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1 SILAC-based quantitative proteomics reveals pleiotropic,
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30

31 **ABSTRACT**

32 Leishmaniasis are major vector-borne tropical diseases responsible for great human morbidity
33 and mortality, caused by protozoan, trypanosomatid parasites of the genus *Leishmania*. In the
34 mammalian host, parasites survive and multiply within mononuclear phagocytes, especially

35 macrophages. However, the underlying mechanisms by which *Leishmania* spp affect their host
36 are not fully understood. Herein, proteomic alterations of primary, bone marrow-derived
37 BALB/c macrophages are documented after 72 h of infection with *Leishmania donovani* insect-
38 stage promastigotes, applying a SILAC-based, quantitative proteomics approach. The protocol
39 was optimised by combining strong anion exchange and gel electrophoresis fractionation that
40 displayed similar depth of analysis (combined total 6189 mouse proteins). Our analyses
41 revealed 86 differentially modulated proteins (35 showing increased and 51 decreased
42 abundance) in response to *Leishmania donovani* infection. The proteomics results were
43 validated by analysing the abundance of selected proteins. Intracellular *Leishmania donovani*
44 infection led to changes in various host cell biological processes, including primary metabolism
45 and catabolic process, with a significant enrichment in lysosomal organisation. Overall, our
46 analysis establishes the first proteome of *bona fide* primary macrophages infected *ex vivo* with
47 *Leishmania donovani*, revealing new mechanisms acting at the host/pathogen interface.

48

49 **Keywords:** quantitative proteomics, bone marrow-derived macrophages, *Leishmania*
50 *donovani*, SILAC, LC-MS/MS

51

52 INTRODUCTION

53 The leishmaniasis include a group of diseases caused by more than 21 protozoan species of the
54 trypanosomatid genus *Leishmania*, which are transmitted by the bite of phlebotomine sandflies
55 [1]. These diseases, prevalent in 98 countries, have different clinical outcomes that range from
56 localised skin ulcers (cutaneous leishmaniasis) to lethal systemic disease (visceral
57 leishmaniasis, VL)[2]. VL is the most serious form of the disease, with 200 000 to 400 000 new
58 cases documented every year [2] and a lethal outcome if left untreated. VL is caused mainly by
59 strains of the *Leishmania (L.) donovani* complex and is characterised by systemic symptoms,
60 such as fever and weight loss [3].

61 All *Leishmania* parasites share a digenetic life cycle and live either as extracellular,
62 motile promastigotes within the midgut of a sand fly vector, or as intracellular, non-motile

63 amastigotes in professional phagocytes, with macrophage phagolysosomes representing the
64 primary niche for proliferation [4]. These phagocytic mononuclear cells carry important,
65 immuno-regulatory functions and accordingly can differentiate into phenotypically distinct
66 subtypes [5], including pro-inflammatory M1 cells with microbicidal properties and
67 alternatively activated, anti-inflammatory M2 cells involved in tissue repair (reviewed in [6]).
68 These M1 and M2 phenotypes represent the extremes of a continuous spectrum of polarisation
69 states [7]. Even though both the initial *Leishmania* infection through the bite of infected sand
70 flies and the later acute stages of the disease are characterised by inflammation of the infected
71 tissue [8, 9], *Leishmania* thrives in these environments by efficiently dampening the pro-
72 inflammatory response of its infected host cell, possibly by exploiting developmental programs
73 underlying macrophage phenotypic plasticity [10].

74 The prevention of *Leishmania* clearance and persistent infection of macrophages is
75 governed by the expression of specific parasite virulence factors, such as highly abundant
76 parasite surface glycolipids [lipophosphoglycan (LPG) and glycoinositol phospholipids
77 (GIPLs)], or the GPI anchored metalloprotease, GP63 [11-15]. These and other factors have
78 been shown to remodel the macrophage phenotype to establish permissive conditions for
79 *Leishmania* intracellular survival. Initially, following entry into the macrophage, *L. donovani*
80 promastigotes delay phagosomal/lysosomal fusion and phagosome maturation [16-18], and
81 subsequently modify host cell signaling and metabolic processes, inhibit apoptosis and suppress
82 antigen presentation (reviewed in [10]).

83 However, this ideal concept of ‘macrophage deactivation’ upon *Leishmania* infection
84 is clearly an oversimplification, as the macrophage response to infection under clinically more
85 relevant conditions can depend on many parameters, including the host cell differentiation state,
86 the tissue context, and parasite species or even strain [19, 20]. Systems-level, ‘omics’
87 approaches revealed the complex interface between the parasite and the macrophage and shed
88 important new light on the host cell response to *Leishmania* infection, particularly at the
89 transcript level [8, 9, 19, 21-27]. However, transcript abundance does not always correlate with
90 protein levels and thus may not give an accurate picture of the cellular phenotype. Proteome

91 analyses certainly allows for a more relevant functional insight, but the few proteomics screens
92 - reviewed in Veras & Menezes, 2016 [28] - were performed on *Leishmania* infected cells using
93 either immortalised, macrophage-like cell lines [29, 30] known to be phenotypically and
94 functionally different from *bona fide* macrophages [31-34], or a semi-quantitative approach
95 based on spectral counting [20] that lacks quantitative performance [35]. Here we overcame
96 these limitations and for the first time quantified proteomic changes in primary, bone marrow-
97 derived macrophages (BMDMs) after 72 h of infection with virulent *L. donovani* promastigotes
98 using stable isotope labeling by amino acids in cell culture (SILAC). SILAC, a technique
99 that relies on the metabolic replacement of a given natural amino acid (referred to as
100 'light') with a non-radioactive, stable isotope (referred to as 'heavy') [36], is considered
101 one of the most accurate quantitative approaches when using cell culture systems to study
102 dynamics of biological processes [35, 36]. Our analysis confirms known signatures of
103 infected macrophages, but also provides new insight into *L. donovani*-induced phenotypic
104 changes of primary macrophages and mechanisms of intracellular parasite survival acting at the
105 host/pathogen interface.

106

107 **MATERIALS AND METHODS**

108 *Ethics statement*

109 All animal experiments complied with the ARRIVE guidelines and were carried out according
110 to the EU Directive 2010/63/EU for animal experiments. All animals were housed in an A3
111 animal facility of Institut Pasteur (Paris). Housing and conditions were in compliance with the
112 protocol approved by the Institut Pasteur (Paris) ethical committee for animal experimentation
113 (CETEA: C2TEA 89)) and procedures were conducted in agreement with the project licenses
114 HA0005and#10587, issued by the Institut Pasteur CETEA and the Ministère de l'Enseignement
115 Supérieur de la Recherche et de l'Innovation (MESRI) respectively.

116

117 *Leishmania culture, BMDM differentiation and infection*

118 *L. donovani* strain 1S2D (MHOM/SD/62/1S-CL2D obtained from Henry Murray (Weill
119 Cornell Medical College, New York, USA)) was maintained in female RjHan:AURA golden
120 Syrian hamsters between four and six weeks of age (Janvier Labs) by serial *in vivo*
121 passaging. Amastigotes were purified from hamster spleens as described [37] and
122 differentiated into promastigotes in culture that were maintained for no more than five *in*
123 *vitro* passages. Parasites were cultivated at 26°C in fully supplemented M199 medium (10%
124 FCS, 25 mM HEPES, 4 mM NaHCO₃, 1 mM glutamine, 1 x RPMI 1640 vitamin mix, 0.2 µM
125 folic acid, 100 µM adenine, 7.6 mM hemin, 8 µM biopterin, 50 U ml⁻¹ of penicillin and 50 µg
126 ml⁻¹ of streptomycin, pH7.4).

127 Bone marrow cell suspensions were recovered from tibias and femurs of BALB/c
128 mice (Janvier) and suspended in bacteriologic Petri dishes (Greiner, Germany) at a
129 concentration of 1.35x10⁶ ml⁻¹ in complete medium i.e. RPMI 1640 supplemented with 50
130 µM β-mercaptoethanol, 15% heat-inactivated fetal calf serum (GIBCO), 50 U ml⁻¹ of
131 penicillin and 50 µg ml⁻¹ of streptomycin and 75 ng mL⁻¹ of macrophage Colony Stimulating
132 Factor-1 (mCSF-1) (Immunotools). Cells were incubated at 37°C and 5% CO₂. Three days
133 after plating, 0.4 volume of fresh complete medium was added to the cells. Six days later,
134 adherent bone marrow-derived macrophages (BMDMs) were washed with Dulbecco's
135 phosphate buffered solution (PBS). Cells were detached by incubation with Dulbecco's PBS
136 without Ca²⁺ and Mg²⁺ (Biochrom AG, Berlin, Germany) containing 25 mM EDTA for
137 30min at 37°C.

138 For *Leishmania* infection, recovered BMDMs were plated in flat-bottom, 6-well
139 plates (Tanner, Switzerland) at a density of 4x10⁶ cells per well and were maintained in
140 complete medium. Six hours after plating, macrophages were infected with 4x10⁷ *L.*
141 *donovani* stationary phase promastigotes (multiplicity of infection of 10:1, *in vitro* passage
142 2 after differentiation from splenic amastigotes) for 4 h and incubated at 37°C in a 5% CO₂.
143 Following infection, macrophages were washed four times with PBS to remove free
144 promastigotes. Infected and non-infected macrophages were then maintained for 72 h in
145 complete medium containing only 25 ng mL⁻¹ mCSF-1.

146

147 *SILAC labelling and sample preparation*

148 For labelling cells by SILAC, equal numbers of BMDMs were differentiated and cultured as
149 described above, but using RPMI 1640 without Lysine and Arginine (Thermo Fisher
150 Scientific) that was supplemented either with natural amino acids (L-Lysine, 0.274 mM; L-
151 Lysine, 1.15 mM; Arginine, 1.15 mM) to obtain “light” cells (referred to as control) or with
152 amino acid isotopes $^2\text{H}_4$ -Lysine (Lys4) and $^{13}\text{C}_6$ -Arginine (Arg6) at the same concentrations
153 to obtain “heavy” cells (referred to as labelled) (Thermo Scientific). Fetal calf serum
154 (GIBCO) was dialysed over night against 100 volumes of sterile PBS, followed by two new
155 rounds of 3 h dialysis, each against a low cutoff membrane (3.5 kD, Gebaflex). The light or
156 heavy RPMI 1640 was supplemented with 15% (v/v) of dialysed and filter-sterilised serum,
157 50 μM β -mercaptoethanol, 50 U ml^{-1} penicillin, 50 $\mu\text{g ml}^{-1}$ streptomycin, and 75 ng mL^{-1} of
158 mCSF-1.

159 BMDMs cultivated in SILAC medium were washed three times in PBS and were lysed
160 with a buffer containing 8 M urea, 50 mM ammonium bicarbonate pH7.5, one tablet per 10 ml
161 of cOmplete™ Protease Inhibitor Cocktail tablets (Sigma), and one tablet of the phosphatase
162 inhibitor cocktail PhosSTOP (Roche). Cell extracts were incubated on ice 30 min, sonicated 5
163 min, and centrifuged 15 min at 14,000 g to eliminate cell debris. Proteins were quantified in the
164 supernatants using the RC DC™ protein assay kit (Bio-Rad), according to the manufacturer’s
165 instructions. For protein identification and quantification, control infected were mixed with
166 labeled non-infected and labeled infected were mixed with control non-infected at an equal
167 ratio.

168

169 *SILAC Sample processing*

170 Samples supernatants as processed above were fractionated by two different methods: (i)
171 polyacrylamide gel electrophoresis fractionation (GEL) and (ii) Strong Anion eXchange
172 (SAX). For GEL fractionation, mixed samples were separated by polyacrylamide gel
173 electrophoresis (PAGE) with the use of Invitrogen NuPAGE 10% Bis-Tris 1.0mm*10 Well

174 gels. Proteins were recovered from 9 gel slices following in-gel digestion as described in
175 standard protocols. Briefly, following the SDS-PAGE and washing of the excised gel slices,
176 proteins were reduced by adding 10 mM DTT (Sigma Aldrich) prior to alkylation with 55 mM
177 iodoacetamide (Sigma Aldrich). After washing and dehydrating the gel pieces with 100%
178 acetonitrile, trypsin (Sequencing Grade Modified, Roche Diagnostics) was added and proteins
179 were digested overnight in 25 mM ammonium bicarbonate at 30°C.

180 SAX-based samples were digested in solution prior to fractionation of peptides. Briefly,
181 the protein extracts obtained by urea extraction were mixed at a 1:1 ratio and subjected to the
182 following process: First, samples were reduced using 10 mM DTT, alkylated with 55 mM
183 iodoacetamide and digested using Trypsin overnight. Second, peptides were desalted through
184 Sep-Pak C18 cartridges (Waters) and dried down prior to their fractionation. Peptide
185 fractionation was carried out through SAX using in-house prepared microcolumns (anion
186 exchange disks, Empore) and a Britton - Robinson buffer (20 mM acetic acid, 20 mM
187 phosphoric acid, 20 mM boric acid) at six decreasing pH values (11, 8, 6, 5, 4, 3) for the elution
188 of peptides [38].

189 Samples were loaded onto homemade C18 SepPak-packed StageTips for desalting
190 (principle by stacking one 3M Empore SPE Extraction Disk Octadecyl (C18) and beads from
191 SepPak C18 Cartridge (Waters) into a 200 µL micropipette tip). Peptides were eluted with 40/60
192 MeCN/H₂O + 0.1% formic acid and lyophilysed under vacuum. Desalted samples were
193 reconstituted in injection buffer (2% MeCN, 0.3% TFA) for LC-MS/MS analysis.

194

195 *LC-MS/MS analysis*

196 Online liquid chromatography (LC) was performed with an RSLCnano system (Ultimate 3000,
197 Thermo Scientific) coupled online to an Orbitrap Fusion Tribrid mass spectrometer (MS,
198 Thermo Scientific). Peptides were trapped on a C18 column (75 µm inner diameter × 2 cm;
199 nanoViper Acclaim PepMap™ 100, Thermo Scientific) with buffer A (2/98 MeCN/H₂O in
200 0.1% formic acid) at a flow rate of 4.0 µL/min over 4 min. Separation was performed on a 50
201 cm x 75 µm C18 column (nanoViper Acclaim PepMap™ RSLC, 2 µm, 100Å, Thermo

202 Scientific) regulated to a temperature of 55°C with a linear gradient of 5% to 25% buffer B
203 (100% MeCN in 0.1% formic acid) at a flow rate of 300 nL/min over 100 min for the SAX and
204 the GEL samples. Full-scan MS was acquired in the Orbitrap analyzer with a resolution set to
205 120,000, a mass range of m/z 400–1500 and a 4×10^5 ion count target. Tandem MS was
206 performed by isolation at 1.6 Th with the quadrupole, HCD fragmentation with normalised
207 collision energy of 30, and rapid scan MS analysis in the ion trap. The MS² ion count target
208 was set to 2×10^4 and only those precursors with charge state from 2 to 7 were sampled for
209 MS² acquisition. The instrument was run in top speed mode with 3 s cycles

210

211 *MS Data Processing and Protein Identification*

212 Data were searched against the Swiss-Prot *Mus musculus* (012016) and *L. donovani* (20150914)
213 databases, using Sequest^{HT} through Thermo Scientific Proteome Discoverer (v 2.1). The mass
214 tolerances in MS and MS/MS were set to 10 ppm and 0.6 Da, respectively. We set
215 carbamidomethyl cysteine, oxidation of methionine, N-terminal acetylation, heavy ¹³C₆-
216 Arginine (Arg6) and ²H₄-Lysine (Lys4) as variable modifications. We set specificity of Trypsin
217 digestion and allowed two missed cleavage sites.

218 The resulting files were further processed by using myProMS (v 3.5) [39]. The Sequest
219 HT target and decoy search result were validated at 1% false discovery rate (FDR) with
220 Percolator. For SILAC-based protein quantification, peptides XICs (Extracted Ion
221 Chromatograms) were retrieved from Thermo Scientific Proteome Discoverer. Global Median
222 Absolute Deviation (MAD) normalisation was applied on the total signal to correct the XICs
223 for each biological replicate. Protein ratios were computed as the geometrical mean of related
224 peptides. To estimate ratio significance, a t-test was performed with the R package limma [40]
225 and the false discovery rate has been controlled using the Benjamini-Hochberg procedure [41].

226

227 *RNA isolation and RT qPCR*

228 Total RNA was isolated from macrophages using the Nucleospin RNA isolation kit
229 (MACHEREY-NAGEL GmbH, Germany) according to the manufacturers' instructions. The

230 integrity of the RNA was assessed using the Agilent 2100 Bioanalyzer system. For RNA reverse
231 transcription to first strand cDNA, 1 µg of total RNA was mixed with random hexamers (Roche
232 Diagnostics) and MMLV-RT reverse transcriptase (Moloney Murine Leukemia Virus,
233 Invitrogen Life Technologies), and the reaction was carried according to the manufacturer's
234 instructions. qPCR was performed with the QuantiTect SYBR Green kit (Qiagen) according to
235 the manufacturers' instructions in a 10 µL reaction mixture containing 1 µL cDNA template
236 and 0.5 µM forward/ reverse primers and 5 µl 2x QuantiTect master mix. The reactions were
237 performed in triplicates in white FrameStar® 384 PCR plates (4titude) on a LightCycler® 480
238 system (Roche Diagnostics, Meylan, France) and gene expression analysis was performed with
239 the use of qBase [42]. The primers used are listed in **Table S1**. Analysis of PCR data and
240 normalisation over the reference genes Yhwaz and RpL19, were performed as previously
241 described [43].

242

243 *Western blot analysis*

244 Total protein extracts were separated on 4–12% Bis-Tris NuPAGE® gels (Invitrogen) and
245 blotted onto polyvinylidene difluoride (PVDF) membranes (Pierce). Western blot analyses
246 were performed as previously described [44]. The following primary antibodies were used at
247 1:1000 dilution: rabbit anti-GSTM (Abcam, ab178684), anti-Asah-1 (ab74469), anti-Rab3IL1
248 (Abcam, ab98839), anti-Plxna1 (Abcam, ab23391), anti-Hmox-1 (Abcam, ab13243) and anti-
249 Ctsd (Abcam, ab75852) and the monoclonal anti-β actin 13E5 (Cell Signaling Technologies,
250 #4970). The anti-rabbit and anti-mouse secondary antibodies (Pierce) were diluted 1:20000.
251 Blots were developed using SuperSignal™ West Pico (Pierce) according to the
252 manufacturer's instructions. Western blots were quantified with Image J software.

253

254 *Counting of intracellular parasites*

255 Infected and non-infected macrophages plated on coverslips were fixed for 20 minutes at RT
256 with 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS) and incubated for

257 15 min with PBS containing the nuclear dye Hoechst 33342 (0.5 µg/ml). Excess dye was
258 removed by rinsing coverslips in PBS before mounting on a microscope slide using Mowiol
259 (Sigma-Aldrich). Two different cover slips per condition were analysed with a Leica
260 DMI6000B microscope or an inverted microscope equipped with ApoTome (ZEISS).

261

262 *Functional enrichment analysis*

263 GO default annotations were parsed from the available NCBI GO information
264 (<http://www.ncbi.nlm.nih.gov/Ftp/>), using as background the whole annotation from Uniprot
265 *Mus musculus* reference proteome (UP000000589). The GO enrichment analysis for biological
266 process, molecular function and cellular component was performed using the proteins listed in
267 **Table 1** and **2** as input. The multiple testing correction was selected to the Benjamini and
268 Hochberg false discovery rate at a significance level of 0.01. Results were visualised using the
269 Cytoscape 3.5.1 software package [45] and the BinGO plug-in [46].

270

271 **RESULTS AND DISCUSSION**

272 *Metabolic labeling of BMDMs in SILAC medium and experimental workflow for SILAC* 273 *experiments*

274 First, we tested the capacity of *L. donovani* promastigotes to infect and proliferate
275 within control and isotope-labelled BMDMs. Macrophages at day 6 post-differentiation in
276 SILAC culture medium were infected at a parasite-to-macrophage ratio of 10:1 with *L.*
277 *donovani* promastigotes at *in vitro* passage 2 after differentiation from lesion-derived
278 amastigotes. Intracellular parasite burden was microscopically assessed at 4 h, 48 h and 72 h
279 post-infection (PI) (**Fig. 1A**). We observed a mean number of 8 parasites per macrophage at 4
280 h PI, which transiently decreased at 48 h but then recovered at 72 h. More than 80% of
281 macrophages were infected at all time points tested (4 h, 48 h and 72 h, data not shown),
282 indicating robust infection and intracellular parasite growth. Infection was similar in both

283 labelled and control macrophages, suggesting that the metabolic labelling did not influence the
284 ability of parasites to survive and proliferate.

285 In a series of control experiments, we assessed the culture system for variations in host cell
286 phenotype, SILAC label incorporation, infection efficiency and protein fractionation technique.

287 Under our experimental conditions (see Materials and Methods), over 90% of the adherent cells
288 differentiated into a homogeneous macrophage population as judged (i) by expression of the
289 macrophage-specific F4/80 surface antigen as determined by fluorescent microscopy [47, 48],
290 and (ii) the ability of the cells to phagocytose fluorescently labeled, yeast zymosan bioparticles
291 (data not shown). We tested the isotope incorporation efficiency in a pilot experiment using
292 Lys4 at day 6 after the differentiation process. Under these conditions, we observed an
293 incorporation rate of “heavy” lysine above 98%, as only 19 proteins out of 1111 proteins were
294 unlabeled. We thus considered that a period of at least six days in SILAC medium is appropriate
295 for performing our proteomics screen (**Fig. 1B**). As shown by the experimental layout in **Fig.**
296 **1C**, we performed label-swap experiments, where the experimental state and stable isotope
297 labels are interchanged. Label swap enriches the list of proteins whose abundance is related to
298 the experimental state as it allows for the identification of systematic errors due to labeling, and
299 to experimental outliers. Bone marrow progenitor cells were differentiated into macrophages
300 in medium supplemented with the ‘heavy’ amino acid isotopes, referred to as ‘labelled’ (L),
301 and medium supplemented with the ‘light’, natural amino acids, referred to as ‘control’ (C).
302 Cells were infected six days post-differentiation with *L. donovani* promastigotes for 72 h. *L.*
303 *donovani*- infected (I) and non-infected (NI), labeled (L) and control (C) macrophages were
304 lysed, mixed in equal amounts and their proteomes were analysed by LC/MS-MS. Our protocol
305 further included two fractionation methods, SAX and GEL fractionation that are based on
306 charge- or size-dependent separation, respectively. The reduction of extract complexity by these
307 methods is increasing the number of quantifications and thus the quality of our proteomics
308 analyses by increasing protein sequence coverage, which facilitates detection of low-abundance
309 proteins. For these analyses, we performed two label swap experiments and two technical
310 replicates.

311

312 *Analysis and validation of proteomics data*

313 The comparative analysis of macrophage proteomes from reciprocal L-I/ C-NI and C-I/L-NI
314 samples allowed the identification of 5322 and 5101 proteins using SAX and GEL
315 fractionation, respectively and a combined total 6189 proteins (**Fig. 2A** and **Fig. 2B, Table**
316 **S2**). Seventy five percent of proteins identified by GEL and SAX fractionation (3871 and 4026
317 proteins, respectively) were shared between L-I/C-NI and C-I/L-NI (**Fig. 2A**), indicating a very
318 good, qualitative reproducibility in the depth of our analysis. Moreover, the overlap between
319 SAX and GEL was also significant, with sixty five percent (3291 proteins) of the proteins
320 identified being shared between the two groups (GEL, SAX: L-I/C-NI and GEL, SAX: L-NI/C-
321 I) (**Fig. 2B**). Volcano plots using GEL and SAX fractionation revealed that a subset of proteins
322 were differentially expressed between in L-I/C-NI and C-I/L-NI (**Fig. 2C**).

323 Amongst the proteins with modulated abundance, only macrophage proteins identified
324 in reciprocal (label swapped) experiments that conformed to raised filtering criteria were
325 selected. To this end, we considered in both forward and reverse experiments a fold change
326 (FC) of 1.25 or higher, with at least 2 peptides identified per quantification. With the exception
327 of “infinite quantifications” – i.e “quantifications” where the proteins were identified in only
328 one of the two compared conditions - only quantifications with confidence level of 0.05 or
329 below ($p \leq 0.05$) in at least one of the experiments were accepted (**Table 1** and **Table 2**).

330 This analysis revealed increased abundance in infected macrophages for 35 proteins
331 (27 proteins with GEL, 8 proteins with SAX), with Glutathione S Transferase mu1
332 (Gstm1/P10649) common in the datasets (**Table 1**). The same analysis revealed reduced
333 abundance for 51 proteins in infected macrophages (42 proteins with GEL, 13 proteins with
334 SAX), with 4 proteins common in the two datasets [the macrophage-expressed gene 1 protein
335 (Mpeg-1/A1L314), Palmitoyl-protein thioesterase 1 (Ppt1/O88531), Cathespin D (Ctsd/
336 P18242) and the Mannose receptor C-type 1 (Mrc-1/Q61830)] (**Table 2**). Thus, for the primary
337 macrophage proteome, these results indicate that SAX and GEL fractionation procedures
338 together increase the level of quantifications that pass the filtering criteria, increasing the depth

339 of protein quantification. Moreover these data were run against an *L. donovani* database. This
340 analysis revealed that the number of parasite proteins identified ranged between 763 and 1580
341 (**Table S3**) and a combined total of 1741 proteins (**Table S4**). 55 proteins were shared in each
342 of the quantification between the mouse and the parasitic proteome (**Table S5** and **Table S6**).
343 Comparing these proteins to the proteins listed in **Tables 1** and **2**, only one protein shared one
344 common peptide between mouse and *L. donovani*, namely the 26S protease regulatory subunit
345 (Psmc3/O88685) (**Table 1**, **Table S5** and **Table S6**), showing that most of the peptides and
346 proteins identified as differentially expressed in this screen are mouse proteins, as 1741 proteins
347 are parasite proteins.

348 We then validated our results by immunoblot analysis of infected and non-infected
349 macrophages using β -actin for normalisation. We confirmed reduced abundance in response to
350 infection for Acid ceramidase 1 (Asah1/Q9WV54), Guanine nucleotide exchange factor for
351 Rab-3A (Rab3IL1, Q8TBN0), Heme oxygenase 1 (Hmox-1/P14901) and Cathepsin D (Ctsd
352 /P18242). Additionally, we confirmed the higher abundance of Glutathione S-transferase Mu 1
353 (Gstm1/P10649) and Plexin A1 (PlxnA1/P70206) (**Fig. 3A**, **Table 1** and **Table 2**). Total RNA
354 of replica samples used for immunoblot analysis was extracted, and reverse transcription (RT)
355 qPCR was performed for selected transcripts. The transcripts of 60S ribosomal protein L19
356 (RpL19/P84099) and 14-3-3 protein zeta/delta (Ywhaz/P63101) were used as normalisation
357 controls, as these reference genes are considered to have stable expression [49, 50] in these
358 culture conditions. Certain changes in transcript levels correlated with changes in protein
359 abundance, including reduced abundance for Pppt1 and increased abundance for PlxnA1 and
360 the copper-transporting ATPase 1 (Atp7/Q64430). Other transcripts did not show any
361 correlation, e.g., Ctsd, Rab3IL1 and Hmox-1 mRNAs (**Fig. 3B**). These data suggest that *L.*
362 *donovani* both affects transcriptional and post-transcriptional regulation of infected BMDMs.
363
364 *Gene Ontology analysis illuminates the landscape of subverted pathways in L. donovani*
365 *infected macrophages*

366 The datasets of differentially modulated proteins were analysed for enriched biological
367 processes, molecular functions and cellular components using the Gene Ontology (GO) and the
368 *Mus musculus* Uniprot databases (<https://www.uniprot.org/proteomes/UP000000589>), and
369 results were visualised with the BinGO plug-in of the Cytoscape 3.5.1 software package [45].
370 Up-regulated biological processes were related to metabolism, including lipopolysaccharide
371 biosynthetic process (GO-ID: 9103), cellular ketone metabolic process (GO-ID: 42180),
372 oxoacid (GO-ID: 43436) and carboxylic acid (GO-ID: 19752) metabolism (**Fig. 4A, Table S7**),
373 the latter enrichment having previously been observed in THP-1 cells infected with *L. donovani*
374 promastigotes [30]. Enrichment was also observed for various molecular functions, including
375 intramolecular oxidoreductase activity transposing S-S bonds (GO-ID: 16864), intramolecular
376 oxidoreductase activity, interconverting keto and enol groups (GO-ID: 16862) and protein
377 disulfide isomerase activity (GO-ID: 3756)(**Fig. 4B, Table S7**). This last activity is important
378 for protein folding, stress response (balancing the effects of oxidative stress) and phagocytosis
379 [51]. Moreover, enrichment in cellular components included the cytoplasm (GO-ID: 5737),
380 intracellular membrane bounded organelle (GO-ID: 43231) and endoplasmic reticulum (GO-
381 ID: 5783), with many of the up-regulated endoplasmic reticulum (ER) proteins involved in
382 protein folding and post-translation modification (**Fig. 4C, Table S7**), revealing a potential
383 impact of *L. donovani* infection on endoplasmic reticulum stress and the unfolded protein
384 response as previously documented [52].

385 Using the data set of proteins showing reduced abundance, enrichment was observed
386 for biological processes involved in vacuole (GO-ID: 7033) and lysosome (GO-ID: 7040)
387 organisation and catabolic process (GO-ID: 9056) (**Fig. 5A, Table S8**). Enriched molecular
388 functions in the down-modulated dataset, included binding (GO-ID-5488), transcription
389 elongation regulator activity (GO-ID: 3711), hydrolase activity hydrolysing o-glycosyl
390 compounds (GO-ID: 4553) and cysteine type endopeptidase activity enriched in cysteine type
391 endopeptidases (GO-ID: 4197) (**Fig. 5B, Table S8**). Moreover, amongst the enriched cellular
392 components were lytic vacuole (GO-ID: 323), lysosome (GO-ID: 5764) and vacuole (GO-ID:
393 5773), and thus the very organelles and structures exploited by the parasite as intracellular

394 niche. Other enriched cellular components included cytoplasm (GO-ID: 5737), intracellular
395 membrane bounded organelle, (GO-ID: 43231) and integrin complex (GO-ID: 43231) (**Fig. 5C,**
396 **Table S8**). .

397

398 *L. donovani* modulate the abundance of proteins involved in the lysosome and lytic vacuole
399 organisation of BMDMs

400 Our study reveals that *L. donovani* promastigote infection has a profound effect on BMDM
401 lysosomal biology, with remodeling of this organelle likely promoting parasite survival in its
402 known intracellular niche, the phago-lysosome [4, 53]. Our data show that the ratios of a total
403 of 15 lysosomal proteins in both forward [L-I/C-NI, ratio 1, (r1)] and reverse experiments [C-
404 I/L-NI, ratio 2, (r2)] were below 0.8 (**Table 2** and **Table S7**). Down-regulated proteins included
405 various hydrolases such as lysosomal cathepsins (Ctsb/P10605, $r_1=0.59$ $r_2=0.54$; Ctsc/P97821,
406 $r_1=0.49$, $r_2=0.41$; Ctsd/P18242, $r_1=0.35$, $r_2=0.59$) or dipeptyl peptidase 2 (Dpp-2/Q9ET22,
407 $r_1=0.4$, $r_2=0.75$) and the Lysosome-associated membrane glycoproteins, Lamp2
408 (Cd107b/P17047, $r_1=0.72$, $r_2=0.7$) and Lamp4 (Cd68/P31996, $r_1=0.68$, $r_2=0.49$) (**Table 2**).

409 Lysosomal proteases, including cathepsins may affect parasite survival as they carry
410 microbicidal activity and are key regulators of the host immune response by modulating Major
411 histocompatibility Class (MHC) II antigen presentation and the trafficking of Toll like receptors
412 (TLRs) [54]. Down-modulation of different cathepsins may cause distinct effects on
413 macrophage activation. For example, deletion or pharmacological inhibition of Ctsb results in
414 inflammatory phenotypes [55, 56] and Ctsd inhibition blocks both Th1 and Th2 responses by
415 strongly suppressing MHCII-associated invariant chain molecule [56]. Different *Leishmania*
416 species and strains, parasite stages (promastigotes/amastigotes) and host species may modulate
417 cathepsins in different ways. For example, Prina *et al* reported a time-dependent increase of
418 Ctsb, Ctsh and Ctsd activity in rat bone marrow-derived macrophages infected with *L.*
419 *amazonensis* [57]. On the other hand, the RNA transcripts of Ctsc and Ctss were reported to be
420 down-modulated in monocyte derived human macrophages 24 h after *L. major* metacyclic
421 promastigote infection [58].

422 Apart from lysosomal peptidases, a number of hydrolases involved in fatty acid
423 metabolism were also affected in macrophages by *L. donovani* infection. Interestingly, the
424 down-modulation of the lyase Palmitoyl-protein thioesterase 1 (Ppt1/O88531, $r_1=0.23$, $r_2=0.3$)
425 involved in the removal of thioester-linked fatty acyl groups (like palmitate) is anticipated to
426 disrupt lysosomal catabolism via the rapid accumulation of palmitoylated proteins [59].
427 Moreover, Ppt1 down-modulation impairs mTOR signaling [59] the repression of which has
428 been also linked to *Leishmania* intracellular survival [60]. Additionally, down-modulation of
429 Acid ceramidase -1 (Asah-1/Q9WV54, $r_1= 0.45$, $r_2=0.5$) (**Table 2**) - an enzyme that hydrolyses
430 the sphingolipid ceramide into sphingosine and free fatty acid - may also impact the
431 macrophage environment to favor parasite survival. *Leishmania* is favored by increase in
432 ceramide concentration and in turn *Leishmania* infection, modulates the up-regulation of
433 ceramide synthesis in a biphasic way [61]. At early time points after *Leishmania* infection,
434 increase is mediated by Acid Sphingomyelinase activation, which catalyses the formation of
435 ceramide from sphingomyelin, promoting the formation of cholesterol rich lipid microdomains
436 [61], required for *L. donovani* uptake by macrophages [62]. In the second phase, the *de novo*
437 biosynthesis of ceramide is induced via ceramide synthase, resulting in the displacement of
438 cholesterol from the membrane and impacting MHC-II class antigen presentation [61].
439 Moreover, ceramide increase leads to severe host immune suppression via the reduction of
440 nuclear translocation of NFkB and AP-1, the up-regulation of immunosuppressive cytokines
441 TGF- β and IL-10, and reduced NO generation [63]. Thus increase in ceramide concentration is
442 required for parasite survival, and the down-modulation of Asah-1 revealed in this screen, could
443 be an additional mechanism contributing to this rise, and hence merits further investigation.

444 Interestingly, Fernandes *et al*, have demonstrated that the lysosome is one of the
445 enriched “down-modulated” KEGG pathways in the transcriptome of murine and human
446 macrophages infected with *L. major* [8]. Thus, these results suggest that more than one species
447 of *Leishmania* induce major modifications of lysosomal components, that might aid the parasite
448 to survive inside the hostile macrophage environment. The mechanism that contributes to
449 lysosomal protein down-modulation after *L. donovani* infection, is elusive. One possible

450 scenario would be a dysregulation of transcription factor EB, which is a master regulator of
451 lysosomal biogenesis that mediates the transcription of many lysosomal hydrolases [64].
452 Another scenario would be that the lysosomal content is modified via the action of lysosomal
453 exocytosis, which takes place to repair host cell plasma wounding caused by the parasite [65]
454 or by the direct interaction of the parasites' exo-proteases with lysosomal proteins, leading to
455 protein degradation.

456

457 *L. donovani* induce alterations in abundance of macrophage proteins involved in
458 immunomodulation

459 Our data demonstrate that *L. donovani* promastigotes inhibit host cell immune functions by
460 down-regulation of several macrophage proteins that participate in immunomodulation and
461 innate immunity (Cd180/Q62192, $r_1=0.66$, $r_2=0.76$; Tlr13/Q6R5N8, $r_1=0.66$, $r_2=0.5$;
462 Grn/P28798, $r_1=0.7$, $r_2=0.62$; Arrb2/Q91YI4, $r_1=0.61$, $r_2=0.4$; Gpr84/Q8CIM5, $r_1=0.64$ and
463 $r_2=1/\infty$; Hmox-1/P14901, $r_1=0.67$, $r_2=0.39$; Mpeg1/A1L314, $r_1=0.31$, $r_2=0.4$) (**Table 2**). These
464 results indicate that infection likely dampens the macrophage pro-inflammatory response given
465 the observed down-modulation of (i) Tlr13, which acts via Myd88 and Traf6 leading to NF- κ B
466 activation [66], (ii) Granulin (Grn) a soluble cofactor for Tlr9 signaling [67], and (iii) the G-
467 protein coupled receptor Grp84 known to be activated by medium-chain free fatty acids to
468 enhance inflammation and phagocytosis in macrophages [68].

469 Moreover, *L. donovani* promastigote infection results in increased abundance of
470 macrophage proteins that act as markers or as regulators of alternatively activated macrophages
471 (M2-like phenotype) (**Table 1**) in agreement with previous reports [69-74]. Amongst these
472 proteins were: (i) the Cd9 antigen (P40240, $r_1=1.41$, $r_2=1.76$), a tetraspanin family member
473 known to form a complex with integrins and to negatively regulate LPS-induced macrophage
474 activation [75], (ii) the highly up-regulated urokinase-type plasminogen activator
475 (Plau/P06869, $r_1=6.02$, $r_2=5.41$) known to polarise macrophages towards a M2-like phenotype
476 [76], (iii) the monoamine oxidase A (Mao-A/Q64133, $r_1=1.31$, $r_2=2.24$) involved in
477 the breakdown of monoamines [77], (iv) the TNF alpha induced protein 8 like 2

478 (Tnfaip8l2/Q9D8Y7, $r_1 = \infty$ and $r_2 = 1.4$) involved in phospholipid metabolism [78, 79], (v) the
479 Cd93 antigen (Cd93/O89103, $r_1 = 1.35$, $r_2 = 1.76$), a C-type lectin transmembrane receptor [80]
480 and (vi) the fatty acid-binding protein (Fabp4/P04117, $r_1 = 1.3$, $r_2 = 1.57$) that delivers long-chain
481 fatty acids and retinoic acid to their cognate receptors in the nucleus [81] (**Table 1**).
482 Interestingly, Fabp4 up-modulation in infected macrophages is in line with other studies after
483 macrophage infection with *L. major* promastigotes [26] and *L. amazonensis* amastigotes [24],
484 and suggest that the increased abundance of this protein maybe independent of the *Leishmania*
485 stage or species.

486 In contrast to proteins that control, or are enriched in alternatively activated
487 macrophages, two proteins less abundant in infected macrophages, Cd180 and Beta-arrestin-2
488 (Arrb2), are known to either promote or reduce inflammation depending on the cell type and
489 infection system [82-85]. Moreover a protein known to be enhanced in alternatively activated
490 macrophages, Hmox-1 that converts pro-oxidant heme to the antioxidant biliverdin and
491 bilirubin, restoring the redox environment [86] was found down-modulated in this screen in
492 response to *Leishmania* infection (**Table 2**). Other studies have shown that Hmox-1 is up-
493 regulated in mouse peritoneal macrophages infected with *L. infantum* (*L. chagasi*)
494 promastigotes and promotes the persistence of the parasite [87]. In our infection system, despite
495 the repressed protein levels, we find Hmox-1 mRNA to be up-regulated in macrophages
496 infected with *L. donovani*. The negative correlation between Hmox-1 RNA and protein levels,
497 amongst other factors, could be due to increased secretion of Hmox-1 in the culture medium,
498 as Hmox-1 can be a secreted protein [88].

499 Interestingly, a recent study of Negrão *et al*, which used a label free proteomics
500 approach of the mouse J774 cell line infected with *L. major*, *L. amazonensis* and *L. infantum*
501 promastigotes, 24 h post infection, revealed that certain modulated proteins were also found to
502 be modulated in this study [29]. In the screen of Negrão *et al* proteins with both inverse but also
503 similar regulation to those identified in our screen, were demonstrated. Arrb2 (*L. major*), B2m
504 (*L. amazonensis*) and Hmox-1 (*L. infantum*, *L. amazonensis* and *L. major*) were more abundant
505 in the screen of Negrão *et al* [29], in contrast the lower abundance of these proteins in this

506 report. Proteins with similar regulation include the less abundant Cd180 (*L. infantum*) and Grn
507 (*L. infantum*) [29]. Overall the study of Negrão and colleagues, showed that many up-regulated
508 proteins were involved in the activation of phagocytes and leukocytes, whereas in this screen
509 the differential abundance of many proteins, is anticipated to dampen inflammation, as
510 mentioned above. These differences, could reflect the time of analysis (24 h for Negrão *et al*
511 versus 72 h for this study), as at earlier time points, it is believed that macrophages are generally
512 polarised to a M1 phenotype and at later time points, pathogens subvert macrophage function
513 to a M2 like macrophage, for ensuring survival [7]. Differences could also reflect the
514 *Leishmania* species or the cell type.

515

516 *L. donovani* up-modulate the abundance of proteins involved in the defense against oxidative
517 and ER stress in BMDMs

518 Proteins related to oxidative stress and ER stress response were more abundant in BMDMs 72
519 h after *L. donovani* infection, including proteins involved in glutathione metabolism, such as
520 Glutamate-cysteine ligase regulatory subunit (Gclm/O09172, $r_1=1.28$, $r_2=1.29$), the Glutathione
521 transferase mu1 (Gstm1/P10649, $r_1=1.36$; $r_2=2.53$), ferritin light (Ftl1/ P29391, $r_1=2.9$, $r_2=2.64$)
522 and heavy (Fth1/P09528, $r_1= 1.37$, $r_2=2.23$) chains, and Bola like 1 (Bola1/Q9D8S9, $r_1=1.73$
523 and $r_2=\infty$), a protein that prevents mitochondrial changes upon glutathione depletion [89] acting
524 as mitochondrial iron-sulfur cluster assembly factor [90] (**Table 1**). All of these proteins are
525 known to normalise the redox state of the cell or protect the cell from oxidants [89, 91-93].
526 Interestingly, up-regulated transcripts involved in the response against oxidative stress and
527 glutathione metabolism were previously shown to be up-regulated in human monocyte derived
528 macrophages infected with *L. (Vianna) panamensis* promastigotes [9] and in mouse BMDMs
529 infected with *L. major* promastigotes [26]. Moreover, up rise in cellular ROS, could be
530 associated with ER stress, the unfolded protein (UPR) response and a concomitant up rise of
531 ER proteins aiding protein folding, as a mechanism of the cell to repair oxidant damage [94].
532 In our system several of these proteins including disulfide-isomerase forms [Protein disulfide-
533 isomerase A3 (Pdia3/ P27773, $r_1=1.39$, $r_2=1.27$), A4 (Pdia4/P08003, $r_1=1.35$, $r_2=1.27$) and A6

534 (Pdia6/Q922R8, $r_1=1.43$ $r_2=1.31$) [95] and Hypoxia up-regulated 1 (Hyou1/Q9JKR6, $r_1=1.86$,
535 $r_2=1.38$ [96]), displayed higher levels in infected macrophages (**Table 1**). Likewise, ER stress
536 and induction of the unfolded protein response was previously documented for RAW 264.7
537 cells infected with *L. amazonensis* [52] and in the proteomic study of Singh et al, who showed
538 in THP-1 macrophages infected with *L. donovani*, an increase in the absolute percentage of up-
539 regulated ER proteins, as infection progressed from 12 h and 24 h to 48 h post-infection [30].
540 Overall, these results suggest that *L. donovani* infection induces ROS production and ER stress
541 [10, 97], with specific proteins involved in this process displaying higher abundance, and likely
542 acting as a defense mechanism against the oxidants' deleterious effects

543

544 *L. donovani modulates the abundance macrophage proteins involved in intracellular*
545 *trafficking and ion movement*

546 The abundance of many macrophage proteins involved in intracellular trafficking was modified
547 in *L. donovani* infected BMDMs. Previous studies have shown that *Leishmania* infection
548 impairs intracellular trafficking by modulating small GTPase signal transduction [30]. This
549 form of signaling is important in host/pathogen interactions as it regulates pathways involved
550 in phagocytosis and oxidative burst, vesicle fusion and actin organisation [98, 99]. The
551 deregulation of small GTPase signal transduction, likely alters these macrophage functions, and
552 produces a favorable environment for intracellular parasite infection [30, 100, 101]. Our
553 screening extends previous studies and provides evidence of altered protein expression data in
554 proteins involved in small GTPase signalling, with four proteins showing reduced abundance
555 during infection (Gmip Q6PGG2, $r_1=0.79$, $r_2=0.49$; Rgl2/Q61193, $r_1=1/\infty$; $r_2=0.35$;
556 Arfp2/Q8K221, $r_1=1/\infty$ $r_2=0.64$; Rab3il1/Q8VDV3, $r_1=0.66$, $r_2=0.39$; Nisch/Q80TM9, $r_1=$
557 0.66 , $r_2=0.46$) (**Table 2**), and one member showing increased abundance (Tbc1d22a/Q8R5A6,
558 $r_1=\infty$ and $r_2=1.89$) (**Table 1**). Moreover, various other proteins affecting intracellular trafficking
559 were modulated in response to *L. donovani* promastigote infection, including a signal
560 recognition particle receptor subunit beta (Srprb/P47758; $r_1=1.36$, $r_2=1.25$) (**Table 1**) that likely
561 restores trafficking imbalances to the ER [102], and two cytoskeletal organisation proteins

562 (Peak1/Q69Z38, $r_1=0.45$, $r_2=0.46$; Ppp2r5e/Q61151, $r_1=1/\infty$, $r_2=0.73$) [103, 104]. Additionally,
563 the abundance of macrophage mannose receptor 1 (Mrc-1) - a protein involved in endocytosis
564 and phagocytosis - was down-modulated (Q61830, $r_1=0.63$, $r_2=0.33$), in agreement with
565 previous studies performed with mouse peritoneal macrophages infected with *L. donovani*
566 promastigotes [105]. The study of Singh et al, has also shown that transport and vesicular
567 trafficking is modified in *L. donovani* infected THP-1 macrophages [30], and thus the
568 modulation of these proteins merits further investigation, as they could be part of the parasite
569 evasion strategy interfering with vesicular fusion events in the host cell, important for parasite
570 survival.

571 Interestingly, another form of trafficking, ion movement, has been associated with
572 inflammatory response [106-108]. For example, zinc/copper imbalance reflects immune
573 dysregulation in human leishmaniasis [108]. In this study, some proteins involved in ion
574 transport were modulated in infected BMDMs, including (i) Atp1a1 (Q8VDN2, $r_1= 1.39$;
575 $r_2=1.26$) a subunit of the sodium potassium ATPase [109](**Table 1**), (ii) stomatin
576 (Stom/P54116, $r_1= 0.78$, $r_2=0.48$) (**Table 2**) known to regulate ion channel activity and
577 transmembrane ion transport [110] and (iii) copper-transporting ATPase 1 (ATP7a/P56542,
578 $r_1=2.61$, $r_2=2.66$) that regulates copper efflux under conditions of elevated extracellular copper
579 concentration [111] (**Table 1**). Overall, these changes may reflect immune function and their
580 influence on host/pathogen interaction merits further investigation.

581

582 *L. donovani* modulate the abundance of extracellular matrix and adhesion proteins in BMDMs
583 Macrophage adhesion and cell-cell interactions with other immune cells play important roles
584 in health and disease, with co-stimulatory molecules regulating T cell activation or cell
585 migration enabling leukocyte recruitment to sites of inflammation. Our proteomics screen
586 revealed changes in expression of extracellular matrix and adhesion proteins that could explain
587 the reported modified adhesion of infected macrophages to connective tissue [112]. We
588 confirmed down-modulation of the co-stimulatory molecule Itgax (Q9QXH4, $r_1=1/\infty$, $-r_2= 0.33$)
589 (**Table 2**). Changes in abundance of the integrin subunit Itg β 5 (O70309, $r_1= 0.73$ and $r_2=0.55$)

590 which acts with Itga5 as a fibronectin receptor, were detected, as well as differences in other
591 adhesion or adhesion signaling molecules (Nisch/Q80TM9, $r_1=0.66$, $r_2=0.56$; Plxna1/ P70206,
592 $r_1= 1.65$, $r_2=2.46$; Lpxn/Q99N69, $r_1= 0.74$, $r_2=0.65$) (**Table 1** and **Table 2**). Thus, our data
593 expand and further emphasise the impact of intracellular *Leishmania* infection on connective
594 tissue remodeling that may modify host adaptive immune responses mediated by infected
595 macrophages [112, 113].

596

597 *L. donovani* modulate the abundance of macrophage proteins involved in gene expression

598 It is well established that *Leishmania* infection modulates macrophage gene expression [21]. In
599 our study, several proteins implicated at different levels of gene expression regulation, showed
600 differential abundance in response to *L. donovani* BMDM infection, including transcriptional,
601 epigenetic or post-transcriptional regulators (Hist4h4/P62806, $r_1=1.44$, $r_2=1.33$; Lpxn/Q99N69,
602 $r_1= 0.75$, $r_2=0.65$; Ddx42/ Q810A7, $r_1=0.74$, $r_2=0.65$; Psmc3/088685, $r_1=1.34$, $r_2=1.25$;
603 Supt5h/O5520, $r_1= 0.67$; $r_2=0.68$; Supt6h/Q62383, $r_1=0.59$, $r_2=0.64$), proteins that modify RNA
604 (Cmtr1/Q9DBC3, $r_1=1.39$ and $r_2=\infty$; Dus3L/Q91XI1, $r_1= 0.34$, $r_2=1/\infty$), as well as translation
605 regulators (eIF-3/Q9QZD9, $r_1= 0.77$, $r_2=0.73$; Rps6ka1-Rsk1/P18653, $r_1=0.43$, $r_2=1/\infty$) (**Table**
606 **1** and **Table 2**). The higher abundance of histone H4 (Hist4h4) in infected macrophages is in
607 accordance with previous reports for *L. donovani* infected THP-1 cells [30], whereas up-
608 regulation of histones is anticipated to have a negative effect on gene expression by the
609 modulation of chromatin structure [114]. Moreover, the lower levels of focal adhesion regulator
610 Lpxn [115, 116] and the increased abundance of the 26S proteasome ATPase component,
611 Psmc3/TBP-1 [117], two proteins that display novel functions and are known to modulate
612 transcription via specific nuclear receptors [118-120]. Thus the modulation of abundance of
613 these proteins in infected macrophages, may affect transcriptional regulation of *Leishmania*
614 infected macrophages. In addition, Psmc3/TBP-1 regulates the transcription of Class II trans-
615 activator (CIITA), the master regulator of the MHC-II transcription complex and a critical
616 factor for the initiation of the adaptive immune response [121]. In addition, the down-
617 modulation of specific transcriptional elongation factors (Supt5h and Supt6h) [122-124] is

618 anticipated to contribute to transcriptomic changes in infected macrophages. The down-
619 modulation of the eukaryotic translation initiation factor eIF-3 and of protein S6 kinase alpha
620 1(Rps6ka1/Rsk1) - a kinase known to stimulate protein synthesis and a key mediator of mTOR
621 function [125] - suggests that protein translation proceeds at lower rates in infected
622 macrophages [125]. Rps6ka1/Rsk1 is known to “connect the stress-induced activation of
623 transcription factors and mitogens to the ribosome” [126]. This connection is mediated by the
624 activation/phosphorylation of c-Fos, I κ B α , cAMP-response element-binding protein (CREB)
625 and CREB-binding protein [127], proteins involved in the inflammatory response. Thus, the
626 modulation of this kinase likely participates in the reprogramming of *Leishmania*-infected
627 macrophages to favor intracellular parasite survival.

628

629 *L. donovani* modulate the abundance of macrophage proteins involved in metabolic processes

630 Previous studies have shown that *Leishmania* parasites exploit host metabolism to survive
631 inside the hostile macrophage environment. It has been demonstrated that, early during
632 *Leishmania* infection, the aerobic glycolysis predominates with inhibition of the TCA cycle
633 [26], whereas at later time points energy metabolism is shifted towards oxidative
634 phosphorylation and TCA cycle [30, 128]. *Leishmania* infection also perturbs lipid metabolism,
635 including sterol biosynthesis and triacylglyceride synthesis in BMDMs [24, 26]. In our screen,
636 the cellular ketone metabolic process (GO-ID: 42180) and oxoacid metabolic process (GO-ID:
637 43436) - a broad metabolic processes that includes TCA cycle and aminoacid biosynthesis,
638 were modified, with 5 proteins showing increased abundance (Fabp4/P04117, $r_1=1.3$, $r_2=1.57$;
639 Sucla2/Q9Z2I9, $r_1=1.27$, $r_2=1.37$; Phyh/O35386, $r_1=\infty$ and $r_2=1.25$; Cmas/Q99KK2; $r_1=1.27$,
640 $r_2=1.72$; Gclm/O09172, $r_1=1.27$, $r_2=1.28$). Indeed, Succinate--CoA ligase [ADP-forming]
641 subunit beta (Sucla2) – an enzyme that couples the hydrolysis of succinyl-CoA to the synthesis
642 of ATP [129] - was one of the TCA cycle proteins shown to be up-regulated in *L. donovani*-
643 infected THP-1 cells [30]. Likewise, Sterol O-acyltransferase 1 (Soat1/Q61263, $r_1=1.29$,
644 $r_2=1.31$) - a cholesterol metabolism enzyme known to form cholesteryl esters from cholesterol

645 [130] – displayed higher levels in infected macrophages (**Table 1**). This change could be related
646 to the increase in cholesterol biosynthesis of macrophages infected with *Leishmania* [24, 26].

647 Finally, fatty acid metabolism, including fatty acid elongation (Ppt1/ O88531, $r_1=0.23$,
648 $r_2=0.3$), phospholipases of the ether lipid metabolic process [(Pla2g7/Q60963, $r_1=0.68$, $r_2=0.35$;
649 Plbd2/ Q3TCN2, $r_1=0.71$, $r_2=0.49$) and hydrolases of the sphingolipid metabolism (Asah-
650 1/Q9WV54, $r_1=0.45$, $r_2=0.5$; Gla/P51569, $r_1=0.63$, $r_2=0.75$; Glb1/P23780, $r_1=0.55$, $r_2=0.76$),
651 were found to be less abundant in infected macrophages (**Table 2**). Fatty acid metabolism,
652 including sphingolipid metabolism [131] has been shown to be modulated in macrophages
653 infected with *Leishmania* [24, 26, 61] and is likely to play an important function in
654 *Leishmania*/macrophage interaction.

655

656 *L. donovani* modulate the abundance of macrophage proteins that regulate glycan and
657 glycoside protein post-translational modifications

658 In addition, proteins regulating protein sugar (glycan and glycoside) post-translational
659 modifications were modulated in *L. donovani* infected macrophages, with decreased levels
660 observed for (i) ribophorin I (RpnI/Q91YQ5, $r_1=1.65$, $r_2=1.28$), an essential subunit of the N-
661 oligosaccharyl transferase (OST) complex which catalyses the N-glycosylation of proteins
662 [132] (**Table 1**), and (ii) alpha and beta galactosidases (Gla/P51569, $r_1=0.63$, $r_2=0.75$;
663 Glb1/Q91YQ5, $r_1=0.55$, $r_2=0.76$). In contrast, increased abundance was observed for cytidine
664 monophospho-N-acetylneuraminic acid synthetase (Cmas/Q99KK2, $r_1=1.27$, $r_2=1.72$) that
665 catalyses the activation of N-acetylneuraminic acid (NeuNAc) to cytidine 5'-monophosphate
666 N-acetylneuraminic acid (CMP-NeuNAc) [133], a substrate required by sialyltransferases for
667 the addition of sialic acid to growing oligosaccharide chains. Conceivably, changes in protein
668 glycosylation may affect protein function and alter immune responses and cell adhesion
669 signaling [134, 135] in infected macrophages, that may favor parasite survival.

670

671 *Conclusions*

672 In conclusion, our analyses establish a novel experimental framework for the quantitative
673 proteomics analysis of *Leishmania* infected primary macrophages. Our results draw a highly
674 complex picture of *Leishmania*/macrophage interaction and highlight the pleiotropic
675 modulation of biological processes and molecular functions in infected BMDMs that likely
676 establish permissive conditions for intracellular parasite survival and chronic infection. The
677 parasite seems to have developed mechanisms to subvert key macrophage functions and trigger
678 changes in host cell metabolism, innate immunity and lysosomal function. Future experimental
679 validation combining systems-level, phenotypic, and functional genetic analyses is required to
680 correlate enrichment/activation or depletion/inhibition of identified host pathways with changes
681 in intracellular *Leishmania* survival.

682

683 **Data linking**

684 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
685 Consortium via the PRIDE partner repository [136] with the dataset identifier PXD013448
686 (username: reviewer19892@ebi.ac.uk, password: 5S8FZXVw).

687

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1099 **FIGURE LEGENDS**

1100 **Fig. 1 *In vitro* macrophage infection model with *L. donovani* promastigotes and SILAC-**
1101 **based macrophage proteomics analysis** (A) Histogram plots showing the number of
1102 intracellular parasites per labeled and control macrophages after 4 h, 48 h and 72 h of infection.
1103 (B) Venn diagram showing the number of labeled proteins versus the number of non labeled
1104 proteins of differentiated macrophages in SILAC medium 6 days post-infection. (C)
1105 Workflow diagram showing the experimental strategy used to reveal the variable proteome of
1106 *L. donovani* infected BMDMs. Initially, an equal number of bone marrow (BM) progenitors
1107 were cultured and differentiated in the presence of natural amino acids (light “control” medium)

1108 or amino acid with heavy isotopes (heavy “labeled” medium) supplemented with 75 ng/mL
1109 mCSF. After 6 days of differentiation, adherent cells were detached and plated with fresh
1110 control or labeled medium supplemented with 25 ng/mL mCSF. Labeled (red) and control
1111 (blue) macrophages were infected or not with stationary phase *L. donovani* promastigotes at a
1112 ratio of 10:1 for 4 h. Macrophages were lysed 72 h post-infection, protein extracts were
1113 quantified and mixed at a 1:1 ratio in pairs (control vs labeled, infected or not), fractionated by
1114 either polyacrylamide gel electrophoresis fractionation (GEL) or strong anion exchange
1115 fractionation (SAX), and processed by LC-MS/MS analysis. Error bars represent standard
1116 error of the mean (SEM).

1117

1118 **Fig. 2 Summary of proteomics results** (A) Venn diagram showing the overlap of identified
1119 proteins between L-I/C-NI and C-I/L-NI after GEL (left panel) or SAX fractionation (right
1120 panel). (B) Venn diagram showing the combined overlap identified proteins of L-I/C-NI, C-
1121 I/L-NI samples in both GEL and SAX fractionation conditions. (C) Volcano plots of the data
1122 presented in (A) showing the fold change (FC, x-axis, log₂) of abundance for proteins with
1123 at least two identified peptides plotted against the p-value (y-axis, -log₁₀). Red vertical and
1124 green horizontal lines reflect the filtering criteria, i.e. $FC \geq 1.25$ or $FC \leq 0.8$, and significance
1125 level of 0.05, respectively.

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1127 **Fig. 3 Differential abundance for BMDM proteins and transcripts at 72 h post *L.***
1128 ***donovani* infection** (A) Immunoblot analysis. 20 µg of total protein extracts from infected (I)
1129 and non-infected (NI) BMDMs were analysed for Asah-1, Rab3IL1, Plxna1, Hmox-1, Gstm,
1130 and Ctsd proteins. β-actin expression was used as a loading control. The intensities of the bands
1131 were analysed using the Image J software. The fold overexpression, represented as a bar
1132 diagram, was calculated by dividing the band intensity representing the protein of interest with
1133 the band intensity of β-actin. Results are representative for three independent experiments. **,
1134 $p < 0.01$; *, $p < 0.05$, compared with the corresponding control value for noninfected
1135 macrophages (two-tail paired Student's t test). (B) RT-qPCR. Column diagrams showing the

1136 mean ratio from three different experiments of RNA abundance in infected versus non-
1137 infected macrophages for Plxna1, Ppt1, Rab3IL1, Asah-1, Atp-7A and Hmox-1 relative to
1138 Ywhaz and RpL19. Error bars represent the standard deviations of three experiments.

1139

1140 **Fig. 4 Gene Ontology analyses for up-regulated proteins in *L. donovani* infected BMDMs**

1141 (A) GO analysis for Biological processes. (B) GO analysis for Molecular functions. (C) GO
1142 analysis for Cellular component. The analyses were performed using the hypergeometric
1143 statistical test, a Benjamini & Hochberg false discovery rate and significance level of 0.01. The
1144 p-value is indicated by the color according to the legend.

1145 **Fig. 5 Gene Ontology analyses for down-regulated proteins in *L. donovani* infected**

1146 **BMDMs.** (A) GO analysis for Biological processes. (B) GO analysis for Molecular functions.
1147 (C) GO analysis for Cellular component. The analyses were performed using the
1148 hypergeometric statistical test, a Benjamini & Hochberg false discovery rate and significance
1149 level of 0.01. The p-value is indicated by the color according to the legend.

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Table 1 Macrophage proteins displaying increased abundance after *L. donovani* infection

Description	Uniprot ID	Gene	GEL:L-I/C-NI			GEL:C-I/L-NI		
			Ratio 1	p-value	No. Peptides	Ratio 2	p-value	No. Peptides
Glutamate--cysteine ligase regulatory subunit	O09172	Gclm	1.28	0.16	9	1.29	0.02	10
Complement component C1q receptor	O89103	Cd93	1.35	0.19	8	1.76	0.02	10
Fatty acid-binding protein, adipocyte	P04117	Fabp4	1.3	0.02	10	1.57	0.001	8
Urokinase-type plasminogen activator	P06869	Plau	6.02	0.04	5	5.41	0.003	6
Protein disulfide-isomerase A4	P08003	Pdia4	1.35	0.01	23	1.27	0.12	26
Ferritin heavy chain	P09528	Fth1	1.37	0.03	23	2.23	1.47E-07	20
Glutathione S-transferase Mu 1	P10649	Gstm1	1.36	0.003	26	2.53	8.21E-07	22
Integrin alpha-5	P11688	Itga5	1.34	0.001	18	1.27	0.3	18
Protein disulfide-isomerase A3	P27773	Pdia3	1.39	1.60E-08	51	1.27	7.97E-07	59
Ferritin light chain 1	P29391	Ftl1	2.9	3.90E-07	18	2.64	0.009	5
CD9 antigen	P40240	Cd9	1.41	0.16	5	1.76	0.004	6
Signal recognition particle receptor subunit beta	P47758	Srprb	1.36	0.04	7	1.25	0.14	8
V-type proton ATPase subunit D	P57746	Atp6v1d	1.29	0.31	9	1.37	0.003	6
40S ribosomal protein S15a	P62245	Rps15a	1.31	0.006	11	1.34	0.499	7
Histone H4	P62806	Hist1h4	1.55	3.40E-06	12	1.33	0.15	14

Plexin-A1	P70206	Plxna1	1.65	3.00E-05	35	2.46	1.81E-10	35
Keratin, type I cytoskeletal 9	Q6RHW0	Krt9	∞		8	∞		3
Sterol O-acyltransferase 1	Q61263	Soat1	1.29	0.13	10	1.31	0.03	13
Transmembrane protein 214	Q8BM55	Tmem214	1.34	0.04	5	1.87	0.21	7
Procollagen galactosyltransferase 1	Q8K297	Glt25d1	2.53	0.12	8	1.5	0.001	11
Na ⁺ /K ⁺ -transporting ATPase subunit alpha-1	Q8VDN2	Atp1a1	1.39	0.001	17	1.26	0.007	22
Ribophorin I	Q91YQ5	Rpn1	1.65	0.0003	26	1.28	0.009	32
Protein disulfide-isomerase A6	Q922R8	Pdia6	1.43	0.0004	19	1.31	0.05	19
N-acylneuramate cytidyltransferase	Q99KK2	Cmas	1.27	0.37	11	1.72	0.002	10
Bola-like protein 1	Q9D8S9	Bola1	1.73	0.45	2	∞		2
Cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 1	Q9DBC3	Cmtr1	1.39	0.72	2	∞		4
Hypoxia up-regulated protein 1	Q9JKR6	Hyou1	1.86	2.90E-05	36	1.38	0.008	45
Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	Q9Z2I9	Sucla2	1.27	0.48	10	1.37	0.007	17

Description	Uniprot ID	Gene	GEL:L-I/C-NI			GEL:C-I/L-NI		
			Ratio 1	p-value	No. peptides	Ratio 2	p-value	No. peptides
Phytanoyl-CoA dioxygenase, peroxisomal	O35386	Phyh	∞		2	1.25	0.53	2
26S protease regulatory subunit 6A	O88685	Psmc3	1.34	0.56	17	1.25	0.03	21
Glutathione S-transferase Mu 1	P10649	Gstm1	1.41	0.14	16	2.18	0.002	22

Amine oxidase [flavin-containing] A	Q64133	Maoa	1.31	0.54	15	2.24	0.005	15
Copper-transporting ATPase 1	Q64430	Atp7a	2.61	0.63	3	2.56	0.03	4
Protein PRRC2A	Q7TSC1	Prc2a	∞		3	3.86	0.08	3
TBC1 domain family member 22A	Q8R5A6	Tbc1d2 2a	∞		2	1.89	0.48	3
Tumor necrosis factor alpha-induced protein 8-like protein 2	Q9D8Y7	Tnfaip8l 2	∞		4	1.44	0.71	2

1154

Table 2 Macrophage proteins displaying decreased abundance after *L. donovani* infection

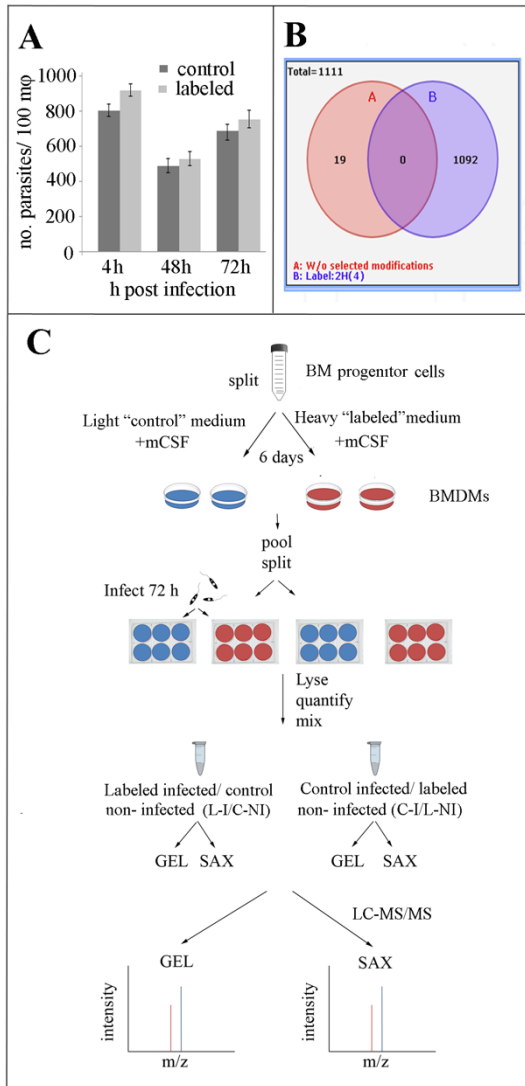
Description	Uniprot ID	Gene	GEL :L-I/C-NI			GEL: C-I/L-NI		
			Ratio 1	p-value	No. Peptides	Ratio 2	p-value	No. Peptides
Macrophage-expressed gene 1 protein	A1L314	Mpeg1	0.31	0.0006	12	0.4	1.00E-06	16
1. Transcription elongation factor SPT5	O55201	Supt5h	0.67	0.01	12	0.68	0.003	17
Integrin beta-5	O70309	Itgb5	0.73	0.31	9	0.55	0.01	5
Palmitoyl-protein thioesterase 1	O88531	Ppt1	0.23	0.001	8	0.3	2.00E-05	11
Tripeptidyl-peptidase 1	O89023	Tpp1	0.65	0.44	4	0.58	0.0181	8
Cathepsin B	P10605	Ctsb	0.59	4.00E-05	22	0.54	9.00E-08	22
Beta-glucuronidase	P12265	Gusb	0.69	9.00E-06	35	0.65	1.00E-05	32
Heme oxygenase 1	P14901	Hmox1	0.67	0.07	7	0.39	2.00E-06	15
Lysosome-associated membrane glycoprotein 2	P17047	Lamp2	0.72	0.04	9	0.7	0.4494	9
Cathepsin D	P18242	Ctsd	0.35	1.00E-13	35	0.59	4.00E-06	35
Ribosomal protein S6 kinase alpha-1	P18653	Rps6ka1	0.43	0.343	3	1/∞		2
Beta-galactosidase	P23780	Glb1	0.55	0.001	18	0.76	0.19	18
Lysosomal acid phosphatase	P24638	Acp2	0.41	0.003	5	0.75	0.36	4
Granulins	P28798	Grn	0.7	0.281	5	0.62	0.02	8
Beta-hexosaminidase subunit alpha	P29416	Hexa	0.74	0.009	20	0.62	0.02	21
Lamp4, Macrosialin	P31996	Cd68	0.68	0.006	8	0.49	0.07	8
Alpha-galactosidase	P51569	Gla	0.63	0.183	14	0.75	0.001	14
Erythrocyte band 7 integral membrane protein, Stomatin	P54116	Stom	0.78	0.057	11	0.48	0.01	6
Deoxyribonuclease-2-alpha	P56542	Dnase2	0.61	0.519	2	0.47	0.05	2
Acid sphingomyelinase-like phosphodiesterase 3a	P70158	Smpdl3a	0.44	0.0001	13	0.44	0.01	12
Dipeptidyl peptidase 1/ Cathepsin c	P97821	Ctsc	0.49	5.00E-05	14	0.41	0.004	10
Putative phospholipase B-like 2	Q3TCN2	Plbd2	0.71	0.009	11	0.49	0.05	9
Platelet-activating factor acetylhydrolase	Q60963	Pla2g7	0.68	0.2	6	0.35	1.00E-05	11
Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit epsilon isoform	Q61151	Ppp2r5e	1/∞		3	0.73	0.14	3
Macrophage mannose receptor 1	Q61830	Mrc1	0.63	1.00E-05	37	0.33	9.00E-16	41
CD180 antigen	Q62192	Cd180	0.66	0.012	15	0.76	0.0246	16
Transcription elongation factor SPT6	Q62383	Supt6h	0.59	0.018	7	0.64	0.02	7
Pseudopodium-enriched atypical kinase 1	Q69Z38	Peak1	0.45	0.127	6	0.47	0.01	9

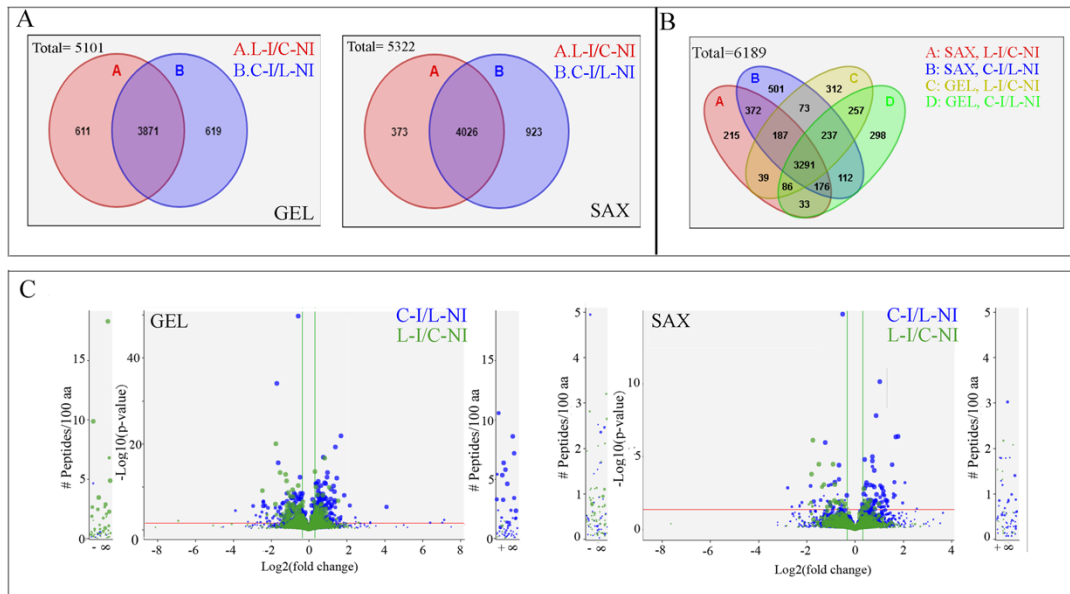
Structural maintenance of chromosomes flexible hinge domain-containing prot. 1	Q6P5D8	Smchd1	0.66	0.172	12	0.51	8,00E-05	15
GEM-interacting protein	Q6PGG2	Gmip	0.79	0.48	7	0.49	0.004	9
Toll-like receptor 13	Q6R5N8	Tlr13	0.66	0.012	15	0.5	8.00E-06	20
E3 ubiquitin-protein ligase UBR2	Q6WKZ8	Ubr2	0.61	0.018	2	0.67	0.014	5
Nischarin	Q80TM9	Nisch	0.66	0.026	17	0.46	6.00E-07	25
ATP-dependent RNA helicase DDX42	Q810A7	Ddx42	0.74	0.122	10	0.65	0.02	7
Guanine nucleotide exchange factor for Rab-3A	Q8VDV3	Rab3il1	0.66	0.144	4	0.29	0.001	6
Beta-arrestin-2	Q91YI4	Arrb2	0.61	0.169	9	0.4	0.05	7
Leupaxin	Q99N69	Lpxn	0.74	0.049	12	0.65	0.003	12
Dipeptidyl peptidase 2	Q9ET22	Dpp7	0.4	0.011	8	0.75	0.23	6
Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1	Q9QUR7	Pin1	0.68	0.198	5	0.5	0.04	3
Integrin alpha-X	Q9QXH4	Itgax	1/∞		2	0.33	0.001	4
Eukaryotic translation initiation factor 3 subunit I	Q9QZD9	Eif3i	0.77	0.399	6	0.73	0.03	9
Acid ceramidase	Q9WV54	Asah1	0.45	5.00E-06	15	0.5	0.1	14

Description	Uniprot ID	Gene	SAX:L-I/C-NI			SAX:C-I/L-NI		
			Ratio 1	p-value	No. Peptides	Ratio 2	p-value	No. Peptides
Macrophage-expressed gene 1 protein	A1L314	Mpeg1	0.43	0.32	7	0.45	0.01	6
Palmitoyl-protein thioesterase 1	O88531	Ppt1	0.29	0.03	7	0.22	0.02	8
Beta-2-microglobulin	P01887	B2m	0.61	0.39	4	1/∞		2
Cathepsin D	P18242	Ctsd	0.30	9.90E-07	26	0.77	0.21	20
Acid sphingomyelinase-like phosphodiesterase 3a	P70158	Smpd13a	0.50	0.03	12	0.80	0.38	10
Caspase-7	P97864	Casp7,Lice2	0.69	0.56	4	0.69	0.03	3
Ral guanine nucleotide dissociation stimulator-like 2	Q61193	Rgl2,Rab2l,Rlf	1/∞		2	0.71	0.74	2
Macrophage mannose receptor 1	Q61830	Mrc1	0.67	0.09	24	0.31	0.01	17
60S ribosomal protein L10	Q6ZWV3	Rpl10,Qm	0.35	0.7	4	0.6	0.01	3
G-protein coupled receptor 84	Q8CIM5	Gpr84	1/∞		3	1/∞		2
Arfaptin-2	Q8K221	Arfp2	1/∞		2	0.64		1

	tRNA-dihydrouridine(47) synthase [NAD(P)(+)]-like	Q91XI1	Dus31	1/∞	2	0.68	0.60	2
1156	Transmembrane 9 superfamily member 1	Q9DBU0	Tm9sf1	1/∞	2	0.64	0.76	2
1157								

Figure 1





1159 **Figure 2**

