TALEN-Based Gene Disruption in the Dengue Vector
Aedes aegypti

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Introduction

Vector-borne diseases such as malaria and dengue fever remain large public health burdens, and novel interventions are still needed. The development of new methods of vector control would be aided substantially by a more detailed genetic and biochemical understanding of many critical behaviors such as development, host seeking, bloodfeeding and vector competence. Though the genomes of several disease vector mosquitoes have been sequenced, many mosquito-specific genes remain without any functional annotation, and there is much still to be learned with regards to understanding the basis for these key behaviors. Of the disease vector mosquitoes that have a sequenced genome, Aedes aegypti, the primary vector for dengue viruses, is probably the most tractable due to the ease of adapting new strains to the laboratory environment and the ability to delay the hatching of developed embryos for months at a time. Progress in the field of site-specific gene editing with meganucleases indicates that these systems relate to the difficulty of assembling/reengineering traditional organisms, including Aedes aegypti.

Though other meganucleases such as homing endonucleases and zinc finger nucleases have been used to perform custom editing of various genomes (reviewed in [1,2]), their adoption by the research community has been limited at best. Limitations with these systems relate to the difficulty of assembling/reengineering these molecules to recognize new target sites due to the strong context-dependence of their DNA-binding regions. In contrast, transcription activator-like elements (TALEs) from the plant pathogenic bacteria Xanthomonas contain a simple, context independent DNA binding region [3,4]. In these molecules, DNA binding is conferred by a series of 34 amino acid repeats, differing only at two positions (the repeat variable diresidue, or RVD), where each RVD specifies a given target nucleotide [3,4]. Fusion of TALE repeat domains to the nuclease editing in any disease vector species.

Abstract

In addition to its role as the primary vector for dengue viruses, Aedes aegypti has a long history as a genetic model organism for other bloodfeeding mosquitoes, due to its ease of colonization, maintenance and reproductive productivity. Though its genome has been sequenced, functional characterization of many Ae. aegypti genes, pathways and behaviors has been slow. TALE nucleases (TALENs) have been used with great success in a number of organisms to generate site-specific DNA lesions. We evaluated the ability of a TALE pair to target the Ae. aegypti kmo gene, whose protein product is essential in the production of eye pigmentation. Following injection into pre-blastoderm embryos, 20–40% of fertile survivors produced white-eyed progeny that failed to complement an existing khw mutation. Most of these individuals produced more than 20% white-eyed progeny, with some producing up to 75%. Mutant alleles were associated with lesions of 1–7 bp specifically at the selected target site. White-eyed individuals could also be recovered following a blind intercross of G1 progeny, yielding several new white-eyed strains in the genetic background of the sequenced Liverpool strain. We conclude that TALENs are highly active in the Ae. aegypti germline, and have the potential to transform how reverse genetic experiments are performed in this important disease vector.
We found that TALEN-based targeting of the *Ae. aegypti* kmo allele was a highly efficient process, with 20–40% of fertile G\textsubscript{0} females producing new kmo mutant alleles in a complementation assay with the *kh\textsuperscript{w}* strain. Mutation rates were sufficiently robust that blind G\textsubscript{1} intercrosses resulted in several new white-eyed strains (*Lvp\textsuperscript{kmo/+}*) developed entirely within the genetic background of the sequenced Liverpool (*Lvp*) strain of *Ae. aegypti*. These results suggest that TALE-based applications are poised to revolutionize the study of *Ae. aegypti* genetics and allow the development of new genetic methods to disrupt disease transmission by this important mosquito vector.

**Results**

**Selection of TALEN target site and transient embryo assay**

Full-length cDNAs for both the wt and *kh\textsuperscript{w} (kmo)* gene (AAEL008879) have been characterized, with an in-frame deletion of 162 bp implicated as the causative mutation in the *kh\textsuperscript{w}* strain [18]. The KMO protein is predicted to contain transmembrane domains near both the N and C termini, with the majority of the protein located on the cytoplasmic face of the membrane (Fig. 1A). Alignment of the *kmo* cDNA described by Han et al [18] to the *Ae. aegypti* genome assembly revealed a structure consisting of seven exons (Fig. 1A). Interestingly, the proposed 162 bp deletion corresponds precisely to exon 6, suggesting that the *kh\textsuperscript{w}* phenotype may in fact be due to the failure to correctly splice in this exon. Indeed, sequencing of genomic DNA from this region from both *kh\textsuperscript{w}* and *Lvp* strain mosquitoes revealed an 11 bp deletion in the splice acceptor site of exon 6 only in the *kh\textsuperscript{w}* strain (Fig. S1). As the loss of exon 6 was sufficient to eliminate KMO activity, we designed our TALEN pair to cleave the region just upstream of the exon 5–6 junction. A frameshift mutation at this deletion in the splice acceptor site of exon 6 only in the *kh\textsuperscript{w}* strain is expected to lead to translation in the +3 frame FF-luc ORF at the FF-luc AUG in the first repeat, resulting in a truncated protein. Fourteen additional AUG codons are present prior to the full-length +2 frame FF-luc ORF to minimize read-through translation. Double-stranded DNA break induction by the introduced TALEN pair (lightning shape) followed by SSA-mediated repair restores the FF-luc ORF.

**Identification of new TALEN-generated kmo alleles through lack of complementation with kh\textsuperscript{w}**

To detect heritable gene editing, we injected the *kmo*-targeting TALEN pair into pre-blastoderm embryos of the black-eyed Liverpool (*Lvp, kmo/+ (kmo/+)* strain and screened the progeny of the surviving individuals for white eyes. As the *kh\textsuperscript{w}* phenotype is completely recessive, injected survivors were mated to *kh\textsuperscript{w} (kmo/ kmo)* mosquitoes in order to detect new mutant alleles. A test cross between untreated *Lvp* and *kh\textsuperscript{w}* strains demonstrated that 100% of progeny retained wild-type eye color (Table 1), confirming that our *Lvp* strain was free from rare *kmo* mutant alleles that might otherwise go undetected. In contrast, following injection of the TALEN constructs, white-eyed progeny were identified in seven of nine pools in experiment 1, and all three pools in experiment 2 (Table 1).

Since most of the pools produced white-eyed progeny, it seemed likely that by pooling G\textsubscript{0} individuals (a strategy common in *Ae. aegypti* transgenic experiments, due to the low rate of transposon-based transformation) we may have been underestimating the rate of mutation accumulation for both wild-type and *kh\textsuperscript{w} (kmo/+)* backgrounds.
amplification of only the deletions that spanned at least one of the PCR primers, allowing (91%) (Fig. 2A). The remaining two cases may represent larger confirmed the existence of deleted bases (1–7 bp) in 21 of 23 cases frame deletions were also found: deletions recovered represented frame-shift mutations, three in- addition to Lvp strain mosquitoes. Offspring from this cross identified that TALEN-based gene editing is a highly efficient process in the strain that failed to provide complementation. However, such a luxury would not be found in most circumstances, where investigations will focus on targeting new genes in order to identify novel phenotypes. Likewise, gene editing experiments will likely need to be performed entirely within the strain of study, without the introgression of confounding genetic material from unrelated and highly inbred strains. To determine if we could identify novel kmo mutations without the assistance of the kmo” complementation assay, we injected the kmo-targeting TALEN pair into Lvp embryos, and this time backcrossed the surviving individuals to Lvp strain mosquitoes. Offspring from this cross were 100% black-eyed; siblings within each family were intercrossed to obtain G2 progeny. From just 10 fertile G0 founders, we identified three that produced white-eyed progeny in the G2 generation (Table 4). The frequency of white-eyed individuals in the G2 generation ranged from 4.6–10.4%. This is consistent with an initial mutant allele frequency of 21–32% in the G1 generation, similar to our prior experiments (Table 3). Sequencing of the TALEN target site in white-eyed G2 individuals revealed genetic lesions consistent with a loss of function phenotype in all cases (Fig. 2B). In fact, we recovered four independent lesions from these three founders, suggesting that a single individual male produced multiple sperm with independent deletion events. Phenotypically, Lvp” individuals were indistinguishable from kmo” strain mosquitoes at all life stages (Fig. 3). Thus, we conclude that TALENs can be used to edit the Ae. aegypti genome in a strain-independent manner at high efficiency, and that individuals homozygous for an expected mutation can be recovered at the G2 stage at useful frequencies, even in the absence of any screening at the G1 (hemizygous) state.

Discussion

Research efforts using model organisms such as D. melanogaster, S. cerevisiae, C. elegans and A. thaliana have benefitted tremendously from the availability of genetic stock centers housing large collections of mutant strains; whereas reverse genetic experiments in non-model organisms have been more limited. While the development of RNAi technology has enabled some such experiments to move forward, this technology is limited by low penetration of injected double-stranded RNA into some tissues [20], gene by gene variation in the degree and timing of knockdown (Adelman, unpublished observations), and off-target effects resulting from the large pool of siRNAs generated from the introduced precursor molecules [21]. In contrast, the ability to directly and specifically disrupt a gene of interest offers the possibility to perform intricate reverse genetic experiments on any gene, in any organism. We confirm that TALEN-based gene disruption can be a highly efficient process in Ae. aegypti, with editing rates between 20–40%. This is an order of magnitude greater than both traditional transposon-based transformation [22] and phiC31-based recombination [23], and offers up the

### Table 1. Generation of new mutant kmo alleles from pooled G0 populations.

| Exp. | # embryos injected | # G0 (%) | G2 gender | Pool ID | Phenotype* |
|------|--------------------|----------|-----------|---------|------------|
|      |                    |          |           | wt      | we         | %we      |
| Neg. control | n.a. | n.a. | n.a. | n.a. | 8970 | 0 | 0 |
| #1   | 1020              | 187 (18.3%) | ♂ | P1    | 1200 | 64 | 5.3% |
|      |                   |          | ♂ | P2    | 1350 | 70 | 5.2% |
|      |                   |          | ♂ | P3    | 250  | 24 | 9.6% |
|      |                   |          | ♂ | P4    | 1700 | 56 | 3.3% |
|      |                   |          | ♂ | P5    | 900  | 23 | 2.6% |
|      |                   |          | ♂ | P6    | 1100 | 11 | 1.0% |
|      |                   |          | ♂ | P7    | 2400 | 0  | 0%  |
|      |                   |          | ♂ | P8    | 2700 | 0  | 0%  |
|      |                   |          | ♂ | P9    | 1400 | 56 | 4.0% |
| #2   | 1010              | 195 (19.3%) | ♂ | B1    | 1800 | 130 | 7.2% |
|      |                   |          | ♂ | B2    | 200  | 31 | 15.5% |
|      |                   |          | ♂ | B3    | 36   | 3  | 8.3% |

*wt, wild-type; we, white-eyed.

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possibility that TALE-based experiments will be much more amenable to moderate or higher throughput applications than what has been achieved over the past decade with these less robust genetic systems.

In our experiments, we only examined a single TALEN pair. Thus, it is possible that not every such pair will achieve the same or similar activity. However, the success rates we observed are similar to those described in many other organisms, including several other insects [6,8,9]. Given the success of others in large scale TALEN pairs screens [24,25,26,27,28], the rate of TALEN failure appears to be acceptably low (≤20%). The primary difficulty with developing new TALEN pairs to target genes of interest is the time and effort required to assemble the numerous TAL repeat constructs. However, the recent availability of many new assembly methods have substantially decreased the time required for developing new TALEN pairs, with full assembly decreasing from 6–8 weeks to 3–24 hrs [24,27,29,30]. We anticipate that if need be groups of TALEN pairs can be screened initially using the SSA assay we described in pre-blastoderm embryos. Others have demonstrated that in vitro SSA results are highly correlated with germline editing activity [7,31]; we have made similar observations with homing endonucleases in Ae. aegypti (Adelman, unpublished). Thus, germline editing experiments can be restricted to those TALEN pairs which perform well in this assay.

TALEN-based editing was associated with small deletions ranging in size from 1–7 bp. This is similar to results obtained in other insects such as the vinegar fly D. melanogaster [6], the silkworm B. mori [7,8] and the cricket G. bimaculatus [9]. This limited deletion size has several favorable consequences; the most significant in our opinion is that we were able to recover essentially the same set of deletions in two independent experiments, where identical 4-bp and 5-bp deletions were recovered in both instances. This indicates that there may be no substantial burden for the long-term maintenance of TALEN-modified mosquito strains. Thus, there is no need for large (expensive) stock centers to house an ever-growing collection of TALEN-modified strains. As long as the TALE-binding sites are made available (or the TALEN constructs themselves), the disruption in question could be re-

Figure 2. TALEN-induced deletions in AAEL008879, the Ae. aegypti kmo gene. Sequenced amplicons obtained from white-eyed individuals were aligned and compared to the wt kmo sequence in the Lvp/khm hybrid genetic background (A) or the Lvp background alone (B). The DNA-binding regions of the right (RH) and left (LH) TALENs are indicated. The three in-frame deletions are indicated (*).
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Figure 3. TALEN-generated kmo alleles phenocopy khm strain mosquitoes. LVP, khm and LVPkm mo mosquitoes imaged as larvae (L4), pupae (P) and adults (A).
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generated at any point in the future, in the most useful genetic background at the time. In the same vein, identical deletions obtained from separate founders could be mixed into a single population, substantially eliminating the influence of any off-target effects possibly occurring within a single founder.

The modularity of TALE-binding domains lends them to applications beyond the generation of double-stranded DNA breaks. Though not addressed directly in our experiments, our data indicate that TALE fusions to other active domains, such as transcriptional activators/repressors [32] or recombinases [33], are certainly worth pursuing in *Ae. aegypti*. Likewise, experiments involving the knock-in of a transgene [31] or single-stranded oligonucleotide [29,34] through homologous recombination may further increase the ever growing utility of TALE-based enzymes in specifically editing the genome of this mosquito.

**Materials and Methods**

**Plasmid construction**

To generate the SSA reporter, a synthetic fragment encoding the first 298 bp of the Firefly luciferase gene and an additional 354 bp spacer region was inserted in between the *PUb* promoter and FF-luc ORF of pGL3Basic/*PUb*-FFluc [16]. The spacer region included a portion of the *Ae. aegypti* *kmo* gene containing the target site. TALEN constructs were obtained from Cellectis Bioresearch (Paris, France). Each TALEN-encoding sequence was placed downstream of the *Ae. aegypti* polyubiquitin promotor through standard cloning procedures. DNA for each of the *PUb*-TALEN plasmids was prepared using the Qiagen Endo-free Maxi-prep kit (Experiment #1) or the Machery-Nagel endo-free midi kit (Experiment #2) as directed by the manufacturer prior to injection into mosquito embryos.

**Mosquito rearing, crosses, and embryonic injections**

*Ae. aegypti* mosquitoes (Lvp and *kk* strains) were maintained in an insectary at 28 °C and 60–70% humidity, with a 14/10 h day/night light cycle. Embryonic injections were performed as described previously [15]. For the transient assay, an injection mix containing the SSA test construct, *PUb*-TALEN and a normalization control in injection buffer [14] were introduced into 1 hr old pre-blastoderm embryos. All plasmids were present at 0.2 mg/ml, for a total DNA concentration of 0.8 mg/ml. Embryos were snap-frozen in liquid nitrogen at 24 hours post injection and lysate prepared for dual luciferase assay (Promega, Madison, WI).

Luciferase activity was determined using the Dual-Luciferase Reporter Assay System with a GloMax-Multi Detection System instrument according to the manufacturer's instructions (Promega, Madison, WI). For germline experiments, *PUb*-TALEN constructs (0.3 mg/ml of each) were similarly introduced into developing embryos. *G₀* survivorship counts were based on the number of individuals emerging as adults. For mating, *G₀* survivors were separated into single vials as pupae; emergent adults were collected each day and transferred into male-only or female-only cages. *G₀* males were anesthetized under CO₂ and mated individually to 5 virgin *kk* or Lvp strain females for two to three days, at which point they were either directly offered a bloodmeal (for Lvp experiments) or combined into families. Groups of *G₀* females were combined with 15–20 males of the appropriate parental strain prior to bloodfeeding and egg collection.

**Table 2. Frequency of TALEN-generated kmo alleles per fertile *G₀* female.**

|          | *G₀* ♀ (total) | *G₀* ♀ (fertile) | # *G₀* ♀ producing kmo progeny | TALEN frequency |
|----------|----------------|-----------------|------------------------------|-----------------|
| P1       | 30             | 12 (40%)        | 3                            | 25%             |
| P2       | 30             | 18 (60%)        | 6                            | 33%             |
| P3       | 25             | 6 (24%)         | 1                            | 17%             |
| Exp1 total | 85             | 36 (42%)        | 10                           | 28%             |
| B1       | 35             | 12 (34%)        | 6                            | 50%             |
| B2       | 30             | 11 (37%)        | 5                            | 45%             |
| B3       | 27             | 6 (22%)         | 2                            | 33%             |
| Exp2 total | 92             | 29 (32%)        | 13                           | 45%             |

**Table 3. Generation of new mutant kmo alleles from single *G₀* females*.**

|          | P1       | P2       | P3       | B1       | B2       | B3       |
|----------|----------|----------|----------|----------|----------|----------|
| wt       | 7        | 16       | 8        | 46       | 34       | 27       |
| we       | 14       | 3        | 24       | 27       | 9        | 29       |
| %        | 67%      | 17%      | 75%      | 59%      | 23%      | 41%      |
| wt       | 15       | 17       | 39       | 19       | 30       | 13       |
| we       | 28       | 5        | 7        | 27       | 9        | 27       |
| %        | 39%      | 13%      | 20%      | 59%      | 23%      | 41%      |
| wt       | 52       | 35       | 39       | 29       | 30       | 13       |
| we       | 6        | 5        | 7        | 7        | 9        | 9        |
| %        | 3%       | 2%       | 20%      | 20%      | 23%      | 41%      |
| wt       | 43       | 35       | 39       | 29       | 30       | 13       |
| we       | 28       | 5        | 7        | 7        | 9        | 9        |
| %        | 39%      | 13%      | 20%      | 20%      | 23%      | 41%      |
| wt       | 9        | 46       | 46       | 19       | 30       | 13       |
| we       | 13       | 27       | 27       | 27       | 9        | 9        |
| %        | 6%       | 59%      | 59%      | 59%      | 23%      | 41%      |

*wt, wild-type; we, white-eyed.
**Each row represents the 1st, 2nd, 3rd, etc... female in each pool that produced one or more kmo mutant progeny.

**Table 2.** Frequency of TALEN-generated *kmo* alleles per fertile *G₀* female.

**Table 3.** Generation of new mutant *kmo* alleles from single *G₀* females*.

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Table 4. Identification of new kmo mutant alleles in the LVP genetic background.

| ID# | G0 gender | G2 gender | G2 eye phenotype  |
|-----|-----------|-----------|------------------|
|     | wt | we | %we |
| 1   | ♀ | 1700 | 0 |
| 2   | ♂ | 1500 | 105 | 6.5% |
| 3   | ♂ | 950 | 110 | 10.4% |
| 5   | ♂ | 650 | 0 |
| 9   | ♂ | 1050 | 0 |
| 12  | ♂ | 750 | 0 |
| 16  | ♂ | 450 | 0 |
| 36  | ♂ | 650 | 0 |
| 50  | ♂ | 800 | 0 |
| 59  | ♂ | 750 | 36 | 4.6% |

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