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Genome editing in *Drosophila melanogaster*: from basic genome engineering to the multipurpose CRISPR-Cas9 system

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Nowadays, genome editing tools are indispensable for studying gene function in order to increase our knowledge of biochemical processes and disease mechanisms. The extensive availability of mutagenesis and transgenesis tools make *Drosophila melanogaster* an excellent model organism for geneticists. Early mutagenesis tools relied on chemical or physical methods, ethyl methane sulfonate (EMS) and X-rays respectively, to randomly alter DNA at a nucleotide or chromosomal level. Since the discovery of transposable elements and the availability of the complete fly genome, specific genome editing tools, such as P-elements, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have undergone rapid development. Currently, one of the leading and most effective contemporary tools is the CRISPR-cas9 system made popular because of its low cost, effectiveness, specificity and simplicity of use. This review briefly addresses the most commonly used mutagenesis and transgenesis tools in *Drosophila*, followed by an in-depth review of the multipurpose CRISPR-Cas9 system and its current applications.

genome editing, *Drosophila melanogaster*, mutagenesis, transgenesis, CRISPR-Cas9

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INTRODUCTION

Since the early days of genome editing, an astonishing set of tools has made *Drosophila melanogaster* a popular model organism for studying gene function (Figure 1). Even before the complete *Drosophila* genome became available, various tools were available for mutagenesis. The early mutagenesis tools relied on chemical or physical methods, ethyl methane sulfonate (EMS) and X-rays respectively, to randomly alter DNA at the nucleotide or chromosomal level (Eeken et al., 1994; Lewis and Bacher, 1968; Mahmoud et al., 1991; Muller, 1927). Since the discovery of transposons, more particularly the P-element, in 1977, other techniques like the FLP/FRT and GAL4-UAS systems quickly emerged (Brand and Dormand, 1995; Engels, 1992; Golic et al., 1997). In the beginning, these systems were mainly used to perform large-scale mutagenesis, obtaining a collection of phenotypes to be further examined for possible roles in various pathways. When in 2000 the complete *Drosophila* genome was elucidated, and the first method for targeted mutagenesis by

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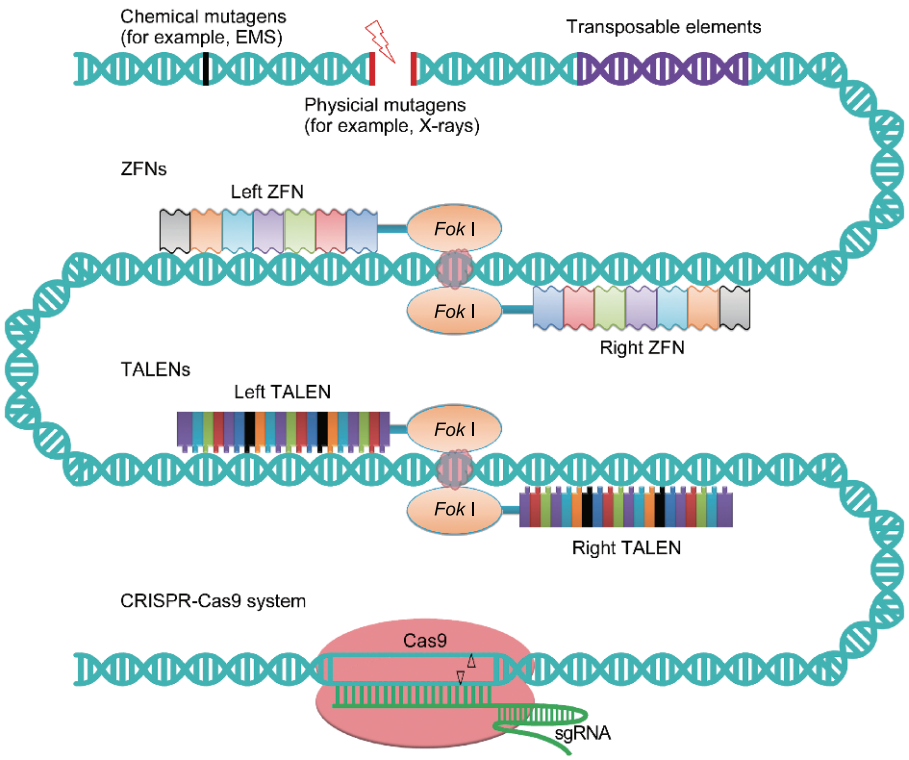

Figure 1 Overview of genome engineering methods in *Drosophila melanogaster* throughout history. The *Drosophila* genome engineering methods allow researchers to modify the genome in a random (chemical mutagens, physical mutagens, transposable elements) or specific fashion (ZFNs, TALENs, CRISPR-Cas9 system). Chemical mutagens, e.g. EMS, provokes single nucleotide transitions leading to random point mutations, whereas physical mutagens, e.g. X-rays, create DNA DSBs introducing inversions, deletions or duplications of genomic fragments. Transposable elements, e.g. P-elements, on the other hand, are mobile DNA fragments that can disrupt the genome by insertion or excision of the fragment at almost random locations. The second group of genome editing methods provides a more specific way of modification by using a designed nuclease-DNA targeting complex to introduce a DSB at a target sequence of interest. ZFN and TALEN, both use the *Fok* I-nuclease which upon dimerization facilitates a DSB. The specificity is defined by the DNA binding domains of the complex, which for ZFNs and TALENs interacts with 3 and 1 bp per module, respectively. The CRISPR-Cas9 system uses the Cas9 nuclease instead of *Fok* I to target specific genomic loci. The Cas9 nuclease is directed to its target sequence by binding of a sgRNA, which has a 20 nucleotide DNA binding sequence at its 5′ end.

homologous recombination (HR) was published, the researchers' toolbox for specific genome engineering increased tremendously (Adams et al., 2000; Rong and Golic, 2000). The first transgenesis methods relied on the introduction, at a target site via HR, of a homologous donor DNA containing the gene of interest or mutations, with resulting low efficiency and biased specificity (Capecchi, 2005). Despite efforts to cope with specificity and efficacy, the techniques reached their limitations. A major break-through occurred when it was realized that, by introducing double strand breaks (DSBs) and thus activating the DNA repair mechanism, the effectiveness of HR could be increased dramatically. From that moment on, new approaches such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and, later on, CRISPR-Cas9 were developed, all based on their ability to induce a DSB at a target site of interest (Beumer et al., 2008; Gratz et al., 2013; Katsuyama et al., 2013). These systems guide a nuclease to a specific site where they induce a DSB, activating the DNA repair mechanism and leading to homology directed repair (HDR) or non-homologous end joining (NHEJ) (Takata et al., 1998).

The purpose of this review paper is to introduce the *Drosophila* scientist to some of the most important genomeediting tools available for mutagenesis and transgenesis. As novel tools are developing at a rapid pace, various new tools have increased in prevalence, due to their low cost, effectiveness, specificity and simplicity of use. One of the leading and most effective contemporary tools is the CRISPR-Cas9 system. Since new applications for this system are rapidly being discovered, we will discuss this technique in-depth.

RANDOM GENOME EDITING: FORWARD GENETIC SCREENS BY CHEMICAL, PHYSICAL OR TRANSPOSON MUTAGENESIS

One way to identify novel players in biological processes of interest is by performing a forward genetic screen. This approach allows for the discovery of genes causing a particular complex phenotype, because a phenotype is often the result of multiple genetic influences (Ashburner, 1989). These screens can lead to the discovery of key players in particular

biological processes, giving insight into disease mechanisms. The generation of various phenotypes in an unbiased way can be achieved by using chemical or physical mutagens, or in a more biased way via transposon mutagenesis (St Johnston, 2002).

Chemical mutagenesis

The most commonly used chemical mutagen in *Drosophila* is EMS, which functions as an alkylating agent, introducing random point mutations throughout the entire genome (Figure 1). This mutagen acts through nucleotide substitution by primarily attacking guanine, mostly resulting in the loss or gain of gene function (Bökel, 2008). In flies, EMS gained popularity because it is inexpensive, effective and simple to use, as it can simply be applied through feeding (Lewis and Bacher, 1968). By using the standard feeding protocol, treatment with 25 mmol L^{-1} EMS, an average gene mutation rate of about 1 out of 1,000 nucleotides can be obtained. This rate is dependent upon the gene coding region size and amount of essential amino acids required for function (Greenspan, 2004). Due to the rather unpredictable and random occurrence of point mutations, a 20-fold higher mutation rate can sometimes be attained in larger genes like *dumpy* (*dp*) (St Johnston, 2002). Despite many advantages, EMS lost popularity because of the difficulty and labor needed to map EMS-induced point mutations to a specific gene responsible for the phenotype of interest. Nowadays, this problem has been largely overcome by the development of single nucleotide polymorphisms (SNP) maps, which allow for the quick meiotic mapping of mutations (Berger et al., 2001; Martin et al., 2001). Moreover, the progeny of EMS-mutagenized males is frequently mosaic, in which only some of the cells carry the mutation. This can be observed due to the segregation of a point mutation in one strand from the unmutated strand during the first zygotic division (when the mismatch is not repaired). Thus, unless the germline cells are mutant, the mutations identified in the first-generation screens will not be passed to the next generation (St Johnston, 2002). Some recent studies have applied EMS to characterize gene defects in diseases, e.g. Parkinson's disease (PD). A study by Dodson et al. in 2014 showed that EMS-induced null alleles of the *Drosophila LRRK2* homolog, a commonly known genetic determinant of PD, revealed its crucial role in endolysosomal function and autophagy *in vivo* (Dodson et al., 2014). Besides EMS, several popular chemical mutagens in other organisms, such as hexamethylphosphoramide (HMPA), which introduces small deletions via cross-linking, or N-ethyl-N-nitrosourea (ENU), which induces point mutations and AT-GC transitions, are not favored in *Drosophila* owing to their high toxicity (Ashburner, 1989; Nairz et al., 2004).

Physical mutagenesis

In 1927, Muller was the first to discover that physical mutagenesis by X-rays also induces genetic alterations in *Drosophila*, similar to chemical mutagens (Muller, 1927). This discovery led to the use of physical mutagens, such as UV-, X- and gamma-rays, to perform forward genetic screens (Figure 1) (Eeken et al., 1994; Mahmoud et al., 1991). DNA can absorb the photon energy of UV light, causing neighboring thymine bases to link into pyrimidine dimers, and creating strand disruptions (Pfeifer et al., 2005). X- and gamma-rays, on the other hand, are ionizing mutagens that induce the formation of hydroxyl radicals, which are highly reactive and can cause DSBs. Instead of the small nucleotide changes induced by chemical mutagens, physical mutagenesis results in rather large genomic rearrangements including inversions, deletions and duplications (St Johnston, 2002). In contrast to chemical mutagenesis, these mutations can easily be detected cytologically in larval polytene chromosomes, allowing mutations to be mapped to a region and further identified on southern blots (Mglinets, 1973). Physical methods have the same advantages as their chemical counterparts, yet they are less popular due to the lower mutation rate and wide mutational window, that generally results in large rearrangements that can influence multiple genes (Antosh et al., 2014; Koana et al., 2007). In *Drosophila*, physical mutagens can overcome the possible disadvantages of chemical mutagens. As mentioned previously, EMS-mutagenized males often have mosaic mutant progeny, which means that the mutation is not transferred to the next generation. To address this problem, F_1 screens are often carried out using X-rays as a mutagen. Despite lower efficacy when compared to EMS, physical mutagens induce DSBs, removing the possibility of mosaic progeny (St Johnston, 2002).

Transposon mutagenesis

Transposable elements (TE), first discovered in maize by McClintock in 1950, are mobile DNA fragments that have the ability to translocate across the genome, laying the basis for various types of chromosomal rearrangement (Figure 1) (McClintock, 1950). TEs, also called transposons, were discovered in *Drosophila* during the study of a phenomenon called hybrid dysgenesis. This event often occurs in wild-type strains when so-called P-cytotype males are crossed to M-cytotype females (Cooley et al., 1988; Engels, 1996; Roberts, 1987). The resulting offspring exhibit a number of genetic defects, including mutations, chromosome breakages and even male recombination, with the latter never being found in wild-type male flies (Vazquez et al., 2002). This rather surprising event occurs because the P-cytotype strains uniquely carry a transposon called the P-element, which is constructed out of two 31-mer terminal inverted repeats and transposase, an enzyme needed to catalyze the transposition (Rio, 1990). Several features of P-elements make them useful for genetic manipulation. Some, such as

transposase, are limited to germline cells by tissue-specific splicing, and the frequency of mobilization can be controlled by the expression of the active transposase (Rio et al., 1986). Although the translocation of P-elements does not need a specific target sequence, it is clearly biased toward the 5′ regions of genes that lie within a 100 bp distance of the transcriptional start site. This makes P-element mutagenesis a less random method compared to both its chemical and physical counterparts (Bellen et al., 2011; Spradling et al., 2011). Nowadays, different transposon types can be used, such as piggyback, Minos or HoBo, which do not have a preference for a promoter region, resulting in a less biased method to perform a forward genetic screen (Hacker et al., 2003; Metaxakis et al., 2005). Besides the transposon backbone, the use of a dominant selection marker is important to identify and characterize the insertion. A number of different selection markers have been used successfully in *Drosophila*, including *rosy*, *vermillion*, *yellow*, *GFP* or *DsRed*; however, the most commonly used marker is *white*, resulting in a dosage sensitive red eye phenotype (Berghammer et al., 1999; Horn and Wimmer, 2000; Patton et al., 1992; Pirrotta, 1988; Venken and Bellen, 2007).

Insertional mutagenesis. To introduce a random insertion, a jump starter strain is used which carries multiple insertions on a balancer chromosome. By crossing these strains with the appropriate construct strain, such as P(white), about 1 out of 150 carries a new insertion. The rate of mutagenesis is dependent upon the sensitivity of the used marker, e.g. P(rosy) results in a new insertion for 1 out of 100 flies (Robertson et al., 1988). Once a P-element is inserted, it can be replaced by another one that allows expression of downstream genes, and can function as an enhancer detector strain driving the expression of a reporter gene such as GAL4 in a specific temporal and spatial pattern. This can be achieved because the cut-and-paste nature of a P-element leaves a double-stranded gap which then is repaired by sequence replacement from a homologous template. These transitions have a 1% efficiency, depending upon the location of the element (Gloor et al., 1991; Sepp and Auld, 1999). When a TE insertion in a gene of interest is not available, it is possible instead to mobilize a nearby P-element, a technique called “local hopping”. When the offspring of a cross between a P-element insertion strain and a stable transposase line are crossed with the appropriate balancer flies, a transposition takes place to integration sites within 0–150 kb. One major drawback is that local hopping mostly induces a deletion around the starting P-element (Zhang and Spradling, 1993).

Deletional mutagenesis. As mentioned above, when a P-element is excised from a genome, a DSB is generated, which can lead to degradation of the ends before repair. This event only occurs in approximately 1% of cases and can result in a deletion of genetic material in the range of several bp to kbp (Gloor et al., 1991; Sepp and Auld, 1999). Such an event can be detected when the offspring lacks the used selection marker, and can be characterized by using PCR or southern blotting. One strategy, called hybrid element insertion (HEI), relies on the mobilization of multiple P-elements at once, generating the possibility for the transposition process to pair different ends (Figure 2A). This event occurs preferentially when two P-elements are in trans on sister chromatids, removing everything in between the P-element regions (Gray et al., 1996). A second strategy is for P-elements to be inserted using a FRT/FLP-construct (Figure 2B). These FRT/FLP regions flanking the P-element can generate a deletion via FLP-recombinase, in the same fashion as the HEI system. The efficiency of both systems is reliant upon the distance between the P-elements that outline the deletion area (Golic, 1991; Golic and Lindquist, 1989). A third, and recent, system makes use of the hybrid P-element construct *P*[*wHy*], which contains a Hobo deletion element flanked by two se-

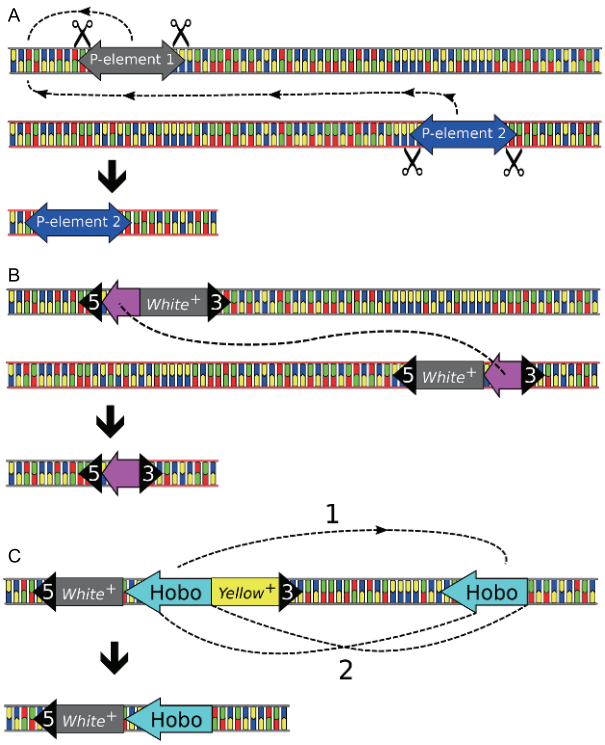

Figure 2 HEI, FRT/FLP and Hobo strategies for deletional mutagenesis with transposable elements. HEI is a strategy that allows for the possibility of the transposition process to pair different ends (A). This event occurs favorably when two P-elements are in trans on sister chromatids, removing everything between the P-element regions. P-elements inserted using a FRT-FLP-cassette, can generate a deletion by FLP-recombinase in a same fashion as the HEI system (B). By strategically using the *white*$^+$ marker gene, the mutant offspring can be easily selected when lacking the *white*$^+$ phenotype. The hybrid P-element construct *P[wHy]* contains a Hobo deletion element flanked by two selection markers, namely *white*$^+$ and *yellow*$^+$ (C). When the Hobo element is duplicated to another genomic region via local hopping (1), the region in-between can be deleted by homologous recombination of the Hobo fragment (2). Mutants can be easily selected because they only show the *white*$^+$ phenotype.

lection markers, namely *white* and *yellow* (Figure 2C). When this hybrid P-element is inserted together with a HoBo element at a chosen location upstream and in the same direction, all genes located in-between the HoBo elements can be removed by recombination. This leads to the loss of one or both marker genes making it easy to identify the deletion mutants. With this system, deletions of up to 400 kb can be generated (Mohr and Gelbart, 2002).

SPECIFIC GENOME ENGINEERING

DNA repair mechanism

A critical discovery that led to the exploitation of specific genome editing is that DSBs can stimulate the endogenous cellular machinery to repair the break (Figure 3). These DSBs are repaired by two major pathways, termed NHEJ and HDR (Takata et al., 1998). The NHEJ repair mechanism simply rejoins the ends without the need of a template, but as this is not a fail-safe process it frequently results in small insertions or deletions at the break site. These indels can inactivate genes as a result of frame shifts or by causing changes in the sequence coding for critical amino acids. Less frequently, a large deletion (>100 bp) or a large insertion can occur (Lieber et al., 2003). The second pathway, HDR, repairs the DSB in a template-dependent manner, which results in the seamless restoration of the original sequence. By providing homologous donor DNA, the researcher can introduce specific modifications, from a single nucleotide change to large insertions or deletions or even the introduction of genetic markers (Szostak et al., 1983). These endogenous cellular repair mechanisms are used by various genome engineering tools to create a wide variety of specific genomic alterations. Besides the techniques described below, physical mutagenesis (such as by X-rays) also induces DSBs, but because of its random nature, it is no longer used for this purpose.

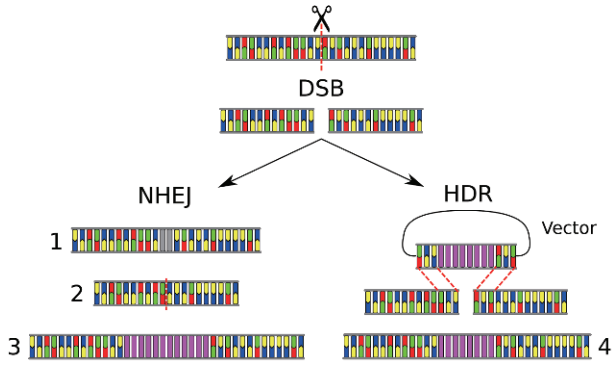

Figure 3 Endogenous cellular DNA repair mechanisms. Generation of a DSB leads to the stimulation of the NHEJ and HDR endogenous cellular repair machineries. NHEJ does not need a template and often results in (1) altered nucleotides at the breakage area, (2) small deletions or (3) inserts. HDR repairs the DSB using a homologous template, which when provided in a vector construct, can be used to induce (4) insertions or deletions at the breakage site.

Transposons

As described in the previous section, P-elements are extremely useful to induce, in a more biased way, random mutagenesis. These P-elements are also useful to induce mutations and large deletions, but are limited in terms of specificity as one cannot specify the alteration in a narrow window (Bellen et al., 2011; Spradling et al., 2011). Despite these limitations, P-elements were initially used to activate the HDR pathway to insert a transgene. This was made possible by constructing a P-element vector containing the gene of interest and a marker for selection, along with a separate vector containing the transposase protein. When both vectors are injected into a fly, the transposase makes a DSB which can insert the transgene into the desired location via the HDR repair mechanism (Spradling and Rubin, 1982). By using a different vector cassette containing FLP, the HDR efficiency can be increased (Golic et al., 1997). A huge drawback of this approach is that a P-element must already be present at the target location in order to use the repair mechanism in an efficient way. Due to these drawbacks, P-elements have been quickly replaced by other techniques that are more efficient and allow for the target location to be specified (Beumer et al., 2013). As using P-elements for precise and specific genome engineering is not favorable, this technique will not be discussed further.

Zinc-finger nucleases

In the early 1990s, Chandrasegaran discovered that *Fok* I, a type IIS restriction enzyme, could be separated by a protease into a DNA-binding and a DNA-cutting nuclease domain (Li et al., 1992). This discovery led to the possibility of creating a novel sequence specific nuclease by fusing the *Fok* I nuclease domain with a specific engineered DNA-binding domain. In 1996, Chandrasegaran was successful in creating such a hybrid nuclease, consisting of the non-specific *Fok* I nuclease domain and zinc finger proteins, which specifies the DNA target site (Figure 1) (Kim et al., 1996). It was the work of Carroll, with the injection of custom designed ZFNs in *Drosophila* embryos, that achieved genome editing in animals for the first time (Bibikova et al., 2003; Bibikova et al., 2002). Each of these ZFNs consists of three Cys_2His_2 zinc finger domains, with each domain interacting with a 3 bp sequence (Pavletich and Pabo, 1991). As ZFNs can be designed to attack a specific site, an 18 bp recognition site can be obtained which is specific enough even for large genomes. In order to cut DNA efficiently, the two cleavage domains must dimerize, and this requires the ZFN-binding domains to be in close proximity to the intended target (Bitinaite et al., 1998; Kim et al., 1996; Smith et al., 2000). As the dimer interface is weak, dimerization does not occur efficiently, leading to

undesired effects such as off-target cleavage at related sites and resultant cytotoxicity. To reduce this possibility, ZFNs are now constructed with *Fok* I variants, that provide for obligate heterodimerization, thus improving specificity (Doyon et al., 2011). Despite considerable additional efforts, genome editing with ZFNs has a rather low cleavage efficiency and there is a high cost to producing reliable and highly specific ZFNs (Beumer et al., 2013; Bhakta et al., 2013; Gupta et al., 2012; Miller et al., 2007; Sander et al., 2011; Szczepek et al., 2007). Nonetheless, ZFNs provide a significant improvement in genome editing when compared to P-elements.

Transcription activator-like effector nucleases

A major step forward in overcoming the problem of cytotoxicity resulted when the groups of Bogdanove and Bonas determined, in the bacterial plant pathogen *Xanthomonas*, the code for a *Xanthomonas*-derived transcription activator-like effector (TALE) (Boch et al., 2009; Moscou and Bogdanove, 2009). These TALEs are injected by bacteria into the host plant, where they recognize DNA targets and activate gene expression for proliferation and thus spreading of the pathogen (Boch and Bonas, 2010). TALEs have a typical tandem array of 15.5 to 19.5 highly conserved repeats, with only two residues, the so-called repeat-variable di-residues (RVDs), that target specific DNA sequences (Boch et al., 2009; Moscou and Bogdanove, 2009). Unlike ZFNs, each DNA targeting domain in a TALE recognizes a single base, improving the specificity and reducing the toxicity of ZFNs (Miller et al., 2011). When these TALEs are fused to the *Fok* I nuclease domain, TALEN, a new chimeric nuclease is created with a high specificity for cleaving a particular target sequence (Figure 1). Both NHEJ and HDR are then used to generate the desired altered genome. In addition, two TALENs can be used simultaneously to create larger deletions, with each TALEN defining the start and endpoint of the deletion (Chen and Gao, 2013; Liu et al., 2016). One drawback of this system is that the high similarity between the multiple repeats necessitated the creation of a unique cloning system. Another drawback is that each TALEN monomer must be engineered individually, making it expensive, especially when only a few TALENs are needed. Furthermore, it is not favorable to use multiple TALENs at the same time to create simultaneous genome modifications. Regardless, TALEN is still a commonly used method for specific genome engineering due to its specificity, efficiency, and minimal toxicity, especially when compared to ZFNs (Beumer et al., 2013; Katsuyama et al., 2013; Liu et al., 2012). However, the development of the CRISPR-Cas9 system has surpassed TALEN because it is a rapid and easy method with a low cost, and can be used to introduce a large variety and multitude of modifications at one time (Bassett et al., 2013). Therefore, an in-depth overview of the versatility of the CRISPR-Cas9 system is addressed in the next section.

THE ALL-IN-ONE CRISPR-CAS9 SYSTEM

Clustered regularly interspaced short palindromic repeats, known as CRISPR, is a natural bacterial defense mechanism used to prevent invading viruses and plasmids, and was first discovered as unknown strange repeats in bacterial genomes (Barrangou et al., 2007; Bolotin et al., 2005; Ishino et al., 1987; Jansen et al., 2002; Mojica et al., 2005; Mojica et al., 2000; Pourcel et al., 2005). Later, it was shown in *Streptococcus pyogenes* that an endonuclease called Cas9, or CRISPR-associated protein 9 can target invading pathogens in a sequence specific manner. This specificity is conferred on the endonuclease by crRNA or CRISPR RNA, which targets a 20 nt complementary sequence in the pathogen DNA. crRNA firstly forms a complex with tracrRNA, or trans-activating crRNA, to ensure the incorporation into the Cas9 endonuclease (Brouns et al., 2008; Gasiunas et al., 2012; Jinek et al., 2012). To simplify the system, the crRNA and tracrRNA have been fused into an approximately 100 nt synthetic single guide RNA (sgRNA) (Figure 1) (Cong et al., 2013; Dahlem et al., 2012; Mali et al., 2013a). Regarding sequence limitation, a protospacer-adjacent motif (PAM) must be located 3′ of the crRNA target sequence. The PAM sequence depends upon the species of Cas9, and in the case of the *Streptococcus pyogenes* Cas9 it is 5′-NGG-3′ (Chylinski et al., 2014; Fonfara et al., 2014; Sternberg et al., 2014). Different Cas9 variants with different PAMs can be engineered, expanding the possible target sequences (Kleinstiver et al., 2015a; Kleinstiver et al., 2015b). Upon its formation, the sgRNA-Cas9 complex undergoes a conformational change making association with the PAM sequence possible. Next, the upstream sequence is checked for complementarity with the crRNA, which, upon matching, then forms a DNA-RNA-hetero-duplex, leading to the cleavage of the target DNA by Cas9 (Anders et al., 2014; Jinek et al., 2014; Josephs et al., 2015; Nishimasu et al., 2014; Sternberg et al., 2014). Since these developments, the CRISPR-Cas9 system has emerged as a powerful tool for genome editing in various organisms, including mammals and *Drosophila* (Chen et al., 2017; Cong et al., 2013; Friedland et al., 2013; Hruscha et al., 2013; Mali et al., 2013b; Ren et al., 2017; Ren et al., 2013; Shen et al., 2017; Wang et al., 2013). Many research groups have applied the system for genome editing in *Drosophila*. Gratz and colleagues from the University of Wisconsin were the first to report that Cas9 can be used to induce germline transmitted genomic modifications. They injected Cas9 and sgRNA plasmids into *Drosophila* preplastoderm embryos to target the *yellow* marker gene, located on the X-chromosome. This resulted in the loss of the *yellow* phenotype, proving that the CRISPR-Cas9 system induces DSBs, and thus could be a suitable tool to specifically target the fly's genomic DNA. Although high specificity was achieved, the overall germline transmission efficiency was low, being 0.25%–1.37% (Gratz

et al., 2013). One month later, one group from the University of Oxford, and another joint group from the Chinese Academy of Sciences and Tsinghua University, reported on a highly efficient CRISPR-Cas9 system via the injection of *in vitro* transcribed Cas9 mRNA and sgRNA into *Drosophila* embryos. By using this method, they achieved much higher overall germline transmission efficiency of up to 34.5% and 10.40%, respectively (Bassett et al., 2013; Yu et al., 2013). Comparing the injection of DNA plasmids encoding Cas9 and sgRNA and the direct mRNA injection of Cas9 and sgRNA, the latter method provides over a 10-fold improvement in efficiency (Table 1). However, injection of RNA is not convenient due to the tedious *in vitro* transcription steps and the wide distribution of RNases. Several groups have successfully overcome this drawback by using a transgenic CRISPR-Cas9 system such as nos-Cas9, vasa-Cas9 and actin5C-Cas9. Using these lines, the overall germline transmission rate improved substantially, reaching 100% in some cases (Table 1) (Gratz et al., 2014; Kondo and Ueda, 2013; Port et al., 2014; Ren et al., 2013; Sebo et al., 2014). These transgenic CRISPR-Cas9 systems demonstrate many advantages compared to the previous *Drosophila* CRISPR-Cas9 system, especially regarding their simplicity and efficacy. This move away from the use of *in vitro* transcribed RNA in the CRISPR-Cas9 system has significantly increased the systems use along with a tremendous reduction in its cost.

CRISPR-Cas9 system mediated precise genomic modifications

To introduce genomic modifications in an effective way, the CRISPR-Cas9 system uses the cell's HDR mechanism to generate precise indel mutations in *Drosophila*. To trigger the HDR system, a donor template including a DNA sequence of interest, such as selection markers (*mini-white*, *vermilion*, 3xP3-mCherry/GFP), protein tags (HA, Flag, V5) or specific alleles (point mutations, truncated alleles), flanked by a left and right homology arm is used (Xu et al., 2015). As mentioned previously, in the first reports of the use of CRISPR-Cas9 system in *Drosophila* by Gratz and colleagues the *yellow* marker gene was targeted. During that study they designed a single-strand oligo deoxynucleotide (ssODN) donor template to replace the *yellow* gene, which together with Cas9 and two sgRNAs was injected into preblastoderm embryos. This resulted in the loss of the *yellow* phenotype, confirming the HDR but with a low overall rate of less than 0.1% (Table 2). To overcome this low HDR efficiency, they made improvements by replacing the ssODN donor with a dsDNA donor containing the visible marker 3xP3-DsRed. Also, transient expression of a Cas9-containing DNA plasmid under the control of the *vasa* promoter resulted in vasa-Cas9 embryos with a stable transgenic Cas9 expression. These improvements resulted in a 100-fold improvement in heritable HDR efficiency (Table 2) (Gratz et al., 2014). Besides changing the

Table 1 Overview of the CRISPR-Cas9 system mediated NHEJ in *Drosophila*[a)]

NHEJ			
Cas9 and sgRNA information (format)	Target gene	Overall germline transmission rate, %*	Reference
hsp70-Cas9 (DNA) U6b-sgRNA (DNA)	*yellow*	0.25%–1.37%	(Gratz et al., 2013)
T7-Cas9 (mRNA) T7-sgRNA (RNA)	*yellowwhite*	0.0%–34.5%	(Bassett et al., 2013)
SP6-Cas9 (mRNA) T7-sgRNA (RNA)	*yellow*	8.10%–10.40%	(Yu et al., 2013)
nos-Cas9 (transgene) U6b-sgRNA (transgene)	*white*	3.4%–93.2%	(Kondo and Ueda, 2013)
nos-Cas9 (transgene) U6b-sgRNA (DNA)	*white*	12.4%–74.2%	(Ren et al., 2013)
vasa-Cas9 (transgene) U6b-sgRNA (DNA)	*rosy*	6.49%–15.11%	(Gratz et al., 2014)
vasa-Cas9 (transgene) U6-sgRNA (DNA)	3×P3-mRFP 3×P3-EGFP	0%–100%**	(Sebo et al., 2014)
actin5C-Cas9 (transgene) vasa-Cas9 (transgene) nos-Cas9 (transgene) nos-Cas9:GFP (transgene) nosG4VP16>UAS-Cas9 (transgene) U6c-sgRNA (transgene)	*yellow* *ebony*	26.1%–100%	(Port et al., 2014)
U6b-sgRNA-hsp70Bb-Cas9 (DNA)***	*ebony*	13.0%	(Gokcezade et al., 2014)

a) *, The overall germline transmission rate is calculated as the number of mutant offspring divided by the total number of offspring from all crosses. **, The efficiency is the individual germline transmission rate instead of overall germline transmission rate. ***, Cas9 under the control of the *hsp70Bb* promoter and sgRNA under the control of *U6b* promoter are encoded on a single DNA plasmid.

Table 2 Overview of the CRISPR-Cas9 system mediated HDR in *Drosophila*[a)]

HDR					
Cas9 and sgRNA information (format)	Donor	Target gene	Genomic modification	Overall germline transmission rata, % (*n*)*	Reference
hsp70-Cas9 (DNA) U6b-sgRNA (DNA)	ssODN donor	*yellow*	End-out	0.04%**	(Gratz et al., 2013)
vasa-Cas9 (transgene) U6b-sgRNA (DNA)	dsDNA donor	*DSH3PX1*	End-out	1.98% (599/7657)*** 7.82% (45/2277)****	(Gratz et al., 2014)
Cas9 (mRNA) sgRNA (RNA)	dsDNA donor	*CG4221*	loxP replacement	4.3% (10/230)	(Yu et al., 2014)
		CG5961	*Hin*d III replacement	3.8% (2/52)	
		Chameau	eGFP tagging	2.7% (8/296)	
		CG4221	Myc tagging	10.4% (24/231)	
nos-Cas9 (transgene) U6:3-sgRNA-wls (transgene)	ssODN	*wntless*	amino acid point mutation (Gly11Ala: GGC-GcC)	28% (13/46)	(Port et al., 2014)
nos-Cas9 (transgene) U6b-sgRNA (DNA)	dsDNA donor	*piwi*	End-out	32.8% (446/1361)	(Ren et al., 2014b)
		hp1a		12% (98/822)	

a) *, The overall germline transmission rate is calculated as the number of mutant offspring divided by the total number of offspring from all crosses. **, The overall germline transmission rate of *yellow* mutation is 1.16% ((24+34)/(2336+2655)=1.16%), and the HDR transmission events only occurred in 2 founders of 61 G0 flies. Therefore, the estimated overall germline transmission rate of HDR is 0.04% (1.16%×3.28%=0.04%). ***, sgRNAS1+S2. ****, sgRNAS1+S3.

Cas9 delivery method, selecting the most efficient sgRNA is another way to improve HDR efficiency. By systematically analyzing sgRNA sequence parameters, Ren and colleagues found a strong positive correlation between genome editing efficiency and the sgRNA GC content of the six PAM-proximal nucleotides. By using these optimized sgRNAs and dsDNA donors, they achieved a significantly higher HDR rate of recessive viable and lethal alleles at 32.8% and 12%, respectively (Table 2) (Ren et al., 2014b). As mentioned previously, the endogenous cellular DNA repair machinery can use both HDR and NHEJ, the latter impairs HDR efficiency. In the past few years, researches have attempted to knockdown or knockout factors involved in the NHEJ pathway, shifting the balance toward HDR, thus increasing HDR efficiency. One of these factors is the ATP-dependent DNA-ligase 4 (*Lig4*) which functions in the *Drosophila* NHEJ pathway. Mutation of *Lig4* produces viable and fertile flies (Gorski et al., 2003; McVey et al., 2004). Kelly J. Beumer and colleagues showed that using the ZFN system in a *Lig4* mutant background resulted in a much higher HDR rate when compared to wild-type flies (Beumer et al., 2008). Based on this, numerous researchers have tried to improve the HDR efficiency of the CRISPR-Cas9 system by disruption of *Lig4* in the NHEJ pathway, but with only limited success. Notwithstanding this, in cultured *Drosophila* S2 cells, the homology targeted integration frequency is increased by a transient depletion of *lig4* using RNA interference (RNAi) together with Cas9 and a HR donor (Böttcher et al., 2014). Several CRISPR experiments carried out in the *Lig4* mutant background *in vivo* suggest evidence for only a slight increase in HDR efficiency, suggesting that depletion of *Lig4* has only a very limited effect on HDR (Gratz et al., 2014; Wen et al., 2016). Research into other DSB repair processes mediated by CRISPR-Cas9 will be important to improve HDR efficiency in the future.

CRISPR-Cas9 system mediated conditional knockout

The generation of different allele types is very important for the dissection of gene function in model organisms. However, many fundamental genes are recessive lethal, while heterozygous mutation alleles have no obvious phenotype. The best way to investigate these recessive lethal genes is to use transgenic RNAi system and conditional knockouts (Choi et al., 2009; Ni et al., 2011). Recently, a new conditional knockout method based on the CRISPR-Cas9 system has been shown to be an effective complement to the RNAi system, allowing for disruption of a specific gene in a conditional manner. Conditional alleles can be generated by incorporation of FRT sites flanking a specific gene through CRISPR-Cas9 mediated HDR (Gratz et al., 2014). Crossing of a conditional allele with conditional Flp or hs-Flp can lead to gene knockout in specific tissues or cells. Another alternative method is the GAL4-triggered UAS-Cas9 system, in which tissue-specific GAL4 or hs-GAL4 controlled Cas9 is combined with a *U6* promoter driving sgRNA expression. This system also leads to targeted gene mutagenesis, occurring only in specific tissues or cells (Xue et al., 2014). Sometimes off-target mutagenesis occurs, resulting from leaky Cas9 expression in combination with the ubiquitous sgRNA expression. Another disadvantage is the varying mutagenesis efficiency found in different flies. To address this, researchers have created a tRNA-sgRNA system, in

which the sgRNA is flanked by tRNAs and controlled by GAL4-UAS, increasing the specificity and efficiency of conditional gene disruption by the CRISPR-Cas9 system (Port and Bullock, 2016) .

CRISPR-dCas9 system mediated transcriptional activation

The use of CRISPR-Cas9 system as a genetic tool for genome editing is only one application of this powerful system. Besides mutagenesis, the system can be re-engineered as a useful transcriptional activation system (Chavez et al., 2015; Konermann et al., 2015). Regarding the atomic structure of the Cas9-sgRNA-target DNA complex, the tetraloop and the second stemloop of the sgRNA protrude outside the complex (Nishimasu et al., 2014). These protrusions can be modified by adding a hairpin aptamer, which selectively binds to specific RNA binding proteins, like the MS2 bacteriophage coating protein (Peabody, 1993). This sgRNA MS2 binding, in complex with the inactive Cas9, or dead Cas9 (dCas9), fused to VP64, results in the CRISPR-Cas9 synergistic activation mediator (SAM) transcription system (Konermann et al., 2015). SAM-activators achieve effective transcriptional activation of endogenous genes and can be used for genome-scale screening in mammal cell lines. An alternative transcriptional activation, referred to as the VPR system, has been made through the direct fusion of multiple transcriptional activators, VP64-p65-Rta (VPR), to dCas9. This changes the Cas9 DNA nuclease into a sgRNA guided transcriptional activator, activating both endogenous coding and noncoding genes (Chavez et al., 2015). An increasing number of research papers show the effective use of the CRISPR-Cas9 mediated transcriptional activation system in cells. The first *in vivo* application comes from the laboratory of Norbert Perrimon, demonstrating use of the VPR system in *Drosophila* cells to up-regulate target genes at endogenous loci (Lin et al., 2015). This system can function in either a single or multiple activation manner. This *in vivo* transcriptional activation using the VPR system provides a simple and effective tool for gain-of-function studies.

CRISPR-Cas9 system specificity and design

As the CRISPR-Cas9 system has been rapidly emerging as a powerful tool for genome editing, mutagenesis, transcriptional activation and repression, the specificity of this tool has been a concern for all researchers. In mammals, a single-base mismatch in the 1–11 bp PAM-proximal region abolished genomic cleavage by Cas9, whereas a single-base mismatch in the PAM-distal region retained Cas9 cleavage activity (Cong et al., 2013). A similar effect is observed when testing double-base mismatches between the guide RNA and target DNA. When two consecutive transversion mismatches occurred in the 11–19 bp PAM-distal region of the sgRNA distinct Cas9 cleavage activity was observed, whereas only weak Cas9 activity was observed when two consecutive transversion mismatches occurred in the 1–11 bp PAM-proximal region of the sgRNA (Slaymaker et al., 2016). As a high rate of off-target effects may influence the application of the CRISPR-Cas9 system, researchers have improved the specificity of SpCas9 by structure-guided amino acid mutagenesis. The combination of mutations in single alanine re-engineered SpCas9 has resulted in the production of an "enhanced specificity" SpCas9 (eSpCas9) variant with reduced off-target effects while maintaining robust on-target cleavage activity. eSpCas9 can be used broadly for many applications which strictly require high specificity with no off-target effects (Slaymaker et al., 2016). In contrast to other organisms, the *Drosophila* CRISPR-Cas9 system is less susceptible to off-target effects. In the *Drosophila* germline specific CRISPR-Cas9 system, stable Cas9 expression in germline cells is under the control of the *nanos (nos)* regulatory sequence (Ren et al., 2013; Ren et al., 2014a; Ren et al., 2014b). Delivering the sgRNA plasmid into nos-Cas9 embryos by microinjection has led to the development of a tool for systematically analyzing the off-target effect of the CRISRP-Cas9 system in flies (Ren et al., 2013). Ren and colleagues introduced sgRNA mutations and detected the mutation rates in off-target sites directly. They found no detectable off-target effects of sgRNAs having three or more mismatches with off-target sites. Based on this new criterion, about 86.9% of sgRNAs targeting the fly genome are specific to the target sequence (Ren et al., 2014b). When designing a personal CRISPR-Cas9 system, the crucial step is to find the appropriate sgRNA to uniquely target the genomic region of interest, hence controlling the specificity and efficiency of the system. There are many online recourses and tools available to help researchers find and construct specific sgRNAs, based on the NGG or NAG PAM-sequence and stringency for off-targets effects (Table 3). Some tools use search methods (Gratz et al., 2014), while others need the desired target DNA sequence to design the most suitable sgRNA (Kondo and Ueda, 2013). "DRSC Find CRISPRs", a tool designed by the Norbert Perrimon lab, has been updated to version 2 in which they have added the efficiency prediction function. This new improvement will provide guidance in finding a highly efficient sgRNA for any application. "E-CRISPR" developed by the Michael Boutros lab and "CRISPR Design" developed by the Feng Zhang lab allow paired sgRNAs for the CRISPR-Cas9 nickase system to be found, inducing two single-stranded breaks each on the opposite strand, resulting in a DSB (Heigwer et al., 2014; Hsu et al., 2013).

Future perspectives

Since the discovery of the CRISPR-Cas9 system, it has rapidly evolved into the preferred tool for specific genomic modifications due to its simplicity, low cost and high speci-

Table 3 Online resources for finding genome-wide sgRNAs

Name	Link	Contributor	Reference
DRSC Find CRISPRs	http://www.flyrnai.org/crispr2/	Norbert Perrimon lab	(Ren et al., 2013)
E-CRISPR	http://www.e-crisp.org/E-CRISP/	Michael Boutros lab	(Heigwer et al., 2014)
CRISPR optimal target finder	http://tools.flycrispr.molbio.wisc.edu/targetFinder/	KateM. O'Connor-Giles lab	(Gratz et al., 2014)
FlyCas9	http://shigen.nig.ac.jp/fly/nigfly/cas9/index.jsp	Shu Kondo lab	(Kondo and Ueda, 2013)
CRISPR Design	http://www.genome-engineering.org/crispr/?page_id=41	Feng Zhang lab	(Cong et al., 2013) (Hsu et al., 2013)

ficity. Also in other research domains it has opened new possibilities to tackle longstanding questions or diseases. Recently, Brenda A. Peterson and colleagues were able to simultaneously target fourteen genomic loci in *Arabidopsis thaliana*, without any off-target effects. By targeting multiple genes at once, researchers can achieve advanced knowledge of plant development and environmental responses. Without any off-target effects, the CRISPR-Cas9 system can improve agricultural crops by counteracting diseases that cause crop losses (Peterson et al., 2016). Andrew Hammond's group has also shown promising results in research to suppress malaria by using CRISPR-Cas9 endonuclease constructs, which function as the gene drive system in *Anopheles gambiae*, the main vector for malaria. They identified three genes that present a recessive female-sterility phenotype when disrupted and inserted them into locus CRISPR-Cas9 gene drive constructs designed to target and edit each gene. Through super-Mendelian inheritance, they can now transmit this sterile phenotype to the progeny with rates of up to 99.6%, leading to the suppression of mosquito populations over a timeframe of a few years (Hammond et al., 2016). In cancer research, CRISPR-Cas9 can be used to specifically target and cleave mutations impairing cell growth, showing the potential of the system for personalized medicine and as well as for broader therapeutic use. Christina Geber and co-workers found that they could theoretically target more than 80% of cancer mutations with CRISPR-Cas9. By using cancer-specific sgRNA, they can specifically cleave alleles encoding common cancer mutations without targeting wild-type alleles (Gebler et al., 2017). The system can also be used therapeutically to treat in diseases such as sickle cell anemia (Dever et al., 2016) and HIV (Kaminski et al., 2016). As applications for the CRISPR-Cas9 system develop at a high rate, they prove once more the capability of this multipurpose system.

CONCLUSION

Drosophila based large arrays of available genome editing tools make the fly a popular model organism for biochemical research. For every research purpose a suitable tool is available with each having its advantages and drawbacks. Older techniques for mutagenesis and transgenesis are still used in current research, but these tools are gradually being replaced by newer tools with improved specificity and a lower cost. Nowadays, because of the emergence of the CRISPR-Cas9 system, the fly has gained even more popularity among researchers. This multipurpose tool is exponentially evolving, and is being chosen more frequently for genome engineering, not only in *Drosophila* but also in various other organisms. CRISPR-Cas9 system is suitable for genome editing, mutagenesis, transcriptional activation and repression. Furthermore, it allows genomic modification at a single nucleotide resolution, as well as multiple mutations all at once. As the CRISPR-Cas9 system opens new opportunities, researchers will be able to tackle previously unresolved biochemical questions and disease mechanisms in the future.

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