THE ROLE OF APOPTOTIC FACTORS IN SINDBIS VIRUS INFECTION AND REPLICATION IN THE MOSQUITO VECTOR *AEDES AEGYPTI*

by

KATELYN LEIGH O'NEILL

B.S., Wayne State College, 2006

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Division of Biology
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Abstract

Mosquitoes are carriers of a variety of harmful human pathogens, including viruses. In order to be successfully transmitted, a virus must evade mosquito immune responses. In this work, the innate immune role of apoptosis in mosquito-virus interactions was examined utilizing the disease vector *Aedes aegypti* and Sindbis virus. *Ae. aegypti* is the main vector for yellow fever and dengue virus, which result in over 100 million infections per year. Sindbis virus (*Togaviridae*) can be transmitted to vertebrates by *Ae. aegypti* in the laboratory. Sindbis is also well characterized molecularly, making it a good model system for understanding virus-vector interactions.

Sindbis MRE-16 recombinant virus clones were utilized to express either an anti-apoptotic or pro-apoptotic gene during virus replication. Mosquitoes were infected with recombinant virus clones during a blood meal or by intrathoracic injection. Midgut tissue and whole body samples were analyzed for virus infection and dissemination. Virus was also quantified in saliva and mosquito survival was assayed. Decreased infection in the midgut and delayed virus replication were observed in mosquitoes that were infected with virus expressing a pro-apoptotic gene. Infection with this virus clone also resulted in less virus in the saliva and reduced survival of infected mosquitoes. In addition, negative selection against pro-apoptotic gene expression during virus replication was observed. Collectively, these data suggest that apoptosis can serve as an antiviral defense in *Ae. aegypti* and may potentially be exploited to control virus transmission.

An additional study included in this dissertation focused on zebrafish development and migration of somitic precursors from the tailbud. The tailbud consists of a population of stem

cells at the posterior tip of the embryonic tail. The exit of these stem cells from the tailbud is required for the formation of tail somites. A novel double mutant was identified that lacked the t-box transcription factor *spadetail* and the BMP inhibitor *chordin*. Double mutants completely lacked somites and had an enlarged tailbud due to accumulation of stem cells that were unable to exit the tailbud. This study indicates the importance of BMP inhibition and *spadetail* expression in the proper exit of muscle precursors from the tailbud.

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Dedication

This work is dedicated to Mrs. Evelyn Hoem, who believed since the time I was 8 years old that I would be a published author and would one day be a doctor.

Chapter 1 - Introduction

Vector Biology of *Aedes aegypti*

Biology of *Aedes aegypti*

Natural History

Mosquitoes are thought to have appeared 200-245 million years ago (Ma) during the Triassic period, along with other Diptera. However, the first fossils resembling mosquitoes are dated at 37-58 Ma. Based on genetic analysis, *Anopheles gambiae* diverged from *D. melanogaster* around 250 Ma. *Aedes* species radiated from *Anopheles* spp. 150 Ma. Today there are more than 3,500 species of mosquitoes (Marquardt and Kondratieff, 2005).

Mosquitoes are from the *Nematocera* suborder which they share with biting midges and sandflies. *Aedes aegypti* belong to the *Culicinae* family and the *Culicidae* subfamily. They are one of the best characterized species within *Culicinae*. This is probably due to their easy transition from the field to the lab and their ability to vector multiple pathogens, such as filarial worms, Plasmodium, and viruses (Marquardt and Kondratieff, 2005).

Geographical distribution

Ae. aegypti survives year round in many tropic and sub-tropic regions. While climatic changes may affect their distribution, domestic nature likely influences their distribution to a greater extent (Jansen and Beebe, 2010). *Ae. aegypti* is native to Africa and is thought to have originally been forest dwelling. They then spread from tropical forests to urban environments in North Africa or Near East countries resulting in domestic populations. They were introduced throughout tropic and subtropic regions through trading activities and were likely imported to the New World during the African slave trade. They were later introduced to Asia and spread to Southeast Asia and the Pacific region during World War II. WWII activities extended the distribution of *Ae. aegypti* to Pacific Islands (Urdaneta-Marquez and Failloux, 2011).

Aedes species often breed in domestic containers and prefer to feed on human blood. They are most commonly found in domestic settings and will readily enter human dwellings to feed and rest (Christophers, 1960; Mackenzie et al., 2004). They have a short flight range of ~800 m per week. Their dispersal occurs mainly at the adult stage and is thought to be due to searching for oviposition sites (Edman et al., 1998; Honorio et al., 2003; Reiter et al., 1995). *Ae. aegypti* will disperse eggs among multiple breeding sites (Apostol et al., 1996). Dispersal rates inversely correlate with the number of available breeding sites (Edman et al., 1998). One study found that most *Ae. aegypti* mosquitoes will oviposit within 90 m of their origin but some will oviposit over 400 m away from their origin (Apostol et al., 1996). *Ae. aegypti* may also be passively dispersed by human transportation. Humans may unintentionally transport eggs, immature mosquitoes or adults, facilitating mosquito distribution (Huber et al., 2003; Jansen and Beebe, 2010).

Aedes spp. populations drastically declined and even disappeared in the Americas during 1946-1970 due to mosquito eradication efforts conducted by the Pan American Health Organization. It has since reinfested these regions due to lack of program sustainability since the 1970's (Gubler, 1997). *Ae. aegypti* was common in the Mediterranean region and Europe prior to WWII and has since disappeared from this area, likely due to malaria eradication efforts and DDT use (Kumm, 1931; Reiter, 2001). Based on these historic instances, control of *Ae. aegypti* is possible yet it is very challenging to sustain. Geographical distribution is certainly not static and has drastically changed in a number of countries over time (Jansen and Beebe, 2010).

Life Cycle

Mosquitoes exist anywhere there is standing water, which is required for egg laying. Approximately three-fourths of mosquito species live in tropic and sub-tropic regions. They

undergo complete metamorphosis consisting of an egg stage, four larval stages, a single pupal stage, and adult (Clements, 1992). *Ae. aegypti* lay 100 or more eggs per batch, laying them at multiple sites. Their eggs can withstand desiccation for up to one year (Leahy et al., 1978; Raminani and Cupp, 1975). *Ae. aegypti* will lay eggs in standing water found in natural (tree or rock holes, ground depressions) or man-made (drums, tires, bird baths) containers (Christophers, 1960; Leahy et al., 1978; Strickman and Kittayapong, 1993). Unfavorable conditions may result in diapause or quiescence during the egg stage. Stimuli for hatching are known to be submersion in water and a drop in dissolved oxygen levels. Solutions containing a range of inorganic or organic compounds or infusion of bacteria have also been shown to stimulate hatching (Barbosa and Peters, 1969; Clements, 1992; Gjullin, 1941; Judson, 1960).

In *Ae. aegypti*, males often hatch first and are not sexually mature at emergence. After 24-48 hours males are ready for copulation (Chevone and Richards, 1977; Elzinga, 1961; Roth, 1948; Steward and Atwood, 1963). Yet, female *Ae. aegypti* are usually unreceptive to mating for 30-60 hours after emergence (Clements, 1999). Mosquitoes mate mid-air in swarms often at dusk. *Ae. aegypti* will mate between ending flight and resting periods, but they will not mate during resting periods (Christophers, 1960). Males are polygamous but females mate only once and are refractory to second matings. Females will store sperm in an organ known as the spermatheca and will use sperm from one male to fertilize eggs throughout their lifetime (Clements, 1992).

Females must take a blood meal to obtain protein for ovary development. *Ae. aegypti* fed high protein solutions have been shown to develop just as many eggs as blood-fed females (Kogan, 1990). Females fed solutions containing other nutrients from blood, such as fats or iron, did not develop eggs (Dimond, 1956; Lea et al., 1956; Singh, 1957). L-isoleucine was found to

be the limiting factor for egg production in *Ae. aegypti* (Briegel and Rezzonico, 1985). Other amino acids may be required for different mosquito species.

Mosquitoes can eat 2-4 times their weight in blood (Nayar and Sauerman, 1975). Estimated blood meal size in *Ae. aegypti* is ~5 µl in well-nourished females and 3.5 µl in smaller females. Blood meal size ranged from 2.2-7 µl (Klowden and Lea, 1978). Once imbibed, the blood meal is stored in the midgut, which is the site of nutrient absorption. Water uptake is also stored in the midgut and carbohydrate or sugars are stored in the crop (Friend, 1978). The midgut consists of single epithelia layer composed of 3 cell types: columnar cells, regenerative cells, and endocrine cells (Brown et al., 1985; Christophers, 1960; Thompson, 1905). The midgut is surrounded by basal lamina, which is an extracellular matrix composed mostly of the proteins collagen and laminin. It is thought to be impassible to particles larger than 10 nm. The basal lamina is able to stretch with immediate stretching of the midgut occurring during a blood meal. The pore size roughly doubles with this stretching, however, it is still too small for even the smallest viruses to pass through (Foy and Olson, 2008; Houk et al., 1980; Houk et al., 1981; Reinhardt and Hecker, 1973).

Blood in the midgut is housed in a peritrophic matrix which begins forming 30 minutes after a blood meal and is fully hardened 4-8 hours later in *Ae. aegypti* (Perrone and Spielman, 1988; Zhuzhikov et al., 1970). This time varies among mosquito species. The peritrophic matrix has been proposed to prevent damage of the midgut epithilia, separate anterior and posterior gut regions to prevent nectar from the crop from mixing with protein digestion, or restrict the ectoperitrophic space to keep digestive enzymes away from inhibitors in the blood meal (Clements, 1992). However, the essential function of the peritrophic matrix is not very well understood, as blood digestion occurs normally without its formation (Billingsley and Rudin,

1992). After blood feeding and digestion, global hormonal regulation and numerous physiological changes lead to maturation of oocytes and eventually oviposition. Egg production in mosquitoes is a cyclic process and a single gonotrophic cycle starts with search for a blood meal and ends with egg laying (Beklemishev, 1940; Clements, 1992). *Ae. aegypti* will feed multiple times during a single gonotrophic cycle. Adult female *Ae. aegypti* usually have a lifespan of 4-8 weeks and will undergo multiple gonotrophic cycles which average 3-5 days in length (Clements, 1992).

Genetics

Mosquitoes can be good models for genetic research. They have a short life cycle and certain species are easy to rear in the lab. They have a small chromosome complement and nurse cells with polytene chromosomes which aids in genetic mapping (Clements, 1992). Mutants are available in some species, including *Ae. aegypti* (Fraser, 2012). *Aedes* spp. have 3 pairs of chromosomes including a homomorphic sex chromosome. The genome of *Ae. aegypti* was sequenced and published in 2007. It is estimated to be ~1300 Mbp in size and 16,789 transcripts were predicted (Nene et al., 2007). The *An. gambiae* genome was published in 2002 and is considerably smaller, with a size of 278 Mbp (Holt et al., 2002). Not surprisingly, the proteome of *Ae. aegypti* is more similar to *An. gambiae* than *D. melanogaster*, with 67% of the proteome having orthologs in *An. gambiae* and being 58% orthologous to *D. melanogaster*. Just about half of the *Ae. aegypti* genome is composed of transposable elements, which accounts for its large size. It also contains increased intron size and quantity compared to *An. gambiae* and *D. melanogaster*. This is also likely due to the high number of transposable elements (Nene et al., 2007). Genome sequencing of *Ae. aegypti* has facilitated a plethora of molecular research and investigation into its behavior, survival and ability to transmit pathogens.

With the power of a sequenced genome, some areas of intese investigation include immune players, viral receptors, candidate genes for vector competence and biological control of mosquito populations. Attempts to transform *Aedes* spp. mosquitoes began in the late 1980s, utilizing P elements from *D. melanogaster*. P elements were successfully integrated into the genetic background but integration was not mediated by P transposase. Therefore, efficiency was too low for this system to be useful (Morris et al., 1989). From there, alternative transposable elements (TE) were used next, such as *Hermes, Mariner*, and *piggyBac*. Successful transposition assays were developed from these (Coates et al., 1998; Jasinskiene et al., 1998; Lobo et al., 1999; Sarkar et al., 1997). *Hermes* has been used most frequently, yet integration is more precise using *Mariner* or *piggyBac*. Transposons were an exciting stride in mosquito transgenics. They are, however, not a perfect system. Integration at random sites, instability of inserted sequences, limited carrying capacity, and low efficiency are all challenges faced when using transposons (Fraser, 2012).

Site specific recombinases are another approach for transgenic engineering. Tyrosine catalyzed integrase systems such as Cre/loxP and FLP/FRP are candidates that have yet to be successfully used in mosquitoes (Jasinskiene et al., 2003; Morris et al., 1991; Nimmo et al., 2006; Schetelig et al., 2011). However, bacteriophage *ϕC31* intregrase has successfully been used for site directed recombination in *Ae. aegypti* (Franz et al., 2011). Homing endonucleases are another potential transformation technique, which have successfully been used in *An. gambiae* and may be utilized in *Ae. aegypti* (Traver et al., 2009; Windbichler et al., 2007; Windbichler et al., 2008). Recently, zinc finger nucleases (ZFN) have been explored in insect systems to insert or delete genetic information. In *Ae. aegypti* a combination of ZFN and

transposases has successfully been used to achieve efficient and targeted gene integration (Maragathavally et al., 2006).

Transgenic research holds great potential for sterile insect techniques, pathogen targeting or blocking pathogen success. Field trials and gene drive systems are being utilized to infiltrate desired transgenics into natural populations in order to achieve protection against vector borne diseases (Hoffmann et al., 2011; Labbe et al., 2012; Lacroix et al., 2012; Marshall and Hay, 2012; O'Connor et al., 2012). We are getting closer to biological control of natural insect populations and making progress in the battle against taxing diseases caused by mosquitoes and other vectors.

Vector competence of *Aedes aegypti*

It is the blood feeding behavior of mosquitoes that has led to their medical and veterinary importance. Blood feeding puts mosquitoes in direct contact with many blood-borne pathogens, some of which have been able to utilize mosquitoes as a vector and be transmitted to secondary hosts during subsequent blood meals. Arboviruses are viruses transmitted by arthropods. *Aedes* and *Culex* species are the most common mosquito species known to transmit viral pathogens (Marquardt and Kondratieff, 2005). Both species are able to transmit flaviviruses, alphaviruses and bunyaviruses (Powers, 2009; Tabachnick, 2013). *Ae. aegypti* is the main vector for yellow fever virus, dengue virus, and chikungunya virus epidemics in Indian Ocean countries (CDC, 2012; Ligon, 2006; Tomori, 2004). *Ae. aegypti* is a very successful vector for a number of reasons. It has a wide global range. It prefers a human blood meal as opposed to feeding on other vertebrates. It inhabits urban areas and is often found in human dwellings (Christophers, 1960; Harrington et al., 2005; Ponlawat and Harrington, 2005). *Ae. aegypti* also bite during the day, making control methods more challenging (Scott et al., 2000). The presence of *Ae. aegypti*

and the pathogens it is known to transmit must coincide in the same location for transmission to occur. Vector competence is dependent on a pathogen's ability to bypass certain physical barriers within the vector and to replicate in tissues required for transmission. In mosquitoes, these include the midgut as the primary site of infection and the salivary glands as the final site of infection. A pathogen must be able to infect the midgut cells and escape the midgut and eventually infect the salivary glands and then be secreted in the saliva (Hardy et al., 1983; Mellor, 2000).

Yellow Fever Virus

Ae. aegypti is often referred to as the yellow fever mosquito. It was identified as the vector for yellow fever virus in 1900. This was the first virus identified to be transmitted by a mosquito (Marquardt and Kondratieff, 2005). There is an effective vaccine available to protect against yellow fever, yet it is still a disease burden in Africa and South America. There are an estimated 200,000 cases annually, with a 20% fatality rate. Yellow fever virus is the prototype member of the family *Flaviviridae*, with "flavus" being the Latin word for "yellow". It is an enveloped, positive-strand RNA virus with a genome size of 10.5-11 kb (Barrett and Higgs, 2007).

The first recorded outbreaks of yellow fever are from 1648 in Mexico (Carter, 1931). However, outbreak of a similar disease dates back to Haiti in 1495. The term yellow fever was first used by Griffin Hughes in 1750. Yellow fever virus was first isolated from West Africa in 1927. Outbreaks in the 17-19th centuries demanded medical attention and research to prevent infection and death. This resulted in improved understanding of vector-borne diseases and development of techniques to study pathogens and vectors and the importance of vector control in public health programs (Barrett and Higgs, 2007).

In 1901 mosquito eradication efforts were initiated in Cuba by Major William Gorgas. Breeding sites were removed and in 6 months yellow fever was eliminated and instances of malaria declined. The same methods were used to eliminate yellow fever in Panama. From there, the Pan American Health Organization eradication efforts were facilitated to prevent urban outbreaks of yellow fever and other vector diseases in the Americas. This was a very successful campaign, eliminating *Ae. aegypti* from several countries and greatly reducing vector diseases (Schliesman and Calheiros, 1974; Soper et al., 1943). However, once the program stopped being maintained, re-infestation of mosquito populations and disease ensued (Gubler, 2004). In South America there are approximately 160 cases of jungle fever, which are cases acquired from people entering the jungle and being bitten by infected mosquitoes from the sylvatic cycle, reported each year with a 65% fatality rate (WHO, 2012b). A greater number is likely not reported. However, no urban yellow fever has been reported in South America since eradication efforts in the early 1900s. Africa, on the other hand, currently has 600 million people at risk for yellow fever and 90% of annual cases are estimated to be in Africa. There is little eradication efforts and vaccine administration in Africa compared to South America (Barrett and Higgs, 2007; WHO, 2012b).

In the 1930's, two live attenuated vaccines were produced against yellow fever. Both were very successful and dramatically reduced the instance of yellow fever. Use of one of the vaccines was discontinued after post-neurotropic disease developed in some patients. The other vaccine is still used today (Barrett, 1997). Eradication of yellow fever is not likely due to its enzootic cycle in rainforests, where it is maintained in lower primates. However, prevention is effective as long as the vaccine is available and administered to those in need. Concerns for future outbreaks are caused by unvaccinated travelers, travel of viremic individuals from infected

regions, and increased rainfall and urbanization in Africa (Barrett and Higgs, 2007; Barrett and Monath, 2003). Introduction of yellow fever in a developing country where *Ae. aegypti* is present could lead to very serious outbreaks. This has been previously demonstrated in North America with the introduction of West Nile Virus in 1999 (Lanciotti et al., 1999).

Dengue Virus

Dengue is the most common arbovirus infection in the world. 2.5 billion people are at risk for dengue and there are 50-100 million cases reported each year. It is endemic in over 100 countries, significantly affecting Southeast Asia and the Western Pacific. There is currently no vaccine available to protect against dengue, leaving mosquito control to be the only option (Murrell et al., 2011; WHO, 2012a).

Dengue also belongs to the *Flaviviridae* family. It is an enveloped positive-strand RNA virus and it has a 10.6 kb genome (Lindenbach and Rice, 2003). There are 4 antigenically distinct serotypes of dengue. Subsequent infections with a second serotype are thought to lead to more severe forms of the disease, termed dengue hemorrhagic fever and dengue shock syndrome (Bravo et al., 1987; Halstead, 1989; Vaughn et al., 2000). The outcome of dengue infection in humans ranges from asymptomatic to very severe. Dengue infection typically results in symptoms such as headache, rash, gastric disorders, fevers, joint/muscle pain and nausea and vomiting. Symptoms vary depending on the virus serotype and the immune status of the individual. Dengue hemorrhagic fever is characterized by fever, hemorrhagic bleeding, plasma leaking and circulatory disruption or failure. Dengue hemorrhagic fever results in 10-100 fold higher levels of virus in the blood (Hammon et al., 1960). Dengue shock syndrome results from shock that occurs when fluid leaks into intestinal space, which can be a life threatening condition (Halstead, 1988; Halstead, 1989).

The first reports of a dengue-like illness are found in a Chinese medical encyclopedia dating back to 265-420 A.D. Descriptions of outbreaks of a similar disease are found from the 17th and 18th century, reaching the U.S. in 1780. During the 19th century, outbreaks occurred in India, Egypt, Greece, Spain, Peru, Brazil and the east and southeast coastal regions of the U.S. (Buchillet, 2012; Gubler, 1997).

Dengue was not linked to *Ae. aegypti* until 1902 (Marquardt and Kondratieff, 2005). The current dengue epidemic is believed to have started in SE Asia during WWII. Lab tests for dengue were developed around this time (Buchillet, 2012; Urdaneta-Marquez and Failloux, 2011). All four serotypes are maintained in most SE Asia cities today. In Asia and the Americas, dengue maintains a human to mosquito to human cycle with humans being the main reservoir. In Africa, a sylvatic enzootic cycle is predominant. Dengue is cycled between nonhuman primates and arboreal *Aedes* spp. and an endemic cycle is also sustained with humans and *Ae. aegypti* (Urdaneta-Marquez and Failloux, 2011). Dengue has an extrinsic incubation period of 8-12 days in the mosquito (Tomashek, 2012).

Other than prevention via mosquito control, not much progress has been made for treatment of dengue. It is challenging to know how to protect against this disease. All serotypes must be covered and both dengue naive and dengue exposed individuals must be considered when administering a treatment or prevention method (Murrell et al., 2011).

Chikungunya Virus

Chikungunya virus is a more recently identified arbovirus that has been responsible for outbreaks in Africa, Asia, Indian Ocean regions and parts of Europe. It is an alphavirus with a positive sense RNA genome consisting of ~11.7 kb. Outbreaks of chikungunya are not as common and less documented than those of dengue (Deller and Russell, 1968; Powers et al.,

2000). The first recorded outbreak of a similar disease dates back to the 18th century in Indonesia (Carey, 1971). The first isolate was obtained from Tanzania in 1953 (Robinson, 1955). East/Central/Southern African (ECSA) and Asian strains diverged roughly 150 years ago. The Asian groups include the Indian line, which may be extinct, the Southeast Asian lineage and the recent Indian Ocean Line (IOL) from 2004. The IOL lineage likely originated from the ESCA group around 2002 (Powers et al., 2000; Schuffenecker et al., 2006; Volk et al., 2010).

Ae. aegypti is the main vector for chikungunya virus, however, *Ae. albopictus* has been the main vector in a few Indian Ocean regions during the last 5 years (Tsetsarkin et al., 2011; Tsetsarkin and Weaver, 2011). Chikungunya virus is maintained by non-human primates and *Aedes* spp. in Africa. In Asia, it maintains a human-mosquito-human cycle. It is considered endemic in rural Africa and epidemic in Asia. It usually peaks and then declines in a region as the population gains immunity (Pialoux et al., 2007). Its extrinsic incubation period in *Ae. aegypti* is only 2-3 days (Dubrulle et al., 2009).

Symptoms in infected humans are similar to dengue symptoms, including a fever, rash, and joint pain, which may last for months (Robinson, 1955). The word "chikungunya" is derived from Makonde dialect and means "to walk bent over". This describes the posture of patients with joint pain during chikungunya infection (Enserink, 2006). Infection seems to produce long-term protective immunity against chikungunya.

There is no current vaccine to protect against chikungunya. A successful vaccine is estimated to be potentially used for 6 million individuals per year (Weaver et al., 2012). Vaccine candidates do exist. The U.S. army had a vaccine in phase III trials several years ago but it has not been approved for administering to the public (Edelman et al., 2000; Levitt et al., 1986).

Others are currently being tested. Until a successful vaccine is produced, protection against *Ae. aegypti* and *Ae. albopictus* vectors is the best prevention method (Weaver et al., 2012).

Sindbis Virus and Alphavirus Transducing Systems

Sindbis Virus

Sindbis virus is the type member of the *Togaviridae* family and belongs to the *Alphavirus* genus. There are over 30 known alphaviruses which include chikungunya virus, o'nyong nyong virus, Ross River virus, Eastern Equine Encephalitis virus, Western Equine Encephalitis virus, and Venezuealan Equine Encephalitis virus. Alphaviruses are grouped into Old World and New World viruses based on where they occur. Sindbis is an Old World virus from the Semilki Forest complex, although is seems to be more closely related to New World alphaviruses (Schlesinger and Schlesinger, 1996; Strauss and Strauss, 1994). Old World alphaviruses are usually less severe and have lower mortality rates compared to the New World group (Paredes et al., 2005; Ryman and Klimstra, 2008).

Pathogenesis

Sindbis was first isolated in 1952 from Cairo, Egypt. Sindbis is naturally vectored by *Culex* species, yet it can be transmitted by *Ae. aegypti* in the laboratory (Echalier, 1965; Taylor et al., 1955). It cycles between mosquitoes and bird reservoirs in nature. Humans are infected with Sindbis when bitten by an infected mosquito (Taylor et al., 1955).

Sindbis infection is generally not life threatening. Symptoms may include joint pain, fever, malaise, and a rash. Sindbis infection is often asymptomatic (Kurkela et al., 2005). However, it has been linked to chronic conditions such as arthritis in certain European regions (Kurkela et al., 2004). Sindbis fever is most common in South and East Africa, Israel, Phillipines, and parts of Australia (Doherty et al., 1969; Malherbe et al., 1963; Olson and Trent,

1985). Due to its mild symptoms, Sindbis has been utilized to study virus replication, structure, genetics, and arbovirus-vector interactions (Foy and Olson, 2008; Strauss and Strauss, 1994).

Genome

The Sindbis genome is ~11.7 kb in size. It is a single-strand, positive sense RNA genome with a 5'cap and a 3'poly-A tail (Strauss et al., 1984). It is composed of two open reading frames. The first ORF encompasses the first two-thirds of the genome and codes for the non-structural viral proteins nsP1, nsP2, nsP3, and nsP4 (Fig 1.1). The second ORF makes up the last one-third of the genome at the 3' end. It contains sequences for the 5 structural viral proteins – nucleocapsid protein C, 6K, E1, E2, and E3 (Fig 1.1). Transcribed mRNA from each open reading frame is used to produce a polyprotein that is processed to produce either the nonstructural or the structural proteins needed for replication and assembly/release of new viruses (Schlesinger and Schlesinger, 1996; Strauss and Strauss, 1994).

The Sindbis genome also contains multiple *cis*-acting elements that are very important for virus replication. The 5' UTR is important for initiating translation of the polyprotein for the non-structural proteins. The 5' UTR and the 3' complementary sequence on the negative RNA strand are core promoter elements for the RNA-dependent RNA polymerase and synthesis of the negative-strand RNA template. The negative-strand template is required for synthesis of full-length genomic RNA and subgenomic mRNA coding for the structural proteins (Frolov et al., 2001; Garmashova et al., 2006). In the nsP1 coding region, there is a 51 nt conserved sequence element (CSE) which enhances virus replication and is critical for virus replication in mosquito cells (Frolova et al., 2002; Garmashova et al., 2006; Ou et al., 1982). There is a second CSE, 24 nt in length, at the start of the subgenomic RNA sequence. Its complement in the negative-strand template is a core promoter for transcription for the structural genes (Frolov et al., 2001). The

non-coding region at the 3' end of the genome is thought to play a role in host specificity, perhaps through interactions with cellular proteins (Kuhn et al., 1990).

Virus Replication

The Sindbis genome acts as an mRNA once it is inside the cytoplasm of an infected cell. Translation of nonstructural genes must take place first. nsP123 and nsP1234 polyproteins are produced and later processed into nsP1, nsP2, nsP3, and nsP4 (Strauss and Strauss, 1994). Replication takes place in intracellular membranes in infected cells. Formation of these membranes is induced by nsP1 and is required by dsRNA replicative intermediates (Frolova et al., 2010). nsP1 also has guanine-7-methyltransferase and guanyltransferase activity needed for capping of genomic RNA. nsP2 plays multiple roles in virus replication. It acts as a protease, helicase, and 5'triphosphatase. nsP3 has a macrodomain that is very important in virus replication. nsP4 is the RNA-dependent RNA polymerase needed to produce negative and positive-strand RNA products (Garmashova et al., 2006; Strauss and Strauss, 1994).

Nonstructural proteins and *cis*-acting elements regulate genomic replication and subgenomic transcription. nsP1234 is processed in *cis* into nsP123 and nsP4 by nsP2. The nsP123/nsP4 complex then forms and is important for synthesis of full length negative-strand RNA. nsP123 cleavage into nsP1, nsP2 and nsP3 by nsP2 in *trans* produces a replicase complex (nsP1/2/3/4) which synthesizes positive-strand RNA (49S) and subgenomic RNA (26S). This complex also stops negative-strand RNA synthesis (Garmashova et al., 2006; Sawicki and Sawicki, 1994; Thal et al., 2007; Wielgosz et al., 2001). Synthesis of negative-strand RNA is required in order to produce a template for genomic and subgenomic RNA. Negative-strand RNA template is usually present by 3-4 hpi but is not found at late stages of replication.

Positive-strand RNA is found at all stages of infection (Frolov et al., 2001; Strauss and Strauss, 1994).

Once 26S RNA is transcribed from negative-strand RNA, translation of structural proteins takes place. The capsid polypeptide is the first to be translated. Autocleavage of the C-terminal tryptophan-serine bond via serine protease activity produces the capsid proteins. Capsid proteins will later assemble to encapsidate genomic viral RNA (49S). Translation of signal peptide from subgenomic mRNA downstream of capsid sequence translocates the downstream polypeptide to the endoplasmic reticulum (Strauss and Strauss, 1990; Strauss and Strauss, 1994). Structural proteins PE2, 6K, and E1 are then translated and transported together to the golgi apparatus. After golgi processing they are transported to the plasma membrane of the host cell (Carleton and Brown, 1996; Carleton et al., 1997). PE2 and E1 are transmembrane proteins and 6k is a small membrane-embedded protein. PE2 is later processed into E2 and E3 by furin-like activity when virus is released from infected cells. Heterodimers of PE2 and E1 are then converted to E1 and E2, releasing E3 which is a small membrane-embedded protein (Gaedigk-Nitschko and Schlesinger, 1990; von Bonsdorff and Harrison, 1975; von Bonsdorff and Harrison, 1978). The final step of viral replication is budding of the capsid through the plasma membrane of the host cell. In doing so, viral particles acquire a lipid bilayer envelope with embedded viral proteins (Strauss et al., 1995).

Virion Structure

Sindbis virions are 69 nm in diameter and tend to be spherical and slightly pleomorphic. 240 individual capsid proteins make up the virus nucleocapsid, which is ~40 nm in diameter. Capsid proteins are assembled into 12 pentamers and 30 hexamers in a T=4 arrangement (Paredes et al., 1993; Paredes et al., 1992; Pletnev et al., 2001). Nucleocapsids in the Old World

alphaviruses have a slight clockwise rotation of pentamers relative to hexamers (Paredes et al., 2005).

The viral envelope is composed of 2 glycoproteins (E1 and E2) and a lipid bilayer acquired from the host cell. The envelope and nucleocapsid are thought to interact through the C-terminal residues of E2 proteins exposed on the inner surface of the lipid bilayer and the C terminus of the capsid proteins (Anthony and Brown, 1991). Approximately 80 E1/E2 heterotrimers make up the outer envelope, consisting of 120 E1 dimers and 80 E2 homotrimers. E1 dimer interactions are important for forming a scaffolding lattice that is thought to help stabilize the icosahedral structure of the virus. E2 homotrimers are the primary component of spikes on the envelope surface and are likely responsible for host cell receptor interactions (Anthony and Brown, 1991).

Finding the host cell receptor for Sindbis is a complex investigation. Alphavirus fusion with host cells is not likely endosome mediated because it does not take place in low pH conditions. Brief exposure to low pH and then a return to neutral pH is required for fusion (Edwards and Brown, 1986). Therefore, penetration may occur by injection of RNA into a pore formed with the host cell receptor (Paredes et al., 2004). Sindbis likely has multiple cell receptors, especially because it thrives in several different hosts, both invertebrate and vertebrate. A laminin receptor was identified to be important in mammalian cells and was found to be important to a lesser extent in mosquito cells (Wang et al., 1992). Laminin receptor and heparan sulfate attachment factors have been shown to enhance infection yet are not required for viral entry (Klimstra et al., 1998; Wang et al., 1992). A recent study identified a natural resistance associated macrophage protein (NRAMP) as a host cell receptor for Sindbis in *Drosophila* cells. Fly and mouse NRAMP mutants were non-permissive to infection. *Ae. aegypti* cells were

refractory to Sindbis infection under high iron conditions, possibly indicating NRAMP, which is a divalent metal ion transporter, as a receptor in the mosquito as well (Rose et al., 2011). However, the search for additional receptors in the mosquito is still underway.

Alphavirus Transducing Systems

Alphavirus transducing systems (ATS) are molecular tools that can be used to study virus and vector interactions via fast and efficient gene transcription and expression in infected cells. They were originally developed as an expression system for transposon-based DNA complement systems in mosquitoes. They consist of an infectious virus clone with an inserted construct to be expressed during virus replication. Expression of genes of interest is limited to tropism of the virus. Expression will only take place in infected cells and the timing of expression during virus replication will be at later stages (Foy and Olson, 2008).

Alphavirus transducing systems exist for Sindbis, o'nyong-nyong, and chikungunya viruses. They have been used for infection and expression of desired genes in *Aedes* and *Culex* species as well as *An. gambiae* (Brault et al., 2004; Foy et al., 2004a; Olson et al., 1994; Pierro et al., 2003; Vanlandingham et al., 2005). They have been utilized to express innate immune factors, such as RNAi and apoptotic factors, insect neurotoxins, single chain antibodies, anti-microbial peptides and reporter genes such as green fluorescent protein (GFP) (Cheng et al., 2001; de Lara Capurro et al., 2000; Higgs et al., 1995; Keene et al., 2004; Olson et al., 1994; Pierro et al., 2003; Wang et al., 2008; Wang and Clem, 2011). ATS can also be used to stimulate RNA interference. They can be used to transcribe pieces of RNA from unrelated viruses that are then able to target and inhibit replication of the unrelated virus in the mosquito (Adelman et al., 2001; Blair et al., 2000; Franz et al., 2006; Higgs et al., 1998; Olson et al., 1996; Sanchez-Vargas et al., 2004). Post-transcriptional silencing of host factors has been achieved for phenoloxidase

and GATA repressor genes via injection of infectious virus clones into adult mosquitoes (Attardo et al., 2003; Shiao et al., 2001).

Infectious virus clones can be used to infect mosquitoes by injection or when administered orally during a blood meal. Oral infections are dependent upon foregut and midgut infection. Once primary tissues are infected the virus must be able to escape the midgut and infect other tissues in order to study the effects of the inserted gene in those tissues (Myles et al., 2004a; Pierro et al., 2003). Injection of the virus clone allows for infection of neural tissue, hemocoelic tissue, fat body, muscle, malphagian tubules and salivary glands (Foy et al., 2004a; Kamrud et al., 1997; Olson et al., 1994; Olson et al., 2000; Shiao et al., 2001; Tamang et al., 2004).

Developing an ATS

Alphavirus transducing systems must first be constructed as a cDNA clone. After reverse transcription of the viral genome, the cDNA must be assembled and inserted into a bacterial plasmid containing an origin of replication and an antibiotic resistant marker. The resulting plasmid can then be manipulated using molecular cloning techniques. An RNA polymerase promoter, such as T7 or SP6, should be inserted at the 5' end of the viral genome and the first nucleotide of the genome needs to be modified to allow for insertion of a capped nucleotide. A unique restriction site after the dT sequence also needs to be added for linearization of the plasmid. At this point the infectious virus clone has been produced and can be transcribed in vitro and transformed into susceptible cells for virus expression (Foy and Olson, 2008). Infectious virus clones exist for Semliki Forest virus, Ross River virus, Sagiyama virus and Venezualian Equine Encephalitis virus as well as Sindbis, o'ynong-ynong, and chikungunya

viruses (Davis et al., 1989; Keene et al., 2004; Kuhn et al., 1991; Liljestrom et al., 1991; Olson et al., 1994; Simpson et al., 1996; Vanlandingham et al., 2005; Yamaguchi and Shirako, 2002).

To produce an alphavirus transducing system from an infectious virus clone, the viral subgenomic promoter must be duplicated and reinserted into the genome as a cassette with a multiple cloning site for gene insertion (Foy et al., 2004a). The subgenomic promoter has been well characterized and consists of ~112 nt at the end of nsP4 and directly upstream of the capsid start codon (LaStarza et al., 1994; Wielgosz et al., 2001). The secondary subgenomic promoter can be inserted into the 3' UTR (3'dsATS) or directly upstream of the original subgenomic promoter (5'dsATS) (Hahn et al., 1992; Raju and Huang, 1991). 3'dsATS systems have been found to be less stable for gene expression but are useful tools for RNAi due to yield of two subgenomic RNA's with the effector sequence. dsRNA of the desired gene post transcriptionally inhibits expression via RNAi and antisense RNA produced will potentially interrupt mRNA translation by binding to endogenous RNA (Foy and Olson, 2008; Johnson et al., 1999). 5'dsATS systems tend to have more stable expression of the gene of interest. This is thought to be due to insertion into the middle of the genome making it less likely that the inserted expression construct will be deleted (Cheng et al., 2001; Foy and Olson, 2008; Pierro et al., 2003). Construction of 5'dsATS is diagrammed in Figure 1.1.

When inserting the secondary subgenomic promoter and the gene of interest, there are size limitations with respect to the viral genome. The virion can only package RNA containing up to 2 kb of additional sequence. Insertions of less than 1 kb are presumed to minimally compromise virus replication (Foy and Olson, 2008). 5'dsATS with GFP have been found to stably express fluorescent protein, yet, virus disseminates more slowly than wild type. This

delay is thought to be due to increased transcription time and slower virus packaging (Foy et al., 2004a).

Sindbis Expression Systems

The first Sindbis virus ATS was TE 3'2J (Hahn et al., 1992). It was based on a neurovirulent Sindbis strain (TE) that had been passaged in mice (Lustig et al., 1988). The expression cassette was placed at the 3' end. It was first used to express chloramphenicol acetyltransferase (CAT) in mosquito cells and the mosquito. When injected it was found to replicate in multiple tissues (Hahn et al., 1992). It has since been used to express exogenous genes in *Aedes*, *Culex* and Anopholine species as well as non-vector insects (de Lara Capurro et al., 2000; Higgs et al., 1995; Higgs et al., 1996; Kamrud et al., 1997; Lewis et al., 1999). A 5'dsATS based on the TE strain was also constructed and was more resistant to recombination and loss of the insert over time (Pugachev et al., 1995; Raju and Huang, 1991). The main problem with TE ATS was that the TE strain has lost the ability to efficiently infect mosquito midgut cells because its envelope proteins are adapted to the nervous system of mice. TE will replicate well in cell culture and hemocoelic tissues in the mosquito after injection, however, it is not useful for studies requiring oral infection (Foy and Olson, 2008; Olson et al., 1996; Olson et al., 1994).

To overcome this, an ATS from the MRE-16 Sindbis strain was developed. 5'dsATS and 3'dsATS constructs were made using the MRE-16 infectious clone (Foy et al., 2004a). MRE16ic was found to contain critical E2 residues required for midgut infection (Myles et al., 2003; Myles et al., 2004b). 5'dsMRE16ic-GFP was characterized in 3 Culicine species and 2 Lepidopteran species, using GFP as a reporter. Foregut and midgut infection was observed in *Ae. aegypti* at 1-2 dpi and initial midgut infection was found to be dose-dependent (Foy et al.,

2004b). A chimeric ATS was made using nonstructural genes from TE5'2J and structural genes from MRE-16. This chimeric construct established infection in *Ae. aegypti* midgut epithelia when orally fed to mosquitoes (Olson et al., 2000; Pierro et al., 2003; Seabaugh et al., 1998).

In addition to Sindbis ATS, an expression system for o'nyong-nyong has shed light on arbovirus mechanisms. O'nyong-nyong is transmitted by *Anopheles* spp., whereas, most alphaviruses are transmitted by Culicine mosquitoes (Lanciotti et al., 1998; Rwaguma et al., 1997). 5'dsONNic-GFP was used to identify atypical sites of infections and very different dissemination patterns compared to *Culex*-alphavirus studies (Brault et al., 2004). It was also used to first demonstrate that RNAi acts as a natural antagonist to alphavirus replication in mosquitoes (Keene et al., 2004).

Implications of ATS

Alphavirus transducing systems have contributed greatly to our knowledge of vector and virus interactions. They have proved to be a quick and efficient way to look at the effects of foreign gene expression or gene knockdown in vector species. Producing transgenic mosquitoes has proven to be very difficult for some species and is a labor intensive process. It is time consuming to characterize constructs, perform mutagenesis, and verify expression in transgenics. Once transgenic lines are made they must be maintained which can demand a lot of time and laboratory space. In addition, complex life cycles can make routine transgenesis challenging (Foy and Olson, 2008).

ATS are an excellent complement to transgenic studies. They are a quick way to provide prior knowledge before transforming mosquitoes, perhaps differentiating useful genes from less promising candidates (Foy and Olson, 2008). There may be timing and stability issues with ATS expression. However, they provide a way to study the mosquito and the virus at the same time.

Overall, alphavirus transducing systems are a useful tool to look at virus determinants of mosquito infection and virus-vector interactions.

Apoptosis Pathways

Apoptosis is a vital and controlled process of programmed cell death in multicellular organisms. It is a crucial process required to delete unwanted, damaged or infected cells. It is vital for development, homeostasis and immune responses (Vaux and Korsmeyer, 1999; Vaux and Strasser, 1996). Excessive apoptosis can contribute to neurodegenerative diseases such as Alzheimer's disease and too little apoptosis can result in autoimmune diseases or cancer (Hanahan and Weinberg, 2000; Thompson, 1995; Yuan and Yankner, 2000). Apoptosis is differentiated from passive cell death (necrosis) by hallmarks such as DNA fragmentation, nuclear condensation, blebbing of the plasma membrane, cell shrinkage and formation of apoptotic bodies (Kerr et al., 1972; Thornberry and Lazebnik, 1998; Wyllie et al., 1980). The plasma membrane is not compromised during apoptosis. There is no cytoplasmic leakage as there is during necrosis thus preventing an inflammatory response (Edinger and Thompson, 2004).

The type of programmed cell death we now call apoptosis was first observed and described by Carl Vogt in 1842 while studying tadpole development. It was further described by Walther Fleming in 1885 while he found nuclei breaking up in rabbit ovarian follicles. Throughout the next several decades, programmed cell death was observed and described in multiple organisms and tissues, both in developmental and adult stages, as well as healthy, damaged/ischemic and cancer tissue (Majno and Joris, 1995). It wasn't until 1972 that the process was recognized to be similar in both vertebrates and invertebrates, given the name "apoptosis", and the hallmarks of this process were clearly defined (Kerr et al., 1972). Early on

the process was thought to be programmed and genetically regulated and maintained (Kerr, 2002). The first evidence for apoptosis being a genetically controlled process came from studies in *Caenorhabditis elegans* in the late 1980s and early 1990s (Ellis and Horvitz, 1986; Hengartner et al., 1992; Miura et al., 1993; Yan and Shi, 2005). The simple pathway involving only 4 genes in *C. elegans* is conserved among animals and has since been characterized in a number of insects and vertebrates, including mammals (Putcha and Johnson, 2004). The complexity of the pathway and the players involved does differ from one system to another.

The Core Apoptosis Pathway

The main executioners of apoptosis are cysteinyl, aspartate-specific proteases called caspases (Hengartner, 2000; Thornberry and Lazebnik, 1998). They are present in cells as inactive zymogens and become active once they are cleaved and/or associated with an adapter protein (Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004). Caspases contain a key cysteine in their active site and cleave substrates after an aspartic acid in a specific cleavage site determined by four amino acids (Nicholson, 1999; Shi, 2002). In *Drosophila*, cleavage takes place after a glutamate residue in some cases (Hawkins et al., 2000). All caspases have a prodomain at their N terminus followed by a large (P20) subunit and a small (P10) subunit (Hengartner, 2000; Riedl and Shi, 2004). Cleavage first occurs between the large and small subunits at an aspartic acid residue, and later the prodomain is removed, resulting in the active form of the caspase (Bratton and Cohen, 2001; Riedl and Shi, 2004). There are two types of caspases: upstream initiator caspases that are characterized by a long prodomain and downstream effector caspases which are characterized by a short prodomain (Shi, 2002).

Initiator caspases are the first to be activated after apoptotic stimuli. Their long prodomain is important for protein interactions, which are facilitated by specific protein-protein

interaction domains. Two examples of such domains are the caspase activation recruitment domain (CARD) or the death effector domain (DED) (Earnshaw et al., 1999; Fuentes-Prior and Salvesen, 2004; Ho and Hawkins, 2005; Park et al., 2007; Weber and Vincenz, 2001). After initial P20/P10 cleavage, initiator caspases form heterotetramers, made up of 2 P20/P10 heterodimers (Earnshaw et al., 1999; Thornberry and Lazebnik, 1998). Initiator caspases are normally auto-activated with the help of adaptor proteins or oligomerizing factors (Bao and Shi, 2007). The true mechanism of activation for initiator caspases is not completely understood. Cleavage is not necessarily required for catalytic activity (Stennicke and Salvesen, 1999). Activation has been proposed to occur once initiator caspases are within close proximity of each other or once they are recruited by adaptor proteins and associated in oligomeric complexes (Boatright et al., 2003; Boatright and Salvesen, 2003; Degterev et al., 2003; Ho and Hawkins, 2005; Shi, 2004). Once activated, initiator caspases will cleave and activate downstream effector caspases (Boatright and Salvesen, 2003; Raff, 1998).

Effector caspases are activated by cleavage at an aspartate residue between their large and small prodomain subunits. They reside as homodimers in the active or inactive form (Chai et al., 2001; Riedl et al., 2001). They must be cleaved by initiator caspases to become active. In their active form their substrates include key cellular factors such as DNA repair enzymes, nuclease inhibitors, chromatin modifying enzymes and structural proteins such as actin and laminin. They may also cleave other pro-apoptotic factors and caspases (Earnshaw et al., 1999; Enari et al., 1998; Fischer et al., 2003; Liu et al., 1997; Yokoyama et al., 2000).

The caspase cascade is regulated by inhibitor of apoptosis (IAP) proteins (Hengartner, 2000). IAP proteins were first discovered in baculoviruses (Birnbaum et al., 1994; Crook et al., 1993). IAP proteins have since been identified in a number of organisms and viruses (Clarke

and Clem, 2003b; Verhagen et al., 2001). They have the crucial role of keeping caspase activity in check. IAP proteins are characterized by N-terminal baculovirus IAP repeat (BIR) domains (Hinds et al., 1999; Miller, 1999). IAP proteins contain 1 to 3 BIR domains which are important for binding to and inhibiting caspases or other proteins (Uren et al., 1998). IAP binding of caspases inhibits substrate cleavage and/or targets them for degradation (Deveraux et al., 1999; Huang et al., 2001; Tenev et al., 2005). However, not all proteins that contain a BIR domain inhibit apoptosis (Richter and Duckett, 2000; Silke and Vaux, 2001). Some IAPs also contain a C-terminal RING finger domain (Yang et al., 2000). The RING domain has E3 ubiquitin ligase activity, allowing it to ubiquitinate itself or its target. This may tag either for proteasome degradation, but ubiquitination does not always lead to degradation; it may also simply modify the location or the activity of a protein (Hicke, 2001; Joazeiro and Weissman, 2000; Vaux and Silke, 2005).

There are upstream regulators of IAP inhibition called IAP antagonists. These are pro-apoptotic proteins that bind to and inhibit IAP proteins by binding to certain BIR domains (Chai et al., 2000; Liu et al., 2000; Silke et al., 2000; Vucic et al., 1998; Wang et al., 1999; Wu et al., 2000; Wu et al., 2001). They are transcriptionally activated by apoptotic stimuli such as stress, developmental signals or steroid hormones (Steller, 2008). They were first discovered in *Drosophila*, when a locus containing 3 closely linked IAP antagonists was deleted and almost all embryonic death was prevented (Grether et al., 1995; White et al., 1994). IAP antagonists possess an N-terminal IAP binding motif (IBM) (Chai et al., 2000; Liu et al., 2000; Silke et al., 2000; Wing et al., 2001). This motif will bind to certain BIR domains of IAP proteins and compete for caspase binding sites. This results in freeing caspases to cleave key apoptotic substrates (Chai et al., 2000; Liu et al., 2000; Silke et al., 2000; Vucic et al., 1998; Wang et al.,

1999; Wu et al., 2000; Wu et al., 2001). IAP antagonists may also induce degradation of IAP by stimulating auto-ubiquitination or they can decrease IAP levels by general post-translational shutdown (Colon-Ramos et al., 2006; Goyal et al., 2000; Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002).

Drosophila Apoptosis

Most of what we know about apoptosis in insects comes from studies involving genetic and biochemical characterization in *Drosophila melanogaster* (Fig. 1.2). In the *Drosophila* genome there are 3 initiator caspases identified by their long prodomain: Dronc, Dredd, and Dream/Strica and four effector caspases with a short prodomain: Drice, Dcp-1, Decay, and Damm (Hay and Guo, 2006; Riedl and Shi, 2004). Dronc is the main apoptotic initiator caspase in *Drosophila*, as it is involved in most apoptotic processes at embryonic, developmental and adult stages (Chew et al., 2004; Daish et al., 2004; Huh et al., 2004; Waldhuber et al., 2005; Xu et al., 2005). Dronc is the only *Drosophila* initiator caspase possessing a CARD domain. This CARD domain is important for recruitment by the adaptor protein Dark. Once Dronc is recruited by Dark, autoprocessing and activation ensue (Muro et al., 2004; Yan et al., 2006).

Upon activation, Dronc will cleave Drice, Dcp-1, DIAP1, and itself. Dronc can cleave at Glu or Asp residues. Dronc cleaves itself at Glu352 resulting in a catalytically active dimer (Hawkins et al., 2000; Muro et al., 2004). This initial cleavage is important for stabilizing the resulting active dimer through apoptosome formation. A subsequent self-cleavage takes place at Glu143. This removes the CARD domain and allows Dronc to be released from the apoptosome (Dorstyn and Kumar, 2008; Snipas et al., 2008; Yan et al., 2006). Dronc also cleaves DIAP1 at a Glu residue, located between its BIR1 and BIR2 domain. This cleavage occurs in normal and

apoptotic cells and is required for efficient inhibition of apoptosis (Muro et al., 2005; Yan et al., 2004).

Dronc cleaves and activates Drice, which in turn can cleave Dronc at Asp135 for further activation of the caspase cascade (Dorstyn and Kumar, 2008; Muro et al., 2005). Drice and Dcp-1 are highly homologous, yet Drice has a more essential role in apoptosis as an effector caspase. Dcp-1 mutants have few defects in apoptosis, whereas Drice mutants have severe defects. Silencing Drice in S2 cells inhibits cell death when exposed to multiple apoptotic stimuli but silencing Dcp-1 has little effect (Muro et al., 2006; Xu et al., 2006).

DIAP1 is the crucial inhibitor of cell death in *D. melanogaster*. Loss of DIAP1 in S2 cells or *Drosophila* embryos results in widespread apoptosis (Hay and Guo, 2006). DIAP1 negatively regulates initiator and effector caspases by binding and/or tagging them for degradation. It has two BIR domains and a RING domain. The BIR2 domain physically interacts with Dronc by binding 12 residues between its CARD and large subunit. The DIAP1 RING domain promotes ubiquitination and degradation of Dronc (Chai et al., 2003). BIR1 interacts with effector caspases, Drice and Dcp-1, through the IBM, which is generated by P20 cleavage (Tenev et al., 2005; Yan et al., 2004). Prior cleavage by Drice is required for binding and inhibition by DIAP1. The N-end rule degradation also requires this cleavage, allowing for proteasome degradation of DIAP1 and its bound substrate (Ditzel et al., 2003). Physical binding of caspases is not efficient enough to inhibit all the caspases present within a cell. This is why the DIAP1 RING domain is important for caspase degradation or interference by ubiquitination (Chai et al., 2003; Wilson et al., 2002). Therefore, the abilities of DIAP1 to sterically inhibit and polyubiquitinate caspases are both important for inhibition of apoptosis.

IAP antagonists in *Drosophila* include Reaper, Hid, Grim, Sickle, and Jafrac-2 (Claveria et al., 2002; Grether et al., 1995; Tenev et al., 2002; White et al., 1996). As previously mentioned, Reaper, Hid, and Grim (RHG) are closely linked and were discovered when a large deletion, H99, resulted in almost no apoptosis (Grether et al., 1995; White et al., 1994). Ectopic expression of RHG proteins induces apoptosis in *Drosophila* or S2 cells and induced activity can be blocked by the baculovirus caspase inhibitor P35, showing it is caspase-dependent cell death occurring (Chen et al., 1996; Grether et al., 1995). These IAP antagonists have a highly conserved N-terminal IBM which binds to BIR1 and BIR2 of DIAP1. They compete for these binding sites to free caspases and allow for activation (Yan et al., 2004). They have varying affinities for BIR1 or BIR2. Reaper and Grim bind BIR1 or BIR2, while Hid, Sickle and Jafrac-2 have higher affinity for BIR2 (Zachariou et al., 2003). IBM/BIR interaction requires exposure of an Ala residue at the N terminus of IAP antagonist proteins. IBM exposure occurs after cleavage of the signal peptide in the case of the ER protein Jafrac-2 and after methionine aminopeptidase-cleavage of Reaper, Hid, and Grim (Bergmann et al., 2003; Tenev et al., 2002). IAP antagonists can stimulate auto-ubiquitination of DIAP1 via its RING domain or ubiquitination by other E3 ligases (Chai et al., 2003; Yoo et al., 2002). Reaper and Grim can also inhibit cellular translation to promote apoptosis (Colon-Ramos et al., 2006). The half-life of Dronc is longer than that of DIAP1 allowing for Dronc to be free and induce apoptosis (Holley et al., 2002; Yoo et al., 2002). Reaper, Hid, and Grim all contain a GH3 domain which is important for mitochondria localization. This mitochondria localization results in mitochondrial fragmentation and is able to stimulate cell death on its own without the IBM (Claveria et al., 2002; Zhou et al., 2005b).

Mosquito Apoptosis

Characterization of apoptosis in mosquitoes is fairly recent and is still a work in progress. The pathway is very similar to what is known in *Drosophila* (Fig. 1.2). Conserved apoptotic proteins have been identified within the genomes of *An. gambiae* and *Ae. aegypti* and *Culex quinquefasciatus* (Beck et al., 2007; Bryant et al., 2008; Liu et al., 2011; Waterhouse et al., 2007; Zhou et al., 2005a). Characterization of some of the main proteins has been performed, with more in depth analysis of players in *Ae. aegypti* (Bryant et al., 2008; Liu and Clem, 2011; Wang et al., 2008; Wang and Clem, 2011; Zhou et al., 2005a).

In *Ae. aegypti*, initiator caspase homologs of Dronc and Dredd were identified and are referred to as AeDronc and AeDredd. AeDronc contains a CARD domain and AeDredd contains two DED domains. Homologs of Decay and Damm have also been identified but are yet to be functionally characterized (Bryant et al., 2008). AeDronc seems to be the main initiator caspase involved in apoptosis in *Ae. aegypti*. It is presumed to be recruited by adaptor protein AeArk. AeArk, a homolog of Dark, contains a CARD, nucleotide binding adaptor (NB-ARC), and WD-40 domain(s). The main effector caspases identified in *Ae. aegypti* are CASPS7 and CASPS8 which are closely related to Drice and Dcp-1. Both of these effector caspases can be activated by AeDronc and have high activity (Bryant et al., 2008; Liu and Clem, 2011). They seem to play equally important roles in apoptosis as compared to *Drosophila*, where the main effector caspase is Drice (Muro et al., 2006; Xu et al., 2006). Predicted effector caspases, Ags7 and Ags8, in *An. gambiae* have not been well studied or characterized at this point (Cooper et al., 2009).

IAP1 homologs have been identified in *Ae. aegypti, Ae. albopictus,* and *Ae. triseriatus* (Beck et al., 2007; Blitvich et al., 2002; Li et al., 2007). *Ae. albopictus* IAP1 was shown to protect against blue tongue virus infection in mammalian cells. This inhibitor also protected SF9 cells from apoptosis when Hid was expressed (Li et al., 2007). AeIAP1 in *Ae. aegypti* was

upregulated in response to apoptotic and stress stimuli such as UV or heat shock treatments (Pridgeon et al., 2008a). Silencing of AeIAP1 in mosquito cells leads to spontaneous apoptosis, indicating it is the main inhibitor of apoptosis in mosquito cells (Liu and Clem, 2011). Knockdown of AeIAP1 by intrathoracic dsRNA injection resulted in compromised morphology in the midgut and death in over half the mosquitoes by 48hpi (Wang et al., 2012). Mortality also occurred when dsRNA targeting AeIAP1 was placed on the thorax of female mosquitoes (Pridgeon et al., 2008b).

IAP antagonists have also been identified in mosquitoes. Michelob_X (Mx) was the first insect IAP antagonist identified outside of *Drosophila*. It has been identified as an ortholog of Reaper in *Ae. aegypti*, *Ae. albopictus, C. quinquefasciatus*, and *An. gambiae* genomes. Mx has been characterized in the *Drosophila* background, where it was able to antagonize DIAP1. It has also been expressed in *Culex* spp. and *Aedes* spp. backgrounds, where it was able to induce apoptosis (Liu et al., 2011; Zhou et al., 2005a). A second IAP antagonist was identified in *Ae. aegypti* and was named IMP (IAP-antagonist Michelob_X-like protein). IMP was identified in the genome by searching for Mx homology (Bryant et al., 2008). Both Mx and IMP contain IBM domains. When the IBM domain was removed from Mx or IMP, their pro-apoptotic activity was lost. Expression of IMP and Mx in *Ae. albopictus* cells results in apoptosis, whereas, silencing IMP and Mx protects cells from apoptotic stimuli (Bryant et al., 2008; Wang et al., 2008; Wang and Clem, 2011).

There are gene duplications that have occurred with several caspase genes in the mosquito. Additional roles in apoptosis and immune function are yet to be characterized and applied to mosquito research. So far, there is an exciting and invaluable start to understanding

the mosquito apoptosis pathway. The following section will discuss apoptosis in more detail as an innate immune response in mosquitoes.

Insect Innate Immunity and Antiviral Defenses

Insects are susceptible to many different pathogens throughout development and as adults. Some of these pathogens are harmful to insects with important ecological or industrial roles such as honey bees or silkworms. Other pathogens may not be harmful to the insect but instead cause devastating human or animal diseases that are transmitted by insect vectors. In this regard, understanding the immune responses in insects is an important task (Rolff and Reynolds, 2009). Immunity may be innate or acquired. Insects are most recognized for innate immune responses. Acquired immune responses in insects are yet to be found or well characterized (Beckage, 2008). Immune responses have been studied in depth, at the molecular and genetic levels, in *Drosophila melanogaster* (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). Most of what is known about insect immunity is based on these studies and is starting to be extended to other insects, including important mosquito vectors (Beckage, 2008; Rolff and Reynolds, 2009). This section will mainly focus on insect innate immune pathways linked to bacterial, fungal, and viral infection in *Drosophila* and mosquito species (Fig. 1.3).

Innate Immune Responses to Bacteria and Fungi

The first step of an immune response is to recognize the presence of a pathogen. In insects, immune responses are regulated on a large scale by pathogen recognition receptors. These receptors sense microbes based on essential pathogen-associated molecular patterns, such as lipopolysaccharide or teichoic acids in bacteria (Broderick et al., 2009; Das et al., 2009). Once bacterial or fungal intruders are recognized, antimicrobial peptides (AMPs) are produced to defend against them. In insects a lot depends on the fact that they have an open circulatory

system and the small volume of hemolymph available for active levels of AMPs to be reached (Broderick et al., 2009).

There are 20 AMPs from 7 different classes identified in *Drosophila*. They are usually expressed only when infection is present. AMPs are very small, usually <10 kDa and they defensively act on microbial membranes. They have different specificities as to what type of pathogens they target (Broderick et al., 2009). Attacin, Drosocin, and Diptericin will act on gram negative bacteria, whereas Defensin targets gram positive bacteria (Asling et al., 1995; Bulet et al., 1993; Dimarcq et al., 1994; Wicker et al., 1990). Fungi are targeted by Drosomycin and Metchnikowin (Fehlbaum et al., 1994; Levashina et al., 1995). Cecropin A1 acts on both bacteria and fungi (Ekengren and Hultmark, 1999). *Drosophila* also encodes 13 different lysozymes; however, they are not involved in systemic immune response but instead are involved in digestion (Hultmark, 1996).

Toll and IMD pathways regulate the expression of AMPs (Lemaitre et al., 1995). The Toll pathway is induced by gram positive bacterial and fungal infection (Lemaitre et al., 1996; Rutschmann et al., 2002; Tauszig-Delamasure et al., 2002). Infection with gram positive bacteria or fungi activates clip-domain serine proteases, which cleave the secreted cytokine, Spaetzle. Cleaved Spaetzle then binds to the transmembrane Toll receptor. Once bound, Toll will dimerize and intracellularly recruit adaptor proteins containing death domains (Hu et al., 2004; Weber et al., 2003). These adaptor proteins activate Pelle which leads to Cactus degradation. Cactus is an inhibitor of Relish proteins, Dif and Dorsal. Now active, Dif and Dorsal will then translocate to the nucleus where they induce the expression of AMPs by binding to κB motifs on DNA promoters (Fig. 1.3) (Busse et al., 2007; Engstrom et al., 1993; Ip et al., 1993; Kappler et al., 1993).

The IMD pathway is activated by gram negative bacterial infection (Lemaitre et al., 1995). Activation is facilitated by the pathogen recognition receptor, PGRP-LC (peptidoglycan recognition protein), which is expressed by the fat body cells (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002). Upon recognition of gram negative bacteria, PGRP-LC recruits cellular IMD which interacts with dFADD (Leulier et al., 2000; Leulier et al., 2002). dFADD recruits Dredd which associates with Relish (Hedengren et al., 1999; Stoven et al., 2003). Relish is cleaved and its inhibitory domains remain in the cytosol while its transactivator domain goes to the nucleus. There it induces expression of AMPs to target gram negative bacteria (Fig. 1.3) (Lu et al., 2001; Rutschmann et al., 2000; Silverman et al., 2000). In addition to systemic immune response, the IMD pathway is also associated with local immune responses elicited by epithelia cells (Zaidman-Remy et al., 2006). It is also involved in nitric oxide immune signaling between the gut cells and fat body cells (Dijkers and O'Farrell, 2007; Foley and O'Farrell, 2004).

Toll and IMD are activated individually but they can act synergistically. Infection usually activates both pathways to some extent. The levels of activation depend on the type of pathogen (Lemaitre et al., 1997). There seems to be cross talk between the two, likely due to the fact that they both rely on NF-κB transcription factors (Busse et al., 2007; De Gregorio et al., 2002; Tanji et al., 2007). Both are involved in response to septic injury. Mutations affecting Toll and IMD pathways often lead to death associated with excessive bacterial and/or fungal proliferation (Broderick et al., 2009; De Gregorio et al., 2001; Irving et al., 2001).

Anti-viral Immune Responses

Anti-viral defenses have been studied in *D. melanogaster* as well. They are still being dissected and characterized but have opened up the doors for looking at anti-viral applications in mosquito vectors. There are over 25 known viruses that infect *D. melanogaster* (Huszar and

Imler, 2008). Defenses against viral infection include an inducible response, which involves the induction of many genes to counter infection; RNA interference, which targets and degrades viral RNA; and apoptosis, which induces cell death to inhibit virus replication and protect other cells from virus infection (Imler and Eleftherianos, 2009).

Receptor Induced Responses

Toll and IMD pathways in *Drosophila* are not induced by Drosophila C Virus (DCV) or Flock House Virus (FHV) infection (Dostert et al., 2005; Go et al., 2006; Sabatier et al., 2003). Drosophila X Virus (DXV) does induce some AMPs to be expressed to similar levels as microbial infections. Knockout of Rel does not sensitize cells to infection with DXV. Dif knockouts are sensitized, but seem to regulate DXV infection in a Toll-independent manner, as loss of upstream factors does not have the same effect (Zambon et al., 2005).

After a genome-wide screen to look at *Drosophila* genes differentially regulated by virus infection, 140 were found to be up-regulated by at least 2-fold and two-thirds of these were not up-regulated by bacterial or fungal infection. The study of one these genes, *virus induced RNA-1* (*vir-1*), led to identification of an immune pathway specific to viral infection. *vir-1* was induced by DCV and FHV but not by bacteria or fungi (Dostert et al., 2005; Hedges and Johnson, 2008). It contained a signal transduction and activators of transcription (STAT)-binding site in its promoter and was found to be induced by the transcription factor, STAT92E. This suggested the involvement of the Janus Kinase (JAK)/STAT pathway in *vir-1* expression (Dostert et al., 2005). In *Drosophila*, JAK kinase is encoded by the *hopscotch* gene and STAT is encoded by *marelle* (Agaisse et al., 2003; Dostert et al., 2005). The pathway is regulated by cytokine receptor, Domeless. Domeless regulates JAK kinase which results in the phosphorylation of STAT. Once phosphorylated, STAT undergoes nuclear transport and induces *vir-1* expression (Fig. 1.3).

Hopscotch mutant flies have been found to have higher viral load. This result could possibly link other genes induced by virus infection and dependence on *hopscotch* to anti-viral defense (Dostert et al., 2005; Imler and Eleftherianos, 2009). How *vir-1* and other potential inducible anti-viral factors protect against virus infection is not yet clear. Anti-viral molecules may interfere with viral replication or target virions. JAK/STAT is not as well characterized as Toll and IMD signaling. Prospectively, it is more complex and probably involves other pathways that are not fully understood or characterized to protect against virus infection (Imler and Eleftherianos, 2009).

RNA Interference

RNA interference (RNAi) has proved to be an important anti-viral defense since its discovery in the 1990s. It was first shown to protect against viral infection in plants and has since been shown to be important in multiple organisms, including *Drosophila* and mosquitoes (Blair, 2011; Fire et al., 1998; Kemp and Imler, 2009; Noad et al., 1997; Ratcliff et al., 1997). RNAi acts as a specific defense reaction, involving base pairing of small RNAs and invading nucleotides. Small RNAs include microRNAs (miRNA), small interfering RNAs (siRNA), and Piwi associated RNAs (piRNA) (Imler and Eleftherianos, 2009).

miRNAs are produced in the nucleus and exit into the cytosol as pre-miRNAs after nuclear processing by RNAse III, Drosha. In the cytosol they are processed by a second RNAse III, Dicer-1, to produce miRNA (Lee et al., 2004). miRNA duplexes are disrupted by the dsRNA binding protein R3D1 and are then incorporated into a complex called miRISC (RNA interference silencing complex). This complex also contains the catalytic protein, Argonaute-1 (AGO-1). miRISC is guided by miRNA to complementary sequences (Jiang et al., 2005;

Okamura et al., 2004). Binding of these sequences either inhibits translation or cleaves the mRNA (Brodersen et al., 2008).

siRNAs are produced from dsRNA molecules which can either be viral or endogenous. Many viruses have double-stranded RNA genomes or produce dsRNA intermediates during replication (Huszar and Imler, 2008; Imler and Eleftherianos, 2009). dsRNA molecules are recognized by Dicer-2 and cleaved into 21-22 bp fragments to produce siRNAs. siRNA then incorporates into siRISC complex which contains argonaute-2 (AGO-2) and requires R2D2 for exogenous RNA or R3D1 for endogenous RNA. siRISC targets complementary single strand RNA for cleavage by AGO-2 (Lee et al., 2004; Obbard and Finnegan, 2008).

piRNAs are involved in heterochromatin maintenance. They are important for control of transposons and endogenous retroviruses. piRNAs are produced from the *flamenco* locus by original amplification by Piwi, Aubergine, and AGO-3. They are usually 24-30 nucleotides in length. piRNAs associate with Piwi proteins, which are from the Argonaute family of proteins, and guide them to transposon and endogenous retrovirus sequences in order to silence them (Girard and Hannon, 2007).

These small RNAs have been shown to be important in *Drosophila* antiviral defense. Dicer-2, AGO-2, and R2D2 mutants are more susceptible infection of RNA viruses such as Sindbis virus, FHV, DCV, and Cricket paralysis virus (CrPV) (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). Also, siRNAs corresponding to viral sequences have been detected in infected flies (Galiana-Arnoux et al., 2006; Imler and Eleftherianos, 2009). In addition, *piwi* has been shown to be involved in controlling West Nile Virus (WNV) and DXV viral load in *Drosophila* (Chotkowski et al., 2008; Zambon et al., 2006).

RNAi is an efficient and important anti-viral defense. In response, viruses have developed ways to successfully replicate and suppress RNAi. Viral suppressors of RNAi (VSR) genes have been identified in plant and insect viruses (Ding and Voinnet, 2007). Plant viruses have been shown to express dsRNA binding proteins that prevent RISC interaction or proteins that prevent RISC assembly, interfere with dsRNA cleavage, or promote ubiquitin dependent degradation of RNAi factors (Imler and Eleftherianos, 2009). In *Drosophila*, FHV encodes a VSR gene called B2. B2 is synthesized in infected cells and binds to both long and short dsRNAs (17 bp and up). This sequence-independent binding prevents Dicer interactions or association with other RNAi components. When B2 is deleted from the virus, it is no longer virulent (Chao et al., 2005). DCV also encodes a protein with a dsRNA binding domain, DCV-1a. DCV-1a binds long dsRNAs, preventing Dicer from processing them (van Rij et al., 2006). A VSR gene has also been identified in the CrPV genome (Wang et al., 2006). Flies infected with Sindbis virus expressing this CrPV VSR exhibited increased infection and mortality. However, when flies were infected with Sindbis expressing DCV-1a, only a modest increase in viral load and pathogenicity was observed (Nayak et al., 2010). Thus these responses and defenses are virus-specific.

Mosquito Immune Responses

Mosquito immune responses have been the target of numerous vector biology studies in order to better understand pathogen-host interactions and give some insight into vector-borne pathogen success and transmission. Most work has looked at virus or parasite interactions with mosquito vectors. These pathogens not only must evade mosquito immune responses, they also must survive in vertebrate hosts and reach high enough titers to be imbibed during a blood meal.

Studies in *Drosophila* and genome sequencing of mosquito vectors have opened the door to the molecular and genetic investigation of these responses.

Anti-viral pathways

Mosquitoes have Dorsal and Rel orthologs but lack Dif-related proteins (Meister et al., 2005; Shin et al., 2002; Shin et al., 2005). STAT orthologs have been found in *An. gambiae* and *Ae. albopictus* and AMPs have been identified in *Ae. aegypti* and *An. gambiae* (Barillas-Mury et al., 1999; Lin et al., 2004; Lowenberger, 2001). Orthologs of Defensin and Cecropin were expressed after immune activation in *Ae. aegypti* and *An. gambiae* (Lowenberger, 2001). Sindbis infections in *Ae. aegypti* resulted in early activation of the Toll pathway, which was later down-regulated as virus titers increased. JNK signaling, thought to be mediated by the IMD pathway, was observed at later stages of Sindbis infection (Sanders et al., 2005). Dengue infection induced AMP expression as well as factors from Toll and JAK/STAT pathways. A genome wide transcription analysis in response to dengue infection in *Ae. aegypti* showed upregulation of Toll genes and 4 JAK/STAT genes. Suppression of Toll proteins resulted in a 2-3 fold increase in dengue infection (Xi et al., 2008). Knockdown of *domeless* and *hopscotch* transcripts also resulted in increased dengue load. In addition, two new AMPs with STAT binding sites and dengue restriction factors were identified (Souza-Neto et al., 2009). STAT DNA binding activity was also seen in *Ae. albopictus* C6/36 cells during Japanese Encephalitis Virus (JEV) infection. In C6/36 cells, lipopolysaccharide induces the phosphorylation of STAT and infection with JEV inhibits STAT activity by blocking this phosphorylation (Lin et al., 2004). It is thought to do so by expression of an NS5 protein, which has been shown to prevent STAT and TYK-2 phosphorylation in vertebrate cells (Lin et al., 2006).

Other mosquito anti-viral proteins have been identified in screens looking at genome-wide expression levels during infection. During Sindbis infection in *Ae. aegypti* an Unc93b ortholog was found to be upregulated (Sanders et al., 2005). Unc93b is involved in Toll-like receptor-mediated antiviral defense in mammals (Brinkmann et al., 2007). A heat shock-related protein, 70B, was shown to have anti-viral activity in o'nyong-nyong virus (ONNV)-infected *An. gambiae*. Silencing of this gene resulted in reduced life span of infected *An. gambiae* (Kang et al., 2008; Sim et al., 2007). These studies are just a start to the investigation of innate immune signaling pathways in mosquitoes. Pathogen recognition receptors, pathogen activated molecular patterns and antiviral effector mechanisms are not well known or understood in mosquitoes.

RNA interference

RNAi is thought to be a very important antiviral defense in mosquitoes. Dcr-2, R2D2, and AGO-2 homologs have been identified in *An. gambiae, C. quinquefasciatus*, and *Ae. aegypti*. Silencing of these factors has shown that they are important for limiting flavivirus and alphavirus replication and spread within the mosquito (Blair, 2011). Knockdown of Dcr-2 in *Ae. aegypti* resulted in a 10-fold increase in viral load at 10 dpi. It also reduced the extrinsic incubation period (EIP) to 7 days instead of 10-12 (Sanchez-Vargas et al., 2009). Knockdown of Piwi protein AGO-3 in *An. gambiae* resulted in increased infection of ONNV (Keene et al., 2004). The RISC protein TSN is up-regulated during Sindbis infection and has been shown to be important for limiting Sindbis dissemination in *Ae. aegypti* (Campbell et al., 2008; Sanders et al., 2005).

Interestingly, detection of small RNAs produced from viral genomes (viRNA) in flavivirus-infected mosquitoes or cells yield very low numbers. Only .01-.05% of small RNAs were dengue viRNAs in infected Aag2 cells and *Ae. aegypti* mosquitoes (Scott et al., 2010).

Numbers were similar in WNV-infected *C. quinquefasciatus* mosquitoes, with .05% of the small RNAs being viRNAs (Brackney et al., 2009). Alphavirus infection had higher yields, with 10% viRNA in Sindbis infected *Ae. aegypti*, 1.2% viRNA in ONNV-infected *An. gambiae* and 2.1% viRNA in SFV-infected *Ae. albopictus* U4.4 cells (Myles et al., 2009; Myles et al., 2008; Siu et al., 2011). These differences may be due to different hosts being infected as well as the different virus types. WNV and dengue may evade RNAi responses more efficiently, as they are thought to replicate in double membrane vesicles in vertebrate and insect cells (Poole-Smith, 2010; Uchil and Satchidanandam, 2003; Welsch et al., 2009). These membranes would likely protect them from being targeted by RISC (Geiss et al., 2005). Also of interest is defective Dcr-2 activity in C6/36 cells (Brackney et al., 2010). Virus is propagated to higher titers in these cells compared to other *Aedes* spp. cell lines (Igarashi, 1978). This is likely due to the fact that their RNAi machinery is defective and enforces the importance of RNAi as a defense for viral infection.

To date, no VSRs have been found in arboviruses. Experiments have been done with expression of VSRs during infection in mosquito cells. Increased spread of SFV in U4.4 cells was seen when tombusvirus p19, a dsRNA binding protein, was expressed (Attarzadeh-Yazdi et al., 2009). Sindbis expressing FHV B2 protein exhibited enhanced virus replication and spread and also caused decreased mosquito survival. Based on this study, a probable reason VSRs have not been found in arboviruses is because they would increase virus success to a point that would negatively affect the fitness of the vector, which is not optimal for further transmission (Cirimotich et al., 2009; Myles et al., 2008).

In the studies done to date, RNAi does not seem to eliminate virus from insect vectors but simply keeps virus replication in check. However, if RNAi is induced prior to or early on during infection it may be used as a mechanism to prevent efficient virus replication or

disseminating required for transmission (Blair, 2011). Prior expression of dengue dsRNA fragments in *Ae. aegypti* mosquitoes was able to inhibit infection and replication later on (Adelman et al., 2001; Olson et al., 1996). Transgenic *Ae. aegypti* have been produced that express inverted repeat RNA derived from dengue using a midgut specific promoter. These mosquitoes expressed dsRNA targeting dengue after a blood meal. This mechanism kept virus from spreading and reaching the salivary glands (Franz et al., 2006). RNAi-impaired transgenic *Ae. aegypti* had higher virus infection at 7 dpi in the midgut and earlier, and also exhibited increased disseminated infection in the mosquito (Khoo et al., 2010). This further reinforced the idea that RNAi is an important midgut defense against virus infection and escape.

Apoptosis

Apoptosis is a type of programmed cell death that is an important process in development and homeostasis, but it is also an innate response to virus infection and can limit replication and spread of virus (Roulston et al., 1999). Apoptosis is often induced in infected cells by stimuli such as viral genetic material, late viral gene expression, or impediment of host transcription and translation. Depending on the virus, apoptosis can be either helpful or harmful to virus replication and spread. Therefore, viruses have developed ways to induce or inhibit apoptosis to their advantage (Roulston et al., 1999). Apoptosis is often induced by arbovirus infection in mammalian cells, yet it is rarely seen in infected mosquito cell cultures, which usually develop a persistent infection compared to lytic infection in mammalian hosts (Karpf and Brown, 1998; Levine et al., 1993; Schlesinger, 1975; Stollar et al., 1975). There are some reports of apoptosis induced by alphavirus and flavivirus infection in mosquitoes (Girard et al., 2005; Girard et al., 2007; Kelly et al., 2012; Weaver et al., 1992; Weaver et al., 1988). However, studies in the actual mosquito vectors are limited.

Apoptosis as an insect antiviral defense and the ability of a virus to inhibit apoptosis in its host is best characterized in baculovirus and its host lepidopteran insect *Spodoptera frugiperda*. The story began when an *Autographa californica* M nucleopolyhedrovirus strain caused apoptosis and reduced virus production in *S. frugiperda* (SF-21) cells. This virus was missing a gene called *p35* (Clem et al., 1991; Friesen and Miller, 1987; Hershberger et al., 1992). The P35 protein was later shown to inhibit caspase activity in infected cells. P35 is cleaved and activated by cellular caspases. Upon cleavage, P35 undergoes a conformational change and then covalently binds to the caspase, inhibiting its activity (Clem, 2005). The ability of P35 to inhibit caspase activity and therefore apoptosis is important for virus success. *S. frugiperda* larvae infected with *p35* deficient AcMNPV exhibited apoptosis in fat body and epithelial cells. This correlated with reduced virus replication and spread compared to wild type AcMNPV (Clarke and Clem, 2003a). Decreased lethality was also observed when *S. frugiperda* larvae were infected with AcMNPV *p35* mutant virus compared to wild type AcMNPV, clearly indicating apoptosis to be an important antiviral defense (Clem and Miller, 1993; Clem and Miller, 1994).

Other anti-apoptotic baculovirus proteins have since been discovered in OpMNPV, CpGV, SlNPVand AcMNPV (Birnbaum et al., 1994; Crook et al., 1993; Du et al., 1999; Griffiths et al., 1999; Jabbour et al., 2002). Anti-apoptotic proteins expressed by other viruses have been identified as well. One example is the adenovirus anti-apoptotic protein EIB 19K, which is a Bcl-2 homolog. It interacts with the cellular pro-apoptotic Bcl-2 genes, Bax and Bak. Infection with adenovirus mutants lacking this gene results in increased apoptosis and impaired virus production in HeLa cells (Chiou et al., 1994; Cuconati et al., 2002; Cuconati and White, 2002). Shrimp Taura syndrome virus also encodes an IAP protein with a BIR domain that is thought to be used to interfere with host apoptosis responses (Mari et al., 2002).

As previously mentioned, arbovirus infection in mosquito cells usually does not induce apoptosis. No cell death or change in IAP1 levels was observed in LACV infected C6/36 cells (Blitvich et al., 2002; Borucki et al., 2002). However, this does not necessarily mean that apoptosis never occurs during arbovirus infection in insect vectors. It may occur at low levels or simply be hard to detect in insect hosts. On the other hand, it may, for some reason, not be induced by virus infection in insects or perhaps it is effectively inhibited by the virus in infected cells (Fragkoudis et al., 2009). Pro-apoptotic gene expression in C6/36 cells has been shown to induce apoptosis, whereas, apoptotic inhibitors protect against cell death. Increased apoptosis caused by expression of IAP antagonists did not affect the early production of Sindbis virus in these cells, although virus production was affected later on as cells died (Wang et al., 2008).

Ae. aegypti salivary glands exhibited pathological effects, such as, depleted cytoplasm, nuclear leakage and shrunken lobes, when infected with SFV (Mims et al., 1966). EEEV can have pathologic effects in the midgut of *C. melanura*, while Sindbis infection can have pathologic effects in midgut and salivary gland tissue of *Ae. albopictus* (Bowers et al., 2003; Weaver et al., 1988). WNV infection in *C. quinquefaciatus* results in increased apoptosis in salivary glands and has been shown to correlate with lower levels of transmission (Girard et al., 2005; Girard et al., 2007). Recent transcriptome analysis found a decrease in IAP expression in the salivary glands of WNV-infected *C. quinquefasciatus*, suggesting that apoptosis may be a potential defense against virus infection in this tissue (Girard et al., 2010). In addition, a strain of *C. quinquefasciatus* that is refractory to WNV infection also undergoes extensive cell death in midgut tissues (Vaidyanathan and Scott, 2006).

A recent study looking at pro-apoptotic gene expression in infected mosquitoes utilized a mosquito baculovirus, CuniNPV. *Ae. aegypti* larvae were used as a refractory host and *C.*

quinquefasciatus larvae as a susceptible host. Upregulation of the pro-apoptotic gene *michelob_X* (*mx*) was seen very early after infection in *Ae. aegypti*, while *mx* expression in *C. quinquefasciatus* was modest and delayed (Liu et al., 2011). Similar results were observed with dengue infected *Ae. aegypti* adult mosquitoes that were either refractory or susceptible to infection. The refractory strain had increased *mx* expression 3 hours after an infectious blood meal compared to blood-fed controls, whereas, susceptible *Ae. aegypti* had no difference in *mx* expression compared to noninfected controls (Liu et al., 2013). This suggests an importance for apoptosis in vector defense and ability of a virus to be transmitted. Another study looked at RNA silencing of the anti-apoptotic factor, *AeIAP1*, in *Ae. aegypti* to increase apoptosis in the host. This resulted in disrupted midgut morphology and lethality in the mosquitoes as well as increased infection and spread of Sindbis virus (Wang et al., 2012). These results indicate widespread apoptosis is detrimental to *Ae. aegypti* and virus infection and spread was likely facilitated by decreased fitness and compromised barriers within mosquitoes (Wang et al., 2012). Cross talk between apoptosis and the RNAi pathway has been indicated in *Drosophila*. RNAi was suppressed in cells adjacent to apoptotic cells (Xie et al., 2011). If this holds in mosquitoes, a decrease in an important host defense such as RNAi could also influence increased virus replication and spread.

Whether apoptosis positively or negatively affects virus success in insect hosts is likely very specific to virus-vector combinations. Apoptosis may be a threshold response or a modest host response to keep virus replication in check. Its effect on most arboviruses is not well understood or known in their insect hosts. Chapter Two will discuss findings linking the impacts of apoptosis in *Ae. aegypti* to Sindbis virus infection.

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Chapter 1 Figures

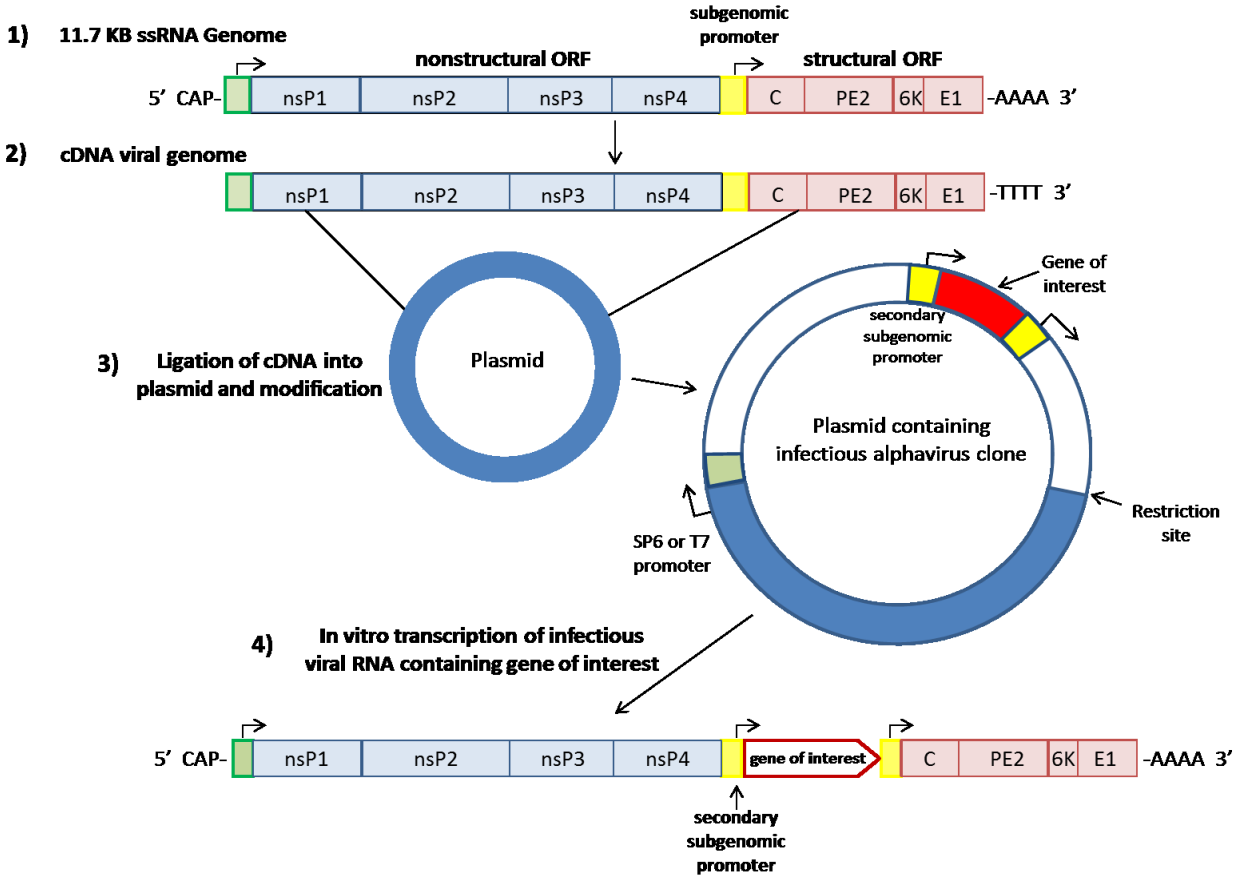

Figure 1.1 Construction of a 5' ds-Alphavirus Transducing System.

(Adapted from Strauss and Strauss, 1994 and Foy and Olson, 2008)

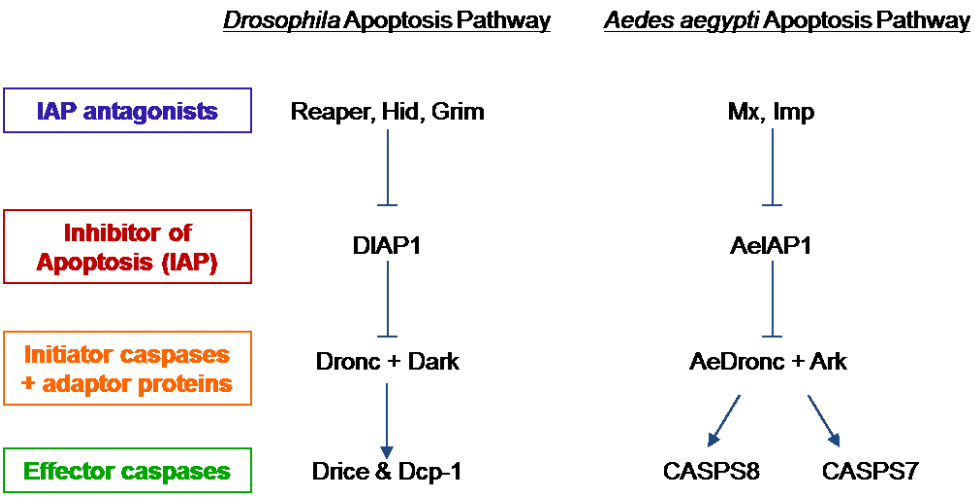

Figure 1.2 Apoptosis Pathways in *Drosophila* and *Ae. aegypti*
(Adapted from Liu et al., 2011)

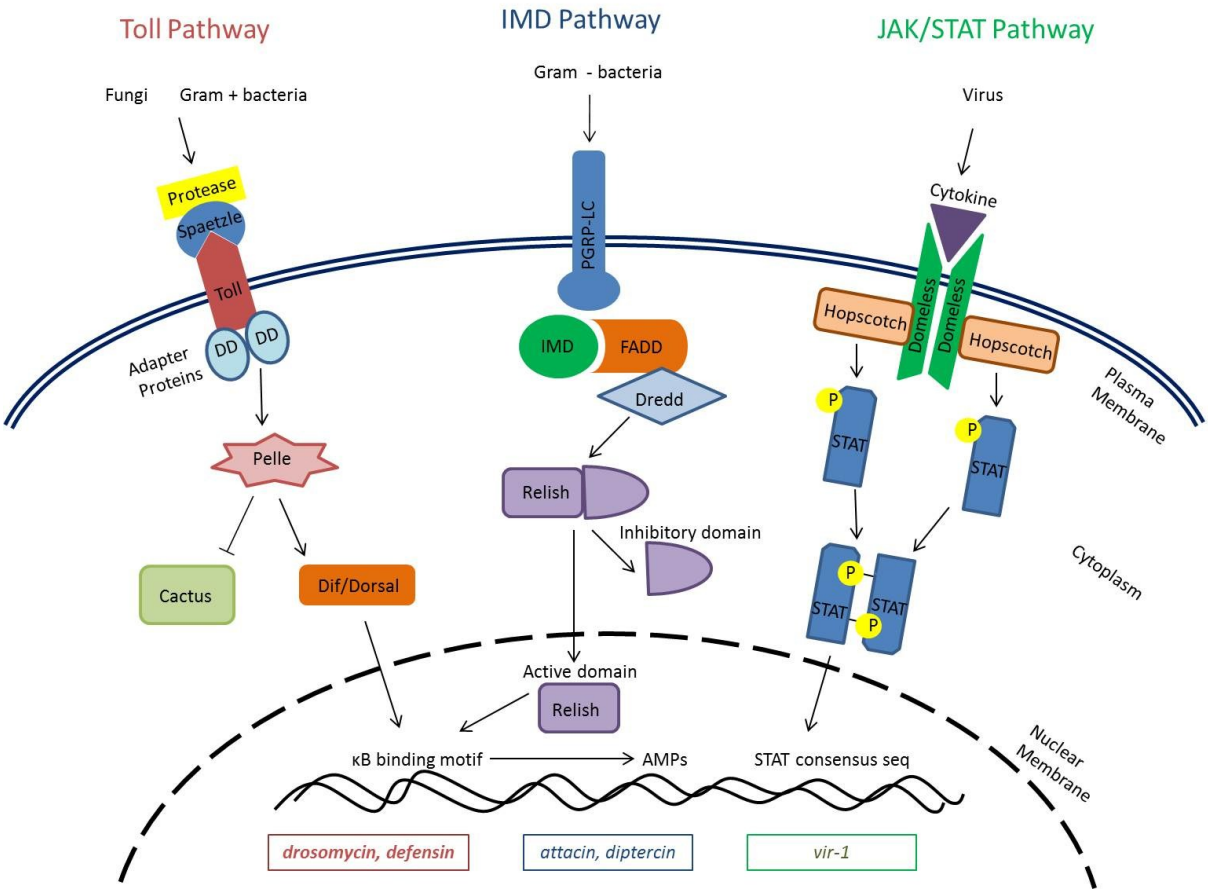

Figure 1.3 Insect Innate Immune Pathways

(Adapted from Broderick et al., 2009 and Imler and Elefinerianos, 2009)

Chapter 2 - Reaper expression negatively affects Sindbis virus infection and dissemination in the mosquito vector *Aedes aegypti*

Abstract

Although apoptosis is known to be a defense against various viruses in insects and mammals, the role of apoptosis in mosquito immunity against arboviruses is largely unexplored. Some studies have suggested a correlation between apoptosis and resistance to infection, but little direct evidence exists supporting a causal relationship. The mosquito *Aedes aegypti* is an important vector for yellow fever and dengue. Because of its ability to be engineered to express foreign genes, Sindbis virus (SINV; *Togaviridae*) was used to study the possible role of apoptosis in *Ae. aegypti* immunity against arboviruses. A series of infectious SINV clones based on the MRE-16 strain were used to express either the pro-apoptotic gene *reaper* from *Drosophila* (MRE/Rpr) or the anti-apoptotic baculovirus gene *p35* (MRE/P35). Control virus clones were used which contained noncoding inserts of similar or greater sizes. Adult female *Ae. aegypti* were orally infected with the recombinant SINV viruses, and midgut infection was analyzed by immunofluorescence assay (IFA). Viral replication was also monitored by titering the amount of infectious virus in individual mosquitoes. MRE/Rpr caused increased caspase activity and TUNEL staining in midguts compared to control viruses, indicating that apoptosis was stimulated by expression of Reaper. IFA and viral titer results indicated that infection with MRE/Rpr resulted in decreased rate and spread of virus infection in mosquitoes compared to control viruses. Infection with MRE/Rpr also decreased the lifespan of infected mosquitoes, even to a greater extent than the other viruses tested. Sequencing of individual plaque-purified viruses from infected mosquitoes revealed loss of the insert sequence in the majority of MRE/Rpr viruses sampled, beginning at early stages of infection. In contrast, the inserts remained intact in control viruses obtained from mosquitoes, suggesting that rapid negative selection occurred against pro-apoptotic gene expression during virus replication in the

mosquito. These results suggest that if apoptosis is induced in infected cells, it can play a role in defense against arbovirus infection in mosquitoes.

Introduction

The yellow fever mosquito, *Aedes aegypti*, is an important disease vector due to its ability to transmit a number of medically important arboviruses. *Ae. aegypti* is the main vector for yellow fever and dengue viruses, which together are responsible for ~50,000 deaths and 50-100 million infections worldwide per year (CDC, 2012a; CDC, 2012c). *Ae. aegypti* is also a vector for chikungunya virus, an emerging pathogen in Africa, Europe, and South Asia (CDC, 2012b; Ligon, 2006). *Ae. aegypti* has a wide global range, being found in tropic and sub-tropic regions around the world. This species primarily lives in close association with humans and is a diurnal feeder, rendering certain control methods, such as bed nets, ineffective (Diallo et al., 2003; Ponlawat and Harrington, 2005; Scott et al., 2000).

Once a virus is ingested by *Ae. aegypti* during an infectious blood meal there are a number of barriers and antiviral defenses the virus must bypass in order to be successfully transmitted to another host. The virus must first infect the midgut epithelial cells and then escape the midgut and infect other tissues in the mosquito. To be transmitted to another host, the virus must infect the salivary glands and be expelled in the saliva during a subsequent blood meal (Hardy et al., 1983; Mellor, 2000). In addition to these physical barriers, the virus must overcome innate immune defenses such as the Toll and JAK/STAT pathways and RNA interference (RNAi) (Campbell et al., 2008; Keene et al., 2004; Sanchez-Vargas et al., 2004; Sanders et al., 2005; Xi et al., 2008).

Another potential innate defense mechanism in mosquitoes is programmed cell death, or apoptosis. There are many examples where apoptosis has been shown to be a defense against viruses in other insects and in higher animals (Barber, 2001; Clarke and Clem, 2003). Apoptosis is often induced in infected cells due to the presence of viral proteins and /or stress on the cell

due to virus replication. Depending on the virus-host combination, apoptosis can be helpful or harmful to virus replication (reviewed in Roulston et al., 1999). When infected cells undergo apoptosis, virions may become packaged in apoptotic bodies, which in some cases has been suggested to protect virus from host immune factors and allow for undetected virus spread. Alternatively, suppression of apoptosis by viral factors can protect infected cells against early cell death and allow for complete virus replication and release to occur (Roulston et al., 1999).

The role of apoptosis in arbovirus-vector interactions is not clear. Arbovirus infection often leads to lytic infection in mammalian cells and persistent infection in mosquito cells. In cell culture, apoptosis is observed in infected mammalian cells but is not commonly seen in infected mosquito cells (Karpf and Brown, 1998; Levine et al., 1993; Nava et al., 1998; Schlesinger, 1975; Stollar et al., 1975). Dengue- or SINV-infected *A. albopictus* C6/36 cells have moderate, if any, cytopathic effects (Stollar et al., 1975; Wang et al., 2008; White, 1987). Although infection does not induce apoptosis in C6/36 cells, expression of pro-apoptotic genes has been shown to lead to cell death whereas expression of anti-apoptotic factors will protect C6/36 and other mosquito cells against apoptotic stimuli (Garcia et al., 2001; Wang et al., 2008; Zhou et al., 2005). Therefore mosquito cells have a functioning apoptosis pathway, but it appears in most cases to either not be induced or to be effectively suppressed by virus infection.

Pathological effects due to arbovirus infection in mosquitoes have been reported in a number of systems including Eastern equine encephalitis virus or West Nile virus (WNV)-infected *Culex* mosquitoes, Semliki Forest virus-infected *Ae. aegypti*, and Sindbis virus (SINV)-infected *A. albopictus* (Bowers et al., 2003; Girard et al., 2005; Mims et al., 1966; Weaver et al., 1988). Apoptosis due to WNV infection in *Culex quinquefasciatus* has been characterized by morphology as well as by down-regulation of anti-apoptotic factors in salivary glands (Girard et

al., 2010; Girard et al., 2005; Girard et al., 2007). This has been proposed to be a defense against infection in salivary gland tissue. Furthermore, WNV infection in a refractory strain of *C. quinquefasciatus* correlated with extensive cell death in midgut tissue (Vaidyanathan and Scott, 2006), but whether apoptosis was the reason for resistance to infection is not known. Another study examined apoptosis in response to infection with a *Culex* baculovirus in *Ae. aegypti*, which are resistant to infection with this virus. Pro-apoptotic gene expression was highly induced early during infection in *Ae. aegypti* larvae compared to delayed expression in susceptible *C. quinquefascitus* larvae, pointing to apoptosis as an effective defense against baculovirus (Liu et al., 2011). Similar results were observed when susceptible and refractory *Ae. aegypti* strains were infected with dengue virus. Refractory *Ae. aegypti* mosquitoes had 2.5 fold higher *mx* expression 3 hours post infectious blood meal compared to controls fed a noninfectious blood meal. No difference in *mx* expression was observed between dengue and control fed susceptible *Ae. aegypti* mosquitoes (Liu et al., 2013). Another study looked at the effects of apoptosis during SINV infection in *Ae. aegypti* by RNAi-mediated silencing of pro- and anti-apoptotic factors. When the initiator caspase *AeDronc* was knocked down, decreased infection and spread was observed, compared to increased infection and spread when apoptosis was increased by knockdown of *AeIAP1* (Wang et al., 2012). These results suggest that apoptosis may actually increase SINV infection; however, secondary effects such as increased lethality and disrupted midgut morphology when *AeIAP1* was silenced could be accountable for increased virus infection and dissemination under these conditions (Pridgeon et al., 2008; Wang et al., 2012).

Until a few years ago, nothing was known about the molecular pathways that regulate apoptosis in the mosquito. Recent studies have shown that the core apoptosis pathway in *Ae. aegypti* closely resembles that seen in *Drosophila melanogaster* (Bryant et al., 2008; Liu and

Clem, 2011; Wang and Clem, 2011). In *Ae. aegypti* the initiator caspase AeDronc and the adaptor protein AeArk are both required for apoptosis, while two effector caspases, CASPS7 and CASPS8 play partially redundant roles (Bryant et al., 2008; Liu and Clem, 2011). AeArk is presumed to activate AeDronc, which then activates the effector caspases CASPS7 and 8. The pathway is negatively regulated by the IAP protein AeIAP1 and positively regulated by the IAP antagonists Imp and Michelob_x (Bryant et al., 2008; Liu and Clem, 2011; Wang and Clem, 2011; Zhou et al., 2005).

SINV, the type member of the genus *Alphavirus* in the family *Togaviridae*, can be transmitted by *Ae. aegypti* under laboratory conditions and has been used extensively as a model to study virus-vector interactions (Foy and Olson, 2008; Olson et al., 1998; Taylor et al., 1955). SINV is well characterized molecularly and has been developed as an alphavirus transducing system, which makes it a powerful research tool. This system can be used to express foreign genes of interest during virus replication (Foy and Olson, 2008; Olson et al., 1994; Olson et al., 2000). Alphaviruses encode nonstructural genes at the 5' end and structural genes at the 3' end of their positive sense, single-stranded RNA genomes. The genomes of alphaviruses are capped and polyadenylated, and upon infection, the nonstructural polyprotein, which includes the viral RNA-dependent RNA polymerase (RDRP), is translated directly from the genome (Strauss and Strauss, 1994). The RDRP then initiates minus strand synthesis using the genome as a template, followed by transcription of positive sense mRNA from two promoters in the minus strand. One of these is located at the 3' end of the minus strand, which results in the translation of more nonstructural polyprotein, while the other is a subgenomic promoter that is responsible for expression of the structural genes (Frolov et al., 2001; Sawicki and Sawicki, 1994).

Various strategies have been used to develop SINV as an expression system, including one in which a duplicated copy of the subgenomic promoter was inserted upstream of the structural genes (Fig. 2.1) (Foy et al., 2004; Pugachev et al., 1995; Raju and Huang, 1991; Seabaugh et al., 1998). SINV expression systems utilizing the MRE-16 strain have high midgut infection and dissemination rates in infected *Ae. aegypti* mosquitoes when administered during a blood meal (Myles et al., 2004b; Olson et al., 2000; Pierro et al., 2003).

In this study, we utilized SINV 5'dsMRE16ic clones that were engineered to express either the pro-apoptotic protein Reaper from *Drosophila* or the baculovirus caspase inhibitor P35 in order to examine the effects of inducing or inhibiting apoptosis on the ability of SINV to infect *Ae. aegypti* mosquitoes. Our results demonstrate for the first time that induction of apoptosis in infected cells during virus replication is detrimental to the ability of an arbovirus to replicate and cause disseminated infection in *Ae. aegypti*.

Materials and Methods

Insect Rearing

Aedes aegypti mosquitoes, Orlando strain, (obtained from Dr. James Becnel at ARS, USDA in Gainesville, Florida) were reared at 27ºC, 80% humidity on a 12 hour light/12 hour dark cycle. They were allowed to feed on raisins and water prior to blood feeding and sucrose, raisins, and water post-blood meal. All experiments with SINV-infected mosquitoes were performed in an ACL-2 level insectary at Kansas State University.

Propagation of recombinant SINV virus and determination of viral titers

Recombinant 5'dsMRE16ic SINV clones containing sense and antisense sequences for baculovirus *p35* (MRE/P35 and MRE/P35-as), sense sequence for *Drosophila reaper* (MRE/Rpr) and antisense controls containing the antisense sequence of *reaper* or

michelob_x (MRE/Rpr-as or MRE/Mx-as) were previously described (Wang et al., 2008). These viruses have insert sizes of 900 nt for MRE/P35 and MRE/P35-as, 198 nt for MRE/Rpr and MRE/Rpr-as, and 339 nt for MRE/Mx-as. Capped viral RNA was produced from linearized plasmids using AmpliScribe^{TM} SP6 HighYield Transcription Kit (Epicentre Biotechnologies) and m^7G(5')ppp(5')G Cap Analog (Ambion). Aliquots of each transcription reaction (10µl) was used to transfect BHK21 cells in 1 ml Opti-MEM Reduced Serum Medium (Invitrogen) with 6 µl of Lipofectamine^{TM} 2000 (Invitrogen) as previously described (Wang et al., 2008). At 2-3 days post-transfection, medium was collected, aliquoted, and stored at -80ºC. Virus stocks were amplified once by using 100 µl of virus to infect a T75 flask containing 90% confluent C6/36 cells, cultured in L-15 (Leibovitz) Medium (Invitrogen) supplemented with 10% FBS. At 2-4 days post-infection (dpi), virus was harvested, aliquoted and stored at -80ºC. Viral titers were determined using $TCID_{50}$ assays in BHK21 cells as described below. Viruses used in this study were only passaged once and thawed once before use.

Oral infection with SINV

Two-three day post-eclosion mosquitoes were sorted for feeding while under cold-induced coma. Cages contained a 1:20 male to female ratio. Mosquitoes were given only water for 24 hrs prior to feeding. Defibrinated sheep blood (Colorado Serum Company) was mixed 1:1 with cell culture supernatant containing SINV for a final virus concentration of $1x10^7$ $TCID_{50}$/ml. Three to four day old mosquitoes were then administered an infectious blood meal using a Hemotek 5W1 feeding system (Discovery Workshops). Mosquitoes were allowed to probe and feed through a parafilm membrane for 30-60 minutes. Mosquitoes were knocked down at 4ºC and sorted for fully engorged females. Blood-fed females were sorted into cages and given sucrose, raisins, and water until experiments were completed.

Intrathoracic infection with SINV

A Nanoinject II injector (Drummond Scientific) was used to inject 3-4 day post-eclosion female mosquitoes with 69 nl of DMEM media containing 10-1000 PFU of SINV. Virus was injected intrathoracically while mosquitoes were knocked down with cold. After injection, mosquitoes were placed in cages and given sugar, raisins and water until experiments were completed. Three independent biological replicates were performed.

Midgut Antibody and TUNEL staining

Dissected midguts were fixed in 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Fixed midguts were washed in PBS + 0.1% Triton X-100 and blocked with PBS + 10% FBS +1% BSA. Anti-E2 SINV mAb (obtained from Carol Blair, Colorado State University) diluted 1:200 was used as a primary antibody and goat anti-mouse Alexa Fluor 488 (Molecular Probes) diluted 1:500 was used as secondary antibody. Washed midguts were mounted on slides using Fluormount-G (Electron Microscopy Sciences). Each midgut was given an infection score as previously described (Myles et al., 2004a; Wang et al., 2008), determined by multiplying the estimated percentage of the midgut surface area that was infected by the brightness of the staining (scale 1-3, 1=dull, 2=moderate, 3=bright). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed using the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science) either after antibody staining was complete or with no antibody staining. TUNEL-stained and antibody-stained midguts were imaged using a laser scanning confocal microscope (Zeiss LSM 5 Pascal, KSU Microscopy Facility). TUNEL positive cells on the epithelial surface of the midgut were counted in two infected regions and one noninfected region (230 µm x 230 µm) of 7 dpi midguts.

Immunoblotting

Pooled midgut lysate samples (10 midguts per sample) from 3, 5, and 7 dpi mosquitoes were dissected, placed in NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-Cl pH 8.0) and disrupted via sonication. Lysate was then mixed with SDS-PAGE loading buffer, incubated at 100ºC for 5 min and resolved by 15% SDS-PAGE. Samples were then transferred to PVDF membrane and detected with 1:3000 anti-p35NF primary antibody, kindly provided by Paul Friesen (University of Wisconsin) (Hershberger et al., 1994), and 1:5000 anti-Rabbit IgG-HRP secondary antibody (Santa Cruz Biotechnology). SuperSignal West Pico Chemiluminescent substrate (Pierce) was used to visualize antibody binding.

$TCID_{50}$ assay with mosquito samples

Individual mosquitoes were placed in 500 µl DMEM media (Invitrogen) supplemented with 15 µg/ml penicillin/streptomycin (Invitrogen) and 1 ng/ml gentamycin (Cellgro). Mosquitoes were triturated in 1.5 ml tubes with a disposable pestle and then debris was spun down. Supernatant from each sample was used to perform a serial dilution. BHK21 cells in supplemented DMEM media were used to seed 96 well plates. Each mosquito sample dilution (10 µl) was added to 8 wells of BHK21 cells ($1x10^4$ cells/well). Plates were scored for infection at 6 dpi by observing cytopathic effects. The number of infected wells per dilution was used to determine the $TCID_{50}$ per mosquito (O'Reilly et al., 1992).

Saliva Collection

Saliva was collected from 10 or 14 dpi mosquitoes. Mosquitoes were starved of a sugar source for 24 hrs prior to saliva collection, then anesthetized by cold treatment and their wings and legs were removed. The proboscis was placed in a pipette tip containing approximately 20 µl FBS + 1 mM ATP. Mosquitoes were allowed to salivate for 60-90 min. After salivation the FBS was

added to 100 µl of DMEM media containing penicillin/streptomycin and gentimycin. Samples were vortexed, spun down, and then used for $TCID_{50}$ assays. Four independent biological replicates were performed per sample.

Longevity assay

Mosquitoes at 3-4 days post-eclosion were allowed to feed on a noninfectious blood meal or a blood meal containing $1x10^7$ PFU/ml of the indicated virus. Blood-fed mosquitoes were then placed in pint sized containers and fed raisins and water throughout the experiment. Mortality was monitored daily for 42 days. Four independent biological replicates were performed.

Caspase activity assay

Pools of 10 midguts were lysed in caspase buffer (20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) using sonication. Debris was spun down and supernatant was used for caspase activity assay. The concentration of protein in each lysate sample was quantified using BCA Protein Assay Kit (Pierce) and samples were diluted to equal protein concentrations. Each sample (50µl) was added to a 96 well white plate (Costar). When incubated with CASPS8, 0.4 µM recombinant purified CASPS8-His_6 protein, purified as previously described (Wang and Clem, 2011), was incubated with midgut lysate. After incubation at 37ºC for 10-15 min, Ac-DEVD-AFC substrate (MP Biomedicals) was added to each well at a concentration of 20 µM. Cleavage of fluorogenic substrate was then measured by fluorescence produced at excitation 405 nm and emission 535 nm using a Victor3 1420 Multi-label Counter (Perkin Elmer). Caspase activity in infected midguts was measured by reading fluorescence every 15 min, while CASP8 + lysate samples were measured every minute. The rate of substrate cleavage in the first 20-30 min was used to compare the amount of activity in each sample. Three independent biological replicates were performed for each experiment.

Plaque collection and sequencing

Plaques were isolated by diluting supernatant from titered individual mosquitoes in DMEM media + 0.5% agarose, 10% FBS, 15 µg/ml penicillin/streptomycin and 1 ng/ml of gentamycin. BHK21 cells were overlayed with a mixture of DMEM, virus and 0.5% agarose. At 3 dpi, 10 plaques from each mosquito or stock virus were collected and amplified once in BHK21 cells cultured in supplemented DMEM media. Plaque sizes varied, but did not correlate with retention of the inserts. At 2-3 dpi, amplified virus was harvested from BHK21 cells. Viral RNA was isolated from each individual amplified plaque using TRIzol LS reagent (Invitrogen). Reverse transcription was performed using ImProm-II™ Reverse Transcription Kit System (Promega) and the virus-specific primer 5'TACTGCGGAGGTCAATTGTT 3'. The region containing the insert was amplified by PCR with sense primer 5'CTGAGACACTGGCTACTGCG 3', antisense primer 5'CGGCCGAGCATATTAAAGAA 3', and GoTaq polymerase (Promega). Purified PCR products were sequenced by Genewiz, Inc (South Plainfield, NJ). Sequences were analyzed using EMBOSS Kalign (http://www.ebi.ac.uk/Tools/msa/kalign/).

Results

Utilizing recombinant SINV to express pro- or anti-apoptotic genes

To examine the effects of inducing or inhibiting apoptosis on arbovirus-vector interactions, we utilized the arbovirus SINV and took advantage of the fact that it has been developed as an alphavirus transducing system (Olson et al., 2000; Pierro et al., 2003). Infectious cDNA clones of the SINV strain 5'dsMRE16ic were used that had been engineered to express either a pro- or an anti-apoptotic gene, via a duplicated subgenomic promoter (Wang et al., 2008) (Fig. 2.1). For each gene of interest, control constructs were used that contained an antisense sequence of similar or greater size, in order to control for the effects of genome size on

virus replication. Plasmids containing the infectious cDNA constructs were transcribed *in vitro* and the capped viral RNA was used to transfect BHK21 cells. The resulting virus stocks were amplified by a single passage in the *A. albopictus* cell line, C6/36.

Virus clones that were used expressed either the anti-apoptotic baculovirus gene *p35* or the pro-apoptotic *Drosophila* gene *reaper* (Wang et al., 2008) (Fig 2.1). P35 is a stoichiometric inhibitor of effector caspases, while Reaper is an antagonist of the inhibitor of apoptosis (IAP) protein DIAP1, and overexpression of Reaper induces apoptosis. Both P35 and Reaper have been shown to function in diverse systems, including mosquito cells (Clem, 2007; Goyal et al., 2000; Wang et al., 2008; White et al., 1996).

The Reaper-expressing virus, MRE/Rpr, has been shown to induce apoptosis in C6/36 cells (Wang et al., 2008), but we first determined whether SINV expression of Reaper (MRE/Rpr) would induce apoptosis in infected mosquitoes. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay was used to label apoptotic cells in midguts at 7 days post-infection (dpi). Midguts were also stained for virus infection using an anti-SINV antibody. MRE/Rpr-infected midgut regions exhibited significantly more TUNEL-positive cells on the epithelial surface of the midgut (indicated by white arrows), compared to the number of TUNEL-positive cells detected in non-infected regions or infected areas of midguts infected with the control viruses MRE/Rpr-as or MRE/Mx-as, which contain inserts of similar or greater size to *reaper* but do not express a foreign protein (Fig. 2.2A-J and data not shown). All midguts, regardless of infection or blood meal status, displayed background staining on certain cells that were associated with trachea (blue arrows). The staining of these trachea-associated cells was not considered indicative of apoptosis.

In addition to TUNEL staining, midgut lysate was analyzed for caspase activity at 7 dpi using the fluorogenic caspase substrate Ac-DEVD-AFC, a substrate of effector caspases. Lysate from midguts infected with MRE/Rpr displayed higher caspase activity than MRE/Mx-as-infected, MRE/Rpr-as-infected, or control blood-fed midgut lysate samples (Fig. 2.2K and data not shown). These results indicate that expression of Reaper via the recombinant SINV clone MRE/Rpr induced effector caspase activation and apoptosis in *Ae. aegypti* midgut.

Next we characterized P35 expression and anti-apoptotic activity in mosquitoes infected with SINV expressing P35 (MRE/P35). Infected midguts were collected from orally infected mosquitoes at 3, 5, and 7 dpi. Midgut lysate was then used for western blot analysis using anti-P35 polyclonal antibody. Mock-infected and MRE/P35-as-infected midguts were used as negative controls. Expression of P35, which migrates at 35kDa, was observed in MRE/P35-infected midguts from each time point (Fig. 2.3A and data not shown). The origin of the additional immunoreactive band seen at approximately 25 kDa is unknown, but a similar band has been observed previously with this antiserum (Cartier et al., 1994) and may be due to cleavage of P35 by caspases.

In order to determine whether P35 expression in midgut could inhibit caspase activity, midgut lysates were tested for their ability to inhibit a recombinant caspase. Midgut lysates collected at 7 dpi from mock-infected, MRE/P35-infected, or MRE/P35-as-infected mosquitoes were incubated with recombinant CASPS8, an *Ae. aegypti* effector caspase that had been produced in bacteria. Ac-DEVD-AFC was then used as a substrate to determine the level of effector caspase activity in samples. CASPS8 incubated with MRE/P35-infected midgut lysate had lower activity compared to controls, further indicating that MRE/P35 expressed functional P35 in midgut (Fig. 2.3B).

We next looked at the stability of the inserted regions of our virus clones by performing sequence analysis with individual plaques collected from stock viruses used to infect mosquitoes. MRE/Rpr and MRE/Mx-as maintained the full inserted sequence in the majority of the plaques that were sequenced. MRE/Rpr stocks had 92% of plaques that maintained the full gene of interest and MRE/Mx-as stocks had 84% of plaques that contained the full antisense sequence (n=25-35, Table 2.1). On the other hand, MRE/P35 and MRE/P35-as inserted regions were poorly maintained in the stock viruses. Only 8% of MRE/P35 plaques containing the full gene cassette and 3% of MRE/P35-as plaques containing the full antisense sequence (n=30-40, Table 2.1), indicating that the *p35* sequence was rapidly lost regardless of whether P35 was expressed, even after only a single virus passage in C6/36 cells. However, due to the fact that P35 expression and activity were observed in MRE/P35 infected midguts, we moved forward with the analysis of MRE/P35 and MRE/P35-as infected mosquitoes alongside MRE/Rpr and MRE/Mx-as infected mosquitoes.

Effects of apoptosis on midgut infection

In order to assess whether increasing or decreasing apoptosis during virus replication affected the ability of SINV to infect the midgut, the primary site of infection in the mosquito, midguts from mosquitoes that had been given a blood meal containing MRE/Rpr, MRE/P35 or control viruses were dissected and examined for levels of infection at 3, 5, and 7 dpi using an anti-SINV antibody. Each midgut was assigned an infection score based on the surface area that was infected and the brightness of the antibody staining, as previously described (Myles et al., 2004a; Wang et al., 2008). Prevalence of infection (the proportion of mosquitoes that were positive for SINV antigen in midgut) did not differ significantly between MRE/P35 and MRE/P35-as-infected mosquitoes at any of the time points examined (Fig. 2.4A). In addition,

there was no significant difference between MRE/P35- and MRE/P35-as-infected midgut infection scores at any of the time points (Fig. 2.4B). However, infection prevalence was lower among mosquitoes infected with MRE/Rpr than control virus MRE/Mx-as at 3 dpi (Fig. 2.4A). This difference was no longer significant at 5 and 7 dpi. Similarly, infection scores were significantly lower in mosquitoes infected with MRE/Rpr than in control-infected midguts at 3 and 5 dpi (Fig. 2.4B). At 7 dpi there was no significant difference between MRE/Rpr and control-infected midguts. These results suggest that increasing apoptosis during virus replication in the midgut resulted in less infection in the midgut at earlier stages of infection, but that the replication of MRE/Rpr caught up to that of control virus by 7 dpi. The results also suggest that expression of P35 did not have an effect on midgut infection. Midgut infection prevalence decreased over time in mosquitoes infected with any of the viruses (Fig. 2.4A), suggesting that midgut infection was being cleared in some of the mosquitoes.

Effects of apoptosis on SINV replication and dissemination in *Aedes aegypti*

We next wanted to address the effects of increasing or decreasing apoptosis on SINV replication and dissemination in *Ae. aegypti*. In order to determine levels of virus replication and dissemination, the amount of infectious virus per individual mosquito was measured using $TCID_{50}$ assays in BHK21 cells. As an additional control, the infectious MRE clone with no insert was also included (MRE/WT). Infection prevalence (the proportion of mosquitoes with detectable virus titers) and virus titers were similar between mosquitoes infected with MRE/P35 and those infected with either MRE/P35-as or MRE/WT at 3, 5 and 7 dpi (Fig. 2.5A and B). However, similar to what was observed by staining for viral antigen, mosquitoes that were infected with MRE/Rpr had a significantly lower prevalence of infection compared to infection with MRE/Mx-as, with the biggest difference being at 3 dpi (Fig. 2.5A). Among mosquitoes that

were infected, virus titers were also lower in mosquitoes infected with MRE/Rpr than MRE/Mx-as- or MRE/WT- infected mosquitoes, with the difference being significant at 5 dpi (Fig. 2.5B). A single experiment performed with MRE/Rpr-as controls yielded similar results (data not shown). These results indicate a delay in the ability of SINV to establish infection in *Ae. aegypti* when *reaper* is expressed during virus replication.

Mosquitoes infected with MRE/Rpr also had a distinct lower group of titers ranging from 10^2-10^4 $TCID_{50}$/ml, that was most pronounced at 5 dpi (Fig. 2.5B). To determine if these lower titers represented mosquitoes in which the virus had not escaped the midgut, we titered individual midguts separately from the rest of the mosquito bodies (Fig. 2.5C). At 3 dpi, half (17/34) of the bodies from mosquitoes fed MRE/Rpr did not contain detectable virus while a quarter (12/49) of the bodies from mosquitoes fed MRE/Mx-as control virus were not infected. Titer values of MRE/Rpr-infected midguts and bodies were significantly lower than control-infected (Fig. 2.5C), correlating with lower midgut infection scores at this time point, although body titers were not significantly different if uninfected bodies were omitted from the analysis. At 5 dpi, virus could be detected in most of the bodies (37/40 MRE/Rpr and 44/46 MRE/Mx-as) and MRE/Rpr body titers had no significant difference compared to control. Midgut titers at 5 dpi also had no significant difference from control (Fig. 2.5C). At 7 dpi, 9/45 mosquitoes that had been fed MRE/Rpr had no detectable body titer, compared to only 2/47 mosquitoes fed with control virus, which was a significant difference in viral dissemination as determined by Fisher's exact test (p=0.02). Body titers were also significantly different at this time point due to these uninfected samples. However, 7 dpi midgut titers were not significantly different (Fig. 2.5C), also consistent with the midgut infection scores at this time point. The titer values seen in the lower group of whole body titer samples in Fig. 2.5B were consistent with the range of titers

seen in midgut samples (Fig. 2.5C). Thus, although the majority of MRE/Rpr-infected mosquitoes had high body titers by 5 dpi, indicating that the virus was able to escape the midgut in most cases, there was a significant difference in disseminated infection rates between MRE/Rpr and control virus at 3 and 7 dpi. It appears that the lower group of whole body titers seen in Fig. 2.5B was due to lower initial infection in the midgut and either decreased or delayed midgut escape. This reinforces the notion that *reaper* expression decreases infection and dissemination of SINV.

Intrathoracic injection of recombinant SINV clones expressing apoptotic regulatory genes

The experiments above all involved oral infection, which is the natural route of infection. To determine whether Reaper expression would also have an effect when the midgut barrier was bypassed, mosquitoes were infected by intrathoracic injection. Doses of 10, 100, or 1000 PFU were injected per mosquito and samples were collected for $TCID_{50}$ assays at 1, 3, and 5 dpi.

Mosquitoes infected with MRE/P35 had titers that were similar to control-infected mosquitoes for each dose injected and at each time point (Fig. 2.6A). However, mosquitoes injected with MRE/Rpr had significantly lower titers than controls at 1 dpi with each of the injected doses. By 3 dpi, replication of MRE/Rpr had caught up with the control virus and there was no significant difference in titer between MRE/Rpr and controls at 3 or 5 dpi (Fig. 2.6B). These results indicate that even when the midgut barrier was bypassed, expression of Reaper during virus replication delayed SINV replication within the mosquito during the early stages of infection.

Quantification of SINV salivated by infected mosquitoes

An important question was whether apoptosis affected the amount of virus in the saliva of infected mosquitoes, since this is what ultimately determines whether a mosquito will be able to

transmit the virus. We were not able to find any published data that quantified SINV in *Ae. aegypti* saliva, and only a few transmission studies have been reported, which vary on how successfully SINV is transmitted by *Ae. aegypti* (Dohm et al., 1995; Myles et al., 2004b). However, the presence of SINV in *Ae. aegypti* saliva has been reported by 9 days after oral infection (Phillips et al., 2010). We collected saliva from orally infected mosquitoes at 10 and 14 dpi and determined virus titers. There was no significant difference in the proportion of mosquitoes having virus-positive saliva for any of the viruses (Fig. 2.7A), with only 10-20% of saliva samples containing virus at 10 dpi and less than 10% being virus-positive at 14 dpi.

MRE/P35 saliva titers were not significantly different from control titers at 10 or 14 dpi (Fig. 2.7B). However, consistent with the delay seen in virus replication in midgut and whole body, MRE/Rpr saliva titers were significantly lower than control virus at 10 dpi (Fig. 2.7B). No significant difference was observed at 14 dpi, however, sample sizes at this time point were small. These results again indicate that *reaper* expression slowed the replication and dissemination of SINV during *Ae. aegypti* infection.

Effects of infection on mosquito longevity

SINV establishes a persistent infection within the mosquito. Once a mosquito is infected, virus will continue to replicate in its tissues for the remainder of the mosquito's life (Strauss and Strauss, 1994). Therefore, we were interested to know whether infecting a mosquito with a virus expressing a pro- or anti-apoptotic factor would affect the life span of the mosquito.

Previous studies in *Ae. aegypti* reported that increasing apoptosis by silencing expression of the anti-apoptotic gene *AeIAP1* resulted in a majority of mosquitoes dying within 1-2 days after dsRNA treatment (Pridgeon et al., 2008; Wang et al., 2012). We also observed decreased mosquito survival after infection with MRE/Rpr, although the effect on longevity was not

evident until much later. Infection with any of the viruses had a small, but statistically significant, negative effect on lifespan compared to blood-fed mosquitoes, with increased mortality beginning at about 35 dpi (Fig. 2.8). However, mosquitoes infected with MRE/Rpr had an even lower survival rate than the other viruses tested, with increased mortality beginning at around 20 dpi (Fig. 2.8). These results, in conjunction with previous studies, indicate that increasing apoptosis reduced the lifespan of the mosquito more than just infection alone.

Strong negative selection against maintenance of the Rpr insert within infected mosquitoes

Given that the MRE/Rpr virus replicated less well than control virus in the early stage of infection, but then seemed to catch up later, we wondered whether the inserted *reaper* gene was intact as SINV continued to replicate in the mosquito. In order to examine virus sequences in individual mosquitoes, we isolated plaques from titered whole body samples. For each virus and time point, 8-10 plaques were isolated from 10 individual mosquitoes and amplified in BHK21 cells. Viral RNA was then isolated and the region of the genome containing the insert sequences was amplified using primers that bind to flanking viral coding regions that are required for virus replication. The resulting amplified DNA was then sequenced. A summary of the strategy and results of the virus sequencing is shown in Fig. 2.9 and Table 2.1.

When individual plaque isolates were sequenced from the MRE/Rpr stock virus, 92% of the isolates contained the complete *reaper* sequence with no mutations or deletions. However, at 3 dpi, the majority of viruses isolated from mosquitoes lacked the intact *reaper* cassette (Fig. 2.8B). In 4/10 mosquitoes that had been infected with MRE/Rpr, 100% of plaques had deletions in the *reaper* cassette (Fig. 2.9B). The types of mutations observed included deletions in the *reaper* coding sequence, start site, and/or promoter, or in some cases the entire insert sequence was deleted. Point mutations were not observed. The remaining 6/10 mosquitoes had mixtures

of virus genotypes, with the proportion of plaques that contained the intact *reaper* cassette ranging from 10-45%. At 5 dpi, 5/10 mosquitoes had deletions in the *reaper* cassette in all plaque samples, while 4 of the remaining 5 mosquitoes analyzed had mixtures of viruses with intact or deleted *reaper* inserts, and 1 mosquito had 10/10 plaques with the intact *reaper* cassette (Fig. 2.9B). Interestingly, at 7 dpi, plaque sequences in individual mosquitoes were homogenous; that is, either all of the plaques contained the intact insert (5/12 mosquitoes) or all of the plaques had deletions eliminating expression of *reaper* (7/12 mosquitoes) (Fig. 2.9B). In contrast, when plaque isolates obtained at 7 dpi from mosquitoes infected with the control virus MRE/Mx-as were sequenced, all of them contained the intact control insert and subgenomic promoter (Fig. 2.9B). Similar results were obtained with MRE/Rpr-as (20/20 plaques had the intact insert at 7 dpi, data not shown). These sequencing results indicate a strong negative selection against *reaper* expression during replication of MRE/Rpr in mosquitoes.

When we analyzed the sequences of MRE/P35 and MRE/P35-as stock viruses and infected mosquitoes, only 8% of MRE/P35 stock plaques and 3% of the MRE/P35-as stock plaques contained the intact insert. Not surprisingly, when infected mosquitoes were examined at 7 dpi, MRE/P35 samples had only 1/10 mosquitoes (with 3/10 plaques) containing the intact *p35* cassette, while none of the 3 dpi MRE/P35 or 7 dpi MRE/P35-as isolates (n = 5 mosquitoes, 8-10 plaques each) contained intact inserted sequences (Fig. 2.9B). Thus, even though P35 protein expression was easily detectable in infected midguts at 5 and 7 dpi (Fig. 2.3A and B), the large majority of the MRE/P35 viruses used to infect the mosquitoes did not express P35.

Discussion

The potential role of apoptosis in virus-vector interactions has come under scrutiny in recent years. Most of the studies on this topic have examined pathology in infected mosquitoes,

in some cases correlating apoptosis with resistance to infection (Bowers et al., 2003; Girard et al., 2005; Girard et al., 2007; Kelly et al., 2012; Liu et al., 2011; Mims et al., 1966; Vaidyanathan and Scott, 2006; Weaver et al., 1988). A recent study looked at the effects of silencing anti-apoptotic or pro-apoptotic genes on SINV infection in *Ae. aegypti* mosquitoes and found that inducing widespread apoptosis exacerbated infection (Wang et al., 2012). In this study, we examined the effects of inducing apoptosis in infected cells as compared to widespread knockdown of apoptotic factors. Using this approach, we eliminated secondary effects of gene knockdown in the entire mosquito and studied the effects of expressing apoptotic factors only in infected cells during virus replication.

The effects of apoptosis appear to be most significant in midgut

Based on our studies, increasing apoptosis during virus replication decreased midgut infection and delayed dissemination of SINV in *Ae. aegypti*. The delay observed in virus dissemination may be due to lower initial infection of the midgut, or to a lower number of viruses escaping from the midgut, or both. Midgut titers and infection scores indicated lower rates of initial virus infection of the midgut by MRE/Rpr compared to control viruses. However, prevalence of SINV in MRE/Rpr-infected whole body samples increased over time and prevalence in body tissues outside the midgut also increased from 3 to 5 dpi. The ability of MRE/Rpr replication to catch up with the control virus at later time points could be due to Reaper expression only causing a delay in replication or cell-to-cell spread of virus, not complete elimination of these events. On the other hand, it could also be due to a replicative advantage of viruses with deletions that eliminated the expression of *reaper*. It is clear that there was strong selective pressure favoring viruses that no longer expressed Reaper, since the *reaper* cassette was frequently deleted in MRE/Rpr but not in the control viruses, which contained inserts of similar

size. It is interesting, however, that at 7 dpi, around half of the mosquitoes contained a high proportion of MRE/Rpr viruses with intact *reaper* inserts, while the other half contained mainly viruses with deleted inserts. This seems to indicate that once the virus escaped from the midgut, the selective pressure against Reaper expression was relaxed, and viruses that expressed Reaper were able to replicate as well as viruses that did not express Reaper. It also appears from these results that only a small number of viruses escape the midgut and are responsible for establishing disseminated infection, since mosquitoes at 5 dpi contained mixtures of viruses, but by 7 dpi, virus populations were much more homogeneous. It is also worth noting that MRE/Rpr virus replication was delayed compared to control virus when the midgut barrier was bypassed, suggesting that apoptosis affects virus replication in other tissues as well. However, MRE/Rpr replication also rapidly caught up with control virus in this scenario. In contrast, prevalence of infection in midgut samples decreased over time and MRE/Rpr midgut titers were only significantly lower at 3 dpi when compared to controls. This loss of significance in midgut titers at later time points may also be due in part to routine clearing of infection from the midgut.

SINV does not appear to induce significant levels of apoptosis during infection of *Ae. aegypti* midgut, at least with this combination of virus and mosquito strains. This was indicated by the lack of TUNEL-positive cells and lower caspase activity in control-infected midguts. Therefore, expression of an apoptosis inhibitor may not be expected to have an effect on virus replication in this case. P35 expression did not seem to have an effect on SINV replication. However, sequence analysis of individual MRE/P35 plaque isolates indicated that P35 was only expressed from a small fraction of the viruses used to infect the mosquitoes, and thus we suspect that P35 was not expressed in most of the infected cells. This makes it difficult to draw conclusions about the effects of P35 expression on SINV infection.

When thinking about apoptosis as an antiviral defense and comparing it to what is known from other virus-host systems, the ability of *reaper* expression to decrease infection makes sense. Other studies have suggested that early induction of apoptosis in the midgut may result in non-permissive infection in the mosquito. *Culex* mosquitoes that were refractory to WNV infection had increased apoptosis in midgut tissues at 3 dpi infection (Vaidyanathan and Scott, 2006). *Ae. aegypti* larvae that were refractory to infection by a *Culex* baculovirus had increased apoptosis as soon as 2 hours post infection in midgut tissues, whereas susceptible *Culex* larvae were slower to express pro-apoptotic factors and displayed necrotic cells late during infection (Liu et al., 2011). Similar results were observed with pro-apoptotic gene expression in the midguts of *Ae. aegypti* adult mosquitoes that were either susceptible or refractory to dengue virus infection (Liu et al., 2013). Therefore, apoptosis appears to be an antiviral defense that may be exploitable in controlling the spread of viruses by mosquito vectors.

Comparing different approaches to manipulating apoptosis during virus infection

A previous study by Wang et al. (2012) examined the effects of apoptosis on SINV infection in *Ae. aegypti* by using RNAi to silence pro- and anti-apoptotic genes in the mosquito. Increasing apoptosis by this method increased infection and spread, while knocking down expression of the initiator caspase AeDronc decreased infection and spread of MRE/GFP, a SINV expressing GFP. The study by Wang et al. (2012) and our study utilized very different approaches to address similar questions. Decreased infection and spread of SINV when AeDronc was silenced indicates that apoptosis, or at least caspase activation, may be occurring at some level during SINV infection, and that either apoptosis or caspases may play a role in assisting infection in the mosquito or in virus escape from the midgut. Caspase activity has been shown to aid baculovirus escape from the midgut of lepidopteran hosts due to their role in

remodeling the basal lamina of the midgut (Means and Passarelli, 2010). The approach used in this study was not able to address this point, since P35 was not expressed in the majority of viruses fed to the mosquitoes.

Additionally, widespread knockdown of a gene can have numerous effects in an organism. For instance, knockdown of *AeIAP1* and the resulting widespread increase in apoptosis killed over 50% of injected mosquitoes by 24 hrs post injection of dsRNA, and midguts from mosquitoes with high levels of apoptosis occurring were much more fragile and displayed disorganized epithelium cells and shorter, gapped microvilli (Wang et al., 2012). Altering the midgut physical barrier, the overall health, or the immune status of mosquitoes could easily lead to increased virus infection and spread. For example, a recent study in *Drosophila* showed that increasing apoptosis suppressed the RNAi pathway, not only in dying cells but in surrounding cells as well (Xie et al., 2011). RNAi is known to be an important anti-viral defense in *Drosophila* as well as *Ae. aegypti* (Attarzadeh-Yazdi et al., 2009; Chotkowski et al., 2008; Khoo et al., 2010; Saleh et al., 2009; Sanchez-Vargas et al., 2009). Suppression of this pathway could have contributed to increased SINV infection and spread in the Wang et al. (2012) study.

Using the virus to express pro-apoptotic or anti-apoptotic genes, apoptosis should only be increased or decreased in cells where virus replication is taking place. Secondary effects seen with gene knockdown should be minimized using this method. However, retention of the inserted genes in the recombinant viruses is an issue when using this approach. Therefore, both approaches are valuable to increase our understanding of the effects of apoptosis on virus-vector interactions.

Effects of apoptosis on SINV transmission

There are several factors to consider when looking at ways to decrease or block virus transmission by mosquitoes, including virus prevalence, the amount and timing of virus salivated, and mosquito lifespan. Although SINV is naturally vectored by *Culex* mosquitoes, it can be transmitted by *Ae. aegypti* in the laboratory setting (Echalier, 1965; Taylor et al., 1955). However, studies that have examined transmission of SINV by *Ae. aegypti* are limited. Transmission studies using SINV-infected *Ae. aegypti* and 1 day old chicks reported a very low transmission rate of 7%, while *Culex pipiens* and *Aedes albopictus* mosquitoes in the same study had 37% and 53% transmission rates, respectively (Dohm et al., 1995). Studies with the SINV strain MRE/GFP, however, saw transmission to 12/14 neonatal mice after being fed on by infected *Ae. aegypti* (Myles et al., 2004a).

We observed very low rates of prevalence of SINV in saliva at 10 dpi and even lower prevalence at 14 dpi. These rates were comparable to some other virus-vector studies with Barmah Forest virus or SINV and *Culex quinquefasciatus* or *Opifex fuscus* (Kramer et al., 2011). The titers of virus we found in saliva samples were also similar to quantities seen in other alphavirus studies (Dubrulle et al., 2009; Smith et al., 2005). Prevalence of SINV in saliva was similar between SINV expressing *reaper* or *p35* and control viruses, but MRE/Rpr-infected mosquitoes did have significantly lower amounts of virus in saliva than control virus at 10 dpi. Lowering the amount of virus in saliva is encouraging but may still allow for transmission to occur. A recent report found increased apoptosis in SINV-infected salivary glands in *Ae. aegypti*. Apoptosis was not observed in infected foregut, midgut or hindgut tissue, however these mosquitoes were infected by intrathoracic injection (Kelly et al., 2012). WNV infection in *Culex* mosquitoes has been shown to lead to apoptosis and cytopathologic changes in salivary glands, which correlated with lower prevalence of virus in saliva (Girard et al., 2007). A second

study saw decreased expression of *AeIAP1* in the salivary glands of WNV-infected *C. quinquefasciatus* (Girard et al., 2010). These studies lend to the notion that apoptosis in salivary glands may be utilized as an antiviral defense. Induction of apoptosis by SINV in salivary gland may also explain why we observed decreased infection prevalence in saliva at 14 dpi compared to 10 dpi with all of the viruses tested. Purposely stimulating apoptosis in salivary glands prior to dissemination from the midgut may be a means of blocking virus from getting into saliva and therefore being transmitted during subsequent blood meals.

In addition, we observed a significant reduction in the lifespan of mosquitoes infected with MRE/Rpr, compared to control viruses. If mosquitoes die before being able to transmit whatever pathogen they have acquired, transmission is blocked. In this case, the extrinsic incubation period (EIP) of SINV in *Ae. aegypti* is around 9 days (Phillips et al., 2010). Infected mosquitoes would need to die before this time point for transmission to be affected. MRE/Rpr-infected mosquitoes died well after 9 dpi and approximately half of them lived past 40 dpi. However, it is not known whether infection with MRE/Rpr would affect their feeding behavior. Also, the length of time required for MRE/Rpr infection to cause lethality may have been affected by the loss of *reaper* expression over time in many of the virus genotypes. In contrast, over 40% of mosquitoes in which *AeIAP1* expression had been silenced died within 24-48 hours after injection or topical application of dsRNA (Pridgeon et al., 2008; Wang et al., 2012). A balance between these two approaches may be able to achieve reduced lifespan prior to when the EIP is complete, without causing rapid lethality or the appearance of resistance. Such a system would also have to ensure overall health of the infected mosquitoes. RNAi defenses and the midgut barrier could not be compromised to the point where virus infection and transmission could be increased if the mosquito survived past the EIP.

Use of SINV as an expression system

TE and MRE SINV strains have been used to develop alphavirus transducing systems over the past several years (Olson et al., 1994; Olson et al., 2000; Pierro et al., 2003; Raju and Huang, 1991; Seabaugh et al., 1998). These recombinant virus clones have been powerful tools in studying virus and vector interactions. However, they are not perfect for all studies. For instance, there is limited plasticity in viral genome length for the alphaviruses. For SINV, the virion will only fit RNA with up to 2 kb of additional length. Around 1 kb is considered to be the functional limit in terms of minimally compromising virus replication (Foy and Olson, 2008). To reduce the chances of the gene of interest being deleted during virus replication, the system used in this study was developed, in which the site of insertion is located 5' of the subgenomic promoter, rather than at the 3' end of the genome (Cheng et al., 2001; Pierro et al., 2003). This expression system has previously been tested for its efficiency using western blot detection of expressed protein or reporter genes such as GFP (Myles et al., 2004b; Olson et al., 2000; Pierro et al., 2003; Wang et al., 2008).

We chose to perform conventional sequencing of virus genomes from individual plaques obtained from individual mosquitoes, rather than pooling samples and using next generation sequencing, because we wanted to compare the virus populations in individual mosquitoes. Given the large number of individual mosquitoes we wanted to obtain separate virus sequences from, next generation sequencing would have been cost prohibitive, even utilizing bar coding approaches. The disadvantage of this approach is that ten sequences out of thousands or millions is just a small window into the sequence diversity in individual mosquitoes. However, we were most interested in finding out what was happening with the majority of the viruses that were present, and not as interested in rare mutations or deletions occurring at low frequency.

Our sequencing results suggest that even if foreign gene expression is observed in infected cells, this may not accurately reflect the proportion of viruses retaining the insert since expression will be observed even if only a small percentage of recombinant viruses express the desired protein. Even with inserts smaller than 1 kb, retention of the foreign insert appears to be strongly influenced by insert size. The MRE/Rpr-as and MRE/Mx-as insertions were 200 and 340 nt in length, respectively, and were highly retained in mosquitoes even after 7 dpi. However, the MRE/P35-as insertion was 900 nt in length, and a high percentage of even the viruses in the original stock had already lost the insert after only a single passage.

The high rate of lost Reaper expression, while the antisense control sequence was maintained, indicates a strong selective pressure against viruses expressing Reaper. This pressure is likely caused by a negative impact of apoptosis on SINV infection. One of the mechanisms by which Reaper induces apoptosis is by inhibiting cap-dependent protein translation (Colon-Ramos et al., 2006). Since SINV mRNAs are capped, inhibition of cap-dependent translation by Reaper may play a role in decreasing SINV replication. Interestingly though, we found that by 7 dpi, the mosquitoes sampled contained homogeneous populations of virus, either containing the *reaper* insert or not. This suggests that the effect of apoptosis is strongest in the initial stages of infection, probably in the midgut, and once the infection has spread beyond the midgut, the selective pressure against expression of *reaper* has relaxed, and whatever virus escapes the midgut first predominates.

Based on our sequencing results, the *p35* insert was retained at extremely low levels even after a single passage in C6/36 cells for amplification. Even though P35 protein was detected by western blot and was able to inhibit caspase activity in MRE/P35 midgut samples, it was likely expressed at very low levels in the mosquito. We do not know how many infected cells

expressed P35 during SINV replication. Therefore, it is difficult to determine whether P35 expression affects SINV infection and replication in the mosquito using this approach.

Conclusions

In conclusion, our data are the strongest indication to date that apoptosis can act as an antiviral defense against arbovirus infection in mosquitoes. Increasing apoptosis by expression of *reaper* during virus replication decreased midgut infection, delayed virus spread, and lowered SINV prevalence. MRE/Rpr infection also reduced the lifespan of infected mosquitoes, likely due to a persistent increase in apoptosis over time. Our results also indicate a strong selection against Reaper expression, likely due to negative effects on virus infection and replication. Expression of P35 did not appear to have an effect on SINV infection and dissemination, but it is difficult to determine the effects of inhibiting apoptosis on virus infection with this system based on the low level of viruses expressing P35. Using plaque sequencing to determine stability of inserts, we reinforce the importance of insert size and study length when using alphavirus transducing systems.

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Chapter 2 Figures and Tables

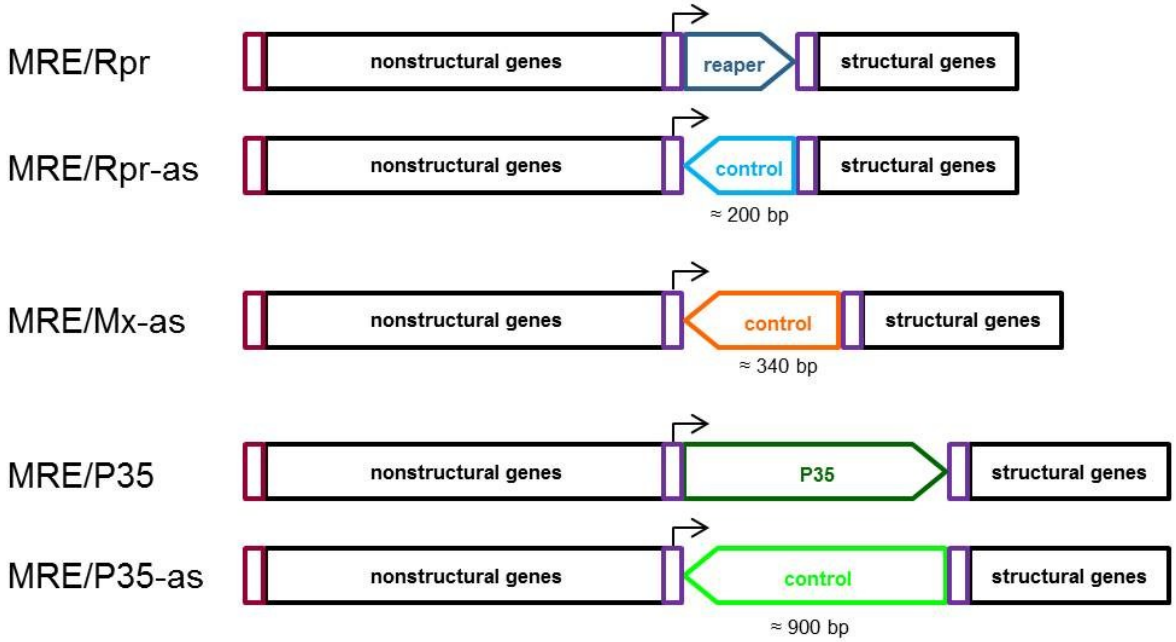

Figure 2.1. **Recombinant SINV clones containing pro or anti-apoptotic genes.** Recombinant SINV clones used in this study expressing either a pro-apoptotic or anti-apoptotic gene during virus replication. Expression was driven by an inserted secondary subgenomic promoter (arrow). Controls for each clone contained an antisense insert of similar or greater size. The pro-apoptotic gene used was the *Drosophila* IAP antagonist *reaper* (Rpr), while the anti-apoptotic gene used was the baculovirus caspase inhibitor *p35*. A clone containing the *michelob_x* (Mx) gene inserted in antisense orientation was used as an additional control.

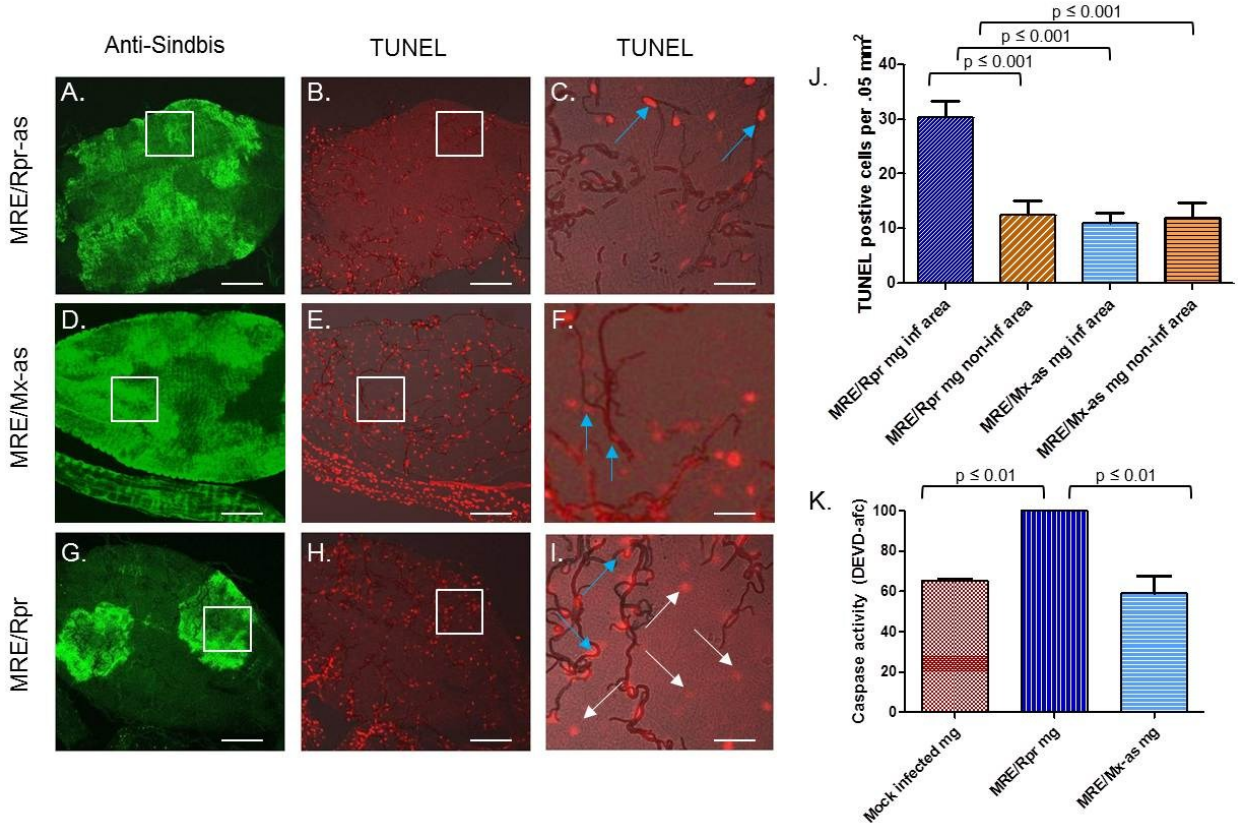

Figure 2.2. Infection with SINV expressing Reaper results in increased apoptosis in the midgut.

Infection with SINV expressing Reaper results in increased apoptosis in the midgut. A-I) Midguts from *Ae. aegypti* infected with MRE/Rpr, MRE/Rpr-as or MRE/Mx-as harvested at 7 dpi. SINV infection (green) was visualized using an anti-SINV antibody (A, D, G). TUNEL staining was used to label apoptotic cells (red). (Scale bars = 200 µm for A,B,D,E,G,H and 50 µm for C,F,I.). MRE/Rpr-infected samples had numerous TUNEL-positive cells (white arrows) on the surface of the infected midguts (I, J), while non-infect areas of midguts and MRE/Rpr-as and MRE/Mx-as-infected midguts rarely exhibited TUNEL-positive staining on the midgut surface (C, F, J). Background staining of trachea-associated cells was observed in all samples (blue arrows) and was not considered indicative of apoptosis. Ten midguts were analyzed for TUNEL cell counts. Error bars indicate the standard error of the mean and significance was determined by one-way ANOVA followed by Bonferroni's post-test. K) Lysates from 7 dpi

midguts infected with MRE/Rpr or control samples were used to analyze the cleavage rate of a fluorogenic caspase substrate (Ac-DEVD-AFC). MRE/Rpr values were arbitrarily set to 100. Three independent biological replicates were performed and error bars indicate the standard error of the mean. Significance was determined by one-way ANOVA followed by Bonferroni's post-test

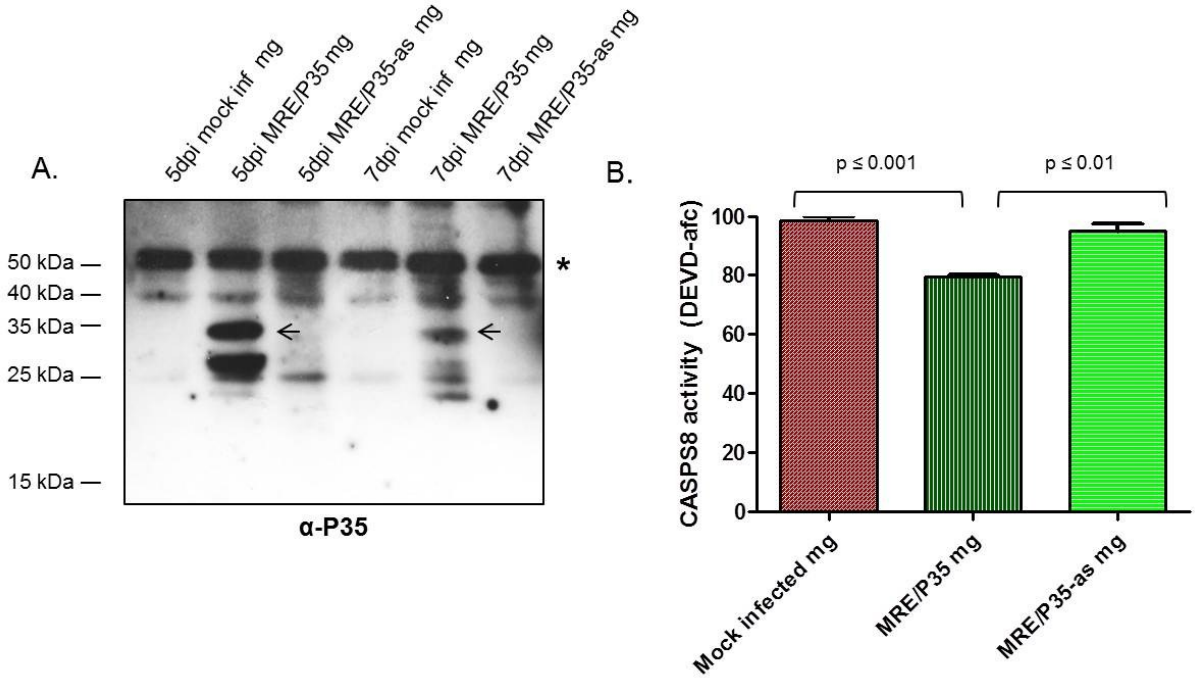

Figure 2.3. P35 expression and function in MRE/P35 infected midguts

A) Western blot analysis of infected midgut lysate showing expression of P35 protein (arrows) in MRE/P35-infected samples at 5 and 7 dpi. Non-specific bands are indicated by asterisks and serve as loading controls. B) Lysate from 7 dpi MRE/P35-infected or control midguts was incubated with recombinant CASPS8 protein and Ac-DEVD-AFC. The rate of enzymatic activity of CASPS8 was compared among samples, with that of mock-infected set at 100. Three independent biological replicates were performed and error bars indicate the standard error of the mean. Significance was determined by one-way ANOVA followed by Bonferroni's post-test.

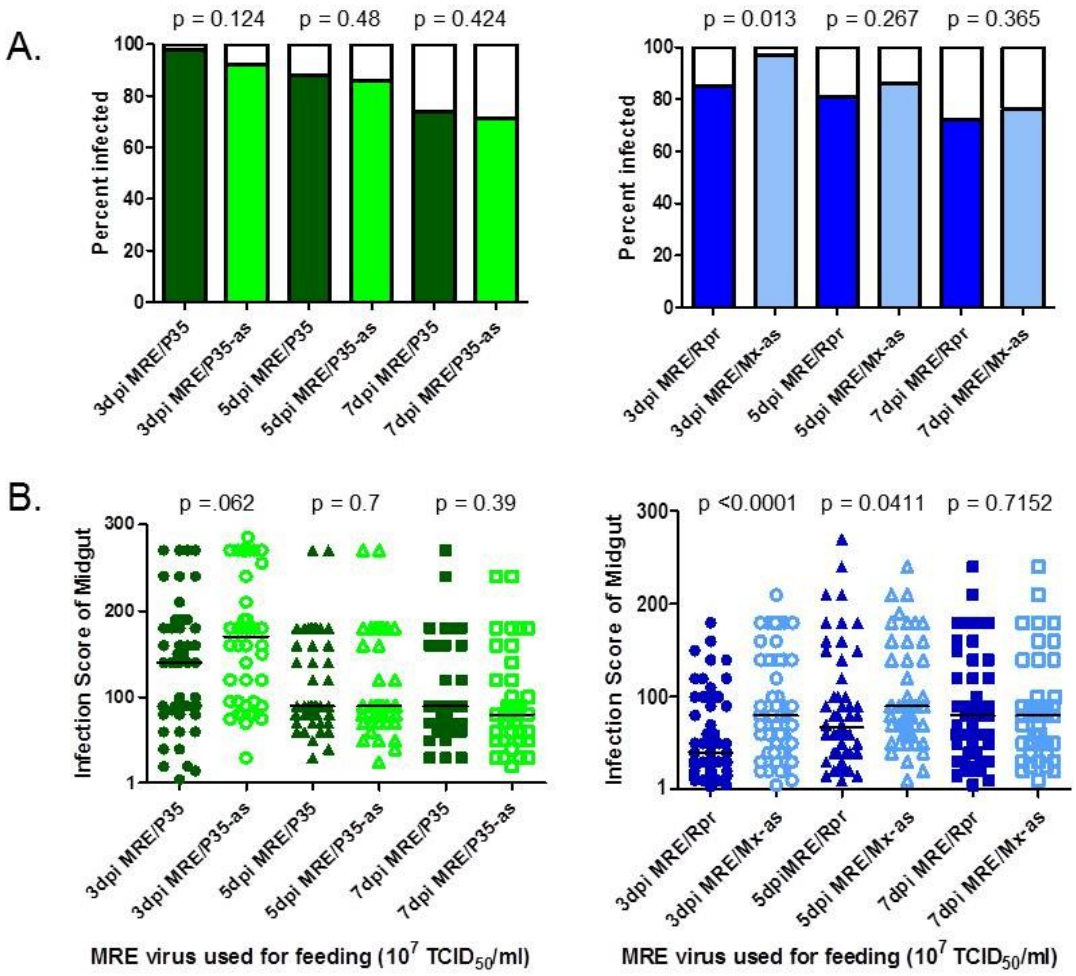

Figure 2.4. Expression of Reaper decreases midgut infection and prevalence at early stages of infection while P35 expression has no effect.

A) Prevalence of infection in midgut at different time points based on presence or absence of staining with an anti-SINV antibody. Prevalence of midgut infection in MRE/P35-infected mosquitoes was similar to controls, while that of MRE/Rpr-infected midguts was lower than controls but only significantly lower at 3 dpi. Fisher's exact test was used to determine one-tailed *p* values. B) Individual midgut infection scores determined by staining with anti-SINV antibody. Infection scores were determined as described in Materials and Methods. MRE/P35-infected midguts were not significantly different from controls. MRE/Rpr-infected midguts had significantly lower infection scores than control virus at 3 and 5 dpi. Only infected midguts were

included in the analysis of infection scores. Mann-Whitney U test was used to determine significance. Graphs represent combined results from at least 3 biologically independent experiments. Black lines indicate the median.

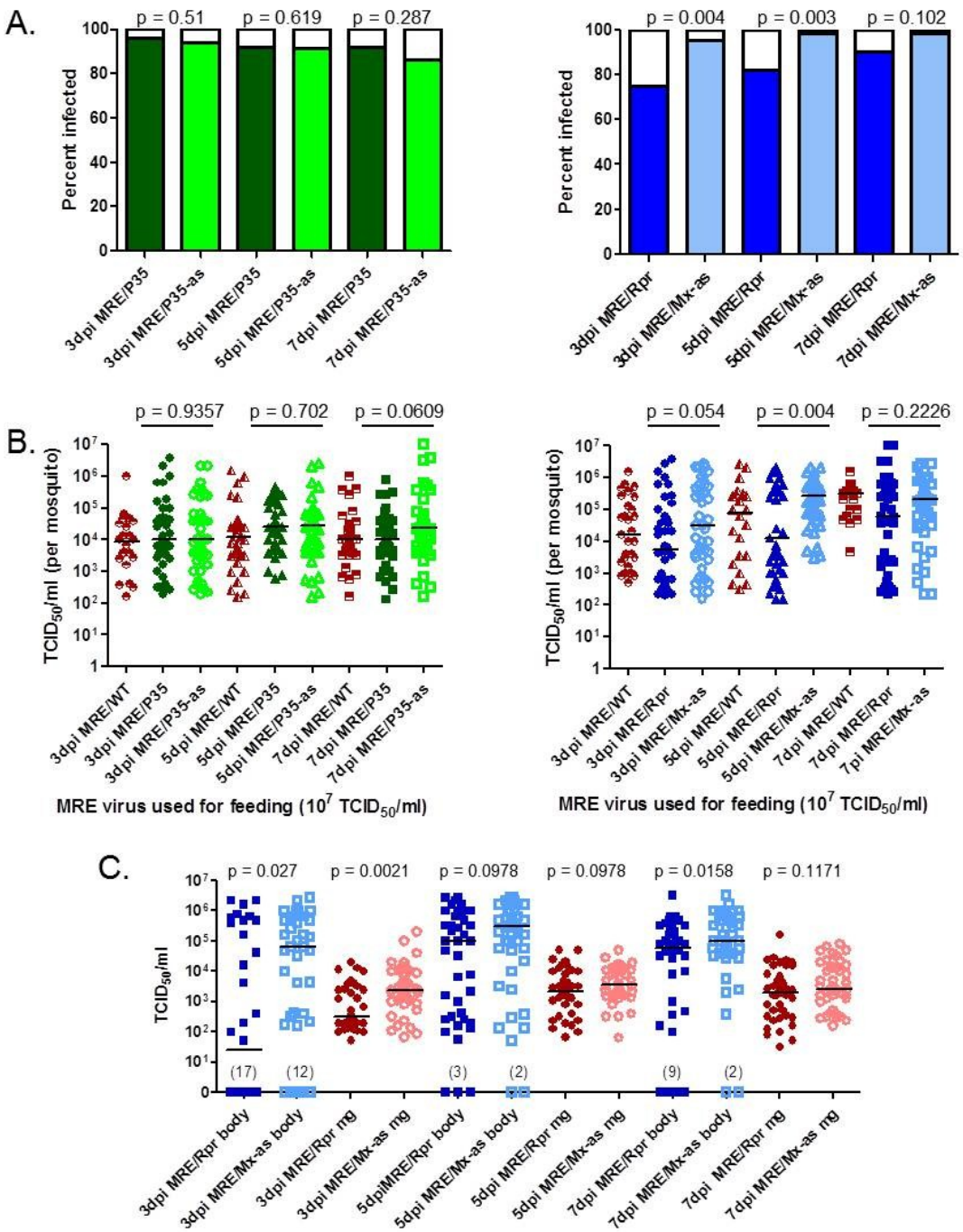

Figure 2.5. Reaper expression during virus replication lowers SINV replication and slows down dissemination in *Ae. aegypti*, while expression of P35 has no effect.

A) Prevalence of infection in titered samples. Fisher's exact test was used to determine one-tailed *p* values. No significant difference was observed between mosquitoes fed MRE/P35 and MRE/P35-as , but mosquitoes fed MRE/Rpr had significantly lower infection prevalence than

control-infected at 3 and 5 dpi and p=0.05 at 7 dpi. B) $TCID_{50}$ assays were performed to measure the amount of virus in individual mosquitoes. Only infected mosquitoes were included in the analysis and Mann-Whitney U tests were used to determine p values. Black lines indicate the median. Mosquitoes fed MRE/P35 had titers that did not vary significantly from controls at any of the selected time points. Mosquitoes infected with MRE/Rpr had lower titers than controls, but titers were only significantly lower at 5 dpi. C) Infected midguts were titered separately from the rest of the body, and results were analyzed as described above. Four to five individual biological replicates were performed for all experiments, and the results were combined.

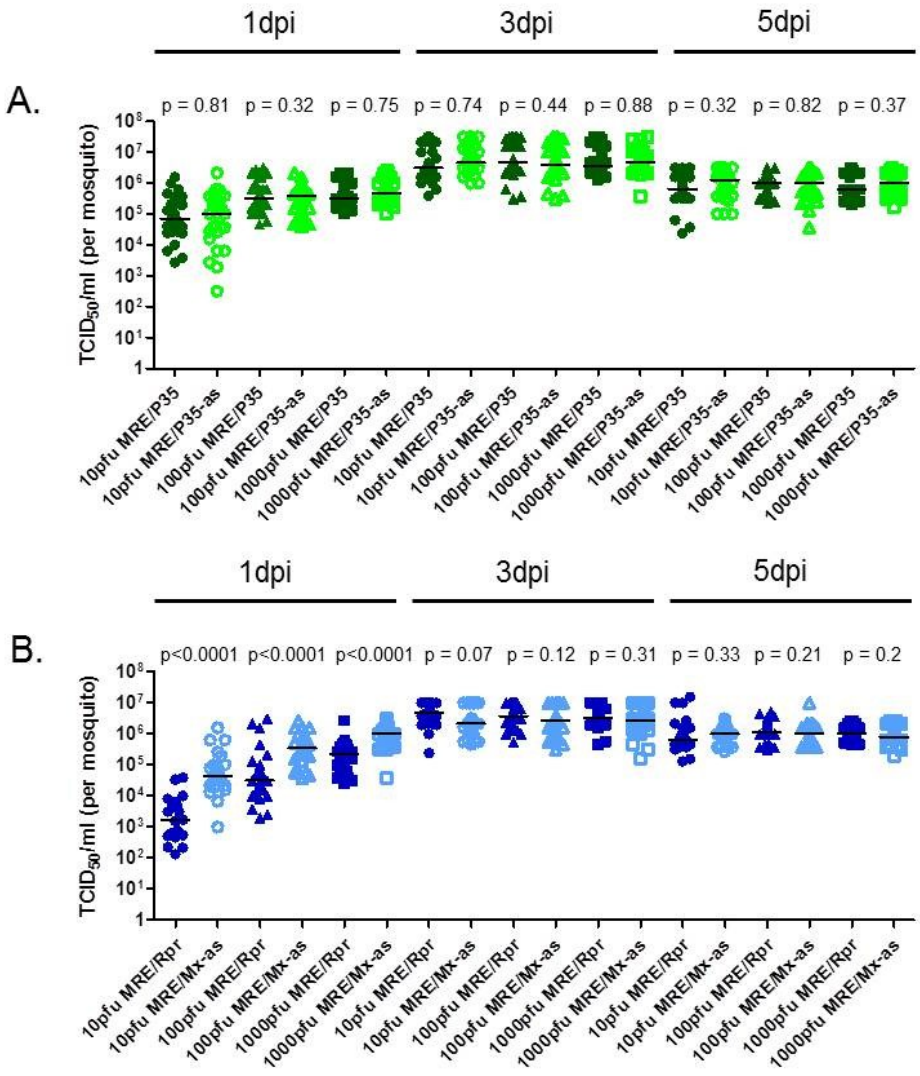

Figure 2.6. Intrathoracic infection of SINV expressing Reaper also results in lower infection and dissemination in *Ae. aegypti*.

The indicated recombinant viruses were intrathoracically injected at 3 different doses (10, 100, or 1000 PFU/mosquito). Mosquitoes were collected for $TCID_{50}$ assays at 1, 3, and 5 dpi. A) MRE/P35 infected mosquitoes had titers similar to MRE/P35-as at each dose and each time point. B) MRE/Rpr-infected mosquitoes had significantly lower titers than controls at 1 dpi for each injected dose. No significant differences were seen at 3 or 5 dpi. Only infected mosquitoes were included in the analysis and *p* values were determined using the Mann-Whitney U test. Black lines indicate the median. Graphs include 3 sets of biologically independent samples.

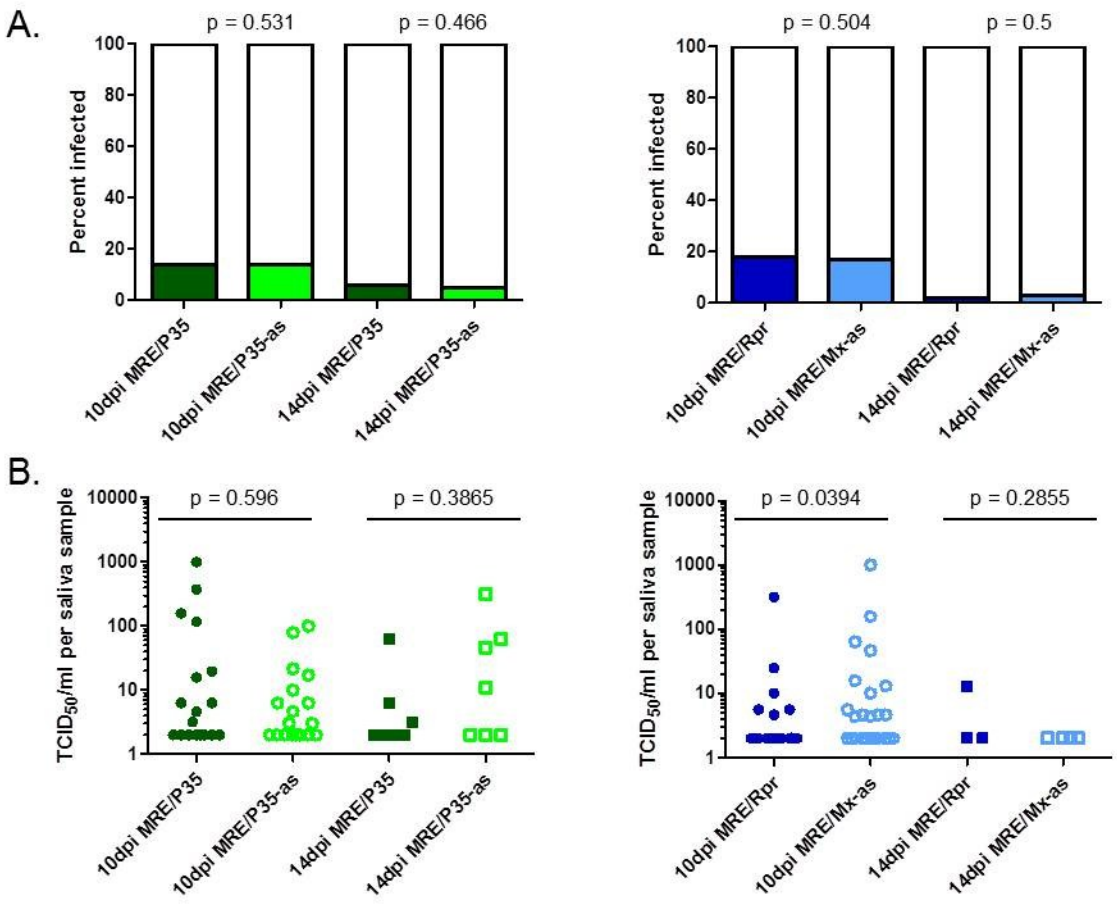

Figure 2.7. Reaper expression results in lower amounts of virus in the saliva early after the extrinsic incubation period.

Saliva was collected from infected mosquitoes at 10 and 14 dpi. A) Prevalence of virus in the saliva was higher at 10 dpi than 14 dpi for both sets of viruses. There was no significant difference in infection prevalence between experimental and control samples at either time point. Fisher's exact test was used to determine one-tailed *p*-values. B) Virus titers in individual saliva samples. Infection with MRE/P35 did not have an effect on the amount of virus in the saliva at either time point when compared to MRE/P35-as, while MRE/Rpr infection resulted in a lower amount of virus than control in the saliva at 10 dpi, but there was no significant difference at 14 dpi. Mann-Whitney U tests were used to determine *p* values for $TCID_{50}$ samples. Four independent biological replicates were performed for these experiments.

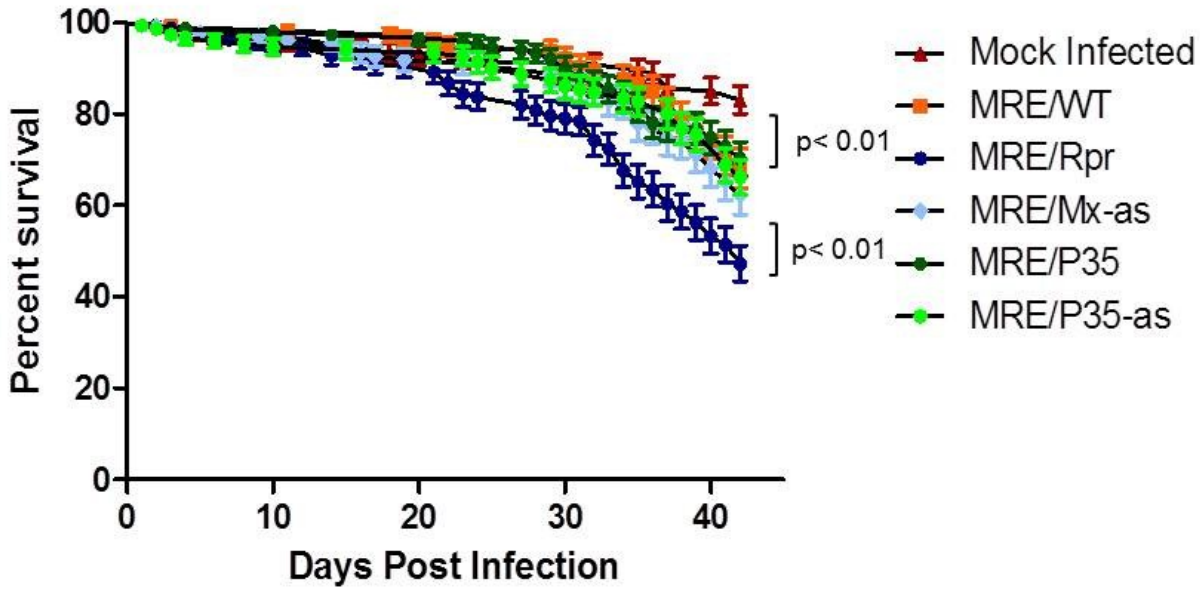

Figure 2.8. Infection with MRE/Rpr reduces the life span of *Ae. aegypti.*

Mosquitoes were orally infected with the indicated viruses or mock-infected (fed a blood meal with no virus) and were analyzed daily for mortality. Longevity assays were performed with 4 independent biological replicates. *p* values were determined by making single comparsions among samples using Mantel-Cox tests.

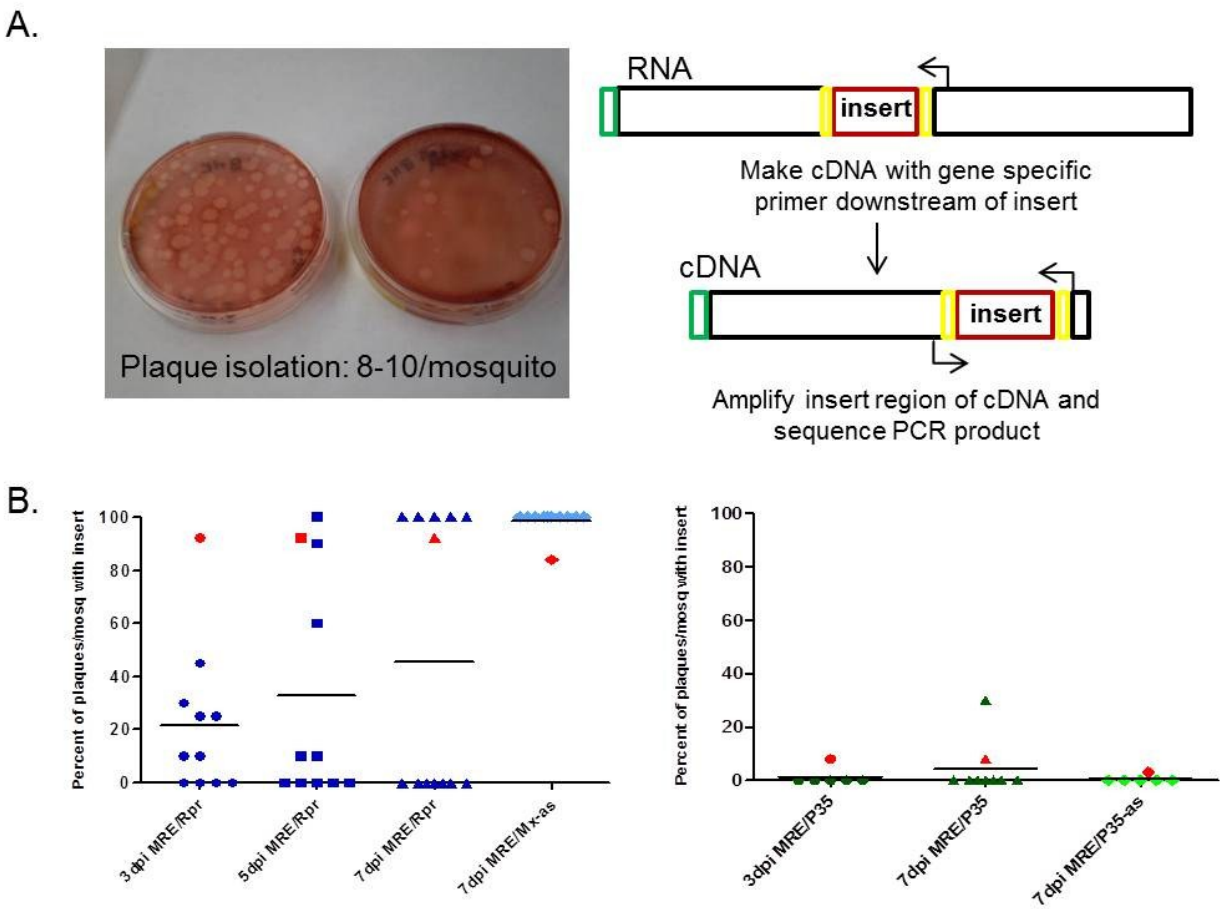

Figure 2.9. Negative selection against maintaining the inserted *reaper* gene in SINV during mosquito infection.

A) Strategy used for virus sequencing. Viruses collected from infected mosquitoes were isolated by plaque assay and the inserted secondary subgenomic region from 8-10 plaque isolates from each of 10 mosquitoes was sequenced. B) Each data point indicates the percentage of sampled viruses (n=8-10) containing intact inserts from a single mosquito. Data obtained from sequencing individual plaques isolated from stock virus are indicated by red shapes (n=25-40 plaques). Black lines indicate the mean.

Table 2.1 Plaque and titer results per mosquito and time point.

Linear regression analysis indicated a lack of correlation between whole body titer and presence or absence of the *reaper* insert ($r^2 = 0.01$, 0.03, and 0.15 at 3, 5 and 7 dpi).

Mosquito	Mosquito titer	Plaque sequence result	Mosquito	Mosquito titer	Plaque sequence result
3dpi MRE/Rpr 1	2.57x10^2	1/10 insert	3dpi MRE/P35 1	7.63x10^3	10/10 no insert
3dpi MRE/Rpr 2	1.95x10^2	10/10 no insert	3dpi MRE/P35 2	1.7x10^3	9/9 no insert
3dpi MRE/Rpr 3	4.39x10^3	9/9 no insert	3dpi MRE/P35 3	4.11x10^4	10/10 no insert
3dpi MRE/Rpr 4	2.43x10^4	8/8 insert	3dpi MRE/P35 4	4.64x10^4	9/9 no insert
3dpi MRE/Rpr 5	6.31x10^2	5/11 w insert	3dpi MRE/P35 5	1.58x10^4	8/8 no insert
3dpi MRE/Rpr 6	6.81x10^3	2/8 insert			
3dpi MRE/Rpr 7	1.58x10^4	3/10 insert	7dpi MRE/P35 1	4.64x10^3	10/10 no insert
3dpi MRE/Rpr 8	6.31x10^2	2/8 insert	7dpi MRE/P35 2	1.13x10^3	3/8 insert
3dpi MRE/Rpr 9	3.73x10^2	9/9 insert	7dpi MRE/P35 3	1.7x10^5	8/8 no insert
3dpi MRE/Rpr 10	2.15x10^5	1/10 insert	7dpi MRE/P35 4	1.58x10^4	9/9 no insert
			7dpi MRE/P35 5	3.73x10^3	10/10 no insert
5dpi MRE/Rpr 1	9.23x10^2	10/10 no insert	7dpi MRE/P35 6	3.16x10^4	8/8 no insert
5dpi MRE/Rpr 2	1.43x10^3	3/5 full insert	7dpi MRE/P35 7	4.64x10^3	8/8 no insert
5dpi MRE/Rpr 3	3.38x10^4	9/10 full insert	7dpi MRE/P35 8	1.95x10^4	10/10 no insert
5dpi MRE/Rpr 4	6.31x10^2	10/10 full insert	7dpi MRE/P35 9	4.64x10^2	10/10 no insert
5dpi MRE/Rpr 5	6.31x10^2	5/5 no insert	7dpi MRE/P35 10	1.7x10^3	10/10 no insert
5dpi MRE/Rpr 6	1.58x10^2	5/5 no subgenomic			
5dpi MRE/Rpr 7	4.64x10^5	10/10 no start site	7dpi MRE/P35-as 1	4.64x10^3	8/8 no insert
5dpi MRE/Rpr 8	3.89x10^5	9/10 deletions	7dpi MRE/P35-as 2	2.15x10^4	9/9 no insert
5dpi MRE/Rpr 9	3.73x10^6	9/10 deletions	7dpi MRE/P35-as 3	5.13x10^5	8/8 no insert
5dpi MRE/Rpr 10	1x10^3	10/10 no subgenomic	7dpi MRE/P35-as 4	1.95x10^5	10/10 no insert
			7dpi MRE/P35-as 5	3.89x10^4	10/10 no insert
7dpi MRE/Rpr 1	3.73x10^4	10/10 full insert			
7dpi MRE/Rpr 2	6.4x10^2	7/7 large deletion	7dpi MRE/Mx-as 1	1.31x10^5	10/10 full insert
7dpi MRE/Rpr 3	4.64x10^5	9/9 full insert	7dpi MRE/Mx-as 2	3.16x10^5	10/10 full insert
7dpi MRE/Rpr 4	1x10^6	10/10 full insert	7dpi MRE/Mx-as 3	2.15x10^6	9/9 full insert
7dpi MRE/Rpr 5	5.62x10^2	9/9 mutations	7dpi MRE/Mx-as 4	2.57x10^5	7/7 full insert
7dpi MRE/Rpr 6	4.64x10^5	10/10 full insert	7dpi MRE/Mx-as 5	7.63x10^5	8/8 full insert
7dpi MRE/Rpr 7	2.6x10^6	8/8 full insert	7dpi MRE/Mx-as 6	5.13x10^5	9/9 full insert
7dpi MRE/Rpr 8	2.57x10^5	10/10 no insert	7dpi MRE/Mx-as 7	3.21x10^5	9/9 full insert
7dpi MRE/Rpr 9	1.58x10^6	9/9 no insert	7dpi MRE/Mx-as 8	3.85x10^5	10/10 full insert
7dpi MRE/Rpr 10	1.08x10^4	10/10 deletion	7dpi MRE/Mx-as 9	3.88x10^5	8/8 full insert
7dpi MRE/Rpr 11	4.34X10^4	10/10 no insert	7dpi MRE/Mx-as 10	7.72x10^5	8/8 full insert
7dpi MRE/Rpr 12	2.69X10^5	10/10 deletion			
Stock Viruses	**Proportion of plaques with insert**				
MRE/Rpr stock	23/25	92%			
MRE/Mx-as stock	27/32	84%			
MRE/P35 stock	3/38	8%			
MRE/P35-as stock	1/33	3%			

Chapter 3 - Conclusions

As researchers we are looking for that piece of the puzzle that will add to the big picture or solve a problem. We each do our part adding to previous knowledge and impacting the world in a positive way. When it comes to vector-borne disease an immense amount of work has been done over many years in the hopes that the millions of people suffering from such diseases may be helped. With technological and intellectual advances we come closer and closer to alleviating the burden of certain vector transmitted pathogens.

In this work we show for the first time that apoptosis acts as an antiviral defense in *Ae. aegypti* mosquitoes. Therefore, apoptosis has potential as a defense against arbovirus transmission. Increasing apoptosis during virus infection did not fully protect against infection or transmission. However, results showing lower virus infection and delayed replication are encouraging. Various things to consider and further investigate certainly remain.

This study was done with non-endogenous apoptotic genes. It would be valuable to look at infection using SINV MRE-16 clone expressing endogenous genes such as IAP antagonists Mx or IMP. Expression of endogenous genes may have a more dramatic antiviral effect. However, there is the possibility that viral replicative intermediates could lead to knockdown of endogenous factors by triggering an RNAi response (Foy and Olson, 2008). It will also be important to look at the effects on the mosquito RNAi pathway when apoptosis is increased in *Ae. aegypti*. Increased apoptosis had suppressive effects on RNAi in *Drosophila* but this has yet to be shown in the mosquito (Xie et al., 2011).

We demonstrated with individual plaque sequences that the pro-apoptotic gene *reaper* was poorly maintained in infected mosquitoes and likely selected against due to its negative effects on virus infection or replication. It may be beneficial to perform deep sequencing with infected mosquito samples as a comparative method to fully analyze how much virus retains the

insert and at which time point it is generally lost. Plaque sequences or deep sequencing of mosquito tissues or saliva would be interesting as well. This may show if the selective pressure is higest in the midgut or if it is present in the salivary glands and if salivated virus was able to retain the insert.

Due to negative effects that occur with widespread gene knockdown and potential loss of the inserted sequences when using the SINV expression system, alternative methods to examine apoptosis on virus infection in *Ae. aegypti* are needed. Transgenic mosquitos that express or knock-down either pro- or anti-apoptotic genes in a tissue specific manner would be an exciting system to study virus-vector interactions. Utilizing the carboxypeptidase A promoter, genes could be expressed or knocked down after a blood-meal in the midgut (Edwards et al., 2000; Khoo et al., 2010; Moreira et al., 2000). Early induction of apoptosis in the midgut has been suggested to be important for inhibiting baculovirus spread in *Ae. aegypti* (Liu et al., 2011). Expression or knock-down in the salivary glands may be more challenging but would be interesting as well. Apoptosis has previously been observed in salivary gland tissue during flavi- or alphavirus infection and is thought to be a possible antiviral defense in the salivary glands (Girard et al., 2007; Kelly et al., 2012). In transgenic mosquitoes other arboviruses could easily be tested as well. SINV is not naturally transmitted by *Ae. aegypti*. Therefore, it will be important to look at the effects of apoptosis on viruses such as dengue or chikungunya, which are naturally transmitted by *Ae. aegypti* (Gubler, 1997; Pialoux et al., 2007).

Considering the big picture, transgenic mosquitoes may bring hopes to reality when it comes to creating a non-transmitting mosquito. As we approach this reality questions arise regarding what to do with these transgenic lines. Transgenic lines most likely have fitness costs that come with the genetic manipulations they have undergone and possible genetic load they

have been given. How will they survive in the wild? How will they ever outcompete and replace transmitting mosquitoes? Releasing transgenic lines into the wild will likely dilute the anti-pathogen genes they possess and diminish their ability to inhibit transmission (James, 2005).

In the past several years gene drive systems have been posed to ensure the success of transgenic non-transmitting mosquitoes. Gene drive systems exhibit non-Mendelian genetics and utilize selfish gene mechanisms (Curtis, 1968; Knipling et al., 1968). Selfish genetic mechanisms will make sure anti-pathogen genes will be expressed in following generations regardless of fitness costs (Dawkins, 1976). Gene drive mechanisms have been directly developed from or simply inspired by naturally occurring selfish genes. Gene drive systems can be used to spread anti-pathogen genes through mosquito populations replacing transmitting mosquitoes with non-transmitting populations. Multiple selfish genetic elements exist that may be utilized as gene drive systems. Ones that are well known and understood will most likely produce an effective drive system. Unfortunately not all of the proposed systems are found in mosquitoes but at this point they cannot be ruled out as possible mosquito drive mechanisms. Several of the proposed mechanisms are transposable elements, homing endonucleases, engineered underdominance, meiotic drive, *Medea* elements, Wolbachia endosymbionts, and RIDL techniques (Marshall, 2009).

Gene drive systems will allow us to apply transgenic research in vector disease biology and administer a cure by targeting the vector. Of course a cure will never be reached using just one technique. It will take multiple approaches and strategies to ever completely eradicate a vector-borne disease. At least by targeting the vector many people may be positively influenced. Certain approaches such as insecticidal sprays, bed nets, window screens, and vaccines have been very successful but not everyone is reached with these approaches due to political,

economical and social barriers. Resistance to insecticides is also a recurring issue. By replacing transmitting mosquito populations with non-transmitting ones, large areas will be reached and the disease burden in these areas lightened. Another benefit of gene drive systems is that they will replace the vector but not eliminate them. By replacing the vector with a different population, negative effects due to dynamics of the vector and its surroundings (i.e., environment, predators, and communal species) will be avoided. Their niche will continue to be filled.

Obviously there is a lot of work yet to be done. Incredible amounts of work have been done in vector disease research in the past years and strides are made daily towards answers and solutions. With multiple minds and talents at work, the pieces to the puzzle will continue to be put in their place.

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Appendix A - BMP signaling and *spadetail* regulate exit of muscle precursors from the zebrafish tailbud.

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Abstract

The tailbud is a population of stem cells in the posterior embryonic tail. During zebrafish development, these stem cells give rise to the main structures of the embryo's posterior body, including the tail somites. Progenitor cells reside in the tailbud for variable amounts of time before they exit and begin to differentiate. There must be a careful balance between cells that leave the tailbud and cells that are held back in order to give rise to later somites. However, this meticulous process is not well understood. A gene that has shed some light on this area is the t-box transcription factor *spadetail (spt*). When *spt* is mutated, embryos develop an enlarged tailbud and are only able to form roughly half of their somites. This phenotype is due to the fact that some of the somitic precursors are not able to leave the tailbud or differentiate. Another factor involved in tail morphogenesis is the Bone Morphogenetic Protein (BMP) pathway. BMPs are important for many processes during early development, including cell migration. *Chordino* (*chd)* is a secreted protein that inhibits BMP signaling. BMPs are upregulated in *chd* mutants, however, these mutants are able to form organized somites. In embryos where *chd* and *spt* are mutated, somites are completely absent. These double mutants also develop a large tailbud due to the accumulation of progenitor cells that are never able to leave or differentiate. To study the dynamics of cells in the tailbud and their role in somite formation we have analyzed the genetic factors and pathway interactions involved, conducted transplant experiments to look at behavior of mutant cells in different genetic backgrounds, and used time lapse microscopy to characterize cell movements and behavior in wildtype and mutant tailbuds. These data suggest that *spt* expression and BMP inhibition are both required for somitic precursors to exit the tailbud. They also elucidate that *chd;spt* tailbud mesodermal progenitor cells (MPC) behave autonomously and their dynamics within the tailbud are drastically different than WT MPCs.

Introduction

In zebrafish, mesodermal precursors are continuously generated both during and subsequent to gastrulation in order to form trunk and tail somites (Agathon et al., 2003; Kimmel et al., 1990; Szeto and Kimelman, 2006). A careful balance between proliferation, migration, and differentiation among these precursors must be struck to ensure that as some progenitors differentiate and contribute to somite formation, others are maintained in an undifferentiated state in order to contribute to somites formed later in development. The BMP signaling pathway has been shown to be essential for proper specification and patterning of mesodermal progenitors (Myers et al., 2002; Row and Kimelman, 2009; Szeto and Kimelman, 2004, 2006). During gastrulation, a gradient of BMP activity is established by the complex interplay between BMP ligands, expressed most highly on the ventral side of the embryo, and secreted BMP inhibitors such as Chordin and Noggin, which are expressed dorsally (Dal-Pra et al., 2006; Furthauer et al., 1999; Myers et al., 2002; von der Hardt et al., 2007). This BMP gradient not only patterns mesodermal cell fates along the dorsal/ventral (DV) axis, but also regulates morphogenetic movements during gastrulation, ensuring that, for example, lateral mesodermal precursors converge towards the dorsal midline where they can contribute to trunk somites, while the ventral-most progenitors are directed to the tailbud, where they will subsequently form tail somites (Ho and Kane, 1990; Kanki and Ho, 1997; Kimmel et al., 1990; Myers et al., 2002; von der Hardt et al., 2007).

Patterning and morphogenesis of trunk and tail mesoderm is also under the control of members of the T box family of transcription factors: *no tail(ntl)*, *brachyury(bra)*, and *spadetail(spt)* (Griffin et al., 1998). *ntl* and *bra* function redundantly to maintain a population of mesodermal progenitors that contribute to the somites of the posterior trunk and tail (Martin and

Kimelman, 2008). *ntl* and *bra* form a positive regulatory loop with two Wnt genes, *wnt3a* and *wnt8a* (Martin and Kimelman, 2008). In embryos lacking both *ntl* and *bra* or both *wnt3a* and *wnt8a*, only the anteriormost 8-10 somites are formed (Martin and Kimelman, 2008; Shimizu et al., 2005; Thorpe et al., 2005). It has been proposed that this phenotype reflects the failure to maintain a population of mesodermal progenitor cells. In the absence of *ntl/bra* or *wnt3a/wnt8a*, the initial population of progenitors is quickly exhausted, leading to a severely truncated embryo (Martin and Kimelman, 2008, 2009; Thorpe et al., 2005).

spt is required for the formation of trunk somites. *spt* mutant embryos have only a few scattered muscle cells in the trunk, but no somites (Kimmel et al., 1989). The tailbud of *spt* embryos is significantly enlarged (the "spade" phenotype) although tail somites are formed normally. Detailed cellular analysis has shown that in *spt* mutants, trunk somite precursors, rather than converging towards the dorsal midline, are instead carried by epiboly movements to the vegetal pole, where they contribute to the enlarged tailbud (Ho and Kane, 1990). Curiously, these misplaced trunk mesodermal progenitors are unable to exit the tailbud and contribute to tail somites, remaining trapped in the tailbud through the completion of tail development. This phenotype indicates a key difference between 'endogenous' tail mesodermal progenitors derived from the ventral margin, which exit the tailbud normally in *spt* mutants, and the misplaced progenitors derived from the lateral margin, which cannot (Ho and Kane, 1990). The nature of the difference between these two populations of mesodermal progenitors remains unclear, although one possibility is that exposure to different levels of BMP during pregastrula stages could play a role. BMP signaling occurring between 4-5 hours post fertilization (hpf) is thought to program a subset of mesodermal progenitors to move to the tailbud and begin forming somites only later, during tail development (Szeto and Kimelman, 2006). Normally, these cells derive

only from the ventral margin, where BMP activity is highest (Ho and Kane, 1990; Kimmel et al., 1990; Myers et al., 2002; Pyati et al., 2005). It may be that cells derived from the lateral margin, where BMP signaling is lower, are in some way not competent to respond to later cues that govern exit from the tailbud.

Some insight into the mechanisms governing tailbud exit comes from genetic analysis of double mutants between *spt* and *one eyed pinhead(oep)*, an essential cofactor in Nodal signaling, which has uncovered a role for *spt* and Nodal in this process (Griffin and Kimelman, 2002; Gritsman et al., 1999; Zhang et al., 1998). As in *spt* embryos, formation of tail somites in *oep* mutants occurs normally (Hammerschmitt et al., 1996a). However, in *spt,oep* double mutant embryos, a dramatic defect in posterior mesoderm development is observed. Not only are the scattered muscle cells observed in *spt* single mutants completely absent, but tail somitic muscle is also missing. These embryos fail to downregulate the expression of mesodermal progenitor marker genes such as *ntl* and *wnt8a* in the tailbud, leading to the suggestion that in *spt*;*oep* embryos, progenitor cells are 'locked' into an undifferentiated state, and, being unable to progress along a differentiation program, are unable to leave the tailbud (Kelly et al., 1995; Griffin et al., 1998; Griffin and Kimelman, 2002; Schulte-Merker et al., 1992, 1994). Intriguingly, it has been shown that induced overexpression of a constitutively active BMP receptor construct (caBMPR) early in tail development resulted in embryos with expanded *ntl* expression in the tailbud and a transient defect in tail extension (Row and Kimelman, 2009). This result suggests that BMP signaling, in addition to an early role setting aside a population of mesodermal progenitors for tail development, might act later, during tail development, in governing the exit of these cells from the tailbud. This effect may not be direct, as overexpression of caBMPR led to a downregulation of secreted Wnt inhibitors in the presomitic

mesoderm just anterior to the tailbud (Row and Kimelman, 2009). There are multiple Wnts expressed in the tailbud, both those that signal through β-catenin, such as *wnt3a* and *wnt8a*, as well as noncanonical Wnts like *pipetail(ppt)/wnt5*, which has been shown to regulate cell movements during gastrulation (Clements et al., 2009; Kelly et al., 1995; Lekven et al., 2001; Liu et al., 1999; Rauch et al., 1997). caBMPR overexpression does lead to increased nuclear β-catenin, indicating that the observed decrease in the expression of secreted Wnt inhibitors does have an effect on Wnt responsiveness in the tailbud (Row and Kimelman, 2009).

However, much remains unclear concerning the role of BMP in regulating exit from the tailbud. Neither *chordino* nor *ogon* mutant embryos, which carry mutations in the secreted BMP inhibitors Chordin and Sizzled, respectively, show any defects in tail somite formation, although it is possible that BMP activity is not sufficiently elevated in these mutants to cause a tailbud exit phenotype (Hammerschmidt et al., 1996b, 1996c; Martyn and Schulte-Merker, 2003; Miller-Bertoglio et al., 1999; Schulte-Merker et al., 1997). Also, it is unknown how BMP signaling ties in with the previously described roles for *spt* and Nodal signaling in governing exit from the tailbud.

To address these open questions, we have undertaken an analysis of tail development in WT and *spt* mutant embryos in which BMP signaling has been altered. We have found that *chd;spt* double mutant embryos exhibit a dramatic defect in somitic mesoderm development highly reminiscent of *spt;oep* embryos, with a nearly complete absence of muscle cells in the trunk and a total block in tail development, leading to the formation of an enormous tailbud. We show that BMP is functioning during postgastrulation stages in this process. We also use transplantation and imaging techniques to show that *spt*;caBMPR-expressing cells are

autonomously unable to leave the tailbud. Lastly, we show *chd;spt* tailbud cells exhibit drastically different behavior and morphology than cells in wildtype zebrafish tailbuds.

Methods and Materials

Fish Lines and Maintenance

Zebrafish were raised using standard techniques. Wildtype fish used were AB. We used *spt*^{b104} and a new allele of *chd* which arose via spontaneous mutation and was phenotypically indistinguishable from previously described *b215* allele (Fisher and Halpern, 1999; Griffin et al., 1998). Transgenic *flh:*eGFP were a generous gift of Marnie Halpern (Gamse et al., 2003). Mutant alleles were maintained in heterozygous fish that were outcrossed to wildtype lines. Single and double mutants were identified based on evident phenotypes.

Morpholino and RNA Injection

Morpholinos, RNA, and fluorescent dyes were injected at the one cell stage. *Spt, chd, oep, p53*, and standard control morpholinos (Lekven et al., 2001; Nasevicius and Ekker, 2000; Ramel et al., 2005) were designed and obtained from Gene Tools LLC, Philomath, OR USA. They were diluted in Danieau's buffer prior to injection. *spt* MO was injected at 3 mg/mL and *chd* MO was injected at 2 mg/mL when injected singly. In combinations they were both injected at 3 mg/mL along with *p53* MO 1 mg/mL. *oep* MO was injected at 2 mg/mL, both individually and in combination with *spt*, though in the latter case, 1 mg/mL of *p53* was added. mRNA's were constructed using mMessage mMachine kit (Ambion). They were diluted in RNAse free water prior to injection. mGFP was injected at 50 µg/mL. caBMPR (Macias-Silva et al, 1998) was injected at 4 µg/mL. Dextran Rhodamine B and dextran Alexa Fluor 488 (Invitrogen) were diluted in 0.2 M KCl. Dextran Rhodamine B was injected at 500 µg/mL and dextran Alexa Fluor 488 was injected at 50 µg/mL.

In Situ Hybridization and Antibody Staining

Digoxigenin labled RNA probes were used for in situ hybridizations using standard methods (Oxtoby and Jowett, 1993). For *ntn1b* and *tbx6* reactions *tbx6* was labeled with fluoroscein and incubated with anti-fluoroscein antibody after *ntn1b* NBT/BCIP color reaction. Embryos were washed and then stained with fast red to detect *tbx6*-expressing cells (Hauptmann and Gerster, 1994). Probes used were *myoD* (Weinberg et al., 1996), *papc* (Yamamoto et al., 1998), *ntn1b* (Strahle et al., 1997), and *tbx6* (Hug et al., 1997). P-smad 1/5/8 antibody (Cell Signaling Technology) was used at 1:100 overnight incubation. Secondary antibody was Alexa Fluor 488 anti-rabbit (Invitrogen) at 1:500.

Dorsomorphin Treatment

Dechorinated embryos were treated at 12 hpf with 63 μM of dorsomorphin using 5 mg/ml stock solution in DMSO (AMPK inhibitor, Compound C : Calbiochem). Embryos were treated overnight at 28^0C and fixed at 24 hpf.

Transplantations

Cell transplants were performed prior to gastrulation (30-50% epiboly). Labeled cells were removed from donor embryos using a manual microinjector (Sutter Instruments Co.). Cells were then transplanted to the ventral lateral margin of transgenic *flh:*eGFP embryos at corresponding stages. Embryos were mounted in 30% methyl cellulose during transplantation and placed in Ringers solution with penicillin and streptomycin for recovery afterward. Transplants were screened post gastrulation to look for fluorescence in the tailbud. Only embryos with fluorescence in the tailbud were screened after somitogenesis.

Time Lapse Imaging and Cell Measurements and Tracking

Time lapse was performed using a laser scanning confocal microscope (Zeiss LSM 5 Pascal, KSU Microscope Facility). Embryos were mounted in low melt agarose and methyl cellulose. Scans were recorded every 90 seconds. Images were merged and compiled into videos using ImageJ. Cell movements were demonstrated using manual cell tracking in ImageJ. Cell length and width were measured using ImageJ draw and measure tools (Rasband, 1997-2009). Cells posterior of the notochord were used for measurement data. Length was measured perpendicular to the notochord and width was measured parallel to the notochord of embryos.

Results

Phenotype of *chd;spt* embryos

To approach the question of what allows cells to exit the tailbud and pattern somites we began by looking at *spadetail* (*spt*) mutants (Fig. A.1B). An outstanding question regarding these mutants is why endogenous muscle precursor cells (those derived from the ventral margin) are able to leave the tailbud when tail somite development commences, but ectopic precursors (those from the lateral margin) remain trapped. Szeto and Kimelman suggested that exposure to high levels of BMP signaling during gastrulation directs muscle progenitors to adopt a tail somite identity (2006). One possible explanation for the *spt* phentotype, then, is that the laterally derived precursors are not exposed to sufficiently high levels of BMP during gastrulation to specify them as tail somite progenitors. These ectopic cells may then be unable to respond to cues within the tailbud that direct their exit during tail somitogenesis.

If this were the case, we reasoned that by increasing the levels of BMP signaling during gastrulation, we might be able to reprogram the lateral muscle progenitors from a trunk somite fate to a tail somite fate, perhaps allowing them to exit the tailbud properly. To test this idea, we

constructed a double mutant line between *spt* and *chordino(chd)*. *chd* mutant embryos exhibit higher levels of BMP signaling during gastrulation, and have slightly smaller trunk somites and enlarged tail somites, though all cells are able to exit the tailbud normally (Schulte-Merker et al., 1997) (Fig. A.1C).

If exposure to higher levels of BMP were able to direct misplaced *spt* muscle precursors to exit the tailbud, we would expect to see rescue of the enlarged 'spade' tail phenotype. In contrast, we observed a dramatic enhancement of the tailbud phenotype in *chd;spt* embryos (Fig. A.1D). These embryos exhibited a significantly enlarged tailbud compared to *spt* single mutants (Fig. A.1B), and a nearly complete failure to generate any tail somites. We confirmed the absence of somites by staining *chd;spt* double mutant embryos with *myoD* (Weinberg et al., 1996), (Fig. A.2A-D). In most embryos (92%, n=65), we observed only a few scattered *myoD*-positive cells in the trunk and tail, and no organized somites at all. In rare cases (8%), *chd;spt* embryos made 2-3 small somites in the tail.

Production of MPCs in *chd;spt* embryos

One possible explanation for the lack of somites in *chd;spt* embryos could be that they are not producing somitic progenitor cells. We scored embryos for the presence of mesodermal progenitor cells (MPCs), as well as differentiating muscle cells outside the tailbud by in situ hybridization. We used *ntn1b* to label MPC's that had exited the tailbud and *tbx6* as a marker for progenitor cells within the tailbud (Hug et al., 1997; Strahle et al., 1997). In wild type, *chd*, and *spt* embryos, we observed MPCs in the tailbud as well as anterior to the tailbud (Fig. A.2E-G). In contrast, in *chd;spt* mutants, we saw an accumulation of MPCs in the tailbud with a complete absence of muscle cells anterior to the tailbud (Fig. A.2H). We also used *papc* to label progenitor cells in WT and mutant embryos at a later stage (Yamamoto et al., 1998). All backgrounds

contained MPCs in the tailbud, with double mutants again having a large accumulation of progenitor cells in the tailbud (Fig. A.2I-L). Therefore, *chd;spt* embryos are able to produce MPCs; these cells are simply not able to leave the tailbud and differentiate to form somites. This indicates that *spt* and *chd* redundantly promote exit of MPCs from the tailbud.

BMP activity levels in *chd* and *oep* mutant backgrounds

Previous studies have shown that a similar tailbud phenotype results when *spt* is knocked down in combination with *one-eyed pinhead (oep)*. *oep;spt* mutants have an enlarged tailbud and lack somites (Griffin and Kimelman, 2002). Oep is a required co-receptor in the Nodal pathway and has been indicated to act as an upstream inhibitor of BMP (Kiecker et al, 2000). Interestingly, *fgf8*, a transcriptional target of Nodal signaling in late blastula stage embryos, has been shown to inhibit the transcription of BMP ligands (Furthauer et al., 1997, 2004). This raises the possibility that Nodal signaling might regulate tailbud exit via inhibition of BMP activity. We therefore tested whether *oep* and/or *oep/spt* embryos exhibited elevated levels of BMP signaling in the tailbud, using an anti-phospho Smad-1,5,8 (p-Smad) antibody.

We looked at levels of active BMP in the tailbuds of WT, single, and double mutants. We observed that both *chd* mutant (Fig. A.3B) and *oep*MO (Fig. A.3F) embryos had higher levels of p-Smad staining in their tailbuds compared to WT (Fig. A.3A,E). *chd-/-;spt-/-* (Fig. A.3D) and *oep;spt*MO (Fig. A.3G) embryos also had high expression in their tailbuds, although this expression seemed concentrated in patches. These data suggest that both *oep* and *chd* mutants have high levels of BMP activity in their tailbuds. However, this result also indicates that merely having high levels of BMP does not interfere with tailbud exit, as *oep* and *chd* single mutants have normal tails. Only when high levels of BMP activity are combined with the absence of *spt* function is tailbud exit impaired.

Timing of BMP requirement in tailbud exit

Our phospho-Smad staining results suggested that elevated BMP levels could contribute to the tailbud phenotype in *oep;spt* embryos. If this were the case, we reasoned that we may be able to rescue the tailbud phenotype of *oep;spt* double mutants by inhibiting BMP by other means. To test this possibility, we used dorsomorphin, a small molecule inhibitor of the BMP pathway. Dorsomorphin selectively inhibits BMP type I receptors, blocking downstream phosphorylation of Smad proteins (Yu et al., 2008). Treating *oep;spt* embryos with dorsomorphin should rescue them to a *spt* phenotype if BMP inhibition is the only role *oep* is playing in tailbud exit. We also treated *chd;spt* embryos in parallel. We treated embryos at several stages with dorsomorphin, then assessed phenotypic rescue by staining embryos with *myoD* to score for the presence of somitic muscle. When treated at gastrulation and pre-somitogenesis stages no rescue was observed in *chd;spt* or *oep;spt* embryos (data not shown). However, when treated during early somitogenesis, at the 6 somite stage, partial rescue was observed in *chd;spt* embryos. Most embryos were able to form some tail somites (Fig. A.3H-I). (23/25 *chd;spt* treated embryos had 5-9 somites whereas 1/26 untreated *chd;spt* had 5-9 somites). In contrast, we observed no significant rescue of the *oep*MO*;spt*MO double mutant by any regimen of dorsomorphin treatment (data not shown, *oep*MO*;spt*MO nontreated, n=215; *oep*MO*;spt*MO dorsomorphin treated n=349). These results suggest that inhibition of BMP by *chd*, in combination with *spt* activity are required for cells to exit the tailbud, and that the role of *oep* is independent of BMP signaling. These results also indicate that inhibition of BMP is required at the 6 somite stage (12 hpf). Inhibition of BMP at this time point is required in order for MPCs to exit the tailbud and form tail somites.

BMP and *spt* regulation of tailbud exit is cell autonomous

Next we addressed the question of whether BMP regulates tailbud exit in a cell-autonomous or non-cell autonomous fashion. We performed transplantation studies to examine cell behavior in genetically mixed backgrounds. To generate *spt* donor cells or host embryos, we used *spt* MO, and to generate cells autonomously experiencing high levels of BMP, we injected embryos with caBMPR mRNA (Macias-Silva et al., 1998). *chd* mutant cells could not be used due to the fact that Chd is a secreted protein and transplanted *chd-/-* cells would be exposed to Chordin secreted from WT neighboring cells. We used doses of caBMPR mRNA that mimicked the *chd* phenotype when injected into embryos at the 1 cell stage (data not shown).

For these experiments, donor embryos were labeled with rhodamine-dextran and donor cells were transplanted at 30-50% epiboly into unlabeled host embryos. We used transgenic *flh*:eGFP host embryos so that the dorsally localized GFP expression could be used as a marker of the dorsal side of the embryo. This enabled us to target donor cells to the ventral margin even at pre-gastrula stages, allowing for efficient incorporation of donor cells into the tailbud. Transplants were screened post-gastrulation to verify that transplanted cells were localized exclusively in the tailbud. Only transplants with obvious fluorescence localized to the tailbud were used for screening after somitogenesis. Once somitogenesis was complete, embryos were examined with confocal microscopy to see if transplanted cells were able to contribute to somites or if they remained in the tailbud.

As expected, caBMPR donor cells were able to leave the tailbud in WT embryos and contribute to tail somites (n=9; Fig. A.4A). Donor cells were found in a range of somites, the most anterior somite containing donor cells was used to categorize recipient embryos. Somites were labeled 1-31 with 1 being the most anterior and 31 being the most posterior somite. Out of

9 recipient WT embryos, 7 had caBMPR donor cells in somites 11-15 and 2/9 had donor cells in the most anterior group of somites, 16-20 (Fig. A.4E). We also observed that transplanted *spt* MO cells were able to leave the tailbud of WT hosts and contribute to anterior and posterior tail somites (n=20; Fig. A.4B, 10/20 had donor cells in somites 11-20 and 10/20 had donor cells in somites 21-31, Fig. A.4F). However, caBMPR mRNA + *spt* MO injected-cells were not able to efficiently leave the tailbud. Most cells remained in the tailbud at 48 hpf (n=14; Fig. A.4C). Occasionally a few fluorescent cells could be found in posterior tail somites and 3 of 14 embryos had a few cells in the anterior tail somites (Fig. A.4G). It is possible that these cells may not have been efficiently expressing caBMPR and/or had sufficient knockdown of *spt*, although we note that most *chd;spt* double mutant embryos have a few cells that are able to exit the tailbud.

To test whether cells expressing *spt* (*spt* +/+) are able to leave the tailbud of *chd;spt* hosts, we used *chd* MO donor cells. In the presence of *spadetail*, *chd* MO should not affect the ability of cells to exit the tailbud. We used these cells to exclude the possibility that wildtype donor cells might secrete enough Chd to create a localized region of relatively normal levels of BMP activity that might affect cell behavior. When *chd* MO cells were placed in a double mutant background, they were able to exit the tailbud (n=15; Fig. A.4D,H). Taken together, our transplant experiments suggest that the ability of a cell to exit the tailbud is an autonomous cell function.

In a separate set of experiments, we characterized behavior of transplanted cells in different backgrounds at time points during and post somitogenesis. For these experiments, donor embryos were labeled by injecting mGFP mRNA at the 1 cell stage, and WT lines were used for host embryos instead embryos from the *flh*:eGFP line. As a control, we examined the behavior of wildtype cells transplanted in wildtype hosts. Donor cells freely intermixed with

host cells and left the tailbud at different times (Fig. A.5A). Embryos were screened at 18 hpf and 48 hpf to determine the placement of transplanted cells. Transplanted cells were differentiated as muscle cells and were found in a range of tail somites (n=11; Fig. A.5C). Double mutant cells (caBMPR mRNA + *spt* MO) in WT background were not able to efficiently exit the tailbud. Further, they did not intermix with wild type host cells, instead remaining together in a tight clump (Fig. A.5B). When scored at 48 hpf, caBMPR mRNA + *spt* MO donor cells were seen in the very tip of the tail, lacking muscle morphology; 14/18 embryos had transplanted cells exclusively in the tailbud at this time (Fig. A.5D). As seen previously, *chd*MO cells in double mutant background (*chd*MO;*spt*MO) did mix with double mutant tailbud cells and some were able to exit the tailbud (n=2, Fig. A.6A). Usually some cells would exit and some would remain behind. The cells that were able to leave the tailbud seemed to be due to tail extension even though extension in double mutants is severely reduced. As expected, double (*chd*MO;*spt*MO) into double mutant (*chd*MO;*spt*MO) transplants showed intermixing of donor cells and host cells, although the donor cells did not exit the tailbud (n=2, Fig. A.6B). Taken together, our observations indicate that exit of MPCs from the tailbud is a cell autonomous process. Further, the clumping behavior of caBMPR mRNA + *spt*MO cells in a wild type background suggests that these cells may differ in their adhesive properties from the surrounding wild type cells.

Cell movement in *chd;spt* mutant tailbuds are perturbed

We next used time lapse imaging to look at detailed cell movements in the tailbuds of WT and mutant embryos. This allowed us to look at dynamics that take place as tail extension and somite formation occurs. Embryos were labeled with membrane targeted GFP by injecting mRNA at the one cell stage. Embryos with strong expression in the tailbud were mounted in low

melt agarose and used for confocal microscopy time lapse. Agarose was cleared from around the tail to allow for proper extension. Tailbud cells posterior of the notochord were measured at early and late somitogenesis stages. Length was measured perpendicular to the notochord and width was measured parallel to the notochord (Fig. A.7). WT movements occurred as previously described by Kanki and Ho (1997). As the tail extends, the notochord moves posteriorly and cells in the posterior tailbud move away from the midline and migrate anteriorly where they form somites. Cells are polarized perpendicular to the midline which corresponds with their movement away from the midline. WT tailbud cells have 1.7:1 length to width ratio just prior to tail somite formation (10 somites; cells=102, 3 individuals, Movie1) and a 2.3:1 ratio during later stages (20-22 somites; cells=55, 2 ind.). *chd* mutants display similar cell movements but are not as drastically polarized if at all. They have a 1.1:1 length to width ratio at 10 somite stage (cells=105, 2 ind, Movie2) and a 1.4:1 ratio at 22 somite stage (cells=60, 2 ind.). This may be attributed to the fact that *chd* tailbuds are larger and contain more cells that WT tailbuds until late stages of somitogenesis. *spt* mutant embryos exhibit similar subduction movements to WT during early tail somite formation; however at later stages (18-25 somites) cells were not as dynamic and didn't exhibit obvious movement away from the midline. Most cells in the tailbud at these stages are likely trunk precursors and will remain in the tailbud. *spt* mutant cells display a 1.2:1 length to width ratio at 10 somite stage (cells=95, 3 ind, Movie3) and a 1.3:1 ratio at 22 somite stage (cells=100, 3 ind). They are not as polarized or active as wildtype cells and the few that are may be ones that will form tail somites. In *chd;spt* mutants the cell movements were dramatically different from WT. The notochord did not penetrate and extend into the tailbud. Instead, it moved the entire mass of cells posteriorly as it extended. Cells in the tailbud seem very cohesive and held together as a unit. They are still dynamic and intermixing but do not

seem to leave the tailbud. These cells do not appear to be polarized. They exhibit a 1:1 length to width ratio prior to when tail somites would usually be starting to form (cells=95, 3 ind. Movie4) and 1.2:1 ratio when later stages of somitogenesis should be taking place (cells=100, 2 ind). Cell movements in WT and double mutants were illustrated by using manual cell tracking in ImageJ (Fig. A.5E-F). These results indicate that cell movements in *chd;spt* mutant tailbuds are perturbed and abnormal. Cells are not polarized nor do they have a uniform migration pattern when *spadetail* is not expressed and BMP signaling is increased.

Discussion

Role of spadetail and BMP signaling in fating tail somites

A number of signaling pathways are involved in the specification of tail somites. The exact roles and complex interactions of these pathways and their components remain unclear. Our results show that *spadetail* function and appropriate levels of BMP signaling are required for cells to properly exit the tailbud and differentiate into tail somites.

In the absence of *spt* function, trunk MPCs migrate inappropriately into the tailbud, where they are never able to leave. Cells that would normally form tail somites are able to leave and do so while cells that would have formed trunk somites are stuck in the tailbud (Ho and Kane, 1990). We show through *chd;spt* mutants that high BMP does not rescue the *spt* phenotype and instead causes a more severe defect. Presomitic trunk cells that are stuck in the tailbud are not reprogrammed to a muscle fate. Instead, high BMP in the *spadetail* background leads to no formation of trunk or tail somites. However, high BMP alone does not lead to such a phenotype. *Chd* mutants form trunk and tail somites but are slightly ventrallized which results in expanded posterior tissues including a few posterior somites (Schulte-Merker et al., 1997). Only in combination with *spt* does this phenotype occur.

Cells that accumulate in the tailbud of *chd;spt* mutants are pluripotent progenitor cells based on in situ hybridizations we performed using progenitor markers such as *ntn1b*, *papc* and *tbx6* (Hug et al., 1997; Strahle et al., 1997; Yamamoto et al., 1998). High BMP may affect proper communication between cells, proper polarization, migration, or a number of molecular cues required for tailbud exit. Presomitic trunk precursors may behave differently in the tailbud due to a sensitive time window that is missed and needed in order to respond to molecular cues that lead to proper migration and differentiation. They may also have unique surface molecules which affect their ability to respond to ligands or molecules in the tailbud or may possess adhesive qualities which inhibit their mobility.

Inhibition of Nodal signaling combined with the *spadetail* mutation leads to a nearly identical phenotype to that observed in *chd;spt* mutants (Griffin and Kimelman 2002). *Oep* dependent Nodal signaling has been shown to inhibit the BMP pathway during gastrulation, and we indeed observe elevated levels of BMP signaling in the tailbuds of both *oep* and *oep;spt* mutants (Kiecker et al., 2000). However, our failure to rescue *oep;spt* mutants by inhibiting BMP signaling with dorsomorphin suggests that Nodal signaling does not regulate tailbud exit via regulation of BMP signaling. Our data are consistent with BMP and Nodal acting through independent mechanisms, though both in conjunction with *spt* function, to control exit of MPCs from the tailbud (Fig. A.8).

Wnt signaling and tailbud exit

Wnt signaling has long been known to play a role in the differentiation and migration of progenitor cells. Canonical Wnt signaling has been found to play a role in determining cell fates within the tailbud (Martin and Kimelman, 2012). However, its exact role in tailbud exit is still unclear. Row and Kimelman used a heat shock inducible, constitutively active BMP receptor

(caBMPR) to show that high levels of BMP signaling impede the exit of MPCs from the tailbud (2009). BMP is proposed to accomplish this, at least in part, by negatively regulating the expression of secreted Wnt inhibitors in the anterior tailbud. The activity of these inhibitors is thought to restrict Wnt activity to the posterior part of the tailbud. High levels of BMP result in the loss of expression of Wnt inhibitors, which is predicted to result in higher levels of both canonical and noncanonical Wnt signaling in the tailbud (Row and Kimelman, 2009). BMP regulates the activity of at least the canonical Wnt signaling pathway in the tailbud, although whether Wnt signaling regulates tailbud exit is still uncertain.

Rac and Rho have been shown to function downstream of non-canonical Wnt signaling (reviewed in Schlessinger et al., 2009). These RhoGTPases can be important for cell polarity, migration, and adhesion. Therefore, they may play a possible role in the ability of cells to exit the tailbud. Rho and Rho-associated kinase are involved in myosin phosphorylation in cell blebbing and migration (Amano et al., 1996; Kimura et al., 1996). However, Rho dependent myosin phosphorylation needed for cell blebbing does not seem to be involved in the regulation of cell blebbing in *spt-/-* tailbuds (Row et al., 2011). Rac and Rho have also been shown to be required for establishment of cadherin dependent cell-cell adhesion and actin recruitment and remodeling (Braga et al., 1997). *spt-/-* tailbud cells have been shown to be more adhesive than wildtype cells, however there is no obvious difference in cadherin levels between the two (Row et al., 2011). At this time, the specific role of Rac and Rho in mesodermal progenitor cells in the tailbud has yet to be characterized.

Cell behavior in the tailbud

Our transplant experiments show that the ability to exit the tailbud is a cell autonomous function. Specifically, transplanted wildtype cells were able to exit the tailbud irrespective of the

host background; they were even able to leave the tailbuds of *chd;spt* host embryos, despite the severe defects in extension of the embryo and nearly complete absence of any host cells exiting the tailbud.

In contrast, transplanted caBMPR mRNA + *spt* MO cells failed to exit the tailbud when transplanted into wild type host embryos. Further analysis showed that wildtype cells transplanted in wildtype background did intermix with other cells in the tailbud and exited the tailbud normally. caBMPR mRNA + *spt* MO cells did not mix with the wildtype cells in the tailbud. Instead, they formed a cluster of cells that never left. This may be due to surface adhesion proteins that caused them to form a cohesive group or their hindered ability polarize, migrate or respond to cues needed to exit the tailbud.

Our time lapse analyses also lend to these possibilities, as they indicate abnormal cell movement within *chd;spt* mutant tailbuds. As described by Kanki and Ho, cells in the posterior tailbud converge away from the midline moving laterally and then anteriorly where they are incorporated into a specific somite (1997). Cells leave the tailbud in a timely manner as tail extension occurs and ones that remain behind actively divide in the tailbud to provide progenitor cells to form later somites. Wildtype tailbud cells exhibited such movement as indicated by cell tracking. WT cells were polarized in the direction of these movements and very dynamic until incorporated into a somite. Cells in *chd* and *spt* tailbuds were not as dramatically polarized as wildtype tailbud cells. This could be due to high BMP in *chd* tailbuds. It may also be due to the large size of *chd* tailbuds prior to tail somite formation. Differences in *spt* tailbuds could be a result of the mixture of presomitic trunk precursors that will remain in the tailbud and presomitic tail precursors that will exit the tailbud. *chd;spt* mutants were not polarized perhaps owing to high BMP, high number of cells in the tailbud, inability to move past a certain point, or inability

to respond to molecular cues in order to exit. *Chd;spt* tailbud cells were slightly polarized at later time points likely because cells are overcrowded and cramped for room as the notochord extended posteriorly and pushed against the anterior border of the tailbud. These cells did not exhibit uniform migration patterns or organized movements when tracked. The exact cellular mechanisms responsible for the ability of cells to exit the tailbud are elusive at this point. Future studies may be able to determine what players are involved and how cell polarity, adhesion, and migration are regulated and employed in this process.

Conclusions

In summary, we have characterized a novel double mutant in which the t-box transcription factor, *spadetail*, and the BMP inhibitor, *chordin*, are non-functional. These double mutants have an enlarged tailbud due to an accumulation of mesodermal progenitor cells. These cells are not able to properly exit the tailbud and differentiate into somitic muscle cells. This phenotype is similar to *oep;spt* mutants. We were able to show that BMP inhibition is crucial at 12 hpf for tailbud exit to occur properly. *Chd;spt* mutants phenotypes were partially rescued at this stage when treated with a small molecule inhibitor of BMP. *Oep;spt* mutants were not rescued suggesting an alternative mechanism for *oep*'s role in this process. Transplantation studies indicate the ability of cells to exit the tailbud is an autonomous function. Time lapse data revealed wild type MPCs polarize away from the midline and move laterally and then anteriorly in order to exit the tailbud and form somites. *Chd;spt* MPCs did not exhibit organized migration patterns and scarcely polarized, if at all. They remain as a unified group of cells throughout tail extension yet they are still dynamic and intermixing within the tailbud. Studies with *chd;spt* mutants will aid in further understanding of tail formation and cues needed for tailbud exit and somitogenesis during embryonic development.

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Appendix A Figures

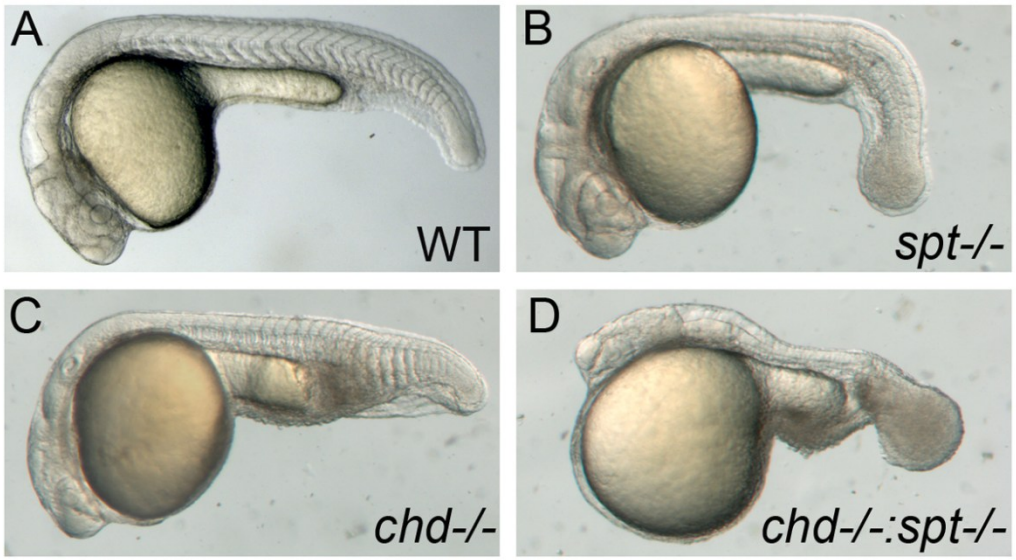

Figure A.1 *Spt* and *chd* expression are required for tail somite formation.
Live embryos were photographed at 24 hpf. All images are lateral views with left being anterior and posterior to the right. WT embryos have somites throughout the trunk and tail (A). *spt* mutants lack trunk somites and have an enlarged tailbud (B). *chd* embryos are ventrallized and have reduced trunk somites (C). *spt* and *chd* were crossed to see if *spt* phenotype could be rescued. Instead *chd;spt* mutants exhibit an even larger tailbud and no visible somites (D).

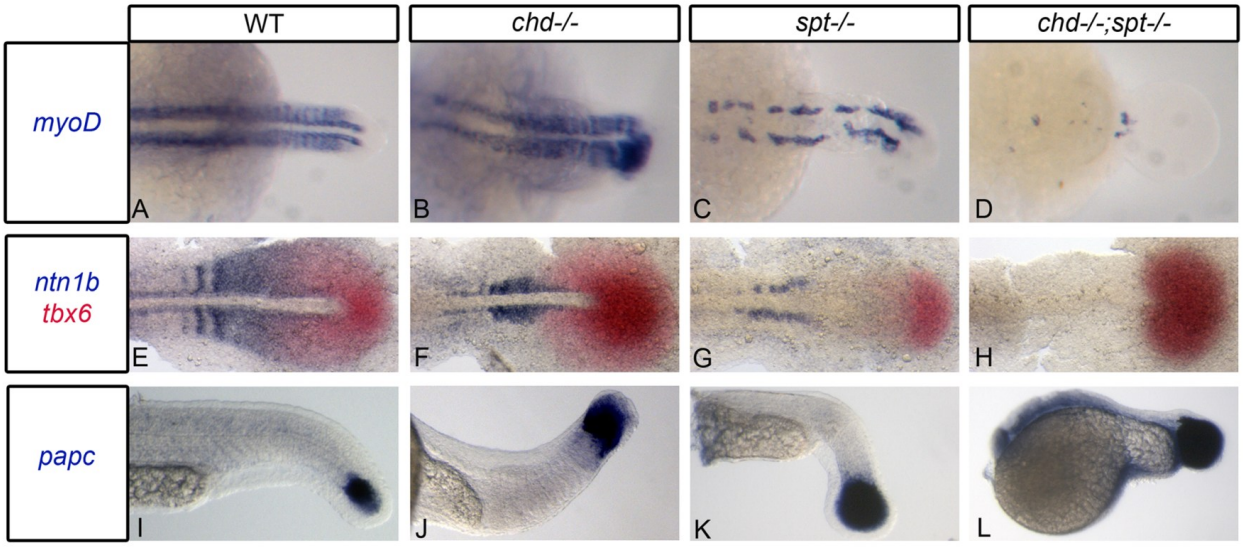

Figure A.2 *Chd;spt* mutants produce mesodermal progenitor cells in the tailbud but are not able to form somites.

A-D, Dorsal view of *myoD* expression in 24 hpf embryos. A-C, WT, *chd*, and *spt* embryos form organized somites even if it is only in the tail (*spt*). *chd;spt* mutants do not form organized somites. (60/65 have no organized somites. The remaining 5 only had 2-3 somites form.) (D). E-H, dorsal view of *ntn1b* expressing muscle cells and *tbx6* expressing MPCs in 11 hpf embryos. E-G, WT, *chd*, and *spt* embryos have MPCs in tailbud as well as differentiated muscle cells outside the tailbud. c*hd;spt* embryos have an accumulation of MPCs in the tailbud and no differentiated muscle cells. I-L, lateral view of *papc* expression in 24 hpf embryos. WT and mutant embryos have MPCs in the tailbud with *spt* and *chd;spt* embryos having a large accumulation of progenitor cells.

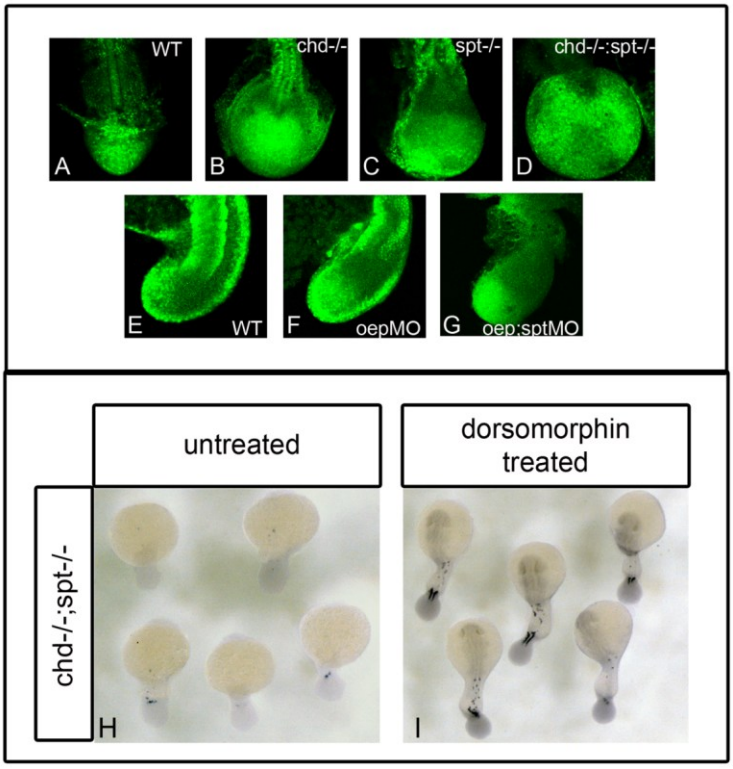

Figure A.3 BMP inhibition is required for cells to exit the tailbud and form somites. P-smad 1/5/8 antibody was used to detect BMP activity in the tailbud of embryos during somitiogenesis. A-D, dorsal view of P-smad 1/5/8 antibody fluorescence in 17 hpf (16 som) embryos; E-G are lateral views of P-smad 1/5/8 staining in 17 hpf embryos. WT embryos have normal levels of active BMP (A, E). *Chd-/-* (B) and *oep* MO (F) embryos have elevated levels of BMP, possibly due to the roles of *chd* and *oep* in BMP inhibition. *chd-/-;spt-/-* (D) and *oep*MO;*spt*MO (G) embryos also have higher levels of BMP in the tailbud, however, elevated levels are found in only some areas in the tailbud. Elevated Bmp activity and lack of *spt* leads to phenotypes where progenitor cells cannot exit the tailbud to form somites. H-I, dorsal view of *myoD* expression in 24 hpf embryos: c*hd;spt* embryos treated with dorsomorphin, a small molecule inhibitor of BMP, at 12 hpf were partially rescued and able to produce tail somites. (23/25 had 5-9 somites) (I), whereas untreated *chd;spt* embryos do not produce somites (1/26 had 5-9 somites) (H).

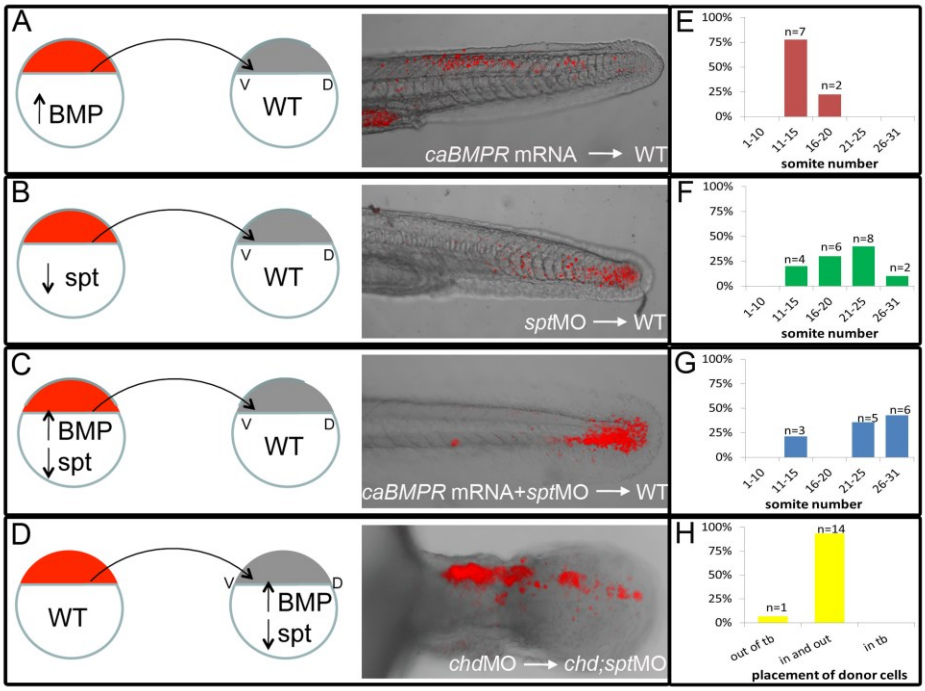

Figure A.4 Ability to exit the tailbud is a cell autonomous fate decision.

Cell transplants were performed at 30-50% epiboly stages as diagramed above. Labeled donor cells were placed on the ventral lateral margin of unlabeled host embryos. A-D, Live embryos were photographed using confocal microscopy at 30-48 hpf. E-H, Recipient embryos were scored based on the most anterior somite containing donor cells. Somite 1 being the most anterior and 31 being the most posterior. Cells injected with caBMPR mRNA 4 µg/mL and placed in a WT host were able to exit the tailbud and differentiate in posterior and anterior tail somites, n=9 (A, E). Cells injected with *spt* MO 3 µg/mL were also able to able to leave the tailbud in WT background and contribute to tail somites, n=20 (B, F). Cells injected with caBMPR mRNA + *spt* MO were not able to efficiently leave the tailbud in WT backgrounds. A few donor cells could be occasionally found in posterior tail somites and 3 embryos had donor cells in anterior tail somites. However, the majority of transplanted cells were still in the tailbud at 48 hpf, n=14 (C, G). When *chd* MO cells were placed in a *chd*MO;*spt*MO background they were able to exit the tailbud and migrate anteriorly, n=15 (D, H).

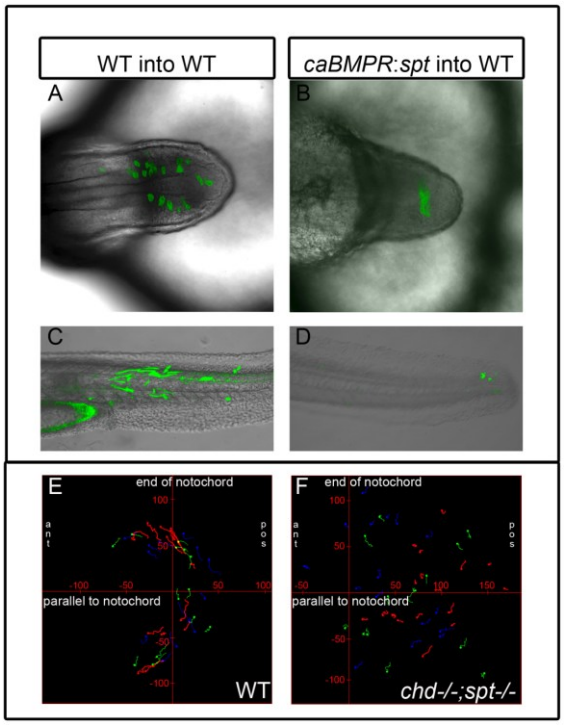

Figure A.5 Cell movements are perturbed in cells lacking spadetail and experiencing high BMP levels.

A-B, Top-down view of tailbud at 18 hpf with transplanted WT(A) and caBMPR mRNA + *spt* MO(B) cells in WT background. C-D Lateral view of 48 hpf embryos with transplanted WT (C, n=11) and caBMPR mRNA + *spt* MO (D, n=18) in a WT background. Transplanted WT cells are able to exit the tailbud and contribute to somites (A, C). caBMPR mRNA + *spt* MO cells remain in the tailbud forming a tight cluster and do not intermix with WT cells or form somites(B,D). E-F, Time lapse images were made of mGFP labeled embryos. Stills were taken every 90 seconds for 1-2 hours of development at 14 somite stage. Manual cell tracking was performed for 10-30 consecutive frames using ImageJ and placed on graphs with X axis parallel to the notochord and Y axis perpendicular to the end of the notochord, tick marks on axis are 50um. Tailbud cells in WT embryos (n=3) display movement away from the midline and anterior migration to form tail somites (E). *chd:spt* tailbud (n=3) cells do not display a uniform pattern of movement and their migration paths are much shorter (F).

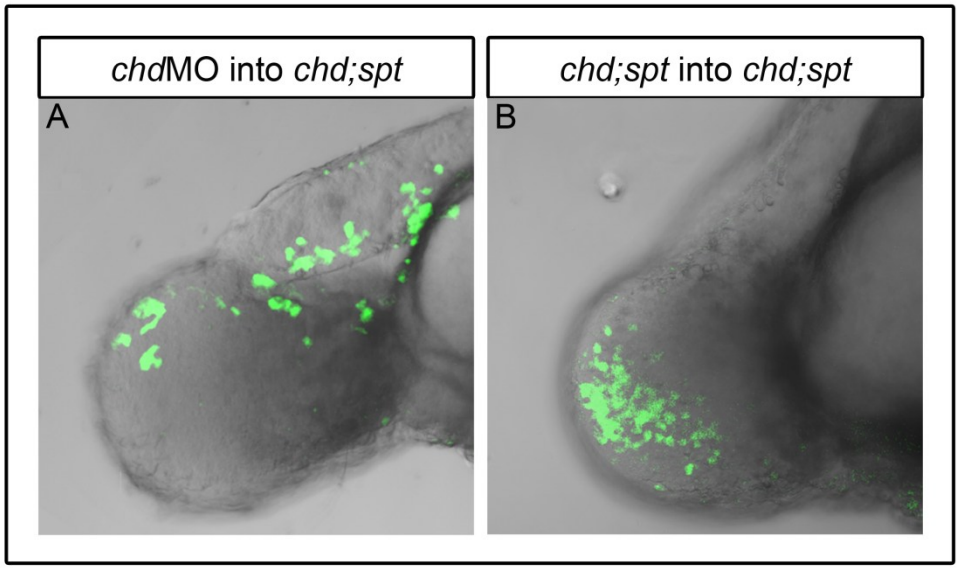

Figure A.6 Controls for mGFP cell transplantation experiments further indicate tailbud exit is a cell autonomous function.

Lateral view of 48 hpf embryos: *chd* MO cells in double mutant (*chd*MO;*spt*MO) background were able to mix with other cells in tailbud and some were able to exit the tailbud (A, n=2). Double (*chd*MO;*spt*MO) mutant cells in double mutant background could intermix with other cells in the tailbud but, as expected, were not able to leave the tailbud (B, n=2).

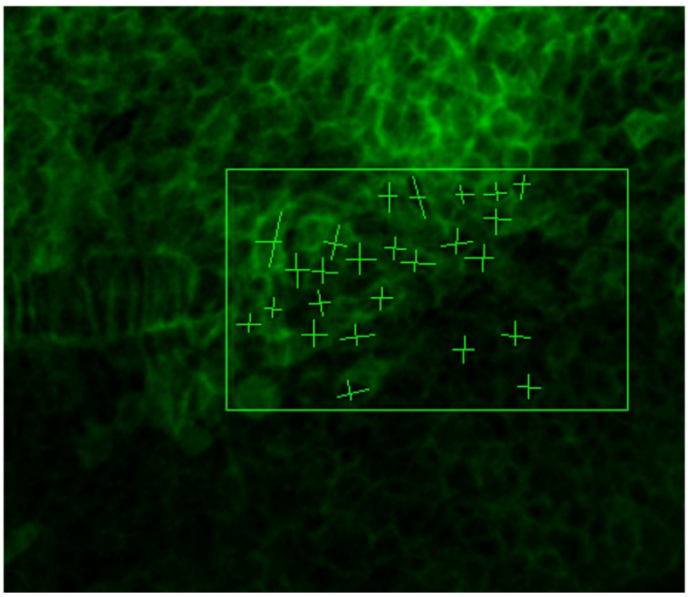

Figure A.7 Length and width measurements in tailbud cells.

Top down view of tailbud cells in a *chd*-/- embryo 14 hpf: Cells just posterior of the notochord were used to calculate length to width ratios of WT and mutant embryos. Length was measured perpendicular to the notochord, while width was measured parallel to the notochord. Cell measurements were made using ImageJ draw and measure tools (Rasband, 1997-2009).

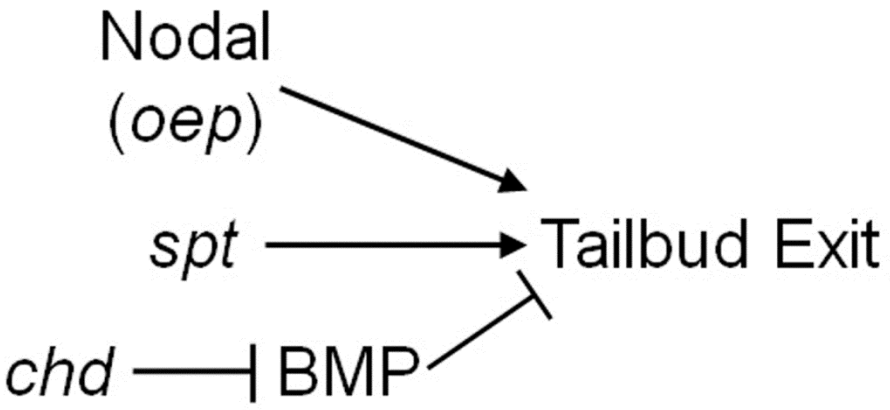

Figure A.8 Tailbud exit requires expression of *spadetail* in combination with BMP inhibition or with Nodal signaling.

Spadetail, Nodal signaling and BMP inhibition are required for MPCs to properly exit the tailbud to form muscle cells. *oep* and *chd* mutant MPCs are able to exit the tailbud and form somites. *spt* mutants are only able to form tail somites while trunk somite precursors remain trapped in their tailbud. Nodal and Chordin each act in conjunction with Spadetail to control this process. Loss of *oep* and *chd* in *spadetail* mutants results in the inability to form somites. However, *oep* and *chd* seem to be using independent mechanisms, with Chordin's crucial role in this process being to inhibit BMP signaling.

Movie 1. mGFP labeled WT tailbud 13hpf

Time lapse confocal imaging of WT zebrafish tailbud at 13 hpf (included as an external file). Dorsal view of mGFP labeled embryo captured just prior to tail somitiogenesis. Images were captured every 90 seconds for a 1.5 hour period. Cells in the posterior tailbud, which is the group of cells posterior to the notochord in the tip of the tail, are preparing to leave the tailbud by migrating anteriorly to form tail somites. As the notochord extends posterior cells move laterally then anteriorly where they will differentiate into muscle cells.

Movie 2. mGFP labeled *chd -/-* tailbud 14hpf

Time lapse confocal imaging of *chd* mutant tailbud at 14 hpf (included as an external file). Dorsal view of mGFP labeled embryo captured prior to tail somite formation. Images were captured every 90 seconds over a span of 40 minutes. *chd-/-* tailbuds are large at this stage as MPCs will contribute to expanded tail somites. Cells will migrate anteriorly and form tail somites as the notochord extends posteriorly.

Movie 3. mGFP labeled *spt-/-* tailbud 14hpf

Time lapse confocal imaging of *spt-/-* tailbud at 14 hpf (included as an external file). Dorsal view of mGFP labeled embryo captured prior to tail somite formation. Images were captured every 90 seconds for 1.5 hours. The notochord is only visible near the end of the video as the tailbud in these mutants are large and the notochord is mostly anterior to the cells instead extending into the tailbud as in wildtype embryos. Cells in this *spt-/-* tailbud are dynamic and intermixing similar those in WT tailbuds. Roughly half of these cells will exit the tailbud to

form tail somites and the other half, which would have given rise to trunk somites, will remain behind in the "spade" like tailbud.

Movie 4. mGFP labeled *chd-/-;spt-/-* tailbud with bright-field image 14 hpf.

Time lapse confocal imaging of *chd-/-;spt-/-* tailbud at 14 hpf (included as an external file). Dorsal view of mGFP labeled embryo captured prior to when tail somites would normally form. Images were captured every 90 seconds for 50 minutes. *Chd;spt* mutants completely lack somites and MPCs accumulate and remain in the tailbud. mGFP labeled image shows tailbud cells actively moving within the tailbud. The bright-field image captures the posterior extension of the notochord. As the notochord extends it pushes the entire mass of tailbud cells posteriorly. The notochord does not extend into the tailbud and the cells do not migrate anteriorly to form muscle cells as they do in single mutants and wildtype embryos.