Efficient Gene Knock-out and Knock-in with Transgenic Cas9 in Drosophila

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ABSTRACT Bacterial Cas9 nuclease induces site-specific DNA breaks using small gRNA as guides. Cas9 has been successfully introduced into Drosophila for genome editing. Here, we improve the versatility of this method by developing a transgenic system that expresses Cas9 in the Drosophila germline. Using this system, we induced inheritable knock-out mutations by injecting only the gRNA into embryos, achieved highly efficient mutagenesis by expressing gRNA from a novel non-coding RNA gene, and recovered homologous recombination-based knock-in of a fluorescent marker at a rate of 4.5% by co-injecting gRNA with a circular DNA donor.

KEYWORDS knock-out knock-in Cas9 gRNA Drosophila

The RNA-guided CRISPR/Cas9 system has been successfully used for the purpose of genome editing in many organisms, including Drosophila (Golic 2013). Cas9 nuclease is guided to its target DNA by a small guide RNA (gRNA), which shares a homologous region of approximately 20 nucleotides (nt) with the cleavage site. By direct injection of synthesized Cas9 mRNA and customized gRNA into wild-type Drosophila embryos, we previously achieved a mutational rate of up to 100% in the germline (Yu et al. 2013). We considered that at least two aspects of our previous technique could be improved upon. First, the generation and handling of in vitro synthesized RNA, particularly the relatively large Cas9 mRNA, could be challenging to inexperienced researchers, which could result in inconsistencies in mutational efficiency. Second, the utility of Cas9 in generating knock-in mutations has not been adequately demonstrated in Drosophila. Here, we report efficient knock-out and knock-in manipulations of the Drosophila genome with a Cas9 transgenic system. We note that several groups have independently developed tools to conduct mutagenesis using transgenic lines expressing Cas9 in the germ-line (Sebo et al. 2014; Ren et al. 2013; Kondo and Ueda 2013) and to generate knock-in mutations (Baena-Lopes et al. 2013; Gratz et al. 2014). In addition, an efficient knock-in system using TALEN has been developed (Katsuyama et al. 2013).

Materials and Methods

Fly stocks
Several different wild-type stocks such as w¹¹¹⁸, y w and Oregon-R (red eye) were used in this study. All balancers were obtained from the Bloomington Stock Center (Indiana). Flies were cultured at 25°C.

Plasmid construction (PiggyBac-vasa-cas9, pUAST-gRNA, and homologous recombination donor plasmid)

PiggyBac-vasa-cas9 construction: PiggyBac-vasa-cas9 construction required three steps. First, a standard entry vector with a visible GFP marker, PiggyBac-9A-GFP, was constructed by inserting a 3xP3GFP fragment with SV40 3′-UTR from the plasmid p3GFP (stock in our laboratory) between the Xmal and SpsomI sites of PiggyBac-9A (Venken et al. 2006). Second, a 2241-bp promoter sequence and a 576-bp 3′-UTR-containing sequence were amplified from the vasa gene by PCR and were cloned into the same p3GFP-GFP (Figure S6). PiggyBac-vasa-cas9 was released from TA-vasa-P3GFP (stock in our laboratory) by NotI/Sall and inserted between the NotI and Xhol sites of TA-vasa-Pro-UTR to generate TA-vasa-Pro-cas9-UTR. Eventually, the larger fragment containing vasa-Pro-cas9-UTR was released from TA-vasa-Pro-cas9-UTR by Xmal/Spel and cloned into PiggyBac-9A-GFP using the same restriction enzymes. This produced the final transgenic vector PiggyBac-vasa-GFP (Figure S6).

pUAST-gRNA construction: Three steps were needed for pUAST-gRNA construction. First, a fragment containing 3xP3RFP and the tubulin 3′ UTR was amplified from the pM3×P3-RFPattP vector (Bischof et al. 2007) and used to replace the white gene between

Copyright © 2014 Xue et al. doi: 10.1534/g3.114.010496
Manuscript received December 22, 2013; accepted for publication March 19, 2014; published Early Online March 21, 2014.
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Supporting information is available online at http://www.g3journal.org/lookup/suppl/doi:10.1534/g3.114.010496/-/DC1
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two EcoRV sites in pUAST; this created the entry plasmid pUAST-RFP. Second, a 400-bp Drosophila U6b promoter sequence (Wakiyama et al. 2005) and the 1133-bp promoter sequence from Drosophila non-coding RNA CR34335 (Graveley et al. 2010) (Figure S4) were separately cloned into the pMD19-TA gRNA scaffold vector (stock in our laboratory) to create TA-U6b-gRNA and TA-CR7T-gRNA, respectively. The KOD mutagenesis kit (TOYOBO, Japan) was used to insert 20-bp target sequences between the promoter and the gRNA scaffold. Third, the target-specific U6b/CR7T-gRNAs were released by SpeI/KpnI and cloned using the same sites in pUAST-RFP, generating the final transgenic gene-specific vectors (Figure S6). In total, five transgenic gRNA vectors were generated: pUAST-U6b-k81-gRNA for targeting the ms(3)k81 locus; pUAST-U6b-y1-gRNA and pUAST-CR7T-y2-gRNA for targeting the yellow locus; and pUAST-CR7T-w1-gRNA and pUAST-CR7T-w2-gRNA for targeting the white locus. All primers are listed in Table S2 and Table S3.

Donor plasmid pHisc-RA-RFP construction: To generate the pHisc-RA-RFP donor plasmid, a synthetic DNA fragment containing the attP, FRT, and 3xP3RFP sequences was cloned into pEASY-T to generate vector pEASY-RFP (available upon request). Subsequently, 1.5-kb and 1.2-kb sequences, respectively, corresponding to the 5′ and 3′ ends of the histone cluster right-arm (Hisc-RA) were inserted flanking the RFP cassette to obtain the final construct pHisc-RA-RFP.

gRNA injections

DNA templates were PCR-amplified from gRNA scaffold vectors for in vitro gRNA transcription (Yu et al. 2013). Transcription was performed with the RibomAX Large Scale RNA Production System-T7 Kit (Promega, USA) according to the manufacturer’s protocol. Purified gRNA (0.2 µg/µl) was injected into vasa-Cas9 fly embryos either directly or after mixing with purified donor plasmid (0.8 µg/µl) according to standard procedures. All primers are listed in Table S1.

Fly transformation and genetics

To obtain transgenic vasa-Cas9 flies, the PiggyBac-vasa-cas9 vector was mixed with helper vector PiggyBac-transposase (Thibault et al. 2004) and injected into w1118 fly embryos. Flies carrying vasa-Cas9 were identified by GFP expression in the eye when viewed under a fluorescence stereomicroscope. Mutation position was mapped to chromosome 2 after crossing with balancer vasa−Cas9−Cas9/ pUAST-U6B-k81-gRNA and its F1 offspring were recovered and analyzed as described above. The mosaic yellow phenotype was evaluated by screening for yellow RFP flies with visible RFP eye markers. The male F0 flies were then crossed to wild-type flies to obtain F1 progeny with germine-transmitted mutations. The mosaic eye phenotype of F0 transgenic vasa−Cas9/pUAST-gRNA males was recovered by collecting F1 progeny expressing both RFP and GFP eye markers. The female red-eye vasa-Cas9 flies were crossed to pUAST-CR7T-gRNA males to generate male F0 flies, which were identified by screening for the GFP and RFP eye markers. The male F0 flies were then crossed to wild-type flies to obtain F1 progeny with germline-transmitted mutations. The mosaic eye phenotype of F0 transgenic vasa−Cas9/pUAST-gRNA was investigated and indel scoring in F1 progeny was performed and analyzed as above. Primers used for PCR and sequencing are listed in Table S3.

Recovery and analysis of germline mutants via homologous recombination

Hisc-RA-gRNA and the donor pHisc-RA-RFP plasmid were co-injected into vasa−Cas9 fly embryos, and germline mutations containing the attP-FRT-RFP fragment were recovered by collecting F1 progeny with visible RFP eye expression. Genomic DNA was prepared from individual heterozygous and some homozygous candidates. The target genome region was amplified using three pairs of primers, including one pair that flanked the locus (H shimmer-001 and H shimmer-002) (Figure 1A). The corresponding products were analyzed by gel electrophoresis and sequenced (Figure 1B). Homozygous knock-in stocks were established after crossing to balancer flies. Primers used for PCR and sequencing are listed in Table S3.

RESULTS AND DISCUSSION

Cas9 knock-out by gRNA injection into Cas9-producing embryos

To eliminate the need to synthesize Cas9 mRNA for injection, we set out to achieve Cas9 expression in the germline by driving the cas9 gene with the vasa promoter (Lasko and Ashburner 1988). We recovered a single PiggyBac-mediated insertion of this transgene in chromosome 2. Transgenic Cas9 flies are homozygous viable and fertile. To investigate whether these transgenic flies can be used to efficiently produce site-specific mutations, we set out to knock-out ms(3)k81 using a previously designed gRNA (Yu et al. 2013). Mutations
in ms(3)k81 cause male sterility (Fuyama 1986). When we injected Cas9 mRNA and ms(3)k81 gRNA simultaneously, all surviving males were sterile and all harbored somatic ms(3)k81 mutations (Yu et al. 2013). We hypothesized that in these males, both copies of the ms(3) k81 gene might have been mutated in male germ cells. Many surviving females were fertile, and all of their progeny were mutants for ms(3) k81, consistent with our proposition that both copies of ms(3)k81 had been mutated as a result of the repair of a Cas9-induced DNA break.

Here, we injected only the ms(3)k81 gRNA into vasa-cas9 embryos. We detected somatic ms(3)k81 mutations in 70% of the surviving males (12/17). All 12 males were fertile and produced a ms(3)k81 mutational rate of 25% in the germline (Table 1). Surviving females...
Table 1 Frequencies of *ms(3)k81*/*U6B-k81* gRNA-mediated mutagenesis

| Locus        | gRNA    | F₀       | Fertile F₀ with Somatic Mutation n/n, (%) | F₁, Mutation Rates n/n, (%) |
|--------------|---------|----------|------------------------------------------|----------------------------|
| *ms(3)k81*   | Injection | Female   | 20/26 (76.9)                              | 11/49 (22.4)               |
|              |         | Male     | 12/17 (70.1)                              | 5/20 (25.0)                |
|              |         | Male     | 22/22 (100)                               | 4/28 (14.3)                |
| *Yellow*     | U6B-y1  | Female   | 36/36 (100)                               | 221/588 (37.6)             |
|              |         | Male     | 8/8 (100)                                 | 158/178 (88.8)             |
|              | CR7T-y2 | Female   | 30/30 (100)                               | 593/628 (94.4)             |
|              |         | Male     | 7/7 (100)                                 | 74/82 (90.2)               |
| *White*      | CR7T-w1 | Female   | NA                                        | 430/664 (64.8)             |
|              |         | Male     | 17/17 (100)                               | NA                         |
|              | CR7T-w2 | Female   | 23/23 (100)                               | NA                         |
|              |         | Male     | 18/18 (100)                               | 86/506 (17.0)              |

NA, not applicable.

a All males are sterile.

b The female progeny of F₁ was counted as total progeny.

produced a similar mutational rate in their germline (Table 1). Therefore, our transgenic system can efficiently induce mutations in the germline (Figure S1A).

**Knock-out by transgenic expression of both gRNA and Cas9**

To completely eliminate the need to inject RNA, we set out to establish a transgenic gRNA system to combine with germline Cas9 expression. The U6B promoter has commonly been used for gRNA expression (Kondo and Ueda 2013; Ren et al. 2013; Sebo et al. 2014). Here, we were interested in testing the efficacy of using the promoter of another non-coding RNA gene to drive gRNA expression. We chose the non-coding *CR34335* gene, which is ubiquitously expressed at high levels based on genome-wide RNA-seq data (Graveley et al. 2010), and established a gRNA expression system that is driven by the *CR34335* promoter, which we named CR7T. gRNA for various targets were put under the control of the U6B or CR7T promoters and introduced into the fly genome by P-element–mediated transformation.

As a test of the functionality of our U6B expression system, we generated flies with *vasa-Cas9* and *U6B-k81*, which expresses a gRNA designed for the *ms(3)k81* used previously. All males (*n* = 26) were sterile, suggesting that both *ms(3)k81* copies had been mutated in male germ cells. Twenty-two females were fertile, from which we obtained a *ms(3)k81* mutational rate of 14.3% (Table 1 and Figure S1B). Therefore, we observed a lower efficiency using the U6B promoter in the female vs. the male germline. We tested the same system on the *yellow* locus and observed a similar effect (y1 gRNA in Table 1). In contrast, for the y2 gRNA against *yellow*, we achieved equally high (more than 90%) frequencies from both germ lines using the CR7T promoter (Table 1 and Figure S2). We further confirmed the utility of CR7T in driving gRNA expression by using it in the generation of mutations at two target locations in *white*, both with reasonably high efficiencies (Table 1 and Figure S3).

In summary, we successfully generated knock-out mutations to three genes by transgenically expressing both Cas9 and gRNA. The germline mutational rate ranged from 14% to 95%. Additionally, we developed a new gRNA expression system using the CR7T promoter.

**Knock-in by Cas9-induced homologous recombination**

The ability to introduce predefined genetic modifications is highly desirable in genome editing. The knock-in approach utilizes the ability of the cell to incorporate exogenously supplied DNA into the host genome by homologous recombination (HR). Previously, a 50-nt modification (an attP attachment site for the phiC31 integrase) was successfully introduced into the *yellow* locus by Cas9-induced HR (Gratz et al. 2013). This modification was accomplished by co-injection of Cas9 mRNA, gRNAs against *yellow*, and single-stranded oligos as donor DNA for recombination [single-strand oligodeoxynucleotide (ssODN)]. The ssODN had two 60 nt homology arms flanking attP.

For the Cas9-induced knock-in method to become routine practice in *Drosophila*, at least two issues need to be further examined. The first issue is whether this approach can be applied to a general locus where an easily screenable phenotype (such as yellow body color in *yellow*-mutant flies) is not available. The second issue is whether double-stranded donor DNA can be used when the size of the desired modification precludes the use of oligos. As an attempt to address the first question, we used ssODN to introduce an FRT site along with an attP to the left side (centromere-distal) of the histone locus on chromosome 2. The oligo is 135 nt in total length with a 24-nt homology arm on the left side and a 27-nt homology arm on the right side. Using PCR as a screening method, we successfully recovered precise knock-in events in 10 of the 112 F₁ progeny screened, suggesting that the ssODN approach can, in principle, be applied without the necessity of a screenable phenotype in the target locus. PCR followed by sequencing confirmed the precise integration of the FRT and attP elements (Figure S3). However, a similar approach applied to the right (centromere-proximal) side of the histone locus failed, with the oligo being 87 nt in total length, with a 26-nt homology arm on left side and a 27-nt homology arm on the right side. The reason for this is unclear.

As an attempt to address the second question, we modified the histone cluster locus using a plasmid as the HR donor. The overall scheme of this targeting experiment is shown in Figure 1A. Our goal was to knock-in an element with attP and FRT along with a 3×P3RFP marker gene to the right side of the histone locus. In the donor plasmid, these three DNA elements were flanked by a 1.5-kb homology arm to the left and a 1.2-kb arm to the right (Figure 1A). The inclusion of these elements into the donor also disrupted a Cas9 cleavage site so that Cas9 will only cut at the chromosome but not the donor. The in vitro synthesized gRNA molecules and donor DNA were injected into *vasa-Cas9* embryos. We set up 89 fertile crosses from flies that survived the injection process. Using the RFP marker, we recovered at least four independent events with a total of 49 F₁ progeny showing RFP expression. PCR amplification followed by sequencing confirmed that in all of these 49 F₁ individuals, a single copy of the 2-kb RFP cassette was precisely targeted to the desired location (Figure 1B). Therefore, we have demonstrated the targeted insertion of a large piece of heterologous DNA (2 kb in total) aided by the use of
a visible marker in Drosophila. Using this RFP-marked insertion and the previously generated attP-FRT insertion to the left of the histone locus, we succeeded in deleting the entire 125-kb histone locus using FLP-mediated recombination (data not shown).

ACKNOWLEDGMENTS
We thank Dr. Hugo J. Bellen and Dr. Johannes Bischoff for providing the PiggyBac-9A and pM 3×P3RFP-RFFattP′ plasmids. We also thank Wei Wei for technical assistance with DNA sequencing and PCR analysis. This work was supported by grants from the National Natural Science Foundation of China (31171278, 31271542). Research in the laboratory of Y.S.R. is supported by the intramural program of National Cancer Institute (NCI).

LITERATURE CITED
Baena-Lopez, L. A., C. Alexandre, A. Mitchell, I. Pasakarnis, and J.-P. Vincent, 2013 Accelerated homologous recombination and subsequent genome modification in Drosophila. Development 140: 4818–4825.
Bischof, J., R. K. Maeda, M. Hediger, F. Karch, and K. Basler, 2007 An optimized transgenesis system for Drosophila using germ-line-specific C31 integrases. Proc. Natl. Acad. Sci. USA 104: 3312–3317.
Fuyama, Y., 1986 Genetics of Parthenogenesis in DROSOPHILA MELANOCASTER. 1. The modes of diploidization in the gynogenesis induced by a male-sterile mutant, ms(3)K81. Genetics 112: 237–248.
Golic, K. G., 2013 RNA-guided nucleases: a new era for engineering the genomes of model and nonmodel organisms. Genetics 195: 303–308.
Gratz, S. J., A. M. Cummings, J. N. Nguyen, D. C. Hamm, L. K. Donohue et al., 2013 Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. Genetics 194: 1029–1035.
Gratz, S. J., F. P. Ukken, C. D. Rubinstein, G. Thiede, L. K. Donohue et al., 2014 Highly Specific and Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in Drosophila. Genetics 196: 161–971.
Graveley, B. R., A. N. Brooks, J. W. Carlson, M. O. Duff, J. M. Landolin et al., 2010 The developmental transcriptome of Drosophila melanogaster. Nature 471: 473–479.
Katsuyama, T., A. Akmannmedov, M. Seimiya, S. C. Hess, C. Sievers et al., 2013 An efficient strategy for TALEN-mediated genome engineering in Drosophila. Nucleic Acids Res 41: e163–e163.
Kondo, S., and R. Ueda, 2013 Highly improved gene targeting by germine-specific Cas9 expression in Drosophila. Genetics 195: 715–721.
Lasko, P. F., and M. Ashburner, 1988 The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A. Nature 335: 611–617.
Liu, J., C. Li, Z. Yu, P. Huang, H. Wu et al., 2012 Efficient and specific modifications of the Drosophila genome by means of an easy TALEN strategy. J. Genet. Genomics 39: 209–215.
Ren, X., J. Sun, B. E. Housden, Y. Hu, C. Roesel et al., 2013 Optimized gene editing technology for Drosophila melanogaster using germ line-specific Cas9. Proc. Natl. Acad. Sci. USA 110: 19012–19017.
Sebo, Z. L., H. B. Lee, Y. Peng, and Y. Guo, 2014 A simplified and efficient germine-specific CRISPR/Cas9 system for Drosophila genomic engineering. Fly (Austin) 8: 52–57.
Thibault, S. T., M. A. Singer, W. Y. Miyazaki, B. Milash, N. A. Dompe et al., 2004 A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. Nat. Genet. 36: 283–287.
Venken, K. J., Y. He, R. A. Hoskins, and H. J. Bellen, 2006 P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in D. melanogaster. Science 314: 1747–1751.
Wakiyama, M., T. Matsumoto, and S. Yokoyama, 2005 Drosophila U6 promoter-driven short hairpin RNAs effectively induce RNA interference in Schneider 2 cells. Biochem. Biophys. Res. Commun. 331: 1163–1170.
Yu, Z., M. Ren, Z. Wang, B. Zhang, Y. S. Rong et al., 2013 Highly efficient genome modifications mediated by CRISPR/Cas9 in Drosophila. Genetics 195: 289–291.