



Functional characterization of LIT1, the *Leishmania amazonensis* ferrous iron transporter

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ABSTRACT

Leishmania amazonensis LIT1 was identified based on homology with IRT1, a ferrous iron transporter from *Arabidopsis thaliana*. $\Delta lit1$ *L. amazonensis* are defective in intracellular replication and lesion formation *in vivo*, a virulence phenotype attributed to defective intracellular iron acquisition. Here we functionally characterize LIT1, directly demonstrating that it functions as a ferrous iron membrane transporter from the ZIP family. Conserved residues in the predicted transmembrane domains II, IV, V and VII of LIT1 are essential for iron transport in yeast, including histidines that were proposed to function as metal ligands in ZIP transporters. LIT1 also contains two regions within the predicted intracellular loop that are not found in *Arabidopsis* IRT1. Deletion of region I inhibited LIT1 expression on the surface of *Leishmania* promastigotes. Deletion of region II did not interfere with LIT1 trafficking to the surface, but abolished its iron transport capacity when expressed in yeast. Mutagenesis revealed two motifs within region II, HGHQH and TPPRDM, that are independently required for iron transport by LIT1. D263 was identified as a key residue required for iron transport within the TPPRDM motif, while P260 and P261 were dispensable. Deletion of proline-rich regions within region I and between regions I and II did not affect iron transport in yeast, but in *L. amazonensis* were not able to rescue the intracellular growth of $\Delta lit1$ parasites, or their ability to form lesions in mice. These results are consistent with a potential role of the unique intracellular loop of LIT1 in intracellular regulation by *Leishmania*-specific factors.

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1. Introduction

The parasitic protozoan *Leishmania* is the causative agent of leishmaniasis, an increasingly serious human infectious disease that ranges from mild, self-healing cutaneous lesions to a lethal visceral form [1]. Individuals become infected with metacyclic promastigotes during the bite of an infected sand fly. After being taken up by macrophages, metacyclic promastigotes are found inside parasitophorous vacuoles (PVs) that have properties of phagolysosomes [2]. Inside PVs the parasites replicate as amastigotes, giving rise to the symptoms of leishmaniasis [3].

In order to survive and replicate within acidified PVs, *Leishmania* has to overcome very challenging conditions, which include the restricted access to essential micronutrients [4]. One such micronutrient, iron, is critical for the intracellular survival of several pathogens, which depend on high affinity membrane transporters for acquiring this metal [5–7]. Nramp1, a transporter located on late

endosomal compartments, is thought to restrict pathogen growth by depleting phagosomes in iron [4,8]. Iron is an essential co-factor for many biologically active molecules, but its acquisition is a major challenge under physiological conditions. Ferrous iron (Fe^{2+}) is soluble at neutral pH, but its accumulation must be tightly controlled to avoid generation of toxic hydroxyl radicals by the Fenton reaction. For this reason, in aerobic environments iron is largely present in the insoluble ferric (Fe^{3+}) form, which relies on carrier proteins such as transferrin, ferritin or lactoferrin for its transport and storage. Reductases thus play a central role in iron acquisition, by converting Fe^{3+} into Fe^{2+} for transmembrane transport [9]. Ferric iron reductase activity was detected in *Leishmania* [10], and recently a plasma membrane molecule with properties of a ferrous transporter was identified for the first time in this parasite [11].

Analysis of the *Leishmania* genome revealed two identical genes encoding a membrane protein that shares 30% identity with IRT1, an iron regulated transporter from *Arabidopsis thaliana* [11,12]. As previously shown for IRT1 [13], LIT1 (*Leishmania* Iron Transporter 1) expression rescued growth of the *Saccharomyces cerevisiae* $\Delta fet3fet4$ strain that is defective in iron transport [14,15], and was capable of stimulating iron import when over-expressed in the parasites [11]. Also similar to *Arabidopsis* IRT1 [13], LIT1 was capable of translocating other divalent cations such as manganese, cad-

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mium and zinc, but showed strong preference for iron [11]. The LIT1 protein was only detected in *Leishmania amazonensis* amastigotes residing intracellularly within PVs, which are predicted to be iron-poor late endosomal compartments [11]. Importantly, deletion of the LIT1 gene from the genome of *L. amazonensis* inhibited the parasite's ability to replicate within macrophages, and to cause cutaneous lesions in mice [11]. These findings established LIT1 as the first putative ferrous iron transporter to be identified in trypanosomatid parasites, and showed that it is an important determinant of *Leishmania* virulence.

The *Leishmania* LIT1 belongs to the ZRT-, IRT-like family (ZIP) of membrane proteins, which includes both zinc and iron transporters [16]. Only very limited information is available on the structure, function, selectivity and localization of ZIP transporters. The *A. thaliana* IRT1 was the first member of this family to be identified through functional complementation studies in *S. cerevisiae* [13]. ZIP proteins are predicted to share a similar membrane topology, with both the amino- and carboxy-terminal ends located on the extracellular side of the membrane [11,12,16]. ZIP family proteins range from 227 to 476 amino acid residues in length, and contain alpha helical regions that span the membrane five to eight times. LIT1 has eight predicted transmembrane domains, and is 432 amino acid residues in length [11]. The length variations within the ZIP family are due to a variable region between transmembrane domains III and IV, which is predicted to form a loop that extends into the cytoplasm [12,16]. Interestingly, the LIT1 variable region is significantly longer than that of IRT1 by 72 amino acid residues [11]. In this study, we used site directed mutagenesis as a tool to investigate the functional role of this unique region in *Leishmania* LIT1, as well as the role of conserved residues that were previously implicated in iron transport in other members of the ZIP family of membrane transporters.

2. Materials and methods

2.1. Parasites

L. amazonensis promastigotes were cultured in M199 media (Invitrogen) supplemented with 20% heat-inactivated fetal bovine serum, 5% penicillin–streptomycin, 0.1% hemin (25 mg/ml in 50% triethanolamine), 10 mM adenine, and 5 mM L-glutamine at 26 °C. Axenic *L. amazonensis* amastigotes were grown in M199 media supplemented with 0.25% glucose, 0.5% trypticase, and 20 mM sodium succinate (pH 4.5) at 32 °C.

2.2. Molecular cloning and mutagenesis to generate LIT1 alleles

Single base pair mutations were generated in the yeast expression vector p426 (URA2⁺) containing the LIT1 gene [11] by site directed polymerase chain reaction (PCR) (Stratagene). The PCR reaction consisted of the following: 5 µl 10× reaction buffer, 1 µl dNTP, 100 ng of plasmid DNA, 125 ng of each oligonucleotide and 2 µl PfuTurbo DNA polymerase (2.5 U/µl) at a final reaction volume of 50 µl. The thermal cycler was programmed as follows: initial denaturation at 95 °C for 30 s; 12 cycles at 95 °C for 30 s, 55 °C for 60 s and 68 °C for 30 min. The PCR product was digested with DpnI to degrade the methylated parental plasmid, the product was transformed into DH5α cells, and plasmid DNA from selected colonies was subjected to sequencing to identify clones containing the mutagenized construct. Wild type and mutant LIT1 constructs were transformed into *S. cerevisiae* Δ *fet3fet4* as previously described [11] and selected on minimal essential plates without uracil (the vector confers the ability to synthesize uracil).

To create truncated LIT1 proteins, site directed polymerase chain reaction (PCR) was used to amplify upstream and down-

stream flanking sequences of the targeted DNA sequence marked for deletion. PCR products were cloned into pGEM-T Easy Vector (Promega). The sequence downstream of the deleted fragment within LIT1 was digested with BglII and XbaI, gel purified and ligated to the BglII and XbaI linearized pGEM-T Easy vector carrying the sequence upstream of the deleted fragment within LIT1. The pGEM-T Easy vector carrying the truncated LIT1 gene was verified through sequencing, the truncated gene was excised using BamHI digestion, ligated into BamHI linearized p426 (URA2⁺) vector, and again confirmed by sequencing. To perform parallel studies in *Leishmania*, the BamHI excised LIT gene was cloned into BglIII digested pF4X1.4 sat vector (Jena Bioscience, Jena, Germany) to create pF4X1.4-LIT1. Wild type and mutant pF4X1.4-LIT1 were transformed into the *L. amazonensis* Δ *lit1* strain and selected on media supplemented with 50 µg/ml nourseothricin [11]. The pF4X1.4 sat vector promotes stable chromosomal integration, resulting in high level expression (Jena Bioscience).

2.3. Functional complementation studies in yeast

For functional studies in yeast, Δ *fet3fet4* *S. cerevisiae* (provided by M. Guerinot, Dartmouth College, Hannover, NH) carrying the *Leishmania* iron transporter were grown in yeast peptone dextrose (YPD), YPD containing 20 µM bathophenanthroline disulfonate (BPS) and in selective medium lacking uracil (SCD-URA), as described [11]. The mutant constructs, vector alone or the vector containing the LIT1 gene were placed in Δ *fet3fet4* using the yeast expression vector p426 (URA2⁺) that enables yeast to synthesize uracil. The cells were grown in minimal media lacking uracil (SCD-URA3) for 2 days at 30 °C and the cultures were then diluted to optical densities (OD) of 10, 1, 0.1, and 0.01. Aliquots (5 µl) of the dilution were spotted on both yeast peptone dextrose (YPD) and on agarose plates containing yeast peptone dextrose supplemented with 20 µM of the iron chelator, bathophenanthroline disulfonate (BPS) (YPD + 20 µM BPS). The plates were incubated for 2 days at 30 °C and digitally photographed.

2.4. Iron transport assays

Iron uptake in Δ *fet3fet4* was carried out as described [17]. Briefly, cells were washed twice in assay buffer containing 10 mM MES, 2% glucose and 1 mM nitriloacetic acid, pH 6.1, resuspended in ice-cold assay buffer, and 50 µl of the cell suspension was added to 450 µl of assay buffer containing 1 mM sodium ascorbate and 1.2 µl of a 1:10 dilution of ⁵⁵Fe (82.69 mCi/mg, or 39 mCi/ml; PerkinElmer). The suspension was incubated at 30 °C or 0 °C for 20 min, vortexed, vacuum filtered through Whatman GF/C filters, washed three times with ice-cold SSW buffer (1 mM EDTA, 20 mM Na₃-citrate, pH 4.2, 1 mM CaCl₂, 5 mM MgSO₄ and 1 mM NaCl) [17]. The 20 min time point chosen for these assays was found to be within the linear ⁵⁵Fe uptake range in kinetic assays. Radioactively labeled cells were detected in a liquid scintillation counter (Wallac 1409; PerkinElmer) as described [11].

2.5. Macrophage infection and *Leishmania* intracellular growth assay

Bone marrow macrophages were isolated from C57BL/6 mice (Charles River Laboratories) as previously described [18]. Macrophages were seeded at 3.8×10^5 cells per well onto 6 well plates containing three coverslips per well in RPMI 1640 supplemented with 10% fetal bovine serum containing 5% L cell supernatant (as a source of M-CSF) at 37 °C and 5% CO₂. 24 h later 3.8×10^5 amastigotes were added to the macrophages for 1 h at 34 °C and 5% CO₂. Cells were washed three times with phosphate buffered saline containing calcium and magnesium (PBS+/+) and

supplemented with 2% L cell supernatant and 5% fetal bovine serum for the duration of the infection. Infected macrophages were fixed with 2% paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100 and stained with DAPI. The number of intracellular parasites was determined microscopically at 1000 \times (Zeiss Axiovert 200) in a minimum of 300 macrophages per coverslip, in triplicate.

2.6. Immunofluorescence microscopy

Exponentially growing *L. amazonensis* promastigotes were fixed in 2% paraformaldehyde (PFA) overnight at 4°C. The cells were washed three times with (PBS+/+), incubated in blocking buffer (10% goat serum and 50 mM ammonium chloride in PBS+/+) for 30 min, followed by anti-LIT rabbit polyclonal antibodies in blocking buffer for 1 h at room temperature. The cells were then washed five times with PBS+/+ and incubated with Alexa Fluor 488 conjugated goat anti-rabbit antibodies (Invitrogen), washed and stained with DAPI for 30 min at room temperature. Images were acquired using a fluorescence microscope (Zeiss Axiovert 200) equipped with a CCD camera (CoolSNAP HQ; Photometrics) controlled by imaging software (MetaMorph; Molecular Devices Corporation).

3. Results

3.1. Functional conservation of residues required for iron transport by *L. amazonensis* LIT1

Residues His-96, His-197, Ser-198, His-224 and Glu-228 of *A. thaliana* IRT1, which correspond to residues His-108, His-283, Ser-284, His-309, Glu-313 in LIT1 respectively, were previously shown to be important for iron transport [19] (Fig. 1, red). These IRT1 residues, postulated to be located on the inner surface of a channel formed by the transmembrane domains, are likely candidates

for binding metal ions during transport. In order to determine if the corresponding residues were also required for iron transport by LIT1, we replaced His-108, His-283, Ser-284, His-309, Glu-313 residues by alanine. The mutant alleles were then cloned into a yeast overexpression vector and transformed into the Δ *fet3fet4* *S. cerevisiae* strain which lacks the FET3 multicopper oxidase required for high affinity Fe²⁺ transport, and the FET4 low affinity Fe²⁺ transporter [14,15]. The auxotroph strains carrying the mutant plasmids were then tested for growth in the presence or absence of the iron chelator BPS. As previously reported for IRT1, the mutated LIT1 alleles failed to rescue the growth phenotype of Δ *fet3fet4* under iron limiting conditions (Fig. 2A and B). To confirm that the growth failure of these mutant strains was due to loss-of-function, and not to overexpression of the mutated alleles, we carried out iron uptake assays to directly measure transporter function. Unlike Δ *fet3fet4* carrying wild type LIT1, which can acquire ⁵⁵Fe, yeast strains carrying the mutated alleles failed to transport ⁵⁵Fe intracellularly (Fig. 2D). In addition to the absolutely conserved residues discussed above, we also investigated the requirement for LIT1 Glu-112, which corresponds to Asp-100 in IRT1, and is predicted to be located on the extracellular loop between transmembrane domains II and III. LIT1 carrying a replacement of Glu-112 by alanine failed to rescue the ability of Δ *fet3fet4* to grow in iron limiting conditions (Fig. 2B), and to incorporate ⁵⁵Fe (Fig. 2D). We also changed LIT1 residues Glu-115, Asp-153, Arg-157 and Ser-319 to alanine. Complementation tests for Δ *fet3fet4* growth and ⁵⁵Fe uptake showed that these residues (of which only Glu-115 is conserved between IRT1 and LIT1) did not disrupt function of the *Leishmania* iron transporter (Fig. 2C and D).

3.2. Requirement for amino acids with aromatic and bulky side groups at the 382 and 391 positions in LIT1

Residues Tyr-382 and Asp-391, which correspond to Tyr-295 and Glu-305 in IRT1, are conserved throughout the ZIP transporter family [19] and predicted to reside within transmembrane domain VII. Replacement of these two residues with alanine was reported to have no effect in the ability of IRT1 to transport iron [19]. In contrast, the alanine replacements of these residues abolished the ability of LIT1 to promote growth in iron-depleted medium, and to uptake iron (Fig. 3A and B). However, when Tyr-382 was replaced by phenylalanine, growth and iron transport were restored, suggesting a requirement at this location for a residue with an aromatic side chain. We also changed Asp-391 to either Asn-391 or Glu-391, and the results of growth and iron uptake assays showed that LIT1 appears to have a preference for a bulky side chain in this position, since the D391N and D391E mutations partially rescued the growth phenotype of Δ *fet3fet4* on iron limiting medium. These strains also showed an intermediate level of ⁵⁵Fe accumulation within cells (Fig. 3A and B).

These results suggested that LIT1 carrying the Y382A and D391A substitutions might have failed to rescue Δ *fet3fet4* iron transport and growth because of defects in protein expression or intracellular folding/targeting. To directly investigate this issue, we transfected Δ *lit1* *L. amazonensis* promastigotes [11] with LIT1 carrying the Y382A and D391A mutations. Both mutated transporters were detected by immunofluorescence on the surface of non-permeabilized Δ *lit1* *L. amazonensis* (Fig. 3C), in a punctate pattern that is very consistent with the pattern of endogenous LIT1 detected in intracellular amastigotes [11]. These observations indicate that the alanine substitutions did not disrupt the intracellular folding and plasma membrane trafficking of the transporter. Thus, the Y382 and D391 residues, which are predicted to reside either within or adjacent to transmembrane VII of LIT1, may be uniquely required for the passing of metal ion substrates across the membrane.

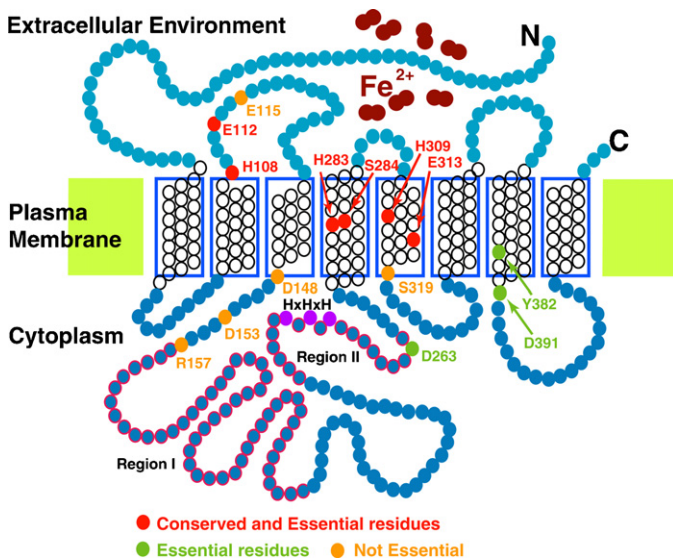


Fig. 1. Summary of the mutations analyzed for phenotypes in iron transport and cellular growth. The figure shows a schematic representation of the *Leishmania* iron transporter LIT1, with the predicted carboxy- and amino-termini in direct contact with the extracellular medium, and the variable region forming an intracellular loop facing the cytosolic side of the plasma membrane. Boxed residues correspond to the predicted transmembrane regions I–VIII. The residues in red (H108, E112, D263, H283, S284, H309 and E313) are conserved between LIT1 and Arabidopsis IRT1, and required for promoting iron acquisition when LIT1 is expressed in the iron transport-deficient Δ *fet3fet4* *S. cerevisiae* strain. In green (Y382, D263 and D391) are the amino acids that are essential for iron transport in LIT1, but not in IRT1. In orange (D148, D153, R157, P259, P260 and S319) are the residues not required for iron transport in LIT1. Blue residues outlined in red were designated as regions 1 and 2 and targeted for deletion in this study. HxHxH indicates the histidine-rich motif within region 1 that is essential for iron transport.

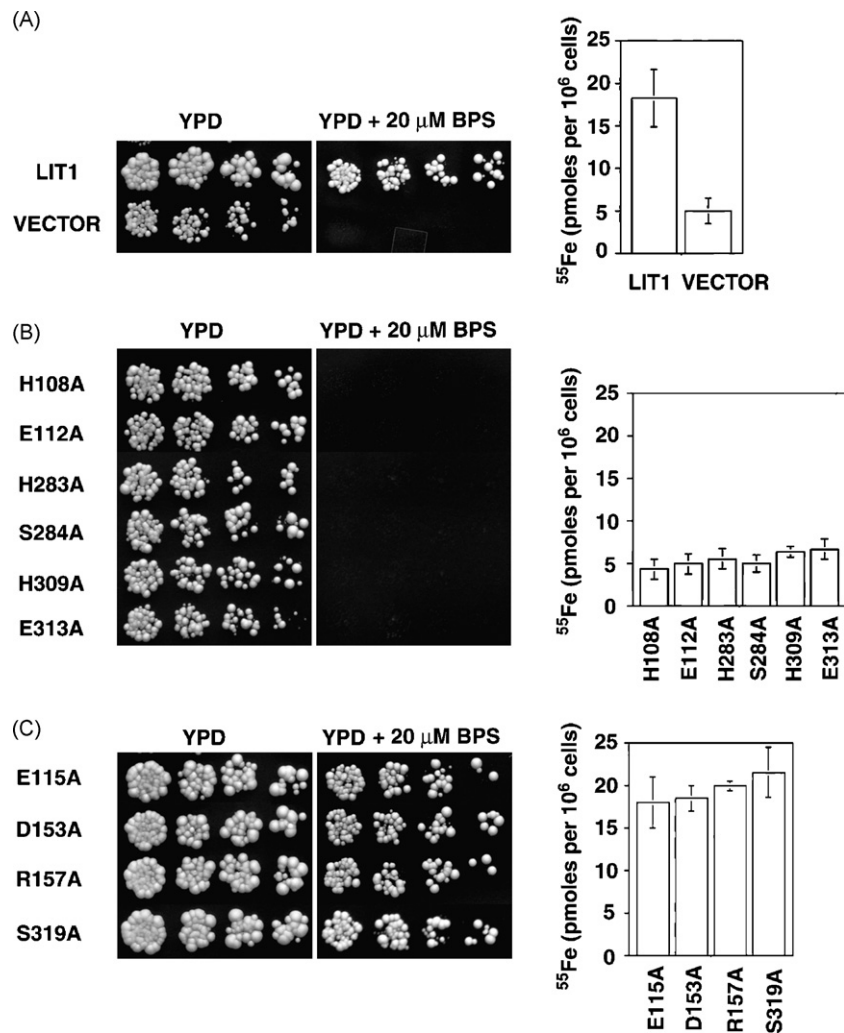


Fig. 2. Conserved residues are critical for LIT1 function. The iron transport-deficient Δ *fet3fet4* strain was transformed with wild type LIT1, empty vector or mutant LIT1 constructs. Transformed strains were spotted on agar supplemented (YPD + 20 μ M BPS) or not (YPD) with the iron chelator, bathophenanthroline disulfonate (BPS), in serial 1:10 dilutions. (A) Wild type LIT1 was able to complement the growth phenotype of Δ *fet3fet4* under iron limit condition, whereas Δ *fet3fet4* transformed with empty vector failed to do so. (B) The LIT1 conserved amino acids H108A, E112A, H283A, S284A, H309A, E313A were mutagenized to alanine. The transformed strains grew on iron rich agar (YPD) at comparable rates to WT LIT1, but these strains failed to grow under iron-deficient conditions (YPD + 20 μ M BPS). (C) Δ *fet3fet4* expressing LIT1 carrying the mutations E115A, D153A, R157A, S319A grew on both YPD and YPD + 20 μ M BPS. (D) The ability of Δ *fet3fet4* carrying wild type LIT1, empty vector, and LIT1 mutant constructs to accumulate iron ^{55}Fe was measured. LIT1 carrying the H108A, E112A, E115A, H283A, S284A, H309A, E313A, Y382A and D391A mutations did not restore the iron transport deficiency of Δ *fet3fet4*, indicating that these residues are essential for iron transport. In contrast, D148A, D153A, R157A, S319A, D391N or D391E restored the iron transport deficiency of Δ *fet3fet4*, indicating that these residues are not essential. The data represent average \pm SD of triplicates.

3.3. Functional role of the cytoplasmic variable region of LIT1

The predicted intracellular loop region of the *Leishmania* LIT1 transporter is longer than that of IRT1 by 72 amino acids: residues 158–211 (53 amino acids, region I) and residues 245–264 (19 amino acids, region II) (Fig. 1). We hypothesized that these two regions might have important roles in regulating LIT1 function. To address this issue, we created two truncated LIT1 constructs: *lit1* (Δ 158–211) and *lit1* (Δ 245–264) and performed yeast complementation experiments. Unlike wild type LIT1, the two deletion alleles (lacking regions I and II) did not complement the Δ *fet3fet4* iron transport and growth defect (Fig. 4A and B). One concern was that the large size of region I deletion *lit1* (Δ 158–211) could have prevented this truncated protein from reaching the plasma membrane. Indeed, when this construct was expressed in *L. amazonensis*, it failed to be detected on the cell surface by immunofluorescence with anti-LIT1 antibodies, in both fixed/non-permeabilized, and live promastigotes (Fig. 4C).

Residues 251–255 of region II consist of the histidine-rich motif HGHQH. Such motifs found in the variable region between transmembrane domains III and IV of ZIP family members were proposed to be potential cytoplasmic metal binding domains [20]. This might explain why *lit1* (Δ 245–264) was not able to complement the Δ *fet3fet4* iron transport defect under iron limiting conditions. To directly verify the contribution of the histidine motif to LIT1 function, we generated a construct lacking only the five amino acids comprising the HxHxH motif. Similar to the *lit1* (Δ 245–264) construct, *lit1* (Δ 251–255) failed to rescue the growth phenotype of Δ *fet3fet4* and showed no iron uptake capacity (Fig. 4B). Both *lit1* (Δ 245–264) and *lit1* (Δ 251–255) mutant proteins were properly localized to the plasma membrane of in both fixed/non-permeabilized, and live *L. amazonensis* (Fig. 4C), indicating that the iron transport defect is likely to have been a consequence of inability of the transporter to bind metal ions through the histidine-rich motif, and not of improper targeting to the plasma membrane.

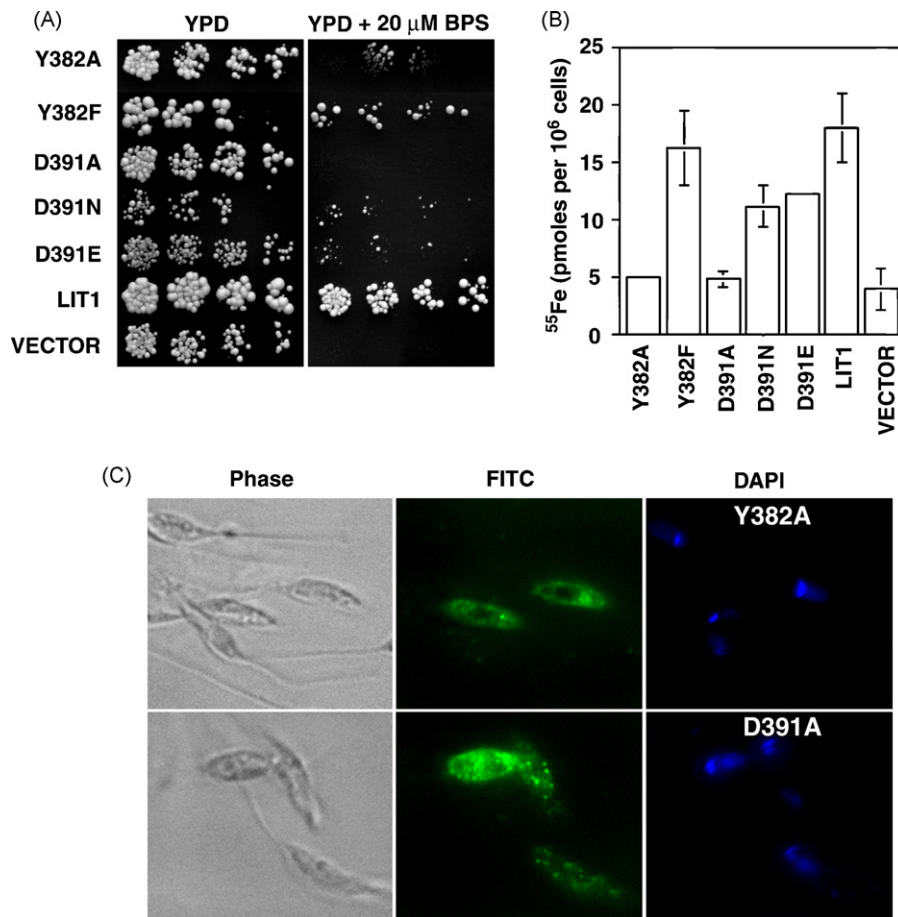


Fig. 3. Aromatic and bulky side groups of LIT1 residues Y382 and D391 are essential for iron transport. Results are shown for growth (A) or ^{55}Fe uptake (B) of $\Delta\text{fet3fet4}$ yeast expressing wild type and mutant LIT1. Yeast carrying Y382F but not Y382A were able to grow in iron limiting media and accumulate ^{55}Fe , indicating a requirement for aromatic side chains required at position 382. Likewise, D391E and D391N were able to rescue the growth and iron uptake phenotypes of $\Delta\text{fet3fet4}$, but not D391A. The ^{55}Fe data represent average \pm SD of triplicates. (C) LIT1 (Y382A) and LIT1 (D391A) were detected by immunofluorescence on the plasma membrane (green) when expressed in Δlit1 *L. amazonensis* promastigotes (green). Δlit1 promastigotes were used as a negative control; the parasite's DNA is stained with DAPI (blue).

3.4. Identification of aspartic acid 263 as critical for LIT1 function

We also investigated the role of a proline-rich region unique for LIT1, located in close proximity to the histidine-rich motif, within region II. The deletion construct *lit1* ($\Delta 259$ –264) was also not capable of rescuing growth and iron uptake by $\Delta\text{fet3fet4}$ (Fig. 5A and B), indicating a requirement for this TPPRDM domain, in addition to the histidine-rich motif. One possibility was that the two proline residues within this region might be required for positioning the histidine-rich motif in a conformation required for iron binding. However, when LIT1 carrying P260A, P261A, or double P260A P261A substitutions were expressed in $\Delta\text{fet3fet4}$ yeast, the ability to rescue iron transport and growth was maintained (Fig. 5A and B). When we investigated the role of Asp-263, a residue that is conserved in some members of the ZIP family, we found that the D263A substitution abolished the ability of LIT1 ability to rescue the iron transport and growth defect of $\Delta\text{fet3fet4}$ in iron limiting conditions. In contrast, LIT1 (D263E) and LIT1 (D263N) were as efficient as wild type LIT1 in supporting growth and iron uptake (Fig. 5A and B). After expression in *Leishmania*, the two constructs that could not rescue iron transport in $\Delta\text{fet3fet4}$, *lit1* ($\Delta 259$ –264) (lacking the HxHXH motif) and LIT1 (D263A), were localized on the plasma membrane by immunofluorescence (Fig. 5C). Thus, the observed loss-of-function appeared to be related to a requirement for Asp-263 (or a similar size residue such as Asn or Glu at that position), and not a defect in trafficking of the mutated transporter to the plasma membrane.

3.5. Identification of LIT1 regions specifically required for *Leishmania* intracellular growth in macrophages

The presence of unique regions within the predicted intracellular loop of LIT1 suggested that this portion of the protein might play a regulatory role through interactions with *Leishmania*-specific proteins. Thus, we examined the effect of additional truncations in this region, with the goal of identifying regions important for LIT1 function in *Leishmania*, but not in yeast. Two additional deletion constructs in proline-rich regions, *lit1* ($\Delta 195$ –201), within region I and *lit1* ($\Delta 230$ –239) (between regions I and II) were generated, and yeast growth complementation and iron uptake assays revealed that they were still able to rescue the growth of $\Delta\text{fet3fet4}$ yeast under iron limiting conditions, and to promote iron transport at rates comparable to strains expressing wild type LIT1 (Fig. 6A and B). When these constructs were expressed in Δlit1 *L. amazonensis*, immunofluorescence of non-permeabilized promastigotes showed that the mutated proteins were correctly targeted to the plasma membrane (Fig. 6C). These parasites were then differentiated into axenic amastigotes and used to infect bone marrow-derived macrophages. The growth rates of parasites expressing the *lit1* ($\Delta 195$ –201) and *lit1* ($\Delta 230$ –239) alleles were followed over the course of 72 h. Amastigotes expressing wild type LIT1 were able to grow intracellularly as previously reported [11] (Fig. 6D). Interestingly, although *lit1* ($\Delta 195$ –201) and *lit1* ($\Delta 230$ –239) did not lead to defects in iron transport in yeast and encoded proteins able to reach normally the plasma

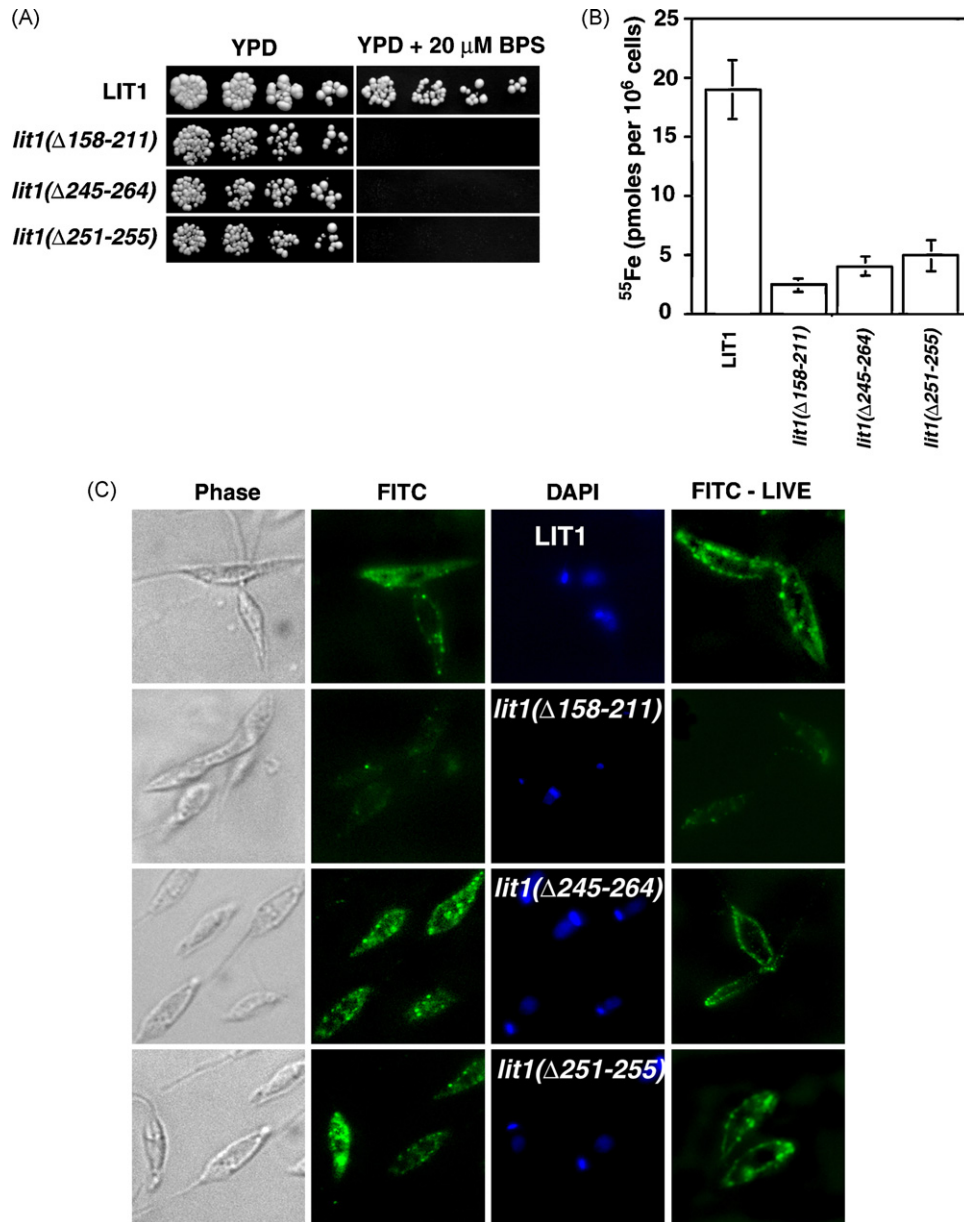


Fig. 4. Unique intracellular regions of *Leishmania* LIT1 are required for iron transport. Iron transport-deficient $\Delta fet3fet4$ yeast carrying wild type LIT1 or the LIT1 truncation constructs *lit1* ($\Delta 158-211$), *lit1* ($\Delta 245-264$) and *lit1* ($\Delta 251-255$) were spotted on YPD agar and YPD agar + 20 μM BPS, in serial 1:10 dilutions. (A) $\Delta fet3fet4$ carrying *lit1* ($\Delta 158-211$), *lit1* ($\Delta 245-264$) and *lit1* ($\Delta 251-255$) grew on iron rich agar (YPD) but failed to grow on iron limiting agar (YPD + 20 μM BPS). (B) The loss-of-function phenotypes observed on agar grow assays were confirmed by iron uptake assays. $\Delta fet3fet4$ carrying wild type LIT1 was able to accumulate ^{55}Fe , whereas *lit1* ($\Delta 158-211$), *lit1* ($\Delta 245-264$) and *lit1* ($\Delta 251-255$) showed significantly lower uptake levels. The data represent average \pm SD of triplicates. (C) Immunofluorescence showed that wild type LIT1, *lit1* ($\Delta 245-264$) and *lit1* ($\Delta 251-255$) constructs were targeted to the plasma membrane when expressed in $\Delta lit1$ *L. amazonensis*. In contrast, *lit1* ($\Delta 158-211$) failed to localize to the plasma membrane of $\Delta lit1$ promastigotes. LIT1 immunofluorescence, green, parasite DNA, blue. FITC, anti-LIT1 IF on fixed/non-permeabilized promastigotes; FITC-LIVE, anti-LIT1 IF on live promastigotes.

membrane in *Leishmania*, the truncated proteins were not able to rescue the ability of $\Delta lit1$ *L. amazonensis* to grow intracellularly in macrophages (Fig. 6D), and to cause cutaneous lesions in mice (Fig. 6F). These results are consistent with the potential existence of *Leishmania*-specific pathways that regulate the iron transport function of LIT1.

4. Discussion

In this study we performed a detailed mutagenesis analysis to functionally characterize LIT1, a ferrous iron transporter required for the intracellular growth and virulence of *L. amazonensis* [11]. LIT1 was identified and proposed to function as a ferrous iron transporter based on its 31% identity with IRT1 from *A. thaliana*. IRT1 is

expressed in the roots of *Arabidopsis* under iron limiting conditions [16], and mutations in this gene cause severe leaf chlorosis and growth defects [21]. Addition of exogenous ferrous iron complements the chlorotic and growth phenotypes of the *A. thaliana* *irt1* mutants, pointing to iron deficiency as the underlying cause of the defects [21]. Mutagenesis studies further defined the role of IRT1 in iron acquisition, identifying key residues involved in metal substrate translocation and selectivity [19]. Since many of the residues show to be involved in iron transport in IRT1 are conserved in *Leishmania* LIT1, we sought to obtain direct evidence for their role in iron acquisition.

We took advantage of the previous observation that LIT1 expression rescues the growth defect of the *S. cerevisiae* mutant strain $\Delta fet3fet4$ under iron-deficient conditions [11], in order to iden-

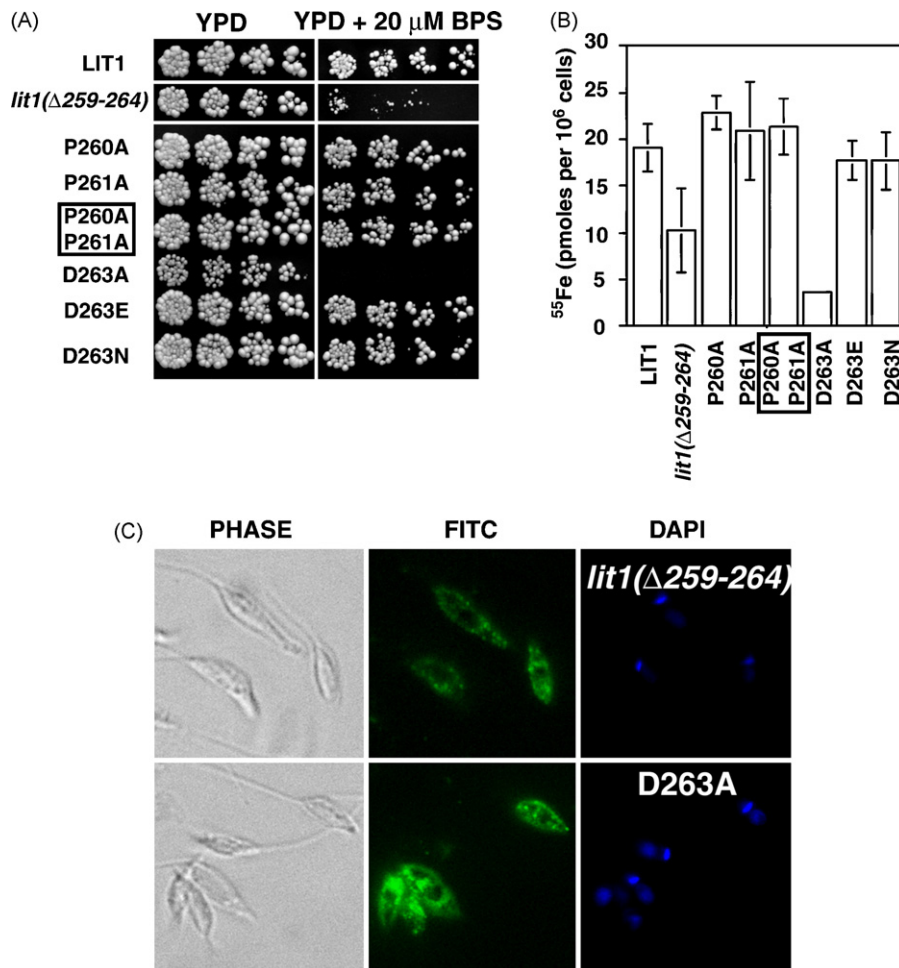


Fig. 5. Residue D263 within region II is critical for LIT1 function. (A) Δ *fet3fet4* carrying wild type LIT1, *lit1* (Δ259–264), *lit1* (P260A), *lit1* (P261A), *lit1* (P260A, P261A), *lit1* (D263A), *lit1* (D263E), and *lit1* (D263N) were spotted on iron rich or iron limiting agar, in serial 1:10 dilutions. All strains were able to grow on iron rich media (YPD), but *lit1* (Δ259–264) and *lit1* (D263A) failed to restore the iron transport deficiency of Δ *fet3fet4* when grown on iron limiting conditions, indicating that these regions are essential for LIT1 function. (B) Similar results were obtained in ⁵⁵Fe uptake assays. The data represent average \pm SD of triplicates. (C) Immunofluorescence showed that *lit1* (Δ259–264) and *lit1* (D263A) localized to the plasma membrane when expressed in Δ *lit1* *L. amazonensis* promastigotes. LIT1 immunofluorescence, green, parasite DNA, blue.

tify residues and motifs in LIT1 required for iron transport. Using alanine mutagenesis we found that residues His-108, Glu-112, His-283, Ser-284, His-309 and Glu-313, which are conserved among ZIP family members [19], are essential for the function of *Leishmania* LIT1 as an iron transporter. These residues, positioned adjacent to or within transmembrane regions II, IV and V, are predicted to function as metal ligands, and thus strongly reinforce the functional role of LIT1 as a ferrous iron transporter. His-283, in particular, along with the adjacent polar Ser-284 residue, correspond to ZIP family residues previously proposed to comprise part of an intramembranous heavy metal binding site along the transport pathway [12]. Our results showing that His-108, Glu-112, His-283, Ser-284, His-309, and Glu-313 are essential for LIT1-mediated iron transport in yeast are very consistent with previous findings implicating the corresponding residues of *Arabidopsis* IRT1: His-96, Asp-100, His-197, Ser-198, His-224 and Glu-228 [19]. Unlike that prior study, which concluded that Tyr-295 and Asp300 in IRT1 were not required [19], we found that residues Tyr-382 and Asp-391 of LIT1 are essential for promoting iron transport in yeast. In addition to alanine replacements, our Y382F, D391N and D391E substitutions suggest that aromatic and bulky side chains are required at the LIT1 382 and 391 positions, respectively. These findings suggest that residues within the transmembrane region VII of ZIP family transporters may also participate in metal transport, although we cannot rule out that these mutations affected only the topology of

LIT1 on the plasma membrane, without directly altering the metal transport pathway.

Members of the ZIP family of metal transporters have a variable region between transmembrane domains III and IV that is predicted to form an intracellular loop [16]. In most transporters from the ZIP family, this variable region contains a histidine-rich domain proposed to function as an extra-membrane metal binding domain [12], and shown to be essential for zinc transport by the human ZIP transporter hZip1 [22]. A similar motif is present in LIT1, although not directly aligned with the HGHGH motif located in the cytoplasmic variable region of *Arabidopsis* IRT1 [11]. Consistent with the proposed role of this motif in metal binding, deletion of the HGHQH motif abolished the ability of LIT1 to promote iron transport in yeast. As previously proposed for the hZip1 human zinc transporter [22], the tertiary structure of LIT1 may bring histidines of variable region loop into spacial proximity to the histidines in the transmembrane domains that are predicted to be located within amphipathic helices that form the metal transport pathway. Our studies also identified an essential aspartic acid residue in the proximity of the HGHQH motif, which may have an important role in stabilizing the metal binding site independently of charge, since it could be replaced by glutamic acid or asparagine without loss in the ability to mediate iron transport in yeast. Database searches indicate that the D391 LIT1 residue is conserved among some members of the ZIP

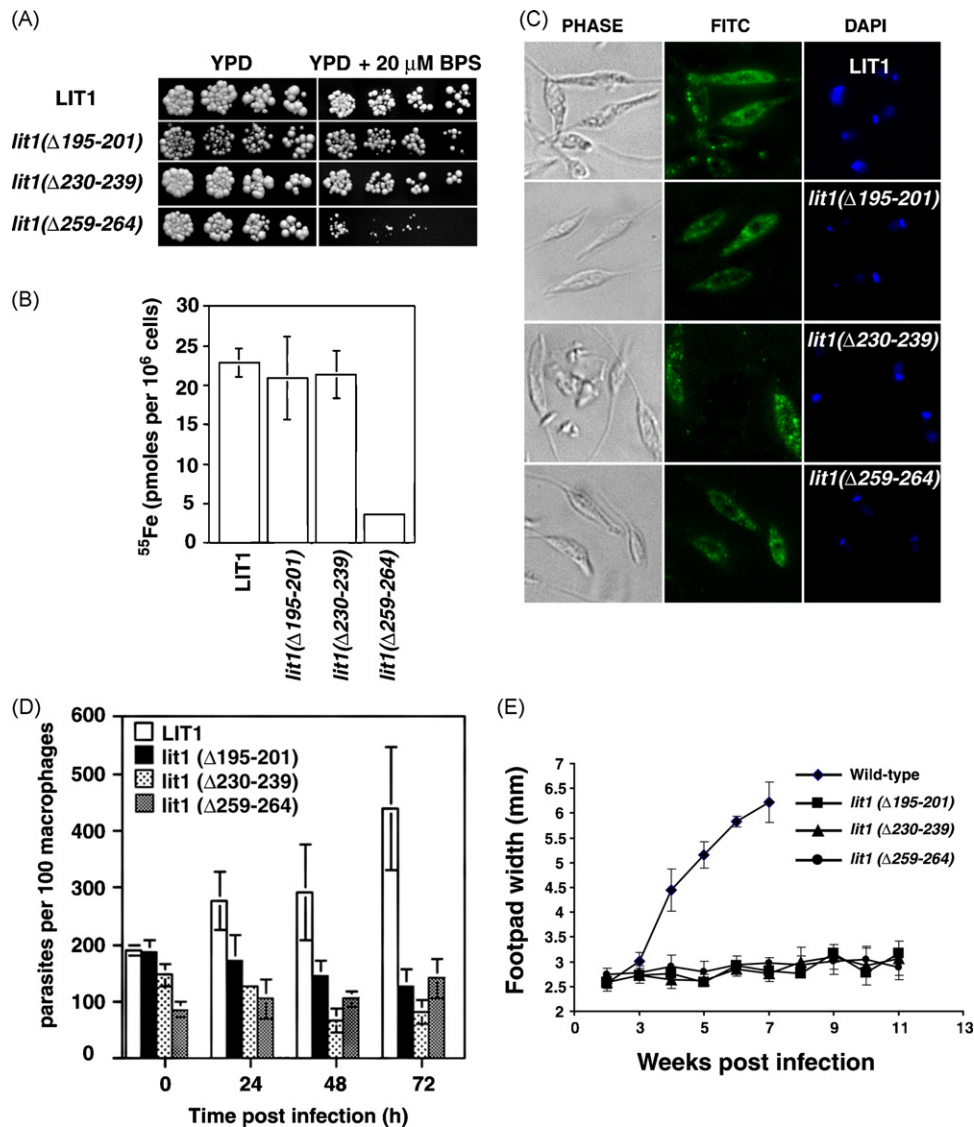


Fig. 6. The diverse intracellular loop of LIT1 contains proline-rich regions that are required for intracellular growth in macrophages and lesion formation in mice. (A) Δ *fet3fet4* carrying wild type LIT1, *lit1*(Δ 195–201), *lit1*(Δ 230–239) and *lit1*(Δ 259–264) were spotted on iron rich and iron limiting agar, in serial 1:10 dilutions. Unlike *lit1*(Δ 259–264), these truncation mutants were able to complement the growth phenotype of Δ *fet3fet4* on iron limiting media. (B) Similar results were obtained in ^{55}Fe uptake assays. The data represent average \pm SD of triplicates. (C) Immunofluorescence showed that *lit1*(Δ 195–201), *lit1*(Δ 230–239) and *lit1*(Δ 259–264) localized to the plasma membrane when expressed in Δ *lit1* *L. amazonensis* promastigotes. LIT1 immunofluorescence, green, parasite DNA, blue. (D) Bone marrow-derived macrophages were infected with Δ *lit1* *L. amazonensis* amastigotes overexpressing wild type or mutated LIT1. The number of intracellular parasites was determined microscopically at 1, 48 and 72 h post-infection. Unlike wild type LIT1, *lit1*(Δ 195–201), *lit1*(Δ 230–239) and *lit1*(Δ 259–264) failed to rescue the growth of Δ *lit1* *L. amazonensis* in macrophages. (E) Balb/c mice were inoculated in the footpad with 10^6 metacyclic promastigotes overexpressing wild type LIT1, *lit1*(Δ 195–201), *lit1*(Δ 230–239) or *lit1*(Δ 259–264), and the development of cutaneous lesions was measured weekly with a caliper. The data represents average \pm SD of five individual mice.

family of metal transporters, reinforcing its possible role in metal binding/transport.

It has been suggested that the predicted intracellular loop region of IRT1 and other ZIP metal transporters might play a role not only in sensing intracellular metal levels, but also in mediating protein turnover [12,13,23]. The extensive divergence within the intracellular loop among members of the ZIP family supports the hypothesis that it might serve as a species-specific regulatory domain. Using deletion constructs lacking specific segments of the variable region of *Leishmania* LIT1, we found that all deletions except the largest one, *lit1*(Δ 158–211), were able to traffic normally to the plasma membrane when expressed in *Leishmania* promastigotes. This observation, taken together with the fact that point mutations abolishing iron transport by LIT1 also did not block its surface expression, indicate that the phenotypes described in this study are not related to

defects in folding and intracellular targeting of the LIT1 transporter.

The phenotype initially observed with the region II deletion, *lit1*(Δ 245–264), was subsequently fully explained by the requirement for the histidine-rich motif (residues 251–255) and the D263 residue adjacent to it, and their predicted roles in assembling a metal binding domain. Interestingly, despite normal function in yeast, *lit1*(Δ 195–201) and *lit1*(Δ 230–239) were not able to rescue the ability of LIT1-deficient *L. amazonensis* to grow intracellularly in macrophages, or to induce cutaneous lesions in mice. The presence of several non-conserved proline residues within these two regions raise the intriguing possibility that the conformation of these domains is important for interaction with regulatory elements unique to *Leishmania*. An alternative explanation is that the expression levels of LIT1 that can be achieved in the infective amastigote stages with the available *Leishmania* expression sys-

tems are incompatible with functional complementation. Further studies are required to clarify these questions, and to search for putative parasite regulatory factors that might interact intracellularly with the variable region of the LIT1 iron transporter.

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