# THE ROLE AND MECHANISM OF APOPTOSIS AS AN INNATE IMMUNE RESPONSE AGAINST VIRAL INFECTION IN INSECTS

By

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To Yanchao, my dear wife and to our family

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LIST OF ABBREVIATIONS

| | |
|---|---|
| AcMNPV | *Autographa californica* nucleopolyhedrovirus |
| BIR | Baculovirus IAP repeats |
| BmNPV | *Bombyx mori* NPV |
| CuniNPV | *Culex nigripalpus* nucleopolyhedrovirus |
| DIAP1 | *Drosophila* inhibitor of apoptosis 1 |
| EBV | Epstein-Barr virus |
| FADD | Fas associated protein with death domain |
| FBS | Fetal bovine serum |
| FHV | Flock house virus |
| FISH | Fluorescent in situ hybridization |
| HBV | Hepatitis B virus |
| IAP | Inhibitor of apoptosis |
| IBM | IAP binding motif |
| PI | Propidium Iodide |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| Q-PCR | Quantitative real-time PCR |
| RING | Really interesting new gene |
| SlNPV | *Spodoptera litura* NPV |
| TNF | Tumor necrosis factor |
| TNFR | Tumor necrosis factor receptor |
| TnMNPV | *Trichoplusia ni* MNPV |
| TRADD | TNF receptor associated protein with death domain |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| XIAP | X-linked inhibitor of apoptosis |

Abstract of Dissertation Presented to the Graduate School
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# THE ROLE AND MECHANISM OF APOPTOSIS AS AN INNATE IMMUNE RESPONSE AGAINST VIRAL INFECTION IN INSECTS

By

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Arthropod-borne pathogens account for millions of deaths each year. Understanding the genetic basis controlling vector susceptibility to pathogens is pivotal to developing novel disease control strategies. The fact that many vertebrate and insect viruses possess anti-apoptotic genes that are required for their infectivity has long led to the hypothesis that induction of apoptosis could be a fundamental innate immune response. However, the cellular mechanisms mediating the induction of apoptosis following viral infection remained enigmatic, which prevented experimental verification of the functional significance of apoptosis in limiting/preventing viral infection in insects. In addition, studies with cultured insect cells showed that either there is a lack of apoptosis, or the pro-apoptotic response happens relatively late, thus casting doubt on the functional significance of apoptosis as an innate immunity.

To prove the hypothesis that apoptosis can act as an innate immunity against viral infection in insects, I developed two kinds of *in vivo* infection systems. The first is a native infection system established in mosquitoes. In this system, the mosquitoes were orally infected with virus mimicing the natural infection route. Using this system, I found that there is a rapid induction of *reaper*-like pro-apoptotic genes and apoptosis within a

few hours following exposure to DNA or RNA viruses specifically in refractory mosquitoes rather than susceptible species. The correlation between apoptosis and mosquito susceptibility strongly suggests that apoptosis can serve as a defense against viral infection.

To elucidate the mechanisms of virus- induced apoptosis, I established two *in vivo* infection systems in the genetic model organism *Drosophila*. Similar to the results in mosquitoes, I found that pro-apoptotic genes (e.g. *reaper* and *hid*) and apoptosis were also rapidly induced in *Drosophila* following viral infection. Moreover, I found that this rapid induction of apoptosis requires the function of transcription factor *p53* and is mediated by a stress –responsive region upstream of *reaper*. More importantly, I showed that the rapid induction of apoptosis is responsible for denying the expression of viral genes and blocking/limiting the infection which proved the role of apoptosis as an anti-viral defense.

# CHAPTER 1
# BACKGROUND AND INTRODUCTION

## Apoptosis and Regulatory Pathways

Apoptosis is a genetically controlled cell suicide process in multicellular organisms to remove obsolete, damaged and potential dangerous cells. Apoptosis is characterized by a series of morphological features and biological processes including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and breaking down into apoptotic bodies. Eventually the apoptotic bodies are engulfed and digested by cells with phagocytotic activity such as microphage without causing inflammation. (Bergmann et al., 1998b; Vaux and Strasser, 1996; White, 1996).

Apoptosis plays important roles in development. For example, during the development of the brain, half of the neurons that are originally generated die later on when the adult brain forms (Hutchins and Barger, 1998). The formation of the individual digits is also due to massive cell death in interdigital mesenchymal tissues(Zuzarte-Luis and Hurle, 2002). Apoptosis is also critical for maintaining homeostasis in adult tissues. Cell proliferation in adult organs must be balanced by cell death to keep cell numbers constant. Moreover, apoptosis also has a protective role by removing the potentially dangerous cells such as those carrying mutations in the genome or cells infected by intracellular pathogens. The dysregulation of apoptosis manifests itself in a variety of human diseases. Insufficient apoptosis can cause cancer, autoimmune disease or the spreading of the viral infection; in contrast, excessive apoptosis can result in neurodegenerative disease (Fadeel et al., 1999).

Most of the current knowledge about apoptosis was derived from the work on the nematode *C.elegans*. The fate of every somatic cell during *C.elegans* development is

traceable (Kimble and Hirsh, 1979; Sulston and Horvitz, 1977). Out of the 1090 somatic cells that are originally generated during development, 131 always die by apoptosis (Ellis et al., 1991; Horvitz et al., 1994). This strongly suggests that apoptosis is a strictly regulated instead of randomly occurring process. Later on, the genetic analysis in *C.elegans* identified the pathway controlling apoptosis which is called CED-9/CED-4/CED-3 pathway (Figure 1-1, top green letters). Animals carrying mutation for *ced-3* lost all cell death that would have occurred in normal development (Yuan et al., 1993; Yuan and Horvitz, 1990). Subsequently, the regulatory pathways have been found to be highly conserved across all metazoans. Figure 1-1 listed three major cell death regulatory pathways in nematode, fly and mammal (Zhou et al., 2003). In all three pathways, the executors of apoptosis are a family of proteases called caspases (cysteine aspartic acid-specific protease). In normal situations, the caspases are inactive zymogens and ubiquitously expressed in both dying and living cells. Upon apoptotic stimuli such as ionizing radiation, a series of cascading events are triggered which can eventually activate the caspases. The activated caspases further cleave multiple cellular components such as the cytoskeleton, enzyme inhibitors and so on which will lead to collapse of the cells (Budihardjo et al., 1999). Thus far, eleven caspases have been described in humans, ten in mice, seven in *Drosophila* and four in *C.elegans* (Lamkanfi et al., 2002).

The mammalian orthologs of CED-9 are the BCL-2 family proteins which can positively or negatively regulate mitochondria membrane integrity (Adams and Cory, 2001; Chao and Korsmeyer, 1998). The members that possess pro-apoptotic activities can cause mitochondria permeabilization and result in the release of cytochrome c

(CYT c) into the cytosol, which further activates downstream APAF-1, the mammalian ortholog of CED-4 (Zou et al., 1997). CYT c, together with activated APAF-1 and ATP can form a complex namely the apoptosome, which, once formed, can recruit and activate caspases (Hu et al., 1998; Pan et al., 1998).

The middle pathway presented with blue letters in figure 1-1 was termed IAP-pathway which was originally characterized in *Drosophila*. The details concerning this pathway will be discussed in next section. Generally speaking, the core regulator in this pathway is a protein called DIAP1 (*Drosophila* Inhibitor of Apoptosis). DIAP1 can inhibit the enzymatic activity of caspases through direct interaction (Salvesen and Duckett, 2002). The IAPs may be considered as a universal "brake" to caspase activation (Figure 1-2). Upon apoptotic stimuli such as steroid hormones, radiation etc., a family of upstream genes including *reaper*, *hid*, *grim* and *sickle* are transcriptionally upregulated (Brodsky et al., 2000; Chen et al., 1996; Christich et al., 2002; Jiang et al., 2000; Lohmann et al., 2002; Nordstrom et al., 1996; Robinow et al., 1997). The protein products of these genes can compete with caspases for DIAP1 binding and thus these genes are called IAP-antagonists (Chai et al., 2003; Yan et al., 2004). In *Drosophila*, unlike DIAP1 and caspases which are ubiquitously expressed in cells, the IAP-antagonists are selectively expressed in cells doomed to die. To date, at least two mammalian proteins, SMAC/DIABLO (Chai et al., 2000; Du et al., 2000) and OMI1/HTRA2 (Martins et al., 2002; Suzuki et al., 2001), have been found to have IBM (IAP Binding Motif) and act as IAP-antagonists. However, unlike IAP-antagonists in *Drosophila*, these mammalian IAP-antagonists are ubiquitously expressed and reside in the mitochondria, from which they are released into the cytosol when apoptosis

happens. Moreover, mice deficient for SMAC/DIABLO or OMI1/HTRA2 did not show increased resistance to apoptosis (Jones et al., 2003; Okada et al., 2002). Thus, the physiological role of these proteins in regulating IAPs is still unclear. So far eight mammalian IAPs have been identified (Salvesen and Duckett, 2002) and the best studied is XIAP (X-linked Inhibitor of Apoptosis). It is regarded as the most potent caspase inhibitor in vitro (Eckelman and Salvesen, 2006). However, XIAP-deficient mice present the normal phenotype, so the physiological role of XIAP in vivo remains to be studied (Harlin et al., 2001).

In addition to those pathways discussed above, the bottom pathway presented in black letters in figure 1-1 is another conserved cell death regulatory pathway, namely death receptor pathway. Death receptors such as TNFR (Tumor Necrosis Factor Receptor), FasR (Fas Receptor) etc. are a subset of transmembrane receptors with intracellular death domain (Daniel and Krammer, 1994; Itoh and Nagata, 1993; Nagata, 1999; Schmitz et al., 2000). After binding with corresponding ligands through the extracellular sequence, the death receptors become oligomerized and recruit caspase 8 through the adaptor proteins such as FADD (Fas Associated Protein with Death Domain) or TRADD (TNF Receptor Associated Protein with Death Domain) (Chinnaiyan et al., 1995; Hsu et al., 1995), which further activate caspase 8 and induces apoptosis.

Although I discussed the cell death regulatory pathways independently, there is complicated crosstalk among different pathways. For example, caspase 8 is activated by the death receptor pathway. Meanwhile, its target, pro-apoptotic gene *bid*, is also involved in regulating mitochondrial membrane integrity (Li et al., 1998). Deciphering the

interactions between different pathways can help to establish the whole network of apoptosis regulation.

## Apoptosis Regulation in *Drosophila* and Mosquito

### *Drosophila*

As a powerful model organism, *Drosophila* provides us with an excellent tool to study apoptosis regulation. Interestingly, when searching *Drosophila* genome for the motifs presented in major cell death regulatory proteins, it seems that the orthologs of the proteins involving in all three major cell death regulatory pathways listed in figure 1 exist in *Drosophila*, which suggests that all three cell death regulatory pathways might be conserved in *Drosophila*. However, the IAP pathway that was discussed in above section has been found to be the most important cell death regulatory pathways in flies. The IAP (Inhibitor of Apoptosis) which was initially identified from insect baculoviruses is a potent inhibitor of apoptosis (Crook et al., 1993). Subsequently, cellular orthologs of IAP were identified in multiple organisms such as *Drosophila* and mammals (Hay, 2000). The core factor in the IAP pathway is DIAP1 which is the *Drosophila* ortholog of IAP. DIAP1 can inhibit the enzymatic activity of caspases through direct interaction which is mediated by its N-terminal BIR domain (Baculovirus IAP Repeats). Moreover, DIAP1 also possesses ubiquitin E3 ligase activity through the C-terminal RING (Really Interesting New Gene) domain which can lead to the ubiquitination and degradation of caspases (Vaux and Silke, 2005). Loss-of-function of *diap1* mutants are embryonic lethal due to the widespread apoptosis (Wang et al., 1999).

Upon apoptotic stimuli, the inhibitory function of DIAP1 can be counteracted by a family of upstream proteins including REAPER, HID, GRIM and SICKLE, all of which are termed IAP-antagonist. IAP-antagonists were initially identified through genetic

studies in flies. Interestingly, the four IAP-antagonists genes are located in proximity at the 75C1-2 chromosome region and share regulatory enhancer regions (Zhang et al., 2008). These IAP-antagonists function by binding to the BIR domain of DIAP1, the selfsame domain normally bound to caspases. As IAP-antagonists displace caspases from DIAP1, the caspases become free to induce apoptosis (Chai et al., 2003; Yan et al., 2004). The N-terminal IBMs (IAP Binding Motif) in IAP-antagonists are responsible for the DIAP1 binding. The sequences of those IAP-antagonists are highly divergent except for the short IBM. After genetically removing these IAP-antagonists, almost all developmental apoptosis was blocked (White et al., 1994). On the other hand, over-expression of these genes can result in potent apoptosis induction (Chen et al., 1996; Grether et al., 1995). This indicates that “releasing the brake” is a major mechanism of cell death regulation in *Drosophila* (figure 1-2). Indeed, in *Drosophila*, while DIAP1 and caspases are ubiquitously expressed in cells, the IAP-antagonists are selectively expressed in cells doomed to die. Those IAP-antagonists act in a partially redundant manner because removing an individual gene only causes a mild apoptosis phenotype (Grether et al., 1995; Peterson et al., 2002; Zhou et al., 1997).

**Mosquito**

The mosquito is a common insect in the order of Diptera, the same as *Drosophila*. They are very important vectors for a variety of pathogens including malaria, dengue fever, yellow fever etc. Although mosquitoes are in the same order as the fruit fly and the apoptosis regulation in *Drosophila* has been extensively studied, the apoptosis regulation in mosquitoes has been a mystery until the completion of the genome project of *Anophele gambiae* in 2002 (Christophides et al., 2002). It seems that at the sequence level, the general organization of cell death regulatory machinery in mosquitoes is highly

comparable to that in *Drosophila* (Table 1-1). Twelve caspases and seven IAPs were identified in the genome sequence of *Anophele gambiae*, representing a significant increase compared to *Drosophila*, which has seven and four respectively (Christophides et al., 2002). The significant increase of caspases and IAPs may reflect the functional requirement of fine-tuning cell death in response to parasites and viruses commonly encountered as a consequence of blood feeding. The specific expansion of IAPs also implies that, as in *Drosophila*, the IAP pathway also plays important roles in cell death regulation in *Anopheles* mosquitoes. However, the genome project did not identify any orthologs of IAP-antagonists (i.e. *reaper*, *hid*, *grim*, *sickle*) due to the fast divergence of these genes (Table 1-1). The orthologs of IAP antagonist were later identified by using a bioinformatics method and functionally characterized (Zhou et al., 2005). More orthologs were then identified by BLAST (Bryant et al., 2008; Wang and Clem, 2011).

The orthologs of core factors in IAP pathway were subsequently identified from other mosquito species such as the yellow fever mosquito *Aedes aegypti* (Bryant et al., 2008; Nene et al., 2007). Functional tests in mosquito cell lines verified the role of those genes in regulating apoptosis, e.g. silencing the mosquito IAP caused spontaneous apoptosis; silencing the mosquito caspases completely or partially inhibited apoptosis induced by different apoptotic stimuli (Liu and Clem, 2011). These findings further suggest the potential roles of IAP pathway in apoptosis regulation in mosquitoes.

## Apoptosis and Viral Infection

It has been well established that apoptosis of host cells are usually associated with viral infection. Many viral gene products can manipulate host cell apoptosis. On the one hand, it appears that many viruses carry anti-apoptotic genes in their genome to prevent premature death of host cells to maximize viral yield. On the other hand, a variety of

viral gene products have been shown to actively promote apoptosis, which was proposed to facilitate viral spreading while evading host inflammatory response. Therefore, the exact role of apoptosis in viral infection is still to be determined.

## Viral Inhibition of Apoptosis

### Adenovirus

Human adenovirus is the first virus that has been shown to manipulate host cell apoptosis (Ezoe et al., 1981). The E1B 19K protein of adenovirus was found to be the homolog of anti-apoptotic protein BCL-2 (Chiou et al., 1994; Pilder et al., 1984). Loss-of-function viral mutants can cause a strong apoptosis phenotype in infected cells (Ezoe et al., 1981). Moreover, E1B 19K is also able to suppress TNFα (White et al., 1992) or Fas ligand (Huang et al., 1997) induced apoptosis. Another adenovirus protein, E4orf6, has been shown to block P53-mediated apoptosis by preventing P53 accumulation in infected cells (Querido et al., 1997). Recently, E4orf6 was found to promote de novo heterochromatin formation at P53-targeted promoters and led to the epigenetic silencing of the P53-targeted genes, many of which can induce apoptosis (Soria et al., 2010). In addition to human adenovirus, anti-apoptotic genes were also identified from other adenoviruses. For example, GAM1 which encodes a 30kDa protein was discovered from chicken adenovirus type 1. Although GAM1 did not show any homology to so-far sequenced proteins at the sequence level, it has been shown to function like BCL-2 and E1B 19K (Chiocca et al., 1997).

### Baculoviruses

Baculoviruses are a family of large rod-shaped viruses which have a very restricted host range. They can only infect certain insect species and are non-pathogenic to humans (Okano et al., 2006). To date, baculovirus might be the one in

which manipulation of host cell apoptosis has been best studied. Two well-known anti-apoptotic proteins, P35 and IAP, were both initially identified from baculovirus (Clem et al., 1991; Crook et al., 1993). P35 can act as bait for a variety of activated effecter caspases such as human caspases 1, 3, 6, 7, 8, 10 (Zhou et al., 1998) and *Drosophila* DrICE (Fraser et al., 1997), but not for initiator caspases such as human caspase 9 (Vier et al., 2000) and *Drosophila* DRONC (Meier et al., 2000). Mutant viruses lacking functional P35 can strongly induce apoptosis in infected cells (Clem et al., 1991). Ever since the initial identification from AcMNPV (*Autographa californica* nucleopolyhedrovirus), *p35* has been subsequently identified from many other baculoviruses including BmNPV (*Bombyx mori* NPV), SlNPV (*Spodoptera litura* NPV), TnMNPV (*Trichoplusia ni* MNPV). However, no cellular *p35* has been identified so far.

Unlike P35 which can only act on effecter caspases, IAP can directly interact with both initiator and effecter caspases and inhibit their activity. Cellular IAP was also later identified from multiple organisms. For example, the DIAP1 in the IAP pathway was the *Drosophila* homolog of IAP. IAP proteins have also been described in other viruses that can infect arthropods such as entomopoxvirus (Li et al., 2005), iridovirus (Crook et al., 1993), Hz-1 virus (Cheng et al., 2002) and African swine fever virus (Chacon et al., 1995). Interestingly, to date, no IAP homologs have been described from viruses that can infect vertebrates. Given the notion that viruses usually target the most important proteins or pathways, this suggests that IAP proteins may be more important for cell death regulation in arthropods than vertebrates.

**Herpesviruses**

Like many other viruses, herpesviruses are also able to inhibit host cell apoptosis by expressing BCL-2 homologs or sequestering P53 activity. Human cytomegalovirus

(CMV) IE2 has been shown to bind to P53 and repress its transcriptional activity (Speir et al., 1994). Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) can up-regulate BCL-2 expression (Henderson et al., 1991) or inhibit P53-mediated apoptosis to prevent B cells from killing themselves (Okan et al., 1995). Moreover, two other EBV proteins, EBNA-5 (Szekely et al., 1993) and BZLF1 (Zhang et al., 1994), have been found to bind with P53, which may play a role in blocking apoptosis. An EBV protein involved in the lytic cycle, BHFR1, has homology with BCL-2. Functional verification indicated that BHFR1 can suppress apoptosis triggered by multiple stimuli such as serum depletion (Henderson et al., 1993), DNA damage or infection by adenovirus mutants lacking E1B 19K (Tarodi et al., 1994). Moreover, BHFR1 has been shown to inhibit TNFα or Fas induced apoptosis in intestinal epithelial cells (Kawanishi, 1997). The product of ORF16 of Herpes simian B virus is a BCL-2 homolog, which can block apoptosis induced by viral infection (Nava et al., 1997). Kaposi sarcoma-associated virus contains a homolog of BCL-2, KSBCL-2, that can inhibit viral infection-induced apoptosis (Cheng et al., 1997). The LAT protein of Herpes Simplex Virus (HSV) was also shown to inhibit cell death by blocking caspse 8 and caspase 9 pathways (Bloom, 2004).

**Poxviruses**

The CRMA (cytokine response modifier) in cowpox is an inhibitor of caspases which can block apoptosis induced by TNFα or Fas signaling (Miura et al., 1995; Talley et al., 1995). Likewise, vaccinia virus also inhibits apoptosis through a gene namely SPI-2 which shows high homology to CrmA (Dobbelstein and Shenk, 1996; Kettle et al., 1997). T2 protein contained in myxomavirus is a homolog of TNF receptor which is used by the virus to antagonize TNFα-induced apoptosis (Macen et al., 1996). Molluscum

contagiosum, another poxvirus encodes a vFLIP (viral FLICE-like inhibitory proteins) to inhibit FADD-mediated caspase activation and apoptosis (Thome et al., 1997).

**Other viruses**

Hepatitis B virus (HBV) encodes a pX protein which can bind to P53 and inhibit the DNA binding property of P53 thus blocking P53-mediated transcriptional activation and suppress P53-dependent apoptosis (Wang et al., 1995). Hepatitis C virus core protein was shown to repress P53 expression to inhibit apoptosis (Ray et al., 1997). In addition, the large T antigen of SV40 has been shown to inactivate P53 to block P53-dependent apoptosis (McCarthy et al., 1994).

**Viral Induction of Apoptosis**

As discussed above, many viral proteins can trigger apoptosis instead of blocking it. This active induction of apoptosis might represent a strategy used by many viruses, particularly non-enveloped, non-lytic viruses, to spread without initiating host immune response. Compared to the abundant knowledge about how viruses inhibit apoptosis, much less information is available about how viruses actively induce apoptosis.

**Adenovirus**

The adenovirus E1A protein has been shown to induce apoptosis in cultured cells when expressed (Rao et al., 1992). E1A has been found to stabilize P53, which further mediates apoptosis. Moreover, the interaction between E1A and the product of E4 transcription complex has been implicated in apoptosis when cells lack functional P53 (Marcellus et al., 1996).

**Human immunodeficiency virus type 1 (HIV-1)**

HIV-1 TAT protein has been found to up-regulate Fas ligand which further activates Fas receptor and apoptosis (Westendorp et al., 1995). When expressed in

haematopoietic cell lines, TAT was shown to down-regulate anti-apoptotic protein BCL-2 and up-regulate pro-apoptotic protein BAX (Sastry et al., 1996).

**Other viruses**

The alphavirus, Sindbis virus, has been described to cause cell death in a variety of mammalian cells (Levine et al., 1993). However, the mechanisms responsible for this effect have not been determined. The B19 human parvovirus has been found to promote apoptosis through the non-structural protein (NSP) (Morey et al., 1993; Ozawa et al., 1988). The PRRSV (Porcine reproductive and respiratory syndrome virus) can induce apoptosis through the P25 protein, encoded by ORF5 (Suarez et al., 1996).

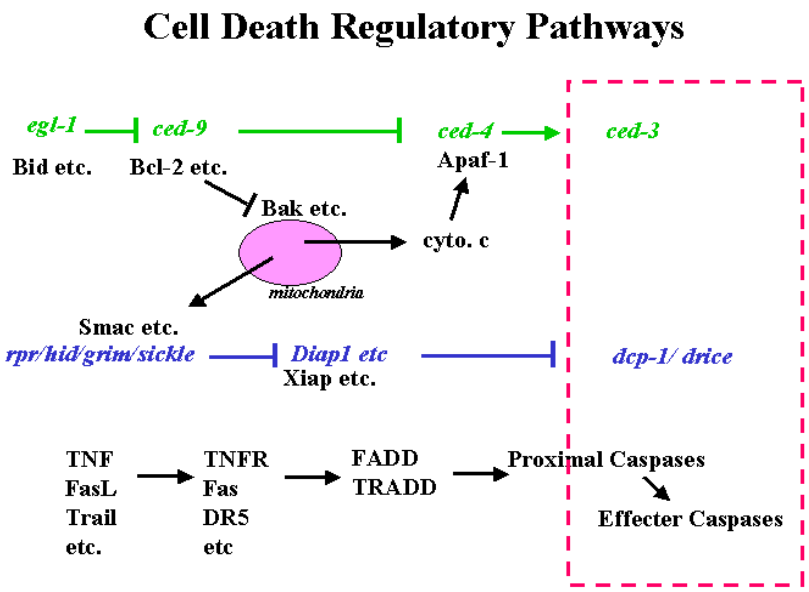


Figure 1-1. Three major cell death regulatory pathways in different organisms. When activated, all pathways lead to the activation of a family of protease termed caspases (encircled by red dashed square). Different colors represent different organisms (Green:C.elegan; blue: Drosophila and black: mammals). Stimulatory and inhibitory interactions are indicated by arrows and T-bar respectively. (Zhou et al., 2003)

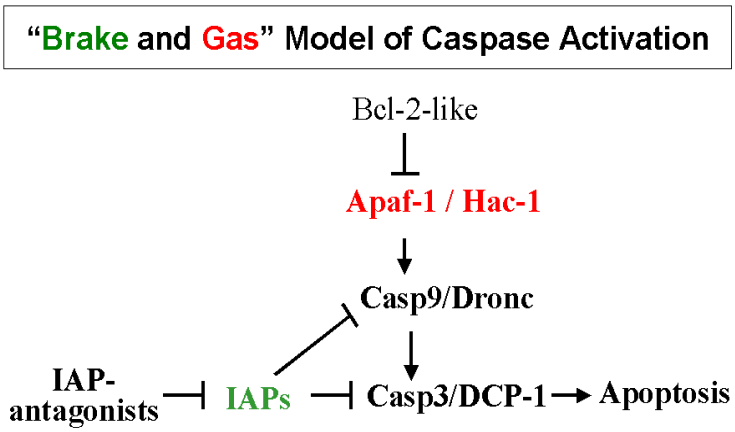


Figure 1-2. Simplified schematic presentation of the integration of cell death regulatory control in the "Gas and Brake" model. Apaf-1-like molecules function as "Gas" for caspase activation, which is checked by the "Brake" IAPs. *Drosophila* gene names are separated from their mammalian counterpart with "/". Due to space limitation only one name is chosen for each gene.

Table 1-1. Comparison of the numbers of identified cell death regulatory genes in *Anopheles* mosquito and *Drosophila*

| Genes | *Anopheles gambiae* | *Drosophila melanogaster* |
|---|---|---|
| Caspases | 12 | 7 |
| IAP | 7 | 4 |
| IAP-antagonists | 0? | 4 |
| Apaf-1/Hac-1 | 1 | 1 |
| Bcl-2 family | 2 | 2 |
| TNFR family | 1 | 1 |
| TNF | 0? | 1 |

Data on *Anopheles* genes are mainly based on sequence similarity analysis (Christophides et al., 2002; Holt et al., 2002) . "0?" indicates that the ortholog was not identified by the genome project, i.e. cannot be identified based on routine similarity search such as BLAST analysis.

# CHAPTER 2
# INDUCTION OF REAPER ORTHOLOG MX IN MOSQUITO MIDGUT CELLS FOLLOWING BACULOVIRUS INFECTION


## Summary

Many vertebrate and insect viruses possess anti-apoptotic genes that are required for their infectivity. This led to the hypothesis that apoptosis is an innate immunoresponse important for limiting virus infections. The role of apoptosis may be especially important in insect antiviral defense because of the lack of adaptive immunity. However, the cellular mechanism that elicits apoptosis in response to viral infection in insects has not been determined. Using an in vivo infection system with the mosquito baculovirus CuniNPV (*Culex nigripalpus* nucleopolyhedrovirus), I demonstrated that *michelob_x* (*mx*), the mosquito ortholog of *Drosophila* pro-apoptotic gene *reaper*, is specifically induced in larval midgut cells following viral infection. Interestingly, the dynamics of *mx* induction corresponds with the outcome of the infection. In the permissive mosquito *Culex quinquefasciatus* (*C. quinquefasciatus*), a slow induction of *mx* failed to induce prompt apoptosis, and the infected cells eventually undergo necrosis with heavy loads of encapsulated viruses. In contrast, in the refractory mosquito *Aedes aegypti* (*A.aegypti*), a rapid induction of *mx* within 30 min p.i. is followed by apoptosis within 2–6 h p.i., suggesting a possible role for apoptosis in limiting viral infection. When the execution of apoptosis was delayed by caspase inhibitors, viral gene expression became detectable in the *Aedes aegypti* larvae.


## Introduction

The theory that apoptosis has a very important role in virus infections was mainly supported by evidence that many viruses possess one or multiple genes that interfere with cellular apoptosis during the infection process (Benedict et al., 2002). Studies on

viruses have uncovered a multitude of viral arsenals that can manipulate cellular apoptotic response in essentially all aspects and levels of the process. For instance, both the intrinsic and extrinsic cell death regulatory pathways are targeted by proteins and/or small RNAs encoded by mammalian viruses (Galluzzi et al., 2008; Thomson, 2001). The extensiveness of viral interference of apoptosis ranges from manipulating upstream sensing and regulatory mechanisms to blocking the enzymatic activity of downstream effectors such as caspases.

Several very important cell death regulators were initially identified in viruses. For instance, IAP (inhibitor of apoptosis) was originally identified in lepidopteran baculoviruses (Clem et al., 1991; Crook et al., 1993). Viruses mutated for *iap* induce rapid cell death in infected cells (Li et al., 2005; Means et al., 2003). Viral IAPs not only can block cell death associated with viral infection but also apoptosis induced by other cytotoxic stimuli. Independently, genetic study in *Drosophila melanogaster* identified *reaper*-like genes as the pivotal regulators of programmed cell death (White et al., 1994). Subsequent genetic and biochemical analysis revealed that *reaper*-like pro-apoptotic genes function as IAP antagonists. One of the cellular *iap* genes, *diap1*, is ubiquitously expressed and required for the survival of cells. Essentially all cells in the developing embryo undergo apoptosis when functional DIAP1 is absent (Goyal et al., 2000; Hawkins et al., 1999). Selective cell death during *Drosophila* development is mainly achieved by specific expression of the IAP antagonists *reaper, hid, grim*, and *sickle*. With the exception of HID, whose pro-apoptotic activity is subject to post-translational modification (Bergmann et al., 1998a), IAP antagonists such as *reaper* are mainly regulated at the transcriptional level. In addition to mediating developmental cell

death, IAP antagonists are also responsible for mediating cell death in response to environmental stimuli. For example, the expression of *reaper* in *Drosophila* can be activated/induced by X ray, UV irradiation, or hormonal surges (Jiang et al., 2000; White et al., 1994; Zhou and Steller, 2003).

As insects lack adaptive immunity, it has been postulated that apoptosis would have an even more important role in antiviral response. Indeed, apoptosis has been observed during pathogen infection of mosquitoes and has been associated with host susceptibility to viral infection. It has been documented that ingestion of blood containing West Nile virus induces apoptosis in the midgut of a refractory *Culex pipiens* strain (Vaidyanathan and Scott, 2006). In contrast, necrosis has been associated with Western Equine Encephalomyelitis virus infection in susceptible *Culex. tarsalis* strains (Weaver et al., 1992). Although these evidences strongly suggest that pro-apoptotic response may have a very important role in determining vector compatibility, detailed mechanistic study has been hindered by the lack of knowledge about the underlying genetic mechanisms mediating pro-apoptotic response against viral infection.

The genome projects of *Anopheles gambiae* and *Aedes aegypti* revealed that, compared with *Drosophila*, these arbovirus vectors have expanded families of IAPs and caspases (Christophides et al., 2002). This was speculated as an adaptation to repeated exposure to pathogens associated with blood ingestion, although expansion of caspases has also been found in other *Drosophila* species (Bryant et al., 2010). The *A. gambiae* genome project did not initially annotate any IAP antagonists because of the fast divergence of their sequences. The missing IAP antagonist was uncovered using an advanced bioinformatics approach, which identified *michelob_x* (*mx*) as the *reaper*-

like IAP antagonist in both *Anopheles* and *Aedes* mosquitoes (Zhou et al., 2005). Another IAP antagonist that is related to *mx* was subsequently characterized in *A. aegypti* (Bryant et al., 2008). Despite the low sequence similarity of the entire gene, the functional domain, that is, the IAP-binding motif (IBM), was very well conserved, and the functional mechanism of MX appears to be very similar to that of REAPER.

The identification of IAP antagonists in mosquitoes allowed us to ask whether *reaper*-like genes are involved in pro-apoptotic response to viral infection. We took advantage of the mosquito baculovirus CuniNPV (*Culex nigripalpus* nucleopolyhedrovirus) because of the accessibility of this system and the established insect pathology associated with CuniNPV infection (Andreadis et al., 2003). CuniNPV is originally isolated from the mosquito *Culex.nigripalpus* (Becnel et al., 2001). It is related to lepidopteran and hymenopteran baculoviruses, but genomic sequence comparison indicated that there is a large evolutionary distance between CuniNPV and lepidopteran baculoviruses (Moser et al., 2001). CuniNPV infects only epithelial cells of the larval midgut, has a restricted host range, and mainly infects *Culex* (Andreadis et al., 2003). None of the examined *Aedes* mosquitoes, including *A. aegypti*, is susceptible to CuniNPV infection (Andreadis et al., 2003). CuniNPV can exist either as the occluded form or the budded form. The virus exists outside the mosquito in the occluded form, which allows the virus to survive under harsh environmental conditions. Ingested occluded virus initiates the infection in the presence of the divalent cation magnesium. Not all larval midgut cells are receptive to CuniNPV infection, which is limited to a particular group of resorbing/secreting cells in the gastric caeca and the posterior

midgut (Moser et al., 2001). Once inside the midgut, the virus can spread from infected cells to neighboring cells via the budded form.

In this study, I showed that *mx* is induced in larval midgut cells following exposure to a mosquito baculovirus CuniNPV. More importantly, the dynamics of this induction is different in the susceptible *C. quinquefasciatus* versus the refractory *A. aegypti*. The relatively timid and delayed response of *mx* in *C. quinquefasciatus* (*mx_Cu.qu*) is associated with necrosis, whereas the robust and immediate induction of *mx* in *A. aegypti* (*mx_Ae.ae*) is followed by apoptosis within 6 h of viral exposure, suggesting that apoptosis may contribute to limiting viral infection in *Aedes*.

## Materials and Methods

### Bioinformatics, Gene Cloning, and Cell Death Assay

Data-mining strategy, plasmid construction, and in vitro cell death assay were performed as described previously (Zhou et al., 2005). Briefly, a motif search program implemented in C was customized to search for the IBM motifs in the genomic and EST sequences from mosquito genomes. An intronless cDNA for *mx_Cu.qu* was then obtained by reverse transcriptase PCR using RNA extracted from CuniNPV-infected mosquito *C. quinquefasciatus* that serves as the template for PCR reaction. The primers used here are 5'-ACCGGCCGGCGCTGGTTACGTGATTCT-3' and 3'-CGGGATCCATACACTTTGCGGAGCAGA-5'. PCR product was then subcloned into the pIE-3 vector for transfection assay, and cloned into pBS for synthesizing cRNA probe. The other plasmids used in this study, including Pie-LacZ, Pie-MX_Ae.ae, Pie-P35, and Pie-DIAP1, are described previously (Zhou et al., 2005).

**Mosquito Rearing and CuniNPV Infection**

*C. quinquefasciatus* (Gainesville, FL strain, maintained since 1995) and *A. aegypti* (Orlando, FL strain, maintained since 1952) were reared in the insectary of the Mosquito and Fly Research Unit at the Center for Medical, Agricultural, and Veterinary Entomology, USDA-ARS, Gainesville, FL, USA. CuniNPV viral OBs were purified from infected *C. quinquefasciatus* larvae as described previously (Moser et al., 2001). The standard assay involves groups of 100 three- to four-day-old *C. quinquefasciatus* and *A. aegypti* larvae obtained from the laboratory colony. Larvae are exposed at a dose of $1.2\times10^9$ OBs per ml in 100 ml of deionized water with 15mM $MgCl_2$ plus 50 mg alfalfa and potbelly pig chow mixture (2 : 1). Groups without the addition of the virus serve as controls.

**RNA Extraction and Quantitative Real-Time PCR (Q-PCR)**

Total RNA was extracted from frozen larvae with RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the protocol provided by the manufacturer. RNA samples were treated with DNase I to remove genomic DNA. cDNA was prepared by reverse transcription of total RNA with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Q-PCR was performed with an ABI 7500 Fast thermocycler (Applied Biosystems) following protocols provided by the manufacturer. Triplicates were measured for each gene/sample combination. To control for variation of *mx* expression levels during normal development, each pool of larvae was hatched on the same day and randomly separated into control and viral treatment groups. A sample of 10–12 larvae was taken from each group at a given time point and frozen at -80 $^oC$. The cellular expression levels of *mx* were measured as relative to the *glyceraldehyde 3-*

*phosphate dehydrogenase* (*GAPDH*) or *actin* levels before the calculation of the ratio between CuniNPV-treated and control samples.

**PI Staining**

Live mosquito larvae were removed from the culture media, washed briefly with water, and transferred to 6-well plates with 5ml of fresh culture media containing 1 mg/ml PI (Sigma, St. Louis, MO, USA). After 10 min of incubation, the staining was stopped by transferring the larvae to fresh media without PI. The larvae were than dissected quickly, and the midgut and attached tissues were fixed in 4% paraformaldehyde in PBS. The fixed samples were washed three times in PBS. Samples were mounted with the Vectorshield mounting medium (Vector Lab, Burlingame, CA, USA) containing DAPI. The pictures were taken with a Leica upright fluorescent microscope (Leica, Bannockburn, IL, USA) using OpenLab software (Improvision, Coventry, UK).

**TUNEL Assay**

Apoptosis was detected using the FragEL Kit (Calbiochem, Gibbstown, NJ, USA) with a modified protocol. Midguts were dissected and fixed with 4% paraformaldehyde, 100mM PIPES buffer, pH 7.4, 2mM MgSO4, and 1mM EGTA for 20 min at room temperature. They were washed with TBS (20mM Tris-HCl, pH 8.0, and 140mM NaCl), followed by 5 min incubations in a TBS-methanol step gradient from 0, 25, 50, 75 to 100% methanol and back to TBS buffer following a reversed methanol series of concentrations. The tissue was then incubated for 5 min at room temperature with 20 mg/ml protease K in TBS, and the reaction was stopped by washing with 4% paraformaldehyde in TBS. This was followed by another 10 min fixation with 4% paraformaldehyde in TBS. The sample was equilibrated for 20 min with equilibration

buffer at room temperature and incubated with TdT labeling reaction mixture for 1 h at 37 $^{o}$C. The samples were then mounted with Vectorshield mounting medium (Vector Lab) containing DAPI. The pictures were taken with a Leica upright fluorescent microscope using OpenLab software.

**Fluorescent in Situ Hybridization (FISH)**

Probes were synthesized using DIG- or Fluorescein-RNA Labeling Mix (Roche, Madison, WI, USA). Midguts were dissected, fixed, and processed as described above. After prefixing with 4% paraformaldehyde in PBT_DEPC (0.3% Triton in PBS made with DEPC pretreated double-distilled water) for 30 min, the tissue was incubated for 7 min with 50 mg/ml protease K in PBT_DEPC, and reaction was stopped by washing with 4% paraformaldehyde. Samples were incubated with probes diluted in hybridization buffer (50% formamide, 25% 2×SSC, 20 mg/ml yeast tRNA, 100 mg/ml ssRNA, 50 mg/ml heparin, and 0.1% Tween-20). Hybridization was performed overnight at 60 $^{o}$C. If necessary, HRP-conjugated anti-DIG or anti-FITC (Roche) antibody (depends on what marker the probes carry) was applied after hybridization, followed by signal amplification using the Tyramid Signal Amplification Kit (PerkinElmer, Waltham, MA, USA).

**Blocking Apoptosis with Z-VAD and Q-VD-OPH**

Immediately following the administration of virus and $MgCl_2$, exposed *A. aegypti* larvae were removed from the culture pan together with the virus containing media to 24-well or 12-well culture dishes. z-VAD-fmk (R&D Bioscience, Minneapolis, MN, USA) and Q-VD-OPH (BioVision, Mountain View, CA, USA) were added to individual set of the wells for final concentration of 100 and 50 mM respectively. The combination of 50 mM z-VAD-fmk and 20 mM Q-VD-OPH was also applied. Meanwhile, equal amounts of DMSO (solvent for z-VAD and Q-VD-OPH) were added to a parallel set of wells as

control. Larvae were collected at discrete time points following the infection and processed for Q-PCR or FISH as described above. To assess the inhibitory efficiency of above caspase inhibitors, cultured C6/36 cells were transfected with 0.2 mg lacZ and 0.2 mg Mx in the presence or absence of corresponding caspase inhibitors. The number of viable (lacZ positive) cells were counted at 24 h after transfection.

## Results

### Molecular Cloning and Characterization of MX in *C. quinquefasciatus*

*mx* was originally identified in *Anopheles* and *Aedes* mosquito genomes as the ortholog of *Drosophila reaper* using an integrated bioinformatics strategy and verified via functional assays (Zhou et al., 2005). A similar bioinformatics approach was applied to identify potential IAP antagonists in the *C. pipiens* genome. Using the sequence information, we were able to clone the *mx* ortholog (*mx_Cu.qu*) gene from a cDNA made from *C. quinquefasciatus* larvae.

MX_Cu.qu is ~80% identical to its orthologs in *A. aegypti* (MX_Ae.ae) or *A. albopictus* (MX_Ae.al). The three *mx* orthologs in the Culicinae tribe share considerable similarity beyond the IAP-binding motif (Figure 2-1A). In contrast, they share little similarity with the *mx* ortholog in *A .gambiae* except the IBM. Given the evolution history of these groups, we would expect a significant difference between the subfamilies Anophelinae and Culicinae, which may also be partially reflected in the MX protein distance tree (Figure 2-1B).

Like the *mx* orthologs in *Aedes* and *Anopheles*, MX_Cu.qu induces rapid cell death when expressed in C3/36 cells (Figure 2-1C). This pro-apoptotic activity is largely, if not totally, dependent on the N-terminal IBM. Removing the first three amino acids of this motif (2–4; ‘AIA’) abolishes most of the killing ability of MX_Cu.qu (Figure 2-1C).

The pro-apoptotic activity of MX_Cu.qu and MX_Ae.ae appear to be very similar when assayed in C6/36 cells. Cell killing induced by either MX_Cu.qu or MX_Ae.ae is significantly suppressed by co-transfection of DIAP1, and to a less degree, by co-transfection of the viral inhibitor P35 (Figure 2-1D). DIAP1 is the major anti-apoptotic cellular protein in *Drosophila* and is highly conserved from insects to mammals. P35 is the viral caspase inhibitor that was originally identified from lepidopteran baculovirus (Clem et al., 1991). The fact that MX_Cu.qu-induced cell death can be blocked by either DIAP1 or P35 indicates that the functional mechanism of MX_Cu.qu is the same as previously characterized for MX orthologs in *Anopheles* and *Aedes* and REAPER in *Drosophila*.

Cell death induced by expressing MX_Cu.qu or MX_Ae.ae in C6/36 has typical apoptotic hallmarks, that is, nuclear condensation, fragmentation, and so on. Interestingly, C6/36 cells killed by expression of MX_Cu.qu or MX_Ae.ae appear to be quickly phagocytosed by neighboring cells (Figure 2-1E). Since pIE-LacZ was co-transfected with pIE-MX_Cu.qu, cells expressing MX_Cu.qu or MX_Ae.ae are also β-galactosidase (β-gal) positive. At 20 h after transfection, most of the remaining blue β-gal-positive cells in MX_Cu.qu-transfected samples appear to be in the later stages of apoptosis. These β-gal-positive cells are often rounded up and/or fragmented. Small phagosomes with β-gal staining can be clearly seen in cells surrounding the fragmented β-gal-positive cell, indicating that dying or dead cells were quickly phagocytosed before the breakdown of the LacZ protein. A similar phenomenon was observed for REAPER and HID-induced cell death in *Drosophila* embryos where most dying cells were quickly phagocytosed by hemocyte/macrophages (Abrams et al., 1993; White et al., 1994; Zhou

et al., 1995). However, not all *Drosophila* cell lines have endocytotic capability (Abrams et al., 1992). The fact that C6/36 cells are actively attracted to apoptotic cells and aggressively phagocytose fragmented apoptotic bodies suggests that this cell line, similar to the *Drosophila* S2 cell line, may have a mesoderm origin and display hemocyte/macrophage functionality.

**The *Mx* Gene Is Induced in Midgut Cells Infected by CuniNPV**

Using quantitative real-time PCR (Q-PCR) I first monitored the expression of *mx* following CuniNPV infection in pools of whole larvae. I found that in *C. quinquefasciatus* there was a lack of significant increase of *mx_Cu.qu* expression during the initial phase of infection, that is, before 8 h p.i. However, 48 h after the initiation of virus infection, the level of *mx_Cu.qu* reached significantly higher levels (Figure 2-2A). At this time, the level of *mx_Cu.qu* in infected larvae was about six times higher than the uninfected controls.

In sharp contrast, Q-PCR analysis indicated that there was a quick response of *mx_Ae.ae* induction in the first 2 h following exposure to CuniNPV in the refractory *A. aegypti* larvae (Figure 2-2A). Significant induction of *mx_Ae.ae* was found within 1 h of exposure to the virus (Data not shown). The elevated level of *mx_Ae.ae* in virus challenged larvae was transient and retreated to background or previous infection level at about 8 h p.i.

The dynamics of *mx* induction in both *C. quinquifasciatus* and *A. aegypti* were verified via fluorescent in situ hybridization (FISH) with cRNA probes to *mx_Cu.qu* and *mx_Ae.ae*, respectively. Consistent with the Q-PCR data, only timid induction of *mx_Cu.qu* could be detected in a few cells in the gastric caeca at early stage of virus infection (2 h) in CuniNPV infected *C. quinquifasciatus* larvae (Figure 2b). However, the

mRNA level of *mx_Cu.qu* is very high at later stages of infection. At 48 h p.i., it appears that most cells in the gastric caeca and the posterior midgut have very high levels of *mx_Cu.qu* (Figure 2-2B). These cells also display necrotic nuclei morphology (described below).

In contrast, there is a rapid induction of *mx_Ae.ae* in the gastric caeca of *A.aegypti* larvae exposed to the same treatment of CuniNPV. The number of cells expressing *mx_Ae.ae*, as detected by FISH, reached a peak at about 2 h p.i. At 4 h p.i., the number of *mx_Ae.ae*-positive cells had decreased significantly. By 8 h p.i., there was no difference in *mx_Ae.ae* FISH signal between virus-treated and control *A. aegypti* larvae (Figure 2-2C).

### Necrosis, Not Apoptosis, Is Associated with CuniNPV Infection in the Susceptible Mosquito *C. quinquefasciatus*

Previous histological analysis has shown that many cells at the gastric caeca and posterior midgut of infected *C. nigripalpus* or *C. quinquefasciatus* larvae were heavily infected with CuniNPV occlusion bodies (OBs) and display signs of necrosis at the final stages of infection (Becnel et al., 2003; Moser et al., 2001). When TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) was applied to monitor possible apoptotic response to viral infection in *C. quinquefasciatus* larvae, there was no significant level of apoptosis at either early stage (2–8 h p.i.) or late stages (24 and 48 h p.i.) of the infection (data not shown).

FISH analysis with cRNA probes for CuniNPV genes allowed us to pinpoint virus-infected cells even before the final stages of infection. Counter stain with DAPI indicated that the nuclei of infected cells, that is, cells with CuniNPV gene expression, were greatly hypertrophied at 48 h p.i. (Figure 2-3A). These nuclei were swollen to 2–4 times

the diameter compared with corresponding cells in uninfected larvae. FISH analysis confirmed that before 8 h p.i., only a small group of cells at the gastric caeca and posterior midgut were infected (i.e., have viral gene expression). However, by 48 h p.i., most cells at the posterior midgut and gastric caeca were positive for viral gene expression and had swollen nuclei (Moser et al., 2001). This indicates that the virus initially infects only a small group of cells but later spreads horizontally to infect other cells, presumably via budded virus. Interestingly, while most of the cells in the posterior midgut were positive for viral gene expression, none of the cells at the anterior midgut was positive for viral gene expression.

To confirm that virus-infected cells in *C. quinquefasciatus* larvae undergo necrotic instead of apoptotic cell death, I examine the membrane permeability of these infected cells. When live infected *C. quinquefasciatus* larvae were incubated for 10 min in normal culture media containing 1 mg/ml propidium iodide (PI), all of these swollen cells at posterior midgut were labeled with PI, whereas none of the uninfected cells in the anterior midgut were labeled (Figure 2-3B). The swollen morphology and the compromised membrane integrity of these cells are characteristics of necrosis. Indeed, tissues of the infected specimens at 48 h p.i. were very fragile and easily disintegrated when touched or pulled during dissection. Eventually, at about 60–72 h p.i., most mosquitoes died and tissues disintegrated into the culture media.

### Rapid Induction of *Mx_Ae.ae* Is Followed by Apoptosis in the Refractory Mosquito *A. aegypti*

The dynamics of induction of *mx* was very different between the susceptible *C.quinquefasciatus* larvae and the refractory *A. aegypti* larvae. Cells expressing high level of *mx_Ae.ae* could be detected within 1 h of infection in the gastric caeca and

reach a peak at 2 h p.i. TUNEL analysis indicated that this rapid induction of *mx_Ae.ae* was followed by a wave of apoptosis in the midgut epithelial cells. TUNEL-positive cells could be observed in the gastric caeca at 2 h p.i. (Figure 2-4). This wave of apoptosis in CuniNPV-infected *A. aegypti* larvae appears to be relatively synchronized. The number of TUNEL-positive cells is the highest at about 2–4 h p.i. (Figure 2-4A). By 8 h p.i., few cells in the gastric caeca or posterior midgut can be found to be TUNEL positive.

The quick apoptotic process following *mx_Ae.ae* induction helps to explain the sharp decline of *mx_Ae.ae*–positive cells after 2 h p.i. Colocalization analysis confirmed that TUNEL positive cells, especially those at early apoptotic stages before the onset of significant nuclei condensation, are also positive for *mx_Ae.ae* (Figure 2-4B). The timeline of the events indicates that cells undergo apoptosis shortly after the expression of *mx_Ae.ae*. This is not surprising given that in *Drosophila* embryogenesis, the expression of *reaper* in cells destined to die is followed immediately by apoptosis within approximately 1–2 h (White et al., 1994).

### Inhibition of Apoptosis Leads to Expression of Viral Genes in the Refractory Mosquito *A. aegypti*

The rapid induction of *mx* and apoptosis following CuniNPV infection in the larval midgut suggests that cells invaded by the virus underwent apoptosis and were quickly eliminated from the midgut epithelium. Under normal conditions, I were not able to detect viral gene expression in *A. aegypti* larvae exposed to CuniNPV at any time point by either FISH or Q-PCR. This could potentially be attributed to at least three possibilities. First, maybe the virus could not enter the cell because of incompatibility of cell surface receptors. Second, virus that entered the cell could not replicate because of incompatibility of the cellular transcription system. Third, it could be that the quick

apoptotic response prevented viral gene expression from reaching a detectable level. To discern these possibilities, I tested whether blocking or delaying apoptosis following CuniNPV infection in *A. aegypti* larvae could have an impact on the infection process.

The refractory *A. aegypti* larvae were infected with CuniNPV and split into two populations after the addition of virus and $MgCl_2$. To one series of samples, pan caspase inhibitors z-VAD-FMK, or Q-VD-OPH, or a combination of both was added to the medium. As a control, the same amount of DMSO (the solvent for both z-VAD and Q-VD) was added to the parallel samples. This treatment of both z-VAD and/or Q-VD was able to suppress the cell death induced by transfection of *mx* in a tissue culture-based assay (Figure 2-5).

From both the caspase inhibitor-treated and control populations, larvae were collected at the indicated time points and processed for Q-PCR and FISH analyses. Immediate early (ie) gene *cun103* is one of the first genes expressed following CuniNPV infection of *C. quinquefasciatus*. Although it is not detected in *A. aegypti* larvae treated with DMSO, it was reliably detectable in caspase inhibitor-treated samples (Figure 2-6A). The expression of *cun103* was detectable via Q-PCR at 8, 24, and up to 30 h p.i. In most of the independent trials, the expression of *cun103* diminished after 48 h p.i. Only in one out of five trials, I detected persistent *cun103* expression in a 72 h p.i. sample. Correspondingly, structural (nucleocapsid) genes *cun024* and *cun035* were detectable in that sample, indicating that at least in one larva, late-stage viral expression was achieved by inhibiting/delaying apoptosis (Figure 2-7).

FISH analysis confirmed that about 10–20% of larvae treated with caspase inhibitors have cells with detectable viral gene expression at 24 h p.i. (Figure 2-6B). The

viral gene signals were confined to the nuclei, similar to what we observed with early-stage infection of CuniNPV in the permissive *C. quinquefasciatus* (Figure 2-8). However, repeated trials with different caspase inhibitors or combinations of caspase inhibitors indicated that the efficacy of caspase inhibitor in transforming the resistant *A. aegypti* larvae was limited. I were never able to improve the infection rate to more than 20%, nor could I reliably detect the expression of virus structural genes in every trial.

## Discussion

A significant contribution to our knowledge about the role of apoptosis in virus–host interaction came from studying lepidopteran baculoviruses such as AcMNPV (*Autographa californica* multicapsid nucleopolyhedrovirus) (Clem, 2007). Several anti-apoptotic genes have been characterized in lepidopteran baculoviruses, including *iap*, *p35*, and *p49* (Clem and Miller, 1994; Zoog et al., 2002). While injecting wild-type virus to moth larvae usually leads to necrosis, injecting viruses with mutated *p35* induced massive apoptosis (Clarke and Clem, 2003). Consequently, the infectivity of the mutant virus was much lower than that of the wild-type virus. Most of these previous studies relied on directly injecting budded viruses into the hemocoel. However, in natural conditions, baculovirus infection of insects is initiated in the midgut epithelia by occluded viruses. Mine is the first evidence showing that infection by the occluded baculovirus induces pro-apoptotic response. In addition, the correlation between the cellular outcome of CuniNPV-infected midgut epithelial cells and the organism susceptibility to viral infection strongly suggests that apoptosis has a very important role in limiting viral infection.

Apoptosis in the mosquito midgut epithelia has long been observed accompanying the infection of a variety of pathogens (Han et al., 2000; Vaidyanathan and Scott, 2006).

However, we know little as to which regulatory pathway is responsible for mediating apoptosis against pathogen infection (Abraham and Jacobs-Lorena, 2004). My study here indicated that the mosquito ortholog of *reaper*, *mx*, is activated following CuniNPV infection. This, to our knowledge, is the first evidence suggesting that *reaper*-like genes, known for their pivotal role in regulating programmed cell death during development, are also involved in the innate immune response against viral infection.

In *Drosophila*, *reaper*-like genes (*hid, grim, and sickle*) are required for most developmental cell death (Steller, 2008). The *reaper*, *grim*, and *sickle* are exclusively expressed in cells destined to die during development or following cytotoxic stimuli such as irradiation. Transcriptional regulation of *mx* also appears to be tightly regulated in the mosquito larval midgut. In the absence of viral infection, only a few cells have detectable levels of *mx* expression and presumably reflects the routine turnover of midgut epithelial cells. The induction of *mx* in CuniNPV infected *C. quinquefasciatus* larval midgut is limited to virus infected tissues, that is, gastric caeca and posterior midgut. As we have reported previously, like *reaper*, *mx* is also transcriptionally responsive to UV irradiation (Zhang et al., 2008). Thus it appears that although the protein sequence of MX has diverged significantly from that of REAPER, their regulation at the transcriptional level share much similarity.

My study strongly indicates that the induction of *mx* is very likely responsible for the pro-apoptotic response following CuniNPV infection. However, direct assessment of this hypothesis was hindered by the lack of technical means to specifically block the surge of *mx_Ae.ae* expression following viral infection. There has not been any reported success with RNAi in *Aedes* larvae. My attempts with double-stranded RNA, using a

strategy that worked in *A. gambiae* larvae (Zhang et al., 2010), failed to produce any damping effect on the induction of *mx_Ae.ae* following CuniNPV infection. Besides potential problems associated with the efficacy of RNAi in *Aedes* larvae, it is also questionable whether the RNAi mechanism, which can bring down the level of constitutively expressed genes, can be effective in suppressing the quick induction of *mx_Ae.ae* associated with viral infection. It is possible that because the induction is so robust and followed quickly by apoptosis, RNAi mechanism could not function fast enough to have a significant, if any, impact on the rapid increase of *mx_Ae.ae*. It might be necessary to first identify the enhancer(s) mediating viral infection-induced *mx_Ae.ae* and applying this to genetically engineered mosquito to truly test the hypothesis that the induction of *mx_Ae.ae* is responsible for the apoptosis of midgut cells following CuniNPV infection.

**A Race to Apoptosis**

My study highlights the importance of the timing of pro-apoptotic response on the cellular and organismal outcome. In the refractory host *A. aegypti*, the induction of *mx_Ae.ae* and apoptosis happened so fast that viral gene expression was never detectable in the process. In contrast, in the susceptible host *C. quinquefasciatus*, the delayed and timid induction of *mx_Cu.qu* did not reach to detectable level until viral genes were detectable (Figure 2-9). The data presented here do not exclusively suggest that the quick induction of apoptosis is responsible for the refractory phenotype, as there are many other differences between the two species. However, the fact that viral gene can be detected in *A. aegypti* when apoptosis is delayed/suppressed suggests that apoptosis is responsible for preventing viral gene expression.

The extremely high level of *mx_Cu.qu* in CuniNPV-infected midgut cells in *C. quinquefasciatus* indicates that the virus has a very efficient way to block *mx*-induced apoptosis. In the tissue culture system, *mx*-induced cell death can be effectively blocked by co-expression of *iap*. However, there was no clear ortholog of either *iap* or *p49* in CuniNPV. A predicted viral protein, CUN75, has marginal similarity to AcMNPV P35, but lacks a conserved reactive site loop that is required for caspase-inhibition activity (dela Cruz et al., 2001). We did not find any anti-apoptotic effect of CUN75 when co-transfected with MX_Cu.qu or MX_Ae.ae. As the only Dipteran baculovirus with genomic information, CuniNPV is distantly related to lepidopteran baculoviruses and lacks identifiable orthologs to many important genes such as the ie genes (Afonso et al., 2001). It is very likely that CuniNPV utilizes a yet unknown, but powerful, mechanism to block the apoptotic pathway downstream of *mx* activation.

**Apoptosis and Mosquito–Host Compatibility**

Correlation between apoptosis and resistant phenotype has been found with refractory *Culex* mosquito exposed to the West Nile virus (Vaidyanathan and Scott, 2006). The comparison of the CuniNPV infection in the *A. aegypti* and *C. quinquefasciatus* also suggests that apoptosis has an important role in eliminating CuniNPV infected midgut cells and convey resistance to viral infection.

Partially blocking apoptosis by feeding the refractory *A. aegypti* larvae with caspase inhibitors allowed reliable detection of several immediate early viral genes. This indicates that the virus was able to enter into the nuclei of the midgut cells and successfully initiate gene expression when apoptosis was blocked or delayed. The fact that the application of caspase inhibitor alone was not reliable in allowing expression of structural genes may be because of several possibilities. First, there may be additional

antiviral responses that can block the developmental program of the virus. However, it is also possible that while caspase inhibitor partially blocks the activity of MX-activated caspases, it did not block the other antivirus mechanisms elicited by MX. It has been found that REAPER can nonspecifically inhibit protein synthesis (Holley et al., 2002), an activity that is independent of caspase activation. It remains to be seen as to whether MX also has this activity and can inhibit viral protein synthesis. It is notable that in the tissue culture system, while co-expressing IAP was very effective in blocking MX-induced cell death (Figure 2-1), application of very high level of caspase inhibitor only partially blocked/delayed cell death (Figure 2-5).

In summary, my study found that *mx*, the mosquito ortholog of the pro-apoptotic gene *reaper*, is transcriptionally activated in cells infected by CuniNPV and that the cellular outcome correlates with organism resistance/susceptibility. This should pave the way for more mechanistic studies addressing the role of apoptosis in determining host susceptibility or vector compatibility for human pathogens. For instance, as controlled expression of *mx_Ae.ae* has been successfully used as an effecter for sterile insect approaches (Fu et al., 2010), it will be very interesting to see whether adding viral responsiveness to such a system could also make the vector more resistant to viral infection.

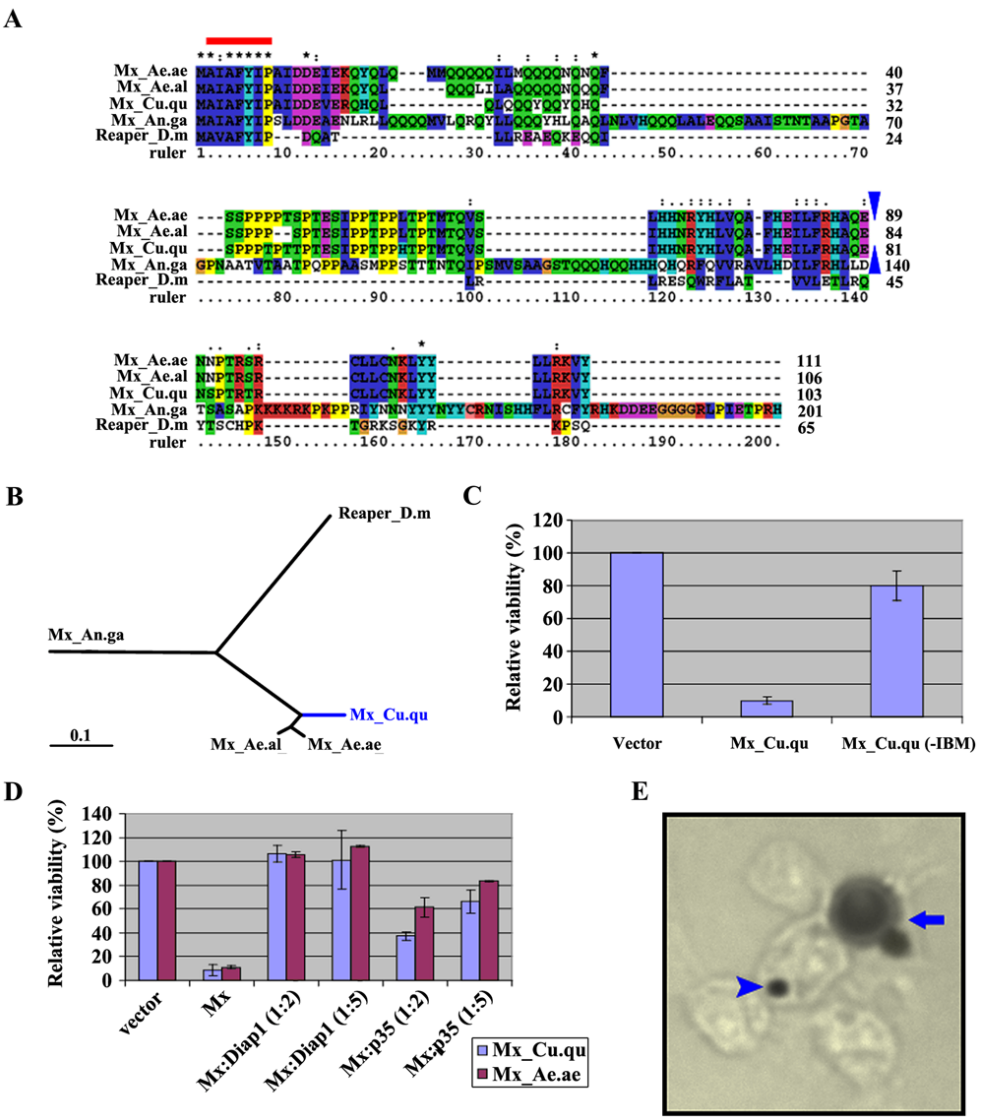


Figure 2-1. Gene cloning and characterization of *mx_Cu.qu*. (A) Alignment of protein sequences of MX orthologs in mosquitoes and REAPER from *D. melanogaster*. The blue triangles denote the relative position of the intron in MX sequences (there is no intron in Reaper). (B) Distance tree of these orthologs. (C) Expression of MX_Cu.qu kills C6/36 cells. However, when amino acids 2–4 are removed (ΔIBM), the protein has little, if any, proapoptotic activity. (D) MX_Cu.qu-induced cell death is almost completely blocked by co-transfection of DIAP1 and significantly inhibited by co-transfection of P35. Numbers in parentheses denote the ratio of respective genes. (E) C6/36 cells killed by expression of MX_Cu.qu or MX_Ae.ae show typical apoptotic morphology including cell fragmentation (arrow) and phagocytosis by neighboring cells (arrow head). Cells co-transfected with pIE-LacZ and pIE-MX_Cu.qu were fixed 20 h later and processed for X-Gal staining.

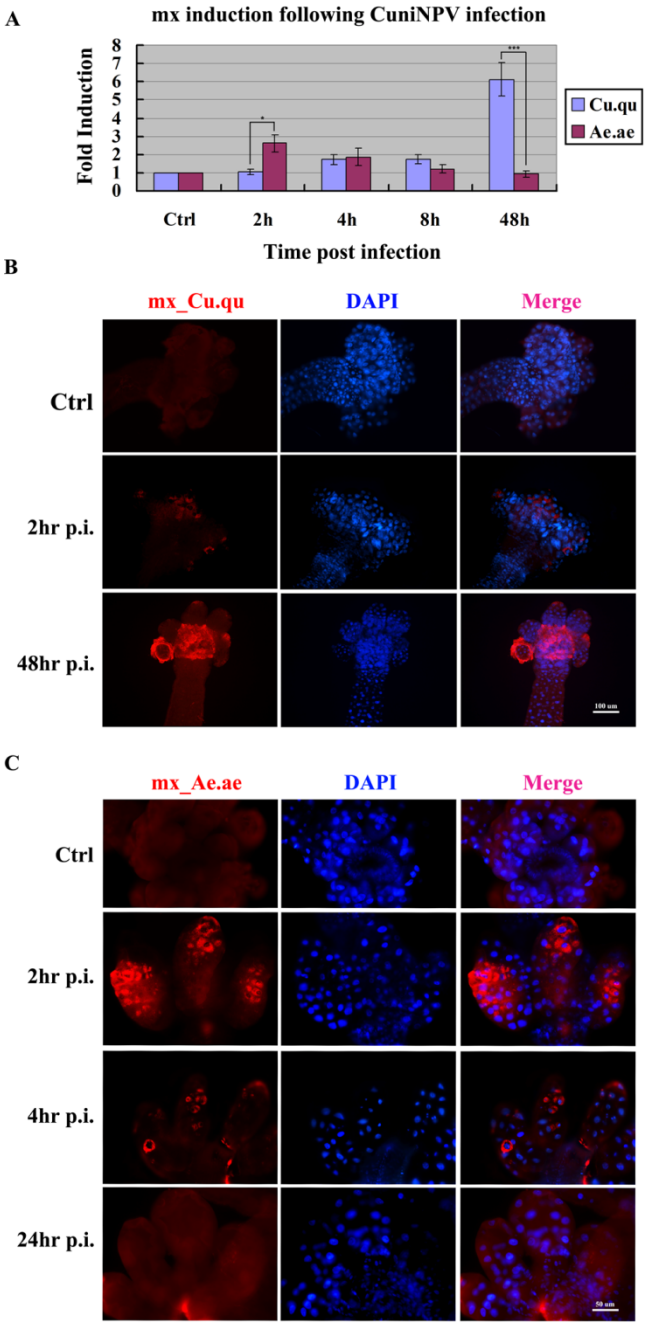


Figure 2-2. Induction of *mx_Cu.qu* and *mx_Ae.ae* following CuniNPV infection. (A). The level of *mx_Cu.qu* and *mx_Ae.ae* in pooled larvae following CuniNPV infection. Expression level was first normalized with house-keeping genes before calculating the fold induction. Data are presented as mean±S.D. of at least three independent experiments. (*P-value<0.05 and ***P-value<0.001.) (B) and (C). Expression of *mx_Cu.qu* and *mx_Ae.ae* in the midguts of CuniNPV-infected or control (Ctrl) larvae was monitored with FISH. Photos are the representatives of three independent experiments.

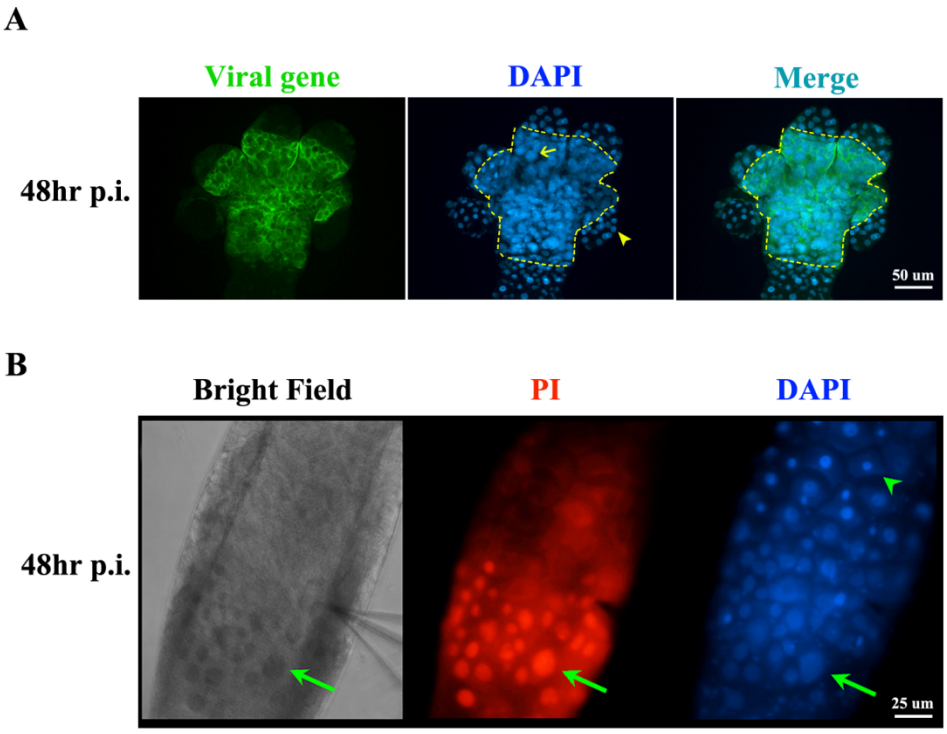


Figure 2-3. Necrosis in CuniNPV-infected C. quinquefasciatus larvae. (A) Cells infected with CuniNPV at 48 h p.i. were identified with a pool of fluorescein-labeled cRNA probes against CuniNPV genes, cun24, cun75, and cun85. Virus-infected cells at this time demonstrated hypertrophied nuclei (arrow) compared with uninfected cells (arrow head). Note that there is clear separation between cells that were positive for viral gene expression versus those that were negative (dashed line). More than 100 midguts were examined in several independent experiments; all cells positive for viral gene expression at this time point have hypertrophied nuclei. (B) Cells with hypertrophied nuclei have compromised cell membrane integrity. Live larvae at 48 h p.i. were exposed to 1 mg/ml PI in culture media for 10 min before the midguts were dissected out, fixed with paraformaldehyde, and counterstained using DAPI. All the cells with hypertrophied nuclei (arrow) are also permeable to PI, indicating necrotic cell death. In contrast, cells in the anterior midgut (arrow head) have normal nuclei and intact membrane. More than 40 midguts were observed in three independent experiments, and essentially all of them showed the described association between nuclei morphology and permeability to PI.

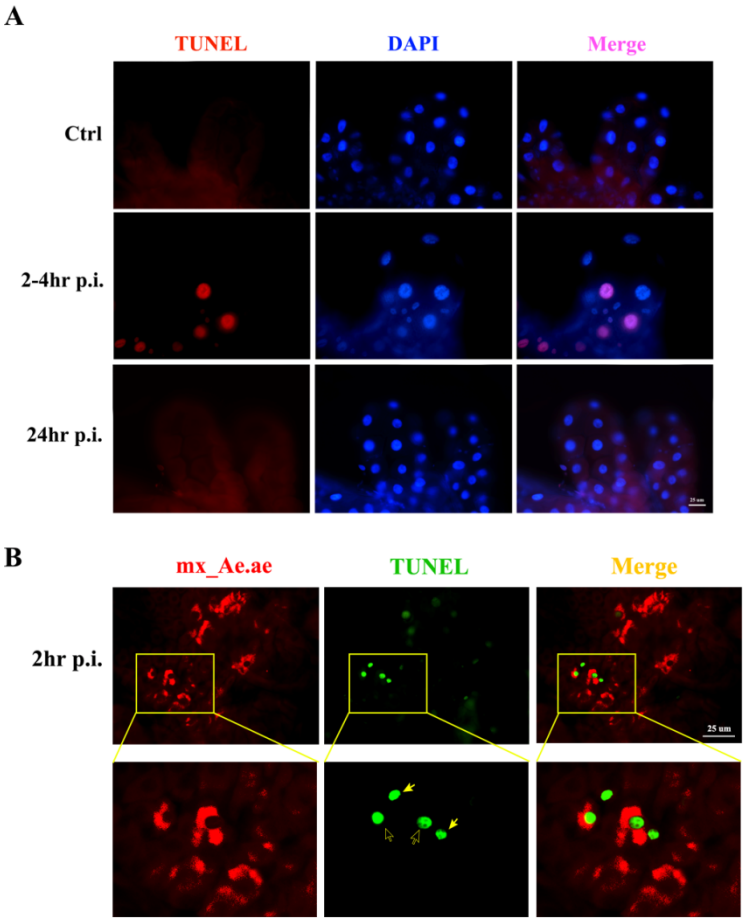


Figure 2-4. Rapid apoptosis following the *mx_Ae.ae* induction in *A. aegypti* larvae exposed to CuniNPV. (A) TUNEL assay in *A. aegypti* midgut. At 2–4 h after CuniNPV infection (2–4 h p.i.), significant increase of TUNEL-positive cells can be detected in over 80% of the examined midguts. In contrast, few TUNEL-positive cells were detectable in the control animal (Ctrl) or the midgut of infected animal at late time points (24 h p.i.). (B) Colocalization analysis indicated that TUNEL-positive cells are the ones that express *mx_Ae.ae*. Early-stage TUNEL-positive cells (open arrow), indicated by relatively normal nuclei morphology and intact cytoplasm, have high levels of FISH signal for *mx_Ae.ae*. The *mx_Ae.ae* signal is not visible in later stage TUNEL-positive cells with significantly condensed nuclei (solid arrow), which is likely because of the shrinkage of cytoplasm and/or degradation of macromolecules at this stage. Some *mx*-expressing cells are not TUNEL positive, which is likely because of the lag between *mx* expression and the activation of caspase-dependent DNase.

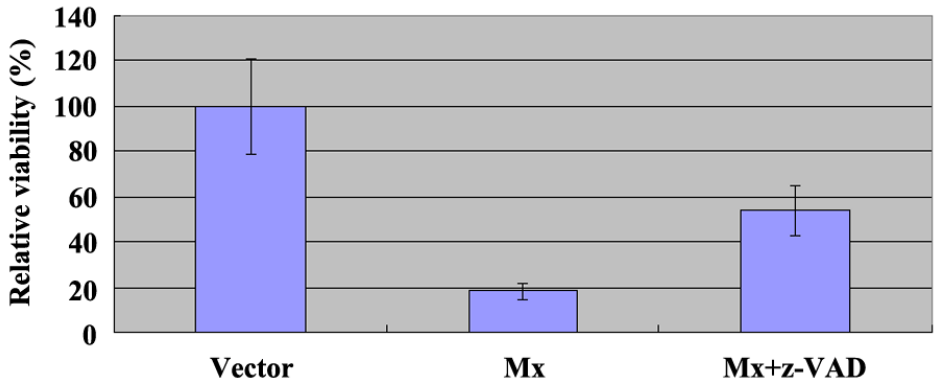


Figure 2-5. Mx_Ae.ae induced cell death could be partially rescued by caspase inhibitor z-VAD-fmk. Cultured C6/36 cell was transfected with Vector or Mx in the absence or presence of z-VAD. 100uM z-VAD partially suppressed MX-induced cell death.

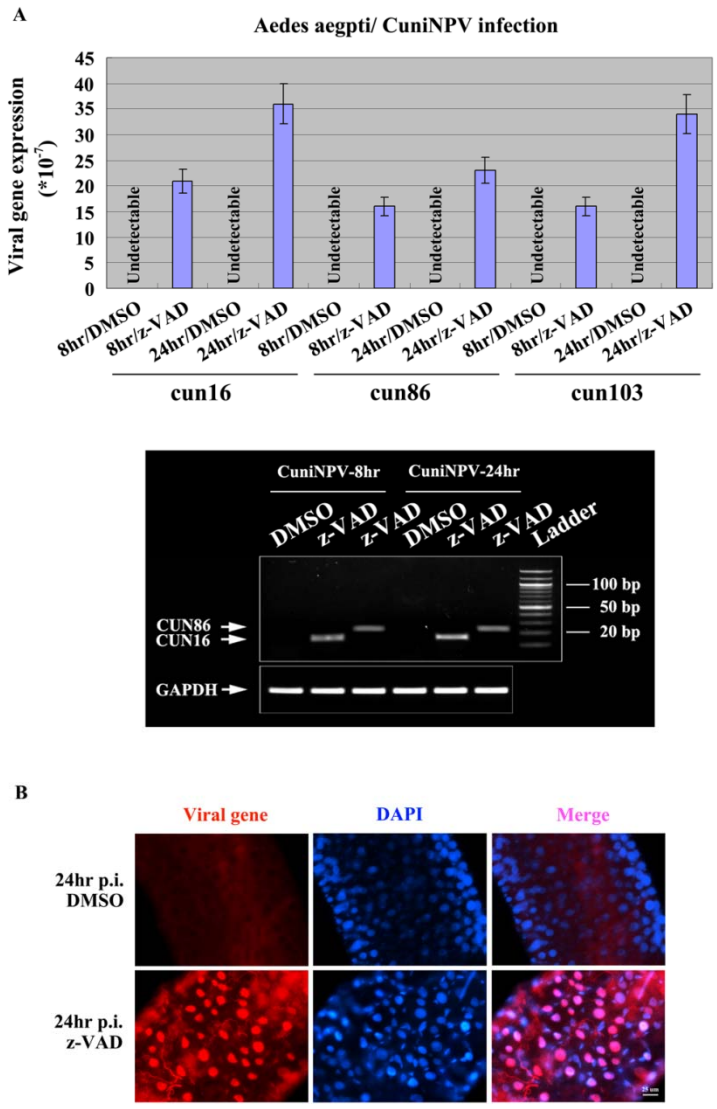


Figure 2-6. Suppressing apoptosis in *A. aegypti* leads to expressions of viral genes. (A) Suppressing apoptosis allowed the expression of ie viral genes. None of the three ie CuniNPV genes, *cun16, cun86, and cun103*, could be detectable by Q-PCR in *A. aegypti* larvae exposed to CuniNPV and treated with DMSO only. In contrast, all the three genes could be detected in *A. aegypti* larvae treated with capsase inhibitors z-VAD-FMK (z-VAD). Bottom panels are gel pictures of the Q-PCR products. (B) FISH was performed with a pool of cRNA probes against CuniNPV genes, *cun16, cun86*, and *cun103*. Top panel, no viral gene could be detected in the midgut of any of the DMSO-treated larvae (over 100 examined) at 24 h after CuniNPV infection. In contrast, viral gene expression was detectable in ~20% of the midguts dissected from caspase inhibitor-treated larvae.

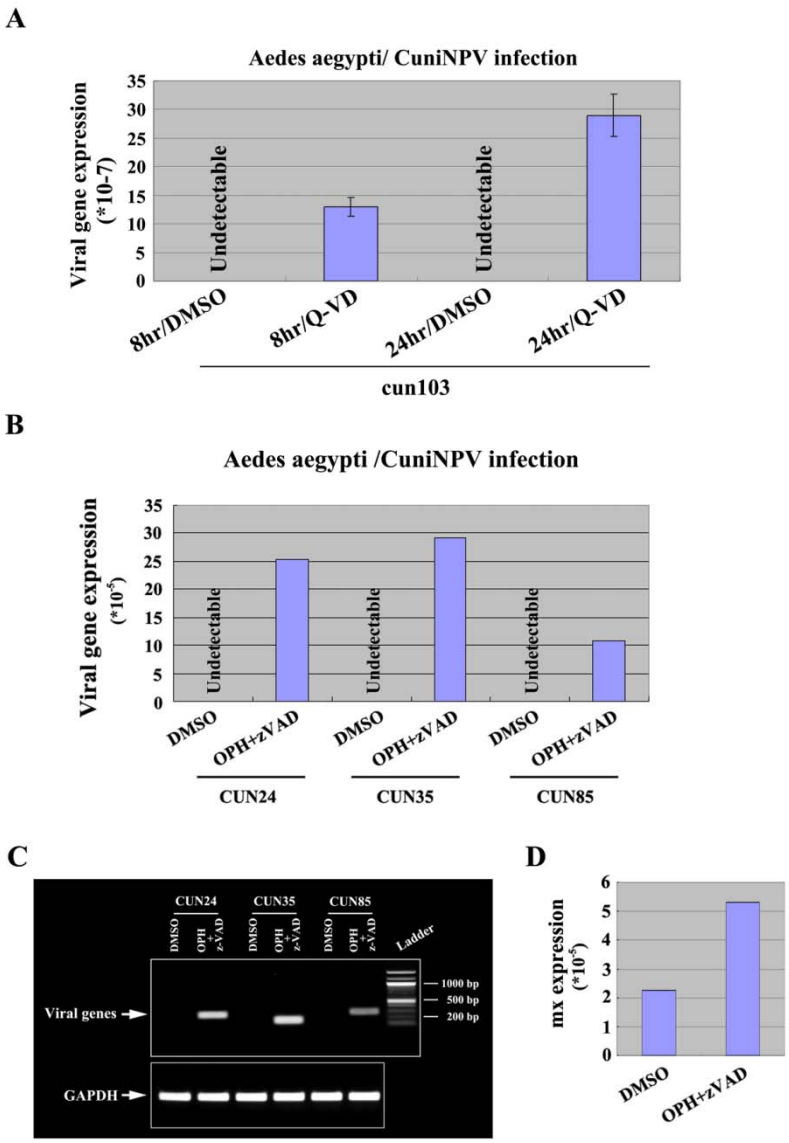


Figure 2-7. Suppressing apoptosis with caspase inhibitor Q-VD and combination of z-VAD and Q-VD allowed expression of viral genes in the refractory *A.aegypti* larvae. (A) Detection of cun103 in virus exposed *A. aegypti* larvae treated with 50uM Q-VD but not in the larvae samples treated only with DMSO. (B-D) At one of the trials, a combined treatment of Q-VD and z-VAD led to the detection of capsid genes *cun24*, *cun35* and occluded body gene *cun85* at 48 hr p.i. The specificity of QPCR analysis (B) was verified by gel electrophoresis (C) and sequencing of the DNA fragments. Interestingly, in this trial, the level of *mx_Ae.ae* at 48hr p.i. is significantly higher than that of the DMSO-treated larvae (D). This could be either due to more cells were infected through secondary infection at this late time point, or, similar to what we observed in *C. quinquefasciatus*, due to the accumulation of *mx_Ae.ae* message in infected cells that failed to undergo apoptosis.

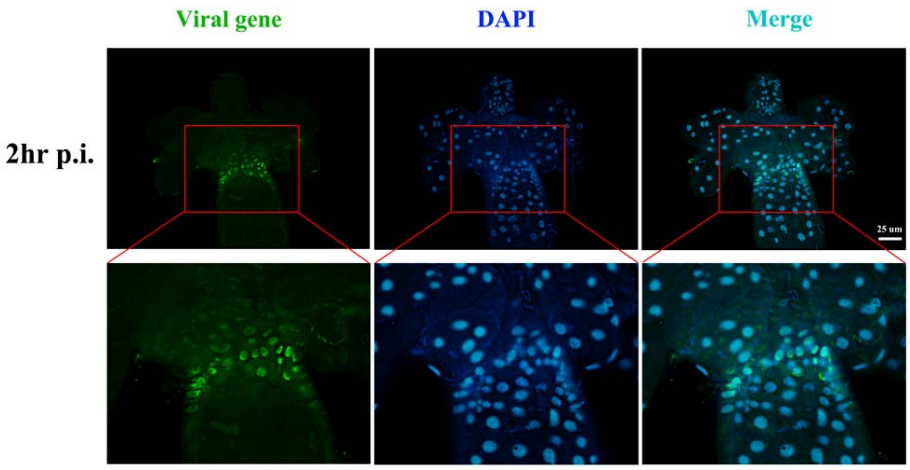


Figure 2-8. Viral gene expression was confined to the nuclei of a few cells at early stage of CuniNPV infection in *Culex quinquefasciatus*. FISH was performed with a pool of cRNA probes against viral genes cun16, cun86 and cun103 in the midgut of the susceptible *C. quinquefasciatus* larvae at 2-4 hr post infection. Bottom panel, higher magnification of insets in top panels.

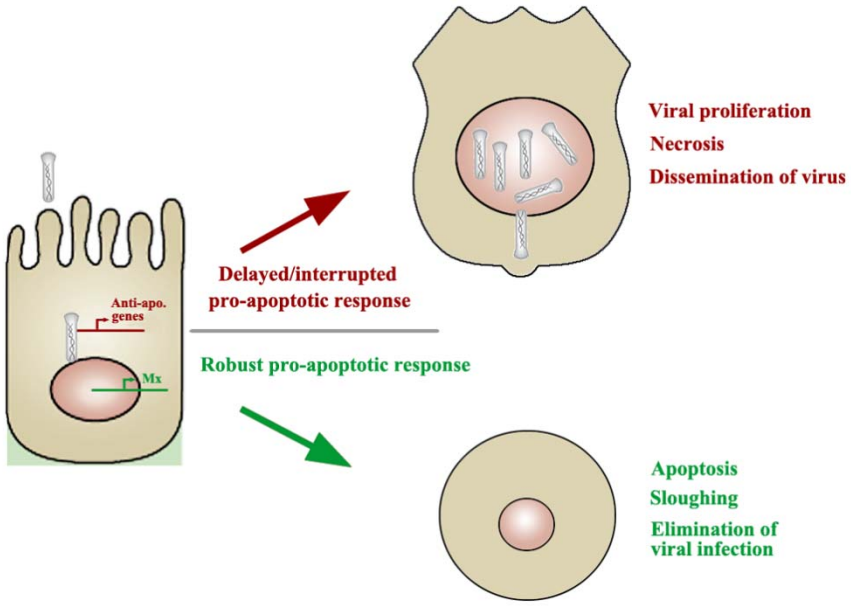


Figure 2-9. The race to apoptosis through the mosquito *reaper*. This is a schematic presentation of our findings. On CuniNPV infection, there is a competing 'race' between the host cell to express cellular pro-apoptotic gene(s) to eliminate the infected cell and the virus to express early genes to block apoptosis and initiate proliferation. A prompt induction of *mx* and apoptosis leads to elimination of the infected cell before the viral genes are detectable in the refractory mosquito *A. aegypti*. A delay in this process, either due to insufficient pro-apoptotic response or interference of caspase inhibitors, could subjugate the cell under viral control and render the organism susceptible to the virus.

# CHAPTER 3
# IDENTIFICATION OF THE ANTI-APOPTOTIC GENE(S) IN CUNINPV GENOME


## Summary

CuniNPV (*Culex nigripalpus* nucleopolyhedrovirus) is a kind of baculovirus which can specifically infect *Culex* mosquitoes. My previous studies suggested that similar to other baculoviruses, CuniNPV is also capable of inhibiting host cell apoptosis. However, sequence analysis did not find any orthologs of known anti-apoptotic genes from the CuniNPV genome, suggesting that CuniNPV carries novel anti-apoptotic gene(s). Identification of the anti-apoptotic gene(s) in the CuniNPV genome can enrich our knowledge about apoptosis regulation. To identify the anti-apoptotic gene(s) in CuniNPV, I extracted intact CuniNPV genome and partially digested it with restriction enzymes to get genomic fragments. A CuniNPV genomic DNA library was subsequently constructed by cloning those genomic fragments into a plasmid vector. Once the library is established, functional tests will be done in cultured cells to identify which genomic fragment possesses the anti-apoptotic activity. After the initial round of screening, the genomic range will be further narrowed down until the anti-apoptotic gene(s) is(are) localized. So far, I have finished ~90% of the library construction work.


## Introduction

Baculoviruses are a family of rod-shaped viruses with double stranded DNA genome (dsDNA). They have a very restricted host range, i.e., they only infect arthropods and are non-pathogenic to humans (Clem, 2007). Therefore, they have been widely used in biological control of pest insects. Moreover, the baculovirus expression system is powerful for foreign gene expression and is being widely used nowadays to produce foreign gene products. Given the fact that baculoviruses can enter almost any

kind of cell but only replicate in certain insect cells, they have been studied to serve as vectors for human gene therapy (Clem, 2001).

In addition to those benefits mentioned above, another important discovery derived from baculovirus study is their ability to suppress host cell apoptosis. Several well-known anti-apoptotic genes have been originally identified from various baculoviruses (relative information can also be found in Chapter 1). For example, *p35* which was identified from baculovirus AcMNPV, can serve as an inhibitor of activated effecter caspases (Bump et al., 1995; Clem et al., 1991; Xue and Horvitz, 1995). It is the only known gene that can inhibit apoptosis in all three major model organisms, the nematode, the fly and the mouse. Later on, the crystal structure of P35 revealed a loop which contains a caspase cleavage site. After being recognized by activated caspase, P35 and caspase can form a stable complex to sequester the caspase activity (Fisher et al., 1999). Below the loop is an α-helix followed by a β-sheet. By disrupting the α-helix structure, the anti-apoptotic activity of P35 can be abolished (Zoog et al., 1999).

So far, no P35 homologs have been reported from cells. However, beside AcMNPV, other baculoviruses have been found to carry *p35*-homologous genes such as BmNPV (*Bombyx mori* NPV) and SlNPV (*Spodoptora littoralis* NPV). BmNPV P35 is ~90% homologous to AcMNPV P35 and can suppress BmNPV-induced apoptosis in *Bombyx mori* cells (Kamita et al., 1993). SlNPV P35 which is called P49 is more divergent. The sequence of P49 is ~49% similar to AcMNPV P35 (Du et al., 1999).

In addition to the *p35*, another anti-apoptotic gene, *iap*, was originally identified from baculovirus CpGV (*Cydia pomonella* granulovirus) using a genetic screen (Crook et al., 1993). To date, *iap*-homologous genes have been identified from more than 10

different baculoviruses. Other than this, many cellular *iap*-homologous genes have been found in multiple organisms. The functional principle of IAP has been discussed previously in Chapter 1.

So far, most of the baculovirus studies that have been done are focused on lepidopteron baculovirus. CuniNPV, which was identified in 2001, is the only sequenced Dipteran baculovirus (Afonso et al., 2001; Becnel et al., 2001). CuniNPV replicates in the nuclei of midgut epithelial cells in the gastric caeca and posterior midgut. Similarly to most of the baculoviruses, the CuniNPV lifecycle also involves two forms of virus: ODV (occlusion-derived virion) and BV (budded virion). ODV is encapsulated in the occlusion bodies that are composed of the proteins polyhedrin or granulin. Upon ingestion by the insects, the occlusion bodies dissolve under the alkaline conditions of the midgut. The ODV is released starting the initial infection. After replication in infected cells, a large number of viruses escape by budding, thereby initiating the secondary infection.

The CuniNPV genome is a circular, double-stranded DNA molecule of 108252 bp containing 252 ORFs, of which 109 are likely to encode proteins. Only 36 of the 109 putative genes show homology to genes from other baculoviruses (Afonso et al., 2001). The 109 putative genes encode proteins that have multiple functions such as DNA polymerase, RNA polymerase, transcriptional factors, structural proteins etc. Moreover, CuniNPV genome contains a gene, *cun075*, which shows homology to anti-apoptotic gene *p35* except that CUN075 lacks the 110-amino-acid C-terminal region of AcMNPV P35. Moreover, no homologs of *iap* has been found in CuniNPV genome.

My previous study indicated that in CuniNPV-infected *Culex* mosquito, at the late stage of infection, the cells become necrosis (Figure 2-3) rather than apoptosis,

although at this point the level of pro-apoptotic gene, *mx*, was very high (Figure 2-2). It has been shown that if the apoptotic machinery has been initiated by certain stimuli such as TNF (Tumor Necrosis Factor) treatment but get inhibited from downstream using caspase inhibitor, the cells eventually undergo necrosis (Nicotera and Melino, 2004). This strongly suggests that CuniNPV is capable of inhibiting apoptosis downstream of *mx* induction. As mentioned, *cun075* shows certain level of homology to *p35*, however, functional test by us did not find any anti-apoptotic activity of *cun075,* which suggests that CuniNPV inhibits apoptosis using a novel gene(s). In this study, I constructed the genomic library of CuniNPV through partial digestion by restriction enzymes and molecular cloning. The following functional test will be performed to identify the novel anti-apoptotic gene(s) carried in CuniNPV genome. Identification of the novel anti-apoptotic gene(s) will increase our understanding of the processes involved in apoptosis regulation.

## Materials and Methods

### Cell Culture and Transfection

The mosquito *Aedes albopictus* cell line C6/36 was maintained with MEM culture medium (Cellgro, Manassas, VA, USA) supplied with 10% FBS (Fetal Bovine Serum) (Sigma, St. Louis, MO, USA), 1% penicillin/streptomycin (Sigma) and 1% nonessential amino acid (Cellgro) at 28$^{o}$C with 5% $CO_2$. The *Drosophila* cell line S2 was maintained with Schneider's medium (Sigma) with 10% FBS, 1% penicillin/streptomycin and 1% nonessential amino acid at 25$^{o}$C without $CO_2$. Transfection of C6/36 and S2 was done with Insect GeneJuice transfection reagent (EMD Millpore, Billerica, MA, USA) and Cellfectin (Invitrogen) respectively following the manual provided by the manufacturer.

**Extraction of CuniNPV Genomic DNA**

CuniNPV genomic DNA was extracted from occlusion bodies following a protocol adapted from a previously reported method (Bruce et al., 1991; Wilson, 2001). Generally speaking, CuniNPV occlusion bodies (~$2.5\times10^{12}$) were dissolved in 60ul of Buffer 1 (0.1M NaOH+10% SDS v/v=5:1) by incubating at room temperature for 8 min. Then the alkaline condition was neutralized with 50ul of 1M Tris (PH 8.0). After treatment with 6ul of 5mg/ml protease K (final conc. 100ug/ml) at 37$^{o}$C for 1 hr, 16ul of Sarkosyl was added (final conc. 2.5mg/ml) and kept incubating at 37$^{o}$C for 1.5 hr. 45.6ul of 5M NaCl was then added to a final concentration of 0.5M and followed by addition of 0.15 volumes of CTAB (Hexadecyl trimethyl-ammonium bromide)/NaCl (~53.6ul). After incubating at 65$^{o}$C for 1 hr, the suspension was extracted once with 412ul of chloroform/isoamyl-alcohol (24:1) and once with an equal volume of phenol/chloroform/isoamyl-alcohol (25:24:1). The DNA was precipitated by adding 0.6 volumes of isopropanol and centrifugation at 15000g for 10 min. The pellet was washed with 70% ethanol and resuspended in a small volume of TE buffer (0.01M Tris, 0.001M EDTA, PH 8.0).

**Construction of CuniNPV Genomic Library**

To construct the CuniNPV genomic library, the purified CuniNPV genomic DNA was digested with restriction enzymes. To obtain large genomic fragments, partial enzyme digestion was performed instead of complete digestion. To ensure a certain level of overlapping among different fragments, the extracted genomic DNA was partially digested with several different restriction enzymes. Specifically, CuniNPV genomic DNA was digested with 0.5u BamH1 or Kpn1 for 10 min at 37$^{o}$C. The digestion products were separated by electrophoresis on 0.7% agarose gel. Another option of

partial digestion is using high unit of restriction enzyme combined with short digestion time (e.g. 10u restriction enzymes for 20 sec at 37$^{o}$C). The fragments larger than 5 kb were recovered from the gel with QIAEXII Gel Extraction Kit (QIAGEN, Valencia, CA, USA) following the manual provided by the manufacturer. The recovered fragments were then ligated into the pBlueScript plasmid vector and transformed into DH5α competent *E.Coli* cells (Invitrogen, Grand Island, NY, USA). The bacterial colonies were grown on the LB (Lysogeny Broth) agar plate containing Ampicillin (100ug/ml) and x-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) in a 37$^{o}$C incubator. White colonies were picked and the contained insertions were verified by sequencing. The fragments were aligned to the CuniNPV genome with BLAST (Basic Local Alignment Search Tool) provided by NCBI (National Center for Biotechnology Information).

**Quantitative Real-Time PCR (Q-PCR)**

Plasmids were transfected into the S2 cells and 24 hr later, the cells were lysed and total RNA was extracted with RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the protocol provided by the manufacturer. RNA samples were treated with DNase I to remove genomic DNA. cDNA was prepared by reverse transcription of total RNA with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Q-PCR was performed with an ABI 7500 Fast thermocycler (Applied Biosystems) following protocols provided by the manufacturer. Triplicates were measured for each gene/sample combination.

**Cloning Full Length *Mx_Culex***

Full length *mx_Culex* was obtained by PCR using *Culex quinquefasciatus* genomic DNA as the template. The primers used for amplifying *mx* are 5'-GGCAAGCAGTCGTGTTTGTA-3' and 5'-GAGCAGGTACTGGCTGGTTC-3'. PCR

product was sub-cloned into pMT vector which contains the copper-inducible promoter to achieve controllable expression of *mx*.

### In Vitro Cell Death Assay

Cell death assay will be performed essentially as previously described (Jones et al., 2000). For each test, a total of 1.0 ug of DNA mixed with 8ug of liposome Cellfectin in serum free media was distributed into 2 wells in a 24-well plate. This included 0.1 ug pIE-lacZ and 0.9 ug of the test DNA sample or a combination of samples in the pIE vector. Intact pIE vector was used as the control. The transfection lasted for 4 hours and was stopped by replacing the transfection mix with culture media supplemented with 10% FBS. At 20 hours post-transfection, cells were fixed and stained with x-Gal/IPTG. Blue cells were counted to calculate the percentage of cell survival.

## Results

### Extraction of High Quality CuniNPV Genomic DNA

To construct the genomic library of CuniNPV, I first extracted intact genomic DNA from occlusion bodies. On the basis of some previously published methods (Bruce et al., 1991; Wilson, 2001), I developed a protocol to extract CuniNPV genome. Figure 3-1 is the agarose gel picture of purified CuniNPV genomic DNA which indicated a clear band of the CuniNPV genome. This demonstrates that our newly developed protocol worked well to extract CuniNPV genomic DNA.

### Construction of CuniNPV Genomic Library

By using restriction enzyme partial digestion strategy, I have been trying to construct the CuniNPV genomic library which can cover the entire CuniNPV genome. The CuniNPV genomic fragments were cloned into the pBlueScript plasmid vector which allows me to do blue-white screening for the colonies containing insertions.

Moreover, the T3/T7 sites at pBlueScript can facilitate the sequencing of the insertions. The sequenced genomic fragments were mapped to the CuniNPV genome using BLAST. Figure 3-2 lists all current available fragments. The red backbone represents the linearized genomic map of CuniNPV and the black lines above indicate the cloned and sequenced fragments. So far, I have covered ~90% of the entire CuniNPV genome and the nearby fragments have certain level of overlapping.

**The Gene Contained in the Plasmid Can Be Successfully Expressed**

Since pBlueScript vector has no promoter in it, the genes inserted were presumably driven by their own promoters. To ensure that the inserted genes can be expressed successfully, I randomly picked several plasmids and transfected into *Drosophila* S2 cells. RNA was then extracted and gene expression was examined by Q-PCR. The plasmids that I transfected were #5 and #10 (Figure 3-2). The corresponding genes that I examined were *cun026* for #5 and *cun068*, *cun071* for #10. Q-PCR results indicated that in #5-transfected cells, viral gene *cun026* was easily detected. Under threshold of 1, the Ct value is ~30. In contrast, *cun026* was undetectable in #10-transfected cells, which proved the specificity of the *cun026* signal (Figure 3-3A). Likewise, *cun068* and *cun071* were easily detected in #10-transfected cells but not #5-transfected cells (Figure 3-3B & C). Those signals cannot be due to the contamination of the transfected plasmid DNA because I cannot detect any signal for any gene from the RNA of #5 or #10-transfected S2 cells (data not shown). Taken together, these data indicates that the genes in the plasmid can be successfully expressed when transfected into the cultured cells.

## Discussion

Baculoviruses have made great contributions to humans. There are several benefits that came from baculoviruses studies. First, baculoviruses have been widely used to express foreign proteins in eukaryotic cells. Second, baculoviruses are useful to control certain insect pests. Last but not least, baculoviruses have been used to study virus-host interactions and to aid in the identification of several anti-apoptotic genes. This has largely increased our understanding of the pathways regulating apoptosis.

CuniNPV is a the first baculovirus isolated from mosquitoes. It is the only sequenced dipteran baculovirus so far. My previous studies suggest that similar to lepidopteran baculoviruses, CuniNPV is also capable of inhibiting host cell apoptosis. However, only *cun075* shows a certain level of homology to AcMNPV *p35*; functional tests found that *cun075* had no anti-apoptotic activity at all. This suggests that CuniNPV carries novel anti-apoptotic genes to inhibit host cell apoptosis. The aim of this chapter is to identify and functionally characterize the anti-apoptotic gene(s) in CuniNPV genome. The strategy is to construct the CuniNPV genomic library and screen for the fragments that possess anti-apoptotic activity.

To date, I have covered ~90% of the entire CuniNPV genome (Figure 3-2). Once the library is established, we will screen for the fragments possessing anti-apoptotic activity by *in vitro* cell death assay. To include the possibility that CuniNPV uses microRNA to inhibit *mx*-induced apoptosis, I will use full length *mx* which contains 5'-UTR (Untranslated Region) and 3'-UTR besides the open reading frame in the *in vitro* cell death assay because 3'-UTR is usually the target region of microRNA.

The plasmid vector that I used to construct the CuniNPV genomic library is pBlueScript. The benefit of this vector is it allows doing blue-white screening which

saves much of the effort in isolating colonies that contain insertions. However, one potential drawback of using this vector is that pBlueScript is not an expressing vector, i.e. there is no promoter in the vector so the expression of the inserted genes cannot be ensured. Therefore, the successful expressions of inserted viral genes need to rely on their own promoters. Q-PCR result indicated that indeed, the inserted genes can be expressed after transfection of the plasmids into the cells (Figure 3-3). So far, I have only test two genes. After finishing construction the genomic library, it might be necessary to examine the expressions of all 109 putative genes using Q-PCR to ensure their expression. If certain genes failed to be expressed, I will clone those fragments to insect cell expressing vectors such as pIE.

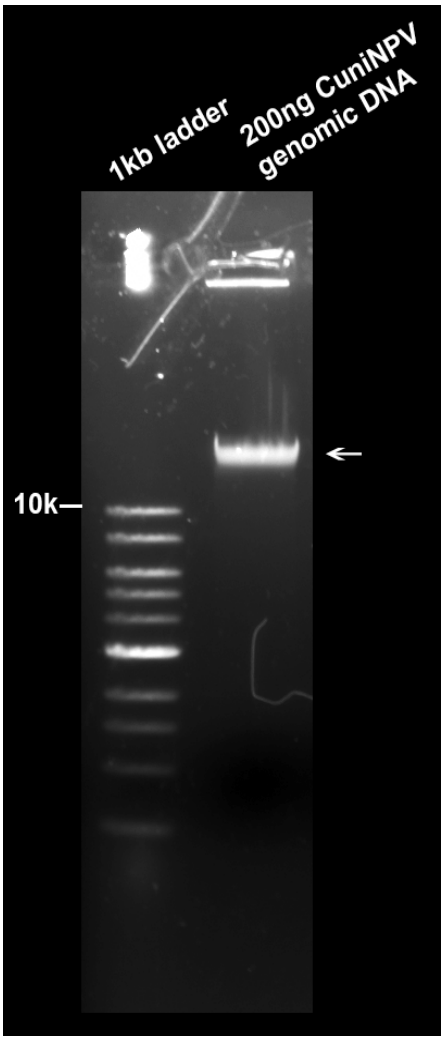


Figure 3-1. Gel picture of extracted CuniNPV genomic DNA. The CuniNPV genomic DNA band is indicated by the white arrow.

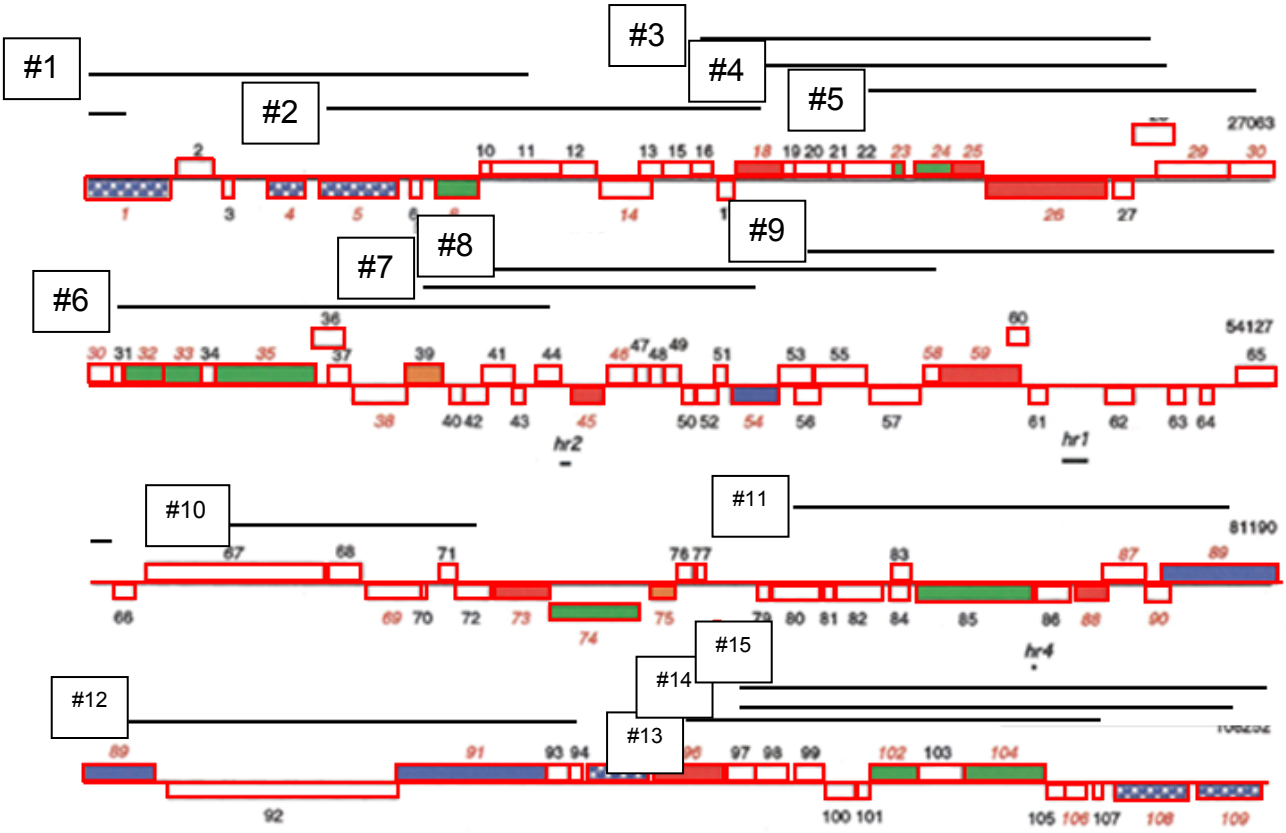


Figure 3-2. Currently sequenced CuniNPV fragments. The red backbone is the linearized genomic map of CuniNPV and the red boxes are the 109 of putative genes. The black lines above indicate the fragments that have been cloned into the plasmid vector and sequenced. The identifier of each plasmid is labeled in front of the fragments (picture adapted from (Afonso et al., 2001))

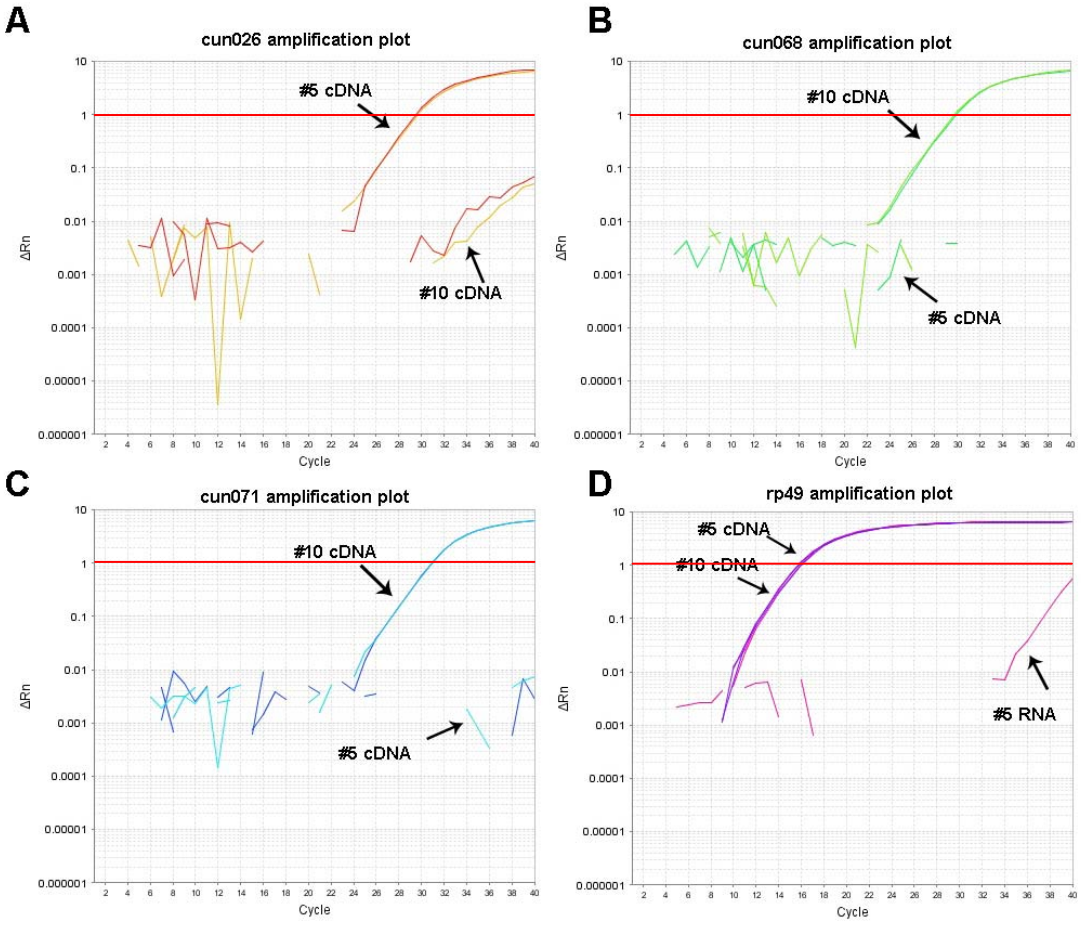


Figure 3-3. Amplification plots of several target genes, *cun026* (A), *cun068* (B), *cun071* (C) and *Drosophila* housekeeping gene *rp49* (D). The red line in each panel represents the threshold of 1.

# CHAPTER 4
# P53-MEDIATED RAPID INDUCTION OF APOPTOSIS CONVEYS RESISTANCE TO VIRAL INFECTION IN INSECTS


## Summary

Arthropod-borne pathogens account for millions of death each year. Understanding the genetic basis controlling vector susceptibility to pathogens has profound implication for developing novel strategies for controlling insect transmitted infectious diseases. The fact that many viral genes have anti-apoptotic activity has long led to the hypothesis that induction of apoptosis could be a fundamental innate immune response. However, the cellular mechanisms mediating the induction of apoptosis following viral infection remained enigmatic, which prevented experimental verification of the functional significance of apoptosis in limiting/preventing viral infection in insects. In addition, studies with cultured insect cells showed that either there is a lack of apoptosis, or the pro-apoptotic response happens relatively late, thus casting doubt on the functional significance of apoptosis as an innate immunity. Using *in vivo* mosquito models mimicking the native route of infection, I found that there is a rapid induction of *reaper*-like pro-apoptotic genes within a few hours following exposure to DNA/RNA viruses. Recapitulating similar response in *Drosophila*, I found that this rapid induction of apoptosis requires the function of P53 and is mediated by a stress –responsive regulatory region upstream of *reaper*. More importantly, I showed that the rapid induction of apoptosis is responsible for denying the expression of viral genes and blocking/limiting the infection. Genetic changes influencing this rapid induction of *reaper*-like pro-apoptotic genes lead to significant differences in susceptibility to viral infection.

## Introduction

As a genetically regulated mechanism of cell elimination, apoptosis plays an important role in maintaining tissue homeostasis through the removal of obsolete or potentially dangerous cells. The controlled collapse of intracellular infrastructures and encapsulation of cell bodies associated with apoptotic cell death has long led to the speculation that apoptosis could function as an efficient innate immune mechanism against intracellular pathogens such as virus (Clouston and Kerr, 1985; Everett and McFadden, 1999; Hardwick, 1998).

The majority of evidences supporting the role of apoptosis as an important anti-viral immune response came from the study of viruses. Many viruses encode genes that can interfere with the regulation of apoptosis at various levels (Benedict et al., 2002). For example, the pivotal upstream regulator *p53* is a frequent target of viral inhibition. It can be sequestered by the SV (Simian virus) 40 T antigen or degraded by proteins encoded by Adenovirus or human papillomaviruses. In addition, it was found recently that adenovirus E4orf3 can block P53-induced gene expression by promoting de novo heterochromatin formation at P53-targeted promoters (Soria et al., 2010). Besides blocking the sensors / upstream regulators, viral proteins can also directly interfere with the apoptotic machinery. For instance, many viruses (including adenovirus, Epstein-Barr virus, Kaposi's sarcoma-associated γ-herpesvirus, and mouse γ-herpesvirus, etc.) encode functional homologs of the anti-apoptotic regulator BCL-2, which can directly inhibit the intrinsic apoptotic pathway. Similarly, key components of the extrinsic pathway are targeted by viruses such as Shope fibroma virus, myxoma virus, and smallpox virus, etc. (Best, 2008). Last but not least, some viruses, particularly insect baculoviruses, encode caspase inhibitors. P35 and IAP (Inhibitor of Apoptosis) were

initially identified in lepidopteran baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) and *Cydia pomonella* granulosis virus (CpGV), respectively (Clem et al., 1991; Crook et al., 1993). It has been very well demonstrated that these two genes are required for the infectivity of baculoviruses (Clem, 2005). All of these evidences strongly suggest that evading/delaying apoptosis is an important mechanism for viruses to succeed in establishing proliferative infection.

Despite of these evidences from virology studies, the functional role of apoptosis in mediating insect immunity has been under debate. Since insects do not have adaptive immunity, induction of apoptosis could conceivably play an even more prominent role in antiviral defense than in mammalian or vertebrate hosts. Indeed, it has been documented that ingestion of blood containing West Nile virus induces apoptosis in the midgut of a refractory *Culex pipiens* strain (Vaidyanathan and Scott, 2006). In contrast, necrosis has been associated with Western Equine Encephalomyelitis virus infection in susceptible *Culex tarsalis* strains (Weaver et al., 1992). Although these observations suggested that apoptosis might play an important role in insect antiviral defense, we know little about what mechanism is involved in the induction of apoptosis following viral infection. Consequently, there is a conspicuous lack of direct evidence supporting the role of apoptosis in insect innate immunity. In the meantime, a series of studies conducted in cultured insect cells reported that apoptosis was either not observed (Blitvich et al., 2002; Borucki et al., 2002), or as is the case for AcMNPV or flock house virus (FHV), only observed relatively late in the infection cycle (i.e. at or after 24 hrs p.i.) (Settles and Friesen, 2008; Vandergaast et al., 2011). More importantly, blocking apoptosis in these infection systems seems to have little effect on

the infection and proliferation of the viruses. These observations raised the question as whether apoptosis is an innate immune response that can prevent/limit the infection, or it is simply one of the cellular outcomes associated with late stage viral infection.

Genetic studies in *Drosophila* revealed that the four IAP-antagonist genes, *reaper*, *hid*, *grim*, and *sickle* (also referred to as the RHG genes) together play a pivotal role in mediating developmental cell death (Steller, 2008). With the exception of HID, whose pro-apopotic activity can be suppressed by the MAP Kinase pathway (Bergmann et al., 1998a), RHG genes are mostly regulated at the transcription level and are selectively expressed in cells destined to die during animal development. Transcriptional activation of the RHG genes is also responsible for mediating the induction of apoptosis following cytotoxic stimuli such as irradiation. Interestingly, the sequences of the RHG genes diverged very fast during evolution. Consequently, no RHG ortholog was identified by the annotation of the genome of *Anopheles gambiae*. The first RHG gene in mosquitoes, *michelob_x* (*mx*), was identified with an advanced bioinformatics approach and verified as a bona fide IAP-antagonist (Zhou et al., 2005). Although the sequence of *mx* has diverged so much from that of *reaper*, to the extent that it is almost beyond recognition, its transcriptional regulation was surprisingly similar in that it is induced rapidly following UV irradiation.

Using a baculovirus-mosquito system mimicking the native route of infection, I found that *mx* is rapidly induced in virus-infected larval midgut cells in a refractory species (Liu et al., 2011). This rapid induction of *mx* in virus –infected cells was followed by quick apoptotic cell death and elimination of the infected cells. In contrast, this rapid induction of apoptosis is absent in a species that is susceptible to this virus (Liu et al.,

2011) (refer to Chapter 2 for details). Interestingly, the rapid induction of *mx* following blood meal containing Dengue virus serotype 2 (DEN-2) was also observed in a refractory *Aedes* mosquito strain but not in a susceptible strain. While these observations strongly suggested that the rapid induction of pro-apoptotic response could be responsible for the resistance phenotype, the lack of genetic tools prevented mechanistic test of this hypothesis.

In this study, by using two *in vivo* virus infection systems in *Drosophila melanogester*, I demonstrated that upon viral injection, several RHG genes are quickly induced at 1-2hr post infection. The induction of the RHG genes requires the function of dP53 and is mediated by a highly conserved regulatory region in the vicinity of the *reaper* gene. More importantly, I showed that, in live animals, the rapid induction of apoptosis is an important innate immune response that is capable of blocking/limiting viral gene expression and infection.

## Materials and Methods

### *Drosophila* Strains

*Drosophila* white 1118 (w1118) strain was used as a standard wild-type strain. *p53* deficient line p53[5A-1-4] which has a 3.3k deletion in *p53* gene (Rong et al., 2002) was obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN, USA). The IRER deficient strain B11 was generated by us as previously reported (Zhang et al., 2008). All strains were maintained on a standard cornmeal medium at room temperature.

### Cell Culture, Viral Production and *Drosophila* Infection

Baculovirus AcMNPV was produced as previously described (Huang et al., 2011). Generally speaking, *Spodoptera frugiperda* cell line sf9 was cultured with sf900 medium

at 28$^{o}$C incubator. Infectious *Autographa californica* nucleopolyhedrovirus (AcMNPV) was obtained by transfection of sf9 cells with bacmid DNA containing AcMNPV genome. Virus was tittered in sf9 cells by standard end point dilution assay. For AcMNPV infection, *Drosophila* 3$^{rd}$ instar larvae were injected with budded AcMNPV at a dosage of 3x10$^{4}$ PFU/larvae from the dorsal-posterior part of larval body. Injected larvae were kept in sf900 medium with or without virus for indicated times before subject to RNA extraction and Q-PCR analysis. Flock house virus particles were purified following established protocols (Schneemann and Marshall, 1998). For flock house virus (FHV) infection, adult flies 4-6 day of age were used. FHV stock was prepared with sf900 medium. Infection was achieved by injection of viral suspension into the thoraces of adult flies. The injected flies were then cultured with standard fly food at room temperature.

**RNA Extraction and Q-PCR**

Larval total RNA was extracted with RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the protocol provided by the manufacturer. Adult RNA was extracted with TRIZol Reagent (Invitrogen, Grand Island, NY, USA) following manufacturer's manual and purified with RNeasy Mini Spin Column (QIAGEN). RNA samples were treated with DNase I to remove genomic DNA. cDNA was prepared by reverse transcription of total RNA with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Q-PCR was performed with an ABI 7500 Fast thermocycler (Applied Biosystems) following protocols provided by the manufacturer. Triplicates were measured for each gene/sample combination. The oligo sequences of the main target genes are as follows: *reaper*: 5'-ACGGGGAAAACCAATAGTCC-3' and 5'-TGGCTCTGTGTCCTTGACTG-3'; *hid*: 5'-CTAAAACGCTTGGCGAACTT-3' and 5'-

CCCAAAAATCGCATTGATCT-3'; *rp49*: 5'-GCTAAGCTGTCGCACAAATG-3' and 5'-GTTCGATCCGTAACCGATGT-3'; AcMNPV *ie0*: 5'- CGAGACGCGTTGAAGCTAAT-3' and 5'- CGCAACATTCTTTTGGCTTT-3'; AcMNPV *ie1*: 5'-GGCAGCTTCAAACTTTTTGG-3' and 5'- TTCACACCAGCAGAATGCTC-3'; FHV RNA1: 5'- CCAGATCACCCGAACTGAAT-3' and 5'-AGGCTGTCAAGCGGATAGAA-3'; FHV RNA2: 5'-CGTCACAACAACCCAAACAG-3' and 5'-GGTCGGTGTTGAAGTCAGGT-3'.

**FHV Genome Estimation**

The amount of FHV genome was estimated according to the pre-generated standard curve and regression equation. To get the standard curve of the viral dosage/Ct value, a serial dilution of known dosage of FHV was mixed with wild type adult male (one fly for one dilution). RNA extraction and Q-PCR were performed as described above to get the Ct value of viral RNA1 or RNA2. Standard curve and regression equation were generated using Microsoft Office Excel (version 2007).

**Fluorescent in Situ Hybridization (FISH)**

Probes were synthesized using digoxin (DIG)-RNA Labeling Mix (Roche, Madison, WI, USA). *Drosophila* 3$^{rd}$ instar larval skins were partially removed to expose inside tissue in 4% paraformaldehyde. After prefixing with 4% paraformaldehyde in PBT_DEPC (0.3% Triton in PBS made with DEPC pretreated double-distilled water) for 30 min, the tissue was incubated for 7 min with 50 mg/ml protease K in PBT_DEPC, and reaction was stopped by washing with 4% paraformaldehyde. Samples were incubated with probes diluted in hybridization buffer (50% formamide, 25% 2xSSC, 20 mg/ml yeast tRNA, 100 mg/ml ssRNA, 50 mg/ml heparin, and 0.1% Tween-20). Hybridization was performed overnight at 60 $^{o}$C. Larvae were incubated with

horseradish peroxidase (HRP)-conjugated anti-DIG (Roche) antibody after hybridization, followed by signal amplification using the Tyramid Signal Amplification Kit (PerkinElmer, Waltham, MA, USA).

**Antibody and Immunostaining**

Rabbit monoclonal antibody to cleaved caspase-3 was purchased from Cell Signaling (Danvers, MA, USA). The antibody was used at a dilution of 1:200. AlexaFluor 488 labeled goat anti rabbit antibody was purchased from Molecular Probes and was used at a dilution of 1:1000. Propidium iodide was purchased from Sigma (St. Louis, MO, USA). To detect cell necrosis, 100nL of PI (1mg/mL) was injected into the thoraces of adult flies. Injected flies were kept culturing with standard fly food at room temperature for 20 min before subjected to immunostaining. Standard procedure was used for immunostaining. Fly fat bodies were dissected in PBS containing 4% paraformaldehyde and fixed for 20min at room temperature. After being washed with PBS containing 0.1% Triton X-100 (PBST), the samples were blocked with PBST containing 5% normal goat serum for 30min. Samples were then incubated overnight with anti-cleaved caspase-3 antibody (1:200 dilution) at 4$^{o}$C. Labeling with secondary antibody was done at 25$^{o}$C for 2hr. Slides were mounted with Vectorshield Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Pictures were taken with a Leica upright fluorescent microscope (Leica, Bannockburn, IL, USA) using OpenLab software (Improvision, Coventry, UK).

## Results

### Rapid Induction of Mx Following DEN-2 Infection in a Refractory, but Not in a Susceptible, Strain of *Aedes aegypti*

My previous work found that following exposure to the mosquito baculovirus CuniNPV, the mosquito *reaper* ortholog *mx* was rapidly induced (within 2 hour p.i.) in infected midgut cells of the *Aedes aegypti* larvae. This induction of *mx* is followed by apoptotic death of the infected midgut cells at about 4-6 hour p.i.. However, this rapid induction of *mx* and apoptosis was absent in a mosquito species that is susceptible to CuniNPV infection (Liu et al., 2011).

Interestingly, similar correlation between the induction of *mx* and the sensitivity to virus infection was also observed in two *Aedes aegypti* strains following exposure to DEN-2. Blood meals containing DEN-2 (dengue virus serotype 2) was introduced to adult mosquitoes of the refractory (MOYO-R) or the susceptible (MOYO-S) strains. When the expression level of *mx* was monitored via Q-PCR, we found that it is significantly induced in the MOYO-R strain following DEN-2 exposure when compared with the control-fed mosquitoes (Figure 4-1). This induction of *mx* in the refractory strain was rapid, since at 3 hrs post blood meal (p.b.m.) the level of *mx* was about 2.5 fold higher in the virus-fed compared to the control-fed. The level of *mx* receded to lower levels at 18 hr p.b.m.. In contrast, there was no difference between control-fed or DEN-2 fed mosquitoes of the MOYO-S strain. This indicates that the susceptible MOYO-S strain lacks the rapid induction of *mx*.

### Rapid Induction of RHG Genes Following Viral Infection in Live Fruit Flies but Not in Cultured Cells

To test whether similar rapid induction of RHG genes and apoptosis can be observed following viral infection in the fruit fly, I tried to infect *Drosophila* larvae and

adults with AcMNPV and FHV, respectively. AcMNPV, a lepidopteran baculovirus with a dsDNA genome of about 134 kb, infects the larvae of susceptible hosts (Vail et al., 1971) but do not replicate in *Drosophila* cells. Budded AcMNPV virus was propagated in SF9 cells and introduced into the abdominal hemocoel of 3rd instar *Drosophila* larvae through micro-injection. Q-PCR analysis indicates that following AcMNPV injection ($3x10^4$ PFU per animal), two RHG genes, *hid* and *reaper*, were quickly induced as early as 1hr post injection (p.i.). By 2hr p.i., the level of pro-apoptotic genes has gone back to normal (Figure 4-2A). This rapid induction of RHG genes likely requires immediate early gene expression from the baculovirus, since UV-inactivated virus failed to induce *hid* expression (Figure 4-3). In parallel, I also introduced AcMNPV to cultured *Drosophila* DL-1 cells. Even at MOI of 20, AcMNPV failed to induce significant induction of *reaper* or *hid* at early stage of the infection. Moderate level of induction was observed at 6 hr p.i. and significant induction was observed at 24 hr p.i. (Figure 4-2B).

Since the RNA was extracted from homogenized whole larvae, the level of gene induction revealed by Q-PCR cannot fully reflect the magnitude of change of gene expression in specific cells. To monitor the level of *hid* in individual cells, FISH was performed with DIG-labeled RNA probes against *hid* and *reaper*. Several tissues, including fat body, midgut, hindgut, malpighian tube, ovary/testis, etc. were examined. I found that the induction of *hid* following AcMNPV infection was mainly observed in scattered fat body cells (Figure 4-2C). The number of *hid*-positive cells in the fat body following AcMNPV injection was about 10-20% of the total fat body cells. A few midgut cells, less than 1% of all cells in that tissue, were also found to be *hid*-positive in the infected larvae (Figure 4-2D).

The flock house virus (FHV) is a positive-sense single strand RNA virus of the nodavirus family (reviewed in (van Rij et al., 2006)). Originally isolated from grass grubs, it has been shown to replicate in plants, yeast, and a variety of insects (Galiana-Arnoux et al., 2006; Lanman et al., 2008). I injected FHV into the thoraces of the adult flies at dosages ranging from $2\times10^2$ to $2 \times 10^6$PFU/adult. Q-PCR result indicated that similar to AcMNPV, at 1-2hr post viral injection, the expression of *reaper* was significantly induced in FHV-injected adults. The level of *reaper* in FHV infected adults was about 1.6 fold of that in the control-injected sample (Figure 4-2E). This result demonstrated that besides DNA virus, infection by RNA virus can also induce rapid induction of RHG genes. This rapid induction of RHG genes was not observed when parallel infection was performed in *Drosophila* DL-1 cells. Addition of FHV, at 40 MOI, to cultured DL-1 cells did not induce *reaper* or *hid* expression until at about 36 hr p.i. (Figure 4-2F). This corresponds well with a previous report which showed that caspase activation and apoptosis happened after 36 hr p.i. when a similar dosage of FHV was applied to the DL-1 cells (Settles and Friesen, 2008). It has been shown that fat body is the major target of FHV in *Drosophila* (Dostert et al., 2005), to prove that the *reaper* induction is indeed due to viral infection, I performed FISH for *reaper* mRNA and immunostaining for FHV capsid protein in the fly strain that has DIAP1 overexpressed in the fat body cells to inhibit apoptosis. Indeed, in the cells that had FHV capsid signal, *reaper* signal is positive. In contrast, in the cells lacking FHV, there is no *reaper* expression (Figure 4-2G). This result indicates that *reaper* induction is indeed due to viral infection.

Taken together, these observations indicated that the rapid induction of RHG genes following viral exposure is a general phenomenon that can be observed in both

mosquitoes and *Drosophila*. In addition, this response is not limited to DNA viruses. Infection by RNA virus such as the FHV can elicit a rapid induction of pro-apoptotic genes as well. The significant difference in the timing of pro-apoptotic response following AcMNPV and FHV infection in animal models vs. that in cultured cells indicated that the dynamics of pro-apoptotic response, and possibly the mechanism, differs significantly. To elucidate the role of rapid induction of apoptosis in limiting/blocking viral infection in animals, I focused the following analysis on in vivo models.

**Rapid Induction of *Reaper* and *Hid* Requires *P53* and the Regulatory Region IRER**

The rapid induction of both *reaper* and *hid* following viral infection was reminiscent of what was observed following ionizing irradiation, in which case the function of the transcriptional factor P53 is required for the rapid induction of the RHG genes (Brodsky et al., 2004). To investigate the mechanism responsible for mediating virus -induced pro-apoptotic gene expression, I introduced AcMNPV infection to larvae of different genotypes and monitored the induction of *hid* following the injection. The P53 loss-of-function allele 5A-1-4 is a deletion generated via homologous recombination (Rong et al., 2002). Animals homozygous to this mutant allele have reduced level of stress-induced apoptosis, but are otherwise viable and have no obvious phenotype. When the level of RHG genes were monitored following injection of AcMNPV infection, I found that the induction of *hid* and *reaper* was completely blocked in this *p53* null mutant (Figure 4-4A).

Somewhat to my surprise, FHV -induced expression of RHG genes in the adult is also *p53*-dependent. Q-PCR results indicated that while in wild type flies, FHV infection can result in about 1.6 fold induction of *reaper* at 1-2hr post infection, FHV injection

failed to induce *reaper* expression in animals lacking p53 function (Figure 4-4B). Taken together, these results indicated that P53 plays a pivotal role in mediating the rapid induction of RHG genes following viral infection.

The induction of RHG genes following ionizing irradiation requires a regulatory region upstream of *reaper/grim/hid*, which was referred to as the IRER (Irradiation responsive enhancer region) (Zhang et al., 2008). In animals deficient for IRER, the induction of RHG genes *reaper* and *hid* following irradiation is either completely blocked or significantly suppressed depending on the tissue and development stage examined. The proximal breaking point of the deletion is about 3 kb upstream of the *reaper* promoter. Thus this regulatory mutant specifically blocks stress-induced expression of the RHG genes without deleting any transcribed region. More importantly, other DNA damage –induced responses, such as the induction of DNA repair proteins KU70/KU80, remains intact in this mutant (Zhang et al., 2008). My data indicated that similar to what was observed in the P53 mutant strain, AcMNPV and FHV -induced RHG gene expression is blocked in Df(IRER) animals, indicating this regulatory region is required for induction of *reaper/hid* following virus infection (Figure 4-4 A & B).

My previous work showed that midgut cells infected by CuniNPV following a native route of infection become TUNEL-positive at 4-6 hr p.i. (Liu et al., 2011). In wild type animals infected by AcMNPV or FHV, the rapid induction of RHG genes in fat body cells is followed by apoptosis at about 2.5 hrs p.i. (Figure 4-4 C & D). Apoptotic cells were recognized with an antibody developed against activated (cleaved) capase-3, which labels cells with activated upstream caspase DRONC (Fan and Bergmann, 2008). There is little if any apoptotic cells in larvae or adult fat bodies injected with control

media or suspension buffer, respectively. However, significant increase of apoptotic cells was observed at 2.5 hours following either AcMNPV or FHV injection. Corresponding with the absence of the induction of the RHG genes following viral infection, the rapid induction of apoptosis in both larval and adult fat body was blocked in the P53 null mutant. The induction of apoptosis was also blocked in homozygous Df(IRER) animals. These evidences indicate that P53 induced expression of the RHG genes following viral infection is responsible for the rapid induction of apoptosis.

Significant induction of RHG genes was not observed in P53-/- animals even at later time point of the infection. At 4-7 days p.i., essentially all cells in the fat body of the P53-/- animals are filled with FHV (containing capsid protein immune-reactivity) (Figure 4-3E). These cells, unlike those in wild type animals, have lost the integrity of cell membrane and become permeable to propidium iodide (PI). Similar loss of membrane integrity, a typical feature of necrotic cells, was also observed in CuniNPV –infected susceptible mosquitoes at 48-72 hr p.i. (Liu et al., 2011).

### Rapid Induction of Apoptosis Blocks/limits Viral Gene Expression and Proliferation

To investigate the functional significance of the rapid induction of apoptosis as an innate immunity against viral infection, I first examined the expression of AcMNPV immediate early genes, *ie0* and *ie1* (Stewart et al., 2005) at 6hr post infection in wild type, *p53* deficient, and IRER deficient strains. For *ie0*, it was not detectable in wild type larvae following virus injection. However, it was reliably detectable in either P53 null or Df(IRER) animals at 6 hr post injection (Figure 4-5 A). For *ie1*, its level of expression was very low, but nonetheless detectable, in wild type animals at 6 hr post infection. Its level of expression was dramatically higher in *p53* mutant or Df(IRER) animals that

lacks the rapid induction of apoptosis. This indicates that the rapid induction of apoptosis mediated by P53 and IRER plays an important role in blocking/ inhibiting viral gene expression.

To test whether the rapid induction of apoptosis is responsible for blocking / limiting viral proliferation, I monitored the genome levels of FHV at 24 hr post injection of 200 PFU per animals. In this assay, a group of 5 animals for each genotype was homogenized and relative abundance of the FHV RNA genome was assayed with Q-PCR primers targeting both RNA1 and RNA2 of the FHV (Figure 4-5B). I found that comparing to wild type animals, the relative levels of FHV genome were significantly higher in P53-/- and Df(IRER) mutant than that in wild type animals.

To verified whether the difference in FHV proliferation is indeed due to the lack (or delay) of cell death, I next injected 200 PFU per animal to flies that carrying LSP-Gal4/UAS-DroncRNAi. DRONC is an upstream caspase that play a pivotal role in mediating cell death induced by RHG genes. I found that knocking down *dronc* specifically in the fat body (where LSP-Gal4 is expressed), allowed the proliferation of FHV viral genome to a level comparable to that observed in Df(IRER) animals at 24 hr p.i. (Figure 4-5C). These evidences indicate that rapid induction of apoptosis is responsible for limiting viral proliferation at early stage of the infection.

**Animals Lacking the Rapid Induction of Apoptosis Are Hyper-susceptible to FHV Infection**

To test whether the lack of rapid induction of apoptosis could lead to established virus proliferation and infection, I monitored the proliferation of viral genomes following FHV injection in individual wild type or mutant animals. At 4 days following FHV injection of 20 PFU per animal, the levels of the viral genome in most wild type animals did not

increase at all (Figure 4-6 A), indicating that there is no successful proliferation of the virus. In contrast, in both P53 mutant and Df(IRER) animals, the level of FHV genome have increased dramatically, indicating successful proliferation of the virus. When 200 PFU per animal of FHV was injected, the level of viral genomes was unchanged in most wild type animals at 4 days post injection. In contrast, the levels of viral RNA in P53-/- and Df(IRER) animals indicated that significant proliferation had occurred in those mutants that lack rapid induction of apoptosis (Figure 4-6B).

The successful proliferation of FHV in animals lacking the rapid induction of apoptosis was also verified by visualizing packaged viruses using an antiserum raised against the FHV coat protein (Figure 4-6C). At 4 days following 200 PFU per animal injection, no cells can be detected positive for FHV in wild type flies. In contrast, all fat body cells in the P53 mutant (or Df(IRER)) animals are positive for FHV. Furthermore, as early as 4 days p.i., cells containing FHV capsid protein can be identified in the salivary gland of P53-/- animals (Figure 4-6D), indicating systematic infection has been established. These evidences indicated that the rapid induction of apoptosis, observed in wild type animals, is capable of blocking the infection when the viral titter is not too high. Conversely, lack of rapid induction of apoptosis, as was observed for the P53-/- and Df(IRER), leads to significantly increased susceptibility.

## Discussion

Although apoptosis was long ago postulated to be a major mechanism against viral infection in insect vectors, the mechanistic detail remained elusive. The lack of mechanistic understanding, and the conflicting results obtained from cultured cells, casted serious doubt on the functional significance of apoptosis as an innate immunity against viral infection. My study revealed that that the RHG genes, known for their

pivotal role in regulating developmental cell death, is also responsible for mediating the rapid induction of apoptosis following viral infection. The induction of the RHG genes following viral infection requires the function of P53 as well as the regulatory region IRER. Furthermore, I showed that the rapid induction of apoptosis is capable of blocking/limiting viral gene expression and infection. Genetic variations in this response likely play an important role in determining the susceptibility of insect host to viral infection.

**Animal Models vs. Cultured Cells for Arbovirus Infection**

So far, the rapid induction of RHG genes and apoptosis following viral infection have only been observed in live animals. Dengue viral infection of the mosquito cells line C6/36 did not induce apoptosis and there appears to be no significant induction of pro-apoptotic genes (Lin et al., 2007; Shih et al., 2010). When AcMNPV or FHV was applied to a *Drosophila* cell line, apoptosis was only observed 24 or 36 hr post infection, respectively (Settles and Friesen, 2008; Vandergaast et al., 2011). Correspondingly, I observed that there was no significant induction of RHG genes before 24 hr p.i. for either virus (Figure 4-2).

It is unclear as to why cell lines lack the rapid pro-apoptotic response observed in both live mosquitoes and fruit flies. One possibility is that only certain types of cells can launch the rapid pro-apoptotic response, and such cell types are not represented in cultured cell lines. Another possibility is that cultured cell lines were selected to have reduced sensitivity to stress-induced cell death. The regulatory region required for mediating viral infection induced pro-apoptotic genes, i.e. IRER, serves as a locus control region mediating the induction of RHG genes in response to a variety of stresses, such as x-ray, UV, oncogenic stresses, etc. In addition, the accessibility of

IRER is controlled by epigenetic regulation. When IRER is epigenetically blocked, i.e. in heterochromatin-like conformation, the RHG genes are no longer responsive to stresses such as DNA damage (Zhang et al., 2008). Our analysis of several *Drosophila* cell lines (S2, Kc167, etc.) indicated that the IRER region in these cell lines is enriched for heterochromatic modifications and resistant to DNase I treatment (Lin et al., 2011)(and unpublished observations). It is possible that cells with reduced sensitivity to stress-induced cell death, either through genetic mutation or epigenetic silencing of IRER, are inadvertently selected during in vitro cell culture processes. As a result, the ability to launch the rapid induction of RHG genes following viral infection could have been lost in long term cultured cells.

**Rapid Induction of Apoptosis is a Key Innate Immunity against Viral Infection**

My data indicated that the rapid induction of apoptosis is capable of blocking the infection at its initiation stage when the animals are exposed to relatively small amount of viruses. This response very likely contribute to the “midgut infection barrier” that has long been observed for arbovirus transmission through insect vectors (Bosio et al., 2000). Apoptosis of midgut cells following viral exposure has been observed before, when a refractor strain of *Culex pipiens* was fed with blood meal containing West Nile virus (Vaidyanathan and Scott, 2006). Similarly, rapid induction of *mx* was observed in refractory *Aedes aegypti* (MOYO-R) fed with blood meal containing DEN-2 (Figure 4-1). There is a conspicuous lack of rapid induction of *mx* in the susceptible strain (MOYO-S). My data with FHV-infected P53-/- and Df(IRER) animals indicated that the lack of rapid induction of apoptosis following viral infection could lead to dramatically increased susceptibility to established systematic infection.

The rapid induction of apoptosis effectively denies the opportunity of viral gene expression and accumulation (Figure 4-8). This has have been previously demonstrated for CuniNPV infection through a native route of infection (Liu et al., 2011), where I showed that viral gene expression was only detected when apoptosis was delayed/suppressed with caspase inhibitors. In this study, I showed the lack of rapid induction of apoptosis in P53 and Df(IRER) animals allowed viral gene expression and proliferation. In addition, significant viral proliferation can be achieved when the level of DRONC in fat body cells was knocked down by tissue-specific RNAi. All of these evidenced indicate that rapid elimination of the infected cell is responsible for blocking the infection at the initiation stage, before significant expression of viral genes could take control of the cellular system.

Rapid induction of apoptosis as an innate immunity against viral infection is not restricted to insects. For instance, rapid induction of apoptosis was observed at 8 hr following infection of human embryonic stem cells (hESCs) with recombinant AAV (Hirsch et al., 2011). The hESCs are extreme sensitivity to various stresses. And the rapid induction of apoptosis in hESC cells following rAAV infection also requires P53. Rapid induction of apoptosis was also observed following the infection of primary dendritic cells by the intracellular pathogen *legionella pneumophila*, which induces apoptosis within the first hour of infection (Nogueira et al., 2009). Similarly, influenza A viruses (IAV) mutated for NS1 can also induces rapid apoptosis in primary macrophages (Stasakova et al., 2005). Similar to what I discovered with *Drosophila*, P53-/- mice are hypersensitive to IAV infection (Munoz-Fontela et al., 2011). However, the anti-viral effect of P53 in mice may include induction of pro-inflammatory genes in

addition to its pro-apoptosis function. Study of innate immunity in *C. elegans* also revealed that P53 has an ancient role as an immune/stress sensor (Fuhrman et al., 2009).

In this study, I found that although FHV virus can proliferate in Df(IRER) that lacks rapid induction of apoptosis, the titer of FHV was consistently lower than that observed for P53 null mutant animals. It is very likely that the observed difference between P53-/- and Df(IRER) animals is due to anti-viral activity of P53 besides its role in the rapid induction of apoptosis. Indeed, a genomic survey of dengue virus-induced changes of gene expression revealed that many P53 targeted genes were activated in the refractory (MOYO-R) strain but not in the susceptible strain (Behura et al., 2011). However, my analysis with Df(IRER) animals and those with DRONC KD indicated that a major mechanism of P53 –mediated anti-viral activity in insects is through the rapid induction of RHG genes and apoptosis.

**What is the Signal Transduction Pathway that Activates P53**

It is not clear as to how is P53 activated in virus -infected cells. In *Drosophila*, the well characterized immune pathways including the Toll pathway, the IMD pathway, and the Jak-STAT pathway (reviewed in (Cherry and Silverman, 2006)). Toll pathway is mainly responsive to fungi and Gram-positive bacteria while IMD pathway is activated by Gram-negative bacteria (Hoffmann and Reichhart, 2002). Recent studies indicated that except for anti-fungal and anti-bacterial functions, the Toll pathway is also involved in antiviral response (Nakamoto et al., 2012; Zambon et al., 2005). The JaK-STAT pathway has been shown to be activated by *Drosophila* C virus. Loss of function of JaK led to increased viral load and decreased survival rate after viral infection (Dostert et al., 2005). However, I found that *vir-1*, a target gene of JaK-STAT, was not induced by

AcMNPV (or FHV) infection when *reaper*/*hid* were significantly induced, which suggested that JaK-STAT pathway is unlikely involved in activating P53 (Figure 4-9).

The fact that both AcMNPV and FHV induced rapid transcriptional activation of RHG genes requires P53 and IRER suggests that a common mechanism may be responsible. The two viruses are quite different, i.e. dsDNA virus vs. ssRNA virus. AcMNPV cannot replicate in *Drosophila* whereas FHV can replicate in a variety of insects including *Drosophila*. The fact that these two viruses, and very likely other viruses such as DEN-2 and CuniNPV, induce rapid induction through the same transcription factor and regulatory region strongly suggests that a more general mechanism is involved. Revealing this mechanism should shed great light on our understanding of arbovirus–vector interaction.

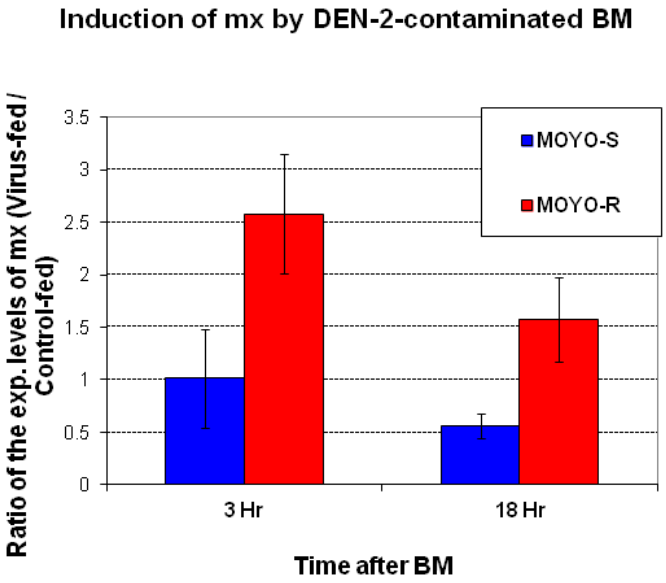


Figure 4-1. Specific induction of *mx* in the refractory strain (MOYO-R), but not in the susceptible strain (MOYO-S), following exposure to DEN-2. Expression level of *mx* was measured with Q-PCR and normalized against housekeeping gene GAPDH before calculating the fold of induction, i.e. the ratio of *mx* in virus-fed vs. the control-fed samples. At 3hr post feeding, the level of *mx* in mosquitoes exposed to DEN-2 was more than 2 fold higher than those fed with control blood meal. This level of *mx* expression goes down at 18hr, possibly due to death of *mx*-expressing cells as we observed for CuniNPV infected *Aedes aegypti* larvae. Data represented as Mean $\pm$ STD.

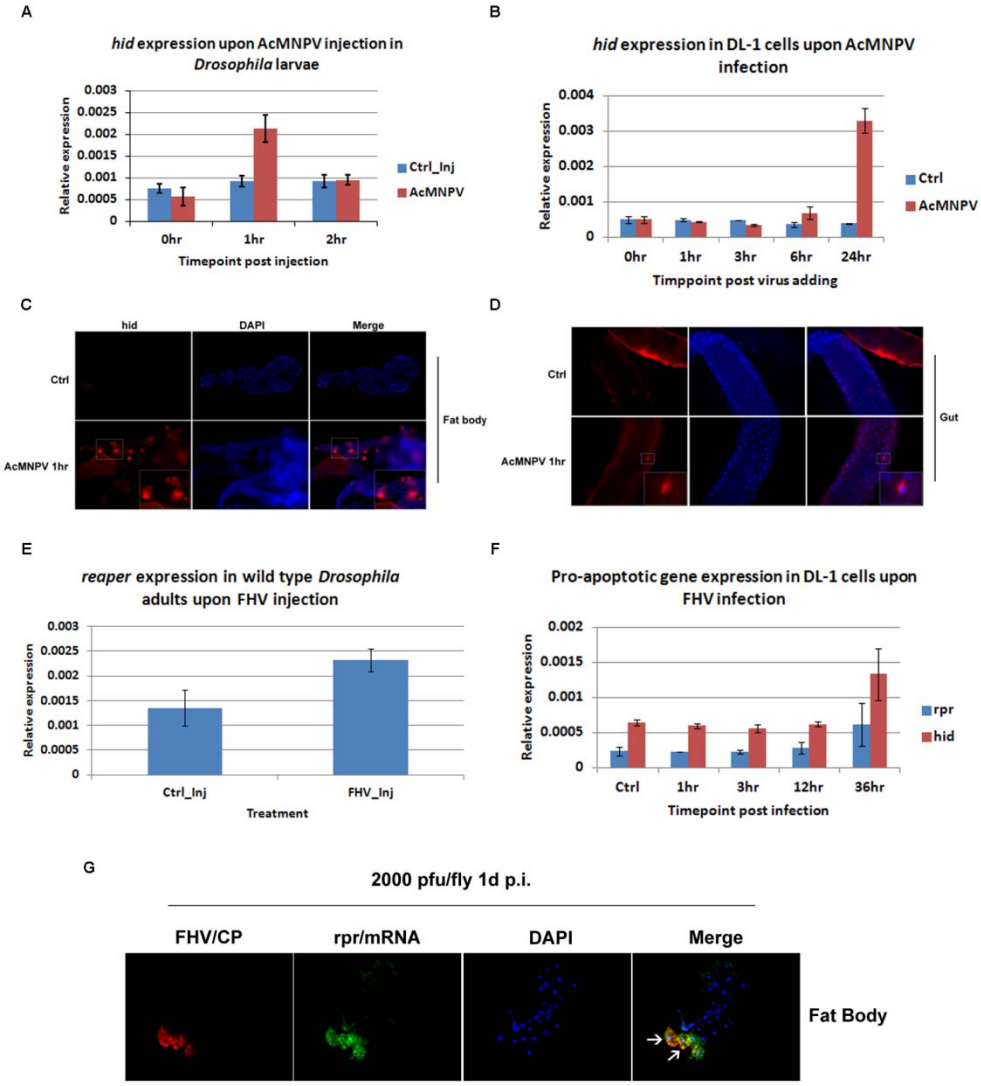


Figure 4-2. Rapid induction of *reaper* and *hid* following viral infection of *Drosophila* larvae or adults. (A). At 1hr post AcMNPV injection (p.i.), *hid* mRNA level in injected larvae was induced ~2 fold. By 2hr p.i., *hid* level has gone back to normal. (B). AcMNPV infection in cultured DL-1 cell line (MOI=20) did not induce *reaper*/*hid* until relative late stage (24hr p.i.). Data are shown as Mean± STD of two to three independent experiments. (C) and (D). Following AcMNPV injection, *hid* is mainly induced in fat body cells and some gut cells as revealed by FISH using digoxin-labeled cRNA probe. (E). At 1hr post FHV injection, *reaper* mRNA level in infected adults was induced ~1.6 fold. (F). FHV infection in cultured DL-1 cell (MOI=40) did not induce *reaper*/*hid* expression until 36hr p.i.. (G). Induced *reaper* is due to FHV infection. Immunostaining for FHV capsid protein (red) and *reaper* FISH (green) were performed in the fly strain that has DIAP1 overexpression in the fat body. Note the colocalization of red and green signal (white arrows). In contrast, the FHV negative cells have no *reaper* signal either (white arrow heads). Photos are representatives of at least 2 independent experiments.

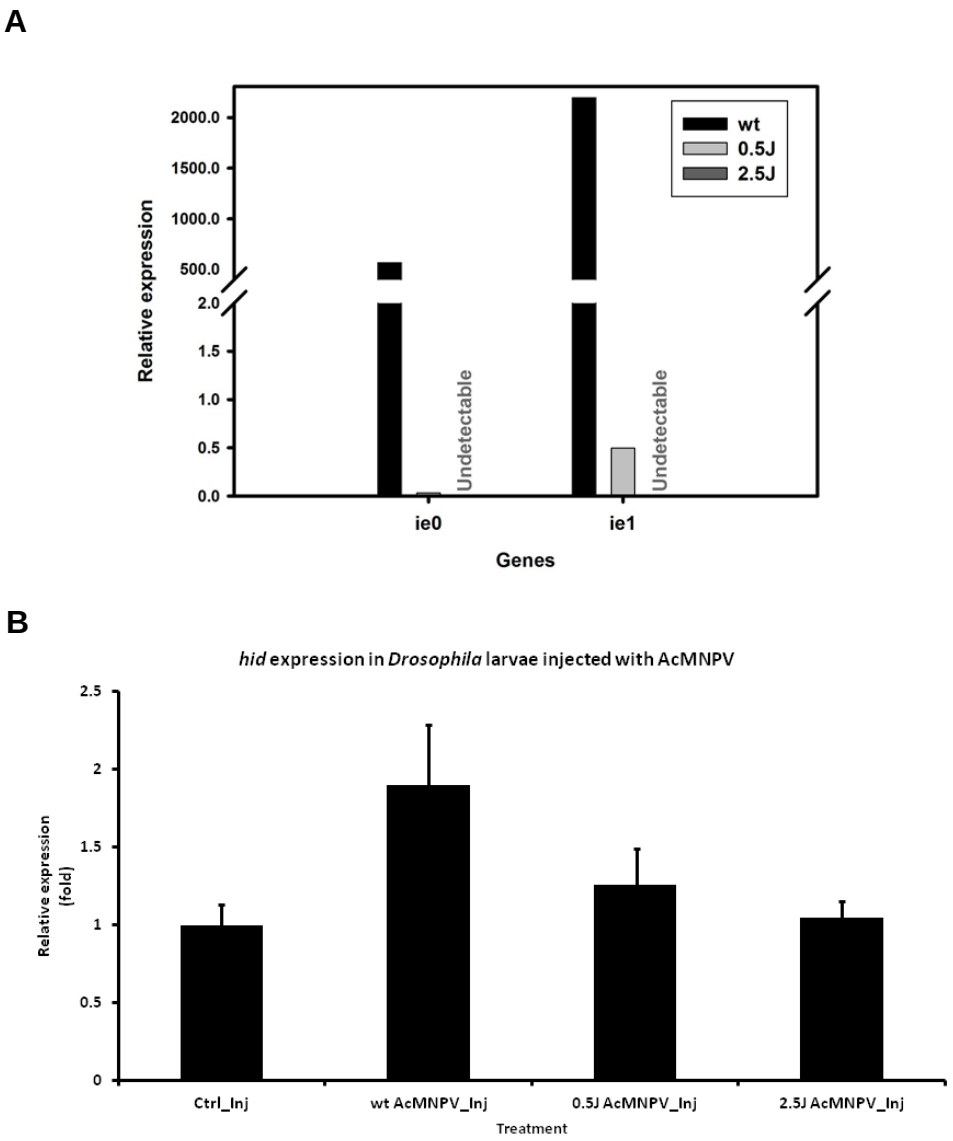


Figure 4-3. Viral gene expression is required for the induction of pro-apoptotic response. (A). UV irradiation can dramatically decrease the early gene transcription of AcMNPV. sf9 cells were infected with wild type and UV-irradiated AcMNPV. Two immediate early genes *ie0* and *ie1* mRNA level were examined with Q-PCR to indicate the UV effect. (B). UV-inactivated AcMNPV has decreased ability to induce *hid* expression. *Drosophila* larvae were injected with wild type and UV-inactivated AcMNPV. mRNA level was detected with Q-PCR.

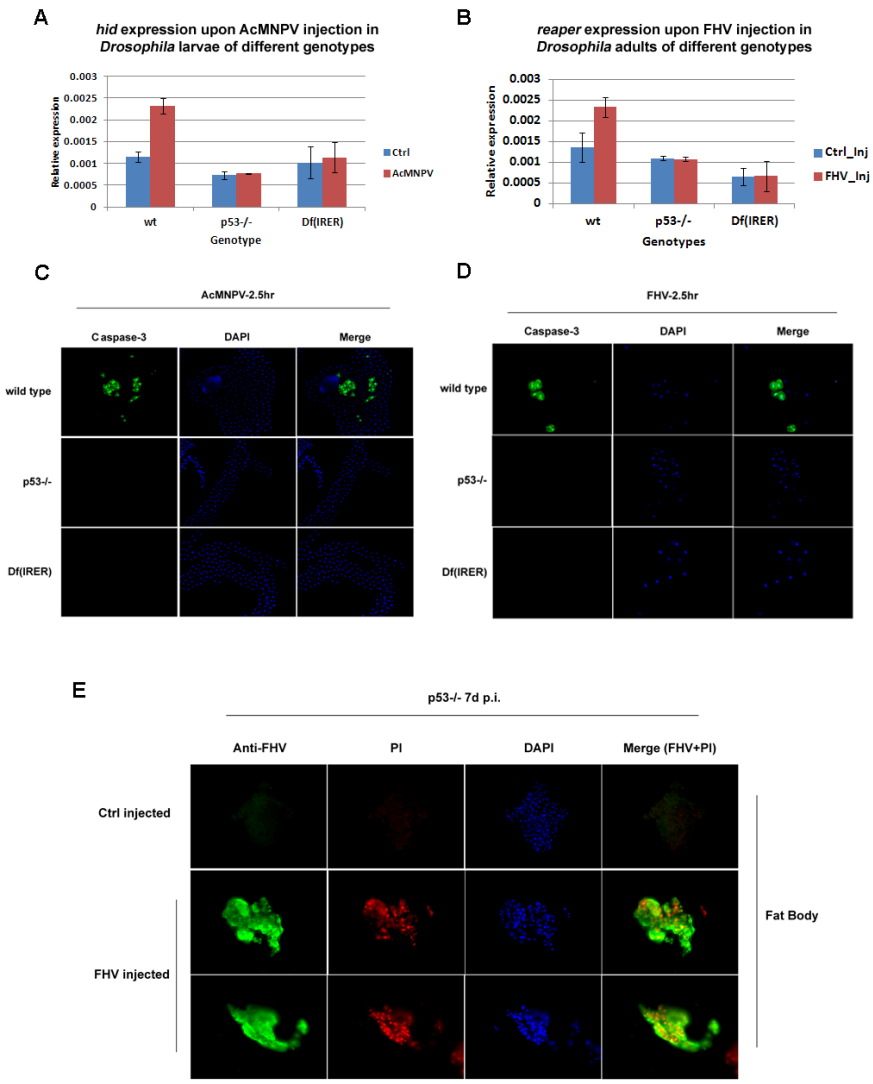


Figure 4-4. Virus -induced *reaper*/*hid* expression and apoptosis requires P53 and IRER. (A). The induction of *hid* following AcMNPV injection is absent in animals homozygous to a P53 null mutation (p53-/-) or deficiency removing the regulatory region IRER (Df(IRER). (B). Likewise, the induction of *reaper* following FHV infection also requires p53 and IRER. Data are shown as Mean± STD of two independent Q-PCR experiments. (C) and (D). Activated caspase 3 can be detected in the fat body cells of wild type fly larvae or adults infected with AcMNPV or FHV, respectively. In contrast, cells with activated caspase 3 were not observed in p53-/- or IRER deficient flies. (E) Fat body cells in P53-/- became necrotic at 4-7 days p.i.. PI was injected to either control-injected or FHV (200 PFU /per animal) –injected animals at 7 days p.i. and the animals were sacrificed and fixed 20 minutes later. The presence of FHV was visualized with an antibody against the capsid protein. The presence of PI indicating the integrity of the cell membrane was compromised in fat body cells infected by FHV. Photos are representatives of two independent experiments.

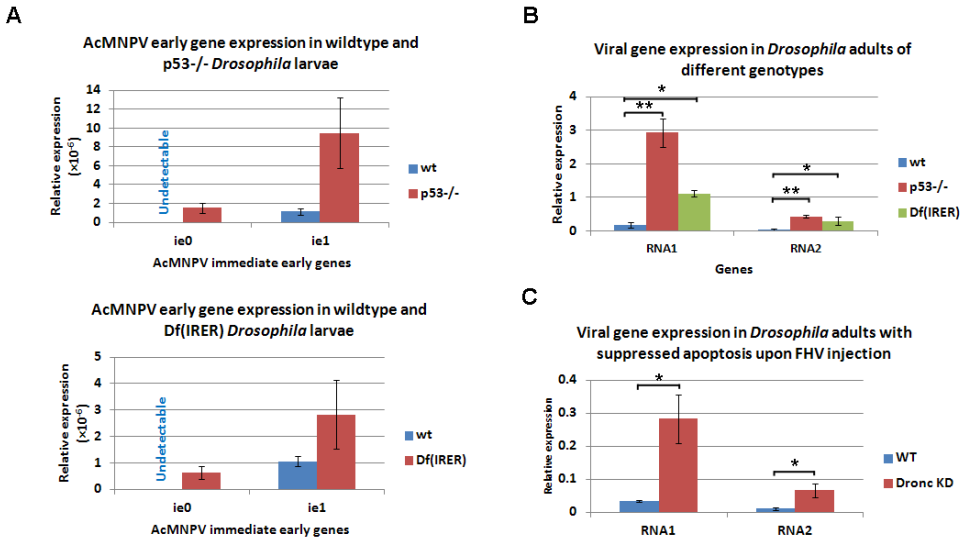


Figure 4-5. Rapid induction of apoptosis functions to block/limit viral gene expression and proliferation. (A). The mRNA levels of AcMNPV immediate early gene *ie0* and *ie1* were examined at 6hr post virus injection by Q-PCR in *Drosophila* larvae. Data indicates that in both p53 -/- and IRER deficient strains that lacks rapid induction of apoptosis, *ie0* and *ie1* expression levels were dramatically higher than that in the wild type strain. Data are shown as Mean± STD. (B) FHV gene *RNA1* and *RNA2* levels were examined at 24hr post injection in wild type, p53-/- and Df(IRER) adults by Q-PCR. Note the significantly higher copy levels of *RNA1* and *RNA2* in p53-/- and Df(IRER) flies than that in wild type flies. * p-value<0.05, ** p-value<0.01, t-test. (C) *RNA1* and *RNA2* levels were also higher when apoptosis was suppressed by knocking down the upstream caspases dronc (Dronc KD) in the fat body cells (genotype LSP2-Gal4;UAS-dronc_RNAi). Data are shown as Mean± STD. * p-value<0.05, t-test.

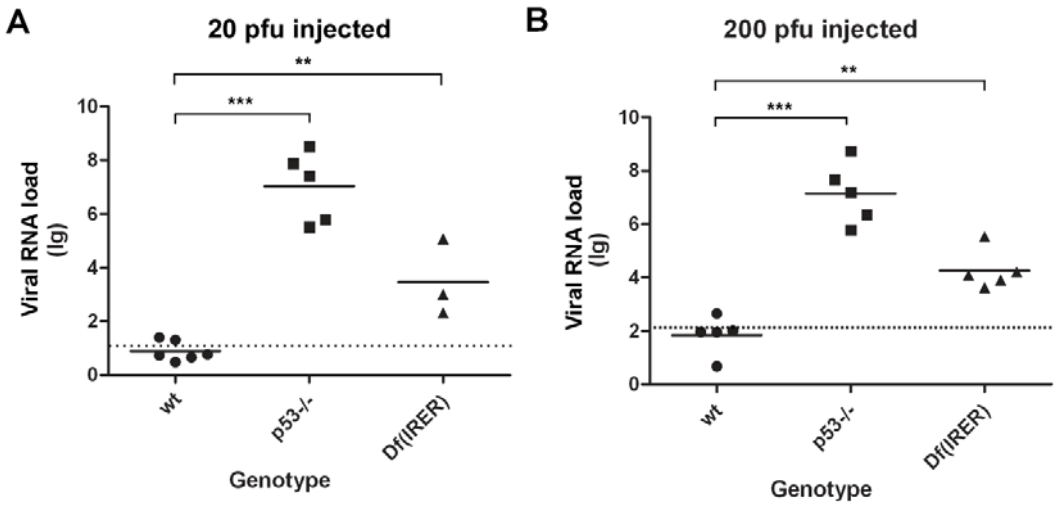


Figure 4-6. Rapid induction of apoptosis functions to block/limit viral proliferation. (A) When a low dosage FHV (20 PFU/per animal) was injected, no viral proliferation was observed at 4 days p.i.. In contrast, significant proliferation of FHV was observed in p53-/- or Df(IRER) flies. The dotted line indicated the amount of virus injected to each animal. Total RNA extraction and Q-PCR were performed in individual fly to estimate the copy numbers of *RNA1* and *RNA2* (Material and Methods and Figure 4-7). ** p-value<0.01, t-test. (B) When 200 PFU/per animal was injected. Most wild type flies has no significant increase of FHV copy numbers, although the virus proliferated significantly in P53-/- and Df(IRER) animals. ** p-value<0.01, t-test. (C) Immunostaining was performed with antiserum against FHV capsid protein and AlexaFluor 488 labeled goat anti rabbit secondary antibody. Cells in the fat body of p53-/- animal are filled with FHV capsid protein at 3 days post 20 PFU injection. No FHV –positive cells can be detected in wild type (wt) animals inject 20PFU FHV. (D) At 4 days p.i., cells in the salivary gland of P53-/- flies are positive for FHV capsid protein immunity, while no spreading of virus can be detected in wild type animals. Photos are representatives of two independent experiments.

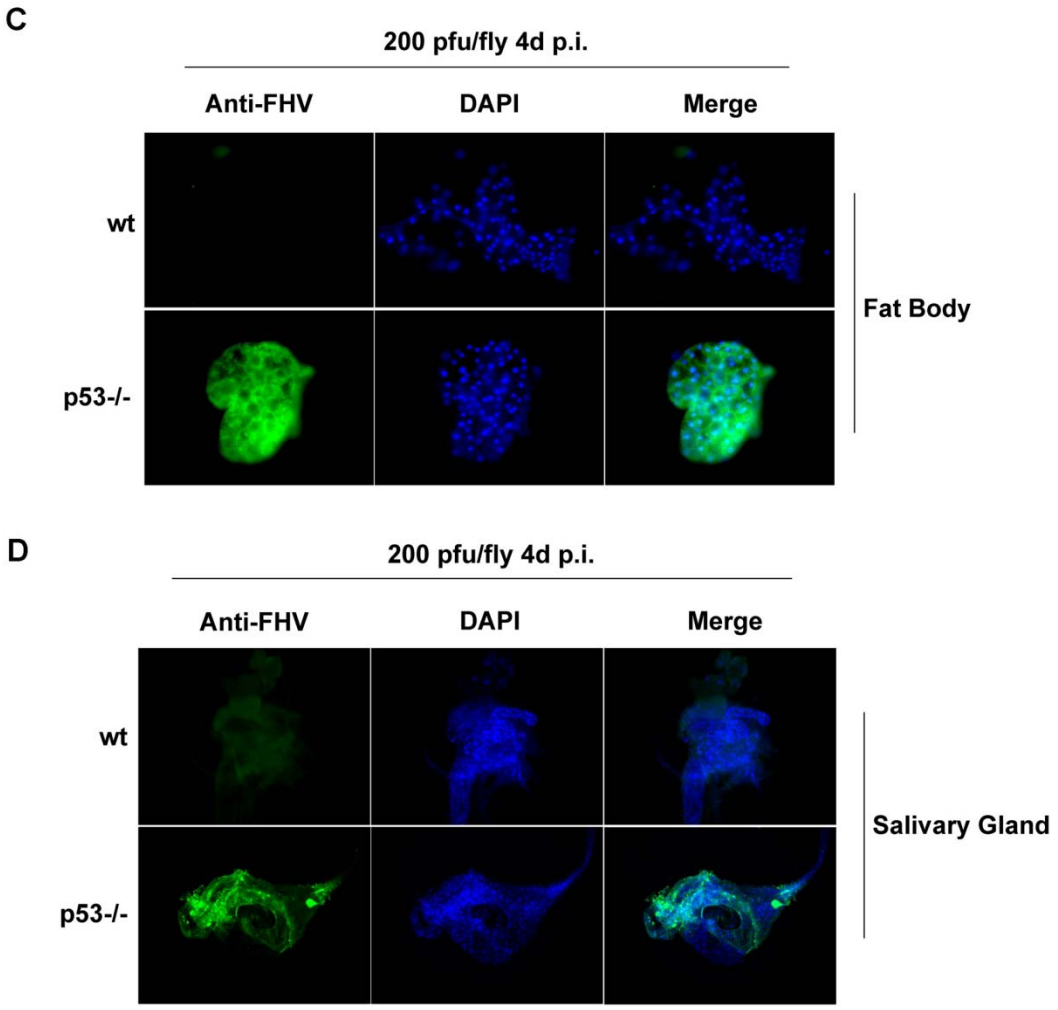


Figure 4-6. Continued.

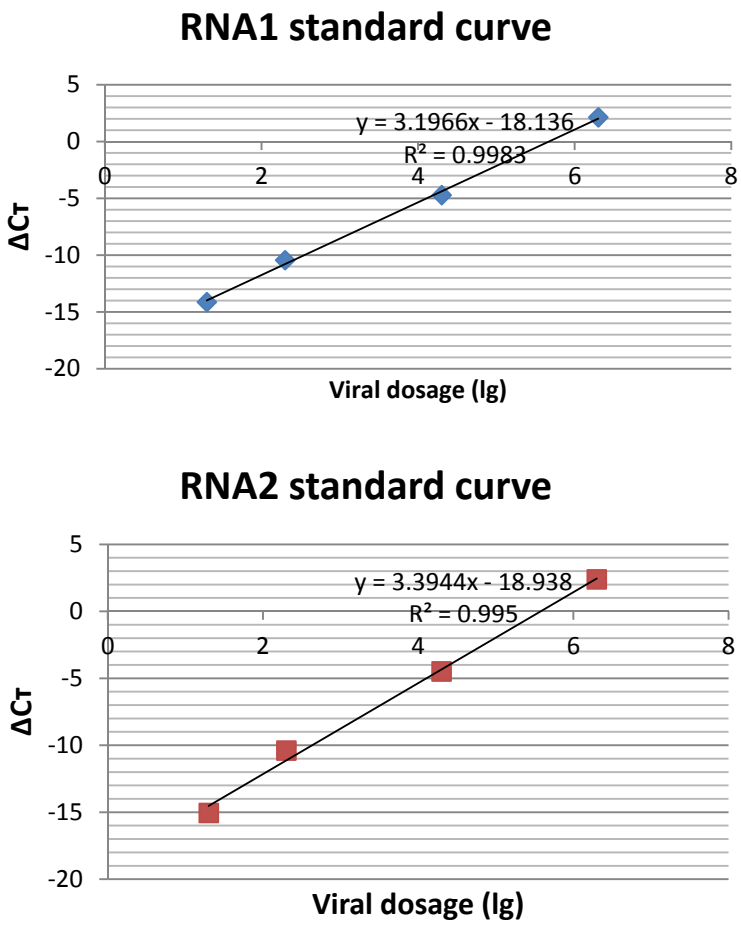


Figure 4-7. Standard curve for estimating FHV genome/titter. FHV of various titter was mixed with 1 adult fly and processed immediate for RNA extraction. Q-PCR were performed for *RNA1* and *RNA2* and the relative CT to GAPDH was calculated plotted against the viral dosage. This information was used to estimate viral genome number / titter in Figure 4-6 A & B.

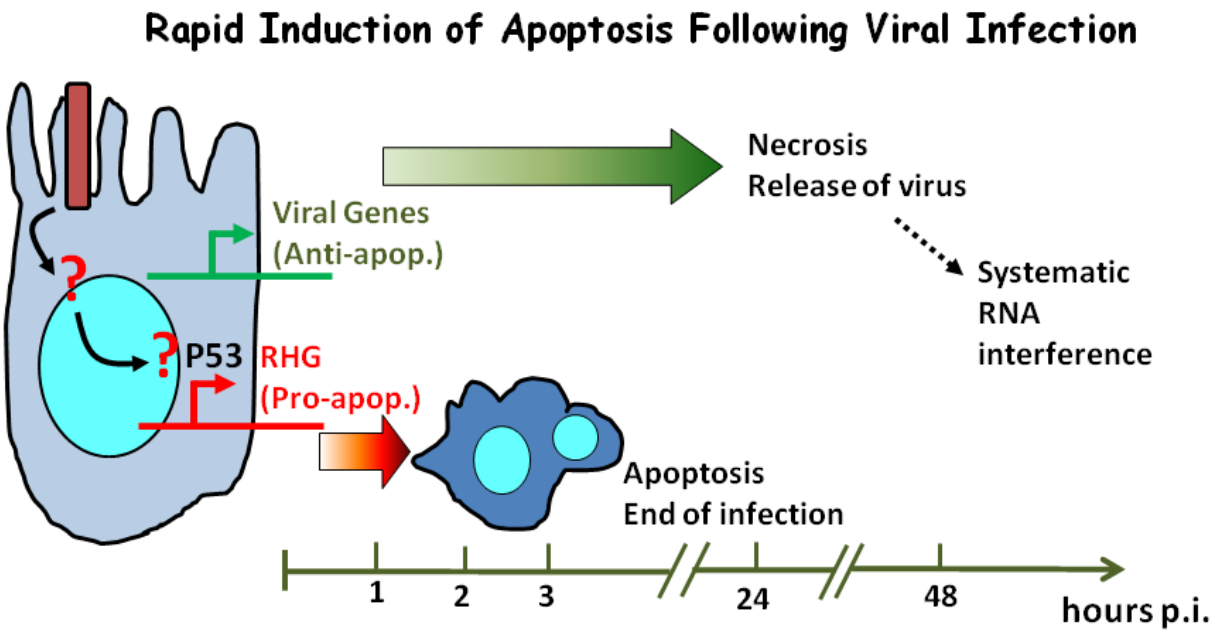


Fig 4-8. Diagram summarizes the role of rapid induction of apoptosis as an innate immune response against viral infection. By eliminating the infected cells before accumulation of viral gene production, the infection can be blocked at the initiation stage.

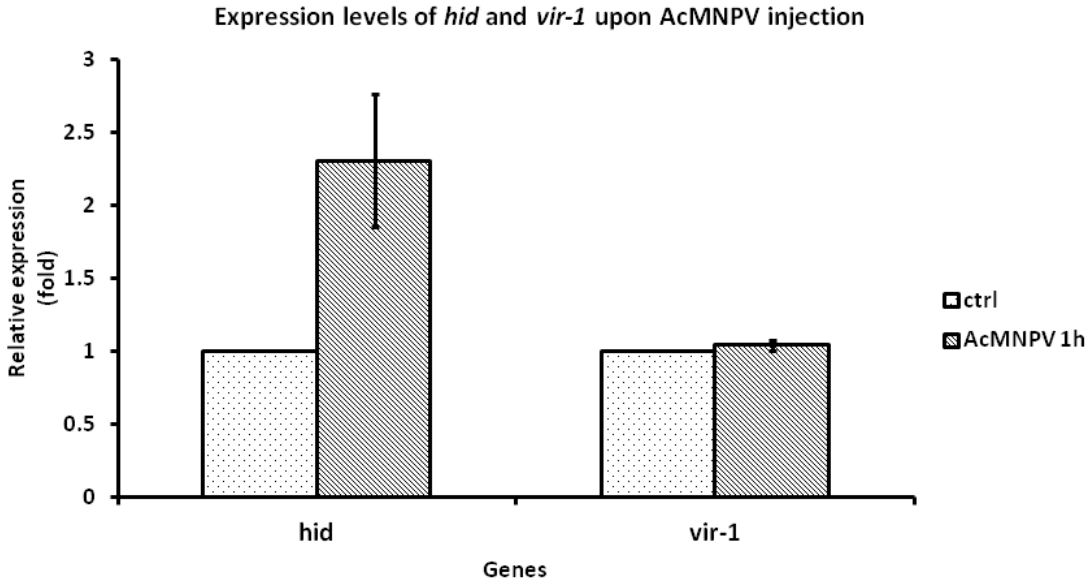


Figure 4-9. Monitoring the status of the JAK/STAT pathway. QPCR measurements of *hid* and *vir-1* mRNA were first normalized against GAPDH before calculating the ratio (AcMNPV injected / Control media injected).

# CHAPTER 5
# DISCUSSION AND PERSPECTIVES

## The Role of Apoptosis as an Innate Immune Response against Viral Infection

The role of apoptosis as a defense against viral infection has long been under debate. On the one hand, a variety of viral genes have been found to inhibit host cell apoptosis (refer to Chapter 1 for details), moreover, it has been found that the viral anti-apoptotic genes are essential for the infectivity of the viruses (Clem, 2001). On the other hand, many viral genes have been found to actively trigger host cell apoptosis (also discussed in Chapter 1). In addition, results derived from cultured insect cells indicated that inhibition of virus-induced apoptosis had no effect on viral yield (Settles and Friesen, 2008; Vandergaast et al., 2011). Due to the lack of efficient experimental systems, the exact role of apoptosis in viral infection has always been a mystery.

In this study, I tried to demonstrate the role of apoptosis in viral infection by establishing two virus-host interaction systems: the mosquito system and the *Drosophila* system.

The mosquito system is a native infection system, i.e. animals were orally infected by viruses which mimicked the native infection route. In this system, mosquito larvae and adults were used respectively. The mosquito larvae were infected by directly adding baculoviruses to the culture medium of the larvae. The mosquito adults were fed with blood meal containing the human pathogen, Dengue virus. In both larvae and adults, I found that upon viral infection, there was rapid induction of pro-apoptotic gene, *mx*, in the refractory *Aedes aegypti* strain but not susceptible strain. In larvae, I further found that the apoptosis correlated with mosquito competence, i.e. rapid apoptosis can be detected in refractory species but not susceptible species. After inhibiting apoptosis in

refractory species, viral genes became detectable. The correlation between apoptosis and mosquito susceptibility strongly suggests that apoptosis serves as a defense against viral infection. Moreover, the correlation between *mx* induction and apoptosis suggests that *mx* might be the mediator of virus-induced apoptosis. To further prove this, I need to specifically knock down *mx* from refractory species and examine if apoptosis still happens upon viral infection. However, to date, no mature RNAi technique has been established in mosquito larvae. Moreover, given the limited genetic tools in mosquito, it is hard to inhibit apoptosis through genetic methods. Therefore, the major drawback of the mosquito system is that it is hard to study the mechanisms of virus-induced apoptosis.

To study the mechanisms of virus-induced apoptosis and further prove the role of apoptosis as an anti-viral response, I established two infection systems in *Drosophila*. As a powerful model system, there are a large number of genetic resources in *Drosophila*. In this system, viruses were injected into *Drosophila* larvae or adults because very few viruses have been reported to infect *Drosophila* through a native route. By using the DNA virus AcMNPV and RNA virus FHV, I found that, similar to the mosquito, pro-apoptotic genes are quickly induced in *Drosophila* upon viral infection. More importantly, I found that virus induced pro-apoptotic gene expression is P53-dependent and mediated by IRER. In P53-deficient or IRER-deficient animals, there was no rapid apoptosis following FHV infection and the animals were hyper susceptible to FHV infection, which proved the role of apoptosis in defending against viral infection.

## Innate Immune Responses in *Drosophila* and Mosquito

### *Drosophila*

The well characterized immune pathways in *Drosophila* include the Toll pathway and the IMD pathway (Figure 5-1) (Hoffmann, 2003). The Toll pathway is mainly activated by gram-positive bacteria and fungi while the IMD pathway is mainly activated by gram-negative bacteria infection. Consequently, the Toll mutants are highly susceptible to fungal infection and the IMD mutants die after Gram-negative bacteria infection (Lemaitre et al., 1995; Lemaitre et al., 1996). In both Toll and IMD pathways, upon pathogen infection, the transcription factor NF-κB-like protein (*Dorsal/Dif* in the Toll pathway and *Relish* in the IMD pathway) is activated. The activated NF-κB then translocates into the nuclei where it binds to the promoters of the target genes and triggers their expression. The known target genes include antimicrobial peptides such as *Diptericin* and *Cecropins*. To date, seven antimicrobial peptides have been identified (Hoffmann, 2003). The secretion of these antimicrobial peptides into the haemolymph (insect blood) is an important aspect of insect humoral immunity. Following the identification of the roles of Toll and IMD pathways in immune response, a third pathway, JaK-STAT, was later reported to be activated by viral infection and involved in anti-viral response (Dostert et al., 2005). One gene, *vir-1*, whose promoter contains two STAT binding sites, has been found to be the target of JaK-STAT pathway and can be strongly induced by DCV (*Drosophila* C virus) infection (Dostert et al., 2005).

Using the *Drosophila* infection system, I found that virus-induced apoptosis is P53-dependent and mediated by an enhancer region IRER. The responsible signaling transduction pathways remain to be identified. The preliminary results indicated that *vir-1* cannot be induced by viral infection, which suggested that the JaK-STAT pathway is

unlikely involved. To elucidate the involved signaling transduction pathways, a nonbiased RNAi screening could be performed. I will take advantage of the available RNAi strains generated by TRiP (Transgenic RNAi Project) to specifically knockdown the key regulators in major signal transduction pathways such as the DNA damage pathways, Toll pathway, IMD pathways, etc. to identify the pathways involved.

**Mosquito**

The sequencing and annotation of major mosquito genomes have largely prompted the understanding of the mosquito immunity. Research on mosquito immunity has been strongly influenced by work on *Drosophila*. Homologs of the major factors in *Drosophila* Toll and IMD pathways have also been found in mosquitoes. However, unlike *Drosophila*, mosquitoes lack *Dif*, but use *Dorsal* ortholog *Rel1* and *Relish* ortholog *Rel2* to induce the expression of antimicrobial peptides (Fragkoudis et al., 2009). The heat-shock protein cognate 70B has been found to be upregulated by virus infection, and the silencing of this gene decreased the lifespan of ONNV-infected *A. gambiae* (Kang et al., 2008; Sim et al., 2007). In DEN-2-infected *A. aegypti*, strong upregulation of the Toll and JaK-STAT pathways can be observed. It was further proved that the Toll pathway played a role in controlling DEN-2 (Xi et al., 2008).

In addition to the classical immune pathways described above, the siRNA-mediated RNAi has been demonstrated to be an important antiviral mechanism in mosquitoes (Keene et al., 2004; Li et al., 2004). Virus-derived long dsRNAs can be cleaved by the RNase DCR-2 to generate short siRNAs (~21-25 bp in length), often called viRNAs. DCR-2 and dsRNA binding protein R2D2 then integrate one strand of viRNA into the RNA-induced silencing complex RISC, which further activates RISC. The activated RISC mediates the sequence specific cleavage of viral RNA through its AGO-

2 component. The orthologs of DCR-2, R2D2 and AGO-2 have all been found in major mosquito genomes, e.g. *A. gambiae*, *C. pipiens* and *A. aegypti.* Moreover, AGO-2 has been found to evolve quickly, which suggests its role in antiviral response (Campbell et al., 2008a). In *Drosophila*, all three genes DCR-2, R2D2 and AGO-2 were found to evolve quickly under positively selection (Obbard et al., 2006). To date, genetic mutants for these siRNA proteins have not been available in mosquitoes, however, the artificial system by injection of long dsRNA for DCR-2, R2D2 or AGO-2 has proved that these genes are important in controlling flaviviruses and alphaviruses infection or spreading (Campbell et al., 2008b; Keene et al., 2004; Sanchez-Vargas et al., 2009).

### Significance of Studying the Defensive Mechanisms in *Drosophila* and Mosquitoes

A striking finding through studying *Drosophila* innate immunity is that most of the genes involved are very similar to genes implicated in mammalian innate immunity. For example, the Toll-like receptor (TLR) in mammals is similar to *Drosophila* Toll and TLR has been found to be able to activate NF-κB (Medzhitov et al., 1997). Likewise, the apoptosis regulatory mechanisms are also conserved between *Drosophila* and mammals. Studying the role of apoptosis as an innate immune response in *Drosophila* may enrich the understanding of innate immunity in mammals.

More importantly, studying the antiviral defensive mechanisms in mosquitoes might be useful to develop novel strategies to control mosquito-borne viruses. The transmission of mosquito-borne viruses requires the established infection in the mosquito vector. When the viruses enter the mosquito body, they infect certain cells and replicate inside those cells. After the initial replication, the viruses spread to the salivary gland (SG) of the mosquito and further replicate in SG. The viruses can then be

transmitted to other hosts upon mosquito biting. Therefore, identifying the antiviral mechanisms in mosquito and genetically manipulating the defensive mechanism can help to break the viral transmission chain.

Nowadays, many labs are focusing on genetic manipulation of vector mosquitoes to make them refractory to viruses and other parasites such as malaria. Then, the genetically manipulated mosquitoes can be released into the wild to replace the wild species. This strategy is termed population replacement. Unlike the traditional population reduction strategy, population replacement can avoid the potential ecological effect elicited by population reduction (Terenius et al., 2008). Indeed, this strategy has successfully generated virus-refractory mosquitoes (Mathur et al., 2010) or malaria-resistant mosquitoes (Ito et al., 2002; Moreira et al., 2002). However, the trangenes usually lead to fitness cost which makes the transgenic mosquitoes less competitive than the wild species (Catteruccia et al., 2003; Irvin et al., 2004; Moreira et al., 2004). Therefore, Mendelian inheritance alone will not be sufficient to drive the spreading of the refractory gene. A maternal-effect selfish genetic element, *medea*, described in *Drosophila*, might be useful to spread the transgenes in the wild (Chen et al., 2007).

The Sterile Insect Technique (SIT) is a method to reduce and eliminate a certain population of the pest insect. After mass rearing and sterilizing the males by exposing them to low doses of radiation, the sterile males are released into the wild to compete with wild male for female mating. Eventually, the population size will be reduced or totally eliminated. SIT has been successfully used against the screwworm fly *cocbliomyia bominivorax* in the USA. SIT in mosquitoes were also conducted during 1970's and 80's on several mosquito species such as *Ae. aegypti*, *Ae. Albopictus*, *C.*

*pipiens*, *C. quinquefasciatus*, *A. gambiae* and *A. albimanus* (Benedict and Robinson, 2003). However, although many of these trials showed a reduction in mosquito population, very few can eliminate the population in the released area (Benedict and Robinson, 2003).

One major problem with SIT is that it is difficult to separate the males from the females. This step is crucial as the females transmit the disease through bite. The pro-apoptotic gene, *mx*, initially identified by our lab, has been successfully used by others to specifically express in the flight muscle of female and cause the flightless phenotype of the female (Fu et al., 2010). This is an efficient way to remove females. Another drawback of traditional SIT is the sterilization. It is very hard to find an ideal method to sterilize males while mitigating fitness cost. To overcome these drawbacks of traditional SIT, an improved technique, RIDL (Release of Insects carrying a Dominant Lethal) was developed. Unlike SIT, which releases the males sterilized with irradiation, RIDL releases males that are homozygous for a dominant lethal gene. Mating with wild populations produces offspring that are heterozygous for the dominant lethal gene, resulting in the death of all progeny.

All in all, the identification of more and more factors involved in the mosquito defensive mechanisms will greatly benefit the development of novel disease-controlling strategies, and the elimination of mosquito-borne pathogens will no longer be a dream.

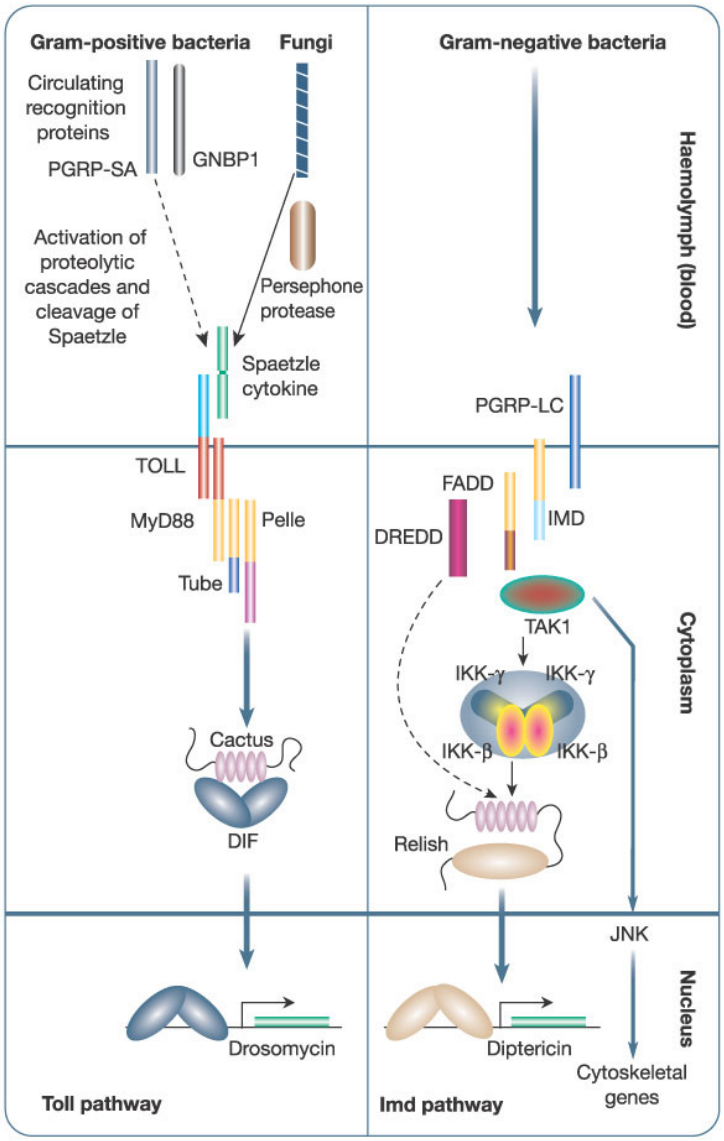


Figure 5-1. The schematic representation of Toll and IMD immune pathways in *Drosophila*. (cited from (Hoffmann, 2003))

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# BIOGRAPHICAL SKETCH

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