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1           **Cysteine Peptidase B Regulates *Leishmania mexicana* Virulence**  
2           **through the Modulation of GP63 Expression**

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## ABSTRACT

26  
27  
28 Cysteine peptidases play a central role in the biology of *Leishmania*. In this work, we sought to  
29 further elucidate the mechanism(s) by which the cysteine peptidase CPB contributes to *L.*  
30 *mexicana* virulence and whether CPB participates in the formation of large communal  
31 parasitophorous vacuoles induced by these parasites. We initially examined the impact of *L.*  
32 *mexicana* infection on the trafficking of VAMP3 and VAMP8, two endocytic SNARE proteins  
33 associated with phagolysosome biogenesis and function. Using a CPB-deficient mutant, we  
34 found that both VAMP3 and VAMP8 were down-modulated in a CPB-dependent manner. We  
35 also discovered that expression of the virulence-associated GPI-anchored metalloprotease GP63  
36 was inhibited in the absence of CPB. Expression of GP63 in the CPB-deficient mutant was  
37 sufficient to down-modulate VAMP3 and VAMP8. Similarly, episomal expression of GP63  
38 enabled the CPB-deficient mutant to establish infection in macrophages, induce the formation of  
39 large communal parasitophorous vacuoles, and cause lesions in mice. These findings implicate  
40 CPB in the regulation of GP63 expression and provide evidence that both GP63 and CPB are key  
41 virulence factors in *L. mexicana*.

42

43

## AUTHOR SUMMARY

44  
45  
46 The parasite *Leishmania mexicana* expresses several cysteine peptidases of the papain family that  
47 are involved in processes such as virulence and evasion of host immune responses. The cysteine  
48 peptidase CPB is required for survival within macrophages and for lesion formation in  
49 susceptible mice. Upon their internalization by macrophages, parasites of the *L. mexicana*  
50 complex induce the formation of large communal parasitophorous vacuoles in which they  
51 replicate, and expansion of those large vacuoles correlates with the ability of the parasites to  
52 survive inside macrophages. Here, we found that CPB contributes to *L. mexicana* virulence  
53 (macrophage survival, formation and expansion of communal parasitophorous vacuoles, lesion  
54 formation in mice) through the regulation of the virulence factor GP63, a *Leishmania* zinc-  
55 metalloprotease that acts by cleaving key host cell proteins. This work thus elucidates a novel  
56 *Leishmania* virulence regulatory mechanism whereby CPB controls the expression of GP63.

57

58

59

## INTRODUCTION

60  
61  
62 The protozoan *Leishmania* parasitizes macrophages and causes a spectrum of human  
63 diseases ranging from self-healing cutaneous lesions to a progressive visceral infection that can  
64 be fatal if left untreated. Infection is initiated when promastigote forms of the parasite are  
65 inoculated into the mammalian host by infected sand flies and are internalized by phagocytes.  
66 Inside macrophages, promastigotes differentiate into amastigotes to replicate within  
67 phagolysosomal compartments also known as parasitophorous vacuoles (PVs). Upon their  
68 internalization, *L. donovani* and *L. major* promastigotes arrest phagolysosomal biogenesis and  
69 create an intracellular niche favorable to the establishment of infection and to the evasion of the  
70 immune system [1, 2]. Disruption of the macrophage membrane fusion machinery through the  
71 action of virulence factors plays an critical role in this PV remodeling. Hence, insertion of the  
72 promastigote surface glycolipid lipophosphoglycan (LPG) into the PV membrane destabilizes  
73 lipid microdomains and causes exclusion of the membrane fusion regulator synaptotagmin V  
74 from the PV [2-4]. Similarly, the parasite GPI-anchored metalloprotease GP63 [5, 6]  
75 redistributes within the infected cells and cleaves key Soluble NSF Attachment Protein Receptors  
76 (SNAREs) and synaptotagmins to impair phagosome functions [1, 7].

77  
78 Whereas *L. major* and *L. donovani* multiply in tight individual PVs, parasites of the *L.*  
79 *mexicana* complex (*L. mexicana*, *L. amazonensis*) replicate within large communal PVs.  
80 Relatively little is known about the host and parasite factors involved in the biogenesis and  
81 expansion of those communal PVs. Studies with *L. amazonensis* revealed that phagosomes  
82 containing promastigotes fuse extensively with late endosomes/lysosomes within 30 minutes  
83 post-infection [8]. At that stage, parasites are located within small individual compartments and

84 by 18 to 24 hours large PVs containing several parasites are observed. The rapid increase in the  
85 size of those PVs requires extensive fusion with secondary lysosomes and correlates with the  
86 depletion of those organelles in infected cells [9-11]. Homotypic fusion between *L.*  
87 *amazonensis*-containing PVs also occurs, but its contribution to PV enlargement remains to be  
88 further investigated [12]. These studies highlighted the contribution of the host cell membrane  
89 fusion machinery in the biogenesis and expansion of large communal PVs and are consistent with  
90 a role for endocytic SNAREs in this process [13]. Interestingly, communal PVs interact with the  
91 host cell's endoplasmic reticulum (ER) and disruption of the fusion machinery associated with  
92 the ER and Golgi inhibits parasite replication and PV enlargement [14-16].

93  
94 The *Leishmania*-derived molecules involved in the expansion of the communal PVs  
95 remains to be identified. LPG and other phosphoglycans do not play a significant role in *L.*  
96 *mexicana* promastigote virulence and PV formation [17], in contrast to *L. major* and *L. donovani*  
97 [2]. Cysteine peptidases (CP) are a large family of papain-like enzymes that play important roles  
98 in the biology of *Leishmania* [18]. Three members of these papain-like proteases are expressed  
99 by *L. mexicana* and the generation of CP-deficient mutants revealed that CPB contributes to the  
100 ability to infect macrophages and to induce lesions in BALB/c mice [19-21]. The precise  
101 mechanism(s) by which CPB participates in the virulence of *L. mexicana* is poorly understood.  
102 Previous studies revealed that CPB traffics within and outside infected macrophages [18]. In  
103 infected macrophages, CPB alters signal transduction and gene expression through the activation  
104 of the protein tyrosine phosphatase PTP-1B and the cleavage of transcription factors responsible  
105 for the expression of genes involved in host defense and immunity [20, 22]. The observation that  
106 CPs interfere with the host immune response through the degradation of MHC class II molecules

107 and invariant chains present in PVs housing *L. amazonensis* [23], raises the possibility that CPB  
108 participates in the modulation of PV composition and function.

109

110 In this study, we sought to gain insight into the mechanism by which CPB contributes to  
111 *L. mexicana* virulence, with a focus on the PV. We provide evidence that CPB participates in PV  
112 biogenesis and virulence through the regulation of GP63 expression.

113

## RESULTS

114  
115  
116 ***CPB enables L. mexicana to down-modulate VAMP3 and VAMP8.*** Formation and expansion  
117 of communal PVs hosting *L. mexicana* involve fusion between PVs and endocytic organelles, as  
118 well as homotypic fusion among PVs [10-12]. To identify the host and parasite factors involved  
119 in this process, we embarked on a study to elucidate the fate of endosomal SNAREs during  
120 infection of macrophages with *L. mexicana*. Given the requirement of CPB for *L. mexicana* to  
121 replicate normally inside macrophages [19], we included a *L. mexicana* CPB-deficient mutant  
122 ( $\Delta cpb$ ) in our investigation. We infected BMM with either WT or  $\Delta cpb$  *L. mexicana*  
123 promastigotes for 2 h and we assessed the distribution of the endosomal SNAREs VAMP3 and  
124 VAMP8 by confocal immunofluorescence microscopy. As previously observed during infection  
125 with *L. major* promastigotes [1], we found a notable reduction in the staining intensity for both  
126 VAMP3 (Figure 1A) and VAMP8 (Figure 1B) in BMM infected with WT *L. mexicana*, but this  
127 was not observed with  $\Delta cpb$ . This reduction in staining intensity correlated with a down-  
128 modulation of VAMP3 and VAMP8 proteins in BMM infected with WT *L. mexicana*, compared  
129 to cells infected with  $\Delta cpb$  (Figure 1C). These results suggested that *L. mexicana* causes the  
130 reduction of VAMP3 and VAMP8 levels in infected BMM through the action of CPB. However,  
131 we considered the possibility that CPB acted indirectly on VAMP3 and VAMP8 because we  
132 previously found that GP63 targets those SNAREs in *L. major*-infected BMM [1]. We therefore  
133 ensured that similar levels of GP63 were present in lysates of BMM infected with WT and  $\Delta cpb$   
134 *L. mexicana* promastigotes. As shown in Figure 2, GP63 was detected in lysates of BMM  
135 infected with WT *L. mexicana* up to 72 h post-infection, when the parasites replicate as  
136 amastigotes. The important reduction in GP63 levels at this time point is consistent with  
137 previously published data showing a 90% reduction in the amount of GP63 detected in



138 amastigotes with respect to promastigotes [24, 25]. Surprisingly, we found that GP63 was barely  
139 detectable in BMM infected with  $\Delta cpb$  at all time points tested. This observation raised the  
140 possibility that the lack of VAMP3 and VAMP8 down-regulation in  $\Delta cpb$ -infected BMM was  
141 due to defective expression of GP63.

142  
143 **Figure 1. Down-modulation of VAMP3 and VAMP8 by *L. mexicana*.** BMM were infected  
144 with serum-opsonized stationary phase *L. mexicana* (WT and  $\Delta cpb$ ) promastigotes for 2 h.  
145 VAMP3 (A) and VAMP8 (B) levels (green) were then visualized by confocal microscopy.  
146 Macrophage and parasite nuclei are shown in blue (DAPI). Internalized parasites are denoted by  
147 white arrowheads. In (C), VAMP3 and VAMP8 levels in total cell extracts were assessed by  
148 Western blot analysis. Each immunofluorescence assay was done on 300 phagosomes on  
149 triplicate coverslips in two independent experiments and Western blot analyses were performed  
150 twice in three independent experiments. VAMP3 and VAMP8/ $\beta$ -actin ratios were determined by  
151 densitometry. Original magnification X63.

152  
153 **Figure 2. GP63 is down-modulated in the *L. mexicana*  $\Delta cpb$  mutant.** BMM were infected  
154 with serum-opsonized stationary phase *L. mexicana* (WT and  $\Delta cpb$ ) promastigotes for 2 h, 24 h,  
155 48 h and 72 h. Total cell extracts were assayed for GP63 levels by Western blot analysis. GP63/  
156  $\beta$ -actin ratios were determined by densitometry. Similar results were obtained in three  
157 independent experiments.

158  
159 ***CPB is required for GP63 expression.*** To address the issue of GP63 down-regulation in *L.*  
160 *mexicana*  $\Delta cpb$ , we first determined whether complementation of  $\Delta cpb$  with the *CPB* gene array

161 ( $\Delta cpb+CPB$ ) restores wild type GP63 levels. As shown in Figure 3A, GP63 levels and activity  
162 are down-modulated in the  $\Delta cpb$  mutant, and complementation with the  $CPB$  array restored  
163 GP63 levels and activity similar to those observed in WT parasites. It was previously reported  
164 that expression of the cell surface glycolipid LPG and of GP63 may share common biosynthetic  
165 steps [26-29]. We therefore evaluated the levels of LPG in lysates of WT,  $\Delta cpb$ ,  $\Delta cpb+CPB$ , and  
166  $\Delta cpb+GP63$  parasites by Western blot analysis. Strikingly, similar to GP63, LPG levels were  
167 also down-modulated in the  $\Delta cpb$  mutant and complementation with either the  $CPB$  array or  
168  $GP63$  restored wild type LPG levels. To further investigate the possible role of CPB in the  
169 regulation of GP63 expression, we determined the levels of  $GP63$  mRNA in WT,  $\Delta cpb$ ,  
170  $\Delta cpb+CPB$ , and  $\Delta cpb+GP63$  parasites by RT-PCR. As shown in Figure 3B,  $GP63$  mRNA levels  
171 were highly down-regulated in  $\Delta cpb$  and complementation with the CPB array restored wild type  
172 levels of GP63 mRNA. Interestingly, complementation of  $\Delta cpb$  with *L. major*  $GP63$  did not  
173 increase endogenous  $GP63$  mRNAs. RT-PCR using *L. major* GP63-specific primers showed that  
174 this gene is expressed only in  $\Delta cpb+GP63$ . Together, these results suggest that CPB controls  
175 GP63 mRNA levels at the post-transcriptional level. Clearly, additional studies will be required  
176 to elucidate the underlying mechanism(s). Our results also raised the possibility that down-  
177 modulation of GP63 in the  $\Delta cpb$  mutant may have accounted for the inability of  $\Delta cpb$  to down-  
178 regulate VAMP3 and VAMP8. The finding that expression of GP63 in  $\Delta cpb$  restored LPG levels  
179 was unexpected and suggested a role for GP63 in the expression of LPG in *L. mexicana*. As it is  
180 estimated that at least 25 genes are required for the synthesis, assembly, and transport of the  
181 various components of LPG [30], it may be difficult to determine whether GP63 acts on the  
182 expression of a LPG biosynthetic gene or on a biosynthetic step. Assessment of  $LPG2$  gene  
183 expression revealed that it was equally expressed WT,  $\Delta cpb$ ,  $\Delta cpb+CPB$ , and  $\Delta cpb+GP63$   
184 parasites. Further studies will be necessary to understand how GP63 expression restores LPG

185 synthesis in  $\Delta cpb$ . Since LPG does not play a major role in the virulence of *L. mexicana* [17], the  
186  $\Delta cpb$  mutant expressing exogenous GP63 provides a unique opportunity to address the impact of  
187 GP63 on SNARE cleavage, as well as on the *in vitro* and *in vivo* virulence of *L. mexicana*.

188  
189 **Figure 3. Expression of GP63 and LPG is impaired in the absence of CPB.** (A) Stationary  
190 phase promastigotes were lysed and total cell extracts were analysed by Western blotting and  
191 zymography for GP63 levels and activity and for LPG levels. Aldolase was used as a loading  
192 control. GP63 and LPG/aldolase ratios were determined by densitometry. (B) Promastigote total  
193 RNA was extracted and reverse transcription followed by PCR was performed to assess mRNA  
194 levels for *L. mexicana* GP63-C1, LPG2, and  $\alpha$ -tubulin, and *L. major* GP63-1. Similar results  
195 were obtained in three independent experiments.

196  
197 **GP63 is responsible for the cleavage of VAMP3 and VAMP8 by *L. mexicana*.** We next  
198 assessed the impact of GP63 on VAMP3 and VAMP8 during *L. mexicana* infection. To this end,  
199 we infected BMM with either WT,  $\Delta cpb$ ,  $\Delta cpb+CPB$ , or  $\Delta cpb+GP63$  *L. mexicana* promastigotes  
200 for various time points, and we assessed VAMP3 and VAMP8 levels and intracellular  
201 distribution. Figure 4A shows that GP63 is present at high levels in lysates of BMM infected for  
202 2 h with WT,  $\Delta cpb+CPB$ , and  $\Delta cpb+GP63$  promastigotes (compared to lysates of BMM infected  
203 with  $\Delta cpb$ ). At 72 h post-infection, GP63 levels are strongly reduced in BMM infected with WT  
204 and  $\Delta cpb+CPB$ , whereas they remain elevated in BMM infected with the  $\Delta cpb+GP63$  (Figure  
205 4A) [25]. The high levels of GP63 present in BMM infected with  $\Delta cpb+GP63$  for 72 h may be  
206 related to the fact that expression of the *L. major* GP63 gene from the pLEXNeo episomal vector  
207 [31] is not under the control of endogenous GP63 3' untranslated regions. Western blot analyses

208 revealed that down-regulation of VAMP3 and VAMP8 correlated with GP63 levels expressed by  
209 the parasites. Consistently, the staining intensity of VAMP3 and VAMP8 was reduced in BMM  
210 infected with GP63-expressing parasites, as assessed by confocal immunofluorescence  
211 microscopy (Figure 4D and E). These results suggest that GP63 is responsible for the down-  
212 modulation of the endosomal SNAREs VAMP3 and VAMP8 in *L. mexicana*-infected BMM.  
213 Interestingly, we observed recruitment of VAMP3 to PVs containing *L. mexicana* parasites at  
214 later time points, when promastigotes have differentiated into amastigotes, with the exception of  
215  $\Delta cpb+GP63$  *L. mexicana* promastigotes (Figure 4B). In contrast, we found that VAMP8 is  
216 excluded from *L. mexicana*-containing PVs both at early and later time points post-infection,  
217 independently of GP63 levels, suggesting that additional mechanisms regulate VAMP8  
218 recruitment to *L. mexicana* PVs.

219  
220 **Figure 4. GP63 is responsible for the down-modulation of VAMP3 and VAMP8 in *L.***  
221 ***mexicana*-infected macrophages.** BMM were infected with serum-opsonized stationary phase  
222 *L. mexicana* (WT,  $\Delta cpb$ ,  $\Delta cpb+CPB$  and  $\Delta cpb+GP63$ ) promastigotes for 2 h and 72 h. Total cell  
223 extracts were analysed by Western blot **(A)**. Similar results were obtained in three independent  
224 experiments. VAMP3 and VAMP8 recruitment to the phagosome was visualized by  
225 immunofluorescence microscopy and quantified for 300 phagosomes on triplicate coverslips **(B**  
226 **and C)**. Representative pictures from each condition are shown **(D and E)**. Immunofluorescence  
227 assays were performed on 300 phagosomes on triplicate coverslips for three independent  
228 experiments. \* $p < 0.0001$ . Original magnification X63.

229  
230 ***GP63* expression restores virulence of  $\Delta cpb$ .** Since GP63 was shown to contribute to *L. major*  
231 virulence [32], we next sought to determine whether expression of GP63 is sufficient to restore

232 the ability of  $\Delta cpb$  to replicate inside macrophages and to cause lesions in mice [19]. To this  
233 end, we first infected BMM with either WT,  $\Delta cpb$ ,  $\Delta cpb+CPB$ , or  $\Delta cpb+GP63$  stationary phase  
234 promastigotes and we assessed parasite burden and PV surface area at various time points post-  
235 infection. We found that  $\Delta cpb$  had an impaired capacity to replicate inside macrophages and to  
236 induce the formation of large communal PVs compared to WT and  $\Delta cpb+CPB$  parasites (Figure  
237 5A, B, C). Strikingly, expression of GP63 in  $\Delta cpb$  restored its ability to replicate in macrophages  
238 and to induce large communal PVs up to 72 h post-infection. These results underline the role of  
239 GP63 in the ability of *L. mexicana* to infect and replicate in macrophages, even in the absence of  
240 CPB. Following inoculation inside the mammalian host, promastigotes are exposed to  
241 complement and both GP63 and LPG confer resistance to complement-mediated lysis [32, 33].  
242 *L. mexicana* promastigotes were therefore analyzed for their sensitivity to complement-mediated  
243 lysis in the presence of fresh human serum. As shown in Figure 6A, over 40% of  $\Delta cpb$  was  
244 killed after 30 min in the presence of 20% serum, whereas  $\Delta cpb+CPB$ , and  $\Delta cpb+GP63$  were  
245 more resistant to serum lysis at 14% and 10%, respectively. Absence of both GP63 and LPG  
246 may be responsible for the serum sensitivity of  $\Delta cpb$ . Finally, to assess the impact of GP63 on  
247 the ability of  $\Delta cpb$  to cause lesions, we used a mouse model of cutaneous leishmaniasis.  
248 Susceptible BALB/c mice were infected in the hind footpad with either WT,  $\Delta cpb$ ,  $\Delta cpb+CPB$ ,  
249 or  $\Delta cpb+GP63$  promastigotes and disease progression was monitored for 9 weeks. Consistent  
250 with its reduced capacity to replicate inside macrophages,  $\Delta cpb$  failed to cause significant lesions  
251 compared to WT parasites [19] and  $\Delta cpb$  complemented with *CPB* (Figure 6B). Remarkably,  
252 expression of GP63 in  $\Delta cpb$  restored its capacity to cause lesions, albeit to a lower level than  
253  $\Delta cpb$  complemented with *CPB*. Lesion size correlated with parasite burden, as measured at 9  
254 weeks post-infection (Figure 6C). Collectively, these results indicate that expression of GP63 is  
255 sufficient to restore virulence of  $\Delta cpb$ .

256  
257 **Figure 5. GP63 enables *L. mexicana*  $\Delta cpb$  to infect macrophages and induce large PVs.**  
258 BMM were infected with stationary phase serum-opsonized *L. mexicana* (WT,  $\Delta cpb$ ,  $\Delta cpb+cpb$   
259 and  $\Delta cpb+GP63$ ) promastigotes for 2 h, 24 h, 48 h and 72 h. Macrophages were stained with the  
260 HEMA 3<sup>TM</sup> kit. Representative pictures from each condition are shown (A) Parasites were  
261 counted in 300 macrophages on triplicate coverslips (B). Macrophages were stained with the  
262 LAMP1 antibody and vacuole sizes were measured with the ZEN 2012 software (C). Parasitemia  
263 and vacuole size was determined on 300 phagosomes in triplicate in three independent  
264 experiments. \* $p < 0.0001$ .

265  
266 **Figure 6. GP63 confers virulence to *L. mexicana*  $\Delta cpb$ .** Stationary phase *L. mexicana* (WT,  
267  $\Delta cpb$ ,  $\Delta cpb+cpb$  and  $\Delta cpb+GP63$ ) promastigotes were incubated in the presence of 20% human  
268 serum for 30 min, stained with a fixable viability dye, and then subjected to flow cytometry (A).  
269 Mice were challenged with  $5 \times 10^6$  late-stationary phase *L. mexicana* (WT,  $\Delta cpb$ ,  $\Delta cpb+cpb$  and  
270  $\Delta cpb+GP63$ ) promastigotes that were injected subcutaneously into the hind footpad. Disease  
271 progression was monitored at weekly intervals, by measuring the thickness of the infected  
272 footpad and the contralateral uninfected footpad. (B). Parasite burden was obtained by limiting  
273 dilution assay from infected homogenized footpads 9 weeks after inoculation (C). Human serum  
274 lyses were performed in two independent experiments and six mice per group were used for the  
275 determination of lesion formation and parasite burden. Each data point represents the average  $\pm$   
276 SEM of 6 mice per group, and statistical significance was denoted by \*  $p \leq 0.01$ , and \*\*\*  
277  $p \leq 0.0001$ .

## DISCUSSION

278  
279  
280 This study aimed at investigating the mechanism(s) by which CPB contributes to *L.*  
281 *mexicana* virulence. To this end, we initially examined PV biogenesis by assessing the impact of  
282 *L. mexicana* infection on the trafficking of VAMP3 and VAMP8, two endocytic SNAREs  
283 associated with phagosome biogenesis and function [1, 34]. We found that both SNAREs were  
284 down-modulated in a CPB-dependent manner, which hampered VAMP3 recruitment to PVs. We  
285 also discovered that expression of GP63, which we previously showed to be responsible for  
286 cleaving SNAREs in *L. major*-infected macrophages [1], was down-modulated in the *L.*  
287 *mexicana*  $\Delta cpb$ . Strikingly, restoration of GP63 expression in  $\Delta cpb$  bypassed the need for CPB  
288 for SNARE cleavage. Similarly, episomal expression of GP63 enabled the  $\Delta cpb$  mutant to  
289 establish infection in macrophages, induce larger PVs and cause lesions in mice. These findings  
290 imply that CPB contributes to *L. mexicana* virulence in part through the regulation of GP63  
291 expression, and provide evidence that GP63 is a key virulence factor for *L. mexicana*.

292  
293 The observation that CPB regulates GP63 expression at the mRNA levels was both  
294 unexpected and intriguing. Insight into the possible mechanism(s) may be deduced from a recent  
295 study on the role of cathepsin B in *L. donovani*, which is homologous to the *L. mexicana* CPC  
296 [35]. Similar to *L. mexicana*  $\Delta cpb$ , *L. donovani*  $\Delta catB$  displays reduced virulence in  
297 macrophages. To investigate the role of cathepsin B in virulence, the authors performed  
298 quantitative proteome profiling of WT and  $\Delta catB$  parasites and identified 83 proteins whose  
299 expression is altered in the absence of cathepsin B, with the majority being down-modulated [35].  
300 Among those were a group of proteins involved in post-transcriptional regulation of gene  
301 expression (RNA stability, processing, translation) [35]. Whether this is the case in  $\Delta cpb$

302 deserves further investigation. Clearly, a detailed analysis of wild-type and  $\Delta cpb$  parasites may  
303 provide the information required to understand the extent of the impact of CBP on the expression  
304 and synthesis of virulence factors and the exact role of CPB in *L. mexicana* virulence. The  
305 observation that episomal expression of GP63 in  $\Delta cpb$  restored LPG synthesis is an intriguing  
306 issue, as it suggests that GP63 acts on a LPG biosynthetic step. This role for GP63 is likely  
307 redundant, since *L. major*  $\Delta gp63$  promastigotes express LPG levels similar to that of wild type  
308 parasites (S1 Fig).

309  
310 It has been proposed that expansion of the PVs hosting parasites of the *L. mexicana*  
311 complex leads to the dilution of the microbicidal effectors to which the parasites are exposed,  
312 thereby contributing to parasite survival [36]. Both host and parasite factors may be involved in  
313 the control of PV enlargement. On the host side, it has been previously reported that *L.*  
314 *amazonensis* cannot survive in cells overexpressing *LYST*, a host gene that restricts *Leishmania*  
315 growth by counteracting PV expansion [37]. Similarly, disrupting the fusion between PVs  
316 housing *L. amazonensis* and the endoplasmic reticulum resulted in limited PV expansion and  
317 inhibition of parasite replication [15, 16]. On the parasite side, virulence of *L. amazonensis*  
318 isolates was shown to correlate with the ability to induce larger PVs [38]. Our results indicate  
319 that the inability of  $\Delta cpb$  to multiply inside macrophages is associated with smaller PV size, and  
320 that expression of GP63 is sufficient to restore the capacity of  $\Delta cpb$  to survive within  
321 macrophages and to induce PV expansion. How does GP63 modulate *L. mexicana* virulence and  
322 PV expansion? In addition to the numerous macrophage proteins known to be targeted by GP63,  
323 it is possible that SNARE cleavage is one of the factors associated with *L. mexicana* virulence  
324 and PV expansion. For instance, we previously reported that VAMP8 is required for phagosomal  
325 oxidative activity [1]. One may envision that its degradation by GP63 is part of a strategy used



326 by *L. mexicana* to establish infection in an environment devoid of oxidants, thereby contributing  
327 to parasite survival. The LYST protein is a regulator of lysosome size and its absence leads to  
328 further PV expansion and enhanced *L. amazonensis* replication [37]. It is interesting to note that  
329 LYST was proposed to function as an adaptor protein that juxtaposes proteins such as SNAREs  
330 that mediate intracellular membrane fusion reactions [39]. In this context, cleavage of SNAREs  
331 that interact with LYST may interfere with its function and promote PV expansion. Further  
332 studies will be necessary to clarify these issues, including the potential role of VAMP3 and  
333 VAMP8 in PV biogenesis and expansion.

334  
335         Previous studies using  $\Delta cpb$  parasites led to the conclusion that CPB enables *L. mexicana*  
336 to alter host cell signaling and gene expression through the cleavage of various host proteins [20,  
337 22]. Hence, CPB-dependent cleavage of PTP-1B, NF- $\kappa$ B, STAT1, and AP1 in *L. mexicana*-  
338 infected macrophages was associated with the inhibition of IL-12 expression and generation of  
339 nitric oxide, both of which are important for initiation of a host immune response and parasite  
340 killing, respectively. Our finding that GP63 expression is down-modulated in the  $\Delta cpb$  mutant  
341 raises the possibility that cleavage of those transcription factors may actually be mediated by  
342 GP63. Indeed, GP63 cleaves numerous host macrophage effectors, including PTP-1B, NF- $\kappa$ B,  
343 STAT1, and AP1 [40]. Revisiting the role of CPB in the context of GP63 expression will be  
344 necessary to elucidate whether, and to which extent, CPB is acting directly on the host cell  
345 proteome.

346  
347         In sum, we discovered that CPB contributes to *L. mexicana* virulence in part through the  
348 regulation of GP63 expression. Complementation of  $\Delta cpb$  revealed the importance of GP63 for

349 the virulence of *L. mexicana*, as it participates in the survival of intracellular parasites, PV  
350 expansion, and the formation of cutaneous lesions.

351

## MATERIALS AND METHODS

352

353

### 354 **Ethics statement**

355 Experiments involving mice were done as prescribed by protocol 1406-02, which was approved  
356 by the *Comité Institutionnel de Protection des Animaux* of the INRS-Institut Armand-Frappier. *In*  
357 *vivo* infections were performed as per Animal Use Protocol #4859, which was approved by the  
358 Institutional Animal Care and Use Committees at McGill University. These protocols respect  
359 procedures on good animal practice provided by the Canadian Council on Animal Care (CCAC).

360

### 361 **Antibodies**

362 The mouse anti-GP63 monoclonal antibody was previously described [41]. The mouse anti-  
363 phosphoglycans CA7AE monoclonal antibody [42] was from Cedarlane and the rabbit polyclonal  
364 anti-aldolase was a gift from Dr. A. Jardim (McGill University). Rabbit polyclonal antibodies for  
365 VAMP3 and VAMP8 were obtained from Synaptic Systems.

366

### 367 **Cell culture**

368 Bone marrow-derived macrophages (BMM) were differentiated from the bone marrow of 6- to 8-  
369 week-old female 129XB6 mice (Charles River Laboratories) as previously described [43]. Cells  
370 were cultured for 7 days in complete medium (DMEM [Life Technologies] supplemented with L-  
371 glutamine [Life Technologies], 10% heat-inactivated FBS [PAA Laboratories], 10 mM HEPES at  
372 pH 7.4, and antibiotics) containing 15% v/v L929 cell-conditioned medium as a source of M-  
373 CSF. Macrophages were kept at 37°C in a humidified incubator with 5% CO<sub>2</sub>. To render BMM  
374 quiescent prior to experiments, cells were transferred to 6- or 24-well tissue culture microplates  
375 (TrueLine) and kept for 16 h in complete DMEM without L929 cell-conditioned medium.

376 Promastigotes of *L. mexicana* wild-type strain (MNYC/BZ/62/M379) and of *L. major* NIH S  
377 (MHOM/SN/74/Seidman) clone A2 were grown at 26°C in *Leishmania* medium (Medium 199  
378 supplemented with 10% heat-inactivated FBS, 40 mM HEPES pH 7.4, 100 µM hypoxanthine, 5  
379 µM hemin, 3 µM biopterin, 1 µM biotin, and antibiotics). The isogenic *L. mexicana* CPB-  
380 deficient mutant  $\Delta cpb^{pac}$  (thereafter referred to as  $\Delta cpb$ ) and its complemented counterpart  
381  $\Delta cpb^{pac}$ [pGL263] (thereafter referred to as  $\Delta cpb+CPB$ ) were described previously [21]. *L.*  
382 *mexicana*  $\Delta cpb$  promastigotes were electroporated as described [44] with the pLEXNeoGP63.1  
383 plasmid [32] to generate  $\Delta cpb+GP63$  parasites. *L. mexicana*  $\Delta cpb+CPB$  and  $\Delta cpb+GP63$   
384 promastigotes were grown in the presence of 50 µg/ml hygromycin or 50 µg/ml G418,  
385 respectively. The *L. major* NIH clone A2 isogenic  $\Delta gp63$  mutant and its complemented  
386 counterpart  $\Delta gp63+gp63$  have been previously described [32]. Cultures of  $\Delta gp63+gp63$   
387 promastigotes were supplemented with 50 µg/ml G418.

388

### 389 **Synchronized phagocytosis assays**

390 Prior to macrophage infections, promastigotes in late stationary phase were opsonized with  
391 DBA/2 mouse serum. For synchronized phagocytosis using parasites, macrophages and parasites  
392 were incubated at 4°C for 10 min and spun at 167 g for 1 min, and internalization was triggered  
393 by transferring cells to 34°C. Macrophages were washed twice after 2h with complete DMEM to  
394 remove the non-internalized parasites and were further incubated at 34°C for the required times.  
395 Cells were then washed with PBS and stained using the Hema 3™ Fixative and Solutions kit  
396 (Fisher HealthCare), or prepared for confocal immunofluorescence microscopy.

397

398

399 **Confocal immunofluorescence microscopy**

400 Macrophages on coverslips were fixed with 2% paraformaldehyde (Canemco and Mirvac) for 40  
401 min and blocked/permeabilized for 17 min with a solution of 0.05% saponin, 1% BSA, 6% skim  
402 milk, 2% goat serum, and 50% FBS. This was followed by a 2 h incubation with primary  
403 antibodies diluted in PBS. Macrophages were then incubated with a suitable combination of  
404 secondary antibodies (anti-rabbit Alexa Fluor 488 and anti-rat 568; Molecular Probes) and DAPI  
405 (Life technologies). Coverslips were washed three times with PBS after every step. After the final  
406 washes, Fluoromount-G (Southern Biotechnology Associates) was used to mount coverslips on  
407 glass slides, and coverslips were sealed with nail polish (Sally Hansen). Macrophages were  
408 imaged with the 63X objective of an LSM780 microscope (Carl Zeiss Microimaging), and  
409 images were taken in sequential scanning mode. Image analysis and vacuole size measurements  
410 were performed with the ZEN 2012 software.

411

412 **Electrophoresis, Western blotting, and zymography**

413 Prior to lysis, macrophages were placed on ice and washed with PBS containing 1 mM sodium  
414 orthovanadate and 5 mM 1,10-phenanthroline (Roche). Cells were scraped in the presence of  
415 lysis buffer containing 1% Nonidet P-40 (Caledon), 50 mM Tris-HCl (pH 7.5) (Bioshop), 150  
416 mM NaCl, 1 mM EDTA (pH 8), 5 mM 1,10-phenanthroline, and phosphatase and protease  
417 inhibitors (Roche). Parasites were washed twice with PBS and lysed in the presence of lysis  
418 buffer containing 0.5% Nonidet P-40 (Caledon), 100mM Tris-HCl (Bioshop) and 150 mM NaCl  
419 at -70° C. Lysates were thawed on ice and centrifuged for 10 min to remove insoluble matter.  
420 After protein quantification, protein samples were boiled (100°C) for 6 min in SDS sample buffer  
421 and migrated in 12% or 15% SDS-PAGE gels. Three micrograms and 15 µg of protein were  
422 loaded for parasite and infected macrophage lysates, respectively. Proteins were transferred onto

423 Hybond-ECL membranes (Amersham Biosciences), blocked for 1 h in TBS 1X-0.1% Tween  
424 containing 5% skim milk, incubated with primary antibodies (diluted in TBS 1X-0.1% Tween  
425 containing 5% BSA) overnight at 4°C, and thence with appropriate HRP-conjugated secondary  
426 antibodies for 1 h at room temperature. Then, membranes were incubated in ECL (GE  
427 Healthcare), and immunodetection was achieved via chemiluminescence. Membranes were  
428 washed 3 times between each step. For zymography, 2 µg of lysate were incubated at RT for 6  
429 min in sample buffer without DTT and then migrated in 12% SDS-PAGE gels with 0.2% gelatin  
430 (Sigma). Gels were incubated for 1 h in the presence of 50 mM Tris pH 7.4, 2,5% Triton X-100,  
431 5 mM CaCl<sub>2</sub> and 1 µM ZnCl<sub>2</sub> and incubated overnight in the presence of 50 mM Tris pH 7.4, 5  
432 mM CaCl<sub>2</sub>, 1µM ZnCl<sub>2</sub> and 0,01% NaN<sub>3</sub> at 37°C [45].

433

#### 434 **FACS analysis**

435 Late stationary phase promastigotes were incubated for 30 min in complete DMEM medium with  
436 20% human serum from healthy donors. Parasites were then incubated in LIVE/DEAD® Fixable  
437 Violet Dead Cell Stain Kit (Life technologies) and fixed in 2% paraformaldehyde. Flow  
438 cytometric analysis was carried out using the LSRFortessa cytometer (Special Order Research  
439 Product; BD Biosciences), and the BD FACSDiva Software (version 6.2) was used for data  
440 acquisition and analysis.

441

#### 442 **Mouse infections**

443 Male BALB/c mice (6 to 8 weeks old) were purchased from Charles River Laboratories and  
444 infected in the right hind footpad with 5x10<sup>6</sup> stationary phase *L. mexicana* promastigotes as  
445 described [46]. Disease progression was monitored by measuring footpad swelling weekly with a

446 metric caliper, for up to 9 weeks post-infection. Footpads were then processed to calculate  
447 parasite burden using the limiting dilution assay.

448

#### 449 **Limiting dilution assay**

450 After 9 weeks of infection, mice were euthanized under CO<sub>2</sub> asphyxiation and subsequently by  
451 cervical dislocation. The infected footpads were surface-sterilized with a chlorine dioxide based  
452 disinfectant followed by ethanol 70% for 5 min. Footpads were washed in PBS, lightly sliced,  
453 transferred to a glass tissue homogenizer containing 6 ml of PBS, and manually homogenized.  
454 The last step was repeated two to three times, until complete tissue disruption was achieved.  
455 Final homogenate was then centrifuged at 3,000 x g for 5 min and resuspended in the appropriate  
456 volume of PBS. 100 µl of homogenate were added in duplicates to 96-well plates containing 100  
457 µl of complete Schneider's medium in each well (twenty-four 2-fold dilutions for each duplicate).  
458 Plates were incubated at 28°C. After 7-10 days, the number of viable parasites was determined  
459 from the highest dilutions at which promastigotes were observed using an inverted microscope  
460 [47].

461

#### 462 **Reverse transcription-PCR (RT-PCR)**

463 Total RNA was extracted from promastigotes using the TRIzol reagent (Invitrogen Life  
464 Technology, Carlsbad, CA) and reverse transcribed. One-tenth of the resulting cDNA was  
465 amplified by PCR on a DNA thermal cycler, version 2.3 (Perkin-Elmer Corporation, Norwalk,  
466 CT), with the following primer pairs: for the *L. mexicana* GP63 C-1 5'-  
467 ACCGTCTGAGAGTCGGA ACT-3' (forward), 5'-GTAGTCCAGGAATGGCGAGT-3'  
468 (reverse); the *L. major* GP63-1 5'-TCTGAGGCACATGCTTCGTT-3' (forward), 5'-  
469 GTCAGTTGCCTTCGGTCTGA-3' (reverse), the *L. mexicana* LPG2

470 5'CATTGGTATCCTGGTGCTG-3' (forward), 5'-GAGGAAGCCACTGTTAGCC-3' (reverse),  
471 and the *L. mexicana*  $\alpha$ -tubulin 5'-CTATCTGCATCCACATTGGC-3' (forward), 5'-  
472 ACTTGTCAGAGGGCATGGA-3' (reverse). The PCR products were analyzed by  
473 electrophoresis on a 3% (wt/vol) agarose gel, and the pictures were taken using AlphaImager  
474 3400 imaging software (Alpha Innotech Corporation, San Leandro, CA).

475

#### 476 **Statistical analyses**

477 Statistically significant differences were analyzed by ANOVA followed by the Tukey post-hoc  
478 test using the Graphpad Prism program (version 5.0). For the limiting dilution assay, the non-  
479 parametric Mann-Whitney or Kruskal-Wallis test was used. Values starting at  $P < 0.05$  were  
480 considered statistically significant. All data are presented as mean  $\pm$  SEM.

481

482



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484 We are grateful to Dr. Armando Jardim (McGill University) for providing the anti-aldolase  
485 antiserum, Jessy Tremblay for assistance in immunofluorescence experiments, and Guillermo  
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487

488

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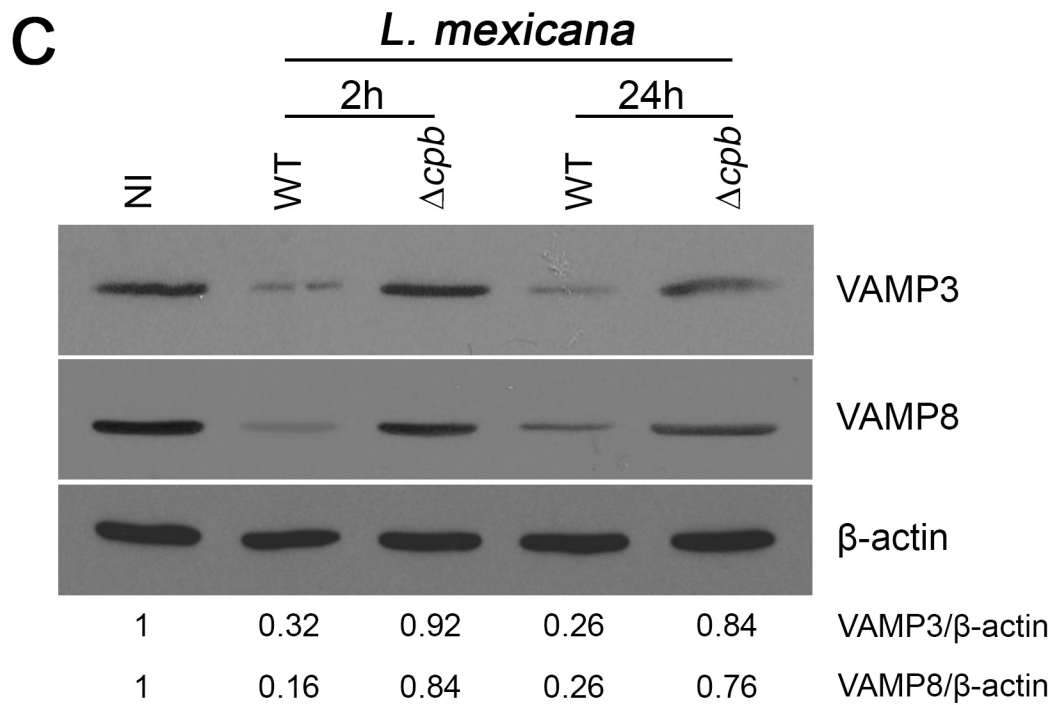
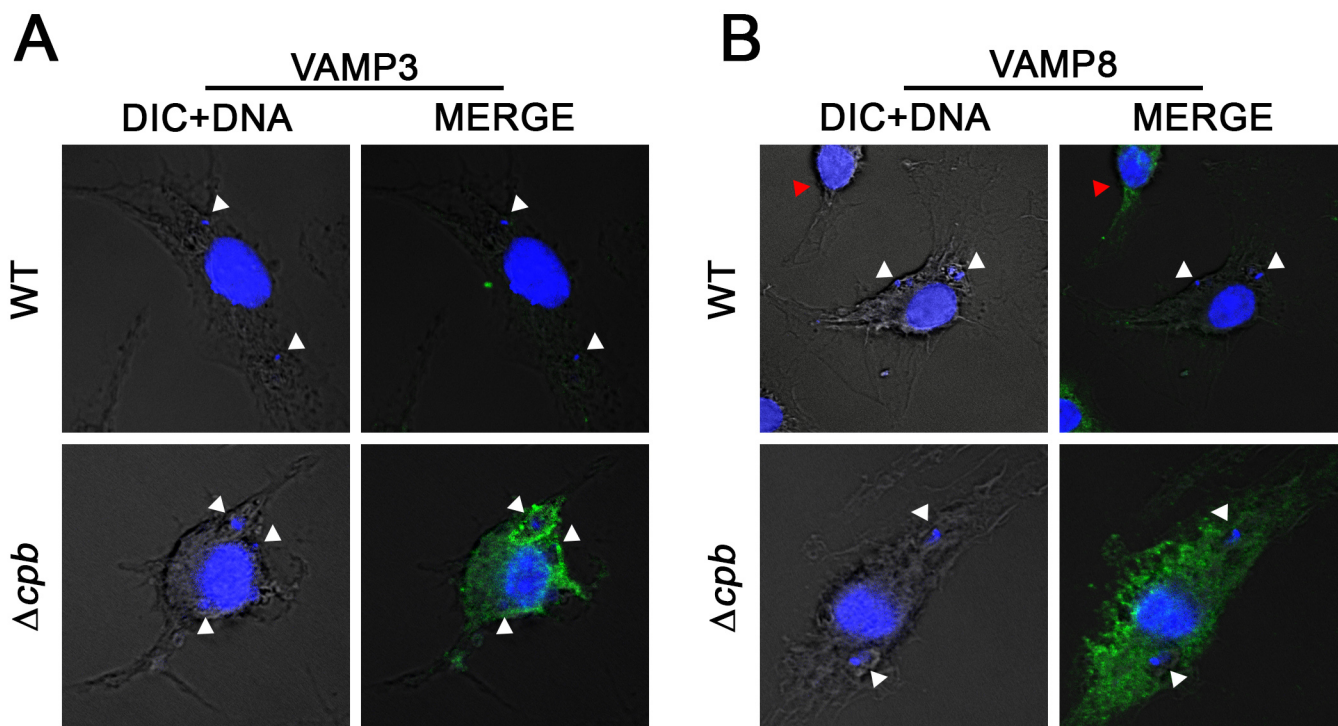
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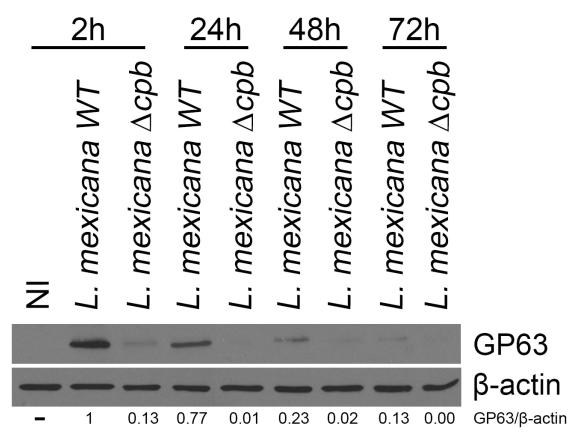
618 **Supporting Information**

619

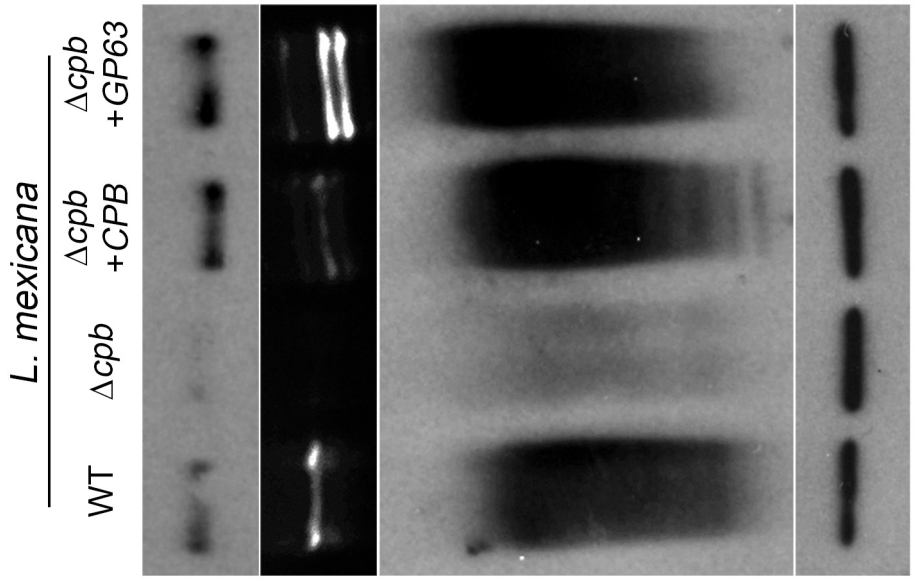
620 **S1 Fig. *L. major*  $\Delta gp63$  promastigotes express normal levels of LPG.** Stationary phase  
621 promastigotes were lysed and total cell extracts were analysed by Western blotting for LPG  
622 levels. Similar results were obtained in two separate experiments.





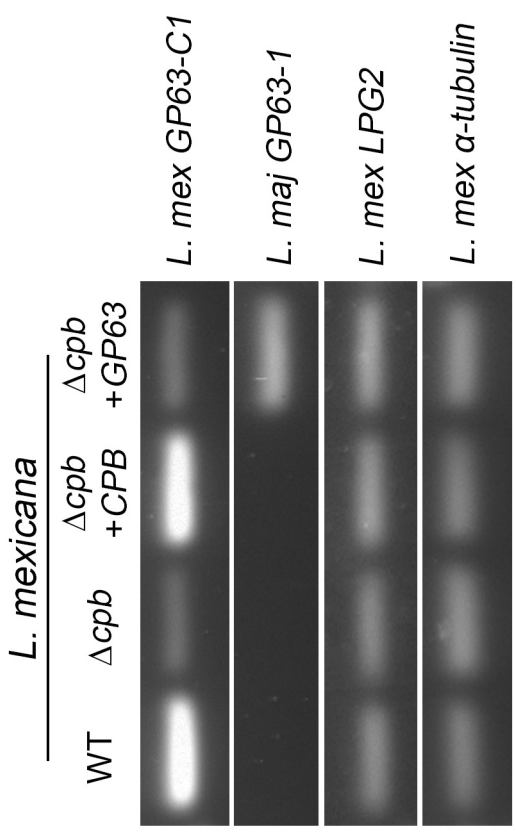


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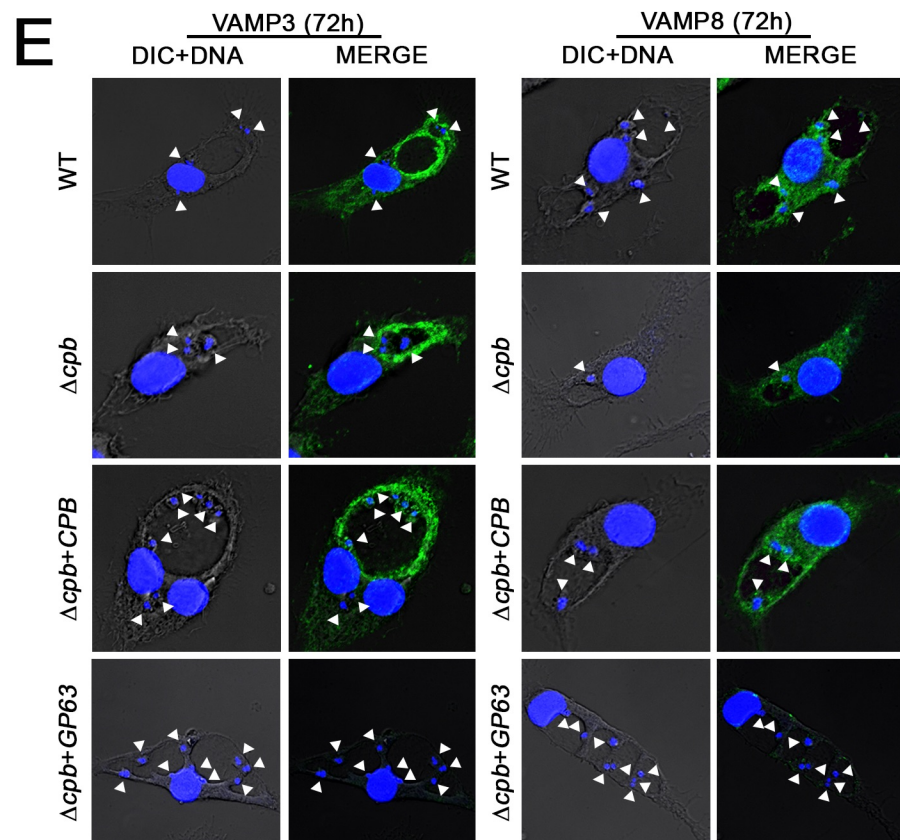
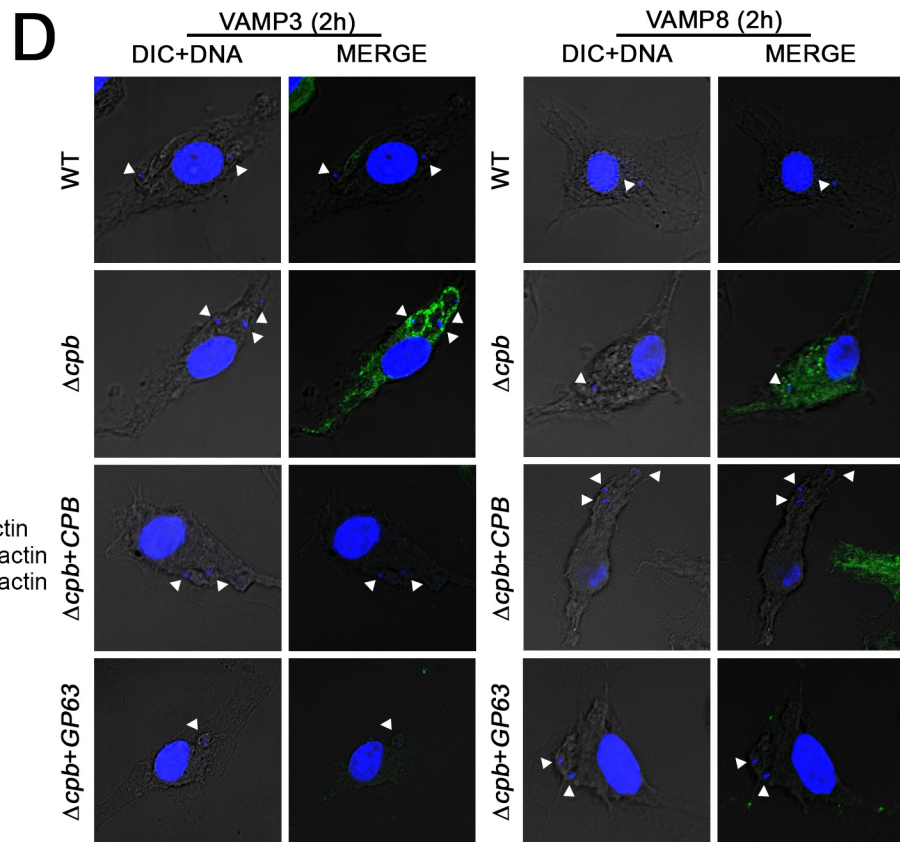
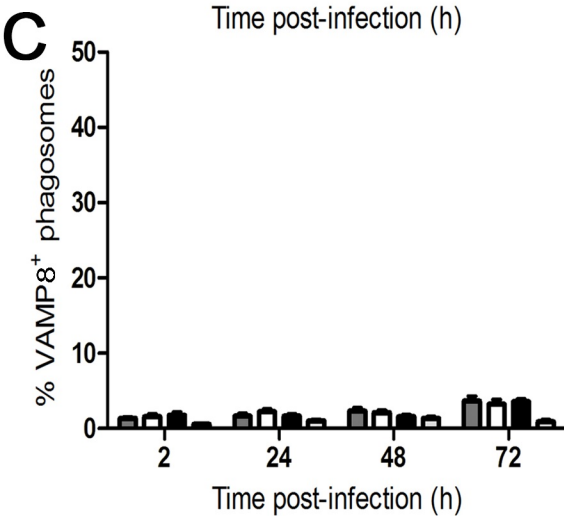
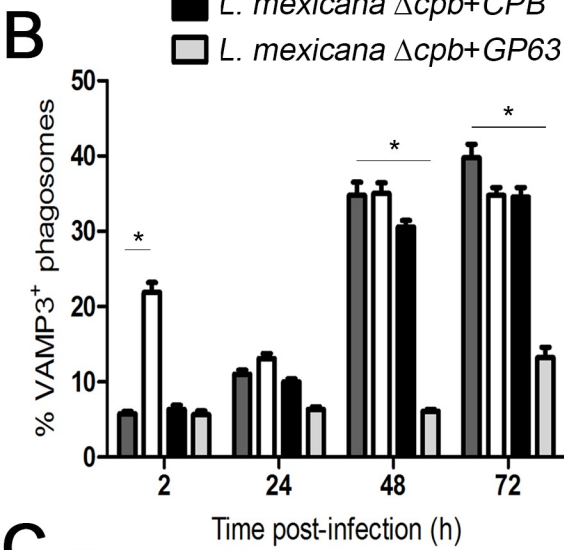
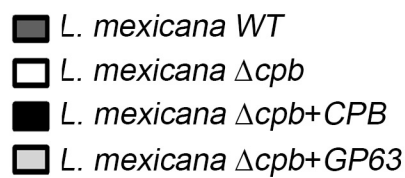
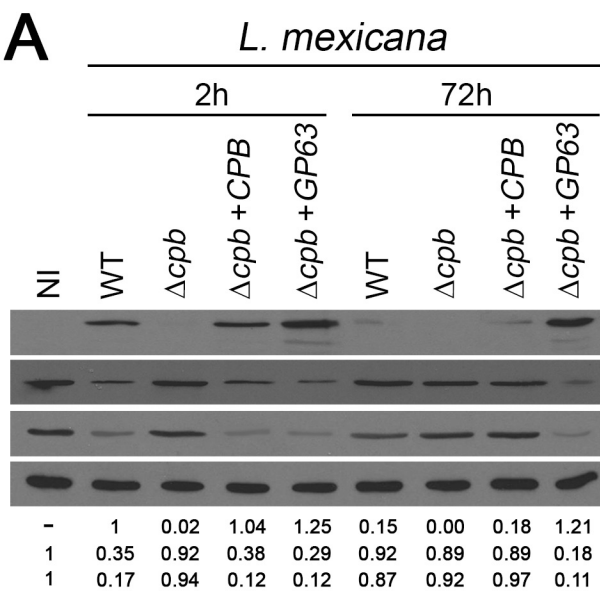


1	0.20	1.20	1.28
1	0.32	1.22	1.21

**B**

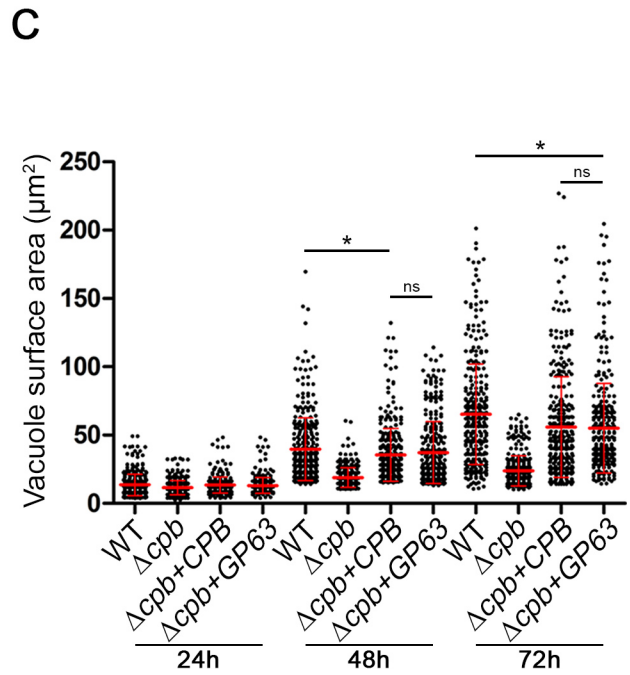
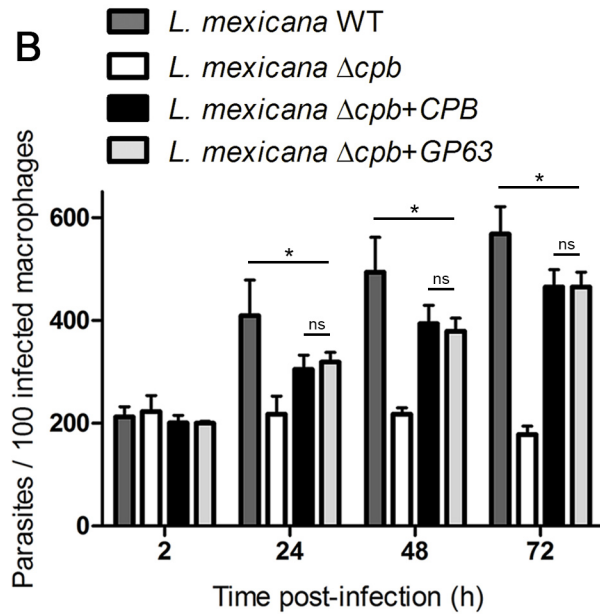
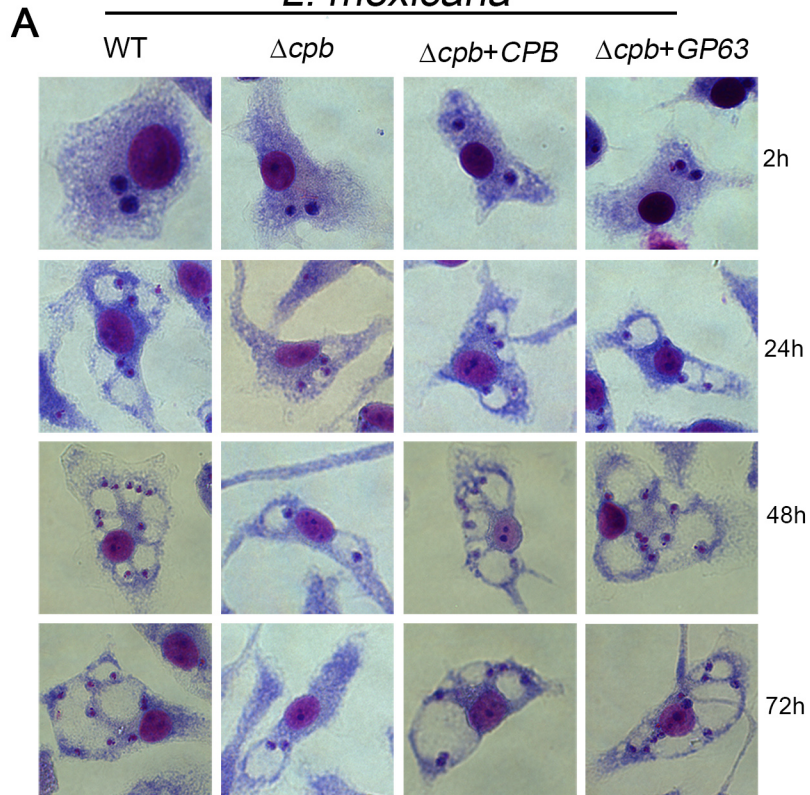


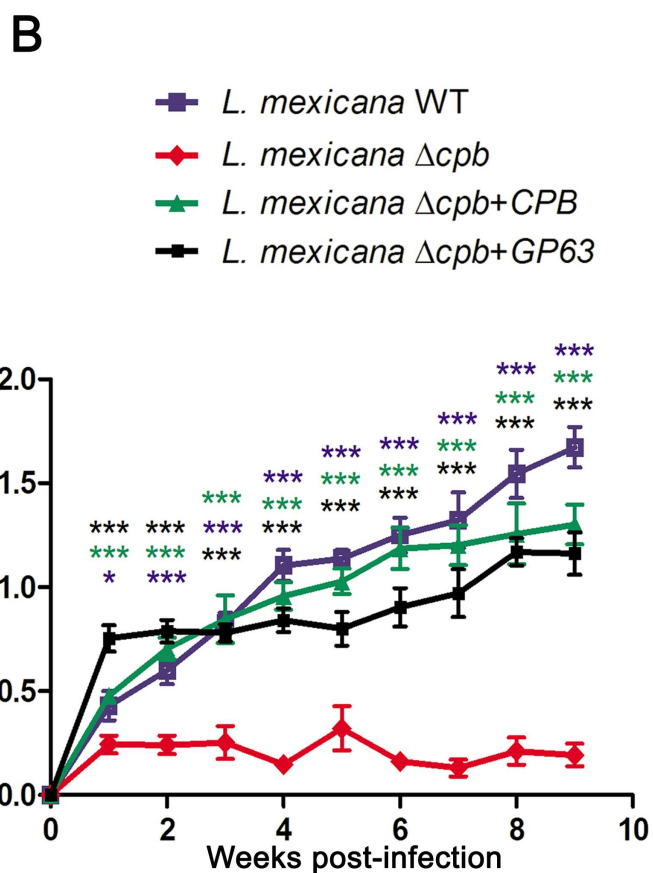
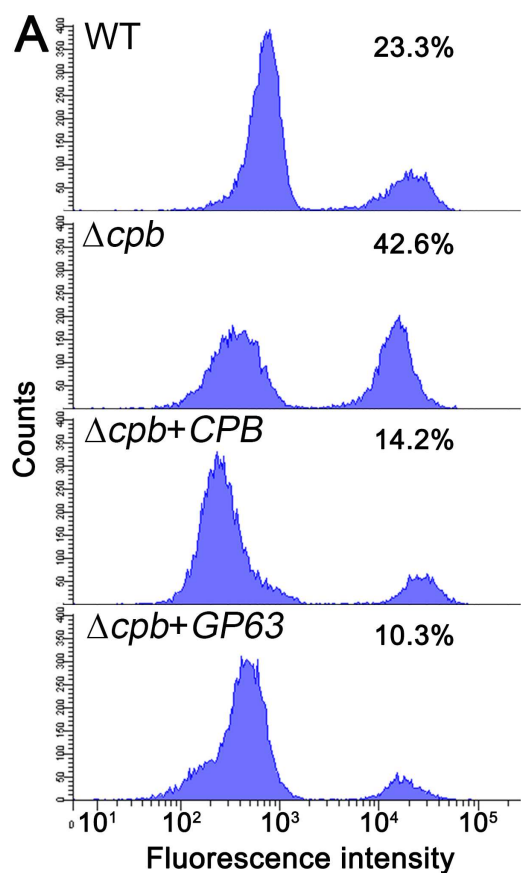
L. mex GP63-C1  
L. maj GP63-1  
L. mex LPG2  
L. mex  $\alpha$ -tubulin



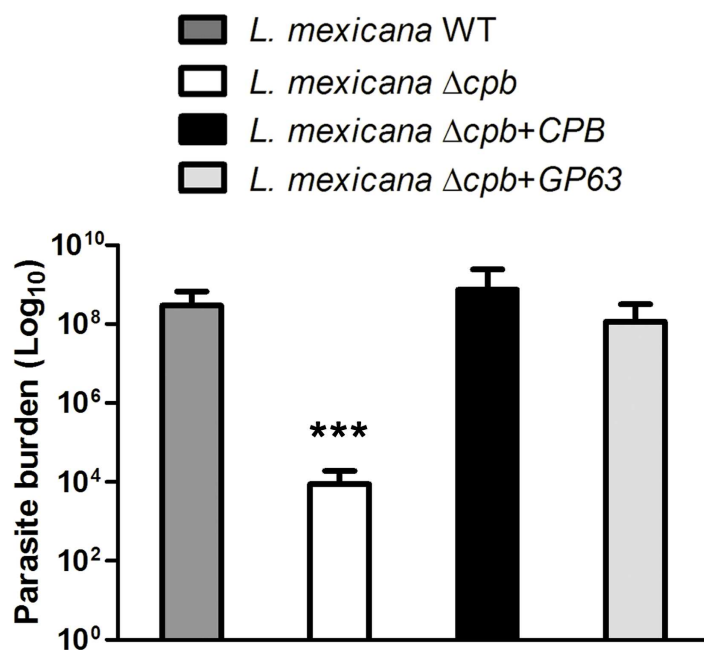


*L. mexicana*



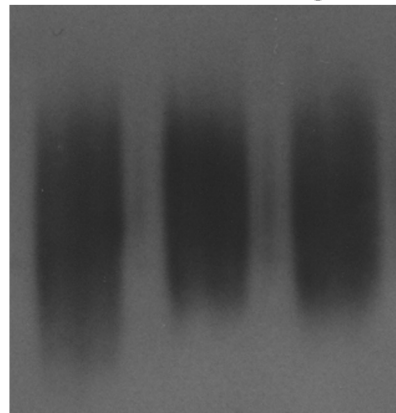


**C**



*L. major*

WT     $\Delta gp63$      $\Delta gp63$   
+GP63



LPG