Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in Aedes aegypti

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Conventional control strategies for mosquito-borne pathogens such as malaria and dengue are now being complemented by the development of transgenic mosquito strains reprogrammed to generate beneficial phenotypes such as conditional sterility or pathogen resistance. The widespread success of site-specific nucleases such as transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 in model organisms also suggests that reprogrammable gene drive systems based on these nucleases may be capable of spreading such beneficial phenotypes in wild mosquito populations. Using the mosquito Aedes aegypti, we determined that mutations in the FokI domain used in TALENs to generate obligate heterodimeric complexes substantially and significantly reduce gene editing rates. We found that CRISPR/Cas9-based editing in the mosquito Ae. aegypti is also highly variable, with the majority of guide RNAs unable to generate detectable editing. By first evaluating candidate guide RNAs using a transient embryo assay, we were able to rapidly identify highly effective guide RNAs; focusing germ line-based experiments only on this cohort resulted in consistently high editing rates of 24–90%. Microinjection of double-stranded RNAs targeting ku70 or lig4, both essential components of the end-joining response, increased recombination-based repair in early embryos as determined by plasmid-based reporters. RNAi-based suppression of ku70 concordant with embryonic microinjection of site-specific nucleases yielded consistent guide insertion frequencies of 2–3%, similar to traditional transposon- or φC31-based integration methods but without the requirement for an initial docking step. These studies should greatly accelerate investigations into mosquito biology, streamline development of transgenic strains for field releases, and simplify the evaluation of novel Cas9-based gene drive systems.

Aedes  |  CRISPR  |  TALEN  |  transgenic  |  recombination

Mosquitoes transmit disease agents that cause malaria, dengue fever, chikungunya, and more. Conventional control strategies based on insecticides, bed nets, source reduction, vaccines, and drug treatments are now being complemented by transgenic approaches, which may soon include pathogen-resistant mosquito strains. Although development of these strategies is ongoing, field releases of first-generation technologies have had some remarkable successes (1, 2). Significant effort is currently being expended to develop transgene-based approaches with so-called “gene drive” systems designed to increase the incidence of inheriting specific alleles in a target population (3). Although a number of theoretical systems have been proposed, only one based on the site-specific homing endonuclease I-SceI has been shown to successfully drive a genetic modification into a mosquito population in large cage studies (4).

Unlike homing endonucleases, which are difficult to re-engineer, site-specific nucleases based on the type II clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 are unrivaled in the ease with which they can be tailored to a wide range of potential target sequences, already leading to the proposal of alternative gene drive designs and regulatory structure based on these more facile genetic tools (5, 6). Identified in type II Streptococcus pyogenes as a heritable immune defense mechanism against viruses and plasmids (7), endogenous CRISPR loci are comprised of 30–40 nucleotide (nt) variable sequences separated by short direct repeats. CRISPR locus transcripts are used to generate short CRISPR RNAs (crRNAs) that act in conjunction with a common transactivating-RNA (trRNA) to guide Cas9 endonuclease to the complementary double-stranded DNA (dsDNA) target site. An advancement in this technique has led to coupling of the crRNA and trRNA into a single chimeric guide RNA (8), resulting in a two-component system to cleave an 18- to 20-nt target sequence. However, the effectiveness of the CRISPR/Cas9 system in disease vector mosquitoes has not yet been evaluated.

Transcription activator-like effector nucleases (TALENs) are also a two-component site-specific nucleic acid system and have been used in genomic editing of a number of different genomes including the mosquitoes Ae. aegypti (9) and Anopheles gambiae (10). TALENs are hybrid proteins consisting of a TAL DNA binding domain (discovered in the plant pathogenic bacteria Xanthomonas) and the FokI nuclease domain (11). An individual TALEN consists of a left and right monomer protein, each with its own FokI nuclease domain that, on dimerization, induces

**Significance**

Mosquitoes are vectors of both parasites and viruses responsible for high-impact diseases including malaria, dengue, and chikungunya. Novel interventions based on genetic modification of the mosquito genome are currently being developed and implemented. To comprehensively exploit such interventions, detailed knowledge of mosquito physiology, genetics, and genome engineering are required. We developed and validated a two-step process for performing high-efficiency site-specific insertion of genetic material into the mosquito genome by first evaluating candidate site-specific nucleases in a rapid format, followed by germ line-based editing where the choice of DNA repair response is constrained. This model should significantly accelerate both basic and applied research concerning disease vector mosquitoes.

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dsDNA breaks. Initially, WT versions of the FokI domain were used in TALEN architectures; however, activity at off-target sites was attributed to the homodimerization of left or right TALEN monomers (left-left or right-right) (12). Targeted mutations of the FokI dimerization plane, R487D (FokI-DD) or D483R (FokI-RR), established a requirement for heterodimerization of the TALEN left and right protein monomers that significantly reduced off-target activity (13, 14). However, these improvements also decreased target activity by reducing dimerization energy (15). Introduction of secondary mutations that added oppositely charged amino acids to each version, R487D and N496D (FokI-DDD) or D483R and H537R (FokI-RRR), restored activity to near WT levels while still maintaining decreased off-target activity (15). The efficacy of each of the different TALEN architectures (WT vs. DD/RR vs. DDD/RRR) has not yet been investigated in either Ae. aegypti or An. gambiae.

Early attempts at generating transgenic mosquitoes using transposon-based systems resulted in some rare successes (16–18), but efficient methods of mosquito transformation were not developed until almost 10 years later (19, 20), ushering in the current era of mosquito genome manipulation. This technology has led to the generation of mosquito strains with pathogen-resistant (21–23), conditional sterile (24, 25), flightless (26), and sex-biased (27) phenotypes. Similarly, rare successes have been reported for homology-directed repair (HDR) of nuclease-induced double-stranded DNA breaks (DSBs) using an exogenous donor template, documented in both the dengue vector, Ae. aegypti (28, 29), and the malaria vector, An. gambiae (30), but at rates less than 0.1%. Although site-specific nucleases may increase both the ease and efficiency with which DSBs can be introduced into the mosquito genome, the fact that both nonhomologous end-joining (NHEJ) and HDR are in competition for DSB repair, with rates less than 0.1%, although site-specific nucleases have similar activity as those with the WT domain. However, in the presence of a matching final repeat, DDD/RRR TALENs had similar activity as those with the WT domain. However, in the presence of a mismatched final 1/2 repeat, the activity of TALENs containing a DD/RR FokI was significantly lower than those with a WT or DDD/RRR mutation (Fig. 1 B and C), consistent with previous findings in Drosophila (37). In the presence of a matching final repeat, DDD/RRR TALENs had similar activity as those with the WT domain. However, in the presence of a mismatched final 1/2 repeat, the presence of the DDD/RRR mutation reduced TALEN activity (Fig. 1 C).
RRR mutation on editing efficiency appears to be small, but if TALEN binding is weakened (in this case through the mismatched position), then the DDD/RRR mutations have a larger influence on the editing rate. With this in mind, six TALENs were reassembled to contain a WT FokI domain. With two of the six TALEN pairs (lig4-A, lig4-B), we were still unable to detect gene editing by HRMA. However, the presence of the WT domain in the remaining four TALEN pairs resulted in a substantial increase in gene editing, both in transient embryo assays (Fig. S1) and in germ-line experiments for the TALEN targeting dcr2-exon5 (Table S1). This indicates that the architecture of the FokI domain influences the rate of TALEN cleavage at target sites in the mosquito genome.

Given the substantially lower cost and ease of use of the CRISPR/Cas9 gene editing system (38), we developed a series of short guide RNAs targeting the *Ae. aegypti kmo* gene (Fig. S2A). Following injection of purified Cas9 protein, crRNA, and trRNA that target two overlapping sites within the previously validated TALEN target site (kmo-exon5), we readily observed gene editing by HRMA (Fig. S2B). Subsequent experiments demonstrated that a single fused sgRNA was more effective at editing an identical target sequence than the crRNA + trRNA combination (Fig. S2C). Targeting six other sequences in the same exon revealed substantial variability in sgRNA-directed editing, with three sgRNAs exhibiting higher levels of editing (Δ fluorescence > 0.15), two sgRNAs exhibiting lower editing rates (Δ fluorescence = 0.04–0.05), and one indistinguishable from those of the control (Fig. S2D and E). Similar results were obtained when substituting Cas9 mRNA for purified protein, although editing rates were generally higher with the latter (Fig. S3).

Although the CRISPR/Cas9 system was clearly effective in *Ae. aegypti*, we considered that editing rates may vary across the genome. Therefore, we designed more than 40 additional sgRNAs targeting five different genes (loqs, rzd2, ku70, lig4, and nix) and evaluated their editing potential in transient embryo assays using HRMA (SI Appendix). Effective sgRNAs (Δ fluorescence > 0.05) were identified for each of the targeted genes, but success rates varied widely (range, 10–70%) on a gene-to-gene basis (Table 1). When compared, highly active sgRNAs did not reveal any consensus (Fig. S4), nor did they conform to previously published design criteria (39). The most effective sgRNAs for each target gene were subsequently used in germ-line experiments to determine rates of heritable mutations. In all cases, sgRNAs that were previously validated in transient embryo assays induced heritable mutations at high frequency (24–90%; Table 2). In the case of *kmo*, somatic activity was readily observed (Fig. 2 A–D), with both insertions and deletions detected at the target site (Fig. 2E). Even in the absence of a visible phenotypic marker, mutations in the other target genes were easily detected in pooled G1 progeny using HRMA (Fig. S5). Thus, we conclude that the CRISPR/Cas9 system is highly efficient at editing the genome of *Ae. aegypti*, and prescreening candidate sgRNAs in transient embryo assays consistently identified highly functional nucleases.

To determine the effect of knocking down *ku70* on HDR-based insertion of a transgene in the germ line, embryos were injected with either the *kmo-exon5* or *dcr2-exon5* TALENs, a donor vector, and either no dsRNA or dsRNA targeting EGFP or *ku70* (Fig. 4A). The donor vector consisted of the *PUb*-EGFP reporter and 1–2 kb of flanking sequence homologous to the respective target sites. EGFP+ progeny were recovered from all experiments where *ku70* dsRNA was present but not from experiments with the same TALENs/donors when no dsRNA or EGFP dsRNA was included in the injection mix (Table 3) in a manner that was highly significant (Fisher’s exact test, P = 0.0119). EGFP fluorescence in transgenic larvae was easily observed (Fig. 4B). Precise insertion of the *PUb*-EGFP cassette at the TALEN target sites was confirmed with PCR (Fig. 4C). The minimum rate of gene knockin over three separate experiments

| Vectorbase ID | Gene | No. sgRNAs | HRMA* | HRMA** (>0.05) |
|---------------|------|------------|-------|----------------|
| AAEL008879    | kmo  | 8          | 7     | 5              |
| AAEL017365    | lig4 | 10         | 2     | 1              |
| None          | ku70 | 4          | 3     | 1              |
| AAEL008687    | loqs | 12         | 11    | 3              |
| AAEL011753    | rzd2 | 10         | 6     | 1              |
| None          | nix  | 8          | 4     | 2              |
| Total         |      | 52         | 33 (63%) | 13 (25%) |

Table 1. Effectiveness of sgRNAs between and within genes

Previous studies that examined the promiscuity of the CRISPR-Cas9 system have found evidence of a seed sequence of 8–12 bp largely responsible for DNA binding (38). Single mismatches between sgRNA and the target site within this region significantly decrease the likelihood of DNA cleavage, whereas those with a conserved seed sequence and protospacer adjacent motif site (NGG) may be subject to off-target dsDNA breaks. Using the flyCRISPR target finder (40), we identified the most likely potential off-target sites in the mosquito genome for four highly active sgRNAs and analyzed them using HRMA. No evidence of off-target cleavage events could be identified (Fig. S6).

The generation of loss-of-function mutant organisms necessitates genotyping of individuals from successive generations, both for propagation and experimentation. Although feasible for a small number of strains, this becomes increasingly onerous as the number of strains increases. Multiple groups have reported the ability to insert exogenous sequences at specific sites in the mosquito genome to facilitate both the maintenance and identification of mutant stock strains (28–30). However, in none of these cases was the efficiency of the homologous recombination sufficient for routine use. We hypothesized that loss of components of the NHEJ pathway would increase the frequency of repair by the *Ae. aegypti* HDR pathway (33), thereby increasing the efficiency of gene insertion into TALEN- or CRISPR-induced DSBs in the mosquito. Unfortunately, *lig4* mutant alleles were quickly lost from our colonies (Fig. S7), suggesting that *Ae. aegypti* may not be able to tolerate complete loss of NHEJ. Thus, for our experiments, we used RNAi to temporarily suppress NHEJ activity in the early embryo. Mosquito embryos were injected with reporter constructs for monitoring either the SSA (a proxy for HDR; Fig. 3A) or NHEJ DSB repair pathways (Fig. 3B), along with dsRNA corresponding to either EGFP (control) or NHEJ components *lig4* or *ku70*. DSB induction was mediated by the homing endonuclease Y2-I-Anil (35). As expected, knockdown of NHEJ components increased recombination-based repair (Fig. 3C). However, depleting *ku70* decreased the rate of NHEJ-based repair, whereas diminished *Lig4* increased both SSA and NHEJ activity (Fig. 3D). Similar effects were observed with a second dsRNA molecule targeting *ku70* or by targeting *ku80* (Fig. S8).

Table 2. CRISPR mutagenesis is highly efficient in *Ae. aegypti*

| Target | sgRNA No. injected | Total G0 | Fertile G0 | Mutant G0 |
|--------|--------------------|----------|------------|-----------|
| kmo    | 519*               | 830      | 170 (20.5%)| 84 (49.4%)| 27/85 (32.1%)|
| lig4   | #7                 | 1,110    | 176 (15.8%)| ND        | 8/23 (34.8%)|
| ku70   | #3                 | 613      | 172 (28%)  | 77 (44.8%)| 10/42 (23.8%)|
| rzd2   | #6                 | 565      | 121 (21.4%)| 51 (42.1%)| 20/33 (60.6%)|
| loqs   | #3                 | 548      | 124 (22.6%)| 62 (50%)  | 27/30 (90.0%)|

ND, not determined.

*Number of G0 individuals that produced mutant progeny/total.

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Suppression of NHEJ components increases recombination activity in...
FokI in Drosophila (37), we observed a substantial decrease in TALEN activity with this architecture in the mosquito Ae. aegypti. Thus, in scenarios where TALENs are to be used to modify the genomes of vector species, the WT FokI domain appears to be the most effective at inducing germ-line mutations and HDR events.

Although both TALENs and CRISPR/Cas9 were highly effective in generating targeted mutations, throughput was substantially higher for the CRISPR/Cas9 system. Although the majority of sgRNAs resulted in some degree of editing, we recommend that only those displaying the highest levels of activity be used in germ-line experiments. Thus, we propose a two-step approach for gene editing experiments in difficult to manipulate organisms such as mosquitoes, whereby a large cohort of sgRNAs are evaluated in vivo (in our case, early embryos), with only highly ranking candidates going forward to germ line-based experiments. Combining this two-step approach with the transient suppression of NHEJ components, such as Ku70, indicates that experiments involving the site-specific integration of transgenes into disease vector mosquitoes should be greatly accelerated.

**Materials and Methods**

**Plasmid Construction.** TALEN plasmids targeting the Ae. aegypti kmo gene were as previously described (46). TALEN expression plasmids targeting dcr2, ago2, and lig4 genes were generated by the University of Utah Mutation Generation and Detection (MGD) Core using the TALEN Golden Gate kit (11), with modifications as previously described (36, 47). Briefly, TALEN target sites were designed using TALEN Effector Nucleotide Targeter 2.0 (48). Design criteria for target site selection were as follows: (i) spacer length of 14–18; (ii) TALE repeat array length of 15–21; and (iii) applying all additional options that restrict TALEN target site choice. The uniqueness of potential TALEN target sequences was determined by performing a BLAST analysis against the Ae. aegypti genome, ensuring that no highly similar left and right binding sites were in close proximity.

**PCR Confirmation of HR Events.** To confirm that the transgene was correctly inserted into the gene of interest (dcr2), genomic DNA was extracted from a single male G0 (EGFP+) using the Macherey-Nagel Tissue Nucleospin Kit. Primers 5′-ttttggatccTGCATCAAAATCCTTCCAATGACGGAAAT-3′ (lowercase bases indicate added restriction site) and 5′-TTCATCTCATCATTGTTGTTTGGT TTGTTCC-3′ (1,795 bp) were used to amplify the target region using Platinum PfX polymerase (Invitrogen) (94°C for 30 s, 35 cycles of 94°C for 10 s, 55°C for 30 s, and 68°C for 2 min, and 68°C for 10 min). The resultant amplicon was gel-purified and sequenced.

**Mosquitoes and Embryonic Injections.** Ae. aegypti mosquitoes from both Liverpool (LVP) and UBCaREFGFP#P17A (49) strains were maintained in an insectary at 28°C and 60–70% humidity, with a 14/10 h day/night light cycle. All photographs of larvae and adult mosquitoes were obtained using a Leica MZ16FL microscope with a Canon Powershot S3 IS digital camera. Embryonic injections were performed as described previously (46). For TALEN-based editing experiments, injection mixes contained 0.3 μg/μL of each TALEN expression construct. For HDR germ-line experiments, dsRNA (see SI Appendix for oligonucleotides and methods used for preparing dsRNA; 0.1 μg/μL was coinjected with TALENs (0.2 μg/μL each) and the respective donor construct (0.2 μg/μL). G0 survivors were mated with the parental strain and progeny screened for EGFP expression. SSA and NHEJ plasmid assays in mosquito early embryos were performed as previously described (46). The NHEJ plasmid assay differed from the SSA assay only in the reporter plasmid used; the Firefly luciferase ORF was interrupted by the insertion of an a-AniI recognition site (see SI Appendix for details). LVP embryos were injected with either SSA or NHEJ assay constructs (0.2 μg/μL), a plasmid construct coding for Y2-I-AniI homing endonuclease (0.2 μg/μL), and a normalization control plasmid (pKhs82-Renilla) encoding Renilla luciferase (0.2 μg/μL) and snap-frozen at 48 h after injection. Embryos were homogenized, and luciferase values were determined. For CRISPR/Cas9, an injection mix was assembled containing 0.1 μg/μL of each sgRNA and the desired concentration of Cas9 mRNA or protein (and trRNA, where applicable) at 0.6 μg/μL unless otherwise stated. The injection mix with Cas9 protein was incubated at 37°C for 20 min and then stored on ice for the duration of the injections. After 24 h, each sample (100–130 embryos) was transferred to a microcentrifuge tube and snap-frozen in liquid nitrogen.

**Table 3. Transgene insertion following suppression of NHEJ**

| Target  | HR arms | dsRNA | No. injected | No. G0 (%) | No. G0 pools | No. G1 screened | EGFP+ (#) | Rate† |
|---------|---------|-------|--------------|------------|--------------|----------------|-----------|-------|
| kmo     | 0.6/0.5 kb | —     | 1,010        | 109 (11%)  | 6            | >11,000        | 0         | <1.8% |
| kmo     | 2.2/1.7 kb | —     | 1,030        | 152 (15%)  | 8            | >12,000        | 0         | <1.3% |
| kmo     | 2.2/1.7 kb | egfp  | 1,130        | 192 (17%)  | 9            | ~33,000        | 0         | <1.0% |
| kmo     | 2.2/1.7 kb | ku70 #1 | 1,090       | 166 (15%)  | 9            | ~33,000        | P1 (1), P3 (36) | 2.4% |
| kmo     | 2.2/1.7 kb | ku70 #2 | 1,035       | 118 (11%)  | 5            | ~16,000        | P2 (8)    | 1.7% |
| dcr2    | 1/1.7 kb | egfp  | 1,138        | 237 (21%)  | 10           | ~46,000        | 0         | <0.8% |
| dcr2    | 1/1.7 kb | ku70 #2 | 1,050       | 144 (14%)  | 7            | ~23,000        | P1 (7), P2 (2) | 2.7% |
| Total   |         |       |              |            |              |                |           |       |
| kmo     | 3,298   | -egfp | 3,175        | 428 (13%)  | 21           | ~72,000        | 0         | <0.3% |
| kmo     |         | ku70  | 3,175        | 428 (13%)  | 21           | ~72,000        | 0         | 2.3% |

†Minimum rate of gene knockin based on 50% fertility.
CRISPR/Cas9 Reagents. Purified Cas9 protein was obtained from PNA Bio. The lyophilized pellet was dissolved in nuclelease-free water to a concentration of 2 μg/μL. Solution was stored at −80 °C. Cas9 mRNA was generated using pRGEN-Cas9-CMV (PNA BIO). One microgram of linearized DNA was used as the template for an in vitro transcription reaction using the mMESSAGE mMACHINE T7 Ultra kit (Ambion). The reaction was incubated at 37 °C for 6 h, DNase treated at 37 °C for 30 min, polyA-tailed at 37 °C for 1 h, and purified using the MEGAClear Clean-Up kit (Life Technologies). sgRNAs19* and sgRNAs19 crRNA, and the corresponding trRNA were obtained from PNA BIO. Lyophilized solution was dissolved in nuclelease-free water containing 1 μg/mL, aliquoted, and stored at −80 °C. Otherwise, guide RNAs were generated as described in ref. 8. Using the oligonucleotides listed in SI Appendix.

HRMA Assays. Genomic DNA was extracted from adults or embryos by using the Nucleospin Tissue kit (Macherey-Nagel). Alternatively, PCR was directly performed on adult female mosquitoes using the TALE-PCR kit (Thermo Scientific) as described previously (46). HRMA primer assays were designed to generate amplicons of 80–120 bp traversing the CRISPR/TALEN target site using the LightScanner Primer Design Software (Biofire Defense). A complete list of HRMA primers used is presented in SI Appendix. PCR was performed in 10-μL reactions with 2 μL genomic DNA, 4 μL 2.5x LightScanner MasterMix (Biofire Defense), 200 nM of each primer, and 2 μL nuclelease-free water. Reactions were conducted within a 96-well plate and sealed with optical film before cycling in a BioRad thermal cycler (95 °C for 2 min; 40 cycles of 94 °C for 30 s, 65 °C for 30 s; 94 °C for 30 s; 25 °C for 30 s; and 4 °C hold). Thermal melt profiles were generated on a LightScanner Instrument (Biofire Defense) (60–95 °C, hold 57 °C) and analyzed using the Lightscanner Cal-it 2.0 software. Samples showing mutations using HRMA were reamplified to generate a larger amplicon that was sequenced directly; Poly Peak Parser (50) was used to identify specific indels.

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