Engineering of Dengue Virus Refractoriness in *Aedes aegypti* and Development of an Underdominant Gene Drive System in *Drosophila melanogaster*

Thesis by

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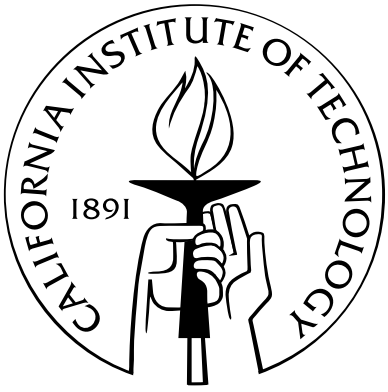

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Without you, this would all have been possible, but much less fun, Jeeves.

Acknowledgements

At the end of my Ph.D journey I feel both great joy and sadness. It has been a truly transformative time, and it could not have been the trip it was without my advisor for of all this work, Bruce Hay. I have learned innumerable 'things', but far more importantly, I have learned to think, to make connections, and even to just plain observe. These lessons were not always learned without trials and tribulations, but I feel they are what have changed me from a student into a scientist. I could have gotten a degree from many places, but I am thankful for Bruce, who is responsible for helping me develop into the researcher I am today.

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Even though much of my work was independent, I was undoubtedly part of a team. I want to especially thank Dr. Chun-Hong Chen for his support, both scientific and moral, in the early years, and Katie Kennedy for listening, learning, and her invaluable help in the lab more recently. Many other members of the Hay lab have also given help, guidance, and puzzles to solve that have kept me busy and engaged during my tenure at Caltech. I also want to thank and congratulate Gal Barak and Margaret Chiu—my star undergraduates who kept up with their projects no matter where their studies took them, and who will graduate alongside me this June. Ladies, you are going amazing places.

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Flores. 'Support staff' doesn't begin to cover it—thank you so much!

I came into the BMB program with a true cast of characters—Phil, Fred, Russ, Johnson, and Cambrian: We won't always have UCC, but maybe that's for the best. I've also made other amazing, lifelong friends and I can't wait to see what we accomplish in the years ahead.

Lastly, I owe the largest thank you to my parents, sister, and brother for seeing me through this journey. Somehow, knowing you can always go home makes it easier to forge ahead. I've also gained new family since starting my degree, so thank you new, lovely husband, Andrew, Parents II, and that baby, Jack!

Abstract

Vector-borne diseases have a profound impact on world health. The two most well-known and costly diseases are dengue fever and malaria, both spread by mosquito vectors. In the last decade, many new solutions to halting the spread of these diseases have been sought, including vector-mediated disease suppression. The work presented here seeks to generate alleles to effect this suppression, and engineer a drive system to replace the native population. Additional work on systems to keep engineered organisms genetically isolated from native populations has also been carried out. Initial studies in *C. elegans* investigated use of the transitive nature of RNAi in this species to genetically isolate one population from another. This type of speciation could be used in plant populations to limit gene flow of engineered crops into local environments.

The next series of studies details work on engineering of refractoriness alleles. Dengue virus has several enzymatic activities that are essential for its replicative cycle, including an RNA-dependednt RNA polymerase (RdRp) responsible for synthesizing both the sense and antisense viral genomes and a protease responsible for several essential cleavages of the viral polyprotein. Artificial substrates for these proteins were created to act as sensors, triggering an apoptotic response when viral infection occurs. Several generations of constructs were tested, but so far no completely functional sensor has been generated.

Lastly, a series of underdominant gene drive architectures were built and tested in *Drosophila melanogaster*. Initial systems utilized a *Drosophila* cell death protein, Hid, as toxin, and engineered microRNAs designed to target the Hid proteins as antidote. Two toxin-antidote pairs were mismatched and positioned on separate chromosomes so that an organism carrying both chromosomes survives, but an organism carrying only a single chromosome is unviable. Construction of a proof-of-principle in the eye was successful, but work in essential tissues is ongoing. Systems using engineered microRNAs as toxins and

resupply of the native protein as antidote were tested in essential tissues. Testing of many components has contributed to the development of these systems, but a complete system has not yet been constructed.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

I decided on Caltech for my Ph.D. after an April visit filled with sunshine and calm—something of a miracle coming from a Boston not-quite-spring. When I began this adventure almost 7 years ago, I knew I wanted to work on research that was somewhat applied. Given that, I really wasn't sure where to settle until I heard Bruce give his research preview talk for the first-year students, presumably to lure us in. I was sold then and there. I was enchanted by the clever projects, but also by the chance to work on something that had a direct application to the lives of those in need.

Fighting Dengue with Mosquitoes

Medea, a powerful gene drive system, was almost complete when I joined the lab, so I leapt at the chance to work on her cargo, the genes that would do the disease fighting in the wild. There were so many ideas and so much work to be done. In my first year in the lab I worked in *C. elegans* and *Drosophila*, with mosquito and fly cell lines, and with dengue and yellow fever—all of this with no prior experience in any of those systems. *Aedes* and mice were to be added to my repertoire later on. The first two years were a battle to keep up with the possibilities of what to work on, and to keep a realistic check on what had to be learned just to get started on the projects. It was a trial by fire, and an empowering time to realize there is knowledge in this community that is there for the asking.

My primary objectives in the first few years were to design and test dengue refractoriness alleles. As I worked in the lab, I also learned about the financial and social costs of the virus: almost a third of the world's population lives in dengue-endemic areas and it is considered a neglected disease by the WHO. Further, the range of the main vector, *Aedes*

aegypti, has been increasing since the 1960s due to many factors including urbanization and globalization, but also possibly due to the loss of DDT as a powerful pesticide and global warming (Wu et al., 2009; Knobler et al., 2006). The four related serotypes have made vaccine generation difficult and slow (Coller and Clements, 2011; Murrell et al., 2011), giving traction to alternative disease-fighting methods. Broad use of genetically modified crops have opened the door to the possibility of releasing engineered organisms into the wild; hopefully one day soon we will use insects to fight the battles against dengue and malaria. I am excited to be part of the team breaking that trail.

Drive

As the field of genetically modified insects has grown and evolved—and it has significantly during my time in the lab—demand for additional drive systems has increased. Eventually, my focus shifted to exploring new methods of delivering these disease-resistance alleles into native, naïve populations.

Refractoriness alleles penalize the animal carrying them by forcing the organism to perform an additional task that is above and beyond their basic genetic mandate of living and reproducing. This fitness cost prevents the alleles from spreading in natural populations: A gene-drive system is required. Some communities may want drive systems that spread beyond their release area, and others may not. Drive should be quick, but many communities may not want irreversible population replacement. This kind of flexibility has not yet been developed, so my work on gene drive aims to expand and diversify the drive systems available to our 'customers', the people living with vector-borne disease.

Genetic Isolation

With the advent of genetically engineered organisms, concerns have arisen about the flow of genes between engineered individuals and the native species from which they were derived. In particular, the plant community has found that public concerns have profound effects on their ability to deliver important products to both first-world businesses and third-world populations in need of better crop yields and nutritionally supplemented foods (Hails, 2000). Community-wide resistance to plants with exogenous genetic material has resulted in bans in some countries and widespread skepticism among the general public domestically (Gaskell

et al., 1999; Fernandez Cornejo and Caswell, 2006). Going forward, it will be increasingly important to have engineered organisms that are tightly genetically isolated if they will be cultivated or released into the wild. Generating fully genetically isolated synthetic species is of increasing interest to many including biologists, agriculturists, and even epidemiologists.

Here I detail work on engineering refractoriness alleles for use in *Aedes aegypti* targeting dengue virus, progress in the development of an underdominant gene drive system, and a small piece on the use of RNAi-based approaches to reproductively isolate genetically modified organisms.

Chapter 2

Harnessing Transitive RNAi to Generate Genetically Isolated *C. elegans*

2.1 Introduction

Project Motivations

The goal of this project is the creation of a genetically isolated *C. elegans* line through creation of an engineered line able to detect wild-type chromosomes and selectively kill heterozygous progeny. The approach is based on the observed phenomenology of transitive RNAi, which affords silencing of genes upstream of the original target through unprimed generation of $\sim$ 22nt RNA molecules by endogenous RNA-dependent RNA polymerases (RdRps) (Alder et al., 2003). The spreading of silencing was first observed bidirectionally in plants through introduction of vectors containing exogenous, promoterless DNAs (Voinnet et al., 1998; Vaistij et al., 2002), but has since been observed in *C. elegans* (Sijen et al., 2001, 2007), and shown to be triggered by shRNAs, including microRNA (miRNA) mimics (Shimamura et al., 2007; Poethig et al., 2006; Manavella et al., 2012). Transitive RNAi is not triggered in either *Anopheles* or *Drosophila* (Roignant et al., 2003; Hoa et al., 2003), however if the proof of principle were accomplished, the strategy could be used in the generation of genetically isolated plants as a method of gene flow control.

Due to its broad existence in eukaryotes (Shabalina and Koonin, 2008), post-transcriptional silencing has become a powerful tool in studying higher-order biological systems. Theoretically, any transcript can be targeted specifically provided it has some unique sequence in

its spliced mRNA, and this provides a wide range of potential toxins in the form of lost essential transcripts. In practice, there seem to be a large variety of factors that influence the ability of a gene to be targeted using a specific sequence and approach. The transitive nature of RNAi-induced silencing in *C. elegans* further expands the flexibility of RNAi-based knockdown by allowing the targeting of a downstream sequence to effect silencing of an upstream target (Figure 2.1).

Figure 2.1: (a) Initial studies using long dsRNA-targeting GFP showed that silencing of upstream transcripts, most often an uncoordinated (Unc) gene, was a result of specific targeting of downstream regions (Alder et al., 2003). (b) Later on, shRNAs were used and functional RNAi molecules were detected which targeted upstream regions. This transitive behavior was shown to be unprimed generation of $\sim$ 22 nt RNAs (Sijen et al., 2007).

The challenge inherent in generating a genetically isolated population is the sensing of the individuals' genetic makeup as self or other. The result of this detection must then trigger a switch that allows viability of those found to be self, and destruction of those progeny which are other. Nature does this continually in the sense that most animals cannot mate outside their own species and create viable, fertile offspring, although vigorous hybrids do exist and may contribute to evolution in their own right (Rieseberg et al., 2003). The courses nature takes for this detection are often complex and not well understood (Kao et al., 2009). For our purposes, however, the population should be as closely related as possible to its parent. Ideally, it will fill an identical biological niche, mate freely with its wild counterpart, and compete well enough to ultimately replace the native population if population replacement is desired. At a minimum, gene flow between engineered and wild populations will be prevented. Key to our approach is the use of RNAi to detect a small difference in a single mRNA such that if an animal bears a single copy (is heterozygous wild type) it will be detected. If the targeted region is not present, RNAi targeting it will

be unable to effect a phenotype, but if one copy of the gene bears the target, both will be silenced, as the upstream siRNAs generated from the target-bearing transcript silence the non-bearing allele via transitive RNAi.

Target Selection

A mutant allele (*ok825*) of *cdc-42*, a cell-division cycle gene in *C. elegans* containing a 632bp deletion beginning in the 3'UTR of the gene. was identified as an appropriate target for this project. MiRNAs designed to target a site within the deletion are innocuous in the mutant strain, but should cause suppression of *cdc-42* in a wild-type background. Loss of Cdc-42 results in disruptions in cell polarity (Etienne-Manneville, 2004). Crossing an *ok825* strain bearing the miRNAs to the wild-type N2 strain should generate lethality in trans-heterozygous progeny (Figure 2.2) if expressed broadly, as with the *858* promoter, or movement defects if expressed under *myo-3*, a body-wall-muscle promoter.

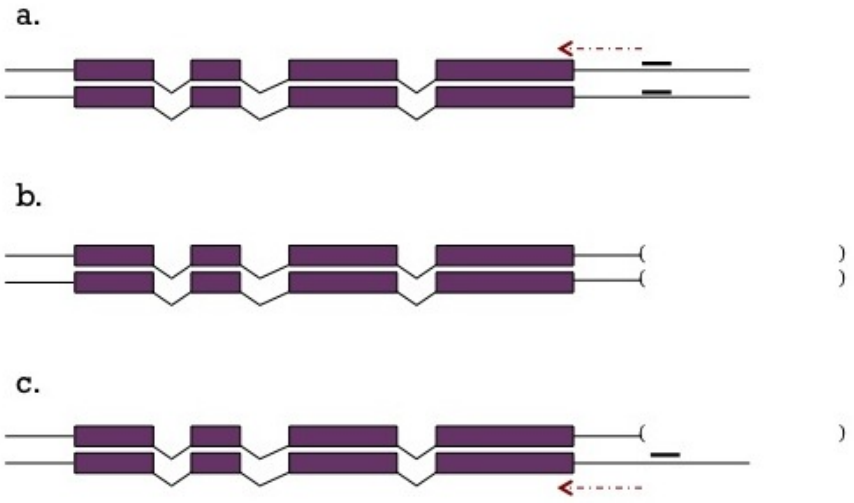


Figure 2.2: (a) In a wild-type animal, silencing of the essential transcript will occur through both sequence-specific targeting and via 5' transitive silencing. (b) In a mutant, the miRNA will be unable to target the transcript and the animal will remain viable. (c) Heterozygous animals will have their wild-type transcript silenced normally, while the mutant chromosome will be silenced only via transitive RNAi produced from the wild-type transcript.

2.2 Results and Discussion

The first task was to generate a male-enriched *ok825* line so that once transgenics were obtained, crosses could be carried out. Attempts were made to generate an *ok825; him-5(e1490)* strain. *ok825* hermaphrodites were mated to *him-5* males. Cross-progeny hermaphrodites, $\frac{ok825}{+};\frac{him-5}{+}$, were self crossed. These self crosses were predominantly sterile. Of the few progeny that did survive, all lacked the *ok825* allele as scored by PCR. It appears that the *him-5* and *ok825* alleles somehow interact, resulting in sterility or embryonic lethality. This was not pursued, although in future other male-enriched lines could be tested for compatibility with the *ok-825* allele. This approach was abandoned and males were generated post transformation through either heat shock or simple collection of naturally occurring male individuals. The *ok825* line obtained from the stock center may be slightly male-enriched based on the ease of finding males when required.

I next assembled a miR targeting the native Cdc-42 sequence. The site targeted is CGATGAGCATGATTCCAGAATT and begins 9bps inside of the ok825 deletion region. Ten transgenic lines were isolated using the bombardment protocol. These carried a myo2:: GFP marker (pharyngeal cells) and the Cdc-42 targeting miR in the pPD95.86 backbone (pPD96.86-Cdc42miR), which drives expression in the body wall muscles under the myo-3 promoter. Unfortunately, no F1s gave F2 GFP positive progeny. Personal communication from John DeModena indicated that use of GFP as a positive marker for transformation via bombardment has not been successful. GFP-positive individuals generated in this way are usually mosaics and the transgene is not heritable. Additionally, isolating transformants is very difficult because of the numbers of progeny generated that must be screened from the bombardment protocol. In the future, the *unc-119* movement-based scoring protocol should be used. For this experiment it was not feasible because it was essential to generate transformants in the mutant background.

The myo2::GFP marker and construct pPD95.86-Cdc42miR were co-precipitated with pBluescriptSK+ as carrier DNA and injected by Harmonee Kim. I screened progeny from these worms and isolated high transmission array lines. Three integrated lines were generated through γ-irradiation. When outcrossed to *unc-119* and N2, no genetic isolation was observed. Progeny were healthy, carried one copy of the deletion, one of the wild-type gene, and the transgene.

I next tried using a different miR backbone that targeted the same site. The *let-7* backbone was selected because of its demonstrated efficacy in generating transitive RNAi in *C. elegans* (Sijen et al., 2007). The myo-2::gfp and pPD118.25-Cdc42/let-7 DNAs were prepped and delivered to Steven Kuntz, who did one round of microinjection. pPD118.25 contains the *let-858* promoter and is broadly expressed. The resulting worms yielded several high-transmission lines. γ-irradiation yielded two integrated lines that were crossed to *unc-119*. Again, these yielded healthy, mobile, GPF-positive progeny, indicating the transgene was being transmitted without killing the heterozygous progeny.

2.3 Future Implementations

Due to increasing interest in genetic isolation in plants and a dearth of truly flexible, modular systems to accomplish it, reopening this project in *C. elegans* or even *A. thaliana* may be worthwhile. Demonstrating genetic isolation via a pathway known to function in plants may offer a viable alternatives to approaches in use that rely on imperfect phenotypes like seed shattering, or physical methods like barrier crops.

MiRNA Design

The biggest unknowns with respect to these results are the efficacy of the miRNAs and the suitability of *cdc-42* as a target. Neither miRNA was tested in a homozygous wild-type background, so it may be that they fail to target efficiently in the first instance, or the transitive nature of the response is weak, or both.

A great deal of work has been done using the let-7 backbone *in vitro* since this project was set aside, and it might be possible to design improved miRNAs based on this backbone. Probably due to the ease of feeding long dsRNA to elicit phenotypes in *C. elegans*, not much work has been done optimizing miRNA function, but lessons might be learned from better-studied systems like *Drosophila*. The testing of multiple backbones targeting a single site known to work might be called for.

Target Selection

Target selection in *Drosophila* seems to be one of the most sensitive parameters in successfully targeting a gene with miRNAs, and this may hold true for *C. elegans* as well.

Phenocopying a known loss-of-function phenotype might be an important control for assessing miRNA efficiency as a function of target site. Additionally, if a haplo-insufficient target could be selected, this would increase the sensitivity of the system immensely, and might reduce the level of knockdown necessary for a functional system.

Knock-in technology also broadens the range of targets available. We worked with an extant mutant, but this is not necessarily the best way to approach target selection at the gene level. Many changes to UTRs can be tolerated, and even changes to the coding region can be benign if the transcript is not especially sensitive to codon bias effects. A best case scenario would involve the targeting of the coding sequence of a gene that is mutated at the DNA level to preserve wild-type protein function but leave it vulnerable to targeting by engineered miRNAs. The modified transcript could be knocked-in to its native locus, thereby creating an 'invisible' change where health and behavior are concerned. The new strain would be wild type in all but its response to the presence of the engineered miRNAs. This type of engineering would also allow the incorporation of a fluorescent marker, and the linking of the miRNA to the un-targetable target.

Unfortunately, work is just beginning on the development of knock-in systems in plants and *C. elegans*. The closest the worm field seems to get is an effort at site-specific integration using a *Drosophila* class II transposon Mos1. Even with the planned insertion library, the type of knock-in required for this project is not possible (Robert and Bessereau, 2007). The field is interested in directed gene manipulation in worms, with FLP-based and Mos1–transposon-based systems recently described (Robert and Bessereau, 2011; Vazquez-Manrique et al., 2010). According to Wormbook, low levels of true knock-in intergrations have been observed via bombardment, but the documentation is minimal. In plants, the situation is better with actual fusions to endogenous genes having been produced in rice (Yamauchi et al., 2009), so perhaps studies would proceed more fruitfully in *A. thaliana*.

2.4 Materials and Methods

C. elegans culture and strains

Worms were cultured according to standard methods (Brenner, 1974) at 20°C on *E. coli* OP-50. Strain RB942, bearing the *ok825* allele, was obtained from the *Caenorhabditis* Genetics Center. Additional strains used in this study—wild-type N2, *him-5(e1490)* (Hodgkin et al.,

1979), and *unc-119(ed3)* (Maduro and Pilgrim, 1995)—were generously provided by the Sternberg laboratory.

The GFP marker, myo-2::gfp, was observed on a fluorescence stereomicroscope in the Sternberg laboratory. Phenotypes induced by miRNA expression were scored based on movement and viability of L3, L4, and adult worms.

Plasmid Construction

Four plasmids were constructed each containing a single miR6.1 miRNA targeting *cdc-42*. The miRNA was constructed through two rounds of PCR. First, two inner primers were annealed and amplified. A second round of PCR was carried out to add either an NheI/KpnI or EcoRI/NotI pair of restriction digest sites to the flanks. These primers also carried internal BglII and BamHI sites for construction of a doublet, but this miRNA was used singly. The miRNA assembly procedure is further documented in Appendix D. Primers are shown below with restriction sites underlined. Primers for EcoRI/NotI cloning are not shown. The site targeted is CGATGAGCATGATTCCAGAATT and begins 9bps inside of the *ok825* deletion region. Cloning of the synthetic miRNA into a vector was carried out via traditional methods following digestion of Fire lab plasmids pPD95.86 (NheI/KpnI), pPD118.25 (NheI/KpnI), pPD134.96 (EcoRI/NotI), and pPD134.99 (EcoRI/NotI) obtained from Addgene (Cambridge, MA). A second generation of plasmid was constructed using a *C. elegans let-7* backbone in place of *Drosophila's* miR6.1, and the same target. Strategic mispairings in the stem were intended to help preserve stem structure. This miR was cloned into the pPD118.25 backbone (NheI/KpnI).

Generation of Transgenics

Transgenics were generated through bombardment (Wilm et al., 1999) and microinjection (Mello et al., 1991). Steven Kuntz's bombardment protocol (see Appendix A) was adapted for use with the *ok825* strain. 6μgs of DNA consisting of a 1:1 ratio of myo2::gfp and pPD95.86-Cdc42miR were used for bombardment. Instead of screening for rescue of the uncoordinated phenotype, as is usual for locating bombardment transformants, screening for GFP-positive transformants was carried out on a fluorescence stereoscope in the Sternberg lab.

Primers for the Assembly of *cdc-42* miRNAs

Inner Primers miR6.1	Fwd	5' - GGCAGCTTACTTAAACTTAATCACAGCCTTTAATGTCGAT GAGCATGATTCCAGACTTTAAGTTAATATACCATATC
	Rev	5' - AATAATGATGTTAGGCACTTTAGGTACCGATGAGCATGAT TCCAGAATTTAGATATGGTATATTAACTTAAAGT
Outer Primers miR6.1	Fwd KpnI/BglII	5' - GGCGGTACCGCCAGATCTGTTTAAAGTCCACAACTCATC AAGGAAAATGAAAGTCAAAGTTGGCAGCTTACTTAAACTTA
	Rev NheI/BamHI	5' - GGCCGCTAGCACGGATCCAAAACGGCATGGTTATTCGTG TGCCAAAAAAAAAAAAAAATTAAATAATGATGTTAGGCAC
Inner Primers let-7	Fwd	5' - GAAAGTTGTGAGAGCAAGACGACGCAGCTTCGTAAGAGT CTGTCTCCGGCGACGAGCATCACCCCAATAATTTCACCGGTG GTAATATTC
	Rev	5' - AGGCAAGCAGGCGATTGGTGGACGGTCTACACTGTGGAT CCGGCGATGAGCATGATTCCAGAATTTTTGGAATATTACCAC CGGTGAAAA
Outer Primers let-7	Fwd KpnI	5' - GGCGGTACCGCCAGATCTAAAATAAAGAAAAACAAAGAG GTGAAAGTAAGAGGAGGAAGAAAACGAAAAGAAAGTTGTGA GAGCAAGACG
	Rev NheI	5' - GGCCGCTAGCCACATCTCCCTTTGAATTTATATGTCTAAT TTAACAACAAGTACTAATCCATTTTTCAGGCAAGCAGGCGAT TGGTGG

Two rounds of microinjections were also carried out by Harmonee Kim and later Steven Kuntz. The first round utilized the same DNAs as above, but the second round utilized the second-generation *let-7* based miRNA in the pPD118.25 backbone. Transformants were isolated and γ-irradiation integrations were performed using a Cs137 source. Integrated transformants were outcrossed to wild-type N2 and *unc-119(ed3)* strains to assess genetic isolation characteristics. Worm genotypes with respect to the *ok825* allele were confirmed via PCR using primers KJD7 and KJD8.

Chapter 3

Engineering Refractoriness to Dengue Virus

3.1 Introduction

Project Motivations

Myriad pathogens are spread to humans via arthropod hosts. This study focuses on dengue virus (DENV), an important reemerging pathogen in tropical regions, and its main insect vector, *Aedes aegypti*. The fight against dengue in humans has mainly focused on vaccination, an approach which often presents an efficient and (relatively) cost-effective method of controlling viral pathogens and the diseases they cause. The creation of a DENV vaccine has proven difficult and, and while there are good candidates in development, there are none ready for deployment. Presented here are efforts to develop a new mode of control for the suppression of insect-borne disease while circumventing the need for vaccine development, individualized vaccine delivery or medical care, and chemical suppression of vector populations which have this far failed to break the transmission cycle and slow the spread of dengue virus.

The project goal is to use endogenous insect proteins, or other engineered biomolecules, to detect the presence of DENV. Coupled with, or integral to, the sensor is an apoptotic signal that triggers cell death only in infected cells. There are two main families of approaches here, the first focusing on using the viral protease to activate components of the cell death pathway and the second using the RNAi pathway coupled with the viral RdRp to induce an apoptotic response. We used *Drosophila* as our model because many of the cell death components are well characterized in that system and because culture and transgenesis are better developed than in *Aedes aegypti*.

Dengue Virus

DENV, which causes dengue fever, is a member of the family flaviviridae, that also includes yellow fever virus, Japanese encephalitis, tick-borne encephalitis, and West Nile virus. Infection with DENV is typically characterized by fever, headache, nausea, vomiting, and joint pain. The World Health Organization estimates that there are approximately 50 million cases of dengue fever each year. Of those, 1% result in dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), much more serious conditions that require medical intervention and result in death $\sim 2.5\%$ of the time (WHO, 2012).

The virus is largely transmitted by the *Aedes aegypti* mosquito in urban and semi-urban environments. It breeds predominantly in man-made containers, and increased urbanization and reduced efficacy and use of pesticides have increased the spread of the virus. Current methods of controlling the virus include chemical suppression of mosquito populations during outbreaks and educating the public to reduce man-made standing water breeding environments such as flower pots. These methods work with only limited success as the virus continues to emerge and become endemic over larger geographic areas.

DENV circulates as four serotypes and exposure to one confers only partial, temporary immunity to any of the other three. Exposure to one type can also cause a more severe infection upon exposure to the other serotypes due to antibody-dependent enhancement. Currently, no vaccine is available and treatment mainly consists of clinical management of symptoms. With proper care, even DHF and DSS are rarely fatal, although the level of treatment required is not always available in areas where dengue is endemic (WHO, 2012).

Viral Features

The DENV genome consists of a single, positive-stranded RNA of ~ 11 kb that encodes 5' and 3' untranslated regions (UTRs) and a single precursor polyprotein. The polyprotein consists of the membrane, envelope, and capsid proteins in addition to seven nonstructural proteins, NS1-NS5 (Chambers et al., 1990). During maturation, the polyprotein undergoes a series of proteolytic cleavages carried out by the viral protease and several cellular proteases (Falgout and Markoff, 1995; Falgout et al., 1991). The other main enzymatic function of the virus is carried out by its RNA-dependent RNA polymerase (RdRp) which is responsible for making both positive and negative strands of viral RNA for replication and packaging (Chambers et al., 1990).

a.

b.

	DENV1	DENV2	DENV3	DENV4
2A/2B	KIWGRK SWPLNE	NTSKRR SWPLNE	DTKLRR SWPLNE	KGASRR SWPLNE
2B/3	QKKKQR SGVLWD	EVKKQR AGVLWD	QKQTQR SGVLWD	QVKTQR SGAWLD
3/4A	FAAGRR SVSGDL	FAAGRK SLTLNL	GAAGRK SIALDL	GASGRK SITLDI
4B/5	LGGGRR GTGAQG	TTNTRR GTGNIG	VGTGKR GTGSQG	AQTPRR GTGTTG

Figure 3.1: (a) The DENV RNA consists of 3 structural, capsid (C), membrane (prM), and envelope (E) proteins, and seven nonstructural (NS) proteins, as shown. They are translated as a single polyprotein that is then cleaved by a combination of viral and cellular proteases. (b) The four cleavage sites in the DENV polyprotein in each of the four serotypes. The residue N-terminal to the cleavage site is shown in red and the residue C-terminal is in orange.

The Viral Protease

The DENV protease is encoded in the 180 amino terminal amino acids of the NS3 protein and it cleaves both in *cis* and in *trans*. It must cleave multiple target sites, all of which share certain sequence characteristics, including charged residues N-terminal to the cleavage site and small uncharged residues C-terminal to it (Figure 3.1). The protease functionality requires NS2B as a cofactor. It is autolytically cleaved from the NS2A and NS3 proteins. NS2B is largely hydrophobic and embeds in the endoplasmic reticulum (ER) as a trans-membrane protein. A set of 40 amino acids of NS2B compose a hydrophilic domain that is necessary and sufficient as a cofactor to the NS3 protease (Falgout et al., 1991; Preugschat et al., 1990; Zhang et al., 1992).

A recombinant protein has been constructed fusing, via a flexible linker, the 40 amino acid hydrophilic region, $NS2B_{40}$, to the NS3 protease, and it cleaves successfully in vitro (Leung, 2001). A cleavage consensus sequence was determined using the cleavage sites of all four serotypes (Li, 2005), and it was adapted for use in this work. Some of the viral protease is sequestered out of the cytoplasm in viral replication complexes that are closely associated with cellular membranes derived from the endoplasmic reticulum (Mackenzie et al., 1998; Westaway et al., 1997). Some studies indicate, that the bulk of the protease is found in a cytoplasmic fraction accessible to trypsin digestion (Uchil and Satchidanandam, 2003).

The Viral RNA-Dependent RNA Polymerase

The RdRp is one of the functionalities of NS5, the largest and most highly conserved of the viral proteins (Chambers et al., 1990). It synthesizes the viral RNA efficiently and specifically, to the exclusion of cellular mRNAs (Grdzelishvili et al., 2005; Ranjith-Kumar et al., 2003). Cyclization of the viral genome is required for replication and is believed to be accomplished by long-range RNA-RNA interactions through highly conserved sequences on either end of the RNA molecule, and a conserved stem-loop on the 3' end as shown in Figure 3.2 (Alvarez et al., 2005). Additionally, it has been shown that when these elements alone are present at the ends of non-viral RNA sequences, the viral RdRp will perform negative strand synthesis (Filomatori et al., 2006). A cytoplasmically expressed mRNA containing the conserved cyclizing sequences has the potential to be transformed into a double-stranded intermediate by the viral RdRp upon viral infection. Studies carried out *in vitro* have shown that the 3' terminal region of the (–) strand alone is sufficient for RNA synthesis as well (Nomaguchi et al., 2004).

NS5 has two other functions: it is a methyl transferase and a guanylyl transferase (Zhou et al., 2007). Like the protease, the majority of the RdRp protein exists cytoplasmically, but enough is sequestered in or near the ER in replication complexes to allow viral replication.

Drosophila as a model

Drosophila has served for over 100 years as a model organism in biology, but has not yet proven its utility as a model for disease-bearing arthropods. For these studies, *Drosophila* is used as a model of one of its own order, diptera. While great differences exist between mosquitoes and flies, we have chosen to work in the better-understood insect. The two species diverged approximately 250 million years ago, as opposed to the most divergent *Drosophila* species that diverged from one another 40–60 million years ago (Severson et al., 2004).

Elements of the Cell Death Pathway

The two classes of sensors explored here take advantage of the known components of the *Drosophila* apoptotic pathway. The initial designs for the protease-based sensors centered on the effector (downstream) caspase, drICE, a protein that carries out the cell death process

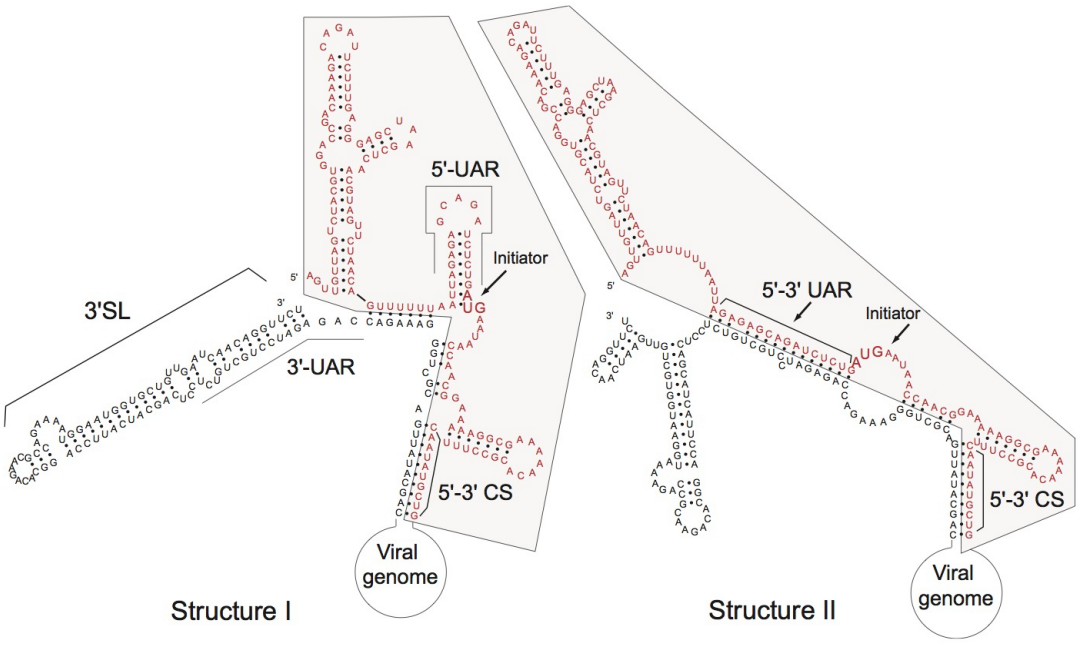


Figure 3.2: Reproduced above is a figure from Alvarez et al. (2005) showing two possible folding structures of a circularized DENV genome. That study also shows that mutations in the 5' end resulting in loss of pairing with the 3' end abolish function that can be rescued by compensatory mutations, indicating it is the structure that the viral RdRp recognises and the exact sequence is somewhat plastic.

by cleaving many cellular proteins. drICE is produced in the cell as a zymogen and is activated upon cleavage by an initiator (upstream) caspase, Dronc (Hawkins et al., 2000). drICE activity can be suppressed by an inhibitor of apoptosis protein, DIAP1, but only after drICEs 20 amino terminal residues have been removed (Yan et al., 2004). Based on tissue culture studies in *Drosophila* S2 cells, drICE has been shown to sensitize cells to apoptosis when overexpressed and to be required for apoptosis (Fraser and Evan, 1997; Fraser et al., 1997; Muro et al., 2004). No phenotype is observed when drICE is overexpressed in the fly eye. Control of drICE cleavage, and thus activation, *in vivo* is a step toward controlling the apoptotic response.

Upstream of both DIAP1 and drICE are Reaper (Rpr), head involution defective (Hid), Grim and Sickle (SKL), the RHG proteins. The RHG proteins are pro-apoptotic and induce caspase-dependent death by disrupting the interactions between the IAPs and initiator and effector caspases (Bergmann et al., 2003; Hay and Guo, 2004). The N terminus of Hid carries a conserved IAP binding motif, AVPF, that has been shown to interact with

DIAP1 and be required for its pro-apoptotic activity (Hay, 2000). If the N-terminal motif is not exposed, the ability of Hid to induce cell death is compromised. Normally, Hid is at least partially inhibited by the Ras pathway, through MAPK-dependent phosphorylation. A mutant form lacking the five natural MAP Kinase phosphorylation sites that evades inhibition by Ras and exhibits a much greater killing activity was used in this study (Bergmann et al., 1998). If the activity of Hid can be precisely triggered, the cell death response can be controlled. A schematic of the *Drosophila* cell death pathway is shown in Figure 3.3.

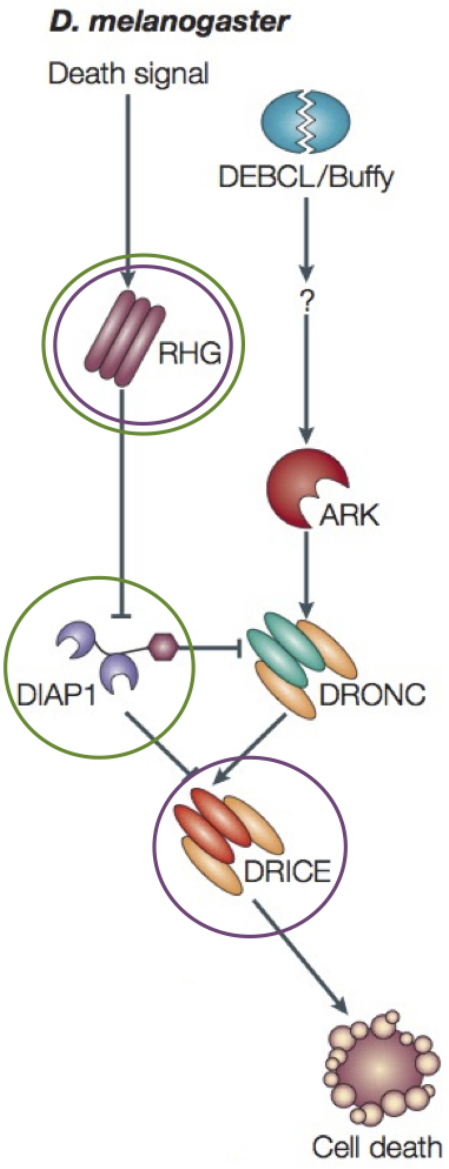


Figure 3.3: This a partial reproduction of a figure from Hay and Guo (2004) showing the cell death pathway in *Drosophila*. Circled in green are the proteins targeted in the RdRp-based approaches and in purple the proteins targeted with the protease-based approaches.

Mosquito Mediated Pathogen Control

For over a decade, the scientific community has been working toward the genetic engineering of mosquitoes as a possible method of disease control, mainly focusing on malaria (James and Collins, 1996), although there has been more work done with dengue and *Aedes aegypti* of late. Fewer than ten years ago, *A. aegypti* was successfully transformed using a method similar to the P-element transformation common in *Drosophila*, allowing the introduction of new traits (Coates et al., 1998; Spradling, 1986). Shortly thereafter, *A. aegypti* was engineered to express an antibacterial factor, Defensin A, under the control of the promoter of a bloodmeal-activated gene, vitellogenin. The expression resulted in antibacterial activity and persisted for at least 20 days (Kokoza et al., 2000). Since the early work, a great deal more progress has been made. Franz et al. (2006) engineered an RNAi based refractoriness cassette and showed suppression of DENV infection in the laboratory, but there may

be issues with the stability of that line. After more than 17 generations, the expression of the RNAi cassette seems to be suppressed and DENV is able to replicate and be detected in the salivary glands of the insects (Franz et al., 2009). Ongoing work may answer questions about how and why this happened, but it certainly must be addressed before this strategy could be implemented in the wild. The other, and most successful, approach is the use of a Wolbachia infected line of mosquitoes. These not only spread Wolbachia, but the bacterial infection itself suppresses DENV replication. The authors do note that the Wolbachia strains that give the best Dengue protection also bear the largest fitness cost, so this must be addressed, but field trials are going forward (Hoffmann et al., 2011; Walker et al., 2011). This stain also side steps the hurdles of public acceptance of genetically modified (GM) animals because the infection of the mosquitoes with a *Drosophila* endosymbiant is not characterized as genetic modification. The insects are not subject to the regulatory frameworks being developed to handle releases of GM animals into the wild (Barro et al., 2011).

Creation of a Genetic Driver

An essential component to the concept of GM mosquitoes fighting disease is the ability to push the genetic components into the insect population to fixation quickly, even if their presence results in a fitness cost. The mainstay of gene drive in the Hay lab is Maternal Effect Dominant Embryonic Arrest (Medea), a selfish genetic element that can drive to fixation in *Drosophila* populations (Chen et al., 2007). I did participate in Medea based projects, but they will not be discussed here. My work on a separate gene drive system, Underdominance, can be found in Chapter 4.

3.2 Results and Discussion

3.2.1 Viral Protease-Based Sensing & Killing

Fly Results

The first pair of flies generated were GMR-drICE^{vp} and GMR-NS2B$_{40}$/NS3$_{185}$. The downstream caspase, drICE, was modified to contain a viral protease consensus sequence LKRR-SGSG (Li, 2005) where its endogenous Dronc cleavage site is located. The natural drICE cleavage sites (D217 and D230) and its IAP binding site (A29) were mutated away to render

it insensitive to IAP-dependent inhibition. This drICE^{vp} was cloned into a transformation vector containing the eye-specific GMR promoter. A fusion of the viral protease domain of NS3, NS3$_{185}$, and its cofactor, NS2B$_{40}$, joined by a flexible linker was also cloned into the GMR vector. The injection of GMR-drICE^{vp} resulted in the generation of 15 lines, and all the flies had healthy, wild-type eyes. From the GMR-NS2B$_{40}$/NS3$_{185}$ injection, 19 lines were isolated, but only 5 showed mild or no phenotype in the eye. Most of the lines gave small-eye phenotypes and were so sick as to be unmaintainable. I worked with lines with wild-type eyes, as we wanted to see the effect of our sensor, not effects of over expression of the protease. When the GMR-drICE^{vp} and GMR-NS2B$_{40}$/NS3$_{185}$ flies were crossed together, death was observed (Figure 3.4), but, as evidenced by the full sized eye, was occurring post-differentiation and only in some cells. This indicated that the protease was able to trigger a drICE-induced cell death phenotype, but that the effect was not

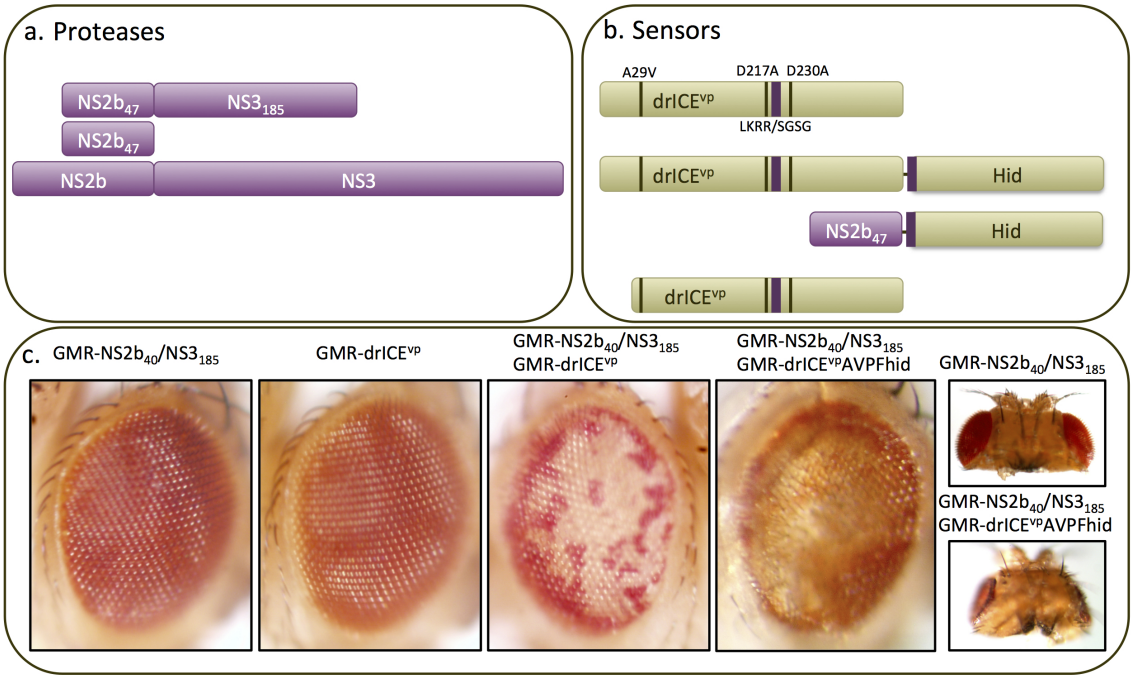


Figure 3.4: Shown in (a) are the three proteases expressed under the GMR promoter. (b) The four substrates tested. (c) Phenotypes of some of the constructs in (a) and (b). Killing late in eye development was achieved with drICE alone, and improved with a Hid fusion.

strong. This effect was observed when protease-bearing flies were crossed to several different GMR-drICE^{vp} lines, so it is unlikely that it was a function of too little sensor in a specific line. Killing could be limited by amount of protease, because I intentionally selected lines with lower and more-specific expression patterns, but I did not assess the level of protease

expression compared to that in a native viral infection in a mosquito.

To improve killing, a second round of flies were generated: GMR-NS2BfAVPFhid, the NS2B co-factor's hydrophilic domain alone fused via a flexible linker containing viral cleavage sites to the cell death protein Hid, GMR-drICE^{vp}AVPFhid, the modified drICE caspase fused via the same linker to Hid, and GMR-pdldrICE^{vp}, a modified drICE that also lacked its prodomain, as sensors, GMR-NS2B/NS3FL, a full-length fusion of the NS2B co-factor and the NS3 protease, and GMR-NS2Bf, the hydrophilic fragment of the NS2B co-factor, as proteases. Schematics of these constructs are shown in Figure 3.4. The flies carrying a full-length NS2B-NS3 fusion surprisingly gave no phenotype, perhaps because the intact protease sequesters itself in the ER membrane and therefore is not damaging to the cell. This was also true of the NS2B hydrophilic fragment alone. The GMR-drICE^{vp}AVPFhid did give some enhancement of the eye phenotype when crossed to the original GMR-NS2B$_{40}$/NS3$_{185}$ protease line, but I never achieved a Hid-like small-eye phenotype from crossing any of the modified drICE constructs to the viral protease lines.

To achieve a more realistic model, I attempted to infect adult *D. melanogaster* through feeding of DENV2-laden media. Both wild-type and Dcr-2 mutant strains were evaluated. If infection was successful, I expected to see viral-infection-induced death in the Dcr-2 mutants, but there was no significant difference in the survivorship of virally exposed flies compared to flies fed only serum after 12 days of observation (Table 3.1).

	w/ virus			w/o virus		
	Dcr-2	Dcr-2/+	w-	Dcr-2	Dcr-2/+	w-
Day 0	97	98	100	98	100	100
Day 1	96	97	100	98	100	100
Day 2	91	97	99	95	99	96
Day 3	90	97	98	96	99	95
Day 4	86	97	98	92	99	93
Day 5	81	96	93	90	99	93
Day 6	80	96	88	85	99	90
Day 7	75	93	88	77	99	88
Day 8	73	91	87	74	92	84
Day 10	71	83	82	65	83	72
Day 12	62	75	72	57	83	66

Table 3.1: Survivorship of flies fed viral-laden MEM

Tissue Culture Studies

I also attempted to infect *Drosophila* S2 cells with DENV2. Infection was monitored via immunofluorescence assay. Initially, infections were carried out identically to those for C6/36 cells. Schneider's media was removed and replaced with 200 μl of virus containing MEM. The cells were incubated for an hour and then the viral media was removed and the cells returned to Schneider's media. Attempts to assay for viral infection were unsuccessful because the less-adherent S2 cells were lost using the extant staining procedures for C6/36 cells. To improve adherence, I treated small, circular glass coverslips with Concanavalin A solution, as described in Appendix B (Sabatini, 2004), and then cultured S2 in wells containing these slides. The slides allowed the cells to adhere and were retained through the staining procedure. Initial immunofluorescence procedures created a great deal of background, but blocking with BSA reduced the autofluorescence or nonspecific binding. The final staining procedure is documented in Appendix B. Unfortunately, no staining was observed, indicating that viral replication was not occurring at significant levels in the S2 cells after 48 hours. I attempted culturing the S2 cells in 1XMEM, but they were not viable in that media. I then reversed the experiment and cultured the C6/36 cells in Schneider's medium, infected with virus-containing MEM media, and replaced the Schneider's media. The cells were not very healthy under this condition, but developed a robust viral infection, so I ruled out Schneider's media as limiting viral competence. Ironically, the C6/36 cells did not adhere well to the Concanavalin-A-treated slides, but I don't believe that the Concanavalin A interfered with infection. In an attempt to sensitize the S2 cells to infection, I pretreated with Dcr-2 dsRNA. The success of the Dcr-2 knockdown was evaluated by co-treatment with DIAP1 dsRNAs, which normally results in massive apoptotic death, but this was suppressed by the Dcr-2 knockdown (Figure 3.5). I was unable to achieve a robust DENV2 infection, even with the knockdown of the RNAi machinery, so I shifted my approach to focus on detecting the viral infection in C6/36 cells.

Tissue culture of *Aedes albopictus* cells (C6/36) was also explored as a method to assay constructs in the presence of a native DENV infection. Transfections were carried out using lipofectin, cellfectin, lipofectamine, and FuGENE6 along with a GFP marker, but I only had good success with FuGENE6. I surveyed ratios of FuGENE to DNA ranging from 2:2 to 9:2. Optimal transfection efficiency of around 15–20% was achieved using a ratio of 3:2. This is not a high enough transfection efficiency to use loss of cells an an accurate assay

1. dsRNA: DIAP1 2. – Cells killed on Day 1	1. dsRNA: Dcr-2 2. dsRNA:DIAP1 dsRNA: Dcr-2 4. Cells healthy No Infection	1. dsRNA: Dcr-2 2. dsRNA: Dcr-2 DENV2 infect. 4. Cells healthy No Infection
1. – 2. – 4. Cells healthy	1. dsRNA: Dcr-2 2. dsRNA: Dcr-2 4. Cells healthy No Infection	1. - 2. DENV2 infect. 4. Cells healthy No Infection

Figure 3.5: Cells were treated with dsRNAs targeting Dcr-2 in an effort to sensitize them to DENV2 infection. (1) On the first day, cells were treated with 40 μg of dsRNA. (2) On day 2, a second dsRNA treatment with or without a subsequent DENV2 infection was carried out. (4) On day 4, cells were observed and immunostained. No evidence of DENV2 infection was found in either Dcr-2-dsRNA-treated cells or untreated cells.

readout. A loss of 15% of the cells, the best case if the killing was absolute, is too subjective an analysis to be reliable. Instead, a GFP co-transfect with any of the sensors allowed us to use GFP as a marker of successful transfection, and then see its loss (dependent on the sensors) as the readout for a successful trial. Transfections shown as part of this study have variable FuGENE ratios as they were conducted as I was still optimizing the system, but no transfection represented had a transfection efficiency below 10–15%, and each was representative of the results I achieved.

I began the testing the same constructs that I had tested in the fly eye in C6/36 tissue culture. The proteases and drICE sensors were cloned into a heat shock plasmid for use in C6/36 cells kindly gifted by Bart Bryant in the Clem lab at Kansas State University (designated KHS in these studies). A sample transfection is shown in Table 3.2. No killing was observed before or after viral infection when using the contructs KHS-drICE^{vp}AVPFhid and pAc5.1-drICE^{vp}. I tried triggering them with both KHS-NS2B$_{40}$/NS3$_{185}$ and a native viral infection. The constructs themselves were well tolerated, but neither co-expression of the viral protease fusion nor infection with DENV2 instigated killing of transfected cells. Staining of cells after scoring for killing indicated that a robust viral infection had been achieved in wells that were treated.

Our first concern was that perhaps *D. melanogaster* cell death proteins were not effective

a.	Marker DNA KHS-GFP (μg)	KHS-drICE^{vp} (μg)	Balance DNA pBSK+ (μg)	FuGENE (μl)	Ratio	GFP signal upon infect
1	0.4		1.6	9	9:2	+++
2	0.4	1.6		9	9:2	+++
		KHS-drICE^{vp} AVPFhid				
3	0.4		1.6	9	9:2	+++
4	0.4	1.6		9	9:2	+++
		KHS-NS2B/ NS3				GFP signal No Infect
5	0.4		1.6	9	9:2	+++
6	0.4	1.6		9	9:2	+++
		KHS-NS2B/ NS3	KHS-drICE^{vp} AVPFhid			
7	0.4	0.8	0.8	9	9:2	+++
8	0.4	0.8	0.8	9	9:2	+++

b.	Marker DNA KHS-GFP (μg)	KHS-RHG (μg)	Balance DNA pBSK+ (μg)	FuGENE (μl)	Ratio	GFP signal No infect
1 Ctrl	0.4		1.6	9	9:2	+++
2 Rpr	0.4	1.6		9	9:2	-
3 Hid	0.4	1.6		9	9:2	-
4 Grim	0.4	1.6		9	9:2	-

Table 3.2: Tissue culture results for transfections using (a) fly-eye protease targets and (b) testing of RHG proteins in C6/36 culture

toxins in *Aedes albopictus* cells, so I took a step back and tested the ability of *D. melanogaster* cell death proteins Rpr, Hid, and Grim to kill in C6/36 cells. Transfection of KHS driving each of the RHG proteins individually gave complete ablation of GFP positive cells in repeated transfections. DNAs were carefully cleaned several times so that I could be sure the DNA treatment itself was not inducing death, and the cell culture itself looked healthy, indicating to us that only the transfected fraction was being killed. This led me to conclude that the RHG proteins as toxins would work in C6/36 cells, but that there was another barrier to killing. The next hurdle was making sure the sensors were exposed to the viral protease, so I made constructs targeting the sensors to the ER.

Initially, two synthetic transmembrane domains were synthesized, and each was fused via a flexible linker containing synthetic protease target sites to either Gal4 or FLP. The first domain, prMcleave, was a fragment of the virus itself, comprised of a small piece of the capsid, the entire prM protein and a small piece of the M protein. This fragment contains the first transmembrane domain of the virus. A fully synthetic fragment, p450cleave, was designed containing the N-terminal signal sequence and ER localization signal from

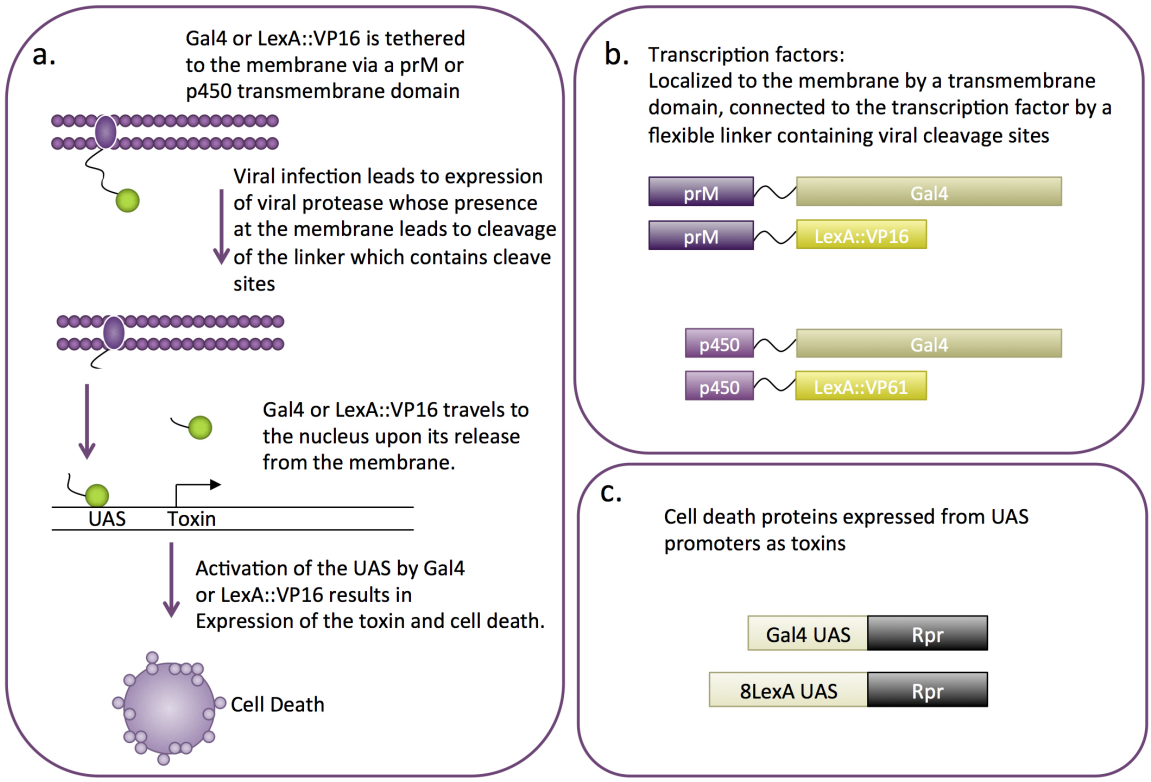


Figure 3.6: (a) This schematic shows the design rationale of the membrane-tethered transcription factor sensors. (b) The transcription factors Gal4 and LexA::VP16 are fused to transmembrane domains meant to target them to the endoplasmic reticulum via a flexible linker containing viral cleavage sites. (c) The toxin, expression of a cell death protein, is only expressed when the transcription factor is cleaved from its membrane tether and activates its UAS in the nucleus.

cytochrome p450, a highly conserved protein (Szczesna-Skorupa and Kemper, 2000). Both of these domains were intended to help the sensors co-localize with the replicating virus, thereby bringing the protease and sensor together. The linker contains multiple cleavage sites, and cleavage would result in the release of either FLP or Gal4 to travel to the nucleus, and by different mechanisms trigger a cell death response. These systems are more complex than the drICE-hid system because they require both the cleavable component and a downstream activity. For the Gal4 constructs, KHS-prMcleave::Gal4 and KHS-p450cleave::Gal4, the downstream activity is simply the expression of a cell death protein driven by Gal4's UAS once they are cleaved by the virus, as shown in Figure 3.6a. The UAS-RHG constructs are quite toxic to the cells when transfected at high concentrations, probably due to leakage from the basal promoter attached to the Gal4 UAS which is derived from hsp70 and known to express in C6/36 without any heat shock. I titrated the doses down to a level where no

killing was detectable, but no lower, as I wanted to use the highest dose possible. I observed no enhancement of killing when cells transfected with KHS-prMcleave and UAS-Hid were subjected to a DENV2 infection (Table 3.3). The trials with p450cleave produced identical results.

a.	KHS-GFP (μg)	UAS-Hid (μg)	prM-Gal4 (μg)	Balance DNA pBSK+ (μg)	FuGENE (μl)	Ratio	GFP signal upon infect
1	0.4			1.6	3	3:2	+++
2	0.4		1.6		3	3:2	++
3	0.4	1.6			3	3:2	-
4	0.4	0.2	0.4	1.0	3	3:2	++
5	0.4	0.15	0.4	1.05	3	3:2	+++
6	0.4	0.1	0.4	1.1	3	3:2	+++

b.	KHS-GFP (μg)	KHS-Gal 4 (μg)	UAS-GFP (μg)	UAS-Rpr (μg)	pBSK+ (μg)	FuGENE Ratio	GFP signal No infect
1	0.4				1.6	3:2	+++
2	0.4	0.4			1.2	3:2	+
3			0.4		1.6	3:2	-
4		0.4	0.4		1.2	3:2	+
5	0.4			0.2	1.0	3:2	+++

Table 3.3: (a) On its own, UAS-Hid is toxic in high concentrations, although this effect can be neutralised through the use of less DNA per transfection. The KHS-prMcleave::Gal4 construct can also confer some toxicity on its own. This is consistent with reports that Gal4 is not a neutral molecule and induces phenotypes in adult *D. melanogaster* as well. When the two molecules are co-transfected and then an infection is performed, no enhancement of killing is observed. (b) Gal4 fails to drive GFP or Rpr significantly in C6/36 cells

I then set out to test the function of Gal4 in C6/36 cells because I could not distinguish from our results whether we were getting cleavage and no UAS activation or whether cleavage was not occurring. I therefore expressed Gal4 on its own from KHS plasmid and used this to drive the RHG proteins and also GFP. Unfortunately, I found that Gal4 functions only very weakly in C6/36 cells (Table 3.3). This result is consistent with a report from the James lab at UC Irvine that Gal4 functions poorly in *Aedes*, although a more recent paper refutes this (Kokoza and Raikhel, 2011). Even if cleavage was occurring, very little cell death protein would be produced and I would observe no killing. I learned very little from this system because I had no way to determine whether no cleavage was occurring, whether cleavage was happening and the Gal4 was unable to reach its target, or whether everything was functioning, but expression off the UAS was just weak.

To circumvent the possible Gal4-UAS system failure in C6/36 cells, I replaced the Gal4 with a LexA::VP16 (LV) fusion, and changed to an 8LexA UAS fused to either a bottleneck-

supercore (bnkSCP) fragment as basal promoter or a fragment of the UASp promoter. The 8lexUASp promoter was leaky in C6/36 cells, resulting in uncontrollable killing, and so I focused my attention on the p8lexbnkSCP promoter driving Rpr and Hid. As a control, KHS-LV was transfected with 8lexbnkSCP and this yielded killing, indicating to us that the LexA::VP16 fusion I was using worked in the C6/36 cells and that I could test the function of our sensor because the output was sound. I carried out a series of control transfections and found that KHS-prMcleave::LV and KHS-p450cleave::LV constructs were well tolerated by the cells and did not result in death on their own as the Gal4 versions had. Co-transfections of the sensors with their targets, however, revealed that killing was occurring even without the addition of virus. It appeared that the LexA::VP16 was not being retained in the ER, but instead was localizing to the nucleus and activating transcription of Rpr. No matter how little of the KHS-prMcleave::LV or KHS-p450cleave::LV was used, killing still resulted. The p450 versions were more toxic than the prM versions.

Concurrently, I was testing the KHS-prMcleave::FLP and KHS-p450cleave::FLP constructs against a different type of reporter showed in Figure 3.7. Work had just begun in the lab on engineered underdominance, and Chun-Hong Chen had built a test construct consisting of a pair of FRT sites flanking a miRNA and two target sites for that miRNA. Downstream of the second FRT and the miRs, was space for an open reading frame to be cloned. I cloned wild-type Rpr and RrpKR which contains no lysines and cannot be ubiquitylated (Vernooy et al., 2002), downstream of the miR and its targets (See Figure 3.6). The design of this substrate ensures that if it is expressed without FLP, the miR is processed, and targets the same transcript it is processed from, ensuring that any expressed mRNAs are cleaved and targeted for degradation. Processessing of the miRNA will also cleave the cap off the transcript, destabilizing it. If flipping occurs, the mRNA produced will carry no miR and no targets and so, a capped, stable mRNA that bears the Rpr ORF is produced, resulting in cell death. Both KHS-synRprWT and KHS-synRprKR were slightly toxic, but could be transfected at reasonable levels (0.2 μg) without observable killing. As with the LV constructs, killing occurred when the KHS-prMcleave::FLP or KHS-p450cleave::FLP construct was co-transfected with its Rpr-bearing target. This also suggests that the transmembrane domains are not retaining the fusion proteins anchored to the ER.

Improved transmembrane targeting seemed to be the next logical improvement as killing

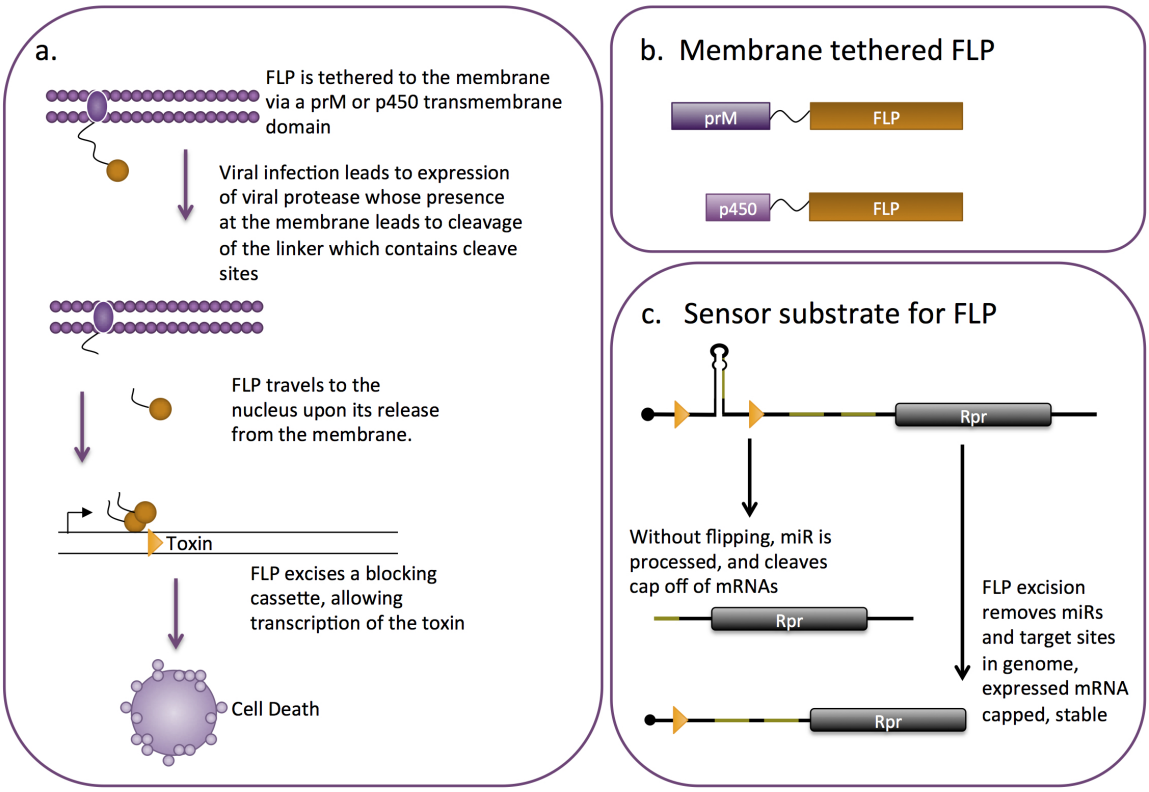


Figure 3.7: (a) This schematic shows the design rationale of the membrane tethered FLP-based sensors. (b) FLP is fused to transmembrane domains meant to target them to the endoplasmic reticulum via a flexible linker containing viral cleavage sites. (c) The KHS-synRprWT and KHS-synRprKR constructs, which bear the toxin, are benign before flipping because a miRNA that targets the transcript it is processed from is placed upstream of the toxin ORF. Once FLP excises the miRNA, expression of the toxin is free to occur.

and the targets were both functional. I again used a transmembrane domain, either from the prM or the envelope protein (E), and fused it downstream of CD4 which has been shown in *D. melanogaster* to keep cleavable fusion tethered to the membrane (Hawkins et al., 1999; Op De Beeck et al., 2004). The use of viral transmembrane domains was intended to help with colocalization of my transcripts with the viral protease. The same linker and LV as in the previous round were used. The function of these constructs is as described in Figure 3.6. The last membrane-targeting strategy I tried was a much smaller fusion of amino acids 1–29 of a protein called Erlin-1 (Browman et al., 2006). This fragment was fused N-terminal to a new set of viral cleavage sites and a LexA::VP16 fusion that had been codon optimized for insects and had several inconvenient restriction sites removed.

This round of transfections yielded us the most perplexing results yet. The Erlin construct activated the 8LexbnkSCP at even minuscule concentrations and so it was abandoned.

a.	GFP (μg)	8lexbnk SCP (μg)	Erlin:: LV (μg)	CD4E:: LV (μg)	CD4prM ::LV(μg)	pBSK+ (μg)	GFP signal No infect	GFP signal Upon infect
1	0.4					1.6	+++	+++
2	0.4	0.4				1.2	+++	++++
3	0.4	0.4	0.1			1.1	+	+
4	0.4	0.4		0.1		1.1	+++	++++
5	0.4	0.4			0.1	1.1	+++	++++

b.	GFP (μg)	8lexbnk SCP (μg)	NS2bf LV (μg)	CD4E:: LV (μg)	CD4prM ::LV(μg)	pBSK+ (μg)	GFP signal No infect	GFP signal Upon infect
1	0.4					1.6	++	++
2	0.4		0.4			1.2	++	+++
3	0.4	0.4		0.2		1.0	++	++
4	0.4	0.4	0.4	0.2		0.6	+	+
5	0.4	0.4			0.2	1.0	+	++
6	0.4	0.4	0.4		0.2	0.6	+	++

Table 3.4: (a) The KHS-Erlin::nLV construct is toxic when co-transfected with 8lexbnkSCP-rpr, indicating it is not being sequestered in the membrane prior to cleavage. The CD4E and M constructs do not localize to the nucleus precociously, but also do not kill upon infection. An increase of GFP signal results, possibly due to stress on the cells activating the KHS promoter, which is responsive to heat shock. (b) Adding a KHS-NS2bf construct to try to increase cleavage did not result in more killing.

In *D. melanogaster* the very N-terminal fragment of Erlin-1 is not enough to localize and retain the protein in the ER. The CD4 constructs were well tolerated, but no killing was induced upon infection. In fact, infection significantly increased our GFP signal. Upon reading more about historical usage of the hsp70 fragment, certain kinds of non-heat shock stress increase expression off these fragments. It is possible that something about these membrane-targeting constructs induces stress in the presence of a viral infection. I still wanted to test whether or not cleavage could be induced and the transcription factor could be released and get to the nucleus, so KHS-NS2bf (which does not carry the NS3 domain) was added to the transfections. This was meant to increase our odds of cleavage. The same result was obtained, however, with an increase in GFP signal upon infection with DENV2. It is important to note that NS2bf transfected alone also induced an increase in GFP and did not seem to be toxic, so it is unclear whether this fragment would cleave the targets in the cells.

At this time, we still do not fully understand these results. It may be valuable to go back to using the CD4-based constructs in *D. melanogaster* S2 cells because progress has been made in achieving infection of both S2 cells and adult *Drosophila* (Chotkowski et al., 2008; Mukherjee and Hanley, 2010). The strategies used in those studies to sensitize *Drosophila* to

flaviviral infection are not very different from those approaches I tried. Knockdown of Dcr-2, Argonaut, and a healthy dose of patience resulted in success where I failed. There is even a flaviviral strain that is adapted to S2 cells and differs at only a few amino acids from the native virus. The better-understood pathways in S2 cells may still make them a fruitful test bed, especially now that the staining protocols and strategies to make them more adherent have been developed. It should be noted that assaying for virus was done more rigorously via plaque assay in both of these studies. Also, in light of the Dcr-2 deficiency of the C6/36 cell line (personal communication, Ken Olson), it may not be a good choice for the testing of our constructs, although this is more true of the constructs in the next section.

3.2.2 RdRp-Dependent Sensing & Killing

Fly Results

A family of constructs designed to be substrates for the viral RdRp were generated. The 5' and 3' ends of dengue virus (D5 and D3), responsible for circularizing and allowing initiation of transcription, were used to flank an antisense fragment of the DIAP1 (D) coding sequence. A HDV ribozyme (R) was cloned downstream of the D3 fragment to cleave it specifically at the known 3' end of the viral RNA genome, and a tubulin 3'UTR was used to help with proper transcription. This generated the construct D5DD3RT, meant to act as a stable mRNA bearing both the 5' and 3' ends of the dengue genome that would be a substrate for minus strand synthesis by the NS5 RdRp expected to be floating in the cytoplasm during a dengue infection. It was cloned into the same GMR promoter bearing transformation vector used previously for expression in the fly eye, producing construct pGMR-D5DD3RT. The 5' end of the mRNA would bear the 5'UTR normally downstream of the GMR transcription start site. The rationale behind these constructs was that the DIAP1 antisense, when transcribed by the RdRp, would produce a fragment of the sense strand of DIAP. Together with the original mRNA produced from the D5DD3RT, this would generate dsRNA molecules of DIAP1. Knockdown of DIAP in *Drosophila* results in efficient apoptotic killing of cells. Individually, the NS5 and its substrate should be benign.

Over ten lines of each GMR-NS5 and GMR-D5DD3RT were generated, and all of them had healthy, wild-type eyes. When crossed together, there was no change in eye size, health or patterning. Tissue culture studies were beginning at that point, and so I focused on those.

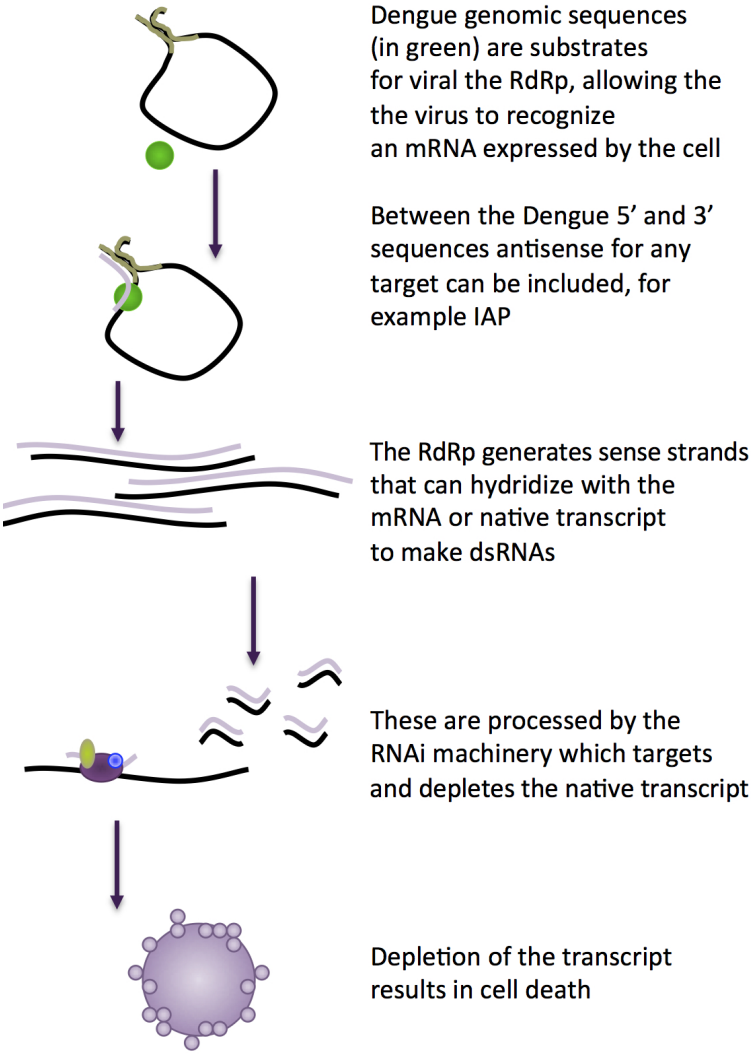


Figure 3.8: A schematic of the proposed function of the RdRp sensor

Cell Culture Results

Versions of the RdRp substrate described above were made for testing in tissue culture. As I was working it C6/36 cells, the anti-sense fragment used was from *Aedes albopictus* IAP (A). They were cloned into the KHS vector used in the viral protease studies and also utilized the 5' and 3' ends of dengue. The pGMR-D5DD3RT was modified so that when cloned into KHS the dengue 5' fragment initiated precisely at its 5' end without any extra nucleotides, producing construct KHS-D5AD3RT. A second construct, KHS-AD3(–)R, was built that carried the AAIAP fragment, a dengue antisense 3' fragment (D3'(–)) and the HDV ribozyme. These constructs rely on the production of dsRNAs that are processed and induce RNAi-based suppression of the AAIAP to kill cells (Figure 3.8). We carried out a

large number of transfections, but never observed killing from these transcripts.

I built and evaluated miRs against AAIAP (targeting sites TCACCGATAACAAAGTTAAAGA and GAAGATGAACCAAATAGAAAAC) to test whether or not suppression of IAP in *Aedes albopictus* cells had the same effect as in S2 cells, but only saw weak induction of apoptosis. I also used stabilized antisense fragments of AAIAP to carry out this investigation, but I will not report the full details of what we did because about two years after these studies were put on hold, we received information from a collaborator, Dr. Ken Olson at Colorado State, about the competence of C6/36 cells for RNAi. He indicated that the cell line has a defective Dcr-2 gene and does not generate the 21 bp dsRNAs characteristic of the RNAi response. Even our miRNAs may have been hampered by the lack of a functioning Dcr-2, despite normal processing of miRs by Dcr-1. Because of their complete pairing through the middle of the stem, they may be loaded preferentially into the Dcr-2 RISC complexes. Mis-pairing in the middle section is thought to direct miRNAs to the Ago1/Dcr-1 machinery (Lee et al., 2004). Some work has been done in the lab to understand how the miR6.1 synthetic miRs are processed, but the data are not clear.

In any case, the experiments would best be repeated using the Aag2 cell line, although it also has limitations. Aag2s are persistently infected with cell fusing agent virus, which incites a large RNAi response of its own, even before a hengue infection is initiated. Although the Aag2 is competent for dengue infection, a deep sequencing run on an Aag2 sample infected with both viruses yielded about 40-fold more small RNAs against CFA virus compared to dengue. Titers of dengue from Aag2 infections 5 days post infection are around 10^5 as compared to C6/36 which are around 10^7. Additionally, the IAP in *Aedes aegypti* has not been identified, although other targets could be utilized. Constructs targeting GFP could offer a way of testing the ability of the Dengue RdRp to replicate the artificial 'genomes' we created without having an apoptotic protein to target.

I also generated a set of RdRp substrates that would not be dependent on the action of the RNAi machinery, but instead would rely on the viral RdRp synthesizing a sense-strand mRNA of a toxic transcript. I used the bacterial toxin, Barnase, as my lethal transcript. Barnase and Barstar are a toxin-antidote pair produced by *Bacillus amyloliquefaciens*. Barnase is a ribonuclease meant to be excreted and Barstar is its intracellular inhibitor that keeps Barnase from killing the cell producing it (Hartley, 1989). The design of these constructs depended on transfection with a reverse-complement of Barnase being innocuous

until the viral RdRp created a sense strand that would be competent for the translation of a functional Barnase molecule, leading to cell death.

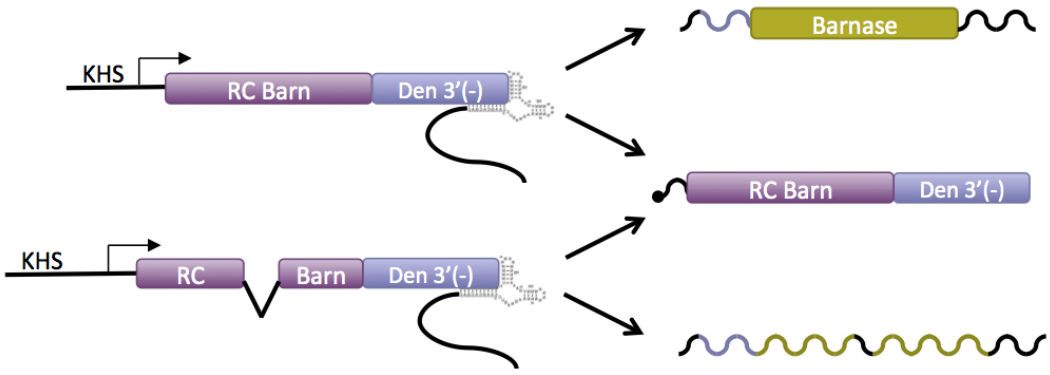


Figure 3.9: The initial Barnase construct was designed to produce a capped mRNA carrying a full-length reverse complement of Barnase that can be reverse transcribed by the viral RdRp, making a protein coding transcript. If the cellular machinery produces the complementary strand, however, the sense strand of Barnase, competent for translation, is produced. By putting an intron in the forward direction with respect to the promoter, any spurious transcription of the 'wrong' strand results in an mRNA without a functional Barnase open reading frame.

The initial design, which relied on the 3' end of the (–) strand of the dengue genome acting as an attractive initiation site for the viral RdRp, carried the full length reverse-complement of Barnase. In concept, this was predicted to be tightly 'off', that is, not expressed, until a viral infection occurred. In practice, transfection of DNAs result in multicopy arrays of DNAs entering the cell, and it appears that expression of both sense and antisense occurred, because a great deal of killing was observed when the original Barnase sensor was transfected. Enough Barnase protein was produced to kill the transfected cells, even when introduced as a small fraction of the transfection mixture.

We never tested this theory directly, but we did synthesize a new Barnase construct. It also carried the 3'(–) strand of the the DENV2 and a full length Barnase reverse complement, but an intron was inserted in the Barnase reverse complement such that if produced from the plasmid in the correct direction (making an mRNA with the reverse complement of Barnase) the intron would be spliced out and a viral sensor would be produced. If, however, spurious transcription occurred which would produce a sense strand of Barnase, the transcript would contain a stretch of nonsense coding RNA with stops and random amino acids produced so that no functional protein would result. A schematic of the two constructs is shown in Figure 3.9.

Transfection of this construct was nontoxic, giving some indirect evidence that expression of a sense mRNA from the first generation Barnase sensor had been produced. dengue infection did not trigger killing of the cells, when an infection was carried out 24 hours post transfection of the new Barnase sensor. In my hands, the 3'(–) fragment of dengue was insufficient for the RdRp to initiate synthesis of the negative strand. A simple further test would be to clone the intron bearing Barnase between the dengue 5' and 3' ends to see if together enough of the (–) strand could be produced to allow translation of Barnase and kill cells. Testing of the RdRp on this substrate *in vitro* would answer the question of whether or not the substrates can be recognized and used by the virus. If they were, the only hurdle remaining would be bringing the NS5 protein and the sensor into contact.

3.2.3 PKR-Mediated Killing

A study in mammalian tissue culture showed that a fusion between the dsRNA binding domain of PKR and apaf-1 resulted in apaf-1 proteins able to be brought into close proximity when dsRNA is present due to the PKR dsRNA binding domain. The binding of apaf-1 to procaspases allows them to reciprocally cleave one another, activating the hand triggering cell death. Viral infections, including a dengue infection, in the presence of this fusion PKR-apaf-1 induced apoptosis in infected cells while leaving uninfected cells untouched (Rider et al., 2011). I built a similar set of constructs including the fusion of the *D. melanogaster* homologue of apaf-1, ark, and the dsRNA binding domain of PKR and both the components separately. Viral infection did not kill the transfected, infected cells. While the cell death pathway architectures are very similar between mammalian systems and *Drosophila*, there does seem to be a difference in which components dominate the function of the pathway, and where in the pathway the key decision points are located (Hay and Guo, 2006). In *Drosophila* IAP1 is a powerful regulator of cell death, and as part of the normal functioning system, suppresses the somewhat constitutive activity of Dronc, the caspase ark activates. Therefore, it may not be surprising that further activation of ark, and therefore Dronc is not enough of a signal to induce apoptotic death in mosquito cells. Figure 3.10 shows how these constructs were expected to function and a summary of the fly cell death pathway.

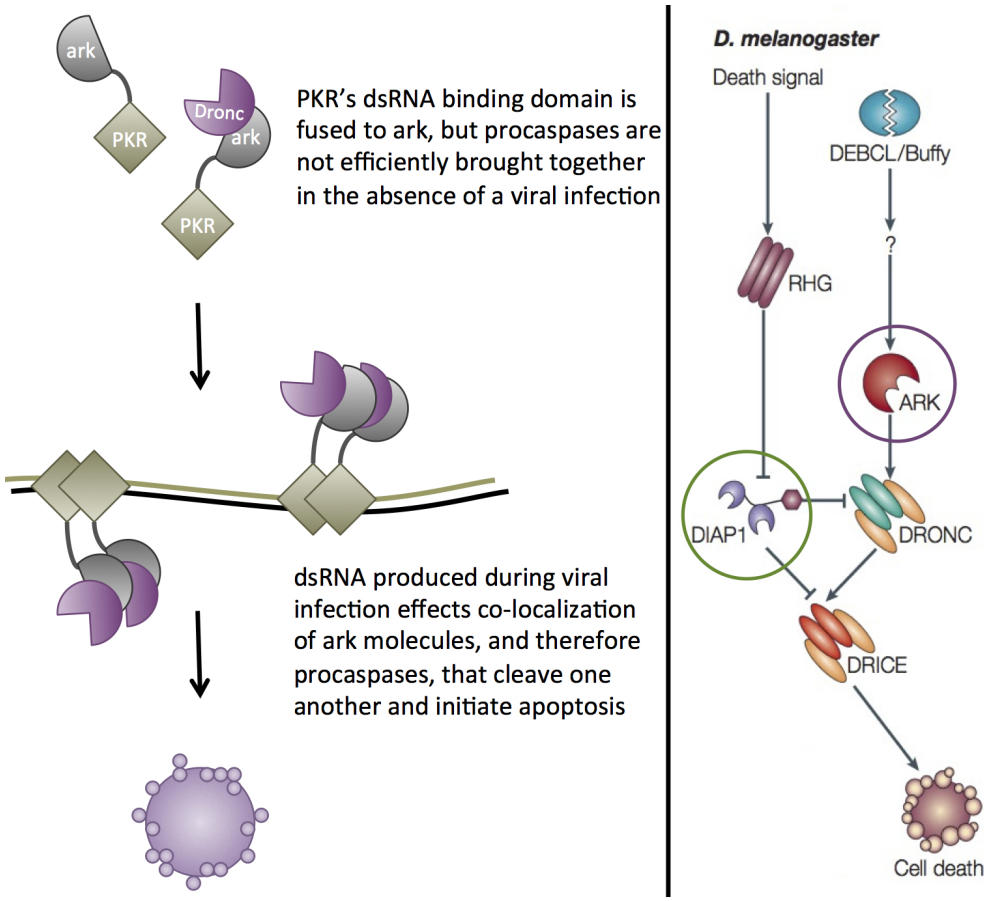


Figure 3.10: Infection with dengue results in dsRNA production. The PKR dsRNA binding domain fused to the ark protein instigates co-localization of multiple arks. These bind the procaspase form of Dronc and allow the molecules to reciprocally cleave each other. This should induce apoptosis. DIAP1 is the master regulator of *D. melanogaster* cell death, and may not allow activation of Dronc to induce apoptosis in the absence of other signals.

3.3 Future Directions

Protease approaches

In light of the advances in S2 and adult *D. melanogaster* infection (Chotkowski et al., 2008; Mukherjee and Hanley, 2010), I think future work on the protease approaches should be continued in S2 cells. Based on the information available, a reliable infection protocol can be developed, and a wide variety of designs could be tested readily because S2 cells are more easily and reliably transfected. Any design that worked in S2 cells would have to be moved to either C6/36 or Aag2 cells for final testing before a mosquito was built, but with both cell lines available in the lab, this should not present a problem.

I believe that the biggest hurdle to the protease approach will be achieving co-localization

of the sensor and the viral protease. The difficulty of tethering the transcription factor to the membrane has been solved by use of the CD4 domain, but with the system I had previously developed, separating the behavior of the other parts of the system was not possible. If GFP were cloned in downstream of the linker with a nuclear localization signal on it, its localization to the membrane and then release upon infection could be monitored. The localization and cleavage could both be monitored using that single construct, and hopefully improved. Once those two variables are sorted out, then the transcription factors could be substituted in for the GFP and the sensor would have a good likelihood of working.

RdRp approaches

For the RdRp approaches, the first and easiest step lies in testing all the constructs that are extant in the Aag2 cell lines. Literature shows that the RdRp is competent to replicate artificial substrates *in vitro* (Nomaguchi et al., 2003, 2004) and produces subgenomic fragments *in vivo* (Pijlman et al., 2008; Li et al., 2011; Pesko et al., 2012). This implies that it should be possible to create an artificial fragment that is able to be amplified in tissue culture. Putting a GFP fragment as the RNAi inducing moiety would give a simple read out that would not be expected to be toxic. It would be worth while to try both sense and antisense between the dengue fragments as its unclear which would be most efficacious. In S2 cell studies, expression of antisense alone has not triggered an RNAi response so it should be 'safe' for tests and with the virus producing both strands it may ultimately induce better suppression.

The Barnase construct bearing the intron would be especially interesting to pursue because the protein is toxic in such low doses and the current construct is tolerated well by the cells. Even a small amount of reverse transcription by the virus might be enough to trigger death. Trying a variety of dengue fragments to elicit reverse transcription by the virus should offer a short term set of trials that could yield a functional sensor and killer in one, small construct.

Additionally, NS5 is known to exist broadly in the cytoplasm, but a significant fraction also localizes to the nucleus (Pryor et al., 2007) and so it could be that trying to retain our sensors in the nucleus is also a viable route toward getting the RdRp to replicate them. Getting the virally produced transcripts to then export for processing would then become a key step, however.

PKR approach

The PKR approach is also worth a little more effort and creativity. While the dimerization of ark may not prove toxic in insects, there are other proteins whose functionalities require dimerization. One class that comes to mind is restriction enzymes. Class II restriction enzymes are often homodimers, and some, such as FokI, require dimerization for function. There is a crystal structure of FokI that shows its dimerization surface (Wah et al., 1998) and there is a known mutant that can no longer dimerize. The cleavage of this mutant is greatly compromised (Bitinaite et al., 1998). If the dsRNA binding domain from PKR were fused to a dimerization deficient version of FokI, this could serve as a sensor and killer. The trouble with this approach is that the enzyme once dimerized would need to be able translate to the nucleus, bind and cleave DNA. There are probably many other candidates, such as RNases, out there that could prove toxic when dimerized as a function of PKR binding of dsRNA.

To test directly the ability of PKR to dimerize protein domains in insect cells in the presence of a viral infection, there is a split GFP that could be utilized (Ghosh et al., 2000). It has been used with small, artificial coiled-coils, but it would be an empirical question as to whether or not it would tolerate the larger PKR dsRNA binding domain.

Despite not succeeding in creating a functional sensor, the field has advanced since I last worked on most of these designs. I think each of the approaches has a way forward that is worth exploring.

3.4 Materials and Methods

Vector Construction: Viral Protease-Based Approach

A modified drICE (drICE^{vp}) with three point mutations, A29V, D217A and D230A, and the region around its endogenous cleavage site (TMQRSQ) replaced with the viral cleavage site consensus (LKRRSGSG) was generated by fusion PCR and site-specific mutagenesis (primers shown in Table 3.5) and cloned into pGMR-1N using EcoRI and NotI restriction sites. The previously described $NS2B_{40}/NS3_{185}$ fusion protease (Li, 2005) derived from DENV2(TSV01) was PCRed to add restriction sites and cloned into pGMR-1N and pCaSpeR-hs using EcoRI and NotI restriction sites. The $NS2B_{40}$ (NS2Bf) cofactor and drICE^{vp} were each fused via PCR to a short linker containing half the viral protease consen-

sus sequence (G_4S-**LKRR**-AVPF) and a *hid* gain-of-function allele, Hid^{Ala3}, from Bergmann et al. (1998), and cloned into pGMR-1N using EcoRI and NotI restriction sites. A full length NS2B-NS3 fusion (NS2B-NS3FL) was assembled from plasmids kindly gifted from Richard Kuhn's lab at Purdue (Bera et al., 2007), and cloned via a three piece ligation into the HpaI and NotI sites of pGMR-1N. NS2Bf was also cloned into GMR-1N on its own. A prodomain-less version of $drICE^{vp}$ that lacks amino acids 1-28 of endogenous drICE was cloned between the BglII and NotI restriction sites in pGMR-1N. All of the constructs described above were used to generate germ line transformants by standard methods (Spradling and Rubin, 1982) producing flies GMR-$drICE^{vp}$, GMR-$NS2B_{40}/NS3_{185}$, hs-$NS2B_{40}/NS3_{185}$, GMR-NS2BfAVPFhid, GMR-$drICE^{vp}$AVPFhid, GMR-NS2BNS3FL, GMR-NS2Bf, and GMR-pdl$drICE^{vp}$.[1] Schematics of the constructs are shown in Figure 3.4.

A series of constructs for use in *Drosophila* S2 and *Aedes albopictus* C6/36 tissue culture were also built. $drICE^{vp}$ and $drICE^{vp}$AVPFhid were cloned into pAc5.1/V5-HisB (Invitrogen, Carlsbad, CA) and an *Aedes albopictus* expression vector, pHsp70 (designated KHS in this work), from the Clem lab at Kansas State University (Clem and Miller, 1994).

Two artificial proteins were synthesized. prMcleave and p450cleave and their sequences can be found in Appendix B. These were cloned into KHS using EcoRI and BglII. Fused in-frame downstream of each were Flippase (FLP), Gal4, and LexA::VP16 (LV) (from the University of Massachusetts Medical School) using cloning sites BglII and NotI yielding constructs KHS-prMcleave::FLP, KHS-prMcleave::Gal4, KHS-prMcleave::LV, KHS-p450cleav::FLP, KHS-p450cleav::Gal4, and KHS-p450cleave::LV. H. Huang originally cloned prMcleave::Gal4 and p450cleave::Gal4 into pCaSpeR-HS Actin, but they were then sub cloned into KHS. FLP was PCRed from BH1465. The LV transcription factor binds to an 8LexA enhancer which was PCRed from pSH18-34 (Invitrogen, Carlsbad, CA) and fused to a fragment of the bottleneck promoter. A basal super core promoter (SCP) (Juven-Gershon et al., 2006), was fused downstream of this fragment generating p8LexbnkSCP. Another 8Lex promoter was constructed by the fusion of the UASp promoter element fused to the 8Lex sites and cloned into KHS XbaI and ApaI with a PstI site between the 8LexA and downstream elements. Rpr and Hid were cloned downstream using restriction sites ApaI and NotI.

The second generation ER targeting proteins were designed to carry amino acids 1-371 of CD4, either a prM or E transmembrane domain and a flexible linker with viral protease

[1] The GMR-$NS2B_{40}/NS3_{185}$ line used in crosses has been lost, as have the original GMR-$drICE^{vp}$ lines.

cleavage sites fused in frame with a recoded LexA::VP16 (nLV) protein. The final construct in this series carried amino acids 1-29 of Erlin-1, a modified linker and nLV which was synthesized. Each of these ORFs was cloned into KHS. The sequences for the components used are given in Appendix B.

Gal4 and LexA::VP16 were cloned EcoRI/NotI and blunt/NotI, respectively, into KHS. GFP was cloned into the pUASt vector (BH1517) EcoRI/NotI. Rpr, Grim, and Hid were sub cloned EcoRI/NotI into KHS from BH stocks 1482, 1483, and 1484 respectively. *H.sapiens* PKR, a kind gift of the Pierce lab at Caltech, was blunt/NotI cloned into KHS, pGMR-1N and pUASt. A portion of the Dcr-2 ORF was cloned into pBlueScriptKS(+) (pBSK) EcoR1/NotI for generation of dsRNA using the Epicentre AmpliScipe High Yield Transcription Kits (Epicentre, Madison, WI).

Name	Sequence 5' to 3'
drICE^{vp} 5' Frag F	GCG GAA TTC CAA CCA AAA TGG ACG CCA CTA ACA ATG GAG AA
drICE^{vp} Viral Site R	GCC GGA GCC GGA GCG CTT CAG CAC TCC GCC AGC CAA TCT GTC GCC CTG GCA GGC CT
drICE^{vp} Viral Site F	CTG AAG CGC CGC TCC GGC TCC GGC ACG GAA ACC GCT GGC GAC TCC TCG ATG AGC TAC AAG
drICE^{vp} 3' Frag R	GCG GCC GCT CAA ACC CGT CCG GCT GGT GCC AA
drICE^{vp} Site specific mut F	CAG CCC AAC GAT CAC ACA GAT GTG CTG GGC TCC GTG GAT CCG GAG
drICE^{vp} Site specefic mut R	CTC CGG ATC CAC GGA GCC CAG CAC ATC TGT GTG ATC GTT GGG CTG
NS2B$_{40}$/NS3$_{185}$ F	CGC GCG AAT TCC AAC CAA AAT GGC TGA TTT GGA ACT GGA GAG
NS2B$_{40}$/NS3$_{185}$ R	CGC GCG CGG CCG CTT ACT TTC GAA AGA TGT CAT CTT CA
NS2B Full length F	GGC GCG GTT AAC CAA CCA AAA TGA GGA GCT GGC CAT TAA ATG AGG CTA TC
NS3 Full length (fusion) R	CCG GGG CGG CCG CCT ACT TTC GGA AAA TGT CAT CTT CGA TCT C
NS2B/NS3 Full length internal F	GCG GCA AAG AAG GAA CAT TCC ATA CAA TGT GG
NS3 Full length R	GGC GGC GGC CGC CTA TTT CTT CCG GCT GCA AAT TCC TTA A
NS2B$_{40}$ fragment F	GGC GAA TTC AAC CAA AAT GGC CGA TCT GGA ACT AGA GAG AGC
NS2B$_{40}$ fragment R	GGC GCG GCC GCC TAC AAT GTT TGC TCT TCC TCT TC
Prodomain(–) drICE^{vp} F	CGC AGA TCT CAA CCA AAA TGG TGC TGG GCT CCG TGG GAT CC
drICE^{vp} (hid fusion) R	GGC GCT TCA GGG ATC CGC CTC CGC CAA CCC GTC CGG CTG GTG CCA ACT G

Name	Sequence 5' to 3'
hid (drICE^{vp} fusion) F	GGG AGG CGG ATC CCT GAA GCG CCG CGC CGT GCC CTT TTC TTT GCC CGA
hid R	GCG GCG GCC GCT CAT CGC GCC GCA AAG AAG CCA CA
NS2Bf (hid fusion) R	GCG CTT CAG GGA TCC GCC TCC GCC AGT CAA TGT TTG CTC TTC CTC TTC
Gal4 F (EcoRI)	GGC GAA TTC ATG AAG CTA CTG TCT TCT ATC G
Gal4 R (NotI)	GCG GCG GCC GCT TAC TCT TTT TTT GGG TTT GGT GGG GTA TCT TCA TC
GFP F (EcoRI)	GGC GAA TTC ATG GTG AGC AAG GGC GAG GA
GFP R (NotI)	GCH GCG GCC GCT TAC TTG TAC AGC TCG TCC A
FLP F (BglII)	GGC AGA TCT ATG CCA CAA TTT GGT ATA TT
FLP R (NotI)	GCG GCG GCC GCT TAT ATG CGT CTA TTT ATG T
LexA F (BglII)	GGC AGA TCT ATG AAA GCG TTA ACG GCC A
SCP Den 5' F	GCG CGG GTA CCG TAC TTA TAT AAG GGG GTG GGG GCG CGT TCG TCC TCA GTT GTT AGT CTA CGT GGA CCG ACA
Den 5' R	CCC CCC TCG AGG AGG TCC TCG TCC CTG CAG CAT TCC
DIAPI Frag F	GCC GCC TCG AGG CGC AGG CGT GCC GTC TCG ATG GCG
DIAPI Frag R	CCG GGA AGC TTT CAA AAA TAA TAT AAA CAA AAC CAG
Den 3' F	GGC CGA AGC TTA GAT TCA GAA AAG AAG AGG AAG AGG
Den 3'/Ribo R	CCC TCG GAA TGT TGC CCA GCC GGC GCC AGC GAG GAG GCT GGG ACC ATG CCG GCC AGA ACC TGT TGA TTC AAC AGC ACC A
Ribo Rev 2nd Frag	CCG CCG AAT TCT GGG TCC CAT TCG CCA TTA CCG AGG GGA CGG TCC CCT CGG AAT GTT GCC CAG CCG GCG
Tubulin F	CGC GGG AAT TCG CGT CAC GCC ACT TCA ACG CTC GAT
Tubulin R	CGG CCG CGG CCG CAA AGA AAA ACA GTG GGG TTT TCT TA
hsp70 F	ATA AAG AAA TTT CCA AAA TAA AGC G
hsp70 (Den 5') R	CTT TGT CGG TCC ACG TAG ACT AAC AAC TCC GTC GAC GAA GCG CCT CTA TTT ATA
Den 5'(hsp70)	TAT AAA TAG AGG CGC TTC GTC GAC GGA GTT GTT AGT CTA CGT GGA CCG ACA AAG
AAIAP F	GCG GCA AGC TTT GTG GGA ACA GAA CGA TAA TGT CCT
AAIAP R	GGC GGC TCG AGT ACT CAC AGT TAC TAT ACC ACA TGG
3'(–) F	GCG CGT CTA GAG AGG TCC TCG TCC CTG CAG CAT TCC
3'(–) R Ribo	CCC TCG GAA TGT TGC CCA GCC GGC GCC AGC GAG GAG GCT GGG ACC ATG CCG GCC AGT TGT TAG TCT ACG TGG ACC GAC AAA G
Ribo R 3'(–) Build	GGC GGG AGC TCT GGG TCC CAT TCG CCA TTA CCG AGG GGA CGG TCC CCT CGG AAT GTT GCC CAG CCG GCG

Table 3.5: Oligonucleotide primers used in this study. This is not a comprehensive list, but defines fragments used repeatedly and not fully annotated elsewhere.

Other constructs used in this study, KHS-synRprWT and KHS-synRprK→R(RprKR)

were derived from Chun-Hong Chen's constructs and subcloned into the KHS vector. UAS-superHid (BH932), UAS-RprKR (BH979), pCaSpeRhsAct-GFP, pBSK and pBKS-IAP1 were pulled from lab stocks.

Vector Construction: Viral RdRp-Based Approach

Two constructs were built for testing in the fly eye, GMR-NS5, the full RdRp and methyltransferase protein from DENV2 and a substrate for it to act on consisting of the DENV2 5' and 3' ends terminated by a self-cleaving ribozyme and then the tubulin 3'UTR. These frame a piece of DIAP1 antisense, and the whole cassette (D5DD3RT) is also driven by the GMR promoter. The DENV2 5' and 3' ends were PCRed from pSY2-pSP64, a gift from R. Padmanabhan at Georgetown. The super core promoter and HDV ribozyme (Walker et al., 2003) were added via PCR. The fragment of DIAP1 was PCRed from BH1091. The tubulin 3'UTR was PCRed from a template provided by Haixia Huang and cloned EcoRI/NotI into pGMR-1N. The remainder of the pieces were ligated together and PCRed to generate a single PCR fragment that was cloned KpnI/EcoRI into pGMR-tubulin. The complete construct D5DD3RT was subcloned into pBSK to allow for the changing out of the antisense fragment. NS5 was subcloned from a vector provided by the Kuhn lab at Purdue. NS5 was cut XbaI, blunted and then cut BamHI to drop it out of the pET15 vector it was in and pGMR-1N was cut BglII/StuI to accept the fragment. These two constructs were used to generate germ line transformants.

Tissue culture constructs were also generated. The pGMR-D5DD3RT was modified so that when cloned into KHS the Dengue 5' fragment initiated precisely at its 5' end without any extra nucleotides and so that the antisense fragment targeted *Aedes albopictus* IAP (AA-IAP), producing construct KHS-D5AD3RT. A second construct, KHS-AD3(–)R, was built that carried the AAIAP fragment, a dengue antisense 3' fragment (D3'(–)) and the HDV ribozyme. A pair of miRs targeting AAIAP at sites TCACCGATAACAAAGTTAAAGA and GAAGATGAACCAAATAGAAAAC were designed using the miR6.1 backbone and cloned into KHS.

Designed for another project that required the stabilization of noncoding RNAs, I had synthesized a construct that carried the Kunjin and dengue 3' ends which are known to be highly structured and resist nuclease degradation (Pijlman et al., 2008), and an intron to help expedite transport to the cytoplasm. Between the two viral fragments are cloning

sites to allow placement of the desired antisense fragment. More information about this scaffold is provided in Appendix B. I cloned in and antisense fragment of AAIAP to generate KHS-TSAAIAP. The addition of a stem loop to prevent 5' to 3' exonuclease activity was included in a separate version of the construct, KHS-SLTSAAIAP. These were designed to help evaluated the effect of knockdown of AAIAP in *Albopictus* cells. A final antisense construct, pU6-D5GFPD3 was built up in the pAc5.1 backbone. The Actin promoter was removed by digesting BglII/KpnI and the components, including the Dengue 5' and 3' ends, GFP, and the U6 promoter and terminator, were PCRed and cloned in a single enzymatic assembly (EA) cloning reaction (Gibson et al., 2009).[2]

The final set of constructs are Barnase (Hartley, 1989) derived. The reverse complement of Barnase was cloned upstream of the D3'(–) and the HDV ribozyme in KHS. EcoRI and Xbal were used to clone Barnase and XbaI and SacI to clone the Den3'(–)R fragment into pBSK and the completed fragment was subcloned into KHS producing KHS-RCBarnD3'(–)R. The cloning of even the reverse-complement of Barnase required cotransformation with a Barstar expressing plasmid to suppress the toxicity of the Barnase bearing plasmids. Barstar was cloned into pRSF-1b (Novagen, Madison, WI) KpnI/NotI. This backbone was selected because it allows IPTG-inducible expression of Barstar during culture and has a RSF origin allowing both it and the pBSK or KHS plasmids to be concurrently expressed. The cloning was carried out in T7 Express I^q cells (New England BioLabs, Ipswich, MA) using kanamycin, ampicillin and IPTG. A separate Barnase construct carrying the reverse-complement of the Barnase ORF with an intron was synthesized.

Vector Construction: PKR Designs

A set of three constructs, pAc-PKRdbd, pAc-PKRaaArk, and pAc-aaArk were built by one-step cloning. PKR was PCRed from the KHS-PKR construct and aaArk was RT-PCRed from a C6/36 RNA preparation.

Cells and Transfections

Drosophila S2 cells were maintained in Schneider's medium 10% FBS, 1% penicillin and streptomycin (P/S) at 27.5°C, passaged every 3-4 days. The *Aedes albopitus* C6/36 cell line from the Strauss Lab was cultured in Dulbecco 1XMEM supplemented with nonessential

[2]See Chapter 4 and referenced appendices for further information on our use of EA cloning techniques.

amino acids, L-glutamate, 10% FBS, and 1% P/S at 29°C with 5% CO_2. Cells were trypsinized when passaged. A culture of C6/36 received from the Clem lab at Kansas State University were cultured in Liebovitz L-15 medium supplemented with 10% FBS and 1% P/S at 29°C without CO_2. KSU-C6/36 can be dislodged physically, so trypsinization is not required for passaging.

C6/36 transfections were carried out using the FuGENE6 reagent. The most successful ratio of FuGENE:DNA was 3μl:2μg. A GFP marker was often used, and always as 20% of the DNA transfected. Use of endotoxin-free DNAs was essential for good transfection efficiency. S2 cell transfections were optimally transfection using FuGENE6 at a ratio of 2.5 μl FuGENE:1 μg DNA. Immunofluorescence assays were carried out to assess viral infection as described in Appendix B.

Drosophila Culture

Fly crosses were carried out under standard conditions at 25°C. Viral infections of *D. melanogaster* were attempted through feeding. Extra Thick Blot Paper (Bio-rad, Hercules, CA) cut to $\sim$ 0.75 cm^2 were soaked in viral containing media supplemented with 10% glucose and provided to adult flies as their only food for 24 hours. The flies were then returned to normal fly diet and rearing conditions.

Chapter 4

Underdominance

4.1 Introduction

Project Motivations

Hand-in-hand with the need to engineer refractoriness cassettes to fight vector-borne disease goes the need to deliver these systems into wild populations. Releases of engineered, lab-reared animals carrying a refractoriness allele alone will not replace native populations of insects. Generation of a genetically diverse laboratory strain has attempted to equalize some of the fitness disparity between wild and lab-reared individuals (Valdez et al., 2010), but with only limited success. This is partly due to the fitness costs associated with the refractoriness alleles, but also because populations of lab-reared individuals (even genetically diverse ones) are no longer as fit for wild survival. To address this problem, refractoriness genes need to be carried by a drive system that will push the engineered chromosomes into wild populations, despite their associated fitness costs.

Naturally occurring selfish genetic elements offer intriguing clues about the best ways of accomplishing gene drive, but for the most part require engineering *a priori*, or at least re-purposing to function in the target organism. Some, like Wolbachia, have a known agent and can be translated across species (Hoffmann et al., 2011; Walker et al., 2011), despite their mode of action at the molecular level remaining mysterious. Others, like homing endonuclease genes and transposons, are understood at the molecular level and can be transferred into organisms of interest (Carareto et al., 1997; Sinkins and Gould, 2006; Windbichler et al., 2011). Lastly, some types of drive have known genetic behavior, but are not easily moved between species either because of their specificity to the organism they are found in

or because the molecular mechanism is completely unknown. Meiotic drive (unpublished), Medea (Chen et al., 2007), and underdominance (Davis et al., 2001; Magori, 2005) are drive systems that must be engineered to give the genetic behavior desired without nature's molecular blueprint. All of these systems offer ways to do population replacement, but each has a unique set of drive characteristic and the types of concerns the public may have about them vary as widely. Here I present work on underdominance (UD), and argue that it represents an important tool in the effort to deliver disease-resistant insect vectors to needful communities.

UD systems can also address another, only partially satisfied, need in the genetically modified organism (GMO) community. It is not always desirable for GMOs released into the environment to interbreed with native organisms. UD can offer a tightly reproductively isolated method of controlling gene flow between engineered and wild organisms. This means that engineered animals could bear traits which might generate concerns about hybridization with wild animals because no genetic exchange would occur between the wild and native individuals. While the work here is not done in plants, the plant GMO community is especially interested in preventing the traits of their products from transferring into unmodified species and is working on ways of achieving genetic isolation (Kwit et al., 2011). Hopefully what we learn and the types of systems we are developing here could be used by that community, and help to reassure the public of the safety of GMOs they would wish to use in their nations and that the integrity of their ecosystems can be protected.

Conceptual Framework

Underdominance, also called heterozygote disadvantage, is defined by selection against the heterozygous state. Animals homozygous for either of a pair of alleles is more fit than an individual heterozygous at that locus in an UD genetic system. It is a genetic behavior observed in natural populations that can be appropriated for use as a gene drive mechanism. In an engineered system, the premise is that the two fit homozygous states are represented by wild type alleles and engineered alleles. When an engineered allele and a wild allele are carried by the same individual, fitness is compromised, or, in extreme underdominant systems, eliminated.

The challenge in engineering an UD system lies in the fact that the heterozygous animal would be the progeny of a wild and engineered mating, and that only one of the parental

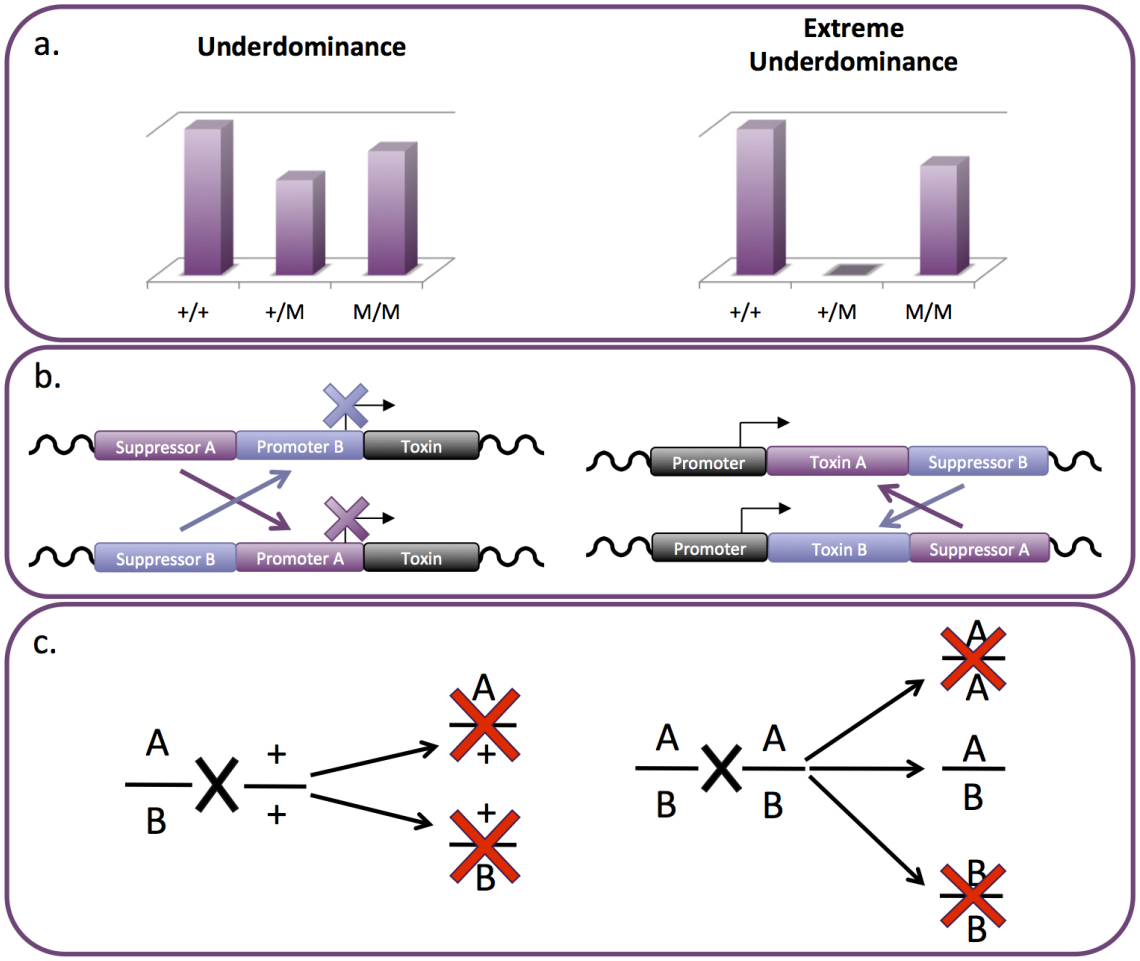


Figure 4.1: (a) Underdominance results when either of two homozygous states is more fit than the heterozygous state. In extreme underdominance, the heterozygous is unviable. (b) In the Davis model (left) the toxin remains constant and a pair of promoters and their suppressors comprise the underdominant system. In the systems described in this work, a pair of toxins and their antidotes are expressed from a promoter held constant. (c) In a single locus, extreme underdominant system, outcrosses to wild individuals produce no viable progeny. When the organisms are crossed to each other, 50% of the progeny survive.

animals (the engineered animal) can have an engineered genome. This means that the allele generating the fitness disadvantage must somehow interact with the wild genome to enforce the cost without doing so in the fully engineered background. Davis et al. suggested a way around this hurdle by using a two allele system to generate a fit 'homozygous' engineered animal, which is really a transheterozygote at the UD locus. The original system proposed consists of a pair of alleles, each bearing a copy of a toxin driven by a different repressible promoter, and the repressor for the complementary promoter (Figure 4.1). In this way, carrying both alleles results in repression of both promoters, so no toxin is expressed and the individual is viable. An individual carrying a single allele (as would be the case if

the engineered transheterozygote mated to a wild organism) results in de-repression of the promoter driving the toxic transcript. Trying to implement this system is difficult because promoter/repressor pairs that are tight enough to remain off, but strong enough to kill are a challenging component to identify and work with. Here we propose a simplified system with toxin and antidote both derive from an expressed transcript, and do not rely on the repression of promoter activity. This distinction seems small, but really opens up a wide variety of toxin-antidote systems that can be worked with.

Drive Characteristics

One benefit of an engineered UD system is that due to its two allele functionality, single and two locus systems can be engineered. These have distinct drive characteristics. Generation of the single locus system requires site-specific integration via the PhiC31 system (Groth et al., 2004) which allows the positioning of the two alleles at the same genomic locus.

Extreme underdominance, resulting in the death of individuals carrying only a single allele, in a single locus system means that no hybrids between engineered animals and native animals are produced. In effect, the wild population and engineered population are genetically isolated from one another and no gene flow occurs. As shown in Figure 4.2, this means that replacing a local population requires a large release compared to the native population, but also implied is that spread from the release area to neighboring populations will be slow or non-existent. Additionally, removal of the system is possible through release of a manageable number of wild-type individuals: A release of wild-type animals will drive the fraction of engineered animals below the threshold required for population replacement.

If more drive is required due to increased fitness costs, or genetic isolation is not a requirement for a specific release, a two locus systems offers slightly different drive characteristics. The initial releases required for population replacement are not as large, the time to fixation is similar for comparable release and there is still a threshold that would allow removal of underdominant animals from the wild if necessary.

This type of drive contrasts greatly with Medea's drive. Medea, when released at low ratios takes some time to drive, but unless eliminated quickly by stochastic events, will always effect complete population replacement when fitness costs are low. Medea is a very aggressive drive system, which also means that once established in an area, it will be difficult, if not impossible, to remove. Also, if there is significant migration, it will spread beyond

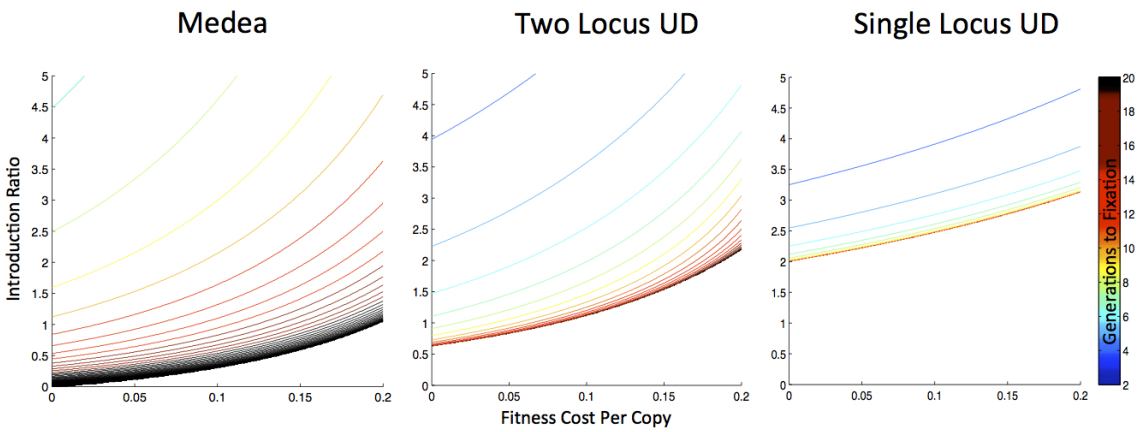


Figure 4.2: Shown here are three graphs produced by Catherine Ward showing the drive dynamics of Medea, and Single and Two Locus Underdominance. Each shows the same parameter space and is a useful framework to compare the aggressiveness of drive and release ratios required to effect population replacement. The space below the lowest contour on each graph represents conditions under which no drive can occur. This area is much larger for both of the underdominant systems than it is for Medea.

the area of initial release.

While replacing all mosquitoes with refractory individuals is attractive in theory, communities may show reluctance about the release of engineered organisms that cannot be recalled or removed (Marshall et al., 2010). The animals will not respect international borders, and so releases in one country could upset neighbors who have not agreed to replace their native populations. Underdominant drive offers a different profile in that single and two locus underdominance both have much less aggressive drive profiles. An additional selling point is that they drive more quickly because of the large initial releases required. The two locus system is not genetically isolated, but does drive quickly at intermediate release thresholds, so in effect engineered underdominance contributes two new tools to the gene drive tool box.

Toxin and Antidote Systems

In the studies that follow, I worked with two distinct classes of toxin and antidote pairs. The first relies on the use of a protein toxin, specifically the *Drosophila* cell death protein, Hid, and miRNAs targeting recoded Hid transcripts as antidote. Other apoptotic proteins such as Rpr or even exogenous toxins such as Barnase could be imagined in this role, but it is important that some leak of the toxin be tolerated. Suppression occurs at the mRNA level and if any protein is produced, its function cannot be suppressed by the miRNA antidote.

Protein toxins have the advantage that their action is predictable, and several toxin-antidote pairs are already extant. Their disadvantage lies in their instability on evolutionary time scales. A single base pair change could ablate function of the toxin, disabling the system.

The second type of system I explore here uses miRNAs to deplete an essential transcript in a sensitive and essential tissue as the toxin. The restoration through use of miRNA-insensitive transcripts able to provide the protein when and where it is required serves as antidote. The challenge in resupplying a necessary transcript is that often dose and timing are important, however, there are several modes of supplying the antidote between two cassettes. The first is through use of a split transcription factor, as is used in the yeast two-hybrid system (Fields and Song, 1989). If each underdominant allele provides half of the transcription factor, one the activation domain and the other the DNA binding domain, bringing them together can instigate transcription from the upstream activation sequence. This approach could likely provide a high dose of rescue, but relies on a tightly off behavior when the alleles are in isolation.

Another way to resupply a targeted protein is through the expression of the target itself in two pieces. If the protein has a natural split point, small, charged coiled-coils can be used to bring the two domains of the proteins together. Even if the protein cannot tolerate coiled-coil reassembly, reconstitution of two expressed pieces into a single protein may be possible through the use of inteins (Lockless and Muir, 2009; Schwartz et al., 2007). If protein splicing is efficient enough, rescue could be effected. The work described here mostly falls into the transcription factor method of rescue, but the other methods are being developed as well.

Approaches to UD: Induced Fitness Cost or Lethality

A functional UD system relies on the induction of a fitness cost in a target organism. Extreme underdominance, the ultimate goal of this project, requires that no animal bearing only a single engineered chromosome could survive in the wild. One significant challenge in the construction of these systems is that both underdominant alleles must be introduced into the genome of the target organism. These chromosomes must be introduced individually, and tolerated until their compensatory allele can be crossed in.

One way to circumvent this challenge is through the generation of single underdominant chromosomes that will not kill the individual. The underdominant system then relies on

the distinction between fitness in the wild and fitness in the laboratory. *Drosophila* has been the geneticists' workhorse for over 100 years, and in that time a multitude of mutants have been generated, many of which can be maintained in the lab but would not survive or compete in the wild. This includes all manner of physical deformities including wingless flies, blind flies and flies without a full complement of, or with too many, appendages. Many of these mutations result from disruption of control sequences, but some are induced by over expression or loss of a known transcript, and these can be used in underdominant systems. If a phenotype that is tolerated in a laboratory system, but would critically compromising in the wild, can be induced and then rescued, a viable toxin-antidote pair has been identified.

The other approach, a true lethals system, requires the use of an conditional or reversible rescue. Each allele as it is generated is suppressed in a way that can be either discontinued or removed from the genome of the animal. The cassette can introduce considerable complexity to the system, but they are only required in construction stages and not for the function of any system during or after release.

4.2 Results and Discussion

4.2.1 Protein Toxins

GMR Constructs

A great amount of work has been done in the fly eye with cell death proteins, so our initial underdominance constructs derived from that work. The expression of the RHG proteins produce small eye phenotypes when over expressed, and this death can be suppressed through the use of miRNAs (Chun-Hong Chen, unpublished). Hid^{Ala3} (Bergmann et al., 1998) was recoded at the DNA level to allow targeting of its mRNA specifically by engineered miRNAs. Two version were made that differed from one another so they could be specifically targeted by synthetic miRNAs, and from the endogenous Hid transcript, so that the normal function of Hid as part of the cell death machinery was not perturbed. Three miRNAs were designed to target each version of Hid, with miRNA set 2 (m2) cloned downstream of Recoded Hid 1 (rH1) and vice-versa, producing GMR-rH1m2 (UD1) and GMR-rH2m1 (UD2). Each miRNA was located in its own intron. This generated constructs each bearing a toxin (Hid) and a rescue (miRNA) that targets the other recoded version of Hid, as shown in Figure 4.3.

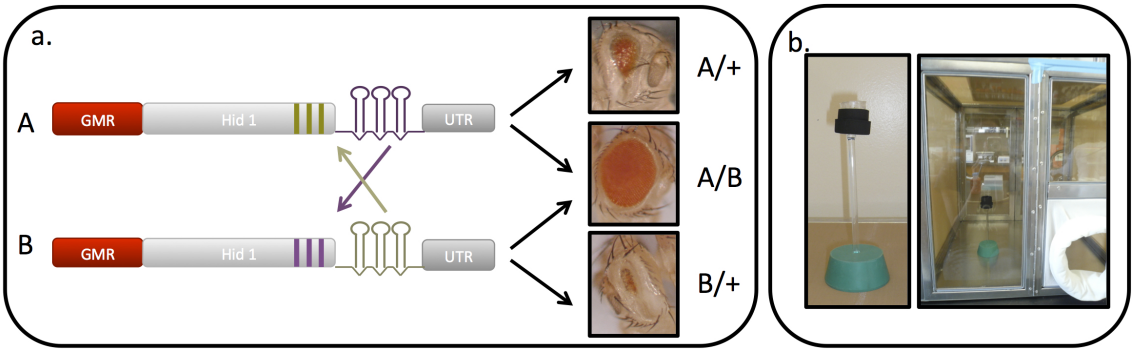


Figure 4.3: (a) Each chromosome bears a toxin, Hid, and a rescue, a set of miRNAs. The Hids have been recoded at the DNA level to produce mRNAs specifically targeted by the miRNAs. When a fly bears only a single chromosome, there is death in the eye, but when it bears both, the eye is restored to its wild-type size, shape and patterning. (b) Cage experiments investigating drive using the GMR-Hid UD system were conducted. The food was elevated on a pedestal treated with SigmaCote and unable to be climbed by the flies, and positioned in either a 12 in^2 or 18 in^2 cage.

Site-specific integration was used in this project so that single locus systems could be developed and to be able to compare experiments to one another. Rainbow Transgenics generated transformants of GMR-UD1 and GMR-UD2 at 86Fa and GMR-UD2 at 96E. Toxicity from injecting these constructs was significant and GMR-UD1 transformants could not be generated at 96E. Eye phenotypes at 96E were more severe, and the GMR-UD1 line was less healthy at 86Fa than the GMR-UD2 line. Generating transformants was difficult, probably due to leakage off the GMR promoter in the embryo following injections. Many of the later injections were injected in-house because so few G_0s were received from commercial injections. When 86Fa GMR-UD1 was crossed to 96E GMR-UD2, a fully rescued eye resulted. This indicated that the miRNAs were functioning catalytically because the expression levels at the two insertion sites differ.

I attempted drive experiments with these flies. Conversations with Dr. Michael Dickinson indicated that blind flies might not initiate flight, so I constructed a cage with an elevated food source only able to be reached by flight. The food was positioned on a pedestal. The pedestal was constructed by fixing a food vial to a glass rod coated with SigmaCote, a material that makes the glass too slippery for the flies to climb up it. A mixture of Oregon R, 86Fa-UD1, and 86Fa-UD2 were introduced into the habitat. Within 6 hours, all the flies had reached the food source and stayed there. Mating, eating and egg laying were all occurring on the food, so the blind flies were not being selected against and drive could not be observed. While they might be compromised in the wild, blind *Drosophila* are not

greatly disadvantaged in the laboratory.

Essential Tissue Constructs

The Hid system was then moved to essential tissues. Generating transformants carrying only one of the alleles required for an underdominant system required a conditional or reversible rescue. The first system I worked with utilized a Tetracycline (Tet) driven rescue. Using the same tissue-specific promoter as was driving Hid, I drove expression of rtTA2^S-M2 (tTAV), a prokaryotic Tet operon binding domain fused to minimal VP16 activation domain repeats. The P_{tight} promoter, which is recognized by tTAV, was cloned upstream of a GFP open reading frame that carried a set of miRNAs able to suppress the toxin in the 3'UTR of GFP. When fed Tet or Doxycline (Dox), the flies express a GFP marker and miRNAs to suppress the Hid transcript. If reared off Tet, no rescue is expressed and Hid induces apoptosis. A schematic is shown in Figure 4.4a.

The Tet-inducible rescue was tested in a Hid-UD construct with GMR as the driver of Hid and the tTAV protein. Despite the Clontech and other literature stating that Tet would not activate tTAV (Gossen et al., 1995), it worked fairly well at high doses in flies. However, many of the promoters I worked with were on during pupal development, and tests with GMR-Hid UD constructs showed that Tet's half-life was too short to effect rescue through this developmental stage. I also found, however that Dox was not well tolerated during larval stages. To address these issues, I treated larvae with Tet in the food, and administered a dose of Dox approximately 24 hours before pupation. This produced complete rescue of the eye (Figure 4.5). The Tet-then-Dox treatment regimen was used in testing all of the Tet-inducible cassette constructs. A second generation of the Tet-inducible rescue was tested. It carried a 5' intron and Kozak sequence (shown in Appendix C) upstream of the tTAV, and these constructs are designated ntTAV. This design made no appreciable change to effectiveness of Tet treatments or degree of rescue.

A set of Hid-UD constructs targeting essential tissues were designed. The original pair of promoters used were from *Drosophila* proteins giant (gt) and EDG84a. The gt enhancer as described in Berman et al. (2002) is expressed significantly only in the early embryo between 2–8 hours. The SCP was fused at the 3' end of the enhancer. EDG84a is a cuticle gene (Kayashima et al., 2005; Murata et al., 1996). The EDG84a enhancer has a short first exon coding for four amino acids followed by an intron. The initiating ATG was mutated

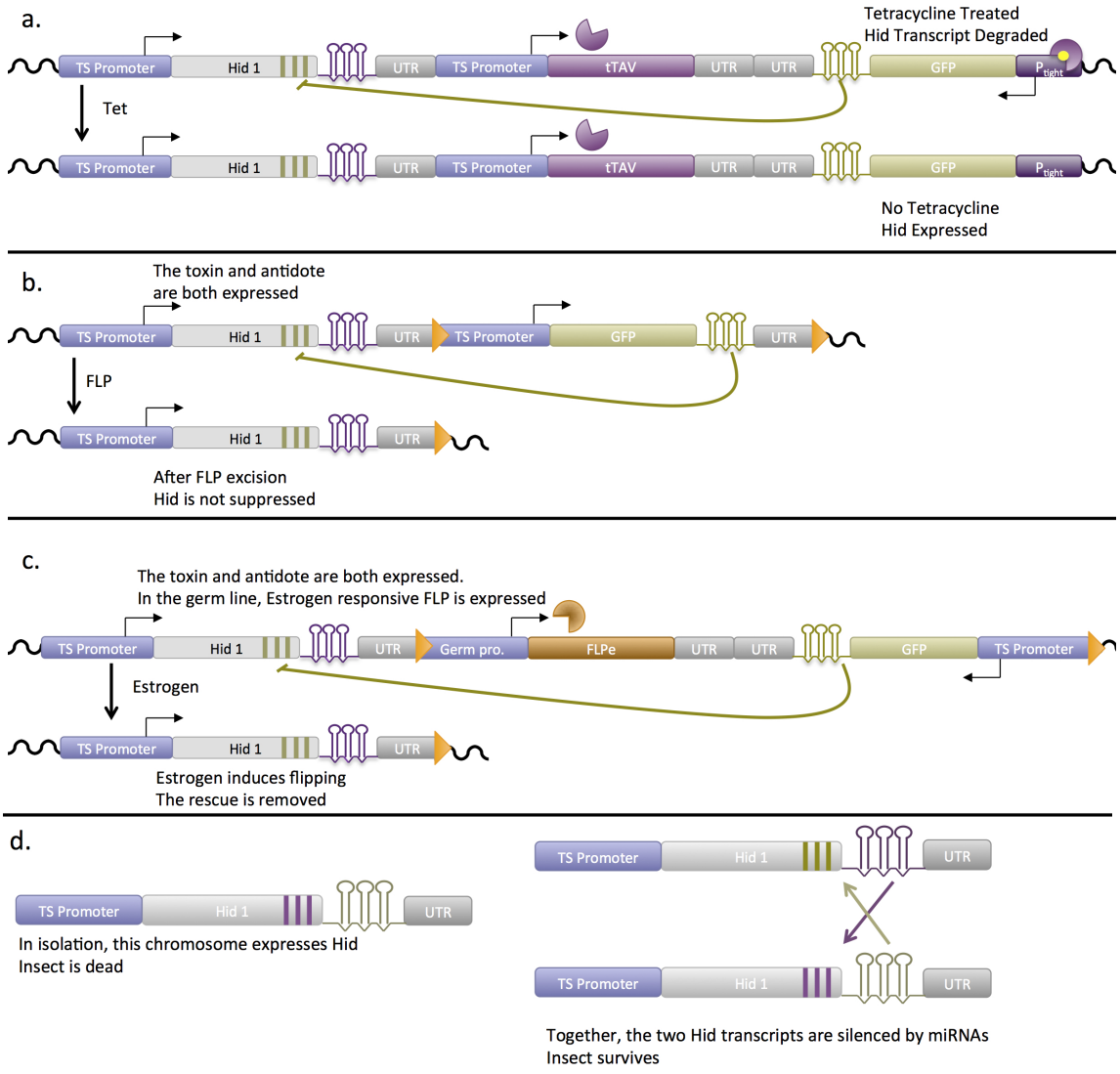


Figure 4.4: Shown are three modes of rescue, and how they are positioned in the underdominant constructs. (a) The first conditional rescues (Tet, ntTAV) used were Tet-inducible rescues. Feeding of Tet-induced expression of GFP and a set of miRNAs able to suppress the Hid in the construct. (b) A second generation of rescue (FRTs) utilized the same GFP-miRNA cassette expressed directly off the tissue-specific promoter used to effect killing. This construct was removable through FLP excision. (c) The last construct shown in this set (FlpeERT2-ex) operates by estrogen-inducible FLP excision in the germ line so that parental animals fed estrogen produce progeny not carrying the cassette. This design was not tested in flies. (d) In isolation, the chromosomes are toxic, but when both are carried, as in the eye, Hid is suppressed and the animal is viable.

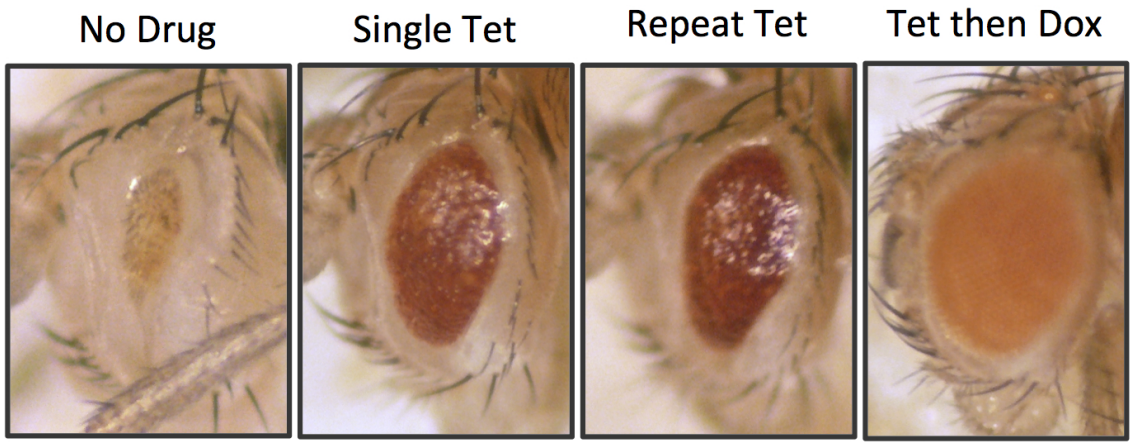


Figure 4.5: GMR-Hid UD flies with a Tet-inducible rescue were used as a model for the function of both the Hid underdominant system and the conditional rescue. An untreated fly has shows the small-eyed phenotype expected from Hid^{Ala} over-expression. Treatment with Tet, either as a single dose in the food or repeatedly, gives only partial rescue of the eye phenotype. Treatment Tet through the larval stages and then Dox shortly before pupation resulted in complete rescue.

via PCR to prevent tagging of the tTAV and Hid proteins with the first four amino acids of the EDG84a protein. These two enhancers were tested with the original Tet-inducible rescue, and gave transformants of both UD alleles when injected in-house, but neither was toxic when the animals were reared off Tet.

Additional enhancers were tested that targeted various tissue. Other embryonic genes tested were serendipity-α and scute (Deshpande et al., 1995). Several proteins involved with nervous system development such as nerfin-1, nervy (Kuzin et al., 2009) and Dfd (Lou et al., 1995) were also tested. In-house injections yielded transformants for at least one Hid-UD chromosome for all 5 of these enhancers, although only Dfd gave some indication that expression of Hid was killing. Co-injecting pAc-miRNAs constructs suppressed most of the toxicity associated with injecting the Hid-UD constructs. Transformants of Dfd-UD constructs were very hard to generate. Three different types of rescue, the original Tet-induced rescue, the ntTAV rescue, and a blocked version like the bottom schematic of Figure 4.4(a.), were tested. Transformants were only ever generated for one chromosome of the FLP-excisable rescue, and I was unable to locate this insertion to the attB site I was targeting. Dfd was revisited later when a final round of Hid constructs were built and tested.

Two enhancers targeting muscle, mef-2 (Nguyen and Xu, 1998) and paramyosin (Hess

Hid-based UD Constructs and Flies				
Promoter	Rescue Type	Transformants	Killing	Rescue
GMR	none	Both	Y	Y
	Tet	UD1	Y	Y
	ntTAV	UD1	Y	Y
	FRTs	UD1	Y	Y
EDG84a	Tet	Both	N	
gt-SCP	Tet	Both	N	
Nerfin-1	Tet	Both	N	
scute-hsp70	Tet	Both	N	
mef-2-hsp70	Tet	UD1	N	
sry-α	Tet	N		
	FRTs	UD1	N	
Ubq	Tet	N		
Nervy	Tet	UD1[1]		
	ntTAV	N		
	FRTs	Y	N	
Dfd-hsp70	Tet	N[3]		
	ntTAV	N[3]		
	FRTS	UD2[1]	N	
	α1-Tub	Both	N	
hand-hsp70	Tet	UD2	N[2]	
	FRTS	UD1	N[2]	
	α1-Tub	Both	Y	N
Fln	FRTs	Both	N	

Table 4.1: Fly results
[1]Insertion was not at attB site.
[2]Killing was likely partial, and this was before we appreciated that ovo-FLP(8727) had a variable activity.
[3]Several vials had dead late pupae.

et al., 2007; Marco-Ferreres et al., 2005) were designed, although the paramyosin constructs were never injected. The mef-2-Hid UD transformants were healthy when reared without Tet. A Ubq-Hid UD construct was built, using a promoter provided by O. Akbari, but no transformants could be generated. The heart enhancer, hand (Sellin et al., 2006), was tested preliminarily at 86Fa without much success but was revisited about two years after the initial phase of this project was put on hold. A comprehensive list of flies generated and tested is shown in Table 4.1.

Testing of Estrogen-Responsive FLP

FlpeERT2 (Hunter et al., 2005) was tested in S2 tissue culture before generating *Drosophila* carrying the estrogen responsive FLP excision cassette. Both FlpeERT2 and a derivative of one of A. Kumar's counting constructs, pAc-NoFlpCount were transfected into S2 cells. The flippable construct is shown in Figure 4.6. When pAc-FlpeERT2 and pAc-NoFlpCount were co-transfected, a great deal of flipping occurred even in the absence of either Tamoxifen (TAM) or Estrogen. I also found both these drugs to be toxic to S2 cells, even in small amounts compared to recommended dosages (27 μM and 37 μM, respectively), although these doses were derived from mammalian protocols. Heres-Pulido et al. (2004) have shown some toxicity associated with TAM, and an estrogen responsive system has been implemented in *Drosophila* (Thackray et al., 2000), although the literature remains incredibly sparse. Doses used in these studies were lower than those I used, and toxicity is not discussed. One concern I had was about the presence of estrogens in the FBS used to supplement the S2 media. In the Thackary study, charcoal stripped FBS is used and so I also used it for my studies, but the background rate of flipping was still extremely high. At this point, I stopped development of the estrogen-responsive FLP because we could not maintain an unflipped state in tissue culture.

Excision of FLP-Removable Rescues

I also built rescues that were not drug-inducible, and acted as blocking sequences that separated the tissue specific promoter from the Hid ORF. These had to be excised by introducing FLP into the germ line of Hid-UD bearing animals. The crossing scheme is shown in Figure 4.7. Separate male and female germ line FLPs on the X chromosome, ovo-FLP and β-tubulin-FLP, were obtained from the Bloomington Stock Center (Bloomington,

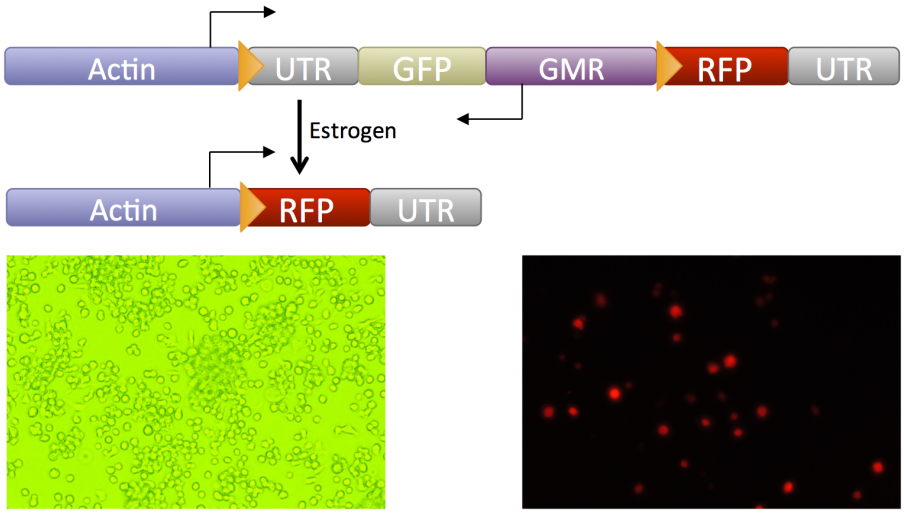


Figure 4.6: The construct shown, NoFlpCount, was designed for use in flies and modified for testing in tissue culture. When no FLP is present, GMR should drive GFP, but in tissue culture GMR is not active, so there is no fluorescent signal. If flipping occurs, RFP is expressed from the Actin5C promoter, which is very active in S2 cells. Shown here are bright-field and RFP-channel photos from a transfection of pAc-FlpeERT2 and pAc-NoFlpCount *without* estrogen or TAM applied. There is a great deal of flipping occurring.

IL), and crossed to homozygous Hid-UD lines. FRT flanked rescues that were expressed from the same promoter as was driving Hid were cloned as well. These are marked and excised in the same way as the blocking rescues, but are less clever because they are cloned as a separate cassette and the toxin is still expressed.

The Hid-UD lines were marked with a somatic GFP showing the presence of the FLP-excisable cassette and the attP site is marked with a 3XP3 (eye) RFP, so the unflipped flies carry two fluorescent markers, scoreable in two separate tissues. Of the tissue specific promoters we used, hand and prm gave strong, easily scored GFP phenotypes. For tissues that did not give a strong GFP expression pattern, prm was used for subsequent sets of constructs, as was the case with Dfd.

Progeny from the initial cross, F_1 individuals, bear a GFP marker in the soma, an RFP marker in their eyes, and FLP is expressed in the germ line. The F_2 flies, if flipping has occurred, will no longer be marked with GFP, but will still be marked with RFP. They should only be viable if they carry both UD chromosomes. This crossing scheme allows analysis of the efficiency of the various FLP lines and efficacy of the toxin. If F_1 individuals bearing a germ line FLP and a Hid-UD chromosome are outcrossed to w- (a wild type line), no RFP positive, GFP-negative flies should be produced if killing is complete. The

fraction of GFP-positive flies allows quantification of FLP efficiency, whether or not killing is complete.

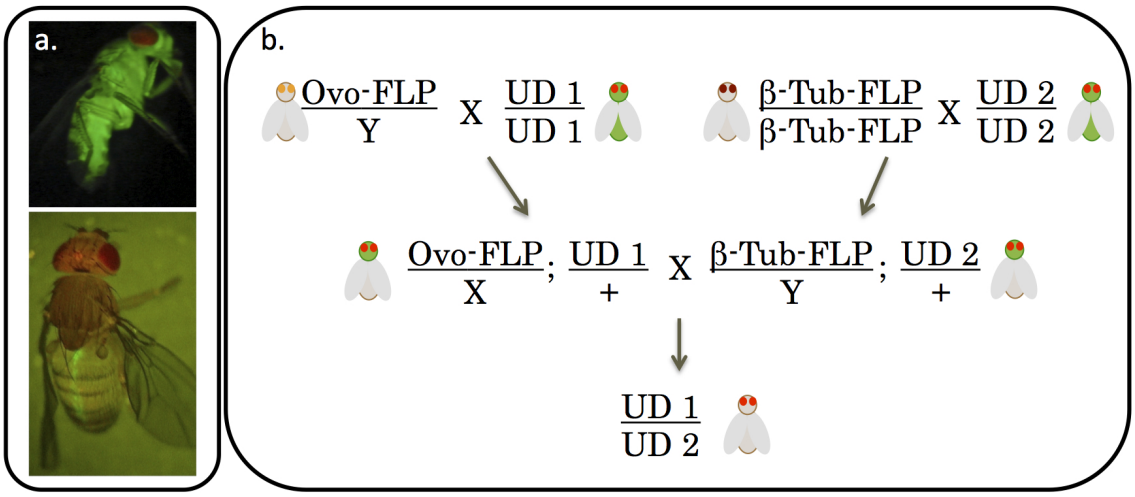


Figure 4.7: (a) GFP expression from the prm-hsp70 (top) and hand-hsp70 promoters at PhiC31 insertion site 86Fa (b) Crossing scheme carried out to FLP-excise blocking rescues

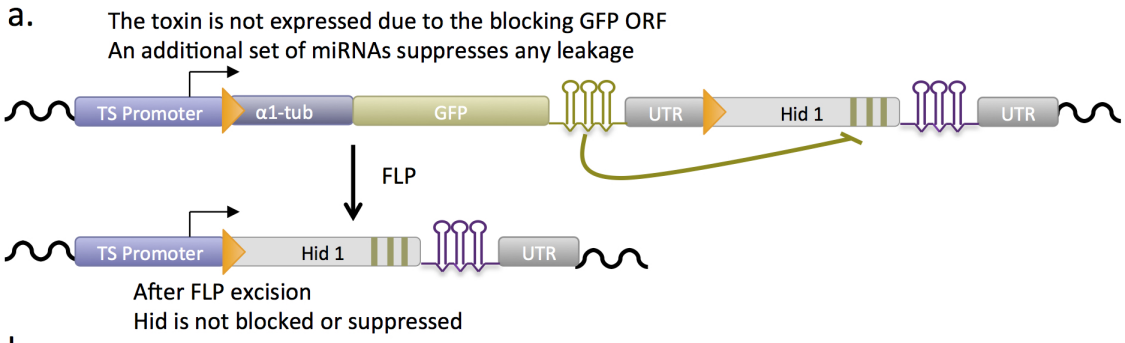
Enhancers that were tested with FLP-excisable rescues were GMR, sry-α, nervy, Dfd, hand and Fln. GMR flies had wild-type eyes when blocked, and FLP excision did result in a small eye phenotype indicating that the system was sound. Sry-α and nervy both produced animals that were viable when flipped. I found one Dfd transformant from the non-blocked FRT removable rescue, and flipping did not result in killing, but GFP was not expressed and I also determined that this insertion was not at 86Fa. This fly was scored an an inconclusive result. Hand seemed to give partial killing at 86Fa, and later studies showed that the ovo-FLP line used had variable efficiency resulting in difficulty scoring the lines. The expected phenotype for Fln was the inability to fly, but expression of Hid off this enhancer did not produce flightless flies.

Final Set of Essential Tissue Constructs

Before this round of flies was generated, studies with miRNA toxin UD constructs began, and the Hid-UD constructs were set aside. Recently, I generated one final round of Hid-based constructs. I used the Dfd and hand enhancers to generate a set of Hid-UD alleles as these two enhancers had given more encouraging results with both the Hid and miRNA UD constructs. These also had the α1-tubulin promoter driving GFP and miRNAS as the conditional rescue in an effort to have a strong, unambiguous GFP marker and ubiquitous

expression of miRNAs to suppress any Hid leakiness. DNAs were injected by Rainbow Transgenics. Dfd insertions were generated at 58A and 86Fa. Hand insertions were achieved at 51D, 58A, and 86Fa.

a.



b.

Enhancer	attP site	Killing	UD1/UD2
	51D	Complete	No
hand	58A	Complete	No
	86Fa	Partial	-
	51D	-	-
Dfd	58A	None	-
	86Fa	None	-

Figure 4.8: (a) The architecture of the final Hid-UD constructs tested (b) Killing and generation of trans-heterozygous underdominant individuals

The α1-tubulin promoter did not give a GFP phenotype scoreable on the dissecting scope, but either there was some expression and the miRNAs were effective in suppressing toxicity or the blocking sequence worked well because I had no difficulty generating transformants. In the F_2 generation, some individuals were outcrossed to w- to assess killing and others were crossed to the complementary UD line to generate transheterozygous individuals. Results are in Figure 4.8. The Dfd constructs failed to kill. The hand constructs killed, but could not be rescued. Studies with this architecture are ongoing.

4.2.2 MiRNA Toxins

The miRNA-toxin-based UD system also requires two alleles, but in this case the toxin is identical in each allele and the rescue is split between the two alleles. I used two architectures that were very similar to one another, differing only in the placement of CTCF insulators, designed to suppress genomic influences on expression. A schematic is shown in Figure 4.10, and the components of the system are explained in detail below. The main

advantage to this system over the protein toxin system is the robustness of the toxin. The miRNAs can be multimerized and made to target multiple sites, so a point mutation in the toxin itself or the targeted transcript should not result in broken alleles. This is desirable for releases into wild populations where selection agains the system will be strong and diversity of the population could result in varying sensitivity to any given toxin. There are many choices for all of the key components in this system, and many of the experiments described occurred in parallel or partially overlapped.

Split Transcription Factors and Promoter Development

The design of the miRNA-toxin UD system as we envision it is more complicated because the rescue needs to be distributed between two alleles. The added complexity does not increase the fragility of the constructs when thinking of a release because as long as the toxin remains intact, any animals with a compromised rescue will simply die. We decided to use a split transcription factor as the two component mode of rescue. The two parts of the transcription factor would associate non-covalently when co-expressed in the targeted tissue. In these designs, each UD allele bears the miRNA toxin and half of the transcription factor. The rescue is expressed from an enhancer driving the replacement transcript as part of each allele only when both halves of the transcription factor are expressed (when the animal carries both UD alleles).

Before any flies were built, I tested the split transcription factors in tissue culture. A split version of Gal4::VP16 had been shown to work *in vivo* in *Drosophila* (Luan et al., 2006), but because of the work I had already done using the LexA::VP16 transcription factor in my studies of the DENV sensors (described in Chapter 3), I tested that system. The 8LexA enhancer was fused to a fragment of the UASp promoter (the basal piece that does not bear the Gal4 UAS). This was cloned into the pAc5.1-HisB in place of the Actin promoter. eGFP was cloned into the resulting plasmid, p8LexUASp, so that co-transfection with the LexA::VP16 fused transcription factor resulted in eGFP expression. The fusion worked very well as a control, shown in Figure 4.9.

I tested LexA with coiled-coils located N- and C-termially to the LexA DBD (Moll et al., 2001; Arndt et al., 2001). I tested both the WinzipA2-WinZipB1 and $EE_{12}RR_{345}L$-$RR_{12}EE_{345}L$ coiled-coil pairs. I saw no expression with the WinZip pair, so in this study,

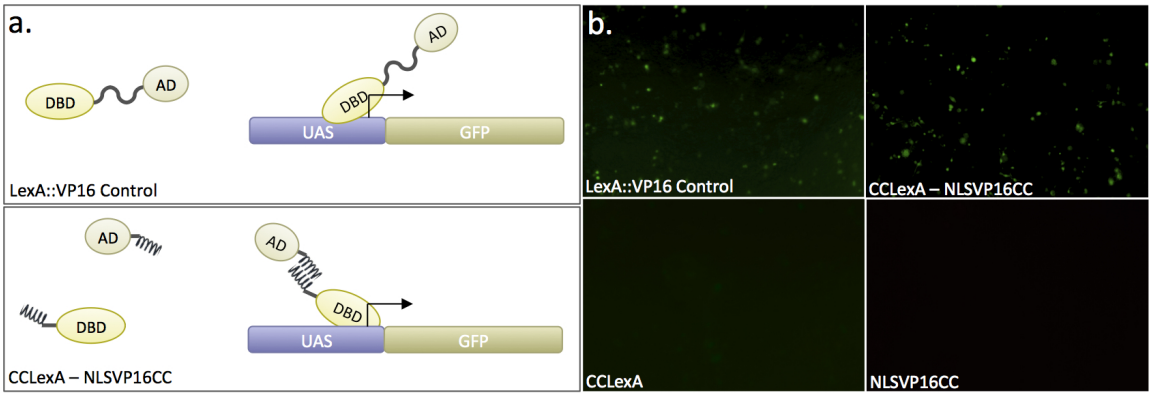


Figure 4.9: In panel (a) are shown how the LexA::VP16 fusion and the LexA and VP16 with coiled-coil domains function. (b) The results of a transfection using the split transcription factor components in isolation, together and with the fusion LexA::VP16 as a control.

CC refers to the $EE_{12}RR_{345}$L-$RR_{12}EE_{345}$L coiled-coil pair. I also used Gal4DBD-CC as a control when testing the LexA split transcription factor. LexA with an N-terminal CC gave the best expression, better than Gal4DBD-CC with NLS-VP16-CC in my hands. Expression induced by the CC-LexA and NLS-VP16-CC was similar to that achieved with a fused transcription factor. One important modification I made to the VP16 was the addition of the nuclear localization signal (AAPAAKKKKLD). Without it, there is no GFP expression from the 8LexA promoters in S2 culture.

As fly studies with complete miRNA-toxin UD constructs progressed, leak off the 8LexA-UASp promoter became a concern. While it was not clear that level of leakiness observed would compromise the function of the system, it was desirable to have other promoters available to test. I assembled three more 8LexA promoters. The first used the same fragment of hsp70 used in the UASt and GMR promoters as both these are broadly and successfully utilized. The 8LexA-hsp70 promoter was tested in tissue culture and gave a very robust response to both the fused and split transcription factors. I also cloned two more minimal promoters, each consisting of the 8LexA repeat, an $\sim$ 60 bp spacer that was part of the yeast 8LexA promoter construct (this was in all the 8LexA promoters tested), and a short basal fragment. One was a short artificial TATAA segment (Butler, 2001), and the other derived from the TATA box and downstream promoter element (DPE) from the *Drosophila* sex combs reduced gene. Neither of these minimal 8LexA promoters gave GFP expression in the tissue culture assay (data not shown).

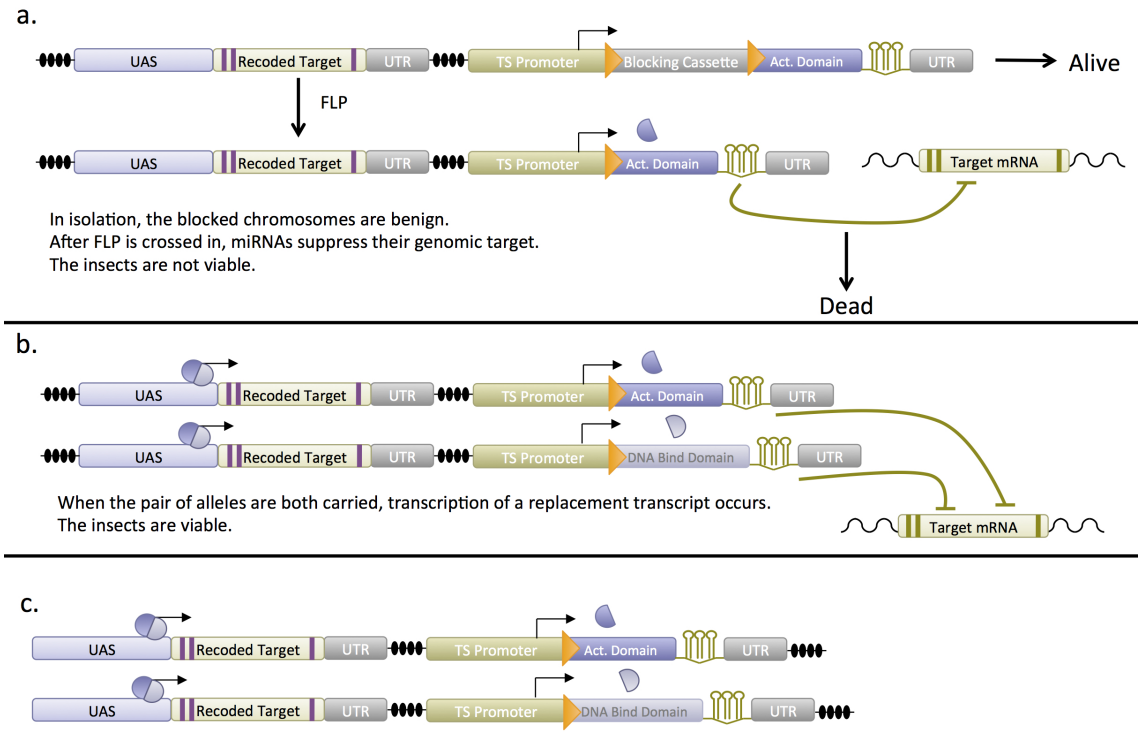


Figure 4.10: (a) The miRNA-based toxin is driven as part of the split transcription factor by a tissue specific promoter. Transcription is blocked by a cassette like that in Figure 4.8. Once FLP is introduced, the blocking cassette is excised and the miRNAs are free to suppress the targeted transcript, resulting in death. Half of the transcription factor is produced, but alone cannot drive expression of the rescue. (b) When both constructs are carried and FLP excision has occurred, the two halves of the transcription factor come together and drive expression of the rescue. The target has been coded so as not to be targeted by the miRNAs. (c) Shown here is a second architecture varying from that in panel (b) by the placement of CTCF insulators, depicted as quadruples of small black ovals.

Target Selection and MiRNA Design

The use of miRNAs as a toxin allows the selection of any transcript as the target, but it is necessary to select a transcript whose depletion in a given tissue or set of tissues is toxic. These studies were commenced after I had had some success with the hand enhancer in the Hid-UD studies, and so I continued working with the heart specific enhancer. An RNAi screen in the heart using the tinman enhancer, which has a similar expression pattern, showed that the depletion of several transcripts resulted in developmental lethality (Neely et al., 2010). From this study, we selected RpL35a, a ribosomal subunit, and CG5266 a proteasome subunit as primary targets. RpL35a is known to be haploinsufficient (Marygold et al., 2007), so it seemed likely that it was a target I would be able to suppress sufficiently using artificial miRNAs. One concern, however, was that it might be difficult to rescue if tissues were very sensitive to dosage. CG5266 is less well studied, but suppression of several proteasome subunits by RNAi gave developmental lethality. CG5266 has a single homologue in *Aedes* making it a good candidate for translating the system to the mosquito upon success in *Drosophila*.

The initial triplets of miRNAs designed to target the RpL35a and CG5266 transcripts were a compromise between good target sites and minimizing the amount of recoding of the transcript necessary to render the rescue insensitive to the toxin. Each set had one miRNA designed to target the 5' UTR, one targeting the 3' UTR and the last targeting the start codon. As in the Hid-UD constructs, the miRNAs were positioned between the end of an ORF, the split transcription factor, and an endogenous *D. melanogaster* 3' UTR. In this case, rather than each miRNA being positioned in its own intron, the triplet as a whole was flanked by strong 5' and 3' splice sites. This ensures that the split transcription factor mRNAs will remain stable even if the miRNAs are efficiently processed.

As understanding of miRNA design progressed in the laboratory, and use of the EA cloning strategy became standard, later sets of miRNAs were designed such that a preference was given to targeting the CDS exclusively. For the last set of CG5266 miRNAs designed, we did not even shy away from clustering the targets all in one part of the transcript. A list of all target sites used in the miRNA-UD constructs is shown in Table 4.2.

Tissue selection is an equally important consideration. In addition to the hand-hsp70 promoter targeting the heart, I also worked with Dfd-hsp70 and prm-hsp70 as the Hid-UD constructs had seemed to indicate killing would be a major hurdle. prm-hsp70 was an espe-

miRNA Target Sites				
Target	Sequence	Location	Killing	Rescue
IAP1	CGAGGCGGGTTTCTTCTATACA GAAAGGAGCCAGAGAAGAAATT	CDS 5' UTR	Yes[1]	D.ana ORF
RpL35a	ATACATTCATCGAACAACAAGC AACAACAAGCTAAGCCATGGCC TAAGGCGAGATACCGATTGAAA	5'UTR ATG 3'UTR	Yes	RpL35a
Fln	CCAACTATTACGATGATGTAAT TCAGTATAAACACTATAGTAAA	CDS 5' UTR	No	Recode Fln
CG5266	TCATTTTACACATCAAATCACT CCAGGAAATATGGCTACCGAAC CCACAAGCTAAGCTTTCTTAAT	5'UTR ATG 3'UTR	No	Recode 5266
CG5266	AAACACAAGTCACCGCTGTATG CCATCCTTACGCTGAAAGAAGG GAATCTGCGATCAGAACGGATT	CDS CDS CDS	Yes[2]	Recode 5266
CG5266	CATGGGCAAGAACGCAGTGAAC CACCGCCATCCTTACGCTGAAA TGAAAGAAGGTTTTGAGGGAAA	CDS CDS CDS	Yes[2]	Recode 5266

Table 4.2: The targeted transcripts and the 22 bp target sites used in miRNA doublets or triplets

[1]Effectiveness reported by Chun-Hong Chen, but not confirmed by my studies

[2]These sets of targets results in suppression, with the last set being most effective (A. Buchman, unpublished)

cially aggressive choice as it is expressed broadly in muscle tissue throughout development and into adulthood. I expected that ablation of any of these three tissues would result in death of the animal.

I also built some non-lethal miRNA-UD constructs. The two tissues I targeted were the flight muscle and the eye. The intention with the eye was again proof-of-principle, testing of miRNAs sets, and observation and analysis of modes of rescue. The GMR promoter expresses for a relatively long window during development, and so is a good model for a proliferative tissue. The generation of flightless flies offered a fitness deficit that I thought could be challenged in the lab. A food source that cannot be reached by walking would allow execution of a drive experiment in a caged environment. The cage we had designed for the blind flies would offer a good habitat in which to observe drive. MiRNA toxins tested in the eye targeted IAP and RpL35a. The flight muscle promoter, Fln, was used to target flightin protein itself, in addition to IAP and RpL35a.

Other Construct Components

An additional worry was having the enhancer driving the toxin so close to the rescue, and so partially artificial CTCF insulators (Kyrchanova et al., 2008) were positioned around the rescuing portion of the construct. These were intended to limit both precocious rescue from non-specific genome driven expression and reach-around by the enhancer.

The final major component to this design is the conditional rescue. Having found the Tet-inducible rescues to be difficult to construct and not any more functional than simply separating the promoter from the toxin, we decided to work with the FLP excisable rescues as shown in Figure 4.4b, but without a miRNA suppressor. All of the constructs discussed here required exogenous FLP to be crossed in as discussed in the previous section, although a Tet-induced FLP cassette was designed and is still being tested in the lab. It is discussed in Appendix E. These components were assembled into the *Drosophila* attB backbone and injected by Rainbow Transgenics, Inc.

Fly Results

The initial set of flies generated carried RpL35a-targeting constructs driven by the hands-hsp70, Dfd-hsp70 and prm-hsp70 promoters. As shown in Table E.1, these constructs all resulted in good killing. When the flies were crossed to germ line FLP flies and

then outcrossed to w-, no survival of FLP-excised, UD construct bearing flies was observed. Unfortunately, when the ovo-FLP/X; UD1/+ flies were crossed to the β-tubulin/Y; UD2/+ flies, no transheterozygous progeny were recovered. The RpL35a rescuing cassette was not sufficient to resupply the protein and rescue.

CG5266 targeting UD constructs were also used to generate flies. Constructs driven by the hand-hsp70 and prm-hsp70 promoters were injected. Unfortunately, the CG5266 flies were healthy, even after FLP excision of the blocking ORF. This, however, gave us an opportunity to examine the function of some of the system components.

I began working with the hand-CG5266-LexA and hand-CG5266-VP16 flies because I noticed that the ovo-FLP/X; UD1/+ X β-tubulin/Y; UD2/+ progeny could be categorized into two groups: those with wild type wings and those with delicate, fluid filled wings. Initially, we hypothesized that perhaps suppressing CG5266 in the heart, while not lethal, did induce a fitness cost (these flies had significantly worse survivorship in addition to the wing phenotype, but were fertile). I sorted the flies into two groups, the healthy flies and the sickly flies, and genotyped individuals. If the miRNAs were inducing the sickly phenotype, I expected to see the healthy winged flies carried both the LexA and the VP16 UD alleles, and that the sickly flies bore only one UD allele. Instead, I observed that the sickly phenotype segregated with the LexA allele, as shown in Figure 4.11. This suggested that expression of the CC-LexA protein in the heart was causing the sickly phenotype. Later experiments by Anna Buchman indicated that suppression of CG5266 in the heart also produced this phenotype, so it is likely a result of compromised heart function in *Drosophila*.

The viable CG5266 flies allowed me to ask two more questions: whether or not the LexA-VP16 split transcription factor was coming together to activate transcription of the rescue, and whether the 8LexA-UASp promoter I had constructed leaked. To do this, I pooled the sickly flies, some of which would carry both LexA and VP16, and the healthy flies that carried only VP16, and carried out RT-PCR to assess the level of expression of the rescue transcript. I was able to sample the rescuing transcript without contamination from the native transcript because the region across the start codon had been recoded so as to be insensitive to the miRNA toxin.

In flies carrying only the VP16 allele, polyT priming did not give rise to a CG5266 band, but gene-specific primers (GSPs) gave a faint band. The endogenous hid transcript was used as a positive control for the RT and PCR reactions. When the mixture of LexA only and

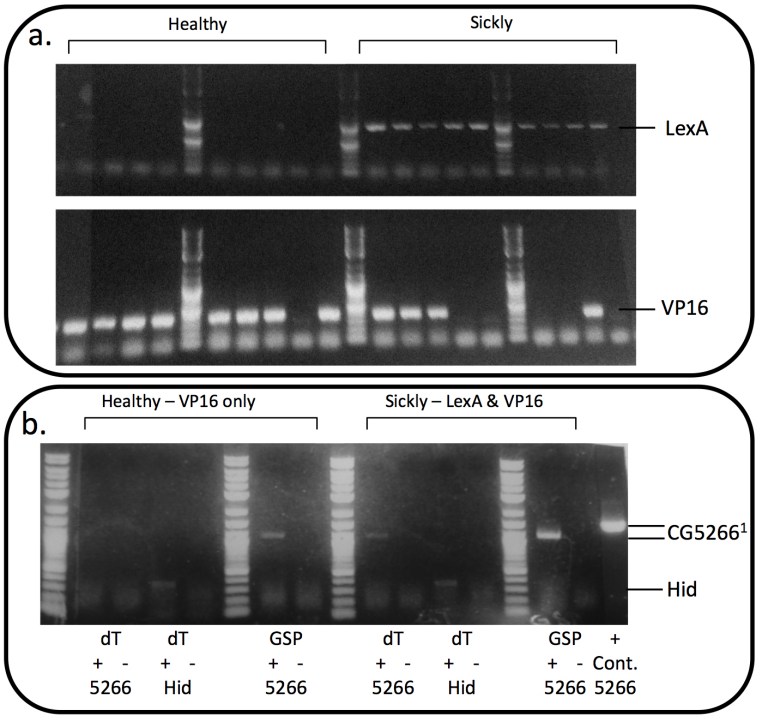


Figure 4.11: (a) The sickly phenotype segregates very cleanly with the LexA allele. Carrying both alleles does not alleviate the fitness effects of LexA expression. (b) Expression of the CG5266 allele is weak, but detectable with gene-specific primers (GSP) in flies carrying only the VP16 allele. Expression is greatly enhanced when some of the flies in the sample carry both LexA and VP16 alleles.
[1]The CG5266 control band is from a plasmid template and gives a larger product than the RT-PCR bands because the plasmid carries a genomic CG5266, which has introns totaling approximately 325 bps.

LexA/VP16 flies was assayed, a CG5266 band was visible with both a polyT primer and GSPs. This is not quantitative, but I felt comfortable concluding that the transcription factor does assemble *in vivo* and initiate transcription of the rescuing transcript. There was some leak occurring from the 8LexA-UASp promoter, however, and it was of concern, so this lead me to test other 8LexA promoters. As mentioned previously, the minimal TATAA and DPE promoters did not function in tissue culture, but fly constructs were built using the 8LexA-hsp70.

Around this time, I also began to notice non-uniform GFP phenotypes and eye colors from my 86Fa lines. While it is still unclear what the cause of this was, I moved away from the 86Fa line and began working with the 8621 attB line. Injections of hand-RpL35a with the 8LexA-hsp70 promoter gave identical results to those achieved at 86Fa with the 8Lex-UASp promoter, but results from other lines indicate that 8621 is not a particularly

miRNA-based UD Constructs and Flies				
Promoter	Target	Rescue Promoter	Injection Site	Killing
hand	IAP1	8LexA-UASp	86Fa	No
	RpL35a	8LexA-UASp	86Fa	Yes
		8LexA-hsp70	8621	Yes
	CG5266	8LexA-UASp	86Fa	Partial[1]
		8LexA-hsp70	8621	No
Fln	Fln	8LexA-hsp70	8621	Partial[2]
	IAP1	8LexA-UASp	8621	No
	RpL35a	8LexA-UASp	8621	No
Dfd	IAP1	8LexA-UASp	86Fa	No
	RpL35a	8LexA-UASp	86Fa	Yes
GMR	IAP1	8LexA-UASp	86Fa	No[3]
	RpL35a	8LexA-UASp	-[4]	
prm	RpL35a	8LexA-UASp	86Fa	Yes
		8LexA-hsp70	8621	Yes
	CG5266	8LexA-hsp70	8621	No

Table 4.3: Fly results
[1]Killing was attributed to expression of LexA.
[2]Only a single LexA line was flightless and had a very dark eye color. This could be due to over-expression of LexA, an off-target insertion, or both.
[3]A surprising result, as this miRNA pair had been shown to kill previously (Chun-Hong Chen, unpublished.)
[4]No transformants were generated.

strong site. A complete list of systems tested is in Table E.1.

With miRNA suppression of RpL35a unable to be rescued, and CG5266 miRNAs unable to kill, I went back to a protein whose depletion is known to induce apoptosis, IAP1. Unfortunately, despite the miRNAs against IAP1 having been reported to work, I did not observe an eye or flight phenotype. Testing with the hand-hsp70 or Dfd-hsp70 promoters also failed to give lethality.

I built a non-lethal system using the Fln promoter driving miRNAs targeting the Fln mRNA. I did generate one flightless line, a LexA allele. Many Fln-VP16 and other Fln-LexA insertions were isolated, but all were able to fly. Given my previous data indicating that expression of LexA on its own can be toxic, it is possible that the flight muscle was not compromised due to loss of the Fln transcript, but only due to LexA expression. Also, the eye color was not as expected for the injection site, so it was likely an ectopic insertion.

Flight was not was not rescuable by crossing Fln-VP16 line to the flightless Fln-LexA line, also indicating that the phenotype might not have been due to the action of the miRNAs.

Untested Constructs

I next needed to determine whether the leakiness from the 8LexA promoter was precociously rescuing the CG5266 constructs or the miRNA toxin was not potent enough to kill. Two additional sets of miRNAs were designed. For the first set I selected three targets, but tried to distribute them throughout the transcript. The second set of targets was selected by Geoff Pittman who took the optimal targets which clustered very near each other, with two of the three actually overlapping. Blocked kill only constructs using the VP16 allele and each of the three sets of miRNAs were sent out for injections (Figure 4.12), but BestGene could not generate transformants. At this point, I moved on, but Anna Buchman later tested the three sets of miRNAs in tissue culture and then in the fly eye. She determed that the original set gave the weakest killing, and that the clustered, overlapping set gave the strongest. These miRNAs are now being used to test alternative methods of rescue such as using inteins to reassemble the rescue protein.

Figure 4.12: This construct bears only the VP16 portion of the split transcription factor and the miRNA toxin. It is nontoxic until the blocking cassette is excised, and allows the testing of miRNA killing in the selected tissue.

The final set of constructs that I built (but did not test) were intended to address the clear fitness cost induced by the expression of CC-LexA. The 8LexA promoter was replaced by the Gal4-UASt promoter, and CC-Lex replaced by a CC-Gal4DBD. Versions containing the most potent set of CG5266 miRNAs and the hand promoter were developed, but not injected and I moved back to working on the Hid-UD chromosomes. If a tissue sensitive to knockdown of CG5266 can be identified, this Gal4 system should be tested.

4.3 Future Studies

Development of underdominance in *Drosophila* remains an important goal in the field of insect engineering. The current tool set is limited, and underdominance offers two different

drive profiles each of which is not currently available. Working in *Drosophila* still offers the best chance of developing a drive system model which can then be moved into pest insect genomes.

Protein Toxin Underdominance

When considering the hurdles to protein-based UD development, one important set of tools that is still being developed are the transcriptional profiling data sets. There is a massive amount of data available now that did not exist when we carried out our initial enhancer selections. Some of the choices we made initially in light of this data look very good, like the hand enhancer. The expression pattern is tight, and it is on during later embryogenesis, all larval stages and the pupal to adult transition giving a good window for killing. Others, like EDG84a, show a strong pulse at a single developmental stage that may or may not be particularly sensitive to killing. A short window with moderate expression levels might not be the best sort of profile to pursue. With what is now known, the enhancer for ACP65a, also a cuticle gene, might be a better choice because its overall expression level is higher and it is on through the pupal-adult transition.

These data sets do not, of course, guarantee that the enhancer selected will give rise to the desired expression pattern, but they can guide enhancer selection. Eliciting the expression pattern desired is more likely when starting with an enhancer for a gene that has the potential to give rise to that pattern. Looking back at the enhancers we screened earlier could reveal some that warrant more study and new candidates could also be identified.

I also think that a stable, strong PhiC31 site has finally been identified in 51D. Conducting comparable experiments is important for understanding the function of our components, and testing enhancers that were previously unable to kill at this site might breathe new life into some of the constructs that have been set aside. Taking constructs that kill at some sites, but not others, like the hand-hsp Hid-UD constructs could also help identify other PhiC31 sites that have strong somatic expression.

As my constructs evolved, refinement of the conditional rescue reduced the overall size and complexity of the UD-alleles. The ‘doubly-off’ system of expressing GFP in place of the toxin and placing rescuing miRNAs in the 3’UTR expression allowed for the easy generation of transformants. In future, use of this rescue paradigm will ease the generation of transformants and speed the experimental cycle.

The Hid-UD system appears to be on the verge of functioning. It's unclear why rescue cannot be effected in the heart when it appears to be so complete in the eye. It is possible that the long expression window from the hand enhancer allows for enough Hid protein to be produced that it is toxic. Using a wild-type version of Hid could solve this problem, but might not prove a potent enough toxin. The miRNAs may also not function well enough through all the developmental stages of the heart to keep Hid suppressed. Increasing the number of miRNAs, the number of sites targeted, or the backbone used may all help. Some of these solutions are already being tested.

MiRNA Toxin Underdominance

The hurdles of the miRNA toxin approach are different. The studies described here have revealed a lot about many of the components of the system, but discovering the difficulties is not the same as finding solutions.

It seems target selection must be done empirically. Despite working from an RNAi study focused on the same tissue I was targeting, killing remained hit or miss. This is likely due to effectiveness of miRNA targeting, sensitivity of the tissue to depletion of the transcript, and stability of the protein produced. Reverting to the use of the promoter in the RNAi study, *tinman*, did not enhance our killing. Redesign of miRNAs has improved killing as observed through their suppression of CG5266 in the eye, but all three sets of miRNAs gave killing in tissue culture. Killing using these miRNAs in the heart, even the best set, is ambiguous as the sickly phenotype is partially penetrant and the adults are fertile. Additionally, expression of the RpL35a miRNAs (which kill quite efficiently *in vivo*) in tissue culture did not kill S2 cells, indicating tissue culture may not be a reliable test bed for miRNA efficacy. Ultimately, this means that each system must be tested directly in flies—a slow and painful process because each set must be blocked by a FLP-excisable rescue that requires genetic removal, unless the Tet-inducible FLP system can be implemented.

The mode of rescue presents more difficulties. It is clear from this work that the LexA-VP16 transcription factor can induce expression *in vivo*, but also that the 8LexA promoters tested so far have significant leak in the heart, and likely other tissues as well. The CC-LexA used in most of my designs also clearly results in a fitness cost on its own. The Gal4 system has not been tested, but is likely to give somewhat weaker expression based on tissue culture experiments, a problem because expression of the rescuing RpL35a transcript already seems

insufficient. Getting away from split transcription factors would eliminate promoter leakage and fitness costs from that rescue approach, but the intein rescue, which allows reassembly of the native protein, is likely to be fairly inefficient. Initial tests in the eye have shown that it cannot rescue the small eye phenotype induced by miRNA suppression of CG5266.

Since I last worked on this project, the PhiC31 insertion site issues seemed to have been resolved and expression at 51D using the hand enhancer is very strong. One more effort with RpL35a seems called for, especially because the constructs needed could be easily assembled with parts available. The very 5' end of the RpL35a rescue could be recoded to be insensitive to the miRNAs with a single primer, and the Gal4-VP16 split transcription factor could be tested. Use of new translational enhancers could boost protein production induced by the Gal4-VP16 transcription factor (Pfeiffer et al., 2012).

Ultimately the difficulty with the miRNA approach lies in the large number of parts that must be individually tuned and the fact that observing their behavior in flies requires a large time investment and a large number of transformants. In-house injections into a stable, strong PhiC31 site will allow progress at modest cost, but on long time scales.

4.4 Materials and Methods

Protein Toxin Constructs

The first generation underdominance constructs were cloned by traditional methods into the *Drosophila* attB vector with an multiple cloning site (MCS) comprised of 8 bp restriction sites. The 5' end of Hid^{Ala3} was PCRed from BH932 and cloned PmeI/AsiSI. The tubulin 3' UTR was PCRed from pGMR-D5DD3RT, described in the previous chapter, and cloned in AscI/SwaI. Two versions of the 3' end of Hid were recoded and synthesized by GeneArt (Regensburg, Germany) as were the two triplets of miRNAs that targeted these recoded Hid fragments. The 3' Hid fragments were cloned AsiSI/FseI and the miRNAs FseI/AscI. The GMR promoter was PCRed from pGMR-1N and cloned PacI/PmeI. Annotated versions of the miRNAs and the recoded Hid 3' ends are shown in Appendix C. The pair generated comprises the original set of underdominance constructs, attB-GMR-rH1m2 (UD1) and attB-GMR-rH2m1 (UD2). UD1 constructs always carry Hid version 1 and miRNA set 2 and vice versa for UD2 constructs.

Conditional rescues were assembled separately. The original Tetracycline-inducible res-

cuing cassette was assembled in pBSK. P_{tight} (XhoI/HindIII) and rtTA2^S-M2 (SpeI/BamHI) were PCRed from plasmids that are part of the Tet-On Advanced Inducible Gene Expression System produced by Clontech (Mountain View, CA). γ-tubulin 23c (EcoRI/PstI) and Actin 88F (BamHI/PstI) 3' UTRs were PCRed from *Drosophila* genomic DNA. eGFP (HindIII/EcoRV) was PCRed from the KHS-GFP construct. The tissue specific promoters were cloned NotI/SpeI. Each set of miRNAs was cloned via the enzymatic assembly (EA cloning) procedure (Gibson et al., 2009) into an EcoRV site. This procedure is more fully documented in Appendix C. Primers for all these components are shown in Table 4.4 and a schematic in Figure 4.4.

The second generation of UD constructs were made targeting essential tissues. Promoters PCRed from genomic DNA and cloned directly into the attB-UD constructs PacI/PmeI were Nervy (Kuzin et al., 2009) and Serendipity-α (sry-α) (Ibnsouda et al., 1995). EDG84a (Kayashima et al., 2005; Murata et al., 1996) was cloned as a complete promoter, but with its native ATG mutated away via PCR. The super core promoter (SCP) (Juven-Gershon et al., 2006) was PCRed onto giant (gt) (Berman et al., 2002). Other enhancers were PCRed from genomic DNA and cloned into pBSK NotI/XbaI. A fragment of the hsp70 promoter was cloned downstream XbaI/SpeI. Complete promoters were then PCRed and cloned into the attB-UD backbone and the Tet-inducible rescue. Enhancers cloned in this manner are scute (sc) (Deshpande et al., 1995), Nerfin-1 (Kuzin et al., 2009), mef-2 (Nguyen and Xu, 1998), hand (Sellin et al., 2006), deformed (Dfd) (Lou et al., 1995), and paramyosin (prm) (Hess et al., 2007; Marco-Ferreres et al., 2005). The primers that define all these fragments are in Table 4.4. These promoters were then sub cloned into the attB-UD constructs. A final promoter, flightin (Fln) (Rider et al., 2011) was cloned as an alternative to essential tissue targeting into the Hid-UD constructs.

Name	Sequence 5' to 3'
P_{tight} F	GGC ATC GAT GCG ATC TGA CGG TTC ACT AAA CG
P_{tight} R	GGC CTC GAG TTA ATT AAG TAT CAC GAG GCC CTT TCG TCT TCA
Gamma Tubulin UTR F	GGC GGA TCC TCG CTT GTG CCA GAA GAA ATG C
Gamma Tubulin UTR R	CTG CAG ATT TAA AAA CTG TTT TTT ATT TAA TGT GTC
Actin UTR F (3' End)	GGC CTG CAG AAA ATA TTT AAT AGT TTT AAT GGA AG
Actin UTR R (5' End)	GGC GAA TTC GTC TTT CGC CCG CCG CGA AAG CTC
tTAV F	GGC ACT AGT ATG TCT AGA CTG GAC AAG AGC A
tTAV R	GGC GGA TCC TTA CCC GGG GAG CAT GTC AAG G
GFP F (EA)	ATC GAT AAG CTT GAT ATG GTG AGC AAG GGC GAG GAG CTG T

Name	Sequence 5' to 3'
GFP R (EA)	AAA GAC GAA TTC GAT ATC TTA CTT GTA CAG CTC GTC CAT GCC
miRNA F (EA)	AAA GAC GAA TTC GAT ATC ATA TTC TAG AGT TGA TGG ATC CGT AG
miRNA R (EA)	CTG TAC AAG TAA GAT ATT AAA AGA TCT ATG GCA TAC CC
Tubulin UTR F	GCG GCG CGC CGC GTC ACG CCA CTT CAA CGC TCG AT
Tubulin UTR R	GGC GAT ATC ATC GAT GCG GCC GCA TTT AAA TAA AGA AAA ACA GTG GGG TTT TCT TA
gt F	GGC TTA ATT AAA GAA ACT TAC CAT CAC TTC GAG ATG
gt R w/ SCP	GCC GTT TAA ACC GGT CCG TAG GCA CGT CTG CTC GGC TCG AGT GTT CGA TCG C
EDG84a F	GGC TTA ATT AAA ATT CTT TTT TAT TAA TTT TAA AGT TAC
EDG84a R I	ACT TGT AAA TAA CAC ATG ATA TAC CTT AAC CAA AAT GCT GAT CGA ATT TTT AGG TTG ATG
EDF84a R II	GGC GTT TAA ACA GTC TGT ACG TGG TTA AAG GAT TAG GAT AAA GGC AAG CCA ACT TGT AAA TAA CAC ATG
hsp70 F	GGC TCT AGA ATC CCA AAA CAA ACT GGT TGT TGC GG
hsp70 R	GGC ACT AGT GGT GGC GAC CTT AGG AGA TCT GC
Dfd F	GGC TTA ATT AAT GAC CTG ACC CAT GTT AGT TCA CAT TTT TC
Dfd R	GGC TCT AGA GCA AGG GGA TGG GTT GGT TAC G
Ubq F	GGC GCG GCC GCC GCG CAG ATC GCC GAT GGG CGT GGC GC
Ubq R	GGC ACT AGT TCT GCG GGT CAA AAT AGA GAT GTG G
Nerfin F	GGC TTA ATT AAG TGT CTG CTA GTC TGT TAG TCT G
Nerfin R	GGC TCT AGA CTC GAG TGT CCT TTT TCG ACG CCG GC
Nervy F	GGC GCG GCC GCC TAA AGC CCT CGA TGT GCC CAT TTC C
Nervy R	GGC ACT AGT TCC GAC CAG TCG TAA GTG GCG TTT G
hand F	GGC GCG GCC GCG TAA GAG AGT AAT TTA CTT TGT CAT GC
hand R	GGC CTG AAA TAT AAA TTG TCA TTA TTA ATT GGA ACA
sc F	GGC TTA ATT AAT ATC TGT ATC TTA GCA TCT TTA CCC ATA TCG
sc R	GGC TCT AGA TGG ACC ATG GCG ACG CGT GGC AGG TGT ATT
mef2 F	GGC GCG GCC GCG AAG AAA CCC CTG CCA AGC AGT TAA
mef2 R	GGC TCT AGA ATT CTG ATT CCC GTT TGC AGT GTC C
Sry-α F	GGC TTA ATT AAG GTA GTC CTT TGC TAG ATT AAT CTA AGA AGC C
Sry-α R	GGC GTT TAA ACG CTG TTC TAT CAG ATG TGC TCC GGG AAA C
prm F	GGC GGC GCG CCT CAA GTA GGC ACA CAC TCT
prm R	GGC CCT AGG GTG AAA ATT CTC GTT CGT CTT CGA GCG

Table 4.4: Oligonucleotide primers used to construct the tetracycline-inducible rescue and to isolate promoter fragments used in protein-kill UD constructs

Additional conditional rescue cassettes, also diagrammed in Figure 4.4, were cloned using a subset of the above parts and a few others. FLP was PCRed from lab sources, and contained a glycine at position 5 of the protein (Nern et al., 2011). The FRT sequence used was GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTT

C (Zhu and Sadowski, 1995). It was PCRed onto components using a pair of primers each time. I assembled them as few times as was possible because they were prone to mutation and the PCRs were difficult to perform because they have a large degree of self complementarity. These later versions of the rescue were assembled using EA cloning (Gibson et al., 2009). Primers had 30 bp of sequence overlapping the neighboring fragment and 30 bps that annealed to the fragment being amplified.

Two other modes of rescuing were also built, but never tested. The first utilized an RU486 inducible Gal4 that drove GFP and miRNAs, just as in the Tet-inducible system. This utilizes the canonical Gal4 UAS, PCRed from lab sources and Gal4 from the P{Switch} system (Roman et al., 2001). These components were cloned into the Tet-inducible rescue in place of P_{tight} and tTAV. The second system utilized a FLP modified to function only in the presence of estrogens to excise the rescuing functionality from the UD construct. This system used the bag-of-marbles germ line promoter, PCRed from genomic DNA, to drive FLPe-ERT2 (Hunter et al., 2005). The cassette also carried the tissue-specific promoter being used to drive the toxin as the driver for a GFP marker and a rescuing set of miRNAs (Figure 4.4b).

The final set of Hid UD constructs bore the α1-tubulin promoter, PCRed from genomic DNA, driving GFP and a rescuing set of miRNAs between FRTs sites (the first positioned downstream of the tissue specific promoter, and the second downstream of the UTR of the GFP-miRNAs cassette). FLP excision required introduction of a germ line FLP via crossing to a germ-FLP bearing fly.

MiRNA Toxin Constructs

The split transcription factor I used consists of the LexA DNA binding domain (DBD) and the VP16 activation domain (AD), each tagged with a coiled-coil (Arndt et al., 2001; Luan et al., 2006). The AD was also tagged with a nuclear localization signal, AAPAAKKKKLD. The two halves, CC-LexA and NLS-VP16-CC, were cloned into the pAc5.1-HisB backbone EcoRI/XbaI. The coiled-coils and nuclear localization signal (NLS) were fused to the DBD and AD by PCR. A short flexible linker (AGSSTGSSTG) was inserted between the coiled-coil and the DBD or AD. The codon optimized LexA::VP16 described in Chapter 3 was also cloned into pAc5.1-HisB. The Actin5C promoter was removed from the pAc5.1-HisB backbone BglII/KpnI and replaced with the LexA UAS (8LexA), PCRed from pSH-18-34,

fused to one of several basal promoters. Basal promoters tested were UASp, hsp70, the sexcombs reduce DPE, and a minimal TATAA fragment described by Butler (2001).

Name	30 bps of the 5' and 3' ends of each fragment, 5' to 3'	Source
UASp	ATACATATCCATATACATATCCATATCTAA GATTAGTTTTTTAGCCTTATTTCTGGGGTA	pSH18-34
8lexA	TCGATAGCCGAAGCTTACCGAAGTATACAC ACAATAACGTGACTGTGCGTTAGGTCCTGT	pCaSpeR5-UASp
hsp70	GCGAAAAGAGCGCCGGAGTATAAATAGAGG AACAAGCGCAGCTGAACAAGCTAAACAATC	pGMR
UASt	TGCATGCCTGCAGGTCGGAGTACTGTCCTC ATTATTGAATACAAGAAGAGAACTCTGAAT	pUASt
DPE	TTATTGATGCTCAGAAATTTGAATTATGCC TTGGCGCTTCGTGCACGTATCCGCGGATAC	Genomic
TATAA	GGCTATAAAAGGGGGTGGGGGCGCGTTCGT GACGTGCCAAATCTCATGTCCGCCGCCGCT	PCR Assem.
CTCF	CCTTGCAGCGCCACCTGGCCGCGAAGAGTT ACAACTCACACCTTGCAGCGCCACCTGGCC	Genomic + PCR Add-on
Actin 3' UTR	GTCTTTCGCCCGCCGCGAAAGCTCTTCAAA ATATCTTCCATTAAAACTATTAAATATTTT	Genomic
γ–tubulin 3' UTR	TCGCTTGTGCCAGAAGAAATGCGTGCCATG TGACACATTAAATAAAAAACAGTTTTTAAA	Genomic
D.ana IAP	ATGGCAAATGTGGTAACCAGCTCGCGGTAT ACCGATGTGATGCGTGTATATTTTTCTTAA	D.ana Genomic
RpL35a	ATGGCCGACACACAAGCCAAGTCCACTACT TTGCAGATGCTGTACCCATCAAGGATTTAA	Genomic
CG5266	ATGGCAACTGAGCGATACAGCTTTTCGTTG[1] ATCAAGGACTACTTGGCCAGCATCCCCTAA	Genomic
Fln ORF	ATGGCAGACGAAGAAGATCCATGGGGTTTC ATCAACCAAAGGTATGCCAGTGTCCTTTAG	Genomic
Fln Promoter	CGTTCCCGTGATAGAGTAACGGTTCCTTTT TCAGTGGACCCAATGTCCTAGTTTTAGCTG	Genomic
NLS-VP16-CC	ATGGCGGCTCCGGCTGCCAAAAAGAAGAAA CAGTATCGGACACGATACGGCCCACTGTAA	pAc-NLS-VP16-CC
CC-LexA	ATGTTGGAAATTCGTAGGGCCGCTTTTCTG GAGCTCCATCTGGACGGCGAAGACGTTTAA	pAc-CC-LexA

Table 4.5: First and last 30 bps of fragments used in the miRNA-toxin constructs [1]The 5' end is recoded to make it insensitive to the miRNA.

The architecture of the full UD constructs utilizing miRNA-based killing and a split transcription factor rescue is shown in Figure 4.10. The assembly of all of these constructs was achieved through EA cloning (Appendix C). They each consist of two halves. One half

bears the LexA-VP16 responsive promoter driving the rescuing mRNA and a 3'UTR. The other part of the constructs bears the tissue specific promoter, half the rescuing transcription factor, the miRNA toxin and a 3'UTR. The first and last 30 bps of each fragment that has not been previously described are listed in Table 4.5.

The GMR, hand-hsp70, Dfd-hsp70, and prm-hsp70 tissue-specific promoters were generated as described. Fln was PCRed from genomic DNA. The split transcription factor components are as described above. The 3'UTR used downstream of the miRNAs is γ-tubulin.

MiRs were cloned as doublets and triplets as described in Appendix D. Identical miR6.1 backbones were used for all miRNAs. Site selection was carried out using rules described by Chun-Hong Chen or using the Dharmacon siRNA design tool (http://www.dharmacon.com/designcenter/). A list of the miRNAs used in this study is provided in Table 4.2. Each set of miRNAs was flanked by splice sites so the doublet or triplet would be spliced from the transcript as as single unit. The consensus sequence used for the 5' site was cagGTaagt and for the 3' site was tactaattcttcttttccctttttttttAGg with the donor and acceptor highlighted in capitol letters.

The rescuing mRNA used was matched to the toxin. The RpL35a rescue was PCRed out of the *Drosophila* genome. For Fln and the first CG5266 constructs, the recoding of the mRNA only required a few point mutations around the start codon and could be accomplished using a single forward primer. Later version of the CG5266 were recoded via EA cloning techniques. The actin 3'UTR was used downstream of each of the rescuing open reading frames (ORFs). Partially artificial CTCFs were generated by PCRing a pair out of the *Drosophila* genome and adding another pair, one on each primer making a tetramer roughly as described in Kyrchanova et al. (2008). One CTCF was place between the two halves of the construct, and the other either 5' to the rescue or 3' to the miRNA bearing section.

RT-PCR

RNA was prepped using Qiagen's RNeasy Kit (Valencia, CA). RT-PCR was carried out using SuperScript III First-Strand Synthesis Kit from Invitrogen (Carlsbad, CA) according to package instructions. The final PCR was conducted using Phusion (Thermo Scientific, Lafayette, CO).

Tissue Culture

Drosophila S2 cells were maintained in Schneider's medium 10% FBS, 1% penicillin and streptomycin (P/S) at 27.5°C, passaged every 3–4 days. S2 cell transfections were carried out using the FuGENE6 reagent. The most successful ratio of FuGENE:DNA was 2.5 μl:1 μg. A GFP or RFP marker was often used, and always as 20% of the DNA transfected. Use of endotoxin-free DNAs was essential for good transfection efficiency. A 2.7 M tamoxifen stock solution and a 3.7 M estradiol stock solution (both from Sigma) were made in EtOH and stored at -20°C. Dilutions were made in ethanol and amounts not greater than 2 μl were added to wells containing 500 μl culturing media. Mock treatments of EtOH containing no drug were used as controls.

Fly Culture and Strains

Fly crosses were carried out under standard conditions at 25°C. Injections were carried out both in-house according to standard methods with Hay lab particulars described in Appendix C, and by several companies including Rainbow Transgenics (Camarillo, CA), BestGene (Chino Hills, CA), Genetic Services Inc. (Sudbury, MA) and Genetivision (Houston, TX). Rainbow Transgenics and Genetivision provided the most reliable transformation results.

A saturated solution of tetracycline (Tet) in 70% EtOH or 50 mg/m doxycycline-hyclate (Dox) in PBS was added directly to our standard fly food at a ratio of 1:100. Fly food was liquefied by microwaving and allowed to cool to $\sim$ 50°C. The concentrate was added directly to the food and mixed, and the food was allowed to resolidify. Because Tet degrades rapidly in the light, food is stored in the dark at 4°C. For rearing, repeated Tet treatments were carried out by adding 10 μl of the drug solutions directly to the food the flies were cultured in and on.

Additional strains used in these studies were ovo-FLP (Bloomington Stock Center # 8727, 8728, 8729), β-tubulin-FLP (# 7196), and PhiC-31 lines 86Fa (# 24486) and 96E (# 24487).

Chapter 5

Conclusions

Engineering Insects is Hard

This is an outrageous oversimplification and poor final conclusion for this body of work, but it summarizes many of the lessons I have learned. *Drosophila* is one of the best understood organisms on the planet, having been studied for over one hundred years. The cell death pathway, one of the systems I primarily worked with, has been intensively investigated (Hay and Guo, 2006). Much of the work has been done in the eye —a non-essential tissue that tolerates a great deal of disruption—using a well-characterized enhancer (see Bergmann et al. (2003, 1998); Hawkins et al. (2000); Hay et al. (1994); Yoo et al. (2002) as examples). Thus, this cell pathway and tissue target was almost an ideal choice for our model drive experiment. Perhaps unsurprisingly, the proof-of-principle system in the eye worked as predicted. Despite the fact that the cell pathway is known to be active in numerous tissues throughout the organism, we were often unable to produce expected phenotypes when the system was moved to other essential tissues, even when other carefully evaluated enhancers were used. While more data is continually being generated that describes expression levels and patterns, we now know that each enhancer will have to be tested empirically. When I did elicit expected phenotypes, suppressing the apoptotic activities was unsuccessful, which implies that miRNA machinery is also not the same in the eye as in all other tissues.

My other studies also encountered similar problems of components not working uniformly from one set of conditions to another. The work on viral sensors utilizing cell death proteins was complicated not by the inability to kill, but by difficulties in keeping the death-inducing proteins from carrying out their activities. Literature reporting success in tethering proteins to membranes (Browman et al., 2006; Op De Beeck et al., 2004; Szczesna-Skorupa

and Kemper, 2000) was not as translatable to the mosquito tissue culture system as we had hoped. The signaling and localization sequences derived from other systems, and studied in various tissues, could not reprise their functions in mosquito tissue culture. Function of the UAS promoters also showed variability when moved between S2 culture, C6/36 culture, and *Drosophila*. My work also highlights the concern that models created in tissue culture may require additional and unpredictable adjustments when implemented in animals.

The overarching challenge is the staggering complexity of cellular metabolism, of interactions between cells, within tissues, and in whole organisms. Carefully designed inputs can interact with thousands of other components in the cellular milieu, and it is impossible to predict exactly how they will behave. We are still largely engineering in a black box, and future progress will rely on empirical results and direct testing of systems *in vivo*.

Progress is Possible

Despite these challenges, we have learned a great deal from my efforts here. Many components of the viral sensors have been tested and refined. I have identified a split-transcription factor that works in both *Drosophila* and C6/36 culture. Tests of cell death proteins from *Drosophila* in C6/36 culture indicate that, in some cases, those components can be moved from one species to another. Experiments investigating the fate of protease-based sensor components will identify the point at which failure is occurring and, hopefully, allow completion of that project. In light of the new information about the mosquito cell lines, work can be resumed on the RdRp-base approaches. Testing of the RdRp substrates, in some ways, never began, so testing of those sensors is a great next step in relaunching that project.

The underdominant systems also need more diagnostic work, but the functioning proof-of-principle system in the eye demonstrates that the protein-based system is viable. Identification of a suitable essential tissue and improvement of miRNA function are the two remaining variables that will allow completion of the lethal system. The miRNA-based system has many more tunable parts, and once an appropriate tissue, or two, are selected, components can be tested individually to identify weak points in the design. Working on one component at a time will be slow, but should allow steady advancement of the project and hopefully the building of a functioning underdominant system suitable for testing in vector species.

Improved genome manipulation techniques in *C. elegans* offer a chance to revisit the

transitive RNAi project. Work on miRNAs in many systems including *C. elegans* will be able to better inform target selection. The systematic perturbations of genes and expression patterns that are carried out in the worm may offer a treasure trove of genes and tissues to work with when trying to genetically isolate one line from another.

Engineering Insects is Important

Despite the challenging and ambitious nature of this work, I am confident that engineered insects can play an important role in reducing the burden of vector-borne disease. As with the development of any new technology, early days have been fraught with setbacks, but the ultimate product makes it worth the time and energy invested. Reduction in efficacy of pesticides and anti-malaria medications (Shah et al., 2011; Trape et al., 2011) means an increasing disease burden where disease is already endemic. As temperatures rise and the ranges of these insects broaden, exposure to these diseases will only rise (Lambrechts et al., 2010). Working to address this problem now is part of our responsibility to the future.

Bibliography

Alder, M. N., Dames, S., Gaudet, J., and Mango, S. E. (2003). Gene silencing in *Caenorhabditis elegans* by transitive RNA interference. *RNA*, 9(1):25–32.

Alvarez, D. E., Lodeiro, M. F., Ludueña, S. J., Pietrasanta, L. I., and Gamarnik, A. V. (2005). Long-range RNA-RNA interactions circularize the dengue virus genome. *Journal of Virology*, 79(11):6631–6643.

Arndt, K. M., Müller, K. M., and Plückthun, A. (2001). Helix-stabilized Fv (hsFv) antibody fragments: substituting the constant domains of a Fab fragment for a heterodimeric coiled-coil domain. *Journal of Molecular Biology*, 312(1):221–228.

Barro, P. J., Murphy, B., Jansen, C. C., and Murray, J. (2011). The proposed release of the yellow fever mosquito, *Aedes aegypti* containing a naturally occurring strain of *Wolbachia pipientis*, a question of regulatory responsibility. *Journal für Verbraucherschutz und Lebensmittelsicherheit*, 6(S1):33–40.

Bera, A. K., Kuhn, R. J., and Smith, J. L. (2007). Functional characterization of *cis* and *trans* protease activity of the flavivirus NS2B-NS3 protease and trans activity of the flavivirus NS2B-NS3 protease. *J Biol Chem*, 282(17):12883–12892.

Bergmann, A., Agapite, J., McCall, K., and Steller, H. (1998). The *Drosophila* gene *hid* is a direct molecular target of ras-dependent survival signalling. *Cell*, 95:331–341.

Bergmann, A., Yang, A. Y., and Srivastava, M. (2003). Regulators of IAP function: Coming to grips with the grim reaper. *Curr Opin Cell Biol*, 15(6):717–24.

Berman, B., Nibu, Y., Pfeiffer, B., Tomancak, P., Celniker, S., Levine, M., Rubin, G., and Eisen, M. (2002). Exploiting transcription factor binding site clustering to identify cis-

regulatory modules involved in pattern formation in the *Drosophila* genome. *Proceedings of the National Academy of Sciences*, 99(2):757–762.

Bitinaite, J., Wah, D. A., Aggarwal, A. K., and Schildkraut, I. (1998). FokI dimerization is required for DNA cleavage. *Proceedings of the National Academy of Sciences*, 95(18):10570–10575.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 77(1):71–94.

Browman, D. T., Resek, M. E., Zajchowski, L. D., and Robbins, S. M. (2006). Erlin-1 and erlin-2 are novel members of the prohibitin family of proteins that define lipid-raft-like domains of the ER. *Journal of Cell Science*, 119(15):3149–3160.

Butler, J. E. F. (2001). Enhancer-promoter specificity mediated by DPE or TATA core promoter motifs. *Genes & development*, 15(19):2515–2519.

Carareto, C. M., Kim, W., Wojciechowski, M. F., O'Grady, P., Prokchorova, A. V., Silva, J. C., and Kidwell, M. G. (1997). Testing transposable elements as genetic drive mechanisms using *Drosophila* P element constructs as a model system. *Genetica*, 101(1):13–33.

Chambers, T. J., Hahn, C. S., Galler, R., and Rice, C. M. (1990). Flavivirus genome organization, experssion and replication. *Annual Reviews Microbiology*, 44:649–688.

Chen, C.-H., Huang, H., Ward, C. M., Su, J. T., Schaeffer, L. V., Guo, M., and Hay, B. A. (2007). A synthetic maternal-effect selfish genetic element drives population replacement in *Drosophila*. *Science*, 316(5824):597–600.

Chotkowski, H. L., Ciota, A. T., Jia, Y., Puig-Basagoiti, F., Kramer, L. D., Shi, P.-Y., and Glaser, R. L. (2008). West Nile virus infection of *Drosophila melanogaster* induces a protective RNAi response. *Virology*, 377(1):197–206.

Clem, R. J. and Miller, L. K. (1994). Control of programmed cell death by the baculovirus genes p35 and IAP. *Molecular and Cellular Biology*, 14(8):5212–5222.

Coates, C. J., Jasinskiene, N., Miyashiro, L., and James, A. A. (1998). Mariner transposition and transformation of the yellow fever mosquito, *Aedes aegypti*. *Proc Natl Acad Sci U S A*, 95(7):3748–51.

Coller, B.-A. G. and Clements, D. E. (2011). Dengue vaccines: progress and challenges. *Curr Opin Immunol*, 23(3):391–398.

Davis, S., Bax, N., and Grewe, P. (2001). Engineered underdominance allows efficient and economical introgression of traits into pest populations. *Journal of Theoretical Biology*, 212(1):83–98.

Deshpande, G., Stukey, J., and Schedl, P. (1995). *scute* (sis-b) function in Drosophila sex determination. *Molecular and Cellular Biology*, 15(8):4430–4440.

Etienne-Manneville, S. (2004). Cdc-42—the centre of polarity. *Journal of Cell Science*, 117(8):1291–1300.

Falgout, B. and Markoff, L. (1995). Evidence that flavivirus NS1-NS2A cleavage is mediated by a membrane-bound host protease in the endoplasmic reticulum. *J Virol*, 69(11):7232–7243.

Falgout, B., Pethel, M., Zhang, Y. M., and Lai, C. J. (1991). Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *J Virol*, 65(5):2467–2475.

Fernandez Cornejo, J. and Caswell, M. F. (2006). The First Decade of Genetically Engineered Crops in the United States. *USDA-ERS Economic Information Bulletin*, 11.

Fields, S. and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature*, 340(6230):245–246.

Filomatori, C. V., Lodeiro, M. F., Alvarez, D. E., Samsa, M. M., Pietrasanta, L., and Gamarnik, A. V. (2006). A 5' RNA element promotes dengue virus RNA synthesis on a circular genome. *Genes and Development*, 20:2238–2249.

Franz, A. W. E., Sanchez-Vargas, I., Adelman, Z. N., Blair, C. D., Beaty, B. J., James, A. A., and Olson, K. E. (2006). Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, 103(11):4198–4203.

Franz, A. W. E., Sanchez-Vargas, I., Piper, J., Smith, M. R., Khoo, C. C. H., James, A. A., and Olson, K. E. (2009). Stability and loss of a virus resistance phenotype over

time in transgenic mosquitoes harbouring an antiviral effector gene. *Insect Mol Biol*, 18(5):661–672.

Fraser, A. G. and Evan, G. I. (1997). Identification of a *Drosophila melanogaster* ICE/CED-3-related protease, drICE. *Embo J*, 16(10):2805–2813.

Fraser, A. G., McCarthy, N. J., and Evan, G. I. (1997). drICE is an essential caspase required for apoptotic activity in *Drosophila* cells. *Embo J*, 16(20):6192–6199.

Gaskell, G., Bauer, M. W., Durant, J., and Allum, N. C. (1999). Worlds apart? The reception of genetically modified foods in Europe and the U.S. *Science*, 285(5426):384–387.

Ghosh, I., Hamilton, A. D., and Regan, L. (2000). Antiparallel leucine zipper-directed protein reassembly: application to the green fluorescent protein. *Journal of the American Chemical Society*, 122(23):5658–5659.

Gibson, D., Young, L., Chuang, R., Venter, J., Hutchison, C., and Smith, H. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, 6(5):343–345.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. *Science*, 268(5218):1766–1769.

Grdzelishvili, V. Z., Garcia-Ruiz, H., Watanabe, T., and Ahlquist, P. (2005). Mutual interference between genomic RNA replication and subgenomic mRNA transcription in brome mosaic virus. *J Virol*, 79(3):1438–51.

Groth, A. C., Fish, M., Nusse, R., and Calos, M. P. (2004). Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31. *Genetics*, 166(4):1775–1782.

Hails, R. S. (2000). Genetically modified plant—the debate continues. *Trends in Ecology and Evolution*, 15(1):14–18.

Hartley, R. W. (1989). Barnase and barstar: Two small proteins to fold and fit together. *Trends in Biochemical Sciences*, 14(11):450–454.

Hawkins, C. J., Wang, S. L., and Hay, B. A. (1999). A cloning method to identify caspases and their regulators in yeast: Identification of *Drosophila* IAP1 as an inhibitor of the *Drosophila* caspase DCP-1. *Proceedings of the National Academy of Sciences*, 96:2885–2890.

Hawkins, C. J., Yoo, S. J., Peterson, E. P., Wang, S. L., Vernooy, S. Y., and Hay, B. A. (2000). The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *Journal of Biological Chemistry*, 275(35):27084–27093.

Hay, B. and Guo, M. (2006). Caspase-Dependent Cell Death in *Drosophila*. *Annual Review of Cell and Developmental Biology*, 22:623.

Hay, B. A. (2000). Understanding IAP function and regulation: A view from *Drosophila*. *Cell Death Differ*, 7(11):1045–56.

Hay, B. A. and Guo, M. (2004). The genetics of cell death: Approaches, insights and opportunities in *Drosophila*. *Nature Reviews Genetics*, 5:911–922.

Hay, B. A., Wolff, T., and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development*, 120(8):2121–2129.

Heres-Pulido, M., Dueñas-García, I., Castañeda-Partida, L., Sánchez-García, A., Contreras-Sousa, M., Durán-Díaz, Á., and Graf, U. (2004). Genotoxicity of tamoxifen citrate and 4-nitroquinoline-1-oxide in the wing spot test of *Drosophila melanogaster*. *Mutagenesis*, 19(3):187–193.

Hess, N., Singer, P., Trinh, K., Nikkhoy, M., and Bernstein, S. (2007). Transcriptional regulation of the *Drosophila melanogaster* muscle myosin heavy-chain gene. *Gene Expression Patterns*, 7(4):413–422.

Hoa, N. T., Keene, K. M., Olson, K. E., and Zheng, L. (2003). Characterization of RNA interference in an *Anopheles gambiae* cell line. *Insect Biochem Mol Biol*, 33(9):949–957.

Hodgkin, J., Horvitz, H. R., and Brenner, S. (1979). Nondisjunction mutants of the nematode *Caenorhabditis elegans*. *Genetics*, 91(1):67–94.

Hoffmann, A. A., Montgomery, B. L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P. H., Muzzi, F., Greenfield, M., Durkan, M., Leong, Y. S., Dong, Y., Cook, H., Axford, J., Callahan, A. G., Kenny, N., Omodei, C., McGraw, E. A., Ryan, P. A., Ritchie, S. A., Turelli, M., and O'Neill, S. L. (2011). Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature*, 476(7361):454–457.

Hunter, N. L., Awatramani, R. B., Farley, F. W., and Dymecki, S. M. (2005). Ligand-activated Flpe for temporally regulated gene modifications. *Genesis*, 41(3):99–109.

Ibnsouda, S., Schweisguth, F., Jullien, D., Kücherer, C., Lepesant, J., and Vincent, A. (1995). Evolutionarily conserved positive and negative cis-acting elements control the blastoderm-specific expression of the *Drosophila serendipity-α* cellularisation gene. *Mechanisms of Development*, 49(1-2):71–82.

James, A. and Collins, F. (1996). Genetic modification of mosquitoes. *Science & Medicine*, 3(6).

Juven-Gershon, T., Cheng, S., and Kadonaga, J. T. (2006). Rational design of a super core promoter that enhances gene expression. *Nature Methods*, 3(11):917–922.

Kao, K. C., Schwartz, K., and Sherlock, G. (2009). A genome-wide analysis reveals no nuclear Dobzhansky-Muller pairs of determinant of speciation between *S. cerevisiae* and *S. paradoxus*, but suggests more complex incompatibilities. *PLoS Genetics*, 6(7).

Kayashima, Y., Hirose, S., and Ueda, H. (2005). Anterior epidermis-specific expression of the cuticle gene EDG84A is controlled by many cis-regulatory elements in *Drosophila melanogaster*. *Development Genes and Evolution*, 215(11):545–552.

Knobler, S., Mahmou, A., Lemon, S., and Pray, L., editors (2006). *The Impact of Globalization on Infectious Disease Emergence and Control: Exploring the Consequences and Opportunities: Workshop Summary.* National Academies Press (US), Washington, D.C.

Kokoza, V., Ahmed, A., Cho, W. L., Jasinskiene, N., James, A. A., and Raikhel, A. (2000). Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. *Proc Natl Acad Sci U S A*, 97(16):9144–9149.

Kokoza, V. A. and Raikhel, A. S. (2011). Targeted gene expression in the transgenic *Aedes aegypti* using the binary Gal4-UAS system. *Insect Biochemistry and Molecular Biology*, 41(8):637–644.

Kuzin, A., Kundu, M., Ekatomatis, A., Brody, T., and Odenwald, W. (2009). Conserved sequence block clustering and flanking inter-cluster flexibility delineate enhancers that regulate nerfin-1 expression during *Drosophila* CNS development. *Gene Expression Patterns*, 9(2):65–72.

Kwit, C., Moon, H. S., Warwick, S. I., and Stewart, C. N. (2011). Transgene introgression in crop relatives: molecular evidence and mitigation strategies. *Trends in Biotechnology*, 29(6):284–293.

Kyrchanova, O., Chetverina, D., Maksimenko, O., Kullyev, A., and Georgiev, P. (2008). Orientation-dependent interaction between *Drosophila* insulators is a property of this class of regulatory elements. *Nucleic Acids Research*, 36(22):7019–7028.

Lambrechts, L., Scott, T. W., and Gubler, D. J. (2010). Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission. *PLoS Negl Trop Dis*, 4(5):e646.

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J., and Carthew, R. W. (2004). Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, 117(1):69–81.

Leung, D. (2001). Activity of Recombinant Dengue 2 Virus NS3 Protease in the Presence of a Truncated NS2B Co-factor, Small Peptide Substrates, and Inhibitors. *Journal of Biological Chemistry*, 276(49):45762–45771.

Li, D., Lott, W. B., Lowry, K., Jones, A., Thu, H. M., and Aaskov, J. (2011). Defective interfering viral particles in acute dengue infections. *PLoS One*, 6(4):–.

Li, J. (2005). Functional Profiling of Recombinant NS3 Proteases from All Four Serotypes of Dengue Virus Using Tetrapeptide and Octapeptide Substrate Libraries. *Journal of Biological Chemistry*, 280(31):28766–28774.

Lockless, S. W. and Muir, T. W. (2009). Traceless protein splicing utilizing evolved split inteins. *Proceedings of the National Academy of Sciences*, 106(27):10999–11004.

Lou, L., Bergson, C., and McGinnis, W. (1995). Deformed expression in the *Drosophila* central nervous system is controlled by an autoactivated intronic enhancer. *Nucleic Acids Research*, 23(17):3481.

Luan, H., Peabody, N. C., Vinson, C. R., and White, B. H. (2006). Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. *Neuron*, 52(3):425–436.

Mackenzie, J. M., Khromykh, A. A., Jones, M. K., and Westaway, E. G. (1998). Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. *Virology*, 245(2):203–215.

Maduro, M. and Pilgrim, D. (1995). Identification and cloning of *unc-119*, a gene expressed in the *Caenorhabditis elegans* nervous system. *Genetics*, 141:977–988.

Magori, K. (2005). Genetically Engineered Underdominance for Manipulation of Pest Populations: A Deterministic Model. *Genetics*, 172(4):2613–2620.

Manavella, P. A., Koenig, D., and Weigel, D. (2012). Plant secondary siRNA production determined by microRNA-duplex structure. *Proc Natl Acad Sci U S A*, 109(7):2461–2466.

Marco-Ferreres, R., Vivar, J., Arredondo, J., Portillo, F., and Cervera, M. (2005). Cooperation between enhancers modulates quantitative expression from the *Drosophila* Paramyosin/miniparamyosin gene in different muscle types. *Mechanisms of Development*, 122(5):681–694.

Marshall, J., Toure, M., Traore, M., Famenini, S., and Taylor, C. (2010). Perspectives of people in Mali toward genetically-modified mosquitoes for malaria control. *Malaria Journal*, 9(1):128.

Marygold, S., Roote, J., Reuter, G., Lambertsson, A., Ashburner, M., Millburn, G., Harrison, P., Yu, Z., Kenmochi, N., Kaufman, T., Leevers, S., and Cook, K. (2007). The ribosomal protein genes and minute loci of *Drosophila melanogaster*. *Genome Biology*, 8(10):R216.

Mello, C. C., Kramer, J. M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in *C. elegans*: extrachromosomal maintenance and integration of transforming sequences. *The EMBO Journal*, 10(12):3959–3970.

Moll, J., Ruvinov, S., Pastan, I., and Vinson, C. (2001). Designed heterodimerizing leucine zippers with a ranger of pIs and stabilities up to 10–15 M. *Protein Science: A Publication of the Protein Society*, 10(3):649.

Mukherjee, S. and Hanley, K. A. (2010). RNA interference modulates replication of dengue virus in *Drosophila melanogaster* cells. *BMC Microbiol*, 10(127).

Murata, T., Kageyama, Y., Hirose, S., and Ueda, H. (1996). Regulation of the EDG84A gene by FTZ-F1 during metamorphosis in *Drosophila melanogaster*. *Molecular and Cellular Biology*, 16(11):6509–6515.

Muro, I., Monser, K., and Clem, R. J. (2004). Mechanism of Dronc activation in *Drosophila* cells. *J Cell Sci*, 117(Pt 21):5035–5041.

Murrell, S., Wu, S.-C., and Butler, M. (2011). Review of dengue virus and the development of a vaccine. *Biotechnol Adv*, 29(2):239–247.

Neely, G. G., Kuba, K., Cammarato, A., Isobe, K., Amann, S., Zhang, L., Murata, M., ElmEn, L., Gupta, V., Arora, S., Sarangi, R., Dan, D., Fujisawa, S., Usami, T., Xia, C.-p., Keene, A. C., Alayari, N. N., Yamakawa, H., Elling, U., Berger, C., Novatchkova, M., Koglgruber, R., Fukuda, K., Nishina, H., Isobe, M., Pospisilik, J. A., Imai, Y., Pfeufer, A., Hicks, A. A., Pramstaller, P. P., Subramaniam, S., Kimura, A., Ocorr, K., Bodmer, R., and Penninger, J. M. (2010). A Global In Vivo Drosophila RNAi Screen Identifies NOT3 as a Conserved Regulator of Heart Function. *Cell*, 141(1):142–153.

Nern, A., Pfeiffer, B. D., Svoboda, K., and Rubin, G. M. (2011). Multiple new site-specific recombinases for use in manipulating animal genomes. *Proceedings of the National Academy of Sciences*, 108(34):14198–14203.

Nguyen, H. T. and Xu, X. (1998). *Drosophila* mef2 expression during mesoderm development is controlled by a complex array of cis-acting regulatory modules. *Developmental Biology*, 204(2):550–566.

Nomaguchi, M., Ackermann, M., Yon, C., You, S., and Padmanabhan, R. (2003). *De novo* synthesis of negative-strand RNA by dengue virus RNA-dependent RNA polymerase in vitro: Nucleotide, primer and template parameters. *Journal of Virology*, 77(16):8831–8842.

Nomaguchi, M., Teramoto, T., Yu, L., Markoff, L., and Padmanabhan, R. (2004). Requirements for West Nile virus (-)- and (+)-strand subgenomic RNA synthesis *in vitro* by the viral RNA-dependent RNA polymerase expressed in *Escherichia coli*. *Journal of Biological Chemistry*, 279(13):12141–12151.

Op De Beeck, A., Rouillé, Y., Caron, M., Duvet, S., and Dubuisson, J. (2004). The transmembrane domains of the prM and E proteins of yellow fever virus are endoplasmic reticulum localization signals. *Journal of Virology*, 78(22):12591–12602.

Pesko, K. N., Fitzpatrick, K. A., Ryan, E. M., Shi, P.-Y., Zhang, B., Lennon, N. J., Newman, R. M., Henn, M. R., and Ebel, G. D. (2012). Internally deleted WNV genomes isolated from exotic birds in New Mexico: Function in cells, mosquitoes, and mice. *Virology*, 427(1):10–17.

Pfeiffer, B. D., Truman, J. W., and Rubin, G. M. (2012). Using translational enhancers to increase transgene expression in *Drosophila*. *Proceedings of the National Academy of Sciences*, 109(17):6626–6631.

Pijlman, G., Funk, A., Kondratieva, N., Leung, J., Torres, S., Vanderaa, L., Liu, W., Palmenberg, A., Shi, P., and Hall, R. (2008). A Highly Structured, Nuclease-Resistant, Noncoding RNA Produced by Flaviviruses Is Required for Pathogenicity. *Cell Host & Microbe*, 4(6):579–591.

Poethig, R. S., Peragine, A., Yoshikawa, M., Hunter, C., Willmann, M., and Wu, G. (2006). The function of RNAi in plant development. *Cold Spring Harb Symp Quant Biol*, 71:165–170.

Preugschat, F., Yao, C. W., and Strauss, J. H. (1990). In vitro processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. *Journal of Virology*, 64(9):4364–4374.

Pryor, M. J., Rowlinson, S. M., Butcher, R. E., Barton, C. L., Waterhouse, T. A., Vasudevan, S. G., Bardin, P. G., Wright, P. J., Jans, D. A., and Davidson, A. D. (2007). Nuclear localization of dengue virus nonstructural protein 5 through its importin α/β-recognized nuclear localization sequences is integral to viral infection. *Traffic*, 8:795–807.

Ranjith-Kumar, C., Gutshall, L., Sarisky, R. T., and Kao, C. (2003). Multiple interactions within the Hepatitis C Virus RNA polymerase repress primer-dependent RNA synthesis. *Journal of Molecular Biology*, 330(4):675–685.

Rider, T. H., Zook, C. E., Boettcher, T. L., Wick, S. T., Pancoast, J. S., and Zusman, B. D. (2011). Broad-spectrum antiviral therapeutics. *PloS One*, 6(7):e22572.

Rieseberg, L. H., Raymond, O., Rosenthal, D. M., Lai, Z., Livingstone, K., Nakazato, T., Durphy, J. L., Schwarzbach, A. E., Donovan, L. A., and Lexer, C. (2003). Major ecological transitions in wild sunflowers facilitated by hybridization. *Science*, 301(5637):1211–1216.

Robert, V. and Bessereau, J.-L. (2007). Targeted engineering of the *Caenorhabditis elegans* genome following *Mos1*-triggered chromosomal breaks. *The EMBO Journal*, 26(1):170–183.

Robert, V. J. P. and Bessereau, J.-L. (2011). Genome engineering by transgene-instructed gene conversion in *C. elegans*. *Methods Cell Biol*, 106:65–88.

Roignant, J.-Y., Carre, C., Mugat, B., Szymczak, D., Lepesant, J.-A., and Antoniewski, C. (2003). Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. *RNA*, 9(3):299–308.

Roman, G., Endo, K., Zong, L., and Davis, R. L. (2001). P[Switch], a system for spatial and temporal control of gene expression in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 98(22):12602–12607.

Sabatini, D. M. (2004). *Concanavalin A Slide Coating Protocol*. Technical report, Whitehead Institute for Biomedical Research.

Schwartz, E. C., Saez, L., Young, M. W., and Muir, T. W. (2007). Post-translational enzyme activation in an animal via optimized conditional protein splicing. *Nature Chemical Biology*, 3(1):50–54.

Sellin, J., Albrecht, S., Kölsch, V., and Paululat, A. (2006). Dynamics of heart differentiation, visualized utilizing heart enhancer elements of the *Drosophila melanogaster* bHLH transcription factor Hand. *Gene Expression Patterns*, 6(4):360–375.

Severson, D. W., deBruyn, B., Lovin, D. D., Brown, S. E., Knudson, D. L., and Morlais, I. (2004). Comparative genome analysis of the yellow fever mosquito *Aedes aegypti* with *Drosophila melanogaster* and the malaria vector mosquito *Anopheles gambiae*. *Journal of Heredity*, 95(2):103–113.

Shabalina, S. A. and Koonin, E. V. (2008). Origins and evolution of eukaryotic RNA interference. *Trends in Ecology & Evolution*, 23(10):578–587.

Shah, N. K., Dhillon, G. P. S., Dash, A. P., Arora, U., Meshnick, S. R., and Valecha, N. (2011). Antimalarial drug resistance of *Plasmodium falciparum* in India: Changes over time and space. *Lancet Infect Dis*, 11(1):57–64.

Shimamura, K., Oka, S.-i., Shimotori, Y., Ohmori, T., and Kodama, H. (2007). Generation of secondary small interfering RNA in cell-autonomous and non-cell autonomous RNA silencing in tobacco. *Plant Mol Biol*, 63(6):803–813.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell*, 107(4):465–476.

Sijen, T., Steiner, F. A., Thijssen, K. L., and Plasterk, R. H. A. (2007). Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science*, 315(5809):244–247.

Sinkins, S. P. and Gould, F. (2006). Gene drive systems for insect disease vectors. *Nature Reviews Genetics*, 7(6):427–435.

Spradling, A. C. (1986). P element-mediated transformation. In Roberts, D. B., editor, *Drosophila: A Practical Approach*, pages 175–197. Oxford Press, Oxford.

Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science*, 218(4570):341–347.

Sulston, J. E. and Brenner, S. (1974). The DNA of *Caenorhabditis elegans*. *Genetics*, 77(1):95–104.

Szczesna-Skorupa, E. and Kemper, B. (2000). Endoplasmic reticulum retention determinants in the transmembrane and linker domains of cytochrome p450 2C1. *Journal of Biological Chemistry*, 275(25):19409–19415.

Tartof, K. and Hobbs, C. (1987). Improved media for growing plasmid and cosmid clones. *Focus*, 9(12):–.

Thackray, V. G., Young, R. H., Hooper, J. E., and Nordeen, S. K. (2000). Estrogen agonist and antagonist action on the human estrogen receptor in *Drosophila*. *Endocrinology*, 141(10):3912–3915.

Trape, J.-F., Tall, A., Diagne, N., Ndiath, O., Ly, A. B., Faye, J., Dieye-Ba, F., Roucher, C., Bouganali, C., Badiane, A., Sarr, F. D., Mazenot, C., Tourè-Baldè, A., Raoult, D., Druilhe, P., Mercereau-Puijalon, O., Rogier, C., and Sokhna, C. (2011). Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bednets and artemisinin-based combination therapies: A longitudinal study. *The Lancet Infectious Diseases*, 11(12):925–932.

Uchil, P. D. and Satchidanandam, V. (2003). Architecture of the flaviviral replication complex. *Journal of Biological Chemistry*, 278(27):24388–24398.

Vaistij, F. E., Jones, L., and Baulcombe, D. C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell*, 14(4):857–867.

Valdez, M. R. W. D., Suchman, E. L., Carlson, J. O., and Black, W. C. (2010). A large scale laboratory cage trial of Aedes Densonucleosis Virus (AeDNV). *Journal of Medical Entomology*, 47(3):392–399.

Vazquez-Manrique, R. P., Legg, J. C., Olofsson, B., Ly, S., and Baylis, H. A. (2010). Improved gene targeting in *C. elegans* using counter-selection and Flp-mediated marker excision. *Genomics*, 95(1):37–46.

Vernooy, S. Y., Chow, V., Su, J., Verbrugghe, K., Yang, J., Cole, S., Olson, M. R., and Hay, B. A. (2002). *Drosophila* Bruce can potently suppress Rpr- and Grim-dependent but not Hid-dependent cell death. *Current Biology*, 12:1164–1168.

Voinnet, O., Vain, P., Angell, S., and Baulcombe, D. C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell*, 95(2):177–187.

Wah, D. A., Bitinaite, J., Schildkraut, I., and Aggarwal, A. K. (1998). Structure of FokI has implications for DNA cleavage. *Proceedings of the National Academy of Sciences*, 95(18):10564–10569.

Walker, S., Avis, J., and Conn, G. (2003). General plasmids for producing RNA in vitro transcripts with homogeneous ends. *Nucleic Acids Research*, 31(15).

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., and Hoffmann, A. A. (2011). The wMel Wolbachia strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature*, 476(7361):450–453.

Westaway, E. G., Mackenzie, J. M., Kenney, M. T., Jones, M. K., and Khromykh, A. A. (1997). Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. *Journal of Virology*, 71(9):6650–6661.

WHO (2012). *Dengue and severe dengue*. Fact Sheet 117, World Heath Organization, http://www.who.int/mediacentre/factsheets/fs117/en/.

Wilm, T., Demel, P., Koop, H.-U., Schnabel, H., and Schnabel, R. (1999). Ballistic transformation of *Caenorhabditis elegans*. *Gene*, 229(1–2):31–35.

Windbichler, N., Menichelli, M., Papathanos, P. A., Thyme, S. B., Li, H., Ulge, U. Y., Hovde, B. T., Baker, D., Monnat, R. J., Burt, A., and Crisanti, A. (2011). A synthetic homing endonuclease-based gene drive system in the human malaria mosquito. *Nature*, 473(7346):212–215.

Wu, P.-C., Lay, J.-G., Guo, H.-R., Lin, C.-Y., Lung, S.-C., and Su, H.-J. (2009). Higher temperature and urbanization affect the spatial patterns of dengue fever transmission in subtropical Taiwan. *Sci Total Environ*, 407(7):2224–2233.

Yamauchi, T., Johzuka-Hisatomi, Y., Fukada-Tanaka, S., Terada, R., Nakamura, I., and Iida, S. (2009). Homologous recombination-mediated knock-in targeting of the MET1a gene for a maintenance DNA methyltransferase reproducibly reveals dosage-dependent spatiotemporal gene expression in rice. *The Plant Journal*, 60:386–396.

Yan, N., Wu, J. W., Chai, J., Li, W., and Shi, Y. (2004). Molecular mechanisms of DrICE inhibition by DIAP1 and removal of inhibition by Reaper, Hid and Grim. *Nat Struct Mol Biol*, 11(5):420–428.

Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang, S. L., Feldman, R. M. R., Clem, R. J., Müller, H.-A. J., and Hay, B. A. (2002). Hid, Rpr, and Grim negatively regulate DIAP1 levels through distinct mechanisms. *Nature Cell Biology*, 4:416–414.

Zhang, L., Mohan, P. M., and Padmanabhan, R. (1992). Processing and localization of Dengue virus type 2 polyprotein precursor NS3-NS4A-NS4B-NS5. *Journal of Virology*, 66(12):7549–7554.

Zhou, Y., Ray, D., Zhao, Y., Dong, H., Ren, S., Li, Z., Guo, Y., Bernard, K. A., Shi, P.-Y., and Li, H. (April 15, 2007). Structure and function of flavivirus NS5 methyltransferase. *Journal of Virology*, 81(8):3891–3903.

Zhu, X. and Sadowski, P. (1995). Cleavage-dependent Ligation by the FLP Recombinase. *Journal of Biological Chemistry*, 270(39):23044.

Appendix A

Transitive RNAi

A.1 Bombardment Protocol, S. Kuntz

Materials

Growth of Bacteria

Per liter of Terrific Broth (TB) (Tartof and Hobbs, 1987):

To 900 mL of dI H_2O add:

12 g tryptone

24 g yeast extract

4 mL glycerol

Mix until the solutes dissolve.

Autoclave.

To 90 mL of dI H_2O add:

2.31 g KH_2PO_4

12.54 g K_2HPO_4

Adjust to 100 mL with dI H_2O.

Autoclave.

Once the two solutions have cooled to 60°C or cooler, combine.

Per 500 mL culture you will need:

1 2800 mL Fernbach flask

1 5 mL O/N culture of HB101

1000 X Streptomycin solution (300 mg Streptomycin in 10 mL ddH_2O)

2 tared 250 mL centrifuge bottles

37°C shaker & Sorval centrifuge

Growth of Worms in Liquid Culture

Per $\sim$ 1 L of Complete S-medium (Sulston and Brenner, 1974):

1 M potassium phosphate, pH 6.0:

136 g KH_2PO_4

Add DI H_2O to 900 mL. Adjust to pH 6.0 with KOH.

Add DI H_2O to 1 L and autoclave.

1 M potassium citrate, pH 6.0:

268.8 g tripotassium citrate

26.3 g citric acid monohydrate

Add DI H_2O to 900 mL. Adjust to pH 6.0 with KOH.

Add DI H_2O to 1 L and autoclave.

Trace metals solution:

1.86 g Na_2EDTA

0.69 g $FeSO_4{\cdot}7H_22O$

0.20 g $MnCl_2{\cdot}4H_2O$

0.29 g $ZnSO_4{\cdot}7H_2O$

0.016 g $CuSO_4$

Add DI H_2O to 1 L, autoclave, and store in the dark.

S-basal medium:

5.9 g NaCl

50 mL of 1 M potassium phosphate, pH 6.0 (above)

1 mL 5 mg/mL cholesterol in EtOH

Add dI H_2O to 1 L and autoclave.

S-complete medium:

1 L S-basal medium (above)

10 mL of 1 M potassium citrate, pH 6.0 (above)

10 mL of trace metals solution (above)

3 mL of 1 M $CaCl_2$ (autoclaved)

3 mL of 1 M $MgSO_4$ (autoclaved)

1 1 L flask

3 Nearly cleared NGM Special plates of gravid *unc-119* worms

Concentrated HB101 bacteria from above

20°C shaker

Gold Microparticle Cleaning

One-time Preparation of beads:

60 mg gold microparticles

70% ethanol

ddH_2O

50% glycerol

Platform vortexer

Keep prepared beads at 4°C.

Gold Microparticle Coating

Coating Beads:

70 μL of 60 mg/mL gold microparticles in 50% glycerol

7–14 μg of DNA

3.5 μg of control DNA (such as *unc-119* rescue vector)

3.5 μg of experimental DNA (such as PCR-products or GFP-markers)

315 μL of 2.5 M $CaCl_2$

Stock made by 138.75 g of $CaCl_2$ with H_2O up to 500 mL

112 μL of 0.1 M spermidine

800 μL of 70% EtOH

800 μL of 100% EtOH (fresh)

70 μL of 100% EtOH (fresh)

7 Macrocarriers (large orange discs) [Bio-Rad #: 1652263]

Platform vortexer

Biolistic Gun Setup

1 tank of UHP Helium (99.999%)

7 macrocarriers seeded with the DNA-coated beads (as described above)

1 2200 psi Rupture Disc (small dark orange-red discs)

1 Hepta Stopping Screen (metal grid)

Post-Bombardment Care of Worms

25 small (5 cm) NGM lite plates liberally seeded with OP-50 24 hours in advance

13 9 cm NG plates seeded with OP-50

Methods

Growth of Bacteria for Food

1. Inoculate 5 mL HB101 culture in LB and streptomycin. Streptomycin final concentration at 30 μg/mL.
2. Grow 10–12 hours at 37°C.
3. Inoculate 500 mL TB and streptomycin culture with the 5 mL culture. Use a 2800 mL Fernbach flask (with baffling if available).
4. Grow 24 hours at 37°C, 250 rpm.
5. Spin at 4000 g in 2 500 mL tared centrifuge bottles for 10 min. 6500 rpm with the GSA rotor.
6. Remove supernatant.
7. Resuspend bacteria in LB to a 50% w/w concentration.
8. Store at 4°C.

Growth of Worms in Liquid Culture

1. Wash off the plates with M9 into 2 15-mL falcon tubes, spin down the worms, remove all but 1 mL of supernatant and then bleach with 7 mL water, 2 mL bleach, and 1 mL 5M KOH in each tube.

2. Divide into aliquots of 1 million eggs. Use serial dilutions and count the number of eggs in 10 μL of solution.
3. Place each aliquot in 100 mL of S-complete medium in a covered 1 L flask.
4. Shake for 24 hours at 20°C at 150–200 rpm.
5. Add 6 mL of concentrated HB101 solution. This is approximately 3 g of bacterial mass.
6. Shake for 48 hours at 20°C at 150–200 rpm. 24 hours into shaking 1 mL of solution should be extracted and the worms checked for contamination and progress of growth.
7. Harvest worms. The exact time of harvest is flexible within a 4 hour buffer, thus 48 hours $\pm$ 4 hours should be observed.

Purification of Worms from Liquid Culture

To purify 100 mL of worms:

1. Pour worm culture into 2 50-mL centrifuge tubes. Check that the worms are L4s or young adults.
2. Gravity sediment the worms for 10 minutes. Only gravity sediment as centrifugation packs the worms too tightly.
3. Remove supernatant to culture flask. Be careful to not disturb the pellet, as it may be very loose.
4. Plate 1–1.5 mL of the worm pellet per 9 cm NG plate. Keep most of the worms at the center, they will naturally spread out.
5. Place NG plates on ice and allow to dry. Placing on ice prevents the worms from clumping together. Failure of the plate to dry completely is not a problem, but it should be fairly dry. If placed at 4°C they should be fine for several hours.
6. Shake supernatant culture for 24 hours to starve it out.

Cyclic Liquid Culture of Worms

1. Pour starved supernatant worm culture into 2 50-mL centrifuge tubes. This is the worm culture from above.
2. Spin down worms and remove supernatant. Using a clinical centrifuge, spin at level 4 for 5 minutes.

3. Aliquot at 1 million worms per 100 mL solution. Use a fresh 1 L flask and fresh S-complete.
4. Add 6 mL HB101 solution.
5. Shake for 48 hours at 20°C at 150–200 rpm.
6. Bleach worms and continue with next cycle. The bleached eggs will be used in the next round of growth, step 2 of the Growth of Worms in Liquid Culture section.

Cleaning Gold Microparticles

One-time preparation of gold microcarrier beads, good for 14 hepta bombardments:

1. Weigh 60 mg of gold in a 1.5 mL microfuge tube. The gold is the consistency of clay, but can be affected by static electricity.
2. Add 1 mL 70% EtOH.
3. Vortex 5 min, settle 15 min, spin 15 seconds.
4. Discard supernatant. The supernatant should be clear. If not, centrifuge longer.
5. Repeat three times:
   (a) Add 1 mL ddH_2O.
   (b) Settle 1 min.
   (c) Centrifuge 15 seconds, discard supernatant. The supernatant should be clear. If not, centrifuge longer.
6. Add 1 mL 50% glycerol.
7. Store at 4°C.

The solution should be good for two months. After two months, re-clean the beads.

Coating Gold Microparticles

All numbers are for hepta-shots. For single shots, divide all figures by seven, except for on step 13. Coating the beads with DNA (performed every time):

1. Vortex gold solution for 10 min.
2. Mix 7–14 μg DNA in fresh microfuge tube. Avoid doing more than a few sets of hepta-shots at a time, as the steps should be performed as quickly as possible. For thorough mixing, vortex and pipette up and down.

3. Place macrocarriers in hepta-adapter macrocarrier holder. Use the red macrocarrier plug to help place the macrocarriers.
4. Add 70 μL gold beads to DNA, vortex 10 seconds. The gold solution should be a homogenous brown solution. Vortex it immediately before removing the 70 μL.
5. Add 315 μL 2.5M $CaCl_2$, vortex 10 seconds.
6. Add 112 μL 0.1M spermidine.
7. Vortex 3 min, settle 1 min, spin 15 sec.
8. Discard supernatant. The supernatant should be clear. If not, spin longer.
9. Resuspend in 800 μL 70% EtOH.
10. Settle 1 min, spin 15 sec, discard supernatant. The supernatant should be clear. If not, spin longer.
11. Add 800 μL fresh 100% EtOH, remove supernatant. The EtOH must be fresh, as non-denatured 100% EtOH quickly becomes $<$ 100% after being opened.
12. Resuspend in 70 μL 100% EtOH. Mix well, both vortexing at low speed and pipetting up and down.
13. Plate 10 μL on each macrocarrier, allow to dry. Be certain that the solution is well mixed while taking aliquots. Always use 10 μL per macrocarrier, regardless of the number of shots. Once dry the gold should appear as a white powder if well-mixed and distributed. The macrocarriers may be stored for up to 12 hours at 4°C, though immediate use is recommended.

Biolistic Gun Setup

1. Turn on helium tank (top valve). Only UHP (ultra-high purity or 99.999%) helium should be used.
2. Remove the hepta-adaptor pressure disc holder.
3. Place 2200 psi rupture disc in hepta-adapter pressure disc holder.
4. Screw in and tighten pressure disc holder (nozzle). Tighten securely, but do not bend in any other direction. The torque wrench may be used but no orthogonal torque should be applied.
5. Add stopping screen to macrocarrier holder, put in macrocarrier holder. Be certain the gold microparticles are facing downward.

6. Line up air-flow pathways.
7. Put in plate of worms immediately below macrocarrier holder. Place the worm plate on the plastic target shelf and remove the plate lid.
8. Close door tightly.

Biolistic Gun Firing

1. Turn on the gun.
2. Turn on the vacuum pump.
3. Switch regulator to Vacuum, pull vacuum past 20. Rocker in the top position. The Fire light should turn on.
4. Switch regulator to Hold. Rocker in the bottom position.
5. Press and hold Fire until the disc pops. The gun pressure should raise until the disc ruptures above 1500 psi.
6. Switch regulator to Vent. Rocker in the middle position. This is necessary for the door to be opened.
7. Remove all disposable components, clean off macrocarrier holder.
8. Switch regulator to Vacuum and allow the vacuum pump to run before shutting down. This step is necessary to not damage the vacuum pump. Leave the door open during this step.
9. Shut off the helium tank (top valve) and turn off the gun.

Post-Bombardment Care of Worms

1. Add 8 mL of M9 to worms.
2. Let sit for 1 hour. Between 45 minutes and 2 hours is acceptable.
3. Distribute worms on 25 seeded 5 cm NGM lite plates. Additional M9 may be added to assist in the distribution. Be sure to label each plate.
4. Dry plates. During the next 2 days additional food may be added if the worms appear to be starving. After that, starvation is encouraged.
5. Seed 13 9 cm NG plates.
6. Check for transgenics after 3 days and chunk small plates onto large plates. If using *unc-119* worms, large plates are a crawling assay. Each F1 is an independent line.

Timeline

Day 0 (Sunday): Prepare all media.

Inoculate overnight HB101 culture.

Day 1 (Monday): Bleach worms.

Inoculate 500 mL HB101 culture.

Add worms to S-complete (cultures A and B).

Day 2 (Tuesday): Harvest bacteria.

Culture A:

Add bacteria to worm culture.

Day 3 (Wednesday): Culture A: Check on worm growth.

Seed 25 5-cm NGM lite plates.

Culture B: Add bacteria to worm culture.

Day 4 (Thursday): Culture A:

Harvest worms.

Coat beads.

Inoculate extra worms in cyclic culture.

Bombard worms.

Replate worms on NGM plates.

Seed 13 9-cm NG plates.

Culture B:

Check on worm growth

Seed 25 5-cm NGM lite plates

Day 5 (Friday): Culture B: Harvest worms.

Coat beads.

Inoculate extra worms for cyclic culture (mix with cyclic culture A).

Bombard worms.

Replate worms on NGM plates.

Seed 13 9-cm NGM plates.

Day 6 (Saturday): Add food to cyclic worm culture.

Day 7 (Sunday): Culture A:

Chunk worms to NG plates.

Begin scoring.

Day 8 (Monday): Start over with bleaching.

Culture B:

Chunk worms to NG plates.

Begin scoring.

Appendix B

Viral Sensing and Killing

B.1 Immunostaining for DENV2 Infection in S2 and C6/36 Cells

Concanavalin A Treatment of Slides

1. Cleaning the slip covers
   (a) Prepare Cleaning Solution:
      i. Dissolve 70 g NaOH in 280 mL ddH_2O.
      ii. Add 420 mL 95% ethanol. Total volume is 700 mL; stir until completely mixed.
      iii. If solution remains cloudy, add ddH_2O until clear.
   (b) Pour solution into container with slip covers; cover chambers with glass lids. Mix on orbital shaker for 2 hr.
   (c) Once slides are clean, they should be exposed to air as little as possible. Dust particles will interfere with coating.
   (d) Quickly transfer slips to a fresh container filled with ddH_2O. Rinse vigorously by agitation.
   (e) Repeat rinses 4X with fresh ddH_2O each time. It is critical to remove all traces of NaOH-ethanol.
2. Concanavalin A Solution
   (a) Measure 0.0625 g of Concanavalin A (ConA).
   (b) In a graduated cylinder, add ConA to 200 mL nuclease-free water.

(c) Mix by inversion.

(d) After ConA is dissolved, bring volume to 250 mL.

(e) Pour ConA into a 250 mL jar. Add a sterilized stir bar to jar, seal top of jar and stir ConA on a stir plate for 4 hours at a speed that creates a sufficient vortex in the solution.

(f) After 4 hours of stirring, filter solution into a new, sterilized container with 0.22 μm vacuum filter.

3. Slide Coating (Performed in tissue culture hood. Use powder free gloves when manually handling slides)

   (a) In a tissue culture hood, array small circular coverslips that will fit in the bottom of the wells in 24-well tissue culture dishes on Kimwipes.

   (b) Spray the fingertips of the gloves used to handle the slides with 100% ethanol.

   (c) Clean slides with 100% ethanol.

   (d) Cover the coverslips with the solution. It has good surface tension and nearly the whole surface can be covered without it spilling off.

   (e) Allow to sit for 2–3 minutes.

   (f) Pipette off and allow to dry for 1 hour.

   (g) Rinse 2–3 times with nuclease free, autoclaved H_2O. Usually I hold them with forceps and rinse with a spray bottle.

   (h) Dry slides for 1 hour as before.

4. Slide Storage

   (a) Normally I use them immediately.

   (b) I have stored them in the fridge for up to about 2 weeks without a noticible decline in quality.

Immunostaining of S2 Cells

This can be carried out in the wells containing treated coverslips. There is no need to remove the slips, although they are better observed outside of wells.

1. Remove media and fix cells in 4% formaldehyde.
2. Wash 5 times with 1XPBS.

3. Incubate 20 minutes in PBT.
4. Wash twice with 1XPBT.
5. Wash twice times with 1XPBS.
6. Incubate 15 minutes in 1XPBS with 10% BSA and 10% PBT.
7. Wash four times with 1XPBS.
8. Incubate 1 hour with 150 μl of primary (DENV2, MAB8702) antibody, diluted 1:400.
9. Wash 3 times with 1XPBS.
10. Incubate 1 hour with 150 μl of secondary (anti-mouse) antibody, diluted 1:200.
11. Wash 3 times with 1XPBS.
12. Observe on an inverted fluorescence scope.

Immunostaining of C6/36 Cells

1. Wash with ice-cold 1XPBS
2. Remove media and fix cells in -20° MeOH.
3. Wash 3 times with ice-cold 1XPBS.
4. Incubate 1 hour with 150 μl of primary (DENV2, MAB8702) antibody, diluted 1:400.
5. Wash 3 times with ice-cold 1XPBS.
6. Incubate 1 hour with 150 μl of secondary (anti-mouse) antibody, diluted 1:200.
7. Wash 3 times with ice-cold 1XPBS.
8. Observe on an inverted fluorescence scope.

B.2 Transmembrane-Targeted Proteins

PrM-Cleave

A portion of the DENV2 poly protein comprising amino acids 102-264 which encompasses a small C-terminal portion of the capsid, the entire prM and a small piece of the M proteins (lowercase) which includes a transmembrane domain is fused to a set of NS2B/NS3 target sites (uppercase). Restriction sites, EcoRI and BglII, are underlined and the Kozak sequence is italicized. This is meant to be in-frame fused to either Flp, Gal4 or LexA::VP16.

gaattc *caaaccaaa* atggggtctg caggcatgat cattatgctg attccaacag tgatggcgtt ccatttaacc acacg-

taacg gagaaccaca catgatcgtc agcagacaag agaaagggaa aagtcttctg tttaaaacag aggatggcgt gaacatgtgt accctcatgg ccatggacct tggtgaattg tgtgaagaca caatcacgta caagtgtccc cttctcaggc agaatgagcc agaagacata gactgttggt gcaactctac gtccacgtgg gtaacttatg ggacgtgtac caccatggga gaacatagaa gagaaaaaag atcagtggca ctcgttccac atgtgggaat gggactggag acacgaactg aaacatggat gtcatcagaa ggggcctgga aacatgtcca gagaattgaa acttggatct tgagacatcc aggcttcacc atgatggcag caatcctggc atacaccata ggaacgacac atttccaaag aGGCG GCGGA GGCGG TGGCG GGGGC GGACT GAAGC GCCGC TCCGG CTCCG GCTTC GCCGC CGGCC GCAAG TCCCT GACCC TGAAC CTGCT TAAAA GACGT TCGGG GAGCG GTaga tct

P450-Cleave

An artificial protein with an N-terminal signal sequence and ER localization signal (uppercase) fused to a set of C-terminal NS2B/NS3 cleavage sites (bold) via a polyglycine linker. The signal sequence and ER localization domains are taken from a cytochrome p450, which is presumed to be conserved. Restriction sites, EcoRI and BglII, are underlined and the Kozak sequence is italicized. This is meant to be in-frame fused to either Flp, Gal4 or LexA::VP16.

Protein Sequence

mdpvvvlglclsclllslw KQSYGGGK gggggggg **lkrrsgsg-faagrk-sltlnl-lkrrsgsg**

DNA Sequence

gaattc *caaaccaaa* atggac cccgtg gtggta ctgggc ctgtgc ctgtcc tgcctg ctgctc ctgtcc ctgtgg AAGCAG TCCTAC GGCGGC GGCAAG GGCggc ggaggc ggtggc gggggc gga**ctg aagcgc cgctcc ggctcc ggcttc gccgcc ggccgc aagtcc ctgacc ctgaac ctgctt aaaaga cgttcg gggagc ggt agatct**

CD4-E-Link

This construct consists of the N-terminal, transmembrane section of CD4 (aa 1-371) which is not shown, a fragment of the DENV envelope (italics), a flexible linker containing viral cleavage sites (in bold), and a LexA::VP16 fusion transcription factor. Note that upstream of the NotI site an extra nucleotide, a, is added in to maintain the open reading frame. This DNA carries the original LV used, but the final constructs carry the recoded LV because

a point mutation when cloning necessitated replacing the part and I used the newer LV to replace it.

Protein sequence: *GAIYGAAFSGVSWTMKILIGVIITWIGMNSRS*EF**GGGGGGGGG LKRRSGSFAAGRKSLTLNLLKRRSGSGS**GRMKALTARQQEVFDLIRDHISQT GMPPTRAEIAQRLGFRSPNAAEEHLKALARKGVIEIVSGASRGIRLLQEEEEGLPLV GRVAAGEPLLAQQHIEGHYQVDPSLFKPNADFLLRVSGMSMKDIGIMDGDLLAVH KTQDVRNGQVVVARIDDEVTVKRLKKQGNKVELLPENSEFKPIVVDLRQQSFTIEG LAVGVIRNGDWLEFPGIRRPAGIPGDLAPPTDVSLGDELHLDGEDVAMAHADALD DFDLDMLGDGDSPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG

(SpeI site) ACTAGT –

(Envelope fragment) GGAGCAATCTATGGAGCTGCCTTCAGTGGGGTTTCATGGA CTATGAAAATCCTCATAGGAGTCATTATCACATGGATAGGAATGAATTCACGC AGC –

(EcoRI site) GAATTC –

(Linker) GGCGGCGGAGGCGGTGGCGGGGGCGGACTGAAGCGCCGCTCCGGCTC CGGCTTCGCCGCCGGCCGCAAGTCCCTGACCCTGAACCTGCTTAAAAGACGTT CGGGGAGCGGTa –

(NotI site) GCGGCCGC –

(LexA::VP16 fusion) ATGAAAGCGTTAACGGCCAGGCAACAAGAGGTGTTTGATC TCATCCGTGATCACATCAGCCAGACAGGTATGCCGCCGACGCGTGCGGAAATC GCGCAGCGTTTGGGGTTCCGTTCCCCAAACGCGGCTGAAGAACATCTGAAGG CGCTGGCACGCAAAGGCGTTATTGAAATTGTTTCCGGCGCATCACGCGGGATT CGTCTGTTGCAGGAAGAGGAAGAAGGGTTGCCGCTGGTAGGTCGTGTGGCTG CCGGTGAACCACTTCTGGCGCAACAGCATATTGAAGGTCATTATCAGGTCGAT CCTTCCTTATTCAAGCCGAATGCTGATTTCCTGCTGCGCGTCAGCGGGATGTC

GATGAAAGATATCGGCATTATGGATGGTGACTTGCTGGCAGTGCATAAAACT
CAGGATGTACGTAACGGTCAGGTCGTTGTCGCACGTATTGATGACGAAGTTA
CCGTTAAGCGCCTGAAAAAACAGGGCAATAAAGTCGAACTGTTGCCAGAAAA
TAGCGAGTTTAAACCAATTGTCGTAGATCTTCGTCAGCAGAGCTTCACCATTG
AAGGGCTGGCGGTTGGGGTTATTCGCAACGGCGACTGGCTGGAATTCCCGGG
GATCCGTCGACCTGCAGGAATTCCCGGGGATCTGGCCCCCCCGACCGATGTCA
GCCTGGGGGACGAGCTCCACTTAGACGGCGAGGACGTGGCGATGGCGCATGC
CGACGCGCTAGACGATTTCGATCTGGACATGTTGGGGGACGGGGATTCCCCG
GGTCCGGGATTTACCCCCCACGACTCCGCCCCCTACGGCGCTCTGGATATGGC
CGACTTCGAGTTTGAGCAGATGTTTACCGATGCCCTTGGAATTGACGAGTACG
GTGGGTAG –

(SacII site) CCGCGG

CD4-M-Link

This construct is the same as the previous except that a section of the prM protein is substituted in place of the envelope fragment.

actagtagaattgaaacttggatcttgagacatccaggcttcaccatgatggcagcaatcctggcatacaccataggaacgacac
atttccaaaga

Protein Sequence
TSRIETWILRHPGFTMMAAILAYTIGTTHFQR

Erlin-Link-nLV

In place of the CD4 and viral fragments is a short N-terminal fragment from Erlin1, codon optimized for insects during gene synthesis. The linker is also updated and the LexA::VP16 has also been recoded.

Protein Sequence
EFQTKMTQARVLVAAVVGLVAVLLYASIH**KIEEGGGGGGGGGGGLKRRSGSG**
FAAGRKSLTLNLLKRRSGSGMKALTARQQEVFDLIRDHISQTGMPPTRAEIAQ

RLGFRSPNAAEEHLKALARKGVIEIVSGASRGIRLLQEEEEGLPLVGRVAAGEPLLA
QQHIEGHYQVDPSLFKPNADFLLRVSGMSMKDIGIMDGDLLAVHKTQDVRNGQVV
VARIDDEVTVKRLKKQGNKVELLPENSEFKPIVVDLRQQSFTIEGLAVGVIRNGDW
LEFPGIRRPAGIPGDLAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGD
SPGPGFTPHDSAPYGALDMADFEFEQMFTDALGIDEYGG

(Erlin 1–29) ATGACTCAAGCACGTGTTTTGGTTGCAGCTGTAGTAGGATTGGTT
GCGGTCTTGTTGTACGCCAGCATCCACAAAATCGAAGAAGGC –

(Linker) GGCGGTGGCGGTGGTGGTGGCGGCGGACTCAAGCGTCGCTCCGGCTC
CGGTTTCGCTGCCGGTCGCAAGTCGCTTACGCTGAACCTGCTGAAACGTCGCA
GCGGTTCCGGT –

(Recoded LexA::VP16) ATGAAGGCTTTGACTGCTCGGCAGCAAGAGGTGTTCGA
CCTGATCCGCGATCACATAAGCCAGACGGGAATGCCACCAACGCGAGCCGAGA
TTGCTCAGCGGCTGGGCTTCCGGTCGCCCAATGCTGCTGAGGAACACCTGAAG
GCACTTGCCCGAAAGGGCGTAATCGAGATCGTCTCGGGTGCCTCGCGGGGCA
TCCGCCTCTTGCAGGAAGAGGAGGAGGGCCTGCCCCTGGTTGGCCGCGTTGC
CGCCGGAGAGCCGCTGCTGGCCCAGCAACATATAGAGGGCCATTACCAGGTC
GATCCATCCTTGTTCAAGCCGAACGCTGACTTCCTCCTGCGAGTATCCGGCAT
GAGTATGAAAGATATTGGCATAATGGATGGCGACCTGCTGGCGGTGCACAAG
ACTCAAGATGTGCGCAATGGCCAGGTTGTAGTGGCCCGTATAGATGATGAGG
TCACTGTGAAGCGGCTGAAGAAGCAGGGTAATAAGGTGGAGCTGCTGCCCGA
GAACAGCGAATTTAAACCTATTGTGGTGGATCTGAGGCAGCAATCCTTCACTA
TCGAGGGACTCGCCGTCGGTGTCATTCGCAACGGCGACTGGTTGGAATTTCCC
GGCATACGACGCCCGGCCGGCATCCCGGGCGACCTGGCGCCACCCACCGATGT
GTCGTTGGGCGACGAGCTCCATCTGGACGGCGAAGACGTTGCCATGGCCCAC
GCAGATGCATTGGATGATTTTGATCTGGATATGTTGGGCGATGGCGACAGTC
CTGGCCCCGGATTCACTCCCCACGACTCCGCCCCGTATGGAGCTCTTGATATG
GCTGATTTTGAGTTCGAGCAGATGTTCACAGACGCACTGGGCATCGACGAGT
ACGGAGGATAA

B.3 Chun-Hong's Flip-Based Killer

This was designed by Chun-Hong Chen as half of an underdominant system. We adapted it for use as a viral sensor. When expressed, the transcript codes for a miRNA that self targets and is flanked by FRT sites. Downstream of the FRT sites and the miR is a Rpr open reading frame. If no flp is present, the miR is expressed, targets the transcript downstream and cleaves it leaving an uncapped Rpr transcript that is degraded. Once flip excision has occurred, Rpr is expressed and cell death occurs. The sequence of each component is shown below and a schematic is in Figure B.1

(PacI site) ATTTAAAT –

(FRT) GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC –

(miR)CACGGCCAATTCCAACGATTTGTCATTTGTGGCACGCATTTGTGTCACC
TCAGTGCGAAAATTGAAAATTGTATTTAATGTATACGCACGATCAACAAAACT
CTAAGTTAATATACCATATCTAGATTTTTGTTGATCGTGCGTATGTACCTAAA
GCAATCGATCTACGTTCAGTGGTTTGCCAGGACATGAAACAGAAATATTTTCC
GTCAACAGACTTCTGATTGCACAAATTCCTC –

(FRT) GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC –

(2X miR Target) CATACGCACGATCAACAAAAATCaaaaaaaaaaCATACGCACGATC
AACAAAAATC –

(FseI site) GGCCGGCC –

(Rpr) ATGGCAGTGGCATTCTACATACCCGATCAGGCGACTCTGTTGCGGGAGG
CGGAGCAGAGGGAGCAGCAGATTCTCCGCTTGCGGGAGTCACAGTGGAGATT
CCTGGCCACCGTCGTCCTGGAAACCCTGCGCCAGTACACTTCATGTCATCCGA
GGACCGGAAGAAGGTCCGGCAGATATCGCAGGCCATCGCAATGA –

(NotI site) GCGGCCGC

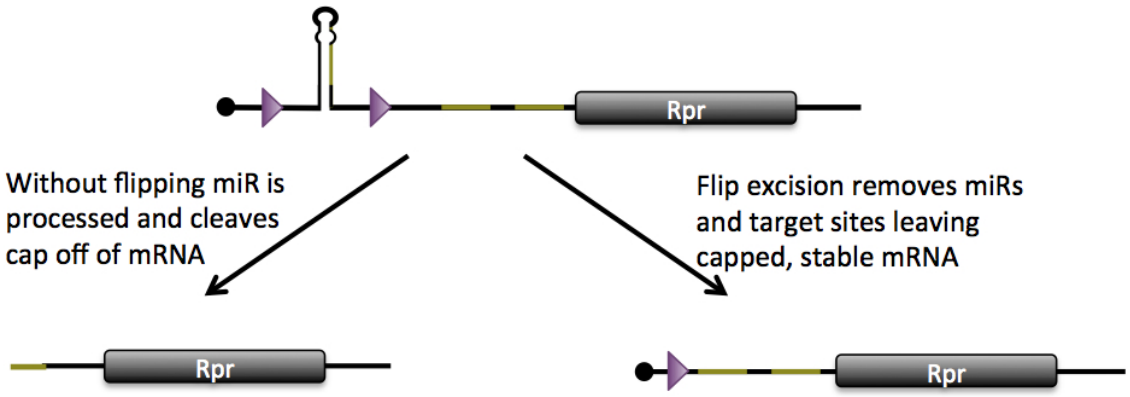


Figure B.1: If flp is tethered to the ER and only released by NS2B/NS3 cleavage when cells are infected with dengue, then viral infection will trigger the cell death cascade.

B.4 RdRp Designs

Titraton Scaffold

The titration scaffold was designed to resist degradation of unstructured, noncoding RNAs in tissue culture experiments. The Dengue 3' and Kunjin 3' sequences that are naturally exonuclease resistant were synthesized, and between them a space was left for cloning. Other features include an intron to help promote export to the cytoplasm, and external cloning sites so the entire scaffold can be moved as one piece once modified.

(EcoRI – PacI – spacer) GAATTC TTAATTAA GAATACAAGCTCACGA –

(Kunjin 3' sequence) ATATTGTTATTATGTGTAGAAGTTTAGCTTTATAATAGTG
TTTAGTGTGTTTAGAGTTAGAAAAATTTTAGTGAGGAAGTCAGGCCGGAAAA
TTCCCGCCACCGGAAGTTGAGTAGACGGTGCTGCCTGCGACTCAACCCCAGGA
GGACTGGGTGAACAAAGCTGCGAAGTGATCCATGTAAGCCCTCAGAACCGTC
TCGGAAAGAGGACCCCACATGTTGTAGCTTCAAGGCCCAATGTCAGACCACGC
CATGGCGTGCCACTCTGCGGAGAGTGCAGTCTGCGACAGTGCCCCAGGAGGA
CTGGGTGAACAAAGGCGAATCAACGTCCCACGCGGCCCTAGCTCTGGCAATG

GTGTTAACCAGAGTGAAAGGACTAGAGGTTAGAGGAGACCCCGCGTTCTGAA
GTGCACGGCCCAGCCTGGCTGAAGCTGTAGGTCAGGGGAAGGACTAGAGGTT
AGTGGAGACCCCGTGCCGCAAAACACCACAACAACACAGCATATTGACACCTG
GGATAGACTAGGAGTCTTCTGCTCTGCACAACCAGCCACACGGCACAGTGCGC
CGACAATGGTGGCTGGTGGTGCGAGAACACAGGATCT –

(Cloning Site Rich Linker) ACGACTTGAACGTTAGCGTTTAAACTTCCAGGCTGCA
GATCTACTCGATGCGGCCGCAGGTTCTCCGGCCGCTTGGG –

(Dengue 2 3' Sequence) TTGGCAATGAGGAATACACAGATTACATGCCATCCATGA
AAAGATTCAGAAGAGAAGAGGAAGAGGCAGGAGTTTTGTGGTAGAAAAACAT
GAAACAAAACAGAAGTCAGGTCGGATTAAGCCATAGTACGGGAAAAACTATG
CTACCTGTGAGCCCCGTCCAAGGACGTTAAAAGAAGTCAGGCCACTTTGATGC
CATAGCTTGAGCAAACTGTGCAGCCTGTAGCTCACCTGAGAAGGTGTAAAAAA
TCCGGGAGGCCACAAACCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCG
GTTAGAGGAGACCCCTCCCTTACAGATCGCAGCAACAATGGGGGCCCAAGGT
GAGATGAAGCTGTAGTCTCACTGGAAGGACTAGAGGTTAGAGGAGACCCCCC
CAAAACAAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCT
CCTCAGCATCATTCCAGGCACAGGACGCCAGAAAATGGAATGGTGCTGTTGAA
TCAACAGGTTCT –

(KpnI) GGTACC –

(Intron) TACCCATACGACGTTCCGGACTACGCTGTTAAGGTAGGCTGGCACACG
AATAACCATGCCGTTTTCAAATCTAATCCCTTTAGTAAAACTGTTTTAATTTC
TTTTCTTTTTTCAGCCA –

(SalI – AscI – XhoI) GTCGAC GGCGCGCC CTCGAG

Appendix C

Underdominance

C.1 Enzymatic Assembly Cloning Method

Traditional cloning methods allow the cloning of one, or at most two, fragments at once. Each step requires at least one restriction enzyme, two to control the direction of insertion. As constructs become large and complicated, finding restriction sites to assemble them can be difficult. In 2009, technology was published that allowed the cloning of multiple fragments at a time into a single restriction site (which could be destroyed or regenerated as desired) in a directionally controlled manner with additional bases between components if desired (Gibson et al., 2009). Clontech also produced the "In-Fusion Dry-Down PCR Cloning Kit," which I tried, but it was orders of magnitude more expensive and produced identical results to the in-house prep based on Gibson et al.'s method. I made the 5X ISO buffer in 2009 and this supply lasted until the end of 2011. Stocks of DTT and NAD made at the same time were used to make the second batch of ISO Buffer which worked perfectly, indicating the components are stable for years at -20°C.

I prepped the necessary materials in-house and followed the recipe provided in the article:

1. 320 μl 5X isothermal (ISO) reaction buffer:
   (a) 3 ml of 1 M Tris-HCl pH 7.5
   (b) 150 μl of 2 M $MgCl_2$
   (c) 60 μl of 100 mM dGTP
   (d) 60 μl of 100 mM dATP
   (e) 60 μl of 100 mM dTTP

(f) 60 μl of 100 mM dCTP

(g) 300 μl of 1 M DTT

(h) 1.5 g PEG-8000

(i) 300 μl of 100 mM NAD

(j) Add water to 6 ml

(k) Aliquot and store at -20°C.

2. 0.64 μl T5 exonuclease (Epicentre)
3. 20 μl Phusion DNA polymerase (New England Biolabs)
4. 160 μl Taq DNA ligase (New England Biolabs)
5. Add H_2O to 1.2 ml.
6. Aliquot 15 μl into PCR tubes and store at -20°C.

The total volume of DNA added into each reaction is 5 μl. Each fragment to be assembled and the backbone are combined in 1:1 molar ratios.

Each PCRed fragment was designed to have 30 bp of overlap to the backbone if it was to overlap the backbone. When overlapping adjacent PCR fragments, the primer carried 30 bp to anneal for annealing during PCR and 30 bps matching the fragment it would be fused to (Figure C.1). This overlap distance was a compromise between maximizing overlap and minimizing cost. PCR products were generated nearly exclusively using Phusion which tolerates fairly significantly different annealing temperatures between the primers in a pair. It would be acceptable and likely just as successful to use backbone overlaps of 20 bps and between PCR fragments, however I think keeping the overlaps to at least 30 bps is advisable. Whenever possible, I cloned into backbones linearized with a single blunt-cutting restriction enzyme because this eases primer design.

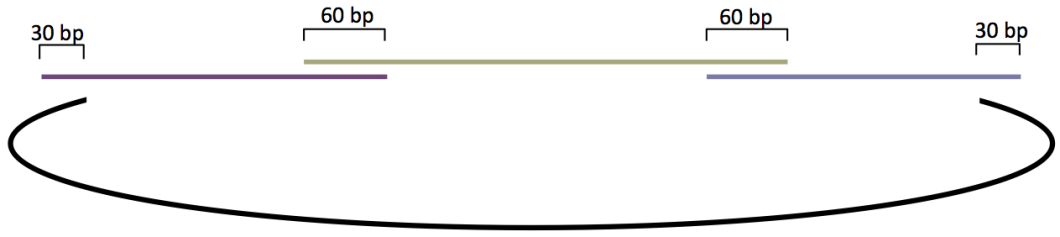


Figure C.1: This is a schematic where three PCR fragments are inserted into a linearized backbone. Overlaps between inserts and the backbone are 30 bp and overlaps between PCR fragments is 60 bp.

C.2 attB Injections

There are a multitude of injection protocols available, so the notes here are intended to help with steps specific to Hay lab equipment and conditions.

Needle Puller Kopf Instruments Model 720 Needle/Pipette Puller. Heater set to $\sim$ 12.0 and solenoid $\sim$ 4. These settings worked well, but it is advisable to play around with them to achieve a needle shape that works well for each set of injections. Avoid touching the filament. Spare parts are available from the company.

Capillaries Drummond 1-000-0500, 50 μl volume. The fit the needle puller and injection apparatus. The larger sizes will fit the needle puller but will not fit into the micro injector. The glass also has the right composition for injection of *Drosophila* embryos still in their chorion. It is not an appropriate glass for the injection of mosquito embryos.

Dye Blue food dye is used. It should be spun twice at 13 K RPM for 30 minutes each time. Each time keep the top half and discard the rest. Stable at 4°C for years. Approximately 1 μl dye per 20 μl DNA solution should be sufficient.

DNA EtOH precipitate, resuspend in nuclease-free H_2O. Spin 30' after resuspension. Total concentration for attB injection 200–250 μl. Any co-inject should be co-precipitated. Sticky/clog-prone DNA is not recoverable. Begin by reprecipitating a new sample and if it is also bad, reprep from a new culture. The Machery-Nagle kits seem to give very clean DNA, good for injections.

Flies Flies should be reared in healthy bottles set less than two weeks before adults will first be collected. Adults should be transferred to grape plates and kept at 25°C for at least 3 days before injections will begin. Adults should be given fresh yeast and plates approximately every 12 hrs for at least 3 days before egg collections begin. Two dense cages should be sufficient for injections.

Embryos Collections should be approximately 45 minutes long. Two one-hour collections should be made and discarded before embryos for injection are collected. It is easy to kill embryos that are too old. The yeast paste is removed with a cotton swab and the

rest of the embryos are loosened from the plate using a cotton swab and PBT (1% TritonX100 in 1XPBS). They are then rinsed very thoroghly through a silk screening mesh in the top of a falcon tube.

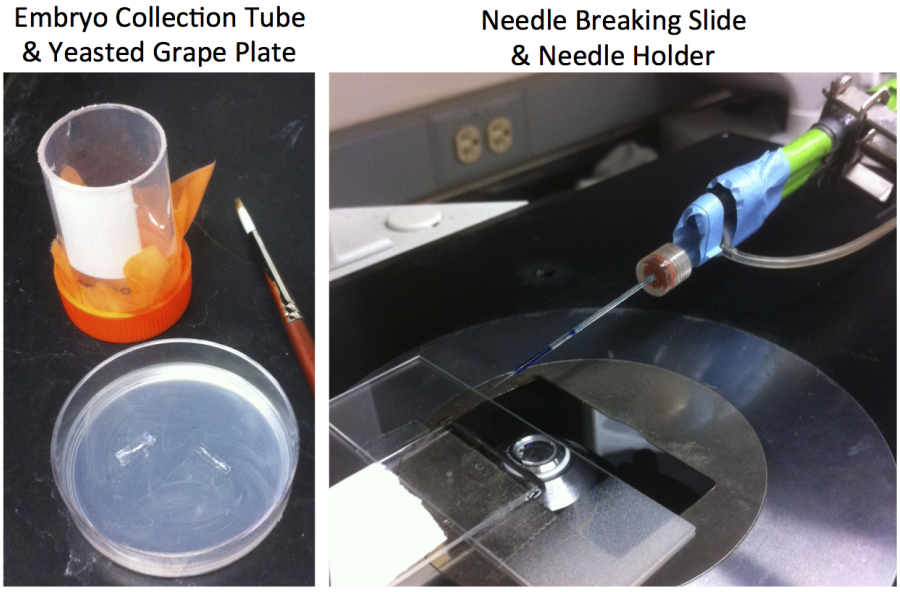


Figure C.2: Shown are a few of the materials used for injection in the lab.

Tape The tape used in the lab is Scotch^{TM} 137 Double-Sided Office Tape. It is available in the stockroom and the packaging is yellow.

Oil We use two oils for injection. The first is Halocarbon Oil 700 and is used as the medium for breaking needles. Halocarbon Oil 27 is used for covering embryos just prior to injection.

Mounting the Needle The needle should be placed into the needle holder and tightened firmly but not severely as this wears on the gaskets. Needle should not move when pressure is applied through the syringe. If the needle breaks off during insertion or removal, disassemble the needle holder and carefully remove all traces of glass to preserve the gaskets.

Breaking Needles Several needles can be opened prior to injections starting, but may dry closed if not used within an hour or two as the oil slides away from the tip. Needles are broken on the edge of a glass slide that has been broken in half and mounted on another glass slide. The hole should be as small as possible for DNA that flows reliably but not freely. DNA will travel back up the needle like beads on a string with

a good break. Trial and error is the only way to achieve a break that works for you reproducibly. If debris is visibly clogging the needle, DNA should be reprepped.

Temperatures Flies should be reared at 25°C. Injections should occur in a room at 18°C and immediately after injection, embryos should be returned to a grape plate at 25°C. If constructs carry a HS promoter and may be toxic, post-injection embryos can be kept at 18°C.

Embryo Alignment This is carried out on a strip of grape plate agar. Thin grape plates are poured in 10 cm petri dishes, allowed to set normally and then allowed to dry on the bench top over night. Cracking of the agar along the edges is fine and an indication the plate has dried significantly. Strips are cut and then blotted thoroughly to remove additional water. A strip that has not dried sufficiently will make picking up the eggs on the tape difficult or impossible.

Post-Injection Oil should be removed from embryos by vacuum until no more oil can be removed. A grape plate is spread with a tiny amount of yeast paste all over its surface. Embryos on tape are cut from the slide to minimize tape larva must crawl across to reach yeast and grape plate, and this tape strip is applied to the grape plate. I usually divide a full morning's injected embryos between two plates. Plates are placed top up in a pipette tip box with a wet, but not dripping, paper towel to maintain moisture.

C.3 Recoding of Hid & Hid MiRNAs

The regions targeted by miRs are highlighted in bold. The sequences below are those synthesized by GeneArt.

Hid 1

TACGACAACTTTACGGCGGGCCGGGAGCGTCTGCAGGAGTTCAATGGACGCA
TCCCGCCCCGGAAGAAGAAGTCGAGCAACTC**GCATAGCTCCTCCTCCAAC**
AACCCAGTCTGCCATACCGATAGCCAGCCCGGTGGTACATCCC**AGGCTGAA**
TCCGGAGCTATTCATGGCCACATCAGTCAGCAGCGACAGGTGGAGCGAGA
ACGACAAAAGGCGAAGGCCGAGAAGAAGAAACCACAGAGCTTCACTTGGC**CT**
ACAGTAGTTACGGTATTTGTTTTGGCCATGGGCTGTGGCTTCTTTGCGGC

GCGATGA

Hid 2

TACGACAACTTTACGGCGGGCCGGGAGCGTCTGCAGGAGTTCAATGGACGCA
TCCCGCCCCGGAAGAAGAAGTCCAGTAATTC**CCACTCCTCGAGTTCGAAC**
AATCCAGTCTGCCATACCGATAGCCAGCCCGGTGGTACATCCC**AAGCAGAG**
AGTGGCGCAATACATGGCCACATCAGTCAGCAGCGACAGGTGGAGCGAGA
ACGACAAAAGGCGAAGGCCGAGAAGAAGAAACCACAGAGCTTCACTTGGC**CG**
ACCGTCGTAACAGTCTTTGTTTTGGCCATGGGCTGTGGCTTCTTTGCGGC
GCGATGA

These are the miRs synthesised to target the Hids shown above. MiR set 1 targets Hid 1 and miR2 set 2 targets Hid 2. Splice junctions are underlined and stem-loops highlighted in bold.

MiR set 1

ATTAAAAGATCTATGGCATACCCATACGACGTTCCGGACTACGCTGTTAAGGT
AGGCATTAACTATTTAAAGTCCACAACTCATCAAGGAAAATGAAAGTCAAAGT
TGGCAGCTTACTTAAACTTAATCACAGCCTTTAATGT**GCATAGCTCCTCCT**
CCAACCACTAAGTTAATATACCATATCTAGTTGTTGGAGGAGGAGC
TATGCGTACCTAAAGTGCCTAACATCATTATTTAATTTTTATTTTTATTGGCA
CACGAATAACCATGCCGTTTTCAATACTAATCCCTTTAGTAAAACTGTTTTAA
TTTCTTTTCTTTTTTCAGCCAAGGACACCCAGCGCTTCATCATGTATGGTGAG
CAAGGGCGAGGAGCTGTTCACCGGGGTAACGTAGCCAATATCTTTTTGAAAT
CAACAACCCAACACGGGAAGTGTAAGTCTAAGTTAGCAGCTTAGTTTAACTTG
ATTACAGCCTTTAATGT**AGGCTGAATCCGGAGCTATCCATAAGTTTCA**
AAATCATATCTATGAATAGCTCCGGATTCAGCCTGTACCTAAAGTGCA
TAGCAGCAGTACTTAATCTTATTATTCTTTGGTACTCGCATCACTATGCCCTT
GTCATTACTAATCAATTAAGAAACACTGTTATACTATATTTTTCTTTTACAGA
TGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAG
CGTGTCCGGAAGGTAAGTTTTCAATATTTAGAGCCCTCATCTCATTAACGATA
ATGATAGGCAGAGTTGTCAGCTTACATATACTTAATCCCAGCCTTTAATGT**CT**
ACAGTAGTTACGGTATTCGTTAAGTTTGTTTTTCATTTCTAACAAAT

ACCGTAACTACTGTAGGTACCTAAAGTGCCAACATCATAATCTAGTTCTTT
TTATTGTTTGCCCATGAGTAGCCTTGCCGCTTCGGTCACAACTAACCCCTTTA
CTAACACTGTCTTACTTATATTTCTTCTGTCAGGACGAGGGCGAGGGCGATGC
CACCTACGGATCCATCAACTCTAGAATAT

MiR set 2

ATTAAAAGATCTATGGCATACCCATACGACGTTCCGGACTACGCTGTTAAGGT
AGGCATTAACTATTTAAAGTCCACAACTCATCAAGGAAAATGAAAGTCAAAGT
TGGCAGCTTACTTAAACTTAATCACAGCCTTTAATGT**CCACTCCTCGAGTT**
CGAACCATTAAGTTAATATACCATATCTAATTGTTCGAACTCGAGG
AGTGGGTACCTAAAGTGCCTAACATCATTATTTAATTTTTATTTTTATTGGC
ACACGAATAACCATGCCGTTTTCAATACTAATCCCTTTAGTAAAACTGTTTTA
ATTTCTTTTCTTTTTTCAGCCAAGGACACCCAGCGCTTCATCATGTATGGTGA
GCAAGGGCGAGGAGCTGTTCACCGGGGTAACGTAGCCAATATCTTTTTGAAA
TCAACAACCCAACACGGGAAGTGTAAGTCTAAGTTAGCAGCTTAGTTTAACTT
GATTACAGCCTTTAATGT**AAGCAGAGAGTGGCGCAATCCATAAGTTTC**
AAAATCATATCTATGTATTGCGCCACTCTCTGCTTGTACCTAAAGTGC
ATAGCAGCAGTACTTAATCTTATTATTCTTTGGTACTCGCATCACTATGCCCT
TGTCATTACTAATCAATTAAGAAACACTGTTATACTATATTTTTCTTTTACAG
ATGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCA
GCGTGTCCGGAAGGTAAGTTTTCAATATTTAGAGCCCTCATCTCATTAACGAT
AATGATAGGCAGAGTTGTCAGCTTACATATACTTAATCCCAGCCTTTAATGT**C**
GACCGTCGTAACAGTCTTCGTTAAGTTTGTTTTTCATTTCTAACAAA
GACTGTTACGACGGTCGGTACCTAAAGTGCCAACATCATAATCTAGTTCT
TTTTATTGTTTGCCCATGAGTAGCCTTGCCGCTTCGGTCACAACTAACCCCTT
TACTAACACTGTCTTACTTATATTTCTTCTGTCAGGACGAGGGCGAGGGCGAT
GCCACCTACGGATCCATCAACTCTAGAATAT

tTAV 5' Intron & Kozak

Splice sites are underlined.

5’ Intron: CACTGGGCAGGTAAGTATCAAGGTTACAAGACAGGTTTAAGGAGAC
CAATAGAAACTGGGCTTGTCGAGACAGAGAAGACTCTTGCGTTTCTGATAGG
CACCTATTGGTCTTACTGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCAC
TCCC

Kozak: CAACCAAA

Appendix D

Synthetic MicroRNA Assembly

D.1 Primer Design

There are a variety of arguments made of how targets and backbones should be selected, but assuming a target and backbone have been selected, this is how primers are designed for the construction of a single stem loop. In this example, the Drosophila microRNA miR6.1 will be used as the backbone, and it is designed to target the open reading frame of the *Drosophila melanogaster* Flightin gene. This microRNA has been designed to mimic the natural structure of 6.1.

First, two outside primers are designed which are not specific to the gene being targeted and can be used in any scheme requiring the same cloning sites and backbone. These are shown in Figure D.1a. The first three bases on each primer serve as a tail to make sure once both rounds of PCR are complete, the restriction enzymes will have enough double stranded DNA to allow digestion. In red are shown the pair of restriction sites on each primer. Between the restriction sites, in blue, are consensus sequences for the 5' and 3' splice sites so that microRNA will be spliced out of any transcript it is part of. This allows the inclusion of microRNAs in UTRs of transcripts containing open reading frames without the truncation of these transcripts. The splice sites themselves are underlined. Recall the 3' end is reverse-complemented. This outer pair can be used in the construct of any miR6.1 backbone that uses the same restriction sites in its cloning scheme. The primers can be easily modified to accommodate any restriction sites required or to remove the splice sites.

The inner primers are shown in Figure D.1b. This pair is self annealing, and is made specific by the four bases shown in orange. The dark and light purple sections are the step and the green represents the loop. To preserve the natural loop in the miR6.1stem, a

mismatch at position 20 of the stem has been included. The guide strand pairs perfectly with the target, however. I have also shown a schematic of the assembled microRNA after both rounds of PCR can be carried out.

a.

5' – GGC GAATTC CAGGTAAGT GGATCC TTT AAA GTC CAC AAC TCA TCA AGG AAA ATG AAA GTC AAA GTT GGC AGC TTA CTT AAA CTT A – 3'

5' – GGCC GCGGCCGC CCTAAAAAAAAGGGAAAAGAAGAAATTAGA AGATCT AA AAC GGC ATG GTT ATT CGT GTG CCA AAA AAA AAA AAA ATT AAA TAA TGA TGT TAG GCA C – 3'

b.

5' – GGCAGCTTACTTAAACTTAATCACAGCCTTTAATGT TCAGTATAAACACTATAGTCAA TA AGT TAA TAT ACC ATA TC – 3'

5' – AATAATGATGTTAGGCACTTTAGGTAC TCAGTATAAACACTATAGTAAATA GAT ATG GTA TAT TAA CTT ATTGA – 3'

c.

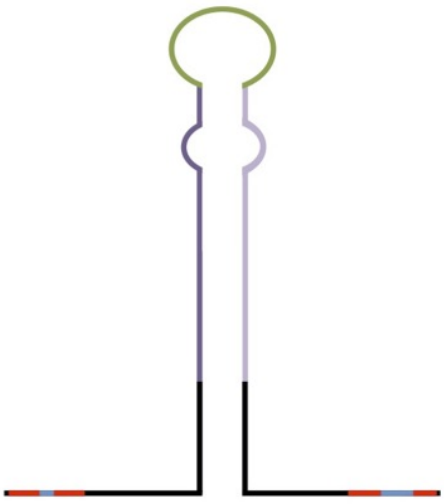


Figure D.1: (a) Outer primer pair. Each primer contains two restriction sites (red), a splice site (blue), and a portion of the microRNA flanking sequence (black). (b) Inner primer pair. These primers anneal to one another. The dark purple sequence corresponds to the sequence targeted apart from an A-to-C base pair substitution at position 20 to preserve a mismatch in the native stem. The light purple sequence is exactly the sequence to be targeted. The four orange bases make this pair specific to one another and help catch mistakes in PCR setup by only allowing the correct pair to be annealed. (c) A schematic of the miR6.1 architecture color coded to match the primers used to construct it.

D.2 MicroRNA Multimerization

The microRNA design above allows for easy cloning and multimerization. This protocol was designed by Haixia Huang and Chun-Hong Chen and was originally carried out through a three piece ligation. Our current standard is to ligate the miRs separately, and then clone in a second step.

For this example, the four enzymes used are EcoRI, NotI, BamHI, and BglII. EcoRI and NotI serve as the outer restriction sites and are used for cloning into the plasmid backbone. BamHI and BglII have compatible ends are are used as the inner pair. First, two aliquots of the microRNA are generated. These may be the same stem-loop or two different stem-loops with the same flanks. One is digested with BamHI and then EcoRI, and the other BglII then NotI. BamHI and BglII digestions leave compatible ends, so the two microRNAs can be ligated together. Phosophatase treatment of one (but not both!) before digesting with the outer enzyme, either NotI or EcoRI, reduces the number of ligations of the microRNAs to themselves. Even if two BamHI or BglII sites do ligate to one another, however, this will not result in background because those fragments will not be able to be ligated in the next step.

Following the initial ligation, we gel excise to isolate the pair which can then be ligated into a backbone digested with the outer enzymes, EcoRI and NotI. This creates the initial vector containing a pair of microRNAs flanked by the full complement of restriction sites that each of the singles had within an intron. If only a pair is desired, cloning is complete. If further multimerization is required, additional stem-loops can be cloned in by opening this vector with either EcoRI/BamHI and cloning in a microRNA digested EcoRI/BglII or the opposite: The backbone is cut BglII/NotI and the microRNA BamHI/NotI. Because the BamHI/BglII ligation results in a fused site that is recognized by neither of the enzymes, there is no multiplication of the BamHI or BglII cut sites and this procedure can be repeated as necessary. Exponential growth of the number of stem-loops is limited only by the tolerance of the plasmid and cells with respect to repetitive DNAs. A schematic showing this example is shown in Figure D.2.

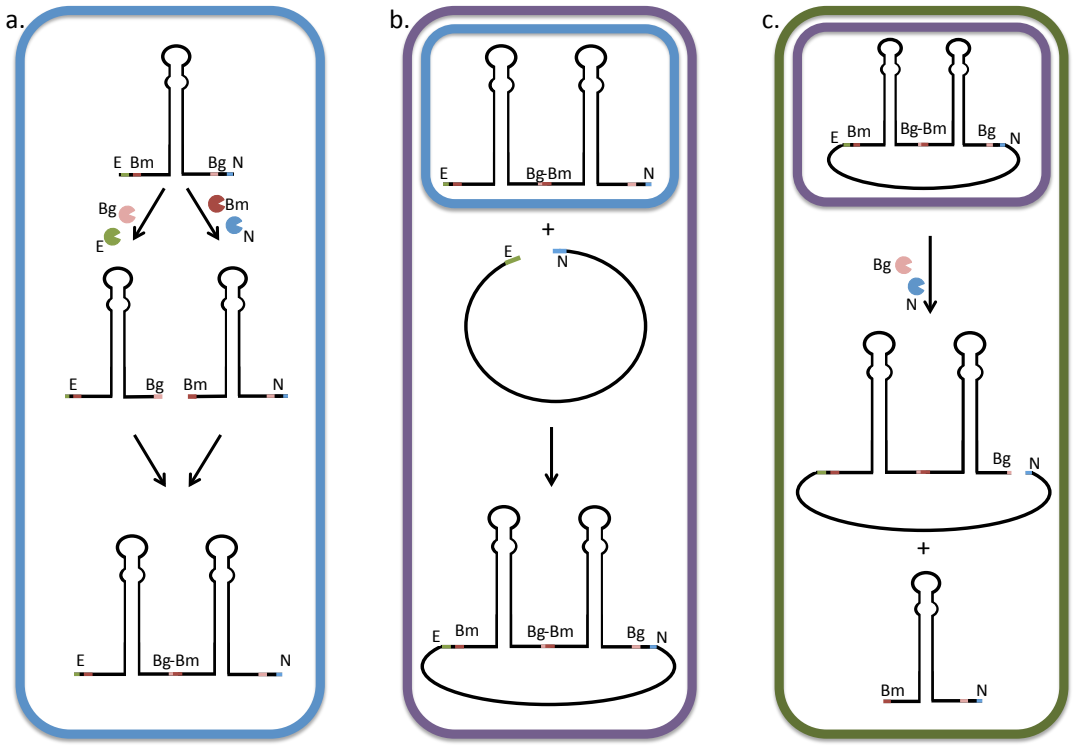


Figure D.2: (a) MicroRNAs are digested with a pair of restriction enzymes such that one over-hang matches the opposite microRNA, but when they are ligated neither restriction site will be recovered. This pair is ligated together making a covalently linked pair. (b) This product also has a pair of ends that allow ligation into a doubly digested plasmid. (c) The newly constructed plasmid can now can be redigested and another set of microRNAs cloned in.

General Protocol

1. Run a 10 cycle PCR with the inner primer pair.
2. Run a second PCR using the first round as template and the outer primer pair.
3. Clean and concentrate the reactions.
4. Digest one microRNA with BglII and the second with BamHI.
5. Phosphatase treat one of these reactions.
6. Heat inactive the phosphatase.
7. Clean an concentrate both reactions.

8. Ligate entire volume of both reactions together.
9. Gel purify (the band may be faint).
10. Digest the gel-purified ligation and backbone EcoRI/NotI.
11. Phosphatase treat backbone.
12. Clean and concentrate both reactions.
13. Ligate, transform, and plate.

Appendix E

FLP Excision

E.1 FLP Excision Lines

All of the FLP excisions carried out genetically in this work were done using lines ordered from the Bloomington Stock Center.

The three ovo-FLP lines I ordered were:

#8727 P[ovo-FLP.]M1A, w[*]

#8728 w[*]; P[ovo-FLP.R]F1B, wgSp-1/CyO; MKRS/TM2

#8729 w[*]; Povo-FLP.RM1B; MKRS/TM2

The β-tubulin line used was:

#7196 P[ry[+t7.2]=betaTub85D-FLP]1; ry[506]

Flipping of all three ovo-FLP lines were tested, but I had trouble maintaining #8728 because it gave nearly all male progeny. I attempted to segregate what was giving rise to the sex ratio distortion, but I could not easily identify even the chromosome causing it and so I opted to not use it.

I used the β-tubulin-FLP line and the 8727 ovo-FLP lines in the excision crossing schemes with the UD flies because of their reasonable flipping efficiencies and position on the X chromosome. The 8727 line sometimes gave very efficient flipping and other times barely flipped at all. I was unable to determine what was influencing this variability. Some of the effect may have been due to the size of the fragment to be excised, but even this did not seem to be consistent. We tried to develop other germ line FLPs with greater and more

Assessment of FLP Lines		
FLP line	Chromosome/PhiC31 Site	Percentage Flipped
ovo-FLP (8727)	X	$\sim 95\%^1, 60\%$
ovo-FLP (8729)	II	$\sim 5\%$?
ovo-FLP (8728)	II	-
ExuMed-FLP	X/9753	$\sim 4\%$
ExuMed-FLP	II/9724	$\sim 36\%$
β-tubulin FLP(7196)	X	$\sim 90\%^1, 60\%$,
ExuLong-FLP	X/9753	$\sim 52\%$
ExuLong-FLP	II/9724	$\sim 52\%$
vasa-FLP	X/9753	0%
vasa-FLP	II/9724	0%

Table E.1: Lines were tested for the percentage flipping in their germ line. [1]These two counts were done using a GMR-Hid UD construct. The other assessments were done using a GMR RFP/GFP assay with nothing toxic involved.

repeatable FLP efficiency.

First I worked with a vasa fragment, hoping to get FLP expression in both male and female germ lines, but at two different PhiC31 insertion sites no flipping was observed. It also came to our attention that there are two FLP versions used in the *Drosophila* literature and our constructs bore the one which had a very low flipping efficiency (Nern et al., 2011).

The next round of germ line FLP constructs used the more efficient FLP allele and were constructed using two different Exu promoter fragments—one meant to express in the female germ line (ExuMed) and the other the male germ line (ExuLong). These two fragments were identified by O. Akbari in his survey of maternal gene promoters. Flipping is still not very efficient.

E.2 Tet-Induced Excision

If generating an UD system in mosquitos for release, removing the conditional rescue through feeding of a drug would be very desirable. This would allow introgression of the UD chromosomes into a genetically diverse background and would alleviate the need to develop efficient germ line FLPs in the mosquito.

To accomplish this, I built up a cassette that could excise itself upon the feeding of tetracycline. Between two FRT sites are the blocking ORF and its 3' UTR (usually GFP,

although anything could be used), a germ-line-promoter driven tTAV and a P_{tight}-driven FLP. In the soma of animals fed Tet, the GFP is expressed from the tissue-specific promoter as usual. In the germ line, tTAV is expressed in both the presence or absence of drug. If the animal is fed Tet, however, tTAV binds to the P_{tight} promoter, driving expression of FLP and excising the cassette. The progeny that grow from germ cells that have undergone FLP excision bear an UD chromosome without the blocking sequence and express the toxin.

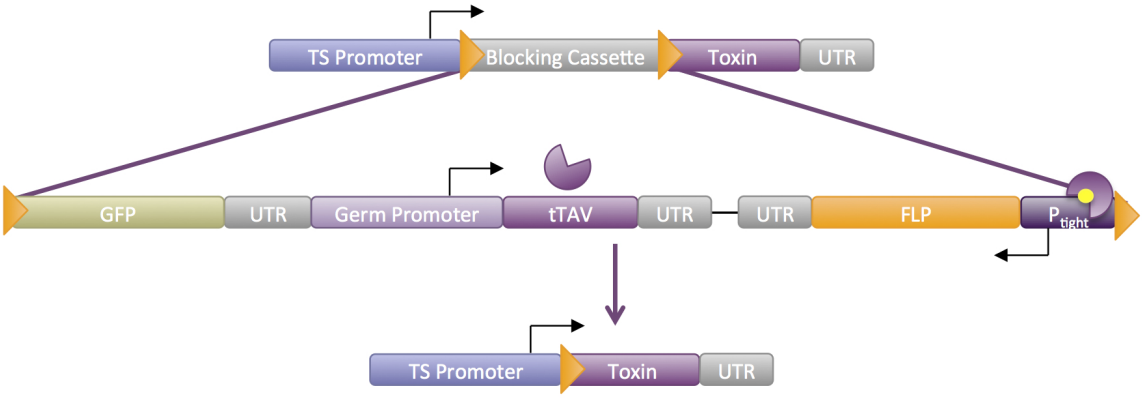


Figure E.1: The blocking cassette is flanked by FRT sites. Also between the sites is a germ line driven tTAV and a P_{tight} driven FLP.

This system was tested using vasa as the germ line promoter and the less efficient FLP, and no flipping was observed. New versions using other germ line promoters and the more efficient allele of FLP are being tested.