ABSTRACT

Title of Document: BIOLOGICAL SIGNIFICANCE OF SELECTED *IXODES SCAPULARIS* TRANSCRIPTION FACTORS REGULATING TICK HEMATOPHAGY AND DEVELOPMENT

Kazi Rifat Antara, Ph.D., 2023

Directed By: Professor Utpal Pal, Department of Veterinary Medicine

Lyme disease is one of the most prominent vector-borne diseases, which is transmitted by the *Ixodes scapularis* tick and related species, and the causative agent is the bacterial pathogen *Borrelia burgdorferi*. Besides *I. scapularis*, many other tick species are also prolific vectors of several bacterial, viral, or eukaryotic pathogens affecting humans and animals. *I. scapularis* possess a large genome of 2.26 Gbp, predominantly featuring repetitive DNA or transposomal elements. Although many orthologous genes are present in other arthropods and blood-borne vectors, the genome also encodes numerous unique tick-specific genes. Despite many advances in *Ixodes* biology and genomics, the molecular basis of their hematophagy and development remains unknown. During feeding on the host, a major tick organ like the gut undergoes remarkable yet poorly understood episodes of cell division and differentiation, accommodating a huge blood meal that can be up to 100-fold greater than their body weight. The gut, therefore, plays a vital role in blood meal acquisition, digestion, and storage, supporting the long-term survival of ticks during prolonged off-host periods of nutrient deprivation. Understanding the

molecular mechanism of gut physiology, including cell division and differentiation, is an essential area of research. As transcription factors are central to the biology and development of metazoan organisms yet remain largely uncharacterized in ticks, the goal of this dissertation is to decipher the biological significance of representative groups of major development-associated transcription factors in *I. scapularis* that are expressed in the gut, especially during blood meal engorgement process. Among them, two of the highly upregulated transcription factors in the gut were chosen for further characterization. We show that both transcription factors, Immunoglobin-fold transcription factor (SuH) and POU domain transcription factor (Nubbin), play essential roles in tick physiology, as their knockdowns impart phenotypic defects, impacting tick feeding, development and life cycle. The latter part of the dissertation will highlight the molecular mechanism of their functions. A fundamental understanding of the molecular basis of tick biology, hematophagy, and development may contribute to developing novel strategies to curb the spread of tick-infection.

BIOLOGICAL SIGNIFICANCE OF SELECTED *IXODES SCAPULARIS* TRANSCRIPTION FACTORS REGULATING TICK HEMATOPHAGY AND DEVELOPMENT

By

Kazi Rifat Antara

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Advisory Committee:
Professor Utpal Pal, Chair
Dr. Osnat Herzberg
Dr. Daniel Nelson
Dr. George Belov
Dr. Yanjin Zhang

Dedication

I am grateful to my father, Kazi Rezaul Haque, who always motivated me to pursue higher studies, and my mother, Kazi Zinnatul Fatima, for her encouragement. I am eager to thank my husband, Safat Siddiqui, who was always by my side to fulfill my wish. I am also grateful to friends and relatives who were by my side on this journey.

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List of Abbreviations

BSA	Bovine serum albumin
cDNA	Complement DNA
ELISA	Enzyme linked Immunosorbent Assay
EMSA	Electrophoretic mobility shift assay
GST	Glutathione S-transferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
mRNA	Messenger RNA
RNAi	RNA Interference
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis
SuH	Suppressor of hairless
Nub	Nubbin
CBF	Core-binding factor
NICD	Notch Intracellular domain
NF	Nuclear factor
Zn	Zinc
µg	microgram
µl	microliter
^{0}C	Degrees Celsius
bp	Base pair
ml	milliliter

LB	Luria Bertani
PBS	Phosphate buffered saline
HLH	Helix-Loop-Helix
HTH	Helix-Turn-Helix
EC	Enterocyte
EE	Entero-endocrine cells
CCHF	Crimean-Congo Hemorrhagic Fever
TBEV	Tick-borne encephalitis virus
FOX	Forkhead box
DEET	N, N-diethyl-meta-toluamide
TFs	Transcription Factors
MAPK	Mitogen-activated protein kinase
FOXO	Forkhead boxO
RF	Relapsing fever
CNS	Central nervous system
DAPC	Discriminant analysis of principal components
ORF	Open reading frame
HMW	High molecular weight
RUNX	Runt-related transcription factor
BMP	Bone morphogenic protein
RHR	Rel homology region
IPT	Immunoglobin plexin transcription factor
BTD	Beta trefoil domain
CTD	C-terminal domain

PTLD	Post-treatment lyme disease
MH	Mad homology
CBE	Core binding elements
NF	Nuclear factor
Lz	Lozenge
GCM	Glial cell missing
EGFR	Epidermal growth factor receptor
InR	Insulin receptor
ISC	Intestinal stem cell
AMP	Antimicrobial peptide
PC	Peripheral cell
POU	Pit-1, Oct 1 and 2, and nematode unc -86
Pdm	POU domain protein

Chapter 1: General Introduction: *I. scapularis* development-associated transcription factors

Ticks

There are a total of 896 identified tick species that belong to three major families: *Argasidae* (soft ticks, 193 species), *Ixodidae* (hard ticks, 702 species), and *Nuttalliellidae* (1 species) (Guglielmone *et al*., 2010; Chen *et al*., 2010). The *Ixodidae* are commonly known as hard ticks, and the *Argasidae* are known as soft ticks. Hard ticks possess a sclerotized scutum and an apically placed gnathosoma. The hard tick family Ixodidae includes around 700 species from different genera: *Ixodes, Rhipicephalus, Dermacentor, Haemaphysalis, Amblyomma, Hyalomma, Anomalohimalaya, Margaropus, Nosomma, Rhipicentor, Bothriocroton, Cosmiomma* and the fossil representatives *Cornupalpatum* and *Compluriscutula*. They can feed for long periods and ingest more than 100 times their body mass. On the other hand, around 193 species are under the *Argasidae* family and are divided into two major genera: *Argas* and *Ornithodoros* (Filippova *et al*., 2008). These soft ticks don't possess a scutum, and their mouthparts are located anterioventrally. Soft ticks also contain a leathery integument that can expand rapidly and allow them to engorge around ten times their body mass within a few hours. *Nuttalliellidae* ticks are also rapid and frequent feeders, achieving engorgement in seconds to hours. The sequence analysis of18S rRNA of this tick species has indicated that is basal to other tick families indicating that it is the closest extant relative of the last ancestral tick lineage (Latif et al., 2012). There are some major differences in the physiological processes between these hard and soft tick families (Hajdusek *et al*., 2013). For example, soft ticks use an ultrafiltration organ, to filtrate blood from the water. In contrast, hard ticks secrete excess water from the blood back into the host through its specialized salivary gland.

Ticks have a unique and complex developmental cycle; many tick species are transient obligate parasites of vertebrate animals. They have three different development stages besides eggs: larvae, nymph, and adult (male and female). However, the stages and duration of the life cycle depend upon the tick's family. Ticks tend to exhibit endophilous behavior since they live close to their hosts or areas nearby, and at least for the soft ticks, their development is mostly dependent on the environment of the burrow. Almost all argasid ticks and some ixodid ticks exhibit endophilous behaviors (Gray *et al*., 2014). The other type of behavior is exophilous, which involves the Ixodidae ticks, where the vector usually waits for a suitable host to attach, exposing itself to the outside environment.

There are uncertainties about the precise origin of hematophagy and the evolution of ticks (Kitsou *et al*., 2021). Ticks are known to have originated in the Carboniferous period (Klompen *et al*., 1993). However, the emergence of diversity occurred afterward. Some studies propose that the different families of hard and soft ticks emerged in the Middle Permian, and some studies propose it occurred in the Early Permian time (Mans *et al*., 2012). The microclimate and the abundance of hosts shape the abundance of ticks, known as phenology. The tick lifecycle largely depends on the seasonal changes, climate, and abundance of hosts. Different studies show that ticks' questing depends on the climate (Ogden *et al*., 2008; Gray *et al*., 2008; Estrada-Pena *et al*., 2007). Before beginning to quest, most ticks remain inactive, and questing occurs in a specific combination of photoperiods and climate (Belozerov *et al*., 1982). Photoperiods can also play a role in molting or questing stages, activating or delaying the onset of these episodes. This can be a part of the natural selection process that allows ticks to find a host, feed, and molt before the winter and stop the activities before the spring. Ticks are susceptible to long-term weather variables, but the response of ticks to changes in climate conditions is not simple and depends on the changes in the population

dynamics of vertebrates, amongst many other factors. Activities of humans that change the landscape can modify the habitats where vectors, pathogens, and hosts co-exist (Agoulon *et al*., 2012).

Tick-borne diseases

Ticks were described as human parasites several hundred-year BC by ancient Greek writers. Tick-borne diseases and Tick-associated pathogens have been observed since the 20th century in many medical descriptions. Climate change and humans have a major impact on ticks and their global emergence. Both deforestation and reforestation have a significant impact on tick populations. The changes in wildlife, such as the introduction of new species and the disappearance of species, can also have significant impacts (Kilpatrick *et al*., 2012).

Nevertheless, the annual losses due to tick infestations and tick-borne diseases can reach up to billions of dollars (Jongejan *et al*., 2004). Ticks are among the most important vectors affecting human and animal health in many countries and continents. Unlike many arthropod vectors, ticks can transmit a plethora of infectious disease agents, including viral (such as tick-borne encephalitis), bacterial (*Borrelia*, *Anaplasma*, etc.), and protozoan (*Babesia*, *Theileria*, etc.) origins. The pathogenesis with these infectious agents is complex and often results in multisystem clinical complications, where humans and many domesticated animals are considered as non-natural or accidental hosts. The disease usually transmits through hematophagus bites and, in some rare cases, through blood transfusions (such as transfusion-transmitted *Babesia*) (Piesman *et al*., 2008). The tick-borne pathogens can persist in hosts or vectors for a long time, and the transmission can occur in many ways depending on the specific microbe. The different types of transmission include tick-to-tick through the host (horizontal), females-to-eggs (vertical), and stage-to-stage

(trans stadial). Some infectious diseases like Lyme disease can be transmitted from one tick to another without bacteremia in the host during co-feeding in the bite site (Randolph *et al*., 1996). However, these are less documented and require further investigation. In ticks infected with bacteria or parasites, the infectious agents need to be transmitted to the salivary gland to transmit to the host, which may take several hours (de La Fuente *et al*., 2017), from where they are swiftly transferred to the dermis along with the saliva. Figure 1 lists six major tick genera which causes human diseases.

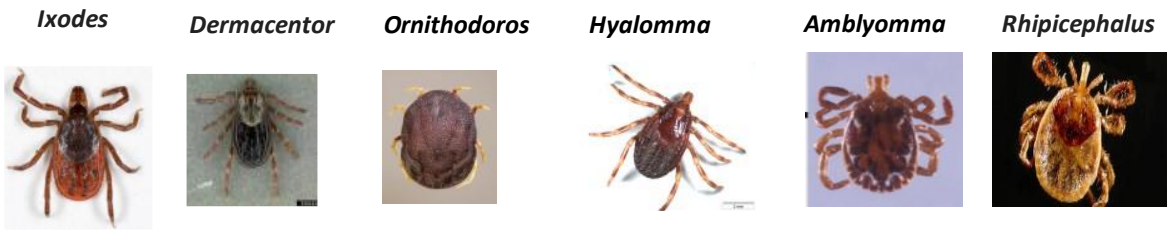

Figure 1: Six major tick genera that cause human diseases.

Viral infections

Numerous viral pathogens can be transmitted to humans and animals by ticks. Many of these viruses belong to three families: Flaviviridae, Bunyaviridae, and Reoviridae, and they are transmitted through hard ticks (Lani *et al.*, 2014). Two of the most important viral infections in humans are tick-borne encephalitis (TBEV) and Crimean-Congo hemorrhagic fever (CCHF). The tick-borne encephalitis virus belongs to the *Flavivirus*, an RNA virus. Ticks acquire the virus from the infected vertebrate host and transmit the virus to another host following a subsequent blood meal. Ticks can acquire the virus during co-feeding on non-viremic hosts (Randolph *et al*., 1996).

TBEV: The infection is mainly a neurological disease transmitted by ticks in Asia and Europe, and thousands of cases are observed each year. There are three subtypes of the virus have been reported. The European subtype is transmitted by *I. ricinus* and less commonly by *I. hexagonus* ticks (Rumer *et al*., 2011). The Eastern subtype is transmitted by *I. persulcatus* (Suss *et al*., 2011). The disease is zoonotic, involving wild and domestic animals (Mansfield et al., 2009). Humans are infected mainly in early spring and summer, coinciding with the seasonal emergence of subadult ticks. Ingestion of dairy products can also cause disease transmission (Mansfield *et al.*, 2009). The symptoms represent febrile diseases like influenza-like illnesses (Huang *et al*., 2014). In around 50% of cases, patients can get encephalitis, and in less than 10% of cases, patients can get myelitis. The case fatality rate is around 3% for European subtypes and 35% for Eastern subtypes (Mansfield *et al*., 2009).

CCHF: The Crimean-Congo hemorrhagic fever was first reported in Crimea. The disease is characterized by severe hemorrhagic fever; hence, the virus is called the Crimean-Congo hemorrhagic fever virus. The virus contains enveloped dsDNA and belongs to the *Bunyaviridae* family. The virus is mainly transmitted via ticks from the *Hyalomma* genus. It can also be transmitted via the *Dermacentor* and *Rhipicephalus* genera. The virus can be transmitted and circulated among vertebrate hosts by remaining asymptomatic and can infect both wild and domestic animals, but in most cases, humans contract the most severe form of the disease. Tick bite is the main route of transmission for humans. However, contamination can take place from infected body fluids (Mertens *et al.*, 2013). The disease is characterized by fever, becoming fatal in around fifty percent of cases.

Bacterial infections

One of the major pathogens transmitted by *Ixodes* tick is *Borrelia* spp. They are extracellular, spiral-shaped spirochetes, and the genus can be divided into two groups: one that causes Relapsing fever and the other that elicits Lyme disease (also known as Lyme borreliosis) and related infections. Hard ticks transmit Lyme borreliosis from the *Ixodes* genus. In contrast, Relapsing fever *Borrelia* can be transmitted by both soft ticks (*Ornithodoros* and *Argas*) and hard ticks (such as transmission of *B. miyamotoi* by *Ixodes* ticks) (Stanek *et al*., 2012). In addition to these major pathogens, ticks also transmit additional pathogens and infections, which are highlighted below:

Lyme disease or Lyme borreliosis: The infection is caused by *B. burgdorferi* sensu lato group, and 21 species have been identified. It is the vector-borne disease that is most prevalent in the northern hemisphere. The disease is transmitted by the vector *I. scapularis* in the eastern coast and *I. pacificus* on the western coast. *I. persulcatus* is more prevalent in eastern Europe and Asia and *I. ricinus* is the main vector in Europe. *B. burgdorferi* sensu stricto (ss) is a pathogenic species in North America for humans. There are several species known to be involved in human pathologies in Europe, such as *B. burgdorferi* ss, *B. afzelii, B. garinii, B. spielmanii* and *B. bavariensis* (Stanek *et al*., 2012). While rodents serve as a major reservoir host, many other vertebrates don't act as a true reservoir of pathogenic bacteria. Still, they can contribute to maintaining the large population of vector ticks. While rodents support the borrelial life cycle in sub-adult ticks, deer hosts adult ticks in the USA. The immune system of hosts, such as deer, can kill *Borrelia*, thus unable to transmit spirochetes. However, these incompetent hosts contribute to maintaining large populations of ticks in the environment as they are the preferential

hosts of adult ticks. The clinical manifestations of the disease include cutaneous inflammation, i.e., erythema migrans. The infection may progress to early or late disseminated stages involving neurological, cutaneous, and cardiac systems (Stanek *et al*., 2012). The pathogenicity is dependent upon the pathogenic species involved. The immunity against Lyme borreliosis is largely non-protective, and one individual can be recurrently infected in subsequently infected tick-bites.

Tick-borne relapsing fever: Soft tick *Ornithodoros* mainly transmit the tick-borne relapsing fever. The causative agent is *B. miyamotoi,* and the disease can also be transmitted by hard tick *I. ricinus* (Platonov *et al*., 2012). This species is mainly present in Europe and is pathogenic to immunodeficient people (Hovius *et al*., 2013; Boden *et al*., 2016). There are around 22 species in the *Borrelia* group. They are present in tropical areas and the United States (Talagrand *et al*., 2018). The infection's symptoms include fever peaks due to changes in bacterial surface antigen. The tick-borne relapsing fever is underdiagnosed in tropical regions, which can be confused with malaria.

Rickettsioses: These bacteria are transmitted by ticks and many other arthropods. The bacteria *Rickettsia* is intracellular, Gram-negative, and are transmitted through ticks all over the world. The geographic distribution is similar to its vector (Parola *et al*., 2013). The pathogen interacts with endothelial cells, increasing the vascular permeability, which infests the clinical manifestations (Blanton *et al*., 2013). The pathogens can be divided into typhus, spotted fever, and transitional groups. Ticks transmit twenty among the twenty-one species of spotted fever group. Some putative examples of *Rickettsia* species include *R. conorii, R. sanguineus* in Europe, Asia, and Africa. The symptoms include a black spot at the bite site and fever for three to five days.

There are several diseases which, such as tick-borne lymphadenopathy (TIBOLA, scalp eschar, and neck lymphadenopathy, and *Dermacentor*-borne necrosis erythema lymphadenopathy are transmitted by *R. slovaca* and *R. raoultii* and the vector is the *Dermacentor* tick. The symptoms include cervical adenopathy and black spots that can be present on the scalp. *R. helvetica* is transmitted by *I. ricinus,* and the pathogenicity varies among patients.

Human granulocytic anaplasmosis: The infection is transmitted by the bacteria from Rickettsiales order, which is strictly a group of intracellular bacteria, which includes *Anaplasma* and *Ehrlichia* and are mostly transmitted by ticks (Dumler *et al*., 2001). Human monocytic ehrlichiosis is a bacterial disease caused by *Ehrlichia chaffeensis* in many parts of the United States. The vector of this disease is *Amblyomma americanum* tick, and the main reservoirs are deer (White-tailed). *Anaplasma phagocytophilum* is a clinically important pathogen responsible for human granulocytic anaplasmosis (Koenen *et al.*, 2013). The pathogen usually invades white blood cells, primarily neutrophils. The disease was first reported in 1994 in the United States (Woldehiwet *et al*., 2010) is known to present in temperate regions, and has been known to cause pathogenesis in both humans and animals. Bacterial occurrence varies among tick species in endemic regions; the prevalence rate can be up to 20% for *I. ricinus* and 16.7% for *I. persulcatus* (Stuen *et al.*, 2013). The onset of the disease can take around one to three weeks, and the symptoms include fever, chills, muscular pain, headache, etc. Doxycycline can be prescribed to patients (Koebel *et al*., 2012). Ticks transmit other bacterial pathogens of different genera, such as *Coxiella, Bartonella, Francisella,* etc.

Q fever: The causative agent of Q fever is a Gram-negative, obligate intracellular bacterium that is mainly found in monocytes and macrophages of infected humans and animals. It's a zoonotic disease that can infect numerous animals and humans (Roest *et al*., 2013). The disease can be transmitted through the respiratory or digestive route when they contact the infected animals, mainly ruminants, or through unpasteurized dairy products. The symptoms include influenza-like illness, pneumonia, or hepatitis. The disease can be severe in chronic infection, such as carditis. Ticks play a secondary role in transmitting the pathogen (mainly endocarditis).

Tularemia: The infection is caused by *F. tularensis*, a Gram-negative, intracellular bacterium. The bacteria can also be transmitted through aerosols, which makes it highly contagious. It's a zoonotic disease, and the hosts are rodents and lagomorphs. The disease has been found in various regions of the world, mainly the Northern Hemisphere (Steiner *et al*., 2014). Two tick genera, *Dermacentor* and *Ixodes* can transmit the disease. The disease can be transmitted by direct contact with infected animals, oral consumption of undercooked meat or inhalation. The disease shows influenza-like symptoms, and the illness ranges from mild to life-threatening.

Bartonellosis: The bacterial genus *Bartonella* is mainly transmitted by sandflies and body lice. It's a Gram-negative bacteria, and around thirteen species are pathogenic to humans, including *B. henselae* and *B. quintana*. Notably, *I. ricinus* ticks are known to transmit various *Bartonella* species, but the vector competency has only been proven with *B. birtlesii* and *B. henselae* (Cotte *et al*., 2008; Reis *et al*., 2011), and, thus, warrants further investigation.

Parasitic infections

Ticks transmit pathogens from the *Babesia* genus. The sporozoites of *Babesia* sp. are present in tick salivary glands and can penetrate red blood cells. There are more than a hundred species have been found for *Babesia* spp. Two species of *Babesia* are more pathogenic for humans, which are *B. microti* and *B. divergens. Ixodes* ticks are the primary vector, and it takes several days for the pathogen to transmit since it remains in the sporozoites phase. A wide range of animals can act as hosts for the pathogen (Vannier *et al*., 2012). The infection in humans is rare but mostly caused in immunocompromised individuals. Besides an infected tick bite, humans can get the disease through blood transfusions, although cases are relatively rare (Koenen *et al.*, 2013). The symptoms include fever, anemia, and jaundice. Figure 2 lists pathogens transmitted by tick *I. scapularis*.

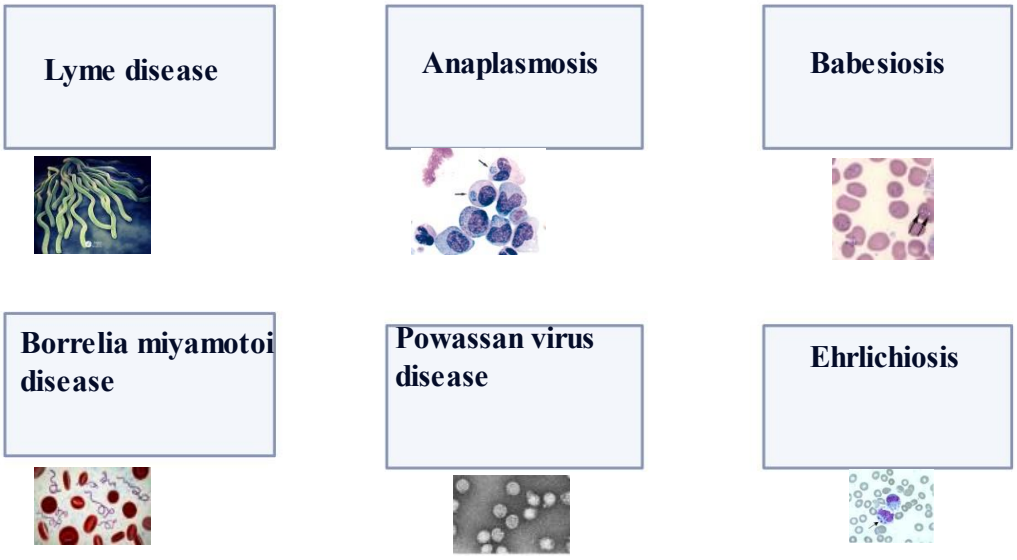

Figure 2: Diseases transmitted by the tick *I. scapularis*.

Prevention of Tick-borne diseases

Suppression of infected ticks

One major approach to control tick-borne diseases includes host-targeted approaches, where the vector is meant to be killed when attached to its host. In Connecticut, the white-footed mice were injected with recombinant OspA vaccine targeted against the surface protein of *B. burgdorferi* (Tsao *et al*., 2004). The vaccination significantly decreased the infection rate of the vector *I. scapularis* nymphs in wild rodent hosts (Tsao *et al*., 2004). In wildlife, an oral vaccination approach could be based on the vaccinia viral vector, which was tested effective against rodents in the laboratory **(**Scheckelhoff *et al*., 2006). Adding antibiotics, such as Doxycycline, can decrease the chances of tick-borne diseases. Doxycycline's role in reducing the transmission of *B. burgdorferi* has been tested in rodents (Zeidner *et al*., 2004).

Vaccination

There can be several approaches to avoid getting diseases from infected ticks. Although the human vaccine against TBEV is available in Europe and Russia, and recently U.S. FDA approved Pfizer's TICOVAC™ vaccine, no other preventive strategy is available for most of the other tick-borne infections. These diseases can be suppressed by administering the vaccine, as shown by the development of a human Lyme disease vaccine, although currently, a vaccine to prevent human infection is unavailable. The previous Lyme disease vaccine for humans is called LYMErixTM, which was introduced in 1998 in North America and was retracted from the market in 2002. (Lathrop *et al*., 2002, Poland *et al*., 2001). There were several reasons for the retraction of the vaccine, which included frequent boosters, musculoskeletal symptoms, high cost, age restrictions, etc. (Hanson *et al*., 2003; Hayes *et al*., 2003). As a prevention approach for human

diseases, the ticks should be checked daily following exposure to high-risk habitats, and quick removal after attachment can prevent pathogen transmission. It has been proven that *B. burgdorferi* is not transmitted before twenty-four hours after attachment of *I. scapularis* and *I. pacificus* ticks (Peavy *et al*., 1995; Piesman *et al*., 1993). For Lyme disease, spirochete antibiotic treatment after a tick bite can decrease the risk of getting the disease or developing symptoms (Korenberg et al., 1996; Nadelman *et al*., 2001).

Avoiding tick bites

Some studies show behavioral risk factors involving ticks and tick-borne pathogens (Guglielmone *et al*., 2010; Mertens *et al*., 2013; Vannier *et al*., 2012). If frequently exposed to tick habitats, some simple measures can be taken to avoid tick bite and subsequent pathogen exposure. Wearing clothing, including long trousers tucked into socks, the long-sleeved shirt can mechanically decrease the chances of ticks finding a feeding site on its host. When applied to clothing or skin, skin repellents can effectively reduce the chances of tick bites (Gassner *et al*., 2016; Mansfield *et al*., 2009). Some DEET (*N*, *N*-diethyl-meta-toluamide) and permethrin-based products can be used as a tick repellent, but in some studies, it has been found toxic (Schouls *et al*., 1999).

Suppression of ticks seeking for hosts

Acaricides are applied in tick-infested areas in different agricultural settings. In the 60s, DDT (dichloro-diphenyl-trichloroethane) was known to substantially decrease tick populations in the Soviet Union, decreasing the incidence of tick-borne encephalitis. The targeted vector was *I. persulcatus*. (Lindquist *et al*., 2008). The Lyme disease-endemic regions in the United States use area-wide acaricides by targeting the vector *I. scapularis*. Second-generation pesticides such as

carbaryl, diazinon, and chlorpyrifos are used to kill the vector. Later, in the US, several synthetic, less-toxic pesticides such as permethrin, deltamethrin, and cyfluthrin came into the market to eradicate the Lyme disease vector. Deltamethrin is very effective in killing *I. scapularis*; a single application in the forest-lawn interface can kill ninety-five percent *I. scapularis* nymphs (Schulze *et al*., 2001). Applying synthetic pesticides can significantly reduce the risk of Lyme borreliosis, babesiosis, or human granulocytic anaplasmosis. But Acaricides cause environmental damage and mammalian toxicity. In highly Lyme borreliosis endemic regions in the North-Eastern United States, most residents are known to not use pesticides in their properties due to significant health risks. The public health department is seeking for alternate interventions to combat the vector.

Public awareness

The prevention of tick-borne diseases can be made in an easily understandable format to reach out to the general public. It is an inadequately studied aspect of tick-borne disease prevention. The web-based information can also be kept updated since, nowadays, the internet is the first option to get information. There are also options for two-way interactions among users and novel web-based resources that help exchange information between the source and the user. Due to increasing global awareness, however, the U.S. Senate recently passed "21st Century Cures Act" that prioritizes the research on tick-borne diseases, including developing novel vaccines and treatment strategies.

***Ixodes scapularis* ticks**

As mentioned earlier, the spirochete *B. burgdorferi* causes Lyme disease, and the disease is transmitted to vertebrate hosts by *Ixodes* spp. ticks. Several species within

the *B. burgdorferi* sensu lato complex have been identified as pathogens to humans. However, in the United States, nearly all Lyme disease cases are caused by *B. burgdorferi* sensu stricto (referred to as *B. burgdorferi)* (Kurokawa *et al*., 2020). Early Lyme disease may appear as a distinctive skin rash called erythema migrans, and the acute illness is characterized by fever, fatigue, muscle and joint aches, swollen lymph nodes that are clinically manifested as Lyme arthritis, Lyme carditis, and a variety of neurological symptoms known as neuroborreliosis. Some patients who are treated with antibiotics may have symptoms of pain, fatigue, or difficulty thinking that last for more than six months and is termed Post-Treatment Lyme Disease Syndrome (PTLDS) (Crowder *et al*., 2014). The precise etiology, pathogenesis, and treatment for PTLDS remains unknown. It is speculated that the Lyme disease spirochaete and ticks have developed dynamic interactions for the pathogen to persist, colonize, or transmit from ticks to other mammalian hosts (Kurokawa *et al*., 2020). A better understanding of the molecular mechanism of tick biology, including blood meal digestion and development, would support the invention of novel therapeutics against tick-borne diseases like Lyme borreliosis.

The black-legged or deer tick *I. scapularis,* which transmit *B. burgdorferi,* has four life stages: egg, larva, nymph, and adult (De *et al,* 2021). Ixodid is a multi-host tick with three life stages (larva, nymph, and adult) where sub-adults and adults feed on a different host. The black-legged tick feeds on a wide array of mammals, birds, and sometimes on incidental hosts like domestic animals and humans. The first stage of the tick lifecycle is the egg, laid by engorged females, typically in the late spring at a site detached from the host. By a month or more, eggs are hatched into six-legged larvae, which feed once on various small mammals or birds. Larvae do not carry the Lyme disease pathogen *B. burgdorferi*. Still, they may acquire the spirochete from an infected host during their first blood meal and eventually transmit those pathogens to another host

during their subsequent feedings as nymphs or adults. By several months, larvae emerge as eight-legged nymphs. The nymph is more likely to transmit tick-borne pathogens to humans due to their relatively small size, making them harder to detect. Both larvae and nymphs can be infected with Lyme disease bacteria (*B. burgdorferi*) and other tick-borne pathogens when they feed on infected white-footed mice, chipmunks, or certain species of birds (www.cdc.gov/ticks/life_cycle_and_hosts). By a few weeks, nymphs molt into male or female ticks. Adult ticks prefer to feed on medium to large mammals, such as deer and other large mammals, when they usually mate, and after several weeks, they lay eggs and eventually die. Figure 3 shows the life-cycle of tick *Ixodes scapularis*.

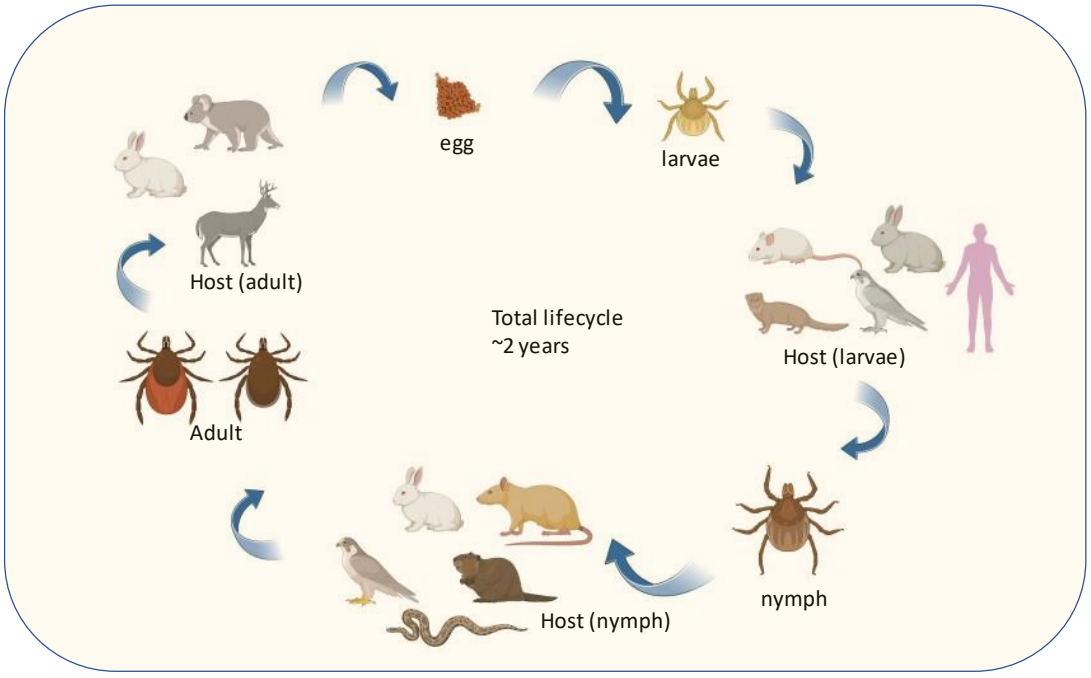

Figure 3: Lifecycle of *Ixodes scapularis* tick.

Like most vector-host relationships, the contact between I. scapularis ticks and hosts is mediated by many conditions. Those could be abiotic parameters like temperature, humidity, day length, etc. The rest depends on biotic parameters such as biological processes within the tick and

the host and interactions between the two (Ostfeld *et al*., 2015). Tick mouthpart is one of the most exciting fields of study, and it has three visible components: the two outside jointed pulps, which are highly mobile and protect the center rod-shaped structure, the hypostome. The rough hypostome has many projections and plunges into the host's skin while feeding. Ixodid tick feed for long periods of time, from a couple of days to weeks (www.cdc.gov/ticks/life_cycle). The cuticle of hard ticks can accommodate a large volume of blood ingested, and in adult ticks, may be up to 100 times their unfed body weight. The study of atypical feeding and development of the Ixodid tick would provide us with critical insight into vector biology.

I. scapularis ticks belongs to subphyla Chelicerata (includes ticks and mites) and Mandibulata (includes insects) shared a common ancestor 543–526 million years ago (De *et al*., 2023). It possesses some unique characteristics such as unusual feeding behavior, sucking large volumes of blood meals (100 times the body weight), long-term survival during the off-host period with limited metabolic quiescence, widespread geographical distribution, being less impacted by seasonal fluctuations, etc. (Herrman *et al.*, 2015). These unique characteristics of ticks were evolved to combat the hostile environment and offered ticks the survival advantage over other arthropods to develop as living fossils. How tick adapted to persist in nature with 2-4 years of life span but only with three blood meals is the point of research interest for many years, and the answer is probably implied in the molecular mechanism of tick hematophagy and blood meal digestion and storage (Simo Ladislav *et al.*, 2017). The primary focus of this chapter will be to highlight significant groups of development-associated transcription factors in *I. scapularis*.

Tick genome

While genomes of most tick species are yet to be developed, such resources for some dominant tick species across the Asian continent are currently available and include high-quality genomes of *Ixodes persulcatus, Hyalomma asiaticum, Rhipicephalus sanguineus, Rhipicephalus microplus, Haemaphysalis longicornis* and *Dermacentor silvarum*. The whole genome sequence of these ticks was compared in a recent study (Jia *et al*., 2020). Most of these six species are three-host ticks except *Rhipicephalus microplus*. The study (Jia *et al*., 2020) analyzed the genomes of six dominant tick genera and 684 species to highlight the genomic variations among tick species. First, they generated DNA libraries with high sequencing depth, prepared short fragment libraries, and sequenced them with Illumina HiSeq X-Ten. It was used to estimate the genome size of each specific tick species. Combining the homology-based approaches, the repetitive elements identified were 52.6% – 64.4% among these three tick species. The transcriptome-based homology search showed 25,718 – 29,857 protein-coding genes in these tick genomes. The average length of genes among these six tick species also varied considerably: the smallest is *H. longicornis,* which is 6466 bp, and the largest is *I. persulcatus* which is 15,067 bp. The authors also compared the chromosome size, gene content, and repetitive elements among those six tick genomes. *D. silvarum* has the largest genome size, and the *I. persulcatus* has the lowest. Among the tick genome, the GC content remained the same. This species of tick (*I. persulcatus)* shows high genetic divergence and low conserved synteny. The evolutionary distance was measured among these tick species and *I. scapularis*. The maximum likelihood tree was constructed based on the coding sequence among these species, and 464 orthologues were compared. The two Ixodid spp. (*I. scapularis* and *I. persulcatus*) shared common ancestor 200 million years ago with the other tick species. The other tick species clustered together from a

common ancestor about 137.8 mya. This also supports the ancient origin and a divergent evolutionary tree of ticks.

The first annotated *I. scapularis* tick genome comprises 1.8 Gbps. 570,640 contigs in 369,495 scaffolds (Gulia-Nuss *et al*., 2016). The annotation of 18,385 scaffolds represents 1.2 Gbps, 57% of the genome with predicted 20, 486 genes. The Ixodid tick usually contains haploid genomes; the repetitive DNA is around seventy percent in this tick species (Gulia-Nuss *et al*., 2016). This is due to a high degree of transposable elements and tandem repeats. The study also describes the genome of the black-legged tick possessing 13 autosomes and 2 sex chromosomes. The study also described the presence of tandem repeat in centromeric or peri-centromeric regions, which is also present in *Ixodes scapularis* and comprises 40% of the genomic DNA. Around sixty percent of tick genes have orthologues in other arthropods, and almost half of them are maintained by the major lineage of arthropods. The study also described around fifty percent of the genes have homologs, and one-fifth of tick genes are unique. There are approximately 22% of paralogous genes are present. Within the last 40 million years, two duplication events have occurred in *I. scapularis* containing hundreds of genes, consistent with the ticks worldwide. The tick mitochondrial genome indicates it as an ancestral arthropod by its phylogenetic analysis.

A recent publication (De *et al.,* 2023) aims to establish a more accurate complete genome containing high molecular weight DNA isolated from a single tick combining PacBio low input DNA-sequencing libraries. The single female tick was used in the sequencing, and the PacBio single-molecule HiFi sequencing platform was used. A total of 3096 ng of HMW DNA was isolated from a single female and used for sequencing. The result generated ~41-fold PacBio HiFi data, including 40.2 Gb of low input and 56.0 Gb of ULI data. Multiple HiFi genome assemblers were used to build a more comprehensive genome, and one that showed the best assembly based

on quality was used. Finally, four additional HMW DNA libraries were generated, and an SMRT-sequencing was performed to developed a fully scaffolded genome. To annotate comprehensive tick transcriptome, Iso-seq was used, an RNA-sequencing technology from PacBio where RNA was isolated from a single tick and used for the genome study. The Iso-seq data identified 142,057 isoforms and protein-coding sequences with complete open reading frames (ORFs). The sequencing also identified multigene families like immune pathway genes. Around 75 immunity genes were annotated; 62 were involved in tick immune response. The RAD-seq analysis was also performed on *I. scapularis* ticks which was collected from various endemic regions. Discriminant analysis of principal components (DAPC) also showed that the ticks are genetically diverse. Next, the single nuclear polymorphism was performed from ticks with more excellent loci, and the annotation data is publicly available as NCBI *Ixodes scapularis* Annotation Release 103.

Tick development

The molecular basis of tick development remains largely unknown. Following successful mating and engorgement, the female tick detaches from the host. Oviposition takes place within 14 days, and the eggs are released from the female tick and are coated with wax. It protects the eggs from desiccation and helps in the egg mass formation. After a month, the eggs embryonate, and hatching occurs afterward. The transparent larvae can become sclerotized within two weeks and quest on the host. Figure 4 shows the tree major stages of tick-life cycle.

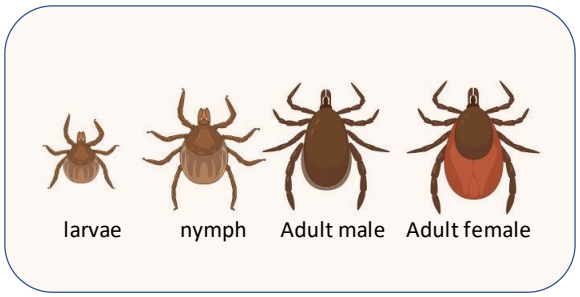

Figure 4: Stages of tick-lifecycle.

The tick larvae usually feed for four days, engorge, fall off, and molt to nymph, which takes around a month. Nymphs feed for four to six days, and they drop off from the host and eventually molt to adult male or female tick. The whole process may require 4-6 weeks. After maturation of around two weeks, the sclerotin forms. At this stage, the adult tick can find its host and feed.

Introduction to transcription factors

In all metazoan organisms, specific cellular functions depend on the differential expression of genes, which occurs primarily at the transcriptional level, controlled by complicated regulatory networks of transcription factors (Rhee *et al*., 2014). A diverse group of transcription factors has been studied in the fruit fly *Drosophila* for decades. However, our knowledge about the development-related transcription factors in vectors that transmit human diseases, particularly in ticks, remains under-explored. Transcription factors bind to a specific site(s) in target DNA, unique interactions with other transcription factors, co-factors, and proteins, and regulate gene expression central to the biological responses.

As there is very limited information about molecular biology of ticks, we choose to focus on transcription factors to understand how these proteins drive major physiological or

developmental episodes in ticks. The wealth of knowledge gathered in model arthropods, primarily covering the development-associated transcription factors, might give some insight into the complex regulatory networks controlled by these molecular effectors, which ultimately enrich our knowledge of molecular and developmental biology of these understudied disease vectors. The primary goal of this chapter is to present a general overview of tick transcription factors. As gathered from the Flybase database (https://flybase.org), the significant groups of transcription factors that are involved in the development were listed in the table, and their orthologs in the tick genome were identified by blasting the protein sequence against the *I. scapularis* genome database. Special attention was given to the groups of transcription factors that are expressed highly during tick feeding, and known to be involved in gut biology and development. A better understanding of *I. scapularis* transcription factor-like genes during feeding can contribute to developing new therapeutics that interfere with the feeding and persistence of this Arthropod in nature.

The high-quality *I. scapularis* genome sequence information is now available in major publicly available databases. The *I. scapularis* genome information was initially searched at NCBI (www.ncbi.gov) and VectorBase database (www.VectorBase.org). Additionally, the supporting literature for identifying and comparing additional transcription factors, including those discovered in the mosquito, fruit fly and mammalian genomes, were also reviewed. Further information was used to sort out potential *Ixodes* orthologs via BLASTP in the NCBI and VectorBase databases. In addition, the potential transcription factors known to be highly involved in the development of organisms through metazoan evolution were also included in the list. In total, 124 genes were articulated and categorized into one of the following eight major groups: **basic domain transcription factors**, **glial cell missing transcription factors**, **forkhead box transcription factors**, **immunoglobin-fold transcription factors**, **MAD homology domain**

transcription factors, **CBF domain transcription factors**, **zn-finger transcription factors**, and **homeobox transcription factors**. Although this list may not include all transcription factors related to published data, there could be potential unidentified transcription factors like genes involved in tick development. A group of transcription factors that fall into this category but are less likely to be associated with growth weren't selected for further studies. Moreover, additional published data may have been inadvertently overlooked. But it still represents a portion of the genes that are highly involved in development. In the following sections, these groups of development-associated transcription factors are discussed briefly regarding their presence in ticks. Their potential impacts on the development of organisms will also be discussed.

Basic domain transcription factors

The protein structure of basic domain transcription factors contains a basic domain in its structural motif (Duek *et al.*, 2005; Feller *et al.*, 2011). The basic motif of a characteristic basic domain transcription factor consists of a cluster of positively charged amino acids, for example, arginine and lysine residues. The positively charged motif of the basic domain transcription factor interacts and binds with the negatively charged DNA phosphate backbone. The basic domain plays an important role in transcriptional regulation and DNA binding. The specific sequence of DNA is recognized and bound by transcription factor response elements within the promoter or enhancer regions of several regulatory genes. The bound transcription factor recruits other co-factors and proteins to regulate the transcription of many target genes.

Several examples of putative basic domain transcription factors include the basic leucine zipper family, the basic helix-loop-helix family, and some homeodomain families. Each of these families of transcription factors has a characteristic basic domain and structural features that

pertain to their target DNA binding properties and functions. Basic domain transcription factors maintain a range of biological phenomena like differentiation, development, cellular signaling, and immune responses. They play crucial roles in regulating gene expression in response to internal or external signals. Studying basic domain transcription factors can help uncover the molecular mechanism that controls numerous biological functions. One of the major groups of basic domain transcription factor bHLH superfamily. It was named on the conserved helix-loop-helix domain. This family of transcription factors is very diverse and present in plants and animals (Duek *et al*., 2005; Feller *et al*., 2011). It contains approximately sixty conserved amino acids, a basic region, and one helix-loop-helix region (HLH region). The basic region plays a role in DNA binding to their target genes; the helix-loop-helix region plays a role in dimerization to change the expression of target genes involved in different signaling pathways. Based on their DNA binding abilities and phylogenetic relationship, the members of basic helix-loop-helix in eukaryotes are classified into six major groups named A, B, C, D, E, and F (Atchley *et al*., 1997). They are known to be involved in diverse functions in Arthropods like in *Drosophila melanogester*. For instance, achaete is a basic helix-loop-helix transcription factor that plays a role in neural precursor formation. It is also a major antagonist in notch signaling that plays roles in the nervous system's development. Asense gets expressed in CNS neuroblasts and sensory organ precursors, also involved in notch-mediated lateral inhibition. Amos plays a major role in dendritic neuron formation. The feedback loop of gene expression is maintained by clockwork orange (cwo), which participates in both activation and repression of transcription. Deadpan (dpn) is a significant regulator of neuroblasts development and a major target in Notch signaling. The list of representative transcription factors of the basic domain is listed in Table 1. Figure 5 shows *Ixodes* the transcription factor hes1.

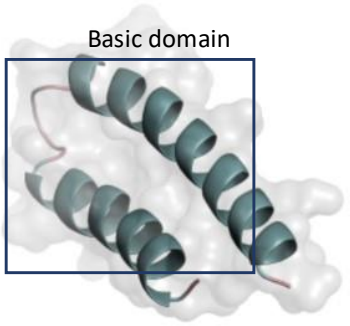

Figure 5: The structure of basic domain transcription factor hes1 (ISCW016537) in *I. scapularis*. The boxed region indicates the representative basic domain. The structure (and others, as presented in subsequent figures (Fig. 6-12), was generated by using I-TASSER software and the structure was visualized through Pymol.

Table 1: The list of representative Basic domain transcription factors identified in the tick genome databases.

Annotation	**Accession no**	**Ortholog annotation in model organism *Drosophila***
Transcription factor hes-1, putative	ISCW016537	Deadpan
Hypoxia-inducible factor 1 alpha, putative	ISCW012137	Dysfusion
DNA-binding protein inhibitor, putative	ISCW014470	Extra macrochaetae
Dhand, putative	ISCW009436	Hand
Uncharacterized protein	ISCW021820	Knot
Pancreas-specific transcription factor 1A, ptfa, putative	ISCW003737	48 related 1
N-twist protein, putative	ISCW024442	48 related 2
Transcription factor hey, putative	ISCW011251	Hairy/E(spl)-related with YRPW motif
Musculin, putative	ISCW019706	HLH54F
Helixloop-helix DNA-binding domain-containing protein	ISCW008805	Methoprene-tolerant
Microphthalmia-associated transcription factor, putative	ISCW011796	Mitf
Max binding protein mnt, putative	ISCW007968	Mnt
Myogenic factor 6, putative	ISCW009440	Nautilus
Predicted protein	ISCW011969	Net

BZIP protein, putative	ISCW019206	Cap-n-collar
BZIP transcription factor domain containing protein	ISCW019219	cryptocephal
Uncharacterized protein	ISCW008037	Cyclic-AMP response element binding protein A
Uncharacterized protein	ISCW001920	Achaete
Transcription factor, putative	ISCW003667	Absent MD neurons and olfactory sensilla
Uncharacterized protein	ISCW009120	Asense
Uncharacterized protein	ISCW004855	Clockwork orange

Forkhead box transcription factors

Forkhead box (FOX) are transcription factors that play a putative role in biological processes like development, immunity, and metabolism (Jackson *et al*., 2010). The term "forkhead box" came from the DNA binding domain called forkhead box or "winged helix" domain. The forkhead box domain contains a DNA-binding region of approximately a hundred amino acids that form a characteristic three-dimensional winged helix structure. The FOX transcription factors bind to target DNA sequences through the forkhead domain, which is termed the forkhead binding site, which is present in the regulatory regions of the genes. Aside from the forkhead domain, each FOX family transcription factor contains additional domains that contribute to their interactions and functions with other proteins. Some well-characterized FOX transcription factors include FOXO, FOXC, FOXP, etc. This group of transcription factors is known to be involved in both activation and repression of target genes. Regulation of a wide array of target genes influences several cellular processes such as apoptosis, metabolism, cell cycle regulation, and development of various tissues and organs.

FOX transcription factor has some implications in some diseases. For instance, mutations of FOX genes cause developmental, cancer, metabolic, and autoimmune diseases. FOXO transcription factors have been known to be linked with diabetes, cancer progression, or aging. FOX transcription factors must be studied in detail since they can provide insight into the

regulatory networks that control different cellular processes. The precise control of FOX target gene expression is maintained by cellular crosstalk among other regulatory molecules, signaling pathways, or transcription factors. So, further study of molecular mechanisms and functions of FOX transcription factors is critical to decipher cellular homeostasis, development, or disease. Around fifty and forty-four genes are encoded by the forkhead box family of transcription factors in humans and mice, respectively (Jackson *et al*., 2010). The forkhead domain consists of three α-helices and β-sheets, which are highly conserved, and the structure variability occurs between the second and third helices. The transcription factor interacts with DNA through its third α-helix (Lehmann *et al*., 2003). The motif that this group of transcription factors recognizes are around 15-17 bp (Carlsson *et al*., 2002).

Forkhead box transcription factors have numerous roles in various signal transduction pathways. FoxH1 has been known for its putative function in mesoderm specification (Watanabe and Whitman, 1999). It binds to the activin response element in the promoter of homeobox gene Mix2, which is meso-endodermal in origin (Chen *et al*., 1996). In the presence of activin, a complex formed by foxH1, Smad2, and Smad4 locates on DNA, and the DNA transcription is activated (Yeo *et al*., 1999). The LIN-31 (Miller *et al*., 1993, 2000) interacts with receptor tyrosine RAS/MAPK signaling cascade (Tan *et al*., 1998). The epidermal growth factor-related gene lin-3 also plays a role in carrying out the signal. When MAP-kinase gets phosphorylated, the interaction between Lin31/Lin1 gets disrupted (Tan *et al*., 1998). FoxO subfamily of forkhead box transcription factors are targets of insulin-like growth factor receptors. They control the target gene regulation, which regulates metabolism or apoptosis. The role of forkhead box transcription factors in metabolism was first delineated in *C. elegans*. The worm gene daf-2 encodes insulin receptor homolog that shifts metabolism toward fat storage and increases the life-span (Kimura *et al*.,

1997). The list of representative forkhead domain transcription factors are listed in Table 2. Figure 6 shows the representative forkhead domain transcription factor in *Ixodes scapularis*.

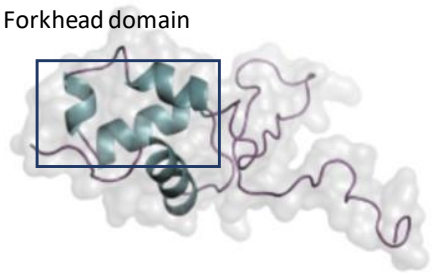

Figure 6: The structure of putative forkhead domain transcription factor (ISCW016268) in *I. scapularis*. The boxed region indicates the representative forkhead domain.

Table 2: The list of representative Forkhead domain transcription factors identified in the tick genome databases.

Annotation	**Accession no**	**Ortholog annotation in model organism *Drosophila***
Forkhead domain-containing protein, putative	ISCW016268	Crocodile
Transcription factor, putative	ISCW006922	Fork head
Zinc finger protein, putative	ISCW015581	Forkhead box, sub-group O
Forkhead domain-containing protein, putative	ISCW008179	Jumeau
Transcription factor, putative	ISCW019820	Sloppy paired 1
Forkhead domain protein, putative	ISCW015937	Forkhead domain 59A
Transcription factor, putative	ISCW010527	Forkhead domain 96Ca
Forkhead domain protein, putative	ISCW003951	Forkhead domain 102C
Forkhead domain protein, putative	ISCW023474	Forkhead box P
Forkhead domain protein, putative	ISCW004287	Checkpoint suppressor 1-like

Homeobox transcription factors

Homeobox transcription factors are known for their essential roles in the development and differentiation of cells (Favier *et al*., 1997; Prince *et al*., 2002). They contain a DNA-binding domain called homeodomain, around sixty amino acids, and a helix-turn helix structural motif.

They control the activation or repression of target genes by binding to their target DNA sequences, and the binding site of the DNA is termed as homeobox binding site.

This large group of transcription factors involves various cellular processes during development, including organ formation, body axis patterning, and cell differentiation. They maintain the fate and identity of cells and tissues in a spatio-temporal manner. The HOX gene family is a well-known homeobox transcription factor. This group of homeobox transcription factors is well-known for their roles in positional identity specification during embryonic development. In addition, dysregulation or mutation of this gene can result in severe developmental defects. Homeobox transcription factors have also been known for their roles in tissue homeostasis, stem cell maintenance, tissue regeneration, etc. The activation or repression of target genes of homeodomain transcription factors is maintained by their functional domains that have specific function and their interactions with other transcription factors. Other than the homeodomain, they may contain protein-protein interaction domains, gene activation or repression domains, etc. Studying this group of transcription factors can provide important insights into the molecular mechanisms of development.

Homeobox transcription factors are critical in maintaining or differentiating human trophoblasts (Quinn *et al*., 1997). The homeobox genes were discovered in the model organism *Drosophila melanogaster*, where they retain morphogenesis during embryonic development at the transcriptional level (Favier *et al*., 1997; Prince *et al*., 2002). Homeobox transcription factors contain a conserved homeodomain and 180 base pairs in length. The homeobox transcription factor's DNA binding region is termed a helix-turn-helix motif. Based on the specificity of the binding of homeodomain transcription factors, it helps activate or repress its downstream target molecules (Levine *et al*., 1988). It has also been known that homeobox

transcription factors are involved in several cancers or developmental defects (Nunes *et al*., 2003). The knockout mice have shown flaws in morphogenesis (Gorski *et al*., 2003; Myers *et al*., 2002). POU domain transcription factor is one of the groups of homeobox transcription factors that control developmental gene expression (Herr *et al*., 1988). These groups of transcription factors have a portion of similar sequences in the DNA binding domain, termed the POU domain. POU stands for mammalian transcription factor Pit-1, Oct 1 and 2, and nematode unc -86 (Herr *et al*., 1988). Around twenty genes have been characterized in vertebrates, and around four genes have been identified in *Drosophila melanogaster* (Stein *et al*., 1996).

The POU domain consists of two significant subdomains: one is the N-terminal POU-specific domain (POUs), and the other is the C-terminal homeodomain (POUh) (Herr *et al*., 1988; Strum *et al.*, 1988). As described earlier, POUH is a classic homeodomain first identified in *Drosophila*'s homeotic gene products (Stein *et al*., 1996). These two domains are crucial for sequence-specific DNA binding (Strum *et al.*, 1988). Both subdomains are considered functional units (Herr *et al.*, 1995). Though structural analyses, it has been known that the POUS and POUH are connected by a linker region and structurally independent (Dekker *et al*., 1993; Assa-Munt *et al*., 1993). The list of representative homeobox domain transcription factors is listed in Table 3. Figure 7 shows the representative homeobox domain transcription factor in *Ixodes scapularis*.

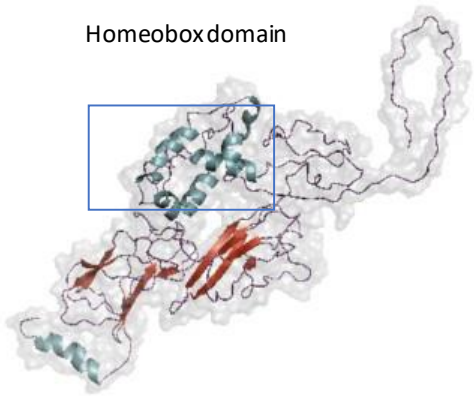

Figure 7: The structure of putative homeobox domain transcription factor (ISCW022240) in *I. scapularis*. The boxed region indicates the representative homeobox domain.

Table 3: The list of representative homeobox transcription factors identified in the tick genome databases.

Annotation	**Accession no**	**Ortholog annotation in model organism *Drosophila***
POU domain protein, putative	ISCW007997	Abnormal chemosensory jump 6
POU 2, putative	ISCW022240	**Nubbin**
N-Oct 3 octamer DNA (ATGCAAAT) binding protein, putative	ISCW020718	**Nubbin**
Pou domain transcription factor, putative	ISCW017821	POU domain motif 3
Cut domain containing protein	ISCW022880	Cut
Uncharacterized transcription factor like protein	ISCW015706	Defective proventriculus
Homeobox protein, putative	ISCW021054	Abdominal A
Homeobox protein Hox-C9 putative	ISCW021042	Abdominal B
Antennapedia, putative	ISCW021060	Antennapedia
Uncharacterized protein	ISCW015706	Defective proventriculus
ONECUT DNA binding protein, putative	ISCW015625	Onecut
Homeobox protein Hox-A4, putative	ISCW021080	Deformed
Homeobox domain containing protein putative	ISCW001025	Even skipped
Homeobox protein gsh, putative	ISCW008920	Intermediate neuroblasts defective
Proboscipedia, putative	ISCW021086	Proboscipedia
Vax1 transcription factor, putative	ISCW008247	Rough
Fushi Tarazu, putative	ISCW021072	Sex combs reduced
Ultrathorax, putative	ISCW021058	Ultrabithorax
Arrowhead, putative	ISCW011461	Apterous
Lim homeobox protein, putative	ISCW023842	LIM homeobox transcription factor 1 alpha
Lhx3, putative	ISCW004213	LIM homeobox 1

Insulin protein enhancer isl, putative	ISCW021829	Tailup
Nk homeobox protein, putative	ISCW014989	bagpipe
Homeobox protein, putative	ISCW018939	BarH1
Brain-specific homeobox protein, putative	ISCW005966	Brain-specific homeobox
Homeobox protein dbx, putative	ISCW020093	Dbx
Homeobox protein distal-less DLX, putative	ISCW020504	Distal-less
Nk-6 homeobox protein, putative	ISCW014430	HGTX
Engrailed, putative	ISCW022797	Invected
Uncharacterized protein	ISCW021484	ladybird early
Retinal homeobox protein, putative	ISCW015819	aristaless
Homeobox protein arx, putative	ISCW021207	Dorsal root ganglia homeobox
Uncharacterized protein	ISCW016210	Ods-site homeobox
Retinal homeobox protein, putative	ISCW014108	Retinal homeobox
Restna and anterior neural fold homeobox protein, putative	ISCW009634	Visual system homeobox 1
Transcription factor, putative	ISCW015893	Zn finger homeodomain 1
Transcription factor Pax6, putative	ISCW003096	Pox meso
Zn finger protein, putative	ISCW010367	Bric a brac 1

Immunoglobin fold transcription factors

Some transcription factors contain immunoglobulin-like fold in their protein structure, similar to the immunoglobin domain found in antibodies. Spi-B is an example of an immunoglobin-like fold transcription factor that is expressed in B cells and plays a role in the immune response, development, and differentiation of B cells (Sasaki *et al*., 2012). Early B cell factor (EBF) proteins are another example of transcription factors involved in developing B cells (Boller *et al.*, 2014). This group of transcription factors has an immunoglobin-like DNA binding domain termed the EBF domain, which regulates the expression of the genes involved in B cell differentiation and lineages. Ikaros family zinc-finger 1 contains multiple zinc-finger domains and immunoglobin-like DNA-binding domains, which are involved in immune cell development and function (Fan *et al.*, 2016).

Among several Rel transcription factors characterized in *Drosophila* are Relish, Dorsal, and Dif. The protein contains approximately three hundred amino acids conserved in the Rel homology domain in the fruit fly. Overall, the Rel proteins show significant variability around fifty percent (Dushay *et al.*, 1996; Ip *et al*., 1993). The Rel homology domain plays a vital role in DNA binding, dimerization, and interaction with the IκB proteins. Cactus has been defined as a putative *Drosophila* IκB inhibitor with six ankyrin repeats. It also establishes interactions with Dorsal and Dif in the cytoplasm (Geisler *et al*., 1992; Kidd, 1992).

Relish is a transcription factor that contains the ankyrin repeats in the C-terminal region. The structural organization of mammalian, vertebrate, and invertebrates is similar, but there are high degrees of variability (Huguet *et al.*, 1997). In vertebrates, the Rel proteins control fetal liver development, neuronal cell functions, immune cell development, etc. (Gerondakis *et al*., 1999). In *Drosophila* , Rel proteins maintain several biological roles such as dorsoventral polarity (Belvin and Anderson, 1996; Drier and Steward, 1997), muscle development (Halfon and Keshishian, 1998) or hematopoiesis (Qiu *et al*., 1998).

CSL consists of three structural and functional domains, each similar to some known structures. The N-terminal domain (NTD) is identical to an RHR-N domain, and the C-terminal domain (CTD) resembles an IPT/TIG domain (RHR-C). Surprisingly, the central domain is a beta-trefoil domain. The unusual Rel domain occurs to place the beta trefoil domain (BTD), and it is placed in the juncture between RHR-N and RHR-C. The list of representative immunoglobin fold transcription factors are listed in Table 4. Figure 8 shows the representative Immunoglobin-fold domain transcription factor SuH in *Ixodes scapularis*.

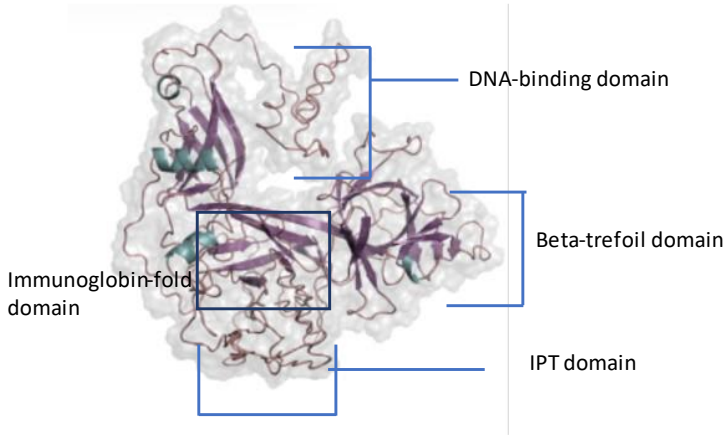

Figure 8: The structure of putative Immunoglobin-fold domain transcription factor (ISCW010197) in *I. scapularis*. The boxed region indicates the representative immunoglobin-fold domain.

Table 4: The list of representative immunoglobin-fold transcription factors identified in the tick genome databases.

Annotation	**Accession no**	**Ortholog annotation in model organism *Drosophila***
Embryonic polarity dorsal, putative	ISCW000140	Dorsal-related immunity factor
Uncharacterized transcription factor like protein	ISCW004493	lozenge
T-box transcription factor, putative	ISCW019283	Bifid
T-box transcription factor, putative	ISCW024150	Brachyenteron
Recombining binding protein, suppressor of hairless	ISCW010197	**Suppressor of Hairless (SuH)**

MAD homology domain transcription factors

The Mad homology (MH) domain transcription factors are essential for Transforming the growth factor-beta signaling pathway (Padgett *et al*., 1999; Raftery *et al*., 1999). These groups of transcription factors are known as Mad family proteins or SMAD proteins. SMAD1 is involved in the BMP signaling pathway, SMAD2, and SMAD3 are associated with TGF-beta and Activin

signaling, and SMAD4 is also involved in signal transduction of the TGF-beta family. The typical Mad homology1 (MH1) domain is present in the N-terminus of the transcription factors. It usually interacts with the DNA sequence known as SMAD binding elements. The SMAD proteins also contain the C-terminus MH2 domain involved in protein-protein interactions. It also interacts with SMAD4 and forms a heteromeric complex. The complex translocates in the nucleus and regulates the expression of the genes involved in TGF-beta signaling.

Both the MH domains of the transcription factors contribute to transduce signals through SMAD proteins where the transcription of target genes is regulated. This transcription factor group is involved in activating and repressing target genes. They are known to regulate tissue development, apoptosis, cell proliferation, etc. The differentiation and development of cells in a wide range of organisms such as flies, worms, mice, or humans are regulated by transforming growth factor beta (TGFβ). TGFβ activates the cell surface receptor serine-threonine kinases, and the signal is carried out through the intracellular signal transducer Smads. The smads complex gets activated by ligand stimulation and translocates to the nucleus, activating or repressing transcription with other transcription factors. Smads contains two conserved domains: Mad homology domains 1 and 2, connected through a linker region. The activity of the complex is mediated by protein-DNA or protein-protein interactions. In mice, smad mutation hinders embryonic development or causes tumorigenesis (Attisano *et al*., 2001).

The Smad family members, Mad and Smad, were first articulated in *Drosophila* and *Caenorhabditis elegans* model organisms. (Padgett *et al*., 1999; Raftery *et al*., 1999). Proteins with similar functions were later identified in vertebrates such as mice, rats, or humans. Smads have been divided into three subfamilies: the inhibitory Smads, the receptor-regulated Smads, and the common Smads; their distinct role in signaling justifies their

specification (Attisano *et al*., 2001). The representative members of this signaling show a high degree of amino acid sequence similarities. The human Smad1 and *Drosophila* Mad are 82% identical in amino acid sequence.

In *Drosophila*, Mad encodes transcription factors that generate cellular response through BMP signaling. It forms a complex with Med and moves to the nucleus, exerting its effect by controlling BMP target gene expression. Med encodes a transcriptional regulator that binds to mad or smox to mediate signal transduction for the activin or dpp ligands. The family of SMAD transcription factors are involved in different cellular processes. Understanding the intricate signaling pathway regulated by this group of transcription factors is essential to understanding various pathological and physiological processes. The list of representative MAD homology domain transcription factors is listed in Table 5. Figure 9 shows the representative MAD-homology domain transcription factor in *Ixodes scapularis*.

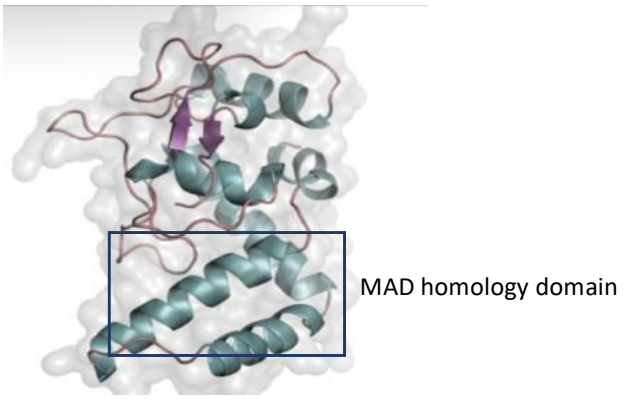

Figure 9: The structure of putative MAD-homology domain transcription factor (ISCW004440) in *I. scapularis*.

The boxed region indicates the representative MAD homology domain.

Table 5: The list of representative MAD homology domain transcription factors identified in the tick genome databases.

Annotation	Accession no	Ortholog annotation in model organism *Drosophila*
Mothers against decapentaplegic homolog	ISCW010869	Mothers against dpp
Uncharacterized protein	ISCW007667	Medea
Nuclear factor I, putative	ISCW004440	Nuclear factor I

The core binding factor (CBF) domain transcription factors

The core binding factor domain is found in certain transcription factors, also called the runt domain first identified in the RUNX (Runt-related transcription factor) family transcription factors. The heterodimeric complex is formed between the CBF-beta subunit, leading to transcriptional regulation and DNA binding. There are three members in the RUNX family transcription factors in mammals, which are RUNX1, RUNX2, and RUNX3. The CBF domain contains approximately 128 amino acids, which assemble a three-dimensional structure that binds the DNA or interacts with other transcription factors.

The DNA binding region of these transcription factors is termed the core binding elements (CBE), and the DNA sequence recognized by the core binding element is termed the runt-binding site. They regulate the expression of target genes by recruiting chromatin-modifying enzymes and transcriptional co-regulators. The RunX transcription factors are crucial in immune response, hematopoiesis, skeletal development, etc. Specifically, RUNX1 differentiates various blood cell lineages, develops hematopoietic stem cells, etc. In mammals, RUNX2 is involved in the regulation of gene expression those are involved in bone formation. RUNX3 is known to be involved in developing the immune and nervous system. In fruit fly *Drosophila,* Lozenge (Lz), and *Caenorhabditis elegans,* LIN-29 also share sequence homology in their DNA binding domain with RUNX family transcription factors.

CBF domain-containing transcription factor is a heterotrimeric transcription factor that binds to the consensus sequence in many eukaryotic target genes. The CBF-B subunit contains two transcription activation domains with glutamine-rich and serine-threonine-rich regions. In the CBF-C subunit, it includes a rich region. CBF is also known as the CCAAT motif binding factor. The primary functions of Nf-YA and Nf-YB transcription factors are thorax and eye development. The NF-Y complex is formed by three distinct subunits, NF-YA, NF-YB, and NF-YC, which bind to the consensus sequence of the DNA (Mantovani, 1999). Among all these three subunits, NF-YB and NF-YC are required for DNA binding. The c-terminal region of NF-YA is also necessary for DNA binding (Mantovani, 1999). The NF-YB and NF-YC form a heterodimer that eventually interacts with NF-YA, and a heterotrimeric NF-Y is created containing all three subunits (Mantovani, 1999). The target sequence CCAAT motif is present in the promoter regions of several genes, especially those involved in regulating cell cycle such as E2F1, cyclin B1, CDC2 etc (Hu et al., 2002; Kao et al., 1999). It has also been known to regulate gamma-globulin genes and Sox-family genes (Fang et al., 2004). The activity of this group of transcription factors has been found in almost all mammalian cells and tissues. The NF-YA knockout mouse dies during embryonic development (Bhattacharya et al., 2003). The orthologues of NF-YA among *Drosophila* and human shows 82% sequence similarity in their DNA binding region, which is known to be conserved among organisms (Yoshioka *et al*., 2007). The interactions and roles of CBF domain transcription factors can provide insight into the intricate regulatory networks that control differentiation and development. The list of representative CBF domain transcription factors is listed in Table 6. Figure 10 shows the representative CBF domain transcription factor in *Ixodes scapularis*.

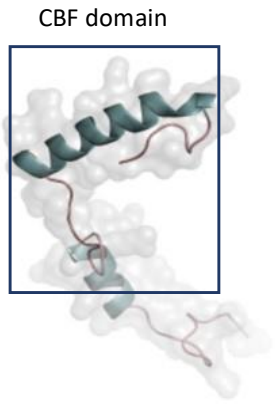

Figure 10: The structure of putative CBF domain transcription factor (ISCW017122) in *I. scapularis*. The boxed region indicates the representative CBF domain.

Table 6: The list of representative CBF domain transcription factors identified in the tick genome databases.

Annotation	**Accession no**	**Ortholog annotation in model organism *Drosophila***
Transcription factor nf-Y alpha, putative	ISCW017122	Nuclear factor Y-box A
Ccaat-binding transcription factor subunit A, putative	ISCW020078	Nuclear factor Y-box B

Zn-finger transcription factors

Zn-finger transcription factors are a large group of proteins that contain one or more zn-finger motifs that bind to the DNA sequences. The motif is formed by a Zn-ion coordinated by histidine and cysteine residues and thus creates a finger-like structure. Among different zinc finger transcription factors, cys2His2 is the most common, where two cysteine and histidine residues coordinate a zinc-ion. They are involved in various biological processes, such as development, immunity, and disease.

One of the families under the Zn-finger transcription factor includes Kruppel-like factors, which control an array of cellular responses such as development cell cycle control. They have also varied functions in different cell and tissue types. GATA transcription factor also falls

in the category of Zn-finger transcription factor, which contains two zinc finger motifs and is involved in development. Another putative group of zn finger transcription factors known is zinc finger E-box binding homeobox transcription factors, which contain multiple zinc finger motifs and interact with E-box DNA.

GATA transcription factors contain Zn-finger domains in all family members and bind to the consensus sequence (A/T)GATA(A/G). One Zn-finger is required for the consensus sequence binding and recognition (Yang and Evans, 1992), and the other Zn-finger stabilizes the interaction established by the other or interacts with other proteins or co-factors (Tsang *et al.*, 1997; Wilkinson-White *et al*., 2015). The N and C terminal regions vary significantly among different GATA factors (Morrisey *et al*., 1997). There are six members in the GATA transcription factor superfamily in vertebrates, and among them, GATA 3 has the highest sequence similarity with both GATA orthologs and paralogs (Trembley *et al*., 2018).

For instance, In *Drosophila*, *Asciz* is involved in somatic chromosome segregation, motor functions, maintenance of cell cycle, etc. *Blimp*-1 plays a significant role in trachea formation and metamorphosis. *Bowl* is important for cell fate specification. The transcription factor GATA are evolutionarily conserved among plant, fungi, and animals. In mammals, some GATA factors have hematopoietic (GATA 1,2,3) and cardiac (GATA 4,5,6) origins; their expression and function are not limited to these tissues. Some GATA transcription factors (GATA 2 and 3) are crucial for the central nervous system, skin, prostate, or kidney development (Grote *et al*., 2008; Kaufman *et al*., 2003). In addition, some GATA transcription factors (GATA 4,5,6) are required to develop the pancreas, lung, or liver (Molkentin *et al*., 2000). Overall, GATA transcription factors have some potential roles in various developing systems. Their role in cell- or tissue-specific manner is very important for organogenesis and embryonic development. The

list of representative Zn-finger domain transcription factors is listed in Table 7. Figure 11 shows the representative Zn-finger domain transcription factor in *Ixodes scapularis*.

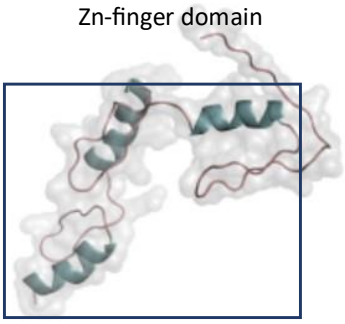

Figure 11: The structure of putative Zn-finger domain transcription factor (ISCW023685) in *I. scapularis*. The boxed region indicates the representative Zn-finger domain.

Table 7: The list of representative Zn-finger domain transcription factors identified in the tick genome databases.

Annotation	**Accession no**	**Ortholog annotation in model organism *Drosophila***
Zinc finger, c2h2 type domain-containing protein	ISCW006409	ASCIZ zinc finger protein
Uncharacterized protein	ISCW023811	Blimp-1
Brother of odd with entrails limited	ISCW017921	Protein krueppel, putative
Zinc finger protein, putative	ISCW023685	Buttonhead
Uncharacterized protein	ISCW009730	Castor
GATA binding factor-1B, putative	ISCW005453	Grain
Uncharacterized protein	ISCW022767	Buttonhead
Zinc finger protein, putative	ISCW017895	Chorion factor 2
Zinc finger, c2h2 type domain containing protein	ISCW019333	Charlatan
Uncharacterized protein	ISCW005491	cubitus interruptus
Zn-finger protein, putative	ISCW015582	Chromatin-linked adaptor for MSL proteins

Zn-finger protein, putative	ISCW010823	Crooked legs
Transcriptional repressor CTCF, putative	ISCW012049	CTCF
Zn-finger protein, putative	ISCW016993	Dendritic arbor reduction 1
Zinc finger, c2h2 type domain containing protein	ISCW018372	Disconnected
Uncharacterized protein	ISCW015526	DZIP1
Zinc finger, C2H2 type, putative	ISCW016275	Earmuff
Zn finger protein, putative	ISCW012727	Escargot
Glass/che-1, putative	ISCW013678	Glass
Zinc finger, c2h2 type domain containing protein	ISCW010781	Hamlet
Hunchback protein, putative	ISCW021385	Hunchback
Zinc finger, C2H2 type, putative	ISCW000054	Huckebein
Zinc finger protein, putative	ISCW019865	jim
Zinc finger protein, putative	ISCW021309	Kahuli
Zinc finger protein, putative	ISCW005617	Ken and barbie
Zinc finger protein, putative	ISCW010690	Kruppel-like factor 15
Zinc finger protein, putative	ISCW010362	Klumpfuss
Zinc finger, c2h2 type domain containing protein	ISCW003496	Lobe
Salivary protein is3, putative	ISCW021698	Lame duck
Zinc finger, C2H2 type, putative	ISCW016356	Longitudinals lacking
Zinc finger protein, putative	ISCW016993	Luna
Uncharacterized protein	ISCW015122	Ouija board
Ras-responsive element binding protein, putative	ISCW009132	Pebbled
Zinc finger protein, putative	ISCW011786	Phaser
Zinc finger protein, putative	ISCW024807	PR/SET domain 13
Zinc finger protein, putative	ISCW009191	Spalt major
Senseless, putative	ISCW001542	Schnurri
Zn-finger protein	ISCW004992	Stripe 2
Zinc finger, C2H2 type, putative	ISCW010121	Stripe

Zinc finger protein, putative	ISCW001569	Suppressor of Hairy wing
Transcription factor, putative	ISCW015893	Zn finger homeodomain 1
Uncharacterized protein	ISCW017915	Zn finger homeodomain 2
Uncharacterized protein	ISCW016368	Tramtrack
Zinc finger protein, putative	ISCW003495	U-shaped

Glial cell missing transcription factors

The nervous system's non-neural cells, termed glial cells, play a crucial role in the function and development of the nervous system. This group of transcription factors was initially discovered in the fruit fly *Drosophila melanogaster,* which is a key regulator of the differentiation of glial cells in neurons. The gene gcm (glial cell missing) encodes the GCM transcription factor in fruit flies. Mutations of the gene lead to a reduction or loss of glial cells, which results in defects in the nervous system development. This transcription factor binds to specific DNA sequences in the promoter regions of the target genes and promotes their expression. The glial cell missing transcription factor has been found in other vertebrates like mice and humans. In the central nervous system, glial cells are involved in developing and differentiating by regulating GCM TFs. They have significant roles in dividing and forming various neural cell types, such as astrocytes, Schwann cells and oligodendrocytes. In humans, the mutation in the GCM2 gene, a glial cell missing transcription factor, causes autosomal recessive congenital hypoparathyroidism (ARHP), a rare genetic disease. It results in an impaired development of the parathyroid glands or hypoparathyroidism.

This group of transcription factors interacts with other transcription factors, epigenetic regulators, or signaling molecules to convey the molecular signal that drives glial cell differentiation, development, or functions in the nervous system. Studying the GCM TFs and their

target genes is essential for understanding the molecular mechanism of glial cell development and operation. It can provide further insights into the interplay between neuronal and glial cells in the nervous system and contribute to our knowledge of neurodegenerative diseases and disorders that involve glial dysfunctions.

The gene gcm encodes for preliminary regulators of glial cell specification in *Drosophila* (Hosoya *et al*., 1995; Jones *et al*., 1995). The gene is expressed in glial cells except for mesectoderm-specific glia. When gcm is highly expressed, neurons turn to glia or vice-versa (Jones *et al*., 2001), which indicates its dual function. The transcription factor gcm is a DNA-binding protein that binds to the octameric DNA sequence found in the regulatory regions of its target genes (Akiyama *et al*., 1996; Schreiber *et al*., 1997). There are two homologs in mammals: Gcm1/GCMa and Gcm2/GCMb (Akiyama *et al*., 1996; Altshuller *et al*., 1996), both shares conserved 153 amino-acid N-terminal region and C-terminus activation domain. Overall, gcm proteins are transcriptional activators that bind to the specific sequence of the DNA. Gcm1 in mice plays a role in the development of trophoblasts, and gcm2 pertains to parathyroid gland development (Schreiber *et al*., 2000; Gunther *et al*., 2000). Gcm1 does get expressed at a low level in the central nervous system, but its role is vital since gcm mutant mice die in early developmental stages due to placental failure. Gcm proteins act as transcriptional regulators of diverse tissue types. In addition, gcm is required in hematopoiesis, which is a master regulator of the development of specific blood cell types (Lebestky *et al*., 2000). Hence, the glial cell development is partially but not entirely dependent on gcm expression. It isn't known if other factors are involved in gcm development. The list of representative glial cell missing transcription factors is listed in Table 8. Figure 12 shows the representative glial cell missing domain transcription factor in *Ixodes scapularis*.

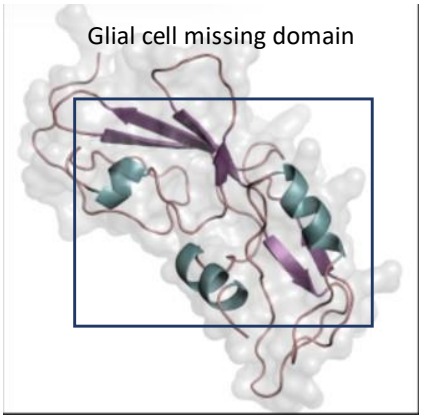

Figure 12: The structure of putative cell missing Glial domain (ISCW017122) in *I. scapularis*. The boxed region indicates the representative glial cell missing domain.

Table 8: The list of representative glial cell missing transcription factors identified in the tick genome databases.

Annotation	Accession no	Ortholog annotation in model organism *Drosophila*
GCM motif protein	ISCW022914	Glial cell missing

Concluding Remarks

The black-legged tick *I. scapularis* can transmit several bacterial, viral, or protozoan diseases, although the molecular basis of their biology and development remains largely unexplored yet important for developing novel anti-tick vaccines or therapeutics. Several studies have explored the transcription factor-mediated development in model organisms, which we seek to address for *I. scapularis* ticks. This study provides important insight into molecular mechanisms of development-associated pathways in ticks, that are regulated by representative transcription factors. With the availability of genomic sequences, a wealth of knowledge in the field of

transcription factors is emerging. Our ultimate goal is to analyze transcription factors in *I. scapularis* ticks to understand better the molecular biology of dynamic blood meal acquisition and development. Several pathways are described in metazoans that execute multiple roles in organism's immunity and development (Belvin *et al*, 1996; Drier and Steward, 1997; Marc and Keshishian, 1998, De Gregorio *et al* 2002; Bray, 2006; Contreras-Cornejo *et al*, 2016). Ticks, compared to other blood-sucking arthropods, have a vast and primitive yet highly adaptable genome encoding an array of transcription factors that potentially plays a critical role in the development and digestion of a huge blood meal, that drive their postembryonic development.

In the following chapters we detail our efforts to elucidate the role of selected key transcription factors in various aspects of the tick life cycle, primarily focusing on tick feeding and subsequent intermolt development. In our study, we used a major vector species prevalent in northeastern U.S. - the *I. scapularis* tick - and a well-established animal model (C3H mice as mammalian host). The knockdowns of the desired gene were achieved through RNA interference method developed two decades ago (Pal *et al*., 2004), performed via microinjection of target dsRNA, and the phenotype of the target transcription factors (Nub or SuH) knockdown ticks would be compared with control (dsGFP-injected ticks). Furthermore, we also incorporated established techniques and tools that are standard in our lab and core facilities, such as qPCR, confocal microscopy, scanning electron microscopy, western blotting, far Western blotting, Co-immuno-precipitation, LC-Mass Spectrometry, RNA-sequencing, Electrophoretic mobility shift assay (EMSA) amongst other standard techniques to address our objectives.

For selection of transcription factors analyzed in our current study, annotation of the transcription factors among different groups in databases (NCBI and VectorBase), based on their functions, was identified through their known involvement in regulatory networks, expression

patterns, and interactions with other proteins. Furthermore, the additional information from peer-reviewed journals covering development-associated transcription factors discovered and delineated in the mosquito, fruit fly, and mammalian genome were also reviewed. The gathered information was used to locate probable orthologs in deer ticks via BLASTP against the VectorBase database (www.vectorbase.org). As a whole, 124 genes were identified as potential transcription factors in *I. scapularis* and categorized into eight major groups, that are further elaborated in the next chapter. As presented in the subsequent chapters, the transcriptomic and proteomic analysis also assisted us to identify potential *I. scapularis* gene-products that are associated with a selected set of transcription factors. These studies are expected to provide evidence-based knowledge of tick biology, development, and vectorial competence, which can contribute to the development of novel preventions against ticks and tick-borne diseases.

Chapter 2: Selection and Characterization of Transcription Factor SuH

Introduction

As emphasized in the general introduction (Chapter 1), ticks are considered as prolific arthropod vectors that transmit many bacterial, viral, or protozoan disease agents. As one example, Lyme borreliosis is a tick-borne illness that occurs worldwide and is caused by the pathogen *B. burgdorferi* sensu lato. Two disparate groups of hosts maintain the pathogen in nature: a vertebrate host and an arthropod vector, for example, *Ixodes scapularis,* or the deer tick in northeastern United States. The spirochete transmission can occur when the bacterium is deposited in the host's skin when the vector takes a bloodmeal. From there, the bacterium disseminates to various internal organs, including the joints, heart, and central nervous system. Humans are considered incidental hosts; if infected and untreated, the subjects can show severe clinical symptoms, such as a variety of neurological illnesses, Lyme arthritis, or carditis (Radolf *et al.*, 2012). However, despite public awareness about ticks as a major disease vector, there is limited evidence of their molecular biology, including how transcriptional regulation of developmental genes pertains to their survival in nature. Notably, tick genome encodes numerous components of a range of development-associated signaling and evolutionarily conserved molecules of many development-associated pathways (De *et al,* 2023). Through the evolution of close interactions and co-option of vector physiology, tick-borne pathogens have gained the ability to persist and be transmitted by tick species. In the vector, unique events of rapid cell division and differentiation of gut tissues support the vector's ability to accommodate large amounts of bloodmeal. In addition, ticks can survive through long periods of nutrient deprivation along with extreme environments and limited metabolic functions. It also maintains dynamic postembryonic and reproductive developmental processes. Moreover, subadult ticks have a wide array of host ranges, while adult ticks display

much narrower host selectivity. All these features are likely to be influenced by transcription factors.

In Chapter 1, a catalog of tick development-associated transcription factors was generated by a comprehensive analysis of the available databases, including the newly sequenced tick genome. The annotated tick development-associated genes were initially searched in the NCBI (https://www.ncbi.nlm.nih.gov/) and VectorBase databases (www.VectorBase.org) to perform this analysis. Our goal was to determine the level of expressions of target transcription factors during feeding (different time points during blood meal engorgement in mice) or the molting process. The tick gut has three functional and anatomical divisions: foregut, midgut, and hindgut (Sonenshine and Roe, 2013). The first portion of the tick digestive system is the foregut, which contains the mouthparts and esophagus. The mouthparts function by piercing the host's skin and sucking the blood. Tick hypostome is inserted through the host skin and thus allows them to anchor to the host. The esophagus enables the connection of the mouthparts and the midgut. The central portion of the tick digestive system is the midgut. The midgut is responsible for the digestion and absorption of nutrients from the blood meal. The highly folded structure of the midgut inner surface increases the surface area and facilitates nutrient absorption (Sonenshine and Roe, 2013). The digestive enzymes are also secreted from the midgut, allowing the breakdown of components of bloodmeal. The hindgut is the posterior part of the tick digestive system involved in liquid absorption and waste disposal. The hindgut connects to the anus, where undigested fecal materials are excreted. Ticks have a unique feeding strategy: they feed on host blood, engorge, and finally detach from the host. Further digestion and storage of bloodmeal takes place during the off-host period. They get their essential macro and micronutrients from the blood meal, which promotes their survival, development, and reproduction. Tick gut structure is adapted to efficiently

differentiate to accommodate and store huge amounts of blood meal. The general structure of the gut containing three major parts is consistent among different tick species (Sonenshine and Roe, 2013). Despite substantial knowledge of gut morphology and gross cellular organization, the molecular basis of its physiology and development remains largely unknown. Herein we seek to study the biological significance of selected transcription factors expressed in the gut, beginning with deciphering their expression profiles in course of blood meal acquisition from the host. Figure 13 shows the picture of unfed and fed *Ixodes scapularis* tick.

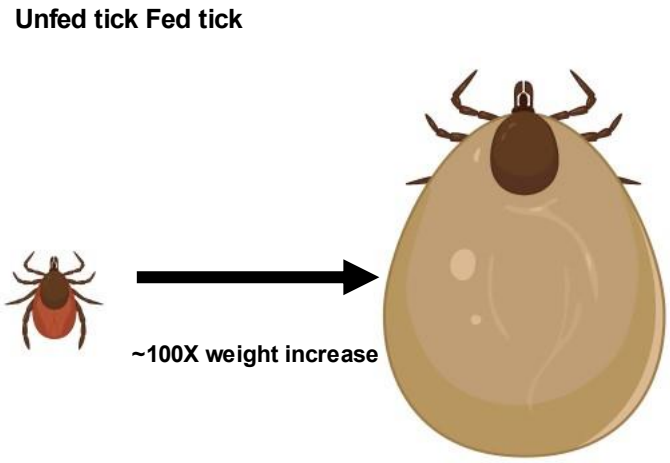

Figure 13: The picture depicts the unfed and fed *Ixodes scapularis* tick. Upon acquisition of blood meal, the tick gut undergoes rapid division and differentiation to accommodate huge amounts of blood meal. It also helps in the storage and survival of the tick in off-host periods.

In metazoans, embryonic development depends on many conserved developmental pathways, such as Notch signaling (Louvi *et al*., 2012). By intensive chromosomal deletion studies, only 26 haplo-insufficient loci associated with developmental phenotypes were discovered in *D. melanogaster* (Cook *et al*., 2012). Among them, only three comprise regions coding for critical factors in the Notch signaling pathway. The transmembrane receptor, Notch, can interact

with one of its two ligands, Delta, and the Notch antagonist Hairless (H). Transcriptional activation or repression of Notch target genes is mediated by the CSL transcription factor [human: CBF1, fly: Su(H), worm: lag-1] and including additional co-activators, notably Mastermind (Artavanis *et al*., 1999; Bray *et al*., 2006; Kopan *et al*., 2009; Kovall *et al*., 2010). Su(H) has pleiotropic biological effects, such as transcriptional regulation, forming corresponding complexes and both activation and repression effects on transcription of Notch target genes (Contreras *et al*., 2016). However, the function of Su(H) has not been identified, which might play a more complex role in highly intricate genomes in ticks. Figure 14 shows gut cell division in model arthropod.

The stem cell characterization in the midgut of adult flies (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006) has revealed several regulatory networks that are conserved between arthropods and vertebrates (Biteau et al., 2011; Jiang & Edgar, 2012). The midgut of the model arthropod *Drosophila*'s epithelium is made up of intestinal stem cells (ISCs), enteroblasts (EBs), absorptive enterocytes (ECs), and enteroendocrine (EE) cells (Fig 14). ISCs divide to self-renew and maintain the stem cell pool, and generate progenitor cells; those acquire either an EC or an EE cell fate. Additionally, intestinal stem cells divide to generate two daughter ISCs or two differentiating cells (O'Brien et al., 2011; Goulas et al., 2012; de Navascues et al., 2012). Intestinal stem cells express the Delta (Dl), which activates Notch (N) in the adjacent enteroblasts to undergo differentiation and determine cell fate decisions. A strong Notch signaling event generates enterocytes, while a weak signal indicates entero-endocrine cell fate (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006, 2007). Hence, Notch's loss of function mutations produce clusters of intestinal stem cell-like cells and EEs caused by the interrupted EB differentiation. In addition, the ectopic expression of Notch in intestinal stem cells results in differentiation mostly toward the enterocyte fate (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006, 2007).

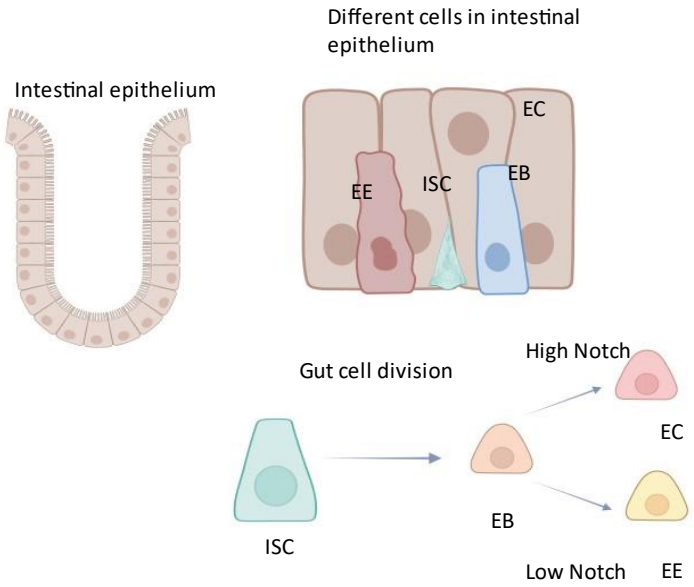

Figure 14: Notch signaling regulate the gut cell division and differentiation.

The differentiation and specification of midgut progenitors during multiple stages of development is highly dependent on Notch signaling. Cell fate decisions during larval or pupal development are also determined by Notch signaling. The larval development is highly dependent upon Notch ligand D1 expression in the central cells of adult midgut progenitors, which in turn activates Notch signaling in the peripheral cells to indicate those as peripheral cells (PCs) (Mathur *et al*., 2010; Takashima *et al*., 2011). These peripheral cells are potentially a niche for adult midgut progenitors (Mathur *et al*., 2010). It has also been proposed that transient pupal midgut epithelium wraps around the larval midgut epithelium during metamorphosis (Takashima *et al*., 2011). These peripheral cells are also predicted to function in the histolysis of the larval midgut epithelium, which is not carried out during metamorphosis. In the adult midgut epithelium, D1 ligand is expressed in low levels. Similarly, low Notch activation plays a role in the expansion of the ISC pools and the later emergence of mature EEs in the midgut epithelium (Takashima *et al*., 2011).

In addition, Notch signaling is the key to differentiating ECs from ISCs (Micchelli *et al*., 2006; Ohlstein *et al*., 2006). At the same time, Dl gets expressed highly in ISCs, but the level of fluctuation is subjected to oscillation. If D1 gets expressed in ISCs, it is assumed to lead to a high level of Notch activation in EBs and promote enteroblasts to differentiate into the ECs. In contrast, low D1 expression has been delineated to induce EE cell fate (Ohlstein *et al*., 2007). Conversely, RNAi-mediated knockdown of *D1* or *Notch* indicates that EEs can be produced without D1/Notch activity. The mechanism behind the change in the *D1* expression in ISCs is not known. However, similar fluctuations in Notch ligand *Dl* expression have also been found in mammalian neurons (Shimojo *et al*., 2008).

Interestingly, absorptive cell fate in gut is conserved between the mammalian intestine and the fly midgut. The impact of Notch signaling on ISC proliferation and renewal is opposite in both systems. In the intestine of mammals, Notch activation indicates the proliferation of intestinal epithelial cells and progenitor cells (Fre *et al*., 2005). However, Notch deactivation inhibits cell proliferation and promotes the formation of goblet cells (Van *et al*., 2005). In contrast, in *Drosophila*, Notch expression in the intestinal stem cells promotes differentiation into mature ECs and suppresses the proliferation of ISCs, leading to stem cell depletion (Micchelli *et al*., 2006; Ohlstein *et al*., 2006). However, in *Drosophila*, loss of Notch causes an increase in Delta-positive ISCs and EE pools and suppresses EC production (Micchelli *et al*., 2006; Ohlstein *et al*., 2006). In other words, Notch activity suppression promotes ISC proliferation in the *Drosophila* midgut. The reason for this duplication and expansion of ISCs might also involve positive feedback regulation by autocrine ligands (such as, Upd, Spi) produced by these cells. In addition, unlike mammals,

Drosophila midgut lacks transient amplifying cells, which explains the different impact of Notch signaling on cell proliferation in the system.

The molecular mechanism of Notch activation is complex. Differential expression of Notch activation in *Drosophila* midgut daughter cells is caused by asymmetric D1 expression (Ohlstein *et al*., 2007; Bardin *et al*., 2010). Both daughter cells express D1 during mitosis, but right after completion of division, only one daughter cell maintains the D1 expression. At the same time, the other one loses Dl expression but activates Notch target genes. The more apically located daughter cells lose Delta expression and turn to enteroblast, while the basally located daughter cells maintain Delta expression and ISC status (Ohlstein *et al.*, 2007). The asymmetrical expression of Dl in both ISCs and EBs is conferred by the inhibition of Dl transcription in the EBs. The exact mechanism for maintaining asymmetric D1 expression in the progenitor cells is unclear, but multiple mechanisms might be involved (Ohlstein *et al.*, 2007; Bardin *et al.*, 2010). In the ISCs, Notch downstream target genes are repressed by the Hairless-Suppress of Hairless [Su(H)] repressor complex; the repression is essential for the stem cell fate (Bardin *et al.*, 2010). In the enteroblast, Notch gets activated and binds to Su(H) and Hairless to suppress the Notch target genes, bHLH transcription factors. Thus, a conserved cascade of transcription factors regulates the differentiation and self-renewal of ISCs in the fly and system. This chapter aims to delineate the

role of SuH transcription factor in *Ixodes scapularis* development. Figure 15 shows the signal transduction via Notch signaling.

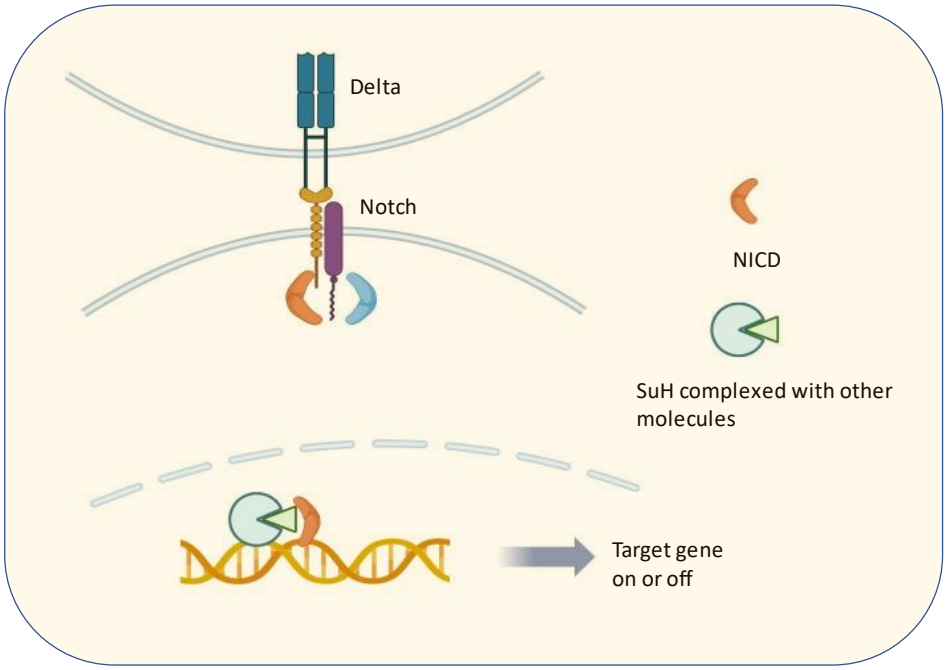

Figure 15: Simplified view of the signal transduction via Notch signaling.

Materials and Methods

B. burgdorferi, mice, and ticks

The bacterial isolate used in this study is *B. burgdorferi*, clone B31-A3 (Elias *et al.*, 2002). C3H/HeN mice aged between four to six weeks were purchased from Charles River. All experiments were performed under the Institutional Animal Care and Use Committee and Institutional Biosafety Committee approval and guidelines. The *I. scapularis* ticks used in the study originated from the colony which is maintained in the laboratory.

Gene expression

Gene expression analysis was performed using published procedures (Rana *et al.*, 2023). The gene-specific primers were designed using SnapGene software, and the annealing temperature was kept around 60 degrees Celsius and the amplified product was about 0.2 Kb. The ticks were reared in the laboratory using published procedures (De *et al*., 2023). Unless noted otherwise, the newly molted nymphs were used in the experiment. Selected internal organs of the tick, such as the gut, salivary gland, and carcass, were dissected and isolated for further analysis. The total RNA was isolated from ticks in each group using TRIzol reagent (Invitrogen). Afterward, the RNA samples were treated with DNase 1 and reverse transcribed to cDNA. The relative gene expression levels were measured by quantitative PCR (qPCR) as detailed (De *et al.*, 2023). Each primer pair was tested for specificity and efficacy through melt curve analysis. The amplification cycle includes initial denaturation at 95^{o} C for 5 min, 45 cycles of 95^{o} C for 10s, 60^{o} C for 20s, and 72^{o} C for 30 sec, followed by a melt curve analysis: 55^{o} C for 30s, increase 0.5^{o} C per cycle to 95^{o} C. The expression was analyzed using SYBR Green Supermix, and the amplification was conducted in an iQ5 or CFX96 Touch real-time thermal cycler (Bio-rad). For screening, the candidate genes were evaluated in 96 well PCR plates, and the wells were duplicated for better accuracy, and negative (no template) control was also added.

Phylogenetic tree

Homologous proteins from humans, mice, fruit fly, mosquitos, and other organisms were compared using BLASTP software. Multiple sequence alignment was performed using T-Coffee or Clustal W2 software. The phylogenetic tree was constructed by Mega 11 software and the result was generated by viewing the distance tree.

RNA interference

The target tick genes were knocked down using RNA interference using published procedures (Kariu *et al.*, 2013; Pal *et al.*, 2004). The total tick cDNA was prepared as detailed above and used as a template to amplify fragments of the target genes. As a control, the fragment of the GFP gene was also amplified using a similar procedure. The primers used to amplify the target DNA sequences are listed in the Table 14 (at the end of the thesis). The dsRNA fragments were synthesized and purified through a commercial RNAi kit (MEGAscript RNAi kit, Ambion, Inc.). Five µl (2 µg/µl) of the target dsRNA was placed into the capillary tubes and eventually microinjected inside the gut of nymphs (30 ticks/group). The injected nymphs were stored overnight in the tick incubator and further used for feeding on naïve mice. 48-hour fed ticks were collected and processed individually for gene silencing using qRT-PCR analysis. Primers that bind the upstream and downstream of the target template cDNA covering the dsRNA sequence were used for qRT-PCR.

Generation of Recombinant Proteins and Antiserum

The target gene was cloned into pET-28a by using specific primers, and the recombinant protein was produced in *Escherichia coli*. Expression and purification of the his-tagged proteins were performed as described (Kariu *et al.*, 2013). To generate polyclonal antiserum, the proteins produced in *E. coli* were emulsified in complete Freund adjuvant and injected into groups of 4 mice (10 µg/animal). The mice were boosted twice every three-week interval with the same antigen dose using incomplete Freund adjuvant. The serum samples were collected two weeks after the second boost. The titer and antiserum's specificity were assessed as described (Kariu *et al.*, 2013).

Co-immunoprecipitation and mass spectrometry

Immunoprecipitation (IP) was performed as detailed by the Pierce Co-Immunoprecipitation Kit (Thermo Scientific). Anti-mouse antibody against target protein was conjugated with Protein G-Sepharose 4B resin (Thermo Fisher Scientific). Precleaned whole tick lysates were incubated with the immobilized target protein for 2 hours and bound proteins were then analyzed by mass spectrometry. The PBS immunized anti-mouse Ab conjugated with Protein G-Sepharose 4B resin binding proteins was used as a negative control. Protein from IP was eluted in two fractions and stored at -80ºC until processed for mass spectrometry (MS). Mass spectrometry processing and protein identification were performed as previously described (De *et al*, 2023).

Confocal immunofluorescent Microscopy

The samples were processed for confocal microscopy using published procedures to localize the target transcription factors in tick organs or cells (Yang *et al*., 2014). The target protein was visualized via primary antibody followed by Alexa 568-labeled anti-mouse secondary antibodies. The specificity of secondary antibodies was checked in samples in the presence of normal mouse IgG (isotype controls), which doesn't generate any non-specific immunofluorescence signals. Tick organs were fixed in 4% paraformaldehyde at 4°C overnight and then permeabilized with acetone for 10 min, followed by three washes with PBS. The samples were blocked with 5% goat serum in PBS-T (PBS with 0.05% Tween 20) for around 2 hours at room temperature and later incubated with primary antibody at 4°C overnight. After three washes with PBS-T, the tissues were incubated overnight with Alexa Fluor secondary antibodies. The nuclei or F-actin were stained with DAPI and actin, respectively (Thermo Fisher Scientific). Later,

it was mounted with an antifade mounting medium (Thermo Fisher Scientific). A 10X dry, 40X water-captured confocal images, or 63X oil objective lens and ZEN imaging software (version 2.6, ZEISS). The microscopic analyses were performed as detailed earlier (Osahor *et al*., 2017). The images of whole ticks were captured using a Canon SX720HS digital camera and the Olympus SZ61 microscope.

Histological analyses

The tick samples were cross-sectioned and were subjected to standard hematoxylin and eosin (H&E) staining (Quentin *et al.*, 2018). The slides were later processed by using a Leica Biosystem anatomic pathology scanner through ImageScope software. All microscopic analyses were conducted in a double-blinded manner. Three microscopic areas from three sections were enumerated for nuclei for quantitative analysis.

Additional bioinformatics and statistical analysis

The protein annotation and blasts were performed using the arthropod database website VectorBase (www.vectorbase.org) and NCBI (https://www.ncbi.nlm.nih.gov). The analyses of protein domains and families were performed using the CDD (http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml) and the Pfam (http://pfam.sanger.ac.uk/) databases. Multiple sequence alignment analysis was conducted by using Clustal W2 or T-coffee software. Results are described as the mean ± standard error of the mean (SEM). The significance level of differences between the control and test groups was analyzed using GraphPad Prism version 8.0 (GraphPad Software). The two-tailed *t*-test or non-parametrical Mann-Whitney tests were used to compare the mean values. The p-value, which is less than 0.05 was considered

significant. One star indicates *p<0.05, two indicate **p<0.01, and three indicate ***p<0.001. Images were generated using a scientific image and illustration Software, BioRender.

Western blots

The immunoblot was performed via the following procedures published in our lab (Kariu *et al.*, 2015). The tick lysate or recombinant protein was electrophoresed using SDS-PAGE, and the protein was transferred via semi-dry transfer to the nitrocellulose membrane. The blocking was performed in PBS with 2% skim milk. Appropriate primary antibody (1:3000) and HRP-labeled anti-mouse secondary antibody (1:10000) were used for detection, respectively.

Results

A sensitive qRT-PCR was performed to compare the level of transcripts of 124 genes in the tick gut to screen out expression of potential genes that play a role in the development of the arthropod vector. The analysis data revealed that around 36 genes show detectable expression when blood meal acquisition started in the tick gut, although except for two, most genes were expressed at extremely low levels. The genes are listed in the table (Table 9), while the expression data is shown in figure 16. The value was normalized against the level of tick beta-actin transcripts. The two groups of transcription factors SuH and Nubbin were highly expressed when feeding starts. Thefore, we opted to focus on these two groups of transcription factors in greater detail.

The SuH protein has three major domains: the lag1 DNA binding superfamily, beta trefoil domain, and IPT superfamily (Figure 17A). This is a conserved protein and there are various orthologues of this protein in different organisms. Alignment of *Ixodes* SuH protein with representative orthologs shows somewhat conserved amino acid sequences or similar domain

structures in other organisms (different species of ticks, mosquitoes, fruit flies, mice, humans, zebrafish, worms etc) although we have detected regions that are unique in *I. scapularis* (Figure 17B). The protein sequence was aligned via ClustalW2 software in different species of ticks, mosquitoes, fruit flies, mice, humans, zebrafish, worms etc. The phylogenetic tree was generated via Mega 11 software (Figure 17C), which suggested that while various tick SuH proteins are clustered together, they indeed reflect evolutionary distances from other arthropods, mice and humans. We next seek to measure the level of SuH expression at different time-points of tick feeding (Figure 18). Ticks were placed on mice, and at each indicated time point of feeding (such as 24, 48, 72 and 96 hours), ticks were forcibly detached from animals and their expression was checked via qRT-PCR.

To understand the importance of SuH in tick biology, we seek to generate knockdown ticks via RNAi, as detailed in the Methods section. Initially, the larvae were knocked down via dsRNA, and the control and the SuH knockdown groups were compared in the next developmental (nymphal) stage. The SuH-knockdown nymphal ticks show severe defects in the development. The ticks were observed for the impact of SuH knockdown (Figure 19A-D). The stored ticks were examined both on the dorsal and ventral side to record the effect of knockdown, which in most cases, were not viable or unable to molt (Figure 19B). A minor percentage of ticks show defects in limb and mouthpart formation (Figure 19D). The quantitative data on molting impacts were shown in Figure 20. The bar graphs show around three percent of ticks were able to molt (Figure 20A). The feeding ticks were examined for knockdown at 48 hrs of feeding in control and knockdown groups (Figure 20B). The significance level was calculated using a two-tailed t-test (p-value<0.05). The level of expression of SuH was also measured at intermolt stages, and the expression level gradually increased from day 5 to day 15 and eventually decreased at day 20

(Figure 20C). SuH-knockdown ticks were not able to molt. To observe any developmental defect in the gut, the ticks were used for histological analysis (Figure 20D). The knockdown group shows severe impairment in the gut histology, as evidenced by presence of potentially undigested blood meal and defect in organ formation, as compared to the control.

Next, the same dsRNA was used to check the impact of SuH knockdown on nymphal ticks (Figure 21 and 22). Similar to larvae, the control dsGFP injected ticks were able to molt but dsSuH knockdown ticks were unable to molt, arresting subsequent development to the adult stage. Images representing the dorsal and ventral sides of the control and knockdown groups are shown (Figure 21A and B). Interestingly, despite these defects in post-feeding stages, the initial weight of fed nymphs (Figure 22A) and their feeding parameters (Figure 22D) were similar in both SuH-knockdown and control groups. However, the fed weight were also measured at day 20 after completion of the feeding which showed significant reduction in their weight in SuH knockdown ticks (Figure 22B); these ticks were also unable to molt (Fig 22C).

To understand the mode of action of SuH, we opted to produce the recombinant protein and use it for subsequent biochemical experiments. The gene SuH was cloned and expressed in pET-28a vector and the recombinant protein was produced in *E. coli* (Figure 23). The polyclonal antibody was generated via injecting the recombinant proteins in mice (Figure 24). We next wanted to analyze the localization of the transcription factor in the tick gut via confocal immunofluorescence microscopy. The image (Figure 25) highlights the localization of the protein showing occurrence of SuH protein around the nucleus of the tick gut. We could not track the protein within nucleus, possibly due to its relative lower abundance, below the limit of detection under the microscope.

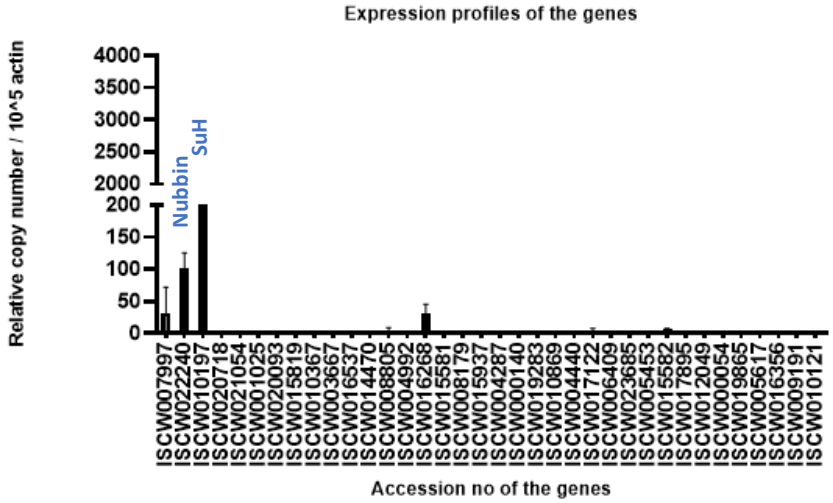

Figure 16: The bar graph showing the expression of transcription factor genes in the tick gut. The tick gut samples were dissected at 48 hours, processed for qRT-PCR, and the value was normalized using tick beta-actin transcripts. The experiment was repeated twice. The two groups, SuH (ISCW010197) and Nubbin (ISCW022240) were highly upregulated in the tick gut. Other thirty-four genes were poorly expressed with high Ct values and were not shown in the graph.

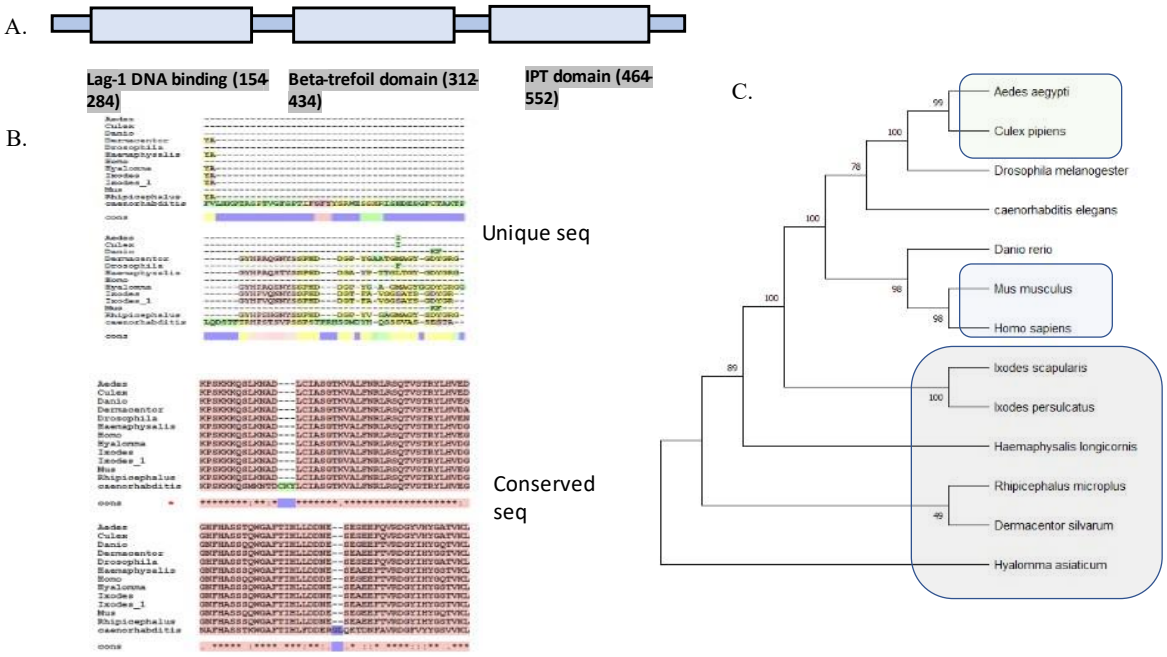

Figure 17: *Ixodes scapularis* SuH protein domains. (A) The SuH protein contains three major domains: lag1 DNA binding superfamily (adopts a beta-sandwich structure with nine strands in two beta sheets and allows DNA binding), beta trefoil domain superfamily (protein fold that consists of six beta hairpins, each formed by two beta strands) and IPT superfamily (Immunoglobulin-like fold, Plexins, Transcription factors). These domains are present in intracellular transcription factors and cell surface receptors such as plexins and scatter factor receptors. (B) Alignment of *Ixodes* SuH protein orthologs in putative well-studied organisms (mosquito, fruit fly, mice, human, zebrafish, and worm). It shows conserved amino acid sequences and similar domains among different organisms. The pictures shown here are representative part of the entire alignments. (C) A phylogenetic tree was generated to show homologous proteins from different species of ticks, mice, humans, mosquitos, *Drosophila* and zebrafish.

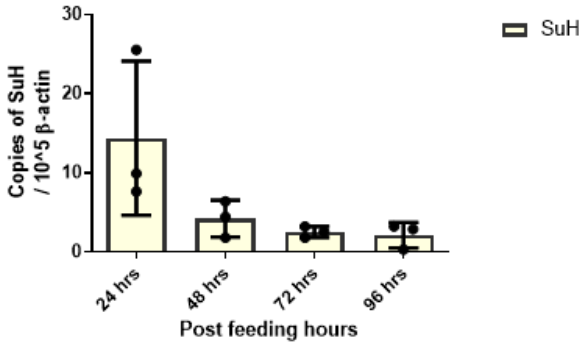

Figure 18: The level of SuH expression at different time points of feeding. Levels of target gene expression at different time points of feeding are shown. Three individual ticks were used to analyze expressions in each time point (24, 48, 72, 96 hrs.).

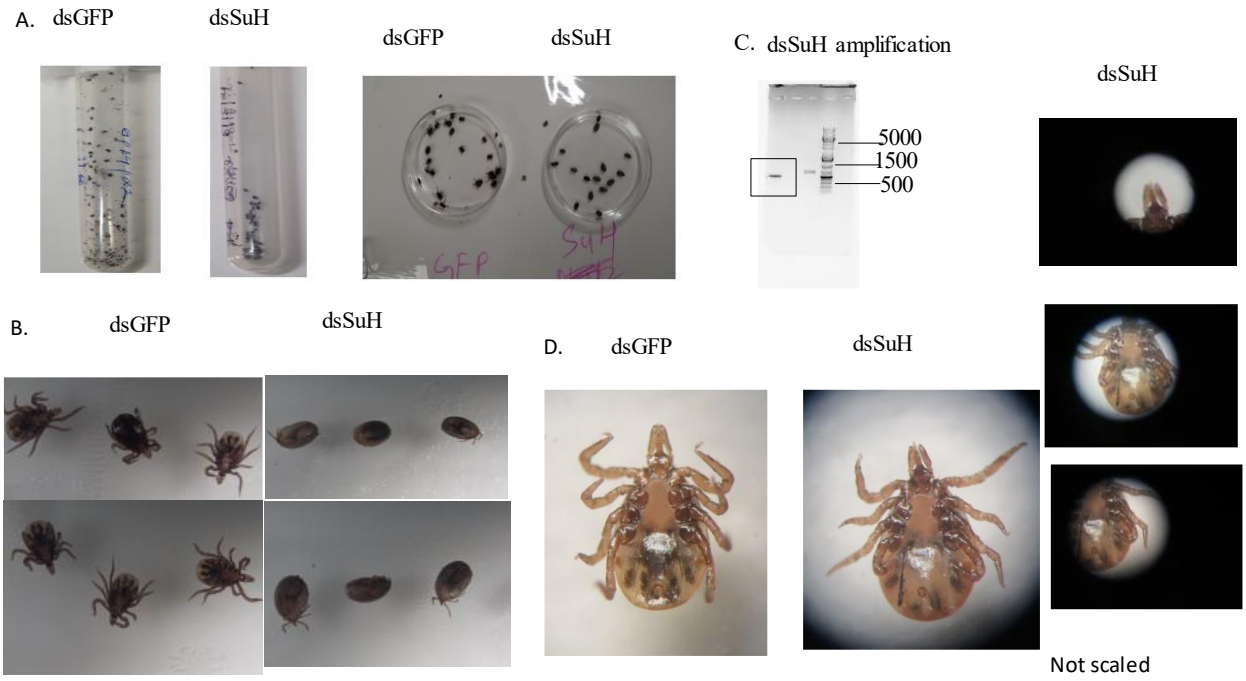

Figure 19: Newly molted nymphal ticks show major phenotypic defects in development. (A) Impact of SuH knockdown on molted dsGFP and dsSuH nymphs. The fully fed larval ticks were microinjected with the dsRNA and stored to observe any knockdown impact. The ticks were examined for major developmental defects. (B) Dorsal and ventral side of molted nymphs. The ticks were not able to molt to the next developmental stage. (C) A gel showing the construction of dsRNA targeting the gene. (D) The picture shows a set of ticks showing impaired development and the formation of defective legs or mouthparts. This data is representative of three individual experiments.

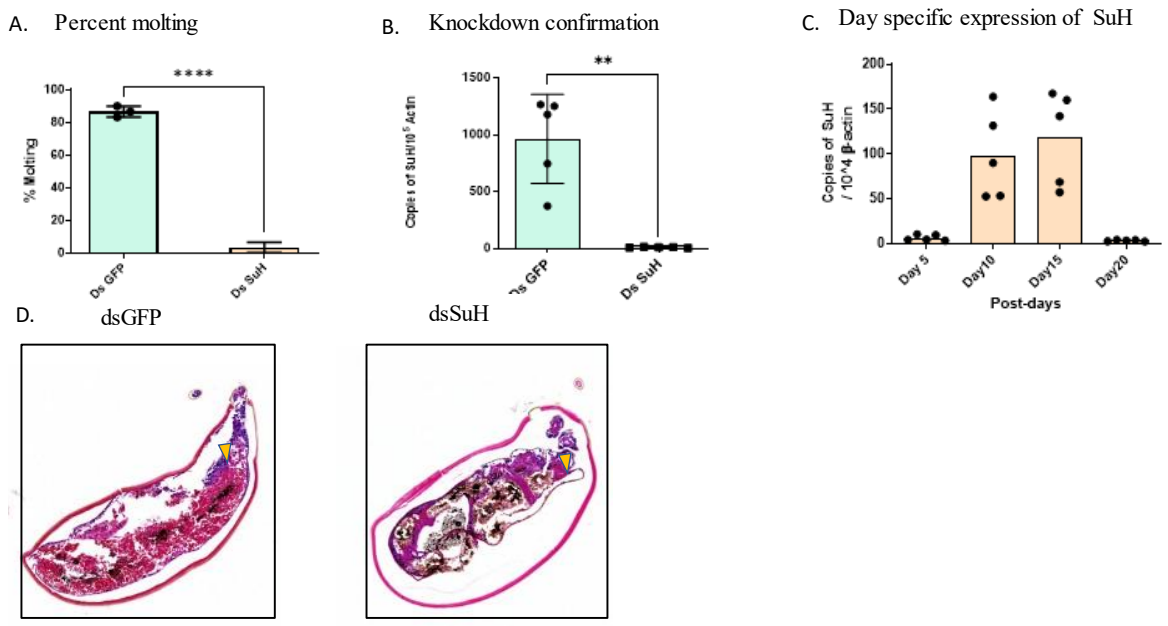

Figure 20: Impact of SuH knockdown on larval-nymphal molt. (A) The panel shows the percentage of molting. Around three percent of SuH knockdown ticks molted. The data represents nymphal ticks that were previously injected in the larval stage. The significance level was calculated using a two-tailed t-test (****p-value<0.001). (B) Confirmation of knockdown at 48 hrs of feeding in dsSuH injected ticks. Five ticks were analyzed for the knockdown confirmation of SuH and compared to control (dsGFP). The significance level was calculated using a two-tailed t-test (**p-value<0.01). (C) The figure represents the expression of SuH, which increases over time and is highly expressed at day 15. As shown on day 20, the level of expression is very low. (D) The intermolt ticks were used for histological analysis (inter-molt day 20). The ticks were cross-sectioned and were subjected to standard hematoxylin and eosin (H&E) staining. The arrow indicates possible undigested blood and necrotic tissues in the gut of the dissected tick.

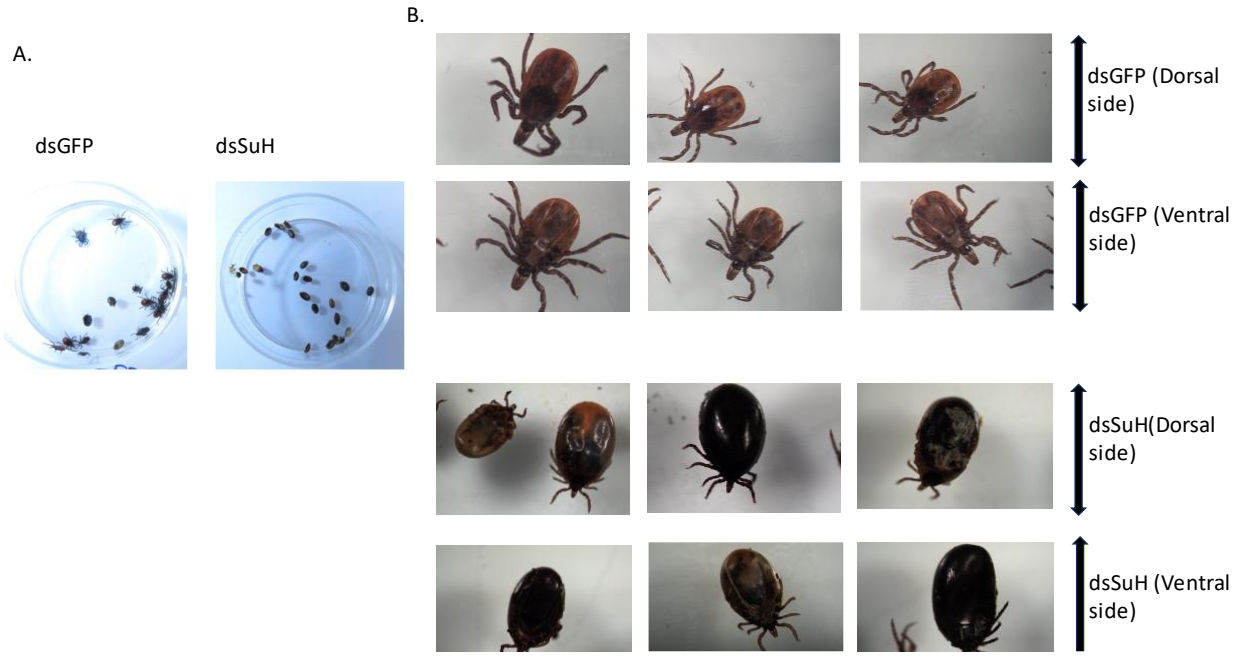

Figure 21: The impact of SuH knockdown on nymphal-adult molt. (A) The image shows representative adult tick groups that were previously microinjected with dsGFP (control) or dsSuH at the nymphal stage. SuH knockdown ticks did not molt to the next developmental stage. (B) The panel represents the image of dsGFP ticks molted to adults while dsSuH nymphs failed to molt to adults. The experiments were repeated three times with similar results.

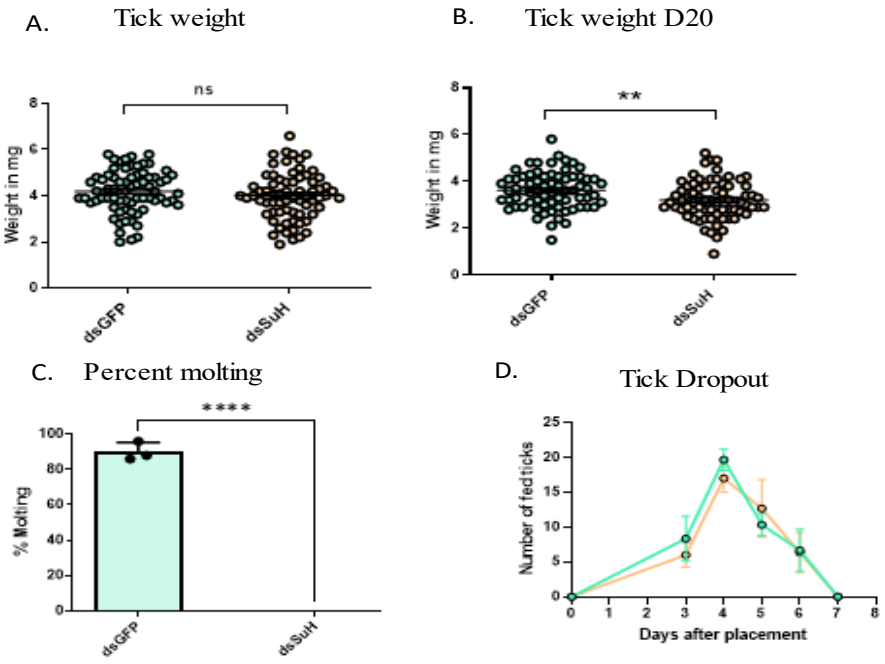

Figure 22: The SuH knockdown nymphal ticks feed completely yet are not able to molt. (A, B) The panels denote fully-fed nymphal weight of post-fed ticks and ones at day 20 (D20) after feeding. The dsGFP-injected ticks were used as a control. The SuH knockdown ticks did not show a significant reduction in their feeding weight as compared to the control. The significance level was calculated using a two-tailed t-test (**p-value<0.01). (C). The SuH knockdown nymphal ticks failed to molt to the next developmental stage. (D) The graph shows the progress of tick feeding. The result is shown as the average of three individual experiments; the control and SuH knockdown groups did not show significant differences in feeding.

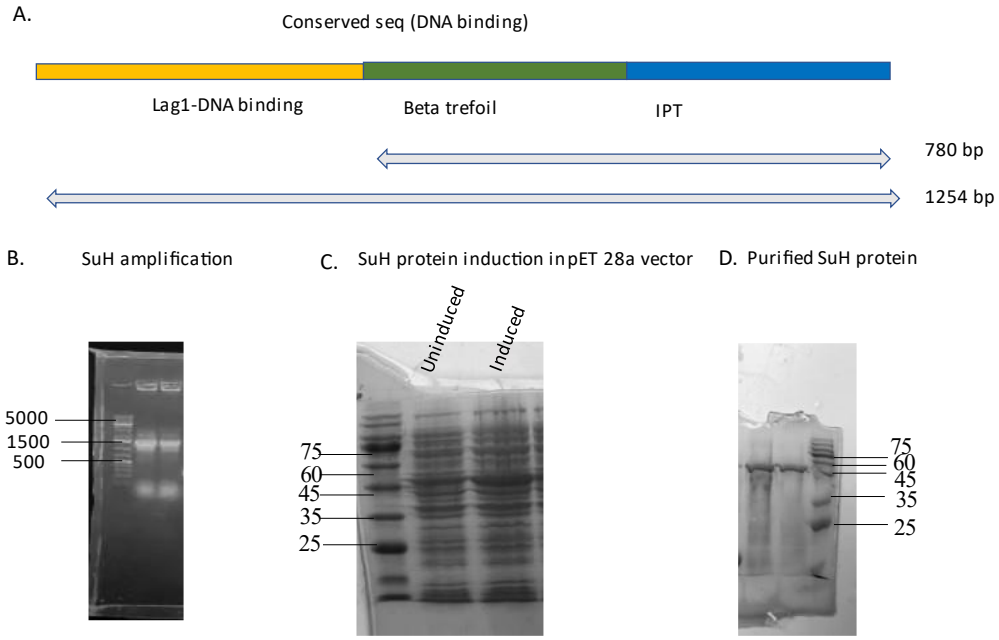

Fig 23: Production of full-length recombinant SuH. (A) The SuH showing different domains. (B, C) The SuH (ISCW010197) gene was PCR-amplified and cloned into pET-28a by using gene-specific primers, and the recombinant protein was produced in *E. coli.* (D) Purified SuH protein. Around 1 microgram of protein was loaded, and the size of the protein is 45 KDa.

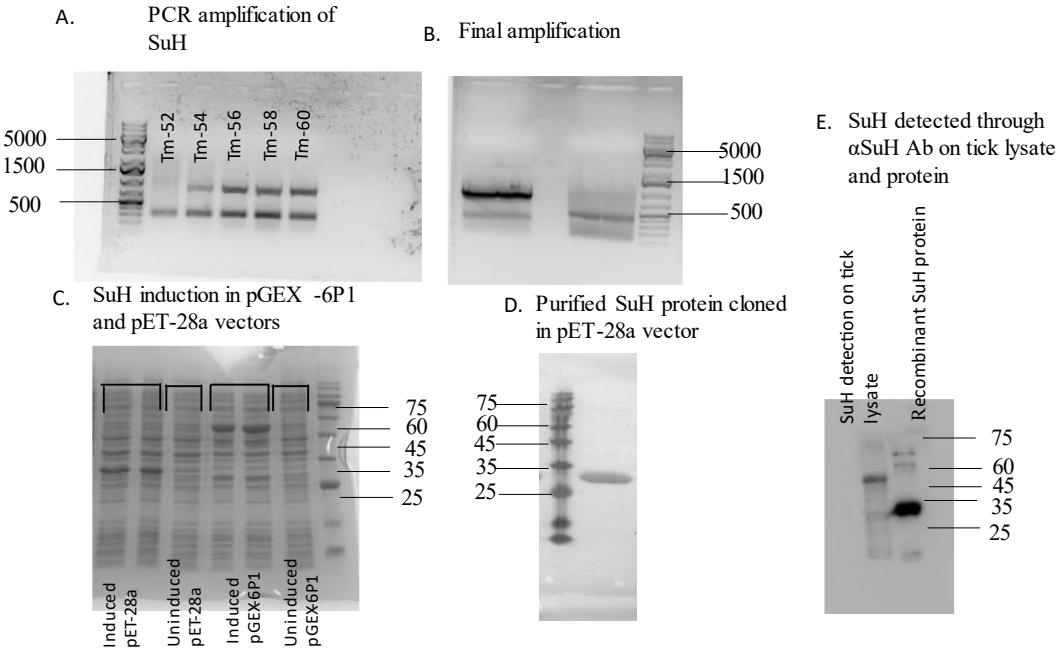

Fig 24: Production of truncated SuH protein and generated antibody. (A, B) A fragment (780 bp) of *SuH* (ISCW010197) *w*as amplified through gradient PCR. (C, D) The SuH fragment was cloned into pGEX-6P1 and pET-28a vectors by using gene-specific primers, and the recombinant protein was produced in *E. coli*. The His-tagged proteins were purified. The size of the protein is around 30 KDa. Around 1 microgram of protein was loaded in the gel. (E) To generate polyclonal antiserum, the proteins were emulsified in complete Freund adjuvant and injected into groups of 4 mice (10 µg/animal). The mice were boosted twice at three-week intervals with the same antigen dose using incomplete Freund adjuvant. The serum samples were collected two weeks after the second boost.

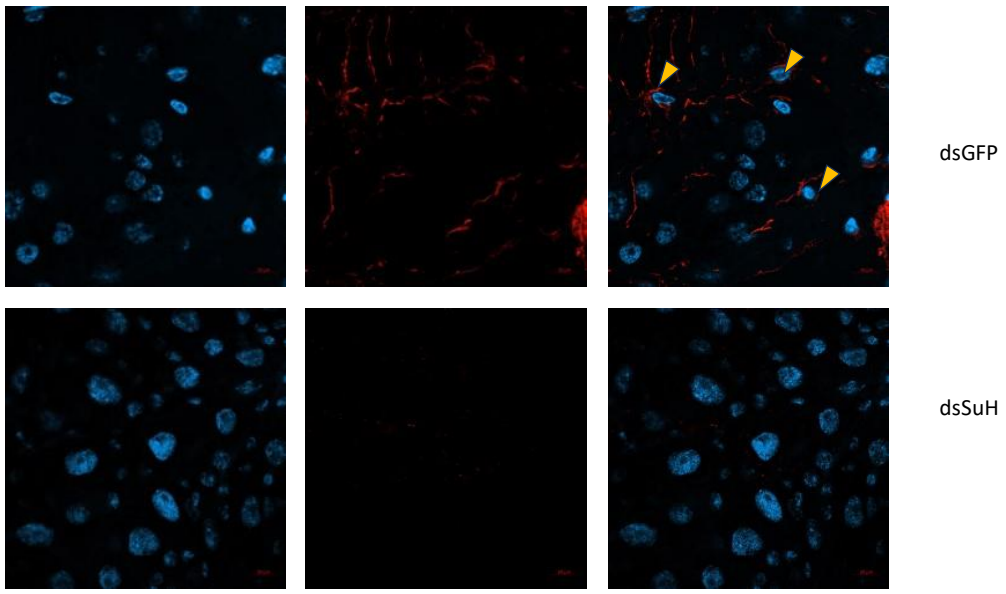

Fig 25: SuH localization in tick gut cells. Nuclei were stained with DAPI. SuH detection in nymphal guts was performed by confocal immunofluorescence microscopy. SuH expression was found mostly around the nucleus and gut epithelial cells. SuH: Red; DAPI: blue.

Table 9: List of TF or related genes expressed in tick gut

Annotation	Accession no.
POU domain protein, putative	ISCW007997
POU 2, putative	ISCW022240
Recombining binding protein suppressor of hairless	ISCW010197
N-Oct 3 octamer DNA binding protein, putative	ISCW020718
Homeobox protein, putative	ISCW021054
Homeobox domain containing protein, putative	ISCW001025
Homeobox protein dbx, putative	ISCW020093
Retinal homeobox protein, putative	ISCW015819
Zn-finger protein, putative	ISCW010367
Transcription factor, putative	ISCW003667
Transcription factor hes-1, putative	ISCW016537
DNA binding protein inhibitor, putative	ISCW014470
Helix-loop-helix DNA binding domain containing protein	ISCW008805
Zn finger protein, putative	ISCW004992
Forkhead domain containing protein	ISCW016268
Zn finger protein, putative	ISCW015581
Forkhead domain containing protein, putative	ISCW008179
Forkhead domain containing protein, putative	ISCW015937
Forkhead domain protein, putative	ISCW004287

Embryonic polarity dorsal, putative	ISCW000140
T-box transcription factor, putative	ISCW019283
Mothers against decapentaplegic homolog	ISCW010869
Nuclear factor I, putative	ISCW004440
Transcription factor Nf-Y alpha, putative	ISCW017122
Zn-finger C2H2 type domain containing protein	ISCW006409
Zn-finger protein, putative	ISCW023685
GATA binding factor 1B, putative	ISCW005453
Zn-finger protein, putative	ISCW015582
Zn-finger protein, putative	ISCW017895
Transcriptional repressor CTCF, putative	ISCW012049
Zn-finger C2H2 type, putative	ISCW000054
Zn-finger protein, putative	ISCW019865
Zn-finger protein, putative	ISCW005617
Zn-finger C2H2 type, putative	ISCW016356
Zn-finger protein, putative	ISCW009191
Zn-finger C2H2 type, putative	ISCW010121

Discussion

The overall goal of this study is to understand biological significance of selected transcription factors, in order to learn more about the molecular basis of tick biology, especially the hematophagy and development. Our hypothesis is that the genes encoding transcription factors that are highly expressed during tick feeding might play an essential role in the gut physiology and the overall development of the organism during subsequent intermolt phases. Though there could be several other gene products that aren't highly expressed during feeding but critical to the development, we focused on selected transcription factors that are highly expressed in feeding tick gut. Based on our literature review, in silico analysis, and expression studies, as detailed in chapter 1, among the 36 expressed transcription factors, we focused on two genes, suppressor of hairless (SuH) and nubbin (Nub), that were observed to be the most dramatically upregulated and hence considered as rational targets that needed further characterization. SuH, which is the focus of the current chapter, is known to play a vital role in development, such as triggering the relevant

pathways like Notch pathway. Although a precise biological function of *I. scapularis* SuH remains unknown, our study highlighted its critical roles in tick postembryonic development. The SuH knockdown ticks showed significant defects in development as they were unable to molt or have some defects in organ formation, suggesting a potential role in regulation of transcription of genes relevant to organogenesis or similar pathways. As the dramatic impact of SuH silencing was observed for both larvae and nymphs, this suggests critical involvement of the protein in indispensable pathways operative in all stages of subadult development. Further research is certainly warranted to understand roles of expressed transcription factors in gut biology and development, such as SuH, which can contribute to identify specific molecular targets for prevention of tick infestation and transmission of tick-borne diseases.

Chapter 3: Role of transcription factor SuH on development associated signaling pathway

Introduction

Arthropods, including *I. scapularis,* have a very high degree of genetic diversity, resulting from complex events of recombination, mutations, and other forms of biological variations (De *et al*., 2023). The rate and speed of these genetic variations is relatively faster in arthropod genomes and can lead to differences in various developmental phenotypes. For instance, differences in the level of expression and regulation of specific genes can influence specific developmental events, including body segmentation and organ formation, which is even more relevant in the biology of blood feeding ectoparasites like *I. scapularis* .

Ticks undergo complex developmental processes that involve numerous signaling pathways (Richards and Degnan 2009). These pathways regulate complex biological processes such as development, molting, reproduction, etc. Several signaling hubs, such as EGF, Notch, Wnt, hedgehog, ecdysone, insulin-like growth factor, and juvenile hormone pathways, are well known for their roles in driving critical processes in metazoan biology (Artavanis-Tsakonas *et al*, 1999; Bray, 2006; Oda *et al.* 2005; Perrimon and Perkins, 1997). In particular, EGF signaling plays a vital role in numerous cellular processes such as development, growth, tissue regeneration, etc (Oda *et al.* 2005; Perrimon and Perkins, 1997). The specific role of this signaling in ticks has not been studied before, however, recent research has highlighted possible importance of EGF signaling in ticks (Koči *et al*, 2021). Figure 26 illustrates simplified view of representative signal transduction pathways in arthropod, possibly operative in Arthropod (Artavanis-Tsakonas *et al*, 1999; Bray, 2006; Oda *et al.* 2005; Perrimon and Perkins, 1997).

Since in studies detailed in our previous chapter demonstrated SuH as a major transcription factor that influences tick postembryonic development, including proper formation of organs, we seek to further examine its mode of action.

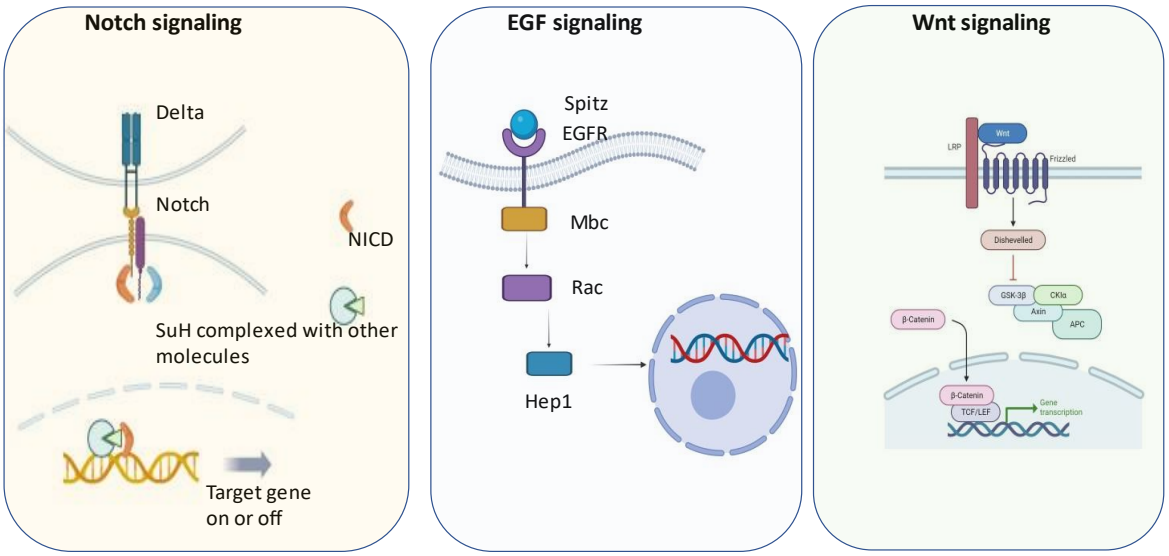

Figure 26: Simplified representation of some major development-associated signaling pathways articulated in Arthropod vector.

Materials and Methods

Enzyme linked immunosorbent assay

The ELISA was performed using routine procedures (Rana *et al*, 2023). Various concentrations of the protein, such as Rac were used to coat the microtiter plates (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 µg/well) in PBS and the plate was incubated overnight and blocked with BSA to saturate non-specific binding sites. The target protein was added subsequently and incubated for 1hr, followed by detection with primary antisera (1:3,000) and secondary anti-mouse HRP-labeled antibody (1:10,000). Later the plate was read at 450 nm wavelength. The result is representative of three individual experiments.

Actin staining

The microscopy was performed using published procedures (Rana *et al*, 2023). The 48-hour fed nymphal gut was dissected and fixed overnight with 4% PFA in PBS. Following washes, the gut was blocked with 5% goat serum in PBS-T (PBS with 0.05% Tween 20) for 2 hours at room temperature, washed with PBS and incubated with fluorescent dye to label actin. Later, it was mounted with an antifade mounting medium. Confocal images were captured by a 63X oil objective lens and ZEN imaging software (version 2.6, ZEISS) (Rana *et al*., 2023).

Electrophoretic mobility shift assay

The assay and reagents used are described in the manufacturer's protocol (ThermoFisher Scientific). The promoter region of the target gene was biotinylated and the labeled probes were incubated with the target protein (SuH) or other control tick proteins (such as Nub). Different concentrations of the biotinylated probe were used to incubate with the protein of interest to test the mobility shift. Later the complex was electrophoresed and transferred to the nylon membrane. The membrane was blocked, washed, and substrate was added to visualize the biotinylated probes using chemiluminescence.

RNA-seq

The TRizol (Thermo Fisher Scientific) reagent was used to extract the total RNA after feeding on mice at 48 hour time point. The extracted total RNA was treated with DNase (PureLink DNase, Thermo Fisher Scientific). The RNA sequencing was performed at the University of Maryland Genomics Core facility using Illumina polyA-enriched strand-specific RNA-Seq library preparation and Illumina paired-end 100bp sequencing targeting 50M read pairs per sample.

Differential expression analyses of transcripts were performed using applicable algorithm, including DESeq2 at the collaborator's (Prof. Juan Anguita's) laboratory at CIC bioGUNE, Spain.

Results

SuH-interacting proteins: In addition to their DNA-binding activities, transcription factors also interact with proteins to regulate gene expression. To identify possible protein(s) that directly interact with SuH, we performed co-immunoprecipitation followed by mass spectrometry. The results showed a set of SuH-interacting proteins that play a role in development-associated signaling, particularly EGF signaling. In particular, these development-associated molecules included RAS-related proteins and GTP-binding nuclear proteins. These proteins are known to be involved in EGF signaling since they work as intracellular signal transporter. Some of the proteins with orthologs involved in muscle development were also found as SuH interacting partners, such as Lim and sh3 domain protein, dynamin, tubulin, etc. The complete list of SuH interacting partners is listed in Table 10.

SuH influence EGF signaling: We next measured the expression levels of selected SuH interacting molecules involved in EGF signaling in control and SuH knockdown ticks. The transcript levels for Spitz, Egfr, Rac, Mbc, and Hep1 copies were measured through qRT-PCR, and their expression level was normalized via the tick beta-actin mRNA (Figure 27). The results show that the downstream target molecules in EGF pathways showed lower expression in knockdown ticks than the control.

Influence of SuH in regulation of additional signaling pathways: To examine the broader impact of SuH knockdown in other representative development-associated signaling pathways, the expression levels of relevant components (such as genes encoding Notch, Hedgehog,

and Frizzled) were also compared between the control and the knockdown groups. As before, their expression level was measured through qRT-PCR, and the expression level was normalized with the tick beta-actin transcripts (Figure 28). In contrast to EGF signaling, these additional signaling pathway molecules did not show any significant differences between the control and the knockdown group.

Biological signifinace of SuH-EGF pathway interactions and impact on tick development: Next, we targeted one of the key ligands of EGF pathway, in particular Spitz, to study its impact in tick biology. To accomplish this, the Spitz dsRNA was prepared following the manufacturer's protocol (Figure 29A). The microinjected fed larval ticks were allowed molt in the incubator. The results show that the Spitz-knockdown tick were unable to molt (Figures 29B and C). The confirmation of knockdown was checked in 48 hours by fed ticks (Figure 29D). The molting percentage of Spitz-silenced ticks was remarkably low (Figure 29E), which was comparable to SuH-knockdown group, as shown earlier.

In addition to larva, we also examined the impact of Spitz knockdown in nymphal ticks using similar procedure. A similar phenotype was also obtained, as Spitz-knockdown fed nymphs were unable to molt (Figure 30).

Similarly, we then used RNAi to examine the role of one of the EGF downstream signaling molecules, particularly Rac, which was also dysregulated in SuH-knockdown ticks. A comparable phenotype of Rac-knockdown ticks was recorded despite an inefficient knockdown of Rac (Figure 31D), which also failed to molt to the next developmental stage, either during larval-nymphal (Figure 31) or nymphal-adult molts (Figure 32).

Furthermore, since Rac GTPase plays a role as a downstream intermediary of EGF signaling and as several Ras-related GTPases are present as the SuH interacting partners, as

revealed by the mass spec results, we directly tested the interaction between Rac and SuH, which was subjected to a protein-protein interaction assay to examine the binding between SuH and Rac proteins .

Mass spec analysis revealed that several GTPases are present as SuH-interacting partners. We targeted one of these abundant major GTPases, Rac (ISCW016255), to directly test the interaction between Rac and SuH. We produced recombinant Rac (Figure 33) which was subjected to a protein-protein interaction assay to examine the binding. The ELISA-based microtiter assay results showed the interaction between SuH and Rac (Figure 34).

SuH impact on tick development or organ development does not include actin reorganization: As actin reorganization in cells are regulated by transcription factors and associated signaling factors (Morita, 2007), and as SuH knockdown reflects apparent dystrophy or abnormalities in gut tissues, we examined possible cellular defects in actin arrangements. To accomplish this, the tick gut samples were dissected from the control (dsGFP) and two knockdown groups, dsSuH and dsRac, ticks. The samples were subjected to confocal immunofluorescence microscopy to monitor the presence of the actin microfibrils in the gut. The results show that knockdown of either target gene did not show any significant difference in the actin filament formation in ticks (Figure 35).

SuH binds to Spitz promoter: Since SuH acts as a transcription factor, we seek to obtain direct evidence of its function, by examining its ability to bind promoter of one of the potential target genes. Since SuH silencing reduces production of EGF signaling components, we opted to examine whether SuH binds to the promoter region of one of the ligands of EGF signaling, particularly Spitz. We used the electrophoretic mobility shift (EMSA) assay, which is routine technique in our lab (Yang *et al*, 2008) to measure the interaction between the target promoter

DNA of Spitz and SuH. The promoter region of Spitz was subjected to biotinylation (200 pM), and different picomolar concentrations of the proteins were used (50 and 100 pM). Varying concentrations of biotin-labeled Spitz oligos were applied in the gel (250, 200, 150, 100, 75, 50, 35 pM), together with a constant amount of SuH or control proteins (100pM). The complex was electrophoresed, transferred, and processed for luminescence that was detected via CCD camera. This assay shows that the protein SuH can bind to the Spitz promoter (Figure 36) which in turn is likely to regulate the EGF signaling.

SuH regulates global transcriptome in *I. scapularis*: Our previous studies show SuH as a transcription factor with global impacts in influencing multiple signaling pathways and events in tick development. Therefore, we opted to assess impact of SuH knockdown on transcriptome of *I. scapularis*. Total RNA samples were collected from SuH-knockdown and control (*dsGFP*-injected) ticks after 48 hours of engorgement on mice and processed for RNA sequencing as detailed in the methods section. The results, as listed in Table 11 and shown in Figure 37, indicated dramatic and global dysregulation of gene expression profiles in SuH deficiency. In particular, several cuticle gene transcripts are downregulated in the knockdown tick (dsSuH), as compared to the control (dsGFP).

Table 10: The listed proteins are SuH interacting partners detected through mass spectrometry.

Accession Number	Description	Abundance Ratio(%): SuH immunized/PBS immunized serum
B7P2F7	RAS-related protein, putative (Fragment) OS=Ixodes scapularis OX=6945 GN=8023649 PE=4 SV=1	5.057
A0A4D5REX3	Putative gtp-binding adp-ribosylation factor-like protein arl1 (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	4.565
B7QIB6	GTP-binding nuclear protein OS=Ixodes scapularis OX=6945 GN=8041684 PE=3 SV=1	4.281
A0A4D5RLV6	Putative g protein (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	4.213
A0A4D5RYG6	Putative ras-related protein rab-11a (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	4.2

A0A4D5RBA2	Putative ras-related protein rab-1a (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	4.062
A0A4D5RPC1	Putative myosin regulatory light chain (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	3.732
B7Q4Q4	ADP/ATP translocase, putative OS=Ixodes scapularis OX=6945 GN=8035879 PE=3 SV=1	3.367
B7PI71	NADH:ubiquinone oxidoreductase, NDUFS2/49 kDa subunit, putative OS=Ixodes scapularis OX=6945 GN=8029323 PE=3 SV=1	3.314
B7PDY5	Vesicle coat complex COPII, GTPase subunit SAR1, putative OS=Ixodes scapularis OX=6945 GN=8028474 PE=3 SV=1	3.268
A0A4D5RGB2	Mitochondrial import inner membrane translocase subunit TIM50 (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	3.265
B7QFX7	RAB-9 and, putative OS=Ixodes scapularis OX=6945 GN=8040663 PE=4 SV=1	3.227
A0A4D5RBH5	Putative gtp-binding adp-ribosylation factor arf6 darf3 (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	2.954
B7P377	Lim and sh3 domain protein 1, lasp-1, putative OS=Ixodes scapularis OX=6945 GN=8023939 PE=4 SV=1	2.877
B7PM12	Dynamin, putative OS=Ixodes scapularis OX=6945 GN=8050678 PE=3 SV=1	2.723
B7PGI8	Tubulin alpha chain OS=Ixodes scapularis OX=6945 GN=8052898 PE=3 SV=1	2.686
B7Q304	Misexpression suppressor of KSR, putative OS=Ixodes scapularis OX=6945 GN=8035131 PE=4 SV=1	2.672
A0A4D5S0W0	Putative cytochrome b-c1 complex subunit 2 mitochondrial-like protein (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	2.642
B7PFU3	Muscle LIM protein isoform A isoform, putative OS=Ixodes scapularis OX=6945 GN=8052043 PE=4 SV=1	2.562
B7Q0X1	Derlin OS=Ixodes scapularis OX=6945 GN=8034250 PE=3 SV=1	2.536
B7PH43	Tubulin alpha chain OS=Ixodes scapularis OX=6945 GN=8028862 PE=3 SV=1	2.5
A0A4D5RET9	Putative mitochondrial import inner membrane translocase subunit tim13 (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	2.305
B7PPI1	Cuticular protein, putative (Fragment) OS=Ixodes scapularis OX=6945 GN=8051553 PE=4 SV=1	2.263
B7Q150	Molecular chaperone, putative OS=Ixodes scapularis OX=6945 GN=8034342 PE=3 SV=1	2.168
A0A4D5S6W2	Fibronectin type-III domain-containing protein (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	2.13
A0A4D5S779	Putative actin (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	2.118
A0A4D5RPT3	Putative myosin regulatory light chain (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	2.07

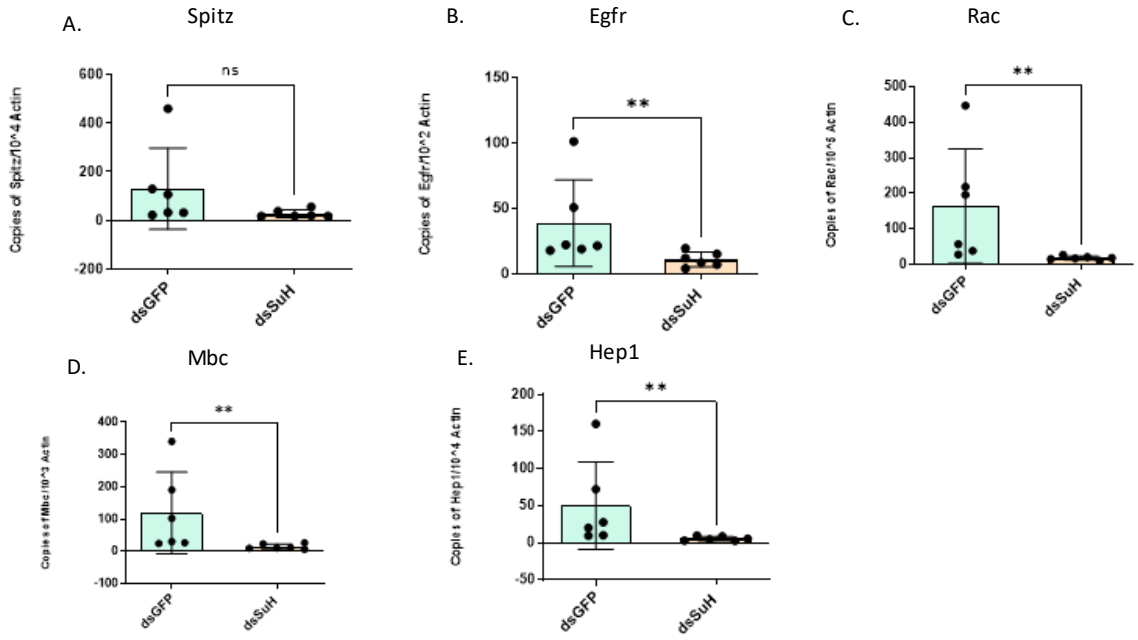

Figure 27: Level of expression of genes that are involved in EGF signaling. Goups of six ticks were examined for expression of the test gene in knockdown ticks, as compared to the control (dsGFP). The significance level was calculated using a two-tailed t-test (**p-value<0.01). The transcript copies of Spitz, Egfr, Rac, Mbc, Hep1 were measured through qRT-PCR, and the level of expression was normalized with tick beta-actin mRNA.

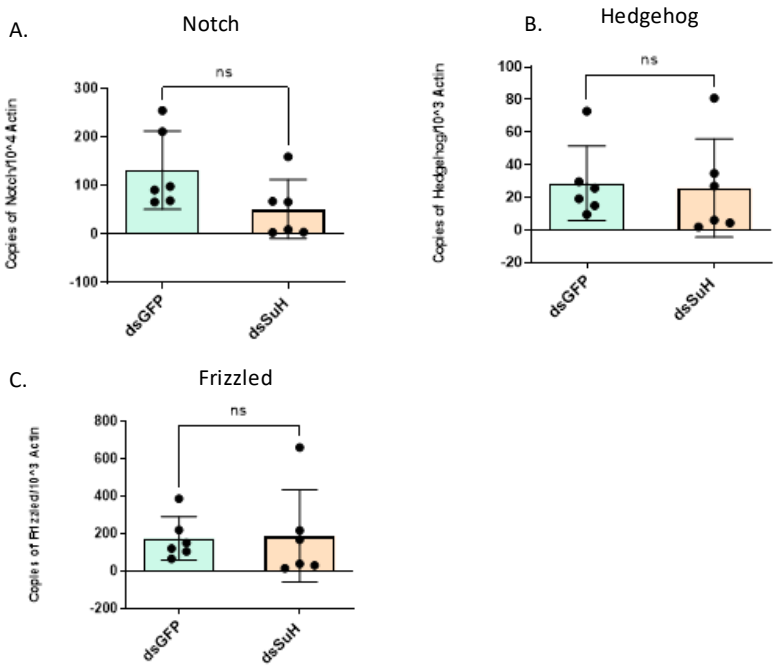

Figure 28: Level of expression of selected genes involved in representative development-associated signaling. The knockdown does not significantly impact above tested signaling pathways. Groups of six ticks were examined for target gene expression and compared to the control (dsGFP). The significance level was calculated using a two-tailed t-test (p-value<0.05). The transcript copies of Notch, Hedgehog, and Frizzled were measured through qRT-PCR, and the level of expression was normalized with tick beta-actin transcripts.

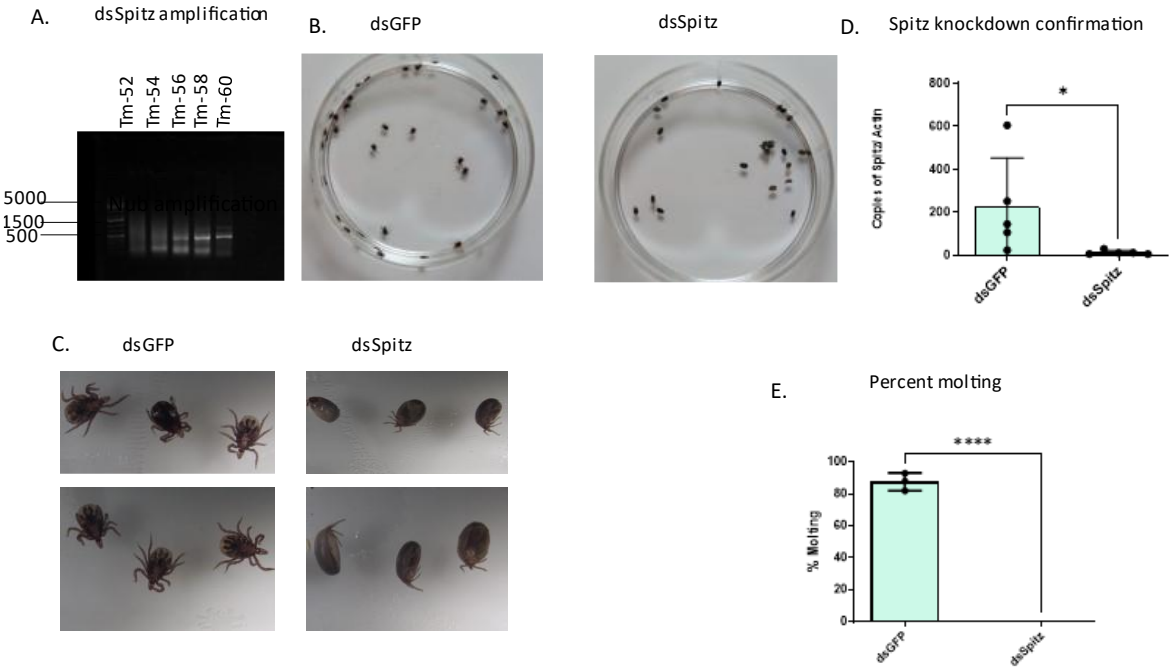

Figure 29: Impact of Spitz knockdown during larval-nymphal molt. (A) The Spitz dsRNA constructs amplification through gradient PCR. (B, C) Dorsal and ventral side of molted nymphs of dsGFP and dsSpitz knockdown ticks are shown. The ticks were unable to molt to the next developmental stage. (D) Confirmation of knockdown at 48 hrs of feeding of dsSpitz injected ticks. The level of significance was calculated by using a two-tailed t-test (*p-value<0.05). (E) The nymphal stage Spitz knockdown ticks failed to molt to the subsequent developmental stage, which showed a significantly different rate (****p<0.001) with the control dsGFP ticks.

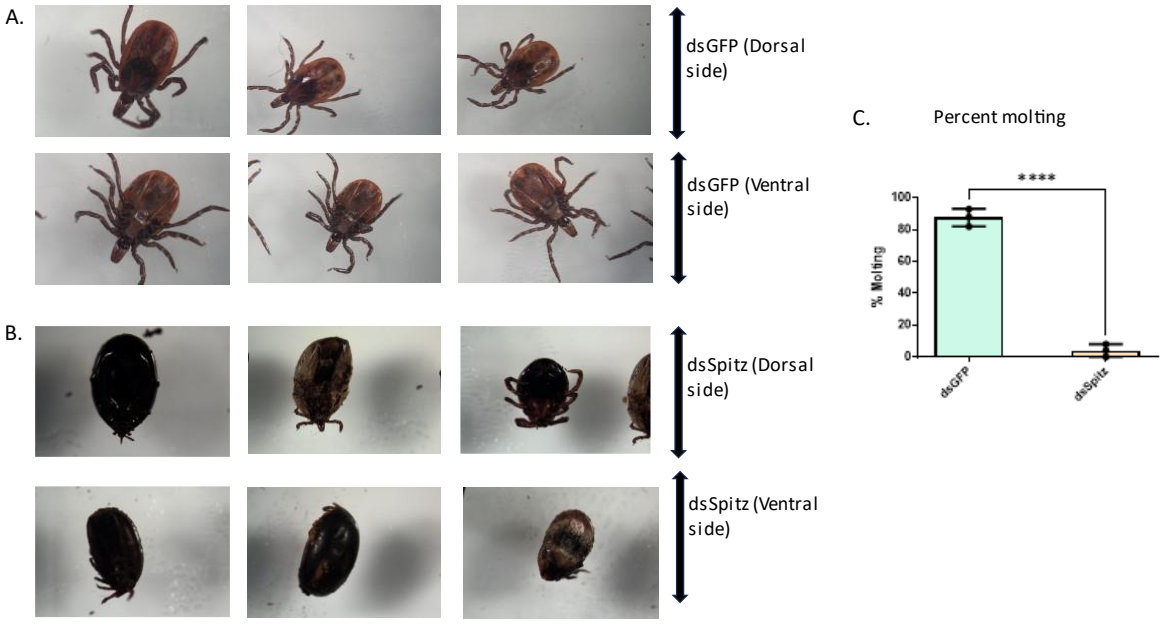

Figure 30: Impact of Spitz knockdown on nymphal-adult molt. (A, B) The upper panels show the control intermolt nymphs (dsGFP), while lower panels denote Spitz-knockdown nymphal ticks that were unable to molt to adults. The data represents three individual experiments with similar results. (C) The molting rate of nymphal Spitz-knockdown ticks, whick largely unable molt to the next developmental stages, showed a significant difference with the control dsGFP ticks. The significance level was calculated using a two-tailed t-test (****p-value<0.001).

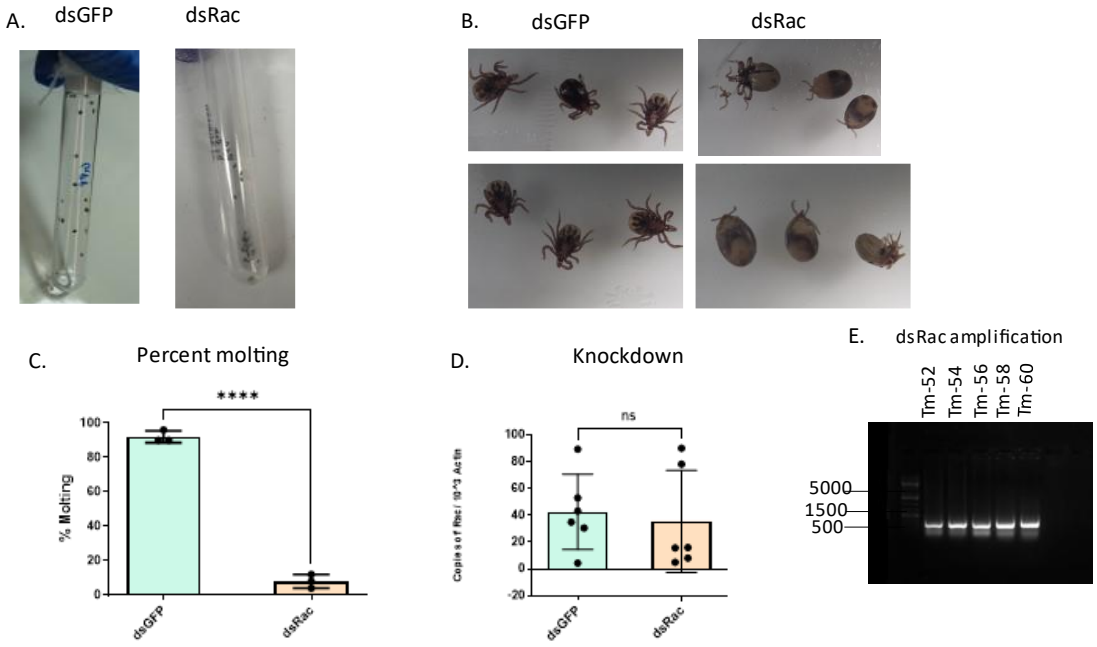

Figure 31: Impact of Rac knockdown on larval-nymphal molt. (A-B) The dorsal and ventral side of Rac-knockdown ticks are shown. (C) Rac-knockdown ticks are largely unable to molt, where only ten percent of ticks were molted. (D) The panel represents knockdown of Rac transcripts. The value was normalized using the beta-actin. The knockdown isn't significant, as shown in the graph. (E) A gel representing the dsRac construct generated via gradient PCR is shown.

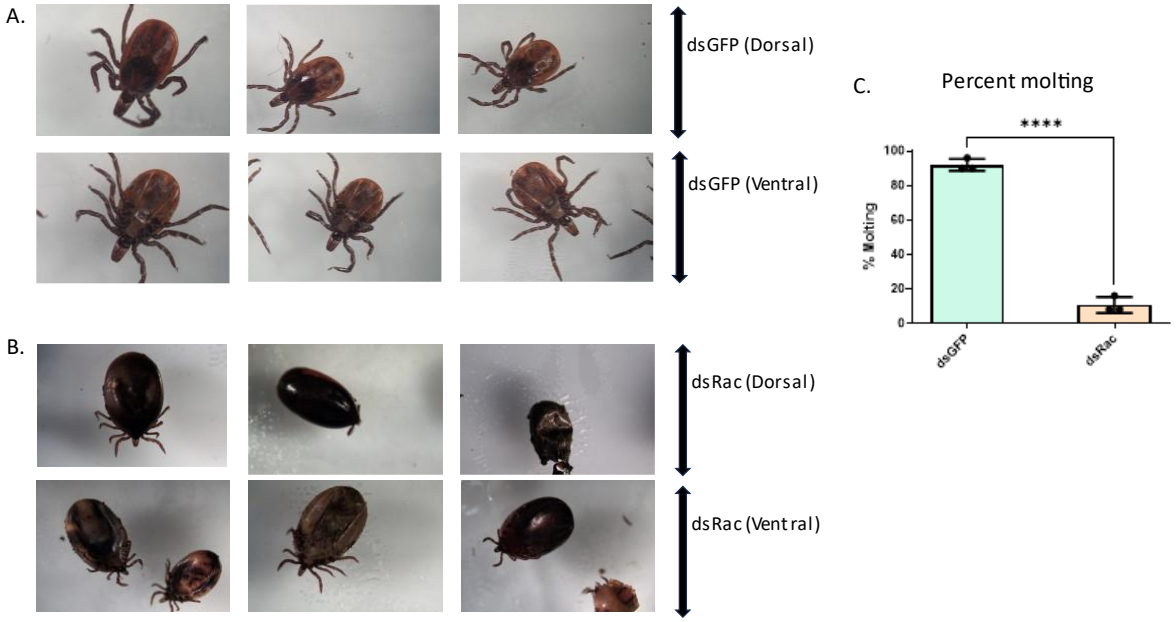

Figure 32 : Impact of Rac knockdown on nymphal-adult molt. (A-B) The panels represent molted or unmolted ticks in the dsGFP and dsRac groups, respectively. The dsGFP group molted to adults while Rac-knockdown ticks were unable to molt at the time of testing. (C) The molting rate of control and the knockdown groups is shown.

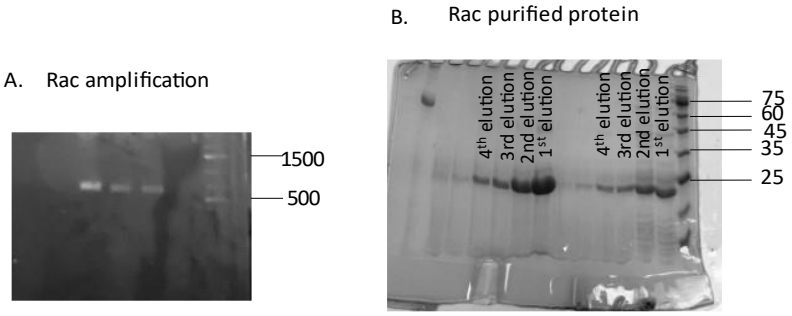

Figure 33: Rac protein production. (A, B) The Rac gene was cloned in the pGEX-6p1 vector and the recombinant protein was purified using GST beads. The size of the protein is around 21kDa.

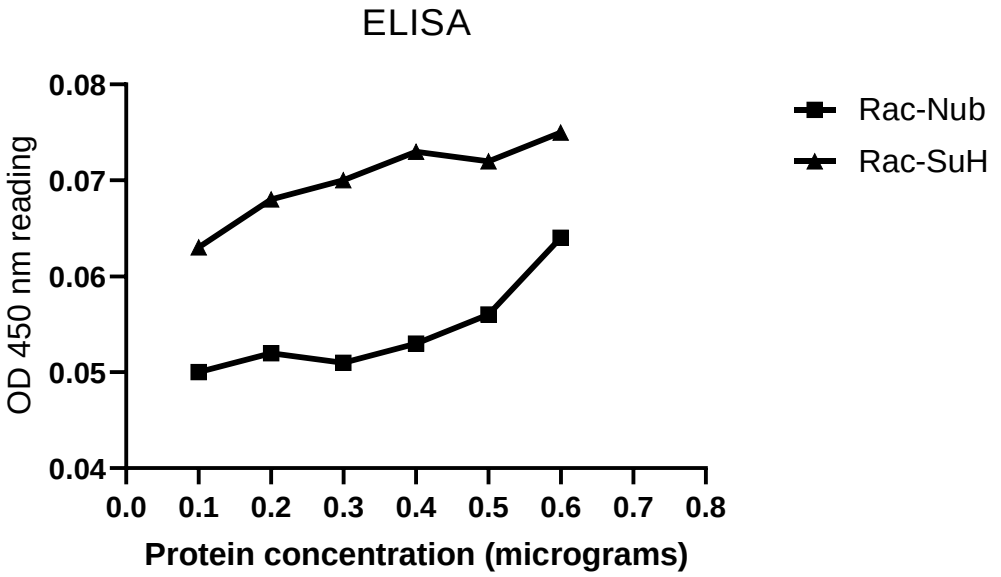

Figure 34: The interaction between Rac and SuH. The Rac was used to coat the plates, and the SuH (His-tagged) was used to assess the interaction with the protein of interest. The His-tagged Nub was used as a negative control.

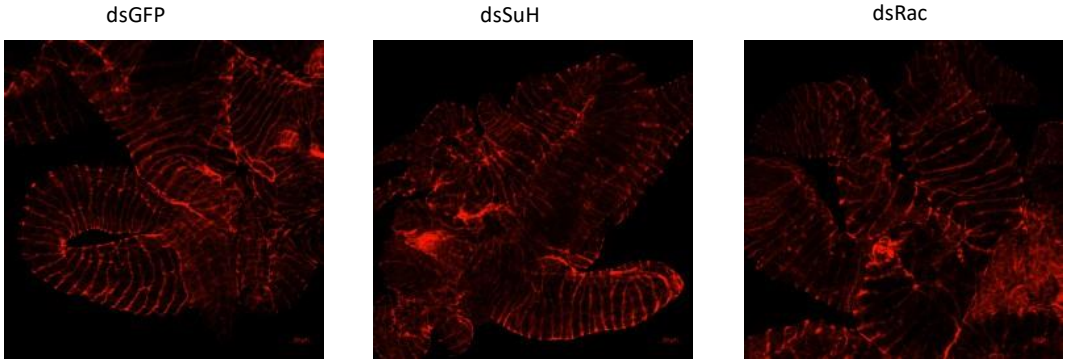

Figure 35: Actin staining of GFP, SuH, and Rac knockdown ticks. The dissected tick gut was subjected to *β-actin* staining (red) to observe the presence of the actin filaments in the gut in control (dsGFP), dsSuH, and dsRac ticks. The knockdown of either target gene did not show any significant difference in the actin microfibril formation, as compared to control. The tick gut was dissected at 48 hours fed tick and was subjected to the staining.

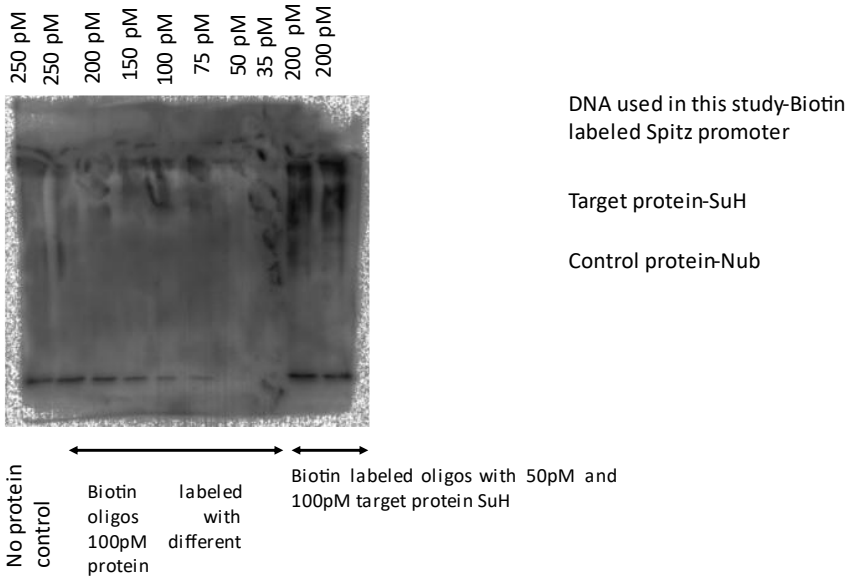

Figure 36: Electrophoretic mobility shift assay showing the SuH bound to the Spitz promoter. The SuH-Spitz complex was electrophoresed, transferred, and processed. Later, the luminescence was detected via CCD camera.

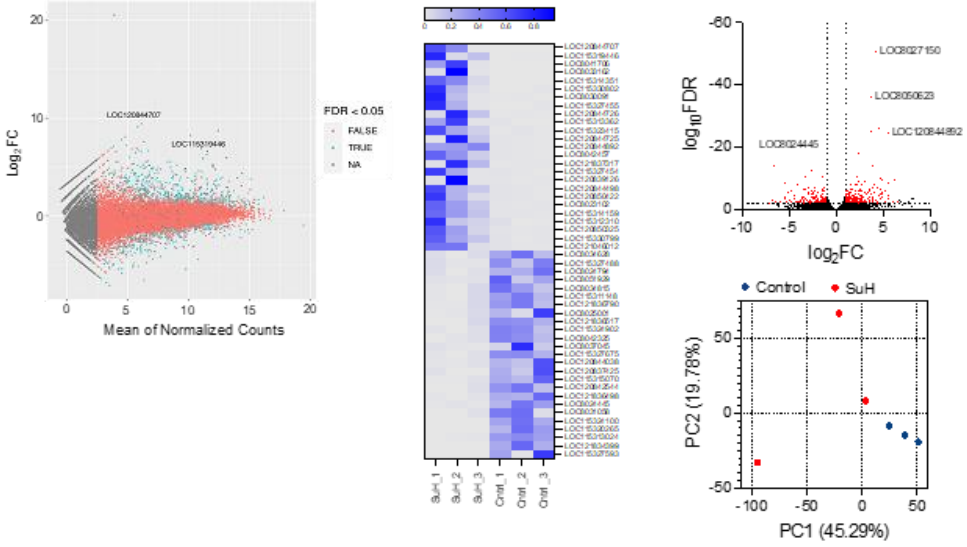

Figure 37: The RNA-seq data showing the highly upregulated genes in the control (dsGFP) as compared to the knockdown (dsSuH) group.

Table 11: Gene transcripts upregulated in control ticks as compared to the knockdown ticks (dsSuH)

Symbol	Description	logFC
LOC120844707	cuticle protein 10.9-like	9.4159314
LOC8033162	antimicrobial peptide ISAMP-like	7.79530179
LOC115330802	cuticle protein 12.5-like	6.73171216
LOC120844726	cuticle protein 10.9-like	6.28569331
LOC115323415	neuropeptide-like protein 31	5.59051126
LOC120844892	cuticle protein 63-like	5.54599527
LOC8042457	cuticle protein 16.5-like	5.5341795
LOC120850422	cuticle protein 64-like	5.1831384
LOC120850325	cuticle protein 38	4.93976615
LOC120840982	cuticle protein 16.8-like	4.67395267
LOC120850400	cuticle protein 38-like	4.37528414
LOC115326800	cuticle protein 10.9-like	4.13103817
LOC8042876	intracellular coagulation inhibitor 2	3.89452581
LOC8032630	antimicrobial peptide microplusin-like	3.65812374
LOC121837268	glycine-rich cell wall structural protein-like	3.59702347
LOC8053521	glycoprotein antigen BM86	3.2925864
LOC120843582	defensin	3.2318059
LOC115330812	cuticle protein 38	3.19743116
LOC115308652	myelin transcription factor 1-like protein	3.06177875
LOC115327980	chitin deacetylase 7-like	3.0537745
LOC8043182	chitin deacetylase 8-like	2.8143073
LOC121045610	cuticle protein 65-like	2.79135514

Discussion

The conserved cell signaling pathways, such as EGF, Notch, and Hedgehog, plays crucial roles in the post-embryonic development of metazoans including arthropods (Artavanis-Tsakonas *et al*, 1999; Bray, 2006; Oda *et al.* 2005; Perrimon and Perkins, 1997). The EGF signaling is also activated during embryogenesis (Lusk *et al*, 2017), and inhibition of the signaling can lead to abnormal development and reduced molting percentage. The functionality of organs involved in tick hematophagy, such as salivary gland, is also known to be regulated by EGF-like growth factor signaling, as shown in a recent study (Denisov *et al*, 2021). It is possibly involved in the

maintenance and development of the salivary gland in *I. scapularis* as well (Koci *et al*, 2021). Our finding that SuH is also involved in the EGF signaling cascade further underscore the critical importance of the transcription factor and the signaling pathway in *I. scapularis* development, encompassing both larval and nymphal stages.

Based on our current study and available literature (Oda *et al.* 2005; Perrimon and Perkins, 1997), we speculate that successful acquisition of blood meals, molting and cuticle formation are all keystone events in the tick lifecycle that can be influenced by EGF signaling. Ticks take a huge blood meal from the mammalian host, which involves complex series of events, including highly-evolved questing behavior, piercing the host's skin through its chitinous mouth parts, as well as highly expandable organs, including gut. Our study showing impaired legs, mouth parts and gut in SuH as well as EGF ligand (Spitz) deficient ticks futher testify a pleiotropic roles of these elements in tick biology. When the transcriptome of control (dsGFP) and knockdown tick (dsSuH) were compared via RNA-seq, several transcripts that were upregulated involved cuticle protein (cuticle protein 12.5-like, cuticle protein 10.9-like, cuticle protein 63-like, cuticle protein 16.5-like, cuticle protein 38 etc) which indicates their involvement in development of tick since the knockdown tick possibly has impaired cuticle and were not able to complete optimal molting process, which could also contributed to the viability of SuH deficient ticks. However, our understanding of EGF signaling in ticks is still limited, and more studies are required to understand the specific mechanisms of this signaling in tick biology. Nevertheless, SuH (or similar factors in EGF signaling pathway), despite its overall conservation, also reflect tick-specific divergence, suggesting its potential as target of anti-tick prevention.

When the Co-IP was performed, several molecules downstream to the EGF-signaling (Artavanis-Tsakonas *et al*, 1999; Bray, 2006; Oda *et al*. 2005; Perrimon and Perkins, 1997) were found as SuH interacting partners, such as RAS-related protein, GTP binding adp-ribosylation factor, GTP binding nuclear protein, and ras-related protein. Several proteins involved in muscle formation, such as Lim domain protein, Dynamin, and Tubulin, are also identified in the mass-spec data, suggesting their regulation by SuH. While we have identified Spitz ligand is important for tick development, as evidenced by impaired larval and nymphal molting events, as well as SuH as a possible regulator, shown by its binding to Spitz promoter, the precise molecular relationship of this protein and others with SuH and how these contribute in tick development remains as a subject of further investigation.

Our study identified possible interaction between SuH and Rac, suggesting their functional association in driving tick development. Although we attempted RNAi experiments using *dsRac* microinjection, the knockdown was inefficient. Notwithstanding, we recorded a dramatic difference in phenotype showing *dsRac*-injected ticks were unable to molt completely, either through larval-nymphal or nymphal-adult stages. Therefore, it is possible that *dsRac* construct is targeting a non-specific yet important gene essential for molting, either another Ras-related GTPase or other unknown genes, which also represents an area of future study.

Chapter 4: Characterization of transcription factor Nubbin

Introduction

Nubbin (Nub) /POU domain protein 1 (Pdm1) is a member of the class II POU transcription factor family and shares homology with the OCT1/POU2F1 and OCT2/POU2F2 proteins in mammals (Tang *et al*., 2018). In *Drosophila*, it is shown to regulate intestinal stem cell (ISC) maintenance and differentiation. Gut epithelium homeostasis is maintained by a complex and well-tuned expression of Nub in progenitor cells (Tang *et al*., 2018). Global transcriptional profiling of adult flies expressing Nub in immunocompetent cells revealed that this protein is a potent transcriptional activator of many immune genes (Lindberg *et al*., 2018). Furthermore, corresponding genetic analyses uncovered that Nub drives expression of both independently or in conjunction with JAK/STAT pathways, nuclear factor kappa B (NF-κB), or JNK pathways. Conversely, they also have roles to repress the expression of the same targets (Lindberg *et al*., 2018). Tick has putative orthologs of Nub although their biological significance in tick development, or more precisely roles in hematophagy or gut tissue development, including ISC maintenance, remain unknown yet important to decipher how a tick can acquire an enormous amount of host blood compared to other blood sucking arthropods. Nub/POU domain proteins are well characterized as transcription factors (Ryan and Rosenfeld, 1997). The DNA-binding region's total structure and helix loop helix is called *bHLH* (Verrijzer *et al*., 2003). In prokaryotes, the helix turn helix is a packed twenty amino acid structure containing two helices connected by a linker (Veenstra *et al*., 1997). The motif consisting of helix turn helix interacts with DNA, and the second alpha-helix binds in the major DNA groove.

The POU-specific domain recognizes and binds the octamer ATGCAAAT with low affinity by using POU_s and the right half by POU homeodomain (Pfaffle *et al*., 1992). The Nub or POU family genes are well studied in *Drosophila* and shown to influence development, cell differentiation, pattern formation or tissue specification (Tang *et al.*, 2018; Lindberg *et al.*, 2018). This chapter aims to highlight the role of POU domain transcription factor Nub in *I. scapularis* tick biology.

Materials and Methods

Materials and Methods used in the current studies were detailed in the previous chapter.

Results

Nub orthologs and their knockdowns: There are two orthologs of Nub protein in *I. scapularis* tick genome (Fig 38A and B). The protein has two superfamilies, the N-terminal POU-specific domain, and the C-terminal subunit homeobox domain with about fifty-two percent sequence similarities (Fig 38C). The first ortholog Nub1 (ISCW22240) is highly expressed during feeding (Figure 16). Since genes encoding both Nubs have a high degree of sequence similarity, we generated a single construct (dsNub1, which has more continuous sequences present in both homologs) effective against both homologs (Fig 39). Nevertheless, we also prepared the dsRNA against Nub2 and checked the expression of Nub1, which was not significantly low in the knockdown group (Figure 40). The subsequent experiments will thus use the dsNub1.

Nub phylogeny: We then performed the multiple sequence alignment to generate the phylogenetic tree for the protein Nub. The figure shows both conserved and variable amino acid sequences in different organisms, as compared to *I. scapularis* (Figures 41).

Nub expression in feeding ticks: Next, we analyzed the expression level of Nub at various time points of tick feeding using murine hosts (Figure 42). Ticks were placed on mice, and the expression was examined at four different time points, such as 24, 48, 72 and 96 hours. The protein is most dramatically expressed at 48 hours of feeding.

Nub plays minor yet significant roles in tick development: We performed the knockdown of the Nub via RNAi using dsRNA microinjection during larval stage. The results show that the Nub knockdown ticks failed to show any major phenotypic or developmental defects during intermolt or as newly molted ticks, as assessed by their morphology (Figure 43). However, we noticed that approximately forty percent of Nub knockdown ticks did not molt to the following developmental stages, suggesting an important role of Nub in supporting tick postembryonic development (Figure 44A and B). We confirmed the knockdown of Nub via qRT-PCR in the feeding tick as detailed before (Figure 44C). Histological analyses were also performed among the control and knockdown groups. Although the knockdown group shows apparent defects in blood meal storage (Figure 44D), the feeding parameters largely did not reflect marked deviation from the controls (Figure 44E). Similarly, we measured tick weights at two different timepoints, one immediately after feeding and the other at day 20 (Figures 45A and B), which remains statistically non-significant between groups. We confirmed the knockdown of the target gene (Figure 45C). We also measured the percent molting after a month and the percent survival at day 60, which show significant decreases in the knockdown group (Figure 45D and E), possibly due a slower feeding progression of knockdown ticks, as compared to control (Figure 45F).

Nub is involved in multiplex protein-protein interactions and expressed in tick gut: To understand molecular function of Nub, including possible involvement in interaction with other tick proteins, we produced recombinant one of the Nub homologs (Nub1). The purification of the

His-tagged Nub is shown (Figure 46A and B). We also produced the polyclonal antiserum against this protein. The antiserum's specificity was assessed via western blot (Figure 46C). As detailed for SuH in previous chapter, the interacting Nub proteins in *I. scapularis* were identified via co-immunoprecipitation and mass spectrometry, as listed in Table 12. We also examined the localization of native Nub in the tick gut. The protein localizes around the nucleus of gut cells (Figure 47).

Nub regulates a limited set of genes in *I. scapularis*: As Nub is likely to be a transcription factor, we assessed the impact of Nub knockdown on transcriptome of *I. scapularis*. Total RNA samples were collected from Nub-knockdown and control (*dsGFP*-injected) ticks after 48 hours of engorgement on mice and processed for RNA sequencing as detailed earlier. The results, as listed in Table 13 and shown in Figure 48, indicated alteration of several metabolism associated genes in the knockdown tick (dsNub), as compared to the control (dsGFP).

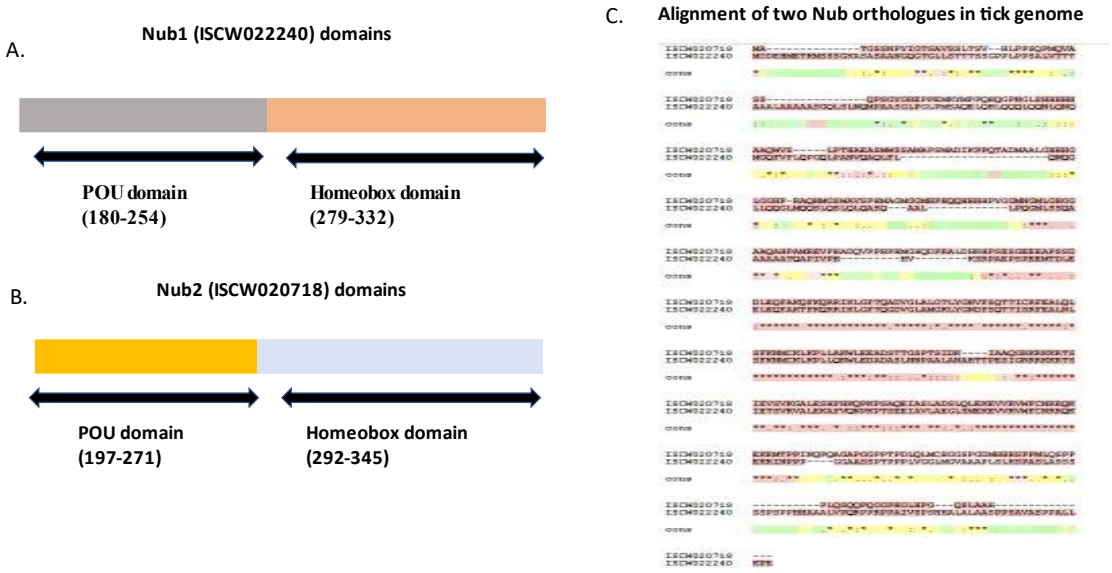

Figure 38: There are two orthologs of Nub in the tick genome. (A, B) *I. scapularis* Nub protein domains. Nub protein contains POU superfamily domain. The POU domain is a bipartite domain composed of two subunits. The N-terminal subunit is known as the POU-specific (POUs) domain, while the C-terminal subunit is a homeobox domain. The tick genome contains two orthologs of the transcription factor Nub1 and Nub2. The first ortholog Nub1 contains the most continuous sequence. (C) When both the sequences were aligned, they showed around 52% sequence homology.

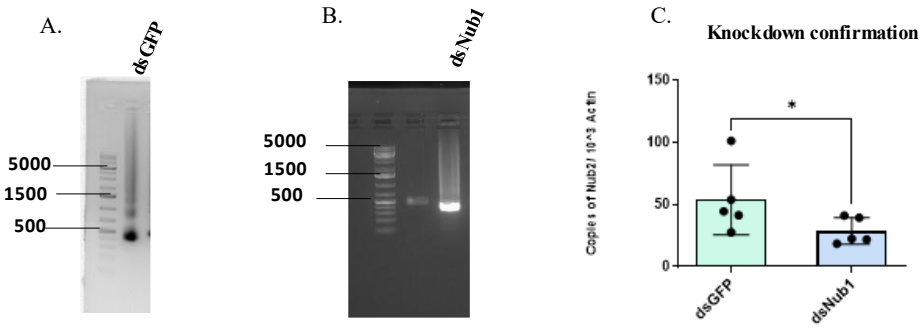

Figure 39: The dsRNA against the gene Nub1 shows knockdown for both Nub orthologs. (A, B) The dsRNA was prepared against the dsGFP and was used as a control. The dsRNA against the first ortholog of Nub, dsNub1, was prepared following the same protocol. The size of the dsRNA constructs is 500 bps. (C) The dsRNA microinjection of Nub1 showed a significant reduction in Nub2 expression level. The level of significance was calculated using a two-tailed t-test (*p-value<0.05).

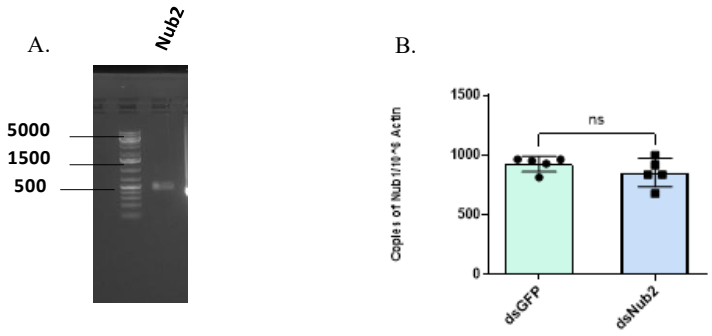

Figure 40: The dsRNA against the Nub2 didn't show knockdown for Nub1. (A) The dsRNA against the second ortholog of Nub, dsNub2 was prepared. The size of the construct is 500 bp. (B) The dsRNA prepared against Nub2 was microinjected in tick nymph, and the nymph was fed on mice. The dsRNA microinjection of Nub2 showed a significant reduction in Nub1 expression level. The significance level was calculated using a two-tailed t-test (p-value<0.05).

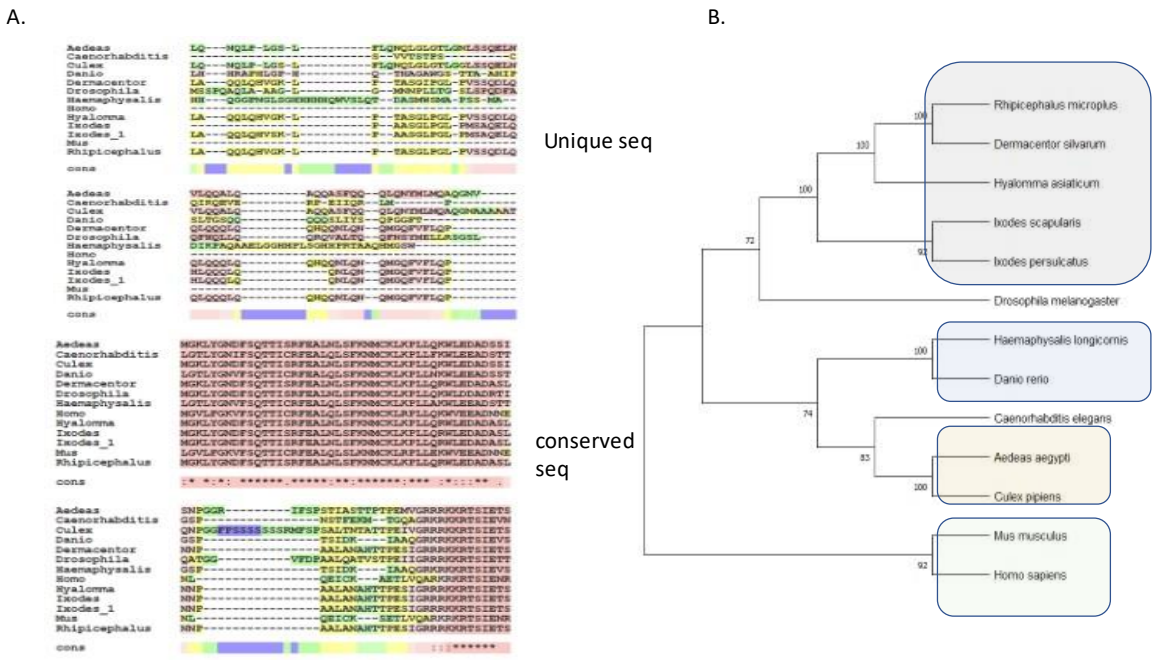

Figure 41: Alignment of Nub1 ortholog and phylogenetic tree. (A) Alignment of *Ixodes* Nub protein orthologs in well-studied organisms (multiple species of ticks, mosquito, fruit fly, mice, human, zebrafish, worms). It shows conserved amino acid sequences among tested organisms. The presence of conserved domains implies their significance through evolution. The pictures shown here are small portions of the entire alignments. (B) Nub phylogenetic tree was generated using the Mega 11 software. Homologous proteins from different species of ticks, mice, humans, mosquitoes, *Drosophila*, zebrafish and mice were compared using BLASTP software.

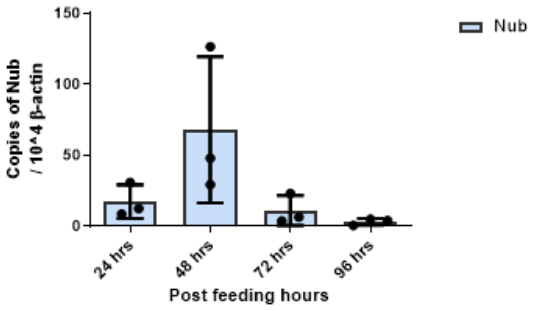

Figure 42: Level of Nub expression at different time points. Ticks were placed on mice and detached at each time point (24, 48, 72, 96 hrs.) for analaysis. Three individual ticks were used to analyze expression in each time point. The expression level is highest at 48 hrs.

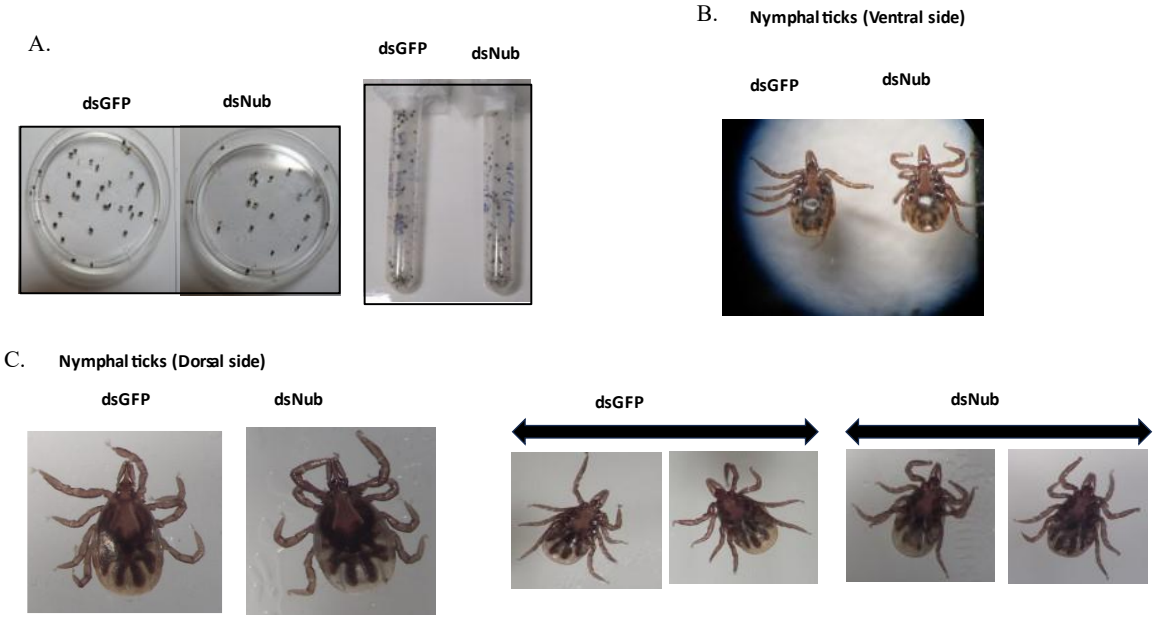

Figure 43: Nub deficiency had negligible influence on tick morphology. (A) Impact of Nub knockdown on molted nymphs (dsGFP and dsNub) that were previously microinjected as larval ticks. The ticks did not show major developmental defects. (B and C) Ventral and dorsal side of molted nymphs, respectively. This data is representative of three individual experiments.

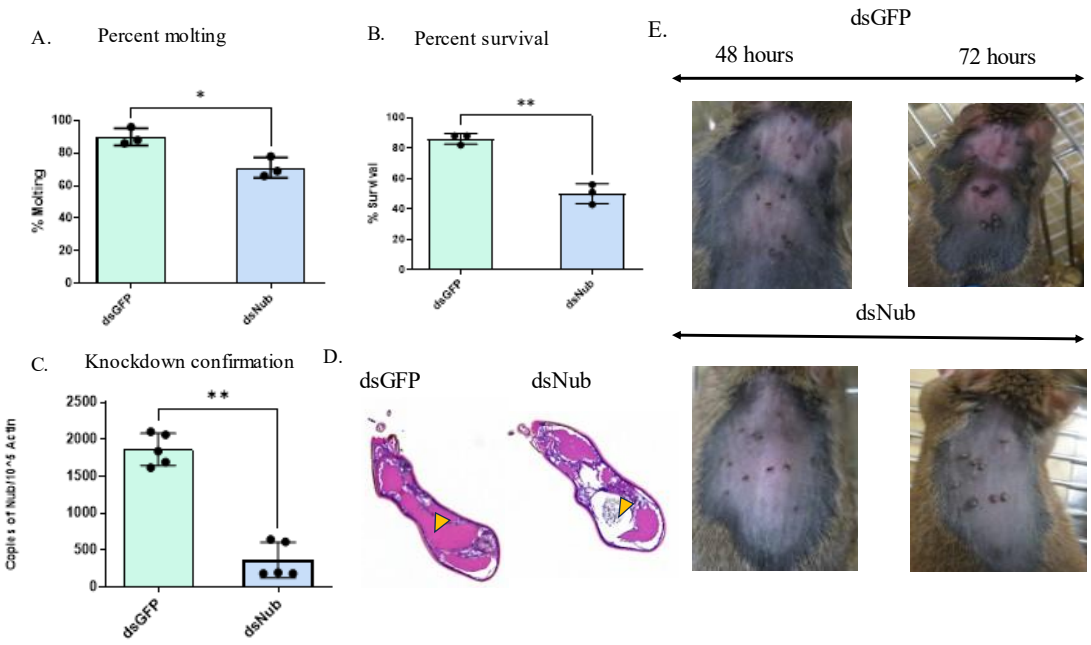

Figure 44: The Nub knockdown impacts larval-nymphal molting. (A, B) The percentage of molting and percent survival at day 60 are shown. Around forty percent of Nub knockdown ticks failed to molt to the next developmental stage. The data shown here represents the average of three individual experiments. The significance level was calculated using a two-tailed t-test (*p-value<0.05 and **p-value<0.01). (C) Confirmation of knockdown at 48 hrs of feeding in the dsRNA injected tick. Five ticks were checked for the test gene's knockdown confirmation and compared to control (dsGFP). The significance level was calculated using a two-tailed t-test (**p-value<0.01). (D) The histology shows the sections of tick gut of dsGFP vs dsNub ticks. The arrows represent possible differences in the tick blood meal storage. (E) Progression of tick feeding at different time points 48 hrs and 72 hrs on mice in control (dsGFP) and Nub knockdown tick (dsNub).

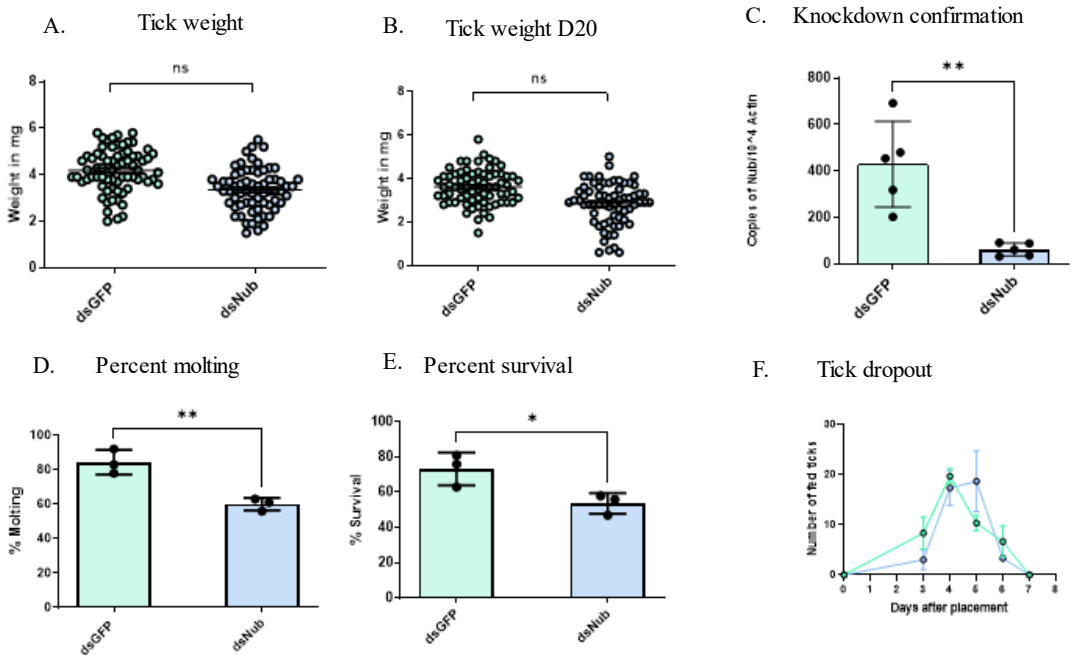

Figure 45: The Nub knockdown impacts nymphal-adult molting. (A, B) The fully-fed nymphal weights, as measured immediately after the feeding and at day 20 (D20) after feeding. The dsGFP-injected ticks were used as a control. The Nub-knockdown ticks did not show a significant reduction in their feeding weight as compared to the control. The level of significance was calculated using a two-tailed t-test. (C) The knockdown was confirmed through qPCR. Five ticks were used to test gene's knockdown and compared to control (ds GFP). The significance level was calculated using a two-tailed t-test (**p-value<0.01). (D, E) Approximately forty percent of nymphal knockdown ticks did not molt to the next developmental stage, showing a significant difference with the control dsGFP injected tick. The survival percentage also decreases at day 60 as compared to control. **p<0.01, *p<0.05. (F) The graph shows the average number of detached ticks in each day of feeding. The result is also shown as the average of three individual experiments and was compared with the control. The control and test groups didn't show much difference in feeding time points in mice.

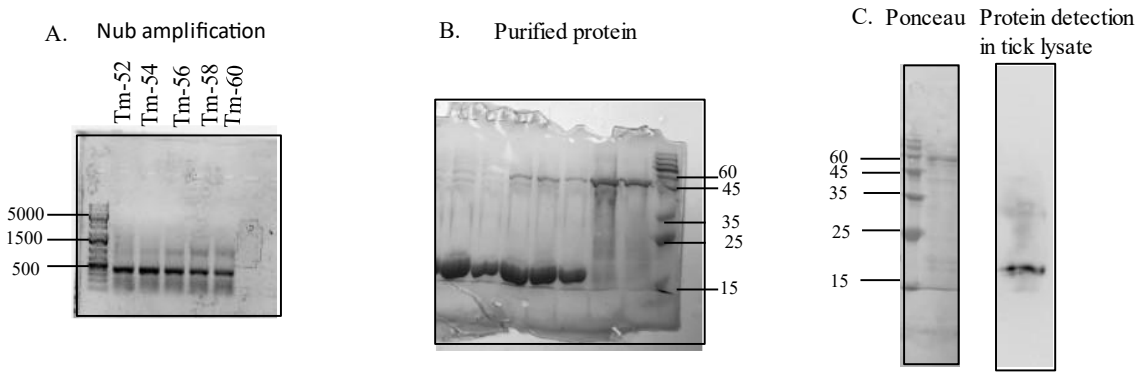

Figure 46: Nub protein and antibody production. (A) The Nub (ISCW022240) gene amplification through gradient PCR. The size of the construct is 450 bps. (B) The Nub was cloned into pET-28a vector and the recombinant protein was produced with His-tag. The protein size is around 16 kDa and 1 microgram protein was loaded. (C) The specificity of Nub antisera was assessed by immunoblotting.

Table 12: The lists of possible Nubbin interacting partners

Accession Number	**Description**	**Abundance Ratio(%): Nub immunized/PBS immunized serum**
B7PA49	Cuticular protein, putative OS=Ixodes scapularis OX=6945 GN= PE=4 SV=1	12.445
A0A4D5RL82	Mannosyl-oligosaccharide glucosidase (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	11.988
A0A4D5RQ98	Exportin-2 (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	11.641
B7PSH6	Ubiquinol-cytochrome-c reductase complex assembly factor 2 OS=Ixodes scapularis OX=6945 GN=8030699 PE=4 SV=1	11.522
B7PTF4	Transmembrane protein 120 homolog OS=Ixodes scapularis OX=6945 GN= PE=3 SV=1	11.387

B7P7F2	Chitin-binding type-2 domain-containing protein (Fragment) OS=Ixodes scapularis OX=6945 GN=IscW_PE=4 SV=1	11.337
B7QIG4	Asparaginyl-tRNA synthetase, putative OS=Ixodes scapularis OX=6945 GN= PE=4 SV=1	11.325
B7PCW5	Oxodicarboxylate carrier protein, putative OS=Ixodes scapularis OX=6945 GN=8028045 PE=3 SV=1	11.137
B7PHK6	Sphingosine phosphate lyase, putative OS=Ixodes scapularis OX=6945 GN=IscW_ PE=3 SV=1	11.034
A0A4D5RJP8	Putative acyl-coa thioesterase (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	11.019
A0A4D5RDQ4	Serine protease HTRA2, mitochondrial (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	10.93
A0A4D5RYI1	Putative cytokinesis actomyosin contractile ring assembly (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	10.871
A0A4D5RQS7	Putative d-alanyl-d-alanine carboxypeptidase protein (Fragment) OS=Ixodes scapularis OX=6945 PE=4 SV=1	10.871
A0A4D5RY52	GDP-mannose 4,6-dehydratase (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	10.844
A0A4D5S3G3	Dolichol-phosphate mannosyltransferase subunit 1 (Fragment) OS=Ixodes scapularis OX=6945 PE=3 SV=1	10.257
F2YHA2	Putative mediator of rna polymerase ii transcription subunit 26 OS=Ixodes scapularis OX=6945 GN=tre31 PE=2 SV=1	9.831
A0A4D5RY70	Putative actin OS=Ixodes scapularis OX=6945 PE=3 SV=1	8.11
B7PEJ6	Ras-related protein Rab-3 OS=Ixodes scapularis OX=6945 GN=8051125 PE=3 SV=1	2.857
B7PPV3	Ribonucloprotein (Fragment) OS=Ixodes scapularis OX=6945 GN= PE=3 SV=1	1.934
B7P5E4	NADH ubiquinone oxidoreductase subunit, putative OS=Ixodes scapularis OX=6945 GN=8024866 PE=4 SV=1	1.883

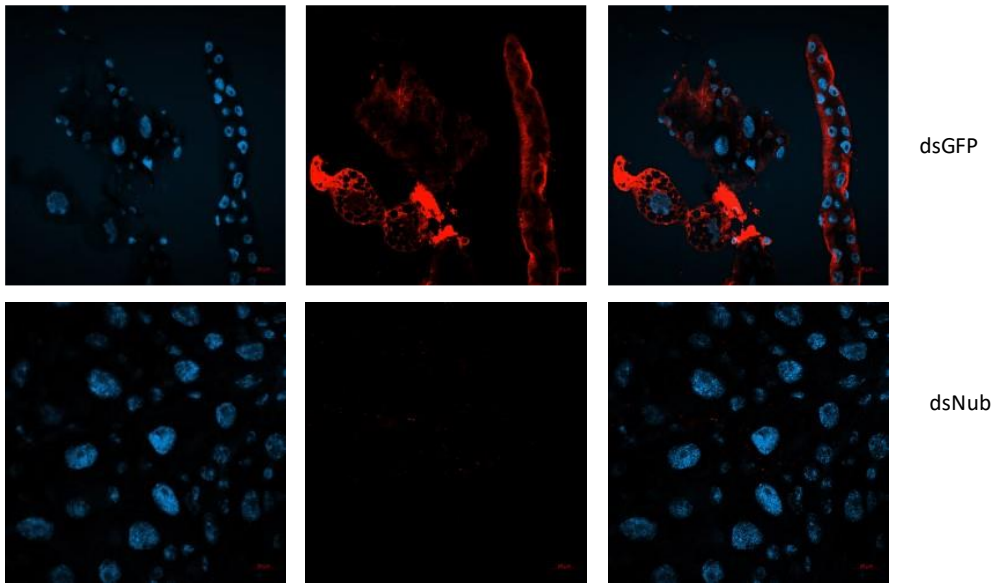

Figure 47: Nub localization in tick gut cells. Nuclei were stained with DAPI. Nub detection of nymphal guts was performed by confocal immunofluorescence microscopy. Nub expression was found around nucleus within gut epithelial cells. Nub: Red; DAPI: Blue.

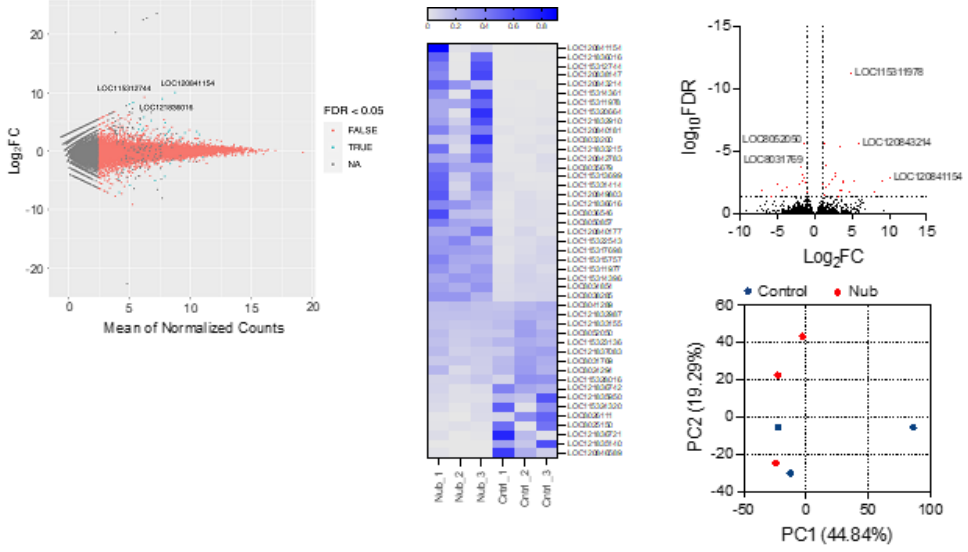

Figure 48: The RNA-seq data showing the highly upregulated genes in the control (dsGFP) as compared to the knockdown (dsNub) group.

Table 13: The listed gene transcripts which gets upregulated in the control as compared to the knockdown ticks (dsNub)

Symbol	Description	logFC
LOC115312744	Spindle pole body component 110	8.31021916
LOC120838147	A disintegrin and metalloproteinase with thrombospondin motifs 7-like	8.0109317
LOC121833215	V-type proton ATPase subunit e 1-like	3.47782739
LOC121836616	epoxide hydrolase 1-like	2.68945393
LOC8050857	sarcoplasmic calcium-binding proteins I, III, and IV	2.49569633
LOC115317698	ribonuclease P protein subunit p25-like protein	1.83601515
LOC115315757	ankyrin-1	1.82683618
LOC115314396	heparan-sulfate 6-O-sulfotransferase 3-B	1.40427367
LOC8038285	sulfotransferase ssu-1-like	1.33791309
LOC8038486	ATP synthase subunit b, mitochondrial	1.11569831

Discussion

Nubbin (Nub) is encoded in genomes of most metazoans, including the fruit fly *D. melanogaster,* which is known to be associated with tissue and organ development during embryonic and post-embryonic stages (Tang *et al*., 2018; Lindberg *et al*., 2018). It has also been known to be involved in forming several sensory organs and limbs. It confers its function by binding to the specific sequence of the DNA, which is present in the promoter region of several regulatory genes. Nub contains both the POU-specific domain and homeodomain, which play a role in the binding to the conserved DNA sequence and hence regulate the expression of the target genes.

Nub is highly expressed in the *I. scapularis* gut with heightened expression at 48 hours after tick placement. Nub-knockdown ticks can feed yet with lesser body weight, and up to 40% are unable to molt, suggesting possible involvement of Nub in acquisition or processing of blood meals.

Although precise molecular functions of Nub remain unknown, we show involvement of this protein in multiplex protein-protein interactions. The fact that these interacting partners encompass cuticular proteins, proteins involved in metabolism (mannosyl-oligosaccharide, cytochrome-c reductase, ribonucleoprotein, NADH-oxidoreductase), and proteins involved in cytokinesis, suggests Nub as multi-functional transcription factor. Similarly, when the transcripts were compared between the control (dsGFP) and the dsNub ticks, there are additional genes transcripts observed to be altered, such as spindle pole body component, metalloproteinase, ATPase subunit e 1-like, epoxide hydrolase, ribonuclease, and ATP synthase – the significance of which remains to be elucidated. The exact molecular mechanism of the feeding behavior of ticks and their subsequent development is likely to be complex, and the genes involved can vary among different species of ticks. Nevertheless, overall, our data indicate that silencing of Nub impacts the tick's feeding behavior and development, warranting future investigation in this poorly-studied yet important area of vector biology.

Chapter 5: Concluding Remarks

Like all metazoan organisms, gut cells in *I. scapularis* are likely maintained by robust homeostasis mechanisms, including cell division, polarization, and regeneration. Although these are likely to be conserved, the specific molecular mechanism of gut cell maintenance may vary among different species of ticks. Tissue differentiation is a crucial step where cells acquire specific lineages and functions (Sonenshine and Roe, 2013). In the tick gut, the cells potentially acquire specific fates required for their specialized functions, such as nutrient absorption and digestion, amongst others. There can be a range of differentiated gut cells, such as digestive, absorptive, and secretory cells, although their precise biology remains largely unknown. The overall activity of gut cells is controlled by molecular mechanisms such as the maintenance of gene expression and signaling pathways (Navascues *et al*., 2012) dictated by specific transcription factors; however, future empirical studies are required to address these questions.

Two transcription factors explored here are SuH and Nub, both of which are involved in tick hematophagic events that ultimately influence the tick postembryonic development. These transcription factors bind to their cognitive DNA promoters (at least as evidenced for SuH) as well as demonstrated involvement in multiplex protein-protein interactions, suggesting their pleiotropic roles in tick biology. In case of SuH, we also show that the transcription factor influence tick developmental signaling pathways, including EGF signaling. We also showed that specific signaling components of EGF pathway, such as Spitz play a critical role in tick development. Additional studies are required to tie these observations, precisely how selected transcription factors like SuH and Nub interface with specific tick signaling pathways or additional cuticular or metabolic components and drive tick hematophagy and development. Tick-borne diseases are on

the rise, along with the discovery of new, more virulent tick-borne pathogens. A continued effort on evidence-based, fundamental knowledge of the molecular basis of tick biology, development, and vectorial capacity will contribute to the future development of potential preventive or therapeutic interventions against tick-borne infections.

Table 14: Oligonucleotide primers used in the study

Primers	Accession no	Sequence
Egfr-RT-F	ISCW021718	GCGAGCACCACTACCAGAACC
Egfr-RT-R	ISCW021718	GCACGTAGCCGGTCACCTC
Fz-RT-F	ISCW004077	CCACCAGAAGCAAGAGGACGC
Fz-RT-R	ISCW004077	CGAGGATGGTGCATACGGGC
Hep1-RT-F	ISCW023081	CGCGCAATTGTCTGTCCGC
Hep1-RT-R	ISCW023081	GTCCGTCAGATCGTCTGCCG
Mbc-RT-F	ISCW021297	GCGAGAGTGGCACCCTGT
Mbc-RT-R	ISCW021297	CCCACATCTGGTCCTGGGTC
Rac1-RT-F	ISCW016255	GGACTACGACCGGCTCAGG
Rac1-RT-R	ISCW016255	CCGCAACTCACCTTGGCTCT
Spitz-RT-F	ISCW002520	GTGGTACTGCCTCAACGGAGC
Spitz-RT-R	ISCW002520	CCCATGTACCCGTCTGCACAC
Wg-RT-F	ISCW020393	TCGCGCAAATGGACTCGTCG
Wg-RT-R	ISCW020393	GCAGGTGACCAGCGAGCA
GATA-2-RT-F	ISCW020081	CAAGCCGTACACCAGGTGG
GATA-2- RT-R	ISCW020081	GAACGCCTTCGGGAGACG
GATA-5-RT-F	ISCW004992	GTTGTGCCAACTGCAAGACG
GATA-5-RT-R	ISCW004992	GCTCTTGGAGGACAGCTTG
GATA-Box A-RT-F	ISCW005453	GGAATGGATGCCAGCGGTG
GATA-Box A-RT-R	ISCW005453	CATAACGGCGTCGAAATGGC
GATA BF-RT- F	ISCW022317	GACAGCAAGGGAGGCCTG
GATA BF-RT-R	ISCW022317	ACGTACTGCTCGTCGAACG
GATA-Zn-10-RT-F	ISCW010833	CTGGCGAGACTCATCTGCA
GATA-Zn-10-RT-R	ISCW010833	GCAGTCTTCGTTCGAACGG
Nub2-RT-F	ISCW020718	CTGACGTCGGTGCACCTG
Nub2-RT-R	ISCW020718	GTGGTGGTGGTGCGAGAG
Nub1-RT-F	ISCW022240	CCTCATCATGTCAAATCGAGACCAG
Nub1-RT-R	ISCW022240	GTTGAGCGCCTCGAACCG
POU5-RT-F	ISCW017821	CACTGCAGGACGACGCCA
POU5-RT-R	ISCW017821	CGCAAAGCCCACCAAGCC
POU7-RT-F	ISCW006423	GTGCCCGGCTAAGATCGG
POU7-RT-R	ISCW006423	GCTGTACTGCTGCACCTCG
POU8-RT-F	ISCW007997	CCCTTCCACCATGGACGG
POU8-RT-R	ISCW007997	GAAGCCGCCCATGCCGTA
POU9-RT-F	ISCW005049	GGCAGTCTCGCTTCGAGC
POU9-RT-R	ISCW005049	CGCATCATCATGTCGCGGA
SuH-RT-F	ISCW010197	CGACATGGTCCTGGTGATTCTAC
SuH-RT-R	ISCW010197	GTCGCGCAGAAGCTGGTC

GATA NR-F	ISCW018843	GCAGCAGCAGCAGGCCAA
GATA-NR-R	ISCW018843	GTACTGGTCCTGCAGCAGG
Fork1-RT-F	ISCW003951	CCTCCAGCGGTCACAGCA
Fork1-RT-R	ISCW003951	GGTCTGGCGCAAGAAGGC
Fork2-RT-F	ISCW015937	CGTGACGCCGTCGAACTC
Fork2-RT-R	ISCW015937	CACGCTGCTGCTGTCTCAC
Fork3-RT-F	ISCW010527	CTCCACGTACGGCGACCA
Fork3-RT-R	ISCW010527	CGGTAGTACGGGAACCGG
Fork11-RT-F	ISCW002976	GGTGTGCAACGGCAAGAAC
Fork11-RT-R	ISCW002976	CCACCAGCGACTGGAACAT
Fork13-RT-F	ISCW016268	CTCCCGGCCGAAACATGG
Fork13-RT-R	ISCW016268	GTGGTGTTGCTGCCGACC
Jumu-RT-F	ISCW008179	GACCGCAGCTACCCGAAG
Jumu-RT-R	ISCW008179	GTTCAGGGACAGGTTGTGC
Nub protein expression-F	ISCW022240	CGC GGA TCC ATG ACG TTG GAA AAT CTG GAT GAC
Nub protein expression -R	ISCW022240	ACG CGT CGA CCT ACT CCG GCT TGA GGA G
SuH protein expression- F	ISCW010197	ATA GGA TCC ATG AGC GAC GTG TAC CTC CC
SuH protein expression- R	ISCW010197	ACGCGTCGACTCAGGACATGTTCTGGTG GTG
SuH-F1	ISCW010197	CGCGGATCCATGCTGGTGATTCTACACG CCAAGG
SuH-F2	ISCW010197	CGCGGATCCATGGACGGCGGCAACTTC CACG
SuH-R	ISCW010197	ACGCGTCGACTTACCTCAGGATGTCGTG CACCAG
Nubbin RNAi FP	ISCW022240	TAATACGACTCACTATAGGGATGGACG ACGAGCACATGGA
Nubbin RNAi RP	ISCW022240	TAATACGACTCACTATAGGGTGCCTGCG AGGACAGCATGC
GATA RNAi_FP	ISCW004992	TAATACGACTCACTATAGGGCAAGCGA GAAGGTACCTTCC
GATA RNAi_RP	ISCW004992	TAATACGACTCACTATAGGGCCTCCTTC TTCATCGTCAGC
SuH_RNAi_FP	ISCW010197	TAATACGACTCACTATAGGGCAACTCGG ACCAGGACATGC
SuH_ RNAi_RP	ISCW010197	TAATACGACTCACTATAGGGAGCAGAC AAGCTTGACGGTG
Rac protein expression-F	ISCW016255	CGCGAATTCATGCTAACGTCCTTGATTC CGCACCTC
Rac protein expression-R	ISCW016255	ACGCGTCGACTTATCAAAGCAGGCAGC AGGG
Rac RNAi-F	ISCW016255	TAATACGACTCACTATAGGGACAACCA ACGCCTTTCCTGG
Rac RNAi-R	ISCW016255	TAATACGACTCACTATAGGGCCTCTTCT TCTTGGGCTTGG
RNAi-F	ISCW020718	TAATACGACTCACTATAGGGATGGCAA CGGGCTCGTCGAACC
RNAi-R	ISCW020718	TAATACGACTCACTATAGGGCCAGCATG CCGTTCATGC
RT-F	ISCW021054	CCATGCCCGAGGCAGAAGC

RT-R	ISCW021054	CCATCAGGCCGGCTTCGT
RT-F	ISCW001025	GGTACAACGCACGTACAAAAATG
RT-R	ISCW001025	CAGACACAGCGCGTGAGC
RT-F	ISCW020093	GGTACAACGCACGTACAAAAATG
RT-R	ISCW020093	CAGACACAGCGCGTGAGC
RT-F	ISCW015819	GTCTGAGCGGTGGCGAGC
RT-R	ISCW015819	CGAGAAGACTGCCCTGCG
RT-F	ISCW010367	GCATCTCGATCGGCATCCC
RT-R	ISCW010367	CCGTGACCCCTGCTCTCT
RT-F	ISCW003667	GGTCCCAGCAGTTCTGCCTC
RT-R	ISCW003667	GACGAACAGCGCCTGGAAG
RT-F	ISCW016537	GACGATGGAACCGATCCCG
RT-R	ISCW016537	CGAAGACATCGTCCCGGT
RT-F	ISCW014470	CGTCCTGAAGACCGGCC
RT-R	ISCW014470	CCAAGATGTCCGCCTTTTCC
RT-F	ISCW008805	CGCCGTCGAGTCGACAAC
RT-R	ISCW008805	CCTGCTGTTCCATCTGCGA
RT-F	ISCW016268	CCTGTTGACGATGCCCGTGA
RT-R	ISCW016268	GGAGGCCATGGACACCATG
RT-F	ISCW015581	CTCGCACGTCAATCCCGC
RT-R	ISCW015581	CGTACGCGTGGTGTTGCTG
RT-F	ISCW008179	CGTAACTTCGGCTGCGCCA
RT-R	ISCW008179	CCGTTCACCTGTGTGTGTCTTC
RT-F	ISCW015937	GCTCTACGAAGACGCCTTCAG
RT-R	ISCW015937	GCGAGATGCCGTGCGAAC
RT-F	ISCW004287	GTGACGCCGTCGAACTCAG
RT-R	ISCW004287	CGTTCTGCTGCGAATGCG
RT-F	ISCW000140	GGCTGTCGTGGCACTCC
RT-R	ISCW000140	GTGCCTTGCCTGCAGACT
RT-F	ISCW019283	CGTCAGCCTCGATGACGTG
RT-R	ISCW019283	CTCGGCGGTGCTGTTGAC
RT-F	ISCW010869	CCTCCGTACAAGGTCAGGGTG
RT-R	ISCW010869	CTGCTCTCCGGTCGACGG
RT-F	ISCW004440	GAGCAATGGCTACAGCGGC
RT-R	ISCW004440	CTTCAGCTTCTTGACGAGCGAG
RT-F	ISCW017122	GGTGGCCTGCGGAATACCG
RT-R	ISCW017122	GGTTGCTGAGCACGCAGG
RT-F	ISCW006409	GACCGCTCCAGGTGCTCC
RT-R	ISCW006409	GGCCAATGGGAATCACCTGC
RT-F	ISCW023685	CCGAAACGCTTCTGCTGCAC
RT-R	ISCW023685	GGACAACCATTCTGCACCG
RT-F	ISCW005453	GCAGTGTAACAAGCTGGCCAG
RT-R	ISCW005453	CCCGACTGAGGCCCGAGT
RT-F	ISCW015582	GCTGGTGACCTTCCCCAAG
RT-R	ISCW015582	GACGGGTGCACCGCTGAC
RT-F	ISCW017895	GGCAGCTCCGGGTGAGTG
RT-R	ISCW017895	GGCACAGTGGACAGCGGG
RT-F	ISCW012049	GATTCTCGCTCCAGAATCTGGC
RT-R	ISCW012049	GTGGGTGCGGAGGTGGAC
RT-F	ISCW000054	CCTGAACACCGGCAACTCG
RT-R	ISCW000054	CCTCACCGCTGTGCTTCTC
RT-F	ISCW019865	GCGTTCAAGGAGCGAGAGG

RT-R	ISCW019865	CTTGAACCGCGTCACCGC
RT-F	ISCW005617	CAGCTGCGACCTCTGCAAC
RT-R	ISCW005617	TGAAGCGCGCCGAGCAGA
RT-F	ISCW016356	GAGGCGAGACGCATCTACGC
RT-R	ISCW016356	CGTCGAGAACCCCACCGG
RT-F	ISCW009191	GGAGGCGATGTCCTTCGG
RT-R	ISCW009191	CCGTACTTGCGACTGCACA
RT-F	ISCW010121	GCTCGCTCGCTGATCTGAAC
RT-R	ISCW010121	GGCACTTGTATGGACGCTCC
GATA Zn1-RT-F	ISCW018843	GCAGCAGCAGCAGGCCAA
GATA-Zn1-RT-R	ISCW018843	GTACTGGTCCTGCAGCAGG

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