ABSTRACT

MUDIGANTI, USHARANI. Insect response to alphavirus infection. (Under the Direction of Prof. Dennis T. Brown.)

Invertebrate cells survive Alphavirus infections to establish viral persistence, in contrast to cell death seen soon after infection in mammalian cells. Invertebrate response to prototype alphavirus, Sindbis, has been studied to a certain extent, using mosquitoes and cell lines derived from mosquitoes. Some of the observations made in studies using mosquito systems include formation of intracellular vesicles soon after infection with Sindbis, identification of antiviral activity in the media used to grow the mosquito cell lines and in Sindbis-infected mosquito cell lysates, controlled levels of virus production as persistence is established and superinfection exclusion by Sindbis-infected cells.

The study presented here is designed to utilize array of genomic and genetic information available in *Drosophila* model to identify the candidate genes / gene products playing a role in establishment of alphavirus persistence. Observations described in Chapter I establish *Drosophila* S2 cells as a suitable invertebrate system to study alphavirus-insect interactions. Gene expression analysis identified increased expression of 18 transcripts coding for membrane trafficking and cytoskeletal components and 10 transcripts coding for Notch pathway components, at 5 days post-infection. Identification of upregulation of Notch pathway suggests similarities between mechanism of establishment of persistence of Alphaviruses and Herpesviruses. Transcript coding for TEP II, a wide-spectrum protease inhibitor is increased in expression at 5 days post-infection and upon superinfection at 5 days post-infection. We probed for inhibition of viral protease activity during early persistence and upon superinfection of Sindbis-infected cells with Sindbis. Inhibition of Sindbis viral protease nsP2 is identified to be involved in establishment of viral persistence and superinfection exclusion in cells derived from *Mosquito* and *Drosophila*.

INSECT RESPONSE TO ALPHAVIRUS INFECTION

by

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To

My Husband, Ramesh

BIOGRAPHY

As a researcher I can introduce myself as a person who is enthusiastic about Science and loves Biochemistry. Excited to say that this interest in science is growing and it is quite interesting to observe that the research is helping me understand life in general or at personal level, in a better way.

I was born in Bhimavaram, a small town in South India. I am the second one of three sisters. Feel fortunate to having been born to Satyanarayana Murty Mudiganti (nannagaru) and Poorna Kameswari Mudiganti (amma) who do more than they can each and everyday to make us sisters happy and encourage us to do what we are interested in. The positive thoughts they inculcated in me have definitely helped me face some difficult situations I encountered in my personal life, during my Ph.D.

My schooling was at different places on the banks of the eternal south Indian rivers of Krishna and Godavari. My interest in Life sciences started in 8^{th} grade and Biology and Chemistry were my natural choice of subjects in B.S. Realized my interest in research while I was in the MS (Biochemistry) program at University of Hyderabad. I got married and came to USA along with my husband Ramesh Sripada, in 1998. My association with NCSU began in 2000 as a Continuing education student doing BCH 703 with Dr Linda Hanley Bowdoin. During the course work she encouraged me to pursue Ph.D and I joined Biochemistry Dept in 2001. I chose my current project as it was very interesting and challenging. It is interesting, as it forms the platform to link Drosophila and Mosquito systems at this right time, at the dawn of post-genomic era and challenging, as it involved a poorly established Drosophila-Sindbis interface. At times I wonder what exactly motivated Dr. Brown to consider me for this assignment. I hope I have met his expectations. Brown Lab will always be special for me as my first work place in US.

I sincerely hope my research work here helps the society in combating infectious diseases. I also hope virology and biochemistry communities will find this work interesting in future.

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I would like to thank Dr. Brown and Dr. Hernandez for their expert guidance, constant support, understanding and co-operation. Thanks to Dr. Brown for designing this project and giving me an opportunity to work on it. Completion of this challenging project wouldn't have been possible if Dr. Brown told me to stop working on it at any point of time. Thanks again to Dr. Brown for not discouraging me even once. I would like to thank my committee members Linda Hanley Bowdoin, Carla Mattos for their suggestions and support. I would like to thank my Committee member Greg Gibson for his timely help and support. I would like to thank the lab members Christopher Whitehurst, John West and Stevenson Nelson for their co-operation and help. I would like to thank my Parents Poorna Kameswari and Satya narayana Murty Mudiganti (Amma and Nannagaru) for a wonderful childhood filled with positive experiences at home and for their constant emotional support. I would like to thank my sisters VenkataLakshmi Mudiganti (Akka) and Rajeswari Mudiganti (Raji) for their support. Thanks a million to my husband, Ramesh for his time and effort to commute 4 hours a day to work and for extraordinary support and encouragement. I appreciate the support from my in-laws.

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LIST OF ABBREVIATIONS

CPE- Cytopathology

dpi - Days Post-Infection

hpi- Hours Post-infection

MOI - Multiplicity of Infection

nsP - nonstructural Protein

PFU - Plaque Forming Unit

p.i or PI - persistently infected

s.p.i or s.i - Hours after superinfection

SV / SVHR – Sindbis Virus / Sindbis Virus Heat Resistant

TEPs - Thiol Ester containing Proteins

ts - Temperature Sensitive

INTRODUCTION

Sindbis Virus (SV) is a membrane containing virus with simple composition and well defined morphology providing an excellent model to study viral replication, viral assembly in the host cell and host cell response to virus infection.

Sindbis Transmission Cycle:

Sindbis and other alphaviruses are transmitted from one vertebrate to the other by blood sucking Mosquitoes. Though mosquitoes cease blood feeding in autumn the virus persistence is maintained in the invertebrate host by vertical transmission (parent to egg) (Fulhorst 1994, Mitchell 1992). Sindbis is carried by *Culex* and *Aedes* mosquitos and passerine birds are the normal vertebrate host. Humans are the incidental hosts and endemics in humans and livestock are seen in regions of world having wide-spread Mosquito population. Mosquitoes don't show any severe symptoms of infection whereas the vertebrates develop high fever, severe Arthritis, Encephalitis that can lead to death.

Sindbis in Mosquitos:

The nucleocapsids of the Alphavirus Western Equine Encephalitis (WEE) are identified 3 hrs after blood meal in the Electron Micrographs of the cells of posterior mesenteron (Houk 1985) of Culex mosquito. By 7dpi (days post-infection), the posterior midgut and thorax of A. albopictus show minimal immunofluorescence with anti-sindbis antibodies whereas the salivary glands, hindgut, fatbodies and hemolymph remain persistently infected (Bowers 1995). Extracts of whole mosquitoes show peak virus production on 2dpi which is reduced 100-fold by 5dpi and remains at that level for long periods (Bowers 1995). Different subcellular morphological changes (appearance of vacuoles, amorphous matrix or membranes associated with nucleocapsids) are observed in different species of Mosquitoes (Houk 1985). The kinetics of virus attachment, penetration and release from Mesenteron depend on virus type, dosage, mosquito species and temperature of incubation (Hardy 1983). All the differences seen in different species and organs of mosquitoes seem to be reflected in cell cultures that are employed.

SV Replication Cycle:

(plus)-sense 49S RNA acts as genome of the virus and can be produced *in vitro* using cDNA clone containing the entire sequence of SV genome. The first 2/3rd of this m-RNA like genome codes for nonstructural polyprotein (*Figure 1*) that is produced as two

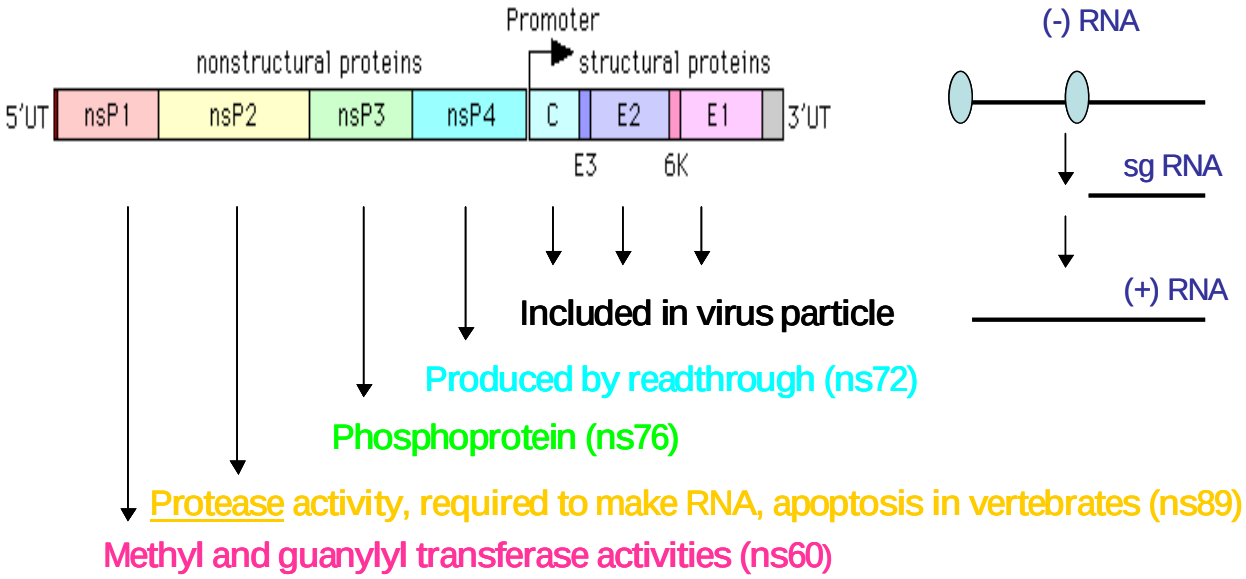
polyproteins nsP123 and nsP1234. The nsP1234 is produced by readthrough of Opal codon between nsP3 and nsP4. The second 1/3rd portion of the RNA genome is transcribed to produce 26S sub-genomic RNA (sgRNA) using a promoter on (minus)-RNA, the later is produced by replication of parental (plus)-RNA. The nonstructural polyproteins are cleaved to produce 4 individual proteins nsP1, nsP2, nsP3, nsP4. The nsPs are the functional proteins of the virus and carry out the enzymatic functions needed for viral RNA replication, transcription and interactions with host (*Figure 1*). Viral Replicases are formed by the polyproteins in the initial stages of infection that are efficient at synthesizing (minus)-RNA that acts as template for sgRNA and the progeny 49S (plus)-RNA genome. At later stages of infection the polyproteins are cleaved to produce the intermediate polyproteins and the individual polyproteins. These cleavage products are more efficient in synthesis of sgRNA and (plus)-RNA (discussed in more detail in chapter Three).

sgRNA codes for structural polyprotein of the virus that produces the structural proteins NH2-C (capsid),E3, E2, 6K, E1-COOH. Capsid protein is first cleaved from the developing polyprotein with a protease activity residing in its own sequence (Choi 1991, Hahn and Strauss, 1990). Capsid binds to 49S (plus)-RNA genome to form the nucleocapsid. These nucleocapsids are found in cytoplasm in the SV-infected mammalian cells and in the membrane-bound cytoplasmic vesicles in invertebrate cells (discussed in Chapter 2). The remaining structural polyprotein containing E2-(E3-E2)-6K-E1 integrate with membranes of the Rough Endoplasmic Reticulum and 6K is cleaved from rest of the polyprotein by activity of endoplasmic signal peptidase (Lilijestrom and Garoff 1991). In vitro translating systems derived from A. albopictus cells and those derived from rabbit reticulocytes produce capsid protein but not the polypeptides PE2 and E1 (Gillies and Stollar 1981), indicating that processes occuring after production of capsid protein need a specific cellular compartment. The envelope proteins are glycosylated and fatty acid acylated (Schlesinger and Malfer, 1982) during the secretory pathway from ER to Golgi in the infected cell. Viral glycoproteins produced in insect cells differ from those produced in vertebrate cells and are sialic-acid free (Sarver and Stollar 1978). The E3 is cleaved from PE2 in the trans-Golgi to produce envelope protein E2 where as E3 is released into the media (DeCurtis and Simons 1988). E1 and E2 form dimers that further

form the heterotrimers to form the spikes on surface of the virus particle. Spikes are capable of recognizing host cells and E2 binds the host receptor that helps during infection. The maturation pathway of structural proteins is relatively well understood in mammalian systems and is not clear in invertebrate systems.

The cytoplasmic tail of E2 in the E1/E2 spike interacts with the capsid protein which helps in initiation of envelopment and budding of the virus in mammalian cells. The mature SV contains a icosohedral nucleocapsid enveloped with E1/E2 spikes (80 spikes) along with the host lipid bilayer.

Figure 1: Organization of 49S (plus)-RNA genome of Sindbis. (plus)-RNA is used as a template for production of (minus)-RNA that acts as template to form sgRNA and progeny (plus)-RNA. Nonstructural proteins are coded as two polyproteins from 5'-2/3rd portion of the (plus)-RNA. Nonstructural proteins are cleaved from the polyprotein and have specific functions required for progeny viral RNA replication.



Aims of the present project:

To identify the candidate genes involved in establishment of Alphavirus persistence in insect host.

1. Establishing *Drosophila* S2 cells as a suitable invertebrate system to study SV infection that would enable us to use the genomic data obtained using this system, to draw inferences about invertebrates in general. This is achieved by comparing response of *Drosophila* S2 cells to SV infection to that of *Mosquito* U4.4 cells, as described in Chapter 1.

2. Identification of transcripts differentially expressed during establishment of SV persistence in invertebrates to identify the biological functions important for establishment of persistent infections. This is achieved in Chapter 2 using Microarrays to obtain the transcriptional profiles from Mock-infected and SV-infected *Drosophila* S2 cells (5dpi). Potential roles of different genes increased in expression in these experiments to bring about changes reported to be associated with alphavirus persistence in Mosquito systems are suggested.
The findings described in chapter 2 have potential to initiate research in several directions to understand the contributions of the host or virus for successful establishment of alphavirus persistence.

3. A protease inhibitor *TEP II* is identified to be upregulated at 5dpi, in *Mosquito* U4.4 cells (*TEP II*-like sequence) and *Drosophila* S2 cells. Inhibition of viral protease activity required for efficient production of viral RNA, coupled with increased levels of *TEP II* is also identified. This is an important finding to explain the mechanism of SV persistence in invertebrates.

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CHAPTER ONE

SINDBIS VIRUS INFECTION OF TWO MODEL INSECT CELL SYSTEMS – A COMPARATIVE STUDY

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Abstract:

Sindbis, the prototype of the Alphaviruses causes mosquito-borne diseases in mammals and replicates in a wide variety of vertebrate and invertebrate cell cultures. This characteristic can be exploited to use the vast array of *Drosophila* genetic information available for investigations of the interaction of Sindbis virus with an alternate invertebrate host. For this purpose, a comparative study of Sindbis virus infection of Schnieder-2 *Drosophila* (S2) cells to cells of the mosquito *Aedes albopictus* (clone U4.4) was undertaken. After infection, vertebrate cells die within 24-48 hrs, while invertebrate cell cultures survive an acute phase of infection and become persistently infected. In this study, infection of a model *Drosophila* system, S2 cells, was compared to U4.4 cells. Virus production, the time course of the establishment of persistence and changes in growth properties of the S2 cells upon infection, were studied in comparison to those of the U4.4 cells. S2 cells survived acute Sindbis infection without any significant cytopathology and continued to produce low levels of virus characteristic of persistently infected cells. S2 cells produced 10 PFU/cell on day 1 post-infection which falls to 2 PFU/cell on day 2. This result is in contrast to U4.4 cells which produce peak virus titer on day 2 post-infection and establish persistence by day 5. Onset of the persistent phase of infection of either U4.4 or S2 cells did not result in any change in morphology or growth characteristics. This study establishes S2 cells as an additional invertebrate model system to study the interactions of an invertebrate host with Sindbis virus.

Introduction:

Viruses belonging to the *Togaviridae* family are responsible for the largest variety of serious diseases of man with symptoms of fever, rash, hemorrhage, arthralgia, and myalgia. Some groups cause encephalitis by targeting the central nervous system. These diseases are spread by mosquitoes to vertebrate hosts of bird and equine species. The genus *Alphavirus* (family *Togaviridae*) is represented by Sindbis virus, which has been

extensively studied. SV has been shown to infect a broad range of vertebrate and invertebrate cell lines (Strauss and Strauss, 1994). Virus growth in invertebrates has been studied in various species of mosquitoes (Bowers et al., 1995; Dohm et al., 1995; Greene et al., 2005; van den Hurk et al., 2002) as well as a variety of invertebrate cell cultures such as mosquito C6-36 and C7/10, which have also been utilized for the study of the mechanisms of virus persistence (Condreay and Brown, 1986; Condreay and Brown, 1988; Karpf et al., 1997a). Vertebrate cell death was shown to be by apoptosis (Karpf and Brown, 1998) and resulted in a lytic infection. All invertebrate cells tested to date survive infection with some cultures displaying varying degrees of cytopathic effect, and all become persistently infected maintaining the viral information for long periods of time (Karpf et al., 1997a; Riedel and Brown, 1979). The ability of such viruses to replicate in host cells of different phyla forms the basis for their pathogenicity in mammals and the method of transmission in nature (Strauss and Strauss, 1994).

The original cultured *Aedes albopictus* cell lines produced from larvae (Singh, 1967) have been used to study the mechanism of infection and related phenomena in invertebrate hosts (Hernandez et al., 2001; Karpf et al., 1997b; Luo and Brown, 1994). However, understanding of the invertebrate system is still limited compared to that of the vertebrate system. Comprehension of mechanisms of virus persistence in the mosquito host is paramount for controlling arthropod-borne disease at the level of the vector. Infection of the mosquito vector is food-borne, acquired after ingestion of an infected blood meal in female mosquitoes and by vertical transmission to the male. Therefore, these viruses are restricted in their host range to invertebrate hosts which require blood meals for reproduction (Doherty et al., 1979; Taylor et al., 1955). As such, other insects do not become infected, although in vitro replication in alternative insect cell cultures has been reported for SV (Foy et al., 2004).

Alphaviruses encode a single stranded + sense polyadenlylated genomic RNA. Several significant differences in the infection process between the vertebrate and invertebrate systems have been reported. Invertebrate cells are known to differ from mammalian cells in the intracellular mechanisms which replicate and assemble the virus during the events of early infection. Virus assembly in mosquito cells occurs within intracellular vesicles or “virus factories” (Gliedman et al., 1975, Miller and Brown,

1992). These cells (Miller and Brown, 1993) have been shown to establish homologous interference (Karpf et al., 1997b; Stollar and Shenk, 1973) by a trans-acting protease activity (Karpf et al., 1997b; Kim et al., 2004), however, are unable to complement the production of temperature sensitive mutants which is readily detected in vertebrate cells (Renz and Brown, 1976; Scheele and Pfefferkorn, 1970). In mammalian cells, the virus matures directly from the plasma membrane by a fission, or "budding" mechanism (Brown, 1980). While SV replication and assembly have been extensively studied in vertebrate hosts (Czarniecki and Sreevalsan, 1980; Froshauer et al., 1988; Kim et al., 2004) these processes are not clearly understood in the invertebrate systems.

SV infection in invertebrate systems has been characterized in studies using mosquito cells (*Aedes albopictus* clones C6/36, C7-10, and U4.4) (Gliedman et al., 1975; Karpf et al., 1997a; Stollar, 1975) revealing some of the basic differences between the vertebrate and invertebrate systems (Brown, 1980). C7-10 cells show significant CPE whereas C6/36 cells display limited CPE while U4.4 cells do not display any visible CPE, although all became persistently infected (Miller and Brown, 1992). As these cultures are clonal isolates which were derived from mosquito larvae, the difference in response to SV infection may reflect the variegated responses to SV infection in different mosquito organs (Bowers et al., 1995; Bowers et al., 2003). All invertebrate cell lines tested to date established virus persistence in which the levels of virus produced were reduced after the initial stages of acute infection (Bowers et al., 1995; Karpf et al., 1997a). Only a small percentage of persistently infected insect cells produced infectious virus as shown by infectious center assay and no viral structural proteins were detected in the U4.4 cells by immunofluorescence (Miller and Brown, 1993; Riedel and Brown, 1979). These cells however, were able to exclude superinfecting virus in a manner similar to the acutely infected cells demonstrating the presence of some viral encoded factor within these cells.

In the present study, data are presented which establish *Drosophila* as an alternate invertebrate model for SV infection. This effort was undertaken for comparative evaluation of *Mosquito,* the natural host for SV and *Drosophila,* a system that can support replication of SV, to accelerate the efforts to understand the response of invertebrate hosts to SV infection, using the vast genetic and genomic information available for *Drosophila.* In particular, identification genetic factors that contribute to the

establishment of the persistent state of infection in invertebrates should be interesting as it shows ability of invertebrate systems to suppress virus production without deleterious effects on cell viability. S2 (also referred to as SL2) cells derived from 20-24 hr old *Drosophila* embryo are considered to represent hemocytes of insects (Ramet et al., 2001) and have been used successfully to study immune related pathways (Hoffmann et al., 1999; Kallioniemi, 2005; Kleino et al., 2005) and to perform RNAi (Clemens et al., 2000) at genomic level and also in protein expression (Scotter et al., 2005) studies. Studies establishing S2 cells as an alternate model system for infection of pathogens such as Chlamydia (Elwell and Engel, 2005) and Listeria (Cheng and Portnoy, 2003) have also been reported and have allowed a genomics based study of cellular response to infection with these agents.

Because of the different cellular responses to SV infections and the different amounts of virus produced in different clonal isolates of mosquito cells, it was essential to compare the characteristics of SV infection of the S2 cells to those of cells derived from the mosquito host. The U4.4 cell line was chosen for this purpose because this cell line displays no CPE upon SV infection and produces the highest titers during the acute phase of infection. In the present report, comparative data on SV production, change in morphology, and cell growth rates upon infection of S2 cells compared to that of U.4.4 cells are presented. These data show that although *Drosophila* is not a natural host, S2 cells become infected with SV and respond with an acute infection and proceed into virus persistence. Previous studies (Bras-Herreng, 1973; Bras-Herreng, 1975; Bras-Herreng, 1976) (Ohanessian and Echalier, 1967) reporting the ability of SV to replicate in *Drosophila* flies and cells of *Drosophila*, concur with our study.

Experimental Materials and Methods:

Cell Culture and Virus Infection:

Schneider's 2 (S2) *Drosophila* cells (ATCC # CRL-1963, Manassas, VA) were grown in Schneider's *Drosophila* media with 20% FBS (Sigma, JRH 12106-500M Lenexa, Kansas), 2mM L- glutamine, in 75 cm^2 culture flasks at 23^0C in a humidified growth chamber maintained in 10% CO_2. The cells grew as semi-adherent cultures that displayed a high affinity for clumping. Cells were split 1:3 every 5 days. Baby hamster

kidney (BHK-21) cells were cultured in Eagle's minimal essential medium (MEM-E) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 5% tryptose phosphate broth (TPB), and 2 mM L-glutamine were maintained at 37°C in 5% CO_2 as described (Renz and Brown, 1976). *Aedes albopictus* U4.4 cells were subcloned in our laboratory from the original *Aedes albopictus* cell line isolates of Singh (Singh, 1967) and were grown in Mitsuhashi and Maramorosch medium (M & M) (Mitsuhashi and Maramorosch, 1964) at 28^0C supplemented with 20% FBS. SV virus strain SVHR (SV – heat resistant) grown in BHK cells served as wild-type virus in all the experiments. All infections of both S2 and U4.4 cells were at a multiplicity of infection (MOI) of 100 plaque forming units (PFU) per cell. For infection, both S2 and U4.4 cells were incubated in serum-free media to convert the cultures into adherent cells and infection was carried out while rocking for 1 hr at 25^0C. After infection, the inoculum was removed and cells were washed twice with media containing 20% serum and incubated for the indicated times. Virus was titered by plaque assay using BHK cells.

Cell and Virus Growth Curves:

Trypan blue exclusion was used to determine cell doubling times of U4.4 and S2 cells. Aliquots of 200µl of each cell line were incubated for 2 minutes with 10% trypan blue dye (Invitrogen, Grand Island, NY) and the number of cells excluding the dye (living cells) determined. For the virus growth curves, S2 cells (on day 3 or 4 after subculture) were incubated for 1 hr at 23^0C in serum-free medium as described above. Cells were infected at an MOI of 100 PFU/cell by rocking for 1 hr at 25^0C. The inoculum was removed and the cell monolayer was washed with media twice prior to incubation in the above described medium. All centrifugations were done at 1000 rpm in an IEC HN-SII centrifuge. Centrifugation speed was found to be critical since these cells were very sensitive to manipulations and the serum lot used. The washed cells were counted at the times indicated in Results, using the same culture for each point of the growth curve for 9 days post-infection. This was done to avoid any discrepancy in titer owing to collection of virus from different batches of cells. It was previously observed that insect cells show different levels of virus production in clonally selected cells (Karpf et al., 1997a) and growth curves for SV in U4.4 cells are well established. Growth curves of SV derived from U4.4 cells were obtained simultaneously by washing the cells three times with M &

M media and collecting the virus at the times indicated. All the virus samples were frozen in 10% glycerol, stored at -80^{0}C and titered on BHK cells as described previously (Renz and Brown, 1976). Virus produced from S2 cells tended to clump, a characteristic which has been observed previously (Hernandez 2003), for some SV mutants. To circumvent problems due to non-uniform virus suspensions, the plaque assay dilutions were made in TM (Trans-Membrane mutant) diluent previously described for the tittering SV mutants prone to aggregation (10% glycerol, 10% FBS, 10mM HEPES, 4μM phenol red indicator in PBS-D pH 7.4) (Hernandez et al., 2003). Plaques were counted after incubation at 28^{0}C for 4 days, or 37^0 C for 3 days. Comparison of the titers at these temperatures was used to evaluate the production of any temperature sensitive variants that might have arisen in S2 (Bras-Herreng 1976) or U4.4 cells (Brown and Condreay, 1986).

Light and Transmission Electron Microscopy:

For light microscopy, the S2 or U4.4 cells, uninfected or infected for 6 days were observed by phase contrast microscopy and photographed (magnification factor 16.5X).

For transmission electron microscopy (TEM) thin sections, cell pellets were washed twice with PBS-D and fixed with 3% glutaraldehyde (Ladd Research Industries, Williston, VT) in 0.1M cacodylic acid buffer (pH 7.4) (Ladd Research Industries, Williston VT). Cells were then washed three times with 0.1 M cacodylic acid buffer and stained with 2% osmium tetroxide in cacodylic buffer for 1 h. Cells were then washed as before and embedded in 2% agarose. The cell sample was then prestained with 1% uranyl acetate (Polaron Instruments, Hatfield, PA) overnight at 4 °C, washed and dehydrated using ethanol and infiltration with SPURR compound (LADD Research Industries, Williston VT). Blocks were trimmed on an LKB NOVA Ultrotome (Leica Microsystems, Deerfield, IL). Ultra-thin sections obtained were stained with 5% uranyl acetate in distilled water for 60 min and in Reynolds lead citrate (pH 12) (Mallinkrodt Baker, Paris, KY.) for 4 min. The samples were examined at 80 kV in a JEOL JEM 100S transmission electron microscope.

Virus Density and Particle / PFU ratio determination:

S2 and U4.4 cells were infected as described above, the media collected 20 hr post infection and purified by centrifugation on a 15% and 35% potassium tartrate step-gradient using a Beckman L-90K ultracentrifuge, in an SW-28 rotor, at 26K RPM and

4^0C overnight. For a second purification step, the virus band was collected, diluted, and loaded onto a continuous 15% - 35% potassium tartrate gradient and centrifuged for 8 hr, as described above. The virus band was again collected and the refractive index (RI) was measured. The virus was then dialyzed against 1X PBS-D at 4^0C and titered by plaque assay as described above. The virus protein concentration was determined by Micro BCA assay (Pierce, Rockford, IL) and together with the virus titer was used to establish the infectivity of the virus population as a particle/PFU ratio.

Results:

Growth of uninfected and persistently infected S2 cells:

To assess cell growth, the number of living cells in mock-infected and SV infected S2 and U4.4 cultures were determined at specific times as described in Methods. Cells were sub cultured at 5 days after SVHR or mock infection and their growth monitored for a total of 16 days. A 10% decrease in the growth rate of the S2 cells in the initial 5 days post infection was observed (Fig. 1A). The infected S2 cells resumed growth rates similar to those measured for uninfected cells during early persistence beginning on day 6 (Fig. 1A). Similar rates of cell growth were obtained with infected or mock infected U4.4 cells (Fig. 1B). These observations agree with previously reported growth rates of U4.4 cells during early persistence (Riedel and Brown, 1979) and show that the S2 cells respond to virus infection similarly to U4.4 cells. These infected cultures were maintained for several months with no obvious changes in morphology or growth rate.

Growth of Sindbis virus in S2 cells:

The quantity of infectious virus released by S2 and U4.4 cells was measured by plaque assay up to 9 days post-infection (Fig. 2). Cells were infected at an MOI of 100 PFU/cell for one hour after which the cells were washed and placed in fresh media. Virus aliquots were collected at the times indicated and the cells were then washed as described in Methods and resuspended in fresh medium. The amount of virus produced in both cell lines was reduced by approximately 2 orders of magnitude by day 4 or 5 post-infection. The reduction in virus production seen in this study reproduces the observations made in previous studies using mosquito cells (Karpf et al., 1997a; Miller and Brown, 1992) and in whole mosquitoes (Bowers et al., 1995; Bowers et al., 2003).

The amounts of virus produced (Fig. 2) were consistently lower in S2 cells compared to U4.4 cells. Peak virus production during the acute phase of infection was found on day 1 post-infection in S2 cells in contrast to day 2 post-infection seen in U4.4 cells. The quantity of virus produced per cell (PFU/cell) was higher from U4.4 cells compared to S2 cells. The S2 cells released 10 PFU/cell on day 1 after infection. The U4.4 cells released about 10^3 PFU/cell at day 2 post infection. These data demonstrate that the S2 cells become infected with SV producing an acute infection by day 1 post-infection and become persistently infected by day 4-5 post infection. These data are in contrast to those seen for the U4.4 cells which entered the acute phase of the infection on day 2 post-infection. The virus particles generated from S2 cells were found to be of comparable structural stability to those generated from U4.4 cells as demonstrated by the similar particle/PFU ratios (Table 1).

Morphology of S2 cells persistently infected with Sindbis virus:

S2 cells become persistently infected with SV and can be maintained for several months. When examined by phase-contrast microscopy the persistently infected cells have morphologies similar to that of the uninfected cells. In Fig. 3, uninfected and 6 dpi (days post-infection) S2 and U4.4 cell morphologies are presented for comparison.

Ultrastructure of uninfected and infected S2 cells:

The ultrastructure of virus within persistently infected S2 cells or U4.4 cells at 9 dpi were analyzed using electron microscopy of thin-sections as described in Materials and Methods. Micrographs of uninfected S2 cells (Fig. 4A) and U4.4 cells (Fig. 4C) illustrate typical invertebrate cell morphology and contained no virus-like structures. At 9 dpi, S2 cells shown in Fig. 4B displayed mature virions within intracellular vesicles. These virus-filled vesicles were surrounded with membrane producing structures similar to those found in U4.4 cells (Gliedman et al., 1975; Miller and Brown, 1992). The mature virions in S2 cells appeared to be more adherent to one another and the envelope structure was less well defined than that of virus produced in mosquito cells (compare Fig. 4B to 4C). The virus particles produced in S2 cells also appear to have a thicker membrane compared to the virus produced by U4.4 cells (Fig 4B) (Gliedman et al., 1975; Miller and Brown, 1992). This may reflect differences in the membrane composition of these two insect cells. This hypothesis may be supported by the observation that the measured

buoyant density of the virions from S2 cells ρ 1.095 was slightly higher than that of virus produced from U4.4 ρ 1.085 (Table 1).

Discussion

Mosquitoes are the natural vector of Alphaviruses in nature. The infection of adult mosquitoes with Alphaviruses results in limited cytopathology and mortality. The benign nature of this host-virus relationship is underscored by the observation that some Alphaviruses can be transmitted vertically (transovarially) to progeny insects (Mitchell et al., 1992). This non lethal interaction is essential to ensure that the insect can transmit the virus to the next available vertebrate host. Within an infected mosquito, virus levels and the dynamics of infection i.e., time taken to establish, reduce or clear virus production and proceed into persistence varies within different organs/tissues (Bowers et al., 1995). This variation in the response of mosquito tissues may reflect SVHR growth in the various clones of *Aedes albopictus* cells (Karpf et al., 1997a; Miller and Brown, 1992). Cultured, mosquito cells survive SV infection and produce reduced levels of virus during persistence, although differences in the amounts of virus produced and CPE are seen in the different clones (Karpf 1997a; Miller and Brown, 1992). Published observations have documented that even different clonal isolates of the cells from a single cell culture demonstrate differences in levels of virus production (Karpf et al., 1997a; Miller and Brown, 1992). The morphology and growth properties of mosquito cells does not change during "early" persistent infection in the cell clones tested by Karpf and co-workers (Karpf 1997a). Karpf however, did report a reduction in growth rates of U4.4 cells that were infected for more than a year.

In this study we have compared the growth of SVHR in the *Drosophila* S2 cell line to growth of the same virus in the U4.4 clone of *Aedes albopictus* cells. The U4.4 clone was selected for this comparison because, of the three existing clones (U4.4, C6-36 and C7-10), this clone transitions through the acute phase into the persistent phase of infection with no obvious CPE. We found that, after infection with SV, the S2 cells transitioned into a persistently infected state with no obvious cytopathology as did the U4.4 cells. Electron microscopy revealed that Sindbis virus matured into internal vesicles in both cell types following a pattern of assembly previously described for virus production in *Aedes albopictus* cells. However, these studies suggested that some subtle

differences on the surface of the virus produced in the S2 cell line produced more adherent particles, which may be related to variations in the composition of *Drosophila* membranes. This difference did not significantly affect the infectivity of the virus but did result in a small increase in buoyant density (ρ). The S2 cells produced lower levels of virus during the acute phase of infection, compared to U4.4 cells. This result was not unexpected since *Drosophila* is not a natural host of the virus. These reduced levels of virus however, correspond to the levels of virus characteristic of persistent infection. The PFU/cell produced by *Drosophila* cells used in this study reproduced the observation made by Bras-Herreng, (Bras-Herreng, 1975), although the levels of virus were lower (2PFU/cell) in the present study (Fig. 4B) compared to 5 PFU/cell observed previously (Bras-Herreng, 1975). Two continuous *Drosophila* cell lineages K and C, were mixed to conduct this study (Bras-Herreng, 1975). The S2 cells used in present study are distinct from these cell lines and are routinely used along with other cell lines (Kc, Dmel2, BG2-C6) (http://flyrnai.org/RNAi_screens.html) in recent studies described above (see introduction).

The present study establishes that virus production in and the cellular response to virus infection of S2 cells is similar to that of U4.4 cells. These data suggest that *Drosophila* cells, while not a natural host for Alphaviruses may respond to virus infection and control virus infection by mechanisms similar to those in *Aedes* cells. If this were not the case, it would not be expected that S2 cells could respond to virus infection by establishing a persistent infection. *Drosophila* genetics has been extensively described and the genome of this insect has been sequenced with well-defined gene ontologies, the information provided to describe the genetic phenotypes, gene products and their biological roles and specific metabolic functions in Drosophila. Further, oligonucleotide arrays commercially available for *Drosophila* can accelerate research involving invertebrate systems. These data presented herein suggest that *Drosophila* S2 cells can be used as an alternate model system to study the genetic response of insect cells to *Alphavirus* infection. We are exploring this possibility.

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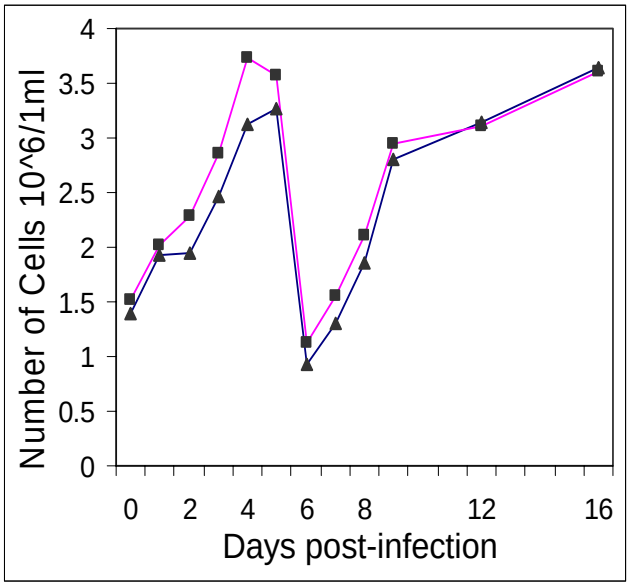
Figure 1.1: Growth of SVHR infected (black) or mock infected (white) S2 (a) or *Aedes albopictus* U4.4 cells (b). The cells were cultured and numbers determined as described in Methods. The cells were sub-cultured at day five. The number at each point is the average of three experiments.

a)



b)

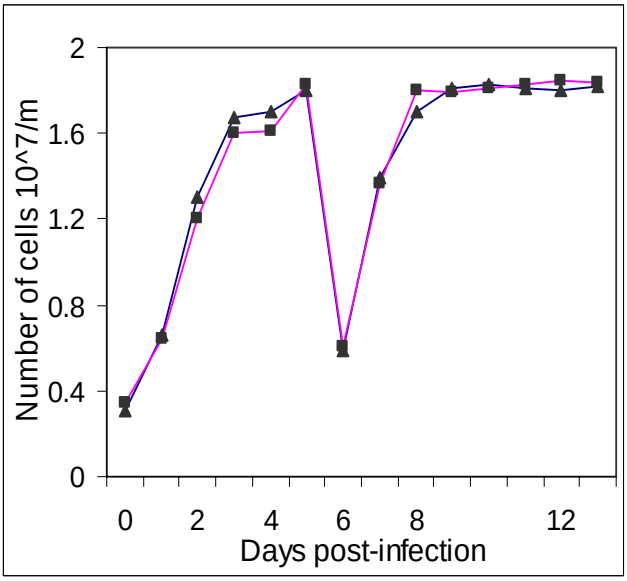


Figure 2.2A: Production of SVHR by cultured S2 (gray) or U4.4 (black) cells. Cells were infected and virus produced from 1-9 days post-infection were determined as described in Methods.

2 a)

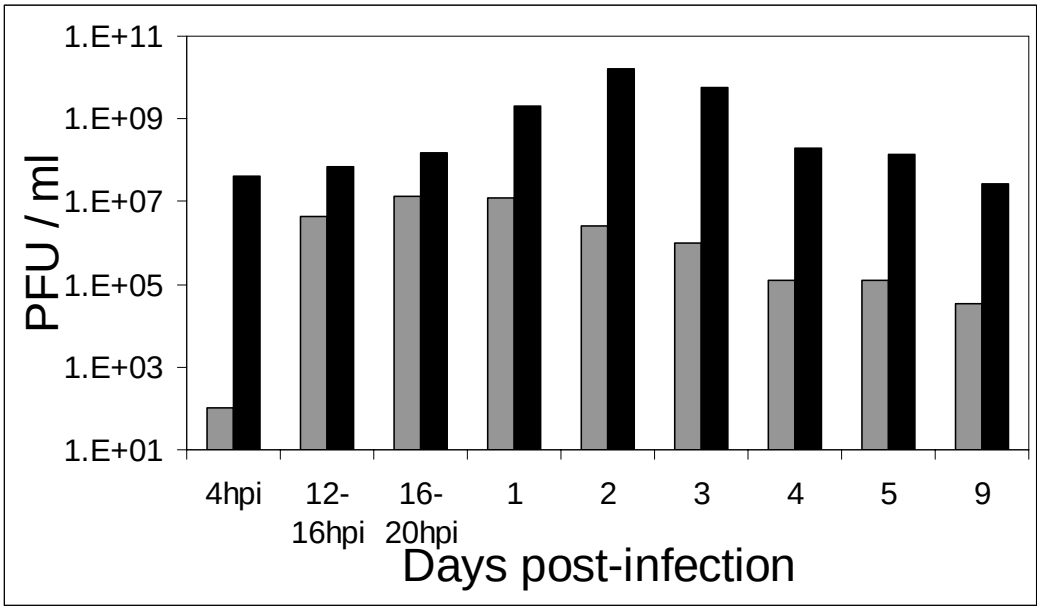
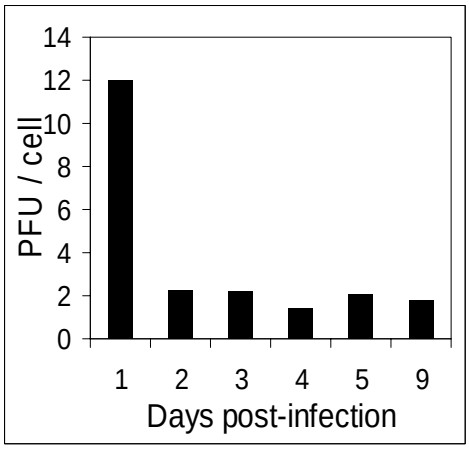


Fig 2 B and C: Reduced levels of virus production <u>per cell</u>, in B – *Drosophila* S2 cells; C- *Mosquito* U4.4 cells.

2 b)



2 c)

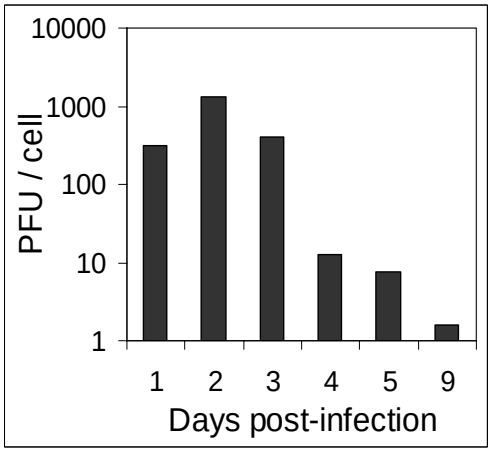


Fig 1.2D: Reduction in Sindbis virus production expressed as percentage of peak amounts of virus produced in S2 cells – Triangles (▲); U4.4 cells – Circles (●). Virus production is reduced by 100 – fold at 5 days post-infection.

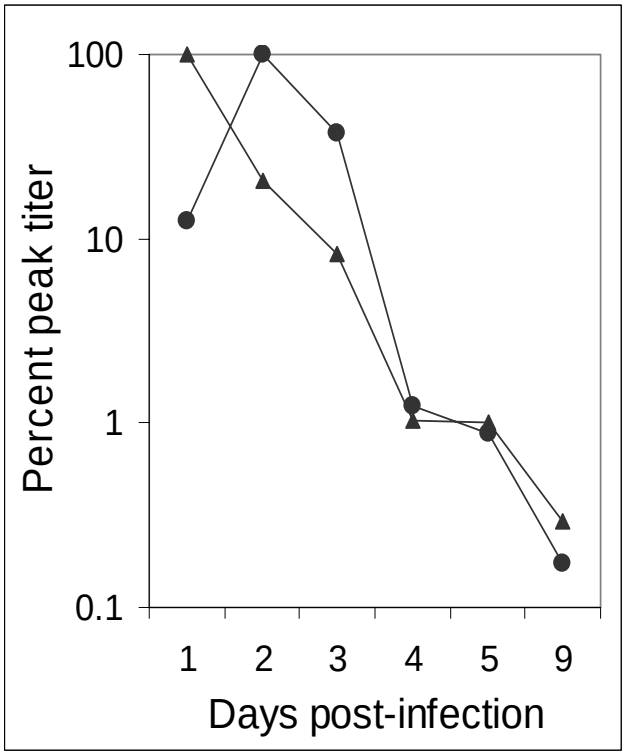
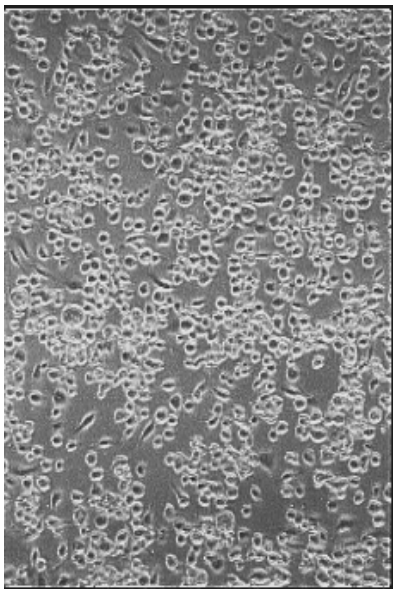
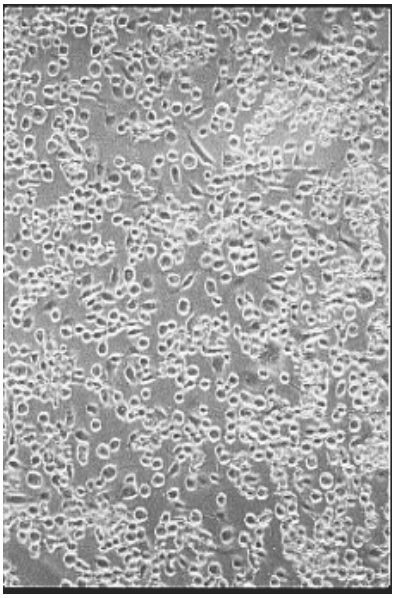


Figure 1.3. Morphology of uninfected (A&C) and infected (B&D) S2 (A&B) or U4.4 cells (C&D). Cells were cultured and infected as described in Methods. Images were made at 6 days after infection at identical magnification.

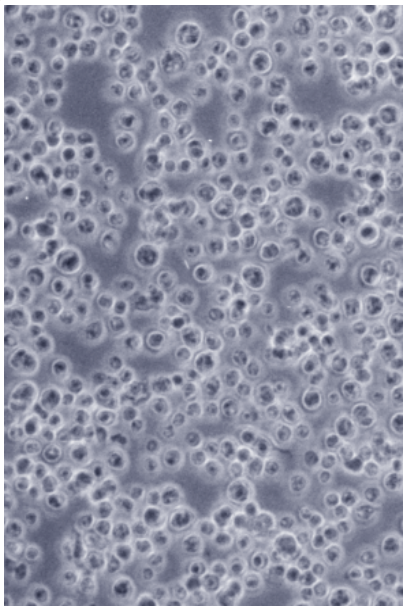
A)



B)



C)



D)

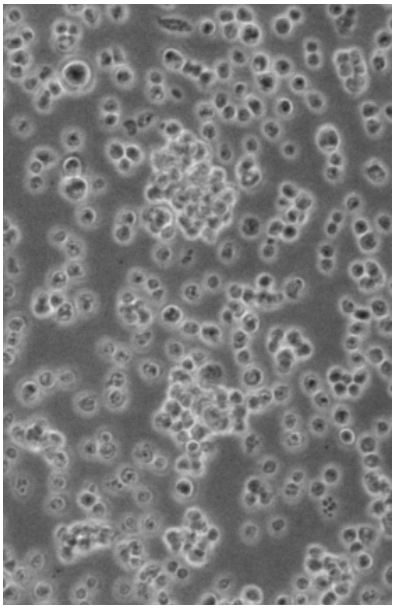


Fig. 1.4: Ultrastructure of S2 (A and B) or U4.4 (C) cells infected (B and C) or uninfected (A) with SVHR. Cells were infected and processed for electron microscopy as described in Section 2. Bars indicate: (A) 1 μm and (B and C) 200 nm.

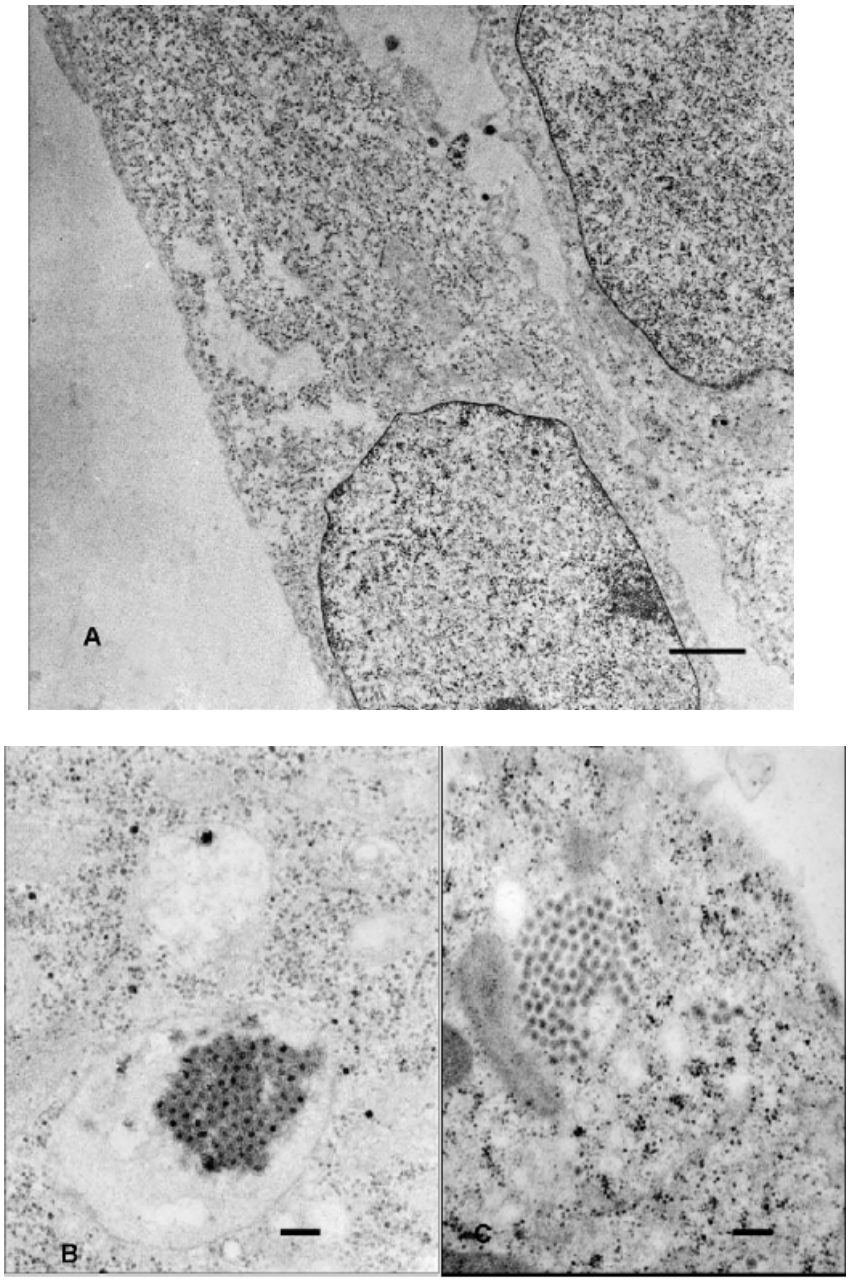


Table1.1: Properties of Sindbis virus produced from Drosophila or Aedine cells

Host for SVHR	Density (g/cm3)	Titer (PFU/ml)	Particle/PFU
U4.4 Mosquito cells	1.0856/ 1.085	1.4 X 109 (2)	12
Schneider-2 Drosophila cells	1.095	1 X 107 (3)	23

CHAPTER TWO

INSECT CELL RESPONSE TO ALPHAVIRUS INFECTION – FACTORS INVOLVED IN ESTABLISHMENT OF PERSISTENCE

Abstract:

Alphavirus persistence in the insect vector is an important element in transmission cycle of the virus and can provide a model to study the biochemical and molecular basis for virus-vector co-existence. Profiling of gene expression in *Drosophila* S2 cells 5 days post-infection with Sindbis virus identified increased levels of transcripts that can help explain previous observations on the interaction of Alphaviruses with insect cells including membrane vesicle formation and changes in cell cycle and cell growth. *numb, nedd4, hook, raps, dlg1, cas, furin2, and osa* that are known to be involved in different aspects of Notch signaling are upregulated. Herpesvirus usurps Notch pathway components to establish latency and activation of the same during establishment of Alphavirus persistence indicates similarities between activities involved in establishment Herpesvirus and Alphavirus latency. Components of the immune-related JNK and JAK-STAT pathways were also upregulated. Upregulation of *ankyrin* and *plap* may indicate similarities between spectrosome / fusome and the vesicles that accumulate mature virus in insect cells and they may play a role in the asymmetric distribution of Notch components which could result in different cell fates. Increased levels of *syx13*, *unc-13*, *csp* may aid in the formation of virus containing vesicles and the release of the virus from the infected cell. Increased levels of *rab1* and *rab8* may be involved in processing of viral structural proteins.

Introduction:

Viruses belonging to genus *Alphavirus* (~30 species. family: Togaviridae) cause diseases with high fever, encephalitis, arthritis and hemorrhagia leading to death in birds and mammals including humans and domestic animals. The family includes species such as Semliki Forest Virus (SFV), Western, Eastern and Venezuelan Equine Encephalitis viruses (WEE, EEE, VEE), Sindbis Virus (SV) and Chikungunya virus. Female *A. aegypti* (*Aedes aegypti*) mosquitoes act as vectors transmitting the virus between the vertebrate hosts with humans being incidental hosts (Strauss and Strauss, 1994).

Epidemics in humans involving Alphaviruses are a serious problem particularly in parts of the world with widespread mosquito populations. The infection of mosquitoes is food-borne and transovarial transmission (Mitchell 1992) maintains the virus in nature without involvement of vertebrates. Following virus infection, Mosquitoes survive initial acute infection and become persistently infected for life, with different amounts of viruses in various organs and tissues (Bowers 1995, Bowers 2003).

Sindbis Virus (SV), the prototype *Alphavirus* infects a wide variety of vertebrate and invertebrate cell lines. Vertebrate cells die by apoptosis (Karpf and Brown 1998) and all the invertebrate cell lines tested to date survive the acute phase of infection leading to persistence with reduced levels of virus production (Riedel and Brown 1977). Further, there are differences in pathways of virus maturation between the vertebrates and invertebrate cells. Upon infection of a cell, the viral plus-sense RNA genome is translated by cellular ribosomes to produce nonstructural polyproteins that function as the viral transcriptase-replicase which in insect cells may be associated with membranes of new vesicles which are formed upon infection (Miller and Brown 1992). Minus strand RNA is produced by a viral replicase activity and is used for production of a sub-genomic RNA that codes for structural proteins and the plus-sense full length RNA that forms the genome of the progeny virion. Virus maturation takes place by envelopment at the cell surface in vertebrate cells. In Mosquito cells virus matures primarily at the membranes of intracellular vesicles with limited envelopment occurring at the cell surface depending on the cell line (Miller and Brown 1992). Infection of *Drosophila* S2 cells with Sindbis virus results in a maturation pathway similar to that seen in Mosquito cells followed by the development of persistent infection also similar to that seen in mosquito cells (Mudiganti 2006).

Different invertebrate cell lines show different dynamics in the establishment of persistence. Three mosquito cell lines, U4.4, C7-10 and C6/36, show differences in their responses to SV infection. During acute infection, the three mosquito cell lines show different degrees of cytopathology (Cytopathic Effect -CPE): The U4.4 clone shows no CPE. While clones C7-10 and C6/36 show significant and moderate CPE respectively. (Miller and Brown 1992). All mosquito cell cultures show peak amounts of virus produced on 2-3dpi (days post-infection) and reduced levels of virus production from day

5 post-infection. *Drosophila* S2 cells show maximum of virus production 1-2 dpi and reduction in virus production starting at 2-3 dpi (Mudiganti 2006). Some of these persistently infected cell lines showed some differences in their cell cycle patterns compared to the uninfected cells. The uninfected and persistently infected U4.4 cells show no difference in cell cycle. The C7-10 cells show G2/M arrest as the cells enter the persistent phase of infection. The C6/36 cells show more apoptotic cells in persistently infected cultures (Karpf 1997a). As these cell lines are derived from mosquito embryos, they may represent progenitors of different tissues of mosquitoes and this may explain differences in their response to SV infection and persistence. Even though there are some differences in particular cellular functions upon infection of different clones of insect cells, persistent infections are established in all invertebrate cells tested to date. Defective Interfering particles of SV produced in BHK cells did not affect viral replication in *Mosquito A. albopictus* cells (Igarashi 1976).

A. aegypti (Peleg and Stollar 1974) or *A. albopictus* cells (Riedel and Brown 1979, Condreay and Brown 1986, Karpf 1997b) which have been treated with cell lysates (Peleg and Stollar 1974) or media from PI (Persistently Infected) cells (Riedel and Brown 1979, Karpf 1997b, Condreay and Brown 1986) exclude super-infecting virus. The media / cell lysate treated cells produce virus at levels that are 2 - 3 logs less than cells that are not treated and these reduced levels correspond to virus levels typically seen in PI *Aedes* cells. Media from the PI *A. albopictus* cells shows antiviral activity that can reduce viral RNA production (Condreay 1988). Specific antiviral factors have been isolated and partially purified from the media of PI cells and pretreatment with these factors reduced viral RNA production (Luo 1993). *Mosquito* cells infected at Multiplicities of Infection (MOIs) ranging from 0.00005 to 50 produce the same amounts of progeny virus, indicating that intracellular factors are responsible for controlling the virus production as SV persistence is established (Karpf 1997a).

In this study, we utilized a genomics approach to identify genes differentially expressed during the development of SV persistence in insect cells and to identify potential regulators of virus production. *Drosophila* S2 (or SL2) cells were used because commercial gene arrays (Affymetrix Genechips) are available for Drosophila. Although not a natural host, S2 cells and the fruit flies themselves can establish persistent

infections with SV and the infected cells show features comparable to *Mosquito* cells (Mudiganti 2006). Therefore the S2 cells provide a suitable system to study insect response to Alphavirus infection. These cells are derived from *Drosophila* embryos and are considered to represent hemocytes. They have been shown to provide a suitable model for detection of immune-related factors (Kallio 2005), signaling by EGFR (Hasson 2004), Notch/Wnt (Frise 1996, Wesley 1999) and immune-related pathways (Boutros 2002). The midgut of *A. aegypti*, the natural vector of SV showed several ion channels and membrane trafficking factors upregulated at 4 days post infection (dpi), in response to SV infection (Sanders 2005). In our studies membrane trafficking factors are also upregulated at 5dpi indicating the importance of these functions during early persistence. We also found upregulation of the *Drosophila* Notch signaling pathway.

Experimental procedures:

Cells and Virus:

Schneider's 2 (S2) *Drosophila* cells (ATCC # CRL-1963, Manassas, VA) were grown in Schneider's *Drosophila* media with 20% FBS (Sigma, JRH 12106-500M Lenexa, Kansas), 2mM L- glutamine, in 75 cm^2 culture flasks at 23°C in a humidified growth chamber maintained in 10% CO_2. The cells grew as semi-adherent cultures that displayed a high affinity for clumping and grew mostly in clumps. Cells were split 1:3 every 4 days.

Infections:

4×10^7 S2 cells are resuspended in serum-free media to form monolayers and infected with SVHR (Sindbis Virus – Heat Resistant) at 100 MOI (Multiplicity of Infection) by incubation with rocking for 1 hr at room temperature.

Preparation of cRNA for hybridization:

Total RNA was isolated from mock infected and 5dpi S2 cells (4×10^7 cells from a 75cm2 flask) using Trizol reagent (Ambion, 9738 Austin, Texas). mRNA was isolated from total RNA using poly (A) kit from Ambion (Ambion, 1916 Austin, Texas). Spike controls (Lys, Dap, Trp, Tyr) were added to total RNA before isolation of mRNA to check for any loss of RNA or mRNA during subsequent labeling and hybridization steps of the protocol. mRNA was processed according to Affymetrix genechip protocol. Briefly, cDNA was obtained by Reverse transcription of the mRNA, using *Superscript II Reverse*

Transcriptase (Invitrogen, 18064-014 Carlsbad, CA) and was transcribed in vitro using Enzo Bioarray kit (Affymetrix, 900182 Santa Clara, CA) using biotinylated rNTPs to obtain 15 µg biotinylated cRNA. cRNA was fragmented to obtain 400-600 nt. long fragments which were hybridized to Affymetrix *Drosophila* genechip (Affymetrix, 900335 Santa Clara, CA) with 14010 *Drosophila* genes (*Drosogenome1*). Hybridization, staining and washing protocols given by manufacturers were followed and the image was scanned using Agilent scanner. All the reagents required for hybridization and staining and washing are obtained from the manufacturers suggested by Affymetrix (*Genechip expression analysis Technical Manual @ Affymetrix.com*).

Preliminary examination of the data using MicroArray Suite (MAS) 5.0:

Array intensity signal data from independent RNA samples (Mock or 5 dpi) showing "Present" call for the house keeping genes and the spike controls (see above) are considered for data analyses steps described below.

Analysis using Genespring 7.2:

Intensity signal values of 3 mock and 2 infected obtained from MAS 5.0 were analysed using genespring 7.2. Per chip normalization was done using distribution of signal intensity values of all genes. Each measurement is divided by 50th percentile of all measurements in that sample. Per gene normalization is done using specific control samples in which intensity signal of each gene is divided by average signal of that gene in control samples. The intensity values corresponding to each gene are scaled around 1. Differentially expressed genes in different Gene Ontology groups were identified with T-test, using Cross-gene error model. Genes showing 1.5 to 2-fold or more change in expression levels as shown by the graph, with p-values below 0.05 are filtered. Genes showing 1.5 – 2 or more change in this scaled value from mock replicas to 5dpi sample replicas are hand-picked and reported here.

Results & discussion:

The complete list of genes up regulated (140 *genes*) and down regulated (39 *genes*) in Genespring analysis, and corresponding *p*-values are included in Appendix. The time point of 5 dpi was chosen because we have previously shown that all the cells in the S2 cell culture were infected and producing virus at levels characteristic of the PI state at this

time (Mudiganti 2006). The gene expression profile of the *Drosophila* genome in Mock and 5dpi samples was analysed using Genespring 7.2 as described in "Experimental Procedures". Our results identified cytoskeletal or membrane trafficking components (18), immune-related components (9), transcription factors (8), factors effecting cell cycle or cell division (9), Ras (6) and Notch pathway components (10) as potentially involved in establishment of viral persistence in insect cells. A number of genes at different levels of significance are given in *Table1*. Genes that showed a normalized P value below 1 (0.3 - 0.9) in Mock samples and 1.4 or above in two 5dpi samples were hand-picked and labeled as changing 1.5 - 2 fold. The results of this study are summarized in figure 2. In the sections that follow different functional classes of genes affected by infection are discussed together.

Signaling pathways:

Among the factors discussed below are cell fate determinants which could play a role in some of the cell fate changes associated with virus persistence in insect cells, for example the 30% reduction in cell growth rate, cell cycle changes reported for *A. albopictus* cells (Karpf 1997a),and the 10% reduction in S2 cell growth rate (Mudiganti 2006).

Notch pathway components involved in establishment of SV persistence in insect cells:

Table2 shows upregulated genes that encode factors that mediate regulatory aspects of cellular function. The Notch pathway is initiated in cells upon binding of the Delta gene product from an adjacent cell. *numb* encodes a protein in the plasma membrane of S2 cells (Frise 1996) and it's differential distribution in dividing SV-infected S2 cells may result in the inhibition of the Notch pathway in one of the two mitotic daughter cells (Frise 1996, Orgogozo 2002, Wan 2000). This asymmetric distribution may influence cell fate as the cells establish persistence. Upregulation of *nedd4* which encodes a regulatory protein involved in the endocytosis of the Notch receptor (Sakata 2004) together with *hook,* which is involved in endocytosis of Delta (Parks 2000) activation of these signaling pathways at 5dpi. *raps,* and *dlg1* are required to form the structural elements that cause the asymmetric distribution of Notch components (Bellaiche 2001, Carmena 1998). The activation of Notch results in the expression of *Ac/Sc* proneural gene supressors (Artavanis-Tsakonas 1999, Bardin 2004) *osa,* which codes for a negative

regulator of *Ac/Sc* (Heitzler 2003) is upregulated also suggesting activation of the pathway., As the SV infected insect cells differ from vertebrate cells by not undergoing apoptosis, stimulation of Notch apparently results in inhibition of apoptosis (Lundell 2003 and references there in) to aid in transition to persistence by suppressing cell death (Mudiganti 2006, Riedel and Brown 1977).

Upregulation of *cas*, which encodes a protein necessary for nuclear transport of Notch components (Tekotte 2002) and *furin* (*Table5*) which encodes an endoprotease that helps in Notch receptor processing (Baron 2002) adds to the evidence that Notch pathway is indeed stimulated and may be an important step in establishment of SV persistence. *Drosophila* Notch, Delta and other proteins identified here have significant similarities with Mosquito *A. aegypti* and *A. gambiae* proteins (*Table2*).

Notch/Wnt/Ras network functions in establishment of SV persistence:

tao-1, that interacts genetically with Fibroblast Growth Factor Receptor (FGFR) (Zhu 2005) and *sugarless* that acts as co-receptor for FGFR and Wnt signaling (Hacker 1997, Ornitz 2000) are up regulated (*Table3*) suggesting the functioning of FGF/Notch/Wnt signaling network at 5dpi. Signals originating from FGFR or EGFR or Wnt interact with Notch signaling in different developmental contexts - Input from Wnt and Notch regulates the cell cycle patterns during imaginal wing disc development (Johnston 1998) and differentiation of follicles (Keller Larking 1999, Lopez-Schier 2001, Morgan 1996, Myster 2000 for review). A combination of signals from the Ras pathways initiated by Epidermal Growth Factor Receptor (EGFR) and FGFR, and the Notch pathway interact in embryonic mesoderm to determine cell fates (Carmena and references therein).

The decrease in expression levels of *bab1* (Galindo 2002), and *toy* suggest an alteration of the EGF pathway, possibly due to input from Notch signaling (Kumar 2001). Thus, input from Ras and Notch pathways (Carmena 2002 and references therein, Kumar 2001, Weijzen 2002) along with Wnt pathway may cause the observed changes in cell growth properties (Mudiganti 2006) leading to alterations similar to differentiation (discussed in following sections). Alternately, these pathways may contribute in parallel towards changes in cell cycle, and growth along with intracellular changes similar to those occurring during differentiation, conferring the ability of insect cells to establish persistence (Karpf 1997a, Mudiganti 2006).

Potential role of Notch/Wnt signaling in SV persistence:

Kaposi's Sarcoma Herpes Virus (KSHV) expresses proteins that affect apoptosis, cell cycle and immune functions of the host early during the infection cycle and these changes are maintained throughout the infection cycle into latency (Yoo 2005). The identification of similar patterns of gene expression at 5dpi in insect cells as Sindbis virus persistence is established may indicate that similar events cause latency in both cases. For example, KSHV usurps Notch components as it establishes latency and treatment with Notch pathway inhibitors induces apoptosis and lysis in KSHV tumor cells (Curry 2005). The activation of Notch signaling in SV infected insect cells may play a similar role, i.e., influencing the cell-fate. Malignancy (increased growth rate) in KSHV infected cells is accompanied by viral LANA (Latency-Associated Nucelar Antigen)-mediated accumulation of intracellular domains of Notch in the nucleus, indicating the activation of Notch (Lan 2006). In contrast to KSHV latency, insect cells persistently infected with SV show a reduction in growth rate (Karpf 1997a). Thus, these two systems may represent two different activities resulting from the stimulation of Notch (Lundell 2003 and references therein) which may in part be due to interaction of Notch signals with FGFR/EGFR/Wnt pathway, as discussed above. Further, cyclic patterns in virus production during SV persistence (Riedel and Brown 1977) and the latency to lysis switch in KSHV infection may involve similar control mechanisms. SV nonstructural proteins (nsPs) have been shown to co-precipitate with host proteins (Barton 1991). nsP3 has been shown to be associated with G3BP (Ras Gap SH3 binding protein) which is in turn found associated with mammalian Gap1 (Zekri and references therein). Hence, upregulation of Drosophila Gap1 in this study (p-value >0.05, *Table3*) may be due to nsP3-dependent activities. Gap1 is a negative regulator of Ras pathway and hence it's upregulation further supports activation of Notch/Wnt/Ras network at 5dpi in Sv-infected cells. nsP2 is shown to be associated with host ribosomal protein S6 (rpS6) through out the infection cycle and nsP1 also is associated with rpS6 (Montgomery 2006), suggesting importance of these proteins to alter host ribosomal functions to cause differential expression of host or viral proteins. Mutations in nsP2 region of SV lead to persistence in mammalian cells, changing the apoptotic cell fate in these cells (Frolova 2002) and nsP2 is shown to enter nuclei using mammalian cells (Rikkonen 1994). SV nsP2 or other

nonstructural proteins may interact with Notch components similar to KSHV viral RTA (Lan 2006). Furthermore, the putative interaction of the intracellular domain of Notch with SV nsP2 or other nonstructural proteins may down regulate host immune modulating factors, as is the case in KSHV infected cells (Chang 2006).

Immune related processes:

JAK-STAT pathway related genes increased in expression in cells at 5dpi:

TEPII which is constitutively expressed in JAK-STAT gain-of-function mutants (Lagueux 2000) and in hemocytes expressing activated Ras (Asha 2003) is up regulated in SV-infected cells at 5dpi, during establishment of persistence (*Table4, Figure4*). Fps85D, implicated in JAK-STAT and JNK pathway (Jasper 2001) is also up regulated (*Table4*). Together with the results of *in vivo* studies (Dostert 2004), this may suggest a role for the JAK-STAT pathway in virus-vector co-existence. Product encoded by *svr* important during melanization is increased in expression in 5dpi (*Table4*). Factors involved in Melanization or prophenol oxidization increased in abundance upon E.coli treatment of hemocytes of *A. aegypti* (Bartholomay 2004), E.coli infection of S2 cells (Kallio 2005) and upregulation of *svr* here might indicate hemocyte-like behavior of S2 cells and may contribute to protect the cell upon SV infection.

Cytoskeleton, Ion Channels and membrane trafficking-related:

Host Factors involved in Vesicle formation and maturation Increased at 5dpi:

Table6 shows factors involved in intracellular transport and vesicle formation that potentially mediate the Intracellular changes observed in SV- infected insect cells (Miller and Brown 1992, Mudiganti 2006). Strikingly, many of the genes up regulated in this category are involved in vesicle formation, ER-Golgi transport or exocytosis; the secretion-like activities needed to accumulate and release SV virions from insect cells (Miller and Brown 1992). We speculate that *csp,* which encodes a component of secretory vesicles (Heckmann 1997) interacts with the product of *syx13* (Nie 1999) to regulate release of the virus and that products of *syx13* and *unc-13* interact to mediate vesicle fusion events (Madison 2005) that ultimately release the virus from the infected cell. The gene product of *syx13* may function in maturation and fusion of vesicles containing SV (Hirling 2000, Collins 2002).

ank that encodes Ankyrin may contribute to formation of spectrosome (Deng 1999), a cytoplasmic organelle in differentiated cells. Or, it might indicate similarities between spectrosome and the vesicles are associated with viral structural and nonstructural proteins in SV-infected *Mosquito* (Miller and Brown 1993) and *Drosophila* cells (Mudiganti 2006). Ank may also function to interact with receptors (Jefford 2000) and / or Sodium channels (Malhotra 2000) to influence the cytoskeletal changes that bring about changes in cell fate to establish SV persistence. Either of these two functions of *ank* may indeed be functional during establishment of persistence. The gene *plap* codes for a protein that interacts with Bam (flybase), an important component of fusome, and is significantly increased in expression (~4-fold) in SV infected cells. Increased levels of *ank* and a potential component of Fusome might function to form an asymmetric mitotic spindle to accomplish asymmetric distribution of viral proteins and / or the signaling components, including notch components (Morgan 1996). This asymmetric distribution of viral proteins may explain presence of viral proteins in only some of the cells in the Mosquito cell cultures (Miller and Brown 1993). As spectrosome and fusome are generally found in well-differentiated organs such as germline cells destined to form oocytes, upregulation of components forming these cytoplasmic entities indicates intracellular changes similar to process of differentiation, at 5dpi, in insect cells.

rab1,rab8 encode the small GTPases crucial for transport from ER to Golgi (Peter 1994). Functions of Rab1 may be essential for fusion of vesicles carrying SV structural proteins from ER to Golgi, similar to the function of this protein during transport of VSV glycoproteins (Pind 1994, Tisdale 1992). Proteins encoded by *rab8* may function to transport SV structural glycoproteins from the Trans-Golgi-Network to Plasma Membrane (Huber 1993) similar to VSV glycoproteins in polarized epithelial cells (Ang 2003). *NPC1* (Nieman-Pick Disease1) (*Table6*) is increased also in midguts of *A. aegypti* upon SV infection particularly on 4dpi (Sanders 2005). *Cytochrome P450* is upregulated during septic injury (De Gregorio 2002) and 24 hr after blood meal in *A. aegypti* midguts (Sanders 2003) and decreased 24 hr post blood meal in A. gambiae (Marinotti 2005). It is, also, increased in expression here (*Table5*).

Cell cycle and Apoptosis regulators:

Increased expression of *pten* (*Table5*) may play a role in cell growth cell cycle changes seen in PI cells (Karpf 1997a) (Huang 1999). Independent functions of *bifocal*, *pp1-87b* and the interactions between these proteins could function to link the changes in mitotic divisions to cytoskeleton, similar to their functions in Drosophila eye (Babu 2005, Helps 2001).

Chitin interacting proteins, Dicer-2, of RNAi pathway and Cyclin G2 are increased in abundance in a previous study with mature *A. aegypti* (Sanders 2005) and are not identified here. Notch and Ras pathway components are identified in our study and not in the previous *in vivo* study (Sanders 2005). These differences in identification of response to SV infection may partly due to *in vivo* or *in vitro* systems that are chosen to perform the study. These differences can further be explained by two factors. First, arrays printed with 2170 ESTs of *A. aegypti* may not include some of the genes identified here using Affymetrix genechip containing probesets corresponding to 14010 genes from completed *Drosophila* genome sequences. Second, some of the genes identified in the *A. aegypti* study may represent a specific response of midgut to SV infection. As the S2 cells used in this study are considered to be hemocytes some of the genes identified here may represent response of specific insect tissues. As membrane trafficking components and JNK components are upregulated in both the in vivo study and the present study, S2 cells seem to reflect the *in vivo* changes occurring upon SV infection in the natural host *A.aegypti.*

Concluding remarks:

Viral persistence represents a complex interaction of virus and host wherein innate immune responses are suppressed to some extent allowing viral replication and specific pathways important to regulate co-existence with virus are activated. The development of infection in insect cells results in suppression of cell death and a proliferation of membranous vesicles and the apparatus that moves them and causes them to fuse with other membranes. The progression of the infection into persistence is accompanied by an ability of the cell to control, suppress and coexist with the virus. Our studies have identified host cell components that may play a role in these events. For example the

immune pathways JNK, JAK-STAT and the cell-fate determining Notch/Wnt/Ras components that can influence cell proliferation and apoptosis are identified to be up regulated in cells that are establishing persistence. Imd or Toll pathway components were not significantly altered suggesting that this portion of immune pathway may not be stimulated by the 5dpi time-point selected here. This is supported by the finding that the Toll pathway component aedif is upregulated on 1dpi in *A. aegypti* infected with SV with no significant change at 4dpi (Sanders 2005). Our study revealed a Notch/Wnt/Ras signaling network in cells destined to enter viral persistence, which may have been masked by signals from other tissues or organs in the previous *in vivo* studies (Sanders 2005). In contrast to the *in vivo* study (Sanders 2005), several of the factors identified in our study influence cell cycle and cell growth changes explaining the changes in same seen in PI insect cell cultures (Karpf 1997). JNK and JAK-STAT signaling are involved in virus-host interactions involving the plus-sense RNA containing viruses as indicated by our study, and by previous studies of Drosophila C Virus and *Drosophila* (Dostert 2004), Tospovirus and WFT (Medeiros 2004). Minus-sense RNA containing Influenza A virus and S. pneumoniae also induce the JAK-STAT pathway (Zhang 2006), and Notch/Wnt signaling indicating that JNK and JAK-STAT mediated responses are involved generally in ssRNA virus-host interactions. Complement related secretory proteins TEPs and Mcr are induced by Tospovirus infection in WFT (Medeiros 2004), fungal infection in S2 cells (Stroschein-Stevenson 2005), E.coli infection in S2 cells (Kallio 2005) and bacterial infection of *Drosophila.* It is interesting to note that expression of complement is increased after 1dpi and continues up to 10dpi in Tospovirus infected WFT (Medeiros 2004) indicating that TEPII identified as upregulated in our study may be important for maintenance of SV persistence in insect cells. One of the exciting findings of this study is identification of activities common in establishment of persistence in two distantly related animal viruses, Alphavirus and Herpesvirus, involving the Notch signaling mechanism. Although there are some differences in the particular genes identified in this study compared to those identified in response to SV infection or bacterial infections in previous studies, striking similarities in activation of specific pathways should improve our understanding of how insects survive microbial infections and maintain them as persistent infections. Further studies of this type will help us

understand the particular mechanisms involved in this transcriptional reprogramming leading to persistence of Alphavirus in the insect host.

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Fig 2.1: Genespring analysis to identify differentially expressed genes in SV-infected S2 cells, at 5dpi. Samples 1,2,3 are Mock infected and 4,5 are infected samples at 5dpi. Graph shows the genes selected from 881 T-test results to show 1.5 fold or more difference in expression levels.

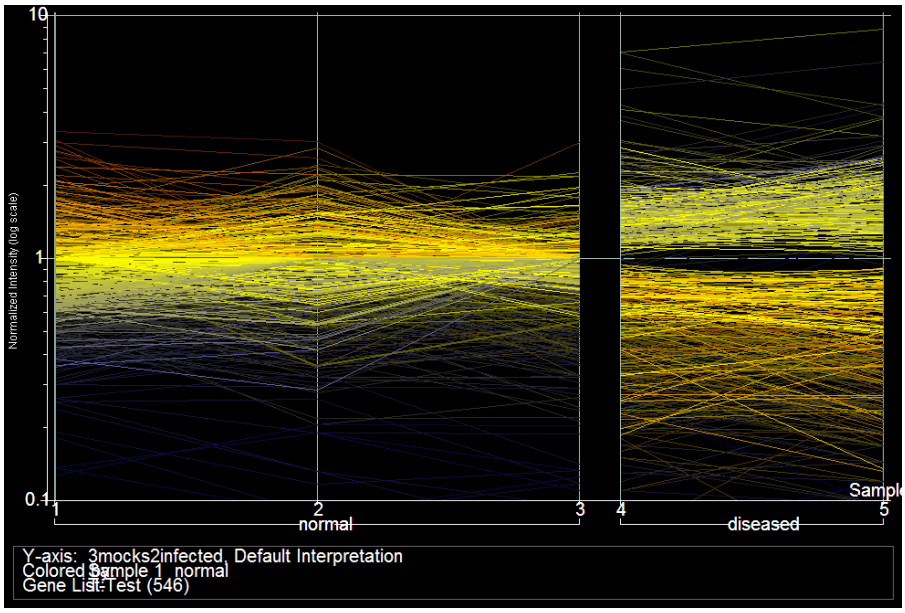


Fig 2.2: Genes up or down-regulated identified in this study, using Genespring 7.2.

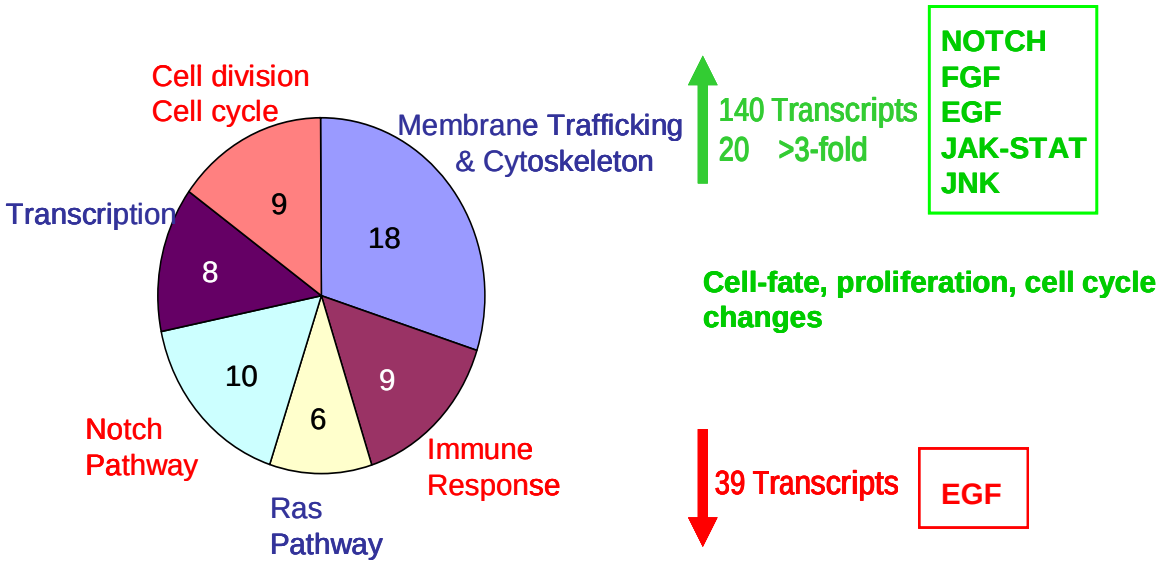
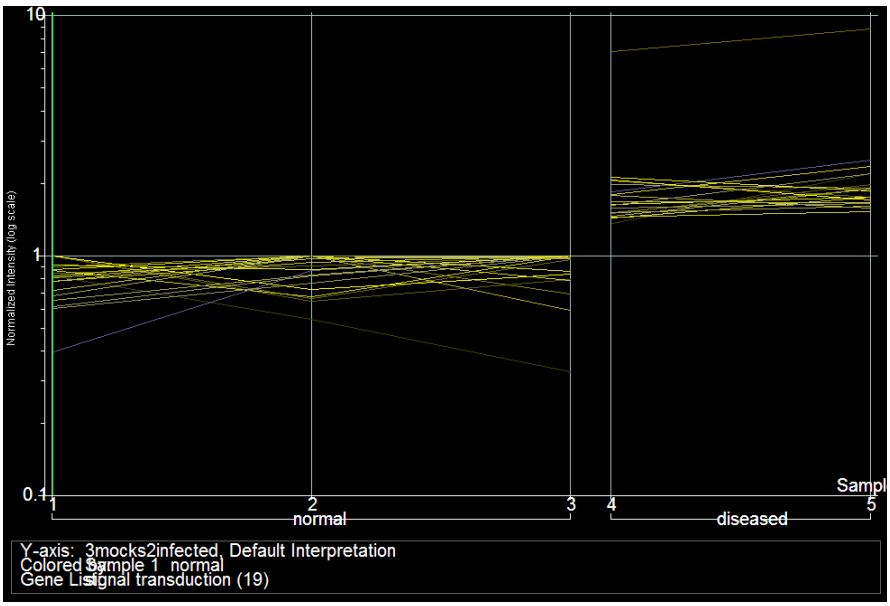


Fig 2.3: Increased levels of genes involved in a) signal transduction processes b) Cytoskeleton and membrane trafficking.

a)



b)

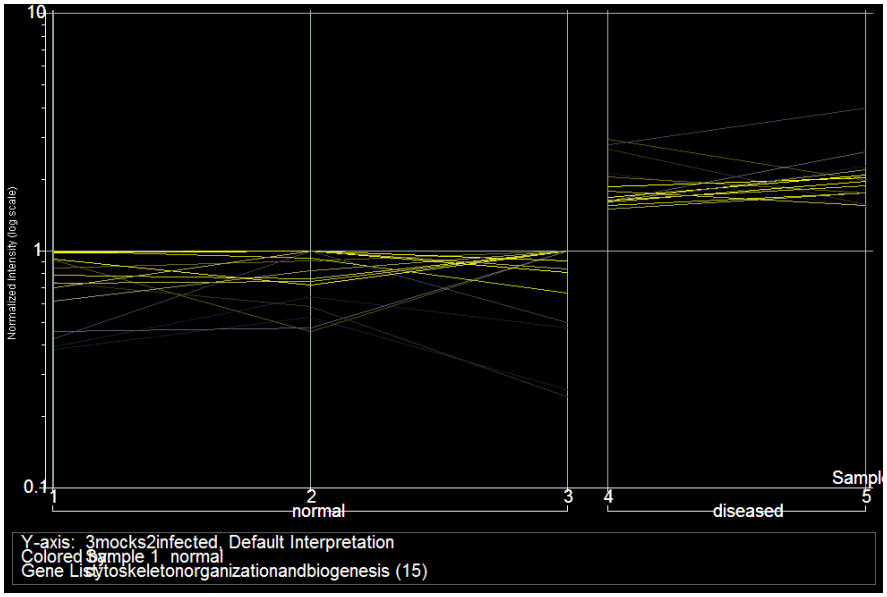


Table 2.1: GENESPRING 7.2 T-test ANALYSIS RESULTS. Number of genes increased, decreased at different significance levels. Complete lists with corresponding gene ontologies can be found in supplementary data.

Category	Number of genes
Filtered with T-test. *p*-value <0.05	881
T-test results. *p*- value <0.01	145
Increase at *p*- value <0.05	140 (Listed in Appendix)
>3 fold increase	20
Bonferroni corrected (2-3 fold increase)	20
Decrease at *p*-value<0.05	39 (Listed in Appendix)

Figure 2: Notch signaling components increased in expression at 5dpi in *Drosophila* S2 cells infected with Sindbis.

Gene ID	Gene product	Function	Fold Change Increase	*p*-value	Identities with *A. gambiae* proteins	*A. gambiae* Gene ID	Identities with *A. aegypti* proteins	*A. aegypti* Gene ID
CG11988	Neur	Ubiquitination of ligand of Notch, Delta	2	>0.05	50%	ENSANGP00000007854		
CG3779	Numb	Interaction with Notch	2	0.02	61%	ENSANGP00000006150		
CG7555	Nedd4	Ubiquitination of Notch and endocytosis	2	0.038	62%	ENSANGP00000025653		
CG10653	Hook	Endocytosis of Delta	1.5	0.02	53%	ENSANGP00000016709		
CG1725	Dlg1	Tumor suppressor interacting with raps	2.5-3.5	0.027	77%	ENSANGP00000012487		
CG5692	Raps	Partner of Inscuteable	>3	0.0003	65%	ENSANGP00000019406	69%	EAT33528
CG13281	Cas	Nuclear export receptor protein	1.5-2	0.0006	63%	ENSANGP00000018477	64%	EAT39461(ranBP)
CG8532	Lqf	Required in signal donor / receptor cell	1.5-2	>0.05	36%	ENSANGP00000002840	66%	EAT39510
CG7467	Osa	Chromatin remodeling to repress *ac/sc*	1.5-2	0.026	47%	ENSANGP00000008445		
CG5203	CHIP	Required for maximal enhancer activity	1.5-2	>0.05	60%	ENSANGP00000020684	62%	EAT36891(Lim BP)

Table 2.3: Genes involved in Wnt or Ras signaling up or down-regulated in SV-infected S2 cells, at 5dpi.

Gene ID	Gene Name	Function	Fold change	*p*-value
Increased:				
CG12019	*cdc37*	Chaperone, cell cycle	1.5-2	0.034
CG14217	*tao-1*	Influences FGF pathway	1.5-2	0.015
CG10072	*sugarless*	Important for Wnt & FGF signaling	2.5	0.014
CG4041		Belongs to Ras family GTPases	2	0.0007
CG6721	*Gap1*	Negative regulation of Ras activity	4	>0.05
Decreased:				
CG11144	*glu-RA*	G-protein coupled receptor activity	2	0.0467
CG9097	*bab1*	Repressed by EGFR signaling	2	0.025
CG11186	*toy*	Inhibitor of EGF pathway	2	0.0179

Table 2.4:
Immune-related genes upregulated during at 5dpi in SV-infected Drosophila S2 cells.

Gene ID	Gene name	Function	Fold change	*p*-value
Defense response:				
CG7668		Predicted receptor, defense response	2.5	0.002
CG18503	*svr*	Melanization	>3	0.018
CG8896	*18w*	Embryonic morphogenesis, receptor activity	2	0.045
CG6395	*csp*	Synaptic vescicle, exocytosis, chaperone	1.5-2	0.042
CG15573	*femcoat*	Insect chorion formation	1.5-2	0.004
CG6895	*Fps85D*	JAK-STAT, Predicted defense response,	1.5-2	0.023
CG7747		Predicted defense response, protein folding	4-6	0.031
CG3373	*hmu*	Mucinous layer on surface of parasitoids	1.5-2	0.045
Complement-related:				
CG7052	*TEP II*	Wide-spectrum protease inhibitor, opsonization, influences JAK-STAT	3.4	Dot Blot

Table 2.5: GENES UPREGULATED IN DROSOPHILA S2 CELLS UPON SINDBIS VIRUS INFECTION AT 5DPI.

Gene ID	***Gene Name***	**Function**	**Fold Change**	***p*- value**
Cell cycle /cell division:				
CG8203	*cdk5*	Synaptic vescicle secretion, cell cycle	2	0.0365
CG5692	*rapsynoid*	Mitotic spindle orientation	3	0.0003
CG8374	*dalmatian*	Nervous system development	2-3	0.001
CG3443	*pecanex*	Nervous system dev and differentiation	2-3	0.005
CG5553	*DNAprim*	DNA primase, successful S phase	>3	
CG1403	*sep1*	Cytokinesis and cell cycle	1.5-2	0.03
CG7749	*fat2*	Negative regulation of cell growth	1.5-2	0.016
CG5650	*pp1-87B*	Necessary for mitosis and chromosome condensation	1.5-2	0.049
CG1822	*bifocal*	Interacts with pp1, actin binding, morphogenesis	1.5-2	0.038
Transcription:				
CG6711	*tafII150*	Hsp70 expression, G2/M transition	2-3	0.0005
CG8815	*sin3A*	Histone deacetylation, transcriptional repression	1.5-2	0.03
CG3458	*top3*	Topoisomerase activity	1.5-2	0.025
CG2679	*gol*	Contains Zinc-finger motif, mesoderm development	2	0.017
CG8625	*iswi*	Component of NURF, Hsp70 expression	2	0.027
CG6539	*dhh1*	RNA helicase activity suggested by seq. similarity	1.5-2	0.036
CG8824	*fdl*	Brain development	1.5-2	0.023
CG10230	*rpn9*	Component of 19S Proteasome regulatory particle	1.5-2	0.035
Ubiquitination:				
CG1782	*uba1*	Ubiquitin activating enzyme	1.5-2	0.047
CG4195	*l(3)73Ah*	Homologous to mammalian oncogenes	1.5-2	0.008
Metabolism:				
CG13927	*GC*	Gamma glutamyl carboxylase	1.5-2	0.038
CG1506	*Ac3*	Adenylate cyclase, potential G-protein signaling	1.5-2	0.033
CG7758	*pumpless*	Glycine catabolism, response to starvation	1.5	0.039
CG11661	*nc73EF*	Probable Succinyl transferring activity, TCA cycle	1.5	0.039
CG10033	*foraging*	cGMP-dependent protein Kinase, larval behaviour	1.5-2	0.015
	SRPK	Alternate splicing , affects viral replication of Hepatitis B virus and Human Immune Deficiency Virus	7	>0.05
Ion Transport:				
CG5594		K-Cl Symporter	2-3	0.013
CG12178	*nhe1*	Na-Hydroden Antiporter	1.5-2	0.006
CG3616	*CytochromeP 450-9c1*	electron transport	1.5-2	0.011

Table 2.6: Genes encoding Cytoskeletal and membrane trafficking factors, upregulated at 5dpi in Sindbis Virus-infected S2 cells.

Gene ID	Gene Name	Function	Fold change	*p*-value
CG5722	*NPC1*	Cholesterol transport, probable hh receptor	1.5-2	0.037
CG1651	*ankyrin*	Actin and, receptor binding, spectrosome	2-3	0.008
CG5105	*plap*	Fusome	>3	0.001
CG8156	*syntaxin13*	Endosomal, excocytosis, vescicle formation	5	0.012
CG3269	*rab1*	Endocytosis	1.5-2	0.012
CG8287	*rab8*	Endocytosis, protein transport	1.5-2	0.037
CG7838	*bub1*	Ser/Thr Kinase, Exit from mitosis	1.5-2	0.042
CG9012	*chc*	Clathrin heavy chain	1.5-2	0.012
CG7210	*peanut*	Cytokinesis, vesicle docking, targeting	2	0.035
CG8266	*sec31*	ER and golgi component, exocytosis	1.5-2	0.012
CG6395	*csp*	Synaptic vesicle exocytosis	2	0.03
CG10653	*hook*	Microtubule binding, endocytosis	1.5-2	0.025
CG3637	*cortactin*	Actin binding, defense response	1.5-2	0.015
CG18734	*furin 2*	Pro-protein processing	1.5	0.046
CG6176	*grip75*	Associates with γ-Tubulin	2	0.012
CG10236	*laminin A*	Mesoderm, heart development, meiosis	1.5-2	0.004
CG2999	*unc-13*	Synaptic vesicle, Calmodulin binding	1.5-2	0.018
CG8240	*rhoGAPp190*	Influences Myosin in neurons	2	0.0042

CHAPTER THREE

ESTABLISHMENT OF PERSISTENCE INVOLVES INHIBITION OF PROTEASE ACTIVITY OF NONSTRUCTURAL POLYPROTEIN

Abstract:

TEP II gene was shown to be upregulated in our initial analysis steps to identify differentially expressed genes in infected cells (5 dpi) using *Affymetrix* MAS (Microarray Suite 5.0) software. *TEP II* codes for a wide-spectrum protease inhibitor that belongs to super family of proteins containing other members like human complement and α-Macroglobulin proteins (AMCOMs). Though the *TEP II* gene showed increase in expression, using Genespring 7.2, the *p*-values for change in expression of this gene did not meet our filtering criteria (i.e., *p*-value >0.05). As TEP II is an immune-related component with protease inhibitory activity, we focused on this factor to detect its upregulation using Dot blots. We also probed for inhibition of viral Protease activity during early persistence and after superinfection to understand the mechanism of establishment of persistence and superinfection exclusion by Alphaviruses. We identified inhibition of viral Protease activity at 5dpi and upon superinfection of SV-infected cells at 5dpi. The cleavage of polyprotein is required for efficient viral RNA replication and SV production and here we identified that inefficient cleavage of the polyprotein leads to reduced levels of viral RNA leading to controlled virus production seen as persistence is established. We propose that TEP II is involved in inhibition of this Protease activity and is one of the host factors involved in establishment of Alphavirus persistence in insect host. TEP I and TEP II are shown to be involved in encapsulation and opsonization of microbes for phagocytosis (Levashina 2001, Stroschein-Stevenson 2006).

Introduction:

THIOL-ESTER CONTAINING PROTEINS:

Four Thiol-Ester containing Proteins (TEPs) TEP I, II, III, IV are identified in genomic clones of Drosophila based on their similarity with human complement C3 and α-Macroglobulin (αM). The Expressed Sequence Tags (ESTs) for all these sequences are available at Berkeley Drosophila Genome Project (BDGP, Howard Hughes Medical Institute EST project). ESTs of some of the alternately spliced products *Drosophila TEP*s are available from Research Genetics (Huntsville, AL).

TEPs are structurally similar to both αM and complement C3 (Lagueux 2000). The classical, alternate and lectin pathways for complement activation result in activation of Mammalian complement C3 that forms covalent bond with microbes to help in its opsonization and phagocytosis. Hence C3 is considered to be the central component of mammalian complement system. αM are wide-spectrum protease Inhibitors and are considered to form the evolutionary precursors of complement C3 (Dodds 1998). Protease inhibitors related to αM and components of alternative and lectin pathways are described in Invertebrates (Nonaka 1999), suggesting a role for these complement-related activities during immune responses in Invertebrates.

Common structural features of Invertebrate TEPs and Mammalian AMCOM proteins:

Though the overall sequence similarity between invertebrate TEPs and mammalian TEPs is 22-30%, they share moderately conserved residues in similar relative positions (Lagueux 2000). AMCOMs have conserved residues in similar relative positions in blocks A to L (Henikoff 1994) and these blocks are found also in Drosophila TEPs in relatively similar positions (Lagueux 2000). Block G of vertebrate and Invertebrate TEPs is conserved and contains the thio-ester motif "GCGEQ". Cysteine (C) and Glutamate (Q) residues of this motif form the characteristic intramolecular thio-ester bond of TEPs. Upon activation of C3, the thio-ester is exposed to the surface of protein and Histidine at equivalent position (~100 residues C-terminal of Thio-ester site) attacks the thio-ester bond to release free Cysteine that acts as nucleophile to bind to "-OH" groups (C3) groups on the microbial surface (Gadjeva 1998). The thio-ester Block D of vertebrates is less conserved and same region in TEPs is highly variable and contains the proteolytic cleavage site "RK". This hyper-variable region in αM corresponds to the bait region containing the cleavage sites for a wide variety of Proteases and is important for activation of the protein to clear the Proteases associated with invading microbes (Armstrong 1998, Chu 1994). αM lacks the Histidine residue down-stream of the Thio-ester bond and forms amide bonds with free "-NH_2" groups on proteins (Dodds and Law 1998). This hypervariable region is differentially spliced in TEP II to give 5 alternately spliced products. Considering the structural similarities between TEPs and Complement

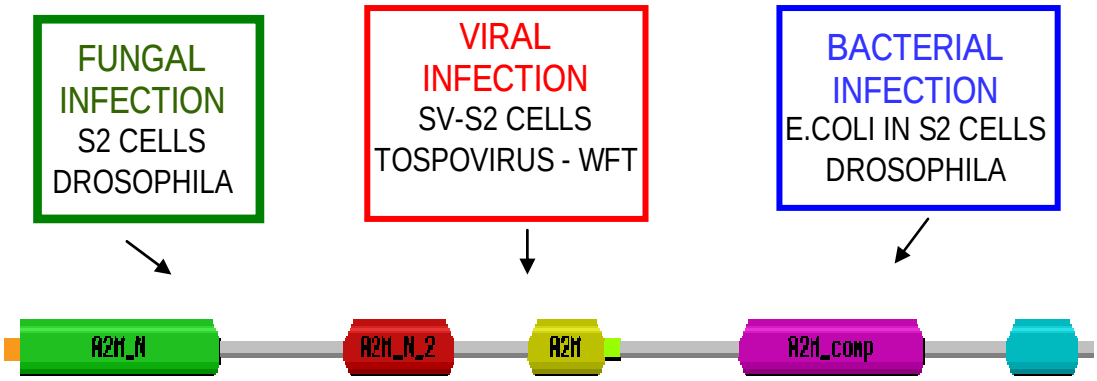
C3 and protease Inhibitor αMs, different functions can be expected for different alternately spliced forms of TEP II.

Drosophila TEPs contain lesser number of Cysteine residues compared to the number of Cysteines in AMCOMs that are distributed throughout the AMCOM proteins in a conserved pattern. All TEPs have hydrophobic signal peptide like sequence near their N-terminus indicating that they can be secreted. TEPI and TEP II have been shown to be important for phagocytosis in cultured cells of *Drosophila* (TEPII- Stroschein-Stevenson 2006) and *A. gambiae* (*TEP I* - Levashina 2001) (Fig. 1). All TEPs contain potential N-glycosylation sites. The calculated Molecular Mass of the monomeric proteins is ~150 KD.

Anopheles gambiae TEPs (*a*TEPs):

*a*TEP I is cloned using degenerate primers corresponding to highly conserved thio-ester coding sequence of the related complement and other thio-ester containing sequences of *D. melanogaster,* on cDNAs of mosquito cell line 5.1*(Levashina 2001). 5' ESTs corresponding to *A. albopictus* TEPs are available. *a*TEP I contains Histidine down-stream of Thio-ester bond, similar to C3 (Levashina 2001), suggesting binding of this protein with –OH groups of target proteins.

Figure 3.1: TEP II is upregulated during microbial infections in Drosophila flies and Drosophila S2 cells.



Alpha-macroglobulin family domains - green, red, yellow, blue.

PROTEASE INHIBITOR - COVALENTLY BINDS THE TARGET PROTEINS
COMPLEMENT – RELATED

SV NONSTRUCTURAL POLYPROTEINS:

SV has a (plus)-sense, 11,703 nt long RNA genome which has a 5' cap and 3' polyadenylated tail. Upon infection, this genome is tranlated by the host ribosomes to synthesize viral proteins. The first 2/3rd portion of the genome codes for the nonstructural proteins whereas the remaining 1/3rd portion acts as a separate cistron to code for viral capsid and envelope proteins. Four non-structural proteins (nsPs) nsP1, nsP2, nsP3 and nsP4 are initially produced as nonstructural polyproteins nsP123 and nsP1234. The nsP1234 is produced by read-through of Opal codon between the nsP3 and nsP4 sequences. The efficiency of this read-through is 5-20% during *in vitro* translation (de Groot 1990). Intermediate nonstructural polyproteins and individual nsPs are produced by cleavage of the polyproteins by Protease activity in nsP2 region of polyprotein or the individual nsP2. nsP123 and nsP4 are produced from nsP1234 by *cis* cleavage and nsP1 nsP23 and nsP4 are produced by cleavage in *trans* (Li and Rice 1989*)*. During initial stages of infection, the viral replication complexes are formed by the polyproteins that are more efficient in (minus)-RNA synthesis, using the (plus)-RNA viral genome as template. The (minus)-strand RNA acts as a template for synthesis of 49S (plus)-RNA genome of the progeny virus and the 26S subgenomic RNA that codes for the viral structural proteins. Replication complexes formed by intermediate polyproteins are efficient at synthesizing both minus and plus-sense RNA whereas replication complexes formed by individual nonstructural proteins cannot synthesize minus strands.

Another Alphavirus Semliki Forest Virus (SFV) is closely related to SV. There is no Opal codon between nsP3 and nsP4 sequences of SFV and hence only nsP1234 is formed initially after infection. SFV is shown to have a replication strategy that is similar to that of SV, where cleavage of polyprotein is important for efficient virus production (Kim 2004).

CLEAVAGE OF POLYPROTEINS IS REQUIRED FOR EFFICIENT VIRAL RNA SYNTHESIS AND PROGENY VIRUS PRODUCTION:

The first 2/3rd portion of mRNA-like RNA genome of SV codes for the polyproteins produced as nsP1234 and nsP123, which form the replicase complexes that synthesize the viral (minus)-RNA. By 4hpi, minus strand synthesis ceases and the number of minus strands determines the amount of (plus)-RNA and sgRNA formed in the infected cells. At

later stages of infection nsP123 is cleaved to produce nsP1, nsP23, nsP4 that can synthesize minus strands and plus strands. Replicases formed during initial stages of infection are unstable and replicases formed with cleaved nsP1, nsP2, nsP3 and nsP4 in later stages of infection are stable and efficiently synthesize the sgRNA and the progeny (plus)-RNA. Infections of Chicken, Mammalian and Mosquito cell lines with SFV or SV mutants defective in cleavages that produce the individual nonstructural proteins show reduced levels of virus production (Shirako and Strauss 1994, Kim 2004). The virus production is reduced by 10-fold when SV or SFV mutants that are defective for cleavage between nsP1&2 (cleavage site 1) and by 100-fold when mutants are defective for cleavage between both nsP1&2 and nsP2&3.

SV cleavage defective mutants are specifically defective in either plus RNA synthesis or in synthesis of both plus and minus strands. Levels of minus and plus RNA after infection of Chicken or Mammalian BHK-21 cells were measured (Shirako and Strauss 1994, Kim 2004). Levels of (minus)-RNA @ 30ºC in Chicken cells infected with SV mutants defective in cleavage between both cleavage sites 1 and 2 were similar to the levels in cells infected with Wild-Type (WT) virus. The (plus)-RNA levels in mutants defective in both the cleavage sites were reduced @ 30ºC and were absent @ 39ºC.

In mammalian cells, nsP1 is associated with plasma membrane and filopodia, nsP2 is enters nucleus, nsP3 is associated with subcellular vesicle-like structures and nsP4 is diffusely distributed in the cytoplasm. When individual or combinations of nonstructural proteins (i.e., nsP12, nsP23) are expressed in HeLa cells using Adenoviral vectors, the nsP1 containing proteins are found associated with plasma membrane and nsP3 containing proteins are associated with vesicle-like structures. Cleavage defective nsP123 proteins distribute partly to plama membrane and partly to the vacuoles and vesicle-like structures, some of which contained the endosomal markers (Salonen 2003).

Functions of nsP2 and other nsPs:

nsPs code the catalytic, polymerase activities and other functions needed for viral RNA production and efficient virus production from the host cell. nsP1 has Methyltransferase and Guanylyltransferase activities that function in capping of the viral 49S and sgRNA (Mi 1991, Wang 1996). Downstream regions of nsP1 function in the initiation of minus-strand RNA synthesis (Sawicki 1991, Wang 1991) and interact with the nsP4 polymerase

(Fata 2002, Shirako 2000). NsP4 has a GDD-containing sequence motif of RNA-dependent RNA polymerases subunit and is required for efficient viral RNA production. The N half to two-thirds of the nsP3 phosphoprotein is conserved among Alphaviruses and provides essential functions for minus-strand and 26S mRNA syntheses (De 2003, Kaariainen 2002, LaStarza 1994). It also includes a conserved macrohistone 2A-like sequence predicted to be an ADP ribose-1 phosphoesterase (De 2003, Koonin 1992). In several SV mutants, the loss of nsP3 phosphorylation led to loss of minus-strand synthesis (De 2003, LaStarza 1994).

nsP2 is a papain-like thiol-protease with Protease activity coded in the C-terminal domain of the protein is responsible for processing the viral nonstructural polyprotein precursors (de Groot 1990, Ding 1989, Hardy and Strauss 1989, 1990) to form the replicase-transcriptases that are essential for efficient synthesis of progeny viral (plus)-RNA and the sgRNA that forms the viral structural proteins. The nsP2 and nsP2 containing polyproteins function as a proteinase to cleave the nonstructural polyproteins in *cis* and *trans* (de Groot 1990) and SV mutants defective in Proteinase activity of nsP2 did not produce any individual nsPs in *in vitro* translation assay (Shirako and Strauss 1994). In temperature-sensitive (*ts*)-mutants of SV that are defective in the nsP2 protease activity (Sawicki 2006) the initial minus strand replicases were unable to change to replicases that can synthesize plus-strands. The N-terminal region of nsP2 expresses RNA 5′-triphosphatase (Vasiljeva 2000). The nsP2 of SFV is shown to have NTPase and helicase activities (Lain 1990, Rikkonen 1994). The *ts* mutants with defects in nsP2 region are defective in sgRNA synthesis, suggesting that it is involved in initiation of sgRNA synthesis (Sawicki and Sawicki 1985). It encodes a nuclear localization signal and localizes to nucleus.

In this study we identified upregulation of TEP II, a wide-spectrum protease inhibitor in Drosophila and Mosquito U4.4 cells at 5dpi and after superinfection of the infected cells at 5dpi. We also identified inhibition of the viral protease activity suggested by higher amounts of polyproteins during early persistence (at 5dpi) and upon superinfection of the infected cells at 5dpi.

Experimental procedures:

Dot Blot: RNA was isolated from 6×10^6 *Drosophila* S2 cells or *Mosquito* U4.4 cells, using the Trizol method. Cellular and viral mRNA was isolated from total RNA from mock-infected, 4.5 hr post-infection or 5 dpi samples, using the poly (A) isolation kit. 5 µg mRNA derived from equal number of cells in each treatment condition was blotted onto positively charged Genescreen membrane (Perkin Elmer, Boston, MA) using a dot blotter (schleicher-schuell, Florham Park, NJ). Each sample was diluted 1.7 – fold, with total of 8 dilutions for each sample to load into the dot blotter. Hybridization was with a probe made from a TEPIIe (alternately spliced form e) cDNA clone. For this, 100ng linear DNA, NEB Buffer 2, (New England Biolabs, Ipswich, MA) 0.5µl 0.1M DTT were boiled for 5' and quick cooled on ice. For preparation of the probe, 0.5µl 50mg/ml BSA, 2.5µl of random oligomers, 0.5µl each of 20mM dTTP, dCTP, dGTP, 5 µl of α-^{32}P dATP (Perkin Elmer, Boston, MA) and 2µl of Klenow enzyme were added to the denaturing reaction (Final reaction volume 50µl) from previous step and incubated for 4 hr at 37°C. Probe was purified using Sephadex G25 column before using for hybridization at 42°C for 16-20 hr (Hybridization buffer - 5X SSPE, 50% deionized Formamide, 5X Denhardt's Solution, 1% SDS, 10% Dextran Sulphate Na salt). Prior to hybridization membrane was prehybridized for 4 hr at 42°C in the hybridization buffer containing 50µl of 100µg/ml denatured Salmon sperm DNA. The membrane was washed twice in 200 ml of 2X SSPE (20X SSPE - 3M NaCl, 0.2M NaH_2PO_4-H_20, 0.02M EDTA-Na_2) at RT. High stringency washes were done at 65^0 C for 1 hr 30 min, twice in buffer containing 2X SSPE and 2%SDS. Low-stringency washes were done twice in 200ml 0.1X SSPE at RT, for 30 min. Autoradiogram was obtained by exposing the membrane to Kodak BioMax XAR film (Source One, Jacksonville, FL) film for 72 hrs.

Western Blot with anti-nsP1 and anti-nsP2 Antibodies:

$1X10^7$ S2 cells were infected with Sindbis Virus Heat Resistant (SVHR) at 100 MOI. To detect the levels of polyproteins in persistently infected cells, the S2 cells were superinfected on 5dpi, at 100 MOI. Cell lysates were prepared from S2 cells Mock-infected, 1.5, 4 hrs after infection, at 5dpi after initial infection and 1.5, 4 hrs after superinfection on 5dpi. Mock infections were done with PBS-D containing 3% FBS (FBS used to grow the S2 cells). All the superinfections were at 100 MOI with SVHR. Cell

pellets were obtained by centrifugation at 1200 rpm, in desktop centrifuge and lysed in 400 ul ice-cold TNT lysis buffer (0.02M Tris pH-7.5, 0.5% NP-40, 150 mM NaCl) containing 0.2mM PMSF, 0.2 mM PMSF). Lysates were incubated at room temperature for 5min and centrifuged at 10K rpm and the supernatants were used for Western blot with Anti-nsP2 antibody. For Western blot with Anti-nsP1 antibody, samples were obtained from Mock-infected S2 cells, 1.5 hrs after infection or superinfection and processed in similar fashion, as described above.

For the western blots, 20µl of the supernatant was mixed with 5ul of 5X sample buffer and run on a 10.8% gel and transfered onto a PVDF (Sequiblot 0.2µ membrane for protein sequencing from Bio-rad) membrane. The blots are incubated in the blocking solution (1X PBS- 70ml, Non-fat dry milk-10g, Goat serum-1ml, make upto 100 ml with 1X PBS) for 1hr, incubated in incubation solution (1X PBS-150 ml, Tween-2-100µl, Non-fat dry milk-20g, Goat serum -2ml, make up to 200ml with 1X PBS) with anti-nsP1 or anti-nsP2 antibody for 1 hr, washed 3 times in washing solution (1X PBS-70ml, Tween-20-100µl, Goat serum-2ml, make upto 200ml) for 5 min each time and then incubated with the I-125 labeled Iodinated anti-rabbit IgG antibody for 1 hr. All incubations were at RT. The blots are washed 3 times in the washing solution and air-dried and exposed to Kodak XAR film for 3-4 days.

Immunoprecipitation using Anti-nsP1 or Anti-nsP2 Antibody:

7 X 10^6 *Drosophila* S2 or *Mosquito* U4.4 cells in a 25-cm^2 flask were infected at 100 MOI with SVHR and superinfected on 5dpi at 1000 MOI. To compare the levels of SV nonstructural polyproteins after infection and superinfection, infections were also done at 1000 MOI. Samples for immunoprecipitations were prepared from cells Mock-infected, 4 hr after infection, superinfection, 5 days after initial infection. Lysates were prepared on ice, using 150 ul of TNT lysis buffer (10mM Tris pH 7.4, 1% NP-40, 150 mM, NaCl, 0.2 mM PMSF) at 4ºC and the samples were incubated for 5 min at RT.

For immunoprecipitations, the lysates were incubated for 20-24 hrs, at 4ºC, with Protein A sepharose beads. The lysates were then incubated with 2µl of primary antibody (Anti-nsP1 or Anti-nsP2), for 36 hrs, at 4ºC. The proteins bound to antibodies in the lysate were separated by incubating the lysates by incubating with a fresh batch of Protein A sepharose beads for 2 hrs, at 4ºC. The proteins bound to Sepharose beads were collected

by spinning the beads + lysate at maximum speed in a tabletop centrifuge. For running the gels, the beads were boiled with loading buffer for 3-5 min and spun for 2 min at maximum speed in a tabletop centrifuge.

Results:

TEP II transcript levels increase upon SV infection:

SV-infected *Drosophila* S2 and *Mosquito* U4.4 cells completely establish infection by day 5 post-infection. Infectious center assays showed that all the cells in the *Mosquito* cultures produce virus by this time-point. These cells produce reduced levels of SV, corresponding to persistent infections, by this time point (Chapter I, Figure 2). *TEP II* levels increased in the 5dpi samples as shown by signal intensities corresponding to two of the Probesets corresponding to this gene during basic analysis using Affymetrix MAS 5.0. The corresponding p-values were more than 0.05 during analysis with Genespring 7.2. As this is a immune-related factor belonging to AMCOM (Alpha Macroglobulin Complement-related) family of proteins (Fig 2), expression levels of TEP II were confirmed by Dot Blot. Comparative levels of TEP II in Mock-infected, 4.5 hpi, 5 dpi, 4.5 hpi after superinfection on 5 dpi were obtained. Probe prepared from Drosophila *TEP II* cDNA clone was used to probe for *TEP II* homologues in Mosquito U4.4 cells. The levels of TEP II increased about 3-fold at 5 dpi and after superinfection with SV (Fig 1a). The levels at 4.5 hpi were similar to the levels of *TEP II* in Mock-infected samples (Fig 1a), indicating that it's functions are important during establishment of persistence. Though there was a high background in the U4.4 blots, the levels of *TEP II* related sequences appeared to increase at 5dpi and 4.5 hpi after superinfection (Fig 1b). In Drosophila, the levels of TEP I and II increased at 6 hpi after infection with E. coli (Lagueux 2001). Here, TEP II levels were not increased at 4.5 hpi after SV infection (Fig 1a), indicating its specific function during establishment of persistent infection.

Hypothesis – TEP II binds to Protease sequence in SV nonstructural Polyprotein and prevents cleavage of the Polyprotein:

TEP II levels increased in general upon bacterial and fungal infections in *Drosophila* flies and S2 cells (Fig 2) and reported to be important for opsonization and phagocytosis of bacteria and fungi. TEP I has been shown to be important for phagocytosis of bacteria by

cells derived from *A. gambiae*. Invertebrate α-Macroglobulins act as inhibitors of proteases associated with microbial infections (Armstrong 1998). There are four sequences coding for TEP I to IV in Drosophila and are 12 potential TEPs in Mosquito genome (Christophides 2002). Expression of multiple similar proteins in Drosophila suggests functional diversity of different members of the TEPs. Further, TEP II is expressed in 5 alternately spliced forms that may be involved in complement-related or protease inhibitory functions in SV-infected insect cells. A recently obtained X-ray crystal structure of Protease domain of Venezuelan equine encephalitis alphavirus (VEEV) nsP2 protease showed two serine residues (at positions 511, 534) positioned on surface of the protein (Russo 2006) that could provide the –OH groups to bind to TEP II. The acidic (Aspartic Acid – 507, 527, 548, 675, 726, 765; Glutamic Acid – 730, 756), or, basic (Lysine -480, Arginine-604) residues on the surface of nsP2 (Russo 2006) could provide the –OH or $–NH_2$ groups respectively, to bind to TEP II. To test the hypothesis that TEP II is involved in inhibition of Protease activity on SV non-structural polyproteins (Fig 3), protease inhibitory functions on SV protease in persistently infected *Drosophila* S2 and *Mosquito* U4.4 cells were probed using Western blots and immunoprecipitations using different anti-nsP antibodies.

Western Blots show inhibition of Protease activity during SV persistence in insect cells:

Sindbis Viral Replicase-Transcriptase complexes preferentially synthesize different viral RNA species in the infected cell, depending on the constituting nonstructural polyproteins or cleaved products. Replicase complexes containing nsP123 and nsP4 efficiently synthesize the viral (minus)-RNA, using the parental (plus)-RNA that enters the cell during infection, as template. During later stages of infection, nsP2 Protease cleaves the polyprotein intially between nsP1 and nsP2 and the derived cleavage products produce the (plus)-RNA of progeny virus and the sgRNA. Inhibition of Protease activity during early persistence was probed by superinfecting the *Drosophila* S2 cells at 5dpi. Western blots using Anti-nsP1 and Anti-nsP2 antibodies showed higher levels of nsP123 in cells at 5dpi, compared to the infected cells that were infected at identical MOI. These levels further increased upon superinfection on 5dpi. These results suggest that the polyprotein nsP123 is cleaved by 4.5 hpi and this protease activity is inhibited by day 5 after infection resulting in detection of the accumulated nsP123 in 5dpi sample. When we

introduced more viral RNA by superinfecting the infected cells, the protease inhibitor active in the infected cells by day 5 after infection, inhibits protease activity on the newly made nsP123. This leads to even higher amounts of nsP123 in the superinfected cells, compared to the 5dpi cells, as detected in Western Blots using anti-nsP2 antibody (Fig. 4b).

Immunoprecipitations show inhibition of Protease activity in U4.4 and S2 cells, during establishment of SV persistence:

Drosophila S2 and *Mosquito* U4.4 cells were infected or superinfected on 5dpi, at 1000 MOI. Immunoprecipitations from S2 samples were done using anti-nsP1 antibody and the immunoprecipitations from U4.4 cells were done using both anti-nsP1 and anti-nsP2 antibody. The results from these experiments are shown in fig.5. The levels of nsP123 (200K) in 5dpi samples were higher compared to infected samples at 1.5 and 4 hpi. These levels further increased upon superinfection. The level of nsP123 in the superinfected samples from S2 (Fig 5b) and U4.4 (Fig 5a) was higher compared to the negligible amounts of polyprotein in the samples infected at same MOI at 4hpi.

In samples from S2 cells, the intermediate polyproteins nsP12 and nsP1 were seen at 4 hpi by immunoprecipitations with Anti-nsP1 antibody (Fig 5b). The nsP123 was not seen at 4 hpi compared to high amounts of nsP123 in the superinfected samples, at 4 hpi on day 5 post-infection. The cleavage product nsP1 was seen in the infected sample and not in the superinfected sample (Fig 5b). These results are similar to those obtained in the Western Blots using Anti-nsP1 and Anti-nsP2 antibodies i.e., the superinfected samples showed high levels of nsP123 compared to negligible amounts at 4 hpi (Fig 4). Immunoprecipitations of S2 samples showed the cleavage products also at 4 hpi, compared to inhibition of the same in superinfected samples.

The above results show that nsP2 protease activity is inhibited as SV during establishment of SV persistence in insect cells, as indicated by higher levels of nsP123 at 5dpi and upon superinfection at 5dpi. As the replicases formed by nsP123 are needed for minus-strand synthesis, these results showed continuous minus-strand syntheis during SV persistence. Infections of mammalian cells with nsP2-cleavage defective mutant SV replicons established persistent infections (Sawicki 2006). These persistently infecting replicons also showed continuous minus strand synthesis in mammalian cells, indicating

that continuous synthesis of minus strands is required for maintenance of SV persistence in both mammalian and insect systems. Further, continuous synthesis of nsP1234 and nsP123 was required to maintain continuous synthesis of minus strands in mammalian cells infected with SV replicons that establish persistent infections (Sawicki 2006).

Increased levels of TEP II, a wide-spectrum Protease Inhibitor (see introduction) at 5dpi and after superinfection with SVHR coupled with inhibition of cleavage of P123 seen at 5dpi and after superinfection suggest involvement of TEP II in inhibition of polyprotein cleavage. TEP II activity can explain a mechanism for establishment of SV persistence and superinfection exclusion by a homologous virus as TEP II would inhibit the cleavage of polyprotein nsP123 from a homologous virus.

nsP2 is involved in induction of apoptotic host responses in vertebrate host and replicons and mutant SV and SV replicons establish persistence in mammalian cells (Frolov 1999). We propose that upregulated TEP II in invertebrate hosts binds to nsP2 and inhibits the apoptosis inducing activities. This may contribute to the absence of apoptosis and cell survival seen in invertebrate hosts in contrast to cell death by apoptosis in vertebrate hosts after Alphavirus infection (Karpf 1998).

Discussion:

Regulation of the amounts of (minus)-RNA is an important step to regulate the total viral RNA production and viral progeny in Alphaviruses. Regulation in these initial stages would be economically advantageous for the virus as (minus)-RNA is the first viral RNA species produced during infection cycle. Regulation of cleavage of the nsPs would be a possible way to achieve this. It was previously proposed that persistent infections showing controlled virus production are maintained because the newly made nsP123 from one infection is immediately cleaved by residual nsP2 from preceding infection cycles (Karpf 1997a, b, Kim 2004). The present study confirms that inhibition of cleavage of nsP123 is involved in maintenance of viral persistent infections, which is in contrast to this previous model proposing immediate cleavage of polyprotein by residual nsP2. nsP123 can cleave in *trans* between nsP1 and nsP2 sequences to produce plus-strand replicase (Li and Rice 1989). This can account for both the minus and plus-strand replicases in the persistently infected cells to produce sufficient amounts of virus to maintain viral infection. We propose that further cleavage of the polyprotein is prevented

by TEP II activity, the plus and sgRNA cannot be produced efficiently in these cells and this would result in controlling the levels of virus as persistence is established. Synthesis of polyproteins are associated with membranes in vesicle-like structures in mammalian cells (see introduction) SV nsPs and structural proteins are associated with membranes of vesicles formed after SV infection in cells derived from *Mosquito*. Higher levels of nsP123 found at 5dpi as identified here might be associated with vesicular membranes in persistently infected insect cells. Increased levels of membrane-related and cytoskeletal factors on 5dpi (discussed chapter II of this report) might aid in this association of replicases to cell membranes.

Inhibition of cleavage of nsP123 would explain superinfection exclusion seen in *Alphavirus* infections. As TEP II is a wide-spectrum Protease inhibitor it can bind to nsP2 region of nsP123 of a homologous virus and inhibit the cleavage leading to reduction in viral RNA levels and virus production in invertebrate cells persistently infected with SV (Karpf 1997b).

Further, SV replicons and viruses with mutations in nsP2 region establish persistence in mammalian cells, a cell fate opposite to apoptosis seen in these cells 24-48 hrs post-infection with SV. (minus)-RNA synthesis ceases at 4-5 hpi in mammalian cells infected with Sindbis replicons expressing wild-type SVHR. SV replicons harboring mutations in nsP2 region show continuous (minus)-RNA production (Sawicki 2006). A mechanism similar to this seems to be involved in establishment of persistence established by WT SV in invertebrate systems.

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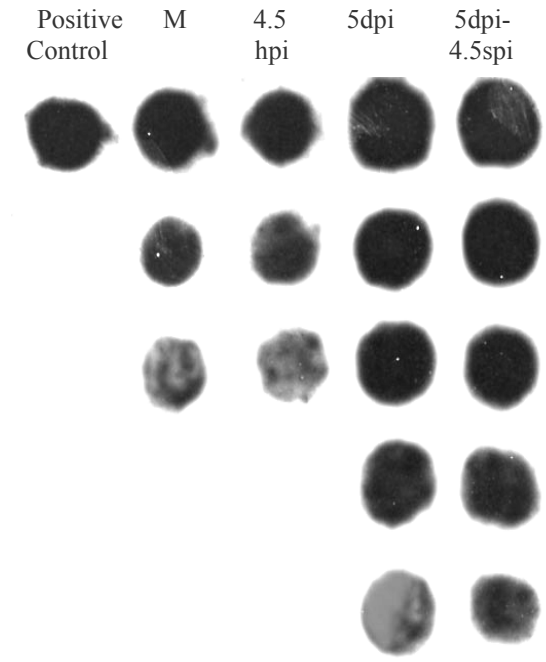
Figure 3. 2: Levels of *TEP II* mRNA in Mock infected and SV-infected cells in a) *Drosophila* S2 cells and b) *Mosquito* U4.4 cells. Levels *TEP II* (S2) or it's mosquito homolog increase at 5dpi and 4.5 hpi after superinfection with SV in both cell lines.

2 a)



2 b)

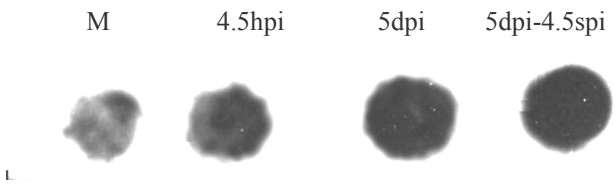


Figure 3.3: Nonstructural polyproteins and cleavage products produced during SV replication cycle in infected cells. TEP II may inhibit the cleavage of polyprotein to affect efficiency of viral RNA production, thus controlling the SV production as viral persistence is established in insect cells.

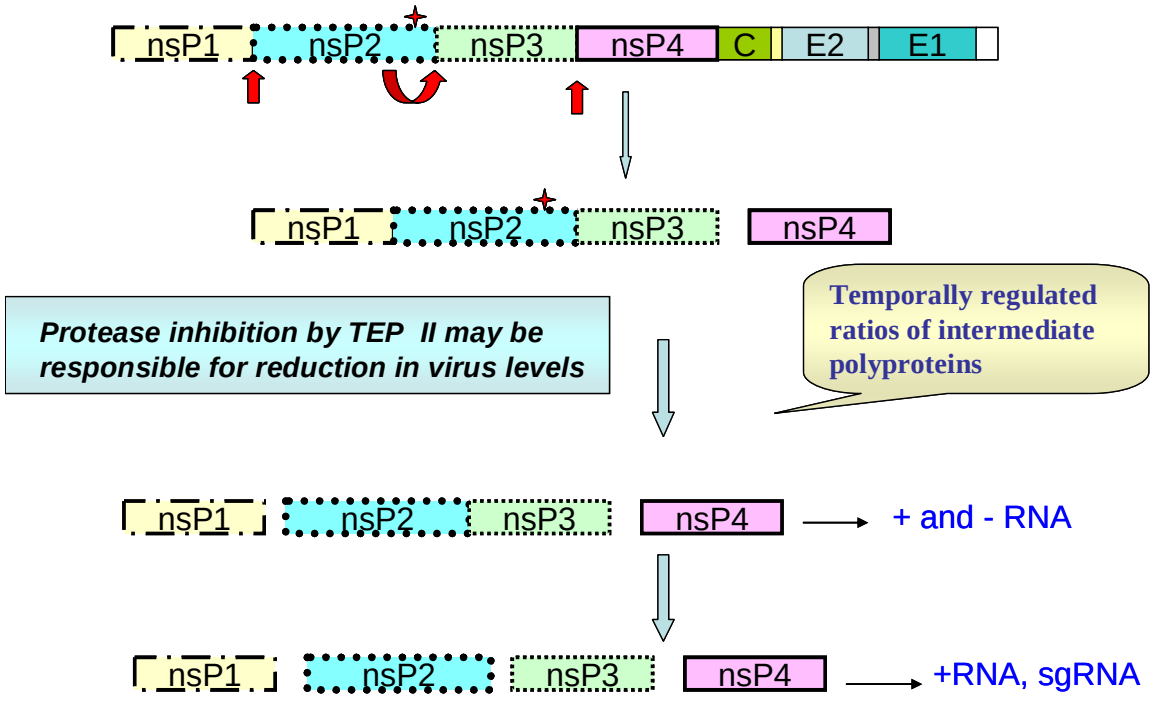


Figure 3.4: Western Blot analysis to identify nonstructural polyproteins and cleavage products to identify inhibition of protease activity. Lysates prepared from Mock-infected, 1.5 and 4 hpi, 1.5 and 4hpi after superinfection (si) with SV at 5 dpi after initial infection and at 5dpi are analysed using a) anti-nsP1; b) and anti-nsP2 antibodies. Western blot is done as described in 'Experimental procedures'.

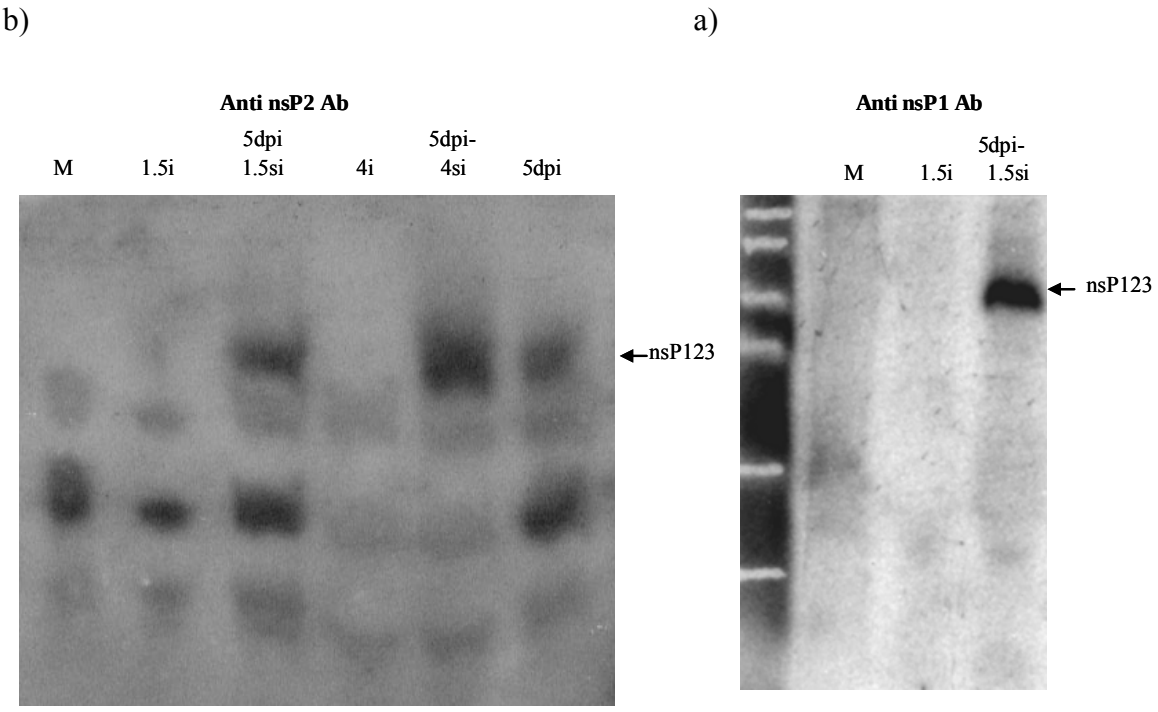
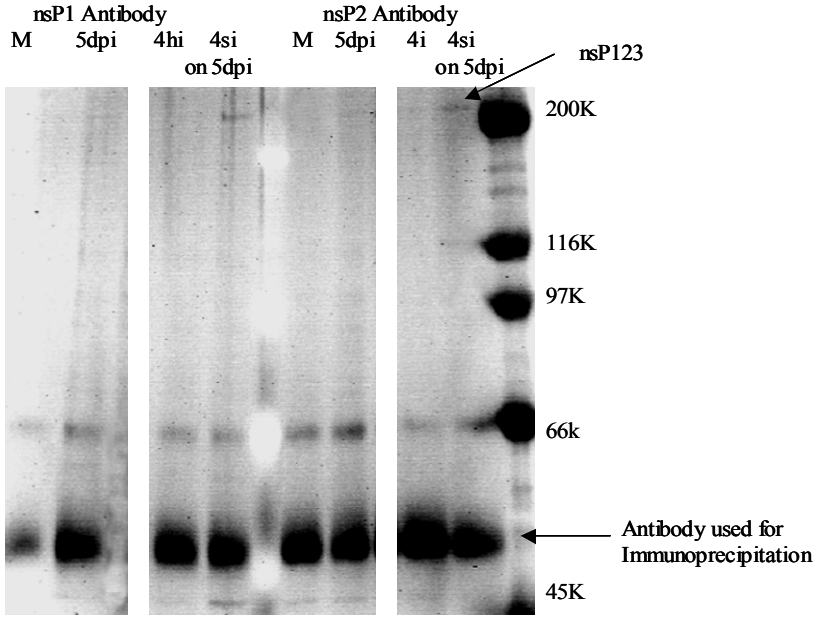
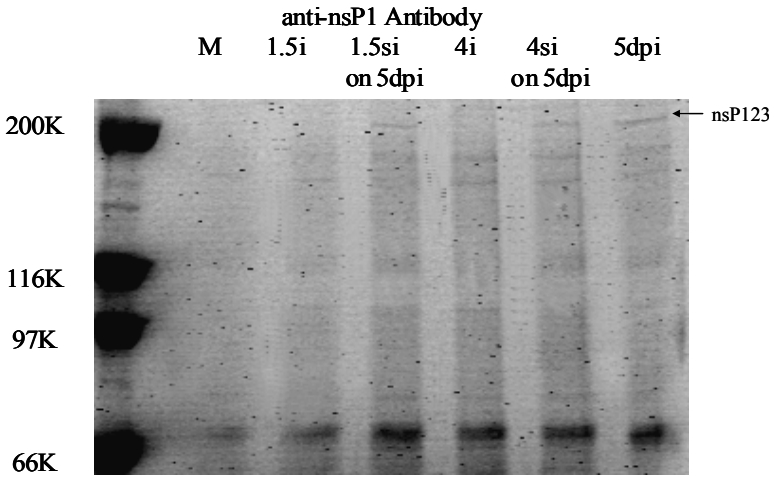


Figure 3.5: Immunoprecipitations with anti-nsP1 or anti-nsP2 antibody showing polyproteins and cleavage products at 1.5 or 4 hpi, 1.5 or 4 hpi after superinfection at 5dpi, and at 5dpi in a) *Mosquito* U4.4 cells and b) *Drosophila* S2 cells. See text for protocols used for Immunoprecipitations.

a)



b) Anti nsP1 antibody



CHAPTER FOUR

A COLORIMETRIC ASSAY FOR VIRAL AGENTS WHICH PRODUCE CYTOPATHIC EFFECTS

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Abstract:

Many animal viruses produce cytopathic effects in their host cells during a productive infection. While some of these virus infections can be assayed by the production of plaques on the appropriate indicator cell line, many viruses, while producing cytotoxicity, do not easily form plaques, or do not form plaques at all. Additionally, viruses from virus families may all have different preferred forms of titration of infectivity making comparative virology difficult even among related groups. Members of the mammalian parvoviruses, belong to such a group. Specific viruses such as porcine parvovirus (PPV), canine parvovirus (CPV), and minute virus of mice (MVM) are usually titered using different infectivity assays to determine the virus titers of stock virus, or experimental samples. We sought to develop an assay which would enable a direct comparison of infectious virus titer between these parvoviruses, as well as to facilitate the measurement of virus infectivity in lieu of, or in the absence of a plaque assay. We have applied the MTT assay, which measures cell viability to measure cytopathic effect produced by viral infection. This is a novel use for the well-known tetrazolium salt assay, MTT. The levels of infectious PPV measured using the MTT method were compared to $TCID_{50}$ measurements, showing an excellent correlation for titers between 1 and 9 logs for these two assays. The titer of CPV and MVM samples were also consistently duplicated. Additionally, this assay was applied to Sindbis virus which is routinely titered by the formation of plaques on indicator plaque monolayers. Titration of Sindbis virus mutants

by the MTT assay was found to be valuable for the purpose of screening large numbers of plaque purified viruses in a 96 well format. This assay can be adapted, by comparison to an accepted titration method to any viral system which produces measurable cytopathic effect.

Keywords: MTT, $TCID_{50}$, plaque assay, PPV, CPV, MVM, Sindbis virus, and cytopathic effect.

CONCLUSIONS AND FUTURE DIRECTIONS

The research presented in this report identified potential role for activation of Notch pathway in establishment of SV persistence. As γ-secretase inhibitor can inhibit Notch pathway (Curry 2005, see chapter 2), treatment of persistently infected S2 cells or U4.4 cells with γ-secretase inhibitor may affect the cell cycle changes and cell growth changes seen in insect cells (Karpf 1997a, see chapter 2) during persistence. To test this hypothesis, the persistently infected U4.4 or S2 cells can be treated with γ-secretase inhibitor and the cell cycle profiles and cell growth curves obtained from the treated cells can be compared to those obtained from untreated persistently infected cells. This must be exciting to test this hypothesis, as it would reveal the similarity between alphavirus and herpesvirus persistence.

As herpesviruses code for proteins that interact with Notch components, interaction of different SV nsPs or polyproteins with Notch components can be tested in Yeast two hybrid assays.

Northern blotting can be done to detect upregulation of the Notch components in U4.4 and other cell lines derived from *A. albopictus*, using the currently available *A. gambaie* cDNA clones (Table2, Chapter Two. Page 47).

Several membrane trafficking and cytoskeletal components are upregulated at 5dpi in gene expression profiling study described here (chapter 2). The levels of these proteins in persistently infected cells can be compared to uninfected cells, using immunofluorescence technique. This should be possible as fluorescent antibodies are available for some of these proteins (for ex: Rab proteins). Further, in vitro interaction maps of the SV nonstructural proteins and different cytoskeletal components can be obtained using Myc, FLAG or GST tagged proteins.

The research presented here also established *Drosophila* S2 cells as a suitable invertebrate host for SV, making it possible to use this cell line to test hypotheses involving factors that are yet to be identified in Mosquito.

TEP II is identified to be upregulated during establishment of SV persistence in insect cells. This was associated with inhibition of viral protease and inhibition of cleavage of polyproteins that form functional viral replicase-transcriptase complexes. Further experiments can be done using Yeast two-hybrid technique to test the hypothesis

that TEP II interacts with nsP2. Potential interaction between replication complexes and TEP II can be studied by in vitro expression of Nonstructural polyproteins and the TEP II. The GCGEQ (Thio-ester forming domain of TEP II) containing region of TEP II and protease domain of nsP2 can be tested to detect any potential interaction between these proteins. The possibility that TEP II interacts with any other region of nonstructural polyprotein or nsP2 cannot be ruled out. Gene silencing (RNAi) can be used to decrease or knock out the TEP II transcript levels to determine its importance in establishment of SV persistence.

Appendices

GENES INCREASED IN EXPRESSION IN DROSOPHILA S2 CELLS UPON SV INFECTION AT 5 DAYS POST INFECTION

Probe Set ID (*p*-value)	Gene Title	Gene Symbol	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description
143503_at (0.0182)	*silver*	*svr*	proteolysis cuticle biosynthesis (sensu Insecta)	metallocarboxypeptidase activity carboxypeptidase A activity carboxypeptidase E activity lysine carboxypeptidase activity metallocarboxypeptidase D activity protein binding carboxypeptidase activity peptidase activity metallopeptidase activity zinc ion binding hydrolase activity structural constituent of cuticle metal ion binding	membrane integral to membrane
141205_at (0.00983)	*CG18177*	*CG18177*		N-acetyltransferase activity	
141207_at (0.0398)	*Nup153*	*Nup153*	protein targeting intracellular protein transport	nucleic acid binding mRNA binding zinc ion binding	nuclear pore nuclear pore
141256_at (0.0139)	*mutagen-sensitive 210*	*mus210*	nucleotide-excision repair DNA repair nucleotide-excision repair response to DNA damage stimulus nucleotide-excision repair	damaged DNA binding DNA binding damaged DNA binding damaged DNA binding	nucleus nucleus
141283_at (0.00709)	*cup*	*cup*	regulation of translation chromosome organization and biogenesis (sensu Eukaryota) female meiosis follicle cell migration (sensu Insecta) oogenesis (sensu Insecta) oogenesis (sensu Insecta) negative regulation of translation meiotic chromosome segregation regulation of pole plasm oskar mRNA localization negative regulation	protein binding translation regulator activity	nuclear envelope lumen cytoplasm plasma membrane plasma membrane

			of oskar mRNA translation transcription regulation of transcription, DNA-dependent development cell differentiation oogenesis		
141780_at (0.0114)	*Cytochrome P450-9c1*	*Cyp9c1*	electron transport	monooxygenase activity electron transporter activity electron transporter activity oxidoreductase activity iron ion binding heme binding metal ion binding electron transporter activity	microsome membrane endoplasmic reticulum microsome membrane
141486_at (0.0428)	*Cysteine string protein*	*Csp*	electron transport protein folding protein folding intracellular protein transport exocytosis response to stress defense response neurotransmitter secretion synaptic vesicle exocytosis synaptic vesicle uncoating vesicle-mediated transport synaptic vesicle exocytosis	electron transporter activity iron ion binding ATPase activity, coupled unfolded protein binding heat shock protein binding	plasma membrane synaptic vesicle membrane plasma membrane
141716_at (0.0318)	*Sin3A*	*Sin3A*	regulation of transcription from RNA polymerase II promoter peripheral nervous system development ATP synthesis coupled proton transport negative regulation of transcription regulation of transcription negative regulation of transcription, DNA-dependent negative regulation of transcription, DNA-dependent regulation of transcription, DNA-dependent regulation of transcription	chromatin binding transcription factor activity RNA polymerase II transcription factor activity transcription cofactor activity ATP binding transcriptional repressor activity deacetylase activity hydrogen-transporting ATP synthase activity, rotational mechanism hydrogen-transporting ATPase activity, rotational mechanism protein binding transcription factor activity	chromatin euchromatin nucleus nucleus nucleus proton-transporting two-sector ATPase complex Sin3 complex nucleus nucleus
141332_at (0.000617)	*Nhe1*	*Nhe1*	cation transport sodium ion	solute:hydrogen antiporter activity	integral to membrane

			transport regulation of pH transport ion transport	sodium:hydrogen antiporter activity sodium:hydrogen antiporter activity antiporter activity sodium:hydrogen antiporter activity sodium:hydrogen antiporter activity	
141643_at (0.00111)	*Phospholipase A2 activator protein*	*Plap*		phospholipase A2 activator activity	fusome
141387_at (0.0186)	*maggie*	*mge*		protein translocase activity	mitochondrion mitochondrial outer membrane translocase complex
141446_at (0.049)	*CG5447*	*CG5447*		protein binding	
141464_at (0.00592)	*pecanex*	*pcx*	ectoderm development nervous system development development cell differentiation		plasma membrane integral to plasma membrane membrane
141765_at (0.0142)	*sugarless*	*sgl*	glycosaminoglycan biosynthesis electron transport lipid metabolism cell surface receptor linked signal transduction smoothened signaling pathway segment polarity determination tracheal epithelial cell migration (sensu Insecta) primary tracheal branching (sensu Insecta) heart development mesoderm migration fibroblast growth factor receptor signaling pathway heparan sulfate proteoglycan biosynthesis heparan sulfate proteoglycan biosynthesis, polysaccharide chain biosynthesis Wnt receptor signaling pathway chondroitin sulfate biosynthesis glycosaminoglycan	UDP-glucose 6-dehydrogenase activity	

			biosynthesis tracheal epithelial cell migration (sensu Insecta) mesoderm migration fibroblast growth factor receptor signaling pathway heparan sulfate proteoglycan biosynthesis, polysaccharide chain biosynthesis chondroitin sulfate biosynthesis tracheal epithelial cell migration (sensu Insecta) fibroblast growth factor receptor signaling pathway		
141370_at (0.0393)	*pumpless*	*ppl*	glycine catabolism metabolism	hydrolase activity lipoic acid binding catalytic activity acetate-CoA ligase activity AMP binding ligase activity acetate-CoA ligase activity	mitochondrion glycine cleavage complex
141663_at (0.0426)	*CG9715*	*CG9715*		nucleic acid binding calcium ion binding zinc ion binding	
141706_at (0.0208)	*CG1371*	*CG1371*	aromatic compound metabolism	catalytic activity ferric iron binding	integral to membrane
141810_at (0.0369)	*Neural conserved at 73EF*	*Nc73EF*	tricarboxylic acid cycle glycolysis metabolism	oxoglutarate dehydrogenase (succinyl-transferring) activity oxoglutarate dehydrogenase (succinyl-transferring) activity oxidoreductase activity, acting on the aldehyde or oxo group of donors, disulfide as acceptor thiamin pyrophosphate binding	oxoglutarate dehydrogenase complex (sensu Eukaryota)
141986_at (0.0481)					
142088_at (0.0276)					
142168_at (0.0241)	*CG12378*	*CG12378*			
142206_s_at (0.00605)	*CG14527* *CG14526*	*CG14527* *CG14526*	proteolysis signal transduction	metalloendopeptidase activity neprilysin activity zinc ion binding endothelin-converting enzyme activity peptidase activity hydrolase activity	membrane
142251_at (0.0157)	*foraging*	*for*	protein amino acid phosphorylation protein amino acid phosphorylation muscle contraction cytoskeleton	protein serine/threonine kinase activity cyclic nucleotide-dependent protein kinase activity cGMP-dependent protein kinase activity	plasma membrane cAMP-dependent protein kinase complex

			organization and biogenesis intracellular signaling cascade feeding behavior regulation of heart contraction larval locomotory behavior transmission of nerve impulse larval feeding behavior larval feeding behavior protein amino acid phosphorylation	cGMP-dependent protein kinase activity receptor signaling protein serine/threonine kinase activity ATP binding cAMP-dependent protein kinase regulator activity nucleotide binding protein kinase activity protein serine/threonine kinase activity transferase activity cGMP binding cyclic nucleotide-dependent protein kinase activity	
142275_at (0.0388)	*gamma-glutamyl carboxylase*	*GC*	protein modification peptidyl-glutamic acid carboxylation peptidyl-glutamic acid carboxylation regulation of transcription, DNA-dependent	gamma-glutamyl carboxylase activity ligase activity protein binding protein binding zinc ion binding	
142306_at (0.00354)	*CG3386*	*CG3386*			
142601_at (0.00567)	*CG5792*	*CG5792*		protein binding	
142749_at (0.0333)	*Ac3*	*Ac3*	cAMP biosynthesis G-protein coupled receptor protein signaling pathway intracellular signaling cascade cyclic nucleotide metabolism cyclic nucleotide biosynthesis	adenylate cyclase activity lyase activity phosphorus-oxygen lyase activity	integral to membrane
142753_at (0.0133)	*CG15536*	*CG15536*			
142758_at (0.0351)	*MAP kinase kinase 4*	*Mkk4*	protein amino acid phosphorylation protein amino acid phosphorylation activation of JNK activity activation of JNK activity protein amino acid phosphorylation protein amino acid phosphorylation activation of JNK activity	receptor signaling protein serine/threonine kinase activity MAP kinase kinase activity ATP binding JUN kinase kinase activity nucleotide binding protein kinase activity protein serine/threonine kinase activity kinase activity transferase activity JUN kinase kinase activity	
142789_at (0.0382)	*Nedd4*	*Nedd4*	proteolysis axon guidance protein ubiquitination axon midline	ubiquitin-protein ligase activity Notch binding protein binding ubiquitin-protein ligase activity ligase activity	intracellular cytoplasm

			choice point recognition protein ubiquitination receptor internalization negative regulation of Notch signaling pathway negative regulation of Notch signaling pathway receptor internalization protein modification ubiquitin cycle Notch signaling pathway development	ubiquitin-protein ligase activity ubiquitin-protein ligase activity	
143164_at (0.0234)	*Fps oncogene analog*	*Fps85D*	protein amino acid phosphorylation defense response JAK-STAT cascade photoreceptor cell morphogenesis (sensu Endopterygota) protein amino acid phosphorylation intracellular signaling cascade development	protein serine/threonine kinase activity protein-tyrosine kinase activity non-membrane spanning ATP binding protein binding nucleotide binding protein kinase activity transferase activity	cytoplasm plasma membrane membrane membrane
143537_at (0.0175)	*goliath*	*gol*	mesoderm formation regulation of transcription, DNA-dependent transcription from RNA polymerase II promoter proteolysis mesoderm development protein ubiquitination regulation of transcription DNA-dependent development	ubiquitin-protein ligase activity peptidase activity zinc ion binding (electronic annotation) transcription regulator activity (expression pattern) DNA binding protein binding metal ion binding	ubiquitin ligase complex nucleus membrane integral to membrane
143662_at (0.034)	*Cdc37*	*Cdc37*	regulation of progression through cell cycle protein folding transmembrane receptor protein tyrosine kinase signaling pathway protein kinase cascade	protein tyrosine kinase activator activity unfolded protein binding chaperone binding unfolded protein binding	cytoplasm
143667_at	*Imitation*	*Iswi*	chromatin	nucleotide binding	transcription factor

(0.0272)	*SWI*		assembly or disassembly nucleosome assembly chromatin remodeling transcription regulation of transcription, DNA-dependent regulation of transcription from RNA polymerase II promoter nucleosome spacing nucleosome mobilization positive regulation of transcription chromatin modification	nucleic acid binding DNA binding DNA helicase activity ATP binding ATP-dependent helicase activity DNA-dependent ATPase activity DNA-dependent ATPase activity general RNA polymerase II transcription factor activity ATPase activity nucleotide binding helicase activity ATP binding hydrolase activity ATP binding general RNA polymerase II transcription factor activity	complex chromatin accessibility complex chromatin accessibility complex chromatin accessibility complex chromatin remodeling complex NURF complex ACF complex brahma complex nucleus chromatin remodeling complex
143681_at (0.0376)	*Septin-1*	*Sep1*	cytokinesis cytokinesis mitosis cellularization cell cycle cell division cytokinesis	GTPase activity GTPase activity structural constituent of cytoskeleton protein binding GTP binding nucleotide binding GTP binding GTP binding	septin ring ring canal (sensu Insecta) septin ring
143692_at (0.036)	*DEAD/DEAH RNA helicase 1*	*Dhh1*	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	nucleic acid binding RNA helicase activity ATP-dependent RNA helicase activity ATP binding protein binding helicase activity hydrolase activity RNA helicase activity (all functions determined from sequence similarity)	
143836_at (0.0451)	*Hemomucin*	*Hmu*	carbohydrate metabolism biosynthesis	mRNA binding serine-type endopeptidase activity receptor activity lyase activity strictosidine synthase activity	cell surface
143879_at (0.0121)	*Rab-protein 1*	*Rab1*	two-component signal transduction system (phosphorelay) regulation of transcription, DNA-dependent intracellular protein transport ER to Golgi vesicle-mediated transport endocytosis actin filament	DNA binding GTPase activity GTP binding ATP binding transcription factor binding GTPase activity	intracellular

			organization cell adhesion signal transduction small GTPase mediated signal transduction regulation of cell shape regulation of exocytosis protein transport		
143953_at (0.00623)	*CAS/CSE1 segregation protein*	*Cas*	protein import into nucleus, docking protein import into nucleus protein export from nucleus protein export from nucleus apoptosis induction of apoptosis cell proliferation transport apoptosis	importin-alpha export receptor activity protein transporter activity protein binding binding importin-alpha export receptor activity	nucleus nuclear pore cytoplasm nucleus cytoplasm
144016_at (0.0371)	*Niemann-Pick Type C-1*	*NPC1*	lipid metabolism lipid transport cholesterol transport	receptor activity hedgehog receptor activity	lysosomal membrane
144064_at (0.0425)	*Bub1*	*Bub1*	protein amino acid phosphorylation chromosome segregation mitotic spindle checkpoint mitotic spindle checkpoint mitotic spindle checkpoint regulation of exit from mitosis	protein kinase activity protein serine/threonine kinase activity receptor signaling protein ATP binding nucleotide binding protein kinase activity transferase activity	kinetochore outer kinetochore of condensed chromosome
144125_at (0.0352)	*Pten*	*Pten*	protein amino acid dephosphorylation protein amino acid dephosphorylation apoptosis apoptosis cytoskeleton organization and biogenesis actin filament organization cell adhesion tracheal epithelial cell fate determination (sensu Insecta) cell proliferation insulin receptor signaling pathway insulin receptor signaling pathway	actin binding phosphoprotein phosphatase activity protein tyrosine phosphatase activity non-membrane spanning serine-type endopeptidase inhibitor activity protein tyrosine/serine/threonine phosphatase activity protein phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase activity PDZ domain binding inositol-1,3,4,5-tetrakisphosphate 3-phosphatase activity phosphatidylinositol-3,4-	cytoplasm

			regulation of cell shape regulation of cell size organ morphogenesis tissue development negative regulation of cell growth regulation of body size negative regulation of body size response to starvation negative regulation of progression through cell cycle negative regulation of organ size negative regulation of insulin receptor signaling pathway negative regulation of insulin receptor signaling pathway phosphoinositide dephosphorylation phosphoinositide dephosphorylation cellular physiological process autophagy protein kinase B signaling cascade regulation of cyclin dependent protein kinase activity negative regulation of cell migration regulation of protein stability inositol phosphate dephosphorylation negative regulation of focal adhesion formation negative regulation of protein kinase B signaling cascade protein amino acid dephosphorylation	bisphosphate 3-phosphatase activity phosphoprotein phosphatase activity protein tyrosine/serine/threonine phosphatase activity hydrolase activity actin binding phosphoprotein phosphatase activity protein tyrosine phosphatase activity non-membrane spanning protein tyrosine phosphatase activity protein tyrosine/serine/threonine phosphatase activity phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase activity	
144145_at (0.0124)	*Grip75*	*Grip75*	DNA repair microtubule nucleation cell cycle microtubule cytoskeleton	DNA binding endonuclease activity structural constituent of cytoskeleton microtubule binding tubulin binding	gamma-tubulin ring complex spindle pole microtubule organizing center microtubule

			organization and biogenesis (electronic annotations)	structural constituent of cytoskeleton	
144182_at (0.0453)	*MLF1-adaptor molecule*	*Madm*	protein amino acid phosphorylation intracellular signaling cascade	protein serine/threonine kinase activity receptor signaling protein serine/threonine kinase activity ATP binding nucleotide binding protein kinase activity kinase activity transferase activity (from electronic annotation or sequence similarity)	
144220_at (0.0412)	*CG15261*	*CG15261*	regulation of translation negative regulation of protein biosynthesis negative regulation of protein biosynthesis		
144468_at (0.00492)	*Femcoat*	*Femcoat*	insect chorion formation	structural constituent of chorion (sensu Insecta)	cytoplasm
144500_at (0.000792)	*CG4041*	*CG4041*	protein amino acid phosphorylation	protein kinase activity protein serine/threonine kinase activity protein-tyrosine kinase activity ATP binding	
144728_at (0.00217)	*CG6999 CG32706*	*CG6999 CG32706*	regulation of alternative nuclear mRNA splicing, via spliceosome	mRNA binding	nucleus
144871_at (0.0278)	*discs large 1*	*dlg1*	regulation of progression through cell cycle morphogenesis of a polarized epithelium morphogenesis of an epithelium protein targeting cytoskeleton organization and biogenesis synaptic transmission dorsal closure dorsal closure nervous system development protein localization asymmetric protein localization asymmetric protein localization cell proliferation negative regulation of cell proliferation establishment and/or	guanylate kinase activity epidermal growth factor receptor binding structural molecule activity structural constituent of cytoskeleton protein binding protein binding signal transducer activity	cytoplasm cytoskeleton plasma membrane septate junction cell cortex membrane basolateral plasma membrane apicolateral plasma membrane apical cortex synapse cytoskeleton tight junction membrane

			maintenance of polarity of embryonic epithelium morphogenesis of follicular epithelium establishment and/or maintenance of polarity of follicular epithelium morphogenesis of larval imaginal disc epithelium establishment and/or maintenance of polarity of larval imaginal disc epithelium establishment and/or maintenance of polarity of larval imaginal disc epithelium septate junction assembly regulation of border follicle cell delamination regulation of cell proliferation asymmetric protein localization during cell fate commitment basal protein localization basal protein localization zonula adherens assembly establishment and/or maintenance of neuroblast polarity establishment and/or maintenance of epithelial cell polarity establishment and/or maintenance of epithelial cell polarity central nervous system development		

			peripheral nervous system development neuron differentiation cell adhesion signal transduction development cell differentiation		
145135_at (0.0131)	*CG8117*	*CG8117*	regulation of transcription, DNA-dependent RNA elongation from RNA polymerase II promoter transcription RNA elongation regulation of transcription RNA elongation from RNA polymerase II promoter	DNA binding transcription factor activity transcriptional elongation regulator activity general RNA polymerase II transcription factor activity protein binding RNA polymerase II transcription factor activity zinc ion binding transcriptional elongation regulator activity	nucleus
145264_at (0.0431)	*CG32554*	*CG32554*		protein binding	
145846_at (0.018)	*CG11030*	*CG11030*			
146121_at (0.0269)	*CG13133*	*CG13133*			
146499_at (0.0252)	*Topoisomerase 3*	*Top3*	DNA metabolism DNA topological change DNA unwinding during replication DNA modification	DNA topoisomerase type I activity DNA binding DNA topoisomerase (ATP-hydrolyzing) activity zinc ion binding	chromosome
146639_at 0.00214	*CG10465*	*CG10465*	potassium ion transport	voltage-gated potassium channel activity protein binding identical protein binding	voltage-gated potassium channel complex membrane
146844_at (0.0343)	*CG30349*	*CG30349*			
146965_at (0.0337)	*CG12341*	*CG12341*			
147099_s_at (0.03)	*achintya vismay*	*achi vis*	regulation of transcription from RNA polymerase II promoter spermatogenesis regulation of transcription	transcription factor activity transcription corepressor activity histone deacetylase binding DNA binding sequence-specific DNA binding	nucleus
147203_at (0.00234)	*CG17388*	*CG17388*			
147819_at (0.0441)	*CG3803*	*CG3803*	electron transport oxidative phosphorylation cytochrome c oxidase complex assembly protein complex		mitochondrial inner membrane

			assembly cytochrome c oxidase complex assembly		
148001_at (0.0093)	*CG13933*	*CG13933*	intracellular signaling cascade		
148325_at (0.0152)	*mutagen-sensitive 312*	*mus312*	resolution of meiotic joint molecules as recombinants nucleotide-excision repair, DNA damage recognition meiotic recombination meiotic chromosome segregation	protein binding	
148564_at (0.0337)	*CG7607*	*CG7607*	development		
148694_at (0.0128)	*Syntaxin 13*	*Syx13*	cytokinesis after mitosis protein targeting mitosis cytokinesis after meiosis I male meiosis female meiosis neurotransmitter secretion synaptic vesicle docking during exocytosis vesicle-mediated transport intracellular protein transport neurotransmitter secretion synaptic vesicle docking during exocytosis vesicle-mediated transport	t-SNARE activity protein transporter activity t-SNARE activity	plasma membrane membrane plasma membrane
148723_at (0.0371)	*bruno-3*	*bru-3*	protein metabolism	mRNA binding protein binding nucleotide binding nucleic acid binding	
148903_at (0.00803)	*lethal (3) 73Ah*	*l(3)73Ah*	nucleobase, nucleoside, nucleotide and nucleic acid metabolism regulation of transcription from RNA polymerase II promoter development protein ubiquitination	ubiquitin-protein ligase activity zinc ion binding transcription regulator activity protein binding	ubiquitin ligase complex
149193_at	*S1P*	*S1P*	proteolysis	serine-type endopeptidase	

(0.0495)			sterol regulatory element binding-protein cleavage (sequence similarity or electronic annotation)	activity subtilisin activity peptidase activity hydrolase activity (electronic annotation)	
149351_at (0.0382)	*CG2051*	*CG2051*	chromatin assembly or disassembly (inferred from sequence similarity with Arabidopsis)	histone acetyltransferase activity	
149991_at (0.00947)	*CG5516*	*CG5516*		protein binding nucleic acid binding zinc ion binding	
150426_at (0.0138)	*CG13830*	*CG13830*		cysteine protease inhibitor activity calcium ion binding protease inhibitor activity (electronic annotation)	
150553_at (0.0374)	*CG11847*	*CG11847*	viral life cycle	No data attributing to function.	
150965_at (0.0175)	*CG2219*	*CG2219*	intracellular protein transport cell surface receptor linked signal transduction small GTPase mediated signal transduction vesicle-mediated transport rRNA processing ribosome biogenesis	GTPase activity GTP binding protein binding (All functions from electronic annotations)	intracellular
151373_at (0.0378)	*CG32795*	*CG32795*	carbon utilization by fixation of carbon dioxide	ribulose-bisphosphate carboxylase activity protein binding	ribulose bisphosphate carboxylase complex (sensu Magnoliophyta)
151406_at (0.0128)	*CG32685*	*CG32685*			
151609_at (0.00163)					
151697_at (0.000365)	*rapsynoid*	*raps*	protein targeting G-protein coupled receptor protein signaling pathway neuroblast fate determination asymmetric protein localization asymmetric cell division establishment of mitotic spindle localization neuroblast division asymmetric protein localization during	guanyl-nucleotide exchange factor activity GTPase activator activity binding protein binding	cell cortex apical part of cell apical cortex

			cell fate commitment basal protein localization maintenance of protein localization signal transduction		
152059_at (0.0118)	*CG2219*	*CG1516*	pyruvate metabolism gluconeogenesis fatty acid biosynthesis metabolism pyruvate metabolism (electronic annotations)	pyruvate carboxylase activity ATP binding biotin binding catalytic activity ligase activity pyruvate carboxylase activity	mitochondrial matrix cytoplasm mitochondrial matrix
152231_at (0.0131)	*CG5594*	*CG5594*	transport cation transport sodium ion transport chloride transport amino acid transport transport (Sequence similarity)	amino acid-polyamine transporter activity cation transporter activity potassium:chloride symporter activity transporter activity cation:chloride symporter activity	integral to membrane membrane
152306_at (0.0256)	*CG32626*	*CG32626*	purine base metabolism purine ribonucleoside monophosphate biosynthesis	AMP deaminase activity (sequence similarity)	
152380_at (0.0265)	*CG1324*	*CG1324*		protein binding	
152544_at (0.0159)	*CG8683*	*CG8683*			
152726_at (0.0397)	*CG8708*	*CG8708*	protein modification protein amino acid glycosylation protein metabolism protein amino acid glycosylation	transferase activity transferring glycosyl groups beta-1,3-galactosyltransferase activity	
152754_at (0.012)	*sec31*	*sec31*	intracellular protein transport exocytosis peptidoglycan metabolism		endoplasmic reticulum Golgi stack synaptic vesicle
152832_at (0.0437)				protein binding	
152907_at (0.0451)	*18 wheeler*	*18w*	defense response immune response antibacterial humoral response (sensu Protostomia) antibacterial humoral response (sensu Protostomia)	transmembrane receptor activity	membrane fraction cytoplasm plasma membrane

			signal transduction development morphogenesis cytokine and chemokine mediated macrophage activation embryonic morphogenesis		
152946_at (0.0475)	*Ubiquitin activating enzyme 1*	*Uba1*	proteolysis protein modification ubiquitin cycle transport autophagy protein transport	ubiquitin activating enzyme activity ubiquitin-protein ligase activity protein binding nucleotide binding catalytic activity ATP binding ubiquitin-like activating enzyme activity ligase activity	
153145_at (0.046)	*Tetratricopeptide repeat protein 2*	*Tpr2*	protein folding protein complex assembly protein metabolism	binding heat shock protein binding unfolded protein binding (electronic annotations)	
153234_at (0.00229)	*CG7668*	*CG7668*	defense response defense response to bacteria	receptor binding	
153251_at (0.016)	*fat2*	*fat2*	protein amino acid phosphorylation homophilic cell adhesion signal transduction tracheal system development (sensu Insecta) salivary gland development foregut morphogenesis hindgut morphogenesis calcium-dependent cell-cell adhesion negative regulation of imaginal disc growth cell adhesion homophilic cell adhesion calcium-dependent cell-cell adhesion	protein-tyrosine kinase activity receptor activity structural molecule activity calcium ion binding ATP binding cell adhesion molecule binding protein binding receptor activity	integral to plasma membrane
153292_at (0.0102)	*CG7861*	*CG7861*	protein folding tubulin folding		
153319_at (0.0202)	*CG3249*	*CG3249*	protein targeting signal transduction protein localization	nucleic acid binding receptor binding protein kinase A binding	
153383_at (0.0354)	*CG17735*	*CG17735*	protein modification	ubiquitin-protein ligase activity ligand-dependent nuclear	intracellular

			ubiquitin cycle	receptor binding	
153421_at (0.0236)	*fused lobes*	*fdl*	polysaccharide metabolism brain development carbohydrate metabolism development (sequence similarities and electronic annotations)	beta-N-acetylhexosaminidase activity hydrolase activity, hydrolyzing N-glycosyl compounds hydrolase activity, acting on glycosyl bonds beta-N-acetylhexosaminidase activity	
153451_at (0.0365)	*Cyclin-dependent kinase 5*	*Cdk5*	regulation of progression through cell cycle protein amino acid phosphorylation protein amino acid phosphorylation cell cycle mitosis neurotransmitter secretion synaptic vesicle transport protein amino acid phosphorylation	protein serine/threonine kinase activity cyclin-dependent protein kinase activity receptor signaling protein serine/threonine kinase activity ATP binding cyclin binding protein binding nucleotide binding protein kinase activity transferase activity cyclin-dependent protein kinase activity	
153494_at (0.0494)	*Protein phosphatase 1 at 87B*	*Pp1-87B*	mitotic sister chromatid segregation protein amino acid dephosphorylation nervous system development learning and/or memory adult locomotory behavior olfactory learning visual learning oogenesis (sensu Insecta) chromosome condensation protein amino acid dephosphorylation	protein phosphatase type 1 activity protein serine/threonine phosphatase activity protein binding myosin phosphatase activity phosphoprotein phosphatase activity iron ion binding hydrolase activity manganese ion binding	protein phosphatase type 1 complex cytoplasm
153540_at (0.0257)	*hook*	*hk*	microtubule cytoskeleton organization and biogenesis intracellular protein transport endocytosis endosome transport cytoskeleton-dependent intracellular transport R7 development endocytosis	structural constituent of cytoskeleton microtubule binding	cytoplasm endosome synapse microtubule
153647_at	*bifocal*	*bif*	female meiosis	actin binding	cytoplasm

(0.0389)			chromosome segregation negative regulation of axon extension	microtubule binding protein phosphatase 1 binding	
153680_at (0.00478)	*Rad9*	*Rad9*	regulation of progression through cell cycle DNA repair electron transport cell redox homeostasis (inferred from electronic annotation)	kinase activator activity electron transporter activity	
153691_at (0.021)	*RhoGAP93B*	*RhoGAP93B*	axon guidance (mutant phenotype)		cytoskeleton
153760_at (0.0259)	*CG18041*	*CG18041*			
153776_at (0.0285)	*dream*	*dream*	proteolysis apoptosis intracellular signaling cascade programmed cell death (Sequence similarities)	caspase activity	
153785_at (0.0386)	*CG7997*	*CG7997*	polysaccharide metabolism carbohydrate metabolism	alpha-galactosidase activity protein binding hydrolase activity, hydrolyzing O-glycosyl compounds	
153803_at (0.0449)	*outspread*	*osp*			
153903_at (0.0353)	*Rpn9*	*Rpn9*	proteolysis regulation of exit from mitosis	endopeptidase activity enzyme regulator activity protein binding	proteasome regulatory particle (sensu Eukaryota) cytosol protein complex
153925_at (0.0289)	*CG5705*	*CG5705*	translational termination RNA processing	translation release factor activity, codon specific protein binding 3'-5'-exoribonuclease activity RNA binding	mitochondrion nuclear exosome (RNase complex) polytene chromosome cytoplasm
153928_at (0.0278)	*CG2201*	*CG2201*	phospholipid metabolism phosphorylation	choline kinase activity ethanolamine kinase activity protein binding kinase activity transferase activity	
153939_at (0.0471)	*CG15439*	*CG15439*	regulation of transcription, DNA-dependent protein ubiquitination (electronic annotation)	DNA binding ubiquitin-protein ligase activity zinc ion binding protein binding	ubiquitin ligase complex

153942_at (0.00499)	*CG8924*	*CG8924*	regulation of transcription from RNA polymerase II promoter sex determination transmission of nerve impulse regulation of transcription	DNA binding protein binding transcription regulator activity protein binding identical protein binding DNA binding	nucleus
153968_at (0.0223)	*S-adenosylmethionine decarboxylase*	*SamDC*	spermine biosynthesis	adenosylmethionine decarboxylase activity carboxy-lyase activity	
153971_at (0.0269)	*osa*	*osa*	regulation of transcription from RNA polymerase II promoter segment specification wing margin morphogenesis Wnt receptor signaling pathway regulation of transcription positive regulation of transcription, DNA-dependent photoreceptor cell differentiation DNA-dependent development chromatin modification	DNA binding chromatin binding transcription coactivator activity transcription regulator activity DNA binding binding transcription coactivator activity DNA binding	nucleus brahma complex intracellular nucleus
153974_at (0.031)	*CG7747*	*CG7747*	protein folding protein targeting defense response	peptidyl-prolyl cis-trans isomerase activity	
153995_at (0.0155)	*Tao-1*	*Tao-1*	MAPKKK cascade protein amino acid phosphorylation induction of apoptosis JNK cascade (all these functions predicted by electronic annotation)	receptor signaling protein serine/threonine kinase activity ATP binding	
154029_at (0.00666)	*CG2875*	*CG2875*			
154044_at (0.00834)	*Ankyrin*	*Ank*	cytoskeletal anchoring signal transduction	actin binding receptor binding structural constituent of cytoskeleton cytoskeletal protein binding protein binding	plasma membrane spectrosome
154252_at (0.00471)	*Laminin A*	*LanA*	proteolysis cell-matrix adhesion signal transduction central nervous	mRNA binding receptor binding structural molecule activity serine-type peptidase activity protein binding	basement membrane basement membrane basal lamina

			system development peripheral nervous system development mesoderm development heart development organ morphogenesis tissue development female meiosis chromosome segregation cell-cell adhesion regulation of cell adhesion regulation of cell migration regulation of embryonic development embryonic morphogenesis axon guidance cell adhesion		laminin-1 membrane extracellular matrix (sensu Metazoa) basement membrane extracellular matrix basement membrane
154346_at (0.0205)	*numb*	*numb*	cell fate determination Notch signaling pathway intracellular signaling cascade ectoderm development neuroblast fate determination neuroblast fate determination central nervous system development ventral cord development peripheral nervous system development heart development rhythmic behavior asymmetric cell division asymmetric cell division regulation of Notch signaling pathway sensory organ precursor cell fate determination sensory organ precursor cell fate determination neuroblast division	nucleic acid binding receptor binding Notch binding protein binding nucleotide binding ATP binding	nucleus cytoplasm basal part of cell basal cortex nucleus

			cell fate commitment negative regulation of Notch signaling pathway negative regulation of Notch signaling pathway development		
154347_at (0.0498)	*falten*	*fal*	gastrulation	GTPase activity protein binding catalytic activity	
154403_at (0.0426)	*CG5466*	*CG5466*	cellular physiological process		
154453_at (0.0181)	*unc-13*	*unc-13*	signal transduction intracellular signaling cascade synaptic transmission neurotransmitter secretion synaptic vesicle exocytosis synaptic vesicle priming synaptic transmission	receptor activity calmodulin binding diacylglycerol binding	synaptic vesicle
154472_at (0.0129)	*CG4266*	*CG4266*	nuclear mRNA splicing, via spliceosome	mRNA binding nucleotide binding	
154497_at (0.0468)	*CG11416*	*CG11416*	protein folding	protein binding	prefoldin complex
154499_at (0.0228)	*CG10492*	*CG10492*		nucleic acid binding zinc ion binding	
154514_at (0.015)	*Cortactin*	*Cortactin*	cytoskeleton organization and biogenesis border follicle cell migration (sensu Insecta) ovarian ring canal formation regulation of cell shape	actin binding structural constituent of cytoskeleton	cytoskeleton
154521_at (0.0464)	*Furin 2*	*Fur2*	protein amino acid phosphorylation proteolysis transmembrane receptor protein tyrosine kinase signaling pathway proteolysis	furin activity subtilase activity transmembrane receptor protein tyrosine kinase activity ATP binding protein binding serine-type endopeptidase activity peptidase activity hydrolase activity	plasma membrane
154684_at (0.0199)	*CG8155*	*CG8155*	intracellular protein transport	small GTPase regulator activity	
154685_at (0.0452)	*Translocase of outer*	*Tom20*	protein targeting to mitochondrion	carrier activity protein translocase activity	mitochondrion mitochondrial

	membrane 20		protein targeting intracellular protein transport		outer membrane translocase complex
154770_at (0.0201)	*CG8289*	*CG8289*	chromatin assembly or disassembly	chromatin binding	chromatin nucleus
154792_at (0.0355)	*peanut*	*pnut*	cytokinesis , contractile ring formation vesicle targeting vesicle docking during exocytosis apoptosis mitosis cellularization eye photoreceptor development (sensu Endopterygota) cytokinesis cell cycle cell division cytokinesis	actin binding GTPase activity structural constituent of cytoskeleton GTP binding microtubule binding hydrolase activity protein binding nucleotide binding	septin ring apical plasma membrane intercellular bridge ring canal (sensu Insecta)
154804_at (0.0496)	*CG8232*	*CG8232*	ubiquitin- dependent protein catabolism	cysteine-type endopeptidase activity ubiquitin thiolesterase activity poly(A)-specific ribonuclease activity nuclease activity exonuclease activity hydrolase activity poly(A)-specific ribonuclease activity	intracellular nucleus
154815_at (0.0161)	*Minichrom osome maintenanc e 7*	*Mcm7*	pre-replicative complex formation and maintenance pre-replicative complex formation and maintenance DNA replication initiation (sequence similarity or electronic annotations)	nucleotide binding DNA helicase activity chromatin binding chromatin binding DNA replication origin binding ATP binding DNA-dependent ATPase activity nucleoside-triphosphatase activity	nucleus pre-replicative complex
154894_at (0.00131)	*dalmatian*	*dmt*	peripheral nervous system development		nucleus nucleus
154983_at (0.0109)	*CG15634*	*CG15634*		protein binding	
155008_at (0.0127)	*Clathrin heavy chain*	*Chc*	intracellular protein transport receptor mediated endocytosis neurotransmitter secretion sperm individualization synaptic vesicle coating	protein binding binding	coated pit synaptic vesicle clathrin vesicle coat clathrin coat of synaptic vesicle clathrin coat of coated pit

155033_at (0.00771)	*CG11120*	*CG11120*		protein binding	
155034_at (0.000529)	*TBP-associated factor 2*	*Taf2*	DNA methylation regulation of transcription, DNA-dependent regulation of transcription from RNA polymerase II promoter transcription initiation from RNA polymerase II promoter	DNA binding N-methyltransferase activity general RNA polymerase II transcription factor activity membrane alanyl aminopeptidase activity general RNA polymerase II	nucleus transcription factor TFIID complex
155078_at (0.0374)	*Rab-protein 8*	*Rab8*	two-component signal transduction system (phosphorelay) regulation of transcription, DNA-dependent intracellular protein transport endocytosis small GTPase mediated signal transduction regulation of exocytosis protein transport	DNA binding GTPase activity GTP binding ATP binding transcription factor binding	intracellular

GENES DECREASED IN EXPRESSION IN DROSOPHILA S2 CELLS UPON SV INFECTION AT 5 DAYS POST INFECTION

Probe Set ID	Gene Title	Gene Symbol	GO Biological Process Description	GO Molecular Function Description	GO Cellular Component Description
141379_at (0.000779)	*Antigen 5-related*	*Ag5r*	defense response		extracellular region
141447_at 0.034		*CG4914*	regulation of transcription, DNA-dependent proteolysis and peptidolysis	transcription factor activity chymotrypsin activity DNA binding serine-type endopeptidase activity trypsin activity	nucleus
141973_at 0.035					
142281_at 0.0197		*CG10188*	hormone secretion cell surface receptor linked signal transduction	Rho guanyl-nucleotide exchange factor activity	
142730_at 0.00562		*pod1*		structural constituent of cytoskeleton actin binding	
143061_at 0.0221	*Actin 88F*	*Act88F*	cytoskeleton organization and biogenesis	structural constituent of cytoskeleton	actin filament actin filament
143416_at 0.0156	*yellow*	*y*	carbon utilization by fixation of carbon dioxide pigmentation melanin biosynthesis from tyrosine ectoderm development pigmentation courtship behavior larval or pupal development (sensu Insecta) adult cuticle biosynthesis (sensu Insecta) wing extension pigmentation	ribulose-bisphosphate carboxylase activity structural molecule activity receptor binding	ribulose bisphosphate carboxylase complex (sensu Magnoliophyta) extracellular region extracellular region
143894_at 0.00247		*Pros28.1B*	ubiquitin-dependent protein catabolism proteolysis and peptidolysis	endopeptidase activity	proteasome core complex (sensu Eukaryota)

143909_at 0.0467	*Metabotropic glutamate receptor*	*Glu-RA*	transmission of nerve impulse metabotropic glutamate receptor signaling pathway	G-protein coupled receptor activity metabotropic glutamate, GABA-B-like receptor activity	integral to membrane integral to plasma membrane plasma membrane
144042_at 0.0309		*CG3777*			
144280_at 0.0253		*CG18095*	transmission of nerve impulse cell adhesion		
144383_at 0.0377					
144395_at 0.0261		*CG14053*			
145695_at 0.0391		*CG3485*	transcription from RNA polymerase II promoter regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism cell proliferation	zinc ion binding nucleic acid binding transcription regulator activity	nucleus
145904_at 0.00728		*CG31638*			myosin
146298_at 0.0127		*CG5781*			
146341_at 0.047		*CG16849*			
146586_at 0.0268	*nervana 3*	*nrv3*	sodium ion transport potassium ion transport cation transport	sodium:potassium-exchanging ATPase activity	sodium:potassium-exchanging ATPase complex plasma membrane
146656_at 0.0308	*Odorant receptor 42b*	*Or42b*	cell-cell signaling G-protein coupled receptor protein signaling pathway perception of smell	odorant binding olfactory receptor activity	integral to membrane
147667_at 0.00203		*CG11291*	metabolism	4-nitrophenylphosphatase activity	
147795_at 0.0231	*Peptidyl-hydroxyglycine-&agr;-amidating lyase*	*Pal*	peptide metabolism protein modification	peptidylglycine monooxygenase activity copper ion binding oxidoreductase activity peptidylamidoglycolate lyase activity peptidylamidoglycolate lyase activity	membrane
147956_at	*bric a brac 1*	*bab1*	transmission of	protein binding	nucleus

0.0257			nerve impulse sex determination regulation of transcription from RNA polymerase II promoter chromatin assembly or disassembly negative regulation of male pigmentation female sex differentiation behavior transcription sex differentiation sex-specific pigmentation negative regulation of pigmentation regulation of pigmentation transcription leg morphogenesis (sensu Endopterygota) leg morphogenesis (sensu Endopterygota) female gonad development female gonad development eye-antennal disc metamorphosis sex differentiation sex differentiation	DNA binding transcription factor activity	
147978_at 0.0282					
148059_at 0.0383	*Prolyl-tRNA synthetase*	*Aats-pro*	prolyl-tRNA aminoacylation	ATP binding mRNA binding proline-tRNA ligase activity	
148501_at 0.0431		*CG6707*			
148942_at 0.0109		*CG13727*			

149310_at 0.0478		*CG12161*	ubiquitin-dependent protein catabolism proteolysis and peptidolysis	endopeptidase activity	proteasome core complex (sensu Eukaryota)
149431_at 0.0491		*CG14606*	carbohydrate transport carbohydrate metabolism	hexose transporter activity	membrane integral to membrane
149911_at 0.00647		*CG7886*			
150072_at 0.0401		*CG5863*	proteolysis and peptidolysis	cathepsin D activity pepsin A activity	
150764_at 0.0396		*CG12872*			
151325_at 0.0395					
151530_i_at 0.0439		*tomosyn*	regulation of cell cycle intracellular protein transport establishment and/or maintenance of cell polarity synaptic vesicle exocytosis synaptic vesicle priming neurotransmitter secretion	structural constituent of cytoskeleton syntaxin-1 binding	synaptic vesicle
152154_at 0.0121		*CG7227*	cell adhesion macrophage activation apoptosis defense response	scavenger receptor activity	integral to plasma membrane
152583_at 0.0179	*twin of eyeless*	*toy*	regulation of transcription from RNA polymerase II promoter neurogenesis muscle development mesoderm development endoderm development cell proliferation eye morphogenesis (sensu Endopterygota)	transcription factor activity DNA binding specific RNA polymerase II transcription factor activity	nucleus

			eye-antennal disc development regulation of transcription		
152871_at 0.0322		*CG1909*	synaptic transmission protein ubiquitination	receptor binding ubiquitin-protein ligase activity zinc ion binding	nicotinic acetylcholine-gated receptor-channel complex ubiquitin ligase complex
153568_at 0.0478		*CG31224*	cell proliferation regulation of transcription from RNA polymerase II promoter	nucleic acid binding transcription regulator activity zinc ion binding	nucleus
154944_at 0.0042		*RhoGAPp190*	transmission of nerve impulse intracellular signaling cascade ectoderm development mushroom body development	Rho GTPase activator activity Rho GTPase activator activity	