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Understanding the DNA damage response in order to achieve desired gene editing outcomes in mosquitoes

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Abstract

Mosquitoes are high-impact disease vectors with the capacity to transmit pathogenic agents that cause diseases such as malaria, yellow fever, chikungunya, and dengue. Continued growth in knowledge of genetic, molecular, and physiological pathways in mosquitoes allows for the development of novel control methods and for the continued optimization of existing ones. The emergence of site-specific nucleases as genomic engineering tools promises to expedite research of crucial biological pathways in these disease vectors. The utilization of these nucleases in a more precise and efficient manner is dependent upon knowledge and manipulation of the DNA repair pathways utilized by the mosquito. While progress has been made in deciphering DNA repair pathways in some model systems, research into the nature of the hierarchy of mosquito DNA repair pathways, as well as in mechanistic differences that may exist, is needed. In this review, we will describe progress in the use of site-specific nucleases in mosquitoes, along with the hierarchy of DNA repair in the context of mosquito chromosomal organization and structure, and how this knowledge may be manipulated to achieve precise chromosomal engineering in mosquitoes.

Keywords

Mosquito; Aedes; Gene editing; TALEN; CRISPR; DNA repair

Mosquito-borne disease

Mosquitoes are important disease vectors, with none more important than the malaria vector *Anopheles gambiae* and the dengue vector *Aedes aegypti. Malaria* is believed to infect more than 200 million annually causing more than half a million deaths (2014a; 2014d), while dengue is thought to infect 50 to 100 million annually, causing mass morbidity and placing a large economic burden on developing countries (2014b;2014c). Other pathogens such as yellow fever virus, chikungunya virus, West Nile virus, eastern equine encephalitis virus, western equine encephalitis virus, and La Crosse virus are also vectored by mosquitoes (Colpitts et al. 2011; Pialoux et al. 2007; Chhabra et al. 2008) and cause substantial human morbidity and mortality.

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Treatment for malaria revolves around the use of insecticide-treated bed nets (Hill et al. 2006), indoor residual insecticides (Pluess et al. 2010), and treatment of infected human hosts with anti-malarial drugs (Schlitzer 2008); for dengue, prevention is primarily through control of the vector by way of source reduction and insecticides (Kamgang et al. 2011). Techniques such as the sterile insect technique (SIT) (Alphey et al. 2010), release of insects harboring intracellular bacteria *Wolbachia* (Walker et al. 2011), and release of insects with dominant lethality (RIDL) (Massonnet-Bruneel et al. 2013; Fu et al. 2010; Labbé et al. 2012) are currently being investigated as additional interventions and appear to hold great promise. Also in development are techniques that aim to replace current mosquito populations with those that are pathogen resistant [reviewed in Burt (2014) and Wang and Jacobs-Lorena (2013)]. A primary technical concern of gene-drive-based control strategies is the integrity and long-term stability of any pathogen resistance transgene. Optimally, such transgenes would be placed in a chromosomal region most likely to be repaired faithfully in the case of DNA damage. However, little is known about mosquito DNA break repair pathways, or how chromosomal structure and repeat content may influence the long-term stability of such transgenes, particularly regions that might be more inclined toward mutations or transcriptional silencing.

More generally, the advent of site-specific gene editing technologies such as transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 is poised to revolutionize the field of mosquito genetics and molecular biology and physiology (Kim and Kim 2014). These highly specific nucleases rely on host DNA repair pathways to fix the broken DNA ends, preferably in a fashion consistent with the hopes of the investigator. An unavoidable complication, however, is that each of the various end-joining and homology-based repair pathways competes with each other for access to the double-stranded DNA break (DSB). Work by others has shown that it is possible to manipulate the repair outcome in insects such as Drosophila (Beumer et al. 2008; Ciapponi et al. 2004; Yoo and McKee 2005; Wei and Rong 2007) and *Bombyx mori* (Ma et al. 2014). However, little is known about the hierarchy of DNA repair choice in mosquitoes, or the protein components most critical for completing each form of DSB repair. In this review, we present our current understanding of DSB repair as gleaned from model organisms such as yeast, flies, and vertebrates in the context of genes and gene families lost and duplicated in the mosquito. Additionally, we present potential strategies for manipulating the DSB response in mosquitoes with respect to protein machinery, chromosomal structure, and sequence content. An understanding of these aspects of genome engineering in mosquitoes will hopefully stimulate further investigation and generate evidence-based questions about how genomic engineering in mosquito disease vectors can be further improved.

Gene editing technologies used in mosquitoes

Homing endonucleases (HEs) were the first site-specific nucleases used to edit the mosquito genome (Windbichler et al. 2007). Known for their extreme target specificity (Stoddard 2005; Stoddard 2011), HEs are naturally occurring selfish genetic elements that can display hyper-Mendelian rates of inheritance due to their ability to be copied from a template to a target chromosome via homology-directed repair following DSB induction (Burt 2003). In

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the malaria mosquito, the HE I-SceI has been used to generate an artificial gene drive system that may one day be used to convert these mosquitoes into a more benign form that no longer transmits malaria parasites (Windbichler et al. 2011). The fortuitous insertion of a transposon bearing an I-SceI recognition sequence onto the Y chromosome of *An. gambiae* has also allowed the homology-dependent integration of additional transgenes to this location (Bernardini et al. 2014). The HE I-PpoI, which recognizes a target ribosomal DNA that is conserved amongst all eukaryotes, was used to develop transgenic strains of *An. gambiae* that display either male-specific sterility (Windbichler et al. 2008; Klein et al. 2012) or male-specific sex distortion (Galizi et al. 2014) phenotypes. While HEs have not been used as extensively to perform chromosomal manipulations in other mosquitoes, several HEs have been shown to recognize and cleave their target sites in a highly specific manner when these sites are present in the *Ae. aegypti* genome (Aryan et al. 2013a; Traver et al. 2009). Despite the fact that several hundred naturally occurring HEs have been described, the difficulty and expense in re-engineering these site-specific nucleases to recognize new and useful targets will likely restrict their application.

In contrast, customizable endonucleases such as zinc finger nucleases (ZFNs) and TALENS are modular and can be much more easily re-engineered to recognize interesting chromosomal targets (Gaj et al. 2013; Carlson et al. 2012). Both systems depend on the generation of two synthetic proteins that when heterodimerized yield an active site-specific nuclease. ZFNs use a variety of zinc finger binding domains linked together, with each binding domain coding for three specific nucleotides (Urnov et al. 2010), while TALENs use a system of linked TALE repeats, where each repeat specifies a single nucleotide (Joung and Sander 2013). Both ZFNs and TALENs couple these repeat domains to a nuclease such as *FokI*. ZFNs have been used successfully in *Ae. aegypti* (Liesch et al. 2013; Degennaro et al. 2013; McMeniman et al. 2014) and TALENs have been used in both *Ae. aegypti* and *An. gambiae* (Aryan et al. 2013b; Smidler et al. 2013). Despite these advances, the cost of synthesizing or assembling new ZFNs or TALENs is likely to prevent their widespread use in mosquito gene editing.

Thus, the most promising future for mosquito chromosomal manipulation may be with CRISPR/Cas9. The CRISPR/Cas9 system is part of the adaptive immune system within certain bacteria (Chakraborty et al. 2009; Karginov and Hannon 2010) and uses short RNA sequences to guide a DNA endonuclease (Cas9), resulting in a DSB (Sander and Joung 2014; Bassett and Liu 2014; Liu and Fan 2014; Mali et al. 2013b). The CRISPR/Cas9 system has been further adapted to use synthetic guide RNAs, further optimizing the process (Bassett et al. 2013; Ma et al. 2013; Upadhyay and Sharma 2014; Bae et al. 2014). Re-engineering new target sites is as simple as synthesizing a new small RNA molecule; this ease of use explains why Cas9-based editing has so rapidly supplanted other technologies. For mosquito gene editing, as for Drosophila, a computational search for potential cross-targeting guide RNAs before the experiment begins (Xie et al. 2014), followed by several generations of out-crossing of edited individuals (Liesch et al. 2013), should be sufficient to minimize confounding off-target effects. Other options include optimizing the concentration of sgRNAs to Cas9, and utilizing pairs of Cas9 nucleases that have been mutated only to allow nicking (or single-stranded breaks) to occur, which when used in conjunction effectively generate a DSB (Mali et al. 2013a). While there have been no published

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incidences of the use of CRISPR/Cas 9 in mosquitoes, unpublished data suggests that the system will in fact work in a highly efficient manner (not shown).

All nuclease-based gene editing and chromosomal manipulation tools rely intrinsically on host-mediated repair processes. In the absence of a visible marker, gene editing events are typically detected via PCR, followed by Sanger or Illumina-based sequencing, digestion of the PCR amplicon with a restriction endonuclease or mismatch-specific nuclease, or through analysis of the amplicon using high-resolution melt curve analysis (HRMA). While these systems have all proven effective to various degrees, these assays all underestimate nuclease activity due to (1) repair that correctly restores the original sequence, or (2) repair that is sufficiently deleterious as to remove one or both primer binding sites. Where the experimental goal is to produce targeted deletions, the experimenter must rely on mistakes made by the classical non-homologous end-joining (C-NHEJ) pathway, while targeted insertions rely on homology-directed repair (HDR) (Liesch et al. 2013; Bassett et al. 2014; Gratz et al. 2014). However, each of these mechanisms is capable of both highly faithful and highly deleterious repair. Thus, all assays and outcomes depend not just on the activity of the site-specific nuclease, but also on the success or failure of the target cell to repair the resultant DSB in a manner consistent with the wishes of the experimenter. Understanding how these repair pathways function, as well as how they interact and compete with each other, is thus critical to optimizing gene editing experiments. This is especially necessary in non-model organisms such as mosquitoes that are more difficult to handle at larger scales.

Double-stranded DNA break repair

Generally speaking, DSBs are repaired by C-NHEJ or HDR, but the more these mechanisms are elucidated, the more complex they appear to be. For the purposes of this review, we will cover HDR, single-strand annealing (SSA), C-NHEJ, and alternative non-homologous end joining (A-NHEJ), in reference to how they repair DSBs and how they may be manipulated to achieve the experimenters' desired results.

Homology-directed repair is initiated when the MRN nuclease complex (Niu et al. 2010) composed of Mre11, Rad50, and Nbs1 resects the DSB (from either end). The MRN complex is aided by the endonuclease Sae2 (Lamarche et al. 2010), as well as secondary endonucleases such as Exo1, Dna2, and Sgs1 (Zhu et al. 2008). Loss of either Mre11 or Rad50 in Drosophila leads to chromosomal instability and higher cell death rates (Ciapponi et al. 2004). Interestingly, *Ae. aegypti* appears to have duplicated both Mre11 and Rad50 (Table 1). Once resection has been accomplished and each end of the DSB has an exposed ssDNA strand, RPA binds the ssDNA (Golub et al. 1998) and is replaced by Rad51 with the aid of mediator proteins such as BRCA2 (Klovstad et al. 2008). In yeast, this mediation is accomplished by Rad52; while Rad52 appears to be less important in some vertebrates, it appears to retain some role in humans (Liu and Heyer 2011) and is completely lost in flies and mosquitoes (Table 1). Rad51 creates a filament complex that has the ability to invade a homologous sequence of dsDNA (Yoo and McKee 2005); Rad54 works in conjunction with the Rad51 invasion filament until the appropriate homologous sequence is found (Kiianitsa et al. 2006). Once homology is detected, either δ or ε (delta or epsilon), polymerase is recruited to accurately repair the DNA lesion (Mehta and Haber 2014). Depending on the

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nature of the break, a Holiday junction is formed (Heyer 2004) and eventually disassembled leading to an "error-free" repair of the DSB. Rad51 and Rad54 mutants have been evaluated in Drosophila using a DNA repair assay; Rad51 was determined to be crucial for HDR, with Rad54 deemed important as well, but to a lesser extent than Rad51 (Wei and Rong 2007). In an alternative approach, RNAi-based suppression of Rad51 mRNA in Drosophila led to higher death rates in the presence of a mutagenic substance (Yoo 2006). Conversely, over-expression of Rad51 in Drosophila using a heat shock promoter also resulted in lethality, suggesting that the amount of homology-based repair must be finely controlled (Yoo and McKee 2004). HDR components have not yet been studied in mosquitoes.

Unlike traditional HDR, which relies on homologous sequences present on sister chromatids or homologous chromosomes, the SSA pathway relies on the use of homologous repeats flanking the DSB (Ivanov et al. 1996). The homologous regions can be as short as 30 bp in yeast (Sugawara et al. 2000), or as short as 18 bp in mosquitoes (Aryan et al. 2013a), and are eventually collapsed, while the genetic information between the two repeats is deleted. SSA is generally categorized as a sub-pathway of HDR because of the use of similar protein machinery in the initial resection steps. As in HDR, resecting occurs via the MRN complex and secondary exonucleases (Mehta and Haber 2014). A protein complex consisting of the scaffolding proteins Slx4 and Saw1 (Li et al. 2008a), the mismatch repair proteins Msh2 and Msh3 (Sugawara et al. 1997), and the Rad1–Rad10 endonuclease complex (Davies et al. 1995) work in conjunction to collapse the two homologous sections and remove excess nucleotides. In yeast, Rad52 then anneals the strands in conjunction with the homo-log Rad59 (Sugawara et al. 1997). Saw1 and Msh2 are not present in flies or mosquitoes, suggesting differences in both scaffolding and mismatch scanning in SSA-based repair, while the absence of Rad52 and Rad59 in flies and mosquitoes suggests that there may be alternative machinery which compensates for the annealing step (Table 1). Mice deficient in Rad1 and Rad10 orthologs showed similar phenotypes with regard to chromosomal damage and ultimately died prematurely (Tian et al. 2004; McWhir et al. 1993), while Drosophila Rad1 and Rad10 mutants are both viable and fertile but largely unstudied (Drysdale and FlyBase 2008).

The C-NHEJ pathway is distinct from HDR and SSA in both its machinery and mechanism. Upon DSB formation, Ku70 and Ku80 form a heterodimeric complex on the ends of the DSB called the Ku complex (Wang and Lees-Miller 2013). Knockout of Ku70 in *Arabidopsis* led to a 5- to 16-fold increase in HDR (QI et al. 2013), while knockdown of Ku70 in *B. mori* increased rates of HDR via a junction PCR assay (Ma et al. 2014), suggesting that Ku proteins may be a target of interest in mosquitoes. While the most current gene set (AaegL3.2) failed to identify a gene model for *Ae. aegypti* Ku70, a homology-based search (tblastn) using the Drosophila or Anopheles Ku70 protein sequences indicates that a likely ortholog is located on scaffold 1.240. The kinase DNA-PKcs tethers the Ku complexes and activates multiple proteins associated with the pathway (Williams et al. 2014), primarily the endonuclease Artemis, which is responsible for removing nucleotides from the DSB (MA et al. 2002, 2013). Subsequently, the polymerases μ and λ play a role in adding any additional nucleotides needed for ligation. Both polymerases *μ* and λ are absent in flies and mosquitoes, while Artemis appears to have been lost in both *Drosophila melanogaster* and *Ae. aegypti* (Fig. 1a and Table 1). In vertebrates, these factors are primarily associated with

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DNA ligation during V(D)J recombination, a process that does not occur in insects, with mutations resulting in a failure to generate proper T and B cells. Following modification of the broken DNA ends, the factors XLF and Xrcc4 form the primary scaffolding components (Mahaney et al. 2013) and recruit Lig4 to the break site, allowing for the final ligation of both ends of the DSB (Williams et al. 2014). While mutation of XLF in mice resulted in radiation sensitivity, impaired V(D)J recombination, and lower levels of lymphocytes (Li et al. 2008b), work by others suggests that Xrcc4 and Lig4 mutants are the most severe in respect to loss of NHEJ (Karanjawala et al. 2002). While in mice the absence of Lig4 results in embryonic death (Karanjawala et al. 2002), Lig4 mutants in *Arabidopsis* show a 3- to 4-fold increase in HDR (QI et al. 2013), and Lig4 Drosophila mutants are viable and produce fertile off-spring with rates of HDR higher than 70 %, where they had previously been less than 15 % (Beumer et al. 2008). Xrcc4 orthologs could not be identified in either *Ae. aegypti* or *An. gambiae* via homology (Blastp) or domain-based (HMMER) searches (not shown), while Lig4 appears to have been duplicated in *Ae. aegypti.* Other proteins such as APLF, APTX, and PNKP are also involved in NHEJ-based repair. APLF is believed to play a role as a scaffolding protein (Grundy et al. 2013) as well as an exonuclease (Li etal. 2011); APTX has been shown to remove AMP from DNA ends (Clements et al. 2004), while PNKP removes and replaces non-ligatable groups from the DSB allowing for Lig4 to complete its function (Weinfeld et al. 2011). While the end joining of this highly complex repair system is believed to be error prone in nature, recent studies suggest that it may be much more faithful than traditionally thought (Betermier et al. 2014).

In addition to C-NHEJ, DSBs may be repaired by a Ku-independent mechanism, termed alternative non-homologous end joining (A-NHEJ). Unlike C-NHEJ, A-NHEJ is suspected to be highly error prone (Betermier et al. 2014; Deriano and Roth 2013). Like the C-NHEJ pathway, A-NHEJ ligates two broken ends of a DSB together, largely without the use of a homologous template. Unlike C-NHEJ, which initiates at the step of binding free-DNA ends, A-NHEJ appears to proceed after initiation of repair by the HDR resection machinery, with the use of the MRN complex, in conjunction with Sae2, to remove undesired nucleotides (Truong et al. 2013). Once resection has initiated, microhomology is utilized in the absence of a competing pathway to ligate the broken ends in an error-prone manner (Soni et al. 2014). The protein PARP1 competes with Ku proteins for binding of the DSB, as well as possibly recruiting other proteins to the break (Wang et al. 2006), tethering the DNA strands (Chiruvella et al. 2013) and possibly mediating translocations (Soni et al. 2014; Simsek and Jasin 2010). The primary ligase involved in this process is believed to be Lig3, which operates in conjunction with Xrcc1 (Oh et al. 2014). However, removal of Xrcc1 via mutation in hamster cells does not stop Lig3 from functioning, instead increasing its susceptibility to competition by Lig1 (Soni et al. 2014). Notably, Lig3 has been lost in mosquitoes, suggesting that Lig1 may be critical for A-NHEJ in these species (Fig. 1b and Table 1).

While repair pathways have been studied in model organisms [reviewed in Lamarche et al. (2010), Williams et al. (2014), and Chiruvella et al. (2013)], little is known about these processes in mosquitoes, which diverged from Drosophila approximately 240 Mya. While the majority of DNA break repair proteins appear to be conserved in flies and mosquitoes, there are some interesting anomalies, as noted above. While models of hierarchical repair

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have been proposed in model organisms (Mansour et al. 2008), similar models must be developed and tested in mosquitoes (Fig. 2). A strong grasp of how pathways are chosen will facilitate better use of endonuclease tools and more confident interpretation of experimental results. How can the various proteins involved in these pathways be manipulated to achieve the experimenter's desired results? The suppression of end-joining repair pathways to increase repair via a homologous template has been accomplished in both flies and silkworm (Beumer et al. 2008; Ma et al. 2014), suggesting that similar results could be achieved in mosquitoes. Alternatively, disabling both HDR and C-NHEJ could potentially increase rates of targeted deletions, favoring the generation of gross repair errors such as translocations and inversions. A more complete understanding of the processes that determine the hierarchy of DNA repair pathways will allow the vector biologist to take full advantage of tools such as CRISPR/Cas9 in the most efficient manner possible.

Chromosomal structure and organization in mosquito genome manipulation

Manipulating the various DNA repair pathways, whether by increasing HDR with Lig4 or Ku70 knockouts (Beumer et al. 2008; Ma et al. 2014; QI et al. 2013) or increasing SSA by removing Rad51 or Rad54 (Wei and Rong 2007) may help generate favorable repair outcomes; however, other factors such as the proteins associated with DNA packaging, as well as sequence content, may also play a role in DNA repair pathway choice. Chromosomal DNA, packed into nucleosomes and wound around a histone octomer, can be generally characterized as either euchromatin or heterochromatin. Euchromatin is typically gene rich, less condensed, and transcriptionally accessible, while heterochromatin is gene poor, highly condensed, and transcriptionally repressed (Tamaru 2010). The highly condensed nature of heterochromatin has been shown to impact repair time and the recruitment of repair proteins such as Ku70 and DNA-PKcs (Lorat et al. 2012). The physical barrier presented by a more condensed heterochromatin may require the selection of a more advantageous target or the use of chemical agents known to relax chromatin structure, such as sodium butyrate or chloroquine (Murr et al. 2006). The existence of chromosome remodeling complexes such as TIP60 acetyltransferase, which acetylates histone H4 permitting the repair of DSBs [reviewed in Price and D'Andrea (2013)], may be manipulated to influence chromatin structure and DNA repair choice. Deletion of TRRAP, a component of the TIP60 remodeling complex, in murine cells leads to a 2-fold reduction in homologous recombination (Murr et al. 2006). Could over-expression of TRRAP lead to an increase in HDR? What other chromosome remodeling complexes can be modulated to impact DNA repair pathway choice?

In addition to the physical obstructions that chromatin proteins may induce, the organization of the chromosome itself also may have a role in the choice of DNA repair pathways. The *An. gambiae* genome is organized with pericentric and intercalary heterochromatic regions, which are gene poor, transposable element (TE) rich, and highly condensed (Sharakhova etal. 2010). The *Ae. aegypti* genome is much larger (~1.4 Gbp), with the typical heterochromatic versus euchromatic demarcation clouded by the presence of large regions of short interspaced repeats (Nene et al. 2007). Gene models in *Ae. aegypti* have expanded intronic regions (nearly four times as long as those of *An. gambiae)*, with repetitive elements distributed throughout; such repeats and TEs make up roughly 50 % of the genome (Nene et

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al. 2007; Severson and Behura 2012). Because SSA-based repair requires flanking repeat sequences (Ivanov et al. 1996) and A-NHEJ appears to utilize microhomology (Truong et al. 2013), the content and organization of repetitive elements around the selected target site may influence the choice of DNA repair pathway. *Ae. aegypti*, which has proportionally more repetitive elements in its genome than does *An. gambiae*, may thus be more likely to use the SSA repair pathway for DNA break repair; we have shown previously that SSA competes favorably with NHEJ when direct repeats are present (Aryan et al. 2013a). Considering the expansion of interspersed repetitive elements in most gene models of *Ae. aegypti*, achieving efficient levels of HDR in this organism may be a challenge due to difficulty identifying unique sequences of sufficient length flanking a DSB site. Initial experiments have used 1–2 kb of homologous sequence on either side of the break (Liesch et al. 2013; McMeniman et al. 2014), but a thorough characterization of minimum requirements for HDR would be beneficial.

Final conclusions

Site-specific nucleases generate controlled DSBs (Carroll 2014; Kim and Kim 2014), which may be selectively used to generate deletions, insertions, inversions, and translocations (van der Weyden and Bradley 2006). While both the C-NHEJ and A-NHEJ pathways can produce deletions (Aryan et al. 2013b; Smidler et al. 2013), the contribution of each of these to perfect repair (undesired) and error-prone repair (desired) remains to be firmly established. Currently, insertion of transgenic constructs into the mosquito genome is a random process associated with the use of transposable elements (Adelman et al. 2002; Kokoza and Raikhel 2011). While site-specific integration based on HDR has been accomplished in mosquitoes (Liesch et al. 2013; McMeniman et al. 2014), efficiencies were less than 1 %. A more efficient system for insertion of a target sequence via HDR would be highly advantageous and represents a critical barrier in mosquito chromosomal engineering. Lastly, the SSA pathway may be utilized opportunistically to remove genetic information such as individual exons or entire genes when located between homologous direct repeats.

Targeted inversions have been generated in human cells via the NHEJ pathway using pairs of ZFNs targeting different sites (Lee et al. 2012); similar results were obtained in Drosophila using HEs (Egli et al. 2004). Inversions in mosquitoes, such as the 2L*a* inversion in *An. gambiae*, have been suggested to confer an advantage to the organism in arid environments (White et al. 2009). Targeted inversions at various locations could be used to confirm phenotypic effects of suspected naturally occurring inversions, or the creation of novel inversion phenotypes. In Drosophila and mice, targeted inversions have been used in the creation of balancer chromosomes that are resistant to homologous recombination (Casso et al. 2000; Zheng et al. 1999). The creation of balancer chromosomes in mosquitoes would simplify screening (by fluorescent makers and homozygous lethal genes) of genetically engineered organisms and reduce rates of homologous recombination stabilizing introduced mutations. These technologies would also facilitate high-throughput methods of screening for the identification of genetic mutants and phenotypic traits. Translocations have been generated with nucleases in both Drosophila and human cells (Egli et al. 2004; Piganeau et al. 2013). Perhaps by upregulating components of the A-NHEJ pathway, which is suspected to play a role in translocations (Soni et al. 2014), targeted translocations may be generated to

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produce novel meiotic gene drive systems (Pearson and Wood 1980). Mosquitoes carrying a targeted translocation could be used in typical SIT fashion for population reduction through the release of males only, or could be used to drive population replacement by the continued release of both males and females. This process could also be reversible with the release of wild-type males and females.

The use of site-specific nucleases in important mosquito vector species is still an emerging field. How these tools work in different mosquito species, as well as the hierarchy of mosquito DNA repair pathways, requires further investigation. Additional inquiries into topics such as the impact of target site location on nuclease selection, the modulation of chromatin protein complexes with respect to DNA repair pathways, and the impact of DNA repair pathway choice on the desired engineering event are required to advance mosquito chromosomal engineering. A firm understanding of how DNA repair pathways can be manipulated will hasten the development of more effective vector control techniques.

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Abbreviations

A-NHEJ Alternative non-homologous end joining

C-NHEJ Classical non-homologous end joining

CRISPR Clustered regulatory interspaced short palindromic repeats

DSB Double-stranded break

HE Homing endonuclease

HDR Homology-directed repair

HRMA High-resolution melt curve analysis

RIDL Release of insects with dominant lethality

SIT Sterile insect technique

SSA Single-strand annealing

TALEN Transcription activator-like effector nuclease

TE Transposable element

ZFN Zinc finger nuclease

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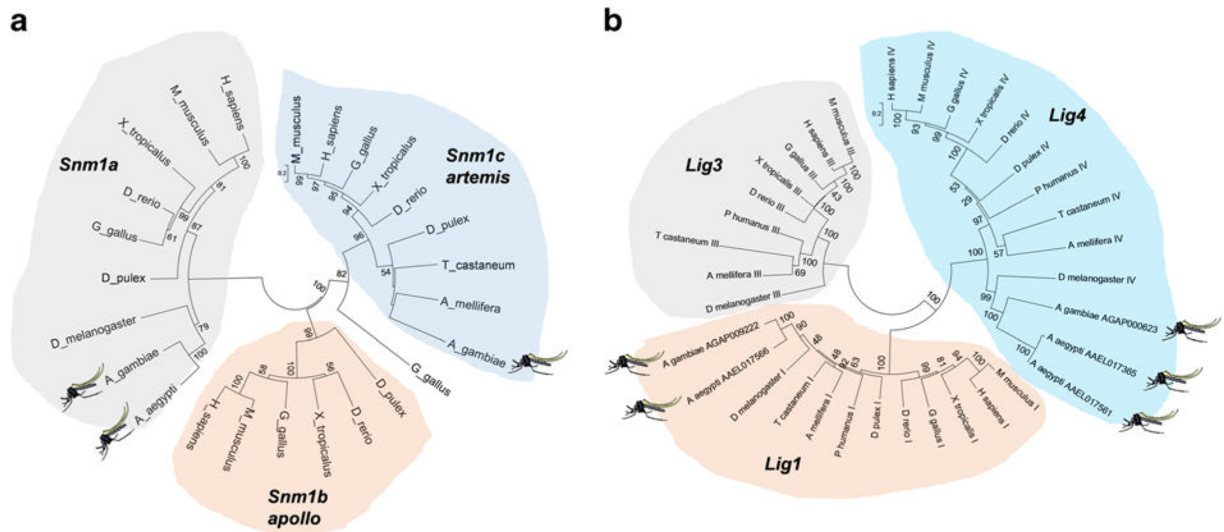

Fig. 1.

Gain and loss of NHEJ components in mosquitoes. Neighboring-joining tree produced from a clustalW alignment of Smn1-family proteins **(a)** or DNA ligases **(b)** using MEGA6 [118]. Bootstrap support (2000 replicates) is indicated on each branch if over 50 %. Abbreviations: *Homo sapiens* (H_sapiens), *Mus musculus* (M_musculus), *Gallus gallus* (G_gallus), *Xenopus tropicalis* (X_tropicalis), *Danio rerio* (D_rerio), *Daphnia pulex* (D_pulex), *Pediculus humanus* (P_humanus), *Tribolium castaneum* (T_castaneum), *Apis mellifera* (A_mellifera), *Drosophila melanogaster* (D_melanogaster), *Anopheles gambiae* (A_gambiae), *Aedes aegypti* (A_aegypti). Mosquito species are indicated with an *icon*

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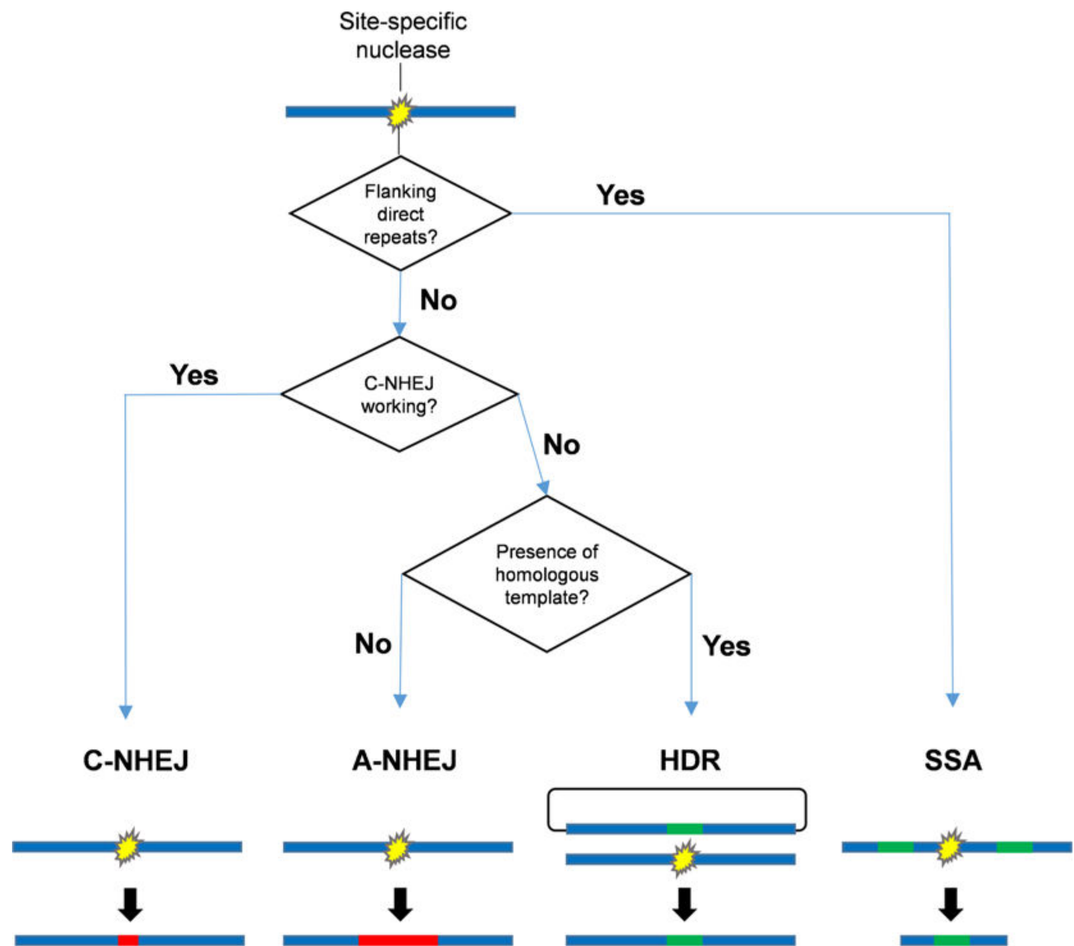

Fig. 2.

Potential hierarchy of DSB repair pathways in mosquitoes. Simplified flowchart of possible repair outcomes following DSB induction in the mosquito genome. Both known (proximity and length of repeat sequences, microhomologies, cell cycle phase, developmental stage, chromosomal organization, etc.) and unknown variables may contribute to each decision fork

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Table 1

Orthologs of DNA break repair components in mosquitoes

Gene	NHEJ	A-NHEJ	HDR	SSA	*D. melanogaster*	*Ae. aegypti*	*An. gambiae*
Ku70	X				FBgn0011774	No gene model	AGAP002690
Ku80	X				FBgn0041627	AAEL003684	AGAP009910
DNA-PKcs	X				Absent	AAEL008123	AGAP003967
Xrcc4	X				FBgn0069301	No ortholog identified	No ortholog identified
XLF	X				No ortholog identified	AAEL002939	No ortholog identified
Ligase 4	X				FBgn0030506	AAEL0173656/AAEL017561	AGAP000623
Polμ/Polλ	X				Absent	Absent	Absent
Artemis	X				No ortholog identified	No ortholog identified	AGAP000597
APLF	X				FBgn0026737	AAEL011254	AGAP004516
PNKP	X				FBgn0037578	AAEL000527	AGAP012174
APTX	X				FBgn0038704	AAEL014945	AGAP004307
Parp1		X			FBgn0010247	AAEL011815	AGAP003230
Ligase 3		X			FBgn0038035	Absent	Absent
Ligase 1		X			FBgn0262619	AAEL017566	AGAP009222
Xrcc3		X			FBgn0003480	AAEL005399	AGAP013180
Xrcc1		X			FBgn0026751	AAEL002782	AGAP002605
ATM		X			FBgn0045035	AAEL014900	AGAP009632
Mre11		X	X		FBgn0020270	AAEL010595/AAEL000034	AGAP006797
Rad50		X	X		FBgn0034728	AAEL014748/AAEL005245	AGAP003676
Nbs1		X	X		FBgn0261530	AAEL014377	AGAP003213
Sae2		X	X		FBgn0029113	AAEL010641	AGAP008637
Exo1			X		FBgn0015553	AAEL006209	AGAP004491
RPA			X		FBgn0010173	AAEL012826	AGAP001421
Sgs1			X		FBgn0002906	AAEL004039	AGAP002967
Dna2			X		FBgn0030170	AAEL000201	AGAP004685
Rad51			X		FBgn0003479	AAEL006080	AGAP013412
Rad54			X		FBgn0002989	AAEL002647	AGAP008748
BRCA2			X		FBgn0050169	AAEL014774/AAEL010133	AGAP007032

Gene	NHEJ	A-NHEJ	HDR	SSA	*D. melanogaster*	*Ae. aegypti*	*An. gambiae*
Polδ			X		FBgn0263600	AAEL014178	AGAP011731
Polσ			X		FBgn0264326	AAEL002800	AGAP004615
Rad52			X	X	Absent	Absent	Absent
Rad1				X	FBgn0026778	AAEL009701	AGAP002255
Rad10/Ercc1				X	FBgn0028434	AAEL008081/AAEL013693	AGAP004029
Msh2				X	FBgn0015546	AAEL014856	AGAP010282
Slx4				X	FBgn0002909	AAEL008482	AGAP007582
Msh3				X	Yeast Only	Yeast Only	Yeast Only
Rad59				X	Absent	Absent	Absent
Saw1				X	Yeast Only	Yeast Only	Yeast Only

NHEJ non-homologous end joining, *A-NHEJ* alternative *NHEJ*, *HDR* homology-directed repair, *SSA* single-strand annealing

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