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Ehrlichia chaffeensis TRP32 is a Nucleomodulin that Directly Regulates Host Gene Expression and Post-Translational Modifications Determine its Localization and Function

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Ehrlichia chaffeensis TRP32 is a Nucleomodulin that Regulates Host Gene Expression and Post-Translational Modifications Determine its Localization and Function

by

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Dedication

To my mother

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Ehrlichia chaffeensis is an obligately intracellular bacterium that reprograms the mononuclear phagocyte through diverse effector-host interactions to modulate numerous host cell processes, including transcription. Previously, we reported that *E. chaffeensis* TRP32, a type 1 secreted effector, interacts with multiple host nucleus-associated proteins and also auto-activates reporter gene expression in yeast. In these studies, I demonstrate that TRP32 is a nucleomodulin that binds host DNA and alters host gene transcription and which is regulated by various post-translational modifications including phosphorylation and ubiquitination. TRP32 enters the host cell nucleus via a noncanonical translocation mechanism that involves phosphorylation of Y179 located in a C-terminal tri-tyrosine motif. Both genistein and mutation of Y179 inhibited TRP32 nuclear entry. I also show that TRP32 is mono and poly-

ubiquitinated on multiple lysine residues during infection by the host E3 Ub ligase NEDD4L. When TRP32 poly-ubiquitin chains were examined by immunoblotting K63-linked chains were detected but not K48 or K11-linked chains and TRP32 was not responsive to treatment with the proteasome inhibitor carfilzomib. An electromobility shift assay (EMSA) demonstrated TRP32 binding to host DNA via its tandem repeat domain. TRP32 DNA binding and motif preference were further confirmed by supershift assays, as well as competition and mutant probe analyses. Using ChIP-Seq, I determined that TRP32 binds a G-rich motif primarily within ±500 bp of the gene transcription start site. An ontology analysis identified genes involved in processes such as immune cell differentiation, chromatin remodeling, and RNA transcription and processing, as primary TRP32 targets. TRP32 bound genes (n=1223) were distributed on all chromosomes and included several global regulators of proliferation and inflammation such as FOS and JUN, AKT3 and NRAS, and non-coding RNA genes, miRNA 21 and miRNA 142. TRP32 target genes were differentially regulated during infection, and direct repression/activation of these genes by TRP32 was confirmed *in vitro* with a cellular luciferase reporter assay. Additionally, I show that treatment with the HECT-ligase inhibitor, heclin, alters TRP32 subnuclear localization and impairs TRP32's ability to repress transcription of target genes in a luciferase assay and that this can be phenocopied using K63R and K123R mutants of TRP32.

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List of Abbreviations

ARDS	Acute respiratory distress syndrome
BED	Browser extensible data
ChIP	Chromatin immunoprecipitation
Cnt	Control
DAPI	4′,6-diamidino-2-phenylindole
DC	Dense-cored cell
DNA	Deoxyribonucleic acid
ESCRT-0	Endosomal sorting complexes required for transport protein complex 0
EM	Electron microscopy
EMSA	Electromobility shift assay
EtpE	Entry triggering protein
FBS	Fetal bovine serum
GREAT	Genomic regions enrichment and annotation tool
HHEX	hematopoietically expressed homeobox
HLH	Hemophagocytic lymphohistiocytosis
HME	Human monocytotropic ehrlichiosis
hpi	Hours post infection
IP	Immunoprecipitation
kDa	Kilodalton
LPS	Lipopolysaccharide

MEME	Multiple EM motif elicitation
PCGF5	Polycomb group protein 5
PCR	Polymerase chain reaction
PTM	Post-translational modification
qPCR	Quantitative reverse transcriptase polymerase chain reaction
RC	Reticulate cell
RNA	Ribonucleic acid
SD	Standard deviation
SnoRNA	Small, nucleolar RNA
SUMO	Small ubiquitin-like modifier
T1SS	Type 1 secretion system
T4SS	Type 4 secretion system
TAD	Transactivation doman
TAL	Transcription activator-like
TR	Tandem repeat
TRP	Tandem repeat protein
Ub	Ubiquitin
WT	Wild-type

Chapter 1. Introduction

EHRLICHIA CHAFFEENSIS

Ehrlichia species are tick borne gram-negative, obligately intracellular bacteria in the alpha subgroup of proteobacteria. They belong to the family Anaplasmataceae within the order Rickettsiales. Along with *Ehrlichia,* Anaplasmataceae includes the genera *Wolbachia, Neorickettsia*, *Anaplasma,* and *Neoehrlichia*. The genus *Ehrlichia* contains six named species: *E. ruminantium, E. canis, E. chaffeensis*, *E. ewingii, E. muris*, *E. minasensis,* all of which are known vertebrate pathogens with the exception of *E. minasensis*, which thus far has only been detected in *Rhipicephalus microplus* ticks [1]. *Ehrlichia* species became known to science in the early 1900s with the discovery of *E. ruminantium,* the etiologic agent of the ruminant disease heartwater [2]. Subsequently, several additional ehrlichial species were identified as veterinary pathogens but it was not until 1987 that the first ehrlichial infection in humans was reported, and 1991 that the new etiological agent *E. chaffeensis* described [3-6]. Since then, *E. chaffeensis* and *E. ewingii* have been the primary human ehrlichial pathogens causing human monocytotropic ehrlichiosis (HME) and human granulocytic ehrlichiosis (HGE), respectively. However, naturally occurring human infections by *E. canis* and *E. ruminantium* have been reported in South and Central America and South Africa, respectively. In addition, serological evidence suggests human infections with *E. muris* occur in Japan [7-9]. Most recently, a new *E. muris*-like agent, possibly a strain of *E. muris,* has

emerged as a human pathogen in the northern US [10]. In addition, human infection with novel ehrlichial strains including the Panola Mountain *Ehrlichia* species have been described [11]. Ehrlichial species are not only globally significant veterinary pathogens, but also a significant cause of life-threatening human tick borne zoonoses. They have limited antibiotic susceptibility, and challenges they present with regard to diagnosis and treatment are reasons that human ehrlichioses continue to be an emerging human health threat.

HUMAN MONOCYTOTROPIC EHRLICHIOSIS

HME is a serious, potentially fatal tick borne disease with a relatively undifferentiated clinical presentation. Approximately half of infected individuals require hospitalization and HME has a case fatality rate of around 3% [12, 13]. There is serological evidence which suggests that some individuals may experience asymptomatic infections; however, the occurrence of cross-reactivity between different ehrlichial species with varying virulence make this a subject of debate [14]. Symptoms of HME are “flu-like” and typically include fever, malaise, myalgias, arthralgias and headache. Gastrointestinal symptoms ranging from abdominal pain to nausea and diarrhea also occur in around 50% of patients [14]. Less common presentations include respiratory symptoms such as cough and pharyngitis, conjunctivitis and changes in mental status due to meningitis [15-23]. Rash is also present in a minority of adults (~30%), but in more than 60% of pediatric patients [24, 25]. Laboratory findings show signs of hematological abnormalities, hepatitis and altered electrolytes. Thrombocytopenia and leukopenia occur in most cases and approximately half of

patients develop anemia. Elevated hepatic transaminases are typical and elevations in bilirubin and alkaline phosphatases may occur with worsening disease severity [14, 24-26]. In cases of severe disease, laboratory findings reflect multisystem impairment and can include signs of impaired clotting, as well as decreased kidney and liver function and acute respiratory distress [19, 27]. This multisystem impairment can progress to organ failure with cardiac, respiratory, hepatic or renal failure and metabolic acidosis and disseminated intravascular coagulopathy having been reported [14, 28, 29]. This multisystem disease has been likened to a toxic or septic shock-like syndrome, a comparison which is supported by various manifestations of the disease [30]. For instance, in immunocompetent patients, disease severity is not correlated with a high bacterial burden, rather only a few bacteria are detected, suggesting an immunopathological mechanism [31]. Further, although *Ehrlichia* lacks lipopolysaccharide (LPS), a classical stimulator of septic shock, during infection high levels of proinflammatory cytokines are produced [32, 33]. Recently, HME has been linked to several cases of hemophagocytic lymphohistiocytosis (HLH), a severe immune hyperactivation syndrome characterized by increased levels of activated macrophages which phagocytize other cells [34-41]. HLH has traditionally been seen as a mimic of sepsis and shows many of the same symptoms of septic/toxic shock and severe HME (fever, shock, capillary leak syndrome, ARDS, cytopenias, abnormal liver tests, disseminated intravascular coagulation, elevated inflammatory markers, multiorgan failure and death) [42,

43]. However, the prevalence of this condition in severe HME is currently not known.

EHRLICHIA CHAFFEENSIS LIFECYCLE

Although *E. chaffeensis* is an important emerging human pathogen, humans are only an incidental host. In nature, *E. chaffeensis* is maintained in a zoonotic cycle consisting of vertebrate hosts, the most important of which is the white-tailed deer (*Odocoileus virginianus*), which can support a persistent infection and the arthropod vector, the lone star tick (*Amblyomma americanum*). Humans are infected through the bite of an infected tick, and the risk factors for infection include outdoor occupational or recreational activities, seasonality (spring and summer) in lone star tick endemic regions (throughout most of the southeastern, south central US). Additionally, men are more commonly infected than women and older adults more than young adults or children [14, 24]. In humans, *E. chaffeensis* preferentially infects mononuclear phagocytes and are seen primarily in monocytes. However, on occasion ehrlichiae have been observed in other cell types including lymphocytes and even occasionally in granulocytes [44, 45].

During infection of mammalian cells, *E. chaffeensis* exists as two distinct ultrastructural forms, a dense-cored cell (DC) form and a reticulate cell (RC) form. The dense-cored cell, named for its dark staining electron dense core seen by electron microscopy (EM), is the infectious form. It attaches to the host monocyte and is internalized in a manner that resembles G-coupled receptor mediated endocytosis and which requires host lipid rafts and caveolin-1[46, 47].

Although the mechanism is not completely defined, several *E. chaffeensis* and host proteins have been shown to be involved. These include the host GPI-anchored protein DNase-X, the ehrlichial entry triggering protein EtpE, and the 120 kDa tandem repeat effector (TRP120) [47, 48]. Other TRPs including TRP120 were also shown to be able to facilitate internalization of TRP-coated beads and internalization of both bacteria and beads resulted in several signaling events including transglutamination, tyrosine phosphorylation and activation of phospholipase Cγ2 (PLC-γ2), inositol 1,4,5-trisphosphate (IP3) production, and release of intracellular calcium [46, 49, 50]. Although, the exact role these proteins play in bacterial entry is unknown, it appears that TRPs interact with unknown Wnt receptor(s) on the host cell membrane, thereby activating noncanoncial Wnt signaling and triggering phagocytosis and entry into the host cell.

Once inside the host cell, the DC remains in a membrane-bound vacuole called a morula (Latin for mulberry), that phenotypically resembles an early endosome [51, 52]. Within this compartment, the DC transitions into a RC, which is the replicative form of *E. chaffeensis,* and begins dividing at around 24 hours post-nfection (24 hpi). The RCs will continue to double every 8.5-12 h until between 48 and 72 hpi when some of the bacteria begin to transition back into DCs. By 72-96 hpi most of the bacteria will have stopped dividing and become DCs, ready to infect a new cell [53-55]. These infectious forms are released by an unknown mechanism that may involve host cell lysis or exocytosis. Throughout this process, the ehrlichiae remain within the morula, which

transitions from small, barely detectable inclusions (24 hpi), into large inclusions which dominate the host cell cytoplasm (48-72 hpi). There are reports that morulae may fuse or divide based on unknown signals, but this has not been well-studied [54, 55]. Many of the mechanisms by which *E. chaffeensis* survives within the host cell, creates and maintains a protective vacuole, acquires nutrients, and avoids host cell killing and immune detection remain undefined. However, early reports show that *E. chaffeensis* infection leads to perturbation of many host processes. Gene transcriptional analysis has determined that 4.5% of all host genes are differentially expressed during *E. chaffeensis* infection. Moreover, of the genes that are differentially expressed many are involved in infection critical cellular processes and the expression patterns of these genes were often unique to *E. chaffeensis* and were not observed during infection with other intracellular bacteria [56]. Further studies into specific pathways targeted by *Ehrlichia* confirmed these results and have shown that secreted effector proteins are often required to mediate these changes.

SECRETION OF BACTERIAL EFFECTORS

Bacterial secretion systems are an important tool that bacteria use for manipulating the extracellular environment. *E. chaffeensis* possess two important types of secretion systems, Type I and Type IV (TISS, T4SS, respectively), that are widespread in gram-negative bacteria and play a role in effector secretion.

Although we will not address the T4SS in detail, it is worth noting because the *E. chaffeensis* T4SS is quite unusual. The traditional T4SS, as characterized

in *Agrobacterium tumefaciens* consists of 12 proteins (VirB1-11 and VirD4) that together form an ATPase motor and a pore through which bacterial proteins or DNA can be exported [57]. However, interestingly in *E. chaffeensis* the loci which code for some of these genes have undergone duplications such that *E. chaffeensis* contains four non-identical versions of VirB4 (ATPase) and VirB6 (inner membrane channel component). All of these proteins are coexpressed during infection and interact with each other and other members of the T4SS. To add further complexity, these VirB6 homologs are all from 3–10-fold larger than the prototypical VirB6. This suggests that *E. chaffeensis* may possess a structurally novel T4SS channel [55, 58, 59]. The *E. chaffeensis* T4SS has not been functionally tested; however, T4SS components are upregulated during the exponential growth phase of the bacteria in monocytes suggesting that this system is functional and may be required for *E. chaffeensis* growth and virulence [58, 59]. Additionally, a T4SS substrate was identified, ECH0825, which interacts with VirD4 and is secreted during infection. ECH0825 was shown to prevent host cell apoptosis by stabilizing host mitochondria and decreasing ROS production [60]. Several other hypothetical T4SS substrates have been identified in *E. chaffeensis* by computational predictions (ECH0261, ECH0767, ECH0389, ECH0653, ECH0684, ECH0877), but have not been experimentally confirmed [60].

More important to our studies is the ehrlichial TISS. It is a typical ATP-binding cassette (ABC) transporter system and is made up of three proteins (ATP-binding protein (ECH0383), HlyD membrane fusion protein (ECH0970) and

TolC outer member protein(ECH1020)) that create a channel allowing for one-step secretion of bacterial effectors from the bacterial cytoplasm into the extracellular environment [61, 62]. This secretion system recognizes substrates that are typically repeat containing proteins with enrichment of specific amino acids [LDAVTSIF] in the final 50 amino acids of their C-termini [63, 64]. Although functional studies of this secretion system have not been performed in *E. chaffeensis*, a study using a heterologous system in *Escherichia coli* was performed that identified several *E. chaffeensis* TISS substrates including the 200 kDa ankyrin repeat protein (Ank200) and several TRPs [65]. These TRPs are important effectors with similarities to the repeats-in-toxins family of secreted proteins.

TANDEM REPEAT PROTEINS

The TRP effectors of *E. chaffeensis* were originally identified as immunodominant proteins against which protective antibody responses were generated to linear epitopes found in the repeat regions [66, 67]. So far, four TRPs have been identified which range from 198 AAs (TRP32) to 584 AAs (TRP75). The TRPs are acidic proteins which migrate more slowly than predicted due to their low pIs and are named based on their apparent molecular weight (TRP32, TRP47, TRP75 and TRP120) [61]. The TRPs possess a variable number of internal repeats which also vary in size and sequence, with TRP32, TRP47 and TRP120 possessing serine-rich, acidic repeats that range from 19 (TRP47) to 80 (TRP120) AAs, while TRP75 possesses lysine-rich and basic repeats that are 24 AAs long [66, 68-71]. The number of repeats varies as

well between strains with TRP32 showing the greatest amount of inter-strain variability possessing from 3 repeats (strain Salpulpa) to 6 repeats (strain Wakulla) [72-74]. This is interesting because *E. chaffeensis* shows strain dependent virulence differences; however, the contribution of the TRPs to this difference is unknown [75].

As previously mentioned, TRPs are secreted by the T1SS into the morula space, eventually escaping the morula by an unknown mechanism. TRPs then traffic to various subcellular locations, including the nucleus, where they interact with a multitude of host proteins involved in important host cell processes [74]. Until recently, despite our knowledge of TRP localization and interacting partners, the function of these TRPs was unknown. However, our laboratory has since shown that TRP120 interacts with host chromatin via its TR domain in a manner that is hypothesized to be like that of the Transcription-activator-like (TAL) effectors *of Xanthomonas* [76, 77]. We showed that TRP120 enters the host nucleus and binds to a GC-rich motif within regulatory regions of specific host genes. TRP120 targets include genes involved in transcriptional regulation, signal transduction, and apoptosis, which were upregulated during infection or when TRP120 was directly introduced into the cell [78]. Although, the mechanisms by which TRP120 regulates host gene expression are not fully understood, TRP120 interactions with host epigenetic regulators such as PCGF5 may play a role [79]. Interestingly, these interactions may be regulated by post-translational modification of TRP120. TRP120 is SUMOylated, and this modification is important for interaction with several host proteins, including

PCGF5 [80]. However, whether this PTM is required for TRP120 transcription factor function is unknown.

PROJECT INTRODUCTION

The previously mentioned studies peaked our curiosity as to whether TRP120 interactions and function were representative of other TRPs. Although the long-term goal of this project was to determine the role of all *E. chaffeensis* TRPs in modulating host responses during infection and the mechanism by which they function, I focused on TRP32 in this project for several reasons. First, the gene coding for TRP32 is one of the most highly upregulated ehrlichial genes during infection of monocytes [81]. This suggests that TRP32 might play an important role in ehrlichial survival within this cell type. Next, both full-length TRP32 and a construct consisting of the amino-terminus and tandem repeats were able to activate transcription of a reporter gene autonomously in yeast two-hybrid studies [82]. This is typical of transcription factors as they often contain transactivation domains (TAD) which are able to recruit members of the transcription initiation complex independently of the "prey" which is simply putative interacting proteins fused to a heterologous TAD. Finally, TRP32 localized to the nucleus of cells when ectopically expressed and was shown to interact with several host nuclear proteins [82]. Together, these findings formed the premise of my hypothesis that *TRP32 is a pathogen encoded transcription factor that modulates transcription of host genes to promote bacterial survival and is regulated in part by post-translational modifications which alter its subcellular localization.* The findings of my research led me to propose the

current project originally titled: "*Ehrlichia* TRP32: Host Transcriptional Modulations and PTMs that Define Function and Subcellular Trafficking." The objective of this project is to demonstrate that TRP32 is a nucleomodulin with transcription factor function and to identify and characterize TRP32 PTMs. Originally, three specific aims were proposed to test this hypothesis. **Aim 1** was to demonstrate that TRP32 modulates host transcription (Ch3). **Aim 2** was to determine the role of mTOR in phosphorylating TRP32 and the impact of phosphorylation on TRP32 nuclear localization (Ch3)**. Aim 3** was to define the mechanism of TRP32 ubiquitination and the role of ubiquitination of TRP32 localization and degradation (Ch4). These aims were completed mostly as planned although as experiments were completed, some changes were made based on the data obtained. Notably, I could not confirm a role for mTOR in phosphorylating TRP32. Additionally, ubiquitination seems to play a more important role in TRP32 transcription factor function than localization and does not seem to be required for nuclear/cytoplasmic shuffling or degradation**.**

However, all's well that ends well.

Chapter 2. Materials and Methods

Cell culture and infection. *Ehrlichia chaffeensis* (Arkansas strain) was propagated in a human monocytic cell line (THP-1; ATCC, Manassas, VA). THP-1 cells were maintained in RPMI 1640 with HEPES (25mM) (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 5 mM L-glutamine, 1% sodium pyruvate, and 12.5 ml 10% glucose (Sigma; St. Louis, MO) at 37°C in a 5% CO_2 atmosphere. *Ehrlichia chaffeensis* infection was maintained by subculturing infected cells with uninfected cells. HeLa cells (human cervical epithelial) for transfection were grown in MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone) and maintained in a 5% CO_2 atmosphere. When stated, cells were incubated with 10mM bortezomib (ApexBio; Houston, TX), PYR41 (Thermo Fisher; Boston, MA), Heclin (Sigma), carfilzomib (ApexBio) or vehicle (DMSO) for 12-24 h before harvesting cells for lysate or luciferase expression measurement.

Expression and purification of recombinant TRP32. Full-length and truncated TRP32 gene fragments were PCR amplified from *E. chaffeensis* genomic material and cloned into pGEX-6p1 vectors (GE Healthcare; Piscataway, NJ). The constructs were transformed into BL21 *E. coli* (Genlantis; San Diego, CA) for protein expression. Briefly, overnight cultures were diluted 1:20 in LB plus ampicillin (Amp) and grown for 3 h with agitation at 37°C then

protein expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM and growing for another for 3-4 h at 37°C. Cells were then suspended in TBS with protease inhibitors (cOmplete mini, EDTA free) (Sigma), lysed by sonication, then cleared by centrifuging for 20 min at 12,000 x g at 4ºC. Cleared lysate was then added to washed Glutathione Sepharose 4B (GE) and recombinant proteins were purified according to the manufacturer's instructions.

***In vitro* microfluidic peptide ubiquitination array**. To determine the ubiquitination potential of TRP32 lysine residues, peptides (12-mer) corresponding to TRP32 putative ubiquitination targets were synthesized by flanking a central lysine residue with 6 N-terminal amino acids and 5 C-terminal amino acids. For each peptide, a corresponding negative control sequence was included with alanine (A) substituted in place of K (A peptide). Peptides were synthesized on chip and an *in vitro* ubiquitination assay performed as previously described [83]. Briefly.ubiquitination reactions were performed using an *in vitro* ubiquination kit (Enzo Life Sciences; Farmingdale, NY) in the presence of THP-1 lysate. Nonspecifically bound proteins were removed by washing with buffer containing 1% SDS, 0.1% β-mercaptoethanol and 100 mM Tris for 30 min, followed by a PBS wash containing 1% Tween-20 (PBST) then ubiquitin was detected using anti-Ub Alexa Fluor 647 antibody (Santa Cruz Biotechnology, 1:300). The signal was then read using an Anon GenePix 4400A (Molecular devices) scanner running GenePix Pro 7 software. Positive signals were determined by comparison with the control peptides and negative controls for

each assay. The data are presented as mean ± SD. Differences between wild-type and control peptides were assessed using the two-tailed Student's t test, and significance was indicated by a *p*-value of <0.05.

***In vitro* ubiquitination assay.** TRP32 ubiquitination was performed with recombinant TRP32 and an *in vitro* ubiquitination kit (Enzo Life Science). Briefly, TRP32 (200 nM) was added to ubiquitination buffer, biotin-tagged ubiquitin protein, E1 ligase and the E2 ligase UBCH5b with and without the E3 NEDD4L and Mg-ATP. Reaction mixtures were incubated at 30°C for 3 h and then boiled with 1x lithium dodecyl sulfate (LDS) sample buffer. Samples were separated by SDS-PAGE and proteins detected by Western blotting with rabbit anti-TRP32 (1:10,000) or anti-Ub (FK2, Enzo, 1:1,000) primary antibodies followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:10,000) (KPL; Gaithersburg, MD). Bound antibodies were visualized after incubation with ECL substrate (Pierce; Rockford, IL).

Ubiquitin immunoprecipitation. In order to determine if TRP32 is ubiquitinated in mammalian cells, HeLa cells were cotransfected with the empty GFP vector (pAC-GFP-CI; Clontech, Mountain View ,CA) or GFP-TRP32, and wildtype or lysine null HA-tagged ubiquitin constructs by use of Lipofectamine 2000 (Invitrogen). Cells were collected and lysed in 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 20 mM NEM (covalent isopeptidase inhibitor; Sigma), 1% Triton X-100, and protease inhibitors (Complete mini, EDTA-free; Sigma) at 24 h posttransfection. Lysates were then centrifuged at 4°C and 16,000 × g (Eppendorf 5430R centrifuge with model FA 45-30-11 rotor) for 20 min, and the

supernatants were transferred to washed anti-HA agarose (Thermo Scientific). Following overnight incubation at 4°C, resin was washed with lysis buffer, boiled in LDS buffer, and analyzed by SDS-PAGE and Western blotting.

Localization studies. TRP32 localization during infection was examined by immunofluorescent and laser confocal microscopy. *E. chaffeensis*-infected and uninfected THP-1 cells were cytocentrifuged unto glass slides at 24, 48 and 72 hpi and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were then permeabilized using 1% Triton X-100 in PBS with 5% bovine serum albumin for 1 h. After permeabilization, cells were incubated with rabbit anti-Dsb (1:1000) and mouse anti-TRP32 (1:1000) for 30 min, then washed and labeled with Alexa Fluor 488-IgG(H+L) conjugated goat anti-mouse and Alexa Fluor 594-IgG(H+L) conjugated goat anti-rabbbit secondary antibodies (1:100; Molecular Probes) for 30 min before mounting with ProLong Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen). Samples were examined using a Zeiss LSM 510 Meta laser scanning confocal microscope configured with an Axiovert 200M inverted microscope using a c-Apochromat 63x/1.2 numerical aperture water immersion lens. UV argon, visible argon ion and green helium neon lasers were used and emissions read using 385-470nm (DAPI), 505-530nm (Alexa Fluor 488-conjugate), 560-615nm (Alexa Fluor 594-conjugate) band pass filters, respectively. Images were analyzed with LSM 510 software Z-stacks were constructed by imaging optical slices at 1 µM intervals. FIJI was used for subsequent image processing and only linear adjustments (i.e. brightness, contrast) were made [84].

In order to identify the domain(s) required for nuclear localization, the full-length, N-terminal and tandem repeat (amino acids 1-138) and C-terminal (amino acids 138-198) constructs were created as previously described [82]. Additional C-terminal truncations were PCR amplified from the full-length plasmid using the following primers:

Table 1: PCR primers for generating C-terminal truncation constructs

Construct (amino acids)	Forward (5'→3')	Reverse (5'→3')
C1 (138-162)	aaaagtcgacGGACAAGACCATGTTAGTTTAT	aaaagaattcAGACAAATAATTCATTGTACTAAAA
C2 (158-182)	aaaagtcgacATGAATTATTTGTCTGGTTAT	aaaagaattcATCAAAATAATAATAAGGATTATAAC
C3 (178-198)	aaaagtcgacTATTATTATTTGATTATGTTACTC	aaaagaattcCTACTCTAAACTACTTTCACTA
C4 (158-177)	aaaagtcgacATGAATTATTTGTCTGGTTAT	aaaagaattcAGGATTATAACAACAATAATGATG
C5 (181-198)	aaaagtcgacTTGATTATGTTACTCCCAGATTAT	aaaagaattcCTACTCTAAACTACTTTCACTA

Non-annealing sequences are shown in lowercase and contain restriction sites for SalI (gtcgac) and EcoRI (gaattc).

TRP32 truncation constructs were cloned into the vector pAC-GFP-C1 (Clontech, Mountain View, CA). TRP32 lysine mutants were created from TRP32 in the pAC-GFP-CI backbone using a QuikChange Mutagensis II kit (Agilent; Santa Clara, CA), or were obtained from a commercial vendor (Gentech). These vectors were amplified in TOP10 *E. coli* and purified using the PerfectPrep EndoFree Plasmid Maxi Kit (5Prime). Purified plasmids were transfected into HeLa cells using Lipofectamine 2000 (ThermoFisher) according to manufacturer's instructions. Cells were acetone fixed and mounted with Antifade reagent with

DAPI (4′,6-diamidino-2-phenylindole)(Invitrogen). Localization of ectopically expressed constructs was examined at 24 h post-transfection using fluorescent microscopy and by immunoblotting of nuclear/cytosolic separations. For immunoblotting, cells were harvested by scraping and washed 2x in ice cold PBS before using NE/PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher) according to the manufacturer's instructions with the addition of two washes of the nuclear pellet before lysing the nucleus. Antibodies for nuclear lamin (Santa Cruz 20682) and tubulin (Santa Cruz 8035) were used as separation controls while TRP32 constructs were detected using anti-TRP32 [85] or anti-GFP (Clontech, 632375).

Additionally, when examining subnuclear localization, cells were incubated with mouse anti-B23 (nucleosome marker) (1:100)(Santa Cruz; Santa Cruz, CA) or mouse anti-coilin (Cajal body marker)(1:100)(Santa Cruz) for 1 h , then washed and incubated with Alexa Fluor 594-IgG(H+L) labeled goat anti-mouse secondary antibodies (1:100) (Molecular Probes; Carlsbad, CA) for 1 h then mounted with ProLong Gold Antifade reagent with DAPI (Invitrogen).

Chromatin immunoprecipitation and sequencing. *E. chaffeensis*-infected THP-1 cells harvested at 3 dpi were used for chromatin immunoprecipitation using EZ Magna ChIP (EMD Millipore). Briefly, cells were cross-linked using a final concentration of 1% formaldehyde for 10 min. Cells were then pelleted, lysed, and lysate was sonicated on ice using Sonics Vibra Cell. Sonication (12 cycles, 30 s at 5 W output, 30 s rest) was used to generate chromatin fragments less than 1 kb in length. TRP32 was immunoprecipitated using rabbit anti-TRP32 and pre-

immune serum was used as a control. Crosslinks were reversed and nucleic acid was purified according to manufacturer's protocol. Library preparation was performed involving PCR amplification with adapter ligation by the UTMB Next Generation Sequencing Core, and samples were tracked using index tags incorporated into the adapters. Library quality was evaluated and quantification of library DNA templates was performed. Samples were sequenced using an Illumina HiSeq 1500 to generate paired-end 50 base pair sequence reads. Sequence reads were analyzed by base calling and sequence quality filtering scripts using Illumina Pipeline software. Sequences were aligned to the human genome (NCBI build 37) using bowtie2 allowing a maximum of two mismatches to the reference genome and a BAM file was generated. MACs was used for peak calling and peaks were visualized using the Integrated Genomics Viewer (IGV) by the Broad Institute.

Gene annotation and ontology. A browser extensible data (BED) file consisting of highly significant peaks ($p<10^{-20}$) was submitted to Genomic Regions Enrichment of Annotations Tool (GREAT v3.0)[86]. These peaks were associated with nearby genes using the rule Basal Plus Extension which assigns genes a proximal regulatory region consisting of -5 kb to +1 kb from the transcription start site and a distal regulatory region extending up to 1000 kb in either direction but ending at any other gene's proximal regulatory region. Additionally, a set of curated regulatory domains from literature was utilized. After the ChIP-Seq regions were assigned to nearby genes, the annotations from those genes were used to calculate statistically enriched categories using hypergeometric and binomial tests. In order to calculate binding site percentages, the Nearest Gene

rule was used to associate peaks to genes. This rule associates peaks to genes similarly to the Basal Plus Extension rule, but only allows one gene to be assigned to any one genomic region. This analysis did not alter the top ten significantly enriched gene ontologies in any category. In order to determine enrichment at the promoter region, experimental data was compared to 10 randomly generated data sets and a one sample *t*-test used to determine significance.

TRP32 binding sites. The DNA-binding motif was identified using the Multiple EM Motif Elicitation (MEME) ChIP software suite [87]. The regions +/- 250 base pairs from the TRP32 binding peak were submitted to MEME in fasta format. Motifs that were statistically enriched in these regions were returned in the form of probability matrices ranked by E-value and similarity. Motifs were tested by EMSA.

EMSA. Whole genomic DNA was isolated from healthy THP-1 cells and sheared using the protocol and enzyme from the ChIP-IT Express Enzymatic Kit (Active Motif) without crosslinking. Resulting sheared genomic material was then purified using the QIAquick PCR Purification Kit (Qiagen) and biotin-labeled using the LabelIT Nucleic Acid Labeling Kit (Mirus). EMSAs were performed using the Lightshift Chemiluminescent EMSA kit (ThermoFisher). Briefly, 5 ng of labeled whole genomic DNA was incubated with 2 µg of purified protein in a 10 mM Tris buffer with 50 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 2.5% glycerol, 0.05% NP-40 and 50 ng/µl Poly (dI•dC). Samples were incubated at 4˚C for 1 h before separation on a 6 % DNA retardation gel (ThermoFisher) at 100V for 90 min. Reactions were then transferred to Biodyne B nylon membranes (ThermoFisher) at 20V for 1 h. Transferred DNA was crosslinked to the membrane by placing the membrane face

down on a transilluminator for 10 min or in a CL-1000 Ultraviolet crosslinker (UVP) for 5 min. Reactions were imaged using streptavidin-HRP and film. Supershift assays were performed using rabbit anti-TRP32 antibody. Antibody at 1:200, 1:50 and 1:20 dilutions was added to the EMSA reaction after 30 min of incubation and allowed to incubate for 30 min. For EMSAs utilizing a probe, 400 pM of labeled DNA was incubated with 1 µg of purified protein. Competition assays were performed using 1000x molar increase of unlabeled nucleic acid. Protein and unlabeled DNA were incubated for 30 min at 4°C before adding labeled DNA and incubating for the remaining 30 min.

Oligonucleotide probes (IDT) modeled after TRP32 bound sequences, were resuspended in annealing buffer (10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA) and complementary strands annealed by heating to 95°C in a heat block for 5 min followed by slowly cooling to room temperature. Before use, probes were diluted to the above mentioned concentrations in annealing buffer.

Table *2:* Probe sequences used for EMSA.

Probe Name	Sequence (5'→3')
MEME Motif 1	CAGCACTTTGGGAGGCTGAGGCAGG
MEME Motif 2	GGGGTTTCACCATGTTGGCCAGGCTGGTC
MEME Motif3 (WT)	GCCGGGCGTGGTGGCGGGCACCTGTAATC
Mutant1 (Δ1)	AAAAAAAAAAGTGGCGGGCACCTGTAATC
Mutant 2(Δ2)	GCCGGGCGTAAAAAAAAAAACCTGTAATC
Mutant 3(Δ3)	GCCGGGCGTGGTGGCGGGCAAAAAAAAAA

qPCR. Expression of TRP32 host gene targets was measured by qRT-PCR. The genes that corresponded to the top 100 most significantly enriched peaks were chosen for initial analysis; however, 25 were excluded because they were histone coding genes which are difficult to differentiate by qPCR due to high sequence identity. Primers were designed for the remaining 75 genes using NCBI Primer Blast. Primers were designed to detect mRNA sequences and were all designed to span exon-exon junction to prevent amplification of corresponding genomic sequences (Table 2). All primers were tested by melt-curve and gel analysis for specificity and primers which amplified multiple products or which amplified a product that was not the predicted size were excluded.

RNA was extracted from healthy THP-1 cells and from cells at 24, 48 and 72 hpi using RNeasy Mini Kit (Qiagen). cDNA was synthesized from 1µg of RNA using qScript cDNA synthesis kit (Quanta Biosciences). qPCR was performed with 5 ng of cDNA in triplicate using iQ SYBR Green Supermix (Bio-Rad) according to manufacturer's instructions. Fold changes were calculated using the $\Delta\Delta_{CT}$ method using GAPDH as the reference gene [88].

Table 3: Primer sequences for qPCR of TRP32 targets

Gene	Name	Forward	Reverse	Tm	Product Size
NM_001037171.1	ACOT9	CCCTCAGGAGCGGAACATTT	GGGCCACTCATGGAGTTGAA	60	384
NM_001101.3	ACTB	GATGATGATATCGCCGCGCTC	TCGTCGCCCACATAGGAATC	61	167
NM_001272071	AP1S2	TGCAGCTTCCTTGAGTGGCGA	ACGTGGGGTTTCAGCTTCTTTCG	64	318
NM_181720	ARHGAP30	GGGCACAGGTCTTGGGGGAT	GGTCTGGCTTCCGCTCTGAC	63.5	287
NM_001199456.1	BRD2	GCTCGTGGAGGGGAATACAG	TTTGGGATTGGACACCTCCG	60	201
NM_001300944	C110rf30	TTGGGTGAAAGACCAAGTTACAGT	CATTGGAGGCAGGACTGGCA	61.4	313
NM_001301837	C12orf57	ACGAGGCTCGGGATAACGCC	GCACAAGGGAGGGCCAACTC	64	205
NM_001256373	C12orf79	GCTTTTTGCTTTTCATCACCTGC	TTGCCAGCCTCCTTCTGACAC	61.3	343
NM_001105530	CAP1	GGAGAGCGGCTGATCGCAGT	ACAGGACCAGCAAGCAGCGA	64.6	200
NM_007276	CBX3	GCTGAAAGTGGTCCCAAAGGGGTA	CGACAAATTCTTCAGGCTCTGCCTC	64.3	361
NM_001300964	CDK19	CCTCGCCTCCCGAGTCACTG	AGATCTTGGCAACTGCATGGGC	64	289
NM_138363.1	CEP95	GGCTCGGATGCTGAGTGGGT	CTGCAAGTAGTCCAAGGCCAGTG	64	228
NM_182523	CMC1	ACCCCGCAGACCAGCATCTC	TGCTTGTTGGAAGCTTCTGTAGCC	64	306
NM_001297733	COX18	ATCCTGGCCAAGCTAGGTGGAAAAT	GTCGGGTGCAGTGAGGTCAGG	64.6	349
NM_001012967	DDX60L	AGGCAAAGGTGTTGTCAGTGC	TCCGGGAGTTCGGCAAGGAT	62.5	400
NM_017925	DENND4c	TTGCCCCAGGAAGAAGCCGT	TGGAGAGCTGATGGAGTGGCTT	63.8	286
NM_019030	DHX29	CCCCCTCCAGGAGTCAGGAAG	AAGCCATCTCTGACCCGCCC	63.9	212
NM_024963	FBXL18	GCCAACGCCCAGTTCTTCCAG	ACCACCACAGGTTCGGCGG	64.6	352
NM_031904	FRMD8	GTGCCCCTGGCTGTGGAGAAC	CACGTCATCGTCTGGGGCACT	65.1	225
NM_017769	G2E3	GGACCAGAAAATACCCAGCCAA	AGGGTGTGTGGACTCTCTGAA	60.5	235
NM_138801.2	GALM	GGCTTCGCCGAGTTGGAAGG	CGCCATTTGACAGCACCCGA	64	208
NM_001256488	GOLGB1	TCCGTCCCAACTGCTAGTGGC	AGCCAGGCGCTCCTGAACAT	64.4	327
NM_021639	GPBP1L1	CGCTGCCCGTTGGTGTCTCA	CACGAACAGCAGCTTTCAAGGCA	64.9	399
NM_005684	GPR52	AGCGTCACTCCTGCCCACTT	ACTCGTGGACACCTGTGGAGT	63.4	264
NM_021958	HLX	CCCCCTCCAGCAAAGACCTCA	GCGCGACCATGAACGCTTCC	64.4	338
NM_001130689	HMGB2	CTTGGCACGATATGCAGCAA	CAGCCAAAGATAAACAACCATATGA		
NM_194247	HNRNPA3	TTGGAAGAGGCGAGTCCGGT	ACACGCCCATCAACCTTGTGT	63.1	337
NM_022465	IKZF4	ATGACGGCGGTTCCCCTCACTT	AAGAGTGCTGGCTGTTGGGGG	65.6	354
NM_001267728	ING1	ACGATTGGTCGCTGAGGCGG	CGCGTTCTCACGGTTCTCGT	64.2	397
NM_001297655	KIF2C	CCTTGCTGACTCTCCGAATGGC	CCTGAGCCGTGATGCGAAGC	63.8	393
NM_182931	KMT2E	GGGGTTCGGGTGTCTCGTGT	GCTCATGACGTTCGCCTCTGG	63.9	227

NM_014708	KNTC1	CGCAGACCATCGAATCCTGCT	CCCGATCGCCGGTACTGGAT	63.4	229
NM_002298	LCP1	TGATTTTTGGTGGGGCGGGGA	GGCAAAGGCAAGCAAGCAGCC	65.2	371
NM_001282460	LRRC63	GTGCCAAGCCCTCCACCTATGA	GGCCAGGTTGGTCATGGCTGTT	64.9	348
NM_005911	MAT2A	GCCCGCTGCTTCGTTCG	TCCTGCTGAAGGTGGGCATCAA	63	278
NM_001130079	MOV10	GTTCAGAGGGCCCCAAGCGGTA	CGAGTCTGAAGAACCTGACCCGA	65.2	369
NM_014046	MRPS18B	CGCCCACACGGGTATCATCT	TCCGCTGGTGTTCTAGGGGG	62.7	351
NM_001099286	MTFR2	TTCCTAACGCCCTCCGCTTGA	CAGAGTTCAAGAGCGGGATCAACT	63.2	303
NM_001123226.1	MTO1	AGTGATTCAGCCAGGCTACG	CACTGGTGCCCAGAGTAGTG	60	386
NM_002475	MYL6B	GGCCAAGAACCGAGGCCAAG	TGGCTGGAACGTGCTTTCGC	64	287
NM_001144030	NAT10	GGTCCCTTCTCGCTCGCCATC	GCAGCACACACATGCCGAAGG	65	393
NM_173638.4	NBPF15	TGGGTCTCAATTTTTACTGTGCCT	ACCCACCAAAACCAATCAGCA	61	207
NM_004289	NFE2L3	CGGATGTGCGAGCGAGGAGA	CTCCCAATGAGATGCCCTCCAGT	64.2	296
NM_207308	NUP210L	CTTGCGTCTACACCGCCTCC	TGAGGCGTATCGGTTGCGTAGA	63.5	260
NM_016081	PALLD	GCTGAGAGGGAAACGAACGG	AGACTTTGTATTCCTGATTAGCTGT	59.5	397
NM_017851	PARP16	TCGCCCTTGCCCCTAACCAG	AATCCAGCTCACCAGGTCCCA	63.8	320
NM_001048183	PHACTR4	CTCCCTTTTTCCCCCTCCCCG	TCTGTAGTGGGCTGGTCTGCT	63.7	200
NM_017934	PHIP	GCTGGTGTAGCCAGTAGGCA	GCCATCACTGTGCCATGCTCT	62.6	342
NM_178136.2	POLDIP3	GGGGTAGCGGAGGTGGTGTT	TCAGGGTCCACTTCGGCAGG	64	245
NM_007215	POLG2	TGGGGACCTAGACCGAGGCA	ACCAGGCCACACAGAAATCCCA	64.3	232
NM_020366	RPGRIP1	CCCTTGAGCAGGATGAACCG	GGCCCCCTCTTGGGTTTCTC	61.6	386
NM_001253382	RPL15	AGATAGGTCTCCCTCCTGTGCGGC	ACAGTGGCGTCCAGCTCGCTC	67.4	373
NM_000986	RPL24	CGTGGAGCTGTCGCCATGAA	TTTGACTGCTCGGCGGGTTC	63.2	246
NM_001030009	RPS15A	CTTTCCGCGCCGCCACAAT	ACTGGCGGGATGGAAGCAGA	64.1	312
NM_001025	RPS23	TTGTGGCTCCTTCCTGCGGT	TGCGACCAAATCCAGCAACCAG	64.1	379
NM_025158	RUFY1	TGAGATCGTGGACCGAAGCCAG	AGCCGACTGGAGCAACACCTT	64.3	286
NM_012240.2	SIRT4	AGTGTCCGTAGAGCTGTGAGAGAA	AGTGTGCAGGGTTAGGCTGGT	63	399
NM_020846	SLAIN2	CCATCCTCAACCCCAGTGCGA	GAACCAGAACTGCGCCGAGA	63.6	269
NM_003049	SLC10A1	ATGCCCCTCACGGCCTTTGT	AGCGCATGTATTGTGGCCGTT	64.1	343
NM_001145250	SP9	ATCCGCCAGCGCCATA	TCAATGTGCTTCTGGGAATCC		
NM_003971	SPAG9	TCAGCCGACTTTTCAGCTCCT	TCCAGCCAAAAGCCTGCACT	62	279
NM_172209	TAPBP	TCTGCTCCTCGCTGTGGCTT	GGGACCCGGAGCCAGAGATG	64.1	322
NM_001205201	TEX28	AGCCCCGTGTGATTGTTGAGGA	AGGGACCGGCATGAAGGCAC	64.6	234
NM_144632	TMEM182	ATGCTCCTGGGGGTAGTTGCT	GAAGGGGTGAAATCCAGGCAGT	62.9	224
NM_018247	TMEM30A	GCGAAGACTCGGAGACCGGA	AGGGACTGGAAGGCTCTGTTCC	63.7	205

NM_021109.3	TMSB4X	GTGCGCCTCGCTTCGCTTTT	CGCGGCCTTCGTTGTCAGTAG	64	329
NM_000550	TYRP1	AGGATGTCGCTCAGTGCTTGGA	TCTGTGTTGGTGACTGGGGGC	64.3	361
NM_001039590	USP9X	AGTCAAACTCAGCGAAGTCCCCA	GCCTGAGGAGCACATAGCCAC	63.5	269
NM_007146	VEZF1	AAAGACCCTTCAAATGCCAAACGTG	AGGTTTGGCACAGGTTAGCAGC	63.6	390
NM_022553	VPS52	AAGGTTCCGGGGCTAGTTTGT	CCCCAAGTTGCAGTGGTTCCTG	62.8	345
NM_032786	ZC3H10	GGGTAGGCGGCTCTTTGTCG	AACGCTTGCCTCGCTTGCAC	64	351

Luciferase Gene Expression Assay. Promoter regions containing the TRP32 binding sites as determined by GREAT were cloned into pGL4.10 (Promega; Madison, WI) a promoterless firefly luciferase vector. Each promoter construct (200 ng) was transfected into HeLa cells using Lipofectamine 2000 (Thermo Fisher) along with varying concentrations of GFP-TRP32, or an empty GFP expressing vector and where indicated a control vector expressing Renilla luciferase under the control of a HSV-thymidine kinase promoter (pRL-TK Vector, Promega). Equal amounts of total DNA were transfected into each cell. After 24 h cells were harvested and Dual Glo Luciferase Reagent (Promega) added to the cells according to the manufacturer's protocol. Relative light output was measured using a Veritas Microplate Luminometer. Relative expression levels were calculated for each gene compared to the control and *p*-value calculated using the Students *t*-test.

Chapter 3. *Ehrlichia chaffeensis* TRP32 is a Nucleomodulin that Directly Regulates Expression of Host Genes Governing Differentiation and Proliferation

INTRODUCTION

Ehrlichia chaffeensis is a gram-negative, obligately intracellular bacterium and the etiologic agent of human monocytotropic ehrlichiosis (HME), an emerging life-threatening tick-borne zoonosis. In humans, *E. chaffeensis* infects mononuclear phagocytes, causing an acute infection that manifests as an undifferentiated febrile illness. The presence of severe symptoms and the absence of high bacterial load suggests that the disease has an immunopathological basis, caused in part by alterations in infected mononuclear phagocyte function [68].

During *E. chaffeensis* infection, there are significant changes in host gene expression which are associated with direct/indirect pathogen interactions that serve to promote bacterial survival and replication within the host cell. Genes most affected are early innate and cell-mediated immune response, cell cycle and cell differentiation, apoptosis programming, membrane trafficking, and intracellular signaling [56, 89]. The mechanisms whereby *E. chaffeensis* directs

these changes in gene transcription are not fully understood. However, manipulation of host transcription appears to be an important mechanism in the overall molecular strategy of ehrlichial subversion of the host cell.

Nucleomodulins are an emerging class of bacterial effectors that function by entering and reprogramming the host cell nucleus. They have been described in a variety of organisms and typically function by mimicking some aspect of the host biology [90-92]. Nucleomodulins may function by directly modulating host cell gene transcription, as is observed with the transcription activator-like (TAL) effectors of the plant pathogens *Xanthomonas* and *Ralstonia* [92]. The TAL effecters directly interact with host DNA using a novel tandem repeat DNA-binding domain to activate expression of genes that facilitate infection [93].

Nucleomodulins were recently identified in tick-borne, obligately intracellular bacteria from the family *Anaplasmataceae*. *A. phagocytophilum* AnkA interacts with AT-rich sequences found in regulatory regions within the promoters of host genes and leads to the downregulation of genes involved in host defense via an epigenetic mechanism [94, 95]. *E. chaffeensis* Ank200 was also found to bind to repetitive AT-rich sequences called *Alu* elements located within the promoters and intergenic regions of genes involved in transcriptional regulation, ATPase activity, and apoptosis, and Ank200 targets were differentially regulated during infection [96]. Most recently, we reported that the *E. chaffeensis* tandem repeat effector, TRP120, has a tandem repeat DNA binding domain similar to that described in the TAL effectors *of Xanthomonas*. During infection TRP120 enters the host nucleus and binds to a GC-rich motif within regulatory

regions of specific host genes. Targets include genes involved with transcriptional regulation, signal transduction, and apoptosis. TRP120 target genes examined were upregulated during infection, or when TRP120 was directly introduced into the cell [78]. Although, the mechanisms by which TRP120 regulates host gene expression are not fully understood, TRP120 interactions with host epigenetic regulators including the polycomb group ring finger 5 protein (PCGF5), the Jumonji domain-containing-3, histone-lysine demethylase (JMJD3) and the nuclear receptor binding SET-domain protein 1 (NSD1) may play a role [79]. Despite lacking classical nuclear localization signals, multiple *E. chaffeensis* TRPs have been detected in the host cell nucleus during infection; however, their functions within the nucleus are not well-defined [78, 79, 82, 97-99].

E. chaffeensis TRPs are type I secretion system substrates that have defined interactions with a wide variety of host cell proteins including many that are associated with the nucleus or host cell transcription [79, 82, 99]. TRP32 interacting host proteins include several nuclear proteins and transcription factors, including DAZAP2, a Wnt target gene activator, the hematopoietically expressed homeobox (HHEX), a transcription factor required for hematopoietic cell differentiation, the elongation factor 1-alpha-1 (EF1A1), a transcription factor in T-cells, and p53 inducible protein 11 (TP53I11), which binds DNA nonspecifically and induces apoptosis [82, 100-102]. Interestingly, TRP32 constructs containing only the tandem repeat domain were independently capable of activating gene transcription in a eukaryotic system [82].

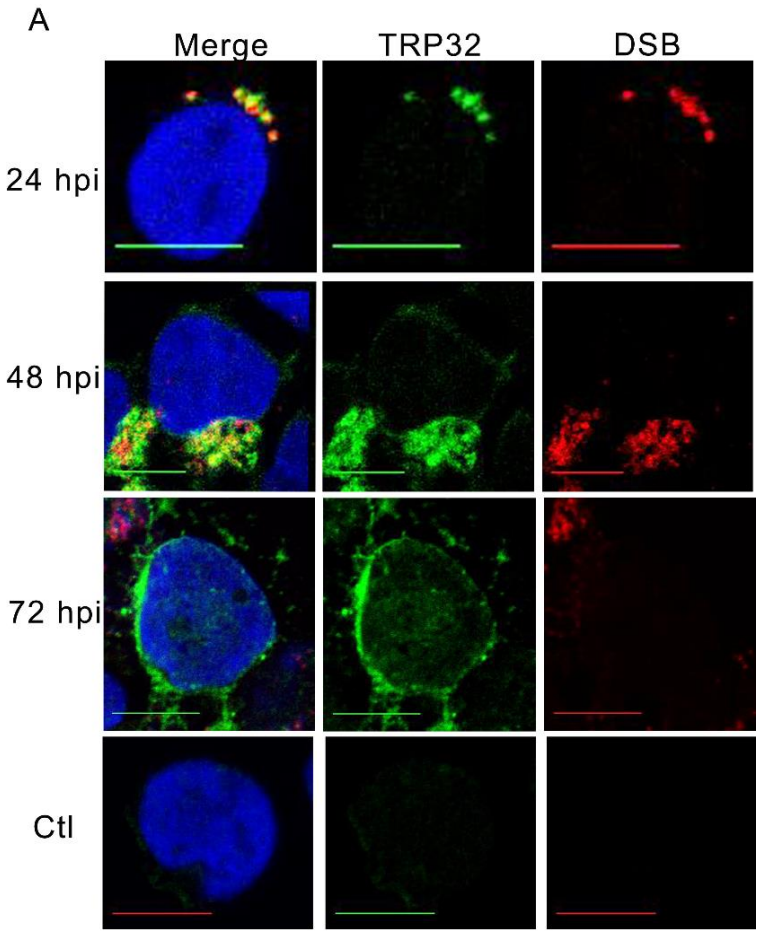
The mechanisms of host transcriptional modulation by pathogens are diverse and complex. In this study, we have determined that *E. chaffeensis* TRP32 is a nucleomodulin that accesses the nucleus through a tyrosine phosphorylation dependent manner and binds host DNA through a tandem repeat domain to modulate host gene transcription.

Results

TRP32 is translocated into the host nucleus in a tyrosine phosphorylation-dependent manner. Although lacking a canonical nuclear localization signal, TRP32 is transported into the host nucleus in a temporally regulated manner. At early points in infection (24 hpi), TRP32 remains around the morulae. However, at 48 hpi TRP32 was observed in the perinuclear region, and by 72 hpi, increasing amounts of TRP32 was detected in the perinuclear region and in the nucleus. Interestingly, TRP32 seems to surround the morula in a manner that suggests that it may be associating with the morular membrane (Fig. 3.1A). TRP32 nuclear localization was confirmed using orthogonal projections of a Z-stack which showed TRP32 diffusely throughout the nucleus and in nuclear puncta (Fig. 3.1B). In order to elucidate the mechanism for TRP32 nuclear translocation, GFP-tagged truncation constructs were created and transfected into HeLa cells and their localization observed. We determined that the C-terminus (60 aa) alone was sufficient for nuclear localization. Additional C-terminal domain truncation constructs were generated to explore the role of two tri-tyrosine motifs in nuclear translocation. These constructs were ectopically expressed and localization observed by fluorescent microscopy. We identified two tri-tyrosine

motifs that were in many of the nuclear translocated constructs (Fig. 3.2). The central tyrosine of each of these motifs was predicted to be phosphorylated, which led to the hypothesis that tyrosine phosphorylation might be involved in TRP32

nuclear localization. Hence, subcellular localization of wild-type TRP32 was examined in HeLa cells treated with vehicle alone (control)



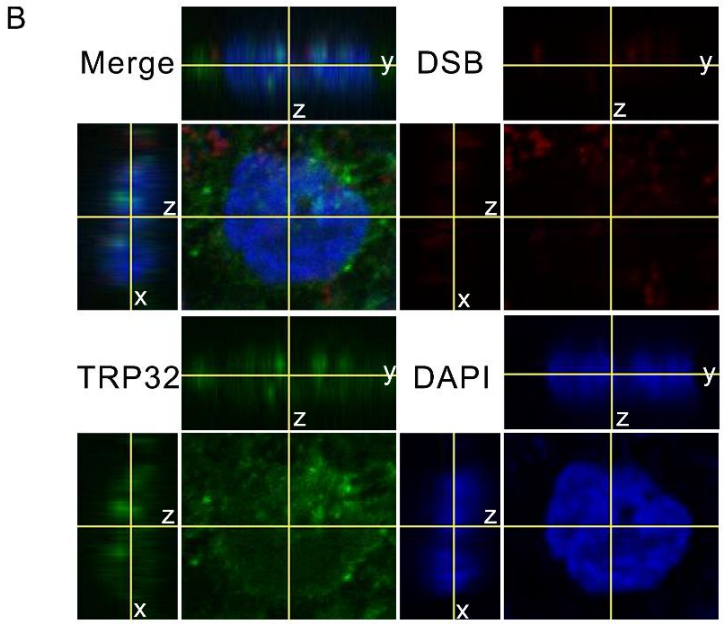


Figure 3-1: TRP32 localizes to the nucleus of *E. chaffeensis* infected THP-1 cells.

(A) *E. chaffeensis* infected and uninfected THP-1 cells were fixed and probed with rabbit anti-TRP32 (green), anti-DSB (red, morula) and DAPI (blue, DNA) then visualized using confocal microscopy. Early (24 hpi) TRP32 is primarily associated with the morulae. At 48 hpi, TRP32 localizes with morulae and in the perinuclear region. At 72 hpi, TRP32 localized with the morulae, but was also observed at the perinuclear region and in nucleus of the host cell. TRP32 was not observed in uninfected cells. (B) Orthogonal projections of optical slices from a Z-stack of an *E. chaffensis* infected THP-1 cell at 72 hpi showed both diffuse and punctate TRP32 within the host nucleus. Top panels: Y-Z projection, left shows X-Z projection. The position of X and Y axes within the projections denotes the Z-depth of the slice shown in center. Scale bar is 10 μM.

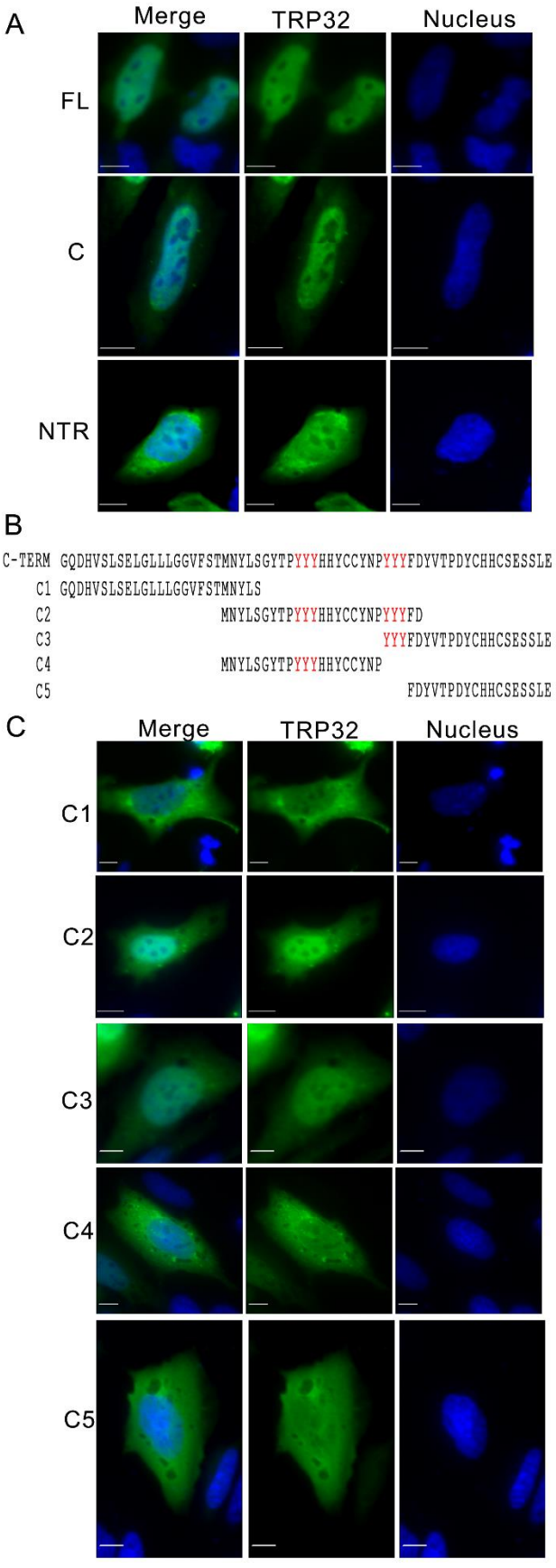


Figure 3-2:C-terminal tri-tyrosine motif is important for *E. chaffeensis* TRP32 nuclear localization.
HeLa cells were transfected with GFP-tagged TRP32 constructs. After 24 h of expression, cells were fixed and visualized using fluorescent microscopy. (A) Both full-length and C-terminal TRP32 constructs (green) localized to the nucleus (blue), but not the N-terminal plus tandem repeat construct. (B) Schematic of TRP32 C-terminal truncation constructs. (C) TRP32 C-terminal truncations, C2 and C, which both contain tri-tyrosine repeats were located primarily in the nucleus, while other C-terminal constructs lacking the tri-tyrosine repeat showed diffuse nuclear and cytosolic localization.

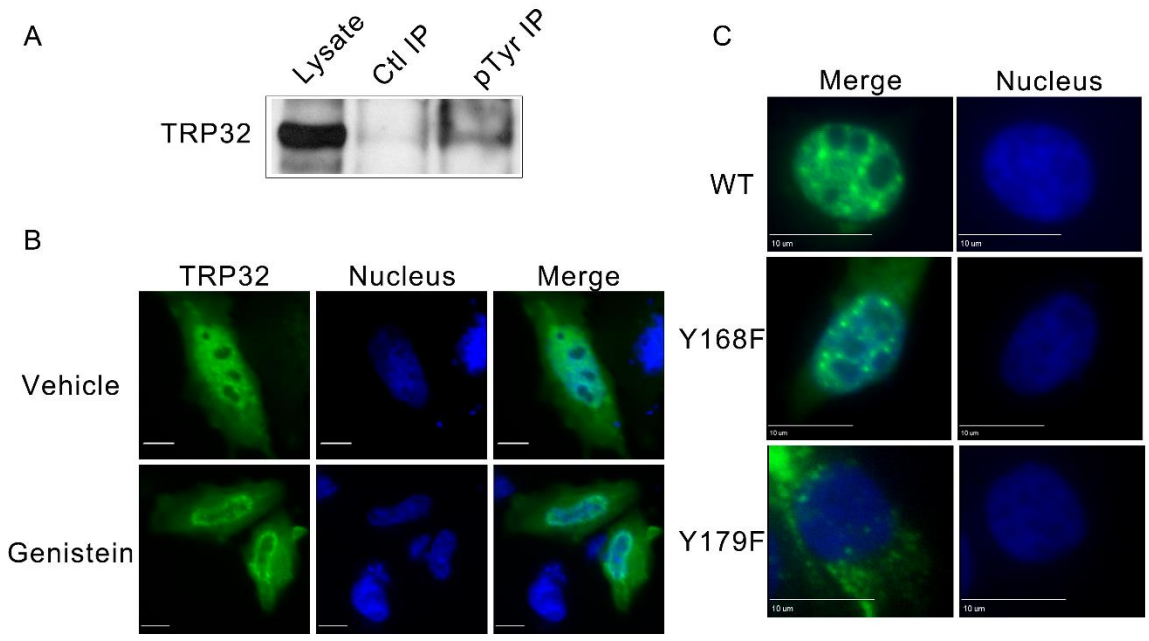


Figure 3-3: Phosphorylation of *E. chaffeensis* TRP32 at tyrosine 179 is required for nuclear localization.

(A) Tyrosine phosphorylation of TRP32 was detected during infection. *E. chaffeensis* infected THP-1 cells were harvested at 72 hpi and immunoprecipitated using an anti-phosphotyrosine antibody (PY99, Santa Cruz) or an IgG control. The blot was probed using rabbit anti-TRP32. (B) Tyrosine kinase inhibitor genistein inhibits TRP32 (green) localization to the nucleus (blue). HeLa cells were transfected with GFP-tagged TRP32, and 24 hpi cells were treated with 10μM Genistein (bottom) or vehicle (DMSO, top) for 15 min then fixed and visualized using fluorescent microscopy. Genistein treated cells show decreased nuclear localization of TRP32 compared to the control. (C) TRP32 tyrosine 179 (Y179) is required for nuclear localization. Wild type TRP32 and tyrosine mutant TRP32 constructs were transfected into HeLa cells, and fluorescent microscopy performed 24 hpi. TRP32 primarily localized to the cell nucleus, but Y179F mutants (3rd down) exhibited predominantly cytosolic localization.

or with the tyrosine kinase inhibitor genistein. In cells treated with genistein, TRP32 was observed primarily in the perinuclear region, while TRP32 in control cells exhibited the characteristic nuclear localization (Fig. 3.3B). In order to examine the role of specific tyrosine residues, single and double phenylalanine mutants of the tyrosine residues (Y168 and Y179) were generated, and localization

of these ectopically expressed constructs was observed. Although both single mutants exhibited reduced nuclear localization, the Y179F mutant showed a greater decrease in nuclear localization compared to the Y168F mutant (Fig. 3.3C). This data supports the conclusion that phosphorylation of Y179 facilitates nuclear localization, and that TRP32 is trafficked to the host cell nucleus in a tyrosine phosphorylation-dependent manner. A similar mechanism has been described with regard to STAT1 nuclear access, which homodimerizes in a phosphorylation-dependent manner to create a dimer-dependent NLS [103]. Moreover, phosphorylation of these residues may be required for interaction with another protein that has an NLS.

TRP32 contains two copies of a nine amino acid transactivation domain. Previously, we showed that full-length TRP32 and its internal tandem repeat domain are able to activate transcription of a reporter gene in a eukaryotic system. Upon further investigation, we identified two, 9 amino acid, trans-activating domains (TADs; AA 52-60 and 82-90) within the tandem repeats (Fig. 3.4). This 9aa TAD is a fuzzy motif consisting of two hydrophobic clusters separated by a hydrophilic cluster, and is found in several yeast and mammalian transcription factors, including VP16, p53, HSF1, NF-IL6, nuclear factor-κB, and NFAT1 [104]. Structurally, these domains are often disordered regions that fold into alpha-helices upon interaction with the KIX domain from a transcriptional coactivator. This motif is reported to be the minimal required element for interaction with several transcriptional cofactors including CBP, p300 and MED15 [105].

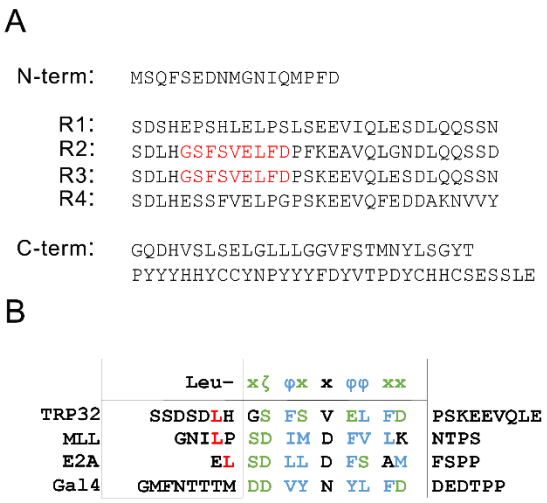


Figure 3-4: *E. chaffeensis* TRP32 putative 9 amino acid transactivation domain.

(A) TRP32 amino acid sequence with predicted transactivating domains (TAD) highlighted in red. (B) Sequence alignment of TRP32 predicted TAD with previously described eukaryotic transactivation domains from Myeloid/Lymphoid Or Mixed-Lineage Leukemia Protein (MLL), Transcription Factor 3 (E2A) and Galactose Induced Regulatory Protein (Gal4). The putative TRP32 TADs are highly similar to previously described eukaryotic transactivating domains with a leucine residue (L, red) followed by an amphipathic motif. Hydrophobic residues (ζ) are shown in green and hydrophilic residues (Φ) in blue.

TRP32 binds to host genomic DNA via its tandem repeat DNA-binding domain. Our previous studies with TRP120, TRP32 activation of reporter gene transcription in a yeast two-hybrid (Y2H) assay, in combination with its localization within the host cell nucleus, led to the conclusion that TRP32 might be binding host genomic DNA through the TR domain, to modulate host cell transcription. We investigated TRP32 DNA binding using EMSAs with recombinant full-length TRP32 and truncation constructs. We determined that the full-length TRP32, as well as the TR domain alone, bound host gDNA (Fig. 3.5). Consistent with *E. chaffeensis* TRP120, which also binds DNA via its TR domain, no homology to

known eukaryotic DNA binding domains was identified by *in silico* modeling. Although the TRP DNA-binding domains are not predicted to be structurally similar to any described DNA-binding domain, the presence of internal tandem repeats with high identity is similar to the *Xanthomonas* transcription activator-like (TAL) effectors which also bind DNA via highly similar internal repeats. This feature suggests that *Ehrlichia* TRP effector DNA-binding domains may interact with DNA similarly. X-ray crystallography studies are needed to determine whether the TRP32 TR DNA binding domain is structurally similar to the *Xanthomonas* tandem repeat DNA-binding domain.

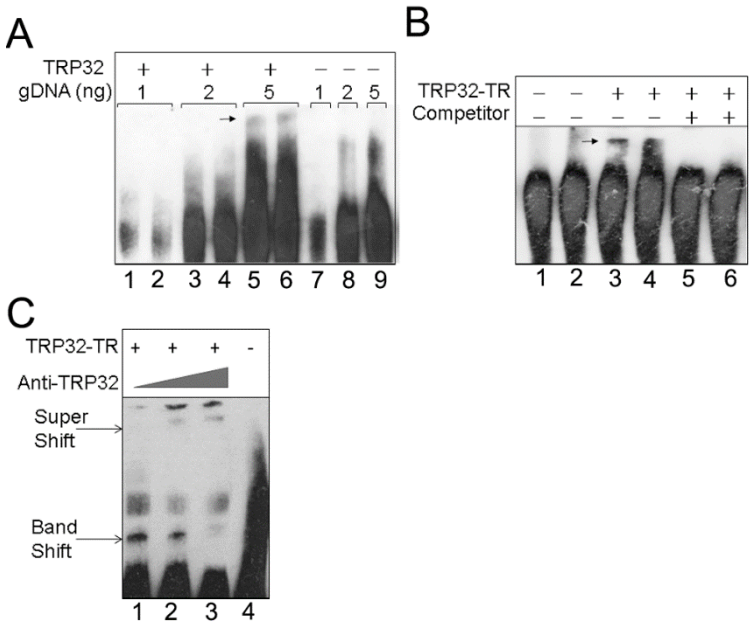


Figure 3-5: *E. chaffeensis* TRP32 binds host gDNA via its tandem repeat domain.

(A) TRP32 binds to host genomic DNA. Varying amounts of purified host gDNA were incubated with recombinant full length TRP32 then separated by EMSA. Band shifts are indicated by arrow (lanes 5 and 6). A band shift was not observed in the corresponding lane to which TRP32 was not added (lane 9). (B) The tandem repeat domain of TRP32 is the DNA-binding domain. EMSA was performed using TRP32 tandem repeat construct (TRP32-TR) and 5 ng of host gDNA. TRP32 band shift is indicated by arrow (lanes 3 and 4). Binding was abolished when excess unlabeled competitor was added (lanes 5 and 6) (C) Supershift assays were performed in which TRP32-TR was incubated with host DNA and increasing concentrations of anti-TRP32 antibody. Band shifts and super shifts are labeled with arrows.

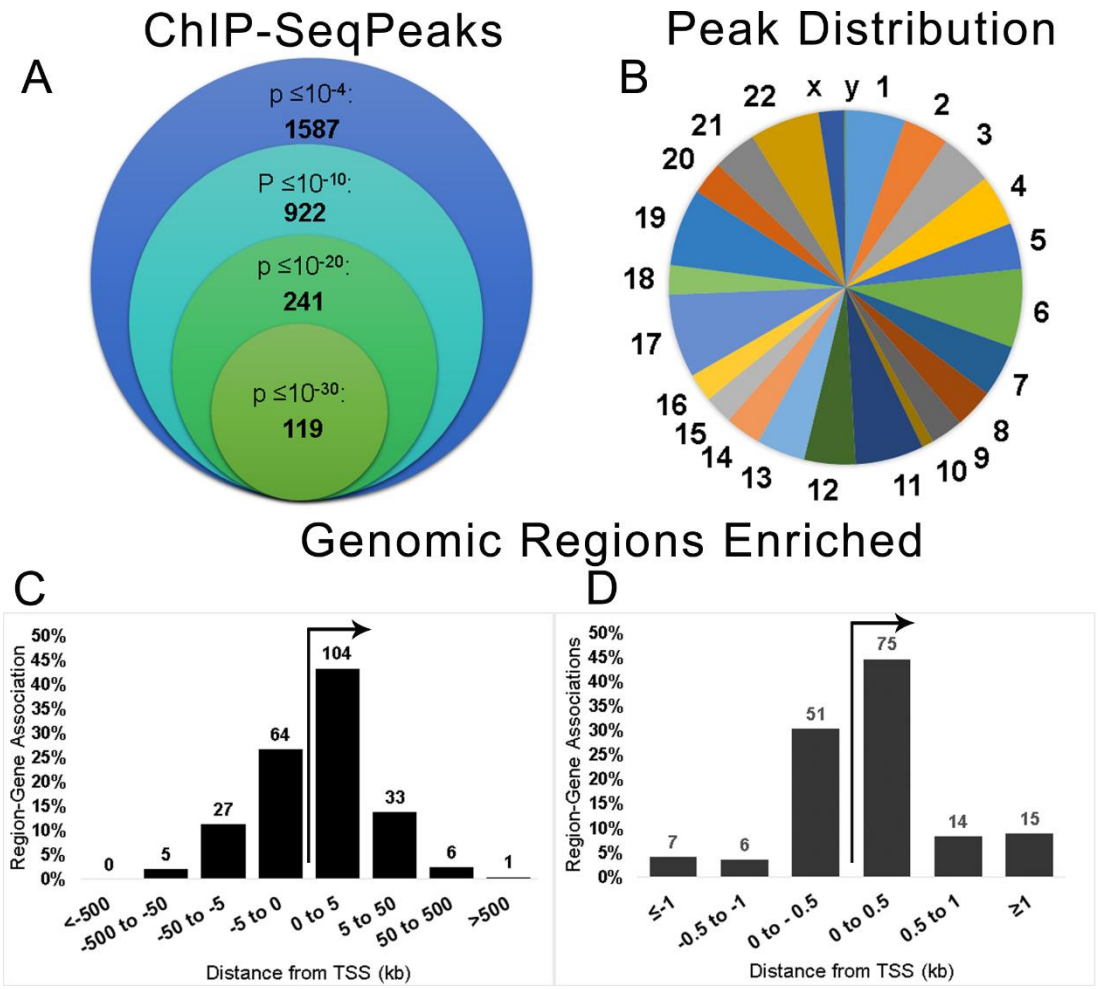


Figure 3-6: *E. chaffeensis* TRP32 binds host genes within promoter regions.

(A) TRP32 chromatin immunoprecipitation resulted in significant enrichment of 1587 regions ($p<10^{-4}$) compared to serum control and 241 peaks were highly significant ($p<10^{-20}$). (B) Number of TRP32 enriched regions/megabase of DNA for each chromosome. (C) TRP32 highly significant peaks are enriched within the promoter regions of host genes. Histogram showing the association of highly significant peaks with host genes demonstrates that TRP32 preferentially associates with host genes within +/- 5kb from transcription start site (TSS). Some peaks corresponded to more than one host gene and were counted multiple times in this histogram. (D) The majority of highly significant peaks clustered within +/- 500 bp from TSS.

TRP32 binds within the promoter region of host genes involved in a variety of cellular processes. ChIP-Seq was used to identify TRP32 genomic targets. We found 1587 genomic regions significantly enriched ($p<10^{-4}$) compared to the negative control. Of these, 241 regions were highly significant, with a p-value less than 10^{-20}. The enriched regions mapped to all chromosomes (Fig. 3.6A-B), and all of the highly significant regions mapped to at least one human gene. These highly significant peaks were significantly enriched within the promoter region (+/- 5 kilobases from the transcription start site) when compared to random control ($p<0.0001$), and the majority 60.8% (146/240) were within +/- 1kb. This binding location is consistent with transcription factor binding [106]. The remaining highly significant peaks were found downstream from the transcription start site (TSS) (22.9%) or further upstream (16.3%) (Fig. 3.6C-D). When spaced motif analysis (SPAMO) was performed on all significant TRP32 bound sequences, binding sites for other transcription factors were significantly enriched at fixed up/downstream intervals, including those for HHEX (17 bp, p=2.98e-52) and TCF7L2 (44 bp, p=2.13e-23) [107]. When significant genomic regions were probed for their association with various biological processes using gene ontology analysis, we observed enrichment of genes associated with cell differentiation, chromatin remodeling, RNA transcription and processing, and regulation of translation. The TRP32 targets were also enriched for genes specific for cellular compartments, most notably the nucleosome and ribosome (Fig. 3.7). Several host cellular pathways were also significantly enriched among TRP32 targets including DNA replication and immune cell activation pathways.

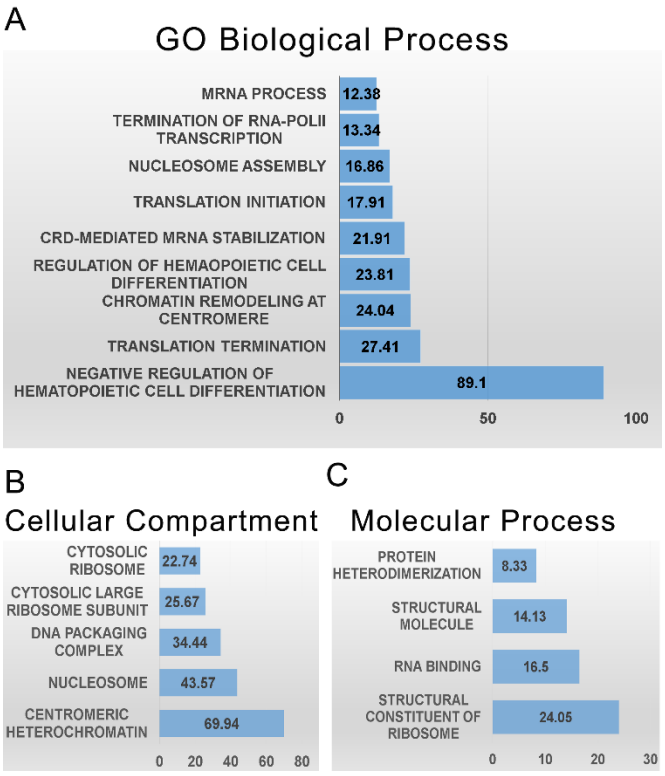


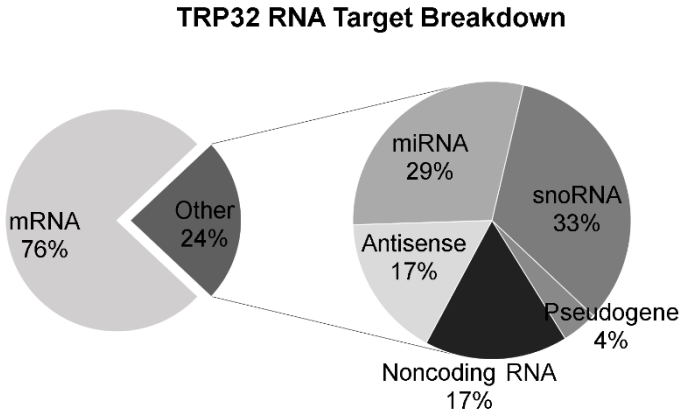
Figure 3-7: *E. chaffeensis* TRP32 targets include genes in several categories relevant to infection.

(A) Terms from gene ontology of biological processes that were significantly enriched by both binomial region and hypergeometric fold enrichment are presented by hypergeometric fold enrichment. (B) Significantly enriched terms of cellular compartments presented by hypergeometric fold enrichment. (C) Significantly enriched gene ontologies of molecular processes presented by hypergeometric fold enrichment.

There was also enrichment of genes associated with apoptotic pathways; however, these did not reach the threshold of significance (FDR *p*-value 0.09) (Table 1). Additionally, it was noted that although the majority of TRP32 target genes were protein-coding, some were also RNA genes. Among TRP32 noncoding RNA targets were several miRNAs, including miRNA 21 and miRNA 142 (Fig. 3.8).

TRP32 DNA motif. In order to define the DNA motif bound by TRP32, the nucleotide sequences of the ChIP peaks were entered into the pattern mining program MEME-ChIP [87]. MEME-ChIP uses multiple algorithms to search for sequences which are enriched in the data set. It returns any statistically significant results in the form of position weight matrices (PWM), and can then compare the resultant sequences with known transcription factor binding motifs.

A



B

Gene ID	Gene Product	Function
NR_027349	Homo sapiens miR-17-92 cluster of 6 miRNAs	Involved in cell survival, proliferation, differentiation (52, 53)
NR_029493	Homo sapiens miRNA 21	Oncomir. Anti-apoptotic. Notable targets include: PTEN, Bcl2, TGFBRII, TAp63, P12/CDK2AP1, MEF2C (54-56)
NR_029683	Homo sapiens miRNA 142	Regulates hematopoietic cell development and function (57-60)
NR_002819	Homo sapiens metastasis associated lung adenocarcinoma transcript 1 (MALAT1)	Controls cell cycle via B-MYB and mRNA processing (61, 62)
NR_029779	Homo sapiens miRNA 200c	MET progression. Regulates TLR-signaling and inflammation. Targets include: IKBKB, KRAS, MYD88, IRAK1 (65-67)
NR_030230	Homo sapiens miRNA 505	Inhibits cell proliferation. Induces apoptosis (68)

Figure 3-8: *E. chaffeensis* TRP32 binds to noncoding RNA genes in the host cell.

(A) TRP32 targets include RNA-coding genes. When all significant TRP32 bound regions of chromatin were examined 24% mapped to RNA coding genes. When the RNA coding genes were examined, the majority were SnoRNAs (33%) followed by miRNAs (29%), antisense RNAs (17%) and noncoding RNAs (17%). A small percentage corresponded to pseudogenes (4%). (B) A table of the characterized noncoding RNAs bound by TRP32 shows that many are involved in cell fate determination.

Using this program, several significant motifs were returned. The three most highly significant motifs were selected for analysis. Notably, all three motifs were highly similar with two containing centrally located GGAGGC and GGTGGC

sequences that may be variants of the same motif. The other motif also contained a similar sequence (CCAGGC), but it was not centrally located. Real sequences representing the three most highly significant motifs were used to construct biotin-labelled probes to test TRP32 binding affinity by EMSA. Using this system, we found that the two probes containing the centrally located GG[A/T]GGC sequence were both bound with high affinity. The probe which contained the peripherally located CCAGGC was bound with much lower affinity. The specificity of these interactions was confirmed using unlabeled probes as specific competitors (Fig. 3.9). The results from the EMSA suggested that a GGTGGC-like sequence approximated the consensus motif for TRP32. Additionally, when the 241 highly significant regions were examined for the presence of this sequence using FIMO (Finding Individual Motif Occurences) with a threshold of $p < 0.01$ they were all found to contain this motif (24). In order to confirm that this was indeed the TRP32 binding motif, wildtype constructs containing this motif and mutant constructs with alanine substitution in the predicted motif, or the ten nucleotides located at the 5' or 3' end were synthesized. These constructs were examined by EMSA and compared to the WT sequence. We found that the Δ1 mutant which had a disrupted GGTGGC sequence showed decreased binding by TRP32 and the Δ2, which entirely lacked the GGTGGC sequence showed an even greater decrease in binding as expected. However, surprisingly the Δ3 mutant also showed decreased binding by TRP32. When this probe sequence was examined further, an inverted repeat

of the predicted motif was identified, which was disrupted in that mutant (Fig. 3.9D).

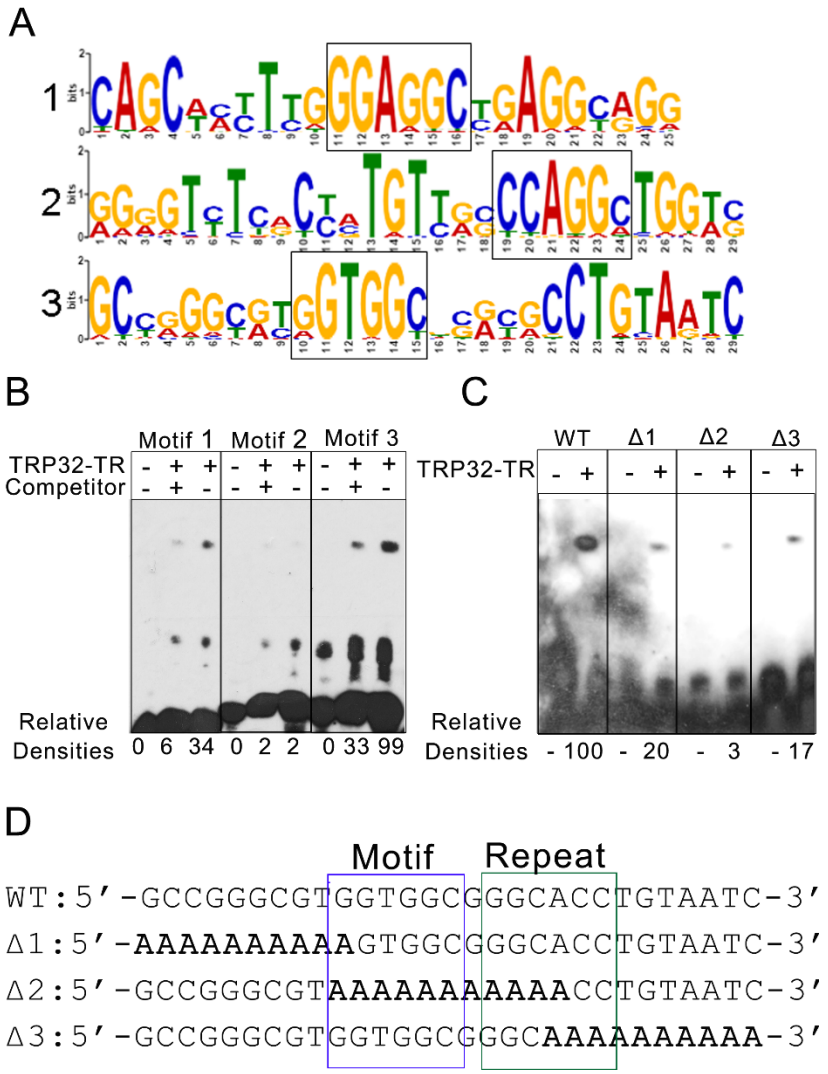


Figure 3-9: *E. chaffeensis* TRP32 binds a G-rich motif.

(A) TRP32 was predicted to bind to multiple, highly similar, G-rich motifs. Probability matrices of the top three predicted TRP32 DNA motifs from MEME-ChIP are shown. (B) These predicted motifs were tested by EMSA. TRP32-TR was incubated with biotin labeled probes in the presence and absence of unlabeled specific competitor. Relative densities are labeled below. Motif 3 was identified as the putative DNA motif due to its strong binding which was significantly decreased ($p = 0.002$) in the presence of competitor. (C) The TRP32-TR construct was incubated with the wild type motif 3 (WT) and with three mutant probes. TRP32 bound the wild type probe with greater affinity than the mutants. All of the mutants showed decreased binding. Relative densities are shown below. (D) Sequences of the wild type (WT) and mutant probes are shown with the putative motif boxed in blue and the inverted repeat boxed in green. Mutated sequences are bolded.

TRP32 modulates expression of target genes during infection and in a luciferase reporter assay. To examine TRP32 effect on transcription of identified target genes, transcriptional activity was measured by qPCR in uninfected and infected cells at 24, 48 and 72 hpi, and relative fold-change of infected compared to uninfected controls was calculated. Of the 57 TRP32 target genes that were measured, 46 (80%) were differentially expressed during infection, with the majority (60%) being highly downregulated; however, some genes were highly upregulated (Fig. 3.10A).

In order to determine the direct role of TRP32 in regulating gene expression, promoters from identified target genes were tested for transcriptional activity using luciferase transcriptional reporter assay. These constructs were transfected into HeLa cells along with varying concentrations of TRP32-GFP and an empty GFP control plasmid, and expression of luciferase was measured at 24 h post transfection. We found that TRP32 expression resulted in differential luciferase expression from the target gene promoters consistent with gene expression observed during infection. This differential regulation occurred in a dose dependent manner, and both activation, and repression of luciferase gene expression due to TRP32 was seen in this system. When the TRP32 binding site was deleted in the JUN promoter (ΔJUN) no differential regulation was seen. (Fig. 3.10B).

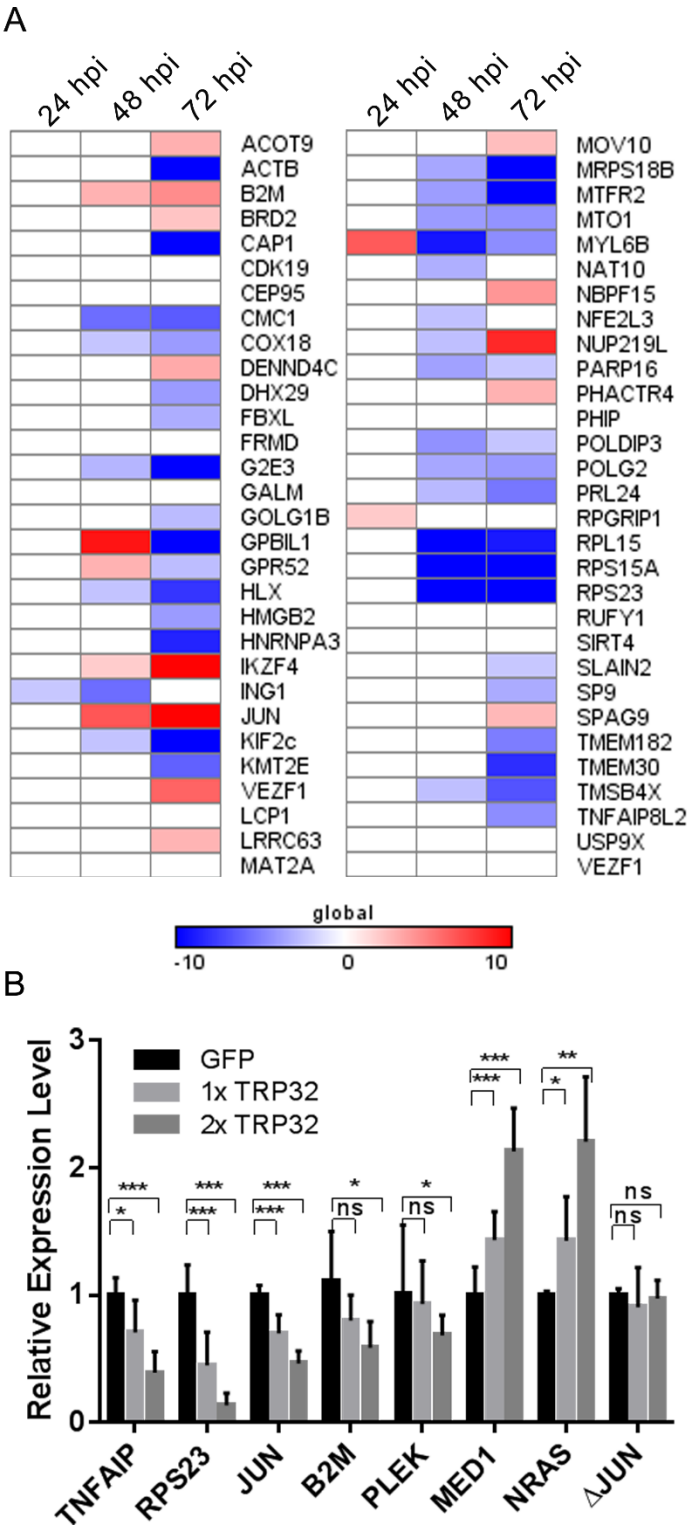


Figure 3-10: *E. chaffeensis* TRP32 has a direct effect on target gene expression.

(A) TRP32 targets are differentially regulated during infection. The most highly enriched genes from the ChIP-seq were assayed by qPCR at 24, 48 and 72 hpi. Data is presented as a fold change from non-infected. Colors represent the fold change from -10 to greater than 10. Data is representative of two (24 and 48 hpi) to three experiments (72 hpi). (B) TRP32 modulates gene expression in a luciferase reporter assay. Several genes were chosen, and the promoter regions including the TRP32 binding site were cloned into a luciferase reporter. Promoter constructs were transfected into HeLa cells along with varying concentrations of a TRP32-expressing or empty GFP vector. Luciferase expression was measured after 24 h. Luciferase expression is reported as fold change from control. Data is representative of 3-5 experiments.

DISCUSSION

All obligately intracellular bacteria including *Ehrlichia* must subvert innate host defenses and exploit normal host processes in order to survive. One of the strategies *E. chaffeensis* uses is to target conserved host pathways that act as nexuses of cell fate through moonlighting effectors, which have multiple roles/functions during infection [49, 108]. Many of these nexuses involve regulating the function of a transcription factor. Recently, bacterial nucleomodulins that directly alter host gene transcription have been described, which target host transcription program at the genetic or epigenetic level and can alter expression of thousands of host genes. This enables intracellular pathogens with small genomes and a limited effector repertoire to efficiently reprogram the host cell [109].

Despite the mounting evidence that *E. chaffeensis* TRPs are a novel family of transcription factor-like effectors that access the host nucleus, a conundrum has been that most lack a classical nuclear localization signal. Our preliminary studies have determined that TRPs enter the host nucleus by diverse means; however, a common theme of “piggy-backing” into the nucleus via protein-protein interactions seems to exist. We found that TRP32 residue Y179 is required for TRP32 nuclear localization via a regulated tyrosine phosphorylation-dependent mechanism. In fact, TRP32 localization at 48 hpi is consistent with the perinuclear localization of TRP32 in genistein treated cells and in Y179 mutants. This is suggestive that phosphorylation of TRP32 is required for interaction with an NLS-containing partner and indeed, the re-localization seen after genistein treatment is consistent

with that of a phospho-regulated nucleocytoplasmic shuttling protein such as ERK1/2 or SMADs [110-112]. Additionally, the PYYY motif seen in TRP32 can be involved in interaction with ubiquitin ligases such as the Cbl, which binds to phosphorylated tyrosines, or the NEDD family ubiquitin ligases, which are themselves regulated by tyrosine phosphorylation [113-115]. It is possible that ubiquitination may be the ultimate signal for TRP32 nuclear localization. The temporal differences seen in TRP32 localization support the idea of a regulated means of nuclear translocation, which is quite unlike TRP120 nuclear localization which begins early during infection (3 hpi) and continues to accumulate in the nucleus as the infection progresses [78]. Identification of the exact mechanism for TRP32 nuclear trafficking, i.e, phosphorylation alone or phosphorylation-triggered ubiquitination and the interacting partners, which potentiate these modifications, remains to be determined.

TRP32 bound 1587 genomic regions significantly, which is similar to the numbers of peaks identified for mammalian transcription factors in other reported ChIP-Seq experiments [116]. This value is approximately an order of magnitude less than the number of peaks bound by TRP120; however, both studies yielded similar numbers of highly significant peaks ($p \leq 10^{-20}$; TRP120: 582, TRP32: 240). When only the highly significant peaks were examined, these peaks mapped to human genes, and 70% were located within the promoter region. Although transcriptional regulatory regions may reside quite distally from the TSS, most well-studied transcription factors bind their target genes within the proximal promoter, and binding in this region has been linked to the regulatory function of transcription

factors [117, 118]. Interestingly, some of the genomic regions contained known promoter elements. For example, TRP32 binds several histone H4 genes within a described suppressor element [119]. Intriguingly, histone H4 gene transcription was downregulated during infection (data not shown). When expression of TRP32 targets was examined we found that 80% were differentially regulated during infection. This is an expected result as transcription factors typically bind many nonfunctional sites with reports of as many as ~50-99.9% of transcription factor bound sites not having a clear functional role [120, 121]. Most gene targets showed differences in expression primarily at 72 hpi, although some showed changes at 48 hpi as well. This is consistent with TRP32 localization data which shows that TRP32 surrounds and begins to enter the nucleus at 48 hpi, and accumulates in the nucleus at 72 hpi.

TRP32 is able to directly modulate transcription of genes during a luciferase gene expression assay. In this assay, targets are both up and downregulated which is similar to what is seen during infection and suggests that TRP32 may act as both a transcription repressor and activator. This differential expression occurs in a dose-dependent manner and is ablated when the TRP32 binding site is deleted. All together this strongly suggests that TRP32 is directly regulating gene expression of its targets. This is different from what was previously seen with TRP120, which primarily activated gene expression of its targets and for which a direct mechanism of action was not confirmed [78]. For a few genes the regulation seen during the luciferase assay did not correspond to changes in gene expression seen during infection. The most notable example of this was JUN which was highly

upregulated during infection, but downregulated by TRP32 in the luciferase assay. This is not unexpected and could be the result of multiple factors. Some differences might be due to cell type; however, other infection related processes are likely involved. Cell stress, the host innate immune response, as well as effector-mediated processes, not only modulate gene transcription, they can also affect post-transcriptional processing of mRNAs. JUN mRNA is highly post-transcriptionally regulated [122].

The ability to act as a transcriptional repressor or activator is a common feature of many eukaryotic transcription factors. Dual function transcription factors can be regulated by several factors including variations in the motif they bind, post translational modifications of the transcription factor, interaction with different transcription coregulators or the general transcriptional milieu (i.e., cell cycle, cell type, cell activation status) [123-125]. Post-translational modification of TRP32 may play a role in regulating TRP32 transcription factor function. TRP32 is predicted to be ubiquitinated at lysine residues adjacent to the putative 9AA TADs. Ubiquitination at these residues could theoretically alter TRP32's ability to interact with specific transcriptional coactivators. Interaction with other transcription factors and transcription initiation complex components can also regulate transcription factor function. TRP32 is known to interact with several host transcription regulators including HHEX and DAZAP2 and SPAMO determined that these interactions likely occur at the promoter, and thus, may be important for TRP32-mediated transcriptional regulation. Hence, their differential activation and recruitment may affect TRP32 function [82, 126].

When TRP32 target genes were examined for cellular process ontologies, several categories with relevance to infection were statistically enriched including hematopoietic cell differentiation and proliferation, chromatin remodeling, transcription and RNA processing and regulation of translation. Additionally, several global regulators of host cell proliferation and inflammation were among TRP32 gene targets including FOS, JUN, AKT3, TNF and NRAS. TRP32 manipulation of host differentiation and proliferation may be a factor in the leukopenia which is a common clinical feature in HME. A previous study examining the host transcriptome during infection showed that genes regulating cell cycle and differentiation, apoptosis and the innate immune response among others were differentially expressed during infection [127]. Although in that study only early time points (up to 24hpi) during infection were examined, the results included many gene categories targeted by multiple ehrlichial nucleomodulins, including TRP32. Overall, although distinct differences in TRP target genes are noted, there is some overlap in functional categories of genes targeted by *E. chaffeensis* nucleomodulins. Previously TRP120 was shown to interact with genes involved in regulating transcription, post-translational modifications and apoptosis [78]. Ank200 targets also include genes involved in transcriptional regulation and apoptosis as well as genes coding for structural proteins associated with the nucleus and membrane-bound organelles [96]. Moreover, there is also overlap in specific gene targets. Both TRP32 and Ank200 target histone coding genes for example and all three nucleomodulins target the inflammatory mediator TNF-α. This poses the question of whether these nucleomodulins may be functioning

cooperatively at the promoter or if they might be regulating gene expression separately in a coordinated manner. When TRP32 and TRP120 common target genes were examined, we found that they both bound within the proximal promoter only ~3% of the time and less than 1% of the time were they within 150 bp. This suggests that they do not typically function as part of the same regulatory complex. Temporal differences in nuclear localization also suggest that TRP32 and TRP120 may function independently, although the possibility of cooperative function cannot be eliminated.

When TRP32 target sequences were examined several highly similar G-rich motifs were statistically over-represented. When the most statistically enriched motifs were examined by EMSA, TRP32 was shown to bind them with differing affinity and specificity. These differences in binding affinity seem to relate to the position of the GG[A/T]GGC-like sequence within the probe and the presence or absence of an imperfect repeat. The probe which contained a peripherally located motif and a truncated repeat showed decreased binding affinity compared to probes with a centrally located GG[A/T]GGC sequence. Additionally, mutational analysis of the probe suggests that TRP32 may bind as a dimer. Although binding to a single GGTGGC was observed, it was considerably weaker than when two motifs were present in the form of imperfect inverted repeats, a finding consistent with other dimeric transcription factors such as EBF [128]. Inverted repeats can also indicate that the DNA is self-annealing to form secondary structures which are recognized by the DNA-binding protein. However, the binding pattern seen in our experiments is consistent with transcription factor

dimers binding to DNA since when either repeat is deleted the DNA-binding affinity decreases; however, it is not abolished as would be expected if the interaction were dependent on DNA secondary structure.

In this work, we present the first report of an ehrlichial effector directly regulating host gene expression. TRP32 was found to bind and regulate specific host genes both during infection and in a cell-based luciferase reporter assay. Additionally, we show that TRP32 enters the host nucleus in a tyrosine phosphorylation-dependent manner primarily after 48 hpi. This is different from what has been reported for previously studied TRPs, which enter the host cell nucleus very early in infection. Further, we have shown that TRP32 interaction with host genes occurs via binding to a repeated GG[A/T]GGG motif found in the promoter region of specific host genes. Additionally, promoter analysis (SPAMO) found significant secondary motifs occurred at defined intervals from the TRP32 binding motif. These secondary motifs corresponded to HHEX and TCF7L2 binding motifs and support that TRP32's interactions with these proteins occur at the promoter and have a functional effect on gene expression. Gene ontology analysis showed that TRP32 targets consist of genes governing immune cell differentiation and activation, chromatin organization, transcription and RNA-processing and translation and suggest that TRP32 may be influencing host cell fate to either promote differentiation, or to prevent a protective immune response. Further studies will define the role of TRP32 in influencing host cell differentiation and function and will identify how the various ehrlichial nucleomodulins interact to regulate the host and promote ehrlichial survival.

Chapter 4. *Ehrlichia chaffeensis* TRP32 nucleomodulin function and localization is regulated by NEDD4L-mediated ubiquitination

INTRODUCTION

Ehrlichia chaffeensis is a gram-negative, obligately intracellular bacterium and the etiologic agent of human monocytotropic ehrlichiosis (HME), an emerging life-threatening tick-borne zoonosis. In humans, *E. chaffeensis* preferentially infects mononuclear phagocytes, causing an acute infection that manifests as an undifferentiated febrile illness. The mechanisms by which *E. chaffeensis* reprograms host cell processes are not fully understood; however, a group of type 1 secreted tandem repeat protein (TRP) effectors similar to the repeats-in-toxin family of exoproteins are involved. TRPs were first identified as *E. chaffeensis* major immunoreactive proteins, and linear antibody epitopes have been mapped to tandem repeat domains that elicit protective antibody responses [67, 85, 129, 130]. It is now established that TRPs are secreted moonlighting effectors that interact with a large array of functionally diverse host cell proteins as well as host cell DNA.

The most well studied TRPs, TRP120 and TRP32, interact with many host cell targets, directly activate cell signaling pathways, and activate/repress host cell transcription. Surface expressed TRPs contribute to ehrlichial entry via WNT pathway activation [49]. Additionally, TRP120 interactions with ADAM17 on the host cell surface activate the Notch pathway resulting in the downregulation of several innate immune molecules including TLR2/4 [131]. TRP120 and TRP32 also act as nucleomodulins and manipulate host gene expression via direct

interactions with target genes. TRP120 binds a GC-rich motif leading to upregulation of specific host genes involved with transcriptional regulation, signal transduction, and apoptosis. While TRP32 also binds a G-rich motif consisting of imperfect repeats of a GGTGGC-like sequence, TRP32 targets genes regulating cell proliferation and differentiation. TRP32 was also shown to both activate and repress expression of targets in a gene specific manner during infection and in a cell based luciferase reporter assay.

A common theme among bacterial pathogens is the hijacking of host post-translational machinery to regulate bacterial effectors. *Ehrlichia* proteins are phosphorylated (TRP32, TRP75, Ank200) and SUMOylated (TRP120) by host enzymes [65, 80, 132, 133]. These PTMS are important for effector function as TRP120 SUMOylation is required for interactions with host proteins, and TRP32 tyrosine phosphorylation is necessary for nuclear import.

Ubiquitination is the covalent attachment of a small peptide modifier, ubiquitin (Ub), to a target lysine or to the N-terminus of the target. This attachment occurs via an enzymatic cascade requiring the sequential action of three classes of enzymes the third of which, the E3 Ub ligase (~600 known), determines substrate specificity [134]. Ubiquitination occurs either singly (monoubiquitination), or as a chain covalently linked via any one of seven lysine residues or the N-termini. All homotypically-linked chains as well as heterotypic and branched polyUb chains have been detected in cells with different chain types targeting the substrate to different fates within the cell (Ub and Ub-like proteins as multifunctional signals). The most studied chains are K48-linked

chains which cause proteasomal degradation of their substrates, while K63-linked chains are involved in cell signaling, receptor endocytosis and protein-protein interactions, and K11-linked chains may play a role in cell cycle specific protein degradation [135].

Although bacteria do not possess an endogenous Ub system, the modification of bacterial effectors by host ubiquitination machinery is well-described [136, 137]. These modifications can influence both physical and temporal localization of effectors, depending on whether the Ub conjugate is a single Ub molecule or a polyUb chain. Mono ubiquitination typically alters protein localization and is involved in both nuclear import and export of eukaryotic proteins [138]. The *Salmonella* effector SopB utilizes monoubiquitination to alter its subcellular localization. SopB is a phosphatase that first localizes to the host membrane, where its enzymatic functions alter actin organization, facilitating bacterial entry. After SopB is multiply mono ubiquitinated, it traffics to the *Salmonella*-containing vacuole where it functions to alter vesicular trafficking to facilitate bacterial replication [139]. The functional consequences of polyubiquitination are dependent on chain type. However, one of the best characterized examples would be in *Legionella pneumophila* which utilizes K48-linked polyubiquitination to temporally regulate the effector SidH [140].

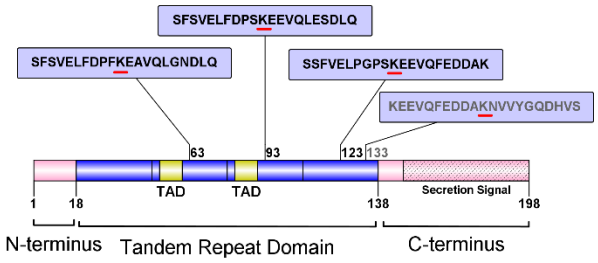
Although a few examples of pathogen effector PTM-regulated function exist, effector regulation by ubiquitination has not been described for any obligately intracellular pathogens such as *Ehrlichia*. In this study, we examined the role of ubiquitination on TRP32 transcription factor function. We demonstrate

that multiple species of mono-and polyubiquitinated TRP32 can be detected during infection, and that the host E3 enzyme, NEDD4L, ubiquitinates TRP32. Moreover, we found that these Ub modifications are required for TRP32 transcription factor function and subnuclear localization.

RESULTS

TRP32 is mono and polyubiquitinated at multiple lysine residues. *In silico* analysis identified multiple lysine residues in TRP32 which were predicted to be sites of ubiquitination (FIG 4.1A) [141]. When 12-mer peptides corresponding to putative TRP32 ubiquitination sites were tested in a microfluidic peptide array to test their ubiquitination potential *in vitro*, two sites were identified (FIG 4.1B). Multiple higher molecular weight TRP32 bands were observed when ectopically expressed TRP32 was co-immuno-precipitated with HA-tagged wildtype or lysine null Ub. Additionally, fewer bands were seen in the Ub null pulldown suggesting that TRP32 was modified by both mono and polyUb chains (FIG 2A). When IP was performed using a TRP32-specific antibody and the eluate probed with antibodies specific for polyUb (FK1) and mono and polyUb conjugates (FK2) higher molecular weight bands were present that were consistent with the presence of both mono and polyubiquitinated TRP32 (FIG 4.2B). Despite the presence of polyubiquitinated TRP32 species, TRP32 does not appear to be degraded by the proteasome as treatment with varying concentrations of the proteasome inhibitor Carfilzomib (IC50< 5nM) did not alter TRP32 proteins levels (FIG 4.2C).

A



Residue	Score	Ubiquitinated	
63	0.72	Yes	Medium confidence
93	0.92	Yes	High confidence
123	0.95	Yes	High confidence
133	0.68	Yes	Low confidence

B

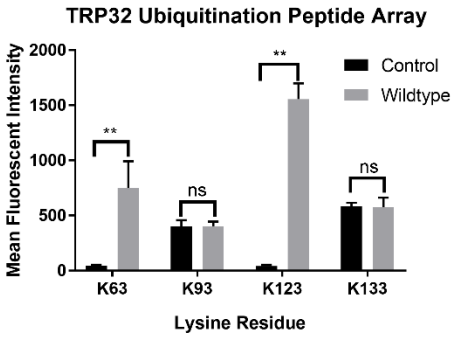


***Figure 4-1: Ehrlichia chaffeensis* TRP32 is predicted to be ubiquitinated at multiple lysine residues.**

A. TRP32 contains four lysine residues, all of which are located within the tandem repeat domain and are predicted to be ubiquitinated with varying confidence levels. B. TRP32 lysine containing peptides were tested in a microfluidic peptide ubiquitination assay. Lysine 63 and 123 showed significantly higher signals than corresponding peptides in which the lysine residues were mutated into alanines.

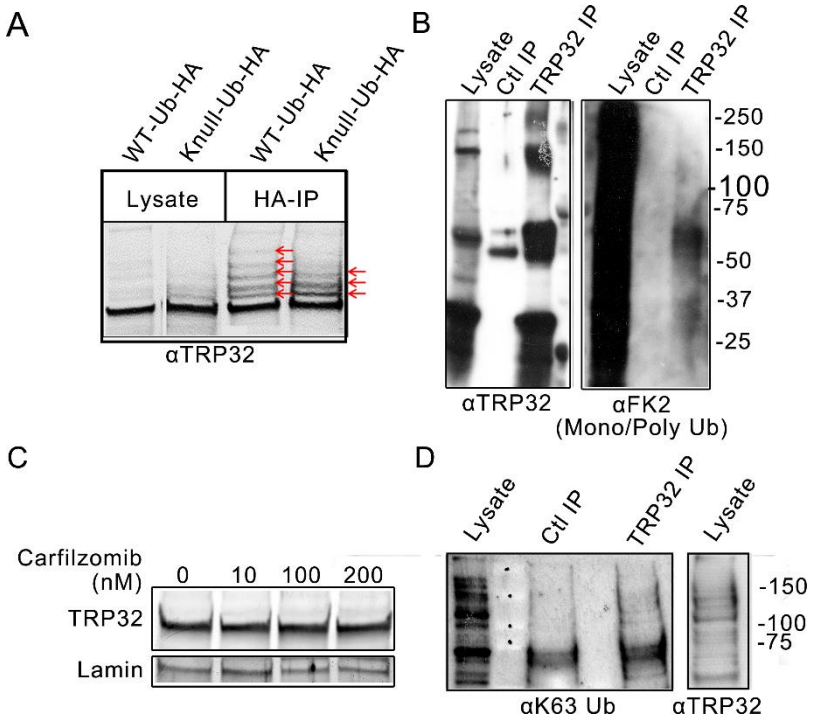


***Figure 4-2: Ehrlichia chaffeensis* TRP32 is mono- and poly-ubiquitinated, but is not degraded by the proteasome.**

A. GFP-tagged TRP32 was cotransfected into HeLa cells with HA-tagged wildtype and lysine null ubiquitin constructs. HA-immunoprecipitation was performed and the resulting eluate was probed with anti-TRP32 specific antibody by immunoblot. Multiple higher molecular weight bands indicative of ubiquitinated species of TRP32 were detected (red arrows). B. Multiple species of ubiquitinated TRP32 were also detected during infection of THP-1 cells. Anti-TRP32 specific antibody was used to immunoprecipitate TRP32 from infected THP-1 lysate. The resultant immunoprecipitated protein was probed with antibodies specific for conjugated mono- and poly-ubiquitin chains (FK2) by immunoblot. Bands were detected between 25-37 kDa, 50-75 kDa and above 100 kDa that colocalized with TRP32 bands. C. TRP32 levels are not responsive to treatment with a proteasome inhibitor. Cells were incubated with varying concentrations of the proteasome inhibitor Carfilzomib for 8 h prior to harvest and immunoblotting with anti-TRP32 and anti-lamin (loading ctrl) antibodies. D. K63 linked ubiquitin was detected on TRP32 during infection of THP-1. IP was performed using anti-TRP32 specific antibodies and the eluate was probed with antibody against K63-linked ubiquitin chains. Bands were detected about 100 kDa that colocalized with TRP32 bands.

TRP32 is polyubiquitinated by K63-linked chains. Because the type of polyUb chain determines the effect of this modification on the substrate protein, we characterized the polyUb chains on TRP32 to gain insight into the function of this modification. Lysate from infected THP1 cells was subject to IP using anti-TRP32 antibody and the resulting eluate probed with antibodies against the three most common polyUb chain types (K48, K63, K11). TRP32 levels were not responsive to treatment with proteasome inhibitors so, as expected, K48-linked polyUb chains attached to TRP32 were not detected; nor did we detect K11-linked chains (not shown). However, several bands reacted with anti-K63-linked Ub antibody that corresponded to bands of similar molecular mass identified with anti-TRP32 antibody, suggesting that TRP32 was modified by K63-linked polyUb chains (FIG 4.2D).

TRP32 interacts with and is ubiquitinated by NEDD4L. NEDD4L is upregulated during infection and associates with ehrlichial morulae; thus, we wanted to determine if NEDD4L ubiquitinates TRP32. Hence, IP was performed on *E. chaffeensis*-infected THP-1 cells using either an anti-TRP32 antibody or IgG control and the resulting eluate was probed with anti-NEDD4L antibody. NEDD4L precipitated with TRP32 suggesting a direct interaction during infection (FIG 4.3A). Additionally, when infected cells were treated with heclin, an inhibitor of HECT-family ligases including NEDD4L, higher molecular weight forms of TRP32 were decreased compared to untreated cells (FIG 4.3B). To investigate this further, we performed an *in vitro* ubiquitination assay on TRP32 in the presence and absence of recombinant NEDD4L. We found an increased

abundance of higher molecular weight forms of TRP32 when NEDD4L was included. These forms colocalized with a broad Ub reactive zone that extended from approximately 50 kDa (the size of unmodifided TRP32-GST) to the top of the gel that was only present in the lane containing both TRP32 and NEDD4L (FIG 4.3C). When these *in vitro* ubiquitination assays were probed with anti-K63 linkage

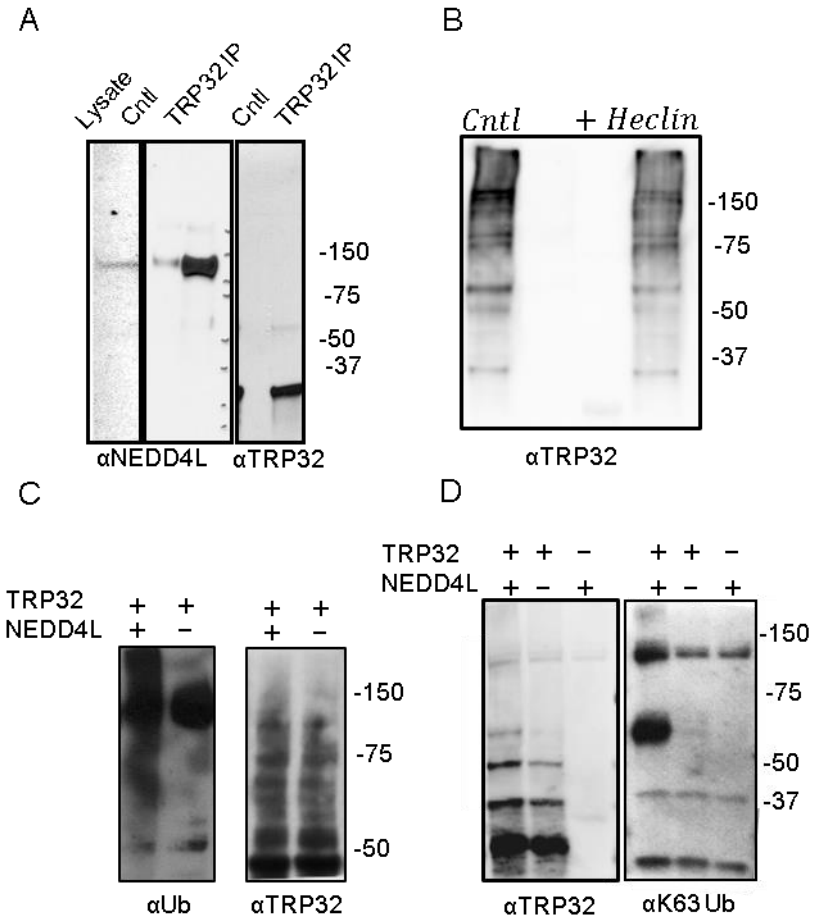


Figure 4-3: Host E3 NEDD4L conjugates TRP32 with K63-linked polyubiquitin chains

A. TRP32 interacts with host NEDD4L during infection. Immunoprecipitation was performed on lysate harvested from *E. chaffeensis*-infected THP-1 cells using anti-TRP32 antibody or pre-immune serum. The resulting eluate was probed with anti-NEDD4L and anti-TRP32 antibody by immunoblot. B. Treatment with heclin decreased TRP32 higher molecular weight bands. *E. chaffeensis*-infected THP-1 cells were incubated with heclin (25uM) for 12 h before harvesting and immunoblotting with anti-TRP32 specific antibodies.

C. NEDD4L facilitates TRP32 ubiquitination during an *in vitro* assay. TRP32 was incubated with E1 and E2 ubiquitin ligases as well ubiquitin and required cofactors in the presence or absence of NEDD4L. The reactions were then separated by SDS-PAGE and probed with anti- mono- and polyubiquitin specific antibody (FK2) or with anti-TRP32 specific antibodies. Greater ubiquitinated TRP32 was detected in the reaction that included NEDD4L. D. NEDD4L conjugates K63-linked polyubiquitin chains to TRP32 in an *in vitro* assay. An *in vitro* assay was performed in the presence or absence of NEDD4L and the resultant reaction was immunoblotted with anti-TRP32 and anti-K63-linked polyubiquitin specific antibodies. Bands corresponding to K-63 linked polyubiquitinated TRP32 were increased in the presence of NEDD4L.

specific antibody, we found that NEDD4L catalyzed TRP32 K63-linked ubiquitination (FIG 4.3D).

Ubiquitination of TRP32 effects localization within the nucleus and transcription factor function. Because both K63-linked polyubiquitinated chains and monoubiquitination have been linked in the literature to regulation of substrate subcellular localization, we examined the role of ubiquitination in TRP32 localization within the host cell. First, we examined the localization of polyubiquitinated TRP32. Infected cells were harvested and nuclear cytosolic fractionation performed before applying the lysates to Ub enrichment beads. The resulting eluate was probed with a TRP32-specific antibody. Higher molecular weight TRP32 was enriched in both the nuclear and cytosolic fractions. However, different species were found in the different compartments, with species above 100 kDa found only in the nuclear fraction (FIG 4.4A). This suggested that ubiquitination may indeed play a role in TRP32 nuclear localization or function. In order to further address this, we first investigated the role of Ub on TRP32 localization by ectopically expressing TRP32 in cells that

had been treated with either a HECT-specific inhibitor (heclin), an E1 inhibitor (Pyr41, pan ubiquitination inhibitor) or a proteasome inhibitor (bortezomib). We found that both the E1 and the HECT-inhibitor resulted in altered localization of TRP32 in the nucleus compared to untreated control. However, the phenotype for the two inhibitors was quite different. Inhibition of total cellular ubiquitination resulted in accumulation of TRP32 within nucleolar regions and in the cytosol. This was not due to accumulation of undegraded protein because treatment with the proteasome inhibitor bortezomib did not alter cellular localization of TRP32. However, treatment with Heclin caused nuclear TRP32 to assume a lacy appearance with rings and puncta concentrated around nucleoli (FIG 4.4B). We hypothesized that the differing subnuclear localization seen with these inhibitors might be due to altered interactions with chromatin or members of the transcription initiation complex. To test this we performed a luciferase assay using a firefly luciferase reporter expressed under control of TRP32 target promoters in the presence or absence of these inhibitors. We found that Pyr41 caused significant decreases in expression of both the constitutively transcribed *Renilla* luciferase and the firefly luciferase suggesting that this inhibitor broadly dysregulated transcription (data not shown). However, when heclin was used we found that TRP32-mediated repression of firefly luciferase was removed and that expression returned to levels similar to control (FIG 4.5).

Next we wanted to confirm that this phenotype was due to direct ubiquitination of TRP32 and to identify if a particular lysine residue was required. To do this, a series of lysine mutants was created, including a mutant in which all four lysines

were mutated (Knull) in order to see if the phenotype seen with heclin could be replicated. When these mutants were ectopically expressed, the K63 and K123 mutants showed phenotypes characterized by peri-nucleolar rings and puncta, similar to that seen in the heclin treatment group. This phenotype was also observed when the Knull mutant was used. Both the K93 and the K133 mutants showed localization similar to wildtype, with the K93 mutant showing slightly increased cytosolic localization (FIG 6). When these mutants were examined in a luciferase assay, we found that the Knull TRP32 was unable to repress transcription. The K63 and K123 mutants also relieved transcriptional repression, as suggested by the localization data. Notably, although the K133 mutant exhibited repression similar to wildtype, the K123 mutant relieved repression (FIG 4.7).

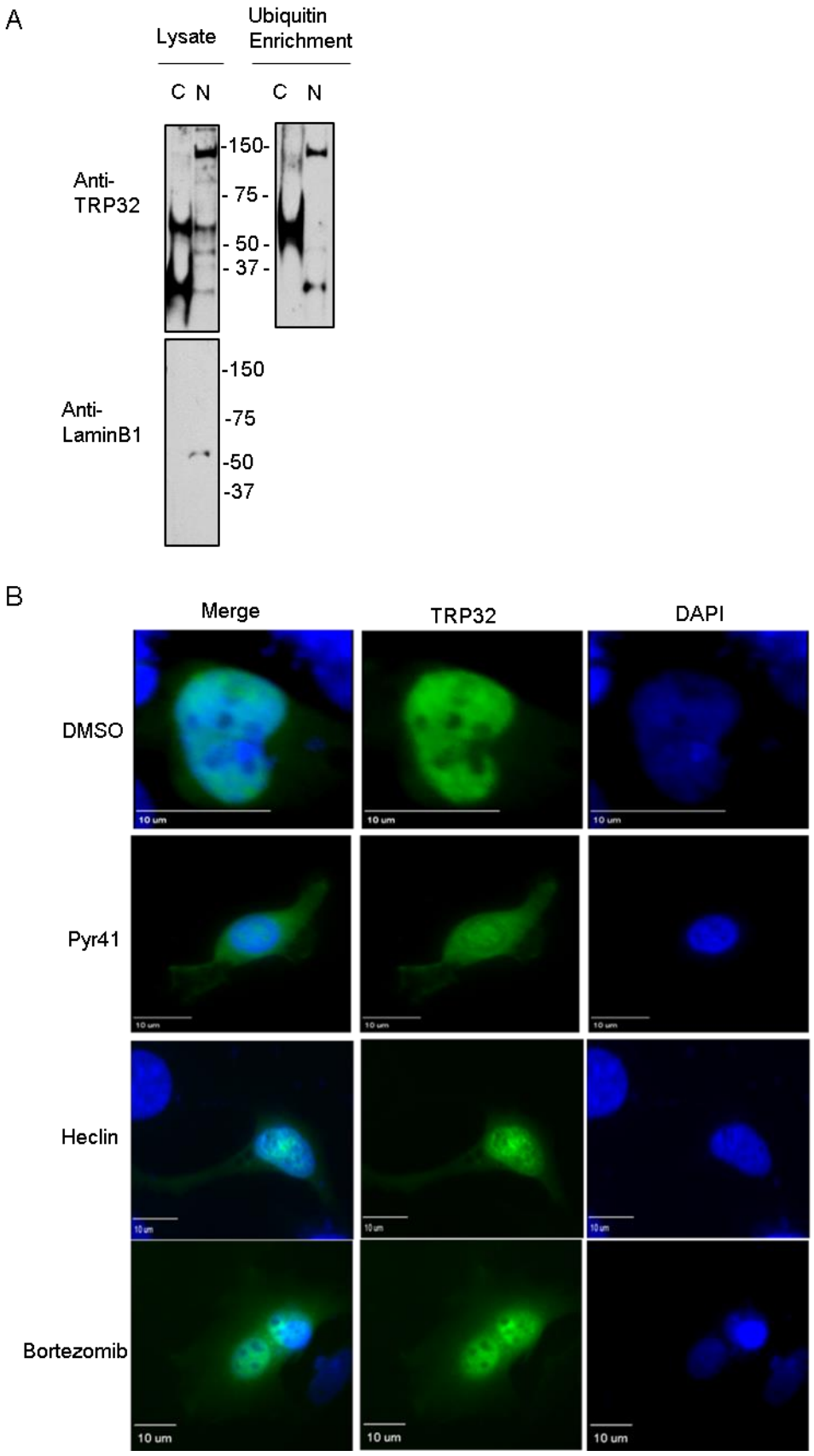


Figure 4-4: Ubiquitination inhibitors cause altered subcellular localization of GFP-tagged TRP32.

A. Polyubiquitinated TRP32 is detected in both the nucleus and cytosol of infected cells. *E. chaffeensis*-infected THP-1 cells were harvested and subject to nuclear cytosolic fractionation before ubiquitin enrichment. Lysate and eluate from Ub enrichment were probed with anti-TRP32 specific antibody by immunoblot. B. HeLa cells were transfected with GFP-tagged TRP32. After 6 h the medium was changed and and Pyr41 (), Heclin(), Bortezomib or DMSO (vehicle) were added. At 24 h post-transfection cells were fixed and visualized using fluorescent microscopy. Nuclei are stained blue (DAPI).

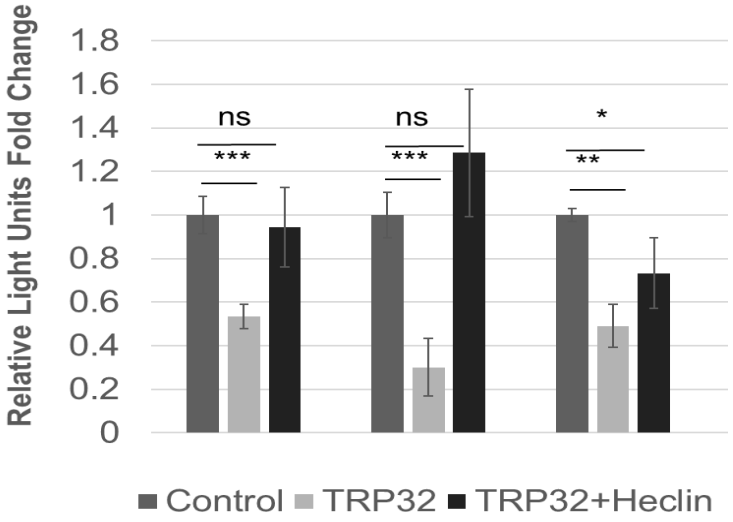


Figure 4-5: TRP32-mediated transcriptional repression is alleviated by the HECT E3 ligase inhibitor heclin

GFP-TRP32 or a GFP control were transfected into HeLa cells with TRP32-target promoter firefly luciferase expression constructs and *Renilla* luciferase expression vectors in the presence of the HECT E3 ligase inhibitor heclin or vehicle. After 24 h luciferase expression was measured and normalized to constitutive *Renilla* luciferase expression and is presented as fold-change from control. Graph is representative of three experiments.

DISCUSSION

The identification of bacterial effectors, their function and regulation within the host cell is essential for understanding how obligately intracellular bacteria such as *E. chaffeensis* create and maintain a viable niche within their host. Previously, we identified TRP32 as a nucleomodulin that regulates transcription of host genes relating to cellular differentiation and proliferation [133]. In this study, we explored how *Ehrlichia* exploits the host ubiquitin system to regulate the function of this effector.

Previously, we have shown that TRP32 is a dual function transcription factor. Although TRP32 possesses putative transactivation domains and was able to activate gene transcription in yeast, during infection of a mammalian cell line the majority of TRP32 targets are downregulated, and only a few are upregulated. This was also seen in a luciferase assay using TRP32 gene targets [133]. Typically, dual function transcription factors which can recruit either coactivators or corepressors function in one of two different ways. In some, their function is promoter dependent while in others it can switch between activation and repression in a signal-dependent manner with the signal being addition of a post-translational modification, typically phosphorylation, SUMOylation or ubiquitination [142, 143]. Although, TRP32 is predicted to be serine/threonine phosphorylated at several sites and tyrosine ubiquitination plays a role in TRP32 nuclear localization, and thus may be required for transcription factor function, we chose to focus on ubiquitination in part because SUMOylation of TRP32 cannot be detected during infection or in an *in vitro assay* (data not shown).

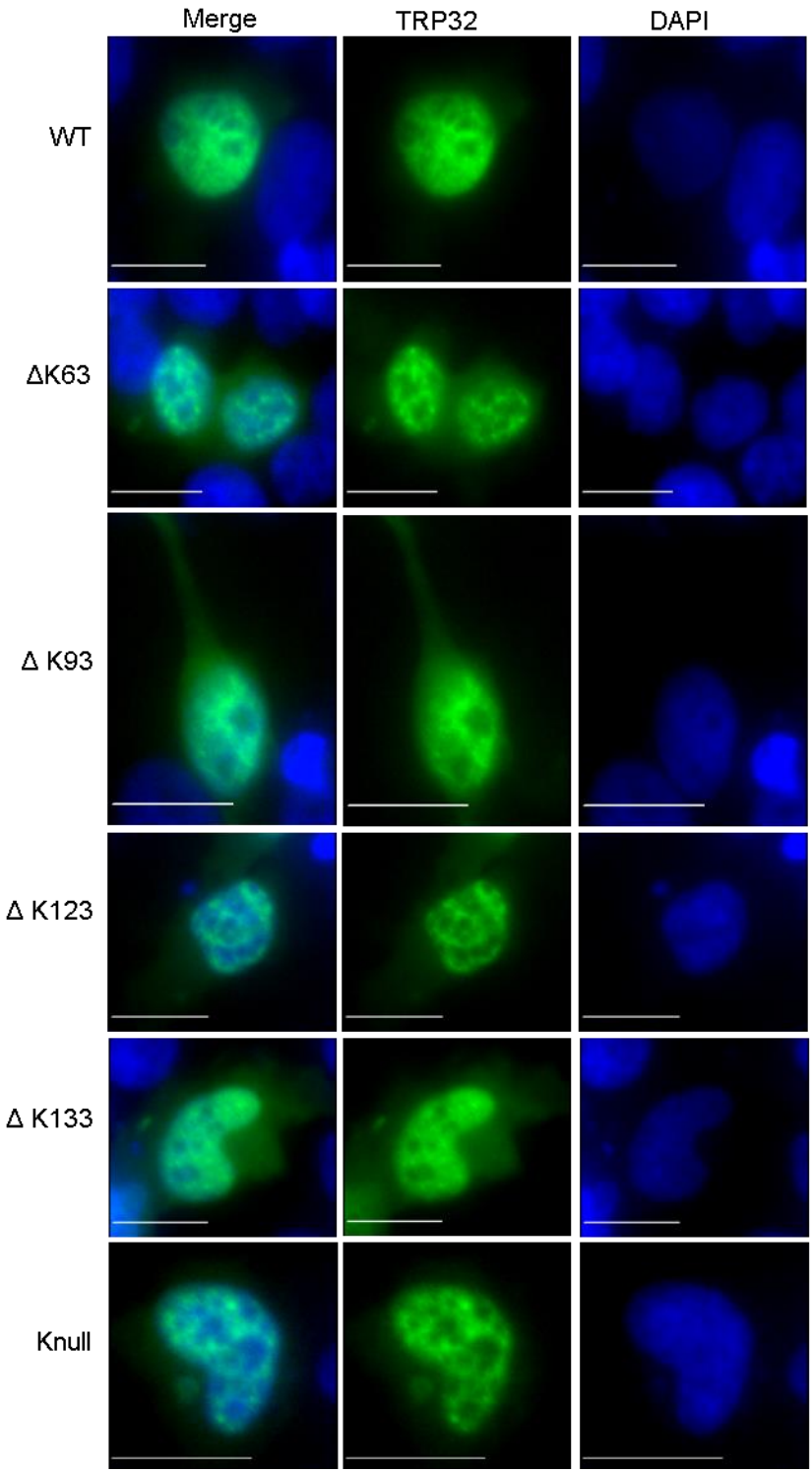


Figure 4-6: Ubiquitination inhibitors cause altered subcellular localization of GFP-tagged TRP32

HeLa cells were transfected with wildtype (WT) and lysine mutant GFP-tagged TRP32. At 24 h post-transfection cells were fixed and visualized using fluorescent microscopy. Nuclei are stained blue (DAPI).

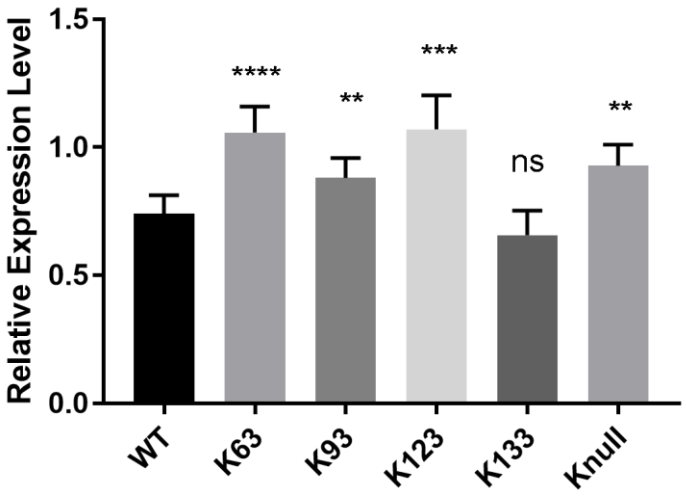


Figure 4-7: TRP32 lysine mutants exhibit decreased repression of target gene transcription.

Wild type and lysine mutant GFP-TRP32 or a GFP control were transfected into HeLa cells with TRP32-target promoter firefly luciferase expression constructs. After 24 h luciferase expression was measured and is presented as fold-change from control. Significance was determined using a student's *t*-test ($p<0.05$). Graph is representative of four experiments.

Additionally, the ubiquitin-proteasome system plays an important role in both positively and negatively regulating several transcription factors. Interestingly, several transcription factors possessing an acidic, 9aa TAD including VP19 and Myc, require polyubiquitination for TAD function [144], and transcription factor activation is intimately linked to proteasomal degradation in a wide variety of eukaryotic transcription factors [145]. Indeed, the proteasome is integral to transcription where it performs both proteolytic and nonproteolytic functions playing a role in coactivator recruitment, transcription elongation and histone modification in addition to protein degradation [146]. The proximity of ubiquitinated lysines to TRP32 9aa TADs led us to speculate that such a

mechanism might be at play here. However, our data showed that ubiquitination is required for TRP32 repressor function. This may be because TRP32 is not ubiquitinated with K48-linked polyUb chains, but instead with K63-linked chains. Indeed, the TRP32 C-terminus (which lacks lysine residues) was previously shown to interact with a member of the 20S proteasome PSMB1 which is known to play a role in transcriptional regulation [147, 148]. Therefore, TRP32 may be able to recruit necessary components of the proteasome independently. Interestingly, K63-linked Ub has been tied to the function of several transcription factors including IRF1, RORyt and FOXO family transcription factors [149-155]. As, K63-linked Ub chains can act as scaffolds for protein-protein interaction, these chains may be required to recruit corepressors or coactivators. Indeed, there is evidence that K63-linked polyUb chains may be required for the recruitment of both histone deacetylases (HDACs) and members of the transcription initiation complex [156, 157]. However, it remains to be determined whether K63-linked ubiquitination is the most important modification of TRP32 in terms of transcriptional regulation. The inhibitor data with heclin support this as NEDD4L primarily conjugates K63-linked Ub chains. However, NEDD4L also has been shown to conjugate monoUb as well as K6, K11, K26, K29 atypical chains [158-160]. Additionally, although we did not address this, a recent study reported that one of the reasons that K63-linked chains are not linked to proteasomal degradation may be due to competitive interactions with members of ESCRT0 [161]. This ties in with other research which links K63-linked chains to multivesicular body biogenesis and cargo sorting and makes it tempting to

hypothesis that K63-linked polyUb could play a role in TRP32 escape from the morula or intracellular trafficking.

Interestingly, our data suggests that ubiquitination at all three lysine residues (K63, K93 and K123) is required for TRP32 repressor function, as mutation of any one of these three lysines alleviated TRP32-mediated transcriptional repression. Whether these lysines are modified by multi monoUb or multi polyUb or a mix of the two remains to be determined. Each of these scenarios has been described in the literature, and indeed a pattern of complex, multi-functional ubiquitin modifications is typical of many eukaryotic transcription factors, and TRP32 seems to be an adept mimic.

We found that TRP32 interacted with and was ubiquitinated by the human E3 ubiquitin ligase NEDD4L during infection and in an *in vitro* assay, respectively. Although, TRP32 lacks the canonical NEDD4L PPxY interaction motif, it does contain three similar C-terminal motifs (TPYY, NPYY, and TPDY) that may mediate interactions with NEDD4L. Other NEDD family members have been shown to interact with motifs containing phosphorylated serine or threonine residues (pS/pT)PxY inaddition to the canonical PPxY motif [162]. Additionally, several known NEDD4L targets lack the PPxY motif completely and interact with NEDD4L via adaptor proteins which may also be the case with TRP32 [163, 164].

Although NEDD4L has not previously been studied in the context of bacterial infection, it has been linked to viral infections including HIV infection where K63-linked ubiquitination of the viral protein Gag by NEDD4L plays an important role

in viral budding, and in enterovirus 71 infection, where it may function via altering IFN-β production [165, 166]. In uninfected human cells, NEDD4L has been most commonly studied in the context of regulation of membrane proteins, primarily sodium and potassium channels where it facilitates sorting into recycling endosomes [167]. However, NEDD4L is also an important regulator of the TGF-β and Wnt signaling pathways via ubiquitination of SMADs and Dishevelled proteins, respectively [160, 168]. Especially interesting in the context of *Ehrlichia* infection is that NEDD4L is phosphorylated and activated by JNK in a WNT5a-dependent manner [160]. Previously, we have shown that both canonical and non-canonical Wnt signaling are vitally important to successful *E. chaffeensis* infection and that WNT5a knockdown results in significantly decreased bacterial levels [49]. Thus, it is likely that WNT5a activation of NEDD4L plays a role in licensing TRP32 transcription factor function.

During infection, we demonstrated that TRP32 interacts with the host E3 Ub ligase NEDD4L, and can be ubiquitinated by NEDD4L in an *in vitro* assay. Multiple mono and polyubiquitinatated forms of TRP32 were seen during infection, including some that did not all disappear when cells were treated with the NEDD4L inhibitor (heclin). Further, we showed that treatment with heclin impaired TRP32's ability to repress target gene transcription, altered TRP32 subnuclear localization, and that mutation of specific TRP32 lysine residues replicated these phenotypes. It is likely that ubiquitination serves multiple functions in regulating TRP32 and that other Ub ligases in addition to NEDD4L are required. Indeed, TRP32 is known to interreact with the putative ring type E3

Ub ligase Roquin [82]. Future directions include identifying the mechanism of altered TRP32 transcriptional function mediated by ubiquitination and fully characterizing the ubiquitin modifications that occur on various TRP32 lysine residues and the potential crosstalk between these various Ub modifications and other PTMs that TRP32 undergoes.

Chapter 5.Summary and Future Directions

In this work, I initially set out to provide support for the hypothesis that *Ehrlichia chaffeensis* tandem repeat proteins are a novel class of nucleomodulins and to increase our understanding of the ways in which these bacterial effectors interact with host proteins including systems of host post-translational modifications to survive within the host monocyte. And although I was successful in my initial aim, during the course of these studies many new questions were uncovered. In Chapter 3, I showed that TRP32 is indeed an ehrlichial effector that functions as a transcription factor. In fact, my luciferase reporter assays present the first report of an ehrlichial effector directly regulating host gene expression. I confirmed that TRP32 does indeed bind host DNA via its TR domain. This provides increasing support for the hypothesis that these ehrlichial TRPs are a novel class of effectors with functional similarities to the TAL effectors of *Xanthomonas*. Using the results of ChIP-seq experiments, I was able to identify and test putative TRP32 DNA motifs. However, during the experiments to confirm the TRP32 DNA motif, I found evidence that TRP32 binds DNA as a multimer unlike the TAL effectors. Previous work by A. Wakeel in our laboratory found that TRP120 tandem repeats contain predicted zinc binding domains [65]. It is not known if TRP120 or TRP32 are ion-binding proteins; however, ion-binding proteins such as zinc-fingers make up a large family of transcription factors and often oligomerize to interact with DNA. Therefore, an interesting question that remains is whether these TRPs are structurally more

similar to TAL DNA-binding proteins, ion-binding “finger” DNA-binding proteins, or to another, possibly novel group of nucleic acid binding proteins.

Additionally, in the ChIP-Seq experiment, I showed that TRP32 bound several genomic regions significantly. When examined these peaks mapped to human genes, preferentially within the promoter. Additionally, I showed that several secondary motifs were significantly enriched at defined intervals from the TRP32 binding motif. The enriched motifs found included the motifs of TRP32-interacting partners HHEX and the DAZAP2 DNA-binding partner TCF7L2. This suggests that TRP32 interacts with these host proteins at the promoter to regulate host gene expression. However, we do not currently know the functional relevance of these interactions. A question that future research could address is whether TRP32 is acting antagonistically with these host transcription factors or is it suborning them to promote transcriptional changes beneficial to *Ehrlichia.*

Finally, in Chapter 3, I showed that TRP32 interacts with genes governing immune cell differentiation and activation, chromatin organization, transcription and RNA-processing and translation. This suggests that TRP32 influences host cell fate to either promote differentiation, or to prevent a protective immune response. However, the phenotypic outcome of TRP32-mediated changes in gene expression are not currently known. Future studies will define the role of TRP32 in host cell differentiation and function and will identify how this relates to disease pathology and ehrlichial survival in the monocyte.

While performing the studies mentioned in Chapter 3, I noticed that TRP32 possesses two TADs that were proximal to predicted ubiquitination sites and that the tyrosine residue which regulated TRP32 nuclear trafficking existed in what another laboratory member had identified in a different TRP as a non-canonical NEDD4L interaction motif. This led me to question the potential impact of TRP32 ubiquitination on its nuclear localization or transcription factor function. In Chapter 4, I showed that TRP32 is multiply ubiquitinated by both single ubiquitin and by polyubiquitin chains during infection. I confirmed my prediction that NEDD4L is an interacting partner of TRP32 and showed that NEDD4L ubiquitinates TRP32 with K63-linked polyubiquitin chains. Additionally, I showed that ubiquitination does not seem to play a role in TRP32 nuclear localization. Instead, I found that ubiquitination is linked to both TRP32 transcriptional regulation and subnuclear localization. It is likely that these two are related and that the redistribution of TRP32 seen when ubiquitination is impaired, either through inhibitor treatment or mutagenesis, likely results from changes in TRP32 interaction with host transcriptional machinery or chromatin. Future studies will address what specific interactions are disrupted that result in this phenotype. Additionally, although I was able to link specific lysine residues on TRP32 to alterations in its function, I was not able to characterize specific ubiquitin modifications on these lysine residues. A question that remains is whether monoubiquitinated or K63 polyubiquitinated TRP32 or both are important for its transcriptional regulatory function. Indeed, the multiple species of ubiquitinated TRP32 seen during infection suggest that ubiquitination may serve multiple functions in regulating TRP32. Hopefully, future

studies will characterize specific modifications on TRP32 lysine residues as well as identifying any potential crosstalk between ubiquitin modifications and other PTMs.

In summary, this investigation set out to explore how *E. chaffeensis* TRP32 functions within the host cell with the hope that this work would uncover novel mechanisms by which *E. chaffeensis* and other intracellular pathogens modulate host cell responses to facilitate their survival and replication. While performing the studies outlined in this dissertation, I found that TRP32 functions within the host cell by acting like a host transcription factor with all of the extraordinary complexity that that entails. Like many eukaryotic transcription factors, TRP32 utilizes phosphorylation to regulate its nuclear entry. Once inside the nucleus, it interacts with other host transcription factors in yet unexplored ways to control host gene expression. Additionally, I found that, like many eukaryotic transcription factors, TRP32 utilizes ubiquitination to regulate its function. Essentially, I showed that a bacterial pathogen with a small genome can increase the functional repertoire of its effectors by hijacking the host's own post-translational modification machinery and using the host's own signaling codes against it.

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Curriculum vitae

I'm currently enrolled in the graduate program in Microbiology and Immunology at the University of Texas Medical Branch in Galveston. My interests include host-pathogen interactions and the role of pathogen effectors in modulating the host response to facilitate their survival and replication.

Education:

University of Texas Medical Branch, Galveston, TX
Current Student in the Graduate School of Biomedical Science, Immunology and Microbiology
2011-Present

Ball State University, Muncie, Indiana
B.A., Biology
May 2009

Research Experience:

Graduate Research Assistant, University of Texas Medical Branch, Galveston TX, August 2012-June 2017

Independent Study, Ball State University, Muncie IN, December 2008-May 2009
Seedbank Study from an Indiana Bog

Publications:

Farris, T., Zhu, B., and McBride, JW. "*Ehrlichia chaffeensis* TRP32 nucleomodulin function and localization is regulated by NEDD4L-mediated ubiquitination." (In preparation)

Farris, T. Dunphy, PS., Zhu, B., Kibler, C., and McBride JW. "*Ehrlichia chaffeensis* TRP32 is a Nucleomodulin that Directly Regulates Expression of Host Genes Governing Differentiation and Proliferation." Infection and Immunity. 2016; 84:11 (Selected by the editors as an article of significant interest-"Spotlight" p.3093)

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Posters:

Farris, T., Zhu, B., and McBride JW. "*Ehrlichia chaffeensis* Nucleomodulin TRP32 is Regulated by Diverse Ubiquitin Modifications During Infection." Poster Presented at: American Society for Rickettsiology, June 11-14, 2016; Big Sky, MT

Farris, T., Dunphy, PS., Zhu, B., Kibler, C., and McBride JW. "*Ehrlichia chaffeensis* TRP32 is a nucleomodulin that directly regulates host gene expression." Poster Presented at: American Society for Rickettsiology, June 11-14, 2016; Big Sky, MT

Farris, T. Dunphy, PS., Zhu, B., Kibler, C., and McBride JW. *Ehrlichia chaffeensis* TRP32 is a nucleomodulin which directly regulates host gene expression. Poster Presented at: Mclaughlin Colloquium on Infection & Immunity, 2016; Galveston, TX

Uribe, G, Zhang, X, **Farris,T** and McBride, JW. Identification of *Ehrlichia chaffeensis* effector proteins targeted for SUMOylation. Poster Presented at: Annual Meeting of the American Society for Microbiology, 2015 May 30- Jun 2; New Orleans, LA

Pohl, NK, Wu, S, Zhu, B, **Farris, T** and McBride, JW. TRP75: A New DNA Binding Protein Identified as an *Ehrlichia chaffeensis* Effector. Poster Presented at: Annual Meeting of the American Society for Microbiology, 2015 May 30- Jun 2; New Orleans, LA

Farris, T, Zhu B and McBride JW. Gene Targets and DNA Binding Motif of *Ehrlichia chaffeensis* Nuclear Effector TRP32. Poster Presented at: Annual Meeting of the American Society for Microbiology, 2015 May 30- Jun 2; New Orleans, LA

Farris, T, Zhu, B and McBride, JW. *Ehrlichia chaffeensis* TRP32 Binds and E-box-like Motif and Interacts with Host Genes of Major Cellular Processes. Poster Presented at: Mclaughlin Colloquium on Infection & Immunity, 2015; Galveston, TX

Uribe**,** G., **Farris, T**, Zhang, X., and Jere W. McBride. Identification of *Ehrlichia chaffeensis* effector proteins targeted for SUMOylation. Fall 2014 General Meeting of the American Society for Microbiology, Houston, TX, November 06-08, 2014; Abstract 44

Farris, T, Dunphy, PS, Zhang, X, Luo, T and McBride, JW. *Ehrlichia chaffeensis* TRP 32 trafficks to the host nucleus in a phosphorylation dependent manner and binds host DNA via a tandem repeat domain. Poster Presented at: Mclaughlin Colloquium on Infection & Immunity, 2014; Galveston, TX

Farris, T, Xie, G and Wang, T. Infection And Innate Cytokine Responses In Mouse Dendritic Cells During Persistent West Nile Virus Infection. Poster Presented at: Mclaughlin Colloquium on Infection & Immunity, 2013; Galveston, TX

Oral Presentations:

Farris, T, Zhu B and McBride, JW. *Ehrlichia chaffeensis* TRP32 Binds and E-box-like Motif and Interacts with Host Genes of Major Cellular Processes. Oral Presentation. Annual Meeting of the American Society for Rickettsiology, 2015 June 20-23; Lake Tahoe, CA

Farris, T and McBride, JW. *Ehrlichia* TISS Effector TRP32 Targets Host Genes Related to Cell Differentiation and Proliferation. Fall Meeting of the Texas Branch American Society for Microbiology, Houston, TX. Nov. 6-8, 2014; Oral Presentation SES 2

Scholarships and Awards:

2012	Field Epidemiology in Northern Peru - Scholarship
2014-2015	T32 NIH Pre-doctoral Training Award, University of Texas Medical Branch, “Emerging and Tropical Infectious Diseases”
2015	American Society for Rickettsiology Travel Award
2015-2016	McLaughlin Pre-doctoral Fellowship
2016	McLaughlin Colloquium Travel Award

Mentoring:

2014	Jennifer Aguilar. High School Summer Research Program Student Project: Creation of truncation mutants to identify the requirements of nuclear localization of *Ehrlichia chaffeensis* effector TRP47
2014-2015 Program	Gabriella Uribe. The Post-Baccalaureate Research Education Project: Characterization of novel *Ehrlichia chaffeensis* SUMOylated effector proteins

2016 Roma Nayyar. High School Summer Research Program Student
Project: The importance of lysine residues in TRP32 function and localization

2016-2017 Nathan Eckhert. High School Scientific Research & Design Bench Tutorials Program
Project: Characterize the impact of *Ehrlichia chaffeensis* effector TRP32 on host cell phenotype

Other Skills :
BSL-2, BSL-3 Certified