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

Graphical abstract

Highlight
- 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

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

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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 (Demauw et al., 2020), also showed that a very small number of gene-edited
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) 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 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;
Inheritance of edited alleles in cockroaches

To test if edited alleles in G₀ 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 (G₁) were heterozygous mutants (Figure 2B), showing very high germline mutation rates in these mosaics. To further confirm this result, G₀ adults with eye color phenotypes (white or mosaic) were crossed and their G₁ progenies were screened for knockout phenotypes (Figure 2C). Notably, when G₀ adults with both eyes white were crossed, all of their G₁ 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 G₀ individuals. Similarly, very high germline mutation rates were also exhibited in mosaic-eyed G₀ 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 G₀ males without genotyping (females = XX and males = XY in T. castaneum) (Grubbs et al., 2015; Shirai and Daimon, 2020).
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-Rodríguez et al., 2018, 2020; Macías 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 (mutating the PAM sequence) 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 G0 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., 2019), 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/mL 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 (Rainhel 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 (Lin 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 G0 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...
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 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 extra-ovarian 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.

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**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2022.100215.

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

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

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**REFERENCES**


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### STAR METHODS

#### KEY RESOURCES TABLE

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### 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 ± 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 ± 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 Dral, 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'): GGCGCACTTGAGGCAGATATG and TTCCCCTACACTTCAATGCGGG. For *T. castaneum cardinal* sgRNA1 (Shirai and Daimon, 2020); CATTAACCAGGGCT for *B. germanica cinnabar* sgRNA1; TTGGAGGCATGCAAAGCTCC for *D. melanogaster white* sgRNA2; GGAACAGATGAACCAAGTGA for *T. castaneum cardinal* sgRNA2; GGCGCACTTGAGGCAGATATG and TTCCCCTACACTTCAATGCGGG.

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 e2 cell reports methods. Injected females were individually reared in containers until the formation of the next nymph (20–50 nymphs hatched from each ootheca). The eye colors of hatched G0 nymphs were examined, and all the nymphs without external phenotypes were subjected to individual genotyping.

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 Micropip Electrophoresis System (MCE-202, Shimadzu). Primer sequences for HMA of *B. germanica cinnabar* 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'): 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'): GCCGCACCTGGAGCAGATATG and TCCCTCACTTCTCAATGCGGG.

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.
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 G0 adults with both eyes whites were subjected to genotyping. For genotyping, genomic DNAs of G0 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 G0 insects were examined during adult stages.

QUANTIFICATION AND STATISTICAL ANALYSIS
Data on gene editing efficiency (GEF) of the G0 progenies (Figure S1C) were analyzed with the Mann-Whitney nonparametric U test. Statistical analyses were performed in the Prism software (Graphpad Software).