

Dengue Virus Host Factors

by

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Date: June 9, 2009

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Dengue fever and dengue hemorrhagic fever are estimated to afflict 50-100 million people annually and are caused by one of the four serotypes of dengue virus. Dengue virus is carried and transmitted to humans by mosquitoes of the *Aedes* genus. Given the broad geographic distribution of *Aedes* mosquitoes, it has been estimated that nearly half the world's population is at risk of contracting the disease. Currently, no vaccine or specific antiviral treatment is available to combat this emerging menace.

A greater understanding of how dengue virus interacts with its insect and human hosts will facilitate the intelligent design of specific antivirals to combat the disease and enable the selective breeding of mosquitoes resistant to the virus. Although the genomes of the two primary mosquito vectors have been sequenced, the molecular tools necessary for conducting a systematic genetic analysis of host factors required for DEN infection are not yet available. These tools do however exist in the closely related fruit fly, *Drosophila melanogaster*. By using a strain of dengue virus that was adapted to propagate in fruit fly cells, we completed a full genetic screen for host factors required for efficient dengue virus propagation. When homologues of these host factors were assayed in a human cell line, over half were also shown to be required for efficient viral propagation. This indicates that while the virus is utilizing many of the same pathways in both of its hosts, the interaction with the insect vector has unique features that may contribute to the observed lack of pathogenesis in mosquitoes.

Dedication

This body of work is dedicated to the light of my life, my companion through thick and thin, my wife, Paola Florez de Sessions.

I would also like to acknowledge here Annemarie Hauschild, Susan Hammerstone, Michael Ferrari, Madaiah Puttaraju, and Mariano Garcia-Blanco for sparking my interest in science and for their excellent mentorship over the years. None of this would have been possible without their guidance along the way.

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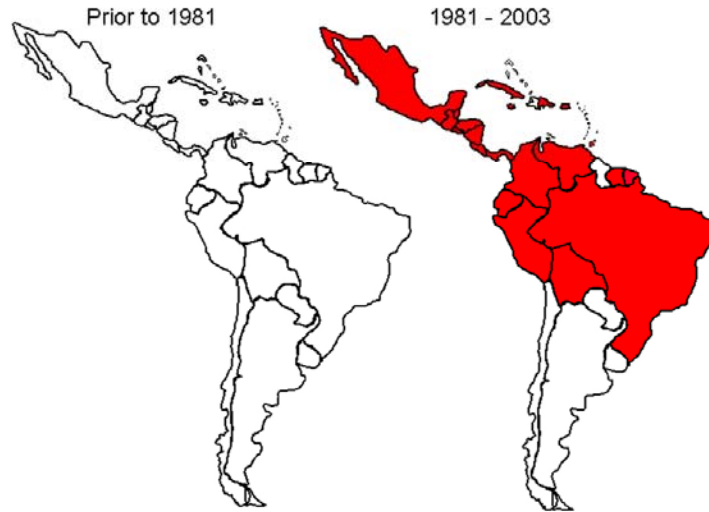
Chapter 1. Introduction

1.1 Background and Significance

Dengue virus (DEN) is the most prevalent arthropod-borne virus affecting humans (Kyle and Harris 2008). It is estimated that 50-100 million people are infected yearly and a staggering 2.5 billion are at risk from contracting the disease caused by DEN (Halstead 2007). Dengue fever (DF) is the most common manifestation of infection which may be caused by any of the four antigenically distinct serotypes of dengue fever virus (DEN1-4)(CDC 2009; WHO 2009). Symptoms of DF range from a mild to severe debilitating febrile illness lasting nearly two weeks and characterized by headache, retro-orbital pain, myalgia, arthralgia, and macular rash (Halstead 2007; Monath 2007; Morens and Fauci 2008; CDC 2009; WHO 2009). The more severe and often fatal forms of the disease, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS), are characterized by capillary leakage accompanied by thrombocytopenia, haemorrhagic manifestations and liver damage (Gubler 2004; Halstead 2007; Kyle and Harris 2008). Fatality rates for DHF and DSS are typically 2.5-5% but can be as high as 20% without proper care (Gubler 2004; CDC 2009; WHO 2009).

DEN is a re-emerging global health threat. In 1779-1780, epidemics of a dengue-like illness were reported in Asia, Africa and North America (Gubler 2006). These accounts indicate that even as early as the 18th century, DEN had already attained a global distribution, likely as a consequence of flourishing international sea-trade (Gubler 2006). The earliest known use of the name *dengue* to describe the disease was recorded

Figure 1-1



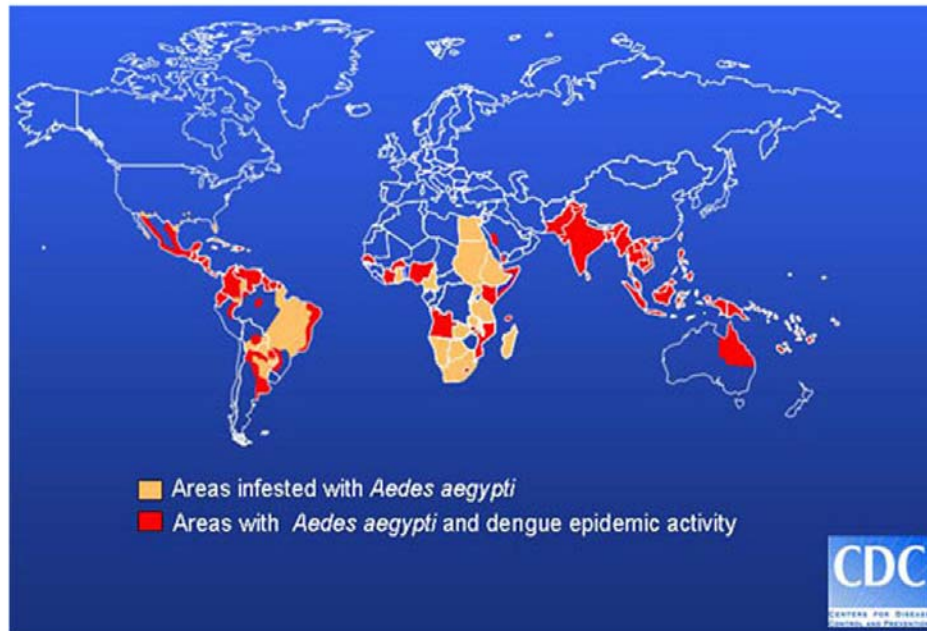
Source: WHO/PAHO/CDC, Aug. 2004

American Countries with laboratory confirmed dengue hemorrhagic fever, prior to 1981 and from 1981 to 2003

in Spain in 1801 although the name likely originates from the Swahili term *Ki-Dinga pepo* (Gubler 2006), which translated means "it is a sudden overtaking by a spirit"(Harper 2009). It is believed that DEN was once a predominantly sylvatic disease that, with the expansion of human settlements and subsequent deforestation of land for farming, DEN was transmitted to humans by a promiscuous, peridomestic mosquito vector such as *Aedes albopictus* or other closely related *Stegomyia* species (Dr. Ken Olson, personal communication and (Gubler 2006)). After the discovery that DEN and the closely related yellow fever virus (YFV) were filterable agents carried by mosquitoes in the early 1900's (Reed and Agramonte 1983), strict mosquito control measures were put in place which greatly reduced the incidence of DF. Following World War II, the use of dichlorodiphenyltrichloroethane (DDT) almost completely eradicated *Aedes aegypti* and consequently DEN from the Western hemisphere (Gubler 2004). Over the last 60 years however, the number of cases of DHF/DSS have risen over 500 fold (Kyle and Harris 2008) (Figure 1-1).

With the decline of strictly enforced vector control efforts over the last 40 years (Gubler 2004), the primary DEN vector, *Aedes aegypti*, has expanded its range to include all of the tropical world (CDC 2009) (Figure 1-2). In 2007, over 890,000 cases of DEN, 26,000 of which progressed to DHF, were reported in the Americas alone (WHO 2009). There are several likely explanations for such a sudden and dramatic rise in the incidence of DF and DHF including profound increases in the human population, especially in developing nations, uncontrolled urbanization with substandard living

Figure 1-2



Global distribution of dengue fever and its principle vector *Aedes aegypti*

Source: <http://www.cdc.gov/ncidod/dvbid/dengue/map-distribution-2005.htm>

conditions, lack of effective vector control, virus evolution, and international travel have all been suggested to play key roles in the global spread of the disease (Gubler 2004; Kyle and Harris 2008; Wilder-Smith and Gubler 2008). Given the high level of infrastructure and socio-economical conditions, namely running water, reliable electric grids for air conditioning and screens in windows and doors, the prospect of widespread re-introduction of DEN into the continental United States is debatable (Kyle and Harris 2008; CDC 2009). Nevertheless, the possibility of re-emergence should be taken seriously (Morens and Fauci 2008). This is underscored by the introduction of endemic DEN into Puerto Rico, Mexico and the southern portions of Florida and Texas over the last 30 years (CDC 2009) and the rapid emergence of the related West Nile virus (WNV) across the North American continent in the last 10 years (Gibbs 2005).

It should also be noted that prosperous, industrialized countries are not immune to the burden of epidemic DEN. In Singapore, strict vector control programs have been in place since the early 1970's and despite an initial 20 year decline in the number of DF cases, the incidence of DF has been steadily rising since the early 1990's to the current level of tens of thousands of cases annually (Ooi, Goh et al. 2006). In contrast to surrounding endemic regions where primary infection predominantly occurs in children and early teenagers, the majority of Singaporean cases are in young adults (Ooi, Goh et al. 2006). DEN infection in this age cohort differs from infection in children in at least two ways. The proportion of clinical to sub-clinical manifestations of the disease is significantly higher although the number of adults progressing to classic DHF is

generally lower (Low, Ooi et al. 2006). Although DF is typically a self-limiting disease, it still causes significant, incapacitating morbidity. This morbidity is of particular economic concern when the majority of those afflicted are in the working population. According to a study of adult DEN infection in Singapore, the average number of work days lost to DF in was five for non-hospitalized cases and ten for those requiring hospitalization (Low, Ooi et al. 2006). It has been estimated that over the next decade DF and DHF will collectively cost Southeastern Asian nations US\$2.36 billion in lost productivity, health clinic visits, hospitalization, medications, travel expenses, and parents' time seeking treatment for their children (Cattand 2006). Additional indirect costs in the form of lost tourism will undoubtedly send these figures far higher (Cattand 2006). When the afflicted regions and populations are considered, it is clear that DEN could result in substantial economic and societal impacts that are born largely by the communities and countries that can least afford it.

Development of a vaccine to combat this global affliction has proven to be difficult (Hatch, Mathew et al. 2008). While infection with one of the DEN serotypes provides life-long protection (Guzman, Kouri et al. 1990; Ooi, Goh et al. 2006), after a short period of cross protection lasting approximately 2-9 months an individual becomes susceptible to infection with one of the other three serotypes of DEN (Sabin 1952; Wearing and Rohani 2006). Subsequent secondary infection with any of the other three serotypes of DEN is strongly correlated with severe disease (Halstead, Nimmannitya et al. 1970). It has been postulated that this severe secondary reaction is a result of sub-

neutralizing levels of antibody specific to the primary infection binding to the secondary DENV creating complexes, which are then directed to mononuclear phagocytes, the favored target for dengue infection (Kou, Quinn et al. 2008). This antibody-dependent enhancement (ADE) model is based on the theory that the more mononuclear phagocytes infected, the more severe the illness in the infected individual (Halstead 1988). *In vitro* studies supporting the ADE model have shown that DEN replicates to higher titers in cultured human monocytes in the presence of sub-neutralizing levels of DEN antibody (Halstead and O'Rourke 1977; Kou, Quinn et al. 2008). ADE has also been demonstrated in a non-human primate model of infection. When rhesus monkeys were infected with any of the four serotypes of DEN and then challenged with a heterologous DEN, the mean peak viremia titers were found to be significantly higher than in primary DEN infections (Halstead 1988).

Given the dangers associated with the ADE phenomenon and the wide circulation of all four serotypes of DEN, immunization against a single serotype might be more dangerous to the recipient than not being immunized at all. Consequently, current research is focused on developing a tetravalent vaccine that can immunize against all four serotypes of DEN simultaneously. However, finding the correct balance of immunogens for a tetravalent vaccine has proven challenging; Sanofi-Pasteur has been working to perfect the formulation of their tetravalent DEN vaccine since the early 1990's and has already spent millions of euros in its development. Currently, the Sanofi-Pasteur three-injection vaccine is in clinical trials in Mexico, Peru, Thailand, Singapore,

Vietnam, and the Philippines, in both children and adults. Initial results from the studies are positive and the company has invested 350 million euros in the construction of a manufacturing facility for the vaccine (Sanofi-Aventis 2009).

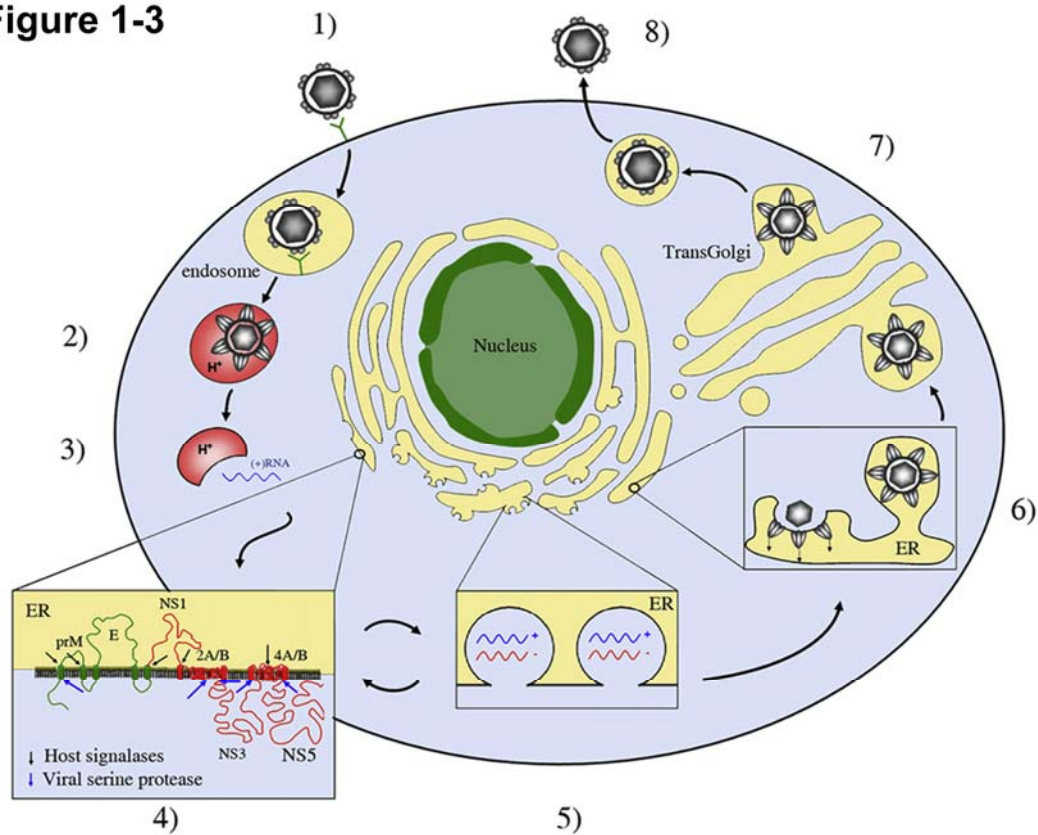
In addition to the current lack of a tetravalent vaccine, there are no specific antiviral treatments available for DF. Given the global impact of DEN, this gap in healthcare service is a desperate deficiency. It is hoped that a more complete understanding of how the virus interacts with its hosts will aid in the development of specific antiviral therapies.

1.2 *Flavivirus Life Cycle*

The first step in the initiation of a successful infection is the entry of the virus into a target host cell. Several different molecules have been suggested to function as the DEN receptor including heparan sulfate, heat shock protein 70 (HSP70), HSP90, GRP78/Bip, a 37/67-kDa high-affinity laminin receptor, the mannose receptor (MR) and the dendritic cell edocytotic receptor dendritic-cell-specific ICAM3-grabbing non-integrin (DC-SIGN) (Chen, Maguire et al. 1997; Salas-Benito and del Angel 1997; Hung, Lee et al. 1999; Martinez-Barragan and del Angel 2001; Navarro-Sanchez, Altmeyer et al. 2003; Tassaneetrithep, Burgess et al. 2003; Jindadamrongwech, Thepparit et al. 2004; Thepparit and Smith 2004; Reyes-Del Valle, Chavez-Salinas et al. 2005; Miller, deWet et al. 2008). Whether any of these serve as the high affinity receptor required for virus internalization or are simply utilized by the virus as tethers to the target cell until the actual high affinity receptor is encountered is a matter of some debate in the field.

Indeed, the latter appears to be the case for DC-SIGN which interacts with glycosylated residues on the viral envelope (E) protein (Mondotte, Lozach et al. 2007). When an endocytosis-defective DC-SIGN molecule was expressed in Langerhans cells it allowed efficient DEN internalization and replication, indicating that DC-SIGN endocytosis is dispensable for the internalization step in DV entry. These results suggest a mechanism by which DC-SIGN enhances DEN entry and infection in combination with a second, as yet unidentified receptor (Lozach, Burleigh et al. 2005). Regardless of which receptor the DENs are using to initiate their entry, it is clear that flaviviruses are internalized via the clathrin-mediated endocytic pathway in both mammalian and insect cells (Gollins and Porterfield 1985; Gollins and Porterfield 1986; Ishak, Tovey et al. 1988; Ng and Lau 1988; Heinz, Stiasny et al. 1994; Andoh, Kawamata et al. 1998; Nawa 1998; Chu and Ng 2004; Chu, Leong et al. 2006; Krishnan, Sukumaran et al. 2007; Acosta, Castilla et al. 2008; Mosso, Galvan-Mendoza et al. 2008) (Figure 1-3). Upon internalization, the virus containing vesicles are transported along the endocytic pathway to early endosomes where they are acidified by the V-ATPase proton pump. Acidification of these endosomal compartments initiates a pH-dependent rearrangement of the E proteins on the surface of the virion. This rearrangement allows the exposed fusion domain to penetrate the endosomal membrane and facilitates the release of the viral nucleocapsid into the cytosol (Rey, Heinz et al. 1995; Kuhn, Zhang et al. 2002; Zhang, Corver et al. 2003; Modis, Ogata et al. 2004). Inhibition of Rab5, a GTPase that regulates trafficking of vesicles to early endosomes (Simonsen, Lippe et al. 1998), restricts viral replication as

Figure 1-3



The Flavivirus Lifecycle Virions are recognized by an as yet unidentified receptor and internalized via receptor-mediated endocytosis (1). Virions are trafficked through the endosomal pathway (2) where the acidic environment induces conformational changes in the E protein leading to fusion of the viral and host membranes and release of the nucleocapsid into the cytoplasm (3). The genomic RNA associates with the host translation machinery and produces a single viral polyprotein which is then cleaved by host and viral proteases (4). The cleavage sites of the structural (green) and nonstructural (red) proteins and their relative positioning in relation to the ER membrane are illustrated. Following translation, replication complexes form in virus-induced membranes and (-) strand copies of the genomic RNA are produced which in turn serve as templates for (+) strand synthesis. Nascent (+) strand RNAs can then return to translation/replication phase for production of more viral proteins/RNAs or can associate with C proteins into nucleocapsids and be assembled into virions (6). Immature virions bud into the lumen of the ER and transit through the host secretory pathway where prM is cleaved by the host protease furin rendering the virions infectious (7). Mature virions are then released from the cell via exocytosis (8).
Figure is adapted from Fernandez-Garcia *et al* 2009.

does inhibition of V-ATPase function (Andoh, Kawamata et al. 1998; Nawa 1998; Krishnan, Sukumaran et al. 2007; Mosso, Galvan-Mendoza et al. 2008; Sessions, Barrows et al. 2009). In contrast, the process of viral genome disassociation from the nucleocapsid upon release into the cytosol is poorly understood and warrants further investigation (Lindenbach and Rice 2003).

Upon entering the cytoplasm, the DEN RNA associates with the host translation machinery and initiates translation of the viral genome. The DEN genome is a positive stranded RNA of ~10.5kb in total length. It has a 5' type I cap (m⁷G5'ppp5'A) structure yet lacks a poly A tail (Chambers, Hahn et al. 1990). The genomic RNA, which also serves as the viral mRNA, has one open reading frame and is translated as a single polyprotein. The viral polyprotein is cleaved by both viral and host proteases to yield three structural proteins: Capsid, preMembrane, Envelope (C, prM, and E) and seven nonstructural proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5. The open reading frame is flanked by a short ~100nt untranslated region (UTR) at its 5' end and a longer UTR of ~ 450nt at its 3' end, both of which associate with host proteins and form structures essential for virus viability. The known and predicted functions and interactions of these viral proteins and RNAs are described in more detail below.

Studies have shown that translation of DEN genomic RNA can be mediated by both cap-dependent and cap-independent mechanisms (Edgil, Polacek et al. 2006). Upon synthesis and maturation of the viral proteins, replication of negative (-) RNA strands ensue and continue throughout the viral life cycle (Wengler, Wengler et al. 1978;

Cleaves, Ryan et al. 1981). These (-) strand RNAs in turn serve as templates for the synthesis of nascent (+) strands (ibid). Viral RNA accumulation is asymmetric; the relative ratio of (+) to (-) strands ranges from 10:1 to 100:1 (Cleaves, Ryan et al. 1981). The replication complexes are membrane-anchored and are primarily localized in the perinuclear region; a feature conserved among the entire Flaviviridae family (Mackenzie and Westaway 2001; Uchil and Satchidanandam 2003). Viral particle assembly is initiated when the homodimeric form of the C protein interacts with viral genomic RNA in the cytoplasm to form the nucleocapsid (Ng, Tan et al. 2001). The nucleocapsid then buds into the ER lumen to form viral particles with the prM and E proteins, a process that has been shown to require the activity of c-SRC protein kinase and alpha-glucosidase (Courageot, Frenkiel et al. 2000; Zhang, Corver et al. 2003; Ma, Jones et al. 2004; Wang, Syu et al. 2004; Chu and Yang 2007). Newly formed virus particles migrate from the peri-nuclear region to the cell surface through the host secretory pathway by a mechanism that involves both actin filaments and microtubules (Chu and Ng 2002; Chu, Choo et al. 2003). As the immature particles transit through the trans-Golgi network, prM is cleaved by the host protease enzyme furin allowing the E proteins to form homodimers and the virion to assume its mature conformation (Stadler, Allison et al. 1997; Li, Lok et al. 2008; Yu, Zhang et al. 2008). Mature virions are then released from the host cell via exocytosis (Ishak, Tovey et al. 1988).

1.3 Molecular biology of flaviruses

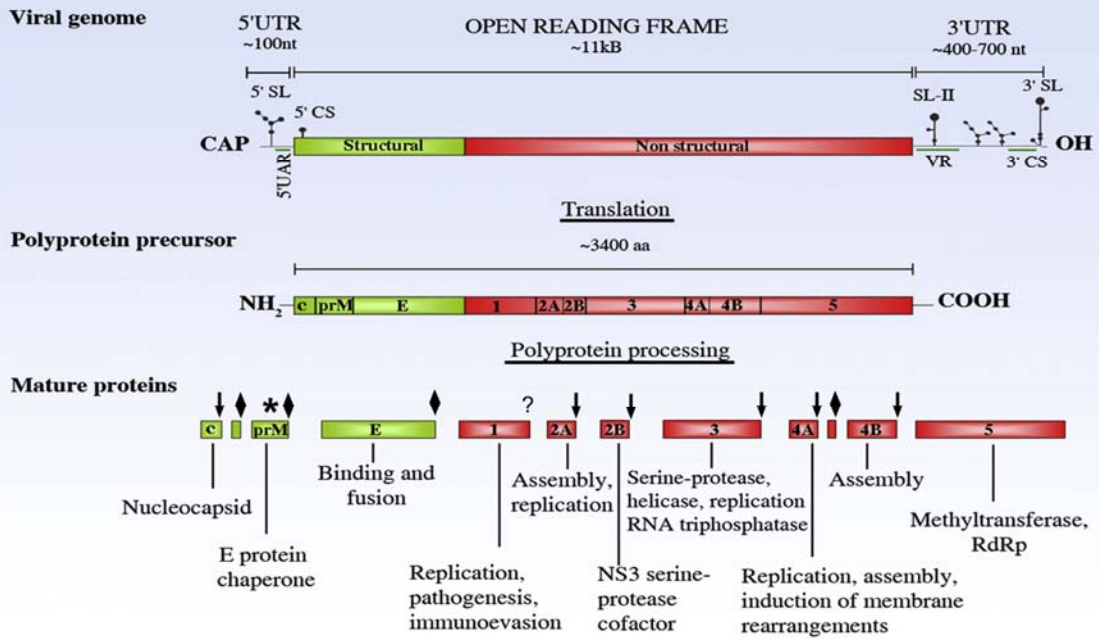
DEN1-4 are members of the flavivirus genus of the *Flaviviridae* family of viruses. Although there has been excellent work done in the more disparate genus within the *Flaviviridae* family of viruses especially in the case of HCV, the functions and interactions described below are primarily referenced from the mosquito and tick-borne genus of *Flavivirus* literature unless otherwise indicated.

1.3.1 Structural Proteins

The structural proteins are located within the amino-terminal portion of the viral polyprotein while the nonstructural proteins are contained in the carboxy-terminal region (Figure 1-4). The most amino-terminal protein is the Capsid (C) which is initially translated in an immature precursor form. Cleavage of a ~20 residue hydrophobic sequence in the carboxy terminus of the precursor C protein by host signal peptidase may serve as a signal sequence required for the proper localization of the prM protein to the lumen of the endoplasmic reticulum (ER) (Amberg, Nestorowicz et al. 1994; Amberg and Rice 1999). A second cleavage by the viral serine protease upstream of this residue produces the mature C protein (Amberg, Nestorowicz et al. 1994; Amberg and Rice 1999).

The mature C protein is 100 amino acids long with highly basic residues at its amino and carboxy termini and contains a hydrophobic domain in its central portion that interacts with cellular membranes and may play a role in virion assembly (Markoff,

Figure 1-4



Flavivirus genome structure. The single open reading frame encodes the viral polyprotein which is then co- and posttranslationally cleaved into the three structural proteins (green) and seven nonstructural proteins (red). Cleavage by the viral serine protease is indicated by (♦), cleavage by the host signal peptidase is indicated by (♦), and cleavage by furin is indicated by (*). Putative functions of the viral proteins are indicated. Figure is adapted from Fernandez-Garcia *et al* 2009.

Falgout et al. 1997). Recombinant C protein exists as a dimer *in vitro* (Lopez, Gil et al. 2009). Approximately 180 copies of the mature C protein interacts with a single viral RNA to form the nucleocapsid complex (Lindenbach and Rice 2003; Zhang, Kostyuchenko et al. 2007). Viral RNA in complex with the C proteins do not appear to be highly structured within the viral particle although the possibility of a unique structure has not been formally disproven (Zhang, Kostyuchenko et al. 2007).

Mutational analyses of the C protein reveal that it is able to retain its nucleating functions and produce viable virus even with relatively large deletions at its amino and carboxy termini and, to a lesser degree, within the hydrophobic region (Kofler, Heinz et al. 2002; Patkar, Jones et al. 2007). This resilience to mutation may be one of the reasons there is low sequence conservation in the C protein across the flavivirus genus (Patkar, Jones et al. 2007). During infection, the C protein has been observed to localize to the nucleus and the nucleolus although exactly what function it is performing in these subcellular locations has yet to be elucidated (Westaway, Khromykh et al. 1997; Wang, Syu et al. 2002; Mori, Okabayashi et al. 2005; Oh, Yang et al. 2006; Sangiambut, Keelapang et al. 2008).

As mentioned above, cleavage of the ~20 hydrophobic amino acid residue in the carboxy terminus of the precursor C protein by the host signal peptidase serves as a signal sequence required for the proper localization of the 26 kDa pre-Membrane (prM) protein to the lumen of the endoplasmic reticulum (ER) (Amberg, Nestorowicz et al. 1994; Amberg and Rice 1999) (Figure 1-4). This cleavage event is temporally connected

to and dependent on the upstream cleavage by the viral serine protease (Amberg, Nestorowicz et al. 1994; Amberg and Rice 1999). Mutations in the prM signal peptide sequence which enhance cleavage by the signal peptidase abolish infectious virion production suggesting that temporal regulation of these processing events is vital to the viral life cycle (Lee, Stocks et al. 2000).

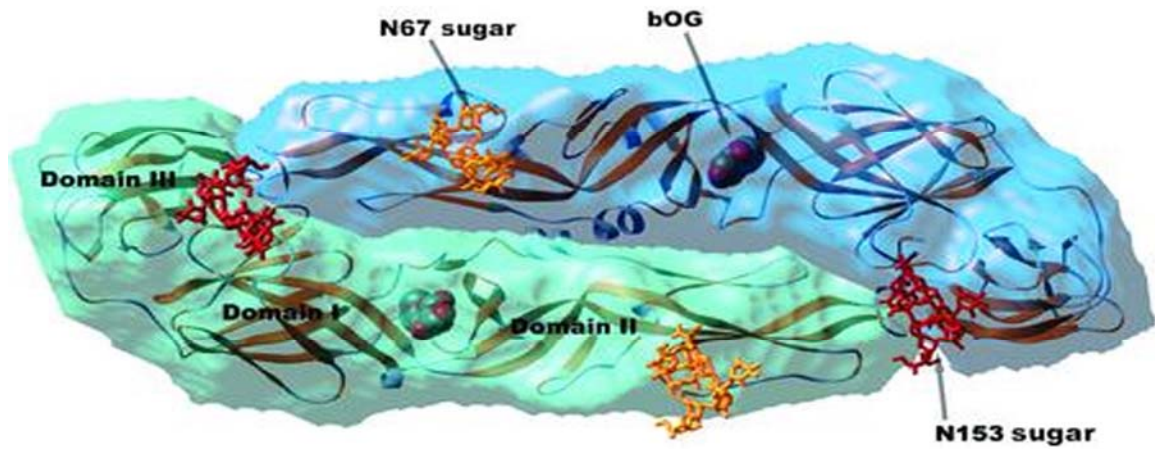
The primary function of the prM protein is to protect the fusogenic domain of the E protein by forming a heterodimer during the assembly and egress of newly synthesized viral particles (Heinz, Stiasny et al. 1994; Li, Lok et al. 2008; Yu, Zhang et al. 2008). This protective function is performed by the hydrophilic “pr” domain of M which is cleaved by the host protease furin as nascent virions transverse through the trans-golgi network and are released into extracellular environment (Stadler, Allison et al. 1997; Li, Lok et al. 2008; Yu, Zhang et al. 2008). Blocking prM cleavage by inhibition of furin results in the exclusive release of non-infectious, immature viral particles (Stadler, Allison et al. 1997). A recent study by Duan *et al* using a yeast two-hybrid screen has identified an interaction of the prM protein with the V-ATPase proton pump, suggesting a novel requirement for this interaction in viral egress (Duan, Lu et al. 2008).

Together with the mature M protein, the membrane associated 50 kDa Envelope (E) glycoprotein form the proteinaceous coat of the flaviviral particle (Lindenbach and Rice 2003). The atomic structures for the soluble ectodomains of several flavivirus E proteins have been solved using X-ray crystallography and comparison has revealed that structurally they are very similar (Rey, Heinz et al. 1995; Modis, Ogata et al. 2003;

Modis, Ogata et al. 2005). The flavivirus E ectodomain consists of three domains (Figure 1-5). Domain I forms the central portion of the E protein structure and contains the amino terminus. Domain II is the primary dimerization domain and contains a small hydrophobic sequence at its distal end that serves to initiate fusion with host cell membranes (Allison, Schalich et al. 2001). A hydrophobic pocket between domains I and II has been proposed as the hinge point in the fusion-activating conformational change (Modis, Ogata et al. 2003; Rey 2003). Glycosylation at two residues in domain II (Asn 67 and Asn 153) have been shown to be important for DEN infectivity in human cells but are dispensable for the infectivity in mosquito cells (Mondotte, Lozach et al. 2007). Domain III is an Ig-like domain that contains the majority of serotype-specific host antibody neutralization sites and has been shown in WNV to be involved in receptor binding (Chu and Ng 2004; Gromowski and Barrett 2007).

As stated previously, E proteins form heterodimers with uncleaved prM during their transit through the golgi network (Heinz, Stiasny et al. 1994; Li, Lok et al. 2008). Upon cleavage of the pr domain from prM (Figure 1-4), 180 E monomers assemble into 90 homodimers in a head-to-tail configuration that lie flat against the surface of the viral particle (Rey, Heinz et al. 1995; Kuhn, Zhang et al. 2002). Upon exposure to low pH, E dimers are postulated to undergo rotational rearrangements around the three- and five-fold axes of symmetry to form fusion competent homotrimeric complexes with their fusion peptides exposed at the tip of the trimer (Heinz, Stiasny et al. 1994; Allison,

Figure 1-5



Dengue virus E protein The two subunits of a dengue virus E homodimer are depicted in different colors for clarity. Domain I, II, and III are labeled as are the two glycosylation sites at N67 and N153. A molecule of the detergent beta-*N*-octylglucoside (indicated by bOG) added during crystallization marks the pocket at the hinge region between domains I and II. Figure is adapted from: Rey 2003

Schalich et al. 2001; Stiasny, Allison et al. 2001; Kuhn, Zhang et al. 2002; Modis, Ogata et al. 2004).

1.3.2 Nonstructural Proteins

The 46 kDa nonstructural protein 1 (NS1) is a highly immunogenic glycoprotein that has been shown to be required for viral replication (Lindenbach and Rice 1997; Hall, Khromykh et al. 1999; Khromykh, Sedlak et al. 1999; Lindenbach and Rice 1999). It has been shown that NS1 is liberated from the carboxy terminus of the E protein by the host signal peptidase however, the protease responsible for cleavage of the NS1-NS2a junction is unknown (Falgout, Chanock et al. 1989; Chambers, McCourt et al. 1990; Falgout and Markoff 1995) (Figure 1-4).

Within twenty to forty minutes following translation, NS1 forms very stable homodimers that are required for its function in replication (Winkler, Maxwell et al. 1989; Hall, Khromykh et al. 1999). In addition to its role in the replication complex, NS1 dimers have been shown to localize to the cell surface and to be secreted from infected mammalian cells (Winkler, Randolph et al. 1988; Post, Carvalho et al. 1991; Crooks, Lee et al. 1994). What role extracellular NS1 may be playing is unclear. However, a study of children in Thailand showed that high amounts of circulating NS1 during DEN infection was positively correlated with the development of DHF suggesting a role for NS1 as a potential virulence factor (Libraty, Young et al. 2002). Interestingly, NS1 is not secreted from infected mosquito cells which may partly explain the lack of overt pathology in the

insect vector (Winkler, Randolph et al. 1988; Mason 1989; Post, Carvalho et al. 1991; Crooks, Lee et al. 1994; Brinton 2002; Macdonald, Tonry et al. 2005).

NS1 has been found to interact with several host factors (Chua, Bhuvanakantham et al. 2005; Chung, Liszewski et al. 2006; Kurosu, Chaichana et al. 2007; Noisakran, Sengsai et al. 2008; Wilson, de Sessions et al. 2008). Co-immunoprecipitation studies with NS1 and human cell extracts followed by mass-spec analysis revealed interactions with factor H and clusterin indicating that NS1 may be actively regulating the complement regulatory pathway (Chung, Liszewski et al. 2006; Kurosu, Chaichana et al. 2007). Intracellular NS1 has been shown both to interact with an N-terminally truncated form of STAT3beta and to block TLR3-mediated signaling suggesting that NS1 antagonizes the innate immune response (Chua, Bhuvanakantham et al. 2005; Wilson, de Sessions et al. 2008). NS1 was also found to interact and co-localize to a limited degree with hnRNP C1/C2 inside infected cells although the significance of this finding remains to be seen (Noisakran, Sengsai et al. 2008).

Nonstructural protein 2a (NS2a) is a small ~22 kDa hydrophobic protein that has been suggested to play a role in virion assembly although the mechanism involved remains unclear (Kummerer and Rice 2002; Liu, Chen et al. 2003). NS2a has been reported to interact with NS3, NS5 and the viral 3'UTR and has been suggested to play a role in the shift between viral RNA replication and RNA packaging (Mackenzie, Khromykh et al. 1998; Lindenbach and Rice 2003) (Figure 1-4).

The ~14kDa membrane-associated nonstructural protein 2b (NS2b) is a required co-factor for the NS3 viral serine protease (Chambers, Grakoui et al. 1991; Falgout, Pethel et al. 1991; Arias, Preugschat et al. 1993; Chambers, Nestorowicz et al. 1993; Falgout, Miller et al. 1993; Jan, Yang et al. 1995) (Figure 1-4). In addition to its role as a cofactor for the viral serine protease, NS2b has also been shown to be involved in membrane permeability although the purpose of this function in terms of the viral life cycle has yet to be elucidated (Chang, Liao et al. 1999).

The ~70kDa nonstructural protein 3 (NS3) has several functions essential for the viral life cycle. The amino terminal domain functions as the viral serine protease in coordination with NS2b and is responsible for cleavage of the viral polyprotein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions (Chambers, Grakoui et al. 1991; Arias, Preugschat et al. 1993; Jan, Yang et al. 1995; Li, Clum et al. 1999; Murthy, Clum et al. 1999) (Figure 1-4). The carboxy terminal domain binds both NS5 and the 3'UTR and has been shown to have helicase (Wengler, Czaya et al. 1991; Warrener, Tamura et al. 1993; Takegami, Sakamuro et al. 1995; Kuo, Chin et al. 1996; Li, Clum et al. 1999; Utama, Shimizu et al. 2000; Utama, Shimizu et al. 2000) as well as triphosphatase activities (Wengler and Wengler 1993), which are required for viral propagation. Full-length NS3 has also been shown through complementation analysis to be required in *cis* for assembly of infectious virions (Liu, Sedlak et al. 2002).

NS3 is located primarily in the perinuclear convoluted membrane (CM) structures which may be derived from the ER, golgi apparatus and/or trans-golgi

network membranes upon flaviviral infection and are assumed to be the sites of viral replication (Westaway, Mackenzie et al. 1997; Mackenzie, Khromykh et al. 1998; Wang, Liao et al. 1998; Mackenzie, Jones et al. 1999; Westaway, Khromykh et al. 1999). Expression of exogenous NS2b/NS3 in the absence of any other viral proteins or RNAs has been shown to be sufficient to initiate reorganization of both microtubule networks and host membranes into CM-like structures (Chiou, Hu et al. 2003). Consistent with this observation, NS3 has been shown to interact with microtubules, possibly through its interaction with tumor susceptibility gene 101 (TSG101) which has been shown to directly interact with tubulin and promote the destabilization of the microtubule structure (Ng and Hong 1989; Cassimeris 2002; Chu and Ng 2002; Chiou, Hu et al. 2003). The interaction of NS3 with TSG101 is intriguing as this protein has also been shown to interact with both the Human Immunodeficiency Virus-1 (HIV-1) Gag protein and the Ebola virus EbVp40 protein to facilitate virion budding and release from cells, suggesting a common mechanism for viral budding and release among disparate RNA viruses (Garrus, von Schwedler et al. 2001; Martin-Serrano, Zang et al. 2001; VerPlank, Bouamr et al. 2001; Chiou, Hu et al. 2003). In addition to microtubules and TSG101, NS3 has also been found to interact with nuclear receptor binding protein (NRBP) and induces its re-localization to CM structures (Chua, Ng et al. 2004). NRBP has been shown to be involved in the processes of ER to Golgi transport possibly through its interaction with Rac3, a member of the small GTPases of the Rho family (De Langhe, Haataja et al. 2002). The La protein has also been demonstrated to be a binding partner

of NS3, NS5 and the viral RNA although the functional significance of this observation remains to be seen (Garcia-Montalvo, Medina et al. 2004).

The small, hydrophobic nonstructural proteins 4a and 4b (NS4a and NS4b) (16 and 27 kDa respectively) are believed to play roles in RNA replication based on their co-localization with replication complexes and the interaction between NS4a and NS1 (Mackenzie, Khromykh et al. 1998; Lindenbach and Rice 1999). Cleavage of the NS4a-NS4b junction appears to follow the same pattern as the C-prM junction; the hydrophobic peptide linking NS4a to NS4b must first be cleaved at its amino terminus on the cytosolic surface by the viral serine protease before it becomes available for cleavage by host signal peptidase (Preugschat and Strauss 1991; Lin, Amberg et al. 1993) (Figure 1-4). A recent study by Roosendaal *et al* has suggested that this regulated cleavage event is essential for rearrangement of host membranes into CM (Roosendaal, Westaway et al. 2006).

In addition to reported role in membrane rearrangement, NS4a and NS4b have been shown to be involved in antagonizing the interferon response by inhibiting the phosphorylation of JAK and Tyk2 thus blocking phosphorylation of the STAT proteins and the activation of IFN-induced genes (Munoz-Jordan, Sanchez-Burgos et al. 2003; Lin, Liao et al. 2004; Best, Morris et al. 2005; Guo, Hayashi et al. 2005; Jones, Davidson et al. 2005; Munoz-Jordan, Laurent-Rolle et al. 2005; Miller, Sparacio et al. 2006). The RNA helicase DDX42 protein was identified as a specific binding partner of NS4A and its overexpression inhibits NS4a ability to antagonize the interferon pathway (Lin, Cheng et

al. 2008). NS4b appears to be the more potent inhibitor of the interferon pathway and this activity has been mapped to the amino terminal region of the protein (Munoz-Jordan, Laurent-Rolle et al. 2005). Proper cleavage by the viral serine protease from the hydrophobic peptide linking it to NS4a is required for this inhibitory activity (Munoz-Jordan, Laurent-Rolle et al. 2005).

NS4b also has a role in viral replication through its ability to enhance the helicase activity of NS3 (Umareddy, Chao et al. 2006). Additionally, NS4b has also been observed to localize to the nucleus during the later stages of infection although the functional significance of this migration is unknown (Westaway, Khromykh et al. 1997).

The ~103kDa nonstructural protein 5 (NS5) is a well-conserved, multifunctional protein containing a methyltransferase domain at its amino terminus which is required for the formation of the cap structure at the 5' end of the viral genome and a domain with an RNA-dependent RNA polymerase (RdRP) domain responsible for genome replication at its carboxy terminus (Rice, Lenches et al. 1985; Grun and Brinton 1986; Guyatt, Westaway et al. 2001; Egloff, Benarroch et al. 2002) (Figure 1-4). NS5 has been shown to directly interact with the 3'UTR and with NS3 (Kapoor, Zhang et al. 1995; Chen, Kuo et al. 1997). The region between the methyltransferase and RdRP domains has been shown to mediate the interaction with NS3 (Kapoor, Zhang et al. 1995).

The NS5 protein has been shown to exist in a phosphorylated form; a feature that is conserved throughout the Flaviviridae family (Reed, Gorbalenya et al. 1998). Phosphorylation appears to disrupt the interaction between NS5 and NS3; moreover,

phospho-NS5 localizes to the nucleus where it has been demonstrated to modulate levels of interleukin-8 (Buckley, Gaidamovich et al. 1992; Kapoor, Zhang et al. 1995; Brooks, Johansson et al. 2002; Pryor, Rawlinson et al. 2007). Localization of hyperphosphorylated NS5 to the nucleus is mediated by its interaction with importin alpha/beta (Johansson, Brooks et al. 2001; Brooks, Johansson et al. 2002; Medin, Fitzgerald et al. 2005; Pryor, Rawlinson et al. 2007). In addition to its interaction with importin alpha/beta, NS5 has also been shown to be exported from the nucleus via its interaction with the exportin, CRM1 (Rawlinson, Pryor et al. 2009). The ability to shuttle between the nucleus and the cytoplasm is required for viral viability; mutation of either the nuclear localization signal or the nuclear export signal are lethal to the virus (Pryor, Rawlinson et al. 2007; Rawlinson, Pryor et al. 2009). Additionally, DEN NS5 has also been shown to bind the tight junction protein zonula occludens-1 (ZO-1) in the nucleus although the functional significance of this finding has yet to be determined (Ellencrona, Syed et al. 2009).

1.3.3 Viral RNAs

The 5' and 3' terminal regions (TRs) of the viral genome which include the 5' and 3' UTRs as well as the adjacent coding regions of (+) strand RNA play an essential role in both translation of the viral polyprotein (Holden and Harris 2004; Chiu, Kinney et al. 2005; Clyde, Kyle et al. 2006) and in RNA replication (Cahour, Pletnev et al. 1995; Ackermann and Padmanabhan 2001; You, Falgout et al. 2001; Yu, Nomaguchi et al. 2008). These regions and the complementary (-) strand sequences are predicted to form

structures that are well conserved among flaviviruses and are known to be functionally significant (Brinton and Dispoto 1988; Mohan and Padmanabhan 1991; Cahour, Pletnev et al. 1995; Proutski, Gould et al. 1997; Leitmeyer, Vaughn et al. 1999; Brinton 2002; Markoff 2003; Thurner, Witwer et al. 2004; Yu, Nomaguchi et al. 2008).

In the 5'UTR, a hair pin structure in the capsid coding region (cHP) is required for proper translation initiation at the suboptimal start site of the polyprotein (Clyde and Harris 2006). In their 3'UTR, all flaviviruses have a conserved stem loop structure known as the 3' terminal stem loop (3'SL) and both the stem and the loop are required for RNA replication (Zeng, Falgout et al. 1998; Khromykh, Kondratieva et al. 2003; Elghonemy, Davis et al. 2005; Tilgner, Deas et al. 2005; Yu and Markoff 2005) although its requirement for translation appears to be species specific (Khromykh, Kondratieva et al. 2003; Holden and Harris 2004; Elghonemy, Davis et al. 2005; Tilgner, Deas et al. 2005; Yu and Markoff 2005; Edgil, Polacek et al. 2006). Consistent with its requirement for replication, the 3'SL is predicted to directly interact with NS3 and NS5 (Khromykh, Sedlak et al. 2000).

In addition to the viral proteins, the 3'UTR has also been shown to functionally interact with TIAR, YB-1, eEF1A, La and PTB (De Nova-Ocampo, Villegas-Sepulveda et al. 2002; Li, Li et al. 2002; Davis, Blackwell et al. 2007; Emara and Brinton 2007; Paranjape and Harris 2007; Anwar, Leong et al. 2009). Long range interactions between sequences located in the capsid ORF and complementary sequences in the 3'UTR termed cyclization sequences (CS) serve to circularize the viral RNA through base pairing. The

CS are required for RNA replication though they appear to be dispensable for translation of the polyprotein (Alvarez, Lodeiro et al. 2005). Although the exact sequences are variant, CS are found throughout the flavivirus genus and are required for replication in each case indicating a conserved replication strategy (Khromykh, Meka et al. 2001; Corver, Lenches et al. 2003; Westaway, Mackenzie et al. 2003; Alvarez, Lodeiro et al. 2005; Villordo and Gamarnik 2009).

Recently, it has been shown that a subgenomic RNA with sequence identity to the 3' TR exists in all mosquito-borne flaviviruses (Lin, Chang et al. 2004; Pijlman, Funk et al. 2008). This subgenomic RNA, or small flavivirus RNA (sfRNA) is generated by incomplete digestion of the full-length viral genome by the 5'-3' exoribonuclease XRN1 in the human host, one of the key enzymes in the cellular mRNA decay pathway (Stevens 2001). sfRNAs have also been found to be produced in infected mosquito cells (Lin, Chang et al. 2004; Pijlman, Funk et al. 2008). Given that the pathways involved in mRNA decay are highly conserved between human and mosquito cells (Opyrchal, Anderson et al. 2005), it is likely that the mechanism of their generation is conserved as well (Pijlman, Funk et al. 2008). Proposed functions for this sfRNA include acting as a decoy, allowing the viral genomic RNA to evade the host antiviral defenses (Pijlman, Funk et al. 2008), and operating as a molecular switch directing the transition of viral RNA from replication to translation (Lin, Chang et al. 2004).

In addition to XRN1, TIAR, YB-1, eEF1A, La and PTB the 5' and 3' TRs of flaviviruses are predicted to functionally interact with other host proteins although the

vast majority of these proteins remain unidentified (Brinton 2001; Brinton 2002). Mutational analyses of the flaviviral TR's have shown differential effects on propagation when analyzed in mammalian or mosquito cell lines indicating that some sequences are important for viability in one host or the other, likely through their interaction with host-specific factors, while others appear to be required in both (Men, Bray et al. 1996; Zeng, Falgout et al. 1998).

1.4 The Dipteran vector

1.4.1 *Aedes aegypti*

Aedes aegypti, the primary arthropod vector for YFV and DEN1-4, is a member of the Culicidae family of mosquitoes in the order Diptera. *Aedes aegypti* originated from the Africa but over the last several hundred years has spread throughout the tropical and subtropical world (Mousson, Dauga et al. 2005; CDC 2009). The mosquito is easily recognizable by the white markings on the legs and thorax (Figure 1-6). *Aedes aegypti* are highly domesticated urban mosquitoes that prefer to lay their eggs in artificial containers such as old tires, flower pots, rainwater buckets, or any other container that will hold water in or around human dwellings (Gubler 2006). Adult mosquitoes are primarily daytime feeders and females will “sample” the same person or several people in the same room several times during a single blood meal (Gubler and Rosen 1976). This nervous feeding behavior and its preference for human dwellings conspire to make *Aedes aegypti* exceedingly efficient vectors for the transmission of DEN and YFV.

Figure 1-6



Aedes aegypti
Source: www.aegypti.vectorbase.org

DEN is transmitted to an adult female mosquito after feeding on an infected individual. Upon taking an infected blood meal, DEN first infects the midgut epithelial cells, where it replicates and produces more virions (Black, Bennett et al. 2002; Xi, Ramirez et al. 2008). It then disseminates into the hemocoel, establishing infection in other tissues such as the trachea, fat body and most importantly for the virus, the salivary glands (ibid). Infection of the salivary glands by DEN is non-pathogenic and persistent, lasting the lifetime of the mosquito (Sanchez-Vargas, Scott et al. 2009). The time required from ingestion of an infected blood meal until it can be transmitted to a human host is called the extrinsic incubation period (EIP). The EIP of DEN in the mosquito varies and is dependent on many factors including mosquito genetics, virus genotype, and environmental factors such as temperature and humidity (Watts, Burke et al. 1987; Armstrong and Rico-Hesse 2001; Black, Bennett et al. 2002; Salazar, Richardson et al. 2007; Xi, Ramirez et al. 2008). In a recently established colony of *Aedes aegypti* collected from Chetumal, Mexico, it was shown that peak viral titers were achieved between 7 and 10 days post infection (dpi) in the midgut, between 7 and 17 dpi in the abdomen and between 12 and 18 dpi in the salivary glands (Salazar, Richardson et al. 2007).

Ingestion of an infected blood meal does not guarantee infection in the mosquito. Upon ingestion, the virus encounters at least two barriers: the mesenteron infection barrier (MIB) preventing infection of the mosquito midgut and the mesenteron escape barrier (MEB) preventing dissemination of the virus into the hemocoel (Kramer and Ebel 2003). These barriers are thought to be controlled by at least two sets of genes, one

controlling the MIB and the other controlling the MEB although the identity of these genes have yet to be determined (Bosio, Fulton et al. 2000; Bennett, Olson et al. 2002; Black, Bennett et al. 2002).

The mosquito innate immunity has also been shown to play an important role in regulating infection by DEN. In a study by Xi et al, it was observed that components of the Toll pathway are specifically activated in both the midgut and the carcass upon infection with DEN (Xi, Ramirez et al. 2008). Consistent with this observation, activation of the Toll pathway via RNAi-mediated depletion of a key Toll pathway inhibitor, cactus, caused a significant decrease in the observed viral titer isolated from treated midguts (ibid).

1.4.2 *Drosophila* as a Model Organism for Mosquito

It has been estimated that the fruitfly, *Drosophila melanogaster* diverged from their mosquito cousins approximately 250 million years ago (Zdobnov, von Mering et al. 2002). *Drosophila melanogaster* is a member of the *Drosophilidae* family in the order Diptera and is one of the most commonly used model organisms in biology. Recent studies have demonstrated that *Drosophila* can support infection by *Plasmodium gallinaceum*, a relative of the human malaria parasite *Plasmodium falciparum*, as well as WNV when injected into the hemocoel (Schneider and Shahabuddin 2000; Brandt, Jaramillo-Gutierrez et al. 2008; Chotkowski, Ciota et al. 2008). These studies strongly support the hypothesis that *Drosophila*, which has been successfully utilized as a model

organism for over a century, can be used as a surrogate mosquito system to accurately identify relevant mosquito host factors.

Chapter 2. Results

2.1 Summary

Given their small genomes and therefore relatively limited coding potential, DEN1-4 and other member of the flavivirus genus are likely to require an extensive number of factors from their human and insect hosts to complete their life cycle. However, as of yet only a limited number of human and an even smaller number of Dipteran factors have been identified (Heinz, Stiasny et al. 1994; Andoh, Kawamata et al. 1998; Nawa 1998; Courageot, Frenkiel et al. 2000; Whitby, Pierson et al. 2005; Chapel, Garcia et al. 2006; Emara and Brinton 2007; Krishnan, Ng et al. 2008). In order to discover insect host factors required for DEN-2 propagation, we completed a genome-wide RNAi screen in DMe12 cells using the well-characterized dsRNA library from the *Drosophila* RNAi Screening Center (DRSC) (DRSC). This screen identified 116 candidate DEN host factors (DVHFs) (Figure 2-1). While some factors have been previously characterized and known to be important for DEN propagation, (e.g., V-ATPases and alpha-glucosidases)(Heinz, Stiasny et al. 1994; Andoh, Kawamata et al. 1998; Nawa 1998; Courageot, Frenkiel et al. 2000; Whitby, Pierson et al. 2005; Chapel, Garcia et al. 2006), the vast majority of DVHFs were newly implicated in DEN propagation. In support of our findings in the *Drosophila* model system, depleting *Aedes aegypti* DVHF homologues via RNAi led to a significant inhibition of DEN propagation in adult mosquitoes. Of the 116 Dipteran DVHFs identified, eighty-two had readily recognizable human homologues. We showed that forty-two of these homologous factors are also human

Figure 2-1

22,632 dsRNAs (~250ng) arrayed in sixty-two 384-well plates in duplicate scored for E protein expression

Filter for dsRNAs affecting final cell number

20,224 dsRNAs analyzed

Sum Rank of duplicate dsRNAs

218 candidate dsRNAs targeting DVHFs

Re-synthesis of dsRNAs for re-screening

179 candidate dsRNAs targeting DVHFs

Re-screen of dsRNAs (>1.5 fold inhibition;p<0.05)

118 dsRNAs identifying 116 DVHFs

Outline of experimental steps taken to identify DVHFs The number of dsRNAs passing through each filter are indicated. Prior to duplicate plate comparison, each dsRNA was assayed for its effect on cell proliferation. Wells with less than 12,500 cells in either duplicate were shown to provide unreliable data and removed from further consideration. The remaining wells were then compared to their duplicates for reproducibility and ranked against the rest of the wells on the plate. Only those dsRNAs duplicates with expectation ≤ 0.065 (218) were considered candidates for further investigation. 179 of the 218 candidates were re-synthesized and tested again for reproducibility of the initial observation with the additional criteria that infectivity had to be inhibited by ≥ 1.5 fold with a p-value < 0.05 . 118 dsRNAs passed these benchmarks identifying 116 unique DVHFs.

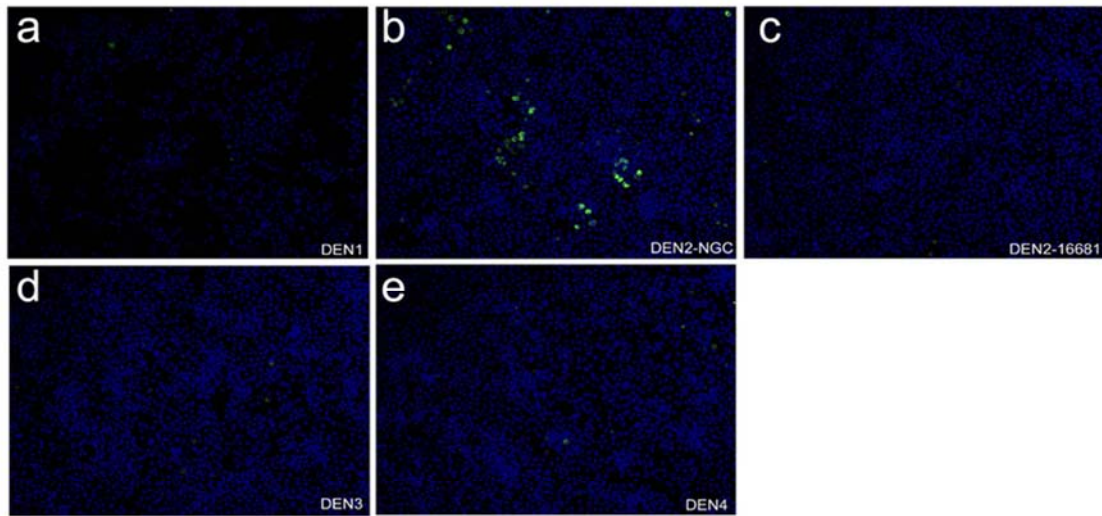
DVHFs using a targeted siRNA screen. This result suggests a substantial conservation of required factors between the Dipteran and human hosts. Preliminary characterization of several human DVHFs revealed that our screen identified factors that function at multiple points in the viral life cycle including those that act prior to accumulation of viral RNA and also factors which are required for later stages of the viral life cycle. Importantly, this work suggests novel interactions that may prove important to efforts to control infection in both insects and humans.

2.2 Adaptation of DEN2-NGC to *Drosophila* cells

DEN 1-4 are transmitted from one human host to another by mosquitoes of the *Aedes* genus, principally *Aedes aegypti* and *albopictus*(Mackenzie, Gubler et al. 2004). While there are important efforts to sequence and annotate the genomes of these vectors(Hubbard, Aken et al. 2007; Lawson, Arensburger et al. 2007; Nene, Wortman et al. 2007), there is currently an unfortunate dearth of resources to carry out systematic functional genomics in *Aedes*. In contrast, there are robust materials and methods to do so in the related Dipteran *Drosophila melanogaster*.

In order to take advantage of resources available in the *Drosophila melanogaster* system, we first had to determine whether a *Drosophila* cell line could support DEN infection. We infected *D. melanogaster* D.Mel-2 cells, a sub-clone of S2 cells, with DEN1-WestPac74, DEN2-NGC, DEN2-16681, DEN3-CH5349, or DEN4-TVP360 (all kindly provided by Dr. A. de Silva UNC-Chapel Hill). Only DEN2-NGC showed significant

Figure 2-2

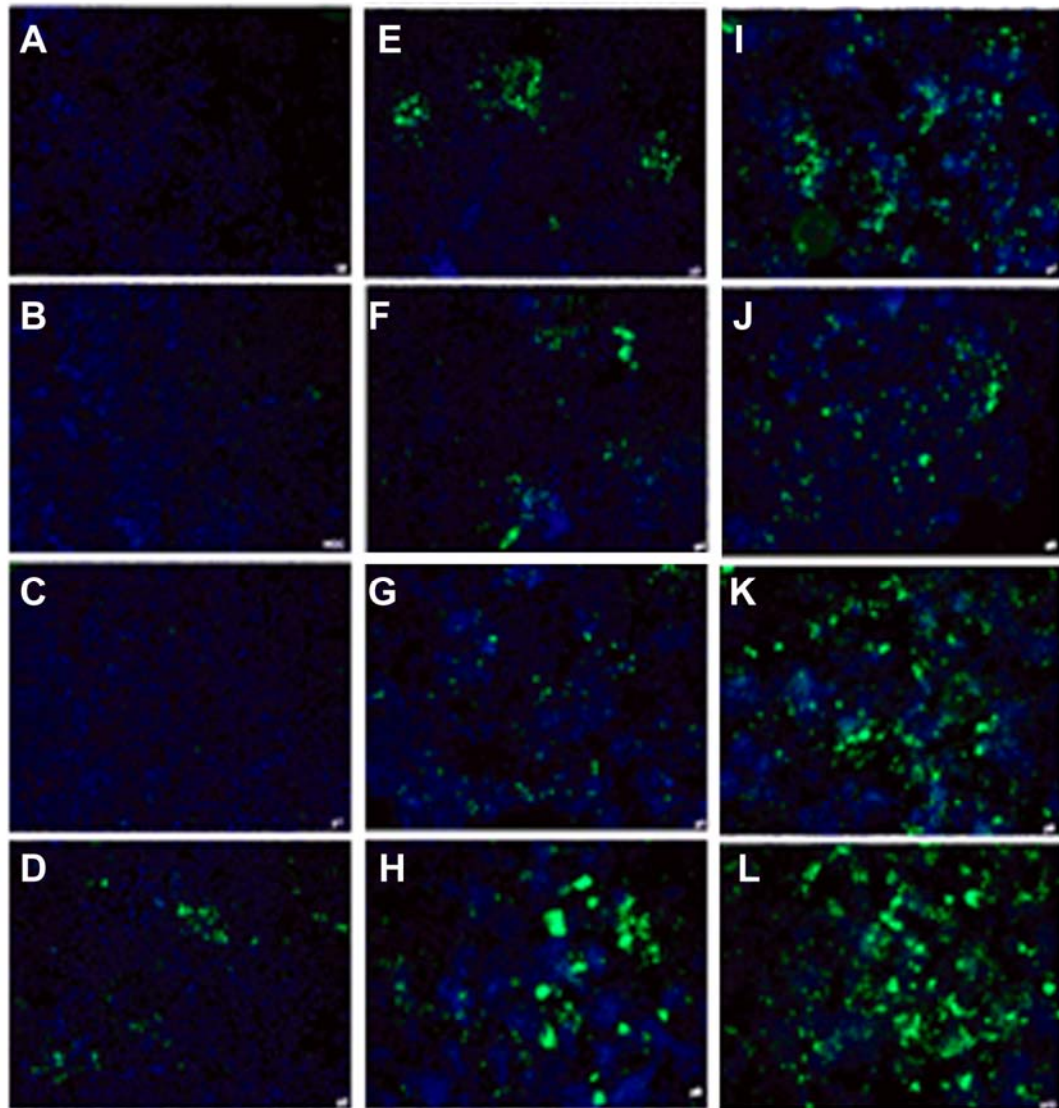


Infection of DMeI2 cells with DEN1-4 DMeI2 cells were infected with DEN1-WestPac74 (a), DEN2-NGC (b), DEN2-16681 (c), DEN3-CH5349 (d), DEN4-TVP360 (d) at an m.o.i. of 1 and infection was allowed to proceed for 72 hours. Cells were fixed, stained for E protein expression and counterstained with Hoescht.

levels of E protein expression and credible evidence of viral propagation, albeit at very low levels (Figure 2-2). Although this was encouraging, these very low levels of infection were not suitable for screening purposes. We therefore sought to take advantage of the relatively high mutation rate of DEN and isolate a strain of DEN2-NGC capable of more robust growth in *Drosophila* cells. DEN2-NGC was adapted by serial passage in D.Mel-2 cells resulting in a viral strain that evidenced a greater propensity for growth in *Drosophila* cells (Figure 2-3). This strain was named dengue 2-S2 (DEN2-S2) and propagated in D.Mel-2 cells 10-100 times better than DEN2-NGC (Figure 2-4b). Passaged virus was then amplified in C6/36 three times to produce a single viral stock for all DEN2-S2 experiments described below.

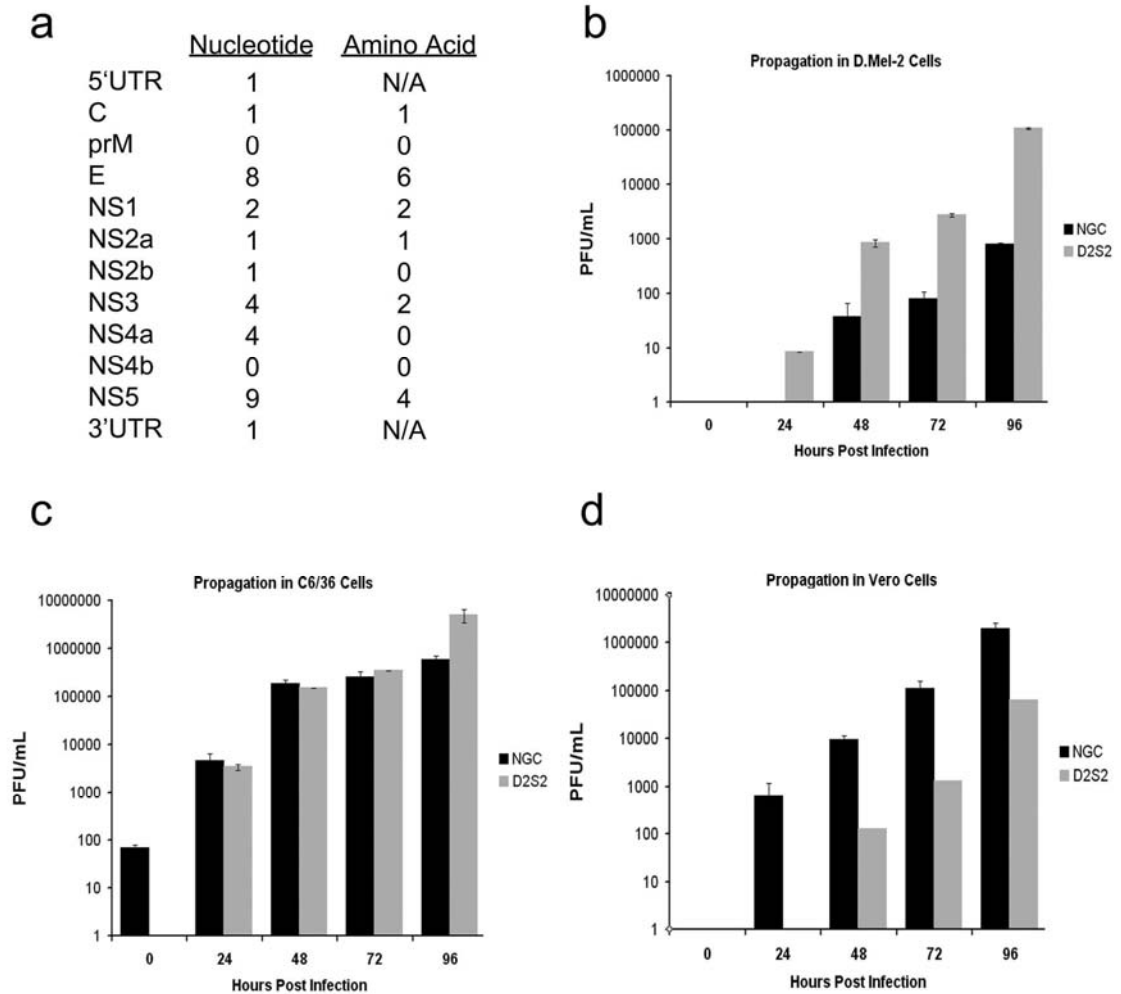
While its ability to productively infect *Aedes albopictus* C6/36 cells was equivalent to that of DEN2-NGC, DEN2-S2 was attenuated in Vero cells (Figure 2-4c,d). Sequence analysis indicates that DEN2-S2 is 99.6% identical to the parental strain at the nucleotide level. Mutations were not distributed equally across the genome; the greatest number of amino acid changes was observed in the E protein (6) followed by the NS5 protein (4) and no mutations were observed in the prM or NS4b coding regions (Figure 2-4a). Interestingly, all of the amino acid changes in the E protein mapped to domain II (Figure 2-5). The significance of this finding is not immediately apparent since receptor binding site is commonly believed to reside in domain III (Chu and Ng 2004; Gromowski and Barrett 2007). Characterization of these mutations by cloning them individually, or in combination, into the parental background may assist in

Figure 2-3



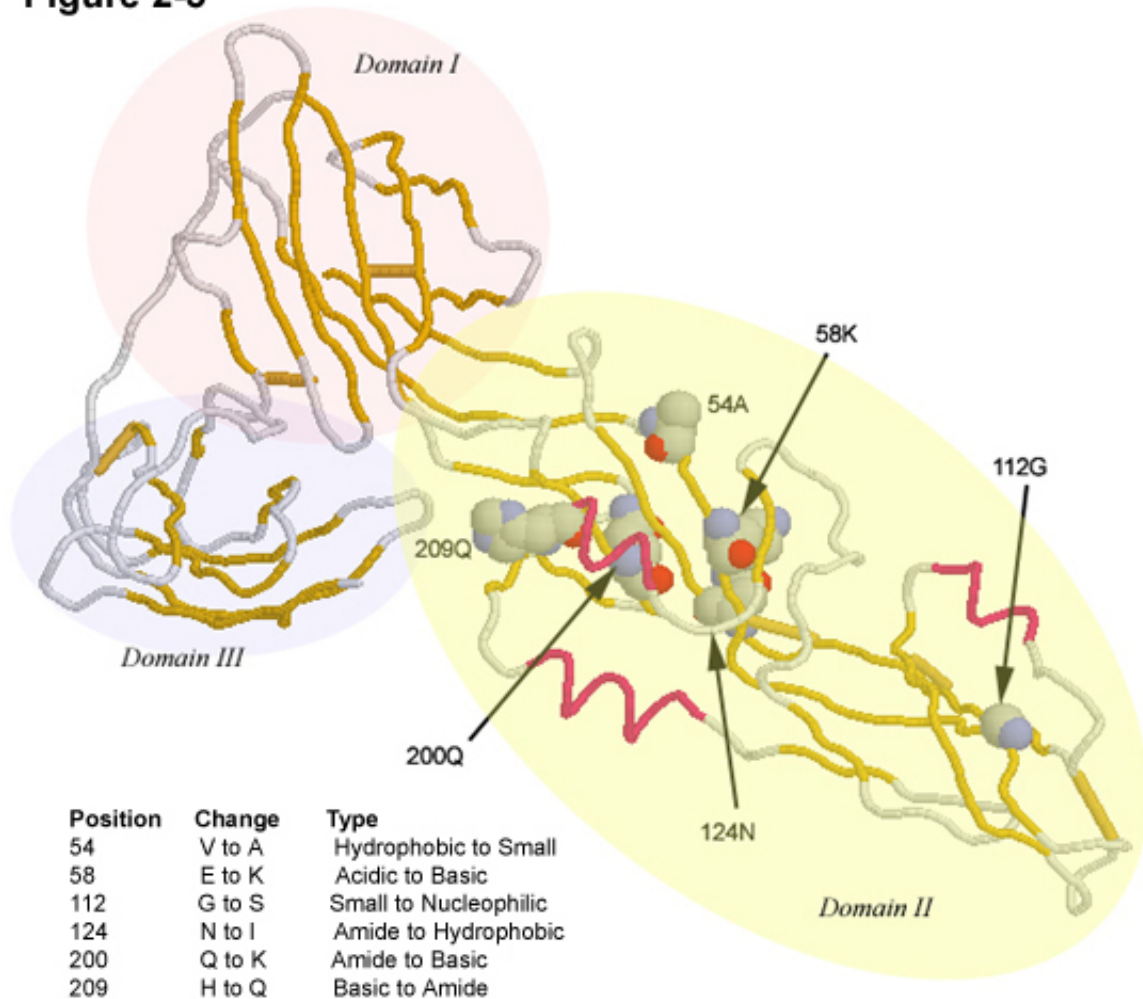
Serial passage of DEN2-NGC in Drosophila cells DMel2 cells were infected with DEN2-NGC at an m.o.i. of 5 and allowed to incubate nine days. Supernatant was then collected, cleared by centrifugation of cellular debris and used to inoculate a fresh flask of DMel2 cells (5mLs) and frozen for later analysis (remainder). After ten passages, the supernatants were titered on Vero cells and the efficacy of the procedure was analyzed by infecting DMel2 cells with viral supernatant from each passage at an m.o.i. of 1. 96 hours post infection, cells were fixed and stained for E protein expression. Representative images from each infection are shown: Uninfected (A), DEN2-NGC (B), passage 1 through 10 (C through L, respectively).

Figure 2-4



Summary of DEN2-S2 mutations and viral propagation curves in *Drosophila*, mosquito and mammalian cell lines. (A) Summary of mutations observed in DEN2-S2 at the nucleotide and amino acid levels compared to the parental DEN2-NGC strain. DEN2-NGC and its D.Mel-2 adapted derivation, DEN2-S2 were tested for their ability to propagate over 96hrs in *Drosophila* a D.Mel-2 cells (B), mosquito C6/36 cells (C), and mammalian Vero cells (D) were infected at a m.o.i. of 1 with DEN2-NGC and DEN2-S2. After one hour adsorption at 28 °C (D.Mel-2 and C6/36 cells) or 37 °C (Vero), inoculation was removed, cells were washed once with PBS and growth media was added. Supernatants were collected every 24 hours, serially diluted and added to Vero monolayers for one hour at 37°C followed by addition of a 1:1 tragacanth gum/2x EMEM overlay supplemented with 2% FBS. Cultures were allowed to incubate for 4-5 days at which point they were fixed, permeabilized, stained for DEN E-protein expression. Foci were then counted and averaged. Error bars represent the standard deviation of three independent samples.

Figure 2-5



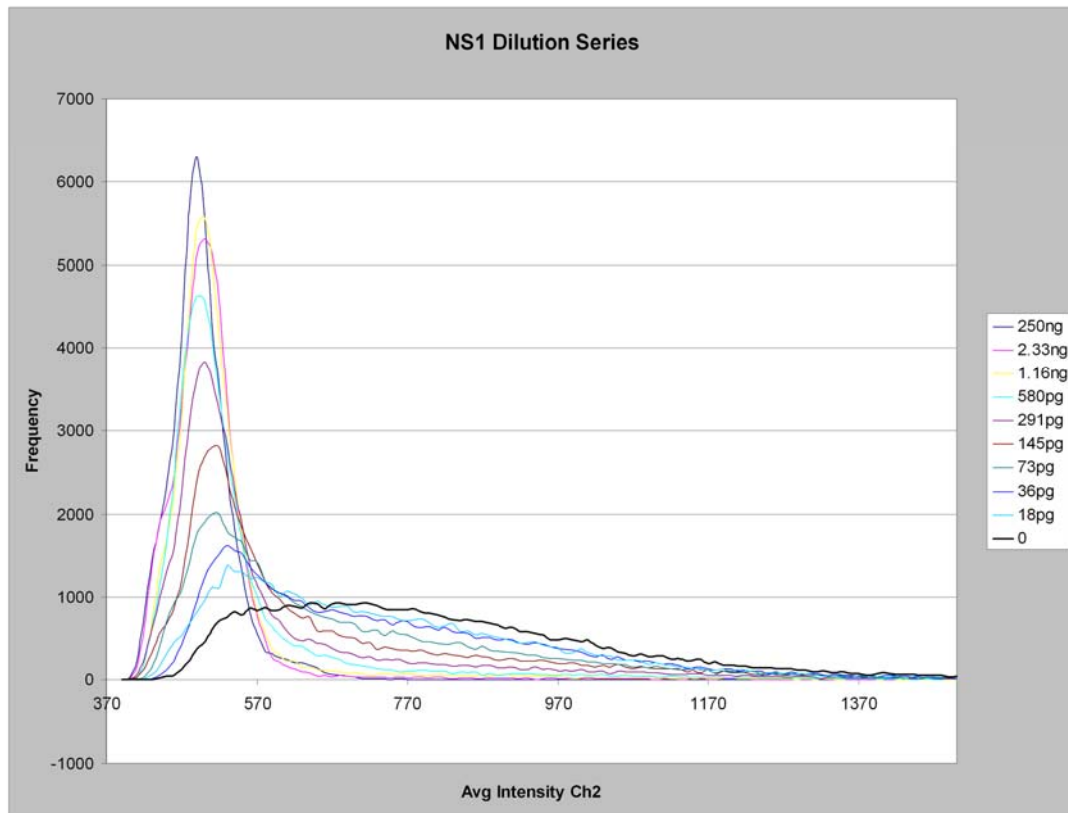
Observed mutations to the E protein in DEN2-S2 The six amino acid changes to the E protein are mapped onto the crystal structure solved by Modis *et al* 2003. The types of amino acid changes are indicated.

elucidating the mechanisms of the observed adaptation (Florez de Sessions, Dobrikova et al. 2007).

2.3 Design of a high throughput RNAi screen for viral host factors

In an RNAi based screen for *Drosophila* host factors required for *Drosophila* C virus replication, the authors utilized a dsRNA targeting the viral genome as a positive control for viral inhibition (Cherry, Doukas et al. 2005). Based on the success of this approach, we designed a dsRNA complementary to the NS1 coding region of the DEN2-S2 viral genome. This double stranded RNA, which is hereafter referred to as dsNS1, was found to be an exceedingly potent inhibitor of viral propagation down to the nanomolar range (Figure 2-6). As a negative control we used a dsRNA targeting GFP which was not endogenous to our system. Infected DMel-2 cells were defined in one of two ways, using the reference wells (RF; wells treated with dsNS1), or a filter we named the daily filter (DaF). RF identification defined infected cells as those cells which displayed an average fluorescent intensity that was three standard deviations higher than the mean of the average fluorescent intensity of the dsNS1 treated cell population in the reference wells within the same plate as determined by an automated software package (Figure 2-7). DaF identification involved manually defining the threshold fluorescent intensity of an infected cell from the wells treated with a dsRNA targeting GFP on a plate by plate basis. Only cells that were unambiguously above background levels of staining were selected. Once the threshold was determined, it was then checked

Figure 2-6



Titration of dsNS1 DMe12 cells were treated for 48 hours with decreasing concentrations of dsNS1 and then infected with the DEN2-S2 virus for 72 hours. Cells were then fixed, stained for E protein expression and counterstained with Hoescht for identification. The average fluorescent intensity for each cell in the treated and untreated wells was calculated using the Cellomics ArrayScan Vti HCS automated fluorescent imaging system. The distribution of cells as function of their average intensity is plotted for each condition.

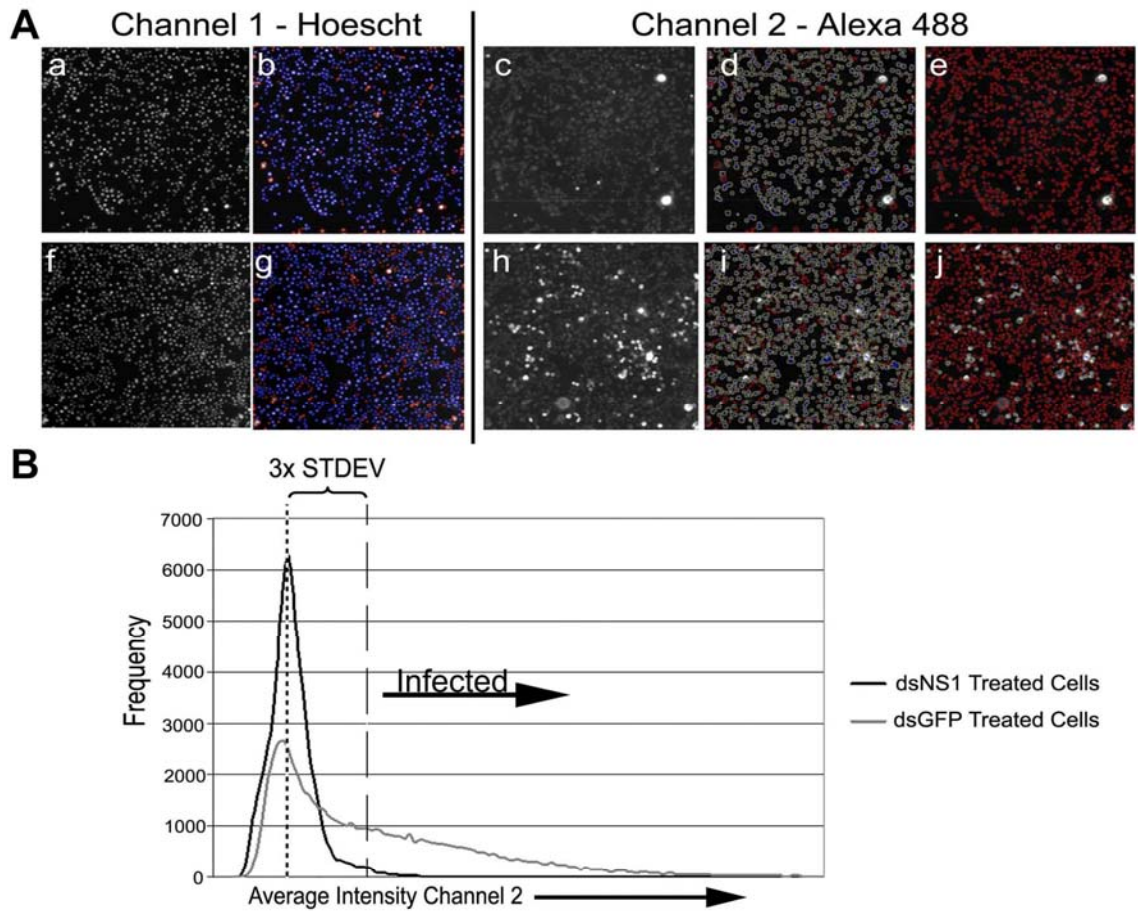
against the dsNS1 treated wells in the same plate to ensure no background level staining remained in the selected population. The DaF was more stringent than the RF method.

A common problem associated with high-throughput screening is the issue of plate effects (Ramadan, Flockhart et al. 2007). During the assay development phase, it was observed that cells in wells on the exterior of the plate were dying or growing at a slower rate than those located on the interior of the plate. Moreover, the average intensity of E protein in cells in sparsely populated wells was significantly different than in those with cells growing normally despite being treated with the same dsRNA and equivalent amounts of virus. Given this limitation of our assay and in consideration of the likelihood that some of the dsRNAs to be tested would have significant effects on cell growth and viability, we instituted a conservative threshold for analysis; if a dsRNA treated well had less than 12,500 cells it was excluded from our analysis. Although it is possible some of the target genes removed by this filter are in fact important for DEN propagation, a different assay or cell type will be required to assess their requirement.

2.4 Identification of DVHFs

Using the 22,632-dsRNA DRSC 2.0 library designed and provided by the *Drosophila* RNAi Screening Center (www.flyrnai.org) (Boutros, Kiger et al. 2004), we carried out a genome-wide RNA interference (RNAi) screen in duplicate for DVHFs in *Drosophila* DMe1-2 cells. Of the 22,632 dsRNAs tested, 2,343 dsRNAs were removed from our analysis due to low cell count (see above). The remaining 20,224 dsRNAs were

Figure 2-7



Automated determination of DEN2-S2 infectivity. dsRNA treated cells exposed to DEN2-S2 for 72 hours were immunofluorescently labeled and imaged at 20x magnification with a Celloomics ArrayScan. Images were then analyzed with the Celloomics Target Activation software to identify infected cells. Panel A: Images of dsNS1 treated cells (a-e) and dsGFP treated cells (f-j) were taken in two channels: Hoescht 33342 (a, b, f, g) and Alexa-488 (c-e, h-j). Nuclear identification parameters were applied to the raw Hoescht 33342 images (a, f). Nuclei that passed these criteria are marked by a blue boundary and those that failed are marked in red (b, g). The blue boundaries around selected nuclei from the Hoescht 33342 channel were then superimposed onto the raw Alexa-488 images (c, h). A second boundary, extending 2 pixels in all directions beyond the inner blue boundary was applied to each selected object (yellow) (selected object = cells) (d, i) and the mean average intensity was calculated for each selected object. A minimum average intensity in the Alexa-488 channel was then calculated (see Panel B) and applied to each selected object. The selected objects that were above this threshold remain yellow while those that were below become red (e, j). Panel B: A frequency distribution for the average intensities of each object was generated for D.Mel-2 cells treated with either dsNS1 or dsGFP were plotted on a histogram. The mean average intensity from four cultures treated with dsNS1 RNA was calculated and the threshold for determining whether a cell was infected was then defined as being ≥ 3 standard deviations away from this average.

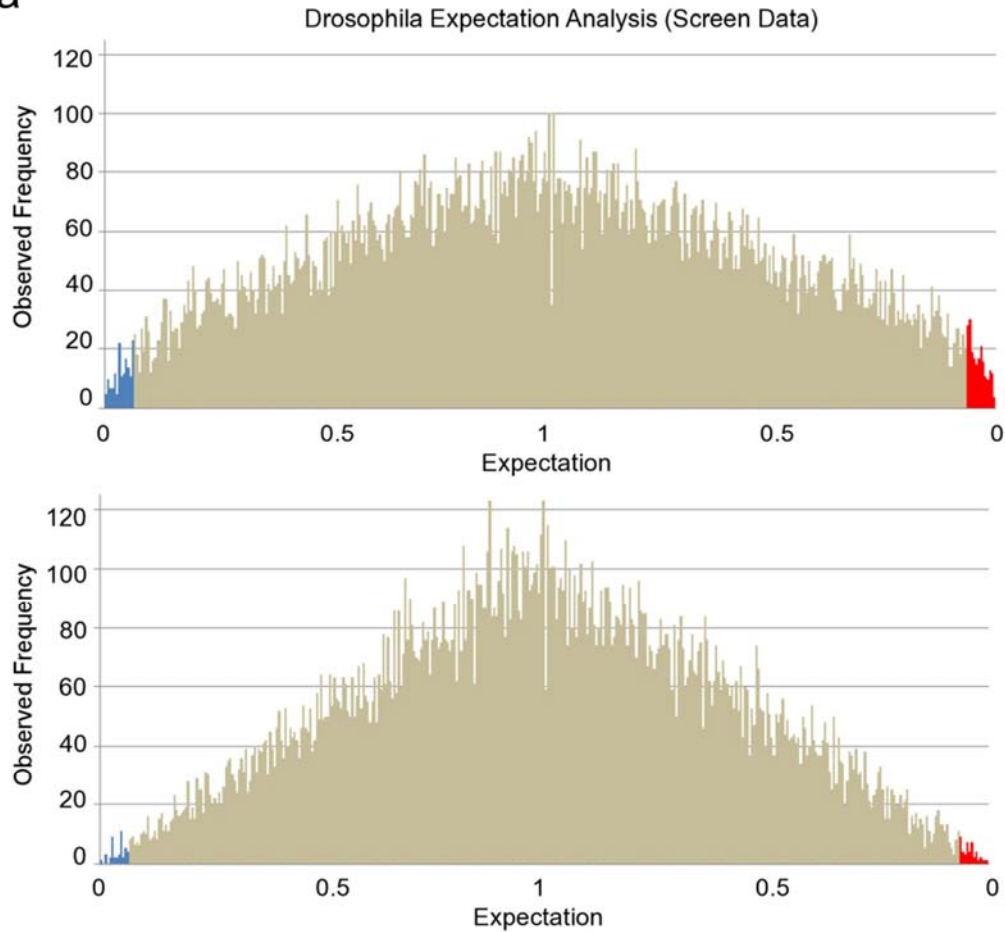
scored by their effect on infection, which was determined by measuring expression of the E protein (Figure 2-7). A Sum Rank score was assigned to each pair of duplicate dsRNAs (Figure 2-8 and methods). Those dsRNAs with SumRank values expected with a frequency ≤ 0.065 by chance alone were selected for further analysis. Of the 218 (1.1%) that met this criterion, we were able to readily re-synthesize and re-screen 179 of the dsRNAs. 118 of the 179 dsRNAs tested (66%), representing 116 unique DVHFs inhibited infectivity by ≥ 1.5 fold with $p < 0.05$ (Table 1). The primary screen also identified 296 dsRNAs that significantly enhanced infectivity (Figure 2-8). Although these dsRNAs may represent interesting factors that act to restrict DEN propagation, we chose to focus first on the factors required for DEN propagation.

2.5 Previously characterized DVHFs

Several of the DVHFs identified in the screen have been previously shown to be required for DEN and other flaviviruses, such as an alpha-glucosidase and the V-ATPase proton pump (CG14476, VhaPP1 and Vha14 in (Figure 2-9b,c) (Andoh, Kawamata et al. 1998; Nawa 1998; Courageot, Frenkiel et al. 2000; Whitby, Pierson et al. 2005; Chapel, Garcia et al. 2006; Duan, Lu et al. 2008; Chang, Wang et al. 2009). The effect on both the V0 and V1 subunits of the V-ATPase provided strong evidence of a requirement for the holoenzyme. In order to obtain independent evidence for this, we tested the effect of bafilomycin, a specific V-ATPase inhibitor previously shown to inhibit flaviviruses (Heinz, Stiasny et al. 1994; Andoh, Kawamata et al. 1998; Nawa 1998), on DEN infection of C6/36 *Aedes albopictus* cells. Bafilomycin treatment dramatically inhibited the propagation of both DEN2-S2 and DEN2-NGC in mosquito

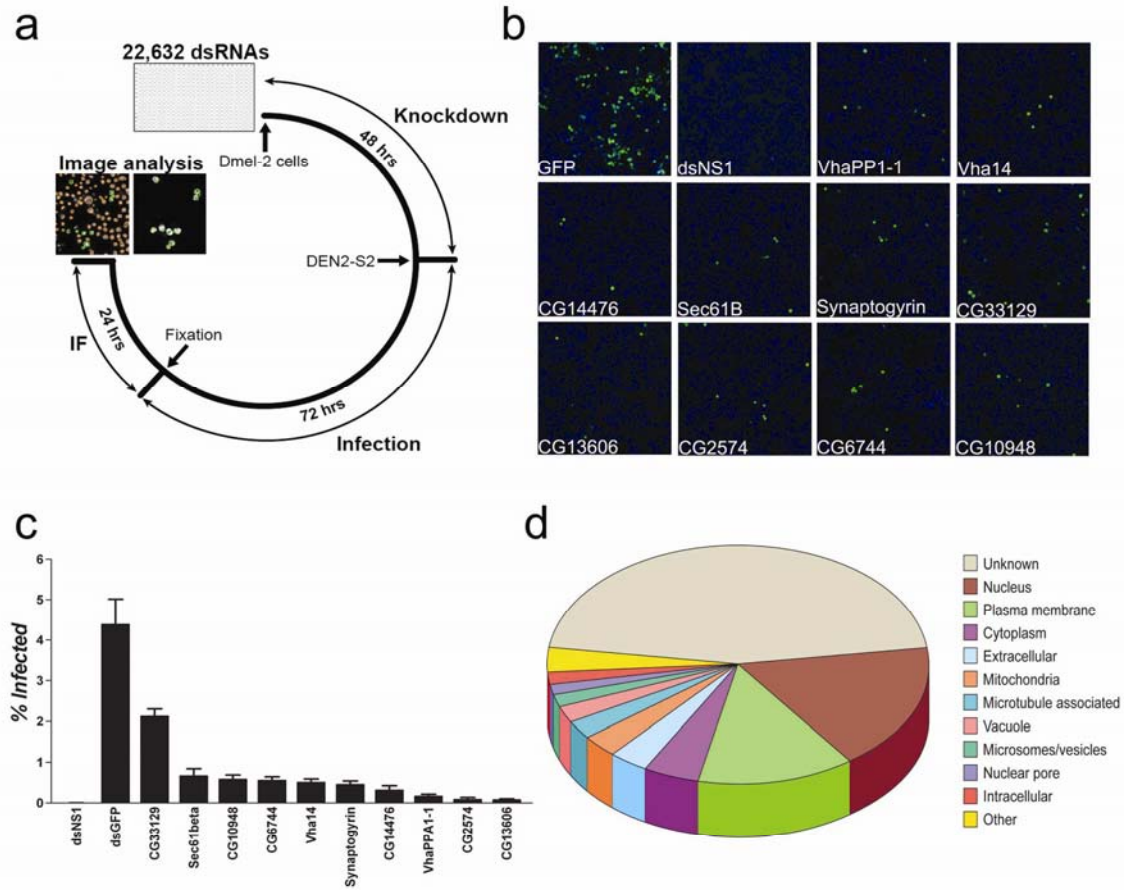
Figure 2-8

a



Histogram of observed and theoretical distributions of expected Sum Rank values. For each paired well in the screen, a Sum Rank statistic was calculated. The expected frequency of each observed Sum Rank is shown on the horizontal axis, with uncommon extremes in low infectivity to the left and high infectivity to the right. The vertical axis indicates the frequency with which each expectation value was observed during the screen in DMeI-2 cells (A) and in a computational simulation of random infectivity (B). Sum Ranks expected to occur fewer than 0.065 times per paired duplicate plate are highlighted in blue and red, representing extremes of low and high infectivity, respectively. Wells from the Drosophila screen (A) yielded a significantly larger number of wells with extremes of low ($\chi^2 = 62.8$, $p < 0.0001$) and high ($\chi^2 = 108$, $p < 0.0001$) infectivity compared to that expected by wells assigned random infectivity (B), suggesting detectable departures from random biological variation upon treatment of cells with dsRNAs. Using the random infectivity analysis to determine the false discovery rate suggested that roughly 24% of detected “hits” (expectation less than 0.065) were due to random chance alone, in rough agreement with the validation rate of putative hits.

Figure 2-9



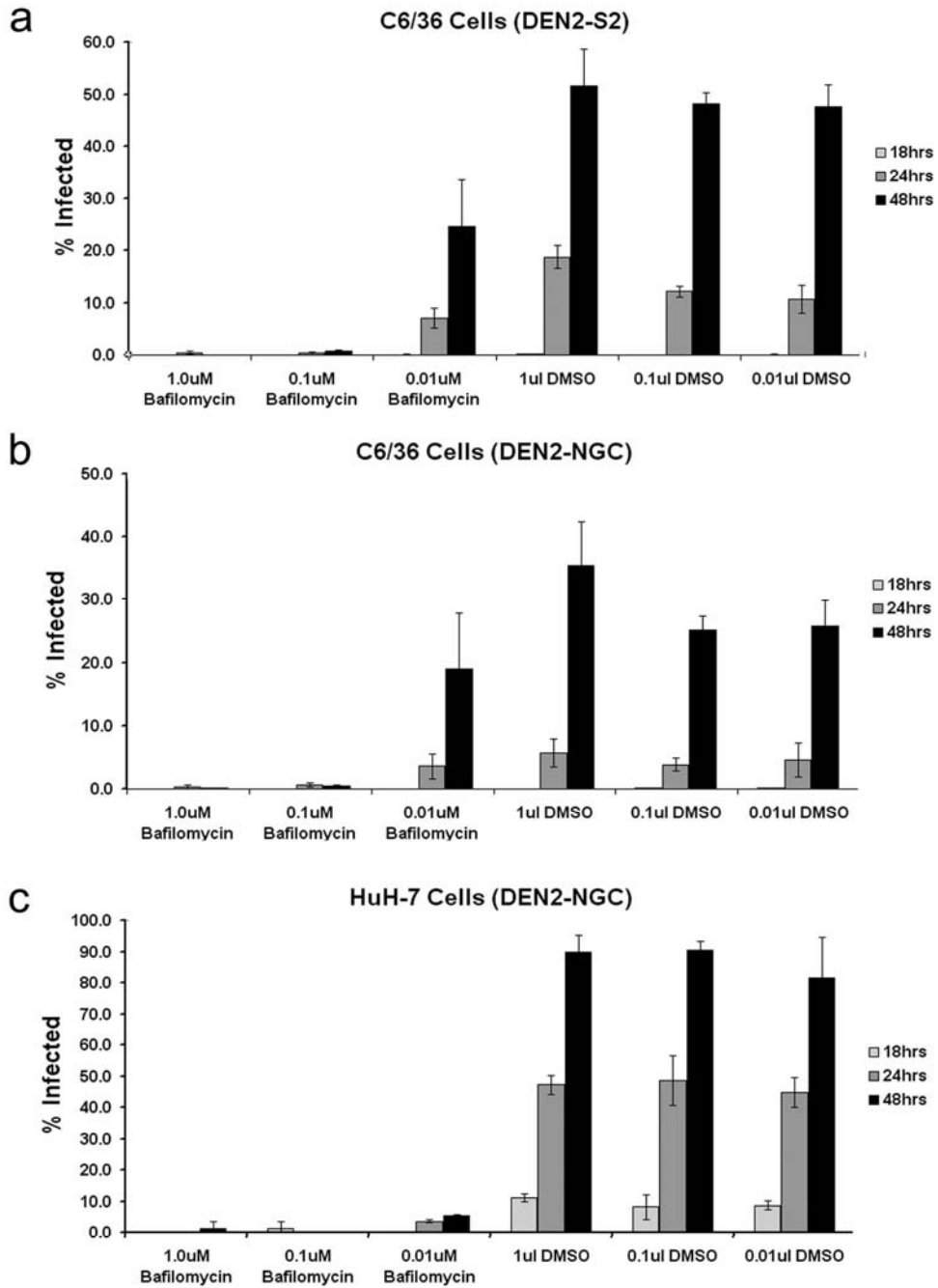
Genome-wide RNAi screen for dipteran DVHFs. 22,632 dsRNAs were assayed in duplicate for their effect on DEN2-S2 viral gene expression in D.Mel-2 cells. (a) Schematic of the experimental protocol used in the screen. (b) Representative images of dsRNA treated D.Mel-2 cells at 20x magnification with nuclei staining (blue) and dengue E protein staining (green). (c) The percentage of DEN2-S2 infected (% Infected) cells is indicated for controls and selected DVHFs. Error bars represent standard error of \geq six independent observations. (d) Cellular localization of the 116 DVHFs identified in the Drosophila screen according to GO cellular component annotation (FlyBase: FB2008_05).

cells (Figure 2-10a and b, respectively) as well as DEN2-NGC in human cells (Figure 2-10c). These data demonstrate that the host factors identified in the *Drosophila* screen can be generalized to the well-studied DEN2 and cells of its natural vector *Aedes albopictus*.

2.6 Classification novel DVHFs

111 of the 116 candidate DVHFs had not been previously identified as such. Existing annotation (FlyBase: FB2008_05) predicted a diverse array of cellular functions for the candidate DVHFs identified in the screen. DnaJ-1 and CG3061 are predicted to be involved in the unfolded protein response, which has been shown to be activated during DEN infection (Yu, Hsu et al. 2006; Umareddy, Pluquet et al. 2007). alpha-Adaptin, cnir, lqf, synaptogyrin, Syx4, and Syx13, are all predicted to be involved in vesicular transport and endocytosis (Littleton 2000). Both these processes have been implicated in the entry and replication of a diverse group of viruses (Pelchen-Matthews, Raposo et al. 2004), including DEN (Krishnan, Sukumaran et al. 2007). The novel DVHF lqf interacts with the *Drosophila* homologue of human EPS15, an essential component in the formation of clathrin-coated vesicles, which is required for WNV and DEN entry (Chu and Ng 2004; Krishnan, Sukumaran et al. 2007; Mosso, Galvan-Mendoza et al. 2008). RNA binding proteins, such as *bol*, *Unr* and CG5205, and the 3'-5' exonuclease-like CG6744, may assist in genome expression, replication and/or packaging (see below). Interestingly, the mosquito homologues of four DVHFs identified in our screen (*LysC*, *pxb*, H15 and *Cyp6a19*) were found to be differentially regulated at the RNA level following DEN infection in adult mosquitoes (Xi, Ramirez et al. 2008).

Figure 2-10



The V-ATPase holoenzyme, a DVHF in dipteran and human cells. Serial dilutions of the specific V-ATPase inhibitor, bafilomycin A1, were tested for their efficacy to reduce viral gene expression in mosquito C6/36 (a & b) and human HuH-7 (c) cell lines. DMSO without bafilomycin A1 serves as a vehicle control. Error bars represent standard deviation of three independent observations.

Gene Ontology (GO) annotation (GATHER 2009) of DVHFs indicated that a large number of proteins are predicted to be membrane-associated: 17 with the plasma membrane and 10 with intracellular membranes (ER, Golgi, vesicles and vacuole-like organelles) (Figure 2-9d). The detection of many membrane associated proteins is consistent with the observations that (+) strand RNA viruses uniformly cause remodeling of cellular membranes (Ahlquist 2006). Also remarkable was the substantial number of nuclear proteins (22). Several DEN gene products are known to transit through the nucleus during the course of infection (Buckley, Gaidamovich et al. 1992; Kapoor, Zhang et al. 1995; Westaway, Khromykh et al. 1997; Johansson, Brooks et al. 2001; Brooks, Johansson et al. 2002; Wang, Syu et al. 2002; Medin, Fitzgerald et al. 2005; Mori, Okabayashi et al. 2005; Oh, Yang et al. 2006; Uchil, Kumar et al. 2006; Pryor, Rawlinson et al. 2007; Sangiambut, Keelapang et al. 2008; Ellencrona, Syed et al. 2009; Rawlinson, Pryor et al. 2009). It is also possible that DEN infection relocalizes many of these factors to the cytoplasm, a mechanism observed in other RNA viruses (Florez, Sessions et al. 2005).

Over half of the nuclear proteins (12) are predicted to function as transcription factors. This is consistent with the findings that DEN infection of mosquitoes significantly alters their RNA expression profile (Xi, Ramirez et al. 2008). GO annotation also indicates that 14 DVHFs are predicted to have functions in the regulation of metabolism. Taken together, these data suggest that DEN may be fine tuning the cellular microenvironment to a degree previously unsuspected.

2.7 Comparison with other genome-wide screens

We compared the results of our screen for DVHFs with previously published genome-wide RNAi screens and found significant overlap with two other DRSC screens that identified gene products required for MAPK signaling (Friedman and Perrimon 2006) and Ca²⁺ influx (Vig, Peinelt et al. 2006). Pathways that mediated Ca²⁺ influx are consistent with the proposed role of Rab5 in viral entry (Krishnan, Sukumaran et al. 2007). In a recent screen for factors affecting influenza infection in *Drosophila* cells the DVHFs *lola*, *Sec61β* and a V-ATPase subunit were also identified to be required for influenza (Hao, Sakurai et al. 2008). In the siRNA screen for HIV dependency factors, *Sec61*, the mitochondrial Complex I and V-ATPase subunits were identified as required factors (Brass, Dykxhoorn et al. 2008).

Noticeably absent among DVHFs were ribosomal proteins, which were the majority of the hits from a genome-wide screen for factors required for efficient *Drosophila* C Virus propagation (Cherry, Doukas et al. 2005). Their absence among DVHFs could be due to an effect on cell number: 65 of the 131 dsRNAs targeting these proteins were removed from analysis due to low cell density. This represented a significant over-representation of dsRNAs targeting ribosomal proteins among those that affected cell density (49.6% for ribosomal proteins vs. 10.3% overall; $\chi^2 = 213.0$, $p = 3 \times 10^{-48}$).

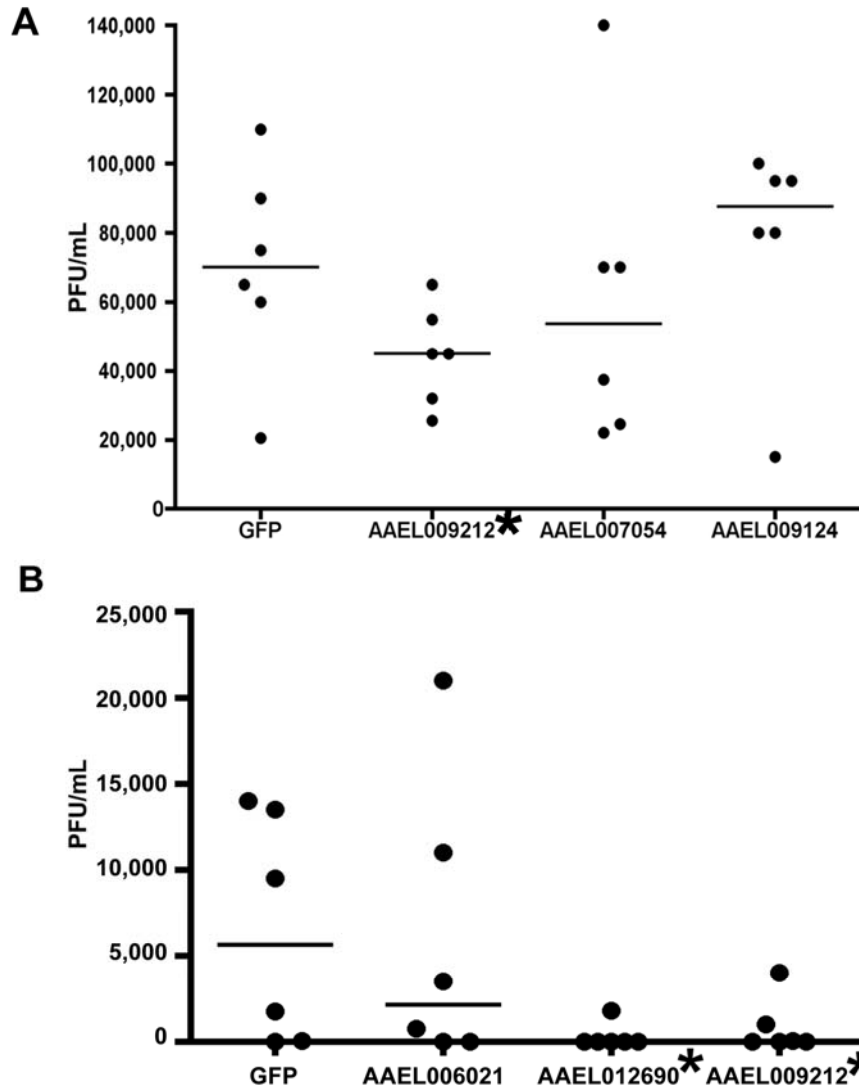
When we examined a list of hepatitis C virus factors identified in a targeted siRNA screen carried out in human cells (Randall, Panis et al. 2007), we did not observe any crossover with our Dipteran DVHFs. A recent siRNA screen for human host factors

required by WNV identified 283 factors required for replication (Krishnan, Ng et al. 2008). When the authors tested these factors for their shared requirement in DEN infection, they identified that 36% (101) were also required for DEN replication. Comparison of DVHFs identified in our study with DEN host factors identified in the Krishnan et al 2008 study revealed that only the Sec61 and V-ATPase complexes were shared between the two screens. The small amount of crossover between our two screens suggests that 1) our screen for DVHFs in Dipteran cells was far from saturating in terms of human DVHFs and/or 2) host factor dependence could vary depending on the experimental details (e.g., cell types and viral construct or strain). It should be noted that our screen was carried out with an infectious DEN2 and assayed multiple rounds of infection in order to detect all stages of the viral life cycle.

2.8 Validation of DVHFs in *Aedes aegypti*

In order to test whether the candidate DVHFs were in fact required for propagation of DEN in the vector mosquito *Aedes aegypti* we tested the impact of depleting the *Aedes aegypti* homologues of lola (AAEL009212), CG10320 (AAEL007054), Cyp6a19 (AAEL009124), Su(Tpl) (AAEL006021), and CG6744 (AAEL012690) through an established method of RNAi-mediated gene silencing (Dong, Aguilar et al. 2006), on DEN infection. dsRNA targeting AAEL009212 (Figure 2-11a, b) and AAEL012690 (Figure 2-11b) reduced the DEN2-NGC capacity to infect the midgut tissue at seven days after ingestion of infected blood. The effect of DVHF gene silencing on DEN infection

Figure 2-11



Injection of *Ae. aegypti* mosquitoes with dsRNA targeting DVHFs inhibits dengue virus propagation. Four-day old female mosquitoes were injected with dsRNAs targeting GFP, AAEL009212, AAEL007054, or AAEL009214 (A) and GFP, AAEL006021, AAEL012690, or AAEL009212 (B). Three days after injection, mosquitoes were fed on a DEN2-NGC supplemented blood meal. Seven days later, 30 mosquitoes for each condition were randomly sorted into six groups of five, their midguts removed, homogenized and titered. Data points indicated the combined titer of five dsRNA-treated mosquito midguts. Dashes indicate the median value of the six biological replicates. Significance at <0.05 level was determined using a one-sided student's T-test of viral titers (dsDVHF vs. dsGFP) and is indicated with an *

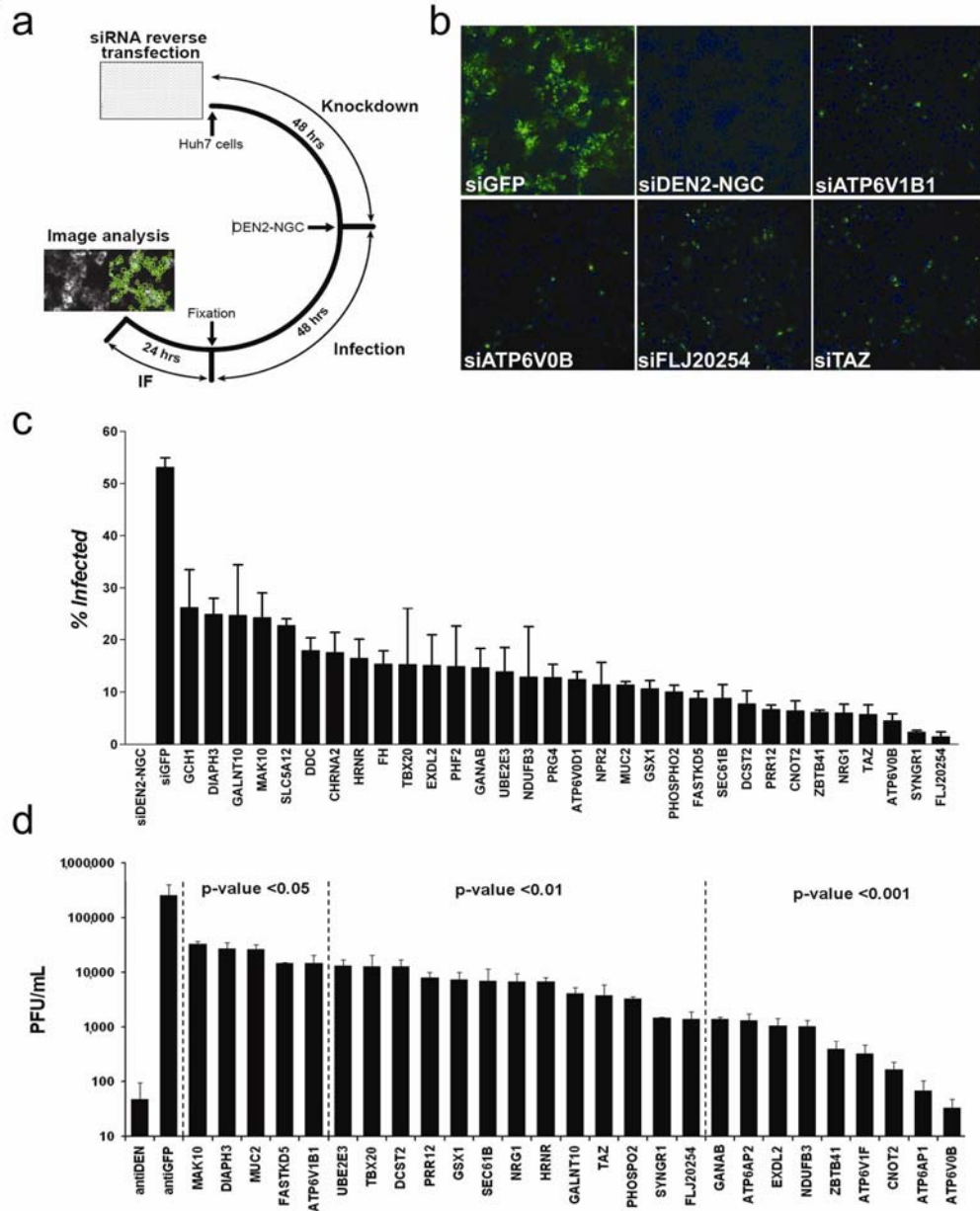
was likely underestimated because of the variability in the dsRNA-GFP injected mosquitoes (Figure 2-11a, b). A dsRNA targeting AAEL009124 did not affect infectivity.

Whereas inhibition of infectivity upon depletion of AAEL007054, a putative NADH dehydrogenase, was not statistically significant, exclusion of the point that appears to be an outlier leads to a reduction of infectivity that approaches significance and suggests that this gene product was likely also a DVHF in *Aedes aegypti* (Figure 2-11a). The same may be true for AAEL006021 however this is less clear (Figure 2-11b). Given the complex spatio-temporal dynamics of DEN infection in the mosquito, the fact that the mosquitoes are genetically polymorphic, the inherent variability of blood meal infections (Franz, Sanchez-Vargas et al. 2006) and the uncertainty of achieving gene product depletion in the appropriate tissue and time after dsRNA injection, it was remarkable to obtain inhibition with these DVHFs. These data, together with those obtained above with *Aedes albopictus* cells, validate the use of the *Drosophila* screen to identify *Aedes* DVHFs.

2.9 Screening for human DVHFs

The 116 DVHFs had 82 readily identifiable human homologues, which we targeted with a small library of siRNAs (Figure 2-12a). We supplemented this library with siRNAs targeting gene products that were functionally associated with the V-ATPase but had not scored as DVHFs in the D.Mel-2 screen, for instance V-ATPase accessory proteins not found in insects (Table 2). Of the 82 homologues of the Dipteran DVHFs, 42 (51%) scored as human DVHFs (Figure 2-12b, c and Table 2). The remarkably high

Figure 2-12



Screen for Human DVHFs. siRNAs targeting the 82 human DVHF homologues were screened in HuH-7 cells for their ability to inhibit DEN2-NGC. (a) Schematic of the experimental protocol. (b) Representative images of siRNA treated HuH-7 cells at 10x magnification with nuclei staining (blue) and dengue E protein staining (green). (c) The percentage of DEN2-NGC infected cells is indicated for controls and selected DVHFs. (d) Viral propagation after treatment with control or DVHF siRNAs was measured 72 hours after DEN2NGC infection and plotted on a logarithmic scale. Error bars in (c) and (d) indicate standard error of three independent observations.

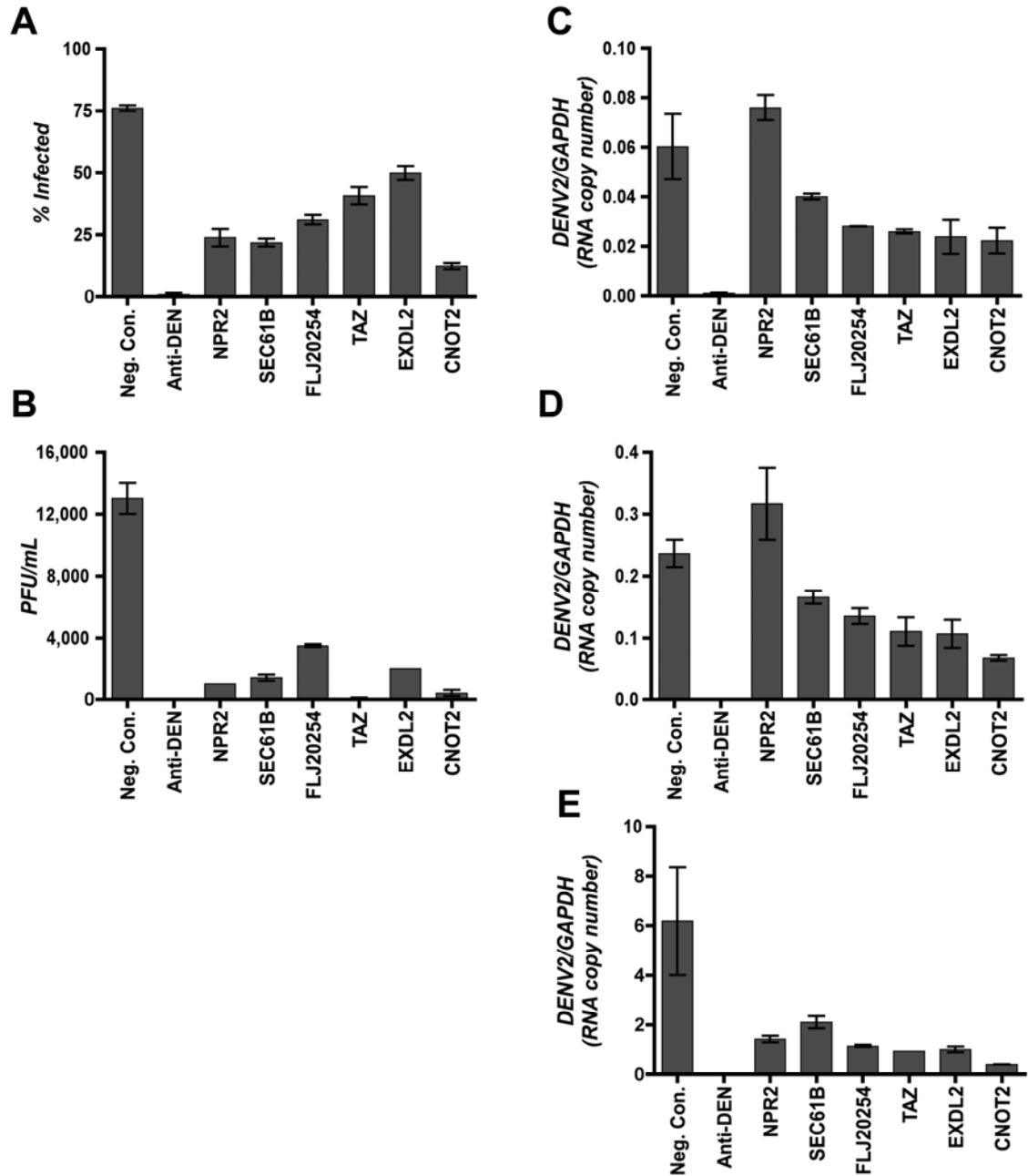
number of Dipteran DVHF homologous required for propagation in human cells provides the first evidence for widespread conservation of flavivirus-host interactions between invertebrates and vertebrates and also serves to further validate the results obtained in the *Drosophila* model system (Figure 2-9 and Table 3).

2.10 Characterization of human DVHFs

RNAi-mediated depletion of human DVHF homologues in HuH-7 cells significantly reduced the formation of DEN2-NGC infectious particles (Figure 2-12d). Depletion of human DVHFs predicted to be involved in entry, post-translational modifications and transcription accounted for the majority of those factors which resulted in greater than 10-fold inhibition of viral propagation. In order to address the question of whether the identified DVHFs were specific to DEN, we assayed for alterations in the gene expression of the related YFV (17D strain) and the unrelated enterovirus Coxsackie B3 (strain 20) in cells depleted of these factors. Of the six DVHFs tested that showed ≥ 2 fold inhibition of DEN2-NGC E protein expression by at least two independent siRNAs, only one (FLJ20254) was required for YFV and three (CNOT2, FLJ20254, TAZ) were required for CBV3 gene expression (Table 4). These data suggest some host factors are shared among these RNA viruses, yet also suggests that some host factors are specific to DEN.

In order to gain some early mechanistic insights and determine whether the factors we had identified affect both early and/or late steps in the viral life cycle, we examined viral RNA accumulation in cells depleted of DVHFs. HuH-7 cells were treated with

Figure 2-13



Analysis of DENV RNA accumulation after DVHF knockdown. (a) The percentage of DENV2-NGC infected (% Infected) cells 48 hours post infection (MOI ~ 1.4) is indicated for controls and for six DVHFs. Error bars represent the standard deviation of six replicates. (b) Viral propagation 48 hours post infection was calculated for controls and six DVHFs. Error bars represent the range of duplicates. (c, d, e) Viral RNA accumulation was measured by RTqPCR at 18, 24, and 48 hours post infection respectively and normalized to GAPDH. Values represent the average of median qPCR measurements. Error bars represent the range of duplicates.

siRNAs targeting NPR2, SEC61B, FLJ20254, TAZ, EXDL2, and CNOT2 transcripts, infected with DEN2-NGC and time points were harvested as shown (Figure 2-13). As previously observed, knockdown of these DVHFs reduced both the number of cells expressing DEN E protein and the titer of infectious virus recovered 48 hours post infection (pi) (Figure 2-13a, b). Consistent with this, we noted a decrease in viral transcripts measured by qRT-PCR at 48 hours pi (Figure 2-13e).

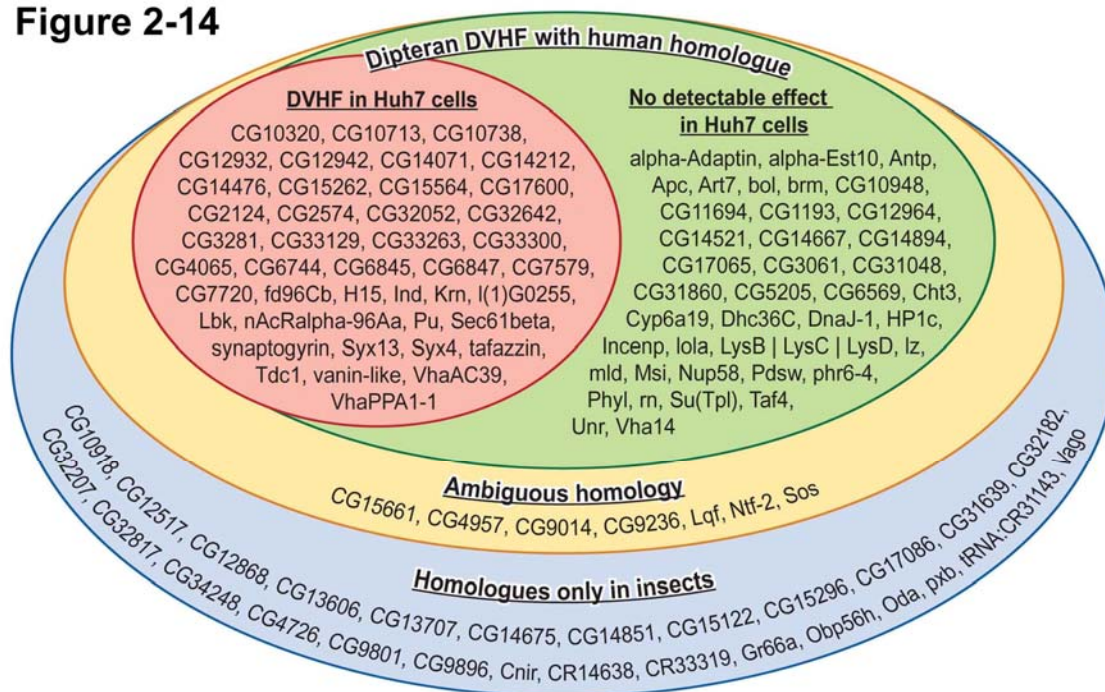
Accumulation of viral transcripts at 18 and 24 hours pi was clearly decreased in cells depleted of FLJ20254, TAZ, EXDL2, and CNOT2 (Figure 2-13c,d), indicating that these DVHFs act on steps required for the accumulation of RNA (e.g., early events). In contrast, knockdown of NPR2 did not result in lower RNA levels at early times pi, indicating that this DVHF acts at steps downstream of RNA accumulation. Given its location in the plasma membrane (Potter, Abbey-Hosch et al. 2006), it is likely that NPR2 is involved in the assembly or exit of DEN.

2.11 Conservation between Dipteran and human hosts

Sixteen Dipteran DVHFs have annotated homologues only among *Drosophila* species and another eleven only among insects (Figure 2-14). These 27 are enriched in genes associated with sensation and response to chemicals, and the transport of ions (FlyMine v12.0) and several belong to families of poorly conserved genes (e.g., gustatory receptors).

DVHFs conserved in Dipterans and humans were significantly enriched for genes associated with secretion, membrane docking and vesicle transport. This may not be

Figure 2-14



Human and dipteran DVHFs. The 116 unique dipteran DVHFs include 27 gene products with homologues found exclusively among insects. The majority of the 116 had readily identifiable human homologues and these are divided here depending on the results of the human screen.

surprising since delivery of DEN genomes to the cytoplasm and assembly of virions in the ER are basic processes required in both insect and human cells. One of the more intriguing discoveries was the conserved requirement for DC-STAMP (TM7SF4). DC-STAMP is a 470-amino acid protein containing seven transmembrane domains, localizes to the cell surface and is specifically expressed by activated dendritic cells (Hartgers, Vissers et al. 2000). Monocyte-derived dendritic cells are believed to be the primary target cells of DEN (Kou, Quinn et al. 2008) and thus we postulate that DC-STAMP may be acting a DEN receptor.

It is difficult to draw definitive conclusions for the 40 human homologues of dipteran DVHFs that did not recapitulate the effect on DEN infection (Figure 2-14). It is likely that some of these failed merely because their knockdown in human cells was ineffective. Nonetheless, these 40 genes that did not score in the human screen are not enriched in any of the aforementioned processes, but show highly significant bias for genes involved in immunity and RNA polymerase II transcription, suggesting that many may represent dipteran-specific DVHFs.

2.12 Significance

Here is reported the results of a genome-wide screen for DVHFs in Dipteran cells, confirming the activity some well-known factors and the discovery of many novel ones. We confirmed the function of several DVHFs in *Aedes albopictus* cells in culture and *Aedes aegypti* mosquitoes *in vivo*. Furthermore, we have provided the first solid evidence for broadly conserved requirements for DEN infection in insects and humans.

Table 2-1 Dipteran DVHFs listed by gene name

Gene	CGs	FBGN	DRSC Amplicon	FOLD DECREASE	p Value	19 bp Matches
alpha-Adaptin	CG4260, CG31654	FBgn0015567	DRSC27715	2.3	1.8E-02	0
alpha-Est10	CG1131	FBgn0015569	DRSC12613	2.7	2.3E-04	1
Antp	CG1028	FBgn0000095	DRSC23104	3.2	6.7E-03	11
Apc	CG1451	FBgn0015589	DRSC14114	1.9	4.1E-03	0
Art7	CG9882	FBgn0034817	DRSC26145	2.1	2.4E-03	0
bol	CG4760, CG4727	FBgn0011206	DRSC08964	2.4	1.3E-03	0
brm	CG18438, CG5942	FBgn0000212	DRSC11330	3.0	2.7E-03	1
CG10320	CG10320	FBgn0034645	DRSC04060	5.4	3.3E-04	0
CG10713	CG10713	FBgn0036360	DRSC24532	734.8	4.3E-04	0
CG10738	CG10738	FBgn0036368	DRSC09792	1.7	7.3E-03	2
CG10918	CG10918	FBgn0031178	DRSC24162	1.6	4.2E-02	0
CG10948	CG10948	FBgn0036317	DRSC09806	3.3	6.9E-05	0
CG11694	CG11694	FBgn0037571	DRSC24061	2.0	1.8E-02	0
CG1193	CG1193	FBgn0037375	DRSC25414	4.0	3.8E-03	0
CG12517	CG12517	FBgn0032311	DRSC02173	1.9	2.6E-02	0
CG12868	CG12868	FBgn0033945	DRSC06207	2.1	2.3E-03	1
CG12932	CG12932	FBgn0033419	DRSC06245	1.6	1.7E-02	1
CG12942	CG12942	FBgn0033569	DRSC06253	10.9	1.1E-04	1
CG12964	CG12964	FBgn0034022	DRSC23210	4.9	2.6E-03	1
CG13606	CG13606	FBgn0039161	DRSC14609	22.9	7.2E-07	0
CG13707	CG13707	FBgn0035578	DRSC08316	2.5	5.1E-04	0
CG14071	CG14071	FBgn0032312	DRSC02374	3.2	1.7E-03	0
CG14212	CG14212	FBgn0031045	DRSC19568	3.9	1.6E-04	1
CG14476	CG14476	FBgn0027588	DRSC21036	4.8	3.4E-04	0
CG14521	CG14521	FBgn0039617	DRSC14873	4.6	2.8E-05	0
CG14667	CG14667	FBgn0037317	DRSC12253	1.7	6.8E-03	2
CG14675	CG14675	FBgn0037385	DRSC12261	2.5	4.1E-04	0
CG14851	CG14851	FBgn0038240	DRSC21500	10.7	9.7E-04	1
CG14894	CG14894	FBgn0038428	DRSC26111	3.3	1.3E-03	0
CG15122	CG15122	FBgn0034457	DRSC26227	3.3	3.2E-04	0
CG15262	CG15262	FBgn0028852	DRSC01975	2.6	4.1E-04	0
CG15296	CG15296	FBgn0030215	DRSC26051	3.2	1.5E-03	0
CG15564	CG15564	FBgn0039833	DRSC15102	4.2	3.3E-05	0
CG15661	CG15661	FBgn0034605	DRSC04252	1.7	3.6E-02	2
CG17065	CG17065	FBgn0031099	DRSC20544	128.1	8.1E-05	1
CG17086	CG17086	FBgn0032310	DRSC02566	1.6	2.8E-02	1
CG17600	CG17600, CG17602	FBgn0031195	DRSC20640	4.1	1.0E-04	0
CG18166	CG18166	FBgn0029526	DRSC17271	2.6	1.3E-02	2
CG3176	CG3176	FBgn0029524				
CG32817	CG32817	FBgn0052817				
CG2124	CG2124	FBgn0030217	DRSC18208	3.6	1.5E-04	0
CG2574	CG2574	FBgn0030386	DRSC19862	16.6	1.7E-05	0
CG3061	CG3061	FBgn0038195	DRSC26419	1.8	7.4E-03	0
CG31048	CG31048, CG14530, CG11754	FBgn0051048	DRSC14373	2.0	1.9E-03	3
CG31639	CG31639	FBgn0051639	DRSC00910	2.9	5.4E-04	1
CG31860	CG31860, CG17215	FBgn0051860	DRSC02591	13.6	3.2E-06	0
CG32052	CG32052, CG8163	FBgn0044328	DRSC27465	2.1	4.7E-03	0
CG32182	CG32182	FBgn0052182	DRSC09508	1.9	1.7E-02	3
CG32207	CG32207	FBgn0052207	DRSC09617	2.0	5.5E-03	11
CG33255	CG33255	FBgn0053255				
CG32642	CG32642	FBgn0052642	DRSC18959	2.0	4.9E-02	1
CG3281	CG3281	FBgn0037967	DRSC28414	2.0	3.2E-02	0

Table 2-1 continued

Gene	CGs	FBGN	DRSC Amplicon	FOLD DECREASE	p Value	19 bp Matches
CG33129 *	CG33129, CG6089, CG6087	FBgn0053129	DRSC02924	2.1	1.0E-02	1
CG33263	CG33263, CG14106	FBgn0053263	DRSC10108	1.5	3.4E-02	0
CG33300	CG33300	FBgn0053300	DRSC01101	7.6	2.3E-05	1
CG34248	CG34248	FBgn0085277	DRSC09426	1.9	7.2E-03	0
CG4065	CG4065	FBgn0034982	DRSC26149	2.0	2.4E-03	0
CG4726	CG4726	FBgn0031307	DRSC00664	3.2	1.2E-04	1
CG4957	CG4957	FBgn0032205	DRSC02793	2.1	2.3E-03	1
CG5205	CG5205	FBgn0038344	DRSC15736	2.9	4.7E-04	0
CG6569	CG6569	FBgn0038909	DRSC16067	1.7	1.6E-02	0
CG6744	CG6744	FBgn0037901	DRSC25938	3.3	1.2E-04	0
CG6845	CG6845	FBgn0035099	DRSC26319	1.8	2.5E-02	0
CG6847	CG6847	FBgn0030884	DRSC20009	9.2	1.2E-05	2
CG7579	CG7579	FBgn0036528	DRSC10885	1.5	2.7E-02	0
CG7720	CG7720	FBgn0038652	DRSC16306	6.2	1.2E-05	0
CG9014	CG9014	FBgn0028847	DRSC01979	5.2	2.1E-05	0
CG9236	CG9236	FBgn0034558	DRSC04535	1.7	1.5E-02	0
CG9801	CG9801	FBgn0037623	DRSC16568	2.3	9.1E-04	0
CG9896	CG9896	FBgn0034808	DRSC04594	1.9	4.1E-03	0
Cht3	CG18140	FBgn0022701	DRSC03766	1.7	3.5E-02	0
cnir	CG17262	FBgn0243513	DRSC26787	3.0	2.5E-03	0
CR14638	CG14638	FBgn0037223	DRSC12224	1.8	4.2E-03	1
CR33319	CG18078	FBgn0053319	DRSC01826	14.6	5.2E-05	2
Cyp6a19	CG10243	FBgn0033979	DRSC07379	4.8	1.8E-04	0
Dhc36C	CG5526	FBgn0013810	DRSC03315	2.5	4.7E-03	0
DnaJ-1	CG10578	FBgn0015657	DRSC11145	2.0	4.0E-03	0
fd96Cb	CG11922	FBgn0004898	DRSC25322	163.3	4.5E-04	0
Gr66a	CG7189	FBgn0035870	DRSC10783	2.0	1.7E-02	0
H15	CG6604	FBgn0016660	DRSC22199	2.0	3.9E-02	28
HP1c	CG6990	FBgn0039019	DRSC28423	2.7	1.1E-02	0
Incenp	CG12165	FBgn0033156	DRSC06116	1.8	3.0E-02	1
ind	CG11551	FBgn0025776	DRSC23095	2.6	1.2E-02	0
Krn	CG32179, CG8056	FBgn0052179	DRSC11202	2.0	2.3E-03	0
l(1)G0255	CG4094	FBgn0028336	DRSC18344	2.2	6.8E-03	1
lbk	CG8434	FBgn0034083	DRSC07178	5.6	4.4E-05	0
lola	CG30013, CG12052, CG18379, CG30014, CG18380, CG18378, CG18376, CG18381, CG30012	FBgn0005630	DRSC05222	17.6	5.8E-06	1
lqf	CG32386, CG8532	FBgn0028582	DRSC11363	2.8	5.4E-04	2
LysB, LysC, LysD	CG1179, CG9111, CG9118	FBgn0004425, FBgn0004426, FBgn0004427	DRSC21249	1.6	2.8E-02	3
lz	CG1689	FBgn0002576	DRSC18790	25.6	3.8E-05	12
mld	CG33343, CG34100, CG9469, CG13620, CG31312	FBgn0083077	DRSC13681	2.7	1.6E-02	26
Msi	CG32178	FBgn0043025	DRSC09507	2.0	9.0E-03	0
nAcRalpha-96Aa	CG5610	FBgn0000036	DRSC13672	2.5	1.5E-02	10

Table 2-1 continued

<u>Gene</u>	<u>CGs</u>	<u>FBGN</u>	<u>DRSC Amplicon</u>	<u>FOLD DECREASE</u>	<u>p Value</u>	<u>19 bp Matches</u>
Ntf-2	CG1740	FBgn0031145	DRSC20552	1.5	4.4E-02	1
Nup58	CG7360	FBgn0038722	DRSC27863	6.4	7.3E-03	0
Obp56h	CG13874	FBgn0034475	DRSC06433	2.3	8.8E-04	1
Oda	CG16747	FBgn0014184	DRSC07626	5.3	4.8E-04	1
Pdsw	CG8844	FBgn0021967	DRSC00715	4.3	5.9E-05	0
phr6-4	CG2488	FBgn0016054	DRSC26873	2.5	4.0E-04	0
phyl	CG10108	FBgn0013725	DRSC07663	1.5	2.5E-02	1
Pu	CG9441	FBgn0003162	DRSC04645	1.7	3.4E-02	1
pxb	CG14874, CG33207, CG14873	FBgn0053207	DRSC22225	2.0	3.9E-02	0
rn	CG32466, CG10040, CG14600, CG14603, CG14601	FBgn0003263	DRSC12544	2.1	8.7E-04	35
Sec61beta	CG10130	FBgn0010638	DRSC21512	3.7	5.0E-03	3
Sos	CG7793	FBgn0001965	DRSC03439	2.0	4.7E-03	5
Su(Tpl)	CG32217, CG8037	FBgn0014037	DRSC10954	5.3	1.3E-04	1
synaptogyrin	CG10808	FBgn0033876	DRSC06003	2.2	8.4E-04	0
Syx13	CG11278	FBgn0036341	DRSC09836	1.8	4.8E-03	1
Syx4	CG2715	FBgn0024980	DRSC27586	2.0	5.8E-03	0
Taf4	CG5444	FBgn0010280	DRSC11297	3.2	1.4E-04	1
tafazzin	CG8766	FBgn0026619	DRSC07704	1.5	3.1E-02	0
Tdc1	CG30445, CG3686	FBgn0050445	DRSC04942	2.2	2.2E-03	0
tRNA:CR31143	CR31143	FBgn0051143	DRSC13633	3.5	6.0E-03	13
Unr	CG7015	FBgn0035895	DRSC10761	2.8	3.1E-04	1
Vago	CG2081	FBgn0030262	DRSC26320	2.2	1.4E-03	0
vanin-like	CG3648, CG32754	FBgn0040069	DRSC17440	2.7	1.1E-02	0
Vha14	CG8210	FBgn0010426	DRSC07571	2.6	2.2E-04	0
VhaAC39	CG2934	FBgn0028665	DRSC27608	3.1	7.3E-03	0
VhaPPA1-1 *	CG7007	FBgn0028662	DRSC16170	10.9	4.3E-06	0

Table 2-2 DVHF identified in HuH-7 cells.

HUGO ID	pValue Infectivity ¹	Fold Change (infectivity)	%Fld Chng/ VO Fld chng ²	siRNA Sequence
ATP6AP1	8.90E-10	7.7	4.4	CACAGTGACATTCAAGTTCAT
ATP6AP2	1.40E-08	3.8	2.3	GGGAACGAGTTTAGTATATTA
ATP6V0A2	7.00E-05	1.6	0.8	CAGGAAATTAATAGAGCTGAT
ATP6V0A4	1.00E-05	1.8	0.9	CACATTTAACAGGACCAATAA
ATP6V0B	3.80E-10	11.8	2.2	CATGGCAATTGTCATTAGCAA
ATP6V0B	1.80E-05	1.7	0.6	CCCAGCCTCTTGTAAGATT
ATP6V0C	1.10E-07	2.6	3.1	CAGCCACAGAATATTATGTAA
ATP6V0C	1.50E-06	2.1	1.9	CTGGATGTTTATTATAAAGA
ATP6V0D1	5.10E-09	4.3	2.9	CACTTTCATGTTCCCTCCTAA
ATP6V0D1	3.60E-07	2.4	0.5	CCGCGCCTTCATCATCCCAT
ATP6V0E1	4.30E-06	1.9	0.7	AACCCTCTCTTGGACCGCAA
ATP6V0E2	2.80E-05	1.6	1.3	AAGGGATATGTGAGATCCAAA
ATP6V1B1	1.30E-10	40.6	13.2	CCGGGTCAAGTTTGCCAGTA
ATP6V1B1	3.30E-10	12.1	7.1	CCCGGCAGTAGCTGCAACCTA
ATP6V1B2	1.50E-10	34.2	15.9	CACGGTTAATGAAGTCTGCTA
ATP6V1B2	1.60E-10	25.5	6	CTCGATTACTCAAATCCCTAT
ATP6V1C2	5.60E-08	4.7	1	CAGGTATGGACTACCAGTGAA
ATP6V1C2	3.90E-07	2.3	1.7	CTGGAGAGGATGAATACTGTA
ATP6V1D	3.30E-05	2.4	0.7	AAAGAAGATAATAGAGACTAA
ATP6V1G2	1.10E-08	3.8	2.5	CACCACCTGCTCACTGGTCAA
ATP6V1G3	2.00E-08	3.5	2.2	CAGAATAATCTCTCAGATGAA
ATP6V1H	1.70E-10	24.8	7.4	CAGCAGAAGTACGATGATGAA
ATP6V1H	5.40E-09	4.1	3.9	CAAGAGATGCTCAAACCTGAA
BTD	3.80E-03	1.6	1.2	ATGCGATTGGTCTCAAGCTAA
CHRNA2	7.70E-08	3	1.7	CAGCCTCTGTTTGACCATGAA
CHRNA2	4.10E-05	1.6	1	CACGGGCACCTACAACAGCAA
CNOT2	7.90E-10	8.3	2.2	CCAGGACTTCTCAATACACAA
CNOT2	2.30E-04	1.5	1.1	CTGGAATATGCAAAATTAGAA
DCST2	1.40E-09	6.9	3.2	CTGCATGATGGTCATACCACA
DCST2	4.70E-03	1.5	0.6	CCACTTCTCTGTGGATCTCAA
DDC	5.00E-08	3	1.1	TCGGCTAAAGGGTTCCAACAA
DDC	1.20E-04	1.6	0.8	CAGGCTTATATCCGCAAGCAT
DIAPH3	1.10E-06	2.1	1.9	AAGAGTGAATATAGCAACTTA
EXDL2	9.40E-08	3.5	1.2	GTGGGTAAATTTGGAAGGCAA
FASTKD5	1.50E-09	6.1	3.1	TACAGATGATTTGATGAATAA
FH	2.00E-08	3.5	2.3	GAGATCTACGATGAACCTTAA
FLJ20254	1.50E-10	37	12.2	CACCTGTGACATGGCCTGCAA
FLJ20254	4.60E-10	12.1	8	TACAGAAGTCTTTGCAAGAAA
FOXB1	1.30E-04	1.8	1.1	TCGCAAACAGCCACCAGCCAA
GALNT10	3.30E-05	2.2	0.5	CAGGCAATTACTGGCCTCAAA
GANAB	2.40E-08	3.6	3.2	GAGGTGTGGTATGACATTCAA
GCH1	1.50E-05	2	1.8	CCCGGTTTCTTTGTGGTCTA
GSX1	2.80E-09	5	1.5	CTCTGTGGACAGCAGCTCTAA
GSX1	4.90E-07	2.2	1.5	CGAGTTCGCTTCTAATATGTA

Table 2-2 continued

HUGO ID	pValue Infectivity ¹	Fold Change (infectivity)	%Fld Chng/ VO Fld chng ²	siRNA Sequence
HRNR	4.70E-08	3.2	1.5	CTGGCTCAGGGTGGTCTTCAA
LRIG3	1.70E-05	1.7	1.5	CAGGAACCTTCATCTCAGCCAA
LRIG3	2.70E-04	1.5	1.5	CAGCTGGACCATAACAACCTA
MAK10	1.80E-06	2.2	1.8	CAAGATTAATAGATAGAATAA
MAK10	4.30E-06	2	1.5	CAGAAAGGCCGTAGTAGTAAA
MUC2	3.10E-09	4.7	3.4	CCCGCTGGGATTCTGAAGTGAA
MUC2	2.50E-04	1.7	0.5	CCGGTTTGGCAACAACACCAA
NDUFB3	5.20E-07	4.1	1.2	CAGATTGAGGATGCACATATA
NPR2	1.10E-08	4.7	1.3	AAGGATGCCCTAGATGAGCTA
NPR2	8.70E-08	3.5	1.6	ACCCAACCTGAATGAAGAGCTA
NRG1	6.50E-10	8.9	4.5	TCGGCTGCAGGTTCCAAACTA
PHF2	2.90E-07	3.6	2.1	AAGATGAATCTTCAACTTTAA
PHOSPHO2	2.20E-09	5.3	2	AAGGGTGTAAGAGAACATGAA
PNLIPRP1	1.90E-04	1.5	0.3	CCCGATGGGTTTGCTGCATAT
PRG4	7.70E-09	4.2	2.6	AAGGAAGAAATCAATAAATAT
PRG4	3.40E-08	3	3.6	CAACATGTAATTATTTAATAA
PRR12	6.80E-10	8	3.4	TTCGGTGTACAGAGAAATTAT
PRR12	2.20E-04	1.5	1.1	AACCGTGTCTCAGGAGCTAA
SEC61B	2.10E-09	6.1	3.8	CCCAACATTTCTTGGACCAA
SEC61B	9.00E-09	3.8	2.9	AAAGTTGGCCCTGTTCCAGTA
SLC5A12	2.60E-07	2.3	2.1	TGGCTTAATCATGTACTCTCA
SLC5A12	4.80E-06	1.9	0.5	ATGGATCTCGACTACATATAT
SMPDL3B	1.30E-04	1.6	0.8	TGGGCGAATTGTGGTCTCTCAA
STX12	3.20E-04	1.8	0.7	TCCCTTAGACATGTACCGGAA
STX4	6.10E-05	1.7	1.1	CAGCTCGGACGAAGAGGACAA
SYNGR1	1.80E-10	23.1	13.8	AGCGTCAAGGACCGCAAGAAA
SYNGR1	2.10E-03	1.5	0.4	CTGGTTCGTGGGATTCTGCTA
TAZ	6.20E-10	9.3	2.8	CCGCCACATCTGGAACCTGAA
TBX20	2.30E-06	3.5	2	AAAGGTGAAACTCACCAACAA
TRIP11	8.50E-05	1.6	1.3	ATCAAGCGTTACAAGAGACTA
UBE2E3	3.00E-08	3.8	1.8	CACAATAAACATGCTCCTGAA
ZBTB41	5.50E-10	8.7	1.5	TCCGTCATGATCACCTTACAA
ZBTB41	8.70E-05	1.6	1.1	AAGGCAGATAGTATATATATA
ZNF91	4.50E-04	1.5	0.6	AAGCATTTATATGGTCTTCAA

¹ Infectivity measured as the percentage of cells infected (see Methods)

² Ratio of infectivity fold change over cell number fold change.

Table 2-3 Dipteran DVHFs and their human homologues

FBGN	Gene	Function (Flybase)	HUGO	Hs protein name	E-value
FBgn0015567	alpha-Adaptin	molecular function is described as: protein transporter activity; protein binding. It is involved in the biological processes: synaptic vesicle transport; asymmetric cell division; neurotransmitter secretion; synaptic vesicle coating; vesicle coating; vesicle-mediated transport; intracellular protein transport; protein complex assembly	AP2A2	adaptor-related protein complex 2, alpha 2 subunit	0
FBgn0015569	alpha-Est10	molecular function is described as: ACHE carboxylesterase activity; cholinesterase activity. The biological processes in which it is involved are not known.		acetylcholinesterase isoform E4-E5 precursor , and others	2.00E-48
FBgn0000095	Antp	molecular function is described as: specific RNA polymerase II transcription factor activity; sequence-specific DNA binding; transcription factor activity. It is involved in the biological processes: specification of segmental identity, antennal segment; midgut development; regulation of transcription from RNA polymerase II promoter; segment specification; heart development; lymph gland development; regulation of transcription, DNA-dependent	HOXB7	homeobox B7	7.00E-35
FBgn0015589	Apc	molecular function is described as: beta-catenin binding; microtubule binding; structural constituent of cytoskeleton. It is involved in the biological processes described with 15 unique terms, many of which group under: anatomical structure development; cell adhesion; regulation of Wnt receptor signaling pathway; organ development; central nervous system development; regulation of biological process; organelle organization and biogenesis; instar larval development; programmed cell death; cell proliferation; cell-cell adhesion; embryonic development via the syncytial blastoderm; cell death; larval chitin-based cuticle development; regulation of signal transduction; microtubule-based process; cell motility; cell communication; gamete generation		adenomatous polyposis coli	5.00E-133
FBgn0034817	Art7	molecular function is described as: protein-arginine N-methyltransferase activity. It is involved in the biological process peptidyl-arginine methylation, to asymmetrical-dimethyl arginine	PRMT7	protein arginine methyltransferase 7	1.00E-131
FBgn0011206	bol	molecular function is described as: mRNA binding; nucleotide binding. It is involved in the biological processes: male meiosis; spermatogenesis; meiotic G2/M1 transition; spermatocyte division; GO:0006445; positive regulation of meiosis	BOLL	boule isoform 1	5.00E-33

Table 2-3 continued

<u>FBGN</u>	<u>Gene</u>	<u>Function (Flybase)</u>	<u>HUGO</u>	<u>Hs protein name</u>	<u>E-value</u>
FBgn0000212	brm	molecular function is described as: DNA-dependent ATPase activity; general RNA polymerase II transcription factor activity; transcription coactivator activity; protein binding; DNA helicase activity; nucleic acid binding; ATP binding; DNA binding; helicase activity. It is involved in the biological processes: imaginal disc-derived wing vein specification; chromatin-mediated maintenance of transcription; regulation of transcription from RNA polymerase II promoter; hemocyte proliferation; muscle development; dendrite morphogenesis; neuron development; phagocytosis, engulfment; oogenesis	SMARCA4	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a4	0
FBgn0034645	CG10320	molecular function is described as: NADH dehydrogenase activity; NADH dehydrogenase (ubiquinone) activity. It is involved in the biological processes: RNA import into nucleus; mitochondrial electron transport, NADH to ubiquinone	NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3	2.00E-08
FBgn0036360	CG10713	molecular function is unknown. The biological processes in which it is involved are not known	HRNR	hornerin	4.00E-08
FBgn0036368	CG10738	molecular function is described as: NPR2 guanylate cyclase activity; receptor activity; ATP binding; protein-tyrosine kinase activity. It is involved in the biological processes: signal transduction; cyclic nucleotide metabolic process; nitric oxide mediated signal transduction; protein amino acid phosphorylation; intracellular signaling cascade; cyclic nucleotide biosynthetic process	NPR2	natriuretic peptide receptor B precursor	4.00E-175
FBgn0031178	CG10918	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	
FBgn0036317	CG10948	molecular function is described as: mRNA binding; nucleic acid binding; nucleotide binding. The biological processes in which it is involved are not known	ENOX2	ecto-NOX disulfide-thiol exchanger 2 isoform b	9.00E-80
FBgn0037571	CG11694	molecular function is unknown. The biological processes in which it is involved are not known	EEA1	early endosome antigen 1, 162kD	3.00E-05
FBgn0037375	CG1193	molecular function is described as: ATPase activity; microtubule binding; ATP binding. It is involved in the biological processes: microtubule severing; microtubule-based process; intracellular protein transport	KATNAL1	katanin p60 subunit A-like 1	7.00E-116
FBgn0032311	CG12517	molecular function is unknown. The biological processes in which it is involved are not known.	N/A	No significant similarity found	
FBgn0033945	CG12868	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	
FBgn0033419	CG12932	molecular function is unknown. The biological processes in which it is involved are not known	HRNR	hornerin	2.00E-04

Table 2-3 continued

FBGN	Gene	Function (Flybase)	HUGO	Hs protein name	E-value
FBgn0033569	CG12942	molecular function is described as: transcription regulator activity; zinc ion binding; nucleic acid binding. It is involved in the biological processes: cell proliferation; nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; regulation of transcription from RNA polymerase II promoter; transcription from RNA polymerase II promoter	ZBTB41	zinc finger and BTB domain containing 41 (NJB Note: lots of ZNF)	7.00E-31
FBgn0034022	CG12964	molecular function is unknown. The biological processes in which it is involved are not known	PAXIP1	PAX interacting protein 1	6.00E-15
FBgn0039161	CG13606	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	
FBgn0035578	CG13707	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	
FBgn0032312	CG14071	molecular function is unknown. The biological processes in which it is involved are not known	TRIP11	thyroid hormone receptor interactor 11	0.003
FBgn0031045	CG14212	molecular function is described as phosphoric monoester hydrolase activity. It is involved in the biological process metabolic process	PHOSPHO	phosphatase, orphan 2	9.00E-25
FBgn0027588	CG14476	molecular function is described as alpha-glucosidase activity. It is involved in the biological processes: polysaccharide metabolic process; carbohydrate metabolic process	GANAB	alpha glucosidase II alpha subunit isoform 2	0
FBgn0039617	CG14521	molecular function is unknown. It is involved in the biological processes: cell adhesion; cell communication; cell-cell adhesion; signal transduction	HNT	neurotrimin isoform 2 (NJB note: lots of orthologs)	1.00E-26
FBgn0037317	CG14667	molecular function is described as: zinc ion binding; nucleic acid binding. The biological processes in which it is involved are not known	ZNF628	zinc finger protein 628	1.00E-08
FBgn0037385	CG14675	molecular function is described as: oxygen binding; heme binding; iron ion binding. It is involved in the biological process oxygen transport.	N/A	No significant similarity found	
FBgn0038240	CG14851	molecular function is unknown. The biological processes in which it is involved are not known	N/A	PREDICTED: hypothetical protein LOC729471	3.00E-05
FBgn0038428	CG14894	molecular function is described as binding. It is involved in the biological processes: protein complex assembly; protein folding; protein metabolic process.	TTC1	tetratricopeptide repeat domain 1	2.00E-59
FBgn0034457	CG15122	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	
FBgn0028852	CG15262	molecular function is described as transcription regulator activity. It is involved in the biological process regulation of transcription	CNOT2	CCR4-NOT transcription complex, subunit 2	5.00E-19
FBgn0030215	CG15296	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	

Table 2-3 continued

<u>FBGN</u>	<u>Gene</u>	<u>Function (Flybase)</u>	<u>HUGO</u>	<u>Hs protein name</u>	<u>E-value</u>
FBgn0034605	CG15661	molecular function is described as glucuronosyltransferase activity. It is involved in the biological process metabolic process	N/A	No significant similarity found	
FBgn0031099	CG17065	molecular function is described as N-acetylglucosamine-6-phosphate deacetylase activity. It is involved in the biological processes: polysaccharide metabolic process; N-acetylglucosamine metabolic process	AMDHD2	amidohydrolase domain containing 2	4.00E-129
FBgn0032310	CG17086	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	
FBgn0031195	CG17600	molecular function is unknown. The biological processes in which it is involved are not known	DIAPH3	diaphanous homolog 3 isoform a	1.00E-04
FBgn0030217	CG2124	molecular function is unknown. The biological processes in which it is involved are not known	FASTKD5	FAST kinase domains 5	1.00E-18
FBgn0030386	CG2574	molecular function is described as: ligase activity; ubiquitin-protein ligase activity. It is involved in the biological processes: protein metabolic process; regulation of protein metabolic process; post-translational protein modification.	UBE2E3	ubiquitin-conjugating enzyme E2E 3	1.00E-46
FBgn0038195	CG3061	molecular function is described as: unfolded protein binding; heat shock protein binding. It is involved in the biological processes: defense response; protein folding; protein metabolic process; response to stress	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12	3.00E-81
FBgn0051048	CG31048	molecular function is described as: small GTPase regulator activity; GTP binding; guanyl-nucleotide exchange factor activity; GTPase binding. It is involved in the biological processes: multicellular organismal development; endocytosis; intracellular protein transport; intracellular signaling cascade; mesoderm development; muscle development; phagocytosis; signal transduction	DOCK3	dedicator of cytokinesis 3, 1, 2, 4, 5	0
FBgn0051639	CG31639	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	
CG18166: FBgn0029526, CG32817: FBgn0052817, CG3176: FBgn0029524	CG3176 CG18166 CG32817	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	
FBgn0051860	CG31860	molecular function is described as zinc ion transmembrane transporter activity. It is involved in the biological process zinc ion transport.	SLC30A2	solute carrier family 30, member 2 isoform 1	2.00E-83
FBgn0044328	CG32052	molecular function is described as sphingomyelin phosphodiesterase activity. The biological processes in which it is involved are not known	SMPDL3B	acid sphingomyelinase-like phosphodiesterase 3B isoform 1	2.00E-64
FBgn0052182	CG32182	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	

Table 2-3 continued

FBGN	Gene	Function (Flybase)	HUGO	Hs protein name	E-value
CG33255: FBgn0053255, CR32205: FBgn0052205, CG32207: FBgn0052207	CG32207 CR32205 CG33255	molecular function is unknown. The N/A biological processes in which it is involved are not known.		No significant similarity found	
FBgn0052642	CG32642	molecular function is unknown. The PRG4 biological processes in which it is involved are not known		proteoglycan 4	1.00E-15
FBgn0037967	CG3281	molecular function is described as: ZNF91 transcription regulator activity; DNA binding; zinc ion binding. It is involved in the biological processes: nucleobase, nucleoside, nucleotide and nucleic acid metabolic process; regulation of transcription from RNA polymerase II promoter; transcription from RNA polymerase II promoter; nucleosome assembly		zinc finger protein 91 (NJB note lots of orthologs)	5.00E-41
FBgn0053129	CG33129	molecular function is unknown. The FLJ20254 biological processes in which it is involved are not known		Hypothetical protein FLJ20254	4.00E-33
FBgn0053263	CG33263	molecular function is described as chitin PHF2 binding. It is involved in the biological process chitin metabolic process		PHD finger protein 2	7.00E-04
FBgn0053300	CG33300	<u>molecular function is unknown. The biological processes in which it is involved are not known. One allele is reported. No phenotypic data is available</u>	MUC2	mucin 2 precursor	2.00E-175
FBgn0085277	CG34248	Its molecular function is unknown. The N/A biological processes in which it is involved are not known.		No significant similarity found	
FBgn0034982	CG4065	molecular function is unknown. The MAK10 biological processes in which it is involved are not known		corneal wound healing- related protein	2.00E-143
FBgn0031307	CG4726	molecular function is described as high N/A affinity inorganic phosphate:sodium symporter activity. It is involved in the biological process transport.		No significant similarity found	
FBgn0032205	CG4957	molecular function is described as: integrase N/A activity; DNA binding; zinc ion binding. It is involved in the biological process DNA integration		No significant similarity found	
FBgn0038344	CG5205	molecular function is described as: helicase ASCC3 activity; RNA helicase activity; ATP- dependent helicase activity; ATP binding; nucleic acid binding. It is involved in the biological process nuclear mRNA splicing, via spliceosome		activating signal cointegrator 1 complex subunit 3 isoform a, and PREDICTED: similar to U5 snRNP-specific protein	0
FBgn0038909	CG6569	molecular function is unknown. The MYH11 biological processes in which it is involved are not known		smooth muscle myosin heavy chain 11 isoform SM1B (njb note lots of orthologes)	9.00E-14
FBgn0037901	CG6744	molecular function is described by 3'-5' EXDL2 exonuclease activity; nucleic acid binding. It is involved in the biological process nucleobase, nucleoside, nucleotide and nucleic acid metabolic process		exonuclease 3'-5' domain-like 2	4.00E-94

Table 2-3 continued

<u>FBGN</u>	<u>Gene</u>	<u>Function (Flybase)</u>	<u>HUGO</u>	<u>Hs protein name</u>	<u>E-value</u>
FBgn0013810	Dhc36C	molecular function is described as: ATPase activity, coupled; motor activity; microtubule motor activity; structural constituent of cytoskeleton; ATP binding; ATPase activity; glycerol-3-phosphate dehydrogenase activity. It is involved in the biological processes: microtubule-based movement; cell motility; glycerol-3-phosphate metabolic process	DNAH7	dynein, axonemal, heavy chain 7, 3, 10, 17, 11, 9, 5, 8	0
FBgn0015657	DnaJ-1	molecular function is described as: unfolded protein binding; heat shock protein binding. It is involved in the biological processes: response to heat; defense response; protein folding; response to stress	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	1.00E-101
FBgn0004898	fd96Cb	molecular function is described as: transcription factor activity; sequence-specific DNA binding. It is involved in the biological processes: embryonic development; regulation of transcription from RNA polymerase II promoter; regulation of transcription, DNA-dependent	FOXB1	forkhead box B1	8.00E-48
FBgn0035870	Gr66a	molecular function is described as taste receptor activity. It is involved in the biological processes: sensory perception of taste; response to caffeine	N/A	No significant similarity found	
FBgn0016660	H15	molecular function is described as transcription factor activity. It is involved in the biological processes: heart development; cardioblast cell fate commitment; embryonic heart tube development; mesoderm development; regulation of transcription from RNA polymerase II promoter; regulation of transcription, DNA-dependent	TBX20	T-box transcription factor TBX20 isoform A, B	1.00E-83
FBgn0039019	HP1c	molecular function is described as chromatin binding. It is involved in the biological processes: chromatin assembly or disassembly; regulation of transcription from RNA polymerase II promoter	CBX1	chromobox homolog 1 (HP1 beta homolog Drosophila) (Njb note: CBX1,3,5)	2.00E-29
FBgn0033156	Incenp	molecular function is described as: microtubule binding; protein binding. It is involved in the biological processes: histone phosphorylation; metaphase plate congression; protein localization; mitotic spindle organization and biogenesis	INCENP	inner centromere protein antigens 135/155kDa isoform 1, and mucin 17	2.00E-20
FBgn0025776	ind	molecular function is described as: transcription factor activity; sequence-specific DNA binding. It is involved in the biological processes: regulation of transcription; ventral cord development; dorsal/ventral pattern formation; neuroblast fate determination; brain development; ectoderm development; central nervous system development; pattern specification process; regulation of transcription, DNA-dependent.	GSX1	GS homeobox 1	1.00E-22

Table 2-3 continued

<u>FBGN</u>	<u>Gene</u>	<u>Function (Flybase)</u>	<u>HUGO</u>	<u>Hs protein name</u>	<u>E-value</u>
FBgn0052179	Krn	molecular function is described as: epidermal growth factor receptor binding; growth factor activity. It is involved in the biological processes: MAPKKK cascade; positive regulation of epidermal growth factor receptor activity; ommatidial rotation; epidermal growth factor receptor signaling pathway; border follicle cell migration.	NRG1	neuregulin 1 isoform ndf43 (njb note: and other isoforms)	6.00E-08
FBgn0028336	l(1)G0255	molecular function is described as fumarate hydratase activity. It is involved in the biological processes: tricarboxylic acid cycle; fumarate metabolic process	FH	fumarate hydratase precursor	0
FBgn0034083	lbk	molecular function is described as protein binding. It is involved in the biological processes: bristle morphogenesis; cell adhesion; transmission of nerve impulse; oogenesis. 4 alleles are reported	LRIG3	leucine-rich repeats and immunoglobulin-like domains 3	5.00E-128
FBgn0005630	lola	molecular function is described as: RNA polymerase II transcription factor activity; transcription activator activity; protein binding; RNA polymerase II transcription factor activity; zinc ion binding; nucleic acid binding; motor activity; structural molecule activity. It is involved in the biological processes: axon guidance; axonogenesis; positive regulation of transcription, DNA-dependent; axon midline choice point recognition; antimicrobial humoral response; regulation of transcription from RNA polymerase II promoter; chromatin assembly or disassembly; sex determination; transmission of nerve impulse; ciliary or flagellar motility	KLHL3	kelch-like 3	2.00E-10
FBgn0028582	lqf	molecular function is unknown. It is involved in the biological processes: neurotransmitter secretion; synaptic vesicle endocytosis; endocytosis; negative regulation of cardioblast cell fate specification; regulation of Notch signaling pathway	N/A	No significant similarity found	
LysD: FBgn0004427, LysC: FBgn0004426, LysB: FBgn0004425	LysB LysC LysD	molecular function is described as lysozyme activity. It is involved in the biological processes: antimicrobial humoral response; cell wall catabolic process	LYZ	lysozyme precursor	2.00E-22

Table 2-3 continued

<u>FBGN</u>	<u>Gene</u>	<u>Function (Flybase)</u>	<u>HUGO</u>	<u>Hs protein name</u>	<u>E-value</u>
FBgn0002576	lz	molecular function is described as: RNA polymerase II transcription factor activity; RNA polymerase II transcription factor activity, enhancer binding; transcription factor activity; DNA binding; sequence-specific DNA binding; electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity; ATP binding. It is involved in the biological processes described with 26 unique terms, many of which group under: anatomical structure development; sensory organ development; hemocyte differentiation; organ development; wound healing; organ morphogenesis; regulation of metabolic process; response to stress; lymph gland hemocyte differentiation; transcription from RNA polymerase II promoter.	RUNX1	runt-related transcription factor 1 isoform b	1.00E-51
FBgn0083077	mld	molecular function is described as: zinc ion binding; nucleic acid binding. It is involved in the biological process ecdysone biosynthetic process	ZNF236	zinc finger protein 236	3.00E-18
FBgn0043025	Msi	molecular function is described as: growth factor activity; deaminase activity. It is involved in the biological processes: spermatid development; purine ribonucleoside monophosphate biosynthetic process.	CECR1	cat eye syndrome critical region protein 1 isoform a precursor	1.00E-62
FBgn0000036	nAcRalpha-96Aa	molecular function is described as: nicotinic acetylcholine-activated cation-selective channel activity; acetylcholine receptor activity; neurotransmitter receptor activity. It is involved in the biological processes: cation transport; muscle contraction; nerve-nerve synaptic transmission; neuromuscular synaptic transmission; ion transport	CHRNA2	cholinergic receptor, nicotinic, alpha polypeptide 2	2.00E-126
FBgn0031145	Ntf-2	molecular function is described as protein transmembrane transporter activity. It is involved in the biological process protein import into nucleus	N/A	No significant similarity found	
FBgn0038722	Nup58	molecular function is described as nucleocytoplasmic transporter activity. It is involved in the biological process nucleocytoplasmic transport	NUPL1	nucleoporin like 1 isoform a, b, c	5.00E-49
FBgn0034475	Obp56h	molecular function is described as odorant binding. It is involved in the biological processes: sensory perception of chemical stimulus; sensory perception of smell; olfactory behavior; response to pheromone; transport	N/A	No significant similarity found	
FBgn0014184	Oda	molecular function is described as ornithine decarboxylase inhibitor activity. It is involved in the biological process cell differentiation	N/A	No significant similarity found	

Table 2-3 continued

FBN	Gene	Function (Flybase)	HUGO	Hs protein name	E-value
FBgn0016054	phr6-4	molecular function is described as: DNA (6-4) photolyase activity; nucleic acid binding. It is involved in the biological process DNA repair	CRY2	cryptochrome (photolyase-like)	2 5.00E-155
FBgn0013725	phyl	molecular function is described as: protein binding; zinc ion binding. It is involved in the biological processes: R1/R6 cell fate commitment; R7 cell fate commitment; Ras protein signal transduction; peripheral nervous system development; R7 cell development; sensory organ boundary specification; sensory organ precursor cell fate determination	C5orf5	hypothetical protein LOC51306 isoform 3	0.01
FBgn0003162	Pu	molecular function is described as: GTP cyclohydrolase I activity; GTP cyclohydrolase activity. It is involved in the biological processes: ommochrome biosynthetic process; tetrahydrobiopterin biosynthetic process; purine base metabolic process; aromatic compound biosynthetic process	GCH1	GTP cyclohydrolase 1 isoform 1	1 9.00E-79
FBgn0053207	pxb	molecular function is unknown. It is involved in the biological processes: smoothed 3 signaling pathway; learning and/or memory; olfactory learning	LOC73113	PREDICTED: hypothetical protein	2.00E-04
FBgn0003263	rn	molecular function is described as: transcription factor activity; zinc ion binding. It is involved in the biological processes: imaginal disc-derived leg morphogenesis; regulation of transcription; cell proliferation; regulation of transcription from RNA polymerase II promoter; compound eye development	ZNF384	nuclear matrix transcription factor 4 isoform b	3.00E-69
FBgn0010638	Sec61beta	molecular function is described as protein transporter activity. It is involved in the biological process SRP-dependent cotranslational protein targeting to membrane, translocation	SEC61B	Sec61 beta subunit	1.00E-30
FBgn0001965	Sos	molecular function is described as: protein binding; Ras guanyl-nucleotide exchange factor activity; DNA binding; Rho guanyl-nucleotide exchange factor activity. It is involved in the biological processes: Ras protein signal transduction; sevenless signaling pathway; actin filament organization; regulation of cell shape; determination of anterior/posterior axis, embryo; torso signaling pathway; nervous system development; nucleosome assembly; regulation of Rho protein signal transduction	N/A	No significant similarity found	
FBgn0014037	Su(Tp)	molecular function is described as: transcription elongation regulator activity; RNA polymerase II transcription elongation factor activity. The biological processes in which it is involved are not known	ELL2	elongation factor, RNA polymerase II, 2	1.00E-30

Table 2-3 continued

<u>FBGN</u>	<u>Gene</u>	<u>Function (Flybase)</u>	<u>HUGO</u>	<u>Hs protein name</u>	<u>E-value</u>
FBgn0036341	Syx13	molecular function is described as SNAP receptor activity. It is involved in the biological processes: synaptic vesicle docking during exocytosis; neurotransmitter secretion; vesicle-mediated transport; cytokinesis after meiosis I; cytokinesis after mitosis; female meiosis; male meiosis; mitosis; protein targeting; intracellular protein transport	STX12	syntaxin 12	2.00E-28
FBgn0024980	Syx4	molecular function is described as SNAP receptor activity. It is involved in the biological processes: neurotransmitter secretion; vesicle-mediated transport; synaptic vesicle docking during exocytosis; protein targeting; regulation of exocytosis	STX4	syntaxin 4	1.00E-26
FBgn0010280	Taf4	molecular function is described as: general RNA polymerase II transcription factor activity; transcription initiation factor activity; transcription factor activity. It is involved in the biological processes: regulation of transcription, DNA-dependent; transcription initiation from RNA polymerase II promoter; positive regulation of transcription from RNA polymerase II promoter; muscle development; dendrite morphogenesis	TAF4B	TAF4b RNA polymerase II, TATA box binding protein associated factor (NJB note: and mucin 2 precursor)	4.00E-72
FBgn0026619	tafazzin	molecular function is described as phosphatidylcholine:cardiolipin O-linoleoyltransferase. It is involved in the biological processes: phospholipid metabolic process; cardiolipin biosynthetic process	TAZ	tafazzin isoform 2	1.00E-64
FBgn0050445	Tdc1	molecular function is described as: tyrosine decarboxylase activity; aromatic-L-amino-acid decarboxylase activity; pyridoxal phosphate binding. It is involved in the biological processes: amino acid metabolic process; transmission of nerve impulse; amino acid and derivative metabolic process; carboxylic acid metabolic process	DDC	dopa decarboxylase (aromatic L-amino acid decarboxylase)	2.00E-136
FBgn0051143	tRNA:CR31143	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found	
FBgn0035895	Unr	molecular function is described as: nucleic acid binding; mRNA binding; protein binding; mRNA 3'-UTR binding; DNA binding. It is involved in the biological processes: negative regulation of translation; dosage compensation, by hyperactivation of X chromosome; regulation of transcription, DNA-dependent	CSDE1	upstream of NRAS isoform 1	7.00E-114
FBgn0030262	Vago	molecular function is unknown. The biological processes in which it is involved are not known	N/A	No significant similarity found.	

Table 2-3 continued

<u>FBGN</u>	<u>Gene</u>	<u>Function (Flybase)</u>	<u>HUGO</u>	<u>Hs protein name</u>	<u>E-value</u>
FBgn0040069	vanin-like	molecular function is described as pantetheinase activity. It is involved in the biological processes: cell motility; cell-cell adhesion; coenzyme metabolic process; cytoskeleton organization and biogenesis; prosthetic group metabolic process; signal transduction; vitamin biosynthetic process; nitrogen compound metabolic process	BTD	biotinidase precursor (NJB note: also Vanin 1 - 3)	5.00E-40
FBgn0010426	Vha14	molecular function is described as: hydrogen exporting ATPase activity, phosphorylative mechanism; hydrogen ion transporting ATPase activity, rotational mechanism; hydrogen ion transporting ATP synthase activity, rotational mechanism. It is involved in the biological processes: proton transport; ATP synthesis coupled proton transport	ATP6V1F	ATPase, H+ transporting, lysosomal 14kD, V1 subunit F (NJB Note: receptor-mediated endocytosis)	7.00E-47
FBgn0028665	VhaAC39	molecular function is described as: hydrogen exporting ATPase activity, phosphorylative mechanism; hydrogen ion transporting ATPase activity, rotational mechanism; hydrogen ion transporting ATP synthase activity, rotational mechanism. It is involved in the biological processes: proton transport; ATP synthesis coupled proton transport	ATP6V0D1	ATPase, H+ transporting, lysosomal, V0 subunit d1	1.00E-170
FBgn0028662	VhaPPA1-1	molecular function is described as: hydrogen exporting ATPase activity, phosphorylative mechanism; hydrogen ion transporting ATPase activity, rotational mechanism; ATP binding; hydrogen ion transporting ATP synthase activity, rotational mechanism. It is involved in the biological processes: cation transport; mitotic spindle organization and biogenesis; ATP synthesis coupled proton transport	ATP6V0B	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit b isoform 1, 2	2.00E-63

Table 2-4 Effect of human DVHF depletion on infection by YFV17D and CBV3 viruses

Virus	DEN2-NGCⁱ	YFV-17Dⁱⁱ	CB3ⁱⁱⁱ
Gene product			
CNOT2	<i>CNOT2_4</i> <i>CNOT2_8</i>	<i>CNOT2_4</i>	<i>CNOT2_4</i> <i>CNOT2_4</i>
NPR2^{iv}	<i>NPR2_2</i> <i>NPR2_5</i>	<i>NPR2_5</i>	<i>NPR2_5</i>
SEC61B^v	<i>SEC61B_5</i> <i>SEC61B_6</i>	<i>SEC61B_5</i>	<i>SEC61B_5</i>
EXDL2	<i>EXDL2_1</i> <i>EXDL2_5^{vi}</i>		
FLJ20254^{vii}	<i>FLJ20254_1</i> <i>FLJ20254_2</i> <i>FLJ20254_3</i>	<i>FLJ20254_2</i> <i>FLJ20254_3</i>	<i>FLJ20254_1</i> <i>FLJ20254_2</i>
TAZ^{viii}	<i>TAZ_2</i> <i>TAZ_5</i>	<i>TAZ_5</i>	<i>TAZ_2</i> <i>TAZ_5</i>

ⁱ siRNAs listed inhibited viral gene expression ≥ 2 fold. Shaded boxes indicate when two or more siRNAs against a gene product resulted in ≥ 2 fold inhibition. DEN2-NGC gene expression was measured by quantifying the percentage of cells positive for E protein expression

ⁱⁱ 17D gene expression was measured using flavivirus group-reactive 4G2 antibody to E protein

ⁱⁱⁱ CBV3 gene products were measured using the anti-enterovirus antibody 5-D8/1

^{iv} A third siRNA inhibited CBV3 but not DEN2-NGC or YFV17D

^v Inhibition of DEN2-NGC was weak

^{vi} Also known as C14orf114_2

^{vii} A fourth siRNA inhibited CBV3 but not DEN2-NGC or YFV17D

^{viii} Diminished cell counts were observed in TAZ depleted cells

2.13 Methods

2.13.1 Cell Culture

Aedes albopictus C6/36 cells (ATCC# CRL-1660) and D. Mel-2 (Invitrogen Cat#10831014) were cultured at 28°C, 5% CO₂. C6/36 cells were grown in MEM (Sigma) supplemented with antibiotic, non-essential amino acids, sodium pyruvate, 5mM HEPES and 10% FBS (Hyclone). D.Mel-2 cells were grown in SF900 II SFM (Gibco) supplemented with antibiotic. Human hepatoma HuH-7 cells and African green monkey kidney Vero cells (ATCC#CCL-81) were grown at 37°C, 5% CO₂ in DMEM (Sigma) supplemented with 10% FBS (Hyclone) and antibiotic. Cells were maintained in accordance with general cell culture practices.

2.13.2 Viral Stock Preparations

DENs

C6/36 cells were passaged into T150 flasks at 1×10^6 cells/mL. The following day, cultures were inoculated with 500µL of virus in 4.5mL serum-free growth media. After 1 hour incubation period with rocking, 15mLs of growth media supplemented with 2% FBS (Hyclone) and 5mM HEPES was added. 48 hours post-inoculation, supernatants were removed and replaced with fresh 2% growth media. 72 hours later, supernatants were collected, cleared of cellular debris by centrifugation, aliquoted and stored at -80°C.

Yellow Fever 17D Virus

Vero cells were passaged into T150 flasks at 1×10^5 cells/mL. The following day, cultures were inoculated with 500µL of virus in 4.5mL serum-free growth media. After 1 hour

incubation period with rocking, 15mLs of growth media supplemented with 2% FBS (Hyclone) and 5mM HEPES was added. 48 hours post-inoculation, supernatants were removed and replaced with fresh 2% growth media. 72 hours later, supernatants were collected, cleared of cellular debris by centrifugation, aliquoted and stored at -80°C.

Coxsackie B3 (Strain 20) Virus

HeLa (R19) cells were passaged into T150 flasks at 1×10^5 cells/mL. The following day, cultures were inoculated with 500 μ L of virus in 4.5mL serum-free growth media. After 1 hour incubation period with rocking, 15mLs of growth media supplemented with 2% FBS (Hyclone) was added. 48 hours later, supernatants were collected, cleared of cellular debris by centrifugation, aliquoted and stored at -80°C.

2.13.3 Plaque Assays

Vero cells were plated onto 24 well plates at a density of 1×10^5 cells/well. The next day, media was aspirated and 100 μ L of serial dilutions of viral supernatant was added to the Vero monolayers for 1hr at 37°C with rocking every 15min. Following adsorption, 0.5mLs of a 1:1 Tragacanth Gum (Sigma)/2x EMEM (CellTech) overlay solution supplemented with 2% FBS (Hyclone) was added to each well and the plates were allowed to incubate for 4-5 days. At the end of this period, overlay was removed and the cells were fixed with fresh 4% paraformaldehyde (Sigma) for 15min at room temperature (RT). The supernatant was then removed and 0.5% Triton X-100 (Sigma) in PBS was added for 15min at RT. Cells were then incubated in block solution (PBS supplemented with 0.1% Tween-20 and 1% Normal Donkey Serum) for 1 hour at RT.

Primary antibody targeting the DEN envelope protein (4G2) diluted 1:2000 in block solution was then added for 1 hour followed by three washes with PBS supplemented with 0.1% Tween. HRP-conjugated anti-mouse secondary (Amersham) diluted 1:2000 in block solution was then added to the cells for 1hr at RT followed by three washes with PBS supplemented with 0.1% Tween. Viral foci were then stained with 0.5mLs of VIP Substrate (Vector Labs) in PBS at RT according to manufacturer's protocol. Once stained, substrate was removed and the monolayers allowed to air dry prior to counting of triplicate samples.

2.13.4 RNAi based screening

Screening in Dipteran cells was performed using the *Drosophila* RNAi Screening Center (DRSC, Harvard Medical School) Genome-wide RNAi Library (DRSC 2.0) in 384-well plate format. The DRSC 2.0 library consists of 22,632 dsRNAs aliquoted into 62 384well plates. Library was screened in duplicate. Library plates, pre-aliquoted with dsRNAs, were thawed at room temperature followed by centrifugation at 180xg in a Beckman GS-6R centrifuge for 2min at 20°C. 100ng of control dsNS1 (targeting the DEN2-S2 genome) in 5µL dH₂O was added to 4 wells per plate. *Drosophila* D.Mel-2 cells, a subclone of S2 cells, (Invitrogen #10831-014) were plated at an initial density of 7000 per well in 40µL Sf-900 II SFM (Gibco#10902) using a Matrix Wellmate automated cell dispenser (Matrix). After 48 hours incubation at 28°C, the cells were infected with 10µL of Dengue-S2 virus (4,780IFU). After 72 hours incubation at 28°C, the cells were processed for immunofluorescence as described below. Screening in HuH-7 human

cells was performed in 384-well format using siRNAs obtained from Qiagen. 1 μ mol of each siRNA was aliquoted into the assay plate in 5 μ L of water. 9.95 μ L of OPTI-MEM I (Gibco #11058) media was complexed with 0.05 μ L of Lipofectamine RNAiMAX (Invitrogen#13778-150) per well and incubated for 30 minutes before addition to siRNA containing well. 1500 HuH-7 cells were then plated into each of the assay wells in 50 μ L DMEM (Gibco #11995) supplemented with 5% FBS (Gibco#16140) and Pen/strep (Gibco#15140) to yield a total volume of 65 μ L and an effective siRNA concentration of 15.4nM. After 48 hrs incubation at 37°C, cells were infected with DEN2-NGC at an MOI ~ 1.4ifu/cell and incubated an additional 48 hrs before fixation and processing for immunofluorescence as described below. Similarly, HuH-7 cells were incubated 72 hours post-siRNA transfection followed by 24 hour yellow fever virus 17D infection at an MOI of ~6.5 ifu/cell or 6 hour Coxsackievirus B3 strain 20 infection at an MOI of ~ 40.9 ifu/cell.

2.13.5 Mosquito rearing and cell line culture

Rockefeller/UGAL strain *Aedes aegypti* mosquitoes were maintained in the insectary facility at 27°C and 80% humidity with a 12-12 hour photoperiod. After egg hatching, larvae were maintained in plastic containers with distilled water and fed with pulverized fish food. Pupae were collected and transferred to cages provided with 10% sucrose, where adults stayed after emersion. The *Aedes albopictus* C6/36 cell line was maintained in 25cm² culture flasks kept inside an incubator at 32°C with 5% CO₂. The

medium utilized to grow the cells was composed by minimal essential medium (MEM), 10% heat inactivated FBS, 1% L-glutamine, and 1% non-essential amino acids.

2.13.6 DEN-2 infection of mosquitoes

DEN-2 from the New Guinea C strain was propagated in C6/36 cells following standard procedures (Troyer, Hanley et al. 2001). For this, C6/36 cells were grown in 75-cm² flasks until they reached 80 % of confluence. Following, the cells were infected with virus with a multiplicity of infection (MOI) of 3.5 virus particles/cell. Infected cells were incubated for 7 days at 32 °C with 5% CO₂, after which they were harvested with a cell scraper and lysed by repeated freezing and thawing in dry CO₂ and a 32°C water bath to release the viral particles. The virus suspension was then mixed with equal amount of commercial human blood and 10% human serum, kept at 32°C for 30 min and immediately used to feed double-stranded RNA injected female *Aedes aegypti* (<http://www.jove.com/index/Details.stp?ID=220>). All procedures involving DEN-2 infections were carried out in a Biological Safety Level 2 laboratory.

2.13.7 Mosquito gene-silencing assays

RNA interference (RNAi)-mediated gene-silencing assays were carried out according to standard methodology (Dong, Aguilar et al. 2006). For this, approximately 69 μ l of a dsRNA suspension (3 μ g/ μ l in water) were injected into the thorax of cold-anesthetized 3- to 4-day-old female mosquitoes using a nano-injector as previously described (<http://www.jove.com/index/Details.stp?ID=230>). Double-stranded RNA injected females were kept in small cups provided with 10% sucrose in the insectary at the

conditions mentioned above. Three days after injection, mosquitoes were fed on a DEN-2-supplemented blood meal. For virus titer measurement, mosquitoes were briefly collected and washed in 70% ethanol, and then rinsed in sterile distilled water, at 7 days after blood meal. Mosquito dissections were done in sterile PBS under a stereo microscope, and the midguts were transferred to microcentrifuge tubes containing 150 μ l of MEM. The tissues were then homogenized with a pellet pestle in a sterile environment. Six independent biological replicate assays were produced for each tested gene.

2.13.8 Immunofluorescence

Cells were fixed with fresh 4% paraformaldehyde (Sigma) for 15min at RT. The supernatant was then removed and 0.5% Triton X-100 (Sigma) in PBS was added for 15min at RT. Cells were then incubated in block solution (PBS supplemented with 0.1% Tween-20 and 1% Normal Donkey Serum) for 1 hour at RT. Primary antibody targeting the DEN envelope protein (4G2) diluted 1:2000 in block solution was then added for 1 hour followed by washes with PBS supplemented with 0.1% Tween. Alexa-488 conjugated, anti-mouse secondary antibody (Invitrogen#A11017) diluted 1:2000 in block solution supplemented with 13.3 μ g/ml of Hoescht 33342 (Sigma) was then added for 1 hour followed by three PBS/0.1%Tween wash steps. Wells were then filled with PBS, sealed and imaged on the Cellomics ArrayScan Vti HCS. Immunofluorescent staining for yellow fever virus 17D was performed exactly the same as above. Immunofluorescent staining for Coxsackievirus B3 strain 20 was similar to above except

the primary antibody was Dako Monoclonal Mouse Anti-Enterovirus Code M7064. The 110mg/L stock was diluted 1:200 in blocking solution.

2.13.9 High-content cell based imaging

Screening plates were imaged and analyzed using a Cellomics ArrayScan Vti HCS automated fluorescent imaging system (ThermoFisher). DMel-2 cell images were acquired at 20x magnification. HuH-7 cells were acquired at 10x magnification. Infected DMel-2 cells were defined in one of two ways, using the RF and DF filters described previously.

2.13.10 Sum Rank Algorithm

During analysis of the D. Mel-2 screen, we observed that infectivity rates 1) were not normally distributed within each plate and 2) varied substantially in magnitude between plates (data not shown). Parametric tests based on the assumption of normality or drawing from similar distributions, such as t-tests or z-scores, were therefore considered inappropriate. We developed a nonparametric approach, the Sum Rank algorithm, in order to produce an appropriate summary statistic of each dsRNA tested in duplicate using two separate 384 well plates. Preliminary analyses also suggested that low cell density (valid object counts as determined by counting nuclei stained with Hoechst) could affect percent infectivity data. Therefore dsRNA wells with fewer than 12500 cells/well were removed from the Sum Rank analysis, along with the 16 control wells present on every plate, to prevent bias within the screen. Within each plate, wells were ranked by the percentage of infected cells, with the well with the lowest percentage

infected cells given rank = 1. For each dsRNA, tested in duplicate, we calculated a Sum Rank statistic (eq 1).

Sum Rank = Rank on plate #1 + Rank on plate #2 (eq 1)

The null distribution for the Sum Rank statistic was mathematically derived, validated through Monte Carlo simulations in R, and experimentally confirmed during the screen (Figure 2-8). The null hypothesis of the Sum Rank test is that infectivity rank within a plate is randomly distributed, or equivalently that all wells are equally infectable. This assumption can be invalid if there are local biases in infectivity within a plate, for example if edges are infected more than center wells. Such effects are present in most genomic screens, including our assay. As a result we may have false positive or false negative rates higher than would be predicted by theory alone. We have not yet assessed the magnitude or implication of such spatial biases within duplicate plates, but believe it is important to acknowledge that such effects are common in genome-wide screens and should be discussed openly to promote improvement of future screens. Spatial biases can be expected to affect any statistical analyses and not just the Sum Rank method. In brief, the Sum Rank score can range from 2 to twice the number of valid wells on a plate (384 – 16 controls = 368 maximum valid wells). Sum Ranks at either extreme are less likely to be observed by random chance. The number of times a given Sum Rank (SR) is expected to occur near the lower extreme (Sum Rank = 2) for a single pair of duplicate plates is given by Equation 2:

$$E[SR] = (SR - 1) / (\# \text{ Valid Wells}) \text{ (eq 2)}$$

The number of times a given Sum Rank is expected to occur near the high extreme (Sum Rank = $2 * \text{Number of Valid Wells}$) follows a symmetrical distribution and is given by Equation 3:

$$E[SR] = ((\# \text{ Valid Wells} * 2) - (SR - 1)) / (\# \text{ Valid Wells}) \text{ (eq 3)}$$

Sum Ranks were calculated for every dsRNA in the *Drosophila* genome, with $E[SR]$ scores below 0.065 used to select potential targets (218 dsRNAs) for further analysis.

2.13.11 qPCR analysis of viral RNA

Total RNA from infected cells was isolated using Trizol (Invitrogen). cDNA was generated using random hexamers to prime reverse transcription reactions using MMLV reverse transcriptase (Invitrogen). cDNAs were then diluted 1:10 with nuclease-free water. RTqPCR was performed with the cDNA using the iQ™ SYBR Green Supermix Kit (Bio-Rad) according to the manufacturer's instructions. Reactions were run on a MyiQ™ iCycler (Bio-Rad) and analyzed with the MyiQ™ Optical System Software (Bio-Rad). qPCR conditions were an initial 95°C for 5 min., followed by 40 cycles of 95°C for 15sec. and 60°C for 30sec. Primers are:

DV2C_FWD 5'-AATATGCTGAAACGCGAGAGA-3'

DV2C-REV 5'-GGGATTGTTAGGAAACGAAGG-3'

GAPDH_FWD 5'-GAGTCAACGGATTTGGTCGT-3'

GAPDH-REV 5'-TTGATTTTGGAGGGATCTCG-3'

Standard curves for copy number determination were generated using DEN2-NGC and

GAPDH containing plasmid DNA. For each sample the DEN2 RNA copy number was normalized to the GAPDH RNA copy number.

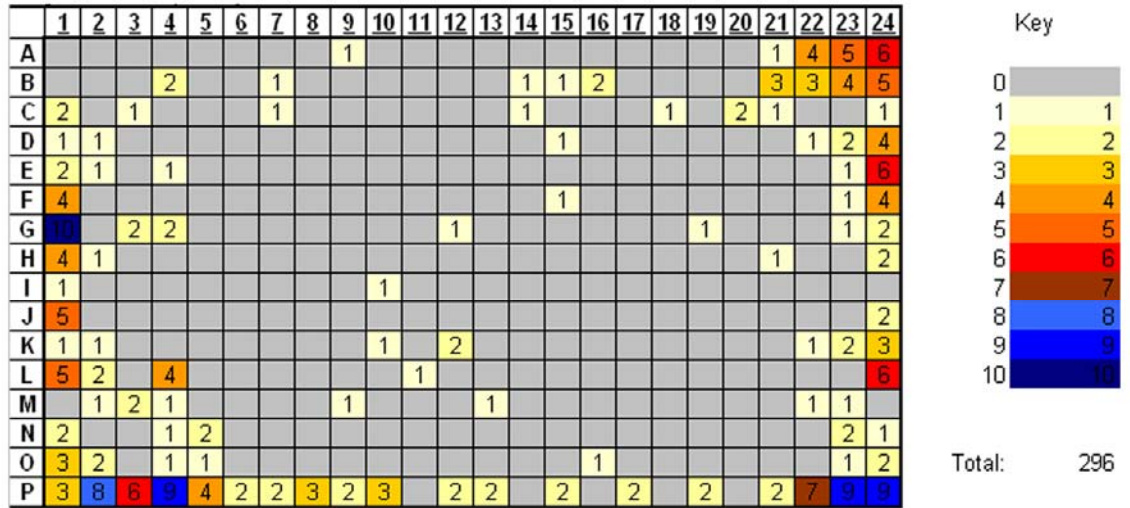
Chapter 3. Conclusion and Perspectives

The work presented here represents the first systematic attempt to identify DVHFs in the Dipteran host. Our screening criteria for these host factors were stringent and thus represent the most conservative analysis of the data generated. Although relaxation of our selection parameters might yield additional authentic DVHFs that were missed in the first analysis, this must be done with the expectation that the false positive rate is likely to increase as well.

In addition to the 116 DVHFs identified in the *Drosophila* screen 296 candidate dengue virus restriction factors (DVRF) were also identified and remain to be validated. We chose to pursue the DVHF candidates before addressing the DVRFs for two reasons: 1) interactions between the viral gene products and host factors required for efficient viral propagation could potentially make attractive targets for small molecule inhibitors of these interactions, 2) many of the dsRNAs targeting candidate DVRFs were non-randomly distributed on the screening plates, suggesting that many of the DVRFs identified may be artifacts of the screening process (Figure 4-1).

Comparison of our results to DEN-host interactions described in the literature and to DEN host factors identified by Krishnan et al 2008, strongly suggests that identification of factors required for DEN propagation, in either the human or insect hosts, has not reached saturation. Indeed, successful identification of all factors required by DEN for propagation by RNAi-mediated depletion of one gene product at a time is unlikely; if a process required by the virus can be accomplished by another protein with

Figure 4-1



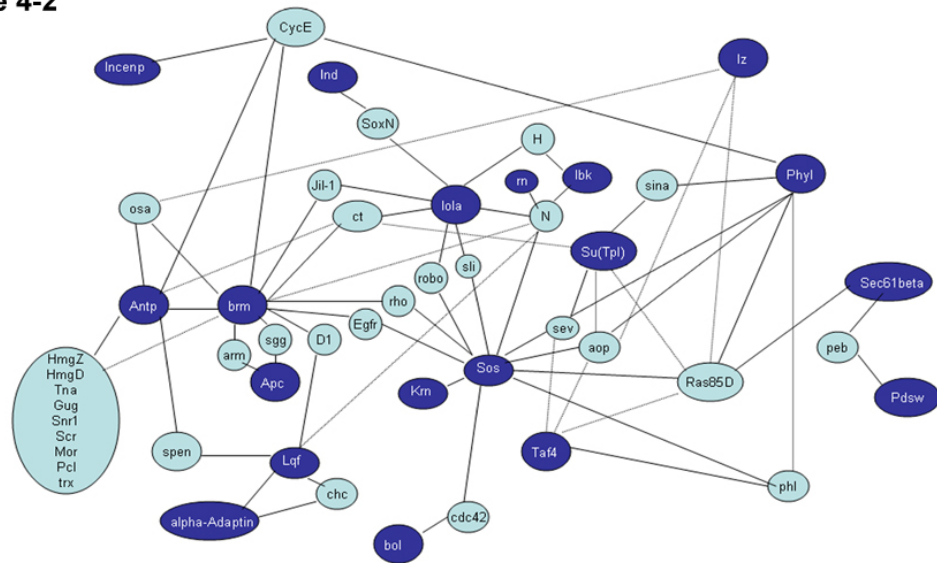
Distribution of candidate DVRFs 296 dsRNAs from the Drosophila screen were identified to increase viral infectivity and represent candidate dengue virus restriction factors (DVRFs). The number of candidate DVRFs found in each location on the 384-well screening plates is indicated on the heat map.

overlapping function, it would not score strongly in this screen. Additionally, RNAi-mediated depletion of transcripts does not necessarily ensure the complete depletion of their gene products and thus candidate factors may be missed, especially if the target is very stable and/or only required in small amounts.

Some of the factors identified as DVHFs are of particular interest. CG6744 (EXDL2, AAEL012690) is predicted to be a 3'-5' exonuclease and was identified to be required for efficient DEN propagation in both a human cell line and in the adult mosquito. Terramoto et al 2008 showed that deletions at the 3' end of the DEN viral genome can be repaired by non-template-based nucleotide addition followed by natural selection of 3'-end sequences that can support viral replication, and it was suggested that this repair process may involve a host 3'-5' exoribonuclease (Terramoto, Kohno et al. 2008). We posit that CG6744 may be this previously unidentified exonuclease and that this function is highly conserved between the human and insect hosts.

Longitudinals lacking (*lola*, AAEL009212), was also of particular interest because it was shown to be a Dipteran specific factor and was one of the DVHFs that caused a significant reduction in DEN propagation when depleted in the adult mosquito. *Lola* is a nuclear factor that is required for axon growth in the *Drosophila* embryo (Madden, Crowner et al. 1999) and normal phagocytosis of bacteria in S2 cells (Ramet, Manfruelli et al. 2002). Interestingly, *lola* was identified in a screen for components of the Imd pathway (Kleino, Valanne et al. 2005), which is one of two major immune pathways in flies. In addition to its reported immuno-modulatory role, *lola* also appears to be one of

Figure 4-2



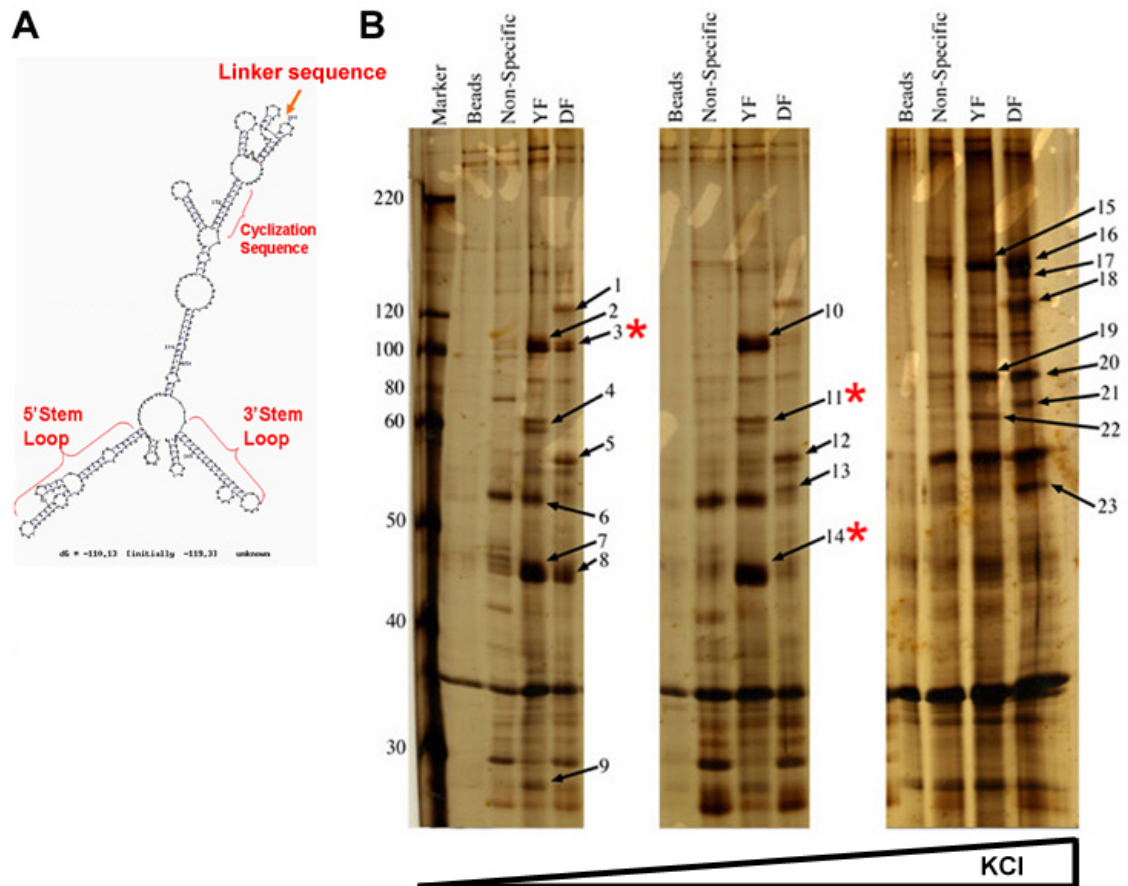
Genetic interactions among DVHFs Flybase annotation describes genetic interactions for 23 of the 116 DVHFs (dark blue circles). 32 interacting proteins (light blue circles) were found to interact with at least two DVHFs. Other interacting proteins (136) that connect to only a single DVHF have been left off the genetic web of interactions for clarity. Solid and dashed edges are also for clarity and are equivalent.

the major nodes in the web of genetic interactions of the twenty-three DVHFs for which this information is available (Flybase 2009) (Figure 4-2).

Characterization of host factors as DVHFs by our definition does not imply a direct interaction with viral gene products. Although much can be inferred from the existing annotation (Flybase 2009), careful biochemical analyses still need to be performed to characterize the exact nature of these DVHF interactions. Combining the results obtained through genome-wide profiling of host factor requirements with established biochemical techniques has the potential to yield important insights. In an RNA affinity chromatography experiment using the 5' and 3' TRs as bait (Figure 4-3a), polypyrimidine tract-binding protein-associated splicing factor (PSF) and nuclear RNA-binding protein 54kDa (p54nrb) from human cell extracts were identified as specific interactors of these structured RNAs (Figure 4-3b) (Sessions and Garcia-Blanco, unpublished results). This is of particular interest because in human cells, p54nrb and PSF have been shown to specifically interact with Brahma (brm) and Brahma-related gene-1 (BRG1) (Ito, Watanabe et al. 2008).

Brm and BRG1 are members of the well-conserved SWI/SNF complex that functions to regulate gene expression by altering chromatin structure (Muchardt and Yaniv 2001; Simone 2006). Brm and BRG1 interact with other members of the SWI/SNF in a mutually exclusive manner and are believed to regulate different sets of genes (Muchardt and Yaniv 2001; Simone 2006). Given the relatively high degree of conservation (72% identity (NCBI 2009)), it is interesting that brm did not score in our

Figure 4-3



PSF and p54nrb specifically interact with flavivirus 5'/3' TRs (A) The predicted structure of the DEN 5' and 3' TRs joined by a linker sequence. (B) Flavivirus and non-specific RNAs were immobilized on beads and incubated in human cell lysates. Matrices were then washed with increasing amounts of KCl and fractions collected. Band 3 was identified as PSF and bands 11 and 14 as p54nrb by MALDI-TOF/TOF.

experiments in human cells. One intriguing possibility is that, in human cells, DEN may be interacting with BRG1 instead of brm through its association with PSF and p54nrb and regulating a different set of genes. As the name suggests, PSF also directly binds PTB, which is known to be required for DEN replication (De Nova-Ocampo, Villegas-Sepulveda et al. 2002; Anwar, Leong et al. 2009). Possible explanations for why PSF and p54nrb were not identified in the Dipteran screen may be that they have overlapping functions (they have been shown to bind brm and BRG1 independently(Ito, Watanabe et al. 2008)) and/or the depletion of the gene products in the screen may have been inefficient.

DEN requires a multitude of factors from its insect and human hosts in order to complete its life cycle. With the advent of RNA interference libraries and automated microscopy, every known and predicted gene product can be assayed one at a time for its requirement in a given system. Using a strain of DEN selected for robust growth in *Drosophila* cells, we have utilized these technologies to identify a multitude of novel DVHFs. As the greater majority of the studies referenced in Chapter 1 have focused on flavivirus-host interactions in mammalian systems, these findings are especially important since they significantly extend our knowledge of the DEN host factor requirement in the Dipteran host. The results from this study serve as a useful list of potential Dipteran targets for vector intervention and aid in evaluating the contribution of specific host factors to disease severity. DVHFs could also serve as effective targets for

therapy, perhaps not only for DF but also for other flaviviral-related illnesses such as West Nile encephalitis/fever and yellow fever.

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Biography

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