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REVIEW

A Toolkit of CRISPR-Based Genome Editing Systems in *Drosophila*

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ABSTRACT

The last couple of years have witnessed an explosion in development of CRISPR-based genome editing technologies in cell lines as well as in model organisms. In this review, we focus on the applications of this popular system in *Drosophila*. We discuss the effectiveness of the CRISPR/Cas9 systems in terms of delivery, mutagenesis detection, parameters affecting efficiency, and off-target issues, with an emphasis on how to apply this powerful tool to characterize gene functions.

KEYWORDS: CRISPR; Genome editing; *Drosophila*; Cas9; sgRNA; Off-target

INTRODUCTION

CRISPRs (clustered regularly interspaced short palindromic repeats) were first discovered in *E. coli* (Ishino et al., 1987). Similar repeats with unknown functions were later found in other species of bacteria and archaea (Mojica et al., 2000). Based on many years of research, it was proposed that CRISPR works as an adaptive immune system against phage infection and foreign DNA (Jansen et al., 2002; Tang et al., 2002; Bolotin et al., 2005; Haft et al., 2005; Mojica et al., 2005; Pourcel et al., 2005; Makarova et al., 2006), and this theory was soon supported by experimental evidence (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). Later, the value of the CRISPR system was quickly realized in the area of genome editing, due to its ability in making DNA double strand breaks (DSBs) with sequence specificity (Jinek et al., 2012, 2013; Cong et al., 2013; Mali et al., 2013b).

The CRISPR system consists of a CRISPR array and Cas proteins that are usually encoded from a single operon (Barrangou and Marraffini, 2014; Bondy-Denomy and Davidson, 2014; van der Oost et al., 2014). The CRISPR array is composed of short palindromic repeats separated by so called proto-spacers, thus giving the name of the system (Brouns et al., 2008). Transcripts from the CRISPR array, termed pre-crRNAs, are processed into small crRNAs, which guide the Cas protein(s) to cut foreign DNA by base-pairing between the spacer sequence in crRNA and the DNA (Brouns et al., 2008). The protospacer adjacent motif (PAM), a small sequence in the foreign DNA, helps to distinguish non-self DNA from self DNA (Mojica et al., 2005; Horvath et al., 2008; Deveau et al., 2010; Shah et al., 2013; Li et al., 2014).

The CRISPR system is divided into three types, mostly depending on the differences in Cas protein function in crRNA biogenesis, and the recognition and destruction of the foreign DNA (Makarova et al., 2011a, 2011b). While type I and type III CRISPR systems require big Cas protein complexes to fulfill the nuclease function, the type II system only requires one Cas protein, Cas9 (Hale et al., 2009; Haurwitz et al., 2010; Hatoum-Aslan et al., 2011; Nam et al., 2012; Rouillon et al., 2013). It is because of this simplicity that the type II CRISPR system has been applied to genome editing technologies.

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In the type II CRISPR system (Fig. 1), the activation of the Cas9 and crRNA ribonucleoprotein also requires a trans-activating crRNA (tracrRNA). It has been demonstrated that a single-guide RNA (sgRNA), which consists of crRNA and tracrRNA modules, can supplement the two RNAs in the type II *Streptococcus pyogenes* CRISPR system, which makes it essentially a two-component system (Gasiunas et al., 2012; Jinek et al., 2012). Because of the simplicity of the *S. pyogenes* CRISPR system and the fact that it only requires a three-nucleotide (NRG) PAM, it has become popular in genome editing. The specificity of the type II *S. pyogenes* CRISPR system is controlled by the targeting sequence of the sgRNA. In addition to genome editing, the *S. pyogenes* CRISPR system can be applied to activate or repress gene expression (Maeder et al., 2013; Mali et al., 2013a; Perez-Pinera et al., 2013; Qi et al., 2013; Konermann et al., 2014), and to label DNA (Chen et al., 2013). This system has been successfully applied in cell lines and many model organisms including *Drosophila* (Bassett et al., 2013; Cong et al., 2013; Friedland et al., 2013; Gratz et al., 2013; Hwang et al., 2013; Kondo and Ueda, 2013; Mali et al., 2013b; Ren et al., 2013; Wang et al., 2013; Yang et al., 2013; Yu et al., 2013; Liu et al., 2014; Port et al., 2014; Sebo et al., 2014).

In this review, we discuss the CRISPR/Cas9 systems in *Drosophila* with an emphasis on their applications. We focus on the delivery methods, mutagenesis detection methods, parameters affecting efficiency, and off-target issues of the powerful CRISPR/Cas9 systems.

METHODS FOR DELIVERING CAS9/sgRNA IN *DROSOPHILA*

In *Drosophila*, quite a few CRISPR-based techniques have been developed to generate designer mutants (Bassett et al., 2013; Gratz et al., 2013, 2014; Kondo and Ueda, 2013; Ren et al., 2013; Wei et al., 2013; Yu et al., 2013; Bassett and Liu, 2014; Gokcezade et al., 2014; Housden et al., 2014; Lin et al., 2014; Port et al., 2014; Sebo et al., 2014; Chen et al., 2015). Multiple methods have been tested in *Drosophila* to deliver the Cas9/sgRNA system, as in cell lines and other model organisms (Cong et al., 2013; Dickinson et al., 2013; Friedland et al., 2013; Hruscha et al., 2013; Hwang et al., 2013; Jiang et al., 2013; Jinek et al., 2013; Mali et al., 2013b; Wang et al., 2013, 2014; Hsu et al., 2014; Kim et al., 2014; Sanjana et al., 2014; Shalem et al., 2014; Sung et al., 2014; Yin et al., 2014). These methods introduced Cas9/sgRNA components into the fly either as *in vitro* transcripts (Fig. 2A), as DNA plasmids (Fig. 2B), or as transgenes (Fig. 2C and D).

A common application of the CRISPR system is the generation of new heritable alleles of interests in *Drosophila melanogaster*. To generate heritable alleles, microinjection of sgRNA plasmids into *Cas9* transgenic flies is the simplest, most rapid, and most cost-effective approach (Fig. 2C), because of the bypass of the laborious *in vitro* RNA synthesis step and the ease of constructing sgRNA plasmids in a high-throughput manner (Ren et al., 2013; Housden et al., 2014; Port et al., 2014). This delivery method allows highly efficient generation of heritable mutants by the CRISPR system, and up to four loci can be successfully mutagenized in one injection in *Drosophila* (Ren et al., 2014b). Establishing stable *Cas9* transgenic animals is relatively easy in *Drosophila*, compared to other multicellular model organisms. Thus, when delivering *Cas9* as a transgene in flies, a few options are available, including general promoters for both germline and somatic expression (Port et al., 2014), and germline specific regulatory elements from *nanos* and *vasa* (Kondo and Ueda, 2013; Ren et al., 2013; Gratz et al., 2014; Port et al., 2014; Sebo et al., 2014; Xue et al., 2014a). It has been demonstrated that germline-specific promoters are more reliable in producing heritable mutations as they can avoid lethality caused by somatic tissue mutation (Ren et al., 2013; Port et al., 2014). *Cas9* transgenes on different chromosomes are available, so these transgenes can be easily outcrossed after the recovery of newly generated alleles. As for sgRNA vectors, different promoters such as *U6b* (Gratz et al., 2013; Kondo and Ueda, 2013; Ren et al., 2013), *U6c* (Port et al., 2014), and *CR7T* (Xue et al., 2014b) are available but they basically share the same sgRNA scaffold design. For U6 promoters, it is important that sgRNA expression starts with a guanine (G). Thus, either an sgRNA targeting sequence starting with a G or an sgRNA vector with a built-in G is required for efficient transcription (Ren et al., 2014b). If necessary, Cas9 and sgRNA reagents from different sources can be used to fulfill specific needs. In addition, crossing sgRNA transgenic flies to Cas9 flies could be an even simpler option (Fig. 2D) (Kondo and Ueda, 2013), but this application is limited by the time spent on establishing sgRNA transgenic fly stocks, as sgRNA flies with the desired targets may not be available.

It is also desirable to apply CRISPR in genetic screens. Under these circumstances, it is inadequate to generate heritable mutants of single loci, but preferable to directly observe mutant phenotypes in a high-throughput manner. Since generating heritable mutations at a few scores of loci could be a major undertaking even using the CRISPR system, a possible alternative is to use a combination of UAS-Cas9 and sgRNA transgenes (Port et al., 2014; Xue et al., 2014b). Combined with the Gal4 drivers (Brand and Perrimon, 1993), the CRISPR system can even achieve tissue-specific mutagenesis of genes. When activated, this system is able to knockout a gene in different cells without variations, which is better than existing gene knockdown methods such as the popular transgenic RNAi system (Ni et al., 2011). However, a couple of problems associated with the method are preventing its large-scale application. First, mutagenesis events occur randomly even when both Cas9 and sgRNA are present (Fig. 3). Without a proper reporter, it is difficult to distinguish between wild-type and mutant cells. Second, to achieve high efficiency and uniformity, multiple sgRNAs might be required to provide consistent results. Thus, the effectiveness and availability of this method are still not comparable with existing gene knockdown methods. Furthermore, a heritable mutant might still need to be generated to verify results and to allow further molecular characterization.

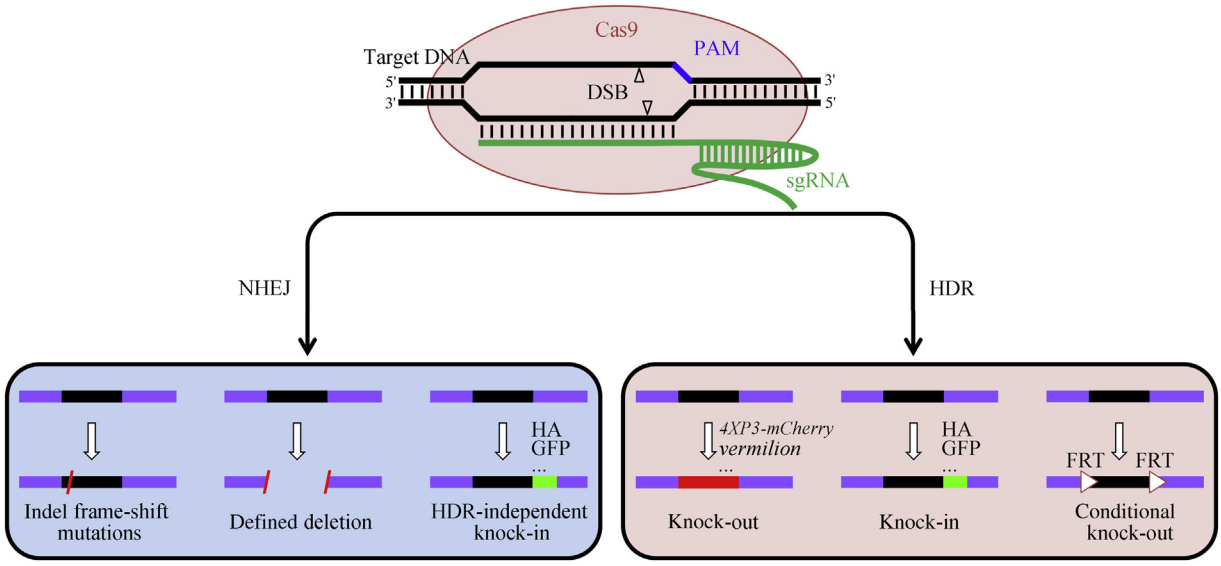


Fig. 1. Common heritable mutant types generated by the CRISPR system.
A model of a CRISPR-induced DNA double strand break is shown at the top. Target DNA is shown in black, Cas9 protein in red, sgRNA in green, and PAM in blue. Common types of mutant alleles generated through the non-homologous end joining (NHEJ) pathway include frame-shift mutations, defined deletions, and knocking-in of tags. The homology-directed repair (HDR) pathway allows more versatile editing practices. Here, the commonly used knock-out and knock-in alleles are shown. With the knocking-in of two FRT sequences, a conditional knock-out allele can be established to allow for spatial and temporal control of gene expression.

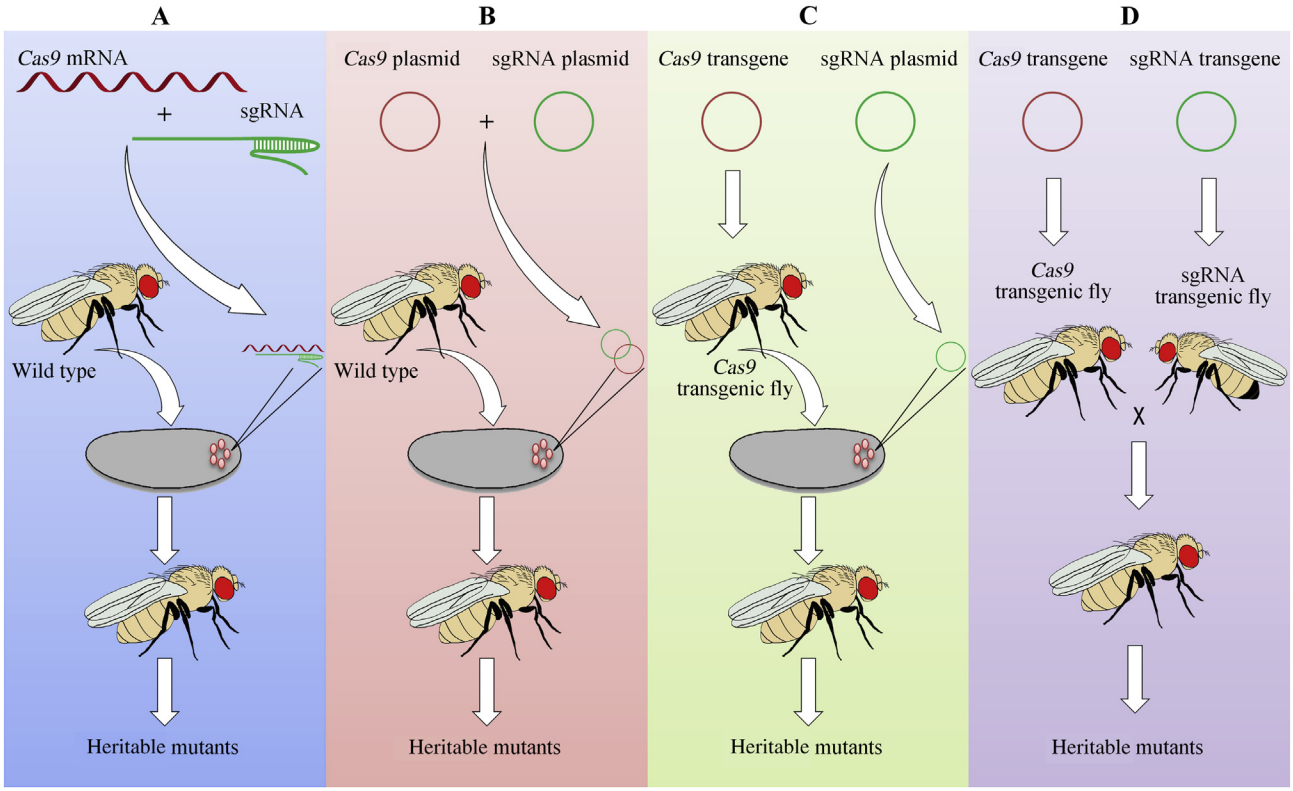


Fig. 2. Commonly used delivery methods of the CRISPR system in *Drosophila*.
A: *Cas9* (red) and sgRNA (green) are both delivered as *in vitro* transcripts through microinjection into fly embryos. **B:** *Cas9* and sgRNA are both delivered as DNA plasmids. **C:** *Cas9* has been previously introduced as a transgene, and sgRNA is injected as a DNA plasmid. **D:** *Cas9* and sgRNA transgenic flies have both been established first, and the two stocks are crossed to allow mutagenesis. In (**A**) to (**D**), heritable mutants are recovered in the F_1 generation, while mutagenesis events can be observed in the G_0 somatic tissues depending on the promoters for *Cas9* and sgRNA expression.

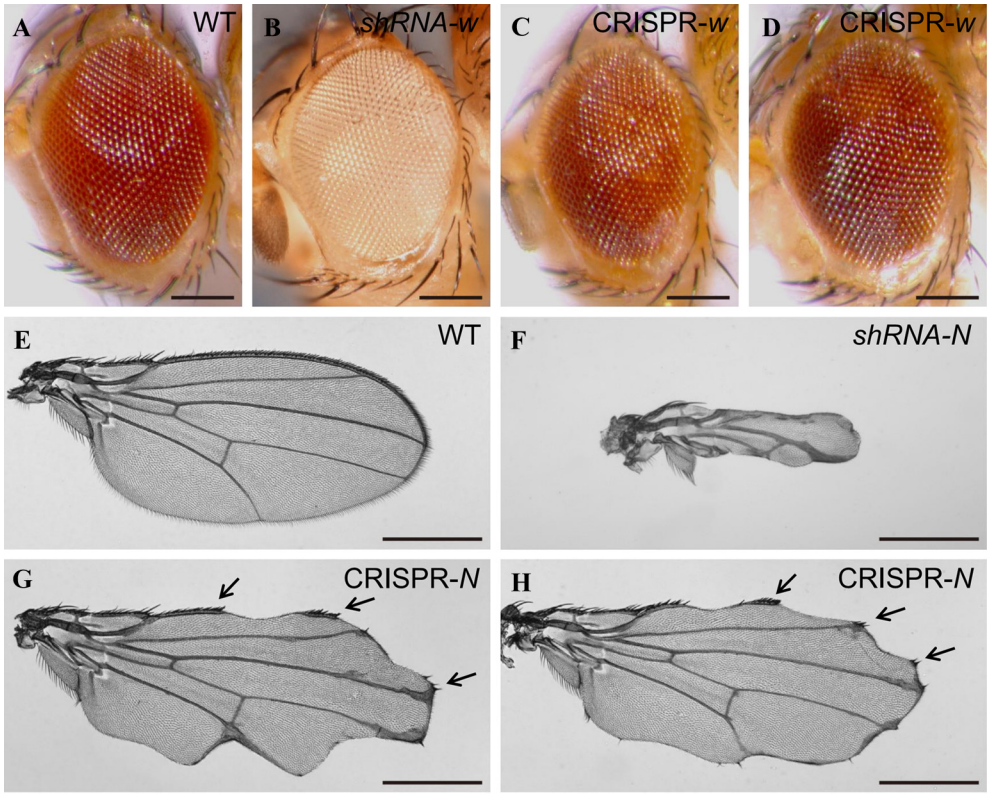


Fig. 3. Gal4/UAS system-induced Cas9 expression generates mutations at random in the somatic tissues when guided by sgRNA.
A−D: Images of eyes from a wild-type fly (**A**), a fly with short hairpin RNA against *white* (**B**), and CRISPR-induced *white* mutagenesis flies (**C** and **D**). CRISPR-induced *white* mutation results in random white ommatidia in flies (**C** and **D**), while transgenic RNAi-induced *white* knocking-down results in a consistent white-eye phenotype (**B**). **E−H:** Images of wings from a wild-type fly (**E**), a fly with short hairpin RNA against *Notch* (**F**), and CRISPR-induced *Notch* mutagenesis flies (**G** and **H**). Transgenic RNAi against *Notch* causes the missing of the whole margin area of the wing (**F**). With the CRISPR somatic mutagenesis system, notched margin areas of the wings differ from fly to fly (**G** and **H**), leaving random intact margins (arrows in **G** and **H**). Activation of the transgenic RNAi and CRISPR systems is triggered by GMR-Gal4 (**B−D**) and C96-Gal4 (**F−H**). Scale bars, 100 μm in **A−D** and 400 μm in **E−H**.

CRISPR also opened new doors for evolutionary developmental biology, where cross-species studies on developmental patterns and mechanisms are critical (Stern, 1998; Turner, 2014), by allowing the generation of new alleles specifically and cheaply, especially in *Drosophila* where the complete genome sequences of multiple species are available. A direct comparison of mutant phenotypes of protein-coding genes or regulatory sequences, such as long non-coding RNAs, across species might reveal unexpected developmental mechanisms or gene functions. In this case, injection of an *in vitro* transcript of the *Cas9* gene and sgRNAs is the method of choice, as this technique can be directly applied to closely related *Drosophila* species without any modifications (Fig. 2A). However, it is difficult to maintain mutant stocks without balancers. Thus generating conditional alleles might be a better choice.

NATURE OF THE DNA EDITING EVENTS AND METHODS OF MUTATION DETECTION

Although screening for successful gene-editing events is one of the last steps in applying the CRISPR system, it should be considered thoroughly even before designing the sgRNAs, since it can easily become the bottleneck in mutagenesis practice. Here, we focus on applying the CRISPR system to generate heritable alleles. Because Cas9 is a DNA nuclease that produces DSBs, a major factor affecting mutation detection is whether the non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathway can be depended upon to produce mutations after DSBs (Fig. 1). The NHEJ pathway does not require adding any extra components to the CRISPR system and is easier to carry out. The HDR pathway allows more possibilities in genome editing but requires an additional donor template.

One major application of the NHEJ pathway is to generate loss-of-function alleles. Since each sgRNA is required to guide Cas9 to make one DSB in the genome, one sgRNA can induce indel frame-shift mutations through the NHEJ pathway, while two sgRNAs can generate a defined deletion (Fig. 1). Generating frame-shift mutations through the NHEJ pathway does not allow for the use of visible reporters, so the most common mutation detection methods are sequencing, high resolution melt analysis (HRMA), and restriction fragment length polymorphism (RFLP), all of which can detect mutagenesis events in the generation 0 animals (G_0s) even when germline-specific Cas9 is applied to trigger mutations in the limited numbers of germ cells (Bassett et al., 2013; Gratz et al., 2013; Kondo and

Ueda, 2013; Ren et al., 2013; Yu et al., 2013; Port et al., 2014; Sebo et al., 2014). HRMA is more sensitive but requires special equipment (Bassett et al., 2013; Ren et al., 2013), while RFLP requires a convenient restriction enzyme recognition site to be abolished in the mutated allele, which may not be applicable for most attempts. On the other hand, defined deletions generated with a pair of sgRNAs can be directly detected by PCR with primers flanking the intended deletion region (Gratz et al., 2013; Ren et al., 2013). It has been demonstrated that a large DNA sequence (>3 kb) can be successfully deleted from the *Drosophila* genome (Gratz et al., 2013; Ren et al., 2013), which is sufficient for most single-gene disruption practices. An alternative approach is to utilize existing neighboring transposons with visible markers to generate defined deletions (Gratz et al., 2014). In this case, the absence of a visible marker such as *white* gene can be readily detected in the F_1 flies after part of the transposon is deleted together with the intended target. Another application of the NHEJ pathway is to insert DNA sequences into the genome (Fig. 1). Although knocking-in of a DNA sequence of up to 5.7 kb through the NHEJ pathway has been demonstrated (Auer et al., 2014), the insertion of these fragments through the HDR pathway is considered to be more reliable as it triggers defined DNA editing events with in-frame insertions, and in theory produces fewer random insertion events.

Through the HDR pathway, it is possible to replace part of the genome with almost any DNA sequence, which allows for the modification of endogenous loci at the single-nucleotide level and brings new insight in terms of molecular biology. The commonly used templates are single-strand oligodeoxyribonucleotides (ssODNs), DNA plasmids, or transgenes; all have homologous arms flanking the target genomic site. Due to the limitations of size and cost involved in ssODN synthesis, most of the ssODNs applied are 100−200 nt long (Cong et al., 2013; Gratz et al., 2013, 2014; Mali et al., 2013b; Bassett and Liu, 2014; Housden et al., 2014). At this length, it is convenient to knock-in detectable small tags such as HA or FLAG in-frame into endogenous loci (Fig. 1). This ssODN method is also good at adding stop codons, FRT sequences, and attP docking sites into the genome, or even replacing part of the gene with donor sequences (Fig. 1) (Gratz et al., 2013; Port et al., 2014; Xue et al., 2014a). DNA plasmids or transgenes as templates are more versatile, but in-house construction of the plasmid or generation of transgenes carries an additional time cost. In our experience, 1 kb of homologous arm sequences is adequate for the replacement of more than 3 kb genome between the homologous sequences (Ren et al., 2014a, 2014b), which is in line with a previous report showing that 200−500 bp of homology between a donor arm and the target is sufficient for HDR (Beumer et al., 2013). We believe that even 1 kb of DNA sequence should be convenient for most cloning attempts. Plasmids with visible markers and flanking multiple cloning sites are ready to use as donor templates (Baena-Lopez et al., 2013; Ren et al., 2014a, 2014b; Xue et al., 2014a; Zhang et al., 2014). There are also plasmids that allow for the selection of desired mutant alleles through suppression of lethality after HDR events (Chen et al., 2015). Furthermore, *lig4* mutants can be combined to allow higher HDR rates (Beumer et al., 2013; Gratz et al., 2014; Zhang et al., 2014).

Utilizing either the NHEJ or the HDR pathway, it is possible to construct conditional mutant alleles (Fig. 1) by inserting small DNA sequences, such as FRT or attP, into the fly genome to allow for induced recombination (Gratz et al., 2014). By crossing these conditional alleles to existing Gal4 or phiC31 driver stocks, temporal and spatial gene knocking-out can be achieved. Here, a visible cellular marker such as GFP or mCherry can be added to detect successfully induced mutations. Big collections of driver stocks are not readily available except in the case of *D. melanogaster*, but with the CRISPR system this may no longer be a major challenge, as tissue-specific driver stocks can be constructed in other *Drosophila* species with methods similar to that described by Chen et al. (2015).

CAS9 AND sgRNA PARAMETERS TO BE CONSIDERED

To successfully apply the CRISPR system in *Drosophila*, a few parameters of Cas9 and sgRNA that affect mutagenesis efficiency and specificity have to be determined. In *Drosophila*, only *S. pyogenes* Cas9 has been introduced so far. In terms of efficiency, specificity, and genome target availability due to restrictions such as the PAM sequence, *S. pyogenes* Cas9 seems adequate for most research needs. Since no other major factors except delivery method significantly affect Cas9 efficiency and different sgRNAs have to be constructed for each mutagenesis event, we focus here on parameters affecting sgRNAs in the two-component CRISPR system.

The sgRNA targeting site in the genome should be carefully chosen depending on the nature of the new alleles desired. For frame-shift indel mutations, an sgRNA site at the beginning of the coding DNA sequence (CDS) is preferred, since frame-shift mutations are less likely to leave residual peptides from the locus. For defined deletions, removing one exon might be sufficient to abolish gene expression. In this case, sgRNAs targeting the two flanking introns can avoid unwanted frame-shift mutations generated by either of them. When knocking-in small DNA sequences through NHEJ or HDR, it is important to destroy the sgRNA target in the desired new allele to avoid CRISPR-induced DSBs of the repaired locus.

The number and sequence of the sgRNA targeting nucleotides are important factors in determining the efficiency of DSBs (Fu et al., 2013; Hsu et al., 2013; Mali et al., 2013a; Pattanayak et al., 2013; Kuscu et al., 2014; Ren et al., 2014b; Wu et al., 2014). The targeting length of sgRNA is usually 20 nt, but in our experience 17−22 nt targeting sequence sgRNAs have comparable mutagenesis efficiency (Ren et al., 2014b). 18 nt sgRNAs have been shown to be as efficient as 20 nt ones, and potentially have lower off-target rates (Fu et al., 2014; Ren et al., 2014b). However, whether 17 nt sgRNAs can be efficiently used in flies requires additional research. High GC content in the seed region of the sgRNA, especially the 6 nt proximal to the PAM, is associated

with high mutagenesis efficiency (Ren et al., 2014b). Thus, high-efficiency sgRNA targets can possibly be chosen *in silico*.

There are quite a few web resources to facilitate sgRNA target selection in *Drosophila* (see a review by Bassett and Liu, 2014). These websites help distribute CRISPR reagents, as well as provide a lot of important information including protocols (http://www.oxfcrispr.org/; http://www.crisprflydesign.org/; http://www.shigen.nig.ac.jp/fly/nigfly/cas9/index.jsp) and web tools for sgRNA target selection and off-target evaluation (http://www.genome-engineering.org/crispr/?page_id=41; http://flycrispr.molbio.wisc.edu/; http://www.flyrnai.org/crispr2/). After deciding the sgRNA target by using one of these online resources, it is important to sequence the fly stock before applying the CRISPR system, since single-nucleotide polymorphism may result in sequence differences between the stocks in hand and the online resource. If such a mismatch happens to be in the PAM or in the seed region of the sgRNA, the system may fail to produce results.

When relying on microinjection to deliver the CRISPR system, the same amount of RNA or DNA plasmids is preferred for each injection into fly embryos, no matter if a pump is used or not. Thus, injection solution concentration is an important factor. For *in vitro* transcription of *Cas9* gene and sgRNAs, the injection concentration of both transcripts should be considered. Some empirical data are available (Bassett et al., 2013) but, depending on the quality of RNA preparation, in-house trials seem unavoidable. For *Cas9* transgenic flies, the level of Cas9 is determined by the design of the Cas9 transgene, and thus the appropriate sgRNA injection concentration can be determined experimentally. In our study, a higher concentration of sgRNA vectors does not necessarily mean a higher DSB rate (Ren et al., 2014b). We believe a similar pattern of mutagenesis efficiency associated with sgRNA concentration exists with other Cas9 transgenic stocks, as they were constructed in similar ways while under different promoters.

OFF-TARGET ISSUES

Because of the small genome size of *Drosophila* and the fact that backcrosses can get rid of unwanted mutations, off-target effects are generally not a major concern, especially when generating heritable alleles. In addition, off-target mutations have not been detected at *Drosophila* genomic regions with three or more mismatches to a pool of sgRNAs, no matter whether regular or bulged (Bassett et al., 2013; Gratz et al., 2013; Kondo and Ueda, 2013; Ren et al., 2013, 2014b; Yu et al., 2013). Although the size of the pool of sgRNAs is limited, these results show that the possibility of off-target effects in *Drosophila* is low. If avoiding off-target DSBs is of great importance to your experimental needs, the application of 18 nt sgRNAs (described above) can be considered. Furthermore, Cas9 nickase that has either one of the nuclease domains mutated has been shown to be able to edit genes in cell lines and in *Drosophila* with almost no off-target effects (Port et al., 2014; Ren et al., 2014a). Cas9 nickase transgenic flies are available, but applications of the Cas9 nickase in generating DSBs in *Drosophila* might be limited since it requires two neighboring offset sgRNAs within a narrow range of distance (Ren et al., 2014a). However, the potential of Cas9 nickase in DNA nick-triggered HDR has not been fully investigated.

Off-target effects pose a bigger concern in the direct observation of mutant phenotypes in somatic tissues, as mutations are evaluated directly and whether the observed phenotypes are due to the desired mutations or to unknown off-target mutations cannot be distinguished clearly. Here, strategies that have been introduced in transgenic RNAi research can be applied (Mohr et al., 2014), such as using multiple sgRNAs to confirm a phenotype or using a CRISPR-insensitive construct to perform the rescue assay. In addition, the efficiency and specificity of 18 nt sgRNAs might be sufficient to be applied in this method.

With more and more labs starting to apply the CRISPR technology for mutagenesis, we expect any systematic off-target events in *Drosophila* will soon be noticed, if there are any. Some unexpected off-target mutations have been observed in cell-line studies (Tsai et al., 2015). However, with a smaller genome and outcrosses after mutagenesis events, the chance of such off-target mutations in the new allele stock is low.

PERSPECTIVES

In order to apply the CRISPR system to generate heritable mutations in *Drosophila*, we expect to see only limited new developments in the methods of Cas9 delivery and exploitation of different versions of Cas9, as the current *S. pyogenes* version seems sufficient in terms of efficiency and specificity. However, a Cas9 that requires a different PAM sequence might be helpful to complement the current version (Cong et al., 2013; Hou et al., 2013). On the other hand, modifications to sgRNA parameters such as delivery method, promoter, scaffold, targeting length, and GC content have been investigated in quite a few studies in *Drosophila*, and might still demand additional research.

In light of the recent development of CRISPR activator/inhibitor systems in cell lines with a modified sgRNA scaffold and corresponding RNA binding transcription activator/inhibitor (Konermann et al., 2014), we expect to see similar methods in *Drosophila*. This method, if successfully developed, will be a great complement to the CRISPR knock-out method to allow the direct observation of phenotypes. Ideally, an sgRNA transgene stock would be used for CRISPR activator, CRISPR inhibitor, and CRISPR-based mutagenesis assays. However, these different applications require sgRNAs targeting multiple regions of a gene. Thus, it is unlikely that a single sgRNA will be able to perform all of these separate functions. We will need a comprehensive understanding of the efficiency and specificity of this method in *Drosophila* to decide whether big undertakings can be carried out.

We expect to see whole-organism and genome-wide applications of the CRISPR-based gene-editing technologies in research involving non-model organisms, as well as in model organisms where vast amounts of knowledge and tools are already available. However, even in the mature model organism *D. melanogaster* more than half of the gene functions are still unknown due to the lack of well-defined mutant alleles. Mutations generated from the CRISPR systems are expected to fill this gap in the near future. Combination of the powerful CRISPR technologies with the existing genetic tools in *Drosophila* will also allow the control of gene expression at unprecedented levels, which will open up new possibilities in genetics, developmental biology, and evolutionary biology. For example, the aforedescribed conditional knockout alleles can allow the temporal and spatial control of gene expression when crossed to existing driver stocks, and artificial exons can be introduced into genes for tagging or other gene manipulations (Wangler et al., 2015). Great opportunities and challenges await us in this exciting field.

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REFERENCES

Auer, T.O., Duroure, K., De Cian, A., Concordet, J.-P., Del Bene, F., 2014. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. Genome Res. 24, 142−153.

Baena-Lopez, L.A., Alexandre, C., Mitchell, A., Pasakarnis, L., Vincent, J.-P., 2013. Accelerated homologous recombination and subsequent genome modification in *Drosophila*. Development 140, 4818−4825.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., Horvath, P., 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315, 1709−1712.

Barrangou, R., Marraffini, L.A., 2014. CRISPR-Cas systems: prokaryotes upgrade to adaptive immunity. Mol. Cell 54, 234−244.

Bassett, A.R., Liu, J.-L., 2014. CRISPR/Cas9 and genome editing in *Drosophila*. J. Genet. Genomics 41, 7−19.

Bassett, A.R., Tibbit, C., Ponting, C.P., Liu, J.-L., 2013. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. Cell Rep. 4, 220−228.

Beumer, K.J.K., Trautman, J.K.J., Mukherjee, K., Carroll, D., 2013. Donor DNA utilization during gene targeting with zinc-finger nucleases. G3 (Bethesda) 3, 657−664.

Bolotin, A., Quinquis, B., Sorokin, A., Ehrlich, S.D., 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 151, 2551−2561.

Bondy-Denomy, J., Davidson, A.R., 2014. To acquire or resist: the complex biological effects of CRISPR-Cas systems. Trends Microbiol. 22, 218−225.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401−415.

Brouns, S.J.J., Jore, M.M., Lundgren, M., Westra, E.R., Slijkhuis, R.J.H., Snijders, A.P.L., Dickman, M.J., Makarova, K.S., Koonin, E.V., van der Oost, J., 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 321, 960−964.

Chen, B., Gilbert, L.A., Cimini, B.A., Schnitzbauer, J., Zhang, W., Li, G.-W., Park, J., Blackburn, E.H., Weissman, J.S., Qi, L.S., Huang, B., 2013. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. Cell 155, 1479−1491.

Chen, H.-M., Huang, Y., Pfeiffer, B.D., Yao, X., Lee, T., 2015. An enhanced gene targeting toolkit for *Drosophila*: Golic+. Genetics 199, 683−694.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., Zhang, F., 2013. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819−823.

Deveau, H., Garneau, J.E., Moineau, S., 2010. CRISPR/Cas system and its role in phage-bacteria interactions. Annu. Rev. Microbiol. 64, 475−493.

Dickinson, D.J., Ward, J.D., Reiner, D.J., Goldstein, B., 2013. Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. Nat. Methods 10, 1028−1034.

Friedland, A.E., Tzur, Y.B., Esvelt, K.M., Colaiácovo, M.P., Church, G.M., Calarco, J.A., 2013. Heritable genome editing in *C. elegans via* a CRISPR-Cas9 system. Nat. Methods 10, 741−743.

Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., Sander, J.D., 2013. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat. Biotechnol. 31, 822−826.

Fu, Y., Sander, J.D., Reyon, D., Cascio, V.M., Joung, J.K., 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat. Biotechnol. 32, 279−284.

Gasiunas, G., Barrangou, R., Horvath, P., Siksnys, V., 2012. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc. Natl. Acad. Sci. USA 109, E2579−E2586.

Gokcezade, J., Sienski, G., Duchek, P., 2014. Efficient CRISPR/Cas9 plasmids for rapid and versatile genome editing in *Drosophila*. G3 (Bethesda) 4, 2279−2282.

Gratz, S.J., Cummings, A.M., Nguyen, J.N., Hamm, D.C., Donohue, L.K., Harrison, M.M., Wildonger, J., O'Connor-Giles, K.M., 2013. Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. Genetics 194, 1029−1035.

Gratz, S.J., Ukken, F.P., Rubinstein, C.D., Thiede, G., Donohue, L.K., Cummings, A.M., O'Connor-Giles, K.M., 2014. Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. Genetics 196, 961−971.

Haft, D.H., Selengut, J., Mongodin, E.F., Nelson, K.E., 2005. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLoS Comput. Biol. 1, e60.

Hale, C.R., Zhao, P., Olson, S., Duff, M.O., Graveley, B.R., Wells, L., Terns, R.M., Terns, M.P., 2009. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell 139, 945−956.

Hatoum-Aslan, A., Maniv, I., Marraffini, L.A., 2011. Mature clustered, regularly interspaced, short palindromic repeats RNA (crRNA) length is measured by a ruler mechanism anchored at the precursor processing site. Proc. Natl. Acad. Sci. USA 108, 21218−21222.

Haurwitz, R.E., Jinek, M., Wiedenheft, B., Zhou, K., Doudna, J.A., 2010. Sequence- and structure-specific RNA processing by a CRISPR endonuclease. Science 329, 1355−1358.

Horvath, P., Romero, D.A., Coûté-Monvoisin, A.-C., Richards, M., Deveau, H., Moineau, S., Boyaval, P., Fremaux, C., Barrangou, R., 2008. Diversity, activity, and evolution of CRISPR loci in *Streptococcus thermophilus*. J. Bacteriol. 190, 1401−1412.

Hou, Z., Zhang, Y., Propson, N.E., Howden, S.E., Chu, L., Sontheimer, E.J., Thomson, J.A., 2013. Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. Proc. Natl. Acad. Sci. USA 110, 15644−15649.

Housden, B.E., Lin, S., Perrimon, N., 2014. Cas9-Based Genome Editing in *Drosophila*. In: first ed. Methods in Enzymology. Elsevier Inc.

Hruscha, A., Krawitz, P., Rechenberg, A., Heinrich, V., Hecht, J., Haass, C., Schmid, B., 2013. Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. Development 140, 4982−4987.

Hsu, P.D., Lander, E.S., Zhang, F., 2014. Development and applications of CRISPR-Cas9 for genome engineering. Cell 157, 1262−1278.

Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., Cradick, T.J., Marraffini, L.A., Bao, G., Zhang, F., 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. 31, 827−832.

Hwang, W.Y., Fu, Y., Reyon, D., Maeder, M.L., Tsai, S.Q., Sander, J.D., Peterson, R.T., Yeh, J.-R.J., Joung, J.K., 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. Nat. Biotechnol. 31, 227−229.

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., Nakata, A., 1987. Nucleotide sequence of the *iap* gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. J. Bacteriol. 169, 5429−5433.

Jansen, R., Embden, J.D., Gaastra, W., Schouls, L.M., 2002. Identification of genes that are associated with DNA repeats in prokaryotes. Mol. Microbiol. 43, 1565−1575.

Jiang, W., Bikard, D., Cox, D., Zhang, F., Marraffini, L.A., 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat. Biotechnol. 31, 233−239.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816−821.

Jinek, M., East, A., Cheng, A., Lin, S., Ma, E., Doudna, J., 2013. RNA-programmed genome editing in human cells. eLife 2, e00471.

Kim, H., Ishidate, T., Ghanta, K.S., Seth, M., Conte, D., Shirayama, M., Mello, C.C., 2014. A co-CRISPR strategy for efficient genome editing in *Caenorhabditis elegans*. Genetics 197, 1069−1080.

Kondo, S., Ueda, R., 2013. Highly improved gene targeting by germline-specific *Cas9* expression in *Drosophila*. Genetics 195, 715−721.

Konermann, S., Brigham, M.D., Trevino, A.E., Joung, J., Abudayyeh, O.O., Barcena, C., Hsu, P.D., Habib, N., Gootenberg, J.S., Nishimasu, H., Nureki, O., Zhang, F., 2014. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 517, 583−588.

Kuscu, C., Arslan, S., Singh, R., Thorpe, J., Adli, M., 2014. Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. Nat. Biotechnol. 32, 677−683.

Li, M., Wang, R., Xiang, H., 2014. *Haloarcula hispanica* CRISPR authenticates PAM of a target sequence to prime discriminative adaptation. Nucleic Acids Res. 42, 7226−7235.

Lin, S.-C., Chang, Y.-Y., Chan, C.-C., 2014. Strategies for gene disruption in *Drosophila*. Cell Biosci. 4, 63.

Liu, D., Wang, Z., Xiao, A., Zhang, Y., Li, W., Zu, Y., Yao, S., Lin, S., Zhang, B., 2014. Efficient gene targeting in zebrafish mediated by a zebrafish-codon-optimized *Cas9* and evaluation of off-targeting effect. J. Genet. Genomics 41, 43−46.

Maeder, M.L., Linder, S.J., Cascio, V.M., Fu, Y., Ho, Q.H., Joung, J.K., 2013. CRISPR RNA-guided activation of endogenous human genes. Nat. Methods 10, 977−979.

Makarova, K.S., Aravind, L., Wolf, Y.I., Koonin, E.V., 2011a. Unification of Cas protein families and a simple scenario for the origin and evolution of CRISPR-Cas systems. Biol. Direct 6, 38.

Makarova, K.S., Grishin, N.V., Shabalina, S.A., Wolf, Y.I., Koonin, E.V., 2006. A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biol. Direct 1, 7.

Makarova, K.S., Haft, D.H., Barrangou, R., Brouns, S.J.J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F.J.M., Wolf, Y.I., Yakunin, A.F., van der Oost, J., Koonin, E.V., 2011b. Evolution and classification of the CRISPR-Cas systems. Nat. Rev. Microbiol. 9, 467−477.

Mali, P., Aach, J., Stranges, P.B., Esvelt, K.M., Moosburner, M., Kosuri, S., Yang, L., Church, G.M., 2013a. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat. Biotechnol. 31, 833−838.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., Church, G.M., 2013b. RNA-guided human genome engineering *via* Cas9. Science 339, 823−826.

Marraffini, L.A., Sontheimer, E.J., 2008. CRISPR interference limits horizontal gene transfer in *staphylococci* by targeting DNA. Science 322, 1843−1845.

Mohr, S.E., Smith, J.A., Shamu, C.E., Neumüller, R.A., Perrimon, N., 2014. RNAi screening comes of age: improved techniques and complementary approaches. Nat. Rev. Mol. Cell Biol. 15, 591−600.

Mojica, F.J., Díez-Villaseñor, C., Soria, E., Juez, G., 2000. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. Mol. Microbiol. 36, 244−246.

Mojica, F.J.M., Díez-Villaseñor, C., García-Martínez, J., Soria, E., 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J. Mol. Evol. 60, 174−182.

Nam, K.H., Haitjema, C., Liu, X., Ding, F., Wang, H., DeLisa, M.P., Ke, A., 2012. Cas5d protein processes pre-crRNA and assembles into a cascade-like interference complex in subtype I-C/Dvulg CRISPR-Cas system. Structure 20, 1574−1584.

Ni, J.-Q., Zhou, R., Czech, B., Liu, L.-P., Holderbaum, L., Yang-Zhou, D., Shim, H.-S., Tao, R., Handler, D., Karpowicz, P., Binari, R., Booker, M., Brennecke, J., Perkins, L.A., Hannon, G.J., Perrimon, N., 2011. A genome-scale shRNA resource for transgenic RNAi in *Drosophila*. Nat. Methods 8, 405−407.

Pattanayak, V., Lin, S., Guilinger, J.P., Ma, E., Doudna, J.A., Liu, D.R., 2013. High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. Nat. Biotechnol. 31, 839−843.

Perez-Pinera, P., Kocak, D.D., Vockley, C.M., Adler, A.F., Kabadi, A.M., Polstein, L.R., Thakore, P.I., Glass, K.A., Ousterout, D.G., Leong, K.W., Guilak, F., Crawford, G.E., Reddy, T.E., Gersbach, C.A., 2013. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nat. Methods 10, 973−976.

Port, F., Chen, H.-M., Lee, T., Bullock, S.L., 2014. Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila*. Proc. Natl. Acad. Sci. USA 111, E2967−E2976.

Pourcel, C., Salvignol, G., Vergnaud, G., 2005. CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. Microbiology 151, 653−663.

Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., Lim, W.A., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152, 1173−1183.

Ren, X., Sun, J., Housden, B.E., Hu, Y., Roesel, C., Lin, S., Liu, L.-P., Yang, Z., Mao, D., Sun, L., Wu, Q., Ji, J.-Y., Xi, J., Mohr, S.E., Xu, J., Perrimon, N., Ni, J.-Q., 2013. Optimized gene editing technology for *Drosophila melanogaster* using germ line-specific Cas9. Proc. Natl. Acad. Sci. USA 110, 19012−19017.

Ren, X., Yang, Z., Mao, D., Chang, Z., Qiao, H.-H., Wang, X., Sun, J., Hu, Q., Cui, Y., Liu, L.-P., Ji, J.-Y., Xu, J., Ni, J.-Q., 2014a. Performance of the Cas9 nickase system in *Drosophila melanogaster*. G3 (Bethesda) 4, 1955−1962.

Ren, X., Yang, Z., Xu, J., Sun, J., Mao, D., Hu, Y., Yang, S.-J., Qiao, H.-H., Wang, X., Hu, Q., Deng, P., Liu, L.-P., Ji, J.-Y., Li, J.B., Ni, J.-Q., 2014b. Enhanced specificity and efficiency of the CRISPR/Cas9 system with optimized sgRNA parameters in *Drosophila*. Cell Rep. 9, 1151−1162.

Rouillon, C., Zhou, M., Zhang, J., Politis, A., Beilsten-Edmands, V., Cannone, G., Graham, S., Robinson, C.V., Spagnolo, L., White, M.F., 2013. Structure of the CRISPR interference complex CSM reveals key similarities with cascade. Mol. Cell 52, 124−134.

Sanjana, N.E., Shalem, O., Zhang, F., 2014. Improved vectors and genome-wide libraries for CRISPR screening. Nat. Methods 11, 783−784.

Sebo, Z.L., Lee, H.B., Peng, Y., Guo, Y., 2014. A simplified and efficient germline-specific CRISPR/Cas9 system for *Drosophila* genomic engineering. Fly (Austin) 8, 52−57.

Shah, S.A., Erdmann, S., Mojica, F.J.M., Garrett, R.A., 2013. Protospacer recognition motifs: mixed identities and functional diversity. RNA Biol. 10, 891−899.

Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelsen, T.S., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G., Zhang, F., 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 343, 84−87.

Stern, D.L., 1998. A role of Ultrabithorax in morphological differences between *Drosophila* species. Nature 396, 463−466.

Sung, Y.H., Kim, J.M., Kim, H.-T., Lee, J., Jeon, J., Jin, Y., Choi, J.-H., Ban, Y.H., Ha, S.-J., Kim, C.-H., Lee, H.-W., Kim, J.-S., 2014. Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases. Genome Res. 24, 125−131.

Tang, T.-H., Bachellerie, J.-P., Rozhdestvensky, T., Bortolin, M.-L., Huber, H., Drungowski, M., Elge, T., Brosius, J., Hüttenhofer, A., 2002. Identification of 86 candidates for small non-messenger RNAs from the archaeon Archaeoglobus fulgidus. Proc. Natl. Acad. Sci. USA 99, 7536−7541.

Tsai, S.Q., Zheng, Z., Nguyen, N.T., Liebers, M., Topkar, V.V., Thapar, V., Wyvekens, N., Khayter, C., Iafrate, A.J., Le, L.P., Aryee, M.J., Joung, J.K., 2015. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat. Biotechnol. 33, 187−197.

Turner, T.L., 2014. Fine-mapping natural alleles: quantitative complementation to the rescue. Mol. Ecol. 23, 2377−2382.

van der Oost, J., Westra, E.R., Jackson, R.N., Wiedenheft, B., 2014. Unravelling the structural and mechanistic basis of CRISPR-Cas systems. Nat. Rev. Microbiol. 12, 479−492.

Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., Jaenisch, R., 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153, 910−918.

Wang, T., Wei, J.J., Sabatini, D.M., Lander, E.S., 2014. Genetic screens in human cells using the CRISPR-Cas9 system. Science 343, 80−84.

Wangler, M.F., Yamamoto, S., Bellen, H.J., 2015. Fruit flies in biomedical research. Genetics 199, 639−653.

Wei, C., Liu, J., Yu, Z., Zhang, B., Gao, G., Jiao, R., 2013. TALEN or Cas9-rapid, efficient and specific choices for genome modifications. J. Genet. Genomics 40, 281−289.

Wu, X., Scott, D.A., Kriz, A.J., Chiu, A.C., Hsu, P.D., Dadon, D.B., Cheng, A.W., Trevino, A.E., Konermann, S., Chen, S., Jaenisch, R., Zhang, F., Sharp, P.A., 2014. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. Nat. Biotechnol. 32, 670−676.

Xue, Z., Ren, M., Wu, M., Dai, J., Rong, Y.S., Gao, G., 2014a. Efficient gene knock-out and knock-in with transgenic Cas9 in *Drosophila*. G3 (Bethesda) 4, 925−929.

Xue, Z., Wu, M., Wen, K., Ren, M., Long, L., Zhang, X., Gao, G., 2014b. CRISPR/Cas9 mediates efficient conditional mutagenesis in *Drosophila*. G3 (Bethesda) 4, 2167−2173.

Yang, H., Wang, H., Shivalila, C.S., Cheng, A.W., Shi, L., Jaenisch, R., 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154, 1370−1379.

Yin, H., Xue, W., Chen, S., Bogorad, R.L., Benedetti, E., Grompe, M., Koteliansky, V., Sharp, P.A., Jacks, T., Anderson, D.G., 2014. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. Nat. Biotechnol. 32, 551−553.

Yu, Z., Ren, M., Wang, Z., Zhang, B., Rong, Y.S., Jiao, R., Gao, G., 2013. Highly efficient genome modifications mediated by CRISPR/Cas9 in *Drosophila*. Genetics 195, 289−291.

Zhang, X., Koolhaas, W.H., Schnorrer, F., 2014. A versatile two-step CRISPR- and RMCE-based strategy for efficient genome engineering in *Drosophila*. G3 (Bethesda) 4, 2409−2418.