Adaptation of *Anaplasma phagocytophilum* to the tick vector is controlled by the transcriptional regulator Tr1

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Running Title:

Master regulator of *Anaplasma* adaptation to the tick

ABSTRACT

Rickettsial pathogens are strictly dependent on the cellular biology of their hosts for survival and replication. Predominantly transmitted by blood-feeding arthropods, these vector-borne pathogens are forced to adapt between the disparate environments of their mammalian host and arthropod vector. To achieve this, the Rickettsial bacteria *Anaplasma phagocytophilum* undergoes extensive transcriptional reprogramming with over 41% of its genes differentially transcribed between mammals and *Ixodes scapularis* ticks. How the bacterium orchestrates this dramatic transcriptional reprogramming is not understood. The gene *tr1* encodes a Helix-Turn-Helix DNA-binding protein that is exclusively expressed during tick infection. Herein, we show that *tr1* is essential for *A. phagocytophilum* survival in ticks and regulates the transcription of other genes necessary to adapt to the arthropod vector. We demonstrate that Tr1 is a DNA-binding protein and recognizes promotors of tick-specific genes in *A. phagocytophilum,* including secreted effector *ateA*, alternate components of type IV secretion system (T4SS), and membrane proteins. Our findings demonstrate that Tr1 is a master regulator of genes that are critical for *A. phagocytophilum* adaptation to the tick.

36 **Keywords:**

37 Anaplasma

38 *Anaplasma phagocytophilum*

39 Ixodes scapularis

40 Tick-borne disease

41 Transcription factor

42 Transcriptional regulation

43 Type IV secretion system

44 T4SS effectors

45 Bacterial effectors

46 Membrane proteins

47

48 **INTRODUCTION**

49 Rickettsial pathogens are strictly dependent on the cellular biology of their hosts
50 for survival and replication, which is reflected by their small genomes and reduced
51 metabolic capacities. Instead these bacteria have evolved factors to mediate host-
52 pathogen interactions and manipulate the intracellular environment[1–4]. These include
53 specialized surface proteins mediating contact and uptake into cells[2,3,5–9] and effector
54 molecules injected through secretion systems to redirect host cell pathways[10–23]. The
55 large majority of Rickettsial host-pathogen interactions are described in the context of
56 mammalian infection[17,20–22,24,25]. However, since Rickettsial bacteria are predominantly
57 transmitted by blood-feeding arthropods, these findings only explore half of the
58 pathogen lifecycle[26]. Mammals and arthropods are separated by over 680 million years
59 of evolution and, as such, present distinct environments that Rickettsial pathogens must
60 adapt to survive intracellularly [27].

61 The most common Rickettsial pathogen in the United States, *Anaplasma*
62 *phagocytophilum*, is transmitted to humans, domestic animals, and wildlife by the tick
63 *Ixodes scapularis*[28]. While in the mammalian host, *A. phagocytophilum* infects and
64 replicates within circulating neutrophils. In the tick, the bacteria initially infect the
65 digestive tract and then migrate to the salivary glands, where they will survive through
66 the molt[29,30]. In response to the disparate biology between the mammalian host and tick
67 vector[31], *A. phagocytophilum* undergoes extensive transcriptional reprogramming.
68 Transcriptomic studies found that over 41% of *A. phagocytophilum* genes are
69 differentially transcribed when comparing infected human monocytes and tick cells[32,33].
70 Indeed, Himar1 transposon disruption of host or vector specific *A. phagocytophilum*

genes reduces bacterial survival in the respective human or tick cell cultures[10,34–37]. Together, this suggests transcriptional reprogramming of *A. phagocytophilum* is necessary for adaptation between the host and vector environments.

While both transcriptomics and proteomics have shown *A. phagocytophilum* and other Rickettsial pathogens undergo extensive retooling during tick infections[5,32,33,38–42], the regulatory mechanisms that control these dramatic shifts are not known. One gene, *tr1*, encodes a putative Helix-Turn-Helix DNA-binding protein (Tr1). *tr1* was first noticed due to its proximity to outer membrane protein (OMP) genes *omp_1X*, *omp_1N* and the *msp2* expression site[43,44], but its impact on this neighboring operon is not defined. Strikingly, of all *A. phagocytophilum* genes *tr1* has the highest transcriptional specificity for tick cell infection[32,33]. Herein, we demonstrate that *tr1* is essential for *A. phagocytophilum* survival in tick cells, colonization of ticks *in vivo*, and expression of many genes necessary for adaptation to the tick. Further, we demonstrate that Tr1 is a DNA-binding protein and recognizes promotors of tick-specific genes in *A. phagocytophilum,* including secreted effector *ateA*, alternate components of type IV secretion system (T4SS), and membrane proteins. From this work we have identified Tr1 as a master regulator of genes that are critical for *A. phagocytophilum* adaptation to the tick.

RESULTS

Transcription of tr1 is specific to tick cell infection.

92 *A. phagocytophilum* HGE1 *tr1* transcription was quantified and compared
93 between bacteria cultured in the human monocyte-like HL60 cell line and embryonic tick
94 ISE6 cells. Consistent with previous tiling array reports, *tr1* transcription was >26 fold
95 higher during growth in tick ISE6 cells over the human HL60 cell line (**Fig 1a**), validating
96 that *tr1* expression is highly specific to growth in tick cells[32,33].

97 *tr1 is necessary for A. phagocytophilum survival in tick cells.*

98 The specificity of *tr1* transcription to ISE6 cell culture prompted us to test if *tr1* is
99 necessary for *A. phagocytophilum* adaptation to ticks. To examine this possibility, we
100 obtained a transposon mutant strain *tr1*::Himar1 from the previously published mutant
101 collection[34]. An established control strain with the Himar1 transposon inserted in an
102 intergenic non-coding region was used for comparison. This control strain is
103 phenotypically comparable to wild-type in both mammalian and tick infection
104 models[10,34,36,37]. *tr1::Himar1* and the control *A. phagocytophilum* strain were purified
105 from HL60 cell culture and used to infect either ISE6s or HL60s. Bacterial burdens were
106 compared over time. During HL60 infection, the control and *tr1*::Himar1 strains grew
107 equivalently (**Fig 1b**). However, during tick cell infection, *tr1*::Himar1 steadily declined
108 (**Fig 1c**), indicating that it is required for *A. phagocytophilum* adaptation to tick cells.

109 *A. phagocytophilum requires tr1 to colonize ticks in vivo.*

110 The *in vitro tr1*::Himar1 phenotype led us to ask if *tr1* is similarly required *in vivo*
111 for *A. phagocytophilum* adaptation to the tick. Mice were infected with either the
112 intergenic control or the *tr1*::Himar1 mutant *A. phagocytophilum*. Seven days post
113 infection bacterial burden in the mice was quantified and found to be equivalent

114 between the strains, indicating *tr1* is not required during murine infection (**Fig 2a**).
115 Burden matched mice from these groups were used to feed *I. scapularis* larval ticks to
116 repletion. We found ticks that fed on mice infected with *tr1*::Himar1 acquired significantly
117 less *A. phagocytophilum* than those that fed on mice infected with the control strain (Fig
118 2b). These findings indicate that *tr1* is dispensable for murine infection but required for
119 colonization of the arthropod vector.

120 *Tr1 is a multimeric Helix-Turn-Helix DNA-binding protein.*

121 Having established that Tr1 is important for adaptation to the tick host by *A.*
122 *phagocytophilum*, we next used bioinformatics approaches to predict the functions of
123 the Tr1 protein. First, a structural model of monomeric Tr1 protein (amino acids 1-183)
124 was generated using ColabFold[45]. The resulting predicted Tr1 monomer model is
125 comprised of two ordered and predominantly α-helical domains that are separated by a
126 central disordered peptide linker and flanked by disordered peptide at both the N and C
127 termini (**Fig 3a**). In total, the model contains approximately 58 residues of predicted
128 disorder. Whilst the global confidence score for the predicted model was low
129 (pTM=0.468), the local confidence scores for the two ordered domains were relatively
130 high (pLDDT greater than 80 for residues ~25-90 and ~130-155, Supplementary figure
131 1).

132 Next, we used the Dali server[46] to search the Protein Data Bank (PDB) for
133 structures similar to Tr1, but which have been the subject of structure-function analyses.
134 Amongst the results of the Dali search, nine of the ten structures most similar to Tr1
135 were DNA-binding proteins with similarity to the N-terminal domain of Tr1 and which
136 function as dimers. Comparing the N-terminal domain of Tr1 with these structures reveal

137 that Tr1 is likely to be a five-helix bundle which contains a DNA-binding helix-turn-helix
138 motif (H-T-H), similar to cI/Cro-like transcription factors (**Fig 3a).**

139 Prompted by this observation, we next used ColabFold Multimer to generate a
140 model of dimeric Tr1 protein. In the resulting model, the N-terminal domains of Tr1 are
141 closely associated with one another, forming a symmetrical dimer (**Fig 3b).** The C-
142 terminal domains of the two Tr1 monomers, which each resemble two antiparallel
143 helices separated by a turn, interdigitate with one another forming an additional point for
144 dimerization between the two protein chains (**Fig 3b)**. Whilst the global confidence
145 score for the dimeric model of Tr1 was higher than that of the monomeric model (dimer
146 pTM 0.557), the confidence score for the interface of the dimeric Tr1 model was low
147 (ipTM=0.545). We considered that the large proportion of disordered protein in Tr1 could
148 skew the interface confidence scores in our predictions, so we tried to predict dimeric
149 models for Tr1 residues corresponding to the N- and C-terminal domains only. Interface
150 confidence scores for dimeric Tr1 N- or C-terminal domains in isolation were high
151 enough to suggest plausible interactions (piTM=0.763 and 0.69, respectively).

152 Structures of dimeric H-T-H proteins identified by our Dali search were
153 superimposed onto the Tr1 dimer model to further assess this predicted structure. The
154 N-terminal domain of Tr1 dimerizes in manner typical of other dimeric H-T-H proteins,
155 including those bound to DNA (**Fig 3c**). Frequently, H-T-H proteins that bind DNA as
156 symmetrical homodimers bind to palindromic DNA sequences with each protein binding
157 to a half-site of the palindrome. From these structural comparisons, we predict this is
158 likely to also be the case for Tr1. Fewer structural homologues of the C-terminal domain
159 of Tr1 were identified but we noted similarity between the Tr1 dimer model and the

160 EspR transcription factor of *Mycobacterium tuberculosis* (**Fig 3d)**. EspR dimerizes via a
161 domain similar to the Tr1 C-terminal domain and this is required for EspR to bind DNA
162 with high-affinity[47].

163 Next, we used gel filtration chromatography to assess whether purified
164 recombinant Tr1 protein could form dimers or other multimers in solution. Recombinant
165 Tr1 protein was purified and affinity tags removed prior to running on a gel filtration
166 column. We reasoned that removing any additional protein sequences from Tr1 would
167 yield protein most similar to that found in its native environment. Since the expected
168 molecular weight of monomeric recombinant Tr1 is 21.3 kDa, we also ran purified MBP
169 and GFP, predominantly monomeric proteins with respective molecular weights of 43.8
170 kDa and 28.5 kDa, as controls. Both MBP and GFP eluted from the gel filtration column
171 as monodisperse peaks whereas Tr1 eluted as two overlapping peaks, indicating that
172 Tr1 exists in multiple forms in solution (**Fig 4a).** SDS-PAGE analysis of fractions
173 collected from these experiments confirmed that the peaks contained the expected
174 proteins (**Fig 4b).** Apparent molecular weights for the peaks were calculated with a
175 standard curve based on their elution volumes. The calculated apparent molecular
176 weights for GFP and MBP were 31.6 and 45.7 kDa, respectively, roughly consistent with
177 their expected weights as monomers. The second peak of Tr1 eluted at an apparent
178 molecular weight of 79.4 kDa, suggesting that Tr1 can form tetramers in solution. The
179 first peak of Tr1 eluted at a volume outside of our standard curve and potentially
180 contained aggregated proteins. We generated a predicted tetrameric model of full-
181 length Tr1, but global confidence scores were noticeably lower than those for other Tr1

182 models (pTM 0.414), so do not present this data here. Further biochemical data are
183 required to guide additional predictive modelling of Tr1 multimers.

184 Taken together, we predict that Tr1 is comprised of H-T-H and dimerization
185 /multimerization domains joined and flanked by regions of disorder. Comparisons
186 indicate that Tr1 might have similarity to cI/Cro transcription factors and EspR from *M.*
187 *tuberculosis.* Gel filtration experiments demonstrate that Tr1 forms multimers in solution,
188 supporting these predictions, however, a high confidence predicted model of a Tr1
189 tetramer could not be generated

190 *Tr1 binds the promotors of neighboring genes omp1X and omp1N.*

191 The *tr1* gene was first noticed in studies examining expression of downstream
192 genes *omp1X*, *omp1N*, and the *msp2/p44* expression site[43,44] (**Fig 5a**). We tested Tr1
193 binding upstream of *tr1*, *omp1X*, *omp1N*, and the *msp2/p44* expression locus by
194 electrophoretic mobility shift assays (EMSA) using DNA probes for sequences
195 preceding each gene (Fig 5a). EMSA shifts indicated Tr1 complexed with promotors p-
196 *tr1* (**Fig 5b**), p-*omp1X* (**Fig 5c**) and p-*omp1N* (**Fig 5d**). At higher Tr1 concentrations p-
197 *tr1* and p-*omp1N* also displayed secondary complexes suggesting multiple Tr1 binding
198 sites or higher order Tr1 oligomer complexes (**Fig 5b,d**). DNA sequence upstream of
199 the *msp2* expression locus did not shift at any concentration of the Tr1 protein,
200 indicating Tr1 does not individually regulate *msp2* (**Fig 5e**).

201 To ask how loss of *tr1* affected these genes' transcription, RNA was collected
202 from control and *tr1*::Himar1 *A. phagocytophilum* infected ISE6 cells. Surprisingly, qPCR
203 measuring *omp1X* and *omp1N* transcription from *tr1*::Himar1 and control *A.*

204 *phagocytophilum* strains (**Fig 5f,g**) found that only *omp1N* displayed a small but
205 significant expression difference during tick cell infection (**Fig 5g**), suggesting Tr1
206 binding alone does fully account for the behavior of these genes.

207 *tr1 is required for A. phagocytophilum transcriptional shift during tick cell infection*

208 Tr1's predicted role as a transcriptional regulator[43,48] and the inability of the
209 *tr1*::Himar1 to survive in tick cells led us to ask how the loss of *tr1* affects the
210 transcriptome during tick cell infection using RNA sequencing (RNAseq). ISE6 tick cells
211 were infected with *A. phagocytophilum tr1::Himar1* or the control strain. Our previous
212 qPCR for *A. phagocytophilum* gDNA from infected ISE6 cells found that the *tr1*::Himar1
213 strain persists up to day 3 before declining relative to the control strain (**Fig 1c**). Since
214 measuring genomic DNA (gDNA) may also reflect dead bacteria, we measured *A.*
215 *phagocytophilum 16s* RNA relative to *I. scapularis actin* transcripts as an indicator of
216 bacterial survival at 12, 24, and 48 hours post infection (hpi). At 24 hours *tr1*::Himar1
217 survival was 63% of the control strain but fell to only 10% by 48 hpi (**Fig S2**). To capture
218 transcriptional differences when *tr1*::Himar1 bacteria remained viable we performed
219 RNAseq 24 hpi.

220 Genes downstream of *tr1*, *omp1X*, *omp1N*, and *msp2*[43] (**Fig 5a**) were all
221 measured in the RNAseq data. Similar to the qRT-PCR measurements (**Fig 5g**), *omp1X*
222 did not significantly differ between *tr1*::Himar1 and the control strain. *omp1N* had a
223 small but significant increase in expression relative to the control (**Table S1**). Measuring
224 *msp2* transcription is complicated by the presence of multiple *msp2* pseudogenes,
225 which *A. phagocytophilum* uses to evade the mammalian antibody response by
226 antigenic variation. For individual *msp2* variants to be transcribed, the pseudogenes

227 recombine into the expression site adjacent to *omp1N*[43,44,49–52] (Fig 5a). In our RNAseq
228 data, 97 *msp2* variants were detected, with 15 having significant transcription
229 differences between *tr1*::Himar1 and the control strain (**Table S2**). To quantify total
230 *msp2* transcription from the expression site, we summed all *msp2* transcripts from the
231 RNAseq data. This found no difference in overall *msp2* transcription between the
232 *tr1*::Himar1 and control strain (**Fig 5h**). Expression differences among individual *msp2*
233 variants likely reflects *msp2* diversity bottle neck during Himar1 library construction
234 and/or *msp2* recombination since the *tr1*::Himar1 and control strains were purified from
235 the larger collection. These finding again suggest the importance of *tr1* extends beyond
236 its immediate genetic neighborhood.

237 Beyond *tr1*'s immediate neighbors, RNAseq identified 177 genes as differentially
238 expressed between *tr1*::Himar1 and the control *A. phagocytophilum* strain (p-adj<0.05)
239 during tick cell infection (**Table S1**). Aside from *msp2* variants, 22 genes were >2 fold
240 upregulated in the *tr1*::Himar1, although none have known host or vector-specific
241 expression[32,33] and are largely ribosomal or other core bacterial genes[34]. Conversely, 14
242 genes had >2 fold reduced transcription from the *tr1*::Himar1 mutant (**Table 1**). Seven of
243 the of the these, (HGE1_03907/APH_0916, *ateA*, *tr1*, HGE1_01872/APH_0406, *msp4*,
244 HGE1_04767/APH_1111, HGE1_03162/APH_0720) have known tick-specific
245 expression patterns[10,32,33], which indicates that *tr1* is needed to upregulate *A.*
246 *phagocytophilum* genes specific for the arthropod vector.

247 *Tr1 binds promotors of genes of tick-specific A. phagocytophilum genes.*

248 We next asked if Tr1 binds promotors of any tick-specific genes impacted by the
249 *tr1*::Himar1 mutation. EMSA were performed with increasing Tr1 protein against

250 promotor sequences preceding; *ateA*, HGE1_06057/APH_1380,
251 HGE1_03162/APH_0720, HGE1_03907/APH_0916, HGE1_01872/APH_0406, and
252 *msp4* (**Fig 6**). Tr1 shifted probes for *ateA* (**Fig 6a**), HGE1_06057 (**Fig 6b**), and *msp4*
253 (**Fig 6c**) at all concentrations, with the majority of the probe band shifted at the highest.
254 HGE1_03162 (**Fig 6d**), HGE1_03907/APH_0916 (**Fig 6e**), and
255 HGE1_01872/APH_0406 (**Fig 6f**) probes also shifted at Tr1 concentrations ≥ 0.125 μM.
256 Similar to EMSAs with probes for *tr1*, *omp1X* and *omp1N,* the higher Tr1 concentrations
257 produced secondary shift bands, suggesting higher order complexes. These findings
258 demonstrate direct Tr1 interaction with promoters for tick-specific *A. phagocytophilum*
259 genes. These EMSA results and differential expression of these genes by the
260 *tr1*::Himar1 mutant strain indicates direct regulation by Tr1.

261 *Tr1 regulated genes are important for A. phagocytophilum survival in tick cells*

262 Inability of the *tr1*::Himar1 to survive in tick cells and the reduced expression of
263 known tick-specific *A. phagocytophilum* genes led us to ask if these genes are important
264 for tick cell infection. We previously showed *ateA*::Himar1 was similarly defective for
265 survival in tick cells and acquisition by ticks[10]. From the *A. phagocytophilum* Himar1
266 mutant library, we identified and isolated three additional mutants disrupted in genes
267 putatively regulated by Tr1: *HGE1_01872*, *HGE1_03162*, and *HGE1_06057*. Growth of
268 all mutant strains in human HL60 cell culture was comparable to the control strain (Fig
269 7a). Accordingly, we infected ISE6 tick cells with *A. phagocytophilum* strains *tr1*::Himar1,
270 *ateA*::Himar1, *HGE1_01872*::Himar1, *HGE1_03162*::Himar1, *HGE1_06057*::Himar1,
271 and intergenic control::Himar1. At 8 days post infection all test strains had significantly
272 reduced *Anaplasma* burden in the tick cells relative to the intergenic Himar1 control,

273 with *tr1::Himar1* being the more severe (**Fig 7b**). From this, we demonstrated three
274 additional tick-specific genes HGE1_01872, HGE1_03162, and HGE1_06057 as critical
275 for infecting ticks. Our results suggest that the tick-specific defect of *tr1*::Himar1 mutant
276 strain stems from its failure to express genes controlled by Tr1 and critical for adaptation
277 to the tick cell environment.

278 *tr1 is necessary for tick-specific alterations in the T4SS apparatus*

279 The promotor sequences of *ateA* and HGE1_06057 had high affinity for Tr1 by
280 EMSA (**Fig 6 a,b**). Previously we identified AteA as the first *A. phagocytophilum* T4SS
281 secreted effector specific to tick infection[10]. HGE1_06057 is also predicted as a T4SS
282 substrate by multiple effector prediction algorithms[12,53]. Tr1 binding to promotors of
283 effectors prompted us to ask if Tr1 influences expression of T4SS machinery itself.

284 An oddity of the *Anaplasma* T4SS is that some components of the apparatus are
285 encoded by multiple paralogs[16,23]. In particular, the needle like pilus of the *A.*
286 *phagocytophilum* T4SS is encoded for by eight paralogs of the pilin gene *virB2* (**Fig 8a**).
287 Transcriptomics studies noticed different combinations of *virB2* paralogs are
288 upregulated when *A. phagocytophilum* infects mammalian or tick cells[32,33]. Because
289 tiling arrays used in these studies can conflate signal among paralogs with close
290 sequence identity[33], we first validated *virB2* expression. We designed primers specific to
291 each *virB2* paralog. We grew *A. phagocytophilum* in human HL60s or tick ISE6 cells
292 and measured transcription of each *virB2* paralog by qRT-PCR. As in the transcriptomic
293 studies, mammalian cultured *A. phagocytophilum* only expressed *virB2* paralogs
294 *virB2_1* and *virB2_2* (**Fig 8b**). Conversely, transcription of *virB2_1* and *virB2_2* was
295 reduced during growth in tick cells, while *virB2*_3 → virB2_8 were upregulated (Fig 8b).

296 To examine if the tick-specific expression pattern among the *virB2* paralogs is
297 dependent on *tr1* we reviewed our RNAseq findings for the *tr1*::Himar mutant. Both
298 *virB2_1* and *virB2_2* expression were significantly elevated (1.47 and 1.38 fold) in the
299 *tr1*::Himar1. While not significant due to low number of total reads *virB2_6* and *virB2_7*
300 had reduced expression (0.55 and 0.218 fold) in the *tr1*::Himar1 relative to the control
301 strain (**Table S1**). Together this indicated that the *tr1*::Himar1 strain fails to adjust
302 expression among the *virB2* paralogs during tick cell infection and retains a *virB2*
303 expression pattern similar to mammalian infection.

304 *Tr1 binds promotors of T4SS virB2 paralogs differentially expressed in tick infection*

305 We asked if Tr1 can directly bind promotor regions among the differentially
306 expressed *virB2* paralogs. DNA probes designed upstream of *virB2_1*, *virB2_6,* and
307 *virB2_8* (Fig 8a) were tested with increasing concentrations of Tr1 protein by EMSA. Tr1
308 shifted the promotors of both *virB2_1/2* (**Fig 8c**) and *virB2_6/7* (**Fig 8d**), with *virB2_1/2*
309 shifting completely at the highest concentration. As with other targets, possible
310 secondary complexes were also visible, suggesting possible higher order interactions.
311 Minimal shifting was seen with probes designed upstream of *virB2_8* (**Fig 8e**)
312 suggesting either independent regulation, or co-regulation with the upstream genes
313 *virB_6/7*. Our findings indicate that Tr1 directly participates in regulation of tick-specific
314 expression patterns of T4SS components.

315 *Tick-specific virB2 paralogs are necessary for A. phagocytophilum survival in tick cells*

316 As with the other Tr1 regulated targets, we asked if the tick-specific *virB2*
317 paralogs are necessary for *A. phagocytophilum* survival in tick cells. From the

318 transposon mutant library, we obtained a mutant strain disrupted in *virB2_5*
319 (*virB2_5*::Himar1) (**Fig 8a**). In the published *A. phagocytophilum* HGE1 genome the
320 *virB2_5* is not annotated and therefore was not initially measured by RNAseq. However,
321 we do detect transcription from the *virB2_5* locus during tick cell infection (**Fig 8b**). The
322 *virB2_5*::Himar1 mutant grew as well as the control strain in human HL60 cells (**Fig 8f**).
323 *A. phagocytophilum virB2_5*::Himar1 and the intergenic control::Himar1 strain were
324 collected from HL60 cell culture and used to infect ISE6 tick cells. Eight days post
325 infection *Anaplasma* burden of *virB2_5*::Himar1 was greatly reduced relative to the
326 control::Himar1 strain in the tick cells (**Fig 8e**). This finding indicates that tick-specific
327 *virB2* paralogs are important for *A. phagocytophilum* adaptation to the tick.

328 *Tr1 homologs are found throughout the Rickettsiales*

329 Having established a role for Tr1 in the survival of *A. phagocytophilum* within the
330 tick host, we queried whether Tr1 homologs are present in other Rickettsiales, since
331 many of these bacteria also have life cycles that involve cycling between vertebrate and
332 arthropod hosts. Using sequence-based searches within the Rickettsiales, we identified
333 candidate homologs of Tr1 in diverse Anaplasmataceae genomes. The *tr1* genes of
334 *Anaplasma*, *Ehrlichia*, and *Neoehrlichia* share a high degree of genomic synteny and
335 are invariably co-localized with *ndk* (encoding nucleoside-diphosphate kinase) and one
336 or more OMP-encoding genes. In *Wolbachia* and *Mesenet* the *tr1* locus is more variable
337 with *ndk* being lost and OMP-encoding genes being co-localized at the *tr1* locus in
338 some, but not all, species.

339 We used Foldseek to extend our search for more distant Tr1 homologs, filtering
340 our results for Rickettsiales bacteria. Using this approach, we identified Tr1 proteins in

341 *Rickettsia* and *Tisiphia* species. Whilst the *tr1* locus in *Rickettsia* species is not
342 syntenous with those from the Anaplasmatacea, the *Rickettsia tr1* gene is most often
343 co-localized with *sca4.* Intriguingly, *sca4* has been identified as having a role in fitness
344 of *Rickettsia parkeri* within the tick host[54]*.* Yet more distant Tr1 homologs were also
345 identified in *Midichloria* and *Orientia*, although in the latter the C-terminal domain is
346 truncated or lost. Candidate *tr1* homologues are co-localized with genes that encode
347 proteins with unknown functions in many Rickettsiales.

348 These results indicate that Tr1 represents a family of transcription factors found
349 broadly across the Rickettsiales, suggesting that some of our findings could be
350 extrapolated to other bacteria of this Order.

351 **DISCUSSION**

352 As a vector-borne pathogen, survival in both the tick and the mammal is essential
353 to complete the transmission cycle of *A. phagocytophilum* and be maintained in the
354 environment. We know the bacteria undergoes extensive transcriptional changes
355 between the two environments[32,33] and have identified individual adaptations that are
356 critical for either mammalian or tick infection[34–37]. Understanding how Rickettsial
357 pathogens switch their host tropism to the arthropod, could inform interventions to
358 disrupt the transmission cycle. In this work we identified Tr1 as a critical switch
359 regulating *A. phagocytophilum* adaptation to the tick and identified Tr1-controlled genes
360 necessary for survival in the arthropod. Collectively our findings uncover a central node
361 in a network of changes necessary for Rickettsial bacteria to complete their vector-
362 borne lifecycle.

363 Due to the obligate intracellular lifecycle and lack of compatible
364 extrachromosomal plasmids, genetic manipulation of *A. phagocytophilum* remains
365 challenging. The Himar1 transposon library has proven to be an invaluable resource,
366 allowing the first phenotypic testing of gene disruptions in *Anaplasma*. The library was
367 generated in HL60 cell culture, which prohibits mutation in genes essential for survival
368 in mammalian cells. However, *A. phagocytophilum* genes specific to tick infection were
369 readily mutated[34,35]. Such mutants have been leveraged to uncover tick-specific roles of
370 the T4SS effector AteA[10], duplicate T4SS component VirB6-4[36], and surface protein
371 modifying O-methyltransferase[37]. In our study we extensively relied on mutants from this
372 library to uncover tick-specific contributions of *tr1* and five of the genes it impacts. Until
373 more targeted genetic tools are available in this system, the *A. phagocytophilum* Himar1
374 transposon library remains a critical resource.

375 Initial interest in the *tr1* gene stemmed from its location upstream of outer
376 membrane proteins genes *omp1X*, *omp1N*, and *msp2/p44* expression site[43,44]. Studies
377 asking what controlled the promotor of *tr1* identified ApxR as the first *Anaplasma* DNA-
378 binding transcriptional regulator. ApxR was later shown to bind its own promotor and
379 upstream of the *p44/msp2* expression site. Similarly, ApxR homolog EcxR in the related
380 Rickettsial pathogen *Ehrlichia chaffeensis*, binds promotors of *tr1* and downstream outer
381 membrane protein genes[55,56]. *E. chaffeensis* Tr1 also binds upstream membrane protein
382 genes *p28* and *omp-1B*[55]. We found *A. phagocytophilum* Tr1 binds DNA sequences
383 upstream of *omp1X* and *omp1N*. However, only expression of *omp1N* differed between
384 the *tr1*::Himar1 mutant and control strain, indicating *tr1* is not the only factor controlling
385 these genes. Though the number, identity, and arrangement of adjacent membrane

386 protein genes downstream of *tr1* in *Anaplasma* and *Ehrlichia* differ greatly[44,55], common
387 models may emerge showing how Tr1 and ApxR/EcxR participate in regulation of the *tr1*
388 adjacent surface protein genes.

389 Looking past its immediate chromosomal neighbors multiple tick-specific
390 membrane protein genes had reduced expression in the Himar1::*tr1* and were directly
391 bound by Tr1. These include; *HGE1_03162* (*Aph_0720*), *HGE1_01872* (*Aph_0406*),
392 *msp4*, and *HGE1_03907* (*Aph_0916*). Transcription of all four is highly specific to *A.*
393 *phagocytophilum* infection in tick cells[32,33]. Reflecting the expression pattern, we found
394 *HGE1_03162* and *HGE1_01872* are necessary for tick cell infection. Adjacent to
395 *HGE1_01872, A. phagocytophilum* encodes two *HGE1_01872* paralogs *asp62*
396 (*HGE1_01862, Aph_0404*) and *asp55* (*HGE1_01867, Aph_0505*). Antibodies against
397 Asp62 and Asp55 were found to limit *A. phagocytophilum* infection of human HL60
398 cells[57]. While Asp62 and Asp55 are expressed during both mammalian and tick cell
399 infection, HGE1_01872 is specific to the arthropod vector[32,33]. Both HGE1_01872 and
400 Msp4 are substrates of an O-methyltransferase required for tick infection and promote
401 binding to *Ixodes* cells[37,58,59]. Similar to HGE1_01872, HGE1_03907 (Aph_0916) is
402 adjacent to the invasion protein gene *aipA*, which is necessary for infection of
403 mammalian cells[60], suggesting HGE1_03907 represents a arthropod-specific AipA
404 alternative. These findings implicate Tr1 as the switch regulator governing this
405 remodeling of the *A. phagocytophilum*'s surface proteome necessary for tick infection.

406 Beyond surface protein genes, Tr1 is playing a broader role controlling genes
407 expressed during infection of the tick. Our findings identify Tr1 as a critical regulator of
408 tick-specific effector AteA and specialization of the T4SS VirB2 pilus necessary for

409 adaptation to the tick. The full repertoire of effectors secreted by *A. phagocytophilum* is
410 unknown, but effector predicting algorithms propose as many as 49[12,53]. So far, few
411 have been experimentally examined[17,21,22,24,61–63], with only AteA investigated in context
412 of the arthropod vector[10]. The *E. chaffeensis* ApxR homolog, EcxR, was also shown to
413 regulate components of the T4SS apparatus during mammalian cell infection[64].
414 However, EcxR has yet to be examined during tick infection, and binding to effector to
415 *virB2* pilin genes is untested. Uncovering the full regulome of Tr1 and how it works with
416 other regulators to manage adaptation between the mammalian and tick environments
417 will likely require larger unbiased binding and regulation studies.

418 How does Tr1 recognize its DNA targets and regulate gene expression? Our
419 bioinformatic analysis indicates that the Tr1 protein has similarity to H-T-H transcription
420 factors and, inferred from this and our protein modelling, is likely to dimerize and bind
421 palindromic DNA motifs to exert influence on transcription on downstream genes.
422 Consistent with this, we also identified a putative dimerization domain in the C-terminus
423 of Tr1 and we observed that recombinant Tr1 protein forms multimers in solution,
424 probably tetramers and possibly other higher order structures. Other H-T-H proteins
425 such as the λ repressor, share this ability to form multimers of dimers, enabling them to
426 bind to multiple DNA sites co-operatively. Indeed, multiple shifts of Tr1-DNA complexes
427 in our EMSAs, supporting that binding of Tr1 might be co-operative at some of its target
428 sites. Understanding if or how the multimerization of Tr1 is regulated during infection,
429 the interplay between Tr1 and other transcription factors at promoter regions, and the
430 identification of a consensus DNA target site and regulon will be crucial for determining
431 how Tr1 mediates *A. phagocytophilum* adaptation to the arthropod host.

432 We identified candidate Tr1 homologs throughout the Rickettsiales. In both
433 *Anaplasma* and some *Rickettsia,* the Tr1 gene is co-localized with genes that are either
434 differentially expressed between the tick and vertebrate host or have been implicated in
435 survival in tick cells. This functional conservation of the *tr1* loci between these two
436 species makes it interesting to consider that Tr1 might have a role in transcriptional
437 remodeling that underpins the cycling between vertebrate and arthropod hosts in
438 diverse Rickettsiales. However, this leaves the question of what the role of Tr1 in
439 Rickettsiales that survive exclusively within arthropods, such as *Wolbachia,* is.
440 Furthermore, in many Rickettsiales, the *tr1* gene is co-localized with genes of no known
441 function. Whilst genomic synteny suggests that Tr1 fulfills similar roles in *Anaplasma,*
442 *Ehrlichia* and *Neoehrlichia,* further study is required to determine how Tr1 functions
443 across diverse Rickettsiales.

444 Altogether, our work identifies Tr1 as an essential master regulator that remodels
445 the *A. phagocytophilum* transcriptome to infect the tick vector. Further, we identified
446 multiple Tr1 regulated genes that contribute to tick cell infection. With most studies
447 focusing on how *A. phagocytophilum* infects mammals, this work provides insights into
448 an understudied aspect of pathogen biology, uncovering wide-reaching transcriptional
449 events that underpin adaption to the arthropod vector. Unable to be transmitted
450 vertically to host progeny, *A. phagocytophilum* must cycle between the host and tick
451 vector to be maintained in the environment. Tr1 and the genes under its control are an
452 essential part of this system. Uncovering how *A. phagocytophilum* and other Rickettsial
453 bacteria achieve this is key to developing interventions to interrupt the transmission
454 cycle.

455

456 **MATERIALS AND METHODS**

457 *Bacterial and eukaryotic cell culture*

458 *Escherichia coli* used for plasmid construction, plasmid amplification, and protein
459 expression was cultured with solid and liquid lysogeny broth (LB) medium with the
460 addition of kanamycin or zeocin 25 μg ml^{-1} antibiotics as needed to select for plasmid
461 encoded resistance genes. For production of recombinant Tr1 proteins, *E. coli* strain
462 DE3 (New England Biolabs) was used with LB or Terrific Broth (TB) supplemented with
463 100 μg ml^{-1} kanamycin.

464 HL60 human promyelocytic cells (ATCC; CCL-240) were maintained in Roswell
465 Park Memorial Institute (RPMI) 1640 medium with 10% FBS and 1× Glutamax. HL60
466 cultures were kept at 37°C with 5% CO_2 in a humidified incubator. HL60 cell cultures
467 were limited to < 20 passages to prevent phenotypic drift and kept between 5×10^4 and
468 1×10^6 cell/ml to prevent differentiation.

469 Wild-type *A. phagocytophilum* strain HGE1 and HGE1 derived Himar1
470 transposon mutants were grown in HL60 cells as previously described[34]. All Himar1
471 mutant strains were obtained from the previously established *A. phagocytophilum*
472 mutant collection [34]. Status of *A. phagocytophilum* infections in HL60 cells was
473 monitored by Diff-Quick Romanowsky–Giemsa staining. To generate host-cell-free *A.*
474 *phagocytophilum,* peak infected HL60 cultures (>95% infected cells) were gently
475 sonicated to disrupt HL60 cell membranes and liberated bacteria were separated from

476 host cell debris by differential centrifugation. *Anaplasma* numbers were estimated as
477 previously described [65,66].

478 *I. scapularis* (Say) embryonic tick cells (ISE6) were maintained in L15C-300
479 medium with 10% FBS (Sigma; F0926), 10% tryptone phosphate broth (TPB; BD;
480 B260300) and 0.1% lipoprotein cholesterol concentrate (MP Biomedicals;
481 219147680)[67], in sealed tissue culture treated flasks, and incubated at 34°C [68]. Infected
482 ISE6 cell cultures were additionally supplemented with 0.25% $NaHCO_3$ and 25 mM
483 HEPES buffer (Sigma), cultured in vented flasks, and kept at 34°C in a humidified
484 chamber with 4% CO_2.

485 *A. phagocytophilum mutant growth curves*

486 *A. phagocytophilum* burden in HL60 and ISE6 cells was evaluated as previously
487 described[36,37]. Briefly, HL60 cells were seeded to 24 well plates at $5x10^4$ cells/well and
488 infected at an MOI of 1. Three to four replicate wells were harvested at the time of
489 inoculation and tested time post inoculation. ISE6 cells were seeded to 24 well plates at
490 $3x10^5$ cells/well. After ISE6 cells adhered to the plate for at least 6 hours, they were
491 inoculated at an MOI of 50 with host-cell-free *A. phagocytophilum* purified from HL60
492 cultures. Bacteria were allowed to infect the tick cells for 4 hours, after which the media
493 was exchanged three times to remove excess extracellular bacteria. Four replicate wells
494 were collected post media exchanged and subsequent time points. Pelleted samples
495 were frozen and later processed for RNA or gDNA using Zymogen Quick-RNA Micro-
496 prep or QIAGEN DNeasy blood and tissue kits. Ratios of bacterial and host cell gDNA
497 copies was measured by qPCR as previously described[10,36,37] Levels of live *A.*
498 *phagocytophilum* Himar1 mutant bacteria in each infected ISE6 or HL60 cell sample

499 was measured by qRT-PCR targeting *A. phagocytophilum* 16S rRNA and *I. scapularis*
500 or human *β-actin* transcripts (**Table S3**) and compared relative to the intergenic Himar1
501 control strain by ΔΔCt[10,65,69].

502 *Measuring A. phagocytophilum gene transcription*

503 HL60 and ISE6 cells in 24 well plates were infected with wild-type *A.*
504 *phagocytophilum* HGE1 as described above. At 24 hours post infections four wells were
505 collected and processed for RNA Zymo Quick-RNA micro-prep kit® (ZymoResearch)
506 according to product protocols for tissue culture samples. cDNA was generated with
507 Verso cDNA Synthesis Kit (ThermoFisher). Transcripts of *A. phagocytophilum* genes of
508 interest were measured by qPCR using gene specific primers (**Table S3**) and SYBR
509 green iTaq universal Supermix (Bio-Rad; 1725125) according to Bio-Rad specified cycle
510 conditions. Genes of interest were measured relative to housekeeping gene *rpoB* and
511 compared between human and tick cell infections by ΔΔCt.

512 *Animal infection*

513 All mice were purchased as six weeks of age from The Jackson Laboratory.
514 Gender balanced groups of ten C57BL/6 mice were infected as previously described[10]
515 with either the *tr1*::Himar1 or the intergenic Himar1 mutant *A. phagocytophilum*. The
516 intergenic Himar1 mutant was previously shown to be phenotypically equivalent to wild-
517 type[10,36,37]. Blood was collected seven days post inoculation and *A. phagocytophilum*
518 burden in the blood was measured by qPCR as in prior studies (16S rRNA / mouse *β-*
519 *actin*[10,65,69]) (Table S3). Larval *I. scapularis* ticks purchased from Oklahoma State
520 University (Stillwater, OK, USA) were kept at 23°C with >95% humidity and a 16/8-h

521 light/dark photoperiods. Mice were selected from the intergenic Himar1 and *tr1*::Himar1
522 strain infected mice with equivalent *Anaplasma* burden. Each mouse was infested with
523 200 naïve larval *I. scapularis* and allowed to feed to repletion. Detached replete ticks
524 were individually processed and assayed for *A. phagocytophilum* burden by qRT-PCR
525 measuring 16S rRNA versus *I. scapularis β-actin* transcripts (Table S3), compared by
526 absolute quantification [10,65,69]. All animal use protocols were approved by the
527 Washington State University Institutional Animal Care and Use Committee (ASAF
528 #6630) and animal housing facilities at Washington State University in Pullman, WA
529 maintains AAALAC-accreditation.

530 *RNA sequencing*

531 ISE6 cells were seeded to 12 well plates at $5x10^5$ cells/well and allowed to
532 adhere for 6 hours. The intergenic transposon control and Himar1::*tr1* mutant *A.*
533 *phagocytophilum* strains were prepared as host-cell-free bacteria from >95% infected
534 HL60 cultures immediately before ISE6 infection. Adhered ISE6 cells were infected at
535 an MOI of 100. Twenty-four hours post infection media was exchanged three times to
536 remove excess extracellular bacteria, and four replicate wells were collected and RNA
537 isolated with Zymogen Quick-RNA Mini-prep kits. RNA was submitted to the WSU
538 Genomics Core for total RNA-seq analysis. Sequencing libraries were prepared using
539 Tru-seq Stranded Total RNA kit, with Ribo-Zero Plus and libraries, sequenced using a
540 HiSeq 2500, and results exported as FASTQ files as previously discribed[70]. RNA-seq
541 data were aligned with the *A. phagocytophilum* HGE1 genome (GCF_000478425.1)[71].
542 Transcript quantification and differential gene expression were analyzed by HTSeq and
543 DESeq2[72].

544 *Overexpression and purification of recombinant Tr1 protein*

545 The *tr1* open reading frame, codon optimized for expression in *E. coli,* was
546 synthesized and cloned by Twist Bioscience into a modified pET28a expression vector.
547 The resultant plasmid encoded the Tr1 protein fused at its N-terminus to a hexa-histidine
548 tagged maltose binding protein (MBP) with a tobacco etch virus (TEV) protease
549 recognition site between Tr1 and MBP. The plasmid was transformed into *E. coli* DE3 for
550 overexpression of the recombinant Tr1 protein. Briefly, overnight cultures, grown at 37 °C
551 in selective LB media, were used to inoculate flasks of TB. Over-expression of MBP-Tr1
552 was induced by the addition of 0.5 mM IPTG once cultures had reached an OD_{600} of 0.5-
553 0.6 and then grown for an additional 18 hours at 25 °C. Cells were harvested by
554 centrifugation and kept at -20 °C for short-term storage.

555 To purify MBP-Tr1 protein, cell pellets were resuspended in Buffer A (20 mM
556 imidazole, pH 8.0; 20 mM Tris-HCl, pH 8.0; 400 mM NaCl; 2 mM beta-mercaptoethanol)
557 and then lysed by sonication. Lysates were clarified by centrifugation and then loaded
558 onto a HisTrap HP purification column (Cytiva). The column was washed extensively with
559 Buffer A prior to elution of the His-tagged MBP-Tr1 with stepwise (10, 20, 100%) washes
560 with Buffer B (as Buffer A but with 400 mM imidazole). Fractions containing MBP-Tr1 were
561 pooled, concentrated by centrifugal-filtration, then further purified by gel-filtration on a
562 Superdex 200 column (Cytiva) equilibrated in SEC Buffer (20 mM Tris-HCl pH 8.0; 250
563 mM NaCl; 2 mM beta-mercaptoethanol). Fractions containing MBP-Tr1, which eluted as
564 a single peak from the gel-filtration column, were supplemented with his-tagged TEV
565 protease and incubated at 4 °C for 16 hours to cleave the His-tagged MBP protein from
566 Tr1. The resulting protein preparation was passed over a HisTrap HP column which was

567 then washed step (5, 10, 100%) with Buffer B. Tr1 protein typically passed through the
568 HisTrap HP column or eluted in 5% Buffer B washes and were thus separated from the
569 His-tagged TEV and MBP proteins, which eluted in 100% Buffer B. This purification
570 strategy yielded highly pure Tr1 protein lacking any tags.

571 *Gel filtration*

572 Purified tagless Tr1 protein, green fluorescent protein (GFP) and maltose binding
573 protein proteins were analyzed by gel filtration using a HiPrep Sephadex 75 16/600
574 column (Cytiva), using Buffer B. GFP and MBP were expressed using pET vectors
575 (modified pET41c and pET28a, respectively) in *E. coli* DE3 and purified by nickel affinity
576 chromatography or amylose affinity chromatography using standard methods.

577 *Electrophoretic-Mobility-Shift-Assays*

578 Promoter proximal DNA sequences for use in Electrophoretic-Mobility-Shift-
579 Assays (EMSAs) were amplified by PCR. Primers for this purpose were designed to
580 yield DNA fragments comprising the ATG start codon of a selected gene plus 250 bp of
581 upstream DNA sequence. In each case, the forwards primer was appended with an M13
582 sequence upstream of the primer sequence (5´- GAG CGG ATA ACA ATT TCA CAC AGG).
583 The resulting DNA fragments were separated by agarose gel electrophoresis and
584 fragments of the anticipated size were excised and purified (QIAquick Gel Extraction
585 Kit). These purified fragments were then used as the templates for a secondary PCR
586 using an M13 sequence primer labelled with FAM at the 5´ end in conjunction with the
587 fragment-specific reverse primer used in the primary PCR. The resulting FAM-labelled

588 PCR products were resolved by agarose gel electrophoresis and purified as above.
589 Sequences of primers used are included in **table S3**.

590 EMSA assays were performed using a modified protocol described elsewhere[73].
591 Briefly, for each DNA fragment an EMSA mixture comprising (100 ng FAM-labelled DNA
592 fragment;200 ng of herring sperm DNA; 5% glycerol; 10 mM Trs-HCl, pH 8.0; 25 mM
593 KCl) was prepared and 8 µl of this dispensed into a series of tubes. These were then
594 supplemented with 2 µl of purified Tr1 protein at varying concentrations, or with buffer
595 alone, and then mixed by gentle pipetting. Tr1 protein was used at final concentrations
596 of 0.25, 0.5, 1, 2, and 4 µM for all EMSAs presented here. Resulting mixtures were
597 incubated at 25 °C for 30 minutes before being loaded onto 7.5 % polyacrylamide gels
598 buffered with 0.25x TBE and 5% glycerol. Gels were run for 60 minutes at 100 V and
599 then fluorescent images (green channel) were collected on a ChemiDoc MP Imaging
600 System (Bio-Rad).

601 *Bioinformatics*

602 Protein structures were analyzed and visualized in Coot[74] and ChimeraX[75]. Modelling
603 and the generation of predicted structures were done using ColabFold and AlphaFold
604 Server with searches for homologues in the PDB being achieved with the Dali
605 Server[45,46].

606

607

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875

876

877 **FIGURE LEGENDS**

878 Figure 1. *tr1* transcription is specific to tick cell infection and necessary for *A. phagocytophilum*
879 survival in tick cells. (**a**) *tr1* transcription normalized to housekeeping gene *rpoB* during *A.*
880 *phagocytophilum* culture in ISE6 tick cells and human HL60 cells. Bar indicate mean of four
881 replicate infections each measured in two technical replicates show as points. (**b and c**) Growth
882 of *A. phagocytophilum tr1*::Himar1 or control strain in cell culture infections of (**b**) human HL60
883 cells and (**c**) tick ISE6 cells. *A. phagocytophilum* burden measured by bacterial gDNA relative to
884 eukaryotic host gDNA via qPCR. Data displayed as mean with ±SD of three biological replicates
885 with two technical replicates each. Data are representative of three experimental replicates.
886 *$P < 0.05$ (Mann-Whitney *t*-test).

887 Figure 2. *tr1* is despicable during murine infection but essential for acquisition by ticks.

888 (**a**) Mouse blood *Anaplasma* burden 7 days post intraperitoneal inoculation with 1×10^8 *A.*
889 *phagocytophilum tr1*::Himar1 or control::Himar1 strains. Blood processed for gDNA and
890 bacterial burden was measured by qPCR of *A. phagocytophilum* 16S rDNA versus mouse actin
891 by ΔΔCt. Each strain was tested in twenty mice (½ male, ½ female). Each sample was tested in
892 technical duplicate reaction. (**b**) Two burden-matched infected mouse pairs were used for
893 *Ixodes scapularis* larvae infestation. Ticks were allowed to feed to repletion and detach. Whole
894 replete *I. scapularis* larvae were processed for RNA. *A. phagocytophilum* bacterial loads were
895 measured via qRT-PCR of *A. phagocytophilum* 16S rRNA levels versus mouse actin transcripts.
896 Data includes ticks from two burden matched mouse pairs. From each mouse, 10–20 individual
897 ticks were collected as biological replicates, and each qRT-PCR was performed in duplicate.
898 *$P < 0.005$ (Welch's *t*-test).

899 Figure 3. Structural modelling of the Tr1 protein. **(a)** Schematic of the predicted Tr1 protein
900 domain architecture. Ordered domains are displayed as boxes and predicted disordered regions

901 as solid black lines. **(b)** Cartoon representation of a predictive Tr1 dimer protein model. For one
902 Tr1 chain the N-terminal DNA binding domain is colored plum, the C-terminal dimerization
903 domain is colored light green, and disordered flanking and linker regions are colored pale
904 yellow. The second Tr1 chain is colored grey. **(c)** Dimeric H-T-H domain of Tr1 (colored
905 magenta) superimposed onto the crystal structure of HipB bound to its DNA operator from
906 *Shewanella oneidensis*[76] (colored light blue; PDB: 4PU4). **(d)** Dimeric C-terminal domain of Tr1
907 (colored green) superimposed onto the crystal structure of EspR from *M. tuberculosis*[77] (colored
908 brown; PDB: 4NDW)

909 Figure 4. Tr1 forms multimers in solution. **(a)** Chromatograms of Tr1, MBP, and GFP separated
910 by gel filtration. The Tr1 protein lacks tryptophan residues and absorbs weakly at 280 nm so the
911 peaks are shown as normalized absorbance. **(b)** SDS-PAGE gel of fractions collected from
912 equivalent volumes in gel filtration experiments. The left-hand lane of each gel contains
913 molecular weight markers with the known weights labelled**.**

914 Figure 5. Tr1 binds promotors of neighboring *omp1X* and *omp1N.* (**a**) Diagram of *tr1* gene with
915 downstream neighboring outer membrane protein genes *omp1X*, *omp1N*, and the *msp2/p44*
916 expression site. (**b through e**) EMSA shifts with increasing rTr1 (0, 0.0625, 0.125, 0.25, 0.5, and
917 1μM) with DNA probes of (**b**) *tr1*, (**c**) *omp1X*, (**d**) *omp1N*, and (**e**) *msp2/p44* promotor
918 sequences. (**f and g**) Transcription of *omp1X* and *omp1R* from control or *tr1*::Himar1 *A.*
919 *phagocytophilum* mutant strains at 24 hours post infection in (**f**) human HL60 or (**g**) tick ISE6
920 cells. Transcripts measured by qRT-PCR and normalized to *rpoB* via ΔΔCt. Data displayed as
921 mean with ±SD of four replicate infections measured with two technical replicates each. *P <
922 0.05 (Mann-Whitney *t*-test). (**h**) *msp2/p44* transcripts totals across all gene variants sequenced
923 by RNAseq from *tr1*::Himar1 or control *A. phagocytophilum* infected ISE6 tick cells. Bars are
924 mean ±SD of four independent infections, each shown as points. n.s. > 0.05 (Mann-Whitney *t*-
925 test).

926 Table 1. Genes with ≥ 2 fold reduced expression in *tr1*::Himar1 mutant during tick cell infection.

927 Figure 6. Tr1 binds the promotors of tick-specific *A. phagocytophilum* genes. EMSA shifts with
928 increasing rTr1 (0, 0.0625, 0.125, 0.25, 0.5, and 1μM) tested against promotor sequences of (**a**)
929 *ateA*, (**b**) *HGE1_06057*, (**c**) *HGE1_01872*, (**d**) *HGE1_03162*, (**e**) *HGE1_03907*, and (**f**) *mps4*.

930 Figure 7. Tr1 impacted genes are necessary for survival during tick cell infection. Survival of
931 indicated Himar1 transposon mutant *A. phagocytophilum* strain relative to intergenic
932 control::Himar1 strain in (**a**) human HL60 (48 hpi) and tick ISE6 cells (8 dpi). Bacterial burden
933 measured by qRT-PCR of *A. phagocytophilum 16s* vs Ixodes actin transcripts. Data displayed
934 as mean ±SD of four replicate infections measured in two technical replicates each. Graph
935 representative of three replicate experiments. * < 0.005 (Mann-Whitney *t*-test).

936 Figure 8. Tr1 is required for tick-specific remodeling of the T4SS pilus. (**a**) Diagram of the *virB2*
937 paralog genes, the location of the *virB2_5*::Himar1 insertion mutation (red), and potential
938 promotor regions subject to EMSA (green). (**b**) Transcription of the *virB2* genes during wild-type
939 *A. phagocytophilum* infection of mammalian HL60 and tick ISE6 cells. Data shown as mean
940 ±SD of four biological replicates. (**c,d,e**) EMSA shifts with increasing rTr1 (0, 0.0625, 0.125,
941 0.25, 0.5, and 1μM) with putative promotor sequences upstream of (**c**) *virB2_1*, (**d**) *virB2_6*,
942 and (**e**) *virB2_8*. (**f and g**) Growth of *A. phagocytophilum virB2_5*::Himar1 or control strain in cell
943 culture infections of (**f**) human HL60 cells and (**g**) tick ISE6 cells. *A. phagocytophilum* burden
944 measured by bacterial gDNA relative to eukaryotic host gDNA via qPCR. Data displayed as
945 mean with ±SD of three biological replicates with two technical replicates each. Data are
946 representative of three experimental replicates. *$P < 0.05$ (Mann-Whitney *t*-test).

947

948

949

950 Figure S1**.** Predicted Local Distance Difference Test (pLDDT) and Multiple Sequence Alignment
951 (MSA) plots from ColabFold of Tr1. (a) pLDDT plot shows that local confidence in the Tr1 model
952 is high in the N- and C-terminal domains, supporting the proposal that Tr1 is comprised of two
953 ordered domains. (b) MSA plot showing that the Tr1 N- and C-terminal domains are
954 evolutionarily conserved.

955 Figure S2. Early survival of *tr1*::Himar1 strain during tick cell infection. *A. phagocytophilum*
956 tr1::Himar1 or control strain in cell culture infections of tick ISE6 cells. *A. phagocytophilum*
957 burden measured by bacterial 16s relative to tick *actin* transcripts via qRT-PCR. Data displayed
958 as mean with ±SD of three biological replicates. Data are representative of three experimental
959 replicates. *P < 0.05 (Mann-Whitney t-test).

960

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967

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AUTHOR CONTRIBUTIONS

EricaRose Warwick: Methodology, Investigation, Visualization, Reviewing & Editing, R**achel Burt:** Methodology, Investigation, Visualization, **Jeffrey T. Badigian:** Methodology, Investigation, **Daniel Howell:** Methodology, Investigation, **Kyle T. Swallow:** Methodology, Investigation, **Chloe Leach:** Methodology, **Azeza M. Falghoush:** Methodology, **Dana K. Shaw:** Methodology, Investigation, Review & Editing. **Ian T. Cadby:** Conceptualization, Supervision, Methodology, Investigation, Writing, Visualization, Funding acquisition. **Jason M. Park:** Conceptualization, Supervision, Methodology, Investigation, Writing, Visualization, Funding acquisition.

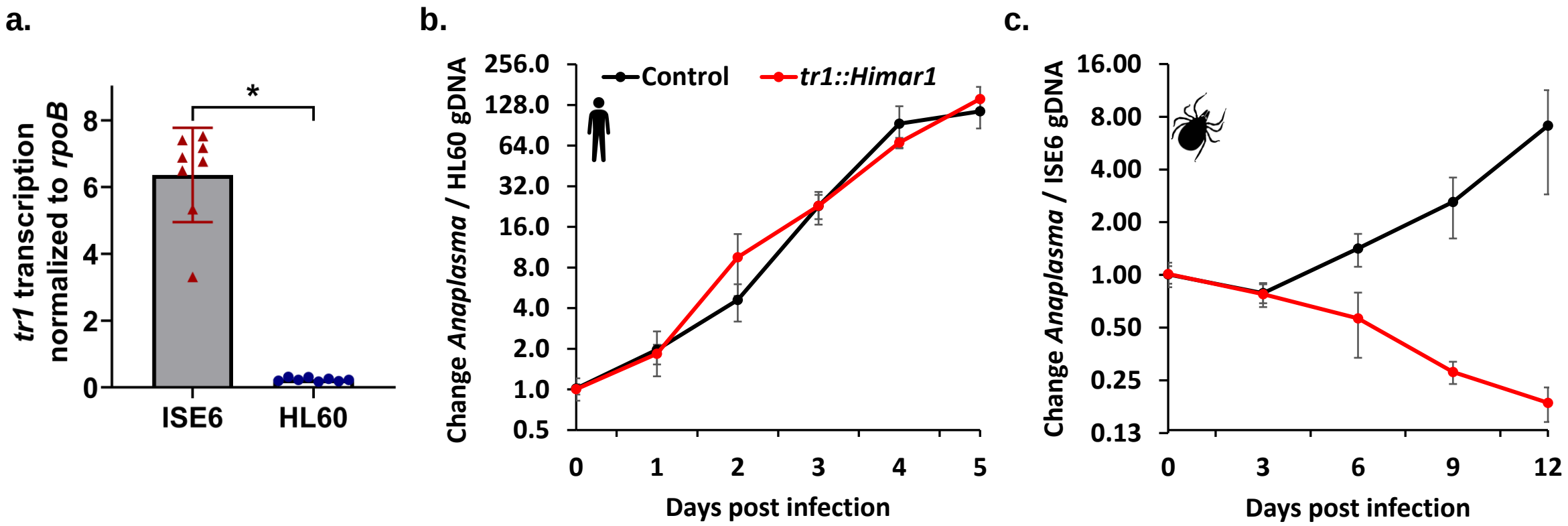
a.
*
tr1 transcription normalized to rpoB
0
2
4
6
8
ISE6
HL60
b.
Control
tr1::Himar1
Change Anaplasma / HL60 gDNA
256.0
128.0
64.0
32.0
16.0
8.0
4.0
2.0
1.0
0.5
0
1
2
3
4
5
Days post infection
c.
Change Anaplasma / ISE6 gDNA
16.00
8.00
4.00
2.00
1.00
0.50
0.25
0.13
0
3
6
9
12
Days post infection

Fig 1

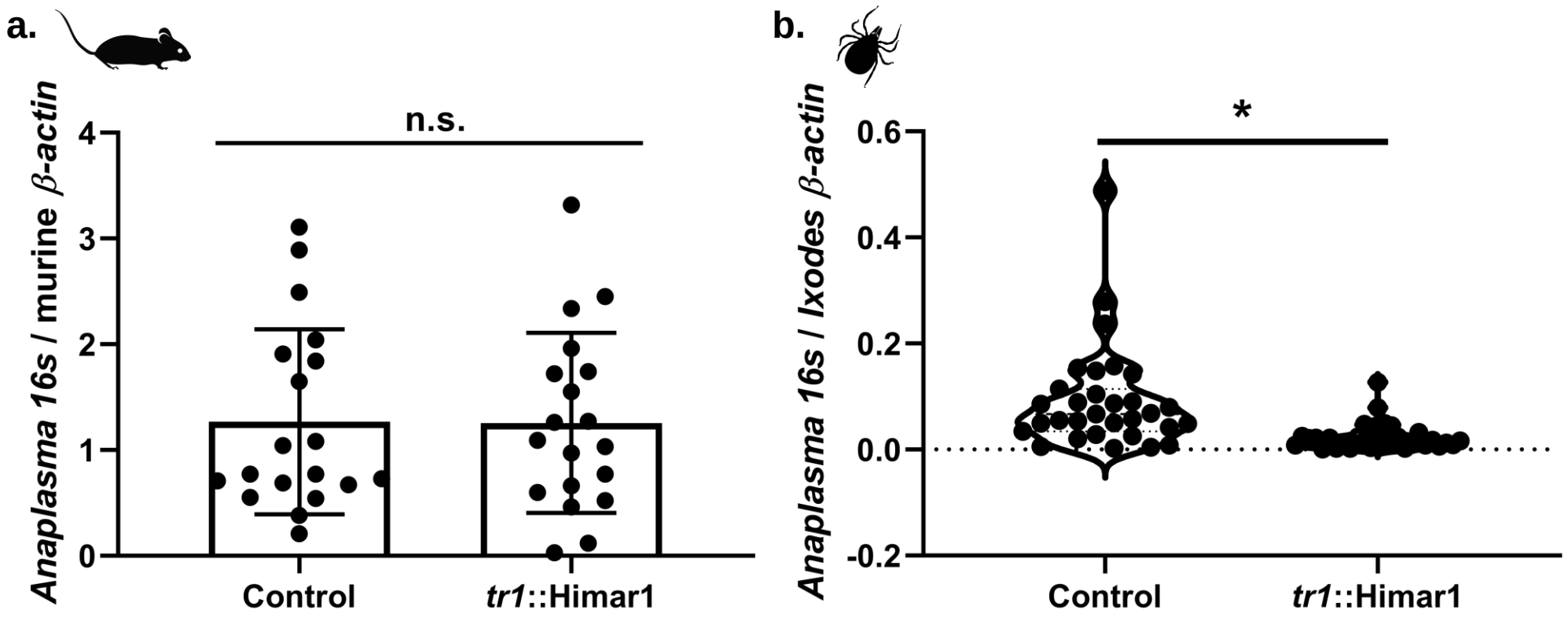
a.
n.s.
Anaplasma 16s / murine β-actin
4
3
2
1
0
Control
tr1::Himar1
b.
*
Anaplasma 16s / Ixodes β-actin
0.6
0.4
0.2
0.0
-0.2
Control
tr1::Himar1

Fig 2

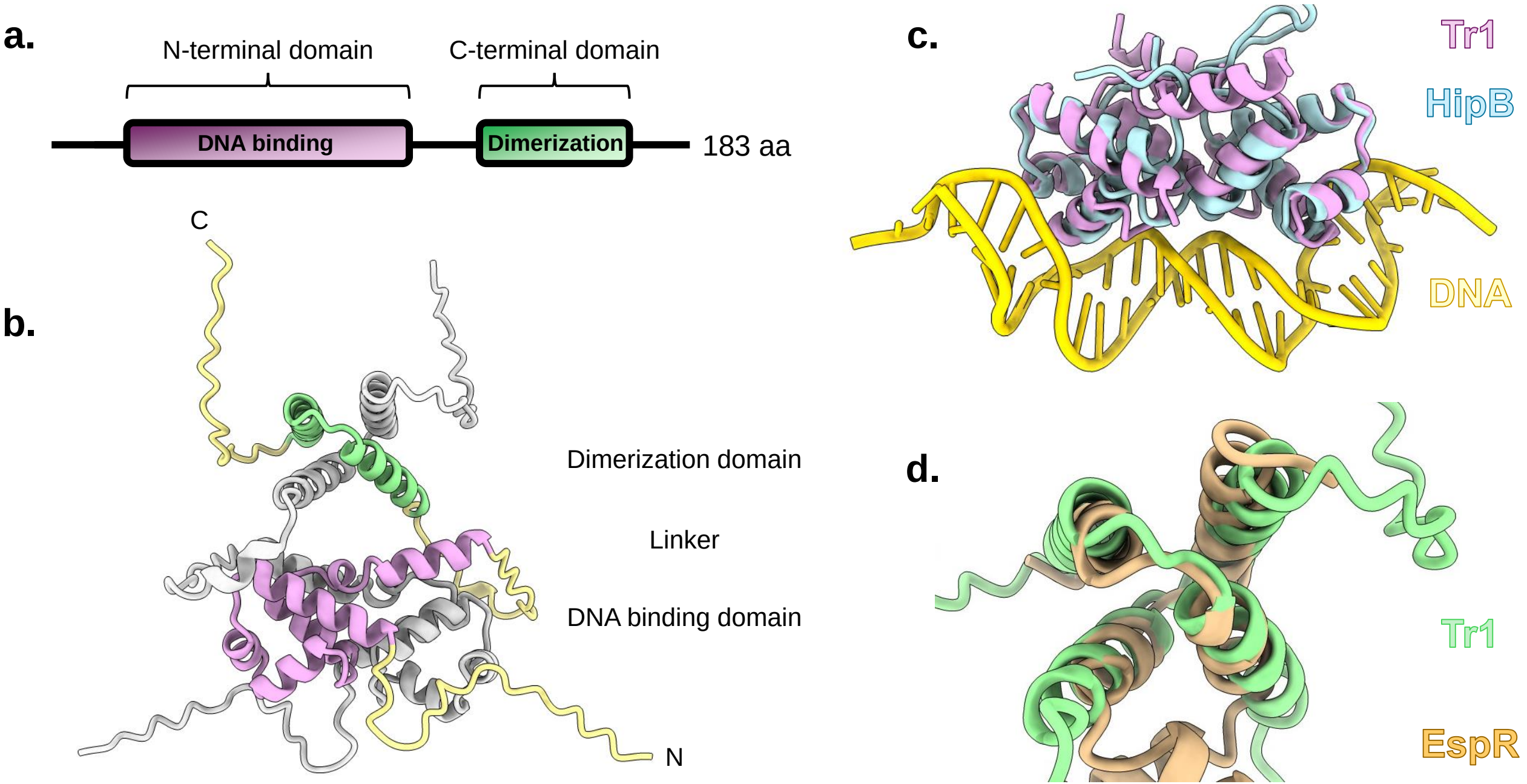
a.
N-terminal domain
C-terminal domain
DNA binding
Dimerization
183 aa
b.
C
Dimerization domain
Linker
DNA binding domain
N
c.
Tr1
HipB
DNA
d.
Tr1
EspR

Fig 3

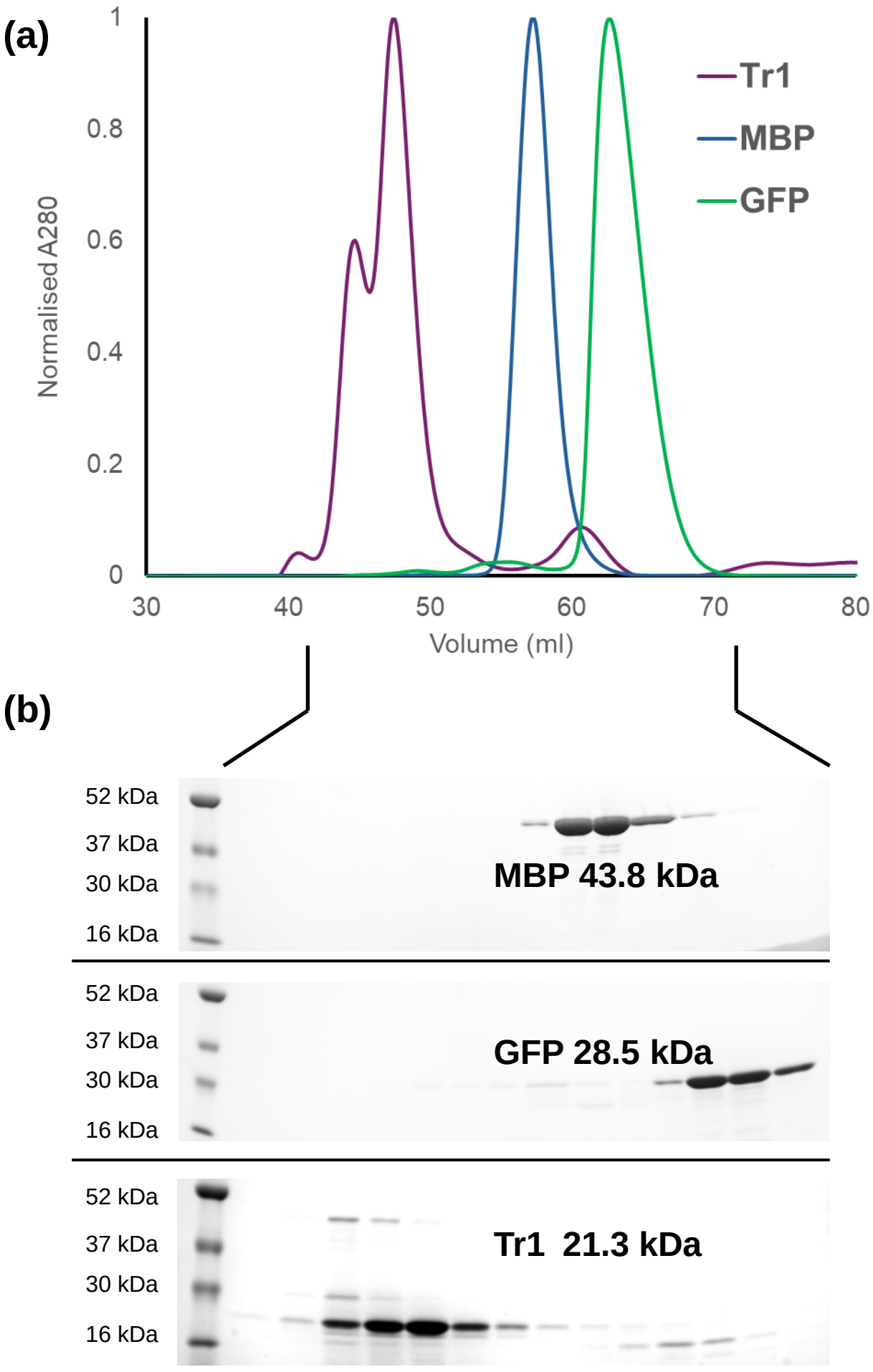
(a)
Normalised A280
1
0.8
0.6
0.4
0.2
0
30
40
50
60
70
80
Volume (ml)
Tr1
MBP
GFP
(b)
52 kDa
37 kDa
30 kDa
16 kDa
MBP 43.8 kDa
52 kDa
37 kDa
30 kDa
16 kDa
GFP 28.5 kDa
52 kDa
37 kDa
30 kDa
16 kDa
Tr1 21.3 kDa

Fig 4

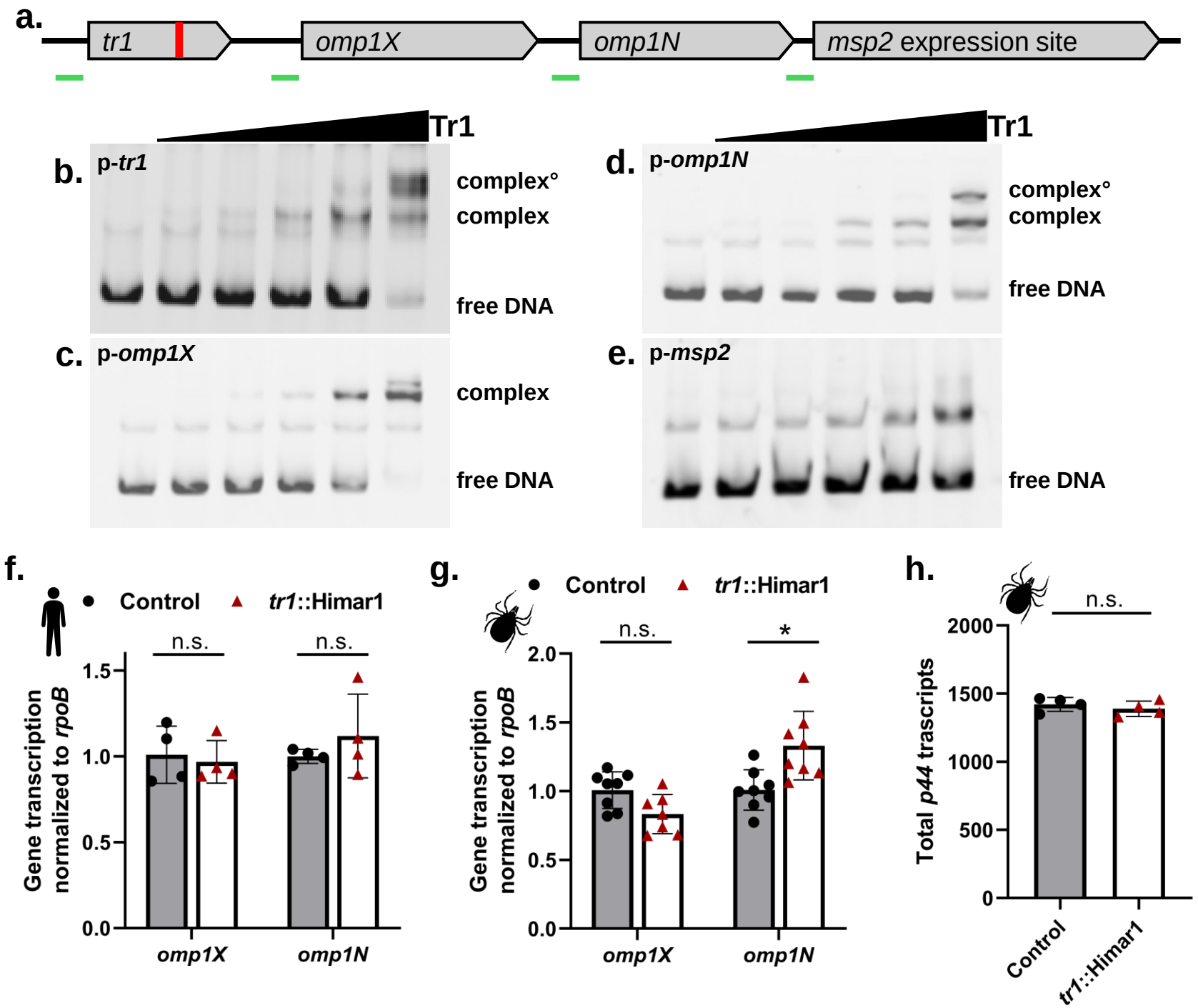
a.
tr1
omp1X
omp1N
msp2 expression site
Tr1
b.
p-tr1
complex°
complex
free DNA
c.
p-omp1X
complex
free DNA
Tr1
d.
p-omp1N
complex°
complex
free DNA
e.
p-msp2
free DNA
f.
Control
tr1::Himar1
n.s.
n.s.
Gene transcription normalized to rpoB
1.5
1.0
0.5
0.0
omp1X
omp1N
g.
Control
tr1::Himar1
n.s.
*
Gene transcription normalized to rpoB
2.0
1.5
1.0
0.5
0.0
omp1X
omp1N
h.
n.s.
Total p44 trascripts
2000
1500
1000
500
0
Control
tr1::Himar1

Fig 5

gene or locus_tag	Note	Base Mean	(-) Fold Change	p-adj	Tn mutant
HGE1_03907	Putative T4SS effector	11	8.2	0.00	
HGE1_02492	T4SS Effector AteA	25	7.9	0.00	Y
tr1	HTH DNA binding	121	5.7	0.00	Y
HGE1_04267	Unknown	93	4.7	0.00	
HGE1_01872	Membrane porin	15	3.9	0.00	Y
msp4	major surface protein	31	3.9	0.00	
HGE1_00340	rRNA methyltransferase	9	3.5	0.01	
HGE1_00460	Unknown	15	2.7	0.01	
HGE1_04767	*mrp* / NBP35 family	12	2.6	0.02	
HGE1_03162	Unknown	49	2.6	0.00	Y
HGE1_04257	*dnaA* / *hda* regulator	16	2.3	0.03	
HGE1_01837	ABC transporter	19	2.1	0.01	
nrdR	Transcription regulator	14	2.1	0.04	
HGE1_06057	Unknown	431	2.0	0.00	Y

Table 1

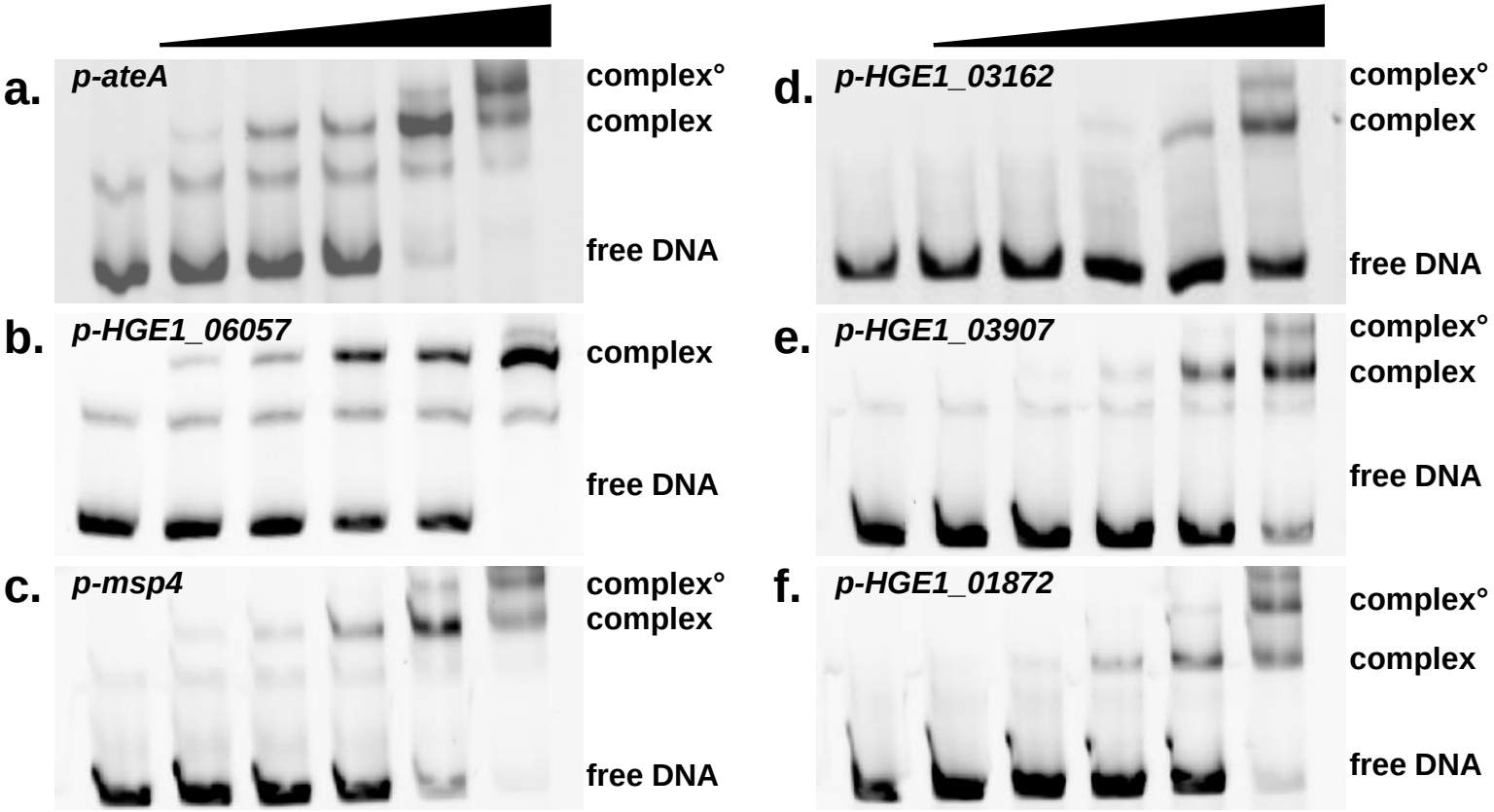
a.
p-ateA
complex°
complex
free DNA
b.
p-HGE1_06057
complex
free DNA
c.
p-msp4
complex°
complex
free DNA
d.
p-HGE1_03162
complex°
complex
free DNA
e.
p-HGE1_03907
complex°
complex
free DNA
f.
p-HGE1_01872
complex°
complex
free DNA

Fig 6

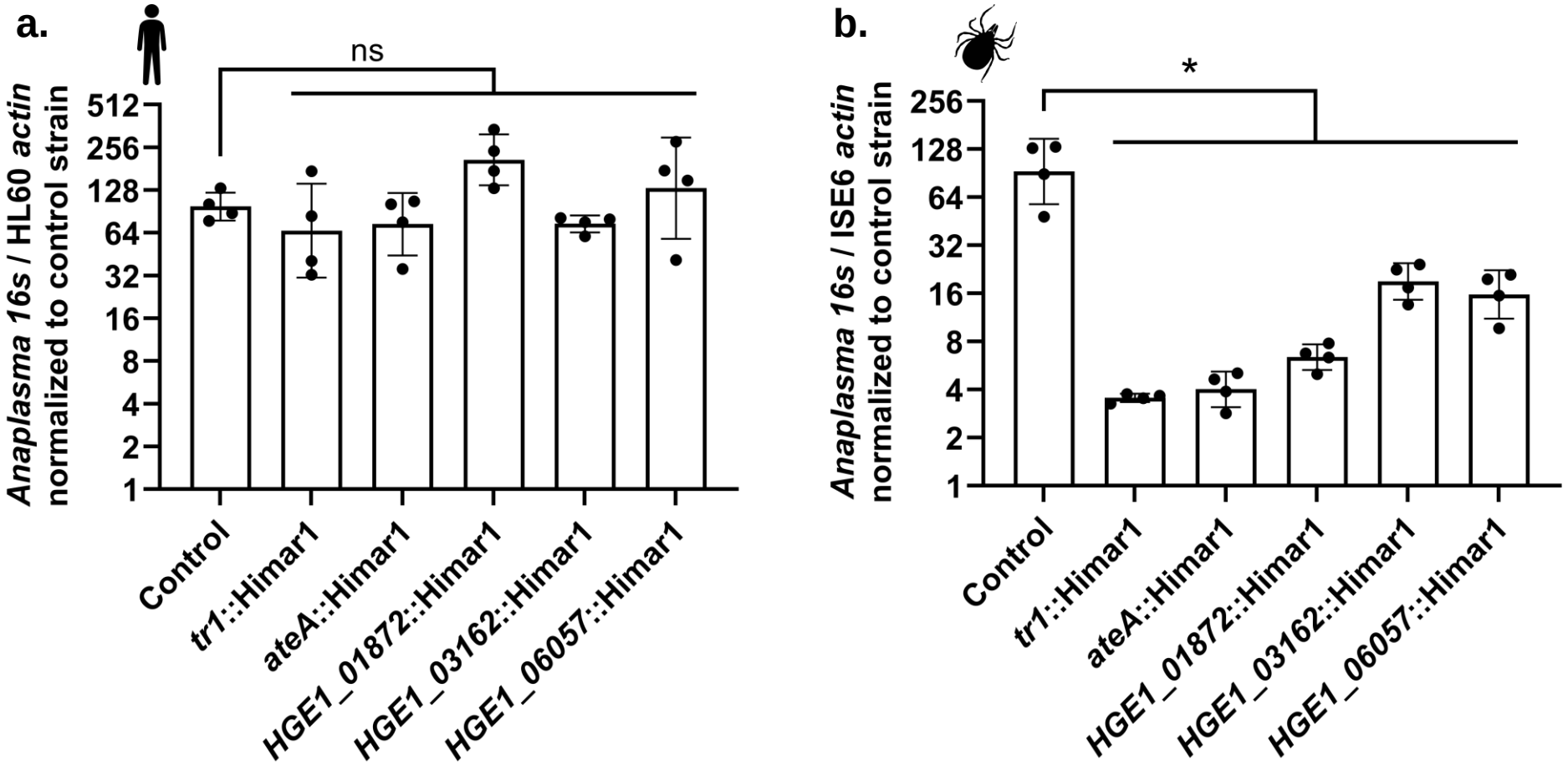
a.
ns
Anaplasma 16s / HL60 actin
normalized to control strain
512
256
128
64
32
16
8
4
2
1
Control
tr1::Himar1
ateA::Himar1
HGE1_01872::Himar1
HGE1_03162::Himar1
HGE1_06057::Himar1
b.
*
Anaplasma 16s / ISE6 actin
normalized to control strain
256
128
64
32
16
8
4
2
1
Control
tr1::Himar1
ateA::Himar1
HGE1_01872::Himar1
HGE1_03162::Himar1
HGE1_06057::Himar1

Fig 7

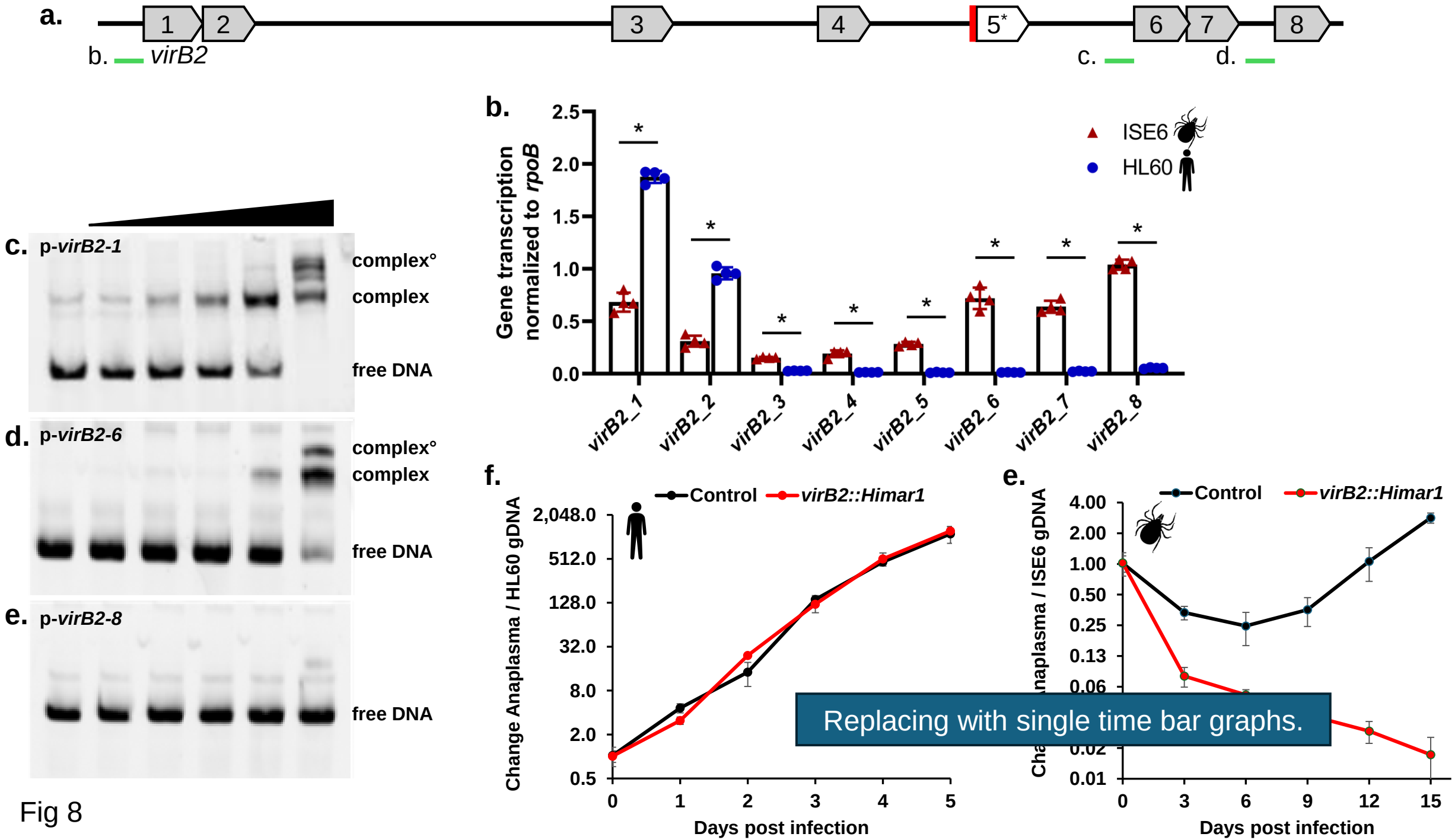
a.
1
2
3
4
5*
6
7
8
b. virB2
c.
d.
b.
Gene transcription normalized to rpoB
ISE6
HL60
virB2_1
virB2_2
virB2_3
virB2_4
virB2_5
virB2_6
virB2_7
virB2_8
c. p-virB2-1
complex°
complex
free DNA
d. p-virB2-6
complex°
complex
free DNA
e. p-virB2-8
free DNA
f.
Control
virB2::Himar1
Change Anaplasma / HL60 gDNA
Days post infection
e.
Control
virB2::Himar1
Anaplasma / ISE6 gDNA
Days post infection
Replacing with single time bar graphs.

Fig 8

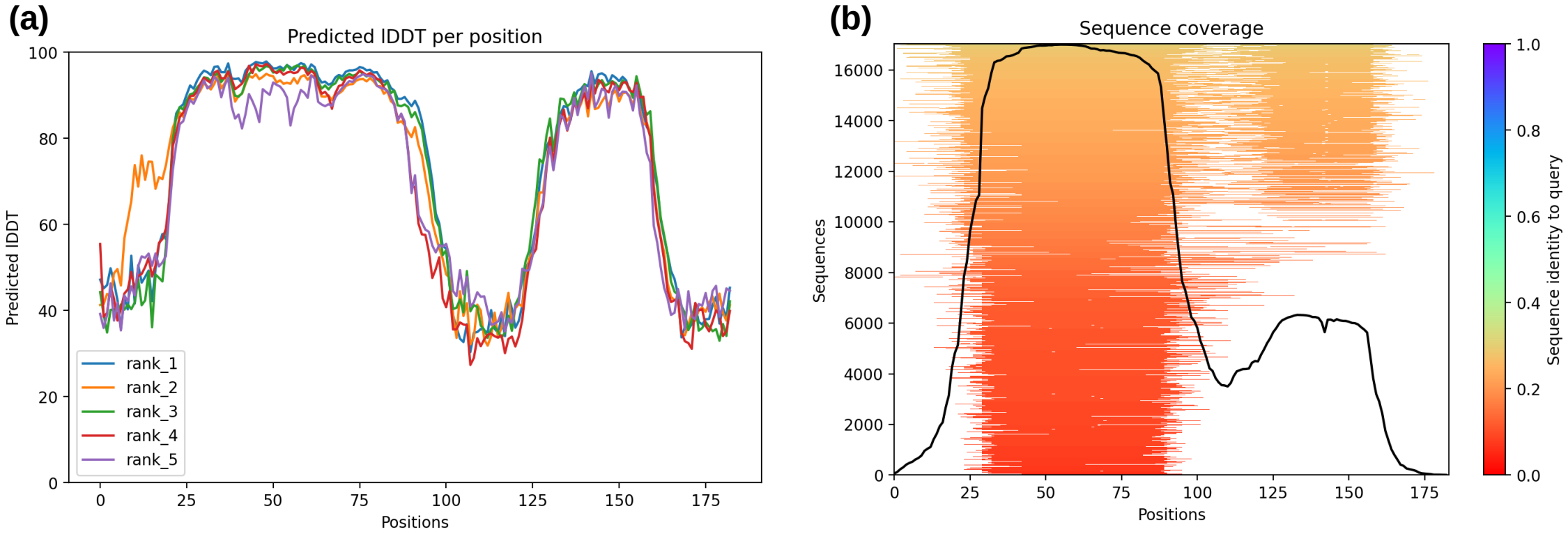
(a)
Predicted lDDT per position
Predicted lDDT
Positions
rank_1
rank_2
rank_3
rank_4
rank_5
(b)
Sequence coverage
Sequences
Positions
Sequence identity to query

Fig S1.

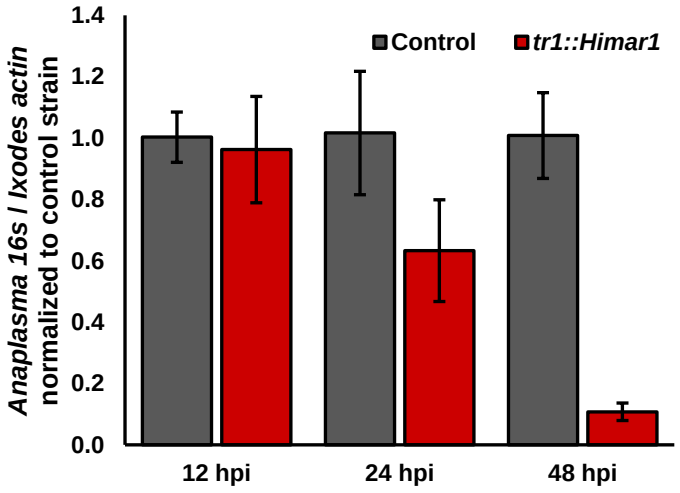
Anaplasma 16s / Ixodes actin
normalized to control strain
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
tr1::Himar1
12 hpi
24 hpi
48 hpi

Fig. S2