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By  Jeffrey Michael Grabowski

Entitled

TICK-FLAVIVIRUS INTERACTIONS: DISCOVERY OF HOST PROTEINS THAT ARE AFFECTED IN VIRUS INFECTION

For the degree of  Doctor of Philosophy

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TICK-FLAVIVIRUS INTERACTIONS: DISCOVERY OF HOST PROTEINS THAT ARE AFFECTED IN VIRUS INFECTION

A Dissertation
Submitted to the Faculty
of
Purdue University
by
Jeffrey Michael Grabowski

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

August 2015
Purdue University
West Lafayette, Indiana
This dissertation is dedicated to my family and friends. I thank Edgar and Mary Grabowski for the utmost support over the years. Also, Jessica and Kevin Napadow have provided great opportunities to help ease one’s mind in stressful times. In addition, I commend my aunts, uncles (present and past), and cousins for the stimulating times. Last, I have so much appreciation for Anastasia Richards and my past grandparents for the sacrifices they made to provide a foundation for the family present today. All in all, words cannot describe the love and the appreciation that I have for my family’s support and sacrifice over the years.

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ABSTRACT

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Tick-borne flavivirus (TBF) infections cause morbidity and mortality worldwide. A primary goal of vector-borne disease research is to identify ways to prevent pathogen and parasite transmission. The black-legged tick, *Ixodes scapularis*, is the only tick species with a sequenced and annotated genome and is a vector of TBFs. This project utilizes Langat virus (LGTV) to model TBF infection in *I. scapularis* ISE6 cells. The aims were to (1) characterize the LGTV-ISE6 system, (2) discover differentially-expressed proteins associated with LGTV infection, and (3) identify key genes of interest that were involved in LGTV infection. Two proteomic analyses by LC-MS/MS identified 486 and 579 ISE6 proteins (total of 764 ISE6 proteins combined). First and second proteomic analyses identified 68 and 246 ISE6 proteins with increased expression and 198 and 188 ISE6 proteins with decreased expression, respectively. Greater than 265 ISE6 proteins were mapped to cellular function/pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG). Greater than 221 ISE6 proteins were identified with no KEGG function/pathway. After filtering based on strength of protein identification, cellular function/pathway involvement, and an increased expression following
LGTV infection, ten proteins were selected. Ten selected proteins/genes of interest included eight with known function (Fumar, ERP29, DPCD, CNHydro, MDH2, PARP, CUKinase, and ACAT1) and two with unknown function (Hypo195 and Hypo576). RNA interference knockdown assays were established in ISE6 cells to determine effect on LGTV infection. Inhibition of genes of interest involved in cellular amino acid, carbohydrate/lipid, and cofactor/vitamin metabolism as well as unknown proteins were shown to affect LGTV genome replication and replication. In addition, LGTV replication was reduced by perturbing ISE6 metabolic function using trichostatin A and oligomycin A and glutamate metabolic function using hexachlorophene and epicatechin gallate small molecules in compound assays. This research suggested that carbohydrate and amino acid metabolic enzymes were involved in LGTV infection in the tick vector and possibly for transmission.
CHAPTER 1: INTRODUCTION

1.1 THE IXODIDAE (HARD TICKS) AS VECTORS OF PARASITES AND PATHOGENS

Hard ticks (Phylum Arthropoda, Subphylum Chelicerata, Subclass Acari, Superorder Parasitiformes, Order Ixodida, Family Ixodidae) are pests of global medical and veterinary importance. These obligate ectoparasites transmit a wide variety of viruses, bacteria and protozoa to humans and animals. Examples of tick-borne disease affecting human health include babesiosis, caused by the parasite *Babesia microti* [1], Lyme disease, caused by the bacterium *Borrelia burgdorferi* [2], Rocky Mountain spotted fever, caused by the bacterium *Rickettsia rickettsii* [3], and granulocytic anaplasmosis, caused by *Anaplasma phagocytophilum* [4]. In addition, domesticated animals can be at risk from borreliosis, babesiosis, spotted fever, ehrlichiosis caused by *Ehrlichia* species, and hepatozoonosis caused by hepatozoon species [5]. In North America, the tick-borne flavivirus (TBF) Powassan virus (POWV) and its genotype Deer Tick virus (DTV) can cause meningitis and encephalitis in humans and potentially in domestic animals [6, 7].

1.2 *IXODES SCAPULARIS; GEOGRAPHIC DISTRIBUTION, LIFE CYCLE, AND DISEASE TRANSMISSION*

In United States (U.S.A.), *I. scapularis* tick is found east of the Rocky Mountains from Canada to Florida, and west to the state of Texas. High populations of *I. scapularis* occur in the upper Midwest and northeast states primarily [8-11].
*Ixodes scapularis* has a two year life cycle in the wild with four developmental stages: egg, larva, nymph, and adult. It is considered a three host tick as the larva, nymph, and adult feed to repletion on different hosts before molting to the next lifestage [12]. Larvae and nymphs feed primarily in the spring and throughout summer on small mammals, reptiles, or birds [13, 14]. Adults feed primarily in the fall on larger mammals such as deer, fox, raccoon, etc and mate during this time. After feeding, adult females drop from the host and lay thousands of eggs that then hatch in the following spring. The nymph and adult stages (primarily adult females) are primarily responsible for transmission of Lyme disease and other tick-borne diseases.

*Ixodes scapularis* is a competent vector for a number of protozoa, bacteria, and viruses that cause disease including *Babesia microti*, *Anaplasma* spp bacteria, *Borrelia* spp bacteria, and POWV/DTV in the U.S.A. [15-18]. The acquisition of parasite and bacteria can occur with the larvae or nymph blood meal, making the subsequent stage infective. POWV/DTV can be acquired by transovarial transmission and therefore the larva, nymph, and adult stages may be infected and capable of virus transmission.

### 1.3 TBFS

TBFs are a complex of 10-11 kb single-stranded, ribonucleic acid (RNA) viruses that cause high human morbidity and mortality [19]. Of the family *Flaviviridae* and genus Flavivirus, TBFs have ten proteins that are translated as a polyprotein and cleaved by a combination of host and viral proteases. Three structural proteins consist of the capsid (C), membrane (M), and envelope (E)
proteins. Seven nonstructural proteins consist of the nonstructural protein 1 (NS1), nonstructural protein 2A (NS2A), nonstructural protein 2B (NS2B), nonstructural protein 3 (NS3), nonstructural protein 4A (NS4A), and nonstructural protein (NS4B).

Many exotic TBFs are identified on the Centers for Disease Control and Prevention “Select Biological Agents and Toxins” list (http://www.selectagents.gov/) as they have the potential to become established zoonotic diseases in the U.S.A. and they are considered possible bioterrorism agents [20]. TBFs result in hemorrhagic fever and encephalitis, and are transmitted to human via bite of infected *Ixodes* species tick [21-23].

Tick-borne encephalitis virus (TBEV) is arguably one of the most important TBFs, and has been reported in Europe, the Far East, and Siberia. Mansfield et al reported a 400% increase in TBEV cases in Europe from 1974 to 2003 [23]. The geographic range of TBEVs may be changing as several TBEVs have recently been reported from a number of “non-endemic” regions, including Norway in 2004 [24]. Each year more than 10,000 cases of TBEV are reported globally. TBEV-associated case fatality rates range from 10-40%. Additionally, survivors of TBEV infection have a high incidence of permanent neurological sequelae. The actual incidence of TBEV is thought to be much higher as many cases are presumably undiagnosed [23, 25]. TBEV is classified as biosafety level 4 (BSL-4), and consequently, most research aimed at understanding TBFs have been conducted using less pathogenic TBFs other than TBEV. Effective prophylactic anti-TBEV vaccines [26] have been developed in the past but there is no direct treatment for infection.
The POWV is the only TBF documented in the U.S.A. POWV can cause encephalitis in humans and domesticated animals, specifically horses, and in rare cases, dogs [27]. The virus was first discovered in 1958. POWV is of considerable concern because the virus has a high case fatality rate of 10-15% [28] in humans. However, confirmed human cases of POWV infection are low in comparison to WNV and other mosquito-borne arboviruses. From 2004 to 2013, an average of six to seven POWV neuroinvasive diseases cases were reported and fifteen confirmed cases were reported from 2013-2014 (CDC; cdc.gov/powassan/statistics; diseasemaps.usgs.gov/). Diagnostic testing is limited for POWV in patients with symptoms of encephalitis. The incidence rate of POWV infection in the U.S.A. is seriously misjudged [29, 30] and more recognition and treatment of this emerging viral infection is necessary. The POWV/DTV has no anti-viral vaccine or specific therapy for treatment. In addition, survivors have a high rate (approximately 50-55% of confirmed cases of infection) of permanent, recurring neurological problems [22]. This situation is similar with DTV, which is also prevalent in mammal reservoirs and transmitted by *I. scapularis* [31]. Both POWV and DTV are classified as BSL-3, limiting research on these viruses to a small number of approved facilities.

Langat (LGTV) virus is classified as BSL-2 and is widely used as a model for TBF research. LGTV is vectored by *Ixodes grandulatus* and *Haemaphysalis* spp. ticks and the natural mammal reservoir is forest rats (Fig 1; [20]). It can cause encephalitis in humans and animals (although LGTV has a low virulence in human beings). LGTV is thought to have originated in either Malaysia, Thailand, or Siberia
Human disease cases of LGTV infection have not been reported but LGTV-antibodies have been detected in blood samples collected off individuals living in endemic regions [20, 23]. In the 1970s, LGTV was used successfully as a live vaccine for TBEV, but its use was terminated when it was discovered that the vaccine caused encephalitis in approximately 1:10,000 vaccinated individuals [21].

1.4 PATHOGENESIS OF TBFS IN THE MAMMALIAN HOST AND TICK VECTOR

1.4.1 TBF life cycle

The flavivirus life cycle begins when the virus binds to the cell surface where receptor-mediated endocytosis occurs with the virus entering the cell in an endocytic vacuole [33]. Fusion of the viral membrane with the endosomal membrane results in the uncoating of the viral nucleocapsid, which releases viral RNA into the cell cytoplasm. Protein translation occurs forming a single polyprotein formed in the cytoplasm and intertwined within endoplasmic reticulum (ER) membrane. Viral genome replication occurs on the ER membrane [34] in replication complexes. Virus assembly and the viral envelope formation occurs within the ER and appears as “budding” under electron microscopy imaging. The immature (noninfectious) viruses bud into the ER and move through the trans-Golgi network (TGN), where eventually they are cleaved by a host protease [35]. This cleavage creates mature (infectious) viruses, which then exit the host cell through exocytosis.

It has been found that TBF maturation differs between mammalian and tick cells. A key difference is that TBEV particles have been detected in the Golgi apparatus in mammalian cells, but were not detected in the Golgi apparatus and
detected in the lumen of vacuoles [36] in tick cells. Yoshii et al has shown that TBEV envelope (E) glycosylation, a modification required in the secretory process of virions in mammalian cells, does not affect infectious TBEV release from tick cells [37]. In addition, inhibiting transport to the Golgi apparatus from the ER affected TBEV release in mammalian cells, but not in tick cells. The model for the TBF life cycle in mammalian and tick cells is shown in Fig 2.

1.4.2 Mammalian host

TBFs first invade epithelial cells at the site of introduction from the bite of an infected tick. Eventually, the virus drains into the lymph nodes and systemic infection ensues [25]. TBFs are neurotropic where neurons are the primary cell type infected [38]. Infection of the brain, spinal cord, and meninges results in encephalitic and meningitis symptoms.

1.4.3 Tick vector

Once a tick stage takes a blood meal that contains infectious TBF, infection of the midgut tissue occurs and subsequently, invasion of the hemolymph and salivary glands. Mitzel et al identified that LGTV titer is an additional factor that determines infection within the I. scapularis larva [39]. No reduction in survival of I. scapularis larvae occurs with LGTV infection, which suggests that TBF infection is not detrimental to tick life stages. LGTV initially infects midgut tissue approximately three days post LGTV exposure and secondary organs (including salivary glands) at approximately ten days post LGTV exposure. Transstadial and transovarial transmission of LGTV and POWV can occur within I. scapularis where larvae, nymphs, and adult ticks can be infected [40, 41].
1.5 *IXODES SCAPULARIS* GENOMIC RESOURCES

The only sequenced and assembled tick genome is that of the black-legged tick, *I. scapularis*, available from Genbank, that currently contains 20,486 gene models predicted via a combination of *ab initio* methods and manual curation [42, 43]. This assembly has been an important resource used to understand both tick biology and tick-borne pathogenesis at the molecular level. Mining this genome assembly has also allowed for identification of new targets for tick control.

1.6 TICK CELL LINES AND *I. SCAPULARIS* ISE6 CELLS

Development of tick cell lines are a valuable tool for studies of tick-borne pathogenesis [44, 45]. Tick cells have been used extensively to explore tick-borne bacterial pathogenesis [46-50]. Tick cell lines have also been utilized as models to explore responses to tick-borne viruses [51, 52]. The *I. scapularis* ISE6 cell line and the model TBF LGTV was used to form an *in vitro* TBF pathogenesis model here. One disadvantage of using tick cell lines is possible contamination with endogenous tick-borne bacteria or viruses. The *I. scapularis* IDE8 cell line is known to have an endogenous orbivirus, St. Croix River virus [53] and virus-like particles have been observed in other tick cell lines as well [54]. Coinfection in tick cell lines with some pathogens has shown variable effects on pathogen replication [55]. Overall, this emphasizes that proper experimental controls need to be developed before conducting TBF research with tick cells.

1.7 IDENTIFICATION OF HOST CELL FACTORS INVOLVED IN TBF INFECTION

Research has shown that proteins and metabolites produced by human [56, 57] and mosquito [58-61] cells (i.e., “host-cell factors”) may facilitate or play roles
in flaviviral infection [62-65]. The mechanisms by which these molecules contribute to the replication of flaviviruses are not well understood. Human host cell factors have been identified with Hepatitis C virus (HCV), Dengue virus (DENV), and West Nile virus (WNV); however, little is known regarding factors involved in flaviviral infection in the vector. Studies conducted in *Drosophila* and a number of mosquito species [58, 62, 66] have identified host factors. However, there has been little attention directed towards understanding host factors in the tick system. Studies dealing with whole tick and tick cells infected with the tick-borne pathogens *Anaplasma* spp. and *Rickettsia montanensis* have shown differentially-regulated genes and differentially-expressed proteins in response to infection [50, 67-71]. These studies give insight into genes and proteins that are upregulated/increased expression or downregulated/decreased expression from a pathogen infection, respectively.

Little work has been done to identify factors produced by the arthropod or mammalian host in response to TBF infection. Many studies have focused on factors produced by mosquitoes in response to flaviviruses. However, there is significant variation between the genomes and host specificities of TBFs and mosquito-borne flaviviruses. Studies have also investigated global changes in the transcriptome in *I. scapularis* and in tick cells following LGTV infection [72], although there is little known about how these responses correlate to changes at the protein level. Tick proteins that facilitate viral infection and replication in the arthropod vector are logical targets for interventions aimed at disrupting transmission of TBF.
1.8 PROTEOMIC ANALYSES OF HARD TICKS

A useful tool includes proteomics, used to understand protein expression and has the utility for improving genome annotations. High throughput proteomic analyses have identified cellular pathways/functions associated with basic tick physiology, such as host attachment and blood feeding [73]. Recently, proteomic analyses of the *I. scapularis* ISE6 and IDE12 cell lines, and synganglia have identified neuronal proteins suggesting ISE6 cells may be a potential *in vitro* model of the tick synganglia/neurons. Proteomics has also been used to investigate interactions between ticks (including tick cells) and bacterial pathogens [70, 71, 74, 75]. Currently, there is limited understanding of how tick protein expression changes following TBF infection.

1.9 THE NEED FOR DEVELOPMENT OF NEW STRATEGIES AGAINST TBFS

One of the biggest challenges in vector biology and the long-term goal of this research is to identify new ways to prevent transmission of TBFs. There are currently no anti-tick vaccines or anti-viral drugs against TBFs [76, 77] and there is limited research to understand the infection process of TBFs in the tick vector. This gap in our knowledge base is an impediment to the development of new strategies against TBFs.

Historically, developing arthropod control approaches have focused on neuronal proteins, enzymes, and receptors. More recently, attention has shifted to novel pest control approaches such as those employing RNAi transcript knockdown of genes in the invertebrate [Macron, Doug; http://www.genomeweb.com/rnai/monsanto-moves-four-rnai-products-through-
pipeline-posts-improved-fiscal-first-q]. RNAi transcript knockdown to identify genes of interest in ticks implicated in bacterial pathogenesis has been undertaken [78, 79]. Genome assemblies are an important source of sequence information but a significant proportion of the gene and conceptual protein repertoire produced by automated annotation of arthropod genomes are considered “orphan” sequences, or proteins of no known function. These orphan proteins are a potential source of novel targets for traditional chemical pesticide development and RNAi-based pest control as they may potentially be unique to the arthropod vector. The impact of experimentally-identified, tick proteins will allow for identification of potential control targets against TBF infection and the tick itself.

An approach to translate the research herein may rely on the use of proteins from this study as antigens to screen to identify small, inhibitory compounds. It was hypothesized that the inhibition/knockdown of tick proteins/genes of interest involved in TBF infection will reduce virus infection and production of progeny virus spread. Upon the identification of involved tick proteins in viral infection, the goal is to use specific therapeutic products (antigen development, small molecules, proteins/peptides, RNAi-based molecules, etc) to disrupt TBF transmission. It is feasible to first identify a select few proteins/genes of interest involved in TBF infection within a tick cell line and then to confirm these proteins are involved in TBF infection with in vivo studies using whole ticks (Fig 3).

1.10 OVERVIEW OF THESIS RESEARCH

The goal of this project was to discover I. scapularis proteins involved in TBF infection in the cell. The study focused on development, characterization, and
analysis of the LGTV-ISE6 cell line model using proteomics to identify tick protein expression levels following LGTV infection. The rationale was that some of these proteins were involved in viral infection in the cell and whole tick and were thus logical targets for strategies to disrupt virus transmission. Overall, it was hypothesized that ISE6 cell proteins with increased expression following LGTV infection were involved in LGTV infection. The aims were (1) to establish a proteomic screen to identify tick proteins associated with TBF infection, (2) to characterize tick cellular pathways and functions of tick proteins with altered expression following TBF infection, and (3) to determine key tick proteins involved in TBF infection.

This multidisciplinary, collaborative project was the first to investigate how flaviviruses perturb the tick proteome. This work was expected to have a broad scientific impact as it will provide an essential basis to understand the molecular processes that operate in tick cells during infection by TBFs. The findings may lead to better medicine to control flavivirus infections, better tick control strategies, improved public health, and countermeasures to protect against bioterrorism. In addition, it was an integral component of an ongoing international effort to understand TBF pathogenesis and analyze the I. scapularis genome.

Chapter 2 introduces the establishment of the LGTV-ISE6 cell model system. This chapter describes the ISE6 cell growth, LGTV growth within ISE6 cells, creation of a noninfectious LGTV control, effect on cell population numbers following LGTV infection, effect on cell viability following LGTV infection, and effect on cell mortality following LGTV infection. Chapter 3 describes two high throughput
proteomic analyses, a differential and quantitative analysis, to identify hundreds of ISE6 proteins with changing expression following LGTV infection associated with cellular pathways. Comparative proteomic analysis to other flavivirus-host systems were presented as well as cellular pathway changes at different time points following LGTV infection, and lysine acetylation of ISE6 proteins associated with LGTV infection. Chapter 4 describes small molecule compound and RNA interference assays to inhibit a subset of ISE6 proteins (discovered from Chapter 3) with increased expression following LGTV infection and identify tick molecular pathways involved in LGTV infection.
Fig 1. **Natural transmission cycle of LGTV.** LGTV is a zoonotic pathogen and is naturally transmitted by *Ixodes* and *Haemaphysalis* hard tick species to forest rats. Rats are the mammalian reservoir for LGTV. Incidental hosts include humans.
Fig 2. LGTV life cycle within the cell. Stages of the LGTV life cycle predicted in the mammalian and tick cell. Red box denotes a different LGTV egress route suggested in the cell tick, but not in the mammalian cell. Black circles denote cellular ribosomes. Blue lines denote positive strand, LGTV RNA genomes. Orange lines denote negative strand, LGTV RNA genomes used as a template for further positive strand, LGTV RNA genome production. Red circles denote viral proteins in association with replication complexes on/in the ER. Purple particles/virions denote immature LGTV before prM-furin cleavage. Green circles denote furin, which cleaves pr from prM, creating mature LGTV. Pre-membrane= prM.
Fig 3. Schematic showing key steps proposed in the translational research pipeline to identify tick proteins to use for development of therapeutics and anti-tick vaccines. Proposed pipeline to identify ISE6 proteins involved with LGTV infection from the present study. Selected proteins can be pursued via functional studies and investigated for potential development of antigens or therapeutics against TBF infection. Selected proteins need to be characterized with *in vivo* studies in the whole tick to confirm reduced transmission of TBF.
CHAPTER 2: CHARACTERIZATION OF ACUTE LGTV INFECTION IN I.
SCAPULARIS ISE6 CELLS

2.1 INTRODUCTION

Tick cell lines have become appropriate models for tick-borne pathogen research [46, 51, 52, 80], especially to identify molecular components involved in pathogen infection. LGTV infections have been carried out in ISE6 cells before to characterize virus replication [81-83], hence ISE6 cells make an effective model for proteomic-based analyses studying tick protein responses with TBF infection.

Identifying tick protein changes following TBF infection in ISE6 cells first requires characterization of the ISE6-LGTV system (Fig 1). For this study, characterization involves (1) identifying ISE6 cell growth, (2) LGTV growth within ISE6 cells, (3) effect on cell population numbers following LGTV infection, (4) effect on cell viability following post LGTV infection, and effect on cell mortality following LGTV infection. It was hypothesized that ISE6 cell viability would not change with LGTV infection in comparison with mock (uninfected) treatment. Characterizing the biological response of the tick cells to LGTV infection was a necessary prerequisite for proteomic analyses.
2.2 MATERIALS AND METHODS

2.2.1 Cell and virus culture

*Ixodes scapularis* embryonic ISE6 cells (provided by T. Kurtti, University of Minnesota, Minneapolis, MN) were cultured at 34°C in L15B-300 media in the absence of CO$_2$ [44, 45]. Baby hamster kidney 15 (BHK15) cells, used for plaque assay and immunofluorescent focus assay (IFA), were cultured with at 37°C in Minimum Essential Media (MEM) supplemented with L-glutamine, non-essential amino acids (NEAA), and 10% heat-inactivated fetal calf serum (FCS) with 5% CO$_2$. Green African monkey kidney Vero cells, used to create LGTV stock and for IFA to determine LGTV stock titer, were cultured at 37°C in MEM supplemented with L-glutamine, NEAA and 10% heat-inactivated FCS with 5% CO$_2$. LGTV TP21 wildtype strain, passage 2 (obtained from A. Pletnev, NIH-NAID, Bethesda, MD [84]) stock was amplified in Vero cells (multiplicity of infection 0.01) [85] and grown as described above, except with 2.5% heat-inactivated FCS, up to passage 4 (p4) to provide a working stock for experimental infections. Serial IFAs were conducted in parallel as previously described [86] in 96-well cell culture plates to determine LGTV stock titers.

2.2.2 Measurement of ISE6 cell concentrations

The concentration of cells in each sample (cells/ml) was estimated by counting cell number on a Scepter 2.0 Automated Cell Counter with 40 µM Scepter sensors (EMD Millipore; PHCC20040) in order to equalize cell numbers between biological replicates and between treatment groups prior to protein extraction. For cell growth analyses, initial cell counts (cells/mL) were determined manually using
a hemocytometer and subsequently verified by sample analysis on the Scepter 2.0 Automated Cell Counter.

**2.2.3 LGTV infection in ISE6 cells and measurement of infectious LGTV**

As described in Grabowski et al [87], serial plaque assays were performed using BHK cells to assess LGTV growth and replication via plaque development. Media collected from culture of LGTV-infected mammalian or tick cells was serially diluted ten-fold in PBS++ (calcium and magnesium) and adsorbed for one hour onto ~80% confluent BHK cells in 6 well cell culture plates. An agarose overlay infused with a 2X MEM, 5% FCS and 2% agarose was added following adsorption. Following solidification of agarose, MEM and 2.5% calf serum was added, and plates were incubated at 37°C in 5% CO₂. Plaque formation was assessed 4-5 days-post infection (dpi) and cells were stained with ~4% Neutral Red solution (Sigma-Aldrich; T6567).

Serial immunofluorescent focus assays were conducted in parallel as previously described [86, 88] in 96 well cell culture plates to determine LGTV stock titers. Ten-fold serial dilutions of media collected from culture of LGTV-infected cells was adsorbed (50 uL total volume) onto BHK cells for one hour at 37°C in 5% CO₂. An infusion of 1.5% carboxymethylcellulose sodium (CMC) solution supplemented with 3% MEM with 2.5% fetal calf serum media was added to each well and plates were incubated for 36 hours post infection at 37°C in 5% CO₂. The CMC and media overlay were removed and cells were rinsed five times with 1X PBS. Cells were fixed using 4% paraformaldehyde for 15 minutes at room temperature. Fixative was removed and 2% Triton-X 100 was added for 10 minutes
at room temperature to permeabilize cells. Following removal of Triton-X 100, cells were rinsed five times and blocked with addition of solution BSA/PBS (10mg/mL BSA in PBS). Cells were labelled with primary and secondary antibodies and immunofluorescent foci were identified at 20x magnification as described above.

IFAs were used to assess the level of LGTV infection in ISE6 cells. Detection of the LGTV non-structural protein 3 (NS3) was performed using YP-conjugated chicken anti-LGTV NS3 (provided by S. Best, NIH-NAID, Hamilton, MT) as primary antibody and IgG-conjugated goat anti-chicken, Alexa Fluor 488 (Invitrogen, Grand Island, NY; A11039) as secondary antibody. Cell nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies, Grand Island, NY; D1306). Glass coverslips were used to culture and infect cells for the IFAs and were placed onto microscope slides, which were viewed on an Olympus model IX81F-3 microscope and images were collected using an Olympus U-CMAD3 camera. Fluorescence excitation was provided by the EXFO X-Cite Series 120PC and Olympus IX2-UCB. Image overlays were produced with Metamorph Basic v7.6.5.0 software.

To establish an MOI and time-point corresponding to optimal LGTV replication in ISE6 cells, three concentrations (MOIs of 7, 13, and 26) of LGTV were used to infect cells. For each, cells were fixed at 3, 9, 24, and 48 hpi with five technical replicates that were imaged under 20x magnification. On the basis of complete infection (>96%) in ISE6 cells between two MOIs (7 and 13) and time points (24 and 48), an MOI of 10 was selected for subsequent experiments for maximum infection.
Separately, an assessment of the cumulative virus release was carried out in LGTV-infected ISE6 cells at an MOI of 10. Media from these LGTV-infected ISE6 cells was harvested at 12 hour intervals for up to 120 hours, and subjected to plaque assays to measure replication. In addition, LGTV one step growth was analyzed in ISE6 cells, where all media were removed and replaced with fresh complete media at each time point. Media removed were then subjected to plaque assays to measure replication at each time point.

2.2.4 Production of non-infectious LGTV

To create non-infectious LGTV (UV-LGTV), LGTV p4 stock media (~4 x 10^6 pfu/mL) was placed in 48 well cell culture plates and treated with UV radiation at a distance of 11 cm from a standard (12.4 watt) UV lamp in a biological safety cabinet (Nuaire Labgard ES, Plymouth, MN) for 30 second intervals over a five minute period. LGTV inactivation was confirmed by blind passage of UV-LGTV on ~2 x 10^7 ISE6 cells and ~80% confluent BHK cells, followed by immunofluorescent and plaque assay as described by Perera et al. [89] to demonstrate lack of infectivity.

2.2.5 Cell viability and mortality assays

To assess cell viability, cells were treated with alamarBlue reagent (AbD Serotec; BUF012A) diluted 1:10 with fresh media for 12 and 2 hours for ISE6 and Vero cells, respectively. Fluorescence (excitation at 560nm, emission at 590 nm) was measured using a Molecular Devices SpectraMax M5 plate reader coupled with SoftMax Pro v4.8 software. Five technical replicates were performed for each time point with biological replicates (n=2).
A trypan blue cell exclusion assay was used to assess mortality in ISE6 cells following LGTV infection. Poly-L-Lysine-treated 96-well plates were seeded with ISE6 cells for 48 hours to a cell density of ~9 x 10^4 cells/well. Cells were treated with LGTV infection (MOI 10; p4 LGTV stock) or condition media as described above. Cells were harvested at 12, 24, 36, and 48 hpi, centrifuged at 1,510 x g for 5 min, media was removed and the cell pellet was re-suspended in 1X PBS. Subsequently, a 1:1 0.4% trypan blue:cell suspension, was prepared, incubated for ~3 min at RT, the cells were immediately counted using a hemocytometer [90] and the percentage of stained ISE6 cells was determined for LGTV and mock treatments. Three technical replicates were collected per treatment with two biological replicates (n=2).

2.2.6 Statistical analyses

Graphpad Prism (v4.03) was used to perform two-tailed t-test analyses. Standard error was completed to show error between technical replicates. At least two biological replicate experiments were completed for each experiment.

2.3 RESULTS

2.3.1 ISE6 cell growth and characterization of LGTV growth in ISE6 cells

_Ixodes scapularis_ ISE6 cell growth was first established before characterization of LGTV infection in ISE6 cells was carried out. ISE6 cell doubling time was determined to be between 4 to 5 days (Fig 2). Timothy J. Kurtti (University of Minnesota) confirmed that the _Ixodes_ species-derived cell lines had a 4-7 day doubling time (personal communication; March 14, 2011). The growth of ISE6 cells
were slow when compared to most mammalian cell cells and ISE6 cells do not form monolayers but grow on top of each other.

IFAs were used in time course experiments to assess levels of LGTV in ISE6 cells (Fig 3). Under the assay conditions described herein, IFA revealed that the maximum level of LGTV infection in the ISE6 cell population (>96%) corresponded to an MOI of 10 as determined by percentage of cells positive with the LGTV NS3 protein (Figs 3A and 3B), and plaque assays revealed that the peak of LGTV release from ISE6 cells occurred at 36 hpi (Fig 4). These conditions were selected for subsequent proteomic analyses. In addition, an LGTV one step growth curve in ISE6 cells with an MOI of 10 identified initial LGTV release at around 12 hpi and a peak LGTV release at 48 hpi (Fig 5). This suggested that the first round of LGTV replication (without reinfection in cells) was ≤12 hpi and that the 36-48 hpi range was the period with maximum infectious LGTV release.

2.3.2 UV inactivated, noninfectious control LGTV

Plaque assays revealed that UV radiation for 30-60 seconds leads to 50% inactivation and ≥120 sec was sufficient to achieve 100% inactivation of LGTV as determined by the lack of plaque formation (Fig 6). The minimum time observed for lack of LGTV NS3 protein appearance was 3.5 minutes (data not shown). UV-LGTV used for proteomic analyses and subsequent assays was inactivated for five minutes.

2.3.3 ISE6 cell population numbers, viability, and mortality following LGTV infection

ISE6 cell population numbers did not change in comparison between LGTV infected and mock treated ISE6 cells (Fig 7). ISE6 cell viability was reduced during
the initial infection with LGTV (i.e., ≤48 hpi) but does not change after 48 hpi (48-60 hpi), as measured based on presence of cellular reducing agents (Fig 8). ISE6 cell mortality, as measured by counting cells treated with trypan blue after LGTV infection or mock treatment, revealed no change with LGTV infection groups (Fig 9).

2.4 DISCUSSION

LGTV can establish persistent infection in ISE6 cells after initial, or acute, infection. After peak infectious LGTV release from ISE6 cells, in combination with published studies [83], LGTV replication remains primarily linear. This also provides phenotypic data that suggested ISE6 cells undergo molecular changes in cellular pathways that LGTV may utilize. However, the hypothesis that ISE6 cell viability does not change with LGTV infection in comparison with mock treatment is rejected since ISE6 cell viability was reduced at ≤48 hpi with LGTV.

2.4.1 Conditions established for proteomic analyses

Characterization of a virus-host system is required before performing any type of genomic, transcriptomic, or proteomic analyses. The effect LGTV infection has on ISE6 cell population numbers, viability, and mortality can be used to explain molecular responses but also can be used to optimize biological sample collection for subsequent proteomic analyses. For example, completing the one step growth analysis of LGTV-infected ISE6 cells identifies the initial release of infectious LGTV around 12 hpi. In addition, cumulative and one step growth analyses identify the peak LGTV release from ISE6 cells between 36 to 48 hpi. In initial acute infection, it was suggested that 12 hpi was early, 24 hpi was early/intermediate, and 36/48
hpi was late. Identification of proteins and corresponding pathways/functions can now be categorized based on the different stages of acute LGTV infection.
Fig 1. LGTV-ISE6 flavivirus-tick cell system. ISE6 cells are derived from *I. scapularis* embryonic eggs. Wildtype TP21 strain LGTV was utilized in this study for the model tick-borne flavivirus of choice to infect ISE6 cells.
Fig 2. *Ixodes scapularis* ISE6 cell population growth. Confluent ISE6 cells are \( \sim 5 \times 10^7 \) cells. ISE6 cells were split and plated in 48 well plates. Cells were consistently flushed off 96 cell surfaces via pipette action and placed on a glass hemocytometer for cell counting under a microscope. Standard error shown in error bars with three technical replicates. Two biological replicate experiments were completed.
Fig 3. Quantification of LGTV infection in *I. scapularis* ISE6 cells via immunofluorescence. (A) Immunofluorescent detection of virus in ISE6 cells at 3, 9, 24 and 48 hours post infection (hpi) with LGTV MOIs of 7, 13 and 26. LGTV NS3 nonstructural protein (green), DAPI-stained nuclei (blue). (B) Percentage of infected ISE6 cells at 3, 9, 24 and 48 hpi following treatment with LGTV at MOIs of 7, 13 and 26 as determined from quantifying ISE6 cells with immunofluorescent LGTV NS3 expression.
Fig 4. LGTV cumulative growth in ISE6 cells. Time course experiment showing amount of infectious LGTV (Log pfu/mL) released from ISE6 cells initially infected with LGTV MOI = 10 (n=3). Small media volume (100 µL) was removed for titration and replaced with the same volume of fresh media at each time point. Titration was performed using both plaque assays and immunofluorescent focus forming assays in BHK cells. Standard error shown in error bars with three technical replicates. Two biological replicate experiments were completed.
Fig 5. LGTV one step growth in ISE6 cells. Time course experiment showing amount of infectious LGTV (pfu/mL) released from ISE6 cells initially infected with LGTV MOI = 10. All media was removed for titration and replaced with fresh media at each time point. Titration was performed using plaque assays in BHK cells. Standard error shown in error bars using three technical replicates. Two biological replicate experiments were completed.
Fig 6. UV-activation of LGTV to create noninfectious LGTV (UV-LGTV). Plaque assays in BHK cells showing the reduction in infectious viral titer (pfu/mL) following treatment with UV-irradiated LGTV viral stocks for up to 300 seconds. Three technical replicates with standard error bars shown. Two biological replicates were completed.
Fig 7. ISE6 cell growth following LGTV infection. LGTV infection (MOI 10) and mock treatment of ISE6 cells were completed on ISE6 cells in a 96 well plate. Cells were consistently flushed off 96 cell surfaces via pipette action, diluted 1/10th the original volume, and placed on a glass hemocytometer for cell counting under a microscope. Standard error shown in error bars with three technical replicates. Two biological replicate experiments were completed.
Fig 8. ISE6 cell viability following LGTV infection. Alteration of ISE6 cell viability at 12-24, 24-36, 36-48 hours post infection are shown with and without LGTV (mock-treated) infection. * denotes p<0.05 and ** denotes p<0.01. RLU denotes relative light units (fluorescent measurement with 560 nm excitation and 590 emission). Standard error of the mean shown in error bars with five technical replicates. Two biological replicate experiments were completed.
Fig 9. ISE6 cell mortality following LGTV infection. Using the trypan Blue cell exclusion assay, cell mortality percentage at 12, 24, 36, 48 hours post infection with and without LGTV (mock-treated) infection was completed. Standard error shown in error bars with five technical replicates. Two biological replicate experiments were completed.
CHAPTER 3: LC-MS/MS PROTEOMIC ANALYSES OF LGTV-INFECTED *I. SCAPULARIS* ISE6 CELLS

3.1 INTRODUCTION

Based on the results presented in Chapter 2 for optimizing/characterizing LGTV infection in ISE6 cells, preparation of biological samples necessary for proteomic analyses was carried out as described in this chapter. Identification of ISE6 proteins with altered levels following LGTV infection was completed at 36 hpi, or peak infectious LGTV release. To compare different biological treatment groups, comparative/quantitative (differential) proteomics was an appropriate method to identify alteration in tick protein expression levels. Once proteins were identified, cellular pathway/function and protein domain analyses were carried out. Key metabolic and gene regulation pathways were identified, along with identification of tick proteins with or without orthology.

The proteomic response in earlier time points post LGTV acute infection was also explored, where key proteins/pathways may be linked with the initial release of LGTV from ISE6 cells. Also, further validation of ISE6 proteins with a second proteomic analysis provided an increased confidence of protein identification. In addition, a recent proteomic study of *I. scapularis* tick cells and synganglia [91] provided identification of tick neuronal proteins that overlap with ISE6 protein identifications. This study has been able show overlapping proteins
to change in expression levels following neurovirulent LGTV infection. Third, by identifying changing ISE6 proteins following different time points post LGTV infection, cellular pathways/functions that were changing/fluctuating can be identified. Fourth, PTMs during TBF infection have not been explored and were hypothesized to be associated, and potentially involved in TBF infection. Specifically, lysine acetylation level change among ISE6 proteins was explored following LGTV infection. Label-free quantification (LFQ) proteomic analysis of ISE6 cells at 12, 24, 36, and 48 hours post LGTV infection was carried out to identify metabolic and gene regulatory proteins, which were further subjected to comparative analyses, pathway/functional cluster analyses, and PTM searches.

Reduced ISE6 cell viability following LGTV infection was identified in Chapter 2, a consequence of reduced cellular energy (a decrease in reducing agents suggest reduced ATP levels). TCA cycle and oxidative phosphorylation pathways were involved in cellular respiration and production of ATP. It was hypothesized that more ISE6 TCA cycle and oxidative phosphorylation proteins have decreased expression than increased expression following 12-48 hpi with LGTV. The rationale was that reduced protein expression was leading to decreased production of cellular reducing agents (NADH, NADPH, etc). The LFQ proteomic analysis of ISE6 cells at 12, 24, 36, and 48 hours post LGTV infection was used to assess this hypothesis.
3.2 MATERIALS AND METHODS

3.2.1 Cell and virus culture

Cells and virus stocks were prepared as described in Chapter 2, Section 2.2.1.

3.2.2 LGTV infection in ISE6 cells and measurement of infectious LGTV

LGTV infection of ISE6 cells and measurement of infectious LGTV was completed as described in Chapter 2, Section 2.2.3.

3.2.3 Production of non-infectious LGTV

Production of non-infectious LGTV was completed as described in Chapter 2, Section 2.2.4.

3.2.4 Preparation of peptide samples and mass spectrometry analyses

3.2.4.1 Preparation of ISE6 cell samples for LC-MS/MS analysis

To establish LGTV infection in ISE6 cells for LC-MS/MS analyses, \( \sim 2 \times 10^6 \) ISE6 cells were adsorbed with LGTV p4 at an MOI of 10 for one hour as previously mentioned. Infection of \( >96\% \) of the cells was confirmed by IFA. In parallel, ISE6 cells were adsorbed with UV-inactivated LGTV and conditioned media (obtained from uninfected Vero cell culture). The production of these three treatment groups representing cells treated with (a) infectious LGTV (LGTV), (b) non-infectious LGTV (UV-LGTV) and (c) conditioned media only (mock control) was replicated five times (n=5 biological replicates). Samples were harvested at 36 hpi and were adjusted to \( \sim 1.7 \times 10^5 \) cells to ensure an equal concentration of cells per replicate. Cells were then pelleted at 1,510 x g for 5 minutes, culture supernatant was removed, and pellets were stored at -80°C. Cell pellets used for the differential
proteomic analysis were thawed, re-suspended with hypotonic 100 mM ammonium bicarbonate buffer, subjected to passive lysis (30 minutes), and mixed manually by pipetting at room temperature (RT). Protein concentration was determined using a NanoDrop 2000c (Thermo Scientific) in protein a280 mode (v1.2.1). Cell pellets used for the LFQ proteomic analysis were thawed, re-suspended with hypotonic 100 mM ammonium bicarbonate buffer, and subjected to freeze-thaw lysis for three cycles with mixing via pipette action at each thaw. Protein concentration was determined using Whatman Protran BA85 Nitrocellulose (Ref: 10402594) with protein standard concentrations. Chloroform:methanol (2:1) extraction was performed and proteins from the aqueous phase was collected [92, 93]. Proteins were precipitated by addition of ice cold 100% acetone to samples, followed by vortexing for five seconds, and incubation at -20°C for one hour. Samples were spun at 16,000 x g for five minutes and the protein pellet was re-suspended in 8M urea supplemented with 10mM DTT and incubated at 37°C for 1.5 hours. Proteins were denatured by addition of a 50mM ammonium bicarbonate solution supplemented with acetonitrile (ACN), triethylphosphine (TEP), and 2-iodoethanol (97.5%:0.5%:2%) [92, 93]. Protein pellets for the differential proteomic analysis were dried on a speed vacuum for two hours at 37°C and digested for 18 hours at 37°C in 50 mM trypsin (Sigma-Aldrich; T6567) solution (diluted in ammonium bicarbonate) at a ratio of 1:50 w/w trypsin:protein [93]. Protein pellets for the LFQ quantitative proteomic analysis were dried on a speed vacuum for two hours at 37°C, resuspended in 50 mM trypsin (Sigma-Aldrich; T6567) solution (diluted in ammonium bicarbonate) at a
ratio of 1:50 w/w trypsin:protein, subjected to barocycler (Pressure BioSciences Inc.; NEP2320) pressure-induced digestion at 50°C for the following conditions: 60 cycles of 20,000 psi for 50 seconds on high pressure and 10 seconds on low pressure, and macrospin C18 columns (The Nest Group, Inc.; SMM SS18V) were used.

3.2.4.2 LC-MS/MS analyses and identification of proteins in ISE6 cell samples

3.2.4.2.1 Differential proteomic analysis

Tryptic, molecular species of peptides were separated on a nanoLC system (1100 Series LC, Agilent Technologies, Santa Clara, CA). Peptides were loaded on an Agilent 300SB-C18 enrichment column for concentration and the column was switched into the nano-flow path for five minutes. Peptides were separated with a C18 reversed phase ZORBAX 300SB-C18 analytical column (0.75 μm x 150 mm, 3.5 μm) from Agilent.

The column was connected to the emission tip from New Objective and coupled to the nano-electrospray ionization (ESI) source of the hybrid ion trap mass spectrometer LTQ-Orbitrap LX (Thermo Scientific). The peptides were eluted from the column using an acetonitrile/0.1% formic acid (FA; mobile phase B) linear gradient. The column was equilibrated with 95% H₂O/0.1% FA (mobile phase A) for 5 min and proteins were eluted using a linear gradient of 5%-35% B for 50 minutes at 0.3 μL/min, followed by a linear gradient of 35%-100% B for 10 minutes. The column was washed with 100% of ACN/0.1% FA and equilibrated with 95% of H₂O/0.1% FA before injection of the subsequent sample. A blank injection run
between every five samples (of each treatment group) was completed to avoid carryover.

Operation was completed in the data-dependent positive acquisition mode in which full MS scan (resolution 30,000) was followed by four MS/MS scans. The four most abundant molecular ions were selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. Raw data was collected via Xcaliber (v 2.0.7). Database searches were conducted using Spectrum Mill (MS Proteomics Workbench v.03.02 software; Agilent Technologies). To identify peptides, a homology search was conducted against the VectorBase *I. scapularis* IscaW1.2 annotation [94] protein dataset (precursor mass tolerance=0.05 Da; fragment mass tolerance=0.6 Da; maximum of two tryptic mis-cleavages) using Spectrum Mill [92, 93]. Only those peptides with a Spectrum Mill score of ≥ 5 and scored peak intensity (% spi) of ≥ 70% were considered [92, 93, 95].

To account for false positives, a decoy (reversed) database search was performed using the MS/MS search option in Spectrum Mill. Peptide scores were compared to those of reversed peptide scores to obtain a delta forward-reverse score. The cut-off was established as >1 for +1 parent charged peptides and +2 parent charged peptides and >2 for +3 (and greater) parent charged peptides [95].

**3.2.4.2.2 LFQ quantitative proteomic analysis**

Peptides were loaded into a C18 trap column (200 µm x 0.5 mm, ChromXP C18-CL, 3 µm, 120 Å, Eksigent) and the separation was carried out in a capillary C18 column (75 µm x 15 cm, ChromXP C18-CL, 3 µm, 120 Å) connected to a
nanoHPLC system (Ekspert nanoLC 400, Eksigent). The elution was performed with the following gradient: 1 min in 5% solvent B (Solvent A: 0.1% FA and solvent B: 100% ACN/ 0.1% FA), 5-35% solvent B in 90 min, 35-80% solvent B in 2 min, 5 min in 80% solvent B, 80-5% B in 2 min, and hold in 5% for 20 min. The flow rate was constant at 200 nL/min over the whole gradient. Eluting peptides were directly analyzed in an electrospray ionization mass spectrometer (TripleTOF 5600+, AB Sciex). Full-MS spectra were collected in the range of 400 to 1600 m/z and the top 50 most intense parent ions were submitted to fragmentation for 50 milliseconds using rolling-collision energy.

Identification and quantification of peptides were performed with MaxQuant software (v 1.5.2.8). Species-specific sequence database of LGTV strain TP21 and *I. scapularis* were downloaded from Uniprot on 01/20/2015. Database searches were performed considering cysteine carbamidomethylation as a fixed modification, protein N-terminal acetylation and oxidation of methionine as variable modifications. Peptide mass tolerance was set to 0.07 and 0.02 Da for first and second database searches, respectively. Identified peptides were filtered with 1% false-discovery rate at peptide-spectrum match and protein levels. For extracting peak areas, peptides that were not found in all the runs were matched based on the precise mass and retention time alignment. Proteins only with two or more peptides identified were selected. To account for false positives and protein contaminants, a decoy (reversed) database search and UniProt contaminant database was searched in parallel with the *I. scapularis* database.
3.2.4.3 LC-MS differential analysis of LGTV-infected ISE6 cell samples

The Omics Discovery Pipeline (ODP; omicsdp.org) was employed for differential protein analyses with mass spectrometry proteomics [92, 93, 96, 97]. Raw data from LC-MS/MS runs were uploaded and total ion chromatograph visualization was completed with each sample run and treatment group. Deconvolution of spectra into peaks was completed for each sample utilizing XMass using the GISTool [98]. Alignment of peaks using XAlign [99] was conducted and normalization was accomplished from a number of different methods [100-102] that best fit the data. Normalized files were subject to statistical analyses and pattern recognition analysis. A heat map was created using the Heatmap.2 function of the ‘gplots’ package in R. Normalized files subjected to statistical analyses also were subject to principal component analysis (PCA), self-organizing maps (SOM), and linear discriminate analysis (LDA) (unpublished data). For more information regarding the specific components of the ODP, refer to [96].

3.2.4.4 LFQ quantitative analysis of LGTV-infected ISE6 cell samples

The average LFQ intensity values for the technical replicates were used for each sample and submitted to InfernoRDN ([103]; http://omics.pnl.gov/software/infernordn). All the values were transformed [log2(x)] and the Liner Regression Normalization and Central Tendancy options were applied. The results table files and GO annotation files were loaded into MS Access and tables were merged into one.
3.2.4.5 Post-translational modification search of ISE6 proteins

MaxQuant software was utilized to search the *I. scapularis* database with lysine acetylation along with the other variable modifications mentioned above. Upon search completion, proteins identified with modified peptides and identified in at least three out of five samples of at least one treatment group were subjected to two sample t-test and ANOVA statistical analyses via GraphPad Prism software (v 4.03) to identify statistically-significant changes in modifications following LGTV infection and UV-LGTV treatment.

3.2.5 Measurement of ISE6 cell concentrations

Measurement of ISE6 cell concentrations was completed as described in Chapter 2, Section 2.2.2.

3.2.6 Statistical analyses

Following alignment and normalization of MS peaks, standard two-sample t-test and Wilcoxon-Mann-Whitney rank test (unpublished data) were used to compare significant differences between mass profiles of the samples. An ANOVA was also utilized to identify significant masses across the three treatment groups and identify group differences of the three-sample analysis. Statistics were incorporated using the R statistical package (http://www.r-project.org/). Application of a false discovery rate based correction method [104] was performed with the significance tests.

For the differential proteomic analysis, the following methodology was used to account for the average fold change of a protein with multiple peptides: (1) Log of each peptide intensity value followed by (2) averaging these Log values,
identified an averaged Log-valued protein intensity fold change; (3) the natural Log (ln) of the averaged Log-valued protein intensity fold change was determined in order to convert values to the original average fold change value for the corresponding protein. The “flattening” of the data was a stringent approach to identify the differential change in MS peak intensities for a particular protein. Fold change of >2 was considered an increase in expression, 0.5-2 fold change denotes no change in expression, and fold change of <0.5 corresponds to a decrease in expression.

3.2.7 Assignment of ISE6 proteins to function, class, and pathway

Peptides with homology to *I. scapularis*, IscaW1.2 gene models were assigned to a putative functional class by searching accession numbers against the KEGG orthology database (http://www.genome.jp/kegg/ko.html) and the KEGG pathway database (http://www.genome.jp/kegg/pathway.html). ISE6 proteins with orthology to KEGG entries were populated within KEGG pathways that also included mammalian and arthropod orthologs.

3.2.8 Biological clustering and assignment of ISE6 proteins to function, class, and pathway

Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/summary.jsp; [105, 106]) was utilized to identify *I. scapularis* proteins with and without orthology (KEGG Orthology). In addition, DAVID was utilized to identify significant, biological clustering from Gene Ontologies and InterPro protein domains corresponding to the *I. scapularis* proteins.
Peptides with homology to *I. scapularis*, IscaW1 gene models were assigned to putative functional class by searching accession numbers against the KEGG orthology database (http://www.genome.jp/kegg/ko.html; [107, 108]) and the KEGG pathway database (http://www.genome.jp/kegg/pathway.html). ISE6 proteins with orthology to KEGG entries were populated within KEGG pathways that also included mammalian and arthropod orthologs.

### 3.3 RESULTS

#### 3.3.1 Differential proteomics: Effects of LGTV infection on the ISE6 proteome

Completion of the virus life cycle as determined by release of infectious virus particles was observed in ISE6 cells infected with LGTV. In comparison, in cells treated with UV-inactivated virus (UV-LGTV) we observed no release of infectious virus particles. Comparative proteomics analyses were used to identify proteins expressed throughout the process of cell infection (LGTV) versus those associated only with viral attachment and entry of the host cell (UV-LGTV). The sequence of proteomic analyses performed using the three treatments (LGTV, UV-LGTV, and mock) are shown in Fig 1 and Table 1.

LC-MS data were compared for LGTV, UV-LGTV and mock samples (Fig 2). The expression patterns of LC-MS peaks for LGTV samples were more similar to that of UV-LGTV samples than to that of mock samples. The t-test and ANOVA were used to identify proteins that exhibited differential expression (p < 0.05) between LGTV and UV-LGTV samples as compared to the mock samples (Fig 3A). In total, 486 ISE6 proteins were identified based by homology to NCBI/VectorBase accessions. Of these, 266 and 248 proteins were identified as differentially
expressed in the LGTV and UV-LGTV samples, respectively, compared to mock samples. Sixty-eight proteins had increased expression, while 198 proteins showed decreased expression in the LGTV samples as compared to mock samples. Additionally, 82 and 166 proteins showed increased and decreased expression (Fig 3B), respectively, in the UV-LGTV samples in comparison to mock samples. Overall, 243 proteins (50%) exhibited decreased expression while 120 (24.7%) showed increased expression in LGTV and UV-LGTV samples as compared to the mock treatment (Fig 4A).

3.3.2 Differential proteomics: Functional analyses of I. scapularis ISE6 proteins

Of the 486 ISE6 proteins identified in this study, 265 (54.5%) mapped to orthologous proteins in the KEGG database, while 221 proteins had no match (KEGG; genome.jp/keg/ko). Of the 265 proteins, 176 (36.2%) mapped to 66 KEGG pathways and 16 KEGG modules (Fig 5A). The KEGG pathways identified in the present study were categorized into five cellular functions: “metabolism”, “genetic information processing”, “environmental information processing”, “cellular processes”, and “organismal systems”. The majority of proteins (52%) were identified to the functional category “genetic information processing”, followed by “metabolic” (38.7%) and “cellular” (6.3%), “environmental information processing” (2%), and “organismal systems” (1%) (Fig 5B).

LGTV samples exhibited the highest number of proteins (53) identified to the KEGG pathway “genetic information processing” (Fig 4B-4D). Within this group, eight proteins exhibited increased expression and were classified in the pathway, “translation” (Fig 4B). For UV-LGTV samples, the majority of ISE6 proteins (57)
were also classified in the pathway, “genetic information processing”. The majority of proteins exhibiting increased expression (17) were classified in the protein processing pathways of “folding, sorting, and degradation” (7 proteins; 41.2%), followed by “translation” (6 proteins; 35.3%) and “transcription” (4 proteins; 23.5%).

Proteins from the LGTV and UV-LGTV samples that lacked a match to KEGG database entries also displayed differential expression. Of these, 30 proteins had increased expression and 91 had decreased expression in LGTV samples in comparison to mock samples (Fig 6). Additionally, 38 and 85 proteins were identified with increased and decreased expression, respectively, in the UV-LGTV samples as compared to mock samples.

### 3.3.3 Differential proteomics: Changes in the ISE6 cell proteome following LGTV infection

Proteins that showed an increase in expression in LGTV samples were mapped onto the KEGG functional categories of cell signaling (CYC, STK3, RPS6), proteolysis (UCHL3, PSMA, UBE2N), carbon-nitrogen hydrolase activity (DDAH, VNN), replication and mRNA processing (PARP, TRA2, CUTL, H2A, CSTF2), translation (RPS6, RPL17, AARS, NARS), glutamate metabolism/glutaminolysis (prostate-specific transglutaminase ISCW011739; Fig 7 and Table 2), pyruvate metabolism and energy association (MDH2; Fig 8). Proteins that exhibited decreased expression were associated with the functional categories of glycolysis (GAPDH; Fig 8), energy processes (ATP5H, ATP5A1), and mRNA surveillance (PABPC, PELO, MSI, THOC4).
3.3.4 **Differential proteomics: Changes in the ISE6 cell proteome following UV-LGTV treatment**

Proteins exhibiting increased expression in UV-LGTV samples were mapped onto the KEGG functional categories of signaling (RHOGDI, RAB35, SIP, LAMC1), cytoskeletal components, (ACTN, TUBA), unfolded protein response and ER-associated degradation (HSPA1_8, RAB7A), lysosomal functions (PSAP), and phagosome functions (RAB7A). Proteins that exhibited a decrease in expression were associated with transport (BAP31), cell survival (BAP31, HYOU1, DERL1, GROEL), cell growth (SUMO, NOP10, MAD1L), translation (NOP10), and protein folding (GROEL).

3.3.5 **Differential proteomics: Changes in the ISE6 cell proteome common to both LGTV infection and UV-LGTV treatment**

Responses common to LGTV and UV-LGTV samples included proteins exhibiting increased expression and associated with signaling (ITGB, MO25), cytoskeletal structure perturbation (TLN), amino acid metabolism (ACAT, DP5CD, GLUD1, CARP, FAH), glutamate metabolism/glutaminolysis (DP5CD, GLUD1, membrane protein (ISCW001521; Fig 7 and Table 2), RNA interference (AUB), and energy-production (ACAT). Proteins with decreased expression and common to both treatment groups were classified to KEGG functions of glycolysis (ALDOA/B/C, ALDH2/1B1/3A2; Fig 8), energy association (ATP5D, ATP5B), RNA interference (VIP), and structural manipulation (ACTB_G1, TUBB).

3.3.6 **Differential proteomics: Comparative proteomics of tick, mosquito, and human-flaviviral infection**

185 of the 265 ISE6 proteins with orthology to KEGG entries (70%) were also identified in a proteomics study of HCV infection of HUH7.5 cells [56] (Fig 9). Sixteen ISE6 proteins (6%) matched orthologs identified in a study of West Nile
virus (WNV) infection of Vero cells [109], 16 proteins matched orthologs in a yeast two-hybrid study of flavivirus-host interactions [110], and 15 proteins (5%) matched orthologs identified in Aedes aegypti infected with dengue virus (DENV) [65]. A subset of proteins that exhibited increased expression following LGTV infection and/or UV-LGTV treatment and matched proteins in the studies above, were associated with protein synthesis and proteolysis (Fig 10). Of the remaining 66 proteins (24.9%), those that exhibited increased expression in LGTV samples were classified in the KEGG functional categories of proteolysis (PMSA, CARP), ATP association/interaction (PSMA, ANMK), cell and matrix adhesion (VNN, ITGB), and as well as oxidative stress and redox homeostasis (VNN and conserved hypothetical protein ISCW020127-PA). Additionally, the cellular function of hydrolase activity was suggested by increased expression of PSMA and VNN.

3.3.7 LFQ quantitative proteomics: ISE6 protein and neuronal protein overlap

The sequence of proteomic analyses performed using the three treatments (LGTV, UV-LGTV, and mock) and the four time points (12, 24, 36, and 48 hpi) is shown in Fig 11 and Table 3. Identification of 579 proteins (Fig 12) from the LFQ proteomic analysis (all ISE6 proteins from 12, 24, 36, and 48 hpi; Fig 13) overlapped with 301 proteins from the differential proteomics analysis (486 ISE6 proteins). A recent study published by Oliver et al [91] identified I. scapularis synganglia proteins where 115 of these proteins overlap with the LFQ and/or differential proteomic analyses (Fig 14). Changes in expression following LGTV infection was initially searched and identified with the overlapped ISE6 and neuronal proteins (data not shown).
**3.3.8 LFQ quantitative proteomics: Biological function clustering of ISE6 proteins changing with LGTV infection**

Of the ISE6 proteins with statistically-significant changing expressions following LGTV infection, DAVID searches revealed greater numbers of biological clusters (similar biological function among proteins identified with statistical significance) within the 12 and 24 hpi time points than in the 36 and 48 hpi time points (Table 4 and Table 5). ISE6 proteins with increased expression identified the most clusters. At 12 hpi, the biological cluster of macromolecular complex assembly and subunit assembly was unique to this time point. At 24 hpi, the biological clusters of protein folding and unfolded protein response, glycosylation activity and ER involvement, protein synthesis/elongation activity, and cofactor/NAD/NADH binding were unique to this time point. At 12 and 24 hpi, the biological clusters of ribonucleotide complex/ribosome/translation, heat shock protein activity, nucleotide/ribonucleotide binding, thioredoxin function/homeostatic response, and GTPase activity were observed. No biological clusters were identified at 36 hpi and the biological cluster of RNA recognition/nucleotide binding was observed at both 24 and 48 hpi. ISE6 proteins with decreased expression identified two biological clusters only at 24 hpi, where RNA recognition/nucleotide binding and ATP/nucleotide/ribonucleotide/nitrogen biosynthetic/metabolic processes/cation transmembrane transporter activity was observed.

**3.3.9 LFQ quantitative proteomics: Cellular pathway fluctuations following LGTV infection**

Pathways with proteins with increased expression were seen in greater amount at 12 and 24 hpi than at 36 and 48 hpi. Pathways with proteins with
decreased expression were seen in great amount at 48 hpi (Fig 15). Pathways with proteins of increased expression (at 12 to 24 hpi) begin to have no to little protein change (identified at 36 hpi) and then have decreased expression later (48 hpi) in LGTV infection (Fig 16).

There were 34 pathways with at least two or more proteins identified with increased and/or decreased expression following LGTV infection. In addition, there were 36 pathways with proteins of increased expression at 12/24 hpi that shifted to decreased expression at 36/48 hpi or vice versa. Metabolic pathways were primarily perturbed across 12 to 48 hpi (Fig 17). Amino acid and carbohydrate metabolic pathways were the top two pathways with proteins of increased expression early in infection at 12 and 24 hpi. Two amino acid pathways (histidine metabolism and lysine biosynthesis) were identified with proteins of increased expression early infection. The third most important metabolic pathways with proteins with increased expression at 12 hpi were lipid, cofactors/vitamins, and xenobiotics biodegradation metabolism. The third most important metabolic pathway with proteins with increased expression at 24 hpi was cofactors/vitamins. Carbohydrate metabolism was identified with proteins with increased expression only early in infection at 12 and 24 hpi. Pathway shifts occur, first with lipid/fatty acid metabolism along with cofactor/vitamin metabolism, which were identified with proteins of increased expression early in infection at 12 and 24 hpi, where lipid/fatty acid metabolism (three pathways: fatty acid elongation, fatty acid degradation, and fatty acid metabolism) was identified late in infection (36 and 48 hpi) with proteins with decreased expression. Energy-associated metabolism (five pathways:
citrate/TCA cycle, oxidative phosphorylation, pyruvate metabolism, carbon metabolism, and 2-oxocarboxylic acid metabolism) which was identified with proteins of increased expression at 12 and/or 24 hpi, where proteins have shifted with decreased expression in these pathways at either 24, 36, and/or 48 hpi. Fig 18 highlights protein expression change in the citrate/TCA cycle following the early and late time points post LGTV infection. Glycan biosynthesis was identified with proteins with increased expression early in infection at 12 and 24 hpi, but have proteins with decreased expression later at 48 hpi. Biosynthesis of amino acids was found only at 12 hpi with increased protein expression, where a shift to proteins with decreased expression occurred later at 24 and 48 hpi.

Genetic information processing (GIP; pathways involved with gene regulation) pathways account for the second most common cellular function being perturbed with LGTV infection across 12 to 48 hpi. Translation pathway was the top GIP pathway with proteins of increased expression at 12 and 24 hpi. Only at 12 hpi, replication/repair pathway was also a shared top pathway with translation with proteins of increased expression. Only three GIP pathways were perturbed across 12 to 48 hpi: Translation, replication/repair, and folding/sorting/degradation. The only GIP pathway identified with proteins of increased expression across 12, 24, 36, and 48 hpi was the folding/sorting/degradation pathway. Nine GIP pathways (ribosome biogenesis in eukaryotes, ribosome, RNA transport, DNA replication, spliceosome, proteasome, nucleotide excision repair, protein processing in endoplasmic reticulum, and protein export) were identified with
proteins of increased expression at 12 and/or 24 hpi and with proteins with decreased expression at 36 and/or 48 hpi or vice versa.

The only cellular processes pathway being perturbed across 12 to 48 hpi was transport and catabolism. A shift was clearly seen with proteins of increased expression in this pathway only at 12 and 24 hpi and with proteins of decreased expression in the pathway only at 36 and 48 hpi (four pathways: lysosome, endocytosis, phagosome, and peroxisome). Endocytosis was the only specific transport and catabolism pathway found with proteins with increased expression only at 12 and 24 hpi.

Signaling transduction pathways (FoxO and Wnt signaling pathways) were only found with proteins of increased expression early in infection, at 12 hpi. Signaling molecules and interaction pathway ECM-receptor interaction was found with proteins with decreased expression at 24 and 48 hpi.

3.3.10 LFQ quantitative proteomics: ISE6 protein lysine acetylation change associated with LGTV infection and UV-LGTV treatment

Changes in expression of lysine acetylation of ISE6 proteins was shown across 12 to 48 hpi with LGTV infection and/or UV-LGTV treatment. Only at 12 and 24 hpi, Histone H3 (ISCW002300 and ISCW003178) was identified with changing expressions of lysine acetylated peptides with LGTV infection and/or UV-LGTV treatment (Fig 19). However, more Histone H3 peptides with lysine acetylation were identified with increased expression following LGTV infection than with decreased expression. In addition, at 12 hpi the secreted protein (ISCW020023) was identified with increased expression of an acetylated peptide following LGTV infection. At 24 hpi, acetylated peptides with increased expression
following LGTV infection were found with the SMC protein (ISCW015559) and the Iron-containing alcohol dehydrogenase (ISCW021748). Additionally, the uncharacterized protein (ISCW006009) was found with an acetylated peptide with decreased expression following UV-LGTV treatment. Histone H4 was found with acetylated peptides with changing expression in all time points of LGTV infection (Histone H3 acetylated peptides observed early in infection). Histone H4 acetylated peptides with increased and decreased expression were found in 12 and 36 hpi. Histone H4 acetylated peptides with increased expression only was found at 48 hpi. Histone H4 acetylated peptides with decreased expression only was found at 24 hpi.

3.4 DISCUSSION

We used an LC-MS/MS proteomics approach to analyze changes in the global protein expression profile of I. scapularis ISE6 cells following infection with LGTV and identified tick proteins tied to flavivirus infection and replication. The present study focused on proteins expressed during 36 hours post infection or the period of peak LGTV release from infected ISE6 cells. In total, 486 ISE6 proteins were identified, and of these, 66 exhibited increased expression and 198 proteins exhibited decreased expression following LGTV infection. Two hundred and sixty-five of the proteins identified (54.5%) had orthology to proteins of known function from a variety of eukaryotes.

The LFQ proteomic analysis was used to identify global protein expression of ISE6 cells at different time points (early to late) following LGTV infection. More ISE6 proteins (579) were identified in the LFQ proteomic analysis compared to the
differential proteomic analysis (486). After characterizing proteins with altered expression (a total of 70 *I. scapularis* pathways with protein expression change) following LGTV infection at each time point, a comparison of pathways with fluctuating protein changes following LGTV infection in early and late time points was completed. In addition, changes in lysine acetylation of ISE6 proteins, specifically histones, following LGTV infection and/or UV-LGTV treatment were identified at early and late time points. Protein/pathway and modification changes provide insight into possible cellular components that may be involved with TBF infection.

3.4.1 Differential proteomics: Impact of LGTV infection on signaling in ISE6 cells

Several proteins were identified in the notch and mTOR signaling pathways. The histone deacetylase 1,2,3 (ISCW007830-PA) exhibited decreased expression in LGTV and UV-LGTV samples. Several studies [111, 112] suggest a link between herpesvirus infection and gene regulation through with the binding of viral proteins to histone deacetylases [112]. We test that LGTV infection may impact the regulation of ISE6 genes via effects on histone deacetylase. In other systems, it has been shown that histone deacetylase can act as a co-repressor in the notch signaling pathway. The 40S ribosomal protein S6 (ISCW024315-PA) and Mo25 (ISCW004710-PA) exhibited increased expression in LGTV cells. These proteins are members of the mTOR signaling pathway, which has been implicated in human cytomegalovirus (HCMV) infection in mammalian cells [113, 114] and DENV infection in *A. aegypti* mosquitoes [115]. Increased expression of Mo25 may reflect a cellular stress response while increased expression of S6 may reflect an increase
in translation to maintain growth of the infected cell or facilitate LGTV replication. Manipulation of mTOR signaling has been noted with WNV infection in mammalian systems [116]. The calcyclin-binding protein CacyBP (ISCW013691-PA) known to function in the Wnt signaling pathway in other systems, had increased expression in UV-LGTV-treated cells and decreased expression in LGTV-infected ISE6 samples. Our observation suggested an increase in proteolysis following virus treatment since the Wnt pathway is associated with the Ca\(^{2+}\)-dependent, ubiquitin-mediated proteolysis pathway. Future investigations regarding roles of post-translational modifications in regulating signaling pathways following tick-borne flavivirus infection is necessary.

### 3.4.2 Differential proteomics: ISE6 anti-viral responses perturbed by LGTV infection

Recently, the piwi-interacting RNA (piRNA) pathway has been implicated in the antiviral response of mosquitoes [117] and tick *I. scapularis* IDE8 cells [118]. Esther et al identified three paralogs (ISCW015916, ISCW0021130, and ISCW011768) of the tick *I. scapularis* argonaute (aubergine) protein as antiviral factors to LGTV infection. The *I. scapularis* aubergine protein possesses the paz and piwi domains [119] associated with RNA binding. Homologs of these proteins were not identified in this study, although a homolog of argonaute (AUB; ISCW011373-PA) was identified that exhibited increased expression in both LGTV and UV-LGTV ISE6 cells and may play an antiviral role in LGTV infection.

Histone (H2A) is involved in DNA binding and chromatin packing of DNA, and therefore likely has a role in gene regulation and downstream host protein translation that may be important for homeostasis. The *I. scapularis* H2A
(ISCW004478-PA) exhibited increased expression in LGTV-infected ISE6 cells. H2A also had increased expression during DENV infection in HUH7 liver cells and binds with the capsid protein to inhibit nucleosome formation in these human cells [120]. This protein has also been found to bind antisense RNA [121], also suggesting a possible anti-pathogen role as a result of changes in gene regulation.

The proteasome subunit alpha type protein (ISCW021572-PA) exhibited increased expression in LGTV samples and the 20S proteasome, regulatory subunit alpha type PSMA7/PRE6 (ISCW007139-PA) had increased expression in both LGTV and UV-LGTV samples. These proteins are subunits of the proteasome-associated 20S core particle and may exert antiviral roles through proteolysis and transcriptional regulation. Protein subunits of the proteasome have been shown to play a role in HCV internal ribosome entry site (IRES)-mediated translation [122] and may also interact with the HIV protein TAT and HBV protein HBX [123, 124].

3.4.3 Differential proteomics: Role of actin polymerization in ISE6 cells following LGTV infection

Decreased expression of actin was observed in both LGTV-infected and UV-LGTV-treated samples. Cofilin (CFN; ISCW006326-PA), an actin-depolymerizing factor, exhibited decreased expression in these samples. CFN was also identified in a proteomic study of HCV-infected HUH7.5 cells [56]. Actin polymerization is involved with formation of actin stress fibers, a process that may facilitate vacuole formation [125] and mammalian neuronal cell entry of Japanese encephalitis virus [126]. UV-LGTV-treated cells exhibited increased expression of the signaling and structural proteins RHOGDI, and ACTN and TUBA, respectively.
RHOGDI has been implicated in actin depolarization [127] and showed increased expression in HCV-infected HUH7.5 cells [56] at an early (12 hpi) infection time point. ACTN showed increased expression in HUH7.5 cells at early (24 hpi) and intermediate (48 hpi) time points post HCV infection and increased expression in UV-HCV-treated cells at a late (72 hr) time point. In the present study, we observed increased expression of ACTN in UV-LGTV samples at the 36 hpi time point. In addition to crosslinking actin fibers and facilitating filament assembly, ACTN has been shown to bind the HCV nonstructural proteins NS3 and NS5 [110, 128]. We hypothesize that this protein may assist LGTV cell entry in ISE6 cells.

3.4.4 Differential proteomics: Maintenance of metabolism in ISE6 cells following LGTV infection

The proteins acetyl-CoA acetyltransferase (ACAT1; ISCW016117) and aldehyde dehydrogenase 4A1 (DP5CD; ISCW015982) exhibited increased expression in LGTV-infected cells. These enzymes operate upstream of the TCA cycle and are associated with the production of acetoacetyl-CoA and pyruvate, respectively during cellular metabolism. This result suggested an increase in acetyl-CoA production following viral infection. Interestingly, citrate synthase (CS; ISCW009586) showed decreased expression following LGTV infection and may reflect a reduction of TCA protein activity late in LGTV infection. We observed a decrease in expression of fumarate hydratase (FH; ISCW020593) that may also similarly reflect reduction of TCA protein activity late in LGTV infection. The increased expression of MDH2 (ISCW003528), a protein involved in the final steps of the TCA cycle, may produce an increase in oxaloacetate, S-malate, and NADH in ISE6 cells. Moreover, increased expression of fumarylacetoacetase (FAH;
ISCW020196) may increase fumarate, also involved in the final steps of the TCA cycle. ACAT1, DP5CD, MDH2, and FAH may aid in maintaining the TCA cycle late in LGTV infection. In parallel, these observations suggest an impact of LGTV on the TCA cycle at 36 hours post infection that may be linked to replication of the virus.

Our observation of a decrease in expression of fructose-bisphosphate aldolase (ALDOA), glyceraldehyde-3-phosphate dehydrogenase (GADH), aldehyde dehydrogenase family 7 member A1 (ALDH3A2), and pyruvate kinase (PKLR) in LGTV-infected and UV-LGTV-treated cells, suggested an impact of LGTV on glycolytic processes. This finding was at odds with that of Patramool et al 2011, who observed that DENV-infected C6/36 A. albopictus cells [64] exhibited increased glycolysis. The study of Tchankouo-Nguetcheu et al identified an increased expression of glycolytic proteins in the in vivo midgut tissues of DENV-infected A. aegypti [65]. Diamond et al 2010 also identified members of the glycolysis pathway that exhibited increased expression at early to intermediate time points (i.e., prior to peak release of infectious virus) following HCV infection, but not at the late (during and following peak release of virus from the cell) time point [56]. Collectively, these data suggest the possible increase in glycolysis at early to intermediate time points post flaviviral infection, but a decrease in glycolysis at later time points.

3.4.5 Differential proteomics: Glutaminolysis associated with LGTV replication

Glutaminolysis can produce an alternative energy source for the cell by generating ATP during the conversion of glutamine to α-ketoglutarate. Increased
expression of proteins associated with glutaminolysis suggested that LGTV infection in ISE6 cells may stimulate glutaminolysis and the production of α-ketoglutarate, a key intermediate in the TCA cycle. Studies suggest that glutaminolysis is manipulated during infection in human cells by both HCMV [129, 130] and HCV [56]. Thus, glutaminolysis and α-ketoglutarate are likely critical not only for maintaining the TCA cycle, but also supporting oxidative phosphorylation and ATP production in the infected cell. Additionally, stimulation of α-ketoglutarate has been shown to increase mTOR activity [131, 132], which operates in parallel with glutaminolysis. Chapter 4 shows an assessment of the manipulation of glutamate dehydrogenase (GDH) activity using inhibitory compounds with the goal of disrupting flaviviral infection.

3.4.6 LFQ quantitative proteomics: Feasibility of ISE6 cells as tick neuronal models for neuroinvasive flavivirus pathogenesis

Neuronal proteins have traditionally been attractive targets for arthropod pest control, including ticks. The proteins identified in the *I. scapularis* tick synganglia [91] that overlap with the ISE6 proteins that are changing with LGTV infection, may provide an *in vitro* model for what may occur with the tick synganglia during flavivirus infection *in vivo*. As soon as the 115 synganglia proteins that overlapped with ISE6 cell proteins identified in the LGTV-ISE6 proteomic analyses are searched for their effect after LGTV infection, further cellular function/pathway analyses can be determined. Implications can then be made that these responses may translate to the neuronal system.
3.4.7 LFQ quantitative proteomics: Perturbed ISE6 cellular functions associated with acute LGTV infection

Biological function clusters identified the ISE6 cellular functions with proteins with increased or decreased expression following LGTV infection. The initial release of infectious LGTV occurs around 12 hpi and continuous release continues from 12 to 36/48 hpi, where peak virus release occurs (Chapter 1). At 12 and 24 hpi, clusters of ribonucleotide complexes, ribosome, GTPase activity, and translation were implicated in LGTV infection. In combination with biological observation, LGTV completes the first translation of viral proteins early in acute infection (<12 hpi) and with cluster observations, manipulates nuclear/genetic functions to help aid in this task. Flavivirus proteins have been known to enter the nucleus during infection [120, 133, 134], so effect on ribonucleotide proteins (histones, histone deacetylases, etc) is imminent although poorly understood. At 24 hpi, protein folding, unfolded protein response, glycosylation activity, endoplasmic reticulum (ER) involvement, RNA recognition, and cofactor/NAD/NADH binding were also implicated in LGTV infection. Protein folding and unfolding response may correlate with viral protein processing, virion assembly, and/or egress/transport. Glycosylation and ER involvement suggested the glycosylation activity of a protein that occurs in the ER right before budding into the Golgi apparatus, where in this case, may be a host and/or viral protein. RNA recognition may possibly correlate with the virus’ RNA replication and/or RNA genome packaging during virion assembly. Stress related responses by the cell were heat shock protein activity, reductase activity, and thioredoxin function at 12 and 24 hpi.
In addition, a cellular environment with reduced energy and biosynthetic metabolic processes was identified at 24 hpi with a cluster formed by proteins with decreased expression. Also, more TCA cycle and oxidative phosphorylation proteins with increased expression than decreased expression following LGTV infection were identified at 12 and 24 hpi. TCA cycle and oxidative phosphorylation proteins were identified with no change in expression at 36 hpi and more TCA cycle and oxidative phosphorylation proteins were identified with decreased expression than increased expression at 48 hpi. This suggested that cellular substrates and components used for energy (ATP) production were used up early (≤ 24 hpi) in LGTV infection, and may depict the cell responding/adjusting to this decreased-nutrient environment. The hypothesis that *more ISE6 TCA cycle and oxidative phosphorylation proteins have decreased expression than increased expression following 12-48 hpi with LGTV was rejected*. Only at 48 hpi was when this hypothesis was observed, not from 12-36 hpi. Further metabolite analyses need to be completed in order to provide insight, in parallel with proteomics, into ISE6 cell responses following LGTV infection.

### 3.4.8 LFQ quantitative proteomics: ISE6 proteins with increased and decreased expression following acute LGTV infection reveal fluctuating cellular pathways

#### 3.4.8.1 Metabolic pathways

A heavy reliance on amino acid and carbohydrate metabolism is crucial for LGTV infection, not only because these pathways would produce substrates for virus particle or protein production, but also because they provide substrates necessary for maintaining cellular energy levels and health. This LFQ proteomic analysis emphasizes this observation by identifying a vast proportion of the altered
ISE6 proteins being identified in amino acid and carbohydrate pathways. With the majority of metabolic proteins with increased expression residing in amino acid and carbohydrate pathways, it may be characteristic of the amino acid supply needed to produce viral proteins but also necessary to provide an alternative carbon energy source (e.g. glutaminolysis) to ISE6 cells (glycolysis/gluconeogenesis found with proteins of increased expression at 24 hpi but found with proteins of decreased levels at 36 hpi in the differential proteomic analysis) as acute infection continues.

The second tier of metabolic pathways identified with proteins of increased expression were lipid, cofactors/vitamins, and xenobiotics biodegradation metabolism. Flavivirus production of replication complexes require lipid membranes and have been known to perturb lipid metabolism [59, 89, 135, 136], so lipid metabolism was suggested to associate with LGTV replication complex formation. The shift of lipid metabolic proteins with increased expression early in infection (12 and 24 hpi) to a decreased expression late in infection (36 and 48 hpi) was suggested to be characteristic of the lipids involved in viral replication complexes and/or for production of cellular energy. Cofactors/vitamins provide the substrates necessary for nucleotide and amino acid production and serve as key intermediates for metabolic enzymes, hence it was suggested these proteins involved in cofactor/vitamin metabolism early in infection (12 and 24 hpi) aid in providing necessary substrates for viral genome and protein production.

Energy metabolism, or oxidative phosphorylation and 2-oxocarboxylic acid metabolism, increased early in infection (12 hpi) and was suggested to be involved
in many biosynthetic processes used by the virus (e.g. viral genome replication complex formation and viral protein production). But they were found to be decreased later (24, 36, and 48 hpi) as the acute infection continues. In addition, three energy-associated (citrate/TCA cycle, pyruvate metabolism, and carbon metabolism) pathways were identified with increased protein expression early in infection and identified with decreased protein expression later in infection. The decrease in energy may be characteristic of the ISE6 cells with decreased substrate/intermediate levels and/or response to limit biosynthetic processes due to viral infection/stress response (e.g. cell survival). Similarly, glycan biosynthesis was increased early at 12 and 24 hpi, but decreased at 48 hpi. Glycans make up some receptors that flaviviruses use for targeted entry receptors but also use glycans as part of their viral proteins. So, increased LGTV production at 12 to 24 hpi was directly correlated with increased glycan biosynthesis.

3.4.8.2 Genetic information processing pathways

Translation, replication/repair, and folding/sorting/degradation pathways were the only GIP pathways identified in the LFQ proteomic analysis. However, proteins with increased expression residing in these pathways were suggested to be involved with LGTV protein production, assembly, and egress/transport. Proteins in the replication and repair pathway may also be increased due to increased DNA damage that may result from LGTV infection and potential nuclear interaction with viral proteins.
3.4.8.3 Cellular processes pathways

Proteins identified with increased expression levels involved in the endocytosis pathway at 12 and 24 hpi suggest this process to be increased early in infection due to LGTV entering ISE6 cells via endocytosis. An increase in phagosome function early in infection may be characteristic of ISE6 cells consuming available cellular substrates to aid the increase in energy needed for biosynthetic processes. An increase in peroxisomes early in LGTV infection was presumably involved with the breakdown of substrates (e.g. fatty acids and amino acids) and formation of acetyl-CoA for use in energy production that may aid in LGTV replication complex formation, LGTV protein production, and/or cellular energy. A decrease in peroxisomes later in LGTV infection (36 and 48 hpi) may suggest that the ISE6 cell was limiting biosynthetic processes and does not need free substrates. An increase of lysosome function may be due to the use of this function to release infectious LGTV and/or due to a response to cellular stress.

3.4.8.4 Environmental information processing pathways

Signaling transduction response was primarily early in LGTV infection. Only 12 hpi revealed proteins with increased expression with signal transduction and as early as 24 hpi signaling became decreased. This suggested that later in infection that metabolic, cellular processes, and signaling all become reduced presumably to limit cellular biosynthetic processes and to maintain a homeostatic condition.

3.4.9 LFQ quantitative proteomics: Histone lysine acetylation changes with LGTV infection and UV-LGTV treatment

As mentioned before, flavivirus proteins have been shown to enter the nucleus of infected cells to manipulate gene regulation-based functions [120, 137,
In addition, histone levels/expressions have been shown to be manipulated with flavivirus infection where viral proteins have been implicated to sequester histone proteins [120]. It was suggested that gene regulation was manipulated via interactions of histones and viral proteins. The mechanism on how viral proteins affect histone acetylation activity yet needs to be elucidated.

Histones have additionally been implicated in connection with controlling metabolic processes by controlling transcription and subsequent translation of host proteins. Acetylation is a modification that activates histones and allows for more genetic material to be transcribed. Altered levels of acetylation of histones directly alters the production of cellular proteins that may be involved with key metabolic pathways [139, 140]. It is hypothesized that ATP-citrate synthase (ISCW009919), increased expression following LGTV infection at 12 hpi, provides an increase flux production of nuclear acetyl-CoA, a required cofactor used to acetylate lysine residues. In addition to nuclear acetyl-CoA levels, cytoplasmic or mitochondrial acetyl-CoA levels were predicted to be increased as well early in LGTV infection.

There were a number of histones identified with increased expression following LGTV infection in the differential and LFQ proteomic analyses: Histone H2A, Histone H1/5, and Histone H4. Also, a histone deacetylase was identified with decreased expression following LGTV infection in the differential and LFQ proteomic analyses. In addition, identified ISE6 proteins with histone acetyltransferase activity and acetyltransferase activity within the differential and LFQ proteomic studies with increased expression following LGTV infection (data not shown) were predicted to affect acetylation activity in multiple cellular
compartments. Finally, the LFQ proteomic analysis identified histones with increased and decreased expressions of lysine acetylation following LGTV infection. Specifically, this included Histone H3 and Histone H4. From this information, it may be possible that LGTV infection may affect activity of ISE6 histones, however the effect on histone levels/activity on ISE6 metabolic enzyme production/activity remains unknown.

3.5 LGTV-ISE6 PROTOMIC ANALYSES SUMMARY

The differential proteomic analysis provided an initial look at cellular pathway/function changes associated with LGTV infection, specifically at peak LGTV replication. The LFQ proteomic analysis provides an additional validation of ISE6 proteins identified from the differential proteomic analysis (with additional ISE6 proteins), quantitated levels of ISE6 proteins following four different time points following LGTV infection, identification of fluctuating cellular pathways from early to late acute LGTV infection, and lysine acetylation changes of ISE6 proteins following LGTV infection. In the future, additional post-translational modifications (glycosylation, phosphorylation, and methylation) can be searched with the same LFQ datasets to identify more modifications on ISE6 proteins associated with LGTV infection.

Identification of 486 tick proteins from the differential proteomic analysis and 579 tick proteins from the quantitative proteomic analysis (301 ISE6 proteins overlap) provided an extensive proteomic characterization of the ISE6 cell, at peak LGTV release and 12-48 hpi acute LGTV infection. In combination with ISE6 proteins identified by Oliver et al (317 identified in total; [91]), 198 proteins overlap
in all three proteomic analyses, 211 overlap with the differential proteomic analysis, 270 overlap with the quantitative proteomic analysis, and 386 overlap with two or more of the three ISE6 protein datasets. For the first time, these analyses provided tick proteome responses to a TBF infection.

The identification of these proteins and their cellular pathways provide a critical resource to improve understanding of the *I. scapularis* proteome, improve gene annotations, and facilitate further studies in the tick cell culture system. In addition, to contribute to an improved understanding of flavivirus-*I. scapularis* interactions, the differential and LFQ proteomic analyses provide proteins and pathways that were changing in association with LGTV. The present study is an important first step toward identifying tick proteins tied to LGTV replication and can be used for anti-tick vaccines and/or for therapeutic screening to disrupt tick-borne flavivirus transmission. Further understanding of protein function can also be achieved using approaches such as IFA, targeted mass spectrometry, small molecule *in vitro* assays, and RNAi. Chapter 4 will go more into depth of the small molecule/RNAi *in vitro* assays and the proteins/cellular pathways that were involved with LGTV infection.
3.6 FIGURES, TABLES, AND LEGENDS

Fig 1. Summary of differential proteomic analysis of LGTV-infected and UV-LGTV-treated I. scapularis ISE6 cells. After whole cell sample harvest of treated ISE6 cells, cell pellet samples were subjected to lipid removal, protein precipitation, peptide denaturation, and tryptic digest of peptides. Samples were prepared for the separation phase (nano LC) by injection, using electrospray ionization (ESI). Mass analysis of the precursor ion spectra was completed, followed by the second fragment ion MS/MS dimension for downstream peptide identification. Two group and three group statistical analyses with ISE6 cells treated with virus (LGTV), UV-inactivated virus (UV-LGTV), and no virus (mock) were compared utilizing a proteomic/metabolite pipeline, Omics Discovery Pipeline (ODP). After identification of significantly-changing (p < 0.05) MS peaks from LGTV-infected and UV-LGTV-treated ISE6 cells, corresponding peptides were identified to specific I. scapularis proteins (VectorBase I. scapularis WIKEL genome IscaW1.2 predicted protein set database). ISE6 proteins were then subject to protein function and pathway analyses (via KEGG). See materials and methods section for more detail.
Table 1. Summary of analyses used to identify proteins from LGTV-infected and UV-LGTV-treated ISE6 cell samples

<table>
<thead>
<tr>
<th>Processing Step</th>
<th>No MS peaks/Peptides/Proteins per Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>LGTV vs. mock</td>
</tr>
<tr>
<td><strong>MS Data (Number of Peaks)</strong></td>
<td>39,241</td>
</tr>
<tr>
<td>Total number of LC-MS peaks observed</td>
<td>39,241</td>
</tr>
<tr>
<td>LC-MS data were aligned and normalized using ODP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27,868</td>
</tr>
<tr>
<td>All normalized peaks that matched to peptides</td>
<td>14,658</td>
</tr>
<tr>
<td>The ODP was used to calculate a P value based on the intensities of each LC-MS peak between groups&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8,154</td>
</tr>
<tr>
<td>Data filtering based on Spectrum Mill score</td>
<td>4,781</td>
</tr>
<tr>
<td>Peptide score&lt;sup&gt;c&lt;/sup&gt; (&gt; 5) and removal of unidentified LC-MS peaks</td>
<td>4,310</td>
</tr>
<tr>
<td>% scored peak intensity&lt;sup&gt;d&lt;/sup&gt; (%SPI≥ 70%)</td>
<td>1,096</td>
</tr>
<tr>
<td>Number of peaks following removal of peptide false positives</td>
<td></td>
</tr>
<tr>
<td>Δ fwd-rev score&lt;sup&gt;e&lt;/sup&gt; &gt; 1 with parent charges +1 and +2</td>
<td></td>
</tr>
<tr>
<td>Δ fwd-rev score &gt; 2 with parent charges +3 and +4</td>
<td></td>
</tr>
<tr>
<td>Removal of replicate MS peaks identified in the multiple samples</td>
<td></td>
</tr>
<tr>
<td><strong>Number of Peptides</strong></td>
<td>772</td>
</tr>
<tr>
<td><strong>Number of Proteins</strong></td>
<td>374</td>
</tr>
</tbody>
</table>

<sup>a</sup>ODP denotes Omics Discovery Pipeline
After normalization of MS peaks in each treatment group (LGTV, UV-LGTV, and mock), t-test and ANOVA analyses were performed in the Significance Test step of the ODP.

Spectrum Mill peptide score and % scored peak intensity are two criteria utilized to identify peptides from *in silico* database searches. See material and methods section for more details.

Spectrum Mill reverse database search was completed to account for possible false positives when identifying peptides from *in silico* database searches. See material and methods section for more details.

≥1 peptide match to a VectorBase gene model predicted peptide sequence was considered a protein identification. See material and methods section for more details.
Fig 2. Hierarchical clustering of MS peak profiles of ISE6 cells treated with LGTV, UV-LGTV, and mock. M1-5, mock1-5 samples; LGTV1-5, LGTV-infected samples 1-5, and UV-LGTV1-5, UV-LGTV samples 1-5. Vertical rows depict n=5 biological replicates. Horizontal rows correspond to significant MS peaks of peptides/proteins at 36 hours post infection/treatment. Clustering analysis shows common patterns of protein expression profiles shared between the three treatment groups. The red-green color scale denotes the Z score fold change with red representing a Z score of -2 and green denoting a Z score of 2 [89].
Fig 3. Identification and expression of ISE6 cell proteins following treatment to LGTV, UV-LGTV, and mock. (A) Four statistical analyses were performed using the ODP output. This included a three-way ANOVA of treatment groups LGTV, UV-LGTV, mock and two-way t-test comparing the LGTV vs. mock, UV-LGTV vs. mock, and LGTV vs. UV-LGTV samples. The Venn diagram shows unique and common ISE6 protein identifications from these datasets. (B) Venn diagrams showing the numbers of ISE6 proteins identified as exhibiting increased or decreased expression following treatment to LGTV or UV-LGTV unique or common to sample groups.
Fig 4. ISE6 proteins identified in KEGG pathways with differential expression following LGTV, UV-LGTV and mock treatment. (A) Total number of ISE6 cell proteins categorized by treatment and change in expression (increase/decrease/no change). Total number of proteins showing (B) increased expression, (C) decreased expression, and (D) no change in expression following LGTV infection and UV-LGTV treatment as compared to mock-treated cells and in LGTV-infected cells as compared to UV-LGTV-treated cells. Proteins were categorized by the KEGG classes for cellular function: metabolism, genetic information processing (GIP), environmental information processing (EIP), cellular processes (CP), and organismal systems (OS).
Fig 5. ISE6 protein orthology and cellular function distribution of proteins found in KEGG pathways and modules. (A) *Ixodes scapularis* ISE6 proteins with KEGG-mapped orthologs (or KEGG orthology [KO]) help to identify cellular pathways in *I. scapularis* (genome.jp/kegg/ko). To be identified in a KEGG pathway, KO is required. ISE6 proteins with KO and not identified in *I. scapularis* (KEGG) pathways are also included. (B) Percent cellular function distribution of proteins found in the 66 identified *I. scapularis* (KEGG) pathways with 16 modules.
Fig 6. Summary of differentially-expressed ISE6 proteins without identified pathways. Expression of ISE6 proteins with (A) or without (B) orthology and no identified pathways. Refer to S2 Table for more specifics on the proteins. Red dotted line denotes differentially-expressed proteins in LGTV-infected ISE6 cells compared to UV-LGTV-treated ISE6 cells (no comparison to mock-treated ISE6 cells).
**Fig 7.** ISE6 proteins associated with the TCA cycle and glutaminolysis. ISE6 glutaminolysis and mTOR signaling proteins altered with LGTV infection are shown. GDH denotes glutamate dehydrogenase enzymes and Glase denotes glutaminase enzymes. ISCWxxxxxx denotes corresponding VB accession ID for corresponding *I. scapularis* protein.
Table 2. ISE6 proteins putatively associated with glutaminolysis.

<table>
<thead>
<tr>
<th>Protein (VB accession name)</th>
<th>KEGG ortholog</th>
<th>Putative functions (GO predictions)</th>
<th>Orthology to glutaminolytic enzyme</th>
<th>Change in expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ISCW011739) prostate-specific transglutaminase, putative</td>
<td>NP*</td>
<td>• peptide cross-linking • protein-glutamine gamma-glutamyltransferase activity • transferase activity, transferring acyl groups • transferase activity</td>
<td>Glutaminase (Glase)</td>
<td>Increased expression with LGTV</td>
</tr>
<tr>
<td>(ISCW000393) glutamate dehydrogenase, putative</td>
<td>K00261</td>
<td>• cellular amino acid metabolic process • oxidation-reduction process • oxidoreductase activity, acting on the CH-NH2 group of donors, NAD or NADP as acceptor • oxidoreductase activity</td>
<td>Glutamate dehydrogenase (GDH)</td>
<td>Increased expression with both LGTV and UV-LGTV</td>
</tr>
<tr>
<td>(ISCW015982) aldehyde dehydrogenase, putative</td>
<td>K00294</td>
<td>• proline biosynthetic process • oxidation-reduction process • metabolic process • oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor • 1-pyrroline-5-carboxylate dehydrogenase activity • oxidoreductase activity</td>
<td>GDH</td>
<td>Increased expression with both LGTV and UV-LGTV</td>
</tr>
<tr>
<td>(ISCW001521) membrane protein, putative</td>
<td>NP</td>
<td>• oxidation-reduction process • saccharopine dehydrogenase (NAD+, L-glutamate-forming) activity • oxidoreductase activity</td>
<td>GDH</td>
<td>Increased expression with both LGTV and UV-LGTV</td>
</tr>
<tr>
<td>(ISCW020361) glutamine synthetase 1, putative</td>
<td>NP</td>
<td>• nitrogen compound metabolic process • glutamine biosynthetic process • ligase activity • glutamate-ammonia ligase activity</td>
<td></td>
<td>Decreased expression with both LGTV and UV-LGTV</td>
</tr>
</tbody>
</table>

*NP denotes does not have a KEGG Ortholog.
Fig 8. Citrate cycle showing ISE6 proteins that exhibited increased/decreased expression following treatment to LGTV and UV-LGTV. The enzymes are indicated with KEGG abbreviated nomenclature and the corresponding substrates are shown in circles. * denotes proteins identified in this study. Dotted lines denote indirect involvement with production. The increased expression of malate dehydrogenase (MDH2) was unique to LGTV-treated cells while increase in the expression of acetyl-CoA acetyltransferase 1 (ACAT1), delta-1-pyrroline-5-carboxylate dehydrogenase 1 (ALDH4A1), glutamate dehydrogenase (GLUD1), and fumarylacetoacetase (FAH) was common to cell samples following LGTV infection and UV-LGTV treatment. Decreased expression of citrate synthase (CS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was observed in LGTV-treated cells and decreased expression of fumarate hydratase (FH), aldolase A/B/C fructose-bisphosphate (ALDOA/B/C), and aldehyde dehydrogenase 2/1B1/3A2 family protein (ALDH2/1B1/3A2) was observed in both LGTV-infected and UV-LGTV--treated cells. ATP citrate lyase (ACLY), aconitase (ACO), isocitrate dehydrogenase 2/3a (IDH2/3a), oxoglutarate/alpha-ketoglutarate dehydrogenase complex (OGDH/DLST), succinyl-CoA synthetase alpha/beta subunit (LSC1/2), succinate dehydrogenase flavoprotein subunit (SDHA), pyruvate kinase (PK), enolase 1/2/3 (ENO1/2/3), and aldehyde dehydrogenase 7A1 family protein (ALDH7A1).
Fig 9. Number of ISE6 proteins corresponding to orthologous proteins identified in proteomic analyses of flavivirus-host systems. Corresponding percentages correspond to the number of ISE6 orthologs identified with orthologs identified in: a S5 Fig, S7 Fig, and S11 Fig of Khadka et al [110]; b S2 Table of Tchankouo-Nguetcheu et al [65]; c Tables 1 and 2 of Pastorino et al [109]; d S1 Table of Diamond et al [56].
Fig 10. ISE6 proteins with increased expression associate with pathways for protein production, transport, assembly, and proteolysis. The ISCW accession numbers corresponding to proteins identified with increased expression in LGTV-infected and UV-LGTV-treated cells are shown (KEGG abbreviated nomenclature provided as well if available in parentheses). The schematic presents potential mechanisms for LGTV-induced perturbation and increase in cell protein expression. Underlined proteins denote ISE6 proteins exhibiting increased expression following flavivirus infection not identified before. “Fld, Srt, & Deg” is the folding, sorting, and degradation KEGG pathway. ** denotes ortholog in human [56]; + denotes human ortholog showing increased expression following HCV infection; -- denotes human ortholog showing decreased expression following HCV infection. Gray boxes denote cellular protein functions. White boxes denote KEGG pathways.
Fig 11. Summary of quantitative proteomic analysis of LGTV-infected and UV-LGTV-treated *I. scapularis* ISE6 cells. After whole cell sample harvest of treated ISE6 cells, cell pellet samples were subject to lipid removal, protein precipitation, peptide denaturation, and tryptic digest of peptides. Samples were prepared for the separation phase (nano LC) by injection, using electrospray ionization (ESI). Mass analysis of the precursor ion spectra was completed, followed by the second fragment ion MS/MS dimension for downstream peptide identification. Two group and three group statistical analyses with ISE6 cells treated with virus (LGTV), UV-inactivated virus (UV-LGTV), and no virus (mock) were compared utilizing a proteomic/metabolite program InfernoRDN. After identification of significantly-changing (p < 0.05) MS peaks from LGTV-infected and UV-LGTV-treated ISE6 cells, corresponding peptides were identified to specific *I. scapularis* proteins (VectorBase *I. scapularis* WIKEI genome IscaW1 predicted protein set database from UniProt). ISE6 proteins were then subject to protein function and pathway analyses (via KEGG). See materials and methods section for more detail.
Table 3. Summary of analyses used to identify proteins from LGTV-infected and UV-LGTV-treated ISE6 cell samples in LFQ analysis.

<table>
<thead>
<tr>
<th>Processing Step</th>
<th>No. Proteins per Time point</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hpi</td>
<td>24 hpi</td>
</tr>
<tr>
<td>Total number of proteins observed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC-MS raw data were aligned and normalized using Inferno RDN(^a) and uploaded to MaxQuant for protein ID output.</td>
<td>960</td>
<td>1,023</td>
</tr>
<tr>
<td>Total number of proteins observed following removal of contaminant(^b) and reverse database proteins(^c)</td>
<td>896</td>
<td>948</td>
</tr>
<tr>
<td>Total number of proteins identified in at least three out of five biological replicate samples</td>
<td>Of three treatments (LGTV, UV-LGTV, and mock), protein identified in at least three out of five biological replicates of at least one treatment group</td>
<td>466</td>
</tr>
</tbody>
</table>

### Proteins with Increased Expression

<table>
<thead>
<tr>
<th></th>
<th>12 hpi</th>
<th>24 hpi</th>
<th>36 hpi</th>
<th>48 hpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGTV infection</td>
<td>34</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>LGTV infection and UV-LGTV treatment</td>
<td>50</td>
<td>74</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>UV-LGTV treatment</td>
<td>20</td>
<td>64</td>
<td>8</td>
<td>70</td>
</tr>
<tr>
<td>Expression with LGTV infection greater than UV-LGTV treatment</td>
<td>33</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

### Proteins with Decreased Expression

<table>
<thead>
<tr>
<th></th>
<th>12 hpi</th>
<th>24 hpi</th>
<th>36 hpi</th>
<th>48 hpi</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGTV infection</td>
<td>23</td>
<td>59</td>
<td>15</td>
<td>74</td>
</tr>
<tr>
<td>LGTV infection and UV-LGTV treatment</td>
<td>3</td>
<td>10</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>UV-LGTV treatment</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\)Inferno RDN (http://omics.pnl.gov/software/infernordn) encompasses R package to complete t-test and ANOVA analyses.

\(^b\)Uploaded, searched Uniprot protein database corresponding to the *I. scapularis* IscaW1 genome assembly has included contaminant peptide sequences to help identify non-*I. scapularis* peptides.

\(^c\)MaxQuant option for reverse database search was selected in combination with database search to identify proteins.
Fig 12. ISE6 proteins identified in quantitative proteomic analysis. ISE6 proteins were identified in (A) at least three out of five biological replicates of at least one treatment group (LGTV, UV-LGTV, and mock) and in (B) statistically-significant ($p \leq 0.05$), changing proteins following LGTV infection and/or UV-LGTV treatment.
Fig 13. Identification of ISE6 cell proteins following LGTV infection and UV-LGTV treatment. For 12 (A), 24 (B), 36 (C), and 48 (D) hpi time points, four statistical analyses were performed. This included a three-way ANOVA of treatment groups LGTV, UV-LGTV, mock and two-way t-test comparing the LGTV vs. mock, UV-LGTV vs. mock, and LGTV vs. UV-LGTV samples. The Venn diagram shows unique and common ISE6 protein identifications from these datasets.
Fig 14. Comparative proteomics analysis identifies ISE6 proteins in *I. scapularis* synganglia tissue. ISE6-LGTV protein identifications (**Grabowski et al differential and LFQ**) and *I. scapularis* synganglia protein identifications (**Oliver et al; [91])** overlap.
Table 4. Summary of proteins used from LGTV-infected and UV-LGTV-treated ISE6 cell samples for LFQ analysis in DAVID functional clustering and KEGG pathway analyses.

<table>
<thead>
<tr>
<th>Processing Step</th>
<th>Description</th>
<th>No. Proteins per Time point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12 hpi</td>
</tr>
<tr>
<td>Total number of proteins searched</td>
<td>Proteins with statistically significant increased/decreased expression following LGTV infection and/or UV-LGTV treatment</td>
<td>128</td>
</tr>
<tr>
<td>Total number of proteins searched by DAVID</td>
<td>DAVID will only search I. scapularis proteins with orthology&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>Total number of proteins not searched by DAVID</td>
<td>I. scapularis proteins with no orthology&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28</td>
</tr>
<tr>
<td>Total number of significant, biological function clusters of all DAVID searched proteins</td>
<td>Enrichment (E)&lt;sup&gt;b&lt;/sup&gt; score of ≥ 1.3 is equal to a ≤ 0.05 p value</td>
<td>5</td>
</tr>
<tr>
<td>Total number of significant, biological function clusters of all DAVID searched proteins</td>
<td>Searched with proteins of increased expression following LGTV infection</td>
<td>5</td>
</tr>
<tr>
<td>Total number of significant, biological function clusters of all DAVID searched proteins</td>
<td>Searched with proteins of decreased expression following LGTV infection</td>
<td>0</td>
</tr>
<tr>
<td>Total number of proteins mapped to KEGG pathways</td>
<td>DAVID will only map proteins with isc-specific&lt;sup&gt;c&lt;/sup&gt;, KEGG pathways</td>
<td>33</td>
</tr>
</tbody>
</table>

<sup>a</sup>Orthology refers to proteins with corresponding KEGG Orthology.

<sup>b</sup>Higher the E score the increased strength or confidence of the cluster of biological function occurring with the included proteins.

<sup>c</sup>Proteins with KEGG Orthology have mapped I. scapularis (isc) pathways or no mapped isc pathways. Proteins with KEGG Orthology and no mapped isc pathways may be mapped to other pathways identified in other eukaryotic systems.
Table 5. Summary of DAVID functional clustering of proteins with increased or decreased expressions following LGTV infection of ISE6 cell samples from LFQ analysis.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Cluster[^]</th>
<th>Cluster Summary</th>
<th>Time point</th>
<th>Cluster[^]</th>
<th>Cluster Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 hpi- Increased Expression</td>
<td>Cluster 1</td>
<td>ribonucleotide complex, ribosome, and translation</td>
<td>12 hpi- Decreased Expression</td>
<td>No Clusters</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cluster 2</td>
<td>heat shock protein, nucleotide binding, and ribonucleotide binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cluster 3</td>
<td>thioredoxin function, homeostatic response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cluster 4</td>
<td>cellular macromolecular complex assembly and subunit organization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cluster 5</td>
<td>GTPase activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hpi- Increased Expression</td>
<td>Cluster 1</td>
<td>ribonucleotide complex, ribosome, and translation</td>
<td>24 hpi- Decreased Expression</td>
<td>Cluster 1</td>
<td>RNA recognition and nucleotide binding</td>
</tr>
<tr>
<td></td>
<td>Cluster 3</td>
<td>RNA recognition and nucleotide binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cluster 4</td>
<td>glycosylation activity and ER involvement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cluster 5</td>
<td>heat shock protein and reductase protein; homeostatic response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cluster 6</td>
<td>nucleotide and ribonucleotide binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cluster 7</td>
<td>GTPase activity along with protein synthesis/elongation activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cluster 8</td>
<td>thioredoxin function; homeostatic response</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Cluster 9</td>
<td>cofactor, NAD, and NADH binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36 hpi- Increased Expression</td>
<td>No Clusters</td>
<td></td>
<td>36 hpi- Decreased Expression</td>
<td>No Clusters</td>
<td></td>
</tr>
<tr>
<td>48 hpi- Increased Expression</td>
<td>Cluster 1</td>
<td>RNA recognition and nucleotide binding</td>
<td>48 hpi- Decreased Expression</td>
<td>No Clusters</td>
<td></td>
</tr>
</tbody>
</table>

[^]: All clusters had an Enrichment score of ≥1.3 and cluster summaries had a p value ≤ 0.05
Fig 15. ISE6 pathway change following acute LGTV infection. Pathways with proteins of increased (A) and decreased (B) expression are identified across 12, 24, 36, and 48 hpi. The Venn diagram shows unique and common ISE6 pathways identified from these time points. Pathways with at least one protein with differential expression included.
Fig 16. ISE6 pathway change following acute LGTV infection at specific time points. Pathways with proteins of increased and decreased expression are identified within each 12, 24, 36, and 48 hpi time point. The Venn diagram shows unique and common ISE6 pathways with proteins of increased and/or decreased expression identified from each time point.
Fig 17. Specified ISE6 pathway change following acute LGTV infection at specific time points. Pathways with proteins with increased or decreased expression are identified at 12, 24, 36, and 48 hpi. Cellular functions categorize the pathways listed and are as follows: Metabolism, Genetic Information Processing (GIP), Cellular Processes, Environmental Information Processing (EIP), and Organismal Systems.
Fig 18. Citrate cycle showing ISE6 proteins that exhibited increased/decreased expression at specific time points following treatment to LGTV infection and UV-LGTV treatment. The enzymes are indicated with KEGG abbreviated nomenclature and the corresponding substrates are shown in circles. * denotes proteins not identified in this study at that specific time point. Dotted lines denote indirect involvement with production. Enzymes listed include: malate dehydrogenase (ISCW003528; MDH2), citrate synthase (ISCW009586; CS), ATP citrate lyase (ISCW009919; ACLY), aconitase (ISCW013457; ACO), isocitrate dehydrogenase 2/3a (ISCW001977, ISCW004116; IDH2/3a), dihydrolipoamide acetyltransferase (ISCW008731; DLST), oxoglutarate/alpha-ketoglutarate dehydrogenase complex (ISCW003165; OGDH), succinyl-CoA synthetase alpha/beta subunit (ISCW011750; LSC2), succinate dehydrogenase flavoprotein subunit (ISCW000555; SDHA), fumarate hydratase (ISCW020593; FH), dihydrolipoamide succinyltransferase (ISCW013308; DLAT), pyruvate dehydrogenase (ISCW019126; PDHA), and branched chain alpha-keto acid dehydrogenase (ISCW009219; PDHB).
Fig 19. ISE6 proteins with lysine acetylation changes following LGTV infection and UV-LGTV treatment. ISE6 proteins with increased or decreased expression of lysine acetylated residues is shown (number listed in parentheses). Red denotes proteins with increased expression of acetylated peptides and blue denotes proteins with decreased expression of acetylated peptides following LGTV infection and/or UV-LGTV treatment. ISCW- - - - - - denotes VectorBase accession ID.
4.1 INTRODUCTION

Grabowski et al (in review) and Chapter 3 provided a set of *I. scapularis* ISE6 proteins differentially-expressed following LGTV infection and identified metabolic processes that may be involved in LGTV infection [87]. Most ISE6 proteins with increased expression were identified as members of amino acid metabolism, carbohydrate metabolism, vitamins/cofactor metabolism, lipid metabolism, nucleotide metabolism, and folding/sorting/degradation pathways. Here, we investigate the hypothesis that ISE6 amino acid/carbohydrate/vitamin/cofactor/lipid/nucleotide metabolic enzymes and folding/sorting/degradation proteins with increased expression following LGTV infection were involved in LGTV infection. In addition to identifying protein expression change following LGTV infection, protein modification change was identified with lysine acetylation change following LGTV infection in Chapter 3. Increased expression of lysine acetylated histones were identified with LGTV infection. From this observation, it was hypothesized that an increase in lysine acetylation of histones increased LGTV infection in ISE6 cells. Currently, there is limited knowledge on how tick proteins, specifically expression and modification levels, effect the LGTV life cycle.
This chapter investigated function of selected proteins identified from Chapter 3 and from Grabowski et al [87]) on LGTV infection by employing two complementary approaches—small molecule compound and RNAi assays. Ten genes of interest were selected for RNAi transcript knockdown and functional studies based on strength of protein identification and orthology. The ten selected genes were fumarylacetoacetase (ISCW020196; Fumar; amino acid metabolism), endoplasmic reticulum protein 29 (ISCW018425; ERP29; folding, sorting, and degradation), 1-pyrroline-5-carboxylate dehydrogenase (ISCW015982; DPCD; amino acid metabolism), pantetheine hydrolase (ISCW004822; CNHydro; metabolism of cofactors and vitamins), malate dehydrogenase (ISCW003528; MDH2; carbohydrate metabolism), poly [ADP-ribose] polymerase (ISCW019519; PARP; replication and repair), UMP-CMP kinase (ISCW012446; CUKinase; nucleotide metabolism), acetyl-CoA acetyltransferase (ISCW016117; ACAT1; carbohydrate, lipid, amino acid, and terpenoids/polyketides metabolism), hypothetical protein (ISCW011195; Hypo195; unknown function), and hypothetical protein (ISCW020576; Hypo576; unknown function). Transcripts for these genes of interest were confirmed in ISE6 cells and female I. scapularis. Transcript knockdowns were conducted and the effect on LGTV genome replication and infectious virus release was evaluated.

Information from these studies could be used to identify tick proteins to control TBFs. Host factors have been used to develop or identify therapeutics against flavivirus infection [141-144] and this study provides new proteins for potential development of therapeutics against TBFs.
4.2 MATERIALS AND METHODS

4.2.1 Cell and virus culture

Cells and virus stocks were prepared as described in Chapter 2, Section 2.2.1.

4.2.2 LGTV infection in ISE6 cells and measurement of infectious LGTV

LGTV infection of ISE6 cells and measurement of infectious LGTV was completed as described in Chapter 2, Section 2.2.3.

4.2.3 Compound and cell viability assays

Trichostatin A (TSA; Sigma-Aldrich; T8552) and Oligomycin A (OligoA; Sigma-Aldrich; 75351) were re-suspended in DMSO to a final concentration of 10 mM and working concentrations of 0.01, 0.1, 1, and 10 µM concentrations. Nicotinamide (NAM; Sigma-Aldrich; 72340) was re-suspended in cell media to a final concentration of 100 mM and working concentrations of 100, 1,000, 10,000, and 100,000 µM concentrations. Famotidine (FAMO; Sigma-Aldrich; F6889) was re-suspended in DMSO to a final concentration of 10 mM and working concentrations of 0.1, 1, 10, 100, 1,000, and 10,000 µM concentrations. Hexachlorophene (Hexa; Sigma-Aldrich; 45526) was re-suspended in DMSO to a final concentration of 100 mM and working concentrations of 0.1, 1, 10, 100, 1,000, and 10,000 µM concentrations. Epigallocatechin (EGC; Sigma-Aldrich; E3768) was re-suspended in DMSO to a final concentration of 10 mM and working concentrations of 0.1, 1, 10, 100, 1,000, and 10,000 µM concentrations. Epicatechin-3-gallate (ECG; Sigma-Aldrich; E3893) was re-suspended in DMSO...
to a final concentration of 100 mM and working concentrations of 0.1, 1, 10, 100, 1,000, and 10,000 µM concentrations.

96 well plates were pretreated with 0.01% Poly-L-Lysine (Sigma Aldrich; P4832) and allowed to air dry in a biosafety cabinet. Plates were separately seeded with ISE6 and Vero cells and incubated for 24 hours to final cell density of ~1 x10^5 cells/96 well, and cells were infected with LGTV passage 4, MOI of 10, and LGTV passage 4, MOI of 3, respectively (TSA, OligoA, NAM, and FAMO were screened in ISE6 and Vero cells; Hexa, EGC, and ECG were screened only in ISE6 cells). Following virus adsorption, compounds diluted in DMSO (1% of total overlay media) or in cell media were added to cells (0 hpi) and cells were incubated at 37°C. Culture supernatants were collected at 36 hpi and used to quantify LGTV replication by plaque assay, and 1:10 dilution of alamarBlue reagent (AbD Serotec; BUF012A) with fresh media was placed onto infected ISE6 and Vero cells for 12 and 2 hours for subsequent cell viability readings. Fluorescence (excitation at 560nm, emission at 590 nm) was measured at 12 and 2 hours post addition using a Molecular Devices SpectraMax M5 plate reader coupled with SoftMax Pro v4.8 software to acquire cell viability readings (see Chapter 2, Section 2.2.5). Mock-treated (pretreated cell media) cells underwent the same treatment but without LGTV infection. Two biological replicates were conducted for each experiment and five technical replicates were performed at with each compound concentration.
4.2.4 Measurement of ISE6 cell concentrations

To equalize cell numbers between biological replicates and between treatment groups prior to protein extraction, the concentration of cells in each sample (cells/ml) was estimated. See Chapter 2, Section 2.2.2 for more detail.

4.2.5 RNA isolation and cDNA synthesis

Single *I. scapularis* female ticks (collected from Tippecanoe State Park, Winimac, IN; October 29, 2013) were flash frozen in liquid N$_2$ and ground in TRIzol reagent (Invitrogen; 15596-026) using mortar and pestle and RNA was extracted as per manufacturer instructions. RNA was isolated from ISE6 cells (passage 96-100) grown in 96 well plates using Buffer RLT lysis buffer from the RNeasy mini kit (Qiagen; 74104) and processed according to kit instructions. cDNA from RNA samples using the iScript cDNA synthesis kit (BIORAD; 170-8891). Thermocycler conditions used for cDNA synthesis were as follows: 25°C for 5 minutes, 42°C for 50 minutes, and 85°C for 5 minutes.

4.2.6 Transcript expression

Primers ranging from 300-607 bp in length were designed for genes of interest using Primer3 software [145] in the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). cDNA template derived from female *I. scapularis* or ISE6 cells, primers (25 nmole; Table 1), and Phusion high-fidelity PCR master mix with HF buffer (NE Biolabs; M0531S) were used for cDNA amplification. The following thermocycler conditions were used: 94°C for 5 minutes, 32 cycles of 94°C for 30 seconds/58°C for 30 seconds/72°C for 2 minutes, and 72°C for 7 minutes. After PCR, gel electrophoresis was performed and bands of
expected sizes were confirmed and excised using the Qiagen gel extraction kit (Qiagen; 28704). cDNA samples were sequenced (using forward primers from Table 1) at the DNA Sequencing Low Throughput Laboratory (Purdue Genomics Core Facility; Purdue University) to confirm identity of genes of interest from both *I. scapularis* female ticks and ISE6 cells.

**4.2.7 Synthesis of dsRNA for genes of interest**

cDNA template and primers with T7 promoter sequence (50 nmole; Table 2) were used in the same manner as in Section 4.2.6 but with the following thermocycler conditions: 94°C for 5 minutes, 5 cycles at 94°C for 30 seconds/58°C for 30 seconds/72°C for 2 minutes (anneals the gene-specific section of the primer to form some PCR product), next 27 cycles at 94°C for 30 seconds/68°C for 30 seconds/72°C for 2 minutes, and final extension at 72°C for 7 minutes (anneals the entire primer site for further amplification of PCR product). PCR product of amplified cDNA with T7 promoter sequence was used to create dsRNA with the MEGAscript RNAi synthesis kit according to Barry et al [146].

**4.2.8 Transfection of ISE6 cells with dsRNA**

OptiMEM Reduced Serum Medium, Glutamax supplement (Invitrogen; 51985-034) and xtremeGENE siRNA transfection reagent (Roche; 4476093001) were used in the transfection mix according to Barry et al [146]. ISE6 cells (~1 x $10^4$-$5$) were grown for 48 hours using 96 well flat-bottom cell culture plates (pre treated with poly-L-lysine), half of the media volume was removed, transfection mix (xtremeGENE siRNA transfection reagent, OptiMEM, and 10 ng dsRNA) was added to replace the removed volume, and 60 hours post transfection of ISE6 cells
was completed. After 60 hours post transfection, media/transfection mix was removed and resazurin salt (alamarBlue reagent) media was placed on the cells for 12 hours growth before fluorescent measurement for cell viability reading. In parallel, media/transfection mix was removed after 60 hours post transfection and RNA was extracted from cells with Buffer RLT for further analysis with qRT-PCR to confirm transcript knockdown of genes of interest.

To measure transcript knockdown effect on LGTV genome replication, media/transfection mix was removed after 60 hours post transfection, cells were treated with LGTV, cells were rinsed 3 x PBS, fresh media was placed on the cells for 12 hpi, and RNA was extracted from cells with Buffer RLT for further analysis with qRT-PCR. To measure transcript knockdown effect on LGTV replication after knockdown, media/transfection mix was removed after 60 hours post transfection, cells were treated with LGTV, cells were rinsed 3 x PBS, fresh media was placed on the cells for 16 hpi, and media was collected for further analysis with plaque assays. To measure transcript knockdown effect on LGTV replication during knockdown, cells were first treated with LGTV, cells were rinsed 3 x PBS, media/transfection mix was placed on the cells for 60 hours post transfection, and media/transfection mix was collected for further analysis with plaque assays.

4.2.9 Cell viability measurement

Resazurin sodium salt solution (27.5 mM; 0.346 g in 50 mL 1XPBS) was used in a 1:100 working stock in media for each cell viability experiment. Fluorescent cell viability readings were obtained as described in section 2.2.5 and in Grabowski et al [87].
4.2.10 **Relative transcript quantification**

Gene expression was quantified by Quantifast SYBR Green qRT-PCR kit (Qiagen; 204054) with cDNA derived from ISE6 cells and qRT-PCR primers (Table 3). This was used to quantify relative expression of genes of interest following dsRNA-mediated transcript knockdown. Additionally, qRT-PCR primers targeting the negative strand of the LGTV intermediate genome were used as in Mitzel et al [39] to identify relative LGTV genome expression. The real-time Applied Biosystems 7300 PCR system (Life Technologies) with MicroAmp Optical 96 well reaction plate with labeled barcode (Life Technologies; 4306737) was used with 7300 system SDS RQ study software (v 1.4.1) to collect raw Ct cycle values. The ΔΔCt method was used to determine transcript expression relative to *I. scapularis β-actin* for the confirmation of transcript knockdown of genes of interest. Also, transcript expression relative to *I. scapularis β-tubulin* was used for determining effect on LGTV negative strand genome replication levels following transcript knockdown of genes of interest [39].

4.2.11 **DAVID functional clustering analyses**

To identify biological function clusters of ISE6 proteins with KEGG orthology and mapped to *I. scapularis* KEGG pathways that exhibitied increased expression following LGTV infection and UV-LGTV treatment [87], Genebank accession IDs were submitted for search in the Function Annotation Tool of DAVID (http://david.abcc.ncifcrf.gov/summary). All Gene Ontology options in the DAVID [147] clustering settings were selected for output. Significant clustering of functions of the uploaded list of proteins was accomplished with a DAVID assignment of an
enrichment score (≥1.3 is equal to a p value ≤0.05). Additionally, within each cluster, a modified Fisher Exact p value (p value ≤0.05 is ideal) was given for each function term with corresponding uploaded accession IDs.

4.2.12 Analysis of predicted protein-chemical interactions using STITCH

The software STITCH (http://stitch.embl.de; [148]) was used to identify predicted *I. scapularis* protein-chemical interactions with compounds (TSA, OligoA, NAM, FAMO, Hexa, EGC, and ECG) screened in ISE6 cells. Compound name/abbreviation was uploaded and actions view was used with a prediction threshold search at >0.500 confidence showing no more than 50 interactants.

4.2.13 Statistical analyses

Unpaired t-test statistical analyses were completed as described in Chapter 2, Section 2.2.6.

4.3 RESULTS

4.3.1 Small molecule compound assays identify energy processes and histone acetylation associated with LGTV infection

Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor and acetylation activator of enzymes involved in intermediate metabolism, including MDH2, decreased viability of Vero cells (with and without LGTV infection) and LGTV replication (measured by infectious virus release pfu) directly with higher concentrations. Conversely, an increase in TSA concentrations directly increased ISE6 cell viability (with LGTV infection) and increased (~0.5 log pfu) LGTV replication (Fig 1 and 2A).

The differential proteomic analysis from Chapter 3 identified candidate histone deacetylase 1, 2, 3 (ISCW007830-PA), histone H4 (ISCW019498-PA),
core histone H2A/H2B/H3/H4 (ISCW002300-PA). The LFQ quantitative proteomic analysis from Chapter 3 identified candidate histone deacetylase 1, 2, 3 (ISCW007830-PA), histone H4 (ISCW019498-PA), histone (ISCW012234-PA), histone H2B (ISCW019496-PA), histone H1 (ISCW016230-PA), histone H2A (ISCW019497-PA and ISCW004478-PA), and histone H3 (ISCW003178-PA and ISCW002300-PA). STITCH predicted TSA to bind to candidate histone deacetylases (ISCW014481-PA, ISCW007923-PA, ISCW008957-PA, ISCW012528-PA, and ISCW007830-PA), bind and inhibit candidate deacetylases (ISCW014481-PA and ISCW008957-PA), and activate candidate histones (ISCW019498-PA and ISCW002300-PA) or candidate histone-modifying proteins (ISCW019432-PA and ISCW007938-PA) (Fig 3).

In an attempt to inhibit the deacetylase activity of sirtuins (proteins that regulate biological pathways via deacetylase, demyristoylase, demalonylase, depalmitoylase, and desuccinylase activity) nicotinamide (NAM) was used and shown to reduce LGTV replication at high concentration \((10^5 \text{ µM})\) in both Vero and ISE6 cells (Fig 4). At lower concentrations \((≤10^2 \text{ µM})\), LGTV replication levels increased in Vero cells (only \(10^2 \text{ µM}\) concentration shown) following NAM treatment, this observation was not seen in ISE6 cells. The differential proteomic analysis from Chapter 3 identified candidate sirtuin type (ISCW023257-PA) and candidate poly [ADP-ribose] polymerase (ISCW019519-PA). The LFQ quantitative proteomic analysis from Chapter 3 identified only candidate poly [ADP-ribose] polymerase (ISCW019519-PA). STITCH predicted that NAM binds to and inhibits candidate sirtuins (ISCW006267-PA, ISCW023257-PA, and ISCW011143-PA).
and candidate poly [ADP-ribose] polymerases (ISCW019519-PA and ISCW017128-PA) (Fig 5).

Oligomycin A (OligoA), a mitochondrial H+ ATPase pump inhibitor, inhibits the end process of the electron transport chain by reducing ATP production. Increasing concentrations of OligoA resulted in a significant decrease in the viability of both Vero cells (~20%) and ISE6 cells (~60%) in the absence of LGTV. We observed a significant reduction (~1.5 log reduction in pfu in Vero cells and ~2 log reduction in pfu in ISE6 cells) in LGTV in both mammalian and invertebrate systems with increasing concentration of OligoA (Fig 1 and 2B). The differential proteomic analysis from Chapter 3 identified candidate ATP synthase D chain (ISCW016217-PA) of the mitochondria ATPase pump. The LFQ quantitative proteomic analysis from Chapter 3 also identified candidate ATP synthase D chain (ISCW016217-PA). STITCH predicted that OligoA inhibits the candidate ATP synthase D chain (ISCW016217-PA) of the H+ ATPase pump in the mitochondria, and activates candidate serine/threonine protein kinase (ISCW020637-PA).

In an effort to perturb the ISE6 glutaminolysis process, compounds were selected and tested in an assay to disrupt glutamate dehydrogenase, the key enzyme involved in creating α-ketoglutarate from glutamate (Fig 6), to evaluate the effect on LGTV infection. The following small molecules were used in functional compound assays: Famotidine (Famo), Hexachlorophene (Hexa), epigallocatechin (EGC), and epicatechin-3-gallate (ECG). A high concentration of Famo (10^5 µM) reduced LGTV replication in Vero (data not shown) and ISE6 cells. Similarly, high concentrations (10^3 - 10^4 µM) of Hexa reduced LGTV replication (10^3
104

µM concentration did not affect cell viability) in ISE6 cells (Fig 7A and 7B). The
differential proteomic analysis from Chapter 3 identified candidate glutamate
dehydrogenase (ISCW000393-PA). The LFQ quantitative proteomic analysis from
Chapter 3 also identified candidate glutamate dehydrogenase (ISCW000393-PA).
STITCH predicted that Hexa binds and inhibits candidate glutamate
dehydrogenase (ISCW000393-PA) and binds candidate mitogen-activated protein
kinase (ISCW021966-PA).

Tea polyphenols EGC and ECG reduce LGTV replication with higher
concentrations \((10^3 - 10^4 \text{ µM})\) and lower concentrations \((10^{-1} - 10^4 \text{ µM})\) in ISE6 cells,
respectively (Fig 8A and 8B). EGC has a higher IC\textsubscript{50} (>50 µM) than ECG (>1.7 µM)
[149-151]. Hexa and tea polyphenols (EGC, ECG, etc) have been found to be
allosteric regulators of GDH. The differential proteomic analysis from Chapter 3
identified candidate glutamate dehydrogenase (ISCW000393-PA). The LFQ
quantitative proteomic analysis from Chapter 3 also identified candidate glutamate
dehydrogenase (ISCW000393-PA). STITCH predicted that ECG binds candidate
 glutamate dehydrogenase (ISCW000393-PA) and activates candidate cAMP-
dependent protein kinase catalytic subunit (ISCW018284-PA).

4.3.2 RNA interference transcript knockdown

4.3.2.1 Identifying and characterizing genes of interest for RNAi-mediated
transcript knockdown

Criteria for choosing genes of interest from hundreds of ISE6 proteins (486
ISE6 proteins from the differential proteomic screen of Chapter 3 and from
Grabowski et al [87]) were derived from protein expression change following LGTV
infection, KEGG orthology or no KEGG orthology (proteins are either orthologous
or not orthologous based on the KEGG analysis), and stronger protein identification (proteins identified with ≥2 peptides). Fig 9 shows the process taken to derive ten genes of interest: FAH (Fumar), ERP29, ALDH4A1 (DPCD), VNN (CNHydro), MDH2, PARP, CMPK1 (CUKinase), ACAT1, hypothetical protein (Hypo195), and hypothetical protein (Hypo576).

First, ISE6 proteins that showed increased expression and with ≥ 2 peptides were selected. Next, the remaining proteins were separated into orthologous and non-orthologous proteins (orthologous refers to KEGG Orthology). Orthologous proteins with putative GO function, InterPro protein domain function, and overlap protein identifications with other flavivirus-host proteomic studies [56, 65, 109, 110] were then separated. Non-orthologous proteins were separated based on putative GO function and InterPro protein domain function, which was also used to identify proteins with no known orthology and orphan proteins.

Cluster analyses provide insight into ISE6 cellular responses following LGTV infection and UV-LGTV treatment. ISE6 proteins mapped to *I. scapularis* KEGG pathways showing increased expression following only LGTV infection, both LGTV infection and UV-LGTV treatment, and only UV-LGTV treatment [87] were used to identify clusters of processes derived from GO descriptions from DAVID. Ribonucleoprotein/ribosomal/translation/protein metabolic functions and genetic/transcriptional control were the primary functions occurring with LGTV infection and UV-LGTV treatment (45 proteins searched, DAVID used 33 for clustering; Fig 10A). Fumar, DPCD, MDH2, PARP, and CUKinase were found to
be involved in the ribonucleoprotein/ribosomal/translation/protein metabolic function.

Functional cluster analyses revealed enriched translation, ribosomal function, and protein metabolic processing (13 proteins searched, DAVID used 8 for clustering) following LGTV infection (Fig 10B). Results revealed enriched protein transport, cytoplasmic ribonucleoprotein control, along with spliceosome function (20 proteins searched, DAVID used 16 for clustering) following UV-LGTV treatment. Results revealed enriched functions of nitrogen metabolic processing as well as amine/amino acid metabolic processing (12 proteins searched, DAVID used 9 for clustering) following both LGTV infection and UV-LGTV treatment. Of the eight ISE6 proteins selected, MDH2 and PARP were found in the enrichment of translation, ribosomal function, and protein metabolic processing functions. CUKinase, DPCD, and Fumar were all found in the enrichment of nitrogen metabolic processing and amine/amino acid metabolic processing. ACAT1 only clustered in nitrogen metabolic processing function.

Four of the eight proteins have <50% amino acid conservation with the human ortholog (Table 4). Additionally, the most common function that the orthologous proteins mapped in was amino acid metabolism. Six of the eight proteins were involved in metabolic response(s). Upon sequencing cDNA amplified from the transcripts for the selected ten genes of interest of the female I. scapularis tick and I. scapularis ISE6 cell line, 96 % and greater conservation was seen from all genes of interest when compared to IscaW1 annotation gene models (Table 5). Five genes of interest had decreased sequence similarity in ISE6 cells
compared to the female tick, where four genes of interest had decreased sequence similarity in the female tick compared to ISE6 cells. CUKinase, Hypo195, and Hypo576 had 2 or less nucleotide mismatches between female tick and/or ISE6 cell line, revealing them to be the three out of ten genes of interest with the least amount of nucleotide change.

4.3.2.2 Confirmation of dsRNA-mediated transcript knockdown of genes of interest

High dsRNA concentrations was detrimental to tick cell viability. Using dsRNA derived from exogenous pGEM, higher concentrations of 100 and 50 ng revealed detrimental effects on ISE6 cell viability, while lower concentrations of 25 and 10 ng dsRNA showed no effect or an increase in ISE6 cell viability (Fig 11).

We observed no significant effect on cell viability of RNAi treated cells (Fig 12). In parallel with cell viability readings, transcript knockdown of all genes of interest was confirmed through qRT-PCR (Fig 13). The 10 ng dsRNA transfections was used for transcript knockdown of the genes of interest.

4.3.2.3 Transcript knockdown effect of genes of interest on LGTV infection

No significant effect on ISE6 cell viability occurred with transcript knockdown of genes of interest and LGTV infection (Fig 14). Three genes of interest (CNHydro, ACAT1, and Hypo576) revealed decreased LGTV negative strand genome levels compared to the negative pGEM control (Fig 15). Transcript knockdown of all genes of interest revealed a statistically-significant decrease in LGTV replication levels compared to negative pGEM control when measuring LGTV replication in pre knockdown ISE6 cells (Fig 16). In addition, transcript knockdown of all but three (Fumar, PARP, and CUKinase) genes of interest
revealed a statistically-significant decrease in LGTV replication levels compared to the negative pGEM control when measuring LGTV replication during transcript knockdown of genes of interest in ISE6 cells (Fig 17). Effects on LGTV infection can be found in Table 6.

Localization of ortholog proteins were predicted as shown in Fig 18. DPCD, MDH2, and ACAT1 are all predicted to be in the mitochondria. Fumar and CUKinase are likely found in the cytosol. PARP and CUKinase are predicted to be found in the nucleus. ERP29 likely maintains a presence in the endoplasmic reticulum as a secreted protein. CNHydro is likely found in the plasma membrane. Since no information is known on Hypo195 and Hypo576, the known locations of these proteins is currently unknown however Hypo576 is suggested to be localized in the cytoplasm since the transcript knockdown of this gene affected LGTV negative strand genome replication.

4.4 DISCUSSION

4.4.1 Impact of LGTV infection on signaling in ISE6 cells

Several proteins identified from Chapter 3 were suggested to impact cellular signaling mechanisms associated with LGTV infection [87]. The candidate histone deacetylase 1,2,3 (ISCW007830-PA) exhibited decreased expression following both LGTV infection and UV-LGTV treatment. Histone acetylation can impact viral replication and virus infection can manipulate histones in order to control host transcriptional regulation [111, 112]. Viral proteins are capable of binding deacetylase enzymes [112] and other host proteins associated with acetylation [152]. In other systems, it has been shown that histone deacetylase can act as a
co-repressor in the notch signaling pathway. It was hypothesized that LGTV infection may be altering this histone deacetylase to better control host gene regulation. Treatment of LGTV-infected ISE6 cells with TSA increased LGTV replication, suggesting that LGTV infection impacts gene regulation through histone deacetylase and associated notch signaling pathway. The candidate 40S ribosomal protein S6 (ISCW024315-PA) and Mo25 protein (ISCW004710-PA) exhibited increased expression in LGTV-infected cells. These proteins may be members of the mTOR signaling pathway (http://www.genome.jp/kegg-bin/show_pathway?map04150). The mTOR signaling pathway has been implicated in Human cytomegalovirus (HCMV) infection in mammalian cells [113, 114] and DENV infection in *A. aegypti* mosquitoes [115]. Increased expression of Mo25 may reflect a stress response and increased expression of S6 may reflect an increase in translation to maintain growth of the infected cell or facilitate LGTV replication. Manipulation of mTOR signaling has been noted with WNV infection in mammalian systems [116] as well. The candidate calcyclin-binding protein CacyBP (ISCW013691-PA) had increased expression in UV-LGTV-treated cells and decreased expression in LGTV-infected cells. In other systems, CacyBP orthologs function in the Wnt signaling pathway. Our observation suggested an increase in proteolysis following virus treatment since the Wnt pathway is associated with the Ca$^{2+}$-dependent, ubiquitin-mediated proteolysis pathway. Future investigations regarding roles of post-translational modifications in regulating signaling pathways following tick-borne flavivirus infection would be a vital research.
4.4.2 **Identification of selected proteins associated with LGTV replication**

TSA was predicted to bind to candidate histone deacetylase 1, 2, 3 (ISCW007830-PA), activates candidate histone H4 (ISCW19498-PA), and activates candidate core histone H2A/H2B/H3/H4 (ISCW002300-PA). From these predictions, it can be further hypothesized that **TSA may increase tick histone acetylation**. This suggested that an increased amount of DNA for transcription was produced by the cell following LGTV infection. Although metabolic enzymes have also shown increased acetylation levels with TSA [153] in mammalian systems, STITCH chemical-protein analyses with TSA did not predict any tick metabolic enzymes interacting. Overall, TSA increases lysine acetylation (histones and metabolic enzymes) and increases LGTV infection in ISE6 cells although exact confirmation of acetylated peptides with TSA treatment was not completed. *This suggested but did not prove that an increase in lysine acetylation of ISE6 histones increases LGTV infection*. Further targeted experiments focusing on the mechanism of histone lysine acetylation and LGTV infection will need to be completed.

NAM has been known to inhibit sirtuins (although NAM was predicted to inhibit poly [ADP-ribose] polymerase, most likely through feedback inhibition), which perform deacetylation activity in the cell. The higher concentrations screened in Vero and ISE6 cells may have saturated the effect on the cell, hence why lower concentrations of the compound may have shown an increased LGTV replication in Vero cells. Additionally, screening of lower concentrations (≤10^2 µM)
of NAM in ISE6 cells needs to be performed before conclusions can be made on the biological effects from the compound on LGTV replication in the tick system.

Our *in vitro* studies have shown that the compounds TSA and OligoA can affect levels of LGTV replication, presumably through impact on a variety of metabolic processes. TSA is thought to inhibit histone deacetylases and stimulate the acetylation of metabolic enzymes [153], while OligoA may inhibit oxidative phosphorylation and electron transport. TSA is known to create effects on transcription and translation that may subsequently impact metabolic pathways. OligoA may activate AMPK activity [154], inhibit ATP production, and affect energy levels. Thus, further studies may be required to determine the mode of action of TSA and OligoA in the LGTV-ISE6 system.

Glutaminolysis is a process that produces an alternative energy source that converts glutamine into the production of ATP. Increased expression of proteins associated with glutaminolysis (Fig 6) suggest that LGTV infection in ISE6 cells may stimulate glutaminolysis that converts glutamate to α-ketoglutarate, which is a key intermediate in the TCA cycle. Studies suggest that glutaminolysis is manipulated by viruses during infection of human cells by both HCMV [129, 130] and HCV infection [56]. These studies suggest that glutaminolysis/glutamate metabolic enzymes and α-ketoglutarate are critical for maintaining the TCA cycle, supporting oxidative phosphorylation, and ATP production in the infected cell. Additionally, stimulation of α-ketoglutarate has been shown to increase mTOR activity [131, 132], which works in parallel with glutaminolysis. Small molecule compound assays assessed the manipulation of glutamate dehydrogenase (GDH)
activity with the goal of inhibiting activities of glutamate metabolic enzymes during flavivirus infection. We hypothesized that Hexa disrupted glutamate metabolism and/or glutaminolysis and decreased LGTV replication. However, assessment of downstream metabolites from glutamate metabolism (glutamate levels) and glutaminolysis (α-ketoglutarate levels) following these compound treatments to cells needs to be completed in order confirm that these processes are being affected.

4.4.3 Silencing of metabolic and protein processing functions decrease LGTV infection

4.4.3.1 Optimization of RNA interference conditions and response in LGTV infection

Transcript knockdown was shown not to cause detrimental effects to ISE6 cells before attempting to measure transcript knockdown effect on LGTV infection. Figs 12 and 14 suggest that the ISE6 cell viability was not significantly affected by transcript knockdown of genes of interest using the optimized 10 ng dsRNA. The 10 ng dsRNA concentration was sufficient for knocking down the ten genes of interest.

By comparing the effect of transcript knockdown of genes of interest to the LGTV 3UTR positive control on the LGTV negative strand genome replication, potential steps of the LGTV life cycle being affected may be suggested. It was predicted that LGTV replication levels would be within the range of the negative pGEM and positive LGTV 3UTR controls. In combination with the effects of transcript knockdown on LGTV negative strand genome replication, effect on LGTV replication contributes a second measurement of the LGTV infection in ISE6 cells.
4.4.3.2 *I. scapularis* metabolic and protein processing enzymes necessary for LGTV infection

Fig 19 shows the possible mechanisms by which seven *I. scapularis* genes of interest (ERP29, DPCD, CNHydro, MDH2, ACAT1, Hypo195, and Hypo576) may affect LGTV replication. Transcript knockdown for two genes of interest with orthology (CNHydro and ACAT1) and one gene of interest with no known function resulted in reduction of both LGTV genome replication and LGTV replication.

CNHydro is involved in cofactors/vitamins and pantothenate/CoA biosynthesis metabolism and location on the plasma membrane that can serve as a GPI-anchored protein suggest deficiency in formation of the replication complex, genome replication, LGTV attachment, endocytosis, release of genome into the cytoplasm.

ACAT1 is involved in carbohydrate, lipid, amino acid, and terpenoids/polyketides metabolism. The identification of a lipid (fatty acid) metabolism pathway (from KEGG) suggested that ACAT1 may affect the formation of replication complexes, which require lipids for membrane formation. Also involved in carbohydrate (pyruvate) metabolism, ACAT1 may affect replication complex formation through reducing necessary energy processes. The ACAT1 ortholog was identified in an HCV-HUH7.5 cell proteomic study [56], but no expression change was observed with HCV infection.

The role of Hypo576 was also unknown. It was also suggested to affect LGTV attachment, endocytosis, virus fusion/release of genome, the formation of replication complexes/vacuoles, or the formation of dsRNA intermediate genome.
RNAi-induced, transcript knockdown of genes of interest that led to no change in LGTV negative strand genome levels compared to the negative pGEM control may have similar effect on LGTV attachment, endocytosis, virus fusion/release of genome, formation of replication complexes/vacuoles, or formation of dsRNA intermediate genome with the negative control. Transcript knockdown of three genes of interest with orthology (ERP29, DPCD, and MDH2) and one gene of interest with unknown function (Hypo195) resulted only in a reduction in LGTV replication.

ERP29 is involved in protein folding/sorting/degradation and its effect on virus infection associated with transcript knockdown suggested deficiency in virion assembly, virion egress, prM cleavage, and/or virus budding. The ERP29 ortholog was identified in an HCV-HUH7.5 cell proteomic study [56], but no expression change was observed with HCV infection. ERP29, an established stress response protein, has been found with increased expression in mouse brain tissue following Japanese Encephalitis virus (flavivirus) infection [155]. In polyomavirus infection, ERP29 was identified to unfold viral proteins [156-158] to aid in subsequent virus binding and release from the ER lumen, which implicates ERP29 does have the ability to aid in virus infection. Although involved in stress response, it is unknown whether ERP29 is directly aiding in flavivirus infection.

DPCD amino acid metabolic involvement may affect the viral protein translation. Additionally, involvement in glutamate metabolism (predicted to influence glutaminolysis) has an effect on energy homeostasis, which may lead to disruption of replication complexes and/or viral proteins. DPCD completes the
conversion of delta-1-pyrroline-5-carboxylate (P5C), which is derived either from proline or ornithine, to glutamate. This is a necessary step in the pathway interconnecting the urea and tricarboxylic acid cycles with glutaminolysis. In addition, DPCD was identified with increased protein expression [56] and gene expression [159] following HCV infection. It is unknown whether the increased expression of DPCD aids flavivirus infection or whether it is the cell response to stress and/or to maintain energy levels.

MDH2 carbohydrate metabolic involvement may affect the translation and/or formation of viral proteins. A key rate-limiting enzyme in the citrate cycle and in pyruvate metabolism may suggest that MDH2 can control energy homeostasis levels and in parallel, affecting cellular but also viral protein production that requires ATP. Increased protein expression of MDH2 has been identified following HCV infection [56]. Increased transcript expression of MDH2 has been identified with bloodfed salivary gland tissue of *I. scapularis* nymphs [72]. In addition, MDH2 protein expression has been identified within *I. scapularis* synganglia tissue [91]. It is unknown whether the increased expression of MDH2 aids flavivirus infection or whether it is the cell response to stress and/or to maintain energy levels.

The role of Hypo195 is currently unknown. It was suggested to affect LGTV viral protein translation, virion assembly, virion egress, prM cleavage, and/or virus budding. In addition, Hypo195 protein expression has been identified within *I. scapularis* synganglia tissue [91].
Overall, a transcript knockdown of genes of interest that lead to decreased LGTV negative strand genome levels and a decreased infectious LGTV release (CNHydro, ACAT1, and Hypo576) reveal that LGTV genome replication may be affected by reduction in viral protein translation, genome replication complexes, or synthesis of dsRNA genome intermediate. Infectious LGTV release was reduced in pre knockdown cells and in cells where transcript knockdown of genes of interest was completed during LGTV infection with seven genes of interest (ERP29, DPCD, CNHydro, MDH2, ACAT1, Hypo195, and Hypo576). This provided confidence that the transcript knockdown of these genes can reduce TBF infection before and during infection. This study was the first report of CNHydro, Hypo195, and Hypo576 with increased expression following flavivirus infection. Also, this was the first report of DPCD, CNHydro, MDH2, ACAT1, Hypo195, and Hypo576 transcript knockdown and effect on flavivirus infection.

Three genes of interest (Fumar, PARP, and CUKinase) did not reduce LGTV replication when transfection was completed during LGTV infection in ISE6 cells, thus were not considered to be involved with LGTV infection. Transcript knockdown of ISE6 amino acid/vitamin/cofactor/carbohydrate/lipid metabolic enzymes (DPCD, CNHydro, MDH2, and ACAT1) and a folding/sorting/degradation protein (ERP29) were correlated with a decrease in LGTV replication. This observation suggested that efficient LGTV replication (denoted as the negative pGEM control) either directly or indirectly involved these these genes of interest. The hypothesis that ISE6 amino acid/carbohydrate/vitamin/cofactor/lipid metabolic
enzymes and folding/sorting/degradation proteins with increased expression following LGTV infection were involved in LGTV infection was accepted.

4.4.3.3 Candidate proteins for future therapeutic development

Approaches to limit transmission of tick-borne pathogens may target either the tick vector or the mammalian reservoir. Small molecule compounds may have application for domestic/companion animals and humans, and, potentially provide the opportunity to reduce pathogen circulation in natural reservoirs. Vaccines could reduce pathogen circulation in domestic/companion animals, humans, and in natural reservoirs. Antigens developed to a pathogen protein have been used for vaccine development targeting the natural reservoir [160, 161], and this may be possible with arthropod vector proteins as well. Currently, oral bait formulations including vaccines targeting pathogen/tick proteins is one way to reduce pathogen circulation and reduce transmission.

de la Fuente and Merino (2013) suggest that RNAi functional analysis of tick proteins associated with pathogen infection are necessary before development of protein vaccines [162]. If successful, vaccines could be developed and vaccine conducted in clinic [163] and field [161, 164] may be completed. This project provides the next step to functionally characterize a candidate protein for development of new therapeutic solutions.
### 4.5 FIGURES, TABLES, AND LEGENDS

**Table 1. PCR primers for selected genes of interest.** Abbreviated names for genes of interest, housekeeping gene (β-actin), and RNAi controls (pGEM and LGTV 3UTR) include Fumar = fumarylacetoacetase; ERP29 = endoplasmic reticulum protein 29; DPCD = 1-pyrroline-5-carboxylate dehydrogenase (aldehyde dehydrogenase 4A1); CNHydro = pantetheine hydrolase (vanin); MDH2 = malate dehydrogenase; PARP = poly [ADP-ribose] polymerase; CUKinase = UMP-CMP kinase; ACAT1 = acetyl-CoA acetyltransferase; Hypo195 = hypothetical protein; Hypo576 = hypothetical protein; β-actin = beta-actin; pGEM = pGEM plasmid cloning vector; LGTV 3UTR = LGTV strain TP21 3’ untranslated region of genome.

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*F denotes forward primer  
*R denotes reverse primer  
*ISCW- - - - - - denotes VectorBase accession ID  
*Derived from [39]  
+GenBank accession identified through Virus Pathogen Resource (viprbrc.org/brc)
Table 2. T7-tagged primers for selected genes of interest. Abbreviated names for genes of interest and RNAi controls (pGEM and LGTV 3'UTR) include Fumar = fumarylacetoacetase; ERP29 = endoplasmic reticulum protein 29; DPCD = 1-pyrroline-5-carboxylate dehydrogenase (aldehyde dehydrogenase 4A1); CNHydro = pantetheine hydrolase (vanin); MDH2 = malate dehydrogenase; PARP = poly [ADP-ribose] polymerase; CUKinase = UMP-CMP kinase; ACAT1 = acetyl-CoA acetyltransferase; Hypo195 = hypothetical protein; Hypo576 = hypothetical protein; pGEM = pGEM plasmid cloning vector; LGTV 3'UTR = LGTV strain TP21 3' untranslated region of genome.

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*F denotes forward primer
*R denotes reverse primer
*ISCW- - - - - - denotes VectorBase accession ID
+GenBank accession identified through Virus Pathogen Resource (viprbrc.org/brc)
Table 3. qRT-PCR primers for selected genes of interest. Abbreviated names for genes of interest, housekeeping genes (β-actin and β-tubulin), and LGTV negative strand primers include Fumar = fumarylacetooacetase; ERP29 = endoplasmic reticulum protein 29; DPCD = 1-pyrroline-5-carboxylate dehydrogenase (aldehyde dehydrogenase 4A1); CNHydro = pantetheine hydrolase (vanin); MDH2 = malate dehydrogenase; PARP = poly [ADP-ribose] polymerase; CUKinase = UMP-CMP kinase; ACAT1 = acetyl-CoA acetyltransferase; Hypo195 = hypothetical protein; Hypo576 = hypothetical protein; β-actin = beta-actin; β-tubulin = beta-tubulin; LGTV negative strand = LGTV strain TP21 negative strand.

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<td>F ERP29 (ISCW018425)</td>
<td>TCAAGCGGTCGTCACAGGCTAC</td>
</tr>
<tr>
<td>R ERP29 (ISCW018425)</td>
<td>GTACGCGCTGCCCTTGTGGTGTG</td>
</tr>
<tr>
<td>F DPCD (ISCW015982)</td>
<td>TTGGAGGCAAGGAGCATCGG</td>
</tr>
<tr>
<td>R DPCD (ISCW015982)</td>
<td>CAGGCTGCTGTGTTTATCGGCTC</td>
</tr>
<tr>
<td>F CNHydro (ISCW004822)</td>
<td>CTACAACACCAACGTCGCT</td>
</tr>
<tr>
<td>R CNHydro (ISCW004822)</td>
<td>AAGGGCTCCACAAACAGGCT</td>
</tr>
<tr>
<td>F MDH2 (ISCW003528)</td>
<td>GGTGTCTCAAGACGCTG</td>
</tr>
<tr>
<td>R MDH2 (ISCW003528)</td>
<td>ACGTTCAGCTGGCTGTC</td>
</tr>
<tr>
<td>F PARP (ISCW019519)</td>
<td>CTCGACGGAGATGATGCAAATG</td>
</tr>
<tr>
<td>R PARP (ISCW019519)</td>
<td>CATCTTGGAGAAGGCCCACAT</td>
</tr>
<tr>
<td>F CUKinase (ISCW012446)</td>
<td>GGCCTCTTGGACCATATGTCG</td>
</tr>
<tr>
<td>R CUKinase (ISCW012446)</td>
<td>AATCTTCTGACACTGGTGTCG</td>
</tr>
<tr>
<td>F ACAT1 (ISCW016117)</td>
<td>CAGGAGTCTCTGGCTG</td>
</tr>
<tr>
<td>R ACAT1 (ISCW016117)</td>
<td>TGAACACCTCTGGACATCG</td>
</tr>
<tr>
<td>F Hypo195 (ISCW011195)</td>
<td>CACCTCGACAAACTGAGCAC</td>
</tr>
<tr>
<td>R Hypo195 (ISCW011195)</td>
<td>CGTCGGTGCAATACTGGGCA</td>
</tr>
<tr>
<td>F Hypo576 (ISCW020576)</td>
<td>TGGCTGAACGGGACATCG</td>
</tr>
<tr>
<td>R Hypo576 (ISCW020576)</td>
<td>TCGGGCTCAACAGGAGGAGC</td>
</tr>
<tr>
<td>F β-actin (ISCW024111)</td>
<td>GCCGGGACCTTACAGACTATC</td>
</tr>
<tr>
<td>R β-actin (ISCW024111)</td>
<td>CAGCAGAATTTCTACGCTG</td>
</tr>
<tr>
<td>F β-tubulin (ISCW005137)</td>
<td>ACCTTCTTGGGCACAGC</td>
</tr>
<tr>
<td>R β-tubulin (ISCW005137)</td>
<td>CTCCTCTGGGTCAGAGT</td>
</tr>
<tr>
<td>F LGTV negative strand</td>
<td>GTCTCCAGTTGAGCATGT</td>
</tr>
<tr>
<td>R LGTV negative strand</td>
<td>CTCGGTCTAGTGATGGGTG</td>
</tr>
</tbody>
</table>

*qRT-PCR consisted of cDNA synthesis of I. scapularis ISE6 RNA first, then conducting qPCR second with cDNA
*F denotes forward primer
*R denotes reverse primer
*ISCW- - - - - - denotes VectorBase accession ID
∞ Derived from [39]
+GenBank accession identified through Virus Pathogen Resource (viprbrc.org/brc)
Fig 1. Predicted effect of Trichostatin A and Oligomycin A in ISE6 cells. Trichostatin A activation of histones and MDH2; Oligomycin A inhibition of H+ ATP synthase pump of the mitochondria. Red denotes TCA Cycle, MDH2 enzyme with increased expression following LGTV infection. Blue denotes TCA Cycle, CS and FH enzymes with decreased expression following LGTV infection.
Fig 2. In vitro ISE6 assay showing effects of (A) trichostatin A and (B) oligomycin A on cell viability and LGTV replication. Release of LGTV was assessed by viral titer (pfu/ml) using plaque assays in BHK cells. Cell viability was determined with alamarBlue reagent and fluorescent assay and percentage was normalized against solvent only control in both LGTV-infected and mock-treated ISE6/Vero cells.
Fig 4. *In vitro* ISE6 assay showing effects of nicotinamide on cell viability and LGTV replication. Release of LGTV was assessed by viral titer (pfu/ml) using plaque assays in BHK cells. Cell viability was determined with alamarBlue reagent and fluorescent assay and percentage was normalized against solvent only control in both LGTV-infected and mock- treated ISE6/Vero cells.
Fig 5. Predicted interactions of nicotinamide on *I. scapularis* proteins. Predictions were completed with STITCH (stitch.embl.de). Circles denote tick proteins with VectorBase accession IDs. Green arrow: denotes nicotinamide (vitamin B) activation of tick protein. Red line: denotes nicotinamide inhibition of tick protein. Blue line: denotes binding activity.
Fig 6. ISE6 proteins associated with glutaminolysis. ISE6 glutaminolysis and mTOR signaling proteins altered with LGTV infection are shown. GDH denotes glutamate dehydrogenase enzymes and Glase denotes glutaminase enzymes.
Fig 7. *In vitro* ISE6 assay showing effects of (A) famotidine and (B) hexachlorophene on cell viability and LGTV replication. Release of LGTV was assessed by viral titer (pfu/ml) using plaque assays in BHK cells. Cell viability was determined with alamarBlue reagent and fluorescent assay and percentage was normalized against solvent only control in both LGTV-infected and mock-treated ISE6/Vero cells.
Fig 8. *In vitro* ISE6 assay showing effects of (A) epigallocatechin and (B) epicatechin-3-gallate on cell viability and LGTV replication. Release of LGTV was assessed by viral titer (pfu/ml) using plaque assays in BHK cells. Cell viability was determined with alamarBlue reagent and fluorescent assay and percentage was normalized against solvent only control in both LGTV-infected and mock-treated ISE6/Vero cells.
Fig 9. Selection of genes of interest from large-scale proteomics screen. ISE6 proteins from the differential proteomic analysis at 36 hpi was searched. Using a combination of protein expression, protein orthology, number of peptides identified for each protein, pathways, and predicted functional analyses, a small percentage of proteins were selected for further downstream RNAi transcript knockdown analyses. ISE6 proteins with increased expression, with ≥2 peptides, were separated via presence/absence of KEGG orthology to where predicted, putative function was used (or not used; orphan proteins) to identify a subset of selected proteins. * denotes ortholog proteins that have been identified in other flavivirus-host proteomic studies. Red denotes the followed filtering path to obtain the final selected protein/genes of interest list.
Fig 10. DAVID enrichment/cluster analysis identifies significant functions of ISE6 proteins with increased expression following LGTV infection and UV-LGTV treatment. ISE6 proteins with increased expression following LGTV infection and/or UV-LGTV treatment from the differential proteomic analysis at 36 hpi was searched. Enrichment (E) score of ≥ 1.3 is equal to a ≤ 0.05 p value; higher the E number, stronger the enrichment of proteins to that particular cluster function. Proteins searched with all virus treatment (A) and proteins searched specific to virus-type treatment (B) are listed. Red denotes six of the eight selected proteins that were selected for RNAi transcript knockdown analyses.
Table 4. Selected ISE6 proteins/genes of interest.

<table>
<thead>
<tr>
<th>Tick Protein/Gene of Interest</th>
<th>Ixodes scapularis Gene Model Accession ID</th>
<th>Function</th>
<th>Human (H. sapien) Ortholog AA % Conservation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumarylacetoacetase (Fumar); K01555</td>
<td>ISCW020196</td>
<td>Amino acid metabolism</td>
<td>65.9 %</td>
</tr>
<tr>
<td>Endoplasmic reticulum protein 29 (ERP29); K09586</td>
<td>ISCW018425</td>
<td>Folding, sorting, &amp; degradation</td>
<td>17 %</td>
</tr>
<tr>
<td>1-pyrroline-5-carboxylate dehydrogenase (DPCD); K00294</td>
<td>ISCW015982</td>
<td>Amino acid metabolism</td>
<td>56.7 %</td>
</tr>
<tr>
<td>Pantetheine hydrolase (CNHydro); K08069</td>
<td>ISCW004822</td>
<td>Metabolism of cofactors &amp; vitamins</td>
<td>33.9 %</td>
</tr>
<tr>
<td>Malate dehydrogenase (MDH2); K00026</td>
<td>ISCW003528</td>
<td>Carbohydrate metabolism</td>
<td>68.9 %</td>
</tr>
<tr>
<td>Poly [ADP-ribose] polymerase (PARP); K10798</td>
<td>ISCW019519</td>
<td>Replication &amp; repair</td>
<td>48.6 %</td>
</tr>
<tr>
<td>UMP-CMP kinase (CUKinase); K13800</td>
<td>ISCW012446</td>
<td>Nucleotide metabolism</td>
<td>40.2 %</td>
</tr>
<tr>
<td>Acetyl-CoA C-acetyltransferase (ACAT1); K00626</td>
<td>ISCW016117</td>
<td>Carbohydrate, lipid, amino acid, terpenoids/polyketides metabolism</td>
<td>60.2 %</td>
</tr>
<tr>
<td>Hypothetical protein (Hypo195)</td>
<td>ISCW011195</td>
<td>Unknown</td>
<td>--</td>
</tr>
<tr>
<td>Hypothetical protein (Hypo576)</td>
<td>ISCW020576</td>
<td>Unknown</td>
<td>--</td>
</tr>
</tbody>
</table>
Table 5. Conservation of genes of interest between tick and tick cell systems.

<table>
<thead>
<tr>
<th>Protein name and transcript length</th>
<th>Female tick transcript expression coverage(^1); nucleotide mismatch(^2)</th>
<th>Alignment expect value</th>
<th>ISE6 cell transcript expression coverage(^1); nucleotide mismatch(^2)</th>
<th>Alignment expect value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumar 551</td>
<td>482/486 (99%); 4</td>
<td>0.0</td>
<td>500/506 (98%); 6</td>
<td>0.0</td>
</tr>
<tr>
<td>ERP29 553</td>
<td>491/495 (99%); 4</td>
<td>0.0</td>
<td>466/470 (99%); 4</td>
<td>0.0</td>
</tr>
<tr>
<td>DPCD 534</td>
<td>460/467 (98%); 7</td>
<td>0.0</td>
<td>466/474 (98%); 8</td>
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</tr>
<tr>
<td>CNHydro 607</td>
<td>520/531 (97%); 11</td>
<td>0.0</td>
<td>500/519 (96%); 19</td>
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</tr>
<tr>
<td>MDH2 310</td>
<td>252/254 (99%); 2</td>
<td>e-134</td>
<td>249/255 (97%); 6</td>
<td>e-125</td>
</tr>
<tr>
<td>PARP 310</td>
<td>241/250 (96%); 9</td>
<td>e-113</td>
<td>257/259 (99%); 2</td>
<td>e-135</td>
</tr>
<tr>
<td>CUKinase 349</td>
<td>265/267 (99%); 2</td>
<td>e-142</td>
<td>249/250 (99%); 1</td>
<td>e-134</td>
</tr>
<tr>
<td>ACAT1 392</td>
<td>240/244 (98%); 4</td>
<td>e-123</td>
<td>253/256 (98%); 3</td>
<td>e-133</td>
</tr>
<tr>
<td>Hypo195 300</td>
<td>245/246 (99%); 1</td>
<td>e-129</td>
<td>244/246 (99%); 2</td>
<td>e-127</td>
</tr>
<tr>
<td>Hypo576 483</td>
<td>424/425 (99%); 1</td>
<td>0.0</td>
<td>430/430 (100%); 0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^1\) coverage denotes genes of interest length of transcript sequenced to corresponding gene model sequence from the Wikel strain *I. scapularis* tick genome W1.

\(^2\) nucleotide mismatch compared to sequence of corresponding gene model from the Wikel strain *I. scapularis* tick genome W1.

*Red* denotes either the highest nucleotide mismatch between a gene of interest expressed in the tick versus in the tick cell line or the lowest expect value between a gene of interest expressed in the tick versus in the tick cell line. This reveals the degree of nucleotide change of each of the genes of interest between wildtype *I. scapularis* female tick and *I. scapularis* tick cells versus the *I. scapularis* Wikel strain, colony-based tick.
Fig 11. Effect of transfection with various dsRNA concentrations on ISE6 cell viability. Xtreme (Xtr) transfection reagent was used to optimize dsRNA (pGEM control) concentrations in ISE6 cells transfected for 60 hours. Cell viability readings were compared with Xtreme + OptiMEM (Xtr + Opti; gray bar) control. Red boxes denote dsRNA concentrations that were not detrimental to ISE6 cell viability. RLU denotes relative light units (fluorescent measurement with 560 nm excitation and 590 emission). Error bars represent standard error of the mean. Statistical unpaired t-test was completed between Xtr + Opti control and specific pGEM dsRNA concentration. Asterisks denote: *p value≤0.05 and **p value≤0.01. Three technical replicates for each treatment were performed within a biological replicate. One biological replicate shown; two biological replicates were completed.
Fig 12. Effect of transfection with dsRNA for genes of interest on ISE6 cell viability. Abbreviated names for genes of interest and RNAi controls (pGEM and LGTV 3UTR) include Fumar = fumarylacetoacetase (ISCW020196); ERP29 = endoplasmic reticulum protein 29 (ISCW018425); DPCD = 1-pyrroline-5-carboxylate dehydrogenase (aldehyde dehydrogenase 4A1; ISCW015982); CNHydro = pantetheine hydrolase (vanin; ISCW004822); MDH2 = malate dehydrogenase (ISCW003528); PARP = poly [ADP-ribose] polymerase (ISCW019519); CUKinase = UMP-CMP kinase (ISCW012446); ACAT1 = acetyl-CoA acetyltransferase (ISCW016117); Hypo195 = hypothetical protein (ISCW011195); Hypo576 = hypothetical protein (ISCW020576); pGEM = pGEM plasmid cloning vector; LGTV 3UTR = LGTV strain TP21 3’ untranslated region of genome. Xtreme (Xtr) transfection reagent was used with 10 ng dsRNA (pGEM, LGTV 3UTR, and gene of interest specific) concentration in ISE6 cell transfections of 96 wells (~1x10^5 cells) for 60 hours. Cell viability readings were conducted at 12 hours after the 60 hours post transfection. Cell viability readings were compared with the negative control pGEM dsRNA (light gray bar). LGTV 3UTR dsRNA is the positive control (dark gray bar) directed towards the LGTV genome that was used for detrimental effect on LGTV genome replication/replication. RLU denotes relative light units (fluorescent measurement with 560 nm excitation and 590 emission). Error bars represent standard error of the mean. Statistical unpaired t-test was completed between negative pGEM dsRNA control and specific I. scapularis dsRNA for gene of interest. Three technical replicates for each treatment were performed within a biological replicate. One biological replicate shown; two biological replicates were completed.
Fig 13. Transcript knockdown of *I. scapularis* genes of interest in ISE6 cells. Abbreviated names for genes of interest and RNAi controls (pGEM and LGTV 3UTR) include Fumar = fumarylacetoacetase (ISCW020196); ERP29 = endoplasmic reticulum protein 29 (ISCW018425); DPCD = 1-pyrroline-5-carboxylate dehydrogenase (aldehyde dehydrogenase 4A1; ISCW015982); CNHydro = pantetheine hydrolase (vanin; ISCW004822); MDH2 = malate dehydrogenase (ISCW003528); PARP = poly [ADP-ribose] polymerase (ISCW019519); CUKinase = UMP-CMP kinase (ISCW012446); ACAT1 = acetyl-CoA acetyltransferase (ISCW016117); Hypo195 = hypothetical protein (ISCW011195); Hypo576 = hypothetical protein (ISCW020576); pGEM = pGEM plasmid cloning vector. Relative expression was compared to *I. scapularis* β-actin gene in pGEM- and specific gene of interest-transfected cDNA. pGEM dsRNA-transfected (gray bars) compared with *I. scapularis*-specific dsRNA-transfected (white bars) ISE6 cells (~1x10^5 cells). After 10 ng dsRNA transfection was completed, ISE6 total RNA was collected at 60 hours post transfection. Relative transcript expression was used to identify fold change (transcript expression of genes of interest were compared to transcript expression of β-tubulin) from ISE6 cells. Error bars represent standard error of the mean. Statistical unpaired t-test was completed between negative pGEM dsRNA control and specific *I. scapularis* dsRNA for gene of interest. Asterisks denote: *p value≤0.05, **p value≤0.01, and ***p value≤0.001. Three technical replicates for each treatment were performed within a biological replicate. Two biological replicates were completed and shown.
Fig 14. Effect of transfection with dsRNA for genes of interest on LGTV-infected ISE6 cell viability. Abbreviated names for genes of interest and RNAi controls (pGEM and LGTV 3UTR) include Fumar = fumarylacetoacetase (ISCW020196); ERP29 = endoplasmic reticulum protein 29 (ISCW018425); DPCD = 1-pyrroline-5-carboxylate dehydrogenase (aldehyde dehydrogenase 4A1; ISCW015982); CNHydro = pantetheine hydrolase (vanin; ISCW004822); MDH2 = malate dehydrogenase (ISCW003528); PARP = poly (ADP-ribose) polymerase (ISCW019519); CUKinase = UMP-CMP kinase (ISCW012446); ACAT1 = acetyl-CoA acetyltransferase (ISCW016117); Hypo195 = hypothetical protein (ISCW011195); Hypo576 = hypothetical protein (ISCW020576); pGEM = pGEM plasmid cloning vector; LGTV 3UTR = LGTV strain TP21 3’ untranslated region of genome. Xtreme (Xtr) transfection reagent was used with 10 ng dsRNA (pGEM, LGTV 3UTR, and gene of interest specific) concentration in ISE6 cell transfections of 96 wells (~1x10^5 cells) for 60 hours, then LGTV infection was completed with transfected cells. Cell viability readings were conducted from 12 hours post infection. Cell viability readings were compared with the negative control pGEM dsRNA (light gray bar). LGTV 3UTR dsRNA is the positive control (dark gray bar) directed towards the LGTV genome that was used for detrimental effect on LGTV genome replication/replication. RLU denotes relative light units (fluorescent measurement with 560 nm excitation and 590 emission). Error bars represent standard error of the mean. Statistical unpaired t-test was completed between negative pGEM dsRNA control and specific *I. scapularis* dsRNA for gene of interest. Three technical replicates for each treatment were performed within a biological replicate. One biological replicate shown; two biological replicates were completed.
Fig 15. LGTV negative strand genome replication levels following knockdown of *I. scapularis* transcripts for genes of interest in ISE6 cells.

Abbreviated names for genes of interest and RNAi controls (pGEM and LGTV 3UTR) include Fumar = fumarylacetoacetase (ISCW020196); ERP29 = endoplasmic reticulum protein 29 (ISCW018425); DPCD = 1-pyrroline-5-carboxylate dehydrogenase (aldehyde dehydrogenase 4A1; ISCW015982); CNHydro = pantetheine hydrolase (vanin; ISCW004822); MDH2 = malate dehydrogenase (ISCW003528); PARP = poly [ADP-ribose] polymerase (ISCW019519); CUKinase = UMP-CMP kinase (ISCW012446); ACAT1 = acetyl-CoA acetyltransferase (ISCW016117); Hypo195 = hypothetical protein (ISCW011195); Hypo576 = hypothetical protein (ISCW020576); pGEM = pGEM plasmid cloning vector; LGTV 3UTR = LGTV strain TP21 3’ untranslated region of genome. Following transcript knockdown of genes of interest (60 hours post transfection with 10 ng dsRNA), effect on LGTV negative strand genome replication is identified. LGTV negative strand genome replication of dsRNA for genes of interest was compared to the negative control pGEM dsRNA (light gray bar). LGTV 3UTR dsRNA is the positive control (dark gray bar) directed towards the LGTV genome that was used for detrimental effect on LGTV genome replication. Relative transcript expression was used to identify fold change (transcript expression LGTV negative strand were compared to transcript expression of *I. scapularis* β-tubulin) of LGTV genome levels from ISE6 cells (~1x10^5 cells). Error bars represent standard error of the mean. Statistical unpaired t-test was completed between negative pGEM dsRNA control and specific *I. scapularis* dsRNA for gene of interest. Asterisks denote: *p value≤0.05, **p value≤0.01, and ***p value≤0.001. Three technical replicates for each treatment were performed within a biological replicate. Two biological replicates were completed and shown.
Fig 16. Infectious LGTV replication after knockdown of I. scapularis transcripts for genes of interest in ISE6 cells. Abbreviated names for genes of interest and RNAi controls (pGEM and LGTV 3UTR) include Fumar = fumarylacetoacetase (ISCW020196); ERP29 = endoplasmic reticulum protein 29 (ISCW018425); DPCD = 1-pyrroline-5-carboxylate dehydrogenase (aldehyde dehydrogenase 4A1; ISCW015982); CNHydro = pantetheine hydrolase (vanin; ISCW004822); MDH2 = malate dehydrogenase (ISCW003528); PARP = poly [ADP-ribose] polymerase (ISCW019519); CUKinase = UMP-CMP kinase (ISCW012446); ACAT1 = acetyl-CoA acetyltransferase (ISCW016117); Hypo195 = hypothetical protein (ISCW011195); Hypo576 = hypothetical protein (ISCW020576); pGEM = pGEM plasmid cloning vector; LGTV 3UTR = LGTV strain TP21 3′ untranslated region of genome. Following transcript knockdown of genes of interest, effect on LGTV replication (infectious virus release) is identified. LGTV replication following transfections with dsRNA for genes of interest was compared to the negative control pGEM dsRNA (light gray bar). LGTV 3UTR dsRNA is the positive control (dark gray bar) directed towards the LGTV genome that was used for detrimental effect on LGTV replication. Plaque assays with BHK15 cells were utilized to identify effect on infectious LGTV release from ISE6 cells (~1x10⁵ cells). Error bars represent standard error of the mean. Statistical unpaired t-test was completed between negative pGEM dsRNA control and specific I. scapularis dsRNA for gene of interest. Asterisks denote: **p value≤0.01 and ***p values≤0.001. Five technical replicates for each treatment were performed within a biological replicate. One biological replicate shown; two biological replicates were completed.
Infectious LGTV replication during knockdown of *I. scapularis* transcripts for genes of interest in ISE6 cells. Abbreviated names for genes of interest and RNAi controls (pGEM and LGTV 3UTR) include Fumar = fumarylacetoacetase (ISCW020196); ERP29 = endoplasmic reticulum protein 29 (ISCW018425); DPCD = 1-pyrroline-5-carboxylate dehydrogenase (aldehyde dehydrogenase 4A1; ISCW015982); CNHydro = pantetheine hydrolase (vanin; ISCW004822); MDH2 = malate dehydrogenase (ISCW003528); PARP = poly [ADP-ribose] polymerase (ISCW019519); CUKinase = UMP-CMP kinase (ISCW012446); ACAT1 = acetyl-CoA acetyltransferase (ISCW016117); Hypo195 = hypothetical protein (ISCW011195); Hypo576 = hypothetical protein (ISCW020576); pGEM = pGEM plasmid cloning vector; LGTV 3UTR = LGTV strain TP21 3’ untranslated region of genome. dsRNA transfection of genes of interest and LGTV infection were carried out at the same time. At 60 hours post transfection/infection, effect on LGTV replication (infectious virus release) was identified. LGTV replication during transfections with dsRNA for genes of interest was compared to the negative control pGEM dsRNA (light gray bar). LGTV 3UTR dsRNA is the positive control (dark gray bar) directed towards the LGTV genome that was used for detrimental effect on LGTV replication. Plaque assays with BHK15 cells were utilized to identify effect on infectious LGTV release from ISE6 cells (~1x10^5 cells). Error bars represent standard error of the mean. Statistical unpaired t-test was completed between negative pGEM dsRNA control and specific *I. scapularis* dsRNA for gene of interest. Asterisks denote: *p value≤0.05, **p value≤0.01, and ***p value≤0.001. Five technical replicates for each treatment were performed within a biological replicate. One biological replicate shown; two biological replicates were completed.
Table 6. Summary of transcript knockdown effect of genes of interest on LGTV infection in ISE6 cells.

<table>
<thead>
<tr>
<th>Selected tick protein</th>
<th><em>Ixodes scapularis</em> gene model accession ID</th>
<th>Function</th>
<th>Human (H. sapien) Ortholog AA % Conservation</th>
<th>Transcript knockdown effect on LGTV negative strand genome replication</th>
<th>Transcript knockdown effect on infectious LGTV replication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumarylacetoacetase (Fumar)$^\Delta$; K01555</td>
<td>ISCW020196</td>
<td>Amino acid metabolism</td>
<td>65.9%</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Endoplasmic reticulum protein 29 (ERP29); K09586</td>
<td>ISCW018425</td>
<td>Folding, sorting, &amp; degradation</td>
<td>17%</td>
<td>No change</td>
<td>Decreased</td>
</tr>
<tr>
<td>1-pyrroline-5-carboxylate dehydrogenase (DPCD); K00294</td>
<td>ISCW015982</td>
<td>Amino acid metabolism</td>
<td>56.7%</td>
<td>No change</td>
<td>Decreased</td>
</tr>
<tr>
<td>Pantetheine hydrolase (CNHydro); K08069</td>
<td>ISCW004822</td>
<td>Metabolism of cofactors &amp; vitamins</td>
<td>33.9%</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Malate dehydrogenase (MDH2)$^\Delta$; K00026</td>
<td>ISCW003528</td>
<td>Carbohydrate metabolism</td>
<td>68.9%</td>
<td>No change</td>
<td>Decreased</td>
</tr>
<tr>
<td>Poly [ADP-ribose] polymerase (PARP)$^\Delta$; K10798</td>
<td>ISCW019519</td>
<td>Replication &amp; repair</td>
<td>48.6%</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>UMP-CMP kinase (CUKinase)$^\Delta$; K13800</td>
<td>ISCW012446</td>
<td>Nucleotide metabolism</td>
<td>40.2%</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Acetyl-CoA C-acetyltransferase (ACAT1); K00626</td>
<td>ISCW016117</td>
<td>Carbohydrate, lipid, amino acid, terpenoids/poly keytides metabolism</td>
<td>60.2%</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Hypothetical protein (Hypo195)$^\Delta$</td>
<td>ISCW011195</td>
<td>Unknown</td>
<td>--</td>
<td>No change</td>
<td>Decreased</td>
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<tr>
<td>Hypothetical protein (Hypo576)</td>
<td>ISCW020576</td>
<td>Unknown</td>
<td>--</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

$^\Delta$ denotes protein identified in *I. scapularis* synganglia [91]

$^\Delta$ denotes Fumar, PARP, and CUKinase transcript knockdowns during LGTV infection in ISE6 cells did not decrease LGTV replication
Fig 18. Predicted cellular locations of selected proteins in ISE6 cells. Selected proteins are shown in cellular locations based off knowledge of orthologous protein locations. Black circles denote ribosomes. Blue lines denote positive strand, LGTV RNA genomes. Orange lines denote negative strand, LGTV RNA genomes used as a template for further positive strand, LGTV RNA genome production. Red circles denote viral proteins in association with replication complexes on/in the ER. Purple particles/virions denote immature LGTV before prM-furin cleavage. Green circles denote furin, which cleaves pr from prM, creating mature LGTV. Different colored proteins denote different expression levels following LGTV infection and UV-LGTV treatment.
Fig 19. Predicted effects of genes of interest transcript knockdown on LGTV life cycle steps. Different colored proteins denote different expression levels following LGTV infection and UV-LGTV treatment.
REFERENCES
REFERENCES


Research Support, U.S. Gov't, Non-P.H.S.


VITA
Jeffrey Michael Grabowski  
(260) 409-6435  
jgrabows@purdue.edu  
(Curriculum Vitae)

Professional Preparation:  
PURDUE UNIVERSITY, Interdisciplinary Life Science Ph.D (PULSe)  
(Expected) August 2015  
(Dept Entomology and Biological Sciences collaboration; Microbiology concentration)

Advisors: Drs. Catherine A. Hill and Richard J. Kuhn  
Thesis Topic: Tick-flavivirus interactions: host proteins affected by virus infection

MANCHESTER UNIVERSITY (as of 2012), Cum Laude, B.S. in Biology  
May 2009  
Cum. GPA: 3.71 / 4.00      Minor in Chemistry  
Undergraduate Thesis: Monitoring Black-legged ticks (*Ixodes scapularis*), American Dog ticks (*Dermacentor variabilis*) and *Borrelia burgdorferi* in southern Kosciusko county, IN  
Advisors: Drs. David P. Kreps (retired) and Jerry E. Sweeten

Appointments:  
NIH Intramural Research Postdoctoral Training Award (IRTA) Scientist  
2015-ongoing  
NIAID/NIH Rocky Mountain Laboratories, Hamilton, MT. Primary Investigator: Dr. Marshall E. Bloom.  
• Biology of vector-borne viruses section

NSF Graduate Research Fellow/ICTSI Predoctoral Trainee (Ph.D graduate student)  
2010-2015  
Research profiles: [http://www.gradschool.purdue.edu/oigp/students/PULSe/profile10.cfm](http://www.gradschool.purdue.edu/oigp/students/PULSe/profile10.cfm)  
[http://www.gradschool.purdue.edu/pulse/profiles/profile12.cfm](http://www.gradschool.purdue.edu/pulse/profiles/profile12.cfm)  
[http://bilbo.bio.purdue.edu/~viruswww/Kuhn_home/research.php#Jeff](http://bilbo.bio.purdue.edu/~viruswww/Kuhn_home/research.php#Jeff)

Purdue University, West Lafayette, IN. Advisors: Drs. Catherine Hill and Richard Kuhn.  
• Flavivirus host cell factor analyses  
  o Mass spectroscopy proteomics  
  o Functional analyses  
  o Molecular Virology
Graduate Assistant (Ph.D graduate student)
2009-2010
Purdue University, West Lafayette, IN. Advisors: Drs. Catherine Hill, Arun Bhunia, Richard Kuhn, and Doug LaCount.

- Bioinformatics of tick heme biosynthesis proteins
- Cloning expressions of LAP protein in Listeria monocytogenes
- Mass spectroscopy lipidomics of virus-infected cells
- Wheat Germ extract analysis of P. falciparum-host proteins

Lyme disease Student Research Scientist (undergraduate student)
2008-2009
Faculty-Student Research, Manchester College, N. Manchester, IN. Monitoring Black-legged ticks (Ixodes scapularis), American Dog ticks (Dermacentor variabilis) and Borrelia burgdorferi in southern Kosciusko county, IN. Collaborators: Dr. David P. Kreps.

- Discovered and designed the study
- Obtained project funding
- Ecological field methods to look at Lyme disease dynamics
- Fluorescent microscopy
- Supervised undergraduates in research study

Lyme Disease REU Student Researcher (undergraduate student)
2007
“Ecology in Context” REU Program, Cary Institute of Ecosystem Studies (CIES), Millbrook, NY. Invasive earthworm species effects on the density and microscale habitat of Ixodes scapularis nymphs in Dutchess County, NY. Advisors: Drs. Mary Killilea and Rick Ostfeld.

- NSF funded through CIES undergraduate grant
- Ecological field methods to look at Lyme disease dynamics
- Fluorescent Microscopy

Awards and Honors:
* PULSe Travel Grant
  2015
* Health and Disease: Science, Culture and Policy 2nd place graduate student poster
  2015
* PULSe Travel Grant
  2014
* ASV Annual Meeting Student Travel Grant Award
  2014
* Entomological Society of America Monsanto Research Grant Award
  2013
*Purdue Interdisciplinary Graduate Program Presentation Excellence Award 2013
- http://www.gradschool.purdue.edu/oigp/calendar/winners.cfm
*Indiana CTSI Annual Meeting Outstanding Poster Presentation 2012
*Purdue Interdisciplinary Graduate Program Most Outstanding Project Award 2012
*Purdue College of Agriculture (Interdisciplinary) TEAM Award 2012
*ASV Annual Meeting Student Travel Grant Award 2012
*ESA North Central Branch Presidential Student Travel Scholarship Award 2012
*Entomological Society of America NCB 3rd place Ph.D graduate student poster 2011
*PULSe 1st Year Poster Competition-Runners Up 2010
*Manchester College Director’s Award 2009
*HCAC Academic All-Conference Athlete 2007-2009
*Manchester College Dean’s List Recipient 2005-2009
*Chi Alpha Sigma ( XA∑ ) National College Athlete Honor Society 2008

Funding History:
*PULSe Travel Grant 2015
*PULSe Travel Grant 2014
*ASV Annual Meeting Student Travel Grant Award 2014
*Entomological Society of America Monsanto Research Grant Award 2013
- Proposal Title: “Identifying novel targets for pest control: elucidating the function of orphan proteins of the Acari (ticks and mites)”
- funding for cell culture and gene knockdown experiments
*Author/coauthor of Indiana CTSI Core Pilot Grant- funded to PI Richard J Kuhn 2013-2015
- Proposal Title: “Comprehensive proteomic analysis of the Ixodes scapularis disease vector during tick-borne flavivirus infection”
- NIH/NCATS- Indiana Clinical and Translational Sciences Institute – #UL1 TR000006
*Indiana CTSI Predoctoral Training Award Program
2012-2014
- Proposal Title: “Tick-borne Langat flavivirus growth and host proteome change following infection: a novel approach to discover host proteins for viral-therapeutic development”
- NIH/NCATS- Indiana Clinical and Translational Sciences Institute – TL1 Program (A. Shekhar, PI), 5/01/08-04/30/13

*ASV Annual Meeting Student Travel Grant Award
2012
*ESA North Central Branch Presidential Student Travel Scholarship Award
2012
*NSF Graduate Research Fellowship Program (DGE 1333468)
2010-2015
- Proposal Title: “Molecular basis of tick-virus infection: novel approaches for flavivirus control”
*Purdue Graduate School Incentive Grant
2010
*Manchester College Faculty-Student Summer Research Grant
2008
*National Science Foundation REU Summer Research Funding (DBI 0552871)
2007
- Website: http://nsf.gov/awardsearch/showAward?AWD_ID=0552871

*Lilly Endowment Inc. Student Grant
2006

Publications (in prep and current):
*Jeffrey M. Grabowski, Monika Gulia-Nuss, Michael F. Oats, Rushika Perera, Richard J. Kuhn, and Catherine A. Hill. Functional knockdown of Ixodes scapularis ISE6 cell metabolic and protein processing genes reduce tick-borne Langat virus infection. In Prep For Insect Biochemistry and Molecular Biology.


*Jeffrey M. Grabowski, Rushika Perera, Ali M. Roumani, Victoria Hedricks, Dorota Inerowicz, Catherine A. Hill, and Richard J. Kuhn. 2015. Global protein changes in


Abstract Publications:

Arthropod Vector: The Controller of Transmission. Sagebrush Inn & Suites, Taos, NM. (poster presentation)


*Grabowski, J, Perera, R, Hill, C, and Kuhn, R. 2012. Properties of tick-borne Langat virus during growth in *Ixodes scapularis* ISE6 cells and host proteome change following infection. Purdue Chapter Sigma Xi Poster Award Competition. Purdue University. (program-published abstract-Poster)


Research Advisor of Lab Personnel:

*Purdue Department of Biological Sciences Rotation Student- Michael F. Oats

2014
Synergistic Activities:
* Coalition for Clinical and Translational Science Capitol Hill Advocacy Day – Indiana Team Member, Washington D.C. 2014


*Bioinformatics Resource for Invertebrate Vectors of Human Pathogens (VectorBase) User Advisor
Website: https://www.vectorbase.org/sug
2014-current

*Influenza Research Database (IRD)/Virus Pathogen Resource (ViPR) User Advisor
Website: http://www.viprbrc.org/brc/staticContent.do?decorator=vipr&type=ViprInfo&subtype=ScienceSupport
2012-current

*Teacher Assistant, ENTM 525: Medical and Veterinary Entomology spring 2013
  - Dr. Catherine A Hill, Purdue University, ENTM Department

*Personal Invitations
  - NIAID/NIH Rocky Mountain Laboratories, Laboratory of Virology Seminar
    - Host: Marshall E. Bloom
    - Seminar Title: The proteomics of tick-flavivirus interactions: studies of the Ixodes scapularis-Langat virus system March 2015
  - Office of Interdisciplinary Graduate Programs Most Outstanding Interdisciplinary Program Award (MOIPA) Reviewer 2014-2015
  - Student presentation at Society for Vector Ecology Meeting (kindly declined) 2014
  - PULSe 1st Year Poster Competition 2014
    - Judge
  - Purdue Entomology Department Booth Exhibitor 2013
    - Entomological Society of America Meeting
  - Office of Interdisciplinary Graduate Programs Student Resource Panel Member 2013
    - Discussion Panelist
  - PULSe Seminar Series Talk (October 15) 2012
  - PULSe “NSF Review Workshop” 2012-2013
    - Co-Host Speaker
  - PULSe Preliminary Exam Panel Member 2012
    - Discussion Panelist
- Purdue Department of Entomology “Survival Skills Workshop”: Applying for NSF 2011
  - Host Speaker
- PULSe 1st Year Poster Competition 2011
  - Judge
- NSF Peer Mentoring-Graduate Fellow Panel Member, Purdue Graduate School 2010-2014
  - Discussion Panelist
  - Provided successful application for public view
  - Liaison for students regarding NSF GRFP questions
- *PGSG Senator 2012-2013
- Advancement Committee
*PULSe Graduate Student Organization (GSO)
- Vice President 2011-2012
- Activities Coordinator 2010-2011
*Purdue SURF Symposium Poster Presentation Judge 2011
*Poster Presentation Title: New tick protein targets to control transmission of tick-borne flaviviruses
  - Interdisciplinary Graduate Program Reception, Purdue University, IN. 2015
  - Health and Disease: Science, Culture and Policy Poster Session, Purdue University, IN. 2015
*Poster Presentation Title: Host proteome and energy/glutaminolysis enzyme change with Langat infection
  - Interdisciplinary Graduate Program Reception, Purdue University, IN. 2014
  - PULSe Welcome Reception and Poster Competition, Purdue University, IN. 2014
*Oral Presentation Title: Host proteome and energy/glutaminolysis enzyme change with Langat infection
  - Annual ASV Meeting: Flavivirus I Section, Fort Collins, CO 2014
  - Rossmann/Kuhn Seminar, West Lafayette, IN 2014
  - ICTSI Spring Reception, West Lafayette, IN 2014
*Oral Presentation Title: Tick-flavivirus analysis and host proteomic change following infection
  - Infectious Disease Research in Progress Seminar: Purdue University, IN 2013
  - ICTSI Presentation Day: Purdue University, IN 2013
  - PULSe Seminar Series Talk: Purdue University, IN 2012
  - Annual ASV Meeting: Flavivirus I Section, Madison, WI. 2012
  - Entomological Society of America NCB Meeting, University of Nebraska, NE. 2012
  - Annual IAS Meeting: Microbio & Mol Bio Section, Purdue University, IN. 2012
  - Entomology Methods Seminar: Purdue University, IN 2012
*Poster Presentation Title: LGTV-ISE6 proteomic screen-Langat virus growth in ISE6 cells and host proteomic change
- ACTS Translational Science Meeting: T0-Basic Scientific Discovery Section, Omni Shoreham Hotel, Washington D.C. 2014
- Entomological Society of America: Austin Convention Center, Austin, TX 2013
- Annual Indiana CTSI Meeting: IUPUI, Indianapolis, IN 2013
- Arthropod Genomics Symposium, University of Notre Dame, IN 2013
- CTSA National Predoctoral Programs Meeting, Mayo Clinic, Rochester, MN 2013
- Interdisciplinary Graduate Program Reception, Purdue University, IN. 2013
- Annual Indiana CTSI Meeting: IUPUI, Indianapolis, IN 2012
- Interdisciplinary Graduate Program Reception, Purdue University, IN. 2012
- Purdue Chapter Sigma Xi Poster Award Competition, Purdue University, IN. 2012
- Biological Sciences Retreat, Swan Lake Resort, Plymouth, IN 2011

*Poster Presentation Title: Langat virus growth in ISE6 cells
- Interdisciplinary Graduate Program Reception, Purdue University, IN. 2011
- Entomological Society of America NCB Meeting, University of Minnesota, MN. 2011

- Purdue Chapter Sigma Xi Poster Award Competition, Purdue University, IN. 2010

*Purdue University Graduate School Exam Proctor 2009-2010

*Oral Presentation Title: Lyme Disease Dynamics of Southern Kosciusko county, IN
- Manchester College Student Research Symposium, N. Manchester, IN. 2009
- Manchester College Science Seminar, N. Manchester, IN. 2008

*Video:
http://users.manchester.edu/Facstaff/CWatson/Seminar/0809/Sept22/Sept22.htm

*Oral Presentation Title: Invasive Earthworms and Nymphal Tick Interaction Dynamics
- Annual IAS Conference: Ecology Section, Indianapolis, IN. 2007
- CIES REU Undergraduate Symposium, Millbrook, NY. 2007
- Manchester College Science Seminar, N. Manchester, IN. 2007

Community/Volunteer Activities:
*Purdue Spring Fest, “Magic Milk,” Purdue University, IN. 2012
Educate students and families on the nature of lipids in milk and how to conduct simple science.

*Purdue zipTrips Speaker, “Disease Detectives” 7th grade episode. 2009-2011

+Association for Communication Excellence in Agriculture, Natural Resources, and Life and Human Sciences Silver Award for the 2011 ACE Critique and Awards Program in the category of Electronic Media, Video: Class 30 programs
+International, world-wide viewed PBS-broadcasted program to encourage interest in STEM sciences
+Recipient of Purdue 2012 College of Agriculture TEAM Award
+2010 show: video.dis.purdue.edu/bns/Agriculture/zipTrips_101118.wmv

*PULSe Science Night Speaker, “DNA Extraction,” Sunnyside Middle School, IN. 2011
Educate elementary students and families on the nature of DNA and how to conduct simple science.

*PULSe Science Night Speaker, “Fun with Microscopy,” Dayton Elementary School, IN. 2011
Educate elementary students and parents on the science of microscopy and microorganisms.
*Big Ten Graduate Expo, Purdue Graduate School.  2010
Graduate student panel discussion for undergraduate students
*“Sharing Science,” Cary Institute of Ecosystem Studies.       2007
Science-based workshop for underprivileged New York City High school students.

Professional Society Student Membership:
*American Society for Virology (ASV)  2012-present
*American Society of Tropical Medicine and Hygiene (ASTMH)  2011-present
*Entomological Society of America  2011-present
*American Association for the Advancement of Science (AAAS)  2011-present
*Association for Clinical & Translational Science
*Indiana Academy of Sciences (IAS)  2012
*Wildlife Disease Association  2011

Workshops Attended:
*VectorBase Workshop, University of Notre Dame, IN  2013

*Virus Pathogen Resource (ViPR) workshop, Purdue University, IN  2011
Resource link: http://www.viprbrc.org/brc/home.do?decorator=vipr
*Annotating a genome sequence: gene models workshop, Purdue University, IN  2011
Resource link: http://academy.nescent.org/wiki/Next-gen_sequencing

Semester Lectures Attended:
*CHEM 69600: Adv Proteomics, Metabolomics, and Bioinformatics, Purdue University, IN  spring 2011-2012
*BIOL 41600: Viruses and Viral Disease, Purdue University, IN  spring 2011-2012
  spring 2012-2013

Animal Care and Use Training
*Mouse and Rat Handling and Restraint           5/28/2013
*Mouse and Rat Basic Injection Techniques (SC, IP, IM)  6/6/2013
*Mouse and Rat Gas Anesthesia           6/25/2013
*Mouse Euthanasia Techniques, Dissection, and Organ Identification  8/5 and 9/19/13
PUBLICATIONS
PLOS Neglected Tropical Diseases
Changes in the proteome of Langat-infected Ixodes scapularis ISE6 cells: metabolic pathways associated with flavivirus infection
--Manuscript Draft--

Manuscript Number:

Full Title: Changes in the proteome of Langat-infected Ixodes scapularis ISE6 cells: metabolic pathways associated with flavivirus infection

Short Title: Tick proteins associated with flavivirus infection

Article Type: Research Article

Keywords: Ixodes scapularis; tick; flavivirus; Langat virus; proteins; proteomics; metabolism; carbohydrate metabolism; amino acid metabolism; gene regulation

Corresponding Author: Richard J. Kuhn, PhD
Purdue University
West Lafayette, IN UNITED STATES

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Corresponding Author’s Institution: Purdue University

Corresponding Author’s Secondary Institution:

First Author: Jeffrey M Grabowski

First Author Secondary Information:

Order of Authors: Jeffrey M Grabowski
Rushika Perera
Ali M Roumani
Victoria E Hedrick
Halina D Inerowicz
Catherine A Hill
Richard J. Kuhn, PhD

Order of Authors Secondary Information:

Abstract:

Background
Ticks (Family Ixodidae) transmit a variety of disease causing agents to humans and animals. The tick-borne flaviviruses (TBFs; family Flaviviridae) are a complex of viruses, many of which cause encephalitis and hemorrhagic fever, and represent global threats to human health and biosecurity. Pathogenesis has been well studied in human and animal disease models. Equivalent analyses of tick-flavivirus interactions are limited and represent an area of study that could reveal novel approaches for TBF control.

Methodology/Principal Findings

High resolution LC-MS/MS was used to analyze the proteome of Ixodes scapularis (Lyme disease tick) embryonic ISE6 cells following infection with Langat virus (LGTV) and identify proteins associated with viral infection and replication. Maximal LGTV infection of cells and determination of peak release of infectious virus, was observed at 36 hours post infection (hpi). Proteins were extracted from ISE6 cells treated with LGTV and non-infectious (UV inactivated) LGTV at 36 hpi and analyzed by mass spectrometry. The Omics Discovery Pipeline (ODP) identified thousands of MS peaks. Protein homology searches against the I. scapularis IscaW1 genome assembly identified a total of 486 proteins that were subsequently assigned to putative functional pathways using searches against the Kyoto Encyclopedia of Genes and Genomes.
(KEGG) database. 266 proteins were differentially expressed following LGTV infection relative to non-infected (mock) cells. Of these, 68 proteins exhibited increased expression and 198 proteins had decreased expression. The majority of the former were classified in the KEGG pathways: "translation", "amino acid metabolism", and "protein folding/sorting/degradation". Finally, Trichostatin A and Oligomycin A increased and decreased LGTV replication in vitro in ISE6 cells, respectively.

Conclusions/Significance

Proteomic analyses revealed ISE6 proteins that were differentially expressed at the peak of LGTV replication. Proteins with increased expression following infection were associated with cellular metabolic pathways and glutaminolysis. In vitro assays using small molecules implicate malate dehydrogenase (MDH2), the citrate cycle, cellular acetylation, and electron transport chain processes in viral replication. Proteins were identified that may be required for TBF infection of ISE6 cells. These proteins are candidates for functional studies and targets for the development of transmission-blocking vaccines and drugs.

Suggested Reviewers:

Ulrike Munderloh
University of Minnesota
munde001@umn.edu
Dr. Ulrike Munderloh focuses her research on ticks and tick-borne pathogens. She utilizes both tick and tick cell systems to explore tick-borne pathogen infection.

Kenneth E Olson
Colorado State University
Kenneth.Olson@colostate.edu
Dr. Kenneth E. Olson focuses on arthropod-borne virus pathogenesis both within arthropod and mammalian systems.

Felix D Guerrero
USDA Agricultural Research Service
felix.guerrero@ars.usda.gov
Dr. Felix D. Guerrero has completed proteomic analyses with the southern cattle tick system. He also is involved with anti-tick vaccine development projects.

Scott F Michael
Florida Gulf Coast University
smichael@gcu.edu
Is a molecular virologist who focuses on vaccine development and methods of control against arthropod-borne flaviviruses.

Opposed Reviewers:

Additional Information:

Question

Data Availability

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available, without restriction and from the time of publication, with only rare exceptions to address legal and ethical concerns (see the PLOS Data Policy and FAQ for further details). When submitting a manuscript, authors must provide a Data Availability Statement that describes where the data underlying their manuscript can be found.

Your answers to the following constitute your statement about data availability and will be included with the article in the event of publication. Please note that simply stating ‘data available on request’
from the author' is not acceptable. If, however, your data are only available upon request from the author(s), you must answer "No" to the first question below, and explain your exceptional situation in the text box provided.

Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?

<table>
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<th>Please describe where your data may be found, writing in full sentences. Your answers should be entered into the box below and will be published in the form you provide them, if your manuscript is accepted. If you are copying our sample text below, please ensure you replace any instances of XXX with the appropriate details.</th>
<th>All relevant data are within the paper and its Supporting Information files. All files are available from VectorBase (<a href="https://www.vectorbase.org/">https://www.vectorbase.org/</a>).</th>
</tr>
</thead>
</table>

If your data are all contained within the paper and/or Supporting Information files, please state this in your answer below. For example, “All relevant data are within the paper and its Supporting Information files.”

If your data are held or will be held in a public repository, include URLs, accession numbers or DOIs. For example, “All XXX files are available from the XXX database (accession number(s) XXX, XXX).” If this information will only be available after acceptance, please indicate this by ticking the box below. If neither of these applies but you are able to provide details of access elsewhere, with or without limitations, please do so in the box below. For example:

“Data are available from the XXX Institutional Data Access / Ethics Committee for researchers who meet the criteria for access to confidential data.”

“Data are from the XXX study whose authors may be contacted at XXX.”

* typeset

Additional data availability information:
Professor Serap Aksoy
Editor-in-Chief
PLoS Neglected Tropical Diseases

May 21, 2015

Dear Serap,

RE: PLoS Neglected Tropical Diseases Submission, Grabowski et al.

I am pleased to submit the manuscript of Grabowski et al. entitled “Changes in the proteome of Langat-infected *Ixodes scapularis* ISE6 cells: metabolic pathways associated with flavivirus infection”, for consideration for publication in PLoS Neglected Tropical Diseases (PLoS NTDs). The manuscript describes a proteomics study to analyze tick-flavivirus interactions. We assessed changes in the proteome of the *Ixodes scapularis* (black legged tick) ISE6 cell line following infection with Langat virus (LGTV; Family Flaviviridae). We identified 486 proteins in total, and proteins that exhibited differential expression following infection. We present data from bioinformatic and *in vitro* studies that suggest virus-induced perturbation of metabolic pathways in the tick cell following infection. From our proteomics screen, we identified proteins and cellular pathways that may be required for TBF infection. These proteins may be targets for the development of anti-tick vaccines and novel therapeutics to disrupt transmission of TBFs.

Co-author, Dr. Catherine Hill was in contact with your office via email on 14 April 2015 as we are keen to have our submission considered as a companion article to a manuscript describing the *I. scapularis* genome (Gulia-Nuss et al., “Genomic clues to a unique parasitic lifestyle in the Lyme disease tick, *Ixodes scapularis*”) submitted today to Nature Communications. Our study provides invaluable protein data to support the annotation and interrogation of the *I. scapularis* genome, the first for a tick, and has been cross-referenced in Gulia-Nuss et al. Please contact Dr. Hill (hillca@purdue.edu) for further information regarding the *I. scapularis* genome manuscript. Dr. Hill is the coordinator of the International *Ixodes scapularis* Genome Consortium and can provide a copy of Gulia-Nuss et al. at the request of Editorial staff.

Our manuscript is an original contribution that we feel meets the scope of PLoS NTDs. Our study is focused on a complex of viruses that impact human health in many parts of the world and have been largely neglected in research, and new approaches to control, two areas of emphasis for the journal. We seek a venue for our manuscript that will reach an audience of scientists broadly interested in neglected vector-borne infectious diseases.

I confirm that all co-authors have read and approved this manuscript for submission. Thank you in advance for your consideration of our work. Please do not hesitate to contact us should you require additional information. We look forward to your favorable response.

Sincerely,

Richard J. Kuhn
Professor and Head, Department of Biological Sciences
Gerald & Edna Mann Director, Bindley Bioscience Center

LILLY HALL OF LIFE SCIENCES • 915 WEST STATE STREET • WEST LAFAYETTE, IN 47907-2054
TELEPHONE: (765) 494-4408 • FAX: (765) 494-8876 • http://www.biology.purdue.edu
Changes in the proteome of Langat-infected *Ixodes scapularis* ISE6 cells: metabolic pathways associated with flavivirus infection

Jeffrey M. Grabowski\(^1,2\), Rushika Perera\(^2\), Ali M. Roumani\(^3\), Victoria E. Hedrick\(^3\), Halina D. Inerowicz\(^3\), Catherine A. Hill\(^1\), Richard J. Kuhn\(^2,3\)*

\(^1\)Department of Entomology, College of Agriculture, Purdue University, West Lafayette, IN 47907,
\(^2\)Markey Center for Structural Biology, Department of Biological Sciences, College of Science, Purdue University, West Lafayette, IN 47907,
\(^3\)Bindley Bioscience Center, Purdue University, West Lafayette, IN 47907

Current addresses:

\(^\gamma\)Department of Microbiology, Immunology, and Pathology, Arthropod-borne & Infectious Diseases Laboratory, Colorado State University, Fort Collins, CO 80523-1692, USA
\(^\delta\)Department of Computer Science, College of Arts & Sciences, Gulf University for Science & Technology, Mishref, Kuwait 32093
\(^\theta\)Office of Indiana State Chemist, Purdue University, West Lafayette, IN 47907

*Correspondence to:
Richard J. Kuhn
Markey Center for Structural Biology
Department of Biological Sciences, Purdue University
915 West State Street, West Lafayette, IN 47907
Email: kuhnr@purdue.edu
ABSTRACT

Background

Ticks (Family Ixodidae) transmit a variety of disease-causing agents to humans and animals. The tick-borne flaviviruses (TBFs; family Flaviviridae) are a complex of viruses, many of which cause encephalitis and hemorrhagic fever, and represent global threats to human health and biosecurity. Pathogenesis has been well studied in human and animal disease models. Equivalent analyses of tick-flavivirus interactions are limited and represent an area of study that could reveal novel approaches for TBF control.

Methodology/Principal Findings

High resolution LC-MS/MS was used to analyze the proteome of *Ixodes scapularis* (Lyme disease tick) embryonic ISE6 cells following infection with Langat virus (LGTV) and identify proteins associated with viral infection and replication. Maximal LGTV infection of cells and determination of peak release of infectious virus, was observed at 36 hours post infection (hpi). Proteins were extracted from ISE6 cells treated with LGTV and non-infectious (UV inactivated) LGTV at 36 hpi and analyzed by mass spectrometry. The Omics Discovery Pipeline (ODP) identified thousands of MS peaks. Protein homology searches against the *I. scapularis* IscaW1 genome assembly identified a total of 486 proteins that were subsequently assigned to putative functional pathways using searches against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. 266 proteins were differentially expressed following LGTV infection relative to non-infected (mock) cells. Of these, 68 proteins exhibited increased expression and 198 proteins had decreased expression. The majority of the former were classified in the KEGG pathways: "translation", "amino acid metabolism", and "protein folding/sorting/degradation". Finally,
Trichostatin A and Oligomycin A increased and decreased LGTV replication in vitro in ISE6 cells, respectively.

Conclusions/Significance

Proteomic analyses revealed ISE6 proteins that were differentially expressed at the peak of LGTV replication. Proteins with increased expression following infection were associated with cellular metabolic pathways and glutaminolysis. In vitro assays using small molecules implicate malate dehydrogenase (MDH2), the citrate cycle, cellular acetylation, and electron transport chain processes in viral replication. Proteins were identified that may be required for TBF infection of ISE6 cells. These proteins are candidates for functional studies and targets for the development of transmission-blocking vaccines and drugs.
AUTHOR SUMMARY

High-throughput proteomics offers an approach to evaluate changes in cell protein levels following arboviral infection. Research to understand the molecular basis of human-flavivirus interactions has advanced significantly over the past decade, but comparatively little is known regarding interactions between ticks and tick-borne flaviviruses (TBFs). Here, we employed a proteomics approach using an *I. scapularis* ISE6 cell line infected with the TBF Langat virus (LGTV) to identify proteins and biochemical pathways affected by viral infection. An LC-MS/MS approach was used to identify proteins that were subsequently assigned to putative cellular pathways based on orthology to proteins in the KEGG database. Biochemical pathways common among arthropods in response to infection with flavivirus and possibly unique to tick-flavivirus interactions, were identified. *In vitro* cellular assays using small molecules suggest the involvement of the ISE6 proteins, malate dehydrogenase (MDH2), and mitochondria in viral replication. These analyses provide a basis for further studies to identify tick proteins associated with viral replication that could be targeted to disrupt TBF transmission.
Characterization of the Proteome of Cytoplasmic Lipid Droplets in Mouse Enterocytes after a Dietary Fat Challenge

Theresa D’Aquilla1, Devika Sirohi2,3, Jeffrey M. Grabowski2,4, Victoria E. Hedrick2, Lake N. Paul3, Andrew S. Greenberg6, Richard J. Kuhn2,3, Kimberly K. Buhman1*

1 Department of Nutrition Science, Purdue University, West Lafayette, Indiana, United States of America, 2 Department of Biological Sciences, Purdue University, West Lafayette, Indiana, United States of America, 3 Bindley Bioscience Center, Purdue University, West Lafayette, Indiana, United States of America, 4 Department of Entomology, Purdue University, West Lafayette, Indiana, United States of America, 5 Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, Massachusetts, United States of America

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Abstract

Dietary fat absorption by the small intestine is a multistep process that regulates the uptake and delivery of essential nutrients and energy. One step of this process is the temporary storage of dietary fat in cytoplasmic lipid droplets (CLDs). The storage and mobilization of dietary fat is thought to be regulated by proteins that associate with the CLD; however, mechanistic details of this process are currently unknown. In this study we analyzed the proteome of CLDs isolated from enterocytes harvested from the small intestine of mice following a dietary fat challenge. In this analysis we identified 181 proteins associated with the CLD fraction, of which 37 are associated with known lipid related metabolic pathways. We confirmed the localization of several of these proteins on or around the CLD through confocal and electron microscopy, including perilipin 3, apolipoprotein A-IV, and acyl-CoA synthetase long-chain family member 5. The identification of the enterocyte CLD proteome provides new insight into potential regulators of CLD metabolism and the process of dietary fat absorption.

Introduction

Dietary fat is the most energy dense macronutrient consumed and is required for the absorption of essential fatty acids and other lipophilic nutrients including fat soluble vitamins. However, when present in excess, dietary fat increases the risk for chronic diseases such as cardiovascular disease and obesity [1–4]. Therefore, understanding the regulators of dietary fat absorption and metabolism is important for both the promotion of health and prevention of disease.

Dietary fat absorption by the small intestine is a multistep process. Triacylglycerol (TAG) is hydrolyzed by pancreatic lipase in the intestinal lumen producing monoaoylglycerol and free fatty acids. These digestive products are taken up by the absorptive cells of the intestine,