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1 ***Legionella pneumophila* modulates mitochondrial dynamics to**
2 **trigger metabolic repurposing of infected macrophages**

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32 **SUMMARY**

33

34 The intracellular bacteria *Legionella pneumophila* encodes a type IV secretion system (T4SS)
35 that injects effector proteins into macrophages in order to establish and replicate within the
36 *Legionella*-containing vacuole (LCV). Once generated, the LCV interacts with mitochondria
37 through unclear mechanisms. We show that *Legionella* uses both T4SS-independent and
38 T4SS-dependent mechanisms to respectively interact with mitochondria and induce
39 mitochondrial fragmentation that ultimately alters mitochondrial metabolism. The T4SS
40 effector MitF, a Ran GTPase activator, is required for fission of the mitochondrial network.
41 These effects of MitF occur through accumulation of mitochondrial DNMI1L, a GTPase
42 critical for fission. Furthermore mitochondrial respiration is abruptly halted in a T4SS-
43 dependent manner, while T4SS-independent upregulation of cellular glycolysis remains
44 elevated. Collectively, these alterations in mitochondrial dynamics promote a Warburg-like
45 phenotype in macrophages that favours bacterial replication. Hence the rewiring of cellular
46 bioenergetics to create a replication permissive niche in host cells is a virulence strategy of
47 *L. pneumophila*.

48

49 INTRODUCTION

50 Macrophages are a first line host defence against bacterial pathogens. Key determinants for
51 their activation and subsequent responses are metabolic changes as shown recently (O'Neill
52 and Pearce, 2016). Important organelles that refine the metabolism of macrophages are
53 mitochondria as critical players in the cellular metabolism and bioenergetics (O'Neill and
54 Pearce, 2016). Moreover, mitochondria have functions in the regulation of cell death and
55 innate immunity (West et al., 2011b). Mitochondrial functions are strongly influenced by the
56 highly dynamic nature of the organelle including organelle localization, morphology, quantity
57 and activity (Labbe et al., 2014). These dynamics are tightly regulated at the single-organelle
58 level to respond to the specific demands of the cell. Whereas localization of mitochondria to
59 specific subcellular positions depends on the mitochondrial Rho GTPases RHOT1/2 (also
60 known as Miro1/2), changes in mitochondrial morphology from elongated networks to single
61 rod-shaped mitochondria, and *vice versa*, are governed by GTPases of the dynamin family
62 such as Mitofusin-1/2, OPA1 and dynamin 1-like protein (DNM1L, also known as DRP1),
63 which are key proteins regulating mitochondrial morphology (Labbe et al., 2014).

64

65 Considering the important functions of mitochondria, intracellular bacteria have evolved
66 mechanisms to target these organelles during infection to exploit the key roles they play in the
67 cell (Escoll et al., 2016). One way to subvert mitochondrial functions is to target
68 mitochondrial dynamics. Indeed, it has been shown that the bacterial effector VopE of *Vibrio*
69 *cholerae* modifies the subcellular localization of mitochondria by interacting with RHOT1/2
70 proteins (Suzuki et al., 2014) while secretion of the *Listeria monocytogenes* toxin listeriolysin
71 O (LLO) during infection induces fragmentation of mitochondrial networks independently of
72 DNM1L, a key component of the mitochondrial fission machinery (Stavru et al., 2011; Stavru
73 et al., 2013). As mitochondrial dynamics impact mitochondrial bioenergetics and *vice versa*,
74 it is expected that this functional crosstalk also operates during infection. However, the
75 interplay of both processes during bacterial infection remains largely unexplored.

76

77 *Legionella pneumophila* is a Gram-negative, intracellular bacterium and the etiologic agent of
78 Legionnaires' disease, a serious pulmonary infection during which the pathogen replicates
79 within human lung macrophages. *L. pneumophila* encodes a type IV secretion system (T4SS),
80 that injects more than 300 bacterial proteins in the host cytoplasm to subvert multiple host
81 functions to ensure intracellular bacterial replication (Isberg et al., 2009). These different
82 effectors have been shown to target different signalling pathways and host organelles, like

83 RomA that induces epigenetic regulations in the nucleus (Rolando et al., 2013) or LpSpl and
84 RavZ that target autophagy in the host cell (Choy et al., 2012; Rolando et al., 2016). However,
85 no effector that targets mitochondrial dynamics has been identified yet, although it was
86 reported that the *Legionella*-containing vacuole (LCV) associates with mitochondria. As early
87 as in 1983 it was reported that within 3 min of phagocytosis 30% of vacuoles were
88 surrounded by mitochondria closely apposed to the vacuolar membrane and that at 1h, an
89 even higher proportion of the LCV was surrounded by mitochondria (Horwitz, 1983). Since
90 this observation, it was reported in textbooks and reviews that depended on the T4SS
91 *L. pneumophila* recruits mitochondria or that mitochondria associate with the LCV. However,
92 this phenomenon has never been explored quantitatively, the bacterial T4SS effectors
93 implicated in mitochondrial recruitment have not been identified nor have the mitochondrial
94 functions probably modulated by *L. pneumophila* been elucidated.

95
96 Here we used high-content analyses of dynamic fluorescence imaging, at the single-cell level
97 and metabolic assays to reveal mitochondrial responses linked to infection. We performed our
98 study in human primary macrophages infected either with virulent or with non-virulent (T4SS
99 deficient) *L. pneumophila* strains. We revealed that T4SS-dependent and T4SS-independent
100 alterations of mitochondrial dynamics and metabolic responses of infected macrophages occur
101 as early as 6h post-infection and prior to bacterial replication. We show that by targeting
102 mitochondrial dynamics in a T4SS- and DNM1L-dependent manner, *L. pneumophila* induces
103 metabolic repurposing of macrophages and provokes a T4SS-induced Warburg-like effect in
104 the infected macrophages that favours bacterial replication.
105

106 RESULTS

107

108 *L. pneumophila* induces highly dynamic mitochondria-phagosome interactions

109 The proximity of mitochondria with the LCV during *Legionella* infection was first observed
110 using electron microscopy on fixed samples of infected human monocytes (Horwitz, 1983)
111 and shortly after also reported in *Legionella*-infected amoeba, its natural hosts (Newsome et
112 al., 1985). However, observations of this phenomenon in living cells have been scarce if any.
113 We thus infected primary human monocyte-derived macrophages (hMDMs) using GFP-
114 expressing *L. pneumophila* strain Philadelphia JR32 (JR32-WT-GFP), and monitored
115 mitochondrial recruitment to the LCV by time-lapse confocal microscopy in over 600 infected,
116 living cells, using automatic image analysis at the single-cell level (Figure 1A). As it was
117 reported that mitochondrial recruitment to the LCV occurs at early times post infection (p.i.),
118 peaking at 2h p.i. (Horwitz, 1983), we performed the time-lapse experiments up to 6h. At any
119 early time point (from 1 to 6h p.i.), more than 80% of the infected cells had at least 1
120 mitochondrion in close proximity ($<1 \mu\text{m}$) of the LCV, with an average of 2.02 ± 0.05
121 mitochondria per LCV (Figure 1B) confirming early observations using fixed cells. When
122 hMDMs were infected with GFP-expressing *L. pneumophila* strain Paris (Lpp-WT-GFP), we
123 obtained similar results, suggesting that interactions of mitochondria with the LCV are a
124 common feature of *L. pneumophila* (Figure 1C). It was reported that mitochondrial
125 recruitment was T4SS depended (Tilney et al., 2001). We therefore monitored mitochondrial
126 association with the LCV during the infection of hMDMs with T4SS-deficient
127 *L. pneumophila* mutants (JR32- $\Delta icmT$ -GFP and Lpp- $\Delta dotA$ -GFP). No differences were
128 observed when comparing JR32- $\Delta icmT$ -GFP and Lpp- $\Delta dotA$ -GFP mutants to the wild-type
129 (WT) strains (Figure 1B and 1C), suggesting that mitochondria surround the LCV in a T4SS-
130 independent manner.

131 To determine the frequency of these interactions we performed 3D confocal time-lapse
132 experiments and recorded the contacts of mitochondria with one LCV over time. Time-lapse
133 confocal 3D reconstructed movies showed that mitochondria-LCV contacts were highly
134 dynamic as these organelles moved to the LCVs, contacted them, tethered during variable but
135 short times, and abandoned the contact with the LCV (Figure 1D and S1, and Movie S1, S2
136 and S3). To assess whether these dynamic mitochondria-LCV contacts were statistically
137 significantly different as compared to contacts by chance, we developed a statistical method
138 for quantitative 3D image analysis (detailed in *STAR Methods*). As shown in Figure 1E, this

139 revealed that contacts between mitochondria and the LCV occurred in a highly dynamic
140 manner during the first 4h p.i. Quantification of the frequency of mitochondria-LCV contacts,
141 showed that they occurred with a frequency of 0.90 ± 0.32 contacts/min per LCV when
142 hMDMs were infected with WT and with a frequency of 0.55 ± 0.38 contacts/min per LCV
143 when hMDMs were infected with the $\Delta icmT$ mutant (Figure 1F). We further compared the
144 frequency of mitochondria-LCV contacts during infection with the frequency of contacts of
145 micro-bead containing phagosomes. As shown in Figure 1F and Movie S4 these contacts
146 occurred in a frequency of 0.73 ± 0.61 contacts/min per LCV, similar to those of the LVC
147 containing WT or $\Delta icmT$ mutant strains. Although a biological trend can be observed, no
148 significant differences were measured in the frequency of mitochondria-LCV contacts during
149 infection with virulent or avirulent *L. pneumophila* strains or beads, suggesting that these
150 highly dynamic contacts mainly occur independently of the *L. pneumophila* T4SS. A transfer
151 of Mitotracker dye from mitochondria to the LCV occurred occasionally during these
152 dynamic contacts (Figure S2 and Movie S5). Due to the bacterial membrane potential,
153 MitoTracker dyes will most likely also stain *L. pneumophila*. Thus our observation might
154 indicate that after contacts with mitochondria changes in the *L. pneumophila* membrane
155 potential occur. However, as Mitotracker dyes form a covalent bond with thiols on
156 mitochondrial proteins (Cottet-Rousselle et al., 2011), these transient but close couplings
157 might also suggest a transfer of proteins from mitochondria to the LCV. Indeed, 24.9% of the
158 proteins found in the proteome of isolated LCVs from macrophages were mitochondrial
159 proteins (Hoffmann et al., 2014).

160 Our results thus indicate that instead of recruiting mitochondria, *L. pneumophila* interacts
161 with host mitochondria in a highly dynamic, transient and mainly T4SS-independent manner.
162

163 ***L. pneumophila* induces T4SS-dependent changes in mitochondrial morphology during** 164 **infection**

165 During physiological conditions, mitochondria perform cycles of fission and fusion that affect
166 overall organelle morphology from an elongated mitochondrial network to a fragmented
167 network of single rod-shaped mitochondria (Labbe et al., 2014). To analyse mitochondrial
168 morphology during *L. pneumophila* infection of hMDMs and to uncover whether the T4SS
169 governs mitochondrial dynamics, we performed time-course experiments and single-cell
170 analyses of the mitochondrial morphology in living macrophages infected with GFP-
171 expressing virulent and T4SS deficient *L. pneumophila* strains (Figure 2A and 2B). Reticular

172 (elongated) or punctuate (fragmented) mitochondria were determined in a high number of
173 infected hMDMs by analysing the images obtained with texture algorithms (Figure 2A and
174 Figure S3) and classical measurements of mitochondrial morphology based on segmentation
175 strategies (Figure S3D). These quantitative, high-content analyses showed that
176 *L. pneumophila* induced mitochondrial fragmentation during the first 6h p.i in a time- and
177 T4SS-dependent manner (Figure 2B), but no alteration of the overall mitochondrial content
178 was observed (Figure S3E). At 6h p.i, macrophages infected with WT strains showed an
179 increased percentage of infected cells with fragmented mitochondria (JR32-WT:
180 $35.53 \pm 6.27\%$ and Lpp-WT: $76.64 \pm 4.33\%$) as compared to the avirulent T4SS-deficient
181 mutants (JR32- $\Delta icmT$: $8.07 \pm 5.39\%$ and Lpp- $\Delta dotA$: $36.94 \pm 2.66\%$). Furthermore,
182 *L. pneumophila*-induced mitochondrial fission was completely restored at 4h p.i. upon
183 complementation of the $\Delta icmT$ mutant (JR32- $\Delta icmT::icmT$, Figure 2C), suggesting the
184 participation of T4SS translocated effectors in the induction of mitochondrial fragmentation.

185 Taken together, *L. pneumophila* modifies the morphology of mitochondria in infected
186 macrophages dependent on its T4SS, suggesting that T4SS secreted effectors are involved in
187 *L. pneumophila*-induced mitochondrial fragmentation.

188

189 **The T4SS effector MitF is involved in *L. pneumophila*-induced fragmentation of** 190 **mitochondria during infection**

191 To identify the effector(s) involved in mitochondrial fragmentation we infected hMDMs with
192 different, *L. pneumophila* mutants that lacked specific T4SS effectors. As mitochondrial
193 dynamics are known to be governed by cellular GTPases (Chan, 2012) we first chose RalF
194 and LegG1 (Lpg1976) as both are known to target host GTPases. Whereas a mutant lacking
195 the T4SS effector RalF (targets ARF1-GTPase) induced mitochondrial fragmentation like the
196 WT strain, a mutant lacking LegG1 (targets Ran-GTPase) showed a significantly reduced
197 ability to fragment the mitochondrial network in hMDMs compared to the WT (Figure 2D).
198 Complementation of $\Delta legG1$ with the *legG1* gene restored the fragmentation phenotype
199 (Figure 2E and 2F), confirming the specific participation of LegG1 in the fragmentation
200 process. Furthermore, single cell texture analysis of mitochondria during infection showed
201 that overexpression of *legG1* in the complemented strain caused even stronger mitochondrial
202 fragmentation than the WT strain (Figure 2G). According to its function we named this
203 effector mitochondrial fragmentation factor, MitF. Thus the T4SS effector MitF promotes
204 mitochondrial fragmentation during *L. pneumophila* infection of human macrophages.

205 ***L. pneumophila*-induced fragmentation depends on DNMI1L**

206 Mitochondrial fusion and fission are processes that are highly regulated in eukaryotic cells.
207 The human protein DNMI1L, a large GTPase of the dynamin family, is a critical player in the
208 process of mitochondrial fission (Chan, 2012). We thus analysed the recruitment of DNMI1L
209 to mitochondria of hMDMs infected with the WT or a $\Delta icmT$ mutant by measuring the
210 number of DNMI1L puncta per μm^2 of host mitochondria (Figure 3A and S4). The number of
211 DNMI1L puncta on mitochondria was significantly lower in non infected than in WT infected
212 hMDMs (Figure 3B), suggesting an increased recruitment of DNMI1L to host mitochondria
213 during *L. pneumophila* infection. Importantly, $\Delta icmT$ -infected macrophages showed a
214 significantly reduced number of DNMI1L puncta as compared to non-infected or WT-infected
215 macrophages. Thus, the recruitment of DNMI1L to host mitochondria coincides with the
216 T4SS-induced fragmentation of mitochondria during *L. pneumophila* infection. Analyses of
217 DNMI1L protein levels and its phosphorylation status showed that DNMI1L levels are
218 downregulated during infection with the $\Delta icmT$ mutant, but remained constant during WT
219 infection (up to 6h, Figure 3C). Furthermore, activation of DNMI1L by the WT seems related
220 to phosphorylation of Ser616, as it is reduced during infection with the $\Delta icmT$ mutant but
221 phosphorylation/dephosphorylation of Ser637 seems not affected by neither WT nor $\Delta icmT$
222 mutant infection (Figure 3C).

223 To further substantiate this finding we used Mdivi1, a compound specifically
224 inhibiting DNMI1L functions during mitochondrial fission (Cassidy-Stone et al., 2008). When
225 Mdivi1-pretreated hMDMs were infected with the WT, no differences in the percentage of
226 infected cells compared to non-pretreated hMDMs were observed, indicating that uptake of
227 *L. pneumophila* by hMDMs is not affected by Mdivi1 pre-treatment (Figure 3D). However,
228 the percentage of infected cells showing a fragmented phenotype was significantly (p-
229 value=0.023) reduced from $23.26\pm 9.48\%$ in the absence of Mdivi1-pretreatment to
230 $9.74\pm 5.09\%$ in Mdivi1-pretreated, infected hMDMs (Figure 3E). Moreover, silencing of
231 DNMI1L with siRNA in RAW264.7 macrophages significantly reduced mitochondrial
232 fragmentation (p-value<0.0001) compared to cells transfected with scramble siRNA (Figure
233 3F, 3G and S4). To ascertain that Mdivi1 has no adverse effects on *L. pneumophila* we
234 monitored bacterial growth in BYE with Mdivi1 treated or non-treated cell lysates,
235 confirming that the DNMI1L inhibitor did not affect bacterial growth (Figure S5). As Mdivi1
236 only partially abolished the *L. pneumophila* induced fragmentation phenotype, additional
237 factors might be implicated in this process. Thus we used siRNAs to silence also Ran GTPase,

238 a host target of MitF (LegG1). Indeed, mitochondrial fragmentation was also reduced in
239 RAW264.7 macrophages compared to cells transfected with scramble siRNA (p-
240 value<0.0001). Silencing of the Ran binding protein 2 (RanBP2) also lead to statistical
241 significantly reduced fragmentation (p-value<0.0001) (Figure 3F, 3G and S4). Collectively,
242 our results show that DNM1L has an important role in *L. pneumophila*-induced mitochondrial
243 fragmentation and that Ran and RanBP2 are involved in this process.

244 Chemical inhibition of DNM1L by pre-treating hMDMs with Mdivi1 reduced
245 intracellular replication of *L. pneumophila* to 35.45% as compared to replication in non-
246 treated hMDMs (Figure 3H and S4), indicating that DNM1L functions are necessary for its
247 optimal intracellular replication. Furthermore, when DNM1L or RanBP2 were silenced in
248 A549 cells (a cell type that also support mitochondrial fragmentation upon *Legionella*
249 infection, Figure S4), intracellular replication of *L. pneumophila* was reduced by 58.66% and
250 37.33%, respectively, as compared to cells treated with scramble siRNA (Figures 3I and S4),
251 further supporting that DNM1L and RanBP2 favour bacterial replication.

252 Quantitative proteomics of proteins pulled down with DNM1L antibodies from
253 hMDMs at 6h p.i with either the WT or the $\Delta icmT$ mutant (triplicates of independent
254 infections of each strain) identified the DNM1L-interacting proteome during infection.
255 Statistical analysis showed that Wiskott-Aldrich Syndrome protein (WASP) interacts with
256 DNM1L (p-value<0.05) during WT-infection of hMDMs, but not during infection with the
257 $\Delta icmT$ mutant (Figure 3J). Interaction with Arp2/3 complex subunit 5 (ARPC5) was non-
258 significant, but followed the same trend. Interestingly, the WASP and Arp2/3 complex are
259 part of the cellular machinery of actin nucleation and recent studies suggested that actin
260 assembly is needed for DNM1L-mediated mitochondrial fragmentation (Hatch et al., 2016; Li
261 et al., 2015; Moore et al., 2016).

262 Collectively, our results show that *L. pneumophila*-induced fragmentation of mitochondria
263 depends on the activity of DNM1L that is required for optimal bacterial replication within
264 host cells and that Ran GTPase is also involved in this process, probably related to the
265 regulation of cytoskeletal dynamics.

266

267 **Despite mitochondrial fragmentation, *L. pneumophila*-infected human macrophages** 268 **exhibit a lack of cell death signs**

269 Mitochondrial fragmentation and elongation are physiological and dynamic states of the
270 organelle that may be altered during disease (Labbe et al., 2014). Fragmentation of
271 mitochondria is considered as a hallmark of cell death although it remains unclear if it is

272 essential for cell death activation (James and Martinou, 2008). However, cell death of
273 macrophages seems to be a defence mechanism against pathogens (Chow et al., 2016). To
274 analyse whether cell death is induced by *L. pneumophila* at this stage of the infection, we
275 measured absolute numbers of total cells and percentage of apoptotic nuclei of WT-infected
276 hMDMs as compared to non-infected cells. Interestingly, at 6h p.i., similar numbers of
277 apoptotic cells were observed in infected and non-infected cells. As expected, macrophages
278 exposed to the pro-apoptotic compound Staurosporin showed high numbers of apoptotic cells
279 (Figure 4A). This suggests that, if signalling leading to cell death was started at this time of
280 infection, it could only be a very early event in the pathway leading to cell death.

281 As release of Cytochrome C (CytC) from mitochondria is a signal leading to cell death,
282 we imaged CytC in WT and $\Delta icmT$ -infected hMDMs. CytC remained in the mitochondria at
283 6h p.i. (Figure 4B) and, interestingly, even until 12h p.i., when *L. pneumophila* has already
284 completed several rounds of replication (Figure 4C). This finding was confirmed by
285 quantifying the mean fluorescence intensity of CytC in mitochondria (Figure 4D) and by
286 analysing CytC quantity by fractionation and western blot (Figure 4E). Thus *L. pneumophila*-
287 induced mitochondrial fragmentation does not trigger the release of CytC and cell death
288 pathways are not activated at the time where mitochondrial fragmentation can be seen in WT-
289 infected cells (6h p.i. Figure 2). We then stained living hMDMs with AnnexinV, a well-
290 known marker of early cell death, and quantified the percentage of AnnexinV⁺ macrophages
291 during infection by flow cytometry. At 6h p.i. only 4.2% of WT-infected cells were
292 AnnexinV⁺ further supporting our results (Figure 4F). We then monitored Caspase-1
293 activation at 6h p.i. but did not detect its activation at this time point by any of the
294 *L. pneumophila* strains (Figure 4G). Similarly, when several Caspases were measured, we
295 found that only 2.3% of WT-infected macrophages had activated caspases (Figure 4H).

296 Taken together, our results demonstrate that *L. pneumophila*-induced DNM1L-dependent
297 mitochondrial fragmentation is not concurrent with cell death in hMDMs at 6h p.i, as there is
298 a lack of cell death signs. This suggests that mitochondrial fission is an *L. pneumophila*-
299 induced cellular event segregated from cell death pathways.

300

301 ***L. pneumophila* impairs mitochondrial respiration during infection in a T4SS- and** 302 **DNM1L-dependent manner**

303 Mitochondrial morphology impacts mitochondrial bioenergetics (Yu et al., 2015). Thus we
304 investigated whether *L. pneumophila* infection modulates mitochondrial respiration. First, we
305 measured the oxygen consumption rate (OCR) in kinetic experiments during infection of

306 living hMDMs with the WT and the $\Delta icmT$ mutant. Infection rapidly upregulated
307 mitochondrial respiration peaking at 1h p.i. suggesting that it is T4SS-independent. As
308 infection progressed, oxygen consumption was abruptly decreased in WT-infected hMDMs
309 compared to non-infected cells, while $\Delta icmT$ -infected hMDMs maintained a high OCR
310 compared to non-infected cells (Figure 5A). We thus propose that a biphasic regulation of
311 mitochondrial respiration during *L. pneumophila* infection takes place, where a T4SS-
312 independent phase of increased oxygen consumption that peaks at 1h p.i. is followed by a
313 later T4SS-dependent phase of impaired mitochondrial respiration with an abrupt reduction in
314 oxygen consumption. To learn whether *L. pneumophila*-induced impairment of mitochondrial
315 respiration depends on DNM1L, we pre-treated hMDMs with Mdivi-1 during 4h prior to
316 infection. Indeed, Mdivi-1 pre-treatment attenuated WT-induced impairment of oxygen
317 consumption (Figure 4A), suggesting that in hMDMs not only T4SS-dependent impairment
318 of mitochondrial morphology but also mitochondrial respiration depends on DNM1L activity.

319 To gain insight into the respiratory status of mitochondria during *L. pneumophila* infection,
320 we analysed cellular respiration in infected macrophages at 2h or 6h p.i. by adding
321 sequentially Oligomycin, an inhibitor of mitochondrial F_1F_0 -ATPase, FCCP, an ionophore
322 that uncouples mitochondrial respiration by increasing H^+ transport across the inner
323 mitochondrial membrane and Rotenone and Antimycin A, inhibitors of mitochondrial
324 complex I and III, respectively to impact mitochondrial oxygen consumption (Figure 5B and
325 S6). At 2h p.i. the basal respiration was higher in $\Delta icmT$ -infected macrophages compared to
326 non-infected and WT-infected cells (Figure 5B), confirming our previous results (Figure 5A).
327 Addition of Oligomycin at 2h p.i. revealed that Oligomycin-induced reduction of cellular
328 OCR was equivalent in non-infected, WT-infected and $\Delta icmT$ -infected macrophages,
329 indicating that ATP production was not affected by the WT at this time point. Subsequent
330 addition of FCCP, that stimulates respiration, showed that maximal mitochondrial respiration
331 was higher in $\Delta icmT$ -infected macrophages than in WT-infected cells, leading in both
332 conditions to higher maximal respiration as compared to non-infected cells. Subsequent
333 addition of Rotenone and Antimycin A showed that non-mitochondrial respiration was
334 slightly higher in both WT- and $\Delta icmT$ -infected cells as compared to non-infected cells. In
335 macrophages the activity of non-mitochondrial NADPH oxidases may dominate cellular
336 oxygen uptake, however our results indicated that this is not the case during *L. pneumophila*
337 infection.

338 At 6h p.i. the WT strain has induced the morphological changes in mitochondria (Figure
339 2A and 2B). At this time-point WT-infected cells showed a reduced slope in the reduction of
340 oxygen consumption caused by the addition of Oligomycin, suggesting that mitochondrial
341 ATP production was compromised in WT-infected macrophages (Figure 5C), a result that
342 was further confirmed by measuring intracellular ATP during infection (Figure 5D). In
343 addition, maximal respiration was severely decreased below the levels of non-infected
344 macrophages, while maximal respiration of $\Delta icmT$ -infected cells remained high.

345 In order to confirm the role of the T4SS in the impairment of mitochondrial respiration and
346 mitochondrial ATP production, we measured oxygen consumption of hMDMs at 6h post-
347 infection with the complemented $\Delta icmT$ mutant (JR32- $\Delta icmT::icmT$) (Figure 5E) and their
348 intracellular ATP content (Figure 5D). Indeed, the ability to impair respiration and to reduce
349 ATP production was restored. Moreover, when other components of the T4SS, such as *icmP*
350 or *dotA* were deleted, the phenotype was consistent with an increased respiration compared to
351 non-infected cells, while complementation ($\Delta icmP::icmP$) impaired respiration (Figure 5E)
352 independent of bacterial respiration and ATP (Figure S6). Infection with *L. pneumophila*
353 Paris (Lpp WT) and its avirulent $\Delta dotA$ mutant yielded analogous results (Figure 5E),
354 showing that respiration impairment is conserved in different *L. pneumophila* strains.

355 In conclusion, *L. pneumophila* infection induces a biphasic regulation of mitochondrial
356 respiration in hMDMs, with a first phase of a T4SS-independent upregulation of oxygen
357 consumption that is followed by a T4SS-dependent phase where mitochondrial respiration is
358 dramatically decreased, mitochondrial ATP production is impaired and the cellular ATP pool
359 is reduced. DNMI1 activity is involved in this second phase, suggesting that *L. pneumophila*-
360 induced mitochondrial fragmentation impairs oxidative phosphorylation during infection.

361

362 ***L. pneumophila* induces glycolysis during infection in a T4SS-independent manner**

363 As glycolysis is an alternative pathway to produce ATP in the cell when mitochondrial
364 respiration is compromised, we measured the extracellular acidification rate (ECAR) during
365 *L. pneumophila* infection as an output of cellular glycolysis. Figure 5F shows that
366 macrophages infected with WT or $\Delta icmT$ upregulated glycolysis 1h p.i.. Next, we pre-treated
367 hMDMs with 2-deoxy-glucose (2-DG), an inhibitor of glycolysis, or with Oligomycin, an
368 inhibitor of mitochondrial F_1F_0 -ATPase. Whereas 2-DG pre-treatment reduced intracellular
369 replication of the WT strain significantly to $46.03 \pm 11.45\%$ (p-value=0.0036), Oligomycin
370 pre-treatment had no significant effect (Figure 5G and S5), highlighting the importance of

371 host cell glycolysis for optimal intracellular replication of *L. pneumophila* early in infection,
372 while mitochondrial oxidative phosphorylation seems dispensable. Thus, our data suggest that
373 *L. pneumophila* infection upregulates cellular glycolysis independently of the T4SS, and that
374 a functional glycolysis is required for optimal intracellular replication in human macrophages,
375 while mitochondrial ATP production seems largely dispensable.

376

377 **DISCUSSION**

378 Although it was reported over 30 years ago (Horwitz, 1983) that mitochondria associate with
379 the bacterial vacuole, the functions of such an association and a possible effector implicated
380 remained unknown. Here we show that *L. pneumophila* regulates mitochondrial dynamics to
381 subvert mitochondrial bioenergetics of infected cells in a process that involves the bacterial
382 T4SS effector MitF and the host fission protein DNM1L. Surprisingly, *L. pneumophila*
383 induced mitochondrial fragmentation is independent of cell death and ultimately impairs
384 mitochondrial respiration, whereas cellular glycolysis is increased. Thus *L. pneumophila*
385 infection of hMDMs induces changes in mitochondrial dynamics that promote a Warburg-like
386 phenotype in the infected cell that favours bacterial replication.

387 Contrary to what was reported before, live cell imaging of mitochondria during infection
388 of hMDMs revealed that *L. pneumophila* does not recruit mitochondria to the LCV, but it
389 interacts with host mitochondria in a highly dynamic manner through close and transient
390 couplings independently of T4SS effectors (Figure 1). We quantified the dynamics of the
391 mitochondria-LCV contacts using statistical analysis of 3D time-lapses of single, infected
392 cells. Physical interactions of the phagosome with mitochondria have been reported for
393 *Chlamydia*, *Salmonella* or *Shigella* infections (Hall et al., 2013; Matsumoto et al., 1991;
394 Sirianni et al., 2016) however, comparisons are difficult as these interactions had not been
395 quantified in time-lapse experiments in living cells. Here we demonstrate that, although
396 mitochondria can be nearly always found in close proximity to the LCV during the first 6h
397 p.i., these contacts are highly dynamic, transient and mainly T4SS-independent. The absence
398 of T4SS participation in this process suggests that mitochondria-LCV couplings might be
399 inherent to phagosome formation and processing. Indeed, associations of mitochondria and
400 phagosomes were also observed when macrophages phagocytized LPS-coated latex beads
401 (West et al., 2011a), pointing to an infection-dependent but virulence-independent mechanism.
402 Our quantitative data therefore support a model where *L. pneumophila* induces highly
403 dynamic and transient interactions with mitochondria without the participation of T4SS
404 effectors. Moreover, as mitochondrial fragmentation without alterations in the mitochondrial

405 mass (Figure S3E) implies an increase in the number of single mitochondria, the probability
406 of mitochondria contacting the LCV might be increased, suggesting that what was reported
407 over 30 years ago might not be recruitment of mitochondria to the LCV but an increase of
408 LCV-mitochondria contacts due to WT-induced mitochondrial fragmentation.

409 While highly dynamic mitochondria-LCV contacts occur mainly T4SS-independent, our
410 analyses of mitochondrial morphology during infection indicate that translocation of T4SS
411 effectors induces mitochondrial fission in the host cell. We found that the T4SS secreted
412 effector MitF is involved (Figure 2), although participation of other effectors cannot be
413 excluded. MitF is a bacterial activator of host Ran-GTPase that has been shown to modulate
414 cytoskeleton dynamics and cell migration of host cells during infection (Rothmeier et al.,
415 2013; Simon et al., 2014). Here we show that the *L. pneumophila* $\Delta mitF$ mutant has an
416 impaired ability to fragment host mitochondria, whereas plasmid-borne expression of MitF
417 complements the phenotype and leads to even more dramatic mitochondrial fragmentation
418 than the WT strain, confirming its participation in *L. pneumophila*-induced changes of
419 mitochondrial dynamics in hMDMs. A BLASTP search with Lpg1976/MitF revealed that
420 homologues of MitF are present in 15 *L. pneumophila* strains and in eight out of 41
421 *Legionella* species analysed, suggesting that also other *L. pneumophila* strains and *Legionella*
422 species induce mitochondrial fragmentation. To our knowledge, to date only the bacterial
423 toxins listeriolysin O (LLO) of *L. monocytogenes* and the vacuolating toxin A (VacA) of
424 *H. pylori* have been implicated in bacterial-induced mitochondrial fragmentation, but no
425 translocated protein has been identified yet (Jain et al., 2011; Stavru et al., 2011). Thus,
426 uniquely, the translocated bacterial effector MitF of *L. pneumophila* promotes mitochondrial
427 fragmentation in the host cell.

428 On the host side, the fission factor DNM1L aggregates on host mitochondria during
429 *L. pneumophila* infection in a T4SS-dependent manner (Figure 3). Pre-treatment of hMDMs
430 with Mdivi1, an inhibitor of DNM1L functions on mitochondrial fission reduced
431 *L. pneumophila*-induced mitochondrial fragmentation, suggesting a participation of DNM1L
432 in the morphological changes of mitochondria. Furthermore, the host factors Ran and RanBP2
433 are involved in the fragmentation process. Targeting of DNM1L by Mdivi1 or siRNA-
434 mediated silencing reduced bacterial intracellular replication, suggesting that DNM1L-
435 induced fragmentation is required for optimal intracellular replication of *L. pneumophila*.
436 These results are in line with data obtained for *L. monocytogenes*, where depletion of DNM1L
437 impaired bacterial replication, suggesting that *L. monocytogenes* needs to induce
438 mitochondrial fission for efficient infection (Stavru et al., 2011). However,

439 *L. monocytogenes*-induced mitochondrial fragmentation occurs independently of DNM1L
440 (Stavru et al., 2013) different from what we find for *L. pneumophila* and what was reported
441 for *H. pylori* that fragment mitochondria through DNM1L (Jain et al., 2011).

442 Our quantitative proteomic approach shows that DNM1L specifically interacts with WASP
443 during *L. pneumophila*-WT-infection, while it also suggests that DNM1L interacts with
444 ARPC5, a subunit of the Arp2/3 complex. As WASP and the Arp2/3 complex are known to
445 be involved in actin nucleation in macrophages (Rougerie et al., 2013) and recent studies
446 suggested that actin assembly is needed to recruit DNM1L to the fission site by direct binding
447 (Hatch et al., 2016; Li et al., 2015; Moore et al., 2016), this indicates that *L. pneumophila*-
448 induced mitochondrial fragmentation may involve WASP-Arp2/3-mediated recruitment of
449 DNM1L to mitochondria. Moreover, the effector MitF (LegG1) for which we have shown
450 here involvement in *L. pneumophila*-induced mitochondrial fragmentation (Figure 3) is an
451 activator of cellular Ran which targets RanBP2 (Rothmeier et al., 2013). Importantly, it has
452 been shown that Ran regulates WASP and Arp2/3 for actin nucleation (Yi et al., 2011), and
453 both Ran and RanBP2 have been found to directly interact with WASP (Sadhukhan et al.,
454 2014). Thus, cytoskeletal dynamics might – at least partly – account for MitF functions on
455 mitochondrial fragmentation during *L. pneumophila* infection through a Ran – RanBP2 –
456 WASP/Arp2/3 – DNM1L functional axe.

457 It has been proposed that DNM1L participates in apoptotic cell death by stimulating Bax
458 oligomerization and thereby enhancing MOM permeabilization and massive efflux of
459 Cytochrome C (Montessuit et al., 2010). Our data support a model where during
460 *L. pneumophila* infection DNM1L functions in mitochondrial dynamics and cell death
461 pathways are segregated, as we observed an increase of DNM1L puncta on mitochondria in
462 the absence of early or late signs of cell death in the infected cells, including Cytochrome C
463 release or Caspase activation. Indeed, these two functions seem to be intrinsically separated in
464 the DNM1L protein, as DNM1L-induced mitochondrial fission relies on its GTPase activity
465 while DNM1L-induced Bax oligomerization and promotion of cell death appears independent
466 of its GTPase activity (Montessuit et al., 2010). Moreover, Bax oligomerization in
467 mitochondria seems not forcibly dependent on DNM1L, as *dnm1l*^{-/-} cells still recruit Bax and
468 Bak to mitochondria (Otera et al., 2016). This may suggest that the role of DNM1L during
469 *L. pneumophila* infection is restricted to its GTPase activity, whereas its functions on MOM
470 permeabilization, Cytochrome C release and finally cell death are inactive. Importantly,
471 *L. pneumophila* specifically modulates several cell death mediators of macrophages at
472 different levels of the apoptotic cascade (Luo, 2011), which may also interact with DNM1L-

473 induced signalling. Interestingly, our results show that cell death is not an obligate outcome of
474 disrupting mitochondrial dynamics. In addition, the lack of cell death induction in the infected
475 cell might benefit intracellular *L. pneumophila*, as the niche where bacterial replication takes
476 place is preserved.

477 A key finding of our study is the discovery that *L. pneumophila*-induced mitochondrial
478 fission alters the bioenergetics of infected macrophages leading to a Warburg-like metabolism
479 in the cells. Induction of glycolysis and initial upregulation of mitochondrial respiration is
480 independent of *L. pneumophila* T4SS effectors, but at 60min p.i. *L. pneumophila* abruptly
481 reduces mitochondrial oxygen consumption, ATP turnover and the intracellular ATP pool by
482 a mechanism dependent on a functional T4SS and involving DNM1L (Figure 5). Our results
483 indicate that *L. pneumophila*-induced DNM1L-mediated mitochondrial fragmentation plays a
484 key role in the regulation of mitochondrial respiration during infection.

485 We propose that as early as 6h p.i. and prior to bacterial replication, *L. pneumophila*-
486 induced mitochondrial changes allow to repurpose the metabolism of the infected cell in a
487 way where oxidative phosphorylation is impaired while cellular glycolysis remains active.
488 This metabolic switch of the infected host cell strikingly resembles the Warburg effect, a
489 metabolic state observed in a wide range of cancer cells where respiration in mitochondria is
490 limited and glycolysis is enhanced (Liberti and Locasale, 2016). Originally defined in tumor
491 cells, Warburg-like processes are also observed during immune responses and inflammation
492 (Wen et al., 2012). In line with this idea is the fact that intracellular replication of
493 *L. monocytogenes* is favoured in cancer cells as compared to infection of primary cells,
494 suggesting that Warburg metabolism might benefit bacterial replication (Gillmaier et al.,
495 2012). Our experiments show that inhibition of oxidative phosphorylation has little effect on
496 *L. pneumophila* replication, while reduction of cellular glycolysis significantly impairs its
497 replication, in line with previous reports (Ogawa et al., 1994), indicating that *L. pneumophila*
498 takes advantage of this Warburg-like metabolism for its intracellular growth in hMDMs.

499 Taken together, we propose a model (Figure 6) where *L. pneumophila*, by targeting
500 mitochondrial dynamics, induces metabolic repurposing of macrophages and provokes a
501 T4SS-induced Warburg-like effect in the infected cell thereby favouring its own replication.
502 Thus, our results strongly suggest that the bacteria-induced modulation of the mitochondrial
503 dynamics during infection shapes host metabolic responses and represents a key virulence
504 strategy used by *L. pneumophila* to successfully replicate within human macrophages and
505 cause disease.

506

507 **AUTHOR CONTRIBUTIONS**

508 PE and CB conceived the study; PE, ORS, FV, BS, FI performed experiments; TL developed
509 data analyses algorithms; PE, ORS, FV, TL, FI analysed the data. JCOM, PB, HH, CB
510 supervised the study and provided critical advice; PE and CB wrote the manuscript.

511

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528

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655

656 **FIGURE LEGENDS**

657

658 **Figure 1. *L. pneumophila* establishes transient and highly dynamic contacts with**
659 **mitochondria of infected human macrophages.** (A) Human monocyte-derived
660 macrophages (hMDMs) stained with Mitotracker (red) and Hoechst (cyan) were infected with
661 GFP-expressing *L. pneumophila* (green) and imaged by confocal microscopy each hour and
662 analysed by HCA (mitochondria at $<1 \mu\text{m}$ of the LCV). Scale bar: $5 \mu\text{m}$. (B) HCA analysis of
663 mitochondria-LCV associations during WT or $\Delta icmT$ infection of hMDMs. The % of infected
664 cells, the % of LCV with mitochondria at $<1 \mu\text{m}$ and number of mitochondria per LCV are
665 shown (n=3). (C) Same as in B but during infection with *L. pneumophila* Paris WT (Lpp-WT)
666 or Lpp- $\Delta dotA$ (n=3). (D) 3D confocal time-lapse images of a WT infected hMDM showing
667 highly dynamic *Legionella*-mitochondria contacts (5 min). Mitochondria (red), *Legionella*
668 (green). Details of the contacts in the inset. See also Figure S1 and Movie S1. (E)
669 Quantification of *Legionella*-mitochondria contacts in a whole hMDM cell during 5 h of WT
670 infection. See also Figure S1 and Movies S1 and S2. (F) Frequency of quantified *Legionella*-
671 mitochondria contacts during WT or $\Delta icmT$ infection of hMDMs or phagocytosed microbeads
672 (n=7 cells, ns=non-significant, Mann-Whitney t-test)

673

674 **Figure 2. *L. pneumophila* induces T4SS-dependent changes in mitochondrial**
675 **morphology during infection, MitF effector is involved.** (A) hMDMs stained with
676 Mitotracker (red) and Hoechst (cyan) were infected with GFP-expressing *L. pneumophila*
677 (green), imaged by confocal microscopy each hour and analysed by HCA. High resolution
678 raw images (inset) were filtered and automatically segmented to detect nuclei, cytoplasm and
679 LCVs. SER-Edge and SER-Ridge algorithms for texture analysis were applied to analyse
680 mitochondrial morphology of infected cells. Scale bars: $5 \mu\text{m}$. (B) HCA analysis of
681 mitochondrial morphology during JR32-WT or JR32- $\Delta icmT$ infection of hMDMs (upper
682 graph) and Lpp-WT or Lpp- $\Delta dotA$ (bottom graph) infection. % of infected cells with
683 fragmented mitochondria (n=7). (C) HCA analysis of mitochondrial morphology at 4h p.i.
684 with CFSE-labelled JR32-WT, $\Delta icmT$ or $\Delta icmT$ -empty vector (JR32- $\Delta icmT::\text{vector}$) or
685 complemented with *icmT* (*icmT::icmT*). % of infected cells with fragmented mitochondria
686 (n=3). (D) HCA analysis of mitochondrial morphology (hMDMs) at 4h p.i. with WT, JR32-
687 $\Delta icmT$, JR32- $\Delta mitF$ or JR32- $\Delta ralF$. % of infected cells with fragmented mitochondria (n=3).
688 (E) RAW264.7 macrophages imaged at 8h p.i. WT, $\Delta icmT$, $\Delta mitF$ or $\Delta mitF::mitF$ (in green).

689 Cells were stained with Mitotracker Deep Red (red) before fixation. Scale bar: 5 μm . (F) % of
690 infected cells with fragmented mitochondria. RAW264.7 macrophages infected with dsRed-
691 expressing WT, $\Delta icmT$, $\Delta mitF$ or complemented strain $\Delta mitF::mitF$ at 8h p.i. (n =2). (G)
692 Single-cell results from F are shown. Each dot represents a cell (n > 50 cells per condition).
693 *p-value \leq 0.05; **p-value \leq 0.01; ***p-value \leq 0.001; ns = non-significant (Mann-Whitney
694 t-test).

695

696 **Figure 3. *L. pneumophila*-induced fragmentation depends on DNMI1L, necessary for**
697 **optimal bacterial replication.** (A) hMDMs infected with GFP-expressing *L. pneumophila*
698 (green). At 6h p.i cells were fixed and stained for Tom20 (red), DNMI1L (yellow) and nuclei
699 (DAPI, cyan) and analysed by HCA: green, DNMI1L puncta on mitochondria; red, DNMI1L
700 puncta on the cytoplasm (see Figure S4). Scale bars: 10 μm . (B) HCA analysis of the number
701 of DNMI1L puncta per area of mitochondria (puncta/ μm^2) at 6h p.i. with WT or $\Delta icmT$. Dots
702 are replicates (each including more than 100 cells for image analysis) of a representative
703 experiment (n=3). (C) hMDMs infected with WT or $\Delta icmT$ during indicated times, lysed and
704 total and phosphorylated DNMI1L levels analysed by Western blot. +: A549 cells treated with
705 20 μM Forskolin for 2h (positive control). (D) hMDMs pre-treated or not with Mdivi1 (50
706 μM) during 4h, washed, stained with Mitotracker and Hoechst and infected with WT during
707 6h. % of infected cells is shown, dots are replicates (each including more than 100 cells for
708 image analysis) of a representative experiment (n=3). (E) Same as D, % of infected cells with
709 fragmented mitochondria. (F) RAW264.7 macrophages treated with siRNA against DNMI1L,
710 Ran or RanBP2 for 48h and infected with WT during 8h and analysed by HCA for
711 mitochondrial morphology. Depletion efficiency was assessed by Western blot (Figure S4). %
712 of infected cells with fragmented mitochondria (n=2). (G) Single-cell results from F. Each dot
713 represents a cell (n > 50 cells per condition). (H) hMDMs pre-treated or not with Mdivi1 (50
714 μM) during 4h, washed and infected with WT. At 24h p.i., cells were lysed with sterile H₂O
715 and GFP-expressing bacteria were quantified by flow cytometry. Relative intracellular
716 replication, expressed as % of the control. Dots are values obtained from independent
717 experiments (n=3). (I) Human A549 cells treated with siRNA oligonucleotides against
718 DNMI1L, ARF1 or RanBP2 for 48 h and infected with WT. At 24h p.i., fluorescence was
719 measured in a plate reader. The depletion efficiency was assessed by Western blot (Figure S4).
720 Dots are the values obtained from independent experiments (n=3). (J) hMDMs infected with
721 WT or $\Delta icmT$ in 3 independent infections. At 6h p.i., cells were lysed and subjected to

722 immunoprecipitation with specific antibodies against human DNMI1L. Immunoprecipitated
723 lysates were analysed by label-free LC-MS/MS quantitative analysis to identify the DNMI1L-
724 interacting proteome during infection. Volcano plot of the proteins identified (black dots).
725 Those interacting significantly with DNMI1L in the three independent experiments are outside
726 the curved volcano lines (p -value ≤ 0.05). * p -value ≤ 0.05 ; ** p -value ≤ 0.01 ; *** p -value \leq
727 0.001; ns = non-significant (Mann-Whitney t-test).

728

729 **Figure 4. *L. pneumophila*-infected human macrophages exhibit a lack of cell death signs**
730 **despite *L. pneumophila*-induced mitochondrial fragmentation.** (A) HCA analysis of
731 apoptotic nuclei at 6h p.i. with WT or $\Delta icmT$. Control: Staurosporine (STS). Counts of cells
732 per well (sum of 16 fields) and % of cells with apoptotic nuclei are shown (n=3). (B) hMDMs
733 infected with GFP-expressing *L. pneumophila* (green) during 6h, fixed and stained for
734 Cytochrome C (CytC, red) and nuclei (DAPI, cyan). Scale bars: 10 μ m. (C) Same as in B but
735 at 12h p.i. (D) Quantification of CytC in mitochondria of hMDM images such as in B and C.
736 Mean Fluorescence Intensity (MFI) of CytC in mitochondria. More than 100 cells per
737 condition were analyzed in each of the (n=3) independent experiments. (E) hMDMs were
738 infected or stimulated as indicated, at 6h or 12h protein content was fractionated. CytC levels,
739 and the quality of the fractionation (GAPDH: cytosolic control, Bcl2: mitochondrial control)
740 were assessed by Western blot (n=2). (F) hMDMs infected with WT or $\Delta icmT$ during 6h,
741 stained with Annexin-V-488 and analysed by flow cytometry. Dot plot Forward Scatter (FSC)
742 vs. Side Scatter (SSC) include hMDMs in the analysis gate. Annexin-V-488 histograms of
743 non-infected cells (red line) vs. infected cells (blue line). Control: 5% DMSO treatment (blue
744 line, upper right histogram). (G) hMDMs infected with WT or $\Delta icmT$, at 6h p.i. cells were
745 incubated with FAM-YVAD-FMK during 1h to reveal active Caspase-1 imaged by confocal
746 microscopy and analysed by HCA. Control: STS. The graph shows % of cells exhibiting
747 active Caspase-1 (n=3). (H) Same as in E but at 6h p.i. cells were incubated with SR-VAD-
748 FMK to reveal active Caspases (n=3).

749

750 **Figure 5. *L. pneumophila* impairs mitochondrial respiration during infection in a T4SS-**
751 **and DNMI1L-dependent manner but activates glycolysis in a T4SS-independent manner.**
752 (A) Oxygen consumption rate (OCR) of hMDMs monitored on a Seahorse XFe96 Analyser.
753 Basal respiration was recorded during 35 min and infection was performed by releasing WT
754 or $\Delta icmT$ into each well through their corresponding ports. Mdivi1 pretreatment during 4h

755 was followed by washing prior to infection. % of OCR with respect to basal respiration is
756 shown. (B) Bioenergetic profiles of mitochondrial respiration of WT or $\Delta icmT$ -infected
757 hMDMs at 2h p.i. Oligomycin (O), FCCP (F) and Rotenone + Antimycin A (R+AA) were
758 added sequentially into each well through their corresponding ports while OCR was
759 monitored. See Figure S6 for interpretation of the graphs. ●: non-infected; ×: WT; ▲: $\Delta icmT$.
760 (C) Same as B but at 6h p.i. (D) Intracellular ATP during infection with WT, $\Delta icmT$,
761 $\Delta icmT::vector$ or complemented $\Delta icmT::icmT$ strains. (E) OCR values of hMDMs infected
762 with different *L. pneumophila* strains and T4SS-deficient mutants (6h p.i.) (F) Extracellular
763 acidification rate (ECAR) of hMDMs during infection with WT or $\Delta icmT$ at 1h, 2h and 5h p.i.
764 (G) hMDMs pretreated or not with 2-DG or Oligomycin during 4h, washed and infected with
765 WT. At 24h p.i., cells were lysed with sterile H₂O and GFP-expressing bacteria were
766 quantified by flow cytometry. Relative intracellular replication is shown, expressed as % of
767 the non-treated control (n=3). *p-value \leq 0.05; **p-value \leq 0.01; ***p-value \leq 0.001 (Mann-
768 Whitney t-test).

769

770 **Figure 6. *L. pneumophila* modulates mitochondrial dynamics for its efficient replication**

771 *L. pneumophila* exerts highly dynamic interactions with host mitochondria and translocates
772 the effector MitF *via* its T4SS into the cytoplasm of the macrophage. MitF is an activator of
773 Ran GTPase, which triggers the activation of WASP and Arp2/3 with the involvement of
774 RanBP2. Actin nucleation by WASP/Arp2/3 progressively facilitates T4SS-induced DNMI1L-
775 mediated fragmentation of host mitochondria. Whereas infection upregulates mitochondrial
776 oxidative phosphorylation (OXPHOS) and glycolysis at 1 h post infection (p.i.) independently
777 of the T4SS (a metabolic state represented by yellow background), *L. pneumophila*-induced
778 T4SS-dependent mitochondrial fragmentation reduces OXPHOS while glycolysis remains
779 active (a reprogrammed metabolic state represented by salmon background). Thus, by
780 modulating mitochondrial dynamics, *L. pneumophila* induces a Warburg-like effect in the
781 infected cell as early as 6 h p.i., thereby favouring its intracellular replication.

782

783 **STAR★METHODS**

784

785 **CONTACT FOR REAGENT AND RESOURCE SHARING**

786 Further information and requests for resources and reagents should be directed to and will be
787 fulfilled by the Lead Contact, Carmen Buchrieser (cbuch@pasteur.fr).

788

789 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

790 **Human Primary Cell Cultures**

791 Human blood was collected by the French National Blood Service (EFS) from healthy
792 volunteers after obtaining their informed consent and under the ethical rules established by
793 the Ethics and Professional Conduct Committee of the EFS, which imply anonymity about the
794 volunteers' gender, sex or age. Peripheral blood mononuclear cells (PBMCs) were isolated by
795 Ficoll-Hypaque density-gradient separation (Lympholyte-H; Cedarlane Laboratories) at room
796 temperature (RT). PBMCs were incubated with anti human CD14 antibodies coupled to
797 magnetic beads (Miltenyi Biotec) and subjected to magnetic separation using LS columns
798 (Miltenyi Biotec). Positive selected CD14⁺ cells were counted and CD14 expression was
799 analysed by flow cytometry, showing a rutinary purity > 90%. CD14 cells were plated in
800 RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal
801 bovine serum (FBS, Biowest) in 6 well multi-dish Nunc UpCell Surface cell culture plates
802 (Thermo Fisher) and differentiated to human monocyte-derived macrophages (hMDMs) by
803 incubation with 50 ng/ml of recombinant human macrophage colony-stimulating factor
804 (rhMCSF, R&D Systems) for 6 days at 37°C with 5% CO₂ in a humidified atmosphere. At
805 day 4, additional rhMCSF (25 ng/ml) was added. After 6 days differentiation, UpCell plates
806 were placed at 20°C during 10 minutes and hMDMs were gently detached, counted and plated
807 in RPMI 1640 10% FBS + rhMCSF (25 ng/ml), accordingly to the specific assay performed.

808

809 **Cell lines**

810 Murine macrophage-like RAW 264.7 cells (male) and human A549 lung epithelial carcinoma
811 cells (male) were cultivated in RPMI 1640 medium (Life Technologies) supplemented with
812 10% heat-inactivated FBS (Life Technologies) and 1% glutamine (Life Technologies). The
813 cells were incubated at 37°C with 5% CO₂ in a humidified atmosphere.

814

815 **Bacterial strains**

816 *L. pneumophila* strain Paris or JR32 and its derivatives were grown for 3 days on N-(2-
817 acetamido)-2-amino-ethanesulfonic acid (ACES)-buffered charcoal-yeast (BCYE) extract
818 agar, at 37°C. For knock out and complementation constructions chloramphenicol (Cam;
819 5 µg/mL) was added.

820

821 **METHOD DETAILS**

822 **Mitochondrial labelling**

823 The lyophilized MitoTracker Red FM (Life Technologies) was dissolved in DMSO at 1 mM
824 and stored it in -20°C until use it. hMDMs were plated in 384-well plates (Greiner Bio-One)
825 at a density of 1.5×10^4 cells per well in 50 μ l of RPMI 1640 medium (Life Technologies)
826 supplemented with heat-inactivated 10% FBS and 20 ng/mL of rhM-CSF (Milteny) one day
827 before the assay. Then 1 mM of MitoTracker Red FM was diluted in pre-warmed RPMI1640
828 without phenol-red (Life Technologies) supplemented with 100 nM glutamine (Life
829 Technologies) and 10% FBS (assay medium). hMDMs were stained prior to infection with 40
830 μ L of 100 nM of MitoTracker Red FM solution. After 30 minutes of incubation at 37°C/5%
831 CO₂, cells were washed 3 times with assay medium. Between each washing step, cells were
832 incubated at 37°C with 5% CO₂ for 5 minutes. Prior to infection the cells were washed once
833 with pre-warmed RPMI 1640 without phenol-red.

834

835 **Infection of hMDMs and automatic confocal imaging**

836 hMDMs were infected with *L. pneumophila* grown for three days on BCYE agar plates.
837 Bacteria were dissolved in 1X PBS (Life Technologies), the optical density (OD) was
838 adjusted to OD₆₀₀ of 2.5 ($2,2 \times 10^9$ bacteria/mL) and the bacteria were then further diluted in
839 FBS-free RPMI 1640 medium (Life Technologies) prior to infection to obtain the respective
840 multiplicity of infection (MOI). hMDMs were washed twice with FBS-free RPMI 1640 and
841 then infected (MOI = 10) under transient FBS-free conditions in 384-well plates (Greiner Bio-
842 One). The infection was synchronized by centrifugation (200 g for 5 min) and the infected
843 cells were incubated at 37°C for 5 min in a water bath and then for 25 min at 37°C/5%CO₂.
844 After three intensive washes with complete medium (RPMI 1640, 10% FBS) the infection
845 proceeded in complete medium with 300 ng/mL of Hoechst H33342 (nuclear staining; Life
846 technologies) for the respective times. Image acquisitions of multiple fields per well were
847 performed on an automated confocal microscopes (OPERA QEHS, Perkin Elmer, or
848 InCellAnalyzer6000, GE Health Care) using 40X or 60X objectives, excitation lasers at 405,
849 488, 561 and 640 nm, and emission filters at 450, 540, 600 and 690 nm, respectively. Finally,
850 confocal images were transferred to the Columbus Image Data Storage and Analysis System
851 (Perkin Elmer) for HCA analyses.

852 Inhibitors of DNM1L (Mdivi1; Tocris Bioscience), glycolysis (2-DG; Seahorse
853 Bioscience) or oxidative phosphorylation (Oligomycin; Enzo) were added to hMDMs at the
854 indicated concentration during 4 h prior to infection. Then, cells were washed 3 times with
855 RPMI 1640 10% FBS and hMDMs were infected as described above.

856

857 **Infection of RAW macrophages and confocal imaging**

858 Fluorescence microscopy of RAW 264.7 macrophages was performed as described previously
859 (Weber et al., 2006). Briefly, exponentially growing macrophages were seeded on sterile
860 coverslips coated with poly-L-lysine (Sigma-Aldrich) in 24-well plates at 2.5×10^5 in
861 supplemented RPMI 1640 medium and let grow over night (37°C/5%CO₂). Meanwhile, liquid
862 cultures of *L. pneumophila* in ACES yeast extract (AYE) medium were inoculated at an
863 OD₆₀₀ of 0.1 and grown at 37°C for 21 h to an early stationary phase (2×10^9 bacteria/mL).
864 Cam 5 µg/mL was added as required. RAW 264.7 macrophages were infected with
865 dsRedExpress-producing (pCR77) *L. pneumophila* (MOI = 10) and the infection was
866 synchronized by centrifugation (450 g, 10 min), and the infected cells were incubated at 37°C
867 (5% CO₂) for a total of up to 8 h. The cells were then washed 1 time with 37°C RMPI and
868 mitochondria were stained prior to fixation with 50 nM MitoTracker Deep Red FM (Life
869 Technologies), following the manufacturer's protocol. Subsequently, the cells were fixed with
870 4% paraformaldehyde (PFA; Electron Microscopy Sciences) for 15 min at RT. Finally, the
871 coverslips were washed 3 times with DPBS and mounted on glass-slides using ProLong
872 Diamond Antifade Mountant with DAPI (Life Technologies) to stain DNA. The samples were
873 analysed with a Leica TCS SP5 confocal microscope (HCX PL APO CS, objective 63×/1.4–
874 0.60 oil; Leica Microsystems). Finally, confocal images were transferred to Columbus Image
875 Data Storage and Analysis System (Perkin Elmer) for HCA analyses of mitochondrial
876 morphology using the “project” mode of the system.

877

878 **Fixed hMDMs and automatic confocal imaging**

879 hMDMs were infected in 384-well plates as described above for living hMDMs. In the
880 Cytochrome C experiments, 30 min before the specified time post infection, mitochondria
881 were stained with 50 nM MitoTracker Deep Red FM (Life Technologies), following the
882 manufacturer's protocol. Then, cells were fixed with 4% PFA for 15 min, washed with DPBS,
883 permeabilized with DPBS-0.1% Triton-X100 for 15 min and blocked with DPBS-0.5% FBS
884 during 30 min. Fixed cells were incubated overnight at 4°C with 30 µL of the following
885 primary antibodies depending on the experiment: Tom20 (1:50, clone 29, BD), DNM1L
886 (1:50; D6C7, Cell Signalling Technology) or Cytochrome C (1:50; clone 6H2.B4, BD). Cells
887 were then washed three times with DPBS and incubated during 2 h with 50 µL of
888 corresponding secondary antibodies: anti-mouse AlexaFluor-555 (1:500; Life Technologies),

889 anti-rabbit AlexaFluor-561 (1:500; Life Technologies) or anti-mouse AlexaFluor-647 (1:500;
890 Life Technologies), and stained with DAPI (Life Technologies). Image acquisition was done
891 as described above for hMDMs.

892

893 **Acquisition of 3D time-lapse confocal movies**

894 hMDMs were labelled for mitochondria and infected as described above using $2,5 \times 10^5$
895 hMDMs plated in 35 mm μ -Dishes (growth area = 3.5 cm²; Ibidi). hMDMs were intensively
896 washed for 5 min after infection with RPMI 1640 10% FBS. In the case of measurements
897 upon micro-beads phagocytosis, polystyrene-based latex 1 μ m Fluoresbrite 486nm
898 microspheres (17154, Polysciences Inc.) were added (10 beads per hMDM) following the
899 same protocol than for infecting cells. 3D time-lapse confocal movies were acquired in a
900 Spinning-disk UltraView VOX (Perkin Elmer) using 488 and 561 lasers with 1% and 5% of
901 laser intensity, respectively, and a maximum of 100 ms of exposure time. The whole volume
902 of the cell was recorded by acquiring z-stack images ($z = 0.3 \mu\text{m}$) of MitoTracker-labelled
903 hMDMs every minute using Volocity 3D imaging software (Perkin Elmer).

904

905 **Western blot analyses of DNM1L and Cytochrome C**

906 hMDMs were plated in 6 well plates (DNM1L) or 10 cm dishes (Cytochrome C), and infected
907 at MOI=10 as described previously. At the indicated time points, cellular protein lysates for
908 DNM1L analyses were obtained by lysis with 100 μ l of 1X Blue Loading Buffer (62.5 mM
909 Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 0.01% bromophenol blue and 41 mM
910 dithiothreitol, DTT) followed by brief sonication, while protein lysates for Cytochrome C
911 analyses were obtained after cellular fractionation using the Mitochondrial/Cytosol
912 Fractionation kit MIT1000 (Millipore) following manufacturer instructions. After boiling
913 lysates at 95°C during 3 min, 30 μ l of protein lysate were subjected to SDS-PAGE
914 electrophoresis and transferred to Nitrocellulose membranes. Antibodies against β -Actin, Bcl2,
915 or GAPDH were used as loading controls. The sources of the human specific antibodies were:
916 DNM1L total (clone 22, BD), Phospho-DNM1L Ser616 (clone D9A1, Cell Signalling
917 Technology), Phospho-DNM1L Ser637 (Sigma Aldrich), β -Actin (Sigma Aldrich), Bcl2
918 (MIT1000 kit, Millipore), GAPDH (MIT1000 kit, Millipore) and Cytochrome C (clone
919 7H8.2C12, BD, kindly supplied by F. Stavru). After 3 washes with PBS-T, membranes were
920 incubated during 1h at RT with IRDye-800CW and IRDye-680RD-labeled secondary
921 antibodies (Li-Cor) and scanned in an Odyssey CLx infrared digital imaging system (Li-Cor).

922

923 **Immunoprecipitation and Label-free LC-MS/MS**

924 hMDMs were plated in six 100 mm dishes (BD Falcon) at a density of 2×10^6 cells per dish
925 and three dishes were infected (MOI = 10) with *L. pneumophila* JR32-WT strain while the
926 other three dishes were infected with JR32- $\Delta icmT$ mutant strain. The six samples were treated
927 independently and in parallel during the entire immunoprecipitation protocol and posterior
928 quantitative analysis. At 6 h post infection, the medium was removed, cells were washed once
929 with ice cold DPBS and hMDMs from each dish were harvested under non-denaturing
930 conditions by adding to each dish 500 μ L of ice cold 1X Cell Lysis Buffer (Cell Signaling
931 Technology) supplemented with a PhosSTOP tablet (Roche) per 10 mL of Cell Lysis Buffer,
932 incubating dishes on ice during 5 min and scrapping cells off the dishes. Cell lysate from each
933 dish were transferred to an ice-cold micro-centrifuge tube, kept on ice, and sonicated three
934 times for three pulses each. Then, cell lysates were centrifuged for 10 min at 4°C and 14,000
935 g and the supernatants were transferred to new tubes. Mouse anti human DNMI1L (clone 8,
936 BD) was added to each sample at 4 μ g of antibody per mg of total protein and incubated with
937 rotation overnight at 4°C. The following day, 20 μ L of Dynabeads Protein G magnetic beads
938 (Life Technologies) were added to each sample and incubated with rotation for 60 min at 4°C.
939 Then, magnetic beads were pelleted using a magnetic separation rack and washed once with 1
940 mL ice cold 1X Cell Lysis Buffer (no PhosSTOP; Cell Signaling Technology) and 3 times
941 with 1 mL ice cold digestion buffer (20 mM Tris pH 8.0, 2 mM $CaCl_2$), keeping samples on
942 ice between washes. Washed beads were re-suspended in 150 μ L digestion buffer and
943 incubated for 4 h with 1 μ g trypsin (Promega) at 37°C. Beads were removed, another 1 μ g of
944 trypsin was added and proteins were further digested overnight at 37°C. Peptides were
945 purified on Omix C18 tips (Agilent), dried and re-dissolved in 20 μ l 0.1% trifluoroacetic acid
946 in water/acetonitrile (98:2, v/v) of which 2 μ l was injected for LC-MS/MS analysis on an
947 Ultimate 3000 RSLCnano system (Thermo Fisher Scientific) in line connected to a Q Exactive
948 mass spectrometer (Thermo Fisher Scientific). Trapping was performed at 10 μ L/min for 4
949 min in solvent A (0.1% formic acid in water/acetonitrile (98:2, v/v)) on a 100 μ m internal
950 diameter (I.D.) \times 20 mm trapping column (5 μ m beads, C18 Reprosil-HD, Dr. Maisch,
951 Germany) and the sample was loaded on a reverse-phase column (made in-house, 75 μ m I.D.
952 \times 150 mm, 3 μ m beads C18 Reprosil-HD, Dr. Maisch). Peptides were eluted by a linear
953 increase from 2 to 55% solvent B (0.08% formic acid in water/acetonitrile (2:8, v/v)) over 120
954 minutes at a constant flow rate of 300 nL/min. The mass spectrometer was operated in data-
955 dependent mode, automatically switching between MS and MS/MS acquisition for the ten

956 most abundant ion peaks per MS spectrum. Full-scan MS spectra (400-2000 m/z) were
957 acquired at a resolution of 70,000 in the orbitrap analyzer after accumulation to a target value
958 of 3,000,000. The ten most intense ions above a threshold value of 17,000 were isolated
959 (window of 2.0 Th) for fragmentation at a normalized collision energy of 25% after filling the
960 trap at a target value of 50,000 for maximum 60 ms. MS/MS spectra (200-2000 m/z) were
961 acquired at a resolution of 17,500 in the orbitrap analyzer The S-lens RF level was set at 50
962 and we excluded precursor ions with single, unassigned and charge states above five from
963 fragmentation selection.

964

965 **RNA interference, efficiency and cytotoxicity**

966 For RNA interference experiments, A549 cells were grown in 96-well plates and treated for
967 48 h with a final concentration of 10 nM of siRNA oligonucleotides (see Key Resources
968 Table). To this end, the siRNA stock (10 μ M) was diluted 1:15 in RNase-free water, and 3
969 μ L of diluted siRNA was added per well. Allstars siRNA (Qiagen) was used as a negative
970 control. Subsequently, 24.25 μ l RPMI medium without FBS was mixed with 0.75 μ L
971 HiPerFect transfection reagent (Qiagen), added to the well, mixed and incubated for 5-10 min
972 at RT. In the meantime, cells were diluted in RPMI medium with 10% FBS, 175 μ l of the
973 diluted cells (2×10^4 cells) were added on top of each siRNA-HiPerFect transfection
974 complex and incubated for 48 h. RAW264.7 macrophages were transfected using AMAXA
975 Cell Line Nucleofector Kit V (Lonza) according to the manufacturer's recommendations, and
976 treated for 24 h with a final concentration of 150 nM of siRNA oligonucleotides.
977 Macrophages or epithelial cells were infected with GFP-producing *L. pneumophila* strains,
978 and intracellular replication was determined by fluorescence as described above. The protein
979 depletion efficiency was assessed by Western Blot. A549 cells were grown in 24-well plates
980 and treated for 48 h with a final concentration of 10 nM of siRNA oligonucleotides. To this
981 end, the siRNA stock (10 μ M) was diluted 1:15 in RNase-free water, and 9 μ l of diluted
982 siRNA was added per well. Allstars siRNA (Qiagen) was used as a negative control.
983 Subsequently, 72.75 μ l RPMI medium without FBS was mixed with 2.25 μ l HiPerFect
984 transfection reagent (Qiagen), added to the well, mixed and incubated for 5-10 min at RT. In
985 the meantime, cells were diluted in RPMI medium with 10% FBS, 525 μ l of the diluted cells
986 (6×10^4 cells) were added on top of each siRNA-HiPerFect transfection complex and
987 incubated for 48 h. RAW 264.7 cells were transfected using the AMAXA Cell Line
988 Nucleofector Kit V (Lonza) according to the manufacturer's recommendations, and treated

989 for 24 h with a final concentration of 150 nM of siRNA oligonucleotides. Protein depletion
990 efficiency was assessed as follows: cells were harvested in ice-cold DPBS (14190144,
991 Thermo Fisher Scientific), lysed with ice-cold NP-40 cell lysis buffer (Thermo Fisher
992 Scientific) and cell extracts subjected to SDS-PAGE. After tank Western blotting, PVDF
993 membranes (GE Healthcare Life Sciences) were blocked with PBS/3% BSA (bovine serum
994 albumin, 8076.2, C. Roth) for 1 h at RT. Subsequently, specific primary antibodies against
995 Arf1 (ab58578, Abcam), Dnm11 (ab56788, Abcam), RanBP2 (ab64276, Abcam) or GAPDH
996 (2118, Cell Signalling) were diluted 1:500 - 1:1000 in blocking buffer and used to stain the
997 indicated proteins (4°C, overnight). Finally, horse radish peroxidase (HRP)-conjugated
998 secondary antibodies (GE Healthcare Life Sciences) were diluted 1:2000 in blocking buffer
999 and incubated for 1 h at RT. After extensive washing, the enhanced chemiluminescence
1000 (EHL) signal was detected with an ImageQuant LAS4000 (GE Healthcare Life Sciences).

1001 To assess cell viability after siRNA treatment, propidium iodide (PI)-uptake was
1002 measured. A549 cells or RAW 264.7 macrophages were grown and treated with siRNA
1003 oligonucleotides (see Key Resources Table) as described above (protein depletion efficiency).
1004 The cells were then harvested in ice cold DPBS and stained for 10 min with 1 µg/mL PI (Life
1005 Technologies) in DPBS and subjected to flow cytometry analysis (BD FACS Canto II). Gates
1006 were set according to forward/sideward scatter properties, and 10,000 events were collected
1007 for each sample. Cells treated for 10 min with 70% sterile-filtered ethanol (EtOH; Roth)
1008 served as positive control for cell death.

1009

1010 **Intracellular replication assays**

1011 Infection of hMDMs (MOI = 10) was done in 96 well-plates and the quantification of the
1012 bacterial burden was performed by flow cytometry analysis. Infection with GFP-producing
1013 *Legionella* strains was performed as previously described for 384 well-plates by adjusting the
1014 working volumes to 96 well-plates. At 24 h post infection, supernatants were removed and
1015 stored at 4°C. Sterile deionized water was added to the cells and the plates were kept at 37°C
1016 for 1 hour until the cells were lysed. Complete cell lysis was achieved by repeated pipetting of
1017 the cell lysates, which were then recovered and mixed with the previously stored supernatants.
1018 Samples were run on a MACSQuant-VIB cytometer (Miltenyi Biotec) and the obtained data
1019 was analysed on FlowJo v 8.8.7 (Tree Star). Alternatively, cells were lysed at 24h p.i. with 50
1020 µl of sterile deionized water and plated in BCYE plates. After 3 days of incubation at 37°C,
1021 colony-forming units (CFU) were counted.

1022 Infection of A549 cells (MOI = 10) with GFP-producing *Legionella* strains was
1023 performed as described above. Briefly, bacteria were grown for 21 h in AYE medium, diluted
1024 in RPMI 1640 supplemented with 10% FBS/1% glutamine, centrifuged (450 g, 10 min, RT)
1025 and incubated for 1 h. The infected cells were washed 3 times with pre-warmed RPMI 1640
1026 containing 10% FBS and incubated for 24 h or the time indicated (well plate was kept moist
1027 with water in extra wells). GFP fluorescence was measured using a microtiter plate reader
1028 (Synergy H1, BioTek) and results were analysed for statistical significance by the two-sample
1029 Student's t-test assuming unequal variance.

1030

1031 **Monitoring of axenic growth of *L. pneumophila***

1032 After 4h pre-treatment with Mdivi1 (Tocris Bioscience), 2-DG (Seahorse Bioscience) or
1033 Oligomycin (Enzo), hMDMs were washed 4 times, in order to use the same protocol as
1034 during the infection experiments with the inhibitors. After 4h pre-treatment and washing,
1035 hMDMs were lysed with 50µl of sterile deionized water. Then, *L. pneumophila* JR32-WT was
1036 added to the lysate in a minimal amount of growth medium (BYE, 5µl) and OD₆₀₀ was
1037 automatically recorded during bacterial axenic growth, continuously shaking at 37°C, in a
1038 Synergy2 reader (BioTek).

1039

1040 **Metabolic Extracellular Flux Analysis**

1041 hMDMs (50,000) were plated in XF-96-cell culture plates (Seahorse Bioscience). For OCR
1042 measurements, XF Assay Medium (Seahorse Bioscience) supplemented with 1 mM pyruvate
1043 and 10 mM glucose was used, and OCR was measured in a XF-96 Flux Analyzer (Seahorse
1044 Bioscience). For kinetic OCR measurements, 20 µL of *L. pneumophila* was injected through
1045 the port A in each well at an MOI = 10. For the mitochondrial respiratory control assay,
1046 hMDMs were infected at MOI =10 and at 2 h or 6 h p.i., different drugs were injected
1047 (Mitostress kit, Seahorse Bioscience) while OCR was monitored. Specifically, Olygomycin
1048 was injected through the port A, then FCCP was injected through the port B, and finally
1049 Rotenone + Antimycin A were injected through the port C, to reach each of the drugs a final
1050 concentration in the well of 0.5 µM. For ECAR measurements, XF Base Medium (Seahorse
1051 Bioscience) was supplemented with 2 mM glutamine and ECAR was monitored in a XF-96
1052 Flux Analyzer (Seahorse Bioscience) at indicated times during infection of hMDMs with
1053 *L. pneumophila*, MOI = 10.

1054

1055 **Labelling of *L. pneumophila* with CFSE**

1056 *L. pneumophila* was stained with 9 μ M Carboxyfluorescein succinimidyl ester (CFSE; Life
1057 Technologies) solution in DPBS during 20 min at 37°C with continuous agitation. Then, ice
1058 cold RPMI 1640 + 10% FBS was added and stained bacteria centrifuged at 3000 g during 10
1059 min at RT. Supernatant was discarded and CFSE-labelled *L. pneumophila* was washed three
1060 times with ice cold DPBS and finally resuspended in FBS-free RPMI 1640 prior to infection
1061 of hMDMs.

1062

1063 **Annexin-V detection**

1064 hMDMs were plated in 24 well multi-dish Nunc UpCell Surface cell culture plates (Thermo
1065 Fisher) at a density of 2×10^5 cells per well and infected with *L. pneumophila* as described
1066 above. At 6 h post infection, UpCell plates were placed at 20°C during 10 minutes and
1067 hMDMs were gently detached, collected in microcentrifuge tubes and stained with Annexin
1068 V-Alexa Fluor 488 (Life Technologies) following the manufacturer's protocol. Samples were
1069 run on a MACSQuant-VIB cytometer (Miltenyi Biotec) and the obtained data was analyzed
1070 on FlowJo v 8.8.7 (Tree Star). hMDMs treated during 6 h with 5% Dimethylsulfoxyde
1071 (DMSO; Euromedex) served as positive control for early apoptosis.

1072

1073 **Caspase-1 and polycaspases FLICA assays and analyses**

1074 hMDMs were infected in 384-well plates, as described above, with GFP- or DsRed-
1075 expressing *L. pneumophila* for SR-VAD-FMK (polycaspases; Life Technologies) or FAM-
1076 YVAD-FMK (Caspase-1; Immunochemisitry Technologies) FLICA assays, respectively. At 6
1077 h post infection, FLICA reagent was added to the cells and incubated during 1 h at 37°C/5%
1078 CO₂. Then, hMDMs were washed once with DPBS, fixed with 4% PFA during 15 min at RT
1079 and stained with DAPI (Life Technologies). Image acquisitions of multiple fields per well
1080 were performed on an automated confocal microscope (OPERA QEHS, Perkin Elmer) using
1081 40X objective, excitation lasers at 405, 488, 561 and 640 nm, and emission filters at 450, 540,
1082 600 and 690 nm, respectively, and images were transferred to Columbus Image Data Storage
1083 and Analysis System (Perkin Elmer).

1084

1085 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1086 **Statistical analyses**

1087 The two-sample Student's t-test (Mann-Whitney test, not assumption of Gaussian
1088 distributions) was used in all data sets unless the contrary is specified in the Methods Details
1089 section. Data analysis was performed using Prism v5 (Graphpad Software). The exact value

1090 of “n” is specified in the figure legends and represents the number of independent
1091 experiments performed except for those panels showing single cell results (1F, 2G and 3G),
1092 where “n” represents the number of analysed cells per condition.

1093

1094 **Automatic High-Content Analyses**

1095 All the analyses were performed on Columbus Image Data Storage and Analysis System
1096 (Perkin Elmer) using in-house developed scripts (shared upon request). For the HCA of the
1097 subcellular localization of mitochondria, the Hoechst signal was used to segment nuclei in the
1098 405/450 channel (excitation/emission), MitoTracker low signal in the 561/600 channel was
1099 used to segment the cytoplasm region and, after filtering the 561/600 channel using the
1100 sliding parabola method for background removal, the high intensity Mitotracker signal was
1101 used to segment mitochondria. *L. pneumophila* was identified by measuring the GFP signal in
1102 the 488/540 channel and a virtual vacuole was established at 1 μm distance around each
1103 bacterium identified. Finally, the single cell analysis of LCV-mitochondria contacts was
1104 performed by quantifying the number of identified mitochondria inside this virtual vacuole
1105 (see also Figure 1).

1106 For the HCA of the mitochondrial morphology, the Hoechst signal was used to
1107 segment nuclei in the 405/450 channel, while the MitoTracker signal in the 561/600 channel
1108 was used to segment the cytoplasmic region. *L. pneumophila* was identified by measuring the
1109 GFP signal in the 488/540 channel. Two populations - the infected cells and the non-infected
1110 bystander cells - were defined by max GFP fluorescent intensity of each cell. The single cell
1111 analysis of the mitochondrial morphology was performed by applying the SER texture
1112 algorithm to define the cytoplasm region (mainly SER Edge and SER Ridge, normalization
1113 by regional intensity) in the 561/600 channel (filtered by sliding parabola method,
1114 background removal). For the *classical* mitochondrial morphological analyses, mitochondria
1115 were segmented using the 561/600 channel (filtered by the sliding parabola method,
1116 background removal) and morphological properties of segmented mitochondria were
1117 measured. For the HCA of the mitochondrial morphology in RAW 264.7 macrophages, the
1118 identification of *L. pneumophila* was performed using the DsRed signal in the 561/600
1119 channel and the single cell analysis of the mitochondrial morphology was performed by
1120 applying SER texture algorithms to define the cytoplasm region (SER Edge and SER Ridge,
1121 normalization by regional intensity) in the 640/690 channel filtered by sliding parabola
1122 method for background removal (see also Figure 2 and S3).

1123 For the HCA of DNM1L recruitment to mitochondria, the DAPI signal was used to
1124 segment nuclei in the 405/450 channel, while the Tom20 low signal in the 640/690 channel
1125 was used to segment the cytoplasm and high intensity Tom20 signal was used to segment
1126 mitochondria. *L. pneumophila* was identified by measuring the GFP signal in the 488/540
1127 channel and thereby infected and non-infected cells were defined. The single cell analysis of
1128 DNM1L puncta on mitochondria was performed by identifying DNM1L puncta using the
1129 561/600 channel. Selection of DNM1L puncta on mitochondria was performed using the
1130 640/690 channel (Tom20). Subcellular structures positive for both signals (DNM1L and
1131 Tom20) were considered DNM1L puncta on mitochondria, and the number of DNM1L
1132 puncta was measured in each infected cell (see also Figure S4).

1133 For the HCA of apoptotic nuclei, the Hoechst signal was used to segment nuclei in the
1134 405/450 channel and the identification of *L. pneumophila* was performed using the GFP
1135 signal in the 488/540 channel. Then the nuclei size and the Hoechst signal intensity was
1136 measured in each infected or non-infected cell, and small and bright nuclei were considered as
1137 apoptotic ones.

1138 For the HCA analyses of Caspase-1 or polycaspase activation, the DAPI signal was
1139 used to segment nuclei in the 405/450 channel. For the Caspase-1 activity assay, the
1140 identification of *L. pneumophila* was performed using the DsRed signal in the 561/600
1141 channel, and the FAM-YVAD-FMK mean fluorescence intensity in the 488/540 channel was
1142 measured in each infected cell. For the polycaspases activity assay, the identification of
1143 *L. pneumophila* was performed using GFP signal in the 488/540 channel, and SR-VAD-FMK
1144 mean fluorescence intensity in the 561/600 channel was measured in each infected cell.
1145 hMDMs treated during 6 h with 2 μ M Staurosporine (Sigma-Aldrich) served as positive
1146 control for caspases activation.

1147

1148 **Analyses of 3D time-lapse confocal movies**

1149 To quantify statistically the contacts between the LCV and mitochondria in 3D time lapse
1150 confocal microscopy we developed a new point-process approach that is based on the
1151 statistical analysis of the Ripley's K function. The first step of this method is the automatic
1152 detection of LCV and mitochondria with a wavelet-based method that extracts the
1153 fluorescence signal that is significantly brighter than background (Olivo-Marin, 2002). This
1154 method is implemented in the plugin *Spot Detector* in the open-source image analysis
1155 software Icy, available at <http://icy.bioimageanalysis.org/> (de Chaumont et al., 2012).

1156 The second step of this method was the statistical characterization of the distances between
 1157 detected spots. To test whether certain mitochondria are significantly closer to the LCV at
 1158 time t , meaning, that mitochondria are not just randomly distributed in the cell cytoplasm and
 1159 close to the LCV by chance, we computed the Ripley's K function at each time t between the
 1160 positions (centres of mass) of LCV ($n_1(t)$ LCV inside the cell at time t , positions $x(t)$) and
 1161 mitochondria ($n_2(t)$ detected mitochondria, positions $y(t)$) :

$$K(x, y, t)(r) = \frac{\text{Cell Volume}}{n_1 n_2} (t) \sum_{x, y} \mathbf{1} \{d(x(t), y(t)) < r\} b(x(t), y(t), r)$$

1162
 1163 For a distance parameter r , the K function counts the number of positions $y(t)$ that are at a
 1164 distance $< r$ from $x(t)$ positions. The term $b(x, y, r)$ is a boundary correction term that accounts
 1165 for the expected fewer neighbours of LCV that are close to the cell boundary. To test whether
 1166 K function is significantly high, we characterized its statistical distribution under the null
 1167 hypothesis of a random distribution of mitochondria. We have previously shown that K
 1168 function is normally distributed (Lagache et al., 2015) and that the expected mean $\mu(r)$ of K
 1169 function, when spots are randomly distributed, is the volume of the ball of radius r ($4/3 \cdot \pi \cdot r^3$).
 1170 We computed here the variance $v(r)$ of the K function and built a statistical test with

$$\max_{0 \leq r \leq r_{max}} \frac{K(x, y, r) - \mu(r)}{\sqrt{v(r)}}$$

1171
 1172 Then, we measured the percentage of mitochondria that are statistically close (coupled) to
 1173 LCV by performing a parametrical fitting of the K function. Our statistical method is
 1174 implemented and freely available in the plugin *Colocalization Studio* in Icy. The analysis of
 1175 the Mitotracker that we saw transferring from mitochondria to LCVs (Figure S2) was
 1176 performed using Volocity 3D imaging software (Perkin Elmer) by identifying
 1177 *L. pneumophila*-GFP in the 488 channel, segmenting 3D bacteria inside the infected cell and
 1178 measuring MitoTracker signal in the 561 channel inside 3D bacteria in each time point of the
 1179 time lapse.

1180

1181 **Label-free LC-MS/MS quantitative analysis**

1182 For protein identification and quantification, data analysis was performed with MaxQuant
 1183 (version 1.5.4.1) (Cox and Mann, 2008) using the Andromeda search engine with default
 1184 search settings including a false discovery rate set at 1% on both the peptide and protein level.
 1185 Spectra were searched against two reference proteome databases downloaded from Uniprot
 1186 (<http://www.uniprot.org/>). The first database contained 2,930 proteins from *Legionella*

1187 *pneumophila* strain Philadelphia 1 (taxID 272624, proteome ID UP000000609) and the
1188 second database contained 20,198 proteins from *Homo sapiens* (taxID 9606, proteome ID
1189 UP000005640). The mass tolerance for precursor and fragment ions was set to 4.5 and 20
1190 ppm, respectively, during the main search. Enzyme specificity was set as C-terminal to
1191 arginine and lysine, also allowing cleavage at proline bonds with a maximum of three missed
1192 cleavages. Variable modifications were set to oxidation of methionine residues, acetylation of
1193 protein N-termini, phosphorylation of serine, threonine and tyrosine residues and diglycine
1194 modification of lysine residues. Only proteins with at least one unique or razor peptide were
1195 retained leading to the identification of 559 proteins. Proteins were quantified by the
1196 MaxLFQ algorithm integrated in the MaxQuant software (Cox et al., 2014). A minimum ratio
1197 count of two unique or razor peptides was required for quantification. Further data analysis
1198 was performed with the Perseus software (version 1.5.2.4) after loading the protein groups
1199 file from MaxQuant. Proteins only identified by site, reverse database hits and contaminants
1200 were removed and replicate samples of both conditions were grouped. Proteins with less than
1201 three valid values in at least one group were removed and missing values were imputed from
1202 a normal distribution around the detection limit. Then, a t-test was performed for pairwise
1203 comparison of both conditions. The results of this t-test is shown by the volcano plot in Figure
1204 3H. For each protein, the $\log_2 (\Delta icmT/WT)$ fold change value is indicated on the X-axis,
1205 while the statistical significance ($-\log p$ -value) is indicated on the Y-axis. Proteins outside the
1206 curved lines, set by an FDR value of 0.05 and an S0 value of 1 in the Perseus software,
1207 represent specific DNML1 interaction partners in macrophages, binding only after infection
1208 with either the JR32-WT or the JR32- $\Delta icmT$ strain.

1209

1210 **Analyses of Flow Cytometry data**

1211 Obtained cytometry data was analysed on FlowJo v 8.8.7 (Tree Star).

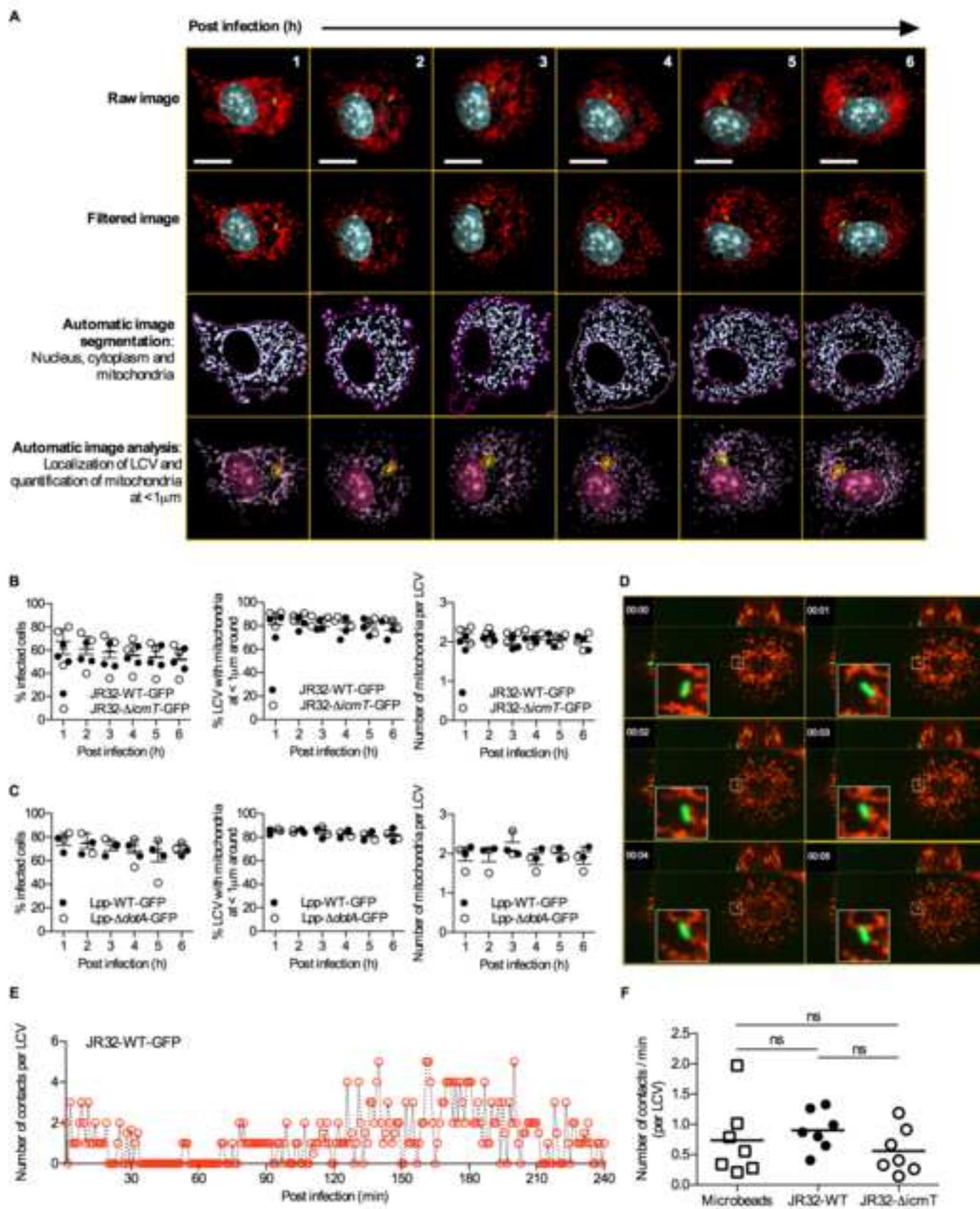
TABLE FOR AUTHOR TO COMPLETE

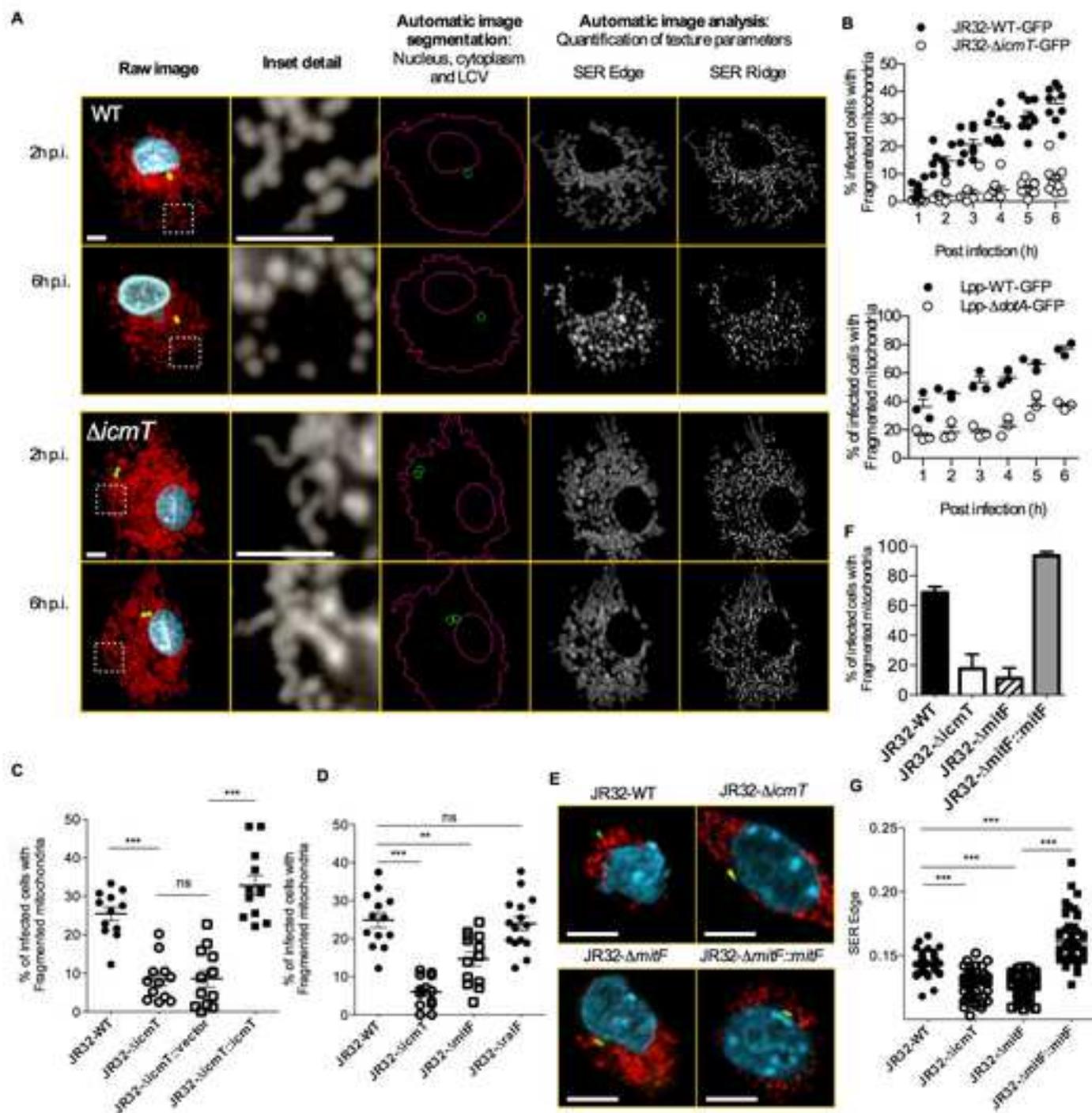
Please upload the completed table as a separate document. **Please do not add subheadings to the Key Resources Table.** If you wish to make an entry that does not fall into one of the subheadings below, please contact your handling editor. (NOTE: For authors publishing in *Current Biology*, please note that references within the KRT should be in numbered style, rather than Harvard.)

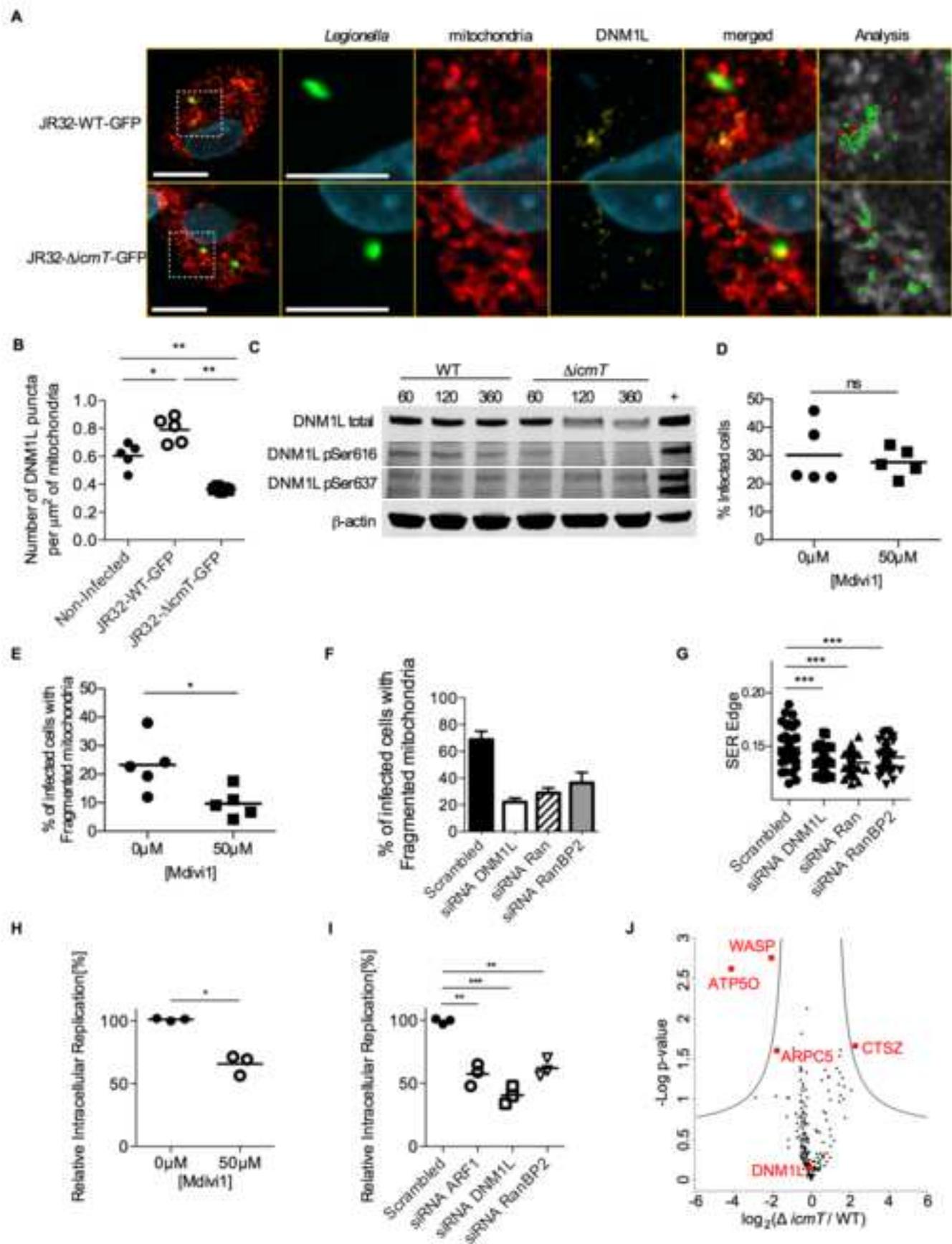
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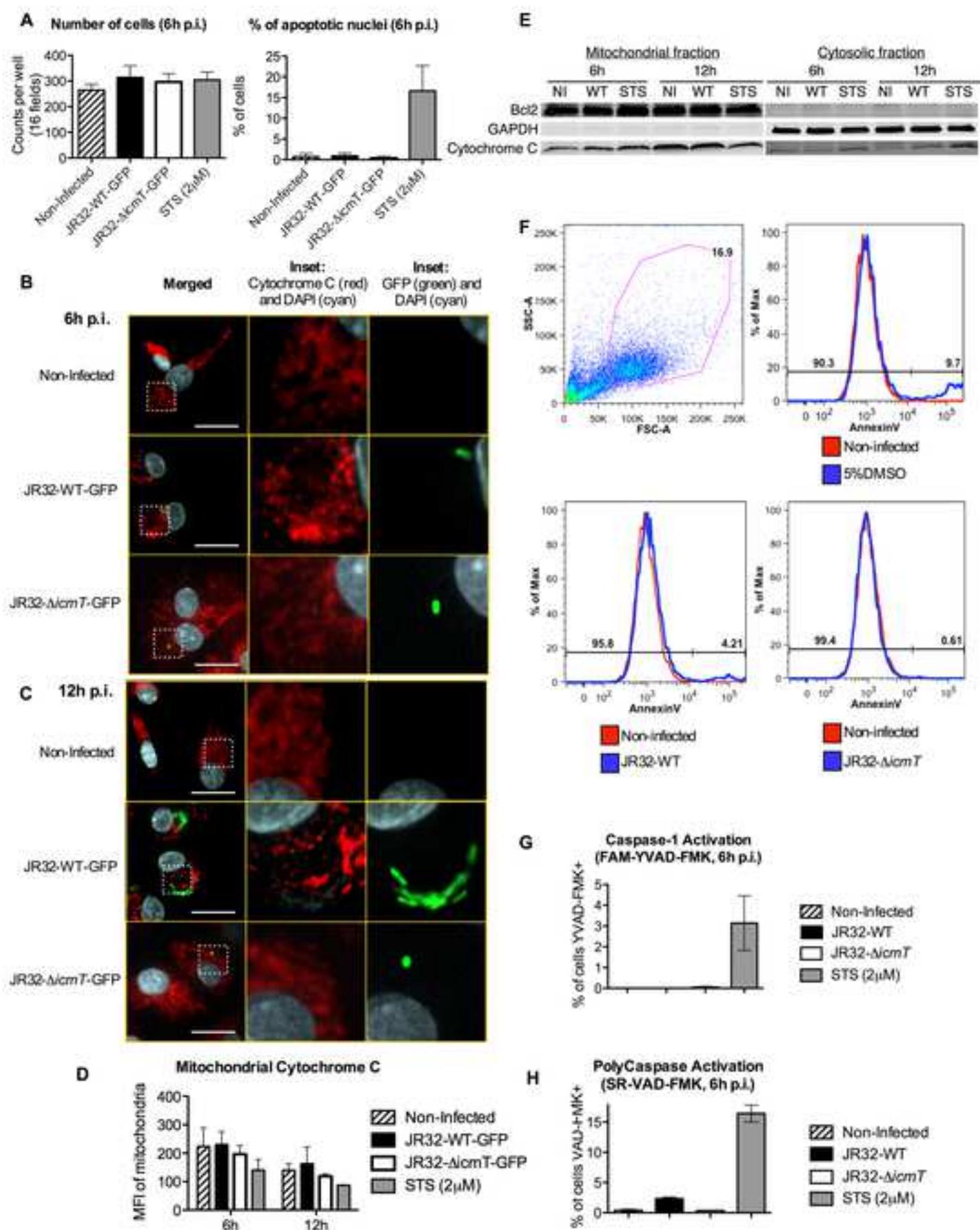
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
MicroBeads conjugated to monoclonal anti-human CD14	Miltenyi Biotec	Cat#130-050-201
Mouse monoclonal anti-Tom20	BD	Clone 29
Rabbit monoclonal DNM1L (IF)	Cell Signalling	Clone D6C7
Mouse monoclonal anti-Cytochrome C (IF)	BD	Clone 6H2.B4
Mouse monoclonal anti-DNM1L (WB)	BD	Clone 22
Mouse monoclonal anti-Phospho-DNM1L Ser616	Cell Signalling	Clone D9A1
Rabbit polyclonal Phospho-DNM1L Ser637	Sigma Aldrich	Cat# SAB4301399
Rabbit polyclonal β -Actin	Sigma Aldrich	Cat# A2066
Mouse monoclonal anti-Bcl2	Millipore	MIT1000 kit
Mouse monoclonal anti-GAPDH	Millipore	MIT1000 kit
Mouse monoclonal anti-Cytochrome C (WB)	BD, kindly supplied by F. Stavru (I. Pasteur)	Clone 7H8.2C12
Goat polyclonal anti-ARF1	Abcam	Cat# ab58578
Mouse monoclonal anti-DNM1L (WB, Figure S7)	Abcam	Cat# ab56788
Rabbit polyclonal anti-RanBP2	Abcam	Cat# ab64276
Rabbit monoclonal anti-GAPDH	Cell Signalling	Clone 14C10
Bacterial and Virus Strains		
JR32-WT, Virulent <i>L. pneumophila</i> serogroup 1 strain Philadelphia	Sadosky A.B., <i>et al.</i> , 1993	N/A
JR32- Δ <i>icmT</i> , <i>L. pneumophila</i> JR32 <i>icmT3011::Kan^R</i>	Segal G. and Shuman H.A., 1998	N/A
JR32- Δ <i>mitF</i> , JR32 <i>legG1::Kan^R</i>	Rothmeier E., <i>et al.</i> , 2013	N/A
JR32- Δ <i>mitF::mitF</i> , JR32 <i>legG1-M45-LegG1::Kan^R</i>	Rothmeier E., <i>et al.</i> , 2013	N/A
Lpp-WT, Virulent <i>L. pneumophila</i> serogroup 1 strain Paris	Cazalet C., <i>et al.</i> , 2004	N/A
Lpp- Δ <i>dotA</i> , <i>L. pneumophila</i> Paris <i>dotA::Kan^R</i>	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
Recombinant human macrophage colony-stimulating factor (rhMCSF)	R&D Systems	Cat# 216-MC-025
Mdivi1	Tocris Bioscience	Cat# 3982
2-DG	Seahorse Bioscience	Cat# 103020-100
Oligomycin	Enzo	Cat# ALX-380-037
Carboxyfluorescein succinimidyl ester (CFSE)	Life Technologies	Cat# C34554
Critical Commercial Assays		
Mitochondrial/Cytosol Fractionation kit	Millipore	MIT1000
Caspase-1 FLICA assay	Immunochemistry Technologies	Cat# 98
Polycaspases FLICA assay	Life Technologies	Cat# I35101

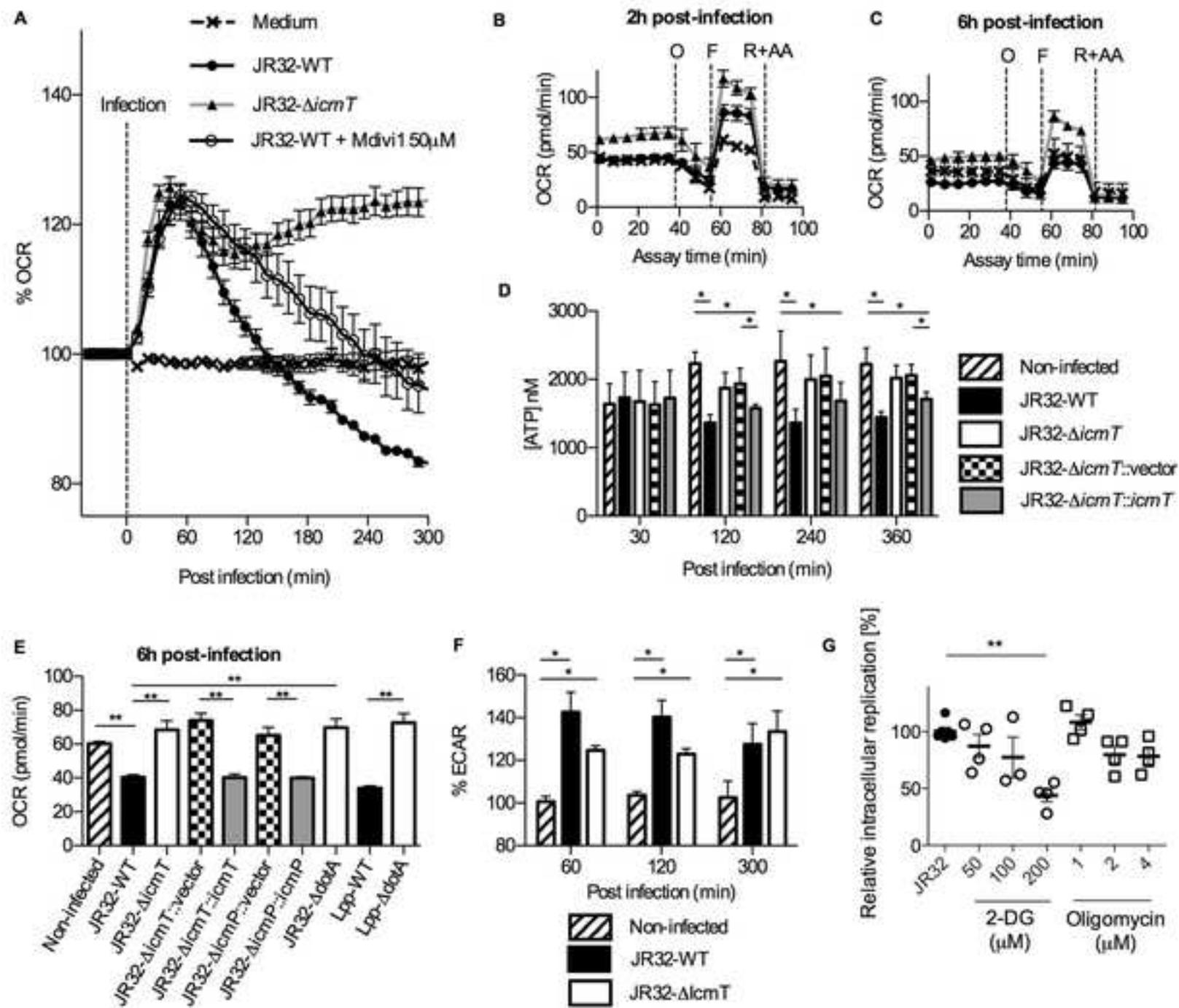
Experimental Models: Cell Lines		
Murine macrophage-like RAW 264.7 cells	ATCC	RRID: CVCL_0493
Human A549 lung epithelial carcinoma cells	ATCC	RRID: CVCL_0023
Biological samples		
Human blood from healthy donors	EFS	N/A
Recombinant DNA		
pCR77, pMMB207C-P _{tac} -RBS- <i>dsred</i> -RBS-MCS	Finsel I., <i>et al.</i> 2013	N/A
pNT28, pMMB207-C, Δ <i>lacIq</i> (constitutive <i>gfp</i> , -GFP)	Tiaden A., <i>et al.</i> 2007	N/A
pER5, pCR77-M45- <i>legG1</i>	Rothmeier E., <i>et al.</i> , 2013	N/A
Oligonucleotides		
<i>See Table S1</i>		
Software and Algorithms		
Columbus Image Data Storage and Analysis System	Perkin Elmer	Part # Columbus
In-house developed HCA scripts for Columbus	This paper	Shared upon request
ICY, open-source image analysis software	http://icy.bioimageanalysis.org/	v. 1.8.6.0
Other		
Nunc UpCell Surface cell culture plates (6W)	Thermo Fisher	Cat# 174901
Nunc UpCell Surface cell culture plates (24W)	Thermo Fisher	Cat# 174899

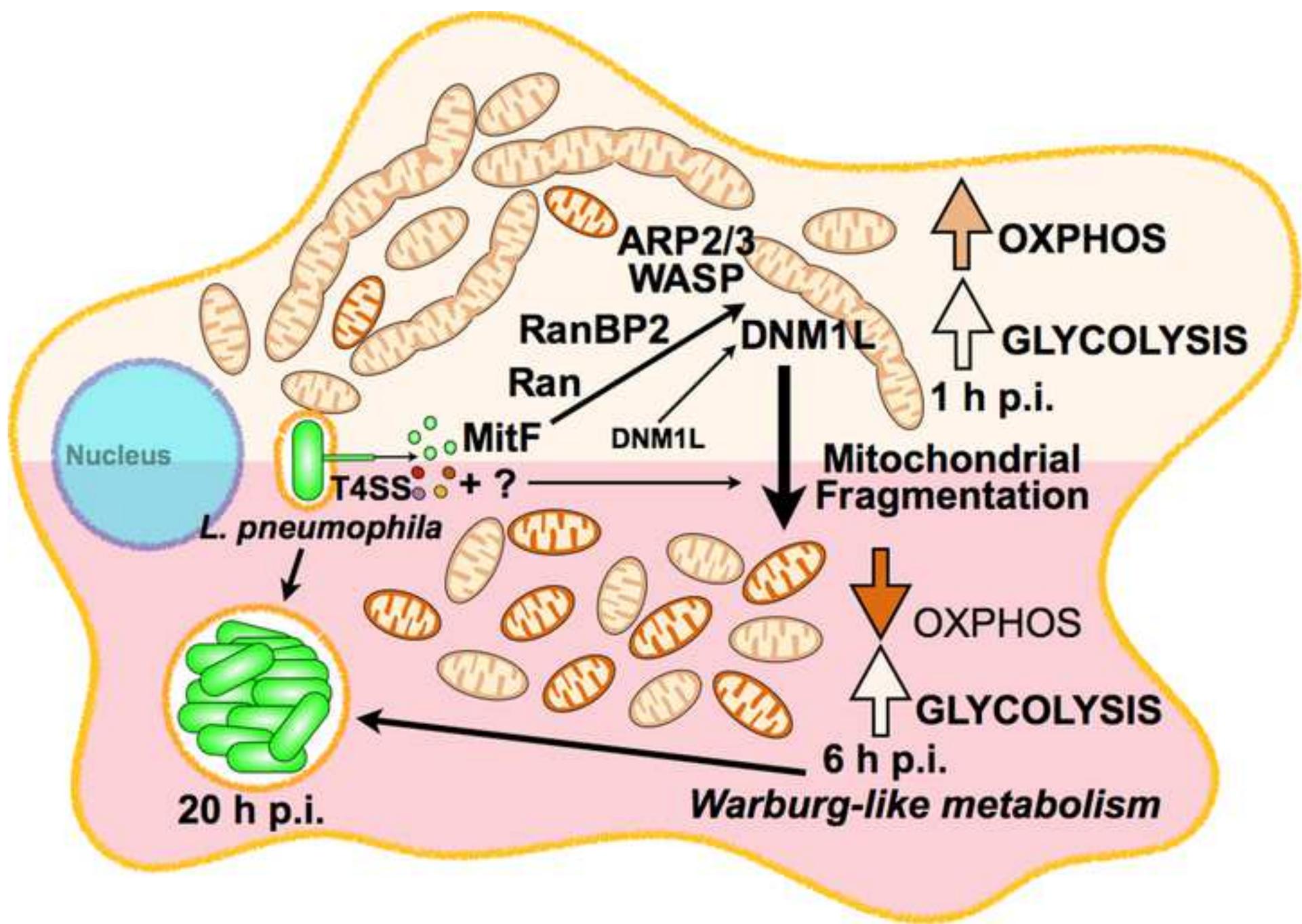












Supplementary Information

***Legionella pneumophila* modulates mitochondrial dynamics to trigger metabolic repurposing of infected macrophages**

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Figure S1: Related to Figure 1. Quantification of *Legionella*-mitochondria contacts in hMDM cells during infection.

Figure S2: Related to Figure 1. Transfer of Mitotracker dye from mitochondria to the LCV occurred occasionally during the dynamic contacts.

Figure S3: Related to Figure 2. Mitochondrial morphology parameters change during infection but not the total mitochondrial mass

Figure S4. Related to Figure 3. DNMI1L is implicated in mitochondrial changes due to *L. pneumophila* infection in different cell types.

Figure S5. Related to Figure 3 and 5. Mdivi-1, 2-DG and Olygomycin pre-treatment has no effect on axenic growth of *L. pneumophila*.

Figure S6. Related to Figure 5. Bioenergetic profiles of the key parameters of mitochondrial respiration change due to *L. pneumophila* infection.

Table S1: Oligonucleotides used for RNA interference.

Movie S1: Related to Figure 1. 3D confocal time-lapse movie of a JR32-WT-GFP-infected hMDM cell.

Movie S2: Related to Figure 1. 3D confocal time-lapse movie of a JR32- Δ *icmT*-GFP-infected hMDM cell.

Movie S3: Related to Figure 1. Reconstruction of 3D confocal z-stack time-lapse movie of a JR32-WT-GFP-infected hMDM cell.

Movie S4: Related to Figure 1. 3D confocal time-lapse movie of a microbeads-“infected” hMDM cell.

Movie S5: Related to Figure 1. Transfer of Mitotracker dye from mitochondria to the LCV occurred occasionally during the dynamic contacts.

Figure Legends:

Figure S1. Related to Figure 1. **Quantification of *Legionella*-mitochondria contacts in hMDM cells during infection.** Number of contacts per LCV during a time course are shown (A) JR32-WT-GFP and (B) JR32- $\Delta icmT$ -GFP. The statistical method used is described in the STAR methods. See also Movies S1 and S2.

Figure S2. Related to Figure 1. **Transfer of Mitotracker dye from mitochondria to the LCV occurred occasionally during the dynamic contacts.** (A) 3D confocal time-lapse images of a JR32-WT-GFP-infected hMDM previously labelled with MitoTracker (Movie S3). Mitochondria (red) and *Legionella* (green) are shown. For the analysis, 3D intracellular bacteria were identified in the 488 channel, labelled as JR32-WT-1, -2 and -3 in the figure. At 4 min p.i., JR32-WT-2 contacted mitochondria (white arrow head). At 29 min p.i. JR32-WT-2 have intensively acquired MitoTracker dye (white asterisk), in contrast to the other two LCVs within the hMDM. Scale bar: 2.40 μm (B) Quantification of the mean fluorescence intensity (MFI) inside 3D bacteria in the 488 and 561 channels. Arrowhead indicates the contact of JR32-WT-2 with mitochondria.

Figure S3. Related to Figure 2. **Mitochondrial morphology parameters change during infection but not the total mitochondrial mass.** (A) HCA Analysis of hMDMs at 2 h (left) or 6h p.i (right) is shown for the same individual cells. Application of texture algorithms to mitochondrial signal converts texture into values, which normally change during changes in mitochondrial morphology. (B) To correlate SER values to specific mitochondrial morphologies, 10 cells are selected in each experiment with clear non-fragmented or fragmented morphologies and SER values are plotted. The cut-off of the fragmented phenotype is set just above the mean of the SER value of the fragmented phenotype. This should be done for each individual experiment, as small differences in parameters such as mean mitochondrial intensity, microscope or magnification used, as well as hMDM from different donors alter SER values between different experiments. (C) Plots of SER values of hMDMs infected with *L. pneumophila* JR32-WT-GFP or JR32- $\Delta icmT$ -GFP in the same experiment as in B. In this experiment, both SER-Edge and SER-Ridge are used to distinguish changes in mitochondrial morphology. (D) Classical morphology measurements using a mitochondrial segmentation strategy in the same experiment as in B. Instead of analysing texture of mitochondrial channel, the channel was used to segment individual mitochondria and different morphological measurements were performed. (E) Mitochondrial mass during *L. pneumophila* infection. hMDMs were infected with *L. pneumophila* JR32-WT, JR32- $\Delta icmT$

mutant or left uninfected. At 6h, cells were stained with the membrane-potential independent dye Mitotracker Green (MTG), detached from the plates and analysed by Flow Cytometry. Mean Fluorescence Intensity (MFI) of MTG is shown. *p-value \leq 0.05; **p-value \leq 0.01; ***p-value \leq 0.001; ns = non-significant (Mann-Whitney t-test).

Figure S4. Related to Figure 3. **DNM1L is implicated in mitochondrial changes due to *L. pneumophila* infection in different cell types.** **(A)** High Content Analysis of DNM1L association with host mitochondria during infection of hMDMs with *L. pneumophila*. The steps of the analysis are depicted. 1: Raw image. 2: Segmentation of nuclei and cytoplasm using DAPI channel (405). 3a: GFP channel (488), localization of LCV. 3b: Mitotracker channel (633), localization of mitochondria. 3c: DNM1L channel (565), localization of DNM1L puncta. Combination of 3a and 3c renders 4: Identification of DNM1L puncta in infected cells. 5: Identification of DNM1L puncta in the cytoplasm (red puncta) and DNM1L puncta co-localizing with mitochondria (green puncta). Quantification of whole cell mitochondria area from step 3b and number of mitochondria-associated DNM1L puncta provide numbers of DNM1L puncta per μm^2 of host mitochondria. **(B)** *L. pneumophila* induces T4SS-dependent changes in mitochondrial morphology during infection of A549 cells. A549 cells were stained with Mitotracker (red), infected with GFP-expressing *L. pneumophila* WT or ΔicmT mutant (green) and their nuclei stained with Hoechst dye (cyan). Living cells were imaged by confocal microscopy each hour and analysed by HCA. **(C)** HCA analysis of mitochondrial morphology during JR32-WT-GFP or JR32- ΔicmT -GFP infection of A549 cells. Percentage of infected cells with fragmented mitochondria is shown (n = 3). **(D)** Related to Figure 3H. hMDMs were pre-treated or not with Mdivi1 (50 or 100 μM) during 4h, washed and infected with *L. pneumophila* JR32-WT or JR32- ΔicmT . At 24h p.i., cells were lysed with 50 μl of sterile distilled water and plated in BCYE plates. After 3 days of incubation at 37°C, colony-forming units (CFU) were counted. Results are expressed as CFU per ml of lysis volume (n=3). **(E)** Western blot analysis of target protein depletion in A549 cells. Human A549 cells were treated with 2 or 4 different siRNA oligonucleotides (Oligo 1-4) against DNM1L, RanBP2 or Arf1 for 48 h. The depletion efficiency was assessed by Western blot using specific antibodies against indicated proteins. GAPDH served as a loading control. Qiagen AllStars oligonucleotides were used as a negative control (scrambled). **(F)** Western blot analysis of target protein depletion in RAW264.7 macrophages. Murine RAW264.7 macrophages were treated with a mix of 4 different siRNA oligonucleotides against DNM1L, Ran, or RanBP2 for 24h. The depletion efficiency was

assessed by Western blot using specific antibodies against indicated proteins. GAPDH served as a loading control. Qiagen AllStars oligonucleotides were used as a negative control (scrambled). **(G)** Cytotoxicity of siRNA treatment. Human A549 epithelial cells were treated with 4 different siRNA oligonucleotides (oligo 1-4) against DNMT1 or RanBP2 for 48h. Cells treated with scrambled siRNA served as negative control, silencing of Arf1 served as positive control. 70% EtOH was used as control for cell death. Qiagen AllStars oligonucleotides (scrambled) were used as baseline for the experiment. Data represent the means and SEM of two independent experiments. PI: propidium iodide.

Figure S5. Related to Figure 3 and 5. **Mdivi-1, 2-DG and Olygomycin pre-treatment has no effect on axenic growth of *L. pneumophila*.** hMDMs were not pretreated (control, A) or pre-treated during 4h with Mdivi-1 (B), 2-DG (C) or Olygomycin (D). After 4h pretreatment, hMDMs were washed, in order to use the same protocol as for the results depicted in Figure 3 and 5. After 4h pretreatment and washing, hMDMs were lysed with 50 μ l of distilled water. Then, *L. pneumophila* WT was added to the lysate in a minimal amount of growth medium (BYE, 5 μ l) and the OD₆₀₀ was automatically recorded during bacterial axenic growth (continuously shaking at 37°C). Panels A, B, C and D show OD₆₀₀ over time of 12 replicates from 2 independent experiments. Panel E shows the comparison of the different conditions by showing the mean of all the replicates of each condition.

Figure S6. Related to Figure 5. **Bioenergetic profiles of the key parameters of mitochondrial respiration change due to *L. pneumophila* infection** **(A)** Bioenergetic profiles of the key parameters of mitochondrial respiration during mitochondrial respiratory control assay using the Seahorse XF Mitostress kit. Sequential compound injections measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration (Source: Seahorse Bioscience). **(B)** Contribution of *L. pneumophila* to oxygen consumption rate (OCR) measurements during infection is minimal. OCR of *L. pneumophila* WT alone (quantity for MOI=10), hMDMs alone or WT-infected hMDMs (4h p.i.) was measured on a Seahorse XFe96 Analyser. Results show 3 independent experiments, each one having 4 replicates. Note the break in the Y axis. The table shows mean and standard deviation of the 3 experiments. **(C)** Contribution of *L. pneumophila* to ATP measurements during infection is minimal. ATP of *L. pneumophila* WT alone (quantity for MOI=10) or hMDMs alone was measured. Results show 2 independent

experiments, each one having 3 replicates. Note the break in the Y axis. The table shows mean and standard deviation of the 2 experiments.

Movie legends:

Movie S1: Related to Figure 1. 3D confocal time-lapse movie of a JR32-WT-GFP-infected hMDM cell. The movie shows the highly dynamic *Legionella*-mitochondria contacts during 24 minutes. Details shown on the left.

Movie S2: Related to Figure 1. 3D confocal time-lapse movie of a JR32- $\Delta icmT$ -GFP-infected hMDM cells. The movie shows highly dynamic *Legionella*-mitochondria contacts during 24 minutes. Details shown on the left.

Movie S3: Related to Figure 1. Reconstruction of 3D confocal z-stack time-lapse movie of a JR32-WT-GFP-infected hMDM cells. The movie shows highly dynamic *Legionella*-mitochondria contacts during 197 minutes. The movie is repeated 3 times. Non-reconstructed time-lapse movie is shown in the inset.

Movie S4: Related to Figure 1. 3D confocal time-lapse movie of a microbeads "infected" hMDM cell. The movie shows the highly dynamic *microbeads*-mitochondria contacts during 24 minutes.

Movie S5: Related to Figure 1. 3D confocal time-lapse movie of a JR32-WT-GFP-infected hMDM cell. The movie shows the transfer of Mitotracker dye from mitochondria to the LCV during 29 min of infection. The movie is repeated 3 times. The arrow indicates the increment of MitoTracker dye in a LCV after the contact with mitochondria

Table S1. Oligonucleotides used for RNA interference

NCBI gene	Gene description	Entrez Gene ID	Product name	Product ID
Unspecific_AllStars_1	AllStars (<i>Scrambled</i>)	0	Unspecific_AllStars_1	SI03650318
ARF1	ADP-ribosylation factor 1	375	Hs_ARF1_1 (1)	SI00299250
			Hs_ARF1_10 (2)	SI02757272
RanBP2	Ran binding protein 2	5903	Hs_RANBP2_6 (1)	SI03117310
			Hs_RANBP2_5 (2)	SI03043901
			Hs_RANBP2_4 (3)	SI00698264
			Hs_RANBP2_2 (4)	SI00698250
DNM1L	Dynamin 1-like	10059	Hs_DNM1L_10 (1)	SI04320092
			Hs_DNM1L_9 (2)	SI04274235
			Hs_DNM1L_8 (3)	SI04202464
			Hs_DNM1L_4 (4)	SI02661365
Arf1	ADP-ribosylation factor 1	11840	Mm_ARF1_4 (1)	SI00901656
RanBP2	Ran binding protein 2	19386	Mm_Ranbp2_1 (1)	SI01396703
			Mm_Ranbp2_2 (2)	SI01396710
			Mm_Ranbp2_3 (3)	SI01396717
			Mm_Ranbp2_4 (4)	SI01396724
DNM1L	Dynamin 1-like	74006	Mm_Dnm1l_1 (1)	SI00982219
			Mm_Dnm1l_2 (2)	SI00982226
			Mm_Dnm1l_3 (3)	SI00982233
			Mm_Dnm1l_4 (4)	SI00982240
Ran	RAN, member RAS oncogene Family	19384	Mm_Ran_1 (1)	SI00184366
			Mm_Ran_3 (2)	SI00184380
			Mm_Ran_4 (3)	SI00184387
			Mm_Ran_7 (4)	SI0271092

Figure S1

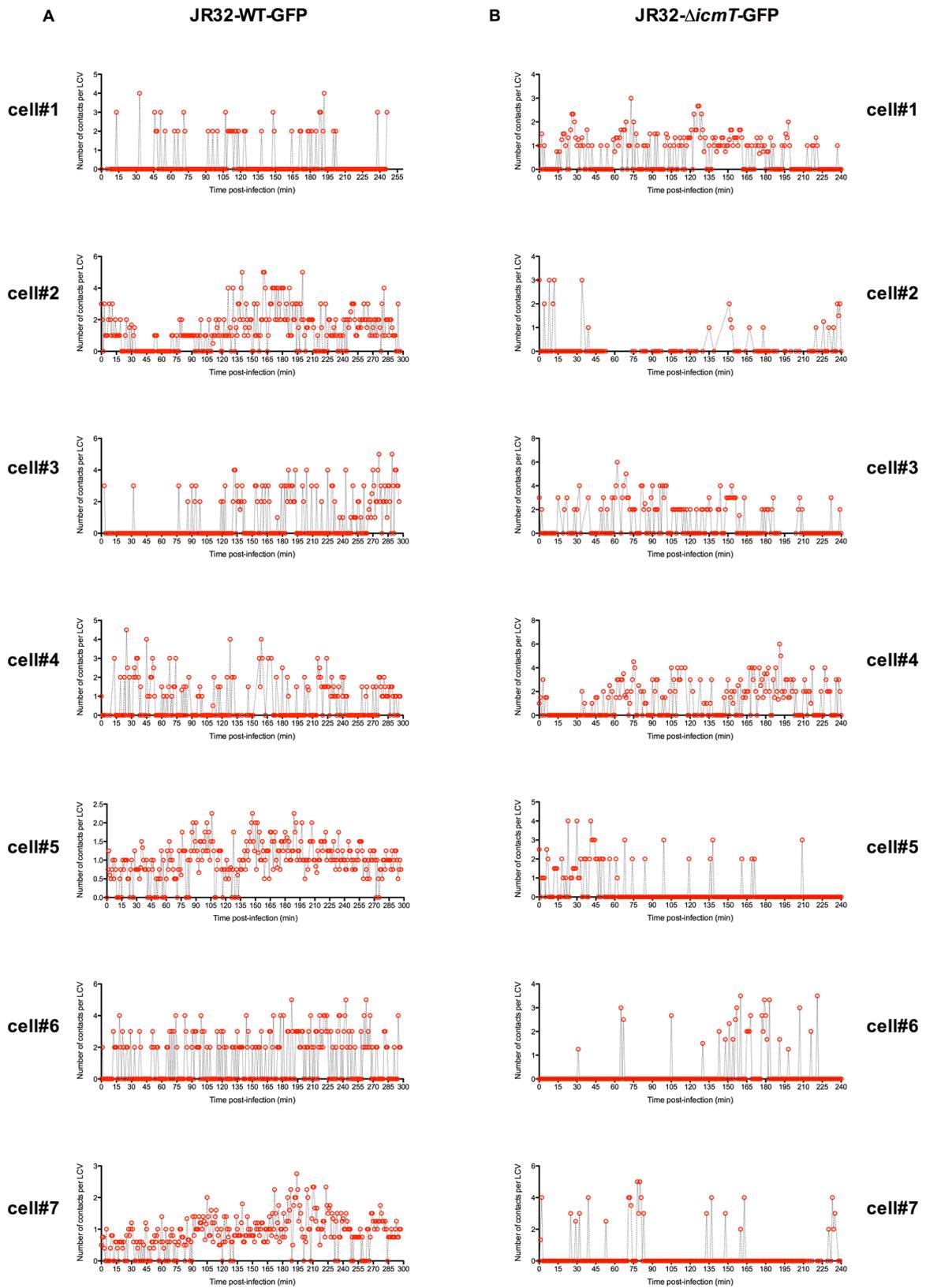


Figure S1. Related to Figure 1.

Figure S2

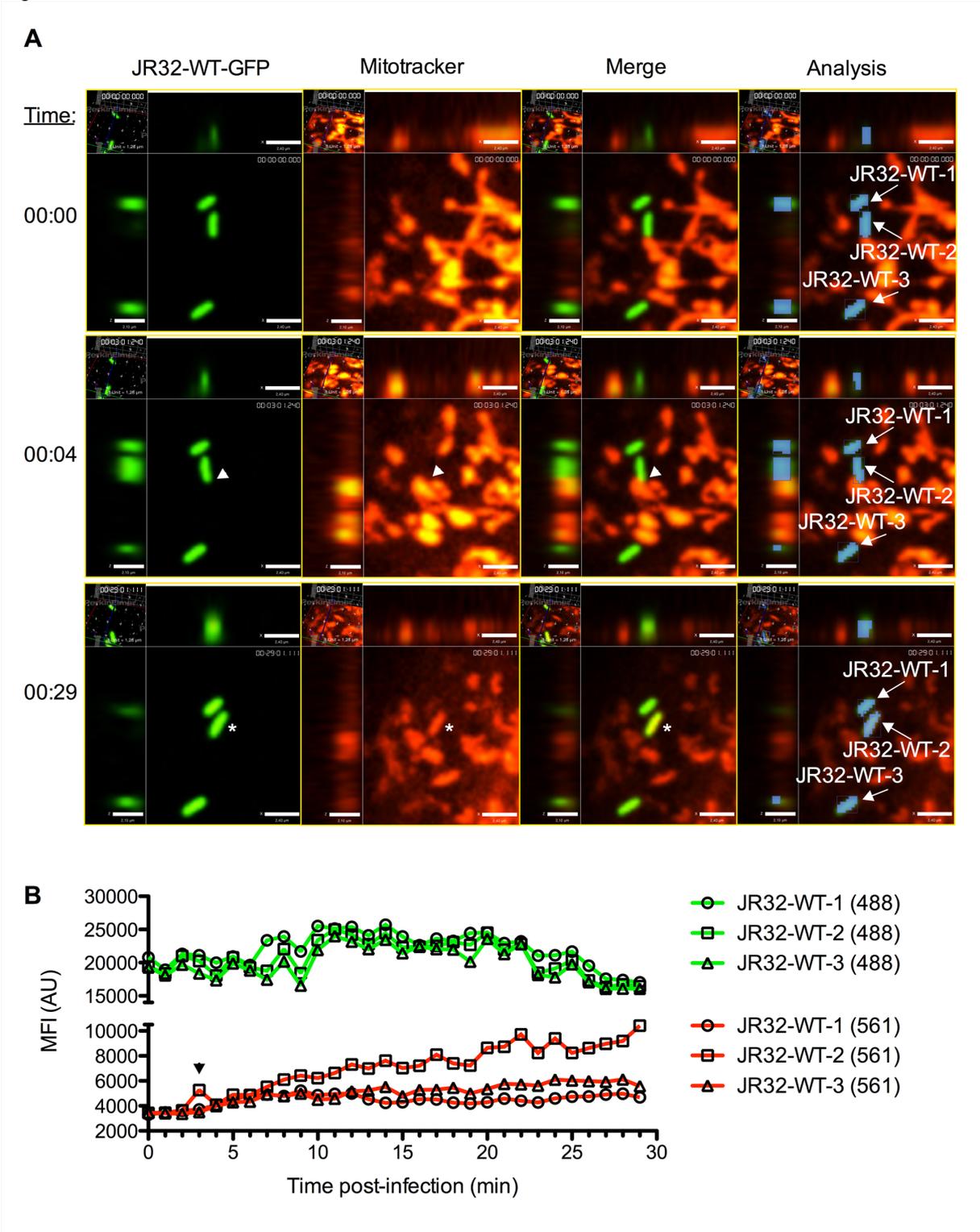


Figure S3

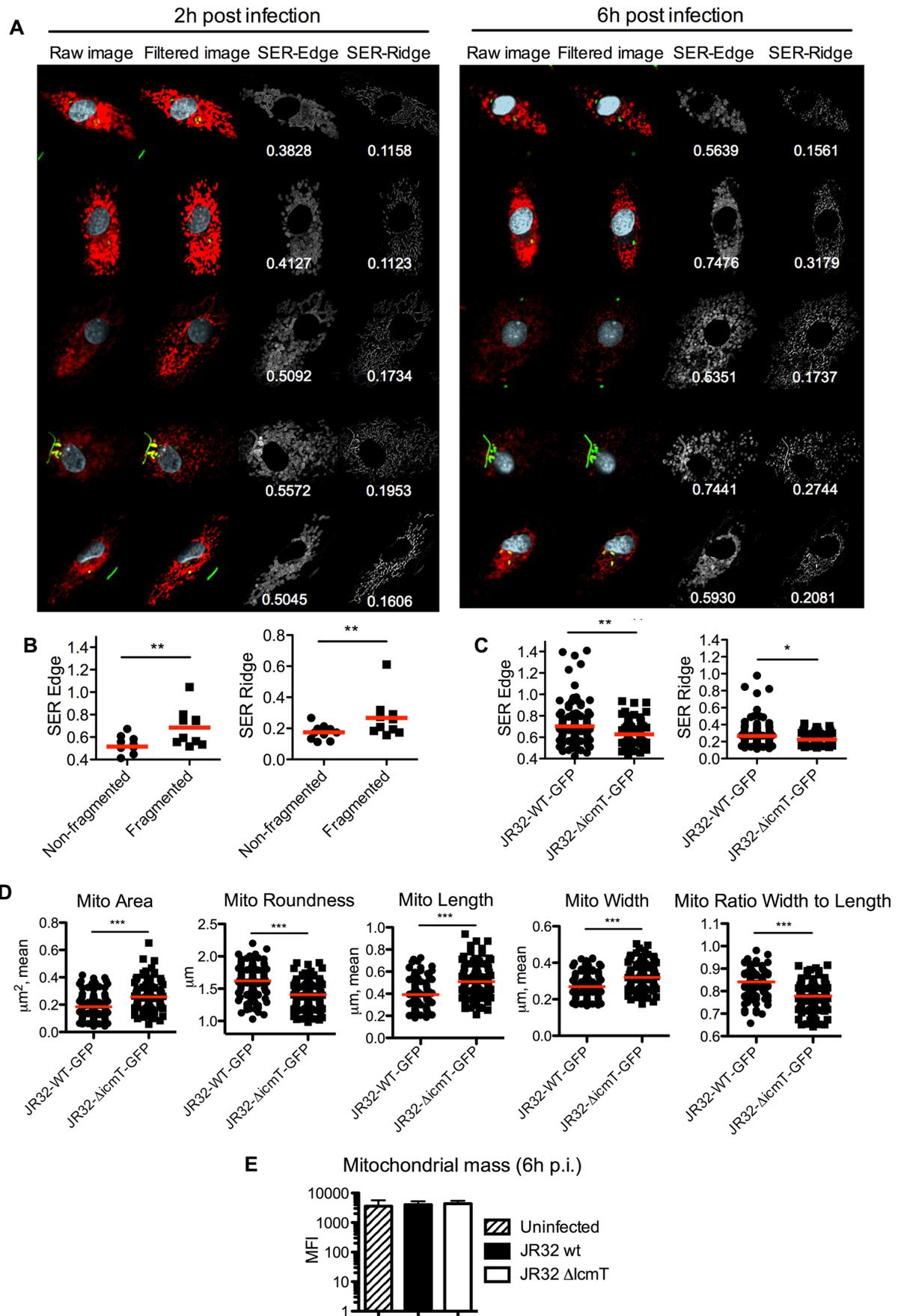


Figure S4

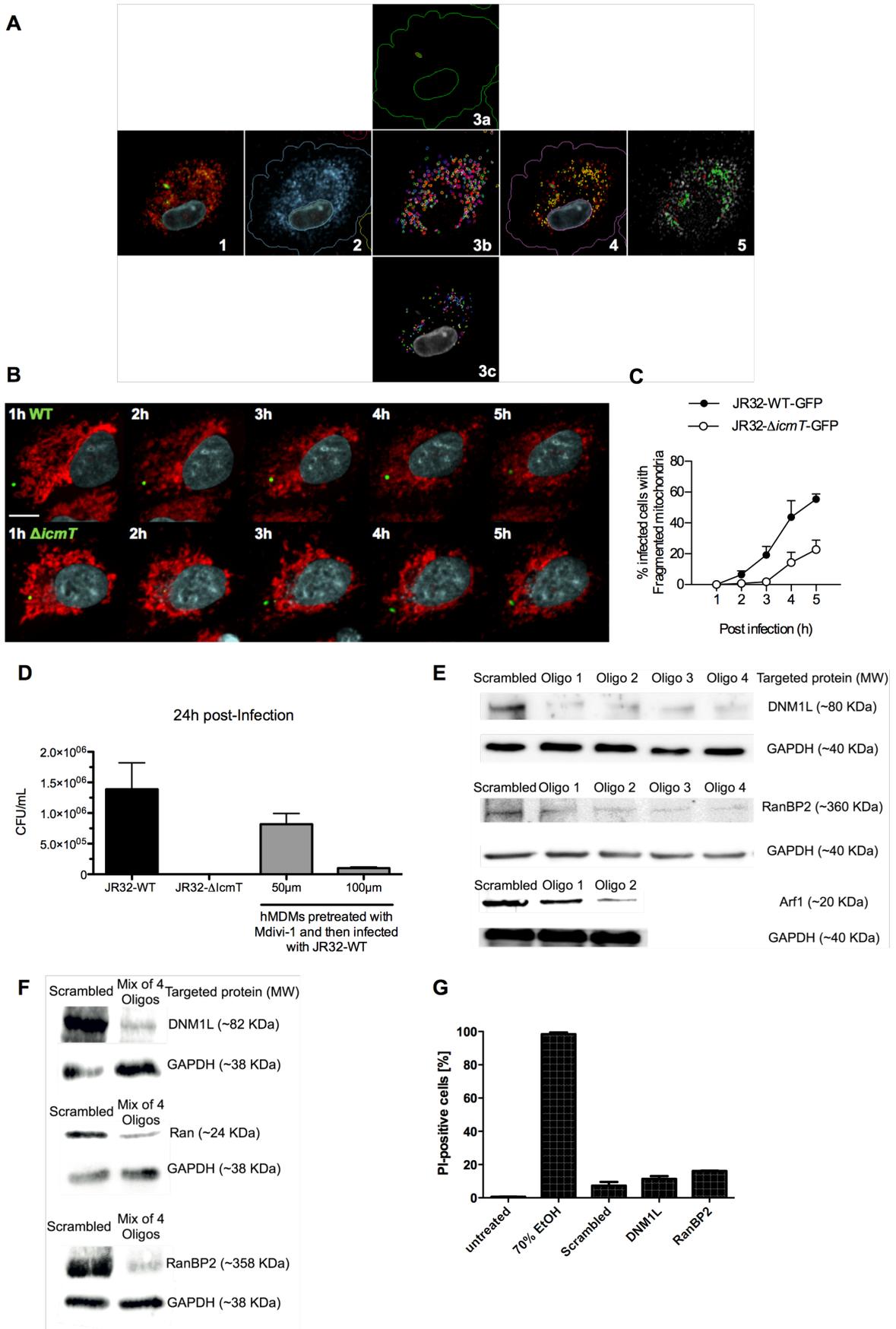


Figure S5

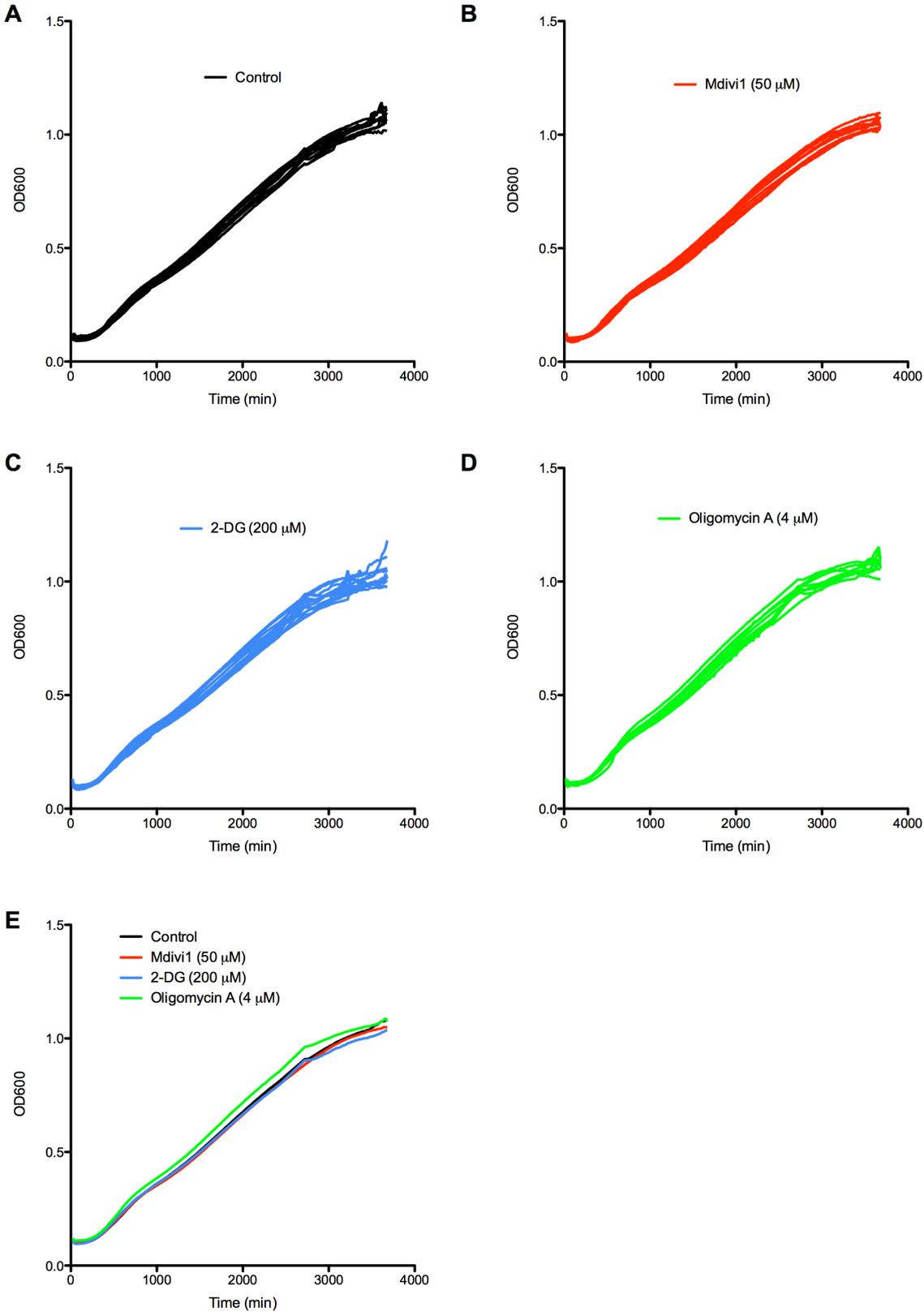


Figure S6

