CRISPR/Cas9-mediated knock-in in *ebony* gene using a PCR product donor template in *Drosophila*

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Abstract

CRISPR/Cas9 technology has been a powerful tool for gene editing in *Drosophila*, particularly for knocking in base-pair mutations or a variety of gene cassettes into endogenous gene loci. Among the *Drosophila* community, there has been a concerted effort to establish CRISPR/Cas9-mediated knock-in protocols that decrease the amount of time spent on molecular cloning. Here, we report the CRISPR/Cas9-mediated insertion of a ~50 base-pair sequence into the *ebony* gene locus, using a linear double-stranded DNA (PCR product) donor template By circumventing the cloning step of the donor template, our approach suggests the PCR product as a useful alternative knock-in donor format.

Keywords

CRISPR; *Drosophila* ; Knock-in; Genome engineering

Description

Since the advent of the CRISPR/Cas9 technology, gene editing in many model organisms, including *Drosophila melanogaster*, has been a common approach for many researchers to study and modify specific gene functions [1,2]. One of the features of the CRISPR/Cas9 system is its ability to disrupt gene function by introducing out-of-frame indel mutations into a target genomic locus [1,3,4]. In addition, the CRISPR/Cas9 system can also be used to precisely insert visible markers (*e.g.*, cassettes encoding fluorescent proteins) to study the localization of live, endogenous proteins within their native environments [5–9].

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Drosophila melanogaster is an important model system for gene editing due to its usefulness to study human diseases since about 2276 genes in flies are conserved and linked to human diseases [10]. Taking advantage of the evolutionarily conserved genes and genetic tractability, the *Drosophila* "Gene Disruption Project" uses a reverse genetics approach to precisely insert reporters or disrupt homologous fly genes to elucidate multiple molecular mechanisms that underlie their disease phenotypes [5,11,12]. To generate these transgenic lines in an efficient and scalable manner, there has been a concerted effort among the *Drosophila* community to characterize more time- and cost-effective approaches and protocols for CRISPR/Cas9-mediated gene editing within the last decade [1,2,5,6,8].

Initially, researchers used plasmid donor templates containing long (>1 kb) homology arms for knock-in *Drosophila* [1,3,4,13]. Constructing these large plasmid donor templates required extensive molecular cloning [14].As a result, there have been approaches that have looked towards decreasing the amount of time towards molecular cloning in preparation for CRISPR/Cas9-mediated knock-in *Drosophila*. One way to bypass cloning the donor template is to use single-stranded oligonucleotide donors – which can be outsourced by a commercial entity [1,12,14–17]. For example, Port et al. successfully introduced an 11 bp mutation containing a restriction enzyme site into the *ebony* genomic locus, using a 50 nt single-stranded oligonucleotide donor and thus, demonstrating a single-stranded oligonucleotide as a cloning-free donor format for CRISPR/Cas9-mediated insertion [1]. Nowadays, custom plasmids can be synthesized by tech companies in a cost-effective man- ner, especially if the gene cassette is short (<1 kb). For instance, Kanca et al. utilized commercially synthesized plasmid donor templates in combination with an *in vivo* linearization strategy – a process in which the specific gRNA cuts linearize the plasmid construct *in vivo* upon injection – for CRISPR/Cas9-mediated knock-in multiple gene loci in *Drosophila* [5].

Using linear double-stranded DNA (PCR product) donors could also decrease the time towards molecular cloning. PCR products require no cloning, can be generated within two hours, and can be readily scaled for high-throughput library generation. PCR product donors have previously been used for CRISPR/Cas9-mediated insertions in *Drosophila in vitro* [8,18] but, so far, not *in vivo*. To demonstrate the PCR product as a cloning-free, alternative donor format, we knock in a short disruptive cassette into the *ebony* gene of fruit flies [5].

To gage gene editing using the CRISPR/Cas9 system in *Drosophila*, previous reports historically have introduced indel mutations into a specific gene locus: *ebony* on the third chromosome [1,3,19,20]. Flies with a homozygous loss-of-function *ebony* mutation (*e.g.,* $TM3,e^1$ /$TM6b,e^1$) are known to display a dark cuticle phenotype – thereby facilitating the screening of successful editing events [1,3,6]. More recently, to benchmark the approach for insertion of a novel donor format, Bosch et al. have used the *ebony* locus to demonstrate CRISPR/Cas9-mediated, homology-independent insertion of a linearized donor plasmid [6].

Similarly, to benchmark CRISPR/Cas9-mediated knock-in using the PCR product donor, we targeted the *ebony* gene by cutting at a site 25 bp downstream of the translational start codon of the *ebony* gene, using *ebony*-gRNA (Fig. 1A), as previously described by Port et al. [1]. We injected a premixed solution of a plasmid expressing the *ebony*-gRNA and the PCR

product donor template into embryos ubiquitously expressing the Cas9 endonuclease. For the knock-in, we used a PCR product donor template that contains a disruptive gene cassette (three tandem stop codons in different reading frames and a mini-PolyA tail, flanked by 90 bp homology arms; adapted from Kanca et al.) [5] (Fig. 1B).

To determine whether the PCR product donor could yield a knock-in event, we extracted the genomic DNA from 700-embryo pools 24 h after injection and amplified a DNA fragment using a set of primers that recognizes a region internal to the knock-in site on the 5′ end and a downstream region of *ebony* on the 3′ end. The knock-in allele-specific PCR should yield an expected amplicon of 991 bp (Fig. 1B).

It has been known that different concentrations of an injected plasmid donor template can affect the frequency of knock-in integration in *Drosophila* [3,6]. To assess the concentration of PCR product donor for knock-in, we tested concentrations from 0 to 700 ng/μL. Using the primer set recognizing the knock-in site within the *ebony* gene, we found that the pooled embryos injected with the PCR product donor template yielded an amplicon corresponding with the expected size of 991 bp, suggesting precise insertions of the PCR product donor into *ebony* (Fig. 2 A). Interestingly, we found that pooled embryos injected with the highest concentration of PCR product donor (700 ng/μL) yielded an additional faint band containing a shorter DNA amplicon. This downshifted band suggests that imprecise integration of the disruptive cassette may occur when a high concentration of PCR product donor is injected.

To avoid imprecise knock-in events, we injected the *ebony*-gRNA with 70 ng/*μL* of the PCR product donor template into the Cas9-expressing embryos for all subsequent microinjections. In total, we injected 2800 embryos, of which 155 flies (5.5%) survived after the microinjection process – which we refer to as G_0 survivors (Fig. 2 B). 75 single G_0 male survivors were then crossed with female *ebony* loss-of-function mutants (*TM3,e^1 / TM6b,e^1*). Only single crosses that generated at least 50 G_1 progenies were counted towards the rate of successful crosses; 43 G_0 male survivors (57.3%) had successful crosses (Fig. 2B).

The G_1 progeny were then screened for the dark cuticle phenotype, indicative of a germline-transmitted CRISPR/Cas9-mediated editing event (Fig. 2C). For this study, we coined "*e^{93C7CRISPR}*" as the allele with the inserted disruptive cassette in the *ebony* gene (located in the 93C7 cytogenetic region). Upon successful integration of the disruptive cassette, G_1 progeny with the dark cuticle phenotype would contain the *ebony ^{93C7CRISPR}* and *ebony^1* alleles (*e^{93C7CRISPR}/TM6b,e^1*) (Fig. 2C). To determine whether a knock-in event occurred, we verified the integration of the disruptive cassette by PCR validation (Fig. 2D). We extracted genomic DNA from each individual fly and amplified the DNA fragment, using the same primer set recognizing the knock-in site within the *ebony* locus. G_1 progeny containing the inserted disruptive cassette (*e^{93C7CRISPR}/TM6b,e^1*) yielded the expected 991 bp band whereas G_1 progeny without the knock-in event (*+/TM6b,e^1*) or female homozygous for the *ebony^1* allele (*TM3,e^1/TM6b,e^1*) did not yield any amplicon (Fig. 2D). Thus, our screening approach could detect the germline-transmitted knock-in event in the *ebony* gene.

Finally, from these G_1 progeny, siblings with the dark cuticle phenotype were crossed to generate homozygous ($e^{93C7CRISPR}$/$e^{93C7CRISPR}$) flies, to which we validated the insertion of the disruptive cassette into the *ebony* gene by Sanger sequencing (Fig. 2E). Based on this representative sequencing data, the insertion of the disruptive cassette was without additional random insertions or deletions in the *ebony* gene. Overall, 4 out of 43 crosses (9.3%) successfully passed down the germline-transmitted knock-in cassette to their progeny (Fig.2B). This founder rate (9.3%) is comparable to that reported by Kanca et al., in which they reported rates roughly ranging from 2 to 12% using their *in vivo* linearized disruptive donor cassette – thus, corroborating with this study's germline-transmitted knock-in rate using PCR product as a donor template [5].

Altogether, these data show that the PCR product as a donor format can be useful for germline-transmitted knock-in into the *ebony* gene in *Drosophila*. While we show that the founder rate using the PCR product donor template is comparable to that of other donor formats from previous literature, we noted that our survival rate is lower than those of previous reports, which roughly range from 10 to 45% after injecting a gene-specific gRNA and donor template [5,6]. To address if the PCR product donor affects survival rate, we injected embryos with ebony-sgRNA alone and saw no significant difference between those injected with or without the PCR product donor (Supplementary Fig. 1). This suggests that the observed survival rate may be due to the laboratory-to-laboratory variation, such as the microinjection process [21]. For example, we dissolved the CRISPR/Cas9 components in TE buffer rather than an in-house formulated injection buffer for microinjections [22,23]. Optimization in injection buffer compositions may further improve the survival rate. However, this parameter remains to be empirically tested in *Drosophila* and is not covered in this study.

Additional studies are required for generating knock-ins using the PCR product donor. These studies would be important for determining whether this approach using a PCR product donor would be useful for scalable generation of a collection of mutant fly lines as the "Gene Disruption Project" is aiming for [5, 11,12]. A previous study has shown that a long gene cassette (> 1000 bp), using a PCR product donor, can be integrated into the *Drosophila* genome in a cell culture system [8]. Whether *in vivo* knock-in of a long gene cassette (> 1000 bp) remains to be tested. Their knock-in strategy also utilized short homology arms (< 100 bp) [8]. Homology arm length is a factor to be considered for this approach as most commercially available primers are capped within a 100 bp length. Currently, the scope of this study is focused on knock-in occurring within the *ebony* gene but paves the way for this knock-in approach to be expanded to other genes in the future.

Methods

Plasmid preparation

For gRNA generation, plasmids were generated using the general fol- lowing protocol. DNA fragments were amplified using Q5 High-Fidelity DNA Polymerase (NEB). Thermocycling conditions are as followed: 98 °C for 30 s (denaturation), 98 °C for 10 s, 52 °C for 10 s, and 72 °C for 45 s for 30 cycles, and 72 °C for 2 min. pCFD4-U6:1_U6:3tandemgRNAs (Plasmid #49411) was digested using BbsI-HF (NEB) at 37 °C for 16 h. The vector

backbone and amplified DNA fragment were then gelpurified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel #740609) and assembled using In-Fusion HD Cloning Plus Kit (Takara Bio #638910).

Chemically competent *E. coli* strain (Takara Bio #636763) was transformed and selected on LB-agar plates with ampicillin. Plasmids were isolated and amplified from colonies using ZymoPURE Plasmid Miniprep Kit (Zymo Research #D4209) and sequenced at Eton Bio-science Inc.

Primer sequences for gRNA plasmid construction are listed below:

ebony -gRNA forward primer: 5′ - TATATAGGAAAGATATCCGGGTG AACTTCGCCACAATTGTCGATCGTCAGTTTTAGAGCTAGAAATAGCAA G-3′

ebony -gRNA reverse primer: 5′- ATTTTAACTTGCTATTTCTAGCTCT AAAACTGACGATCGACAATTGTGGCGACGTTAAATTGAAAATAGGTC-3′

The disruptive donor cassette was synthesized and cloned into pU-CIDT vector at Integrated DNA Technologies. Templates for dsDNA construct were ordered as followed:

Disrup-dsDNA: 5′ -GTAGTACGATCATAACAACGCGGTCCGACTGAGA TTCTAAGCCCAAAACTAACAAAGTATTCCCCACAGTTAATATATCTTCA AGATGGGTTCGCTGCCACAATTGTCGATCGTTCGAATAACGTAACCTAG GAAATAAAATACGAAATGAATTCTACCCAATTCGAATCAAGGGTCTGCA GCAAGACTTCGTGCCTAGAGCTCTGCACCGCATCTTCGAGGAGCAGCAG CTGCGGCATGCCGACAAGGTGGCTCTGACCGCGTTGTTATGATCGTACT AC-3′

Upon synthesis of the plasmid template donor, we amplify a DNA fragment to use as the PCR product donor. Primer sequences for PCR product donor construction are listed as followed:

Disrup-PCRdonor forward primer: 5′-TCCGACTGAGATTCTAAGCC-3′

Disrup-PCRdonor reverse primer: 5′-TCAGAGCCACCTTGTCGG-3′

Embryo microinjection

The CRISPR/Cas9 components were prepared in TE buffer at these final concentrations:

ebony-gRNA plasmid: 300 ng/μL; Disruptive donor cassette (PCR product): 70 ng/μL

CRISPR/Cas9 components were mixed and co-injected into 700 $y[1] = qM\{w[+ mC] = Act5C - Cas9 . P\}ZH - 2 A \quad w[*]$ (BDSC# 54590) em- bryos during the syncytial stage of development for each trial. 4 mi- croinjection trials were performed. Microinjections were performed at 18 °C, and embryos were moved to 25 °C, following injection. Flies were cultured on standard fly food at 25 °C. Injected G_0 males were crossed with $y[1] y[1] w[*]$; TM3, e^1 Sb[1]/TM6 b, e^1 Tb[+] (BDSC# 3720) females, and their subsequent G_1 progeny were screened and validated for knock-in.

Genomic DNA extraction

Genomic DNA was extracted from individual flies using DNAzol (Invitrogen #10503027) or PureLink Genomic DNA Mini Kit (Invitrogen #K182001), according to the manufacturer's protocol. DNA concentration was measured using the NanoPhotometer C40 (Implen).

PCR validation

Primer set (Knockin_F and Ebony_R) recognizing the knock-in region on the 5′ end and the downstream 3′ region of *ebony* was used to amplify a 991 bp product. Primer set (Nrk_F and Nrk_R) was used for *Nrk* gene-specific PCR as a control for DNA quality. PCR validation used the genomic DNA prepared from individual flies and was amplified using Taq 2X Master Mix (NEB #M0270L). Thermocycling conditions are as followed: 95 °C for 30 s (denaturation), 95 °C 30 s, 60 °C 30 s, 68 °C 1 min for 30 cycles, and 68 °C 5 min.

Primer sequences for PCR validation are listed below:

Knockin_F primer: 5′ -CGATCGAAGCTTTAACGTAACCT-3′

Ebony_R primer: 5′ -GATAGGGGTTCTCCGGAGCAGACC-3′

Nrk_F primer: 5′ -GCACATGGCGGTAAAGATCG-3′

Nrk_R primer: 5′ -GTGAGATCAGAGGGGCATCT-3′

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Data availability

No data was used for the research described in the article.

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A

Cut site

5' ATGGGTTCGCTGCCACAATTGTCGATCGTCAAGGGTCTGCAGCAAGACTTCGTGCC 3'
3' TACCCAAGCGACGGTGTTAACAGCTAGCAGTTCCCAGACGTCGTTCTGAAGCACGG 5'

ebony
3R:21,229,839..21,237,177 [-]

1kb

B

90 bp left homology arm   3XSTOP   mini-PolyA tail   90 bp right homology arm   PCR product donor (235 bp)

TAACGTAACCTAGGTAAATAAAATACGAAATGAATTCTACCCAA
ATTGCATTGGATCCATTTATTTTATGCTTTACTTAAGATGGGTT

Knockin_F

5'
3'
ebony genomic locus

Ebony_R

991 bp amplicon

Fig. 1.
Schematic of strategy using PCR product as a donor for CRISPR/Cas9-mediated knock-in *ebony* gene. (A) Schematic of the target site of *ebony*-gRNA in the second exon of the *ebony* gene. Boxes indicate exons (light gray) and untranslated regions (dark gray). Highlighted texts are the cut site targeted by Cas9 endonuclease (gray) and start codon (green). Texts in dark blue and light blue indicate the sequences that flank the cut site. (B) The PCR product donor containing the disruptive gene cassette for insertion into the cut site of *ebony*. The disruptive donor cassette contains three stop codons in all reading frames (red) and a mini-PolyA tail (dark red), flanked by 90 bp homology arms (dark blue and light blue). Black arrows indicate the primer set (Knockin_F and Ebony_R) used to amplify the 991 bp region to confirm the knock-in event in the *ebony* genomic locus.
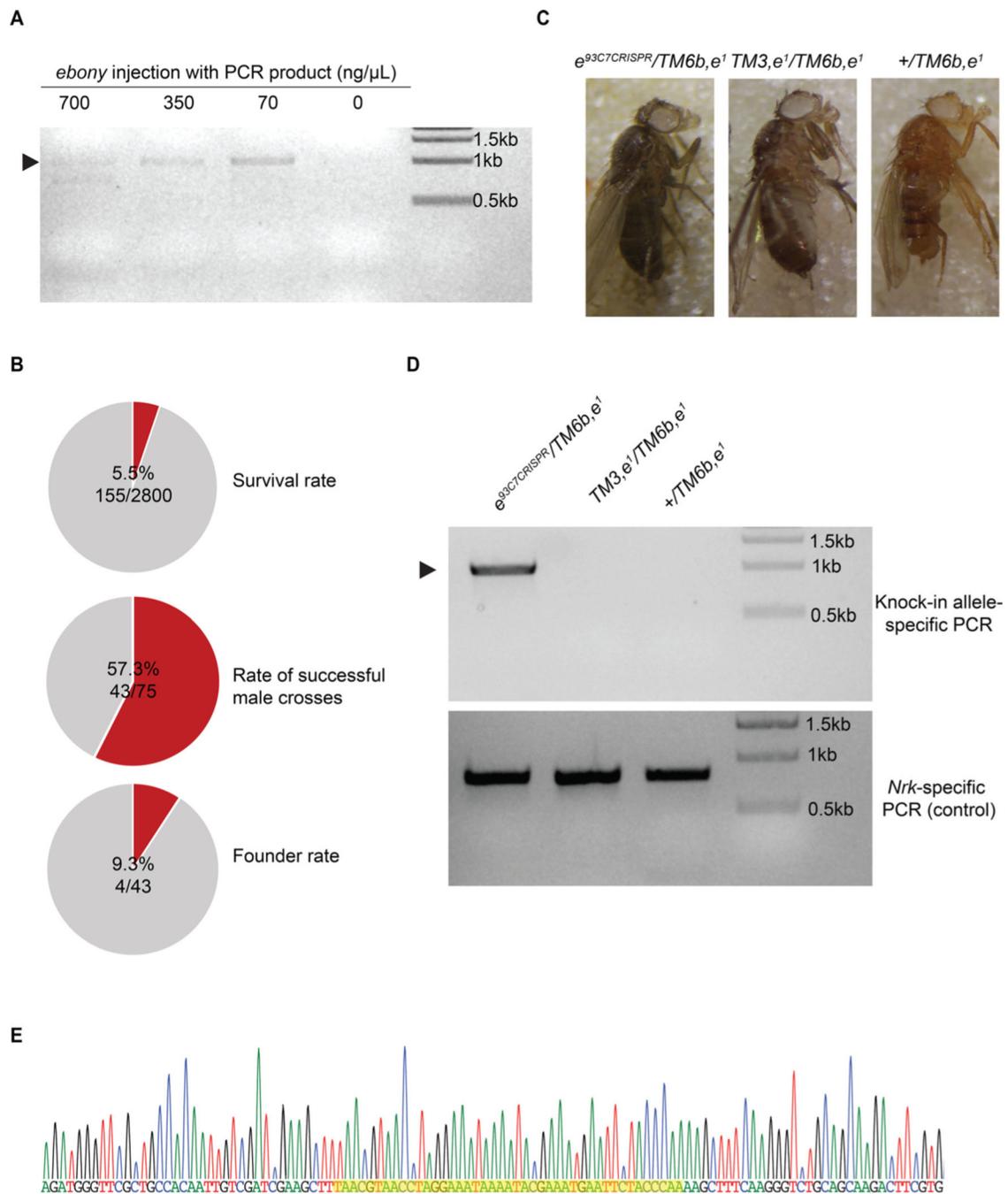
Fig. 2.

Germline-transmitted CRISPR/Cas9-mediated knock-in *ebony* gene using a PCR product donor template. (A) Knock-in events detected using different concentrations (700, 350, 70, 0 ng/µL) of PCR product donor template injected into 700-embryo pools. 1% agarose gel image shows the amplicons from knock-in allele-specific PCR, using 15 ng of genomic DNA and primer set Knockin_F and Ebony_R (see Fig. 1B). Arrowhead indicates the band of the expected 991 bp amplicon. (B) Survival, successful cross, and founder rates, using PCR product donor for CRISPR/Cas9-mediated insertion of the disruptive cassette into

ebony. Drosophila embryos were injected with a pre-mixed solution of the PCR product donor template and gRNA plasmid (see Embryo Microinjection in Materials and Methods). (C) Images of a G_1 $e^{93C7CRISPR}$ / *TM6b,e^1* fly with knock-in of the disruptive donor cassette (left) compared to a *TM3,e^1* / *TM6b,e^1* fly (middle) and a sibling G_1 + /*TM6b,e^1* fly without the knock-in event (right). (D) Top and bottom agarose gel images show the amplicon products from PCR. For the top gel image, shown is the amplified fragment of the knock-in region in an individual G_1 $e^{93C7CRISPR}$ / *TM6b,e^1* fly, resulting from the successful incorporation of the PCR product (first lane) into the *ebony* genomic locus. No amplified fragment from the *TM3,e^1* / *TM6b,e^1* fly (second lane) or the sibling G_1 + /*TM6b,e^1* fly (third lane), as a result of no incorporation of the PCR product donor. Primer set (Knockin_F and Ebony_R) is used for knock-in allele-specific PCR (see Fig. 1 B). For the bottom gel image, a primer set (Nrk_F and Nrk_R) is used for *Neurospecific receptor kinase (Nrk)* gene-specific PCR (control for DNA quality). Arrowhead indicates the band of the 991 bp amplicon from knock-in allele-specific PCR. Each lane contains the PCR amplification from the genomic DNA of a single fly. (E) Sequencing chromatogram of the PCR product amplified from a homozygous fly derived from successful knock-in of PCR product donor. The yellow highlighted region indicates the incorporated three stop codons and mini-PolyA tail in the *ebony* genomic locus.