The ankyrin repeat protein RARP-1 is a periplasmic factor that supports *Rickettsia parkeri* growth and host cell invasion

Rickettsia RARP-1 supports growth and host invasion

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Abstract

Rickettsia spp. are obligate intracellular bacterial pathogens that have evolved a variety of strategies to exploit their host cell niche. However, the bacterial factors that contribute to this intracellular lifestyle are poorly understood. Here, we show that the conserved ankyrin repeat protein RARP-1 supports *Rickettsia parkeri* infection. Specifically, RARP-

1 promotes efficient host cell entry and growth within the host cytoplasm, but it is not necessary for cell-to-cell spread or evasion of host autophagy. We further demonstrate that RARP-1 is not secreted into the host cytoplasm by *R. parkeri*. Instead, RARP-1 resides in the periplasm, and we identify several binding partners that are predicted to work in concert with RARP-1 during infection. Altogether, our data reveal that RARP-1 plays a critical role in the rickettsial life cycle.

Importance

Rickettsia spp. are obligate intracellular bacterial pathogens that pose a growing threat to human health. Nevertheless, their strict reliance on a host cell niche has hindered investigation of the molecular mechanisms driving rickettsial infection. This study yields much needed insight into the *Rickettsia* ankyrin repeat protein RARP-1, which is conserved across the genus but has not yet been functionally characterized. Earlier work had suggested that RARP-1 is secreted into the host cytoplasm. However, the results from this work demonstrate that *R. parkeri* RARP-1 resides in the periplasm and is important both for invasion of host cells and for growth in the host cell cytoplasm. These results reveal RARP-1 as a novel regulator of the rickettsial life cycle.

Introduction

Intracellular bacterial pathogens face considerable challenges and opportunities when invading and occupying their host cell niche. The host cell membrane physically

occludes entry and the endolysosomal pathway imperils invading microbes. Moreover, host cell defenses like autophagy create a hostile environment for internalized bacteria. If a bacterium successfully navigates these obstacles, however, it can conceal itself from humoral immunity, commandeer host metabolites, and exploit host cell biology to support infection. Not surprisingly, the host cell niche has provided fertile ground for the evolution of diverse lifestyles across many well-studied bacterial pathogens such as *Shigella*, *Listeria*, *Salmonella*, and *Legionella* (1, 2). The prospect of uncovering unique infection strategies invites a thorough investigation of these adaptations in more enigmatic pathogens.

Members of the genus *Rickettsia* include emerging global health threats that can cause mild to severe diseases such as typhus and Rocky Mountain spotted fever (3). These Gram-negative bacterial pathogens are transmitted from arthropod vectors to vertebrate hosts where they primarily target the vascular endothelium. As obligate intracellular pathogens, *Rickettsia* spp. define the extreme end of adaptation to intracellular life and are completely dependent on their hosts for survival (4). Consequently, they have evolved a complex life cycle to invade, grow, and disseminate across host tissues.

As the first step of their life cycle, *Rickettsia* spp. adhere to and invade host cells by inducing phagocytosis (5–7). Once inside, these bacteria rapidly escape the phagocytic vacuole to access the host cytoplasm (8, 9). To establish a hospitable niche for proliferation, *Rickettsia* spp. scavenge host nutrients, modulate apoptosis, and thwart antimicrobial autophagy (10–13). Successful colonization of the host cytoplasm allows

Rickettsia spp. to spread to neighboring cells. Members of the spotted fever group (SFG) *Rickettsia* hijack the host actin cytoskeleton, forming tails that propel the bacteria around the cytoplasm, and then protrude through cell-cell junctions to repeat the infection cycle (14, 15).

Recent work using the model SFG member *Rickettsia parkeri* has highlighted a short list of surface-exposed proteins and secreted effectors that manipulate host cell processes during infection (4). For example, the surface protein Sca2 nucleates actin at the bacterial pole and promotes motility by mimicking host formins (14). Sca4, a secreted effector, interacts with host vinculin to reduce intercellular tension and facilitate protrusion engulfment (15). Additionally, methylation of outer membrane proteins like OmpB protects *R. parkeri* from ubiquitylation and autophagy (13, 16). Despite these advances, our knowledge of the factors that govern the multi-step rickettsial life cycle is still limited. Indeed, *Rickettsia* spp. genomes are replete with hypothetical proteins that are conserved even among less virulent members of the genus (17), but a paucity of genetic tools has stunted investigation of these proteins. Such factors could support infection directly, by targeting host processes, or indirectly, by controlling the bacterial mediators at the host-pathogen interface. Thus, it is critical to reveal how these uncharacterized proteins contribute to infection.

In a recent transposon mutagenesis screen in *R. parkeri* (18), we identified over 100 mutants that exhibited defects in infection. Although several hits from this screen have been functionally characterized (13–16), many play unknown roles during infection. One

such unexplored hit is the *Rickettsia* ankyrin repeat protein 1 (RARP-1), which is conserved across the genus and predicted to be secreted into the host cytoplasm (19). To better understand the factors that influence the rickettsial life cycle, we investigated the function of RARP-1 during *R. parkeri* infection. We demonstrated that RARP-1 promotes both efficient host cell invasion and growth in the host cytoplasm, but it is otherwise dispensable for cell-to-cell spread and avoidance of host autophagy. Although prior work indicated that RARP-1 is secreted into the host cytoplasm (19), we found instead that it localizes to the *R. parkeri* periplasm. Furthermore, we showed that RARP-1 interacts with a variety of factors that are predicted to support bacterial fitness. Our results suggest that RARP-1 is a *Rickettsia*-specific tool that promotes the obligate intracellular life cycle.

Results

Transposon mutagenesis of *rarp-1* impairs *R. parkeri* infection

In a previous *mariner*-based transposon mutagenesis screen (18), we identified a number of *R. parkeri* mutants that displayed abnormal plaque sizes after infection of Vero host cell monolayers. We hypothesized that the plaque phenotypes for these mutants were due to defects in growth, cell-to-cell spread, or other steps of the rickettsial life cycle. Two such small plaque (Sp) mutants contained a transposon (Tn) insertion within the *rarp-1* gene, giving a predicted truncation of RARP-1 at residues 305 (Sp116) and 480 (Sp64) (Figure 1A). RARP-1 is a 573 amino acid protein conserved across the *Rickettsia* genus, but the lack of loss-of-function mutants has thus far

prevented characterization of RARP-1 function. Due to the upstream position of its Tn insertion within the *rarp-1* CDS, we focused on Sp116 (herein referred to as *rarp-1*::Tn) for all subsequent studies and confirmed that it formed smaller plaques than GFP-expressing wild-type bacteria (WT, Figure 1B). We generated polyclonal antibodies against a RARP-1 peptide upstream of the Tn insertion site to assess RARP-1 expression in the mutant. As expected, the *rarp-1*::Tn mutant did not express the full-length protein by immunoblotting (Figure 1C). Furthermore, we were unable to detect an obvious band consistent with the expected 30 kDa product resulting from Tn insertion. Altogether, these results suggest that the loss of RARP-1 expression in the *rarp-1*::Tn mutant leads to a small plaque phenotype.

RARP-1 supports bacterial growth and is dispensable for cell-to-cell spread

The small plaques formed by the *rarp-1*::Tn mutant could be the result of defects in one or more steps of the rickettsial life cycle, and determining when RARP-1 acts during infection would support characterization of its function. We first performed infectious focus assays in A549 host cell monolayers to assess the growth and cell-to-cell spread of the *rarp-1*::Tn mutant on a shorter timescale than is required for plaque formation (28 h versus 5 d post-infection). A549 cells support all known aspects of the *R. parkeri* life cycle, and the use of gentamicin prevents asynchronous invasion events (15). Consistent with the small plaque phenotype, the *rarp-1*::Tn mutant generated smaller foci than WT bacteria (Figure 2A). To confirm that this phenotype was due specifically to the disruption of *rarp-1*, we complemented the *rarp-1*::Tn mutant with a plasmid expressing 3xFLAG-tagged RARP-1 (*rarp-1*::Tn + 3xFLAG-RARP-1, Supplementary

Figure 1A). Since *rarp-1* is predicted to be part of an operon (19), we selected a 247 bp region immediately upstream of the first gene in the operon (encoding the outer membrane channel TolC) as a putative promoter to drive *rarp-1* expression. This construct was sufficient for expression of epitope-tagged RARP-1 in the *rarp-1*::Tn mutant (Figures 1C and D). Importantly, the complement strain exhibited infectious focus sizes comparable to WT (Figure 2A), indicating that the putative promoter and epitope-tagged RARP-1 are functionally relevant. Thus, RARP-1 specifically supports the size of *R. parkeri* infectious foci.

A reduction in infectious focus size could be caused by defects in cell-to-cell spread. For example, Tn mutagenesis of *sca2* and *sca4* specifically disrupts spread by limiting actin tail formation and protrusion resolution, respectively, leading to smaller infectious foci (14, 15). Loss of RARP-1 did not alter the frequency of actin tails or protrusions (Figures 2B and C), suggesting that spread may not be regulated by RARP-1. As an orthogonal approach, we also evaluated the efficiency of spread by performing a mixed-cell infectious focus assay (15). In this assay, donor host cells stably expressing a cytoplasmic marker are infected, mixed with unlabeled recipient host cells, and then infection of the mixed monolayer is allowed to progress. Bacteria that spread to unlabeled recipient cells can thus be distinguished from bacteria that remain in the labeled donor cell for each focus. As expected, a *sca2*::Tn mutant failed to spread from infected donor cells (Figure 2D). In contrast, the *rarp-1*::Tn mutant exhibited similar efficiency of spread from donors to recipients as compared to WT bacteria. Altogether, these results indicate that RARP-1 is dispensable for cell-to-cell spread.

157

158 Alternatively, a reduction in infectious focus size could be caused by defects in bacterial
159 growth. When performing the infectious focus assays, we noted that the number of *rarp-*
160 *1*::Tn mutant bacteria within the infectious foci was reduced compared to WT (Figure
161 2E). This was in contrast to Tn mutants of *sca2* and *sca4*, which do not exhibit reduced
162 bacterial loads despite forming smaller foci (14, 15). Restoring RARP-1 expression in
163 the complement strain rescued the bacterial load defect (Figure 2E), suggesting that
164 RARP-1 regulates bacterial growth. To determine if the *rarp-1*:Tn mutant displayed
165 altered growth behavior over the course of infection, we used qPCR to monitor bacterial
166 genome equivalents during infection of Vero host cell monolayers. In agreement with
167 the bacterial load defect observed in the infectious focus assay, the *rarp-1*::Tn mutant
168 exhibited a growth defect compared to WT (Figure 2F). Together, our data support a
169 role for RARP-1 during bacterial growth in multiple cell types.

170

171 **RARP-1 is dispensable for evasion of autophagy**

172 Given the *rarp-1*::Tn mutant growth defect, we hypothesized that RARP-1 might
173 promote bacterial growth by preventing clearance from the host cell. *R. parkeri* avoids
174 recognition and destruction by the host cell autophagy machinery using the abundant
175 outer membrane protein OmpB (13). Bacteria lacking OmpB are readily
176 polyubiquitinated by the host cell and associate with LC3-positive autophagic
177 membranes. We tested whether the *rarp-1*::Tn mutant likewise associates with LC3
178 during infection of A549 cells. In contrast to an *ompB*::Tn mutant, the *rarp-1*::Tn mutant
179 failed to mobilize host LC3 (Figure 3A). Thus, loss of RARP-1 expression does not

render this mutant more susceptible to autophagic clearance, indicating that RARP-1 supports growth through a different mechanism.

RARP-1 supports host cell invasion

We next wanted to determine if RARP-1 plays other roles in the infection cycle upstream of growth inside the host cytoplasm. We tested whether the *rarp-1*::Tn mutant exhibited defects during invasion of A549 host cells using differential immunofluorescent staining (6). In this assay, bacteria are stained both before and after host cell permeabilization to distinguish external and internal bacteria, respectively. Invasion of the *rarp-1*::Tn mutant was delayed compared to WT but otherwise recovered within 30 min post-infection (Figure 3B). We observed similar invasion kinetics for WT bacteria and the complement strain, indicating that the delayed invasion of the *rarp-1*::Tn mutant is due to loss of RARP-1 expression. Thus, RARP-1 supports efficient host cell invasion. We therefore turned our investigation to the localization and binding partners of RARP-1 so that we could reveal how this factor contributes to infection.

RARP-1 is not secreted into the host cytoplasm by *R. parkeri*

RARP-1 contains an N-terminal Sec secretion signal and several C-terminal ankyrin repeats. Ankyrin repeats are often involved in protein-protein interactions (20), and various intracellular pathogens secrete ankyrin repeat-containing proteins to target an array of host cell processes (21, 22). Previous work with the typhus group *Rickettsia* species *R. typhi* suggested that RARP-1 is delivered into host cells through a non-

202 canonical mechanism mediated by the Sec translocon and TolC (19). We originally
203 hypothesized that *R. parkeri* also secretes RARP-1 to target host cell functions and
204 ultimately promote bacterial growth and invasion. To monitor secretion of RARP-1
205 during infection of A549 cells, we used selective lysis to separate supernatants
206 containing the infected host cytoplasm from pellets containing intact bacteria. A protein
207 that is secreted during infection should be detected in both the supernatant and pellet
208 fractions by immunoblotting, as was observed for the secreted effector Sca4 (Figure 4A,
209 middle panel). The absence of the bacterial RNA polymerase subunit RpoA in the
210 supernatant fraction confirmed that our lysis conditions did not cause bacterial lysis and
211 release of non-secreted bacterial proteins (Figure 4A, bottom panel). Unexpectedly, we
212 detected 3xFLAG-RARP-1 in the bacterial pellet but not in the supernatant fraction of
213 cells infected with the *rarp-1*::Tn + 3xFLAG-RARP-1 complement strain (Figure 4A, top
214 panel). Similar results were observed for a 3xFLAG-RARP-1 construct containing an
215 additional Ty1 epitope tag inserted proximal to the C-terminus (Supplementary Figure
216 1A), suggesting that the lack of detection was not due to proteolytic processing of the
217 RARP-1 protein. As with the 3xFLAG-RARP-1 construct, this dual-tagged variant
218 rescued the *rarp-1*::Tn mutant infectious focus defects (Supplementary Figures 1B and
219 C), demonstrating the functional relevance of the tagged RARP-1 construct. Moreover,
220 endogenous RARP-1 protein was detectable in the WT bacterial pellet but not in the
221 supernatant fraction with our polyclonal antibody (Supplementary Figure 1D), confirming
222 that the epitope-tagged constructs recapitulate the behavior of the endogenous protein.
223 Together, these results suggest that RARP-1 is not secreted by *R. parkeri* into the host
224 cytoplasm.

225

226 As an alternative strategy to evaluate RARP-1 secretion, we introduced glycogen
227 synthase kinase (GSK)-tagged constructs into *R. parkeri*. This system has been used to
228 assess secretion of effector proteins by *Rickettsia* spp. and other bacteria, and it does
229 not rely on the selective lysis of infected samples (23, 24). GSK-tagged proteins
230 become phosphorylated by host kinases upon entering the host cytoplasm, and
231 secretion of the tagged protein can be validated by phospho-specific antibodies (25).
232 Although GSK-tagged RARP-2, a known secreted effector (23), was phosphorylated,
233 GSK-tagged RARP-1 and a non-secreted control (BFP) were not phosphorylated during
234 infection (Figure 4B). These results provide further evidence that RARP-1 is not
235 secreted into the host cytoplasm by *R. parkeri*.
236

237 **Heterologously expressed RARP-1 is not secreted by *E. coli***

238 We were surprised by the results above since previous work suggested that RARP-1 is
239 delivered into host cells by *R. typhi*. Heterologous expression in *Escherichia coli*
240 provided evidence that *R. typhi* RARP-1 is secreted in a Sec- and TolC-dependent
241 manner (19). Following the methodology described by that work, we assessed secretion
242 of *R. parkeri* and *R. typhi* RARP-1 by WT and Δ*tolC E. coli*. In this assay, *E. coli*
243 cultures expressing RARP-1 are pelleted and the culture supernatant is then filtered and
244 precipitated to concentrate proteins released into the extracellular milieu. Although *R.*
245 *parkeri* RARP-1 was clearly detectable in the bacterial pellets of both strains, it was not
246 observed in the supernatants for either strain (Figure 4C). Likewise, we were unable to
247 detect secretion of *R. typhi* RARP-1 by either strain, in contrast to the previously

248 described secretion pattern for this protein. To confirm that our use of an N-terminal
249 3xFLAG tag did not disrupt secretion by *E. coli*, we generated an *R. typhi* RARP-1
250 construct with a C-terminal Myc-6xHis tag, as described in the previous work. Again, we
251 were unable to detect secretion of *R. typhi* RARP-1 (Figure 4D). To validate our ability
252 to detect secreted proteins in the culture supernatant, we assessed secretion of 6xHis-
253 tagged YebF, a protein known to be exported into the medium by *E. coli* (26). As
254 expected, YebF was observed in both the bacterial pellet and culture supernatant. The
255 lack of RARP-1 secretion by *E. coli* is consistent with our immunoblotting results for
256 infection with *R. parkeri*, suggesting that RARP-1 is not a secreted effector.
257

258 **RARP-1 localizes to the *R. parkeri* periplasm**

259 Given that RARP-1 is not secreted by *R. parkeri*, we next investigated where it localized
260 during infection using differential immunofluorescent staining (15). In this assay,
261 infected A549 host cells are first selectively permeabilized such that only the host cell
262 contents and bacterial surface are accessible for staining. Then, the bacteria are
263 permeabilized with lysozyme and detergent to permit immunostaining of proteins inside
264 the bacteria. By staining with a FLAG tag-specific antibody either with or without this
265 second permeabilization step, we can distinguish the localization of tagged proteins
266 inside or outside the bacteria, respectively. We predicted that epitope-tagged RARP-1
267 expressed by the *rarp-1*::Tn + 3xFLAG-RARP-1 complement strain would be absent
268 from the host cytoplasm but present inside permeabilized bacteria. In agreement with
269 our immunoblotting results above, we did not detect specific FLAG staining in the host
270 cytoplasm after infection with the complement strain, similar to results with the *rarp-*

271 *1*::Tn mutant (Figure 5A). We also did not detect the protein on the bacterial surface.
272 Instead, 3xFLAG-RARP-1 was only detectable after permeabilizing bacteria with
273 lysozyme and detergent. Under these conditions, the 3xFLAG-RARP-1 signal
274 surrounded the bacteria with variable localization patterns and often formed bipolar
275 puncta (Figure 5B). Line scan analysis of permeabilized bacteria confirmed that
276 3xFLAG-RARP-1 localized adjacent to the bacterial cytoplasm (Figure 5C). These
277 localization patterns, together with the presence of an N-terminal Sec secretion signal,
278 suggest that RARP-1 is not secreted into the host cytoplasm but instead localizes to the
279 *R. parkeri* periplasm.
280

281 **RARP-1 interacts with other bacterial factors that access the periplasm**

282 Based on the 3xFLAG-RARP-1 localization pattern, we hypothesized that RARP-1
283 might interact with other factors in the *R. parkeri* periplasm to support growth and host
284 cell invasion. To test this hypothesis, we isolated *rarp-1*::Tn + 3xFLAG-Ty1-RARP-1
285 bacteria and treated them with lysozyme-containing lysis buffer to release non-secreted
286 proteins for pulldown. As a control, we also prepared lysates from WT bacteria that do
287 not express tagged RARP-1. We then immunoprecipitated the lysates with a FLAG tag-
288 specific antibody, performed an acid elution to release bound proteins, and analyzed the
289 eluates by mass spectrometry to identify putative RARP-1 binding partners
290 (Supplementary Figures 2A and B). Proteins that were present in the tagged lysate
291 pulldown but absent from the untagged lysate pulldown were called as hits (Table 1 and
292 Data Set 1).
293

294 Of the hits identified, only Sca2 has been functionally characterized in *R. parkeri* (14).
295 Although Sca2 promotes late-stage actin-based motility, the *rarp-1*::Tn mutant formed
296 actin tails at frequencies comparable to WT (Figure 2A), indicating that the loss of
297 RARP-1 does not dramatically impair Sca2 function. However, it is possible that RARP-
298 1 functions in a more subtle way to influence Sca2 activity. To test this hypothesis, we
299 used immunoblotting to assess Sca2 expression in the *rarp-1*::Tn mutant (Figure 6A).
300 The abundance of full-length Sca2 and its processed products was comparable
301 between the *rarp-1*::Tn mutant and the complement strain, suggesting that RARP-1
302 does not grossly impact Sca2 levels. Likewise, we observed similar patterns of Sca2
303 localization between strains (Figure 6B), suggesting that RARP-1 does not play a role in
304 the polar positioning of Sca2. Taken together, these results suggest that RARP-1 does
305 not regulate the activity of its putative binding partner Sca2.
306
307 Additional hits identified in our analysis include the type IV secretion system outer
308 membrane components RvhB9 and RvhB10 as well as several hypothetical lipoproteins
309 and porins (Table 1). At this time, none of these proteins have been functionally
310 characterized in *R. parkeri*. Consistent with RARP-1 localization to the periplasm,
311 however, nearly all of these hits are predicted to reside in the periplasm or otherwise
312 access and transit the periplasm *en route* to the bacterial surface. Thus, it remains
313 possible that RARP-1 acts with one or more of these binding partners to support growth
314 and host cell invasion.
315

316 **Discussion**

317 After host cell invasion, obligate intracellular bacteria must scavenge host nutrients,
318 proliferate, and avoid destruction by their hosts (2). Disruption of one or all of these
319 activities will diminish intracellular bacterial loads and ultimately reduce pathogenicity.
320 While many studies have revealed important regulators of invasion, nutrient acquisition,
321 and bacterial growth for other species, little is known about the factors that support
322 rickettsial physiology during infection, and only recently have we begun to uncover the
323 protective strategies *Rickettsia* spp. employ to ward off host cell defenses (13, 16).
324 Consequently, we sought to better understand the genetic determinants of rickettsial
325 infection using our functional genetic approaches in *R. parkeri*. We found that RARP-1
326 likely resides in the periplasm where it interacts with proteins predicted or known to
327 drive bacterial fitness or interactions with the host. Furthermore, our results suggest that
328 RARP-1 supports the *R. parkeri* life cycle by promoting bacterial growth as well as
329 efficient host cell invasion.
330
331 Loss of RARP-1 expression led to a transient invasion delay, suggesting that RARP-1
332 plays a role in host cell entry. Several studies have identified rickettsial surface proteins
333 and candidate secreted effectors that facilitate invasion. For example, the outer
334 membrane protein OmpA and OmpB respectively interact with α2β1 integrin and Ku70
335 at the host cell surface (5, 7), while the effectors RalF and Risk1 modulate host
336 membrane phosphoinositides during entry (27, 28). Nevertheless, there is incomplete
337 conservation across the *Rickettsia* genus for many of these proteins (27), and invasion
338 is not abolished when the activity of any one protein is inhibited (13, 29); thus, it is likely
339 that *Rickettsia* spp. use several redundant strategies to enter their hosts. Although

RARP-1 itself is not exported from the bacterium, one or more of the RARP-1 interaction partners may contribute to efficient internalization as discussed above. Loss of RARP-1 expression would therefore have pleiotropic effects on infection by hindering both invasion and growth. Alternatively, it is possible that the *rarp-1*::Tn mutant invasion delay is the result of defective growth in the preceding infection cycles when the bacteria were harvested. Indeed, invasion competency of the intracellular bacterial pathogen *Brucella abortus* is linked to cell cycle progression (30). Perhaps rickettsial invasion efficiency relies on robust growth, without which the invasion program is impaired.

Loss of RARP-1 expression also reduced bacterial loads, persisting long after the initial invasion delay was overcome. This defect suggests that RARP-1 plays a role in bacterial growth through the regulation of bacterial physiology or avoidance of host defenses. Normally, *R. parkeri* shields itself from autophagy receptors by methylating outer membrane proteins such as OmpB (13). Loss of OmpB or the methyltransferases PKMT1 and PKMT2 promotes autophagy of *R. parkeri* and reduction of intracellular bacterial burdens (16). Since the *rarp-1*::Tn mutant did not display enhanced recruitment of the autophagy marker LC3, we concluded that the loss of RARP-1 does not render this mutant more susceptible to autophagy. Nevertheless, we cannot rule out that growth of the *rarp-1*::Tn mutant is restricted by other host defense strategies employed by the cell lines used in this study.

362 Prior work reported that RARP-1 was robustly secreted into the host cytoplasm by *R.*
363 *typhi*, and experiments in *E. coli* suggested that RARP-1 relied on a non-canonical Sec-
364 and TolC-dependent pathway for export (19). We were unable to detect secretion of
365 endogenous or epitope-tagged RARP-1 into the host cytoplasm by *R. parkeri*, even
366 though the tagged constructs functionally complemented the *rarp-1*::Tn mutant
367 phenotype. Similarly, we were unable to detect phosphorylation of GSK-tagged RARP-1
368 in infected cell lysates as an orthogonal secretion assay. Notably, this lack of secretion
369 was observed during infection of multiple host cell types and for both WT and *rarp-1*::Tn
370 backgrounds. We also could not detect secretion of RARP-1 by *E. coli*, despite testing
371 both *R. parkeri* and *R. typhi* homologs under the same conditions previously published
372 (19). Nevertheless, it is formally possible that our use of a different *E. coli* K-12 strain
373 (BW25513 rather than C600) prevented release of RARP-1 into the culture supernatant.
374 Since *R. typhi* is a BSL-3 pathogen, we are not able to assess secretion of *R. typhi*
375 RARP-1 by *R. parkeri*, and a loss-of-function *rarp-1* mutant does not exist in *R. typhi*.
376 Altogether, our data suggest that RARP-1 is not secreted into the host cytoplasm by *R.*
377 *parkeri*; instead, it is likely targeted to the periplasm by its Sec secretion signal where it
378 stays to support bacterial growth and invasion.
379
380 RARP-1 is not predicted to possess enzymatic activity, but it does contain a large
381 central intrinsically disordered region (IDR) and several C-terminal ankyrin repeats
382 (ANKs). Although IDRs do not form ordered structures on their own, the structural
383 plasticity of IDRs affords them diverse biological functions (31). For example, the IDRs
384 of bacterial proteins facilitate chaperone recruitment, passage through narrow protein

channels, and binding of multiple partners as part of a signaling hub (32–34). In *Caulobacter crescentus*, the IDR of PopZ serves as a scaffold for concentrating cell cycle regulators at the cytoplasmic cell poles (34). Given the localization pattern of RARP-1 and its interactions with various factors in the periplasm, it is possible that the IDR of RARP-1 performs a similar scaffolding role and concentrates binding partners at the *R. parkeri* periplasmic cell poles. Although our results suggest that the function and polar localization of the surface actin nucleator Sca2 is unaffected by the absence of RARP-1, additional studies will be necessary to assess the activity and localization of other RARP-1 binding partners in the *rarp-1*::Tn mutant.

ANKs are among the most common protein-protein interaction modules and ANK-containing proteins govern a variety of cellular processes (20). Many intracellular bacterial pathogens secrete ANK-containing effectors to target host cell functions, including protein trafficking, ubiquitination, and transcription (21, 22). Nevertheless, ANKs have also been shown to support the activity of bacterial proteins that are not secreted into the extracellular milieu. For example, AnkB localizes to the periplasm of *Pseudomonas aeruginosa* where it protects against oxidative stress (35), and Bd3460 of *Bdellovibrio bacteriovorus* complexes with endopeptidases in the periplasm to prevent degradation of its own cell wall (36). Although ANKs are best known for mediating protein-protein interactions, recent work has demonstrated that ANKs can also bind sugars and lipids (37, 38). Future mutational and biochemical analyses may reveal if the RARP-1 ANKs are necessary for interactions with its putative binding partners or if this domain also binds non-protein substrates in the periplasm to support

408 RARP-1 activity.

409

410 Our data suggest that RARP-1 resides in the periplasm where it interacts with several
411 classes of proteins to support growth and invasion. Since many of these binding
412 partners have not been functionally characterized, we focused our attention on the
413 interaction between RARP-1 and Sca2. Sca2 is required for late-stage actin-based
414 motility in mammalian and tick cells (14, 39), and it is necessary for virulence in animal
415 models of SFG rickettsial infection (40). Tn mutagenesis of *rarp-1* did not reduce actin
416 tail frequency or Sca2 localization to the cell poles, suggesting that RARP-1 does not
417 govern Sca2 function. Nevertheless, it is possible that Sca2 supports the localization or
418 function of RARP-1 in the periplasm as it acts on other factors to regulate invasion and
419 growth.

420

421 We also detected interactions between RARP-1 and components of the Rickettsiales *vir*
422 homolog type IV secretion system (*rvh* T4SS). In the canonical *vir* T4SS of
423 *Agrobacterium tumefaciens*, substrates are delivered from the bacterial cytoplasm into
424 the host cell through a channel that spans the inner and outer membranes (41). VirB9
425 and VirB10, together with VirB7, form a core complex positioned in the periplasm and
426 outer membrane (42). It is unknown to what extent the *rvh* subunits play similar roles as
427 their *vir* counterparts, but it is possible that RARP-1 interacts with RvhB10 and both
428 paralogs of RvhB9 in the periplasm to regulate T4SS assembly or export of effectors. At
429 this time, few *rvh* T4SS effectors are known and none of them have been shown to
430 modulate growth (23, 27, 28). Recent work has suggested that the putative *rvh* T4SS

effector Risk1 promotes host cell invasion by *R. typhi* (28); whether Risk1 plays a similar role in *R. parkeri* or if its secretion is impacted in the *rarp-1*::Tn mutant is unknown. As new effectors are characterized, it will be important to determine if their secretion depends on the interaction between RARP-1 and the *rvh* T4SS. Alternatively, it is possible that the function or localization of RARP-1 is influenced by its interaction with RvhB9 and RvhB10. The T4SS of the intracellular bacterial pathogen *Legionella pneumophila* localizes to the poles (43); if the *rvh* T4SS behaves similarly, RARP-1 may be recruited to the periplasmic cell poles by its core complex binding partners.

Interestingly, many of the RARP-1 binding partners we identified include predicted porins (MC1_RS06520, MC1_RS00535, MC1_RS00570, and MC1_RS06525) and lipoproteins (MC1_RS00420 and MC1_RS02895) of unknown function, as well as the 17 kDa surface antigen. Porins are major components of the outer membrane and regulate the transport of hydrophilic compounds such as nutrients, toxins, and antibiotics (44). Homologs of MC1_RS00535 and MC1_RS00570 have been identified on the surface of the related SFG member *R. rickettsii* (45), but the substrates for these and other rickettsial porins have yet to be characterized. Lipoproteins are lipid-modified proteins that anchor to the membrane and support many aspects of bacterial physiology, including nutrient uptake, protein folding, signal transduction, and cell division (46). Based on remote homology predictions (via HHpred (47)), the hypothetical lipoproteins identified in this study appear to be unique to the *Rickettsia* genus and remain uncharacterized. Similarly, the 17 kDa surface antigen is unique to the genus and Tn mutagenesis of this gene reduces *R. parkeri* plaque size (18, 48), but its

454 function is unknown. In future studies, it will be important to investigate how disruption
455 of one or more of these factors contributes to the invasion and growth defects we
456 observed for the *rarp-1*::Tn mutant.
457
458 The remaining RARP-1 interaction partners include homologs of proteins with known
459 roles in bacterial physiology. For example, the peptidoglycan-associated lipoprotein Pal
460 is concentrated at division septa through its interaction with the Tol machinery, which
461 supports constriction of the outer membrane and remodeling of septal peptidoglycan
462 during *E. coli* cell division (49). Although we did not observe any obvious morphological
463 defects for the *rarp-1*::Tn mutant, the interaction between RARP-1 and Pal could
464 influence rickettsial growth in a more subtle manner. PcaH, a subunit of
465 protocatechuate-3,4-dioxygenase, was also identified as a RARP-1 binding partner. As
466 part of the beta-ketoadipate pathway, this enzyme is involved in the conversion of
467 aromatic compounds to TCA cycle intermediates (50). Homologs for all other enzymes
468 in this pathway, however, are absent in the reduced genome of *R. parkeri* (via the
469 KEGG pathway database (51)); thus, a role for PcaH and its interaction with RARP-1
470 during infection is unclear. Finally, we also detected an interaction between RARP-1
471 and HflC. HflC complexes with HflK in the periplasm to modulate the activity of the
472 integral membrane protease FtsH (52). If RARP-1 provides an additional layer of
473 regulation over FtsH through its interaction with HflC, it is possible that disruption of
474 membrane protein quality control underlies the *rarp-1*::Tn mutant invasion and growth
475 defects.
476

477 Our work uncovers an important role for RARP-1 in supporting the *R. parkeri* life cycle.
478 Through its targeting to the periplasm, we propose that RARP-1 regulates invasion and
479 growth by acting in concert with one or more of the factors revealed in our study.
480 Further work is needed to characterize these interactions since many of the RARP-1
481 binding partners we identified have unknown functions in the *Rickettsia* genus.
482 Expansion of the rickettsial toolkit could facilitate these efforts as well as help determine
483 if there is temporal or spatial control of RARP-1 activity during the *R. parkeri* life cycle.
484 Moreover, structure-function analyses of RARP-1 could provide valuable insight into its
485 mechanism of action in particular and the function of ANK- and IDR-containing proteins
486 in general. Homologs of RARP-1 are notably absent outside the genus, despite
487 conservation of the protein across *Rickettsia* spp. (19). We therefore speculate that
488 RARP-1 represents a core and unique adaptation to the demands of the host cell niche,
489 and future studies may extend its relevance to infection of arthropod vectors. The
490 success of *Rickettsia* spp. hinges on their ability to access and thrive within the complex
491 environment of the host cytoplasm. Continued investigation into the factors that support
492 these fundamental processes will not only improve our understanding of rickettsial
493 biology, but will also highlight the diverse strategies underpinning obligate intracellular
494 bacterial life.
495

496 **Materials and Methods**

497 **Cell culture**

498 A549 human lung epithelial and Vero monkey kidney epithelial cell lines were obtained
499 from the University of California, Berkeley Cell Culture Facility (Berkeley, CA). A549
500 cells were maintained in DMEM (Gibco #11965118) containing 10% FBS. Vero cells
501 were maintained in DMEM containing 5% FBS. A549 cells stably expressing
502 cytoplasmic TagRFP-T (A549-TRT) were generated by retroviral transduction as
503 previously described (15). Cell lines were confirmed to be mycoplasma-negative by
504 MycoAlert PLUS Assay (Lonza #LT07-710) performed by the Koch Institute High
505 Throughput Sciences Facility (Cambridge, MA).
506

507 **Plasmid construction**

508 pRAM18dSGA-3xFLAG-RARP-1 was generated from pRAM18dSGA[MCS] (kindly
509 provided by Dr. Ulrike Munderloh) and contains the 247 bp immediately upstream of the
510 *tolC* start codon (MC1_RS01570), the first 23 aa (amino acids) of *R. parkeri* RARP-1
511 (MC1_RS01585) containing the Sec SS, a HVDYKDHDGDYKDHDIDYKDDDDKHV
512 sequence (3xFLAG epitope tag underlined), the remaining 550 aa of RARP-1, and the
513 *R. parkeri ompA* terminator (MC1_RS06480). pRL0079 is identical to pRAM18dSGA-
514 3xFLAG-RARP-1 but contains GSGGEVHTNQDPLDGGT (Ty1 epitope tag underlined)
515 between residues 396 and 397.
516

517 pRL0284 was generated from pRAM18dSGA[MCS] and contains the *R. parkeri ompA*
518 promoter, an N-terminal MSGRPRTTSFAESGS sequence (GSK epitope tag

519 underlined), TagBFP from pRAM18dRA-2xTagBFP (15), and the *ompA* terminator.
520 pRL0285 is identical to pRL0284 but contains *R. parkeri* RARP-2 (MC1_RS04780) in
521 place of TagBFP. Similarly, pRL0286 contains *R. parkeri* RARP-1 in place of TagBFP,
522 but GSMSGRPRTTSFAESGS was inserted after the Sec SS (as in pRAM18dSGA-
523 3xFLAG-RARP-1) instead of at the N-terminus.
524
525 pRL0287 was generated from pEXT20 (kindly provided by Dr. Michael Laub) and
526 contains the *R. parkeri* RARP-1 insert with intervening 3xFLAG epitope tag from
527 pRAM18dSGA-3xFLAG-RARP-1. pRL0288 is identical to pRL0287, except the 23 aa
528 Sec SS of *R. typhi* RARP-1 (RT0218) and the remaining 563 aa of *R. typhi* RARP-1
529 were used. In contrast, pRL0289 contains the full 586 aa of *R. typhi* RARP-1 with a C-
530 terminal KGEFEAYVEQKLISEEDLNSAVDHHHHHH sequence (Myc and 6xHis epitope
531 tags underlined) as previously described (19). For pRL0290, a C-terminal VDHHHHHH
532 sequence (6xHis epitope tag underlined) was added to *E. coli* YebF (NCBI b1847).
533
534 **Generation of *R. parkeri* strains**
535 Parental *R. parkeri* str. Portsmouth (kindly provided by Dr. Chris Paddock) and all
536 derived strains were propagated by infection and mechanical disruption of Vero cells
537 grown in DMEM containing 2% FBS at 33 °C as previously described (15, 18). Bacteria
538 were clonally isolated and expanded from plaques formed after overlaying infected Vero
539 cell monolayers with agarose as previously described (18). When appropriate, bacteria
540 were further purified by centrifugation through a 30% MD-76R gradient (Mallinckrodt
541 Inc. #1317-07) as previously described (15). Bacterial stocks were stored as aliquots at

542 -80 °C to minimize variability due to freeze-thaws. Titers were determined for bacterial
543 stocks by plaque assay (15), and plaque sizes (Figure 1B) were measured with ImageJ
544 after 5 d infection.
545
546 Bacteria were transformed with plasmids by small-scale electroporation as previously
547 described (18), except infections were scaled down to a T25 cm^2 flask and bacteria
548 were electroporated with 1 µg dialyzed plasmid DNA. When appropriate, rifampicin (200
549 ng/mL) or spectinomycin (50 µg/mL) were included to select for transformants. The *rarp-*
550 *1*::Tn and *sca2*::Tn mutants were generated as previously described (18), and the
551 genomic locations of the Tn insertion sites were determined by semi-random nested
552 PCR and Sanger sequencing. The expanded strains were verified by PCR amplification
553 of the Tn insertion site using primers flanking the region. The $ompB^{STOP}$::Tn mutant
554 (referred to as *ompB*::Tn in this work; kindly provided by Dr. Matthew Welch) was
555 generated as previously described (13).
556

557 ***R. parkeri* infections**

558 For the infectious focus assays (Figures 2A and E and Supplementary Figures 1B and
559 C), confluent A549 cells grown on 12 mm coverslips in 24-well plates were infected at
560 an MOI of 0.005-0.025, centrifuged at 200 x g for 5 min at RT, and incubated at 33 °C
561 for 1 h. Infected cells were washed three times with PBS before adding complete media
562 with 10 µg/mL gentamicin. Infections progressed for 28 h at 33°C until fixation with 4%
563 PFA in PBS for 10 min at RT.
564

565 To measure actin tail and protrusion frequencies (Figures 2B and C), confluent A549
566 cells grown on 12 mm coverslips in 24-well plates were infected at an MOI of 0.3-0.6,
567 centrifuged at 200 x g for 5 min at RT, and incubated at 33 °C for 1 h. Infected cells
568 were washed three times with PBS before adding complete media with 10 µg/mL
569 gentamicin. Infections progressed for 28 h at 33°C until fixation with 4% PFA in PBS for
570 10 min at RT.
571
572 For the mixed-cell assays (Figure 2D), A549-TRT donor cells were plated in 96-well
573 plates and unlabeled A549 recipient cells were plated in 6-well plates and grown to
574 confluency. Donors were infected at an MOI of 9-10, centrifuged at 200 x g for 5 min at
575 RT, and incubated at 33 °C for 1 h. Infected donors and uninfected recipients were
576 washed with PBS, lifted with citric saline (135 mM KCl, 15 mM sodium citrate) at 37 °C
577 to preserve cell surface receptors, recovered in complete media, washed twice with
578 complete media to remove residual citric saline, and resuspended in complete media
579 with 10 µg/mL gentamicin (6 x 10^5 cells/mL donors and 8 x 10^5 cells/mL recipients).
580 Cells were then mixed at a 1:125 ratio (5.3 µL donors and 500 µL recipients) and plated
581 on 12 mm coverslips in 24-well plates. Infections progressed for 31 h at 33 °C until
582 fixation with 4% PFA in PBS for 1 h at RT.
583
584 To measure growth (Figure 2F), confluent Vero cells grown in 24-well plates were
585 infected in triplicate at an MOI of 0.025, centrifuged at 200 x g for 5 min at RT, and
586 incubated at 33 °C for 1 h. Infected cells were washed three times with serum-free
587 DMEM before adding complete media and allowing infections to progress at 33 °C. To

588 harvest samples at the indicated time point, infected cells were scraped into the media
589 and centrifuged at 20,000 x g for 5 min. The resulting pellets were resuspended in 600
590 µL Nuclei Lysis Solution (Promega #A7941), boiled for 10 min to release genomic DNA,
591 and processed with a Wizard Genomic DNA Purification Kit (Promega #A1125)
592 according to manufacturer instructions. After air-drying, the DNA pellets were
593 resuspended in 100 µL H_2O, incubated at 65 °C for 1 h, and allowed to completely
594 rehydrate overnight at RT. For qPCR, runs were carried out on a LightCycler 480
595 (Roche) at the MIT BioMicro Center (Cambridge, MA). Primers to the *R. parkeri* 17 kDa
596 surface antigen gene (MC1_RS06550; 5'-TTCGGTAAGGGCAAAGGACA-3' and 5'-
597 GCACCGATTTGTCCACCAAG-3') and to *Chlorocebus sabaeus GAPDH* (5'-
598 AATGGGACTGAAGCTCCTGC-3' and 5'-ATCACCACCCCTCTACCTCC-3') were used
599 to determine bacterial and host genome equivalents, respectively, relative to a standard
600 curve prepared from a pooled mixture of the 96 h time point WT infection samples.
601 Results from each biological replicate were normalized to the 1 h time point and fold-
602 change was calculated.
603
604 To evaluate LC3 recruitment (Figure 3A), confluent A549 cells grown on 12 mm
605 coverslips in 24-well plates were infected at an MOI of 1.8-3.6, centrifuged at 200 x g for
606 5 min at RT, and incubated at 33 °C for 2 h until fixation with 4% PFA in PBS for 10 min.
607
608 To measure invasion efficiency (Figure 3B), confluent A549 cells grown on 12 mm
609 coverslips in 24-well plates were placed on ice and the media was replaced with 500 µL
610 ice-cold complete media. The cells were then infected at an MOI of 0.7-1.2, centrifuged

611 at 200 x g for 5 min at 4 °C, 500 µL 37 °C complete media was added, and the plates
612 were immediately moved to 37 °C until fixation with 4% PFA in PBS for 10 min.
613
614 To evaluate secretion of RARP-1 (Figure 4A and Supplementary Figures 1A and D),
615 confluent A549 cells grown in 24-well plates were infected at an MOI of 0.5-1.0,
616 centrifuged at 200 x g for 5 min at RT, and incubated at 33 °C until the indicated harvest
617 time point (Figure 4A) or for 48 h (Supplementary Figures 1A and D).
618
619 To evaluate secretion of GSK-tagged constructs (Figure 4B), confluent Vero cells grown
620 in 24-well plates were infected with the indicated strains, centrifuged at 200 x g for 5 min
621 at RT, and incubated at 33 °C with spectinomycin for 72 h (when infected cells were
622 approximately 90% rounded) before harvesting.
623
624 To evaluate the localization of epitope-tagged RARP-1 (Figures 5A-C), confluent A549
625 cells grown in 24-well plates were infected at an MOI of 0.3-0.6, centrifuged at 200 x g
626 for 5 min at RT, and incubated at 33 °C for 27 h until fixation with 4% PFA in PBS for 1
627 h.
628
629 To evaluate the localization of Sca2 (Figure 6B), confluent A549 cells grown in 24-well
630 plates were infected at an MOI of 0.3-0.6, centrifuged at 200 x g for 5 min at RT, and
631 incubated at 33 °C for 28 h until fixation with 4% PFA in PBS for 10 min.
632

633 ***E. coli* secretion assays**

634 *E. coli* K-12 BW25113 (WT) and JW5503-1 (Δ*tolC*) from the Keio Knockout Collection
635 (53) were obtained from Horizon Discovery. SDS sensitivity and the KanR cassette
636 insertion site were confirmed for the Δ*tolC* strain. Secretion assay samples were
637 collected and processed as previously described (19). Bacterial pellets and precipitated
638 proteins were boiled in loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol,
639 0.1% bromophenol blue, 5% β-mercaptoethanol).
640

641 **RARP-1 antibody production**

642 The RARP-1 peptide antigen (SNEMHEAQVASNEHND, corresponding to residues
643 159-174) was selected and synthesized by New England Peptide (Gardner, MA). The
644 peptide antigen was conjugated to KLH and used for immunization by Pocono Rabbit
645 Farm and Laboratory (Canadensis, PA) according to their 70 day rabbit polyclonal
646 antibody protocol.
647

648 **Immunoblotting**

649 To assess RARP-1 and Sca2 expression (Figures 1C, 1D, and 6A), purified bacteria
650 were boiled in loading buffer and analyzed by western blot using rabbit RARP-1 peptide
651 antisera, rabbit anti-FLAG (Cell Signaling Technology #2368), rabbit anti-Sca2 (kindly
652 provided by Dr. Matthew Welch), and mouse anti-OmpA 13-3 (kindly provided by Dr.
653 Ted Hackstadt). For Figures 1C and 6A, the parental WT *R. parkeri* strain lacking
654 pRAM18dRGA+OmpApr-GFPuv was used. In Figures 1C and D, the apparent MW of

655 RARP-1 is greater than its predicted MW (60 kDa). This aberrant migration by SDS-
656 PAGE is typical of proteins with IDRs (54).

657 To evaluate secretion of RARP-1 (Figure 4A and Supplementary Figures 1A and D),
658 infected cells were washed three times with PBS, lifted with trypsin-EDTA, and
659 centrifuged at 2,400 x g for 5 min at RT. The resulting pellets were resuspended in
660 selective lysis buffer (50 mM HEPES pH 7.9, 150 mM NaCl, 1 mM EDTA, 10% glycerol,
661 1% IGEPAL) containing protease inhibitors (Sigma-Aldrich #P1860), incubated on ice
662 for 15 min, and centrifuged at 11,300 x g for 10 min at 4 °C. The resulting pellets were
663 washed with PBS and boiled in loading buffer. The resulting supernatants were passed
664 through a 0.22 µm cellulose acetate filter (Thermo Scientific #F2517-1) by centrifugation
665 at 6,700 x g for 10 min at 4 °C, combined with loading buffer (to a final volume equal to
666 the final pellet volume), and boiled. Lysates were analyzed by western blot using rabbit
667 anti-FLAG, rabbit anti-Sca4 (15), mouse anti-Ty1 (kindly provided by Dr. Sebastian
668 Lourido), rabbit RARP-1 peptide antisera, and mouse anti-RpoA (BioLegend #663104).
669

670 To evaluate secretion of GSK-tagged constructs (Figure 4B), infected cells were
671 washed with ice-cold serum-free DMEM, directly lysed in loading buffer, and boiled.
672 Lysates were analyzed by western blot using rabbit anti-GSK-3β-Tag (Cell Signaling
673 Technology #9325) and rabbit anti-phospho-GSK-3β (Cell Signaling Technology
674 #9336).
675

676 For the *E. coli* secretion assays (Figures 4C and D), bacterial pellet lysates (equivalent
677 to 0.025 OD_{600}-mL of cultured cells) and precipitated culture supernatants (equivalent to

678 2 mL of culture supernatant prior to precipitation) were analyzed by western blot using
679 rabbit anti-FLAG and HRP-conjugated mouse anti-His (ABclonal #AE028).
680
681 For the co-immunoprecipitation assays (Supplementary Figures 2A and B), samples
682 were analyzed by western blot using rabbit anti-Sca4 and rabbit anti-FLAG.
683

684 **Immunofluorescence microscopy**

685 All micrographs were acquired on an Olympus IXplore Spin microscope system, and
686 image analysis was performed with ImageJ unless otherwise stated.
687
688 For the infectious focus assays (Figures 2A and E and Supplementary Figures 1B and
689 C), fixed samples were incubated with 0.1 M glycine in PBS for 10 min at RT to quench
690 residual PFA. Samples were then washed three times with PBS, permeabilized with
691 0.5% Triton X-100 in PBS for 5 min at RT, and washed another three times with PBS.
692 Samples were then incubated with blocking buffer (2% BSA in PBS) for 30 min at RT.
693 Primary and secondary antibodies were diluted in blocking buffer and incubated for 1 h
694 each at RT with three 5 min PBS washes after each incubation step. The following
695 antibodies and stains were used: mouse anti-β-catenin (Cell Signaling Technology
696 #2677) to detect host membrane, rabbit anti-*Rickettsia* I7205 (kindly provided by Dr.
697 Ted Hackstadt), goat anti-mouse conjugated to Alexa Fluor 568 (Invitrogen #A-11004),
698 goat anti-rabbit conjugated to Alexa Fluor 488 (Invitrogen #A-11008), and Hoechst
699 (Invitrogen #H3570) to detect host nuclei. Coverslips were mounted using ProLong Gold
700 Antifade Mountant (Invitrogen #P36934). Images were acquired using a 60X

701 UPlanSApo (1.30 NA) objective. For each strain, 20-35 foci were imaged and the
702 number of infected cells and bacteria per focus was calculated.
703
704 To measure actin tail and protrusion frequencies (Figures 2B and C), fixed samples
705 were processed as above, except phalloidin conjugated to Alexa Fluor 647 (Invitrogen
706 #A22287) was included to detect actin. For each strain, ≥ 380 bacteria were imaged
707 using a 100X UPlanSApo (1.35 NA) objective and the percentage of bacteria with tails
708 (> 1 bacterial length) and the percentage of bacteria within protrusions were calculated.
709
710 For the mixed-cell assays (Figure 2D), fixed samples were processed as above, except
711 the following antibodies and stains were used: mouse anti-*Rickettsia* 14-13 (kindly
712 provided by Dr. Ted Hackstadt), goat anti-mouse conjugated to Alexa Fluor 647
713 (Invitrogen #A-21235), and phalloidin-iFluor 405 Reagent (Abcam #ab176752). For
714 each strain, 20 foci were imaged using a 60X objective and the percentage of bacteria
715 per focus that had spread to recipient cells was calculated.
716
717 To evaluate LC3 recruitment (Figure 3A), fixed samples were processed as above,
718 except cells were permeabilized with 100% methanol for 5 min at RT instead of Triton
719 X-100 and the following antibodies and stains were used: rabbit anti-LC3B (ABclonal
720 #A7198), mouse anti-*Rickettsia* 14-13, goat anti-rabbit conjugated to Alexa Fluor 568,
721 goat anti-mouse conjugated to Alexa Fluor 488, and Hoechst. Representative images
722 were acquired using a 100X objective.
723

724 To measure invasion efficiency (Figure 3B), fixed samples were incubated with 0.1 M
725 glycine in PBS for 10 min at RT to quench residual PFA. Samples were then washed
726 three times with PBS and incubated with blocking buffer for 30 min at RT. To stain
727 external bacteria, primary and secondary antibodies were diluted in blocking buffer and
728 incubated for 30 min each at RT with three 5 min PBS washes after each incubation
729 step. The following antibodies and stains were used: mouse anti-*Rickettsia* 14-13 and
730 goat anti-mouse conjugated to Alexa Fluor 647. The samples were then fixed with 4%
731 PFA in PBS for 5 min at RT, washed three times with PBS, and quenched with 0.1 M
732 glycine in PBS for 10 min at RT. Samples were then washed three times with PBS,
733 permeabilized with 0.5% Triton X-100 in PBS for 5 min at RT, and washed another
734 three times with PBS. To stain both external and internal bacteria, primary and
735 secondary antibodies were diluted in blocking buffer and incubated for 30 min each at
736 RT with three 5 min PBS washes after each incubation step. The following antibodies
737 and stains were used: mouse anti-*Rickettsia* 14-13 and goat anti-mouse conjugated to
738 Alexa Fluor 488. For each strain, 20 fields of view each containing ≥ 45 bacteria were
739 imaged using a 60X objective. To facilitate analysis, internal and external bacteria were
740 quantified using ilastik (55); the pixel classifier was trained to distinguish bacteria from
741 background, and then the object classifier was trained to distinguish between internal
742 (single-stained) and external (double-stained) bacteria.
743
744 To evaluate the localization of epitope-tagged RARP-1 (Figures 5A-C), fixed samples
745 were incubated with 0.1 M glycine in PBS for 10 min at RT to quench residual PFA.
746 Samples were then washed three times with PBS, permeabilized with 0.5% Triton X-

747 100 in PBS for 5 min at RT, and washed another three times with PBS. Samples were
748 then incubated with goat serum blocking buffer (2% BSA and 10% normal goat serum in
749 PBS) for 30 min at RT. To stain host cell contents and bacterial surface proteins,
750 primary and secondary antibodies were diluted in goat serum blocking buffer and
751 incubated for 3 h at 37 °C and 1 h at RT, respectively, with three 5 min PBS washes
752 after each incubation step. For Figure 5A, rabbit anti-FLAG, mouse anti-*Rickettsia* 14-
753 13, goat anti-rabbit conjugated to Alexa Fluor 647 (Invitrogen #A-21245), and goat anti-
754 mouse conjugated to Alexa Fluor 488 were used, and coverslips were mounted after
755 washing. For Figure 5B, only mouse anti-*Rickettsia* 14-13 and goat anti-mouse
756 conjugated to Alexa Fluor 488 were used in the first round of staining, and coverslips
757 were instead fixed with 4% PFA in PBS for 5 min at RT after washing. These samples
758 were then incubated with 0.1 M glycine in PBS for 10 min at RT to quench residual PFA
759 and washed three times with PBS. To expose proteins inside the bacteria for staining,
760 these samples were incubated with lysozyme reaction buffer (0.8X PBS, 50 mM
761 glucose, 5 mM EDTA, 0.1% Triton X-100, 5 mg/mL lysozyme (Sigma #L6876)) for 20
762 min at 37 °C and then washed three times with PBS. Rabbit anti-FLAG and goat anti-
763 rabbit conjugated to Alexa Fluor 647 were diluted in goat serum blocking buffer and
764 incubated for 3 h at 37 °C and 1 h at RT, respectively, with three 5 min PBS washes
765 after each incubation step. Coverslips were mounted after the second round of staining.
766 For Figure 5C, the same procedure was used as in Figure 5B, except goat anti-mouse
767 conjugated to Alexa Fluor 488 was replaced with Alexa Fluor 405 (Invitrogen #A-31553)
768 to permit imaging of bacterial GFP. Representative images were acquired using a 100X
769 objective. Images in Figure 5C were deconvolved by performing five iterations of the

770 cellSens (Olympus) advanced maximum likelihood estimation algorithm, and a 0.26 μm
771 width pole-to-pole linescan was performed with ImageJ.
772
773 To evaluate the localization of Sca2 (Figure 6B), fixed samples were processed as
774 above, except the following antibodies and stains were used: rabbit anti-Sca2, mouse
775 anti-*Rickettsia* 14-13, goat anti-rabbit conjugated to Alexa Fluor 568 (Invitrogen #A-
776 11011), goat anti-mouse conjugated to Alexa Fluor 488 (Invitrogen #A-11001),
777 phalloidin conjugated to Alexa Fluor 647, and Hoechst. For each strain, ≥ 350 bacteria
778 were imaged using a 100X objective and the Sca2 localization pattern was determined
779 (following the classification scheme from (14)).
780

781 **Co-immunoprecipitation assays**

782 Two replicate samples each of WT and *rarp-1*::Tn + 3xFLAG-Ty1-RARP-1 bacteria
783 were processed in parallel for FLAG co-immunoprecipitation. For each sample, bacteria
784 purified from a fully infected T175 cm^2 flask were centrifuged at 16,200 x g for 2 min at
785 RT, resuspended in 1 mL immunoprecipitation lysis buffer (50 mM Tris-HCl pH 7.4, 150
786 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing 50 U/μL Ready-Lyse Lysozyme
787 (Lucigen #R1804M) and protease inhibitors, incubated for 25 min at RT, and centrifuged
788 at 11,300 x g for 15 min at 4 °C. The resulting supernatants were pre-cleared twice by
789 incubation with 28 μL 50% mouse IgG agarose slurry (Sigma #A0919) for 30 min at 4
790 °C. The pre-cleared input lysates were then incubated with 28 μL 50% anti-FLAG M2
791 magnetic bead slurry (Sigma #M8823) overnight at 4 °C. The bound complexes were
792 washed four times with 500 μL ice-cold immunoprecipitation wash buffer (50 mM Tris-

793 HCl pH 7.4, 150 mM NaCl) containing protease inhibitors, eluted by incubation with 65.2
794 µL 0.1 M glycine (pH 2.8) for 20 min at RT, and neutralized with 9.8 µL 1 M Tris-HCl (pH
795 8.5). The neutralized eluates were then combined with loading buffer and submitted to
796 the Whitehead Institute Proteomics Core Facility (Cambridge, MA) for sample workup
797 and mass spectrometry analysis. Equivalent bacterial input was confirmed by
798 immunoblotting for Sca4 (Supplementary Figure 2A).
799

800 **Mass spectrometry**

801 Samples were run 1 cm into an SDS-PAGE gel, excised, and then reduced, alkylated,
802 and digested with trypsin overnight at 37 °C. The resulting peptides were extracted,
803 concentrated, and injected onto a nanoACQUITY UPLC (Waters) equipped with a self-
804 packed Aeris 3.6 µm C18 analytical column (20 cm x 75 µm; Phenomenex). Peptides
805 were eluted using standard reverse-phase gradients. The effluent from the column was
806 analyzed using an Orbitrap Elite mass spectrometer (nanospray configuration; Thermo
807 Scientific) operated in a data-dependent manner. Peptides were identified using
808 SEQUEST (Thermo Scientific) and the results were compiled in Scaffold (Proteome
809 Software). RefSeq entries for *R. parkeri* str. Portsmouth (taxonomy ID 1105108) and
810 *Homo sapiens* (taxonomy ID 9606) were downloaded from NCBI and concatenated with
811 a database of common contaminants. Peptide identifications were accepted at a
812 threshold of 95%. Protein identifications were accepted with a threshold of 99% and two
813 unique peptides. Rickettsial proteins that were present in both replicates of the tagged
814 (*rarp-1*::Tn + 3xFLAG-Ty1-RARP-1) lysate pulldown but absent from both replicates of
815 the untagged (WT) lysate pulldown were called as hits.

816

817 **Statistical analyses**

818 Statistical analysis was performed using Prism 9 (GraphPad Software). Graphical
819 representations, statistical parameters, and significance are reported in the figure
820 legends. Data were considered to be statistically significant when $p < 0.05$, as
821 determined by an unpaired Student's *t* test or one-way ANOVA with post-hoc Dunnett's
822 test.

823

824 **Data Availability**

825 Mass spectral data and the protein sequence database used for searches have been
826 deposited in the public proteomics repository MassIVE (https://massive.ucsd.edu,
827 MSV000088867).

828

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Figures

Figure 1. Transposon mutagenesis of *rarp-1* impairs *R. parkeri* infection. (A) *R. parkeri* RARP-1 contains an N-terminal Sec secretion signal (SS, orange), a central intrinsically disordered region (IDR, light blue), and C-terminal ankyrin repeats (ANKs, dark blue). Tn insertions at residues 305 (Sp116) and 480 (Sp64) are indicated (arrowheads). (B) Plaque areas in infected Vero cell monolayers. Means from two independent experiments (squares) are superimposed over the raw data (circles) and were used to calculate the mean ± SD and p-value (unpaired two-tailed *t* test, *$p < 0.05$ relative to WT). (C) Western blot for RARP-1 using purified *R. parkeri* strains. 3xFLAG-tagged and endogenous RARP-1 are indicated (arrowheads). OmpA, loading control.

1018 (D) Western blot for FLAG using purified *R. parkeri* strains. 3xFLAG-tagged RARP-1 is
1019 indicated (arrowhead). OmpA, loading control.

1020

1021 **Figure 2. RARP-1 supports bacterial growth and is dispensable for cell-to-cell**
1022 **spread.** (A) Infected cells per focus during infection of A549 cells. The means from
1023 three independent experiments (squares) are superimposed over the raw data (circles)
1024 and were used to calculate the mean ± SD and p-value (one-way ANOVA with post-hoc
1025 Dunnett's test, **$p < 0.01$ relative to WT). (B) Percentage of bacteria with actin tails
1026 during infection of A549 cells. (C) Percentage of bacteria within a protrusion during
1027 infection of A549 cells. In (B) and (C), the percentages were determined from three
1028 independent experiments (≥ 380 bacteria were counted for each infection) and were
1029 used to calculate the mean ± SD and p-value (one-way ANOVA with post-hoc Dunnett's
1030 test, n.s. relative to WT). (D) Percentage of bacteria per focus that spread from infected
1031 donor cells to uninfected recipient cells by mixed-cell assay in A549 cells. The means
1032 from three independent experiments (squares) are superimposed over the raw data
1033 (circles) and were used to calculate the mean ± SD and p-value (one-way ANOVA with
1034 post-hoc Dunnett's test, ****$p < 0.0001$ relative to WT). The *sca2*::Tn mutant was used
1035 as a positive control. (E) Bacteria per focus during infection of A549 cells. The means
1036 from three independent experiments (squares) are superimposed over the raw data
1037 (circles) and were used to calculate the mean ± SD and p-value (one-way ANOVA with
1038 post-hoc Dunnett's test, *$p < 0.05$). These data correspond to the same set of infectious
1039 focus assays displayed in (A). (F) Growth curves as measured by *R. parkeri* (17 kDa
1040 surface antigen) genome equivalents per Vero host cell (*GAPDH*) genome equivalent

1041 normalized to 1 h post-infection. The mean ± SD for triplicate samples from a
1042 representative experiment were compared at each timepoint after $\log_2$ transformation
1043 (unpaired two-tailed *t* test, *p < 0.05 and **p < 0.01 relative to WT).
1044

1045 **Figure 3. RARP-1 is dispensable for evasion of host cell autophagy and supports**
1046 **host cell invasion.** (A) Recruitment of LC3 during infection of A549 cells. Samples
1047 were stained for LC3 (magenta) and bacteria (cyan). The *ompB*::Tn mutant was used as
1048 a positive control, and bacteria associated with LC3-positive membranes are indicated
1049 (arrowheads). Scale bar, 2 μm. (B) Efficiency of invasion into A549 cells. The means ±
1050 SD from a representative experiment (n = 20 fields of view each with ≥ 45 bacteria)
1051 were compared at each timepoint (one-way ANOVA with post-hoc Dunnett's test, **p <
1052 0.01 and ****p < 0.0001 relative to WT).
1053

1054 **Figure 4. RARP-1 is not secreted.** (A) Western blots for FLAG (top) and Sca4 (middle)
1055 during infection of A549 cells with *rarp-1*::Tn + 3xFLAG-RARP-1 bacteria. Infected host
1056 cells were selectively lysed at various timepoints to separate supernatants (S)
1057 containing the infected host cytoplasm from pellets (P) containing intact bacteria. RpoA
1058 (bottom) served as a control for bacterial lysis or contamination of the infected
1059 cytoplasmic fraction. L, ladder. (B) Western blot for GSK-tagged constructs during
1060 infection of Vero cells. Whole cell infected lysates were probed with antibodies against
1061 the GSK tag (left) or its phosphorylated form (P~GSK, right) to detect exposure to the
1062 host cytoplasm. BFP (non-secreted) and RARP-2 (secreted) were used as controls.

1063 Uninf, uninfected whole cell lysate. (C) Western blot for FLAG using N-terminal FLAG-
1064 tagged *R. parkeri* (*Rp*) or *R. typhi* (*Rt*) RARP-1 expressed by WT or Δ*tolC E. coli*. (D)
1065 Western blot for His using C-terminal Myc-6xHis-tagged *R. typhi* RARP-1 or C-terminal
1066 6xHis-tagged *E. coli* YebF expressed by WT *E. coli*. For (C) and (D), cultures were
1067 pelleted (P) and the culture supernatant (S) was filtered and precipitated to concentrate
1068 proteins released into the medium.

1069

1070 **Figure 5. RARP-1 localizes to the *R. parkeri* periplasm.** (A) Images of *rarp-1*::Tn
1071 (top) and *rarp-1*::Tn + 3xFLAG-RARP-1 (bottom) bacteria during infection of A549 cells.
1072 Samples were stained for FLAG (magenta) and the bacterial surface (cyan) without
1073 permeabilization of bacteria. Scale bars, 20 μm. (B) Images of *rarp-1*::Tn (top) and *rarp-*
1074 *1*::Tn + 3xFLAG-RARP-1 (bottom) bacteria during infection of A549 cells. The bacterial
1075 surface (cyan) was stained prior to permeabilization by lysozyme and detergent and
1076 subsequent staining for FLAG (magenta). Scale bar, 5 μm. (C) Subcellular localization
1077 of 3xFLAG-RARP-1 in a representative *rarp-1*::Tn + 3xFLAG-RARP-1 bacterium during
1078 infection of A549 cells. The bacterial surface (cyan) was stained prior to
1079 permeabilization by lysozyme and detergent and subsequent staining for FLAG
1080 (magenta). GFP (yellow) demarcates the bacterial cytoplasm. Scale bar, 1 μm. A pole-
1081 to-pole 0.26 μm width linescan (right) was generated for FLAG, GFP, and the bacterial
1082 surface.

1083

1084 **Table 1. Co-immunoprecipitation of lysozyme-permeabilized bacteria reveals that**
1085 **RARP-1 interacts with other bacterial factors that access the periplasm.[a,b]**

1086 [a]Putative RARP-1 binding partners are ordered by decreasing spectral count.
1087 [b]MC1_RS05020 is the only hit not predicted to access the periplasm.
1088

1089 **Figure 6. RARP-1 does not regulate the abundance or localization of Sca2.** (A)
1090 Western blot for Sca2 from purified *R. parkeri* strains. Full-length Sca2 (arrowhead),
1091 Sca2 cleavage products (bracket), and the truncation product in the *sca2*::Tn mutant
1092 (open arrowhead) are indicated. (B) Percentage of bacteria with the indicated Sca2
1093 localization pattern during infection of A549 cells. Percentages were determined from
1094 two independent experiments (≥ 350 bacteria were counted for each infection) and were
1095 used to calculate the mean ± SD and p-value (one-way ANOVA with post-hoc Dunnett's
1096 test, n.s. relative to WT).
1097

1098 **Supplemental Material**
1099

1100 **Supplementary Figure 1. Tagged RARP-1 constructs and endogenous RARP-1 are**
1101 **not secreted.** (A) *R. parkeri* RARP-1 with insertion sites for 3xFLAG and Ty1 epitope
1102 tags indicated (arrowheads). Western blots for FLAG (top) and Ty1 (middle) after
1103 infection of A549 cells with *rarp-1*::Tn + 3xFLAG-RARP-1 (single-tagged) or *rarp-1*::Tn +
1104 3xFLAG-Ty1-RARP-1 (dual-tagged) bacteria. (B) Infected cells per focus during

1105 infection of A549 cells. (C) Bacteria per focus during infection of A549 cells. In (B) and
1106 (C), the means from three independent experiments (squares) are superimposed over
1107 the raw data (circles) and were used to calculate the mean ± SD and p-value (one-way
1108 ANOVA with post-hoc Dunnett's test, **$p < 0.01$ relative to WT). (D) Western blots for
1109 RARP-1 (top) and Sca4 (middle) after infection of A549 cells with WT or *rarp-1*::Tn
1110 bacteria. Note the specific RARP-1 band in the pellet sample for WT bacteria only, in
1111 contrast to the identical non-specific bands in the supernatant samples for WT and *rarp-*
1112 *1*::Tn bacteria. In (A) and (D), infected host cells were selectively lysed after 48 h to
1113 separate supernatants (S) containing the infected host cytoplasm from pellets (P)
1114 containing intact bacteria. RpoA (bottom) served as a control for bacterial lysis or
1115 contamination of the infected cytoplasmic fraction.

1116

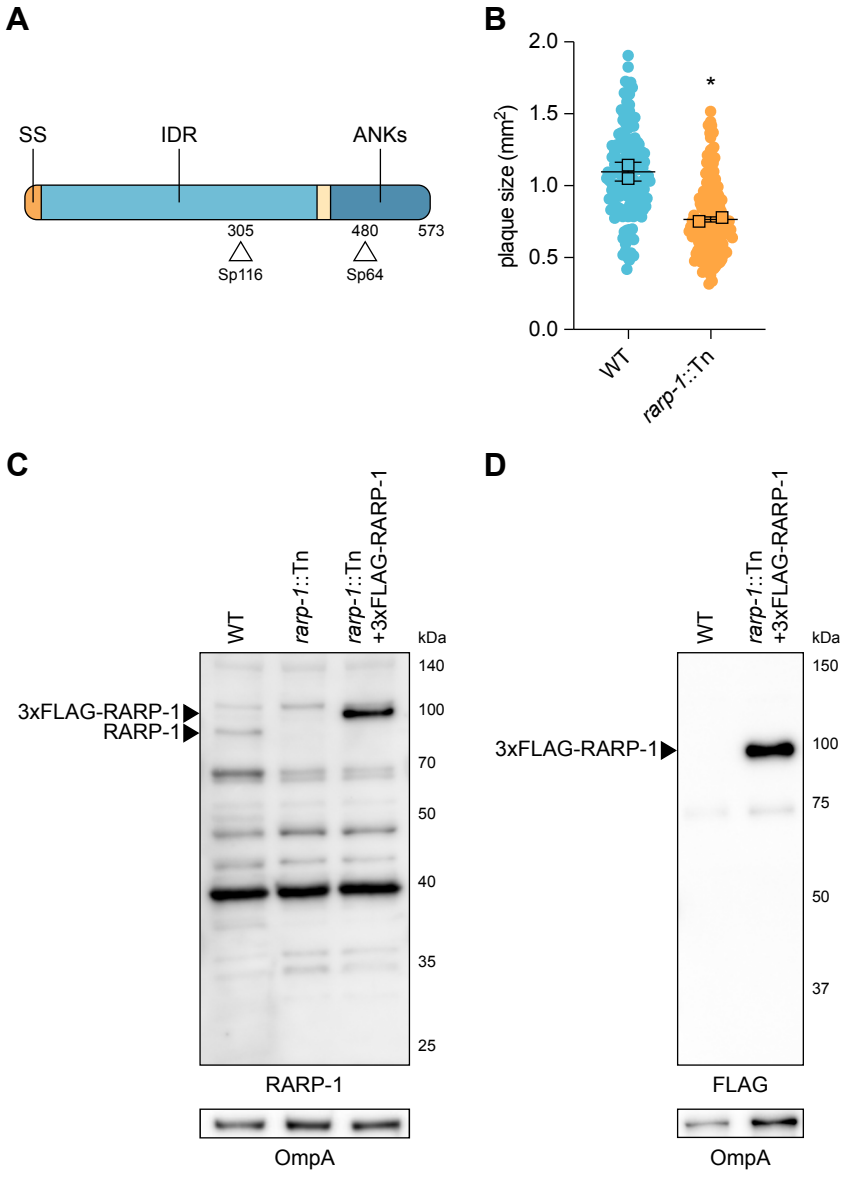
1117 **Supplementary Figure 2. Inputs and eluates from co-immunoprecipitation of**
1118 **lysozyme-permeabilized bacteria.** (A) Western blot for Sca4 (loading control) in input
1119 lysates. (B) Western blot for FLAG in input lysates and FLAG immunoprecipitation
1120 eluates. In (A) and (B), bacteria expressing tagged (+) or untagged (–) RARP-1 were
1121 purified and then permeabilized by lysozyme prior to immunoprecipitation. Two replicate
1122 samples were harvested from each strain.

1123

1124 **Data Set 1. Full co-immunoprecipitation / mass spectrometry results.**

1125

1126 **Supplementary Table 1. Strains and plasmids used in this study.**



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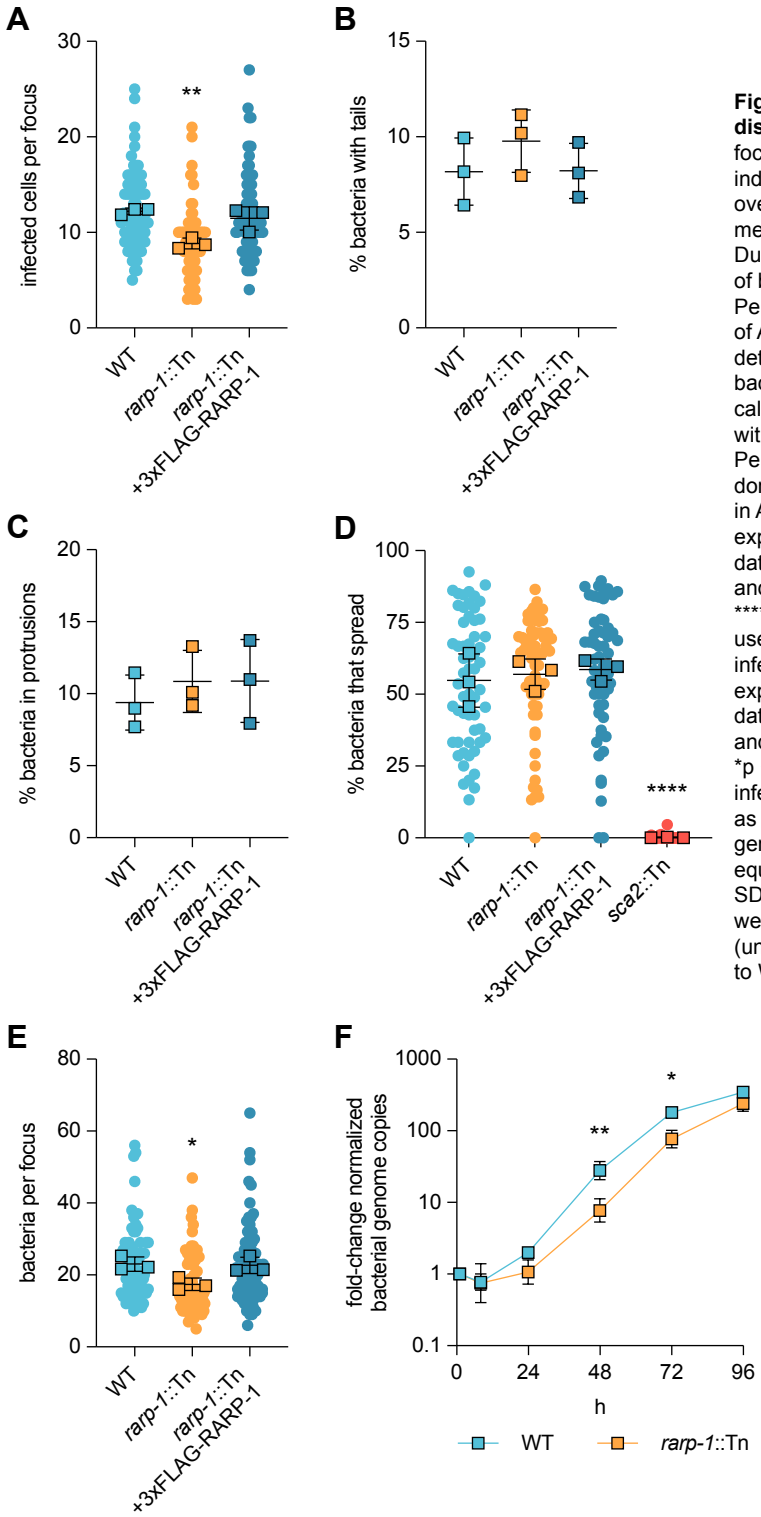

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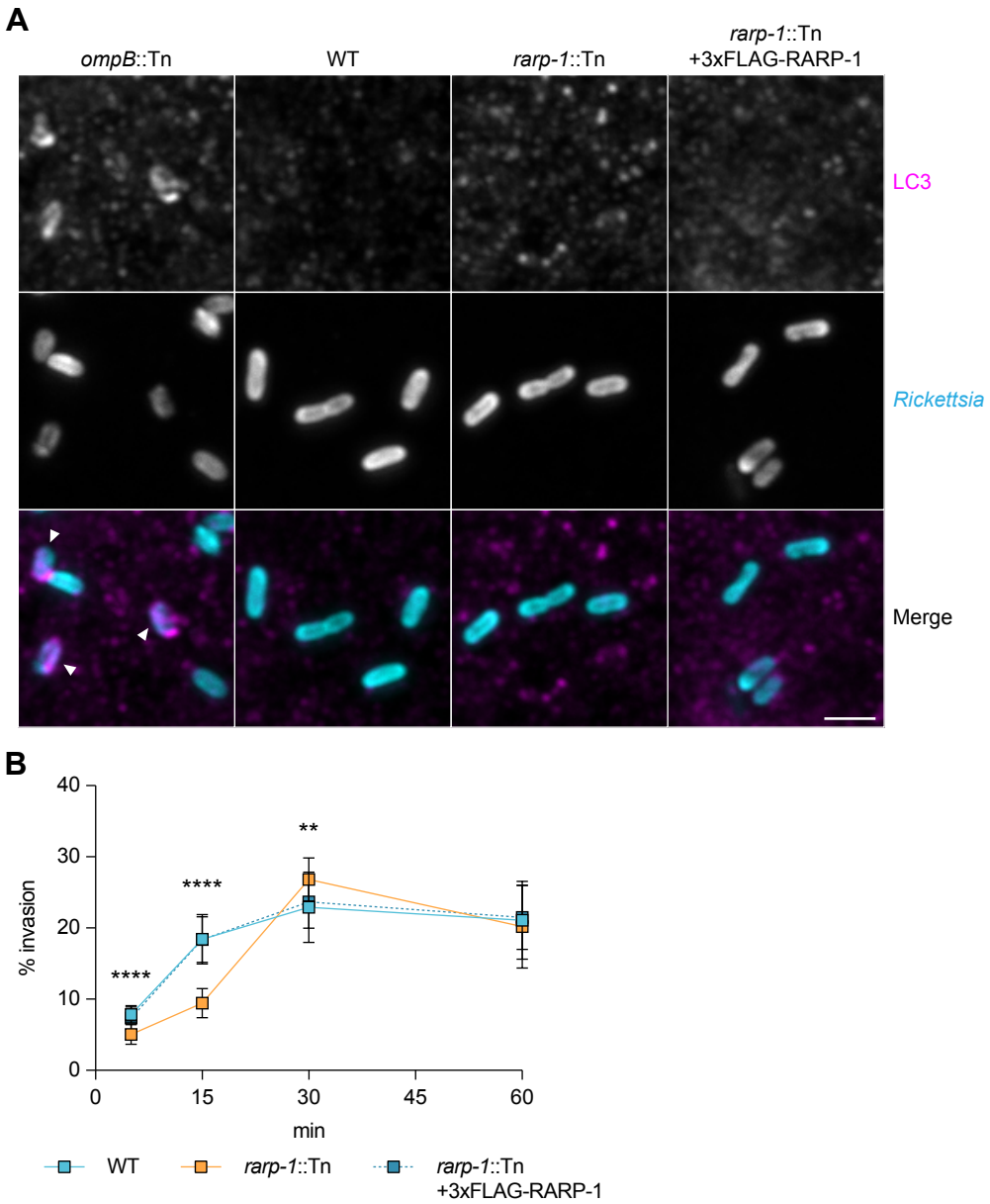

Figure 3. RARP-1 is dispensable for evasion of host cell autophagy and supports host cell invasion. (A) Recruitment of LC3 during infection of A549 cells. Samples were stained for LC3 (magenta) and bacteria (cyan). The *ompB*::Tn mutant was used as a positive control, and bacteria associated with LC3-positive membranes are indicated (arrowheads). Scale bar, 2 µm. (B) Efficiency of invasion into A549 cells. The means ± SD from a representative experiment (n = 20 fields of view each with ≥ 45 bacteria) were compared at each timepoint (one-way ANOVA with post-hoc Dunnett's test, **$p < 0.01$ and ****$p < 0.0001$ relative to WT).

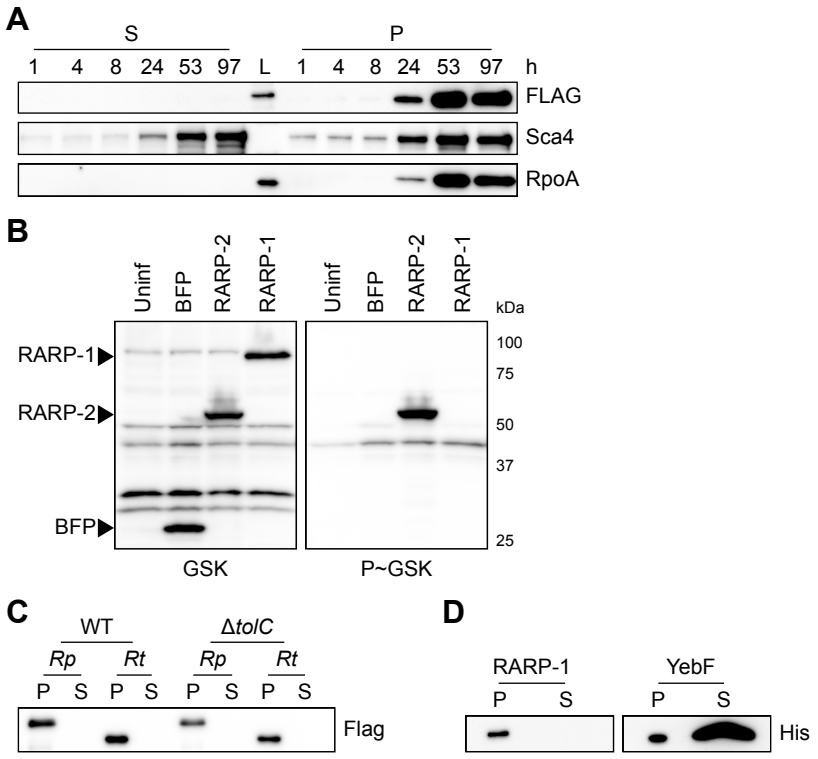

Figure 4. RARP-1 is not secreted. (A) Western blots for FLAG (top) and Sca4 (middle) during infection of A549 cells with *rarp-1*::Tn + 3xFLAG-RARP-1 bacteria. Infected host cells were selectively lysed at various timepoints to separate supernatants (S) containing the infected host cytoplasm from pellets (P) containing intact bacteria. RpoA (bottom) served as a control for bacterial lysis or contamination of the infected cytoplasmic fraction. L, ladder. (B) Western blot for GSK-tagged constructs during infection of Vero cells. Whole cell infected lysates were probed with antibodies against the GSK tag (left) or its phosphorylated form (P~GSK, right) to detect exposure to the host cytoplasm. BFP (non-secreted) and RARP-2 (secreted) were used as controls. Uninf, uninfected whole cell lysate. (C) Western blot for FLAG using N-terminal FLAG-tagged *R. parkeri* (*Rp*) or *R. typhi* (*Rt*) RARP-1 expressed by WT or Δ*tolC E. coli*. (D) Western blot for His using C-terminal Myc-6xHis-tagged *R. typhi* RARP-1 or C-terminal 6xHis-tagged *E. coli* YebF expressed by WT E. coli. For (C) and (D), cultures were pelleted (P) and the culture supernatant (S) was filtered and precipitated to concentrate proteins released into the medium.

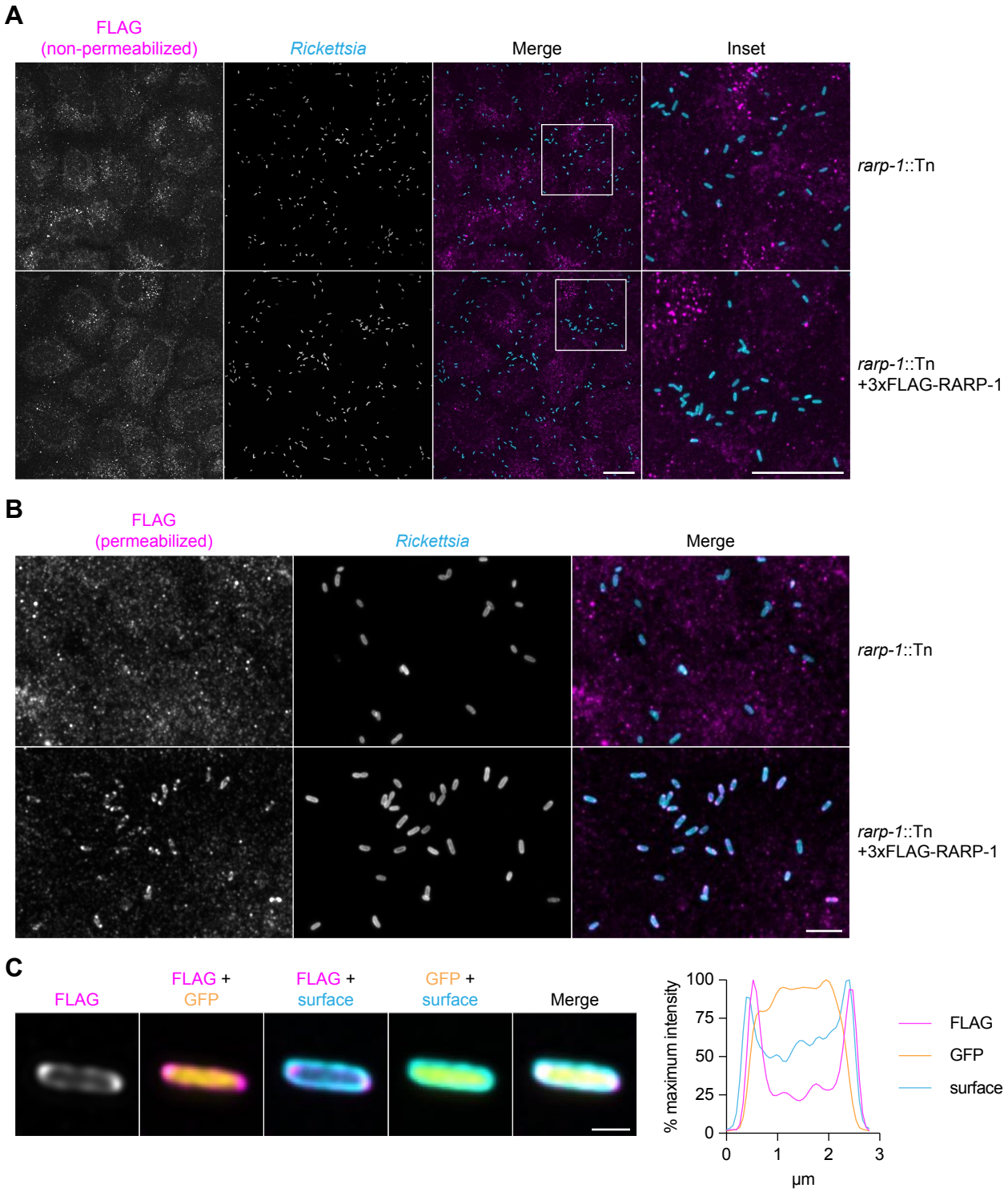

Figure 5. RARP-1 localizes to the *R. parkeri* periplasm. (A) Images of *rarp-1*::Tn (top) and *rarp-1*::Tn + 3xFLAG-RARP-1 (bottom) bacteria during infection of A549 cells. Samples were stained for FLAG (magenta) and the bacterial surface (cyan) without permeabilization of bacteria. Scale bars, 20 µm. (B) Images of *rarp-1*::Tn (top) and *rarp-1*::Tn + 3xFLAG-RARP-1 (bottom) bacteria during infection of A549 cells. The bacterial surface (cyan) was stained prior to permeabilization by lysozyme and detergent and subsequent staining for FLAG (magenta). Scale bar, 5 µm. (C) Subcellular localization of 3xFLAG-RARP-1 in a representative *rarp-1*::Tn + 3xFLAG-RARP-1 bacterium during infection of A549 cells. The bacterial surface (cyan) was stained prior to permeabilization by lysozyme and detergent and subsequent staining for FLAG (magenta). GFP (yellow) demarcates the bacterial cytoplasm. Scale bar, 1 µm. A pole-to-pole 0.26 µm width linescan (right) was generated for FLAG, GFP, and the bacterial surface.

Gene ID	Description
MC1_RS01995	RvhB10; T4SS outer membrane core complex
MC1_RS00605	Sca2; autotransporter; surface actin nucleation
MC1_RS00420	hypothetical lipoprotein
MC1_RS06520	hypothetical porin
MC1_RS02895	hypothetical lipoprotein
MC1_RS00535	hypothetical porin
MC1_RS00570	OmpW family protein; porin
MC1_RS01970	RvhB9a; T4SS outer membrane core complex
MC1_RS01990	RvhB9b; T4SS outer membrane core complex
MC1_RS06075	Pal; peptidoglycan-associated lipoprotein
MC1_RS05020	50S ribosomal protein L17
MC1_RS06525	hypothetical porin
MC1_RS02795	PcaH; protocatechuate-3,4-dioxgenase
MC1_RS00865	HflC; protease modulator
MC1_RS06550	17 kDa surface antigen

Table 1. Co-immunoprecipitation of lysozyme-permeabilized bacteria reveals that RARP-1 interacts with other bacterial factors that access the periplasm.[a,b]

[a]Putative RARP-1 binding partners are ordered by decreasing spectral count.

[b]MC1_RS05020 is the only hit not predicted to access the periplasm.

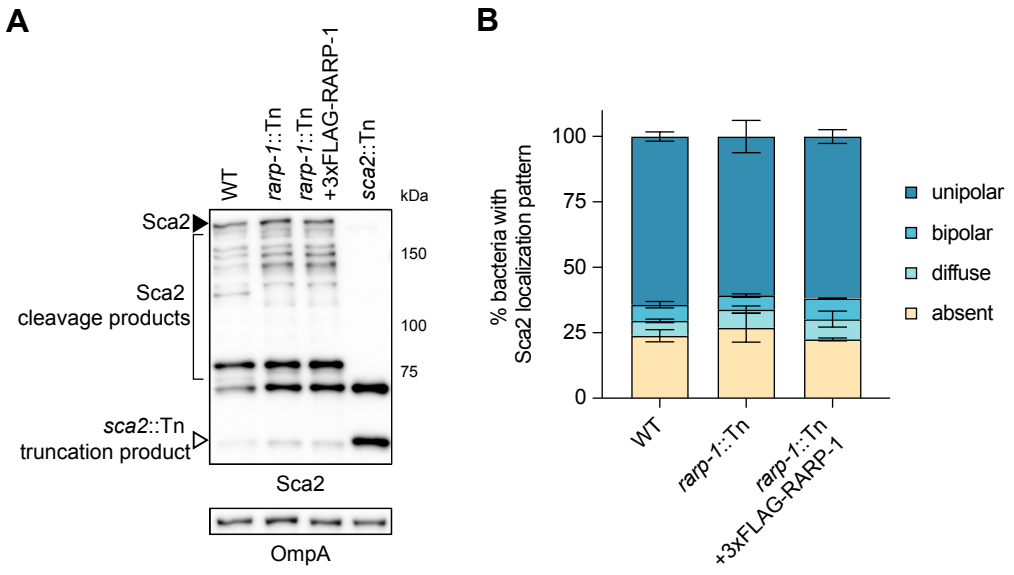

Figure 6. RARP-1 does not regulate the abundance or localization of Sca2. (A) Western blot for Sca2 from purified *R. parkeri* strains. Full-length Sca2 (arrowhead), Sca2 cleavage products (bracket), and the truncation product in the *sca2*::Tn mutant (open arrowhead) are indicated. (B) Percentage of bacteria with the indicated Sca2 localization pattern during infection of A549 cells. Percentages were determined from two independent experiments (≥ 350 bacteria were counted for each infection) and were used to calculate the mean ± SD and p-value (one-way ANOVA with post-hoc Dunnett's test, n.s. relative to WT).