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

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Summary

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

Legionella species – amoebae-resistant agents of Legionnaires' disease

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

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

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

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

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

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

In addition to small GTPases of several different families, large oligomeric GTPases also play a role in *L. pneumophila* infection (Escoll *et al.*, 2017, Steiner *et al.*, 2017). Atlastin3 (Atl3/Sey1) localizes to ER tubules and catalyzes homotypic membrane fusion events. This large GTPase promotes ER remodeling around the LCV, pathogen vacuole expansion and intracellular bacterial replication (Steiner *et al.*, 2017). Furthermore, the mitochondrial Dynamin1-like large GTPase (Dnm1l) mediates mitochondrial fragmentation during *L. pneumophila* infection (Escoll *et al.*, 2017).

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

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

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

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

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

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

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

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

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

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

Legionella species harbor eukaryotic-like RCC1 repeat coding genes

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

L. pneumophila RCC1 repeat effectors localize to distinct cellular compartments

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

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

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

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

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

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

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

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

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

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

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

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

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

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

364 *Coxiella burnetii* is the causative agent of a zoonotic disease called Q ("querry") 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-κB. 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*

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

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Conflict of Interest

The authors declare no conflict of interest.

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

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

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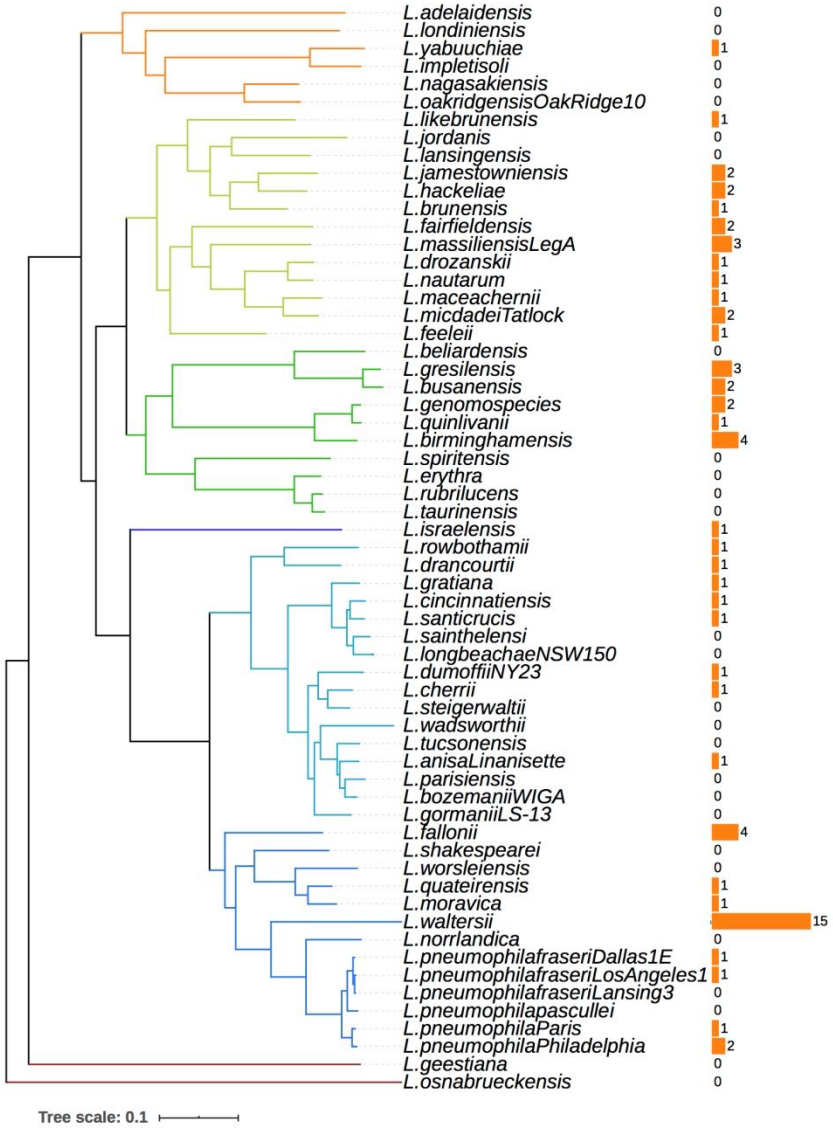
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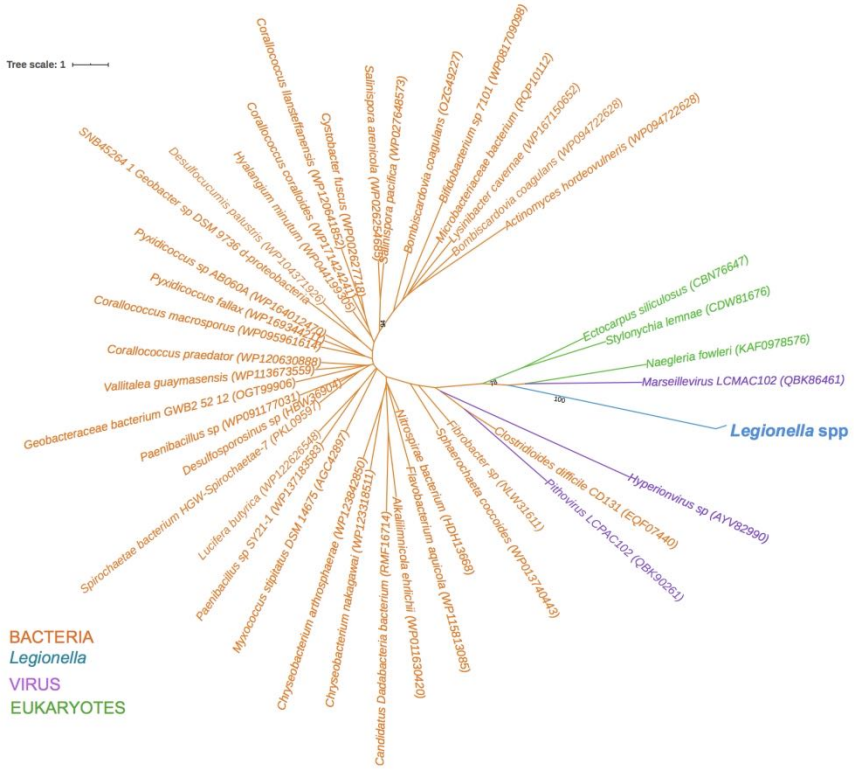
Figure 1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Corallococcus praedator (WP120630888)
Vallitalea guaymasensis (WP113673559)
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Paenibacillus sp (WP091177031)
Desulfosporosinus sp (HBW36904)
Spirochaetae bacterium HGW-Spirochaetae-7 (PKL09597)
Lucifera butyrica (WP122626548)
Paenibacillus sp SY21-1 (WP137183583)
Myxococcus stipitatus DSM 14675 (AGC42897)
Chryseobacterium arthrosphaerae (WP123842850)
Chryseobacterium nakagawai (WP123318511)
Candidatus Dadabacteria bacterium (RMF16714)
Alkalilimnicola ehrlichii (WP011630420)
Flavobacterium aquicola (WP115813085)
Nitrospirae bacterium (HDH13668)
Sphaerochaeta coccoides (WP013740443)
Flavobacter sp (NLW31611)
Clostridioides difficile CD131 (EQF07440)
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Salinispora pacifica (WP022648573)
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100
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BACTERIA
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VIRUS
EUKARYOTES

Figure 2

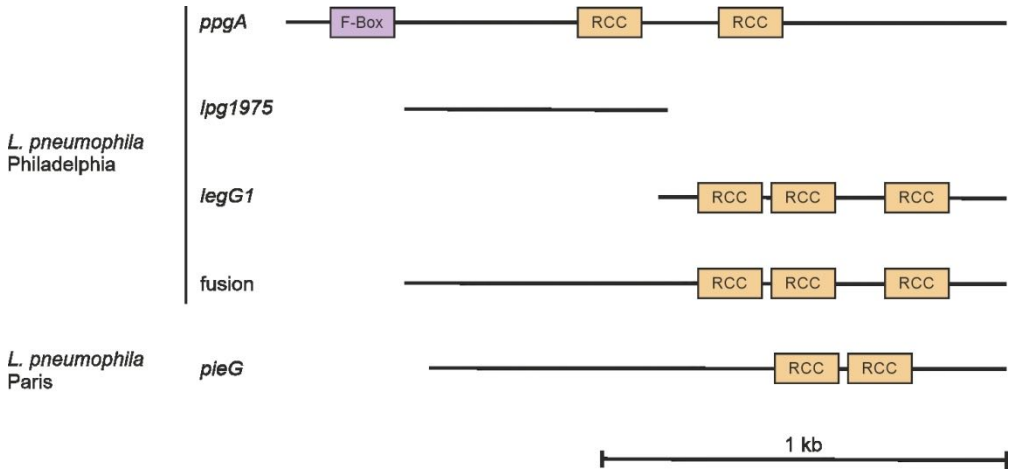
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Figure 3

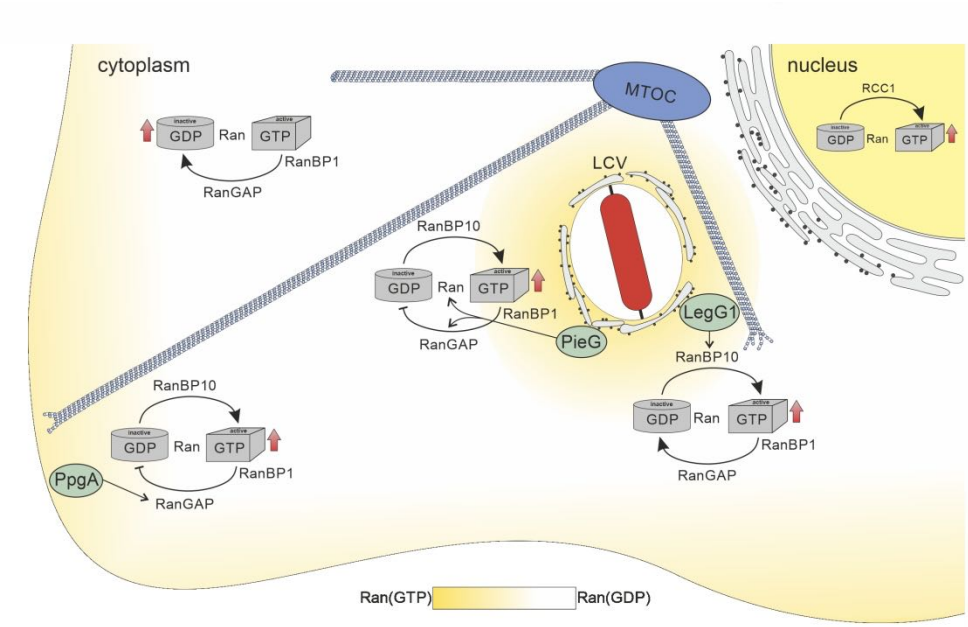
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Figure 4