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1 **Evolution and function of bacterial RCC1 repeat effectors**

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11

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13 protein, endoplasmic reticulum, endosome, host-pathogen interaction, *Legionella*,
14 macrophage, microtubule, pathogen vacuole, phosphoinositide lipid, small GTPase, type IV
15 secretion, vesicle trafficking.

16

17 **Abbreviations:** Icm/Dot, intracellular multiplication/defective organelle trafficking; GAP,
18 GTPase activating protein; GEF, guanine nucleotide exchange factor; LCV, *Legionella*-
19 containing vacuole; *leg*, *Legionella* eukaryotic gene; MTOC, microtubule organizing center;
20 PI, phosphoinositide; PtdIns, phosphatidylinositol, RCC1, regulator of chromosome
21 condensation 1; T4SS, type IV secretion system TGN, *trans*-Golgi network.

22

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24

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27 **Summary**

28 Intracellular bacterial pathogens harbor genes, the closest homologues of which are found in
29 eukaryotes. Regulator of chromosome condensation 1 (RCC1) repeat proteins are
30 phylogenetically widespread and implicated in protein-protein interactions, such as the
31 activation of the small GTPase Ran by its cognate guanine nucleotide exchange factor, RCC1.
32 *Legionella pneumophila* and *Coxiella burnetii*, the causative agents of Legionnaires' disease
33 and Q fever, respectively, harbor RCC1 repeat coding genes. *L. pneumophila* secretes the
34 RCC1 repeat "effector" proteins LegG1, PpgA and PieG into eukaryotic host cells, where
35 they promote the activation of the pleiotropic small GTPase Ran, microtubule stabilization,
36 pathogen vacuole motility and intracellular bacterial growth as well as host cell migration.
37 The RCC1 repeat effectors localize to the pathogen vacuole or the host plasma membrane and
38 target distinct components of the Ran GTPase cycle, including Ran modulators and the small
39 GTPase itself. *C. burnetii* translocates the RCC1 repeat effector NopA into host cells, where
40 the effector localizes to nucleoli. NopA binds to Ran GTPase and promotes the nuclear
41 accumulation of Ran(GTP), thus perturbing the import of the transcription factor NF- κ B and
42 innate immune signaling. Hence, divergent evolution of bacterial RCC1 repeat effectors
43 defines the range of Ran GTPase cycle targets and likely allows fine-tuning of Ran GTPase
44 activation by the pathogens at different cellular sites.

45

46 ***Legionella* species – amoebae-resistant agents of Legionnaires' disease**

47 *Legionella* species are Gram-negative, ubiquitous environmental bacteria (Newton *et al.*,
48 2010, Whiley *et al.*, 2011, Mondino *et al.*, 2020). The bacteria thrive in natural and
49 anthropogenic water systems, including lakes, ponds, rivers, hot springs, cooling towers,
50 thermal spas and drinking or industrial water supplies. In these aquatic niches, *Legionella* can
51 survive in planktonic form, in sessile biofilms or within phagocytic protozoa (Declerck, 2010,
52 Hilbi *et al.*, 2011).

53 A total of at least 65 different *Legionella* species has been identified to date (Parte, 2018),
54 of which almost half have been associated with human infections (Newton *et al.*, 2010).
55 Among the many *Legionella* species, *Legionella pneumophila* and *Legionella longbeachae*
56 are the clinically most relevant and together account for approximately 95% of the reported
57 Legionnaires' disease cases. In most parts of the world, *L. pneumophila* causes more than
58 90% of the infections; however, in Australia and New Zealand, *L. pneumophila* and *L.*
59 *longbeachae* are responsible for approximately 46% and 30% of the cases, respectively (Yu *et*
60 *al.*, 2002, Newton *et al.*, 2010, Whiley *et al.*, 2011). Among the 16 different known *L.*
61 *pneumophila* serogroups, the large majority of the human infections are caused by serogroup
62 1, even though this serogroup is not overrepresented in the environment (Doleans *et al.*,
63 2004).

64 *Legionella* species are opportunistic if not “accidental” human pathogens, which are almost
65 exclusively transmitted through contaminated aerosols from natural or technical water sources
66 (Mercante *et al.*, 2015). While the human lung is considered a dead-end for the pathogen, an
67 isolated incidence of a person-to-person transmission of *L. pneumophila* has been reported
68 (Correia *et al.*, 2016). In this instance, the transmission of a clonal strain from an infected
69 person to another caused the death of the two people involved due to legionellosis.

70

71 **Intracellular growth of *L. pneumophila* and formation of the pathogen vacuole**

72 *L. pneumophila* grows in free-living protozoa and macrophages of the innate immune system
73 employing an apparently conserved mechanism (Gomez-Valero *et al.*, 2011a, Al-Quadani *et*
74 *al.*, 2012). Macrophage resistance is a prerequisite for *Legionella* pathogenesis. In order to
75 replicate within host cells, *L. pneumophila* forms a unique, degradation-resistant
76 compartment, the *Legionella*-containing vacuole (LCV). In the course of its maturation, the
77 LCV does not acidify or fuse with lysosomes, but the compartment extensively communicates
78 with several vesicle trafficking pathways including the endosomal, secretory and retrograde

79 routes (Isberg *et al.*, 2009, Asrat *et al.*, 2014, Personnic *et al.*, 2016, Bärlocher *et al.*, 2017b,
80 Steiner *et al.*, 2018). The initial and decisive stage of LCV formation is characterized by a
81 phosphoinositide lipid conversion, where endosomal phosphatidylinositol 3-phosphate
82 (PtdIns(3)P) is replaced by secretory PtdIns(4)P (Weber *et al.*, 2006, Weber *et al.*, 2014,
83 Weber *et al.*, 2018, Swart *et al.*, 2020a). This process is followed by the recruitment,
84 activation and modification of small GTPases of the Arf (Nagai *et al.*, 2002, Goody *et al.*,
85 2013), Rab (Kagan *et al.*, 2004, Brombacher *et al.*, 2009, Hoffmann *et al.*, 2014, Sherwood *et*
86 *al.*, 2016), Rap (Schmölders *et al.*, 2017) and Ran family (see below). Finally, at late steps of
87 LCV maturation, the pathogen vacuole tightly associates but does not fuse with the
88 endoplasmic reticulum (ER) in macrophages (Swanson *et al.*, 1995, Tilney *et al.*, 2001) and
89 amoebae (Abu Kwaik, 1996, Lu *et al.*, 2005, Weber *et al.*, 2014).

90 In addition to small GTPases of several different families, large oligomeric GTPases also
91 play a role in *L. pneumophila* infection (Escoll *et al.*, 2017, Steiner *et al.*, 2017). Atl3
92 (Atl3/Sey1) localizes to ER tubules and catalyzes homotypic membrane fusion events. This
93 large GTPase promotes ER remodeling around the LCV, pathogen vacuole expansion and
94 intracellular bacterial replication (Steiner *et al.*, 2017). Furthermore, the mitochondrial
95 Dynamin1-like large GTPase (Dnm1l) mediates mitochondrial fragmentation during *L.*
96 *pneumophila* infection (Escoll *et al.*, 2017).

97 Arguably the most important *L. pneumophila* virulence factor essential for LCV formation
98 is the Icm/Dot (intracellular multiplication/defective organelle trafficking) type IV secretion
99 system (T4SS) (Segal *et al.*, 1998, Vogel *et al.*, 1998, Kubori *et al.*, 2016). The Icm/Dot T4SS
100 is conserved among *Legionella* spp. (Burstein *et al.*, 2016, Gomez-Valero *et al.*, 2019c), and
101 in the case of *L. pneumophila* secretes more than 300 different “effector” proteins into
102 eukaryotic host cells (Burstein *et al.*, 2009, Zhu *et al.*, 2011, Lifshitz *et al.*, 2013, Qiu *et al.*,
103 2017). The effectors subvert pivotal process in target cells, including membrane trafficking,
104 cytoskeleton dynamics or signal transduction, and some of them catalyze hitherto unknown

105 biological reactions (Hubber *et al.*, 2010, Hilbi *et al.*, 2012, Haneburger *et al.*, 2013,
106 Sherwood *et al.*, 2013, Finsel *et al.*, 2015, Qiu *et al.*, 2017). A number of effectors target
107 components commandeering eukaryotic membrane dynamics. These include small GTPases
108 and PI lipids, but also the vacuolar H⁺-ATPase (Xu *et al.*, 2010), the autophagy machinery
109 (Choy *et al.*, 2012, Horenkamp *et al.*, 2015, Rolando *et al.*, 2016, Arasaki *et al.*, 2017, Yang
110 *et al.*, 2017), or the retromer coat complex and other components of the retrograde trafficking
111 pathway (Weber *et al.*, 2009, Finsel *et al.*, 2013, Bärlocher *et al.*, 2017a, Romano-Moreno *et*
112 *al.*, 2017, Welin *et al.*, 2018, Yao *et al.*, 2018).

113

114 **Co-evolution of *Legionella* with protozoa and acquisition of eukaryotic genes**

115 Free-living protozoa represent a privileged environmental niche of *Legionella* species. While
116 some protozoa kill and digest *L. pneumophila* (Amaro *et al.*, 2015, Boamah *et al.*, 2017),
117 there is indeed a tremendous diversity of environmental hosts, and *L. pneumophila* has been
118 shown to replicate in at least 30 different protozoan species, including amoebae (e.g.,
119 *Acanthamoeba*, *Hartmanella*, *Naegleria*, *Vahlkampfia* and *Dictyostelium* spp.) and ciliates
120 (e.g., *Tetrahymena* and *Paramecium* spp.) (Fields, 1996, Boamah *et al.*, 2017, Swart *et al.*,
121 2018). The intimate contact and co-evolution with these primordial predators is believed to
122 render environmental protozoa an “evolutionary crib” for pathogen evolution (Greub *et al.*,
123 2004, Molmeret *et al.*, 2005, Gomez-Valero *et al.*, 2013). In fact, it was recently
124 experimentally demonstrated that the cumulative selective pressures of multiple and diverse
125 amoebal hosts shapes the virulence of *L. pneumophila* for macrophages, and – as a corollary –
126 determines pathogenesis in humans (Park *et al.*, 2020).

127 The genus *Legionella* belongs to the γ -proteobacteria and is characterized by a large
128 genetic diversity (Gomez-Valero *et al.*, 2013). To date, the genomes of 58 *Legionella* species,
129 including *L. pneumophila* and *L. longbeachae*, have been fully sequenced and annotated by
130 bioinformatics means (Burstein *et al.*, 2016, Gomez-Valero *et al.*, 2019b). The genomes show

131 a high plasticity, harbor many mobile genetic elements and contain around 10% strain-
132 specific genes. The large diversity among *Legionella* species is owing to widespread
133 horizontal gene transfer among members of the genus, other bacterial species, and – given the
134 omnipresence of “eukaryotic-like” genes – presumably also with eukaryotic host cells
135 (Cazalet *et al.*, 2004, de Felipe *et al.*, 2005, Gomez-Valero *et al.*, 2011b, Gomez-Valero *et al.*,
136 2019a).

137 Upon sequencing the genome of *L. pneumophila*, a wide variety of genes encoding
138 eukaryotic-like proteins and eukaryotic domain-carrying proteins were identified, reflecting
139 the long-standing co-evolution of the bacterium with eukaryotic host cells and highlighting
140 potential virulence factors (Cazalet *et al.*, 2004, de Felipe *et al.*, 2005). Indeed, many of these
141 proteins turned out to be substrates of the Icm/Dot T4SS and likely target host cell processes
142 by functioning analogously to their eukaryotic homologues. However, only a few of these
143 effector proteins have been biochemically and cell biologically characterized in detail to date.

144 The first Icm/Dot substrate identified, RalF, contains a Sec7 domain and functions as a
145 guanine nucleotide exchange factor (GEF) for the ADP ribosylation factor (Arf) family of
146 small GTPases, which recruits and activates Arf1 to LCVs (Nagai *et al.*, 2002, Amor *et al.*,
147 2005, Alix *et al.*, 2012, Folly-Klan *et al.*, 2013). RomA (LegAS4) contains a SET domain and
148 covalently modifies histones through its methyltransferase activity (Li *et al.*, 2013, Rolando *et*
149 *al.*, 2013). LpSpl (LegS2) is a close relative of the eukaryotic sphingosine 1-phosphate lyase
150 (Spl), additionally harbors a C-terminal type IV secretion signal, and inhibits the host cell
151 autophagy pathway in *L. pneumophila*-infected cells (Degtyar *et al.*, 2009, Abu Khweek *et*
152 *al.*, 2016, Rolando *et al.*, 2016).

153 LubX (Kubori *et al.*, 2008), LegU1 (Ensminger *et al.*, 2010) and AnkB (Al-Khodori *et al.*,
154 2008, Price *et al.*, 2009, Ensminger *et al.*, 2010, Lomma *et al.*, 2010) are Icm/Dot-translocated
155 U-box or F-box-containing E3 ubiquitin ligases that catalyze ubiquitination reactions.
156 Furthermore, AnkB (Ivanov *et al.*, 2010, Price *et al.*, 2010), as well as LegG1 (*alias* MitF,

157 Lpg1976) and PieG (Lpp1959) (Ivanov *et al.*, 2010, Rothmeier *et al.*, 2013, Swart *et al.*,
158 2020b) contain a C-terminal CAAX motif, which is lipidated by the host prenylation
159 machinery to facilitate membrane localization.

160

161 ***Legionella* species harbor eukaryotic-like RCC1 repeat coding genes**

162 The human regulator of chromatin condensation 1 (RCC1) is the founding member of the
163 RCC1 repeat family (<http://pfam.xfam.org/family/PF00415>). RCC1 harbors seven RCC1
164 repeats of 51-68 residues and is a GEF for the small Ran (Ras-like nuclear) GTPase (Bischoff
165 *et al.*, 1991). Using the internal repeat profiles of the 7-bladed propeller structures of
166 eukaryotic RCC1 and the prokaryotic β -lactamase inhibitor protein II (BLIP II) allowed the
167 definition of the RCC1-like repeat family of propeller proteins (Stevens *et al.*, 2008). The
168 RCC1 repeats harbor only very little primary amino acid sequence identity and occur in 3-7
169 repeats per protein. RCC1 repeats are wide-spread among metazoans (animals, plants, fungi),
170 unicellular eukaryotes, archaea and prokaryotes. The wide distribution of RCC1 repeats and
171 their conservation among a range of organisms likely reflects the versatile nature of this motif
172 (Stevens *et al.*, 2008).

173 Bioinformatics analysis revealed that among the genomes of *Legionella* species sequenced
174 more than 57% (32 out of 56) are predicted to produce one or multiple RCC1 repeat proteins
175 (Gomez-Valero *et al.*, 2019b) (Figure 1). The seemingly random distribution of RCC1 repeat
176 proteins throughout the genus *Legionella*, together with the observation that not all species
177 within the same clade contain RCC1 repeat proteins, suggests that the proteins were
178 independently acquired rather than originating from a common ancestor. Furthermore, recent
179 studies concluded that DNA interchange between different *Legionella* species is rare
180 (Burstein *et al.*, 2016, Joseph *et al.*, 2016). An independent and exogenous acquisition of
181 RCC1 repeat coding genes is in agreement with a vital role for *Legionella*-host cell
182 interactions.

183 Nonetheless, RCC1 repeat effectors are apparently not essential for *Legionella*
184 pathogenicity, since there is no correlation between the presence of RCC1 repeat proteins and
185 the source of the isolate (clinical or environmental). *L. longbeachae*, the second most
186 common etiological agent of Legionnaires' disease, apparently lacks RCC1 repeat effectors
187 (Cazalet *et al.*, 2010) (Figure 1). In contrast, *L. waltersii*, which has also been linked to a
188 clinical case (König *et al.*, 2005), harbors as many as 15 RCC1 repeat coding genes.
189 Phylogenetic analyses of these homologues revealed a cluster, indicating that they might have
190 evolved through gene duplication. However, the functional significance of this high number
191 of RCC1 repeat coding genes is not known.

192 Little is known about the origin of RCC1 repeat proteins, although it is suggested that
193 prokaryotes acquired their RCC1 repeats via horizontal gene transfer from eukaryotes
194 (Gomez-Valero *et al.*, 2013). In order to obtain further insights into the origin of *L.*
195 *pneumophila* RCC1 repeat effectors, we constructed a phylogenetic tree for one of the
196 proteins, PieG (Figure 2). Phylogenetic trees obtained with either distances and likelihood
197 methods were similar and indicated clustering of *L. pneumophila* PieG with eukaryotic
198 proteins closer than with prokaryotic proteins. The closest eukaryotic sequence belongs to the
199 amoeba *Naegleria fowleri*, an experimentally validated host of *L. pneumophila* (Boamah *et*
200 *al.*, 2017). Moreover, amoebae-infecting viruses such as Marseillevirus (La Scola, 2014), also
201 harbor RCC1 repeat proteins, which group close to *L. pneumophila* PieG (Figure 2). These
202 findings support the notion of horizontal gene transfer from a protozoan hosts or from a co-
203 infecting virus as the origin of *Legionella* RCC1 repeat coding genes.

204

205 ***L. pneumophila* RCC1 repeat coding genes are distributed in two main strain clusters**

206 RCC1 repeat coding genes are conserved in the genomes of all 59 *L. pneumophila* strains
207 sequenced to date (Swart *et al.*, 2020b). While some *L. pneumophila* strains contain two
208 distinct RCC1 repeat coding genes (*e.g.*, strain Philadelphia-1: *legG1/lpg1976* and

209 *ppgA/lpg2224*), others contain only one single gene (e.g., strains Paris or Lens: *pieG/lpp1959*)
210 or additionally a duplicated *ppgA* gene. Accordingly, the RCC1 repeat coding genes are
211 distributed in two main clusters: the “Philadelphia-1” cluster and the “Paris-Lens” cluster,
212 respectively (Swart *et al.*, 2020b).

213 Another emerging pattern is that all but one strains in the “Philadelphia-1” cluster contain a
214 split *pieG* gene (yielding *lpg1975* and *legG1*). Based on the distribution pattern, it is more
215 likely that the *ppgA* gene was acquired by an ancestral *L. pneumophila* strain harboring *pieG*
216 rather than that the *ppgA* gene was lost from a genome. This further suggests that the split of
217 *pieG* (or duplication and modification of *ppgA*) happened as a subsequent step, and possibly
218 as a result of the acquisition of *ppgA* (Swart *et al.*, 2020b).

219 Many RCC1-repeat proteins adopt the complex β -propeller fold (Renault *et al.*, 1998, Lim
220 *et al.*, 2001, Stevens *et al.*, 2008), possibly aided by specific eukaryotic chaperones. PpgA and
221 PieG are predicted to have a similar seven-bladed tertiary structure, whereas LegG1 is
222 truncated and thus lacks three blades. Arguably, production of two full-length, eukaryotic-like
223 RCC1 repeat proteins is challenging for the bacterial folding machinery, which resulted in the
224 split of *pieG* and production of the more easily foldable LegG1. In agreement with this notion,
225 LegG1 is soluble and can easily be purified from *E. coli*, whereas PpgA and PieG form
226 aggregates upon heterologous production in *E. coli* (Swart *et al.*, 2020b).

227

228 ***L. pneumophila* RCC1 repeat effectors target different Ran GTPase cycle components**

229 The 25 kDa Ran protein is the most abundant small GTPase in the cell and a pleiotropic
230 regulator of different processes in eukaryotic cells (Joseph, 2006, Yudin *et al.*, 2009). Ran
231 GTPase is the master regulator of nucleo-cytoplasmic transport (Stewart, 2007), controls
232 mitotic spindle assembly during cell division and post-mitotic nuclear envelope formation
233 (Goodman *et al.*, 2006, Clarke *et al.*, 2008), and governs non-centrosomal microtubule
234 dynamics in the cytoplasm (Schulze *et al.*, 2008). Ran is activated by the GEF RCC1, which

235 facilitates the exchange of GDP with GTP (Bischoff *et al.*, 1991). RCC1 is regulated by
236 phosphorylation, *e.g.*, during mitosis by the tumor suppressor RASSF1A, which induces
237 Ran(GTP)-dependent microtubule hyperstability. Moreover, the cytoplasmic Ran GEF
238 RanBP10 positively regulates the stability of non-centrosomal microtubules in non-mitotic
239 cells, and accordingly, depletion of RanBP10 disrupts the microtubule cytoskeleton (Schulze
240 *et al.*, 2008). Ran is inactivated by the Ran GTPase-activating protein 1 (RanGAP1) together
241 with Ran binding protein 1 (RanBP1), which exclusively binds to activated Ran (Joseph,
242 2006, Yudin *et al.*, 2009).

243 *L. pneumophila* strains produce the Icm/Dot-translocated RCC1 repeat proteins LegG1 (31
244 kDa, 3 repeats) (de Felipe *et al.*, 2005, de Felipe *et al.*, 2008), PpgA (66 kDa, 2 repeats)
245 (Ninio *et al.*, 2009), and PieG (53 kDa, 2 repeats) (Ninio *et al.*, 2009, Ivanov *et al.*, 2010)
246 (Figure 3). LegG1, PpgA and PieG play important roles in pathogen-host interactions, since *L.*
247 *pneumophila* mutant strains lacking the corresponding genes (single or double mutants) are
248 impaired for intracellular replication in macrophages and *Dictyostelium discoideum*, and
249 outcompeted by the parental strain in amoebae competition assays using *Acanthamoeba*
250 *castellanii* as a host cell (Rothmeier *et al.*, 2013, Swart *et al.*, 2020b).

251 LegG1, PpgA and PieG target distinct components of the Ran GTPase cycle, thereby
252 promoting Ran activation (Rothmeier *et al.*, 2013, Swart *et al.*, 2020b). Namely, LegG1
253 interacts with the GEF RanBP10, PpgA with RanGAP1, and PieG with both Ran GTPase as
254 well as RanGAP1 (Swart *et al.*, 2020b). Since all three *L. pneumophila* RCC1 repeat effectors
255 increase the cellular amount of Ran(GTP), we hypothesize that LegG1 activates the GEF
256 RanBP10, PpgA inhibits the GAP RanGAP1, and PieG stabilizes Ran(GTP) by binding to the
257 active GTPase, and/or inhibiting RanGAP1 (Figure 4). By targeting a Ran GEF or a Ran
258 GAP, the *L. pneumophila* RCC1 repeat effectors act differently from other Icm/Dot
259 substrates, which directly show GEF or GAP activity or covalently modify small GTPases (of

260 the Rab family) to modulate their activity (Machner *et al.*, 2006, Ingmundson *et al.*, 2007,
261 Goody *et al.*, 2013, Sherwood *et al.*, 2013).

262 Unexpectedly, LegG1 (the N-terminal part of PieG) targets a different component of the
263 Ran GTPase cycle than full-length PieG. Hence, the split of *pieG* – the hallmark of the *L.*
264 *pneumophila* “Philadelphia-1” cluster – alters the target of the corresponding protein. This
265 substrate switch can be experimentally reversed upon fusion of *legG1* with the upstream ORF,
266 *lpg1975* (Swart *et al.*, 2020b) (Figure 3). By targeting the Ran modulator RanBP10, instead of
267 the small GTPase itself, LegG1 might exert an additional level of control in the modulation of
268 the Ran GTPase cycle. Furthermore, through acquisition of PpgA, strain Philadelphia-1
269 targets not only a Ran GEF, but additionally also a Ran GAP. Overall, divergent evolution of
270 *L. pneumophila* RCC1 repeat effectors expands the range of target components of the Ran
271 GTPase cycle and thus might fine-tune Ran activation in *L. pneumophila*-infected cells.

272

273 ***L. pneumophila* RCC1 repeat effectors localize to distinct cellular compartments**

274 The small GTPase Ran has pleiotropic functions in different cellular compartments (Joseph,
275 2006, Yudin *et al.*, 2009). Hence, the unrestricted activation of Ran is likely detrimental to
276 host cells. To avoid interference with Ran-regulated processes at different subcellular sites,
277 Ran modulation needs to be spatially controlled. Nucleo-cytoplasmic transport, *e.g.*, is tightly
278 regulated by chromatin-bound RCC1 and cytoplasmic RanGAP1, and a re-distribution of the
279 regulators leading to disruption of the Ran(GTP) gradient across the nuclear envelope has
280 disastrous consequences for RNA export from the nucleus (Izaurralde *et al.*, 1997).

281 The *L. pneumophila* RCC1 repeat effectors LegG1, PpgA and PieG localize to different
282 subcellular compartments (Figure 4). Upon ectopic production in mammalian cells, *D.*
283 *discoideum* or yeast, LegG1 and PieG are prenylated at their C-terminal CAAX site and
284 localize to vesicular structures in the cells, or to LCVs in *L. pneumophila*-infected *D.*
285 *discoideum* (Ninio *et al.*, 2009, Ivanov *et al.*, 2010, Swart *et al.*, 2020b). Moreover, upon

286 translocation by the Icm/Dot T4SS in *L. pneumophila*-infected cells, LegG1 also localizes to
287 LCVs and activates Ran in the vicinity of the pathogen vacuole (Rothmeier *et al.*, 2013). In
288 contrast, ectopically produced PpgA exclusively localizes to the plasma membrane in *D.*
289 *discoideum* amoebae as well as in the yeast model (Swart *et al.*, 2020b). Analogously to a
290 LegG1-dependent Ran(GTP) gradient emanating from the LCV, PpgA might activate Ran at
291 the plasma membrane and promote the formation of a Ran(GTP) gradient and microtubule
292 stabilization at the cell cortex (Figure 4).

293 In agreement with a distinct subcellular activity of LegG1 on LCVs, the RCC1 repeat
294 effector does not seem to affect the nuclear localization of the Icm/Dot substrate RomA. The
295 *L. pneumophila* effectors RomA (LegAS4) is transported across the nuclear envelope
296 dependent on its nuclear localization signal (NLS), likely driven by the Ran(GTP) gradient
297 (Li *et al.*, 2013, Rolando *et al.*, 2013). Overall, these observations suggest that the modulation
298 of Ran by *L. pneumophila* RCC1 repeat effectors is strictly controlled by their distinct cellular
299 localization.

300

301 ***L. pneumophila* RCC1 repeat effectors promote LCV motility and host cell migration**

302 The host components of LCVs are instrumental for its architecture and function. In order to
303 determine these components, proteomics analysis was performed using pathogen vacuoles
304 purified from infected *D. discoideum* amoebae (Urwyler *et al.*, 2009, Schmölders *et al.*,
305 2017), murine RAW 264.7 macrophage-like cells (Hoffmann *et al.*, 2014) and primary bone-
306 marrow-derived macrophages from A/J mice (Naujoks *et al.*, 2016). This approach revealed
307 that α - and β -tubulin, Ran GTPase, RanBP1, RanBP2, RanGAP1, RCC1 and RCC2 are LCV
308 components, and accordingly, might be host components functionally implicated in pathogen
309 vacuole formation and function. Indeed, Ran and RanBP1 were validated as LCVs
310 components by fluorescence microscopy, and Ran, RanBP1 as well as RanGAP1 were

311 implicated in intracellular replication of *L. pneumophila* by RNA interference (Rothmeier *et*
312 *al.*, 2013, Swart *et al.*, 2020b).

313 Microtubules, alongside with actin and intermediate filaments, form the cytoskeleton that
314 maintains cell shape and internal organization and plays an essential role for intracellular
315 trafficking processes and cell migration (Etienne-Manneville, 2013). Microtubules are tubular
316 polymers of α - and β -tubulin heterodimers, which originate from a membrane-less organelle
317 called MTOC (microtubule organizing center) located in the vicinity of the nuclear envelope.
318 Long-range vesicle movements inside a cell as well as the distribution and stability of
319 organelles and endocytic compartments require microtubules and associated motor proteins
320 called kinesins, dyneins and myosins, which *via* specific adaptors transport cargoes along the
321 microtubular tracks (Etienne-Manneville, 2013).

322 The *L. pneumophila* RCC1 repeat effectors LegG1, PpgA and PieG promote the
323 intracellular motility of LCVs, and for LegG1 it has been shown that this occurs by stabilizing
324 microtubules on the LCV membrane and also throughout the host cell (Rothmeier *et al.*, 2013,
325 Hilbi *et al.*, 2014, Swart *et al.*, 2020b). LegG1 interacts with the Ran GEF RanBP10 (see
326 above), which also harbors a β -tubulin binding domain (Schulze *et al.*, 2008). Accordingly,
327 LegG1 might directly link Ran activation and microtubule stabilization through this
328 cytoplasmic Ran GEF.

329 Besides their involvement in trafficking of endocytic compartments and the LCV,
330 microtubules are also of key importance for the structure, distribution and function of
331 organelles such as the mitochondria and the Golgi apparatus. Accordingly, microtubule
332 stabilization likely accounts for the effect of LegG1 (MitF) on mitochondrial dynamics and
333 function during *L. pneumophila* infection (Escoll *et al.*, 2017), as well as for the LegG1-
334 dependent disruption of Golgi cisternae in *L. pneumophila*-infected cells (Rothmeier *et al.*,
335 2013). Taken together, the studies reveal that the modulation of the microtubule cytoskeleton

336 by LegG1 affects pathogen vacuole dynamics, host cell vesicle trafficking and organelle
337 integrity.

338 Another cellular process depending on the microtubule cytoskeleton is cell migration
339 (Friedl *et al.*, 2009). Migration is a polarized cellular process, where a protrusive leading edge
340 is opposed to a retracting trailing edge. At the cell front, cortical actin is the primary stimulus
341 for motion, while the microtubule network undertakes a regulatory function in coordinating
342 rear retraction. During migration, the MTOC undergoes reorientation towards the side facing
343 the direction of motion, allowing the microtubules to polarize from the center to the periphery
344 of the cell (Wehrle-Haller *et al.*, 2003, Kaverina *et al.*, 2011).

345 *L. pneumophila* inhibits chemotaxis as well as random host cell migration by means of the
346 small signaling molecule LAI-1 (*Legionella* autoinducer-1, 3-hydroxypentadecane-4-one)
347 (Simon *et al.*, 2015b) as well as through Icm/Dot-translocated effectors (Simon *et al.*, 2014,
348 Simon *et al.*, 2015a). While the effector(s) inhibiting cell migration are not known, LegG1,
349 PpgA and PieG rather promote the random and chemotactic migration of protozoan or
350 mammalian phagocytes (Rothmeier *et al.*, 2013, Simon *et al.*, 2014, Swart *et al.*, 2020b). At
351 least in the case of LegG1, the inhibition of phagocyte migration proceeds through Ran.
352 Similar to LegG1, overproduction of PpgA positively affects host cell motility (Swart *et al.*,
353 2020b). Moreover, migration of amoeba infected with the $\Delta legG1$ - $\Delta ppgA$ double mutant is
354 “hyper-inhibited”, suggesting that both proteins function in concert to stimulate host cell
355 motility. However, LegG1 and PpgA probably employ distinct mechanisms to promote host
356 migration since the former localizes to the LCV membrane and the latter to the plasma
357 membrane, where the local accumulation of Ran(GTP) is expected to increase microtubule
358 stability and hence, promote forward movement. LegG1-dependent microtubule modulation
359 affects vesicular trafficking and thus, might indirectly enhance host cell migration. In
360 summary, the *L. pneumophila* RCC1 repeat effectors promote cell migration either directly or
361 perhaps indirectly through the activation of Ran and microtubule stabilization.

362

363 *Coxiella burnetii* – an obligate intracellular pathogen causing Q fever

364 *Coxiella burnetii* is the causative agent of a zoonotic disease called Q (“query”) fever, which
365 is transmitted through aerosols and manifests as an acute or chronic ailment (Dragan *et al.*,
366 2020). The obligate intracellular bacterium replicates in a large lysosomal/autophagosomal
367 compartment, the *Coxiella*-containing vacuole (CCV), characterized by an acidic pH, acid
368 hydrolases and cationic peptides (Voth *et al.*, 2007). Like *L. pneumophila*, *C. burnetii*
369 employs an Icm/Dot T4SS to translocate ca. 150 effector proteins into host cells (Qiu *et al.*,
370 2017). In fact, T4SS-dependent secretion appears to function very similarly in the two
371 pathogens (Zamboni *et al.*, 2003, Lifshitz *et al.*, 2013), and *L. pneumophila* can be used as a
372 surrogate host to deliver *C. burnetii* effectors (Carey *et al.*, 2011).

373

374 The *C. burnetii* RCC1 repeat effector NopA activates Ran GTPase in host cell nucleoli

375 Among the few characterized *C. burnetii* effectors one is called NopA (Nucleolar protein A)
376 (Burette *et al.*, 2020). NopA is a Icm/Dot-translocated effector, which contains 4 RCC1
377 repeats in the C-terminal part. As only few amino acids define an RCC1 repeat, the repeats
378 share only little sequence identity. Accordingly, *C. burnetii nopA* does not show significant
379 homology with the *L. pneumophila* RCC1 repeat genes. Upon infection or ectopic production,
380 NopA localizes to nucleoli in the host nucleus, where it binds to and activates the small
381 GTPase Ran. The nucleolar accumulation of Ran(GTP) perturbs nucleocytoplasmic transport
382 and the import of the transcription factor NF- κ B. As a result, the production of cytokines and
383 innate immune signaling is impaired by the *C. burnetii* effector NopA (Burette *et al.*, 2020).

384

385 Conclusions and outlook

386 Intracellular bacterial pathogens produce RCC1 repeat effectors, which localize to different
387 subcellular compartments and subvert the host cell Ran GTPase cycle. *L. pneumophila*

388 produces the RCC1 repeat effectors LegG1, PpgA and PieG, which divergently evolved to
389 target distinct components of the Ran GTPase cycle. The RCC1 repeat effectors localize to
390 different subcellular compartments, such as the LCV or the plasma membrane, allowing
391 spatial regulation of Ran activation and the generation of local Ran(GTP) gradients leading to
392 microtubule stabilization. *C. burnettii* produces the RCC1 repeat effector NopA, which
393 localizes to nucleoli, binds to and activates Ran GTPase, and perturbs nucleocytoplasmic
394 transport and NF- κ B-dependent gene expression. These features of bacterial RCC1 repeat
395 effectors – the expansion of host cell targets as well as distinct subcellular localizations –
396 likely allow the pathogens to fine-tune Ran activation rather than risking unrestricted
397 activation of the pleiotropic small GTPase. Future studies will address the biochemical
398 characterization of bacterial RCC1 repeat effectors and the mechanism(s), by which these
399 effectors subvert pivotal Ran-dependent host processes, such as LCV motility, vesicle
400 trafficking, organelle integrity, cell migration and gene expression.

401

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409

410 **Conflict of Interest**

411 The authors declare no conflict of interest.

412

413

414 **Figure legends**

415 **Figure 1. Phylogenetic tree of the genus *Legionella* and the distribution of RCC1 repeat**
416 **proteins.** Distribution and number (orange bars) of proteins containing RCC1 repeats in 58
417 different species/subspecies of the *Legionella* genus according to the Pfam database (El-
418 Gebali *et al.*, 2019). The phylogenetic tree of select strains is based on the *Legionella* core
419 genome (Gomez-Valero *et al.*, 2019b), and branches are colored according to the clade they
420 belong to. The scale bar represents the estimated evolutionary distance (number of amino acid
421 substitutions per site).

422
423 **Figure 2. Phylogenetic tree of the RCC protein Lpp1959 from *Legionella* species and**
424 **homologous sequences.** The sequences were retrieved with blastp searches, and the unrooted
425 phylogenetic tree was constructed by likelihood using the method PhyML implemented in the
426 program SeaView (Gouy *et al.*, 2010). Numbers on the branches indicate bootstrap support
427 for nodes from 100 bootstrap replicates (only values above 75 are shown). The scale bar
428 represents the estimated evolutionary distance (number of amino acid substitutions per site).

429
430 **Figure 3. *L. pneumophila* RCC1 repeat proteins.** Schematic overview and position of
431 RCC1 repeats in *L. pneumophila* PpgA, LegG1/Lpg1976, Lpg1975, Fusion (LegG1-
432 Lpg1975) and PieG/Lpp1959. Scale bar (corresponding gene size), 1 kb.

433
434 **Figure 4. Distinct subcellular localization of *L. pneumophila* RCC1 repeat effectors and**
435 **Ran GTPase cycle targets.** The Icm/Dot T4SS-translocated *L. pneumophila* RCC1 repeat
436 effectors LegG1, PpgA and PieG target distinct members of the Ran GTPase cycle to activate
437 Ran, namely LegG1 interacts with RanBP10, PpgA with RanGAP1 and PieG binds both Ran
438 and RanGAP1. The effectors localize to the LCV or the plasma membrane, respectively, thus
439 likely creating local Ran(GTP) gradients leading to microtubule stabilization. The endogenous

440 Ran(GTP) gradient across the nuclear envelope is created by the Ran GEF RCC1 and
441 RanGAP1 localizing in the nucleus or cytoplasm, respectively. The Ran(GTP) gradient is
442 depicted ranging from yellow (high Ran(GTP) concentration) to white (high Ran(GDP)
443 concentration). MTOC: microtubule organizing center.

444

445

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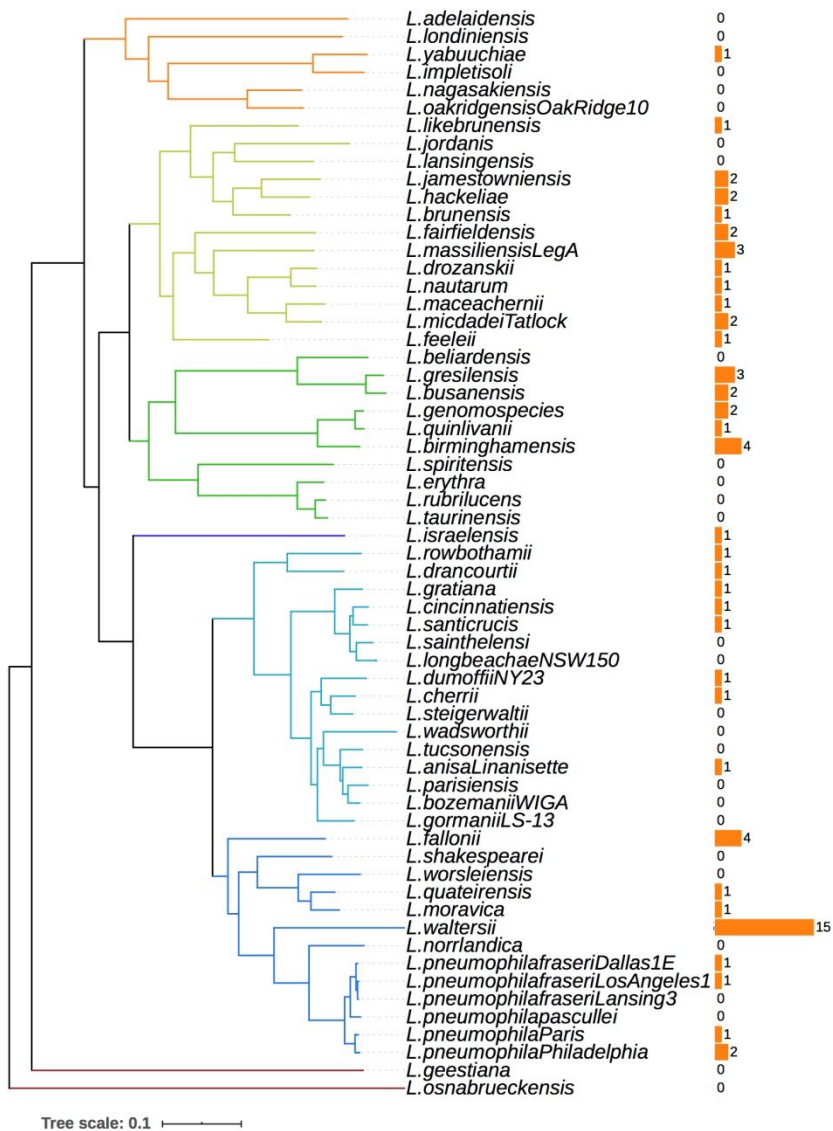


Figure 1

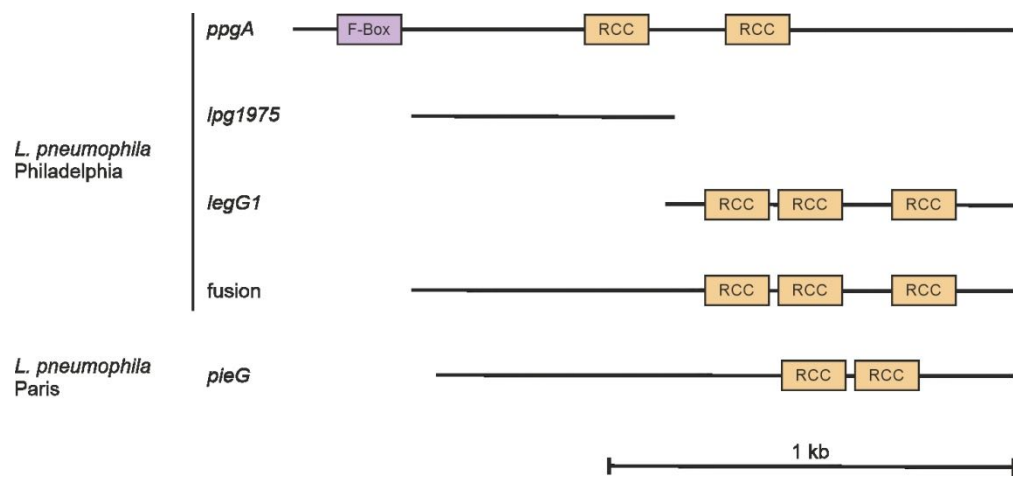


Figure 3

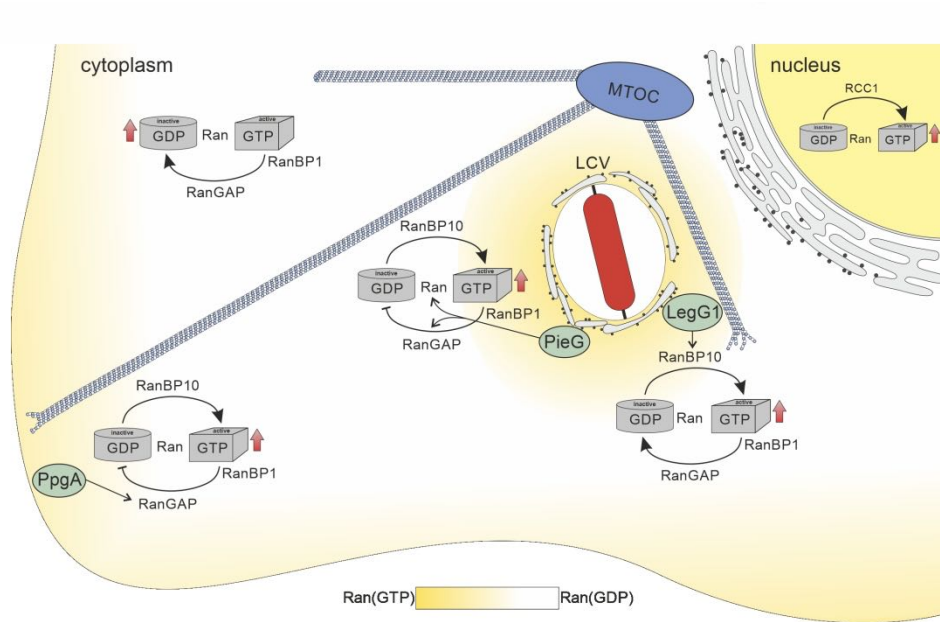


Figure 4