1 **ATF6 enables pathogen infection in ticks by inducing *stomatin* and altering**

2 **cholesterol dynamics**

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4 Kaylee A. Vosbigian[1], Sarah J. Wright[1], Kristin L. Rosche[1,#], Elis A. Fisk[1],

5 Elisabeth Ramirez-Zepp[1], Eric A. Shelden[2], Dana K. Shaw[1]

6

7 [1]Department of Veterinary Microbiology and Pathology, Washington State University,

8 Pullman, WA, USA

9 [2]School of Molecular Biosciences, Washington State University, Pullman, Washington,

10 USA.

11

12 [#]Present address: Carbone Cancer Center, School of Medicine and Public Health,

13 University of Wisconsin – Madison, Madison, WI, USA

14

15 **Corresponding Author**: *Dana K. Shaw; Dana.Shaw@wsu.edu

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17 Running Title: Pathogen success in ticks is promoted by ATF6 regulation of *stomatin*

18

19 **ABSTRACT**

20 How tick-borne pathogens interact with their hosts has been primarily studied in

21 vertebrates where disease is observed. Comparatively less is known about pathogen

22 interactions within the tick. Here, we report that *Ixodes scapularis* ticks infected with

23 either *Anaplasma phagocytophilum* (causative agent of anaplasmosis) or *Borrelia*

24 *burgdorferi* (causative agent of Lyme disease) show activation of the ATF6 branch of

25 the unfolded protein response (UPR). Disabling ATF6 functionally restricts pathogen

26 survival in ticks. When stimulated, ATF6 functions as a transcription factor, but is the

27 least understood out of the three UPR pathways. To interrogate the *Ixodes* ATF6

28 transcriptional network, we developed a custom R script to query tick promoter

29 sequences. This revealed *stomatin* as a potential gene target, which has roles in lipid

30 homeostasis and vesical transport. *Ixodes stomatin* was experimentally validated as a

31 bona fide ATF6-regulated gene through luciferase reporter assays, pharmacological

32 activators, and RNAi transcriptional repression. Silencing *stomatin* decreased *A.*

33 *phagocytophilum* colonization in *Ixodes* and disrupted cholesterol dynamics in tick cells.

34 Furthermore, blocking *stomatin* restricted cholesterol availability to the bacterium,

35 thereby inhibiting growth and survival. Taken together, we have identified the *Ixodes*

36 ATF6 pathway as a novel contributor to vector competence through Stomatin-regulated

37 cholesterol homeostasis. Moreover, our custom, web-based transcription factor binding

38 site search tool "ArthroQuest" revealed that the ATF6-regulated nature of *stomatin* is

39 unique to blood-feeding arthropods. Collectively, these findings highlight the importance

40 of studying fundamental processes in non-model organisms.

44 **IMPORTANCE**

45   Host-pathogen interactions for tick-borne pathogens like *Anaplasma*

46 *phagocytophilum* (causative agent of Anaplasmosis) have been primarily studied in

47 mammalian hosts. Comparatively less is known about interactions within the tick.

48 Herein, we find that tick-borne pathogens activate the cellular stress response receptor,

49 ATF6, in *Ixodes* ticks. Upon activation, ATF6 is cleaved and the cytosolic portion

50 translocates to the nucleus to function as a transcription factor that coordinates gene

51 expression networks. Using a custom script in R to query the *Ixodes* ATF6 regulome,

52 *stomatin* was identified as an ATF6-regulated target that supports *Anaplasma*

53 colonization by facilitating cholesterol availability to the bacterium. Moreover, our

54 custom, web-based tool "ArthroQuest" found that the ATF6-regulated nature of *stomatin*

55 is unique to arthropods. Given that lipid hijacking is common among arthropod-borne

56 microbes, ATF6-mediated induction of *stomatin* may be a mechanism that is exploited

57 in many vector-pathogen relationships for the survival and persistence of transmissible

58 microbes. Collectively, this study identified a novel contributor to vector competence

59 and highlights the importance of studying molecular networks in non-model organisms.

INTRODUCTION

The North American deer tick, *Ixodes scapularis*, can transmit up to seven

different pathogens that impact human and animal health including *Anaplasma*

phagocytophilum (causative agent of Anaplasmosis) and *Borrelia burgdorferi* (causative

agent of Lyme Disease)[1]. The continuous rise in reported cases of tick-borne disease[2–

10] underscores the need for novel intervention strategies. Although the intricacies of

mammalian host-pathogen interactions have been well-studied, comparatively little is

known about tick-pathogen interactions.

Recently we have shown that *A. phagocytophilum* and *B. burgdorferi* activate the

unfolded protein response (UPR) in ticks, which influences microbial colonization and

persistence in the arthropod[11,12]. The UPR is a cellular response network that is initiated

by three endoplasmic reticulum (ER) transmembrane receptors IRE1α, PERK, and

ATF6. Each branch of the UPR initiates a signaling cascade and coordinates gene

expression networks by activating specific transcription factors. We have shown that the

IRE1α-TRAF2 pathway leads to microbe-restricting immune responses in arthropods by

activating the NF-κB-like molecule, Relish[11]. We have also demonstrated that

stimulating PERK activates the antioxidant transcription factor, Nrf2, which facilitates

pathogen persistence in ticks[12]. Out of the three UPR receptors, ATF6 is the least

understood[13]. When activated, site-1 and site-2 proteases cleave the cytosolic portion of

ATF6, which allows it to translocate to the nucleus and act as a transcriptional regulator

(nATF6)[14]. The role of ATF6 has never been explored in arthropod vectors.

Here, we demonstrate that *Ixodes* ATF6 is activated by tick-borne pathogens and

supports *A. phagocytophilum* colonization in ticks. To determine how ATF6 impacts

83   vector competence, we used protein modeling and a custom transcription factor binding

84   site query to probe the ATF6 regulatory network in *I. scapularis.* Gene ontology (GO)

85   and Reactome analyses identified Stomatin, a lipid homeostasis and vesical transport

86   protein, as a potential gene regulated by ATF6 in ticks. Using pharmacological

87   manipulations, RNA interference (RNAi), and quantitative fluorescent assays, we found

88   that Stomatin supports pathogen colonization in ticks by facilitating cholesterol

89   acquisition by the bacterium. These findings demonstrate that *stomatin* is induced

90   during the arthropod-phase of the pathogen life cycle to enable survival and persistence

91   in the vector.

92   Programs that predict transcription factor regulatory networks are generally

93   restricted to model organisms, leaving out many arthropod vectors. We used our

94   custom R script to develop a publicly available, web-based tool termed "ArthroQuest"

95   that currently allows users to query 20 different arthropod vector genomes, in addition to

96   *Drosophila* and humans. Queries with ArthroQuest revealed that the ATF6-regulated

97   nature of *stomatin* appears to be unique to arthropods. Given that lipid hijacking and

98   cholesterol incorporation is common in many arthropod-borne microbes[15], ATF6-

99   mediated induction of *stomatin* may be a shared phenomenon among many vector-

100   pathogen relationships that is exploited for the survival and persistence of transmissible

101   pathogens.

102

103   **RESULTS**

104   *ATF6 is induced by tick-borne pathogens and supports microbial survival*

105   Previously, we found that *A. phagocytophilum* and *B. burgdorferi* activate the

106   UPR through ER receptors IRE1α and PERK, which influence bacterial colonization of

107   the tick[11,12]. The UPR coordinates a variety of transcription factors that modulate the

108   expression of genes involved in cellular stress responses, immunity, and several other

109   physiological conditions[16–18]. To determine which UPR-associated transcription factors

110   respond to infection, we employed a surrogate luciferase reporter plasmid assay[12] and

111   found that ATF6 was significantly activated by both *A. phagocytophilum* and *B.*

112   *burgdorferi* (Fig 1A-B). We next asked if *atf6* is induced in ticks during infection. *I.*

113   *scapularis* larvae were fed to repletion on either *A. phagocytophilum* or *B. burgdorferi*-

114   infected mice and gene expression was analyzed by quantitative real-time PCR (qRT-

115   PCR). In both groups of infected larvae, *atf6* expression was significantly increased (Fig

116   1C-D). To determine how this may impact pathogen colonization, we silenced *atf6* in

117   tick cells using RNAi prior to infecting with *A. phagocytophilum* and found that knocking

118   down *atf6* reduced bacterial survival and replication in tick cells (Fig 1E-F). Collectively,

119   this indicates that ATF6 is activated during infection and supports pathogen survival.

120

121   *Identifying genes putatively regulated by ATF6 in ticks*

122   Out of the three UPR pathways, ATF6 and the gene network it coordinates is the

123   least understood[13]. To examine how ATF6 is mechanistically impacting pathogen

124   dynamics in the tick, we first aligned sequences from *I. scapularis* and humans. We

125   found that, although there was low overall conservation (31.56% identity), there was

126   good conservation in the basic leucine zipper (bZIP) domain which is the DNA-

127   interacting portion of ATF6 (Supplementary Fig 1A; Supplementary Table 1). Moreover,

128 the amino acid residues defined as "essential for DNA binding", K304, N305, and R306,

129 were 100% conserved between the two sequences[19]. We next used AlphaFold to

130 predict the protein structure of *Ixodes* ATF6 and then aligned it with the human

131 structure[20,21] using ChimeraX[22] (Fig 2A, Supplementary Fig 1B). We found that the bZIP

132 domain was structurally well-conserved and that the DNA-binding amino acid residues

133 were found in the same orientation between the two proteins (Fig 2B).

134   The conservation in DNA-binding domain structure and residues between

135 humans and ticks provided the impetus to interrogate the *Ixodes* genome for ATF6-

136 regulated genes. Non-model organisms, such as ticks, have a limited number of

137 genome and proteome analysis tools. Moreover, the tools that are available are often

138 not well-developed. To circumvent this issue, we created a custom query in R to search

139 for ATF6-regulated genes in *I. scapularis* (Fig 2C). Predicted promoter regions in the *I.*

140 *scapularis* genome were defined as 1,000 base pairs upstream from all coding regions.

141 We then searched for ATF6-binding motifs in all promoters. ATF6 functions as a

142 homodimer that promotes gene expression either by itself or in combination with the

143 general transcription factor NF-Y (nuclear transcription factor Y). By itself, ATF6 can

144 drive gene expression by binding TGACGTG within a promoter sequence[19]. In

145 combination, ATF6 and NF-Y can drive transcription by binding CCACG and CCAAT,

146 respectively[23,24]. We scanned all predicted *Ixodes* promoters for either TGACGTG or

147 CCACG in the presence of CCAAT. For comparison, we also predicted ATF6-binding

148 sites in humans and the model insect *D. melanogaster.* Genes downstream from

149 promoters containing ATF6-binding sites were compiled, identified, and compared (Fig

150 2D; Supplementary Table 2). This revealed that known ATF6-regulated genes, including

151   *binding immunoglobulin protein* (*BiP*) and *x-box binding protein 1* (*xbp1*)*,* were present

152   in all three organisms, demonstrating that our custom R script correctly identifies ATF6-

153   regulated genes. We also observed that more genes were shared between *Ixodes* and

154   *Drosophila* (866) than humans and *Ixodes* (290), but the large majority of genes were

155   unique to each organism (Fig 2D).

156   We next sought to analyze the ATF6-regulated gene network in ticks. However,

157   when compared to humans or *Drosophila*, relatively little gene information is available

158   for *Ixodes.* To overcome this obstacle, we identified human and *Drosophila* orthologs for

159   tick gene targets and then extracted corresponding gene information (Supplementary

160   Table 2). 71% of the *Ixodes* genes putatively regulated ATF6 mapped to human and/or

161   *Drosophila* orthologs. Corresponding Gene Ontology (GO) and Reactome information[25–

162   27] were used to perform pathway enrichment analysis[28] (Supplementary Fig 2), which

163   revealed that GO terms associated with the UPR were enriched (Supplementary Table

164   3). This also returned categories of interest with the potential to impact microbial

165   colonization including "Immunity", "Positive Regulation of Bacterial or Viral Processes",

166   "Signal Transduction", and "Associated with the ER"[14,29]. From *Ixodes*, we found 185

167   genes associated with Immunity (GO:0006955, R-HSA-768256), 24 with Positive

168   Regulation of Bacterial or Viral Processes (GO:0048524, GO:1900425), 181 with Signal

169   Transduction (R-HSA-162582), and 179 Associated with the ER (GO:0005783) (Fig

170   2E). The genes *stomatin, neurogenic locus notch homolog protein 1 (notch1),* and

171   *protein disulfide isomerase (pdi)* were found in all four GO categories. *Pdi* is a known

172   ATF6-regulated gene in mammals and assists in the formation of disulfide bonds[30].

173   *Notch1* is also known to be regulated by ATF6 and is involved in immunity, cellular

174   development, and apoptosis[31,32]. Stomatin has roles in lipid homeostasis and vesical

175   transport[33], but has never been linked to ATF6. Since *A. phagocytophilum* incorporates

176   lipids and cholesterol into its membrane, we hypothesized that regulation of *stomatin*

177   expression could be how ATF6 is supporting *A. phagocytophilum* in ticks.

178

179   *Tick stomatin is upregulated by ATF6 during infection*

180   We next used AlphaFold3[34] to predict structural interactions between ATF6 and

181   the *stomatin* promoter (Fig 3A; Supplementary Fig 3A). We found that the *Ixodes* ATF6

182   homodimer (Fig 3A, tan) and DNA-interacting residues K304, N305, and R306 (Fig. 3A,

183   purple) were predicted to be in direct contact with the ATF6-binding nucleotide motif

184   found in the *stomatin* promoter, CCACG (Fig 3A, yellow). To test whether ATF6

185   activation increases *stomatin,* we used a drug, AA147, that selectively activates ATF6

186   independent of ER stress[35]. AA147 did not impact cell viability (Supplementary Fig 3B),

187   but did cause a dose-dependent increase in *stomatin* expression (Fig 3B). Since

188   pharmacological manipulators can have off-target effects, we also silenced *atf6* in tick

189   cells using RNAi. We found that decreasing *atf6* levels (Fig 1E) caused a significant

190   decline in *stomatin* expression (Fig 3C), altogether demonstrating that ATF6 positively

191   correlates with *stomatin* expression.

192   To experimentally validate that ATF6 binds the *stomatin* promoter and drives

193   expression, we designed a Luciferase reporter assay. First, we cloned the *Ixodes*

194   *stomatin* promoter upstream from a *luciferase* gene. Next, plasmids were constructed

195   that constitutively expressed recombinant versions of *Ixodes* nATF6 and NF-Y

196   (Supplementary Fig 4A-4B). All three plasmids were then co-transfected into HEK 293T

197   cells for 24 hours (Fig 3D). A positive control plasmid containing *luciferase* driven by an

198   *atf6* promoter was also co-transfected with *Ixodes* nATF6 and NF-Y constructs

199   (Supplementary Fig 4C). After 24 hours, D-luciferin was added to the cells and

200   Luciferase activity was assayed. We found a significant increase in Luciferase activity

201   when cells were expressing both *Ixodes* nATF6 and NF-Y, indicating *stomatin* promoter

202   activity (Fig 3D). Altogether, these data demonstrate that ATF6, in combination with the

203   ubiquitously expressed transcription factor NF-Y, positively regulates the expression of

204   *stomatin* in *Ixodes* ticks*.*

205   We next evaluated *in vivo stomatin* expression in infected ticks. *Stomatin* was

206   quantified in larvae that had been fed on either *A. phagocytophilum* (Fig 3E) or *B.*

207   *burgdorferi* (Fig 3F) infected mice, and then rested for 7 days or 14 days post-repletion,

208   respectively. This time period correlates with the expansion of microbes in the tick post-

209   repletion[36]. We also quantified *stomatin* expression in *A. phagocytophilum* or *B.*

210   *burgdorferi* infected, flat nymphs (Fig 3G). From both life stages, we found that ticks

211   infected with *A. phagocytophilum* had elevated levels of *stomatin* expression when

212   compared to uninfected ticks. Ticks infected with *B. burgdorferi* had no differences at

213   the rested larval stage, but significantly increased levels of *stomatin* at the unfed nymph

214   stage. It is not clear why *stomatin* is induced in *A. phagocytophilum*-infected, rested

215   larvae*,* but not *B. burgdorferi.* It is possible that differences in niche colonization and

216   subcellular location between the two pathogens are responsible for this observation.

217   This data, together with infection-induced ATF6 transcriptional activity (Fig 1D)

218   demonstrates that ATF6 positively regulates *stomatin* during *A. phagocytophilum* and *B.*

219   *burgdorferi* infection in ticks.

220

Arthropod infection is supported by stomatin

222   Since Stomatin expression increased with *A. phagocytophilum* acquisition in

223 ticks, we next asked what impact it has on microbial colonization. To address this, we

224 used RNAi to silence *stomatin* in tick cells prior to infecting with *A. phagocytophilum*.

225 We found that blocking *stomatin* expression caused a decrease in *Anaplasma* numbers

226 (Fig 4A), similar to what was observed when silencing *atf6* (Fig 1E). To determine how

227 Stomatin impacts microbial colonization *in vivo*, we immersed *Ixodes* larvae[11,12] in

228 siRNA targeting *stomatin.* Ticks were then allowed to feed to repletion on *A.*

229 *phagocytophilum*-infected mice. We found a 2-fold decrease in *Anaplasma* numbers

230 when *stomatin* expression was knocked down relative to the scrambled control (Fig 4B),

231 demonstrating that *A. phagocytophilum* infection and colonization of ticks is supported

232 by Stomatin.

233

Stomatin supports cholesterol incorporation into Anaplasma

235   Stomatin is a member of the stomatin/prohibitin/flotillin/HflK/C (SPFH) family

236 found in lipid rafts on cell membranes, lipid droplets, and endosomes and is known to

237 be involved in vesicle trafficking[37]. When comparing human and *Ixodes* Stomatin, we

238 found that the amino acid sequences and structures are well conserved (Supplementary

239 Fig 5A-C). The *Ixodes* Stomatin SPFH domain (residues 32-191) contains four

240 cholesterol recognition/interaction amino acid consensus sequences, CRAC and CARC

241 (Fig 4C, pink; Supplementary Fig 5D)[38]. Outside of the SPFH domain, there are two

242   additional CARC domains. This suggests that tick Stomatin may bind cholesterol and

243   function similarly to other SPFH family members[37].

244   Cholesterol is essential to the development of *A. phagocytophilum* infection[39]. In

245   mammals, *A. phagocytophilum* will intercept free cholesterol from the low-density

246   lipoprotein uptake pathway[39] and incorporate it for structural support of its membrane.

247   We therefore asked if *A. phagocytophilum* impacts cholesterol dynamics in ticks. Using

248   an Amplex Red cholesterol assay, cholesterol was quantified in uninfected tick cells

249   relative to tick cells that were persistently infected with *A. phagocytophilum*. We found

250   that infection was associated with significantly more cholesterol compared to uninfected

251   cells, indicating that *Anaplasma* induces cholesterol accumulation in ticks (Fig 4D).

252   SPFH-domain containing proteins regulate cholesterol uptake, distribution within

253   the cell, and exportation when levels become too high[40,37]. To understand how Stomatin

254   may be impacting cholesterol dynamics within tick cells, we transfected silencing RNAs

255   targeting *stomatin* into uninfected cells and then quantified cholesterol. We found that

256   when *stomatin* expression is reduced there is an increase in total cholesterol, implying

257   that Stomatin is involved in proper distribution, localization, and export of cholesterol

258   from the cell (Fig 4E).

259   Given the *Anaplasma*-supporting role that Stomatin plays, we asked if it could be

260   facilitating infection and colonization by shuttling cholesterol to the bacterium. To

261   address this possibility, we silenced *stomatin* in persistently infected tick cells for five

262   days (Supplementary Fig 5E). *A. phagocytophilum* were then mechanically lysed from

263   tick cells, washed, and separated from host cell debris. Bacterial cholesterol levels were

264   then quantified using the Amplex Red cholesterol assay. We found that when *A.*

265   *phagocytophilum* is grown in cells with depleted Stomatin, there is significantly less total

266   cholesterol incorporated into the bacteria compared to non-silenced controls (Fig 4F).

267   Therefore, although knocking down *stomatin* caused increased total cellular cholesterol

268   in tick cells, our data shows that it is not accessible by *Anaplasma.* This finding depicts

269   a model where *Anaplasma* activates ATF6 in *Ixodes*, which upregulates Stomatin

270   expression and functionally supports the microbe by facilitating cholesterol delivery,

271   likely for cell wall structure and growth (Fig 5A).

272

273   *ArthroQuest: ATF6-regulation of stomatin is unique to arthropod vectors*

274   Programs that predict transcription factor regulatory networks have generally

275   been restricted to model organisms, leaving out most arthropod vectors[41,42]. To address

276   this deficiency, we created a web-based tool termed "ArthroQuest" to serve as a

277   resource for the vector biology community

278   (https://datahub.vetmed.wsu.edu/Shaw/ArthroQuest/). This tool can query 22 pre-

279   loaded arthropod vector genomes for transcription factor binding motifs. Pre-loaded

280   genomes include those from ticks, mosquitoes, lice, sand flies, mites, fleas, *Drosophila*,

281   and humans. Using NCBI genomic FASTA and annotation files, we defined promoter

282   regions for all 22 genomes and created a user-friendly interface with Shiny App. Users

283   can enter a DNA-binding motif of interest and then select a genome to query. Annotated

284   genes found downstream from positive-hit promoter regions can then be downloaded as

285   a table. Using this tool, we queried all pre-loaded genomes for ATF6-binding sites. In

286   contrast to humans, the large majority of arthropods (18 out of 21) had positive hits in

287   the promoter regions of *stomatin* orthologs, including other ticks, mosquitos, and sand

288   flies (Fig 5B, Supplementary Table 4). Altogether, these findings suggest that the ATF6-

289   regulated nature of *stomatin* may be a common feature among blood-feeding

290   arthropods.

291

292   **DISCUSSION**

293   Pathogen persistence in vectors can be attributed to both microbe and host-

294   responses, although the underlying mechanisms orchestrating this remain incompletely

295   understood. Recently, the UPR receptors IRE1α and PERK have been connected to

296   arthropod immunity and vector competence of ticks[11,12]. The third pathway orchestrated

297   by ATF6 remains the least understood out of the UPR circuits[13]. In this article, we show

298   that transmissible pathogens *A. phagocytophilum* and *B. burgdorferi* activate ATF6 in *I.*

299   *scapularis,* which supports pathogen colonization and persistence in the tick. We

300   provide evidence that the ATF6-regulated transcriptional network supports pathogen

301   survival by inducing Stomatin, which facilitates cholesterol delivery to the bacterium.

302   Moreover, queries using our custom, web-based transcription factor binding site tool

303   ArthroQuest suggest that the ATF6-regulated nature of *stomatin* is unique to

304   arthropods. Collectively, our findings provide mechanistic insight into how cellular stress

305   responses influence vector competence of arthropods and further highlight the

306   fundamental differences in molecular networks between mammals and ticks.

307   To our knowledge, this is the first study to mechanistically investigate how ATF6

308   influences pathogen dynamics in an arthropod vector. The ATF6 regulatory network

309   controls the expression of genes involved in maintaining protein homeostasis, misfolded

310   protein degradation[16,43], development, tissue homeostasis, and cytoprotection[14,43]. Our

311   R script querying the *I. scapularis* genome not only predicted known ATF6-regulated

312   genes, but also many others that have not been previously implicated in the ATF6

313   regulome. This approach led us to the novel finding that ATF6 transcriptionally regulates

314   tick *stomatin*. In addition to *stomatin,* there are several other genes that are unique to

315   the *Ixodes* ATF6 regulome that are not yet characterized (Supplementary Table 2). How

316   these unique targets may be impacting vector competence is an area for future study.

317   ATF6 has previously been connected to lipid metabolism in mammals[44]. *Ixodes*

318   ticks only feed once per life stage and must adapt to an influx of proteins and lipids

319   when taking a blood meal, which can confer stress to the tick[16]. It is possible that stress-

320   responses are activated during blood feeding and that the ATF6-regulated nature of

321   *stomatin* in ticks is needed for the proper coordination of lipid homeostasis and

322   metabolism. Increased Stomatin expression could stabilize lipid rafts, improve

323   membrane integrity, and/or protect the cell membrane against oxidative stress that is

324   associated with a blood meal. This linkage between ATF6 and lipid homeostasis

325   through Stomatin in arthropods may be something that vector-borne pathogens target

326   and actively manipulate to satisfy their own cholesterol requirements for growth and

327   survival[15].

328   Our script in R led to the development of a customizable tool, ArthroQuest, to

329   predict transcription factor binding sites in non-model arthropods

330   (https://datahub.vetmed.wsu.edu/Shaw/ArthroQuest/). Other tools, such as Transfac or

331   TFtarget, exist but are only available for model organisms[41,45]. In addition to humans

332   and *Drosophila,* ArthroQuest currently allows users to query 20 different arthropod

333   vector genomes. This led to the finding that humans do not have an ATF6 binding site in

334   the predicted promoter region of *stomatin*, but the large majority of vectors genomes on

335   ArthroQuest do. This includes several other species of ticks, mosquitos, and sandflies,

336   which suggests that ATF6-mediated regulation of *stomatin* could be a common

337   phenomenon among blood-feeding arthropods. When considering the rapid nutrient

338   influx and temperature shift that hematophagous arthropods endure during blood

339   feeding, a stress-sensing network to cue lipid metabolism would be evolutionarily

340   advantageous, particularly if Stomatin influences cholesterol homeostasis and/or

341   membrane fluidity[33,37,46,47].

342   As a lipid-raft associated protein, Stomatin performs several functions within the

343   cell. It has been implicated in the host cell immune response to fungal pathogens by

344   assisting phagolysosome fusion[48]. Despite this association with immunity, Stomatin has

345   also been shown to promote viral infection. For example, Stomatin assists Hepatitis C

346   virus (HCV) replication by supporting assembly of the viral RNA replicase complexes on

347   detergent-resistant membrane structures[49]. Our study is the first to link Stomatin to

348   bacterial infection and replication. Previous reports have found that another SPFH

349   family protein, Flotillin, facilitates cholesterol transport to the *Anaplasma*-containing

350   vacuole in mammalian cells[50,51]. Our findings show that Stomatin-depleted tick cells

351   have increased cholesterol accumulation within the cells, but decreased cholesterol

352   delivery to the bacterium, suggesting that the role of *Ixodes* Stomatin during *Anaplasma*

353   infection could be similar to mammalian Flotillin. This finding highlights that while the

354   cholesterol requirement for *Anaplasma* growth is conserved between mammalian and

355   tick environments, the molecular host targets that facilitate this process are distinct

356   between the two organisms.

357   While our findings show that both *A. phagocytophilum* and *B. burgdorferi*

358   infection activate ATF6 transcriptional activity, a recent study reported that ATF6 is not

359   activated in RF/6A cells during *Anaplama* infection[52]. The discrepancy in results may be

360   attributable to differences in cell types and/or experimental approaches used to test for

361   ATF6 activation. Wang *et al.* used a transfected RF/6A cell line expressing recombinant,

362   GFP-tagged ATF6 and nuclear accumulation was assessed by fluorescence

363   microscopy after infection[52]. Our assay used a Luciferase reporter plasmid to

364   quantitatively detect transcriptional activity by endogenous, activated ATF6 in infected

365   HEK 293T cells. Another report observed an increase in cleaved ATF6 by immunoblot

366   in *A. phagocytophilum*-infected THP-1 cells, which also suggests activation[53]. It is

367   possible that ATF6 activation may vary between stages of infection. For example, our

368   group previously reported that there was no difference in *atf6* transcript levels between

369   uninfected and *Anaplasma*-infected *Ixodes* nymphs immediately post-repletion[11].

370   However, in infected larvae that were rested for 7 days post-repletion, we found

371   increased *atf6* expression (Fig 1C-D). The discrepancy in expression at different

372   timepoints post-repletion could indicate that ATF6 is only activated after *Anaplasma* has

373   established infection and expanded in population within the tick. This hypothesis is in

374   line with our findings that ATF6 facilitates cholesterol delivery to the *Anaplasma*-

375   containing vacuole for bacterial growth and survival.

376   Many vector-borne pathogens are reliant on host cholesterol for membrane

377   structure and growth. For example, *B. burgdorferi, Ehrlichia* spp., and *Anaplasma* spp.

378   incorporate cholesterol into their outer membranes for structural integrity. Other vector-

379   borne parasites and viruses, such as *Plasmodium* spp. and flaviviruses, are also reliant

380   on host lipids for survival[15]. Based on this knowledge and the data presented herein, we

381   hypothesize that ATF6-regulation of Stomatin expression is a mechanism used by a

382   wide variety of blood-feeding arthropods to control lipid homeostasis during times of

383   stress, which is exploited by vector-borne pathogens to effectively colonize arthropods.

384

385   **METHODS**

386   *Cell culture.*

387   The *I. scapularis* embryonic ISE6 cells (received as gift from Ulrike Munderloh)

388   were cultured at 32°C with 1% CO_2 in L15C300 media supplemented with 10% heat-

389   inactivated FBS (Sigma, F0926), 10% tryptose phosphate broth (BD, B260300) and

390   0.1% lipoprotein bovine cholesterol (MP Biomedicals, 219147680).

391   The human embryonic kidney cell line, HEK 293T cells were cultured in flasks

392   (Corning, 353136) at either 33°C or 37°C in 5% CO_2 in Dulbecco's modified Eagle

393   medium (DMEM; Sigma, D6429) supplemented with 10% heat-inactivated FBS (Atlanta

394   Biologicals, S11550) and 1x Glutamax (Gibco, 35050061).

395

396   *Bacteria and animal models.*

397   *A. phagocytophilum* strain HZ was cultured in HL60 cells (ATCC, CCL-240) in

398   Roswell Park Memorial Institute 1640 (Cytiva SH30027.LS) medium supplemented with

399   10% heat-inactivated FBS and 1x Glutamax. Cultures were kept between 1×10^5 and 1

400    $\times 10^6$ cells mL^{-1} and maintained at 37°C in the presence of 5% CO_2. Persistently

401   infected ISE6 cells were cultured in L15C300 media supplemented with 25 µM 4-(2-

402   Hydroxyethyl)piperazine-1-ethane-sulfonic acid (Sigma, H4034-100G), 0.25% sodium

403   bicarbonate (Sigma, S-5761), pH 7.5 in unvented flasks (GeneseeSci, 25-207) at 37°C,

404   5% CO_2. *A. phagocytophilum* counts were performed as previously described[11,54]. Host

405   cell-free *A. phagocytophilum* was isolated by syringe lysis with a 27-gauge needle.

406   *B. burgdorferi* B31 (strain MSK5) was grown in modified Barbour-Stoenner-Kelly

407   II (BSK-II) medium supplemented with 6% normal rabbit serum (NRS; Pel-Freez;

408   31126-5) at 37°C, 5% CO_2. Dark-field microscopy was used to monitor the density and

409   growth phase of the spirochetes. Plasmid profiles of *B. burgdorferi* cultures were

410   screened by PCR before infection[55].

411   *Escherichia coli* cultures were grown overnight at 37°C with shaking between 230

412   and 250 rpm in lysogeny broth (LB) supplemented with 100 μg μl^{-1} ampicillin.

413   *Ixodes scapularis* ticks were acquired at the larval stage from either the

414   Biodefense and Emerging Infectious Diseases Research Resources Repository for the

415   National Institute of Allergy and Infectious Disease at the National Institutes of Health

416   (https://www.beiresources.org/) or from Oklahoma State University (Stillwater, OK,

417   USA). Ticks were maintained with 16:8-h light:dark photoperiods and 95-100% relative

418   humidity at 23°C.

419   Male C57BL/6 mice were obtained from colonies at Washington State University

420   at ages six to ten weeks old. For *A. phagocytophilum* infection experiments, mice were

421   infected intraperitoneally with 1 x 10^7 host cell-free bacteria in 100 μl of PBS

422   (Intermountain Life Sciences, BSS-PBS) as previously described[11,54]. *A.*

423   *phagocytophilum* burdens of each mouse were assessed six days post-infection by

424   collecting 25 to 50 μl of blood from the lateral saphenous vein, as previously

425   described[11,12]. *A. phagocytophilum* burdens were quantified by quantitative PCR (*16s*

426   relative to mouse *β-actin*). For *B. burgdorferi* infections, mice were inoculated

427   intradermally with 1 x 10^5 low-passage spirochetes. Seven days post-infection, blood

428   was collected from the lateral saphenous vein of each mouse and subcultured in BSK-II

429   medium. The presence of spirochetes were confirmed by dark field microscopy[11,12]. All

430   experiments with mice were carried out according to the guidelines and protocols that

431   are approved by the American Association for Accreditation of Laboratory Animal Care

432   (AAALAC) and by the Office of Campus Veterinarian at Washington State University

433   (Animal Welfare Assurance A3485-01). The mice were housed in an AAALAC-

434   accredited facility at Washington State University in Pullman, WA. The Washington

435   State University Biosafety and Animal Care and Use Committees approved all

436   procedures.

437

438   *RNAi silencing and pharmacological treatments.*

439   The Silencer siRNA Construction Kit (Invitrogen, AM1620) was used to

440   synthesize silencing RNAs (siRNA) and scrambled RNAs (scRNA). For RNAi

441   knockdown experiments, ISE6 cells were seeded at 1 x 10^6 cells per well in a 24-well

442   tissue culture plate. siRNA or scRNA (3 μg) were transfected into tick cells with 2.5 μl of

443   Lipofectamine 3000 (Invitrogen, L3000008) for 24 hours. Plates were centrifuged at

444   room temperature for 1 hour at 450 x g and then incubated overnight. The following day,

445   cells were infected with *A. phagocytophilum* (MOI 50) for 18 hours. Cells were collected

446   in TRIzol (Invitrogen, 15596026) for RNA isolation. The Direct-zol RNA Microprep Kit

447   (Zymo; R2062) was used to extract RNA. cDNA was synthesized using the Verso cDNA

448   Synthesis Kit (Thermo Fisher Scientific; AB1453B) using 300 to 500 ng total RNA per

449   reaction. Gene silencing, bacterial burden, and gene expression were assessed by

450   quantitative reverse transcription PCR (qRT-PCR) with iTaq universal SYBR Green

451   Supermix (Bio-Rad, 1725125) (Supplementary Table 5). Cycling conditions were as

452   recommended by the manufacturer.

453   For pharmacological experiments, ISE6 cells were seeded at 1 x 10^6 cells per

454   well in a 24-well tissue culture plate and treated with 5-50 μM of AA147 (Focus

455   Biomolecules, 10-3973) for 24 hours. RNA isolation, cDNA synthesis, and qRT-PCR

456   were performed as described above. All data are expressed as means ± standard error

457   of the mean (SEM).

458

459   *Protein structure predictions and alignments.*

460   *Ixodes* ATF6 and *Ixodes* NF-Y were identified using National Center for

461   Biotechnology Information protein Basic Local Alignment Tool (BLAST) and querying

462   the tick genome with human protein sequences (ATF6: AAB64434.1. NY-F:

463   ALX00018.1). All protein alignments were visualized with JalView[56]. Shading indicates

464   physiochemical property conservation between amino acids. AlphaFold was used to

465   model *Ixodes* ATF6 and *Ixodes* Stomatin[20,21]. Alignments with human orthologs were

466   performed in UCSF ChimeraX[22]. *Ixodes* ATF6 binding to the *Ixodes stomatin* promoter

467   was predicted using AlphaFold3 and visualized in ChimeraX[34]. Sequences used for

468   AlphaFold predictions are found in Supplementary Table 1.

469

470   *Predicting ATF6 binding sites in the tick genome.*

471   All binding site analyses were conducted using R version 4.2.2 and RStudio

472   2022.07.2.576[57,58]. The *I. scapularis* genome (GCF_01692078) was obtained from the

473   NCBI database in General Feature Format (GFF) and FASTA format[59]. Putative

474   promoter sites were defined as the region 1000 base pairs upstream from each gene.

475   Coding domain sequence (CDS) coordinates from the GFF file were used to identify

476   promoter end sites. Promoter start sites were identified as 1000 nucleotides from the

477   promoter end site on both sense and antisense DNA strands. Using Biostrings[60] and

478   seqinR[61] packages, the nucleotide sequence of each of the predicted promoter regions

479   was obtained from the FASTA file. Using the stringR package[62], ATF6 binding sites

480   within predicted promoter sites were detected using the following motifs: TGACGTG

481   and CCACG with CCAAT. The resulting data included predicted promoter site

482   coordinates and the corresponding gene annotation. To create ArthroQuest, the R script

483   described above was reformatted to create a user-friendly interface with the R package

484   shiny[63].

485   To identify orthologs to the putatively ATF6-regulated genes, corresponding

486   protein accession numbers were used to find amino acid sequences from the *I.*

487   *scapularis* genome using the Rentrez package[64]. Human (GCF_000001405.40) and

488   *Drosophila* (GCF_000001215.4) proteome files were obtained from NCBI as FASTA

489   files. Using the rBLAST package[65], each protein of interest was queried against human

490   or *Drosophila* proteins. The top 2 hits for each protein of interest were retrieved. All R

491   scripts are available in the GitHub repository (https://github.com/Shaw-Lab/Vosbigian-

492   et-al-2025)

493

494   *Gene Enrichment Analysis Visualization.*

495   For pathway analysis, ortholog accessions were queried in Gene Ontology and

496   Reactome databases[25–27]. Using the enrichplot package[66], pathways that were

497   significantly represented (Supplementary Table 3) were plotted. Adjusted *P*-value is

498   indicated by color, Ratios of enriched genes per total annotated genes are indicated by

499   size.

500

501   *Plasmid construction.*

502   Primers (Supplementary Table 5) were used to amplify the *stomatin* promoter

503   sequence by PCR from ISE6 DNA. The resulting fragment was cloned into a pTE

504   luciferase plasmid (Signosis, LR-2200) using BgIII. *Ixodes nf-y* and the active region of

505   *atf6* (amino acids 1-365) were synthesized by GenScript. *Ixodes atf6* was cloned into

506   pCMV-HA (New MCS) (gift from Christopher A. Walsh; Addgene plasmid number

507   32530) using EcoRI and EcoRV. *Ixodes nf-y* was cloned into pCMV/hygro-Negative

508   control vector (SinoBiological; CV005) using HindIII and KpnI. All constructs were

509   confirmed by sequencing (Plasmidsaurus).

510

511   *HEK 293T cell transfection.*

512   1×10^6 HEK 293T cells were seeded into a 6-well plate. The next day, cells were

513   either singly or co-transfected with pCMV-ATF6-HA and pCMV-NFY-FLAG using 10 µl

514   Lipofectamine 3000, 10 µl P reagent (Fisher Scientific; L30000015), in Opti-MEM I

515   reduced-serum medium (Gibco; 31985062). After 5 hours, the medium was replaced

516   with complete DMEM and cells were incubated at 33°C, 5% CO_2 for 24 hours. Cells

517   were lysed and collected in RIPA (radioimmunoprecipitation assay; Fisher Scientific;

518   PI89901) supplemented with 1 x protease and phosphatase inhibitors

519   (ThermoScientific; 78440)[11,12].

520

521   *Polyacrylamide gel electrophoresis and Western blotting.*

522   Protein concentrations were determined by BCA assay (Bicinchoninic acid assay;

523   Pierce; 23225). For each sample, 20 µg of protein was separated using a 4-15% MP

524   TGX precast cassette (Bio-Rad; 4568084) at 100 V for approximately 1 hour and 30

525   minutes before being transferred to a PVDF (polyvinylidene difluoride) membrane.

526   Membranes were blocked with 5% milk in 1 x PBS-T (phosphate-buffered saline

527   containing 0.1% Tween 20) for approximately 1 hour at room temperature. All primary

528   antibodies were diluted in 5% milk, PBS-T and incubated with the blot overnight at 4°C.

529   The primary antibodies used were anti-HA anti-mouse (Invitrogen 26153; 1:1000), anti-

530   FLAG-HRP diluted (Sigma A8512; 1:2000), and anti-Actin (Sigma A2105; 1:1000).

531   Membranes were then washed 3 times with 0.5% milk, PBS-T before adding a

532   secondary antibody. Secondary antibodies included rabbit anti-mouse (Star13B; 1:2000

533   dilution) and donkey anti-rabbit (Sigma; A16023; 1:5000). Secondary antibodies were

534   incubated with membranes for 2 hours at room temperature. Membranes were then

535   washed and imaged using an ECL western blotting substrate (Enhanced

536   chemiluminescence; Fisher Scientific; P13216).

537

538   *Luciferase Reporter Assays.*

539   For the ATF6 activation assay, 1 x 10^4 HEK 293T cells were seeded into white-

540   walled, clear-bottom 96-well plates (Greiner Bio-One, 655098). The following day, cells

541   were transfected with 0.05 µg of the ATF6 luciferase reporter plasmid using 0.5 µl of

542   Lipofectamine 3000 in Opti-MEM I reduced-serum medium (Gibco, 31985062).

543   Transfections were allowed to proceed overnight. The following day, cells were infected

544   for 18 hours with *A. phagocytophilum* (MOI 50) or *B. burgdorferi* (MOI 200) or left

545   uninfected. Luminescence was measured the following day by adding 5 mg mL^{-1} of D-

546   Luciferin potassium salt (Promega, E1500) to each well and quantifying with a plate

547   reader. Data is represented as relative luciferase units (RLU) normalized to non-infected

548   controls $\pm$ SEM.

549   To assess *stomatin* activation by ATF6, cells were triple-transfected with the

550   reporter plasmid containing Luciferase under control of the *stomatin* reporter and

551   plasmids expressing *Ixodes* ATF6 and NF-Y (0.05 µg of each). Luminescence was

552   measured as described above. Data is normalized to the control containing only the

553   *stomatin* reporter plasmid without ATF6 and NF-Y expression plasmids.

554

555   *Gene expression analysis of whole ticks.*

556   Gene expression profiling was performed on ticks at both larval and nymph

557   stages. Replete larvae were collected after being fed on either clean mice, an *A.*

558   *phagocytophilum*-infected mouse, or *B. burgdorferi*-infected mouse. *A.*

559   *phagocytophilum*-infected larvae were collected and maintained in an incubator for

560   either 7 days or were allowed to molt to nymphs. *B. burgdorferi*-infected larvae were

561   collected and either maintained for 14 days post-repletion or were allowed to molt to

562   nymphs. When isolating RNA, individual ticks were flash frozen in liquid nitrogen and

563   mechanically pulverized before adding TRIzol. RNA was isolated and cDNA was

564   synthesized as described above. Primers listed in Supplementary Table 5 were used to

565   measure gene expression by qRT-PCR as described above. All samples were

566   normalized to uninfected controls. Data is expressed as means ± SEM.

567

568   *RNAi silencing and analysis of whole ticks.*

569   *I. scapularis* larvae were silenced with RNAi as previously described[11,12]. Around

570   150 larvae were placed in a 1.5 mL tube with 40 µl of siRNA or scrambled controls and

571   incubated overnight at 15°C. Larvae were then dried and allowed to recover overnight

572   before being placed on infected mice. Replete larvae were collected over a period of 3-5

573   days and frozen. To assess feeding efficiency, larvae were weighed in groups of three.

574   RNA was isolated from individual ticks as described above. To generate absolute

575   numbers of the target sequences, qRT-PCR was performed with a standard curve.

576   Standard curves were generated with a plasmid containing either *A. phagocytophilum*

577   *16s, B. burgdorferi flab, Ixodes β-actin, or Ixodes stomatin* (Supplementary Table 5).

578

579   *Cholesterol Accumulation Assays.*

580   To quantify cholesterol in tick cells, the Amplex Red Cholesterol Assay Kit

581   (Invitrogen, A12216) was used. 6.5×10^7 of ISE6 cells were seeded in T-25 tissue

582   culture flasks (Greiner Bio-one 07-000-226). The next day, the monolayer was washed

583   three times with PBS and resuspended in 1 mL of PBS. An aliquot was set aside for

584   RNA isolation and *β-actin* measurement. The rest of this sample was used for

585 cholesterol quantification. 50 µl of each sample was added to a 96 well clear-bottom,

586 black-sided plate (Thermo Scientific, 12-566-70). Total cholesterol was quantified via

587 the Amplex Red Cholesterol Assay Kit (Invitrogen, A12216) according to the

588 manufacturer's instructions and absorbance was measured at 590 nm. A standard

589 curve was used to calculate total cholesterol concentration. Data is normalized to

590 absolute copies of *β-actin.*

591   To quantify cholesterol in *A. phagocytophilum,* 4 x 10^6 cells of persistently

592 infected ISE6 cells that were silenced for *stomatin* (12 µg of siRNA or scRNA with 10 µl

593 Lipofectamine 3000) were seeded into 6-well plates (STARSTEDT, 83.1839). 5 days

594 post-transfection cells were collected in PBS. Host cell-free *A. phagocytophilum* were

595 isolated by sonication. Briefly, cells were centrifuged at 2300 x g for 10 minutes at 4°C

596 and resuspended in 500 µl of PBS. Samples were sonicated four times for 15 seconds

597 at an amplitude of 30V and lysates were centrifuged at 710 x g for 5 minutes to remove

598 host cell debris. The supernatant was collected and an additional spin at 2300 x g for 10

599 minutes was performed to collect *A. phagocytophilum*. Samples were resuspended in

600 200 µl of PBS. 100 µl was used to quantify cholesterol as described above. The

601 remaining sample was used to quantify bacteria by isolating RNA, synthesizing cDNA,

602 and quantifying *phagocytophilum 16s* by qRT-PCR. Total cholesterol was normalized to

603 *A. phagocytophilum 16s.* One well was collected in TRIzol and analyzed by qRT-PCR to

604 assess silencing efficiency.

605

606 *Statistical analysis.*

607   *In vivo* experiments used at least 10-20 ticks and *in vitro* experiments had at

608   least three to five replicates. Data was analyzed with a non-parametric Mann-Whitney

609   test or an unpaired Student's t-test respectively and expressed as means $\pm$ SEM.

610   GraphPad Prism was used for calculations and creating graphs. Statistical significance

611   was determined by a *P* value of <0.05.

612

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776

777   **FIGURE LEGENDS**

778   <u>**Figure 1.**</u> **ATF6 is induced by tick-borne pathogens during infection and supports**

779   **vector colonization.** (A) HEK 293T cells were transfected with an ATF6 luciferase

780   reporter plasmid. Transfected cells were infected with *A. phagocytophilum* (MOI 50) or

781   *B. burgdorferi* (MOI 200) for 18 hours and normalized to no infection (-). RLU is relative

782   luminescence units. (B) Schematic depicting ATF6 activation. (C-D) Gene expression of

783   *atf6* in *I. scapularis* larvae rested for 7 days after feeding on *Anaplasma*-infected mice

784   (C) and rested for 14 days after feeding on *Borrelia*-infected mice (D). Expression was

785   assessed via qRT-PCR. Each data point is representative of 1 larva. (-) is no infection.

786   *A.p.* is *A. phagocytophilum*. *B.b.* is *B. burgerdorferi*. (E) RNAi knockdown of *atf6* in ISE6

787   cells for 5 days and infected with *A. phagocytophilum* (MOI 50). scRNA, scrambled

788   RNA; siRNA, small interfering RNA. Experiments are representative of at least 2

789   experimental replicates. *P < 0.05.

790

791   <u>**Supplemental Figure 1.**</u> *I. scapularis* **ATF6 sequence alignment and structural**

792   **prediction with AlphaFold.** (A) Human and *I. scapularis* ATF6 protein sequences were

793   aligned and visualized with Jalview. Shaded amino acid residues indicate conservation

794   and percent identity between the two proteins. DNA binding residues are highlighted in

795   gold. *H.s.* is *Homo sapiens*. *I.s.* is *I. scapularis*. (B) *I. scapularis* ATF6 predicted by

796   AlphaFold. Color of each residue represents the model confidence score, pLDDT. Blue

797   indicates regions predicted with high confidence. Yellow to orange indicates regions of

798   low confidence.

799

800   **Figure 2.** **Predicting the ATF6 regulatory network in ticks.** (A) The *I. scapularis*

801   ATF6 (orange) predicted with AlphaFold has a conserved DNA binding domain

802   compared to the human ortholog (green). (B) A portion of the bZIP domain and the DNA

803   binding residues on ATF6 are visualized. (C) Schematic of the developed R script. (D)

804   Comparison of genes predicted to be regulated by ATF6 from the *I. scapularis*, *D.*

805   *melanogaster,* and *H. sapiens*. (E) GO terms and Reactome pathways represented in

806   predicted ATF6-regulated genes from *I. scapularis* genome.

807

808   **Supplementary Figure 2.** **Predicted ATF6 regulatory network across organisms**.

809   GO enrichment analysis comparison between predicted ATF6-regulated genes from *I.*

810   *scapularis*, *D. melanogaster,* and *H. sapiens*.

811

812   **Figure 3.** **ATF6 upregulates *stomatin* during infection in ticks.** (A) The AlphaFold3

813   predicted DNA-protein complex with *I. scapularis* nATF6 (tan) and the predicted

814   promoter of *stomatin* (teal). The ATF6 binding site is highlighted in yellow and DNA

815   binding residues highlighted in purple. (B-C) *stomatin* expression quantified from (B)

816   ISE6 cells treated with 5 µM-50 µM of AA147 for 24 hours and (C) ISE6 cells

817   transfected with siRNA targeting *atf6* or a scrambled control. (D) HEK 293T cells were

818   co-transfected with plasmids constitutively expressing *I. scapularis* nATF6, *I. scapularis*

819   NF-Y, and a luciferase reporter plasmid with the *stomatin* promoter. Luciferase activity

820   was normalized to the control containing only the luciferase reporter plasmid. RLU is

821   relative luminescence units. (E-F) Gene expression of larvae rested for 7 days after

822   being fed on *Anaplasma*-infected mice (E) or rested for 14 days after being fed on *B.*

823   *burgdorferi*-infected mice (F). (G) *stomatin* gene expression from unfed, infected *I.*

824   *scapularis* nymphs. *A.p.* is *A. phagocytophilum. B.b.* is *B. burgerdorferi.* Expression

825   assessed via qRT-PCR. *P < 0.05.

826

827   <u>**Supplementary Figure 3.**</u> **Structural interaction of ATF6 and cell viability post**

828   **AA147 treatment.** (A) AlphaFold3 prediction of ATF6 dimer binding to *stomatin*

829   promoter. Color of each residue represents the model confidence score, pLDDT. Blue

830   indicates regions predicted with high confidence. Yellow to orange indicates regions of

831   low confidence. (B) Cell viability measured for ISE6 cells treated with 5 µM-50 µM of

832   AA147 for 24 hours.

833

834   <u>**Supplementary Figure 4.**</u> **ATF6 schematic and recombinant protein expression**

835   **with NF-Y.** (A) ATF6 linear structure. Green bars indicate glycosylation sites at 98-102,

836   216-220, 334-338, 470-474, 621-625. Purple bars indicate S1 and S2 protease sites at

837   412-415 and 382-385 respectively. (B) Immunoblot against HEK 293T cells transfected

838   with FLAG-tagged pCMV-NF-Y and HA-tagged pCMV-ATF6 (C) HEK 293T cells were

839   co-transfected with an ATF6 luciferase reporter plasmid and plasmids constitutively

840   expressing *I. scapularis* ATF6 and *I. scapularis* NF-Y. Luciferase activity was

841   normalized to the control containing only the luciferase reporter plasmid. RLU is relative

842   luminescence units.

843

844   <u>**Figure 4.**</u> **Stomatin supports infection by facillitating *A. phagocytophilum***

845   **cholesterol uptake.** (A) ISE6 cells were transfected with siRNA or a scrambled control.

846   Cells were infected with *A. phagocytophilum* (MOI 50) for 18 hours. (B) siRNA-treated

847   or scrambled control-treated larvae were fed on *A. phagocytophilum*-infected mice.

848   Each data point is representative of 1 larva. Open and closed dots represent

849   experimental replicates 1 and 2. Gene silencing and bacterial burden were measured by

850   qRT-PCR. (C) AlphaFold-predicted *I. scapularis* Stomatin with SPFH domain

851   highlighted in blue and cholesterol binding domains highlighted in pink. (D) Total

852   cholesterol quantified from *A. phagocytophilum*-infected ISE6 compared to uninfected

853   cells (-). Each dot represents an experimental replicate. (E) Cholesterol quantified from

854   cells treated with silencing RNAs targeting *stomatin* or scrambled controls. (F)

855   Cholesterol quantified in *A. phagocytophilum* grown in *stomatin*-depleted ISE6 tick cells.

856   Gene silencing was measured by qRT-PCR. scRNA, scrambled RNA; siRNA, small

857   interfering RNA. Experiments are representative of at least 2 experimental replicates. *P

858   < 0.05.

859

860   **Supplemental Figure 5. *I. scapularis* Stomatin sequence alignment and structural**

861   **prediction with AlphaFold.** (A) Human and *I. scapularis* Stomatin protein sequences

862   were aligned and visualized with Jalview. Shaded amino acid residues indicate

863   conservation and percent identity between the two proteins. *H.s.* is *Homo sapiens*. *I.s.* is

864   *I. scapularis*. (B) *I. scapularis* Stomatin predicted by AlphaFold. Color of each residue

865   represents the model confidence score, pLDDT. Blue indicates regions predicted with

866   high confidence. Yellow to orange indicates regions of low confidence. (C) Predicted *I.*

867   *scapularis* Stomatin (indigo) aligned with human Stomatin (magenta). (D) *I. scapularis*

868   Stomatin sequence with highlighted cholesterol binding domains. Protein sequence

869   visualized with Jalview. The region in blue indicates the conserved SPFH domain.

870   Cholesterol binding domains, CRAC (purple) and CARC (teal) are highlighted. (E)

871   Supporting information for Fig 4F showing *stomatin* silencing levels compared to the

872   scrambled control. scRNA, scrambled RNA; siRNA, small interfering RNA.

873

874   **Figure 5.** ATF6 supports arthropod infection through Stomatin-regulated

875   **cholesterol delivery to the pathogen.** (A) Infection activates ATF6 which translocates

876   to the nucleus and upregulates *stomatin* expression. Stomatin regulates cholesterol

877   distribution in the cell and to the *Anaplasma* containing vacuole for growth and survival

878   in ticks. (B) ArthroQuest was used to identify organisms containing ATF6 binding sites

879   in the promoter regions of Stomatin orthologs. Stomatin orthologs were identified using

880   the *Ixodes* protein sequence. Green checkmarks indicate that the top ortholog contains

881   an ATF6 binding site in the promoter region. Yellow checkmarks indicate that a

882   significant ortholog hit contains an ATF6 binding site in the promoter region. Red "X"

883   indicates all BLAST hits lack an ATF6 binding site.

884

912

Author Contributions

914   K.A.V and D.K.S. designed the study. K.A.V., S.J.W., K.L.R., E.A.F, E.R-Z., and D.K.S.

915   performed experiments. K.A.V. and E.A.S. developed ArthroQuest. K.A.V. and D.K.S.

916   analyzed data. All authors provided intellectual input into the study. K.A.V., S.J.W., and

917   D.K.S. wrote the manuscript. All authors contributed to editing.

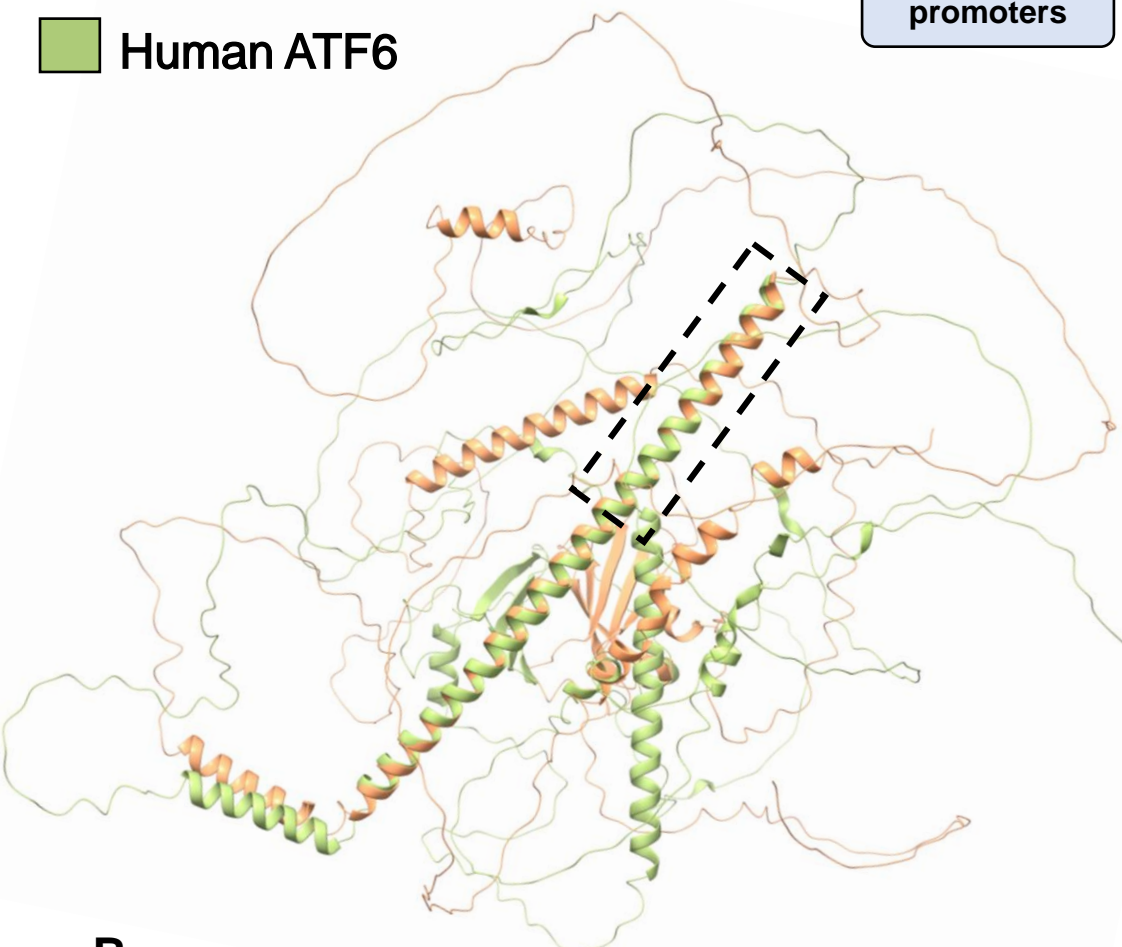**Figure 1.** ATF6 is induced by tick-borne pathogens during infection and supports vector colonization
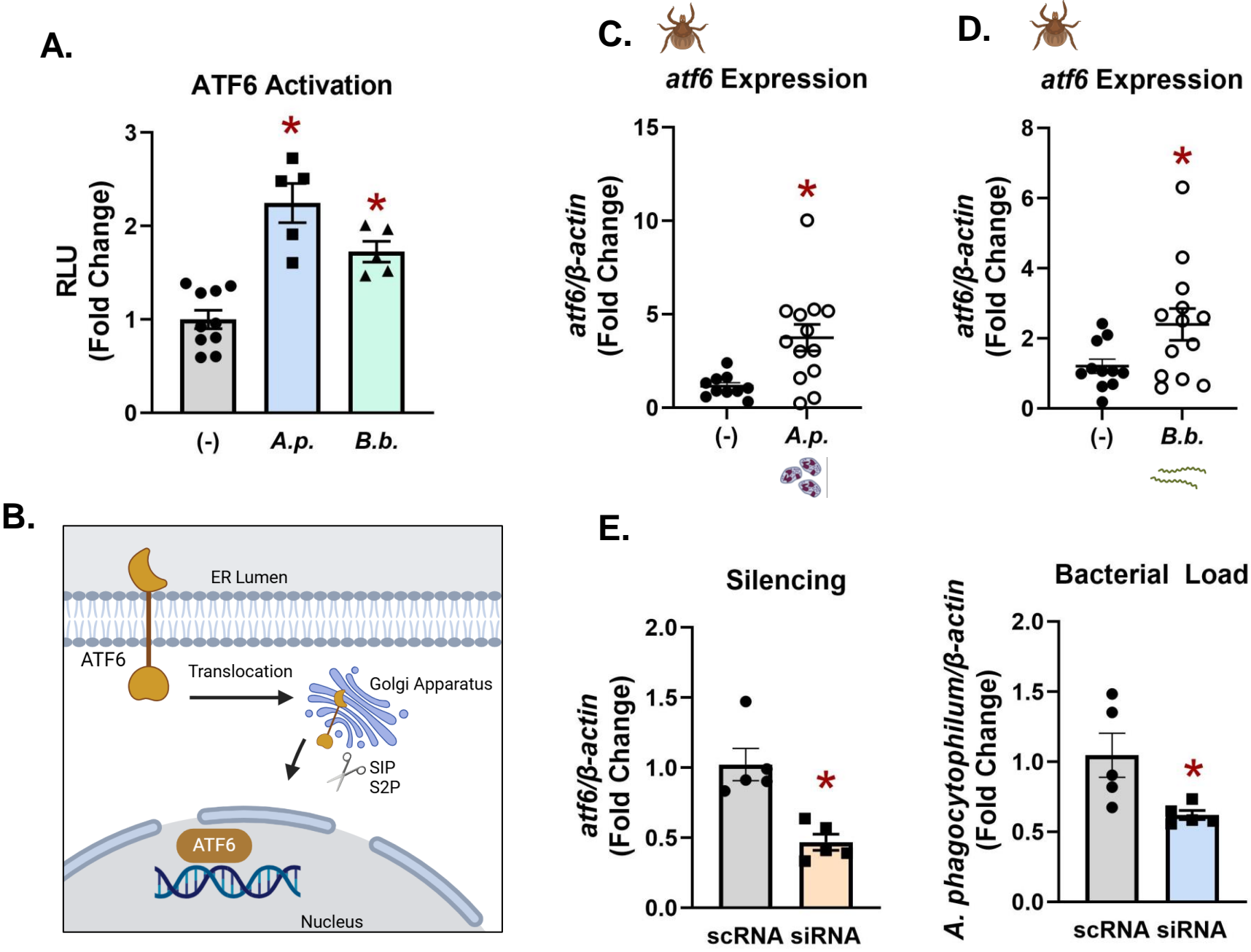
Figure 2. Predicting the ATF6 regulatory network in ticks.

A.

I. scapularis ATF6

Human ATF6

B.

K304

N305

R306

C.

Define predicted promoters → Detect ATF6 binding sites → Extract genes with promoter hits → Find orthologs → Extract pathway information

D.

I. scapularis

H. sapiens

5145

290

5014

124

866

343

2718

D. melanogaster

E.

Immunity

112

Signal Transduction

122

Associated with the ER

20

43

3

Positive Regulation of Bacterial or Viral Processes

146

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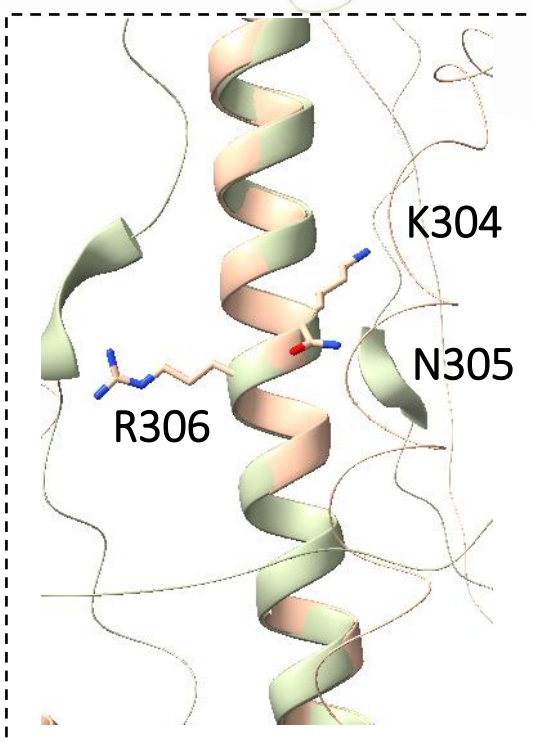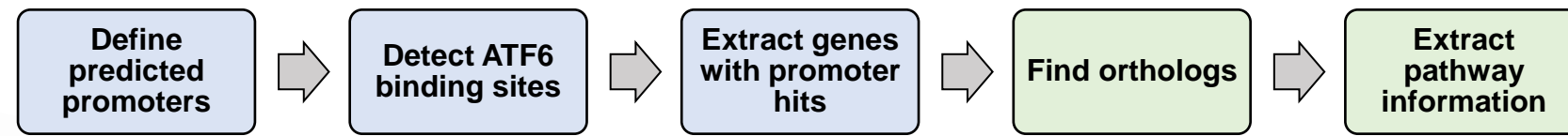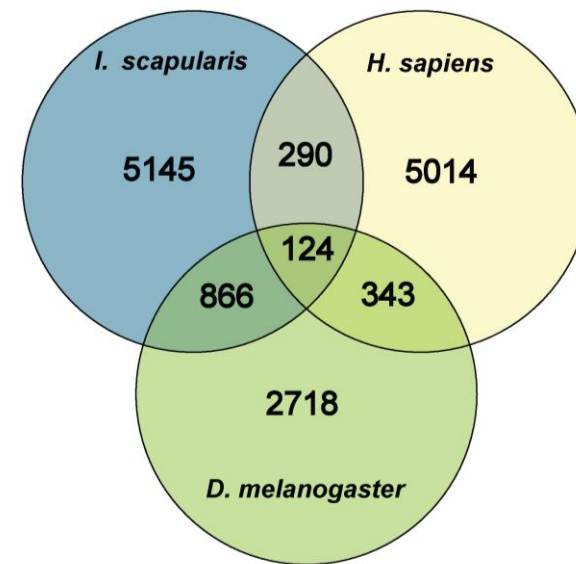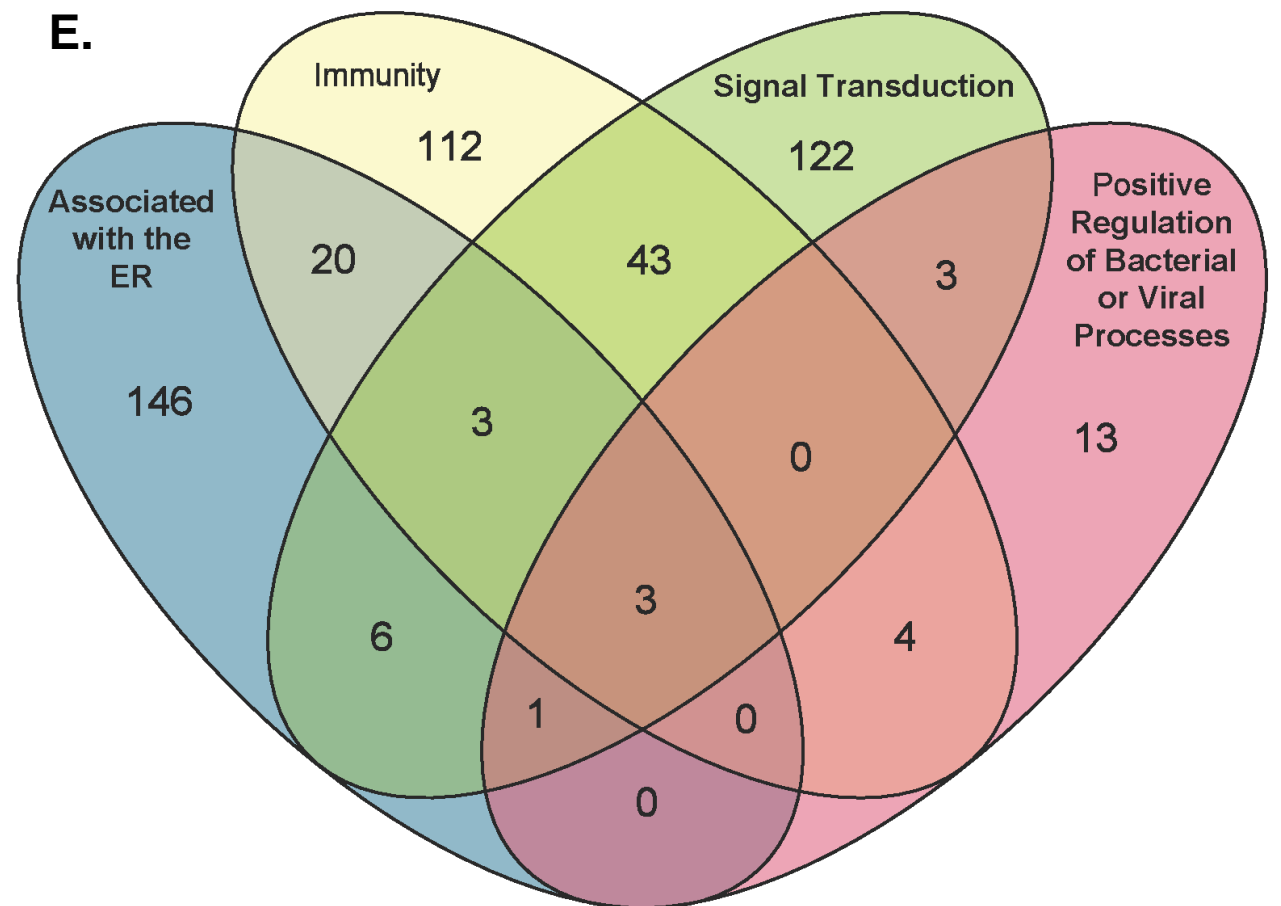
Figure 3. ATF6 upregulates *stomatin* during infection in ticks.
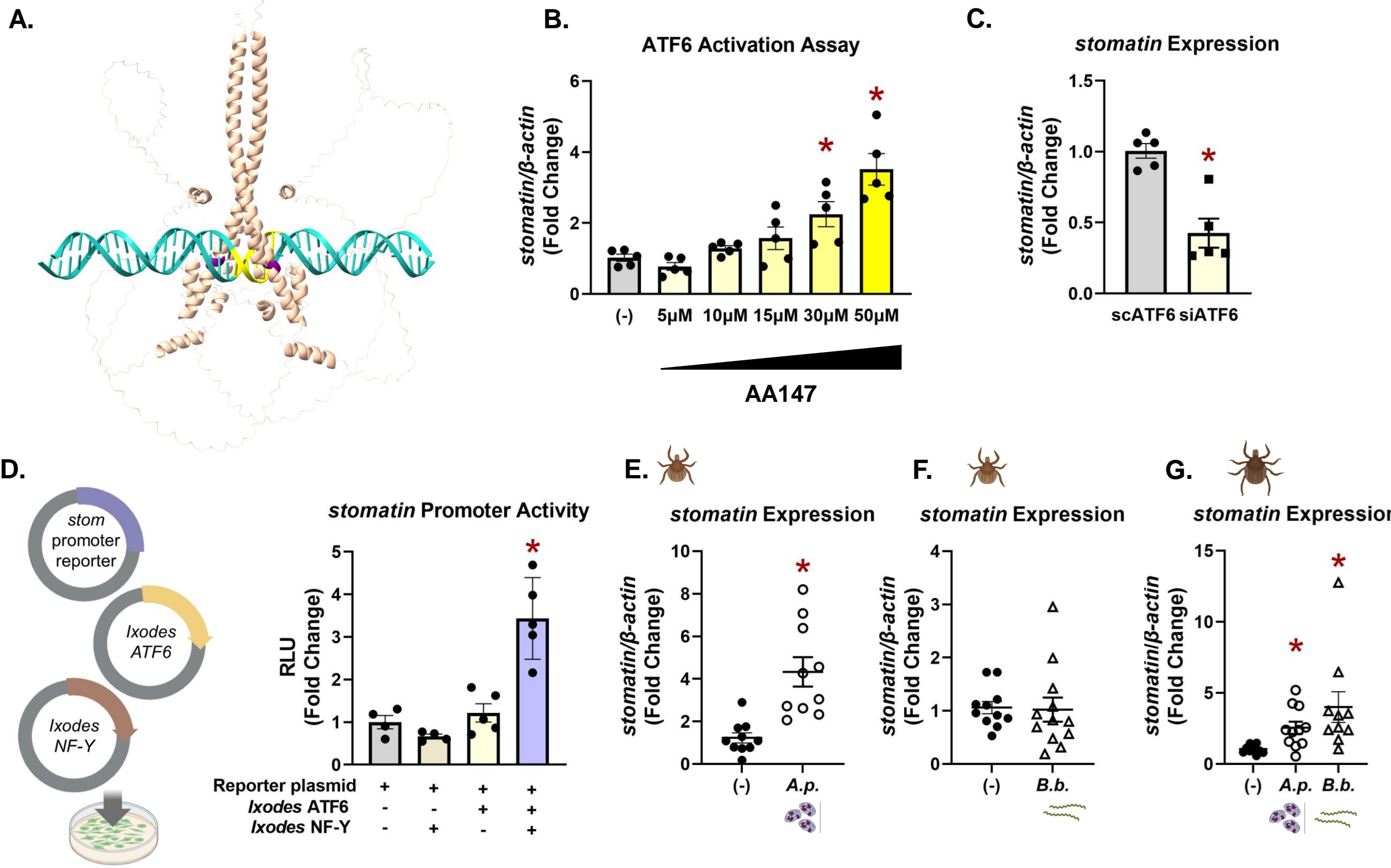
Figure 4: Stomatin supports infection by facillitating *A. phagocytophilum* cholesterol uptake.
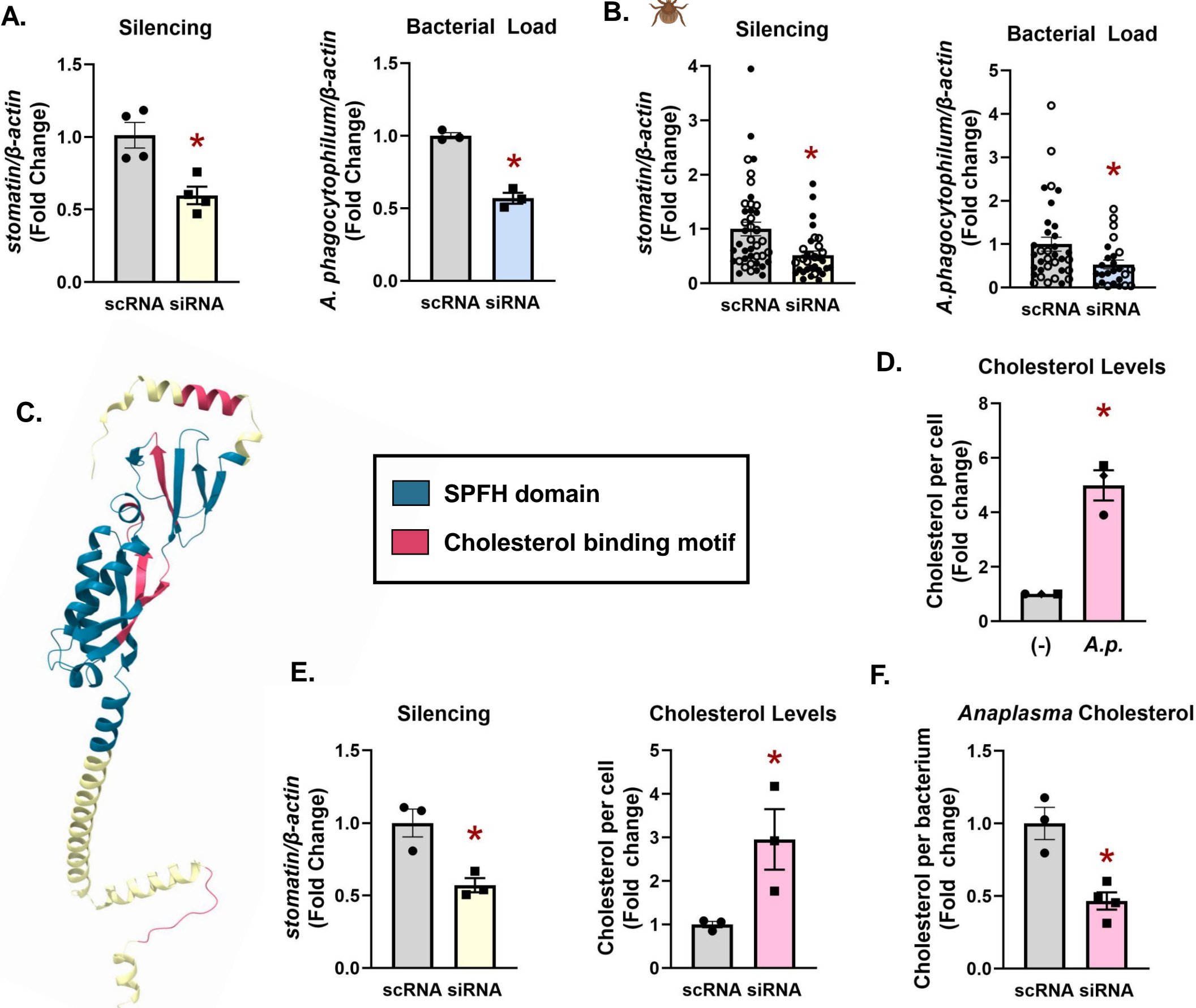
Figure 5. ATF6 supports arthropod infection through Stomatin-regulated cholesterol delivery to the pathogen.

A.

B.