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HUH-tagged Cas9 as a platform for efficient ssODN-mediated knock-in via embryo and adult injection in insects

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Abstract

Recent advances in adult injection-based insect genome editing have enabled genetic manipulation of a wide range of insect species, including those previously considered difficult or even impervious to genetic modification. However, achieving efficient knock-in remains a significant challenge with this approach. Here, we demonstrate that fusing a HUH endonuclease tag to Cas9 significantly enhances both non-homologous end joining (NHEJ)-mediated knockout and homology-directed repair (HDR)-mediated knock-in via adult injection. This fusion increased knockout efficiency by up to fivefold in the beetle *Tribolium castaneum* through adult injection, likely due to its previously unrecognized nuclear localization activity. It also improved single-stranded oligodeoxynucleotide (ssODN)-mediated knock-in efficiency, which we attribute to its characteristic ssDNA-tethering activity. To evaluate its versatility, we tested the HUH-tagged Cas9 in conventional embryo injection, which significantly enhanced HDR-mediated knock-in of an epitope tag in cricket and milkweed bug embryos. Our findings establish the HUH-tag as a versatile platform for improving both NHEJ- and HDR-based genome editing, providing a robust framework to advance genetic engineering across a broad spectrum of arthropods.

Introduction

The CRISPR-Cas9 system is a powerful tool for investigating the biology and evolution of insects, among the most diverse and abundant organisms on Earth. However, conventional gene editing depends on embryo microinjection, which requires specialized optimizations such as laboratory culture conditions, injection setup, and technical expertise^{1,2}. This complexity limits its accessibility across various insect species, underscoring the need for simplification to fully harness the potential of CRISPR-Cas9 in insect science.

To broaden the applicability of gene editing across diverse insect species, adult injection-based methods, such as ReMOT Control, DIPA-CRISPR, and SYNCAS have emerged as practical and versatile alternatives to conventional embryo injection³⁻⁵. Among these, DIPA-CRISPR stands out as the simplest approach due to its minimal requirements. This method involves injecting only two components—commercial Cas9 proteins and single-guide RNAs (sgRNAs) into the body cavity of adult females, enabling gene editing in their offspring. Initially developed for the German cockroach, *Blattella germanica*, and the red flour beetle, *Tribolium castaneum*, DIPA-CRISPR has since been extended to mosquitoes⁶, hemipteran bugs⁷⁻⁹, wasps¹⁰, mites¹¹, and even tardigrades¹², underscoring its versatility across a wide range of species. However, achieving precise gene knock-in via homology-directed repair (HDR) remains challenging with these adult injection-based methods, as HDR efficiency is typically lower than that of non-homologous end joining (NHEJ), highlighting the ongoing need for continued innovation in genetic tool development.

Various strategies have been developed to improve the precision of exogenous DNA knock-in^{13,14}. One such method is the microhomology-mediated end joining (MMEJ)-dependent approach which is applied across various systems^{15,16}. Additionally, many HDR-based strategies

have emerged, including inhibiting NHEJ and enhancing HDR through chemical reagents or genetic modulation^{17–22}, as well as employing Cas9 fused to proteins involved in DNA repair pathways^{23–25}. Techniques such as cell synchronization^{26–28}, since HDR occurs during the late S and G2 phases, and chemical modification of donor DNA have also proven effective^{29–32}. Other promising methods involve the tethering of donor DNA^{33–39}. Among these, we envisioned that using HUH-tagged Cas9 tethering for donor DNA tethering^{35,36,39} could offer a particularly amenable approach due to its simplicity and suitability for adult injection.

HUH-tags were developed as fusion tags to enable covalent conjugation between DNA and proteins, leveraging the intrinsic mechanisms of HUH-endonuclease domains. HUH-endonucleases, named for their conserved histidine-hydrophobic residue-histidine (HUH) motif, are involved in processes such as rolling cycle replication of circular DNA and plasmid conjugation⁴⁰. HUH-endonucleases nick single-stranded DNA at specific sequences at the origin of replication (ori), forming a covalent phosphotyrosine intermediate with a particular tyrosine residue in the enzyme^{39,41}. Aird and colleagues (2018)³⁶ demonstrated that tethering single-stranded oligodeoxynucleotides (ssODNs) to Cas9 using a HUH endonuclease tag from the porcine circovirus 2 (PCV2) Rep protein (i.e., PCV-tag) significantly improved HDR-mediated knock-in efficiency in cultured human cells. The PCV-tag requires only a short 13-nt specific sequence at the 5' end of ssODNs to form a covalent bond, and its utility for genome engineering has been validated in several studies using human cultured cells^{42,43}.

In this study, we aimed to enhance knock-in efficiency by covalently tethering ssODNs with the PCV-tag, thereby establishing a more advanced method for adult injection-based genome editing. We hypothesize that the HUH-tag's covalent bonding ability would markedly improve the precise integration of donor DNA, as shown in cultured human cells. DIPA-CRISPR

is a remarkably simple system, requiring only Cas9 proteins and sgRNAs⁴. By integrating these two strategies, we aimed to create a method that is both straightforward and highly effective, thereby expanding the available toolkit for precise genome editing in insects.

Our results demonstrate that PCV-tagged Cas9 significantly enhances both knockout and knock-in efficiency, underscoring its potential as a robust and practical tool for genome editing in insects. We first optimized Cas9 purification using a three-step chromatography process to express recombinant Cas9 with N-terminal SUMO- and PCV-tags. Testing in the beetle *Tribolium* via adult injection revealed a fivefold increase in NHEJ-mediated knockout efficiency compared to non-tagged Cas9. Additionally, we observed a threefold increase in ssODN knock-in frequency in beetles via adult injection. When applied to the cricket *Gryllus bimaculatus* and the milkweed bug *Oncopeltus fasciatus* via conventional embryo microinjection, this approach enhanced HDR-mediated knock-in of an epitope tag by up to 3.5-fold and twofold, respectively, demonstrating its wide range of uses. HUH-tagged Cas9 thus improves both NHEJ- and HDR-mediated genome editing, advancing the development of genome editing techniques across diverse insect species via both embryo and adult injections.

Results

Optimization of Cas9 purification procedures

The ability to express and purify engineered Cas9 proteins, such as HUH-tagged variants, is a key technical requirement for this study. As a preliminary step, we optimized purification protocols using non-tagged Cas9, which were subsequently adapted for HUH-tagged Cas9 proteins. The final protocol involved a three-step purification process: (1) Ni-NTA affinity chromatography, (2) cation exchange chromatography, and (3) size exclusion chromatography (Fig. 1a; detailed protocol in Supplementary Data 1). SDS-PAGE analysis confirmed that each successive step substantially improved Cas9 purity (Fig. 1b).

To assess the functionality of Cas9 at different purification stages, we evaluated its performance in the beetle *T. castaneum*. Using optimized DIPA-CRISPR conditions, we targeted the X-linked eye color gene *cardinal*, a useful marker for phenotypic screening of mutants in the offspring of injected adult females (females = XX, males = XY) (Fig. 1c)^{4,44}. The performance of purified Cas9 was assessed using two key metrics: "gene editing efficiency (GEF)" and "effort efficiency (EEF)" (Fig. 1d), consistent with previous studies³. GEF represents the proportion of edited G₀ animals within the screened G₀ population, while EEF is the ratio of edited G₀ animals to the number of injected females.

When Cas9 and sgRNAs were injected into females 4–5 days after adult emergence, offspring with white or mosaic eyes were obtained (Fig. 1e). Importantly, increasing the number of purification steps consistently enhanced both GEF and EEF (Figs. 1f and g, and Supplementary Fig. 1). In contrast, Cas9 purified with a single step failed to yield a sufficient number of G₀ offspring, likely due to contamination with undesired materials (Supplementary Fig. 1). These

findings demonstrate that optimized Cas9 expression and purification protocols are essential for achieving high-efficiency gene editing via adult injection.

Recombinant Cas9 with SUMO- and HUH-tags

To develop an efficient knock-in method via adult injection, we first evaluated the performance of recombinant HUH-tagged Cas9 proteins in NHEJ-mediated knockout experiments in *Tribolium*. As a representative HUH-tag, we selected the 115-amino acid (aa) PCV-tag derived from the PCV2 Rep protein, based on its well-characterized ssDNA-tethering activity^{36,39}.

During the purification of Cas9 fusions, we observed that fusion with the PCV-tag significantly reduced solubility, leading to lower yields of purified protein. To overcome this limitation, we introduced an additional SUMO-tag⁴⁵, which improved both the solubility and yield of Cas9 fusion proteins (Fig. 2a).

We next compared the performance of three lab-made variants—non-tagged Cas9, SUMO-Cas9, and SUMO-PCV-Cas9 in *Tribolium*. Among them, SUMO-PCV-Cas9 exhibited the highest GEF and EEF. Specifically, SUMO-PCV-Cas9 resulted in a four- to fivefold increase in both GEF and EEF compared to non-tagged Cas9. These findings suggest that the PCV-tag may enhance editing efficiency through a previously unidentified mechanism (Figs. 2b and c, and Supplementary Fig. 2). Notably, the GEF values of SUMO-PCV-Cas9 were comparable to those of commercially available Cas9 proteins from various vendors (Fig. 2b and Supplementary Fig. 2), while its EEF values were the highest among all tested Cas9 variants (Fig. 2c and Supplementary Fig. 2). Collectively, these results demonstrate that fusion with the PCV-tag improves NHEJ-mediated knockout efficiency, a novel observation not previously reported to our knowledge.

Nuclear localization activity of the PCV-tag in cultured insect cells

To investigate the mechanism by which PCV-tag fusion enhances knockout efficiency, we considered known properties of Rep proteins from porcine circoviruses. These proteins are transported from the cytosol to the nucleus in host cells, a process mediated by nuclear localization signals (NLSs) located near the HUH motifs. Sequence analysis of the PCV-tag used in this study identified three NLS-like motifs (Fig. 3a), resembling those found in other Rep proteins⁴⁶. To test whether the PCV-tag confers nuclear localization activity to Cas9, potentially contributing to the enhanced knockout efficiency of SUMO-PCV-Cas9 (Figs. 2b and c), we conducted transient transfection assays using insect cultured cells (Figs. 3b–e). We first transfected constructs encoding GFP or PCV-tagged GFP, with or without NLSs derived from the SV40 large T antigen (Fig. 3b and Supplementary Fig. 3). Quantification of the nuclear-to-cytoplasmic (N/C) ratio of GFP fluorescence clearly demonstrated that the PCV-tag exhibits strong nuclear localization activity (Fig. 3c).

Next, we examined whether this activity also extends to Cas9 fusions. Constructs encoding Cas9 or PCV-tagged Cas9 were transfected into cells, and their subcellular localization was analyzed (Fig. 3d and e, and Supplementary Fig. 3). Our results revealed that fusion with the PCV-tag significantly enhanced the nuclear localization of Cas9. These findings suggest that the nuclear localization activity of the PCV-tag contributes to its ability to boost knockout efficiency, underscoring its potential as a platform for enhancing genome editing efficiency in insects and other biological systems.

Targeted gene knock-in via adult injection in beetles

We then assessed whether PCV-tagged Cas9 could improve ssODN-mediated knock-in efficiency

in *Tribolium* via adult injection. We designed an ssODN to introduce a 2-nt insertion containing a novel HindIII restriction site to the *cardinal* locus. The ssODN also included 40-nt homology arms at both the 5'- and 3'- ends, a 1-nt substitution to disrupt the PAM sequence, and a 13-nt recognition sequence specific to the PCV-tag (Figs. 4a and b).

To confirm successful conjugation, we performed a gel shift assay using SDS-PAGE, which verified the covalent binding of the PCV-tagged Cas9 to the ssODN (Fig. 4c). We then injected a mixture of SUMO-PCV-Cas9 RNPs and ssODNs into adult females, screened G₀ individuals based on *cardinal* phenotypes, and validated knock-in events by HindIII digestion of PCR products (Fig. 4d).

When control ssODNs having a scrambled 13-nt recognition sequence were used, knock-in frequencies ranged from 1.7% to 2.2%. In contrast, ssODNs with the specific 13-nt PCV recognition sequence yielded knock-in frequencies between 4.0% and 5.3% (Fig. 4e–g), representing a two- to threefold increase. This enhancement was also evident in knock-in effort efficiency (Fig. 4h), indicating that tethering ssODNs to the PCV-tag is responsible for the improved knock-in frequency. We also tested a commercially available HDR enhancer reagent, and found that it did not improve knock-in frequency and in fact decreased egg laying and the overall number of edited animals recovered (Fig. 4e–h). We note that this reagent is designed for mammalian systems and thus may not be effective in insects. Further research will be needed to explore efficient HDR enhancers with lower toxicity for use in insects.

To quantitatively assess the frequency of knock-in alleles in G₀ animals, we performed amplicon sequencing on eight edited G₀ animals carrying knock-in alleles (Fig. 4f). The results revealed high frequencies of knock-in alleles, ranging from 44.73% to 93.84% (Figs. 4i and j, and Supplementary Fig. 4). Taken together, these findings demonstrate that PCV-tagged Cas9 enables

a substantial improvement in ssODN knock-in efficiency via adult injection.

PCV-tag boosts knock-in efficiency via embryo microinjection in crickets

To further explore the versatility of the PCV-tagged Cas9 approach for knock-in applications, we evaluated the performance of SUMO-PCV-Cas9 in a conventional embryo injection setting using the cricket *G. bimaculatus* (Fig. 5a). Our objective was to insert a 3xFLAG epitope tag (22-aa) in-frame immediately downstream of the start codon of the *E93* gene, which encodes a transcription factor essential for adult metamorphosis^{47,48} (Fig. 5b).

We injected a mixture of SUMO-PCV-Cas9, sgRNA, and ssODNs into early embryos and subsequently performed genotyping of individual developing embryos (embryonic stages >12) or hatchlings (Fig. 5a and Supplementary Fig. 5). Genotyping employed three distinct primer sets: one for the 5'-junction (F1/R2 in Fig. 5b), another for the 3'-junction (F2/R1), and a third set flanking the cut site (F1/R1).

Injection of commercial Cas9 yielded an 11.9% success rate in detecting bands at both 5'- and 3'-junctions of the predicted insertion site. In contrast, injection of SUMO-PCV-Cas9 achieved a significantly higher efficiency of 38.1%, representing a 3.5-fold improvement (Figs. 5c and d). Notably, with SUMO-PCV-Cas9, we identified several G₀ individuals carrying exclusively the knock-in allele, with no detectable indels. Sanger sequencing of these knock-in alleles from four such G₀ individuals (#1–#4 in Fig. 5c) revealed that three out of four exhibited clean electropherogram peaks matching the expected sequence, indicating that nearly perfect knock-in individuals can be obtained even in the G₀ generation (Fig. 5e and Supplementary Fig. 5). These results establish that PCV-tagged Cas9 is a robust and effective tool for enhancing ssODN knock-in efficiency in crickets.

Establishment of an epitope-tagged knock-in line in the milkweed bug

We also applied SUMO-PCV-Cas9 via embryo microinjection in the milkweed bug, *O. fasciatus* (Fig. 6a). In this experiment, we aimed to insert a 3xHA epitope tag (31-aa) in-frame immediately upstream of the stop codon of the *traffic jam* (*tj*) gene, which is known to regulate gonad morphogenesis⁴⁹ (Fig. 6b).

Consistent with the results of cricket experiments, we found that the knock-in efficiency of SUMO-PCV-Cas9 outperformed that of commercial Cas9. Using commercial Cas9, we observed a 40.9% success rate in detecting bands at both 5'- and 3'-junctions. In contrast, injection with SUMO-PCV-Cas9 resulted in a significantly higher efficiency of 72.9%, representing a nearly twofold improvement (Figs. 6c and d).

We next assessed germline transmission of the knock-in alleles by performing another round of injections (Fig. 6e). Following embryonic microinjection of 150 eggs, we obtained 20 G₀ adults and genotyped them to determine whether they carried the desired knock-in allele. Of these, six adults carried potential knock-in alleles (Fig. 6f). When crossed individually with wildtype adults, three of the six produced G₁ broods containing individuals with knock-in alleles (Fig. 6f and Supplementary Fig. 6). From these three G₁ broods, we recovered three distinct types of knock-in alleles: one precise knock-in allele, one with a 1-bp substitution, and one with a 78-bp insertion, each originating from a different G₁ brood (Fig. 6f and g, and Supplementary Fig. 6). Finally, using heterozygotes of the precise knock-in allele (*tj-3xHA/+*), we performed immunostaining with an anti-HA antibody in adult ovary and testis, and found that the observed localization patterns corresponded to those of mRNAs detected by *in situ* hybridization chain

reaction (HCR), confirming that the epitope tag was expressed under the control of the *tj* regulatory region (Fig. 6h).

Collectively, these results demonstrate that PCV-tagged Cas9 is applicable not only in adult injection methods but also in embryo injection, and holds great potential for broader use in diverse insect models and other biological systems.

Discussion

In this study, we demonstrated the advantageous properties of the HUH-tag in insect genome editing. First, optimization of Cas9 purification procedures allowed us to explore the potential of engineered Cas9 proteins. Using the red flour beetle, we showed that SUMO- and PCV-tagged Cas9 is applicable to an adult injection-based gene editing approach, and that the PCV-tag also acts as an NLS, significantly enhancing NHEJ-mediated knockout efficiency. Additionally, tethering ssODNs to PCV-tagged Cas9 increased knock-in frequencies. Furthermore, using cricket and milkweed bug embryos, we found that this strategy is readily applicable to conventional embryo injection. Collectively, PCV-tagged Cas9 offers a valuable platform that improves both knockout and knock-in efficiencies, paving the way for more refined genome editing technologies in insects.

Since the development of ReMOT Control, where adult injection of recombinant Cas9 fused with a fragment of yolk protein enables the production of gene-edited offspring³, many successful applications have been reported across a wide range of arthropod species^{3,44,50–55}. Building on this, DIPA-CRISPR was developed as a simpler and more accessible method for gene editing, using adult injection of commercially available Cas9⁴. This approach has since been applied to various insects^{4,6–10}, mites¹¹, and even to a tardigrade, a non-arthropod species¹². In this

study, we further advanced the adult injection-based genome editing approach using PCV-tagged Cas9. The dual functionality of the PCV-tag, as both an NLS and a tether for ssODNs, provides a more efficient and versatile platform for adult-injection-based genome editing across diverse insects and arthropod species.

Since the development of embryo injection-based genome editing methods in insects, achieving efficient gene knock-in in insects has remained a persistent challenge. This difficulty continues with recently introduced adult injection approaches. For example, our previous attempt at ssODN-mediated knock-in using DIPA-CRISPR resulted in very low efficiency⁴, rendering the approach impractical. In this study, we demonstrate that using PCV-tagged Cas9 to tether ssODNs markedly enhances knock-in efficiency. A key advantage of this strategy is its applicability to both embryo and adult injection systems. Another advantage is its simplicity, requiring only the addition of a 13-nt recognition sequence to the ssODN donor. While PCV-tagged Cas9 must be produced in-house, the method is readily applicable across a wide array of insect and arthropod species. Moreover, the PCV-tag improves the nuclear localization of Cas9, and resulted in enhanced cleavage activity at the target site. This feature is expected to be beneficial for gene knockout and ssODN-mediated knock-in, as shown in this study, and is likely useful for other strategies, such as NHEJ- or MMEJ-mediated gene knock-in.

Another key finding is that optimizing the Cas9 purification process is essential for achieving efficient genome editing via adult injection. We found that increasing the number of purification steps consistently led to higher editing efficiency, whereas Cas9 purified with a single step failed to yield a sufficient number of G₀ offspring for practical use. Although the precise factor responsible for this decrease in efficiency remains unknown, it is likely attributable to contamination with undesired materials. With our detailed step-by-step purification protocol

(Supplementary Data 1), we aim to enable others to easily reproduce this process. Collectively, our results represent a notable technical advance for the adult injection approach and emphasize the critical role of protein quality in determining editing efficiency. Building on this insight, several directions can be pursued to further refine adult injection-based genome editing.

First, engineering Cas9 for improved performance in insect systems holds promise. Although the PCV-tag contributes to nuclear localization activity, we observed that SUMO-PCV-Cas9 containing two copies of the SV40 NLS remained predominantly in the cytoplasm in transfected cultured cells, suggesting that nuclear import is still suboptimal. Incorporating alternative or insect-optimized NLS sequences may enhance nuclear import and thereby further boost editing efficiency. This idea is supported by several previous studies examining the relationship between the number of NLS motifs and genome-editing activity. For example, Shui and colleagues⁵⁶ tested Cas9 proteins fused with 0–4 NLSs in cultured human cells and found that increasing the number of NLSs enhanced genome-editing activity without increasing off-target cleavage. Similar observations were reported for Cas12a, another Class 2 single-effector CRISPR nuclease, in which 3xNLS variants outperformed 1xNLS variants without increasing off-target activity⁵⁷.

Second, the precise role and functionality of ovary transduction tags warrant further investigation. Although these tags have been central to the ReMOT Control strategy, previous ReMOT studies have almost exclusively employed single-step purification and have not examined the potential impact of purification methods on editing efficiency^{3,53}. Thus, further studies should evaluate whether the integration of an ovary transduction tag into the PCV-Cas9 platform could further improve genome editing efficiency.

Third, expanding the use of HUH endonucleases may yield new tools for template

tethering. In this study, we utilized the PCV-tag derived from the PCV2 Rep protein, which forms a covalent bond with ssDNA via its conserved catalytic mechanism. While this system has been established and primarily used in cultured human cells³⁶, we further demonstrated that HUH-tagged Cas9 retains its function even when fused with a SUMO-tag, which facilitates recombinant Cas9 production in a soluble form. Furthermore, HUH proteins exhibit considerable structural and functional diversity, and other family members may offer varying tethering efficiencies, recognition sequence requirements, or substrate specificities⁴⁰. Investigating HUH domains from a broader range of sources⁴² may reveal novel tags with enhanced or complementary properties for covalent donor attachment in genome editing applications. It is also noteworthy that the relatively broad sequence specificity of HUH-endonucleases may increase the risk of off-target effects. For example, the PCV-tag requires only a 13-nt sequence for its ssDNA binding, which is much shorter than that required for gRNA recognition³⁶. However, previous studies using PCV-tagged Cas9 reported no substantial increase in off-target activity^{36,42}.

In conclusion, our findings demonstrate that the use of PCV-tagged Cas9 markedly enhances genome editing outcomes, improving both NHEJ-mediated knockout and HDR-mediated knock-in in insects. Importantly, this strategy is compatible with both adult injection and embryo microinjection approaches. These results underscore the utility of PCV-tagged Cas9 as a versatile and effective tool, offering practical benefits for genome editing across diverse insect and arthropod species. By broadening both methodological accessibility and potential application, this approach advances functional genomics in a wide range of insects and arthropods, including emerging model species, economically important species, and agricultural or medical pests.

Methods

Insects and cell culture

A *Tribolium castaneum* (Okinawa strain) colony was maintained on wheat flour containing 5% (w/w) brewer's dry yeast at $30 \pm 1^\circ\text{C}$ and 50%–70% relative humidity as described previously^{4,44}. A *Gryllus bimaculatus* colony derived from the *gwhite* strain at Tokushima University⁵⁸ was maintained on artificial fish food (Spectrum Brands) at 28–30°C with 12L:12D as described previously⁵⁹. An *Oncopeltus fasciatus* colony was maintained on sunflower seeds at 28–30°C with 12L:12D as described previously⁶⁰. The BmN cell line originally from the silkworm *Bombyx mori* ovary was cultured in SF-900 II medium (ThermoFisher, Cat#10902088) containing 10% fetal bovine serum at 27°C.

Cas9 expression and purification

Expression vectors pET28a-Cas9-His (Addgene plasmid #98158)⁶¹ and pTD68-PCV-Cas9 (Addgene plasmid #123643)³⁶ were used to produce Cas9 and SUMO-PCV-Cas9 proteins, respectively. To express SUMO-Cas9 protein, the expression vector pTD68-SUMO-Cas9 was generated by removing the PCV-tag sequence from pTD68-PCV-Cas9 using KOD -plus-Mutagenesis Kit (TOYOBO, Cat#SMK-101) (For the detailed Cas9 expression and purification protocol, refer to Supplementary Data 1). Following purification, the quality of Cas9 proteins was compared by loading them on an SDS-PAGE gel, followed by CBB staining with Quick-CBB (FUJIFILM Wako, Cat#178-00551) or silver staining with EzStain Silver (ATTO, Cat#AE-1360). Amino acid sequences of recombinant Cas9 proteins are available in Supplementary Data 2.

gRNA preparation

sgRNAs targeting *T. castaneum cardinal* (XP_008200769), were synthesized as described previously^{4,44}. sgRNAs targeting *G. bimaculatus E93* and gRNAs (crRNAs + tracrRNAs) targeting *O. fasciatus tj* were chemically synthesized by Integrated DNA Technologies (IDT). The target sequences of the gRNAs are (5'- to -3'): GGAACAGATGAACCAAGTGA for *T. castaneum cardinal*^{4,44}, TTAGGGTGAAGCAAAAATGG for *G. bimaculatus E93* and AGGATGTTTACATGTGACAC for *O. fasciatus tj*.

Adult injection and mutant screening in beetles

In *T. castaneum*, female adults at optimized stages (i.e., 4–5 days after adult emergence), separated from males at the time of injection, were injected with approximately 0.5 μL of the RNP solution containing 3.3 $\mu\text{g}/\mu\text{L}$ Cas9 protein and 1.3 $\mu\text{g}/\mu\text{L}$ sgRNA as described previously^{4,44}. To compare the performance of Cas9 protein, commercial Cas9 products from IDT (Cat#1081059), Sigma (Cat#Cas9PROT), FUJIFILM Wako (Cat#316-08651), Fasmac (Cat#GE-005-S), and TaKaRa (Cat#632679) were used. Injected females were grouped with males in a container with wheat flour and transferred to a new container every 24 hours. To screen gene-edited individuals, the eye colors of the G_0 insects were examined during pupal and adult stages. As the *cardinal* gene is located on the X-chromosome (female = XX, male = XY), 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 *cardinal* were likely underestimated.

Transfection and transient expression in cultured insect cells

Plasmid constructs were generated via ligation or In-Fusion cloning (TaKaRa, Cat#639648).

Fragments were synthesized using GenScript Inc. DNA synthesis service or PCR amplified and then subsequently into pIEx-4 vector (Novagen, USA). BmN cells were seeded on glass coverslips in 16-well plates and grown for 48 h. All transfections were performed with the TransFast transfection reagent (Promega, Cat#E2431) using 1 μ g of plasmid DNA. For fluorescence detection, cells were fixed with 4% formaldehyde in 1 \times phosphate buffered saline (PBS) and washed. The coverslips were mounted in VECTASHIELD Antifade Mounting Medium (Vector Laboratories, Cat#H-1000). For immunocytochemistry, cells were fixed with 4% formaldehyde in PBS for 20 minutes at room temperature, permeabilized with 0.25% Triton X-100 in 1 \times PBS, blocked with 0.1% Tween 20 in 1 \times PBS containing 2% bovine serum albumin and incubated with mouse anti-Cas9 antibody (1:100; Abcam, #ab191468) at 4°C overnight. After washing, cells were incubated with Alexa488-conjugated goat anti-mouse IgG (H+L) (1:1000; Thermo Fisher, #A28175) for 1 hour at room temperature. Coverslips were mounted in VECTASHIELD. Fluorescence confocal images were acquired using an FV3000 confocal laser scanning microscope (Evident). ImageJ was used to calculate the N/C ratio. For quantification, confocal images with magnified nuclear regions were taken. We defined the region of interest of nuclear, cytoplasm, and background regions within the captured images. The average intensity of each registered region was measured, and the N/C values were calculated. Welch's t-test was used for statistical tests. Amino acid sequences of all recombinant GFP and Cas9 proteins are available in Supplementary Data 2.

Gene knock-in experiments in beetles

ssODNs purchased from IDT (Alt-R HDR donor oligos) contain a 13-nt PCV recognition sequence at the 5'-end, left and right homology arms (40-nt each), a 2-nt insertion producing a HindIII site,

and a 1-nt substitution mutating the PAM sequence. ssODNs were knocked into the *cardinal* gene as described previously⁴. Equimolar amounts of SUMO-PCV-Cas9 and the ssODN with PCV recognition sites were incubated for 10–15 minutes at room temperature with 1 mM MgCl₂. Validation of the binding was checked by SDS-PAGE analysis. The ssODN sequence is (5'- to -3'):

AAGTATTACCAGCCGCCACTTGTGTCTGGGGCCCAGGGAACAGATGAACCAAGCTT
GACCGCGTTTATAGACGGGTTCGGTTATTTACGGGGTGG, with the 13-nt PCV recognition

sequence underlined. As a control, we also used the ssODNs containing a scrambled PCV recognition sequence³⁶. The control ssODN sequence is (5'- to -3'):

CTATTGTACTAATCGCCACTTGTGTCTGGGGCCCAGGGAACAGATGAACCAAGCTTG
ACCGCGTTTATAGACGGGTTCGGTTATTTACGGGGTGG, in which the scrambled 13-nt PCV

recognition sequence is underlined. The injection solution contained 3.3 µg/µL SUMO-PCV-Cas9,

1.3 µg/µL sgRNA, and 0.7 µg/µL ssODNs with 1 mM MgCl₂ and 500 mM NaCl. When using Alt-

R HDR enhancer V2 (IDT, Cat#10007910), its concentration was adjusted to either 5 or 20 µM in

the injection solution. Injected females were allowed to lay eggs for 2 days. The resulting G₀ adults

with white and mosaic eyes were used for subsequent genotyping experiments. Genomic DNAs

were individually extracted as described previously⁶². Genomic PCR was performed using KOD

FX Neo (TOYOBO, Cat#KFX-201). PCR products were digested with HindIII and analyzed by

microchip electrophoresis using the MultiNA Microchip Electrophoresis System (MCE-202,

Shimadzu). Primer sequences are (5'- to -3'): GGCCAAAACCGGGGCGCTTCC and

CCGGAAGTTCGTGGGTACAAGCCCG^{4,44}. To explore the nucleotide sequences of knock-in

alleles, PCR products were subcloned and sequenced by Sanger sequencing. To further investigate

the variations of edited alleles, PCR products were subjected to deep sequencing with the MiSeq

system (Illumina). CRISPResso2 was used to analyze the amplicon sequencing data⁶³.

Embryonic microinjection and mutant screening in crickets

ssODNs from IDT (Alt-R HDR donor oligos) contain a 13-nt PCV recognition sequence at the 5'-end, left and right homology arms (55-nt each), and 3xFLAG (66-nt). ssODNs were knocked into the 5' end of the coding sequence of the *E93* gene. For embryo microinjections in crickets, eggs laid within 1-2 h were collected and aligned in wells made of 2% agarose gel dissolved in water. A pulled glass capillary was connected to a Femtojet 4i (Eppendorf). A small droplet of the mixture of 300 ng/μL Cas9 fusion protein, 100 ng/μL sgRNA and 100 ng/μL ssODN with 500 mM NaCl and 1 mM MgCl₂ was injected into eggs. Injection procedures were completed within 4 h after egg oviposition. Genomic DNA was extracted from individual embryos (after stage 12⁶⁴) or hatchlings and used for genotyping. Genomic PCR was performed with different primer sets using KOD FX Neo (TOYOBO, Cat#KFX-201). Primer sequences for HMA were (5'- to -3'): CCGACCAGTCGTTCCGCTAAAG (F1) and CCATATTCTTCGTCCATCTTTGC (R1). Primer sequences for 5'- or 3'-junction PCR were (5'- to -3'): GACTACAAAGACCATGACGGTGA (F2) and CTTGTCATCGTCATCCTTGTAATCG (R2). To determine the nature of the knock-in allele of *E93*, PCR products were subjected to Sanger sequencing. Primer sequences for Sanger sequencing were (5'- to -3'): GTCATTAGTGGAGTAGCGGAAG and TCCACAAAACACGTGTACACACG. Fisher's exact test was used to determine the p-value. Control (non-target) PCR products were amplified at the 3'-end of the *E93* locus. Primer sequences for control PCR were (5'- to -3'): GCCAAAGACTTACAGCAAGAAGAAGG and CTGTCACATAACATCTTGCTTCCTAC.

Embryo microinjection and mutant screening in milkweed bugs

ssODNs from IDT (Alt-R HDR donor oligos) contain a 13-nt PCV recognition sequence at the 5'-end, left and right homology arms (40-nt each), and 3xHA (93-nt). ssODNs were knocked into the 3' end of the coding sequence of the *tj* gene. For embryo microinjections in milkweed bugs, eggs laid within 4 h were collected and aligned on a glass slide with double-sided tape. Then, eggs were submerged in water for 30 minutes to reduce the inner pressure before injection. A pulled glass capillary was connected to a IM300 microinjector (Narishige). A small droplet of the mixture of 300 ng/ μ L Cas9 fusion protein, 100 ng/ μ L gRNA (crRNA + tracrRNA) and 100 ng/ μ L ssODN with 500 mM NaCl and 1 mM MgCl₂ was injected into eggs. Injection procedures were completed within 6 h after egg oviposition. Genomic DNA was extracted from individual embryos 4 days after egg laying and used for genotyping. For crossing experiments, genotyping was performed using gDNA extracted from one leg. Genomic PCR was performed with different primer sets using KOD Fx Neo (TOYOBO, Cat#KFX-201). Primer sequences for HMA were (5'- to -3'): ACAACGTATCCTGCGAACGCGA (F1) and TGTACATAACAGCCTAAACAGGCGTG (R1). Primer sequences for 5'- or 3'-junction PCR were (5'- to -3'): GGCTACCCATACGATGTTTCCTGAC (F2) and TCAAGCGTAATCTGGAACGTCATATGG (R2). Fisher's exact test was used to determine the p-value. Control (non-target) PCR products were amplified at the *E93* locus. Primer sequences for control PCR are (5'- to -3'): GTACCGGAACTACGACCGAG and CACCTTGTATTCAAGTGTGGAGTG.

***in situ* hybridization chain reaction and immunofluorescence in milkweed bugs**

For fluorescence *in situ* hybridization chain reaction (HCR), ovaries and testes were fixed with 4% formaldehyde in 1×PBS for 30 minutes at room temperature, followed by three washes with PBT (1×PBS with 0.1% Triton X-100). HCR probe sets were designed with *insitu_probe_generator*⁶⁵. After washing, samples were incubated in hybridization buffer (Molecular Instruments) at 37°C for at least 30 minutes and then incubated with HCR probes (10 nM) in hybridization buffer at 37°C overnight. The following day, samples were washed four times for 15 minutes with wash buffer (Molecular Instruments) at 37°C and washed two times for 5 minutes with 5×SSCT (5×SSC with 0.1% Triton X-100) at room temperature. After washing, samples were incubated in amplification buffer (Molecular Instruments) for at least 30 minutes at room temperature and incubated with hairpin solutions (HCR amplifier B5 555, Molecular Instruments) (1:50 each) in amplification buffer at room temperature overnight. The following day, samples were washed four times for 15 minutes with 5×SSCT at room temperature and mounted in 100% glycerol. For antibody staining, samples were washed three times with PBT after *in situ* hybridization. After washing, samples were incubated in blocking solution (PBT with 5% normal goat serum) for 1 hour and then incubated with rat anti-HA antibody (1:50; Roche, Cat#11867423001) in blocking solution at 4°C overnight. The following day, samples were washed four times over four hours with PBT, incubated for an hour in blocking solution, and then incubated with Alexa488-conjugated goat anti-rat IgG (H+L) (1:400; Invitrogen, Cat#A-11006) at 4°C overnight. After washing, samples were mounted in 100% glycerol. Images were taken using a Zeiss LSM 980 with Airyscan.

Statistics and reproducibility

Details on sample sizes and the number of replicates are provided in Figures, Supplementary

Figures, Supplementary Data, and their respective legends. In the nuclear localization activity assays, the number of cells used for quantification ranged from 45–51 for GFP fusion proteins, and from 53–60 for Cas9 fusion proteins. Statistical significance was determined using an unpaired *t*-test with Welch's correction. For the knock-in experiments in crickets and milkweed bugs, statistical significance was determined using Fisher's exact test.

Data availability

The amplicon-seq datasets are available at: https://figshare.com/articles/dataset/Tribolium_castaneum_amplicon-seq/29228705.

Additional supporting data in this study are available from the corresponding author upon request.

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Author contributions

Y.S. and T.D. conceived the idea and designed the project; Y.S. conducted biochemical experiments with assistance and supervision from R.N., and O.N.; Y.S. conducted gene editing experiments in beetles and crickets with assistance from N.M.; Y.S. conducted transfection experiments with assistance from T.D.; Y.S., O.N., C.G.E., and T.D. obtained funding; Y.S., J.A.K., and T.K. conducted gene editing experiments in milkweed bugs with supervision from C.G.E.; Y.S. and T.D. wrote the paper with input from all authors; and T.D. supervised this research.

Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1. Optimization of Cas9 purification and adult injection in beetles.

- (a) Schematic overview of Cas9 expression and purification steps. Detailed procedures are described in Supplementary Data 1.
- (b) SDS-PAGE analysis of Cas9 samples collected at sequential purification steps, visualized by CBB or silver staining. Molecular weight markers are indicated on the left. Each lane was loaded with 1 μ g of Cas9 protein.
- (c) Experimental scheme for gene editing via adult injection in the beetle *Tribolium*.
- (d) Definitions of gene editing efficiency (GEF) and effort efficiency (EEF).
- (e) Eyes of G_0 individuals showing *cardinal* mutant phenotypes. White arrowheads indicate loss of black pigmentation in mosaic eyes.
- (f) GEF values of Cas9 purified using 2-step or 3-step protocols. G_0 eggs were collected at 0–24 h and 24–48 h after injection. Pooled values for the 0–48 h period are also shown. Bars represent means of two independent experiments.
- (g) EEF values calculated from the same dataset as in (f).

Figure 2. Performance of Cas9 fusion proteins for adult injection in beetles.

- (a) Schematic of Cas9 fusion proteins containing a SUMO-tag and/or the PCV-tag. Yellow boxes indicate NLSs from SV40 large T-antigen. Red boxes indicate (EAAAK)₈ linkers. Amino acid sequences of these proteins are shown in Supplementary Data 2.
- (b) GEF values of in-house Cas9 fusion proteins and commercial Cas9 proteins from five vendors. G_0 eggs were collected at 0–24 h and 24–48 h after injection. Pooled values for the 0–48 h period are also shown. Bars represent means of two independent experiments.
- (c) EEF values calculated from the same dataset as in (b).

Figure 3. Nuclear localization activity of the PCV-tag.

- (a) Amino acid sequence of the PCV-tag. Three predicted NLS motifs are highlighted in yellow, with basic amino acid residues shown in bold. The HUQ motif (a variant of the canonical HUH motif) and the catalytic tyrosine are marked in red.
- (b) Subcellular localization of GFP fusion proteins in transfected BmN cells. Amino acid sequences of the GFP fusion proteins are shown in Supplementary Data 2.
- (c) Nuclear-to-cytoplasmic (N/C) ratio of GFP fusion proteins. Bars and error bars represent the mean and standard deviation (SD) ($N = 45$ – 51 cells per group). Statistical significance was

determined using an unpaired *t*-test with Welch's correction. Numerical data are shown in Supplementary Data 3.

(d) Subcellular localization of Cas9 fusion proteins in BmN cells, visualized with the “orange hot” lookup table in Fiji. Amino acid sequences of the Cas9 fusion proteins are shown in Supplementary Data 2.

(e) N/C ratio of Cas9 fusion proteins. Bars and error bars represent the mean and SD ($N = 53$ – 60 cells per group). Statistical significance was determined using an unpaired *t*-test with Welch's correction. Numerical data are shown in Supplementary Data 3.

Figure 4. Efficient knock-in using SUMO-PCV-Cas9 via adult injection in beetles.

(a) Schematic of the covalent attachment of ssODN to the PCV-tag. The 13-nt PCV recognition sequence is underlined in orange.

(b) Schematic of ssODN-mediated knock-in into the *cardinal* gene. In the precise knock-in allele, a novel HindIII site is integrated at the target site.

(c) SDS-PAGE gel shift assay. The arrowhead indicates the band corresponding to the covalent attachment between the Cas9 fusion protein and the ssODN.

(d) Experimental scheme of knock-in experiments via adult injection in *Tribolium*.

(e) Results of knock-in experiments using SUMO-PCV-Cas9. An ssODN with a scrambled 13-nt recognition sequence was used as a control. Knock-in frequency was calculated as the proportion of G_0 knock-in animals among G_0 animals showing eye color phenotypes. A commercial HDR enhancer reagent was also tested under the same experimental conditions.

(f) Representative genotyping results of G_0 adults carrying knock-in alleles. Eight G_0 individuals (#1–#8), indicated with asterisks in (e), were analyzed individually. PCR products were analyzed by microchip electrophoresis with (left) or without (right) HindIII digestion.

(g) Knock-in frequencies using SUMO-PCV-Cas9. Raw data are shown in (e). Bars represent the mean of two independent experiments. The dotted line indicates the previously reported knock-in frequency using DIPA-CRISPR⁴.

(h) Knock-in effort efficiency using SUMO-PCV-Cas9. Raw data are shown in (e). The dotted line shows the recalculated knock-in effort efficiency of DIPA-CRISPR⁴.

(i) Results of amplicon sequencing for the same individuals analyzed in (f) (#1–#8). Detailed results are shown in Supplementary Fig. 4.

(j) Representative knock-in and indel alleles identified in G_0 individual #5 by amplicon-seq. Notably, this individual harbored a high proportion (~50%) of the precise knock-in allele.

Figure 5. Efficient knock-in using SUMO-PCV-Cas9 via embryo injection in crickets.

- (a) Experimental scheme for embryo microinjection in the cricket *G. bimaculatus*. Developing embryos (stage >12) or hatchlings were used for genotyping.
- (b) Schematic illustration of HDR-mediated knock-in using tethered ssODNs, introducing a 22-aa epitope tag (3xFLAG) into the *E93* gene. Primer positions used for genotyping are also shown.
- (c) Results of individual genotyping. Asterisks indicate bands derived from knock-in alleles: black asterisks indicate positive bands on one side (5' or 3'), and green asterisks indicate positive bands on both sides. Green dots indicate individuals (#1–#4) selected for further analysis by Sanger sequencing.
- (d) Summary of knock-in experiments in crickets. Statistical significance was determined using Fisher's exact test.
- (e) Sanger electropherogram of genomic PCR product of a knock-in animal carrying a single precise knock-in allele, without detectable wt or indel alleles.

Figure 6. Efficient knock-in using SUMO-PCV-Cas9 via embryo injection in milkweed bugs.

- (a) Experimental scheme for embryo injection in the milkweed bug *O. fasciatus*. Embryos at 4 days after egg laying (AEL) or hatchlings were used for genotyping.
- (b) Schematic illustration of HDR-mediated knock-in using tethered ssODNs, introducing a 31-aa epitope tag (3xHA) into the *tj* gene. Primer positions used for genotyping are also shown.
- (c) Results of individual genotyping. Asterisks indicate bands derived from knock-in alleles: black asterisks indicate positive bands on one side (5' or 3'), whereas orange asterisks indicate positive bands on both sides.
- (d) Summary of knock-in experiments in milkweed bugs. Statistical significance was assessed using Fisher's exact test.
- (e) Experimental scheme of germline transmission. Legs of G₀ adults were used for genotyping before individual crosses.
- (f) Results of embryo microinjection and screening of G₁ individuals.
- (g) Sanger sequencing electropherogram of genomic PCR product from a G₁ knock-in animal carrying a precise knock-in allele.
- (h) *in situ* hybridization chain reaction (HCR) and immunofluorescence images of the ovary and testis of heterozygous knock-in adults (*tj-3xHA/+*). In the ovary, *tj* is expressed in oocytes but not in the tropharium. In the testis, *tj* is expressed in a region of the anterior tip (white arrowheads). White dashed boxes in the overview images indicate regions shown at higher magnification in the lower panels.

Editor's Summary

An HUH endonuclease–tagged Cas9 markedly enhances both knockout and knock-in efficiency in insects. By enabling efficient genome editing via adult and embryo injection, this platform expands precise genetic manipulation across diverse arthropods.

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