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Role of Leishmania membrane nutrient transporters in attenuation

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Summary.

The disease leishmaniasis is caused by protozoa parasites of the genus Leishmania, with the sand fly acting as a vector for their transmission to the human host, where they colonise the phagolysosomes of macrophages. However, this location does not afford the parasites the nutrients they need in sufficient amounts. Furthermore, similar to other eukaryotic parasites, Leishmania cannot undertake de novo synthesis of specific nutrients, so must acquire them from the host. In this regard, cell viability depends crucially on membrane transporters, which eliminate the obstacle of permeability, making it possible for nutrients to pass through lipid membranes. Therefore, the purpose of the present project is to knock out four presumed membrane transporters whose expression is modulated in a gentamicin attenuated Leishmania strain. Attenuation phenotypes may be related to expression regulation of a cohort of genes; however, impairment of nutrient acquisition may result in diminished growth and nutrient transport genes may be related to the attenuation phenotypes. We propose to evaluate the growth rate of the single null mutants of these transporters generated using a CRISPR-Cas9 system and compared it with the wild type (WT) strain to assess the relative importance of these transporters in the attenuation phenotype. To this end, we have obtained all the necessary clones to carry out the transfection that will result in null mutants.

1. Introduction.

Leishmania protozoa cause the disease leishmaniasis, with both in the old world and in the new, sand flies acting as transmission vectors. Similar to other parasitic protozoa, the Leishmania life cycle comprises multi-phase evolution to enable colonisation of the mammalian host. The ability of the parasite to absorb nutrients within the host is crucial to its survival. In mammalian hosts, the phagolysosomes of macrophages constitute the living environment of Leishmania but do not supply all the necessary nutrients in sufficient amounts. Furthermore, as with other eukaryotic parasites, Leishmania depends on the host to obtain specific necessary nutrients that it is unable to synthesise de novo. To overcome this problem, Leishmania relies on membrane transporters with substrate specificities, such as hexose, pentose, iron, polyamines, and amino acid transporters [1]. The presence of transmembrane hydrophobic domains allows to predict putative transport proteins through bioinformatics methods [2]. The problem is that such methods cannot predict substrate specificity and functional characterisations have been produced for just a few obvious membrane transporters. In this work we aimed to characterise the role of some

of these transporters in Leishmania attenuation; therefore, the information available on Leishmania membrane transporters is summarized below.

Among the critical nutrients for cells are hexoses, which primarily consist of glucose. The concentration in which they are available is subject to variation according to the environment colonised by Leishmania. The life cycle of these parasites involves a multi-phase evolution, taking the form of flagellated promastigotes in the sand fly vector, while their location in the human host is the acidified phagolysosome vesicles of macrophages, as shown in Figure 1. As reported by ter Kulle [3], the primary source of metabolic energy for promastigotes in culture is glucose or proline, whereas amino acids and fatty acids are utilized in the macrophage phagolysosomes, where the concentration of carbohydrates is not always high enough [1,4,5]. Burchmoroe and Landfear [6] examined the genome of *Leishmania mexicana* and found a group of three glucose transporter genes dubbed LmxGT1, LmxGT2, and LmxGT3. It is worth noting that GT1 and GT3 mRNAs appear to have a constitutive expression as their levels remain unchanged in promastigotes and intracellular amastigotes. In contrast, the expression levels of GT2 mRNA in amastigotes are about 15 times lower than the levels in promastigotes. The permeases coded by these genes are capable of transporting glucose, fructose, mannose, and galactose [7]. Furthermore, ribose can be transported by GT2 and GT3 [8]. The pellicular plasma membrane (PPM) contains the GT2 and GT3 proteins, whilst the

flagellar membrane is the primary site where GT1 is found [9]. A null mutant was created through targeted gene replacement to afford a more detailed characterisation of such transporters [10]. The assimilation of hexose substrates was no longer possible with the phenotype obtained in this way [7], leading to slower promastigote growth and intracellular amastigotes became less viable [11].

As in other eukaryotes, a particular subgroup of crucial amino acids impossible to synthesise is present in Leishmania. Amino acid transporters figure among the eukaryote-encoded extensive permease families; for instance, *L. major* has up to 40 genes encoding such transporters. The AAP3 arginine carrier from *L. donovani* (LdAAP3) from the amino acid auxin permease (AAP) superfamily [12] was among the first amino acid carriers to receive a characterisation [13]. Since it is a key amino acid, arginine is assimilated by Leishmania from the host [14], with the intracellular arginine concentration being reported to act as transport regulator [15]. A recent study isolated a lysine transporter gene from *L. donovani* (LdAAP7), without which *L. donovani* cannot survive since it is the sole lysine transporter, as implied by the fact that attempts to knock it out have been unsuccessful.
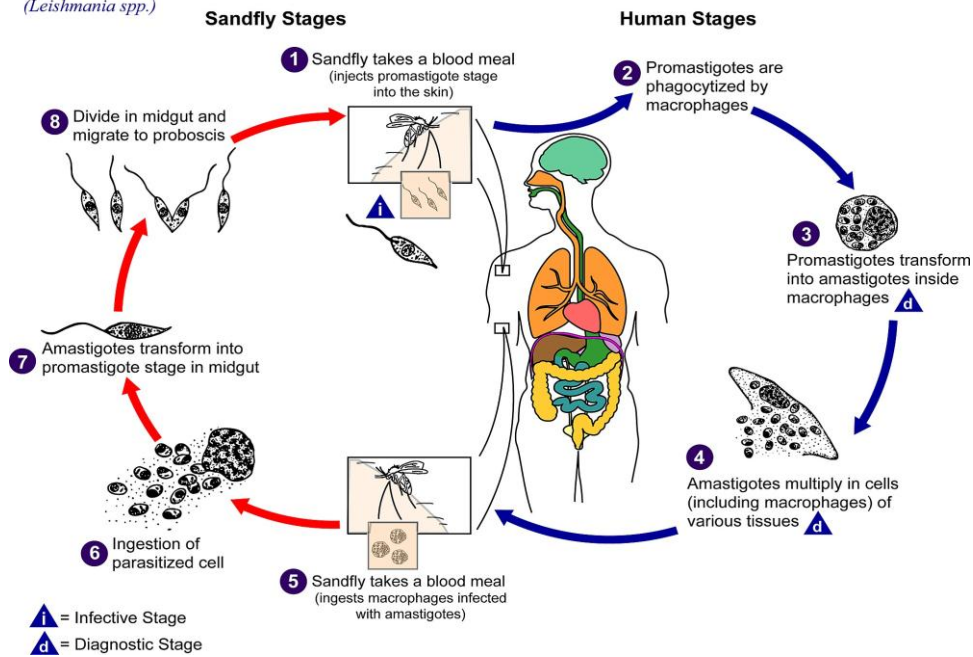
Figure 1. **Leishmania life cycle**. Leishmaniasis is transmitted by the bite of female sand-flies, which inject the infectious form of Leishmania parasites (promastigotes) into the human host. Promastigotes are ingested by the macrophages where they transform into amastigotes, that can divide and infect other mononuclear cells. In the sand-fly gut, amastigotes transform into promastigotes completing the Leishmania life cycle. Figure taken from [16].

Pteridine salvage mechanisms in Leishmania depend on a network of transporters and enzymes that are related to the resistance to antifolate therapy [17]. For instance, mutations that impair folate transporters activity result in the increased activity of pteridine transporters in *L. tarentolae* [18]. Unlike other organisms that use folate for purine biosynthesis, Leishmania is incapable of *de*

novo synthesis of purines, being dependent on the host for the acquisition of these nutrients, for which purine nucleosides must be transported over the membrane [19]. *L. donovani* nucleoside transporters (NTs) were the original genes of protozoan purine transporters that were discovered. More specifically, NT1 undertakes the transport of adenosine and pyrimidine nucleosides [20], while NT2 is responsible for the transport of inosine, guanosine, and xanthosine [21]. NT3 [22] and NT4 [23] is required for transporting hypoxanthine, xanthine, adenine, and guanine nucleobases. These purine transporters are active proton-coupled symporters that function by relying on the electrochemical gradient over the plasma membrane to yield the necessary energy, concentrating their substrates in the parasite [23,24]. It is worth noting that neither promastigotes nor amastigotes depend on any of these transporters, as every single-gene knockout null mutants remain viable [22,25]. This observation implies that these transporters may exhibit functional redundancy. Nonetheless, to be viable, Leishmania parasites are dependent on purine nucleobases, since the generation of NT3 and NT4 double-null mutant has not been achieved.

Numerous proteins employ iron as a cofactor, so its assimilation is vital. Fe^{2+} is stored in complexes alongside specialised proteins because of its toxicity to biological systems. Fe^{2+} transporters are particularly important for the Leishmania amastigotes present in macrophage phagolysosomes, where the

already poor concentration of Fe^{2+} is restricted even more by the lysosomal Fe^{2+} pump Nramp1 [26], which exhausts ferrous iron from phagosomes. Wilson et al. [27] demonstrated the presence of a membrane-bound NADPH-dependent iron reductase in *L. chagasi*, yielding Fe^{2+} from Fe^{3+}, most likely from iron-binding proteins like transferrin. Iron transporters (IT), two of which are so far known, can subsequently import the produced Fe^{2+}. The expression of LIT1, and possibly other ITs, appears to be limited to circumstances in which the phagolysosome is completely deficient in iron [28].

Cationic polyamines (e.g. putrescine, cadaverine and spermidine), which help cells to grow and develop and also underpin the cell cycle, are obtained by Leishmania either through synthesis or acquisition from the host. Despite being capable of undertaking the synthesis of such compounds, Leishmania encodes for polyamine transporters; to give an example, the plasma membrane polyamine transporter (POT1) of *L. major* is a member of the amino acid/polyamine/organocation (APC) superfamily, being responsible for the transport of putrescine/spermidine and has expression solely in the promastigote stage of development [29]. The extent to which polyamines are available may have a regulatory effect on POT1 expression.

Knocking out the gene encoding a particular Leishmania nutrient transporter is a common approach for investigating the role fulfilled by that transporter and how important it is. Apicomplexan parasites are not readily

amenable to genetic manipulation, making genome editing more challenging [30,31]. The genome editing system of Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated protein-9 (CRISPR/Cas9) relies on the prokaryote defence mechanism targeting invading viruses and plasmids [32,33] and is advantageous because it targets particular sequences, thus enabling gene editing without many off-target editions. The components of CRISPR/Cas9 are a prokaryotic endonuclease Cas9 and an engineered single-guide RNA (sgRNA) capable of detecting the desired DNA sequence and generating double-strand breaks (DSB), which results in genome sequence alterations. The DSB repair mechanism of the organism determines how extensive such alterations are [34].

Research into the functions of genes in parasitic protozoa has been considerably widened by the introduction of the targeted genome editing system CRISPR/Cas9. However, in the formulation of an implementation approach, consideration must be given to the DSB repair mechanism that the parasite in question possesses [35,36]. A two-vector approach is used to adapt CRISPR/Cas9 for genome editing in L. major, with the two vectors respectively enabling Cas9 nuclease expression and expressing sgRNA based on U6 promoter alongside DNA donor molecule. Nevertheless, use of DNA donor for genome editing in Leishmania is less than optimal, which is why mixed

populations are usually generated in Leishmania in the presence of episomal vectors.

Sollelis et al. [37] provided an overview of gene knockout with the use of CRISPR/Cas9 in *L. major*. A two-vector approach was also employed by Zhang and Matlashewski [38] to conduct genome editing on *L. donovani* with CRISPR/Cas9; the first vector encoded for Cas9, while the other vector involved transcription of the sgRNA sequence by the ribosomal promoter (sRNAP). Furthermore, to prevent sgRNA polyadenylation and make sure the sequence was long enough, insertion of a Hepatitis Delta Virus (HDV) ribozyme downstream of the sgRNA was conducted in one version of the second vector. The gene that encoded the miltefosine transporter (LdMT) was knocked out with both vectors. Results revealed that efficiency was enhanced by the introduction of the ribozyme downstream of the sgRNA sequence. Thus, it was concluded that the main DSB repair mechanism in *L. donovani* was interchromosomal homologous recombination. Additionally, end-joining mediated by microhomology was identified as well, but non-homologous end-joining was not.

Knockout of expression of presumed Leishmania membrane transporters will be achieved in the present project with CRISPR/Cas9 [37,38]. To determine the substrate specificity of the transporters, nutrient requirement and

metabolomics assays will be undertaken to be able to describe the phenotype of

the obtained null mutants (Figure 2).
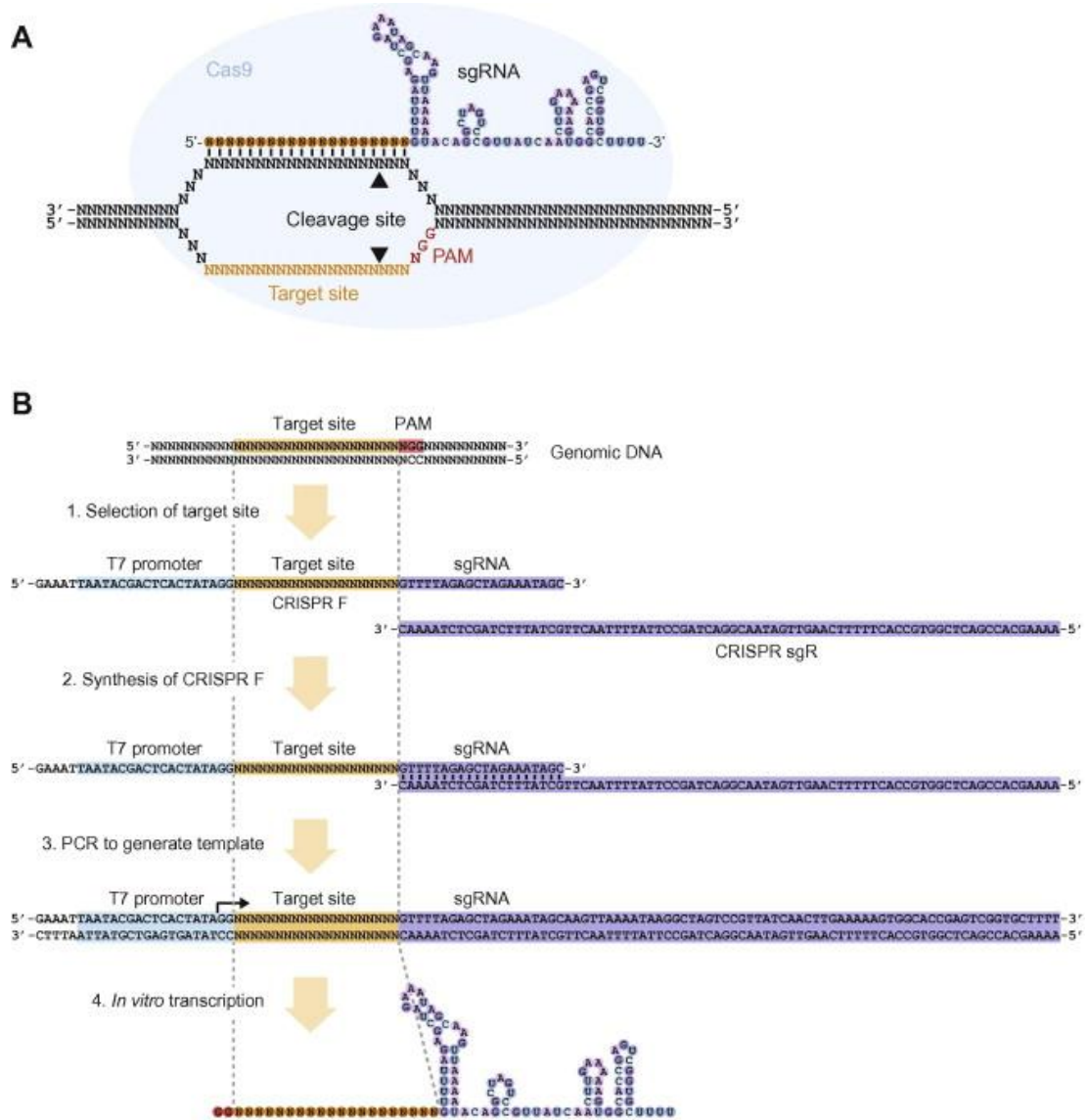
Figure 2. Synthetic guide RNA (sgRNA) template design. In panel (**A**) the formation of the Cas-9-sgRNA complex is shown. The target site is shown in orange, the protospacer adjacent motif in red, the sgRNA in purple, and the cleavage sites are indicated with black triangles. Panel (**B**) shows the strategy to produce the template by in-vitro transcription. First, the CRISPR-F primer contains the target site downstream of the T7 polymerase promoter and includes a sequence that overlaps with the CRISPR sg primer. PCR is used to generate the template for in vitro transcription. Figure taken from [39].

## 2. Materials and Methods.

2.1. Bioinformatics analysis.

The transmembrane transporters that were investigated in this work were selected from data of three omics (metabolomics, transcriptomic and proteomics) correlation studies (Kabli and Burchmore, unpublished) of gentamicin attenuated *L. mexicana* (H-line). Transcriptomic data revealed that an amino acid transporter (LmxM.27.1580), as well as a membrane transport protein (LmxM.16.0800) were significantly up-regulated in H-line, whereas glucose transporter 3 (LmxM.36.6280) and a putative pteridine transporter (LmxM.06.1260) were down-regulated in H-line, as shown in Table 1. The

selected transporters were significantly modulated in H-line, and these might be involved in the attenuation of this strain since it was also found that the encoded proteins also alter their expression and that many metabolites related to the transporters function changed their levels. To understand the function of each transporter and their role in H-line attenuation, the genes encoding for these transporters were knocked out. The structural homology models obtained using the portal Phyre2 [40] are shown in Figure 3.

Table 1. Fold change of the expression level of putative membrane transporters selected for this study. The Gene ID, the standard error, and the P value along with the adjusted P are shown for each of the selected genes.

Gene	Gene ID	log2(FC)	StdErr	P-value	P-adj
Amino acid transporter putative	LmxM.27.1580	0.61	0.09	2.1 E-11	3.08E-10
Glucose transporter 3	LmxM.36.6280	-0.54	0.12	8.49E-06	5.81E-05
Membrane transport protein putative	LmxM.16.0800	0.86	0.06	5.84E-54	1.21E-51

Pteridine transporter putative	LmxM.06.1 260	-0.85	0.04	5.53E-84	2.35E-85
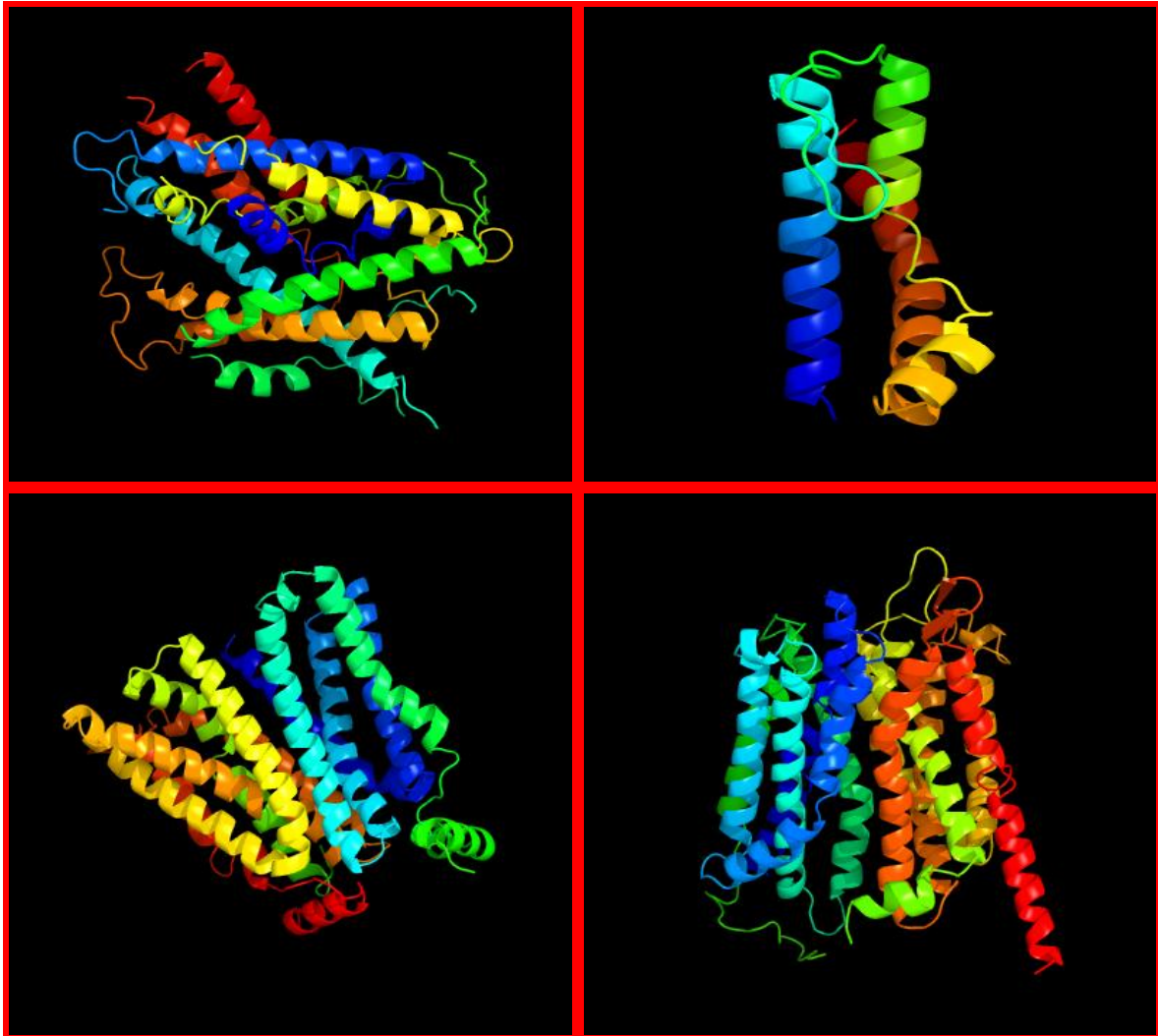
Figure 3. Model of the protein transporter proteins colored by rainbow N C.

Upper left panel correspond to the amino acid transporter 9 sodium-coupled.

Upper right membrane transporter protein structure sodium/bile acid symporter

family. Lower left, glucose transporter 3. Lower right, pteridine putative

transporter.

2.2. Parasite Culture.

Promastigotes from two strains of Leishmania were used, *L. Mexicana* WT (WHO strain M379) and *L. Mexicana* cas9 strain, which was generated by Cong L et al. [41]. Parasites were stored in liquid nitrogen at the Welcome Centre for Molecular Parasitology in the University of Glasgow. Wild-type *L. mexican* M379/WT and *L. mexican* M379/T7RNAP (Cas9) were cultivated in complete haemoflagellate minimal essential medium (HOMEM) (GIBCO®) and supplemented with 10% (vol/vol) heat-inactivated foetal calf serum (HI-FCS) (Labtech International). For transfection the same medium with 10% HI-FCS was supplemented with 10 µg/mL Puroumycine and +5 µg/Ml blastacline was used as selection antibiotic to make sure that the only thing we have in the media. Each culture was initially set up at a parasite density of 1×10^5 parasites/mL and sub-passaged every three days. Growth rate was assessed through daily counting for ten days.

2.3. Growth curve.

To monitor for changes in parasite growth rate a growth curve was determined using a Haemocytometer. Viable (live) were counted in five small squares in the centre of haemocytometer, using 2% formaldehyde, and the cell

counts were recorded every 24 hours. To ensure an accurate cell count we diluted the cells in order to achieve around 40 to 70 cells. The cell number per ml was calculated as follows:

Average number of cells in Five small squares square x dilution factor* x 10^5

*dilution factor is usually 2 (1:1 dilution with 1 X phosphate buffer saline (PBS) or 2% formaldehyde), but depends on whether the cells were further diluted.

2.4. Gene selection.

To edit the genes of interest, primers were designed and downloaded using the web tool kit (http://leishgedit.net/) [42].The sequences of these primers are shown in Table 2. Then, to carry out the knock out generation, a CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids was employed [42], which is available from (https://www.eurofinsgenomics.eu/).

Table 2. Sequences of the primers used in this study.

Gene	Primer name	Sequence
Amino acid transporte	Upstream forward	GCTCTCCCTCTTTCCGTTCACTGACGGCCG gtataatgcagacctgctgc
	Upstream	GTCCGGGCAGCGGGTGCGCTGCGTCATCAT

r (LmxM.27.1580)	reverse	actacccgatcctgatccag
	Downstream forward	ACGGTTGCCTCGATCTACGGCGAGGTGCACggttctggtagtggttccgg
	Downstream reverse	TCTCCTCCACACGTGTGAAACAAACACGCAccaatttgagagacctgtgc
	5' sgRNA	gaaattaatacgactcactataggCAGCCAATACTGTCTGCAGTgttttagagctagaaatagc
	3'sgRNA	gaaattaatacgactcactataggGCGACCACCGCAGACAAGCGgttttagagctagaaatagc
Glucose transporter 3 (LmxM.36.6280)	Upstream forward	GCCGACGCTGTTTCTGCTGACCGCTATCCTgtataatgcagacctgctgc
	Upstream reverse	CTGCACGTTCGCCTCCAACTTGTCGCTCATactacccgatcctgatccag
	Downstream forward	AGCGGGGAATTCGTCGGGAAGAAAGAAATGggttctggtagtggttccgg
	Downstream reverse	TCGCGATGCTGCCAATGCAGTCGAAACAGCccaatttgagagacctgtgc
	5' sgRNA	gaaattaatacgactcactataggGGGTTGGTTGCACGAAAGAAgttttagagctagaaatagc
	3'sgRNA	gaaattaatacgactcactataggGCGACCACCGCAGACAAGCGgttttagagctagaaatagc
Membrane transport protein (LmxM.16.0800)	Upstream forward	ATTTTTCCTCGTTGTTCCCCCCCTCCTCCCgtataatgcagacctgctgc
	Upstream reverse	GGCGATGAATGCCACGCCTAGAATGTCCATactacccgatcctgatccag
	Downstream forward	TCCATCGGTTTGAGCTACGTGACGTCGTTGggttctggtagtggttccgg
	Downstream reverse	ATGCATTCCTGGGCTGCTGCCGAGAATCTTccaatttgagagacctgtgc

	5' sgRNA	gaaattaatacgactcactatagg**CAAGCTTTCGCTTGG GGAGA**gttttagagctagaaatagc
	3'sgRNA	gaaattaatacgactcactatagg**TCTCTGACCCGTCCC CCTCG**gttttagagctagaaatagc
Pteridine transporter (LmxM.06.1260)	Upstream forward	**CGGTTTGACTCGTAGTTTGTGTCTTTGCTC**g tataatgcagacctgctgc
	Upstream reverse	**CATCTCCTCAATCGGCTGTGCGGACGCCAT**a ctacccgatcctgatccag
	Downstream forward	**CAGGGGGACTCACGGGAACCAAAGGCGG CC**ggttctggtagtggttccgg
	Downstream reverse	gaaattaatacgactcactatagg**AGTTCCGCGGTTGCC CCTTC**gttttagagctagaaatagc
	5' sgRNA	gaaattaatacgactcactatagg**ACATTTTTTGTACAA CTTT**gttttagagctagaaatagc
	3'sgRNA	**ATGGCAGGCCTGCGTCGGCTGAGCGCTCC G**ccaatttgagagacctgtgc

2.5.   Isolation of low-copy plasmids, P1 constructs, or cosmids.

To purify the constructs, the kit NucleoSpin (Macherey-Nagel) was employed according to the manufacturer's instructions. Briefly, 10-15 μL of pT/ pPLOT plasmid was transferred into 10–15 mL of a saturated *E. coli* LB culture supplemented with 10-15 μL ampicillin was incubated with shaking at 37°C overnight. Cells were then pelleted in a micro centrifuge for 10 min at 11,000 x *g,* the supernatant was discarded, and 500 μL of lysis buffer was added. Next, the pellet was resuspended in 500 μL of buffer A1, and then 500 μL of Buffer A2 were added. Afterward, the suspension was mixed gently and incubated at room temperature for 5 min. Finally, 600 μL of Buffer A3 were added and

mixed until blue samples turn colorless completely. Then, the lysate was clarified by centrifugation for10 min at 11,000 x g at room temperature. The clarified lysate was passed through a NucleoSpin column and washed using 600 µL Buffer A4 (supplemented with ethanol). Finally, the plasmid was eluted with 50 µL Buffer AE, and NanoDrop was used to determine the DNA concentration of each plasmid.

2.6. *Polymerase chain reaction (PCR).*

2.6.1. *PCR amplification of targeting fragments*.

The PCR reaction was carried out using HiFi polymerase (Roche), 30 ng of the pT puro or pT blast plasmid and 2µM of each primer. The PCR program used was 5min at 94°C followed by 40 cycles of 30sec at 94°C, 30sec at 65°C, 2min 15sec at 72°C. For the final elongation, the PCR reaction was incubated for 7min at 30sec at 72°C.

2.6.2. *PCR amplification of sgRNA templates*.

The template was 2µM of 3 or 5 sgRNA, 2µM of primer G00, and 2µM of the specific forward primer. The PCR program was 30sec at 98°C followed by 35 cycles of 10sec at 98°C, 30sec at 60°C, 15sec at 72°C.

The PCR products were examined by electrophoresis using 1% (for plasmid) or 2% (for sgRNA) agarose gels in 1x Tris Buffered Saline (TBS), and

adding 5 µL Sybr safe DNA gel stain (Invitrogen). The samples were loaded using 2µl of Gel loading dye purple (Biolabs), and a 1Kb (for plasmid) or 100 bp (for sgRNA) DNA ladder was used as a reference. The gel was running at 95 volts for ~40-60 min.

2.7. *Transfection.*

LeishGEdit Transfection Protocol.

A transfection mix was prepared using 100 µl of PCR amplicons to which 150 µl of transfection buffer [25 µl $CaCl_2$, 83 µl 3x Tb-BSF, 42 µl water] was added.

Two exponentially growing cultures of *L. mexicana* M379/WT as negative control and *L. mexicana* M379/T7RNAP (Cas9) construct were obtained over several passages until the day of transfection. For each transfection 1 x 10^7 cells were resuspended in transfection buffer to which 2x sgRNA templates (3' and 5' primers) and 2x KO cassettes (pT puro/ pT blast) were added. The mixture was transferred to electroporation cuvettes (Bio-Rad) and transfected with one pulse with X-001 (AmaxaNucleofector 2b). After that, the cells were transferred into the pre-warmed medium and incubated for 8-16 h, then 20 µg/mL Puromycin and 5 µg/Ml blasticidin were added and the cells incubated until drug-resistant populations emerged (10-14 days).

2.8. Diagnostic PCRs.

To screen for loss of the target gene in KO cell lines (amino acid transporter, glucose transporter 3, membrane transport protein, and pteridine transporter), genomic DNA was isolated 10–14 days' post-transfection with the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's instructions. For each transfection cell line, 5×10^7 cells were employed. DNA was eluted by adding 50 µl Buffer AE directly onto the centre of the spin column membrane, which then was incubated at room temperature for 1 min, and then centrifuged for 1 min at 7000 x g.

A PCR reaction was carried using 100ng of the isolated DNA with 2 µM of each primer, and 1 unit of HiFi Polymerase (Roche). The PCR steps used were 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 5 s at 60°C, 50 s at 72°C followed by

a final elongation step for 7 min at 72°C. 5µl of this reaction was run on a 2% agarose gel to check for the presence of the expected product.

3. Results.

3.1. Wild type and CAS 9 *L. mexicana* Growth Curve.

A comparative growth profile was estimated for WT and cas9 *L. mexicana* cultured under the same conditions (Figure 4). Leishmania promastigotes undergo three differentiation phases: mid-logarithmic or procyclic promastigotes ($\sim5\times10^6$ parasites/mL), late-logarithmic ($\sim1\times10^7$ parasites /mL) and stationary ($\sim2.5\times10^7$ parasites/mL) (32). Late-log promastigotes were seen between days 4 to 6 for both lines (Figure 4), these parasites had longer flagellum and were more motile when compared to mid-log promastigotes. As shown in figure 1, no significant differences in growth rate were found between these two strains.
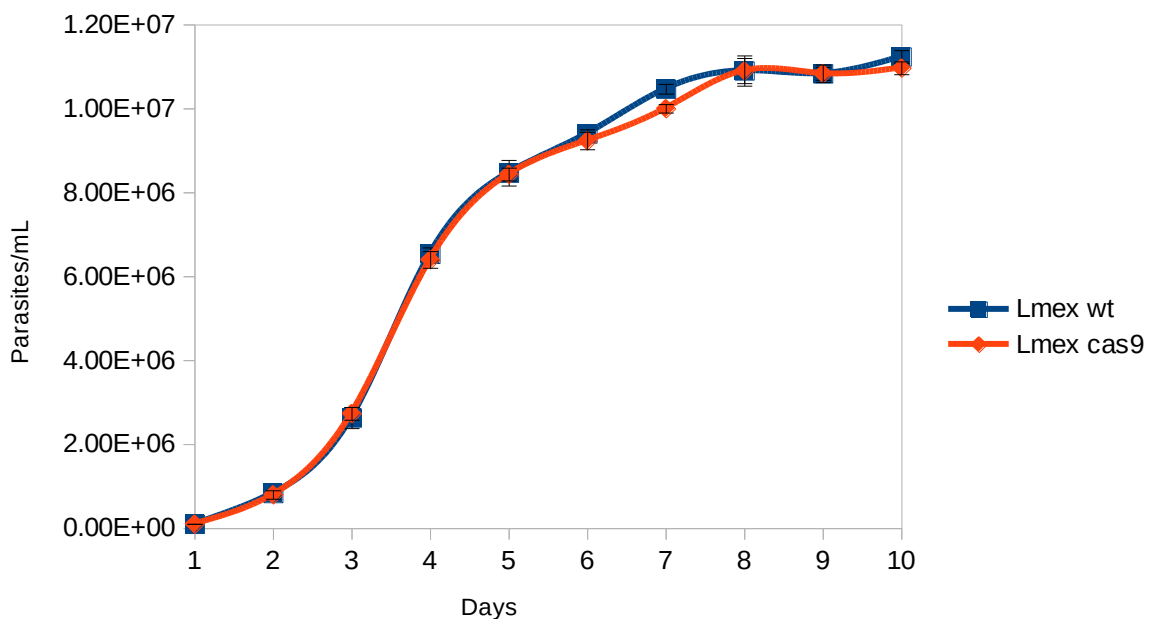

Figure 1. *Leishmania mexicana* **Growth Curve.** Wild type and Cas9 promastigotes were cultured under the same conditions and parasite density was determined by daily counting in a Neubauer Chamber, starting with 1×10^5 parasites on day 0. Error bars indicate standard error of the mean based on 3

biological replicates. This graph shows that WT parasite density is higher during the first 3 days and it reaches late-log phase (~1×10^7 parasites /mL) between days 4-6 as same the Cas9. After day 7 the cell density in both cell lines was similar and maintain a constant growth.

3.2. PCR Amplification Gene Plasmid.

In order to obtain the necessary material to generate the sgRNAs for each of the target genes a PCR amplification was carried out form the plasmids encoding for the different transporters. In Figure 5 the PCR reaction for the amino acid transporter in pT blasticidine (Upper row) or pT puromycine (Lower row) is shown. The PCR product correspond to the expected size. In Figure 6 the PCR amplification reaction for the glucose transporter 3 in pT blasticidine or pT puromycine is shown. In figure 7 the PCR reaction for the pteridine transporter in pT puromucine (Upper row) or pT blsticidine (Lower row) is shown. Finally, in figure 8 the PCR reaction for the putative membrane transport protein in pT blasticidine (Upper row) or pT puromycine (Lower row) is shown.
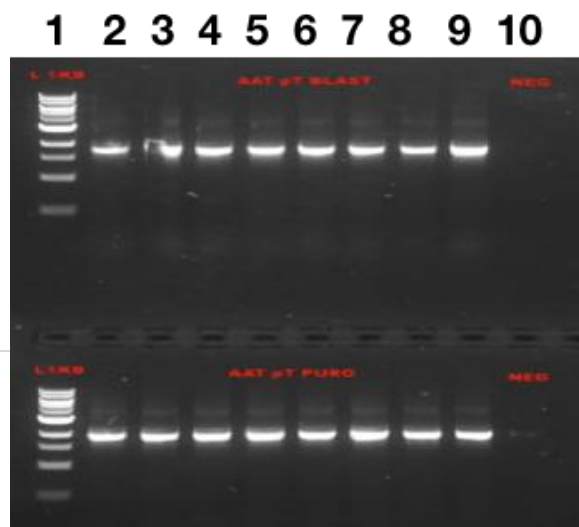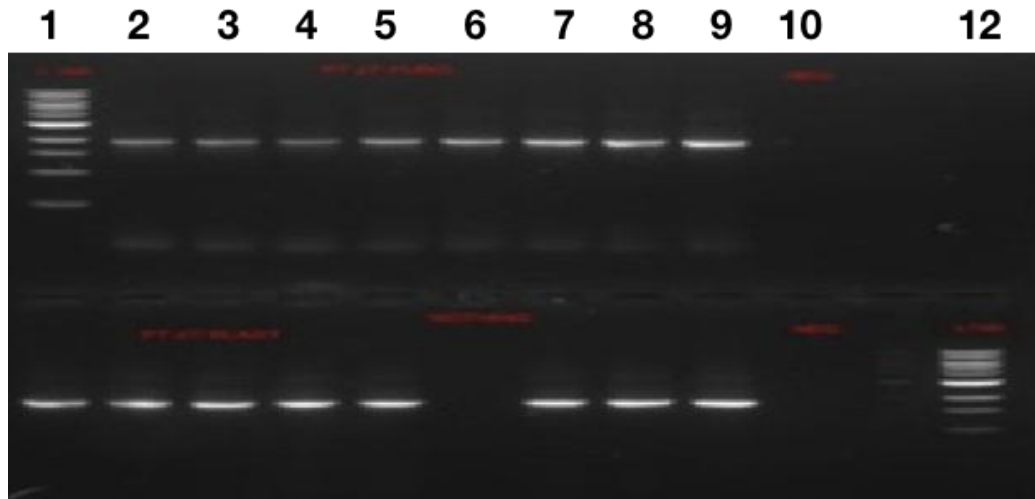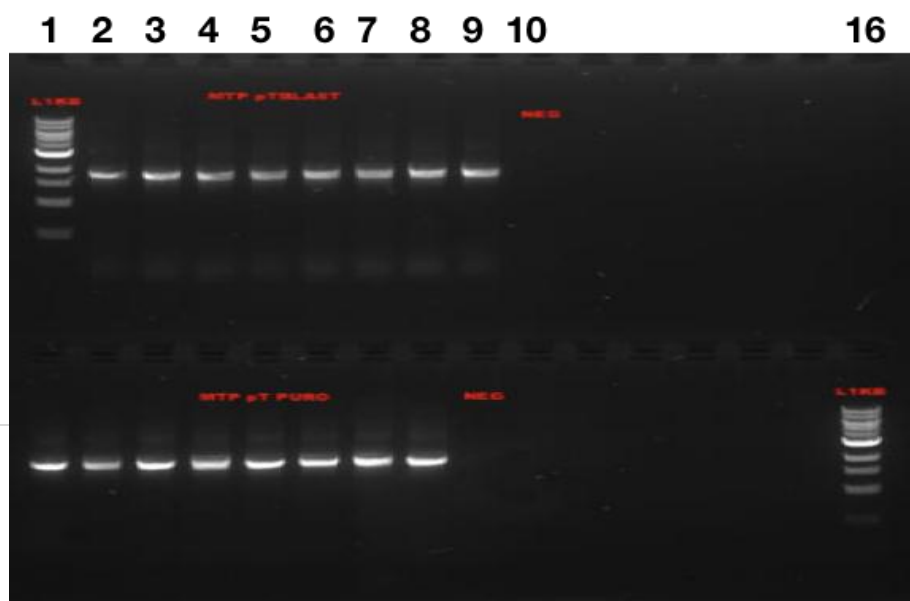
Figure 5. 1% agarose gel electrophoresis of the PCR amplification products of the putative amino acid transporter (lanes 2-9) in pT blasticidine (upper row) or pT puromycin (lower row). Lane 1 show the DNA ladder and lane 10 show the negative control.
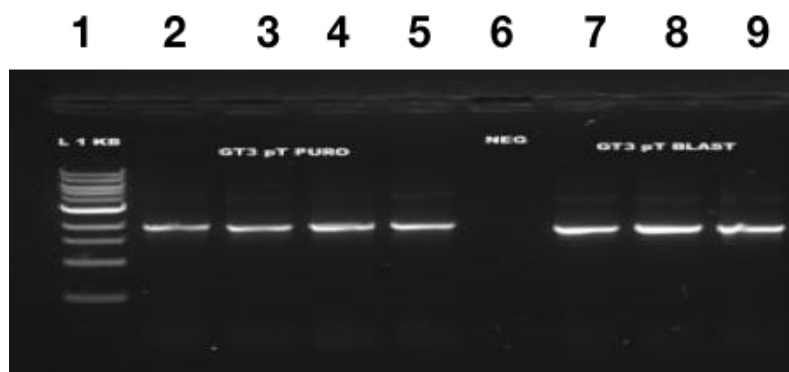
Figure 6. 1% agarose gel electrophoresis of the PCR amplification products of the glucose transporter 3 in pT puromycin (lanes 2-5) or pT blasticidine (lanes 7-9). Lane 1 show the DNA ladder and lane 6 show the negative control.
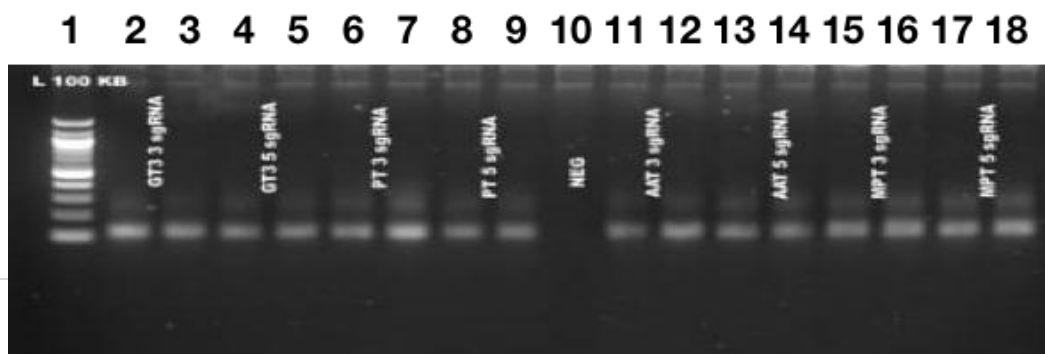

Figure 7. 1% agarose gel electrophoresis of the PCR amplification products of the putative pteridine transporter in pT blasticidine (lanes 2-9 upper row) or pT puromycin (lanes 1-9 lower row). Lane 1 or 12 show the DNA ladder and lane 10 show the negative control.

Figure 8. 1% agarose gel electrophoresis of the PCR amplification products of the membrane transport protein in pT blasticidine (lanes 2-9 upper row) or pT puromycin (lanes 1-8 lower row). Lane 1 or 12 show the DNA ladder and lanes 10 to 9 show the negative control.

After amplifying by PCR the sequences all of the targeted genes a PCR reaction to obtain the sgRNA was carried out according to the protocol described in the methods section. In figure 9 the product of the sgRNA amplification for the all the targeted genes is shown.

Figure9. 2% agarose gel electrophoresis of the PCR amplification products of 3' and 3' sgRNA for the targeted genes. Lane1. DNA ladder, lanes 2-3 glucose transporter 3'sgRNA, lanes 4-5 glucose transporter 3'sgRNA, lanes 6-7 pteridine transporter 3'sgRNA, lanes 8-9 pteridine transporter 5'sgRNA, lane 10 Negative control, lanes 11-12 amino acid transporter 3'sgRNA, lanes 13-14 amino acid transporter 5'sgRNA, lanes 15-16 membrane transport protein 3'sgRNA, lanes 17-18 membrane transport protein 5'sgRNA.

3.3. *Transfection*.

After several transfection attempts we failed to obtain the transfectants of any of the genes, and therefore we could not continue with the characterisation of the phenotype of null mutants, In Table 3 the summary of the transfection attempts is shown.

Table 3. Transfection experiments.

Gene	Transfection date	State after transfection	Observation
All genes	12/6/2018	Contaminated	The contamination source was the media.
All genes	19/6/2018	Contaminated	Unknown contamination origin.
All genes	27/6/2018	Contaminated	The contamination observe after starting the growth curve.
All genes	12/7/2018	Contaminated	After three days post transfection contamination was observed.

*All gene [Amino acid transporter (LmxM.27.1580), membrane transport protein (LmxM.16.0800), glucose transporter 3 (LmxM.36.6280) and a putative pteridine transporter (LmxM.06.1260)].

4. Discussion.

In this work we aimed to establish the role that nutrient acquisition may have in Leishmania attenuation, to this end we first identified four membrane nutrient transporters in *L. mexicana* that seem to be involved in attenuation since their expression is modulated in this strain. After identifying these targets, we prepared a strategy for knocking down these genes using CRISPR-Cas9 technology [33,34]. We have successfully amplified by PCR all the needed sgRNAs. However, we have not been able to determine the functional importance of their targeted genes in the attenuated phenotype given that during transfection the cultures have become repeatedly contaminated.

The source of contamination of the first attempt was identified to be the media, but for the last three attempts, it has not been identified. To address this issue it is necessary to improve our culture handling techniques and to prepare a new batch of reagents and material to ensure that the next attempt is successful.

Since the knocked out strains are necessary to carry out the phenotypic characterization of the null mutants, some of the specific aims of this project will not be addressed because there is no time left to carry out the additional experiments. However, we expect that knocking down nutrient transporters will inhibit the growth of the null mutants since Leishmania is unable to synthesized *de novo* many nutrients. Albeit some transporter redundancy is expected, and therefore we anticipate that the reduction in growth rate will differ depending on

the relative importance of the specific nutrient that is transported by the knocked out gene, as well as the presence of alternative nutrient acquisition mechanisms. For instance, it has been established that single mutants of glucose transporters remain viable indicating some functional redundancy among this family of proteins [10,11], although gene expression of these proteins is stage specific. Also, alternative sources of metabolites can change their regulation to compensate for the lack of a specific transporters, for instance, it has been shown that when impairing folate uptake with drugs the pteridine transport system becomes up-regulated [17,18]. However, it remains possible that if one of the transporters targeted in this study is the only one with a particular substrate specificity present in Leishmania we expect that it will be impossible to obtain a null mutant since it will be lethal to the parasite. Of the genes selected for this study, the putative amino acid transporter and the membrane transport protein are up-regulated in the attenuated *L. mexicana*; therefore, it is possible that their overexpression is a compensation mechanism for the function of the genes that are directly affected in attenuation. On the other hand, the glucose transporter 3 and the pteridine transporter are already down-regulated in the attenuated strain employed in this study, probably interfering with the nutrient acquisition mechanisms of Leishmania. We expect that knocking out any of these genes will impair growth and further the attenuated phenotype.

In summary, to understand how Leishmania attenuation occurs is important because it can give us insight into the relation genotype-phenotype in association with virulence. Also, this strategy can be useful in identifying genes whose function can be successfully blocked, which can point toward novel drug targets. It is very likely that the attenuation phenotype is in most cases a consequence of the modification of the expression of a cohort of genes. Therefore, it can also be useful to identify other proteins that participate in the same biological processes as the target gene to establish what are the key switches in this process that can render a Leishmania strain attenuated. This remains as a perspective of the work presented here.

5. References.

1   Burchmore, R. J. S. and Barrett, M. P. (2001) Life in vacuoles – nutrient acquisition by Leishmania amastigotes. Int. J. Parasitol., Pergamon **31**, 1311–1320.

2   Bridges, D. J., Pitt, A. R., Hanrahan, O., Brennan, K., Voorheis, H. P., Herzyk, P., de Koning, H. P. and Burchmore, R. J. S. (2008) Characterisation of the plasma membrane subproteome of bloodstream formTrypanosoma brucei. Proteomics, Wiley-Blackwell **8**, 83–99.

3   ter Kulle, B. H. (1993) Glucose and proline transport in kinetoplastids. Parasitol. Today, Elsevier Current Trends **9**, 206–210.

4   McConville, M. J., de Souza, D., Saunders, E., Likic, V. A. and Naderer, T. (2007) Living in a phagolysosome; metabolism of Leishmania amastigotes. Trends Parasitol., Elsevier **23**, 368–75.

5   Naderer, T. and McConville, M. J. (2007) The Leishmania-macrophage interaction: a metabolic perspective. Cell. Microbiol., Wiley/Blackwell (10.1111) **10**, 301–308.

6   Burchmore, R. J. and Landfear, S. M. (1998) Differential regulation of multiple glucose transporter genes in Leishmania mexicana. J. Biol. Chem., American Society for Biochemistry and Molecular Biology **273**, 29118–26.

7 Rodriguez-Contreras, D., Feng, X., Keeney, K. M., Bouwer, H. G. A. and Landfear, S. M. (2007) Phenotypic characterization of a glucose transporter null mutant in Leishmania mexicana. Mol. Biochem. Parasitol., Elsevier **153**, 9–18.

8 Naula, C. M., Logan, F. J., Logan, F. M., Wong, P. E., Barrett, M. P. and Burchmore, R. J. (2010) A glucose transporter can mediate ribose uptake: definition of residues that confer substrate specificity in a sugar transporter. J. Biol. Chem., American Society for Biochemistry and Molecular Biology **285**, 29721–8.

9 Piper, R. C., Xu, X., Russell, D. G., Little, B. M. and Landfear, S. M. (1995) Differential targeting of two glucose transporters from Leishmania enriettii is mediated by an NH2-terminal domain. J. Cell Biol., Rockefeller University Press **128**, 499–508.

10 Burchmore, R. J. S., Rodriguez-Contreras, D., McBride, K., Barrett, M. P., Modi, G., Sacks, D., Landfear, S. M. and Landfear, S. M. (2003) Genetic characterization of glucose transporter function in Leishmania mexicana. Proc. Natl. Acad. Sci. **100**, 3901–3906.

11 Feng, X., Rodriguez-Contreras, D., Buffalo, C., Bouwer, H. G. A., Kruvand, E., Beverley, S. M. and Landfear, S. M. (2009) Amplification of

an alternate transporter gene suppresses the avirulent phenotype of glucose transporter null mutants in *Leishmania mexicana*. Mol. Microbiol., Wiley/Blackwell (10.1111) **71**, 369–381.

12   Akerman, M., Shaked-Mishan, P., Mazareb, S., Volpin, H. and Zilberstein, D. (2004) Novel motifs in amino acid permease genes from Leishmania. Biochem. Biophys. Res. Commun., Academic Press **325**, 353–366.

13   Shaked-Mishan, P., Suter-Grotemeyer, M., Yoel-Almagor, T., Holland, N., Zilberstein, D. and Rentsch, D. (2006) A novel high-affinity arginine transporter from the human parasitic protozoan Leishmania donovani. Mol. Microbiol., Wiley/Blackwell (10.1111) **60**, 30–38.

14   Opperdoes, F. R. and Coombs, G. H. (2007) Metabolism of Leishmania: proven and predicted. Trends Parasitol., Elsevier **23**, 149–58.

15   Darlyuk, I., Goldman, A., Roberts, S. C., Ullman, B., Rentsch, D. and Zilberstein, D. (2009) Arginine homeostasis and transport in the human pathogen Leishmania donovani. J. Biol. Chem., American Society for Biochemistry and Molecular Biology **284**, 19800–7.

16   Prevention, C.-C. for D. C. and. (2017) CDC - Leishmaniasis - Biology.

17   Cunningham, M. L. and Beverley, S. M. (2001) Pteridine salvage throughout the Leishmania infectious cycle: implications for antifolate chemotherapy. Mol. Biochem. Parasitol. **113**, 199–213.

18   Kündig, C., Haimeur, A., Légaré, D., Papadopoulou, B. and Ouellette, M. (1999) Increased transport of pteridines compensates for mutations in the high affinity folate transporter and contributes to methotrexate resistance in the protozoan parasite Leishmania tarentolae. EMBO J., European Molecular Biology Organization **18**, 2342–51.

19   Hammond, D. J. and Gutteridge, W. E. (1984) Purine and pyrimidine metabolism in the trypanosomatidae. Mol. Biochem. Parasitol., Elsevier **13**, 243–261.

20   Vasudevan, G., Carter, N. S., Drew, M. E., Beverley, S. M., Sanchez, M. A., Seyfang, A., Ullman, B. and Landfear, S. M. (1998) Cloning of Leishmania nucleoside transporter genes by rescue of a transport-deficient mutant. Proc. Natl. Acad. Sci. U. S. A., National Academy of Sciences **95**, 9873–8.

21   Carter, N. S., Drew, M. E., Sanchez, M., Vasudevan, G., Landfear, S. M. and Ullman, B. (2000) Cloning of a novel inosine-guanosine transporter gene from Leishmania donovani by functional rescue of a transport-

deficient mutant. J. Biol. Chem., American Society for Biochemistry and Molecular Biology **275**, 20935–41.

22   Ortiz, D., Sanchez, M. A., Pierce, S., Herrmann, T., Kimblin, N., Archie Bouwer, H. G. and Landfear, S. M. (2007) Molecular genetic analysis of purine nucleobase transport in Leishmania major. Mol. Microbiol., Wiley/Blackwell (10.1111) **64**, 1228–1243.

23   Ortiz, D., Sanchez, M. A., Koch, H. P., Larsson, H. P. and Landfear, S. M. (2009) An acid-activated nucleobase transporter from Leishmania major. J. Biol. Chem., American Society for Biochemistry and Molecular Biology **284**, 16164–9.

24   Stein, A., Vaseduvan, G., Carter, N. S., Ullman, B., Landfear, S. M. and Kavanaugh, M. P. (2003) Equilibrative nucleoside transporter family members from Leishmania donovani are electrogenic proton symporters. J. Biol. Chem., American Society for Biochemistry and Molecular Biology **278**, 35127–34.

25   Liu, W., Boitz, J. M., Galazka, J., Arendt, C. S., Carter, N. S. and Ullman, B. (2006) Functional characterization of nucleoside transporter gene replacements in Leishmania donovani. Mol. Biochem. Parasitol., Elsevier **150**, 300–307.

26   Fortier, A., Min-Oo, G., Forbes, J., Lam-Yuk-Tseung, S. and Gros, P.

      (2005) Single gene effects in mouse models of host: pathogen interactions.

      J. Leukoc. Biol. **77**, 868–877.

27   Wilson, M. E., Lewis, T. S., Miller, M. A., McCormick, M. L. and

      Britigan, B. E. (2002) Leishmania chagasi: uptake of iron bound to

      lactoferrin or transferrin requires an iron reductase. Exp. Parasitol.,

      Academic Press **100**, 196–207.

28   Jacques, I., Andrews, N. W. and Huynh, C. (2010) Functional

      characterization of LIT1, the Leishmania amazonensis ferrous iron

      transporter. Mol. Biochem. Parasitol., Elsevier **170**, 28–36.

29   Heby, O., Roberts, S. C. and Ullman, B. (2003) Polyamine biosynthetic

      enzymes as drug targets in parasitic protozoa. Biochem. Soc. Trans.,

      Portland Press Limited **31**, 415–9.

30   Burle-Caldas, G. de A., Grazielle-Silva, V., Laibida, L. A., DaRocha, W.

      D. and Teixeira, S. M. R. (2015) Expanding the tool box for genetic

      manipulation of Trypanosoma cruzi. Mol. Biochem. Parasitol. **203**, 25–33.

31   Docampo, R. (2011) Molecular parasitology in the 21st Century. Essays

      Biochem. **51**, 1–13.

32   Horvath, P. and Barrangou, R. (2010) CRISPR/Cas, the Immune System

of Bacteria and Archaea. Science (80-.). **327**, 167–170.

33   Rath, D., Amlinger, L., Rath, A. and Lundgren, M. (2015) The CRISPR-

Cas immune system: biology, mechanisms and applications. Biochimie

117, 119–28.

34   Doudna, J. A. and Charpentier, E. (2014) The new frontier of genome

engineering with CRISPR-Cas9. Science (80-.). **346**, 1258096–1258096.

35   Lander, N., Chiurillo, M. A. and Docampo, R. (2016) Genome Editing by

CRISPR/Cas9: A Game Change in the Genetic Manipulation of Protists. J.

Eukaryot. Microbiol. **63**, 679–90.

36   Vinayak, S., Pawlowic, M. C., Sateriale, A., Brooks, C. F., Studstill, C. J.,

Bar-Peled, Y., Cipriano, M. J. and Striepen, B. (2015) Genetic

modification of the diarrhoeal pathogen Cryptosporidium parvum. Nature

523, 477–480.

37   Sollelis, L., Ghorbal, M., MacPherson, C. R., Martins, R. M., Kuk, N.,

Crobu, L., Bastien, P., Scherf, A., Lopez-Rubio, J.-J. and Sterkers, Y.

(2015) First efficient CRISPR-Cas9-mediated genome editing in

Leishmania parasites. Cell. Microbiol. **17**, 1405–1412.

38   Zhang, W.-W. and Matlashewski, G. (2015) CRISPR-Cas9-Mediated

      Genome Editing in *Leishmania donovani*. MBio **6**, e00861-15.

39   Bassett, A. and Liu, J.-L. (2014) CRISPR/Cas9 mediated genome

      engineering in Drosophila. Methods **69**, 128–136.

40   Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N. and Sternberg, M. J.

      E. (2015) The Phyre2 web portal for protein modeling, prediction and

      analysis. Nat. Protoc. **10**, 845–58.

41   Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D.,

      Wu, X., Jiang, W., Marraffini, L. A., et al. (2013) Multiplex Genome

      Engineering Using CRISPR/Cas Systems. Science (80-.). **339**, 819–823.

42   Beneke, T., Madden, R., Makin, L., Valli, J., Sunter, J. and Gluenz, E.

      (2017) A   CRISPR Cas9 high-throughput genome editing toolkit for

      kinetoplastids. R. Soc. Open Sci. **4**, 170095.