

CL strain by intravenous injection in BALB/c mice followed by isolation of surviving parasite from the spleen. The resulting strain with increased visceral survival, termed "IV", is described herein.

Using the three strains previously described (CL, VL, and IV), we then investigated the factors that mediate these changes in tropism. Transcriptomic analysis does not predict protein levels in *Leishmania*,<sup>11,12</sup> so we performed proteomic analysis of these isolates. Comparison between CL and IV strains identifies protein abundance profiles associated with cutaneous versus visceral survival and virulence in the BALB/c mouse model of leishmaniasis. Because the mouse models of leishmaniasis do not fully replicate human disease,<sup>13,14</sup> we further compared the human CL strain with a human visceral leishmaniasis clinical isolate ("VL" strain). Comparison between the IV and VL strains could identify specific factors associated with survival in visceral organs as well as highlight differences between human and animal visceral leishmaniasis.

This work further has public health implications: a major concern in Sri Lanka is whether the cutaneous Sri Lankan *L. donovani* endemic there may revert to a visceral phenotype. Such a reversion could also challenge the visceral leishmaniasis elimination efforts on the Indian subcontinent. Our results indicate that this is indeed possible over the course of multiple infections and support close monitoring of leishmaniasis in Sri Lanka. More broadly, our results indicate that *L. donovani* is adaptable and can remodel its proteome to fit its environmental niche.

## EXPERIMENTAL PROCEDURES

### Ethics Statement

Isolation of *L. donovani* from the patients for this study was approved by the ethics review committee of the faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka (approval no 482/09). Written informed consent was obtained prior to parasite isolation from the two adult patients. All BALB/c mouse infections (permit no. 7395) were approved by the McGill University Animal Care Committee following guidelines from the Canadian Council on Animal Care (CCAC).

### Parasite Strains and Cultures

The *L. donovani* CL and VL strains were isolated from cutaneous and visceral leishmaniasis patients in Sri Lanka, as described in refs 9 and 15. The VL patient presented with chronic fever, hepatosplenomegaly, lymphadenopathy, and low hemoglobin characteristic of visceral leishmaniasis and was positive by the rK39 serodiagnostic test. The CL patient was rK39 negative and had parasites present in the lesion on his nose.

Parasites were isolated and maintained in M199 medium supplemented with 10% FBS, 40 mM HEPES, streptomycin, penicillin, adenine, hemin, biotin, biopterin, and folic acid at pH 7.2. Strains were immediately frozen after recovery and aliquots freshly thawed for each subsequent experiment. The IV strain was generated by serial passaging as follows. BALB/c mice (17–20 g, Charles River Breeding Laboratories) were infected by tail vein injection of  $1 \times 10^8$  stationary-phase promastigotes. Spleen parasite burden was assessed 28 days following infection by limiting dilution of organ homogenates. Liver parasite burden was assessed by determining Leishman–Donovan Units (LDU): number of amastigotes per 1000 cell nuclei  $\times$  liver weight from Giemsa-stained liver impressions.<sup>16</sup> To recover parasites for subsequent serial infections, spleens were homogenized in *Leishmania* culture media.<sup>15</sup> Parasites were separated from red

blood cells by two rounds of centrifugation at 100g and then at 1500g. Red blood cells were then lysed in 0.17 M  $\text{NH}_4\text{Cl}$  pH 7.2, following which amastigotes were recovered by centrifugation, washed, and resuspended in fresh promastigote culture media. Recovered promastigotes were then used to reinfect mice. Overall, 10 rounds of serial infection were performed.

### Sample Preparation, Fractionation, and LC–MS/MS Analysis of Whole Cell Lysate

To reflect protein expression that occurs early on following exposure to conditions in the mammalian host, mid log phase (day 5 cultures) CL, IV, and VL promastigotes ( $2 \times 10^9$ ) were resuspended at  $5 \times 10^7$  cells/mL in amastigote culture media (M199 media supplemented with 25% FBS, 2 mM  $\text{MgCl}_2$ , 10 mM succinic acid, 0.1 mg/mL streptomycin, 100 U/mL penicillin, pH 5.5) and incubated at 37 °C, 5%  $\text{CO}_2$  for 18 h to induce promastigote to amastigote differentiation. Cells were collected by centrifugation, washed three times with PBS, and resuspended at  $2.5 \times 10^6$  cells/ $\mu\text{L}$  in Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.125 M Tris-HCl pH 6.8, 0.004% bromophenol blue). Two independent biological replicates were produced and used for analysis. Approximately 40  $\mu\text{g}$  of protein from the samples was loaded onto a 10% precast SDS-PAGE gel, followed by staining and destaining of the gel. The gel lane was then cut into 10 slices and processed. Each slice was then reduced with dithiothreitol (DTT) in ammonium bicarbonate and alkylated with iodoacetamide. The samples were then trypsinized overnight at 37 °C.<sup>17</sup> The peptides were extracted from the gel the following day and dried down under vacuum. The dried samples were resuspended in the 5% acetonitrile and 0.5% formic acid.

Samples were separated by Agilent 1260 nanoflow high-performance liquid chromatography. Peptides were loaded on a Thermo Scientific Acclaim (R) Pepmap100 column (2 cm, 75  $\mu\text{m}$  (ID) column of 3  $\mu\text{m}$  C18 reverse-phase material). The analytical column was a Thermo Scientific Acclaim (R) Pepmap RSLC column (15 cm, 75  $\mu\text{m}$  (ID) column of 2  $\mu\text{m}$  reverse-phase material). Peptides were chromatographed on an 0–40% organic phase gradient (aqueous phase: 5% acetonitrile, 0.5% acetic acid in Milli-Q water; organic phase: 90% acetonitrile, 0.5% acetic acid in Milli-Q water) over 150 min (total run time: 180 min), at a flow rate of 350 nL per minute (pressure range: 300–350 bar). The eluate was introduced into an Agilent 6550 QTOF mass spectrometer (operated by Agilent MassHunter Data Acquisition software). Ion source was an Agilent dual nano ESI source with Thermo Scientific 30  $\mu\text{m}$  (ID) steel needle emitter. MS parameters were as follows: precursor selection: mass range, 290–1250  $m/z$ ; acquisition rate, 1 spectra per second. MS/MS parameters were as follows: duty cycle, 1 MS/8 MSMS; mass range, 100–1700  $m/z$ ; acquisition rate, 4 spectra per second; excluded: single charge and charge unassigned peaks; dynamic exclusion parameters: exclude after 1 spectra for 0.25 min. QTOF nitrogen collision cell fragmentation energy settings were as per manufacturers recommendations: slope \* ( $m/z$ )/100 + offset: charge = 2: slope = 3.1; offset = 1; charge >2: slope = 3.6; offset = -4.8.

### Database Searching

Tandem mass spectra were extracted by Mascot Distiller version 2.4. Charge-state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, U.K.; version 2.4.1). Mascot was set up to search the full *u\_ld\_BPK282A1* database (selected for All Entries, *Leishmania donovani* (strain BPK282A1), UniProt,

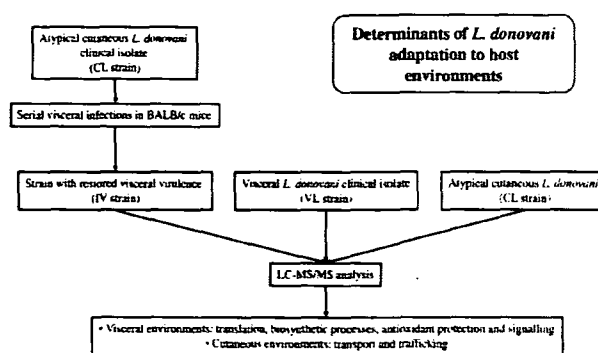
## Adaptation of *Leishmania donovani* to Cutaneous and Visceral Environments: in Vivo Selection and Proteomic Analysis

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### Supporting Information

**ABSTRACT:** Leishmaniasis is a neglected tropical disease caused by *Leishmania* protozoa. Two main forms are found in the Old World, self-limited cutaneous leishmaniasis and potentially fatal visceral leishmaniasis, with parasite dissemination to liver, bone marrow, and spleen. The *Leishmania donovani* species complex is the causative agent of visceral leishmaniasis worldwide, but atypical *L. donovani* strains can cause cutaneous leishmaniasis. We hypothesized that *L. donovani* can adapt to survive in response to restrictions imposed by the host environment. To assess this, we performed in vivo selection in BALB/c mice with a cutaneous *L. donovani* clinical isolate to select for parasites with increased capacity to survive in visceral organs. We then performed whole cell proteomic analysis and compared this visceral-selected strain to the original cutaneous clinical isolate and to a visceral leishmaniasis clinical isolate. Overall, there were no major shifts in proteomic profiles; however, translation, biosynthetic processes, antioxidant protection, and signaling were elevated in visceral strains. Conversely, transport and trafficking were elevated in the cutaneous strain. Overall, these results provide new insight into the adaptability of *Leishmania* parasites to the host environment and on the factors that mediate their ability to survive in different organs.

**KEYWORDS:** visceral leishmaniasis, proteomic analysis, virulence, pathogenesis



### INTRODUCTION

Leishmaniasis is a neglected tropical disease caused by *Leishmania* protozoa. These parasites have a digenetic lifecycle, with a flagellated promastigote stage found in the sandfly vector and an aflagellate amastigote stage in the mammalian host. In the Old World (Europe, Africa, and Asia), this disease is found in two main forms, self-healing cutaneous leishmaniasis with lesions at the site of the sandfly bite and visceral leishmaniasis, in which parasites disseminate from the bite site to the visceral organs, leading to potentially fatal fever, wasting, anemia, and hepatosplenomegaly.<sup>1</sup> While host genetic background, immune status, and infecting *Leishmania* species all determine disease phenotype, with infecting species playing a determining role in immunocompetent hosts,<sup>2,3</sup> some atypical cases in which *Leishmania* species cause different disease manifestations have now been reported. In particular, the normally cutaneous *L. tropica* can cause visceral leishmaniasis. (See, for instance, refs 4 and 5.) Likewise, while *L. donovani* is considered predominantly visceral, it can also cause cutaneous leishmaniasis. (See, for instance, refs 6–9.) In particular, there have been over

2000 reports of cutaneous leishmaniasis caused by *L. donovani* MON-37 in Sri Lanka, while visceral leishmaniasis is very rare there.<sup>10</sup> Moreover, cure of visceral leishmaniasis can also be followed by post kala azar dermal leishmaniasis (PKDL).<sup>1</sup> On the basis of these clinical observations, we therefore hypothesized a previously underappreciated plasticity of *Leishmania* parasites.

To investigate experimentally whether *L. donovani* can adapt to different environments, we used three different strains. The first strain, “CL” was obtained from a cutaneous lesion on the nose of a Sri Lankan cutaneous leishmaniasis patient. The second strain, “VL” was cultured from a bone marrow aspirate from an Sri Lankan visceral leishmaniasis patient. CL and VL strains were confirmed to be *L. donovani* MON-37 by whole isoenzyme typing and whole genome sequencing.<sup>9</sup> Finally, to determine whether it was possible to obtain a strain with increased virulence in the visceral organs and to assess the plasticity of the CL strain, we performed 10 serial passages of the

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### Validation of Mass Spectrometry Data

Mid log phase (day 5) promastigote cultures were used for all validation experiments. Viability following menadione treatments for sensitivity to superoxide was assessed as previously described.<sup>27</sup> In brief, OD600 was measured 72 h after treatment of promastigotes with menadione (Sigma) dissolved in ethanol. To take into account the differences in growth rates between strains, normalized growth was determined as (OD600 value of menadione-treated sample/OD600 value of control sample) × 100. EC50 values were calculated using Collaborative Drug Discovery Vault software.

SDS-PAGE and Western blotting were performed as previously described.<sup>28</sup> Meta 1 was detected with a 1:1000 dilution of mouse anti-meta 1 serum (a gift from Dr. Silvia Uliana, U. of Sao Paulo), tubulin with a 1:2000 dilution of mouse monoclonal anti-tubulin antibody (Oncogene), aldolase with 1:1000 dilution of rabbit anti-aldolase serum (generated in our laboratory), and LDBPK\_367070 with a 1:1000 dilution of rabbit anti-LDBPK\_367070 serum (generated in our laboratory). The secondary antibody was a 1:2500 dilution of peroxidase-conjugated anti-mouse IgG antibody (Rockland) and anti-rabbit IgG antibody.

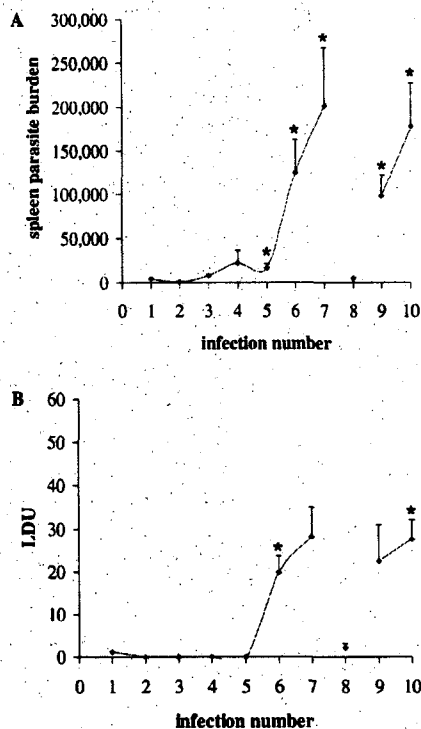
### RESULTS

#### Cutaneous to Visceral Remodelling of *Leishmania* Parasites

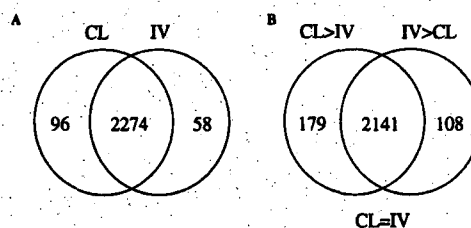
To assess the plasticity of human cutaneous *L. donovani* parasites (CL) and to test whether they could gain the ability to survive in visceral organs, we performed in vivo serial infections in BALB/c mice. Mice were infected intravenously with the initial CL isolate; amastigotes were then recovered from the spleen after 4 weeks and used to reinfect mice intravenously in the same manner for 10 rounds of reinfection. By this in vivo selection, liver, and spleen parasite burden were significantly increased compared with the initial infection, starting at serial infection number five (Figure 1). The drop in virulence following serial infection number eight was due to contamination of the cultures recovered from serial infection number seven, which was cleared following reinfection. Following serial infection number 10, we obtained a strain (termed IV) with a 25-fold increase in the liver parasite burden (LDU values) and a 45-fold increase in the spleen, compared with the initial CL isolate (Figure 1). The cutaneous *L. donovani* from Sri Lanka, avirulent in the visceral organs following intravenous injection, therefore has the potential to convert to a more virulent form, demonstrating its adaptability to host environments.

We next investigated the factors that mediated this restoration of visceral virulence by LC-MS/MS analysis. This identified 277 360 spectra at a 0.63% false discovery rate (FDR) (95% peptide identification confidence). Only proteins with minimum two peptides were considered for analysis. We identified 1959 proteins in CL and 2116 in IV (2428 proteins total), with a 0.0% protein FDR (Tables S1 and S4–S9 in the Supporting Information). Given the large number of differences identified between CL and IV, a five-fold or greater change in empAI values was used as cutoff (with  $p \leq 0.05$ ) to be considered differentially expressed.

Overall, the proteomes of these virulent and avirulent strains were very similar, although it was possible to detect 108 proteins elevated in IV, including 58 that were not detected in CL (Table 1, Figure 2A,B). Of these 108 IV-elevated proteins, 75 were classified as uncharacterized proteins, including of 40 the 58 IV-specific proteins. 179 proteins were elevated in CL, including



**Figure 1.** Generation of IV strain with increased visceral virulence by serial passaging in mouse visceral organs. (A) Spleen parasite burden. (B) Leishman-Donovan Units (LDU). LDU values were only determined for the first serial infections and for infections number 6–10. Values plus standard error are shown. \*,  $p < 0.05$ .



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96 specific to CL (Table 2, Figure 2A,B). Of these 179 CL-elevated proteins, 121 were classified as uncharacterized proteins. 71 of the CL-specific proteins were elevated in IV compared with CL, including the glycosomal superoxide dismutase FeSODB1, meta 1, a protein associated with virulence in *L. amazonensis*,<sup>29</sup> and two ecotins. Ecotins are serine peptidase inhibitors that can inhibit neutrophil elastase and prevent TLR4 activation.<sup>30</sup>

In vivo selection can lead to strains with faster growth rates,<sup>31</sup> and an accelerated growth rate was observed for the IV strain axenic amastigotes compared with the CL strain (Figure S1 in the Supporting Information). Consistent with the faster growth rate, gene ontology terms associated with biosynthetic process (GO:0009058), including cellular biosynthetic process (GO:0044249), cellular macromolecule biosynthetic process (GO:0034645), energy generation (GO:0006091), and translation (GO:0006412), were over-represented in the IV-elevated group. Elevated levels of these metabolic proteins may be required to support the faster growth rate of the IV strain.

7961 entries) assuming the digestion enzyme trypsin and allowing one missed cleavage. Mascot was searched with a fragment ion mass tolerance of 0.100 Da and a parent ion tolerance of 10.0 ppm. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification.

#### Criteria for Protein Identification

Scaffold (version Scaffold\_4.0.6.1, Proteome Software, Portland, OR) was used to validate MS/MS based peptide and protein identifications.<sup>18</sup> Peptide identifications were accepted if they could be established at >95.0% probability by the Peptide Prophet algorithm<sup>19</sup> with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at >99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.<sup>20</sup> Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

#### Data Analysis

A principal component (PC) analysis was performed using the stats package in R with a `prcomp` function. The "x" attribute of the `prcomp` object was used to generate the scatter plots. Normalization was performed by Scaffold by summing the unweighted spectrum counts for each MS sample. Normalized emPAI values were generated in Scaffold and used for data analysis. emPAI values are reported to be directly proportional to protein abundance and can be used for relative quantitation between samples.<sup>21</sup> Fold changes, false discovery rates, and *p* values were calculated using built-in functions in Scaffold.<sup>18,22</sup> Fold change values represent the ratio of average emPAI values between the two strains. The mean emPAI values were used to calculate *p* values.

Gene ontology (GO) annotations were performed using Blast2GO.<sup>23</sup> Default parameters were used for all steps. The initial Blastp step was performed against NCBI nonredundant database with *E* value of  $1 \times 10^{-3}$ . Twenty top blast hits were retrieved and used for annotation. For the annotation step, the pre-eValue-Hit-Filter was  $1 \times 10^{-6}$ , annotation cutoff was 55, and GO weight was 5.

#### Exosome Collection, Purification, and LC-MS/MS Analysis

Purification of *Leishmania* exosomes was performed according to standard methods for mammalian cells in two separate replicate experiments.<sup>24</sup> Stationary phase promastigotes were resuspended at  $4 \times 10^8$  cells/mL in RPMI 1640 without HEPES, L-glutamine, and phenol red and incubated for 4 h at 37 °C to stimulate exosome production as described in refs 25 and 26. Parasite viability was measured by propidium iodide staining before and after incubation at 37 °C. Culture supernatant was recovered by two rounds of centrifugation at 4000 rpm for 10 min. The supernatant was filtered through a 0.45  $\mu$ m filter and then a 0.22  $\mu$ m filter to remove cellular debris, and exosomes were collected by centrifugation at 29 000 rpm for 1 h. Pelleted exosomes overlaid on a 0–2 M gradient of sucrose and centrifuged for 90 min at 65 000 rpm. Ten fractions of 1 mL were collected and washed in exosome buffer (137 mM NaCl 20 mM HEPES pH 7.2) at 29 000 rpm for 1 h. Exosomes were recovered in fractions 5 and 6, corresponding to the concentrations of 1 to 1.3 M of sucrose and densities of 1.13 to 1.15 g/mL. Collected exosomes were precipitated first with Exoquick-TC (System Biosciences) according to manufacturer's instructions then with 15% trichloroacetic acid (TCA)/acetone.

LC-MS/MS was performed at the Institute de Recherches Cliniques de Montréal (IRCM, University of Montreal, Canada). TCA-precipitated samples were resolubilized in 10  $\mu$ L of a 6 M urea buffer. Proteins were reduced by adding 2.5  $\mu$ L of reduction buffer (45 mM DTT, 100 mM ammonium bicarbonate) for 30 min at 37 °C and then alkylated by adding 2.5  $\mu$ L of alkylation buffer (100 mM iodoacetamide, 100 mM ammonium bicarbonate) for 20 min at 24 °C in dark. Prior to trypsin digestion, 20  $\mu$ L of water was added to reduce the urea concentration to 2 M. Ten  $\mu$ L of the trypsin solution (5 ng/ $\mu$ L of trypsin sequencing grade from Promega, 50 mM ammonium bicarbonate) was added to each sample. Protein digestion was performed at 37 °C for 18 h and stopped with 5  $\mu$ L of 5% formic acid. Protein digests were dried down in vacuum centrifuge and stored at –20 °C until LC-MS/MS analysis.

Prior to LC-MS/MS, protein digests were resolubilized under agitation for 15 min in 10  $\mu$ L of 0.2% formic acid. Desalting/cleanup of the digests was performed by using C<sub>18</sub> ZipTip pipet tips (Millipore, Billerica, MA). Eluates were dried down in vacuum centrifuge and then resolubilized under agitation for 15 min in 10  $\mu$ L of 2%ACN/1% formic acid. The LC column was a C18 reversed-phase column packed with a high-pressure packing cell A 75  $\mu$ m i.d. Self-Pack PicoFrit fused silica capillary column (New Objective, Woburn, MA) 15 cm long was packed with C18 Jupiter 5  $\mu$ m 300 Å reverse-phase material (Phenomenex, Torrance, CA). This column was installed on an Easy-nLC II system (Proxeon Biosystems, Odense, Denmark) and coupled to the LTQ Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany) equipped with a Proxeon nanoelectrospray ion source. The buffers used for chromatography were 0.2% formic acid (buffer A) and 100% acetonitrile/0.2% formic acid (buffer B). Peptides were loaded on-column at a flow rate of 600 nL/min and eluted with a 2 slope gradient at a flow rate of 250 nL/min. Solvent B first increased from 1 to 40% in 82 min and then from 40 to 80% B in 28 min. LC-MS/MS data acquisition was accomplished using an 11 scan event cycle composed of a full-scan MS for scan event 1 acquired in the Orbitrap. The mass resolution for MS was set to 60 000 (at *m/z* 400) and used to trigger the 10 additional MS/MS events acquired in parallel in the linear ion trap for the top 10 most intense ions. The mass over charge ratio range was from 360 to 2000 for MS scanning with a target value of 1 000 000 charges and from ~1/3 of parent *m/z* ratio to 2000 for MS/MS scanning with a target value of 10 000 charges. The data-dependent scan events used a maximum ion fill time of 100 ms and 1 microscan. Target ions already selected for MS/MS were dynamically excluded for 25 s. Nanospray and S-lens voltages were set to 0.9 to 1.8 kV and 50 V, respectively. Capillary temperature was set to 225 °C. MS/MS conditions were: normalized collision energy, 35 V; activation *q*, 0.25; activation time, 10 ms.

The peak list files were generated with Proteome Discoverer (version 1.4) using the following parameters: minimum mass set to 500 Da, maximum mass set to 6000 Da, no grouping of MS/MS spectra, precursor charge set to auto, and minimum number of fragment ions set to 5. Protein database searching was performed with Mascot 2.3 (Matrix Science) against the full *Leishmania* *Donovani* `txid5661_20121218` (17 451 entries). The mass tolerances for precursor and fragment ions were set to 15 ppm and 0.6 Da (replicate 1) or 10 ppm and 0.50 Da (replicate 2). Trypsin was used as the enzyme allowing for up to one missed cleavage. Cysteine carbamidomethylation was specified as a fixed modification, and methionine oxidation was specified as a variable modification. Data analysis was performed using Scaffold as previously described.

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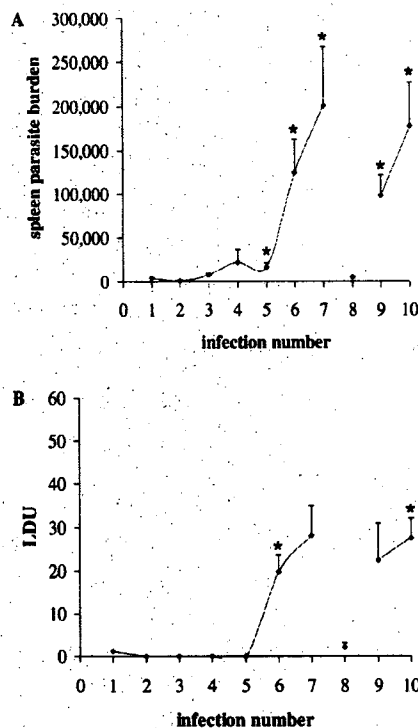
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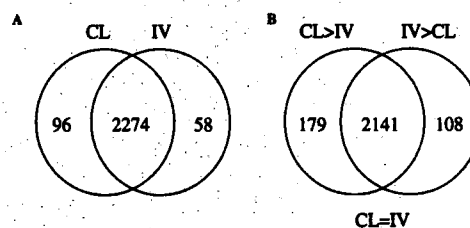
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Table 1. Proteins Elevated in the IV Strain Compared with the CL Isolate

no.	accession number	gene ID	description	molecular weight	fold change IV to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>	IV <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>
1	E9BF19	LDBPK_212030	uncharacterized protein	48 kDa	5	0.014	0.19	0.07	0.64	0.63
2	E9BM55	LDBPK_303120	homoserine kinase, putative	36 kDa	5.1	0.012	0.09	0.16	0.60	0.69
3	E9BMT9	LDBPK_311650	uncharacterized protein	19 kDa	5.2	0.029	0.08	0.06	0.40	0.30
4	E9B7V6	LDBPK_030860	uncharacterized protein	212 kDa	5.4	0.025	0.08	0.06	0.42	0.32
5	E9BRB2	LDBPK_343140	uncharacterized protein	24 kDa	5.5	0.017	0.06	0.17	0.68	0.59
6	E9BKZ3	LDBPK_291960	uncharacterized protein	38 kDa	5.5	0.041	0.09	0.08	0.52	0.37
7	E9B7Z1	LDBPK_040250	uncharacterized protein	28 kDa	5.5	0.047	0.09	0.00	0.26	0.26
8	E9BAS8	LDBPK_110900	60S ribosomal protein L24, putative	23 kDa	5.8	0.044	0.00	0.05	0.14	0.16
9	E9BPT7	LDBPK_330080	uncharacterized protein	20 kDa	5.8	0.044	0.00	0.06	0.16	0.17
10	E9B1L4	LDBPK_262030	uncharacterized protein	80 kDa	5.9	0.00049	0.08	0.06	0.41	0.42
11	E9BTM5	LDBPK_361660	uncharacterized protein	13 kDa	6.1	0.039	0.00	0.10	0.28	0.30
12	E9BQD3	LDBPK_332030	uncharacterized protein	22 kDa	6.2	0.037	0.00	0.11	0.34	0.38
13	E9BMR4	LDBPK_311400	uncharacterized protein	97 kDa	6.3	0.015	0.20	0.61	2.40	2.73
14	E9BH46	LDBPK_241840	inhibitor of cysteine peptidase	13 kDa	6.4	0.028	0.26	0.09	0.97	1.24
15	E9BH94	LDBPK_242330	uncharacterized protein	41 kDa	6.7	0.011	0.06	0.13	0.61	0.71
16	E9BCC7	LDBPK_150870	uncharacterized protein	114 kDa	6.7	0.03	0.00	0.07	0.24	0.26
17	E9BCY2	LDBPK_161280	uncharacterized protein	200 kDa	6.9	0.0051	0.14	0.15	1.03	0.91
18	E9BL20	LDBPK_292230	uncharacterized protein	192 kDa	6.9	0.017	0.13	0.28	1.29	1.57
19	E9B8H2	LDBPK_050820	uncharacterized protein	102 kDa	6.9	0.028	0.06	0.00	0.20	0.21
20	E9BLS2	LDBPK_301790	DNAJ domain protein, putative	79 kDa	7.2	0.0036	0.05	0.11	0.58	0.56
21	E9BRR2	LDBPK_350300	uncharacterized protein	36 kDa	7.2	0.049	0.00	0.16	0.67	0.51
22	E9BK97	LDBPK_280590	uncharacterized protein	32 kDa	7.4	0.029	0.08	0.08	0.69	0.51
23	E9BDF7	LDBPK_171350	uncharacterized protein	117 kDa	7.4	0.032	0.00	0.22	0.90	0.76
24	E9BPW1	LDBPK_330320	autophagy-related protein 3	31 kDa	7.5	0.0037	0.10	0.19	1.07	1.14
25	E9B9B2	LDBPK_071180	peptidyl-prolyl <i>cis-trans</i> isomerase/rotamase, putative	13 kDa	7.7	0.024	0.70	0.00	2.56	2.82
26	E9BG80	LDBPK_230600	5'-3' exonuclease XRNC, putative	116 kDa	7.8	0.032	0.05	0.00	0.22	0.18
27	E9BLR9	LDBPK_301760	uncharacterized protein	211 kDa	7.8	0.037	0.09	0.06	0.49	0.69
28	E9B7H6	LDBPK_020290	ribosomal RNA processing protein, putative	34 kDa	7.9	0.031	0.00	0.08	0.36	0.30
29	E9BDB6	LDBPK_170950	uncharacterized protein	13 kDa	8.2	0.021	0.11	0.00	0.45	0.49
30	E9BDC0	LDBPK_170990	uncharacterized protein (meta 1)	12 kDa	8.9	0.017	0.48	0.00	2.10	2.23
31	E9BMR7	LDBPK_311430	uncharacterized protein (fragment)	77 kDa	9	0.01	0.13	0.11	1.16	0.97
32	E9BKF4	LDBPK_290070	QA-SNARE protein putative	30 kDa	9	0.039	0.00	0.09	0.34	0.45
33	E9B7U0	LDBPK_030720	cytochrome <i>c</i> oxidase copper chaperone, putative	9 kDa	9.1	0.018	0.16	0.00	0.71	0.78
34	E9BKR6	LDBPK_291200	uncharacterized protein	68 kDa	9.1	0.038	0.05	0.00	0.21	0.27
35	E9BQ89	LDBPK_331580	uncharacterized protein	91 kDa	9.3	0.026	0.04	0.00	0.23	0.18
36	E9BIN9	LDBPK_262280	uncharacterized protein	29 kDa	9.5	0.02	0.00	0.10	0.42	0.49
37	E9BBD4	LDBPK_130600	uncharacterized protein	46 kDa	9.6	0.02	0.03	0.05	0.45	0.35
38	E9BJL7	LDBPK_281460	haloacid dehalogenase-like hydrolase-like protein	35 kDa	9.8	0.013	0.00	0.07	0.35	0.36
39	E9BLH3	LDBPK_300800	4-methyl-5(beta-hydroxyethyl)-thiazole monophosphate synthesis protein, putative	21 kDa	10	0.038	0.15	0.29	1.86	2.67
40	E9BUM3	LDBPK_365150	uncharacterized protein	77 kDa	11	0.044	0.04	0.03	0.47	0.32
41	E9BS67	LDBPK_351940	uncharacterized protein	27 kDa	11	0.047	0.00	0.04	0.19	0.28
42	E9BBY5	LDBPK_141030	uncharacterized protein	32 kDa	12	0.0027	0.21	0.17	2.41	2.20
43	E9BIV0	LDBPK_270150	uncharacterized protein	19 kDa	13	0.0069	0.00	0.06	0.39	0.39
44	E9BM02	LDBPK_302590	formate-tetrahydrofolate ligase	67 kDa	13	0.0081	0.05	0.16	1.34	1.55
45	E9BAA8	LDBPK_100630	uncharacterized protein	85 kDa	13	0.042	0.05	0.00	0.24	0.35
46	E9BD09	LDBPK_161580	uncharacterized protein	70 kDa	14	0.0058	0.00	0.04	0.27	0.26
47	E9BG10	LDBPK_221450	Ser/thr protein phosphatase, putative	86 kDa	14	0.041	0.05	0.06	0.58	0.86
48	E9B8U9	LDBPK_060890	dihydrofolate reductase-thymidylate synthase	59 kDa	17	0.012	0.00	0.06	0.48	0.56
49	E9BAD0	LDBPK_100850	uncharacterized protein	136 kDa	21	0.019	0.06	0.00	0.53	0.68
50	E9BE96	LDBPK_191030	uncharacterized protein	132 kDa	33	0.02	0.07	0.13	3.81	2.89
51	E9BEM0	LDBPK_200730	uncharacterized protein	29 kDa	INF <sup>b</sup>		0.00	0.00	0.16	0.16
52	E9BHM3	LDBPK_251190	alpha-tubulin <i>N</i> -acetyltransferase	27 kDa	INF		0.00	0.00	0.17	0.17
53	E9BL51	LDBPK_292540	uncharacterized protein	81 kDa	INF		0.00	0.00	0.99	0.98

Table 1. continued

no.	accession number	gene ID	description	molecular weight	fold change IV to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>	IV <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>
54	E9BMA0	LDBPK_303570	S-adenosylmethionine synthetase	30 kDa	INF		0.00	0.00	0.24	0.24
55	E9BN65	LDBPK_312950	ribosomal protein L7/L12-like protein	22 kDa	INF		0.00	0.00	0.21	0.21
56	E9BBC1	LDBPK_130470	uncharacterized protein	180 kDa	INF		0.00	0.00	0.16	0.16
57	E9BGG4	LDBPK_231500	uncharacterized protein	166 kDa	INF		0.00	0.00	0.12	0.11
58	E9BMR3	LDBPK_311390	uncharacterized protein	157 kDa	INF		0.00	0.00	0.53	0.51
59	E9BNS7	LDBPK_312870	uncharacterized protein	26 kDa	INF		0.00	0.00	0.24	0.23
60	E9BJQ7	LDBPK_281860	uncharacterized protein	65 kDa	INF		0.00	0.00	0.10	0.11
61	E9BHF7	LDBPK_250530	RNA-binding protein, putative	25 kDa	INF		0.00	0.00	0.69	0.66
62	E9BFW7	LDBPK_221010	uncharacterized protein	31 kDa	INF		0.00	0.00	0.12	0.11
63	E9BTS6	LDBPK_362170	serine/threonine-protein phosphatase	97 kDa	INF		0.00	0.00	0.21	0.22
64	E9BPY0	LDBPK_330510	uncharacterized protein	33 kDa	INF		0.00	0.00	0.16	0.15
65	E9B7P1	LDBPK_030230	uncharacterized protein	17 kDa	INF		0.00	0.00	0.19	0.21
66	E9B8E4	LDBPK_050540	uncharacterized protein	14 kDa	INF		0.00	0.00	0.25	0.27
67	E9BC76	LDBPK_150350	ecotin, putative	16 kDa	INF		0.00	0.00	0.92	1.01
68	E9BRE0	LDBPK_343420	cluster of E9BRE0_LEIDB uncharacterized protein	18 kDa	INF		0.00	0.00	0.19	0.20
69	E9BTC7	LDBPK_360680	uncharacterized protein	30 kDa	INF		0.00	0.00	0.10	0.11
70	E9BU71	LDBPK_363620	uncharacterized protein	23 kDa	INF		0.00	0.00	0.14	0.15
71	E9B9W4	LDBPK_090770	cleavage and polyadenylation specificity factor 30 kDa subunit, putative	36 kDa	INF		0.00	0.00	0.21	0.23
72	E9BEW5	LDBPK_201670	uncharacterized protein	29 kDa	INF		0.00	0.00	0.56	0.49
73	E9BIN1	LDBPK_262200	uncharacterized protein	44 kDa	INF		0.00	0.00	0.12	0.14
74	E9BEB8	LDBPK_191260	RNA binding protein, putative	25 kDa	INF		0.00	0.00	0.23	0.26
75	E9BGR3	LDBPK_240510	uncharacterized protein	26 kDa	INF		0.00	0.00	0.22	0.25
76	E9BH92	LDBPK_242310	uncharacterized protein	43 kDa	INF		0.00	0.00	0.19	0.23
77	E9BUC5	LDBPK_364170	uncharacterized protein	41 kDa	INF		0.00	0.00	0.13	0.15
78	E9B9A0	LDBPK_071090	uncharacterized protein	261 kDa	INF		0.00	0.00	0.09	0.07
79	E9B8H9	LDBPK_050890	uncharacterized protein (fragment)	75 kDa	INF		0.00	0.00	0.56	0.69
80	E9B7U3	LDBPK_030750	uncharacterized protein	59 kDa	INF		0.00	0.00	0.31	0.25
81	E9BE74	LDBPK_190790	signal recognition particle protein, putative	81 kDa	INF		0.00	0.00	0.24	0.19
82	E9BMS7	LDBPK_311530	uncharacterized protein	120 kDa	INF		0.00	0.00	1.08	0.85
83	E9BNL2	LDBPK_321090	uncharacterized protein	25 kDa	INF		0.00	0.00	0.15	0.19
84	E9B889	LDBPK_041240	uncharacterized protein	121 kDa	INF		0.00	0.00	0.13	0.18
85	E9BUV2	LDBPK_365950	uncharacterized protein	86 kDa	INF		0.00	0.00	0.16	0.11
86	E9BNN2	LDBPK_321290	ADP-ribosylation factor GTPase activating protein 1, putative	43 kDa	INF		0.00	0.00	0.19	0.14
87	E9BRL8	LDBPK_344190	RNA-binding protein-like protein	43 kDa	INF		0.00	0.00	0.19	0.14
88	E9B7D0	LDBPK_010660	HSP70-like protein	117 kDa	INF		0.00	0.00	0.17	0.12
89	E9BCT9	LDBPK_160860	uncharacterized protein	24 kDa	INF		0.00	0.00	0.30	0.43
90	E9BRR7	LDBPK_350350	uncharacterized protein	77 kDa	INF		0.00	0.00	0.18	0.26
91	E9BSV9	LDBPK_354390	uncharacterized protein	61 kDa	INF		0.00	0.00	0.30	0.21
92	E9B8B0	LDBPK_050200	uncharacterized protein	88 kDa	INF		0.00	0.00	0.32	0.22
93	E9B8S4	LDBPK_040890	uncharacterized protein	140 kDa	INF		0.00	0.00	0.14	0.21
94	E9BCZ4	LDBPK_161420	DNA-directed RNA polymerase	200 kDa	INF		0.00	0.00	0.10	0.14
95	E9BLM9	LDBPK_301360	U3 small nuclear ribonucleoprotein (SnRNP), putative	33 kDa	INF		0.00	0.00	0.48	0.33
96	E9BMQ2	LDBPK_311280	uncharacterized protein	69 kDa	INF		0.00	0.00	0.15	0.10
97	E9BGL2	LDBPK_231980	uncharacterized protein	17 kDa	INF		0.00	0.00	0.32	0.22
98	E9B966	LDBPK_070740	uncharacterized protein	55 kDa	INF		0.00	0.00	0.17	0.11
99	E9BNC7	LDBPK_320220	uncharacterized protein	58 kDa	INF		0.00	0.00	0.39	0.26
100	E9BC95	LDBPK_150540	ecotin, putative	42 kDa	INF		0.00	0.00	1.17	0.76
101	E9B7Q1	LDBPK_030330	protein kinase, putative	154 kDa	INF		0.00	0.00	0.19	0.12
102	E9BQR3	LDBPK_341120	uncharacterized protein	78 kDa	INF		0.00	0.00	0.06	0.09
103	E9BIB4	LDBPK_261020	uncharacterized protein	60 kDa	INF		0.00	0.00	0.10	0.15
104	E9B9Z0	LDBPK_091040	uncharacterized protein	103 kDa	INF		0.00	0.00	0.72	0.45
105	E9BNF6	LDBPK_320520	Ras-related rab-4, putative	22 kDa	INF		0.00	0.00	0.59	0.37
106	E9BS11	LDBPK_351350	uncharacterized protein	23 kDa	INF		0.00	0.00	0.32	0.20
107	E9BN14	LDBPK_312440	uncharacterized protein	58 kDa	INF		0.00	0.00	0.12	0.19

Table 1. continued

no.	accession number	gene ID	description	molecular weight	fold change IV to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>		IV <sup>a</sup>	
108	E9BP87	LDBPK_323370	NADH dehydrogenase subunit N18M, putative	19 kDa	INF	0.00	0.00	0.29	0.46	

<sup>a</sup>Values from two biological replicates. <sup>b</sup>A ratio of "INF" is returned when dividing by zero (protein not detected in CL).

Signaling (GO:0023052) and cell communication (GO:0007154) were also over-represented in IV. Elevation of signaling proteins has been observed in amastigotes<sup>32</sup> and may be an adaptation to allow the parasite to respond to a changing host environment. Elevation of signaling in the IV strain may be required to help the parasite defend itself against the host immune system. In contrast, many proteins involved in trafficking were upregulated in CL compared with IV, including 16 dyneins and one kinesin.

#### Visceral to Cutaneous Remodelling of *Leishmania* Parasites

Sri Lankan *L. donovani*, while causing cutaneous rather than visceral leishmaniasis, is closely related to Indian visceral *L. donovani* strains,<sup>33</sup> and the mechanisms by which the parasite evolved this novel tropism in Sri Lanka are poorly understood but are related to genetic differences.<sup>9</sup> To identify the changes at the protein level associated with the conversion of a predominantly viscerotropic *L. donovani* parasite to a cutaneous parasite, we compared two human clinical isolates from Sri Lanka, the cutaneous CL strain previously described and a visceral clinical isolate, VL, by performing LC-MS/MS analysis on these isolates as detailed in methods. This identified 276 009 spectra at a 0.63% FDR cutoff (95% peptide identification confidence). Only proteins with minimum two peptides were considered for analysis. We identified 1959 proteins in CL and 1802 in VL (2114 proteins in total) with a 0.0% protein FDR (Tables S1 and S4–S9 in the Supporting Information).

We compared protein abundance between CL and VL using a two-fold or greater change in emPAI values with  $p \leq 0.05$  as a cutoff. Overall, the proteomes of the CL and VL strains were very similar: only 35 proteins elevated in VL, including 6 not detected in CL (Table 3, Figure 3A,B). This is consistent with our previous study, which revealed that there were no gene deletions of pseudogenes specific to the CL or VL strains.<sup>9</sup> Of these 35 VL-elevated proteins, 18 were classified as uncharacterized hypothetical proteins. All but one of the 6 VL-specific proteins were uncharacterized. 148 proteins were elevated in CL, including 39 detected exclusively in CL (Table 4, Figure 3A,B). Of these 148 CL-elevated proteins, 81 were classified as uncharacterized proteins. Twenty-one of the 39 CL-specific proteins were uncharacterized.

Both the mitochondrial peroxidoxin (E9BG25) and a superoxide dismutase (E9B9D5) were elevated in VL (Table 3). The mitochondrial peroxidoxin is essential for *Leishmania infantum* virulence but via a chaperone activity rather than through antioxidant functions.<sup>34</sup> E9B9D5 is the mitochondrial iron superoxide dismutase FeSODA that protects against oxidative stress.<sup>35</sup> Indeed, GO analysis indicates over-representation of proteins involved in stress responses (GO:0006950) in VL-elevated proteins. Finally, as with the comparison between CL and IV, signaling (GO:0023052) and signal transduction (GO:0007165) were over-represented in VL.

Several proteins involved in transport were elevated in CL. These include motor proteins (seven members of the dynein

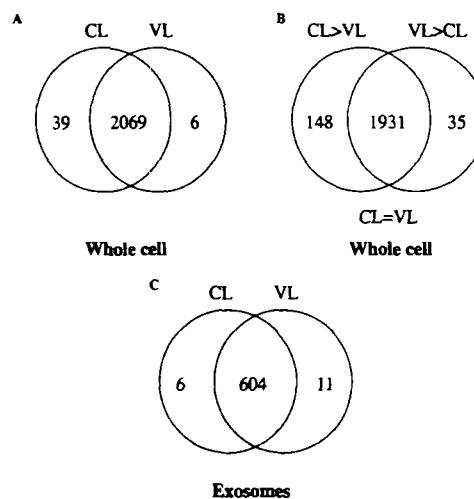


Figure 3. Proteomic comparison of CL and VL. (A) Venn diagram of common proteins and strain-specific proteins in whole cell lysate. (B) Venn diagram of proteins upregulated in CL or in VL whole cell lysate. (C) Venn diagram of common proteins and strain-specific proteins in CL and VL exosomes.

heavy chain family and three kinesins) and proteins that are involved in vesicle assembly and in trafficking. A number of transporters were also upregulated in CL, including three ABC transporters and five cation transporters. These observations were confirmed by gene ontology analysis: the terms localization (GO:0051179), including establishment of localization (GO:0051234), transport (GO:0006810), and ion transport (GO:0006811) were over-represented in the CL-elevated proteins. These processes may be especially important for the establishment of cutaneous infection. Indeed, *L. major* lacking the delta subunit of adaptor protein AP3 were less virulent during subcutaneous infection in mice,<sup>36</sup> and we observed elevated levels of a subunit of AP3 delta in CL. Comparison of mRNA expression across mammalian tissues indicated that GO terms associated with energy and metabolism were enriched in the liver compared with the skin, suggesting that the liver may be a more nutrient-rich environment than the skin.<sup>37</sup> Increased transport in the CL strain may therefore be an adaptation that allows it to survive in the nutrient-poor environment of the skin where other *L. donovani* strains are normally unable to do so.

Secreted proteins released by CL or VL parasites could also modulate the host environment and determine progression to cutaneous or visceral leishmaniasis. In *Leishmania* parasites, most proteins are secreted via a nonclassical pathway; in particular, over 50% of secreted *Leishmania* proteins may be found in exosomes.<sup>38</sup> Exosomes from *Leishmania* parasites alter macrophage signaling pathways,<sup>39</sup> macrophage gene expression,<sup>39</sup> and monocyte and dendritic cell cytokine production.<sup>38,40</sup> Moreover, pre-exposure of mice to *Leishmania* exosomes increased the severity of cutaneous and visceral leishmaniasis.<sup>26</sup> We therefore



Table 2. Proteins Elevated in the CL Isolate Compared with the IV Strain

no.	accession number	gene ID	description	molecular weight	fold change IV to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>	CL <sup>b</sup>	IV <sup>a</sup>	IV <sup>b</sup>
1	E9BC09	LDBPK_141270	uncharacterized protein	58 kDa	0		0.13	0.13	0.00	0.00
2	E9BGZ7	LDBPK_241340	uncharacterized protein	28 kDa	0		0.20	0.19	0.00	0.00
3	E9BH25	LDBPK_241630	uncharacterized protein	67 kDa	0		10.31	10.41	0.00	0.00
4	E9BHP7	LDBPK_251430	uncharacterized protein	29 kDa	0		0.19	0.19	0.00	0.00
5	E9BNT7	LDBPK_321840	uncharacterized protein	54 kDa	0		0.91	0.91	0.00	0.00
6	E9BV46	LDBPK_366900	uncharacterized protein	75 kDa	0		0.63	0.63	0.00	0.00
7	E9BA64	LDBPK_100160	leucine-rich repeat protein, putative	27 kDa	0		2.24	2.19	0.00	0.00
8	E9BTX7	LDBPK_362680	uncharacterized protein	42 kDa	0		0.21	0.21	0.00	0.00
9	E9B9U5	LDBPK_090580	leucine-rich repeat protein, putative	106 kDa	0		1.23	1.26	0.00	0.00
10	E9BSR1	LDBPK_353910	nucleoside diphosphate kinase, putative	37 kDa	0		0.23	0.23	0.00	0.00
11	E9BGM7	LDBPK_240150	uncharacterized protein	32 kDa	0		0.48	0.49	0.00	0.00
12	E9BGH1	LDBPK_231570	dynein heavy chain, putative	544 kDa	0		1.07	1.10	0.00	0.00
13	E9BES2	LDBPK_201230	calpain-like cysteine peptidase, putative	78 kDa	0		0.83	0.81	0.00	0.00
14	E9BBZ0	LDBPK_141080	uncharacterized protein	36 kDa	0		0.33	0.32	0.00	0.00
15	E9BKQ4	LDBPK_291080	uncharacterized protein	105 kDa	0		0.27	0.26	0.00	0.00
16	E9BGC1	LDBPK_231020	uncharacterized protein	57 kDa	0		0.31	0.33	0.00	0.00
17	E9BK45	LDBPK_280050	uncharacterized protein	56 kDa	0		0.35	0.33	0.00	0.00
18	E9BPP8	LDBPK_333100	uncharacterized protein	69 kDa	0		0.36	0.39	0.00	0.00
19	E9BSS5	LDBPK_351800	trypanin-like protein	54 kDa	0		0.13	0.12	0.00	0.00
20	E9BIQ1	LDBPK_262400	uncharacterized protein	558 kDa	0		0.21	0.23	0.00	0.00
21	E9BM84	LDBPK_303410	calmodulin-related protein, putative	81 kDa	0		2.51	2.71	0.00	0.00
22	E9BJA5	LDBPK_271750	paraflagellar rod protein-like protein	89 kDa	0		0.81	0.74	0.00	0.00
23	E9BP85	LDBPK_323350	uncharacterized protein (fragment)	131 kDa	0		0.45	0.49	0.00	0.00
24	E9BAI3	LDBPK_101410	uncharacterized protein	35 kDa	0		0.27	0.24	0.00	0.00
25	E9BG87	LDBPK_230670	uncharacterized protein	348 kDa	0		0.09	0.10	0.00	0.00
26	E9BTL7	LDBPK_361580	cluster of E9BTL7_LEIDB uncharacterized protein	57 kDa	0		5.27	5.82	0.00	0.00
27	E9BCH4	LDBPK_151350	actin-like protein, putative	47 kDa	0		0.42	0.38	0.00	0.00
28	E9BUN3	LDBPK_365250	uncharacterized protein	98 kDa	0		0.19	0.21	0.00	0.00
29	E9BL89	LDBPK_292920	ribonuclease inhibitor-like protein	81 kDa	0		0.10	0.09	0.00	0.00
30	E9BPK1	LDBPK_332630	uncharacterized protein	36 kDa	0		1.02	1.14	0.00	0.00
31	E9BIC8	LDBPK_261160	uncharacterized protein	71 kDa	0		0.34	0.39	0.00	0.00
32	E9BTE0	LDBPK_360810	uncharacterized protein	137 kDa	0		0.14	0.12	0.00	0.00
33	E9BSY1	LDBPK_354620	uncharacterized protein	161 kDa	0		0.15	0.13	0.00	0.00
34	E9BE17	LDBPK_190210	polyprenyl synthase, putative	82 kDa	0		0.22	0.19	0.00	0.00
35	E9BE47	LDBPK_190520	uncharacterized protein	58 kDa	0		1.01	1.19	0.00	0.00
36	E9BBR1	LDBPK_140260	uncharacterized protein	75 kDa	0		0.19	0.22	0.00	0.00
37	E9BUY6	LDBPK_366290	uncharacterized protein	260 kDa	0		0.31	0.36	0.00	0.00
38	E9BC97	LDBPK_150560	uncharacterized protein	45 kDa	0		5.67	4.77	0.00	0.00
39	E9BD16	LDBPK_161650	uncharacterized protein	139 kDa	0		0.85	1.01	0.00	0.00
40	E9BFX6	LDBPK_221100	uncharacterized protein	41 kDa	0		0.63	0.52	0.00	0.00
41	E9BPS6	LDBPK_323050	uncharacterized protein	70 kDa	0		1.92	2.29	0.00	0.00
42	E9BQK4	LDBPK_340500	calcium channel protein, putative	291 kDa	0		0.32	0.39	0.00	0.00
43	E9B7Y6	LDBPK_040200	surface antigen-like protein	29 kDa	0		0.41	0.33	0.00	0.00
44	E9BR85	LDBPK_342870	uncharacterized protein	64 kDa	0		1.67	2.04	0.00	0.00
45	E9BNV4	LDBPK_322020	uncharacterized protein	50 kDa	0		2.81	2.29	0.00	0.00
46	E9BUF2	LDBPK_364440	uncharacterized protein	123 kDa	0		9.75	11.94	0.00	0.00
47	E9BKA3	LDBPK_280650	dynein heavy chain, putative	478 kDa	0		0.89	1.11	0.00	0.00
48	E9BJK0	LDBPK_281290	uncharacterized protein	24 kDa	0		0.88	0.71	0.00	0.00
49	E9BSN3	LDBPK_353620	uncharacterized protein	102 kDa	0		0.37	0.46	0.00	0.00
50	E9BM64	LDBPK_303210	uncharacterized protein	49 kDa	0		0.90	0.71	0.00	0.00
51	E9BU02	LDBPK_362930	uncharacterized protein	295 kDa	0		0.48	0.38	0.00	0.00
52	E9BUP2	LDBPK_365340	uncharacterized protein	29 kDa	0		0.19	0.24	0.00	0.00
53	E9B7H5	LDBPK_020280	uncharacterized protein	73 kDa	0		3.70	4.74	0.00	0.00
54	E9BSZ6	LDBPK_354770	MCAK-like kinesin, putative	53 kDa	0		0.24	0.19	0.00	0.00
55	E9BE03	LDBPK_190070	uncharacterized protein	51 kDa	0		0.81	0.63	0.00	0.00
56	E9BTG1	LDBPK_361010	dynein heavy chain, putative	473 kDa	0		0.65	0.50	0.00	0.00

Table 2. continued

no.	accession number	gene ID	description	molecular weight	fold change IV to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>	IV <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>
57	E9BA45	LDBPK_091600	uncharacterized protein	174 kDa	0		1.36	1.78	0.00	0.00
58	E9BBC8	LDBPK_130540	uncharacterized protein	66 kDa	0		0.38	0.29	0.00	0.00
59	E9BRJ8	LDBPK_343900	uncharacterized protein	79 kDa	0		0.12	0.09	0.00	0.00
60	E9BUX0	LDBPK_366130	uncharacterized protein	86 kDa	0		5.18	6.83	0.00	0.00
61	E9B897	LDBPK_050070	dynein light chain, putative	11 kDa	0		1.06	0.80	0.00	0.00
62	E9BHH6	LDBPK_250720	uncharacterized protein	39 kDa	0		0.25	0.33	0.00	0.00
63	E9BN99	LDBPK_313290	uncharacterized protein	162 kDa	0		0.83	0.62	0.00	0.00
64	E9BN22	LDBPK_312520	uncharacterized protein	24 kDa	0		0.37	0.27	0.00	0.00
65	E9BPH6	LDBPK_332370	uncharacterized protein	36 kDa	0		0.75	0.55	0.00	0.00
66	E9BG37	LDBPK_230170	uncharacterized protein	39 kDa	0		0.17	0.24	0.00	0.00
67	E9BKS2	LDBPK_291260	uncharacterized protein	40 kDa	0		3.07	2.23	0.00	0.00
68	E9BPZ2	LDBPK_330630	uncharacterized protein	101 kDa	0		1.23	1.70	0.00	0.00
69	E9BTB1	LDBPK_360520	uncharacterized protein	36 kDa	0		0.29	0.40	0.00	0.00
70	E9BK71	LDBPK_280310	ribonuclease II-like protein, putative	105 kDa	0		0.12	0.16	0.00	0.00
71	E9BLS4	LDBPK_301810	uncharacterized protein	590 kDa	0		0.24	0.34	0.00	0.00
72	E9B942	LDBPK_070470	uncharacterized protein	89 kDa	0		5.86	8.25	0.00	0.00
73	E9BJ78	LDBPK_271460	diacylglycerol acyltransferase, putative	171 kDa	0		0.23	0.17	0.00	0.00
74	E9BC35	LDBPK_141530	uncharacterized protein	193 kDa	0		0.45	0.64	0.00	0.00
75	E9BP33	LDBPK_232820	uncharacterized protein	42 kDa	0		0.31	0.22	0.00	0.00
76	E9BAA0	LDBPK_100550	uncharacterized protein	713 kDa	0		0.13	0.09	0.00	0.00
77	E9BNU3	LDBPK_321900	protein kinase, putative	71 kDa	0		0.22	0.31	0.00	0.00
78	E9B810	LDBPK_050900	surface antigen-like protein	30 kDa	0		0.41	0.28	0.00	0.00
79	E9BSS6	LDBPK_354060	protein kinase A catalytic subunit isoform 1 (fragment)	19 kDa	0		0.35	0.51	0.00	0.00
80	E9BF01	LDBPK_210160	calpain, putative (fragment)	83 kDa	0		0.33	0.23	0.00	0.00
81	E9B9K3	LDBPK_081030	uncharacterized protein	97 kDa	0		0.44	0.65	0.00	0.00
82	E9BRG7	LDBPK_343690	dynein heavy chain, putative	529 kDa	0		0.84	1.24	0.00	0.00
83	E9B8K0	LDBPK_051100	uncharacterized protein	28 kDa	0		0.17	0.26	0.00	0.00
84	E9BD52	LDBPK_170310	uncharacterized protein	82 kDa	0		0.16	0.24	0.00	0.00
85	E9BJC4	LDBPK_271940	D-lactate dehydrogenase-like protein	54 kDa	0		0.39	0.26	0.00	0.00
86	E9BK12	LDBPK_290360	uncharacterized protein	150 kDa	0		1.26	1.90	0.00	0.00
87	E9BUW5	LDBPK_366080	uncharacterized protein	25 kDa	0		0.10	0.16	0.00	0.00
88	E9BIP5	LDBPK_262340	uncharacterized protein	79 kDa	0		0.77	1.19	0.00	0.00
89	E9BR79	LDBPK_342810	uncharacterized protein	37 kDa	0		0.35	0.23	0.00	0.00
90	E9BE30	LDBPK_190340	uncharacterized protein	50 kDa	0		0.37	0.24	0.00	0.00
91	E9BU58	LDBPK_363490	uncharacterized protein	44 kDa	0		0.41	0.27	0.00	0.00
92	E9BNNS	LDBPK_321320	uncharacterized protein	54 kDa	0		2.21	1.41	0.00	0.00
93	E9BCH5	LDBPK_151360	uncharacterized protein	40 kDa	0		0.16	0.10	0.00	0.00
94	E9BL72	LDBPK_292750	uncharacterized protein	48 kDa	0		0.52	0.33	0.00	0.00
95	E9B7P8	LDBPK_030300	uncharacterized protein	116 kDa	0		0.19	0.30	0.00	0.00
96	E9BSW6	LDBPK_354460	uncharacterized protein	45 kDa	0		0.14	0.23	0.00	0.00
97	E9BT50	LDBPK_355310	uncharacterized protein	39 kDa	0.01		3.14	3.01	0.07	0.00
98	E9BA24	LDBPK_091390	paraflagellar rod component, putative	68 kDa	0.01		4.54	4.79	0.07	0.05
99	E9B894	LDBPK_050040	paraflagellar rod component par4, putative	69 kDa	0.01		1.57	1.92	0.05	0.00
100	E9BK27	LDBPK_283110	dynein heavy chain, putative	474 kDa	0.01		1.20	1.54	0.03	0.00
101	E9BR76	LDBPK_342780	uncharacterized protein	47 kDa	0.01	0.034	2.23	1.53	0.05	0.00
102	E9B9C6	LDBPK_080210	uncharacterized protein	72 kDa	0.01	0.0059	2.24	2.61	0.00	0.05
103	E9BMD2	LDBPK_310100	uncharacterized protein	56 kDa	0.02	0.00057	1.43	1.46	0.06	0.00
104	E9BUK9	LDBPK_365010	uncharacterized protein	34 kDa	0.02	0.00082	5.72	6.04	0.16	0.09
105	E9B7W5	LDBPK_030950	uncharacterized protein	36 kDa	0.02	0.0017	1.42	1.51	0.07	0.00
106	E9BKY6	LDBPK_291890	paraflagellar rod protein 1D, putative	69 kDa	0.02	0.0069	103.62	122.08	3.03	1.61
107	E9BIB2	LDBPK_261000	dynein heavy chain, putative	458 kDa	0.02	0.02	1.35	1.79	0.03	0.03
108	E9BME6	LDBPK_310240	uncharacterized protein	100 kDa	0.02	0.02	1.05	1.40	0.00	0.05
109	E9BM89	LDBPK_303460	uncharacterized protein	67 kDa	0.02	0.042	2.80	4.27	0.00	0.15
110	E9BPF2	LDBPK_324020	myosin XXI	119 kDa	0.02	0.044	3.70	5.69	0.00	0.18
111	E9BJH6	LDBPK_272460	dynein heavy chain (pseudogene), putative	490 kDa	0.03	0.00069	0.85	0.89	0.03	0.03

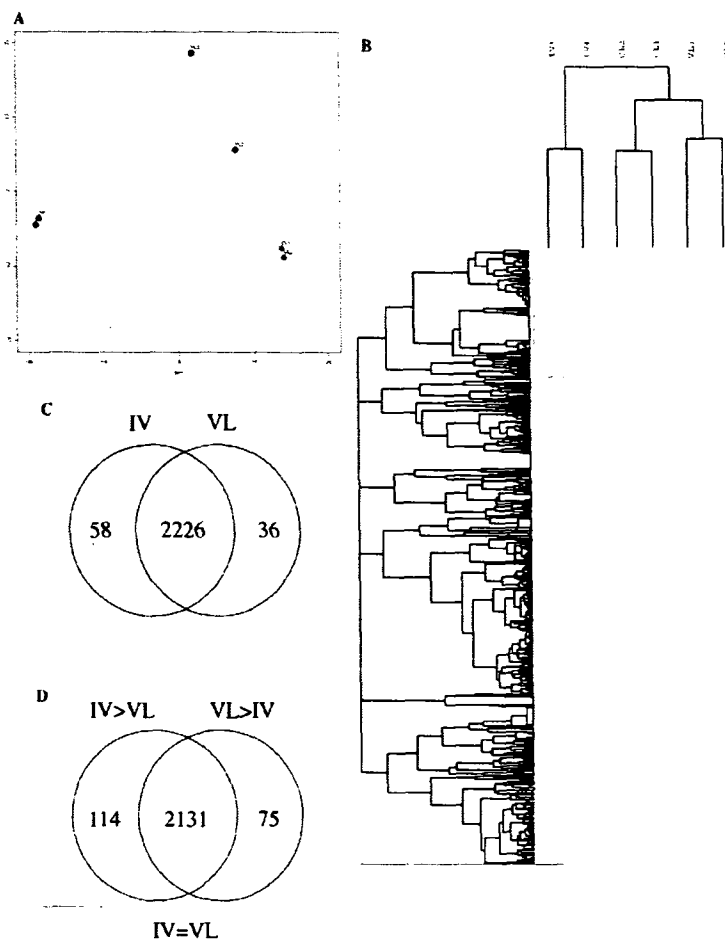
Table 2. continued

no.	accession number	gene ID	description	molecular weight	fold change IV to CL	t test (P value)	normalized empAI values			
							CL <sup>a</sup>	IV <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>
112	E9BIQ5	LDBPK_262440	uncharacterized protein	71 kDa	0.03	0.0029	0.75	0.70	0.00	0.05
113	E9BD03	LDBPK_161510	paraflagellar rod protein 2C	69 kDa	0.03	0.0032	70.95	79.08	2.94	1.98
114	E9BBH5	LDBPK_131010	uncharacterized protein	60 kDa	0.03	0.0056	1.82	1.60	0.11	0.00
115	E9BHQ6	LDBPK_251520	uncharacterized protein	88 kDa	0.03	0.0063	0.83	0.95	0.00	0.05
116	E9BE29	LDBPK_190330	uncharacterized protein	67 kDa	0.03	0.012	1.10	1.35	0.07	0.00
117	E9BV22	LDBPK_366660	uncharacterized protein	84 kDa	0.03	0.016	0.96	0.76	0.05	0.00
118	E9BL84	LDBPK_292870	uncharacterized protein	65 kDa	0.04	0.021	1.89	2.50	0.16	0.00
119	E9BBA4	LDBPK_130290	flagellar radial spoke protein, putative	67 kDa	0.04	0.03	3.44	4.86	0.17	0.15
120	E9BNV6	LDBPK_322040	uncharacterized protein	85 kDa	0.05	0.0021	1.54	1.68	0.08	0.08
121	E9BB91	LDBPK_130160	protein kinase A regulatory subunit, putative	56 kDa	0.05	0.013	18.64	23.15	1.42	0.81
122	E9BJD9	LDBPK_272090	uncharacterized protein	174 kDa	0.05	0.039	0.85	1.23	0.11	0.00
123	E9BML9	LDBPK_310950	sodium stibogluconate resistance protein, putative	171 kDa	0.06	0.00036	1.31	1.29	0.10	0.05
124	E9BGN9	LDBPK_240270	dynein intermediate-chain-like protein	75 kDa	0.06	0.03	2.29	3.20	0.22	0.08
125	E9BHS0	LDBPK_251660	uncharacterized protein	135 kDa	0.06	0.037	0.48	0.68	0.07	0.00
126	E9B8L1	LDBPK_051210	surface antigen-like protein	30 kDa	0.06	0.042	0.65	0.44	0.07	0.00
127	E9BKB7	LDBPK_280790	uncharacterized protein	44 kDa	0.07	0.014	0.39	0.47	0.06	0.00
128	E9BBZ5	LDBPK_141130	dynein heavy chain, putative	473 kDa	0.07	0.021	0.44	0.57	0.05	0.03
129	E9BNE1	LDBPK_320360	uncharacterized protein	51 kDa	0.08	0.00022	4.28	4.18	0.38	0.33
130	E9BHK5	LDBPK_251010	dynein heavy chain, putative	537 kDa	0.08	0.023	7.82	10.41	0.68	0.69
131	E9B9L0	LDBPK_081100	uncharacterized protein	139 kDa	0.09	0.00053	1.41	1.36	0.12	0.11
132	E9BSL4	LDBPK_353430	uncharacterized protein	62 kDa	0.09	0.01	0.26	0.25	0.00	0.05
133	E9BBL3	LDBPK_131390	dynein heavy chain, putative	529 kDa	0.09	0.024	8.03	10.72	1.00	0.72
134	E9BN40	LDBPK_312700	serine/threonine-protein phosphatase	41 kDa	0.09	0.04	0.40	0.29	0.06	0.00
135	E9BAY5	LDBPK_120100	ornithine decarboxylase, putative	34 kDa	0.1	<0.00010	0.47	0.46	0.06	0.06
136	E9BFD5	LDBPK_211490	adenylate kinase, putative	30 kDa	0.1	0.00025	27.31	26.56	2.84	2.67
137	E9BG43	LDBPK_230230	ATP-binding cassette protein s subfamily C, member 1, putative (fragment)	147 kDa	0.1	0.00034	0.45	0.46	0.05	0.04
138	E9B8J8	LDBPK_051080	uncharacterized protein	80 kDa	0.1	0.0024	1.64	1.63	0.11	0.25
139	E9BG17	LDBPK_231730	uncharacterized protein	30 kDa	0.1	0.0028	0.85	0.84	0.15	0.07
140	E9BCJ3	LDBPK_151540	cAMP specific phosphodiesterase, putative	104 kDa	0.1	0.0046	7.28	8.22	0.74	0.87
141	E9BAG8	LDBPK_101240	uncharacterized protein	57 kDa	0.1	0.0048	1.12	1.20	0.22	0.11
142	E9BTG6	LDBPK_361060	uncharacterized protein	145 kDa	0.1	0.012	1.61	1.94	0.27	0.20
143	E9BBK2	LDBPK_131280	endomembrane protein, putative	67 kDa	0.1	0.024	0.63	0.82	0.11	0.09
144	E9BC12	LDBPK_141300	uncharacterized protein	99 kDa	0.1	0.024	4.79	6.15	1.06	0.51
145	E9BDX3	LDBPK_181420	uncharacterized protein	27 kDa	0.1	0.024	0.33	0.29	0.08	0.00
146	E9BGU8	LDBPK_240860	dynein arm light chain, putative	27 kDa	0.1	0.024	0.46	0.60	0.08	0.08
147	E9BCZ5	LDBPK_161430	uncharacterized protein	66 kDa	0.1	0.028	0.17	0.21	0.04	0.00
148	E9BLY4	LDBPK_302410	uncharacterized protein	31 kDa	0.1	0.029	0.18	0.18	0.00	0.05
149	E9BR57	LDBPK_342590	uncharacterized protein	34 kDa	0.1	0.032	0.31	0.39	0.08	0.00
150	E9BUQ9	LDBPK_365520	uncharacterized protein	23 kDa	0.1	0.033	0.31	0.37	0.09	0.00
151	E9BF80	LDBPK_210940	uncharacterized protein	42 kDa	0.1	0.034	0.55	0.47	0.14	0.00
152	E9BF29	LDBPK_210440	uncharacterized protein	25 kDa	0.1	0.035	6.21	8.70	0.70	1.06
153	E9BPU0	LDBPK_330110	uncharacterized protein	39 kDa	0.1	0.036	0.24	0.30	0.07	0.00
154	E9BJ06	LDBPK_270720	uncharacterized protein	85 kDa	0.2	<0.00010	8.99	9.08	1.38	1.41
155	E9BEU4	LDBPK_201450	axoneme central apparatus protein, putative	55 kDa	0.2	0.00091	3.18	3.03	0.51	0.45
156	E9BTC0	LDBPK_360610	uncharacterized protein	36 kDa	0.2	0.0015	1.76	1.70	0.27	0.36
157	E9BKX1	LDBPK_291730	uncharacterized protein	77 kDa	0.2	0.004	0.67	0.68	0.10	0.17
158	E9BUM2	LDBPK_365140	uncharacterized protein	43 kDa	0.2	0.0058	3.70	3.29	0.53	0.68
159	E9BEL2	LDBPK_200650	uncharacterized protein	45 kDa	0.2	0.0075	2.61	2.83	0.81	0.53
160	E9BK64	LDBPK_280240	glycerol-3-phosphate dehydrogenase (FAD-dependent), mitochondrial	67 kDa	0.2	0.0076	0.94	0.93	0.27	0.15
161	E9BST2	LDBPK_354120	uncharacterized protein	32 kDa	0.2	0.0097	2.11	2.37	0.64	0.41
162	E9B9J2	LDBPK_080900	uncharacterized protein	63 kDa	0.2	0.011	3.01	3.53	0.79	0.69

Table 2. continued

no.	accession number	gene ID	description	molecular weight	fold change IV to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>	IV <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>
163	E9BCH0	LDBPK_151310	DNA topoisomerase 2	139 kDa	0.2	0.012	0.75	0.84	0.21	0.10
164	E9BHU4	LDBPK_251900	uncharacterized protein	78 kDa	0.2	0.016	4.99	6.02	1.23	0.73
165	E9BDD3	LDBPK_171120	uncharacterized protein	72 kDa	0.2	0.017	0.50	0.60	0.09	0.15
166	E9BNLS	LDBPK_321120	dynein, putative	68 kDa	0.2	0.018	1.85	1.47	0.29	0.22
167	E9BU33	LDBPK_363240	uncharacterized protein	89 kDa	0.2	0.02	2.73	3.41	0.57	0.72
168	E9BNE2	LDBPK_320370	uncharacterized protein	38 kDa	0.2	0.021	3.16	4.03	0.71	0.55
169	E9B9Q9	LDBPK_090230	uncharacterized protein	33 kDa	0.2	0.023	1.20	1.35	0.17	0.43
170	E9BJ35	LDBPK_271010	intraflagellar transport protein IFT88, putative	91 kDa	0.2	0.023	1.30	1.65	0.33	0.22
171	E9BNV7	LDBPK_322050	heat shock protein-like protein, putative	35 kDa	0.2	0.026	3.23	2.49	0.62	0.36
172	E9BA59	LDBPK_100110	uncharacterized protein	80 kDa	0.2	0.028	2.23	2.97	0.46	0.35
173	E9BRJ7	LDBPK_343990	dynein heavy chain, putative	478 kDa	0.2	0.028	0.85	1.07	0.28	0.18
174	E9BAI5	LDBPK_101430	uncharacterized protein	35 kDa	0.2	0.029	1.10	1.46	0.25	0.18
175	E9BS85	LDBPK_352150	vesicle-associated membrane protein, putative	23 kDa	0.2	0.029	1.13	0.91	0.32	0.15
176	E9BAQ9	LDBPK_110710	ATPase ASNA1 homologue	44 kDa	0.2	0.035	2.59	1.90	0.42	0.46
177	E9BKQ5	LDBPK_291090	uncharacterized protein	68 kDa	0.2	0.038	2.97	4.17	0.60	0.48
178	E9BDC2	LDBPK_171010	uncharacterized protein	55 kDa	0.2	0.044	0.33	0.41	0.00	0.12
179	E9BNC9	LDBPK_320240	dynein light chain, flagellar outer arm, putative	11 kDa	0.2	0.049	5.68	3.91	0.94	1.00

<sup>a</sup>Values from two biological replicates.



**Figure 4.** Human VL strain is distinct from the IV strain selected in mice. (A) Principal component analysis. (B) Hierarchical clustering (HCL). (C) Venn diagram of common proteins and strain-specific proteins in whole cell lysate. (D) Venn diagram of proteins upregulated in IV or in VL whole cell lysate.

Table 3. Proteins Elevated in the VL Isolate Compared with the CL Isolate

no.	accession number	gene ID	description	molecular weight	fold change VL to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>		VL <sup>a</sup>	
1	E9BGD0	LDBPK_231120	cytosolic leucyl aminopeptidase	60 kDa	2	0.016	1.10	1.24	2.50	2.23
2	E9BT18	LDBPK_354990	uncharacterized protein	23 kDa	2	0.04	0.74	0.73	1.64	1.33
3	E9BGB2	LDBPK_230930	mitochondrial RNA binding protein, putative	40 kDa	2.2	0.016	4.07	3.21	8.53	7.77
4	E9BJL9	LDBPK_281480	haloacid dehalogenase-like hydrolase, putative	32 kDa	2.2	0.021	0.36	0.29	0.68	0.78
5	E9BG25	LDBPK_230050	peroxidoxin	25 kDa	2.2	0.027	6.12	6.71	15.01	12.62
6	E9BKD6	LDBPK_280980	ribonucleoside-diphosphate reductase	91 kDa	2.3	0.0023	0.69	0.71	1.68	1.59
7	E9B8N8	LDBPK_060270	uncharacterized protein	34 kDa	2.3	0.035	0.42	0.69	1.24	1.27
8	E9B8D5	LDBPK_050450	uncharacterized protein	22 kDa	2.4	0.044	0.67	0.42	1.19	1.42
9	E9BES5	LDBPK_201260	uncharacterized protein	16 kDa	2.5	0.045	0.08	0.07	0.17	0.21
10	E9BRE0	LDBPK_343420	uncharacterized protein	18 kDa	2.5	0.045	0.08	0.06	0.15	0.20
11	E9BF84	LDBPK_210980	hypoxanthine-guanine phosphoribosyltransferase	24 kDa	2.6	0.0053	0.35	0.33	0.93	0.85
12	E9B9D5	LDBPK_080300	superoxide dismutase	26 kDa	2.6	0.015	0.34	0.27	0.84	0.75
13	E9BBQ9	LDBPK_140240	uncharacterized protein	15 kDa	2.6	0.024	0.34	0.40	0.86	1.03
14	E9BLJ6	LDBPK_301030	p22 protein, putative	23 kDa	2.8	0.04	0.20	0.10	0.41	0.46
15	E9B8D9	LDBPK_050490	uncharacterized protein	29 kDa	2.9	0.0038	0.10	0.08	0.26	0.25
16	E9BG42	LDBPK_230220	endoribonuclease L-PSP (Pb5), putative	17 kDa	2.9	0.02	2.22	1.81	6.29	5.29
17	E9BEW4	LDBPK_201660	soluble N-ethylmaleimide sensitive factor, putative	32 kDa	3	0.012	0.56	0.46	1.44	1.64
18	E9BSV1	LDBPK_354310	uncharacterized protein	33 kDa	3	0.045	0.04	0.03	0.13	0.10
19	E9BN07	LDBPK_312360	phosphatidylethanolamine-methyltransferase-like protein	23 kDa	3.1	0.029	0.06	0.05	0.18	0.14
20	E9BTB0	LDBPK_360510	uncharacterized protein	26 kDa	3.1	0.04	0.53	0.54	1.41	1.87
21	E9BEK8	LDBPK_200610	uncharacterized protein	26 kDa	3.2	0.026	0.09	0.18	0.42	0.47
22	E9BTT8	LDBPK_362290	uncharacterized protein	15 kDa	3.3	0.036	0.09	0.08	0.32	0.24
23	E9BJL0	LDBPK_281390	phenylalanine-4-hydroxylase, putative	52 kDa	3.6	0.02	0.51	0.23	1.33	1.37
24	E9BEY7	LDBPK_200110	phosphoglycerate kinase C, glycosomal	51 kDa	3.7	0.0044	17.85	13.86	61.22	57.03
25	E9BV81	LDBPK_367250	uncharacterized protein	30 kDa	3.7	0.022	0.13	0.17	0.61	0.50
26	E9BFJ4	LDBPK_212080	cytochrome c oxidase subunit VI, putative	19 kDa	4.4	0.0059	1.07	0.85	4.48	4.02
27	E9BKL7	LDBPK_290710	RNA-binding protein, putative	82 kDa	4.5	0.0028	0.07	0.06	0.29	0.27
28	E9BA47	LDBPK_091620	uncharacterized protein	18 kDa	5	0.024	0.17	0.13	0.66	0.86
29	E9BGB7	LDBPK_230980	actin interacting protein-like protein	57 kDa	7.7	0.035	0.10	0.04	0.61	0.44
30	E9BKQ9	LDBPK_291130	uncharacterized protein	203 kDa	INF <sup>b</sup>		0.00	0.00	0.06	0.07
31	E9B8E4	LDBPK_050540	uncharacterized protein	14 kDa	INF		0.00	0.00	0.20	0.26
32	E9BQL4	LDBPK_340600	uncharacterized protein	14 kDa	INF		0.00	0.00	0.20	0.25
33	E9BEM0	LDBPK_200730	uncharacterized protein	29 kDa	INF		0.00	0.00	0.15	0.11
34	E9BLM9	LDBPK_301360	U3 small nuclear ribonucleoprotein (SnRNP), putative	33 kDa	INF		0.00	0.00	0.20	0.13
35	E9BRG8	LDBPK_343700	uncharacterized protein	34 kDa	INF		0.00	0.00	0.20	0.13

<sup>a</sup>Values from two biological replicates. <sup>b</sup>A ratio of "INF" is returned when dividing by zero (protein not detected in CL).

purified exosomes from the CL and VL strains and performed LC-MS/MS analysis. Exosome purity was confirmed by TEM, silver staining, and Western blot (Figure S2 in the Supporting Information).

Overall, we identified 26 239 spectra at a 0.00% FDR, corresponding to 621 proteins with a 0% protein FDR (Tables S2 and S10 in the Supporting Information). Most of these were common to CL and to VL, but 11 proteins were detected only in VL exosomes (Table 5, Figure 3C). Two of these VL-specific proteins could play a role in signaling, a receptor-type adenylate cyclase-like protein, and an uncharacterized protein with a rho-like domain. Only six proteins were specific to CL exosomes (Table 6, Figure 3C). These include a glycosomal glyceraldehyde 3-phosphate dehydrogenase, a putative GTP-binding protein, and a putative 40S ribosomal protein S12, all three of which have

been previously detected in *Leishmania* exosomes,<sup>38</sup> and the uncharacterized protein E9B7J0, which has a porin domain. The detection of a transporter in CL exosomes is especially interesting in view of the elevation of transport processes in CL whole cell lysate.

Interestingly, only 2.9% of proteins identified in exosomes were strain-specific (0.9% specific to CL and 1.7% specific to VL). This is comparable to the 2.1% strain-specific proteins in the whole cell lysate (1.8% specific to CL and 0.3% specific to VL). This suggests that exosome composition is largely reflective of the physiological state of the cells from which they are derived.

#### Adaptability to Rodent and Human Environments

We expected that the VL and IV strains would be more similar to each other than to the CL strain because VL and IV both could

Table 4. Proteins Elevated in the CL Isolate Compared with the VL Isolate

no.	accession number	gene ID	description	molecular weight	fold change VL to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>	VL <sup>a</sup>	CL <sup>a</sup>	VL <sup>a</sup>
1	E9BC09	LDBPK_141270	uncharacterized protein	58 kDa	0		0.12	0.12	0.00	0.00
2	E9BHN4	LDBPK_251300	uncharacterized protein	54 kDa	0		0.43	0.43	0.00	0.00
3	E9BSL4	LDBPK_353430	uncharacterized protein	62 kDa	0		0.24	0.24	0.00	0.00
4	E9BES2	LDBPK_201230	calpain-like cysteine peptidase, putative	78 kDa	0		0.78	0.75	0.00	0.00
5	E9BD75	LDBPK_170540	uncharacterized protein	59 kDa	0		0.11	0.12	0.00	0.00
6	E9BK45	LDBPK_280050	uncharacterized protein	56 kDa	0		0.33	0.31	0.00	0.00
7	E9BF22	LDBPK_210370	uncharacterized protein	35 kDa	0		0.17	0.16	0.00	0.00
8	E9BPP8	LDBPK_333100	uncharacterized protein	69 kDa	0		0.34	0.36	0.00	0.00
9	E9BIQ1	LDBPK_262400	uncharacterized protein	558 kDa	0		0.20	0.21	0.00	0.00
10	E9BAI3	LDBPK_101410	uncharacterized protein	35 kDa	0		0.25	0.23	0.00	0.00
11	E9BCU2	LDBPK_160890	uncharacterized protein	75 kDa	0		0.26	0.29	0.00	0.00
12	E9BL89	LDBPK_292920	ribonuclease inhibitor-like protein	81 kDa	0		0.09	0.08	0.00	0.00
13	E9BSS8	LDBPK_354080	ATP-dependent RNA helicase, putative	79 kDa	0		0.09	0.08	0.00	0.00
14	E9BUP7	LDBPK_365390	uncharacterized protein	65 kDa	0		0.11	0.10	0.00	0.00
15	E9BIC8	LDBPK_261160	uncharacterized protein	71 kDa	0		0.32	0.37	0.00	0.00
16	E9BA72	LDBPK_100240	uncharacterized protein	54 kDa	0		0.57	0.68	0.00	0.00
17	E9B7Y6	LDBPK_040200	surface antigen-like protein	29 kDa	0		0.38	0.31	0.00	0.00
18	E9BE65	LDBPK_190700	kinesin, putative	248 kDa	0		0.11	0.14	0.00	0.00
19	E9BIY2	LDBPK_270480	ATP-binding cassette protein subfamily D, member 1, putative	77 kDa	0		0.15	0.18	0.00	0.00
20	E9BE23	LDBPK_190270	sarcoplasmic reticulum glycoprotein, putative	69 kDa	0		0.15	0.18	0.00	0.00
21	E9BSN3	LDBPK_353620	uncharacterized protein	102 kDa	0		0.34	0.43	0.00	0.00
22	E9BU92	LDBPK_363840	nudix hydrolase-like protein	24 kDa	0		0.27	0.33	0.00	0.00
23	E9BSZ6	LDBPK_354770	MCAK-like kinesin, putative	53 kDa	0		0.23	0.18	0.00	0.00
24	E9BQB1	LDBPK_331800	transcription activator	126 kDa	0		0.15	0.19	0.00	0.00
25	E9BRI8	LDBPK_343900	uncharacterized protein	79 kDa	0		0.11	0.08	0.00	0.00
26	E9BH72	LDBPK_242100	tubulin folding cofactor D, putative	155 kDa	0		0.37	0.27	0.00	0.00
27	E9BIW5	LDBPK_270310	methylmalonyl-coenzyme a mutase, putative	79 kDa	0		0.09	0.13	0.00	0.00
28	E9BK71	LDBPK_280310	ribonuclease II-like protein, putative	105 kDa	0		0.11	0.15	0.00	0.00
29	E9BMA4	LDBPK_303610	cytochrome p450-like protein	58 kDa	0		0.21	0.15	0.00	0.00
30	E9B7N3	LDBPK_030150	ATP-binding cassette protein subfamily F, member 1, putative	82 kDa	0		0.26	0.18	0.00	0.00
31	E9BAA0	LDBPK_100550	uncharacterized protein	713 kDa	0		0.12	0.08	0.00	0.00
32	E9B810	LDBPK_050900	surface antigen-like protein	30 kDa	0		0.38	0.26	0.00	0.00
33	E9BNU3	LDBPK_321900	protein kinase, putative	71 kDa	0		0.20	0.29	0.00	0.00
34	E9BSS6	LDBPK_354060	protein kinase A catalytic subunit isoform 1 (fragment)	19 kDa	0		0.33	0.47	0.00	0.00
35	E9BP89	LDBPK_323390	uncharacterized protein	111 kDa	0		0.10	0.07	0.00	0.00
36	E9BRU5	LDBPK_350680	uncharacterized protein	457 kDa	0		0.05	0.07	0.00	0.00
37	E9BAS3	LDBPK_110850	uncharacterized protein	52 kDa	0		0.13	0.19	0.00	0.00
38	E9BBC7	LDBPK_130530	uncharacterized protein	145 kDa	0		0.19	0.29	0.00	0.00
39	E9BCC5	LDBPK_150850	uncharacterized protein	61 kDa	0		0.11	0.18	0.00	0.00
40	E9BJ68	LDBPK_271360	uncharacterized protein	93 kDa	0.03	0.0062	0.71	0.62	0.05	0.00
41	E9BQK4	LDBPK_340500	calcium channel protein, putative	291 kDa	0.03	0.0094	0.30	0.36	0.02	0.00
42	E9BNN5	LDBPK_321320	uncharacterized protein	54 kDa	0.03	0.049	2.06	1.32	0.10	0.00
43	E9BIF2	LDBPK_261400	uncharacterized protein	257 kDa	0.05	0.0043	0.20	0.19	0.02	0.00
44	E9BIB0	LDBPK_260980	uncharacterized protein	46 kDa	0.05	0.032	0.55	0.39	0.05	0.00
45	E9BLE8	LDBPK_300540	uncharacterized protein	99 kDa	0.06	0.014	0.10	0.09	0.01	0.00
46	E9BF80	LDBPK_210940	uncharacterized protein	42 kDa	0.07	0.013	0.51	0.43	0.07	0.00
47	E9BDC9	LDBPK_171080	uncharacterized protein	83 kDa	0.07	0.021	0.52	0.41	0.07	0.00
48	E9BML9	LDBPK_310950	sodium stibogluconate resistance protein, putative	171 kDa	0.08	0.00057	1.22	1.21	0.13	0.08
49	E9BHF8	LDBPK_250540	ATP-binding cassette protein subfamily B, member 1, putative	72 kDa	0.08	0.0066	0.49	0.49	0.07	0.00
50	E9BG87	LDBPK_230670	uncharacterized protein	348 kDa	0.08	0.011	0.09	0.09	0.02	0.00
51	E9BIP9	LDBPK_262380	Sec1 family transport protein, putative	73 kDa	0.08	0.013	0.29	0.25	0.04	0.00

Table 4. continued

no.	accession number	gene ID	description	molecular weight	fold change VL to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>	VL <sup>a</sup>	CL <sup>a</sup>	VL <sup>a</sup>
52	E9BJ22	LDBPK_270880	uncharacterized protein	23 kDa	0.09	0.036	1.06	1.43	0.23	0.00
53	E9BGH1	LDBPK_231570	dynein heavy chain, putative	544 kDa	0.1	0.0015	0.99	1.02	0.16	0.10
54	E9BE69	LDBPK_190740	uncharacterized protein	48 kDa	0.1	0.014	0.26	0.27	0.06	0.00
55	E9BP85	LDBPK_323350	uncharacterized protein (fragment)	131 kDa	0.1	0.016	0.42	0.46	0.09	0.00
56	E9BHE8	LDBPK_250440	uncharacterized protein	110 kDa	0.1	0.018	0.56	0.68	0.05	0.13
57	E9BCH4	LDBPK_151350	actin-like protein, putative	47 kDa	0.1	0.019	0.39	0.35	0.00	0.08
58	E9BMJ4	LDBPK_310710	uncharacterized protein	32 kDa	0.1	0.022	0.43	0.39	0.10	0.00
59	E9BH95	LDBPK_242340	fatty-acid desaturase, putative	49 kDa	0.1	0.024	0.42	0.48	0.11	0.00
60	E9BSY1	LDBPK_354620	uncharacterized protein	161 kDa	0.1	0.026	0.14	0.12	0.03	0.00
61	E9B9C0	LDBPK_071330	uncharacterized protein	276 kDa	0.1	0.027	0.27	0.29	0.08	0.00
62	E9BF68	LDBPK_210830	uncharacterized protein	79 kDa	0.1	0.028	0.23	0.24	0.07	0.00
63	E9BKN9	LDBPK_290920	guanine deaminase, putative	49 kDa	0.1	0.028	0.77	0.57	0.10	0.08
64	E9BRB9	LDBPK_343210	cleavage and polyadenylation specificity factor, putative	84 kDa	0.1	0.029	0.17	0.19	0.05	0.00
65	E9BCZ5	LDBPK_161430	uncharacterized protein	66 kDa	0.1	0.03	0.16	0.20	0.04	0.00
66	E9BQU8	LDBPK_341470	uncharacterized protein	277 kDa	0.1	0.031	0.30	0.41	0.04	0.05
67	E9BCJ0	LDBPK_151510	phosphomevalonate kinase-like protein	49 kDa	0.1	0.035	0.18	0.24	0.05	0.00
68	E9BSR9	LDBPK_353990	uncharacterized protein	73 kDa	0.1	0.036	0.32	0.24	0.00	0.06
69	E9BCX8	LDBPK_161240	uncharacterized protein	158 kDa	0.1	0.043	0.10	0.13	0.03	0.00
70	E9BSS0	LDBPK_354000	PRP8 protein homologue, putative	278 kDa	0.1	0.045	0.17	0.13	0.04	0.00
71	E9BN99	LDBPK_313290	uncharacterized protein	162 kDa	0.1	0.046	0.77	0.58	0.00	0.18
72	E9BN15	LDBPK_312450	uncharacterized protein	56 kDa	0.1	0.017	0.45	0.51	0.10	0.00
73	E9BJH6	LDBPK_272460	dynein heavy chain (pseudogene), putative	490 kDa	0.2	0.0016	0.79	0.83	0.19	0.17
74	E9BNP3	LDBPK_321400	cleavage and polyadenylation specificity factor-like protein	167 kDa	0.2	0.0042	0.80	0.73	0.17	0.16
75	E9B7W7	LDBPK_040010	calcium-translocating P-type ATPase	111 kDa	0.2	0.0056	2.11	1.97	0.46	0.25
76	E9BB14	LDBPK_120340	uncharacterized protein	44 kDa	0.2	0.01	2.05	2.04	0.63	0.30
77	E9BHM0	LDBPK_251160	aldehyde dehydrogenase, mitochondrial	54 kDa	0.2	0.011	5.43	4.60	1.01	1.16
78	E9BD16	LDBPK_161650	uncharacterized protein	139 kDa	0.2	0.013	0.80	0.95	0.20	0.20
79	E9BIQ5	LDBPK_262440	uncharacterized protein	71 kDa	0.2	0.013	0.70	0.65	0.07	0.19
80	E9BCA5	LDBPK_150640	uncharacterized protein	59 kDa	0.2	0.014	0.62	0.61	0.05	0.18
81	E9BNL9	LDBPK_321160	exportin 1, putative	118 kDa	0.2	0.014	0.41	0.48	0.08	0.12
82	E9BTG6	LDBPK_361060	uncharacterized protein	145 kDa	0.2	0.014	1.50	1.81	0.32	0.19
83	E9BKA3	LDBPK_280650	dynein heavy chain, putative	478 kDa	0.2	0.017	0.83	1.04	0.15	0.17
84	E9BRJ7	LDBPK_343990	dynein heavy chain, putative	478 kDa	0.2	0.023	0.79	1.00	0.13	0.20
85	E9B9C6	LDBPK_080210	uncharacterized protein	72 kDa	0.2	0.026	2.09	2.44	0.68	0.19
86	E9BTG1	LDBPK_361010	dynein heavy chain, putative	473 kDa	0.2	0.027	0.61	0.47	0.11	0.11
87	E9BDD3	LDBPK_171120	uncharacterized protein	72 kDa	0.2	0.028	0.47	0.56	0.17	0.07
88	E9BE47	LDBPK_190520	uncharacterized protein	58 kDa	0.2	0.028	0.94	1.11	0.31	0.09
89	E9B9M1	LDBPK_080010	adaptor complex protein (AP) 3 delta subunit 1, putative	135 kDa	0.2	0.029	0.67	0.51	0.13	0.10
90	E9BTZ0	LDBPK_362810	uncharacterized protein	112 kDa	0.2	0.036	0.39	0.39	0.13	0.00
91	E9BHL7	LDBPK_251130	MGT2 magnesium transporter	46 kDa	0.2	0.04	0.34	0.25	0.05	0.09
92	E9BK30	LDBPK_283140	glutamate dehydrogenase	49 kDa	0.2	0.041	25.52	32.53	9.90	3.95
93	E9BFD7	LDBPK_211510	uncharacterized protein	91 kDa	0.2	0.042	0.30	0.31	0.11	0.00
94	E9BE17	LDBPK_190210	polyprenyl synthase, putative	82 kDa	0.2	0.047	0.21	0.17	0.06	0.00
95	E9BB55	LDBPK_140400	uncharacterized protein	34 kDa	0.2	0.048	0.53	0.77	0.15	0.09
96	E9B8L1	LDBPK_051210	surface antigen-like protein	30 kDa	0.2	0.049	0.60	0.41	0.07	0.10
97	E9BTE0	LDBPK_360810	uncharacterized protein	137 kDa	0.2	0.049	0.13	0.12	0.04	0.00
98	E9BV64	LDBPK_367080	beta-adaptin, putative	82 kDa	0.3	0.0013	0.62	0.60	0.19	0.16
99	E9BC27	LDBPK_141450	myo-inositol-1-phosphate synthase	46 kDa	0.3	0.0023	4.42	4.39	1.65	1.37
100	E9BEL2	LDBPK_200650	uncharacterized protein	45 kDa	0.3	0.004	2.43	2.64	0.86	0.83
101	E9BJ95	LDBPK_271650	dynein heavy chain, putative	499 kDa	0.3	0.0078	0.63	0.72	0.19	0.19
102	E9BM53	LDBPK_303100	kinesin, putative	134 kDa	0.3	0.0091	0.68	0.63	0.13	0.21
103	E9BKV8	LDBPK_291600	uncharacterized protein	74 kDa	0.3	0.012	1.08	1.18	0.22	0.37
104	E9BM72	LDBPK_303290	glutamyl-tRNA synthetase, putative	68 kDa	0.3	0.018	2.58	2.20	0.90	0.69

Table 4. continued

no.	accession number	gene ID	description	molecular weight	fold change VL to CL	t test (P value)	normalized emPAI values			
							CL <sup>a</sup>	VL <sup>a</sup>	IV <sup>a</sup>	VI <sup>a</sup>
105	E9BGK5	LDBPK_231910	vacuolar proton translocating ATPase subunit A, putative	88 kDa	0.3	0.019	2.64	2.38	1.05	0.67
106	E9B9A4	LDBPK_071210	cation-transporting ATPase, putative	140 kDa	0.3	0.026	0.81	0.71	0.16	0.30
107	E9BD21	LDBPK_161700	eukaryotic translation initiation factor 4 gamma, putative	71 kDa	0.3	0.026	0.22	0.18	0.07	0.07
108	E9BRL2	LDBPK_344140	coatomer alpha subunit, putative	133 kDa	0.3	0.028	3.27	3.05	0.74	1.42
109	E9BIH2	LDBPK_261600	phosphatidate cytidyltransferase	49 kDa	0.3	0.029	0.71	0.66	0.11	0.27
110	E9BSY9	LDBPK_354700	uncharacterized protein	56 kDa	0.3	0.029	2.72	2.28	0.96	0.54
111	E9BR85	LDBPK_342870	uncharacterized protein	64 kDa	0.3	0.03	1.56	1.91	0.36	0.63
112	E9BI42	LDBPK_260290	aminopeptidase-like protein	98 kDa	0.3	0.032	1.91	2.41	0.78	0.55
113	E9B923	LDBPK_070270	uncharacterized protein	236 kDa	0.3	0.037	0.14	0.14	0.02	0.06
114	E9BBZ5	LDBPK_141130	dynein heavy chain, putative	473 kDa	0.3	0.037	0.41	0.53	0.11	0.15
115	E9BAQ9	LDBPK_110710	ATPase ASNA1 homologue	44 kDa	0.3	0.043	2.42	1.77	0.61	0.52
116	E9BL84	LDBPK_292870	uncharacterized protein	65 kDa	0.3	0.043	1.76	2.34	0.71	0.45
117	E9BNT7	LDBPK_321840	uncharacterized protein	54 kDa	0.3	0.044	0.85	0.85	0.12	0.38
118	E9BNX2	LDBPK_322200	uncharacterized protein	36 kDa	0.3	0.046	0.71	0.63	0.30	0.12
119	E9BDJ6	LDBPK_180150	serine/threonine protein phosphatase type 5, putative	53 kDa	0.4	0.00097	1.12	1.16	0.43	0.43
120	E9BNC3	LDBPK_320180	U2 small nuclear ribonucleoprotein 40K, putative	35 kDa	0.4	0.0017	0.35	0.35	0.14	0.13
121	E9B9L0	LDBPK_081100	uncharacterized protein	139 kDa	0.4	0.0024	1.32	1.26	0.45	0.51
122	E9BIE0	LDBPK_261280	uncharacterized protein	126 kDa	0.4	0.0026	0.56	0.57	0.19	0.22
123	E9BSL3	LDBPK_353420	uncharacterized protein	117 kDa	0.4	0.0053	2.13	2.22	0.82	0.98
124	E9BK94	LDBPK_280560	RAD50 DNA repair-like protein	154 kDa	0.4	0.0074	0.51	0.46	0.20	0.18
125	E9BKP9	LDBPK_291030	uncharacterized protein	87 kDa	0.4	0.0094	1.55	1.59	0.71	0.53
126	E9BBX9	LDBPK_140970	glutathione synthetase, putative	67 kDa	0.4	0.011	1.04	0.96	0.47	0.39
127	E9BHS9	LDBPK_241970	uncharacterized protein	88 kDa	0.4	0.011	0.27	0.30	0.12	0.10
128	E9BT11	LDBPK_354920	uncharacterized protein	39 kDa	0.4	0.02	1.88	1.66	0.65	0.84
129	E9B7I0	LDBPK_020330	casein kinase II, alpha chain, putative	44 kDa	0.4	0.024	0.61	0.59	0.31	0.20
130	E9BBR3	LDBPK_140280	uncharacterized protein	109 kDa	0.4	0.025	0.77	0.92	0.36	0.39
131	E9BPS3	LDBPK_333360	beta prime cop protein, putative	98 kDa	0.4	0.025	3.62	4.39	1.47	1.64
132	E9BM84	LDBPK_303410	calmodulin-related protein, putative	81 kDa	0.4	0.029	2.34	2.53	1.27	0.83
133	E9BJ94	LDBPK_271630	uncharacterized protein	38 kDa	0.4	0.032	8.89	7.12	3.19	2.46
134	E9BPK4	LDBPK_332660	uncharacterized protein	145 kDa	0.4	0.033	1.20	0.94	0.36	0.39
135	E9BUH0	LDBPK_364620	ATP-dependent RNA helicase-like protein	107 kDa	0.4	0.033	0.28	0.35	0.11	0.13
136	E9BH25	LDBPK_241630	uncharacterized protein	67 kDa	0.4	0.034	9.61	9.71	4.99	2.80
137	E9BUF2	LDBPK_364440	uncharacterized protein	123 kDa	0.4	0.035	9.09	11.14	4.78	3.95
138	E9BR57	LDBPK_342590	uncharacterized protein	34 kDa	0.4	0.037	0.28	0.36	0.13	0.13
139	E9BUY6	LDBPK_366290	uncharacterized protein	260 kDa	0.4	0.038	0.29	0.34	0.16	0.11
140	E9BQH5	LDBPK_340210	uncharacterized protein	56 kDa	0.4	0.04	1.30	1.05	0.33	0.52
141	E9BKV5	LDBPK_291570	uncharacterized protein	75 kDa	0.4	0.046	0.89	0.69	0.34	0.35
142	E9BLD2	LDBPK_300380	phosphatase 2C, putative	42 kDa	0.4	0.047	2.60	2.01	0.97	0.70
143	E9BF15	LDBPK_210300	cluster of E9BF15 hexokinase, putative	52 kDa	0.5	0.0062	76.05	77.59	32.69	39.00
144	E9BME8	LDBPK_310260	ATP-dependent RNA helicase, putative	103 kDa	0.5	0.0095	1.00	1.00	0.51	0.40
145	E9BAI4	LDBPK_101420	cluster of E9BAI4 uncharacterized protein	35 kDa	0.5	0.02	1.04	1.09	0.57	0.41
146	E9BK79	LDBPK_280410	phosphoadenosine phosphosulfate reductase-like protein	25 kDa	0.5	0.045	0.21	0.21	0.08	0.13
147	E9BQS1	LDBPK_341200	uncharacterized protein	30 kDa	0.5	0.046	0.18	0.17	0.07	0.11
148	E9BPB0	LDBPK_323600	uncharacterized protein	67 kDa	0.5	0.047	0.24	0.25	0.09	0.15

<sup>a</sup>Values from two biological replicates.

survive better in visceral organs than the CL isolate. To investigate this, we performed a principal component analysis (PCA) that revealed that the three strains were distinct from each other and the VL and IV strains did not cluster together as expected (Figure 4). This reveals that at the detectable

proteome level the IV and VL strains are as distinct from each other as they are from the CL strain. This suggests that the acquisition of visceral virulence is under different selective pressures in experimental mice and in humans. Indeed, 114 proteins were elevated five times or higher in IV compared with



Table 5. Proteins Specific to VL Exosomes

no.	accession number	gene ID	description	molecular weight	normalized emPAI values			
					CL <sup>a</sup>		VL <sup>a</sup>	
1	E9BQK2	LDBPK_340480	uncharacterized protein, unknown function, partial	77 kDa	0.00	0.00	0.27	0.11
2	E9BAW2	LDBPK_111260	ATP-binding cassette protein subfamily A, member 5, putative	200 kDa	0.00	0.00	0.07	0.21
3	E9BU42	LDBPK_363330	receptor-type adenylate cyclase a-like protein	151 kDa	0.00	0.00	0.14	0.11
4	E9BTV8	LDBPK_362490	tyrosine aminotransferase	50 kDa	0.00	0.00	0.43	0.17
5	E9BAA7	LDBPK_100620	zinc-binding dehydrogenase-like protein	51 kDa	0.00	0.00	0.13	0.52
6	E9BGC0	LDBPK_231010	uncharacterized protein	45 kDa	0.00	0.00	0.11	0.07
7	E9BP91	LDBPK_323410	uncharacterized protein, conserved	24 kDa	0.00	0.00	0.22	0.45
8	E9B988	LDBPK_070970	uncharacterized protein, conserved, partial	75 kDa	0.00	0.00	0.18	0.23
9	E9BPI9	LDBPK_332510	Golgi reassembly stacking protein (GRASP homologue), putative	58 kDa	0.00	0.00	0.24	0.14
10	E9BUY9	LDBPK_366320	uncharacterized protein, conserved	85 kDa	0.00	0.00	0.06	0.04
11	E9BJ35	LDBPK_271010	intraflagellar transport protein IFT88, putative	91 kDa	0.00	0.00	0.06	0.03

<sup>a</sup>Values from two biological replicates.

Table 6. Proteins Specific to CL Exosomes

no.	accession number	gene ID	description	molecular weight	normalized emPAI values			
					CL <sup>a</sup>		VL <sup>a</sup>	
1	E9B7J0	LDBPK_020430	uncharacterized protein	32 kDa	0.31	0.23	0.00	0.00
2	E9BM42	LDBPK_303000	glyceraldehyde 3-phosphate dehydrogenase, glycosomal	39 kDa	0.08	0.36	0.00	0.00
3	E9BL28	LDBPK_292310	GTP-binding protein, putative	78 kDa	0.08	0.06	0.00	0.00
4	E9BPJ5	LDBPK_332570	uncharacterized protein	56 kDa	0.23	0.03	0.00	0.00
5	E9BI46	LDBPK_260330	uncharacterized protein	38 kDa	0.08	0.09	0.00	0.00
6	E9BBC0	LDBPK_130460	40S ribosomal protein S12, putative	16 kDa	0.20	0.23	0.00	0.00

<sup>a</sup>Values from two biological replicates.

VL, and 75 were elevated in VL (Figure 4, Table S3 in the Supporting Information).

Differences between IV and VL notwithstanding, comparing these two visceral strains to the CL strain can help identify factors that are essential for the establishment of visceral infection and survival in visceral organs in both human and murine environments. We compared protein abundance between CL and visceral strains (IV and VL combined) using a two-fold or greater change in emPAI values with  $p \leq 0.05$  as a cutoff. Overall, 65 proteins were elevated in visceral strains, including 15 not detected in CL (Table 7, Figure 5A,B). 212 proteins were

proteins. In contrast, catabolic processes (GO:0009056) were over-represented in CL-elevated proteins.

#### Validation of Proteomic Data

As previously identified, several antioxidant protective proteins were elevated in the VL and IV visceral strains relative to the CL strain. In particular, both the mitochondrial peroxidoxin and a superoxide dismutase were elevated in VL (Table 3). Likewise, a different superoxide dismutase was elevated in IV compared with CL. To validate these results, we compared the resistance of the CL, IV, and VL strains to treatment with menadione, an agent that results in the production of superoxide in *Leishmania*.<sup>41</sup> The VL strain survived and proliferated better than CL strains at all menadione concentrations tested ( $p < 0.05$ ). The IV strain also survived better than CL at menadione concentrations 0.25 to 1  $\mu\text{M}$  ( $p < 0.05$ ) (Figure 6A), thereby supporting the mass spectrometry data.

We also compared the expression of a select number of proteins by Western blot. In accordance with the mass spectrometry data, Western blot analysis showed comparable levels of tubulin, fructose bisphosphate aldolase, and the uncharacterized protein LDBPK\_367070 across all three strains (Figure 6B). In comparison, meta 1 was detected at much higher levels in IV promastigotes compared with CL and VL (Figure 6B). This is consistent with the meta 1 emPAI values which were 8.9 fold higher in IV compared with CL, while the difference was non-significant for CL compared with VL.

#### DISCUSSION

Although the infecting species of *Leishmania* is a major determinant of disease phenotype,<sup>2</sup> these parasites seem to be more plastic than previously appreciated with regards to their

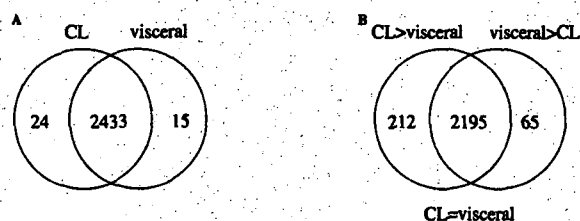


Figure 5. Proteomic comparison of CL and visceral strains (IV and VL combined). (A) Venn diagram of common proteins and strain-specific proteins. (B) Venn diagram of proteins upregulated in CL or in visceral strains.

elevated in CL, including 24 detected exclusively in CL (Table 8, Figure 5A,B). Proteins elevated in the visceral strains include the mitochondrial superoxide dismutase, ribosomal proteins, and proteins involved in energy metabolism (subunits of cytochrome c oxidase and ATP synthase). In addition, biosynthetic processes (including GO:0009058 and GO:0044249) and regulation of gene expression were over-represented in visceral-elevated

Table 7. Proteins Elevated in Visceral Isolates (IV and VL) Compared with the CL Isolate

no.	accession number	gene ID	description	molecular weight	fold change CL to visceral	t test (P value, CL to visceral (IV+VL))	normalized emPAL values					
							CL <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>	IV <sup>a</sup>	VL <sup>a</sup>	VL <sup>a</sup>
1	E9BTN0	LDBPK_361710	poly zinc finger protein 2, putative	15 kDa	2	0.027	0.77	0.62	1.43	1.57	1.02	1.62
2	E9B9D8	LDBPK_080330	mitochondrial associated ribonuclease, putative	25 kDa	2.1	0.047	0.86	0.56	1.66	1.83	1.08	1.32
3	E9BTF9	LDBPK_360990	40S ribosomal protein S18, putative	19 kDa	2.1	0.033	5.28	3.73	6.96	11.15	10.60	8.69
4	E9BBQ0	LDBPK_140150	60S ribosomal protein, putative	19 kDa	2.1	0.029	0.37	0.21	0.52	0.57	0.77	0.59
5	E9BI11	LDBPK_261690	cytochrome c oxidase subunit V, putative	22 kDa	2.1	0.023	2.21	2.42	5.63	4.49	3.61	5.31
6	E9BG73	LDBPK_230530	uncharacterized protein	37 kDa	2.1	0.021	0.19	0.10	0.29	0.36	0.33	0.25
7	E9BAK1	LDBPK_110130	NGG1 interacting factor 3-like protein	30 kDa	2.2	0.046	0.08	0.08	0.23	0.14	0.15	0.23
8	E9B822	LDBPK_040570	spermidine synthase, putative	33 kDa	2.2	0.034	0.97	0.86	2.04	2.56	1.64	1.62
9	E9BTH9	LDBPK_361200	short chain 3-hydroxyacyl-CoA dehydrogenase, putative	33 kDa	2.2	0.034	0.55	0.57	1.40	1.34	1.37	0.81
10	E9BTT7	LDBPK_362280	COP-coated vesicle membrane protein erv25, putative	30 kDa	2.2	0.013	0.68	0.62	1.42	1.51	1.07	1.61
11	E9BKD6	LDBPK_280980	ribonucleoside-diphosphate reductase	91 kDa	2.2	0.0012	0.72	0.73	1.44	1.47	1.73	1.64
12	E9BMA9	LDBPK_303660	ATP synthase, epsilon chain, putative	20 kDa	2.3	0.043	0.64	0.94	2.01	2.19	1.79	1.18
13	E9BFS9	LDBPK_220600	NADH-cytochrome b5 reductase, putative	32 kDa	2.3	0.041	0.52	0.51	1.57	1.24	0.93	0.96
14	E9BET2	LDBPK_201330	uncharacterized protein	31 kDa	2.3	0.012	0.89	0.48	1.68	1.90	1.39	1.49
15	E9BH76	LDBPK_242140	60S ribosomal protein L26, putative (fragment)	13 kDa	2.3	0.0083	2.26	1.01	4.15	3.45	4.03	3.68
16	E9BBF1	LDBPK_130770	uncharacterized protein	76 kDa	2.3	0.003	0.92	1.58	2.79	3.05	2.61	2.92
17	E9BNH0	LDBPK_320660	uncharacterized protein	21 kDa	2.4	0.038	0.96	0.94	2.11	1.67	2.18	3.02
18	E9BBQ9	LDBPK_140240	uncharacterized protein	15 kDa	2.4	0.0066	0.35	0.41	0.74	0.95	0.88	1.06
19	E9BEW4	LDBPK_201660	soluble N-ethylmaleimide sensitive factor, putative	32 kDa	2.5	0.045	0.57	0.48	1.07	0.93	1.48	1.69
20	E9B8D5	LDBPK_050450	uncharacterized protein	22 kDa	2.6	0.03	0.69	0.44	1.92	1.17	1.23	1.46
21	E9B9Y3	LDBPK_090970	calmodulin, putative	17 kDa	2.7	0.043	3.06	2.33	6.25	6.99	5.78	10.43
22	E9B994	LDBPK_071030 (E9B994)	cluster of E9B994_LEIDB Serine/threonine kinase, putative	34 kDa	2.7	0.023	0.54	0.56	1.82	1.47	1.01	1.62
23	E9BG42	LDBPK_230220	endoribonuclease L-PSP (Pb5), putative	17 kDa	2.7	0.0089	2.29	1.86	6.15	4.28	6.47	5.44
24	E9BF84	LDBPK_210980	hypoxanthine-guanine phosphoribosyltransferase	24 kDa	2.8	0.035	0.36	0.34	0.70	1.32	0.95	0.87
25	E9BLJ6	LDBPK_301030	p22 protein, putative	23 kDa	2.8	0.02	0.21	0.10	0.54	0.33	0.42	0.47
26	E9BBQ3	LDBPK_140180	cluster of E9BBQ3_LEIDB carboxypeptidase, putative	57 kDa	2.8	0.012	2.66	1.39	5.40	6.62	6.58	4.47
27	E9B9D5	LDBPK_080300	superoxide dismutase	26 kDa	2.8	0.0068	0.35	0.27	1.06	0.76	0.87	0.77
28	E9BRH9	LDBPK_343810	nucleolar protein family a member-like protein	17 kDa	3	0.033	0.85	0.37	2.28	1.58	1.27	2.06
29	E9B8I7	LDBPK_050970	3-mercaptopyruvate sulfurtransferase	40 kDa	3	0.032	0.00	0.13	0.20	0.20	0.18	0.19
30	E9BEK8	LDBPK_200610	uncharacterized protein	26 kDa	3	0.031	0.10	0.19	0.27	0.51	0.43	0.49
31	E9BT92	LDBPK_360330	uncharacterized protein	25 kDa	3.1	0.015	0.27	0.31	0.88	0.82	0.68	1.14
32	E9BN07	LDBPK_312360	phosphatidylethanolaminemethyltransferase-like protein	23 kDa	3.2	0.0081	0.06	0.05	0.21	0.14	0.19	0.15
33	E9B8D9	LDBPK_050490	uncharacterized protein	29 kDa	3.2	0.0016	0.10	0.08	0.30	0.33	0.27	0.26

Table 7. continued

no.	accession number	gene ID	description	molecular weight	fold change CL to visceral	t test (P value, CL to visceral (IV+VL))	normalized emPAI values					
							CL <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>	IV <sup>a</sup>	VL <sup>a</sup>	VL <sup>a</sup>
34	E9BTE0	LDBPK_360510	uncharacterized protein	26 kDa	3.2	0.0016	0.55	0.56	1.88	1.81	1.46	1.92
35	E9BF85	LDBPK_210990	xanthine phosphoribosyltransferase	27 kDa	3.4	0.03	0.00	0.20	0.36	0.37	0.37	0.26
36	E9BSV1	LDBPK_354310	uncharacterized protein	33 kDa	3.7	0.039	0.04	0.03	0.21	0.13	0.13	0.10
37	E9BP63	LDBPK_323130	tubulin binding cofactor A-like protein	14 kDa	3.7	0.027	0.23	0.08	0.59	0.65	0.35	0.67
38	E9BEF3	LDBPK_191630	uncharacterized protein	19 kDa	3.8	0.015	0.00	0.21	0.40	0.44	0.35	0.45
39	E9BV81	LDBPK_367250	uncharacterized protein	30 kDa	3.9	0.024	0.13	0.18	0.82	0.43	0.63	0.51
40	E9BFJ4	LDBPK_212080	cytochrome c oxidase subunit VI, putative	19 kDa	3.9	0.014	1.10	0.88	2.52	4.00	4.61	4.13
41	E9BGK0	LDBPK_231860	uncharacterized protein	58 kDa	3.9	0.0076	0.12	0.12	0.53	0.44	0.35	0.55
42	E9BFH1	LDBPK_211850	uncharacterized protein	46 kDa	4	0.014	0.03	0.06	0.11	0.19	0.19	0.20
43	E9BKL7	LDBPK_290710	RNA-binding protein, putative	82 kDa	4.4	0.0012	0.07	0.06	0.32	0.24	0.30	0.28
44	E9BNA6	LDBPK_320010	uncharacterized protein	16 kDa	5.5	0.046	0.09	0.00	0.20	0.37	0.18	0.23
45	E9BG80	LDBPK_230600	5'-3' exonuclease XRNC, putative	116 kDa	6.1	0.038	0.05	0.00	0.22	0.18	0.10	0.12
46	E9BLS2	LDBPK_301790	DNAJ domain protein, putative	79 kDa	6.8	0.0028	0.05	0.10	0.55	0.54	0.39	0.58
47	E9BTM5	LDBPK_361660	uncharacterized protein	13 kDa	7.8	0.016	0.00	0.09	0.26	0.29	0.47	0.40
48	E9BLH3	LDBPK_300800	4-methyl-5(beta-hydroxyethyl)-thiazole monophosphate synthesis protein, putative	21 kDa	9.1	0.016	0.15	0.28	1.78	2.56	1.23	2.18
49	E9BIV0	LDBPK_270150	uncharacterized protein	19 kDa	12	0.0034	0.00	0.06	0.38	0.38	0.25	0.37
50	E9BAD0	LDBPK_100850	uncharacterized protein	136 kDa	24	0.038	0.06	0.00	0.51	0.66	0.45	1.07
51	E9BMK0	LDBPK_310770	uncharacterized protein	35 kDa	INF <sup>b</sup>		0.00	0.00	0.21	0.12	0.06	0.19
52	E9BHE2	LDBPK_250380	uncharacterized protein	20 kDa	INF		0.00	0.00	0.15	0.08	0.06	0.08
53	E9BRG8	LDBPK_343700	uncharacterized protein	34 kDa	INF		0.00	0.00	0.34	0.18	0.21	0.13
54	E9BL66	LDBPK_292690	uncharacterized protein	46 kDa	INF		0.00	0.00	0.10	0.21	0.10	0.19
55	E9BEU7	LDBPK_201480	uncharacterized protein	27 kDa	INF		0.00	0.00	0.34	0.16	0.25	0.16
56	E9BTC7	LDBPK_360680	uncharacterized protein	30 kDa	INF		0.00	0.00	0.10	0.11	0.14	0.05
57	E9BEB8	LDBPK_191260	RNA binding protein, putative	25 kDa	INF		0.00	0.00	0.22	0.25	0.10	0.18
58	E9BSZ0	LDBPK_354710	uncharacterized protein	14 kDa	INF		0.00	0.00	0.11	0.12	0.21	0.12
59	E9BKE4	LDBPK_281060	uncharacterized protein	18 kDa	INF		0.00	0.00	0.08	0.09	0.16	0.09
60	E9BBC1	LDBPK_130470	uncharacterized protein	180 kDa	INF		0.00	0.00	0.16	0.15	0.13	0.24
61	E9BU71	LDBPK_363620	uncharacterized protein	23 kDa	INF		0.00	0.00	0.13	0.15	0.12	0.07
62	E9BKQ9	LDBPK_291130	uncharacterized protein	203 kDa	INF		0.00	0.00	0.05	0.04	0.06	0.07
63	E9BEM0	LDBPK_200730	uncharacterized protein	29 kDa	INF		0.00	0.00	0.15	0.15	0.15	0.11
64	E9BRE0	LDBPK_343420 (E9BRE0)	cluster of E9BRE0_LEIDB uncharacterized protein	18 kDa	INF		0.00	0.00	0.18	0.20	0.16	0.20
65	E9B8E4	LDBPK_050540	uncharacterized protein	14 kDa	INF		0.00	0.00	0.24	0.26	0.21	0.27

<sup>a</sup>Values from two biological replicates. <sup>b</sup>A ratio of "INF" is returned when dividing by zero (protein not detected in CL).

Table 8. Proteins Elevated in CL Compared with Visceral Isolates (IV and VL)

no.	accession number	gene ID	description	molecular weight	fold change CL to visceral	t test (P value, CL to visceral (IV+VL))	normalized emPAI values					
							CL <sup>a</sup>	CL <sup>b</sup>	IV <sup>a</sup>	IV <sup>b</sup>	VL <sup>a</sup>	VL <sup>b</sup>
1	E9BJ01	LDBPK_270680	uncharacterized protein	347 kDa	0		0.02	0.06	0.00	0.00	0.00	0.00
2	E9BNE7	LDBPK_320420	uncharacterized protein	83 kDa	0		0.16	0.06	0.00	0.00	0.00	0.00
3	E9BAV9	LDBPK_111210	cluster of E9BAV9_LEIDB ATP-binding cassette protein subfamily A, member 2, putative	197 kDa	0		0.22	0.54	0.00	0.00	0.00	0.00
4	E9B8N0	LDBPK_060190	uncharacterized protein	62 kDa	0		0.08	0.04	0.00	0.00	0.00	0.00
5	E9BPQ0	LDBPK_333120	uncharacterized protein	80 kDa	0		0.40	0.20	0.00	0.00	0.00	0.00
6	E9B9K8	LDBPK_081080	uncharacterized protein	50 kDa	0		0.07	0.13	0.00	0.00	0.00	0.00
7	E9BGN0	LDBPK_240180	uncharacterized protein	91 kDa	0		0.17	0.09	0.00	0.00	0.00	0.00
8	E9B8I0	LDBPK_050900	surface antigen-like protein	30 kDa	0		0.39	0.27	0.00	0.00	0.00	0.00
9	E9BSS6	LDBPK_354060	protein kinase A catalytic subunit isoform 1 (fragment)	19 kDa	0		0.34	0.49	0.00	0.00	0.00	0.00
10	E9BNU3	LDBPK_321900	protein kinase, putative	71 kDa	0		0.21	0.30	0.00	0.00	0.00	0.00
11	E9BAA0	LDBPK_100550	uncharacterized protein	713 kDa	0		0.12	0.09	0.00	0.00	0.00	0.00
12	E9BK71	LDBPK_280310	ribonuclease II-like protein, putative	105 kDa	0		0.11	0.16	0.00	0.00	0.00	0.00
13	E9BRJ8	LDBPK_343900	uncharacterized protein	79 kDa	0		0.11	0.09	0.00	0.00	0.00	0.00
14	E9BSZ6	LDBPK_354770	MCAK-like kinesin, putative	53 kDa	0		0.23	0.18	0.00	0.00	0.00	0.00
15	E9BSN3	LDBPK_353620	uncharacterized protein	102 kDa	0		0.36	0.44	0.00	0.00	0.00	0.00
16	E9B7Y6	LDBPK_040200	surface antigen-like protein	29 kDa	0		0.39	0.32	0.00	0.00	0.00	0.00
17	E9BAJ3	LDBPK_101410	uncharacterized protein	35 kDa	0		0.26	0.23	0.00	0.00	0.00	0.00
18	E9BC09	LDBPK_141270	uncharacterized protein	58 kDa	0		0.12	0.12	0.00	0.00	0.00	0.00
19	E9BES2	LDBPK_201230	calpain-like cysteine peptidase, putative	78 kDa	0		0.80	0.78	0.00	0.00	0.00	0.00
20	E9BIC8	LDBPK_261160	uncharacterized protein	71 kDa	0		0.33	0.38	0.00	0.00	0.00	0.00
21	E9BIQJ	LDBPK_262400	uncharacterized protein	558 kDa	0		0.20	0.22	0.00	0.00	0.00	0.00
22	E9BK45	LDBPK_280050	uncharacterized protein	56 kDa	0		0.34	0.32	0.00	0.00	0.00	0.00
23	E9BL89	LDBPK_292920	ribonuclease inhibitor-like protein	81 kDa	0		0.09	0.08	0.00	0.00	0.00	0.00
24	E9BPP8	LDBPK_333100	uncharacterized protein	69 kDa	0		0.35	0.37	0.00	0.00	0.00	0.00
25	E9BAS5	LDBPK_100070	dehydrogenase-like protein	45 kDa	0.01	0.024	0.74	1.96	0.06	0.00	0.00	0.00
26	E9BQK4	LDBPK_340500	calcium channel protein, putative	291 kDa	0.01	<0.00010	0.31	0.37	0.00	0.00	0.02	0.00
27	E9BNN5	LDBPK_321320	uncharacterized protein	54 kDa	0.02	0.002	2.12	1.36	0.00	0.00	0.11	0.00
28	E9BG87	LDBPK_230670	uncharacterized protein	348 kDa	0.04	0.00016	0.09	0.10	0.00	0.00	0.02	0.00
29	E9BPQ5	LDBPK_333170	uncharacterized protein	120 kDa	0.05	0.0051	0.59	1.02	0.00	0.00	0.05	0.12
30	E9BK65	LDBPK_280250	propionyl-coa carboxylase beta chain, putative	57 kDa	0.05	0.0042	0.18	0.29	0.00	0.00	0.05	0.00
31	E9BL72	LDBPK_292750	uncharacterized protein	48 kDa	0.05	0.004	0.50	0.31	0.00	0.00	0.00	0.08
32	E9BP85	LDBPK_323350	uncharacterized protein	131 kDa	0.05	0.00036	0.43	0.47	0.00	0.00	0.10	0.00
33	E9BSL4	LDBPK_353430	uncharacterized protein	62 kDa	0.05	0.00017	0.25	0.24	0.00	0.05	0.00	0.00
34	E9BP33	LDBPK_322820	uncharacterized protein	42 kDa	0.06	0.0023	0.30	0.21	0.00	0.00	0.06	0.00
35	E9BSY1	LDBPK_354620	uncharacterized protein	161 kDa	0.06	0.00079	0.15	0.13	0.00	0.00	0.03	0.00
36	E9BCH4	LDBPK_151350	actin-like protein, putative	47 kDa	0.06	0.00047	0.40	0.36	0.00	0.00	0.00	0.09

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Table 8. continued

no.	accession number	gene ID	description	molecular weight	fold change CL to visceral	t test (P value, CL to visceral (IV+VL))	normalized emPAI values					
							CL <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>	IV <sup>a</sup>	VL <sup>a</sup>	VL <sup>a</sup>
37	E9BPP5	LDBPK_333070	uncharacterized protein	204 kDa	0.07	0.016	0.75	1.54	0.00	0.00	0.03	0.30
38	E9BPP7	LDBPK_333090	uncharacterized protein	155 kDa	0.07	0.012	0.33	0.64	0.00	0.00	0.04	0.09
39	E9BN99	LDBPK_313290	uncharacterized protein	162 kDa	0.07	0.002	0.79	0.60	0.00	0.00	0.00	0.18
40	E9BGH1	LDBPK_231570	dynein heavy chain, putative	544 kDa	0.07	<0.00010	1.02	1.05	0.00	0.00	0.17	0.10
41	E9BML9	LDBPK_310950	sodium stibogluconate resistance protein, putative	171 kDa	0.07	<0.00010	1.26	1.24	0.09	0.05	0.13	0.08
42	E9BQB6	LDBPK_331850	macrophage migration inhibitory factor-like protein	13 kDa	0.08	0.012	2.76	1.39	0.11	0.13	0.39	0.00
43	E9BJS2	LDBPK_271200	uncharacterized protein	42 kDa	0.08	0.009	0.22	0.13	0.00	0.00	0.06	0.00
44	E9BE17	LDBPK_190210	polyprenyl synthase, putative	82 kDa	0.08	0.0025	0.21	0.18	0.00	0.00	0.07	0.00
45	E9BU02	LDBPK_362930	uncharacterized protein	295 kDa	0.09	0.0036	0.46	0.37	0.00	0.00	0.00	0.15
46	E9BTE0	LDBPK_360810	uncharacterized protein	137 kDa	0.09	0.0027	0.14	0.12	0.00	0.00	0.04	0.00
47	E9BKA3	LDBPK_280650	dynein heavy chain, putative	478 kDa	0.09	0.00076	0.86	1.07	0.00	0.00	0.15	0.17
48	E9BRL5	LDBPK_344160	phosphatidylinositol 3-kinase (Tor2)-like protein	274 kDa	0.1	0.049	0.06	0.13	0.00	0.00	0.00	0.05
49	E9BI59	LDBPK_260460	uncharacterized protein	105 kDa	0.1	0.047	0.06	0.14	0.00	0.04	0.00	0.00
50	E9BPS9	LDBPK_333420	fatty acid desaturase, putative	45 kDa	0.1	0.043	0.16	0.31	0.00	0.13	0.00	0.00
51	E9BHS7	LDBPK_251730	uncharacterized protein	104 kDa	0.1	0.041	0.33	0.87	0.00	0.00	0.16	0.14
52	E9BR26	LDBPK_342280	uncharacterized protein	120 kDa	0.1	0.038	0.16	0.36	0.00	0.00	0.00	0.12
53	E9BK28	LDBPK_283120	uncharacterized protein	43 kDa	0.1	0.021	0.38	0.20	0.00	0.00	0.12	0.00
54	E9BE52	LDBPK_190570	uncharacterized protein	43 kDa	0.1	0.016	0.25	0.44	0.00	0.00	0.14	0.00
55	E9BUP2	LDBPK_365340	uncharacterized protein	29 kDa	0.1	0.016	0.18	0.23	0.00	0.00	0.00	0.11
56	E9BUQ4	LDBPK_365470	glycosyl hydrolase, putative	129 kDa	0.1	0.013	0.51	0.26	0.07	0.05	0.04	0.00
57	E9BIY2	LDBPK_270480	ATP-binding cassette protein subfamily D, member 1, putative	77 kDa	0.1	0.011	0.15	0.19	0.08	0.00	0.00	0.00
58	E9BM89	LDBPK_303460	uncharacterized protein	67 kDa	0.1	0.0088	2.70	4.11	0.00	0.14	1.30	0.43
59	E9BUN3	LDBPK_365250	uncharacterized protein	98 kDa	0.1	0.0072	0.18	0.20	0.00	0.00	0.00	0.09
60	E9BHS0	LDBPK_251660	uncharacterized protein	135 kDa	0.1	0.0065	0.46	0.65	0.07	0.00	0.05	0.21
61	E9BKI2	LDBPK_290360	uncharacterized protein	150 kDa	0.1	0.0065	1.21	1.82	0.00	0.00	0.20	0.50
62	E9BPF2	LDBPK_324020	myosin XXI	119 kDa	0.1	0.0062	3.56	5.47	0.00	0.17	1.06	1.20
63	E9BNT7	LDBPK_321840	uncharacterized protein	54 kDa	0.1	0.006	0.88	0.87	0.00	0.00	0.13	0.40
64	E9BUP7	LDBPK_365390	uncharacterized protein	65 kDa	0.1	0.0059	0.12	0.11	0.05	0.00	0.00	0.00
65	E9BBR1	LDBPK_140260	uncharacterized protein	75 kDa	0.1	0.0058	0.18	0.21	0.00	0.00	0.08	0.00
66	E9BHC4	LDBPK_250200	uncharacterized protein	151 kDa	0.1	0.0051	0.89	1.45	0.19	0.18	0.19	0.00
67	E9BLS4	LDBPK_301810	uncharacterized protein	590 kDa	0.1	0.0051	0.23	0.32	0.00	0.00	0.05	0.09
68	E9BRG7	LDBPK_343690	dynein heavy chain, putative	529 kDa	0.1	0.005	0.80	1.19	0.00	0.00	0.28	0.18
69	E9BS55	LDBPK_351800	trypanin-like protein	54 kDa	0.1	0.0049	0.13	0.12	0.00	0.00	0.05	0.00
70	E9BJA5	LDBPK_271750	paraflagellar rod protein-like protein	89 kDa	0.1	0.0043	0.78	0.72	0.00	0.00	0.05	0.31
71	E9BJ68	LDBPK_271360	uncharacterized protein	93 kDa	0.1	0.0042	0.74	0.64	0.29	0.05	0.05	0.00
72	E9BN40	LDBPK_312700	serine/threonine-protein phosphatase	41 kDa	0.1	0.0042	0.38	0.27	0.06	0.00	0.00	0.10
73	E9BR85	LDBPK_342870	uncharacterized protein	64 kDa	0.1	0.0042	1.61	1.96	0.00	0.00	0.37	0.65

Table 8. continued

no.	accession number	gene ID	description	molecular weight	fold change CL to visceral	t test (P value, CL to visceral (IV+VL))	normalized emPAI values					
							CL <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>	IV <sup>a</sup>	VL <sup>a</sup>	VL <sup>a</sup>
74	E9B8L1	LDBPK_051210	surface antigen-like protein	30 kDa	0.1	0.0025	0.62	0.43	0.07	0.00	0.07	0.11
75	E9BTG1	LDBPK_361010	dynein heavy chain, putative	473 kDa	0.1	0.0019	0.62	0.48	0.00	0.00	0.12	0.12
76	E9BCZ5	LDBPK_161430	uncharacterized protein	66 kDa	0.1	0.0017	0.17	0.21	0.04	0.00	0.04	0.00
77	E9BD16	LDBPK_161650	uncharacterized protein	139 kDa	0.1	0.0015	0.82	0.98	0.00	0.00	0.21	0.21
78	E9BF80	LDBPK_210940	uncharacterized protein	42 kDa	0.1	0.0014	0.53	0.45	0.14	0.00	0.07	0.00
79	E9B9C6	LDBPK_080210	uncharacterized protein	72 kDa	0.1	0.0013	2.15	2.51	0.00	0.05	0.70	0.19
80	E9BIQ5	LDBPK_262440	uncharacterized protein	71 kDa	0.1	0.00063	0.73	0.67	0.00	0.05	0.08	0.19
81	E9BJH6	LDBPK_272460	dynein heavy chain (pseudogene), putative	490 kDa	0.1	0.0005	0.81	0.86	0.03	0.02	0.20	0.17
82	E9BTG6	LDBPK_361060	uncharacterized protein	145 kDa	0.1	0.00017	1.55	1.86	0.26	0.19	0.33	0.20
83	E9BE47	LDBPK_190520	uncharacterized protein	58 kDa	0.1	0.0016	0.97	1.14	0.00	0.00	0.32	0.09
84	E9B9I9	LDBPK_080870	protein kinase, putative	64 kDa	0.2	0.048	0.56	1.03	0.00	0.00	0.27	0.45
85	E9BSJ7	LDBPK_353260	uncharacterized protein	74 kDa	0.2	0.046	0.05	0.09	0.00	0.04	0.00	0.00
86	E9BE30	LDBPK_190340	uncharacterized protein	50 kDa	0.2	0.04	0.36	0.23	0.00	0.00	0.11	0.17
87	E9BER4	LDBPK_201150	DNAj-like protein	75 kDa	0.2	0.039	0.40	0.22	0.18	0.04	0.00	0.00
88	E9BUW5	LDBPK_366080	uncharacterized protein	25 kDa	0.2	0.038	0.10	0.15	0.00	0.00	0.08	0.00
89	E9BA45	LDBPK_091600	uncharacterized protein	174 kDa	0.2	0.036	1.31	1.72	0.00	0.00	0.36	1.00
90	E9BJD9	LDBPK_272090	uncharacterized protein	174 kDa	0.2	0.033	0.81	1.18	0.11	0.00	0.25	0.63
91	E9BAB3	LDBPK_100680	flagellar glycoprotein-like protein	63 kDa	0.2	0.032	1.37	0.76	0.07	0.00	0.56	0.22
92	E9B999	LDBPK_071080	uncharacterized protein	37 kDa	0.2	0.03	1.99	1.12	0.07	0.00	0.68	0.56
93	E9BPF4	LDBPK_324040	AUT2/APG4/ATG4 cysteine peptidase, putative	42 kDa	0.2	0.029	0.14	0.21	0.06	0.00	0.00	0.10
94	E9BSB5	LDBPK_352450	uncharacterized protein	193 kDa	0.2	0.029	0.92	1.70	0.00	0.00	0.25	0.63
95	E9BSW6	LDBPK_354460	uncharacterized protein	45 kDa	0.2	0.029	0.14	0.22	0.00	0.00	0.06	0.09
96	E9B7Y2	LDBPK_040160	uncharacterized protein	53 kDa	0.2	0.028	1.20	0.65	0.00	0.00	0.44	0.19
97	E9BPZ2	LDBPK_330630	uncharacterized protein	101 kDa	0.2	0.028	1.18	1.63	0.00	0.00	0.77	0.52
98	E9BD27	LDBPK_161760	uncharacterized protein	283 kDa	0.2	0.026	0.16	0.08	0.03	0.02	0.02	0.05
99	E9BLY4	LDBPK_302410	uncharacterized protein	31 kDa	0.2	0.025	0.17	0.17	0.00	0.05	0.00	0.11
100	E9BBB4	LDBPK_130390	RNA helicase, putative	244 kDa	0.2	0.024	0.35	0.63	0.06	0.07	0.07	0.25
101	E9BDC2	LDBPK_171010	uncharacterized protein	55 kDa	0.2	0.023	0.32	0.39	0.00	0.11	0.21	0.00
102	E9BIP5	LDBPK_262340	uncharacterized protein	79 kDa	0.2	0.022	0.74	1.14	0.00	0.00	0.45	0.29
103	E9BR76	LDBPK_342780	uncharacterized protein	47 kDa	0.2	0.021	2.14	1.47	0.05	0.00	0.89	0.62
104	E9BUX0	LDBPK_366130	uncharacterized protein	86 kDa	0.2	0.021	4.98	6.57	0.00	0.00	2.93	2.09
105	E9BB86	LDBPK_130110	uncharacterized protein	40 kDa	0.2	0.019	0.40	0.24	0.06	0.00	0.14	0.00
106	E9BHF8	LDBPK_250540	ATP-binding cassette protein subfamily B, member 1, putative	72 kDa	0.2	0.019	0.50	0.50	0.00	0.30	0.08	0.00
107	E9BNI0	LDBPK_320760	uncharacterized protein	55 kDa	0.2	0.019	0.78	0.42	0.20	0.11	0.15	0.00
108	E9BUF2	LDBPK_364440	uncharacterized protein	123 kDa	0.2	0.017	9.37	11.48	0.00	0.00	4.93	4.07
109	E9BUY6	LDBPK_366290	uncharacterized protein	260 kDa	0.2	0.017	0.29	0.35	0.00	0.00	0.16	0.11
110	E9BM84	LDBPK_303410	calmodulin-related protein, putative	81 kDa	0.2	0.016	2.41	2.60	0.00	0.00	1.31	0.86
111	E9BN15	LDBPK_312450	uncharacterized protein	56 kDa	0.2	0.014	0.46	0.53	0.04	0.28	0.10	0.00

Table 8. continued

no.	accession number	gene ID	description	molecular weight	fold change CL to visceral	t test (P value, CL to visceral (IV+VL))	normalized emPAI values					
							CL <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>	IV <sup>a</sup>	VL <sup>a</sup>	VL <sup>a</sup>
112	E9BQZ9	LDBPK_342000	uncharacterized protein	132 kDa	0.2	0.014	0.32	0.17	0.07	0.05	0.04	0.00
113	E9BH25	LDBPK_241630	uncharacterized protein	67 kDa	0.2	0.013	9.91	10.01	0.00	0.00	5.15	2.89
114	E9BE29	LDBPK_190330	uncharacterized protein	67 kDa	0.2	0.012	1.05	1.29	0.07	0.00	0.62	0.20
115	E9BIB2	LDBPK_261000	dynein heavy chain, putative	458 kDa	0.2	0.012	1.30	1.72	0.03	0.03	0.52	0.64
116	E9BNV4	LDBPK_322020	uncharacterized protein	50 kDa	0.2	0.012	2.70	2.20	0.00	0.00	1.24	0.33
117	E9BJ78	LDBPK_271460	diacylglycerol acyltransferase, putative	171 kDa	0.2	0.011	0.23	0.16	0.00	0.00	0.03	0.09
118	E9BTB9	LDBPK_360600	cell division related protein kinase 2	36 kDa	0.2	0.01	0.16	0.22	0.07	0.00	0.06	0.00
119	E9B7W8	LDBPK_040030	cAMP-specific phosphodiesterase, putative	79 kDa	0.2	0.0073	1.22	0.97	0.25	0.35	0.45	0.00
120	E9BK27	LDBPK_283110	dynein heavy chain, putative	474 kDa	0.2	0.0065	1.15	1.48	0.03	0.00	0.22	0.55
121	E9BM44	LDBPK_303010	uncharacterized protein	45 kDa	0.2	0.0064	0.20	0.26	0.05	0.06	0.00	0.09
122	E9BE23	LDBPK_190270	sarcoplasmic reticulum glycoprotein, putative	69 kDa	0.2	0.0062	0.15	0.19	0.06	0.05	0.00	0.00
123	E9BKX1	LDBPK_291730	uncharacterized protein	77 kDa	0.2	0.0059	0.65	0.65	0.09	0.16	0.30	0.00
124	E9BIF2	LDBPK_261400	uncharacterized protein	257 kDa	0.2	0.0043	0.21	0.19	0.09	0.05	0.02	0.00
125	E9BL84	LDBPK_292870	uncharacterized protein	65 kDa	0.2	0.0043	1.82	2.41	0.15	0.00	0.73	0.47
126	E9B9L0	LDBPK_081100	uncharacterized protein	139 kDa	0.2	0.0037	1.36	1.30	0.12	0.11	0.47	0.53
127	E9BHE8	LDBPK_250440	uncharacterized protein	110 kDa	0.2	0.0031	0.57	0.70	0.08	0.26	0.05	0.13
128	E9BBZ5	LDBPK_141130	dynein heavy chain, putative	473 kDa	0.2	0.0027	0.42	0.55	0.05	0.03	0.11	0.16
129	E9BAQ9	LDBPK_110710	ATPase ASNA1 homologue	44 kDa	0.2	0.0016	2.49	1.83	0.41	0.44	0.63	0.54
130	E9BDD3	LDBPK_171120	uncharacterized protein	72 kDa	0.2	0.00086	0.48	0.57	0.09	0.14	0.17	0.07
131	E9BRJ7	LDBPK_343990	dynein heavy chain, putative	478 kDa	0.2	0.00083	0.81	1.03	0.27	0.17	0.13	0.21
132	E9BTL7	LDBPK_361580 (E9BTL7)	Cluster of E9BTL7_LEIDB uncharacterized protein	57 kDa	0.3	0.049	5.06	5.60	0.00	0.00	3.97	1.54
133	E9BJJ2	LDBPK_281210	uncharacterized protein	68 kDa	0.3	0.049	3.02	5.31	0.25	0.40	2.40	1.79
134	E9BSR9	LDBPK_353990	uncharacterized protein	73 kDa	0.3	0.046	0.33	0.25	0.14	0.19	0.00	0.07
135	E9BT00	LDBPK_354810	glyceraldehyde-3-phosphate dehydrogenase-like protein	36 kDa	0.3	0.045	0.58	0.39	0.07	0.08	0.35	0.12
136	E9BT47	LDBPK_355280	GTP-binding protein, putative	42 kDa	0.3	0.045	0.50	0.27	0.06	0.07	0.17	0.20
137	E9B9I3	LDBPK_080810	uncharacterized protein	48 kDa	0.3	0.043	0.31	0.30	0.14	0.07	0.00	0.21
138	E9BBH5	LDBPK_131010	uncharacterized protein	60 kDa	0.3	0.042	1.75	1.54	0.11	0.00	0.52	1.18
139	E9B8S7	LDBPK_060670	lanosterol synthase, putative	114 kDa	0.3	0.04	0.89	0.49	0.06	0.05	0.30	0.34
140	E9BFD7	LDBPK_211510	uncharacterized protein	91 kDa	0.3	0.039	0.31	0.32	0.00	0.21	0.11	0.00
141	E9BE69	LDBPK_190740	uncharacterized protein	48 kDa	0.3	0.038	0.26	0.28	0.11	0.19	0.06	0.00
142	E9BV27	LDBPK_366710	protein transport protein Sec23-like protein	94 kDa	0.3	0.036	0.26	0.51	0.11	0.05	0.16	0.15
143	E9BKY6	LDBPK_291890	parafagellar rod protein 1D, putative	69 kDa	0.3	0.035	99.64	117.43	2.91	1.55	64.50	51.37
144	E9BC97	LDBPK_150560	uncharacterized protein	45 kDa	0.3	0.034	5.45	4.59	0.00	0.00	2.07	3.05
145	E9BJZ8	LDBPK_282780	splicing factor 3B subunit 1, putative	114 kDa	0.3	0.031	1.06	1.60	0.36	0.83	0.10	0.38
146	E9B9B6	LDBPK_071290	uncharacterized protein	40 kDa	0.3	0.03	0.23	0.30	0.06	0.15	0.12	0.00
147	E9BAYS	LDBPK_120100	ornithine decarboxylase, putative	34 kDa	0.3	0.028	0.45	0.45	0.06	0.06	0.13	0.32

Table 8. continued

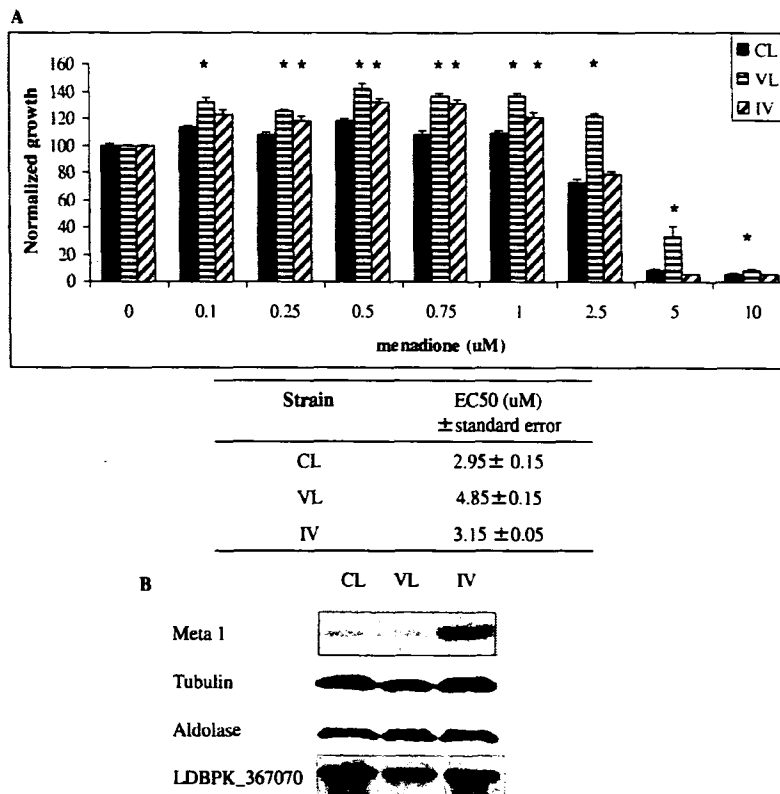
no.	accession number	gene ID	description	molecular weight	fold change CL to visceral	t test (P value, CL to visceral (IV+VL))	normalized emPAI values					
							CL <sup>a</sup>	CL <sup>d</sup>	IV <sup>a</sup>	IV <sup>d</sup>	VL <sup>a</sup>	VL <sup>d</sup>
148	E9BNX1	LDBPK_322190	ABC transporter-like protein	79 kDa	0.3	0.025	0.30	0.32	0.00	0.16	0.07	0.17
149	E9BQS1	LDBPK_341200	uncharacterized protein	30 kDa	0.3	0.024	0.19	0.18	0.00	0.07	0.07	0.11
150	E9BTD7	LDBPK_360780	protein kinase, putative	50 kDa	0.3	0.023	0.16	0.10	0.05	0.06	0.04	0.00
151	E9BAS9	LDBPK_100110	uncharacterized protein	80 kDa	0.3	0.021	2.14	2.86	0.45	0.34	1.56	0.29
152	E9BBL3	LDBPK_131390	dynein heavy chain, putative	529 kDa	0.3	0.021	7.72	10.31	0.96	0.69	3.19	5.18
153	E9BN42	LDBPK_312720	acetoin dehydrogenase e3 component-like protein	60 kDa	0.3	0.021	0.24	0.17	0.10	0.05	0.09	0.00
154	E9BBK2	LDBPK_131280	endomembrane protein, putative	67 kDa	0.3	0.02	0.60	0.79	0.11	0.09	0.08	0.43
155	E9BKN9	LDBPK_290920	guanine deaminase, putative	49 kDa	0.3	0.02	0.79	0.59	0.36	0.33	0.11	0.08
156	E9BUZ2	LDBPK_366350	uncharacterized protein	61 kDa	0.3	0.02	0.64	0.40	0.23	0.24	0.09	0.09
157	E9BDE3	LDBPK_171210	kinesin, putative	158 kDa	0.3	0.018	0.87	1.58	0.37	0.36	0.12	0.38
158	E9BAG9	LDBPK_101250	Rab1 small GTP-binding protein, putative	25 kDa	0.3	0.017	3.39	5.74	1.22	1.61	1.97	0.86
159	E9BIN6	LDBPK_262250	kynureninase	51 kDa	0.3	0.016	1.48	0.98	0.66	0.44	0.22	0.35
160	E9BJ35	LDBPK_271010	intraflagellar transport protein IFT88, putative	91 kDa	0.3	0.016	1.25	1.59	0.31	0.21	0.86	0.46
161	E9BNL9	LDBPK_321160	exportin 1, putative	118 kDa	0.3	0.013	0.42	0.50	0.28	0.10	0.08	0.12
162	E9B9W6	LDBPK_090790	ubiquitin ligase, putative	241 kDa	0.3	0.012	0.34	0.55	0.13	0.18	0.17	0.12
163	E9BCH0	LDBPK_151310	DNA topoisomerase 2	139 kDa	0.3	0.011	0.72	0.80	0.20	0.10	0.44	0.10
164	E9BM76	LDBPK_303330	serine/threonine phosphatase, putative	40 kDa	0.3	0.01	0.16	0.22	0.06	0.07	0.06	0.00
165	E9BSS0	LDBPK_354000	PRP8 protein homologue, putative	278 kDa	0.3	0.01	0.17	0.13	0.05	0.07	0.04	0.00
166	E9BS22	LDBPK_351460	uncharacterized protein	324 kDa	0.3	0.0099	0.08	0.11	0.02	0.02	0.02	0.05
167	E9B9C0	LDBPK_071330	uncharacterized protein	276 kDa	0.3	0.0098	0.27	0.30	0.13	0.11	0.08	0.00
168	E9BUX2	LDBPK_366150	uncharacterized protein	209 kDa	0.3	0.0097	0.38	0.54	0.12	0.22	0.06	0.14
169	E9BD38	LDBPK_170140	receptor-type adenylate cyclase b (fragment)	75 kDa	0.3	0.0096	0.77	0.86	0.39	0.25	0.07	0.39
170	E9BR57	LDBPK_342590	uncharacterized protein	34 kDa	0.3	0.0096	0.29	0.37	0.07	0.00	0.13	0.13
171	E9BBS5	LDBPK_140400	uncharacterized protein	34 kDa	0.3	0.0091	0.55	0.79	0.24	0.30	0.16	0.09
172	E9BIR1	LDBPK_262500	phosphatidyltransferase, putative	25 kDa	0.3	0.008	3.80	5.98	1.47	1.66	1.67	1.14
173	E9BFM7	LDBPK_220080	uncharacterized protein	172 kDa	0.3	0.0069	0.99	1.47	0.52	0.27	0.27	0.26
174	E9BNP3	LDBPK_321400	cleavage and polyadenylation specificity factor-like protein	167 kDa	0.3	0.0047	0.83	0.75	0.39	0.35	0.17	0.17
175	E9BHL7	LDBPK_251130	MGT2 magnesium transporter	46 kDa	0.3	0.004	0.35	0.26	0.05	0.13	0.05	0.09
176	E9BM90	LDBPK_303470	uncharacterized protein	97 kDa	0.3	0.004	0.47	0.62	0.20	0.19	0.21	0.09
177	E9B7W7	LDBPK_040010	calcium-translocating P-type ATPase	111 kDa	0.3	0.0027	2.18	2.03	0.96	0.67	0.48	0.26
178	E9BCA5	LDBPK_150640	uncharacterized protein	59 kDa	0.3	0.0014	0.64	0.63	0.23	0.21	0.06	0.18
179	E9BB14	LDBPK_120340	uncharacterized protein	44 kDa	0.3	0.00088	2.11	2.10	0.86	0.57	0.64	0.31
180	E9BSY9	LDBPK_354700	uncharacterized protein	56 kDa	0.3	0.00081	2.81	2.35	0.85	0.75	0.99	0.55
181	E9BEL2	LDBPK_200650	uncharacterized protein	45 kDa	0.3	0.00021	2.51	2.72	0.78	0.51	0.89	0.86
182	E9BJ95	LDBPK_271650	dynein heavy chain, putative	499 kDa	0.3	0.00012	0.65	0.74	0.24	0.23	0.20	0.20
183	E9BTZ5	LDBPK_362860	uncharacterized protein	102 kDa	0.4	0.048	0.18	0.14	0.10	0.05	0.00	0.09



Table 8. continued

no.	accession number	gene ID	description	molecular weight	fold change CL to visceral	t test (P value; CL to visceral (IV+VL))	normalized emPAI values					
							CL <sup>a</sup>	CL <sup>a</sup>	IV <sup>a</sup>	IV <sup>a</sup>	VL <sup>a</sup>	VL <sup>a</sup>
184	E9BAP7	LDBPK_110590	uncharacterized protein	118 kDa	0.4	0.047	1.36	2.01	0.87	1.05	0.55	0.12
185	E9BQB8	LDBPK_331880	uncharacterized protein	182 kDa	0.4	0.047	0.09	0.13	0.05	0.05	0.06	0.00
186	E9BIK2	LDBPK_261910	uncharacterized protein	134 kDa	0.4	0.044	0.82	1.24	0.51	0.69	0.27	0.21
187	E9BRH8	LDBPK_343800	adaptor gamma-1 chain, putative	91 kDa	0.4	0.026	0.85	0.73	0.27	0.42	0.49	0.10
188	E9BRE3	LDBPK_343450	transmembrane/endomembrane-like protein	71 kDa	0.4	0.023	1.08	1.81	0.44	0.70	0.62	0.61
189	E9BPD2	LDBPK_323820	cluster of E9BPD2_LEIDB 3-hydroxyisobutyryl-coenzyme a hydrolase-like protein	38 kDa	0.4	0.022	5.43	9.02	3.52	3.17	1.82	2.24
190	E9BN36	LDBPK_312660	uncharacterized protein	103 kDa	0.4	0.022	0.59	0.83	0.40	0.35	0.32	0.14
191	E9B809	LDBPK_040430	calpain-like cysteine peptidase, putative	95 kDa	0.4	0.02	0.80	1.04	0.36	0.22	0.61	0.40
192	E9BQ57	LDBPK_331260	uncharacterized protein	73 kDa	0.4	0.02	0.24	0.35	0.06	0.09	0.17	0.12
193	E9BAI1	LDBPK_101390	uncharacterized protein	36 kDa	0.4	0.019	0.79	0.50	0.23	0.23	0.36	0.21
194	E9BV64	LDBPK_367080	beta-adaptin, putative	82 kDa	0.4	0.016	0.64	0.62	0.41	0.33	0.19	0.17
195	E9BJ03	LDBPK_270710	uncharacterized protein	70 kDa	0.4	0.013	0.41	0.56	0.27	0.14	0.18	0.26
196	E9BJW5	LDBPK_282450	DNA topoisomerase 2	168 kDa	0.4	0.0096	1.56	1.93	0.94	0.65	0.43	0.93
197	E9BLG2	LDBPK_300680	uncharacterized protein	80 kDa	0.4	0.0083	1.09	1.26	0.35	0.58	0.70	0.35
198	E9BU08	LDBPK_362980	uncharacterized protein	126 kDa	0.4	0.0054	1.04	1.02	0.36	0.24	0.61	0.34
199	E9BRL2	LDBPK_344140	coatamer alpha subunit, putative	133 kDa	0.4	0.0042	3.37	3.15	1.76	1.45	0.77	1.46
200	E9BJ94	LDBPK_271630	uncharacterized protein	38 kDa	0.4	0.0034	9.16	7.34	4.15	3.87	3.29	2.53
201	E9BLD2	LDBPK_300380	phosphatase 2C, putative	42 kDa	0.4	0.0027	2.68	2.07	0.97	1.04	1.00	0.72
202	E9BK79	LDBPK_280410	phosphoadenosine phosphosulfate reductase-like protein	25 kDa	0.4	0.0026	0.22	0.22	0.08	0.08	0.08	0.13
203	E9BAI4	LDBPK_101420	cluster of E9BAI4_LEIDB uncharacterized protein	35 kDa	0.4	0.0022	1.08	1.12	0.39	0.27	0.58	0.42
204	E9BPS3	LDBPK_333360	beta prime cop protein, putative	98 kDa	0.4	0.0015	3.73	4.52	1.54	2.08	1.51	1.69
205	E9BGK5	LDBPK_231910	vacuolar proton translocating ATPase subunit A, putative	88 kDa	0.4	0.00036	2.73	2.46	0.99	0.88	1.08	0.69
206	E9BRZ2	LDBPK_351160	oligosaccharyl transferase-like protein	89 kDa	0.5	0.048	0.57	0.65	0.27	0.31	0.12	0.48
207	E9BNX2	LDBPK_322200	uncharacterized protein	36 kDa	0.5	0.046	0.74	0.65	0.42	0.49	0.31	0.13
208	E9BK90	LDBPK_280520	uncharacterized protein	42 kDa	0.5	0.026	0.91	0.79	0.43	0.29	0.61	0.22
209	E9BS80	LDBPK_352080	calcium motive p-type ATPase, putative	122 kDa	0.5	0.022	5.36	6.64	2.60	2.30	1.78	4.27
210	E9BDY2	LDBPK_181510	P-type H <sup>+</sup> -ATPase, putative	107 kDa	0.5	0.016	17.98	20.34	8.74	10.13	5.97	13.33
211	E9BJN7	LDBPK_281660	DEAD box RNA helicase, putative	44 kDa	0.5	0.013	1.16	0.88	0.49	0.52	0.60	0.30
212	E9B710	LDBPK_020330	casein kinase II, alpha chain, putative	44 kDa	0.5	0.011	0.62	0.60	0.41	0.21	0.32	0.21

<sup>a</sup>Values from two biological replicates.



**Figure 6.** Validation of mass spectrometry analysis. (A) Visceral strains are more resistant to superoxide than CL. Promastigotes were treated with menadione and OD600 measured 72 h after treatment. Top: Growth in menadione-treated samples was normalized to growth in untreated samples for each strain. \*,  $p < 0.05$  compared with CL. Bottom: EC50 values for menadione treatment ( $\mu\text{M}$ ). (B) Western blot validation of the mass spectrometry data. Protein levels of meta 1, tubulin, aldolase, and the uncharacterized protein LDBPK\_367070 were compared by Western blot. Meta 1 was elevated in IV, whereas all other proteins were expressed at comparable levels across strains.

tropism. Indeed, we were able to restore parasite ability to survive in visceral organs from an initial avirulent cutaneous clinical isolate: five serial passages were sufficient to observe a statistically significant increase in spleen parasite burden, and by serial passage ten, this had increased 45-fold. The relative quick readaptation from avirulent to virulent in visceral sites also supports close monitoring of the clinical situation in Sri Lanka and suggests that a newly visceral strain may re-emerge there.

In this study, it was possible to identify approximately 2000 proteins in each strain out of over 8000 potential protein genes in the *L. donovani* genomes. Fewer than 12% of detected proteins had different levels when comparing the IV and parental CL strains, and fewer than 5% of the proteins had a higher level of expression in the IV strain than the CL strain. This argues that the detectable proteomes of these strains are very similar. It is, however, possible that there are important differences in the proteins levels or primary sequences, which are beyond the limit of detection in this type of analysis. It will therefore be necessary to compare the genomes of the IV and CL strains, as was recently reported for the VL and CL strains.<sup>9</sup>

Considering the detectable proteomes revealed here, several factors were associated with adaptation to cutaneous or visceral infection sites. Transport and trafficking processes were upregulated in the cutaneous strain, perhaps allowing the parasite to survive in the nutrient-poor environment of the skin. In contrast, biosynthetic processes, translation, signaling, and stress response processes were elevated in strains with high visceral virulence, supporting our previous hypothesis that the visceral environment is more stressful for the parasites.<sup>2</sup>

However, many of the proteins identified in this study are classified as hypothetical uncharacterized proteins. These may be key to *Leishmania*-specific processes and as such to the establishment of leishmaniasis. Interestingly, 15 of the 40 IV-specific uncharacterized proteins absent in CL could not be annotated in Blast2GO and had no Interpro domain information. Similarly, all five of the uncharacterized proteins specific to VL and absent in CL did not have any GO annotations using Blast2GO, although some domains could be identified for three of them in Interpro. Further study of these uncharacterized proteins is therefore warranted.

While the IV strain had increased ability to survive in visceral organs, the intravenous injection route used for selection does not assess this strain's potential to visceralize. Factors differing between the CL and IV strains therefore represent proteins required for parasite survival in the visceral organs. Additional selection experiments using intradermal injection or transmission by the natural sandfly vector would help further clarify visceralization factors.

Another interesting observation of this study is that the strain selected for increased visceral virulence in mice differed from the human visceral *L. donovani* strain, although they did have several traits in common including an increase in antioxidant proteins. This suggests, however, that early responses to host conditions and by extent *Leishmania* survival in the spleen are governed by different processes in mice than in humans and as such represents a cautionary note for mouse studies of *L. donovani* virulence in general. However, infection also proceeds via different routes in these two hosts, with experimental visceral infection being

ected by direct injection in the bloodstream, while in humans, infection is initiated at the sandfly intradermal bite site, and this could also contribute to the differences we observed. The expansion in A2 family observed in VL but not in IV compared with CL (Figure S3 in the Supporting Information) could be related to this process, given that A2 has been shown to play a role in parasite visceralization.<sup>42</sup>

Overall, this work represents the first proteomic comparison of *L. donovani* strains with different tropisms and of their adaptation to different host environments. These results provide new insight into the adaptability of *Leishmania* parasites to the host environment and on the factors that mediate disease tropism.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Table S1: Proteins identified in CL, IV, and VL whole cell lysate. Table S2: Proteins identified in CL and VL exosomes. Table S3: Proteins differentially expressed in the IV and VL strains. Table S4: Peptide identification in CL whole cell lysate, replicate 1. Table S5: Peptide identification in CL whole cell lysate, replicate 2. Table S6: Peptide identification in IV whole cell lysate, replicate 1. Table S7: Peptide identification in IV whole cell lysate, replicate 2. Table S8: Peptide identification in VL whole cell lysate, replicate 1. Table S9: Peptide identification in VL whole cell lysate, replicate 2. Table S10: Peptide identification in CL and VL exosomes. Figure S1: Growth curves: promastigotes and amastigotes. Figure S2: Purification of exosomes. Figure S3: Induction of A2 expression by 18 h transfer to amastigote culture conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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