

# *Leishmania* Repression of Host Translation through mTOR Cleavage Is Required for Parasite Survival and Infection

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## SUMMARY

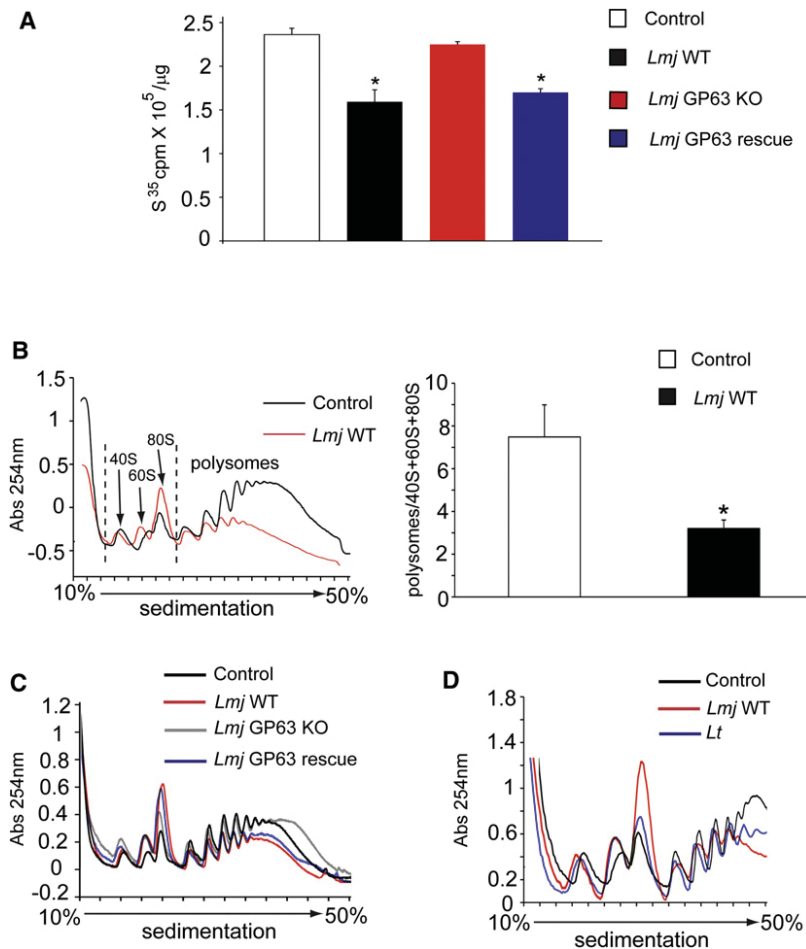
The protozoan parasite *Leishmania* alters the activity of its host cell, the macrophage. However, little is known about the effect of *Leishmania* infection on host protein synthesis. Here, we show that the *Leishmania* protease GP63 cleaves the mammalian/mechanistic target of rapamycin (mTOR), a serine/threonine kinase that regulates the translational repressor 4E-BP1. mTOR cleavage results in the inhibition of mTOR complex 1 (mTORC1) and concomitant activation of 4E-BP1 to promote *Leishmania* proliferation. Consistent with these results, pharmacological activation of 4E-BPs with rapamycin, results in a dramatic increase in parasite replication. In contrast, genetic deletion of 4E-BP1/2 reduces parasite load in macrophages *ex vivo* and decreases susceptibility to cutaneous leishmaniasis *in vivo*. The parasite resistant phenotype of 4E-BP1/2 double-knockout mice involves an enhanced type I IFN response. This study demonstrates that *Leishmania* evolved a survival mechanism by activating 4E-BPs, which serve as major targets for host translational control.

## INTRODUCTION

Translational control of gene expression provides the cell with a rapid response to external triggers or cues. Thus, it constitutes an important regulatory step in processes such as cell growth and proliferation (Schneider and Sonenberg, 2007). In eukaryotes, translational control occurs mostly at the initiation step at which the ribosome is recruited to the messenger RNA

(mRNA). This process is facilitated by the recognition of the mRNA 5' cap structure by the eukaryotic initiation factor 4F (eIF4F), which includes the cap-binding subunit, eIF4E (Gingras et al., 1999). The assembly of eIF4F is blocked by the reversible association of eIF4E with the 4E-binding proteins (4E-BPs), among which 4E-BP1 is the best characterized (Lin et al., 1995; Pause et al., 1994). The activity of 4E-BPs is controlled through their phosphorylation by the serine/threonine kinase mammalian/mechanistic target of rapamycin (mTOR) complex 1 (mTORC1). The protein raptor, which associates with mTOR, defines mTORC1 and is required for mTOR substrate binding (Hara et al., 2002). The mTORC1 signaling pathway is activated in response to a variety of stimuli (e.g., serum, insulin), leading to the hyperphosphorylation of 4E-BPs and their dissociation from eIF4E. Consequently, the formation of the eIF4F complex and the initiation of translation are facilitated (Gingras et al., 2001). Rapamycin blocks mTORC1-mediated 4E-BP phosphorylation, which results in increased 4E-BP binding to eIF4E and inhibition of cap-dependent translation (Beretta et al., 1996a).

Translational control by mTORC1 is a key component of the innate immune response (Cao et al., 2008; Colina et al., 2008; Costa-Mattioli and Sonenberg, 2008). Synthesis of type I IFN (IFN- $\alpha$  and IFN- $\beta$ ) is regulated by mTORC1 and its downstream targets p70 ribosomal S6 protein kinases 1 and 2 (S6K1/2) (Alain et al., 2010; Cao et al., 2008), and 4E-BP1/2 (Colina et al., 2008). Mice and cells lacking 4E-BP1/2 are resistant to viral infections owing to increased type I IFN production (Colina et al., 2008). Type I IFN is also important for host defense against parasite infections, such as leishmaniasis (Bogdan et al., 2004). First, a single administration of IFN- $\beta$  stimulates *Leishmania* killing by their host cells (Mattner et al., 2000; Passwell et al., 1986) and leads to early parasite containment in infected mice (Diefenbach et al., 1998; Laskay et al., 1995). Second, repeated injection of IFN- $\beta$  significantly reduces the progression of cutaneous leishmaniasis (Mattner et al., 2004). Finally, mice treated with



**Figure 1. *Leishmania major* Downregulates Macrophage Protein Synthesis**

(A) B10R M $\phi$  were infected with promastigotes (1:20 ratio) of *Lmj* WT, *Lmj* GP63 KO, or *Lmj* GP63 rescue for 6 hr in methionine-free DMEM. Thirty minutes before sample collection,  $^{35}\text{S}$ -methionine protein labeling mix was added to the culture media. Protein synthesis was measured as the radioactivity incorporated into TCA-precipitable material and was expressed as cpm normalized to total protein concentration ( $\mu\text{g}$ ). Results are the mean of two different experiments performed in duplicate (mean + SEM,  $n = 4$ ).

(B) B10R M $\phi$  were infected or not with *L. major* for 6 hr. Cell lysates were sedimented on 10% to 50% sucrose gradients. Gradients were fractionated and absorbance at 254 nm was continuously recorded. The dashed lines delimit the two areas under the curve that were quantified and compared on the right panel. The area on the left corresponds to the 40S, the 60S, and the 80S subunits, whereas the area on the right corresponds to the polysomes (left panel). Quantification of the area of polysomes over 40S+60S+80S with Image-J software (right panel), mean + SEM,  $n = 3$ .

(C and D) Cells were infected with one of the following species: *L. major* WT, *L. major* GP63 KO, *L. major* GP63 rescue (C); *L. major* WT, *L. tarentolae* (D). Polysome profile analysis was performed as described above. Control equals noninfected M $\phi$ . Data displayed represent one of two separate experiments.

Statistically significant differences (\*) were considered when  $p < 0.05$ . See also Figure S1.

neutralizing antibodies against type I IFN are susceptible to *Leishmania* infection (Diefenbach et al., 1998). Protozoans of the genus *Leishmania* sp. are intracellular parasites that replicate inside macrophages (M $\phi$ ), which are one of the first lines of defense against microbial invasion. To survive, *Leishmania* suppresses the main microbicidal and immune functions of the M $\phi$  through the activation of host phosphatases. This in turn leads to the inactivation of a variety of kinases necessary for the expression of immune-responsive genes (Olivier et al., 2005). The *Leishmania* surface glycoprotein GP63 is an important virulence factor in leishmaniasis. During the initial contact between the parasite and the M $\phi$ , GP63 causes rapid cleavage and activation of three host phosphatases: SHP-1, PTP1B, and TCPTP, thereby contributing to the progression of cutaneous leishmaniasis (Gomez et al., 2009). Moreover, GP63 is involved in parasite evasion of complement-mediated lysis as well as in parasite binding and phagocytosis by the M $\phi$  (Joshi et al., 2002; McGwire et al., 2003). Of notice, cutaneous mouse infection with GP63 knockout (KO) parasites results in delayed lesion formation (Joshi et al., 2002).

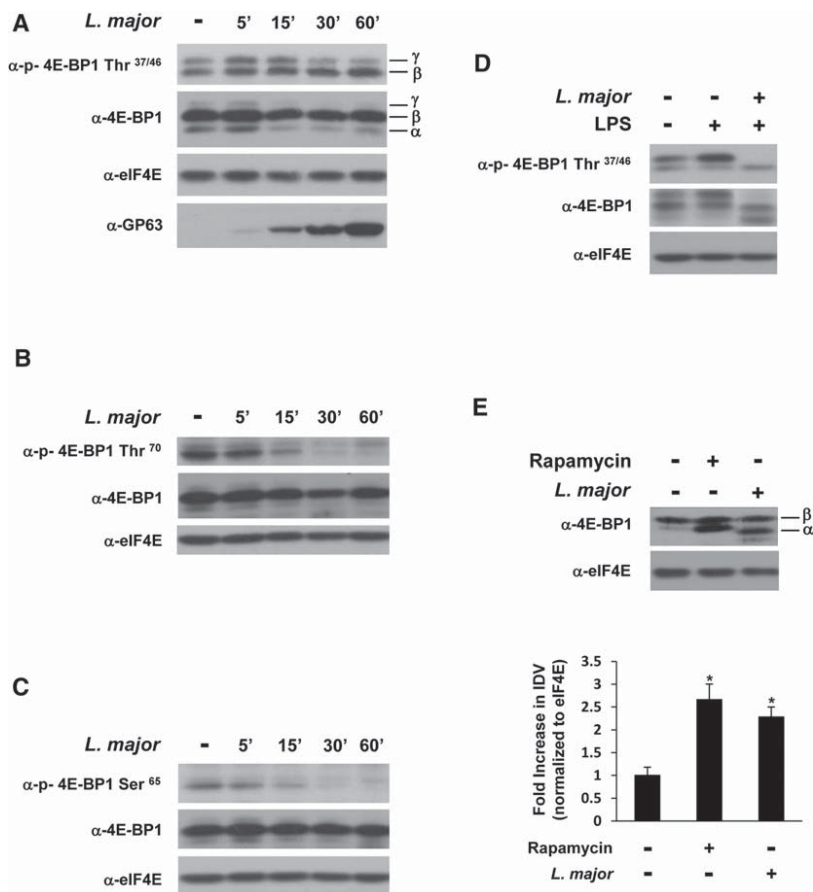
Obligate intracellular pathogens have evolved strategies to hijack the host translation initiation machinery for their own replication. The underlying mechanisms have been extensively studied for viral infections (Mohr et al., 2007) and include the dephosphorylation of eIF2 $\alpha$  (Gale and Katze, 1998) and

machinery during their replication is unknown. Here, we show that *Leishmania* promotes its survival through downregulation of M $\phi$  protein synthesis. We also demonstrate that 4E-BP1/2 are key factors in the progression of cutaneous leishmaniasis, underscoring the importance of mTORC1 signaling in the innate immune response to *Leishmania*.

## RESULTS

### *Leishmania major* Inhibits Macrophage General Translation

To investigate the effect of *L. major* on host protein synthesis, B10R M $\phi$ , a mouse cell line of bone marrow-derived M $\phi$  (BMM $\phi$ ), was infected with *L. major* promastigotes for 6 hr. Infection resulted in a 35% decrease in global translation, as determined by  $^{35}\text{S}$ -methionine incorporation into TCA-precipitable material (Figure 1A). To investigate the involvement of the *Leishmania* glycoprotein GP63 in M $\phi$  translational repression, translation rates were measured in M $\phi$  infected with *L. major* GP63 KO. In contrast to the WT strain, *L. major* GP63 KO parasites failed to downregulate M $\phi$  protein synthesis. To rule out the possibility that these differences were strain or clone specific rather than the effects of the *gp63* deletion per se, the *L. major* GP63 KO line was rescued by reintroducing the *gp63* gene 1 (*L. major* GP63 rescue) (Joshi et al., 2002). Restoring GP63 in

**Figure 2. 4E-BP1 Activation by *Leishmania major***

(A–C) B10R M $\phi$  were infected with *L. major* for various time periods (5 to 60 min). After three washes with cold PBS, total protein extracts were separated by SDS-PAGE and subjected to western blot analysis. Phosphorylation status of M $\phi$  4E-BP1 was monitored with specific phospho-4E-BP1 antibodies against Thr<sup>37/46</sup> (A), Thr<sup>70</sup> (B), and Ser<sup>65</sup> (C). An antibody against *Leishmania* GP63 was employed to monitor M $\phi$  infection with the parasite (Figure 2A). Total amounts of 4E-BP1 and eIF4E were used as loading controls.

(D) B10R M $\phi$  were infected or not with *L. major* for 1 hr and were then stimulated with 100 ng/ml LPS for 30 min. 4E-BP1 phosphorylation status was monitored by western blot with phospho Thr<sup>37/46</sup> and total anti-4E-BP1 antibodies.

(E) Protein lysates from M $\phi$  untreated, exposed to 20 nM rapamycin, or infected with *L. major* for 3 hr were incubated with a m<sup>7</sup>GDP resin. Cap-bound proteins were eluted and 4E-BP1 and eIF4E levels were examined by western blot (upper panel). Densitometric analysis of the 4E-BP1 bands was performed with Image-J software (lower panel). IDV, integrated density value. mean  $\pm$  SEM, n = 3. Statistically significant differences (\*) were considered when p < 0.05. Data are representative of three independent experiments.

See also Figure S2.

GP63 KO parasites, decreased host cell translation to WT levels (Figure 1A). Polysome profile analysis demonstrated a reduction in cellular translation initiation, as infection with *L. major* led to a shift of mRNAs from heavy to light polysomes with a concomitant increase in 80S ribosomes (Figure 1B, left panel). The extent of the defect in translation initiation was obtained by calculating the ratio of polysomes versus 40S+60S+80S (7.5 in control cells versus 3.2 in *L. major*-infected M $\phi$ ) (Figure 1B, right panel). Comparative analysis of the polysome profiles of *L. major*-infected M $\phi$  and *L. major* promastigote cultures showed that the parasite-induced increase in 80S ribosomes was not caused by a contamination with *L. major* ribosomes, since the polysome profile of the parasite was barely detected and did not overlap with the profile of the infected M $\phi$  (Figure S1 available online). Unlike the WT and the GP63 rescue strains, *L. major* GP63 KO did not affect M $\phi$  translation initiation (Figure 1C). Of notice, a nonpathogenic species of *Leishmania*, *L. tarentolae*, failed to cause a shift of host mRNAs from polysomes to monosomes (Figure 1D). Taken together, these findings show that *L. major* infection inhibits host translation initiation in a GP63-dependent manner. This inhibition is likely to cause a reduction in proteins involved in host defense against *L. major* infection.

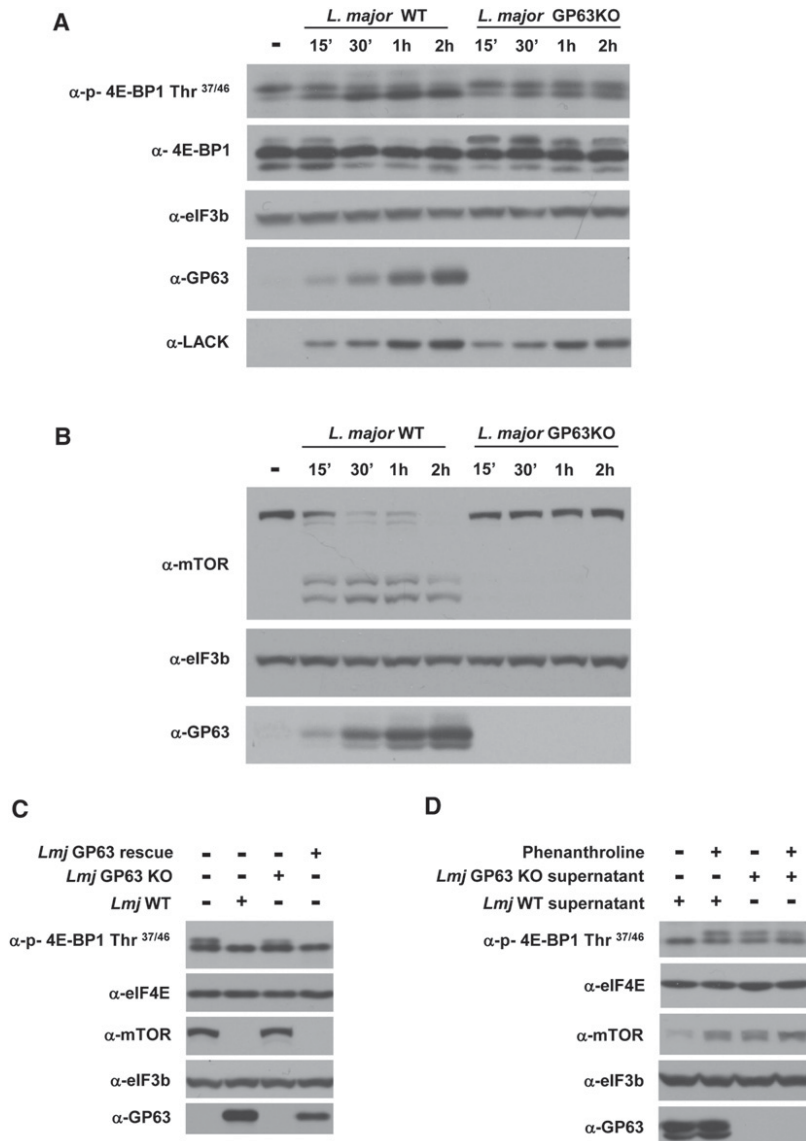
### ***Leishmania major* Activates the Macrophage Translational Repressor 4E-BP1**

One of the major strategies by which *Leishmania* suppresses M $\phi$  functions is the dephosphorylation of host targets (Olivier et al.,

2005). To elucidate the mechanism underlying M $\phi$  translation inhibition by *Leishmania*, we examined the ability of the parasite to modulate the activity of the translational repressor 4E-BP1. There are three different forms of 4E-BP1, which are observed in polyacrylamide fractionation: the slow-migrating or hyperphosphorylated form ( $\gamma$ ), the middle form ( $\beta$ ), and the fastest-migrating or least phosphorylated form ( $\alpha$ ) (Figures S2A and S2B). At 30 min postinfection, *L. major* caused a reduction in phosphorylation of 4E-BP1 on Thr<sup>37/46</sup> (Figure 2A), which are the priming residues in its hierarchical phosphorylation (Gingras et al., 2001). *L. major* also caused 4E-BP1 dephosphorylation on Thr<sup>70</sup> (Figure 2B) and Ser<sup>65</sup> (Figure 2C), indicating full activation of 4E-BP1. It is noteworthy that 4E-BP1 was detected only in M $\phi$  extracts, but not in parasite cultures (Figure S2C), ruling out the possibility that the anti-4E-BP1 antibody interacts with putative *L. major* 4E-BP1 homologs.

Stimuli that trigger mTORC1 signaling cause the hyperphosphorylation of 4E-BPs (Gingras et al., 2001). *L. major* infection dramatically reduced M $\phi$  4E-BP1 phosphorylation in response to the bacterial endotoxin *E. coli* lipopolysaccharide (LPS) (Figure 2D). This observation indicates that after 4E-BP1 dephosphorylation, the M $\phi$  is no longer able to respond to stimuli that would otherwise elicit the phosphorylation of 4E-BP1 and promote protein synthesis. Because LPS activates mTORC1 signaling and thereby induces the production of nitric oxide (NO) via type I IFN (Weinstein et al., 2000), our data suggest that in addition to a direct inhibitory effect on host translation, 4E-BP1 dephosphorylation by *L. major* is likely to block an efficient M $\phi$  response during co-infections (e.g., *L. major* and *E. coli*).

Dephosphorylated 4E-BP1 has a higher affinity for eIF4E, thereby precluding the formation of a functional eIF4F complex,



**Figure 3. *Leishmania major* Glycoprotein GP63 Mediates 4E-BP1 Dephosphorylation and mTOR Cleavage**

(A and B) B10R M $\phi$  were infected with *L. major* WT or *L. major* GP63 KO from 15 min to 2 hr. Phosphorylation status of 4E-BP1 was monitored with a phospho-4E-BP1 Thr<sup>37/46</sup> antibody (A) and mTOR cleavage was investigated with an anti-mTOR antibody (B). An antibody against the *Leishmania* antigen LACK was employed to monitor M $\phi$  infection. Equal loading was verified with anti-4E-BP1 and anti-eIF3b antibodies. An anti-GP63 antibody confirmed the absence of this protein in the GP63 KO parasites.

(C) M $\phi$  were infected for 1 hr with *L. major* WT, *L. major* GP63 KO, or *L. major* GP63 rescue and western blot was carried out as described above.

(D) Parasite culture supernatant was obtained by high-speed centrifugation of *L. major* WT and *L. major* GP63 KO promastigote cultures. Supernatants were incubated or not with 1 mM phenanthroline for 1 hr at room temperature. Then M $\phi$  were treated for 30 min with parasite supernatant, previously incubated or not with phenanthroline, and 4E-BP1 phosphorylation and mTOR cleavage were investigated by western blot. The results displayed are representative of two separate experiments. See also Figure S3 and Table S1.

whereas the *L. major* GP63 rescue strain had the same effect as the WT (Figure 3C). LACK, a *Leishmania* antigen, was used to show that the inability of GP63 KO parasites to dephosphorylate 4E-BP1 is not caused by their incapacity to infect the M $\phi$  (Figure 3A). Two of the main M $\phi$  phosphatases activated by *Leishmania* through GP63-mediated cleavage are SHP-1 and PTP1B (Gomez et al., 2009). However, 4E-BP1 dephosphorylation by *L. major* required neither SHP-1 (Figure S3A) nor PTP1B (Figure S3B).

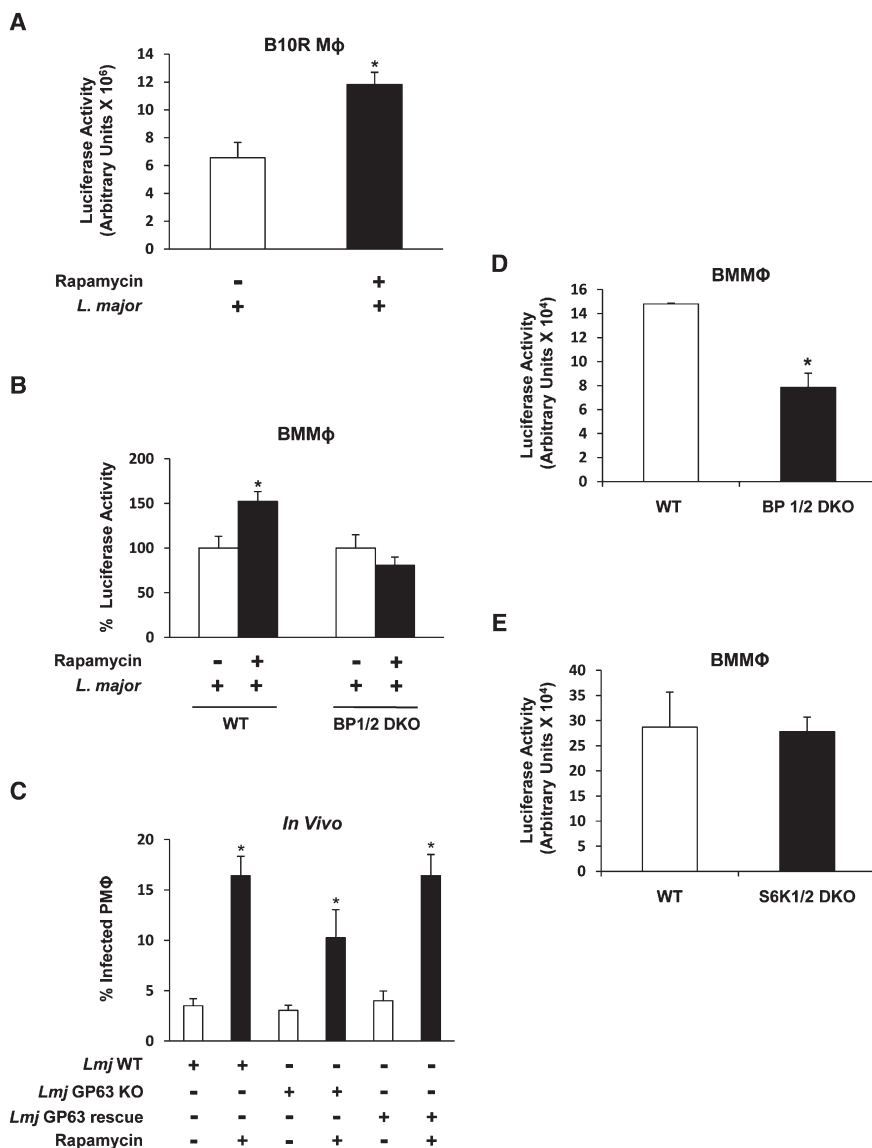
***Leishmania major* Cleaves mTOR**

How does GP63 regulate 4E-BP1 phosphorylation? One possibility is that GP63 cleaves

which is required for the initiation of translation (Haghighat et al., 1995). We therefore wished to confirm that *L. major*-inducible 4E-BP1 dephosphorylation was accompanied by increased affinity for eIF4E. After M $\phi$  infection with *L. major*, 4E-BP1 binding to cap-bound eIF4E was augmented (Figure 2E, upper panel). This increase was accompanied by a dramatic reduction in the association of eIF4G1 with cap-bound eIF4E (Figure S2D). Densitometric analysis of the bands detected by western blotting revealed a 2-fold increase in the amounts of 4E-BP1 bound to eIF4E in infected M $\phi$  as compared to untreated cells. As a control, we used the mTORC1 inhibitor rapamycin, which is known to block the phosphorylation of 4E-BP1 and thereby cause its activation (Beretta et al., 1996a). As expected, a similar effect was caused by rapamycin (Figure 2E).

GP63 is required for M $\phi$  translation inhibition by *L. major*. Thus, we next investigated whether GP63 is necessary for 4E-BP1 dephosphorylation. Infection with *L. major* GP63 KO failed to cause 4E-BP1 dephosphorylation (Figures 3A and 3C),

mTOR, which would lead to reduced 4E-BP1 phosphorylation. The protease GP63 recognizes a consensus sequence in its targets: P<sub>1</sub>↓ P'<sub>1</sub>- P'<sub>2</sub>- P'<sub>3</sub>, where the arrow represents the site of cleavage, P<sub>1</sub> is preferentially a polar amino acid, P'<sub>2</sub> is a hydrophobic amino acid and P'<sub>3</sub> is a basic amino acid (Bouvier et al., 1990). mTOR possesses 13 potential GP63 cleavage sites (Table S1). M $\phi$  infection with *L. major* WT or GP63 rescue, but not with GP63 KO parasites, caused mTOR cleavage (Figures 3B and 3C). It should be noted that the antibody used to monitor mTOR does not recognize *L. major* TOR homologs because mTOR was only detected in M $\phi$  protein extracts (Figure S3C). Further evidence that 4E-BP1 dephosphorylation and mTOR cleavage are mediated by GP63 was obtained by showing that phenanthroline, a compound that inhibits GP63 activity (Tzinia and Soteriadou, 1991), prevents M $\phi$  4E-BP1 dephosphorylation and mTOR cleavage by supernatant of *L. major* WT cultures (Figure 3D). Supernatant from *L. major* GP63 KO cultures (preincubated or not with phenanthroline)



**Figure 4. 4E-BP1/2 Activity, but Not S6K1/2, Is Required for *Leishmania major* Survival**

(A and B) B10R M $\phi$  (A) and BMM $\phi$  isolated from WT and 4E-P1/2 DKO BALB/c mice (B) were treated or not with 20 nM rapamycin and were infected with *L. major*-LUC for 6 hr, washed three times with PBS and incubated for 24 hr in cell culture media. Parasite burden was calculated by measuring luciferase activity.

(C) BALB/c mice were treated with rapamycin or vehicle alone injected i.p. and were infected i.p. with *L. major* WT, *L. major* GP63 KO, or *L. major* GP63 rescue. Parasite burden in resident PM $\phi$  was determined as described in the Experimental Procedures. Results are representative of two independent experiments (mean + SEM, n = 3 mice).

(D and E) BMM $\phi$  isolated from WT, 4E-BP1/2 DKO (D) and S6K1/2 DKO (E) mice were infected with *L. major*-LUC, as described in (A), and parasite burden was calculated.

In (A), (B), (D), and (E), data represent one of three independent experiments (mean + SEM, n = 3). Statistically significant differences (\*) were considered when p < 0.05. See also Figure S4.

injected with rapamycin (or vehicle) and infected (i.p.) with *L. major* WT, *L. major* GP63 KO or *L. major* GP63 rescue. In agreement with the ex vivo data, rapamycin promoted parasite survival in peritoneal M $\phi$  (PM $\phi$ ) in situ (Figure 4C).

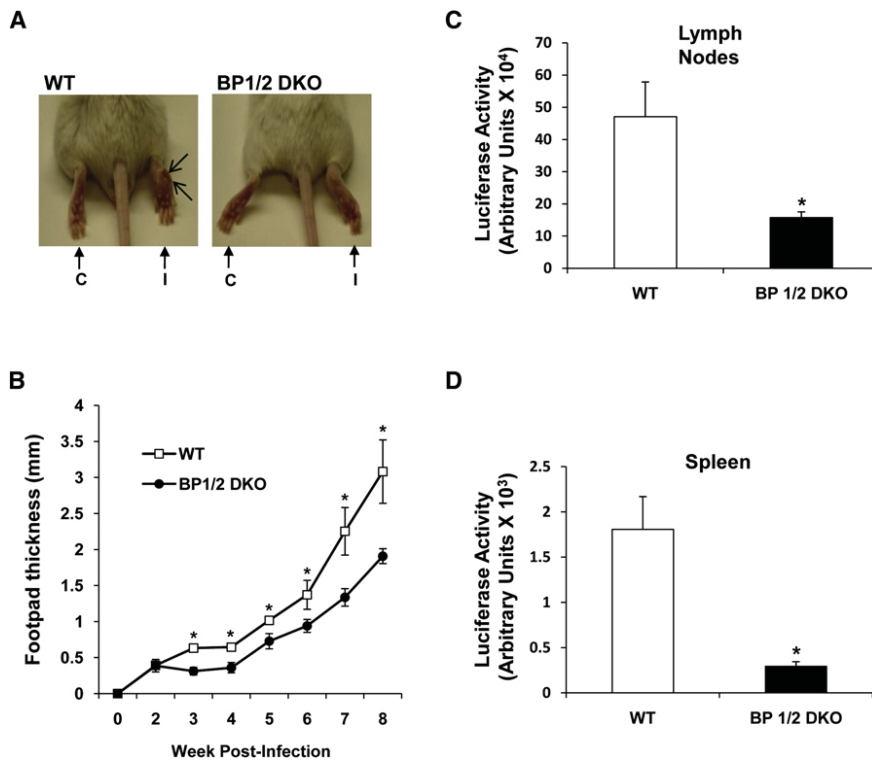
Given that enhanced 4E-BP1 activity favors parasite survival, it is anticipated that the lack of 4E-BPs would confer resistance to parasite replication. To test this hypothesis, we infected WT and 4E-BP1/2 DKO BMM $\phi$  with *L. major*-LUC. A significant decrease (~45%) in parasite load was observed at 24 hr postinfection in cells derived

from 4E-BP1/2 DKO mice (Figure 4D). Time course experiments revealed that at 6 hr postinfection there were no differences in the amount of parasites internalized by WT and 4E-BP1/2 DKO BMM $\phi$  (Figure S4A). In contrast, from 24 to 72 hr after infection, parasite numbers were reduced in 4E-BP1/2 DKO BMM $\phi$  (Figure S4B). A similar reduction was detected in PM $\phi$  isolated from 4E-BP1/2 DKO mice, either susceptible (BALB/c) or resistant (C57BL/6) to *L. major* infection (Figures S4C and S4D). These data provide conclusive evidence that the absence of 4E-BP1/2 impairs *L. major* survival in M $\phi$ , regardless of the host genetic background and M $\phi$  origin. In addition to 4E-BPs, S6K1/2 are other major downstream targets of mTORC1. Thus, we investigated the ability of *L. major* to replicate in the absence of S6K1/2. S6K1/2 DKO BMM $\phi$  exhibited a similar susceptibility to parasite replication as WT (Figure 4E), demonstrating that 4E-BP1/2, and not S6K1/2, are the critical players downstream of mTORC1 in the establishment of *L. major* infection.

### ***Leishmania major* Survival in the Macrophage Is Dependent on 4E-BP1/2**

We next asked whether 4E-BP1 activation by *L. major* contributes to the survival of the parasite inside the host cell. To test this, we treated M $\phi$  with rapamycin and infected with *L. major*-LUC. Rapamycin treatment increased parasite load in B10R M $\phi$  (80%; Figure 4A). Furthermore, rapamycin enhanced parasite burden only in WT (>50%), but not in 4E-BP1/2 DKO primary BMM $\phi$  (Figure 4B). Hence, activation of 4E-BPs by rapamycin promotes parasite survival. To replicate the ex vivo data in vivo, mice were intraperitoneally (i.p.)

injected with rapamycin (or vehicle) and infected (i.p.) with *L. major* WT, *L. major* GP63 KO or *L. major* GP63 rescue. In agreement with the ex vivo data, rapamycin promoted parasite survival in peritoneal M $\phi$  (PM $\phi$ ) in situ (Figure 4C).



**Figure 5. 4E-BP1/2 DKO Mice Are Less Susceptible to *Leishmania major* Infection**

Groups of five to six BALB/c mice either carrying (WT) or lacking (DKO) the *Eif4e-bp1* and *Eif4e-bp2* genes were infected subcutaneously in the right hind footpad with  $5 \times 10^6$  *L. major*-LUC promastigotes.

(A) Representative pictures of the footpad inflammation and the necrotic cutaneous lesions monitored in WT (left image) and 4E-BP1/2 DKO (right image) mice after 8 weeks of infection. C, control; I, infected. Necrotic cutaneous lesions are pointed out by arrows.

(B) Cutaneous leishmaniasis progression was monitored for 8 weeks by measuring the increase in footpad thickness.

(C and D) The popliteal draining LN (C) and the spleens (D) were extracted from WT and 4E-BP1/2 DKO mice infected for 8 weeks and the parasite load was determined by luciferase activity. Results are representative of two independent experiments (mean + SEM, n = 5–6 mice). Statistically significant differences (\*) were considered when  $p < 0.05$ .

**Genetic Deletion of 4E-BP1/2 Confers Resistance to Cutaneous Leishmaniasis**

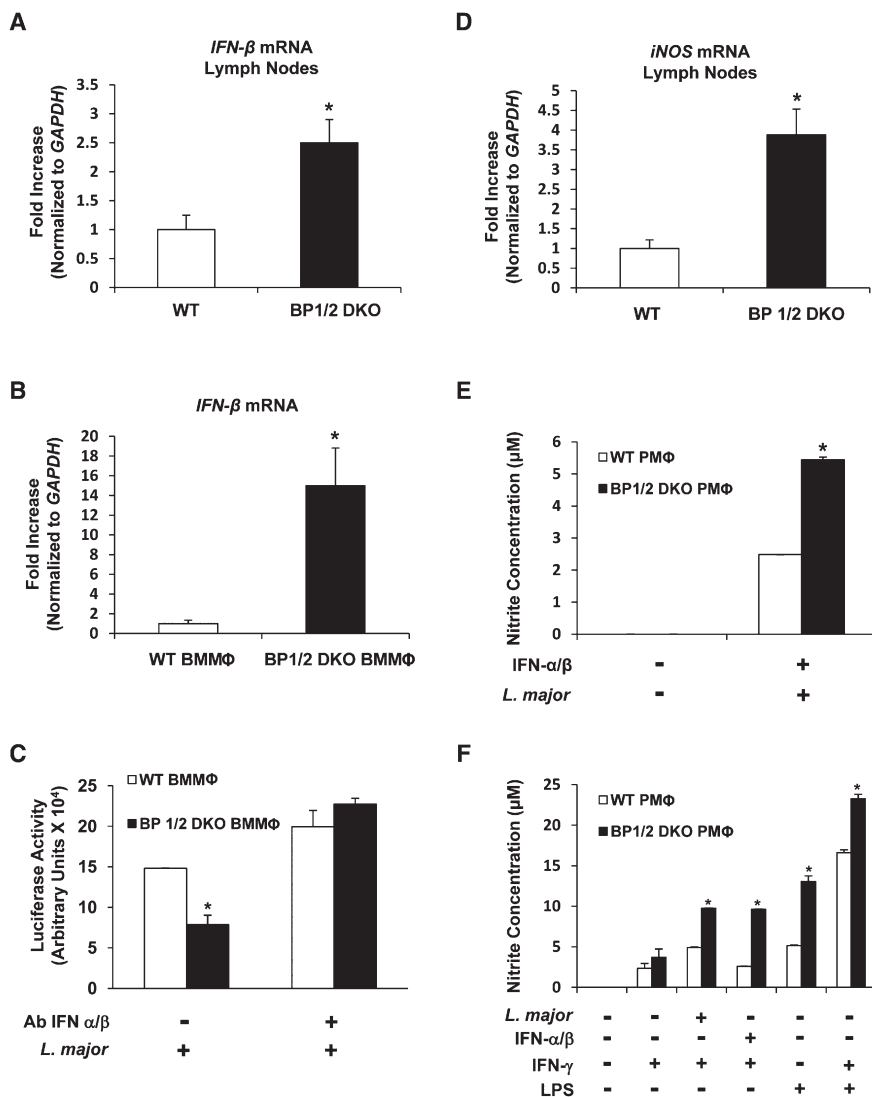
To determine whether 4E-BP1/2 play a role in *L. major* infection in vivo, we employed a mouse model of cutaneous leishmaniasis (Gomez et al., 2009). This model is characterized by footpad swelling and the appearance of necrotic cutaneous lesions at the site of inoculation that do not heal in susceptible mice (BALB/c). WT and 4E-BP1/2 DKO BALB/c mice were infected in the hind footpad with *L. major*-LUC and disease progression was monitored for 8 weeks. WT mice displayed necrotic lesions beginning at 4 and 5 weeks postinfection (Figure 5A). In marked contrast, 4E-BP1/2 DKO mice did not show any perceptible necrotic footpad tissue, even at 8 weeks postinfection (Figure 5A). WT mice developed severe inflammation after 3 weeks, which exacerbated with time, as determined by measurement of footpad thickness (Figure 5B). In the infected footpad from WT mice, the degree of inflammation increased by 2-fold between weeks 2 and 3 and reached up to 8-fold from week 2 to week 8 postinfection. In sharp contrast, inflammation in the footpads from 4E-BP1/2 DKO mice remained low and stable from week 2 to week 4 and was only half of that of the WT by week 8. The reduced footpad inflammation in DKO mice correlated with changes in parasite burden. The parasite load (as determined by luciferase activity) in popliteal draining lymph nodes (LNs) and spleens from 4E-BP1/2 DKO mice was dramatically lower (~70% and ~80%, respectively) than in the WT controls after 8 weeks of infection (Figures 5C and 5D). In contrast to WT mice, in which *L. major* visceralized to the spleen, luciferase activity was barely detectable in the spleen of 4E-BP1/2 DKO mice, indicating containment of the parasite to the infection site. Taken together, these data

show that deletion of 4E-BP1/2 precludes the progression of cutaneous leishmaniasis.

**Lack of 4E-BP1/2 Facilitates the Immune Response against *Leishmania major***

We next sought to determine the mechanism by which the lack of 4E-BP1/2 prevents the progression of *Leishmania* infection. We have previously demonstrated that the virus-resistant phenotype in mice and cells lacking 4E-BP1/2 was due to increased type I IFN production (Colina et al., 2008). Therefore, we investigated whether elevated levels of type I IFN were associated with the resistance of 4E-BP1/2 DKO mice to *L. major* infection. A key step in the inhibition of cutaneous leishmaniasis progression is the confinement of the parasite to the site of infection and to the popliteal draining LN (Laskay et al., 1995), where type I IFN and inducible nitric oxide synthase (iNOS) are locally produced (Diefenbach et al., 1998; Mattner et al., 2004; Stenger et al., 1994). Expression of *IFN-β* mRNA increased ~2-fold in the popliteal draining LN from *L. major*-infected 4E-BP1/2 DKO mice, as compared to the WT (Figure 6A). Consistent with these data, expression of *IFN-β* mRNA was enhanced in naive 4E-BP1/2 DKO BMMφ (~15-fold), as compared to their WT counterparts (Figure 6B). Neutralizing antibodies against IFN-α/β promoted parasite replication (Figure 6C), indicating that the parasite-resistant phenotype of 4E-BP1/2 DKO cells was due, at least in part, to elevated type I IFN. These data demonstrate that type I IFN is: (1) upregulated in *L. major*-infected 4E-BP1/2 DKO mice and (2) is involved in the *L. major*-resistant phenotype displayed by 4E-BP1/2 DKO mice and cells.

How does type I IFN inhibit *L. major* replication? Nitric oxide (NO) plays a key role in the innate immune response against



**Figure 6. A Type I IFN-Mediated Immune Response against *Leishmania major* Is Enhanced in Absence of 4E-BP1/2**

(A and D) Total RNA was extracted from the popliteal draining LN of WT and 4E-BP1/2 DKO mice after 4 weeks of cutaneous infection with *L. major*-LUC. The levels of *IFN- $\beta$*  (A) and *iNOS* (D) mRNA were examined by RT-qPCR.

(B) BMM $\phi$  were isolated from WT and 4E-BP1/2 DKO mice and after total RNA extraction, *IFN- $\beta$*  mRNA was quantified by RT-qPCR.

(C) WT and 4E-BP1/2 DKO BMM $\phi$  were exposed or not to 10 U/ml neutralizing antibodies against IFN- $\alpha$  and IFN- $\beta$ . Subsequently, cells were infected with *L. major*-LUC. Parasite counts were calculated by conducting luciferase assays.

(E) PM $\phi$  from WT and 4E-BP1/2 DKO mice were exposed to 250 U/ml IFN- $\alpha/\beta$  + *L. major*. Forty eight to 72 hr poststimulation cell supernatants were collected and NO production was measured as the accumulation of nitrites using the colorimetric Greiss reaction.

(F) PM $\phi$  from WT and 4E-BP1/2 DKO mice were exposed to different stimuli as follows: 500 U/ml IFN- $\gamma$ ; 500 U/ml IFN- $\gamma$  + *L. major*; 500 U/ml IFN- $\gamma$  + 250 U/ml IFN- $\alpha/\beta$ ; 100 ng/ml *E. coli* LPS; 20 ng/ml *E. coli* LPS + 100 U/ml IFN- $\gamma$ . NO production was evaluated as described above.

In (A) and (D), data are representative of two separate experiments (mean + SEM, n = 5–6 mice). In (B), (C), (E), and (F), results are representative of two experiments (mean + SEM, n = 3). Statistically significant differences (\*) were considered when p < 0.05.

See also Figure S5.

*Leishmania* (Bogdan et al., 2004). Type I IFN promotes *iNOS* expression and NO production, thus protecting mice and M $\phi$  from *Leishmania* infection (Diefenbach et al., 1998; Mattner et al., 2000; Mattner et al., 2004). Because 4E-BP1/2 DKO mice and M $\phi$  exhibit an enhanced type I IFN response, it was pertinent to determine *iNOS* expression in mice lacking 4E-BP1/2. *iNOS* mRNA levels were dramatically increased (~4-fold) in the LN of *L. major*-infected 4E-BP1/2 DKO mice as compared to WT (Figure 6D). Similarly, 4E-BP1/2 DKO PM $\phi$  produced more NO (>40%) than WT controls when costimulated with type I IFN and *L. major* (Figure 6E). To further study the effect of 4E-BP1/2 deletion on M $\phi$  activation, WT and 4E-BP1/2 DKO PM $\phi$  were subjected to different proinflammatory stimuli: *E. coli* LPS alone, IFN- $\gamma$  alone, IFN- $\gamma$  + *L. major*, IFN- $\gamma$  + IFN- $\alpha/\beta$ , IFN- $\gamma$  + LPS. In all cases, except for IFN- $\gamma$  alone, NO levels were higher in 4E-BP1/2 DKO PM $\phi$  as compared to WT cells (Figure 6F). These data are consistent with the findings that type I IFN is necessary for NO production in response to LPS (Weinstein et al., 2000)

and that it acts in synergy with IFN- $\gamma$  for maximal NO generation (Mattner et al., 2000; Mattner et al., 2004). Thus, it is likely that in addition to their role in *L. major* infection, 4E-BPs might be important factors in the progression of other nonviral infections, including other protozoan parasites and intracellular bacteria.

## DISCUSSION

In the current study, we provide evidence that the intracellular protozoan parasite *L. major* subverts the translation machinery of the M $\phi$  through activation of the translational repressor 4E-BP1. Such a strategy is shared with some viruses (e.g., poliovirus and encephalomyocarditis virus [EMCV]) whose infection leads to 4E-BP1 dephosphorylation (Gingras et al., 1996). However, the molecular mechanism remains unknown. We describe a mechanism of 4E-BP1 activation that involves mTOR cleavage and the consequent inhibition of mTORC1 by *L. major*. During poliovirus infection, 4E-BP1 activation is accompanied by cleavage of eIF4G (Gradi et al., 1998). This renders the eIF4F complex inactive for cap-dependent translation of cellular mRNAs while remaining functional for translation of uncapped viral mRNAs (Scheper et al., 1992). In contrast to viruses,

*Leishmania* possesses its own translation initiation machinery (Yoffe et al., 2009). However, by sequestering eIF4E, the parasite inhibits the assembly of the eIF4F complex of the M $\phi$  and promotes its own survival. Thus, *L. major* should repress translation of microbicidal and immune-responsive M $\phi$  mRNAs via activation of 4E-BP1. In support of this idea, mice and M $\phi$  lacking 4E-BP1/2 showed reduced susceptibility to parasite infection, whereas pharmacological activation of 4E-BPs by rapamycin promoted *L. major* replication ex vivo and in vivo. Accordingly, rapamycin increases the intracellular load of *L. amazonensis* in PM $\phi$  (Pinheiro et al., 2009). Rapamycin also enhances viral replication during poliovirus and EMCV infection and augments the shutoff of host protein synthesis (Beretta et al., 1996b). Thus, the combined effect of rapamycin and the virus/parasite in 4E-BP1 activation appears to favor the survival of intracellular pathogens that do not require the host eIF4F complex to translate their own mRNAs.

Inhibitors of mTORC1, including rapamycin, block cell proliferation (Dowling et al., 2010). Because TOR homologs are present in *L. major* (Madeira da Silva and Beverley, 2010), it is conceivable that rapamycin represses parasite replication. However, rapamycin treatment does not affect either growth or proliferation of *L. major* promastigote cultures (Madeira da Silva et al., 2009). In addition, we and others (Pinheiro et al., 2009) observed that rapamycin exerts a positive effect on parasite survival in the M $\phi$ . A likely explanation for this discrepancy is that the TOR isoforms identified in *L. major* bear a Trp substitution at the residue corresponding to mTOR Ser<sup>2035</sup> (Madeira da Silva and Beverley, 2010), which is required for FKBP12/rapamycin binding to mTOR (Chen et al., 1995). Therefore, it is not surprising that *L. major* replication is not inhibited by rapamycin.

Our data show that type I IFN is involved in the protective phenotype against *L. major* in 4E-BP1/2 mice and M $\phi$ . IFN- $\gamma$  (type II IFN) also contributes to the immune response against *Leishmania* (Olivier et al., 2005). However, the LN of *L. major*-infected WT and 4E-BP1/2 DKO mice contained similar amounts of IFN- $\gamma$  mRNA (Figure S5). Consistent with these findings, we previously demonstrated through a comparative microarray analysis that there are no differences in the levels of IFN- $\gamma$  mRNA between WT and 4E-BP1/2 DKO cells, whereas those of IFN- $\alpha$  and IFN- $\beta$  are significantly increased in 4E-BP1/2 DKO cells. We also showed that this is caused by the translational derepression of *Irf7* mRNA (Colina et al., 2008). Importantly, IRF-7 was recently described as a key component of the innate microbicidal activity against *Leishmania* (Phillips et al., 2010). Thus, we conclude that the resistance to *L. major* in absence of 4E-BP1/2 is, at least in part, mediated by an enhanced type I IFN immune response.

The *Leishmania* surface protease GP63 cleaves several proteins. This can result in the activation of the targeted proteins, e.g., the phosphatases PTP1B and SHP-1 (Gomez et al., 2009). However, it can also render them inactive, such as the c-Jun subunit of the transcription factor AP-1 (Contreras et al., 2010). Through these events, GP63 attenuates the innate immune response against the parasite. We show that GP63 is responsible for mTOR cleavage and mTORC1 inactivation in *L. major*-infected M $\phi$ . Interestingly, mTORC2 activity was also affected, as evidenced by a significant reduction in Akt Ser<sup>473</sup> phosphorylation in LPS-stimulated cells (Figure S3D). This observation is

consistent with a previous report showing that *L. donovani* infection decreases LPS-mediated Akt Ser<sup>473</sup> phosphorylation in PM $\phi$  (Dey et al., 2007). While mTOR degradation has been described in response to phospholipids (Fu et al., 2009), here we provide an example of mTOR proteolysis by a pathogen component. Thirteen potential GP63 target sites were identified in mTOR, suggesting that GP63 could cleave mTOR directly. However, we cannot rule out the possibility that through the activation of another target, GP63 indirectly leads to mTOR degradation. Further investigation will shed light on the molecular mechanism of mTOR cleavage by *L. major*.

Parasites that lack GP63 do not inhibit M $\phi$  translation initiation. Thus, we uncovered a key role for GP63 in the inactivation of mTORC1 signaling and inhibition of M $\phi$  protein synthesis by *L. major*. It is noteworthy that *L. tarentolae*, a nonpathogenic species of *Leishmania* in which GP63 is catalytically inactive (Campbell et al., 1992), failed to cause a shift of mRNAs from heavy to light polysomes. These observations indicate that inhibition of host translation by the parasite via GP63 contributes to the pathogenesis of leishmaniasis. In conclusion, our data provide genetic and pharmacological evidence that translational control plays a crucial role in a parasite infection. In addition, these findings raise the interesting possibility that agents that block 4E-BP1/2 could be therapeutically useful as an approach for the treatment not only of leishmaniasis but also of other protozoan parasitic diseases.

## EXPERIMENTAL PROCEDURES

### Mammalian Cell Lines and Parasite Cultures

The mouse M $\phi$  cell lines were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). Promastigotes of the various *Leishmania* strains were maintained in SDM-79 medium at 25°C. Details are given in the Supplemental Experimental Procedures.

### Mice

Mice were kept in pathogen-free housing. The research involving animals was carried out according to the regulations of the Canadian Council of Animal Care and approved by the McGill University Animal Care Committee. Details are given in the Supplemental Experimental Procedures.

### Western Blot Analysis

Cells lysates were subjected to SDS-PAGE and the separated proteins transferred onto a PVDF membrane. Proteins were detected by Western Lightning-ECL. Details are given in the Supplemental Experimental Procedures.

### Chromatography on m<sup>7</sup>GDP-Agarose

B10R M $\phi$  were seeded in 75 cm<sup>2</sup> flasks (2.5 × 10<sup>6</sup> cells) and the next day were treated with 20 nM rapamycin or infected with *Leishmania* promastigotes (1:20) for 3 hr. Cell lysis was conducted in 200  $\mu$ l cold buffer containing 50 mM HEPES-KOH (pH 7.5), 150 mM KCl, 1 mM EDTA, 2 mM DTT, 0.2% Tween, 1 mM PMSF, 25  $\mu$ g/ml leupeptin, and 25  $\mu$ g/ml aprotinin. Protein extracts (0.5 mg) were incubated for 1 hr with m<sup>7</sup>GDP (Sigma-Aldrich)-coupled agarose-adipic resin (Sigma-Aldrich), 50  $\mu$ l packed beads were used per reaction and were prepared as previously described (Edery et al., 1988). Reactions were carried out in a final volume of 500  $\mu$ l lysis buffer. Beads were then spun down in a microcentrifuge (3000 rpm for 30 s) and washed three times in 1 ml lysis buffer. After a final centrifugation step, sample pellets (bound fractions) were resuspended in 50  $\mu$ l Laemmli sample buffer, boiled, and subjected to SDS-PAGE and western blotting. Band intensities were quantified with the image analysis software Image-J, a Java program inspired by NIH-Image (<http://rsb.info.nih.gov/ni-image/about.html>).

**Polysome Profile Analysis**

B10R M $\phi$  were cultured in 15 cm diameter plates and were infected with *Leishmania sp.* for 6 hr. Cells were washed with cold PBS containing 100  $\mu$ g/ml cycloheximide and collected by centrifugation at 1000 rpm for 10 min at 4°C. Pellets were lysed in hypotonic lysis buffer containing 5 mM Tris-HCl (pH 7.5), 2.5 mM MgCl<sub>2</sub>, 1.5 mM KCl, 100  $\mu$ g/ml cycloheximide, 2 mM DTT, 0.5% Triton X-100, and 0.5% sodium deoxycholate, and cell debris was removed by centrifugation at 13,000 rpm for 2 min at 4°C. Lysates were loaded onto 10% to 50% sucrose density gradients (20 mM HEPES-KOH [pH 7.6], 100 mM KCl, 5 mM MgCl<sub>2</sub>) and centrifuged in a Beckman SW40 rotor at 36,000 rpm for 2 hr at 4°C. After centrifugation, gradients were fractionated (30 s and 500  $\mu$ l per fraction) and the absorbance at 254 nm was continuously recorded using an ISCO fractionator (Teledyne, ISCO).

**Metabolic Cell Labeling**

B10R M $\phi$  were seeded in 24-well plates, and <sup>35</sup>S-methionine labeling was performed as described previously (Beretta et al., 1996a). After labeling, cells were washed in cold PBS and lysed in buffer containing 50 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 0.1 mM EGTA, 0.1%  $\beta$ -mercaptoethanol, and 1% NP-40 (v/v). Radioactivity incorporated into trichloroacetic acid (TCA, 5%)-precipitable material was measured.

**Extraction of Bone Marrow-Derived Macrophages**

Bone marrow cells were extracted from the femurs and tibias of uninfected mice between 6 and 8 weeks of age, as previously described (Gomez et al., 2009). In brief, cell differentiation into BMM $\phi$  was carried out by culturing them during 7 to 9 days in DMEM containing 30% L929 cell conditioning medium (LCCM) and 10% FBS, and fresh media was added after 5 days of culture.

**Isolation of Peritoneal Macrophages**

Six- to 8-week-old mice were i.p. injected with 1.5 ml of 3% thioglycollate Brewer modified medium (BD, Mississauga, ON, Canada). After 4 days, the cells that had been recruited into the peritoneal cavity were collected by i.p. injection and further recovery of 7 ml endotoxin-free PBS. Cells of the same genotype were pooled, counted, and seeded in 12-well plates (1  $\times$  10<sup>6</sup> cells/well) or in 25 cm<sup>2</sup> flasks (3.5  $\times$  10<sup>6</sup> cells). After 5 hr of culture, extensive washes with PBS were carried out to eliminate the nonadherent cells. Fresh media was added, and next day the peritoneal M $\phi$  (PM $\phi$ ) were used for experiments.

***Leishmania* Survival Assays In Vitro**

M $\phi$  were infected with stationary phase *L. major*-LUC promastigotes (1:10 ratio). After 6 hr of infection, the nonphagocytosed parasites were removed by three washes in PBS. Fresh media was added and cells were further incubated overnight. Parasite burden was calculated by measuring the luciferase activity. Details are given in the Supplemental Experimental Procedures.

**Intraperitoneal Rapamycin Injection and *Leishmania* Infection**

M $\phi$  were recruited to the peritoneal cavity of BALB/c mice (6 to 8 weeks old) by i.p. injection of 3% thioglycollate Brewer modified medium, as described above (Isolation of Peritoneal Macrophages). Four days later, animals were i.p. injected with 100  $\mu$ l 5 mg/Kg rapamycin prepared in the following vehicle solution: 0.9% saline containing 5% Tween-80 and 5% PEG-400. As negative controls, mice were i.p. injected with 100  $\mu$ l vehicle solution. Mice were i.p. infected with *L. major* WT, *L. major* GP63 KO, or *L. major* GP63 rescue promastigotes (12  $\times$  10<sup>7</sup>). Twenty-four hours postinfection, the animals were sacrificed and the PM $\phi$  collected. Cells were allowed to adhere for 4 hr in 4-well cell culture slides (BD Falcon), three replicas per mouse. PM $\phi$  were washed three times in cold PBS, fixed in methanol, and Giemsa stained. For determination of parasite load, 300 PM $\phi$  per well were counted and monitored for parasite internalization. Counts were expressed as percentage of infected PM $\phi$ .

**Mouse Cutaneous Infections**

WT and 4E-BP1/2 DKO mice 6 to 8 weeks old were infected in the right hind footpad with *L. major*-LUC promastigotes, as previously described

(Gomez et al., 2009). Details are given in the Supplemental Experimental Procedures.

**Quantitative RT-PCR**

Total RNA was extracted from the organs of infected mice and from the M $\phi$ . RNAs were reverse transcribed (RT) and quantitative PCR was performed. Details are given in the Supplemental Experimental Procedures.

**Nitric Oxide Measurements**

WT and 4E-BP1/2 DKO PM $\phi$  were seeded in 12-well plates (1  $\times$  10<sup>6</sup> cells/well). The next day, cells were incubated for 48 to 72 hr with one of the following treatments in triplicate: 250 U/ml IFN- $\alpha/\beta$  + *L. major* (1:20); 250 U/ml IFN- $\alpha/\beta$  + 500 U/ml IFN- $\gamma$ ; 500 U/ml IFN- $\gamma$  + *L. major* (1:20); 100 ng/ml *E. coli* LPS; 500 U/ml IFN- $\gamma$ ; 20 ng/ml *E. coli* LPS + 100 U/ml IFN- $\gamma$ . Then, NO production was assessed by measuring the accumulation of nitrites in the cell culture medium with the colorimetric Griess reaction, as previously described (Gomez et al., 2009).

**Statistical Analysis**

Data were analyzed by one-way ANOVA. Statistically significant differences between groups were considered when  $p < 0.05$ . All data are presented as the mean + standard error of the mean (SEM).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at doi: 10.1016/j.chom.2011.03.008.

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