INVESTIGATING THE CLASSICAL NON-HOMOLOGOUS END JOINING PATHWAY IN THE VIRAL VECTOR *AEDES AEGYPTI*

A Dissertation

by

JUSTIN MICHAEL OVERCASH

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Chair of Committee, Zachary N Adelman
Committee Members, Kevin M Myles
Dorothy Shippen
Christine Merlin
Chair of Interdisciplinary Faculty, David Threadgill

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ABSTRACT

Aedes aegypti is a critical viral vector and is known to transmit yellow fever, dengue and Zika viruses. Current control mechanisms rely heavily on source removal and insecticide use making novel genetic control mechanisms an attractive alternative. The CRISPR/Cas9 system enhances basic and applied research by allowing for sequence specific genetic modification. Utilization of the CRISPR/Cas9 system relies on repair of DNA double stranded breaks by endogenous DNA repair pathways such as the classical non-homologous end joining (C-NHEJ) or homology directed repair (HDR). Manipulating the C-NHEJ pathway to increase rates of HDR would expedite experiments which rely on site specific integration of genetic constructs. To investigate the C-NHEJ pathway we aimed to create a luminescence-based assay to quantify shifts in end joining based repair and knockout the C-NHEJ genes *ku70* and *lig4* in the *Ae. aegypti* LVP strain. Using a GFP-firefly luciferase fusion protein, we created a luminescence-based assay which quantifies shifts in error-prone end joining repair in both *Ae. aegypti* embryos and cell lines (Aag2 and A20). Knockout of *ku70* resulted in female-specific pupal lethality, reduced longevity in both male and female adult populations and delayed chorion melanization. After maintaining mutants $ku70^{-/-}$ for 25 generations, no shift in either the single strand annealing pathway (SSA) or NHEJ was detected in $ku70^{-/-}$ embryos. While no shift in SSA or C-NHEJ was detected in $ku70^{-/-}$ embryos, $ku70^{-/-}$ larvae were significantly more sensitive DNA DSB generated by the DNA DSB inducing antibiotic bleomycin when compared to our LVP strain ($p<0.0001$, Chi-square). Additionally, micro-injections targeting the eye pigment gene *kmo* in LVP and $ku70^{-/-}$ embryos resulted in LVP G_0 with a significantly higher rate of mosaic mutation when compared to $ku70^{-/-}$ G_0s (66% vs 4.1%). The results of knockout of *lig4* and *ku70* suggest that C-NHEJ plays an important role in both fitness and DNA damage sensitivity. Additionally, creation

of the NHEJ assay will allow for future high throughput analysis of additional genes involved in DNA DSB repair in both *Ae. aegypti* embryos and cells. This work will hopefully aid in future DNA damage repair studies which may be used to improve both basic and applied research in *Ae. aegypti*.

DEDICATION

This dissertation is dedicated to my grandfather James Thomas Jenkins who was my first mentor and the man who pushed me to value truth and wisdom. To do good and avoid evil.

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NOMENCLATURE

C-NHEJ	Classical Non-homologous End Joining
HDR	Homology Directed Repair
A-NHEJ	Alternative Non-homologous End Joining
DSB	Double Stranded Break
CRISPR	Clustered Regularly Interspaced Palindromic Repeat
HRMA	High Resolution Melt Curve Analysis
HE	Homing Endonuclease
SIT	Sterile Insect Technique
SSA	Single Strand Annealing
TALEN	Transcription Acitvator-Like Effector Nuclease
ZFN	Zinc Finger Nuclease
GD	Gene Drive
RMCE	Recombinase Mediated Cassette Exchange
TE	Transposable Element

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CHAPTER I

INTRODUCTION

Aedes aegypti is a critical viral vector with the capacity to transmit dengue, chikungunya, yellow fever and Zika viruses (WHO, 2018, CDC, 2019). *Ae. aegypti* are primarily located in tropical and sub-tropical regions of the world, infect millions of people annually and place heavy economic burdens on developing countries. Current vector control approaches rely on the use of insecticides, source removal and biological control strategies (Huang et al., 2017). Development of novel genetic control mechanisms, such as gene drives, may provide attractive alternatives for limiting the dissemination of arboviruses vectored by *Ae. aegypti*.

The application of genetic engineering to create transgenic mosquitoes could advance generation of new control strategies to reduce local *Ae. aegypti* population levels or create virally refractive strains resulting in a reduction of viral transmission. The sterile insect technique (SIT) has been proposed to reduce local populations of *Ae. aegypti* by releasing large quantities of sterile males to compete with wild type males for female mates (Alphey et al., 2010, Bond et al., 2019). A modified version of SIT, known as release of an insect carrying a dominant lethal (RIDL), may increase the effectiveness of population reduction by delaying lethality to later life stages (Fu et al., 2010, Phuc et al., 2007). Delaying lethality to later life stages would allow released transgenics to compete with wild populations for resources at multiple life stages. The RIDL technique has been tested in controlled releases in both the Cayman Islands and Brazil, resulting in population reduction (Carvalho et al., 2015, Harris et al., 2012). An alternative to SIT and RIDL are gene drives, which spread effector genes through a population at hyper-mendelian rates. This allows for rapid dissemination of genes, which can either provide viral resistance or lead to population collapse. Gene drive was initially demonstrated in dipterans in

Drosophila melanogaster (Gantz and Bier, 2015). Since then, drive mechanisms have been demonstrated in *Ae. aegypti*, *Anopheles gambiae*, and *Anopheles stephensi* (Hammond et al., 2016, Gantz et al., 2015, Li et al., 2019). These synthetic gene drives rely on the highly specific clustered regularly interspaced palindromic repeats and Cas9 endonuclease (CRISPR/Cas9) gene editing system.

Highly specific endonucleases have revolutionized genetic engineering in several species including *Aedes aegypti*. Initially, zinc finger nucleases (ZFN) and transcription activator like effector nucleases (TALENs) were utilized in *Ae. aegypti* to target genes of interest for knockout (Aryan et al., 2013b, McMeniman et al., 2014). The Cas9 endonuclease was identified in the bacteria *Streptococcus pyogenes* and was determined to play a role in bacterial adaptive immunity (Karginov and Hannon, 2010). The discovery of the Cas9 endonuclease in bacterial adaptive immunity led to cheaper high throughput gene editing in several dipteran species including *Drosophila melanogaster*, *Anopheles stephensi*, *Anopheles gambiae* and *Aedes aegypti* (Bassett et al., 2013, Gantz and Bier, 2015, Basu et al., 2015, Kistler et al., 2015, Dong et al., 2015, Hammond et al.,2016, Gantz et al., 2015). In mosquitoes, the CRISPR/Cas9 system can be used in basic science to generate knockout lines and insert transgenes at highly specific loci making studying genes with no easily observable phenotype practical. Additionally, the CRISPR/Cas9 system is used in gene drive strategies making the CRISPR/Cas9 system and the DNA damage response relevant to applied science [Reviewed in (Overcash and Adelman, 2016)]. The Cas9 endonuclease generates DNA double stranded breaks (DSB) which are repaired by endogenous DNA DSB repair pathways of the targeted organism. Understanding how DNA DSB are repaired and how DNA DSB repair pathways can be manipulated could enhance basic and applied science further by increasing rates of the desired repair pathways.

The DNA DSB damage response in mosquitoes is composed of several pathways which compete to fix DNA DSB [Reviewed in Chapter II]. The classical non-homologous end joining pathway (C-NHEJ) is believed to repair DSBs in an error prone fashion [Reviewed in (Williams et al., 2014)]. In contrast, the homology directed repair pathway (HDR) utilizes a homologous DNA sequence to accurately repair DNA double stranded breaks in an error free fashion [Reviewed in (Lamarche et al., 2010)]. Additional pathways include the single strand annealing pathway (SSA) and alternative non-homologous end joining (A-NHEJ) pathway. The SSA pathway utilizes homologous sequences up and downstream of the DNA DSB to repair breaks resulting in the loss of genetic information located between the homologous sequences. The A-NHEJ results in error prone repair (Ivanov et al., 1996, Betermier et al., 2014, Bhargava et al., 2016). The C-NHEJ pathway and the HDR pathway are primary competitors, with the C-NHEJ Ku complex and HDR MRN complex directly competing for the ends of double stranded breaks. Understanding the components of the C-NHEJ pathway in *Ae. aegypti* may allow for its inhibition by targeting genes for knockout, knock-in, or chemical inhibition. Inhibition of C-NHEJ could increase rates of HDR for the purpose of increasing the efficiency of genetic engineering, transgene insertion and gene drive in *Ae. aegypti*.

The C-NHEJ pathway relies on several proteins, including Ku70, Ku80, DNA-PKcs, XLF and Lig4, to repair DSB [reviewed in (Chang et al., 2017, Williams et al., 2014) and Chapter II]. Interestingly, certain known components of the C-NHEJ pathway such as μ polymerase, λ polymerase, Xrcc4 and Artemis, are not present in *Ae. aegypti* (Overcash et al. 2015). The DSB is initially bound by the heterodimeric ku-complex which is comprised of Ku70 and Ku80. The ku-complex binds to the broken DNA ends and prevent resection from occurring, thus inhibiting DNA DSB repair pathways which rely on the MRN complex (including HDR,

SSA and A-NHEJ). The DNA-PKcs protein plays a role in tethering the ku-complex and phosphorylates several proteins involved in the DNA DSB repair process. Once the ends of the DSB are within proximity, the ligation complex, which is composed of Lig4 and XLF, ligate the broken ends together and fixes the break. Since the C-NHEJ pathway is a primary DNA DSB repair pathway and directly competes with repair pathways which rely on MRN (such as HDR), targeting components of the C-NHEJ pathway may lead to increased utilization of the HDR pathway.

Several methods to alter rates of NHEJ in favor of HDR have been described [Reviewed in CHAPTER II]. In human and mice cell lines, utilization of SCR7, a ligase 4 inhibitor, led to an increase in HDR (Maruyama et al., 2015, Chu et al., 2015). In insects, targeting components of the C-NHEJ pathway for removal resulted in increased rates of HDR in both *Drosophila melanogaster* and *Bombyx mori* (Beumer et al., 2008, Ma et al., 2014). Our lab previously published a luminescence based assay to detect changes in SSA (Aryan et al., 2013a, Basu et al., 2015). To determine if rates of NHEJ have been altered after inhibition of the C-NHEJ pathway, we generated a luminescence-based assay which detects shifts in error prone repair (Basu et al 2016, CHAPTER III). Targeting the transcripts of *ku70* with dsRNA lead to a decrease in NHEJ, while targeting *lig4* resulted in an increase in error prone repair (Basu et al., 2015). While our initial assay (NHEJv1.0) was able to detect shifts in error prone repair in *Ae. aegypti* embryos, it was unable to do so in *Ae. aegypti* cell lines. To address this issue, as well as a change to a more accurate but less sensitive luminometer, we developed a second version of the NHEJ assay (NHEJv2.0) which allowed us to detect shifts in end joining based repair in both embryos and cells. Targeting *ku70*, *ku80* and *lig4* in two mosquito cell lines (A20 and Aag2) resulted in shifts in end joining based repair. Knockdown of Ku80 and Lig4 resulted in increased error prone

repair in the A20 cell line and Ku70 and Lig4 resulting in decreased rates of error prone repair in the Aag2 cell line, opening up the opportunity to target other NHEJ genes in cell culture (Peleg, 1968, Pudney et al. 1979). To permanently inhibit the C-NHEJ pathway, we targeted the *ku70* and *lig4* genes with CRISPR/Cas9 to generate frameshift mutation knockout strains. After generation and selection for frame shift mutations, we evaluated each knockout line impacts on fitness by determining population genotype distributions. In the *ku70* $^{-/-}$ line, we evaluated several other fitness impacts including longevity, fertility, fecundity, pupal lethality and decreased chorion melanization rates. To determine if we could detect a quantifiable shift in the DNA damage response in our *ku70*$^{-/-}$ mutant, we injected the *ku70*$^{-/-}$ embryos with both the C-NHEJv2.0 and SSA assays, detecting no observable shift in the DNA damage response. We then assessed DNA DSB damage susceptibility by employing the DNA DSB causing agent bleomycin (Murray et al., 2018). The resulting experiments showed that the *ku70*$^{-/-}$ line was more susceptible to DNA DSB induced by bleomycin. Lastly, we quantified rates of mutagenesis in the *ku70*$^{-/-}$ strain by targeting the *kmo* gene and scoring mosaic mutagenesis (Han et al., 2003). The mutagenesis experiments resulted in higher rates of mutagenesis in our WT strain and suggests survival of *ku70*$^{-/-}$ embryos after micro-injection is significantly lower. In this dissertation, we have generated several tools and methods for quantifying shifts in the DNA damage response and detecting DNA damage sensitivity, laying the groundwork for future research of the DNA damage response in *Ae. aegypti*.

REFERENCES

ALPHEY, L., BENEDICT, M., BELLINI, R., CLARK, G., DAME, D., SERVICE, M. & DOBSON, S. 2010. Sterile-insect methods for control of mosquito-borne diseases: an analysis. Vector Borne Zoonotic Dis, 10, 295 - 311.

ARYAN, A., ANDERSON, M. A., MYLES, K. M. & ADELMAN, Z. N. 2013a. Germline excision of transgenes in *Aedes aegypti* by homing endonucleases. Sci Rep, 3, 1603.

ARYAN, A., ANDERSON, M. A., MYLES, K. M. & ADELMAN, Z. N. 2013b. TALEN-based gene disruption in the dengue vector *Aedes aegypti*. PLoS One, 8, e60082.

BASSETT, A. R., TIBBIT, C., PONTING, C. P. & LIU, J. L. 2013. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. Cell Rep, 4, 220-8.

BASU, S., ARYAN, A., OVERCASH, J. M., SAMUEL, G. H., ANDERSON, M. A., DAHLEM, T. J., MYLES, K. M. & ADELMAN, Z. N. 2015. Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. Proc Natl Acad Sci U S A, 112, 4038-43.

BETERMIER, M., BERTRAND, P. & LOPEZ, B. S. 2014. Is non-homologous end-joining really an inherently error-prone process? PLoS Genet, 10, e1004086.

BEUMER, K. J., TRAUTMAN, J. K., BOZAS, A., LIU, J. L., RUTTER, J., GALL, J. G. & CARROLL, D. 2008. Efficient gene targeting in *Drosophila* by direct embryo injection with zinc-finger nucleases. Proc Natl Acad Sci U S A, 105, 19821-6.

BHARGAVA, R., ONYANGO, D. O. & STARK, J. M. 2016. Regulation of Single-Strand Annealing and its Role in Genome Maintenance. Trends in Genetics, 32, 566-575.

BOND, J. G., OSORIO, A. R., AVILA, N., GÓMEZ-SIMUTA, Y., MARINA, C. F., FERNÁNDEZ-SALAS, I., LIEDO, P., DOR, A., CARVALHO, D. O., BOURTZIS, K.

& WILLIAMS, T. 2019. Optimization of irradiation dose to *Aedes aegypti* and *Ae. albopictus* in a sterile insect technique program. PLOS ONE, 14, e0212520.

CARVALHO, D. O., MCKEMEY, A. R., GARZIERA, L., LACROIX, R., DONNELLY, C. A., ALPHEY, L., MALAVASI, A. & CAPURRO, M. L. 2015. Suppression of a Field Population of *Aedes aegypti* in Brazil by Sustained Release of Transgenic Male Mosquitoes. PLOS Neglected Tropical Diseases, 9, e0003864.

CDC. 2019. *Dengue and the Aedes aegypti mosquito* [Online]. Centers for Disease Control and Prevention. Available: https://www.cdc.gov/dengue/resources/30jan2012/aegyptifactsheet.pdf [Accessed 4/10/19 2019].

CHU, V. T., WEBER, T., WEFERS, B., WURST, W., SANDER, S., RAJEWSKY, K. & KUHN, R. 2015. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nat Biotechnol, 33, 543-8.

DONG, S., LIN, J., HELD, N. L., CLEM, R. J., PASSARELLI, A. L. & FRANZ, A. W. 2015. Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti*. PLoS One, 10, e0122353.

FU, G., LEES, R. S., NIMMO, D., AW, D., JIN, L., GRAY, P., BERENDONK, T. U., WHITE-COOPER, H., SCAIFE, S., KIM PHUC, H., MARINOTTI, O., JASINSKIENE, N., JAMES, A. A. & ALPHEY, L. 2010. Female-specific flightless phenotype for mosquito control. Proc Natl Acad Sci U S A, 107, 4550-4.

GANTZ, V. M. & BIER, E. 2015. Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. Science, 348, 442-4.

GANTZ, V. M., JASINSKIENE, N., TATARENKOVA, O., FAZEKAS, A., MACIAS, V. M., BIER, E. & JAMES, A. A. 2015. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. Proc Natl Acad Sci U S A, 112, E6736-43.

HAMMOND, A., GALIZI, R., KYROU, K., SIMONI, A., SINISCALCHI, C., KATSANOS, D., GRIBBLE, M., BAKER, D., MAROIS, E., RUSSELL, S., BURT, A., WINDBICHLER, N., CRISANTI, A. & NOLAN, T. 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. Nat Biotechnol, 34, 78-83.

HAN, Q., CALVO, E., MARINOTTI, O., FANG, J., RIZZI, M., JAMES, A. A. & LI, J. 2003. Analysis of the wild-type and mutant genes encoding the enzyme kynurenine monooxygenase of the yellow fever mosquito, *Aedes aegypti*. Insect molecular biology, 12, 483-490.

HARRIS, A. F., MCKEMEY, A. R., NIMMO, D., CURTIS, Z., BLACK, I., MORGAN, S. A., OVIEDO, M. N., LACROIX, R., NAISH, N., MORRISON, N. I., COLLADO, A., STEVENSON, J., SCAIFE, S., DAFA'ALLA, T., FU, G., PHILLIPS, C., MILES, A., RADUAN, N., KELLY, N., BEECH, C., DONNELLY, C. A., PETRIE, W. D. & ALPHEY, L. 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nat Biotech, 30, 828-830.

HUANG, Y.-J. S., HIGGS, S. & VANLANDINGHAM, D. L. 2017. Biological Control Strategies for Mosquito Vectors of Arboviruses. *Insects,* 8**,** 21.

IVANOV, E. L., SUGAWARA, N., FISHMAN-LOBELL, J. & HABER, J. E. 1996. Genetic requirements for the single-strand annealing pathway of double-strand break repair in *Saccharomyces cerevisiae*. Genetics, 142, 693-704.

KARGINOV, F. V. & HANNON, G. J. 2010. The CRISPR system: small RNA-guided defense in bacteria and archaea. Mol Cell, 37, 7-19.

KISTLER, K. E., VOSSHALL, L. B. & MATTHEWS, B. J. 2015. Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. Cell Rep, 11, 51-60.

LAMARCHE, B. J., ORAZIO, N. I. & WEITZMAN, M. D. 2010. The MRN complex in double-strand break repair and telomere maintenance. FEBS Lett, 584, 3682-95.

LI, M., YANG, T., KANDUL, N. P., BUI, M., GAMEZ, S., RABAN, R., BENNETT, J., SÁNCHEZ C., H. M., LANZARO, G. C., SCHMIDT, H., LEE, Y., MARSHALL, J. M. & AKBARI, O. S. 2019. Development of a Confinable Gene-Drive System in the Human Disease Vector, *Aedes aegypti*. bioRxiv, 645440.

MA, S., CHANG, J., WANG, X., LIU, Y., ZHANG, J., LU, W., GAO, J., SHI, R., ZHAO, P. & XIA, Q. 2014. CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of BmKu70 in *Bombyx mori*. Sci Rep, 4, 4489.

MARUYAMA, T., DOUGAN, S. K., TRUTTMANN, M. C., BILATE, A. M., INGRAM, J. R. & PLOEGH, H. L. 2015. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotech, 33, 538-542.

MCMENIMAN, C. J., CORFAS, R. A., MATTHEWS, B. J., RITCHIE, S. A. & VOSSHALL, L. B. 2014. Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell, 156, 1060-71.

MURRAY, V., CHEN, J. K. & CHUNG, L. H. 2018. The Interaction of the Metallo-Glycopeptide Anti-Tumour Drug Bleomycin with DNA. International journal of molecular sciences, 19, 1372.

OVERCASH, J. & ADELMAN, Z. N. 2016. Progress in Gene Editing Transgenesis Genome Manipulation in Mosquitoes. Advances in Insect Physiology, 51, 1-35.

PELEG, J. 1968. Growth of arboviruses in monolayers from subcultured mosquito embryo cells. Virology, 35, 617-619.

PHUC, H. K., ANDREASEN, M. H., BURTON, R. S., VASS, C., EPTON, M. J., PAPE, G., FU, G., CONDON, K. C., SCAIFE, S., DONNELLY, C. A., COLEMAN, P. G., WHITE-COOPER, H. & ALPHEY, L. 2007. Late-acting dominant lethal genetic systems and mosquito control. BMC Biol, 5, 11.

PUDNEY, M., VARMA, M. G. R. & LEAKE, C. J. 1979. Establishment of cell lines from larvae of culicine (Aedes species) and anopheline mosquitoes. TCA manual / Tissue Culture Association, 5, 997-1002.

WHO. 2018. *Dengue and severe dengue* [Online]. World Health Organization. Available: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue [Accessed 4/10/19 2019].

CHAPTER II

UNDERSTANDING THE DNA DAMAGE RESPONSE IN ORDER TO ACHIEVE DESIRED GENE EDITING OUTCOMES IN MOSQUITOES[*]

INTRODUCTION

Mosquitoes are important disease vectors, with none more important than the malaria vector *Anopheles gambiae* and the dengue vector *Aedes aegypti*. Malaria is believed to infect more than 200 million annually causing more than half a million deaths (CDC 2012, WHO 2014b), while dengue is thought to infect fifty to one hundred million annually, causing mass morbidity and placing a large economic burden on developing countries (WHO 2014a, CDC 2012). Other pathogens such as yellow fever virus, chikungunya virus, West Nile virus, eastern equine encephalitis virus, western equine encephalitis virus, and La Crosse virus are also vectored by mosquitoes (Colpitts et al., 2011; Pialoux et al., 2007; Chhabraet al., 2008) and cause substantial human morbidity and mortality.

Treatment for malaria revolves around the use of insecticide treated bed nets (Hill et al. 2006), indoor residual insecticides (Pluess et al., 2010), and treatment of infected human hosts with anti-malarial drugs (Schlitzer, 2008); for dengue, prevention is primarily through control of the vector by way of source reduction and insecticides (Kamgang et al., 2011). Techniques such as the sterile insect technique (SIT) (Alphey et al., 2010), release of insects harboring intracellular bacteria *Wolbachia* (Walker et al., 2011), and release of insects with dominant lethality (RIDL) (Massonnet-Bruneelet al., 2013, Fu et al. 2010, Labbé et al., 2012) are currently

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being investigated as additional interventions and appear to hold great promise. Also in development are techniques that aim to replace current mosquito populations with those that are pathogen resistant [reviewed in Burt (2014) and Wang and Jacobs-Lorena (2013)]. A primary technical concern of gene-drive based control strategies is the integrity and long-term stability of any pathogen-resistance transgene. Optimally, such transgenes would be placed in a chromosomal region most likely to be repaired faithfully in the case of DNA damage. However, little is known about mosquito DNA break repair pathways, or how chromosomal structure and repeat content may influence the long-term stability of such transgenes, particularly regions that might be more inclined towards mutations or transcriptional silencing.

More generally, the advent of site-specific gene editing technologies such as Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)/Cas9 is poised to revolutionize the field of mosquito genetics, molecular biology and physiology (Kim and Kim, 2014). These highly specific nucleases rely on host DNA repair pathways to fix the broken DNA ends, preferably in a fashion consistent with the hopes of the investigator. An unavoidable complication, however, is that each of the various end-joining and homology-based repair pathways compete with each other for access to the double-stranded DNA break (DSB). Work by others has shown that it is possible to manipulate the repair outcome in insects such as Drosophila (Beumer et al., 2008, Ciapponi et al., 2004, Yoo and McKee, 2005, Wei and Rong, 2007) and *Bombyx mori* (Ma et al., 2014). However, little is known about the hierarchy of DNA repair choice in mosquitoes, or the protein components most critical for completing each form of DSB repair. In this review, we present our current understanding of DSB repair as gleaned from model organisms such as yeast, flies and vertebrates in the context of genes and gene families lost and duplicated in the

mosquito. Additionally, we present potential strategies for manipulating the DSB response in mosquitoes with respect to protein machinery, chromosomal structure, and sequence content. An understanding of these aspects of genome engineering in mosquitoes will hopefully stimulate further investigation and generate evidence-based questions about how genomic engineering in mosquito disease vectors can be further improved.

GENE EDITING TECHNOLOGIES USED IN MOSQUITOES

Homing endonucleases (HEs) were the first site-specific nucleases used to edit the mosquito genome (Windbichler et al., 2007). Known for their extreme target specificity (Stoddard, 2005; Stoddard, 2011), HEs are naturally occurring selfish genetic elements that can display hyper-Mendelian rates of inheritance due to their ability to be copied from a template to a target chromosome via homology-directed repair following DSB induction (Burt 2003). In the malaria mosquito, the HE I-SceI has been used to generate an artificial gene drive system that may one day be used to convert these mosquitoes into a more benign form that no longer transmits malaria parasites (Windbichler et al., 2011). The fortuitous insertion of a transposon bearing an I-SceI recognition sequence onto the Y chromosome of *An. gambiae* has also allowed the homology-dependent integration of additional transgenes to this location (Bernardini et al., 2014). The HE I-PpoI, which recognizes a target ribosomal DNA that that is conserved amongst all eukaryotes, was used to develop transgenic strains of *An. gambiae* that display either male-specific sterility (Windbichler et al., 2008, Klein et al., 2012) or male-specific sex distortion (Galizi et al., 2014) phenotypes. While HEs have not been used as extensively to perform chromosomal manipulations in other mosquitoes, several HEs have been shown to recognize and cleave their target sites in a highly specific manner when these sites are present in the *Ae. aegypti*

genome (Aryan et al., 2013a; Traver et al., 2009). Despite the fact that several hundred naturally occurring HEs have been described, the difficulty and expense in re-engineering these site-specific nucleases to recognize new and useful targets will likely restrict their application.

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

Thus, the most promising future for mosquito chromosomal manipulation may be with CRISPR/Cas9. The CRISPR/Cas9 system is part of the adaptive immune system within certain bacteria (Chakraborty et al., 2009, Karginov and Hannon, 2010) , and uses short RNA sequences to guide a DNA endonuclease (Cas9), resulting in a DSB (Sander and Joung, 2014, Bassett and Liu, 2014, Liu and Fan, 2014, Mali et al., 2013b). The CRISPR/Cas9 system has been further adapted to use synthetic guide RNAs, further optimizing the process (Bassett et al., 2013, Ma et al., 2013, Upadhyay and Sharma, 2014, Bae et al., 2014). Re-engineering new target sites is as simple as synthesizing a new small RNA molecule; this ease of use explains why Cas9-based

editing has so rapidly supplanted other technologies. For mosquito gene editing, as for *Drosophila*, a computational search for potential cross-targeting guide RNAs before the experiment begins (Xie et al., 2014), followed by several generations of out-crossing of edited individuals (Liesch et al., 2013) should be sufficient to minimize confounding off-target effects. Other options include optimizing the concentration of sgRNAs to Cas9, and utilizing pairs of Cas9 nucleases that have been mutated only to allow nicking (or single-stranded breaks) to occur, which when used in conjunction effectively generate a DSB (Mali et al., 2013a). While there have been no published incidences of the use of CRISPR/Cas 9 in mosquitoes; unpublished data suggests that the system will in fact work in a highly efficient manner (not shown).

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

wishes of the experimenter. Understanding how these repair pathways function, as well as how they interact and compete with each other, is thus critical to optimizing gene-editing experiments. This is especially necessary in non-model organisms such as mosquitoes that are more difficult to handle at larger scales.

DOUBLE-STRANDED DNA-BREAK REPAIR

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

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

McKee, 2005). Rad54 works in conjunction with the Rad51 invasion filament until the appropriate homologous sequence is found (Kiianitsa et al., 2006). Once homology is detected, either δ or ε (delta or epsilon), polymerase is recruited to accurately repair the DNA lesion (Mehta and Haber, 2014). Depending on the nature of the break, a Holiday junction is formed (Heyer, 2004) and eventually disassembled leading to an "error free" repair of the DSB. Rad51 and Rad54 mutants have been evaluated in Drosophila using a DNA repair assay; Rad51 was determined to be crucial for HDR, with Rad54 deemed important as well, but to a lesser extent than Rad51 (Wei and Rong, 2007). In an alternative approach, RNAi-based suppression of Rad51 mRNA in Drosophila led to higher death rates in the presence of a mutagenic substance (Yoo, 2006). Conversely, over-expression of Rad51 in Drosophila using a heat shock promoter also resulted in lethality, suggesting that the amount of homology-based repair must be finely controlled (Yoo and McKee, 2004). HDR components have not yet been studied in mosquitoes.

Table 1. Orthologs of DNA break repair components in mosquitoes

Gene	*NHEJ*	*A-NHEJ*	*HDR*	*SSA*	*D. melanogaster*	*Ae. aegypti*	*An. gambiae*
Ku70	X				FBgn0011774	No gene model	AGAP002690
Ku80	X				FBgn0041627	AAEL003684	AGAP009910
DNA-PKcs	X				Absent	AAEL008123	AGAP003967
Xrcc4	X				FBgn0069301	No ortholog identified	No ortholog identified
XLF	X				No ortholog identified	AAEL002939	No ortholog identified
Ligase 4	X				FBgn0030506	AAEL0173656/AAEL017561	AGAP000623
Polμ/Polλ	X				Absent	Absent	Absent
Artemis	X				No ortholog identified	No ortholog identified	AGAP000597
APLF	X				FBgn0026737	AAEL011254	AGAP004516
PNKP	X				FBgn0037578	AAEL000527	AGAP012174
APTX	X				FBgn0038704	AAEL014945	AGAP004307
Parp1		X			FBgn0010247	AAEL011815	AGAP003230
Ligase 3		X			FBgn0038035	Absent	Absent
Ligase 1		X			FBgn0262619	AAEL017566	AGAP009222

Table 1. Continued

Gene	*NHEJ*	*A-NHEJ*	*HDR*	*SSA*	*D. melanogaster*	*Ae. aegypti*	*An. gambiae*
Xrcc3		X			FBgn0003480	AAEL005399	AGAP013180
Xrcc1		X			FBgn0026751	AAEL002782	AGAP002605
ATM		X			FBgn0045035	AAEL014900	AGAP009632
Mre11		X	X		FBgn0020270	AAEL010595/AAEL000034	AGAP006797
Rad50		X	X		FBgn0034728	AAEL014748/AAEL005245	AGAP003676
Nbs1		X	X		FBgn0261530	AAEL014377	AGAP003213
Sae2		X	X		FBgn0029113	AAEL010641	AGAP008637
Exo1			X		FBgn0015553	AAEL006209	AGAP004491
RPA			X		FBgn0010173	AAEL012826	AGAP001421
Sgs1			X		FBgn0002906	AAEL004039	AGAP002967
Dna2			X		FBgn0030170	AAEL000201	AGAP004685
Rad51			X		FBgn0003479	AAEL006080	AGAP013412
Rad54			X		FBgn0002989	AAEL002647	AGAP008748

Table 1. Continued

Gene	*NHEJ*	*A-NHEJ*	*HDR*	*SSA*	*D. melanogaster*	*Ae. aegypti*	*An. gambiae*
BRCA2			X		FBgn0050169	AAEL014774/AAEL010133	AGAP007032
Polδ			X		FBgn0263600	AAEL014178	AGAP011731
Polσ			X		FBgn0264326	AAEL002800	AGAP004615
Rad52			X	X	Absent	Absent	Absent
Rad1				X	FBgn0026778	AAEL009701	AGAP002255
Rad10/Ercc1				X	FBgn0028434	AAEL008081/AAEL013693	AGAP004029
Msh2				X	FBgn0015546	AAEL014856	AGAP010282
Slx4				X	FBgn0002909	AAEL008482	AGAP007582
Msh3				X	Yeast Only	Yeast Only	Yeast Only
Rad59				X	Absent	Absent	Absent
Saw1				X	Yeast Only	Yeast Only	Yeast Only

Unlike traditional HDR, which relies on homologous sequences present on sister chromatids or homologous chromosomes, the SSA pathway relies on the use of homologous repeats flanking the DSB (Ivanov et al., 1996). The homologous regions can be as short as 30 bp in yeast (Sugawara et al., 2000), or as short as 18 bp in mosquitoes (Aryan et al., 2013a), and are eventually collapsed, while the genetic information between the two repeats is deleted. SSA is generally categorized as a sub-pathway of HDR because of the use of similar protein machinery in the initial resection steps. As in HDR, resecting occurs via the MRN complex and secondary exonucleases (Mehta and Haber, 2014). A protein complex consisting of the scaffolding proteins Slx4 and Saw1 (Li et al., 2008a), the mismatch repair proteins Msh2 and Msh3 (Sugawara et al., 1997), and the Rad1-Rad10 endonuclease complex (Davies et al., 1995) work in conjunction to collapse the two homologous sections and remove excess nucleotides. In yeast, Rad52 then anneals the strands in conjunction with the homolog Rad59 (Sugawara et al., 1997). Saw1 and Msh2 are not present in flies or mosquitoes, suggesting differences in both scaffolding and mismatch scanning in SSA-based repair, while the absence of Rad52 and Rad59 in flies and mosquitoes suggests that there may be alternative machinery which compensates for the annealing step (Table 1). Mice deficient in Rad1 and Rad10 orthologs showed similar phenotypes with regard to chromosomal damage and ultimately died prematurely (Tian et al., 2004, McWhir et al., 1993); while Drosophila Rad1 and Rad10 mutants are both viable and fertile, but largely understudied (Drysdale and FlyBase2008).

The C-NHEJ pathway is distinct from HDR and SSA in both its machinery and mechanism. Upon DSB formation, Ku70 and Ku80 form a heterodimeric complex on the ends of the DSB called the Ku complex (Wang and Lees-Miller, 2013). Knockout of Ku70 in *Arabidopsis* led to a five to sixteen fold increase in HDR (Qi et al., 2013), while knockdown of

Ku70 in *Bombyx mori* increased rates of HDR via a junction PCR assay (Ma et al., 2014), suggesting that Ku proteins may be a target of interest in mosquitoes. While the most current gene set (AaegL3.2) failed to identify a gene model for *Ae. aegypti* Ku70, a homology-based search (tblastn) using the *Drosophila* or *Anopheles* Ku70 protein sequences indicates that a likely ortholog is located on scaffold 1.240. The kinase DNA-PKcs tethers the Ku complexes and activates multiple proteins associated with the pathway (Williams et al., 2014), primarily the endonuclease Artemis, which is responsible for removing nucleotides from the DSB (Ma et al., 2002). Subsequently, the polymerases μ and λ play a role in adding any additional nucleotides needed for ligation. Both polymerases μ and λ are absent in flies and mosquitoes, while Artemis appears to have been lost in both *D. melanogaster* and *Ae. aegypti* (Figure 2.1 A, Table 1). In vertebrates, these factors are primarily associated with DNA ligation during V(D)J recombination, a process that does not occur in insects, with mutations resulting in a failure to generate proper T and B cells. Following modification of the broken DNA ends, the factors XLF and Xrcc4 form the primary scaffolding components (Mahaney et al., 2013) and recruit Lig4 to the break site, allowing for the final ligation of both ends of the DSB (Williams et al., 2014). While mutation of XLF in mice resulted in radiation sensitivity, impaired V(D)J recombination and lower levels of lymphocytes (Li et al., 2008b), work by others suggests that Xrcc4 and Lig4 mutants are the most severe in respect to loss of NHEJ (Karanjawala et al., 2002). While in mice the absence of Lig4 results in embryonic death (Karanjawala et al., 2002), Lig4 mutants in *Arabidopsis* show a three to four fold increase in HDR (Qi et al., 2013), and Lig4 Drosophila mutants are viable and produce fertile offspring with rates of HDR higher than 70%, where they had previously been less than 15% (Beumer et al., 2008). Xrcc4 orthologs could not be identified in either *Ae. aegypti* or *An. gambiae* via homology (Blastp) or domain-based (HMMER) searches

(not shown), while Lig4 appears to have been duplicated in *Ae. aegypti*. Other proteins such as APLF, APTX and PNKP are also involved in NHEJ-based repair. APLF is believed to play a role as a scaffolding protein (Grundy et al., 2013) as well as an exonuclease (Li et al., 2011); APTX has been shown to remove AMP from DNA ends (Clements et al. 2004), while PNKP removes and replaces non-ligatable groups from the DSB allowing for Lig4 to complete its function (Weinfeld et al., 2011). While the end joining of this highly complex repair system is believed to be error prone in nature, recent studies suggest that it may be much more faithful than traditionally thought (Betermier et al., 2014).

In addition to C-NHEJ, DSBs may be repaired by a Ku-independent mechanism, termed alternative non-homologous end joining (A-NHEJ). Unlike C-NHEJ, A-NHEJ is suspected to be highly error prone (Betermier et al., 2014, Deriano and Roth, 2013). Like the C-NHEJ pathway, A-NHEJ ligates two broken ends of a DSB together, largely without the use of a homologous template. Unlike C-NHEJ, which initiates at the step of binding free-DNA ends, A-NHEJ appears to proceed after initiation of repair by the HDR resection machinery, with the use of the MRN complex, in conjunction with Sae2, to remove undesired nucleotides (Truong et al., 2013). Once resection has initiated, microhomology is utilized in the absence of a competing pathway to ligate the broken ends in an error-prone manner (Soni et al., 2014). The protein PARP1 competes

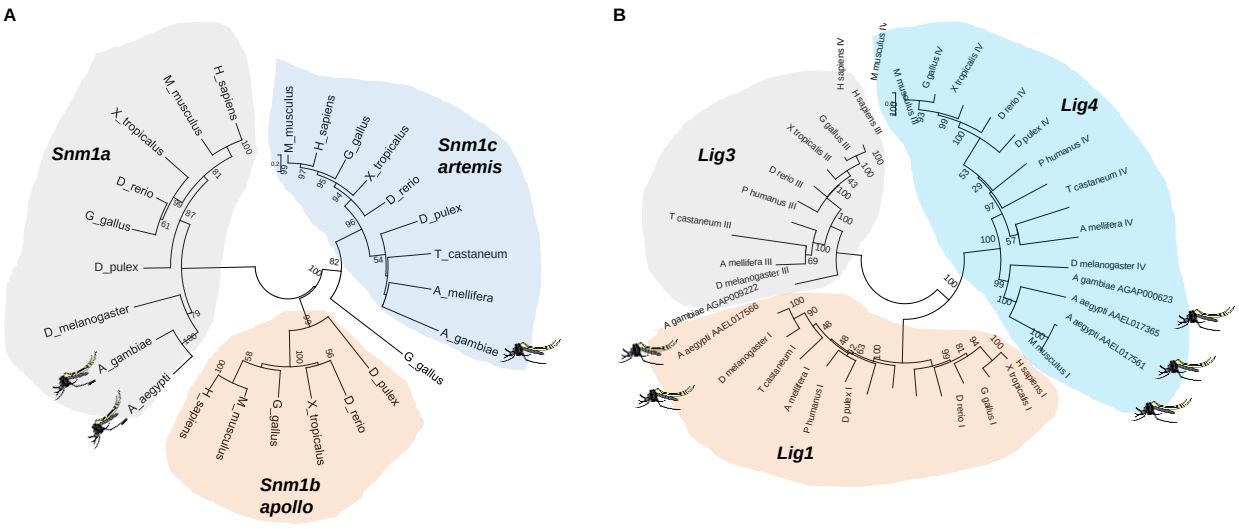

Figure 2.1 Gain and loss of NHEJ components in mosquitoes. Neighboring-joining tree produced from a clustalW alignment of Smn1-family proteins (**A**) or DNA ligases (**B**) using MEGA6 [118]. Bootstrap support (2000 replicates) is indicated on each branch if over 50%. Abbreviations: *Homo sapiens* (H_sapiens), *Mus musculus* (M_musculus), *Gallus gallus* (G_gallus), *Xenopus tropicalis* (X_tropicalis), *Danio rerio* (D_rerio), *Daphnia pulex* (D_pulex), *Pediculus humanus* (P_humanus), *Tribolium castaneum* (T_castaneum), *Apis mellifera* (A_mellifera), *Drosophila melanogaster* (D_melanogaster), *Anopheles gambiae* (A_gambiae), *Aedes aegypti* (A_aegypti). Mosquito species are indicated with an icon.

with Ku proteins for binding of the DSB, as well as possibly recruiting other proteins to the break (Wanget al., 2006), tethering the DNA strands (Chiruvella et al., 2013) and possibly mediating translocations (Soni et al., 2014, Simsek and Jasin, 2010). The primary ligase involved in this process is believed to be Lig3, which operates in conjunction with Xrcc1 (Oh et al., 2014). However, removal of Xrcc1 via mutation in hamster cells does not stop Lig3 from functioning, instead increasing its susceptibility to competition by Lig1 (Soniet al., 2014). Notably, Lig3 has

been lost in mosquitoes, suggesting that Lig1 may be critical for A-NHEJ in these species (Figure 2.1 B, Table 1).

While repair pathways have been studied in model organisms [reviewed in Lamarche et al., (2010),Williams et al., (2014), and Chiruvella et al., (2013)], little is known about these processes in mosquitoes, which diverged from *Drosophila* approximately 240 MYA. While the majority of DNA break repair proteins appear to be conserved in flies and mosquitoes, there are some interesting anomalies, as noted above. While models of hierarchical repair have been proposed in model organisms (Mansour et al., 2008), similar models must be developed and tested in mosquitoes (Figure 2.2). A strong grasp of how pathways are chosen will facilitate better use of endonuclease tools and more confident interpretation of experimental results. How can the various proteins involved in these pathways be manipulated to achieve the experimenter's desired results? The suppression of end-joining repair pathways to increase repair via a homologous template has been accomplished in both flies and silkworm (Beumer et al., 2008, Ma et al., 2014), suggesting that similar results could be achieved in mosquitoes. Alternatively, disabling both HDR and C-NHEJ could potentially increase rates of targeted deletions, favoring the generation of gross repair errors such as translocations and inversions. A more complete understanding of the processing that determines the hierarchy of DNA repair pathways will allow the vector biologist to take full advantage of tools such as CRISPR/Cas9 in the most efficient manner possible.

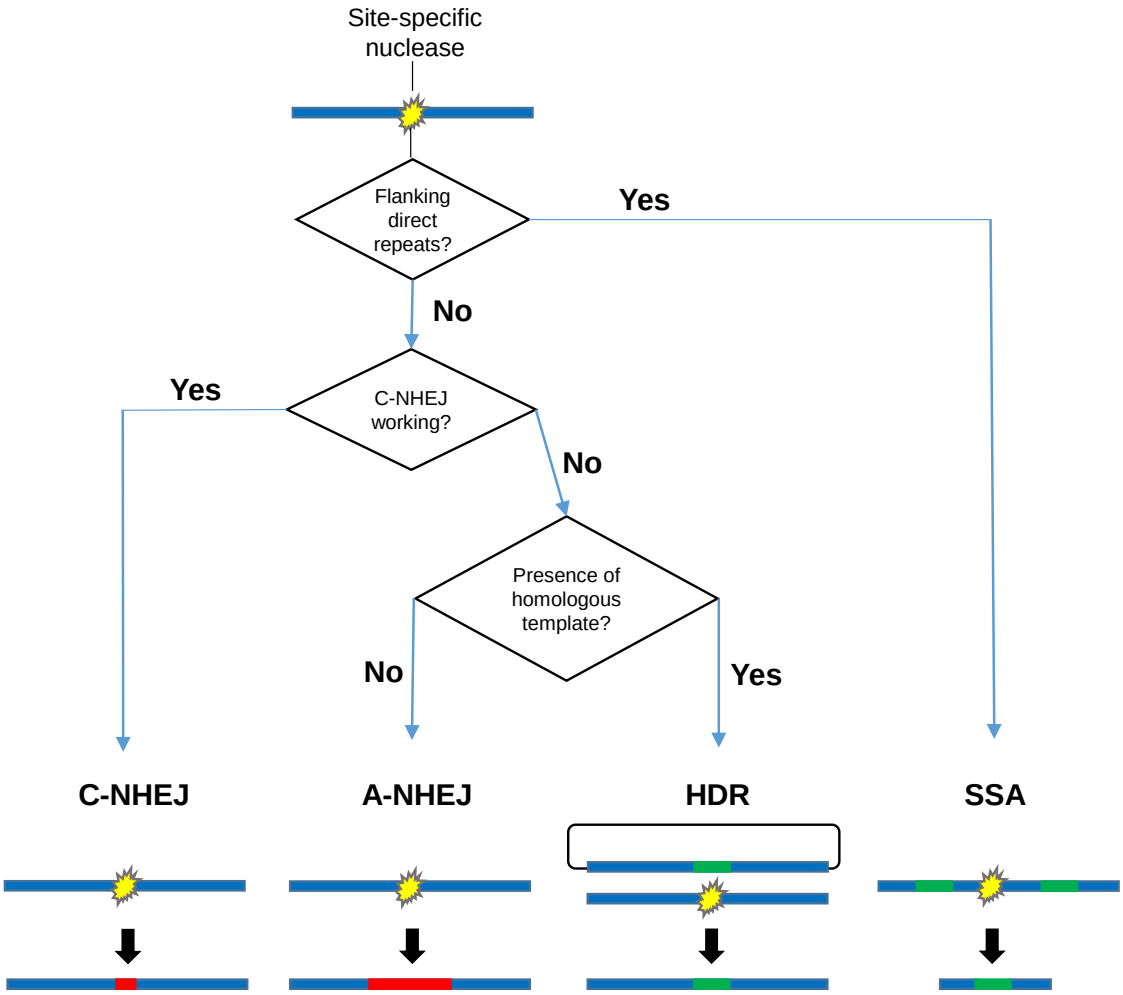

Figure 2.2 Potential hierarchy of DSB repair pathways in mosquitoes. Simplified flowchart of possible repair outcomes following DSB induction in the mosquito genome. Both known (proximity and length of repeat sequences, microhomologies, cell cycle phase, developmental stage, chromosomal organization, etc...) and unknown variables may contribute to each decision fork.

CHROMOSOMAL STRUCTURE AND ORGANIZATION IN MOSQUITO GENOME MANIPULATION

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

In addition to the physical obstructions that chromatin proteins may induce, the organization of the chromosome itself also may have a role in the choice of DNA repair pathways. The *An. gambiae* genome is organized with pericentric and intercalary heterochromatic regions, which are gene-poor, transposable element (TE) rich, and highly condensed (Sharakhova et al., 2010). The *Ae. aegypti* genome is much larger (~1.4 Gbp), with the typical heterochromatic vs. euchromatic demarcation clouded by the presence of large regions of short interspaced repeats (Nene et al., 2007). Gene models in *Ae. aegypti* have expanded intronic regions (nearly four times as long as those of *An. gambiae),* with repetitive elements distributed throughout; such repeats and TEs make up roughly 50% of the genome (Nene et al., 2007, Severson and Behura, 2012). Because SSA-based repair requires flanking repeat sequences (Ivanov et al., 1996) and A-NHEJ appears to utilize microhomology (Truong et al., 2013), the content and organization of repetitive elements around the selected target site may influence the choice of DNA repair pathway. *Ae. aegypti*, which has proportionally more repetitive elements in its genome than does *An. gambiae*, may thus be more likely to use the SSA repair pathway for DNA break repair; we have shown previously that SSA competes favorably with NHEJ when direct repeats are present (Aryan et al., 2013a). Considering the expansion of interspersed repetitive elements in most gene models of *Ae. aegypti* , achieving efficient levels of HDR in this organism may be a challenge due to difficulty identifying unique sequences of sufficient length flanking a DSB site. Initial experiments have used 1-2kb of homologous sequence on either side of the break (Liesch et al., 2013, McMeniman et al., 2014), but a thorough characterization of minimum requirements for HDR would be beneficial.

FINAL CONCLUSIONS

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

Targeted inversions have been generated in human cells via the NHEJ pathway using pairs of ZFNs targeting different sites (Lee et al., 2012); similar results were obtained in Drosophila using HEs (Egli et al., 2004). Inversions in mosquitoes, such as the 2L*a* inversion in *An. gambiae*, have been suggested to confer an advantage to the organism in arid environments (White et al., 2009). Targeted inversions at various locations could be used to confirm phenotypic effects of suspected naturally occurring inversions, or the creation of novel inversion phenotypes. In Drosophila and mice, targeted inversions have been used in the creation of balancer chromosomes that are resistant to homologous recombination (Casso et al., 2000, Zheng et al, 1999). The creation of balancer chromosomes in mosquitoes would simplify screening (by

fluorescent makers and homozygous lethal genes) of genetically engineered organisms and reduce rates of homologous recombination stabilizing introduced mutations. These technologies would also facilitate high-throughput methods of screening for the identification of genetic mutants and phenotypic traits. Translocations have been generated with nucleases in both *Drosophila* and human cells (Egli et al., 2004, Piganeau et al., 2013). Perhaps by up-regulating components of the A-NHEJ pathway, which is suspected to play a role in translocations (Soni et al., 2014), targeted translocations may be generated to produce novel meiotic gene drive systems (Pearson and Wood, 1980). Mosquitoes carrying a targeted translocation could be used in typical SIT fashion for population reduction through the release of males only, or could be used to drive population replacement by the continued release of both males and females. This process could also be reversible with the release of wild type males and females.

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

REFERENCES

ADELMAN, Z. N., JASINSKIENE, N. & JAMES, A. A. 2002. Development and applications of transgenesis in the yellow fever mosquito, *Aedes aegypti*. *Mol Biochem Parasitol,* 121**,** 1-10.

ALPHEY, L., BENEDICT, M., BELLINI, R., CLARK, G., DAME, D., SERVICE, M. & DOBSON, S. 2010. Sterile-insect methods for control of mosquito-borne diseases: an analysis. *Vector Borne Zoonotic Dis,* 10**,** 295 - 311.

ARYAN, A., ANDERSON, M. A., MYLES, K. M. & ADELMAN, Z. N. 2013a. Germline excision of transgenes in *Aedes aegypti* by homing endonucleases. *Sci Rep,* 3**,** 1603.

ARYAN, A., ANDERSON, M. A., MYLES, K. M. & ADELMAN, Z. N. 2013b. TALEN-based gene disruption in the dengue vector *Aedes aegypti*. *PLoS One,* 8**,** e60082.

BAE, S., PARK, J. & KIM, J. S. 2014. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics,* 30**,** 1473-5.

BASSETT, A. & LIU, J. L. 2014. CRISPR/Cas9 mediated genome engineering in *Drosophila*. *Methods,* 69, 128-136.

BASSETT, A. R., TIBBIT, C., PONTING, C. P. & LIU, J. L. 2013. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep,* 4**,** 220-8.

BASSETT, A. R., TIBBIT, C., PONTING, C. P. & LIU, J. L. 2014. Mutagenesis and homologous recombination in *Drosophila* cell lines using CRISPR/Cas9. *Biol Open,* 3**,** 42-9.

BERNARDINI, F., GALIZI, R., MENICHELLI, M., PAPATHANOS, P. A., DRITSOU, V., MAROIS, E., CRISANTI, A. & WINDBICHLER, N. 2014. Site-specific genetic

engineering of the *Anopheles gambiae* Y chromosome. *Proc Natl Acad Sci U S A,* 111**,** 7600-5.

BETERMIER, M., BERTRAND, P. & LOPEZ, B. S. 2014. Is non-homologous end-joining really an inherently error-prone process? *PLoS Genet,* 10**,** e1004086.

BEUMER, K. J., TRAUTMAN, J. K., BOZAS, A., LIU, J. L., RUTTER, J., GALL, J. G. & CARROLL, D. 2008. Efficient gene targeting in *Drosophila* by direct embryo injection with zinc-finger nucleases. *Proc Natl Acad Sci U S A,* 105**,** 19821-6.

BURT, A. 2003. Site-specific selfish genes as tools for the control and genetic engineering of natural populations. *Proc Biol Sci,* 270**,** 921-8.

BURT, A. 2014. Heritable strategies for controlling insect vectors of disease. *Philos Trans R Soc Lond B Biol Sci,* 369**,** 20130432.

CARLSON, D. F., FAHRENKRUG, S. C. & HACKETT, P. B. 2012. Targeting DNA With Fingers and TALENs. *Mol Ther Nucleic Acids,* 1**,** e3.

CARROLL, D. 2014. Genome Engineering with Targetable Nucleases. *Annu Rev Biochem*.

CASSO, D., RAMIREZ-WEBER, F. & KORNBERG, T. B. 2000. GFP-tagged balancer chromosomes for *Drosophila melanogaster*. *Mech Dev,* 91**,** 451-4.

CDC. 2012. *Dengue and the Aedes aegypti mostquito* [Online]. The Centers for Disease Control and Prevention. [Accessed 8/15/19].

CDC. 2014. *CDC and Malaria* [Online]. The Centers for Disease Control and Prevention.[Accessed 8/13/19].

CHAKRABORTY, S., WAISE, T. M., HASSAN, F., KABIR, Y., SMITH, M. A. & ARIF, M. 2009. Assessment of the evolutionary origin and possibility of CRISPR-Cas (CASS) mediated RNA interference pathway in *Vibrio cholerae* O395. *In Silico Biol,* 9**,** 245-54.

CHHABRA, M., MITTAL, V., BHATTACHARYA, D., RANA, U. & LAL, S. 2008. Chikungunya fever: a re-emerging viral infection. *Indian J Med Microbiol,* 26**,** 5-12.

CHIRUVELLA, K. K., LIANG, Z. & WILSON, T. E. 2013. Repair of double-strand breaks by end joining. *Cold Spring Harb Perspect Biol,* 5**,** a012757.

CIAPPONI, L., CENCI, G., DUCAU, J., FLORES, C., JOHNSON-SCHLITZ, D., GORSKI, M. M., ENGELS, W. R. & GATTI, M. 2004. The *Drosophila* Mre11/Rad50 complex is required to prevent both telomeric fusion and chromosome breakage. *Curr Biol,* 14**,** 1360-6.

CLEMENTS, P. M., BRESLIN, C., DEEKS, E. D., BYRD, P. J., JU, L., BIEGANOWSKI, P., BRENNER, C., MOREIRA, M. C., TAYLOR, A. M. & CALDECOTT, K. W. 2004. The ataxia-oculomotor apraxia 1 gene product has a role distinct from ATM and interacts with the DNA strand break repair proteins XRCC1 and XRCC4. *DNA Repair (Amst),* 3**,** 1493-502.

COLPITTS, T. M., COX, J., VANLANDINGHAM, D. L., FEITOSA, F. M., CHENG, G., KURSCHEID, S., WANG, P., KRISHNAN, M. N., HIGGS, S. & FIKRIG, E. 2011. Alterations in the *Aedes aegypti* transcriptome during infection with West Nile, dengue and yellow fever viruses. *PLoS Pathog,* 7**,** e1002189.

DAVIES, A. A., FRIEDBERG, E. C., TOMKINSON, A. E., WOOD, R. D. & WEST, S. C. 1995. Role of the Rad1 and Rad10 proteins in nucleotide excision repair and recombination. *J Biol Chem,* 270**,** 24638-41.

DEGENNARO, M., MCBRIDE, C. S., SEEHOLZER, L., NAKAGAWA, T., DENNIS, E. J., GOLDMAN, C., JASINSKIENE, N., JAMES, A. A. & VOSSHALL, L. B. 2013. orco

mutant mosquitoes lose strong preference for humans and are not repelled by volatile DEET. *Nature*, 498, 487.

DERIANO, L. & ROTH, D. B. 2013. Modernizing the nonhomologous end-joining repertoire: alternative and classical NHEJ share the stage. *Annu Rev Genet,* 47**,** 433-55.

DRYSDALE, R. & FLYBASE, C. 2008. FlyBase : a database for the *Drosophila* research community. *Methods Mol Biol,* 420**,** 45-59.

EGLI, D., HAFEN, E. & SCHAFFNER, W. 2004. An efficient method to generate chromosomal rearrangements by targeted DNA double-strand breaks in *Drosophila* melanogaster. *Genome Res,* 14**,** 1382-93.

FU, G., LEES, R. S., NIMMO, D., AW, D., JIN, L., GRAY, P., BERENDONK, T. U., WHITE-COOPER, H., SCAIFE, S., KIM PHUC, H., MARINOTTI, O., JASINSKIENE, N., JAMES, A. A. & ALPHEY, L. 2010. Female-specific flightless phenotype for mosquito control. *Proc Natl Acad Sci U S A,* 107**,** 4550-4.

GAJ, T., GERSBACH, C. A. & BARBAS, C. F., 3RD 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol,* 31**,** 397-405.

GALIZI, R., DOYLE, L. A., MENICHELLI, M., BERNARDINI, F., DEREDEC, A., BURT, A., STODDARD, B. L., WINDBICHLER, N. & CRISANTI, A. 2014. A synthetic sex ratio distortion system for the control of the human malaria mosquito. *Nat Commun,* 5**,** 3977.

GOLUB, E. I., GUPTA, R. C., HAAF, T., WOLD, M. S. & RADDING, C. M. 1998. Interaction of human rad51 recombination protein with single-stranded DNA binding protein, RPA. *Nucleic Acids Res,* 26**,** 5388-93.

GRATZ, S. J., UKKEN, F. P., RUBINSTEIN, C. D., THIEDE, G., DONOHUE, L. K., CUMMINGS, A. M. & O'CONNOR-GILES, K. M. 2014. Highly Specific and Efficient CRISPR/Cas9-Catalyzed Homology-Directed Repair in *Drosophila*. *Genetics,* 196, 961.

GRUNDY, G. J., RULTEN, S. L., ZENG, Z., ARRIBAS-BOSACOMA, R., ILES, N., MANLEY, K., OLIVER, A. & CALDECOTT, K. W. 2013. APLF promotes the assembly and activity of non-homologous end joining protein complexes. *EMBO J,* 32**,** 112-25.

HEYER, W. D. 2004. Recombination: Holliday junction resolution and crossover formation. *Curr Biol,* 14**,** R56-8.

HILL, J., LINES, J. & ROWLAND, M. 2006. Insecticide-treated nets. *Adv Parasitol,* 61**,** 77-128.

IVANOV, E. L., SUGAWARA, N., FISHMAN-LOBELL, J. & HABER, J. E. 1996. Genetic requirements for the single-strand annealing pathway of double-strand break repair in *Saccharomyces cerevisiae*. *Genetics,* 142**,** 693-704.

JOUNG, J. K. & SANDER, J. D. 2013. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol,* 14**,** 49-55.

KAMGANG, B., MARCOMBE, S., CHANDRE, F., NCHOUTPOUEN, E., NWANE, P., ETANG, J., CORBEL, V. & PAUPY, C. 2011. Insecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* in Central Africa. *Parasit Vectors,* 4**,** 79.

KARANJAWALA, Z. E., ADACHI, N., IRVINE, R. A., OH, E. K., SHIBATA, D., SCHWARZ, K., HSIEH, C. L. & LIEBER, M. R. 2002. The embryonic lethality in DNA ligase IV-deficient mice is rescued by deletion of Ku: implications for unifying the heterogeneous phenotypes of NHEJ mutants. *DNA Repair (Amst),* 1**,** 1017-26.

KARGINOV, F. V. & HANNON, G. J. 2010. The CRISPR system: small RNA-guided defense in bacteria and archaea. *Mol Cell,* 37**,** 7-19.

KIIANITSA, K., SOLINGER, J. A. & HEYER, W. D. 2006. Terminal association of Rad54 protein with the Rad51-dsDNA filament. *Proc Natl Acad Sci U S A,* 103**,** 9767-72.

KIM, H. & KIM, J.-S. 2014. A guide to genome engineering with programmable nucleases. *Nat Rev Genet,* 15**,** 321-334.

KLEIN, T. A., WINDBICHLER, N., DEREDEC, A., BURT, A. & BENEDICT, M. Q. 2012. Infertility resulting from transgenic I-PpoI male *Anopheles gambiae* in large cage trials. *Pathog Glob Health,* 106**,** 20-31.

KLOVSTAD, M., ABDU, U. & SCHUPBACH, T. 2008. *Drosophila* brca2 is required for mitotic and meiotic DNA repair and efficient activation of the meiotic recombination checkpoint. *PLoS Genet,* 4**,** e31.

KOKOZA, V. A. & RAIKHEL, A. S. 2011. Targeted gene expression in the transgenic *Aedes aegypti* using the binary Gal4-UAS system. *Insect Biochem Mol Biol,* 41**,** 637-44.

LABBÉ, G. M. C., SCAIFE, S., MORGAN, S. A., CURTIS, Z. H. & ALPHEY, L. 2012. Female-Specific Flightless (fsRIDL) Phenotype for Control of *Aedes albopictus*. *PLoS Negl Trop Dis,* 6**,** e1724.

LAMARCHE, B. J., ORAZIO, N. I. & WEITZMAN, M. D. 2010. The MRN complex in double-strand break repair and telomere maintenance. *FEBS Lett,* 584**,** 3682-95.

LEE, H. J., KWEON, J., KIM, E., KIM, S. & KIM, J. S. 2012. Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. *Genome Res,* 22**,** 539-48.

LI, F., DONG, J., PAN, X., OUM, J. H., BOEKE, J. D. & LEE, S. E. 2008a. Microarray-based genetic screen defines SAW1, a gene required for Rad1/Rad10-dependent processing of recombination intermediates. *Mol Cell,* 30**,** 325-35.

LI, G., ALT, F. W., CHENG, H. L., BRUSH, J. W., GOFF, P. H., MURPHY, M. M., FRANCO, S., ZHANG, Y. & ZHA, S. 2008b. Lymphocyte-specific compensation for XLF/cernunnos end-joining functions in V(D)J recombination. *Mol Cell,* 31**,** 631-40.

LI, S., KANNO, S., WATANABE, R., OGIWARA, H., KOHNO, T., WATANABE, G., YASUI, A. & LIEBER, M. R. 2011. Polynucleotide kinase and aprataxin-like forkhead-associated protein (PALF) acts as both a single-stranded DNA endonuclease and a single-stranded DNA 3' exonuclease and can participate in DNA end joining in a biochemical system. *J Biol Chem,* 286**,** 36368-77.

LIESCH, J., BELLANI, L. L. & VOSSHALL, L. B. 2013. Functional and Genetic Characterization of Neuropeptide Y-Like Receptors in *Aedes aegypti*. *PLoS Negl Trop Dis,* 7**,** e2486.

LIU, J. & HEYER, W. D. 2011. Who's who in human recombination: BRCA2 and RAD52. *Proc Natl Acad Sci U S A,* 108**,** 441-2.

LIU, L. & FAN, X. D. 2014. CRISPR-Cas system: a powerful tool for genome engineering. *Plant Mol Biol,* 85**,** 209-18.

LORAT, Y., SCHANZ, S., SCHULER, N., WENNEMUTH, G., RUBE, C. & RUBE, C. E. 2012. Beyond repair foci: DNA double-strand break repair in euchromatic and heterochromatic compartments analyzed by transmission electron microscopy. *PLoS One,* 7**,** e38165.

MA, M., YE, A. Y., ZHENG, W. & KONG, L. 2013. A guide RNA sequence design platform for the CRISPR/Cas9 system for model organism genomes. *Biomed Res Int,* 2013**,** 270805.

MA, S., CHANG, J., WANG, X., LIU, Y., ZHANG, J., LU, W., GAO, J., SHI, R., ZHAO, P. & XIA, Q. 2014. CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of BmKu70 in *Bombyx mori*. *Sci Rep,* 4**,** 4489.

MA, Y., PANNICKE, U., SCHWARZ, K. & LIEBER, M. R. 2002. Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell,* 108**,** 781-94.

MAHANEY, B. L., HAMMEL, M., MEEK, K., TAINER, J. A. & LEES-MILLER, S. P. 2013. XRCC4 and XLF form long helical protein filaments suitable for DNA end protection and alignment to facilitate DNA double strand break repair. *Biochem Cell Biol,* 91**,** 31-41.

MALI, P., AACH, J., STRANGES, P. B., ESVELT, K. M., MOOSBURNER, M., KOSURI, S., YANG, L. & CHURCH, G. M. 2013a. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat Biotechnol,* 31**,** 833-8.

MALI, P., ESVELT, K. M. & CHURCH, G. M. 2013b. Cas9 as a versatile tool for engineering biology. *Nat Methods,* 10**,** 957-63.

MANSOUR, W. Y., SCHUMACHER, S., ROSSKOPF, R., RHEIN, T., SCHMIDT-PETERSEN, F., GATZEMEIER, F., HAAG, F., BORGMANN, K., WILLERS, H. & DAHM-DAPHI, J. 2008. Hierarchy of nonhomologous end-joining, single-strand

annealing and gene conversion at site-directed DNA double-strand breaks. *Nucleic Acids Res,* 36**,** 4088-98.

MASSONNET-BRUNEEL, B., CORRE-CATELIN, N., LACROIX, R., LEES, R. S., HOANG, K. P., NIMMO, D., ALPHEY, L. & REITER, P. 2013. Fitness of Transgenic Mosquito *Aedes aegypti* Males Carrying a Dominant Lethal Genetic System. *PLoS One,* 8**,** e62711.

MCMENIMAN, C. J., CORFAS, R. A., MATTHEWS, B. J., RITCHIE, S. A. & VOSSHALL, L. B. 2014. Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. *Cell,* 156**,** 1060-71.

MCWHIR, J., SELFRIDGE, J., HARRISON, D. J., SQUIRES, S. & MELTON, D. W. 1993. Mice with DNA repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. *Nat Genet,* 5**,** 217-24.

MEHTA, A. & HABER, J. E. 2014. Sources of DNA Double-Strand Breaks and Models of Recombinational DNA Repair. *Cold Spring Harb Perspect Biol,* 6, a016428-a016428.

MURR, R., LOIZOU, J. I., YANG, Y. G., CUENIN, C., LI, H., WANG, Z. Q. & HERCEG, Z. 2006. Histone acetylation by Trrap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. *Nat Cell Biol,* 8**,** 91-9.

NENE, V., WORTMAN, J. R., LAWSON, D., HAAS, B., KODIRA, C., TU, Z. J., LOFTUS, B., XI, Z., MEGY, K., GRABHERR, M., REN, Q., ZDOBNOV, E. M., LOBO, N. F., CAMPBELL, K. S., BROWN, S. E., BONALDO, M. F., ZHU, J., SINKINS, S. P., HOGENKAMP, D. G., AMEDEO, P., ARENSBURGER, P., ATKINSON, P. W., BIDWELL, S., BIEDLER, J., BIRNEY, E., BRUGGNER, R. V., COSTAS, J., COY, M. R., CRABTREE, J., CRAWFORD, M., DEBRUYN, B., DECAPRIO, D., EIGLMEIER, K., EISENSTADT, E., EL-DORRY, H., GELBART, W. M., GOMES, S. L.,

HAMMOND, M., HANNICK, L. I., HOGAN, J. R., HOLMES, M. H., JAFFE, D., JOHNSTON, J. S., KENNEDY, R. C., KOO, H., KRAVITZ, S., KRIVENTSEVA, E. V., KULP, D., LABUTTI, K., LEE, E., LI, S., LOVIN, D. D., MAO, C., MAUCELI, E., MENCK, C. F., MILLER, J. R., MONTGOMERY, P., MORI, A., NASCIMENTO, A. L., NAVEIRA, H. F., NUSBAUM, C., O'LEARY, S., ORVIS, J., PERTEA, M., QUESNEVILLE, H., REIDENBACH, K. R., ROGERS, Y. H., ROTH, C. W., SCHNEIDER, J. R., SCHATZ, M., SHUMWAY, M., STANKE, M., STINSON, E. O., TUBIO, J. M., VANZEE, J. P., VERJOVSKI-ALMEIDA, S., WERNER, D., WHITE, O., WYDER, S., ZENG, Q., ZHAO, Q., ZHAO, Y., HILL, C. A., RAIKHEL, A. S., SOARES, M. B., KNUDSON, D. L., LEE, N. H., GALAGAN, J., SALZBERG, S. L., PAULSEN, I. T., DIMOPOULOS, G., COLLINS, F. H., BIRREN, B., FRASER-LIGGETT, C. M. & SEVERSON, D. W. 2007. Genome sequence of *Aedes aegypti*, a major arbovirus vector. *Science,* 316**,** 1718-23.

NIU, H., CHUNG, W. H., ZHU, Z., KWON, Y., ZHAO, W., CHI, P., PRAKASH, R., SEONG, C., LIU, D., LU, L., IRA, G. & SUNG, P. 2010. Mechanism of the ATP-dependent DNA end-resection machinery from *Saccharomyces cerevisiae*. *Nature,* 467**,** 108-11.

OH, S., HARVEY, A., ZIMBRIC, J., WANG, Y., NGUYEN, T., JACKSON, P. J. & HENDRICKSON, E. A. 2014. DNA ligase III and DNA ligase IV carry out genetically distinct forms of end joining in human somatic cells. *DNA Repair (Amst),* 21**,** 97-110.

PEARSON, A. M. & WOOD, R. J. 1980. Combining the Meiotic Drive Gene-D and the Translocation-T1 in the Mosquito, *Aedes-Aegypti* (L) .1. Sex-Ratio Distortion and Fertility. *Genetica,* 51**,** 203-210.

PIALOUX, G., GAUZERE, B. A., JAUREGUIBERRY, S. & STROBEL, M. 2007. Chikungunya, an epidemic arbovirosis. *Lancet Infect Dis,* 7**,** 319-27.

PIGANEAU, M., GHEZRAOUI, H., DE CIAN, A., GUITTAT, L., TOMISHIMA, M., PERROUAULT, L., RENE, O., KATIBAH, G. E., ZHANG, L., HOLMES, M. C., DOYON, Y., CONCORDET, J. P., GIOVANNANGELI, C., JASIN, M. & BRUNET, E. 2013. Cancer translocations in human cells induced by zinc finger and TALE nucleases. *Genome Res,* 23**,** 1182-93.

PLUESS, B., TANSER, F. C., LENGELER, C. & SHARP, B. L. 2010. Indoor residual spraying for preventing malaria. *Cochrane Database Syst Rev***,** CD006657.

PRICE, B. D. & D'ANDREA, A. D. 2013. Chromatin remodeling at DNA double-strand breaks. *Cell,* 152**,** 1344-54.

QI, Y., ZHANG, Y., ZHANG, F., BALLER, J. A., CLELAND, S. C., RYU, Y., STARKER, C. G. & VOYTAS, D. F. 2013. Increasing frequencies of site-specific mutagenesis and gene targeting in Arabidopsis by manipulating DNA repair pathways. *Genome Res,* 23**,** 547-54.

SANDER, J. D. & JOUNG, J. K. 2014. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol,* 32**,** 347-55.

SCHLITZER, M. 2008. Antimalarial drugs - what is in use and what is in the pipeline. *Arch Pharm (Weinheim),* 341**,** 149-63.

SEVERSON, D. W. & BEHURA, S. K. 2012. Mosquito genomics: progress and challenges. *Annu Rev Entomol,* 57**,** 143-66.

SHARAKHOVA, M. V., GEORGE, P., BRUSENTSOVA, I. V., LEMAN, S. C., BAILEY, J. A., SMITH, C. D. & SHARAKHOV, I. V. 2010. Genome mapping and characterization of the *Anopheles gambiae* heterochromatin. *BMC Genomics,* 11**,** 459.

SIMSEK, D. & JASIN, M. 2010. Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4-ligase IV during chromosomal translocation formation. *Nat Struct Mol Biol,* 17**,** 410-6.

SMIDLER, A. L., TERENZI, O., SOICHOT, J., LEVASHINA, E. A. & MAROIS, E. 2013. Targeted Mutagenesis in the Malaria Mosquito Using TALE Nucleases. *PLoS ONE,* 8**,** e74511.

SONI, A., SIEMANN, M., GRABOS, M., MURMANN, T., PANTELIAS, G. E. & ILIAKIS, G. 2014. Requirement for Parp-1 and DNA ligases 1 or 3 but not of Xrcc1 in chromosomal translocation formation by backup end joining. *Nucleic Acids Res,* 42**,** 6380-92.

STODDARD, B. L. 2005. Homing endonuclease structure and function. *Q Rev Biophys,* 38**,** 49-95.

STODDARD, B. L. 2011. Homing endonucleases: from microbial genetic invaders to reagents for targeted DNA modification. *Structure,* 19**,** 7-15.

SUGAWARA, N., IRA, G. & HABER, J. E. 2000. DNA length dependence of the single-strand annealing pathway and the role of *Saccharomyces cerevisiae* RAD59 in double-strand break repair. *Mol Cell Biol,* 20**,** 5300-9.

SUGAWARA, N., PAQUES, F., COLAIACOVO, M. & HABER, J. E. 1997. Role of *Saccharomyces cerevisiae* Msh2 and Msh3 repair proteins in double-strand break-induced recombination. *Proc Natl Acad Sci U S A,* 94**,** 9214-9.

TAMARU, H. 2010. Confining euchromatin/heterochromatin territory: jumonji crosses the line. *Genes Dev,* 24**,** 1465-78.

TIAN, M., SHINKURA, R., SHINKURA, N. & ALT, F. W. 2004. Growth retardation, early death, and DNA repair defects in mice deficient for the nucleotide excision repair enzyme XPF. *Mol Cell Biol,* 24**,** 1200-5.

TRAVER, B. E., ANDERSON, M. A. & ADELMAN, Z. N. 2009. Homing endonucleases catalyze double-stranded DNA breaks and somatic transgene excision in *Aedes aegypti*. *Insect Mol Biol,* 18**,** 623-33.

TRUONG, L. N., LI, Y., SHI, L. Z., HWANG, P. Y., HE, J., WANG, H., RAZAVIAN, N., BERNS, M. W. & WU, X. 2013. Microhomology-mediated End Joining and Homologous Recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. *Proc Natl Acad Sci U S A,* 110**,** 7720-5.

UPADHYAY, S. K. & SHARMA, S. 2014. SSFinder: high throughput CRISPR-Cas target sites prediction tool. *Biomed Res Int,* 2014**,** 742482.

URNOV, F. D., REBAR, E. J., HOLMES, M. C., ZHANG, H. S. & GREGORY, P. D. 2010. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet,* 11**,** 636-46.

VAN DER WEYDEN, L. & BRADLEY, A. 2006. Mouse chromosome engineering for modeling human disease. *Annu Rev Genomics Hum Genet,* 7**,** 247-76.

WALKER, T., JOHNSON, P. H., MOREIRA, L. A., ITURBE-ORMAETXE, I., FRENTIU, F. D., MCMENIMAN, C. J., LEONG, Y. S., DONG, Y., AXFORD, J., KRIESNER, P., LLOYD, A. L., RITCHIE, S. A., O/'NEILL, S. L. & HOFFMANN, A. A. 2011. The wMel Wolbachia strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature,* 476**,** 450-453.

WANG, C. & LEES-MILLER, S. P. 2013. Detection and Repair of Ionizing Radiation-Induced DNA Double Strand Breaks: New Developments in Nonhomologous End Joining. *International Journal of Radiation Oncology*Biology*Physics,* 86**,** 440-449.

WANG, M., WU, W., WU, W., ROSIDI, B., ZHANG, L., WANG, H. & ILIAKIS, G. 2006. PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. *Nucleic Acids Res,* 34**,** 6170-82.

WANG, S. & JACOBS-LORENA, M. 2013. Genetic approaches to interfere with malaria transmission by vector mosquitoes. *Trends in Biotechnology,* 31**,** 185-193.

WEI, D. S. & RONG, Y. S. 2007. A genetic screen for DNA double-strand break repair mutations in *Drosophila*. *Genetics,* 177**,** 63-77.

WEINFELD, M., MANI, R. S., ABDOU, I., ACEYTUNO, R. D. & GLOVER, J. N. 2011. Tidying up loose ends: the role of polynucleotide kinase/phosphatase in DNA strand break repair. *Trends Biochem Sci,* 36**,** 262-71.

WHITE, B. J., CHENG, C., SANGARE, D., LOBO, N. F., COLLINS, F. H. & BESANSKY, N. J. 2009. The population genomics of trans-specific inversion polymorphisms in *Anopheles gambiae*. *Genetics,* 183**,** 275-88.

WHO. 2014a. *Dengue and severe dengue* [Online]. World Heath Organization [Accessed 8/13/19].

WHO. 2014b. *Malaria*. [Online].World Health Organization. [Accessed 8/21/19].

WILLIAMS, G. J., HAMMEL, M., RADHAKRISHNAN, S. K., RAMSDEN, D., LEES-MILLER, S. P. & TAINER, J. A. 2014. Structural insights into NHEJ: building up an integrated picture of the dynamic DSB repair super complex, one component and interaction at a time. *DNA Repair (Amst),* 17**,** 110-20.

WINDBICHLER, N., MENICHELLI, M., PAPATHANOS, P. A., THYME, S. B., LI, H., ULGE, U. Y., HOVDE, B. T., BAKER, D., MONNAT, R. J., JR., BURT, A. & CRISANTI, A. 2011. A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. *Nature,* 473**,** 212-5.

WINDBICHLER, N., PAPATHANOS, P. A., CATTERUCCIA, F., RANSON, H., BURT, A. & CRISANTI, A. 2007. Homing endonuclease mediated gene targeting in *Anopheles gambiae* cells and embryos. *Nucleic Acids Res,* 35**,** 5922-33.

WINDBICHLER, N., PAPATHANOS, P. A. & CRISANTI, A. 2008. Targeting the X chromosome during spermatogenesis induces Y chromosome transmission ratio distortion and early dominant embryo lethality in *Anopheles gambiae*. *PLoS Genet,* 4**,** e1000291.

XIE, S., SHEN, B., ZHANG, C., HUANG, X. & ZHANG, Y. 2014. sgRNAcas9: A Software Package for Designing CRISPR sgRNA and Evaluating Potential Off-Target Cleavage Sites. *PLoS ONE,* 9**,** e100448.

YOO, S. 2006. Characterization of *Drosophila* Rad51/SpnA protein in DNA binding and embryonic development. *Biochem Biophys Res Commun,* 348**,** 1310-8.

YOO, S. & MCKEE, B. D. 2004. Overexpression of *Drosophila* Rad51 protein (DmRad51) disrupts cell cycle progression and leads to apoptosis. *Chromosoma,* 113**,** 92-101.

YOO, S. & MCKEE, B. D. 2005. Functional analysis of the *Drosophila* Rad51 gene (spn-A) in repair of DNA damage and meiotic chromosome segregation. *DNA Repair (Amst),* 4**,** 231-42.

ZHENG, B., SAGE, M., CAI, W. W., THOMPSON, D. M., TAVSANLI, B. C., CHEAH, Y. C. & BRADLEY, A. 1999. Engineering a mouse balancer chromosome. *Nat Genet,* 22**,** 375-8.

ZHU, Z., CHUNG, W. H., SHIM, E. Y., LEE, S. E. & IRA, G. 2008. Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell,* 134**,** 981-94.

CHAPTER III

PROGRESS IN GENE EDITING, TRANSGENESIS AND GENOME MANIPULATION IN MOSQUITOES[*]

PROGRESS IN MOSQUITO GENE EDITING

Mosquitoes act as vectors of some of the world's most burdensome disease-causing pathogens including malaria and dengue (CDC 2014, WHO 2014a, WHO 2014b). Advancements in genomics have given molecular biologists and geneticists a wealth of information from which to further understand the basic biology of these vectors with the hope of generating novel solutions to reduce or beneficially modify vector populations. While the technology to generate transgenic mosquitoes has been established for almost two decades, recent advancements stand to completely revolutionize the specificity and efficacy with respect to gene editing, transgenesis and genome manipulation. The most recent, and perhaps the most exciting of these advancements comes as part of the CRISPR revolution.

CRISPR/CAS9

Clustered regularly-interspaced palindromic repeats (CRISPR) in conjunction with the Cas proteins are found in a wide-range of bacteria and play a role in anti-viral immunity (Marraffini and Sontheimer, 2010). CRISPR sequences are variable in nature, 30-40 nucleotides in length, and are interspersed by direct repeats. These sequences are utilized by a variety of Cas

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nuclease proteins in conjunction with two small RNAs to target viral sequences which have been previously integrated into the bacterial genome (Jinek et al., 2012). Transcription of CRISPR loci followed by processing generates these short RNA sequences which then form a complex with the Cas protein(s) to target viral DNA for double-stranded DNA break (DSB) induction. A key requirement for the Cas nuclease generate DSBs at its target site is the protospacer adjacent motif (PAM). While present on the viral genome, the PAM is absent in the corresponding bacterial sequence, allowing for specific cutting of foreign viral DNA. The most common variant of the Cas proteins used to date is Cas9 (derived from *S. pyogenes*), which has a PAM site of NGG (Cong et al., 2013). While there are several classes of Cas proteins, the Cas9 variant is particularly useful because it only requires a single protein to function. Furthermore, it has been shown that the Cas9 PAM site can be modified to accept alternative nucleotide sequences (Kleinstiver et al., 2015). While initial gene editing efforts utilized two small RNA molecules (crRNA & tracrRNA), as in bacteria, they are now virtually always fused together for synthetic use (synthetic guide RNA, sgRNA). The CRISPR system has been adapted for use in a range of insects including *Tribolium castaneum* (Gilles et al., 2015), *Gryllus bimaculatus* (Awata et al., 2015), *Bombyx mori* (Wei et al., 2014, Zeng et al., 2016), *Danaus plexippus* (Markert et al., 2016), *Drosophila suzukii* (Li and Scott, 2016), *Drosophila melanogaster* (Bassett et al., 2013), and of particular interest for the purposes of this review, the mosquitoes *Aedes aegypti* (Basu et al., 2015), *Anopheles stephensi* (Gantz et al., 2015), and *Anopheles gambiae* (Hammond et al., 2016). The CRISPR system utilizes two key components, the synthetic guide RNA and the Cas9 protein, to search out a specific sequence within the genome of interest and generate a blunt end double stranded break [Figure 3.1].

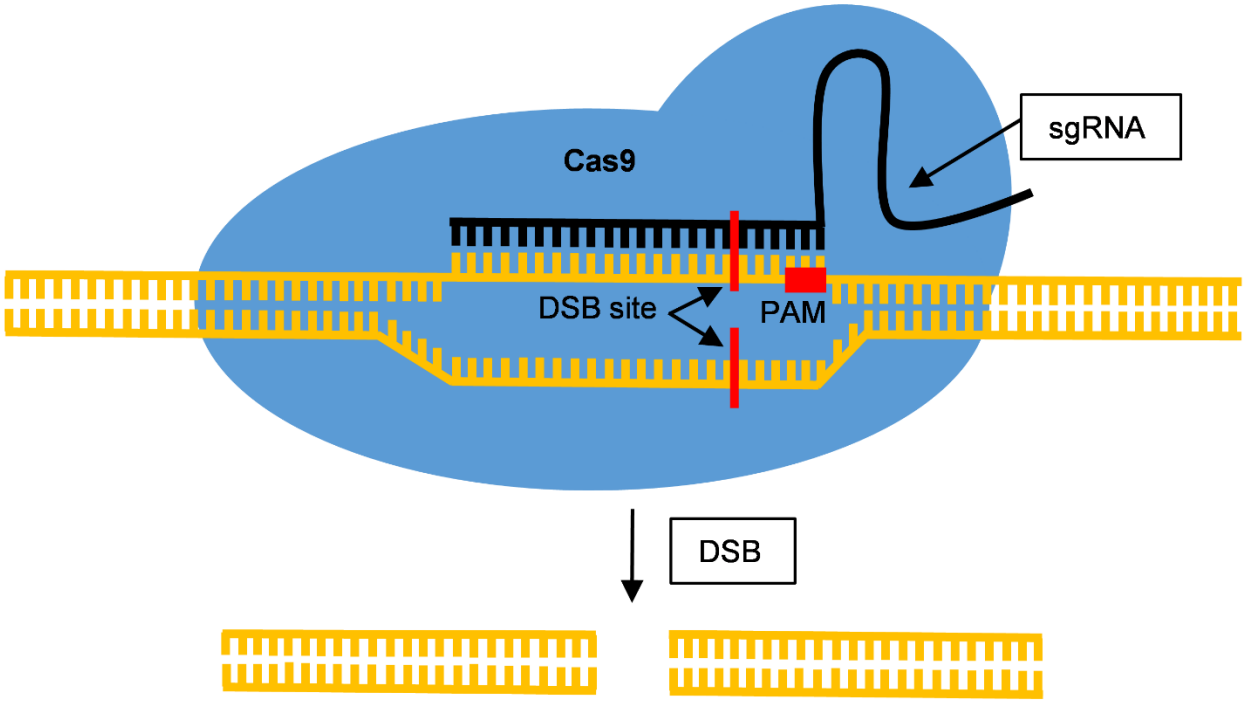

Figure 3.1. Model of sgRNA-directed Cas9 binding to DNA target. DSB (red vertical lines) are 3 bp upstream of the PAM site. The Cas9 protein cuts both strands of the targeted DNA generating a blunt ended double stranded break, which will be repaired by one of several DSB repair pathways.

Following DNA cleavage, DSBs are repaired by one of several endogenous cellular repair pathways including the classical non-homologous end joining pathway (C-NHEJ) and the homology directed repair pathway (HDR). Any resulting mutation, or lack thereof, is dependent upon which pathway is utilized. Mistakes made during C-NHEJ repair may result in small insertions or deletions (Williams et al., 2014). These indels may generate frameshift mutations within the open reading frame of a gene of interest, effectively generating knockout mutants or truncated proteins (desired location within the ORF). It is also plausible that portions of a gene may be removed by utilizing several guide RNAs in tandem. In contrast, if the DSB is repaired utilizing the HDR pathway then perfect repair of the break may be accomplished using the homologous chromosome or an exogenously introduced template (Lamarche et al., 2010). Providing a templated sequence for use in HDR may allow the experimenter to remove undesired alleles from a population or insert foreign DNA in the form a transgenic construct [Figure 3.2]. In addition to its power as a highly specific endonuclease, alternative uses and varieties of the CRISPR system will be discussed later in the chapter.

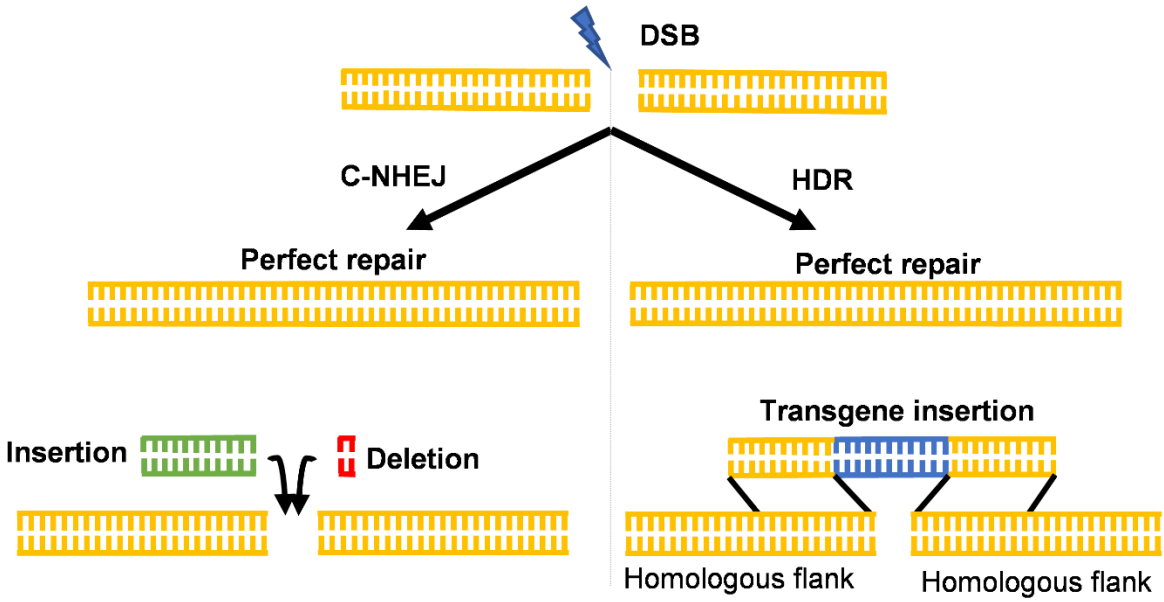

Figure 3.2 DNA repair following DSB induction is a competitive process. Two primary repair pathways are the classical non-homologous end joining pathway (C-NHEJ, left) and the homology directed repair pathway (HDR, right). The C-NHEJ pathway does not require a homologous template to repair a double stranded break, but may result in the generation of indels (insertions or deletions). The homology directed repair pathway requires a homologous template to complete the repair of a double stranded break. If a synthetic template with homologous flanks is present, the HDR pathway can be used to insert transgenes into a highly specific location.

PROGRESS IN CRISPR IN *AEDES*

The ability to utilize CRISPR was first demonstrated by Kistler *et al* (2015) in *Ae. aegypti* with the goal of investigating the genetic basis of innate chemosensory behaviors (Kistler et al., 2015). Kistler *et al* (2015) provide a step by step bench manual for the utilization of CRISPR/Cas9 in *Ae. aegypti*, as well as providing data on the optimization of injection mixes (suggesting that 400ng/ul protein displays the highest cutting efficiency), and testing the

efficiency of C-NHEJ and HDR based repair mechanisms with respect to six different genes. These researchers noted correlation between Cas9 concentration and embryo survival, with lower injection concentrations (333 ng/ µl) yielding survival rates between 46-63% and higher injection concentrations (500 ng/µl) yielding an average survival of 18.6%. Other reports (Basu et al., 2015, Dong et al., 2015) indicate typical survival rates lower than 30%, again using higher concentrations of Cas9 (summarized in Table 2).

After optimization of Cas9 concentration, Kistler *et al* (2015) injected embryos with sgRNAs and single stranded oligodeoxynucleotides (ssODN) 200 bp in length to quantify mutation rates attributed to indel creation by the C-NHEJ pathway and insertion of the ssODN via the HDR pathway. In G_0 survivors, an indel generation rate of 24% and an ssODN insertion rate of 0.71% were observed, suggesting a dominating preference for C-NHEJ repair. In G_1 progeny, an indel inheritance of 18.9% and ssODN inheritance of 0.6% were also observed, suggesting that somatic mutation rates may be a viable way to guide future germline experiments. Basu *et al* (2015) also successfully utilized CRISPR/Cas9 in *Ae*. *aegypti* targeting a range of genes associated with DNA repair, RNAi, and sex determination. Basu *et al* (2015) observed mutation rates as high as 90% following pre-validation of sgRNAs in embryo-based assays (Basu et al., 2015). This may have been due to a higher concentration of Cas9 (600ng/µl vs 400 ng/µl), the larger number of sgRNAs screened in a pre-validation process, or might simply reflect variation between independent genomic target sites. Hall *et al* (2015) further investigated the targeting of the sex determining gene *nix* which is believed to play a role as the master regulator of *doublesex* and *fruitless* splicing, and hence sexual dimorphism in *Ae*. *aegypti* (Hall et al., 2015). Using the same injection specifications as Basu *et al* (2015), two-thirds of transgenic male survivors that had been injected with CRISPR/Cas9 as embryos showed some

level of feminization and deformities in sexually dimorphic organs, indicating that useful gene disruptions can be obtained in a single generation (albeit from a haploid target in this case). Lastly, Dong *et al* (2015) also validated CRISPR/Cas9 in *Ae*. *aegypti* by targeting the enhanced cyan fluorescent protein (ECFP) gene which had been previously introduced as a transgene (Dong et al., 2015). In contrast to previous studies, Dong *et al* (2015) only observed a 5.5% knockout efficiency of the targeted fluorescent gene. While this may have been due to the low number of sgRNAs utilized and screened, the vast variation in CRISPR/Cas9 mosquito mutagenesis within a single species suggests that target selection, gRNA design, and optimization can be further improved.

Table 2. Mosquito gene editing experiments using CRISPR/Cas9

Reference	Cas9 Source	Mosquito	Cas9 (ng/µl)	sgRNA (ng/µl)	NHEJ mutation rate (%)	DNA repair manipulation	HDR insertion size (kb)	Flanks (kb)	Insertion Rate* (%)
Kistler et al (2015)	Protein	*Ae. aegypti*	300	40	24	N/A	2.15-2.32	.8/1.5	0.2-8.7
Basu et al (2015)	mRNA	*Ae. aegypti*	600	100	24-90	Ku70 dsRNA	2.4	2.2/1.7, 1/1.7	1.7-2.7
Dong et al (2015)	mRNA	*Ae. aegypti*	1000	50	5.5	N/A	N/A	N/A	N/A
Hall et al (2015)	mRNA	*Ae. aegypti*	600	100	~70	N/A	N/A	N/A	N/A
Gantz et al (2015)	mRNA	*An. stephensi*	100	100	N/A	Ku70 dsRNA	~17	~1	1.6
Hammond et al (2016)	Plasmid	*An. gambiae*	300	300 (on same plasmid)	N/A	N/A	~1	2/2	11.1-18.8

*Insertion rates were estimated as a percentage of founders of G_0 survivors. When G_0 survivors were pooled 50% fertility was assu

PROGRESS IN CRISPR EDITING IN *ANOPHELES*: GENE DRIVE

In contrast to the basic research described for *Aedes*, in *An. stephensi* and *An. gambiae*, CRISPR has been utilized to generate an active gene drive system as part of applied strategies to combat malaria (Gantz et al., 2015, Hammond et al., 2016). These papers quickly followed the initial utilization of a CRISPR gene drive first reported in *Drosophila melanogaster*, where CRISPR/Cas9 invaded the *yellow* gene at hyper-mendelian rates (Gantz and Bier, 2015). In each case, the CRISPR/Cas9 gene drive was constructed encoding the gene for Cas9 driven by a gonad specific promoter and the sgRNAs utilized for targeting the homologous sequence driven by a pol III promoter into the target site of the sgRNA. In this fashion, the Cas9-sgRNA complex targets the homologous chromosome repair via the HDR pathway the gene drive construct is copied to the homologous chromosome, essentially doubling its copy number each generation. This technique can be used to interrupt genes of interest, or by including transgenes into the gene drive construct, drive a transgene into a population at hyper-Mendelian rates. For further information on CRISPR gene drives, the reader is directed to reviews by (Champer et al., 2016, Esvelt et al., 2014, Gantz and Bier, 2016).

Building on previous work in *An. stephensi*, Gantz *et al* (2015) utilized CRISPR Cas9 along with a DNA template to introduce via the HDR pathway a ~17kb multi-gene unit (Gantz et al., 2015). Once integrated, the gene drive cassette was able to copy itself to its homologous chromosome with greater than 98% efficiency in initial generations. However, G_4 males and females derived from transgenic G_2 females showed inheritance rates more in line with expected Mendelian inheritance rates (1.33:1). In contrast, the offspring from G_2 males retained hyper-mendelian rates of inheritance (96.9-98.5%). Gantz *et al* (2015) suggested that this may have to do with C-NHEJ based repair occurring during embryogenesis prior to the availability of the

homologous chromosome, and recommends further restricting the expression of Cas9 from the gene drive construct to the parental germline. These findings provide insight into the use of gene drives in mosquitoes suggesting that the misexpression of Cas9 expression can undermine the efficiency of gene drive constructs, and reinforces the necessity to survey promoters capable of controlling Cas9 to maximize HDR within a gene drive system. Similarly, Hammond *et al* (2016) utilized CRISPR and TALENS (transcription activator-like effector nucleases) to insert docking constructs into 3 haplosufficient female sterility genes (Hammond et al., 2016). In contrast to insertion of the multi-gene gene drive into a known phenotypic gene, Hammond et al (2016) used a flexible two-step process to build their multi-gene unit. In the first step, they generated EGFP-marked HDR constructs loaded with φC31 attB sites (This docking system is discussed in more detail later in the chapter). The EGFP-marked HDR constructs were then inserted into their respective target genes and the impact on female fertility assessed. Once reduced fertility was confirmed (suggesting the target site was useful), the φC31 docking mechanism was used to insert the gene drive portion (Cas9 and sgRNA expression cassettes). Of the three genes targeted, one provided to be a viable candidate for a sustainable release program (AGAP007280), with the average homing rate per generation being 98% for transgenic males and 98% transgenic females. One very interesting finding that Hammond et al observed was a distinct drop in fertility after the gene drive construct was exchanged into the targeted genes; they suspect that this is due to somatic expression of Cas9. Both Gantz *et al* (2015) and Hammond *et al* (2016) prove that CRISPR based gene drives can function efficiently in mosquitoes. Each study raises questions which require further investigation, specifically which promoters will provide the highest gene drive efficiency and how does the presence of Cas9 within a transgenic line effect the overall fitness of a given transgenic line.

While there have been no reports of a successful gene drive in *Ae*. *aegypti*, the construct(s) in principle should be the same with respect its utilization in *An*. *stephensi* and *An*. *gambiae*. Perhaps one of the more interesting suggestions for its use is to generate a system which drives maleness through a population utilizing male determining factors such as the Nix gene (Adelman and Tu, 2016, Hall et al., 2015). This sex changing gene drive system could lead to rapid population and collapse.

FUTURE PROSPECTS OF MOSQUITO GENE DRIVE USING CRISPR

The validation of highly efficient CRISPR-based gene drive constructs, while extremely promising for novel malaria and dengue control strategies, poses special problems with how such transgenic mosquito strains can be safely evaluated in a field or semi-field environment where the release (intentional or otherwise) may result in the permanent fixation of the transgene in nature. Thus, there has been substantial theorizing as to how to further refine or redesign gene drive constructs to make them more controllable, or to inactivate them from wild populations. Two recently proposed concepts are those of the ERASR (Elements for Reversing the Autocatalytic Chain Reaction) and the CHASR (Construct Hitchhiking on the Autocatalytic Chain Reaction), described by Gantz and Bier (2016). ERASRs are constructs which can be introduced into a transgenic line harboring a gene drive mechanism in an attempt to effectively "erase" its gene drive capabilities. By introducing an sgRNA expression cassette targeting the Cas9 open reading frame itself at the same locus as the gene drive construct, Cas9 is predicted to disrupt its own ORF and allowing for the ERASR sequence to be copied to the chromosome which the CRISPR gene drive previously inhabited. In this fashion the gene drive system may drive itself out of a population though the broken Cas9, though any associated transgenic

sequences would remain. In contrast, CHASRs are proposed to piggyback on the presence of an already present CRISPR gene drive system to drive themselves into a population, while also possibly targeting other genes. While both ERASRs and CHASRS need to be validated experimentally, a similar construct has already been validated in Drosophila (Wu et al., 2016). Termed the CATCHA construct, it works by inserting a guide RNA targeting the Cas9 protein flanked by Cas9 homologous sequences on either side. If Cas9 is present then it will target itself, and drive the CATCHA construct into the population. Using a system involving the *ebony* gene, Wu *et al* (2016) showed that the CATCHA construct was 93.4% efficient at disrupting Cas9 activity and had a conversion rate of 85%. The ERASR, CHASR and CATCHA constructs provide just a few of what promises to be many creative ways to utilize gene drive systems in the future. Another factor which may play a critical role in the future of CRISPR gene drives is alternative endonucleases. All studies to date have used Cas9 endonuclease, but one issue that may arise is the proximity of the induced DSB site to the critical PAM site. As NHEJ competes with HDR, small indels at the DSB site may destroy the PAM and prevent the possibility of the endonuclease to generate another DSB, generating a drive-resistant allele. To circumvent this issue, one might use an endonuclease which induces DSB formation further downstream of the PAM site (DSB induction at 18 and 23 bp from PAM), such as Cpf1 (Zetsche et al., 2015). Cpf1 differs from Cas9 in that it lacks the need of a trans-activating RNA, it has an alternative PAM site (TTN vs NGG), and the cut site is staggered and further upstream of the PAM site. These traits, which differ Cpf1 from Cas9, may help to facilitate HDR (Zetsche et al., 2015). A recent assessment of Cpf1 in Drosophila suggested that it is less efficient than Cas9, with phenotypic mutant offspring only occurring at rates between 4 and 6% (Port and Bullock, 2016); suggesting further improvements may be necessary before the technology can be applied to mosquito gene

drive strategies. Other possibilities could include but are not limited to, systems which transiently modify DNA repair pathways to boost HDR and limit error prone fixes and additional endonucleases which may prove to be better candidates for gene drive (Shmakov et al., 2015). Current studies, as well as hypothetical constructs and untested endonucleases, suggest that there is much to be tested before we can fully maximize the potential of CRISPR gene drive systems, yet they appear to have a bright future in the advancement of basic and applied mosquito research.

PROGRESS IN CRISPR EXPRESSION SGRNAS AND MULTIPLEXING

The wide range of efficiencies seen by the CRISPR/Cas9 in mosquitoes leaves room for much to be learned. One interesting question is what source of Cas9 should be used to deliver the highest DSB efficiency while keeping costs down. Providing Cas9 to embryos via a DNA plasmid would provide cheap stable constructs for immediate use in injections. However, Kistler *et al* (2015), Basu *et al* (2015) and Dong *et al* (2015) all tested the use of Cas9 expression plasmids using strong constitutive promoters but in all cases failed to observe mutations. In contrast, Hammond *et al* (2016) was able to utilize a plasmid construct to generate DSBs utilizing the gonad specific promoter Vasa2. Another question which should be addressed are the advantages and disadvantages associated with introducing multiple guide RNAs targeting a single gene (multiplexing). Utilizing several sgRNAs targeting a single gene can lead to large deletions (Ren et al., 2013), this was confirmed in mosquitoes by Kistler *et al* (2015). Large deletions may or may not be advantageous depending upon the targeted gene. It may also be that the ability to multiplex increases efficiency so much that G_0 survivors display sufficient biallelic disruptions become viable experimental subjects. Port *et al* (2016) showed that the utilization of

4 sgRNAs targeting the sepia eye gene in Drosophila lead to 100% removal of sepia in almost all cases (Port and Bullock, 2016). While undoubtedly not all sgRNAs can achieve this efficiency, if such high efficiency is seen with most multiplexing attempts, the screening process is likely to be much less laborious in the future. Not only may multiplexing lead to high efficiency single target mutant generation but may also be used to target several genes simultaneously (Cong et al., 2013).

PROGRESS IN CRISPR TARGET SELECTION

CRISPR/Cas9 target selection is a multidimensional process requiring the experimenter to appropriately target the gene of interest (keeping in mind functional domains, alternative start codons/splice variants), while also considering the possibility of off-target effects. An initial consideration to take into account is the so called "seed sequence" found directly upstream of the PAM site (between 8-12 bp) (Doudna and Charpentier, 2014). The seed sequence typically shows reduced tolerance for mismatches with respect to the target of interest; others factors which may be worth including are the GC content of the particular sgRNA and the underlying chromatin state (Wu et al., 2014b) of the targeted region, as there is a high correlation between the GC content of the first five bases upstream of the PAM site and mutagenesis efficiency (Ren et al., 2014). Kistler *et al* (2015) designed their sgRNAs manually by searching sequences of interest and locating PAM sites. While this group did not look empirically examine regions for off target effects, they utilized two web tools to avoid sgRNAs with predicted off-target binding sites: http://zifit.partners.org/ZiFiT and http://crispr.mit.edu. Both Dong et al (2015) and Hammond et al (2015) also utilized ZiFiT to predict and avoid sgRNAs with strong off-target potential. Similarly, Basu *et al* 2015 manually designed sgRNAs and searched for off-target

effects by utilizing the software flyCRISPR (Gratz et al., 2014). In this latter study, the top four predicted off-targets for each gene were screened for mutations via high resolution melt curve analysis; no evidence of off-target effects was observed. Basu *et al* (2015) also showed that in *Ae*. *aegypti* highly active sgRNAs did not conform to previously described optimal design criteria of avoiding high and low GC content and targeting the transcribed vs non-transcribed strand (Wang et al., 2014). These differences could be due low numbers (dozens, not thousands) of sgRNAs screened in mosquitoes compared to those screened in mammalian cell lines, or to elements of sgRNA design not yet fully understood. As further studies continue to emerge on how sgRNA design (as well as other factors) impacts efficiency, a more extensive set of rules guiding sgRNA optimization is likely to form. Similarly, Dong *et al* (2015) selected their sgRNAs using ZiFiT, as opposed to flyCRISPR, and searched selected sgRNAs against the *Ae*. *aegypti* genome to minimize off-target effects.

At a certain level, the importance of predicting and avoiding all off-target effects may very well depend upon the goals of the experimenter. For basic research investigating gene function, off-target effects on experimental conclusions may be minimized with standard genetic practices such as out-crossing for multiple generations and restricting analysis to transheterozygotes derived from independent mutation events/sgRNAs. However, for gene drive constructs built for field application, stability and off-target analysis may be crucial to the success or failure of the technology. Hammond *et al* (2016), used both ZiFiT and Chop Chop (https://chopchop.rc.fas.harvard.edu) to search for predicted off-target sites with respect to their genes of interest. An alternative to software suites which search for predicted off-target effects to examine individually, techniques like GUIDE-seq and Digenome-seq allow for unbiased assessment of DSBs occurring within the genome following CRISPR treatment (Kim et al.,

2015, Tsai et al., 2015). GUIDE-seq utilizes double-stranded oligodeoxynucleotide tags to "capture" a DSB; genome fragmentation and amplification with a tag-specific primer followed by high-throughput sequencing can reveal an extensive catalog of off-target sites that is distinct from computational prediction. Diagenome-seq utilizes a comparison of sequence reads after digestion of the genome. If no mutation is present, a clear delineation between 5 and 3' reads is apparent at digestion locations, if sequences overlap the digestion location an indel is present. Neither of these techniques have been utilized in mosquitoes and may provide unbiased insight into the impact off-target effects may generate. For further review of CRISPR prediction software suites, the reader is directed to several thorough reviews (Brazelton et al., 2015, Peng et al., 2016, Wu et al., 2014a).

In conjunction with sgRNA design and off-target prediction, significant advancements have been made with respect to the fidelity of the Cas9 protein itself. Recently, a highly specific version of the Cas9 protein has been developed by altering four amino acids which directly interact with the target DNA's phosphate backbone (Kleinstiver et al., 2016) which in combination with improved sgRNA selection may remove any off-target activity. This high fidelity Cas9 variant was tested against its wild type Cas9 variant using the non-biased GUIDE-seq to test 8 sgRNAs for genome wide off-target effects. While the wild type Cas9 variant induced multiple off-target cuts in 7 of the 8 sgRNAs tested, the high fidelity Cas9 variant only induced a single off-target effect in 1 of the 8 sgRNA tested. This high fidelity Cas9 may extremely beneficial for basic and applied research in mosquitoes. The precision it provides may allow for stable Cas9 driver lines to be generated and boost the confidence that can be placed on knockout experiments. Similarly, it may reduce any fitness costs associated with the introduction

of Cas9 in gene drive systems while ensuring that any inserted sgRNAs are only able to cleave the desired targeted sequence.

PROGRESS IN MUTATION IDENTIFICATION

Following introduction of the CRISPR reagents, the desired mutation must be identified and selected for. In the absence of a physical marker, common methods for doing this including PCR amplification of the target region followed by Surveyor or T7 endonuclease treatment (Reyon et al., 2012), Sanger sequencing (Brinkman et al., 2014), high resolution melt curve analysis (Dahlem et al., 2012), and deep sequencing (Gagnon et al., 2014). Kistler *et al* (2015) suggests deep sequencing as the most cost effective for the high resolution data which is provided. To identify if CRISPR/Cas9 was indeed generating indels in each target gene, these researchers sequenced amplicons covering the site of interest derived from G_0 pupae or adults; barcoding amplicons allowed for substantial multiplexing and cost savings. Once the deep sequencing reads were aligned to the reference sequence, deletions, insertions (including insertions of ssODN), and inversions could be readily identified. However, not every laboratory will be equipped to generate and analyze large volumes of sequence data, and this method does not lend itself to situations where genotype information is required on shorter timescales. Basu *et al* (2015) utilized high resolution melt curve analysis (HRMA) to identify mutations in *Ae. aegypti*. This technique uses intercalating dyes which fluoresce strongly when bound to dsDNA; when the strands melt the dye dissociates and fluoresces at a much lower level. Using the proper equipment, the dissociation of the intercalating dye can be observed under "high resolution" (ie, 10-20 data points per ^{o}C) and a melt curve can be generated for a specific amplicon. Differences in melt curves between similar amplicons can detect single nucleotide polymorphisms as well as

indels as small as 1bp. The strength of HRMA is that it allows relatively high throughput genotyping of mutant individuals with a delay of only a few hours (Pasay et al., 2008). Amplicons displaying differences in melt curves can be compared to known deletions (genotyping a previously identified mutation in the absence of a physical marker) or grouped and sequenced to identify novel out of frame mutants [Figure 3.3]. One drawback of HRMA (and other amplicon-based genotyping methods such as T7 and Surveyor) is that they can give false positive results in the presence of pre-existing genetic polymorphisms. Thus, assays should be designed around regions devoid of such pre-existing polymorphisms whenever possible. A comparison of the sensitivity, cost and time associated with each technique is listed in Figure 3.4.

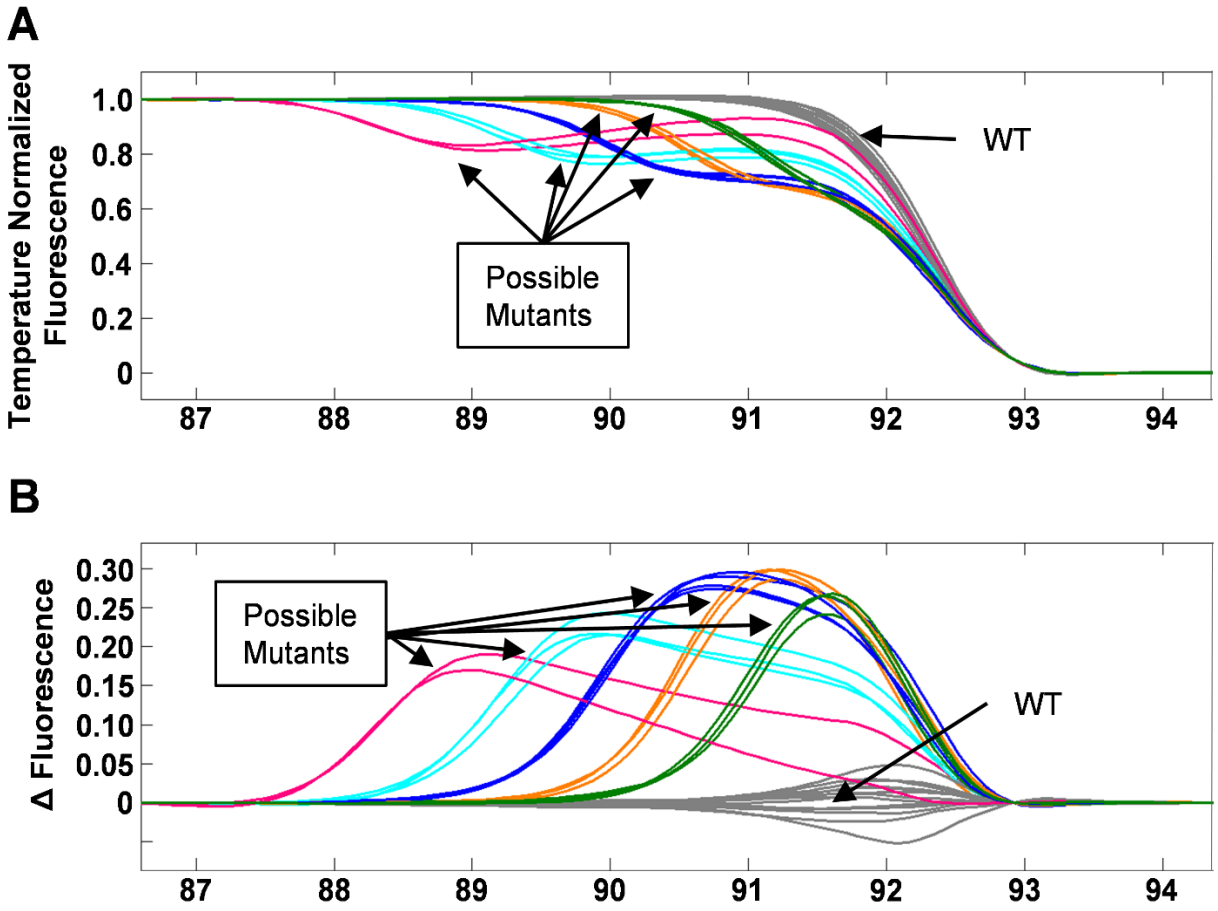

Figure 3.3 High resolution melt curve analysis identification of mutations. PCR amplification of mosquito tissue from single individual (typically from a clipping of a leg) followed by melting in the presence of a fluorescent dye. As the dsDNA melts, the fluorescent dye dissociates from the dsDNA generating a melt curve. Melt curves can be viewed directly with different curves segregating from WT (**A**). Alternatively, WT curves can be subtracted from unknowns to generate difference curves (**B**). After putative mutant groups are identified, sequencing is required to confirm any insertions or deletions, as differences from WT may also be caused by pre-existing polymorphisms.

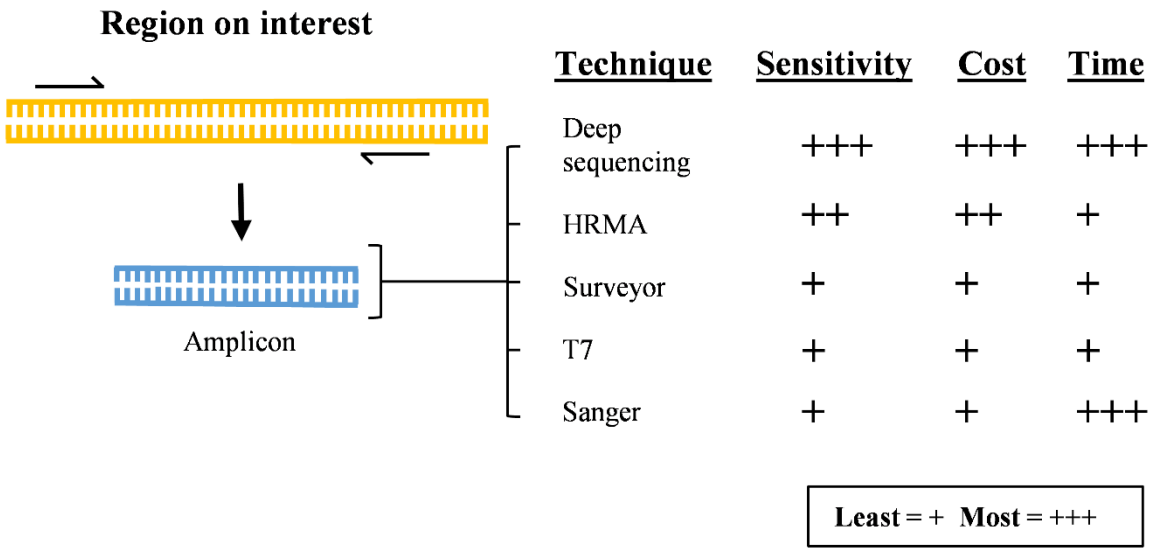

Figure 3.4 Comparison of amplicon-based mutation detection techniques. Following amplification of a target region, the resulting product can be queried for the presence mutations through digestion with mismatch-specific nucleases (T7 or Surveyor), analysis of differences in melting properties (HRMA), or through traditional (Sanger) or next generation sequencing technologies. Each technique is associated with its own strengths and weaknesses, which should be considered based on the goals and resources of the experimenter.

In addition to deep sequencing and HRMA, Kistler *et al* (2015), Basu *et al* (2015), Dong *et al* (2015), Gantz *et al* (2015), and Hammond *et al* (2016), all utilized phenotypic markers incorporated through HDR to tag disrupted regions. Given the capacity of a laboratory to screen for fluorescent markers, screening mutations generated by HDR insertion of a fluorescent marker may be the easiest way to screen and maintain mutant groups. Given the examples provided, not only can fluorescent markers allow for easy maintenance of lab colonies for basic research, but they may be required for proper tracking of gene drive strains. Given the variety of methods which can be utilized for mutation tracking, there are numerous ways in which experimenters

can begin to apply CRISPR to their research. The continued advancement of HDR techniques (which will be discussed in more depth in the next section) suggests this technology may be the most appealing way to identify and monitor mutations within a mosquito population.

PROGRESS IN GENE INSERTION VIA HOMOLOGY DIRECTED REPAIR

The ease of use of inserted fluorescent markers suggests an attractive future for CRISPR-based basic and applied research. As mentioned earlier, the HDR pathway utilizes a homologous template to repair a DSB. If these homologous sequences are placed on either end of a marker gene (along with other cargo), then the cargo can be inserted within the genome. HDR has been utilized with alternative endonucleases in mosquitoes within the past few years (Bernardini et al., 2014, Liesch et al., 2013, McMeniman et al., 2014) with rates of insertion less than 0.1%. Kistler *et al* (2015) was able insert ssODN at a rate of 0.71% in G_0 somatic tissue, and 0.6% in germline tissue but the ssODN was only 200bp in length with the majority of the construct (excluding the restriction enzyme site) consisting of the homologous flanks. When testing the ability to insert larger constructs (such as those that harbor a fluorescent marker) Kistler *et al* (2015) saw highly variable frequencies of insertion, while making the observation that lower G_0 survival rates correlated with higher levels of HDR. Specific characteristics of the construct design and injection mix concentration have been shown to play a role in insertion rate via HDR. First, studies have shown that the larger the construct to be inserted, the larger the homologous sequence must be (Kung et al., 2013). Manipulation of DNA DSB repair pathways via inhibition of key C-NHEJ components in the G_0 embryos may also increase HDR outcomes. For example, in *Drosophila* the knockout of Lig4 was utilized to enhance HDR (Beumer et al., 2008) while the knockout of Ku70 was used to enhance HDR in *Bombyx mori* (Ma et al., 2014). Basu *et al*

(2015) utilized dsRNA transiently targeting Ku70 which increased insertion rates to 1.7-2.7%. Basu *et al* (2015) also utilized dsRNA targeting Ligase IV but found that its inhibition in *Ae. aegypti*, while leading to an increase in HDR, also lead to an increase in indel generation (by what is suspected to be an alternative non-homologous end-joining pathway). Whether this observation is paralleled in other mosquitoes is still unknown, but is an interesting question which may provide insight into the diversity of double stranded break DNA repair pathways in mosquitoes. Gantz *et al* (2015) also utilized dsRNA transiently targeting Ku70 when they introduced their gene drive construct into the kh^{w} locus. Of the 680 embryos injected, 251 survivors were collected which yielded two transgenic progeny. Assuming 50% fertility, an insertion rate of 0.8-1.6% was observed, comparable to that reported by others (Basu et al., 2015, Hammond et al., 2016, Kistler et al., 2015). Interestingly, Hammond *et al* (2016) utilized HDR without the use of a NHEJ inhibitor to insert their EGFP docking constructs and saw rates similar to TE insertion. This may be due to other variables not yet identified within the injection mix, variation in microinjection technique, or differences in the species used (*An. gambiae* vs *Ae. aegypti*). Regardless, it suggests that continued collection and analysis of injection mix data may yield even higher rates of insertion via HDR. Recently it has been suggested that the nature of the homologous sequence also plays a role in how often HDR will occur. The 3' end of the double stranded break is released prior to the 5' end, suggesting that asymmetric templates which favor the 3' section of DNA lead to higher rates of HDR (Richardson et al., 2016).

As suggested above, a primary difficulty associated with the utilization of HDR is competing DSB repair pathways. While in some organisms, such as yeast, the utilization of the homology directed repair pathway is highly prevalent, in other organisms, such as mammals, the C-NHEJ pathway may be the most prevalent pathway (Mansour et al., 2008). DNA repair

pathway distinctions in cell cycle phase and tissue type will also play a role. The ability for the experimenter to manipulate the DNA double stranded break repair hierarchy is key for the utilization of the HDR pathway in organisms in which other pathways may be utilized prior to the HDR pathway. One possible way to achieve this is the successful identification and utilization of molecules which have been found to inhibit a desired DNA repair pathway. One molecule which has been shown to be effective at inhibiting C-NHEJ in vertebrates is SCR-7 (Chu et al., 2015, Maruyama et al., 2015). An inhibitor of the critical C-NHEJ protein Ligase IV, SCR-7 has been shown to increase the rate of HDR in human and mouse cell lines. It remains to be determined whether SCR-7 will be useful in invertebrates such as mosquitoes, but given that many molecules that inhibit DNA repair are researched for their anti-cancer therapeutic properties, it is possible that new molecules will surface which may play a role in aiding the genetic engineering of mosquitoes including inhibitors of other key repair proteins and chromosomal relaxers (Murr et al., 2006, Pinder et al., 2015, Yu et al., 2015).

Chemical inhibitors may prove to be advantageous additives to the genetic toolbox in mosquitoes but traditional techniques which involve the use of knockout lines and RNA interference are still highly relevant to DNA repair manipulation. For these techniques to be implemented a firm understanding of how DNA repair pathways work and which genes/proteins are the most appropriate to target. Another thought to be considered is whether a specific NHEJ component should be removed transiently or permanently. While transient knockdown of Ku70 resulted in an increase in HDR in *Ae*. *aegypti*, such mutations may or may not be sustainable in mosquito populations and may even heavily depend upon the species of mosquito. For instance, Basu *et al* (2015) found that Ligase IV mutants were unstable as heterozygotes, with mutations disappearing from the population by the 3rd generation even after selective breeding. While

Ligase IV and Ku70 are good starting points, there is a long list of genes which may be targeted to the same effect, or to greater effect (Overcash et al., 2015, Zhu et al., 2015). In summary, HDR allows for the insertion of easily detectable and trackable marker genes which can be highly beneficial to both basic and applied mosquito research. Some basic tenets, such as large homologous flanks on transgenic constructs and DNA repair manipulation increase the rate of insertion via HDR. Lastly, there several more steps which can be taken to further increase insertion rate including identification of possible chemical modulators, identification of alternative DNA repair genes for targeting, and the overall continued optimization of injection mixes.

FUTURE PROSPECTS OF GENE EDITING: CRISPR VARIANTS

The CRISPR revolution has taken hold and is opening up several new avenues for genetic engineering. While on a basic level the CRISPR systems acts as a highly specific nuclease, there are several modifications and variants which promise to further enhance genetic engineering in mosquitoes. Since the discovery of the Cas9 system much effort has been put into finding alternative versions of Cas9 which may be more specific (such as the high fidelity Cas9 mentioned earlier), or allow for different target sites (different PAM site) (Leenay et al., 2016). There have been a variety of the proteins found with some having alternative PAM sites and others being smaller in size (Garneau et al., 2010, Kleinstiver et al., 2015, Zhang et al., 2013). These proteins could be used to generate smaller gene drive constructs or expand the targetable regions within mosquito genomes. The current Cas9 protein has also been modified to provide enzymatic action as a nickase, generating only a single stranded cut (instead of a DSB) (Mali et al., 2013a). This allows for two sgRNAs targeting opposite strands in close proximity to generate

a DSB, generating higher specificity and removing some of the risk of off-target effects. A variety of catalytically inactive Cas9 fusion proteins have been developed to take advantage of sgRNA-directed DNA binding (Mali et al., 2013b). For instance, Cas9 fusion proteins containing an activator have been shown to up regulate the expression of human genes (Maeder et al., 2013). The CRISPR system has also been retooled to interfere with mRNA (Qi et al., 2013). It should also be noted that creativity within the CRISPR system is not limited Cas9 protein modifications. An interesting example is the generation of synthetic constructs which provide tissue specific expression of sgRNAs of interest. Ubiquitous expression of the sgRNA is typically achieved via a strong pol III-directed promoter so that the sgRNA is not given a 5' cap, a poly A tail and maintains its nuclear localization. A disadvantage with such pol III-based transcription is that they are not tissue specific. Port *et al* (2016) recently described a method that allows for experimenters to link a tissue-specific pol II-based promoter to a synthetic construct whereby sgRNAs of interest are flanked by tRNAs *in cis* (Port and Bullock, 2016). Following initial transcription, RNases P & Z cleave the tRNAs releasing the intact sgRNAs within the nucleus. Port *et al* (2016) observed that the pol III-based sgRNA production resulted in leaky expression and incomplete reduction of the targeted protein in imaginal wing discs of developing *Drosophila* (only 60% imaginal wing discs were deficient in protein of target gene and 60% of imaginal wing discs targeted showed some level of protein reduction of the targeted gene protein in adjacent tissue). In contrast, utilizing tRNA-flanked sgRNAs, no detectable protein of the targeted gene was observed in targeted tissue and only 10% imaginal wing discs showed some protein removal in adjacent tissue. These observations suggest that there is still room for improvement with respect to tissue specific expression of transgenes and that the tRNA flanking technique is worth exploring in mosquitoes for generating sgRNAs. The creativity with which

the CRISPR system can be used is almost limitless. Now that the utilization of CRISPR in mosquitoes is becoming more well established, the Cas9 variants which provide the most promise with respect genetic manipulation of disease vectors should be assessed and characterized with respect to mosquitoes. This burgeoning field should provide ample research opportunities which could drastically enhance mosquito research.

PROGRESS IN TRANSPOSABLE ELEMENT-BASED MOSQUITO TRANSFORMATION

Routine transformation with transposable elements in mosquitoes was first established in *Ae*. *aegypti* a decade after the successful use in Drosophila with the use of Class II (DNA elements) *MosI*, *Hermes* and *piggyBac* transposable elements (Coates et al., 1998, Jasinskiene et al., 1998, Kokoza et al., 2001). Shortly afterwards, TEs were adapted for use in *An*. *stephensi* using *minos* and *An*. *gambiae* using *piggyBac* (Catteruccia et al., 2000, Grossman et al., 2001). Transposable elements have to this point served as the work horse of transgenic experiments in mosquitoes allowing vector biologists to confer phenotypes such as pathogen resistance (Corby-Harris et al., 2010, Kokoza et al., 2010, Meredith et al., 2011), sex bias (Galizi et al., 2014), flightlessness (Fu et al., 2010), and conditional sterility (Phuc et al., 2007) on laboratory mosquito populations. While routine and reliable, TEs are limited by the fact that their integration site in the genome cannot be predetermined. For many applications such as the development of anti-viral/malarial or gene drive, it is critical that transgenes are integrated in stable locations within the genome which do not impact fitness (Amenya et al., 2010) and avoid transcriptional gene silencing (Vaucheret and Fagard, 2001). What transposable elements do allow for, is for the experimenter to conduct forward genetic screens where an optimal location is not known *a priori*. By inserting genetic elements which may have anti-viral or anti-parasitic

properties at random within the genome, the investigator can sample locations to identify those that might be most favorable. For example, Labbe *et al* (2012) generated 18 transgenic lines (9 for *Ae*. *aegypti* and 9 for *Ae*. *albopictus*) carrying the same construct, but reported varying results with respect to female specific flightlessness in each (Labbé et al., 2012). Ultimately, screening a number of unique insertions was critical to finding an optimal position, emphasizing the impact of TE integration site on construct efficiency. In addition to embryonic injection, another way to sample multiple locations is remobilization of the TE housing the transgenic construct. O'Brochta *et al* (2012) provides an excellent example of forward genetic screens by remobilizing the *piggyBac* TE for the generation of Gal4-UAS lines (O'Brochta et al., 2012). Utilizing TEs to generate multiple genome-wide insertions, the researchers observed various expression profiles which were then selected from for future Gal4-UAS experiments; these experiments will be discussed in more detail in Section 3.

Sufficient mosquito transformation experiments have been performed to allow meta-analysis of various parameters and success rates. Gregory *et al* (2016) analyzed metadata on *piggyBac* usage in a range of insects with the goal of assessing appropriate transformation efficiency (Gregory et al., 2016). To this end Gregory et al (2016) developed a decision making model which what they termed "Goldilocks" (https://mammykins.shinyapps.io/App-gold), allowing researchers to predict the number of embryos they need to inject to achieve a desired number of transgenics. These researchers found that experimenters can utilize analysis of survival rates and transformation efficiency to minimize wasted efforts and resources resulting from too few or too many injections. Their method could also be extrapolated to other germline transformation methods given that the proper data is available for analysis.

Recently, TEs have been used in combination with highly specific DNA binding domains to increase transposition efficiency in *Bombyx mori* (Ye et al., 2015). Ye *et al* (2015) found that when a transcription activator-like effector was fused to the *piggyBac* transposase a stable transposition efficiency as high as 63.9% was seen, as well as a nearly 6-fold increase in transgene positive individuals. While no site specific integration of the TE was observed, the significant increase in overall TE integration warrants further investigation. If applied in mosquitoes, such a large increase in transformation efficiency could greatly reduce the labor and cost associated with forward genetic screens. While no reports have been published to this date, it may very well be the case that CRISPR and TEs can be utilized in a similar fashion with the advantage of being able to multiplex sgRNAs. In summary, TEs still have an important role to play in mosquito transgenesis. They are still a viable option when it comes to inserting transgenes within the genome at a reliable rate, and allow for the systematic testing of stability, fitness, and transgene expression in an unbiased manner. Assessment of current and future TE studies with respect to integration rates should be conducted to optimize injection parameters and potentially unnecessary labor. Lastly, ways that TE can be used in conjunction with the expanding genetic toolbox (especially CRISPR) should be explored.

CONTROLLING TRANSGENE EXPRESSION USING BIPARTITE SYSTEMS

Bipartite systems involve the use two independent transgenes in conjunction to achieve an activity that neither alone possesses. The separate nature of the transgenes allows for spatiotemporal control of a gene of interest via proper crossing of transgenic lines. Typically, one portion of the bipartite system acts as a “driver” which produces a specific transcription factor

and the other portion acts as a “responder” which is the target of that particular transcription factor; those mosquitoes with both the driver and the responder will express the particular gene that the responder promotes. For the purposes of this chapter, we will discuss the Gal4-UAS system, the Q system, and the Tet On/Off systems.

The Gal4-UAS system derives from yeast, with the Gal4 transactivator promoting the transcription of the target gene by binding to an upstream activation sequence (UAS). The workhouse of Drosophila genetics [Reviewed in (Griffin et al., 2014)], the Gal4-UAS system has been successfully deployed in several mosquito species including *Ae. aegypti, An. gambiae*, and *An. stephensi* (Kokoza and Raikhel, 2011, Lynd and Lycett, 2012, O'Brochta et al., 2012, Zhao et al., 2014). Kokoza *et al* (2011) utilized the fat-body specific, bloodmeal-induced Vg promoter to drive Gal4 expression. When crossed to a UAS-EGFP responder line, expression of EGFP was observed within the fat bodies of bloodfed females. The Vg-Gal4 driver subsequently allowed for the interrogation of 20E activation of the Vg promoter. Lynd *et al* (2012) utilized the midgut-specific bloodmeal-induced carboxypeptidase promoter to control Gal4; these driver lines were crossed with UAS-luciferase and UAS-YFP responders re-capitulating the sex, tissue, and stage specific expression of carboxypeptidase in *An. gambiae*. Similarly, Zhao *et al* (2014) also established an *Ae. aegypti* Gal4 driver line based on the homologous midgut-specific carboxypeptidase A gene promoter; this was also validated by crossing to a UAS-EGFP responder line. Despite the power of the Gal4-UAS system to control transgene expression and the number of mosquito gene promoters that have already been characterized [reviewed in (Adelman, 2015)], relatively few have been utilized to generate re-usable Gal4-drivers. Increasing the number of driver lines available to the mosquito genetics and physiology community would likely accelerate basic research and should be pursued when possible.

In addition to reverse genetic approaches described above, bipartite systems such as Gal4-UAS represent a powerful tool to perform forward genetic screens in mosquitoes. O'Brochta *et al* (2012) took advantage of the fact that *piggyBac* can be efficiently remobilized in *An. stephensi* (O'Brochta et al., 2011) to build a *piggyBac*-based enhancer trap using the Gal4-UAS system. Remobilization of the *piggyBac* element containing the Gal4 enhancer trap in the presence of UAS-tdTomato (red fluorescent protein) allowed the identification of novel insertion sites yielding tissue specific expression patterns in the midgut, fat body and salivary glands. The identification of unique enhancer elements provides a catalog of valuable insertion sites; combined with the power of CRISPR and site-specific gene insertion, such sites can be re-used even if the initial strains cannot be propagated indefinitely. The unique ability to perform such screens is so far restricted to *An. stephensi*, as such transposon remobilization has not yet been possible in *Ae. aegypti* or *An. gambiae* (Sethuraman et al., 2007, Wilson et al., 2003, Palavesam et al., 2013). Thus, this work is extremely valuable in identifying enhancer elements which may be utilized for basic or applied research approaches, as the successful introduction of anti-pathogen genes in mosquitoes is dependent upon utilizing an expression profile which maximizes their effect on the pathogen, while minimizing negative effects on host fitness or behaviors.

Recently the Gal4/UAS system has also been used to generate a driver line in drosophila which allow for tissue specific expression of the Cas9 endonuclease (Xue et al., 2014). Such a driver line removes the need to include Cas9 mRNA from any future gene editing experiments reducing the cost and labor needed for gene editing. The development of UAS-Cas9 mosquito strains that could be combined with existing or novel Gal4-drivers would give unprecedented control over tissue-specific gene disruption, and would be of great value to understanding the

genetic basis of essential traits, particularly in regards to genes that are critical for both early developmental stages and adult stages and thus are recalcitrant to ubiquitous disruption. Thus, characterization of novel promoters and generation of Gal4-UAS lines harnessing promoters already characterized would allow the mosquito genetics community to fully take advantage of this bipartite system [Figure 3.5].

The Q system, identified in the fungus *Neurospora crassa*, utilizes genes associated with the *qa* gene cluster and is regulated by the concentration of glucose via the transcription factor (QF) and the transcription repressor (QS). While this system has not yet been used in mosquitoes, it is effective in Drosophila and in combination with the Gal4/UAS system, may lead to even more precise control of transgenes (Potter and Luo, 2011). The ability to utilize both the GAL4-UAS system and Q system in conjunction could vastly expand the toolbox of bipartite systems in mosquitoes allowing for the testing of two independent genes of interest.

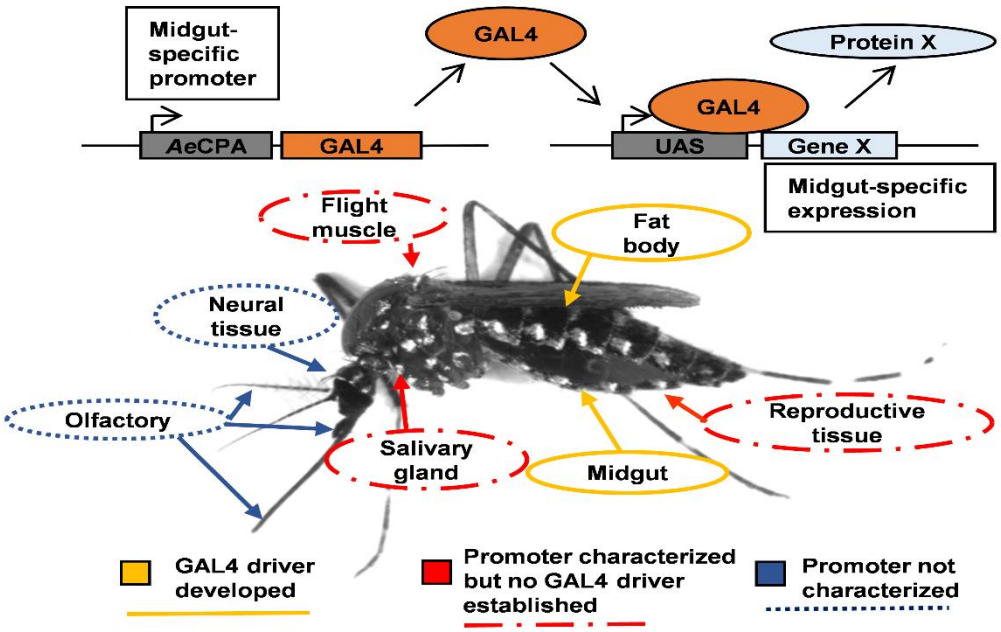

Figure 3.5 The GAL4-UAS system allows for tissue specific expression of effector genes in mosquitoes. The upper portion of the figure shows a typical driver-responder pair, in this case a midgut specific promoter (*Ae*CPA) driving the expression of GAL4; GAL4 in turn interacts with the upstream associated sequence or UAS to promote the production of a second gene X (gene of interest). The bipartite nature of the GAL4-UAS system allows for the experimenter to interchange either the promoter of GAL4 expression or the gene being promoted by the UAS promoter. This allows for quick and easy crosses to be made to test the impact of a specific effector gene within a targeted tissue. The bottom portion of the figure shows specific tissue types in which the GAL4-UAS system has been successfully utilized in mosquitoes (yellow; solid line), tissue types in which possible promoters have been identified but the GAL4-UAS system has not been utilized yet (red; dash-dot line), and tissue types in which no promoters have been identified and the GAL4-UAS system has not been utilized yet (blue; dotted line).

Lastly, tetracycline-controllable gene expression systems are very well established in mosquitoes in both Tet-Off and Tet-On forms (Lycett et al., 2004). Both of these systems rely on an external effector molecule (tetracycline or one of its analogs) which can be added or removed

from the mosquito's diet. Lycett *et al* (2004) was able generate both Tet-On and Tet-Off transgenic lines in *An. stephensi* using the tet analog doxycycline to regulate the expression of β-galactosidase with its expression being promoted in the transgenic Tet-On line and its expression being repressed in the corresponding Tet-Off lines. Following this initial proof of principle, the Tet-Off system was used in transgenic strains of *Ae. aegypti*, *Ae. albopictus* and *An. stephensi* (Fu et al., 2010, Labbé et al., 2012, Marinotti et al., 2013, Phuc et al., 2007, Wise de Valdez et al., 2011). Phuc *et al* (2007) generated transgenic strains of *Ae. aegypti* carrying a gene which when expressed lead to lethality late in development (dominant lethality). In the presence of tetracycline the transgenic lines yielded survival rates comparable to wild type controls (87-89%) but in its absence survival rates at adult emergence was severely reduced (2.6-4.2%). The late acting effect of the transgene allowed released transgenics to compete with the wild population, which provides an advantage when compared to traditional sterile insect techniques. The best of the strains developed by Phuc *et al* (2007) were tested in field releases in the Cayman Islands (Harris et al., 2012), with eventual suppression rates calculated at around 80%. Currently, these techniques are being utilized by in Brazil to great effect, as Carvalho *et al* (2015) found that release of transgenic male mosquitoes (OX513A) led to a 95% reduction of *Ae. aegypti* in the release area (Carvalho et al., 2015). Initially, 2,800 transgenic males per hectare per week were released, but upon conducting a rangefinder study, production was optimized to release many more transgenic males than previously hypothesized (25,000 transgenic males per hectare per week). Given the recent outbreak of the Zika virus in South America (Imperato, 2016), the expedient use of these transgenic lines have become increasingly important. Fu *et al* (2010) attempted a similar strategy using the Tet-Off system under the control of a female-specific indirect flight muscle promoter (Actin-4) to produce female *Ae. aegypti* which lack the ability to

fly. Follow up cage trials indicated some success (Wise de Valdez et al 2011). In a similar design, Labbe *et al* (2012) and Marinotti *et al* (2013) generated transgenic strains with female-specific flightless phenotypes in *Ae. albopictus* and *An. stephensi,* respectively. These experiments reinforce the utility of the Tet-Off system to control the expression of deleterious genes capable of conferring useful phenotypes in mosquitoes.

PROGRESS IN ΦC31 & RMCE RECOMBINATION IN MOSQUITOES

The use of "docking" mechanisms allows the experimenter to re-utilize a useful position within a genome to introduce additional genetic constructs. The insertion of a docking location can be accomplished via the random initial insertion of a transposable element, or a pre-specified location utilizing HDR. The φC31 system [isolated from the φC31 bacteriophage (Thorpe and Smith, 1998)] has been shown to be particularly useful in mosquitoes, and is based on the recombination reaction between attachment sites in the phage (*att*P) and target bacterium (*att*B). [For detailed background information on the φC31 system see (Fogg et al., 2014, Knapp et al., 2015, Smith et al., 2010)].

First adapted for use in human cells and in Drosophila (Groth et al., 2000, Groth et al., 2004), the φC31system has been adapted for use in several mosquito species including *Ae. aegypti, Ae. albopictus, An. stephensi* and *An. gambiae* (Amenya et al., 2010, Labbe et al., 2010, Meredith et al., 2011, Nimmo et al., 2006). Nimmo *et al* (2006) generated 5 *att*P φC31 docking strains in *Ae. aegypti* utilizing *piggyBac*, with between 1 and 4 docking sites per strain. Of the five strains generated, four were tested for site specific integration by insertion of an *att*B construct containing a red fluorescent marker (DsRed); integration was observed at rates between 16.7 and 31.8% (average 23%). Labbe *et al* (2010) utilized the φC31 docking system in *Ae.*

albopictus, using a *piggyBac* element to first insert an *att*P docking site. After the recovery of five independent docking lines, three were chosen for integration of DsRed via an *att*B docking site, with reported integration rates between 2-6%. While significantly lower than that reported by Nimmo *et al* (2006), these rates were similar to that observed in *Ae*. *aegypti* by Franz *et al* (2011), who found integration rates of an EGFP marker gene of 1.7-4.9%. It is possible that the location of the docking site integration may play a large role in φC31 integration rates, and that rates obtained by Labbe *et al* (2010) and Franz *et al* (2011) may be more typical of what can be expected.

Amenya *et al* (2010) generated a cohort of integrated φC31 docking sites into *An*. *stephensi* to measure the impact of docking sites (in conjunction with a fluorescent marker gene) on mosquito fitness (transformation efficiency not reported). These researchers monitored fecundity, percentage of females that did not lay eggs, pupal development time, larval viability, male and female longevity, and male and female wingspan. While there were individual differences between strains, Amenya *et al* (2010) found no statistically significant difference, under the parameters measured, between mosquitoes containing the docking site and those without. This suggests that φC31 docking sites can be inserted into the genome without causing inherent fitness costs to the mosquito host. Meredith *et al* (2011) utilized a φC31 docking site to integrate Vida3, an anti-malarial peptide, in *An*. *gambiae*. Four independent docking strains were generated via *piggyBac*, a single strain was selected for integration of the corresponding *att*B sequence which was accompanied by the Vida3 anti-malarial peptide and the DsRed marker gene, with reported efficiency of 10 %. Windbichler *et al* (2011) utilized a φC31 docking strain to provide the first proof of principle of a homing endonuclease (HEG) gene drive system (Windbichler et al., 2011). Using the docking site, they generated donor, reporter, and target

strains of *An. gambiae,* all at the same genetic locus. The donor strain contained EGFP interrupted with an HEG gene, the reporter contained an interrupted EGFP open reading frame (which could be correctly expressed after incurring frameshift indels via the NHEJ pathway) and the target strain contained EGFP not interrupted via an HEG. Since the genetic constructs where driven by a male specific promoter, female crosses were used as a control. When males were crossed with WT females, a homing rate of 56% was observed with 97% of progeny containing the HEG cassette. The ability to utilize the φC31 docking system to generate a variety of transgenic lines within transgenic constructs in homologous portions of the chromosome, provide a unique a useful asset for assessing possible future gene drive components as well as interrogating mechanisms of DSB break repair.

Hammond *et al* (2016) also utilized the φC31 docking system to improve their ability to generate complex multi-gene insertions for gene drive applications. Docking sites were initially integrated utilizing HDR in conjunction with an EGFP marker gene following DSB induction by a site-specific nuclease. This allowed for identification of lines with the docking site, and allowing for confirmation of the position of the docking site via PCR as well as for the characterization of the targeted genes with respect to their suspected role in female fertility. Once their role in female fertility was established, the gene drive construct was injected along with the recombinase construct allowing for recombination mediated cassette exchange to occur. This relatively simple work flow allows for a smaller construct (the marker gene and φC31 docking site) to be inserted before introduction of a larger gene drive construct. An advantage of this technique could be the avoidance of the requirement to generate the large homologous flanks needed to insert a HDR construct which carries extensive of genetic cargo. It would also be

advantageous for genetic targets which have only a small region which is targetable for HDR insertion (possibly due to repetitive elements).

Other recombinases such as the cre recombinase system and the FLP/FRT have been postulated for use in mosquitoes based on their successful use in Drosophila (Oberstein et al., 2005, Venken and Bellen, 2012). Jasinskiene *et al* (2003) showed that the cre system catalyzes transgene excision at extremely high rates (up to 99.4%) but was unable to exchange cassettes (Jasinskiene et al., 2003). Recently the FLP and cre systems have been used in conjunction with the φC31 system in *Ae. aegypti* successfully (Haghighat-Khah et al., 2015). Haghighat-Khah *et al* (2015) generated a system which they termed iRMCE which utilizes the integration ability of the φC31 system and the excision abilities of the FLP and cre systems. This integrase-recombinase mediated cassette exchange (iRMCE) technique allows for the removal of any unnecessary genetic components found in the plasmid backbone of the integrase. Utilizing this system, Haghighat-Khah *et al* (2015) observed integration rates of 4.1-4.8% and minimum excision rates of 2.4% for cre mediated excision and 1.3% for FLP mediated excision in *Ae. aegypti*. These docking mechanisms provide for a unique tool which ultimately can be used in conjunction with all other previous tools and techniques mentioned in this chapter. The φC31system may be used in association with TEs to insert transgenes throughout the mosquito genome to assess expression and stability and can also be utilized with HDR to insert docking sites in highly specific locations. The φC31, FLP, and cre systems can also be utilized in conjunction with bipartite systems to achieve RMCE of new promoter sequences and effector genes into docking based lines. While the φC31 system is relatively well established in mosquitoes, more research and development is encouraged on the FLP and cre systems so that they can be utilized independently or in conjunction with φC31 to facilitate more highly efficient

RMCE. For an excellent description of current best practices and protocols regarding the use of φC31 in mosquitoes, please see (Pondeville et al., 2014).

CONSIDERATIONS AND OUTLOOK

The toolkit used for the manipulation and genetic modification of mosquitoes has expanded tremendously in the past two decades (Volohonsky et al., 2015). First transposable elements opened the door for the introduction of transgenic constructs. Next came in the introduction of docking systems such as φC31, which increased the specificity of genetic manipulation. Then came the first wave of specific nucleases including homing endonucleases, zinc finger nucleases, and transcription activator-like effector nucleases (these were not covered here due to recent advancements in CRISPR) in conjunction with utilization of the homology directed repair pathway. Lastly, the most recent advancements have come with the development of CRISPR technology. CRISPR stands to continually revolutionize gene editing not only in mosquitoes but in other model organisms of interest. In parallel with the advancements in the variety and efficiency of CRISPR a continued advancement in the understanding of how DNA double stranded break repair promises to enhance the specificity and control associated with genetic modifications of mosquitoes. While older techniques such as the use of TEs and docking sites will continue to play a role in years to come, the CRISPR revolution and associated advancements stand to change gene editing and transgenesis in crucial disease vectors.

REFERENCES

ADELMAN, Z. N. AND Z. TU (2016). Control of Mosquito-Borne Infectious Diseases: Sex and Gene Drive. Trends Parasitol 32(3): 219-229.

ADELMAN, Z. N., BASU, S. AND MYLES, K.M. (2015). Gene Insertion and Deletion in Mosquitoes. Genetic Control of Malaria and Dengue. Z. N. Adelman, Elsevier**:** 139-168.

AMENYA, D. A., M. BONIZZONI, A. T. ISAACS, N. JASINSKIENE, H. CHEN, O. MARINOTTI, G. YAN AND A. A. JAMES (2010). Comparative fitness assessment of *Anopheles stephensi* transgenic lines receptive to site-specific integration. Insect Mol Biol 19(2): 263-269.

AWATA, H., T. WATANABE, Y. HAMANAKA, T. MITO, S. NOJI AND M. MIZUNAMI (2015). Knockout crickets for the study of learning and memory: Dopamine receptor Dop1 mediates aversive but not appetitive reinforcement in crickets. Scientific Reports 5: 15885.

BASSETT, A. R., C. TIBBIT, C. P. PONTING AND J. L. LIU (2013). Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. Cell Rep 4(1): 220-228.

BASU, S., A. ARYAN, J. M. OVERCASH, G. H. SAMUEL, M. A. ANDERSON, T. J. DAHLEM, K. M. MYLES AND Z. N. ADELMAN (2015). Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. Proc Natl Acad Sci U S A 112(13): 4038-4043.

BERNARDINI, F., R. GALIZI, M. MENICHELLI, P. A. PAPATHANOS, V. DRITSOU, E. MAROIS, A. CRISANTI AND N. WINDBICHLER (2014). Site-specific genetic

engineering of the *Anopheles gambiae* Y chromosome. Proc Natl Acad Sci U S A 111(21): 7600-7605.

BEUMER, K. J., J. K. TRAUTMAN, A. BOZAS, J. L. LIU, J. RUTTER, J. G. GALL AND D. CARROLL (2008). Efficient gene targeting in *Drosophila* by direct embryo injection with zinc-finger nucleases. Proc Natl Acad Sci U S A 105(50): 19821-19826.

BRAZELTON, V. A., S. ZARECOR, D. A. WRIGHT, Y. WANG, J. LIU, K. CHEN, B. YANG AND C. J. LAWRENCE-DILL (2015). A quick guide to CRISPR sgRNA design tools. GM Crops & Food 6(4): 266-276.

BRINKMAN, E. K., T. CHEN, M. AMENDOLA AND B. VAN STEENSEL (2014). Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res 42(22): e168.

CARVALHO, D. O., A. R. MCKEMEY, L. GARZIERA, R. LACROIX, C. A. DONNELLY, L. ALPHEY, A. MALAVASI AND M. L. CAPURRO (2015). Suppression of a Field Population of *Aedes aegypti* in Brazil by Sustained Release of Transgenic Male Mosquitoes. PLoS Negl Trop Dis 9(7): e0003864.

CATTERUCCIA, F., T. NOLAN, T. G. LOUKERIS, C. BLASS, C. SAVAKIS, F. C. KAFATOS AND A. CRISANTI (2000). Stable germline transformation of the malaria mosquito *Anopheles stephensi*. Nature 405(6789): 959-962.

CDC. 2014. *CDC and Malaria* [Online]. The Centers for Disease Control and Prevention.[Accessed 8/13/19].

CHAMPER, J., A. BUCHMAN AND O. S. AKBARI (2016). Cheating evolution: engineering gene drives to manipulate the fate of wild populations. Nat Rev Genet 17(3): 146-159.

CHU, V. T., T. WEBER, B. WEFERS, W. WURST, S. SANDER, K. RAJEWSKY AND R. KUHN (2015). Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nat Biotechnol 33(5): 543-548.

COATES, C. J., N. JASINSKIENE, L. MIYASHIRO AND A. A. JAMES (1998). Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. Proc Natl Acad Sci U S A 95(7): 3748-3751.

CONG, L., F. A. RAN, D. COX, S. LIN, R. BARRETTO, N. HABIB, P. D. HSU, X. WU, W. JIANG, L. A. MARRAFFINI AND F. ZHANG (2013). Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121): 819-823.

CORBY-HARRIS, V., A. DREXLER, L. WATKINS DE JONG, Y. ANTONOVA, N. PAKPOUR, R. ZIEGLER, F. RAMBERG, E. E. LEWIS, J. M. BROWN, S. LUCKHART AND M. A. RIEHLE (2010). Activation of Akt signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in *Anopheles stephensi* mosquitoes. PLoS Pathog 6(7): e1001003.

DAHLEM, T. J., K. HOSHIJIMA, M. J. JURYNEC, D. GUNTHER, C. G. STARKER, A. S. LOCKE, A. M. WEIS, D. F. VOYTAS AND D. J. GRUNWALD (2012). Simple methods for generating and detecting locus-specific mutations induced with TALENs in the zebrafish genome. PLoS Genet 8(8): e1002861.

DONG, S., J. LIN, N. L. HELD, R. J. CLEM, A. L. PASSARELLI AND A. W. FRANZ (2015). Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti*. PLoS One 10(3): e0122353.

DOUDNA, J. A. AND E. CHARPENTIER (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346(6213): 1258096.

ESVELT, K. M., A. L. SMIDLER, F. CATTERUCCIA AND G. M. CHURCH (2014). Concerning RNA-guided gene drives for the alteration of wild populations. Elife: e03401.

FOGG, P. C. M., S. COLLOMS, S. ROSSER, M. STARK AND M. C. M. SMITH (2014). New Applications for Phage Integrases. Journal of Molecular Biology 426(15): 2703-2716.

FU, G., R. S. LEES, D. NIMMO, D. AW, L. JIN, P. GRAY, T. U. BERENDONK, H. WHITE-COOPER, S. SCAIFE, H. KIM PHUC, O. MARINOTTI, N. JASINSKIENE, A. A. JAMES AND L. ALPHEY (2010). Female-specific flightless phenotype for mosquito control. Proc Natl Acad Sci U S A 107(10): 4550-4554.

GAGNON, J. A., E. VALEN, S. B. THYME, P. HUANG, L. AKHMETOVA, A. PAULI, T. G. MONTAGUE, S. ZIMMERMAN, C. RICHTER AND A. F. SCHIER (2014). Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. PLoS One 9(5): e98186.

GALIZI, R., L. A. DOYLE, M. MENICHELLI, F. BERNARDINI, A. DEREDEC, A. BURT, B. L. STODDARD, N. WINDBICHLER AND A. CRISANTI (2014). A synthetic sex ratio distortion system for the control of the human malaria mosquito. Nat Commun **5**: 3977.

GANTZ, V. M. AND E. BIER (2015). Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. Science 348(6233): 442-444.

GANTZ, V. M. AND E. BIER (2016). The dawn of active genetics. Bioessays 38(1): 50-63.

GANTZ, V. M., N. JASINSKIENE, O. TATARENKOVA, A. FAZEKAS, V. M. MACIAS, E. BIER AND A. A. JAMES (2015). Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. Proceedings

of the National Academy of Sciences of the United States of America 112(49): E6736-E6743.

GARNEAU, J. E., M. E. DUPUIS, M. VILLION, D. A. ROMERO, R. BARRANGOU, P. BOYAVAL, C. FREMAUX, P. HORVATH, A. H. MAGADAN AND S. MOINEAU (2010). The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 468(7320): 67-71.

GILLES, A. F., J. B. SCHINKO AND M. AVEROF (2015). Efficient CRISPR-mediated gene targeting and transgene replacement in the beetle *Tribolium castaneum*. Development 142(16): 2832-2839.

GRATZ, S. J., F. P. UKKEN, C. D. RUBINSTEIN, G. THIEDE, L. K. DONOHUE, A. M. CUMMINGS AND K. M. O'CONNOR-GILES (2014). Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. Genetics 196(4): 961-971.

GREGORY, M., L. ALPHEY, N. I. MORRISON AND S. M. SHIMELD (2016). Insect transformation with *piggyBac*: getting the number of injections just right. Insect Mol Biol. doi: 10.1111/imb.12220

GRIFFIN, R., R. BINARI AND N. PERRIMON (2014). Genetic odyssey to generate marked clones in *Drosophila* mosaics. Proceedings of the National Academy of Sciences 111(13): 4756-4763.

GROSSMAN, G. L., C. S. RAFFERTY, J. R. CLAYTON, T. K. STEVENS, O. MUKABAYIRE AND M. Q. BENEDICT (2001). Germline transformation of the malaria vector, *Anopheles gambiae*, with the *piggyBac* transposable element. Insect Mol Biol 10(6): 597-604.

GROTH, A. C., M. FISH, R. NUSSE AND M. P. CALOS (2004). Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. Genetics 166(4): 1775-1782.

GROTH, A. C., E. C. OLIVARES, B. THYAGARAJAN AND M. P. CALOS (2000). A phage integrase directs efficient site-specific integration in human cells. Proc Natl Acad Sci U S A 97(11): 5995-6000.

HAGHIGHAT-KHAH, R. E., S. SCAIFE, S. MARTINS, O. ST JOHN, K. J. MATZEN, N. MORRISON AND L. ALPHEY (2015). Site-Specific Cassette Exchange Systems in the *Aedes aegypti* Mosquito and the *Plutella xylostella* Moth. PLoS ONE 10(4): e0121097.

HALL, A. B., S. BASU, X. JIANG, Y. QI, V. A. TIMOSHEVSKIY, J. K. BIEDLER, M. V. SHARAKHOVA, R. ELAHI, M.A. ANDERSON, X. G. CHEN, I. V. SHARAKHOV, Z. N. ADELMAN AND Z. TU (2015). A male-determining factor in the mosquito *Aedes aegypti*. Science 348(6240): 1268-1270.

HAMMOND, A., R. GALIZI, K. KYROU, A. SIMONI, C. SINISCALCHI, D. KATSANOS, M. GRIBBLE, D. BAKER, E. MAROIS, S. RUSSELL, A. BURT, N. WINDBICHLER, A. CRISANTI AND T. NOLAN (2016). A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. Nat Biotechnol 34(1): 78-83.

HARRIS, A. F., A. R. MCKEMEY, D. NIMMO, Z. CURTIS, I. BLACK, S. A. MORGAN, M. N. OVIEDO, R. LACROIX, N. NAISH, N. I. MORRISON, A. COLLADO, J. STEVENSON, S. SCAIFE, T. DAFA'ALLA, G. FU, C. PHILLIPS, A. MILES, N. RADUAN, N. KELLY, C. BEECH, C. A. DONNELLY, W. D. PETRIE AND L.

ALPHEY (2012). Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nat Biotech 30(9): 828-830.

IMPERATO, P. J. (2016). The Convergence of a Virus, Mosquitoes, and Human Travel in Globalizing the Zika Epidemic. Journal of Community Health 41(3): 674-679.

JASINSKIENE, N., C. J. COATES, A. ASHIKYAN AND A. A. JAMES (2003). High efficiency, site-specific excision of a marker gene by the phage P1 cre-loxP system in the yellow fever mosquito, *Aedes aegypti*. Nucleic Acids Res 31(22): e147.

JASINSKIENE, N., C. J. COATES, M. Q. BENEDICT, A. J. CORNEL, C. S. RAFFERTY, A. A. JAMES AND F. H. COLLINS (1998). Stable transformation of the yellow fever mosquito, *Aedes aegypti*, with the *Hermes* element from the housefly. Proc Natl Acad Sci U S A 95(7): 3743-3747.

JINEK, M., K. CHYLINSKI, I. FONFARA, M. HAUER, J. A. DOUDNA AND E. CHARPENTIER (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096): 816-821.

KIM, D., S. BAE, J. PARK, E. KIM, S. KIM, H. R. YU, J. HWANG, J.-I. KIM AND J.-S. KIM (2015). Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. Nat Meth 12(3): 237-243.

KISTLER, K. E., L. B. VOSSHALL AND B. J. MATTHEWS (2015). Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. Cell Rep 11(1): 51-60.

KLEINSTIVER, B. P., V. PATTANAYAK, M. S. PREW, S. Q. TSAI, N. T. NGUYEN, Z. ZHENG AND J. K. JOUNG (2016). High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature 529(7587): 490-495.

KLEINSTIVER, B. P., M. S. PREW, S. Q. TSAI, V. V. TOPKAR, N. T. NGUYEN, Z. ZHENG, A. P. GONZALES, Z. LI, R. T. PETERSON, J. R. YEH, M. J. ARYEE AND J. K. JOUNG (2015). Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 523(7561): 481-485.

KNAPP, J.-M., P. CHUNG AND J. H. SIMPSON (2015). Generating Customized Transgene Landing Sites and Multi-Transgene Arrays in *Drosophila* Using phiC31 Integrase. Genetics 199(4): 919-934 .

KOKOZA, V., A. AHMED, E. A. WIMMER AND A. S. RAIKHEL (2001). Efficient transformation of the yellow fever mosquito *Aedes aegypti* using the piggyBac transposable element vector pBac[3xP3-EGFP afm]. Insect Biochem Mol Biol 31(12): 1137-1143.

KOKOZA, V., A. AHMED, S. WOON SHIN, N. OKAFOR, Z. ZOU AND A. S. RAIKHEL (2010). Blocking of Plasmodium transmission by cooperative action of Cecropin A and Defensin A in transgenic *Aedes aegypti* mosquitoes. Proc Natl Acad Sci U S A 107(18): 8111-8116.

KOKOZA, V. A. AND A. S. RAIKHEL (2011). Targeted gene expression in the transgenic *Aedes aegypti* using the binary Gal4-UAS system. Insect Biochem Mol Biol 41(8): 637-644.

KUNG, S. H., A. C. RETCHLESS, J. Y. KWAN AND R. P. ALMEIDA (2013). Effects of DNA size on transformation and recombination efficiencies in *Xylella fastidiosa*. Appl Environ Microbiol 79(5): 1712-1717.

LABBE, G. M., D. D. NIMMO AND L. ALPHEY (2010). *Piggybac*- and PhiC31-mediated genetic transformation of the Asian tiger mosquito, *Aedes albopictus* (Skuse). PLoS Negl Trop Dis 4(8): e788.

LABBÉ, G. M. C., S. SCAIFE, S. A. MORGAN, Z. H. CURTIS AND L. ALPHEY (2012). Female-Specific Flightless (fsRIDL) Phenotype for Control of *Aedes albopictus*. PLoS Negl Trop Dis 6(7): e1724.

LAMARCHE, B. J., N. I. ORAZIO AND M. D. WEITZMAN (2010). The MRN complex in double-strand break repair and telomere maintenance. FEBS Lett 584(17): 3682-3695.

LEENAY, R. T., K. R. MAKSIMCHUK, R. A. SLOTKOWSKI, R. N. AGRAWAL, A. A. GOMAA, A. E. BRINER, R. BARRANGOU AND C. L. BEISEL (2016). Identifying and Visualizing Functional PAM Diversity across CRISPR-Cas Systems. Mol Cell 62(1): 137-147.

LI, F. AND M. J. SCOTT (2016). CRISPR/Cas9-mediated mutagenesis of the white and Sex lethal loci in the invasive pest, *Drosophila suzukii*. Biochemical and Biophysical Research Communications 469(4): 911-916.

LIESCH, J., L. L. BELLANI AND L. B. VOSSHALL (2013). Functional and Genetic Characterization of Neuropeptide Y-Like Receptors in *Aedes aegypti*. PLoS Negl Trop Dis **7**(10): e2486.

LYCETT, G. J., F. C. KAFATOS AND T. G. LOUKERIS (2004). Conditional Expression in the Malaria Mosquito *Anopheles stephensi* With Tet-On and Tet-Off Systems. Genetics 167(4): 1781-1790.

LYND, A. AND G. J. LYCETT (2012). Development of the bi-partite Gal4-UAS system in the African malaria mosquito, *Anopheles gambiae*. PLoS One 7(2): e31552.

MA, S., J. CHANG, X. WANG, Y. LIU, J. ZHANG, W. LU, J. GAO, R. SHI, P. ZHAO AND Q. XIA (2014). CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of BmKu70 in *Bombyx mori*. Sci Rep 4: 4489.

MAEDER, M. L., S. J. LINDER, V. M. CASCIO, Y. FU, Q. H. HO AND J. K. JOUNG (2013). CRISPR RNA-guided activation of endogenous human genes. Nat Methods 10(10): 977-979.

MALI, P., J. AACH, P. B. STRANGES, K. M. ESVELT, M. MOOSBURNER, S. KOSURI, L. YANG AND G. M. CHURCH (2013). CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol 31(9): 833-838.

MALI, P., K. M. ESVELT AND G. M. CHURCH (2013). Cas9 as a versatile tool for engineering biology. Nat Methods 10(10): 957-963.

MANSOUR, W. Y., S. SCHUMACHER, R. ROSSKOPF, T. RHEIN, F. SCHMIDT-PETERSEN, F. GATZEMEIER, F. HAAG, K. BORGMANN, H. WILLERS AND J. DAHM-DAPHI (2008). Hierarchy of nonhomologous end-joining, single-strand annealing and gene conversion at site-directed DNA double-strand breaks. Nucleic Acids Res 36(12): 4088-4098.

MARINOTTI, O., N. JASINSKIENE, A. FAZEKAS, S. SCAIFE, G. FU, S. T. MATTINGLY, K. CHOW, D. M. BROWN, L. ALPHEY AND A. A. JAMES (2013). Development of a population suppression strain of the human malaria vector mosquito, *Anopheles stephensi*. Malar J 12: 142.

MARKERT, M. J., Y. ZHANG, M. S. ENUAMEH, S. M. REPPERT, S. A. WOLFE AND C. MERLIN (2016). Genomic Access to Monarch Migration Using TALEN and

CRISPR/Cas9-Mediated Targeted Mutagenesis. G3: Genes|Genomes|Genetics 6(4): 905-915.

MARRAFFINI, L. A. AND E. J. SONTHEIMER (2010). CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nat Rev Genet 11(3): 181-190.

MARUYAMA, T., S. K. DOUGAN, M. C. TRUTTMANN, A. M. BILATE, J. R. INGRAM AND H. L. PLOEGH (2015). Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotech 33(5): 538-542.

MCMENIMAN, C. J., R. A. CORFAS, B. J. MATTHEWS, S. A. RITCHIE AND L. B. VOSSHALL (2014). Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell 156(5): 1060-1071.

MEREDITH, J. M., S. BASU, D. D. NIMMO, I. LARGET-THIERY, E. L. WARR, A. UNDERHILL, C. C. MCARTHUR, V. CARTER, H. HURD, C. BOURGOUIN AND P. EGGLESTON (2011). Site-specific integration and expression of an anti-malarial gene in transgenic *Anopheles gambiae* significantly reduces Plasmodium infections. PLoS One 6(1): e14587.

MURR, R., J. I. LOIZOU, Y. G. YANG, C. CUENIN, H. LI, Z. Q. WANG AND Z. HERCEG (2006). Histone acetylation by Trrap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. Nat Cell Biol 8(1): 91-99.

NIMMO, D. D., L. ALPHEY, J. M. MEREDITH AND P. EGGLESTON (2006). High efficiency site-specific genetic engineering of the mosquito genome. Insect Mol Biol 15(2): 129-136.

O'BROCHTA, D. A., R. T. ALFORD, K. L. PILITT, C. U. ALUVIHARE AND R. A. HARRELL (2011). *piggyBac* transposon remobilization and enhancer detection in

Anopheles mosquitoes. Proceedings of the National Academy of Sciences 108(39): 16339-16344.

O'BROCHTA, D. A., K. L. PILITT, R. A. HARRELL, 2ND, C. ALUVIHARE AND R. T. ALFORD (2012). Gal4-based enhancer-trapping in the malaria mosquito *Anopheles stephensi*. G3 (Bethesda) 2(11): 1305-1315.

OBERSTEIN, A., A. PARE, L. KAPLAN AND S. SMALL (2005). Site-specific transgenesis by Cre-mediated recombination in *Drosophila*. Nat Methods 2(8): 583-585.

OVERCASH, J. M., A. ARYAN, K. M. MYLES AND Z. N. ADELMAN (2015). Understanding the DNA damage response in order to achieve desired gene editing outcomes in mosquitoes. Chromosome Res 23(1): 31-42.

PALAVESAM, A., C. ESNAULT AND D. A. O'BROCHTA (2013). Post-Integration Silencing of *piggyBac* Transposable Elements in *Aedes aegypti*. PLoS ONE 8(7): e68454.

PASAY, C., L. ARLIAN, M. MORGAN, D. VYSZENSKI-MOHER, A. ROSE, D. HOLT, S. WALTON AND J. MCCARTHY (2008). High-resolution melt analysis for the detection of a mutation associated with permethrin resistance in a population of scabies mites. Med Vet Entomol 22(1): 82-88.

PENG, R., G. LIN AND J. LI (2016). Potential pitfalls of CRISPR/Cas9-mediated genome editing. FEBS Journal 283(7): 1218-1231.

PHUC, H. K., M. H. ANDREASEN, R. S. BURTON, C. VASS, M. J. EPTON, G. PAPE, G. FU, K. C. CONDON, S. SCAIFE, C. A. DONNELLY, P. G. COLEMAN, H. WHITE-COOPER AND L. ALPHEY (2007). Late-acting dominant lethal genetic systems and mosquito control. BMC Biol 5: 11.

PINDER, J., J. SALSMAN AND G. DELLAIRE (2015). Nuclear domain 'knock-in' screen for the evaluation and identification of small molecule enhancers of CRISPR-based genome editing. Nucleic Acids Res 43(19): 9379-9392.

PONDEVILLE, E., N. PUCHOT, J. M. MEREDITH, A. LYND, K. D. VERNICK, G. J. LYCETT, P. EGGLESTON AND C. BOURGOUIN (2014). Efficient ΦC31 integrase–mediated site-specific germline transformation of *Anopheles gambiae*. Nat. Protocols 9(7): 1698-1712.

PORT, F. AND S. L. BULLOCK (2016). Expansion of the CRISPR toolbox in an animal with tRNA-flanked Cas9 and Cpf1 gRNAs. bioRxiv doi: http://dxdoi.org/10.1101/046417

POTTER, C. J. AND L. LUO (2011). Using the Q system in *Drosophila melanogaster*. Nat Protoc 6(8): 1105-1120.

QI, L. S., M. H. LARSON, L. A. GILBERT, J. A. DOUDNA, J. S. WEISSMAN, A. P. ARKIN AND W. A. LIM (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152(5): 1173-1183.

REN, X., J. SUN, B. E. HOUSDEN, Y. HU, C. ROESEL, S. LIN, L. P. LIU, Z. YANG, D. MAO, L. SUN, Q. WU, J. Y. JI, J. XI, S. E. MOHR, J. XU, N. PERRIMON AND J. Q. NI (2013). Optimized gene editing technology for *Drosophila melanogaster* using germ line-specific Cas9. Proc Natl Acad Sci U S A 110(47): 19012-19017.

REN, X., Z. YANG, J. XU, J. SUN, D. MAO, Y. HU, S. J. YANG, H. H. QIAO, X. WANG, Q. HU, P. DENG, L. P. LIU, J. Y. JI, J. B. LI AND J. Q. NI (2014). Enhanced specificity and efficiency of the CRISPR/Cas9 system with optimized sgRNA parameters in *Drosophila*. Cell Rep 9(3): 1151-1162.

REYON, D., S. Q. TSAI, C. KHAYTER, J. A. FODEN, J. D. SANDER AND J. K. JOUNG (2012). FLASH assembly of TALENs for high-throughput genome editing. Nat Biotechnol 30(5): 460-465.

RICHARDSON, C. D., G. J. RAY, M. A. DEWITT, G. L. CURIE AND J. E. CORN (2016). Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotech 34(3): 339-344.

SETHURAMAN, N., M. J. FRASER JR, P. EGGLESTON AND D. A. O'BROCHTA (2007). Post-integration stability of *piggyBac* in *Aedes aegypti*. Insect Biochemistry and Molecular Biology 37(9): 941-951.

SHMAKOV, S., O. O. ABUDAYYEH, K. S. MAKAROVA, Y. I. WOLF, J. S. GOOTENBERG, E. SEMENOVA, L. MINAKHIN, J. JOUNG, S. KONERMANN, K. SEVERINOV, F. ZHANG AND E. V. KOONIN (2015). Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. Mol Cell 60(3): 385-397.

SMITH, MARGARET C. M., WILLIAM R. A. BROWN, ANDREW R. MCEWAN AND PAUL A. ROWLEY (2010). Site-specific recombination by φC31 integrase and other large serine recombinases. Biochemical Society Transactions 38(2): 388-394.

THORPE, H. M. AND M. C. SMITH (1998). *In vitro* site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. Proc Natl Acad Sci U S A 95(10): 5505-5510.

TSAI, S. Q., Z. ZHENG, N. T. NGUYEN, M. LIEBERS, V. V. TOPKAR, V. THAPAR, N. WYVEKENS, C. KHAYTER, A. J. IAFRATE, L. P. LE, M. J. ARYEE AND J. K. JOUNG (2015). GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat Biotech 33(2): 187-197.

VAUCHERET, H. AND M. FAGARD (2001). Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet 17(1): 29-35.

VENKEN, K. J. AND H. J. BELLEN (2012). Genome-wide manipulations of *Drosophila melanogaster* with transposons, Flp recombinase, and PhiC31 integrase. Methods Mol Biol 859: 203-228.

VOLOHONSKY, G., O. TERENZI, J. SOICHOT, D. A. NAUJOKS, T. NOLAN, N. WINDBICHLER, D. KAPPS, A. L. SMIDLER, A. VITTU, G. COSTA, S. STEINERT, E. A. LEVASHINA, S. A. BLANDIN AND E. MAROIS (2015). Tools for *Anopheles gambiae* Transgenesis. G3: Genes|Genomes|Genetics 5(6): 1151-1163.

WANG, T., J. J. WEI, D. M. SABATINI AND E. S. LANDER (2014). Genetic Screens in Human Cells Using the CRISPR-Cas9 System. Science 343(6166): 80-84.

WEI, W., H. XIN, B. ROY, J. DAI, Y. MIAO AND G. GAO (2014). Heritable Genome Editing with CRISPR/Cas9 in the Silkworm, *Bombyx mori*. PLoS ONE 9(7): e101210.

WHO. 2014a. *Dengue and severe dengue* [Online]. World Heath Organization [Accessed 8/13/19].

WHO. 2014b. *Malaria*. [Online].World Health Organization. [Accessed 8/21/19].

WILLIAMS, G. J., M. HAMMEL, S. K. RADHAKRISHNAN, D. RAMSDEN, S. P. LEES-MILLER AND J. A. TAINER (2014). Structural insights into NHEJ: building up an integrated picture of the dynamic DSB repair super complex, one component and interaction at a time. DNA Repair (Amst) 17: 110-120.

WILSON, R., J. ORSETTI, A. D. KLOCKO, C. ALUVIHARE, E. PECKHAM, P. W. ATKINSON, M. J. LEHANE AND D. A. O'BROCHTA (2003). Post-integration

behavior of a *Mos*1 mariner gene vector in *Aedes aegypti*. Insect Biochemistry and Molecular Biology 33(9): 853-863.

WINDBICHLER, N., M. MENICHELLI, P. A. PAPATHANOS, S. B. THYME, H. LI, U. Y. ULGE, B. T. HOVDE, D. BAKER, R. J. MONNAT, A. BURT AND A. CRISANTI (2011). A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. Nature 473(7346): 212-215.

WISE DE VALDEZ, M. R., D. NIMMO, J. BETZ, H. F. GONG, A. A. JAMES, L. ALPHEY AND W. C. T. BLACK (2011). Genetic elimination of dengue vector mosquitoes. Proc Natl Acad Sci U S A 108(12): 4772-4775.

WU, B., L. LUO AND X. J. GAO (2016). Cas9-triggered chain ablation of cas9 as a gene drive brake. Nat Biotech 34(2): 137-138.

WU, X., A. J. KRIZ AND P. A. SHARP (2014). Target specificity of the CRISPR-Cas9 system. Quantitative biology 2(2): 59-70.

WU, X., D. A. SCOTT, A. J. KRIZ, A. C. CHIU, P. D. HSU, D. B. DADON, A. W. CHENG, A. E. TREVINO, S. KONERMANN, S. CHEN, R. JAENISCH, F. ZHANG AND P. A. SHARP (2014). Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. Nat Biotech 32(7): 670-676.

XUE, Z., M. WU, K. WEN, M. REN, L. LONG, X. ZHANG AND G. GAO (2014). CRISPR/Cas9 mediates efficient conditional mutagenesis in *Drosophila*. G3 (Bethesda) 4(11): 2167-2173.

YE, L., Z. YOU, Q. QIAN, Y. ZHANG, J. CHE, J. SONG AND B. ZHONG (2015). TAL effectors mediate high-efficiency transposition of the *piggyBac* transposon in silkworm *Bombyx mori* L. Sci Rep 5: 17172.

YU, C., Y. LIU, T. MA, K. LIU, S. XU, Y. ZHANG, H. LIU, M. LA RUSSA, M. XIE, S. DING AND L. S. QI (2015). Small molecules enhance CRISPR genome editing in pluripotent stem cells. Cell Stem Cell 16(2): 142-147.

ZENG, B., S. ZHAN, Y. WANG, Y. HUANG, J. XU, Q. LIU, Z. LI, Y. HUANG AND A. TAN (2016). Expansion of CRISPR targeting sites in *Bombyx mori*. Insect Biochemistry and Molecular Biology 72: 31-40.

ZETSCHE, B., J. S. GOOTENBERG, O. O. ABUDAYYEH, I. M. SLAYMAKER, K. S. MAKAROVA, P. ESSLETZBICHLER, S. E. VOLZ, J. JOUNG, J. VAN DER OOST, A. REGEV, E. V. KOONIN AND F. ZHANG (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163(3): 759-771.

ZHANG, Y., N. HEIDRICH, B. J. AMPATTU, C. W. GUNDERSON, H. S. SEIFERT, C. SCHOEN, J. VOGEL AND E. J. SONTHEIMER (2013). Processing-independent CRISPR RNAs limit natural transformation in *Neisseria meningitidis*. Mol Cell 50(4): 488-503.

ZHAO, B., V. A. KOKOZA, T. T. SAHA, S. WANG, S. ROY AND A. S. RAIKHEL (2014). Regulation of the gut-specific carboxypeptidase: a study using the binary Gal4/UAS system in the mosquito *Aedes aegypti*. Insect Biochem Mol Biol 54: 1-10.

ZHU, L., H. MON, J. XU, J. M. LEE AND T. KUSAKABE (2015). CRISPR/Cas9-mediated knockout of factors in non-homologous end joining pathway enhances gene targeting in silkworm cells. Scientific Reports 5: 18103.

CHAPTER IV

DEVELOPMENT OF AN END-JOINING SENSOR

INTRODUCTION

Aedes aegypti is a blood feeding mosquito with the capacity to transmit dengue, Zika, yellow fever and chikungunya viruses (WHO, 2018, CDC, 2019). These viruses place a significant health and financial load on many developing countries in tropical and sub-tropical regions of the world. Many of the viruses which are vectored by *Ae. aegypti* currently have no vaccines available with the exception of yellow fever and a limited vaccine for dengue (Gotuzzo et al., 2013, Torresi et al., 2017, Frierson, 2010). While biological, source and insecticide-based control mechanisms are currently utilized, labor and financial costs make these strategies difficult to implement (Huang et al., 2017). An alternative to the use of traditional controls are genetic control techniques to make mosquitoes refractory to viral infection or to reducing mosquito population densities to the point of population collapse that could halt or reduce rates of human arbovirus infection.

. The use of a CRISPR/Cas9 gene drive in *Drosophila melanogaster* spurred research to implement gene drives for vector control of mosquitoes (Gantz and Bier, 2015). Several instances of gene drives which result in population reduction or viral refraction have been achieved in Anopheline mosquitoes (Hammond et al., 2016, Gantz et al., 2015), with gene drive eliminating mosquito populations in small cage trials reported (Kyrou et al., 2018). While models for gene drive in *Ae. aegypti* have been proposed (Adelman and Tu, 2016) no peer reviewed reports have yet been published. Currently, work to expand the number of gene drive components is ongoing (Buchman et al., 2019). Many current and proposed gene drives rely on

the CRISPR/Cas9 system, the DNA double stranded break caused by the Cas9 endonuclease, and the hosts DNA DSB repair pathways propagating genes at hyper-mendelian rates.

The CRISPR Cas9 system was adapted from bacterial antiviral proteins that targeted viral DNA inserted within the bacterial genome (Marraffini and Sontheimer, 2010). Initially, the CRISPR Cas9 system was adapted for use in the dipteran *Drosophila melanogaster* (Bassett and Liu, 2014, Bassett et al., 2013). This system was then quickly adapted for use in *Ae. aegypti* to target genes for knockout and homology directed insertion of marker genes (Basu et al., 2015, Kistler et al., 2015, Dong et al., 2015). The CRISPR/Cas9 system induces double stranded breaks which must be repaired by DSB repair pathways. The experimenter may prefer end joining base repair to generate knockout mutants or the homology directed repair (HDR) pathway to generate knock-in transgenics. Use of the classical non-homologous end joining pathway (C-NHEJ) is believed to be error prone and quickly repairs double stranded breaks with the *ku* complex and *lig4* complex [reviewed in (Williams et al., 2014)] (Figure 4.1).

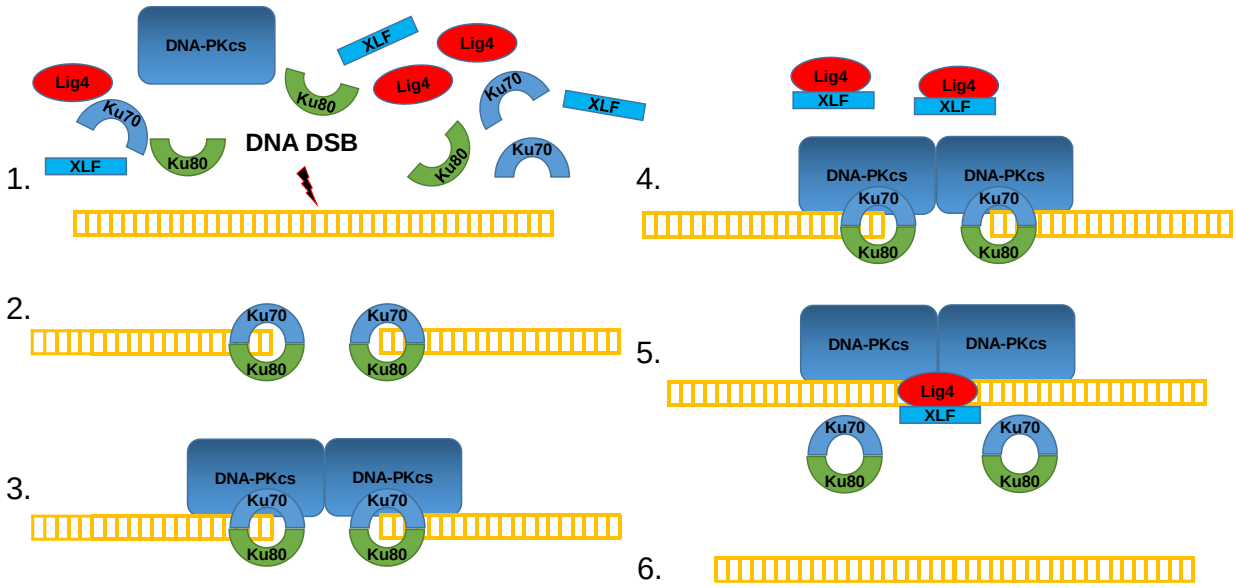

Figure 4.1 Components of the C-NHEJ pathway Critical components of the Classical non-homologous end joining pathway include ku70, ku80, DNA-PKcs, lig4, and XLF. Hypothetical progression of DSB repair via the C-NHEJ pathway in *Aedes aegypti*. Initially a DNA double stranded break occurs, then the ku complex is formed (with Ku70 and Ku80 forming a heterodimer). Afterwords the protein kinase DNA-PKcs is recruited to the break site. Lastly, Lig4 and XLF are recruited to the break site to ligate the broken DNA ends.

In contrast, the HDR pathway utilizes a homologous template to initiate a "perfect repair" [reviewed in (Lamarche et al., 2010)]. Both NHEJ and HDR can be used advantageously in mosquitoes, with NHEJ used to knockout genes and HDR used to insert marker genes or transgenic constructs at highly specific locations (Overcash et al., 2015). To increase rates of homology directed repair, components of the classical non-homologous end joining pathway can be knocked out shifting the DNA repair hierarchy. In insects, knockout of *lig4* in *Drosophila melanogaster* (Beumer et al., 2008) and *ku70* in *Bombyx mori* (Ma et al., 2014) led to significant

increases in HDR and transgene insertion. While quantifying rates homology directed insertion can be utilized to directly measure HDR efficiency, in *Ae. aegypti* the process of generating transgenic lines technical and labor intensive. The list of possible DNA repair genes to target for knockdown or knockout is substantial and will likely grow as new proteins associated with the DNA damage response are discovered (Overcash et al., 2015). This makes identifying gene targets which will generate the greatest shift towards HDR and away from other DNA DSB repair pathways critical. To address this issue we sought to develop a high throughput assay that would allow for the swift determination of end-joining capacity. In cells, both fluorescence and luminescence based assays can be utilized to quantify rates of end joining repair. Given that we were developing an assay that could be utilized both *in vitro* and *in vivo*, we choose to develop a luminescence based assay due to the increased sensitivity and large dynamic range it provides. Luminescence-based assays have been previously used to quantify DNA damage in human cells (Fan et al., 2013). Our lab has also developed a dual luciferase assay to quantify rates of single strand annealing (SSA) in *Ae. aegypti* embryos (Aryan et al., 2013). The SSA pathway is a sub-pathway of homology directed repair pathway, so generating a NHEJ luminescence-based assay would allow us to detect both a shift away from end-joining and a shift towards HDR using a similar assay. We found that an initial version of the assay failed to detect end-joining repair in the embryonic *Ae. aegypti* Aag2 cell line but was able to detect a significant shift in end-joining repair in *Ae. aegypti* embryos (Peleg, 1968, Basu et al., 2015). To generate a more sensitive NHEJ assay, a EGFP-firefly luciferase fusion gene was created with endonuclease cleavage sites located between the two genes. This second assay was substantially more sensitive and was able to quantify shifts in end joining repair in both *Ae. aegypti* embryos and the Aag2 embryonic and alternative larval A20 *Ae. aegypti* cell lines (Peleg, 1968, Pudney et al., 1979).

MATERIALS AND METHODS

NHEJv1 construction: The non-homologous end-joining construct was generated by digesting plasmid pGL3-PUb-FFluc (Anderson et al., 2010) with BsrGI, SphI, XcmI, or AvaI. Oligonucleotides that included the Y2-I-AniI site (ZA2355: 5'GTACATTATTTACAGAGAA ACCTCCTCAAAT3' with ZA2356: 5'GTACATTTGAGGAGGTTTCTC TGTAAATAAT3' at BsrGI; ZA2290: 5' TTATTTACAGAGAAACCTCCTCAAATCATG'3 with ZA2291: 5'ATTTGAGGAGGTTTCTCTGTAAATAACATG'at SphI; ZA2359: 5'AAATAAAT GTCTCTTTGGAGGAGTTTAAAG3' with ZA2360: 5'TTTAAACTCCTCCAAAGAGA CATTTATTTC3' at XcmI; ZA2403: 5'CCGAAATAAATGTCTCTTTGGAGGAGTTTA3' with ZA2404: 5'TCGGTAAACTCCTCCAAAGAGACATTTATT3' at AvaI) were annealed and ligated into their respective restriction enzyme sites of the pGL3-Pub-FF plasmid using T4-DNA ligase and Promega T4-DNA ligase protocol (Promega, Madison, WI).

NHEJv2.0 construction: The EGFP open reading frame was amplified using primers (NHEJ-GFP-F: 5'TTTCAACCATGGTGAGCAAGGGCGAGG3' and NHEJ-GFP-R: 5'TTTTTTCATGAATACCGCGGGATTCTTTCTTGTACAGCTCGTCCATG3'). The resulting amplicon, as well as plasmid pGL3-PUb-FFluc were digested with EcoRI. Linearized plasmid was purified using the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Duren, Germany), and was ligated with the EGFP PCR product using T4-DNA ligase and Promega T4-DNA ligase protocol (Promega, Madison, WI). The sequence of completed plasmid pGL3-EGFP-FFluc or NHEJ transition plasmid (NHEJ-T) was confirmed via sanger sequencing. To generate the final plasmid (NHEJv2.0), two additional oligonucleotides (Ani-IN-F: 5' AATTCCGCGTTATTTAC**AGAGAAACCTCCTCAAATAG**AGTGGAGGTGGCG

GAGGTGGC3' and Ani-IN-R:5'CAC CTCCGCC**ACCTCCACCTCTATTTGAGG**AGGTTT CTCTGTAAATAACGCGG3') were annealed and ligated with T4-DNA ligase (Promega, Madison, WI) into the EcoRI- SacII digested NHEJ-T plasmid to insert AniI/CRISPR/Cas9 binding sites (I-AniI site indicated by underlined sequence, bold letters in forward primer indicates sgRNA A binding site, bold letters in reverse primer indicates binding sgRNA binding site B).The I-AniI target site insert was ligated using T4-DNA ligase and Promega T4-DNA ligase protocol (Promega, Madison, WI). Positive colonies were identified by via PCR for presence of insert. Sequence was confirmed via sanger sequencing.

Cell maintenance: A20 and Aag2 cells were maintained in C02 series 3 incubator (ThermoFisher Scientific, Waltham, MA) at 28℃ in L-15 media [Gibco 1X Leibovitz Media + Gibco 1X TPB (2% final concentration) (BioSciences, Dublin, Ireland) + fetal bovine serum (10% final concentration) (Atlanta Biologicals, Flowery Branch, GA) + penicillin-streptomycin (1% final concentration) (Corning, Corning, NY)]. Mosquito cell lines were passaged twice a week in T-75 flasks. Cells were counted using counted using phase counting chamber (Hausser Scientific Partnership, Horsham, PA). Antibiotic-free media was generated using same components excluding penicillin-streptomycin [Gibco 1X Leibovits Media + Gibco 1X TPB (2% final concentration) (BioSciences, Dublin, Ireland) + fetal bovine serum (10% final concentration) (Atlanta Biologicals, Flowery Branch, GA)].

Dual luciferase Assays: Dual luciferase assays were completed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), with a slightly modified protocol. Passive lysis buffer (PLB) was used to lyse snap-frozen tissue (100µl per 100 embryos or 45 ml per well 96

well plate). After addition of PLB, samples were frozen at -80℃ and allowed to thaw (2X) in -80℃ freezer. Before dual luciferase assay 20µl of each sample was placed in white bottom 96 well plate. Reagents (LARII and Stop&Glo) were removed from -80℃ freezer and allowed to thaw. A volume of 100ul of both LARII and Stop&Glo was allotted for each sample, with an additional 1ml allotted to prime the SpectraMax i3 automatic injector system. The SpectraMax i3 dual injector (Molecular Devices, Jose, CA) was primed with luciferase substrates (LARII and Stop and Glo) loaded in separate reservoirs, and the assay plate was read using the dual luciferase program in SoftMax Pro 6.5.1 software suite [Samples analyzed one at a time, assay protocol: Inject 80ul LARII, 2s delay, 10s read, Inject Stop&Glo, 2s delay, 10s read].

dsRNA Transcription: Exon regions from the genes of interest (*ku70, ku80* and *lig4* were identified (about 500bp) and T7 transcription sites were added to each primer (both 5' and 3') to allow for dsRNA transcription (Ku70-ZA2474: 5'taatacgactcactatagggCATGAGAAACA AGATCATCAG3' and dsKu70R_T7: 5'taatacgactcactatagggATCTTCGTCGGCTACCGTA CA; Ku80-dsKu80F1: 5'taatacgactcactatagggGTACGGCGGGTACCATCATA3' and dsKu80R1: 5'taatacgactcactatagggCGGCTCTGGTAGCTTTCTTC3'; Lig4-dsRNALig4-F1: 5'taatacgactcactatagggCGGCATCCGGGTAAAAACTCTGC3' and dsLig4R1_T7: 5'taatacga ctcactatagggAGCCA TTCCCACGCCAGGTTCC3'; Bgal-Bgal F2 T7: 5'taatacgactcactataggg TGGCAGGCGTTTCGTCAGTATCC3' and BGalr2: 5'taatacgactcactatagggTACCCGTAGGT AGTCACGCA3'). The cDNA used to generate each dsRNA template was generated using Super Script IV VILO Master Mix (ThermoFisher Scientific, Waltham, MA) from RNA extracted from the A20 cell line. Amplification was completed utilizing Phusion Polymerase (NEB, Ipswich MA) [2min 98℃, 30s AT (ku80&Bgal-62℃, ku70-59℃, lig4-72℃), 30s 72℃,

cycle X34, 5min 72℃, hold at 4 ℃] and 1µl cDNA. Amplicons were separated through a 2% agarose gel, excised using a scalpel, and extracted utilizing NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Duren, Germany). Purified amplicons were used as a template to transcribe dsRNA with the MEGAscript RNAi kit (ThermoFisher Scientific, Waltham, MA). The resulting dsRNA products were then purified with the MEGAclear transcription clean up kit (ThermoFisher Scientific, Waltham, MA).

dsRNA Transfections: Six well plates were seeded with 1 million cells per well (2.5 ml of antibiotic free media total), followed by a wait period of 72 hours before transfection. Transfections were performed using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad CA) using dsRNA (3.2µg per well), 125ul OPTIMEM + dsRNA and 125ul OPTIMEM + L2000 (12.5 per well). The transfection mix was allowed to incubate for five minutes, during which time wells were washed with 1ml of PBS and 2ml of antibiotic-free media [Gibco 1X Leibovits Media + Gibco 1X TPB (2% final concentration) (BioSciences, Dublin, Ireland) + fetal bovine serum (10% final concentration) (Atlanta Biologicals, Flowery Branch, GA) was added to each well. After a 5-minute incubation period, L-2000/dsRNA mix was added to each well using a spiral motion. Cells were checked daily and 1ml of media was replaced with antibiotic free media. After 72 hours, the dsRNA transfected cells were harvested from each individual well with 1ml of antibiotic free media. Cells were counted and 200,000 cells were added per well in a 96 well (8 replicates per target gene). Remaining cells were retained for RNA extraction and cDNA synthesis. After cells were seeded plasmids pNHEJv2.0, pPUb-Y2-I-AniI and PUb-Renilla were transfected (100ng per well of each plasmid). Each transfection mix included 100ng of each plasmid diluted in 25 µl OPTIMEM and 25 ul OPTIMEM + L2000 (.5 per well). The

mixture of plasmid and L-2000 was added, mixed and allowed to incubate for 5 minutes at room temperature. After the incubation period, each transfection mix was added to seeded cells (50µl per well). After 48 hours media was removed, and wells were washed with 100µl of PBS. Once PBS was removed, 45ul of passive lysis buffer (Promega, Madison, WI) was added to each well and the plate was allowed to shake for 15 minutes using an advanced vortex mixer (VWR, Radnor, PA). After the shake period cells were placed directly at -80^{o}C for two freeze thaw cycles. After freeze thaw cycles, dual luciferase assays were completed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) immediately using Spectramax i3x (Molecular Devices, Jose, CA) following Promega's protocol and using Molecular Devices dual luciferase assay software.

qPCR: To determine the optimal annealing temperature, qPCR primer sets were run across a gradient of annealing temperatures (55-65˚C). Those primers with clear amplification at 59˚C were given preference as this was the annealing temperature of primers for the housekeeping gene S7. Next, a linear amplification plot was generated through a series of 10-fold dilutions (1/10 – 1/10,0000). Primer amplification efficiency was calculated using the slope of each linear amplification plot, with primers with the best primer amplification efficiency selected for quantification. Simultaneously, Primer sets utilized to quantify the transcript targets in qPCR experiments (ku70-ku70QF2: 5'GTTTCGCTGGGT GTCAACAT3' and ku70QR2: 5'CTTG GCCCGGATTGATCATG3'; Ku80-ku80QF2: 5'AGTCTTCTCGCTCGGAACAA3' and ku80QR2: 5'GTCGCTTTCACTTGCTCCAA3'; Lig4-Lig4QF2: 5'TGTAACGAGCAAGG ACGAGT3' and Lig4QR2: 5'TTTGCTACCTT GGTGTTGGC3'). To complete qPCR (for linear amplification plot and quantification), SsoAdvanced Universal CYBR Green Supermix

(BioRad, Hercules, California) was used in conjunction with 0.66μM of primer mix (final concentration) combined with cDNA template. PCR conditions were: 30s 95˚C, 15s 95˚C, 30s 59˚C cycle X44, Melt Curve 65˚C -95˚C fluorescent read every 0.5˚C for 5s. Each quantitative PCR reaction was completed utilizing the CFX96 Touch and Real-Time PCR Detection System (BioRad, Hercules, California).

RESULTS

Strategies to shift the DSB DNA repair hierarchy towards one outcome (NHEJ or HDR) at the expense of the other may facilitate reverse genetic approaches to either knock-out or knock-in genetic material. In order to detect these shifts in repair outcomes, we sought to develop a luminescence-based NHEJ assay in mosquito cells and embryos. To generate the initial test plasmid, NHEJv1.0, we modified a previously described luciferase expression vector (pGL2-PUb-FFLuc) by adding the recognition site for the homing endonuclease I-AniI within the firefly luciferase open reading frame. We hypothesized that if DSB induction by I-AniI was followed by NHEJ repair, the open reading frame of the firefly luciferase gene would shift, resulting in either frame restoration or disruption depending on the initial configuration (Figure 4.2 A). To identify a region of the firefly ORF that could tolerate the in-frame addition of the 23 bp I-AniI site, a series of modified firefly luciferase expression plasmids was generated through insertion of the I-AniI recognition site into the firefly luciferase open reading frame at several restriction enzyme sites. Transfection of each plasmid into mosquito cells and measurement of the resulting firefly luciferase activity demonstrated that all modified luciferase coding regions had substantially reduced activity compared with the native version (Figure 4.2 B). However, insertion of the I-AniI recognition site into the luciferase BsrGI restriction enzyme site had the

least impact on luciferase activity (Figure 4.2 B). Based on these results, we developed a variant plasmid where insertion of the I-AniI site shifted the firefly luciferase ORF out of frame (NHEJ-FS). Initial tests in Aag2 mosquito cells showed no statistical difference in relative luciferase levels following transfection of PUb-FF, NHEJ-IF or NHEJ-FS in the presence or absence of I-AniI homing endonuclease (Figure 4.2 C). To assess the ability of plasmid NHEJ-FS to detect NHEJ-based repair *in vivo*, NHEJ-FS and NHEJ-IF were injected into wild type *A. aegypti* embryos in the presence or absence of plasmid PUb-Y2-AniI. In contrast to our findings in cultured cells, we detected a decrease in luminescence ($p=0.0079$, Mann-Whitney) in NHEJ-IF in the presence of PUb-Y2-AniI (suggesting indel generation) and a significant increase in luciferase activity from NHEJ-FS in the presence of I-AniI. (Figure 4.2 D, $p=0.0057$, t-test). This showed that the NHEJ-FS plasmid in the presence of I-AniI is able to detect error prone end-joining based repair.

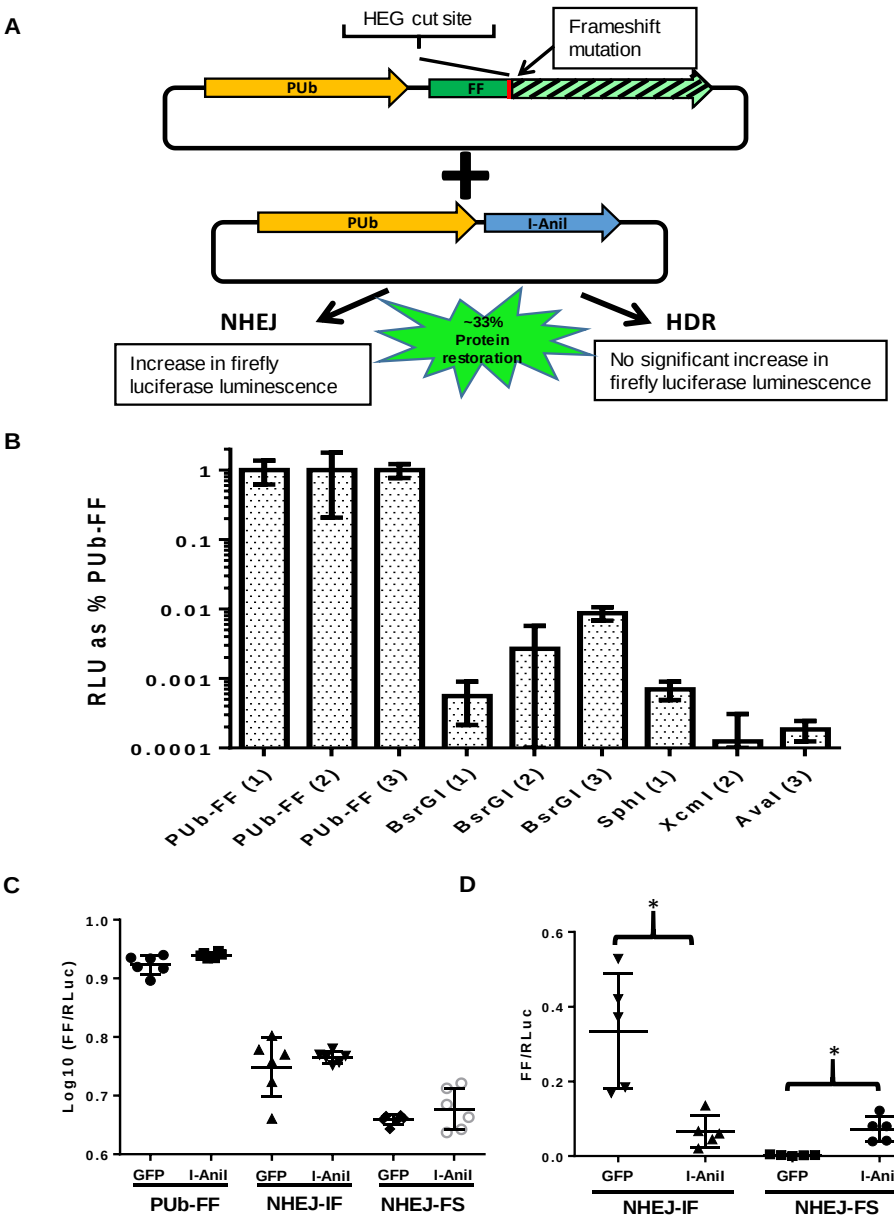

Figure 4.2 Development of an initial plasmid reporter to detect levels of NHEJ in mosquito cells. (**A**) Schematic representation of the NHEJv1.0 plasmid and experimental plan. (**B**) Relative luciferase units (RLU) from Aag2 cells shown as a proportion of the unmodified starting plasmid (PUb-FF). For each test plasmid, numbers in parentheses indicate independent transfection dates. Error bars indicate standard deviation from the mean (n=6). (**C**+**D**) Ratio of firefly (FF) luciferase values by the Renilla luciferase (Rluc) transfection control for the starting construct (PUb-FF) or constructs modified to contain an I-AniI recognition site either in frame (NHEJ-IF) or out-of-frame (NHEJ-FS) after transfection into Aag2 cells (**C**) or following injection into mosquito embryos (**D**).

As our goal was to detect a shift in the DNA repair hierarchy *in vivo*, embryos were injected with the frame shift mutated NHEJ-FS sensor plasmid and dsRNA targeting either the *ku70* or EGFP gene transcripts. In the presence of dsRNA targeting *ku70* there was a statistically lower level of luminescence than wild type LVP embryos, suggesting a shift away from end-joining repair upon knockdown of *ku70* (Figure 4.3). In contrast, knockdown of *lig4* resulted in a significant increase in end joining repair (p=.011, Mann-Whitney).

Despite these initial successes, upon changing luminometers (from the GloMax to the Spectramax i3x), we found that raw firefly luciferase values obtained from the NHEJ-FS sensor (from this point forward referred to as NHEJv1.0), were below the level of detection on the new instrument. Thus, we sought to create a more sensitive end joining assay. To accomplish this, plasmid NHEJv2.0 was generated by fusing the EGFP and firefly luciferase open reading frames, with the target site for I-AniI inserted between the two genes to generate a frameshift prior to the firefly luciferase open reading frame instead of within its open reading frame (Figure 4.4 A).

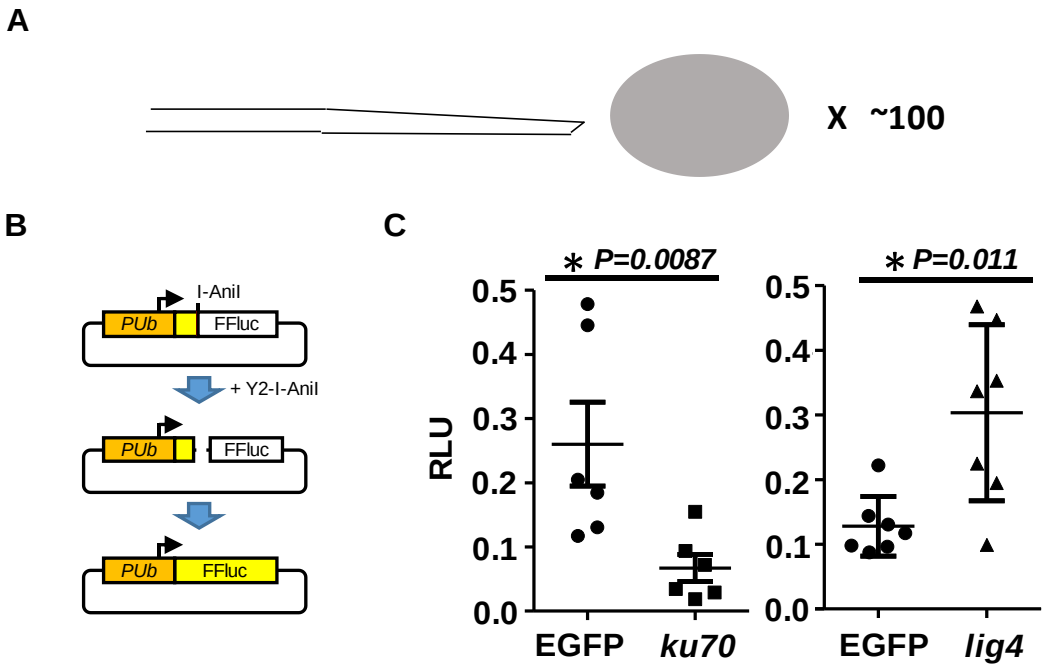

Figure 4.3 Targeting classical non-homologous end joining genes for knockdown to detect shifts in end joining repair. **(A)** *Aedes aegypti* embryos were micro-injected in groups of about 100 with PUb-Renilla, PUb-I-AniI, NHEJ-FS and dsRNA targeting either the control gene EGFP or NHEJ gene (*ku70* or *lig4*). **(B)** Model of the NHEJ assay relies on the I-AniI homing endonuclease to cut an inserted target site, generating indels and restoring the firefly luciferase open reading frame. **(C)** Ratio of firefly (FF) luciferase to Renilla luciferase (Rluc) 48 hours after injection of plasmid mix into *Ae. aegypti* embryos along with the indicated dsRNA. Each point represents ~100 embryos. Horizontal lines indicate mean, error bars indicate standard deviation from the mean (n=6-7) (Adapted from Basu et al. 2015).

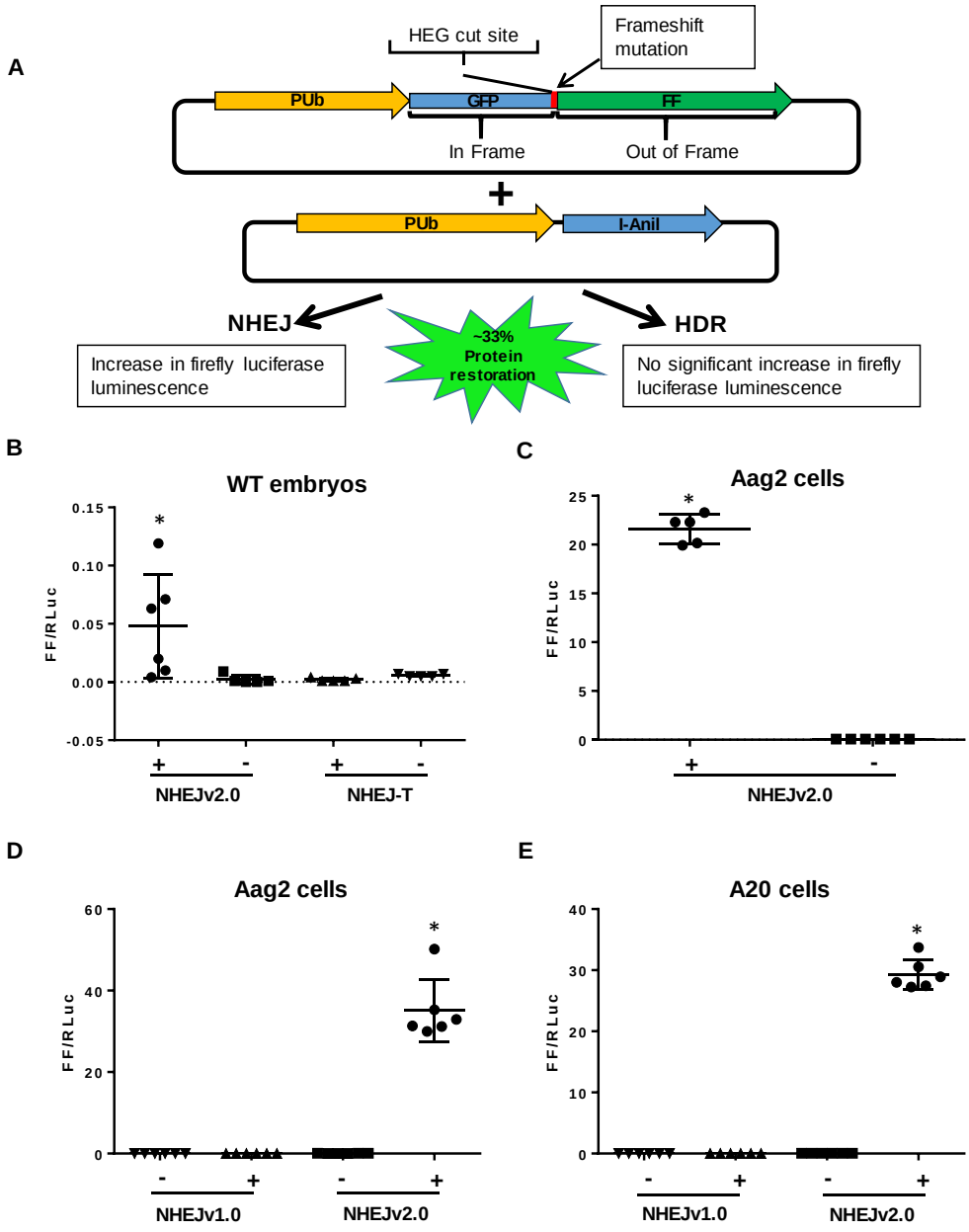

Figure 4.4 A more sensitive report for NHEJ in mosquito cells and embryos. **(A)** Representation of the NHEJv2 plasmid and experimental after DSB expectations after DSB induction by I-AniI. **(B-E)** Horizontal bars represent relative light unit mean and error bars represent standard deviation (n=6) in the presence (+) or absence (-) of PUb-Y2-I-AniI plasmid Ratio of firefly to Renilla luciferase after injecting each test plasmid into *Ae. aegypti* embryos **(B)** or following transfection into cells (**C**). Ratio of firefly to Renilla luciferase of the NHEJv2.0 versus the original version of the NHEJ construct (NHEJv1.0) in the Aag2 **(D)** and A20 **(E)**. Statistical significance is indicated by asterisk (*).

The NHEJv2.0 and NHEJ-T plasmids were injected into *Ae. aegypti* embryos in the presence or absence of PUb-Y2-I-AniI. As compared to NHEJ-T, injection of plasmid NHEJv2.0 resulted in significantly higher rates of luminescence in the presence of PUb-Y2-I-AniI (Figure 4.4 B, p=.0087 Mann-Whitney). Since the NHEJv2.0 plasmid appeared to be more sensitive at detecting end-joining repair in embryos, we assessed its ability to detect NHEJ repair in A20 and Aag2 *Ae. aegypti* cell lines. Transfection of NHEJv2.0 into Aag2 cells with or without PUb-Y2-I-AniI demonstrated an I-AniI-dependent increase in luminescence (Figure 4.4 C, $p<0.0001$ t-test), whereas no such difference was discernable for NHEJv1.0 in either Aag2 (Figure 4.4 D) or A20 (Figure 4.4 E) cells.

Both NHEJv1.0 and NHEJv2.0 end joining sensors are dependent upon the I-AniI homing endonuclease to generate a targeted double stranded break. In addition to the I-AniI binding site we added two sgRNA target sites to the NHEJv2.0 plasmid, designating these sgRNAs as sgRNA A&B. To assess ability to detect a shift in end joining repair by using Cas9 instead of I-AniI, a transgenic *Ae. aegypti* strain expressing maternally provided Cas9 (Li et al., 2017) was injected with plasmid NHEJv2.0 in conjunction with sgRNA A, sgRNA B or sgRNA A&B (Figure 4.5 A). While no significant difference in mean luminescence was observed, there was a significant difference in standard deviation distribution between sgRNA-injected and no sgRNA controls (p=0.0003, Bartlett's test) (Figure 4.5 B).

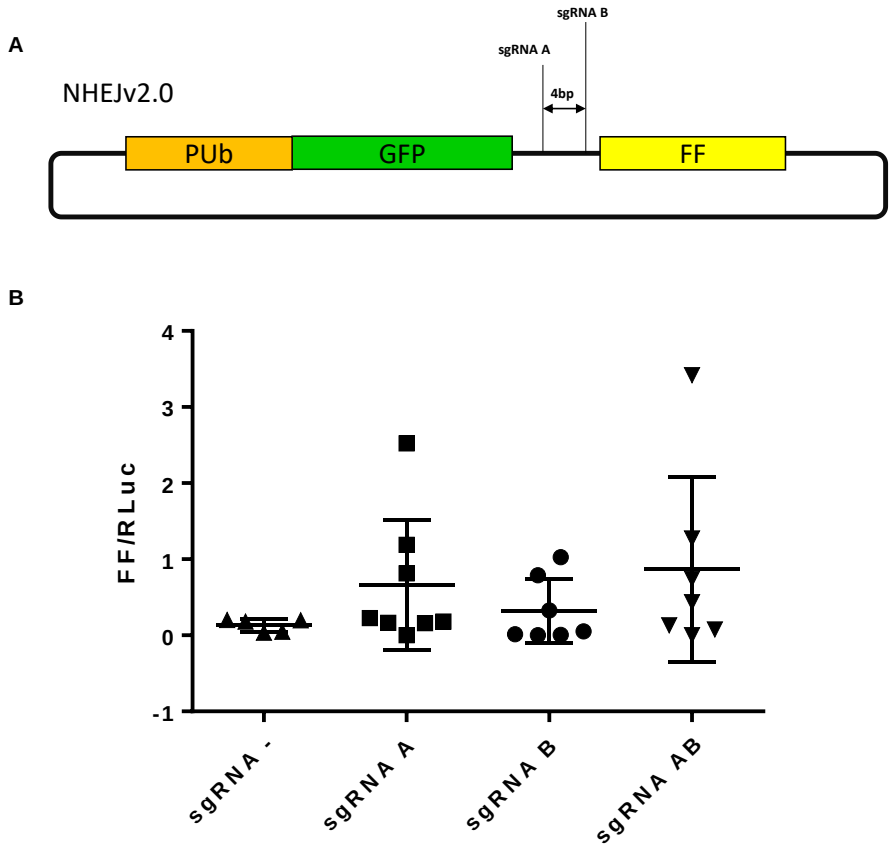

Figure 4.5 NHEJv2.0 can utilize the CRISPR/Cas9 system in place of the homing endonuclease I-AniI. **(A)** Schematic of the location of the sgRNA target sites in the NHEJv2.0 construct. **(B)** Ratio of firefly to Renilla luciferase of *Ae. aegypti* embryos injected with combinations of sgRNA's targeting the NHEJv2.0 plasmid. Each data point represents ~100 embryos. Horizontal lines represent relative light unit mean and error bars indicate standard deviation from the mean.

The ability to detect shifts in end joining repair in cells using the NHEJv2.0 construct reopened the possibility of detecting shifts in end joining repair via RNAi knockdown of C-NHEJ genes in *Ae. aegypti* cell lines. To determine if knockdown of C-NHEJ genes led to a detectable shift in end joining repair, dsRNAs targeting *Bgal* (control), *ku80, ku70* and *lig4* were

transfected into cells, followed by transfection of the NHEJv2.0, PUb-Y2-I-AniI and PUb-Renilla plasmids and measurement of luciferase (Figure 4.6).

Each experiment was performed three times independently for each cell line. In the first transfection in the A20 cell line end joining based repair was significantly different ($p=0.0167$, one-way ANOVA) between target genes with multiple comparisons showing that knockdown of *ku80* significantly increased luminescence as compared to the *Bgal* controls (Figure 4.7 A). In the second transfection, shifts in end joining based repair were also significant ($p=0.001$, one-way ANOVA) with multiple comparisons showing that knockdown of either *ku80* or *ku70* resulted in significantly higher rates of luminescence than the control (Figure 4.7 B).

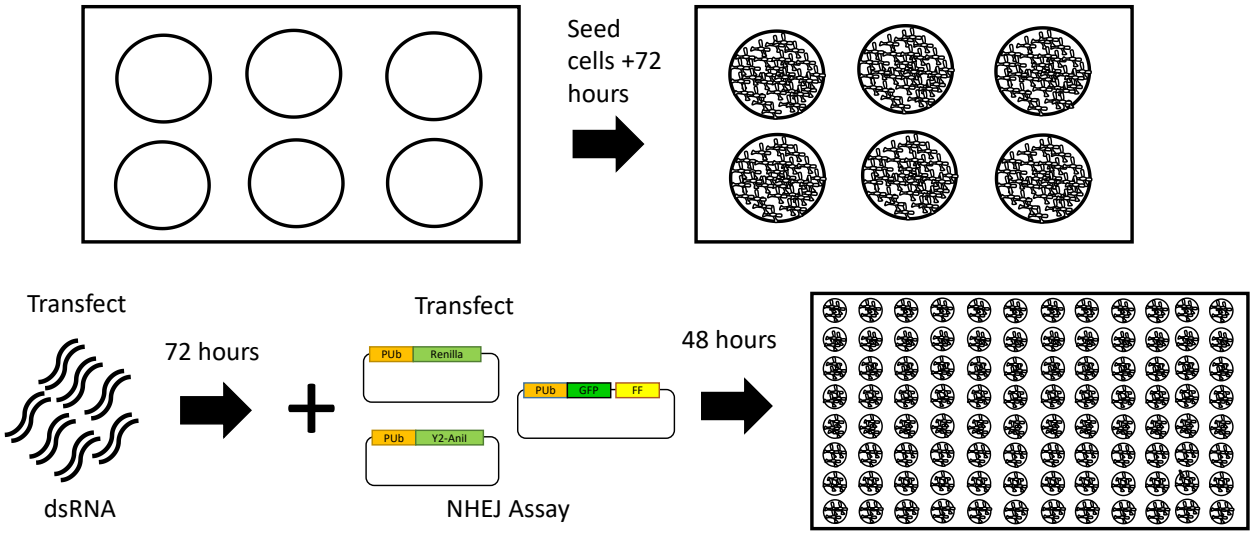

Figure 4.6 Experimental plan for evaluating the NHEJ-based repair in *Ae. aegypti* cells. Cells (Aag2 or A20) were seeded into six well plates. After a 72-hour growth period, cells were transfected with double stranded RNA. After 72 hours cells were seeded into a 96 well plate (8 replicates per transfection) and given 48 hours for luciferase expression (remaining cells were harvested for RNA extraction and cDNA production).

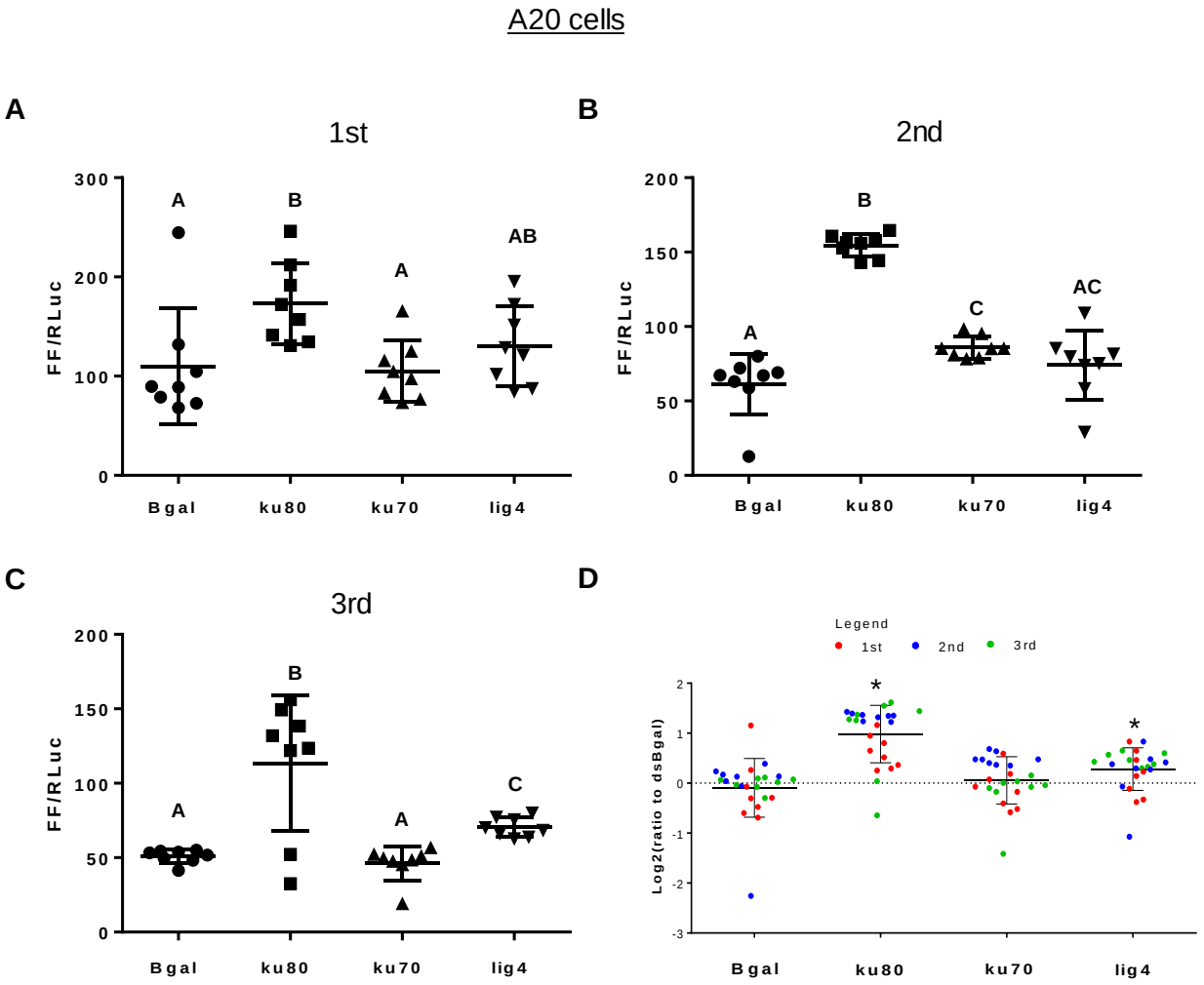

Figure 4.7 Knockdown of C-NHEJ genes *ku80* and *lig4* increases end joining repair in the *Ae. aegypti* A20 cell line. (A-C) Ratio of firefly to Renilla luciferase in A20 cells transfected from three replicate experiments following transfection with the indicated dsRNA. Each data point represents one well from a 96 well plate transfected with plasmid NHEJv2.0 76 hours post dsRNA transfection**.** Horizontal lines represent the mean; error bars indicate standard deviation. **(D)** Values from all three experiments were normalized to dsRNA *Bgal* controls Horizontal bars represent mean relative light units and error bars indicate standard deviation. Asterisks (*) indicate statistical significance by linear multiple effects analysis. Colors indicate data from independent experiments.

In the final replicate, there was a significant difference in end joining based repair ($p=0.0007$, one-way ANOVA) with knockdown of *ku80* resulting in significantly higher levels of luciferase than the control (Figure 4.7 C). When compiling all three replicates by normalizing to the *Bgal* control, transfection of *ku80* double stranded RNA resulted in a marked increase in end joining based repair ($p<0.0001$ LME), transfection of *ku70* dsRNA resulted in no significant change and dsRNA targeting *lig4* resulted a slight but significant increase in end joining based repair ($p=0.01036$ LME) (Figure 4.7 D).

In the first replicate of transfections in the Aag2 cell line, rates of end joining based repair were significantly different (Figure 4.8 B, $p<0.0001$ one-way ANOVA) with multiple comparisons showing knockdown of *ku80* resulted in significantly higher rates of luminescence and knockdown of *lig4* showing significantly lower rates of luminescence in comparison to the *Bgal* control. In the second round of transfections, in contrast to all previous transfections, the *ku80* knockdown group was the only one not significantly different than the *Bgal* controls (Figure 4.8 C, $p<0.0001$ one-way ANOVA). The final experiment was similar to the second, with knockdown of *lig4* resulting in significantly lower rates of luminescence than the control, while knockdown of *ku70* resulted in lower but not significantly different rates of luminescence and *ku80* showing no significant difference (Figure 4.8 D, $p=0.0004$ one-way ANOVA). When data

Aag2 cells

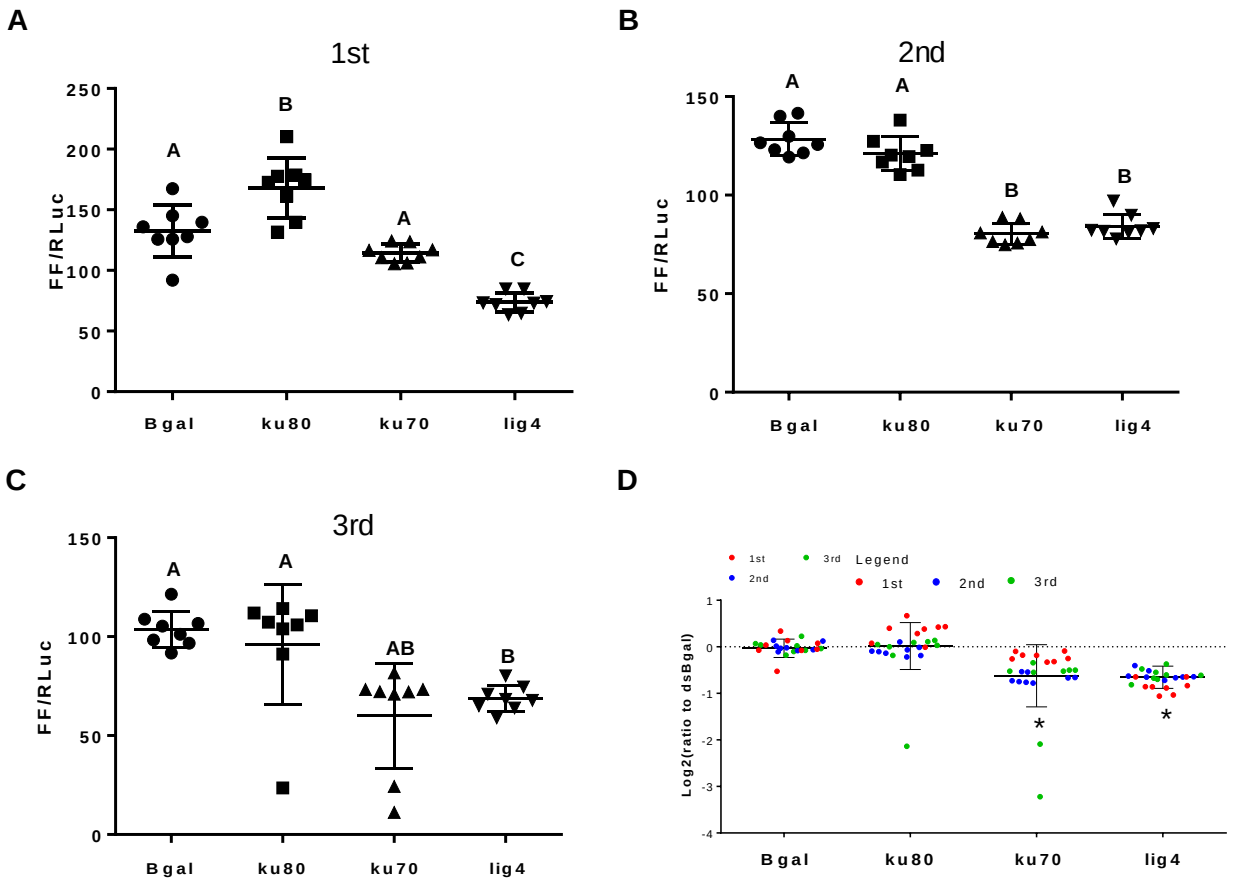

Figure 4.8 Transfecting dsRNA targeting C-NHEJ genes *ku70* and *lig4* result in a decrease in end joining repair in the *Ae. aegypti* Aag2 cell line. **(A-C)** Ratio of firefly to Renilla luciferase in Aag2 cells transfected with dsRNA targeting *ku80*, *ku70*, *lig4* and control *Bgal*. Each data point represents one well from a 96 well plate transfected with plasmid NHEJv2.0 76 hours post dsRNA transfection. Horizontal lines represent relative light unit means and error bars indicate standard deviation. **(D)** Values from all three transfections normalized to dsRNA *Bgal* controls and log2 values graphed to compare biological replicates. Horizontal bars represent mean relative light units and error bars indicate standard deviation. Asterisks (*) indicate statistical significance by linear multiple effects analysis. Colors indicate data from independent experiments.

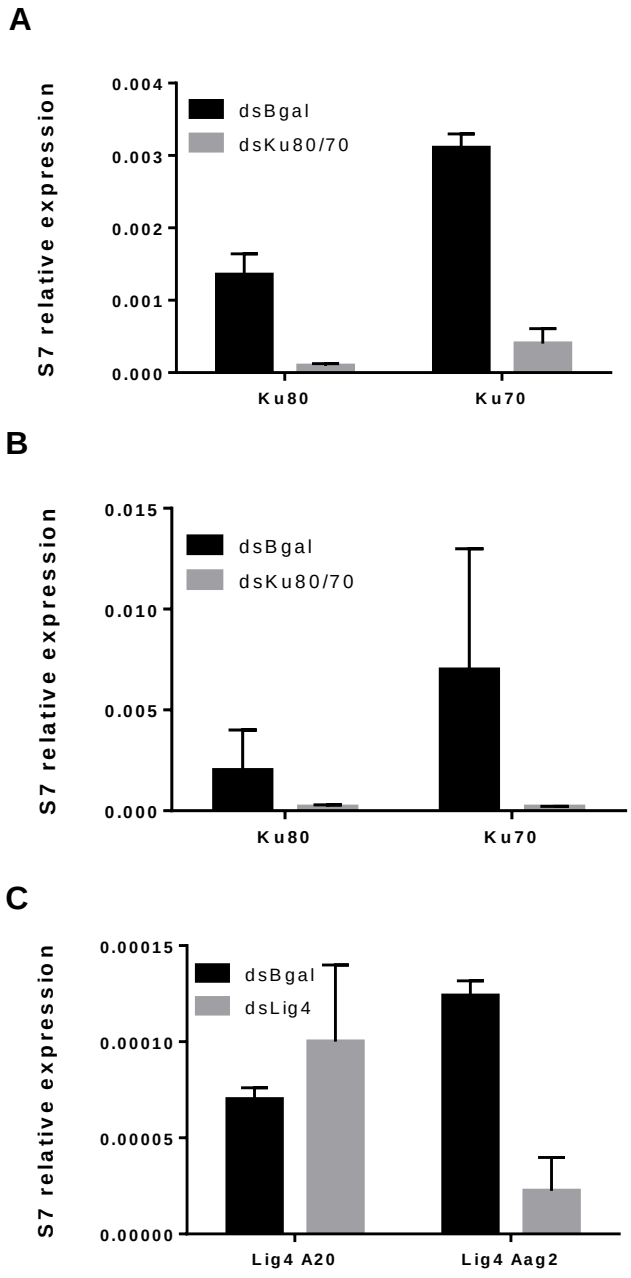

Figure 4.9 S7 relative expression of *ku80*, *ku70* and *lig4*. **(A-C)** Bars are mean values of three replicate experiments, error bars indicate standard deviation. **(A)** S7 relative expression of *ku80* and *ku70* in A20 cells. **(B)** S7 relative expression of *ku80* and *ku70* in Aag2 cells.

from all three experiments were combined, knockdown of *ku80* in resulted in no significant change in end joining based repair, while knockdown of *ku70* ($p<0.0001$ LME) and *lig4* ($p<0.0001$ LME) resulted in a significant decrease in end joining based repair.

To determine how effective the knockdown of *ku80*, *ku70* and *lig4* was, we performed qPCR to determine relative transcript rates between the experimental groups (*ku80*, *ku70* & *lig4*) and the *Bgal* control using cells harvested from each replicate of dsRNA transfections. In A20 cells, a significant reduction was not observed for both the *ku80* ($p=0.12$, multiple t-tests) and *ku70* ($p=0.194$, multiple t-tests) transcripts with respect to the *Bgal* dsRNA transfected controls when normalized to the S7 housekeeping gene (Figure 4.9 A). Calculating rate of knockdown showed that *ku80* displayed 89% knockdown while *ku70* displayed 79% knockdown. In Aag2 cells both the *ku80* ($p>0.00085$, multiple t-tests) and *ku70* ($p=0.0017$, multiple t-tests) transcripts were also significantly reduced with respect to their *Bgal* counterparts when normalized to the S7 housekeeping gene (Figure 4.9 B). Calculating the rate of knockdown showed *ku80* displayed 91% knockdown while *ku70* displayed 85% knockdown. In A20 cells there were high levels of deviation making the results unclear but *lig4* transcripts were significantly reduced ($p=0.00075$, multiple t-tests) in the Aag2 cell line and displayed 80% knockdown (Figure 4.9 C).

DISCUSSION

There have been several reporter systems developed to assay DNA damage (Fan et al., 2013), single strand annealing (Aryan et al., 2013) and overall rates of end joining repair (Bennardo et al., 2008). We developed the NHEJv1.0 assay allowing quantification of end joining repair in *Ae. aegypti* embryos. As was reported in (Basu et al., 2015), we observed a significant reduction in end joining and increase in SSA when using RNAi to target the *ku70* gene. Given that SSA is a sub pathway of HDR (Bhargava et al., 2016) and utilizes the same initial genes Mre11, RAD50 and Nbs1 (Lamarche et al., 2010), we hypothesized that a reduction of end joining, and an increase in SSA may indicate increased utilization of the HDR. Indeed, compared to controls, rates of homology directed gene insertion rose rates of insertion by several fold to (~2.3%) with the inclusion *ku70* dsRNA. In contrast, *lig4* dsRNA resulted in a reduction of SSA and an increase in end joining repair (Basu et al 2015). These results provided evidence that our end-joining sensor could also be used to detect an increase in error prone repair (possibly from A-NHEJ). Thus, the plasmid sensor developed here may be useful in analyzing mosquito components of the A-NHEJ pathway such as the MRN complex, Xrcc1 or PARP1 (Dueva and Iliakis, 2013).

While transitioning labs from Virginia to Texas, we switched luminometers to the SpectraMax i3. Initial microinjections into *ku70*$^{-/-}$ embryos (discussed in the next chapter) resulted in data values below background luminescence suggesting that the NHEJv1.0 assay was not sensitive enough when used in combination with this instrument. The reduced sensitivity of the NHEJv1.0 plasmid was most likely due to the I-AniI binding site disrupting the ability of firefly luciferase protein to fold correctly. The insertion of the I-AniI site also reduced the dynamic range of the assay, making its use in cells and embryos lacking *ku70* limited. To address

these issues, we generated the NHEJv2.0 plasmid by moving the I-AniI binding site upstream of the firefly open reading frame in a GFP-FF fusion protein. The NHEJv2.0 plasmid allowed for measurements of NHEJ activity in both embryos and the Aag2 and A20 cell lines.

Transfection of dsRNAs targeting C-NHEJ genes *ku80* and *lig4* in the A20 cell line resulted in an increase in end joining repair, while knockdown of *ku70* was not different from controls. This contrasts with our prior work where dsRNA targeting *ku70* resulted in a decrease in error-prone end-joining while dsRNA targeting *lig4* resulted in an increase in error prone repair (Basu et al, 2015). In contrast to the A20 cells, knockdown of the same genes in the Aag2 cell line resulted in no change in error prone repair when *ku80* was targeted but decreases in end joining repair when dsRNAs for *ku70* and *lig4* were transfected. While it has been reported that knockdown of *ku80*, *ku70* and *lig4* can result in increased rates of single strand annealing repair (Basu et al, 2015), how knockdown or knockout of these genes impacts A-NHEJ is limited. In human cells it has been reported that the ku complex inhibits A-NHEJ, and the loss of *ku80* resulted in no change in the rate of DNA DSB repair, with DSB-repair relying on microhomology mediated repair (A-NHEJ) (Betermier et al., 2014). In contrast, knockout of *lig4*, *xlf* and *DNA-PKcs* resulted in reduced rates of end joining repair (Fattah et al., 2010). The ku complex may inhibit error prone repair through competition with the MRN complex to bind the ends of double stranded breaks (Shibata et al., 2018, Lamarche et al., 2010). The reason for why the knockdown of *ku80* would increase rates of end joining in the A20 cell line, but not in Aag2 cells is interesting. One possibility for differences in rates of end joining repair is differential expression of proteins which are involved in upstream regulation of DSB repair pathway selection such as ATM, ATR or 53BP1 (Kang and Yan, 2018).

While different rates of end joining repair could be due upstream effector proteins, the observation that knockdown of *ku70* or *ku80* resulted in different effectson the rate of end joining repair within the same cell line could suggest that ku-complex formation had not been interrupted to the same extent in both cases. The ku-complex is highly conserved in eukaryotes and once formed binds double stranded DNA with high affinity [Reviewed in (Downs and Jackson, 2004, Walker et al., 2001)]. In the absence of either Ku80 or Ku70, the complementary protein should not be able to bind to DNA with high affinity. Inability to bind DNA without the complementary ku protein (either Ku70 or Ku80) should result in consistent phenotypes between *ku80* and *ku70* knockdowns. While we analyzed relative transcripts rates of ku80 and ku70 after dsRNA knockdown, we did not determine if the Ku80 and Ku70 proteins were absent. Given that we do not know if the respective proteins were depleted, a difference in half-life between Ku80 and Ku70 proteins could result in the retention of ku-complex units following dsRNA transfection. Any remaining ku-complexes could then compete for DSBs skewing the results of the assay. This question can be addressed by western blots analysis of Ku70 and Ku80 72hrs after transfection with dsRNA to confirm protein depletion.

Lastly, as the Aag2 and A20 cell lines have been maintained in culture for a very long time (>40yrs) distinct preferences for DSB repair pathways may have developed. The SSA, A-NHEJ and HDR repair pathways utilize the MRN complex to initiate resection and compete for DNA ends (Lamarche et al., 2010, Bhargava, et al 2016). Given that cell lines do not require the same number of genes to survive as a multicellular organism, the number of acceptable mutations within *Ae. aegypti* cell lines in likely much larger than in *Ae. aegypti* itself. In combination with the strong selection for rapid cellular replication, tolerance of mutations may have allowed for increased utilization of mutagenic repair pathways such as A-NHEJ instead of

less mutagenic repair pathways such as HDR. The preference for A-NHEJ in the A20 cell line may explain why knockdown of *ku80* results in increased rates of error prone repair, while in the Aag2 cell line it does not. If HDR is less active in the A20 cell line, we would expect overall rates of mutagenic error prone repair to be higher when compared to the Aag2 cell line which corresponds with what we observed. While the differences between error prone repair in the A20 and Aag2 cell line provides insight into competition between DSB pathways and the opportunity to investigate how acquired mutations between the A20 and Aag2 cell lines may result in different DSB repair pathway selection, they do not necessarily reflect DSB repair pathway choice *in vivo*.

While the underlying reasons for the differential effects of *ku70* or *ku80* knockdown on repair between cell lines require further investigation, the data obtained demonstrate that the NHEJv2.0 reporter plasmid can be used to detect shifts in end joining based repair in each of these cell lines. We have previously published a list of suspected genes associated with C-NHEJ, A-NHEJ, SSA and HDR in *Ae. aegypti* (Overcash et al 2015). While the ku complex and *lig4* gene provided initial targets for assessment, we now have the ability to determine which genes associated with both the classical and alternative NHEJ lead to an increase or decrease in end joining repair. The knockdown of *DNA-PKcs, xlf, 53bp1* in both the A20 and Aag2 cells lines should provide more insight into how these genes impact end-joining repair. Additionally, genes associated with A-NHEJ such as *parp1* and *xrcc1*, can be targets to quantify a reduction in error prone repair (Betermier et al., 2014). In conjunction with the NHEJ assay, the previously described SSA assay could further be used to track how targeting C-NHEJ and A-NHEJ genes impacts SSA (Aryan et al., 2013). To more fully elucidate how these repair pathways are

competing for DSBs in cells will require a more comprehensive look than just rates of error prone end joining repair or alternative MRN repair pathways such as SSA or HDR.

As mentioned above, the NHEJv2.0 assay allows for high throughput investigation of DNA repair pathways in *Ae. aegypti* cell lines. While the utilization of CRISPR/Cas9 in *Ae. aegypti* has increased the potential for reverse genetics *in vivo* (Basu et al., 2015, Kistler et al., 2015, Dong et al., 2015), the ability to screen for genes with RNAi in cells narrows the field of potential targets to knockout *in vivo*. In addition to quantifying shifts in end joining repair after knockdown of target genes, determining how chemicals known to impact DNA repair can also be investigated in a high throughput manner. The *lig4* inhibitor SCR7 has been reported to increase rates of HDR in mice (Maruyama et al., 2015), while the PARP1 inhibitor MK4827 has been reported to shift relative mRNA transcript levels of genes associated with DNA double stranded breaks in the absence of the C-NHEJ gene *xrcc4* (Kang and Yan, 2018). Given that DNA double stranded break repair genes are studied for their prevalence/importance in human cancers (Nickoloff et al., 2017), several other molecules have been developed/identified to target genes associated with DNA DSB. The NEHJv2.0 assay can be used to determine if these compounds have a similar impact on shifting end joining repair in *Ae. aegypti*, and may identify potential chemical modulators which can be used to enhance in vitro gene knockout or increase rates of marker gene knock in. The use of DNA damage agents has also shown to shift levels of relative mRNA transcript numbers of DNA DSB related genes (Kang and Yan, 2018). By treating cells with DNA damage inducing agents such as bleomycin (Chen et al., 2008) or doxorubicin, we may be able to screen for genes related to DNA DSB repair genes in *Ae. aegypti* by differential transcriptome analysis.

The results we present in this chapter provide evidence that we generated two separate luminescence-based assays (NHEJv1.0 and NHEJv2.0) which can be used to detect shifts in error prone end joining based repair in *Ae. aegypti* embryos (NHEJv1.0 and NHEJv2.0) as well as in *Ae aegypti* cell lines Aag2 and A20 (NHEJv2.0). Furthermore, the NHEJ-T plasmid can be used as the initial construct for developing alternative versions of the NHEJv2.0 plasmid to investigate alternative DSB mechanisms as well as the resulting type of damage (blunt vs overhang).

REFERENCES

ADELMAN, Z. N. & TU, Z. 2016. Control of Mosquito-Borne Infectious Diseases: Sex and Gene Drive. *Trends Parasitol,* 32**,** 219-29.

ALPHEY, L., MCKEMEY, A., NIMMO, D., NEIRA OVIEDO, M., LACROIX, R., MATZEN, K. & BEECH, C. 2013. Genetic control of *Aedes* mosquitoes. *Pathogens and global health,* 107**,** 170-179.

ANDERSON, M. A. E., GROSS, T. L., MYLES, K. M. & ADELMAN, Z. N. 2010. Validation of novel promoter sequences derived from two endogenous ubiquitin genes in transgenic *Aedes aegypti*. *Insect Molecular Biology,* 19**,** 441-449.

ARYAN, A., ANDERSON, M. A., MYLES, K. M. & ADELMAN, Z. N. 2013. Germline excision of transgenes in *Aedes aegypti* by homing endonucleases. *Sci Rep,* 3**,** 1603.

BASSETT, A. R. & LIU, J. L. 2014. CRISPR/Cas9 and Genome Editing in *Drosophila*. *J Genet Genomics,* 41**,** 7-19.

BASSETT, A. R., TIBBIT, C., PONTING, C. P. & LIU, J. L. 2013. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep,* 4**,** 220-8.

BASU, S., ARYAN, A., OVERCASH, J. M., SAMUEL, G. H., ANDERSON, M. A., DAHLEM, T. J., MYLES, K. M. & ADELMAN, Z. N. 2015. Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. *Proc Natl Acad Sci U S A,* 112**,** 4038-43.

BENNARDO, N., CHENG, A., HUANG, N. & STARK, J. M. 2008. Alternative-NHEJ Is a Mechanistically Distinct Pathway of Mammalian Chromosome Break Repair. *PLOS Genetics,* 4**,** e1000110.

BERNARDINI, F., HAGHIGHAT-KHAH, R. E., GALIZI, R., HAMMOND, A. M., NOLAN, T. & CRISANTI, A. 2018. Molecular tools and genetic markers for the generation of transgenic sexing strains in Anopheline mosquitoes. *Parasites & vectors,* 11**,** 660-660.

BETERMIER, M., BERTRAND, P. & LOPEZ, B. S. 2014. Is non-homologous end-joining really an inherently error-prone process? *PLoS Genet,* 10**,** e1004086.

BEUMER, K. J., TRAUTMAN, J. K., BOZAS, A., LIU, J. L., RUTTER, J., GALL, J. G. & CARROLL, D. 2008. Efficient gene targeting in *Drosophila* by direct embryo injection with zinc-finger nucleases. *Proc Natl Acad Sci U S A,* 105**,** 19821-6.

BHARGAVA, R., ONYANGO, D. O. & STARK, J. M. 2016. Regulation of Single-Strand Annealing and its Role in Genome Maintenance. *Trends in Genetics,* 32**,** 566-575.

BUCHMAN, A., GAMEZ, S., LI, M., ANTOSHECHKIN, I., LI, H.-H., WANG, H.-W., CHEN, C.-H., KLEIN, M. J., DUCHEMIN, J.-B., PARADKAR, P. N. & AKBARI, O. S. 2019. Engineered resistance to Zika virus in transgenic *Aedes aegypti* expressing a polycistronic cluster of synthetic small RNAs. *Proceedings of the National Academy of Sciences,* 116**,** 3656.

CARVALHO, D. O., MCKEMEY, A. R., GARZIERA, L., LACROIX, R., DONNELLY, C. A., ALPHEY, L., MALAVASI, A. & CAPURRO, M. L. 2015. Suppression of a Field Population of *Aedes aegypti* in Brazil by Sustained Release of Transgenic Male Mosquitoes. *PLOS Neglected Tropical Diseases,* 9**,** e0003864.

CDC. 2019. *Dengue and the Aedes aegypti mosquito* [Online]. Centers for Disease Control and Prevention. Available: https://www.cdc.gov/dengue/resources/30jan2012/aegyptifactsheet.pdf [Accessed 4/10/19 2019].

CHEN, J., GHORAI, M. K., KENNEY, G. & STUBBE, J. 2008. Mechanistic studies on bleomycin-mediated DNA damage: multiple binding modes can result in double-stranded DNA cleavage. *Nucleic acids research,* 36**,** 3781-3790.

DONG, S., LIN, J., HELD, N. L., CLEM, R. J., PASSARELLI, A. L. & FRANZ, A. W. 2015. Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti*. *PLoS One,* 10**,** e0122353.

DOWNS, J. A. & JACKSON, S. P. 2004. A means to a DNA end: the many roles of Ku. Nature Reviews Molecular Cell Biology, 5, 367-378.

DUEVA, R. & ILIAKIS, G. 2013. Alternative pathways of non-homologous end joining (NHEJ) in genomic instability and cancer. *Translational Cancer Research,* 2**,** 163-177.

FAN, L., NIU, Y., ZHANG, S., SHI, L., GUO, H., LIU, Y. & ZHANG, R. 2013. Development of a screening system for DNA damage and repair of potential carcinogens based on dual luciferase assay in human HepG2 cell. *Mutagenesis,* 28**,** 515-524.

FATTAH, F., LEE, E. H., WEISENSEL, N., WANG, Y., LICHTER, N. & HENDRICKSON, E. A. 2010. Ku Regulates the Non-Homologous End Joining Pathway Choice of DNA Double-Strand Break Repair in Human Somatic Cells. *PLOS Genetics,* 6**,** e1000855.

FRIERSON, J. G. 2010. The yellow fever vaccine: a history. *Yale J Biol Med,* 83**,** 77-85.

FU, G., LEES, R. S., NIMMO, D., AW, D., JIN, L., GRAY, P., BERENDONK, T. U., WHITE-COOPER, H., SCAIFE, S., KIM PHUC, H., MARINOTTI, O., JASINSKIENE, N., JAMES, A. A. & ALPHEY, L. 2010. Female-specific flightless phenotype for mosquito control. *Proc Natl Acad Sci U S A,* 107**,** 4550-4.

GANTZ, V. M. & BIER, E. 2015. Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science,* 348**,** 442-4.

GANTZ, V. M., JASINSKIENE, N., TATARENKOVA, O., FAZEKAS, A., MACIAS, V. M., BIER, E. & JAMES, A. A. 2015. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proc Natl Acad Sci U S A,* 112**,** E6736-43.

GOTUZZO, E., YACTAYO, S. & CÓRDOVA, E. 2013. Efficacy and duration of immunity after yellow fever vaccination: systematic review on the need for a booster every 10 years. *The American journal of tropical medicine and hygiene,* 89**,** 434-444.

HAMMOND, A., GALIZI, R., KYROU, K., SIMONI, A., SINISCALCHI, C., KATSANOS, D., GRIBBLE, M., BAKER, D., MAROIS, E., RUSSELL, S., BURT, A., WINDBICHLER, N., CRISANTI, A. & NOLAN, T. 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nat Biotechnol,* 34**,** 78-83.

HARRIS, A. F., MCKEMEY, A. R., NIMMO, D., CURTIS, Z., BLACK, I., MORGAN, S. A., OVIEDO, M. N., LACROIX, R., NAISH, N., MORRISON, N. I., COLLADO, A., STEVENSON, J., SCAIFE, S., DAFA'ALLA, T., FU, G., PHILLIPS, C., MILES, A., RADUAN, N., KELLY, N., BEECH, C., DONNELLY, C. A., PETRIE, W. D. & ALPHEY, L. 2012. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. *Nat Biotech,* 30**,** 828-830.

HUANG, Y.-J. S., HIGGS, S. & VANLANDINGHAM, D. L. 2017. Biological Control Strategies for Mosquito Vectors of Arboviruses. *Insects,* 8**,** 21.

KANG, Y.-J. & YAN, C. T. 2018. Regulation of DNA repair in the absence of classical non-homologous end joining. *DNA Repair,* 68**,** 34-40.

KISTLER, K. E., VOSSHALL, L. B. & MATTHEWS, B. J. 2015. Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. *Cell Rep,* 11**,** 51-60.

KYROU, K., HAMMOND, A. M., GALIZI, R., KRANJC, N., BURT, A., BEAGHTON, A. K., NOLAN, T. & CRISANTI, A. 2018. A CRISPR–Cas9 gene drive targeting doublesex causes complete population suppression in caged *Anopheles gambiae* mosquitoes. *Nature Biotechnology,* 36**,** 1062.

LABBÉ, G. M. C., SCAIFE, S., MORGAN, S. A., CURTIS, Z. H. & ALPHEY, L. 2012. Female-Specific Flightless (fsRIDL) Phenotype for Control of *Aedes albopictus*. *PLoS Negl Trop Dis,* 6**,** e1724.

LAMARCHE, B. J., ORAZIO, N. I. & WEITZMAN, M. D. 2010. The MRN complex in double-strand break repair and telomere maintenance. *FEBS Lett,* 584**,** 3682-95.

LI, M., BUI, M., YANG, T., BOWMAN, C. S., WHITE, B. J. & AKBARI, O. S. 2017. Germline Cas9 expression yields highly efficient genome engineering in a major

worldwide disease vector, *Aedes aegypti*. *Proceedings of the National Academy of Sciences,* 114**,** E10540.

MA, S., CHANG, J., WANG, X., LIU, Y., ZHANG, J., LU, W., GAO, J., SHI, R., ZHAO, P. & XIA, Q. 2014. CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of BmKu70 in *Bombyx mori*. *Sci Rep,* 4**,** 4489.

MARRAFFINI, L. A. & SONTHEIMER, E. J. 2010. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet,* 11**,** 181-90.

MARUYAMA, T., DOUGAN, S. K., TRUTTMANN, M. C., BILATE, A. M., INGRAM, J. R. & PLOEGH, H. L. 2015. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotech,* 33**,** 538-542.

NICKOLOFF, J. A., JONES, D., LEE, S.-H., WILLIAMSON, E. A. & HROMAS, R. 2017. Drugging the Cancers Addicted to DNA Repair. *JNCI: Journal of the National Cancer Institute,* 109.

OVERCASH, J. & ADELMAN, Z. N. 2016. Progress in Gene Editing Transgenesis Genome Manipulation in Mosquitoes. *Advances in Insect Physiology,* 51**,** 1-35.

OVERCASH, J. M., ARYAN, A., MYLES, K. M. & ADELMAN, Z. N. 2015. Understanding the DNA damage response in order to achieve desired gene editing outcomes in mosquitoes. *Chromosome Res,* 23**,** 31-42.

PELEG, J. 1968. Growth of arboviruses in monolayers from subcultured mosquito embryo cells. Virology, 35, 617-619.

PHUC, H. K., ANDREASEN, M. H., BURTON, R. S., VASS, C., EPTON, M. J., PAPE, G., FU, G., CONDON, K. C., SCAIFE, S., DONNELLY, C. A., COLEMAN, P. G.,

WHITE-COOPER, H. & ALPHEY, L. 2007. Late-acting dominant lethal genetic systems and mosquito control. *BMC Biol,* 5**,** 11.

PUDNEY, M., VARMA, M. G. R. & LEAKE, C. J. 1979. Establishment of cell lines from larvae of culicine (Aedes species) and anopheline mosquitoes. TCA manual / Tissue Culture Association, 5, 997-1002.

SHIBATA, A., JEGGO, P. & LÖBRICH, M. 2018. The pendulum of the Ku-Ku clock. *DNA Repair,* 71**,** 164-171.

TORRESI, J., EBERT, G. & PELLEGRINI, M. 2017. Vaccines licensed and in clinical trials for the prevention of dengue. *Human vaccines & immunotherapeutics,* 13**,** 1059-1072.

WALKER, J. R., CORPINA, R. A. & GOLDBERG, J. 2001. Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. Nature, 412, 607-614.

WHO. 2018. *Dungue and severe dengue* [Online]. World Health Organization. Available: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue [Accessed 4/10/19 2019].

WILLIAMS, G. J., HAMMEL, M., RADHAKRISHNAN, S. K., RAMSDEN, D., LEES-MILLER, S. P. & TAINER, J. A. 2014. Structural insights into NHEJ: building up an integrated picture of the dynamic DSB repair super complex, one component and interaction at a time. *DNA Repair (Amst),* 17**,** 110-20.

CHAPTER V

KNOCKOUT OF THE OF C-NHEJ GENES LIG4 AND KU70 IN THE DISEASE VECTOR *AEDES AEGYPTI*

INTRODUCTION

Aedes aegypti is a crucial vector of dengue, chikungunya and Zika viruses (WHO, 2018, CDC, 2019). These viruses cause mortality and morbidity in developing countries in tropical and subtropical regions. Current mechanisms of control include biological control, source reduction and the use of insecticides (Huang et al., 2017). In conjunction with the cost and labor required for current control methods, the inability to vaccinate populations for most of the viruses (with the exception of yellow fever) makes novel genetic control mechanisms an attractive alternative (Frierson, 2010, Torresi et al., 2017).

The Cas9 endonuclease was identified in bacteria (*Streptococcus pyogenes*) and plays a role in anti-viral defense (Karginov and Hannon, 2010). The Cas9 protein targets viral DNA sequences and produces a double stranded break (DSB) to disrupt the DNA sequence (Sander and Joung, 2014). The CRISRP/Cas9 system has been adopted for gene editing in several insect species including *Bombyx mori* (Wei et al., 2014), *Danaus plexippus* (Markert et al., 2016), *Tribolium castaneum* (Gilles et al., 2015), *Drosophila melanogaster* (Bassett et al., 2013), *Anophelines* (Gantz et al., 2015, Hammond et al., 2016) and *Aedes aegypti* (Basu et al., 2015, Kistler et al., 2015, Dong et al., 2015). Genome editing with the CRISPR/Cas9 system relies on creation of indels, small insertions or deletions, by error prone repair of an end-joining pathway (Williams et al., 2014, Betermier et al., 2014) or insertion of a sequence by homology directed repair (HDR) (Lamarche et al., 2010). Use of HDR to insert marker genes within a gene of

interest to disrupt transcription enhances basic research by reducing the workload required to track genes with no easily observable phenotype. Using HDR to insert markers at highly specific genomic locations is limited by the rate of construct integration. Competition for the DNA DSB between the classical non-homologous end joining pathway (C-NHEJ) and the MRN complex, which is required to initiate HDR, is one reason integration events are rare (Chang et al., 2017). Shifting the DNA damage response to increase rates HDR would be advantageous for expediting basic science in *Ae. aegypti* and provide potential insight into how to enhance future gene drive-based genetic control strategies.

By transiently or permanently removing components of the C-NHEJ pathway, rates of homology directed insertion were increased from 15 to 70% in *Drosophila melanogaster* and from less than 1% to 1.7-2.7% in *Ae. aegypti* (Basu et al., 2015, Beumer et al., 2008,). This makes the generation of mosquito strains deficient in components of the C-NHEJ an attractive proposition for facilitating site-specific gene insertion. C-NHEJ is carried out by proteins including Ku70, Ku80, Lig4 and DNA-PKcs [reviewed in (Chang et al., 2017, Williams et al., 2014)]. *Aedes aegypti* encodes orthologs for many of the components of the NHEJ pathway (Overcash et al., 2015) and the genes *ku70*, *ku80* and *lig4* have previously been knockdown to shift the DNA damage response in *Ae. aegypti* (Basu et al., 2015). Several components of the C-NHEJ pathway, including *ku70*, *ku80* and *lig4*, have been studied in the dipteran *Drosophila melanogaster* (Cenci et al., 2005, Ciapponi et al., 2004, Boutanaev et al., 2007, Beumer et al., 2008).

Deficiency of *lig4* and *ku70* can result in a range of deleterious phenotypes dependent upon the host organism. In mice, loss of *lig4* causes embryonic lethality (Karanjawala et al., 2002), while in *Drosophila melanogaster* and *Arabidopsis thaliana* this leads to an increase in

rates of HDR (Beumer et al., 2008, Qi et al., 2013). Similarly, in mice the loss of *ku70* results in premature aging (Li et al., 2007), while in *Bombyx mori* and *Arabidopsis thaliana* loss of *ku70* led to an increase in rates of HDR (Ma et al., 2014, Qi et al., 2013). Given that knockout of *lig4* and *ku70* in *Ae. aegypti* leads to increased rates of homology directed repair in the early embryo, we considered them good initial targets modifying repair choice to stimulate transgene integration in *Ae. aegypti* (Basu et al., 2015).

To attempt to more effectively increase rates of homology directed repair, we targeted the C-NHEJ genes *lig4* and *ku70* with the CRISPR/Cas9 system. By microinjecting *Ae. aegypti* embryos with Cas9 and sgRNAs targeting *lig4* and *ku70,* we were able to generate frameshift mutations in both of these genes. Of these, detailed characterization of a homozygous $ku70^{-/-}$ strain of *Ae. aegypti* showed female specific pupal lethality, no significant difference in fecundity and fertility, reduced life span and delayed chorion melanization. This suggests that the removal $ku70^{-/-}$ of impacts the fitness of the mosquito at multiple points in the life cycle.

MATERIALS AND METHODS

Mosquito rearing and maintenance: Mosquitoes were reared at 28˚C, 60-70% humidity and 10/14 dark light cycle. Mosquito larvae were reared in 4 liters of RO H_2O with ~400 larvae per pan. Larvae were given finely ground Tetra fish food (~1/8 teaspoon a day) (Tetra, Blacksburg, VA). Adult females were blood feed on defibrinated sheep's blood warmed to 37˚C (LVP on parafilm, *ku70* on hog-gut to entice feeding) (Colorado Serum Co., Denver, CO).

Ku70 and Lig4 knockout mutants: Micro-injection of *Ae. aegypti* embryos was completed using the protocol described in (Aryan et al., 2014). Adult females were blood fed three to four days prior to embryo collection. Gravid female mosquitoes were collected from containers and placed in 50ml conical tubes containing a cotton ball saturated with RO water topped with chromatography paper for egg deposition. Conical tubes containing females were moved to a dark container for 45minutes to encourage egg deposition. After 45 minute egg deposition period, females were returned to their container. Embryos were lined up and injected before their chorions fully darkened. Injection needles were created using 4" long, 1.00mm outside diameter borosilicate glass capillaries (World precision instruments, Sarasota, FL) and pulled using Sutter micropipette puller P-2000 (Sutter Instrument, Novato, CA) using settings listed: HE-270, FIL-3-, VEL-37, DEL-250, PUL-140. Embryos were injected using Eppendorf FemtoJet 4i (Eppendorf, Hamburg, Germany) under a Leica DM-1000 microscope (Leica Microsystems, Wetzlar Germany). Injection mixes consisted of Cas9 protein from PNABio (PNABio, Newbury Park, CA) previously aliquoted at 2ug/ul and guide RNAs. Cas9 protein was diluted to the concentration of 400ng/ul using DEPC-treated H20 and mixed with guide RNAs at 100ng/µl, then allowed to incubate at 37˚C for 20m. Guide RNAs used in injections were synthesized using

primers: rev ZA2267: 5'AAAAGCACCGACTCGGTGC CACTTTTTCAAGTTGATAACGGACTAGCC TTATTTTAACTTGCTATTTCTAGC TCTAAAAC3', KU70#3 ZA2487: 5'GAAATT AATACGACTCACTATAGGAACGAT GAGCCGCATTCGGCGTTTTAGAGCTAGAAA TAGC3', Lig4#7 ZA2463: 5'GAAA TTAATACGACTCACTATAGGGATGCGCGGAC GTTGCCCGGGTTTTAGAG CTAGAAATAGC3'. Guide RNAs were synthesized as reported in (Bassett et al., 2013). Guide RNA transcription was completed using Megascript T7 kit (ThermoFisher Scientific, Waltham, MA) and purified using MEGAclear kit (ThermoFisher Scientific, Waltham, MA). Injections targeting *ku70* and *lig4* genes were completed by Dr. Azadeh Aryan (Figure 5.1).

Genotyping & High Resolution Melt Curve Analysis: Genotyping was completed through gene amplification and high resolution melt curve analysis (HRMA) of amplicon products. Amplicons were generated using Phire Animal Tissue Direct PCR kit (ThermoFisher Scientific, Waltham, MA) and LcGreen (BioFire Defense, Salt Lake City, UT). Tissue samples were collected for genotyping by removing a single hind leg from adult mosquitoes and placing in Phire Animal Tissue PCR Buffer. The mosquitoes were then moved into individual *Drosophila* vials until completion of HRMA. Tissue samples in Phire Animal Tissue PCR Buffer were incubated at 98°C for 2m prior to setting up PCR. The PCRs were set up using the protocol described in (Basu et al 2015) and run in a BioRad C-1000 thermal cycler [PCR: 98°C 5min, 98°C 10s, AT 30s, 72°C 30s, 72°C 2min; AT: *ku70* -60°C, *lig4*-69°C]. Primers for amplicon reaction: [*ku70*: ZA2481: 5'ATCCAGCATTGTCCTGTTC3'; ZA2483: 5'ATCAAAACTATGT TGATGTCCAG3'; *lig4*: ZA2469: 5'GGTAGCAATGGGAGCAGCG3'; ZA2470: 5' ATGAGCTCGTCCTGCGTTT3']. HRMA was completed using the Idaho Technology Inc.

Light Scanner (Idaho Technology Inc., Salt Lake City, UT). Individuals with melt curves unique to controls were then transferred from individual tubes to secondary containers for back-crossing. Outcrossed groups were genotyped via HRMA every generation to retain mutations and set up future crosses.

Pupae Survival Assay: When female larvae initiated pupation, they were sequestered into groups every two hours over a 14hr period. Over a three-day period, lethality of each group of female pupae groups was recorded every two hours.

Longevity Assays: Female and male mosquito groups were placed separately in soup cup containers. Each container was marked with the day of emergence, and groups of ten adults (either male or females) were moved to new soup cup containers. Adults mosquitoes were restricted to groups which emerged within the same 24hr period. Adult mosquitoes were given 10% sucrose and checked for lethality daily, lethality was scored based on lack of movement and irregular body shape due to desiccation.

Fecundity Assays: Adult females were given a blood meal four to seven days post emergence. In the first replicate, *ku70*$^{-/-}$ females were only fed once. In the second replicate *ku70*$^{-/-}$ females were offered a bloodmeal on three consecutive days to compensate for poor feeding success. Twenty-four hours after the blood meal, replete females were moved to a separate container with WT males. Four days post bloodmeal, females were moved to individual containers for laying embryos. After twenty-four hours, female mosquitoes were removed from the tubes, and embryos were counted two to three days after being deposited. Five days after laying, reverse-

osmosis purified H2O and a few drops of diluted fish food (Tetra, Blacksburg, VA) were added to tubes containing embryos. Embryos were given two to four days to hatch at which point larvae were counted.

Embryo development assays: Crosses were set up with fifty females per group crossed with fifteen males, genotypes crossed: LVP♀ x LVP♂, LVP♀ x *ku70*$^{-/-}$♂, *ku70*$^{-/-}$♀ x LVP♂ and *ku70*$^{-/-}$♀ x *ku70*$^{-/-}$♂. Four to seven days after emergence, females were blood fed for 20 minutes through a hog gut membrane instead of parafilm. After a three-day period for vitellogenesis for LVP females, and four-day vitellogenesis period for *ku70*$^{-/-}$ females, 5-10 females were placed in a tube for 45 minutes to deposit embryos. After the 45-minute laying period, females were moved back into their containers and fifty random embryos were selected and positioned for photographs. Photographs of embryos were taken using AmScope Mu300 (AmScope, Irvine, CA). Photographs were taken at 1, 1.5, 2, 2.5, and 3 hours after the initiation of oviposition. Embryo pigmentation was analyzed using Fiji Image J (Laboratory for Optical and Computational Instrumentation, Madison, WI).

RESULTS

To determine if removal of C-NHEJ repair genes could increase rates of HDR in *Ae. aegypti*, we utilized the CRISPR Cas9 system to generate loss-of-function mutations in the *ku70* and *lig4* genes (Figure 5.1 A). Micro-injection of CRISPR/Cas9 protein and guide RNAs embryos targeting the *ku70* and *lig4* genes into *Ae. aegypti* resulted in mutations that were detected by high resolution melt curve analysis (HRMA) (Figure 5.1 B, C). Amplicons bearing suspected mutations were sequenced (Figure 5.1 D). Several *lig4* mutants were generated with CRISPR/Cas9 microinjections and identified via HRMA (Figure 5.2 A). From the individuals selected from HRMA two distinct mutant groups appeared, one which was sex linked, suggesting that it is on the first chromosome of *Ae. aegypti* (Chromosome 1 carries the sex determining M locus) and the second was not sex linked. The identification of two distinct mutant groups matched the predicted number of *lig4* gene copies present in the third assembly of the *Ae. aegypti* genome. A 5 bp deletion ($M^{\Delta 5}m$) was detected on the male first chromosome with sex linkage determined by rates of inheritance (100% of male progeny retaining the

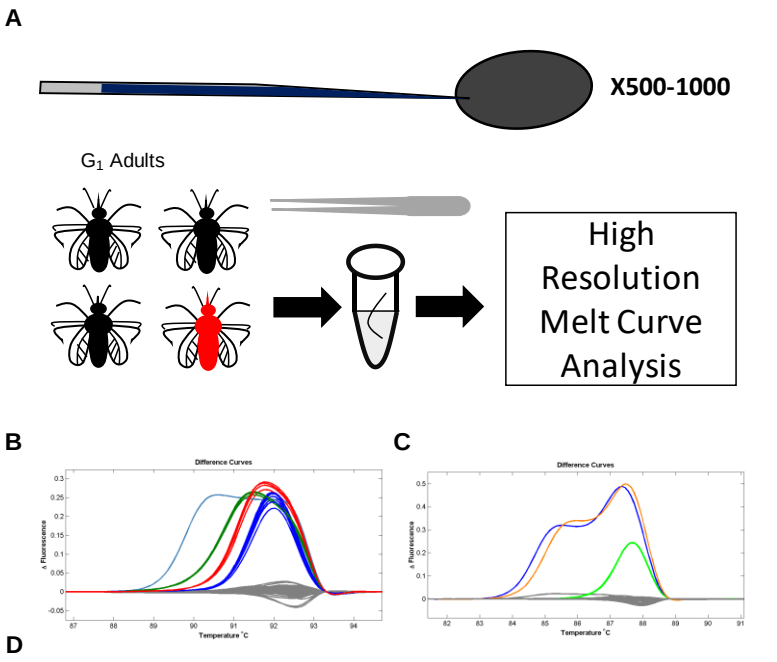

Figure 5.1 CRISPR/Cas9 generated *lig4* and *ku70* knockout mutants. **(A)** Embryos were injected with Cas9 targeting either *lig4* or *ku70*. Offspring from surviving individuals were genotyped by high resolution melt curve analysis (HRMA). **(B&C)** Melt curves with a difference in fluorescence (red, green, blue) from controls (gray) were used to identify potential *lig4* **(B)** and *ku70* **(C)** mutants. **(D)** Sequence of wild-type (WT) and *ku70*$^{\Delta 10}$ (M5) and *ku70*$^{\Delta 1}$ (M2) mutations.

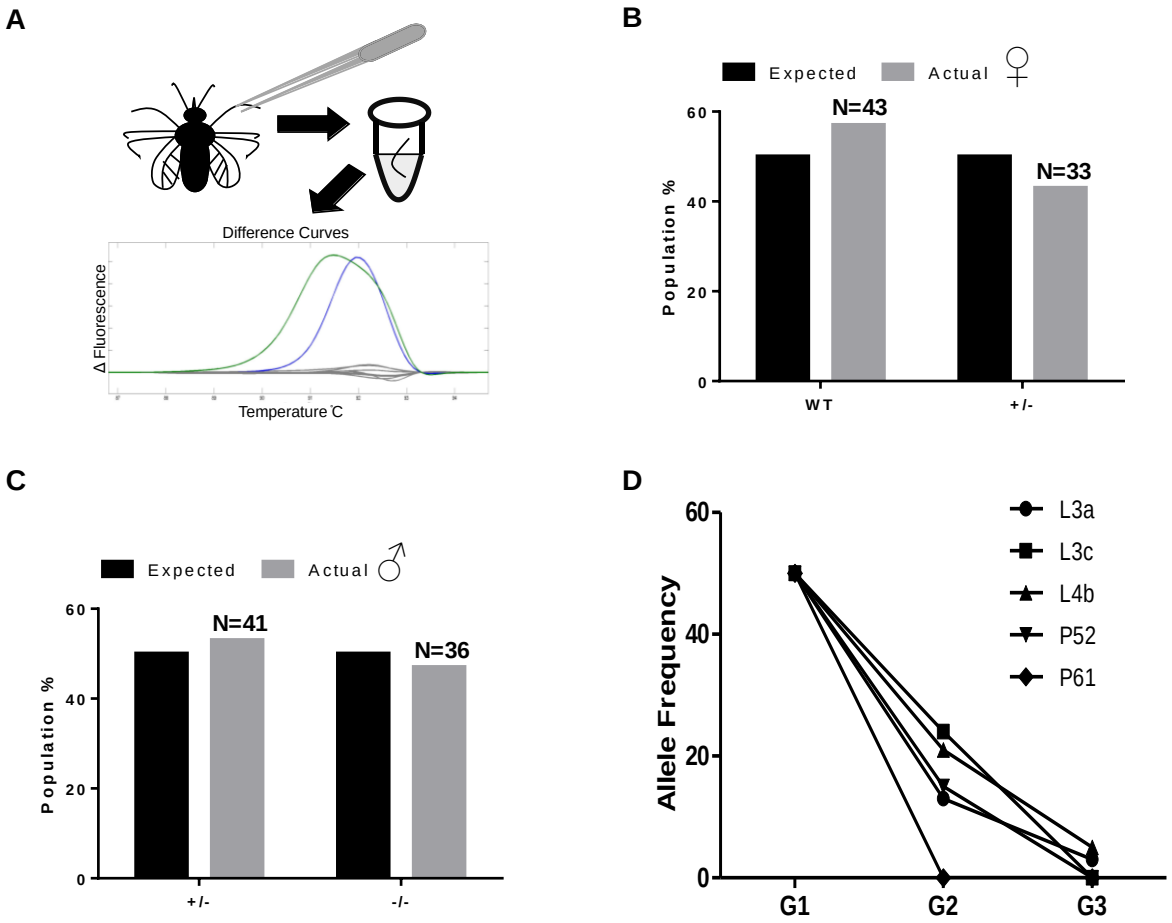

Figure 5.2 Two different *lig4* mutant groups display distinct phenotypes. **(A)** Schematic of genotyping via HRMA to identify mutant groups. **(B+C)** Sex linked heterozygous *lig4* mutants were self-crossed. Progeny of the self-cross were genotyped and rates of female **(B)** and male **(C)** genotype distributions were tabulated. **(D)** Non-sex linked *lig4* mutants were tracked over several generations via active selection of genotype by HRMA for outcrossing. The legend identifies different *lig4* frameshift mutations which were tracked.

mutation and no female progeny inheriting mutation). A second strain contained a 14 bp insertion (mm^{I14}) that was identified as linked to the female chromosome one. Sex linkage was determined by rates of inheritance with inheritance rates dependent on the sex of the parent. By crossing the mutants $M^{\Delta 5}m$ and mm^{I14} we were able to generate heterozygous (mm^{I14}) females (Figure 5.2 B, p=0.3950 Chi-square), heterozygous ($M^{\Delta 5}m$) males and homozygous ($M^{\Delta 5}m^{I14}$) males at expected mendelian rates (Figure 5.2 C, p=0.7773 Chi-square). In contrast, mutant strains which were not sex linked were progressively reduced within the population and had completely disappeared by the third generation (Figure 5.2 D, Basu et al.,2015).

Similarly to *lig4*, we identified two mutations in the *ku70* gene predicted to result in a premature stop codon. These mutations were identified as a 1 base pair and 10 base pair deletion ($ku70^{\Delta 1/+}$ and $ku70^{\Delta 10/+}$). As each *ku70* mutant strain was derived from a single founder female, both were backcrossed to the parental strain for five generations to reduce fitness costs associated with the associated genetic bottleneck. This also served to reduce the potential impact of off-target mutations induced by the CRISPR/Cas9 complex.

During the process of backcrossing, a subgroup of the third-generation *ku70* mutants ($ku70^{\Delta 1/+}$ and $ku70^{\Delta 10/+}$) were self-crossed to monitor rates of genotype distribution. The progeny were genotyped via HRMA as adults to assess the relative proportions of wild type ($ku70^{+/+}$, heterozygote ($ku70^{\Delta 1/+}$ and $ku70^{\Delta 10/+}$) and transheterozygote ($ku70^{\Delta 1/\Delta 10}$) genotypes within the population (Figure 5.3 A). Progeny of the $ku70^{\Delta 1/+}$ x $ku70^{\Delta 10/+}$ cross showed statistically lower proportion of transheterozygote genotypes than expected (Figure 5.3 B, $p<0.0001$, Chi-square). This observation led us to suspect a potential fitness cost associated with the loss of *ku70*. To confirm these results and determine if the lack of $ku70^{\Delta 1/\Delta 10}$ transheterozygous individuals was consistent amongst males and females, the intercross between $ku70^{\Delta 1/+}$ and $ku70^{\Delta 10/+}$

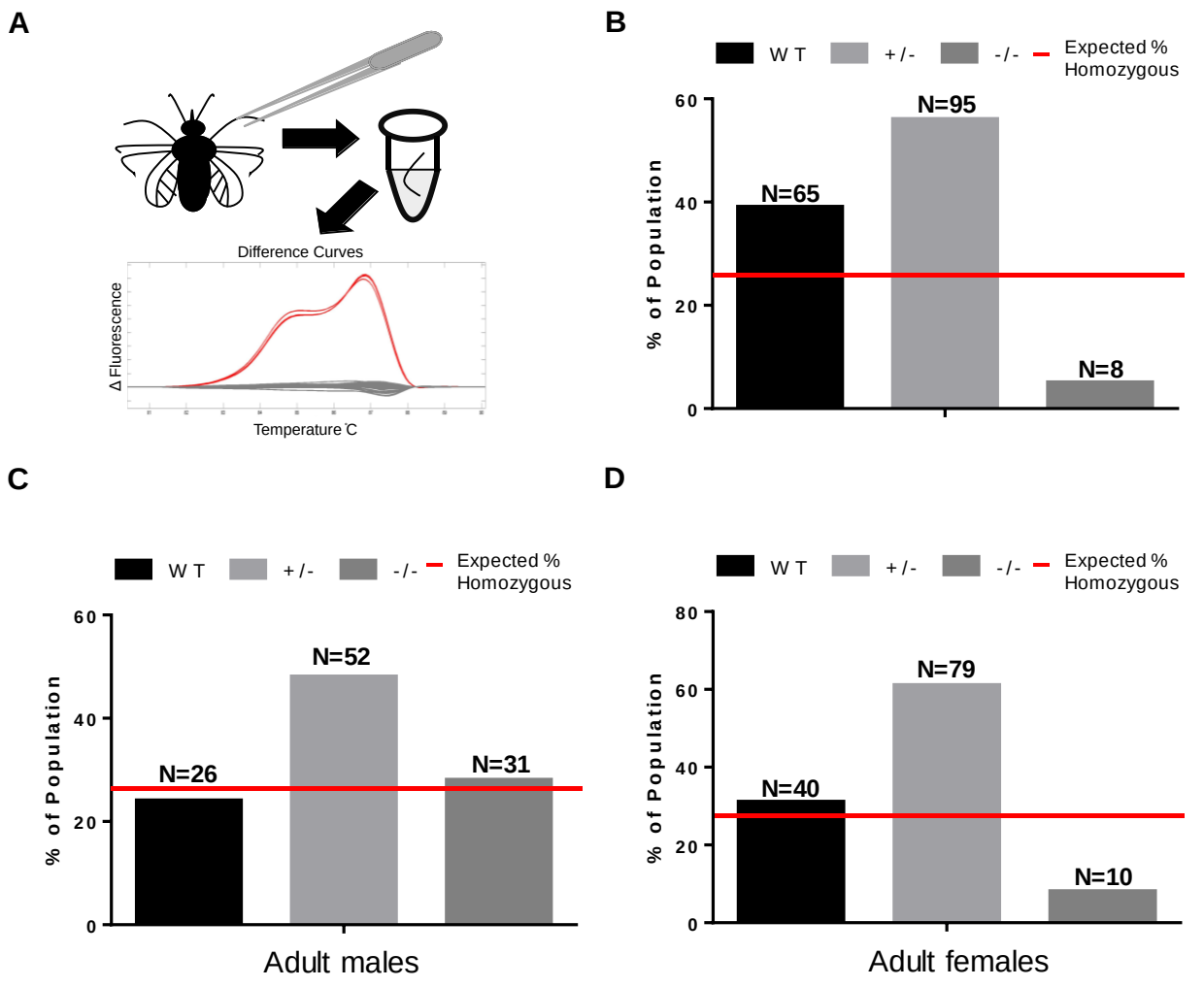

Figure 5.3 Early *ku70* self-crosses resulted in lower than expected rates of homozygous females. (A) Three to seven-day old adults were genotyped by HRMA. **(B)** *ku70* heterozygotes (G_2) were self-crossed and a random subset of progeny (G_3) were genotyped. **(C&D)** *ku70* heterozygotes (G_4) were crossed and resulting male **(C)** and female **(D)** offspring (G_5) were genotyped.

heterozygotes was repeated at the fifth generation and populations of males and females were genotyped separately. While male offspring from the test cross displayed the expected mendelian

rate of *ku70*$^{\Delta 1/\Delta 10}$ transheterozygotes (Figure 5.3 C, p=0.85, Chi-square), the female progeny had significantly lower levels of transheterozygotes than expected (Figure 5.3 D, p=0.001 Chi-square). This suggested that female fitness alone was impacted by the knockout of *ku70*.

To determine when female *ku70*$^{\Delta 1/\Delta 10}$ transheterozygotes were lost from the population, we determined the frequency of each genotype at the next earliest stage, the pupal stage. Female *ku70* pupae were collected within 16 hours of the initiation of pupation for genotyping, as well as within 24 hours of adult emergence. The frequency of wild type (*ku70*$^{+/+}$), heterozygote (*ku70*$^{\Delta 1/+}$ or *ku70*$^{+/\Delta 10}$) and transheterozygote (*ku70*$^{\Delta 1/\Delta 10}$) genotypes were as expected in pupae (Figure 5.4 A, p=0.77 Chi-square), but *ku70*$^{\Delta 1/\Delta 10}$ transheterozygotes were again significantly underrepresented in the one day-old female adult group (Figure 5.4 B, p=0.0076 Chi-square), suggesting that lethality occurred between the initiation of pupation and eclosion. To further clarify the time of lethality, we observed the *ku70*$^{-/-}$ mutants (*ku70*$^{\Delta 1/\Delta 10}$, *ku70*$^{\Delta 10/\Delta 10}$ and *ku70*$^{\Delta 1/\Delta 1}$) as pupae and recorded time of lethality (hereafter referred to as *ku70*$^{-/-}$). Female *ku70*$^{-/-}$ pupae were observed in groups every 2 hours with overlapping time points to confirm when in the process of pupation females were dying. Lethality appeared to be most frequent at the initiation and termination of pupation (Figure 5.4 C, p<0.0001 Mantel-Cox).

While we observed Mendelian rates of inheritance in *ku70*$^{-/-}$ males, we next evaluated the effect of ku70 deficiency on fertility. Nine homozygous *ku70*$^{-/-}$ males (G_5) were crossed individually with wild type females and the number of viable progeny determined (Table 3). All test crosses produced viable progeny; a subset of the progeny from three of these founders was genotyped. As expected, all the progeny were *ku70*$^{-/+}$. These data confirm that *ku70*$^{-/-}$ males are fertile.

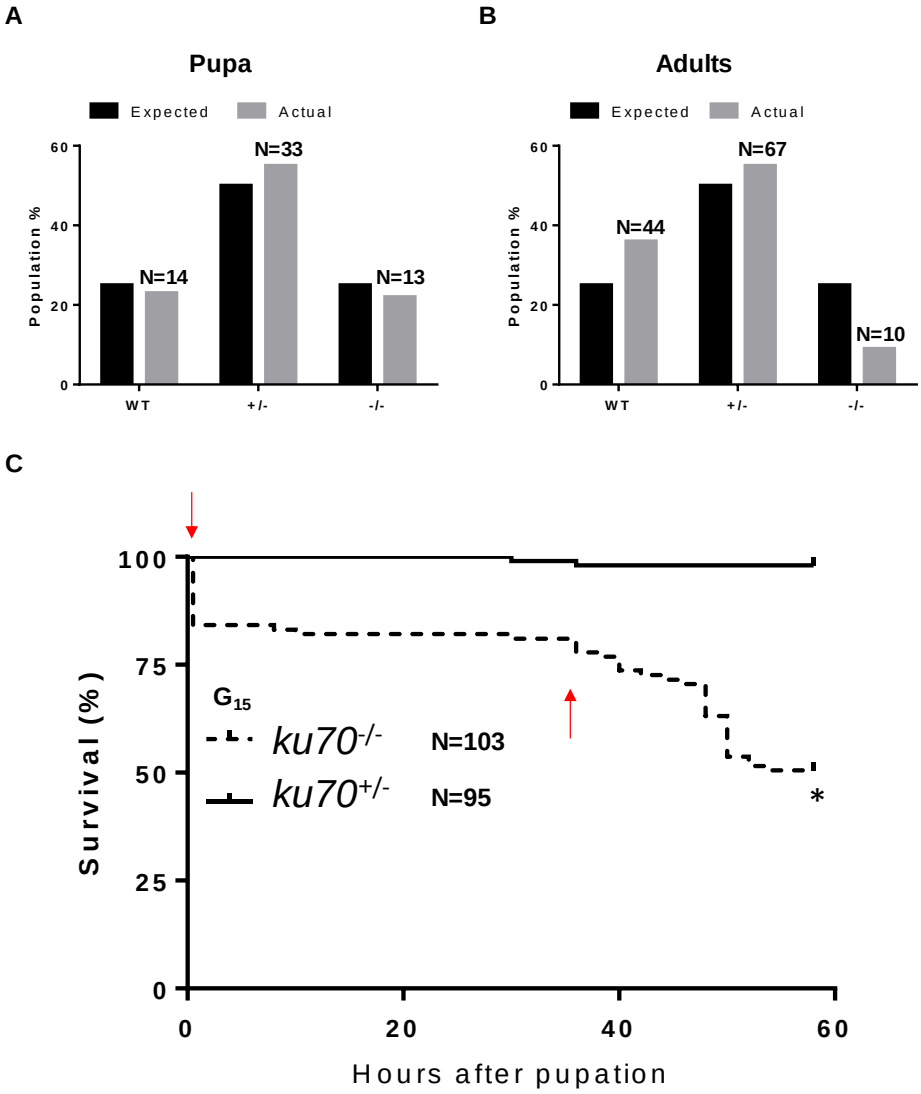

Figure 5.4 Females lacking *ku70* die at the initiation and termination of pupation. (A&B) Heterozygous *ku70* mutants were self-crossed. The female progeny was genotyped within 15 hours of pupation **(A)** and within one day post emergence **(B). (C)** Time of pupal lethality was determined over the pupation process with red arrows indicating the initiation and termination of the pupal stages.

Table 3. Male *Ae. aegypti* lacking *ku70* are fertile.

Group	Fertile	# offspring	% of offspring heterozygous
K1	**Yes**	120	100
K2	**Yes**	238	-
K3	**Yes**	160	-
K4	**Yes**	188	-
K5	**Yes**	248	-
K6	**Yes**	331	-
K7	**Yes**	73	100
K8	**Yes**	232	100
K9	**Yes**	251	-

While *ku70*$^{-/-}$ females suffered substantial pupal lethality, half of the population did survive to adulthood. Since *ku70*$^{-/-}$ males were fertile, this allowed us to establish a transheterozygous/homozygous line deficient in ku70 (termed *ku70*$^{-/-}$). Once this *ku70*$^{-/-}$ line had been established, longevity, fecundity, fertility and embryo melanization time were assessed to further establish phenotypes associated with the *ku70*$^{-/-}$ deficiency.

The longevity of both *ku70*$^{-/-}$ males and females were assessed over the course of three experiments and multiple generations. The first replicate utilized *ku70*$^{-/+}$ as controls while the second and third replicates utilized wild type (*ku70*$^{+/+}$; LVP) as controls. At generation 15, there was a significant difference between the survival of *ku70*$^{-/-}$ females and males as compared to each control group (Figure 5.5 A&B, $p<0.0001$ & $p=0.0131$ respectively, Mantel-Cox).

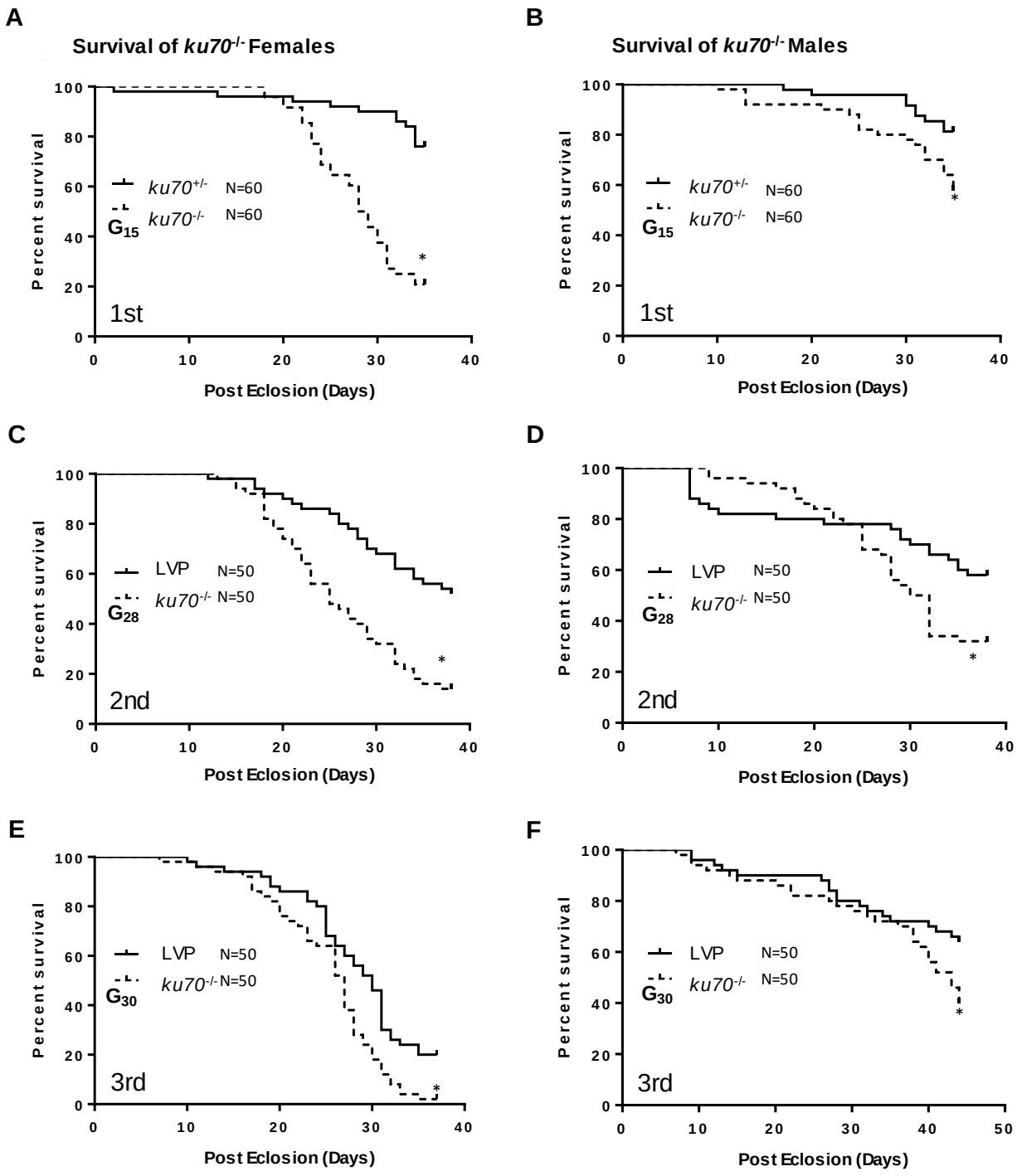

Figure 5.5 Knockout of *ku70* reduces longevity in both male and female mutants. (A+B+C+D+E+F) Asterisk (*) indicates significant difference. **(A+C+E)** Comparison of longevity between female *ku70*$^{-/-}$ vs control mosquitos**. (B+D+F)** Comparison of longevity between male *ku70*$^{-/-}$ vs control mosquitoes.

While both male and female *ku70*$^{-/-}$ mutants lived significantly shorter time than the control group, lifespan was more substantially reduced in the female group. In the second experiment, the results were consistent with both male and female groups of *ku70*$^{-/-}$ at generation 28 having significantly shorter lifespans (Figure 5.5 C&D, $p<0.0001$ & $p=0.0191$ respectively, Mantel-Cox). The third experiment conducted at generation 30, yielded similar results to the first and second replicate (Figure 5.5 E&F, $p=0.0013$ & $p=0.0339$ respectively, Mantel-Cox). While in all replicates *ku70*$^{-/-}$ mutants displaced significantly shorter lifetimes, the magnitude of the difference and thus the statistical significance decreased as generation number increased. This may be due to differences between reared cohorts or minor environmental differences.

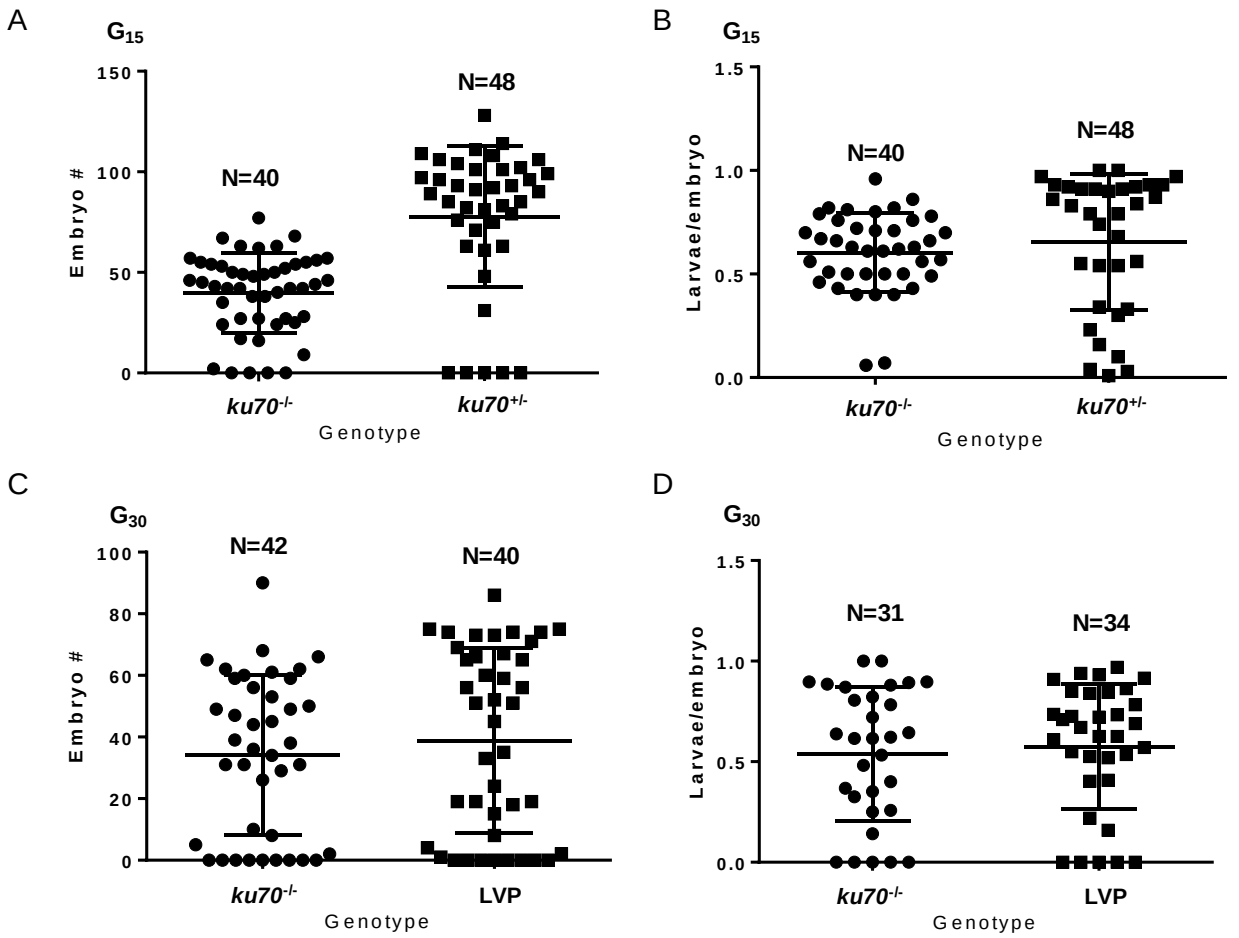

Figure 5.6 Female fecundity and fertility are not impacted by knockdown of ku*70*. **(A+C)** Fecundity assays of *ku70*$^{-/-}$ females in comparison to controls. **(B+D)** Fertility assays of *ku70* null females in comparison to controls. Larvae to embryo ratio is calculated by the initial number of embryos present divided by the number of larvae hatched. For each, horizontal bars indicate mean value and error bars indicate standard deviation.

The fecundity and fertility of females was assessed at the 15th generation and again at the 30th generation. In the first replicate, *ku70*$^{-/-}$ females laid fewer eggs ($p<0.0001$ t-test) than the *ku70* heterozygous controls, while fertility levels were not significantly different (Figure 5.6

A&B, p=0.3848, t-test). In the second experiment, we observed no significant difference between the fecundity of *ku70* females and LVP controls (p=0.4440 t-test) or fertility (Figure 5.6 C&D, 0.7707 t-test). However, we note that the mean number of embryos produced from *ku70*$^{-/-}$ females was similar between the two experiments (mean <50 in exp. 1, <40 in exp. 2), where the mean number of control embryos dropped substantially in the second experiment (mean ~70 in exp. 1 vs ~40 in exp. 2). This suggests a potential problem with the colony strain in this experiment and indicates more experiments are required before any firm conclusions can be drawn with regard to the effect of loss of *ku70*$^{-/-}$ on female fecundity/fertility.

While injecting embryos collected from *ku70*$^{-/-}$ females (the results of which will be discussed in the next chapter) we noticed that chorion melanization appeared to occur much more slowly in *ku70*$^{-/-}$ embryos than in *ku70*$^{+/+}$ individuals (Figure 5.7 A). To quantify this observation, we monitored embryos collected from *ku70*$^{+/+}$ or *ku70*$^{-/-}$ female mosquitoes. In the first and second replicates of the experiment three crosses were set up. Wild type LVP females were crossed with LVP males, while *ku70* females were crossed with either LVP males or *ku70*

males. In both replicates the results were significant ($p<0.0001$, 2-way ANOVA) with both replicates showing that the *ku70*$^{-/-}$ groups were significantly different than the LVP groups by multiple comparison analysis (Figure 5.7 B & C). Given the significant results of the first and second replicates, we expanded the number of crosses from three to four to include LVP females crossed with *ku70*$^{-/-}$ males; additionally, we expanded the time point from 4 (1-2.5hr) to 6 (1-3.5hr). The results of the third experiment were similar to those of the first and second with multiple comparison analysis showing significant difference between the LVP and *ku70* groups at the last time point ($p<0.0001$, 2-way ANOVA) (Figure 5.7 D). The fourth replicate was similar with the exception of multiple analysis showing that LVP females crossed with *ku70* males melanized significantly faster than LVP females crossed with LVP males ($p<0.0001$, 2-way ANOVA) (Figure 5.7 E). Even with the results of the fourth experiment, the results of all experiments in conjunction suggest that the maternal genotype impacts melanization rates more than paternal factors.

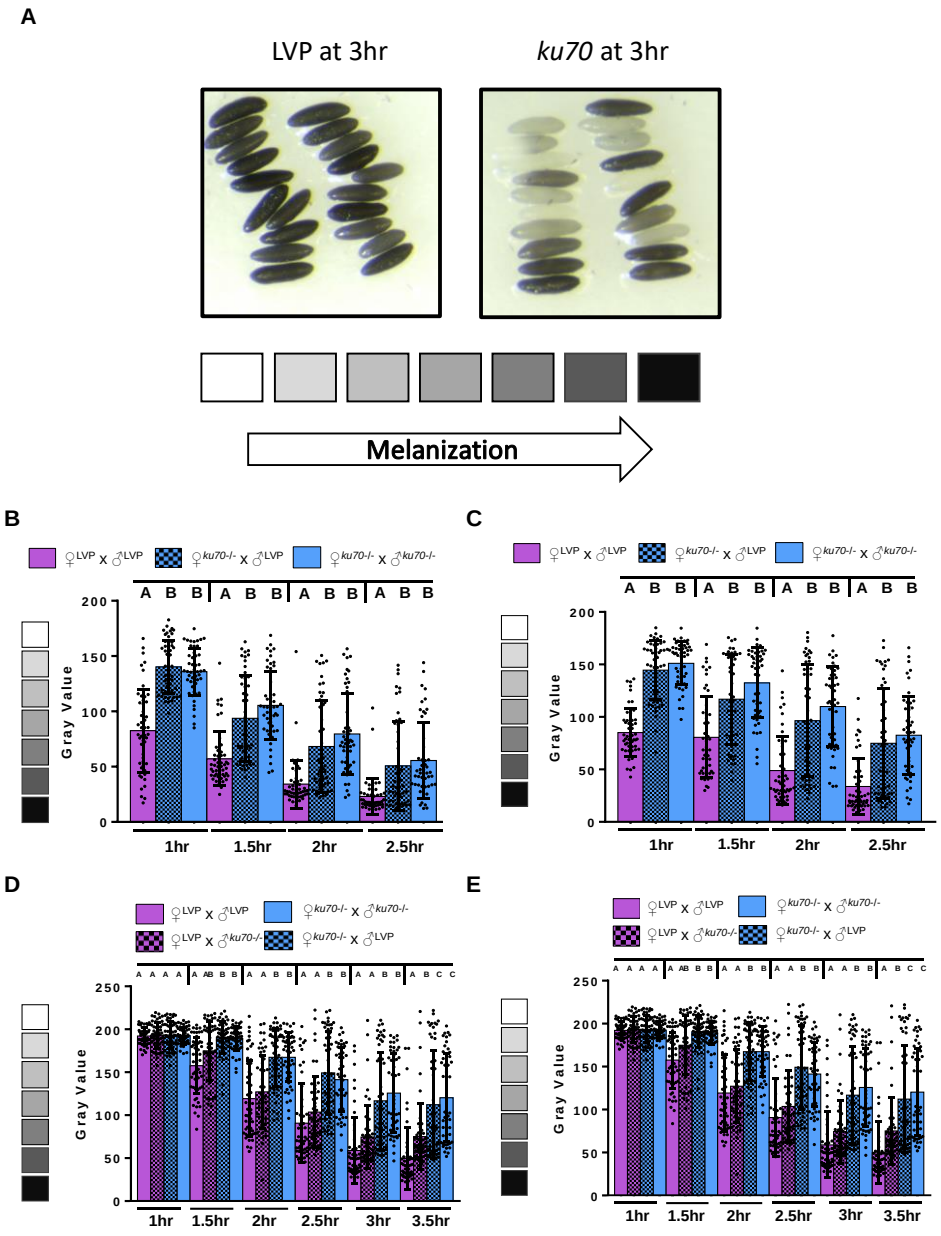

Figure 5.7 Embryos lacking *ku70* melanize at a slower rate. **(A)** LVP and *ku70* deficient embryos melanize at differential rates. **(B+C)** Photos of embryos between 1-2.5hrs (n=50 per group). **(D+F)** photos of embryos between 1-3.5 hours (n=50 per group). Data points are representative of individual embryos gray score. Error bars indicate standard deviation.

DISCUSSION

Knockout of *ku70* resulted in several observable phenotypes, some of which impacted fitness. One phenotype associated with knockout of *ku70* was female specific lethality of 50% of pupae. We observed that lethality was predominantly observed at the initiation of pupation and eclosion. Given that transition from one life stage to another in insects is regulated by 20-hydroxyecdysone and juvenile hormone in insects, we hypothesized that misregulation of these hormones may play a role in the lethality observed in the $ku70^{-/-}$ line. Increases in 20-hydroxyecdysone are responsible for molting and eclosion (Margam et al., 2006) while levels of juvenile hormone inhibits metamorphosis (Riddiford et al., 2010). It is possible that stress associated from DNA damage impacts hormone production/regulation, in turn impacting pupation.

We observed that both male and female $ku70^{-/-}$ individuals had a reduced lifespan. One possible explanation for this may be a role for Ku70 in preventing apoptosis. The removal of *ku70* in mice results in premature aging (Li et al., 2007). In $ku70^{-/-}$ mice, the removal of the apoptosis gene *bax* increased lifespans of *ku70* mutants suggesting Ku70 may play a role in regulating apoptosis (Ngo et al., 2015). It has also been reported that the ku complex plays a role in telomere regulation (Nugent et al., 1998), and telomere length has been linked to an organisms age (Greider, 1991) due to the removal of the telomeres eventually leading to chromosomal destabilization. That being said, telomere length has been studied in *D. melanogaster* with shortened telomeres not resulting in shorter lifespan, but instead impacting fecundity and fertility (Walter et al., 2007). Telomeres in the dipteran *Drosophila melanogaster* are significantly different from telomeres found in mammalian cells [Reviewed in (Mason et al., 2008)]. Telomeres can be generally categorized as being comprised of three regions: the cap, the

terminal DNA sequence and telomere-associated sequences. In mammals, the telomere-associated sequence is comprised of short repetitive elements (not conserved between species) which are maintained by the reverse transcriptase telomerase. In contrast, in *Drosophila melanogaster* the telomere-associated sequences are composed of the long (up to 12kb) retrotransposons Het-A, TART and TAHRE. Additionally, these retrotransposons do not rely on telomerase (which is absent in the *D. melanogaster* genome) to maintain telomere length, but instead utilize targeted transposition which is currently not well understood. While the components of the telomere-associated sequences are distinct between mammals and *D. melanogaster*, in both cases the ku-complex is required for telomeric length stability (Melnikova et al., 2005). This is most likely due to the sequence independent fashion in which the ku-complex binds to dsDNA. While the highly conserved nature of the ku-complex and its known role in the dipteran *D. melanogaster* might suggest it fills a similar role in *Ae. aegypti*, confirmation of the ku-complex's role in telomere regulation with respect to telomere stability and life span in *Ae. aegypti* will require further investigation.

Embryos derived from *ku70*$^{-/-}$ mosquitoes also displayed slower rates of chorion melanization. Melanization of the chorion is dependent upon follicle cells in the embryo. Typically, melanization of the embryo occurs within the first 1-2 hours after oviposition (Li and Li, 2006). *ku70*$^{-/-}$ embryos displayed abnormally long melanization times, with a subset of embryos taking more than 6 hours to fully melanize. In *D. melanogaster*, over-replication of genes associated with chorion development occurs in follicle cells by reinitiating of the S phase of the cell cycle (Calvi et al., 1998). One of the most common sources of double stranded DNA breaks are replication fork stalls which occur in DNA replication. Over replication of genomic regions associated with chorion development may result in high rates of DNA double stranded

breaks. More specifically, those DNA double stranded breaks would be more likely to occur in regions which hold genes which could play a key role in chorion development. Alternatively, a mutation linkage with chorion associated genes such as cross-linking proteins may have been isolated when generating the *ku70*$^{-/-}$ line (Li and Li, 2006). To test both these hypothesizes further investigation is required. One possible route of investigation would be to perform a bioinformatic survey of genes within the same chromosomal region to determine if genes known to be associated with chorion melanization are present. Additionally, to determine if over-replication of chorion associated proteins is inhibited in *ku70*$^{-/-}$ mosquitoes, transcript and protein levels of known chorion cross linking proteins and their biochemical activities could be quantified.

While up until this point we have relied on genomic sequences of mutants to evaluate the state of knockout of *ku70*, we have not determined if partial ku70 transcripts or proteins are being produced. We are currently testing an *Ae. aegypti* Ku70 specific antibody to determine the presence or absence of any Ku70 associated peptides. By using the CRISPR/Cas9 system we generated premature stop codons which should produce a truncated protein expected to be unable to bind to DNA. It is possible that initiation at downstream start codon may produce a N-terminally truncated Ku70 protein from the parent transcript. Generally, mRNA which contains premature stop codons will be treated as a nonsense RNA and will undergo nonsense mediated decay [Reviewed in (He and Jacobson, 2015)]. To determine if nonsense mediated decay is occurring, an evaluation of ku70 transcripts of several life stages, including but not limited to embryos, larvae, male and female pupae, and male and female adults is required. The presence or absence of transcripts will elucidate if any partial protein transcript is being produced and translated.

Unlike with *ku70*, two nearly identical copies of *lig4* appeared to be present in *Ae. aegypti* with knockout resulting in distinct phenotypes. The removal of *lig4* resulted in two phenotypes, one which was sex linked and appeared to have no impact on genotype distribution, and the other which was not sex linked could not be maintained past the third generation as heterozygotes (Basu et al., 2015). Given that in mice the absence of *lig4* results in embryonic lethality (Karanjawala et al., 2002) and the fact that our non-sex linked *lig4* mutants were disappearing even after artificial selection by HRMA, we initially believed that the non-sex linked version of the gene was most likely a functional copy of *lig4* while the sex-linked *lig4* was the result of a duplication event which resulted in the generation of a pseudogene. At the time we had generated the *lig4* mutants, the current version of the *Ae. aegypti* genome supported the presence of two genes which contained similar functional domains. In the most recent version of the *Ae. aegypti* genome only one copy of *lig4* is present and it is located on chromosome 1 (making it sex linked) (Matthews et al., 2018). This makes interpretation of our current results difficult. Knockdown of *lig4* in D. melanogaster resulted in increased rates of HDR with no reported reduction in fitness (Beumer et al., 2008). Evolutionarily, *D. melanogaster* is more closely related to *Ae. aegypti* than *Mus musculus*. While evolutionary distance does not correlate to importance of a gene for an organism's fitness, it was curious to see the fitness cost associated the absence of the non-sex linked *lig4* mutant caused in *Ae. aegypti*. While we are currently generating new *lig4* mutant strains to reevaluate our previous results, there are a few explanations as to why we may have seen two distinct groups while the current genome only identifies one copy of *lig4*. The first explanation could be that the current map is not complete, and the presence of two copies of lig4 are present but not accounted for. A second explanation is that within our wild type strain (LVP) a duplication event has occurred in which the lig4 gene

which is not located on chromosome one has retained its activity, while the sex-linked copy has lost its function. Currently we are in the process of genotyping, but though tracking mendelian rates of inheritance we will be able to determine if we see the same trends. Additionally, we have developed DNA sensitivity assays which will allow us to test both mutant types to determine if one or both are sensitivity to DNA DSBs.

REFERENCES

ARYAN, A., MYLES, K. M. & ADELMAN, Z. N. 2014. Targeted genome editing in *Aedes aegypti* using TALENs. *Methods*, 69, 38-45.

BASSETT, A. R., TIBBIT, C., PONTING, C. P. & LIU, J. L. 2013. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep,* 4**,** 220-8.

BASU, S., ARYAN, A., OVERCASH, J. M., SAMUEL, G. H., ANDERSON, M. A., DAHLEM, T. J., MYLES, K. M. & ADELMAN, Z. N. 2015. Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. *Proc Natl Acad Sci U S A,* 112**,** 4038-43.

BETERMIER, M., BERTRAND, P. & LOPEZ, B. S. 2014. Is non-homologous end-joining really an inherently error-prone process? PLoS Genet, 10, e1004086.

BEUMER, K. J., TRAUTMAN, J. K., BOZAS, A., LIU, J. L., RUTTER, J., GALL, J. G. & CARROLL, D. 2008. Efficient gene targeting in *Drosophila* by direct embryo injection with zinc-finger nucleases. *Proc Natl Acad Sci U S A,* 105**,** 19821-6.

BOUTANAEV, A. M., MIKHAYLOVA, L. M. & NURMINSKY, D. I. 2007. Up-regulation of the Ku heterodimer in *Drosophila* testicular cyst cells. *FEBS Letters,* 581**,** 1707-1715.

CALVI, B. R., LILLY, M. A. & SPRADLING, A. C. 1998. Cell cycle control of chorion gene amplification. *Genes & development,* 12**,** 734-744.

CHANG, H. H. Y., PANNUNZIO, N. R., ADACHI, N. & LIEBER, M. R. 2017. Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nature Reviews Molecular Cell Biology, 18, 495.

CDC. 2019. *Dengue and the Aedes aegypti mosquito* [Online]. Centers for Disease Control and Prevention. Available:

https://www.cdc.gov/dengue/resources/30jan2012/aegyptifactsheet.pdf [Accessed 4/10/19 2019].

CENCI, G., CIAPPONI, L. & GATTI, M. 2005. The mechanism of telomere protection: a comparison between *Drosophila* and humans. *Chromosoma,* 114**,** 135-145.

CHANG, H. H. Y., PANNUNZIO, N. R., ADACHI, N. & LIEBER, M. R. 2017. Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature Reviews Molecular Cell Biology,* 18**,** 495.

CIAPPONI, L., CENCI, G., DUCAU, J., FLORES, C., JOHNSON-SCHLITZ, D., GORSKI, M. M., ENGELS, W. R. & GATTI, M. 2004. The *Drosophila* Mre11/Rad50 complex is required to prevent both telomeric fusion and chromosome breakage. *Curr Biol,* 14**,** 1360-6.

DONG, S., LIN, J., HELD, N. L., CLEM, R. J., PASSARELLI, A. L. & FRANZ, A. W. 2015. Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti*. PLoS One, 10, e0122353.

GANTZ, V. M., JASINSKIENE, N., TATARENKOVA, O., FAZEKAS, A., MACIAS, V. M., BIER, E. & JAMES, A. A. 2015. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. Proc Natl Acad Sci U S A, 112, E6736-43.

GILLES, A. F., SCHINKO, J. B. & AVEROF, M. 2015. Efficient CRISPR-mediated gene targeting and transgene replacement in the beetle *Tribolium castaneum*. Development, 142, 2832-2839.

FRIERSON, J. G. 2010. The yellow fever vaccine: a history. *Yale J Biol Med,* 83**,** 77-85.

HAMMOND, A., GALIZI, R., KYROU, K., SIMONI, A., SINISCALCHI, C., KATSANOS, D., GRIBBLE, M., BAKER, D., MAROIS, E., RUSSELL, S., BURT, A., WINDBICHLER, N., CRISANTI, A. & NOLAN, T. 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nat Biotechnol,* 34**,** 78-83.

HE, F. & JACOBSON, A. 2015. Nonsense-Mediated mRNA Decay: Degradation of Defective Transcripts Is Only Part of the Story. *Annual review of genetics,* 49**,** 339-366.

HUANG, Y.-J. S., HIGGS, S. & VANLANDINGHAM, D. L. 2017. Biological Control Strategies for Mosquito Vectors of Arboviruses. *Insects,* 8**,** 21.

KARANJAWALA, Z. E., ADACHI, N., IRVINE, R. A., OH, E. K., SHIBATA, D., SCHWARZ, K., HSIEH, C. L. & LIEBER, M. R. 2002. The embryonic lethality in DNA ligase IV-deficient mice is rescued by deletion of Ku: implications for unifying the heterogeneous phenotypes of NHEJ mutants. *DNA Repair (Amst),* 1**,** 1017-26.

KARGINOV, F. V. & HANNON, G. J. 2010. The CRISPR system: small RNA-guided defense in bacteria and archaea. Mol Cell, 37, 7-19.

KISTLER, K. E., VOSSHALL, L. B. & MATTHEWS, B. J. 2015. Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. Cell Rep, 11, 51-60.

LAMARCHE, B. J., ORAZIO, N. I. & WEITZMAN, M. D. 2010. The MRN complex in double-strand break repair and telomere maintenance. FEBS Lett, 584, 3682-95.

LI, H., VOGEL, H., HOLCOMB, V. B., GU, Y. & HASTY, P. 2007. Deletion of Ku70, Ku80, or both causes early aging without substantially increased cancer. *Molecular and cellular biology,* 27**,** 8205-8214.

MA, S., CHANG, J., WANG, X., LIU, Y., ZHANG, J., LU, W., GAO, J., SHI, R., ZHAO, P. & XIA, Q. 2014. CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of BmKu70 in *Bombyx mori*. *Sci Rep,* 4**,** 4489.

MARKERT, M. J., ZHANG, Y., ENUAMEH, M. S., REPPERT, S. M., WOLFE, S. A. & MERLIN, C. 2016. Genomic Access to Monarch Migration Using TALEN and CRISPR/Cas9-Mediated Targeted Mutagenesis. G3 (Bethesda, Md.), 6, 905-915.

MARUYAMA, T., DOUGAN, S. K., TRUTTMANN, M. C., BILATE, A. M., INGRAM, J. R. & PLOEGH, H. L. 2015. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotech,* 33**,** 538-542.

MASON, J. M., FRYDRYCHOVA, R. C. & BIESSMANN, H. 2008. *Drosophila* telomeres: an exception providing new insights. BioEssays : news and reviews in molecular, cellular and developmental biology, 30, 25-37.

MATTHEWS, B. J., DUDCHENKO, O., KINGAN, S. B., KOREN, S., ANTOSHECHKIN, I., CRAWFORD, J. E., GLASSFORD, W. J., HERRE, M., REDMOND, S. N., ROSE, N. H., WEEDALL, G. D., WU, Y., BATRA, S. S., BRITO-SIERRA, C. A., BUCKINGHAM, S. D., CAMPBELL, C. L., CHAN, S., COX, E., EVANS, B. R., FANSIRI, T., FILIPOVIĆ, I., FONTAINE, A., GLORIA-SORIA, A., HALL, R., JOARDAR, V. S., JONES, A. K., KAY, R. G. G., KODALI, V. K., LEE, J., LYCETT, G. J., MITCHELL, S. N., MUEHLING, J., MURPHY, M. R., OMER, A. D., PARTRIDGE, F. A., PELUSO, P., AIDEN, A. P., RAMASAMY, V., RAŠIĆ, G., ROY, S., SAAVEDRA-RODRIGUEZ, K., SHARAN, S., SHARMA, A., SMITH, M. L., TURNER, J., WEAKLEY, A. M., ZHAO, Z., AKBARI, O. S., BLACK, W. C., CAO, H., DARBY, A. C., HILL, C. A., JOHNSTON, J. S., MURPHY, T. D., RAIKHEL, A. S.,

SATTELLE, D. B., SHARAKHOV, I. V., WHITE, B. J., ZHAO, L., AIDEN, E. L., MANN, R. S., LAMBRECHTS, L., POWELL, J. R., SHARAKHOVA, M. V., TU, Z., ROBERTSON, H. M., MCBRIDE, C. S., HASTIE, A. R., KORLACH, J., NEAFSEY, D. E., PHILLIPPY, A. M. & VOSSHALL, L. B. 2018. Improved reference genome of *Aedes aegypti* informs arbovirus vector control. *Nature,* 563**,** 501-507.

MELNIKOVA, L., BIESSMANN, H. & GEORGIEV, P. 2005. The Ku Protein Complex Is Involved in Length Regulation of *Drosophila* Telomeres. Genetics, 170, 221-235.

OVERCASH, J. M., ARYAN, A., MYLES, K. M. & ADELMAN, Z. N. 2015. Understanding the DNA damage response in order to achieve desired gene editing outcomes in mosquitoes. *Chromosome Res,* 23**,** 31-42.

QI, Y., ZHANG, Y., ZHANG, F., BALLER, J. A., CLELAND, S. C., RYU, Y., STARKER, C. G. & VOYTAS, D. F. 2013. Increasing frequencies of site-specific mutagenesis and gene targeting in Arabidopsis by manipulating DNA repair pathways. *Genome Res,* 23**,** 547-54.

RIDDIFORD, L. M., TRUMAN, J. W., MIRTH, C. K. & SHEN, Y.-C. 2010. A role for juvenile hormone in the prepupal development of *Drosophila melanogaster*. *Development,* 137**,** 1117.

SANDER, J. D. & JOUNG, J. K. 2014. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotech, 32, 347-355.

TORRESI, J., EBERT, G. & PELLEGRINI, M. 2017. Vaccines licensed and in clinical trials for the prevention of dengue. *Human vaccines & immunotherapeutics,* 13**,** 1059-1072.

WEI, W., XIN, H., ROY, B., DAI, J., MIAO, Y. & GAO, G. 2014. Heritable Genome Editing with CRISPR/Cas9 in the Silkworm, *Bombyx mori*. PLoS ONE, 9, e101210.

WHO. 2018. *Dungue and severe dengue* [Online]. World Health Organization. Available: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue [Accessed 4/10/19 2019].

WILLIAMS, G. J., HAMMEL, M., RADHAKRISHNAN, S. K., RAMSDEN, D., LEES-MILLER, S. P. & TAINER, J. A. 2014. Structural insights into NHEJ: building up an integrated picture of the dynamic DSB repair super complex, one component and interaction at a time. *DNA Repair (Amst),* 17**,** 110-20.

CHAPTER VI

ASSESSING IMPACTS OF C-NHEJ DEFICIENCY ON DNA DSB REPAIR IN *AEDES AEGYPTI*

INTRODUCTION

Aedes aegypti is a critical viral vector that impacts tropical and sub-tropical regions. The *Ae. aegypti* mosquito vectors yellow fever, Zika, dengue and chikungunya viruses (WHO, 2018, CDC, 2019). Reduction of local mosquito populations is typically achieved through removal of habitat (standing water), biological controls, insecticide use or a combination of all three (Huang et al., 2017, Benelli et al., 2016). Advances in gene editing technology have created the opportunity for the development of more efficient genetic based control mechanisms (Overcash and Adelman, 2016, Alphey, 2014, Bernardini et al., 2018).

The homology directed repair pathway (HDR) can be utilized to enhance basic science through the insertion of engineered gene sequences into the mosquito chromosome (Basu et al., 2015, Kistler et al., 2015, Dong et al., 2015). HDR is also utilized in CRISPR/Cas9 gene drive mechanisms to drive transgenic constructs though a population at hyper-mendelian rates (Hammond et al., 2016, Gantz et al., 2015). The use of the HDR pathway is limited by the requirement of a homologous template and other competing pathways such as the classical non-homologous end joining pathway (C-NHEJ), resulting in low rates of HDR gene insertion (Liesch et al., 2013). These limiting factors result in very low rates of homology directed repair, which is often cell cycle dependent. To achieve higher rates of HDR, we utilized the RNAi pathway to target the *ku70* gene and observed knock-in rates comparable to transposable element transgenesis techniques (Basu et al., 2015). Understanding how inhibition of C-NHEJ impacts

the DNA damage response and influences repair pathway selection is un can provide insight into increasing rates of HDR and advancing basic and applied research.

The DNA damage response can be broadly characterized as repair of DNA damage to either single stranded or double stranded DNA. Single stranded DNA damage can be repaired by the mismatch repair pathway (MMR), the nucleotide excision repair pathway (NER) or the base excision repair pathway (BER) [Reviewed in (Mani et al., 2019]. While single strand repair pathways are critical for maintaining genomic integrity, recent gene editing techniques in *Ae. aegypti* utilized double stranded break inducing endonucleases such as zinc finger nucleases (ZFN), transcription activator like effector nucleases (TALEN) and the clustered regularly interspaced palindromic repeats (CRISPR) in conjunction with the Cas9 endonuclease (Aryan et al., 2013b, McMeniman et al., 2014, Dong et al., 2015, Kistler et al., 2015). As previously mentioned, the utilization of the DSB repair pathway HDR would be beneficial for both basic and applied research, but without manipulation of the DNA damage response, rates of HDR are extremely low in *Ae. aegypti* (McMeniman et al., 2014). Utilization of the CRISPR/Cas9 system has quickly surpassed ZFNs and TALENs in *Ae. aegypti* due to cost and the ability to multiplex guide RNAs. This makes understanding how DNA DSB induced by the CRISPR/Cas9 system are repaired critical for expanding the gene editing tool kit in *Ae. aegypti*.

Both homology directed gene insertion and Cas9-mediated gene drive rely on the use of homologous sequences to be used as a template for repair after initiation of a DSB by the Cas9 endonuclease. A DSB induced by Cas9 can be repaired by several different pathways including single strand annealing SSA (Bhargava et al., 2016), NHEJ [Reviewed in (Williams et al., 2014, Chang et al., 2017)], HDR (Lamarche et al., 2010) or the alternative non-homologous end joining pathway (A-NHEJ) (Betermier et al., 2014). How the genomic sequence is repaired

(indel generation vs error free repair) after damage is induced is dependent upon which pathway is utilized to fix the break. In the event of SSA, homologous sequences are collapsed with the intermediate sequence deleted. C-NHEJ may repair perfectly or result in the introduction of small indels. The HDR pathway uses a homologous template to repair the break, leading to insertion of new genetic material. Lastly, the A-NHEJ pathway is believed to necessitate error prone repair and can act independent of homologous sequences. Error prone repair as a result of C-NHEJ, SSA or A-NHEJ can inhibit gene drive mechanisms by destroying sgRNA target sites (Hammond et al., 2017). The C-NHEJ pathway competes directly with the initiation factors of HDR, SSA and A-NHEJ. By removing the ability for the C-NHEJ pathway to engage in DSB repair we hypothesized that we could increase utilization of HDR.

To further understand how inhibition of the NHEJ impacts DSB DNA hierarchy in *Ae. aegypti* we decided to target the NHEJ gene *ku70*. After generation of a *ku70*$^{-/-}$ mosquito strain, we characterized various effects on mosquito fitness (previous chapter). However, it remains unclear how loss of *ku70* impacts other competing DNA DSB repair pathways. Here, we observed that *ku70*$^{-/-}$ larvae were significantly more sensitive to DSB induced by bleomycin than wild type, providing functional confirmation that these mosquitoes were deficient in DNA damage repair. However, using SSA and NHEJv2.0 plasmid assays, we observed no significant difference in either form of DNA repair in *ku70*$^{-/-}$ embryos as compared to the wild type strain. We observed that micro-injection of CRISPR/Cas9 reagents targeting the kynurenine 3-monooxygenase gene (*kmo*) in the *ku70*$^{-/-}$ mosquitoes resulted in lower injection survival rates as compared to control embryos. Additionally, at 100ng/µl of Cas9 LVP injected embryos had high levels of *kmo* mosaicism (66%) when compared to *ku70*$^{-/-}$ injected embryos (4.1%) suggesting higher rates of homology directed repair may be occurring in *ku70*$^{-/-}$ embryos.

MATERIALS AND METHODS

Mosquito rearing and maintenance: Mosquitoes were reared at 28˚C, 60-70% humidity and 10/14 dark light cycle. Mosquito larvae were reared in 4 liters of reverse osmosis-purified H_2O with ~400 larvae per pan. Larvae were given finely ground Tetra fish food (~1/8 teaspoon a day) (Tetra, Blacksburg, VA). Adult females were blood feed on defibrinated sheep's blood warmed to 37˚C (LVP on parafilm, *ku70* on hog-gut to entice feeding) (Colorado Serum Co., Denver, CO).

Dual luciferase Assays: Dual luciferase assays were completed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), with a slightly modified protocol. Passive lysis buffer (PLB) was used to lyse snap-frozen tissue (100µl per 100 embryos). After addition of PLB, samples were frozen at -80˚C and allowed to thaw (2X) in -80˚C freezer. Before dual luciferase assay 20µl of each sample was placed in white bottom 96 well plate. Reagents (LARII and Stop&Glo) were removed from -80˚C freezer and allowed to thaw. A volume of 100ul of both LARII and Stop&Glo was allotted for each sample, with an additional 1ml allotted to prime the SpectraMax i3 automatic injector system. The SpectraMax i3 dual injector (Molecular Devices, Jose, CA) was primed with luciferase substrates (LARII and Stop and Glo) loaded in separate reservoirs, and the assay plate was read using the dual luciferase program in SoftMax Pro 6.5.1 software suite [Samples analyzed one at a time, assay protocol: Inject 80ul LARII, 2s delay, 10s read, Inject Stop&Glo, 2s delay, 10s read].

NHEJ assay injections: NHEJ embryo injection mix consisted of three plasmids: PUb-Renilla, PUb-Y2-AniI and NHEJv2.0. Assay plasmids were mixed together at a final injection

concentration of 200ng/µl per plasmid (600ng/ul total). Injection mix was centrifuged at maximum speed for ~45 minutes at 4°C (Eppendorf, Hamburg, Germany). Injections were completed in groups of ~100 embryos. Each group of embryos was harvested 48 hours post injection and were snap frozen in liquid nitrogen for dual luciferase assays.

SSA assay injections: SSA embryo mix consisted of plasmids PUb-Renilla, PUb-Y2-AniI and SSA sensor plasmid (Aryan et al., 2013a). Assay plasmids were mixed at a final injection concentration of 200ng/µl per plasmid (600ng/µl total). Injection mix was centrifuged at maximum speed for ~ 45 minutes at 4°C. Injections were completed in groups of ~100 embryos. Each group of embryos was harvested 48 hours post injection and were snap frozen in liquid nitrogen for dual luciferase assays.

Bleomycin Assay: Bleomycin was obtained from Adooq Bioscience (Adooq Bioscience, Irvine, CA). Bleomycin powder was dissolved in DMSO to generate a 10mM stock solution. For each experiment, dilutions of 100µm, 10µm, 1µm, and 0.1µm were made from the stock solution using DEPC-treated H_20. Diluted bleomycin was placed in a 12 well cell plate with one dilution per well, and four dilutions per genotype (2ml of solution per well). Mosquito embryos (LVP, *ku70*$^{-/-}$ and *ku70*$^{+/-}$) were hatched in water under vacuum for 20-30 minutes, and L_1 larvae collected with 1000µl pipette and passed through a 100µm cell strainer (Corning, Corning, NY) to minimize dilution of the bleomycin. At 48 hrs after immersion in bleomycin, larvae were scored as dead (complete lack of movement), or alive (movement observed) following probing to prod larvae to check for residual movement.

Variable Injection of Cas9 Protein Injections were completed as described in (Aryan et al.,2014). Injection mixes were made using stock Cas9 endonuclease protein (PNA Bio, Newbury Park, CA) aliquoted at 2ug/µl. Cas9 was diluted to concentration using DEPC H20. Guide RNAs were synthesized as reported in (Bassett et al., 2013). Guide RNA transcription was completed using Megascript T7 kit (ThermoFisher Scientific, Waltham, MA) and purified using MEGAclear kit (ThermoFisher Scientific, Waltham, MA). The primers for sgRNAs synthesis are as listed: [KMO144 ZA2268: 5'GAAATTAATACGACTCACTATAGGACTTAA TACTGTTGAAGTGTTTTAGAGCTAGAAATAGC3'; kmo144-2: 5'GAAATTAAT ACGACTCACTATAGGGTTTAGTGATACGCGTTGGGGTTTTAGAGCTAGAAA3'; kmo144-3: GAAATTAATACGACTCACTATAGGATGCCATGGTTCCCTTCTACGTT TTAG AGCTAGAAA3']. All sgRNAs targeted the *kmo* gene and were named KMO-144, KMO-144-2, and KMO144-3. The sgRNAs were combined with Cas9 endonuclease at a final concentration of 75ng/µl per sgRNA with either 100 or 50ng/µl of Cas9; the final injection mix volume was 10ul. After injection, embryos were transferred to chromatography paper within 10 minutes and excess halo carbon oil 27 (Sigma-Aldrich, St. Louis, MO) was rinsed off. After 24 hours embryos were counted, with dead embryos discarded from consideration (dead embryos were identified by chorion discoloration and flattened appearance). At five days post injection, embryos were hatched under standard rearing conditions.

RESULTS

We initially hypothesized that the use of homology-based repair would have increased in *ku70*$^{-/-}$ embryos with respect to wild type LVP controls. The plasmid-based SSA assay previously described in our lab (Aryan et al 2013) allows quantification of any shift in SSA, a form of HDR (Figure 6.1 A). The SSA assay plasmid was injected into both wild type (LVP) and *ku70*$^{-/-}$ embryos; the entire experiment was repeated twice. In the first experiment there was no significant difference in SSA-based repair between *ku70*$^{-/-}$ and LVP embryos (Figure 6.1 B, p=0.3204 Mann-Whitney) with quantifiable rates of both firefly and Renilla luciferase luminescence (Figure 6.1 C&D). In the second experiment, there was a significant reduction in SSA-repair, in *ku70*$^{-/-}$ embryos (Figure 6.1 E, p=0.0379 Mann-Whitney) with quantifiable rates of both firefly and Renilla luciferase luminescence (Figure 6.1 F&G). When results from both replicates were combined there was no significant difference in SSA-repair between *ku70*$^{-/-}$ embryos and LVP controls (Figure 6.1 H, p=0.3843 Mann-Whitney).

We hypothesized that end joining in *ku70*$^{-/-}$ embryos would be reduced as compared to LVP controls. To determine if this was in fact the case, we injected the NHEJv2 assay (Chapter III) into either LVP or *ku70*$^{-/-}$ embryos in two replicate experiments. In the first experiment, there was no significant difference in end-joining repair between *ku70*$^{-/-}$ and LVP embryos (Figure 6.2 B, p=0.1263 Mann-Whitney) with quantifiable rates of both firefly and Renilla luciferase (Figure 6.2 C&D). In the second experiment, there was again no significant difference (Figure 6.2 E, p>0.99 Mann-Whitney) with quantifiable rates of both firefly and Renilla luciferase (Figure 6.2 F&G). Analysis of the combined data from the two experiments resulted in no significant difference between *ku70*$^{-/-}$ and LVP embryos (Figure 6.2 H, p=0.1644 Mann-Whitney).

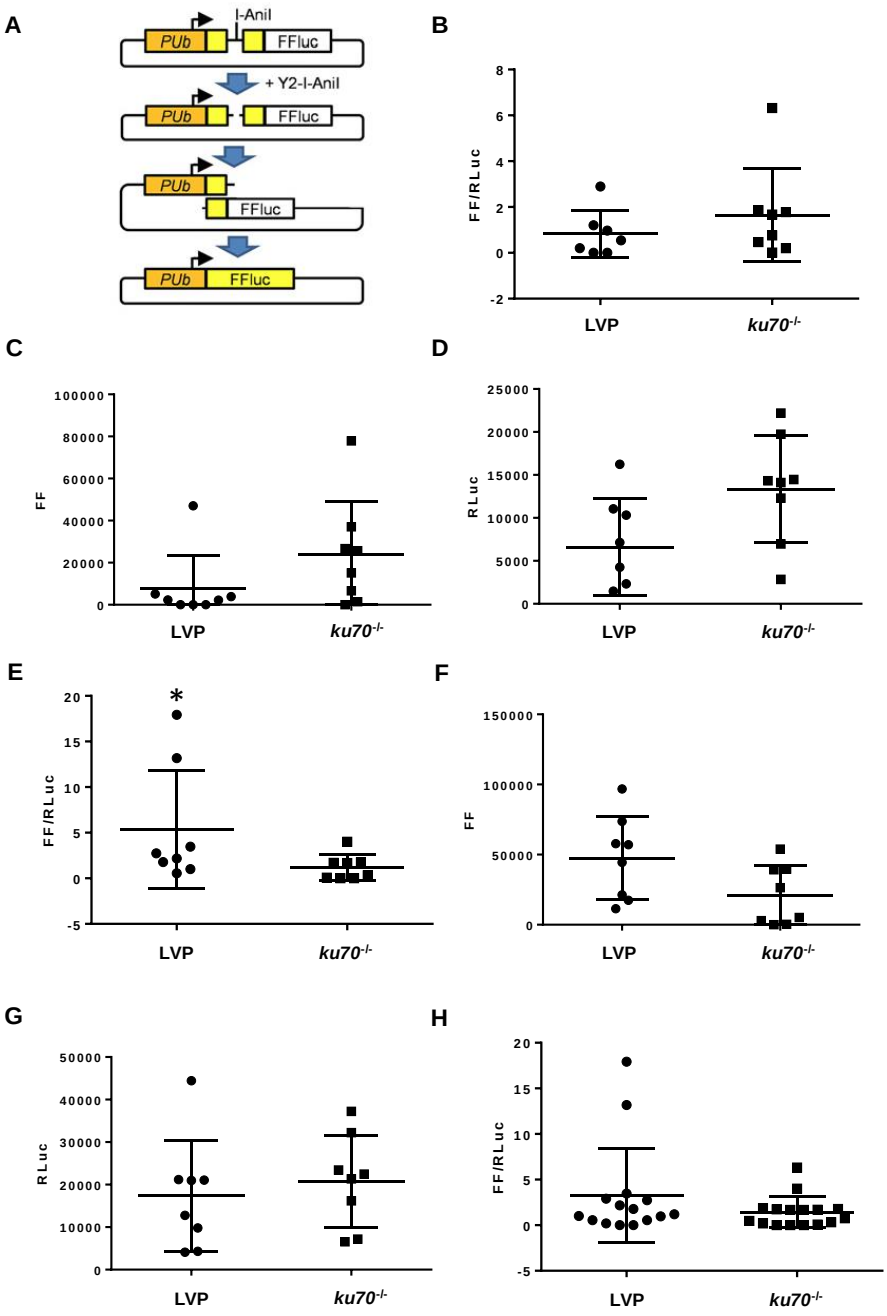

Figure 6.1. Single strand annealing is unchanged in *ku70* null mosquito embryos. (A) Schematic of SSA assay. **(B&E)** Generation of *ku70*$^{-/-}$lines at the time of injection G_{27} **(B)** and G_{28} **(E). (B-H)** The first **(B),** second **(E)**, and combined (**H**) experiments displaying the ratio of firefly to Renilla luciferase. The raw firefly values for the first **(C)** and second **(F)** experiments. The raw Renilla luciferase values for the first **(D)** and second experiments **(G)**. Horizontal bars indicate mean; error bars indicate standard deviation. Each data point represents a pool of ~100 injected embryos. Statistical significance is indicated by asterisk (*).

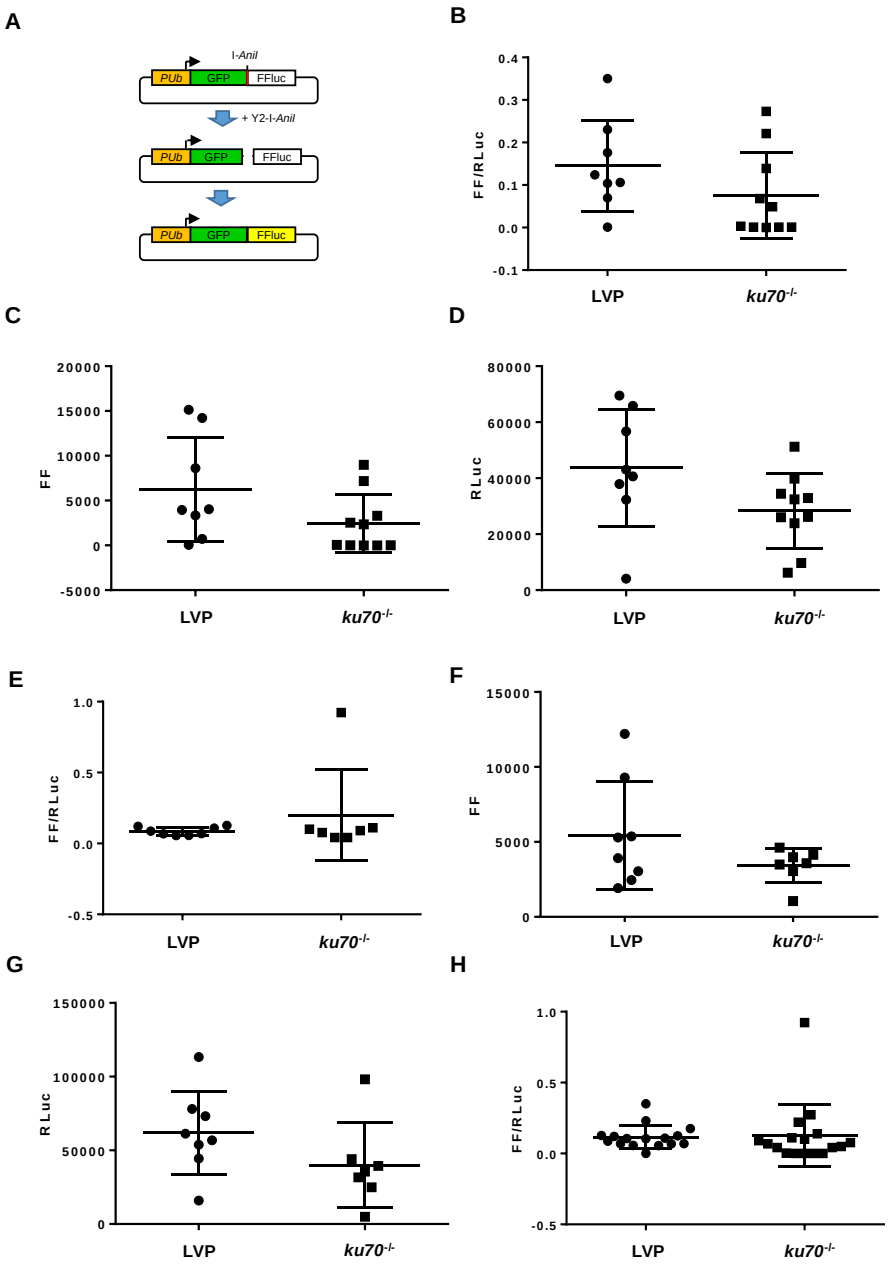

Figure 6.2 Non-homologous end joining is unchanged in *ku70* null mosquito embryos. (A) Schematic of NHEJ assay. **(B&E)** Generation of *ku70*$^{-/-}$ lines at time of injection G_{26}. **(B-H)** The first **(B),** second **(E)**, and combined (**H**) experiments displaying the ratio of firefly to Renilla luciferase. The raw firefly values for the first **(C)** and second **(F)** experiments. The raw Renilla luciferase values for the first **(D)** and second experiments **(G)**. Horizontal bars indicate mean; error bars indicate standard deviation. Each data point represents a pool of ~100 injected embryos. Horizontal bars indicate mean; error bars indicate standard deviation. Each data point represents a pool of ~100 injected embryos.

As these results did not support our initial hypothesis, we considered the possibility that despite the genetic lesions introduced into the *ku70* gene, that C-NHEJ was able to process and repair DSBs normally. Alternatively, it was possible that other alternative pathways may have been selected to compensate for any deficiency in DSB repair. In order to determine if DNA repair was inhibited more generally, we designed a DSB sensitivity assay utilizing the DSB inducing antibiotic bleomycin.

Larvae from both LVP and *ku70*$^{-/-}$ strains were placed in bleomycin of varying concentrations in a 12 well plate (Figure 6.3 A). The entire experiment was replicated three times, with each experiment containing four 10-fold dilutions of bleomycin from 100μm to 0.1μm. All three replicates showed significant differences between groups (Figure 6.3 B-D, $p<0.0001$ Chi-square).

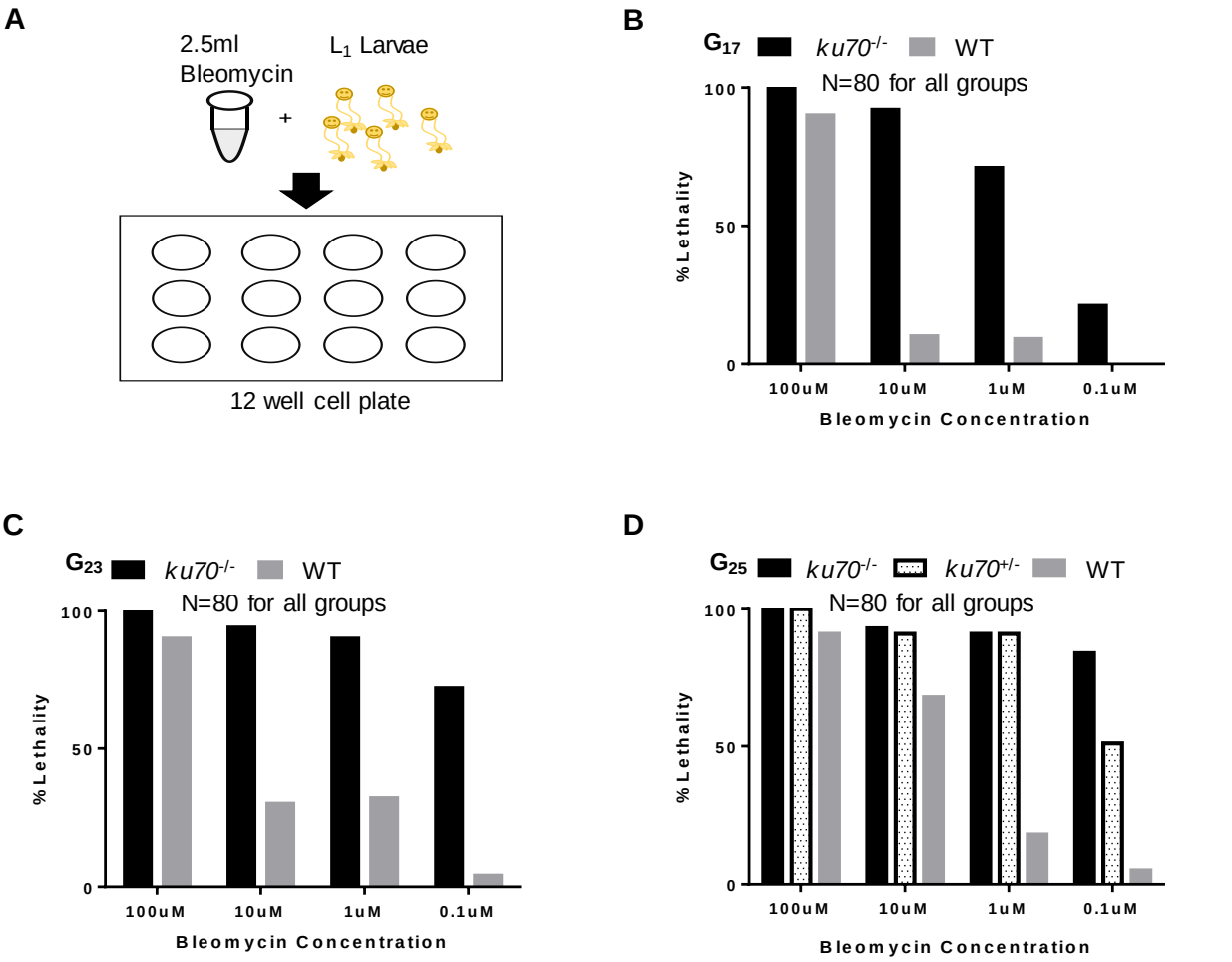

Figure 6.3 *ku70* null larvae are sensitive to DNA double stranded break agent bleomycin. (A) Schematic of bleomycin DNA damage sensitivity assays. **(B+C+D)** The first **(B),** second **(C)** and third **(D)** replicates of bleomycin assays.

Statistical analysis of both *ku70*$^{-/-}$ and LVP groups across all three replicates showed that the lethal dose concentration (LD50) was at 10.5µM for LVP L_1 larvae and 0.0103 µM for *ku70*$^{-/-}$ L_1 larvae, suggesting that *ku70*$^{-/-}$ are 100-fold more sensitive to bleomycin induced DSB than LVP controls. Lastly, the *ku70*$^{+/-}$ larvae showed lower rates of lethality than *ku70*$^{-/-}$ suggesting

one copy of *ku70*$^{-/-}$ can compensate for bleomycin DSB at lower concentrations. Together, these experiments suggest that while we were unable to detect a shift in either SSA or NHEJ in the *ku70*$^{-/-}$ embryos, *ku70*$^{-/-}$ mosquitoes are in fact sensitive to DNA DSB damage.

We hypothesized that *ku70*$^{-/-}$ mosquitoes may not present a difference in SSA or C-NHEJ due to compensation by the A-NHEJ pathway. Similarly, the results from the SSA assay could be due to preference of either HDR or A-NHEJ pathways. To test this, we injected *ku70*$^{-/-}$ embryos with Cas9 along with sgRNAs targeting the *kmo* gene under the hypothesis that if A-NHEJ was elevated in *ku70*$^{-/-}$ mosquitoes we would observe increased rates of *kmo* gene disruption by scoring mutagenic mosaicism (Figure 6.4).

Contrary to this expectation, two-thirds (66.6%) of LVP strain mosquitoes treated as embryos with 100ng/µl of Cas9 displayed some level of disruption in eye pigmentation following injection as compared with just 4% in the *ku70*$^{-/-}$ strain (Table 4). While the rate of mosaicism was higher (21%) at the lower concentration of Cas9, this was associated with very few surviving individuals. We note that at both concentrations of Cas9 protein, embryo survival

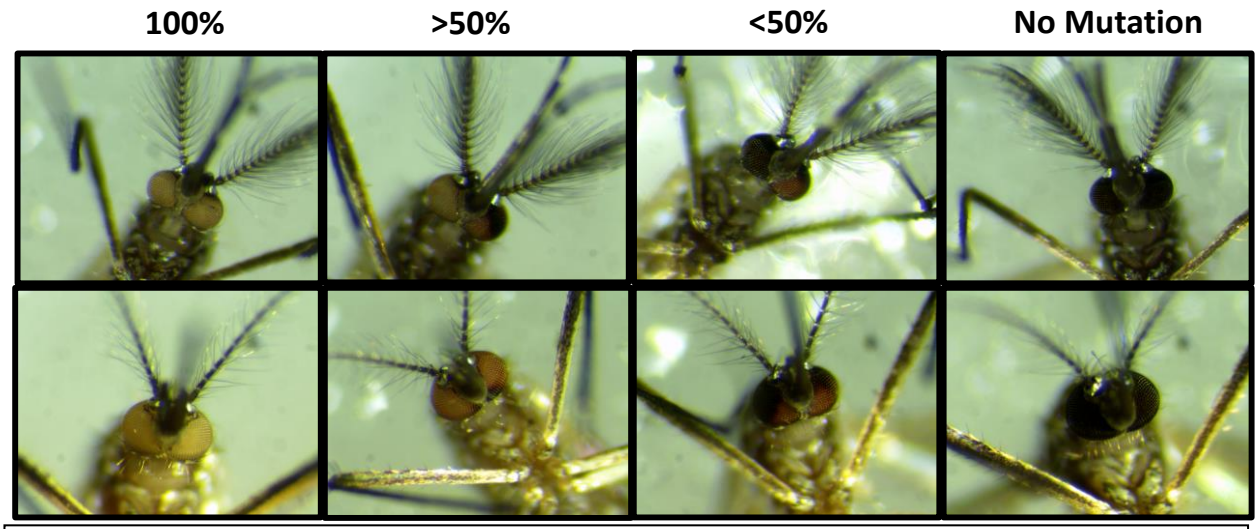

Figure 6.4 Scoring *Ae. aegypti* mosquitoes injected with CRISPR/Cas9 targeting the *kmo* eye pigment gene. Representation of scoring mechanism used in Table 4.

Table 4. Injection of CRISRP/Cas9 targeting the *kmo* eye pigment gene in LVP and *ku70* null embryos.

Genotype	Cas9 Concentration	G_0 Adults	# Injected	Survival (%)	Complete WE	>50% mosaic	<50% Mosaic	0% Mosaic	Mosaic mutant (%)
LVP	50ng	87	455	19	0	0	8	79	8
LVP	100ng	75	421	17.8	21	18	11	25	66.6
ku70$^{-/-}$	50ng	14	427	3.3	0	1	2	11	21
ku70$^{-/-}$	100ng	24	439	5.4	0	0	1	23	4.1

was substantially higher in the LVP control as compared to *ku70*$^{-/-}$ (17-19% vs 3-5%). To determine if the low survival rates observed were due to overall fitness *ku70*$^{-/-}$ embryos, or decreased ability to repair Cas9-induced DSBs, we repeated the experiment but this time compared injected Cas9 or DEPC-treated water into *ku70*$^{-/-}$ embryos. Importantly, once prepared, the samples were anonymized so that at the time of injection the experimenter (JMO) was unaware of the identity of each test solution. We obtained an average survival of 6.9% following injection of 50 ng/µl Cas9, versus 9.1% when injecting only DEPC-treated water. When rates of survival were compared the presence of Cas9 did not significantly decrease total survival % (Table 5, p=0.3876, t-test). While the results do not provide clear evidence for either an increase HDR or A-NHEJ in the *ku70*$^{-/-}$ line, it does show that the fitness cost associated with the *ku70* line directly impacts *ku70*$^{-/-}$ G_0's ability to survive micro-injection.

Table 5. Blind study injections of *ku70* embryos resulted in minor difference between survival in between embryos injected with Cas9 or DEPC water.

Injection group	Injection mix	# Injected	# G_0	Survival %	# Mosaic (%)	Total Survival %
1A	50ng/µl	126	7	5.5	0	6.9
2A		151	14	9.2	1 (7)	
3A		127	8	6.2	0	
1B	H_20	181	12	6.6	0	9.1
2B		188	24	12.8	0	
3B		190	15	7.9	0	

DISCUSSION

The knockout of *ku70* resulted in no detectable shift in SSA or NHEJ using plasmid-based sensors. Results from our previous publication showed that dsRNA targeting *ku70* resulted in an increase in SSA (Basu et al., 2015). The distinction between our previous experiment and the one reported in this chapter is with regard to transient knockdown versus knockout of *ku70*. It has been previously reported in mice cells that knockout of the C-NHEJ by targeting *xrcc4* resulted in shifts in transcript rates towards alternative end joining pathways. The SSA pathway is considered a sub-pathway under HDR and relies on the same initial protein machinery (MRN complex) to initiate DNA repair (Lamarche et al., 2010, Bhargava et al., 2016). How pathways dependent upon the MRN complex compete in *Ae. aegypti* is not yet known. To investigate how these pathways interact with one another, quantifying transcript levels of all genes associated with MRN sub pathways in *ku70*$^{-/-}$ and wild type LVP should provide evidence on any preferential shift toward one of the three pathways.

Similarly, we previously reported a shift away from error prone end joining based repair when dsRNA targeting *ku70* was microinjected into embryos (Basu et al., 2015). Injecting the NHEJv2.0 plasmid into *ku70*$^{-/-}$ embryos resulted in no detectable shift when compared to the LVP controls. While with the SSA assay competition within the MRN sub pathways could result in a selection of alternative pathways, error prone repair after inhibition of C-NHEJ may be due to compensation by A-NHEJ. In mice cells, a shift toward A-NHEJ after knocking out *xrcc4* lead to a direct increase of relative mRNA transcript numbers of genes associated with A-NHEJ (Kang and Yan, 2018). The Ku70 gene is involved in the initiation of C-NHEJ repair. Given that all current reports suggest that the ku complex is an obligate heterodimer, loss of Ku70 alone should be sufficient to stop initiation by this complex (Shibata et al., 2018, Fattah et al., 2010). A

secondary point to be considered is the unknown rate of error prone repair in C-NHEJ. It has been suggested that the C-NHEJ pathway may not be as error prone as previously believed (Betermier et al., 2014). The NHEJv2.0 plasmid allows us to detect shifts in error prone repair but does not specify which pathway is responsible for the error prone repair itself. Once again, quantifying shifts in transcripts associated with the MRN sub pathway may provide more insight into which of those pathways is transcriptionally favored in our *ku70*$^{-/-}$ line.

Both the SSA and NHEJv2.0 are plasmid-based assays; this format provides both benefits and drawbacks. The benefits of these assays include that both assays are luminescence-based, which allows us to quantify shifts in end joining over a large dynamic range in both cells and embryos, and the extrachromosomal nature of the assays allows for high throughput screening of shifts in DNA repair across any number of mutagenic lines or cell types. A primary drawback of these assays is that they may not capture the full scope of DNA repair selection, as the plasmid templates are not in the context of a mosquito chromosome. It is been well reported that a crucial component in DNA repair selection is the histone proteins surrounding the DNA DSB (Janssen et al., 2019). The nature of euchromatin which is less condensed and transcriptionally accessible versus heterochromatin which is more condensed and less accessible could play a role in DNA damage repair selection (Tamaru, 2010). The absence of chromatin associated proteins in both the SSA and NHEJv2.0 assays may also impact competition between pathways for DNA break repair. To address this issue, we are currently developing chromosome-based assays which utilize transgenic constructs to quantify rates of repair. These assays will be beneficial not only because they more fully integrate all components involved in DNA repair selection, but also will allow for quantifying rates of HDR between our *ku70*$^{-/-}$ and LVP mosquito strains.

The use of mutagenic reagents and ionizing radiation to cause DNA damage has been in practice for decades. Ionizing radiation can be used to detect sensitivity to DNA damage, but also has applications in cancer treatment and SIT (Nickoloff et al., 2017, L. and Fen, 2014, Bond et al., 2019). Ionizing radiation can result in several types of DNA damage and requires access to an irradiator as well as an ionization chamber to quantify radiation dosage. To induce DSB specific DNA damage we developed an assay based on the antibiotic bleomycin (Chen et al., 2008). Bleomycin was discovered in the mid 20th century and has been used as a chemotherapeutic agent to treat cancer [Reviewed in (Murray et al., 2018)]. Once in the cell, bleomycin interacts with iron Fe (II) and oxygen (O_2) to bind to DNA and cause DSBs. The type of DNA damage induced by bleomycin and its solubility in H20 at low concentrations allowed us to target *Ae. aegypti* in its early life stages. The initial stages of the *Ae. aegypti* life cycle are aquatic, allowing us to soak both *ku70*$^{-/-}$ and LVP controls in a solution which induces double stranded breaks at high rates. The results from the bleomycin assays showed that in comparison to our LVP control groups, *ku70*$^{-/-}$ larvae are significantly more sensitive ($p<0.0001$, Chi square) to DSB incurred from bleomycin. While the A-NHEJ pathway may be compensating for the lack of C-NHEJ, the A-NHEJ pathway is reported to be more error prone and associated with translocations (Soni et al., 2014, Betermier et al., 2014, Wray et al., 2013). This suggests that while DNA DSB repair can be managed, the organism's overall sensitivity to DNA damage is increased.

The results of the NHEJ and bleomycin assays suggest that the A-NHEJ pathway may be compensating for the C-NHEJ pathway, and thus we hypothesized that DNA repair may be more mutagenic in the *ku70*$^{-/-}$ line than the LVP control line. To test this hypothesis, we used the *kmo* gene, which encodes a protein required for eye pigment biosynthesis (Han et al., 2003), as a

target to gauge rates of mutagenesis. Our lab has experience with targeting the *kmo* gene with TALENs (Aryan et al., 2013b) and CRISPR (Basu et al., 2015). Targeting the *kmo* gene in both our *ku70*$^{-/-}$ and LVP lines showed reduced rates of mutagenesis in the *ku70*$^{-/-}$ line at a Cas9 concentration of 100ng/µl (66.6% in LVP vs 4.1% in ku70). The fitness of the *ku70*-/- embryos, which can be gauged by survival rates, was much lower than the LVP control making the sample size too small to draw conclusions. The possibility that A-NHEJ and HDR use similar cellular machinery initially (Bennardo et al., 2008) may indicate that there is direct competition between these two pathways for repair of the double stranded break. The low rate of mutagenesis in the *ku70*$^{-/-}$ line may suggest that increased rates of HDR are occurring. By utilizing the indel free copy of *kmo* to repair any damage instead of A-NHEJ, the *ku70*$^{-/-}$ embryos may avoid generation of a mosaic phenotype. To address this finding more experimentation will be required. One way to compensate for the low survival rate of the *ku70*$^{-/-}$ embryos would be to increase the size of the experiment. Increasing the number of injected embryos from several hundred, to several thousand should strengthen the evidence supporting this finding. Additionally, other genes such as *yellow* and *ebony* (Li et al., 2017) could be targeted with CRISPR/Cas9 to negate any impact of chromosomal location on DNA repair choice.

To further expand on shifting the DNA repair hierarchy, the *ku70*$^{-/-}$ line may be an ideal candidate for knockdown of A-NHEJ genes. There are several genes associated with the A-NHEJ pathway such as parp1, *xrcc1*, *xrcc3* and *lig3*, by generating dsRNA targeting genes and co-injecting them with sgRNAs that result in a visible phenotype we may be able to quantify shifts away from mutagenic damage associated with A-NHEJ. As we have reported, an ortholog for *lig3* is absent in the *Ae. aegypti* genome, making *parp1*, *xrcc1*and *xrcc3* possible targets for shifts in repair (Overcash et al., 2015). This information would be beneficial for increasing rates

of HDR for basic science and could provide insight into how to reduce mutagenic gene drive resistance which has been reported (Hammond et al., 2017).

REFERENCES

ALPHEY, L. 2014. Genetic control of mosquitoes. *Annu Rev Entomol,* 59**,** 205-24.

ARYAN, A., ANDERSON, M. A., MYLES, K. M. & ADELMAN, Z. N. 2013a. Germline excision of transgenes in *Aedes aegypti* by homing endonucleases. *Sci Rep,* 3**,** 1603.

ARYAN, A., ANDERSON, M. A., MYLES, K. M. & ADELMAN, Z. N. 2013b. TALEN-based gene disruption in the dengue vector *Aedes aegypti*. *PLoS One,* 8**,** e60082.

BASSETT, A. R., TIBBIT, C., PONTING, C. P. & LIU, J. L. 2013. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep,* 4**,** 220-8.

BASU, S., ARYAN, A., OVERCASH, J. M., SAMUEL, G. H., ANDERSON, M. A., DAHLEM, T. J., MYLES, K. M. & ADELMAN, Z. N. 2015. Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. *Proc Natl Acad Sci U S A,* 112**,** 4038-43.

BENELLI, G., JEFFRIES, C. L. & WALKER, T. 2016. Biological Control of Mosquito Vectors: Past, Present, and Future. *Insects,* 7**,** 52.

BERNARDINI, F., HAGHIGHAT-KHAH, R. E., GALIZI, R., HAMMOND, A. M., NOLAN, T. & CRISANTI, A. 2018. Molecular tools and genetic markers for the generation of transgenic sexing strains in Anopheline mosquitoes. *Parasites & vectors,* 11**,** 660-660.

BETERMIER, M., BERTRAND, P. & LOPEZ, B. S. 2014. Is non-homologous end-joining really an inherently error-prone process? *PLoS Genet,* 10**,** e1004086.

BEUMER, K. J., TRAUTMAN, J. K., BOZAS, A., LIU, J. L., RUTTER, J., GALL, J. G. & CARROLL, D. 2008. Efficient gene targeting in Drosophila by direct embryo injection with zinc-finger nucleases. *Proc Natl Acad Sci U S A,* 105**,** 19821-6.

BHARGAVA, R., ONYANGO, D. O. & STARK, J. M. 2016. Regulation of Single-Strand Annealing and its Role in Genome Maintenance. *Trends in Genetics,* 32**,** 566-575.

BOND, J. G., OSORIO, A. R., AVILA, N., GÓMEZ-SIMUTA, Y., MARINA, C. F., FERNÁNDEZ-SALAS, I., LIEDO, P., DOR, A., CARVALHO, D. O., BOURTZIS, K. & WILLIAMS, T. 2019. Optimization of irradiation dose to *Aedes aegypti* and *Ae. albopictus* in a sterile insect technique program. *PLOS ONE,* 14**,** e0212520.

CDC. 2019. *Dengue and the Aedes aegypti mosquito* [Online]. Centers for Disease Control and Prevention. Available: https://www.cdc.gov/dengue/resources/30jan2012/aegyptifactsheet.pdf [Accessed 4/10/19 2019].

CHANG, H. H. Y., PANNUNZIO, N. R., ADACHI, N. & LIEBER, M. R. 2017. Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature Reviews Molecular Cell Biology,* 18**,** 495.

CHEN, J., GHORAI, M. K., KENNEY, G. & STUBBE, J. 2008. Mechanistic studies on bleomycin-mediated DNA damage: multiple binding modes can result in double-stranded DNA cleavage. *Nucleic acids research,* 36**,** 3781-3790.

DONG, S., LIN, J., HELD, N. L., CLEM, R. J., PASSARELLI, A. L. & FRANZ, A. W. 2015. Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, *Aedes aegypti*. *PLoS One,* 10**,** e0122353.

FATTAH, F., LEE, E. H., WEISENSEL, N., WANG, Y., LICHTER, N. & HENDRICKSON, E. A. 2010. Ku Regulates the Non-Homologous End Joining Pathway Choice of DNA Double-Strand Break Repair in Human Somatic Cells. *PLOS Genetics,* 6**,** e1000855.

GANTZ, V. M., JASINSKIENE, N., TATARENKOVA, O., FAZEKAS, A., MACIAS, V. M., BIER, E. & JAMES, A. A. 2015. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proc Natl Acad Sci U S A,* 112**,** E6736-43.

HAMMOND, A., GALIZI, R., KYROU, K., SIMONI, A., SINISCALCHI, C., KATSANOS, D., GRIBBLE, M., BAKER, D., MAROIS, E., RUSSELL, S., BURT, A., WINDBICHLER, N., CRISANTI, A. & NOLAN, T. 2016. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nat Biotechnol,* 34**,** 78-83.

HAMMOND, A. M., KYROU, K., BRUTTINI, M., NORTH, A., GALIZI, R., KARLSSON, X., KRANJC, N., CARPI, F. M., D'AURIZIO, R., CRISANTI, A. & NOLAN, T. 2017. The creation and selection of mutations resistant to a gene drive over multiple generations in the malaria mosquito. *PLoS genetics,* 13**,** e1007039-e1007039.

HAN, Q., CALVO, E., MARINOTTI, O., FANG, J., RIZZI, M., JAMES, A. A. & LI, J. 2003. Analysis of the wild-type and mutant genes encoding the enzyme kynurenine monooxygenase of the yellow fever mosquito, *Aedes aegypti*. *Insect molecular biology,* 12**,** 483-490.

HUANG, Y.-J. S., HIGGS, S. & VANLANDINGHAM, D. L. 2017. Biological Control Strategies for Mosquito Vectors of Arboviruses. *Insects,* 8**,** 21.

JANSSEN, J. M., CHEN, X., LIU, J. & GONÇALVES, M. A. F. V. 2019. The Chromatin Structure of CRISPR-Cas9 Target DNA Controls the Balance between Mutagenic and Homology-Directed Gene-Editing Events. *Molecular Therapy - Nucleic Acids,* 16**,** 141-154.

KANG, Y.-J. & YAN, C. T. 2018. Regulation of DNA repair in the absence of classical non-homologous end joining. *DNA Repair,* 68**,** 34-40.

KISTLER, K. E., VOSSHALL, L. B. & MATTHEWS, B. J. 2015. Genome engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*. *Cell Rep,* 11**,** 51-60.

L., S. W. & FEN, X. 2014. Ionizing Radiation-Induced DNA Damage, Response, and Repair. *Antioxidants & Redox Signaling,* 21**,** 251-259.

LAMARCHE, B. J., ORAZIO, N. I. & WEITZMAN, M. D. 2010. The MRN complex in double-strand break repair and telomere maintenance. *FEBS Lett,* 584**,** 3682-95.

LI, M., BUI, M., YANG, T., BOWMAN, C. S., WHITE, B. J. & AKBARI, O. S. 2017. Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, *Aedes aegypti*. *Proceedings of the National Academy of Sciences,* 114**,** E10540.

LIESCH, J., BELLANI, L. L. & VOSSHALL, L. B. 2013. Functional and Genetic Characterization of Neuropeptide Y-Like Receptors in *Aedes aegypti*. *PLoS Negl Trop Dis,* 7**,** e2486.

MA, S., CHANG, J., WANG, X., LIU, Y., ZHANG, J., LU, W., GAO, J., SHI, R., ZHAO, P. & XIA, Q. 2014. CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of BmKu70 in *Bombyx mori*. *Sci Rep,* 4**,** 4489.

MANI, C., REDDY, P. H. & PALLE, K. 2019. DNA repair fidelity in stem cell maintenance, health, and disease. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease.

MARUYAMA, T., DOUGAN, S. K., TRUTTMANN, M. C., BILATE, A. M., INGRAM, J. R. & PLOEGH, H. L. 2015. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotech,* 33**,** 538-542.

MCMENIMAN, C. J., CORFAS, R. A., MATTHEWS, B. J., RITCHIE, S. A. & VOSSHALL, L. B. 2014. Multimodal integration of carbon dioxide and other sensory cues drives mosquito attraction to humans. Cell, 156, 1060-71.

MURRAY, V., CHEN, J. K. & CHUNG, L. H. 2018. The Interaction of the Metallo-Glycopeptide Anti-Tumour Drug Bleomycin with DNA. *International journal of molecular sciences,* 19**,** 1372.

NICKOLOFF, J. A., JONES, D., LEE, S.-H., WILLIAMSON, E. A. & HROMAS, R. 2017. Drugging the Cancers Addicted to DNA Repair. *JNCI: Journal of the National Cancer Institute,* 109.

OVERCASH, J. & ADELMAN, Z. N. 2016. Progress in Gene Editing Transgenesis Genome Manipulation in Mosquitoes. *Advances in Insect Physiology,* 51**,** 1-35.

OVERCASH, J. M., ARYAN, A., MYLES, K. M. & ADELMAN, Z. N. 2015. Understanding the DNA damage response in order to achieve desired gene editing outcomes in mosquitoes. *Chromosome Res,* 23**,** 31-42.

QI, Y., ZHANG, Y., ZHANG, F., BALLER, J. A., CLELAND, S. C., RYU, Y., STARKER, C. G. & VOYTAS, D. F. 2013. Increasing frequencies of site-specific mutagenesis and gene targeting in Arabidopsis by manipulating DNA repair pathways. *Genome Res,* 23**,** 547-54.

SHIBATA, A., JEGGO, P. & LÖBRICH, M. 2018. The pendulum of the Ku-Ku clock. *DNA Repair,* 71**,** 164-171.

SONI, A., SIEMANN, M., GRABOS, M., MURMANN, T., PANTELIAS, G. E. & ILIAKIS, G. 2014. Requirement for Parp-1 and DNA ligases 1 or 3 but not of Xrcc1 in chromosomal translocation formation by backup end joining. *Nucleic Acids Res,* 42**,** 6380-92.

TAMARU, H. 2010. Confining euchromatin/heterochromatin territory: jumonji crosses the line. *Genes Dev,* 24**,** 1465-78.

WHO. 2018. *Dungue and severe dengue* [Online]. World Health Organization. Available: https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue [Accessed 4/10/19 2019].

WILLIAMS, G. J., HAMMEL, M., RADHAKRISHNAN, S. K., RAMSDEN, D., LEES-MILLER, S. P. & TAINER, J. A. 2014. Structural insights into NHEJ: building up an integrated picture of the dynamic DSB repair super complex, one component and interaction at a time. *DNA Repair (Amst),* 17**,** 110-20.

WRAY, J., WILLIAMSON, E. A., SINGH, S. B., WU, Y., COGLE, C. R., WEINSTOCK, D. M., ZHANG, Y., LEE, S. H., ZHOU, D., SHAO, L., HAUER-JENSEN, M., PATHAK, R., KLIMEK, V., NICKOLOFF, J. A. & HROMAS, R. 2013. PARP1 is required for chromosomal translocations. *Blood,* 121**,** 4359-65.

CHAPTER VII

CONCLUSIONS

In this dissertation, I have covered experimentation on end joining based repair, and more specifically the C-NHEJ pathway in the disease vector *Aedes aegypti*. *Ae. aegypti* is a crucial disease vector which is responsible for diseases such as dengue, yellow fever, chikungunya, and Zika. The ability to control this vector currently relies on the use of biological, source and pesticide control strategies (Huang et al., 2017). While these control strategies can be utilized, alternative genetic control approaches may reduce cost and labor required to reduce local mosquito populations (Alphey et al., 2013).

The development of the CRISPR/Cas9 system in *Ae. aegypti* allows for mutant generation by gene knockout or transgene insertion (Basu et al., 2015). The CRISPR/Cas9 system is necessarily dependent on endogenous DNA double stranded break repair pathways. Understanding how the DNA repair hierarchy is utilized in the event of a double stranded break in *Ae. aegypti* could help to fine tune both the basic and applied aspects of genetic manipulation required for novel genetic control methods. To increase our knowledge of the classical non-homologous end joining pathway, as well as how manipulation of that pathway results in DNA repair shifts in *Ae. aegypti*, we designed luminescence-based assays for quantification of error prone end joining repair, assessed phenotypic results of knockout of *ku70*$^{-/-}$, quantified shifts in C-NHEJ and SSA in *ku70*$^{-/-}$ embryos, quantified DNA damage sensitivity to the DSB inducing antibiotic bleomycin and observed differential mutation rates within *ku70*$^{-/-}$ and wild type *Ae. aegypti*.

The initial sensor for the NHEJ pathway (NHEJv1.0) was shown to be able to quantify shifts in end joining repair at the embryonic stage, though was not sensitive enough for

utilization in cell lines, or in mutant embryos. To make the assay more sensitive we moved the location of the I-AniI target upstream of the firefly open reading frame by generating a GFP-FF fusion protein. This increased firefly luciferase activity and allowed for experimentation in *Ae. aegypti* cell lines (Aag2 and A20) and *ku70*$^{-/-}$ mutant embryos. The generation of NHEJv2.0 allowed for the quantification of shifts in end joining based repair after knockdown of the C-NHEJ genes *ku80, ku70* and *lig4* and provides an excellent tool for assessment of other C-NHEJ genes in the future. The NHEJv2.0 assay also incorporates sgRNA target locations allowing for direct experimentation of how DSB from the CRISPR/Cas9 system impact DNA DSB repair.

We are currently expanding the end joining repair assays that we can use to quantify the fidelity of C-NHEJ DNA DSB repair. In addition to NHEJv2.0 we have generated plasmid named NHEJv2.1 which we hypothesized would allow us to quantify rates of perfect versus imperfect repair by excision of nucleotide sequences between two CRISPR/Cas9 target sites (Figure 7.1).

Additionally, we are working on designs for the NHEJ assay based on restriction enzymes to generate double stranded breaks in place of the I-AniI homing endonuclease or Cas9. Current work in our lab suggests that the efficiency, fidelity and cost of Cas9 in vitro digests will limit the ability to perform large scale experiments in the A20 and Aag2 cell lines.

In contrast the use of restriction enzymes has a lower cost, higher fidelity and will allow us to test both blunt and overhang DSB (or a combination of different DSB ends). This will allow for an assessment of how a variety of DNA DSB impact error prone end joining repair in *Ae. aegypti* cells in a high throughput manner.

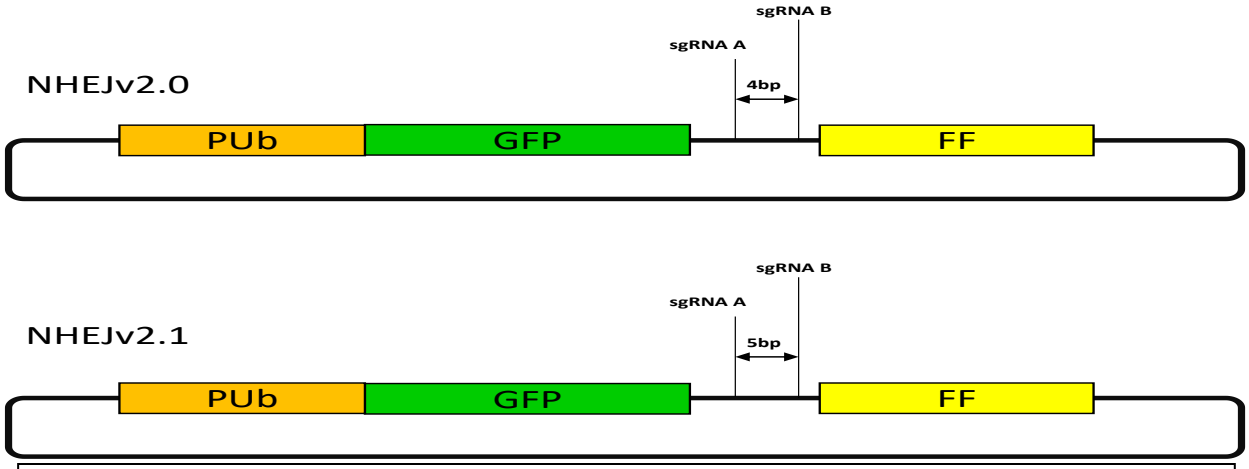

Figure 7.1. Expanding tools to detect perfect versus imperfect repair. Schematic of NHEJv2.0 and NHEJv2.1.

The generation of *lig4* and *ku70* mutants provided insight into how relevant these genes are for mosquito fitness. Our initial results from knockout of *lig4* show that there was both a haplosufficient and haploinsufficient copy of the gene in our LVP lab strain. These results were in agreement with the available genome assembly at the time, but as mentioned previously, the new genome assembly suggests that only one copy of *lig4* is present (Matthews et al., 2018). Our results suggest that there is an additional question that needs to be answered: Is there only one copy of the *lig4* gene in *Ae. aegypti*? Or is the most current genome assembly missing a copy? Alternatively, it could be the case that our LVP strain has acquired a duplicate copy which serves as the functional copy. We are currently generating new *lig4* mutants to address both of these questions.

The *ku70*$^{-/-}$ mutants displayed several phenotypes including sex-specific pupal lethality, reduced longevity and slowed chorion melanization. While we currently know when female pupal lethality is occurring, we have not been able to describe why it might be happening. As we discussed in the fourth chapter, the pupal lethality may be occurring because of irregular hormonal regulation in the *ku70*$^{-/-}$ line. Further experiments to determine levels of both 20-hydroxyecdysone and juvenile hormone in both *ku70*$^{-/-}$ and LVP lines should be completed to determine if hormones play a role in the DNA damage response. If there are different levels of hormones between *ku70*$^{-/-}$ and the LVP lines, this may suggest that the ability to repair DSB either directly or indirectly impacts hormonal regulation.

While we measured both fecundity and fertility at two different time points, the number of embryos which were deposited in our LVP controls were lower than we would have expected. In the first experiment the fecundity of the *ku70*$^{-/-}$ line was significantly lower, but it was not in the second replicate. While the reduced fecundity of the LVP in the second experiment is most likely due to environmental factors, both fecundity and fertility should be assessed again.

One question which has not been addressed is the long-term impact of removal of or inhibition of a crucial DNA repair pathway on the genomic stability of an organism. Our initial hypothesis was that the C-NHEJ plays a pivotal role in the inhibition of other deleterious repair pathways such as SSA or A-NHEJ (Betermier et al., 2014, Kang and Yan, 2018). In the reduction/absence of the C-NHEJ pathway it may be that these deleterious pathways are used at increased rates, especially given the requirement for a homologous template to be present for HDR to occur. The increased use of SSA and A-NHEJ may have resulted in an increase in deletions, insertions, inversions and translocations. By sequencing the full genome of our *ku70*$^{-/-}$ line the full extent of any genomic damage can be quantified by comparison to our LVP strain.

This would provide direct insight into how mutations within the DNA repair genes impact mutation rates. The relevant question which is addressed by investigating the accumulation of DNA damage in our *ku70*$^{-/-}$ line is the role of DNA repair pathways in evolution and mutation accumulation. Typically, evolution via mutation is broken down to be a result of errors in DNA synthesis, base pair mismatches duplication via mitotic recombination, viral mutagenesis and environmental stimuli (oxidative stress and radiation). Evidence of the impact of mutations within DNA repair pathways may shed light on how an organism's own endogenous DNA DSB repair pathways affect mutations commonly associated with evolution.

In addition to sequencing the whole genome, quantitative PCR on NHEJ related genes in the *ku70*$^{-/-}$ line may provide insight into how an organism compensates for the loss of a primary DNA double strand break repair pathway. An increase in relative rates of other C-NHEJ genes (such as *ku80*) may suggest that some form of compensation is occurring. Alternatively, it is plausible that increased generation of A-NHEJ, SSA or HDR related transcripts could compensate the reduction of C-NHEJ based repair. As we mentioned in the fifth chapter, identifying genes which are associated with all these pathways and quantifying relative transcript rates would provide insight into how the *ku70*$^{-/-}$ line has adapted to repair DSB.

One additional step to confirm the observed phenotypes in the *ku70*$^{-/-}$ line are a result of the knockout of *ku70* is to generate a rescue line. Our lab is currently working to accomplish this. Additionally, creation of a rescue line which only transcribes Ku70 in stages after embryo development may allow us to increase rates of HDR in embryos while avoiding phenotypes such as reduced longevity and pupal lethality.

Another experiment which is currently on going is knockout of other C-NHEJ genes including *ku80, xlf, DNA-PKcs* and *53BP1*. Generation of these mutant lines will allow us to

derive a fitness hierarchy for C-NHEJ genes in *Ae. aegypti*. This will contribute to the basic knowledge of the role of C-NHEJ in mosquitoes and will allow us to determine which knockout has the highest increased rates of HDR with the lowest impact on fitness. Given that we have created high throughput methods for determining rates of end joining repair and DNA DSB sensitivity we will be able to quickly quantify phenotypes in these mutant lines.

In the final research chapter, we quantified rates of NHEJ, SSA, DNA damage sensitivity and mutagenesis in our *ku70*$^{-/-}$ strain. The results from the SSA and NHEJ assays were interesting because of how they conflict with the results we observed when knocking down *ku70* (Basu et al., 2015). They suggest that flexibility within pathway selection allows for adaptability in fixing DSB. As mentioned above, transcriptomic analysis of the *ku70*$^{-/-}$ line should provide more insight into how our *ku70*$^{-/-}$ line is compensating for the absence of Ku70.

To quantify DNA DSB damage sensitivity, we utilized the antibiotic bleomycin to generate DSB in our *ku70*$^{-/-}$ and LVP lines. The results showed that our *ku70*$^{-/-}$ line is significantly more sensitive to bleomycin than the LVP line. The third replicate also suggests that while having one functional copy of *ku70* reduces sensitivity at lower concentrations of bleomycin, at higher concentrations *ku70* is haplosufficient. Lastly, the bleomycin assays show that we can quickly assess DNA damage sensitivity in mutants and will be useful in quantifying DNA damage sensitivity in future C-NHEJ mutants.

Lastly, we looked at rates of mutagenesis between our *ku70*$^{-/-}$ line and our LVP line. Our results suggest that knockout of *ku70* reduces fitness in embryos resulting in drastically lower survival rates after microinjection. Additionally, the *ku70*$^{-/-}$ line showed significantly lower rates of mutagenesis at 100ng/µl Cas9 concentration, hinting that rates of HDR may be higher in *ku70*$^{-/-}$ than in LVP. Future experiments should include increasing the number of *ku70*$^{-/-}$ embryos

injected, as well as targeting A-NHEJ genes such as *parp1*, *xrcc1* and *xrcc3* to assess how removal of A-NHEJ genes impact rates of mutagenesis.

In conclusion, our results show that we have created a luminescence-based tool which can be used in both embryos and cells to identify shifts in error prone DNA damage. Targeting C-NHEJ genes in cells for knockdown via RNAi results in shifts in error prone repair. The removal of *ku70* results in reduced longevity, female pupal lethality, and slowed chorion melanization. Rates of SSA and NHEJ were similar between our $ku70^{-/-}$ and LVP lines. While we were unable to detect a shift in either SSA or NHEJ, the $ku70^{-/-}$ line is sensitive to DNA DSB induced by bleomycin. Lastly, the $ku70^{-/-}$ embryos displays lower rates of mutagenesis when compared to our LVP line.

REFERENCES

ALPHEY, L., MCKEMEY, A., NIMMO, D., NEIRA OVIEDO, M., LACROIX, R., MATZEN, K. & BEECH, C. 2013. Genetic control of *Aedes* mosquitoes. Pathogens and global health, 107, 170-179.

BASU, S., ARYAN, A., OVERCASH, J. M., SAMUEL, G. H., ANDERSON, M. A., DAHLEM, T. J., MYLES, K. M. & ADELMAN, Z. N. 2015. Silencing of end-joining repair for efficient site-specific gene insertion after TALEN/CRISPR mutagenesis in *Aedes aegypti*. Proc Natl Acad Sci U S A, 112, 4038-43.

BETERMIER, M., BERTRAND, P. & LOPEZ, B. S. 2014. Is non-homologous end-joining really an inherently error-prone process? PLoS Genet, 10, e1004086.

HUANG, Y.-J. S., HIGGS, S. & VANLANDINGHAM, D. L. 2017. Biological Control Strategies for Mosquito Vectors of Arboviruses. Insects, 8, 21.

KANG, Y.-J. & YAN, C. T. 2018. Regulation of DNA repair in the absence of classical non-homologous end joining. DNA Repair, 68, 34-40.