Lysine methylation shields an intracellular pathogen from ubiquitylation

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Abstract

Many intracellular pathogens avoid detection by their host cells. However, it remains unknown how they avoid being tagged by ubiquitin, an initial step leading to anti-microbial autophagy. Here, we show that the intracellular bacterial pathogen *Rickettsia parkeri* uses two protein-lysine methyltransferases (PKMTs) to modify outer membrane proteins (OMPs) and prevent their ubiquitylation. Mutants deficient in the PKMTs were avirulent in mice and failed to grow in macrophages due to ubiquitylation and autophagy. Analysis of the lysine-methylome revealed that PKMTs modify a subset of OMPs by methylation at the same sites that are recognized by host ubiquitin. These findings show that lysine methylation is an essential determinant of rickettsial pathogenesis that shields bacterial proteins from ubiquitylation to evade autophagic targeting.

12 **Main**

13 Intracellular pathogens generally evade the host immune surveillance machinery. This
14 includes avoidance of surface targeting by the host ubiquitylation machinery and subsequent
15 formation of a polyubiquitin coat, a first step in cell-autonomous immunity (*1-5*). The ubiquitin
16 coat recruits autophagy receptors that engage with the autophagy machinery to target cytosol-
17 exposed microbes for destruction (*1, 4-8*). Bacterial outer membrane proteins (OMPs) are targets
18 for the host ubiquitylation machinery (*9, 10*), and the tick-borne obligate intracellular pathogen
19 *Rickettsia parkeri* requires the abundant surface protein OmpB to protect OMPs from
20 ubiquitylation (*11*). However, the detailed mechanisms that *R. parkeri* and other pathogens use to
21 block lysine ubiquitylation of surface proteins, including OMPs, are unknown. We hypothesized
22 that cell surface structures or modifications could provide protection at the molecular level. One
23 such modification is lysine methylation, which is widespread in all domains of life. This
24 modification involves the transfer of one, two, or three methyl groups to the amino group of lysine
25 side chains, the same amino group that can also be modified by ubiquitin (*12*). Whether lysine
26 methylation of bacterial surfaces prevents host detection and promotes intracellular survival has
27 not been explored.

28 To identify bacteria-derived surface modifications that protect against ubiquitin coating,
29 we screened a library of *R. parkeri* transposon mutants (*13*) (**Table S1**) for increased
30 polyubiquitylation (pUb) relative to wild-type (WT) in Vero cells (an epithelial cell line commonly
31 used to propagate and study intracellular pathogens) by immunofluorescence microscopy (**Fig.
32 1A**). We subsequently analyzed individual mutants and identified 4 that were ubiquitylated, similar
33 to *ompB* mutant bacteria (*11*) (**Fig. 1B** and **D**). Importantly, these 4 strains expressed OmpB (**Fig.
34 S1**). Two of the strains had insertions in the protein-lysine methyltransferase genes *pkmt1* and

35 *pkmt2*, which are located in two distinct chromosomal regions. The remaining two strains had
36 insertions in the *wecA* and *rmlD* genes, which are required for the biosynthesis of O-antigen (**Fig.**
37 **S2**), a common surface molecule in Gram-negative bacteria. The O-antigen also protects
38 *Francisella tularensis* from ubiquitylation (*5*), suggesting it performs a conserved function in this
39 process. As a control, we analyzed a strain with a mutation in the *mrdA* gene, which is required
40 for peptidoglycan biosynthesis and cell shape in other bacteria (*14*). This mutant strain had altered
41 shape but was not polyubiquitylated (**Fig. 1B** and **D**), suggesting that not all bacterial cell envelope
42 structures are required to avoid ubiquitylation. In addition, we further quantified the pUb levels
43 and observed that the *pkmt1*::tn mutant, followed by the *ompB* and *pkmt2*::tn mutants, had the
44 highest levels (**Fig. 1E**). These data indicated that OmpB, PKMTs, and the O-antigen protect *R.*
45 *parkeri* from ubiquitylation.

46 The O-antigen was previously shown to be required for rickettsial pathogenesis (*15*), and
47 OmpB was previously found to be required for *R. parkeri* to cause lethal disease in *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$
48 mice lacking the type I interferon receptor (IFNAR) and IFN-g receptor (IFNGR) (*16*). We
49 therefore examined whether PKMT1 or PKMT2 are important for causing disease *in vivo*. We
50 observed that *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice succumbed to infection with WT but not to *pkmt1*::tn or *pkmt2*::tn
51 bacteria (**Fig. 1F**). Mice infected with the *pkmt1*::tn mutant showed no signs of disease, whereas
52 mice infected with *pkmt2*::tn showed a transient loss in body weight (**Fig. S3**). This indicates that
53 PKMT1 and PKMT2 are determinants of *R. parkeri* pathogenesis.

54 Because PKMT1 or PKMT2 had previously been shown to methylate OmpB *in vitro* (*17-*
55 *20*), we next examined whether methylation protects OmpB, or another abundant outer membrane
56 protein OmpA (*21*), from ubiquitylation. First, Vero cells overexpressing 6xHis-tagged ubiquitin
57 were infected with WT and the *ompB*, *pkmt1*::tn, and *pkmt2*::tn strains. 6xHis-tagged ubiquitin

58 was recruited to the surface of all of the mutants, but not WT bacteria, as observed by
59 immunofluorescence microscopy (**Fig. 2A**). Then, 6xHis-ubiquitylated proteins were affinity-
60 purified from infected cells, and OmpA and OmpB were detected by western blotting. OmpA was
61 shifted towards higher molecular weights in cells infected with mutant, but not WT bacteria,
62 indicating OmpA is ubiquitylated (**Fig. 2B**). Similarly, in comparison with WT bacteria, OmpB
63 was also shifted towards higher as well as lower molecular weights in cells infected with the
64 *pkmt1*::tn and *pkmt2*::tn mutants, suggesting that OmpB is ubiquitylated (**Fig. 2B**). To confirm that
65 methylation protects OmpB and OmpA from ubiquitylation on the bacterial surface, we performed
66 pUb-enrichments of surface fractions from purified bacteria followed by western blotting. This
67 revealed that both OmpB and OmpA shifted towards higher molecular weights in the
68 methyltransferase mutants but not in WT bacteria (**Fig. 2C**). Thus, methylation is critical to protect
69 OMPs from ubiquitylation on the bacterial surface.

70 Based on our observation that methylation protects both OmpA and OmpB from
71 ubiquitylation, we set out to determine how frequently, and to what extent, lysines of *R. parkeri*
72 OMPs are methylated. Peptides with methylated lysines from whole WT bacteria were quantified
73 using label-free liquid chromatography-mass spectrometry (LC-MS). We then analyzed lysine
74 methylation frequency in abundant OMPs as well as other abundant *R. parkeri* proteins (**Table**
75 **S2**). This analysis revealed that *R. parkeri* OmpB, OmpA, and surface cell antigen 2 (Sca2)
76 proteins had the highest abundance of methylated peptides. Lysine methylation was also detected
77 in the outer-membrane assembly protein BamA and in a predicted outer membrane protein porin
78 (WP_014410329.1; from here on referred to as OMP-porin) (**Fig. 3A** and **Table S3**). We next
79 mapped both methylated and unmethylated lysines on the above-mentioned OMPs and found that
80 more than 50% of lysines detected from OmpB and OmpA were methylated, and a significant

81 fraction of lysines were also methylated in Sca2 (31%), OMP-porin (30%), and BamA (27%) (**Fig.**
82 **3B** and **Fig. S4**). Thus, in *R. parkeri*, lysine methylation of OMPs is common.

83 To identify OMPs that are methylated by PKMT1 and PKMT2 during infection, we
84 compared lysine methylation frequencies in WT with those of *pkmt1*::tn or *pkmt2*::tn mutant
85 bacteria using LC-MS. We found that monomethylation of OmpB, OmpA, the predicted OMP-
86 porin, and another surface cell antigen protein Sca1 were reduced in *pkmt1*::tn compared to WT
87 bacteria (**Fig. 3C** and **Fig. S5**). Dimethylation of rickettsial surface proteins was not reduced in the
88 mutants (**Fig. S6**). Although trimethylation was rare and therefore difficult to analyze at the
89 individual protein level (**Fig. S6**), OmpB had reduced trimethylation levels in both
90 methyltransferase mutants (**Fig. 3C**). Notably, the frequency of unmethylated lysines in OmpB
91 was specifically increased in *pkmt1*::tn bacteria (**Fig. 3C** and **Fig. S5**). Lysine methylation of five
92 other surface proteins (Sca1, Sca2, BamA, LomR, and Pal-lipoprotein), and 21 of 23 abundant
93 proteins with different predicted subcellular distributions, was not affected by mutations in the
94 *pkmt1* or *pkmt2* genes (**Fig. S5**). These data indicate that the PKMTs are required for methylation
95 of a specific subset of OMPs including OmpB.

96 To determine which OmpB residues are modified by PKMT1 and PKMT2 during *R.*
97 *parkeri* infection, we analyzed the methylation frequency of individual lysines in the mutants
98 compared to WT using LC-MS. We observed reduced monomethylation frequencies of OmpB
99 K418, K623, K634, K902, K1061, K1294, and K1323 in *pkmt1*::tn bacteria compared with WT,
100 and reduced trimethylation frequencies on K1061 and K388 in the *pkmt2*::tn strain (**Fig. 3D**).
101 These data indicate that several lysines in *R. parkeri* OmpB are methylated by PKMT1 and
102 PKMT2 during infection. Although these results were consistent with previous biochemical results
103 indicating that PKMT1 monomethylates and PKMT2 trimethylates OmpB's lysines (*17, 19*), we

104 found that total OmpB-methylation is unaffected in the *pkmt2*::tn mutant. This suggests that
105 PKMT1 is a primary methyltransferase for OmpB and that it can compensate, at least partly, for a
106 deficiency in PKMT2.

107 To test the hypothesis that methylation of specific lysines in OmpB shields the same
108 residues from ubiquitylation, we performed pUb-enrichments of bacterial surface fractions
109 followed by LC-MS to quantify lysines with diglycine (diGly) remnants, a signature for ubiquitin
110 after trypsin digestion. A prediction of this hypothesis is that individual lysines that are heavily
111 methylated in OmpB of *Rickettsia* species (*17*), including WT *R. parkeri* (**Fig. 3D**), are targets for
112 ubiquitylation in *pkmt1*::tn bacteria. In support of this hypothesis, OmpB K634 and K623 in
113 *pkmt1*::tn exhibited 7 to 10,000-fold increased ubiquitylation compared with WT bacteria (**Fig.
114 3E, F** and **Fig. S7**; **Table S4**). Furthermore, we observed a 13-fold increase in ubiquitylation of
115 the OMP-porin in *pkmt1*::tn bacteria (**Table S4**), indicating that methylation also protects
116 additional OMPs. However, in the *pkmt2*::tn mutant, differential OMP-ubiquitylation was below
117 detection limits (**Fig. 3E, F** and **Table S4**). Together, these data indicate that methylation by
118 PKMT1 camouflages lysines in OMPs from ubiquitylation.

119 Because polyubiquitylation promotes recruitment of the autophagy receptors
120 p62/SQSTM1 and NDP52 (*7, 8*), we hypothesized that lysine methylation shields OMPs from
121 ubiquitylation to block recruitment of these proteins. Consistent with this hypothesis, we observed
122 that the majority of *pkmt1*::tn, *pkmt2*::tn, as well as *ompB* mutant bacteria, co-localized with p62
123 and NDP52 by immunofluorescence microscopy (**Fig. S8**). These data demonstrate that PKMTs
124 protect *R. parkeri* from autophagy recognition.

125 Many pathogenic bacteria including *R. parkeri* grow in immune cells such as macrophages
126 (*11*), despite the fact that microbial detection in such cells triggers anti-bacterial pathways. We

127 therefore investigated whether PKMT1 or PKMT2 were required for evading autophagy targeting
128 and bacterial growth in cultured bone marrow-derived macrophages (BMDMs), as was observed
129 for OmpB (*11*). BMDMs were generated from control mice and mice lacking the gene encoding
130 for autophagy related 5 (ATG5), a protein required for optimal membrane envelopment around
131 pathogens targeted by autophagy, and for their subsequent destruction (*6*). We observed that
132 *pkmt1*::tn mutant bacteria were unable to grow in control BMDMs (*Atg5*$^{flox/flox}$), and that growth
133 was rescued in *Atg5*-deficient BMDMs (*Atg5*$^{-/-}$). Further, >91% of *pkmt1*::tn bacteria were labeled
134 with both pUb and p62 in *Atg5*-deficient BMDMs (**Fig. 4A, B, C** and **D**), suggesting that ubiquitin-
135 tagged bacteria were not restricted when the autophagy cascade was prevented. In contrast, the
136 *pkmt2*::tn mutant did not have a major growth defect compared with WT bacteria and 50% of the
137 bacteria were labeled with p62, irrespective of host genotype (**Fig. 4A, B, C** and **D**), consistent
138 with less pronounced ubiquitylation phenotypes compared to *pkmt1*::tn bacteria. Collectively,
139 these data indicate that methylation is required for *R. parkeri* growth in macrophages by avoiding
140 autophagic targeting.

141 Our work reveals a detailed molecular mechanism that camouflages bacterial surface
142 proteins from host detection. In particular, we found that lysine methylation was essential for
143 blocking ubiquitylation, a first step in cell-autonomous immunity (*1-4*). This highlights an intricate
144 evolutionary arms race between pathogens and hosts and reveals a strategy that pathogens can
145 adapt to counteract host responses. The lysine methyltransferases PKMT1 and PKMT2 are
146 conserved between rickettsial species (**Fig. S9** and **Fig. S10**) and contain a core Rossmann fold
147 found in the broader superfamily of class I methyltransferases that exist in diverse organisms (*12,*
148 *18*). Thus, we propose that lysine methylation, and potentially other lysine modifications, could be
149 used by pathogens, symbionts, and perhaps even in eukaryotic organelles, to prevent undesirable

150 surface ubiquitylation and downstream consequences including elimination by autophagy. Further
151 study of microbial surface modifications will continue to enhance our understanding of the
152 pathogen-host interface and could ultimately lead to new therapeutic targets for treating human
153 diseases including those caused by infectious agents.

References and Notes

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240 **Acknowledgements**

241 We thank Prof. Dan Portnoy (UC Berkeley) and Dr. Lilliana Radoshevich (University of
242 Iowa) for providing comments on this manuscript, and Neil Fischer (UC Berkeley) for critically
243 reading the manuscript. We also thank Dr. Hwan Kim (Stony Brook University) for kindly
244 providing the O-antigen antibody and for fruitful discussions. We are also grateful for the
245 *Atg5*$^{flox/flox}$ and *Atg5*$^{-/-}$ BMDMs provided by Dr. G. Golovkine (UC Berkeley), and Profs. Jeffery
246 S. Cox and Michael Rape (both UC Berkeley) for fruitful discussions. **Funding:** P.E. was
247 supported by a postdoctoral fellowship from the Sweden-America Foundation. M.D.W. was
248 supported by NIH/NIAID grants R01 AI109044 and R21 AI138550. A mass spectrometer used in
249 this study was purchased with support from NIH grant 1S10 OD020062-01. **Author**
250 **contributions:** P.E. conceived the study with the assistance of M.D.W. P.E. performed laboratory
251 work and analysis, except for mass spectrometry, which was conducted together with A.T.I, and
252 animal experiments, which were conducted by T.P.B. P.E. drafted the initial manuscript, and A.T.I,
253 T.P.B, and M.W.D provided editorial feedback. **Data and materials availability:** All data used
254 in the analysis are provided in the main text or supplementary materials. Materials are available
255 upon request.

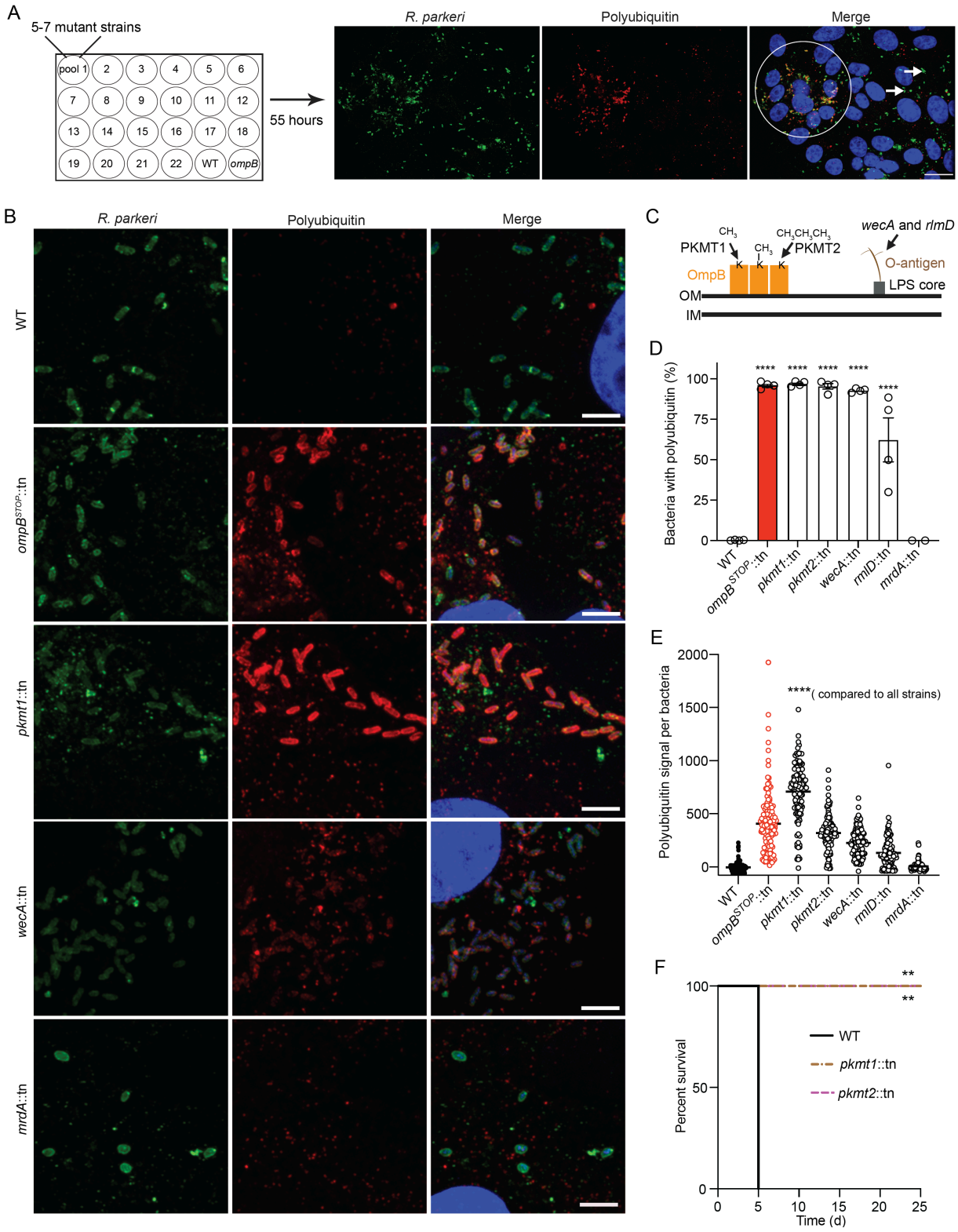


Fig. 1. The O-antigen and lysine methylation are virulence factors that protect *R. parkeri* from ubiquitylation. (**A**) Pools of *R. parkeri* (green) mutants screened for increased pUb (red) via immunofluorescence microscopy. DNA, blue. *ompB* mutant (*11*), positive control; WT,

negative control. Circle, pUb-positive bacteria; arrows pUb-negative bacteria. Scale bar, 20 µm. (**B**) Infected cells at 72 hours post-infection (h.p.i.) stained as in **A**. Scale bar, 5 µm (n = 4). (**C**) Biological function of the genes identified. (**D**) Percentage of bacteria co-localized with pUb at 72 h.p.i. (Data are the mean ± s.e.m.; n = 4 for WT and mutant bacteria; *mrdA*::tn; n = 2). Statistical comparisons between WT and mutants were performed using a one-way ANOVA with Dunnett's post-hoc test; **** $P < 0.0001$. (**E**) pUb signal per bacteria. (Lines indicate the means; n = 3 fields of vision). Statistical comparisons were performed using a Kruskal-Wallis test with Dunn's post-hoc test; **** $P < 0.0001$. (**F**) Survival of *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice intravenously infected with 5×10^6 WT or mutant bacteria (n = 5 mice, WT; n = 6 mice, *pkmt1*::tn and *pkmt2*::tn). Statistical comparison between WT and mutants was performed using a two-way ANOVA; ** $P < 0.01$.

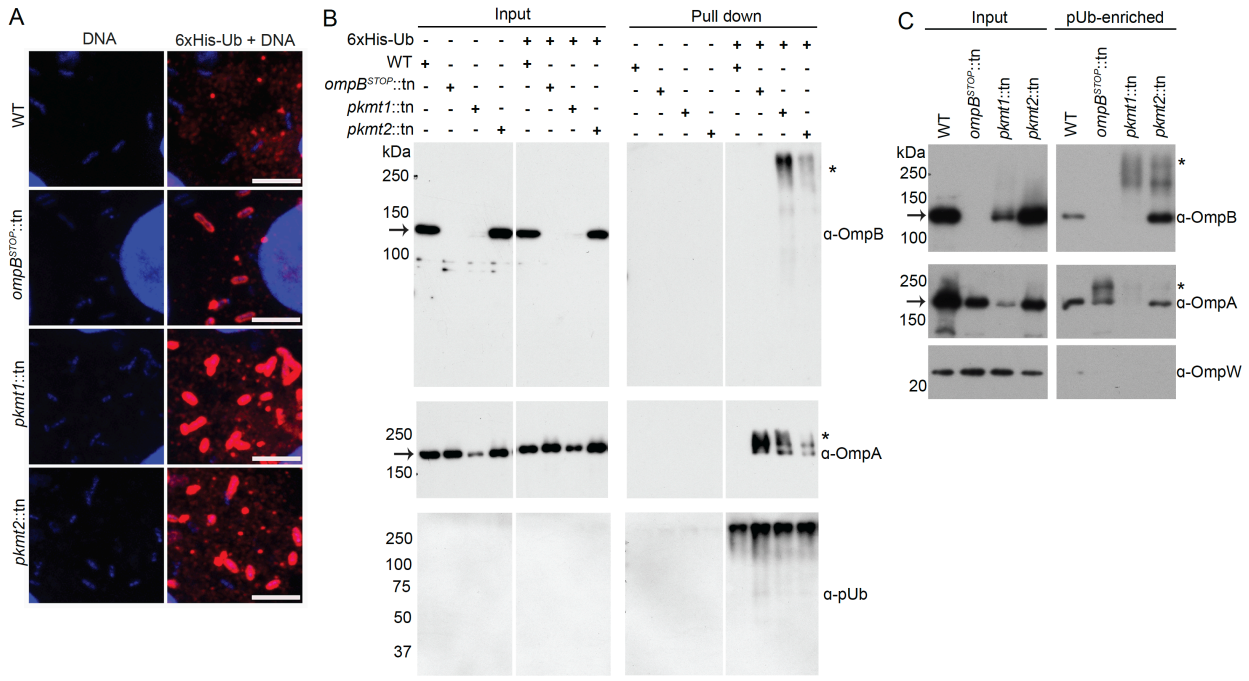


Fig. 2. Lysine methylation protects OMPs from ubiquitylation. (**A**) Micrographs of infected Vero cells expressing 6xHis-ubiquitin stained with anti-His antibody (red) and Hoechst (blue, bacterial and host DNA), at 28 h.p.i. Scale bar, 5 µm (representative of $n = 2$). (**B**) Western blot of His-Ub input and pull-down samples from infected control and 6xHis-ubiquitin expressing cells, probed for OmpB, OmpA, and pUb (representative of $n = 3$). (**C**) pUb-enriched (TUBE-1, pan specific) samples from purified bacteria probed for OmpB, OmpA, and OmpW (OmpB and OmpA of endogenous molecular weight represent non-specific binding to TUBE-1 beads) (representative of $n = 3$). Asterisks indicate OmpB and OmpA that exhibit increased molecular weight, indicating ubiquitylation. Arrows indicate OmpB and OmpA of endogenous molecular weight.

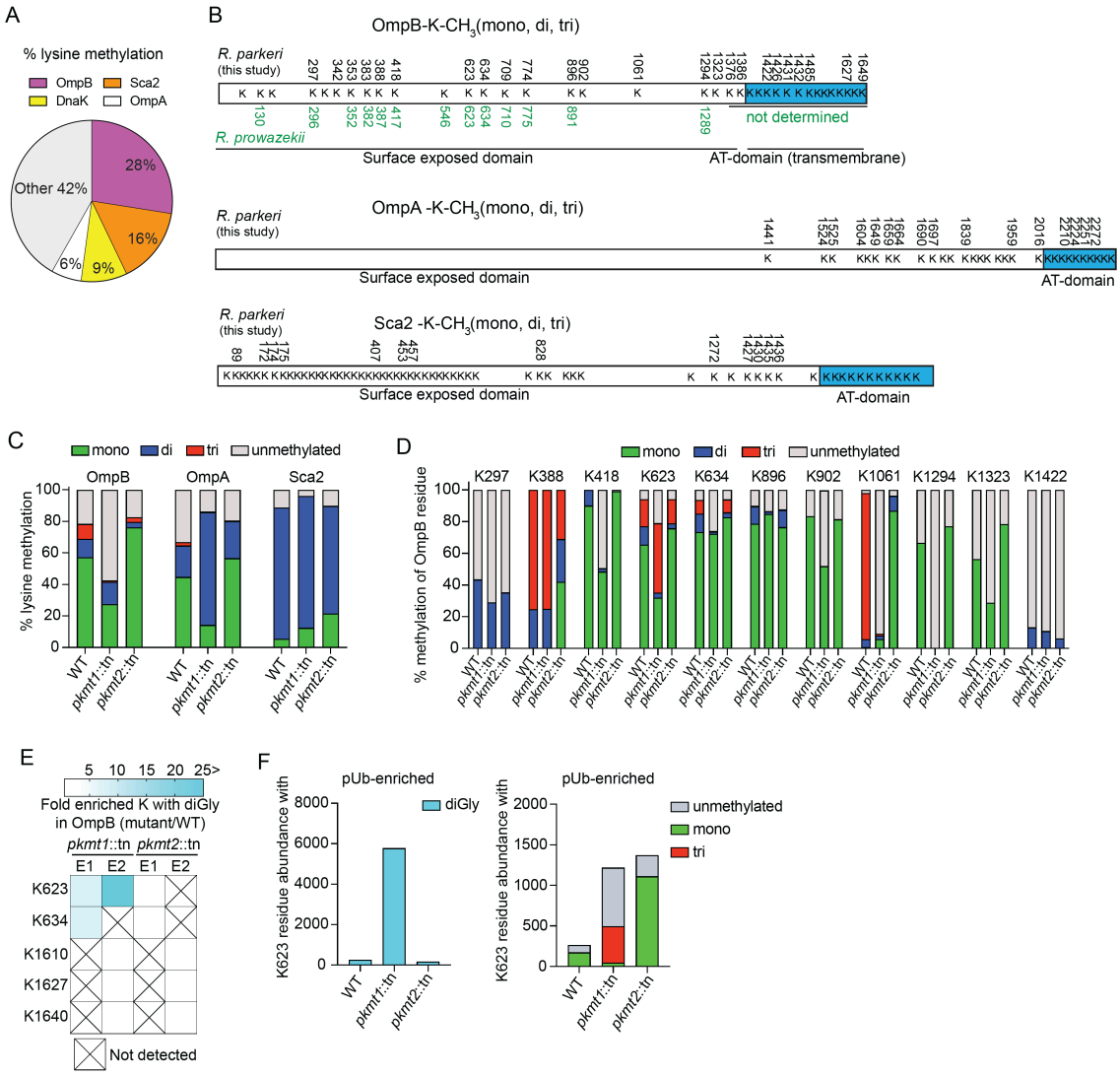


Fig. 3. Methylation camouflages lysines from ubiquitylation. (**A**) Percentage lysine methylation abundance of total among the abundantly detected proteins in WT *R. parkeri* determined by LC-MS (data are combined from $n = 2$). (**B**) Methylated lysines (K) are indicated with residue number in each OMP, and lysines in *R. prowazekii* OmpB known to be methylated in green (*17*). K78, K131, K149, K312, and K547 in OmpB were unmethylated ($n = 5$, using data-independent and data-dependent acquisition modes). (**C**) Percentage of total abundances of the lysines methylated in **B**, that is unmethylated, mono-, di-, or tri-methylated (data are the mean of $n = 2$). (**D**) Percentage of individual lysines in OmpB that are unmethylated, mono-, di- or tri-methylated. Only residues repeatedly detected in all strains were analyzed (data are the mean of $n = 2$). (**E**) Heat map representation of OmpB residues from *pkmt1*::tn or *pkmt2*::tn that have similar levels of K-diGly peptides (white boxes) compared to WT, or with a 5-fold, or more, increase of K-diGly peptides (cyan) after pUb-enrichments ($n = 2$). (**F**) Abundances of ubiquitylated (diGly), unmethylated, mono-, di-, or tri-methylated OmpB K623 (data are the mean of $n = 2$). Each experiment (n) was performed in technical triplicate.

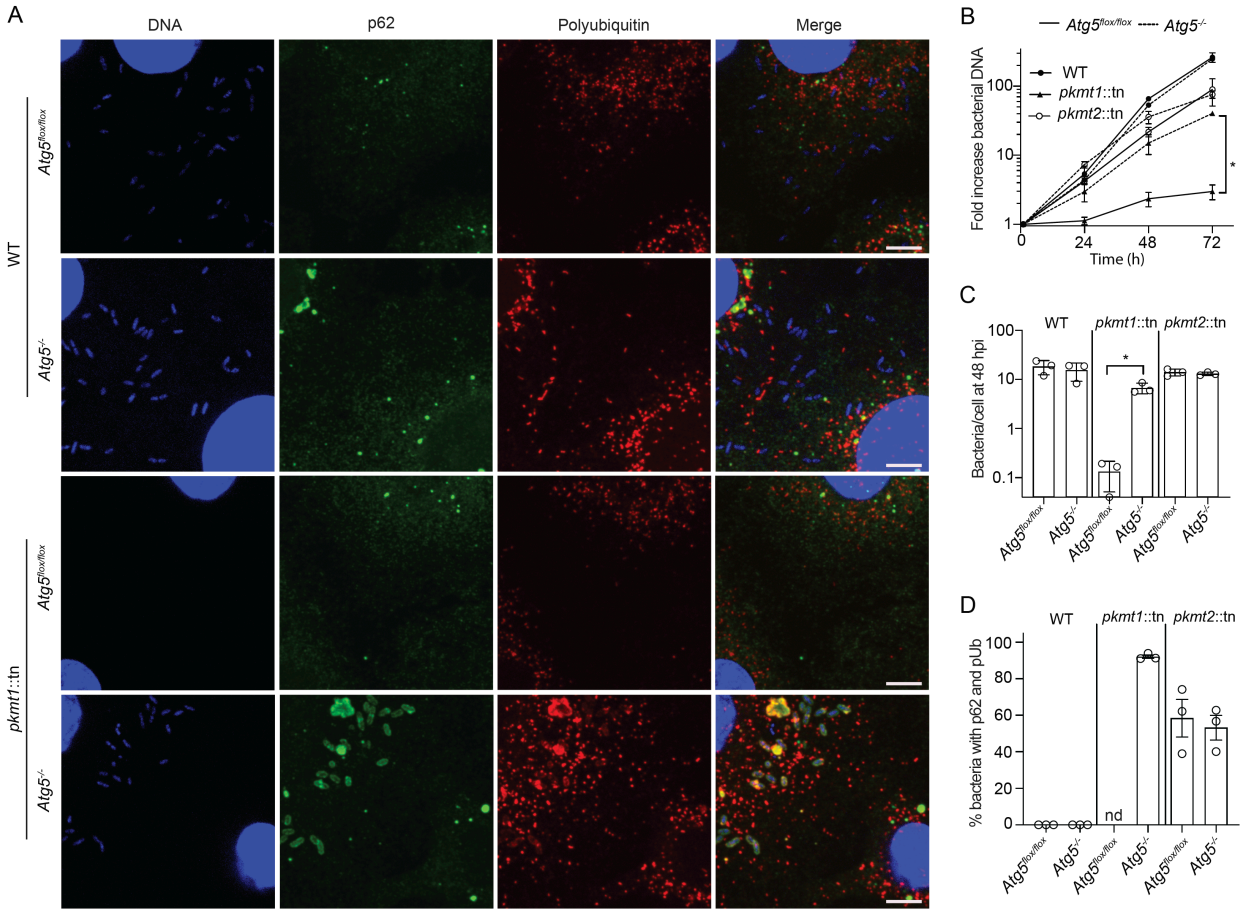
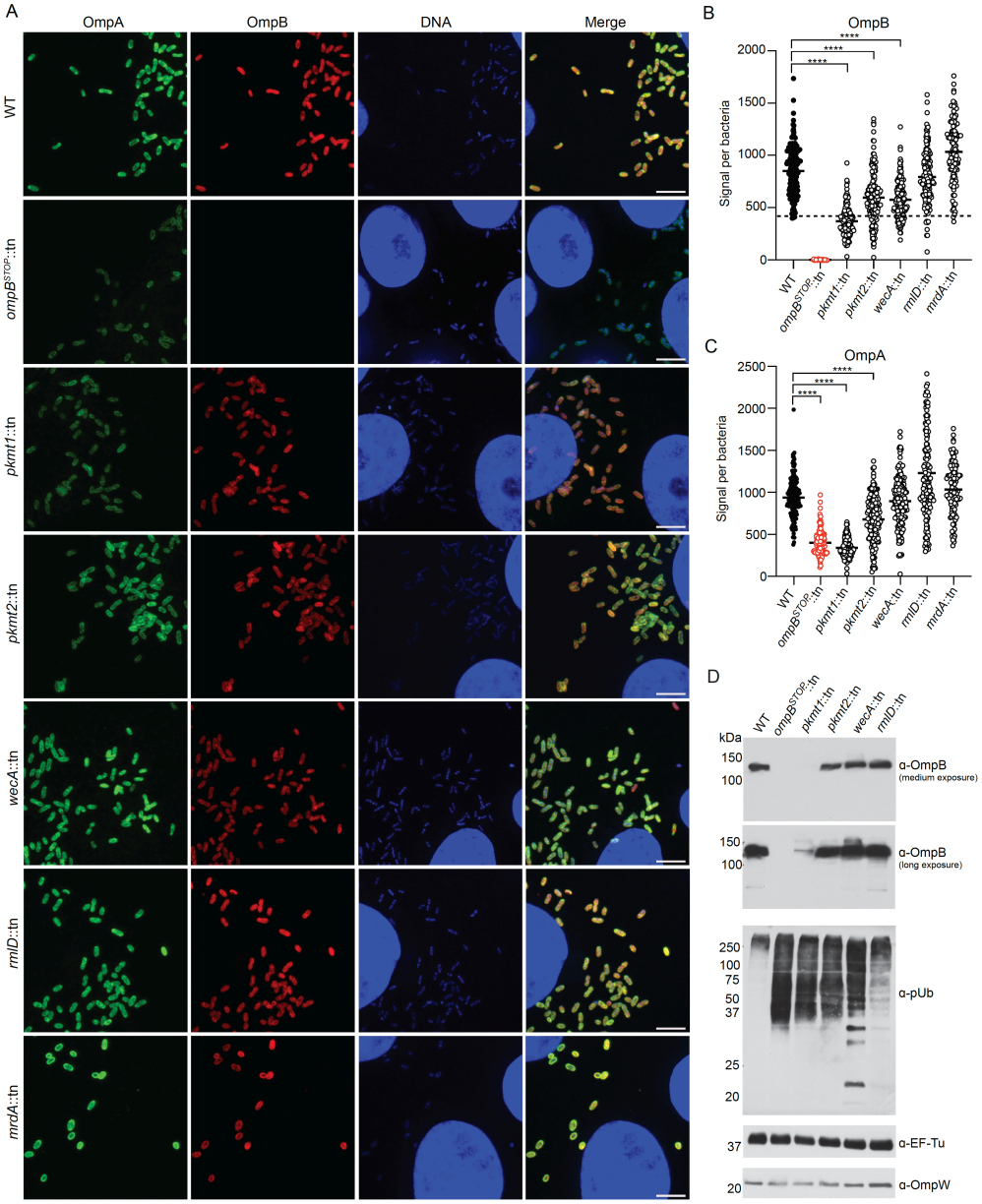


Fig. 4. Methylation prevents ATG5-dependent *R. parkeri* killing in macrophages. (**A**) Micrographs of infected control (*Atg5*$^{flox/flox}$) and *Atg5*-deficient (*Atg5*$^{-/-}$) BMDMs at 48 h.p.i., stained for DNA (blue), pUb (red), and p62 (green). Arrow indicate a bacterium positive for both pUb and p62. Scale bars, 5 µm (n = 3). (**B**) Bacterial growth curves in control and *Atg5*$^{-/-}$ BMDMs, as measured by genomic equivalents using qPCR (n = 3). (**C**) Quantification of the mean number of bacteria per cell (n = 3). (**D**) Quantification of the percentage of bacteria with p62 and pUb (n = 3). nd, not determined. Statistical comparisons in **B** and **C** were performed using a Brown-Forsyth and Welch ANOVA with Dunnett's post-hoc test; *, $P < 0.05$. Presented data are the mean ± s.e.m.

306 **Supplementary Figures**



307

308 **Fig. S1. Polyubiquitylated mutant *R. parkeri* strains are positive for OmpA and OmpB. (A)**
309 Micrographs of Vero cells infected with the indicated strains stained for OmpA (green, 13-3
310 antibody), OmpB (red, OmpB-antibody), and DNA (blue, Hoechst) at 72 h.p.i. (representative of
311 $n = 3$). Scale bar 5 µm. (**B**) Quantification of OmpB signal per bacteria. (Lines indicate the means;
312 $n = 3$ fields of vision; ≥ 50 bacteria per field of vision were analyzed). Statistical comparisons were
313 performed using a Kruskal-Wallis test with Dunn's post-hoc test; **** $P < 0.0001$ between
314 indicated strains. Dashed line indicates that the majority of mutant bacterial populations, except
315 *pkmt1*::tn bacteria, have OmpB levels comparable to WT bacteria. (**C**) Quantification of OmpA
316 signal per bacteria as in **B**. (**D**) Western blot of $5x10^6$ purified bacteria probed for OmpB, pUb,
317 OmpW and EF-Tu (bacterial loading controls) (representative of $n = 2$).

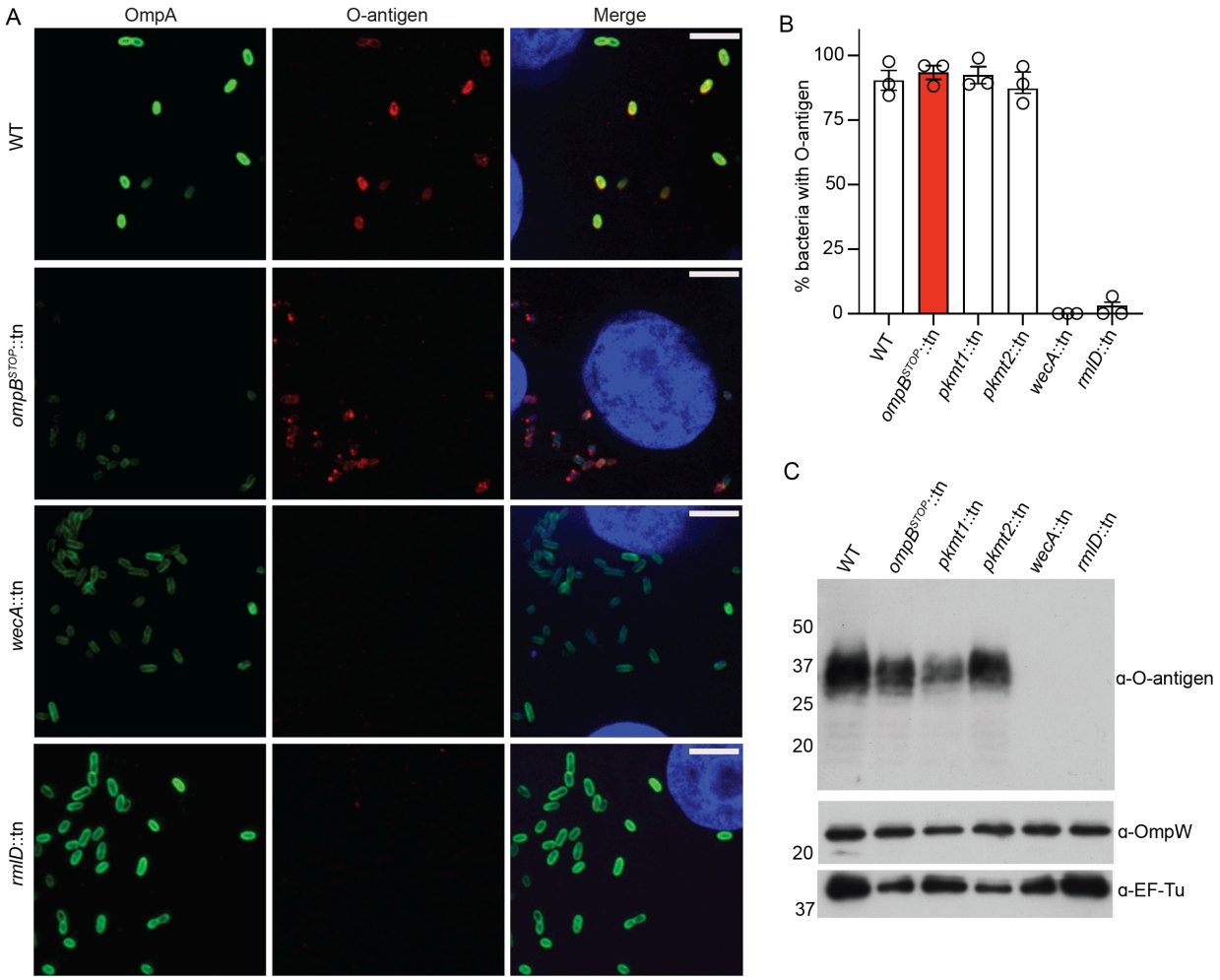
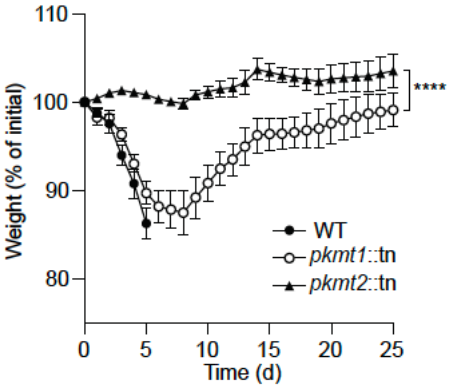


Fig. S2. The *wecA*::tn and *rmlD*::tn bacteria lack the O-antigen. (**A**) Micrographs of Vero cells infected with the indicated strains stained for OmpA (green, 13-3 antibody), the O-antigen (red, O-antigen antibody(*15*)) and DNA (blue, Hoechst) at 72 h.p.i. (representative of $n = 3$). Scale bars, 5 µm. (**B**) Percentage of bacteria positive for the O-antigen at 72 h.p.i. $n = 2$; ≥ 90 bacteria were counted in each infection (data are the mean ± s.e.m.; $n = 3$). (**C**) Western blot of 5×10^6 purified WT and mutant bacteria probed for the O-antigen. OmpW and EF-Tu were used as bacterial loading controls (representative of $n = 3$).



326

327 **Fig. S3. PKMT1 plays a more significant role in causing disease *in vivo* compared to PKMT2.**
328 (**A**) Weight changes of *Ifnar*$^{-/-}$*Ifngr*$^{-/-}$ mice intravenous infected with 5x10^{6} WT, *pkmt1*::tn, or
329 *pkmt2*::tn bacteria (data are the mean ± s.e.m. $n = 5$, WT; $n = 6$, *pkmt1*::tn and *pkmt2*::tn, combined
330 from two independent experiments). A two-way ANOVA from 0 to 25 days post-infection (d.p.i.)
331 was used to statistically compare the weight changes between the *pkmt1*::tn and *pkmt2*::tn mutants.
332 **** $P < 0.0001$.

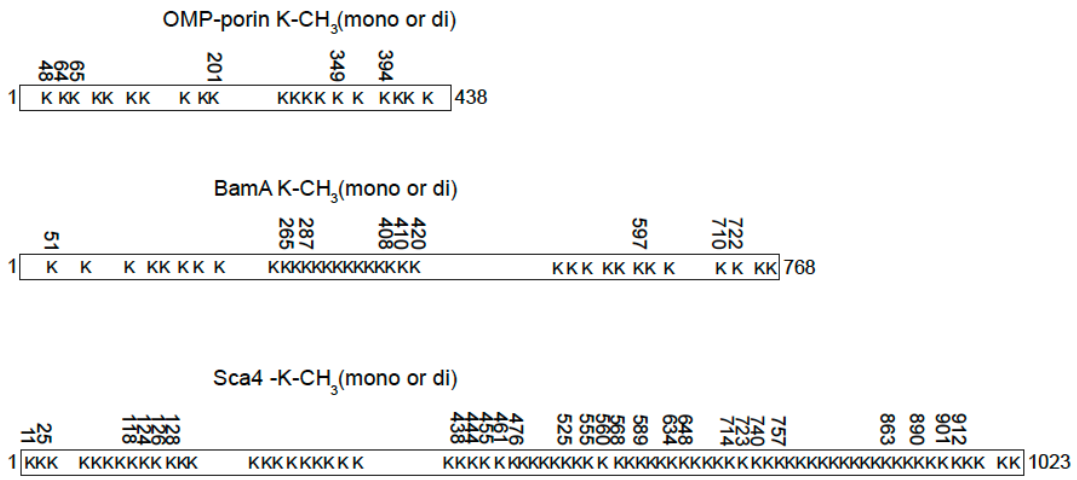


Fig. S4. Lysine-methylome reveals that *R. parkeri* OMP-porin, BamA, and the released factor Sca4 are methylated. Methylated lysines are indicated with residue number, and unmethylated residues without, determined by LC-MS as in **Fig. 3B** (data is combined from five independent experiments). See also **Table S2**.

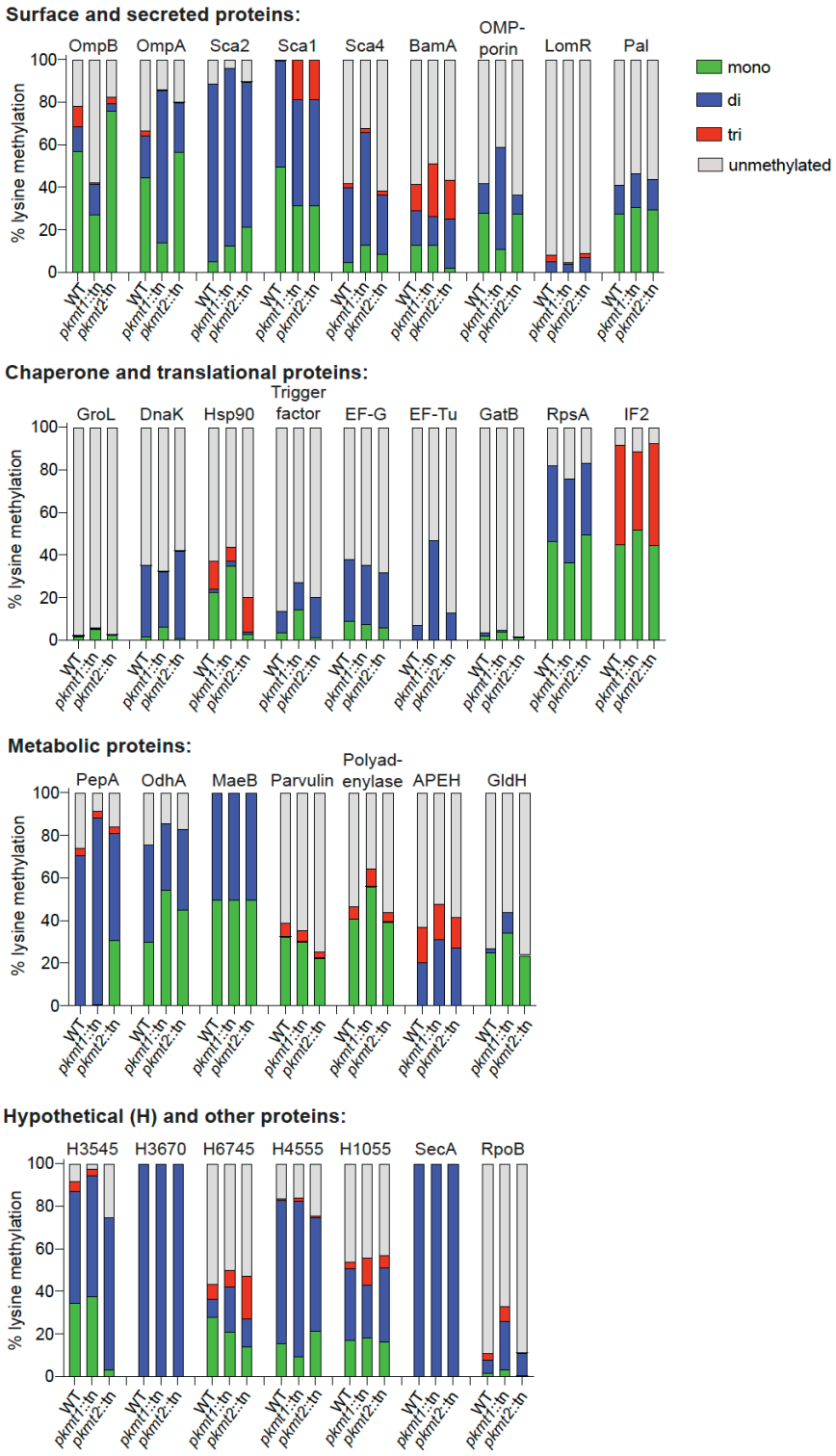


Fig. S5. PKMT1 primarily modifies bacterial OMPs. Percentage of total abundances of the lysines methylated in WT, that is unmethylated, mono-, di-, or tri-methylated in respective strain. Proteins with ≥3 lysines methylated detected in independent experiments are shown (mean of n = 2, performed in technical triplicates). OmpB, OmpA and Sca2 data are the same as in **Fig. 3C**. See also **Table S2**.

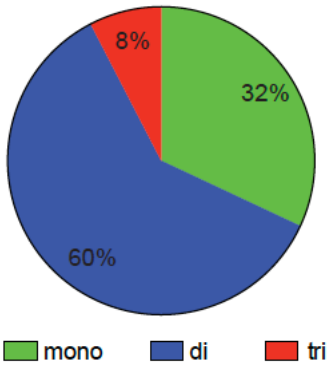


Fig. S6. Dimethylation is a common methylation state among the abundant *R. parkeri* proteins. Percentage of total lysines abundances detected to as mono-, di-, or tri-methylated in WT bacteria of the proteins indicated in **Table S2** (mean of n = 2, performed in technical triplicates).

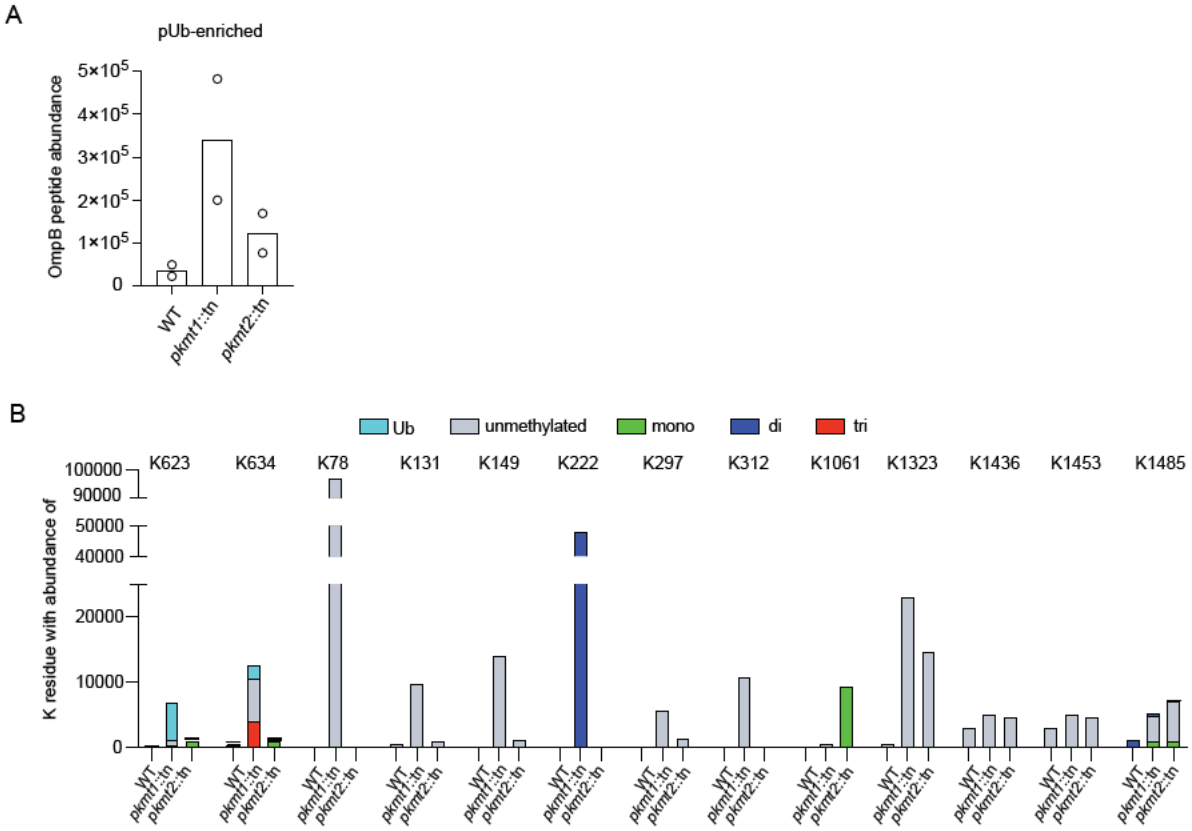


Fig. S7. OmpB K623 and K634 are frequently ubiquitylated when PKMT1-mediated methylation is reduced. (**A**) OmpB peptide abundance values after pUb-enrichments from respective strain, as determined by LC-MS (data are mean of $n = 2$, performed in triplicates). (**B**) Abundance values of lysines in OmpB that are ubiquitylated (diGly), unmethylated, mono-, di-, or tri-methylated, in respective strain after pUb-enrichments as in **Fig 2C.** Only residues detected in independent experiments are shown (data are the mean of $n = 2$, performed in technical triplicates). K623 data in **B** are the same as in **Fig. 3F**.

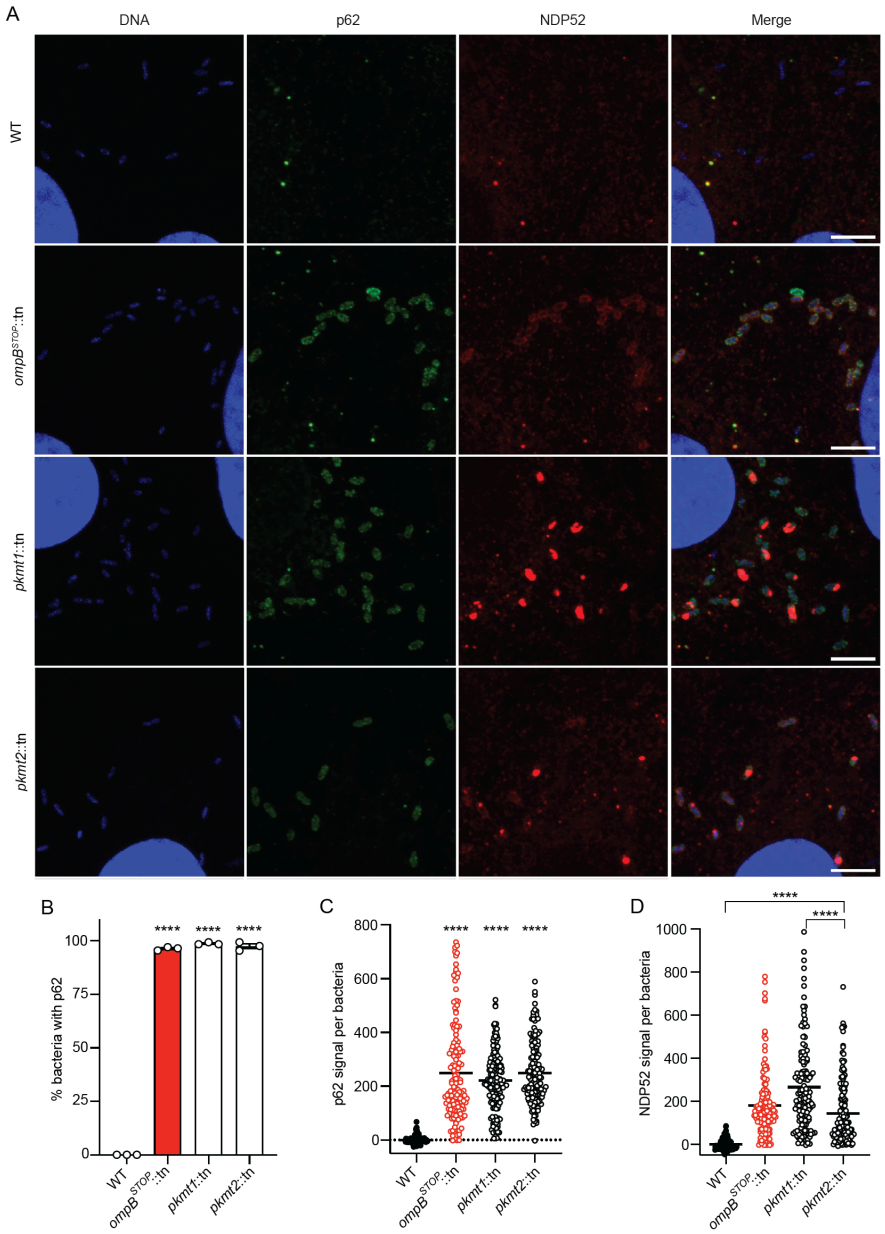


Fig. S8. OmpB and lysine methylation block recruitment of autophagy receptors to *R. parkeri*. (**A**) Micrographs of Vero cells infected with the indicated strains: stained for bacterial and host DNA (blue, Hoechst), p62 (green, p62-antibody), and NDP52 (red, NDP52-antibody) at 72 h.p.i ($n = 4$ for p62 staining; $n = 2$ for NDP52 staining). (**B**) Percentage of bacteria that show rim-like surface localization of p62 at 72 h.p.i. (Data are the mean ± s.e.m.; $n = 3$; ≥ 142 bacteria were counted for each infection). Statistical comparisons between WT and *ompB*^{STOP}::tn, *pkmt1*::tn, *pkmt2*::tn were performed using a one-way ANOVA with Tukey's post-hoc test; **** $P < 0.0001$. (**C**) Quantification of p62 signal per bacteria from a representative experiment. Lines indicate the means. (n = 3 fields of vision; ≥ 50 bacteria per field of vision were analyzed). Statistical comparisons were performed using a Kruskal-Wallis test with Dunn's post-hoc test; **** $P <$ 0.0001 between indicated strains. (**D**) Quantification of NDP52 signal per bacteria as in **C**.

```
Rickettsia parkeri                                   1   MSPKVTNSSSTPNGHDKMAKKTHSAQSVVN--GAVSDHNTYDEIPYESYPYALTNPYHLSTLATLFGVNAPEVENAKILE  78
Rickettsia rickettsii                                1   MSPKATNSSSTPNGHDKMAKKTHSAQSVVN--GAVSDHNTYDEIPYESYPYALTNPYHLSTLATLFGVNAPEVENAKILE  78
Rickettsia conorii                                   1   MSPKATNSSSTPNGHDKMAKKTHSAQSVVN--GAVLDHNTYDEIPYESYPYALTHPYHLSTLATLFGVNAPEVENAKILE  78
Rickettsia typhi                                     1   MSLKSST----TNDHDK-TTKINSIQSLVNctDTVADHNTYDEIPYESYPYAITNPYHLSTLATLFGINAPEVENSKILE  75
Rickettsia prowazeki                                 1   MSLKSTTSSLTTNNHDK-T--INSVQSLVNgtGTVADHNPYDEVPYESYPYAITNPYHLSTLATLFGINAPEVENSKILE  77
Rickettsia bellii                                    1   MSAKASNSSNLPNGHDKTTKEKHNTQPVIN--GAIK-HNTYDEVPYESYPYSFTNPFHLSTLATLFGVDAPNVETAKILE  77
Rickettsia endosymbiont of Proechinophthirus fluctus 1   MSPKATNSSSTPNSHDKMAKKTHSVQSVVN--SAVSDHNTYDEIPYESYPYAFTHPYHLSTLATLFGVNAPEVENAKILE  78

Rickettsia parkeri                                   79  LGCAAGGNLIPHAVLYPKAYFVGVDLSKVQIDEANKNVKALGLKNIEFHHCSITDINDSFGKFDYIICHGVISWVPKTVR  158
Rickettsia rickettsii                                79  LGCAAGGNLIPHAVLYPKAYFVGVDLSKVQIDEANKNVKALGLKNIEFHHCSITDINDSFGKFDYIICHGVISWVPKTVR  158
Rickettsia conorii                                   79  LGCAAGGNLIPHAVLYPKAYFVGVDLSKVQIDEANKNVKALGLKNIEFHHCSITDINDSFGKFDYIICHGVISWVPKTVR  158
Rickettsia typhi                                     76  LGCAAGGNLIPHAVLYPKAHFVGVDLSKVQIDEANKNVRALGLKNIEFHHCSITDINDSFGKFDYIICHGVISWVPKIVR  155
Rickettsia prowazeki                                 78  LGCAAGGNLIPHAVLYPNAHFVGVDLSKVQIDEANKNVRALGLKNIEFHHCSITDIDDSFGKFDYIICHGVISWVPKIVR  157
Rickettsia bellii                                    78  LGCAAGGNLIPHAVLYPKAHFVGIDLSKVQIDEANKTVKELGLKNIEFHHCSILDIDDSLNKFDYIICHGVMSWVSKNVR  157
Rickettsia endosymbiont of Proechinophthirus fluctus 79  LGCAAGGNLIPHAVLYPKAYFVGVDLSKVQIDEANKNVKALGLKNIEFHHCSITDINDSFGKFDYIICHGVISWVPKTVR  158

Rickettsia parkeri                                   159 DKIFEVCNKNLSPNGIAYISYNTLPGWNMVRTIRDMMMYHSSSFANVRDRIAQSRLLLEFVKDSLENSKTPYAEALKTEA  238
Rickettsia rickettsii                                159 DKIFEVCNKNLSPNGIAYISYNTLPGWNMVRTIRDMMMYHSSSFANVRDKIAQSRLLLEFVKDSLENSKTPYAEALKTEA  238
Rickettsia conorii                                   159 DKIFEVCNKNLSPNGIAYISYNTLPGWNMVRTIRDMMMYHSSSFANVRDRIAQSRLLLEFVKDSLENSKTPYAEALKTEA  238
Rickettsia typhi                                     156 DKIFKVCNSNLSTNGIAYISYNTLPGWNMVRTIRDMMLYHSSSFTNVRDRIAQSRLLLEFVKDSLENSKTPYAEVLKTEA  235
Rickettsia prowazeki                                 158 DKIFKVCNRNLSTNGIAYISYNTLPGWNMVRTIRDMMLYHSSSFTNIRDRIAQSRLLLEFVKDSLEHSKTPYAEVLKTEA  237
Rickettsia bellii                                    158 DKIFDVCNKNLSKNGIAYISYNTLPGWNMVRTVRDMMLYHSSSFVNVRDKIAQSRLLLDFVKDSLENSKTPYAEVLKTEA  237
Rickettsia endosymbiont of Proechinophthirus fluctus 159 DKIFEVCNKNLSPNGIAYISYNTLPGWNMVRTIRDMMMYHSSSFANVRDRIAQSRLLLEFVKDSLENSKTPYAEALKTEA  238

Rickettsia parkeri                                   239 GLLAKQTDHYLRHDHLEEENAQFYFHEFMNEARKHNLQYLADCNLSTMYLGNMPPKVVEQLKAVNDIVRTEQYMDFITNR  318
Rickettsia rickettsii                                239 GLLAKQTDHYLRHDHLEEENAQFYFHEFMNEARKHNLQYLADCNLSTMYLGNMPPKVVEQLKAVNDIVRTEQYMDFITNR  318
Rickettsia conorii                                   239 GLLAKQTDHYLRHDHLEEENAQFYFHEFMNEARKHNLQYLADCNLSTMYLGNMPPKVVEQLKAVNDIVRTEQYMDFITNR  318
Rickettsia typhi                                     236 GLLAKQTDHYLRHDHLEEENAQFYFHEFMNEARKYNLQYLADCNISTMYLGNMPPKVVEQLKAVNDIVRTEQYMDFITNR  315
Rickettsia prowazeki                                 238 GLLAKQTDHYLRHDHLEEENAQFYFHEFMNEARKHNLQYLADCNISTMYLGNMPPKVVEQLKAVNDIVRTEQYMDFITNR  317
Rickettsia bellii                                    238 GLLSKQTDHYLRHDHLEEENAQFYFHEFMDEARKHNLQYLADCNLSTMYLGNMPQKVVEQLKVVNDIVRTEQYMDFITNR  317
Rickettsia endosymbiont of Proechinophthirus fluctus 239 GLLAKQTDHYLRHDHLEEENAQFYFHEFMNEARKHNLQYLADCNLSTMYLGNMPPKVVEQLKAVNDIVRTEQYMDFITNR  318

Rickettsia parkeri                                   319 RFRTTLLCHSDVKINRNINNDDITKFNIIFNIVPEKPLKEVDLNNASENLKFFLNGNQDSNLTTSSPYMKAILYTFSENL  398
Rickettsia rickettsii                                319 RFRTTLLCHSDVKINRNINNDDITKFNIIFNIVPEKPLKEVDLNNASENLKFFLNGNQDSNLTTSSPYMKAILYTFSENL  398
Rickettsia conorii                                   319 RFRTTLLCHSDVKINRNINNDDITKFNIIFNIVPEKPLKEVDLNNASENLKFFLNGNQDSNLTTSSPYMKAILYTFSENL  398
Rickettsia typhi                                     316 RFRTTLLCHNDLKINRNINNEDIKKFNIIFNVVPEKPLQEVDLDNAAENLQFFLNGNKECNLSTTSPYMKAILYTFSENL  395
Rickettsia prowazeki                                 318 RFRTTLLCHNDLKINRNINNDDIKKFNIIFNVIPEKPLKEVDLNNATENLQFFLNGNKESNLSTTSPYMKAILYTFSENL  397
Rickettsia bellii                                    318 RFRSTLLCHNDVKINRNINNEDIMKFNMIFNVVPEKSLKEVDLNNSSESLAFFLNGNKDSNLTTASPYMKAILYTFSEHL  397
Rickettsia endosymbiont of Proechinophthirus fluctus 319 RFRTTLLCHSDVKINRNINNDDITKFNIIFNIVPEKPLKEVDLNNASENLKFFLNGNQDSNLTTSSPYMKAILYTFSENL  398

Rickettsia parkeri                                   399 NNPLSFEKITTEANKKLHNTKLNEIKAEFLNNAMKLVLQGYISITNQKHRNNPELDKPKTTKMVIHQATHTPSMWVTNLK  478
Rickettsia rickettsii                                399 NNPLSFEKITTEANKKLHNTKLNEIKAELLNNAMKLVLQGYISITNQKHRNNPELDKPKTTKMVIHQATHTPSMWVTNLK  478
Rickettsia conorii                                   399 NNPLSFEKITTEANKKLHNTKLNEIKAELLNNAMKLVLQGYISITNQKHRNNPELDKPKTTKMVIHQATHTPSMWVTNLK  478
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Rickettsia prowazeki                                 398 NNPLSFKQVTSEANKKLNNTKLNEIKNELLNNAMKLVLQGYISITNQKHRSKPVLDKPKTTQMVIYQAKYTPSMWVTNLK  477
Rickettsia bellii                                    398 NNPLSFEKITEEANKKLHGTKLNEIKAELLENAMKLVLQGYINITTQKHRETPELNKPKTTKLVIHQAIHTPYMWVTNLK  477
Rickettsia endosymbiont of Proechinophthirus fluctus 399 NNPLSFEKITTEANKKLHNTKLNEIKAELLNNAMKLVLQGYISITNQKHRNNPELDKPKTTKMVIHQATHTPSMWVTNLK  478

Rickettsia parkeri                                   479 HEPIGVNFFEKFALRYMDGKHDKKAIIEAVLGHVEKGELTLSKEGQKVENKEEIRKELESLFIPMIKKFSSNALLV  554
Rickettsia rickettsii                                479 HEPIGVNFFEKFALRYMDGKHDKKAIIEAVLGHVEKGELTLSKEGQKVENKEEIRKELESLFIPMIKKFSSNALLV  554
Rickettsia conorii                                   479 HEPIGVNFFEKFALRYMDGKHDKKAIIEAVLGHVEKGELTLSKEGQKVENKEEIRKELESLFIPMIKKFSSNALLV  554
Rickettsia typhi                                     476 HEPIGVNFFEKFALRYMDGKNDKKAIIEAILGHVEKGELTLSKEGQKIENKEEIRKELESLFTPMIEKFASNALLV  551
Rickettsia prowazeki                                 478 HEPIGVNFFEKFALRYMDGRNDKKAIIEAILGHVEKGELTLSREGQKIENKEEIRKELESLFTPMIEKFCSNALLV  553
Rickettsia bellii                                    478 HEPVGVNFFEKLAIRYMDGKHDKKAIIDAVMGHVVKGEINLSKDGQKIEDQEVIRKQLEVLFMPMIDKFAANALLV  553
Rickettsia endosymbiont of Proechinophthirus fluctus 479 HEPIGVNFFEKFALRYMDGKHDKKAIIEAVLGHVEKGELTLSKDGQKVENKEEIRKELESLFIPMIKKFSSNALLV  554
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Fig. S9. The PKMT1 enzyme is highly conserved between diverse rickettsial species. Amino acid sequence alignment of PKMT1 from *R. parkeri* (WP_014411082.1), *R. rickettsii* (WP_012262600.1), *R. conorii* (WP_016926592.1), *R. typhi* (WP_011191207.1), *R. prowazekii* (WP_004596928.1), *R. bellii* (WP_045799810.1), and a *Rickettsia* endosymbiont (WP_062811822.1), using COBALT. Amino acids indicated in red are identical; blue, variation between species; grey, one or more of the analyzed proteins are lacking this residue(s).

```
Rickettsia parkeri      1   MTKQANKISYDEVPYSPFTFSYTSPPYLRTIGKLFGLNPPPLETAKILELGCGIGVNLLNFAETYPKSQSLGVDLSKTQI  80
Rickettsia rickettsii   1   MTKQANKISYDEVPYSPFTFSYTSPPYLRTIGKLFGLNPPPLETAKILELGCGIGVNLLNFAETYPKSQSLGVDLSKTQI  80
Rickettsia conorii      1   MTKQANKISYDEVPYSPFTFSYTSPPYLRTIGKLFGLNPPPLETAKILELGCGIGVNLLNFAETYPKSQSLGVDLSKTQI  80
Rickettsia typhi        1   MIKKANKISYDEVPYPPFTFSYTYPPYLRTIGKLFGLNPPLLETAKVLDIGCGIGVNLLNFAETYPKSQSLGVDLSKTQI  80
Rickettsia prowazeki    1   MIKKTNKISYDEVPYPPFTFSYTYPPYLRTIGKLFGLNPPPLETAKILDIGCGVGVNLLNFAETYPKSQSLGVDLSKTQI  80

Rickettsia parkeri      81  ELGKKFISDLKIKNAELKALSILDLDESYGKFDYIVCHGVYSWVPEEVQDKILKVCNKLLNPNGIAFVSYNTLPGWNMQR  160
Rickettsia rickettsii   81  ELGKKIISDLKIKNAELKALSILDLDESYGKFDYIICHGVYSWVPEEVQDKILKVCNKLLNPNGIAFVSYNTLPGWNMQS  160
Rickettsia conorii      81  ELGKKIISDLKIKNAELKALSILDLDESYGKFDYIVCHGVYSWVPEEVQDKILKICNKLLNPNGIAFVSYNTLPGWNMQR  160
Rickettsia typhi        81  ELGKKTISDAKINNVELKALSILDLDESYGKFDYIVCHGVYSWVSQEVQDKILEVLNKLLNPNGIAFVSYNTLPGWNMQN  160
Rickettsia prowazeki    81  EIGKKTISDSKIKNVGLKALSILDLDESYGKFDYIVCHGVYSWVSKEVQDKILEVLNKLLNPNGIAFISYNTLPGWNMQN  160

Rickettsia parkeri      161 TIREMIMFHSELFNTSHDKLQQAKLLLKFINDSLESSTTPYSNFLRDETKLLSAYTDSYVLHEYLGEINTGIYFHQFIEK  240
Rickettsia rickettsii   161 TIREMIMFHSELFNTSHDKLQQAKLLLKFINDSLESSTTPYSNFLRDETKLLSAYTDSYVLHEYLGEINTGIYFHQFIEK  240
Rickettsia conorii      161 TIREMIMFHSELFNTSHDKLQQAKLLLKFINDSLESSTTPYSNFLRDETKLLSAYTDSYVLHEYLGEINTGIYFHQFIEK  240
Rickettsia typhi        161 TIREMMMFHSESFNTSHDKLQQARLLLKFINDSLGNSTTPYANFLRDEAKLISTYDDSYVLHEYLGEINTGTYFHQFIEK  240
Rickettsia prowazeki    161 TIREMMMFHSESFNTSHDKLQQSKLLLKFINDSLENSTTPYANFLREEAKLISTYADSYVLHEYLGEINTGTYFHQFIEK  240

Rickettsia parkeri      241 AQKNHLNYLGDTSLTAMFIGNLPTQAAEKLQAVNDIVRTEQYMDFITNRKFRSTLLCHQNIPINRKIEFNNLKEFFTSLN  320
Rickettsia rickettsii   241 AQKNHLNYLGDTSLTAMFIGNLPTQAAEKLQAVNDIVRTEQYMDFITNRKFRSTLLCHQNIPINRKIEFNNLKEFFTSLN  320
Rickettsia conorii      241 AQKNHLNYLGDTSLTAMFIGNLPTQAAEKLQAVNDIVRTEQYMDFITNRKFRSTLLCHQNIPINRKIEFNNLKEFFTSLN  320
Rickettsia typhi        241 AQKNHLNYLGDTSIAAMFIGNLPTKAASKLQAINDIVCTEQYMDFITNRKFRSTLLCHQNIPINRKIEFDNLKDFYTTFN  320
Rickettsia prowazeki    241 AQKNHLNYLGDTSITAMFIGNLPTKAAEKLQAINDIVRTEQYMDFITNRKFRSTLLCHQNIPINRKIEFENLKDFYTTFN  320

Rickettsia parkeri      321 IRPVILEKAVDLTNEQENVSFYYENLPEPFISTTSPIMKAILYVYAENISNPISLEQVAKEAFKKLGKYQLQDFLAALEQ  400
Rickettsia rickettsii   321 IRPVILEKAVDLTNEQENVSFYYENLPEPFISTTSPIMKAILYVYAENISNPISLEQVAKEAFKKLGKYQLQDFLAALEQ  400
Rickettsia conorii      321 IRPVILEKAVDLTNEQENVSFYYENLPEPFISTTSPIMKAILYVYAENISNPISLEQVAKEAFKKLGKYQLQDFLAALEQ  400
Rickettsia typhi        321 IRPISPENKIDLNNEQENISFYYENLPEPFISTTSAIMKAILYVYAENISNPIRLEQVAKEAFKKLGKYRLQDFLATLEQ  400
Rickettsia prowazeki    321 IRPISSENKIDLNNEQENISFYYENLPEPFISTTSAIMKAILYVYAENISNPIRLEQVAKEAFKKLGKYQLQDFLAILEQ  400

Rickettsia parkeri      401 HFIIFIFQGYLKIFETKPHAIATITEKPKTSEFARYQAKQAYFNNVTSVFSVTNRLNDMVGIPIHEKYILEMLDGTHNID  480
Rickettsia rickettsii   401 HFIIFIFQGYLKIFETKPHAIATITEKPKTSEFARYQAKQAYFNNVTSVFSVTNRLNDMVGIPIHEKYILEMLDGTHNID  480
Rickettsia conorii      401 HFIIFIFQGYLKIFETKPHAIATITEKPKTSEFARYQAKQAYFNNVTSVFSVTNRLNDMVGIPIHEKYILEMLDGTHNID  480
Rickettsia typhi        401 HFITLIFQGYLKIFETKPHAIATITEKPKTSQFARYQAKHAHFNNVTNMFSITNRLNDMIGIPIHEKYILEMLDGTHNID  480
Rickettsia prowazeki    401 HFITFIFQGYLKIFETKPHAIATITEKPKTSQFVRYQAKHAHFNNVTNMLSVTNRLNDMIGIPIHEKYILEMLDGTHNID  480

Rickettsia parkeri      481 DIKKGVLEKINSKLLTARDDKGQEVTDPKLLKEFVDYVVNTSLEKFRINYLLVE  534
Rickettsia rickettsii   481 DIKKGVLEKINSKLLTARDDKGQEVTDPKLLKEFVDYAVNTSLEKFRMNYLLVE  534
Rickettsia conorii      481 DIKKGVLEKINSKLLTARDDKGQEVTDPKLLKEFVDYVVNTSLEKFRINYLLVE  534
Rickettsia typhi        481 DIKKSIIEKINSKLLTACDNKGQVVTDPKLLKEFVDYVVAVSLEKFRINYLLVG  534
Rickettsia prowazeki    481 DIKKGMIEKINSKLLIACDNKGQAVTDPKLLKEFVDYIVNISLEKFRINYLLIG  534
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Fig. S10. The PKMT2 enzyme is highly conserved between virulent rickettsial species but absent from *R. bellii* and the *Rickettsia endosymbiont Proechinophthirus fluctus*. Amino acid sequence alignment of PKMT2 from *R. parkeri* (WP_014410272.1), *R. rickettsii* (WP_012150259.1), *R. conorii* (WP_016925880.1), *R. typhi* (WP_011190574.1), and *R. prowazekii* (WP_004596662.1) using COBALT. PKMT1 of *R. bellii* and the *Rickettsia endosymbiont Proechinophthirus fluctus* showed 51% identity to PKMT2 of *R. parkeri* using single alignment BLAST at NCBI; however, no PKMT2 variants could be found in these organisms, and therefore they were excluded from this analysis. Amino acids indicated in red are identical; blue, variation between species.

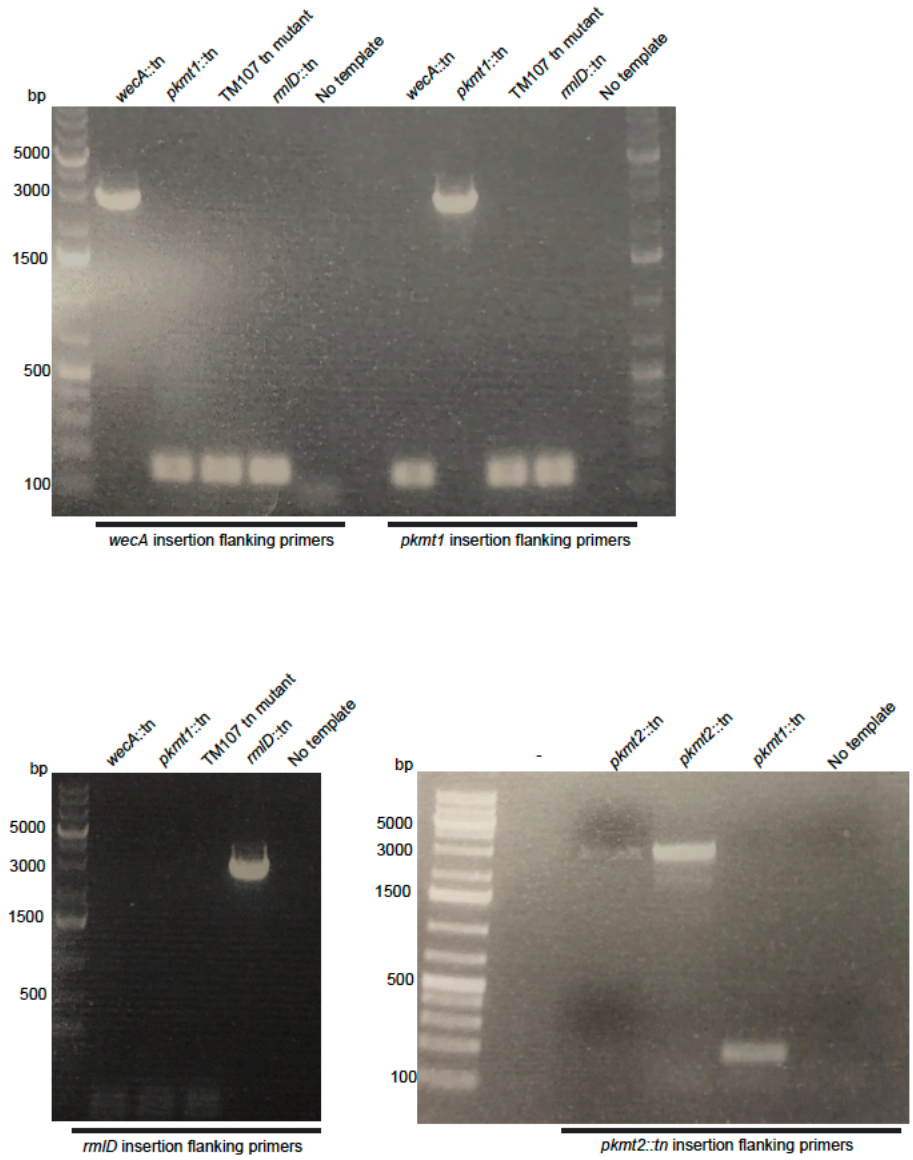

Fig. S11. Strain validation of clonality and insertion site using primers for flanking chromosomal regions (n = 1).

389 **Materials and Methods**

390 **Cell lines and primary mouse macrophages**

391 Vero cells were purchased from the UC Berkeley Cell Culture Facility and the identity was
392 repeatedly confirmed by mass-spectrometry analysis. Cells were grown at 37 °C and 5% CO_2 in
393 DMEM (Gibco, cat. no. 11965) with high glucose (4.5 g l^{-1}) and 2% heat-inactivated (30 min, 56
394 °C, in a water-bath) fetal bovine serum (Gemcell). Vero cells were confirmed to be mycoplasma
395 negative by DAPI staining and fluorescence microscopy screening at the UC Berkeley Cell Culture
396 Facility.

397 BMDMs generated from the femurs of mutant *Atg5*$^{flox/flox}$ and matched *Atg5*$^{-/-}$ C57BL/6
398 mice were a kind gift from the laboratory of Jeffery S. Cox (UC Berkeley), and they were prepared
399 as previously described (*11*) although in the absence of antibiotics. Genotypes were confirmed by
400 PCR and Sanger sequencing at the UC Berkeley DNA Sequencing Facility, as previously
401 described (*11*).

402

403 ***Rickettsia parkeri* strain generation and validation**

404 WT *R. parkeri* strain Portsmouth (NCBI accession no. NC_017044.1; originally a gift from
405 C. Paddock, Center for Disease Control and Prevention) and bacterial stocks of *ompB*^{STOP}::tn (the
406 genome sequences of these bacterial strains are available at the Sequence Read Archive as
407 accession no. SRP154218 (WT, SRX4401164; *ompB*^{STOP}::tn, SRX4401167)), *pkmt1*::tn,
408 *pkmt2*::tn, *wecA*::tn and *rlmD*::tn mutants were propagated and purified every ~6-10 months as
409 described below. Side-by-side experimental comparisons were made between stocks prepared at
410 similar times.

411 *R. parkeri pkmt1*::tn, *pkmt2*::tn, *wecA*::tn, and 114 other transposon insertion mutant

412 strains, screened for pUb, were previously isolated in a screen for small plaque mutants (*13, 22*).
413 The *ompB*^{STOP}::tn was previously isolated in a suppressor screen and lacked expression of OmpB
414 (*11*). The *rmlD*::tn, and 132 other transposon insertion mutant strains, screened for pUb, were
415 isolated in an independent screen in which mutants were isolated without regard for plaque size
416 (**Table S1**). The genomic locations of transposon insertion sites for all mutants were determined
417 by semi-random nested PCR. To verify the insertions and clonality, we used PCR reactions that
418 amplified the transposon insertion site using primers for flanking chromosomal regions:
419 5'GCTCACTAGATAGCACTCG'3 and 5'GCTCGATTTATCTCACTTTATG'3 for *rlmD*::tn,
420 5'CGTTTAATAGTCCAGTTAATTTGT'3 and 5'CCGTCTATACCGTCCATAAAAT'3 for
421 *wecA*::tn, 5'GCATCGAAATAACCCTGAG'3 and 5'GCAAACTTCTCAAAGAAATTAACG'3
422 for *pkmt1*::tn, 5'GCTAAGAAATCTTCTAATTTGATATTTTAC'3 and
423 5'CGAAAATTTACCTGAGCCTT'3 for *pkmt2*::tn, 5'CGACACATAATAGCACAAACTAC'3
424 and 5'GCGGAGGCGGTAGTAAAG'3 for *mrdA*::tn (**Fig. S11**).
425

426 **Screening for pUb-positive strains**

427 To prepare the mutant library for screening, passage 1 (P1) transposon insertion mutants
428 were amplified one time in Vero cells using 24-well cell culture plates. At 5-12 d.p.i, when 50-
429 70% of the infected cells appeared to be rounded up (as a sign of infection) by visual inspection
430 using a light microscope, cell culture media were completely removed, and cells were subsequently
431 lysed in 500 µL cold sterile water for 2-3 minutes (min). Next, 500 µL of 2x cold sterile brain-
432 heart-infusion (BHI) broth (BD Difco, 237500) was added to the lysed cells, resuspended, and P2
433 bacteria were transferred to cryogenic storage vials and frozen at -80 °C.

434 To screen for pUb-positive strains, 10-40 μL of each of five to seven P2 mutant bacterial
435 strains were diluted in 1 mL of room temperature (RT) cell culture media supplemented with 2%
436 FBS. Subsequently, the pooled bacterial suspension was centrifuged at 250*g* for 4 min at RT onto
437 confluent Vero cells grown on coverslips in 24-well plates. Cells were then incubated at 33 °C and
438 fixed at 50-55 h.p.i. with pre-warmed 4% paraformaldehyde (PFA) (Ted Pella Inc., 18505) for 10
439 min at RT. If cells were over-infected (i.e., individual infection foci had grown together) as
440 determined by immunofluorescence microscopy, infections of that specific pool were repeated
441 using reduced volumes of P2 bacteria. Next, fixed cells were permeabilized with 0.2% Triton-X
442 for 5 min and then stained with the anti-*Rickettsia* I7205 antibody (1:500 dilution; gift from Ted
443 Hackstadt) and anti-polyubiquitin FK1 antibody (Enzo Life Sciences, BML-PW8805-0500; 1:250
444 dilution), followed by Alexa 488 anti-rabbit antibody (Invitrogen, A11008; 1:500 dilution) or goat
445 anti-mouse Alexa-568 (Invitrogen, A11004). Whole coverslips were manually inspected on a
446 Nikon Ti Eclipse microscope with 60x (1.4 numerical aperture) Plan Apo objective. The initial
447 screen revealed that five out of 39 mutant pools contained pUb-positive areas.

448 In a secondary screen, individual strains from the pUb-positive pools were used to infect
449 Vero cells, as stated above. Infected cells were also fixed and stained as above except that a post-
450 fixation step using 100% methanol for 5 min was included and an OmpB antibody (*11*) and
451 Hoechst (Sigma, B2261, 1:2500 dilution) was used instead of the anti-*Rickettsia* I7205 antibody.
452 Samples were inspected as above and strains were scored as follows: **1**) pUb-negative (49 strains),
453 **2**) a few infection foci were pUb-positive (2 strains: Sp mutant 24, insertion at bp position 753916;
454 Sp mutant 94, insertion at bp position 774831), **3**) bacteria in the center of foci were pUb-positive
455 but not on the edges (2 strains: Sp mutant 43, insertion at bp position 651602-651604; Sp mutant
456 45, insertion at bp position 751156), **4**) almost all bacteria in all foci were pUb-positive (4 strains:

457 *pkmt1*::tn, insertion at bp position 1161553 (gene *MC1_RS06185*); *pkmt2*::tn, insertion at bp
458 position 34100 (*MC1_RS00180*); *wecA*::tn, insertion at bp position 1223170 (*MC1_RS06510*); and
459 *rmlD*::tn, insertion at bp position 455753 (*MC1_RS02345*).
460

461 ***Rickettsia* purification**

462 *R. parkeri* strains were propagated as described previously (*11*). "Purified bacteria" were
463 from five T175 flasks of Vero cells that after 5-8 days of infection (normally ~75% infected as
464 observed by light microscopy) were harvested in the media using a cell scraper. Bacteria were tehn
465 centrifuged 12000*g* for 15 min at 4 °C in pre-chilled tubes. Pellets were resuspended in cold K-36
466 buffer (0.05 M KH_2PO_4, 0.05 M K_2HPO_4, pH 7, 100 mM KCl and 15 mM NaCl) and a pre-chilled
467 dounce-homogenizer (tight fit) were used for 60 strokes to release bacteria from host cells. The
468 homogenate was then centrifuged at 200*g* for 5 min at 4 °C to remove cellular debris. The
469 supernatant was overlaid onto cold 30% v/v MD-76R (Mallinckrodt Inc., 1317-07) diluted in K-
470 36, and centrifuged at 58300*g* for 20 min at 4 °C in an SW-28 swinging-bucket rotor. The bacterial
471 pellet was resuspended in cold 1x BHI broth (0.5 mL BHI per infected T175 flask), and after letting
472 DNA precipitates sediment to the bottom of the tubes, bacterial suspensions were collected,
473 aliquoted and frozen at -80 °C.

474 "Gradient-purified bacteria" were from ten T175 flasks of Vero cells, purified as above
475 with the addition of a 40/44/54% v/v MD-76R (diluted in K-36 buffer) gradient step centrifuged
476 at 58300*g* for 25 min at 4 °C using the SW-28 swinging bucket rotor. The bacteria were then
477 collected from the 44-54% interface, diluted in K-36 buffer, and pelleted by centrifugation at
478 12000*g* for 15 min at 4 °C. The pellet was resuspended in cold 1x BHI broth and subsequently
479 aliquoted and frozen at -80 °C.

480

481 **OmpW and EF-Tu antibody production**

482 The sequence encoding amino acids 22-224 of outer membrane protein W (OmpW;
483 WP_014411122.1) (a protein that lacks the signal peptide), or full-length Elongation factor Tu
484 (EF-Tu; WP_004997779.1), were amplified by PCR from *R. parkeri* genomic DNA, and
485 subsequently cloned into plasmid pETM1, which encodes N-terminal 6xHis and maltose-binding
486 proteins (MBP) tags. From the resulting plasmids, fusion proteins were expressed in *E. coli* strain
487 BL21 codon plus RIL-Cam^r (DE3) (QB3 Macrolab, UC Berkeley) by induction with 1 mM
488 isopropyl-β-D-thio-galactoside (IPTG) for 2-2.5 hours at 37 °C. Bacterial pellets were resuspended
489 in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 1mM EDTA, and 1 mM dithiothreitol
490 (DTT)) and stored at -80 °C. For protein purification, bacteria were thawed, lysozyme was added
491 to 1 mg/mL (Sigma, L4919), and lysis was carried out by sonication. Lysates containing 6xHis-
492 MBP-OmpW and 6xHis-MBP-EF-Tu were incubated on amylose resin (New England Biolabs,
493 E8021L) (Qiagen, 1018244) and bound proteins were eluted in lysis buffer lacking EDTA and
494 DTT but containing 10 mM maltose. Fractions were analyzed by SDS-PAGE and those with the
495 highest concentrations of fusion proteins were pooled to generate rabbit antibodies against OmpW
496 and EF-Tu. 1.2 mg of purified 6xHis-MBP-OmpW and 6xHis-MBP-EF-Tu proteins were sent to
497 Pocono Rabbit Farm and Laboratory (Canadensis, PA), and immunization was carried out
498 according to their 91-d protocol.

499

500 **Western blotting**

501 To determine the levels of bacterial and host proteins in purified bacterial samples, 30%-
502 purified bacterial samples were boiled in 1x SDS loading buffer (150 mM Tris pH 6.8, 6% SDS,

503 0.3% bromophenol blue, 30% glycerol, 15% β-mercaptoethanol) for 10 min, then $5x10^6$ PFUs
504 were resolved on an 8-12% SDS-PAGE gel and transferred to an Immobilon-FL polyvinylidene
505 difluoride membrane (Millipore, IPEL00010). Membranes were probed for 30 min at room
506 temperature or 4°C overnight with antibodies as follows: affinity-purified rabbit anti-OmpB
507 antibody (*11*) diluted 1:200-30000 in TBS-T (20 mM Tris, 150 mM NaCl, pH 8.0, 0.05% Tween
508 20 (Sigma, P9416)) plus 5% dry milk (Apex, 20-241); mouse monoclonal anti-OmpA 13-3
509 antibody diluted 1:10000-50000 in TBS-T plus 5% dry milk; rabbit anti-OmpW serum diluted
510 1:8000 in TBS-T plus 5% dry milk; mouse monoclonal FK1 anti-polyubiquitin antibody diluted
511 1:2500 in TBS-T plus 2% BSA; rabbit anti-EF-Tu serum diluted 1:15000 in TBS-T plus 5% dry
512 milk; or rabbit anti-O-antigen serum 1:5000 in TBS-T plus 5% dry milk. Secondary antibodies
513 were: mouse anti-rabbit horseradish peroxidase (HRP) (Santa Cruz Biotechnology, sc-2357), or
514 goat anti-mouse HRP (Santa Cruz Biotechnology, sc-2005), all diluted 1:1000-2500 in TBS-T plus
515 5% dry milk. Secondary antibodies were detected with ECL Western Blotting Detection Reagents
516 (GE, Healthcare, RPN2106) for 1 min at room temperature, and developed using Biomax Light
517 Film (Carestream, 178-8207).
518
519 **Immunofluorescence microscopy**
520 *R. parkeri* infections were carried out in 24-well plates with sterile circle 12-mm coverslips
521 (Thermo Fisher Scientific, 12-545-80). To initiate infection, 30%-purified bacteria were diluted in
522 cell culture media at room temperature to a MOI of 0.01 for Vero cells, and a MOI of 0.1 for
523 BMDMs. Bacteria were centrifuged onto cells at 300*g* for 5 min at room temperature and
524 subsequently incubated at 33 °C. Next, infected cells were fixed for 10 min at room temperature
525 in pre-warmed (37 °C) 4% PFA diluted in PBS, pH 7.4, then washed 3 times with PBS. Primary

526 antibodies were the following: for staining with the guinea pig polyclonal anti-p62 antibody
527 (Fitzgerald, 20R-PP001; 1:500 dilution), mouse polyclonal anti-NDP52 antibody (Novus
528 Biologicals, H00010241-B01P; 1:100 dilution), a rabbit anti-*Rickettsia* I7205 antibody (1:500
529 dilution; gift from Ted Hackstadt), or anti-polyubiquitin FK1 antibody (1:250 dilution), cells were
530 permeabilized with 0.5% Triton-X100 for 5 min prior to staining. For staining with mouse
531 monoclonal anti-OmpA 13-3 antibody (1:5000 dilution), anti-OmpB antibody (*11*) (1:1000
532 dilution), or rabbit anti-O-antigen serum (*15*) (1:500 dilution), infected cells were post-fixed in
533 methanol for 5 min at RT (no Triton-X). Cells were then washed 3 times with PBS and incubated
534 with the primary antibodies for 30 min at RT. To detect the primary antibodies, secondary goat
535 anti-rabbit Alexa-568 (Invitrogen, A11036), goat anti-mouse Alexa-568 (Invitrogen, A11004), or
536 goat anti-guinea pig Alexa-568 (Invitrogen, A11075), Alexa 488 anti-rabbit antibody (Invitrogen,
537 A11008; 1:500 dilution), Alexa 488 anti-mouse antibody (Invitrogen, A11001) antibodies were
538 incubated at room temperature for 30 min (all 1:500 in PBS with 2% BSA). Images were captured
539 as 15-25 z-stacks (0.1-µm step size) on a Nikon Ti Eclipse microscope with a Yokogawa CSU-XI
540 spinning disc confocal with 100x (1.4 NA) Plan Apo objectives, and a Clara Interline CCD Camera
541 (Andor Technology) using MetaMorph software (Molecular Devices). Images were processed
542 using ImageJ using z-stack average maximum intensity projections and assembled in Adobe
543 Photoshop. For quantification of the percentage of bacteria with pUb and p62, only bacteria that
544 co-localized with rim-like patterns of the respective marker were scored as positive for staining.
545 To quantify pUb, p62, NDP52, OmpB, and OmpA signal per bacteria, z-stacks were projected as
546 stated above, and the edges of individual bacteria were marked by the freehand region of interest
547 (ROI) function in ImageJ. Subsequently, the average pixel intensity within that ROI was measured.
548 pUb/p62/NDP52 signal intensities were calculated by subtracting the average pUb/p62/NDP52

549 signal of WT bacteria from the pUb/p62/NDP52-value of each bacterium. OmpB signal intensity
550 was calculated by subtracting the average OmpB-signal of *ompB*^{STOP}::tn bacteria from the OmpB-
551 value of each bacterium. OmpA signal intensity was calculated by subtracting the average
552 background-signal (areas with no bacteria) from the OmpA-value of each bacterium.
553

554 **Sample preparation for mass spectrometry to determine the lysine methylome**

555 $5x10^7$ gradient-purified WT (Passage 6), *pkmt1*::tn (P4) and *pkmt2*::tn (P4) bacteria were
556 centrifuged at 11,000*g* for 3 min. Each pellet was resuspended in 50 µL Tris (10 mM)-EDTA (10
557 mM), pH 7.6, and incubated for 45 min in a 45 °C water bath. Bacterial surface fractions were
558 recovered from the supernatant after centrifugation at 11,000*g* for 3 min. Pellet was resuspended
559 as above and incubated for additional 45 min at 45 °C before resuspension in 50 µL Tris (10 mM)-
560 EDTA (10 mM). Both pellet and surface fractions were boiled at 95 °C for 10 min. Samples were
561 cooled to RT prior to addition of 20 µL 50 mM NH_4HCO_3, pH 7.5, and 50 µL of a 0.2% solution
562 of RapiGest (diluted in NH_4HCO_3, Waters, 186001861). Next, samples were heated at 80 °C for
563 15 min and cooled to RT before addition of 1 µg of trypsin (Promega, V511A). Samples were
564 digested at 37 °C overnight. To hydrolyze the RapiGest, 20 µL of 5% trifluoroacetic acid (TFA)
565 was added to samples which were incubated at 37 °C for 90 min prior centrifugation at 15000*g* for
566 25 min at 4 °C. Samples were desalted using C18 OMIX tips (Agilent Technologies, A57003100)
567 according to the manufacturer's instructions and sample volume was decreased to 20 µL using a
568 SpeedVac vacuum concentrator. Samples were stored at 4 °C prior to analysis.
569

570 **TUBE assay and sample preparation for mass spectrometry**

571 To enrich for polyubiquitylated proteins, 3x10^8 PFUs of "purified" WT (P6), *ompB*^{STOP}::tn
572 (P6) and *pkmt1*::tn (P4) and *pkmt2*::tn (P3) bacteria were centrifuged at 14,000*g* for 3 min at room
573 temperature. Next, to release the surface protein fraction, the bacterial pellets were resuspended in
574 lysis buffer (50 mM Tris-HCI, pH 7.5, 150 mM NaCl, 1 mM EDTA and 10% glycerol),
575 supplemented with 0.0031% v/v lysonase (Millipore, 71230), the deubiquitylase inhibitor PR619
576 (Life Sensor, SI9619) at a final concentration of 20 μM, and 0.8% w/v octyl β-D-glucopyranoside
577 (Sigma, O8001), and incubated on ice for 10 min with occasional pipetting of samples to break
578 pellet into smaller pieces. Subsequently, the lysate was cleared by centrifugation at 14,000*g* at 4
579 ˚C for 5 min and incubated with equilibrated agarose TUBE-1 (Life Sensor, UM401) for 3 h, at 4
580 ˚C. After binding of polyubiquitylated proteins to TUBE-1, agarose beads were washed 1 time
581 with TBS supplemented with 0.05% Tween and 5 mM EDTA, and subsequently 3 times with TBS
582 only (no Tween or EDTA) and centrifuged at 5,000*g* for 5 min. To prepare samples for MS
583 analysis, enriched proteins were digested at 37 ˚C overnight on agarose beads in RapiGest SF
584 solution (Waters, 186001861) supplemented with 0.75 μg trypsin (Promega, V511A). The reaction
585 was stopped using 1% TFA (Sigma, T6508). Octyl β-D-glucopyranoside was extracted using
586 water-saturated ethyl acetate (Sigma, 34858). Prior to submission of samples for mass
587 spectrometry analysis, samples were desalted using C18 OMIX tips (Agilent Technologies,
588 A57003100), according to the manufacturer's instructions.
589
590 **Liquid chromatography-mass spectrometry**
591 Samples of proteolytically digested proteins were analyzed using a Synapt G2-S*i* ion
592 mobility mass spectrometer that was equipped with a nanoelectrospray ionization source (Waters).
593 The mass spectrometer was connected in line with an Acquity M-class ultra-performance liquid

594 chromatography system that was equipped with trapping (Symmetry C18, inner diameter: 180 µm,
595 length: 20 mm, particle size: 5 µm) and analytical (HSS T3, inner diameter: 75 µm, length: 250
596 mm, particle size: 1.8 µm) columns (Waters). Data-independent, ion mobility-enabled, high-
597 definition mass spectra and tandem mass spectra were acquired in the positive ion mode (*23-25*).
598 Raw data acquisition was controlled using MassLynx software (version 4.1), and tryptic peptide
599 identification and relative quantification using a label-free approach (*26, 27*) were performed using
600 Progenesis QI for Proteomics software (version 4.0, Waters). Raw data were searched against
601 *Rickettsia parkeri* and *Chlorocebus sabaeus* protein databases (National Center for Biotechnology
602 Information, NCBI) to identify tryptic peptides, allowing for up to three missed proteolytic
603 cleavages, with diglycine-modified lysine (i.e., ubiquitylation remnant) and methylated lysine as
604 variable post-translational modifications. Calculation of the percentage of lysine methylation
605 (mono, di, tri or unmethylated), for each bacterial strain, was performed by dividing the abundance
606 of a residue/protein bearing a modification by the total abundance and multiplying by 100. Data-
607 dependent analysis was performed using an UltiMate3000 RSLCnano liquid chromatography
608 system that was connected in line with an LTQ-Orbitrap-XL mass spectrometer equipped with a
609 nanoelectrospray ionization source, and Xcalibur (version 2.0.7) and Proteome Discoverer
610 (version 1.3, Thermo Fisher Scientific, Waltham, MA) software, as described elsewhere (*28*).
611

612 **Localization of tagged ubiquitin and ubiquitin pull-downs**

613 To assess localization of 6xHis-ubiquitin during infection, confluent Vero cells grown in
614 24-well plates with coverslips were transfected with 2 µg of pCS2-6xHis-ubiquitin plasmid DNA
615 using Lipofectamine 2000 (Invitrogen, 11668-019) for 6 h in Opti-MEM (Gibco, 31985-070).
616 Subsequently, media were exchanged to media without transfection reagent, and cells were

617 incubated overnight at 37 ˚C and 5% CO_2. The following day (~16 h after transfection), transfected
618 cells were infected with purified WT or mutant bacteria at a MOI of 1. At 28 h.p.i., infected cells
619 were fixed with 4% PFA diluted in PBS, pH 7.4 for 10 min, then washed 3 times with PBS. Primary
620 anti-6xHis monoclonal mouse antibody (Clontech, 631212, diluted 1:1,000) was used to detect
621 6xHis-ubiquitin in samples permeabilized with 0.5% Triton-X100, and a goat anti-mouse Alexa-
622 568 (Invitrogen, A11004) to detect the primary 6xHis antibody. Hoechst (Thermo Scientific,
623 62249, diluted 1:2500) was used to detect host and bacterial DNA. Samples were imaged as
624 already described.

625 For ubiquitin pull-downs, confluent Vero cells grown in 6-well plates were transfected and
626 infected as described above. At 28 h.p.i., cells were washed once with 1x PBS, pH 7.4, and
627 subsequently lysed in urea lysis buffer (8 M urea, 50 mM Tris-HCI, pH 8.0, 300 mM NaCI, 50
628 mM Na_2HPO_4, 0.5% Igepal CA-630 (Sigma, I8896)) for 20 min at RT. Subsequently, samples
629 were sonicated, and lysate was cleared by centrifugation at 15000*g* for 15 min at room temperature.
630 Prior to incubation with Ni-NTA resin, an aliquot was saved for the input sample. 6xHis-ubiquitin
631 conjugates were purified by incubation and rotation with Ni-NTA resin for 3 h, at RT, in the
632 presence of 10 mM imidazole. Beads were washed 3 times with urea lysis buffer and 1 time with
633 urea lysis buffer lacking Igepal CA-630. Ubiquitin conjugates were eluted at 65 ˚C for 15 min in
634 2x Laemmli buffer containing 200 mM imidazole and 5% 2-mercaptoetanol (Sigma, M6250),
635 vortexed for 90 seconds, and centrifuged at 5000*g* for 5 min at RT. Eluted and input proteins were
636 detected by SDS-PAGE followed by western blotting, as described above.

637

638 **Animal experiments**

639 Animal research using mice was conducted under a protocol approved by the UC Berkeley
640 Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare
641 Act. The UC Berkeley IACUC is fully accredited by the Association for the Assessment and
642 Accreditation of Laboratory Animal Care International and adheres to the principles of the Guide
643 for the Care and Use of Laboratory Animals. Infections were performed in a biosafety level 2
644 facility. Mice were age-matched between 8 and 18 weeks old. Mice were selected for experiments
645 based on their availability, regardless of sex. All mice were healthy at the time of infection and
646 were housed in microisolator cages and provided chow and water. Littermates of the same sex
647 were randomly assigned to experimental groups. For infections, *R. parkeri* was prepared by
648 diluting 30%-prep bacteria into cold sterile 1x PBS to $5x10^6$ PFU per 200 mL. Bacterial
649 suspensions were kept on ice during injections. Mice were exposed to a heat lamp while in their
650 cages for approximately 5 min, and then each mouse was moved to a mouse restrainer (Braintree,
651 TB-150 STD). The tail was sterilized with 70% ethanol, and 200 µL of bacterial suspensions were
652 injected using 30.5-gauge needles into the lateral tail vein. Body temperatures were monitored
653 using a rodent rectal thermometer (BrainTree Scientific, RET-3). Mice were monitored daily for
654 clinical signs of disease, such as hunched posture, lethargy, or scruffed fur. If a mouse displayed
655 severe signs of infection, as defined by a reduction in body temperature below 90 ˚F or an inability
656 to move around the cage normally, the animal was immediately and humanely euthanized using
657 CO_2 followed by cervical dislocation, according to IACUC-approved procedures (*16*).
658

659 **Statistical analysis, experimental variability and reproducibility**

660 Statistical parameters and significance are reported in the legends. Data were considered
661 to be statistically significant when $p < 0.05$, as determined by a one-way ANOVA with Dunnett's

662 post-hoc test, a Kruskal-Wallis test with Dunn's post-hoc test, a Brown-Forsyth and Welch
663 ANOVA with Dunnett's post-hoc test, or a two-way ANOVA (all two-sided). Statistical analysis
664 was performed using PRISM 6 software (GraphPad Software). If not otherwise described, *n*
665 indicates the number of independent biological experiments executed at different times.