

CRISPR/Cas9 Genome Editing System in Drosophila

Mingkuan Sun, Iyla Oulabi and Liam Chen*

Neuropathology Division, Department of Pathology, Johns Hopkins University, School of Medicine, Baltimore, Maryland, USA

Abstract

To introduce changes into a genome, traditional methods relied on large-scale screen from randomly induced mutations or time-consuming gene targeting by homologous recombination. The recently developed CRISPR/Cas9 system has revolutionized the field of genetic engineering, allowing genome editing to be accomplished relatively easily. In this short review, we discuss the effectiveness, advantages and limitations of applying this powerful technique to genetic analysis in Drosophila.

Keywords: Drosophila; Genome; Nucleotides; Mutant phenotypes

CRISPR/Cas9 Overview

CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) and the CRISPR-associated (Cas) proteins are essential parts of adaptive immune system in bacteria and archaea against invading viruses and plasmids [1-3]. Three types of CRISPR systems exist, of which type II is the simplest one, only requires one Cas protein, Cas9 [4]. Because of its ability in making DNA Double Strand Breaks (DSBs) with sequence specificity, the CRISPR/Cas9 system was adapted into gene targeting in 2012, hence triggered the “CRISPR explosion” in many model organisms including Drosophila [5-9].

The type II CRISPR/Cas9 system consists of three components (Figure 1). Cas9 endonuclease is the key element and is responsible for the digestion of the target DNA [10]. To achieve site-specific DNA recognition and cleavage, Cas9 must be complexed with both a CRISPR RNA (crRNA) and a separate trans-acting crRNA (tracrRNA). The tracrRNA is partially complementary to the crRNA and is required for targeting its incorporation into the Cas9 complex [10,11]. The specificity is determined by a 20-nucleotide (nt) sequence within the crRNA, which can be altered to match any desired sequence in the endogenous DNA. This three-component system is now simplified

by fusing together crRNA and tracrRNA, creating a single chimeric “guide” RNA (abbreviated as sgRNA) [12,13]. The ease of using a single RNA has led to the widespread adoption of CRISPR/Cas9 for genome engineering.

One of the major advantages of the CRISPR system over other existing genome editing technologies is the relative lack of sequence limitations in the targeted sites. The only requirement is the presence of a Protospacer Adjacent Motif (PAM) at the 3' end of the DNA target site, which optimal sequence is 5'-NGG, or less effectively, NAG [14]. Cas9 cleaves at 3-4 nucleotides from the PAM sequence on both strands of the DNA. The DSBs at the target site can be repaired by either Non-Homologous End Joining (NHEJ) or Homology-Directed Recombination (HDR), and both repair mechanism can be used to manipulate the genome in a defined manner.

Applications in Drosophila

Multiple methods have been developed to supply the Cas9/sgRNA components into the fruit fly Drosophila melanogaster [15-23]. As a matter of fact, since only two components need to be supplied, the RNA injection techniques can apply the CRISPR/Cas9 system into essentially any genetic background. So far, the most cost-effective and rapid approach uses microinjection of plasmids encoding the sgRNA into transgenic flies in which Cas9 expression is controlled by a germline specific promoter. Alternatively, a second line with ubiquitous expression of the sgRNA can be crossed to Cas9-expressing flies. This approach produces the highest efficiency but is a lengthy process due to the need to establish a new fly stock for every sgRNA and to remove the Cas9 and sgRNA transgenes after mutant generation.

The most common application of the CRISPR/Cas9 system in Drosophila utilizes its ability to make DSBs at specific sites to introduce new heritable alleles of interests. Here, the major determining factor is whether the NHEJ or HDR pathway is depended upon to produce mutations.

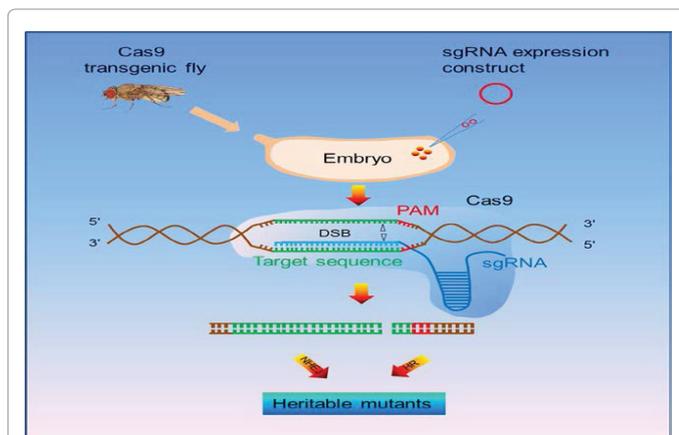


Figure 1: The CRISPR/Cas9 system in Drosophila.

To generate heritable alleles, the most cost-effective and rapid method is microinjection of sgRNA plasmids into Cas9 transgenic flies. DSBs generated by Cas9 at the target site can be repaired by Non-Homologous End Joining (NHEJ) or Homology-Directed Recombination (HDR). Frame-shift mutations or deletion of a larger region can be generated through the NHEJ pathway, whereas it is possible to replace part of the genome with almost any DNA sequence through the HDR pathway.

*Corresponding author: Liam Chen, Neuropathology Division, Department of Pathology, Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205 USA, Tel: 4109558102; E-mail: lchen99@jhmi.edu

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NHEJ-Mediated Mutation

One sgRNA can induce small insertions or deletions through the NHEJ pathway, resulting in frame-shift mutations in the protein coding region. NHEJ repair can also be used to disrupt functional elements within the genome such as transcription regulatory sequence, splice donor or acceptor sites. On the other hand, two sgRNAs guide pairs of Cas9 to trigger deletion or inversion of a larger intervening fragment, which can be very helpful to make defined deficiencies or remove larger functional regions such as protein domains, non-coding RNA genes. In addition, coinjections of two sgRNAs can simultaneously mutate two target genes, making genetic analyses of redundancy, epistasis or genetic interactions much easier to achieve.

HDR-Mediated Mutation

The HDR pathway requires the supply of an additional donor template, but it enables gene targeting to modify the genome at single-nucleotide level and to replace the target with almost any DNA sequence. The repair template can be either a 100-200 nt long single stranded DNA (ssDNA) convenient to integrate short sequences such as defined mutations or small detectable HA or FLAG tags, or longer double stranded DNA (dsDNA) with a larger homologous arm at either side of the integration site [24,25]. In practice, 1 kb of homologous sequence is adequate for most editing attempts, capable of replacement of more than 3 kb DNA sequence. This will allow integration of a GAL4 transcriptional reporter to track expression patterns of endogenous genes, or a GFP protein to visualize expression of endogenous proteins.

Utilizing either the NHEJ or the HDR pathway, it is possible to insert site-specific recombinase sites such as the attP sites to allow subsequent modification at the same position [26]. Other recombinase sites such as Flippase Recognition Target (FRT) sites could be introduced as well to allow somatic mosaic analysis. Perhaps one of the most exciting possibilities of applying CRISPR/Cas9 system in Drosophila is genome wide libraries of sgRNAs [27]. This would allow rigorous genetic screens that knock out the function of every protein coding gene or to perform genetic modifier screening far more efficiently. Recently a genome-wide CRISPR library which contains 40,279 sgRNAs targeting 13,501 genes has been constructed in Drosophila cells and is available to [28]. This will be a valuable resource for the Drosophila community for cellular phenotype screening, and as a source of sgRNAs for functional studies *in vivo*.

Other than introducing DSBs, recent developments have extended the CRISPR/Cas9 system to regulate gene expression. Transcription activation or repression of target genes from their native genomic locus is made possible by using a nuclease-dead Cas9 (dCas9) fused to transcriptional activation or repression domains [29,30]. Activating genes from their endogenous transcription start site provides several benefits over traditional over-expression methods based on cloned cDNAs. For example, dCas9-mediation activation technique is preferable for genes that are difficult to clone, e.g., if they occur in multiple splice isoforms and/or are very large. In addition, there is evidence that dCas9-mediated activation leads to target gene activation at physiologically relevant levels, as opposed to many existing techniques. Perrimon lab has recently shown that this technique can activate target genes and cause dominant phenotypes in fly, offering a simple and broadly applicable technique for over-expression studies in a multicellular animal [31].

Limitations of the CRISPR/Cas9 System

The major limitation of the CRISPR/Cas9 system is its off-target

effects due to the fact that several mismatches within the 20 nt recognition site can still be tolerated [32]. This poses a particular concern in the direct observation of mutant phenotypes in somatic tissues, as whether the observed phenotypes are due to unknown off target mutations cannot be clearly excluded. However, due to the small genome size of Drosophila and the fact that short generation time of Drosophila also allows backcrossed to remove such off-target mutations relatively easily, this is less of a problem in Drosophila than in other model systems, and sequences can be carefully chosen to minimize such off-target effects. If avoiding off-target effects is of paramount importance, it is also possible to use the "double-nick" approach to improve specificity. A mutated Cas9 protein that is only able to make single strand nicks in the DNA, coupled with two neighboring sgRNAs that target nearby sequences to double the specificity, it has been shown to be able to edit genes in Drosophila with almost no off-target effects [33].

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