

1 ***Legionella pneumophila* regulates host cell motility by targeting Phldb2 with a 14-**
2 **3-3 ζ -dependent protease effector**

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Abstract

The cytoskeleton network of eukaryotic cells is essential for diverse cellular processes, including vesicle trafficking, cell motility and immunity, thus is a common target for bacterial virulence factors. A number of effectors from the bacterial pathogen *Legionella pneumophila* have been shown to modulate the function of host actin cytoskeleton to construct the Legionella-containing vacuole (LCV) permissive for its intracellular replication. In this study, we identified the Dot/Icm effector Lem8 (Lpg1290) as a protease that interferes with host motility. We show that the protease activity of Lem8 is catalyzed by a Cys-His-Asp motif known to be associated with diverse biochemical activities. Intriguingly, we found that Lem8 interacts with the host regulatory protein 14-3-3 ζ , which activates its protease activity. Furthermore, Lem8 undergoes self-cleavage in a process that requires 14-3-3 ζ . We identified the PH domain-containing protein Phldb2 involved in cell migration as a target of Lem8 and demonstrate that Lem8 plays a role in the inhibition of host cell migration. Our results reveal a novel mechanism of inhibiting host cell motility by *L. pneumophila* for its virulence.

Key words: bacterial virulence, cell migration, cytoskeleton, cysteine protease, self-cleavage

41 Introduction

42 *Legionella pneumophila* is a Gram-negative intracellular bacterial pathogen
43 ubiquitously found in freshwater habitats, where it replicates in a wide range of amoebae
44 (Richards et al., 2013). It is believed that these natural hosts serve as the main replication
45 niches for *L. pneumophila* in the environment and provide the primary evolutionary
46 pressure for the acquisition and maintenance of virulence factors necessary for its
47 intracellular lifecycle. Infection of humans by *L. pneumophila* occurs when susceptible
48 individuals inhale aerosols generated from contaminated water, which introduces the
49 bacterium to the lungs where it is phagocytosed by alveolar macrophages. Instead of
50 being digested and cleared, internalized bacteria replicate within a membrane-bound
51 compartment termed Legionella-containing vacuole (LCV), leading to the development
52 of Legionnaires' disease, a form of severe pneumonia (Cunha et al., 2016).

53 One feature associated with the LCV is its ability to evade fusion with the lysosomal
54 network in the early phase (<8 h post-infection in mouse bone marrow-derived
55 macrophages(BMDMs)) of its development and the quick acquisition of proteins of the
56 endoplasmic reticulum (ER) origin (Kagan and Roy, 2002; Sturgill-Koszycki and Swanson,
57 2000; Swanson and Isberg, 1995). Biogenesis of the LCV requires the Dot/Icm type IV
58 secretion system that injects more than 300 effector proteins into host cells (Qiu and Luo,
59 2017). These effectors function to modulate a wide cohort of host processes, including
60 vesicle trafficking (Tan et al., 2011), protein synthesis (Shen et al., 2009), lipid metabolism
61 (Gaspar and Machner, 2014), and autophagy (Choy et al., 2012) by diverse biochemical
62 mechanisms. Coordinated activity of these effectors leads to the formation of the LCV
63 which largely resembles the ER in its morphology and protein composition (Qiu and Luo,
64 2017).

65 The cytoskeleton of eukaryotic cells is composed of microfilaments derived from
66 actin polymers, intermediate filaments and microtubules, which play distinct roles in
67 maintaining cell shape, migration, endocytosis, intracellular transport and the association
68 of cell with the extracellular matrix and cell-cell interactions (Jones et al., 2019). Due to
69 its essential role in these cellular processes, components of the cytoskeleton, particularly
70 the actin cytoskeleton is a common target for infectious agents. For example, *Salmonella*

71 *enterica* Typhimurium utilizes a set of type III effectors, including SipC, SopE and SptP
72 to reversibly regulate the rearrangement of host actin cytoskeleton to facilitate its entry
73 into non-phagocytic cells (Kubori and Galan, 2003). Other bacterial pathogens such as
74 *Chlamydia*, *Orientia tsutsugamushi*, and *Listeria* also exploit the actin cytoskeleton and
75 microtubule networks to promote their movement in the cytoplasm of host cells and cell
76 to cell spread (Cheng et al., 2018; Grieshaber et al., 2003; Kim et al., 2001).

77 Growing evidence indicates that manipulation of the actin cytoskeleton dynamics
78 plays an important role in the intracellular lifecycle of *L. pneumophila*. It has been
79 documented that chemical interference of the actin cytoskeleton structure impedes
80 bacterial entry and replication (Charpentier et al., 2009). A number of Dot/Icm effectors
81 have been shown to impose complex modulation of the host actin cytoskeleton. Among
82 these, VipA promotes actin polymerization by functioning as a nucleator (Franco et al.,
83 2012). LegK2 appears to inhibit actin nucleation by phosphorylating the Arp2/3 complex
84 (Michard et al., 2015). The protein phosphatase WipA participate in this regulation by
85 dephosphorylating several proteins involved in actin polymerization, including N-WASP,
86 NCK1, ARP3, and ACK1, leading to dysregulation of actin polymerization (He et al., 2019).
87 RavK is a metalloprotease that cleaves actin in host cells, abolishing its ability to form
88 polymers (Liu et al., 2017). Ceg14 also appears to inhibit actin polymerization by a yet
89 unrecognized mechanism (Guo et al., 2014). Interestingly, LegG1 has been
90 demonstrated to promote microtubule polymerization and host cell migration by
91 functioning as a guanine nucleotide exchange factor (GEF) for the Ran GTPase
92 (Rothmeier et al., 2013; Simon et al., 2014). Counterintuitive to the role of LegG1, cells
93 infected by *L. pneumophila* display defects in migration in a way that requires a functional
94 Dot/Icm system (Simon *et al.*, 2014), suggesting the existence of effectors that function
95 to block cell migration.

96 Herein, we demonstrate that the *L. pneumophila* effector Lem8 (Lpg1290) (Burstein
97 et al., 2009) is a cysteine protease that functions to inhibit host cell migration by targeting
98 the microtubule-associated protein Phldb2 via a mechanism that requires the regulatory
99 protein 14-3-3 ζ .

100

101 **Results**

102

103 **Lem8 is a *Legionella* effector with putative cysteine protease activities**

104 One major challenge in the study of bacterial effectors is their unique primary
105 sequences that share little similarity with proteins of known function. Bioinformatics
106 analysis has been proven useful in the identification of putative cryptic functional motifs
107 embedded in their structures. We used PSI-BLAST to analyze a library of the *L.*
108 *pneumophila* Dot/Icm effectors (Zhu et al., 2011) and found that Lem8 harbors a putative
109 Cys₂₈₀-His₃₉₁-Asp₄₁₂ catalytic triad present in a variety of cysteine proteases (**Fig. 1A**).
110 Further analysis by HHpred (Soding et al., 2005) revealed that Lem8 has high probability
111 to have structural similarity with HopN1 and AvrPphB from *Pseudomonas syringae*
112 (Rodriguez-Herva et al., 2012; Shao et al., 2002), as well as YopT from *Yersinia*
113 *enterocolitica* (Shao et al., 2002) and PfhB1 from *Pasteurella multocida* (Shao et al., 2002)
114 (**Fig. S1**).

115 Lem8 is a protein of 528 residues coded for by the gene lpg1290 in *L. pneumophila*
116 strain Philadelphia 1, it was first identified as a substrate of the Dot/Icm transporter by a
117 machine learning approach (Burstein et al., 2009). The translocation of Lem8 by the
118 Dot/Icm system into host cells during *L. pneumophila* infection was later validated by two
119 independent reporter systems (Huang et al., 2011; Zhu et al., 2011). Consistent with
120 these results, we observed Dot/Icm-dependent translocation of Lem8 into host cells using
121 the β -lactamase- and CCF2-based reporter assay. Approximately 60% of the cells
122 infected with a Dot/Icm competent strain expressing the β -lactamase-fusion emitted blue
123 fluorescence signals. No translocation was detected when the same fusion was
124 expressed in the *dotA*⁻ mutant defective in the Dot/Icm system (Berger and Isberg, 1993)
125 (**Fig. 1B**).

126 The expression of many Dot/Icm substrates peaks in the post-exponential phase,
127 probably due to the demand for high quantity of effectors to thwart host defense in the
128 initial phase of LCV construction (Luo and Isberg, 2004; Segal, 2013). Thus, we
129 evaluated the expression pattern of *lem8* throughout the entire growth cycle of *L.*
130 *pneumophila* in broth. Intriguingly, unlike most of effectors, the expression of *lem8* is

131 detected at high levels in the lag phase of its growth cycle in bacteriological medium. A
132 decrease in protein abundance is detected 9 h after the subcultures have started and is
133 maintained constant throughout the remaining 15 h experimental duration (**Fig. 1C**).
134 These results suggest that Lem8 may play a role in the entire intracellular lifecycle of
135 *L. pneumophila*.

136 Next, we attempted to determine whether the putative cysteine protease motif is
137 important for the effects of Lem8 on eukaryotic cells. We first tested whether Lem8 is
138 toxic to yeast and if so, whether the Cys₂₈₀-His₃₉₁-Asp₄₁₂ motif is required for such toxicity.
139 Expression of Lem8 from the galactose-inducible promoter caused cell growth arrest (**Fig.**
140 **1D**). Mutations in Cys₂₈₀, His₃₉₁ or Asp₄₁₂ did not affect the stability of the protein in yeast,
141 but abolished such toxicity (**Fig. 1D**). Thus, the putative cysteine protease activity
142 conferred by the predicted Cys₂₈₀-His₃₉₁-Asp₄₁₂ catalytic triad very likely is important for
143 the effects of Lem8 on eukaryotic cells.

144 Genomic analysis reveals that in addition to *L. pneumophila*, *lem8* or its homolog is
145 present only in *L. waltersii*, one of the 40 Legionella species whose genome had been
146 fully sequenced (Burstein et al., 2016). Such a low prevalence suggests that Lem8 plays
147 a role in the survival of the bacteria in specific niches, or its role in other Legionella
148 species is substituted by genes of little sequence similarity that may have arisen by
149 convergent evolution. We probed the role of *lem8* in *L. pneumophila* virulence by
150 examining intracellular replication of the Δ *lem8* mutant in the protozoan host
151 *Dictyostelium discoideum* and in BMDMs. In both host cells, intracellular growth of the
152 Δ *lem8* mutant was indistinguishable to that of the wild-type strain (**Fig. S2**), which is akin
153 to most mutants lacking one single Dot/Icm substrate gene (Qiu and Luo, 2017).

154

155 **Lem8 directly interacts with the regulatory protein 14-3-3 ζ**

156 To identify the host target of Lem8, we performed a yeast two-hybrid screening
157 against a mouse cDNA Library (Clontech) using Lem8_{C280S} fused to the DNA binding
158 domain of the transcriptional factor GAL4 as bait. Plasmid DNA of the library was
159 introduced into the yeast strain PJ69-4A (James et al., 1996) expressing the bait fusion
160 and colonies appeared on selective medium were isolated and the inserts of the rescued

161 plasmids capable of conferring the interactions were sequenced. We found that 50 out of
162 the 96 independent clones analyzed harbored portions of the gene coding for 14-3-3 ζ , a
163 member of a chaperone family important for the activity of a wide variety of proteins in
164 eukaryotic cells (Pennington et al., 2018). Robust interactions occurred in the yeast two-
165 hybrid system when full-length 14-3-3 ζ was fused to the AD domain of Gal4 (**Fig. 2A**).

166 We further explored the interactions between 14-3-3 ζ and Lem8 by reciprocal
167 immunoprecipitation (IP) assays. Flag-tagged 14-3-3 ζ was coexpressed with GFP-
168 tagged Lem8 or GFP in HEK293 cells. IP using the Flag antibody specifically precipitated
169 GFP-Lem8, whereas GFP was not detectable in similar experiments. Reciprocally, IP
170 with GFP antibodies specifically pulled down Flag-tagged 14-3-3 ζ (**Fig. 2B**). These
171 results suggest that Lem8 forms a complex with 14-3-3 ζ in mammalian cells.

172 To determine whether Lem8 directly binds to 14-3-3 ζ , we purified recombinant
173 proteins and used GST pulldown assay to analyze their interactions. We found that
174 mixing His₆-Lem8 and GST-14-3-3 ζ in reactions led to the formation of stable protein
175 complexes that can be retained by GST beads (**Fig. 2C**).

176 Members of the 14-3-3 family commonly recognize phospho-serine and/or phospho-
177 threonine sites of client proteins for binding (Muslin et al., 1996). Yet, we did not detect
178 phosphorylation on Lem8 purified from mammalian cells or *E coli* using a pan phospho-
179 serine/threonine antibody. As a control, this antibody detected phosphorylation on
180 CTNNB1, a known phosphorylated target of 14-3-3 ζ (Tian et al., 2004). As expected, no
181 signal was detected for ExoS, a non-phosphorylated 14-3-3 interacting effector from *P.*
182 *aeruginosa* (Henriksson et al., 2002) (**Fig. S3**).

183 To determine the region in Lem8 involved in binding 14-3-3 ζ , we constructed a series
184 of Lem8 deletion mutants and examined their interactions with 14-3-3 ζ by
185 immunoprecipitation. Whereas removing as few as 25 residues from the amino terminal
186 end of Lem8 abolished its ability to bind 14-3-3 ζ , a Lem8 mutant lacking the last 50
187 residues can still robustly interact with 14-3-3 ζ , and deleting an additional 50 residues
188 from this end abolished the binding (**Fig. 2D**). Thus, either 14-3-3 ζ recognizes a large
189 region of Lem8 or deletion from either end of this protein caused significant disruptions
190 in its structure and abolished its ability to interact with 14-3-3 ζ .

191

192 **Lem8 undergoes 14-3-3 ζ -dependent auto-cleavage**

193 Since Lem8 harbors the predicted Cys₂₈₀-His₃₉₁-Asp₄₁₂ catalytic triad associated with
194 proteases from diverse bacterial pathogens, we next investigated whether Lem8 cleaves
195 14-3-3 ζ . Incubation of recombinant His₆-Lem8 with His₆-14-3-3 ζ at room temperature for
196 2 h did not lead to detectable cleavage of 14-3-3 ζ . Unexpectedly, a protein with a
197 molecular weight slightly smaller than that of Lem8 was detected in this reaction (**Fig.**
198 **3A**). The production of this smaller protein did not occur in reactions that contained the
199 Lem8_{C280S} mutant or when the cysteine protease-specific inhibitor E64 was included in
200 the reactions (**Fig. 3A**), suggesting that this band represents a fragment of Lem8
201 produced by self-cleavage. Intriguingly, the self-cleavage did not occur in samples
202 containing only Lem8, suggesting that the self-cleavage activity of Lem8 requires 14-3-
203 3 ζ as a co-factor.

204 *Dictyostelium discoideum*, the protozoan host of *L. pneumophila* codes for one 14-
205 3-3 protein with 66% identity and 78% similarity to that of human 14-3-3 ζ (Eichinger et
206 al., 2005), we investigated whether the *D. discoideum* 14-3-3 (14-3-3Dd) can activate
207 Lem8 by incubating His₆-Lem8 with GST-14-3-3Dd or human 14-3-3 ζ (14-3-3 ζ HS). In
208 each case, we observed the production of a protein with a size clearly smaller than Lem8
209 as early as 2 h after the reaction has started. (**Fig. 3B**). Thus, Lem8 can be activated by
210 14-3-3 from both humans and a protist.

211 To determine the self-cleavage site of Lem8, we incubated His₆-Lem8 with His₆-14-
212 3-3 ζ at room temperature for 16 h. Proteins resolved by SDS-PAGE were stained with
213 Coomassie brilliant blue and bands corresponding to full-length and cleaved Lem8 were
214 excised, digested with trypsin and sequenced by mass spectrometry, respectively (**Fig.**
215 **3C**). Analysis of the tryptic fragments identified a semi-tryptic fragment -
216 A₄₆₈PQPTPQRQ₄₇₆- present in the cleaved protein but not in the full-length protein,
217 suggesting that the cleavage occurs between Gln₄₇₆ and Arg₄₇₇ (**Fig. 3C**). To narrow
218 down the potential self-cleavage site, we compared the abundance of identified tryptic
219 peptides from the full-length and cleaved Lem8 and found that the abundance of -
220 A₄₆₈PQPTPQR₄₇₅- was similar between two sets of samples, whereas peptide -

221 A₄₇₈QSLSAETER₄₈₇- was present only in full-length samples but not in the cleaved ones
222 (**Fig. S4A**), suggesting that the cleavage site was between R475 and R487. Consistent
223 with this notion, the signal of a semi-tryptic fragment -L₄₆₄CEKAPQPTPQRQ₄₇₆- was
224 identified in the cleaved protein but not in the full-length protein, suggesting that the
225 cleavage occurs between Gln476 and Arg477 (**Fig. 3C**).

226

227 To determine whether Lem8 undergoes auto-cleavage via the recognition of the
228 protein sequence around Gln₄₇₆, we introduced mutations to replace residues Pro₄₇₃,
229 Gln₄₇₄, Arg₄₇₅ and Gln₄₇₆ with alanine and incubated this Lem8 mutant (called 4A) with
230 14-3-3ζ. Unexpectedly, although at a lower rate, self-cleavage still occurred in this mutant
231 (**Fig. 3D**). We further examined the self-cleavage of Lem8 by fusing GFP to the carboxyl
232 end of Lem8, Lem8_{C280S} and Lem8_{ΔC50}, respectively. These fusion proteins were
233 expressed in HEK293T cells and the cleavage was probed by immunoblotting with GFP-
234 specific antibodies. We found that a fraction of Lem8-GFP and Lem8_{ΔC52}-GFP has lost
235 the GFP portion of the fusions, an event that did not occur in Lem8_{C280S}-GFP (**Fig. S4B**).
236 Thus, although the amino acids adjacent to Gln₄₇₆ play a role in its self-cleavage, other
237 factors such as the overall structure of Lem8 may contribute to the recognition of the
238 cleaving site.

239

240 **Lem8 targets Phldb2 for cleavage**

241 It has been reported that some bacterial cysteine proteases cleave both themselves
242 and their substrates in the host by recognizing sites with similar sequences. For instance,
243 AvrpphB and Avrrpt2, two type III effectors from *P. syringae* cleave themselves as well
244 as their host targets PBS1 and RIN4, respectively (Chisholm et al., 2005; Shao et al.,
245 2003). Importantly, in each case, the sequences of the recognition sites for both self-
246 cleavage and cellular target cleavage are very similar. In fact, this feature has been
247 exploited to predict the potential host substrates of these effectors by bioinformatic
248 analyses. Therefore, we performed BLAST searches and obtained 10 candidate proteins
249 that contain sequence elements resembling the self-cleavage site of Lem8, including
250 Phldb2, Rasgrp2, Pak6, Exoc8, Ankrd13B, Chkb, Ppp6R1, Kiaa1033, Gnal and Gpr61.

251 The predicted recognition sites in these proteins locate in the middle or at sites close to
252 either their amino or carboxyl ends (**Fig. 4A**). Further experiments revealed that one of
253 the candidates, Pleckstrin homology-like domain family B member 2 (Phldb2) can be
254 cleaved by Lem8 in a process that requires an intact Cys₂₈₀-His₃₉₁-Asp₄₁₂ catalytic triad.
255 The predicted Lem8 recognition site locates between the 1106th residue and the 1119th
256 residue in this protein of 1253 amino acids (**Fig. 4A**). In HEK293T cells, expression of
257 Lem8 led to a considerable reduction of endogenous Phldb2 (**Fig. 4B**). To confirm this
258 finding, we added an HA and a Flag tag to the amino and carboxyl end of Phldb2
259 respectively, and co-expressed the double tagged protein in HEK293T cells with Lem8
260 or each of the mutants with mutations in one of the three sites (C280S, H391A and D412A)
261 predicted to be critical for catalysis. Detection of tagged Phldb2 by immunoblotting with
262 the Flag-specific antibody indicated that the protein levels in cells expressing Lem8 were
263 reduced comparing to samples in which the catalytically inactive mutants were expressed.
264 Although to a lesser extent, reduction in Phldb2 was also observed in experiments in
265 which the tagged protein was detected with the HA antibody (**Fig. 4C**).

266 Phldb2 is a phosphatidylinositol-3,4,5-triphosphate (PIP3) binding protein and is
267 associated with the plasma membrane (Paranavitane et al., 2003), we next examined
268 how Lem8-mediated cleavage impacts its cellular localization. In HEK293T cells, when
269 GFP-Phldb2 was ectopically expressed, the GFP signals mainly were associated with
270 the plasma membrane, and this pattern of distribution remains unchanged in cells co-
271 expressing enzymatically inactive Lem8 mutants (**Fig. 4D**). In contrast, in cells co-
272 expressing wild-type Lem8, the GFP signals redistributed to occupy the entire cytoplasm,
273 including the nuclei of transfected cells, a pattern similar to that of GFP itself (**Fig. 4D**).
274 These observations suggest that GFP tag had been cleaved from the GFP-Phldb2 fusion
275 to assume its typical localization in these cells. We also analyzed how Lem8 impacts the
276 subcellular localization of endogenous Phldb2. In cells expressing mCherry-Lem8_{C280S},
277 Phldb2 is mainly associated with the plasma membrane. In contrast, in cells expressing
278 mCherry-Lem8, the association of Phldb2 with the plasma membrane almost became
279 undetectable (**Fig. S5A**).

280 We next examined whether the cleavage of Phldb2 by Lem8 occurs in a cell-free

281 reaction. HA-Phldb2-Flag expressed in HEK293T cells isolated by immunoprecipitation
282 was incubated with Lem8 or its inactive mutants with or without 14-3-3 ζ . Cleavage of
283 Phldb2 occurred only in reactions containing wild-type Lem8 and 14-3-3 ζ (**Fig. 4E**). Taken
284 together, these results establish Phldb2 as a target of Lem8.

285 Our results using the double tagged Phldb2 suggest that Lem8 likely cleaves Phldb2
286 not only at the predicted site located in the carboxyl end of the protein, but also targets
287 its amino terminal portion (**Fig. 4C-E**). To test this hypothesis, we constructed two Phldb2
288 mutants by replacing residues Arg₁₁₁₁ and Gln₁₁₁₂ (Phldb2_{AA1}) or Gln₁₁₁₂ and Arg₁₁₁₃
289 (Phldb2_{AA2}) within the predicted recognition sequence with alanine. Each of these
290 mutants was co-expressed with Lem8 in HEK293T cells by transfection. Comparing to
291 samples expressing enzymatically inactive Lem8, the amounts of protein detected by the
292 amino terminal Flag epitope and the carboxyl end HA tag both decreased in cells co-
293 expressing wild-type Lem8 (**Fig. S5B**). We validate this notion by making constructs in
294 which GFP was fused to the amino terminal end of Phldb2 and three of its truncation
295 mutants, Phldb2 Δ N50, Phldb2 Δ N100 and Phldb2 Δ N200, respectively. Each of these fusion
296 proteins was co-expressed with Lem8 or Lem8_{C280S} in HEK293T cells and the protein
297 level of these fusions was probed by immunoblotting with GFP-specific antibodies. In
298 each case, a fraction of the protein has lost the GFP portion of the fusions when co-
299 expressed with Lem8 but not with Lem8_{C280S} (**Fig. S5C**). Intriguingly, the cleavage also
300 occurred in fusion proteins in which the GFP is fused to the carboxyl terminus of Phldb2
301 or Phldb2 Δ C153 (**Fig. S5C**). These results are consistent with the notion that Lem8 targets
302 Phldb2 at multiple sites.

303 We also attempted to detect Lem8-mediated cleavage of endogenous Phldb2 in cells
304 infected with *L. pneumophila*. Although Lem8 translocated into infected cells by a
305 Dot/Icm-competent strain expressing Lem8 from a multicopy plasmid is readily detectable,
306 we were unable to detect Phldb2 cleavage in these samples (**Fig. S5D**). The most likely
307 reason for the inability to detect Lem8 activity against Phldb2 in infected cells is the low
308 abundance or instability of the cleaved protein or a combination of both.

309
310 **14-3-3 ζ binds Lem8 by recognizing a coiled-coil motif in its amino terminal region**

311 Using the online MARCOIL sequence analysis software (Gabler et al., 2020), we
312 identified a putative coiled coil motif located in the amino region of Lem8. Coiled coil is a
313 common structural element in proteins, particularly those of eukaryotic origin; it is formed
314 by 2-7 supercoiled alpha-helices (Liu et al., 2006), and often is involved in protein-protein
315 interactions, thus playing an important role in the formation of protein complexes
316 (Burkhard et al., 2001). To determine the role of this region in the activity of Lem8, we
317 introduced mutations to replace Leu₅₈ and Glu₅₉, the two sites predicted to be essential
318 for the coiled coil structure in Lem8, with glycine (called Lem8_{GG}) (**Fig. 5A**). When tested
319 in yeast, these mutations have completely abolished the toxicity of Lem8 without affecting
320 its expression or stability (**Fig. 5B**). These mutations may affect the cysteine protease
321 activity of Lem8, its interaction with the regulatory protein 14-3-3 ζ or its ability to
322 recognize substrates.

323 We examined the ability of Lem8_{GG} to cleave Phldb2 by coexpressing them in
324 HEK293T cells. Whereas wild-type Lem8 consistently cleaves this substrate, Lem8_{GG}
325 has lost such activity despite a similar expression level (**Fig. 5C**). To test the self-cleavage
326 of Lem8_{GG}, we expressed Lem8-GFP or Lem8_{GG}-GFP in HEK293T cells and probed the
327 fusion proteins by immunoblotting with GFP-specific antibodies. Comparing to Lem8-GFP,
328 the protein levels of Lem8_{GG}-GFP and in Lem8_{C280S}-GFP were similarly higher, indicating
329 that the loss of the GFP portion of the fusion occurred in Lem8-GFP but not in Lem8_{GG}-
330 GFP (**Fig. 5D**). Finally, we examined the impact of these mutations on the interaction
331 between Lem8 and 14-3-3 ζ . Albeit Lem8_{GG} expressed similarly to the wild-type, it has
332 largely lost the ability to bind 14-3-3 ζ in immunoprecipitation assays (**Fig. 5E**). Together
333 with the observation that Lem8 mutants lacking as few as 25 residues from its amino
334 terminal end are unable to bind 14-3-3 ζ , these results suggest that the regulatory protein
335 most likely bind Lem8 by recognizing the coiled coil motif located in its amino end region.

336

337 **Auto-cleaved Lem8 maintains the cysteine protease activity**

338 It has been well-established that some proteins, particularly enzymes are made as
339 precursors or zymogens that need either auto-processing or cleavage by other enzymes
340 to exhibit their biological functions. One such example is caspases involved in cell death

341 regulation and other important cellular functions. These enzymes are synthesized as
342 zymogens before being activated by proteolytic cleavage in response to stimulation
343 (Shalini et al., 2015). In some cases, auto-processing leads to changes or even loss of
344 their enzymatic activity (Kapust et al., 2001; Zhang et al., 2018). To investigate whether
345 Lem8 that has undergone self-cleavage still possesses the cysteine protease activity, we
346 tested the cleavage of Phldb2 by Lem8 Δ C52, its self-processed form. Similar to full-length
347 Lem8, Lem8 Δ C52 was able to reduced the protein levels of Phldb2. In contrast, other
348 truncation mutants, including Lem8 Δ N25, Lem8 Δ N50 and Lem8 Δ C100 have lost the capacity
349 to cleave Phldb2 (**Fig. 6A**). In addition, Lem8 Δ C52, but not Lem8 Δ N25 or Lem8 Δ C100, cleaved
350 the GFP tag from from the GFP-Phldb2 fusion and released the GFP signals from the
351 plasma membrane (**Fig. 6B**). Intriguingly, although their ability to cleave Phldb2
352 appears similar, under our experimental conditions, the protein level of Lem8 Δ C52 is
353 considerably lower than that of Lem8 (**Fig. 6A**), suggesting that the self-processed form
354 has higher activity.

355 We next examined whether the protease activity of Lem8 Δ C52 still requires 14-3-3 ζ
356 binding. Results from immunoprecipitation and pulldown assays with purified protiens
357 clearly showed that Lem8 Δ C52 robustly binds 14-3-3 ζ (**Fig. 6C-D**). Furthermore,
358 incubation of Lem8 Δ C52 with Phldb2 isolated from cells did not lead to its cleavage, but
359 the inclusion of 14-3-3 ζ allowed the cleavage to occur (**Fig. 6E**), indicating that Lem8 Δ C52
360 still requires 14-3-3 ζ for its protease activity.

361

362 **Lem8 inhibits migration in mammalian cells**

363 Phldb2 is a PIP3 binding protein involved in microtubule stabilization (Lansbergen et
364 al., 2006; Parnavitane *et al.*, 2003), thus playing a pivotal role in cell motility. Depletion
365 of Phldb2 significantly reduces the migration of MDA-231 cells in the haptotactic
366 migration assay (Astro et al., 2014). As Lem8 cleaves Phldb2, we hypothesized that
367 Lem8 may affect cell migration. To test this, we first established HEK293T-derived cell
368 lines that stably express GFP, GFP-Lem8 or GFP-Lem8 Δ C280S. Immunoblotting confirmed
369 that Lem8 and Lem8 Δ C280S robustly expressed in the respective cell lines. Furthermore, in
370 the cell line expressing Lem8, the level of Phldb2 was drastically reduced comparing to

371 that in the line expressing GFP or Lem8_{C280S} (**Fig. 7A**). We then used the wound-healing
372 scratch assay (De Ieso and Pei, 2018) to examine the impact of ectopic Lem8 expression
373 on cell motility. Confluent monolayer of each cell lines was scratched using a pipette tip
374 and the migration of cells into the gap was monitored over a period of 24 h. Results from
375 this experiment showed that the percentage of wound closure at 24 h after wounding was
376 around 50% in samples using cells expressing GFP or GFP-Lem8_{C280S}. In the same
377 experimental duration, cells expressing GFP-Lem8 only filled the gap by 26%, which was
378 significantly slower than that of the controls (**Fig. 7B**). Thus, ectopic expression of Lem8
379 inhibits mammalian cell migration.

380 An earlier study has shown that in the under-agarose migration assay, *L.*
381 *pneumophila* inhibits the chemotaxis of mouse macrophages towards cytokines CCL5
382 and TNF- α in a Dot/Icm-dependent manner (Simon *et al.*, 2014). Yet, the Dot/Icm
383 substrates responsible for this inhibition remain elusive. We further studied whether Lem8
384 contributes to the inhibition of infected cell migration. To test this, we performed the
385 scratch assay with cells infected with wild-type *L. pneumophila* or several strains relevant
386 to *lem8*. The percentage of wound closure by cells infected with wild-type *L. pneumophila*
387 or Δ *lem8*(pLem8) was significantly lower than that with cells infected with the Δ *lem8*
388 mutant. Consistent with its lack of the protease activity, Lem8_{C280S} was unable to
389 complement the defects displayed by the Δ *lem8* mutant (**Fig. 7C**). Thus, the inhibition of
390 cell migration by *L. pneumophila* during infection is caused at least in part by the activity
391 of Lem8.

392

393 Discussion

394 Intracellular bacteria manipulate cellular processes to create a niche that supports
395 their survival and replication in host cells by virulence factors that target proteins
396 important for the regulation of these processes. These virulence factors often attack host
397 regulatory proteins by diverse posttranslational modifications (PTMs) such as
398 phosphorylation (Krachler *et al.*, 2011), ubiquitination (Zhou and Zhu, 2015), AMPylation
399 (Yarbrough *et al.*, 2009), acetylation (Mukherjee *et al.*, 2006) and ADP-ribosylation
400 (Cohen and Chang, 2018). Proteolytic processing is a type of PTM that can lead to the

401 activation, inactivation or destruction of target proteins, causing alterations in cellular
402 structure or signaling that benefit the pathogen. For instance, the type III effector EspL
403 from enteropathogenic *Escherichia coli* functions as a cysteine protease that antagonizes
404 host inflammatory response by degrading several proteins involved in necroptotic
405 signalling (Pearson et al., 2017). Our results herein establish Lem8 as a cysteine
406 protease that directly targets the microtubule associated protein Phldb2, therefore
407 contributing to the inhibition of host cell migration by *L. pneumophila*. Lem8 joins a
408 growing list of Legionella effectors with protease activity, including the serine protease
409 Lpg1137 that inhibits autophagy by cleaving syntaxin 17 (Arasaki et al., 2017) and the
410 metalloprotease RavK that attacks actin to disrupt the actin cytoskeleton of host cells (Liu
411 et al., 2017).

412 One interesting feature associated with Lem8 is the requirement of 14-3-3 ζ for its
413 activity. In line with the notion that amoebae are the primary host of *L. pneumophila*, the
414 sole 14-3-3 protein from *D. discoideum* similarly activates Lem8. In mammals, members
415 of the 14-3-3 family, including 14-3-3 ζ often bind their client proteins by recognizing
416 phosphorylated pockets with relatively conserved sequences such as RSX[pS/pT]XP
417 (mode I) and RXXX[pS/pT]XP (mode II) (pS, phospho-serine, pT, phospho-threonine, X,
418 any residue) (Morrison, 2009). Intriguingly, neither of these two motifs is present in Lem8.
419 Consistently, using a pan phospho-serine/threonine antibody capable of detecting
420 phosphorylation of vimentin, another 14-3-3 ζ binding protein in mammalian cells, we
421 cannot detect phosphorylation on Lem8 purified from mammalian cells or *E. coli* (**Fig.**
422 **S3**). The binding of 14-3-3 proteins to unmodified clients is not unprecedented. All
423 isoforms of 14-3-3 bind non-phosphorylated ExoS of *P. aeruginosa* by recognizing the
424 DALDL element (Henriksson et al., 2002), which bears sequence similarity to the
425 unphosphorylated target WLDLE, an artificial R18 peptide inhibitor derived from a phage
426 display library (Petosa et al., 1998). Elements with a sequence similar to these
427 established recognition sites are not present in Lem8 nor is there one resembling those
428 in other nonphosphorylated binding targets of 14-3-3, including GPIb- α (Gu and Du,
429 1998), p75NTR-associated cell death executor (NADE) (Kimura et al., 2001) and CLIC4
430 (Suginta et al., 2001).

431 Two lines of evidence suggest that 14-3-3 ζ recognizes a coiled coil motif in the amino
432 terminal portion of Lem8. First, deletion of as few as 25 residues from the amino terminus
433 end of Lem8 abolished its interaction with 14-3-3 ζ (**Fig. 2D**). Second, the integrity of a
434 predicted coiled coil motif in the amino terminal portion of Lem8 is required for its binding
435 to the regulatory protein (**Fig. 5**). Coiled coil motifs have long been known to be important
436 for protein-protein interaction but its involvement in binding 14-3-3 has not yet been
437 established. The binding of 14-3-3 to TRIM25 had been suggested to be mediated by
438 recognizing a coiled coil domain, but the mechanism of such binding or whether
439 phosphorylation is required remains unclear (Gupta et al., 2019). Future study,
440 particularly structural analysis of the Lem8-14-3-3 ζ complex may allow a definite
441 identification of the region in Lem8 recognized by 14-3-3 ζ , which will surely shed light on
442 the additional features of the sequences recognizable by these important regulatory
443 proteins.

444 The self-cleavage of Lem8 has allowed us to identify its recognition sequence and
445 several candidate cellular targets. One unexpected observation is that mutations in the
446 identified recognition element reduced but did not abolish self-cleavage (**Fig. 3D**).
447 Thus, the primary sequence may not be the only factor that dictates the specificity of Lem8
448 in substrate recognition. Other factors such as the overall structure of substrates may
449 contribute to the determination of the cleavage site. The low level of promiscuity in
450 cleavage site selection may allow Lem8 to more effectively bring down the protein level
451 of its cellular targets, which may explain the requirement of 14-3-3 ζ for its activity. If a
452 host co-factor is not needed for its activity, Lem8 may cleave itself or even other proteins
453 in *L. pneumophila* cells. For Lem8, self-cleavage in the absence of 14-3-3 ζ in bacterial
454 cells will be disastrous because the cleaved product will lose the portion of the protein
455 that harbors translocation signals recognized by the Dot/Icm system (Luo and Isberg,
456 2004; Nagai et al., 2005). Likewise, the requirement of CaM by the Dot/Icm effector SidJ
457 to inhibit the activity of members of the SidE family is to ensure that such inhibition does
458 not occur in bacterial cells (Bhogaraju et al., 2019; Black et al., 2019; Gan et al., 2019;
459 Sulpizio et al., 2019). The promiscuity in cleavage site recognition by Lem8 is also
460 supported by the observation that this protease appears to cleave Phldb2 at multiple sites

461 **(Figs. 4 and S5).**

462 Interference with host cell motility appears to be a common strategy used by
463 bacterial pathogens. For example, *Salmonella enterica* Typhimurium inhibits the
464 migration of infected macrophages and dendritic cells in a process that requires its type
465 III effector SseI, which binds to IQGAP1, an important regulator of cell migration
466 (McLaughlin et al., 2009). Similarly, the phosphatidylinositol phosphatase IpgD from
467 *Shigella flexneri* contributes to the inhibition of chemokine-induced migration of human T
468 cells (Konradt et al., 2011). The observation that cells infected with the wild-type *L.*
469 *pneumophila* or strain $\Delta Lem8$ (pLem8) migrated significantly slower than those infected
470 with the $\Delta Lem8$ mutant or its complementation strain expressing the Lem8_{C280A} mutant
471 suggests a role of Lem8 in cell mobility inhibition (Simon et al., 2014).

472 Akin to most *L. pneumophila* Dot/Icm effectors, Lem8 is not required for proficient
473 bacterial intracellular growth in commonly used laboratory hosts such as *D. discoideum*.
474 Lem8 may be required for the survival of the bacteria in some specific habitats or other
475 Dot/Icm effectors may substitute its role by distinct mechanisms, thus contributing to such
476 inhibition. Future studies aiming at the identification and characterization of Dot/Icm
477 effectors involved in attacking host cells motility will continue to provide insights into the
478 mechanisms of not only bacterial virulence but also the regulation of eukaryotic cell
479 migration.

480

481 **Materials and Methods**

482 **Bacterial stains, plasmids and cell culture**

483 *E. coli* strain DH5 α was used for plasmid construction and strain BL21(DE3) or
484 XL1blue was used for recombinant protein production and purification. All *E. coli* strains
485 were grown on LB agar plates or in LB broth at 37°C. For maintenance of plasmids in *E.*
486 *coli*, antibiotics were added in media at the following concentrations: ampicillin, 100 μ g/mL;
487 kanamycin, 30 μ g/mL. All *L. pneumophila* strains were derived from the Philadelphia 1
488 strain Lp02 and the *dotA*⁻ mutant strain Lp03 (Berger and Isberg, 1993) and were listed
489 in **Table S1**. *L. pneumophila* was cultured in N-(2-acetamido)-2-aminoethanesulfonic
490 acid buffered yeast extract medium (AYE) or on charcoal buffered yeast extract plates
491 (CYE). When necessary, thymidine was added into AYE at a final concentration of 0.2
492 g/mL. pZL507 and its derivatives which allow expression of His₆-tagged proteins (Xu et
493 al., 2010) in *L. pneumophila* were maintained by thymidine autotrophic. Deletion of the
494 Lem8 coding gene lpg1290 from the genome of *L. pneumophila* was performed as
495 described previously (Liu and Luo, 2007).

496 Plasmids used in this study are listed in **Table S1**. Genes were amplified by
497 polymerase chain reactions (PCR) using Platinum™ SuperFi II Green PCR mix
498 (Invitrogen, cat# 12369050). The PCR product was digested with restriction enzymes
499 (New England Biolabs, NEB), followed by ligated to linearized plasmid using T4 DNA
500 ligase (NEB). For site-directed mutagenesis, plasmid was reacted with primer pairs
501 designed to introduce the desired mutations using Quikchange kit (Agilent, cat# 600670).
502 After digestion with the restriction enzyme *DpnI* (NEB, cat# R0176), the products were
503 transformed into *E. coli* strain DH5 α . All substitution mutants were verified by double
504 strand DNA sequencing. The sequences of primers used for molecular cloning are listed
505 in **Table S1**.

506 HEK293T and Hela cells purchased from ATCC were cultured in Dulbecco's modified
507 minimal Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS).
508 Bone marrow cells were isolated from 6- to 10-week-old female A/J mice
509 (GemPharmatech, Co., Ltd.) and were differentiated into BMDMs using L929-cell
510 conditioned medium as described previously (Conover et al., 2003). PCR-based test

511 (Sigma, cat# MP0025) was used to validate the absence of potential mycoplasma
512 contamination in all mammalian cell lines. pAPH-HA, a derivative of pVR1012 (Wang et
513 al., 2018) suitable for expressing proteins with an amino HA tag and a carboxyl Flag tag.

514

515 **Yeast manipulation**

516 Unless otherwise indicated, yeast strains used in this study were derived from W303
517 (Thomas and Rothstein, 1989); yeast was grown at 30°C in yeast extract, peptone,
518 dextrose medium (YPD) medium or in appropriate amino acid dropout synthetic media
519 supplemented with 2% of glucose or galactose as the sole carbon source.

520 For assessment of inducible protein toxicity, Lem8 or its derivatives were cloned into
521 pYES2/NTA (Invitrogen) in which their expression is driven by the galactose-inducible
522 promoter. Yeast transformation was performed using the lithium acetate method (Gietz
523 et al., 1995). After growing in selective liquid medium with 2% raffinose, yeast cultures
524 were serially diluted (five-fold) and 10 µL of each dilution was spotted onto selective
525 plates containing glucose or galactose. Plates were incubated at 30°C for 3 days before
526 image acquisition.

527 To screen Lem8-interacting protein(s), Gal4-based two-hybrid screening against the
528 mouse cDNA library (Clontech) was performed as described before (Mitsuzawa et al.,
529 2005). Briefly, Lem8_{C280S} was inserted into pGBKT7 (Banga et al., 2007) to give
530 pGBKLem8, which was transformed into the yeast strain PJ-64A (James *et al.*, 1996) and
531 the resulting strain was used for yeast two-hybrid screening. The mouse cDNA library
532 was amplified in accordance with the manufacturer's instructions and the plasmid DNA
533 was transformed into strain PJ-64A (pGBKLem8). Transformants were plated onto a
534 selective synthetic medium lacking adenine, tryptophan, leucine, and histidine, colonies
535 appeared on the selective medium were verified for interactions by reintroducing into
536 strain PJ-64A (pGBKLem8) and inserts of those that maintained the interaction
537 phenotype were sequenced to identify the interacting proteins.

538 To validate the interactions between 14-3-3ζ and Lem8, its full-length gene was
539 inserted into pGADGH (Banga *et al.*, 2007) and the plasmids were introduced yeast strain

540 PJ-64A (pGBKLem8). Yeast strains harboring the indicated plasmid combinations were
541 streaked on Leu⁻ and Trp⁻ synthetic medium to select for plasmids and the transformants
542 were transferred to Leu⁻, Trp⁻, Ade⁻, and His⁻ medium to examine protein-protein
543 interactions measured by cell growth.

544

545 **Antibodies and immunoblotting**

546 Polyclonal antibody against Lem8 were generated according to the protocol
547 described before (Guide for the Care and Use of Laboratory Animals, 1996; J. Derrell
548 Clark, 1997). Briefly, 1 mg of emulsified His₆-Lem8 with complete Freund's adjuvant was
549 injected intracutaneously into a rabbit 4 times at 10-day intervals. Sera of the immunized
550 rabbit containing Lem8-specific antibodies were used for affinity purification of IgG with
551 an established protocol (Harlow, 1999).

552 Samples from cells or bacteria lysates were prepared by adding 5×SDS loading
553 buffer and heated at 95°C for 10 min. The soluble fraction of the lysates was resolved by
554 SDS-PAGE, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes
555 (Pall Life Sciences). The membranes were blocking with 5% nonfat milk for 30 min,
556 followed by incubated with primary antibodies at the indicated dilutions: α-Phldb2 (Sigma,
557 cat# HPA035147, 1:1000), α-HA (Sigma, cat# H3663, 1:3000), α-Flag (Sigma, Cat#
558 F1804, 1: 3000), α-GFP (Proteintech, cat# 50430-2-AP, 1:5000), α-GST (Proteintech,
559 cat# 66001-2, 1:10000), α-His (Sigma, cat# H1029, 1: 3,000), α-ICDH (1: 10,000) (Xu *et*
560 *al.*, 2010), α-Lem8 (1: 5,000), α-PGK (Abcam, cat# ab113687, 1:2,500) and α-Tubulin
561 (Bioworld, cat# AP0064, 1:10,000). After washed 3 times, the membranes were
562 incubated with appropriate HRP-labeled secondary antibodies and the signals were
563 taken and analyzed by Tanon 5200 Chemiluminescent Imaging System.

564

565 **Transfection and immunoprecipitation**

566 When grown to approximately 80% confluence, HEK293T cells were transfected
567 using Lipofectamine 3000 (Invitrogen, cat# L3000150) according to the manufacturer's
568 protocol. Twenty-four hours after transfection, cells were lysed using a lysis buffer (50
569 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, PH 7.5) for 10 min on ice, followed by

570 centrifugation at 12,000g at 4°C for 10 min. Beads coated with Flag- (Sigma, cat# F2426),
571 HA- (Sigma, cat# E6779) or GFP-specific antibodies (Sigma, cat# G6539) were washed
572 twice with lysis buffer and then mixed with the prepared cell supernatant. The mixture
573 was incubated on a rotatory shaker at 4°C overnight. The resin was washed with the lysis
574 buffer for five times, followed by boiling in the Laemmli buffer at 95°C for 10 min to release
575 the bound Flag- or HA-tagged proteins. For proteins used in biochemical reactions, the
576 Flag- or HA-tagged proteins were eluted with Flag peptide (Sigma, cat# F4799) or HA
577 peptide (Sigma, cat# I2149), respectively.

578

579 **Protein expression and purification**

580 Lem8 and its mutants were amplified by PCR and cloned into pQE30 to express
581 His₆-tagged proteins. The plasmids were transformed into in *E. coli* strain XL1blue and
582 grown in LB broth. When the cell density reached an OD₆₀₀ of 0.8, isopropyl-β-D-
583 thiogalactopyranoside (IPTG) was added into the cultures at a final concentration of 0.2
584 mM to induce the expression of target proteins for 14 h at 16°C. Cells collected by
585 centrifugation were re-suspended in a lysis buffer (1×PBS, 2 mM DTT and 1 mM PMSF),
586 and were lysed with a cell homogenizer (JN-mini, JNBIO, Guangzhou, China). The
587 lysates were centrifugated at 20,000g for 30 min at 4°C twice to remove cell debris. The
588 supernatant was incubated with Ni²⁺-NTA beads (QIAGEN) at 4°C for 1 h, followed by
589 washed with 50x bed volumes of 20 mM imidazole to remove unbound proteins. The
590 His₆-tagged proteins were eluted with 250 mM imidazole in PBS buffer. Purified proteins
591 were dialyzed in a storage buffer (30mM NaCl, 20 mM Tris, 10% glycerol, pH 7.5)
592 overnight at 4°C and then stored at -80°C.

593 14-3-3ζ and its homologous genes were cloned into pGEX6p-1 to express GST-
594 tagged proteins. The plasmids were transformed into *E. coli* strain BL21(DE3). Protein
595 expression induction and purification was carried out similarly with Glutathione
596 Sepharose 4B (GE Healthcare) beads. The resin was collected and washed for with wash
597 buffer (lysis buffer plus 200 mM NaCl). The GST-tagged proteins were eluted with 10 mM
598 glutathione and stored at -80°C after dialysis.

599

600 ***In vitro* cleavage assays**

601 For auto-cleavage assays, 5 µg His₆-Lem8 or its mutants was incubated with or
602 without 2.5 µg 14-3-3ζ in 50 µl reaction buffer (50 mM Tris, 150 mM NaCl, PH 7.5) at
603 room temperature for the indicated time points. For Phldb2 cleavage, Flag-Phldb2
604 purified from HEK293T cells were added into reactions with or without Lem8 and 14-3-
605 3ζ at room temperature for the indicated time. In each case, samples were analyzed by
606 SDS-PAGE followed by immunoblotting or Coomassie brilliant blue staining.

607

608 **GST pulldown assay**

609 GST-14-3-3ζ or GST bound to Glutathione Sepharose 4B was incubated with His₆-
610 Lem8 in a binding buffer (50 mM Tris, 137 mM NaCl, 13.7 mM KCl) for 2 h at 4 °C. After
611 washing three times with the binding buffer, beads were boiled in the Laemmli buffer at
612 95°C for 10 min and the samples were resolved by SDS-PAGE. Proteins were detected
613 by Coomassie brilliant blue staining.

614

615 **Bacterial infection, immunostaining and image analysis**

616 For infection experiments, *L. pneumophila* strains were grown in AYE broth to the
617 post-exponential growth phase (OD₆₀₀=3.3-3.8). When necessary, complementation
618 strains were induced by 0.1 mM IPTG for another 4 h at 37°C before infection.

619 To determine intracellular bacterial growth, *D. discoideum* or BMDMs of A/J mice
620 were infected with relevant *L. pneumophila* at a multiplicity of infection (MOI) of 0.05. 2 h
621 after adding the bacteria, the cells were washed using warm PBS to remove the
622 extracellular bacteria. *D. discoideum* and BMDMs were maintained in 22°C and 37°C,
623 respectively. At the indicated time points, cells were lysed with 0.2% saponin and
624 appropriately diluted lysates were plated on CYE plates. After 4-day incubation at 37°C,
625 the counts of bacterial colonies were calculated to evaluate the growth.

626 To determine the impact of the infection on cell migration, HEK293T cells transfected
627 to express FcγRII receptor (Qiu et al., 2016) were infected with the indicated bacterial
628 strains. 2 h after infection, cells were washed using warm PBS and were used for the
629 wound healing assay.

630 To determine the cellular localization of Lem8 in infected cells, BMDMs were infected
631 with relevant *L. pneumophila* strains at an MOI of 10 for 2 h. The samples were
632 immunostained as described earlier (Haenssler et al., 2015). Briefly, we washed the
633 samples 3 times with PBS to remove extracellular bacteria, and fixed the cells with 4%
634 paraformaldehyde at room temperature for 10 min. After three times washes, cells were
635 permeabilized using 0.1% Triton X-100 and then were blocked with 4% goat serum for 1
636 h. Samples were incubated with rat anti-*Legionella* antibodies (1:10,000) and rabbit anti-
637 Lem8 antibodies (1:100) at 4°C overnight. followed by incubated with appropriate
638 fluorescence-labeled secondary antibodies at room temperature for 1 h. After stained by
639 Hoechst 33342 (Invitrogen, cat# H3570, 1:5000), samples were inspected using an
640 Olympus IX-83 fluorescence microscope.

641 To detect the cleavage of endogenous Phldb2 by Lem8, Hela cells transfected to
642 express mCherry-Lem8 or mCherry-Lem8_{C280S} were stained with Phldb2-specific
643 antibodies (1:100) as described above. The images were taken using a Zeiss LSM 880
644 confocal microscope. The determine the impact on ectopically expressed Phldb2,
645 mCherry-Lem8 or mutants each was co-transfected with GFP-Phldb2 into HEK293T cells
646 seeded onto glass coverslips (Nest, cat# 801001). Fixed samples were stained with
647 Hoechst, cell images were acquired by a confocal microscope.

648

649 **Production of lentiviral particles and transduction**

650 For production of lentiviral particles carrying *lem8* or its mutants, the *gfp-lem8* fusion
651 was inserted into pCDH-CMV-MCS-EF1a-Puro (System Biosciences, cat# CD510B-1).
652 The plasmids were co-transfected with pMD2.G (gift from Dr. Didier Trono, Addgene
653 #12259) and psPAX2 (gift from Dr. Didier Trono, Addgene #12260) into HEK293T cells
654 grown to about 70% confluence. Supernatant was collected after 48 hours incubation,
655 followed by filtration with 0.45- μ m syringe filters. After measuring the titers using qPCR
656 with the Lentivirus Titer Kit (abm, cat# LV900), the packed lentiviral particles were used
657 to infect newly prepared HEK293T cells at an MOI of 10. After incubation for 2 days, cells
658 were sorted by BD Influx™ cell sorter to establish cell lines stably expressing the gene
659 of interest.

660

661 **Mass spectrometry analysis of Lem8 self-cleavage site**

662 Recombinant His₆-Lem8 was incubated with His₆-14-3-3 for 8 h and the samples were
663 separated by SDS-PAGE. After Coomassie brilliant blue staining, bands corresponding
664 full-length His₆-Lem8 or cleaved were excised and subjected to in-gel digestion with
665 trypsin. Peptides were loaded into a nano-LC system (EASY-nLC 1200, Thermo Scientific)
666 coupled to an LTQ-Orbitrap mass spectrometer (Orbitrap Velos, Thermo Scientific).
667 Peptides were separated in a capillary column (75 µm x 15 cm) packed with C18 resin
668 (Michrom BioResources Inc., 4 µm, 100 Å) with the following gradient: solvent B (100
669 ACN, 0.1% FA) was started at 7% for 3 min and gradually raised to 35% in 40 min, then
670 rapidly increased to 90% in 2 min and maintained for 10 min before column equilibration
671 with 100% solvent A (97% H₂O, 3% ACN, 0.1% FA). The flow rate was set at 300 nL/min
672 and eluting peptides were directly analyzed in the mass spectrometer. Full-MS spectra
673 were collected in the range of 350 to 1500 *m/z* and the top 10 most intense parent ions
674 were submitted to fragmentation in a data-dependent mode using collision-induced
675 dissociation (CID) with the max injection time of 10 milliseconds. MS/MS spectra were
676 searched against the *Legionella pneumophila* (strain Philadelphia 1) database
677 downloaded from UniProt using Mascot (Matrix Science Inc.). The signals of Lem8 tryptic
678 peptides were compared between full-length and cleaved samples to narrow down the
679 potential cleavage site(s) within specific peptides, cleaved Lem8 semi-tryptic peptides
680 were inspected manually.

681

682 **Wound healing assay**

683 Wound healing assays were performed as previously described (Liang et al., 2007).
684 Briefly, HEK293T cells were seeded into 6-well plates and incubated until the confluency
685 reached about 90%. The cell monolayer was scraped in a straight line using a p200 pipet
686 tip to create a “wound”, followed by washing with growth medium to remove the debris.
687 Reduced-serum medium (1% serum) was added and the cells were placed back in a
688 37°C incubator. 2 h and 24 h after making the scratch, images of the cell monolayer
689 wound were taken using an Olympus IX-83 fluorescence microscope. For each image,

690 distances between one side of the wound and the other were quantitated by Image J
691 (<http://rsb.info.nih.gov/ij/>). The wound healing rate was calculated by the following
692 formula: % wound healing = (0 h distance – 24 h distance)/24 h distance × 100.

693

694 **Data quantitation, statistical analyses**

695 All data were represented as mean ± standard deviation (SD). Student's *t*-test was
696 applied to analyze the statistical difference between two groups each with at least three
697 independent samples.

698

699 **Acknowledgements**

700 The authors thank Dr. Shaohua Wang for plasmids, the study was funded in part by Jilin
701 Science and Technology Agency grant 20200403117SF (LS), 20200901010SF (DL),
702 National Natural Science Foundation of China grant 21974002 (XL), Beijing Municipal
703 Natural Science Foundation grant 5202012 (XL), and the National Institutes of Health
704 grant R01AI127465 (ZQL).

705

706 **Author contributions**

707 LS, ZQL, YL and YT conceived the projects, LS, YL, YT, JL, DH, and YZ performed the
708 experiments. KY and XL performed the mass spectrometric analysis. SL, YT, YL, XL, and
709 ZQL analyzed data. SL drafted the first version of the manuscript, and ZQL revised the
710 manuscript with input from all authors.

711

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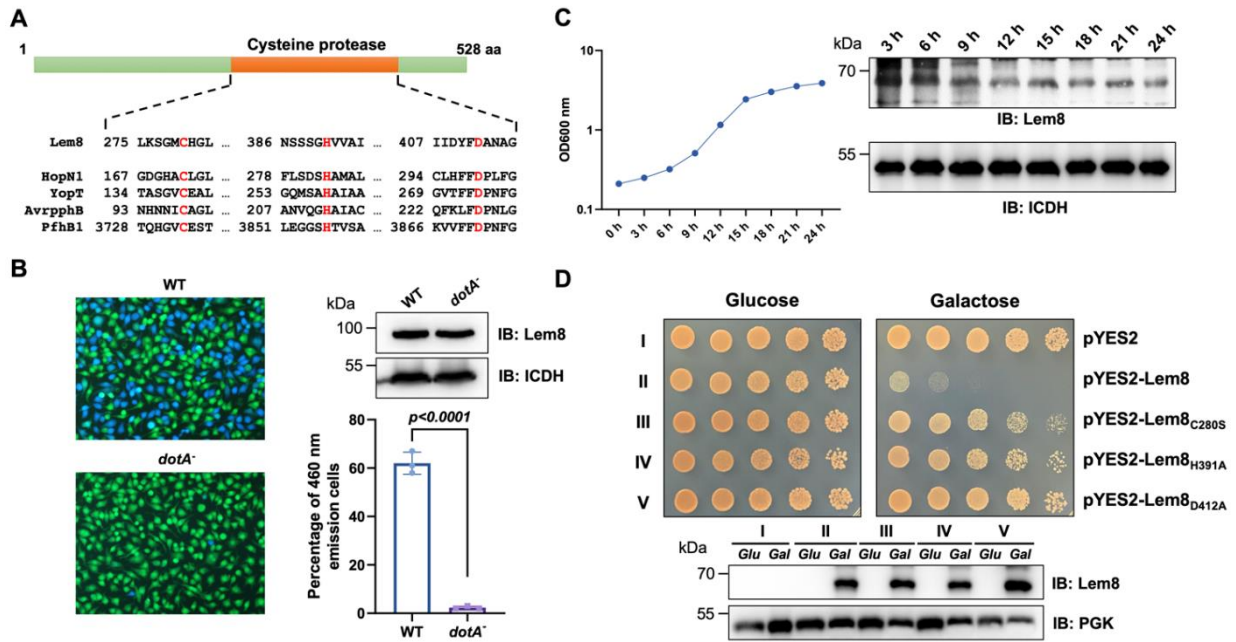
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934

935 **Figures and legends**

936



937 **Fig. 1 Lem8 is a cysteine protease-like Dot/Icm effector toxic to yeast**

938 **A.** Alignment of Lem8 with several known cysteine proteases obtained by PSI-BLAST
 939 analysis. The strictly conserved catalytic residues are marked in red. Shown cysteine
 940 proteases are HopN1 and AvrpphB from *P. syringae*, YopT from *Y. enterocolitica*, and
 941 PfhB1 from *P. multocida*.

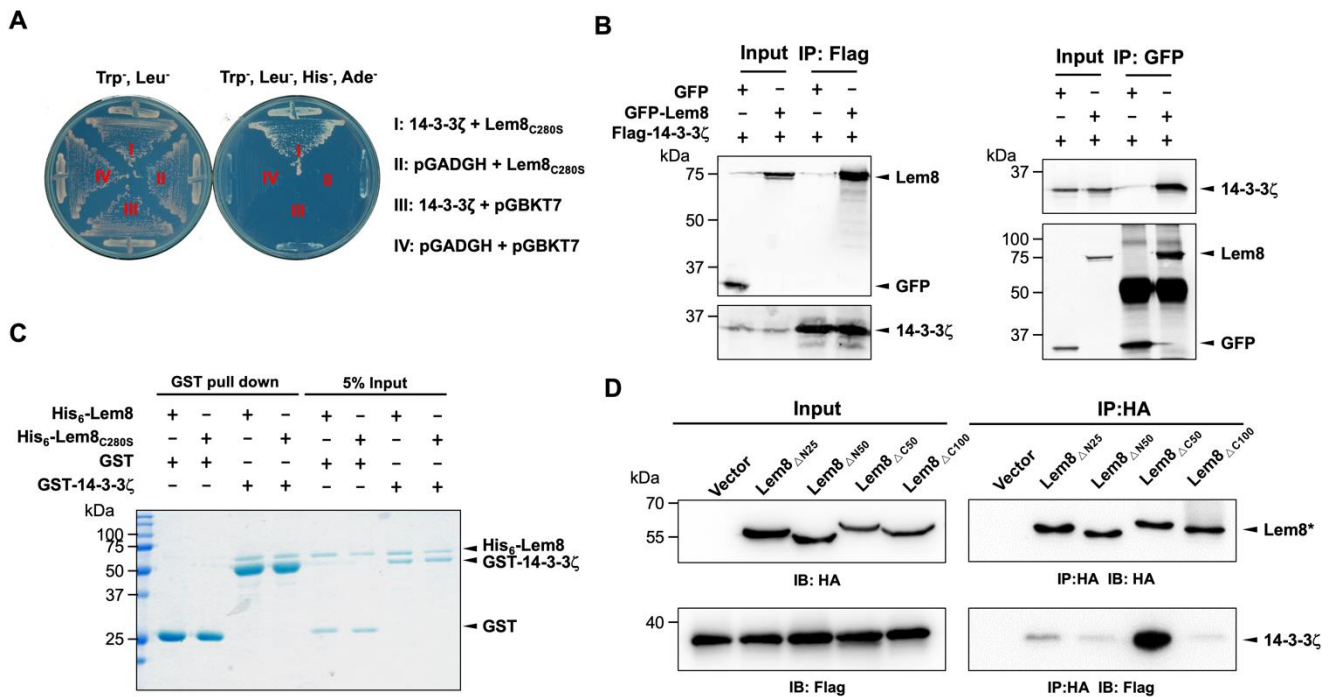
942 **B.** Lem8 is translocated into mammalian cells via the Dot/Icm transporter. U937 cells
 943 were infected with wild-type *L. pneumophila* or a *dotA*⁻ mutant expressing a β-lactamase-
 944 Lem8 fusion. One hour after infection, the CCF4-AM fluorescence substrate was added
 945 into the cultures and the cells were incubated for another 2 h at room temperature before
 946 image acquisition. Cells emitting blue fluorescence signals were quantitated by counting
 947 at least 500 cells in each experiment done in triplicate. Results shown are mean ± s.e.
 948 from one representative experiment.

949 **C.** Expression profile of *lem8* in *L. pneumophila* grown in AYET broth. Bacteria grown to
 950 stationary phase were diluted at 1:20 in fresh medium and subcultures were grown in a
 951 shaker. Bacterial growth was monitored by measuring OD₆₀₀ at the indicated time points.
 952 Equal amounts of bacterial cells were lysed for measurement of Lem8 levels by
 953 immunoblotting with Lem8-specific antibodies. The metabolic protein isocitrate

954 dehydrogenase (ICDH) was probed as loading control.

955 **D.** Lem8 is toxic to yeast in a manner that requires the predicted Cys-His-Asp motif. Yeast
956 strains expressing Lem8 or the indicated mutants from the galactose-inducible promotor
957 were serially diluted and spotted on the indicated media. The plates were incubated at
958 30°C for 48 h before image acquisition. The expression of Lem8 and its mutants induced
959 by galactose were determined by immunoblotting with Lem8-specific antibodies. The 3-
960 phosphoglycerate kinase (PGK) was detected as loading control.

961



962 **Fig. 2 The interactions between Lem8 and 14-3-3ζ**

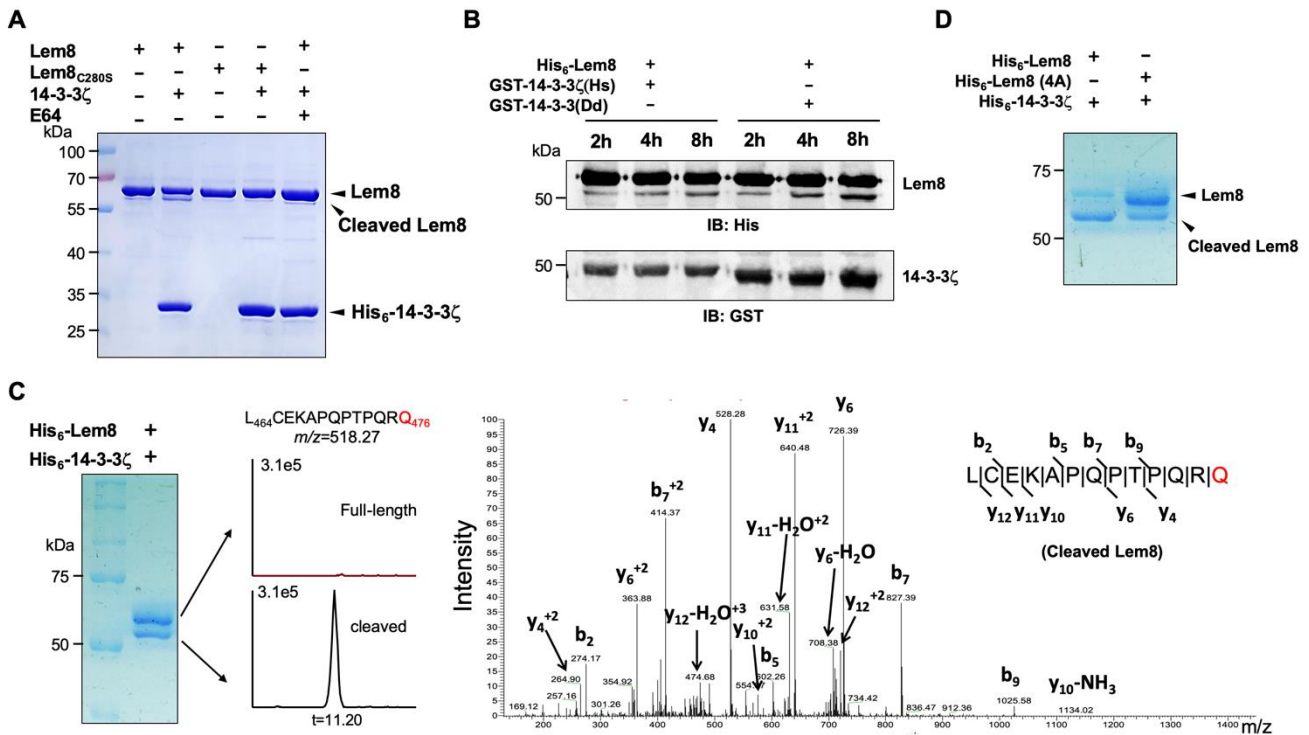
963 **A.** Interactions between Lem8 and 14-3-3ζ detected by yeast two-hybrid assay. Yeast
 964 strains harboring the indicated constructs were streaked on Leu⁻ and Trp⁻ medium to
 965 select for plasmids (left) or on Leu⁻, Trp⁻, Ade⁻, and His⁻ medium to assess the interactions
 966 (right). Images were acquired after 3-d incubation at 30°C.

967 **B.** Lem8 and 14-3-3ζ form a protein complex in mammalian cell. Total lysates of
 968 HEK293T cells transfected with indicated plasmid combinations were
 969 immunoprecipitated with a Flag-specific antibody (left panels) or GFP-specific antibodies
 970 (right panels), and the precipitates were probed with both Flag and GFP antibodies.
 971 Similar results were obtained from at least three independent experiments and the data
 972 shown here were from one representative experiment.

973 **C.** Lem8 directly interacts with 14-3-3ζ. GST-14-3-3ζ was incubated with His₆-Lem8 or
 974 His₆-Lem8_{C280S}, and the potential protein complex was captured by glutathione beads for
 975 1 h at 4°C. After extensive washing, bound proteins were solubilized with SDS loading
 976 buffer, and proteins were detected by Coomassie brilliant blue staining after being
 977 resolved by SDS/PAGE.

978 **D.** Interactions between 14-3-3ζ and Lem8 deletion mutants. Lysates of 293T cells
 979 expressing Flag-14-3-3ζ and each of the HA-tagged deletion Lem8 were subjected to

980 immunoprecipitation with the anti-HA antibody and the presence of 14-3-3 ζ in the
981 precipitates was probed with the Flag-specific antibody.
982



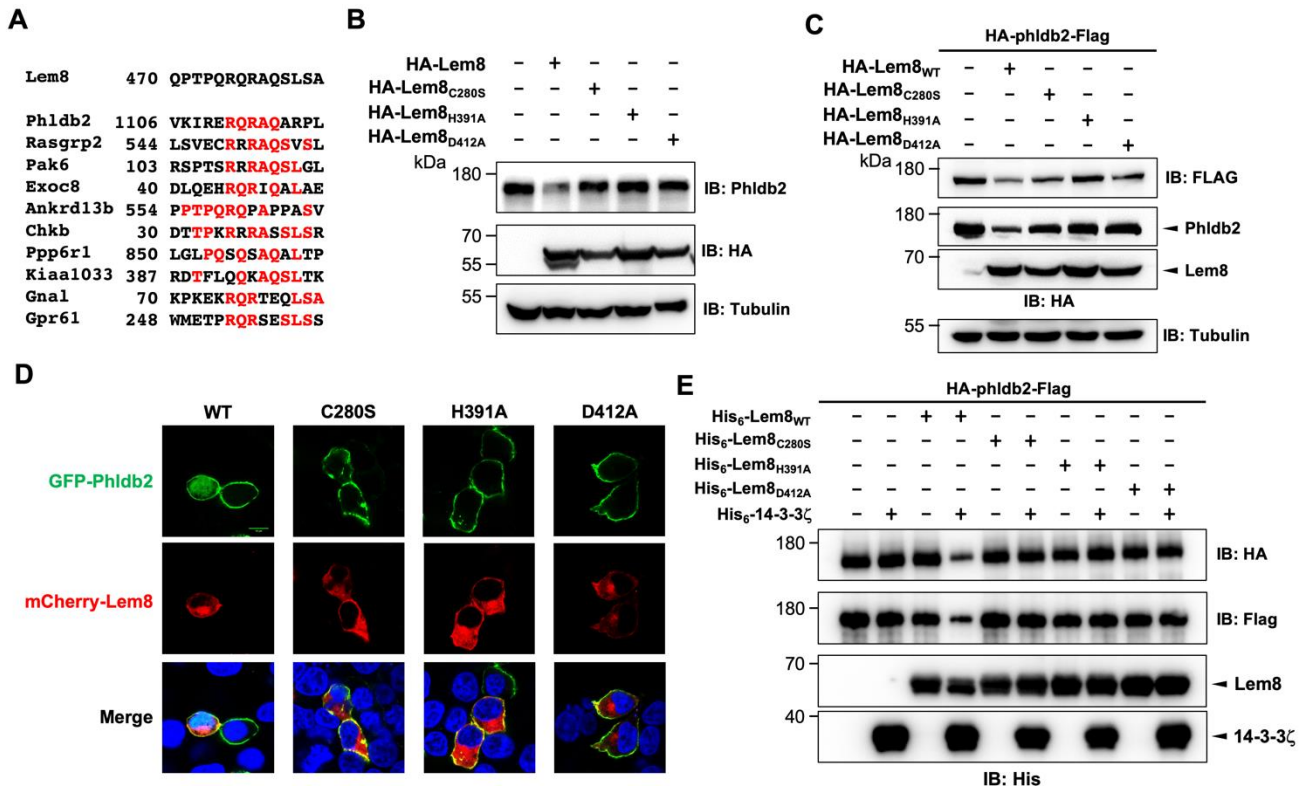
983 **Fig. 3 14-3-3 ζ induces Lem8 to undergo self-cleavage**

984 **A.** Self-processing of Lem8 requires 14-3-3 ζ . His₆-Lem8 or His₆-Lem8^{c280s} was
 985 incubated with His₆-14-3-3 ζ for 2 h, proteins resolved by SDS-PAGE were detected by
 986 Coomassie brilliant blue staining. The cysteine protease inhibitor E64 was added to the
 987 indicated samples.

988 **B.** The 14-3-3 protein from *D. discoideum* induces the self-cleavage of Lem8. His₆-Lem8
 989 was incubated with GST-14-3-3 ζ or GST-14-3-3Dd for the indicated time and the mixtures
 990 separated by SDS-PAGE were detected by immunoblotting with antibodies specific for
 991 Lem8 and GST, respectively.

992 **C.** Determination of the self-cleavage site of Lem8. His₆-Lem8 was incubated with His₆-
 993 14-3-3 ζ for 16 h, proteins were resolved by SDS-PAGE, stained with Coomassie brilliant
 994 blue. Protein bands corresponding to full-length and cleaved Lem8 band was excised,
 995 digested with trypsin and analyzed by mass spectrometry. The detection of the semi-
 996 tryptic peptide -L₄₆₄CEKAPQPTPQRQ₄₇₆- in cleaved samples suggested that the
 997 cleavage site lies between Gln476 and Arg477.

998 **D.** Mutations in cleavage site does not abolish Lem8 self-processing. Recombinant
 999 protein of Lem8 and the 4A mutant were each incubated with His₆-14-3-3 ζ for 4 h.
 1000 Proteins resolved by SDS-PAGE were detected by Coomassie brilliant blue.



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Fig. 4 Lem8 cleaves Phldb2 in a manner that requires 14-3-3ζ.

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A. Multiple alignments of the self-cleavage site of Lem8 with potential targets in human cells identified by bioinformatic analysis. Identical residues are highlighted in red.

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B. Lem8 reduces the protein levels of endogenous Phldb2 in mammalian cells. Lem8 and the indicated mutants were individually expressed in HEK293T cells by transfection. 24 h after transfection, the samples were resolved by SDS-PAGE and detected by immunoblotting with anti-Phldb2 antibodies. Tubulin was used as a loading control. Results shown were one representative from three independent experiments with similar results.

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C. Lem8 cleaves exogenous Phldb2 in mammalian cells. HA and Flag tag were fused to the amino and carboxyl end of Phldb2 respectively and the double tagged protein was co-expressed in HEK293T cells with Lem8 or each of the mutants. 24 h after transfection, the samples were resolved by SDS-PAGE and probed by a HA-specific antibody and a Flag-specific antibody, respectively. Tubulin was detected as a loading control. Results shown were one representative from three independent experiments with similar results.

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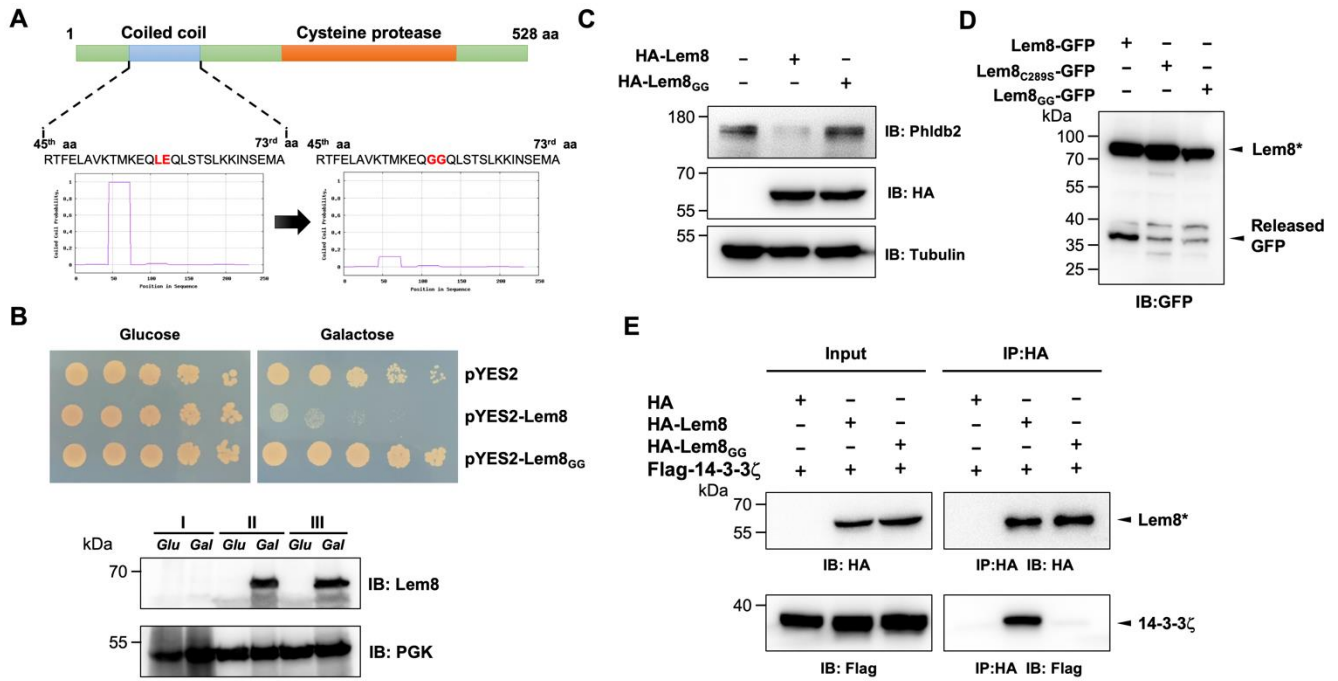
D. Lem8 alters the subcellular distribution of GFP fused to Phldb2. GFP was fused to the amino end of Phldb2 and the protein was co-expressed in HEK293T cells with mCherry-

1018

1019 Lem8 or each of the mutants. 24 h after transfection, cells were fixed and nucleus were
1020 stained by Hoechst 33342. The fluorescence images of GFP (green), mCherry (red) and
1021 Hoechst (blue) were acquired with a Zeiss LSM 880 confocal microscope. Bar, 5 μ m.

1022 **E.** 14-3-3 ζ is required for the cleavage of Phldb2 by Lem8. HA-Phldb2-Flag was
1023 expressed in HEK293T cells, immunoprecipitated with a Flag-specific antibody, and
1024 eluted with 3 \times Flag peptides. Purified Phldb2 was incubated with His₆-Lem8 or each of
1025 the mutants in reactions with or without His₆-14-3-3 ζ . Total proteins of all samples were
1026 resolved with SDS-PAGE, and probed by immunoblotting with a HA-specific antibody, a
1027 Flag-specific antibody and a His-specific antibody.

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1030 **Fig. 5 14-3-3ζ binds to Lem8 through a Coiled coil motif.**

1031 **A.** Lem8 harbors a putative coil motif. A predicted coiled coil motif located in the amino
 1032 end of Lem8 (top panel). The sequence ranges from the 45th residue to the 73rd residue
 1033 with a coiled-coil probability of 100% according to MARCOIL (lower panel, left).
 1034 Replacement of Leu₅₈ and Glu₅₉ with glycine (highlighted in red) is predicted to reduce
 1035 the coiled-coil probability to about 10% (lower panel, right).

1036 **B.** The predicted coiled coil motif is critical for Lem8-mediated yeast toxicity. Yeast cells
 1037 inducibly expressing Lem8 or mutant Lem8_{GG} were serially diluted and spotted onto the
 1038 indicated media for 48 h (top panel). The expression of Lem8 and Lem8_{GG} was examined
 1039 and PGK1 was probed as a loading control (lower panel).

1040 **C.** Lem8_{GG} loses the capacity to cleave Phldb2 in mammalian cells. Lysates of HEK293T
 1041 cells expressing Lem8 or Lem8_{GG} were resolved by SDS-PAGE and detected by
 1042 immunoblotting with antibodies specific for Phldb2 and Lem8, respectively. Tubulin was
 1043 used as a loading control. Results shown were one representative from three
 1044 independent experiments with similar results.

1045 **D.** The predicted coiled coil motif is required for self-processing of Lem8. The indicated
 1046 alleles of Lem8-GFP were individually expressed in HEK293T cells by transfection.

1047 Samples resolved by SDS-PAGE were detected by immunoblotting with GFP-specific
1048 antibodies. Results shown were one representative from three independent experiments
1049 with similar results.

1050 **E.** Interactions between 14-3-3 ζ and the Lem8_{GG} mutant. Lysates of 293T cells
1051 expressing Flag-14-3-3 ζ with HA-Lem8 or HA-Lem8_{GG} were subjected to
1052 immunoprecipitation with the anti-HA antibody and the presence of 14-3-3 ζ in the
1053 precipitates was probed with the Flag-specific antibody.

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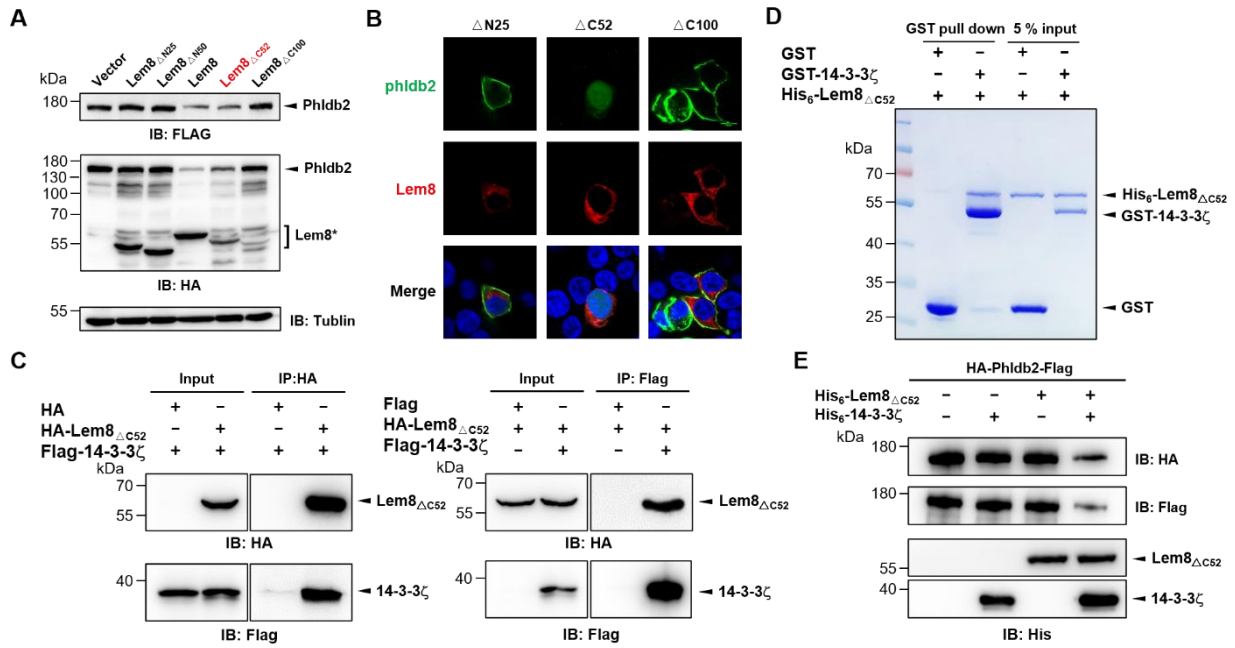


Fig. 6 The cysteine protease activity of auto-processing Lem8

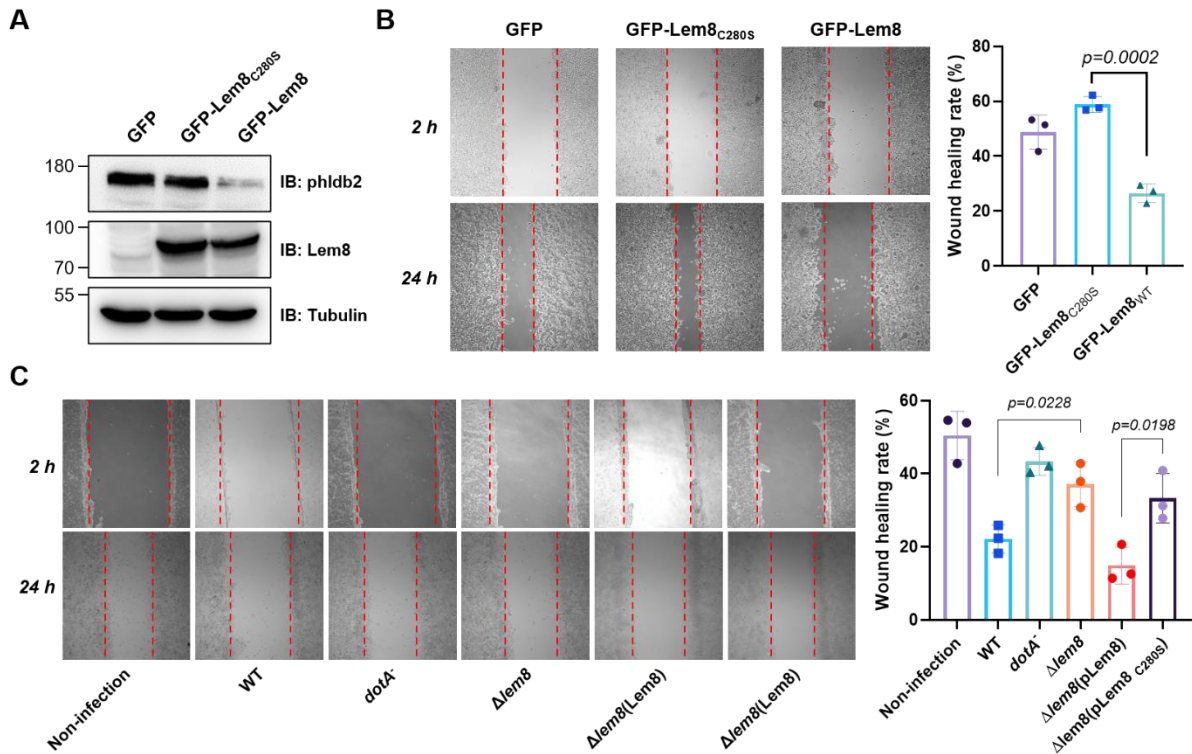
A. The auto-processed form of Lem8 cleaves Phldb2 in cells. HA-Phldb2-Flag was co-expressed in HEK293T cells with Lem8 or the indicated truncation mutants including the self-processed form, Lem8_{ΔC52}. 24 h after transfection, the samples were resolved by SDS-PAGE and probed by a HA-specific antibody and a Flag-specific antibody. Tubulin was used as a loading control. Results shown were one representative from three independent experiments with similar results.

B. Lem8_{ΔC52} causes redistribution of GFP-Phldb2. Truncations of Lem8, including Lem8_{ΔN25}, Lem8_{ΔC52} and Lem8_{ΔC100} fused to mCherry was individually expressed in HEK293T cells with GFP-Phldb2. 24 h after transfection, the fluorescence images were acquired with a Zeiss LSM 880 confocal microscope. Bar, 5 μm.

C. The interaction between Lem8_{ΔC52} and 14-3-3ζ. Total lysates of HEK293T cells transfected with indicated plasmid combinations were immunoprecipitated with antibodies specific for HA (left panel) or Flag (right), and the precipitates were probed with both HA and Flag antibodies. Similar results were obtained from at least three independent experiments and the data shown here were from one representative experiment.

D. Lem8_{ΔC52} directly interacts with 14-3-3ζ. Mixtures containing GST-14-3-3ζ and His₆-Lem8_{ΔC52} were incubated with glutathione beads for 1 h at 4°C. After washing, samples

1075 resolved by SDS/PAGE were detected by Coomassie brilliant blue staining.
1076 **E.** The cleavage of Phldb2 by Lem8 Δ C52 requires 14-3-3 ζ . Purified HA-Phldb2-Flag from
1077 HEK293T was incubated with His₆-Lem8 Δ C52 in reactions with or without His₆-14-3-3 ζ .
1078 Total proteins of all samples were resolved with SDS-PAGE, and probed by
1079 immunoblotting with antibody specific for HA, Flag and His₆, respectively.
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1082 **Fig. 7 Lem8 contributes to cell migration inhibition by *L. pneumophila***

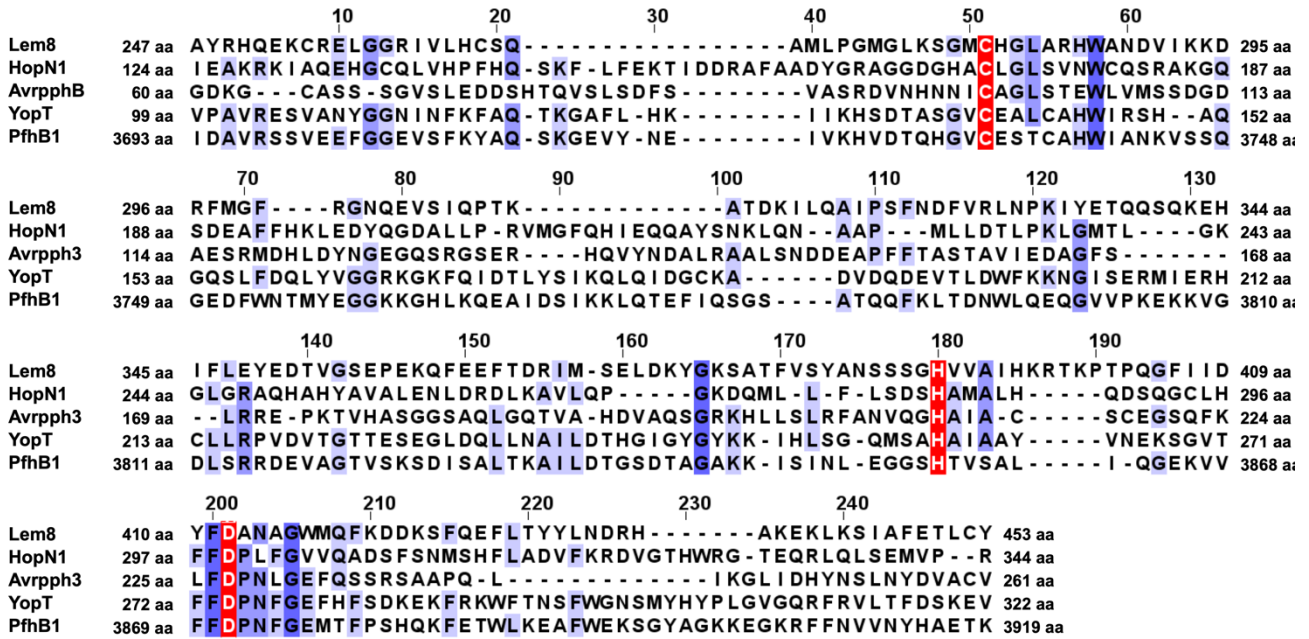
1083 **A.** Establishment of cell lines stably expressing Lem8 or its enzymatically inactive mutant.
 1084 HEK293T cells were transduced with lentiviral particles harboring the indicated plasmid
 1085 at an MOI of 10 for two days, and the GFP-positive cells were isolated by a BD Influx™
 1086 cell sorter. Lysates of each cell lines were probed by immunoblotting with antibodies
 1087 specific for Phldb2 or GFP. Tubulin was used as a loading control.

1088 **B.** Wound-healing scratch assay of the three stable cell lines. The three cell lines were
 1089 individually seeded into 6 well plates. When reached confluency, cell monolayer of each
 1090 cell lines was scratched using a pipette tip. Images of the wounds were captured using
 1091 an Olympus IX-83 fluorescence microscope at 2 h and 24 h after making the scratches.
 1092 Images of a representative experiment were shown (left panel). The wound healing rates
 1093 from three independent experiments was quantitated by Image J (right panel).

1094 **C.** Evaluation of the impact of Lem8 on cell migration in cells infected with *L. pneumophila*.
 1095 HEK293T cells expressing the FcγII receptor were infected with opsonized bacteria of
 1096 the indicated *L. pneumophila* strains at an MOI of 50 for 2 h. After washes, the wound-
 1097 healing scratch assay was performed to evaluate the impact of infection on cell migration.
 1098 Images of a representative experiment were shown (C, left panel) and the wound healing

1099 rate was analyzed by Image J (C, right panel).

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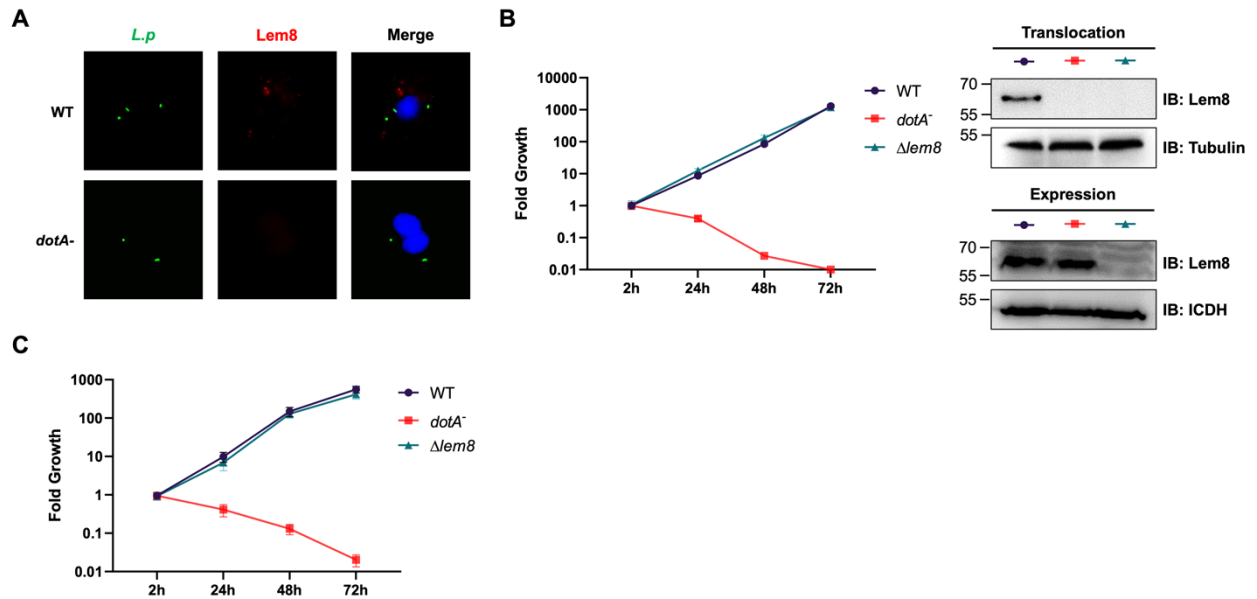
1102 **Fig. S1 Sequence alignment of Lem8 with four bacterial cysteine protease effectors**

1103 The strictly conserved residues were shown in dark purple background and the Cys-His-

1104 Asp motif are marked with white letters in a red background. HopN1 and Avrpph3 are

1105 from *P. syringae*. YopT and PfhB1 are from *Y. pestis* and *P. multocida*, respectively.

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Fig. S2 Lem8 is dispensable for intracellular growth of *L. pneumophila*

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A. Subcellular distribution of Lem8. 2h after infected with the indicated bacterial strains, BMDMs were immunostained using anti-*Legionella* antibody to identify the bacterial vacuoles (green), followed by staining with the Lem8 specific antibody (red). The nucleus was stained using Hoechst 33342 (blue).

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B. Intracellular growth of the $\Delta Lem8$ strain in *D. discoideum*. *D. discoideum* were infected with the indicated bacterial strains at an MOI of 0.1, and the intracellular growth was determined at a 24-h interval for 72 h (left panel). The expression and translocation of Lem8 in each strain was probed with Lem8 specific antibodies (right panel). ICDH and Tubulin were used as loading controls for bacterial and host cells, respectively. Similar results were obtained in three independent experiments.

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C. Intracellular growth of $\Delta Lem8$ strain in BMDMs. The bacterial strains were used to infect BMDMs at an MOI of 0.1 and the intracellular growth was monitored at the indicated time points (left panel). Similar results were obtained in three independent experiments.

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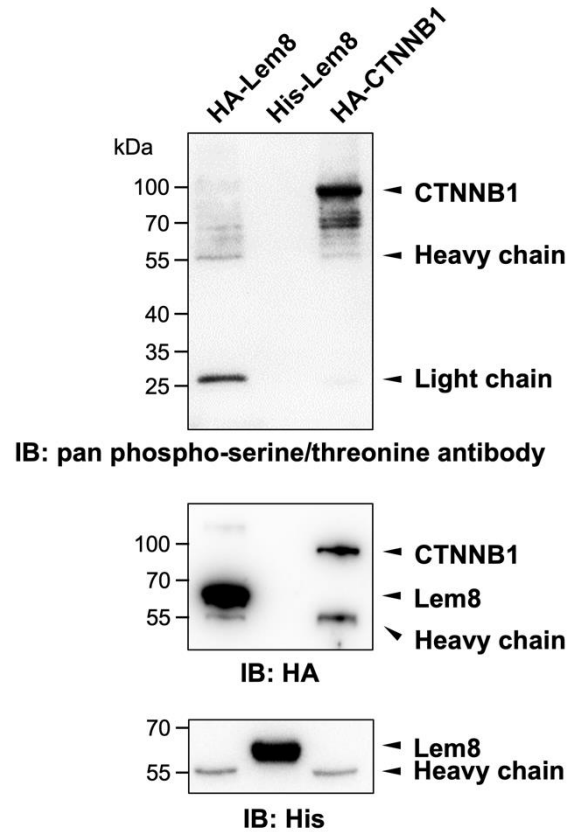
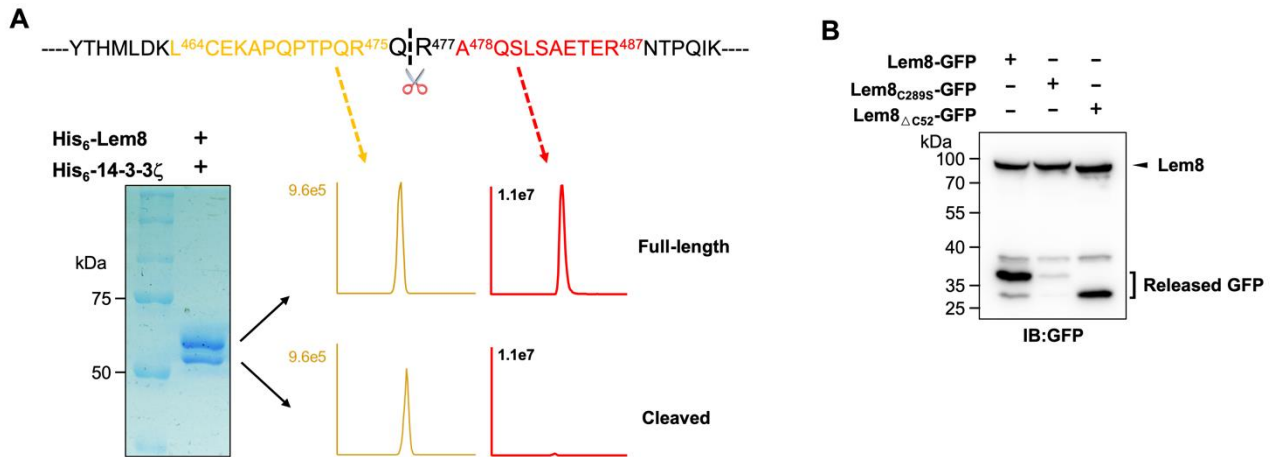


Fig. S3 Phosphorylation of Lem8 is not required for 14-3-3 ζ binding. Lysates of HEK293T cells expressing indicated HA tagged proteins were subjected to immunoprecipitation with agarose beads coated with the HA antibody. The precipitates, as well as His₆-Lem8 purified from *E coli* were resolved by SDS-PAGE and probed by immunoblotting with a pan phospho-serine/threonine antibody, the HA specific antibody and the His₆-specific antibody, respectively.



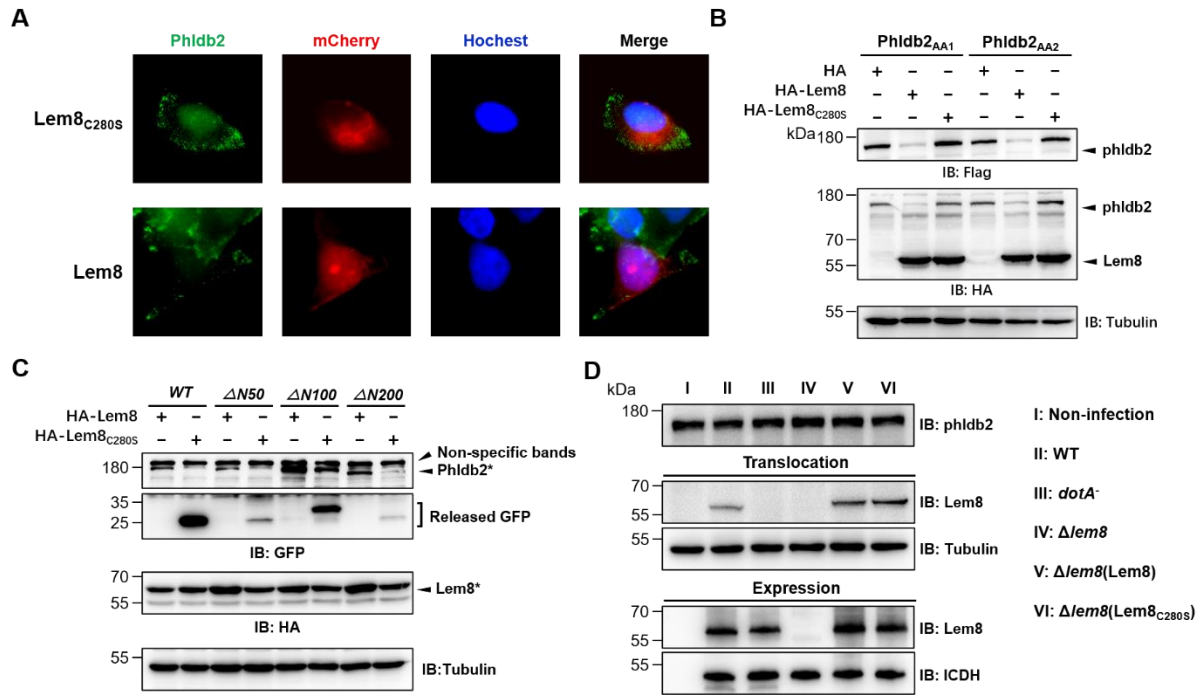
1145

1146 **Fig. S4 Identification of the self-cleavage site of Lem8.**

1147 **A.** Determination of the self-cleavage site of Lem8 by mass spectrometry. A diagram of
1148 the sequence containing the recognition site with the two diagnostic peptides used to
1149 determine the cleavage site (top panel). Protein bands corresponding to full-length and
1150 cleaved Lem8 band was excised (lower left panel), digested with trypsin and analyzed
1151 by mass spectrometry. The semi-tryptic peptide -L₄₆₄CEKAPQPTPQRQ₄₇₆- is present in
1152 cleaved samples but not in samples of full-length Lem8, whereas the fragment -
1153 A₄₇₈QSLSAETER₄₈₇- was only detected in samples of the full-length protein (lower right
1154 panel), supporting the notion that the cleavage site lies between Gln476 and Arg477
1155 described in Fig.3C.

1156 **B.** Self-cleavage of Lem8 removes GFP fused to its carboxyl end. GFP was fused to the
1157 indicated alleles of Lem8 and the fusion proteins were individually expressed in HEK293T
1158 cells by transfection. Samples resolved by SDS-PAGE were detected by immunoblotting
1159 with GFP-specific antibodies. Results shown were one representative from three
1160 independent experiments with similar results.

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Fig. S5 Lem8 cleaves phldb2 at multiple sites.

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A. Lem8 causes redistribution of Phldb2 in cells. HeLa cells were transfected to express the indicated mCherry fusion proteins. 24 h after transfection, cells were fixed and immunostained with anti-Phldb2 antibodies. The nuclei were stained by Hoechst 33342. Images were acquired with a Zeiss LSM 880 confocal microscope. Phldb2, green (GFP); Lem8 and its mutants, red (mCherry); nuclei, blue (Hoechst). Bar, 5 μm. Mutations in the cleavage site of Phldb2 cannot completely prevent its degradation by Lem8.

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B. Mutations were introduced into HA-Phldb2-Flag to replace residues Arg₁₁₁₁ and Gln₁₁₁₂ (Phldb2_{AA1}) or Gln₁₁₁₂ and Arg₁₁₁₃ (Phldb2_{AA2}) with alanine, respectively. The two mutants were co-expressed in HEK293T cells with Lem8 or Lem8_{C280S}. Samples were resolved with SDS-PAGE, and probed by immunoblotting with the antibody specific to HA and Flag, respectively.

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C. Lem8 removes the GFP tag fused to the amino end of Phldb2 deletion mutants. GFP was fused to the amino end of Phldb2 and the indicated truncation mutants. The fusion proteins were individually co-expressed with HA-Lem8 or HA-Lem8_{C280S} in HEK293T cells by transfection. Samples resolved by SDS-PAGE were detected by immunoblotting with GFP-specific antibodies. Results shown were one representative from three independent experiments with similar results. Mutations in the cleavage site of Phldb2 cannot completely prevent its degradation by Lem8.

1181

1182 **D.** Cleavage of Phldb2 is undetectable during *L. pneumophila* infection. HEK293T cells
1183 transfected to express FcγRII receptor were infected with the indicated bacterial strains.
1184 2 h after infection, the protein levels of Phldb2, as well as the translocation and
1185 expression of Lem8, were probed with the appropriate antibodies with Tubulin and ICDH
1186 as loading control, respectively. Bacterial strains: I, Non-infection; II, Lp02 (WT); III, *dotA*-
1187 (defective in Dot/Icm); IV, Lp02Δlem8; V, Lp02Δlem8(pLem8); VI,
1188 Lp02Δlem8(pLem8_{C280S}).

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1191 **Table S1 Bacterial strains, plasmids and primers used in the study**

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Bacterial Strains	Source	Identifier
<i>L. pneumophila</i> (Philadelphia-1) LP02	(Berger and Isberg, 1993)	N/A
<i>L. pneumophila</i> LP03	(Berger and Isberg, 1993)	N/A
LP02Δ <i>lem8</i>	This study	N/A
LP02Δ <i>lem8</i> (pZL507)	This study	N/A
LP02Δ <i>lem8</i> (pLem8)	This study	N/A
LP02Δ <i>lem8</i> (pLem8 _{C280S})	This study	N/A
<i>E. coli</i> BL21 (DE3)	Transgen	CAT# CD601
<i>E. coli</i> XL1-Blue	Transgen	CAT# CD401

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Plasmids	Source	Identifier
pZL507	(Qiu <i>et al.</i> , 2016)	N/A
pZL507:: <i>lem8</i>	This study	N/A
pZL507:: <i>lem8</i> _{C280S}	This study	N/A
pXDC61m:: <i>lem8</i>	This study	N/A
pAPH	This study	N/A
pAPH:: <i>GFP</i>	This study	N/A
pAPH:: <i>lem8</i>	This study	N/A
pAPH:: <i>lem8</i> _{C280S}	This study	N/A
pAPH:: <i>lem8</i> _{H391A}	This study	N/A
pAPH:: <i>lem8</i> _{D412A}	This study	N/A
pAPH:: <i>lem8</i> _{L58G/E59G}	This study	N/A
pAPH:: <i>lem8</i> ΔN25	This study	N/A
pAPH:: <i>lem8</i> ΔN50	This study	N/A
pAPH:: <i>lem8</i> ΔC50	This study	N/A
pAPH:: <i>lem8</i> ΔC52	This study	N/A
pAPH:: <i>lem8</i> ΔC100	This study	N/A
pAPH:: <i>PHLDB2-Flag</i>	This study	N/A
pAPH:: <i>PHLDB2</i> _{R1101/Q1102A} - <i>Flag</i>	This study	N/A
pAPH:: <i>PHLDB2</i> _{Q1102/R1103A} - <i>Flag</i>	This study	N/A
pAPH:: <i>lem8-GFP</i>	This study	N/A
pAPH:: <i>lem8</i> _{C280S} - <i>GFP</i>	This study	N/A

pAPH:: <i>lem8ΔC52-GFP</i>	This study	N/A
pAPH:: <i>lem8^{L58G/E59G}-GFP</i>	This study	N/A
pEGFP-C1	Clontech	CAT#6084-1
GFP:: <i>lem8</i>	This study	N/A
GFP:: <i>PHLBD2</i>	This study	N/A
GFP:: <i>PHLBD2ΔN50</i>	This study	N/A
GFP:: <i>PHLBD2ΔN100</i>	This study	N/A
GFP:: <i>PHLBD2ΔN200</i>	This study	N/A
pmCherry-C1	Clontech	CAT#632524
mCherry-C1:: <i>lem8</i>	This study	N/A
mCherry-C1:: <i>lem8^{C280S}</i>	This study	N/A
mCherry-C1:: <i>lem8^{H391A}</i>	This study	N/A
mCherry-C1:: <i>lem8^{D412A}</i>	This study	N/A
mCherry-C1:: <i>lem8ΔN25</i>	This study	N/A
mCherry-C1:: <i>lem8ΔC52</i>	This study	N/A
mCherry-C1:: <i>lem8ΔC100</i>	This study	N/A
pQE30	Qiagen	CAT#32915
pQE30:: <i>mmu-YWHAZ</i>	This study	N/A
pQE30:: <i>lem8</i>	This study	N/A
pQE30:: <i>lem8^{C280S}</i>	This study	N/A
pQE30:: <i>lem8^{H391A}</i>	This study	N/A
pQE30:: <i>lem8^{D412A}</i>	This study	N/A
pQE30:: <i>lem8^{P473A/Q474A/R475A/Q476A}</i>	This study	N/A
pQE30:: <i>lem8ΔC52</i>	This study	N/A
pGEX6p-1	Cytiva	CAT#28-9546-48
pGEX6p-1:: <i>mmu-YWHAZ</i>	This study	N/A
pGEX6p-1:: <i>hsa-YWHAZ</i>	This study	N/A
pGEX6p-1:: <i>ddi-fttB</i>	This study	N/A
pCDH-CMV-MCS-EF1-Puro	System Biosciences	CAT#CD510B-1
pCDH-CMV-MCS-EF1-Puro:: <i>GFP</i>	This study	N/A
pCDH-CMV-MCS-EF1-Puro:: <i>GFP-Lem8</i>	This study	N/A
pCDH-CMV-MCS-EF1-Puro:: <i>GFP-Lem8^{C280S}</i>	This study	N/A
pYES2CT	Invitrogen	CAT#V825120
pYES2CT:: <i>lem8</i>	This study	N/A
pYES2CT:: <i>lem8^{C280S}</i>	This study	N/A
pYES2CT:: <i>lem8^{H391A}</i>	This study	N/A
pYES2CT:: <i>lem8^{D412A}</i>	This study	N/A
pYES2CT:: <i>lem8^{L58G/E59G}</i>	This study	N/A
pGADGH	Clontech	CAT#638853

pGADGH:: <i>mmu</i> - <i>YWHAZ</i>	This study	N/A
pGBKT7	Clontech	CAT#630489
pGBKT7:: <i>lem8</i> _{C280S}	This study	N/A
pMD2.G	Addgene	Cat#12259
psPAX2	Addgene	Cat#12260

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Primers	Sequence (Restriction enzyme sites are underlined)	Note
pSL1001	cgcg <u>gatcc</u> atgcctcaaatcctaaatg	<i>lem8</i> 5F BamHI
pSL1002	acgc <u>gtcgact</u> taaataggaaatcctctgtatttc	<i>lem8</i> 3R Sall
pSL1003	ctgg <u>agctca</u> ataaattttttcactcaaggaagg	<i>lem8</i> up 5F SacI knockout
pSL1004	cattctcagtgctcagctcaatgacagcc	<i>lem8</i> up 3R knockout
pSL1005	tgaagctgacactgagaatgagcaagcag	<i>lem8</i> down 5F knockout
pSL1006	acgc <u>gtcgacg</u> ataggctggctccatgg	<i>lem8</i> down 3R Sall knockout
pSL1007	cctagctaagccgtgactcataccactcttaaac	<i>lem8</i> _{C280S-1}
pSL1008	gtttaagagtggtatgagtcacggcttagctagg	<i>lem8</i> _{C280S-2}
pSL1009	gtttgtgatagcaacaacagctccagaactgctattagcatagc	<i>lem8</i> _{H391A-1}
pSL1010	gctatgctaatagcagttctggagctgtgttgctatccacaaac	<i>lem8</i> _{H391A-2}
pSL1011	gcatccaacctgcattagcagcaaaatagcgcgatgataaa	<i>lem8</i> _{D412A-1}
pSL1012	tttcatcatcgactattttgctgctaatagcaggttgatgc	<i>lem8</i> _{D412A-2}
pSL1013	tttcaatgaagtagataattgtccgctgtctttcatggttttactgctaactc	<i>lem8</i> _{L58G/E59G-1}
pSL1014	gagttagcagtaaaaaccatgaaaagcaagggcagcaattatctacttcattgaaaa	<i>lem8</i> _{L58G/E59G-2}
pSL1015	gtttctgctgacaagctctgtgctctgtgctccgcagcagtaggtgtggtgcttttcacagag	<i>lem8</i> _{P473A/Q474A/R475A/Q476A-1}
pSL1016	ctctgtgaaaaagcaccacaacctactgctgctgagcaagagcacagagctgtcagcagaaac	<i>lem8</i> _{P473A/Q474A/R475A/Q476A-2}
pSL1017	cgcg <u>gatcc</u> attgttgataaatactctgaca	<i>lem8</i> Δ N25 5F BamHI
pSL1018	cgcg <u>gatcc</u> gtaaaaaccatgaaagagca	<i>lem8</i> Δ N50 5F BamHI
pSL1019	acgc <u>gtcgact</u> atgctctttgtctctgagg	<i>lem8</i> Δ C50 3R Sall

pSL1020	acgcgtcgacttattgtctctgaggagtagg	<i>lem8ΔC52</i> 3R Sall
pSL1021	acgcgtcgacttactcttgaaaactcttatcgt	<i>lem8ΔC100</i> 3R Sall
pSL1022	acgcgtcgacaataggaatcctctgtatttc	<i>lem8-GFP</i> 3R Sall
pSL1023	acgcgtcgacttgtctctgaggagtagg	<i>lem8ΔC52-GFP</i> 3R Sall
pSL1024	cgcggatccatggataaaaatgagctggt	<i>mmu-YWHAZ</i> 5F BclI
pSL1025	acgcgtcgacttaattttcccctcctctc	<i>mmu-YWHAZ</i> 3R Sall
pSL1026	cgcggatccatggataaaaatgagctggt	<i>hsa-YWHAZ</i> 5F BamHI
pSL1027	acgcgtcgacttaattttcccctcctctc	<i>hsa-YWHAZ</i> 3R XhoI
pSL1028	cgcggatccatgaccagagaagaaaatgt	<i>ddi-fttB</i> 5F BamHI
pSL1029	acgcgtcgacttacattcctggtcattttg	<i>ddi-fttB</i> 3R Sall
pSL1030	cgcggatccatggaagagcatagctaca	<i>phldb2</i> 5F BamHI
pSL1031	acgcgtcgacctacaacaagaagtgagtgt	<i>phldb2</i> 3R Sall
pSL1032	cgcggatccatggccaatggagactattctg	<i>phldb2ΔN50</i> 5F BamHI
pSL1033	cgcggatccatgaaaaatattcctatgaaacctcaa	<i>phldb2ΔN100</i> 5F BamHI
pSL1034	cgcggatccatgccttcaagccca	<i>phldb2ΔN200</i> 5F BamHI
pSL1035	acgcgtcgaccaacaagaagtgagtgtaac	<i>phldb2-Flag</i> 3R Sall
pSL1036	aaggacgagcctgtgccctgtcctccctatttttactcctttct	<i>Phldb2_{R1101/Q1102A}-1</i>
pSL1037	agaaaaggaagtaaaaataagggaggcagcaagggcacaggctcgcctt	<i>Phldb2_{R1101/Q1102A}-2</i>
pSL1038	aggacgagcctgtgccgctgctctccttatttttactcctttctatt	<i>Phldb2_{Q1102/R1103A}-1</i>
pSL1039	aatagaaaaggaagtaaaaataagggagagagcagcggcacaggctcgcctt	<i>Phldb2_{Q1102/R1103A}-2</i>
pSL1040	acgcgtcgacatggtgagcaagggcga	<i>GFP</i> 5F Sall
pSL1041	ataagaatgcgccgcctgttacagctcgtccatgc	<i>GFP</i> 3R NotI
