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ORIGINAL INVESTIGATION

Leishmania donovani chaperonin 10 regulates parasite internalization and intracellular survival in human macrophages

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Abstract Protozoa of the genus *Leishmania* infect macrophages in their mammalian hosts causing a spectrum of diseases known as the leishmaniases. The search for leishmania effectors that support macrophage infection is a focus of significant interest. One such candidate is leishmania chaperonin 10 (CPN10) which is secreted in exosomes and may have immunosuppressive properties. Here, we report for the first time that leishmania CPN10 localizes to the cytosol of infected macrophages. Next, we generated two genetically modified strains of *Leishmania donovani* (Ld): one strain overexpressing CPN10 (CPN10+++) and the second, a CPN10 single allele knockdown (CPN10+/−), as the null mutant was lethal. When compared with the wild-type (WT) parental strain, CPN10+/− Ld showed higher infection rates and parasite loads in human macrophages after 24 h of infection. Conversely, CPN10+++ Ld was associated with lower initial infection rates. This unexpected apparent gain-of-function for the knockdown could have been explained either by enhanced parasite internalization or by enhanced intracellular survival. Paradoxically, we found that CPN10+/− leishmania were more readily internalized than WT Ld, but also displayed significantly impaired intracellular survival. This suggests that leishmania CPN10 negatively regulates the rate of parasite uptake by macrophages while being required for intracellular survival. Finally, quantitative proteomics identified an array of leishmania proteins whose expression was positively regulated by CPN10. In contrast, many macrophage proteins involved in innate immunity were negatively regulated by CPN10. Taken together, these findings identify leishmania CPN10 as a novel effector with broad based effects on macrophage cell regulation and parasite survival.

Keywords Leishmania donovani · Host–pathogen interaction · Parasitic infection · Chaperonin · Heat shock protein · Virulence factor

Introduction

The leishmaniases—identified as neglected tropical diseases by the World Health Organization—are a group of diseases caused by protozoan parasites of the genus *Leishmania*. Flagellated and motile leishmania promastigotes replicate within the alimentary tracts of their sandfly vectors and are transmitted to their mammalian hosts when an infected sandfly takes a blood meal. Promastigotes are then taken up by mononuclear phagocytes, especially macrophages. After ingestion by phagocytosis, leishmania differentiate into non-flagellated amastigotes and replicate within acidified phagolysosomes. Macrophages are highly specialized phagocytic cells, whose primary function is to engulf and digest pathogens, dying cells and cell debris. Leishmania exploit macrophages to their benefit as they get internalized by phagocytosis, and use the cell as a safe haven, scavenging nutrients and replicating while *de facto* hidden from other immune effectors. The mechanisms by

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which leishmania survive within these powerful immune cells are beginning to be unraveled. It is believed that leishmania pathogenesis is related to the ability of the parasite to prevent macrophage activation—a necessary step to induce digestion and killing of macrophage prey—by hijacking critical intracellular signaling pathways [1–3]. Another requirement for leishmania is to withstand the extreme conditions within acidified phagolysosomes [4].

Upon transmission from the fly to mammalian hosts, leishmania experience heat shock and must respond accordingly. The heat shock response is a conserved homeostatic mechanism aimed at protecting cells against deleterious effects of heat and other environmental stressors. It is especially important in parasites such as leishmania because their digenetic life cycle requires adaptation to higher temperature, as well as other stressors such as hypoxia, nutrient deficiency, low pH and oxidative attack [5]. Among the cell stress response elements, heat shock proteins (HSPs) play a key role in adaptation and survival especially during transfer from the poikilothermic sandfly vector to a mammalian host. Many HSPs are molecular chaperones, which function to support the folding of nascent proteins, to direct the translocation of proteins to their correct cellular localization, and to facilitate the refolding of damaged proteins. Alternatively, when the latter are irreparable, chaperonins may direct them to the protein degradation machinery where they are degraded and recycled [6]. Heat shock proteins are, therefore, required for normal homeostasis, in addition to their role in response to different stressors.

The change in temperature that leishmania experience upon introduction into their mammalian hosts is accompanied by strong induction of HSPs [7]. Moreover, several heat shock proteins have been shown to support leishmania pathogenesis [7, 8]. For example, HSP100 was found to be essential for intracellular survival, but was dispensable for axenic growth of *Leishmania donovani*, both as promastigotes and amastigotes [9]. HSP90 (HSP83) was found to play a role in promastigote to amastigote differentiation, in the cellular stress response in *L. donovani* [10] and in intracellular survival [11]. HSP70 abundance was shown to correlate with the heat shock response and with resistance to macrophage induced oxidative stress in *Leishmania chagasi* [12]. In addition, the small heat shock protein HSP23 was found to be essential for thermotolerance and intracellular survival of *L. donovani* [11], and chaperonin 60 showed increased expression during in vitro promastigote to amastigote differentiation [13].

Type I chaperonins, such as CPN60 and CPN10, are essential for protein folding under both normal and stressful conditions. They are induced by stress, especially heat shock, where they contribute to the refolding of damaged or misfolded proteins [14]. The bacterial GroES is the best characterized CPN10 homolog, and was used to characterize the structure and mode of action of the GroES/GroEL complex (CPN10/CPN60) [15]. This chaperonin complex consists of a cylindrical cage made of two rings of seven subunits each of GroEL and a lid made of seven subunits of the co-chaperonin GroES, interacting in an ATP-dependent manner. The main function of the complex is to assist with the folding of newly synthesized proteins and in the refolding of denatured proteins, as well as to prevent intermolecular aggregation by sequestering unfolded proteins in the cage-like structure. It is estimated that approximately 12% of all newly synthesized proteins in *E. coli* associate with this chaperonin complex, and that this increases two to threefold during heat shock [16].

In humans, CPN10 was first described as early pregnancy factor, an extracellular protein with immunosuppressive and growth factor properties, necessary for embryonic survival and detected in serum of pregnant women by the rosette inhibition test [17, 18]. Thus, it appears that despite the fact that the CPN10/CPN60 complex is found in relative abundance in the mitochondrial matrix of eukaryotic cells, CPN10 is neither exclusively mitochondrial nor even intracellular, and any differences between secreted and intracellular CPN10, and the secretion system involved are not known.

Notably, CPN10 was shown to have immunosuppressive activity for murine macrophages and human peripheral blood mononuclear cells where it attenuated lipopolysaccharide-induced NF-kB activation and secretion of inflammatory cytokines [19]. Recombinant CPN10 also inhibited inflammatory responses in rats and mice with experimental autoimmune encephalomyelitis as well delayed-type hypersensitivity [20, 21]. CPN10 from *Mycobacterium tuberculosis* has also been shown to downregulate several animal models of autoimmunity [22, 23]. A recombinant form of CPN10, known as XToll (CBio Ltd) is currently being tested in phase IIa clinical trials of rheumatoid arthritis, psoriasis and multiple sclerosis [24]. However, these immunosuppressive activities do not appear to be conserved across all species of CPN10. Notably, HSP10 of *Chlamydophila pneumoniae* was found to induce proinflammatory cytokines in THP-1 cells [25].

In leishmania, CPN10 is found primarily in the mitochondrion in a complex with CPN60. Its expression is significantly increased upon heat stress and in axenic amastigotes as compared to promastigotes in *L. donovani* [26]. This suggests a role for CPN10 in the differentiation to the amastigote stage. Moreover, CPN10 was among the proteins identified in the secretome of leishmania [27] as well as in leishmania exosomes, the latter having been show to induce a suppressive macrophage phenotype, permissive for infection [28, 29].

Based upon this array of findings supporting immunemodulatory roles for CPN10 in a variety of settings, in the

current study, we asked whether leishmania CPN10 plays a role in pathogenesis. To study the role of leishmania CPN10 in infection, we generated two genetically modified *leishmania donovani* (Ld) strains expressing more or less of CPN10: a single allele knock-out (CPN10+/− Ld) and a CPN10 overexpressing strain (CPN10+++). Unexpectedly, CPN10+/− Ld showed higher infection rates than wild-type (WT) Ld, while CPN10+++ displayed lower infection rates than the control strain. However, when macrophages were lysed after infection, CPN10+/− Ld were significantly impaired in their survival and recovery, as compared to WT. Quantitative proteomic analyses identified a diverse array of leishmania proteins whose expression was positively regulated by CPN10. Conversely, many proteins involved in innate immunity, intracellular trafficking and transport were negatively regulated in macrophages infected with WT leishmania as compared with CPN10+/− Ld. These findings which are discussed in detail below validate important roles for CPN10 in leishmania pathogenesis and identify it as an attractive target for further study.

Materials and methods

L. donovani strains and culture

Leishmania donovani Sudan strain 2S was obtained from Dr Kwang Poo Chang (at the time Rockefeller University, NY, USA), routinely cultured in the lab, and virulence was maintained by regular passages through Syrian Golden hamster from which fresh amastigotes were purified and transformed in vitro into promastigotes. Promastigotes were cultured at 26 °C in M199 (Sigma–Aldrich) complemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 10 mM HEPES (Stemcell), 6 μg/ml hemin (Sigma–Aldrich), 10 μg/ml folic acid (Sigma–Aldrich), 2 mM L-Glutamine (Stemcell), 100 U/ml penicillin/streptomycin (Stemcell) and 100 mM adenosine (Sigma–Aldrich). The parasites were subcultured every 3 days for a maximum of 20–25 passages.

Axenic amastigotes were obtained by placing stationary phase promastigotes in complete M199 (as described above) at 37 °C, 5% CO_2 for 16–18 h, at 1×10^6 cells/ml. The cells were then spun down at 2000 RPM for 10 min and resuspended in acidic medium. This medium consisted of RPMI-1640 (HyClone) with 25% heat-inactivated fetal bovine serum (FBS), 2 mM L-Glutamine (Stemcell), 100 mM adenosine (Sigma–Aldrich), 100 U/ml penicillin/streptomycin (Stemcell) and 20 mM MES buffer (Sigma–Aldrich). The pH of this medium was adjusted to pH 5.5. Axenic amastigotes were cultured for a maximum of 5 days in pH 5.5 medium at 37 °C, 5% CO_2.

Ethics statement

All animals were handled according to The Canadian Council on Animal Care (CCAC) guidelines, and prior approval for animal experiments was obtained from the Animal Care Committee of University of British Columbia (protocol approval# A14-00218).

Anti-CPN10 antibody

Anti-leishmania CPN10 antibodies were raised by Genemed Synthesis, Inc (San Antonio, Texas, United States), using a CPN10 peptide (H-Cys-Val-Glu-Gly-Glu-Glu-Leu-Phe-Leu-Tyr-Asp-Glu-Ser-Val-Leu-Leu-Gly-Val-Leu-Ser-Ser-OH) as an antigen in two rabbits. Serum was tested for the specificity using total cell lysates of THP-1 cells and Ld promastigotes in a Western blot assay and was found to be specific for leishmania CPN-10. Protein A Sepharose was used to purify IgGs.

Generation of CPN10 knockdown and CPN10 over expressing *L. donovani*

A single allele knockout was generated using a specific construct. *L. donovani* CPN10 is encoded by two genes (GeneDB accession numbers LdBPK_260590.1 and LdBPK_260610.1) situated on both sides of an unknown gene (GeneDB LdBPK_260600.1). In order not to delete this gene of unknown function together with the two CPN10 genes, a construct carrying the LdBPK_260600.1 gene and a puromycin resistance gene flanked by approximately 1000 bp of the 5′ and 3′ UTRs of the *L. donovani* CPN10 genes was used to replace the whole region and reintroduce the LdBPK_260600.1 gene simultaneously.

Genomic DNA of *L. donovani* was extracted with Gentra Systems Puregene Tissue Core Kit A (Qiagen). A large fragment containing LdBPK_260600.1 was amplified using CPN10-P1fwd and CPN10-P2rev primers that added BamHI and XbaI sites. This fragment was digested using BamHI and SalI and ligated into pUC19 vector previously digested with the same enzymes. The primers CPN10-P3rev and CPN10-P4fwd were used to delete approximately 1000 bp of pUC-260600 by PCR, and the PUC19-260600 vector was recircularized with ligase.

The UTRs of CPN10 were amplified by PCR: CPN10-5′UTR ahead of LdBPK_260590 with the primers CPN10-P5fwd and CPN10-P6rev that added EcoRI and KpnI restriction sites to the fragment, while CPN10-3′UTR behind LdBPK_260610 with the primers CPN10-P7fwd and CPN10-P8rev added SalI and HindIII sites. The amplified fragments were digested with EcoRI/KpnI and SalI/HindIII and ligated in pUC19 vector previously digested with the same enzyme pairs to obtain the constructs

pUC19-CPN10-5′UTR and pUC19-CPN10-3′UTR. The 5′UTR was then cut out using EcoRI and KpnI digestion, ligated into pUC19-260600 digested with the same enzyme pair to obtain pUC19-CPN10-5′-260600. Similarly, the 3′UTR was cut out of the puC19 using SalI and EcoRI and ligated into pUC19-CPN10-5′-260600 to yield pUC19-CPN10-5′-260600-CPN10-3′. A puroAC gene was digested with KpnI and BamHI out of a pUC-PAC vector and ligated into the construct previously digested with the same enzyme pair to yield pUC19-CPN10-5′-puroAC-260600-CPN10-3′.

Primers and sequences:

CPN10-P1fwd: GGGAAGGATCCGAAACGGGTG GAGAAGAGAC
CPN10-P2rev: GGGAGTCTAGAGCGACTGGTGCGT CCCCCTC
CPN10-P3rev: ACATCAATTTCCGCACCACC
CPN10-P4fwd: GGCGAGTGTGGTCGTCATG
CPN10-P5fwd: GGGGAATTCATTTAAATTACACAG ATCGATGCACCGAG
CPN10-P6rev: GGGGGTACCGAGAAAAAGTTGTAA AGGTTTAGG
CPN10-P7fwd: GGGGTCGACGGTGGAGAAGGG ACCGACAC
CPN10-P8rev: GGGGTCGACATTTAAATCCATATC AAGAGAGAATACG
CPN10-P9fwd: GGGGGTACCATGCTCCGCTTCACC ATCC
CPN10-P10rev: GGGAGATCTTCAGCTTGACAG CACGCCAAG

The plasmids were extracted from transformed E. coli DH5a, and purified using CsCl continuous gradient centrifugation [30]. The pUC19-CPN10-5′-puroAC-260600-CPN10-3′ plasmid was linearized with SwaI and digestion fragments were separated by agarose electrophoresis and purified. The targeting fragments were transfected into log phase promastigotes by electroporation as described previously [31]. Briefly, stationary phase promastigotes were washed two times with ice-cold PBS and once with electroporation buffer, before being resuspended at 1×10^8 cells/ml in electroporation buffer. 2 µg of linearized DNA was mixed on ice with 400 µl of cell suspension. The electroporation was then performed using the program U-033 on an Amaxa Nucleofector (Lonza). Mock transfection was done in parallel in the absence of DNA to obtain a negative control of antibiotic selection. Cells were kept on ice for 10 min and transferred to culture flasks containing 10 ml of complete M199. Antibiotics were added after 24 h of incubation at 26 °C (puromycin at 25 µg/ml and G418 at 35 µg/ml), and cultivation continued until the mock-transfected population was completely eliminated. To select for individual clones, parasites were seeded at 0.5 cells per well in a 96-well plate. After 10–14 days, the content of wells in which growth was detected were transferred to culture flasks.

For over expression, the CPN10 open reading frame (LdBPK_260610.1) was PCR amplified using primers CPN10-P9fwd and CPN10-P10rev adding KpnI and BglIII sites to either side of the open reading frame. The PCR fragment was digested with these enzymes and ligated into pCL2N, a variant of pCLN [11] with additional restriction sites in the multiple cloning region, digested with KpnI and BglIII to yield pCL2N-CPN10. The circular plasmid was transfected as an episomal vector into log phase promastigotes by electroporation (5 µg of plasmid DNA per 1×10^8 cells).

WT leishmania was the parasite strain used to create the other three strains. Moreover, they were submitted to the same procedures as the other strains: mock transfection, freeze/thaw cycles and in vitro passages.

After checking for expression of CPN10 by Western blot, aliquots of each newly created leishmania were frozen down, and fresh batches of leishmania were thawed every two months, in order not to exceed 20–25 passages.

Purification and culture of human cells

The THP-1 monocytic cell line was purchased from ATCC, and cultured in RPMI-1640 (HyClone) supplemented with 10% heat-inactivated FBS (Gibco) and 2 mM L-Glutamine (Stemcell). THP-1 cells were subcultured every 2–3 days and kept between 0.2×10^6 and 1×10^6 cells/ml. Differentiation was induced using phorbol 12-myristate 13-acetate (PMA) at 10 ng/ml for 16–18 h. Attached cells were then washed three times with warm HBSS. Fresh supplemented RPMI without PMA was then added and the cells were rested for 24 h.

Human monocytes were purified from buffy coats of healthy donors by Ficoll density purification followed by plastic adherence, and differentiated into macrophages using GM-CSF. Buffy coats were diluted 1:2.5 with sterile PBS with 2 mM EDTA, and Ficoll was underlayered (GE Healthcare). Tubes were centrifuged for 20 min at 500×*g* at room temperature with brakes off. The mononuclear cell layer was collected, washed 3–4 times with PBS-2 mM EDTA to remove platelets. Cells were then resuspended in complete RPMI and allowed to adhere in 150 cm^2 culture flasks for 1 h at 37 °C. Flasks were carefully washed with HBSS 3 times to remove unattached cells. Complete RPMI was added to the flasks, and adherent cells were scraped off using a cell scraper. Türk's solution was used to stain the cell suspension and count monocytes. Cells were then seeded into 6-well plates (1.5×10^6 cells per well in 2 ml) or 24-well plates containing sterilized glass coverslips (0.3×10^6 cells per well in 0.5 ml), in complete

RPMI containing 10 ng/ml human GM-CSF. Plates were incubated for 3 days at 37 °C with 5% CO_2. Then cells were washed three times with warm HBSS, and fresh RPMI with 10 ng/ml GM-CSF was added. After another 3 days, cells were washed three times with warm HBSS, and fresh RPMI was added. Macrophages were used the day after.

In vitro infection experiments

Day 5 stationary phase promastigotes were counted, washed in HBSS, resuspended in HBSS and used to infect human cells at 10:1 and 20:1 multiplicities of infection (MOI).

Infection rates were assessed in infected cells grown on coverslips. At given timepoints, coverslips were washed three times with PBS and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. After fixation, coverslips were washed in PBS and mounted on glass slides using ProLong Diamond Antifade Mountant with DAPI (ThermoFischer). Fluorescence microscopy was performed using an Axioplan II epifluorescence microscope (Carl Zeiss Inc.), an AxioCam MRm Camera and the AxioVision software Version 4.8.2 (Carl Zeiss Inc.) to record images. Infection rates were obtained by taking at least ten images at 40X magnification per coverslip. DAPI stained both the macrophages nuclei and the leishmania nuclei and kinetoplast, which allowed the number of infected cells per total cells (infection rate) and the number of leishmania per infected macrophage (parasite load) to be counted on each image and the averages were calculated.

To assess intracellular survival and recovery after infection, a parasite rescue and transformation assay was performed [32]: differentiated macrophages were infected for 4h or 24h, after which the wells were washed three times with HBSS to remove unbound leishmania. The plates were then returned to the incubator. After 24h or 48h, the wells were washed three times with HBSS, and 150 µl of 0.01% SDS in HBSS was added to the wells to lyse the macrophages and free the leishmania from the phagolysosomes. After 30 min of incubation at 37 °C, the lysis of cells was visually verified under the microscope and 300 µl of complete M199 was added to each well. The plates were then incubated at 26 °C for 2 days, and the leishmania were counted.

Western blotting

Leishmania were washed two times with cold PBS, and resuspended in protein lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton-X-100, 1 mM EDTA, 2.5 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM PMSF, 5 µg/ mL aprotinin, 5 µg/mL leupeptin). After 5 min of incubation on ice, 4x Laemmli loading buffer was added and samples were boiled for 7 min. Samples were loaded on a 15% Tris-Tricine gel, run at 100 V followed by a semi-dry transfer to a PVDF membrane (37 mA for 90 min). Immunoblotting was performed using anti-Leishmania CPN10 antibody for 3 h at room temperature in 5% milk in TBS with 0.05% Tween-20. Anti-alpha tubulin (Sigma–Aldrich, T6074) was used for 2 h at room temperature in 5% milk in TBS with 0.1% Tween 20. After washings, blots were incubated with secondary Alexa Fluor 700 antibodies (anti-rabbit and anti-mouse, respectively, Life Technologies) for 1 h at room temperature, washed again and read using Odyssey CLx Imaging Systems (Li-Cor). Densitometry analysis was performed using ImageJ.

Immunofluorescence microscopy on infected macrophages

THP-1 cells were grown on coverslips and differentiated with PMA, as described above (Generation of CPN10 knockdown and CPN10 over expressing *L. donovani*). The cells were infected with WT *Leishmania donovani* for 18 h, at which point they were washed three times with PBS, and fixed in 2% PFA in PBS for 15 min at room temperature. The cells were then permeabilized using 0.3% Triton X-100 in PBS for 10 min. The coverslips were then washed three times in PBS 0.1% Tween-20 (PBST). A blocking step was performed using 1% BSA in PBST for 1 h, before an overnight incubation with the primary anti-leishmania CPN10 antibody. The coverslips were washed three times in PBST, and incubated for 1 h with anti-rabbit Alexa Fluor 594 (Life Technologies). The coverslips were washed again, and mounted on glass slides using ProLong Diamond Antifade Mountant with DAPI (ThermoFischer). The slides were then analyzed using a Zeiss LSM 780 confocal microscope (Carl Zeiss, Thornwood, NY). Controls for staining were performed using only the secondary antibody, as well as full staining with both primary and secondary antibodies on uninfected macrophages, both of which led to no detectable fluorescence.

Immunofluorescence microscopy on leishmania

Leishmania were washed twice in PBS, resuspended in PBS at 10×10^6 cells/ml. 100 µl of cell suspension was placed on top of sterile coverslips. 400 µl of ice-cold methanol was added on each coverslip. After 15 min of incubation at room temperature, the coverslips were washed with PBS + 0.1% triton X-100, and stored in PBS. For tubulin staining: the coverslips were blocked in 2% BSA 0.1% triton X-100 in PBS for 1 h at room temperature. Anti-tubulin (sigma) was used at 1:1000 in blocking buffer for 1 h at room temperature. The coverslips were washed three times with 0.1% triton X-100 in PBS.

Secondary anti-mouse FITC was used at 1:100 in blocking buffer, and incubated for 1 h at room temperature. The coverslips were washed three times with 0.2% triton X-100 in PBS, then once with PBS. Coverslips were then mounted on microscopy slides using DAPI-mounting media. Fluorescence microscopy was performed using an Axioplan II epifluorescence microscope (Carl Zeiss Inc.), an AxioCam MRm Camera and the AxioVision software Version 4.8.2 (Carl Zeiss Inc.) to record images.

Electron microscopy

Carbon coated 3-mm sapphire discs (Leica) were coated with poly-L-lysine for 30 min, rinsed five times with sterile water, and dried for 1 h at 37 °C. 5×10^4 THP-1 cells in supplemented RPMI with 10 ng/ml PMA were added on each disc, and incubated at 37 °C for 16–18 h. The discs were washed three times with Hanks and supplemented RPMI was added to the discs. After a 24 h incubation, *Leishmania donovani* were added at a MOI of 10:1. After 18 h of incubation, the cells were fixed with 4% paraformaldehyde in PBS for 30 min and, washed three times with PBS.

Samples were then high-pressure frozen using a Leica HPM 100 high pressure freezer and hexadecane as a space filler between the sapphire disk and the 100um side of a Leica type A HPF specimen carrier. The samples were then freeze-substituted in acetone using a Leica AFS freeze-substitution machine at −90 °C for 3 days, then infiltrated with and low-temperature embedded in UV polymerized HM20 resin at −50 °C. The samples were sectioned with a Leica UC7 ultramicrotome at 70 nm section thickness with sections deposited on 200 mesh formvar coated nickel grids.

The grids were immunolabeled using anti-LCPN10 antibodies. Grids were first incubated in 50 mM NH_4Cl for 30 min, then in PBS with 1% BSA and 0.05% Tween 20 (PBS-BSAT) for 1 h at room temperature. Grids were incubated with anti-LCPN10 in PBSBSAT for 1 h then washed three times for 5 min each in PBS-BSAT and incubated in secondary anti-rabbit antibody coupled to 15 nm gold particles for 1 h. Grids were rinsed for 10 min in PBS with 3% BSA, then 10 min in PBS with 1% BSA, then in PBS for 10 min and finally in distilled water for 10 min. A control using only secondary antibodies was prepared in parallel, as well as staining on uninfected samples, which did not show any labeling after incubation with anti-leishmania CPN10 and secondary antibodies. Grids were then dried and analyzed using a Hitachi H-7600 TEM operating at 80 kV and equipped with an AMT XR50 side mount CCD camera.

Leishmania stable isotope labeling by amino acids in cell culture (SILAC)

Leishmania were grown in special SILAC medium, prepared using powdered M199 minus L-Arginine and L-lysine with Earle's salts (Caisson labs), supplemented with 10% heat inactivated dialyzed fetal bovine serum (Gibco), 10 mM HEPES (Stemcell), 6 µg/ml hemin (Sigma–Aldrich), 10 µg/ml folic acid (Sigma–Aldrich), 2 mM L-Glutamine (Stemcell), 100 U/ml penicillin/streptomycin (Stemcell) and 100 mM adenosine (Sigma–Aldrich). The medium was then split into two, and the "light" medium was supplemented with normal isotopic abundance arginine (42 mg/l) and lysine (73 mg/l), while the "heavy" medium was supplemented with $^{13}C_6$-arginine (43.5 mg/l) and $^{2}H_4$-lysine (75 mg/l).

Fresh WT and CPN10+/− promastigotes (less than 2 passages) were spun down and resuspended in light and heavy SILAC medium, respectively. Selection pressure was maintained on CPN10+/− leishmania by adding 25 µg/ml of puromycin to their media. The leishmania were cultured at 26 °C, diluted 1:10 every 3–4 days, and their growth was monitored. After 2 weeks, during which the cells have gone through at least four doublings, stationary phase promastigotes were collected, spun down, washed three times in PBS, and pellets were frozen at −80 °C.

Macrophage SILAC

Special SILAC media was prepared using RPMI1640 minus L-Glutamine and L-Lysine (Caisson labs), supplemented with 10% heat inactivated dialyzed fetal bovine serum (Gibco) and 2mM L-glutamine. Light SILAC medium was made by addition of normal isotopic abundance arginine (42 mg/l) and lysine (73 mg/l), while heavy SILAC medium was supplemented with $^{13}C_6$-arginine (43.5 mg/l) and $^{2}H_4$-lysine (75 mg/l). Fresh THP-1 cells (less than 2 passages) were spun down and half of which was resuspended in each medium. THP-1 were cultured at 37 °C, subcultured every 3 or 4 days for a minimum of 5 passages. Cells were counted every day to monitor growth.

After 2 weeks, cells were differentiated using 10 ng/ml of PMA for 16–18 h, before being washed three times with HBSS and rested for 24 h in fresh supplemented SILAC media. On the day of infection, stationary phase WT and CPN10+/− promastigotes were spun down, washed two times in PBS, resuspended at 50×10^6 cells/ml in PBS. CFSE (Life Technologies) was added at 5 µM, and the cells were incubated at 37 °C for 10 min in the dark. Cells were washed two times in large volumes of PBS with 10% fetal bovine serum, and used to infect differentiated THP-1 (WT Ld for heavy THP-1, and CPN10+/− for light THP-1). After 24 h of infection, the plates were washed two times in

PBS, and THP-1 cells were collected using a cell scraper, spun down, and resuspended in PBS-2% fetal bovine serum. Samples were then run on a BD FACSAria cell sorter, and CFSE+ macrophages were collected as infected cells for each of the two samples. Cells were spun down and pellets were frozen at −80 °C.

Mass spectrometry

Cell pellets were lysed in RIPA buffer (1% NP40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris pH8) and proteins were quantified using BCA assay (Pierce). Fifteen microgram of protein from each label were mixed and run on a short 10% SDS–PAGE gel. Proteins were visualized by colloidal coomassie [33] and digested out of the gel as described [34]. Peptide samples were purified by solid phase extraction on C-18 STop And Go Extraction (STAGE) Tips [35], and run on LC-MS/MS (Bruker IMpact II Qtof).

Purified peptides were analyzed using a quadrupole—time of flight mass spectrometer (Impact II; Bruker Daltonics) online coupled to an Easy nano LC 1000 HPLC (ThermoFisher Scientific) using a Captive spray nanospray ionization source (Bruker Daltonics) including a 2-cm-long, 100-µm-inner diameter fused silica fritted trap column, 75-µm-inner diameter fused silica analytical column with an integrated spray tip (6–8 µm-diameter opening, pulled on a P-2000 laser puller from Sutter Instruments), fixed on an in-house constructed column heater set at 50 °C. The trap column was packed with 5 µm Aqua C-18 beads (Phenomenex, http://www.phenomenex.com) while the analytical column was packed with 1.9 µm-diameter Reprosil-Pur C-18-AQ beads (Dr. Maisch, http://www.Dr-Maisch.com). Buffer A consisted of 0.1% aqueous formic acid, and buffer B consisted of 0.1% formic acid and 80% acetonitrile in water. Samples were resuspended in buffer A and loaded with the same buffer. A standard 120 min run gradient was from 10% B to 35% B over 105 min, then held at 100% B for 15 min. Before each run the trap column was conditioned with 20 µL buffer A, the analytical with 4 µL of the same buffer and the sample loading was set at 5 µL. The LC termostat temperature was set at 7 °C. The analysis was performed at a flow rate of 0.25 µL/min. The Impact II was set to acquire in a data-dependent auto-MS/MS mode with inactive focus fragmenting the 20 most abundant ions (one at the time at 18 Hz rate) after each full-range scan from *m/z* 200 Th to *m/z* 2000 Th (at 5 Hz rate). The isolation window for MS/MS was 2–3 Th depending on parent ion mass to charge ratio and the collision energy ranged from 23 to 65 eV depending on ion mass and charge. Parent ions were then excluded from MS/MS for the next 0.4 min and reconsidered if their intensity increased more than five times. Singly charged ions were excluded since in ESI mode peptides usually carry multiple charges. Strict active exclusion was applied. Mass accuracy: error of mass measurement was typically within 5 ppm and was not allowed to exceed 10 ppm. The nano ESI source was operated at 1700 V capillary voltage, 0.20 Bar nano buster pressure, 3 L/min drying gas and 150 °C drying temperature.

Proteomics analysis

Peak lists were created using Compass 1.9 for OTOF version 4.0.15.3248 (Bruker Daltonik GmbH). MaxQuant 1.5.1.0 was used for database searching. Searches were performed against a database comprised the protein sequences from the source organism plus common contaminants using the following parameters: peptide mass accuracy 10 parts per million; fragment mass accuracy 0.05 Da; trypsin enzyme specificity, fixed modifications—carbamidomethyl, variable modifications—methionine oxidation and *N*-acetyl proteins; allowing for up to two miscleavages. Only those peptides exceeding the individually calculated 99% confidence limit (as opposed to the average limit for the whole experiment) were considered as accurately identified.

The sequence databases used in searches were the human database from MaxQuant group, and the *Leishmania major* Friedlin database (custom—8324 proteins). False positive determination was done by "revert" decoy search; in infected THP-1 cells, 12 decoy proteins were found out of 1121.

Statistically significant differences in the expression of individual proteins between heavy and light were determined using a *z* test on Excel, with a threshold of p value <0.05.

Statistical analysis

Statistical analyses were performed using Prism 6.0 (GraphPad) or Excel 2011 (Microsoft). Data are presented as mean +/− standard deviation. A p value <0.05 was considered statistically significant.

Results

Leishmania CPN10 is delivered to the cytosol of infected macrophages

Leishmania CPN10 was initially described as a mitochondrial protein [26], but it was also identified in the secretome of *L. donovani* [27], and in *L. donovani* exosomes [28]. Given that CPN10 is not restricted to an intracellular location, a key question was whether CPN10 is delivered to the cytosol of infected macrophages. THP-1 cells infected with *L. donovani* were stained and analyzed by confocal

microscopy (Fig. 1d). Leishmania CPN10 staining was the strongest in intracellular leishmania, which can also be identified with DAPI staining (small dots are leishmania nuclei and kinetoplasts). A diffuse staining was also seen in the cytosol of both infected and uninfected macrophages. Of note, approximately 70% of macrophages were infected, but the leishmania may be out of focus and not seen in the confocal image. Nevertheless, it was expected that some cells that did not harbor DAPI-stained leishmania nuclei and kinetoplasts but were labeled by anti-leishmania CPN10 might contain killed leishmania, since DAPI would not stain dead parasites. Moreover, uninfected macrophages could also be CPN10 positive, as CPN10 is known to be secreted and delivered in exosomes produced by leishmania that are still free in culture media. Secondary antibody-only and uninfected samples controls were performed, for which there was no CPN10 staining at all.

The same infected THP-1 cells were analyzed by transmission electron microscopy, with anti-CPN10 gold immunostaining (Fig. 1a–c). CPN10 was found in the mitochondrion of leishmania as expected (Fig. 1b), but also accumulated close to the leishmania plasma membrane, and in the cytosol of infected macrophages (Fig. 1c).

Engineering leishmania for reduced expression and overexpression of CPN10

In *L. donovani*, CPN10 is encoded by two genes on chromosome 26 (GeneDB number LdBPK_260590.1 and LdBPK_260610.1), separated by approximately 5000 bp, which comprises a hypothetical protein-coding sequence (LdBPK_260600.1). Each of these genes is 303 bp and they share 99% identity. To replace CPN10 in *L. donovani*, a construct was designed to replace both CPN10 genes and to reintroduce the intervening hypothetical protein-coding sequence at the same time, in case this gene would have an important role. Three attempts were made to replace both alleles simultaneously as described [31], but a viable double-allelic replacement could not be obtained. This suggested that CPN10 is essential

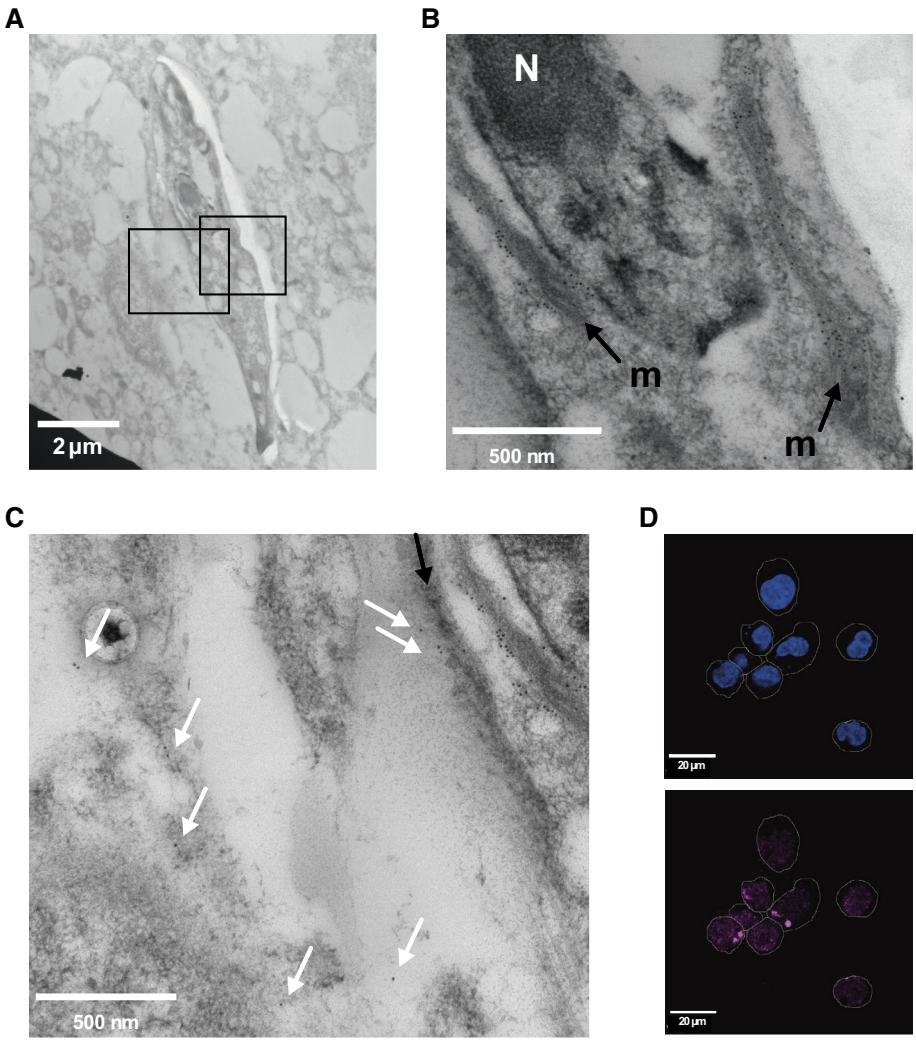

Fig. 1 Leishmania CPN10 is present in the cytosol of leishmania-infected macrophages. PMA-differentiated THP-1 cells infected for 18h with WT *L. donovani*. **a**–**c** Samples were processed by high-pressure freezing and embedding. The cut sections on grids were labeled using anti-leishmania CPN10 antibodies and secondary antibody labeled with gold 15 nm particles. The grids were then analyzed by transmission electron microscopy. **a** Intracellular leishmania surrounded by the macrophage's cytosol. **b** Close-up zoom of the *right square* on image **a**; "*N*" is the nucleus, "*m*" indicate parts of the mitochondrion that stretches on either side of the nucleus. **c** Close-up zoom of the *left square* on image **a**; the *black arrow* points at the membrane between the leishmania and the macrophage's cytosol, *white arrows* point at leishmania CPN10 in the macrophage's cytosol. **d** Infected THP-1 cells on coverslips were stained with anti-leishmania CPN10 antibodies followed by anti-rabbit Alexa Fluor 594 (*bottom*), and DAPI (*top*). The *white line* is drawn based on brightfield, showing the macrophages' outline

to leishmania. Alternatively, one of the two alleles was replaced to obtain a partial knockdown of CPN10, called CPN10+/− Ld, that expressed 50% of the amount of CPN10, as compared to WT Ld (Fig. 2a, c).

To determine whether the partial deletion was effective at all life stages of leishmania, promastigotes were cultured at 37 °C for 3 days, in pH = 7 medium to obtain "heat shocked promastigotes", and axenic amastigotes were prepared by culturing the leishmania at 37 °C in pH 5.5 medium for 3 days, to mimic the environment of phagosomes. First, we found that CPN10 was more highly expressed in leishmania cultured at 37 °C for both promastigotes and axenic amastigotes (Fig. 2a, c), consistent with what has been found previously [26]. More importantly, CPN10+/− Ld expressed approximately half the amount of CPN10 as compared to WT Ld at all of these three stages (Fig. 2a, c).

In parallel, we produced an episomal vector with the intention of creating a CPN10 over expressing Ld (CPN10+++), while the same vector without the CPN10 gene was used to transfect control cells thereafter called 'empty vector control' (Fig. 2b, c).

Both the CPN10 knockdown and overexpressing strains demonstrated normal growth, either as promastigotes or axenic amastigotes (Fig. 3). Moreover, their promastigote growth was similar to WT Ld and empty vector controls when cultured for three days at 37 °C, suggesting that reduced abundance of CPN10 in leishmania does not increase their sensitivity to heat shock (Fig. S1).

Finally, their morphology, as promastigotes, heat shocked promastigotes and axenic amastigotes was mostly unchanged, except for the CPN10+/− promastigotes that were overall slightly shorter than WT promastigotes (Fig. S2). Thus, a higher or lower amount of CPN10 in *L. donovani* did not affect either their axenic growth, both as promastigotes and amastigotes, or their morphology, and did not make them more or less sensitive to heat shock.

CPN10+/− growth is inhibited by tunicamycin-induced ER stress

CPN10+/− leishmania were not found to be more susceptible to heat shock stress than their WT counterparts (Fig. S1). To examine their sensitivity to other kinds of stress, Tunicamycin was used as it is known to induce

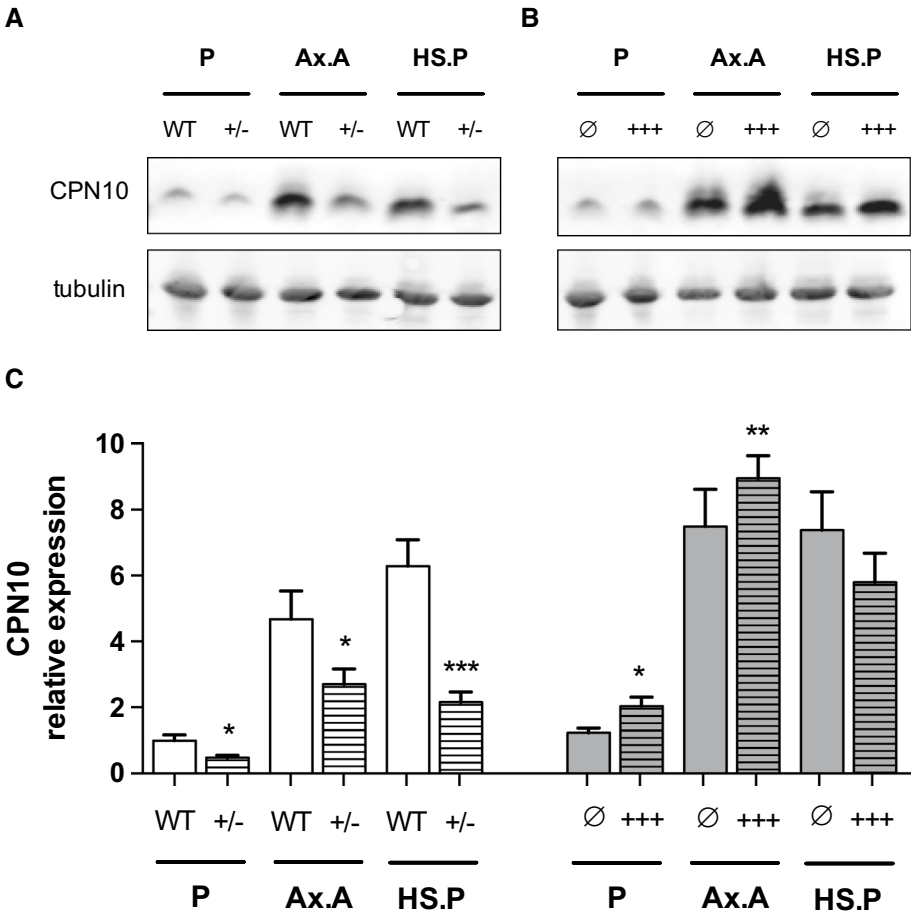

Fig. 2 Leishmania CPN10 expression in promastigotes (P), axenic amastigotes (*Ax.A*) and heat shocked promastigotes (*HS.P*) of WT Ld (*WT*), CPN10+/− Ld (+/−), empty vector control Ld (Ø) and CPN10+++ Ld (+++). **a, b** Leishmania were cultured in pH 7 medium at 26 °C for 3 days (promastigotes), in pH 5.5 medium at 37 °C for 3 days (axenic amastigotes), or in pH 7 medium at 37 °C for 3 days (heat shocked promastigotes), lysed, and WB was performed on lysate using anti-CPN10 and anti-tubulin antibodies. **c** the relative amount of CPN10 was determined by densitometry, using tubulin as a control. Mean and standard deviation of six independent experiments. Unpaired *t* test, compared to WT at each respective stage (**p* value < 0.05, ***p* value < 0.01, ****p* value < 0.001)

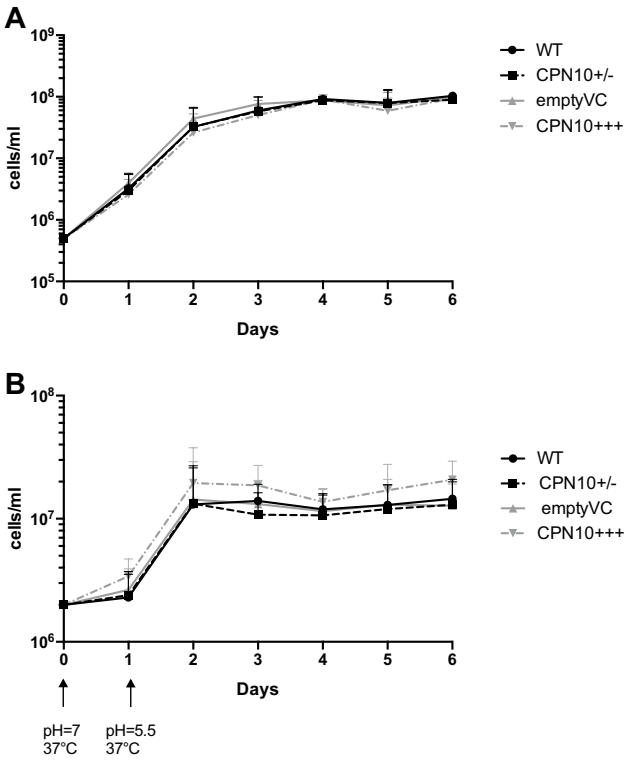

Fig. 3 Promastigote and axenic amastigote growth are not altered by CPN10 knockdown or overexpression. Growth curves of promastigotes (**a**) and axenic amastigotes (**b**). Promastigotes were set at 5×10^5 cells/ml in pH 7 medium at 26 °C. Axenic amastigotes were set at 1×10^6 cells/ml in pH 7 medium at 37 °C for 24 h and then transferred to pH 5.5 at 37 °C. Cells were counted every 24 h for 6 days. Mean and standard deviation of three independent experiments

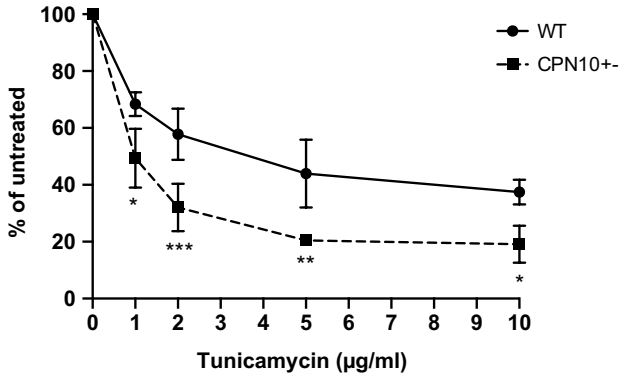

Fig. 4 CPN10+/− promastigotes are more susceptible to tunicamycin-induced stress than WT Ld. Promastigotes were treated with increasing concentrations of tunicamycin (1, 2, 5 and 10 µg/ml) and counted after 48 h of treatment. Mean and standard deviation of three independent experiments. Tukey's multiple comparisons test (*p value < 0.05, **p value < 0.01, ***p value < 0.001)

endoplasmic reticulum (ER) stress and the ER-unfolded protein response (UPR). The latter triggers an increase in mitochondrial metabolism that contributes to the cellular adaptation to ER stress. Moreover, beyond a high degree of ER damage, it also triggers apoptotic pathways. Altogether, the cellular response to ER stress relies on functional mitochondria, and especially on ER-to-mitochondria Ca^{2+} transfer [36, 37].

Tunicamycin treatment at 10 µg/ml inhibited growth of WT leishmania by up to 50% as compared to untreated controls. CPN10+/− leishmania displayed an even lower growth than WT leishmania, at all concentrations tested, down to only 20% growth when treated with 10 µg/ml of tunicamycin (Fig. 4). Thus, CPN10+/− leishmania were significantly more sensitive to tunicamycin-induced stress than WT cells.

Proteomic profiling of CPN10+/− leishmania

To characterize CPN10+/− Ld further, to understand their phenotype and increased sensitivity to tunicamycin, a proteomics study comparing WT and CPN10+/− Ld was performed. Using stable isotope labeling by amino acids in cell culture (SILAC), followed by mass spectrometry, a total of 2886 proteins were identified in three independent experiments. This was done using the *Leishmania major* Friedlin genome as a reference, it being the best annotated amongst all leishmania genomes thus far. A quantitative ratio comparing WT and CPN10+/− Ld was obtained for 2307 proteins. A z test with Bonferroni multiple testing correction allowed the identification of 123 proteins significantly changed between WT and CPN10+/− Ld across all three replicates. Amongst these proteins, a majority (80.5%) was downregulated in CPN10+/− Ld as compared to WT cells ($n = 99$ proteins), while only 19.5% were upregulated ($n = 24$ proteins) (Fig. 5a).

Examining the annotation of the proteins whose expression was changed, 38 were hypothetical unknown proteins, which had no known ortholog in any organism and 84 corresponded to known proteins in the database. Of note, CPN10 was one of the significantly downregulated proteins (log2 ratio CPN10+/−/WT is −0.712), similar to the Western blot data, which confirmed this as a *bona fide* knockdown strain (Table 1).

A gene ontology analysis was performed using PantherDB, starting with all the significantly changed proteins (p value < 0.05 after z test), and the biggest GO term for biological processes was metabolic process: 46 proteins were downregulated in CPN10+/− Ld as compared to WT (Fig. 5b, Table S1). Proteins involved in cellular processes, localization, biological regulation as well as response to stimulus were also mainly downregulated. When looking at the protein classes, several enzyme classes were downregulated, e.g., hydrolases, transferases, oxidoreductases and phosphatases. A number of nucleic acid binding proteins were also downregulated, as well as transfer/

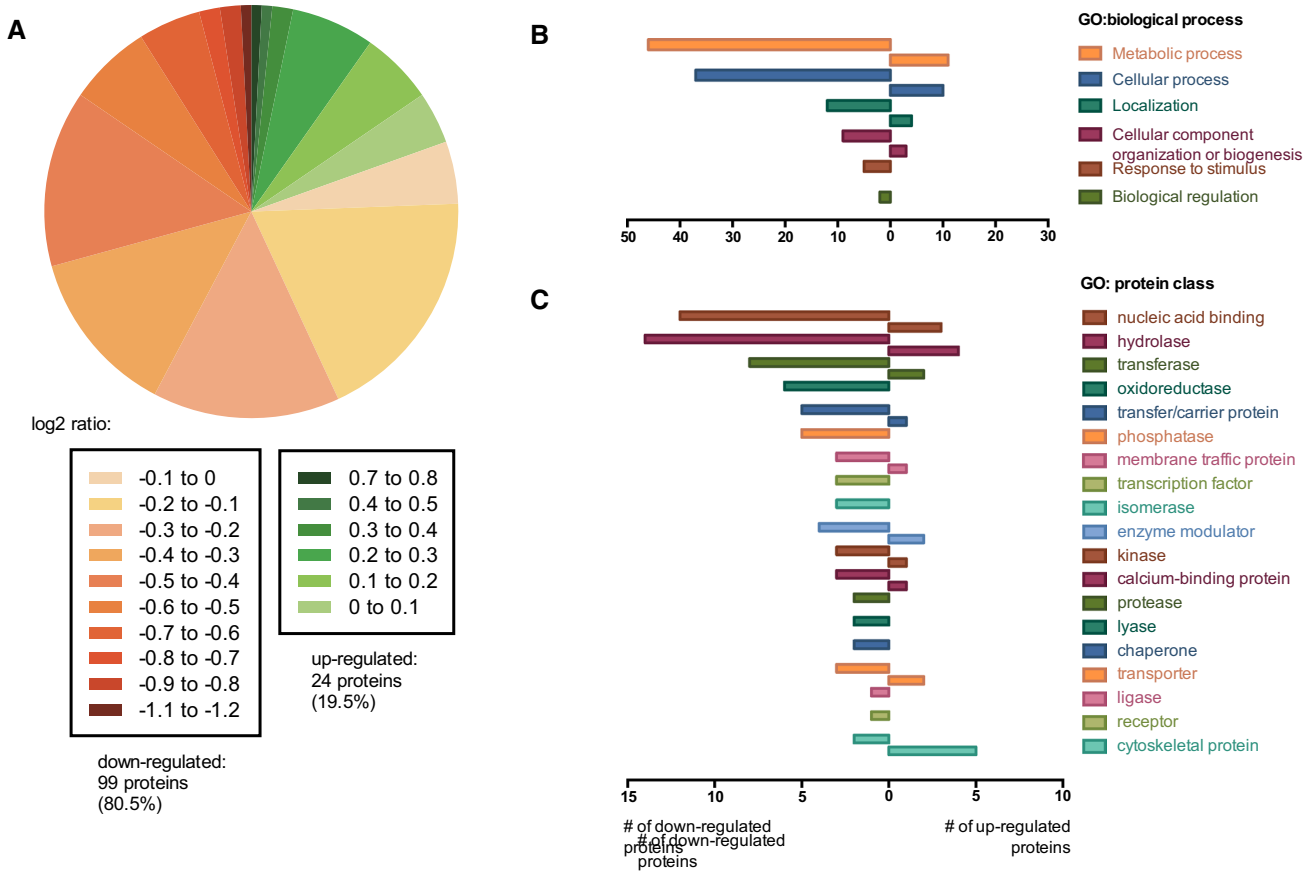

Fig. 5 Comparative proteomics of CPN10+/− Ld and WT Ld. Promastigotes were cultured in light and heavy SILAC medium, and mass spectrometry was performed on a mix of both lysates allowing for a quantitative comparison between the two strains (3 independent experiments). **a** log2 ratio of significantly changed proteins: proteins with a negative ratio were downregulated in CPN10+/− Ld, whereas proteins with a positive ratio were upregulated in CPN10+/− Ld, as compared to WT Ld. **b** Gene ontology analysis of significantly changed proteins. The *X* axis represents the number of proteins up- or downregulated in CPN10+/− Ld for each Gene Ontology term (PantherDB: protein class and biological process)

carrier proteins. On the other hand, cytoskeletal proteins was the only protein class for which the number of upregulated proteins was found to be higher than those that were downregulated.

Concerning the details of proteins significantly changed in CPN10+/− Ld, the most highly changed are presented in Table 1. No proteins known to be involved in pathogenesis, such as gp63, Kinetoplastid Membrane Protein-11 [38] or enolase [39] were found to be either up- or downregulated. No other heat shock proteins or proteins known to be involved in unfolded protein response or in heat shock response were found to be increased to compensate for reduced abundance of CPN10.

CPN10 modulates the infection rates and parasite load in human macrophages

Next, we examined the impact of CPN10 on macrophage infection in vitro. Infection experiments were performed on human monocyte-derived macrophages (HMDMs) purified from buffy coats and on the THP-1 monocytic cell line, for 24 h. Two multiplicities of infection (MOI) were used, 20:1 and 10:1, that yielded similar infection rates for a given strain, suggesting that the MOI of 10:1 represents a saturation of the in vitro model (Fig. 6). Nevertheless, CPN10+/− Ld yielded higher infection rates when compared with WT Ld: 75% of HMDMs were infected with CPN10+/− Ld at a MOI of 20:1, versus 47% with WT Ld and 64 versus 37%, respectively, with a MOI of 10:1 (Fig. 6a). The same trend was observed with THP-1 cells; 56% of cells were infected with CPN10+/− versus 38% infected with WT Ld with a MOI of 20:1, and 53 versus 36%, respectively, for a MOI of 10:1 (Fig. 6b). Parasite loads (number of leishmania per infected cell) also tended to be higher with CPN10+/− Ld as compared to WT, both in HMDMs and THP-1 (Fig. 6c, d). On the other hand, the overexpressing strain CPN10+++ was associated with lower infection

Table 1 Comparative proteomics of CPN10+/− Ld and WT Ld

Gene ID	Protein name—ortholog (Tritrypdb)	log2 ratio
Metabolic process		
LmjF.06.0460	ATP-NAD kinase-like protein	−0.628
Lipid metabolic process		
LmjF.06.0880	Putative acyl-coenzyme a dehydrogenase	−0.416
LmjF.27.0290	Acyl carrier protein	−0.444
LmjF.24.1840	Lysophospholipase, putative	−0.655
LmjF.05.0180	Dihydrolipoamide branched chain transacylase, putative	−0.552
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process		
LmjF.05.0830	Methylthioadenosine phosphorylase	−0.507
LmjF.17.0360	Cytidine deaminase-like protein	−0.413
RNA metabolic process		
LmjF.16.1350	DNA-directed RNA polymerase I largest subunit	−0.434
LmjF.31.0250	ATP-dependent RNA helicase, putative	−0.491
LmjF.11.0470	Pumilio protein 10, putative	−0.394
LmjF.27.2300	Transcription elongation regulator-like protein SPT5	−0.661
Amino acid metabolic process		
LmjF.05.0970	MST mercaptopyruvate sulfurtransferase	−0.420
Translation		
LmjF.35.2190	60S ribosomal protein L12, putative	−0.385
Protein metabolic and modification process		
Protein folding		
LmjF26.0620	10 kDa heat shock protein (Chaperonin 10)	−0.712
LmjF.10.0890	Peptidyl-prolyl cis–trans isomerase	−0.615
Peptidases		
LmjF.33.1610	Peptidase M20/M25/M40, putative	−0.536
LmjF.13.0680	Metallopeptidase family M24, putative	−0.766
LmjF.29.2300	Ubiquitin carboxyl-terminal hydrolase, putative	−0.409
Protein phosphatases		
LmjF.05.0100	Serine/threonine phosphoprotein phosphatase	−0.449
LmjF.34.2510	Protein phosphatase 2c-like protein	−0.607
LmjF.32.0100	NLI interacting factor-like phosphatase, putative	−0.446
Ubiquitination		
LmjF.27.2480	WD-repeat protein	−0.531
Protein transport		
LmjF.19.1600	Signal Recognition Particle subunit SRP19 protein, putative	−0.525
LmjF.04.0710	Tir chaperone protein (CesT) family/PDZ domain containing protein, putative	−0.437
Transport		
LmjF.23.0380	ATP-binding cassette protein subfamily G, member 5 (ABCG5)	−0.389
Microtubule-based process		
LmjF.30.1450	Kinesin, putative	0.731
LmjF.35.2090	Kinesin, putative	−0.552
Unclassified/uncharacterized		
LmjF.33.3090	Hypothetical protein, conserved—contains a holliday junction resolvase-like of SPT6/SH2 domain	−0.545
LmjF.19.1010	Hypothetical protein, conserved—contains a WD domain, G-beta repeat	−0.377
LmjF.19.1400	Hypothetical protein, conserved—contains a sel1 repeat/tetratricopeptide repeat	−0.442
LmjF.20.0580	Hypothetical protein, conserved—contains MORN repeat	0.446
LmjF.19.0020	Hypothetical protein, conserved	−1.109
LmjF.28.1060	Hypothetical protein, conserved	−0.828
LmjF.20.0930	Hypothetical protein, conserved	−0.638

Table 1 (continued)

Gene ID	Protein name—ortholog (Tritrypdb)	log2 ratio
LmjF.34.0750	Hypothetical protein, conserved	−0.555
LmjF.04.0990	Hypothetical protein, conserved	−0.472
LmjF.13.0520	Protein of unknown function (DUF1014 family), putative	−0.458
LmjF.29.0270	Hypothetical protein, conserved	−0.444
LmjF.15.0810	Hypothetical protein, conserved	−0.412
LmjF.26.0730	Hypothetical protein, conserved	−0.409

The most highly changed proteins (log2 ratio <−0.3 and >0.3) are listed here. CPN10+/− and WT promastigotes were cultured in light and heavy SILAC medium, and mass spectrometry was performed on a mix of both lysates. Only proteins that were significantly changed across three replicates are listed here (*t* test, adjusted *p* value < 0.05). Proteins with a negative ratio were downregulated in CPN10+/− Ld, whereas proteins with a positive ratio were upregulated in CPN10+/− Ld, as compared to WT Ld

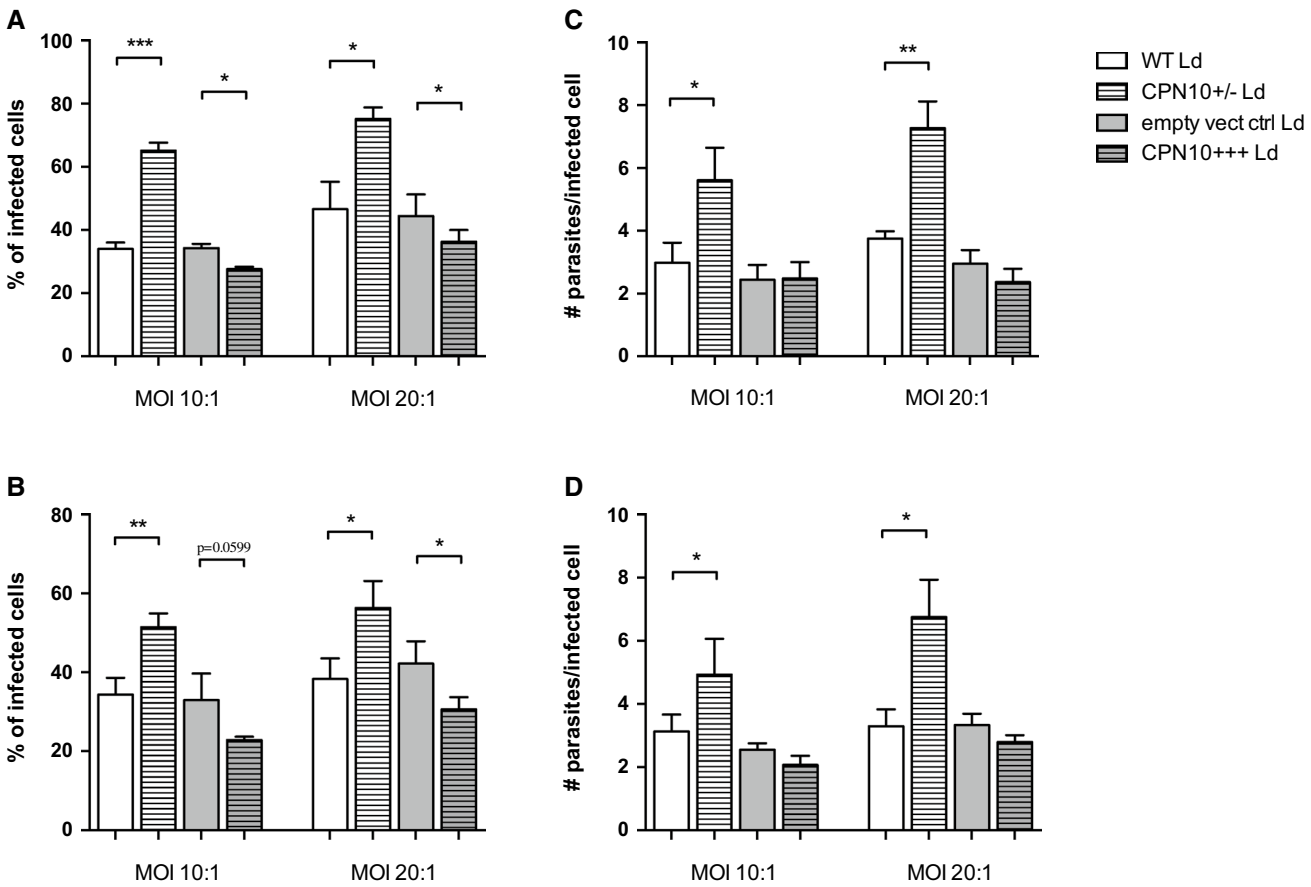

Fig. 6 CPN10 modulates the infection rate and parasite load in human macrophages: HMDMs (**a, c**, respectively) and THP-1 cells (**b, d**, respectively). Monocytes were purified from healthy donors buffy coats and differentiated on coverslips. THP-1 cells were differentiated on coverslips using PMA. HMDMs and THP-1 cells were then infected with stationary stage promastigotes at MOI of 10:1 and 20:1 for 24 h. Cells were washed, fixed, and mounted on microscopy slides using DAPI-mounting media. The infection rate and parasite loads were assessed using a fluorescence microscope. Mean and standard deviation of three independent experiments. Paired *t* test (*p* values: *<0.05, **<0.01, ***<0.001)

rates when compared with both empty vector control and WT Ld (Fig. 6a, b), both in HMDMs (10–15% decrease, $p < 0.05$ with MOI 20:1 and 10:1), and in THP-1 cells (10–20% decrease with MOI 20:1 and 10:1). The parasite load of the CPN10 over expressing strain was also lower than the empty vector control and WT Ld in THP-1s, but similar to the controls in HMDMs (Fig. 6c, d).

These results were unexpected since they suggested a gain-of-function phenotype of increased infection rates for CPN10+/− Ld. Establishing chronic infection is dependent

both on efficient parasite uptake and subsequent intracellular survival. Thus, the increased infection rates and parasite loads displayed by CPN10+/− Ld could have been due to either more efficient internalization of the parasite by macrophages or to better survival once internalized.

CPN10 modulates the internalization of leishmania

To address the question of internalization, infection rates and parasite loads were monitored at 2, 4 and 6 h post-addition of leishmania, as it is during this time period that most of the uptake of leishmania by macrophages occurs. At every timepoint, the infection rates and parasite loads of CPN10+/− were significantly higher than those of WT Ld, by approximately 1.5-fold (Fig. 7a, b). Using a nonlinear regression equation and comparison of fits, we showed that the internalization rate itself was significantly higher for CPN10+/− than WT, both in term of infection rate and parasite load (Fig. 7a, b graphic inserts). Concerning CPN10+++, its infection rate was significantly lower than that of both empty VC and WT, but not its parasite load (Fig. 7a, b). It seems therefore that CPN10 restricts the internalization of leishmania by macrophages.

To investigate why CPN10+/− were more efficiently internalized than WT Ld, the apoptotic phenotype of stationary phase promastigotes was analyzed, as it is believed that leishmania use apoptotic mimicry to get internalized without activating macrophages [40, 41]. Leishmania were stained with annexin-V-FITC that recognizes surface exposed phosphatidylserine. However, no difference in staining was observed between WT and CPN10+/− Ld (Fig. S3).

CPN10 is required for intracellular survival

To address the question of intracellular survival, we first performed a 4 h infection of THP-1 cells, followed by thorough washings to remove all unbound leishmania, and monitored the number of parasites per 100 macrophages at 4, 24 and 48 h post-infection. The numbers for WT Leishmania were constant between 4 and 24 h, after which they started increasing, revealing that they were able to multiply in infected macrophages, between 24 and 48 h post-infection (Fig. 8a). On the other hand, CPN10+/− Ld numbers decreased markedly and steadily from 4 h, down to half their starting numbers at 48 h post-infection, showing that their intracellular survival was significantly impaired. Finally, CPN10+++ numbers started increasing earlier than emptyVC and WT, between 4 and 24 h, after which their numbers were stable, suggesting that overexpression of CPN10 allows them to start multiplying intracellularly faster than WT leishmania.

Another way to look at intracellular survival, paired with recovery after infection is to perform a parasite rescue and transformation assay. This assay allows us to compare survival rates based upon the recovery of viable leishmania

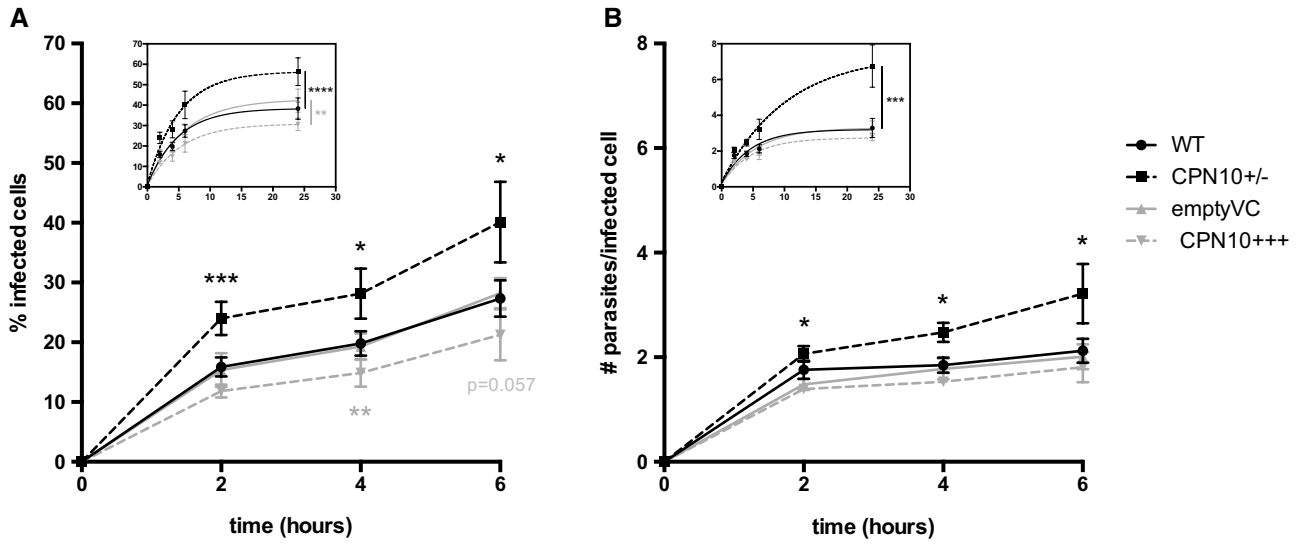

Fig. 7 CPN10 modulates the internalization of leishmania. PMA-differentiated THP-1 cells were infected for 2, 4 and 6 h, at which point the coverlips were washed, fixed and mounted on microscopy slides using DAPI-mounting media. The infection rate and parasite load were assessed using a fluorescence microscope. **a** Infection rate, **b** parasite load. Mean and standard deviation of three independent experiments. Paired *t* test, *black stars* are *p* values for WT versus CPN10+/−, *gray stars* for emptyVC versus CPN10+++ (*<0.05, **<0.01, ***<0.001, ****<0.0001). Graphic inserts represent the nonlinear regression of the same data, from 0 to 24 h of infection (variable slope, four parameters). A comparison of fits was performed on semilog fits of the curves, using graphpad prism (*p* values are represented as stars). Represented are mean and standard deviation

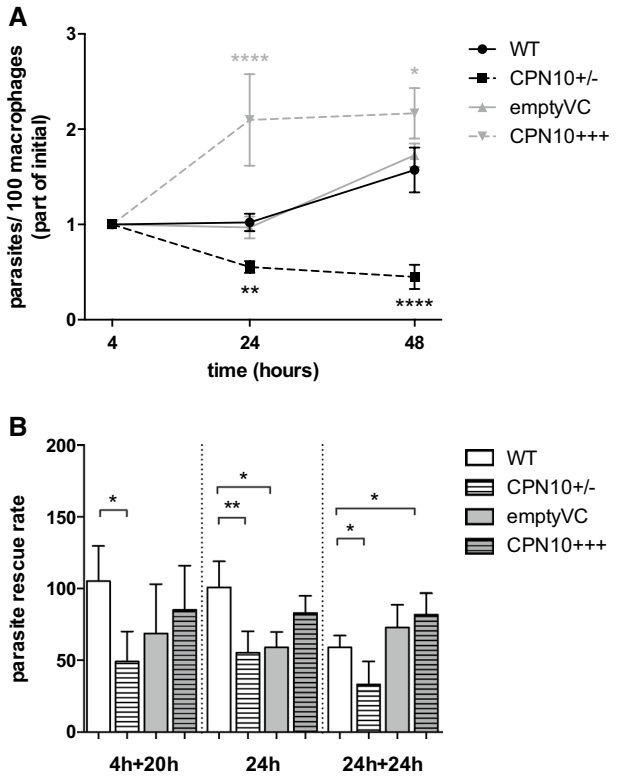

Fig. 8 CPN10 modulates the intracellular survival and recovery of leishmania after infection. **a** Intracellular survival: PMA-differentiated THP-1 cells were infected for 4 h with leishmania, at which point the cells were thoroughly washed to remove uninternalized leishmania. At 4, 24 and 48 h post-infection, the coverslips were washed, fixed, and mounted on microscopy slides using DAPI-mounting media. The number of parasites per 100 macrophages was assessed using a fluorescence microscope. The values are represented as ratio of initial (4 h). Mean and standard deviation of three independent experiments. Two-way ANOVA, black stars represent the p-value of WT versus CPN10+/−, *gray stars* are emptyVC versus CPN10+++ (*<0.05, **<0.01, ****<0.0001). **b** Parasite rescue and transformation assay: PMA-differentiated THP-1 cells were infected with leishmania promastigotes, and at different timepoints THP-1 cells were lysed to release internalized leishmania, which were then grown for 2 days. "4 + 20h": uninternalized leishmania were washed away after 4h and THP-1 cells were incubated for another 20h, lysis was performed at 24 h post-infection. "24 h": THP-1 cells were infected with leishmania for 24 h when cells were washed and lysed. "24 + 24h": uninternalized leishmania were washed away after 24 h, and cells were incubated for another 24 h, and lysed 48 h post-infection. Recovered leishmania were counted after 2 days of growth in M199 to assess their recovery. In parallel, the number of internalized leishmania was assessed by microscopy. Represented here is the survival rate (recovered/internalized ratio), mean and standard deviation of four independent experiments. Paired *t* test (*p* value: *<0.05, **<0.01, ***<0.001, ****<0.0001)

from macrophages infected with each leishmania strain. To examine how well CPN10+/− Ld survive inside macrophages, infected cells were selectively lysed at different time points to free the intracellular leishmania and grow them up for 2 days to compare their recovery (viability), as described in [32]. Since CPN10+/− Ld started with more internalized organisms inside macrophages before lysis, the counts of recovered leishmania were normalized by dividing them by the number of internalized leishmania at each time point, and this ratio was called parasite rescue rate (Fig. 8b). When cells were infected for 4 h, washed to remove uninternalized leishmania, and incubated for another 20 h before lysis, the parasite rescue rate of CPN10+/− Ld was only 49 versus 105 for WT Ld, meaning that for every leishmania internalized, 105 WT promastigotes were counted 2 days after release from the macrophages, but only 49 when the same procedure was done with CPN10+/− Ld. When cells were infected for 24 h before washing and lysis, CPN10+/− Ld had a parasite rescue rate of 55 versus 100 for WT Ld. After 24 h of infection, washing, and an extra 24 h incubation before cell lysis, WT Ld had a parasite rescue rate of 59, significantly higher than CPN10+/− Ld at only 33. Altogether, these results indicate that CPN10+/− Ld had significantly impaired intracellular survival when compared to WT.

The parasite rescue rate of emptyVC and CPN10+++ Ld were unchanged when compared to WT at 4 + 20 h (uninternalized leishmania washed away after 4 h, lysis at 24 h post-infection), largely because of the high variation obtained at this early timepoint, which was due to the low numbers of internalized leishmania (Fig. 8b). After 24 h of infection, the parasite rescue rate of CPN10+++ was not significantly different from that of WT Ld, while at 24 + 24 h, CPN10+++ had an increased parasite rescue rate compared with WT (82 versus 59), while emptyVC was similar to WT. These findings indicate that 48 h post-infection CPN10+++ Ld are more successful at surviving inside macrophages. Taken together, the data in Fig. 8 suggest that leishmania CPN10 is required for optimal intracellular survival.

Macrophages produce reactive oxygen species to kill microbes, including leishmania [42, 43]. During phagocytosis, leishmania promastigotes are exposed to reactive oxygen species such as hydrogen peroxide (H_2O_2) which was shown to induce apoptosis-like death in *L. donovani* [44]. However, CPN10+/− did not appear to be any more or less resistant to H_2O_2 treatment, all four strains showing similar growth reduction by H_2O_2 (Fig. S4). Thus it would appear that the requirement for CPN10 for optimal intracellular survival of leishmania is not related to resistance to H_2O_2.

Proteomic profiling of infected macrophages

As shown above, the CPN10 knockdown did not appear to affect axenic growth of either promastigote or amastigotes, but it clearly increased parasite uptake by macrophages while at the same time reducing intracellular survival.

These findings suggested that CPN10+/− Ld may interact with macrophages in a fundamentally different manner than WT Ld, resulting in enhanced phagocytosis and increased intracellular killing. To understand the molecular basis of this phenotype, a proteomic study was performed, comparing macrophages infected with WT Ld and CPN10+/− Ld. THP-1 cells were cultured in two different SILAC media for at least five doublings, differentiated using PMA and infected with leishmania. To account for the difference in infection rates, and to compare the proteomic profiles of infected cells only, infected macrophages were sorted based upon using CFSE-labeling of leishmania prior to infection. The "light" THP-1 were infected with CPN10+/− Ld and the "heavy" with WT Ld. After 24 h of infection, cells were sorted and CFSE+ infected cells were collected, combined and processed for mass spectrometry.

1109 distinct proteins were identified: 999 human proteins, 50 bovine proteins and other contaminants, and 62 leishmania proteins. Using a z test, 45 proteins were identified as being significantly changed between macrophages infected with WT Ld or CPN10+/− Ld (p value < 0.05). A majority of changed proteins were upregulated in CPN10+/− infected cells as compared to WT infected cells (82.2%, 37 proteins) (Fig. 9a). The details of proteins significantly changed is presented in Table 2.

A gene ontology analysis was done using PantherDB, and proteins involved in "innate immune response", "gene expression", "membrane organization", "apoptotic process" were found to be upregulated in CPN10+/− infected cells (Fig. 9b, Table S2). When looking at the gene ontology terms concerning molecular function, the most common one was "protein binding" (25 proteins upregulated, 6 proteins downregulated), the following is "poly(A) RNA binding" (8 proteins upregulated, 6 downregulated) (Fig. 9b; Table S2).

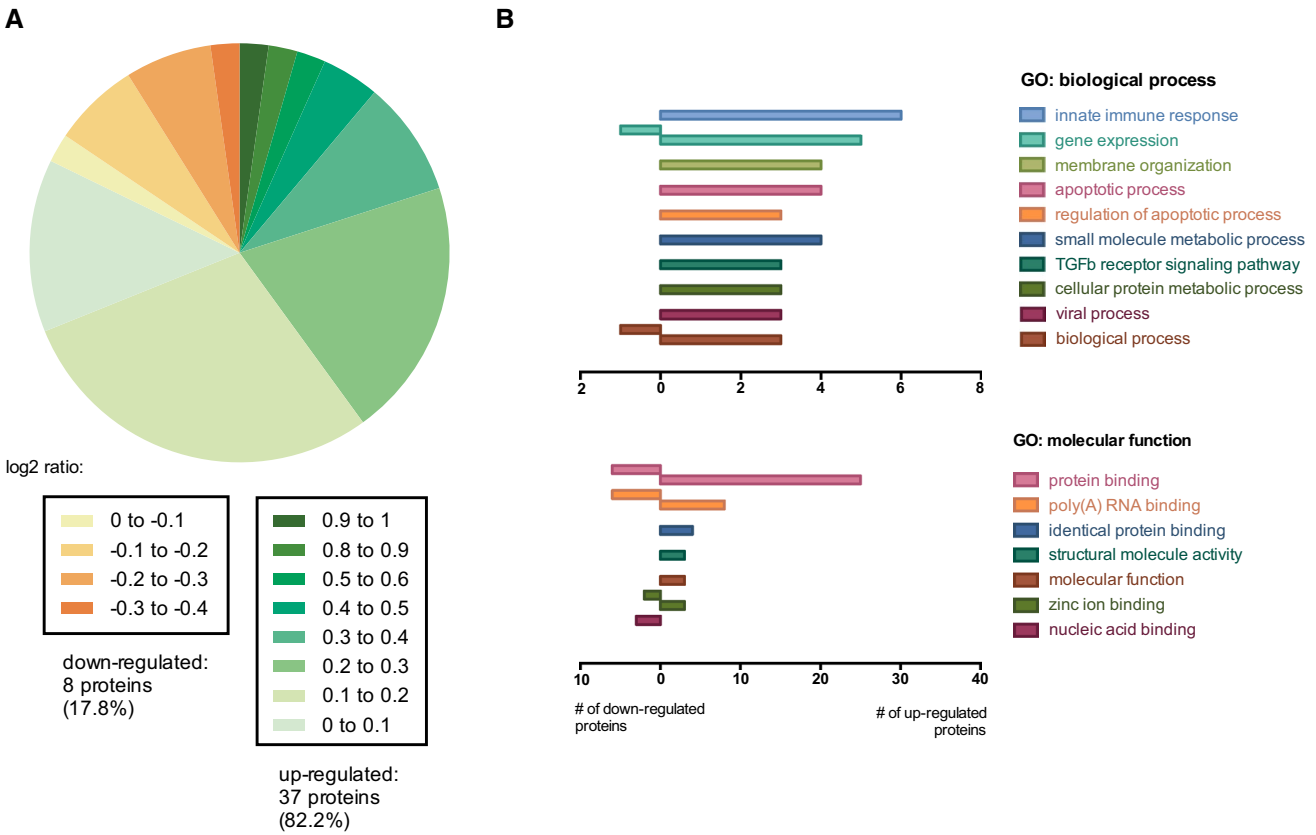

Fig. 9 Comparative proteomics of infected macrophages with CPN10+/− Ld and WT Ld. THP-1 cells were grown in SILAC media for 2 weeks, then differentiated with PMA and infected with CFSE-labeled WT Ld or CPN10+/− Ld. Infected CFSE+ macrophages were sorted out and mass spectrometry analysis was performed on a mixture of both—"heavy" infected with WT Ld and "light" infected with CPN10+/− Ld—to allow for quantitative analysis. A Z test was performed to select the proteins significantly changed across all three independent replicates. **a** log2 fold change significantly changed proteins: proteins which ratio < 0 are downregulated in cells infected with CPN10+/− Ld, whereas proteins which ratio > 1 are upregulated in cells infected with CPN10+/− Ld, as compared to cells infected with WT Ld. **b** Gene ontology analysis of significantly changed proteins. The X axis represents the number of proteins up- or downregulated in cells infected with CPN10+/− Ld as compared with cells infected with WT Ld for each Gene Ontology term (InnateDB: biological process and molecular function)

Table 2 Comparative proteomics of macrophages infected with either CPN10+/− Ld or WT Ld

Gene ID	Gene name	log2 ratio
Cellular processes and signaling		
Multiple roles in cellular processes		
P29590	Promyelocytic leukemia (PML)	0.432
Cell cycle control, cell division		
P27816	Microtubule-associated protein 4 (MAP4)	0.232
Q9Y266	Nuclear distribution C homolog (NUDC)	0.100
Cell adhesion		
Q14956	Glycoprotein (transmembrane) nmb (GPNMB)	0.262
Post-translational modification, protein turnover, and chaperones		
P04080	Cystatin B (stefin B) (CSTB)	0.473
P61960	Ubiquitin-fold modifier 1 (UFM1)	0.172
Q15819	Ubiquitin-conjugating enzyme E2 variant 2 (UBE2V2)	0.035
P62987, P62979, P0CG47, Q96C32, P0CG48	Polyubiquitin-B (UBB), Polyubiquitin-C (UBC), Ubiquitin-40S ribosomal protein S27a (RPS27A), Ubiquitin-60S ribosomal protein L40 (UBA52)	0.270
Signal transduction mechanisms		
O15173	Progesterone receptor membrane component 2 (PGRMC2)	0.087
P26447	S100 calcium binding protein A4 (S100A4)	−0.232
Intracellular trafficking, secretion, and vesicular transport		
Q9NZZ3	Charged multivesicular body protein 5 (CHMP5)	0.291
Q5SNT6, Q9Y4E1, Q641Q2, Q5T1D7, Q5SRD0	WASH complex subunits FAM21C; FAM21B; FAM21A	0.290
Q9Y5L4	Translocase of inner mitochondrial membrane 13 homolog (TIMM13)	0.198
P53367	ADP-ribosylation factor interacting protein 1 (ARFIP1)	0.170
P09496	Clathrin, light chain A (CLTA)	0.161
Defense mechanisms		
P04792	Heat shock 27 kDa protein 1 (HSPB1)	0.184
P55145	Mesencephalic astrocyte-derived neurotrophic factor (MANF)	0.085
Q92597	N-myc downstream regulated 1 (NDRG1)	−0.333
Immunity		
P08571	CD14 molecule	0.815
P17813	Endoglin (ENG) - CD105	0.282
Q6GTX8, Q6ISS4	Leukocyte-associated immunoglobulin-like receptor 1&2 (LAIR1, LAIR2)	0.291
Q13123	IK cytokine, protein Red (IK)	0.051
P20138	CD33 molecule	−0.100
Nuclear structure		
Q03252	Lamin B2 (LMNB2)	0.112
Cytoskeleton		
P29966	Myristoylated alanine-rich protein kinase C substrate (MARCKS)	0.918
Q9UHB6	LIM domain and actin binding 1 (LIMA1)	0.178
Q9NYL9	Tropomodulin 3 (TMOD3)	0.160
Q99439, Q15417	Calponin 2 & 3 (CNN2, CNN3)	0.136
P21291	Cysteine and glycine-rich protein 1 (CSRP1)	−0.280
Apoptosis		
Q15121	Phosphoprotein enriched in astrocytes 15 (PEA15)	0.168
Information storage and processing		
RNA processing and modification		
Q01130, Q9BRL6	Serine/arginine-rich splicing factor 2 & 8 (SRSF2, SRSF8)	0.547
O75937	DnaJ (Hsp40) homolog, subfamily C, member 8 (DNAJC8)	0.297

Table 2 (continued)

Gene ID	Gene name	log2 ratio
Q9UPT8	Zinc finger CCCH-type containing 4 (ZC3H4)	0.072
Q12874	Splicing factor 3a, subunit 3 (SF3A3)	−0.124
Translation, ribosomal structure and biogenesis		
Q14444	Cell cycle associated protein 1 (CAPRIN1)	0.323
O75822	Eukaryotic translation initiation factor 3, subunit J (EIF3J)	0.183
Q9BRP8	Within bgcn homolog (WIBG)	0.152
Transcription		
Q99729	Heterogeneous nuclear ribonucleoprotein A/B (HNRNPAB)	−0.055
P49750	YLP motif-containing protein 1 (YLPM1)	−0.153
O14979	Heterogeneous nuclear ribonucleoprotein D-like (HNRPDL)	−0.211
Metabolism		
Amino acid transport and metabolism		
P19440, P36268	Gamma-glutamyltransferase 1 (GGT1), 3P (GGT3P) and 2 (GGT2)	0.379
O94903	Proline synthetase co-transcribed homolog (PROSC)	0.080
Lipid transport and metabolism		
Q99541, Q5SYF3, Q5SYF5, Q5SYF4	Perilipin 2 (PLIN2)	0.338
Q9Y2B0	Canopy 2 homolog (CNPY2)	0.237
Inorganic ion transport and metabolism		
P02792	Ferritin, light polypeptide (FTL)	0.383

The log2 ratio of proteins significantly changed across all three replicates (adjusted p value < 0.05) is represented here. A positive log2 ratio means the protein is upregulated in CPN10+/− infected macrophages, if negative the protein is downregulated in CPN10+/− infected macrophages as compared to macrophages infected with WT Ld

Discussion

To examine the role of CPN10 in pathogenesis, we repeatedly attempted to generate a null mutant of CPN10 in *L. donovani*. This involved a one-step double-allele replacement strategy [31], but no viable strains could be obtained. This suggested that -similar to many other organisms- CPN10 is essential in *Leishmania* and it is consistent with the fact that to date, no viable complete knockout of CPN10 has been described in any organism.

As an alternative strategy, we generated a partial CPN10 knockdown in *L. donovani* (CPN10+/− Ld), via single allele replacement (Fig. 2), and used this to study the role of CPN10 in pathogenesis. Reduced expression of CPN10 did not impair axenic growth, either as promastigotes or amastigotes (Fig. 3). CPN10+/− Ld promastigotes displayed a normal ability to differentiate into amastigotes under axenic conditions, having similar growth and morphology when compared with WT Ld (Fig. 3; Fig. S2). Moreover, CPN10+/− Ld were able to resist heat shock as well as WT organisms (Fig. S1). These findings indicate that reduced amounts of CPN10 are sufficient for axenic growth or for resistance to heat shock. Resistance to heat stress is known to depend on at least two other chaperones, HSP23 and HSP100 [11, 45].

In contrast to their resistance to heat shock, CPN10+/− Ld were much more sensitive to tunicamycin-induced stress than WT Ld (Fig. 4). Tunicamycin is an antibiotic that inhibits N-linked glycosylation during glycoprotein synthesis thereby inducing endoplasmic reticulum (ER) stress, the unfolded protein response and apoptosis, as well as increased mitochondrial metabolism. ER stress occurs in response to adverse environmental changes, such as shifts in temperature, oxidative stress, changing pH, hypoxia, and nutrient deficiency, all of which leishmania have to confront to establish a productive infection. This suggests that even though they can tolerate heat shock, CPN10+/− Ld are more sensitive to ER stress than WT Ld, underlining the need for leishmania to express WT amounts of CPN10 for optimal fitness.

CPN10+/− Ld displayed an interesting, novel phenotype of increased uptake by macrophages, but decreased intracellular survival when compared to WT Ld (Figs. 6, 7, 8). Leishmania's preferred host cells are macrophages, and leishmania exploit macrophage phagocytosis to enter the cells and replicate within phagolysosomes. Using CPN10+/− Ld, we found that reducing the amount of CPN10 in leishmania surprisingly led to increased infection rates due to increased parasite internalization (Figs. 6, 7). This may have been due to a difference in promastigote

velocity or to increased flagellar length or both. As for now, the mechanism accounting for this is unknown and may very well be multifactorial, but it is interesting to note that macrophages infected with CPN10+/− Ld demonstrated higher abundances of multiple proteins involved in membrane organization (Fig. 9b), perhaps accounting for the increased uptake of CPN10+/− Ld as compared to WT Ld.

The finding of increased macrophage internalization of CPN10+/− Ld implies that CPN10 normally acts to restrict the rate of uptake of leishmania promastigotes. This seems somewhat paradoxical since leishmania that fail to invade macrophages are quickly eliminated by cytotoxic NK cells, neutrophils and eosinophils in the host [46]. This raises the question of why high levels of CPN10 were maintained evolutionarily in Ld if they act to reduce pathogen internalization.

In considering this apparent paradox, it must be kept in mind that invading macrophages is not all leishmania need to do to establish chronic infection, as they must subsequently survive in phagolysosomes. We examined the ability of CPN10+/− Ld to survive intracellularly and be recovered after lysis of their host cells, and indeed their intracellular survival and recovery were strongly impaired compared to WT Ld (Fig. 8a, b). Consistent with this, over expressing CPN10 in Ld, even modestly, led to decreased internalization of leishmania by macrophages, but improved intracellular survival and recovery after 48 h of infection, as compared to WT Ld (Figs. 7, 8). These findings show clearly that CPN10 is required for intracellular survival, explaining its evolutionary conservation.

An important question that arises here is what advantage if any would there be for leishmania CPN10 restricting parasite internalization. This may relate to the fact that to establish a long term infection, leishmania prefer—as much as possible—to enter macrophages silently without activating them as the latter would promote parasite killing. It is interesting to hypothesize, therefore, that higher amounts of leishmania CPN10 in the cell—by restricting the rate of internalization—would favor "silent" entry, so as not to promote macrophage activation and death of leishmania. This hypothesis is supported by the lower intracellular survival shown by CPN10+/− Ld in host macrophages (Fig. 8). Thus, CPN10+/− Ld are internalized more efficiently by macrophages, but also are more readily killed, perhaps related to their propensity to promote greater degrees of macrophage activation. This latter argument is supported further by the upregulation of multiple proteins involved in the innate immune response—which may contribute to parasite killing—in macrophages infected with CPN10+/− Ld as compared to macrophages infected with WT Ld (Fig. 9b; Table 2).

Quantitative proteomics comparing WT-Ld and CPN10+/− Ld showed clearly that lowering the amount of leishmania CPN10 available in the cell resulted in changed abundance of many leishmania proteins. The majority (80.5%) of leishmania proteins that showed altered abundance were downregulated in CPN10+/− Ld as compared to the parental WT strain (Fig. 5a). This suggests that a lower pool of CPN10 in leishmania leads to a less efficient protein folding or refolding, resulting in increased degradation of these unfolded proteins and their reduced expression. The importance of CPN10 in protein folding is, therefore, underlined here, revealing that despite normal axenic growth and resistance to heat shock, many proteins are affected by reduced levels of CPN10.

Gene ontology analysis revealed that a large number of the downregulated proteins are involved in metabolic processes and cellular processes (Fig. 5b). In particular, multiple proteins involved in oxidation–reduction processes were downregulated in CPN10+/− Ld, with 6 oxidoreductases showing reduced expression compared to WT Ld (Fig. 5b). A robust system to regulate the cellular redox state is essential for optimal fitness in leishmania, especially in intracellular amastigotes when they are targeted by the macrophage effectors within phagolysosomes. For example, proline oxidase—also called proline dehydrogenase—which was reduced in CPN10+/− Ld (Table 1) contributes to the regulation of redox state and oxidative metabolism in another trypanosomatid, *Trypanosoma cruzi*, and its overexpression led to resistance to hydrogen peroxide [47]. Also, downregulated in CPN10+/− Ld was cytochrome-b5-reductase. Lack of this protein resulted in decreased linoleate synthesis, increased oxidative stress and apoptosis in *Leishmania major* [48]. Taken together, these findings suggest that CPN10+/− Ld would not be able to deal with oxidative stress as well as WT Ld, and this is consistent with their lowered resistance to tunicamycin-induced stress (Fig. 4).

We also found that many proteins involved in fatty acid metabolism (Fig. 5b; Table 1) were downregulated in CPN10+/− Ld and reduction of any one or more of these may contribute to its reduced fitness. For example, dihydrolipoamide branched chain transacylase is involved in fatty-acyl-coA biosynthesis. This is an important function as acyl-coA dehydrogenase catalyses the initial step of fatty acid beta-oxidation, playing roles both breaking down fatty acids and in energy production. Also, reduced in abundance were Acyl carrier protein and C-5 sterol desaturase, two proteins involved in fatty acid biosynthesis and their products are used to form cell and organellar membranes. Notably, the lipid composition of the leishmania plasma membrane plays a role in pathogenesis as well as resistance to drugs [49, 50]. Altered lipid composition of the leishmania plasma membrane might also modify its interactions with the macrophage plasma membrane resulting in the phenotype of higher internalization of CPN10+/− Ld as compared to WT Ld.

Several proteins involved in nucleoside, nucleotide and nucleic acid metabolic processes were also downregulated in CPN10+/− Ld (Fig. 5b). For example, methylthioadenosine phosphorylase was reduced in CPN10+/− Ld (Table 1). This is an important drug target, through its key function in purine and polyamine metabolism and the methionine salvage pathway [51]. Together with downregulation of cytidine deaminase which is involved in pyrimidine salvage, this suggests that both purine and pyrimidine metabolism might be impaired in CPN10+/− Ld [52].

Concerning RNA metabolic processes, DNA-directed RNA Pol I, transcription elongation regulator-like protein SPT5, and an ATP-dependent RNA helicase were all reduced in abundance suggesting that transcription may be affected in CPN10+/− Ld (Fig. 5b; Table 1).

Several protein phosphatases were also downregulated in CPN10+/− Ld (Fig. 5b). Protein phosphatases allow the reversible protein phosophorylation that is essential for the regulation of signaling pathways controlling a variety of processes including metabolic pathways, cell–cell communication, cell growth and proliferation and gene transcription. Other proteins involved in protein modifications and transport were also downregulated in CPN10+/−. Two peptidases were also strongly downregulated. All these proteins act on other proteins to change their activity, their stability, their localization, thereby playing important roles in many metabolism processes, and essential to cellular homeostasis.

Proteins involved in localization were also downregulated, especially transfer/carrier proteins, membrane traffic proteins and transporters (Fig. 5b). The only protein class being overall upregulated in CPN10+/− Ld was cytoskeletal proteins.

Taken together, this proteomic analysis establishes leishmania CPN10 as a pleiotropic regulator of the leishmania proteome. This likely promotes the expression of leishmania effectors and contributes to pathogenesis and warrants further study.

To allow for their survival and propagation within their hosts, leishmania target signaling pathways to inhibit macrophage activation and block the induction of innate and adaptive immunity [53]. How this is orchestrated is a focus of significant interest. Here, using quantitative proteomics we found that macrophages infected with CPN10+/− Ld—in contrast to WT leishmania—were strongly biased towards the increased expression of proteins associated with innate immune responses (Fig. 9b; Table 2). These findings suggest that during infection with WT leishmania expressing WT levels of CPN10, that this heat shock protein acts in some fashion to inhibit the expression of critical host defense proteins.

Among the most highly upregulated proteins in CPN10+/− Ld-infected cells was CD14, an innate immunity pattern recognition receptor expressed specifically by monocytes and macrophages (Table 2). CD14 works in concert with other proteins to recognize a variety of ligands, including potentially leishmania-derived ligands such as *Leishmania pifanoi* P8 glycolipid complex, which it delivers to TLR4. This in turn promotes MD2 and MyD88-dependent signaling and the production of proinflammatory cytokines [54]. Consistent with our finding of enhanced expression of CD14 in CPN10+/− Ld-infected cells, cell surface expression of CD14 was previously found to be downregulated in monocyte-derived macrophages when infected with *Leishmania major* [55]. Taken together, our findings suggest that WT levels of CPN10 act to tonically limit CD14 expression thereby restricting the innate immune response.

Like CD14, leukocyte-associated immunoglobulin-like receptors 1 and 2 (LAIR1, LAIR2) were also upregulated in CPN10+/− Ld-infected macrophages (Table 2). LAIR1 and 2 belong to a family of immune inhibitory receptors that regulate immune system balance. LAIR-1 is a collagen-receptor that inhibits immune cell function, while LAIR-2 is expressed as a soluble receptor able to antagonize the collagen/LAIR-1 inhibitory immune interaction [56]. LAIR-1 and LAIR-2 have 74–100% identity, depending on isoforms, which likely explains why mass spectrometry did not allow us to differentiate between them. Nevertheless, it is interesting to speculate that downregulation of the expression of either LAIR1 or LAIR2 or both by leishmania CPN10 may contribute to defective induction of innate or adaptive anti-leishmania immunity.

Endoglin (CD105), also upregulated in macrophages infected with CPN10+/− Ld (Table 2), is a transmembrane protein component of the TGF-β receptor complex. It seems to have variable modulatory roles in TGF-β signaling in macrophages: in some cases inhibitory [57] and in others enhancing it [58]. A direct link between endoglin and leishmania infection has not been described, but TGF-β is known to promote parasite survival.

Promyelocytic leukemia protein (PML) is a multifunctional protein, that plays critical roles in growth suppression, induction of apoptosis and cellular senescence, but also in innate immunity, potentially through the TLR/NF-kB prosurvival pathway [59]. Here we show that PML is upregulated in macrophages infected with CPN10+/− Ld (Table 2). This may lead to activation of the TLR/NF-kB pathway, which contributes to host defense against leishmania [60], but it could also play a role in inducing apoptosis.

Proteins of the ubiquitin family were also upregulated in CPN10+/− Ld-infected macrophages (Table 2). Ubiquitin proteins are central to a variety of cellular processes in which they attach covalently to other proteins thereby

changing the stability, activity or localization of their target proteins. Amongst others, protein ubiquitination regulates the activaties of toll-like receptors (TLRs), which are targeted by leishmania as a mean to escape the host immune response [61]. In this context, it was shown that *L. donovani* upregulates the host deubiquitinating enzyme A20, leading to a drastic reduction of TRAF6 ubiquitination, explaining modulation of the TLR2-mediated signaling pathway in infected macrophages [62]. Ubiquitination is involved in many different pathways, and the increased abundance of ubiquitin in the cell may indicate that protein ubiquitination is a more frequent event in cells infected with CPN10+/− leishmania compared with WT. How this may influence intracellular survival of leishmania is not clear at this stage, but the identification of proteins that undergo changes in ubiquitination seems to be a logical next step.

Other miscellaneous proteins previously linked to the fate of leishmania infection were found to be upregulated in cells infected with CPN10+/−Ld. One example is ferritin (Table 2), a protein responsible for cytosolic storage of iron in a redox-inert form, decreasing access of the parasite to free iron, which is essential for the intracellular replication of leishmania [63].

MARCKS, a protein kinase C substrate, was one of the most strongly upregulated proteins in macrophages infected with CPN10+/− Ld (Table 2). MARCKS has been described as being strongly downregulated in leishmania-infected cells, together with its partner MARCKS-related protein (MRP) [64]. Leishmania has been shown to interfere with many host cell signaling pathways, including protein kinase C (PKC)-dependent signaling [65, 66], a pathway required for optimal macrophage activation [67]. These findings suggest that CPN10+/− Ld may not interfere with host PKC-dependent signaling to the same extent as WT leishmania. This would allow for more effective macrophage activation resulting in decreased intracellular survival of CPN10+/− Ld as we observed (Fig. 7).

Another protein of interest found to be upregulated in cells infected with CPN10+/− Ld was cystatin, a cysteine protease inhibitor (Table 2). Cystatin targets parasite cysteine proteases thought to play a role in infection by directing the immune response towards a Th2 response, favorable to parasite proliferation [68]. The ability of leishmania CPN10 to repress macrophage cystatin expression may confer a survival advantage and this is consistent with reduced intracellular survival of CPN10+/− Ld as compared to WT (Fig. 7). This notion is supported further by the finding that chicken cystatin has been used successfully as a treatment against murine visceral leishmaniasis by activating an NF-kB-mediated proinflammatory responses [69, 70].

In summary, the results of the present study define novels roles for leishmania CPN10 and identify it as is a novel *bona fide* virulence factor. CPN10 is shown to both attenuate the rate of leishmania internalization—which may limit the degree of macrophage activation—while at the same time promoting their intracellular survival. Using a systems biology based approach, we show that leishmania CPN10 is a pleiotropic regulator of the leishmania proteome. Numerous leishmania proteins belonging to diverse classes were found to be positively regulated by CPN10 and any one or more of these candidate effectors may contribute to pathogenesis. Not only did leishmania CPN10 regulate expression of the leishmania proteome, but it also influenced the expression of a large and diverse group of macrophage proteins in infected cells. In particular, leishmania CPN10 was strongly biased towards downregulation of an interesting array of proteins linked to innate immunity and host defense. This notable and unanticipated property of leishmania CPN10 to reciprocally up regulate candidate leishmania effectors while down regulating various host innate immune effectors places it at a critical nidus at the host-pathogen interface. These findings identify leishmania CPN10 as an interesting candidate virulence factor. Combined with its probable essentiality, CPN10 also appears to be an attractive drug target and such a notion is supported by the precedent that GroES and GroEL inhibitors have already been identified as potential antibiotics, and tested against a panel of Gram-positive and Gram-negative bacteria [71, 72].

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