IDT (Alt-R® S.p. Cas9 Nuclease V3, Cat#1081059), which has nuclear localization signals and a C-terminal 6-His tag (further details were not disclosed to the authors), was used in this study. Cas9 protein and sgRNAs were mixed at a molar ratio of approximately 1:2, and incubated for 10–15 min at room temperature to allow Cas9 RNP formation. In some experiments, freshly-prepared chloroquine (FUJIFILM Wako, Cat#038-17971) or saponin (Sigma, Cat#S4521) was added as an endosomal escape reagent (EER) (Chaverra-Rodriguez et al., 2018). Concentrations of Cas9 RNPs and EERs in the injection solution were adjusted with RNase-free water, without adding any other reagents (e.g., buffers or salts). The target sequences of sgRNAs are (5'- to -3'): GGTCTGGCTGTAGTCAAACA for *B. germanica cinnabar* sgRNA1; TTGGAGGCATGCAAAGCTCC for *B. germanica cinnabar* sgRNA2; GGAACAGATGAACCAAGTGA for *T. castaneum cardinal* sgRNA1 (Shirai and Daimon, 2020); CATTAACCAGGGCTTCGGGC for *D. melanogaster white* sgRNA1 (Ren et al., 2014); and AGCGACACATACCGGCGCCC for *D. melanogaster white* sgRNA2 (Ren et al., 2014).

Adult injection and mutant screening in *Blattella germanica*
Female adults carrying the ootheca were collected from a stock colony, monitored daily for ootheca drop, and were staged based on the day after the ootheca drop. The injection was performed using a glass capillary needle equipped with Femtojet 4i (Eppendorf). The females used for injection were anesthetized on ice. Approximately 4 μL of the Cas9 RNP solution containing 3.3 μg/μL Cas9 (IDT, Cat#1081059) and 1.3 μg/μL sgRNAs (a mixture of sgRNA1 and sgRNA2, Figure S1A) with or without chloroquine (2 mM) was injected into the ventral abdomen of the female adults. Injected females were individually reared in containers until the formation of the next ootheca and hatching of G_0 nymphs (nymphs hatched ~20–30 days after injection with ~20–50 nymphs hatched from each ootheca). The eye colors of hatched G_0 nymphs were examined, and all the nymphs without external phenotypes were subjected to individual genotyping. *B. germanica cinnabar* is an autosomal gene, as we found heterozygous males [male = XO and female = XX in *B. germanica* (Meisel et al., 2019)].

Genotyping of *Blattella germanica*
Genomic DNAs were extracted individually as described previously (Daimon et al., 2015). Genomic PCR was conducted using KOD FX Neo (TOYOBO, Cat#KFX-201). Mutations were screened by analyzing the PCR products using the heteroduplex mobility assay (HMA) using the MultiNA Microchip Electrophoresis System (MCE-202, Shimadzu). Primer sequences for HMA of *B. germanica cinnabar* are (5'- to -3'): GAAGGCGGATTTGATCATAGGAGC and CAATCACTTACCTCACCATCTTCTG. To determine the nucleotide sequences of mutant alleles, Sanger sequencing chromatograms were analyzed with Poly Peak Parser program (Hill et al., 2014). Primer sequences for Sanger sequencing of *B. germanica cinnabar* are (5'- to -3'): GGCGCACTTGAGGCAGATATG and TTCCCCTACACTTCAATGCGGG.

Adult injection and mutant screening in *Tribolium castaneum*
Female adults at selected days after adult emergence, separated from males at the time of injection, were injected with approximately 0.5 μL of the Cas9 RNP solution containing 3.3 μg/μL Cas9 (IDT, Cat#1081059) and 1.3 μg/μL sgRNA, with or without saponin

(100 ng/μL), as described previously (Shirai and Daimon, 2020). The injected females were grouped with males in a container with wheat flour and transferred to a new container every 24 hours to examine the relationship between the day of egg laying and the gene editing efficiencies in the hatchlings. To screen gene-edited individuals, the eye colors of the G_0 insects were examined during pupal and adult stages. We also examined and compared the performance of Cas9 products from three companies additional to IDT: Sigma (Cat #CAS9PROT), FUJIFILM Wako (Cat#316-08651), and Fasmac (Cat#GE-005-S), which have a single or multiple nuclear localization signals, by targeting *cardinal* under the same condition (i.e., the same stage of injection and concentration of reagents). As *cardinal* gene locates on the X chromosome (female = XX, male = XY) (Shirai and Daimon, 2020), mutant phenotypes are not visible in heterozygous females. As we screened G_0 insects based on phenotypes but not on genotypes, the GEF values for *T. castaneum cardinal* in this study were most likely underestimated. Primer sequences for Sanger sequencing of *T. castaneum cardinal* are (5'- to -3'): GGCCAAAACCGGGGCGCTTCC and CCGGAAGTTCGTGGGTACAAGCCCG (Shirai and Daimon, 2020).

Gene knock-in experiments in *Tribolium castaneum*
Female adults at optimized stages (i.e., 4–5 days after adult emergence) were injected as above. Injection solution contained 3.3 μg/μL Cas9 (IDT, Cat#1081059), 1.3 μg/μL sgRNA (sgRNA1 for *cardinal*), and ssODNs (1.6 μg/μL). ssODNs were purchased from IDT (Ultramer DNA Oligonucleotides). Injected females were allowed to lay eggs for two days, and the recovered G_0 adults with both eyes whites were subjected to genotyping. For genotyping, genomic DNAs of G_0 adults were individually extracted, and used as a template for PCR. PCR products were digested with HindIII and analyzed by microchip electrophoresis using the MultiNA Microchip Electrophoresis System (MCE-202, Shimadzu). Primer sequences for *T. castaneum cardinal* are (5'- to -3'): GTCACACATCCGGAGTGCTTTCC and GAGTTCACCCCCTGACATCGTC. To determine the nucleotide sequences of knock-in alleles, PCR products were subcloned and subjected to Sanger sequencing.

Adult injection and mutant screening in *Drosophila melanogaster*
Female adults at selected times after adult emergence, separated from males at the time of injection, were injected with approximately 0.5 μL of the Cas9 RNP solution containing 3.3 μg/μL Cas9 (IDT, Cat#1081059) and 1.3 μg/μL sgRNA (a mixture of sgRNA1 and sgRNA2 for white), with or without chloroquine (0.5 or 2.0 mM). The injected females were grouped with males in a vial and transferred to a new vial every 24 hours. To screen gene-edited individuals, the eye colors of the G_0 insects were examined during adult stages.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data on gene editing efficiency (GEF) of the G_0 progenies (Figure S1C) were analyzed with the Mann-Whitney nonparametric U test. Statistical analyses were performed in the Prism software (Graphpad Software).

Supplemental information

DIPA-CRISPR is a simple and accessible method

for insect gene editing

Yu Shirai, Maria-Dolors Piulachs, Xavier Belles, and Takaaki Daimon

A

cinnabar   sgRNA 12

B

G0 nymph (without phenotype, indiv. A)

CAGTGAGGAGGCATATGATGAAACAATCCATACTACACTCCNACCTAACCTGC

wt ▶

G0 nymph (without phenotype, indiv. B)

AGGCATATGATGAAACAATCCATGTTGGTTTGCATGCCTACCACCNAAGATGG

wt ▶

```
                    sgRNA1                                        sgRNA2
wt: ACAATCCATGTTTGACTACAGCCAGACCTACATTGAACATGGATACCTGGAGCTTTGCATGCCTCCAACAGAA
A : ACAATCCA-----GACTACAGCCAGACCTACATTGAACATGGATACCTGGAGCTTTGCATGCCTCCAACAGAA -5
B : ACAATCCATGTT-------------------------------------GCTTTGCATGCCTCCAACAGAA -39
```

C

p = 0.0060   p = 0.070   p = 0.31

EER+
EER-

Gene editing efficiency (%)

Days after ootheca drop

D

```
                    sgRNA1                                        sgRNA2
wt: ACAATCCATGTTTGACTACAGCCAGACCTACATTGAACATGGATACCTGGAGCTTTGCATGCCTCCAACAGAA
a : ACAATCCATGTT-------------------------------------GCTTTGCATGCCTCCAACAGAA -39 (20)
b : ACAATCCAT---TGACTACAGCCAGACCTACATTGAACATGGATACCTGGAGCTTTGCATGCCTCCAACAGAA  -3 (11)
c : ACAATCCATG----ACTACAGCCAGACCTACATTGAACATGGATACCTGGAGCTTTGCATGCCTCCAACAGAA  -4  (3)
d : ACA--------------------GACCTACATTGAACATGGATACCTGGAGCTTTGCATGCCTCCAACAGAA -21 (19)
e : ACAATCCA---GATGAAACAGCCAGACCTACATTGAACATGGATACCTGGAGCTTTGCATGCCTCCAACAGAA  -3  (2)
f : ACAATCCATGTTTGACTACAGCCAGACCTACATTGAACATGGATACCTGGAGCTTTGCATGCCTCCAACAGAA  +4  (4)
                    TCCA
g : ACAATCCATGTTTGACTACAGCCAGACCTACATTGAACATGGATACCT------TTGCATGCCTCCAACAGAA  -6  (2)
```

Figure S1. Disruption of *Blattella germanica cinnabar* by DIPA-CRISPR. Related to Figure 1.

(A) CRISPR target sites of *B. germanica cinnabar* (PSN36199).

(B) Representative results of genotyping of G_0 edited nymphs. Two G_0 nymphs that carried mutations (judged by heteroduplex mobility assay) but do not show eye color phenotypes were subjected to direct Sanger sequencing of genomic PCR products. Red arrows indicate the presence of double peaks caused by indel mutations. The DNA sequences of recovered mutant alleles are shown below the panel with sgRNA (underlined) and PAM (orange letters) sequences.

(C) Gene editing efficiency (GEF) in the G_0 progenies. Each point represents an individual adult female injected. Bars indicate mean ± SD (n = 6–8). The GEF values were analyzed with the Mann-Whitney nonparametric U test.

(D) The DNA sequences of *cinnabar* mutant alleles shown in Figure 2B (i.e., alleles a–g) with sgRNA (underlined) and PAM (orange letters) sequences. The length of indels and the number of G_1 insects (in parenthesis) are shown on the right.

A — start / sgRNA1 / stop / *cardinal*

B

Gene	Stage of injection	EER	Females injected	Survival rates	Screened G0	G0 edited animals		Gene editing efficiency (GEF)
						white	mosaic	
cardinal	3 days AE	+	84	100%	85			0%
		+	68	100%	17			0%
		-	65	100%	34			0%
		-	87	98.9%	91			0%
	4 days AE	+	84	98.8%	9	5		55.6% [a]
		+	76	100%	4	1	2	75.0% [a]
		-	73	100%	63	25	7	50.8%
		-	36	97.2%	54	12	5	31.5%
	5 days AE	-	76	98.7%	21	11	4	71.4%
		-	92	100%	132	31	15	34.9%
	10 days AE	-	106	98.1%	66	3	2	7.6%
		-	97	99.0%	119	5	2	5.9%
	30 days AE	+	94	100%	133	3	2	3.8%
		+	88	97.7%	132			0%
		-	79	94.9%	117	6	2	6.8%
		-	80	97.5%	230	4	1	2.2%

C

```
                sgRNA1
WT   1:GGCCCAGGGAACAGATGAACCAA------------GTGACGGCGTTTATAGACGG
#1   1:GGCCCAGGGAACAGATGAACCAA-------------TGACGGCGTTTATAGACGG      -1
#4   1:GGCCCAGGGAACAGATGAACCAA----------------GGCGTTTATAGACGG      -5
#5   1:GGCCCAGGGAACAGATGAACCAA-----------------GGCGTTTATAGACGG     -5
#6   1:GGCCCAGGGAACAGATGAACCAA-----------GATGACGGCGTTTATAGACGG      +1
#7   1:GGCCCAGGGAACAGATGA--------------------CGGCGTTTATAGACGG      -9
#9   1:GGCCCAGGGAACAGATGAACCAA-------------TGACGGCGTTTATAGACGG      -1
#20  1:GGCCCAGGGAACAGATGAACCAACA----------GATGACGGCGTTTATAGACGG     +3
#27  1:GGCCCAGGGAACAGATGAACCAAGAACAGGGAACAGATGACGGCGTTTATAGACGG     +13
#28  1:GGCCCAGGGAACAGATGA--------------------CGGCGTTTATAGACGG      -9
#29  1:GGCCCAGGGAACAGATGAACCACGGCGTTCATCGCGTTCACGGCGTTTATAGACGG     +13
#32  1:GGCCCAGGGAACAGATGAACAGA-------------TGACGGCGTTTATAGACGG      -1
#34  1:GGCCCAGGGAACAGATGA--------------------CGGCGTTTATAGACGG      -9
#37  1:GGCCCAGGGAACAGATGA--------------------CGGCGTTTATAGACGG      -9
#42  1:GGCCCAGGGAACAGATGAACAGA-------------TGACGGCGTTTATAGACGG      -1
#43  1:GGCCCAGGGAACAGATGAAC----------------------GTTTATAGACGG     -11
```
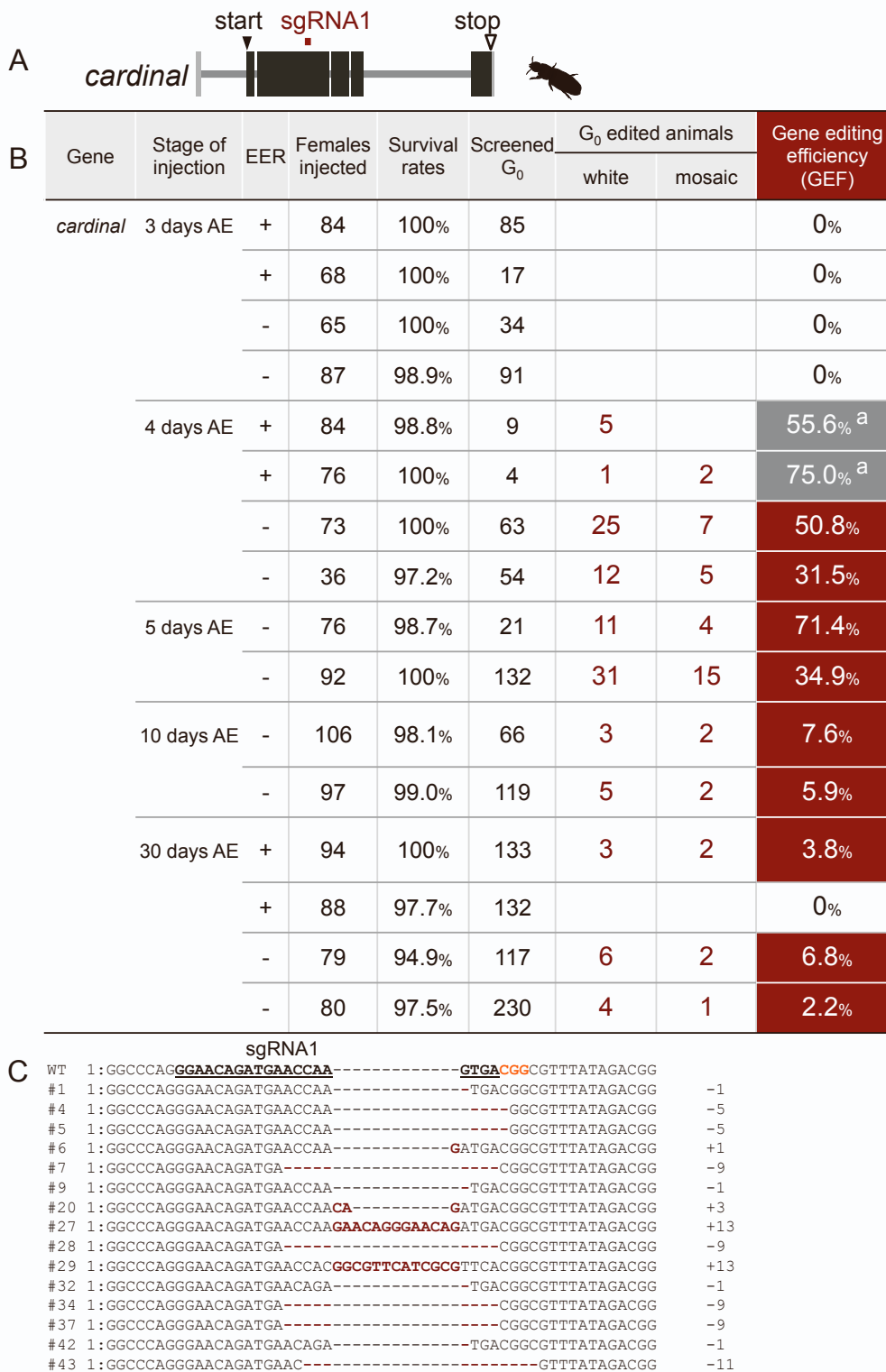
Figure S2. Disruption of *Tribolium castaneum* cardinal by DIPA-CRISPR. Related to Figures 3 and 4.

(A) The CRISPR target site of *T. castaneum cardinal* (XP_008200769). The sgRNA1 targeting the exon 3 of *cardinal* (Shirai and Daimon, 2020) was used.

(B) The detailed results of DIPA-CRISPR in *T. castaneum*. Cas9 ribonucleoprotein (RNP) solution containing 3.3 µg/µL Cas9 (IDT, Cat#1081059) and 1.3 µg/µL sgRNA were injected into adult females of selected days (i.e., 3, 4, 5, 10 or 30 days) after adult emergence (AE). The injected females were pooled, and the results are from the eggs laid during the first 24 h (females at 4 , 5, 10 or 30 days AE) or 48 h (females at 3 days AE, as

they were too young to lay eggs during the first 24 h) after injection. The results from two independent experiments are shown. EER, presence (+) or absence (-) of an endosomal escape reagent saponin (100 ng/μL) in injection solution. a: the gene-editing efficiency (GEF) values are very high, but these values may not be reliable as they are calculated based on very small numbers of G_0 insects hatched and/or survived.

(C) The DNA sequences of *cardinal* mutant alleles in G_0 edited insects. Hemizygous G_0 males with white eyes were randomly chosen and subjected to direct Sanger sequencing of genomic PCR products. Each row represents each G_0 mutant, and the length of indel is shown on the right.
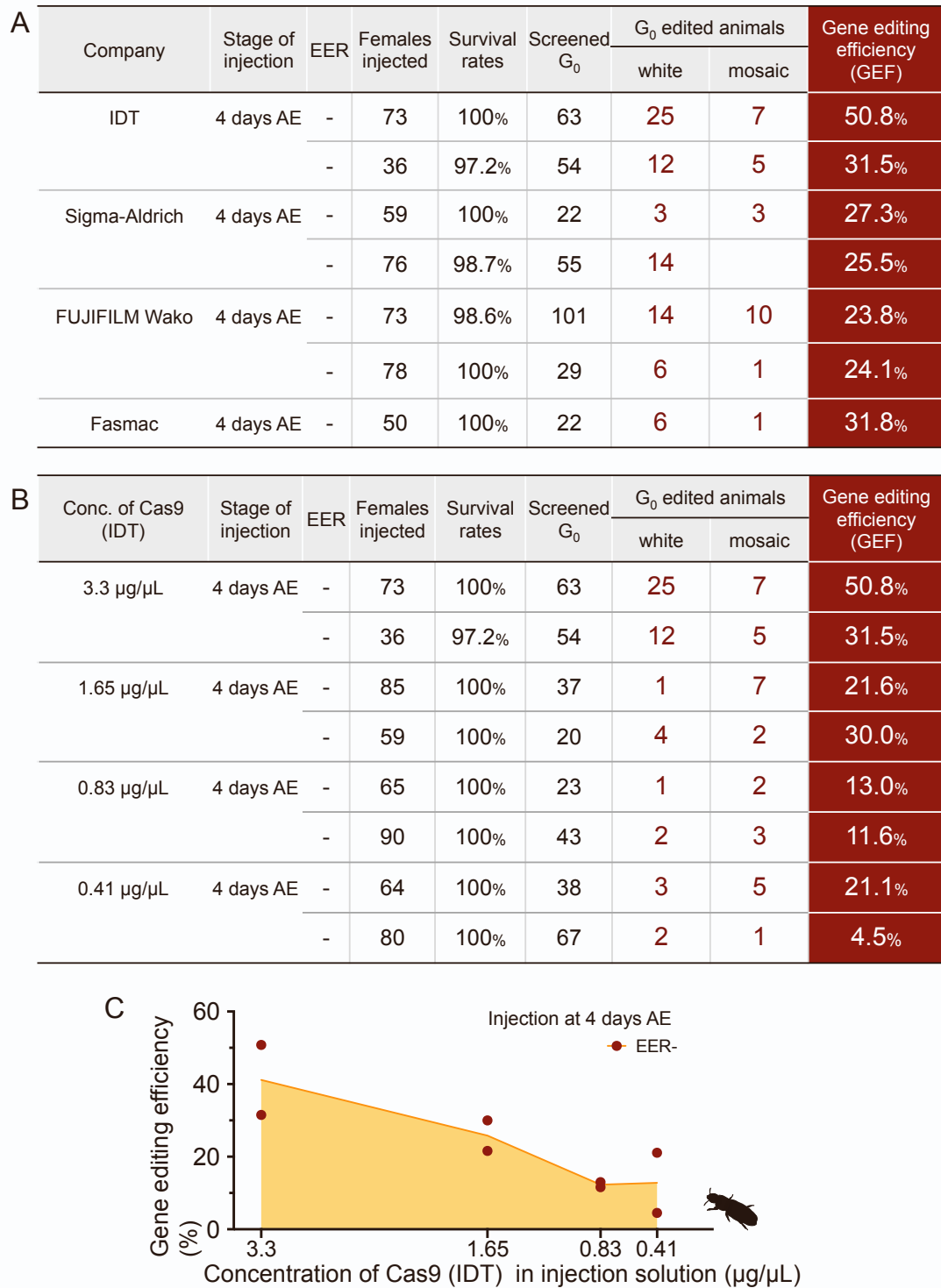
A

Company	Stage of injection	EER	Females injected	Survival rates	Screened G_0	G_0 edited animals		Gene editing efficiency (GEF)
						white	mosaic	
IDT	4 days AE	-	73	100%	63	25	7	50.8%
		-	36	97.2%	54	12	5	31.5%
Sigma-Aldrich	4 days AE	-	59	100%	22	3	3	27.3%
		-	76	98.7%	55	14		25.5%
FUJIFILM Wako	4 days AE	-	73	98.6%	101	14	10	23.8%
		-	78	100%	29	6	1	24.1%
Fasmac	4 days AE	-	50	100%	22	6	1	31.8%

B

Conc. of Cas9 (IDT)	Stage of injection	EER	Females injected	Survival rates	Screened G_0	G_0 edited animals		Gene editing efficiency (GEF)
						white	mosaic	
3.3 µg/µL	4 days AE	-	73	100%	63	25	7	50.8%
		-	36	97.2%	54	12	5	31.5%
1.65 µg/µL	4 days AE	-	85	100%	37	1	7	21.6%
		-	59	100%	20	4	2	30.0%
0.83 µg/µL	4 days AE	-	65	100%	23	1	2	13.0%
		-	90	100%	43	2	3	11.6%
0.41 µg/µL	4 days AE	-	64	100%	38	3	5	21.1%
		-	80	100%	67	2	1	4.5%

Figure S3. Performance of different Cas9 products and different doses of Cas9 in *Tribolium castaneum*. Related to Figures 3 and 4.

(A) Comparisons of Cas9 products from four vendors. Cas9 RNP solution containing 3.3 µg/µL Cas9 and 1.3 µg/µL sgRNA were injected into adult females of 4 days after adult eclosion (AE).

(B) A dilution series of Cas9 RNPs (diluted by water, the molar ratio of Cas9 and sgRNA was fixed to be 1 : 2) were injected into females.

(C) Gene editing efficiency shown in (B) was plotted against the concentration of Cas9 in injection solution. Each point represents the result of each replication.
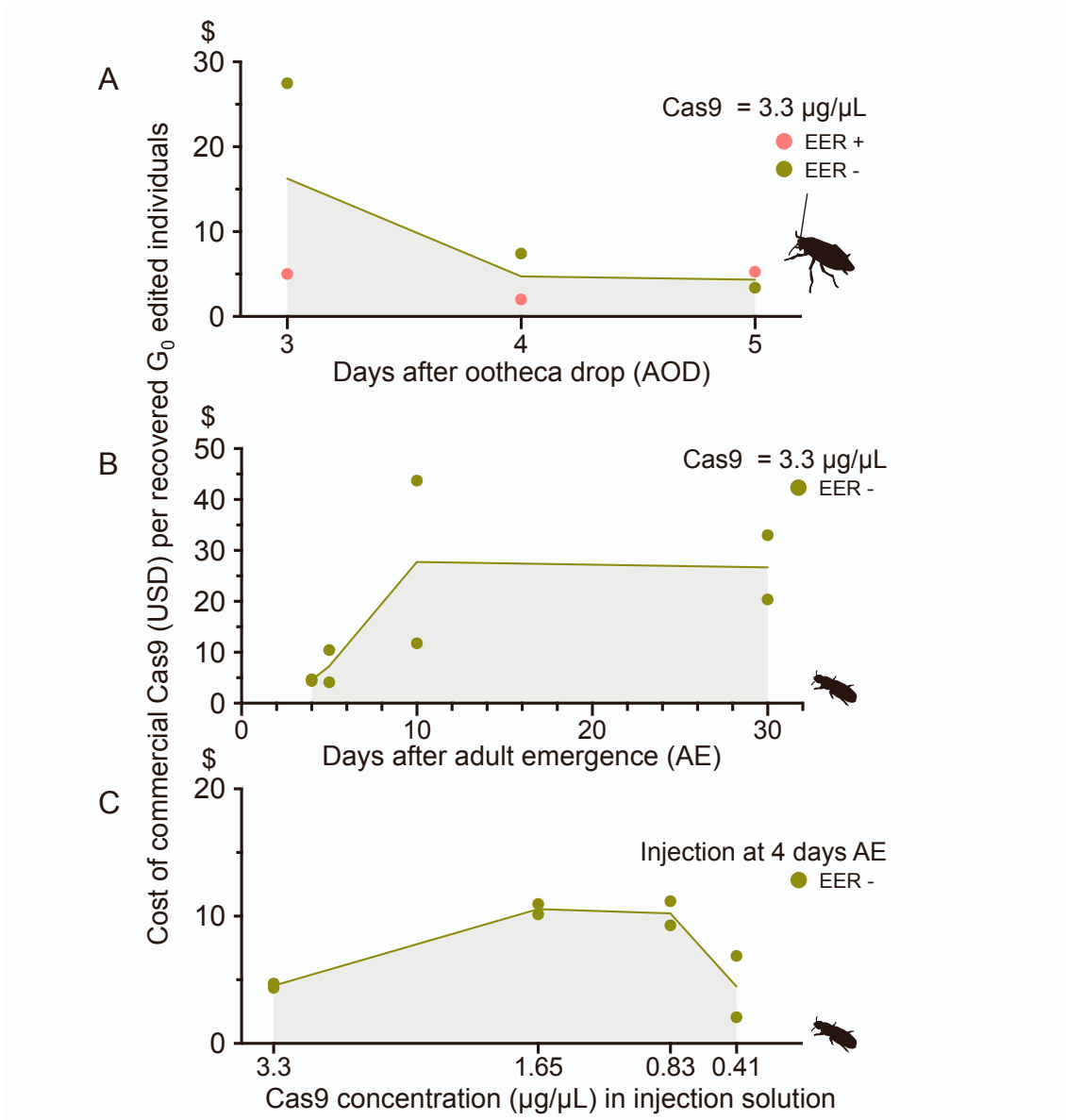
Figure S4. Cost of commercial Cas9 in DIPA-CRISPR. Related to Figures 1 and 3.

(A and B) Cost of commercial Cas9 (IDT, cat#10000735, 1.25 USD/μg) per recovered G_0 edited individuals was plotted against the time of injection for *B. germanica* (A) and *T. castaneum* (B). The results show that the cost can be significantly reduced by injecting at an appropriate timing in both species. Cost was calculated as follows: 1.25 (USD/μg) × amount (μg) of Cas9 injected per adult × total number of adults injected / total number of G_0 edited individuals recovered. Note that this calculation does not include the cost for sgRNA synthesis, as it greatly varies depending on the method (i.e., *in vitro* transcription or chemical synthesis).

(C) Relationship of the cost of Cas9 and the concentration of Cas9 in injection solution in *T. castaneum*. The results shown in Figure S3B were used for calculation.

Supplementary Table 1. DIPA-CRISPR experiments in *Drosophila melanogaster*. Related to Figure 4.

Gene	sgRNA	Stage of injection	EER	Females injected	Survival Rates	Screened G_0	G_0 edited animals white	mosaic	Gene editing efficiency (GEF)
white	chemical synthesis	2 h AE	-	59	81.4%	212			0%
		2 h AE	-	29	96.6%	101			0%
		10 h AE	-	31	87.1%	2			0%
		10 h AE	-	26	96.2%	33			0%
		10 h AE	-	80	96.3%	270			0%
		24 h AE	-	61	85.2%	481			0%
		24 h AE	-	71	91.5%	426			0%
		2 d AE	-	47	53.2%	324			0%
		2 d AE	-	58	77.6%	437			0%
		4 d AE	-	53	60.4%	329			0%
		4 d AE	-	63	76.2%	421			0%
		7 d AE	-	62	66.1%	841			0%
		7 d AE	-	65	80.0%	573			0%
		Random	Chl 2 mM	113	93.1%	891			0%
	in vitro transcription	Random	-	71	91.5%	340			0%
		Random	Chl 0.5 mM	74	95.9%	417			0%
		Random	Chl 2 mM	70	88.6%	662			0%

Cas9 ribonucleoprotein (RNP) solution containing 3.3 µg/µL Cas9 (IDT, Cat#1081059) and 1.3 µg/µL sgRNA (a mixture of sgRNA1 and sgRNA2) were injected into adult females of selected hours or days after adult emergence (AE), or females that were randomly chosen from vials (Random). sgRNAs were purchased (chemical synthesis) or synthesized by in vitro transcription. The injected females were pooled, and the results are from the eggs laid during the first 48 h after injection. EER, presence or absence (-) of an endosomal escape reagent chloroquine (Chl) in injection solution.