



Knockout of the MIS0 Gene in the Plasmodium Falciparum Vector Anopheles Gambiae Using the CRISPR-Cas9 System

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Knockout of the *MISO* Gene in the *Plasmodium falciparum* Vector
Anopheles gambiae Using the CRISPR-Cas9 System

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A Thesis in the Field of Biology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

In the course of this thesis I have sought to engineer a line of *Anopheles gambiae* mosquitoes, the primary vector of the African malaria parasite *Plasmodium falciparum*, with the Mating Induced Stimulator of Oogenesis (*MISO*) gene functionally knocked out of the genome. A *MISO* knockout line (*misoKO*) will enhance our ability to detect subtle phenotypes and improve the significance of earlier findings regarding *MISO* influence on egg production, egg development, and tolerance to *Plasmodium falciparum* infection. I have designed and implemented a piggyBac transgenic system to create a CRISPR/Cas9 mediated knockout of the reproductive gene *MISO* in *Anopheles gambiae*. I have isolated one mutant, *misoA3ΔA76*, which will likely be a hypomorphic allele of *MISO*. Mutant *misoA3ΔA76* features a 290bp deletion of 49 amino acids constituting a removal of 25% of the amino acids in the total *MISO* sequence. *MISO* is a highly unstructured protein with no known domains besides an N-terminal secretion sequence. The *misoA3ΔA76* mutant's deletion removes 49 amino acids from the N-terminal unstructured region of the protein, including the putative secretion peptide sequence. The *MISO* knockout lines we are generating will revolutionize the ability of Professor Flaminia Catteruccia's research group to interrogate the function of *MISO* protein activity.

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Chapter I

Introduction

Malaria, a mosquito borne disease caused by the *Plasmodium* parasite, is among the most devastating diseases faced by humans throughout history. After years of consecutive reduction in the incidence of such disease globally, the rate of decline has stalled and for the first time in 15 years, cases have gone up in certain regions of the world (WHO 2017).

In 2016, there was an estimated 216 million reported cases of malaria worldwide, with 445,000 deaths; 68% of them reported in children under 5 years of age (WHO 2017). Additionally, 3.2 billion people were living in regions at risk of malaria transmission, an area spanning 106 countries.

While there are several species in the genus *Plasmodium*, 99% of the fatalities associated with malaria are caused by the species *Plasmodium falciparum*, transmitted by the infectious bite of a female *Anopheles gambiae* mosquito (WHO 2017). Due to the lack of an effective vaccine against *Plasmodium* parasites, and to mixed therapeutic outcomes (WHO, 2017), control of *Anopheles* mosquito vector populations remains the most important avenue for malaria prevention (Bhatt et al., 2015).

Females of the *Anopheles gambiae* are monandrous – mating only once in their lives – a phenomenon that could be leveraged for innovative vector control strategies. Preliminary efforts aimed at interfering with mosquito reproduction have demonstrated a

reduction in disease burden therefore, additional research into the molecular processes governing mosquito reproduction may aid in the development of additional vector control technologies (Benedict et al., 2003, Papathanos et al., 2009).

During copulation, the male *A. gambiae* mosquito transfers a coagulated mating plug into the female. The mating plug is composed of proteins generated by the male auxiliary sexual organ, the male accessory glands (MAGs). The mating plug is transferred along with a hormone cargo named 20-Hydroxyecdysone (20E) into the atrium of the female together with sperm (Baldini et al., 2012). Transferred 20E triggers profound physiological changes in the female Anopheline mosquito (Gabrielli et al., 2014). The onset of these 20E dependent, mating-induced behavioral and molecular changes is referred to as the post-mating switch (Mitchell et al., 2015). These changes are further associated with a reproductive priority shift, from finding a mate to producing offspring (Gillott, 2003). 20E is intimately associated with egg production, egg laying, and conferring a refractory state to further mating (Mitchell et al., 2015, Baldini et al., 2013, Gabrielli et al., 2014). These functions are vital to the *Anopheles* mosquito reproductive cycle and their capacity as vectors for the *Plasmodium* parasite (Mitchell et al., 2015).

The mechanisms by which 20E initiates the variety of phenotypes associated with the post-mating switch are largely uncharacterized. One gene postulated to play a role in the post-mating switch is the gene Mating Induced Stimulator of Oogenesis (*MISO*). Prof. Flaminia Catteruccia's research group has shown that *MISO* has a role in fertility, egg development, and resistance to *P. falciparum* infection (Baldini et al., 2013, Gabrielli et al., 2014, Mitchell et al., 2015). Thus far, all studies on *MISO* by the Catteruccia

Laboratory have been carried out using thoracic injections of RNA interference (RNAi, also known as dsRNA); however, dsRNA injections are time consuming as each individual requires injection, knockdown efficacy can vary across tissues, and injections can be lethal at early stages of development. Generating a targeted knockout mutation in the *MISO* gene would benefit the Catteruccia Laboratory's efforts to study this important putative reproductive gene and support research into the roles the MISO protein plays in mosquito reproductive biology. The focus of this thesis is to develop genetic knockout lines in the *Anopheles gambiae* *MISO* gene using CRISPR-Cas9 technology.

1.1 The Role of MISO in Female Reproductive Biology

The Mating-Induced-Stimulator of Oogenesis protein (MISO) interacts with the 20E that is transferred from the male during mating (Baldini et al., 2013). RNA interference (RNAi) knockdowns of the MISO protein have been shown to decrease egg production to virgin levels in mated adult females (Figure 1.1). The observed decrease in egg production among *MISO* knockdown females stems from a drop in egg development, thus implicating MISO as an important regulator of egg development.

Further, transcript levels of the yolk precursor protein *Lipophorin*, which is loaded into developing oocytes following a blood-meal, were strongly reduced in mated *MISO* knockdown females when compared to mated controls as shown in figure 1.2, providing additional support for the involvement of MISO in egg development (Baldini et al., 2013).

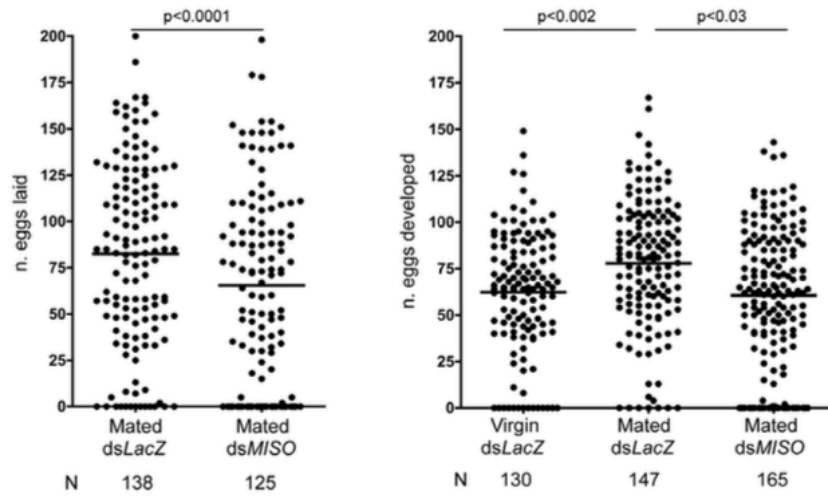


Figure 1.1 RNAi Knockdown of MISO Effects on Fecundity and Egg Development. Mated females injected with dsRNA were blood fed and MISO knockdown decreases egg production to virgin allowed to lay eggs 3 d post-blood-feeding for 4 nights. Control females (dsLacZ) laid on average 82.5 eggs, while dsMISO oviposited a statistically significant lower number of eggs (65.4). The data are representative of three independent replicates. (B) Virgin or mated females injected with dsRNA were blood fed, and eggs developed inside the ovaries were counted 3 d post-blood-feeding without allowing oviposition. Mated dsLacZ produced on average 77.8 eggs, while virgin dsLacZ and mated dsMISO produced a statistically significant lower number of eggs (62.3 and 60.4, respectively). The data are representative of six independent replicates. (Modified from Baldini et al., 2013)

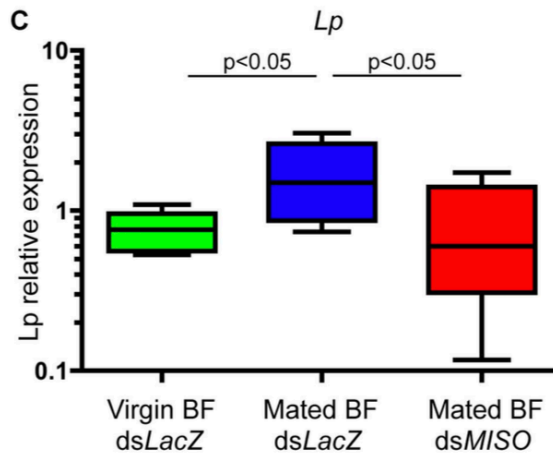


Figure 1.2. RNAi knockdown Effects on Lipophorin mRNA expression levels. Lipophorin mRNA expression in virgin and mated blood fed females injected with dsMISO, control dsRNA (dsLacZ). Expression levels (shown in logarithmic scale) were normalized to the housekeeping gene Rpl19. The box-and-whisker diagrams represent five replicates of pools of 6–10 tissues. (Modified from Baldini et al., 2013)

Perrine Marcenac from Prof. Flaminia Catteruccia's research group has recently shown that MISO also influences tolerance to parasitic infection in the mosquito host. Canonically, *Plasmodium falciparum* infection in females has been correlated with reproductive fitness costs in the form of decreased egg development in many unnatural mosquito-parasite infection pairs; however, the reproductive tradeoff was never observed in natural infection of *A. gambiae* with *P. falciparum* (Hogg & Hurd, 1996, Mendes et al., 2011). Nonetheless, unpublished data from PhD. Student Perrine Marcenac has revealed that MISO-depleted females produce fewer eggs with increasing *P. falciparum* oocyst loads. (Figure 1.3) In healthy wild-type females, infection does not affect egg production. In contrast, knockdown of *MISO* in females results in decreased egg production that is reduced proportional to infection levels, suggesting that MISO plays a possible balancing role in tolerance to *P. falciparum* infection thus, enabling a possible fitness advantage. The development of the *P. falciparum* parasite may have a higher fitness cost in females that lack the MISO protein.

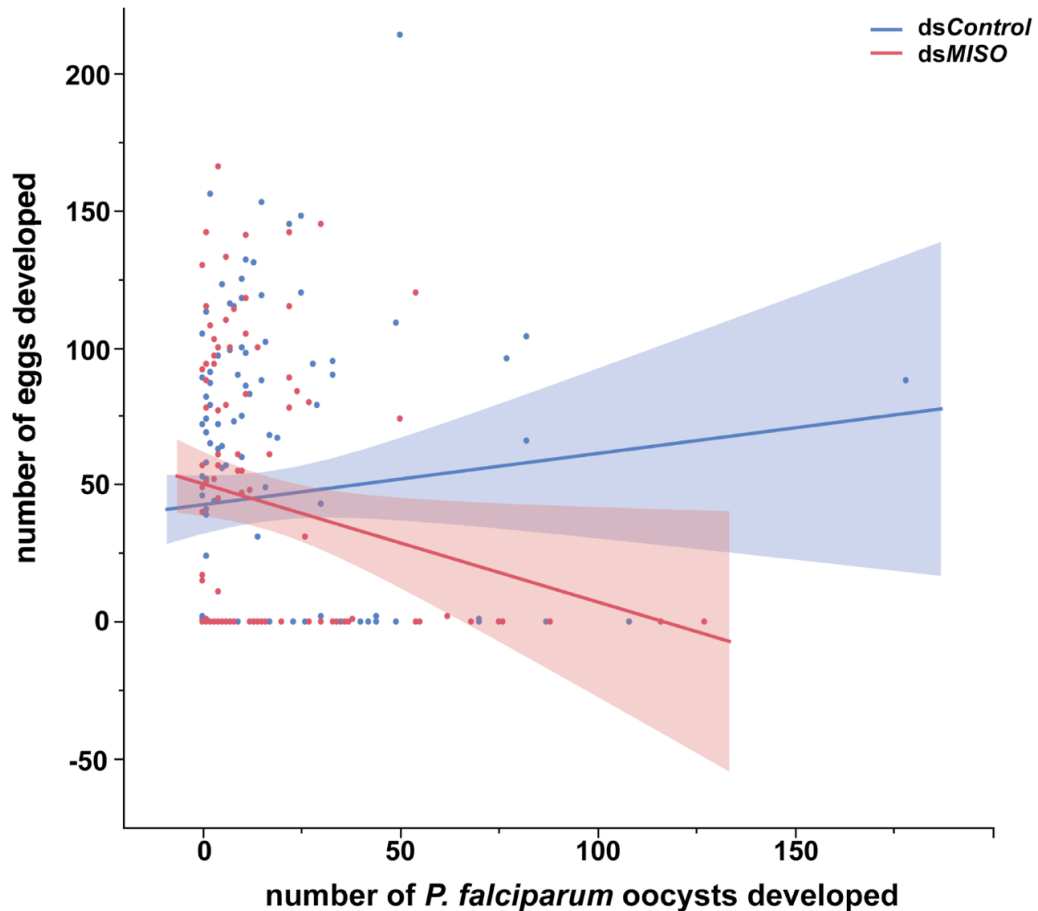


Figure 1.3. MISO Effects on tolerance to *P. falciparum* infection. Females with a *MISO* knockdown produce fewer eggs with an increase in parasite oocysts compared to control. In healthy wild-type females, infection does not affect egg production. In *MISO* knockdown females, egg production is greatly reduced proportional to infection levels. (Modified from unpublished data by Perrine Marcenac)

1.2 Using CRISPR/Cas9 to Create Knockouts in *Anopheles gambiae*

Much of the early work with CRISPR transgenesis in Dipterans was conducted in *Drosophila melanogaster* (Bassett et al., 2013). Genetic manipulation in *Anopheles gambiae*, the primary African Malaria mosquito vector, has historically been notoriously difficult for many reasons. The species' highly polymorphic genome, egg fragility, and a very narrow embryonic injection window can all contribute to making embryonic

transgenesis procedures in *A. gambiae* a difficult proposition for researchers (Vologonsky et al., 2015). Despite these difficulties transgenic tools have been developed in *A. gambiae*, including transgenesis via transposable elements, knockout by TAL-endonucleases, and complex gene drive systems based on CRISPR-Cas9 (Catteruccia et al., 2000, Catteruccia et al., 2005, Hammond et al., 2016, Smidler et al., 2013). I will be applying many of these innovations to generate CRISPR mutations in *Anopheles gambiae*. The knockouts generated during this thesis work will be amongst the very few CRISPR/Cas9 knockouts in *A. gambiae*, as only two are currently in the literature (Hammond et al. 2016, Dong et al. 2018).

In order to generate mutations in *Anopheles*, I used a type II CRISPR/Cas9 system to knockout *MISO* via dual endonuclease cleavage. Successful gene knockouts in *Anopheles* have been previously achieved by generating two transgenic lines, one expressing the Cas9 protein in the germ-line, and the other ubiquitously expressing guide RNAs (Smidler et al., 2013). When the Cas9 and gRNA lines are crossed together, the Cas9 will be armed and targeted by the guide RNAs and can begin to cleave the target locus within the adult germ-line, thus giving rise to mutant individuals in subsequent generations. The Catteruccia Laboratory has adopted the bipartite CRISPR/Cas9 mutagenesis principle and has developed a transgenic line expressing Cas9 nuclease in the adult germ-line that when crossed to lines expressing gRNAs designed to specific target genes will result in targeted mutagenesis. Following the established bipartite principle, I designed a gRNA cassette containing two gRNAs targeting the *MISO* gene in two different regions of its coding sequence (CDS). When a gRNA cassette containing these two gRNAs is inserted into the genome of *Anopheles gambiae*, those mosquitoes

will then express the two guide RNAs included in the cassette. These mosquitoes will then be used to target the *MISO* locus when they are crossed with the Cas9-expressing line, thus generating double-stranded breaks in the *MISO* gene. When repaired, these breaks can produce either large deletions or generate a set of insertions/deletions (indels) within the genes' open reading frame (ORF) (Figure 1.4). Such indels can give rise to frameshift mutations, generating early stop codons in the coding sequence, which are preferred for generating knockout lines.

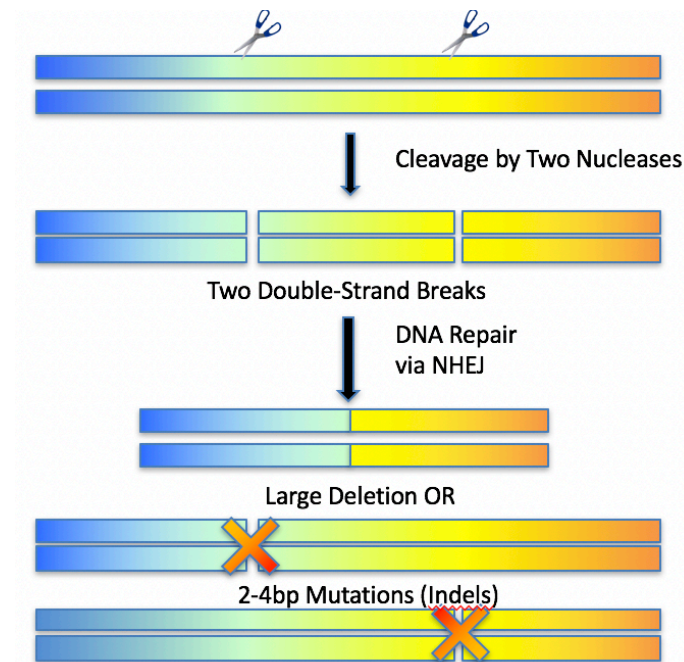


Figure 1.4. Dual endonuclease cleavage results in double stranded breaks that give rise to large deletions or small deletions at the cleavage sites.

Thus far, all the aforementioned efforts by Prof. Flaminia Catteruccia's research group to study *MISO* have utilized RNAi injections to knockdown the *MISO* gene.

Unpublished work from Perrine Marcenac shows a knockdown efficiency of over 85%

for dsMISO in the atrium. The very high atrial efficacy of dsMISO knockdown is contrasted with a 10% to 20% protein knockdown for dsMISO in the ovaries. I hypothesize that a knockout of *MISO* would greatly enhance our research group's ability to interpret and validate the significance of earlier studies using RNAi knockdown techniques, regarding MISO's influence on egg production, egg development, and tolerance to *P. falciparum* infection in *A. gambiae*.

Chapter II

Materials and Methods

2.1. gRNA Target Site Identification

The highly polymorphic nature of the *A. gambiae* genome preemptorily requires checking for SNPs at potential gRNA target sites to ensure efficient and effective binding of putative gRNAs. I screened possible gRNA target sites by amplifying the *MISO* locus via PCR (section 2.1.1) and sequenced the regions of the *MISO* gene in multiple individuals from the laboratory lines X1 (for Φ C31 transgenesis) and G3 (for piggyBac transgenesis). I identified regions with few polymorphisms that could be targeted more efficiently by gRNAs in a greater number of individuals. Based on this analysis of conserved regions in the *A. gambiae* genome, I selected gRNA candidate target sites in more conserved regions of the *MISO* gene in order to avoid regions of higher polymorphism frequency. The candidate target site pool was limited to sites with a Protospacer Adjacent Motif (PAM) sequence, NGG –a critical requirement for Cas9 recognition- within the protein-coding region of the gene. I also searched for target sites that were not too close to the 3' end of the gene to maximize the probability of triggering mRNA degradation by NMD. Candidate target sites were further winnowed down by having to co-localize with a restriction enzyme recognition site to facilitate future PCR confirmation of mutagenesis (section 2.1.5).

I further prioritized gRNAs with two guanines immediately upstream of the PAM

(NGG), as this alternative PAM motif has been shown to facilitate more robust Cas9 cleavage (Deltchava et al., 2011, Roberson, 2015). Only one gRNA target site featured the GGNGG motif and met all other aforementioned parameters. I further used the publicly available computer programs (available at <http://crispr.mit.edu> and <http://www.broadinstitute.org>) to verify that these gRNAs have properties conducive to efficient gRNA cutting. After performing gRNA efficiency analysis, gRNAs were chosen for transgenic expression within our system. gRNA1 targets the start codon region, while gRNA2 targets within exon 2 as shown in figure 2.1 (gRNA1, 5'-TAGAGAGAGATGCGCGCTTT-3'; gRNA2 5'-TACGGCAACGGTGGTGGCGC-3'). gRNA target site 2 features the optimal GGNGG motif and thus had a higher predicted efficacy for DNA cleavage.

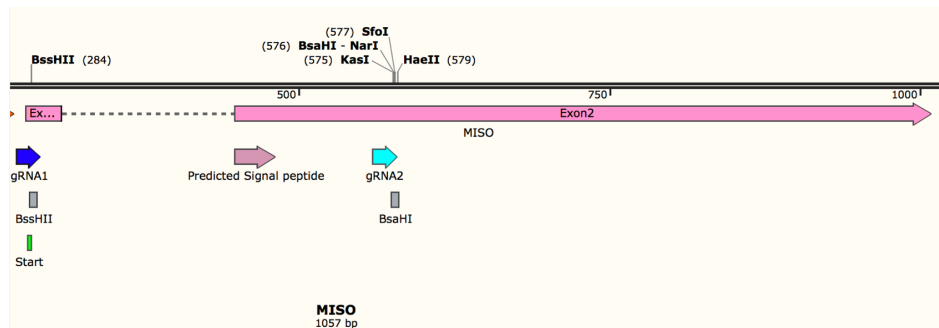


Figure 2.1. gRNA Loci in *MISO*. gRNA target site 1 is over the start codon in exon 1. gRNA target site 2 is in exon 2. Both target sites are co-localized with restriction enzyme recognition sites.

2.2. gRNA Expression Cassette Design and Synthesis

Because gRNAs must be transcribed, but neither translated into protein, nor allowed to leave the nucleus, they must be expressed via a Polymerase III promoter. One such promoter is the U6 promoter consisting of the 5' upstream regulatory sequence from AGAP013557. Use of the U6 promoter for gRNA expression has been extensively validated by Prof. Flaminia Catteruccia's research group and has been used to robustly express transgenic gRNAs in *Anopheles* gene drive constructs (Hammond et al., 2015, unpublished data from Andie Smidler). We designed two DNA fragments for synthesis and ordered them pre-assembled as gBlocks from IDT. These gBlocks contain the U6 promoter upstream of each gRNA and terminal restriction enzyme sites to facilitate golden-gate cloning into *Anopheles* transgenesis vectors (see section 2.3).

2.3. Golden Gate Cloning

Golden Gate cloning is a cloning technique, first described in 2008 by Engler et al., by which many cassettes can be assembled into a construct simultaneously in a "one-pot" synthesis reaction in a single tube. Multiple publicly available and well-characterized mosquito transgenesis plasmid backbones, including pDSAY (section 2.4.1), rely on the Golden Gate cloning method (Vologonsky et al., 2015).

Golden Gate plasmids are designed to contain BsaI restriction enzyme recognition sites because these enzymes cleave their targets in a manner which enables efficient and irreversible cloning of constructs. In Golden Gate ready transgenesis plasmids, the BsaI enzyme sites flank the 5' and 3' ends of a LacZ cassette where it recognizes a 6-base pair

(bp) sequence (5'-GGTCTC-3'). The enzyme then cleaves 5bp downstream of the restriction site, leaving unique 4bp overhangs whose sequence is independent from the recognition sequence.

When the designed gBlocks (section 2.2) are cleaved by BsaI, gBlock 1 has a 3' overhang (GTTG) that is complimentary with the 5' overhang of gBlock 2 (CAAC). The 5' overhang of gBlock 1 (ATCC) is complimentary with the 3' overhang (TAGG) produced by BsaI cleavage of the golden gate ready LacZ-cassette in the destination plasmid. Likewise, the 3' overhang of gBlock 2 (CGAA) is complimentary to the 5' overhang (GCTT) also generated by BsaI cleavage of the golden gate LacZ-cassette in the destination plasmid (Figure 2.2). All components are cleaved by BsaI and have complimentary sticky ends generated by BsaI cleavage.

Thus 0.3 μ L of gBlock 1 and 0.3 μ L of gBlock 2, 1 μ L pDSAY plasmid, 1 μ L BsaI restriction enzyme, 2 μ L T4 ligase, 2 μ L T4 ligase buffer, 0.2 μ L bovine serum albumin, and 13.2 μ L water were reacted in one tube (20 μ L total volume). The gBlock ends were cleaved by BsaI revealing their unique overhangs, and the LacZ cassette was digested out of the plasmid leaving its 5' and 3' unique overhangs complimentary to those of gBlock 1 and 2. Each component then self-assembled in a linear fashion according to overhang complementarity. The piggyBac vector pXL:gMISO was also assembled in using the Golden gate method, with 1 μ L of pXL plasmid replacing the 1 μ L of pDSAY in the reaction mixture.

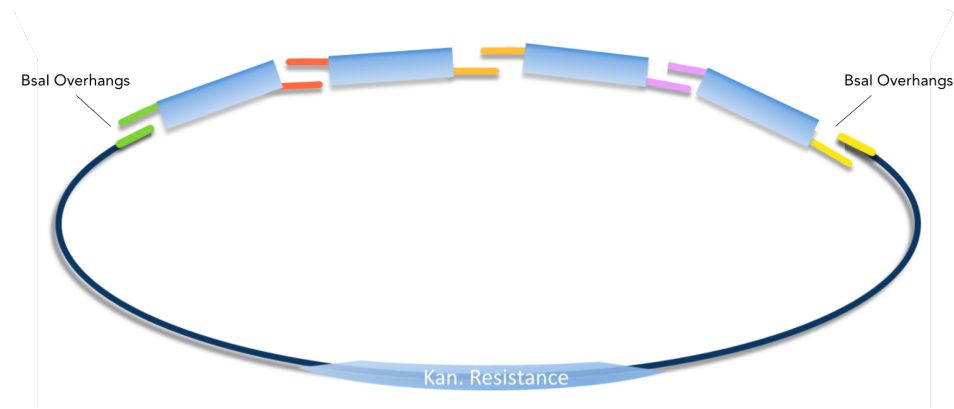


Figure 2.2. Golden Gate Cloning. Multiple fragments with BsaI cleavage sites can be combined in a single reaction tube and ligated based on their BsaI-generated four base pair overhangs. (Adapted with permission of Andie Smidler)

2.4. Φ C31 Transgenesis Plasmid Design

The first step to generating CRISPR knockout lines in *A. gambiae* is using transgenesis to integrate the gMISO cassette from pDSAY:gMISO into the genome. My first attempt was using the Catteruccia Laboratory's X1 docking site line of mosquitoes. The X1 was originally described in 2015 by Volohonsky et al. and features a Φ C31 attP docking site on Chromosome 2L in the *A. gambiae* genome. The attP site in the insert in the presence of an integrase combines with the attB site in the pDSAY transformation vector, thus integrating the vectors gRNA cassette cargo into a known site in the genome.

2.4.1. Φ C31 Plasmid pDSAY:gMISO Construction

The pDSAY:gMISO transformation vector was derived from the pDSAY transgenesis plasmid outlined in Volohonsky *et. al.* The pDSAY plasmid features and

EYFP fluorescent reporter under a 3xP3 promoter (*D. melanogaster*), for fluorescent selection of transgenic larvae, a LacZ cassette for blue white screening of insertion during cloning, and an Ampicillin resistance cassette for colony selection.

Two pre-synthesized DNA gBlock were purchased from IDT (section 2.2), each consisting of a set of gRNAs targeting the *MISO* gene expressed via the Pol III U6 promoter with Golden Gate cloning compatible overhangs. TRACR RNAs were cloned using Golden Gate cloning technique in the pDSAY plasmid (Engler et al., 2009). In a 20 μ l reaction, 50ng/ μ l solutions of the two gblocks (0.3 μ l each) were added with a 1 μ l (50ng/ μ l) solution of pDSAY plasmid to a reaction mixture consisting of 13.2 μ l water, 0.2 μ l bovine serum albumen, 2 μ l T4 Ligase buffer, 2 μ l T4 ligase, and 1 μ l BsaI-HF restriction enzyme. The gblocks were designed with BsaI-HF restriction sites at 5' and 3' ends to facilitate Golden Gate cloning. The transformation vector reaction mixture was then transformed into *E. coli* (NEB Turbo) and plated (section 2.6). Positive colonies from blue/white screening were further screened via colony PCR using M13-pUC and als664 primers (M13-pUC, 5'-GTTTTCCCAGTCACGAC-3'; als663, 5'-CCACCGTTGCCGTACAAG-3'). Positive colonies from colony PCR were cultured overnight and the DNA purified and sequence verified by MacroGenUSA (sections 2.12 and 2.7). The resulting plasmid can be seen in figure 2.3.



Figure 2.3. Φ C31 Transformation Vector pDSAY:gMISO. Fluorescent reporter: 3xP3-EYFP.

2.5. PiggyBac Transgenesis Plasmid Design

PiggyBac transposon transgenesis uses the 13bp perfect inverted repeats from a transposable element first discovered in a *Trichoplusia* cell line, which inserts DNA cargoes into the center of the tetranucleotide TTAA and has been used successfully to achieve *A. gambiae* transgenesis (Grossman et al., 2001, Catteruccia et al., 2005).

2.5.1. Piggybac Plasmid pXL:gMISO Construction

The pXL:gMISO transformation vector was derived from the pXL-BACII-LoxP-3xPDsRed-LoxP plasmid backbone publicly available from Addgene (plasmid #26852). pXL:gMISO was generated by removal of the dsRed fluorescent reporter gene from pXL_BACII_3xP3_dsRed_Amp via restriction digest using restriction enzymes NcoI and NotI. I then subcloned the EYFP fluorescent reporter gene from PDSAY via restriction digest using the restriction enzymes NcoI and NotI, resulting in overhangs matching the destination vector, and gel purified the resulting product. Ligation of EYFP into the pXL backbone was accomplished using T4 ligase in an overnight ligation reaction following standard ligation protocols provided by New England Biolabs (NEB). The LacZ cassette was then subcloned from PDSAY and inserted into the pXL_BACII_3xP3_EYFP_Amp plasmid backbone via Gibson assembly outlined in Gibson et al., 2009, using primers als749 and als750 (als749, 5'-TAACTTCGTATAATGTATGCTATACGAAGTTATGGATCCAATCCAGAGACCCGCAACGC-3'; als750 5'-GAGCTCGAATTAACCATTGTGGGAACACTAGAAAAGCGGAGACCCGCGTTAAATTTTTG-3').

The resulting reaction mixture was transformed into *E. coli* (NEB Turbo) and plated on Ampicillin plates. When blue/white screened, blue colonies were selected and cultured overnight at 37°C in 2XYT media and plasmid DNA purified (section 2.7). The DNA was sequence verified MacroGenUSA.

The two pre-synthesized gBlocks were then ligated into the pXL_BACII_3xP3_EYFP_LacZ_Amp plasmid backbone via Golden Gate assembly

(section 2.3). The resulting reaction mixture was transformed into *E. coli* (NEB Turbo), plated, blue/white screened, purified and sequence verified as previously described. The resulting piggyBac transformation vector can be seen in figure 2.4.

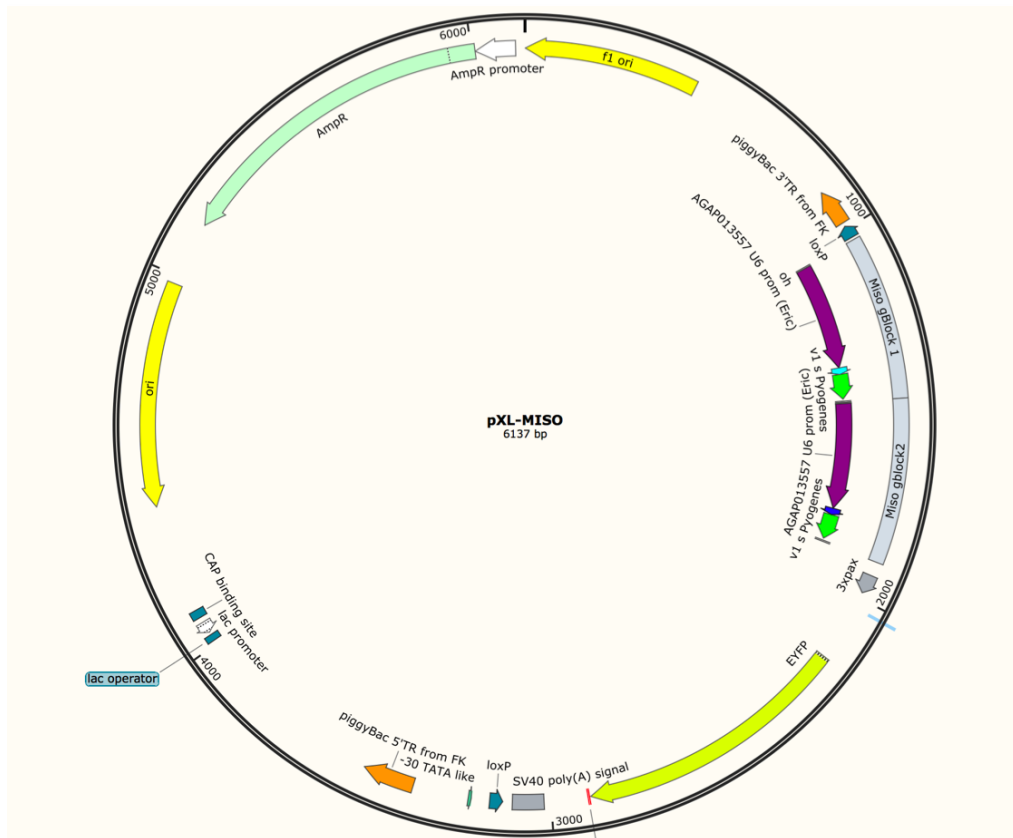


Figure 2.4. PiggyBac Transformation Vector pXL:gMISO. Fluorescent reporter: 3xP3-EYFP.

2.5.2. PiggyBac Plasmid pB:VTK:gMISO Construction

The pB:VTK:gMISO transformation vector was derived from the pB:VTK backbone publicly available from Addgene (plasmid #26852). pB:VTK was generated by subcloning the *piggyBac* transposase gene downstream of the VASA promoter outside of a 5' and 3' piggyback transposon repeat region via Gateway cloning technique, thus

enabling a single-vector transgenesis system. EYFP was then inserted downstream of the actin 5c promoter from *D. melanogaster* using seamless cloning (Invitrogen). To produce pB:VTK:gMISO, pB:VTK:ActEYFP was linearized with BamHI (NEB). *MISO* gblocks containing two sets of gRNA's targeting *MISO* under U6 promoters and TRACR RNAs (V1 *S. pyogenes*) were amplified from pXL:gMISO using the following primers: (Miso Seamless FWD, 5' CACTAGATCCAGAGAATCCCCACATATACTGAAGCG-3'; Miso Seamless REV, 5'-AACTAGAAGCGGAGAAAGCAAAAAAGCACCGACTC-3'). The gMISO fragment was then cloned into BamHI linearized pB:VTK:ActEYFP using the following seamless reaction conditions: a 3:1 ratio of insert to vector, 30ng of pB-VTK vector (2µl) was added to 45ng of gblock insert fragment (1µl) and 3µl of 2x Seamless enzyme master mix (Invitrogen). The reaction was then incubated at room temperature for 30 minutes. The reaction mix was then transformed into *E. coli* (XL-10 Gold Ultra-competent), plated on 2XYT/Agar plates dosed with ampicillin. Colony PCR of the resulting colonies was completed using primers DGA5 and als434 (DGA5, 5'-tacggcaacggtggtgg-3'; als434,). Colonies positive for insertion were selected and cultured overnight at 37°C in 2XYT media. Plasmid DNA was purified (section 2.7) and the DNA was sequence verified by MacroGenUSA. The resulting plasmid can be seen in figure 2.5.

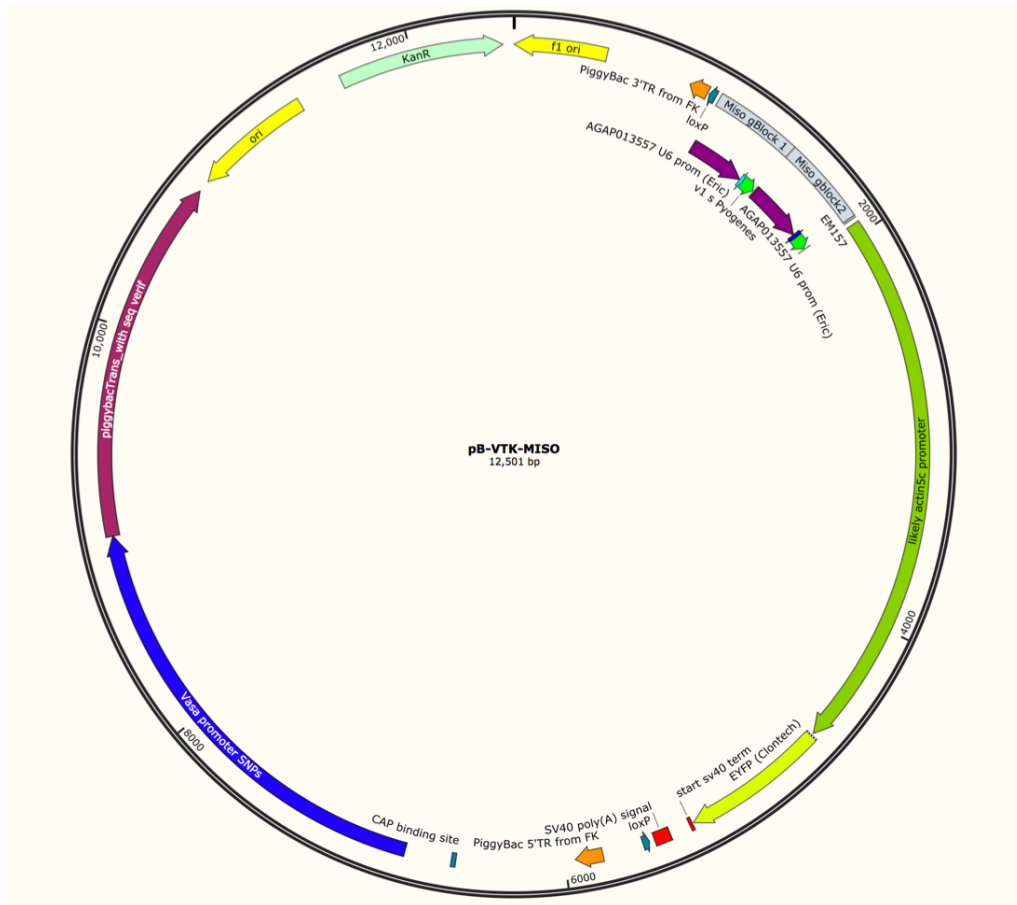


Figure 2.5. PiggyBac Single-Vector Transgenesis System Plasmid pB:VTK:gMISO. Fluorescent reporter: Actin-EYFP.

2.6. Bacterial Transformation

All bacterial transformations, with the exception of pB:VTK:gMISO, were performed by incubating 0.7 μ L of the plasmid mixture in 10 μ L of NEB Turbo Competent *E. coli*. XL-10 Gold ultra-competent cells were used in pB:VTK:gMISO plasmid transformation. The plasmid and bacteria were incubated for 30 minutes on ice and then heat shocked for 30 seconds in a 42°C water bath. One hundred microliters of

SOC growth media was added to the mixture followed by a one-hour incubation at 37°C. After final incubation, the bacteria were plated on 2XYT agar plates containing the corresponding antibiotic for the plasmid and 40µL of XGAL if blue-white screening was to be conducted. Plates were then incubated overnight at 37°C. I then used a colony PCR to screen the colonies for putative colonies containing the desired plasmid (section 2.12).

2.7. Plasmid Purification

Putative positive colonies containing the desired plasmid screened by colony PCR were cultured overnight in 6mL or 100mL of 2XYT liquid media (depending on the amount of plasmid needed). Plasmids were then isolated and purified from the 6mL cultures using the Denville Scientific SpinSmart® Plasmid Purification Kit. In cases where larger quantities of plasmid were needed, I used the Machery-Nagel NucleoBond® Xtra Midi kit to isolate and purify 100mL cultures. The sequence of the purified plasmids was then verified by MacroGenUSA.

2.8. Injection Mix Preparation

Two 1.5µL Eppendorf tubes were prepared for the injection mix by washing with PCR grade de-ionized water, the removal of all water except for one small droplet, and centrifugation at 20000 rpm for 90 minutes. The wash process was repeated twice in

order to ensure the complete removal of any dust particles that could clog the quartz microinjection needle. 120 μ L of injection mix (section 2.8.1) was then added to the tubes and centrifuged at 20000 rpm for 90 minutes. The top 110 μ L was then transferred to the second cleaned tube and spun again at 20,000 rpm for 90 minutes immediately before microinjecting.

2.8.1. Φ C31 injection mix

The injection mix was comprised of pDSAY:gMISO at a concentration of 350ng/ μ L and the integrase helper plasmid, p130, at a concentration of 150ng/ μ L. The total volume of injection mix was 120 μ L.

2.8.2. PiggyBac pXL:gMISO injection mix

The injection mix was comprised of 400ng/ μ L of pXL:gMISO and the transposase helper plasmid, pB Δ Sac, at a concentration of 100ng/ μ L. The total volume of injection mix was 120 μ L.

2.8.3. PiggyBac pB:VTK:gMISO injection mix.

The injection mix was comprised of 350ng/ μ L of pB:VTK:gMISO. The total volume of injection mix was 120 μ L.

2.9. Embryo Microinjections

Blood-fed *A. gambiae* mosquitoes (strain G3) were given an oviposition cup to lay eggs in 72 hours after the blood meal. The oviposition cup remained in the cage for 15-20 minutes to facilitate synchronous egg lays. Females were allowed to lay eggs for 30 minutes. Embryos were then transferred onto nitrocellulose membranes covered by Whatman filter paper on glass slides and aligned at a 45-degree angle to facilitate microinjection (Volohonsky et al., 2015). Embryos were injected according to standard protocols established in 2000 by Catteruccia *et al.*

2.10. Genomic DNA Preparation

Genomic DNA preparations were performed using Qiagen DNeasy[®] Blood and Tissue kit and protocols. Whole mosquitoes or dissected tissues are suspended in 180 μ L of phosphate-buffered saline (PBS) and homogenized using sterile glass beads and a tissue homogenizer before column purification.

2.11. *MISO* PCR

The *MISO* locus was amplified via PCR using PhireTD polymerase (ThermoFisher) from genomic DNA obtained from genomic preparations of whole mosquitoes or leg DNA (sections 2.10 and 2.14). One microliter of DNA sample was added to a master mix of 10 μ L Phire polymerase, 1 μ L of primer als844, 1 μ L of primer als845, and 7 μ L water to make a total reaction volume of 20 μ L (als844 5'-GCGCATCTTCCGATCGTTAC-3'; als845 5'-CTTTTTCGCTCCTCTCCTCC-3'). If a larger quantity of amplified DNA was required than a larger ratio of the reaction master mix was used (e.g. 2x, 5x, etc.). For PCR screening a 40 μ L reaction volume was used, featuring a master mix at 2x volume.

2.12. Colony PCR

Bacterial lawns are sampled using sterile 10 μ L pipette tips and added to 20 μ L of PCR master mix consisting of 10 μ L GoTaq Green polymerase, 1 μ L each of forward and reverse primers, and 7 μ L of water. pDSAY:gMSIO colony PCR features primers als253 and als461 (als461, 5'-GGTGTGGGAGGTTTTTTAAAGCAAG-3'; als253 5'-CAGGTCTCACTAGCCACATATACTGAAGCGG-3'). pB:VTK:gMISO colony PCR uses primers DGA5 and als434 (DGA5, 5'-TACGGCAACGGTGGTGG-3'; als434 5'-CATCGCTCAGGTGGTATAGTATTTGG-3').

2.13. Gel Extraction

PCR products needed for cloning or sequencing were extracted from agarose gel using the SpinSmart PCR Purification and Gel Extraction kit from Denville Scientific. Extracted PCR products were eluted in 20 μ L of elution buffer to yield increased DNA concentrations.

2.14. Leg-PCR

I anaesthetized 24-48-hour old adult mosquitoes with CO₂. Using a fine-haired paintbrush and forceps I removed a single leg (preferably the middle leg) from each individual mosquito and placed the leg in a 96-well plate containing 20 μ l of leg buffer (19.5 μ l solution of *dilution buffer* and 0.5 μ l DNA Release reagent from the ThermoFisher Phire Tissue Direct kit). The corresponding mosquito was then placed in a numbered *Drosophila* fly conical tube containing cotton soaked in 10% sucrose water and topped with a foam stopper. After collecting 48 legs, the leg/leg buffer solution was incubated at room temperature for 5 minutes and then placed in a pre-heated thermocycler at 98°C for 2 minutes. 1 μ l was used for the *MISO* PCR screen as per protocols in the Phire Tissue Direct kit.

2.15 Screening for mutations in *MISO*

Screening for individual mosquitoes with mutations in *MISO* was a vital process for isolating clonal knockouts of *MISO*. Due to the polymorphic nature of the *Anopheles* genome, standard genotyping methods such as Surveyor are inadequate for our purposes (Qiu et al., 2004). To facilitate identification of CRISPR-generated mutations, gRNA target sites were chosen such that the gRNA cleavage site overlaps a naturally occurring semi-unique restriction enzyme site within the wild type genome. Many mutant alleles generated by CRISPR mutagenesis will result in a loss of the restriction enzyme site, while wild-type alleles will retain the sequence. The loss and retention of restriction sites allows straightforward identification of mutant and wild type alleles by PCR and restriction enzyme digest, as outlined in 2013 by Smidler et al.

Following legPCR described in section 2.14, 1 μ L of the leg DNA extract was used to amplify the *MISO* locus via PCR described in section 2.11. The amplicon volume was then divided into three fractions: two 10 μ L fractions that were subjected to restriction enzyme digest, and a 20 μ L undigested fraction. One 10 μ L fraction was digested with BssHII corresponding with gRNA1 target site, and the other fraction with BsaHI corresponding with gRNA2 target site. For each digest, 0.5 μ L of restriction enzyme was mixed with 2 μ L Cutsmart buffer, 7.5 μ L water, and 10 μ L of PCR product.

The digested products and undigested product were then run on a 1% agarose gel. Samples with no mutation would result in normal cleavage of the PCR product, yielding bands at 700bp and 300bp. Samples where a putative mutation at the target site deleted the restriction site for the enzyme would yield a 1000bp band. Samples that featured a putative large deletion would be revealed in the undigested sample lane, e.g. a 400bp deletion would be indicated by a 600bp band in the undigested lane (Figure 2.6).

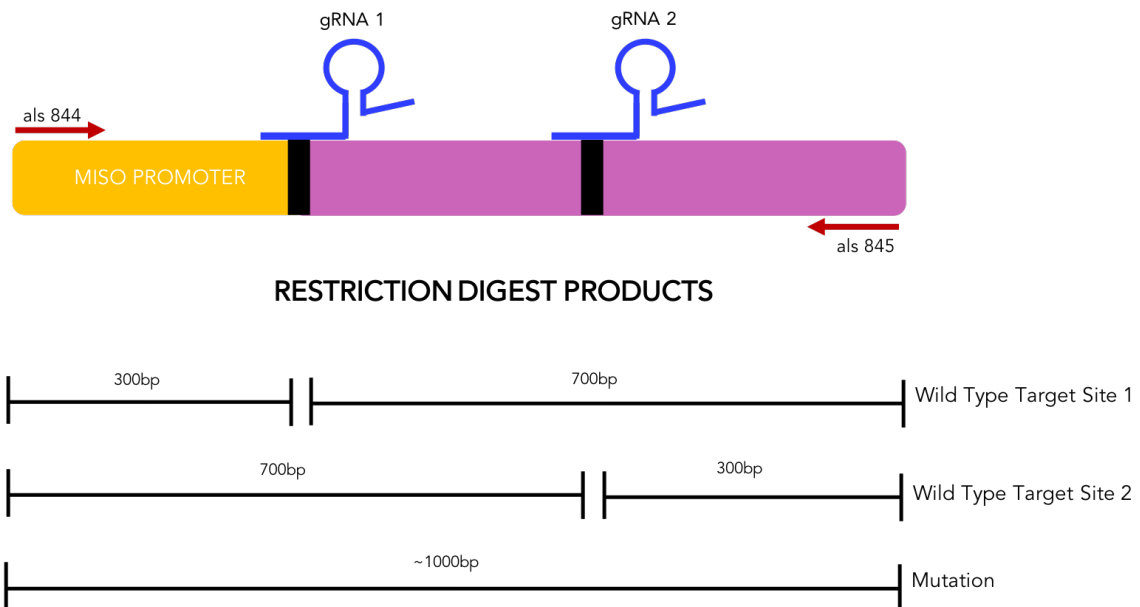


Figure 2.6. Screening for Mutations in *MISO*. *MISO* *leg*PCR product of 1,023bp is digested by restriction enzymes BssHIII for target site 1 and BsaHI for target site 2. Samples with no mutation would result in normal cleavage of the PCR product, yielding bands at 700bp and 300bp. Samples where a putative mutation at the target site deleted the restriction site for the enzyme would yield a 1000bp band.

2.16. Generating Mutations in the *MISO* Gene

F1 transgenic gMISO adult females were sex-separated as pupae and crossed with the Catteruccia Laboratory's homozygous Vasa-Cas9 expressing line of mosquitoes. The Vasa-Cas9 line is distinguished by neuronal 3xP3-DsRED fluorescence while the gMISO line is distinguished by Actin- EYFP fluorescence, therefore progeny from the gMISO/Cas9 cross containing both transgenes could be identified by evidence of both fluorescent markers (Figure 3.4). These dual transgenics were termed gMISO/Cas9. The gMISO/Cas9 mosquitoes were separated by sex as pupae and were outcrossed correspondingly to male and female wild type G3.

As adults, these individuals express the Cas9 protein within their germ-line, and the gRNAs targeting *MISO* ubiquitously throughout all their tissues, thus enabling mutagenesis where the two overlap – the germ tissues. Each follicle or sperm cell is capable of independent unique mutagenesis in these individuals, therefore the germ tissues contain a mosaic mutation pattern and the resulting offspring are highly polymorphic and pleiotropic.

To identify the types of mutations made within a single female we dissected the ovaries from an individual gMISO/Cas9 female, PCR amplified the *MISO* locus, and sequenced individual mutant alleles (sections 2.11 and 2.15).

2.17. Isolation of *MISO* Mutants

To generate heterozygotes with mutations in *MISO*, we crossed male gMISO/Cas9 mosquitoes to G3 wild-type. Female gMISO/Cas9 were not used to generate homozygotes as the Cas9 protein they possess is maternally deposited into the developing egg by nature of the vasa promoter used in the Cas9 transgenic line, thereby leading to unplanned further mutagenesis in their offspring. These offspring would potentially possess different mosaic mutations in the *MISO* gene throughout their body, making isolation of a clonal line difficult.

Putative heterozygous *MISO* knockout offspring from the [gMISO/Cas9 x G3] cross were sorted by fluorescence and sexed to segregate females. Individuals that had no fluorescence were selected as they did not exhibit the selective fluorescent reporter for

either the gMISO cassette (actin-EYFP) or the Cas9 transgene (3xP3-dsRed). Null-fluorescent adults females were mated with G3 wild type males then isolated in oviposition cups. LegPCR mutant screens outlined in section 2.14 were performed on the mated females and positive putative mutants were blood fed and permitted to lay eggs in the oviposition cups. After two successive egg lays I performed genomic DNA preparation on the isolated PCR positive putative heterozygote females and characterized putative mutations via DNA sequencing.

Mutants expected to yield true miso knockouts will feature base pair deletions in the ORF that are not a multiple of three, e.g. a deletion of 4 base pairs or a deletion 17 base pairs. Such mutations will cause a frameshift mutation along the ORF generating premature stop codons downstream from the deletion, thus truncating the normally 196 amino acid protein. Premature stop codons will trigger the mRNA transcripts of the mutated MISO protein to be degraded via NMD making a null mutant.

Progeny from individuals with favorable mutations are maintained and reared for further legPCR mutant screening and subsequent intercross. to generate homozygous mutant line(s). A full crossing scheme is illustrated in figures 2.7 and 2.8.

Crossing Scheme: gMISO and misoKO

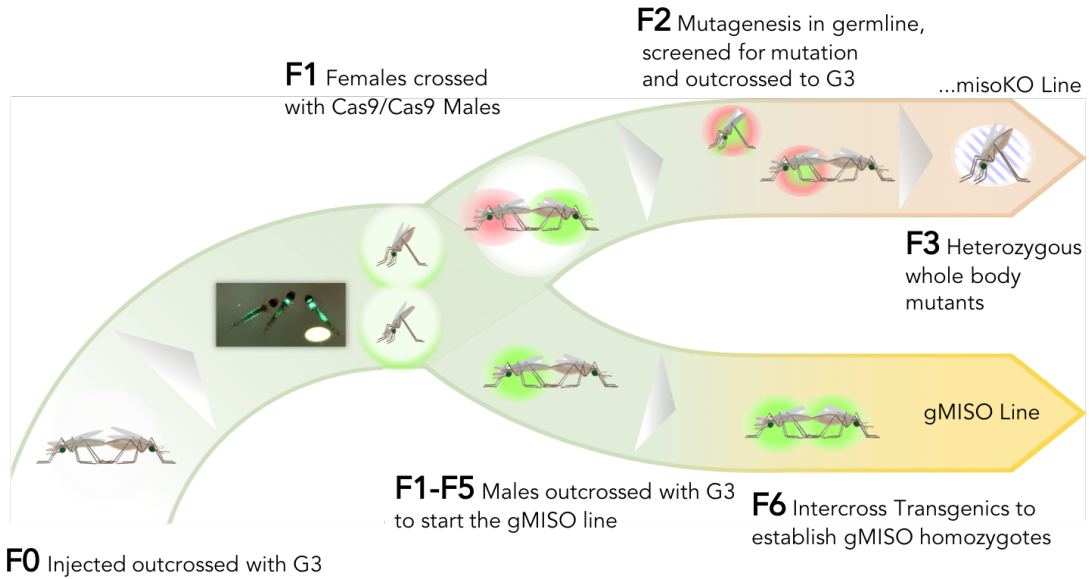


Figure 2.7. Crossing Scheme for Generation of gMISO and miso Knockout Lines

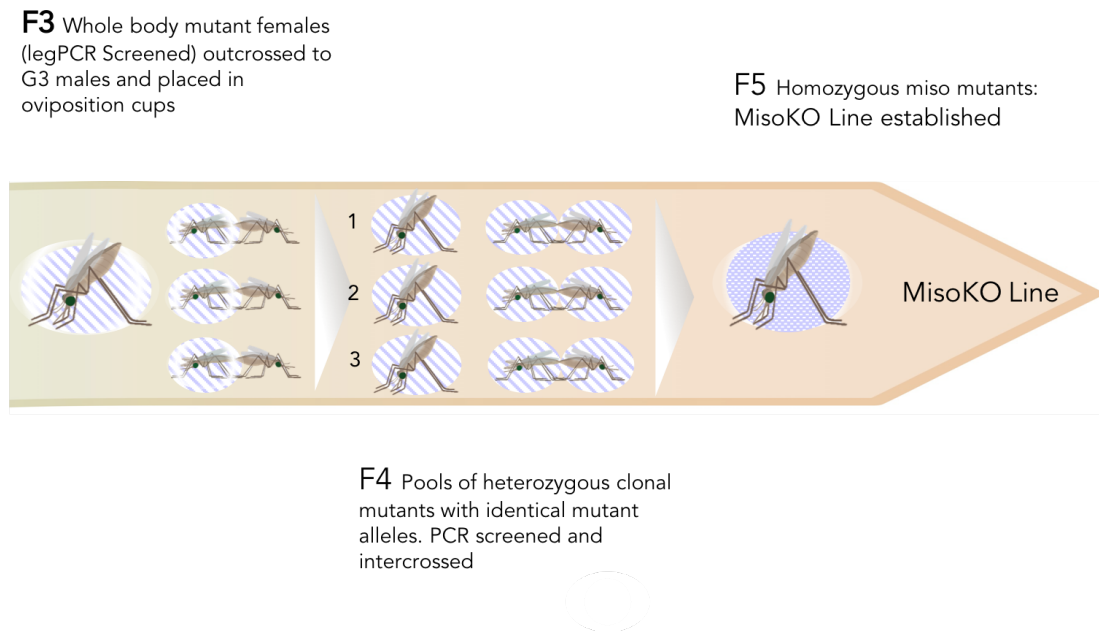


Figure 2.8. Crossing Scheme for Iso-female Families of MISO mutants

Chapter III

Results

3.1 Generating gMISO transgenics

I successfully generated a line of *A. gambiae* that putatively expresses two guide RNAs (gRNA), each targeting the *MISO* gene. Putative transgenesis was achieved after design, assembly and injection of three transgenesis plasmid variants into *Anopheles* embryos. Hereafter the *A. gambiae* MISO-targeting gRNA expressing line will be called the gMISO line. The gMISO line exhibits predicted fluorescence (actin-EYFP) and has generated mutagenesis when crossed with a Cas9 expressing line; however, confirmation of the gRNA cassette insertion via PCR and subsequent sequencing of the insert has yet to be completed.

3.1.1. Unsuccessful Transgenesis with pDSAY:gMISO into the X1 Docking Site

Transgenesis was initially attempted using the pDSAY:gMISO plasmid injected into X1 docking site embryos. Three hundred and eighty six embryos were injected with pDSAY:gMISO and an Φ C31 integrase-cassette plasmid, helper plasmid #130. Thirty-one embryos survived microinjection to hatch (8% survival rate) (Volohonsky, *et. al.*

2015). Epi-fluorescence was observed in three larvae, suggesting successful injection of the plasmid mix into embryos (Figure 3.1). Seventeen surviving adults were outcrossed to G3 after eclosion following standard transgenesis protocols. After 5 subsequent rounds of blood feeding, no transgenic individuals were observed among the progeny of the injected X1. Transgenesis was unsuccessful.

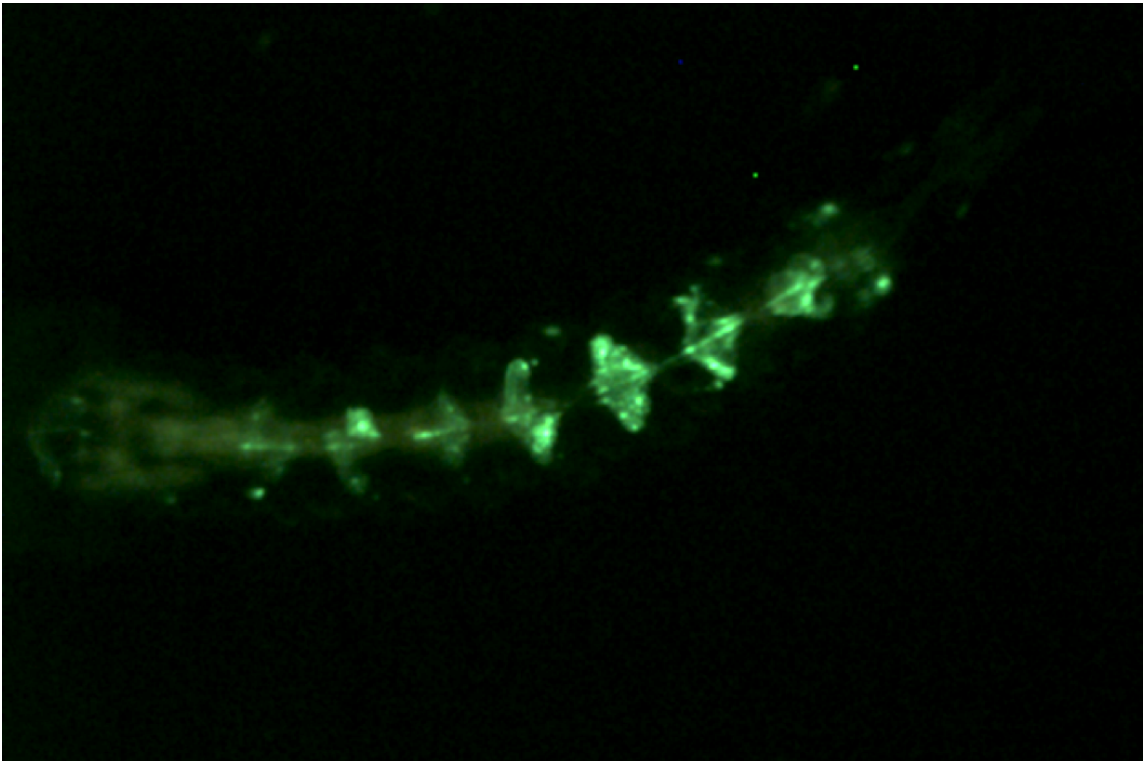


Figure 3.1. Epi-fluorescence in Φ C31 Injected Larva. Larvae shows expected 3xP3-EYFP neuronal expression pattern.

3.1.2 PiggyBac Transgenesis with pXL:gMISO Yielded No Adult Transgenics

Given issues that others in the Catteruccia research group had recently experienced with the Φ C31 transgenesis system, I attempted transgenesis via another

method, piggyBac transgenesis. For the piggyBac transgenesis method, the same gRNA expression cassette used in pDSAY:gMISO was assembled into a piggyBac compatible transgenesis plasmid, pXL:gMISO, devised by Andie Smidler and myself.

Transgenesis was achieved by microinjecting 1,773 embryos with a mixture of pXL:gMISO and p Δ Sac helper plasmid containing a piggyBac transposase driven by heat shock promoter *hsp70* regulation (a gift from Prof. Antony James). One-hundred-seventy-one embryos survived microinjection and hatched (10% survival rate), with epifluorescence observed in hatched larvae. After 4 subsequent rounds of blood feeding and egg collection, a single 3xP3-EYFP expressing transgenic larva was observed among the progeny. This lone transgenic died at the third instar, well before reaching the adult stage.

This experiment demonstrated that while we were successfully able to integrate *MISO*-targeting gRNA cassettes into the wild-type G3 genome, it was likely a rare event with low survivability. Similar experiments previously performed by Dr. Daniel Abernathy suggested that many piggyBac insertion events are only rarely observable by the 3XP3-EYFP promoter, due to neuronal silencing, suggesting that we may indeed have been missing many transgenesis events due to a problem with the regulation of the selectable marker. Further, the use of a secondary discrete helper plasmid, with the transposase cassette necessary for integration, also decreases the probability of generating transgenics as cells within injected embryos would need to receive both plasmids in order to achieve integration of the gRNA cassette into the genome. With these concerns in mind, a new transgenic plasmid vector was designed.

3.1.3 Successful Transgenesis of MISO gRNA Cassette Achieved Using a PiggyBac Transgenesis Plasmid pB:VTK:gMISO with Transposase Cloned into the Backbone and an Actin-EYFP Selectable Marker

To achieve successful PiggyBac-based transgenesis of a MISO gRNA containing transgene, we modified the plasmid pB:VTK:Act:dsRED to contain a ubiquitous Actin-EYFP selectable marker as well as piggyBac transposase integrated on the plasmid backbone in order to remedy issues encountered using the pXL:gMISO transformation vector. We hypothesized that the Actin-EYFP cassette would enable identification of more transgenic larvae due to the intensely ubiquitous expression of the fluorescent protein, thus fewer would be missed during fluorescent screening. I also hypothesized that cloning the transposase onto the plasmid backbone would increase transgenesis rates by guaranteeing that cells that receiving the transgenesis plasmid would also contain requisite mechanism for insertion of the plasmid into the genome. The same gRNA expression cassette used in pXL:gMISO was removed from the pXL:gMISO plasmid and subcloned into the piggyback compatible transgenesis plasmid, pB:VTK:gMISO, devised by Andie Smidler, Dr. Daniel Abernathy, and myself.

To generate transgenics, 489 embryos were microinjected with pB:VTK:gMISO and 82 embryos survived to hatch into larvae (16.7% survival rate). Of these larvae, 54 larvae survived to eclose and were outcrossed to G3 separately by sex. Fluorescent screening revealed that the injected F0 adults produced 137 transgenic F1 progeny collectively between both the male and female outcross cages (Figure 3.2).



Figure 3.2. Transgenic Larvae. F1 transgenic progeny from injected (F0) adults.

The larvae displayed differing patterns of Actin-EFYP fluorescence, consistent with the transgene position effect and multiple insertions (Figure 3.3). Transgenic larvae were pooled, and the line was henceforth termed gMISO. Transgenic pupae were segregated by sex, and males were crossed with G3 to establish and maintaining a gMISO line while females were crossed to the Catteruccia Laboratory's homozygous Vasa-Cas9 line to begin crosses for *MISO* mutagenesis.

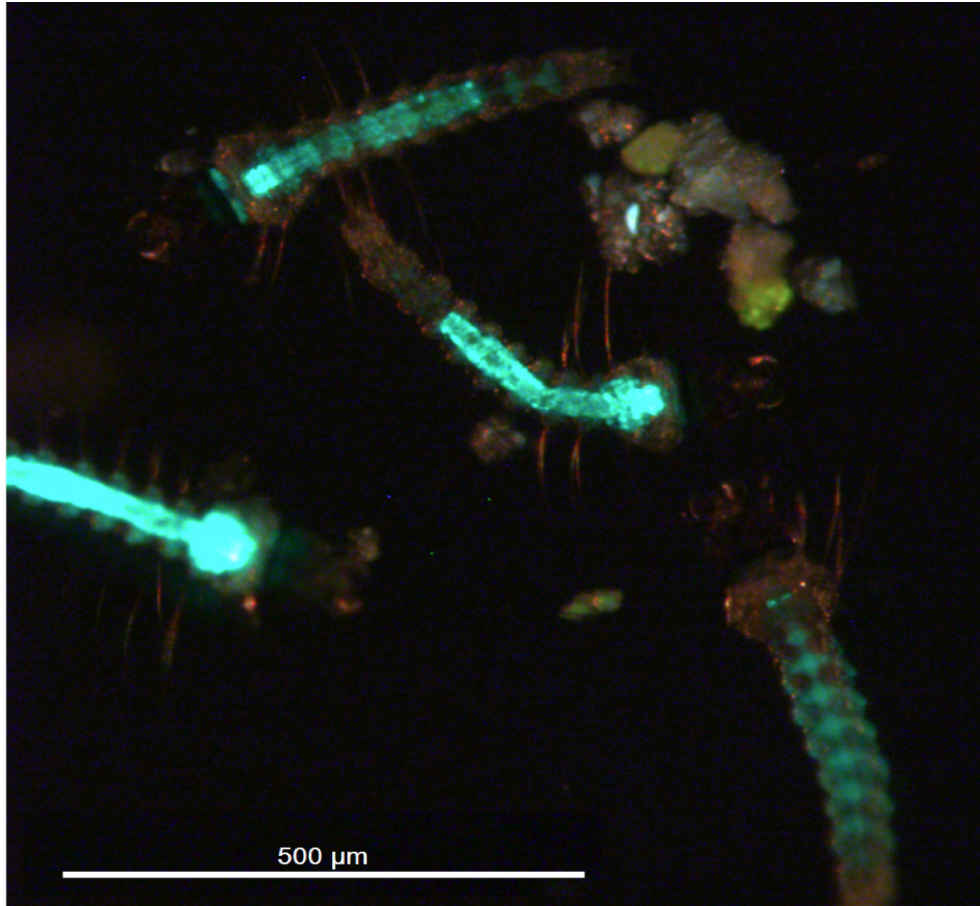


Figure 3.3. The larvae displayed differing patterns of Actin-EFYP fluorescence, consistent with the transgene position effect and multiple insertions position effect and multiple insertions.

3.2 Generating Knockout Mutations in *MISO*

Mutagenesis in *A. gambiae* was accomplished by crossing the gRNA-expressing gMISO line and the Cas9-expressing line generating progeny that possessed both transgenes and leading to mutagenesis at the *MISO* target loci in their germ-line (Figure 3.4).

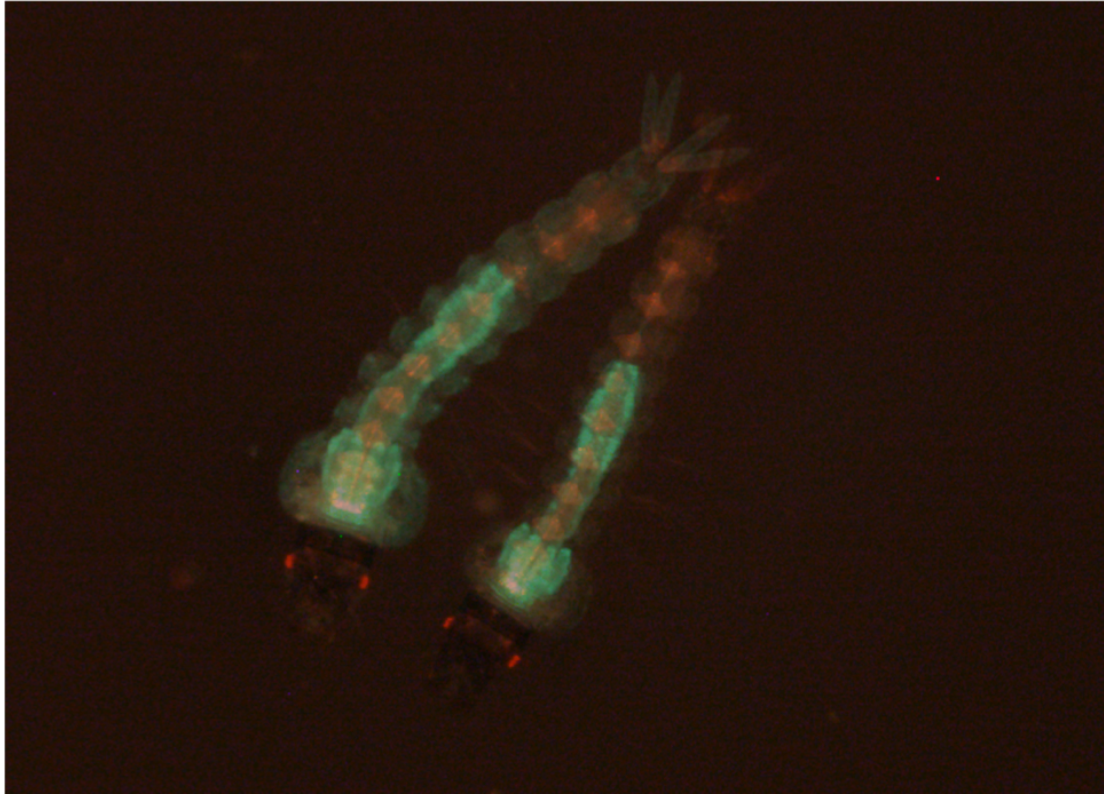


Figure 3.4. gMISO/Cas9 Dual Transgenic Larvae. The Vasa-Cas9 line is distinguished by neuronal 3xP3-DsRED fluorescence while the gMISO line is distinguished by Actin-EYFP fluorescence, therefore progeny from the gMISO/Cas9 cross containing both transgenes could be identified by evidence of both fluorescent markers in single individuals.

Mutagenesis in the gMISO/Cas9 mosquitoes was confirmed when sequencing DNA extracted from the dissected ovaries of one individual gMISO/Cas9 female revealed two different deletions at gRNA target site 2 (located in exon 2 of *MISO*) of 6bp and 18bp, as well as a large 404bp deletion spanning through both exons (Figures 3.5 and 3.6). These identified mutations demonstrated that mutagenesis was indeed occurring in the gMISO/Cas9 mosquitoes, and those mutations were mosaic. These findings suggested that we would be able to isolate mutant larvae in the subsequent generation derived from these mutant germ cells.

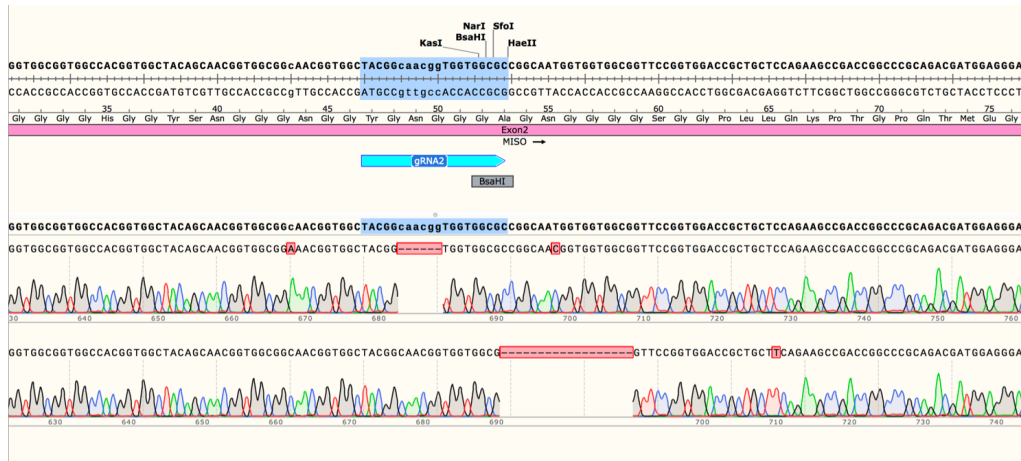


Figure 3.5. 6 base pair and 18 base pair Deletions Observed in Dissected from a Single gMISO/Cas9 Female’s Ovaries.

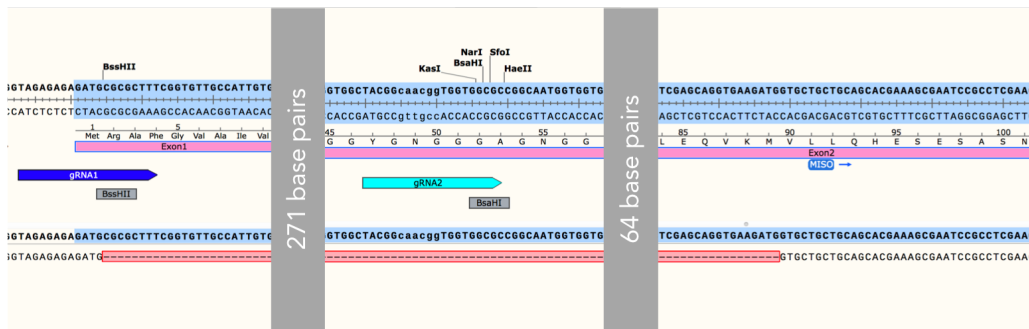


Figure 3.6. Large 404 base pair Deletion Observed in Dissected from a Single gMISO/Cas9 Female’s Ovaries.

3.3 Isolating *MISO* Knockout Mutants

Selected individuals were subjected to legPCR and screened for mutations as outlined in section 2.15. PCR mutant screening revealed that 15 out of 34 screened individuals had putative mutations in *MISO*, a frequency of mutation of 44% (Figures 2.6 and 3.8).

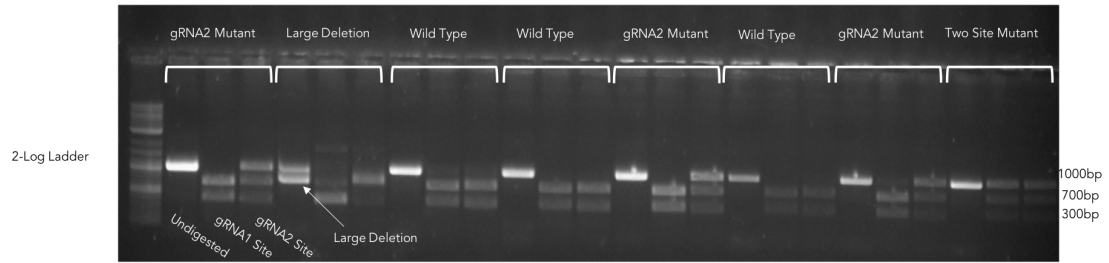


Figure 3.8. legPCR Screen Gel. Of these 8 samples from leg DNA extraction, 4 samples show putative heterozygous mutants at gRNA site 2, 1 sample at gRNA site 1, and the second sample shows a large deletion of ~300bp. 3 samples show wild-type bands.

Every putative mutant screened harbored a mutation at gRNA site 2, while 5 of the 15 also possessed a putative mutation at gRNA target site 1. These preliminary findings suggest that mutagenesis at gRNA target site 2 is more efficient than the gRNA targeting site 1. Putative heterozygous mutant females from PCR screens were mated to G3 wild-type mosquitoes and placed in individual oviposition cups so that lines of clonal mutants could be raised from their progeny.

I have isolated one mutant, *misoA3ΔA76*, which will likely be a hypomorphic allele of *MISO*. The *misoA3ΔA76* mutant features a 290bp deletion of 49 amino acids constituting a removal of 25% of the amino acids in the total *MISO* sequence (Figure 3.9).

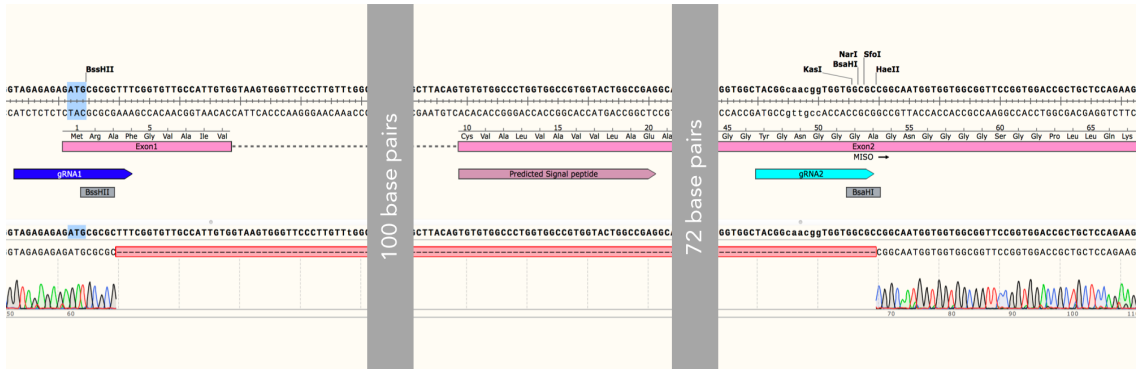


Figure 3.9. Large 290bp Deletion in Putative Heterozygous Mutant *misoA3ΔA76*. *misoA3ΔA76* has a deletion of 49 amino acids. Included in the deletion is the putative extracellular signal peptide. This mutant established the *misoA3ΔA76* mutant line.

MISO is a highly unstructured protein with no known domains besides an N-terminal secretion sequence. The *misoA3ΔA76* mutation removes 49 amino acids from the central unstructured region of the protein. Depending on the critical nature of these amino acids, the phenotype may range from not affecting the phenotype at all, to behaving like a non-functional mutant. As soon as the *misoA3ΔA76* line is stabilized, it will be necessary to assay the mutants for reproductive phenotypes observed in *MISO* RNAi experiments, as well as Western blots to assay for MISO presence, regulation, and localization.

Chapter IV

Discussion

I have successfully designed and implemented a CRISPR transgenic system to generate knockout mutations in the potentially important reproductive gene *MISO* in the major malaria vector, *Anopheles gambiae*. The *A. gambiae MISO* mutants generated during this thesis research are among the first examples of successful implementation of CRISPR in Anopheline mosquitoes. The exposed limitations of the 3xP3 promoter as a driver of selective reporter expression in piggyBac transgenesis will contribute to the design of future transgenic constructs by our research group. The insights to be gleaned from this project promise to expand our engineering toolkit in this notoriously genetically intractable species.

I have successfully demonstrated that *MISO* gRNA-expressing transgenic lines crossed to a Cas9 expressing transgenic line can generate knockout mutations in the target gene at high frequency. I have begun isolation of iso-female lines that are currently heterozygous that will imminently be intercrossed for homozygosity. Following the generation of homozygotes, we will begin characterizing the phenotypes associated with the loss of the *MISO* gene. The *MISO* knockout lines we are generating have the potential to amplify the Catteruccia research group's capacity to interrogate the function of *MISO* activity.

To date, all sequenced isolated mutations feature deletions that do not cause a frameshift mutation or NMD. The identified and isolated hypomorphic *misoA3ΔA76* mutant does not cause a frameshift mutation, but the deletion is so large as to truncate the normally 196 amino acid protein down to 147 amino acids, a loss of 25% of the total amino acid sequence. Further screening for mutants is required to determine if filtering selection is affecting detection of individuals possessing frameshift mutations.

As MISO is expressed in the late embryo as well as the female reproductive system, we suspect that the knockout lines may exhibit significant phenotypes in both of these stages. It is unknown whether the *MISO* gene is essential to larval development. Successful rearing of homozygous *MISO* knockouts will support the possibility that MISO is non-essential. The essential or non-essential nature of MISO will be further characterized by means of a competition assay conducted with heterozygous *misoKO* larvae and homozygous *misoKO* larvae. Larval development between the two lines will be compared to each other and to the Catteruccia Laboratory's wild type *A. gambiae* line, G3, to distinguish marked delays in development, lethality, and other developmental changes in the mutant lines.

Previous studies performed by Baldini et al., Gabrielli et al., Mitchell et al., and unpublished work by PhD candidate Perrine Marcenac have suggested that MISO plays a role in important female reproductive phenotypes such as fertility, egg development and resistance to *P. falciparum* infection. To validate these findings, I will recapitulate their experiments on assay fertility and fecundity in virgin and mated females.

I will compare groups of virgin and mated G3 wild-type and *MISO* knockout mosquitoes after blood feeding. Blood feeding will occur the day after mating. After two

days, I will dissect some the mated and unmated mutant and wild-type females to assay any changes in egg development, while a cohort of each group will be placed into oviposition cups so they can lay eggs. Egg development will be assayed by counting the eggs in the ovaries of dissected mutant females.

For those females set aside to lay eggs, I will perform single female oviposition assays. I will count the number of individual eggs laid in the oviposition cups for mutants and wild-type G3 controls. From these egg clutches, I will also assay for percent fertility. I hypothesize that we will observe a decrease in eggs developed compared to G3 controls, thus supporting findings in dsMISO shown in Baldini et al. and Perrine Marcenac's unpublished work.

Any null mutant line could be invaluable in characterizing MISO function. In particular, null mutant lines could form the basis of a number of transgenic complementation studies to characterize the mechanism by which MISO acts. For example, MISO mRNA has been shown to be massively up-regulated in the atrium following mating, but protein accumulation is mostly localized to the ovaries. We hypothesize that the MISO protein may be traveling between these tissues. We could explore this MISO motility hypothesis by complementing the *miso* knockout line with a transgenic expressing the *MISO* gene fused to a fluorescent protein, which would allow us to follow the localization of the MISO protein in females following mating. Further, a transgenic complimentary copy of the *MISO* gene could be fused to a small epitope such as a FLAG or HA tag, which would enable co-immunoprecipitation experiments to isolate and identify directly interacting protein partners with MISO protein.

The gMISO line originally established by crossing the adult male injected transgenics detailed in section 3.1.3 requires further characterization. While the selective fluorescent reporter gene actin-EYFP is indeed observed, and successful mutagenesis using the gMISO line was achieved, confirmation of the insertion of the gMISO cassette into the *A. gambiae* genome has not been directly verified. The putative site of integration is not known. The tetranucleotide TTAA integration site for piggyBac transgenesis occurs approximately once in every 250 bases. The surfeit of docking sites can lead to thousands of potential sites for insertion and the potential for multiple insertions. While insertional mutagenesis is a concern, discerning the location of the gMISO insert and sequencing the region in situ will be necessary to confirm the gRNA cassette has truly integrated into the gMISO line. I have begun optimization of a random primer PCR protocol under the guidance of Dr. Duo Peng (Zhang et al., 2011). Concurrently, I have continually crossed the gMISO progeny with G3 at each generation to dilute multiple insertions via naturally occurring recombination as well as winnowing out any deleterious insertional mutants.

Conclusions and Impact

During the course of this thesis I was able to generate CRISPR/Cas9 mediated mutagenesis in the *MISO* gene of *Anopheles gambiae*. Many potential *MISO* null mutant individuals remain to be identified and isolated. Furthermore, I was able to isolate a single heterozygous hypomorphic mutant line, *misoA3ΔA76*, that features a deletion of

49 amino acids from the N-terminal region of the *MISO* gene. Further exploration of the phenotype of the *misoA3ΔA76* mutant may yield new insights into the character of the MISO protein.

The impact of this work will add a significant level of support to the Catteruccia research group's interrogation of MISO's role in *Anopheles* reproductive biology and resistance to *Plasmodium falciparum* infection. Before this work, all previous studies performed by our research group have supported a role for MISO in oviposition, egg development, and a possible role in resistance to *P. falciparum* infection using RNAi knockdowns of the *MISO* gene. No characterization of *MISO*'s role in reproductive biology has been performed using a knockout of the *MISO* gene. In the future, a *MISO* knockout line can be used as an experimental cohort that aid the Catteruccia Laboratory's efforts to detect MISO phenotypes and improve the significance of earlier findings regarding the MISO protein's influence on egg production, egg development, and tolerance to *P. falciparum* infection.

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