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Signal transduction and Rickettsial infection of tick cells

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SIGNAL TRANSDUCTION AND RICKETTSIAL INFECTION OF TICK CELLS

A Dissertation

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ABSTRACT

Spotted fever group (SFG) *Rickettsia* are obligate intracellular bacteria that are carried by ticks. One such tick, *Dermacentor variabilis* is a vector for the etiologic agent of Rocky Mountain spotted fever, *R. rickettsii*. These ticks also carry a non-pathogenic *R. montanensis*, the agent used in this study. Interestingly, field data collected from infected *D. variabilis* throughout the United States revealed that the majority of *Rickettsia* in ticks are non-pathogenic species such as *R. montanensis*. Although ticks serve as both vector and reservoir hosts for SFG *Rickettsia*, many questions regarding tick-*Rickettsia* interaction remain unresolved. Therefore, the overall goal of this research was to study the relationship between ticks and *Rickettsia*, specifically examining the molecular mechanisms of rickettsial infection of tick host. As SFG *Rickettsia* can move between vertebrate and invertebrate hosts, the hypothesis is that conserved mechanisms are utilized for invasion of both types of host cell. Biochemical inhibition assays revealed that the tick molecules, PI 3-kinase, protein tyrosine kinases, Src, FAK, Rho GTPase Rac1, N-WASP, Arp2/3 complex, actin, and V-ATPase are important for *R. montanensis* invasion. Further studies were executed to molecularly and functionally characterize the tick molecules, Arp2/3 complex and V-ATPase, which are central to rickettsial internalization. Full length cDNA of Arp2/3 complex subunits and V-ATPase from *D. variabilis* were isolated. Transcriptional profiles of Arp2/3 complex subunits and V-ATPase showed greater expression of the mRNA in tick ovaries compared to midgut and salivary glands. In addition, to gain insight into rickettsial invasion in nature, Arp2/3 complex inhibition assays were performed in tick tissues. The results demonstrated the involvement of Arp2/3 complex in rickettsial entry into midgut, ovary, and salivary glands. The tick molecules identified in this study may provide novel points of intervention in the transmission of tick-borne rickettsial diseases.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 General overview of tick-borne rickettsial disease: Rocky Mountain spotted fever

It has been more than a hundred years since Howard Taylor Ricketts discovered the etiologic agent of the prototypical tick-borne rickettsial disease (TBRD) (Gross and Schäfer, 2011), Rocky Mountain spotted fever (RMSF); however, rickettsial diseases are still prevalent throughout the world. TBRDs are zoonoses in which the epidemiology of the diseases are limited by the geographic ranges and seasonal activities of the vectors and reservoirs (Schriefer et al., 1994; Azad and Beard, 1998); human behaviors that may increase the risk for tick exposure and subsequent infection also influence the epidemiologic characteristics of TBRDs (Comer et al., 2001; Chapman et al., 2006). In the United States, the most common TBRDs include RMSF, human monocytotropic ehrlichiosis (HME), human granulocytic anaplasmosis (HGA) and *Ehrlichia ewingii* infection, which are caused by *Rickettsia rickettsii*, *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*, respectively. During the past decade, the prevalence of TBRD has increased in North America, South America and Europe (Figure 1.1) (Dumler, 2010), and cases attributed to RMSF have increased by more than 300 % (Hall-Baker et al., 2010).

1.1.1 Epidemiology

Although Maxey first reported RMSF in medical literature in 1899 (Ricketts, 1991), surveillance data has only been collected since 1920 (Hattwick, 1971). During the past ten years, the annual incidence of RMSF has dramatically increased (Hall-Baker et al., 2010). In 2008, annual documented cases of RMSF exceeded that of any other year and the disease was distributed throughout most of the United States (Figure 1.2) (www.cdc.gov).

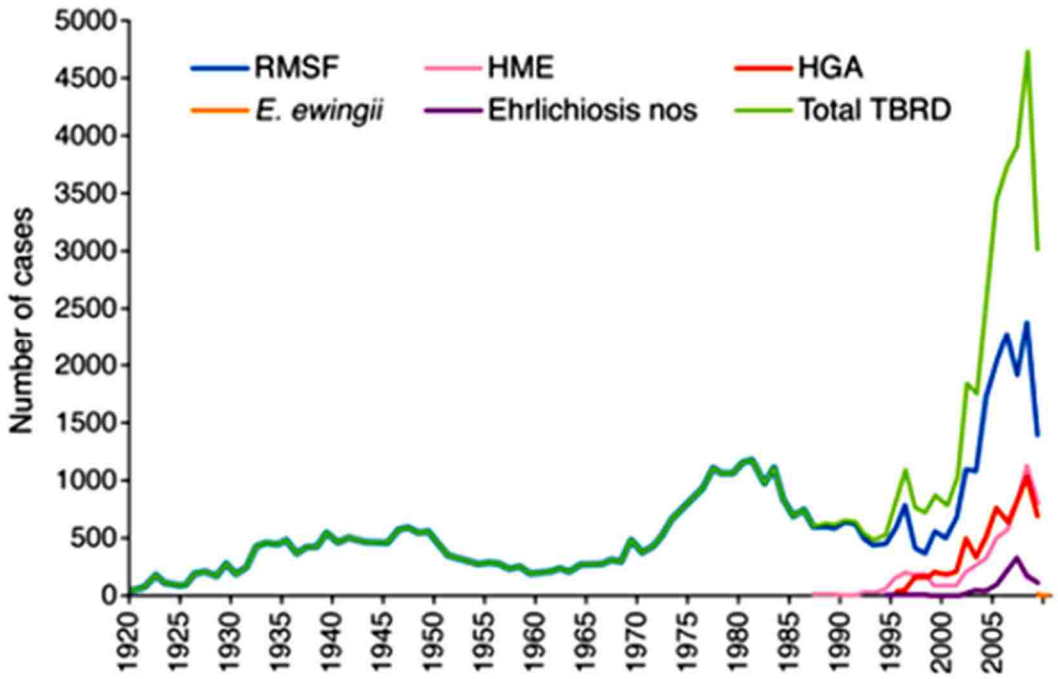

Figure 1.1 Reported cases of tick-borne rickettsial disease (TBRD) in the United States from 1920-2009 (Dumler, 2010). RMSF = Rocky Mountain spotted fever; HME = human monocytic ehrlichiosis; HGA = human granulocytic anaplasmosis; *E. ewingii* = *Ehrlichia ewingii* infection; nos = not otherwise specified. Figure from Dumler, 2010.

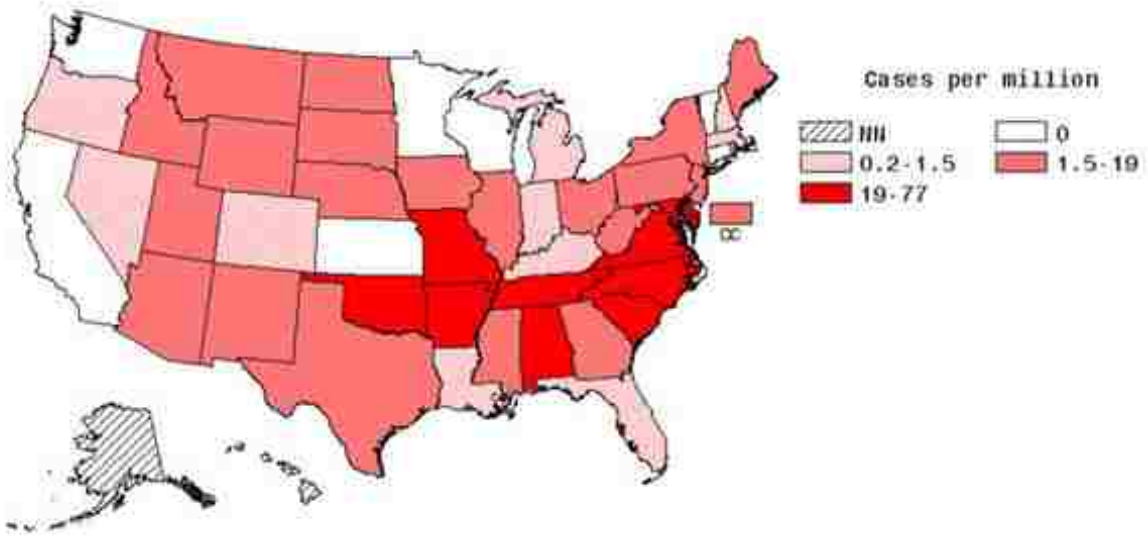

Figure 1.2 Geographic distribution and annual reported incidence (per 1,000,000 persons) of Rocky Mountain spotted fever (RMSF) in the United States, 2008. NN= not notifiable. (Courtesy of Centers of Disease Control and Prevention)

The upward trend of RMSF cases suggests ecological changes affecting disease transmission, increased diagnostic testing and/or changes in surveillance and reporting (Hall-Baker et al., 2010). In addition, the wide distribution of RMSF might be the result of the widespread range of the primary tick vectors; the American dog tick, *Dermacentor variabilis* (East of the Rocky Mountains and in limited area on the Pacific Coast) (Figure 1.3.1), the Rocky Mountain wood tick, *Dermacentor andersoni* (in the Rocky Mountain states) (Figure 1.3.2) and the brown dog tick, *Rhipicephalus sanguineus* (throughout the United States) (Figure 1.3.3) (Hall-Baker et al., 2010). The American dog tick and the Rocky Mountain wood tick have been recognized as vectors in the transmission of *R. rickettsii* for a long time; however, the brown dog tick was found to be a natural carrier of *R. rickettsii* in 2005 (Demma et al., 2005).

Figure 1.3.1 Approximate distribution of the American dog tick, *Dermacentor variabilis* in the United States. (Courtesy of Centers of Disease Control and Prevention)

Figure 1.3.2 Approximate distribution of the Rocky Mountain Wood tick, *Dermacentor andersoni* in the United States. (Courtesy of Centers of Disease Control and Prevention)

Figure 1.3.3 Approximate distribution of the Brown dog tick, *Rhipicephalus sanguineus* in the United States. (Courtesy of Centers of Disease Control and Prevention)

In the United States, RMSF cases are reported every month of the year with the majority of the cases reported during late spring and summer coinciding with the period of highest tick feeding activity. In most states, RMSF cases peak during the months of June and July; nevertheless, in Arizona, where *R. sanguineus* is the common tick vector, the most cases occur during August and September (Hattwick et al., 1976; www.cdc.gov).

The epidemiological data collected by the CDC (2000-2008) have shown that adult males, American Indians and people aged 50-69 contract rickettsial disease more frequently than other groups. This distribution is related to the greater recreational or occupational exposures to ticks and is not due to the different susceptibility of various ages and sexes of human (Hattwick et al., 1976; Demma et al., 2005**;** www.cdc.gov). In January 2010, the disease category of "Rocky Mountain spotted fever" was classified under a new category of "Spotted Fever Rickettsiosis, including Rocky Mountain spotted fever" (www.cdc.gov). This new category is less specific, more inclusive and more accurate; many of the cases previously reported under the "Rocky Mountain spotted fever" category were not specifically caused by *R. rickettsii*. In the United States, in addition to RMSF, two more tick-borne spotted fever rickettsial diseases show similar clinical features to RMSF. These rickettsial diseases are caused by *R. parkeri* and *R.* species 364D (Table 1.1).

Table 1.1 Other tick-borne spotted fever rickettsial infections in the United States.

Species	Tick vector	Geographic area	Clinical features
R. parkeri	*A. maculatum* (Gulf Coast tick)	Eastern and southern U.S., particularly along the coast	Fever, headache, eschar (s), variable rash
***R.* species 364D**	*D. occidentalis* (Pacific Coast tick)	Northern California, Pacific Coast	Fever, eschar (s)

(www.cdc.gov)

1.1.2 Routes of transmission

Ticks become infected with *R. rickettsii* either by transovarial transmission or by feeding on the blood of infected animals. Human infection occurs accidentally through the bite of infected ticks. When the tick attaches and feeds on a human host, a nonvirulent form of *R. rickettsii*, present in salivary glands, is reactivated by the fresh blood meal and is transformed into one with high pathogenicity (Hayes and Burgdorfer, 1982). In addition, humans may become infected through abrasion of skin or through mucous membrane contact with infected tick feces or bodily fluid during removal of the tick from human or animals. Inhalation of large aerosols in the laboratory is also a potential source of infection (Milstone and Dumler, 2009).

1.1.3 Clinical manifestations and treatments

Today, RMSF is still the most lethal and most commonly reported TBRD in the United States. Initial signs and symptoms of highly lethal RMSF include severe headache, high-grade fever (102-104 °F), generalized myalgia (especially in back and leg muscles), nausea and vomiting, beginning within the first several days after *R. rickettsii* is inoculated into skin during blood feeding of infected ticks. If left untreated, severe injury can occur sometimes leading to multi-organ failure for example acute renal disease. In common with other rickettsial diseases, vascular lesions are responsible for clinical manifestations of this illness. The widespread infection of vascular endothelium leads to encephalitis, interstitial pneumonia, non-cardiogenic pulmonary edema and adult respiratory distress syndrome. *Rickettsia rickettsii* invasion of the endothelial cell network at the site of the tick bite begins a process of local dermal and epidermal necrosis that results in an eschar. Dissemination of the infection further damages vascular networks and leads to vasodilation, and maculopapular rash. The rash is a characteristic of RMSF and helpful in establishing a diagnosis; however, some people do not develop the rash

(Egermayer, 2001; Parola and Raoult, 2006; Dantas-Torres, 2007; Walker and Ismail, 2008; Milstone and Dumler, 2009; Walker and Valbuena, 2009).

Antimicrobials in which the agent enters the host cells and is active in the intracellular environment are ideal treatment for RMSF. The first line and effective treatment of *R. rickettsii* infection in adults and children of all ages is doxycycline; the mode of action of doxycycline is the inhibition of protein synthesis thus bacterial growth by binding to 30S and possibly 50S ribosomal subunits of bacteria. The use of doxycycline is recommended due to the potential of tetracycline, also an effective treatment of RMSF, to stain permanent teeth, especially in children younger than 8 years (Milstone and Dumler, 2009). In patients allergic to doxycycline or pregnant patients, chloramphenicol can be used; however, the risk for a lethal outcome is higher in the patients treated with chloramphenicol than doxycycline. Although fluoroquinolones and macrolides have been suggested as effective treatment in some studies, they have not been proven in patients.

RMSF is problematic because of resulting high mortality rates if the disease is left untreated. Also, the control of the animal reservoirs or vectors is very impractical. Vaccination as a prevention of RMSF might be helpful; however, a licensed vaccine for RMSF is currently not available. Thus, understanding the pathogenesis by which rickettsiae causes disease as well, as the development of immune response, is useful for the development of vaccines, pharmacological design and other types of intervention which prevent the damage caused by rickettsiae.

1.2 Tick vectors of rickettsiae

Ticks are ectoparasitic, hematophagous arthropods that transmit a broad range of microorganisms, many of which cause diseases in humans and livestock (Sonenshine and Hynes,

2008). They are classified into three families: hard-bodied ticks (Ixodidae), soft-bodied ticks (Argasidae) and the Nuttalliellidae. Ixodid ticks are vectors and reservoirs for SFG rickettsiae. Their life cycle involves three post-embryonic parasitic stages: larva, nymph and adult. Each developmental stage of hard ticks takes a blood meal in order to reach the next stage. Ticks use their chelicerae to puncture human skin and their hypostome to anchor themselves. Then the chelicerae rupture superficial small vessels in order to take the blood meal. The duration of feeding is 3-12 days depending on stage, species and conditions. Female ticks feed for several days, whereas male ticks feed repeatedly in small amounts. Following each blood meal, ticks molt and undergo morphological development into the next stage. In contrast, Argasid or soft ticks undergo shorter feeding periods and more complex life cycles (Sonenshine et al., 2005; Piesman and Gage, 2005).

1.2.1 Tick-host immune interactions

Attachment of the tick to the vertebrate host elicits both innate and adaptive defenses against tick infestation. Adaptive resistance to tick feeding involves both humoral and cell-mediated immunities that impair tick engorgement, ova production and viability. Nevertheless, ticks modulate host immune responses by the secretion of a wide range of physiologically active components through their saliva (Wikel, 1999; Brossard and Wikel, 2004; Hovius, 2009). Tick saliva contains anti-inflammatory molecules, anti-haemostatic substances that promote blood feeding, and salivary immunomodulatory components that mediate bacterial transmission and prevent the vertebrate host from rejecting them (Figure 1.4) (Wikel, 1999; Brossard and Wikel, 2004; Nuttall and Labuda, 2004).

Tick-mediated host immunosuppressive countermeasures impair natural killer (NK) cells, dendritic cells (DCs), macrophages and neutrophil functions; inhibit complement system; bind to

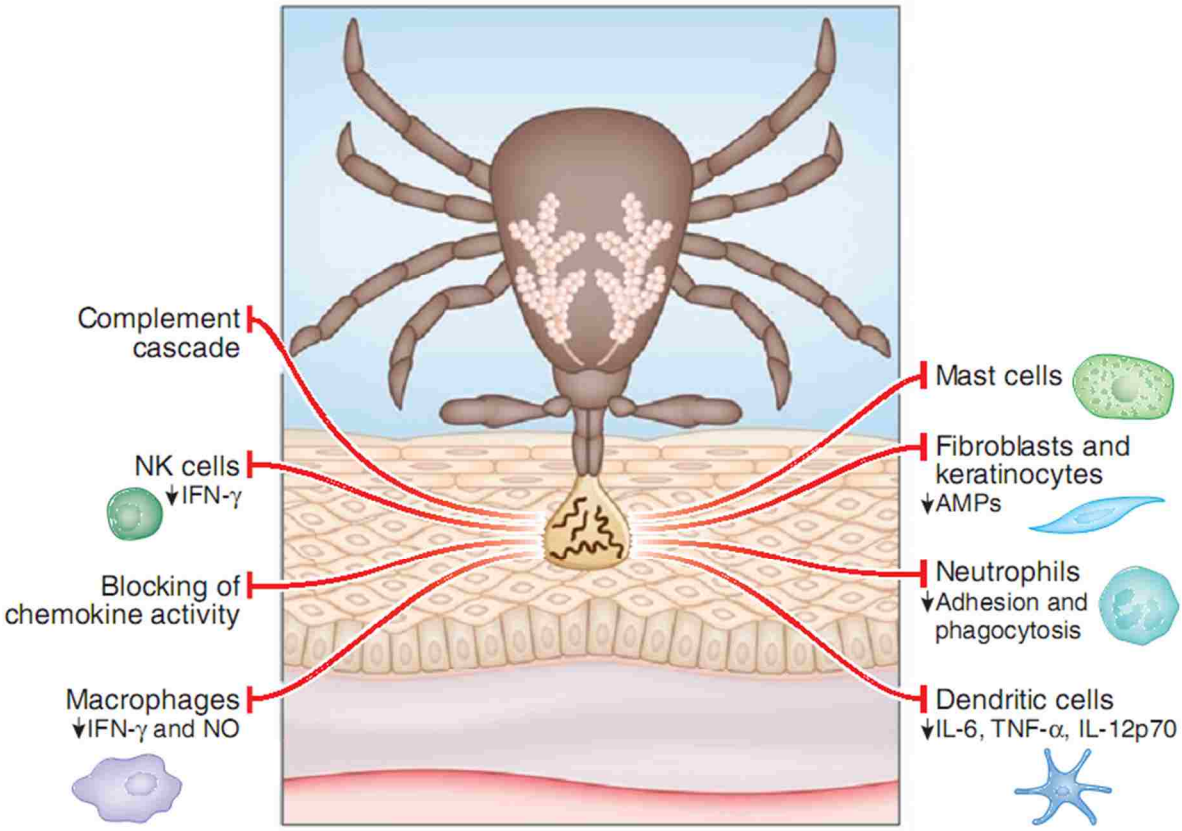

Figure 1.4 Schematic representation of immunosuppressive activities of tick saliva. During feeding, ticks inoculate their saliva containing several immunosuppressive proteins into host skin. The figure depicts the functions of tick saliva in response to host innate immunity. IFN-γ = interferon-γ, NO = nitric oxide, AMP = antimicrobial peptide, IL = interleukin, TNF-α = tumor necrosis factor-α. Figure is modified from Hovius, 2009.

histamine; reduce antibody titer; decrease the production of cytokines such as interleukine-12 (IL-12) and interferon-γ (IFN-γ); block chemokine activity; and inhibit T-cell proliferation and host antibody responses (Walker and Ismail, 2008; Hovius, 2009). In addition, tick salivary gland extract has been shown to inhibit antimicrobial peptide production induced by *Borrelia burgdorferi* (Marchal et al., 2009). Furthermore, tick saliva has been shown to block DCs maturation by inhibiting expression of co-stimulatory (CD40, CD80 and CD86) and adhesion (CD54) molecules. The inhibition of DC maturation by tick saliva affects T cell effector

functions in that initial activation of T cells by DCs would be impaired (Cavassani et al., 2005; Sa-Nunes et al., 2007).

1.2.2 Tick transmission and reservoir of rickettsiae

Ticks are often infected with obligate intracellular bacteria which are primarily transmitted to the vertebrate host by direct inoculation into host blood vessels. The ability of ticks to transmit rickettsiae was first shown in Ricketts's classic studies of RMSF (Ricketts 1906). Ricketts also demonstrated that female ticks previously fed on infected guinea pigs could transmit rickettsiae to uninfected guinea pigs in what he coined horizontal transmission. Additionally, ticks could pass rickettsiae from one developmental stage to the next by either transstadial or transovarial transmission (Ricketts 1907a; Ricketts 1907b). From these results, Ricketts concluded that ticks were the primary reservoirs of rickettsiae. In nature, the transovarial and transstadial transmission of rickettsiae in tick vectors is a highly efficient mechanism ensuring rickettsial survival without requiring the complexity inherent in multi-host reservoir systems. The efficiency of transstadial transmission is close to 100% whereas transovarial transmission is 30-100% efficient (Piesman and Gage, 2005). In contrast, horizontal transmission of rickettsiae by feeding of uninfected ticks on infected host animals is not as efficient as transstadial and transovarial transmission as some hosts are naturally resistant to rickettsiae, immune from earlier infection and/or rickettsemic for only a few days ingesting an insufficient quantity of rickettsiae (Piesman and Gage, 2005). For horizontal transmission, uninfected ticks must feed on rickettsemic hosts to ensure that sufficient quantities of rickettsiae are ingested to establish infection (Burgdorfer et al., 1966)

1.2.3 Vector competence

Vector competence refers to the ability of arthropod vectors to acquire and transmit the pathogen to the new host (Sonenshine et al., 2005). Vector competence is important for the vector-pathogen cycle. Both genetic and environmental factors contribute to the variation of vector competence (Tabachnick, 1994; Beerntsen et al., 2000); however, these factors are undetermined for ticks transmitting rickettsiae.

For the pathogens transmitted by the bite of infected vectors, the midgut is an important barrier that prevents the vector from becoming infected. It is necessary for rickettsiae transmitted by the bite of infected ticks to breach this barrier in order to establish a successful infection. After entering the digestive tract of the tick, rickettsiae invade and multiply within cells of the midgut epithelium where they escape from the midgut to the hemocoel infecting hemocytes. Infected hemocytes carry rickettsiae to other tissues within the ticks, including salivary glands and reproductive organs. Transovarial transmission only occurs after ovaries become infected. On the other hand, for transmission of rickettsiae to vertebrate hosts during blood feeding, tick salivary glands must first -be infected (Sonenshine et al., 2005; Piesman and Gage, 2005).

1.2.4 *Dermacentor variabilis* tick

Dermacentor variabilis, commonly called the American dog tick is a seasonally active tick that is the primary vector of *R. rickettsii* in the eastern and south central United States (Sonenshine et al., 2005). This species of tick also transmits *Francisella tularensis* and *Anaplasma marginale*, the causative agent of tularemia and anaplasmosis, respectively. In addition, non-pathogenic *R. montanensis* has been reported to be carried by *D. variabilis* in areas

of the United States (Feng et al., 1980; Anderson et al., 1986; Pretzman et al., 1990; Ammerman et al., 2004).

Dermacentor variabilis is a three-host tick in which each developmental stage in the life cycle feeds on different hosts (Figure 1.5) (Sonenshine, 2005). Larvae and adults overwinter in the forest or soils. During spring, *D. variabilis* larvae emerge from winter diapauses and feed on small mammals. Larval activity peaks in mid-April in the southeastern United States and in mid-May or early June in the northern United States. After feeding, larvae molt into nymphal ticks that feed on small mammals and peak a few weeks after the larval peak. Thereafter, unfed adults begin to emerge and seek medium- or large-sized animals. Adult ticks become active, culminating in June or early July.

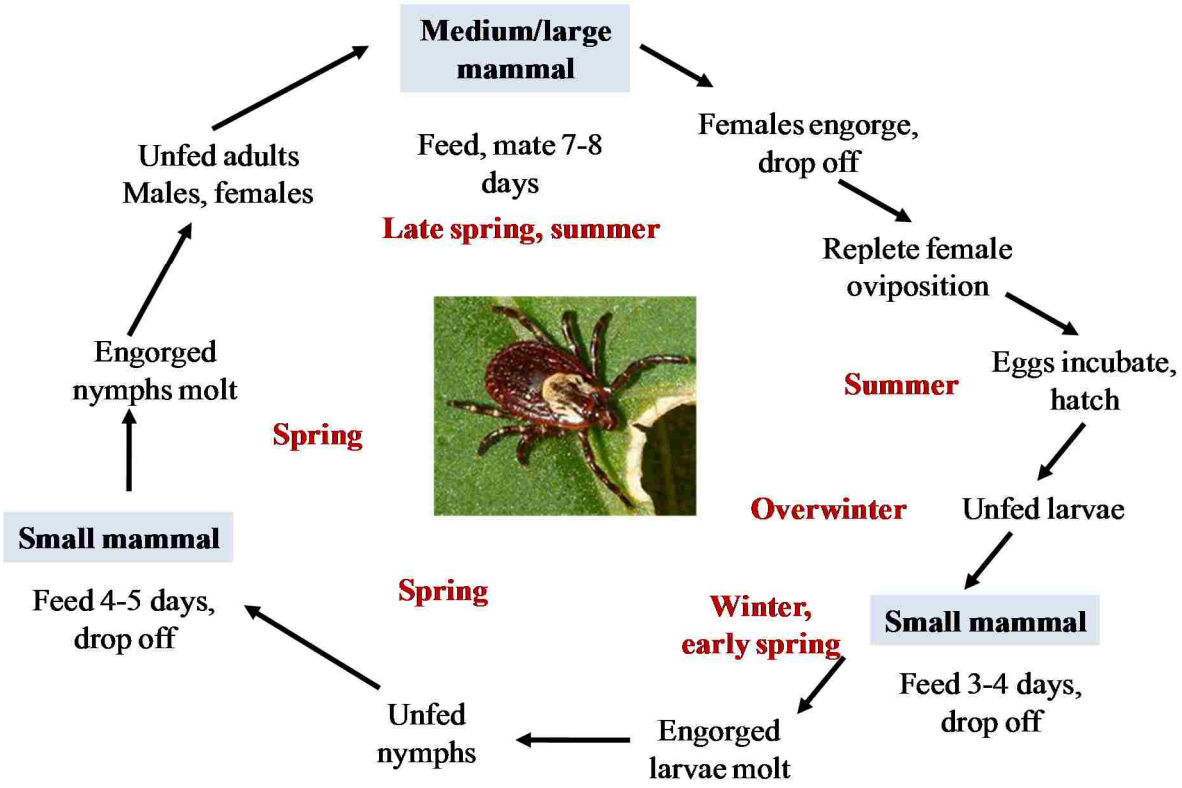

Figure 1.5 Life cycle of a three-host tick. In each developmental stage, a three-host tick feed on different hosts. Larvae and nymphs feed on small mammals. Adults feed on medium- or large-sized animals. Figure is modified from Sonenshine, 2005.

1.3 Rickettsial agents and their characteristics

Although members of the genus *Rickettsia* are primarily associated with arthropod vectors such as ticks, fleas, lice and mites, their life cycle also involve vertebrate hosts (Figure 1.6) (Azad and Beard, 1998; Perlman, 2006). In nature, rickettsiae are adapted to survive within arthropod hosts in which they are maintained by vertical (transstadial and transovarial) transmission. Horizontal transmission of rickettsiae can occur during feeding of uninfected arthropods, such as ticks, on rickettsemic rodents and other animals (Walker and Ismail, 2008); however this process contributes little to the life cycle of rickettsiae. With the exception of *R. prowazekii*, the role of human hosts in the rickettsial life cycle is only secondary (Azard and Beard, 1998).

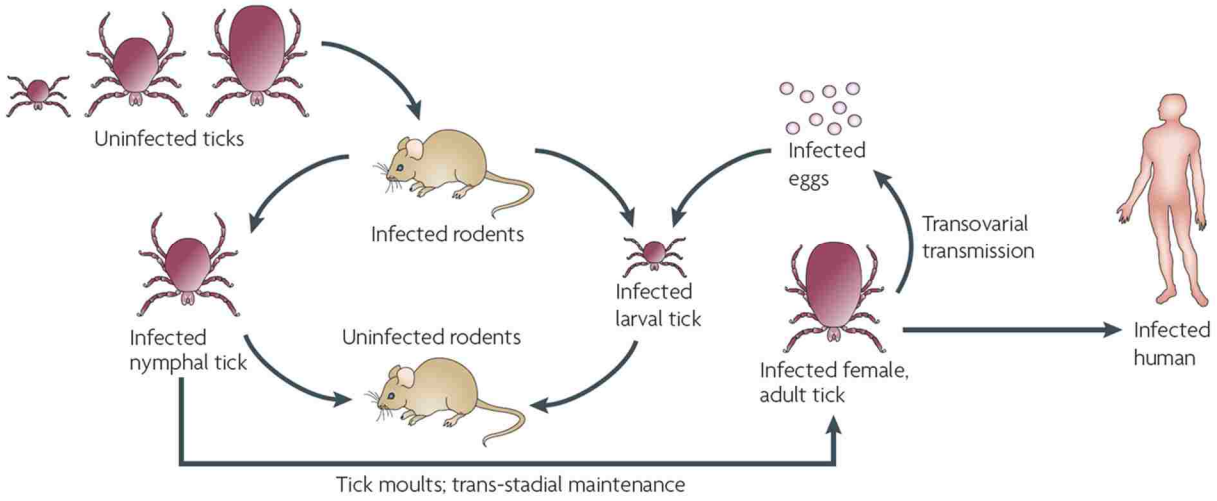

Figure 1.6 Transmission cycle of tick-borne rickettsiae. Spotted fever group rickettsiae are maintained in nature by transovarial and transstadial transmission in ticks and horizontal transmission to uninfected ticks that feed on rickettsemic rodents and other animals. Figure from Walker and Ismail, 2008

1.3.1 Rickettsial classification

Rickettsiae are a group of bacteria belonging to the class *Alphaproteobacteria*. They are members of the order *Rickettsiales* and the family Rickettsiaceae. The family Rickettsiaceae is

comprised of two genera, *Orientia* and *Rickettsia* (Tamura et al., 1995). *Orientia tsutsugamushi* is the only member in genus *Orientia*. The organism is the agent of scrub typhus which is transmitted by the larvae of mites (Mullen et al., 2002).

The genus *Rickettsia* is composed of several species, some of which are apparently harmless (e.g. *R. montanensis*, *R. rhipicephali*, *R. bellii*, *R. canadensis*), and others that are responsible for a number of diseases in human (e.g. *R. rickettsii*, *R. conorii*, *R. parkeri*, *R. africae*, *R. slovaca*, *R. sibirica*, *R. honei*, *R. japonica*, *R. akari*, *R. felis*) (Parola et al., 2005; Walker and Ismail, 2008). The list of human diseases caused by the members of the genus *Rickettsia* along with their associated arthropod vector(s), transmission cycle(s), geographic area and mortality rate are provided in Table 1.2 (Walker and Ismail, 2008).

Based on whole-genome analysis data (Gillespie et al., 2007; Gillespie et al., 2008), the genus *Rickettsia* are classified into four groups: the spotted fever group (*R. rickettsii*, *R. conorii*, *R. sibirica*, *R. helvetica* and several others), typhus group (*R. prowazekii* and *R. typhi*), transitional group (*R. felis*, *R. akari* and *R. australis*), and ancestral group (*R. bellii* and *R. canadensis*) (Figure 1.7). From bioinformatics analysis of the representative rickettsial genomes, these four aforementioned rickettsial groups revealed the number of genes with shared evolutionarily histories as shown by the Venn diagram in Figure 1.8 (Gillespie et al., 2008).

1.3.2 Biological characteristics of rickettsiae

Members of the genus *Rickettsia* possess general characteristics that are shared among species. These organisms are obligate intracellular, gram-negative bacteria that exist freely in the cytoplasm and nuclei of eukaryotic cells (Raoult and Roux, 1997; Azad and Beard, 1998; Perlman et al., 2006). They are small with an average size of 0.3-0.5 μm in width but vary

Table 1.2 Rickettsial diseases in humans.

Disease	Organisms	Arthropod vector	Life cycle	Geographic area	Mortality rate*
Tick-transmitted spotted fevers					
Rocky Mountain spotted fever	*R. rickettsii*	*D. variabilis, D. andersoni, R. sanguineus, A. cajennense* and *A. aureolatum*	Transovarian in ticks and rodent ticks	Western hemisphere	High
Boutonneuse fever	*R. conorii*	*R. sanguineus* and *R. pumilio*	Transovarian in ticks	Southern Europe, Africa and southern Asia	Mild to Moderate
African tick-bite fever	*R. africae*	*A. hebraeum* and *A. variegatum*	Transovarian in ticks	Africa and the West Indies	None reported
Maculatum disease	*R. parkeri*	*A. maculatum* and *A. triste*	Ticks	Western hemisphere	None reported
Flea-transmitted diseases					
Flea-borne spotted fever	*R. felis*	*C. felis*	Transovarian in the cat flea	Worldwide	None reported
Murine typhus	*R. typhi*	*X. cheopis* and *C. felis*	Rat-flea for *X. cheopis* and Opossum flea for *C. felis*	Worldwide	Low
Louse-transmitted diseases					
Epidemic typhus	*R. prowazekii*	*P. humanus humanus*	Human louse	Worldwide	High
Epidemic typhus	*R. prowazekii*	Fleas and lice of flying squirrels and *G. volans volans*	Flying squirrel, flea and louse ectoparasite	United States	Low
Mite-transmitted diseases					
Rickettsialpox	*R. akari*	*L. sanguineus*	Transovarian in mites	Worldwide	None reported

* High mortality is >15%; moderate mortality is 7-15%; mild-to-moderate mortality is 2-7% and low mortality is ≤1% (Walker and Ismail, 2008)

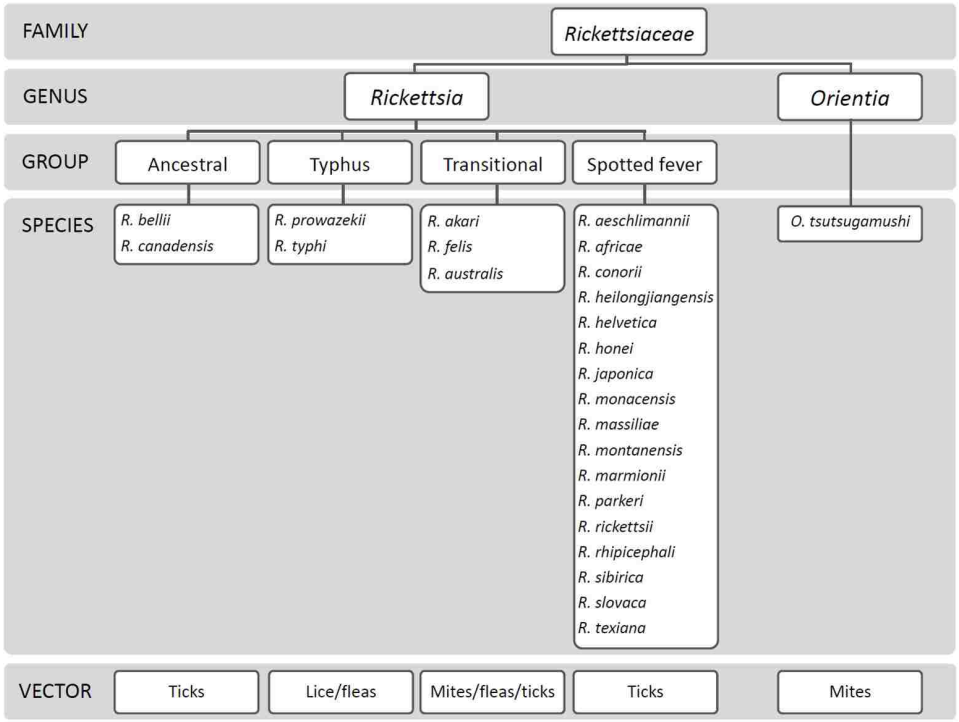

Figure 1.7 Classification of the genus *Rickettsia* and their arthropod vectors. Figure from Sunyakumthorn dissertation, 2011.

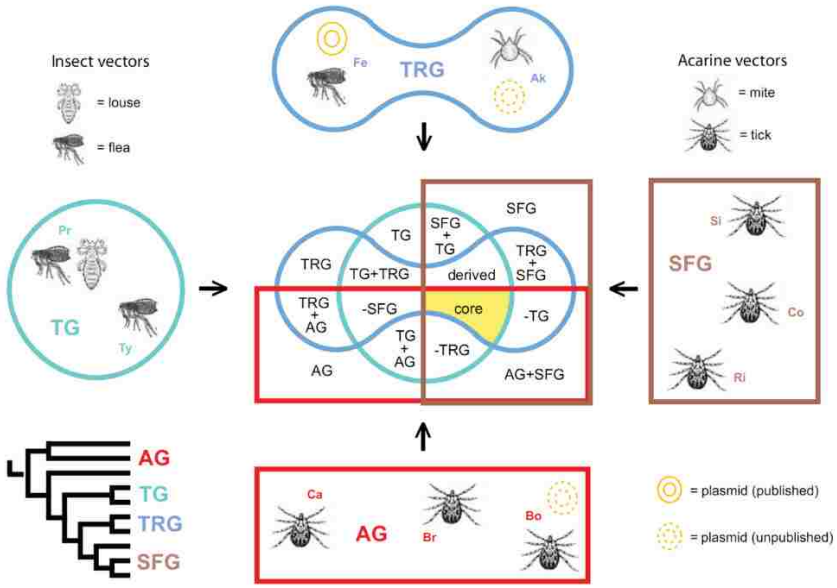

Figure 1.8 Venn diagram represents the intersections for the four rickettsial groups. Molecular phylogenetic analysis of the four groups of *Rickettsia* is shown in the lower left. Arthropod vectors of each genome and bacteria known to have plasmids are shown. AG = ancestral group, TG = typhus group, TRG = transitional group, SFG = spotted fever group, Br and Bo = the two different strains of *R. bellii*, Ca = *R. Canadensis*, Pr = *R. prowazekii*, Ty = *R. typhi*, Ak = *R. akari*, Fe = *R. felis*, Ri = *R. rickettsii*, Co = *R. conorii*, and Si = *R. sibirica*. Figure from Gillespie et al., 2008.

considerably in length; the length of *R. prowazekii* and *R. rickettsii* are 2-4 μm and 2 μm, respectively (Weiss, 1973).

Rickettsiae are nonmotile, coccobacilli, pleomorphic and appear to multiply by binary fission (Sunyakumthorn et al., 2008; Milstone and Dumler, 2009). Unlike other bacteria, rickettsiae are difficult to dye by Gram stain procedures; however, the electron micrographs of rickettsiae (Figure 1.9) reveal a typical gram-negative-like cell wall (Anacker et al., 1967; Anacker et al., 1985; Silverman, 1991; Walker and Ismail, 2008). When properly stained by Giemsa, Castaneda, Macchiavello or Gimenez techniques, the rickettsiae are purple, light blue, red or bright red, respectively (Milstone and Dumler, 2009).

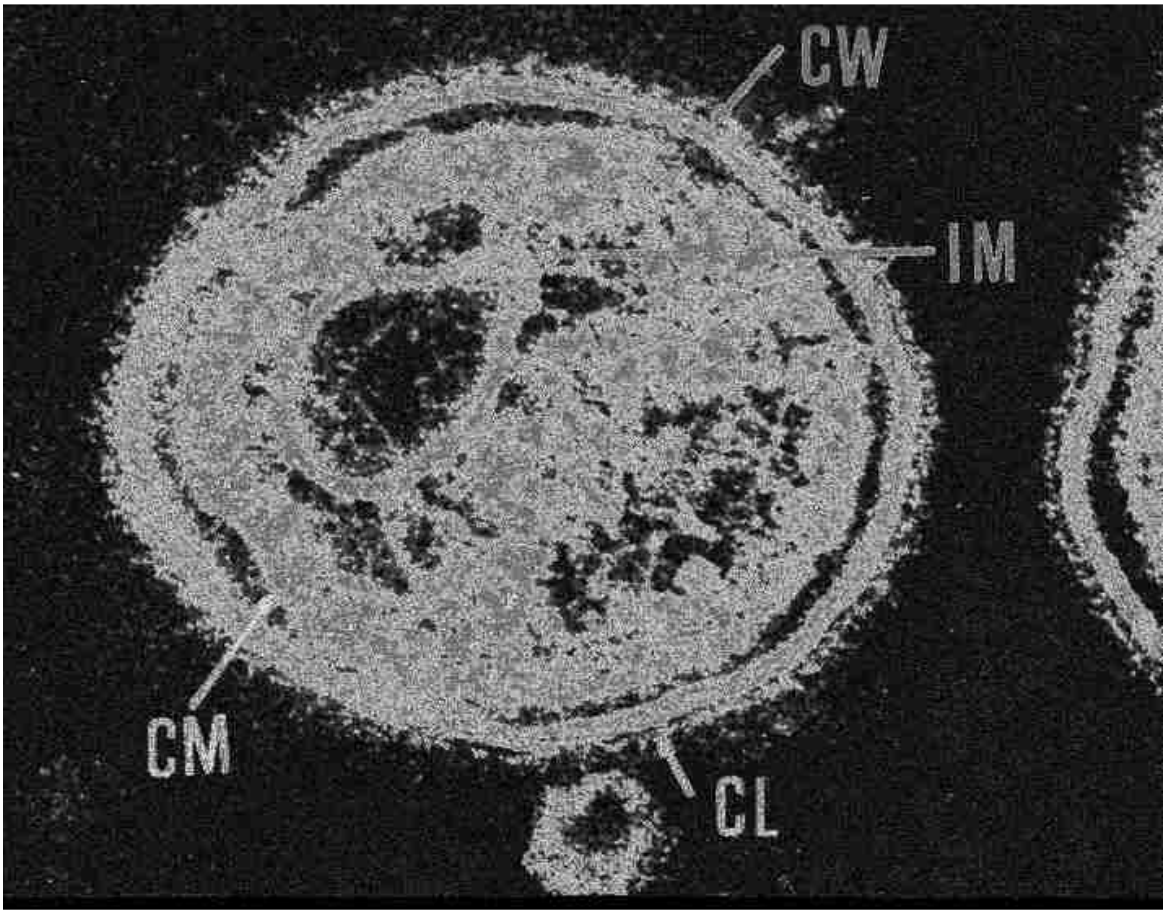

Figure 1.9 The fine structure of *R. prowazekii* as revealed in the thin section of the chick yolk sac. CL = capsule-like structure, CM = cytoplasmic membrane, CW = cell wall, IM = Intracytoplasmic membrane. Figure from Anacker et al., 1967.

Rickettsia has a small genome with size ranging from 1.1-1.6 Mb (Ogata et al., 2000). In order to survive, rickettsiae have evolved to exploit essential machineries from the cytoplasm of the host cells. By doing this, the rickettsial genome has undergone a severe reduction in size in which many genes encoding proteins used in pathways, such as amino acid and nucleotide synthesis, and lipid and carbohydrate metabolism, have been lost (Blanc et al., 2007; Darby et al., 2007; Fuxelius et al., 2007); thus they can survive only in the host cells.

Like other gram-negative bacteria, rickettsiae also contain lipopolysaccharide (LPS) in the cell wall. The LPS in rickettsiae play a role in a group-common antigenicity; antibody to the LPS of *R. rickettsii* cross react with LPS from other spotted fever group rickettsiae, and a slight cross-reactivity of antibody in the LPS of rickettsiae from typhus group is seen (Chen and Sexton, 2008; Jones, 1993). In addition to LPS, rickettsiae contain two immunogenic proteins, outer-membrane protein A (OmpA) and outer-membrane protein B (OmpB). Although OmpA and OmpB elicit a strong response to human antibody in RMSF patients, OmpB is found in all groups of rickettsiae but OmpA is found only in spotted fever group rickettsiae (Parola, 2005; Walker and Ismail, 2008; Milstone and Dumler, 2009).

1.3.3 Pathogenicity of tick-borne rickettsiae

The genus *Rickettsia* comprises of intracellular bacteria that are either pathogenic or non-pathogenic to humans. Although they are not considered to be pathogenic, many non-pathogenic rickettsiae demonstrate pathogenic effects in experimentally infected laboratory animals (Table 1.3) (Paddock, 2009). In the past decade, some rickettsiae that were previously characterized as non-pathogenic organisms have been reported to cause disease in humans (Table 1.4) (Paddock, 2009). Various factors contributing to the pathogenicity of rickettsiae have been described; however, many questions regarding rickettsial pathogenicity still need to be answered.

Table 1.3 Features of rickettsiae of undetermined pathogenicity in humans.

Rickettsia species or strain	Tick(s) infected with *Rickettsia* in nature	Frequency with which tick(s) will bite humans	Demonstrated pathogenicity of *Rickettsia* in animals	Route of infection
R. bellii	Multiple genera, including *Dermacentor* and *Amblyomma*	Frequent	Eschars in rabbits and guinea pigs	ID
R. canadensis	Multiple genera, including *Haemaphysalis* and *Dermacentor*	Infrequent to frequent	Fever in guinea pigs	IP
Strain 364D	*D. occidentalis*	Frequent	Scrotal erythema in guinea pigs	IP
Strain Parumapertus	*D. parumapertus*	Infrequent	Fever and scrotal erythema in guinea pigs	IP
Strain Tillamook	*I. pacificus*	Frequent	Death in mice	IP
R. rhipicephali	Multiple genera, including *Dermacentor* and *Rhipicephalus*	Frequent	Fever, scrotal swelling and death in meadow voles	IP

ID = intradermal; IP = intraperitoneal (Paddock, 2009)

Table 1.4 List of pathogenic rickettsiae previously determined as non-pathogenic organisms to humans.

Agent	Year of discovery (initial designation)	Year reported as a confirmed pathogen (interval from discovery)	Initial diagnosis of index patient(s)
R. parkeri	1937 (maculatum agent)	2004	Rickettsialpox
R. honei	1962 (TT-118)	1992	Queensland tick typhus
R. slovaca	1968 (strain B, D)	1997	Lyme borreliosis
R. felis	1990 (ELB agent	1994	Murine typhus
R. massiliae	1992 (strains Mtu1, Mtu5)	2006	Mediterranean spotted fever
R. aeschlimannii	1995 (strain PoTiR8	2002	Mediterranean spotted fever
R. raoultii	1999 (genotypes RpA4, DnS14, DnS28)	2006	Tick-borne lymphadenopathy
R. monacensis	2002(*R. monacensis*	2007	Mediterranean spotted fever

(Paddock, 2009)

Pathogenic rickettsiae are transmitted to and cause disease in humans via the bite of their respective arthropod vectors; therefore both rickettsiae-human and rickettsia-arthropod interactions must be taken into consideration regarding pathogenicity of rickettsiae. In this section, only factors and their potential functions that influence the pathogenicity of rickettsiae are mentioned. Sequential steps in the pathogenesis of rickettsial infection will be discussed in section 1.4 (Interaction of rickettsiae with host cells).

For tick-borne rickettsiae, bacteria are transmitted mainly through salivary gland secretion during feeding of an infected tick. Thus, the bacteria must be able to localize into tick salivary glands in order to be transmitted to humans (Parola, 2005). The rickettsiae that do not establish infection in tick salivary glands seem unlikely to be pathogenic. For example, *R. peacockii*, originally found in *D. andersoni* ticks, produce infection in ovaries, midguts and malpighian tubules, but not in salivary glands (Niebylski, 1997); therefore limiting subsequent transmission to humans. Rickettsial molecules involved in dissemination of rickettsiae to tick salivary glands might contribute to the pathogenicity of rickettsiae; however, these molecules are still unknown. In *Borrelia afzelii*, a causative agent of the arthropod-borne Lyme disease in humans, the OspC protein plays a role in borrelial invasion of tick salivary glands and also infection of the mammalian hosts (Fingerle et al., 2007). Thus it is possible that rickettsial molecules, which are involved in human infection, are also associated with rickettsial dissemination to tick salivary glands.

In addition, the species of tick vector in which rickettsiae reside is also important to the pathogenicity of rickettsiae (Parola, 2005). In this context, virulent rickettsiae might not be able to cause disease in humans if their arthropod vectors do not take blood meals from humans. Ticks serve as vectors and reservoirs for SFG rickettsiae, but studies often focus primarily on

mammalian hosts while the molecular basis for tick-rickettsiae interaction remains poorly understood. Thus, many aspects regarding the tick-rickettsiae relationship need to be investigated.

When transmitted to human hosts, rickettsiae use several factors in order to establish successful infection. The candidate rickettsial virulence factors and their potential functions are listed in Table 1.5 (Walker and Ismail, 2008; Riley et al., 2010; Haglund et al., 2010). The expression of these particular proteins in pathogenic rickettsiae likely contributes to pathogenicity of these organisms.

Table 1.5 Candidate rickettsial virulence factors.

Virulence factors	Potential function
Patatin B1 precursor	Membranolytic phospholipase A host cell escape
Haemolysin A	Membranolytic traversal of host cell membrane
Haemolysin C	Membranolytic phagosomal escape
Phospholipase D	Membranolytic phagosomal escape
Dinucleoside polyphosphate hydrolase	Hydrolysis of toxic dinucleoside polyphosphates to ATP
Cytochrome c oxidase	Aerobic respiration under optimal aerobic conditions
Superoxide dismutase	Neutralizes oxidative stress of reactive oxygen species
Lipopolysaccharide	Endotoxin-mediated inflammation
Surface cell antigens (Sca), except for sca4, which is an intracellular protein	Autotransporter outer-membrane proteins, actin based motility (Sca2), cell adherence and invasion (Sca1)
Outer-membrane protein A (OmpA)	Spotted-fever-group rickettsial attachment to host cell
Outer-membrane protein B (OmpB)	Rickettsial attachment to host cell
Type IV secretion system (T4SS)	Transport of rickettsial proteins or DNA into host cytosol
Actin-tail polymerization proteins (RickA)	Formation of actin tail and mediation of intracellular and intracellular rickettsial spread

(Walker and Ismail, 2008; Riley et al., 2010; Haglund et al., 2010)

In the genomic era, the knowledge of rickettsial genome sequences allows researchers to identify potential virulence associated proteins. Comparative genomic analysis of non-pathogenic *R. peacockii* and pathogenic *R. rickettsii* revealed genes that are deleted or mutated in *R. peacockii*. These genes, which include those coding for an ankyrin repeat containing protein, DsbA, Protease II, a putative phosphoethanolamine transferase, RickA, Sca1 and OmpA, may be involved in the loss of virulence of *R. peacockii*, suggesting a role in the pathogenicity of rickettsiae (Felsheim et al., 2009). For examples, in *Legionella pneumophila* and *Coxiella burnetii*, many different proteins containing ankyrin repeat domains were identified. These proteins are, in fact, effector proteins delivered to eukaryotic cells by type IV secretion system (Pan et al., 2008). In *Shigella flexneri*, DsbA is important for intracellular survival and virulence of the bacteria (Yu, 1998; Yu et al., 2000). In addition, Protease II is responsible for the entry of *Trypanosoma cruzi* into host cells (Bastos et al., 2005) and phosphoethanolamine transferase is required for *Neisseria meningitidis* binding to endothelial cells (Cox et al., 2003; Takahashi et al., 2008). In *R. conorii*, a surface protein RickA was identified as an activator of the Arp 2/3 (actin-related protein 2/3) complex (Gouin et al., 2004). This process is important in actin polymerization, a strategy rickettsiae use to move within the host cells and spread from cell-to-cell. In 2005, Simser et al. demonstrated that the *rickA* gene in non-pathogenic *R. peacockii* is disrupted by an insertion sequence (IS) element, ISRpe1. This rickettsial IS element was also shown to disrupt the *sca1* gene in *R. peacockii* (Simser et al., 2005). An autotransporter protein, Sca1, has recently been demonstrated to enhance *R. conorii* adherence to mammalian cells (Riley et al., 2010).

Genome comparison of *R. peacockii* and *R. rickettsii* has revealed the presence of the *ompA* gene in both species; however, sequence analysis of *R. peacockii ompA* gene demonstrated

three frameshift mutations resulting in an expression of truncated OmpA protein (Baldridge, 2004; Felsheim et al., 2009). The three premature stop codons of the *ompA* gene were also found in avirulent strain *R. rickettsii* Iowa. The immunodominant, surface-exposed protein, OmpA, plays a critical role in the attachment of *R. rickettsii* to host cells (Li and Walker, 1998).

In addition to virulence factors, the physiologic requirements of particular rickettsiae may also contribute to the pathogenicity of rickettsiae (Paddock, 2009). Studies of the growth of rickettsiae have illustrated that some pathogenic *Rickettsia* species including *R. rickettsii* and *R. conorii* can be cultured at the human body temperature of 37° C, whereas some of the non-pathogenic bacteria including *R. bellii*, *R. helvetica* and *R. peacockii*, grow poorly or not at all in mammalian cell culture (Schaechter et al., 1957; Oaks and Osterman, 1969; Beati et al., 1994; Policastro et al., 1997; Labruna, 2004; Kurtti et al., 2005). Due to the strictly intracellular lifestyle of rickettsiae, genetic manipulation of this pathogen is a laborious task, thus hampering the identification of virulence factors necessitating further research in order to completely understand the pathogenicity of rickettsiae.

1.4 Interaction of rickettsiae with host cells

As previously described, pathogenic tick-borne rickettsiae are transmitted to humans by the bite of an infected tick. The transmission usually occurs several hours after tick attachment to the skin. From the portal of entry in the skin, rickettsiae spread via the blood stream (Figure 1.10) to body organs including skin, heart, liver, kidneys, lungs, pancreas, gastrointestinal tract and brain. In each site, they attach, enter and proliferate in vascular endothelial cells (Walker, 1996).

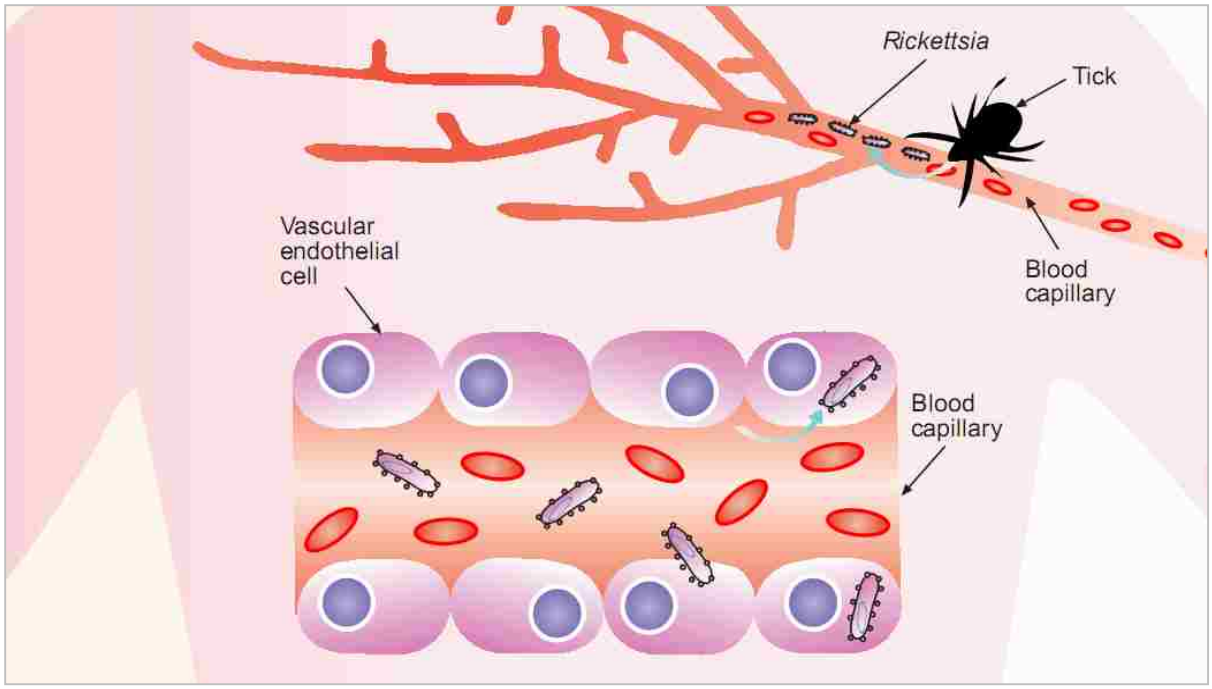

Figure 1.10 Transmission of rickettsiae to humans. Rickettsiae are inoculated into human skin during the bite of infected tick. Rickettsiae spread through the blood stream to body organs and eventually infect the endothelial cells. Figure from www.immunopaedia.org.

1.4.1 Rickettsial infection of mammalian host cells

Although the principal target cells of the genus *Rickettsi*a are endothelial cells, they have been shown to invade several cell types in vitro such as mouse lymphoblasts (Cohn et al., 1959), sheep erythrocytes (Ramm and Winkler, 1973), chicken embryo cells, mouse fibroblast L-929 cells (Wisseman et al., 1976), enucleated L-929 and chicken embryo cells (Stork and Wisseman, 1976), macrophage-like-cell lines (Turco and Winkler, 1982), human endothelial cells (Walker et al., 1982), Vero cells (Teysseire et al., 1995), XTC-2 toad tadpole cells (Raoult et al., 2001), ISE6 tick cell line (Pornwiroon et al., 2006) and mosquito cell lines (Horta et al., 2006; Sakamoto and Azad, 2007). The main steps of rickettsial infection including rickettsial entry, intracellular survival and host actin-based rickettsial cell-to-cell spread are described in the following sections.

1.4.1.1 Rickettsial entry

Spotted fever group rickettsiae have been shown to actively induce their own uptake into mammalian host cells by a receptor-mediated invasion strategy. By using this strategy, which is also called the zipper mechanism, rickettsiae express surface proteins able to interact with host-cell surface receptors and induce host intracellular signals. These signals lead to cytoskeleton rearrangements and membrane extension which result in bacterial uptake through a "zippering" mechanism (Figure 1.11) (Alonso and Portillo, 2004).

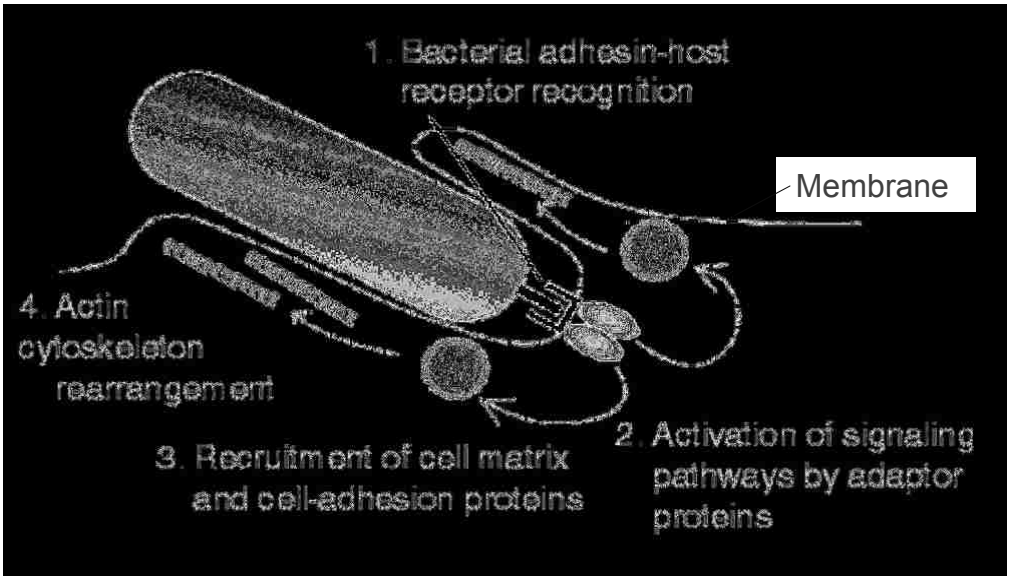

Figure 1.11 Zipper mechanism mediated bacterial entry into eukaryotic cells. Figure from Alonso and Portillo, 2004.

Two well-known bacteria species have been found to use this zipper mechanism of entry; *Yersinia pseudotuberculosis* uses YadA and invasin while *Listeria monocytogenes* uses InlA and InlB to bind host transmembrane proteins and activate the signal cascades that facilitate their internalization into mammalian cells (Alonso and Garcia-del Portillo, 2004; Cossart and Sansonett,i 2004; Veiga and Cossart, 2005; Veiga and Cossart, 2007). In 1995, transmission electron microscopy was used to observe *R. conorii* entry into Vero cells. As shown in figure

1.12, *R. conorii* used a strategy that resemble the zipper mechanism to facilitate its entry into the host cells (Teysseire et al., 1995).

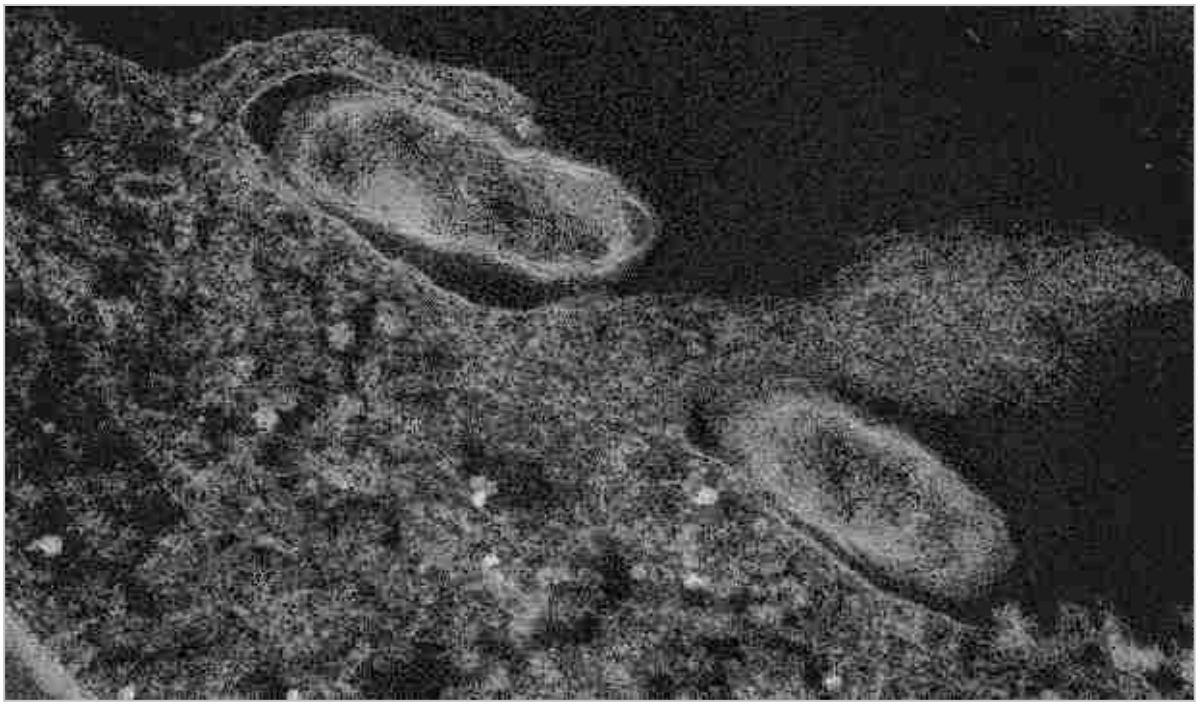

Figure 1.12 *Rickettsia conorii* entry into Vero cell. Figure from Teysseire et al., 1995.

1.4.1.1.a Rickettsial ligands

Since the zipper mechanism was proposed as the mechanism of rickettsial entry into host cells, much research has focused on identification and characterization of rickettsial surface proteins. Four outer membrane-associated rickettsial proteins which include OmpA (Sca0), OmpB (Sca5), Sca1 and Sca2 have been reported to facilitate rickettsial adherence to and/or invasion of mammalian host cells. These proteins are described as follows:

(1) Rickettsial OmpA (rOmpA): rOmpA is an immunodominant surface-exposed protein that is found in only spotted fever group rickettsiae. The size of this protein is found to vary from species to species; *R. rickettsia* strain Sheila Smith expresses a 247 kDa OmpA while *R. conorii* strain Malish 7 expresses 224 kDa OmpA. The critical role of rOmpA in rickettsial

adherence has been demonstrated in *R. rickettsii* using antibody inhibition assays (Li and Walker, 1998). The results showed that monoclonal antibodies against *R. rickettsii*, specifically the Fab fragment of these antibodies, blocked rickettsial attachment to L-929 cells. In addition, rOmpA extracted from *R. rickettsii* competitively inhibited the attachment of *R. rickettsii* to host cells (Li and Walker, 1998). Recently, Hillman et al. (2012) have demonstrated the role of rOmpA in *R. conorii* invasion of human endothelial cells using *Escherichia coli* as an expression host for heterologous rOmpA production. The *E. coli* expressing rOmpA at the outer membrane was then used to infect host cells and the ability of the bacteria to bind and invade the cells were assessed. The results showed that binding of rOmpA to host cells facilitate the invasion of the bacteria.

(2) Rickettsial OmpB (rOmpB): rOmpB is a surface rickettsial protein that is associated with both antigenicity and pathogenicity of rickettsiae. The protein is conserved among all species of *Rickettsia* and is expressed as a 168 kDa protein. The pre-protein then undergoes post-translational protease modification to the mature 120 kDa domain which associates with the outer leaflet of rickettsial outer membrane (Hackstadt et al., 1992). The role of rOmpB in bacterial adherence and invasion of host cells was investigated by expression of rOmpB on inert *E. coli*. The expression of rOmpB allows bacterial adherence to and invasion of non-phagocytic cells (Uchiyama et al., 2006). In 2005, rOmpB was identified as a ligand interacting with mammalian host cell receptor, Ku70 (Martinez et al., 2005).

(3) Sca1: Sca1 is a surface-exposed autotransporter protein that is found in nearly all rickettsiae. The predicted size of Sca1 protein ranges from 594 to 1976 amino acids (Ngwamidiba et al., 2006). Recently, Sca1 has been shown to be expressed on the surface of *R. conorii* with an approximate size of 120 kDa (Riley et al., 2010). The role of *R. conorii* Sca1 has

been investigated by heterologous expression of the protein in *E. coli* cells. The results have shown that *E. coli* expressing *R. conorii* Sca1 has the ability to attach to but not invade host cells (Riley et al., 2010). The function of Sca1 in *R. conorii* adherence to host cells has been confirmed by protein blocking assay. In this assay, pre-incubation of Sca1 protein fragment with *R. conorii* inhibited bacterial attachment to host cells (Riley et al., 2010).

(4) Sca2: Rickettsial Sca2 is found in most of spotted fever group rickettsiae. The size of the protein is approximately 200-220 kDa. The protein has a conserved autotransporter domain, predicted to be exposed on the rickettsial surface, and is expressed during infection of mammalian cells (Ngwamidiba et al., 2005; Cardwell and Martinez, 2009). The Sca2 protein has been shown to mediate rickettsial adherence to and invasion of host cells by heterologous expression in *E. coli*. *E. coli* expressing Sca2 was shown to have ability to adhere and invade many non-phagocytic mammalian cell types including human endothelial cells which are the main targets of rickettsial infection. Furthermore, the pre-incubation of Sca2 protein fragment with *E. coli* or *R. conorii* has been shown to inhibit the invasion of both bacterial species (Card well and Martinez, 2009).

In addition to the four rickettsial surface proteins described above, rickettsial adhesins, Adr1 and Adr2, have been identified as putative interactors of cellular membrane proteins (Renesto et al., 2006). These 30 kDa rickettsial proteins are conserved and ubiquitously found in the genome of rickettsiae. BLAST analysis showed the homologies between these proteins and some of other bacterial adhesins including the adhesin/virulence factor Hek of *E. coli*, the putative invasin of *Lawsonia intracellularis*, and the possible outer membrane adhesin of *Salmonella enteric* subsp. Moreover, Adr1 has been demonstrated to facilitate *R. conorii* entry by antibody blocking assay (Baraj et al., 2009).

1.4.1.1.b Host cell receptors and proteins involved in rickettsial invasion

As mentioned above, the only mammalian host cell receptor identified to interact with rickettsial ligand (rOmpB) is Ku70. Involvement of Ku70 in *R. conorii* invasion was demonstrated by fluorescence microscopic analysis in which localization of Ku70 to bacterial entry sites was observed. Furthermore, the inhibition of endogenous Ku70 by siRNA impaired rickettsial internalization into host cells (Martinez et al., 2005). Ku70 is a subunit of DNA-dependent protein kinase that is found in the nucleus, cytoplasm, plasma membrane and lipid raft microdomains. The expression of Ku70 in the plasma membrane is limited to specific cell types such as endothelial cells, monocytes and macrophages, which are the primary target cells of rickettsiae during infection. Ku70 is also found to be expressed in the plasma membrane of cell lines such as HeLa and Vero cells (Muller et al., 2005). Recently, studies using *E. coli* expressing *R. conorii* OmpA revealed α2β1 integrin as a mammalian ligand that interact with rOmpA. The OmpA-α2β1 integrin interaction was illustrated as the OmpB-Ku70 independent pathway (Hillman et al., 2012).

Besides a receptor for rickettsial ligand, a number of mammalian host cell proteins required for *R. conorii* invasion have been identified by various methods: Immunofluorescence microscopic analysis revealed the localization of the small GTP-binding protein, Cdc42, and the actin nucleating protein, Arp2/3 complex to the sites of rickettsial invasion. Functional inhibition of Arp2/3 complex or Cdc42 by transfection of an Arp2/3 binding derivative of the WASP family proteins, Scar or a dominant-negative variant of Cdc42, respectively impaired rickettsial invasion (Martinez and Cossart, 2004). Together, these findings indicate the involvement of Cdc42 and Arp2/3 complex in the *R. conorii* invasion process. Further examination of signaling pathways modulating the activity of Arp2/3 complex and Cdc42 by

pharmacological inhibition demonstrated that *R. conorii* invasion is dependent on phosphatidylinositol (PI) 3-kinase, protein tyrosine kinase (PTK), Src-family kinase and actin polymerization. In addition, immunofluorescence microscopy showed that c-Src and its downstream target, cortactin were recruited to rickettsial entry sites. Moreover, tyrosine phosphorylation of focal adhesion kinase (FAK), a protein mediating cytoskeleton reorganization and invasion of other pathogen (Persson et al., 1997; Martinez et al., 2000) was detected during rickettsial invasion of the host cells. Recently, a study of rOmpB-Ku70 interaction revealed additional host proteins that facilitate invasion of *R. conorii* into mammalian cells. Depletion of the components of endocytic pathway, c-Cbl ubiquitin ligase, clathrin and caveolin-2 by siRNA diminished rOmpB-mediated rickettsial invasion of host cells (Chan et al., 2009). By a biochemical inhibition approach, microtubule infrastructure has also been reported in association with the invasion of *R. conorii* into mammalian cells.

1.4.1.1.c Model of rickettsial invasion of mammalian host cells

After inoculation into the blood vessels by the bite of infected ticks, rickettsiae spread via the blood stream to infect endothelial cells. The surface membrane proteins (e.g. OmpB, OmpA, Sca1 and/or Sca2 of rickettsiae) bind to ligands on mammalian host cells; OmpB and OmpA bind to Ku70 and α2β1 integrin, respectively and the receptors for Sca1 and Sca2 are unknown. The interaction of rickettsial protein(s) and host cell ligand(s) activate a cascade of signaling events. Initially, c-Cbl ubiquitin ligase ubiquitinates Ku70. After ubiquitination, the signaling molecules, including Cdc42, PTKs, PI 3-Kinase, Src-family tyrosine kinases, FAK, and cortactin, coordinately activates the actin nucleating complex, Arp2/3. This results in the induction of actin polymerization and predicted localized recruitment of the components of endocytic pathway including clathrin and caveolin-2 at the bacterial entry site (Chan et al.,

2010). Ultimately, the reorganization of the actin cytoskeleton results in membrane extrusion leading to the bacterial uptake (Figure 1.13).

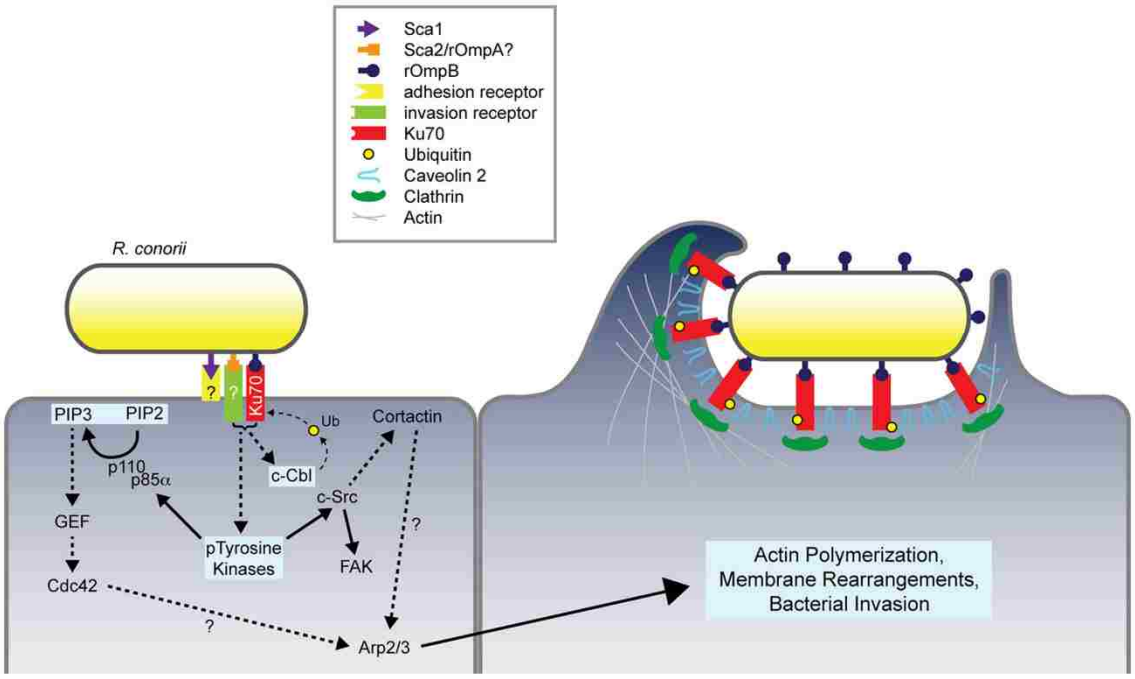

Figure 1.13 Schematic diagram demonstrated *R. conorii* invasion of non-phagocytic mammalian cells. The interaction of *R. conorii* protein surfaces and host cell receptors leads to the activation of signaling cascade resulting in actin polymerization at the bacterial entry foci. The blue boxes show the pathways and proteins activated by OmpB-Ku70 interaction. Dashed arrows and question marks represent putative protein interplay during bacterial invasion. Figure from Chan et al., 2010.

1.4.1.2 Intracellular survival of rickettsiae

After entering into mammalian cells, rickettsiae avoid destruction within the endosomal pathway by escaping from phagosomal vacuoles into the host cytoplasm. The molecular mechanism involved in phagosomal escape of rickettsiae still remains unknown; however, hemolysin and phospholipase enzymes have been hypothesized to facilitate this process (Silverman et al., 1992; Radulovic et al., 1999; Renesto et al., 2003). The involvement of phospholipase A_2 in phagosomal escape has been proposed for *R. rickettsii*, *R. conorii* and *R. prowazekii* (Winkler and Miller, 1982; Walker et al., 2001). In genomic analyses, the genes with

potential membranolytic activity including *tlA*, *tlC*, *pat1* and *pld*, respectively encoding for hemolysin A, hemolysin C, patatin B1 precursor (endowed for phospholipase A_2) and phospholipase D have been found in *R. conorii*, *R. prowazekii* and *R. typhi* genomes (Andersson et al., 1998; Ogata et al., 2001; McLeod et al., 2004). Nevertheless, only *tlC* and *pld* were shown to be transcribed during the period of active phagosomal escape (Whitworth et al., 2005). In addition, heterologous expression of *tlC* and *plD* genes in *Samonella*, which replicates in the endosome, facilitated bacterial escape from the phagosomal vacuole to the host cell cytosol (Whitworth et al., 2005). In *R. prowazekii*, the *plD* mutant, generated by site-directed mutagenesis, exhibited attenuated virulence in infected guinea pig (Driskell et al., 2009). These evidences suggest that rickettsiae use hemolysin C and phospholipase D in order to escape from the phagosome.

1.4.1.3 Actin-based motility

After invasion of the host and entry into the cytosol, spotted fever group *R. conorii*, *R. rickettsii*, *R. montanensis*, *R. parkeri*, *R australis*, and *R. monacensis* have been shown to induce actin polymerization at their surface to promote motility and cell-to-cell spread (Figure 1.14) (Teysseire and Raoult, 1992; Heinzen et al., 1993; Baldridge et al., 2005).

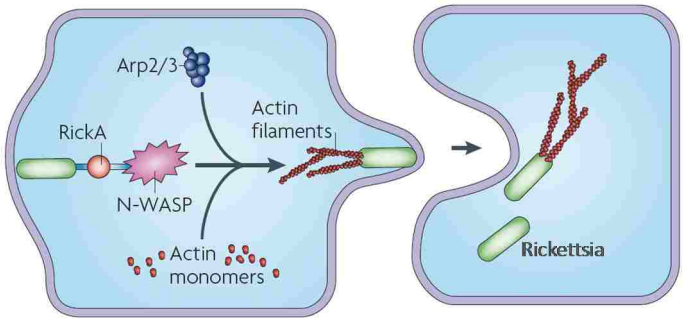

Figure 1.14 Schematic diagram of actin-based motility system and cell-to-cell spread of spotted fever group rickettsiae. The activation of Arp2/3 complex by RickA initiate actin polymerization on rickettsial surface resulting in the movement in the cytosol and spread to adjacent cell of rickettsiae. Figure is modified from Walker and Ismail 2008.

Genomic analysis of *R. conorii* has identified the *rickA* gene which encodes a protein that can activate the activities of actin nucleating and Y-branching of the Arp2/3 complex; however this gene was not found in *R. prowazekii*, which does not have actin tails (Gouin et al., 2004; Jeng et al., 2004). The RickA protein contains a proline-rich domain, WASP (Wiskott-Aldrich syndrome protein) homology 2 (WH2) domain, which binds to actin monomers, and central and acidic regions which bind to the Arp2/3 complex (Figure 1.15) (Gouin et al., 2004; Jeng et al., 2004).

Figure 1.15 Schematic representation of RickA protein. Rick A contain a proline-rich domain (PP) as well as WASP homology 2 (WH2), central (C) and acidic (A) domains. Figure from Goley and Welch, 2006.

The activation of the Arp2/3 complex leads to actin polymerization and induction of the reorganization of the Y-branched network into unbranched filaments (Jeng et al., 2004). The propulsive force generated by actin tail assembly push the rickettsiae to the surface of the host cell and enables the host cell membrane to protrude into the adjacent cell. The protrusion is taken up, along with the rickettsiae within it and results in the formation of the two membrane vacuoles. Disruption of the double membrane vacuoles then allows the rickettsiae to enter into the cytoplasm of the adjoining cell (Goldberg, 2001). However, there is conflicting evidence which includes the unbranched organization of *Rickettsia* actin tails (Heinzen et al., 1999; Van Kirk et. al., 2000), the failure to observe the Arp2/3 complex in the actin filaments (Gouin et al., 1999; Harlander et al., 2003; Heinzen, 2003; Serio et al., 2010), and the ability of rickettsiae to move in Arp2/3 complex knock down cells (Harlander et al., 2003; Heinzen, 2003; Serio et al., 2010) that indicate an Arp2/3 complex independent manner of *Rickettsia* actin-based motility.

Recently, a transposon mutagenesis study has shown that a *R. rickettsii* Sca2 mutant is unable to generate an actin tail or spread directly from cell to cell (Kleba et al., 2010). The sequence analysis of Sca2 revealed domains similar to those found in eukaryotic nucleating proteins including putative actin-binding WH2 domains and formin homology 1-like domain (Haglund et al., 2010; Kleba et al., 2010); formin is another class of actin-nucleating proteins that assemble unbranched actin filaments (Evangelista et al., 2002; Pruyne et al., 2002). Moreover, functional study of *R. parkeri* Sca2 has shown the localization of the protein to actin-polymerizing surfaces of the bacteria as well as the ability to polymerize actin monomers and to elongate actin filaments *in vitro* (Haglund et al., 2010). These finding suggest that *R. parkeri* use their surface protein, Sca2 to mimic the activity of host actin nucleating protein, formin, in order to facilitate actin-based motility. Whether rickettsiae polymerize their actin tails by using host actin nucleators or mimic the activity of the nucleating proteins or both needs to be further investigated.

1.4.1.4 Host defense against rickettsiae

Since rickettsiae are transmitted through the dermis, resident dendritic cells (DCs) are thought to play a role in both innate and adaptive immunity against rickettsiae. Jordan et al., (2009) has proposed the role of DCs in initiation of innate immunity during *in vivo* infection with *R. conorii*. As shown in Figure 1.16, DCs recognize rickettsiae through Toll-like receptor (TLR) 4 and TLR4 ligation promotes the activation and proliferation of natural killer (NK) cells. Subsequently, interferon-γ (IFN-γ) secreted by NK cells and tumor necrosis factor-α (TNF-α) secreted by endothelium and macrophages cooperatively activate nitric oxide synthase 2 (NOS2) leading to the production of rickettsiacidal nitric oxide (Jordan et al., 2009).

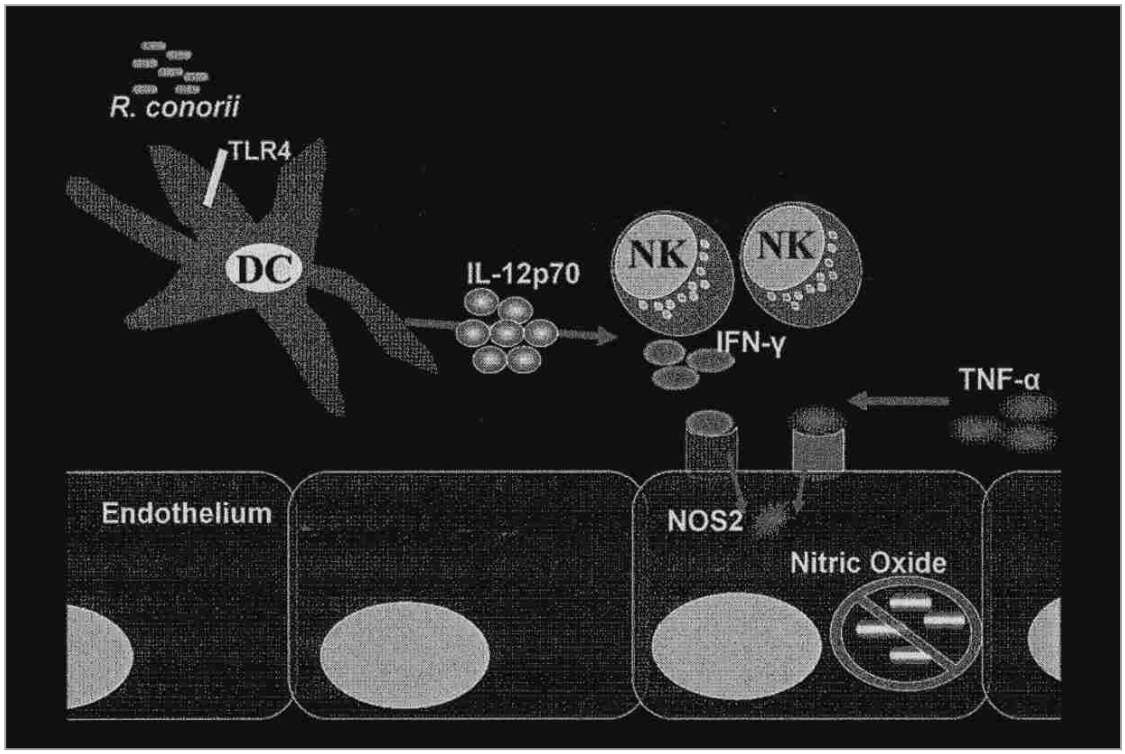

Figure 1.16 Schematic representation of proposed mechanisms by which dendritic cells (DCs) mediate activation of natural killer (NK) cells *in vivo*. Toll-like receptor (TLR) 4-stimulated DCs produce IL-12 that promotes NK cells activation and proliferation and subsequent IFN-γ production. Together with TNF-α (secreted by endothelium and macrophage), IFN-γ activate nitric oxide synthase 2 (NOS2) that induce intracellular killing of rickettsiae by nitric oxide production. Figure from Jordan et al., 2009.

Recently, a study of cell activation in response to *R. akari* has shown the involvement of TLR2 in addition to TLR4 in recognition of rickettsiae (Quevedo-Diaz et al., 2010).

Currently, the concept of rickettsial immunity is not completely defined; however, a model of protective immunity in rickettsial infections has been proposed based on *in vitro* and in *vivo* studies in an animal model of mild disseminated spotted-fever rickettsial disease caused by sublethal rickettsial infection (Walker and Ismail, 2008). As shown in Figure 1.17, once encountering rickettsiae in peripheral tissues (for example skin and lung), immature DCs undergo maturation, in which the major histocompatibility complex (MHC) and the co-stimulatory molecules CD40, CD80 and CD86 are upregulated. They then migrate to secondary

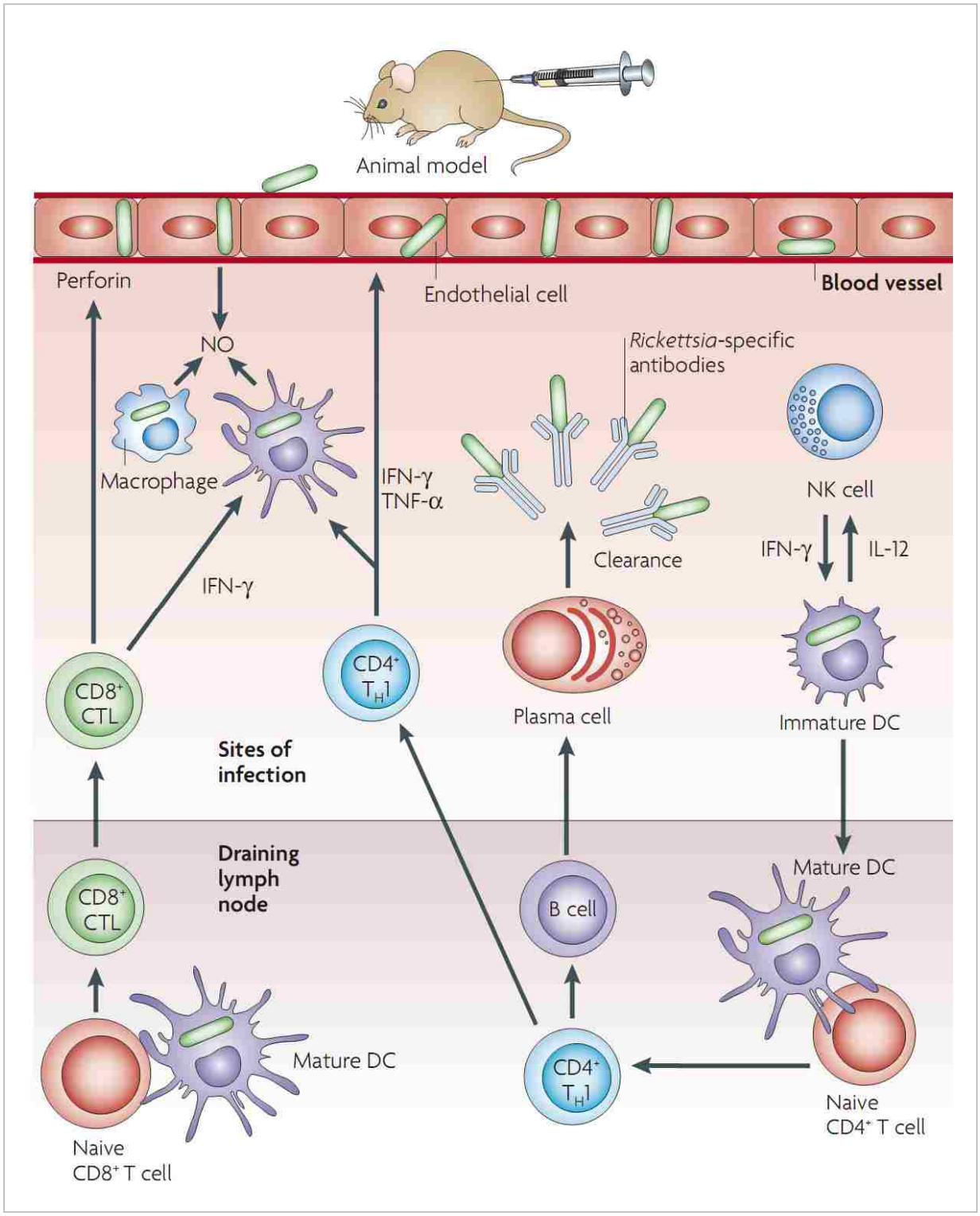

Figure 1.17 Hypothetical model of host response to rickettsial infection. Figure from Walker and Ismail, 2008.

lymphoid tissues such as draining lymph nodes. Additionally, IFN-γ and TNF-α secreted by NK cells or infected endothelial cells could enhance DCs maturation which is indicated by interleukin-12 (IL-12) secretion. This IL-12, in turn, activates NK cells to produce IFN-γ; IL-12 mediated activation of NK cells is important for initial clearance of rickettsiae.

At lymphoid tissues, rickettsiae-infected DCs present rickettsial antigen to naïve $CD4^+$ and $CD8^+$ T lymphocytes and provide co-stimulatory signals that activate T cells. After activation, $CD4^+$ and $CD8^+$ T cells undergo proliferation and differentiation into effector cells that produce IFN-γ and TNF-α. In addition to activation by DCs, $CD4^+$ T helper 1 (T_H1) cells could be induced, in part, by IL-12. T_H1 cells provide help for B-cell proliferation and differentiation into antibody-secreting cells (plasma cells) thus induces rickettsiae specific antibody production which is important for host defense against re-infection. Interestingly, rickettsiae-infected DCs has been shown to activate $CD8^+$ T cells *in vitro* in the absence of $CD4^+$ T_H1 cells (Jordan et al., 2007); however, the experiments in which T cells were depleted or adoptively transferred into mice demonstrated that $CD4^+$ T_H1 cells facilitate protective immunity against rickettsiae through the activation of $CD8^+$ cytotoxic T lymphocytes (CTL) (Feng et al., 1997).

Clearance of rickettsiae in blood and sites of infection are mediated by differentiated B and T lymphocytes that migrate back from lymphoid tissues. Lymphocytes migration is promoted by chemokines production e.g. CXC-chemokine ligand 9 (CXCL9) and CXCL10 produced by infected endothelial cells. Additionally, IFN-γ and TNF-α, secreted from $CD4^+$ T_H1 cells and CTL, enhance the production of intracellular rickettsiacidal molecules (such as nitric oxide) by infected target cells (principally endothelial cell, DCs and macrophages) and therefore promote killing capacity of these cells. Furthermore, killing mechanism of $CD8^+$ T cells by perforin-mediated pathway is required for elimination of rickettsiae-infected target cells.

1.4.2 Rickettsial infection of tick cells

While ticks serve as vectors and reservoirs for many *Rickettsia* species, little research has directly explored tick-rickettsiae interaction. By differential display-display PCR, nine putative tick proteins including clathrin-coated vesicle ATPase, peroxisomal farnesylated protein, Ena/vasodilator-stimulated phosphoprotein-like protein, α-catenin, tubulin α-chain, copper-transporting ATPase, salivary gland protein SGS-3 precursor, glycine-rich protein, and Dreg-2 protein were indentified in response to *R. montanensis* infection of *D. variabilis* ticks; however, the role of these proteins in rickettsial invasion are currently undetermined (Macaluso et al., 2003).

In 2010, Thepparit et al. demonstrated the involvement of histone H2B in *R. felis* internalization of a tick cell line. Histone H2B is one of the core histones that forms the nucleosome complex with histone H1. Although the primary location of histone is the cell nucleus, there is evidence that shows the localization of histone H2B in other cellular compartment including the cytoplasm and cell surface (Zlatanova et al., 1990; Khan et al., 1998; Herren et al., 2006; Das et al., 2007; Theparrit et al., 2010). Co-immunoprecipitation of histone H2B and *R. felis* protein lysate showed the interaction of histone H2B and *R. felis* OmpB protein. RNAi-mediated histone H2B-depletion or enzymatic treatment of histones reduced *R. felis* invasion of a tick cell line. Collectively, these results suggest that histone H2B mediate invasion of *R. felis* into tick cells; however, the molecular mechanism(s) whereby histone H2B mediates the internalization of rickettsiae into tick cells is still unknown.

As mentioned earlier, SFG *Rickettsia* is maintained in nature via transovarial transmission in ticks and transstadial transmission during tick infestation (Sonenshine et al., 2005). Although ticks serve as both vectors and reservoirs for SFG *Rickettsia*, many questions

regarding SFG *Rickettsia*-tick cell interaction remain unresolved. Because *Rickettsia* is maintained vertically by tick vectors, it is necessary to study the interactions between ticks and *Rickettsia* to completely understand the epidemiology of tick-borne rickettsial diseases.

The overall goal of this research is to study the interaction between *Rickettsia* and tick vectors, specifically the molecular mechanisms underlying the entry of *Rickettsia* into tick cells. Previous studies in mammalian and *Drosophila* cells have revealed the signaling molecules involved in the uptake of *Rickettsia* into host cells (Martinez and Cossart, 2004; Martinez et al., 2005; Chan et al., 2009; Hillman et al., 2012; Reed et al., 2012). As SFG *Rickettsia* can move between vertebrate and invertebrate hosts, the hypothesis for this study is that conserved mechanisms are utilized for host cell invasion. Toward the tested hypothesis, the objectives of this study are to identify (chapter 2) and further characterize (chapter 3 and chapter 4) the tick molecules central to *Rickettsia* invasion of host cells. Ultimately, pathway activated during *Rickettsia* internalization of tick cells is proposed (chapter 5).

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CHAPTER 2
SIGNAL TRANSDUCTION EVENTS INVOLVED IN RICKETTSIAL INVASION OF TICK CELLS

2.1 Introduction

Tick-borne *Rickettsia* species are obligate intracellular bacteria with varying pathogenicity in humans. A major component of pathogenesis is transmissibility by the tick host. One such tick, *Dermacentor variabilis*, is a known vector for the etiologic agent of Rocky Mountain spotted fever, *Rickettsia rickettsii*, (Sonenshine et al., 2005) and non-pathogenic *R. montanensis* (Feng et al., 1980; Anderson et al., 1986; Pretzman et al., 1990; Ammerman et al., 2004). Interestingly, the majority of *Rickettsia* detected in infected *D. variabilis* throughout the United States are non-pathogenic *Rickettsia* such as *R. montanensis* (Azad and Beard, 1998). Likewise, the results from field studies demonstrated a lower infection rate of *R. rickettsii* compared to non-pathogenic *Rickettsia* in wild-caught *D. andersoni*, the Rocky Mountain wood ticks (Burgdorfer et al., 1981; Burgdorfer, 1988). Because ticks can acquire *Rickettsia* during feeding and non-pathogenic *Rickettsia* are prevalent in infected ticks in nature, the current research was designed to study the interaction between non-pathogenic *R. montanensis* and tick cells.

As described above, *Rickettsia* are obligate intracellular bacteria, therefore invasion of host cells is a critical step for subsequent bacterial survival. Invasion of host cells by *Rickettsia* has been studied and signaling molecules have been identified in mammalian and *Drosophila* cell lines. After binding to mammalian host receptors, *R. conorii* induces signaling cascades in which Ku70 is initially ubiquitinated by c-Cbl ubiquitin ligase. Subsequently, signaling molecules including Cdc42, protein tyrosine kinases (PTKs), phosphatidylinositol-3'-kinase (PI 3-Kinase), src-family tyrosine kinases, focal adhesion kinase (FAK), and cortactin, coordinately activate the actin nucleating complex, Arp2/3. This leads to actin polymerization and

recruitment of the components of endocytic pathway including clathrin and caveolin-2 at the bacterial entry site. Hereafter, the rearrangement of the actin cytoskeleton results in membrane extrusion leading to bacterial internalization into host cells (Martinez and Cossart, 2004; Martinez et al., 2005; Chan et al., 2009; Chan et al., 2010). Furthermore, studies of *R. parkeri* invasion have demonstrated differences between host factors that are important for rickettsial uptake in *Drosophila* versus mammalian cells. In *Drosophila* cells, the GTPases Rac1 and Rac2, the WAVE nucleation-promoting factor complex and the Arp2/3 complex were identified to be important. The requirement of the Arp2/3 complex was also found in mammalian cells, while the acquirement of WAVE2 and Rho GTPases depended on the specific cell type (Reed et al., 2012).

Although ticks serve as both vectors and reservoirs for the spotted fever group (SFG) *Rickettsia*, little is known about rickettsial interactions with tick vectors at the cellular level. Delineating the molecular mechanisms of rickettsial infection in tick vectors is critical to understanding the ecology of tick-borne rickettsial diseases. Due to the ability of SFG *Rickettsia* to move between vertebrate and invertebrate hosts, the hypothesis for this study is that conserved mechanisms are utilized for cell invasion in both hosts. By using biochemical inhibition assays, the tick signaling molecules involved in *R. montanensis* invasion were identified. Ultimately, the pathway activated during the uptake of *R. montanensis* into tick cells is proposed.

2.2 Materials and methods

2.2.1 Materials

All inhibitors used in this study were purchased from EMD chemical (Billerica, MA). The inhibitors are listed in Table 2.1. DMSO was obtained from Sigma-Aldrich (St. Louis, MO).

Table 2.1 Inhibitors used in inhibition assays.

Inhibitor name	Tick target molecule(s)
Zygosporium mansonii Cytochalasin D	Actin polymerization
CK-666	Arp2/3 Complex
187-1	N-WASP
Clostridium difficile Toxin B	Rho GTPases (Rho, Cdc42, Rac)
Rac1 inhibitor	Rac1
Genistein	General PTKs
PP2	Src family PTKs
Focal Adhesion Kinase Inhibitor I	FAK
PI 3-K Inhibitor XI, HWT	PI 3-kinase

2.2.2 Tick cell culture

D. variabilis (DVE1) cells (kindly provided by Dr. Timothy Kurtti, University of Minnesota) were cultured in L15C medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Waltham, MA), 5% tryptose phosphate broth (Difco, Sparks, MD), 0.1% lipoprotein-cholesterol concentrate (LPC, MP Biomedicals, Santa Ana, CA), 0.6% HEPES solution (1 M, Sigma-Aldrich), and 1.2% sodium bicarbonate solution (5%, Sigma-Aldrich). The cells were maintained in a humidified incubator at 32 °C. Vero cells were grown in DMEM high glucose (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum and maintained in a humidified incubator with 5% CO_2 at 34 °C. For both cell lines, conditioned medium was replaced with new medium once a week. DVE1 cells were subcultured (1:3) once a month and Vero cells were passaged (1:6 or 1:12) every 1-2 weeks.

2.2.3 *Rickettsia* culture and purification

As described by Sunyakumthorn et al. (2012), *R. montanensis* was grown in Vero cells and maintained in a humidified 5% CO_2 incubator at 34 °C. Half of the conditioned medium was replaced with new medium once a week. Infected cells were subcultured (1:12) every 2 weeks by inoculating *Rickettsia*-infected cells to uninfected Vero cells. For each experiment, *Rickettsia* were purified as described by Weiss et al. (1973) with minor modification. Briefly, *Rickettsia* infected cells were detached using a sterile cell scraper (Sarstedt, Newton NC) and lysed by vortexing with sterile 3 mm borosilicate glass beads (Sigma-Aldrich) for 5 min. Cell lysate was then transferred aseptically to 15 ml centrifuge tubes and centrifuged at 4ºC, 16000 x g for 3 min to pellet cellular debris. The supernatant was transferred to a 10 ml syringe and filtered through a 2 µm syringe filter. For all bioassays, the number of *Rickettsia* was determined by counting *Rickettsia* stained with a LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Carlsbad, CA) in a Petroff–Hausser bacterial counting chamber (Hausser Scientific, Horsham, PA) and examined with a Leica microscope (Buffalo Grove, IL).

2.2.4 Rickettsial internalization assays

DVE1 cells (1 x 10^5) were seeded onto 96-well plates and incubated at 32 °C for 48 h. The cells were treated with three different concentrations of inhibitor targeting tick molecules of interest or inhibitor vehicle (complete L15C medium or medium containing 0.1% DMSO) for 2 h. *Rickettsia montanensis* was then inoculated onto the treated cells at a MOI of 10 and the plate was centrifuged at 700 x g for 2 min to facilitate the binding of *Rickettsia* to host cells. After 1 h, *Rickettsia* were removed and the cells were added with 150 µl PBS. The samples were centrifuged at 275 x g for 4 min to collect only infected host cells. After removal of supernatant, the cell pellet was washed with 1 ml PBS and centrifuged at 275 x g for 4 min. The samples were

stored at -20 ºC until used for genomic DNA (gDNA) isolation. According to the manufacturer's instructions, DNeasy Blood & Tissue Kit (QIAGEN, Germantown, MD) was used to extract gDNA from the samples. At the final step, gDNA was eluted in 35 µl DNase/RNase free water. Numbers of *Rickettsia* and tick cells were then quantified by probe-based quantitative PCR (qPCR). The experiments were performed in quadruplicate for each concentration of the inhibitor used and the results were the combination of two independent experiments.

To determine the effect of DMSO on rickettsial internalization of tick cells, different concentrations of DMSO (0.1% versus 1%) were tested prior to performing biochemical inhibition assays. The experiment was done one time with triplicate wells.

To examine whether rickettsial invasion of tick cells occur through an active process, the internalization assays were carried out, in triplicate, using either live or formalin-fixed *Rickettsia*. The result is the combination of the two independent experiments.

2.2.5 Construction of a standard reference plasmid for qPCR

A standard reference plasmid containing portions of *R. montanensis* ompB (RmOmpB) and *D. variabilis* calreticulin (DvCRT) genes was generated and used to create a standard curve in qPCR assays as described by Sunyakumthorn (dissertation, 2011). Briefly, fragments of DvCRT (132 base-pair, bp) and RmOmpB (106 bp) were amplified using CRT*Dv*321F$_{xbaI}$ (5'-AAAAAATCTAGAAGGAGAAAAGCAAGGGACTG-3')/CRT*Dv*452R (5'CAATGTTCTGC TCGTGCTTG-3') and OmpB*Rm*2832F (5'GCGGTGGTGTTCCTAAT AC-3')/ OmpB*Rm*2937R$_{xbaI}$ (5'-AAAAAATCTAGACCTAAGTTGTTATAGTCTGTAGTG-3') primer pairs, respectively (Sunyakumthorn; dissertation, 2011). The amplicons were then digested with *Xba*I (New England BioLabs, Ipswich, MA) and ligated together. The ligation product was

amplified using OmpB*Rm*2832F (5'-GCGGTGGTGTTCCT AATAC-3') and CRT*Dv*452R (5'-CAATGTTCTGCTCGTGCTTG-3') primers, cloned into pCR4-TOPO vector (Invitrogen) and transformed into TOP10 *E. coli* (Invitrogen). The plasmid was isolated using FastPlasmid Mini Kit (Eppendorf, Hauppauge, NY) according to manufacturer's instruction and submitted to Genelab at Louisiana State University, School of Veterinary Medicine for sequencing. A schematic plasmid map is shown in Figure 2.1.

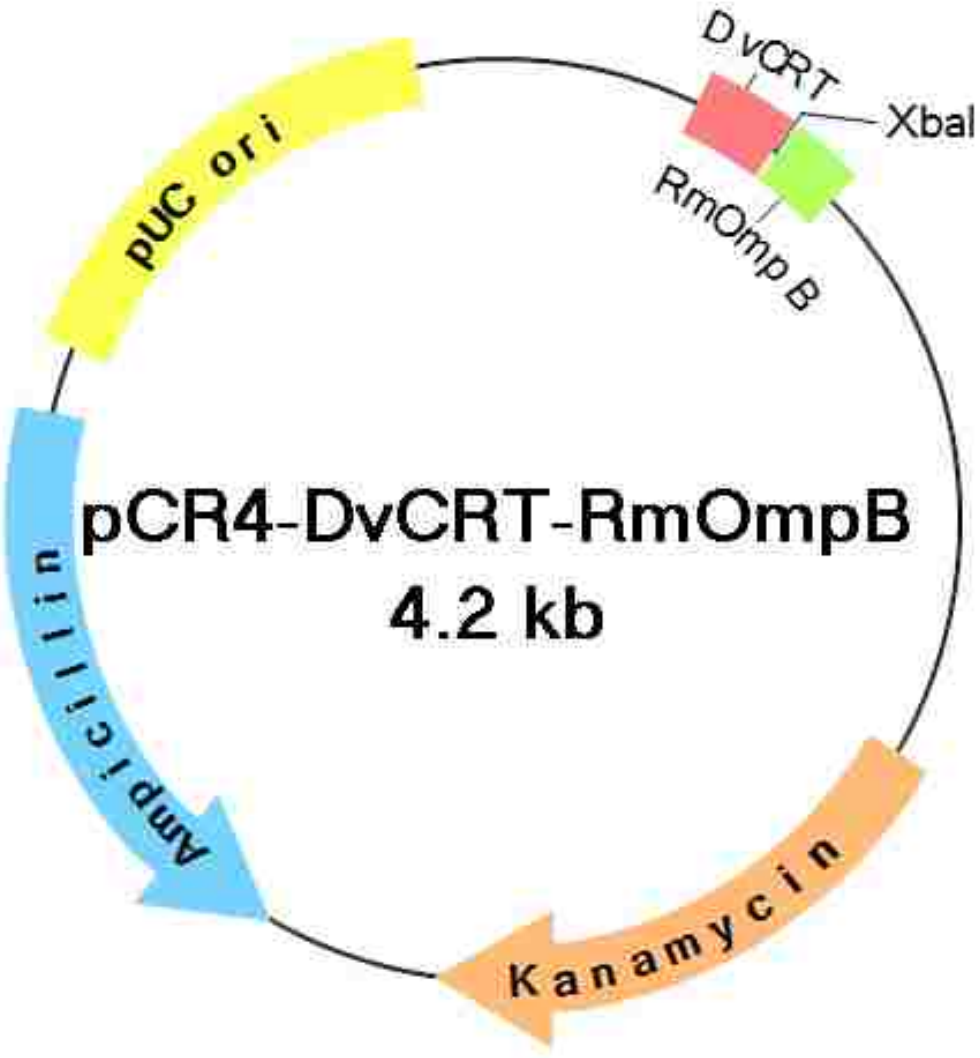

Figure 2.1 A schematic map of a standard reference plasmid (pCR4-DvCRT-RmOmpB) used in qPCR assays. Fragments of DvCRT (132 bp) and RmOmpB (106 bp) were amplified, digested with *Xba*I and ligated together. The ligation product was amplified before cloning into pCR4-TOPO vector. The schematic plasmid map was created using BVTech Plasmid software.

Before using as a standard curve in qPCR assays, the plasmid was linearlized by *Xba*I and diluted to create a ten-fold serial dilution of a standard reference plasmid.

2.2.6 Quantification of *Rickettsia* and tick cells by qPCR

To quantify a number of *Rickettsia* and tick cells in samples, probe based qPCR was used as described by (Thepparit et al., 2011). Briefly, qPCR reactions were prepared in a volume of 35 µl composed of 2X LightCycler® 480 Probe Master (Roche, Indianapolis, IN), 0.3 µM each *Dv*CRT_TYE665 (TYE665/5'-TGGAGAAGGGCTCGAACTTGGC-3'/IAbRQSp) and *Rm*OmpB_HEX (HEX/5'-CGGGGCAAAGATGCTAGCGCTTCACAGTTACCCCG-3'/IABk FQ) probes (Sunyakumthorn, dissertation 2011), 0.1 µM each CRT*Dv*321F, CRT*Dv*452R, OmpB*Rm*2832F and OmpB*Rm*2937R primers, DNase/RNase-free water and 5 µl of gDNA template (samples), water (negative control) or standard reference plasmids. Aliquots of each qPCR reactions were transferred from 96-well plate to 384-well plate (10 µl each, Roche) and run on LightCycler 480® system II (Roche) using the following conditions; a pre-incubation step of 95 ^{0}C for 10 min, 45 amplification cycles of 95 ^{0}C for 10 sec, 60 ^{0}C for 30 sec, and 72 ^{0}C for 1 sec. The average ratio of RmOmpB and DvCRT genes from the control group was used as a reference to calculate the percent relative infection of each sample as shown in the formula below.

$$\% \text{ Relative infection} = \left(\frac{\text{Number of RmOmpB}_{\text{sample}}}{\text{Number of DvCRT}_{\text{sample}}} \times 100\right) / \text{Average of } \frac{\text{Number of RmOmpB}_{\text{control}}}{\text{Number of DvCRT}_{\text{control}}}$$

2.2.7 Statistical analysis

Data were analyzed using GraphPad Prism 5. A two-way Anaysis of Variance (ANOVA) with Bonferroni post test was conducted to examine the effect of rickettsial viability and concentration of inhibitor vehicle on *Rickettsia* invasion of tick cells. Student's paired t-test

was used to determine the influence of each inhibitor, compared to inhibitor vehicle, on rickettsial internalization. *P*-values of ≤ 0.05 were considered significantly different.

2.3 Results

2.3.1 Invasion of tick cells occurs through a process dependent on live *Rickettsia*

The objective of this study is to use biochemical inhibition assays to elucidate the molecular mechanisms underlying rickettsial internalization of tick cells. The concentration of inhibitors used in each experiment was based on literature, solubility, and viability effect of the inhibitors on the tick cells. Most inhibitors were reconstituted in DMSO; therefore the concentration of DMSO was optimized to minimize the influence of the chemical on the assays. Tick cells were seeded, treated with 0.1% or 1% DMSO, infected with *Rickettsia*, and extracted for gDNA. Quantitative PCR was performed to enumerate number of intracellular *Rickettsia* and tick cells. The results showed that neither 0.1% nor 1% DMSO affect percent relative internalization of tick cells by *R. montanensis* (Figure 2.2).

To examine whether invasion of tick cells occurs through a process that is initiated by *Rickettsia*, internalization assays were performed using either live or formalin-fixed *R. montanensis.* The results of qPCR showed that live *R. montanensis* invaded tick cells significantly more efficient ($P < 0.0001$) than non-viable *Rickettsia* suggesting that rickettsial entry into tick cells occur through an active *Rickettsia* specific process (Figure 2.2).

2.3.2 Host actin is required for *R. montanensis* invasion of tick cells

Many species of invasive bacteria such as *Listeria*, *Shigella*, *Rickettsia*, *Burkholderia*, and *Mycobacterium* have ability to manipulate the host actin cytoskeleton in order to invade the cells (Dramsi and Cossart, 1998; Gouin et al., 2004; Sousa et al., 2005; Hamaguchi et al., 2008). This study was carried out to determine whether actin polymerization is important for

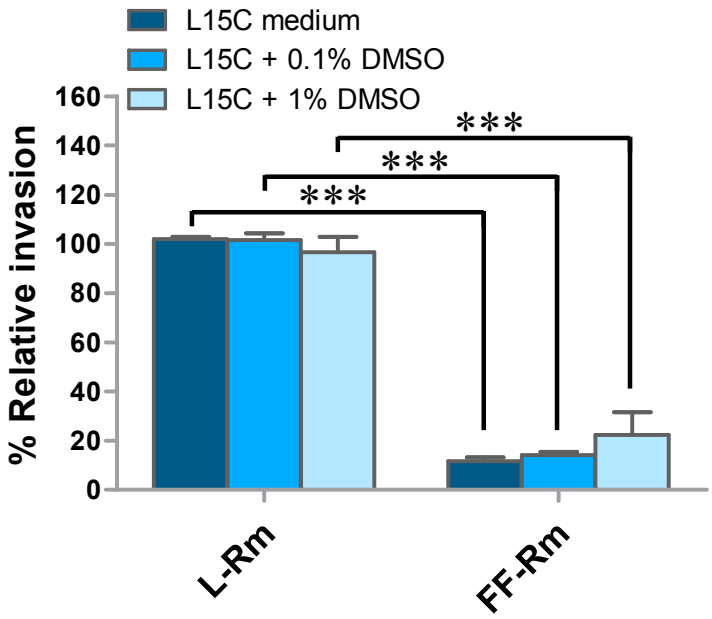

Figure 2.2 Invasion of tick cells occurs through a process dependent on live *Rickettsia*. Live (L-Rm) and formalin-fixed (FF-Rm) *R. montanensis* were used to infect DVE1 cells pre-incubated in complete L15C medium or medium supplemented with 0.1% or 1%DMSO. After 1 h, *Rickettsia* was removed and the cells were washed twice with PBS. The samples were collected by low-speed centrifugation and gDNA was then extracted. Quantitative PCR assay performed to quantify copies of RmOmpB and DvCRT genes. Percent relative invasion of each FF-Rm was compared to L-Rm in each treatment. The experiment was performed in triplicate for each group. For cells pre-incubated in medium only and medium containing 1% DMSO, the result shown is a representative of two independent experiments. Invasion assay of cell pre-incubated in medium supplemented with 0.1% DMSO was performed once. $P \leq 0.05$ was considered significantly different. Three asterisks represent $P < 0.0001$.

R. montanensis internalization of tick cells as previously described in *Drosophila* and mammalian cells (Martinez and Cossart, 2004; Reed et al., 2012). Actin polymerization inhibitor, cytochalasin D, was used to treat DVE1 cells for 2 h prior to infection with *R. montanensis* for 1 h. The results showed that disruption of actin polymerization in tick cells significantly decreased percent relative rickettsial invasion to 40% ($P < 0.0001$), 51% ($P < 0.0001$) and 70% ($P = 0.0003$) at 100, 10 and 1 µM of the inhibitor used, respectively (Figure 2.3). This indicates that *R. montanensis* uptake occur through a process that depends on tick actin.

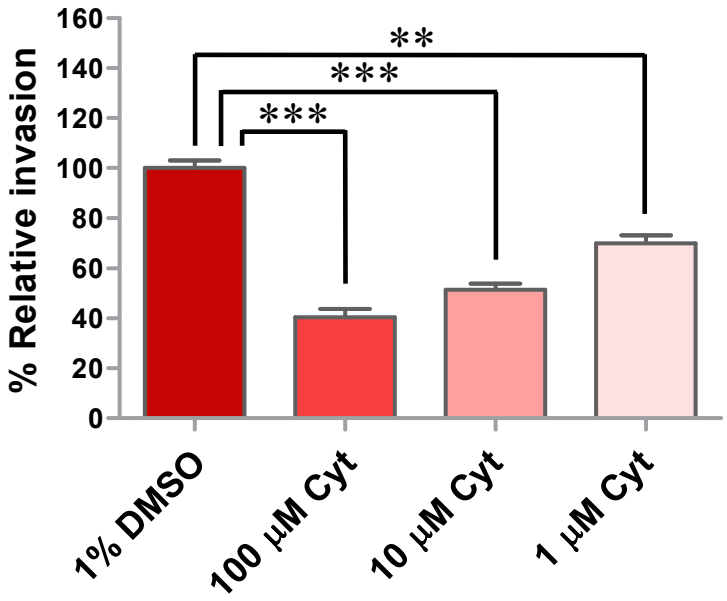

Figure 2.3 Actin polymerization is essential for *R. montanensis* invasion of tick cells. Tick cells were treated with actin depolymerizing agent, cytochalasin-D (Cyt) at 100, 10 and 1 μM 2 h prior to infecting with *R. montanensis* at MOI of 10 for 1 h. After removal of bacteria, the cells were washed twice with PBS and harvested by low-speed centrifugation. Genomic DNA was then extracted from the samples and the numbers of invading *Rickettsia* and host cells were quantified by qPCR. Percent relative invasion for each concentration of the inhibitor used was compared to untreated control. All treatments were performed in quadruplicate for each experiment. Results were the combination of the two independent experiments. $P \leq 0.05$ was considered significantly different. Two and three asterisks represent $P = 0.0003$ and $P < 0.0001$, respectively.

2.3.3 Arp2/3 complex is important for *R. montanensis* invasion of DVE1 cells

The actin related protein 2/3 (Arp2/3) complex is a seven subunit protein capable of nucleating actin filament (Mullins and Pollard, 1999; Goley and Welch, 2006). Previous studies in *Drosophila* and mammalian cells revealed the importance of the Arp2/3 complex in rickettsial internalization (Martinez and Cossart, 2004; Reed et al., 2012). The aim of this study was to examine whether the molecule is essential for the entry of *R. montanensis* into tick cells. The cells were treated with CK-666, an Arp2/3 complex inhibitor, at 500, 50, and 5 μM for 2h, before infecting with *R. montanensis* for 1 h. As shown in Figure 2.4, inhibition of Arp2/3 complex significantly reduced ($P < 0.0001$) percent relative invasion to 8% at the highest

concentration of the inhibitor used. This result suggests an important role of Arp2/3 complex on rickettsial internalization of tick cells.

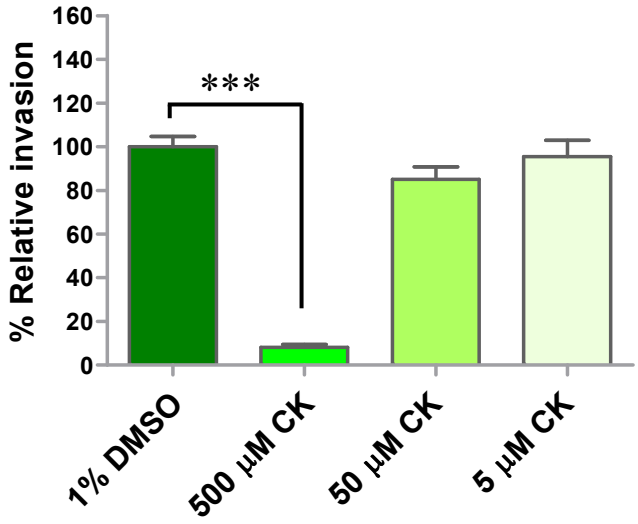

Figure 2.4 Arp2/3 complex is important for rickettsial internalization of DVE1 cells. DVE1 tick cells were treated with CK-666 (CK), an inhibitor of the Arp2/3 complex, at 500, 50, and 5 μM. After 2 h, *R. montanensis* (MOI of 10) was added and allowed to infect the cells for 1 h. *Rickettsia* was then removed and the cells were washed twice with PBS. Samples were collected by low-speed centrifugation and extracted for gDNA. Numbers of intracellular *Rickettsia* and host cells were quantified by probe-based qPCR and percent relative invasion of each treatment was compared to untreated control. All treatments were performed in quadruplicate for each experiment. Results were the combination of the two independent experiments. $P \leq 0.05$ was considered significantly different. Three asterisks represent $P < 0.0001$.

2.3.4 Inhibition of N-WASP has a slight effect on *R. montanensis* invasion of DVE1 cells.

Neural (N) Wiskott-Aldrich syndrome protein (WASP) is a cytoskeleton regulator that promotes actin nucleation by binding to and activation of the Arp 2/3 complex (Takenawa and Suetsugu, 2007; Bershadsky, 2004). Although N-WASP is not required for *R. parkeri* invasion of *Drosophila* and mammalian cells (Reed et al., 2012), in some pathogens, such as *Yersinia pseudotuberculosis* (McGee et al., 2001) and *Listeria monocytogenes* (Hamon et al., 2006), this actin nucleation-promoting factor (NPF) facilitates internalization into host cells. Therefore, the role of N-WASP in *R. montanensis* entry into tick cells was investigated in this study. N-WASP inhibitor, 187-1, was used to treat tick cells at three different concentrations (100, 10 and 1 μM)

for 2 h. The cells were then infected with *R. montanensis* for 1 h. Although inhibition of N-WASP at the highest concentration of the inhibitor used had a slight effect on *R. montanensis* entry (decreased to 80%), the result was significantly different ($P = 0.0183$) compared to control. This suggests that N-WASP is involved in *R. montanensis* invasion of tick cells (Figure 2.5).

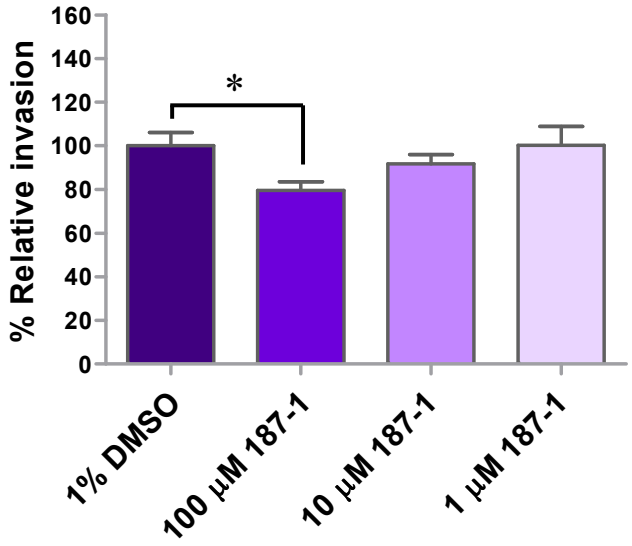

Figure 2.5 Inhibition of N-WASP affects the ability of *R. montanensis* to invade tick cells. *Rickettsia montanensis* (MOI of 10) was used to infect DVE1 cells pretreated for 2 h with 100, 10, and 1 μM N-WASP inhibitor, 187-1. After 1 h, *Rickettsia* was removed and the cells were washed twice with PBS. The samples were collected by low-speed centrifugation and extracted for gDNA. Quantitative PCR was performed to quantify number of invading *Rickettsia* and host cells and percent relative rickettsial invasion of each treatment group was compared to untreated control group. All treatments were performed in quadruplicate for each experiment. Results were the combination of the two independent experiments. $P \leq 0.05$ was considered significantly different. An asterisk represents $P = 0.0183$.

2.3.5 Rho GTPase, Rac1, mediates *R. montanensis* uptake into tick cells

Of the 22 Rho family GTPases, Cdc42 and Rac are the upstream signaling molecules that activate WASP and WASP-family verprolin-homologous protein (WAVE) family proteins (Hall, 1998; Sit and Manser, 2011); WASP and WAVE contain five members which are WASP, N-WASP, WAVE1, WAVE2, and WAVE3 (Takenawa and Miki, 2001; Takenawa and Suetsugu, 2007). Cdc42 has been shown to trigger N-WASP and Rac activates WAVE. Interactions of these molecules lead to Arp2/3 complex-mediated actin polymerization (Ridley, 2006). In *R.*

conorii, Cdc42 facilitates bacterial entry into mammalian cells (Martinez and Cossart, 2004); however, in *R. parkeri*, Rho GTPases Rac1 and Cdc42 were proposed to cooperatively stimulate actin polymerization leading to rickettsial internalization (Reed et al., 2012). Therefore, a broad-spectrum Rho family GTPase inhibitor, *Clostridium difficile* toxin B, which inhibits Rho, Rac, and Cdc42, was used to investigate the role of Rho GTPases in *R. montanensis* entry into tick cells. The results showed that inhibition of Rho GTPases by *C. difficile* toxin B did not affect *R. montanensis* invasion of DVE1 cells (Figure 2.6).

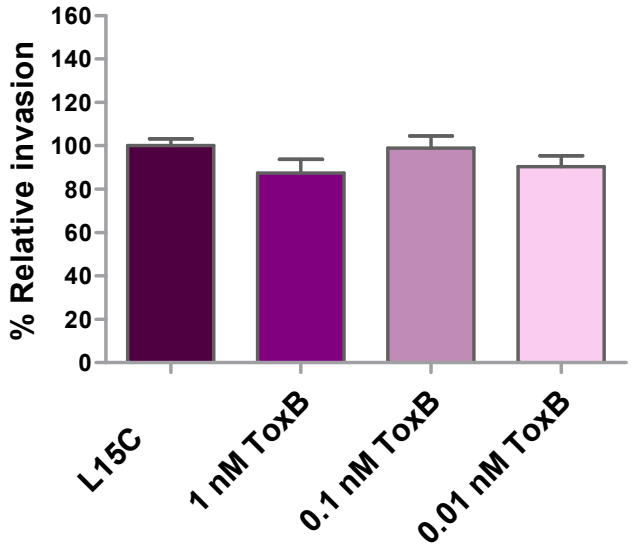

Figure 2.6 Disruption of Rho GTPases by Toxin B did not affect the process of *R. montanensis* uptake into DVE1 cells. Tick cells were treated for 2 h with *C. difficile* toxin B (ToxB), a general inhibitor for Rho GTPases, at 1, 0.1, and 0.01 nM. The cells were then infected with *R. montanensis* at MOI of 10 for 1 h. After removal of bacteria, the cells were washed twice with PBS and collected by low-speed centrifugation. Genomic DNA was extracted from the samples and qPCR was performed to enumerate number of invading bacteria and host cells. Percent relative rickettsial invasion of each treatment group was compare to control group. All treatments were performed in quadruplicate for each experiment. Results were the combination of the two independent experiments.

Next, Rac1, one of the regulators of actin cytoskeleton rearrangement, was specifically targeted using Rac1 inhibitor. As shown in Figure 2.7, inhibition of Rac1 significantly decreased percent relative *R. montanensis* invasion of tick cells compared to untreated control; decreased to

62% ($P < 0.0001$) and 90% ($P = 0.0242$) at 1 and 0.1 mM of the inhibitor used, respectively. Thus it can be concluded that Rac1 facilitated rickettsial entry into tick cells.

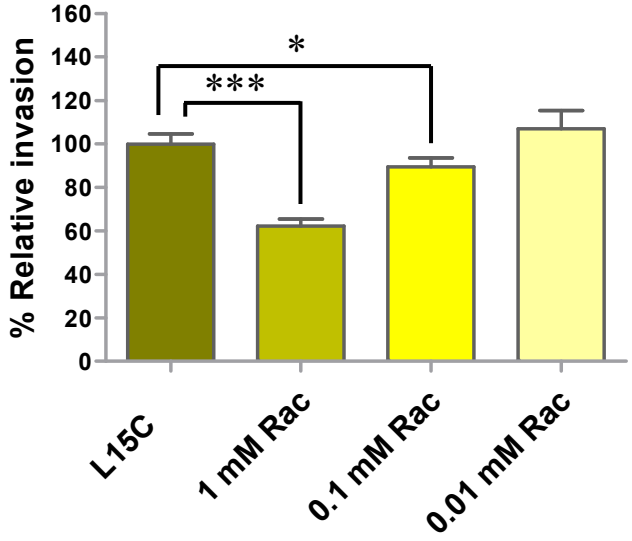

Figure 2.7 Rho GTPase, Rac1, facilitates *R. montanensis* entry into tick cells. DVE1 cells were treated with Rac1 inhibitor (Rac) at 1, 0.1, and 0.01 mM for 2 h prior to infecting with *R. montanensis* (MOI of 10). After 1 h, *Rickettsia* was removed and the cells were washed twice with PBS. The cells were harvested by low-speed centrifugation and gDNA was extracted from. Copies of RmOmpB and DvCRT genes were then quantified by qPCR. Percent relative rickettsial invasion of each treatment was compared to untreated control. All treatments were performed in quadruplicate for each experiment. Results were the combination of the two independent experiments. $P \leq 0.05$ was considered significantly different. Three asterisks and one asterisk represent $P < 0.0001$ and P = 0.0242, respectively.

2.3.6 Protein tyrosine kinases play a role in *R. montanensis* invasion of DVE1 cells

Studies in many pathogens such as *Listeria* (Shen et al., 2000; Mostowy and Cossart, 2009) and *Rickettsia* (Martinez and Cossart, 2004; Reed et al., 2012) have illustrated that phosphorylation of proteins on tyrosine residues mediates internalization of bacteria into host cells. In this study, three different concentrations (500, 50, and 5 µM) of genistein, a specific inhibitor of tyrosine-specific protein kinases, was used to treat DVE1 cells prior to infection with *R. montanensis*. The results (Figure 2.8) showed that inhibition of general tyrosine kinases significantly reduced the ability of rickettsiae to invade tick cells compared to untreated control; reduced to 31% ($P < 0.0001$) and 77% ($P = 0.0229$) at 500 and 50 µM of the inhibitor used,

respectively. This indicated a role for protein tyrosine kinases (PTKs) in R. montanensis internalization of tick cells.

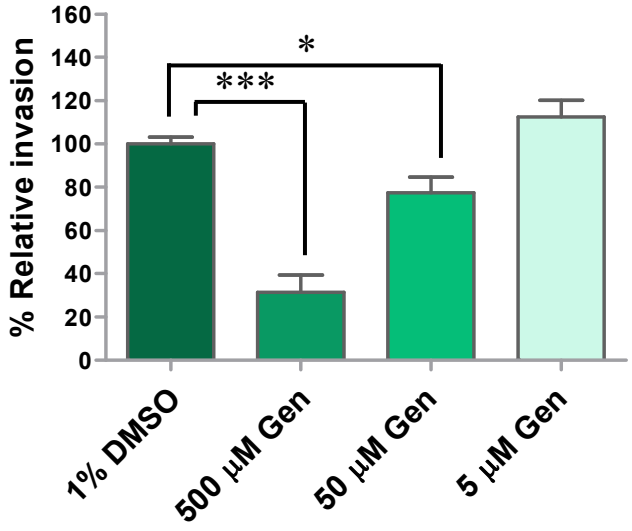

Figure 2.8 Protein tyrosine kinases play a role in *R. montanensis* invasion of DVE1 cells. Three different concentrations (500, 50 and, 5 μM) of genistein (Gen), the inhibitor of general protein tyrosine kinases were used to treat tick cells for 2 h. *Rickettsia montanensis* was then inoculated and allowed to infect the cells for 1 h. After removal of *Rickettsia*, the cells were washed twice with PBS and collected by low-speed centrifugation. Genomic DNA was then extracted from the cells and qPCR was performed to quantify number of invading *Rickettsia* and host cells. Percent relative rickettsial invasion of each treatment group was compared to untreated control. All treatments were performed in quadruplicate for each experiment. Results were the combination of two independent experiments. $P \leq 0.05$ was considered significantly different. Three asterisks and one asterisk represented $P < 0.0001$ and $P = 0.0229$, respectively.

Furthermore, studies of *R. conorii* invasion of mammalian cells revealed the involvement of the Src family PTKs and the focal adhesion kinase (FAK) in bacterial uptake (Martinez and Cossart, 2004). The Src and FAK family kinases were shown to regulate actin cytoskeleton reorganization (Mitra et al., 2005; Seong et al., 2011); therefore, the importance of these molecules in *R. montanensis* invasion of tick cells was examined in this study. As shown in Figure 2.9, inhibition of Src family PTKs by PP2 significantly decreased ($P < 0.0001$) percent relative rickettsial invasion to 52% at 250 μM of the inhibitor used.

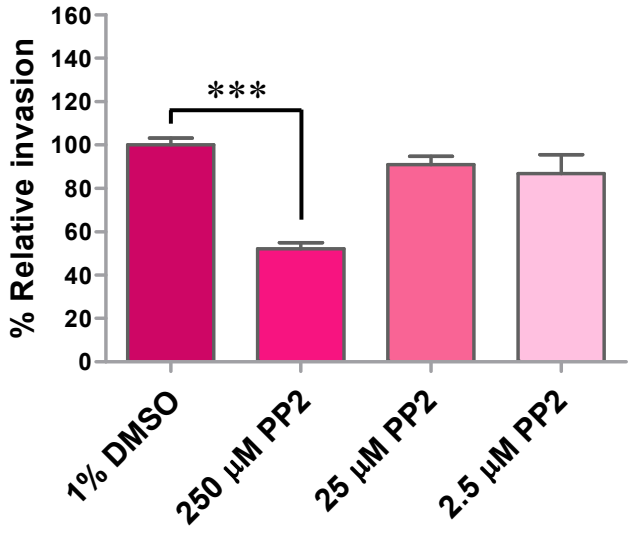

Figure 2.9 Src family PTKs facilitate *R. montanensis* entry into DVE1 cells. *Rickettsia montanensis* (MOI of 10) was used to infect tick cells pretreated for 2h with PP2 (250, 25, and 2.5 µM), an inhibitor of Src family PTK. After 1 h, *Rickettsia* was removed and the cells were washed twice with PBS. The samples were harvested by low-speed centrifugation and extracted for gDNA. Quantitative PCR was then performed to quantify number of intracellular *Rickettsia* and host cells. Percent relative rickettsial invasion of each treatment group was compared to untreated control. All treatments were performed in quadruplicate for each experiment. Results were the combination of the two independent experiments. $P \leq 0.05$ was considered significantly different. Three asterisks represent $P < 0.0001$.

Likewise, disruption of FAK significantly reduced the ability of *R. montanensis* to invade tick cells to 23% ($P < 0.0001$) and 72% ($P < 0.0001$) at 500 and 50 µM of the inhibitor utilized, respectively, compared to untreated control (Figure 2.10). The results suggest that Src family PTKs and FAK are important for *R. montanensis* internalization into DVE1 tick cells.

2.3.7 Phosphatidylinositol-3'-kinase mediates *R. montanensis* invasion of DVE1 cells

The last molecule investigated in this study was the phosphatidylinositide 3-kinase (PI 3-kinase); the molecules have been shown to be involved in actin cytoskeleton remodeling (Kotula, 2012). In addition, the study of *R. conorii* invasion of mammalian cells has demonstrated the association of the proteins with rickettsial uptake (Martinez and Cossart, 2004).

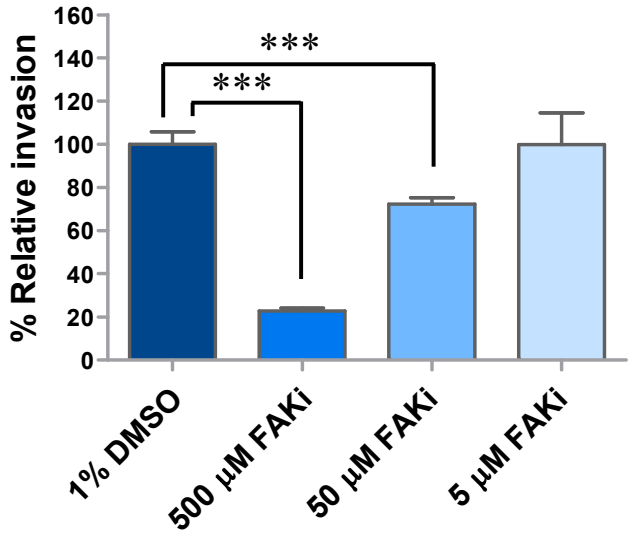

Figure 2.10 Focal adhesion kinase mediates *R. montanensis* internalization of tick cells. DVE1 cells were treated with 500, 50, and 5 µM focal adhesion kinase inhibitor (FAKi) for 2 h. *R. montanensis* was then used to infect the cells at MOI of 10. After 1 h, *Rickettsia* were removed and the cells were washed twice with PBS. Samples were collected by low-speed centrifugation and gDNA was extracted. Quantitative PCR was performed to quantify numbers of invading bacteria and host cells. Percent relative rickettsial invasion of each treatment group was compared to the untreated control. All treatments were performed in quadruplicate for each experiment. Results were the combination of the two independent experiments. $P \leq 0.05$ was considered significantly different. Three asterisks represent $P < 0.0001$.

Therefore, this study aimed to determine the role of the PI 3-kinase in *R. montanensis* invasion of tick cells. After treating with three different concentrations (500, 50 and 5 µM) of PI 3-kinase inhibitor XI, *R. montanensis* was added to infect the cells for 1h. The results showed a significant decrease ($P < 0.0001$) in *R. montanensis* internalization of DVE1 cells compared to untreated control; percent relative invasion was reduced to 40% at the highest concentration of the inhibitor used (Figure 2.11). The results indicate that PI 3-kinase facilitates *R. montanensis* uptake into tick cells.

2.4 Discussion

For SFG *Rickettsia*, ticks serve as both vector and reservoir; however, most molecular mechanisms underlying tick vector competence for *Rickettsia* such as rickettsial invasion of tick cells are undetermined. As obligate intracellular bacteria, invasion of host cells is very important

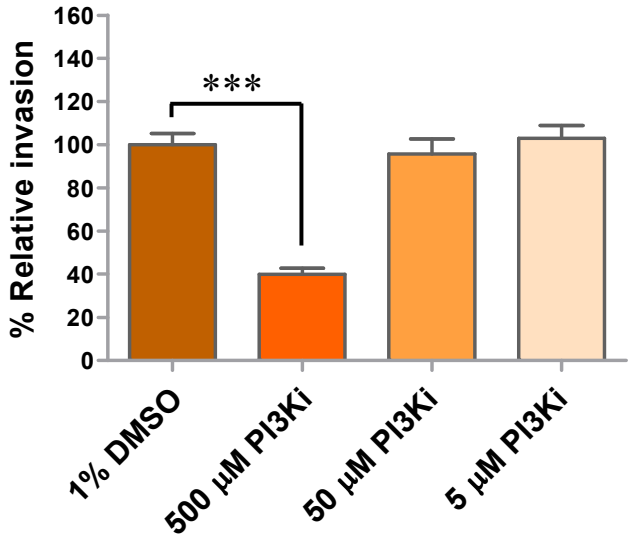

Figure 2.11 Phosphatidylinositol-3'-kinase plays a role in *R. montanensis* entry into tick cells. DVE1 cells were treated with PI 3-kinase inhibitor, HWT, at 500, 50, and 5 µM for 2 h. *Rickettsia montanensis* was then used to infect the cells at MOI of 10 for 1 h. After removal of *Rickettsia*, the cells were washed twice with PBS and samples were collected by low-speed centrifugation. Genomic DNA was then extracted from the samples and qPCR assay was performed to quantify numbers of invading *Rickettsia* and host cells. Percent relative rickettsial invasion of each treatment group was compared to the untreated control. All treatments were performed in quadruplicate for each experiment. Results were the combination of the two independent experiments. $P \leq 0.05$ was considered significantly different. Three asterisks represent $P < 0.0001$. PI3Ki = PI 3-kinase

for subsequent survival of *Rickettsia*. This study aimed to identify tick signaling molecules involved in rickettsial uptake into host cells using biochemical inhibition assays. Because of its capability to move between vertebrate and invertebrate hosts, the hypothesis is that conserved mechanisms are utilized for rickettsial invasion. Therefore, the molecules that have been identified to be involved in the internalization of *Rickettsia* into vertebrate cells were targeted in this study. The results showed that inhibition of tick actin polymerization resulted in a decrease in *R. montanensis* invasion of host cells in dose dependent manner (Figure 2.3). Manipulation of host actin was found to be important for not only the uptake of *Rickettsia* but also the entry of several other pathogens such as *Listeria*, *Shigella*, *Burkholderia*, and *Mycobacterium* (Dramsi and Cossart, 1998; Gouin et al., 2004; Sousa et al., 2005; Hamaguchi et al., 2008). Once bound

to host cell receptors, invasive bacteria induce signaling cascades that lead to actin polymerization, membrane rearrangement, and bacterial internalization.

The next protein examined was Arp2/3 complex, a major actin nucleator (Mullins and Pollard, 1999; Goley and Welch, 2006). Similar to the studies in *R. conorii* and *R. parkeri* invasion of mammalian cells (Martinez and Cossart, 2004; Reed et al., 2012), the results of the current study demonstrate that Arp2/3 complex is important for *R. montanensis* entry into tick cells (Figure 2.4).

Although not required by *R. parkeri* in order to invade mammalian and *Drosophila* cells, N-WASP, a cytoskeleton regulator that promotes actin nucleation by binding to and activation of the Arp 2/3 complex (Takenawa and Suetsugu, 2007; Bershadsky, 2004), facilitates the uptake of *Yersinia pseudotuberculosis* (McGee et al., 2001) and *Listeria monocytogenes* (Hamon et al., 2006). Therefore, the role of N-WASP in *R. montanensis* internalization of tick cells was investigated in this study. The results indicate that disruption of the protein had a slight effect on rickettsial entry into tick hosts (Figure 2.5); however, the effect was shown to be significantly different from untreated cells. The modest effect of the inhibitor on rickettsial invasion might be because the inhibitor is ineffective at inhibiting tick N-WASP. Likewise, the poor solubility of the inhibitor in the medium might result in a trivial effect on inhibiting the molecule. However, the highest concentration of the N-WASP inhibitor used in this study was the maximum solubility of the inhibitor in medium supplemented with 1% DMSO. On the other hand, there is a possibility that the alternate pathway (Rac dependent) might be cooperatively utilized to regulate actin cytoskeleton remodeling during rickettsial invasion of tick cells. Thus *C. difficile* toxin B was used first to inhibit general Rho GTPases including Rho, Rac and Cdc42; however, general disruption of these molecules using toxin B did not affect rickettsial internalization into

tick cells (Figure 2.6). Besides what was described above regarding an ineffective inhibition by the inhibitor, it is possible that the inhibitor that targeted molecules non-specifically might not be as powerful as a specific inhibitor. Therefore, a Rac1 inhibitor was used to specifically study the function of Rac1 on *R. montanensis* invasion of tick cells. The results demonstrate that Rac1 plays a role in rickettsial entry into tick cells (Figure 2.7), as is the case of the uptake of *R. parkeri* into mammalian cells (Reed et al., 2012).

In tick cells, the function of PTKs in *Rickettsia* internalization is not known. Similar to that found in *R. conorii* and *R. parkeri* invasion of mammalian cells (Martinez and Cossart, 2004; Reed et al., 2012), the current study identified that PTKs were important for *R. montanensis* entry into tick cells (Figure 2.8). Next, to determine whether the non-receptor PTKs, Src family PTKs and FAK were essential for the uptake of *R. montanensis* into tick cells as is the case of *R. conorii* internalization into mammalian cells (Martinez and Cossart, 2004), specific inhibitors were used. The results illustrated that both Src family PTKs and FAK played a role in rickettsial invasion of DVE1 cells (Figure 2.9 and 2.10).

The last molecule that was investigated in this study was PI 3-kinase, which is important in actin cytoskeleton reorganization (Kotula, 2012) and is required for *R. conorii* uptake into mammalian cells (Martinez and Cossart, 2004). Similar to Martinez and Cossart (2004), PI 3-kinase was involved in *R. montanensis* entry into tick cells (Figure 2.11).

In summary, tick signaling molecules associated with *R. montanensis* invasion were identified, for the first time, in this study. A pathway of invasion initiated by the binding of *R. montanensis* to unidentified receptor(s) on the tick cells was postulated and shown in Figure 2.12. As discussed above, using inhibitors to disrupt functions of targeted proteins has some limitations; therefore utilizing other powerful techniques, such as RNA interference (RNAi) to

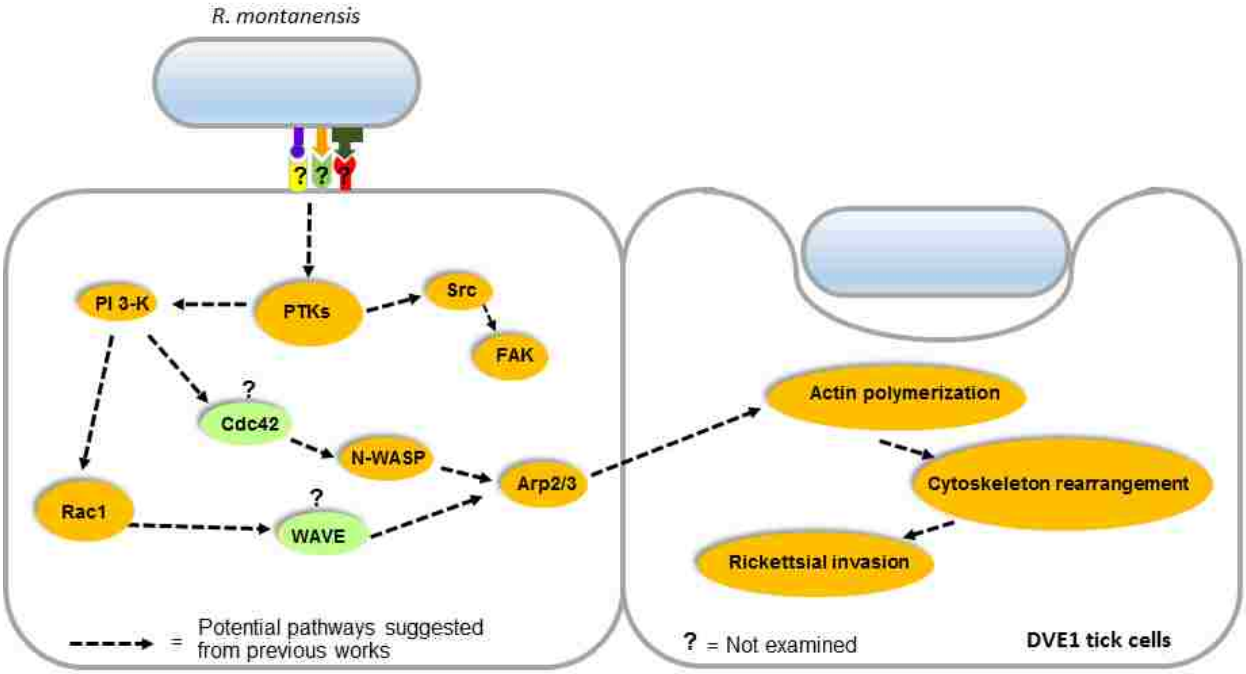

Figure 2.12 The proposed pathway activated during rickettsial invasion of tick cells. After binding of *R. montanensis* to unidentified receptor (s) on tick cells, multiple tick molecules (colored in orange) including phosphatidylinositol-3'-kinase (PI 3-kinase), protein tyrosine kinases (PTKs), Src family PTKs, focal adhesion kinase (FAK), Rho GTPase Rac1, Neural Wiskott-Aldrich syndrome protein (N-WASP), and actin-related protein 2/3 (Arp2/3) complex, are activated. The activation of these molecules leads to actin polymerization resulting in membrane rearrangements and rickettsial invasion into tick cells. The molecules and pathways with question marks were not examined in this study. Figure was modified from Chan et al., 2010.

specifically silence the expression of genes of interest, will confirm the role of these molecules in *R. montanensis* invasion. RNAi also has disadvantage such as the potential off-target effects which compromise the specificity of RNAi (Boutros and Ahringer, 2008). However, the lack of a complete *D. variabilis* genome sequence slows down the functional studies of tick molecules using RNAi. Therefore the next studies (see chapter 3 and 4) were carried out to functionally characterize and provide molecular details of tick genes involved in SFG *Rickettsia* internalization of tick cells.

Compared to the studies in mammalian and *Drosophila* cells (Martinez and Cossart, 2004; Reed et al., 2012), Arp2/3 complex and actin are the central molecules that activate during

the entry of *Rickettsia* into all three types of cells including tick. The upstream signaling proteins cooperating to regulate Arp2/3 complex are similar but not identical for these cells. Although different species of *Rickettsia*, molecules targeted, and techniques used in this and previous studies make comparison of invasion of mammalian, *Drosophila*, and tick cells by *Rickettsia* difficult, it can be concluded from what described above that conserved mechanisms with a degree of variation are utilized in *Rickettsia* invasion of vertebrate and invertebrate cells.

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CHAPTER 3
MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE TICK ARP2/3 COMPLEX DURING RICKETTSIAL INFECTION

3.1 Introduction

Diseases transmitted by ticks, including Rocky Mountain spotted fever (RMSF), are a significant concern throughout the United States. RMSF is a potentially fatal disease caused by *Rickettsia rickettsii*, an obligate intracellular bacterium which is transmitted to humans through the bite of infected ticks (Sonenshine et al. 2005). *Dermacentor variabilis* is a primary vector for pathogenic *R. rickettsii* (Sonenshine et al. 2005). This species of tick is also known to carry non-pathogenic *R. montanensis* (Feng et al., 1980; Anderson et al., 1986; Pretzman et al., 1990; Ammerman et al., 2004). To date, interactions between spotted fever group (SFG) *Rickettsia* and its host have been extensively studied in mammalian cells (Martinez and Cossart, 2004; Martinez et al., 2005; Chan et al., 2009; Hillman et al., 2012; Reed et al., 2012). Previous studies (see chapter 2) from this body of work have revealed the tick molecules involved in *R. montanensis* invasion of host cells. These molecules, including phosphatidylinositol-3'-kinase (PI 3-kinases), protein tyrosine kinases (PTKs), Src family PTKs, FAK, Rac1, and N-WASP, cooperatively activate the Arp2/3 complex leading to actin polymerization that drives rickettsial internalization into tick cells.

The goal of this study was to functionally characterize and provide the molecular detail of the tick Arp2/3 (Actin-related 2/3) complex. Arp2/3 complex is a multi-subunit protein complex that is composed of Arp2, Arp3, ARPC1, ARPC2, ARPC3, ARPC4 and ARPC5 (Higgs and Pollard, 2001; Pollard and Beltzner, 2002). The complex nucleates a new actin filament from the site of an existing filament. Arp2 and Arp3 are actin-related proteins that fold and bind ATP like actin does. These two subunits were suggested to form a dimer that could bind the pointed end

and nucleate the filaments that grow in the barb end direction. The binding of ATP to Arp2 and Arp3 and ATP hydrolysis are required for Arp2/3 complex-mediated actin cytoskeleton remodeling (Dayel et al., 2001; Le Clainche et al., 2001; Le Clainche, 2003; Dayel et al., 2004; Martin et al., 2006).

Several strategies have been used to describe the interaction among subunits of the Arp2/3 complex. In *Acanthamoeba*, the interactions between Arp2/3 subunits are as follows: Arp2 and ARPC1, Arp2 and ARPC4, Arp3 and ARPC2, Arp3 and either ARPC3 or ARPC4, and ARPC4 and ARPC5 (Mullins et al., 1997). In addition, a crystal structure of bovine Arp2/3 complex revealed the interactions between ARPC2 and ARPC4 in which the dimer was predicted to form at a structural backbone of the complex. ARPC4 was suggested to interact with Arp2, ARPC1 and ARPC5, whereas ARPC2 interacted only with Arp3 (Robinson et al., 2001). Furthermore, ARPC1 was described as being in contact with Arp3 in an adjacent complex and have the potential to bind an actin subunit in either the mother or daughter filament. ARPC3 interacted with Arp3, whereas ARPC5 interacted with ARPC1, ARPC4 and Arp2 (Robinson et al., 2001).

Reed et al. (2012) revealed a function for individual Arp2/3 complex subunits from *Drosophila* cells in response to *R. parkeri* infection using RNA interference (RNAi). Depletion of Arp2/3 complex subunits (Arp2, Arp3, ARPC1, ARPC2, ARPC4 and ARPC5) resulted in a decrease in *R. parkeri* internalization.

In chapter 2, it was shown that the Arp2/3 complex is essential for the uptake of *R. montanensis* into tick cells. To gain insight into the molecular mechanisms of *Rickettsia* infection in nature, the present study was carried out at the tissue level to test the same hypothesis that the complex is important for rickettsial invasion of *D. variabilis*. Genes for all

seven subunits of Arp2/3 complex from *D. variabilis* were isolated and described for the first time. Transcriptional profiles of the Arp2/3 complex subunits in unexposed and *R. montanensis* exposed tick tissues (midgut, ovary, and salivary glands) were investigated. Ultimately, biochemical inhibition assays were conducted to examine the function of tick Arp2/3 complex in rickettsial internalization at the tissue level. The functional study of tick Arp2/3 complex at the tissue level provides an insight into the molecular mechanisms of *Rickettsia* infection in nature.

3.2 Materials and methods

3.2.1 Tick infection

D. variabilis colonies were maintained on guinea pigs and rats at Louisiana State University, School of Veterinary Medicine as described by Macaluso et al. (2001). To infect ticks with *R. rickettsii*, a needle inoculation technique was used as described by Sunyakumthorn (dissertation 2011) with minor modification. Briefly, four unfed female ticks were washed with 1% bleach (5 min), 70% ethanol (2 min), and 1% benzalkonium chloride (5 min). Between each wash, the ticks were rinsed once with sterile water. After the final wash, they were rinsed three times and air-dried. *Rickettsia rickettsii* infected Vero cells were then thawed and centrifuged at 16000 x g for 10 min. The cell pellet was reconstituted in 500 µl sterile phosphate buffered saline (PBS, pH7.4) and an equal aliquot was used to inject ticks at the area between Coxa I and basis capituli. After 1 h, the tick's mouthparts and legs were excised. The ticks were then cut along the perimeter of the alloscutum and the dorsal cuticle was removed. All tissues were then collected and added to 600 µl buffer RLT (QIAGEN, Germantown, MD) for RNA and subsequently full-length cDNA isolation. The tissues were lysed by passing through a 27G needle several times. The lysates were kept at -80 ºC until used for RNA extraction and

subsequent gene isolation using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA).

3.2.2 Tick dissection for full-length cDNA isolation and Arp2/3 complex inhibition assays

Unfed or partially fed female ticks were washed as mentioned above. Tick tissues, including midgut, ovary, and salivary glands, were dissected out of the ticks and washed in PBS.

For RNA extraction and subsequent full-length cDNA isolation, TRIzol reagent (Invitrogen, Carlsbad, CA) was added and the tissues were passed through 27G needles or homogenized by grinding with plastic pestles for several minutes. The lysates were kept at -80 ºC until used.

For invasion assays, each tissue was transferred into a 1.7 ml centrifuge tube containing L15C medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Hyclone, Waltham, MA), 5% tryptose phosphate broth (Difco, Sparks, MD), 0.1% lipoprotein-cholesterol concentrate (LPC, MP Biomedicals, Santa Ana, CA), 0.6% HEPES solution (1 M, Sigma, St. Louis, MO), and 1.2% sodium bicarbonate solution (5%, Sigma). The samples were kept on ice and all experiments were carried out on the same day.

3.2.3 Primer design for full-length cDNA isolation

To obtain the primers for the isolation of full-length cDNA from *D. variabilis* (Dv) Arp2/3 complex subunits (Arp2, Arp3, ARPC2, ARPC3, and ARPC5), conserved regions of the genes from various organisms, such as *Ixodes scapularis*, *Drosophila melanogaster*, *D. yakuba*, *Pediculus humanus corporis*, *Aedes aegypti*, *Anopheles gambiae* and *Amblyomma maculatum* (Transcriptome Shotgun Assembly, TSA), were used as templates to design primers for short DNA fragment amplifications. PCRs were then carried out in the presence of *D. variabilis* cDNA. The amplicons were cloned into pCR4-TOPO vector (Invitrogen) and transformed into

TOP10 *E. coli* (Invitrogen). The plasmid was isolated using FastPlasmid Mini Kit (Eppendorf, Hauppauge, NY) according to manufacturer's instructions and submitted to Genelab at Louisiana State University, School of Veterinary Medicine for sequencing. Analysis of DNA sequence was performed using BioEdit software. Similarity comparison was carried out against Genbank nucleotide database using Blastn. Ultimately, gene specific (GSP) primers used for full-length cDNA isolation were designed from the partial sequences of the DvArp2/3 complex subunits (Table 3.1). For ARPC1 and ARPC4, the GSP primers used for cDNA isolation were obtained from *D. variabilis* TSA database (Table 3.1).

3.2.4 Cloning of tick Arp2/3 complex subunit full-length cDNAs

The full-length cDNAs for DvArp2, DvArp3, DvARPC1, DvARPC3, and DvARPC4 were generated using the SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer's protocol. Briefly, total RNA was extracted from tissues collected from four unfed female *R. rickettsii* infected ticks using RNeasy mini kit (QIAGEN) as described in the manufacturer's instructions. The RNA was treated with TURBO DNase (Ambion, Austin, TX) and purified using RNA cleanup kit (Zymo Research, Irvine, CA) according to manufacturers' manuals. One microgram of RNA was used as a template to create 5'- and 3'-RACE-Ready cDNAs. 5'- and 3'- RACE fragments of DvArp2/3 complex subunits were then amplified utilizing GSP primers shown in Table 3.1.

For DvARPC2 and DvARPC5, the GeneRacer Kit (Invitrogen) was used to produce full-length cDNAs according to the manufacturer's recommendation. Briefly, tissues (midgut, ovary and, salivary glands) were dissected out of fifteen partially fed (4 days) female ticks and total RNA was extracted using TRIzol reagent as described in the manufacturer's instructions. Messenger RNA was then purified from total RNA (200 µg) pooled from all tissues and used to

Table 3.1 Primers used in full-length cDNA isolation of DvArp2/3 complex (all subunits).

Primers	Sequences (5' to 3')	Fragment obtained
DvArp2 Fw	CCGCCGATGAACCCGCTCAAAAACC	3'DvArp2
DvArp2 Rev	CTCGATCATGCGCTCGCGGTTTTTG	5'DvArp2
DvArp3 Fw	GTCCCCCTGGGGCTGAGGGCTATGT	3'DvArp3
DvArp3 Rev	ATGCCAACATGCTGCCACCAAACCA	5'DvArp3
DvARPC1 Fw	TCACCTGCCACGCATGGAACAAGGA	3'DvARPC1
DvARPC1 Rev	CTTGGCACCTGACCCCACTGCAAAC	5'DvARPC1
DvARPC2 Fw	AACAAAGATAAGGGTCAGCA	3'DvARPC2
DvARPC2 Rev	TGCGCGCAATCTCCTCTGTGTTCT	5' DvARPC2
DvARPC3 Fw	GTGGGCAACGTCTATGCGACAAGGT	3' DvARPC3
DvARPC3 Rev	TTGTCGCATAGACGTTGCCCACACT	5'DvARPC3
DvARPC4 Fw	GATTCATGATGATGCGTGCCGAGAA	3'DvARPC4
DvARPC4 Rev	GCACAAATACGTGCCCTTGCATTGAG	5'DvARPC4
DvARPC5 Fw	GCTCCCATAGGCTCCAAATGCCAAA	3'DvARPC5
DvARPC5 Rev	GCTGGCTGCATCCTTTACACTTTGG	5'DvARPC5

create RACE-ready first-strand cDNA using NucleoTrap mRNA Mini Kit (Clontech) and SuperScript III Reverse Transcriptase (Invitrogen), respectively, according to the manufacturers' protocols. Both 5' and 3' end fragments of DvARPC2 and DvARPC5 were then amplified using the GSP primers listed in Table 3.1. Amplicons were cloned into pCR4-TOPO vector and transformed into TOP10 *E. coli*. The plasmid was isolated using the FastPlasmid Mini Kit

according to manufacturer's instruction and submitted to Genelab at Louisiana State University, School of Veterinary Medicine for sequencing. Sequence of DNA was analyzed using BioEdit software. Similarity comparison was carried out against protein database in Genbank using BlastX. Amino acid sequence analyses were conducted using web-based software suits. Multiple sequence alignment, MUSCLE, was used to create sequence alignment files and calculated percent identity (pairwise alignment). The alignment output was created using Multiple Align Show. ATP binding sites were predicted using NsitePred - web server (Chen et al., 2012) and the conserved regions in proteins were identified by using the Simple Modular Architecture Research Tool (SMART).

3.2.5 Cell culture

Vero cells were cultured in DMEM high glucose (Invitrogen) containing 5% fetal bovine serum (Hyclone) and maintained in a humidified 5% CO_2 incubator at 34 °C. Conditioned medium was replaced with new medium once a week and the cells were subcultured (1:6 or 1:12) every 1-2 weeks with 0.05% trypsin-EDTA (Invitrogen).

3.2.6 *Rickettsia* culture and purification

Rickettsia montanensis were maintained in Vero cells as described by Sunyakumthorn et al. (2012) with minor modifications. Briefly, half of the conditioned medium was replaced with new medium once a week and the bacteria were subcultured every 2 week by passing 1 ml of infected cells to uninfected Vero cells. For rickettsial invasion assays, *Rickettsia* was purified from host cells as described by Weiss et al. (1973) with minor modifications. Briefly, infected Vero cells were detached by scraping and lysed by vortexing with sterile 3 mm borosilicate glass beads for 5 min (Sigma-Aldrich, St. Louis, MO). To pellet cellular debris, cell lysate was centrifuged at 4 ºC, 275 x g for 3 min. The supernatant was then transferred to a 10 ml syringe

and filtered through a 2 µm syringe filter. Prior to infection, the number of *Rickettsia* was enumerated by counting *Rickettsia* stained with a LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Carlsbad, CA) in a Petroff–Hausser bacterial counting chamber (Hausser Scientific, Horsham, PA) and examined with a Leica microscope (Buffalo Grove, IL).

3.2.7 Expression of DvArp2/3 complex subunit mRNAs in tick tissues infected *ex vivo*

Tissues, including midgut, ovary, and salivary glands, were dissected out of unfed female *D. variabilis* ticks and washed once with PBS. The tissues were then transferred into sterile 1.7 ml centrifuge tubes containing 200 µl complete L15C medium and kept on ice until used (within the day) in the assays. Prior to infection, the tubes were centrifuged at 4 ºC, 700 x g for 5 min to bring the tissues down to the bottom of the tubes. One hundred eighty microliters of the medium was then removed under a dissecting microscope and *R. montanensis* (8×10^7 in 30 µl medium per tissue) was inoculated into the tubes. The samples were centrifuged at 4 ºC, 700 x g for 2 min to facilitate the biding between *Rickettsia* and tick tissues. *Rickettsia* were allowed to infect the tissues at 32 ºC for 1 h. The samples were then washed twice with 1 ml PBS and collected by centrifugation at 4 ºC, 275 x g for 4 min. After each wash, the supernatant was removed carefully under a dissecting microscope. Three samples of the same tissues were pooled and 800 µl TRIzol reagent was added. The samples were then lysed by passing through 27G needle 20 times. The lysate was used immediately or store at -80 ºC for later use for RNA extraction. The experiments were performed twice independently.

3.2.8 Total RNA isolation and relative quantitative RT-PCR (qRT-PCR)

To determine the transcriptional profiles of the Arp2/3 complex subunit genes (all subunits) in tick tissues (unexposed and *Rickettsia* exposed), total RNA and DNA were isolated using TRIzol reagent as described in the manufacturer's protocol. The RNA was treated with

TURBO DNase and extracted with UltraPure Phenol:Chloroform:Isoamyl Alcohol (25:24:1, Invitrogen) to inactivate DNase according to the manufacturers' instruction manuals. First-strand cDNA was then synthesized from 75 ng total RNA using iScript reverse transcription kit (Bio-Rad, Hercules, CA) according to manufacturer's instruction. GSP primers were then used to amplify each subunit of the DvArp2/3 complex and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All qPCR reactions were prepared in 96-well plates in a 35 µl volume composed of 0.1 µM each forward and reverse primers, DNase/RNase-free water, 2 µl of cDNA (sample) or water (negative control) and 2X LightCycler 480 SYBR Green I Master (Roche, Indianapolis, IN) as described by Sunyakumthorn et al., 2012 with minor modification. The mixtures were aliquoted in triplicate 10 µl reactions onto 384-well plates and run on LightCycler 480 system II (Roche). Quantitative PCR assay conditions consisted of a 95 °C pre-incubation for 10 min, 35 amplification cycles of 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 5 sec followed by a melting curve step of 95 °C for 5 sec and 65 °C for 1 min. Primers used in qRT-PCR assays were designed from either 5'- or 3'-RACE fragments of DvArp2/3 complex subunits obtained from this study (Table 3.2). Analyses of the crossing point (Cp) ratio of target (DvArp2, DvArp3, DvARPC1, DvARPC2, DvARPC3, DvARPC4, and DvARPC5) and reference (GAPDH) gene values were conducted with LightCycler 480 (1.5.0) software (Roche) using Basic Relative Quantification analysis (ΔΔCT-Method, Roche,). The ratio of a target DNA sequence to a reference DNA sequence was calculated. The reference was used for normalization of sample-to-sample differences. No RT reaction (water was added instead of Reverse transcriptase) was performed to confirm an absence of genomic DNA. To confirm the infection of tissues in the assays, DNA was extracted from the same sample after RNA isolation

Table 3.2 Primers and probes used in qRT-PCR and qPCR assays.

Primers	Sequences (5' to 3')	Experiment	Reference
DvArp2_123_Fw DvArp2_231_Rev	GGGGTTTGTCAAATGTGGTT TTCGATGTCACCTATCTTGTGG	mRNA expression	This study
DvArp3_593_Fw DvArp3_693_Rev	ATACGCTGTCTGGTTTGGTG GATGCTCGGTCCACACTCTT	mRNA expression	This study
DvArpc1_226_Fw DvArpc1_338_Rev	GCAGCTCTACAAGCGAGAGG TCACAATGCGGTTACTGTGTG	mRNA expression	This study
DvArpc2_356_Fw DvArpc2_460_Rev	AGGAACTGCTTTGCGTCTGT CATGGTTTCATCGTCCCTGT	mRNA expression	This study
DvArpc3_477_Fw DvArpc3_588_Rev	TTCCAGGAGAAGCTGGATTT TGCCCACACTCT TGTCGTAG	mRNA expression	This study
DvArpc4_262_Fw DvArpc4_474_Rev	GCAACGAGAAGGAGAAGGTG TCTGCTCCGTATGGAAGT TTG	mRNA expression	This study
DvArpc5_375_Fw DvArpc5_476_Rev	AGCCTTCCTCCTCCTTGTAGT T CCGTTT CTCTGCTCA CTATGT CT	mRNA expression	This study
DvGAPDH-926For DvGAPDH-1024Rev	ACTCCCACAGCAGCATCTTT TGCTGTAGCCGTACTCGTTG	mRNA expression	Sunyakumtthorn (dissertation, 2011
CRT*Dv*321F CRT*Dv*452R DvCRT_TYE665	AGGAGAAAAGCAAGGGACTG CAATGTTCTGCTCGTGCTTG TYE665/TGGAGAAGGGCTCGAACT TGGC/IAbRQSp	mRNA expression, inhibition assays	Sunyakumtthorn (dissertation, 2011)
OmpB*Rm*2832F OmpB*Rm*2937R *Rm*OmpB_HEX	GCGGTGGTGTTCCTAATAC CCTAAGTTGTTATAGTCTGTAGTG HEX/CGGGGCAAAGATGCTAGCGC TTCACAGTTACCCCG/IABkFQ	mRNA expression, inhibition assays	Sunyakumtthorn (dissertation, 2011)

using TRIzol reagent. Copies of rickettsial genes (RmOmpB) were quantified using qPCR as described in the next section.

3.2.9 DvArp2/3 complex inhibition assays and qPCR

Tissues (midgut, ovary, and salivary glands) were dissected out of unfed female ticks and washed once with PBS as previously described. The tissues were then transferred into microtubes containing 200 µl complete L15C medium and kept on ice until used in the same day for inhibition assays. After centrifugation at 4 ºC, 700 x g for 5 min to bring the tissues down to the bottom of the tubes, the medium was removed under a dissecting microscope. The samples were then treated with 500 µM CK-666, an Arp2/3 complex inhibitor, and incubated at 32 ºC. After 3 h, 180 µl of the inhibitor solution was removed and *R. montanensis* in 30 µl volume of 500 µM inhibitor solution was used to infect tick tissues (8×10^7 per tissue) for 1 h. The tissues were then washed twice with 1 ml PBS and collected by low-speed centrifugation as previously described. Genomic DNA (gDNA) was then extracted from the samples using the DNeasy Blood & Tissue Kit (QIAGEN) and eluted with 35 µl DNase/RNase free water. The numbers of *Rickettsia* and tick cells were then quantified using probe-based quantitative PCR (qPCR) as described by Thepparit (2011). Briefly, qPCR mixtures (35 µl per reaction) were prepared in a 96-well plate. PCR reactions consisted of 0.3 µM each *Dv*CRT_TYE665 and *Rm*OmpB_HEX probes, 0.1 µM each CRT*Dv*321F, CRT*Dv*452R, OmpB*Rm*2832F and OmpB*Rm*2937R primers, DNase/RNase-free water, and 5 µl of gDNA template (samples), water (negative control), water (environmental control during DNA extraction), or standard reference plasmids. Ten microliters of each reaction mixture were transferred into 3 wells of a 384-well plate and run on LightCycler 480 system II. Quantitative PCR assay conditions composed of a pre-incubation step of 95 ºC

for 10 min, 45 amplification cycles of 95 ºC for 10 sec, 60 ºC for 30 sec, and 72 ºC for 1 sec. Percent relative invasion was calculated using a formula shown below.

$$\% \text{ Relative invasion} = \left(\frac{\text{Number of RmOmpB}_{sample}}{\text{Number of DvCRT}_{sample}} \times 100\right) / \text{Average of } \frac{\text{Number of RmOmpB}_{control}}{\text{Number of DvCRT}_{control}}$$

The experiments were performed in quadruplicate for each treatment group and the results were the combination of the three independent experiments.

3.2.10 Statistical analysis

Analysis of Variance (ANOVA) was conducted using the SAS statistical package (Version 9.3) GLM procedure. For mRNA expression in tick tissues, relative gene expression was analyzed using a two-way interaction (rickettsial infection and tick tissues). Pairwise t tests of least-squares means were used to examine the interaction effects of relative mRNA expression of each subunit of the DvArp2/3 complex between unexposed- and *Rickettsia* exposed- tissues or between tissues. For biochemical inhibition assays, the same tests were used to study a role of DvArp2/3 complex during rickettsial invasion of tick cells. *P*-values of ≤ 0.05 were considered significantly different.

3.3 Results

3.3.1 Cloning and sequence analysis of DvArp2/3 complex subunits

Full-length cDNA clones corresponding to the transcript of DvArp2/3 complex subunit genes (DvArp2, DvArp3, DvARPC1, DvARPC2, DvARPC3, DvARPC4, and DvARPC5) from *D. variabilis* were isolated. Analysis of DNA sequence using BioEdit revealed a 2718, 1780, 1852, 1497, 857, 714, and 912 base pair (bp) of DvArp2, DvArp3, DvARPC1, DvARPC2, DvARPC3, DvARPC4, and DvARPC5, respectively. The open reading frame (ORF) lengths,

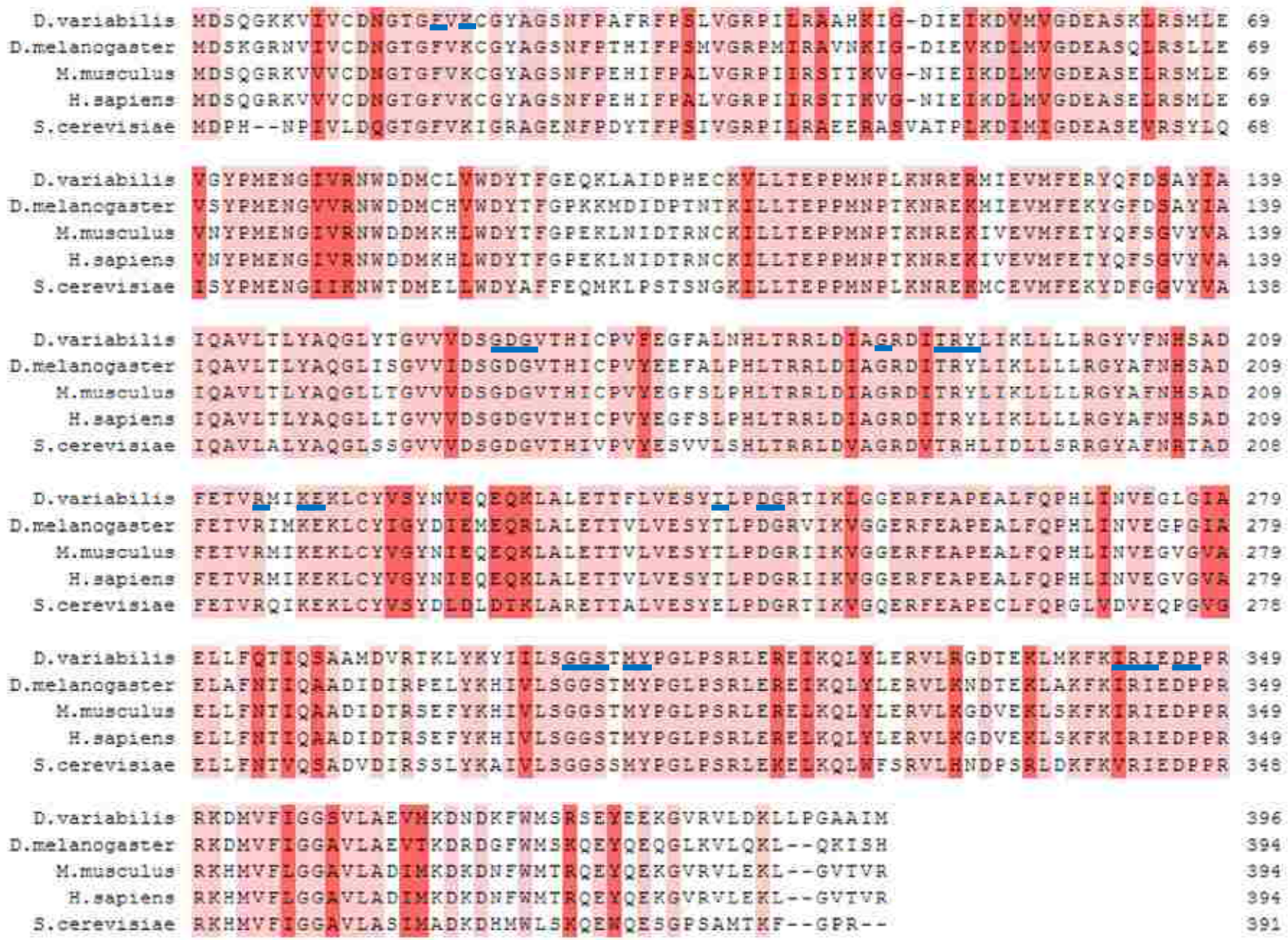
number of deduced amino acid sequences, and estimated molecular weights (MW) of each DvArp2/3 complex subunits are shown in Table 3.3.

Table 3.3 DNA, ORF, amino acid sequence lengths and estimated MW of DvArp2/3 complex subunits.

Subunit	DNA sequence length (bp)	ORF length (bp)	Numbers of amino acids	Estimated MW (kDa)
DvArp2	2718	1191	396	45
DvArp3	1780	1230	409	46
DvArpc1	1852	1014	337	38
DvArpc2	1497	903	300	35
DvArpc3	857	546	181	20
DvArpc4	714	507	168	20
DvArpc5	912	555	152	17

Amino acid sequence analyses of DvArp2/3 complex subunits (Figure 3.1-3.8) were performed using a web-based multiple sequence alignment (MUSCLE) and the percent identity compared to the corresponding subunits of the Arp2/3 complex from *Drosophila melanogaster*, *Mus musculus*, *Homo sapiens*, and *Saccharomyces cerevisiae* are shown in Table 3.4. As mentioned earlier, Arp2 and Arp3 bind to ATP; the proteins were thus searched for ATP binding sites using NsitePred - web server. The predicted ATP binding sites on both proteins are underlined in red in Figure 3.1 and 3.2.

Figure 3.1 (Following page) Multiple sequence alignment of Arp2 subunit sequences. Multiple sequence comparison by log-expectation (MUSCLE) software was used to create a sequence alignment of Arp2 subunits from *D. variabilis*, *D. melanogaster*, *M. musculus*, *H. sapiens*, and *S. cerevisiae*. Identical and similar amino acids are highlighted in light red and dark red, respectively. Figure was created using Multiple Align Show program. ATP binding sites predicted by NsitePred - web server are underlined in blue.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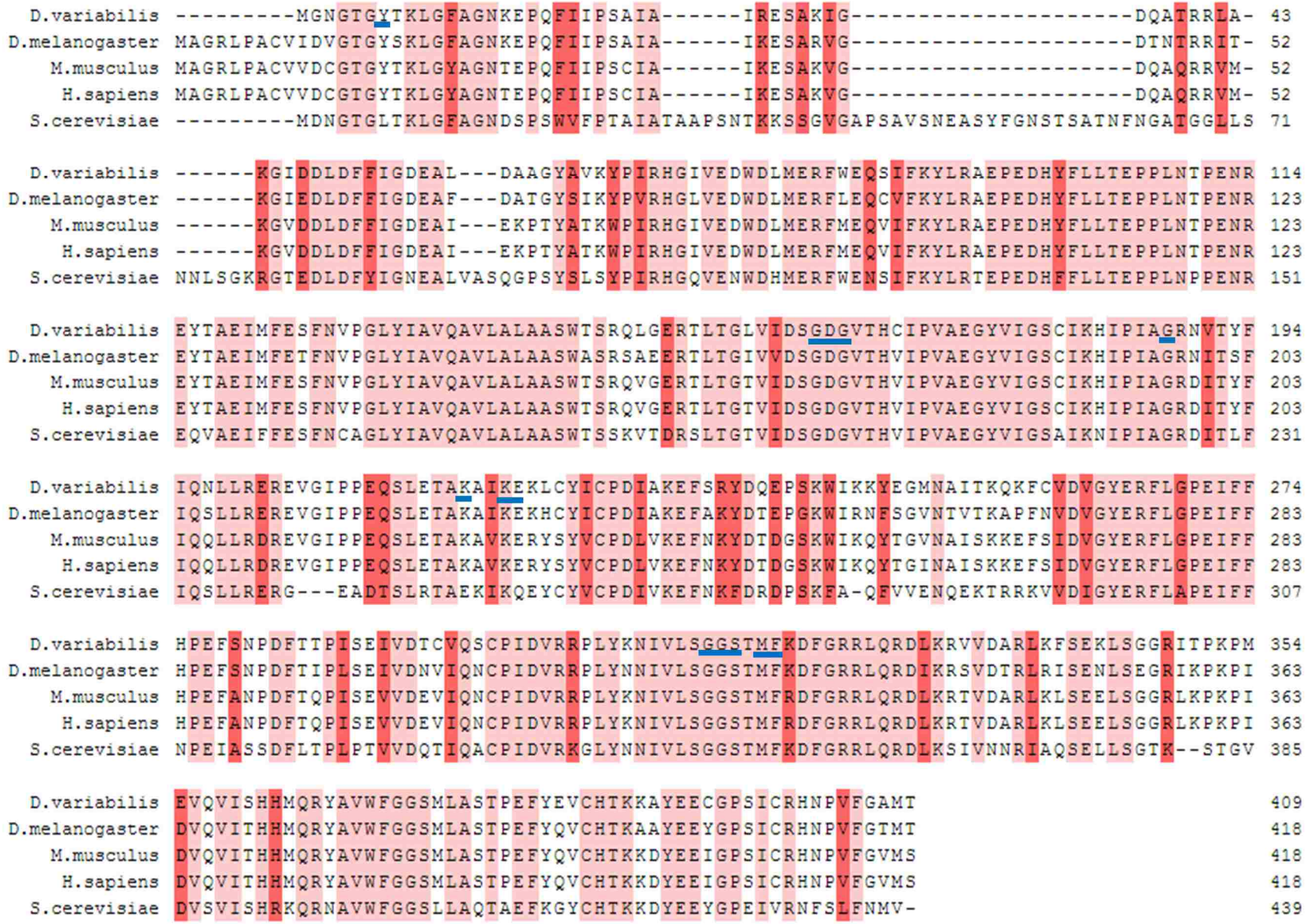
D.melanogaster MDSKGRNVIVCDNGTGFVKCGYAGSNFPTHIFPSMVGRPMIRAVNKIG-DIEVKDLMVGDEASQLRSLLE 69
M.musculus MDSQGRKVVVCDMGTGFVKCGYAGSNFPEHIFPALVGRPIIRSTTKVG-NIEIKDLMVGDEASELRSMLE 69
H.sapiens MDSQGRKVVVCDNGTGFVKCGYAGSNFPEHIFPALVGRPIIRSTTKVG-NIEIKDLMVGDEASELRSMLE 69
S.cerevisiae MDPH--NPIVLDQGTGFVKIGRAGENFPDYTFPSIVGRPILRAEERASVATPLKDIMIGDEASEVRSYLQ 68
D.variabilis VGYPMENGIVRNWDDMCLVWDYTFGEQKLAIDPHECKVLLTEPPMNPLKNRERMIEVMFERYQFDSAYIA 139
D.melanogaster VSYPMENGVVRNWDDMCHVWDYTFGPKKMDIDPTNTKILLTEPPMNPTKNREKMIEVMFEKYGFDSAYLA 139
M.musculus VNYPMENGIVRNWDDMKHLWDYTFGPERLNIDTRNCKILLTEPPMNPTKNREKIVEVMFETYQFSGVYVA 139
H.sapiens VNYPMENGIVRNWDDMKHLWDYTFGPEKLNIDTRNCKILLTEPPMNPTKNREKIVEVMFETYQFSGVYVA 139
S.cerevisiae ISYPMENGIIKNWTDMELLWDYAFFEQMKLPSTSNGKILLTEPPMNPLKNRENMCEVMFEKYDFGGVYVA 138
D.variabilis IQAVLTLYAQGLYTGVVVDSGDGVTHICPVFEGFALNHLTRRLDIAGRDITRYLIKLLLLRGYVFNHSAD 209
D.melanogaster IQAVLTLYAQGLISGVVIDSGDGVTHICPVYEEFALPHLTRRLDIAGRDITRYLIKLLLLRGYAFNHSAD 209
M.musculus IQAVLTLYAQGLLTGVVVDSGDGVTHICPVYEGFSLPHLTRRLDIAGRDITRYLIKLLLLRGYAFNHSAD 209
H.sapiens IQAVLTLYAQGLLTGVVVDSGDGVTHICPVYEGFSLPHLTRRLDIAGRDITRYLIKLLLLRGYAFNHSAD 209
S.cerevisiae IQAVLALYAQGLSSGVVVDSGDGVTHIVPVYESVVLSHLTRRLDVAGRDVTRHLIDLLSRRGYAFNRTAD 208
D.variabilis FETVRMIKEKLCYVSYNVEQEQKLALETTFLVESYILPDGRTIKLGGERFEAPEALFQPHLINVEGLGIA 279
D.melanogaster FETVRIMKEKLCYIGYDIEMEQRLALETTVLVESYTLPDGRVIKVGGERFEAPEALFQPHLINVEGPGIA 279
M.musculus FETVRMIKEKLCYVGYNIEQEQKLALETTVLVESYTLPDGRIIKVGGERFEAPEALFQPHLINVEGVGVA 279
H.sapiens FETVRMIKEKLCYVGYNIEQEQKLALETTVLVESYTLPDGRIIKVGGERFEAPEALFQPHLINVEGVGVA 279
S.cerevisiae FETVRQIKEKLCYVSYDLDLDIKLARETTALVESYELPDGRTIKVGQERFEAPECLFQPGLVDVEQPGVG 278
D.variabilis ELLFQTIQSAAMDVRTKLYKYIILSGGSTMYPGLPSRLERELKQLYLERVLRGDTEKLMKFKIRIEDPPR 349
D.melanogaster ELAFNTIQAADIDIRPELYKHIVLSGGSTMYPGLPSRLERELKQLYLERVLKNDTEKLAKFKIRIEDPPR 349
M.musculus ELLFNTIQAADIDTRSEFYKHIVLSGGSTMYPGLPSRLERELKQLYLERVLKGDVEKLSKFKIRIEDPPR 349
H.sapiens ELLFNTIQAADIDTRSEFYKHIVLSGGSTMYPGLPSRLERELKQLYLERVLKGDVEKLSKFKIRIEDPPR 349
S.cerevisiae ELLFNTVQSADVDIRSSLYKAIVLSGGSSMYPGLPSRLEKELKQLWFSRVLHNDPSRLDKFKVRIEDPPR 348
D.variabilis RKDMVFIGGSVLAEVMKDNDKFWMSRSEYEEKGVRVLDKLLPGAAIM 396
D.melanogaster RKDMVFIGGAVLAEVTKDRDGFWMSKQEYQEQGLKVLQKL--QKISH 394
M.musculus RKHMVFLGGAVLADIMKDKDNFWMTRQEYQEKGVRVLEKL--GVTVR 394
H.sapiens RKHMVFLGGAVLADIMKDKDNFWMTRQEYQEKGVRVLEKL--GVTVR 394
S.cerevisiae RKHMVFIGGAVLASIMADKDHMWLSKQEWQESGPSAMTKF--GPR-- 391

Figure 3.2 (Following page) Multiple sequence alignment of Arp3 subunit sequences. Sequence alignment of Arp3 subunits from *D. variabilis*, *D. melanogaster*, *M. musculus*, *H. sapiens*, and *S. cerevisiae* was obtained from multiple sequence comparison by log-expectation (MUSCLE) software. Identical and similar amino acids are highlighted in light red and dark red, respectively. Figure was created using Multiple Align Show and ATP binding sites predicted by NsitePred - web server are underlined in blue.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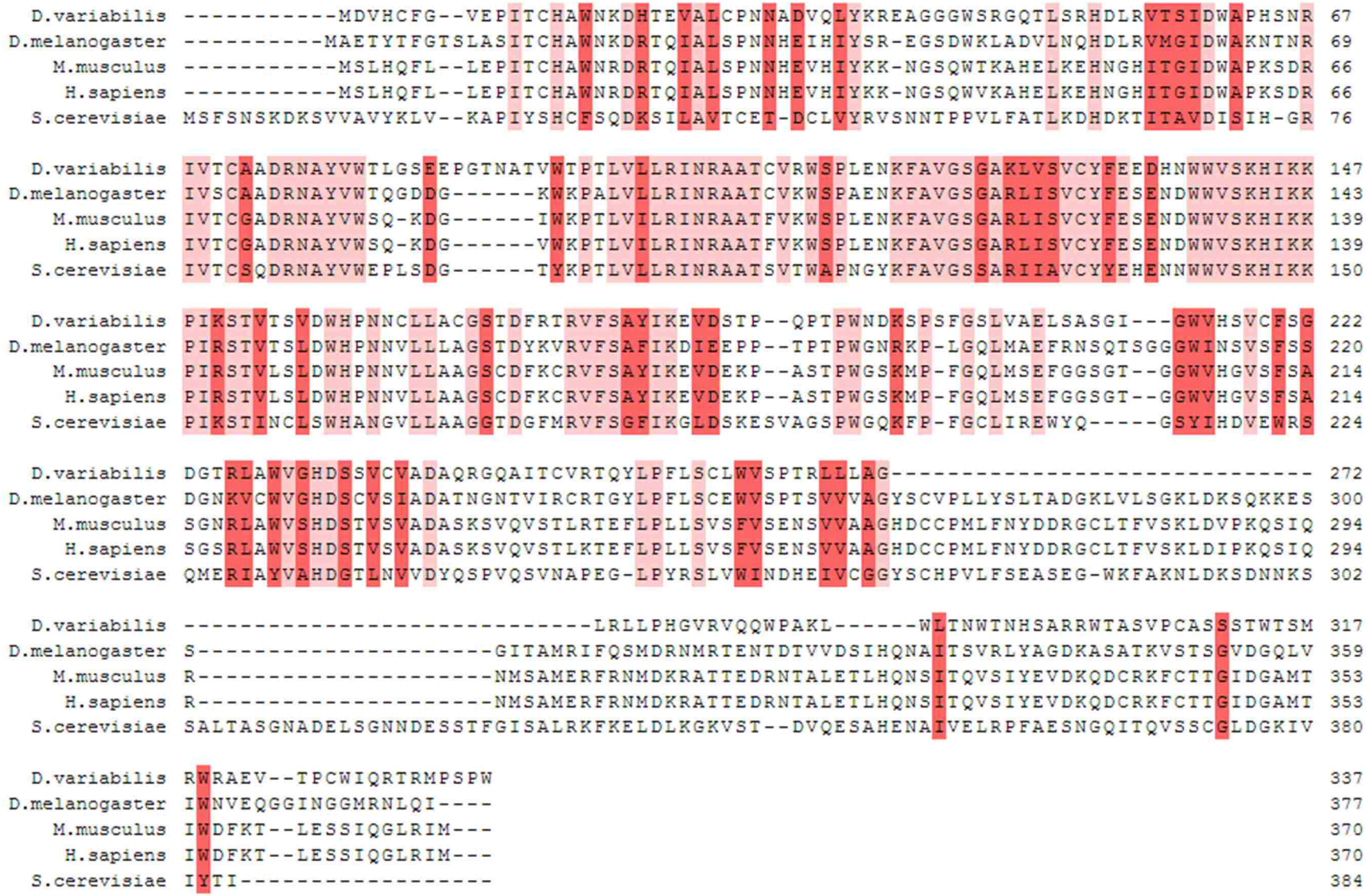
D.melanogaster MAGRLPACVIDVGTGYSKLGFAGNKEPQFIIPSAIA------IKESARVG----------DTNTRRIT- 52
M.musculus MAGRLPACVVDCGTGYTKLGYAGNTEPQFIIPSCIA------IKESAKVG----------DQAQRRVM- 52
H.sapiens MAGRLPACVVDCGTGYTKLGYAGNTEPQFIIPSCIA------IKESAKVG----------DQAQRRVM- 52
S.cerevisiae ---------MDNGTGLTKLGFAGNDSPSWVFPTAIATAAPSNTKKSSGVGAPSAVSNEASYFGNSTSATNFNGATGGLLS 71
D.variabilis ------KGIDDLDFFIGDEAL---DAAGYAVKYPIRHGIVEDWDLMERFWEQSIFKYLRAEPEDHYFLLTEPPLNTPENR 114
D.melanogaster ------KGIEDLDFFIGDEAF---DATGYSIKYPVRHGLVEDWDLMERFLEQCVFKYLRAEPEDHYFLLTEPPLNTPENR 123
M.musculus ------KGVDDLDFFIGDEAI---EKPTYATKWPIRHGIVEDWDLMERFMEQVIFKYLRAEPEDHYFLLTEPPLNTPENR 123
H.sapiens ------KGVDDLDFFIGDEAI---EKPTYATKWPIRHGIVEDWDLMERFMEQVIFKYLRAEPEDHYFLLTEPPLNTPENR 123
S.cerevisiae NNLSGKRGTEDLDFYIGNEALVASQGPSYSLSYPIRHGQVENWDHMERFWENSIFKYLRTEPEDHFFLLTEPPLNPPENR 151
D.variabilis EYTAEIMFESFNVPGLYIAVQAVLALAASWTSRQLGERTLTGLVIDSGDGVTHCIPVAEGYVIGSCIKHIPIAGRNVTYF 194
D.melanogaster EYTAEIMFETFNVPGLYIAVQAVLALAASWASRSAEERTLTGIVVDSGDGVTHVIPVAEGYVIGSCIKHIPIAGRNITSF 203
M.musculus EYTAEIMFESFNVPGLYIAVQAVLALAASWTSRQVGERTLTGTVIDSGDGVTHVIPVAEGYVIGSCIKHIPIAGRDITYF 203
H.sapiens EYTAEIMFESFNVPGLYIAVQAVLALAASWTSRQVGERTLTGTVIDSGDGVTHVIPVAEGYVIGSCIKHIPIAGRDITYF 203
S.cerevisiae EQVAEIFFESFNCAGLYIAVQAVLALAASWTSSKVTDRSLTGTVIDSGDGVTHVIPVAEGYVIGSAIKNIPIAGRDITLF 231
D.variabilis IQNLLREREVGIPPEQSLETAKAIKEKLCYICPDIAKEFSRYDQEPSKWIKKYEGMNAITKQKFCVDVGYERFLGPEIFF 274
D.melanogaster IQSLLREREVGIPPEQSLETAKAIKEKHCYICPDIAKEFAKYDTEPGKWIRNFSGVNTVTKAPFNVDVGYERFLGPEIFF 283
M.musculus IQQLLRDREVGIPPEQSLETAKAVKERYSYVCPDLVKEFNKYDTDGSKWIKQYTGVNAISKKEFSIDVGYERFLGPEIFF 283
H.sapiens IQQLLRDREVGIPPEQSLETAKAVKERYSYVCPDLVKEFNKYDTDGSKWIKQYTGINAISKKEFSIDVGYERFLGPEIFF 283
S.cerevisiae IQSLLRERG---EADTSLRTAEKIKQEYCYVCPDIVKEFNKFDRDPSKFA-QFVVENQEKTRRKVVDIGYERFLAPEIFF 307
D.variabilis HPEFSNPDFTTPISEIVDTCVQSCPIDVRRPLYKNIVLSGGSTMFKDFGRRLQRDLKRVVDARLKFSEKLSGGRITPKPM 354
D.melanogaster HPEFSNPDFTIPLSEIVDNVIQNCPIDVRRPLYNNIVLSGGSTMFKDFGRRLQRDIKRSVDTRLRISENLSEGRIKPKPI 363
M.musculus HPEFANPDFTQPISEVVDEVIQNCPIDVRRPLYKNIVLSGGSTMFRDFGRRLQRDLKRTVDARLKLSEELSGGRLKPKPI 363
H.sapiens HPEFANPDFTQPISEVVDEVIQNCPIDVRRPLYKNIVLSGGSTMFRDFGRRLQRDLKRTVDARLKLSEELSGGRLKPKPI 363
S.cerevisiae NPEIASSDFLTPLPTVVDQTIQACPIDVRKGLYNNIVLSGGSTMFKDFGRRLQRDLKSIVNNRIAQSELLSGTK--STGV 385
D.variabilis EVQVISHHMQRYAVWFGGSMLASTPEFYEVCHTKKAYEECGPSICRHNPVFGAMT 409
D.melanogaster DVQVITHHMQRYAVWFGGSMLASTPEFYQVCHTKAAYEEYGPSICRHNPVFGTMT 418
M.musculus DVQVITHHMQRYAVWFGGSMLASTPEFYQVCHTKKDYEEIGPSICRHNPVFGVMS 418
H.sapiens DVQVITHHMQRYAVWFGGSMLASTPEFYQVCHTKKDYEEIGPSICRHNPVFGVMS 418
S.cerevisiae DVSVISHRKQRNAVWFGGSLLAQTAEFKGYCHTKKDYEEYGPEIVRNFSLFNMV- 439

Figure 3.3 (Following page) Multiple sequence alignment of ARPC1 subunit sequences. Multiple sequence comparison by log-expectation (MUSCLE) software was utilized to generate sequence alignment of ARPC1 subunits from *D. variabilis*, *D. melanogaster*, *M. musculus*, *H. sapiens*, and *S. cerevisiae*. Identical and similar amino acids are highlighted in light red and dark red, respectively. Figure was created using Multiple Align Show.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D.melanogaster ----------MAETYTFGTSLASITCHAWNKDRTQIALSPNNHEIHIYSR-EGSDWKLADVLNQHDLRVMGIDWAKNTNR 69
M.musculus ----------MSLHQFL--LEPITCHAWNRDRTQIALSPNNHEVHIYKK-NGSQWTKAHELKEHNGHITGIDWAPKSDR 66
H.sapiens ----------MSLHQFL--LEPITCHAWNRDRTQIALSPNNHEVHIYKK-NGSQWVKAHELKEHNGHITGIDWAPKSDR 66
S.cerevisiae MSFSNSKDKSVVAVYKLV--KAPIYSHCFSQDKSILAVTCET-DCLVYRVSNNTPPVLFATLKDHDKTITAVDISIH-GR 76
D.variabilis IVTCAADRNAYVWTLGSEEPGTNATVWTPTLVLLRINRAATCVRWSPLENKFAVGSGAKLVSVCYFEEDHNWWVSKHIKK 147
D.melanogaster IVSCAADRNAYVWTQGDDG------KWKPALVLLRINRAATCVKWSPAENKFAVGSGARLISVCYFESENDWWVSKHIKK 143
M.musculus IVTCGADRNAYVWSQ-KDG------IWKPTLVILRINRAATFVKWSPLENKFAVGSGARLISVCYFESENDWWVSKHIKK 139
H.sapiens IVTCGADRNAYVWSQ-KDG------VWKPTLVILRINRAATFVKWSPLENKFAVGSGARLISVCYFESENDWWVSKHIKK 139
S.cerevisiae IVTCSQDRNAYVWEPLSDG------TYKPTLVLLRINRAATSVTWAPNGYKFAVGSSARIIAVCYYEHENNWWVSKHIKK 150
D.variabilis PIKSTVTSVDWHPNNCLLACGSTDFRTRVFSAYIKEVDSTP--QPTPWNDKSPSFGSLVAELSASGI---GWVHSVCFSG 222
D.melanogaster PIRSTVTSLDWHPNNVLLLAGSTDYKVRVFSAFIKDIEEPP--TPTPWGNRKP-LGQLMAEFRNSQTSGGGWINSVSFSS 220
M.musculus PIRSTVLSLDWHPNNVLLAAGSCDFKCRVFSAYIKEVDEKP--ASTPWGSKMP-FGQLMSEFGGSGT--GGWVHGVSFSA 214
H.sapiens PIRSTVLSLDWHPNNVLLAAGSCDFKCRVFSAYIKEVDEKP--ASTPWGSKMP-FGQLMSEFGGSGT--GGWVHGVSFSA 214
S.cerevisiae PIKSTINCLSWHANGVLLAAGGTDGFMRVFSGFIKGLDSKESVAGSPWGQKFP-FGCLIREWYQ-----GSYIHDVEWRS 224
D.variabilis DGTRLAWVGHDSSVCVADAQRGQAITCVRTQYLPFLSCLWVSPTRLLLAG------------------------------ 272
D.melanogaster DGNKVCWVGHDSCVSIADATNGNTVIRCRTGYLPFLSCEWVSPTSVVVAGYSCVPLLYSLTADGKLVLSGKLDKSQKKES 300
M.musculus SGNRLAWVSHDSTVSVADASKSVQVSTLRTEFLPLLSVSFVSENSVVAAGHDCCPMLFNYDDRGCLTFVSKLDVPKQSIQ 294
H.sapiens SGSRLAWVSHDSTVSVADASKSVQVSTLKTEFLPLLSVSFVSENSVVAAGHDCCPMLFNYDDRGCLTFVSKLDIPKQSIQ 294
S.cerevisiae QMERIAYVAHDGTLNVVDYQSPVQSVNAPEG-LPYRSLVWINDHEIVCGGYSCHPVLFSEASEG-WKFAKNLDKSDNNKS 302
D.variabilis ----------------------------LRLLPHGVRVQQWPAKL------WLTNWTNHSARRWTASVPCASSSTWTSM 317
D.melanogaster S----------------------GITAMRIFQSMDRNMRTENTDTVVDSIHQNAITSVRLYAGDKASATKVSTSGVDGQLV 359
M.musculus R----------------------NMSAMERFRNMDKRATTEDRNTALETLHQNSITQVSIYEVDKQDCRKFCTTGIDGAMT 353
H.sapiens R----------------------NMSAMERFRNMDKRATTEDRNTALETLHQNSITQVSIYEVDKQDCRKFCTTGIDGAMT 353
S.cerevisiae SALTASGNADELSGNNDESSTFGISALRKFKELDLKGKVST--DVQESAHENAIVELRPFAESNGQITQVSSCGLDGKIV 380
D.variabilis RWRAEV--TPCWIQRTRMPSPW 337
D.melanogaster IWNVEQGGINGGMRNLQI---- 377
M.musculus IWDFKT--LESSIQGLRIM--- 370
H.sapiens IWDFKT--LESSIQGLRIM--- 370
S.cerevisiae IYTI---------------- 384

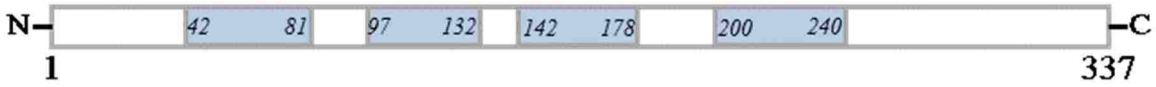

Figure 3.4 Schematic diagram represented the structure of DvARPC1 subunit. Numbers correspond to amino acids of the protein sequence. Shaded blue regions are putative WD domains predicted by SMART software.

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  D.variabilis MILLEINNRIIEETLRLKFKNAQSANKPEAVDVKIADFDGVLYHISNPNGDKTKIRVSIS 60
D.melanogaster MILLEINNRIIEETLLVKYRNAQAGLKPESIDIRIADFDGVLYHISNVNGDKTKVRISIS 60
    M.musculus MILLEVNNRIIEETLALKFENAAAGNKPEAVEVTFADFDGVLYHISNPNGDKTKVMVSIS 60
     H.sapiens MILLEVNNRIIEETLALKFENAAAGNKPEAVEVTFADFDGVLYHISNPNGDKTKVMVSIS 60
  S.cerevisiae MLHLQPQNLLIQKTLNEXIEALRKGS-PLTMDRIVSDFDYTTYHISNTAEDKSILLLSVK 59

  D.variabilis LKFYKELQEHGAD------ELLKREYGPL-----LTTTEDGYNVSLLFDLENLPENTEEI 109
D.melanogaster LKFYKQLQEHGAD------ELLKREYGSL-----LTDTEEGYNVSVLINLEEIPEDCEQI 109
    M.musculus LKFYKELQAHGAD------ELLKRVYGSF-----LVNPEPGYNVSLLYDLENLPASKDSI 109
     H.sapiens LKFYKELQAHGAD------ELLKRVYGSF-----LVNPESGYNVSLLYDLENLPASKDSI 109
  S.cerevisiae TKAWVSVSECQLDGSLTLLKFLADHYSSLGGVTIPSEXEPGYDYTLXITLAEL---VQES 116

  D.variabilis ARKASHLKRNCFASVFE----KYFDFQER----------GEIGDKRA----VIPYRDDE 150
D.melanogaster AKRIGLLKRNCFASVFE----KYFDYQEQ----------GEEGQKRA----VINYRNDE 150
    M.musculus VHQAGMLKRNCFASVFE----KYFQFQEE----------GKEGENRA----VIHYRDDE 150
     H.sapiens VHQAGMLKRNCFASVFE----KYFQFQEE----------GKEGENRA----VIHYRDDE 150
  S.cerevisiae ILQLSVLKTIILSFPFELAISKFIELSQQQPAPVEAEITGGEVAANGDNTLFTIKYRDEE 176

  D.variabilis TMYVEAKPDRVTVVFSTMFKDEGDIVLGKVFMQEFKEGRKAS---HTAPQVLFSHREPPK 207
D.melanogaster TLYVEAKPDRVTVVFSTIFRDEDDVIIGKVFMQELREGRRAS---HTAPQVLFSHREPPL 207
    M.musculus TMYVESKKDRVTVVFSTVFKDDDDVVIGKVFMQEFKEGRRAS---HTAPQVLFSHREPPL 207
     H.sapiens TMYVESKKDRVTVVFSTVFKDDDDVVIGKVFMQEFKEGRRAS---HTAPQVLFSHREPPL 207
  S.cerevisiae NIFIKPSNDRVTIIFETIFQDETDKIFGKVFLQEFVDARKRNRQIQSAPQVLYSH-EPPL 235

  D.variabilis ELE---NTDARVGNSIGYITFVLFPRH-TNRQARDNTINLIHIFRDYLHYHIKCSKAYIH 263
D.melanogaster ELA---NTDARVGDNIGYVTFVLFPRH-TNKETRDNTINLIHMFRDYLHYHIKCSKAYIH 263
    M.musculus ELK---DTDAAVGDNIGYITFVLFPRH-TNATARDNTINLIHTFRDYLHYHIKCSKAYIH 263
     H.sapiens ELK---DTDAAVGDNIGYITFVLFPRH-TNASARDNTINLIHTFRDYLHYHIKCSKAYIH 263
  S.cerevisiae ELKRLYQPPKVAEQSRRFITFVLFPRHFQTKELQFHSICQLTLFRNYFHYHIKCSKAYMH 295

  D.variabilis SRMRTKTSDFLKVLNRARPDPK------SVEKKTITGRTFTRN---- 300
D.melanogaster SRMRAKTSDFLKVLNRARPEPK------NTEKKTITGRTFKRID--- 301
    M.musculus TRMRAKTSDFLKVLNRARPDAE------KKEMKTITGKTFSSR---- 300
     H.sapiens TRMRAKTSDFLKVLNRARPDAE------KKEMKTITGKTFSSR---- 300
  S.cerevisiae SRMRFRVDSFIKVLNRAKVDEDDENDELSAEGRQQARRTFTGRKIVY 342
```

Figure 3.5 Multiple sequence alignment of ARPC2 subunit sequences. Sequence alignment of ARPC2 subunits from *D. variabilis*, *D. melanogaster*, *M. musculus*, *H. sapiens*, and *S. cerevisiae* was generated using multiple sequence comparison by log-expectation (MUSCLE) software. Identical and similar amino acids are highlighted in light red and dark red, respectively. Figure was created using Multiple Align Show.

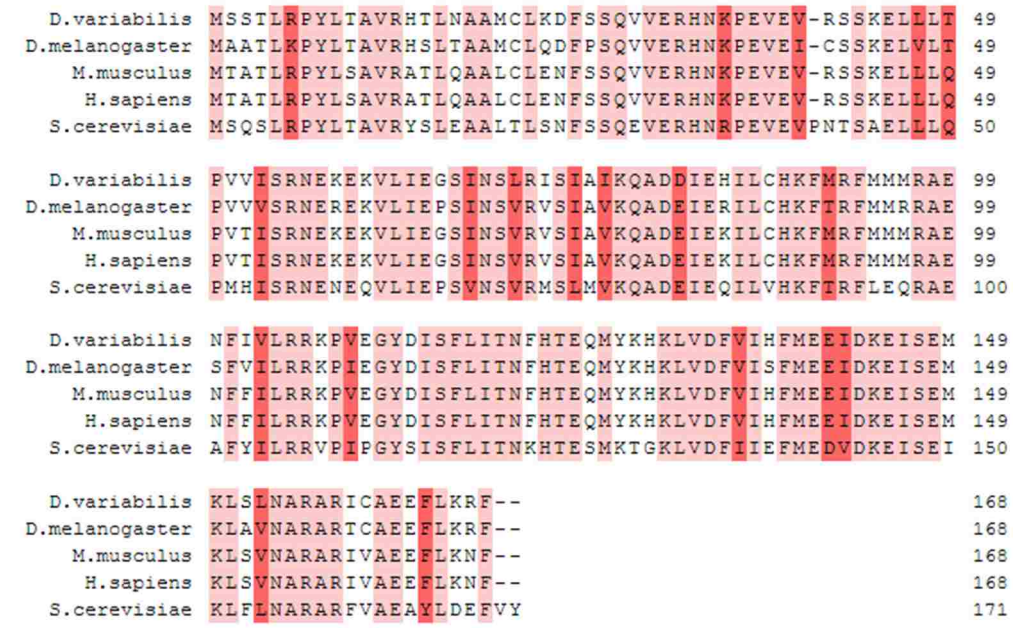

Figure 3.6 Multiple sequence comparison of ARPC3 subunit. The DvARPC3 deduced amino acid sequence was aligned *D. variabilis*, *D. melanogaster*, *M. musculus*, *H. sapiens*, and *S. cerevisiae*. Alignment was performed using multiple sequence comparison by log-expectation (MUSCLE) software. Shaded light red and dark red indicate identical and similar amino acid residues, respectively.

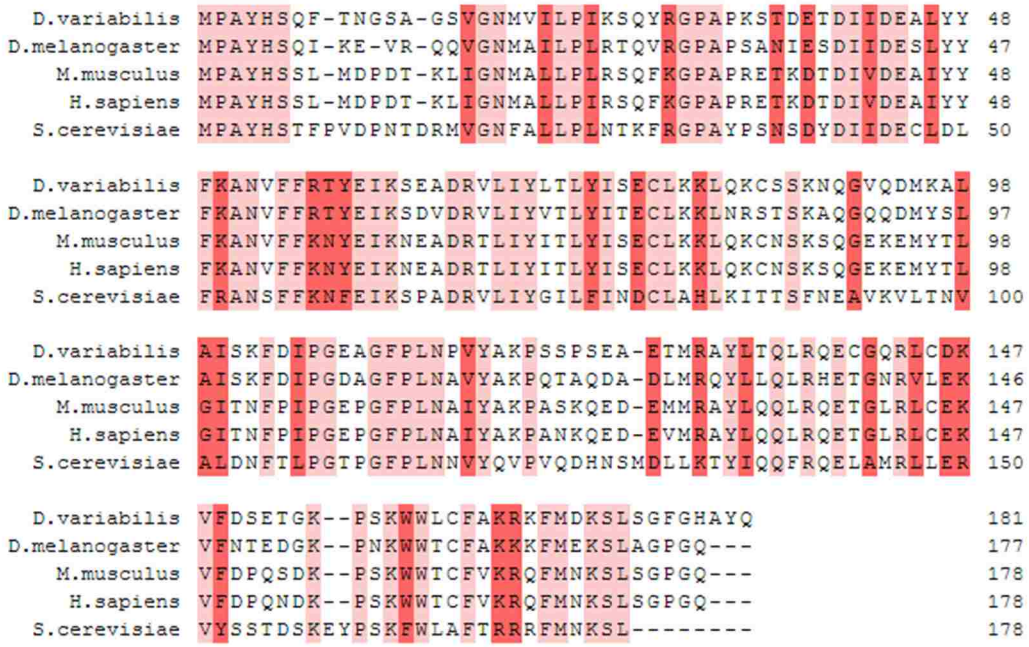

Figure 3.7 Multiple sequence alignment of ARPC4 subunit sequences**.** Sequence alignment of ARPC4 subunits from *D. variabilis*, *D. melanogaster*, *M. musculus*, *H. sapiens*, and *S. cerevisiae* was conducted using multiple sequence comparison by log-expectation (MUSCLE) software. Identical and similar amino acids are shaded in light red and dark red, respectively. Figure was created using Multiple Align Show program.

Figure 3.8 Multiple sequence comparison of ARPC5 subunit of Arp2/3 complex. Multiple sequence comparison by log-expectation (MUSCLE) software was used to produce sequence alignment of ARPC5 subunits from *D. variabilis*, *D. melanogaster*, *M. musculus*, *H. sapiens*, and *S. cerevisiae*. Identical and similar amino acids are highlighted in light red and dark red, respectively. Figure was created using Multiple Align Show.

As shown in Figure 3.4, putative WD motifs, conserved domains in ARPC1 protein (Welch et al., 1997), were also observed in ARPC1 subunit from *D. variabilis* (4 domains).

3.3.2 Expression of DvArp2/3 complex subunit mRNAs in tick tissues infected *ex vivo*

To explore transcriptional profiles of DvArp2/3 complex (all subunits) in *D. variabilis* tissues (midgut, ovary and salivary glands) in response to *R. montanensis* infection, tick tissues were dissected out of the ticks and infected with *Rickettsia*. After 1 h, the tissues were washed and collected by low speed centrifugation. Total RNA was extracted from the samples and the levels of DvArp2, DvArp3, DvARPC1, DvARPC2, DvARPC3, DvARPC4, and DvARPC5 mRNA were measured by qRT-PCR.

Table 3.4 Percent identity of DvArp2/3 complex subunits compared to the corresponding subunits of Arp2/3 complex from different organisms.

DvArp2/3 complex subunits	*D. melanogaster*	*M. musculus*	*H. sapiens*	*S. cerevisiae*
DvArp2	80 %	81 %	81 %	60 %
DvArp3	83 %	83 %	83 %	58 %
DvARPC1	51 %	51 %	51 %	36 %
DvARPC2	79 %	78 %	78 %	36 %
DvARPC3	67 %	66 %	66 %	46 %
DvARPC4	86 %	92 %	92 %	65 %
DvARPC5	59 %	56 %	56 %	26 %

As shown in Figure 3.9 – 3.15, the mRNA of all DvArp2/3 complex subunits was detectable in all tick tissues and in both unexposed- and *Rickettsia* exposed- tissues. Interestingly, the mRNA levels were expressed higher in the ovary than in the midgut and salivary glands with significant differences for DvArp3 ($P = 0.0496$ and 0.0534 in uninfected ovary compared to midgut and salivary glands, respectively; $P = 0.0031$ and 0.0105 in infected ovary compared to midgut and salivary glands, respectively), DvARPC4 ($P = 0.0217$ and 0.0270 in uninfected ovary compared to midgut and salivary glands, respectively; $P < 0.0001$ and $P = 0.0012$ in infected ovary compared to midgut and salivary glands, respectively), and DvARPC5 ($P < 0.0001$ in uninfected ovary compared to both midgut and salivary glands; $P < 0.0001$ in infected ovary compared to

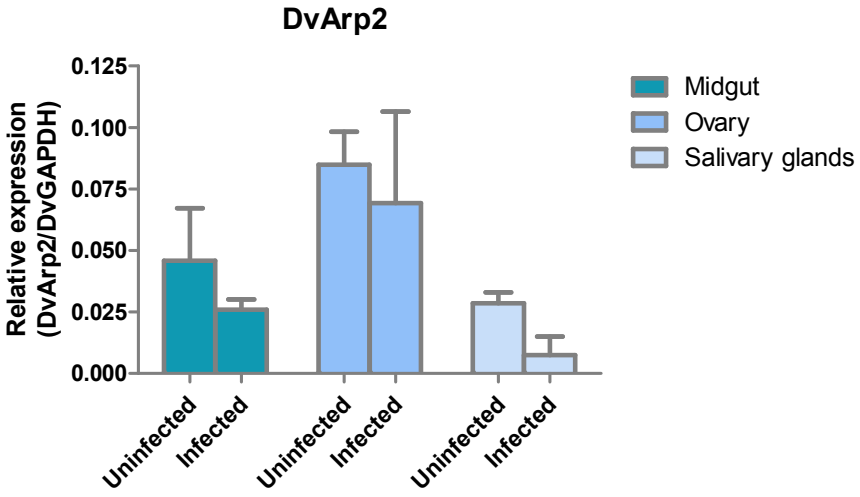

Figure 3.9 Transcriptional profile of Arp2 subunit in *D. variabilis* tissues. The tissues (midgut, ovary, and salivary glands) were dissected out of the ticks then exposed to *R. montanensis* (8 x 10^7 per tissue) for 1 h. After washing, the samples were collected by low-speed centrifugation and extracted for total RNA. The level of DvApr2 mRNA was measured by qRT-PCR and normalized with tick GAPDH mRNA. Data shown are mean relative expression from two independent experiments. Error bar represents SEM values.

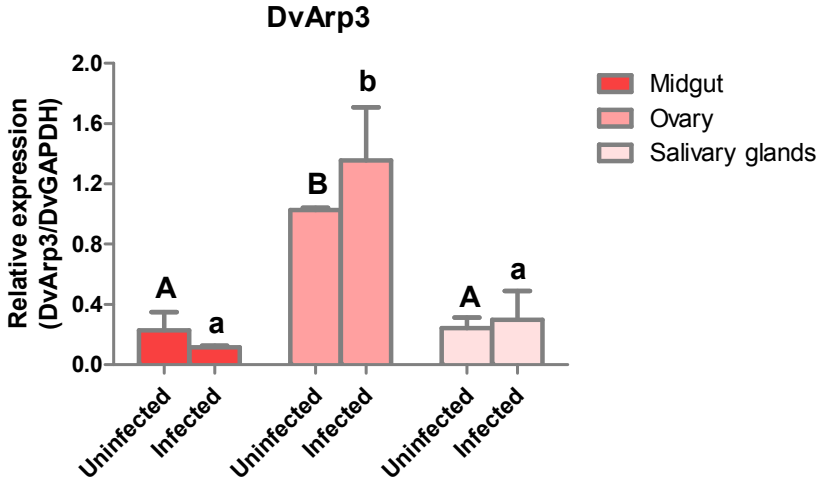

Figure 3.10 Expression of Arp3 subunit mRNA in D. variabilis tissues. After being dissected out, tick tissues including midgut, ovary, and salivary glands were infected with *R. montanensis* (8 x 10^7 per tissue) for 1 h. The tissues were then washed and harvested by low-speed centrifugation. Total RNA was extracted from the tissues and the level of mRNA expression was measured by qRT-PCR. DvGAPDH mRNA was used for normalization of sample-to-sample differences. Data shown are mean relative expression from two independent experiments. Error bar represents SEM values. Graphs with different letters in the same treatment group (unexposed- or *Rickettsia* exposed- tissues) were considered significantly different ($P \leq 0.05$). *P*-values of 0.0496 and 0.0534 represent uninfected ovary compared to midgut and salivary glands, respectively. *P*-values of 0.0031 and 0.0105 represent infected ovary compared to midgut and salivary glands, respectively.

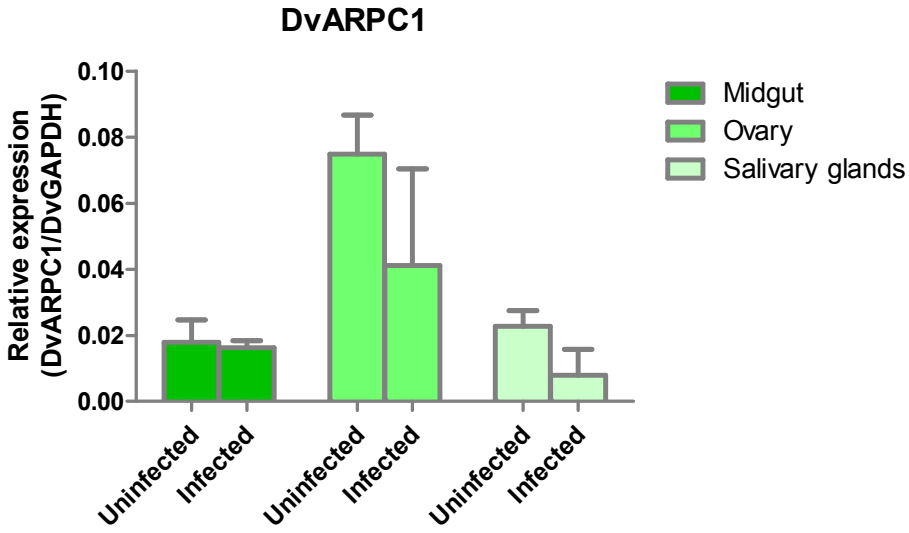

Figure 3.11 Transcriptional profile of ARPC1 in tick tissues. Midgut, ovary, and salivary glands were dissected out of the ticks and infected with *R. montanensis* (8 x 10^7 per tissues) for 1 h. After removal of *Rickettsia*, the tissues were washed and collected by low-speed centrifugation. Total RNA was then extracted from the samples and qRT-PCR was performed to measure DvARPC1 mRNA level. DvGAPDH mRNA was used for normalization of sample-to-sample differences. Data shown are mean relative expression from two independent experiments. Error bar represents SEM values.

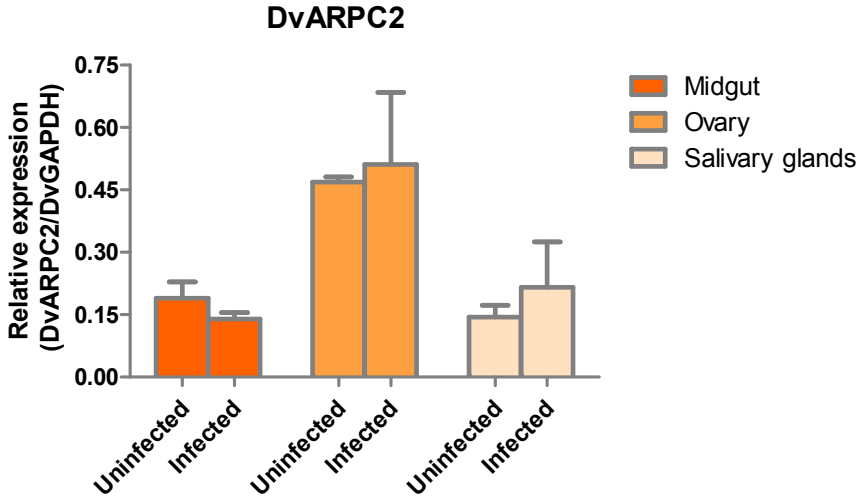

Figure 3.12 Transcriptional profile of ARPC2 in *D. variabilis* tissues. Ticks were dissected and tissues including midgut, ovary, and salivary glands were separated out. *Rickettsia montanensis* (8 x 10^7) was then used to infect tick tissues for 1 h. After washing, the samples were harvested by low-speed centrifugation and total RNA was extracted from the tissues. qRT-PCR was then performed to measure the level of DvARPC2 expression. DvGAPDH mRNA was used for normalization of sample-to-sample differences. Data shown are mean relative expression from two independent experiments. Error bar represents SEM values.

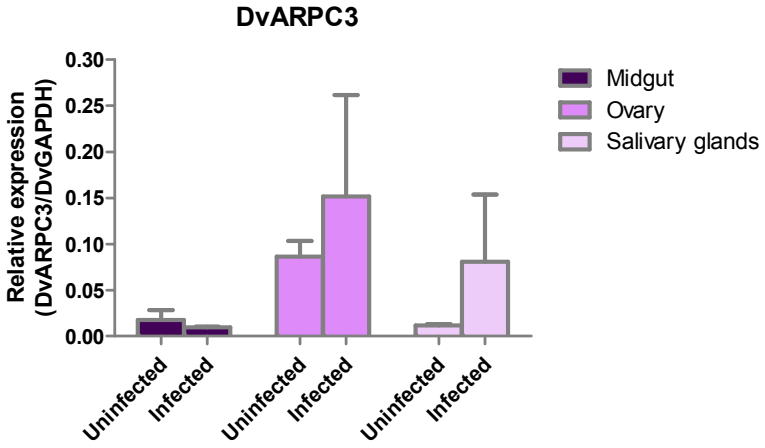

Figure 3.13 Expression of DvARPC3 mRNA in tick tissues. Midgut, ovary, and salivary glands were dissected out of *D. variabilis* ticks and infected with *R. montanensis* (8 x 10^7 per tissue) for 1 h. After removal of the bacteria, the tissues were washed and collected by low-speed centrifugation. Total RNA was then extracted from the samples and the level of DvARPC3 mRNA was determined by qRT-PCR. To normalize the differences of samples, DvGAPDH mRNA was used. Data shown are mean relative expression from two independent experiments. Error bar represents SEM values.

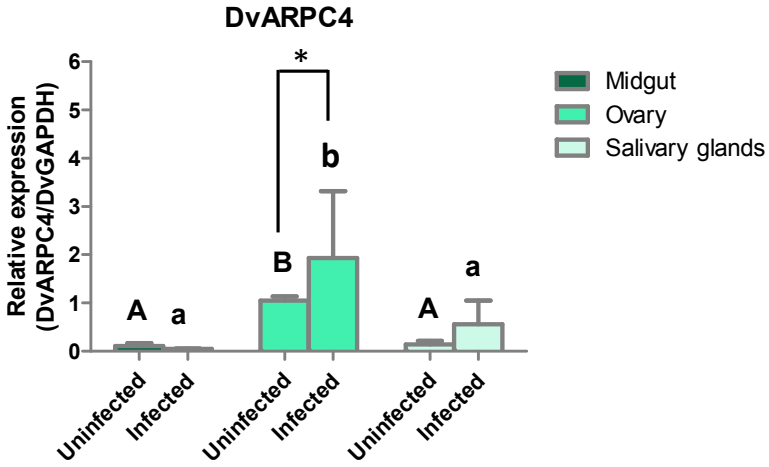

Figure 3.14 Expression of DvARPC4 mRNA in *D. variabilis* tissues. *Rickettsia montanensis* was used to infect tick midgut, ovary, and salivary glands (8 x 10^7 *Rickettsia* per tissue) for 1 h. After removal of *Rickettsia*, tick tissues were washed and collected by low-speed centrifugation. Total RNA was then extracted from the tissues and the levels of DvARPC4 mRNA were measured by qRT-PCR. DvGAPDH mRNA was used to normalize the differences among samples. Data shown are mean relative expression from two independent experiments. Error bar represents SEM values. An asterisk remarks a significant difference ($P = 0.0311$) between treatment group (unexposed- or *Rickettsia* exposed- group) in the same tissue. Graphs with different letters in the same treatment group are significantly different ($P \leq 0.05$). *P*-values of 0.0217 and 0.0270 represent uninfected ovary compared to midgut and salivary glands, respectively. *P*-values of less than 0.0001 and equal to 0.0012 represent infected ovary compared to midgut and salivary glands, respectively.

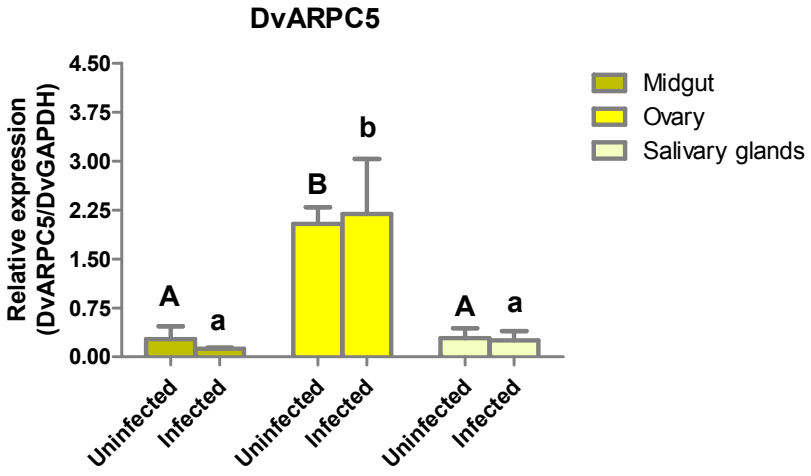

Figure 3.15 Transcriptional profile of DvARPC5 in *D. variabilis* tissues. Tick tissues including midgut, ovary, and salivary glands were dissected out and infected with *R. montanensis* (8×10^7 per tissue). After 1 h, *Rickettsia* was removed and the tissues were washed with PBS. The samples were then collected by low-speed centrifugation and total RNA was extracted from the tissues. The level of DvARPC5 mRNA was measured by qPCR and normalized with DvGAPDH mRNA. Data shown are mean relative expression from two independent experiments. Error bar represents SEM values. Graphs with different letters in the same treatment group (unexposed- or *Rickettsia* exposed- tissues) are significantly different ($P \leq 0.05$). *P*-value of less than 0.0001 represent uninfected ovary compared to both midgut and salivary glands. *P*-value of less than 0.0001 represent infected ovary compared to both midgut and salivary glands

both midgut and salivary glands). The mRNA level of DvARPC4 was shown to be upregulated in response to *R. montanensis* infection of the ovary (Figure 3.14) and the result was significantly different ($P = 0.0311$) compared to uninfected tissues.

To confirm the infection of tick tissues in the assays, DNA was extracted from the same samples after RNA isolation and the copies of rickettsial gene (RmOmpB) in infected tissues were quantified by qPCR. The average numbers of invading *Rickettsia* from two independent experiments are 1.56×10^4, 1.09×10^4, and 1.93×10^4, in midgut, ovary, and salivary glands, respectively.

3.3.3 DvArp2/3 complex inhibition assay

In chapter 2, an involvement of the tick Arp2/3 complex in *R. montanensis* internalization was investigated at the cellular level in DVE1 tick cells. To gain insight into the molecular

mechanisms of *Rickettsia* invasion in nature, an inhibition assay was performed in tissues. Tick tissues, including midgut, ovary, and salivary glands, were removed and treated with 500 µM CK-666, an Arp2/3 complex inhibitor, for 3 h. *Rickettsia montanensis* was then used to infect the tissues (8 x 10^7 per tissue) for 1 h. After removal of *Rickettsia*, the tissues were washed twice with PBS and collected by low speed centrifugation. Genomic DNA was then extracted from the samples and number of invading *Rickettsia* and tick cells were quantified by qPCR. As shown in Figure 3.16, inhibition of DvArp2/3 complex resulted in a decrease in *R. montanensis* invasion of all tissues with significant difference ($P = 0.0477$) in the ovary.

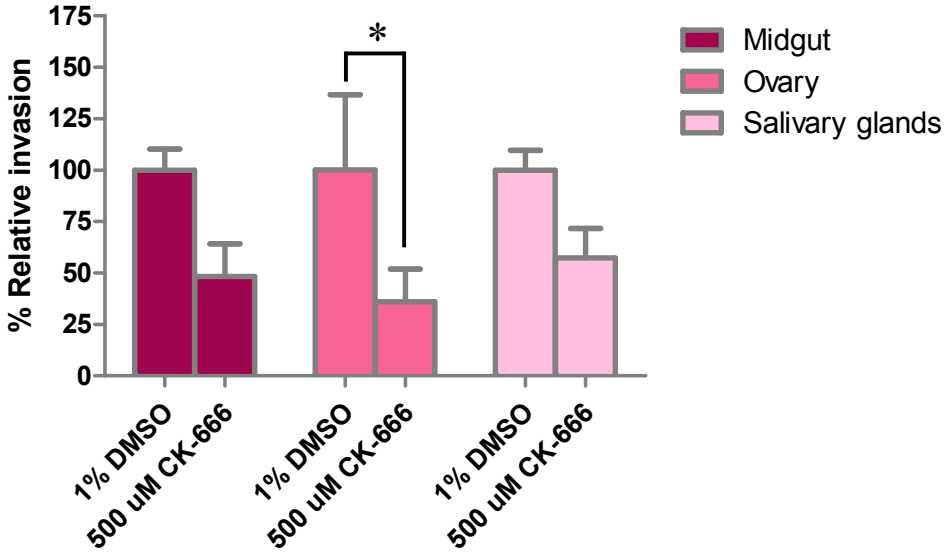

Figure 3.16 Effect of Arp2/3 complex inhibitor on *R. montanensis* invasion of *D. variabilis* tissues. Tick tissues including midgut, ovary, and salivary glands were dissected out prior to infection with *R. montanensis* (8 x 10^7 per tissue). After 1 h, Rickettsia was removed and the tissues were washed once with PBS. The samples were collected by low-speed centrifugation and extracted for gDNA. Quantitative PCR assay was then performed to quantify numbers of invading *Rickettsia* and tick cells. The experiments were performed in quadruplicate for each treatment group and the results were the combination of the three independent experiments. An asterisk indicates a significant difference ($P = 0.0477$).

3.4 Discussion

The Arp2/3 complex is an actin nucleator that is found in most eukaryotic cells. This seven-subunit protein is important in the regulation of the actin polymerization, a key process

utilized by SFG *Rickettsia* to invade host cells. The current study has identified the full-length cDNAs encoding all subunits of Arp2/3 complex from *D. variabilis* (DvArp2, DvArp3, DvARPC1, DvARPC2, DvARPC3, DvARPC4, and DvARPC5). Multiple sequence alignment showed variation in percent identity compared to the corresponding subunits of the Arp2/3 complex from *D. melanogaster*, *M. musculus*, *H. sapiens*, and *S. cerevisiae* (Table 3.4). Ranked in order from the most to the least, DvARPC1 is the most diverged of the seven subunits. Nonetheless, putative WD domains, the conserved motif in ARPC1, (Welch et al., 1997), were observed in ARPC1 isolated from *D. variabilis*. WD repeat, also known as the Trp-Asp or WD40 motif, is involved in a wide variety of cellular processes such as RNA processing, signal transduction, cytoskeleton assembly, and macromolecular protein complex formation (Hudson and Cooley, 2000; Smith et al., 1999). Welch et al. (1997) have suggested the role of ARPC1 subunit on assembly and maintenance of the Arp2/3 complex structure correlated with the capability of WD motif containing proteins in the coordination of multiprotein complexes. In addition, the study postulated that ARPC1 facilitated the binding of Arp2/3 complex with the proteins that regulate its functions (Welch et al., 1997).

On the other hand, amino acid sequence analysis of DvArp2 and DvArp3 revealed the putative ATP binding sites which is consistent with the studies demonstrated that ATP binding on both Arp2 and Arp3, and ATP hydrolysis on Arp2 were required for Arp2/3 complex-mediated actin cytoskeleton rearrangement (Dayel et al., 2001; Le Clainche et al., 2001; Le Clainche, 2003; Dayel et al., 2004; Martin et al., 2006).

The next attempt which focused on elucidating the transcriptional profiles of DvArp2/3 complex subunits (all subunits) in both uninfected- and *Rickettsia* infected- tick tissues revealed that mRNAs of all subunits of the Arp2/3 complex were expressed greater in the tick ovary (both

in uninfected- and *Rickettsia* infected-ovary) than in midgut and salivary glands. This increase in mRNA expression in the ovary compared to the midgut and salivary glands was significantly different for DvArp3, DvARPC4, and DvARPC5 mRNAs. Interestingly, in response to *R. montanensis* infection of the tick ovary, DvARPC4 mRNA level was shown to be significantly upregulated.

Because the level of mRNA expression is not reflective of the protein function(s) and tick tissues including midgut, ovary, and salivary glands are essential for both horizontal and vertical transmission of SFG *Rickettsia* (Munderloh and Kurtti, 1995), the role of DvArp2/3 complex was further studied at the protein level during *Rickettsia* infection of the mentioned tick tissues. In chapter 2, the importance of the tick Arp2/3 complex during the uptake of *R. montanensis* was examined at the cellular level in DVE1 tick cells; percent relative rickettsial invasion of tick cells was reduced to 8% in cells pre-treated with CK-666, the Arp2/3 complex inhibitor, compared to untreated control. In the current study, the inhibition assays were performed *ex vivo* not only to study the function of DvArp2/3 complex at the protein level but also to gain insight into the molecular mechanisms of *Rickettsia* invasion in the nature. After treatment with CK-666, tick tissues (midgut, ovary, and salivary glands) were exposed to *R. montanensis* for a short period of time (1h). Excitingly, the results showed that disruption of host Arp2/3 complex decreased the ability of *Rickettsia* to invade all tick tissues compared to untreated control with significant difference in the tick ovary. However, the mechanisms by which the Arp2/3 complex functions in response to rickettsial infection is still unknown and requires further characterization.

In summary, the current study provides the first description of all seven subunits of the tick derived Arp2/3 complex and the importance of the protein in facilitating the uptake of *Rickettsia* into the tick tissues. It is interesting to note from this study that mRNAs of DvArp3,

DvARPC4, and DvARPC5 were expressed significantly higher in the tick ovary (both in uninfected- and *Rickettsia* infected-ovary) compared to the other tissues, DvARPC4 mRNA was significantly upregulated in response to rickettsial invasion of the tick ovary, and inhibition of DvArp2/3 complex significantly decreased the entry of *Rickettsia* into the tick ovary. These findings, in which the results are significantly different in the tick ovary and the multiple functions of the Arp2/3 complex, lead to the suggestion that the protein not only play a role during *Rickettsia* infection but also has function(s) in uninfected ovary. Nevertheless, the roles of Arp2/3 complex in the uninfected tick ovary are still unknown and need to be further investigated.

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CHAPTER 4
MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF VACUOLAR ATPASE FROM THE AMERICAN DOG TICK *DERMACENTOR VARIABILIS*

4.1 Introduction

Ticks are important vectors of human pathogens, including bacteria, viruses, and parasites (Sonenshine, D. E., 2005; Piesman and Gage, 2005). Among the prokaryotic pathogens transmitted by ticks are the obligate intracellular spotted fever group of *Rickettsia*. Although the specific mechanisms by which *Rickettsia* invade tick cells is unclear, induced endocytosis and phagosomal escape facilitate intracytoplasmic living of *Rickettsia*. Toward an understanding of the mechanisms of rickettsial survival in the arthropod, previous studies have employed molecular techniques such as differential display and subtractive hybridization-polymerase chain reaction (PCR) to identify several tick-derived molecules thought to be associated with tick response to bacterial infection (Macaluso et al., 2003; Mulenga et al., 2003). Among the putatively identified molecules found to be differentially transcribed in response to rickettsial infection is a vacuolar ATPase (V-ATPase).

The V-ATPase is a vital component of eukaryotic cells as it mediates the acidification of intracellular compartments (Nishi and Forgac, 2002; Inoue et al., 2005). The molecule is comprised of multiple subunits separated into the water-soluble V_1 and membrane-associated V_0 domains. The V_0 complex contains the proton translocating pore and the V_1 complex uses ATP to drive proton translocation across the membrane (Nishi and Forgac, 2002; Inoue et al., 2005). Together, the enzyme uses ATP hydrolysis to acidify compartments for receptor-mediated endocytosis, intracellular trafficking, and protein degradation (Nishi and Forgac, 2002; Inoue et al., 2005).

The structure of V-ATPase has been extensively studied in *Saccharomyces cerevisiae* (Wieczorek et al., 2009; Forgac, 2000). The yeast V_1 domain is a 570 kDa complex consisting of

eight different subunits (A-H). The V_0 domain is composed of five different subunits (a, c, c′, c″ and d) with a total molecular mass about 260 kDa (Wieczorek et al., 2009; Forgac, 2000). Although it possess eight different subunits of the V_1 domain, studies of V-ATPase in the midgut of the tobacco hornworm showed that the V_0 domain contains only four subunits, which are a, c, d and e. (Merzendorfer et al., 2000).

To gain a better understanding of the interactions between *Rickettsia* and tick vectors, tick-derived molecules must be characterized at the molecular and functional level. The role of V-ATPase in the tick and the influence of *Rickettsia* on the molecule are unclear. The aims of this study were to test the hypothesis that V-ATPase is involved in rickettsial infection of tick cells. This study is the first description of a tick-derived V-ATPase. The role of V-ATPase during rickettsial infection in tick cells was also assessed using the V-ATPase inhibition assays.

4.2 Materials and methods

4.2.1 Tick dissection and tissue preparation

As previously described by Macaluso et al. (2001), *Dermacentor variabilis* colonies were routinely maintained on rats and rabbits at Old Dominion University. After feeding for 3-5 days, unmated female ticks were removed from host animals, washed twice with 70% ethanol and rinsed with distilled water. Tick tissues, including midgut, ovary, and salivary glands, were removed from the ticks and washed in sterile phosphate buffered saline (PBS, 0.137 mM NaCl, 2.7 mM KCl and 8 mM Na_2HPO_4, pH 7.4) or 0.1% diethyl pyrocarbonate (DEPC) treated water. Ovaries were transferred into microtubes containing RNA*later* (Ambion, Austin, TX) for RNA extraction. For protein preparation, all organs were placed in protease inhibitor cocktail (PIC, Roche, Indianapolis, IN). Tissues were immediately processed or stored at -80 ºC until used for extraction.

4.2.2 Protein and mRNA extraction from tick tissues

The ovaries from at least five *D. variabilis* were rinsed with DEPC treated water before total RNA and mRNA were extracted from using the NucleoSpin RNAII and NucleoTrap mRNA Mini kits (Clontech, Mountain View, CA), respectively, according to the manufacturer's instructions. For protein extraction, the tissue samples were thawed on ice for 15 min and washed once with PBS supplemented with PIC. The tissues were then lysed by adding 100 µl of lysis buffer (100 mN NaH_2PO_4, 10 mM Tris-Cl, 8M urea, pH 8.0), homogenizing with plastic pestle for 15 min and sonicating in a bath sonicator (Crest Ultrasonics, Trenton, NJ) for 10 min. The lysate was centrifuged at 16000 x g for 10 min at 4 °C. The supernatant was then transferred to 1.7 ml centrifuge tubes. The protein solution was diluted two fold and the protein concentration was measured by *DC* protein assay (Bio-Rad, Hercules, CA) according to manufacturer's recommendation. Thirty micrograms of protein from midgut, ovary and salivary glands were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot.

4.2.3 Cloning of the tick V-ATPase V_0 subunit a full-length cDNA

The full-length cDNA for *D. variabilis* (Dv) V-ATPase V_0 subunit a (VATPaseV_0a) was generated using the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's protocol. Briefly, 1 µg of mRNA extracted from ovary was used to create 3' and 5'-RACE-Ready cDNA. Gene specific (GSP) primers designed from a partial sequence of DvVATPaseV_0a gene (Macaluso et al., 2003) were used to amplify the 3' and 5' end fragments. PCR products were cloned into TOPO TA cloning vectors (Invitrogen, Carlsbad, CA) and the plasmids were isolated using the SV miniprep kit (Promega, Madison, WI) according to the manufacturers' protocols. The cloned plasmid inserts were sequenced by the dye terminator

method on a 373 automated fluorescence sequencing system (Applied Biosystems, Carlsbad, CA) in the biopolymer laboratory at the University of Maryland, Baltimore. For DNA sequence analysis, the MacVector software program (Accelrys, San Diego, CA) was used. Similarity comparison of DNA sequence was carried out against the protein database in Genbank using BlastX. Amino acid sequence analyses were conducted using web-based software suits. Multiple sequence alignment, MUSCLE, was used to create a sequence alignment file and calculated percent identity (pairwise alignment). The alignment output was created using Multiple Align Show. The topological and transmembrane regions were predicted using TopPred. A potential N-glycosylation site was obtained using NetNGlyc 1.0 Server.

4.2.4 Construction of plasmid and expression of recombinant DvVATPaseV_0a

The Baculovirus Expression System with Gateway Technology (Invitrogen) was used to express recombinant (r) DvVATPaseV_0a according to manufacturer's protocol. Briefly, the open reading frame (ORF) of DvVATPaseV_0a was subcloned into the pENTR/D-TOPO entry vector (Invitrogen) and then transferred into the cloning cassette of the pDEST10 vector (N-terminal His fusion vector, Invitrogen). The cloned ORF DvVATPaseV_0a plasmid was transformed into DH10Bac *E. coli* (Invitrogen), which contains the baculovirus shuttle vector (bacmid), to produce recombinant bacmid harboring DvVATPaseV_0a. The white colonies of recombinant bacmid DNA in a background of blue colonies containing the unaltered parent bacmid were selected and isolated from small-scale selective (50 μg/ml kanamycin, 7 μg/ml Gentamycin, 10 μg/ml tetracycline, 100 μg/ml Bluo-gal, and 40 μg/ml isopropyl-beta-D-thiogalactopyranoside) medium. These recombinant baculoviruses (1μg) were used to transfect 9 x 10^5 Sf9 insect cells (Gibco, Carlsbad, CA) using Cellfectin reagent (Invitrogen) according to manufacturer's recommendation. The transfected cells were maintained at 27 ^{0}C and the culture medium

containing the recombinant baculoviruses was collected at 7 days post-transfection as a primary viral stock (P1).

The P1 viral stock (8 µl) was used to infect fresh Sf9 (4 x 10^5) cells for 2, 3, 4, 5 and 6 days to determine the optimal harvest time for viral amplification. After analyzed by SDS-PAGE and Western blotting, the viral stock was amplified by infecting Sf9 cells (2 x 10^6) with 40 µl of the recombinant baculoviruses and incubated at 27 ^{0}C for 4 days. The amplified virus was diluted 10 fold from 10^{-3} to 10^{-8} and the viral titer was determined by end-point dilution as described by O'Reilly et.al (1994). The multiplicity of infection (MOI) was optimized by infecting Sf9 cells (8 x 10^5) with P2 viral stock at MOI of 0.1, 0.3, 0.5, 1, 2, 3, 5 and 10 for 4 days. Likewise, the optimal time for harvesting virus was determined by infecting Sf9 cells with P2 viral stock at MOI of 1 for 2, 3, 4, 5 and 7 days. To express the protein, Sf9 cells were infected with rDvVATPaseV_0a baculoviruses at MOI of 1 and maintained at 27 ^{0}C for 4 days. The cells were then harvested and washed once with PBS. Cell pellet was resuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl and 8 M urea, pH 8.0) and stirred at room temperature for 1 h. The lysate was centrifuged at 16000 x g for 30 min and the pellet was analyzed by SDS-PAGE and Western immunoblot.

4.2.5 Purification of DvVATPaseV_0a from polyacrylamide gel

The infected cells were directly added with lysis buffer and loaded onto NuPAGE 4-12% Bis-Tris Zoom gels (Invitrogen). The gels were then negatively stained with E-zinc Reversible Stain Kit (Pierce, Rockford, IL) according to the instructions of the manufacturer. The unstained band of rDvVATPaseV_0a was then excised from the gel and the residual stain from the edges of the excised gel pieces was erased by soaking them in Tris-glycine buffer (25 mM Tris and 192 mM glycine). The excised gel pieces were crushed using a clean pestle and added with 0.5 ml

elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5). The mixture was then incubated in a rotary shaker at 30 ^{0}C overnight and the protein was collected after centrifugation.

4.2.6 Protein identification

To confirm peptide sequences of purified rDvVATPaseV_0a protein, SDS-PAGE coupled with mass spectrophotometry (MS) analysis was performed as described previously by Sunyakumthorn et al. (2008). Briefly, the protein band was excised using the Proteome Works Spot Cutter (Bio-Rad), and a MassPrep Station (Waters, Milford, MA) was used as the digestion robot. The peptides were then extracted from the gel plugs and separated by liquid chromatography using an Atlantis dC_{18} column (75 μm by 100 mm; Waters/Micromass, Milford, MA). For analysis, a Q-Tof (quadrupole time-of-flight) Micro (Waters/Micromass) hybrid mass spectrometer was used and Electrospray analysis (positive mode) was performed. ProteinLynx Global Server, version 2.0 (Waters/Micromass) was used for data acquisition and analysis. Database comparative analysis with an online Mascot (Matrix Science, Boston, MA) tandem MS (MS/MS) ion search against the NCBInr/Proteobacteria was carried out.

4.2.7 Preparation of polyclonal antibody specific for rDvVATPaseV_0a

Polyclonal antibodies to purified rDvVATPaseV_0a were generated in BALB/c mice as described by Mulenga et al. (2003). Briefly, three mice were immunized subcutaneously with rDvVATPaseV_0a protein (~30 μg protein per mouse) emulsified with equal volume of TiterMax Gold adjuvant (Sigma-Aldrich, St. Louis, MO), followed by a second injection (~80 μg protein per mouse) two weeks later, and a third injection (~60 μg protein per mouse) in two weeks later. The mice were bled one week after second and third injection to test for antibodies. Antiserum

from the mouse with the best reactivity toward rDvVATPaseV_0a was collected two weeks after the third injection and stored at 4 ºC for the immunological studies.

4.2.8 SDS-PAGE and Western blot analysis

Transcriptional analysis of the putative DvVATPaseV_0a identified mRNA expression in tick salivary, midgut, and ovary (Macaluso et al., 2003). To determine whether DvVATPaseV_0a was expressed as a protein in the same tick tissues, protein extracts of salivary gland, midgut, and ovary were assessed by Western blot using antibodies to rDvVATPaseV_0a. Protein were separated by NuPAGE 4-12% Bis-Tris gel (Invitrogen) and electronically transferred to PVDF membrane (Bio-Rad). The membrane was blocked with 5% (w/v) skim milk in tris buffer saline (TBS)-Tween (T) buffer (20 mM Tris-HCl, 500 mM NaCl and 0.1% (v/v) Tween-20; pH 7.5) at room temperature for 1 h and incubated with either 1: 5000 dilution of anti-6xHis monoclonal antibody (Clontech) or a 1:200-500 dilution of polyclonal anti-rDvVATPaseV_0a in TBS-T for 2 h. Following three washes, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody diluted (Pierce) 1: 20000 at room temperature for 1 h and then washed three times with TBS-T. The bound antibody complexes were detected using an enhanced chemiluminescent system (Pierce).

4.2.9 Cell culture

Sf9 cells (Gibco) were cultured in SF900 II serum-free medium (Gibco) supplemented with penicillin/streptomycin (50 U/ml and 50μg/ml, respectively, Gibco). DVE1 cells (from *D. variabilis,* kindly provided by Dr. Timothy Kurtti, University of Minnesota) were grown in L15C medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Hyclone, Waltham, MA), 5% tryptose phosphate broth (Difco, Sparks, MD), 0.1% lipoprotein-cholesterol concentrate (LPC, MP Biomedicals, Santa Ana, CA), 0.6% HEPES solution (1 M, Sigma-Aldrich), and 1.2%

sodium bicarbonate solution (5%, Sigma-Aldrich). Vero cells were grown in DMEM high glucose (Invitrogen) containing 5% fetal bovine serum (Hyclone). For all cell lines, conditioned medium was replaced with new medium once a week. Sf9 and DVE1 cells were passaged (1::6) at about every 2 and 4 weeks, respectively. Vero cells were subcultured (1:6 or 1:12) every 1-2 weeks with 0.05% trypsin-EDTA (Invitrogen). Sf9 and DVE1 cells were grown in a humidified incubator at 27 and 32 ºC, respectively. Vero cells were maintained in a humidified 5% CO_2 incubator at 34 ºC.

4.2.10 *Rickettsia* culture and purification

Rickettsia montanensis were maintained in Vero cells as described by Sunyakumthorn et al. (2012) with minor modification. Briefly, *R. montanensis* were fed weekly by replacing half of the conditioned medium with new medium. The organisms were subcultured (1:12) every 2 weeks by inoculating *Rickettsia*-infected cells to uninfected Vero cells.

The isolation of *R. montanensis* from infected Vero cells was carried out as previously described by Weiss et al. (1973) with minor modification. Briefly, *Rickettsia* infected cells were detached by scraping and lysed by vortexing with sterile 3 mm borosilicate glass beads for 5 min (Sigma-Aldrich). Cell lysate was then transferred aseptically to 15 ml centrifuge tubes and centrifuged at 4ºC, 275 x g for 3 min to pellet cellular debris. The supernatant was transferred to a 10 ml syringe and filtered through a 2 µm syringe filter. For all bioassays, the number of *Rickettsia* was determined by counting *Rickettsia* stained with a LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, Carlsbad, CA) in a Petroff–Hausser bacterial counting chamber (Hausser Scientific, Horsham, PA) and examined with a Leica microscope (Buffalo Grove, IL).

4.2.11 Expression of VATPaseV_0a mRNA in backless *D. variabilis*

The backless *D. variabilis* was used as a model to study an expression of DvVATPaseV_0a mRNA in tick tissues (midgut, ovary, salivary glands) in response to *R. montanensis* infection as previously described by Sunyakumthorn et al. (2012). Briefly, six unfed female ticks were washed with 70% ethanol followed by 1% benzalkonium chloride solution and rinsed three times with sterile water. The ticks were air-dried and afterwards the mouthparts and the legs were excised. The ticks were then cut along the perimeter of alloscutum and the dorsal cuticle was removed. The backless ticks were individually transferred onto 96-well plate containing complete L15B medium and incubated at 34 ºC. After 24 h, three ticks were infected with *R. montanensis* (2.4 x 10^8 per tick) for 1 h while the other three were kept in the medium without exposure to *Rickettsia*. Tick tissues were collected by removing and pooling the tissues from three different ticks. The tissues were placed in RNA*later* (Ambion, Austin, TX) and the samples were kept at -20 ºC until used for RNA extraction. The experiments were performed independently twice.

4.2.12 Total RNA isolation and relative quantitative RT-PCR (qRT-PCR)

Total RNA from uninfected- and *R. montanensis* infected- tick tissues was extracted using an RNeasy Mini Kit (QIAGEN, Germantown, MD), treated with TURBO DNase (Ambion, Austin, TX), and purified using an RNA cleanup kit (Zymo Research, Irvine, CA) as described in the manufacturers' manuals. First-strand cDNA was synthesized from 40 ng total RNA using iScript reverse transcription kit (Bio-Rad) according to manufacturer's instruction. All qPCR reactions were performed in 96-well plates in a 35 µl volume containing 100 nM of each primer (5'-CTCCTGGCCGTGATTTGTAT- 3' and 5'-GCTGCTCCGTCCTCTGTATC- 3' for V-ATPase, 5'-CTCGTTCTTGGGAATGGAAG-3' and 5'-CTTGATCTTCATGGTGGAAG

G- 3' for actin), DNase/RNase-free water, 2 µl of cDNA template (samples) or water (negative control) and 2X iTaq SYBR Green Supermix with ROX (Bio-Rad). The mixtures were then aliquoted in triplicate 10 µl reactions onto 384-well plates and run on an ABI 7900HT unit (Applied Biosystems, Carlsbad, CA) at Louisiana State University, School of Veterinary Medicine. No RT reaction (water was added instead of Reverse transcriptase) was performed to confirm an absence of genomic DNA. Results were analyzed with ABI 7900HT sequence detection system (SDS v2.3) software. Data are presented as the percent difference in threshold cycle (CT) value (ΔCT = CT *Actin* – CT *V-ATPase*).

4.2.13 DvVATPase inhibition assay

DVE1 cells (1 x 10^5) were seeded onto 96 well-plates (Greiner Bio-One, Monroe, NC) and incubated at 32 ºC for 48 h. The cells were treated with 5, 0.5, and 0.05 µm of the V-ATPase inhibitor, bafilomycin A1 (EMD Millipore, Billerica, MA), or medium containing 0.1% DMSO (inhibitor vehicle control) for 2 h. *Rickettsia montanensis* was then inoculated onto the treated cells at a MOI of 10 and the plate was centrifuged at 700 x g for 2 min to facilitate the binding of *Rickettsia* to host cells. After 1 h, *Rickettsia* was removed and the cells were added with 150 µl PBS. The samples were centrifuged at 275 x g for 4 min to collect only infected host cells. After removal of supernatant, the cell pellet was washed with 1 ml PBS and centrifuged at 275 x g for 4 min. The samples were stored at -20 ºC until used for genomic DNA (gDNA) isolation. According to the manufacturer's instructions, gDNA was extracted from the samples using DNeasy Blood & Tissue Kit (QIAGEN). Genomic DNA was eluted in 35 µl DNase/RNase free water. The number of *Rickettsia* and tick cells were then quantified by quantitative PCR (qPCR). The experiments were performed in quadruplicate for each

concentration of the inhibitor used and the results were the combination of two independent experiments.

4.2.14 Quantification of *Rickettsia* and tick cells by qPCR

To quantify copies of *R. montanensis* and DVE1 genes in samples, probe-based qPCRs were performed as described in chapter 1. Briefly, serial dilutions of plasmids containing RmOmpB and DvCRT genes were used to create a standard curve. Quantitative PCRs reactions were prepared in volume of 35 µl comprised of LightCycler 480 Probe Master (Roche), 0.3 µM each *Dv*CRT_TYE665 and *Rm*OmpB_HEX probes, 0.1 µM each CRT*Dv*321F, CRT*Dv*452R, OmpB*Rm*2832F and OmpB*Rm*2937R primers, DNase/RNase-free water and 5 µl of gDNA template (samples), water (negative control) or standard reference plasmids. Ten microliters of each reaction mixture were transferred into 3 wells of a 384-well plate and run on LightCycler 480 system II (Roche). Quantitative PCR assay conditions consisted of a pre-incubation step of 95 ºC for 10 min, 45 amplification cycles of 95 ºC for 10 sec, 60 ºC for 30 sec, and 72 ºC for 1 sec. Percent relative invasion was calculated using a formula shown below.

$$\% \text{ Relative invasion} = \left(\frac{\text{Number of RmOmpB}_{sample}}{\text{Number of DvCRT}_{sample}} \times 100\right) / \text{Average of } \frac{\text{Number of RmOmpB}_{control}}{\text{Number of DvCRT}_{control}}$$

4.2.15 Statistical analysis

Analysis of Variance (ANOVA) was conducted using the SAS statistical package (Version 9.3) GLM procedure. For mRNA level measurement in backless ticks, the relative gene expression was analyzed using a two-way interaction (rickettsial infection and tick tissues). Pairwise t tests of least-squares means were used to examine the interaction effects of relative mRNA expression of DvVATPaseV_0a between unexposed- and *Rickettsia* exposed- ticks. For V-ATPase inhibition assays, the same tests were used to elucidate a role of the V-ATPase inhibitor,

bafilomycin A1, on rickettsial invasion of tick cells. *P*-values of ≤ 0.05 were considered significantly different.

4.3 Results

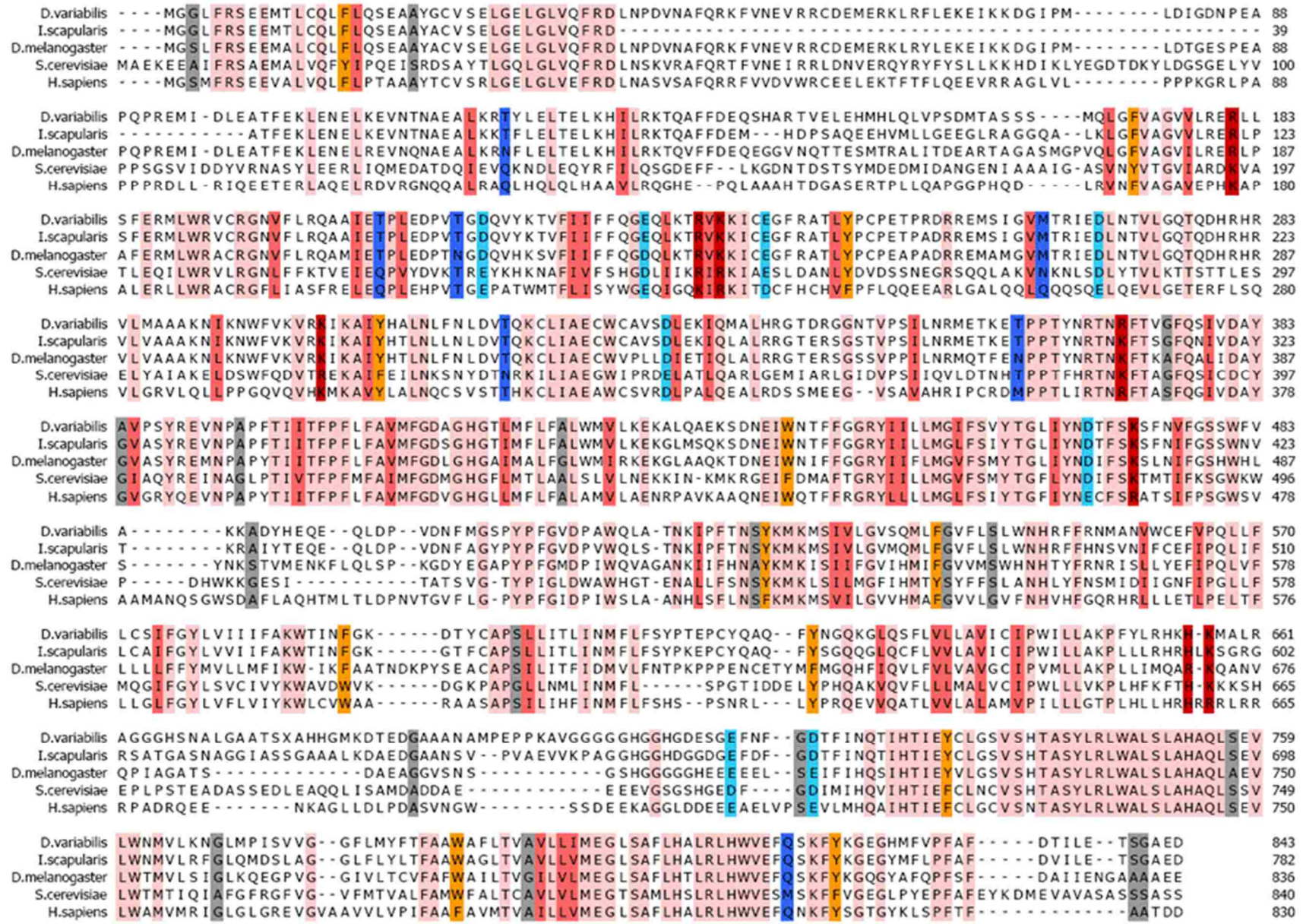
4.3.1 Cloning and sequence analysis of DvVATPaseV_0a

A full-length cDNA clone corresponding to the transcript of the V-ATPase V_0 subunit a gene in *D. variabilis* was isolated using the GSP primers designed from differential display PCR (Macaluso et al., 2003). DNA sequence analysis using MacVector revealed a 2856 bp with 2532 bp ORF (Genbank accession number HM185485). The deduced amino acid sequence comprised 843 residues with an estimated MW of 96 kDa. Amino acid sequence analysis of DvVATPaseV_0a using a web-based multiple sequence alignment, MUSCLE, showed 77%, 66%, 44% and 40% identity to VATPaseV_0a from *Ixodes scapularis*, *Drosophila melanogaster*, *Homo sapiens*, and *Saccharomyces cerevisiae*, respectively, as shown in Figure 4.1. In the figure, the red open box represents the Asn-Xaa-Ser/Thr (asparagine-any amino acid-serine/threonine) sequon, a potential N-glycosylation site, analyzed by NetNGlyc 1.0 Server where Asn was predicted to be glycosylated. Similar to other organisms, DvVATPaseV_0a possesses transmembrane (eight segments), topological segments which were predicted using TopPred software (Figure 4.2).

4.3.2 Expression, purification and antibody production of rDvVATPaseV_0a

In this study, full-length of rDvVATPaseV_0a was produced as hexahistidine (6xHis) fusion protein using the baculovirus expression system in Sf9 insect cells. The size of the protein produced corresponded to an estimated molecular weight (MW) analysis of full-length cDNA sequence i.e. ~ 96 kDa. As the N-terminal of rDvVATPaseV_0a contains 6xHis-tag, this allows us to detect the 6xHis-tag fused recombinant protein using anti-6xHis monoclonal

Figure 4.1 (Following page) Multiple sequence alignment of VATPaseV_0a amino acid sequences. Multiple sequence comparison by log-expectation (MUSCLE) software was used to create a sequence alignment and calculate percent identity of VATPaseV_0a from *D. variabilis* (Genbank accession number HM185485), *I. scapularis* (Genbank accession number XP002414796), *D. melanogaster* (Genbank accession number NP733274), *S. cerevisiae* (Genbank accession number NP014913.3), and *H. sapiens* (Genbank accession number NP006010). Identical amino acids are highlighted in pink. Different composition of similar amino acids are highlighted in red (R, H, K), grey (G, S, A), orange (F, Y, W) light red (L, I, V), dark blue (T, N, Q, M), medium blue (Q, M) or light blue (D, E). Figure was created using Multiple Align Show program. The red open box is Asn-Xaa-Ser/Thr sequon in which asparagine residue was predicted to be glycosylated using NetNGlyc 1.0 Server.


D.variabilis
I.scapularis
D.melanogaster
S.cerevisiae
H.sapiens

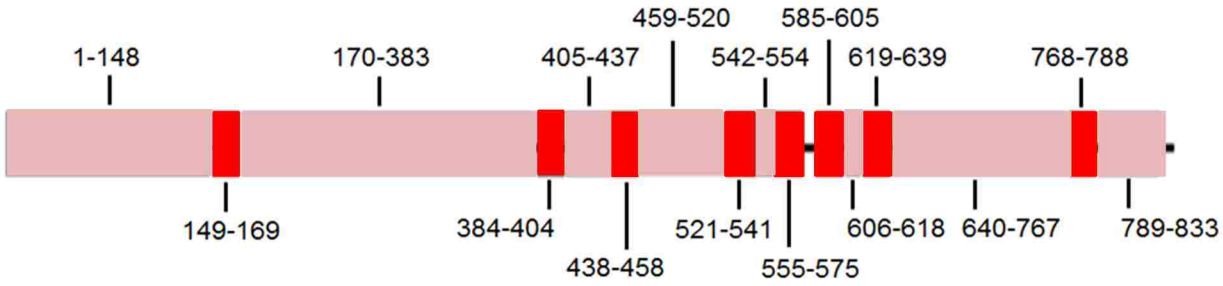


Figure 4.2 Schematic diagram represented the topological and transmembrane regions of DvVATPaseV_0a protein. Start and end amino acid positions of each domain are indicated. Topological and transmembrane domains are shaded in pink and red, respectively.

antibody. The recombinant bacmid containing rDvVATPaseV_0a was determined for optimal harvest times for amplifying baculovirus stock and then amplified to a high titer for further studies. The result showed the highest level of expression at 4 days post-infection (Figure 4.3) and ~ 10^7 pfu/ml of viral titer.

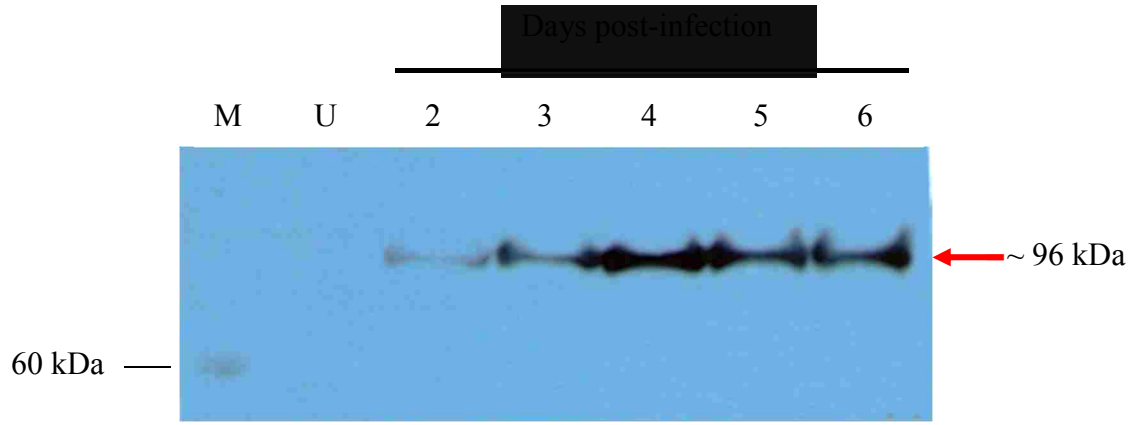


Figure 4.3 Optimization of harvest times for amplifying rDvVATPaseV_0a baculovirus stock. The primary stock of rDvVATPaseV_0a baculovirus was used to infect Sf9 cells and the cells were collected at 2, 3, 4, 5 and 6 days-post infection. Whole cell lysates were analyzed by Western immunoblot probed with anti-6xHis monoclonal antibody. M is His-tagged protein standard (BIONEXUS). U is uninfected Sf9 cells.

The optimal MOI and the time course induction of rDvVATPaseV_0a produced in Sf9 were studied and the results showed no different both at different MOI and different times post-infection (Figure 4.4).

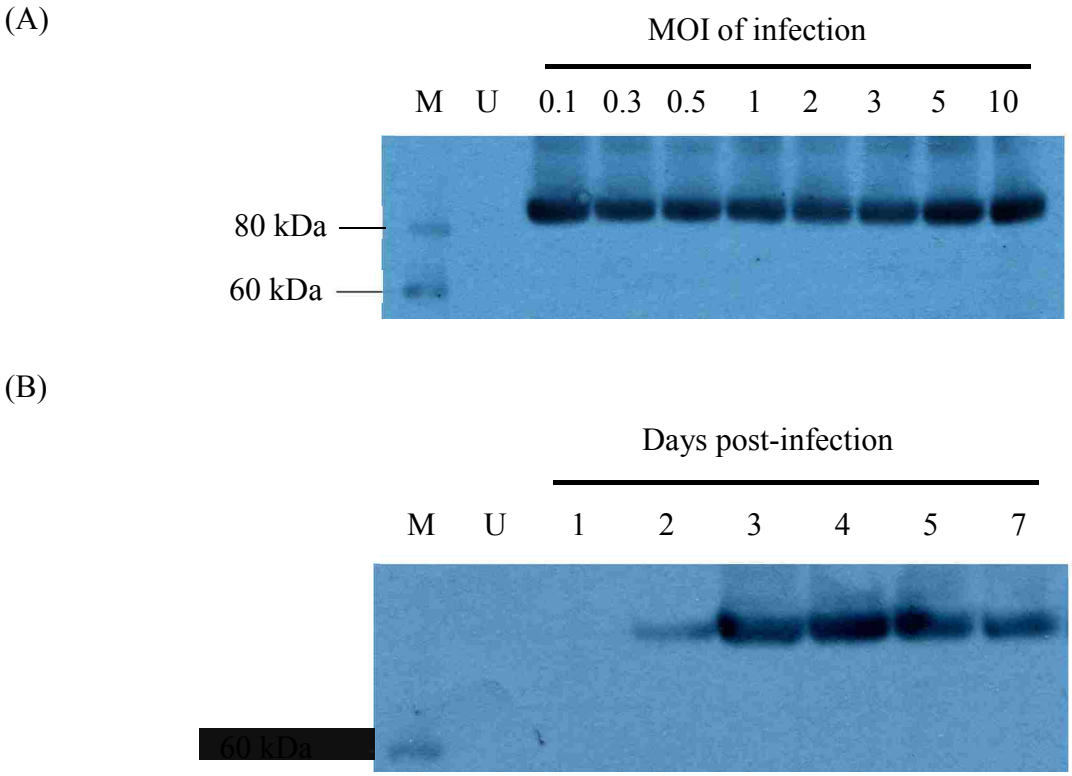


Figure 4.4 Optimization of MOI and time course expression of rDvVATPaseV_0a baculovirus. (A) Sf9 cells were infected with rDvVATPaseV_0a baculovirus at MOI of 0.1, 0.3, 0.5, 1, 3, 5, and 10. After 4 days, cells were collected and subjected to Western blot analysis. (B) rDvVATPaseV_0a baculovirus was used to infect Sf9 cells at MOI of 1 and the cells were harvested at 1, 2, 3, 4, 5 and 7 days post-infection. Who cells lysates (from A and B) were analyzed by Western immunoblot using anti-6xHis monoclonal antibody. M is His-tagged protein standard (BIONEXUS). U is uninfected Sf9 cells.

As V_0 a subunit of V-ATPase is a membrane embedded protein which results in an insoluble protein, so the protein was purified using denaturing purification method. After running SDS-PAGE and negatively staining, the major band (indicated by arrow in Figure 4.5A) of rDvVATPaseV_0a protein was excised and eluted from the gel. The protein was then concentrated and subjected to SDS-PAGE and Western Blot analysis probed with anti-6xHis monoclonal antibody. The results showed the band of purified rDvVATPaseV_0a (~ 96 kDa) and two upper bands with sizes greater than 140 kDa (Figure 4.5B). All three protein bands were excised and subject to MS analysis. The resultant peaks of the 96 kDa band searched against the

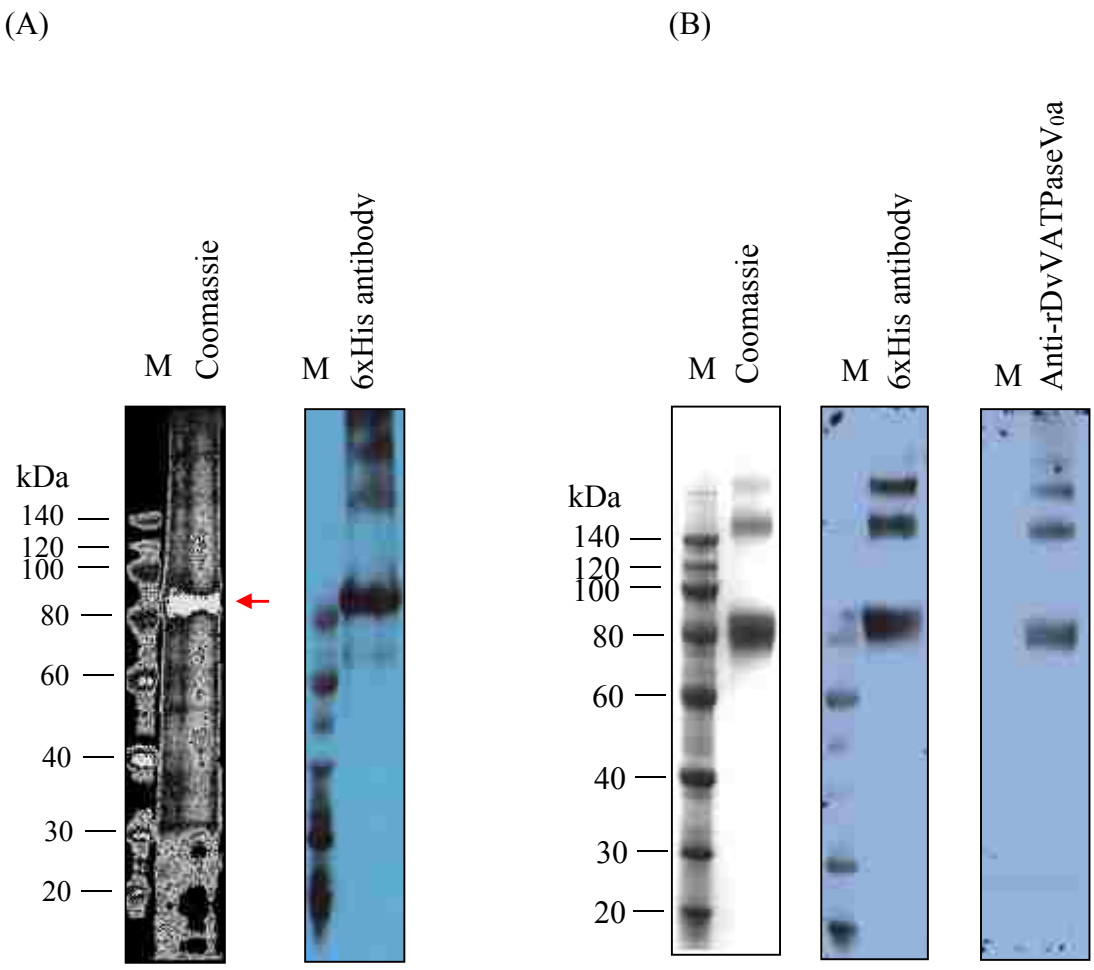


Figure 4.5 SDS-PAGE and Western blot analysis of insoluble portion of Sf9 infected with recombinant baculovirus harboring rDvVATPaseV_0a and purified rDvVATPaseV_0a. Sf9 cells were infected with rDvVATPaseV_0a baculovirus at MOI of 1 for 4 days. Insoluble fragment (A) was analyzed by SDS-PAGE and stained with Coomassie blue or subjected to Western blot probed with anti-6xHis monoclonal antibody. After negatively staining, a major band (indicated by arrow) of rDvVATPaseV_0a was excised then extracted from the gel. The purified protein (B) was analyzed by SDS-PAGE and stained with Coomassie blue or subjected to Western blot probed with anti-6xHis monoclonal antibody or anti-rDvVATPaseV_0a polyclonal antibody. M is His-tagged protein standard (BIONEXUS).

non-redundant Swissport protein sequence database using ProteinLynx Global SERVER was shown to match the V-ATPase protein while the others showed no similarity to any protein in this database. The occurrence of multiple protein bands after purifying and concentrating may result from self-binding in the more concentrated protein preparation. To exclude the possibility of any protein contamination, the ~96 kDa of purified rDvVATPaseV_0a was excised from the gel

and subjected to SDS-PAGE and Western blotting. The results showed the three bands appeared in the same positions as they were observed in the first purification. In this study, a polyclonal antibody against rDvVATPaseV_0a was generated in BALB/c mice. Western blotting analysis has shown that the rDvVATPaseV_0a polyclonal antibody specifically recognized three protein bands of rDvVATPaseV_0a, but not His-tagged protein standard (Figure 4.5B). This result suggested that the generated antibody can be used for further immune-detection of DvVATPaseV_0a.

4.3.3 Detection of VATPaseV_0a protein expression in tick tissues

To examine whether VATPaseV_0a is expressed in *D. variabilis*, the protein was extracted from tick tissues (midgut, ovary and salivary glands) and separated by SDS-PAGE followed by Western blotting with anti-rDvVATPaseV_0a polyclonal antibody. The result of the Western blot analysis presented in Figure 4.6 revealed a detectable DvVATPaseV_0a protein band in the ovary.

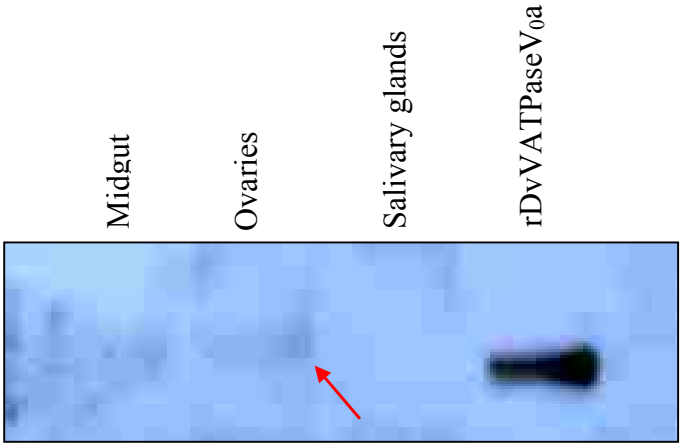


Figure 4.6 Detection of VATPaseV_0a in midgut, ovary and salivary glands from *D. variabilis* using rDvVATPaseV_0a polyclonal antibody. Tick tissues, including midgut, ovary, and salivary glands, were dissected out of the female unfed ticks and the protein was extracted. Thirty microgram of total proteins from each tissue were then analyzed by SDS-PAGE followed by Western immunoblot using anti-rDvVATPaseV_0a polyclonal antibody. The arrow indicates a DvVATPaseV_0a protein band detected in tick ovary.

The apparent molecular mass of this detected band was a little bigger than a 96 kDa band of the recombinant DvVATPaseV_0a which reflected the presence of post-translational modification on the protein in tick ovary. No band was detected in midgut and salivary glands at the same amount of protein loading (30 μg) indicated a much lower amount of DvVATPaseV_0a in midgut and salivary glands.

4.3.4 Expression of VATPaseV_0a mRNA in backless *D. variabilis*

To determine a transcriptional profile of VATPaseV_0a in *D. variabilis* tissues (midgut, ovary and salivary glands) in response to an early stage of *R. montanensis* infection, backless ticks were generated and exposed to *Rickettsia*. After 1 h, the tissues were dissected out of ticks, extracted for total RNA and measured for DvVATPaseV_0a mRNA level by qRT-PCR. As shown in Figure 4.7, the mRNA was detectable in all tick tissues and in both unexposed and *Rickettsia*-exposed ticks. In correspondence with the protein expression pattern, which the protein was detected only in the ovary at 30 μg of total protein loading, DvVATPaseV_0a mRNA was expressed higher in the ovary than in the other tissues with significant difference in unexposed-ovary compared to midgut ($P = 0.0154$) and salivary glands ($P = 0.0155$). However, the mRNA level remained unchanged in *Rickettsia*-exposed ticks (all tissues) compared to unexposed control.

4.3.5 Involvement of tick V-ATPase in *R. montanensis* infection.

To assess the function of tick V-ATPase in response to *Rickettsia* infection, the V-ATPase inhibition assays were performed in DVE1 tick cell line. Tick cells were treated for 2 h with different concentration (5, 0.5, and 0.05 μM) of V-ATPase inhibitor, bafilomycin A1, prior to infection with *R. montanensis* (MOI of 10). After 1 h, *Rickettsia* was removed and the cells

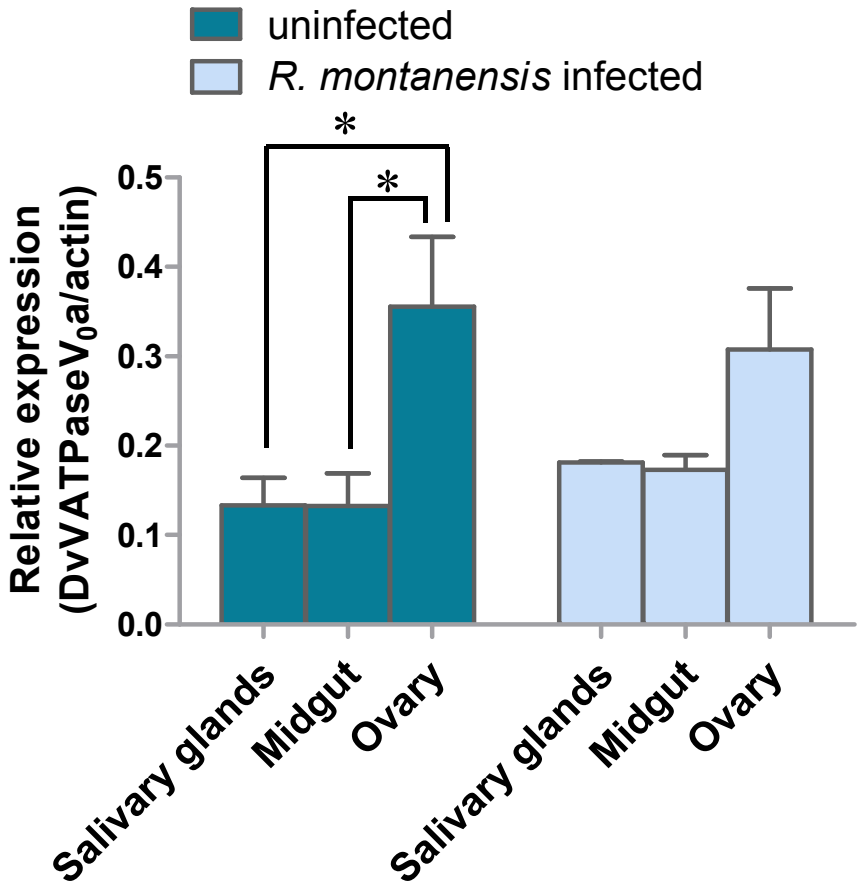


Figure 4.7 Transcriptional profile of VATPaseV_0a in *D. variabilis* tissues. Backless ticks were generated by taking off the dorsal cuticle and were exposed to *R. montanensis* for 1 h. The tick tissues (midgut, ovary and salivary glands) were then dissected out and extracted for total RNA. The level of DvVATPaseV_0a mRNA was measured by qRT-PCR and normalized to tick actin mRNA. Data shown are mean relative expression from two independent experiments. Error bar represents SEM values and $*P \leq 0.05$ was considered significantly different. *P*-values of 0.0154 and 0.0155 represent uninfected ovary compared to midgut and salivary glands, respectively.

were washed twice with PBS. To exclude the possibility of collecting extracellular *Rickettsia*, the samples were harvested by low-speed centrifugation. Genomic DNA was then extracted from the cells and the percentage of rickettsial infection in comparison to control cells was assessed by qPCR. As shown in Figure 4.8, inhibition of DvVATPase reduced percent relative invasion, compared to untreated control, by 27%, at 5 µM ($P = 0.0005$) and 0.5 ($P = 0.0005$) µM and by 13% at 0.05 µM ($P = 0.0877$) of inhibitor used.

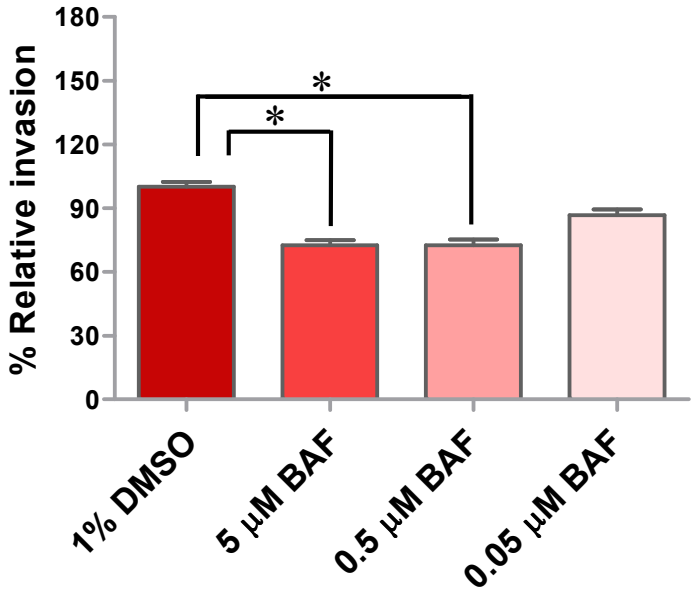


Figure 4.8 Effect of V-ATPase inhibitor on *R. montanensis* infection of DVE1 cells. DVE1 cells were treated for 2h with bafilomycin A1 (BAF) prior to infection with *R. montanensis* at MOI of 10. After 1 h, *Rickettsia* was removed. The cells were washed twice with PBS and collected by low-speed centrifugation. Genomic DNA was then isolated and percent relative invasion was assessed by qPCR. Data shown are mean percent relative invasion from two independent experiments. Error bar represents SEM values. The asterisks mark significant difference from untreated control cells (*P = 0.0005).

4.4 Discussion

V-ATPase is ubiquitously found in and is responsible for acidification of a variety of intracellular organelles in eukaryotic cells, such as endosomes and lysosomes. The enzyme is essential for several biological processes e.g. protein sorting, protein processing and degradation, coupled transport of small molecule and receptor-mediated endocytosis (Nishi and Forgac, 2002). This study was carried out to characterize the V-ATPase V_0 subunit a from *D. variabilis*. A full-length cDNA of DvVATPaseV_0a (2856 bp with 2532 bp ORF) was isolated and expected to code for 843 amino acid protein with an estimated MW of 96 kDa. Multiple sequence alignment (Figure 4.2) showed variation in amino acid sequences among species (77%, 66%, 44% and 40% identity to *I. scapularis*, *D. melanogaster*, *H. sapiens*, and *S. cerevisiae*, respectively). Thus, it is interesting to investigate the protein both in molecular and functional

level. The protein was expressed, purified and the polyclonal antibody was raised against DvVATPaseV_0a. The antibody was then used to confirm the presence of V-ATPase in tick tissues. The results showed a detectable protein band (only in ovary) with the MW slightly bigger than 96 kDa reflecting post-translational modification in the tick ovary (Figure 4.6). Amino acid sequence analysis revealed transmembrane segments and also a potential site for N-glycosylation which is consistent with the studies of calf VATPaseV_0a in which the protein was illustrated as a transmembrane glycoprotein (Adachi et al., 1990). However, further investigation is needed to confirm the presence of N-glycosylation of DvVATPaseV_0a in the tick. The detection of DvVATPaseV_0a band in the tick ovary but not in midgut and salivary glands, at 30 µg of protein loading, correspond to the transcriptional pattern (both in unexposed- and *Rickettsia* exposed- ticks) in which the mRNA in the tick ovary was expressed greater (significant difference in unexposed-ticks) compared to other tissues (Figure 4.8).

The functions of V-ATPase in tick ovary are still unknown and need to be characterized. However, the increased expression of V-ATPase in tick ovary might be associated with receptor-mediated endocytosis and protein transport, the two processes that are important for ovulation and embryogenesis in which V-ATPase are involved. In many organisms, such as *Caenorhabditis elegans* (Grant and Hirsh 1999), *D. melanogaster* (Schonbaum et al. 1995), *Aedes aegypti* (Sappington et al. 1995), and also *D. variabilis* (Mitchell et al. 2007), vitellogenin, a yolk precursor protein, is taken up through receptor-mediated endocytosis pathway during oocyte growth. Studies using RNAi in *C. elegans* showed that V-ATPase is required for ovulation and oogenesis. Specifically, inhibition of V_1 subunit C (Oka and Futai, 2000) and V_0 subunit a (Oka et al. 2001) genes, *vha-11*, *unc-32*, of V-ATPase in *C. elegans* caused embryonic lethality. Moreover, studies of wnt/β-catenin signaling, a cascade important for embryonic

development, revealed an involvement of V-ATPase in acidification of the pathway (Cruciat et al. 2010). Taken together, these findings suggest the importance of V-ATPase in the ovary, but the mechanisms in which V-ATPase function in the ovary need to be elucidated.

As the level of mRNA expression is not reflective of the protein function(s) and tick tissues including midgut, ovary, and salivary glands are essential for both horizontal and vertical transmission of SFG *Rickettsia* (Munderloh and Kurtti, 1995), the role of V-ATPase was then investigated at the protein level during *R. montanensis* invasion of backless *D. variabilis*. The results showed no difference of DvVATPaseV_0a mRNA level between unexposed- and *Rickettsia*-exposed ticks which is inconsistent with the studies in ticks chronically infected with *R. montanensis* (Macaluso et al. 2003), in which V-ATPase was upregulated in response to *Rickettsia* infection. In the current studies, DvVATPaseV_0a mRNA was measured in an early stage of rickettsial infection (1h) thus making comparison to the previous study by Macaluso et al. (2003) difficult. However, the level of mRNA expression is not reflective of protein function. In the current study, the role of V-ATPase in acute rickettsial infection of DVE1 tick cells was examined using inhibition assays. The results showed a significant decrease (dose dependent) in percent relative invasion in cells treated with V-ATPase inhibitor (bafilomycin A1) compared to untreated control. Bafilomycin A1 is a well characterized inhibitor for V-ATPase and previous studies have shown that V_0 domain subunit a (Hanada et al., 1990; Crider et al., 1994; Zhang et al., 1994; Wang et al., 2005) and c (Rautiala et al., 1993; Bowman and Bowman, 2002; Bowman et al., 2006) are targets of the inhibitor. The mechanisms in which tick V-ATPase functions in response to rickettsial infection is unknown. Because the role of V-ATPase was examined in the early stage of infection, it is possible that V-ATPase might play a role in receptor-mediated endocytosis, a process in which *Rickettsia* enter the cells. Besides intracellular compartments,

V-ATPase is also found on the cell surface. V-ATPase in clathrin-coated vesicles involves in receptor-mediated endocytosis. In this process, the enzyme undergoes trafficking to endosomes and functions in recycling receptors (Forgac, 2000). In addition, the studies of the V-ATPase V1 domain showed that subunit B (Holliday et al., 2000) and C (Vitavska et al., 2003) of the protein bind to actin filament (F-actin). In the tobacco hornworm V-ATPase subunit C ($VATPaseV_1C$) not only binds to F-actin but also bind to a monomeric G-actin (Vitavska et al., 2005). The biological function of $VATPaseV_1C$ and F-actin interaction was speculated to be involved in the organization of actin dynamics (Wieczorek et al., 2009). As previously characterized in vertebrate cells, SFG *Rickettsia* manipulate host actin in order to invade host cells (Dramsi and Cossart 1998; Gouin et al., 2004). These findings lead to the suggestion that V-ATPase is involved in rickettsial infection of tick cells via receptor-mediated endocytosis.

Because V-ATPase plays multiple roles in eukaryotic cells, it was necessary to investigate the role of V-ATPase in both uninfected and *Rickettsia* infected ticks. The present study provides a first description of tick V-ATPase and examined the role of the protein during rickettsial invasion. Further studies are required to investigate the mechanism(s) in which *Rickettsia* manipulate tick V-ATPase.

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CHAPTER 5
DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

Spotted fever group (SFG) *Rickettsia* is a group of gram negative obligate intracellular bacteria that are maintained in nature via transovarial transmission in ticks and through transstadial transmission during tick infestation (Sonenshine et al. 2005). One species from this group, *R. rickettsii* is the causative agent of a potentially fatal disease known as Rocky Mountain spotted fever (RMSF). The primary tick vector for pathogenic *R. rickettsii* is *Dermacentor variabilis* (Sonenshine et al. 2005). This species of ticks also carry *R. montanensis* (Feng et al., 1980; Anderson et al., 1986; Pretzman et al., 1990; Ammerman et al., 2004), an agent that was used in this study. Ticks not only serve as vectors but also reservoir for SFG *Rickettsia*; however, many questions concerning SFG *Rickettsia* tick-cell interaction remain unresolved. Because *Rickettsia* is maintained vertically by the tick hosts, understanding the interactions between ticks and *Rickettsia* is important for full comprehension of the epidemiology of tick-borne rickettsial diseases. Thus, the overall goal of this research is to study the interaction between *Rickettsia* and tick vector, specifically the molecular mechanisms involved in the invasion of SFG *Rickettsia* in tick cells. Previous studies have revealed the signaling molecules involved in the uptake of *Rickettsia* into mammalian and *Drosophila* cells (Martinez and Cossart, 2004; Martinez et al., 2005; Chan et al., 2009; Hillman et al., 2012; Reed et al., 2012). Considering the movement of SFG *Rickettsia* between vertebrate and invertebrate hosts, the hypothesis is that conserved mechanisms are utilized for host cell invasion. Toward the tested hypothesis, the objectives for this study are to identify and further characterize the tick molecules central to *Rickettsia* invasion of host cells.

Being an obligate intracellular bacterium, invasion of host cells is a critical step for subsequent rickettsial survival. In order to elucidate the host signaling cascade activated during

the entry of *Rickettsia*, it was necessary to identify the tick molecules involved in *Rickettsia* internalization into the host cells first. This study has employed biochemical inhibition assays to identify the tick proteins involved in the uptake of *R. montanensis* into DVE1 tick cells. The results revealed tick proteins important for rickettsial invasion and the pathway initiated by *R. montanensis* in order to invade DVE1 cells was proposed. After binding to unidentified receptors on tick cells, *R. montanensis* utilized host proteins including phosphatidylinositol-3'-kinase (PI 3-kinases), protein tyrosine kinases (PTKs), Src family PTKs, focal adhesion kinase (FAK), Rho GTPase Rac1, and Neural Wiskott-Aldrich syndrome protein (N-WASP). These molecules cooperatively stimulate actin related protein 2/3 (Arp2/3) complex leading to actin polymerization, cytoskeleton rearrangement and ultimately *Rickettsia* invasion into tick cells. As mentioned above, *D. variabilis* is a vector for both non-pathogenic *R. montanensis* and pathogenic *R. rickettsii*. Interestingly, the majority of *Rickettsia* that has been detected in infected ticks (*D. andersoni* and *D. variabilis*) throughout the United States is non-pathogenic *Rickettsia* such as *R. montanensis*, *R. peacockii*, and *R. rhipicephali* (Azad and Beard, 1998). The studies (both field and laboratory) in *D. andersoni*, the Rocky Mountain wood tick, have demonstrated a lower infection rate of *R. rickettsii* compared to *R. peacockii* (Burgdorfer et al., 1981; Burgdorfer, 1988). The mechanisms underlying the low infection rate of *R. rickettsii* are elusive although the interference phenomenon, in which the secondary infection was blocked by the primary infection of different *Rickettsia* species, was described in *D. variabilis* infected with the two species of non-pathogenic *Rickettsia* (Macaluso et al., 2002). Therefore it is important to further study the molecular mechanisms involved in *R. rickettsii* invasion of tick cells.

In the current study, biochemical inhibitors were used to target selected tick molecules in order to elucidate their functions in *Rickettsia* internalization. However, the effect of inhibitors

on tick cell viability and the solubility of the chemical in culturing medium are limiting factors of the assays. RNAi is a powerful technique that is widely used to silence gene expression for functional study. Further investigation will utilize RNAi to confirm the role of the identified tick molecules during *Rickettsia* infection. However, RNAi carries its own disadvantages, such as off-target effects in which an introduced RNA can non-specifically bind multiple genes at the same time. To knock down gene function using RNAi, the sequence of the target gene is needed. Because a complete *D. variabilis* genome sequence is not available, isolating *D. variabilis* gene sequences will be performed next.

In addition to identifying cDNA sequences, this study aimed to functionally characterize and provide the molecular details of tick signaling molecules involved in rickettsial invasion. Actin-related protein 2/3 (Arp2/3) complex, an important regulator for actin polymerization, was initially identified as a requirement for bacterial entry. Full-length cDNA of all subunits of *D. variabilis* (Dv) Arp2/3 complex including DvArp2, DvArp3, DvARPC1, DvARPC2, DvARPC3, DvARPC4, and DvARPC5 were isolated. Amino acid sequence analysis of DvARP2 and DvARP3 revealed potential ATP binding sites, which is consistent with other studies that have shown ATP binding to both Arp2 and Arp3, as well as ATP hydrolysis on Arp2. These reactions are required for Arp2/3 complex-mediated actin cytoskeleton remodeling (Dayel et al., 2001; Le Clainche et al., 2001; Le Clainche, 2003; Dayel et al., 2004; Martin et al., 2006). Furthermore, four putative WD motifs were observed in DvARPC1 amino acid sequence. One of the roles of WD motif containing protein is to form macromolecular protein complex; therefore, it was suggested that ARPC1 subunit functions in assembly and maintenance of the Arp2/3 complex structure and also in mediating the interaction of the complex with the proteins that regulate its functions (Welch et al., 1997).

The study of Arp2/3 complex mRNA expression in *D. variabilis* tissues revealed a higher expression of all seven DvArp2/3 complex subunit mRNAs in the tick ovary (both in uninfected- and *Rickettsia* infected- ovary) than in the other tissues (midgut and salivary glands); the levels of DvArp3, DvARPC4, and DvARPC5 mRNA expression in tick ovary were significantly greater compared to midgut and salivary glands. In response to *R. montanensis* infection, DvARPC4 mRNA was shown to be significantly upregulated in the tick ovary compared to uninfected tissue. Furthermore, functional study of Arp2/3 complex during *R. montanensis* invasion of tick tissues showed that inhibition of the complex by CK-666 generally decreased the ability of *Rickettsia* to invade tick tissues with a statistically significant invasion decrease in tick ovary.

The role of Arp2/3 complex in tick ovary has not been described and merits further investigation. In *Drosophila*, Arp2/3 complex is essential for oogenesis; Hudson and Cooley (2002) demonstrated that *arp3* and *arpc1* mutants had defects in flies' egg chambers in which the germ line nurse cells failed to transport the cytoplasmic contents to the oocytes.

Arp2/3 complex is a multisubunit protein and is critical for *Rickettsia* invasion of tick cells; our results show that disruption of Arp2/3 complex almost completely abolished (92% decrease) *Rickettsia* internalization into tick cells. Therefore the connection between tick organisms and rickettsial transovarial transmission with respect to Arp2/3 complex requires further study.

The next molecule characterized in this study is V-ATPase. V-ATPase is a multisubunit enzyme that can be found not only in intracellular organelles but also on the surface of eukaryotic cells. In cellular bodies such as endosomes and lysosomes, the enzyme is responsible for acidification of the organelles whereas in clathrin-coated vesicles located on cell surface, it

functions in receptor-mediated endocytosis; the process in which *Rickettsia* enters host cells. A full-length cDNA of V-ATPase V_0 subunit a from *D. variabilis* (DvVATPaseV_0a) was cloned and the amino acid sequence analysis revealed eight transmembrane segments and a potential site for N-glycosylation. These findings are consistent with studies conducted on calf VATPaseV_0a in which the protein was illustrated as a transmembrane glycoprotein (Adachi et al., 1990). The protein was expressed and purified and then polyclonal antibody was produced. Western blot analysis probed with anti-DvVATPaseV_0a antibody revealed a detectable protein band (at 30 µg total protein loaded) in tick ovary with a MW slightly bigger than the recombinant protein which corresponds to the predicted N-glycosylation of the protein.

A transcriptional profile of DvVATPaseV_0a demonstrated a greater mRNA expression in tick ovary than in midgut and salivary glands (significant difference in uninfected-tissues) which is consistent with the protein expression pattern in which the DvVATPaseV_0a band was detected in only the ovary. To examine the influence of *Rickettsia* on DvVATPaseV_0a transcription, mRNA expression was measured during *R. montanensis* infection of backless ticks. The result showed no difference between uninfected- and *Rickettsia* infected- tissues. In contrast, the functional study using a V-ATPase inhibitor, bafilomycin A1, revealed a significant decrease in percent relative rickettsial invasion of tick cells in comparison to untreated control cells. Although the results between mRNA expression and inhibition assays were contrasting, it can be concluded that V-ATPase is involved in the uptake of *Rickettsia* into tick cells because the level of mRNA expression is not always reflective of protein function.

The underlying mechanisms in which tick V-ATPase functions during the entry of *Rickettsia* is unknown. Considering the experimental design in which the role of V-ATPase was measured during the early stages of infection, V-ATPase was speculated to play a role in

receptor-mediated endocytosis, As indicated previously, V-ATPase is also found in clathrin-coated vesicles on the cell surface, where it functions in receptor-mediated endocytosis. In this process, the low pH of endosomes stimulate the dissociation of ligand-receptor interactions leading to recycling of the receptors to the cell surface (Forgac, 2000). Furthermore, there is evidence demonstrating that V-ATPase V1 subunit B and C bind to actin (Holliday et al., 2000; Vitavska et al., 2003; Vitavska et al., 2005) and the interaction by subunit C was speculated to be involved in actin organization dynamics. As described in vertebrate and invertebrate cells (previous and this study, respectively) SFG *Rickettsia* manipulate host actin in order to invade host cells (Dramsi and Cossart 1998; Gouin et al., 2004). These findings are suggestive of V-ATPase's involvement in rickettsial infection of tick cells via receptor-mediated endocytosis. Besides receptor-mediated endocytosis, V-ATPase was shown to be involved in the escape of some pathogens, such as *Listeria monocytogenes,* from the phagosome into the cytosol of host cells (Beauregard et al., 1997). Because phagosomal escape is the process that facilitates rickettsial survival after entering mammalian cells (Winkler and Miller, 1982; Walker et al., 2001; Whitworth et al., 2005), it is necessary to further study the escape of *Rickettsia* from phagosome in tick cells.

Altogether, this dissertation represented, for the first time, the description of tick proteins (Arp2/3 complex and V-ATPase) important for the uptake of *Rickettsia* into tick cells and the pathway activated during *Rickettsia* invasion of tick hosts was proposed as shown in Figure 5.1.

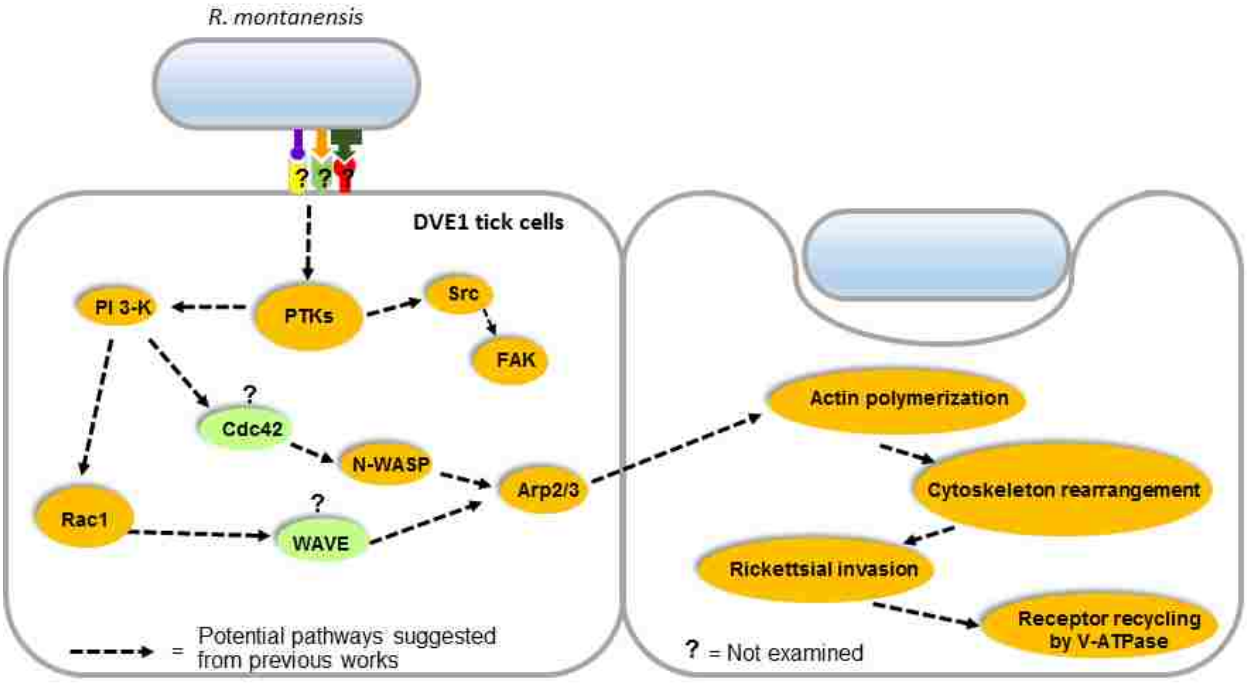

Figure 5.1 The proposed pathway activated during rickettsial invasion of tick cells. After binding of *R. montanensis* to unidentified receptor (s) on tick cells, multiple tick molecules (colored in orange) including phosphatidylinositol-3'-kinase (PI 3-kinases), protein tyrosine kinases (PTKs), Src family PTKs, focal adhesion kinase (FAK), Rho GTPase Rac1, Neural Wiskott-Aldrich syndrome protein (N-WASP), and actin-related protein 2/3 (Arp2/3) complex, are activated. The activation of these molecules leads to actin polymerization resulting in membrane rearrangements and rickettsial invasion into tick cells. V-ATPase then facilitates recycling of the receptor to the cell surface. The molecules and pathways with question marks were not examined in this study. Figure was modified from Chan et al., 2010.

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APPENDIX A
COMMONLY USED ABBREVIATIONS

μM – Micromolar

^{0}C – Degree Celsius

^{0}F – Degree Fahrenheit

AG – Ancestral group

AMP – Antimicrobial peptide

ANOVA – Analysis of Variance

Arp2/3 – Actin related protein 2/3

ATP – Adenosine tri-phosphate

CRT – Calreticulin

CDC – Centers for Disease Control and Prevention

cDNA – Complementary DNA

CL – Capsule-like structure

CM – Cytoplasmic membrane

CW – Cell wall

IM – Intracytoplasmic membrane.

CO_2 – Carbon dioxide

CRT – Calreticulin

CT – Threshold cycle

Cyt – Cytochalasin

CTL – Cytotoxic T lymphocytes

Da – Dalton

DC – dendritic cell

DEPC – Diethylpyrocarbonate

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

dsRNA – Double-stranded RNA

DVE1 – *D. variabilis* cell line

EDTA – Ethylenediaminetetraacetic acid

F-actin – Filamentous actin

FAK – Focal adhesion kinase

FBS – Fetal bovine serum

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

gDNA – Genomic DNA

GSP – Gene specific primer

GTP – Guanosine triphosphate

HGA – Human granulocytic anaplasmosis

HME – Human monocytotropic ehrlichiosis

ID – Intradermal

IL – Interleukin

IS – Insertion sequence

IFN – Interferon

IP – Intraperitoneal

ISE6 – *Ixodes scapularis* cell line

LPS – lipopolysaccharide

LSU – Louisiana State University

MHC – Major histocompatibility complex

Mb – Mega bases

MOI – Multiplicity of infection

mRNA – Messenger RNA

MW – Molecular weights

NK – Natural killer

N-WASP – Neural -Wiskott-Aldrich syndrome protein

NOS – Nitric oxide synthase

NN – Not notifiable

NO – Nitric oxide

N-terminus – Amino-terminus

OmpA – Outer membrane protein A

OmpB – Outer membrane protein B

ORF – Open reading frame

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PI 3-kinase – Phosphatidylinositol-3'-kinase

PTK – Protein tyrosine kinase

PVDF – Polyvinylidene fluoride

qRT-PCR – Quantitative reverse transcriptase PCR

qPCR – Quantitative PCR

RACE – Rapid amplification of cDNA ends

RMSF – Rocky Mountain spotted fever

RNA – Ribonucleic acid

RNAi – RNA interference

RT-PCR – Reverse transcriptase PCR

Sca – Surface cell antigens

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Standard error of means

SFG – Spotted fever group

siRNA – Small interfering RNA

TBRD – Tick-borne rickettsial diseases

TBST – Tris-buffered saline containing Tween-20

TE – Tris-EDTA

TG – Typhus group

TNF-α – Tumor necrosis factor-α

TLR – Toll-like receptor

TRG – Transitional group

T4SS – Type IV secretion system

V-ATPase – Vacuolar ATPase

WAVE – WASP-family verprolin-homologous protein

APPENDIX B
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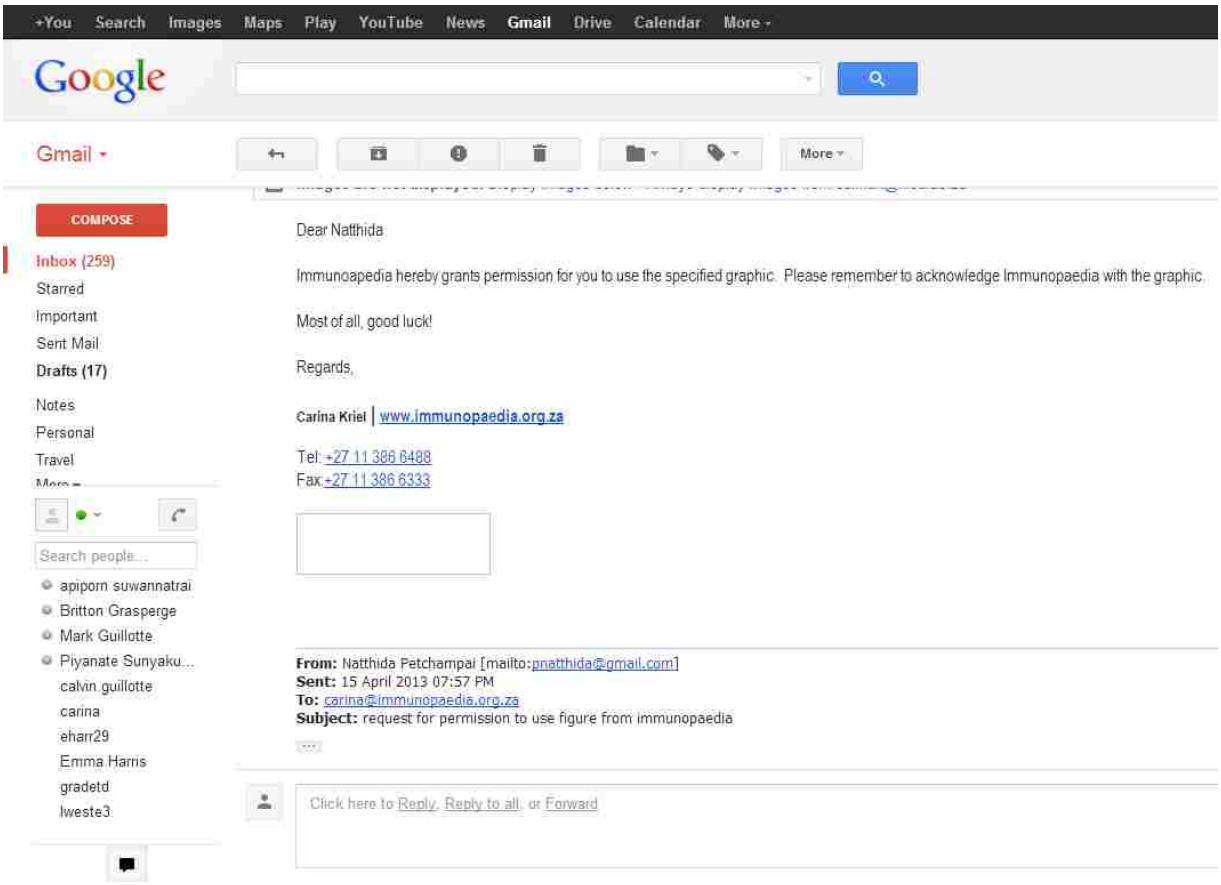
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Natthida Petchampai was born in Phetchabun, Thailand. She is the daughter of Mr. Chinawitchya Petchampai and Mrs. Issarapron Suriyamart, and she is the middle sister of Natthapong Petchampai and Natthawut Petchampai. Natthida graduated with a bachelor of sciences in biochemistry at Kasetsart University in 2000, and enrolled in the master's program in biochemistry at Mahidol University. After completing her Master's degree in 2003, Natthida joined Center of Excellence for Shrimp Molecular Biology and Biotechnology, studying on a yellow head virus (YHV); a pathogen of the black tiger shrimp. She later joined Armed Forced Research Institute of Medical Sciences (AFRIMS), working on a project involving dengue, a mosquito-borne viral disease affecting humans. During her time at AFRIMS she conducted research on detection and identification of the dengue virus from patient sera using reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR. Natthida became interested in the biology of vector-borne diseases and wanted to pursue a research career in the field. So, in 2006 Natthida moved to Baton Rouge, Louisiana to join Dr. Kevin Macaluso's laboratory where she learned about the important role of arthropods as reservoirs and vectors for bacteria.In the spring of 2009 Natthida began her doctorate degree in the Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University under the guidance of Dr. Kevin Macaluso. Natthida completed her dissertation research and will graduate in May, 2013. After graduation Natthida will continue to pursue her research interests in the field of vector-borne diseases in the United States.