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Nutrient Sensing in *Leishmania*: Flagellum and Cytosol

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Summary

Parasites are by definition organisms that utilize resources from a host to support their existence, thus promoting their ability to establish long term infections and disease. Hence, sensing and acquiring nutrients for which the parasite and host compete is central to the parasitic mode of existence. *Leishmania* are flagellated kinetoplastid parasites that parasitize phagocytic cells, principally macrophages, of vertebrate hosts and the alimentary tract of sand fly vectors. Because nutritional supplies vary over time within both these hosts and are often restricted in availability, these parasites must sense a plethora of nutrients and respond accordingly. The flagellum has been recognized as an 'antenna' that plays a core role in sensing environmental conditions, and various flagellar proteins have been implicated in sensing roles. In addition, these parasites exhibit non-flagellar intracellular mechanisms of nutrient sensing, several of which have been explored. Nonetheless, mechanistic details of these sensory pathways are still sparse and represent a challenging frontier for further experimental exploration.

Introduction

Leishmania parasites experience a wide array of environmental conditions during their life cycle and must be able to sense and respond to the changing milieu to successfully navigate each step in this journey. These microorganisms live inside the phagolysosomal vesicles of vertebrate macrophages as disease causing non-motile amastigotes, and they live in the alimentary tract of sand fly vectors as flagellated motile forms, including variously developmentally distinct forms of promastigotes (Fig. 1 and Box 1). Parasitized macrophages are initially taken up by sand flies as part of a blood meal, and amastigotes are subsequently released from parasite-laden macrophages and transform into dividing promastigote forms. Promastigotes live within the peritrophic matrix of the sand fly, a glycan and proteoglycan rich composite that surrounds the blood meal and protects the insect midgut epithelium from deleterious components. Within the peritrophic matrix, the parasites are exposed to nutrients from the blood meal, including glucose, and are hence in a

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Conflict of interest

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relatively nutrient rich environment. Following digestion of the blood meal, the parasites transform into nondividing nectomonads (Sunter & Gull, 2017) that adhere to the villi of the midgut by their flagella to avoid expulsion with the digested blood meal. They subsequently differentiate into long, highly-motile, dividing leptomonads that migrate to the foregut, and these forms differentiate into nondividing infectious metacyclic parasites with small bodies and long flagella, which are delivered to the mammalian host tissue following initiation of another blood meal by the parasitized sand fly. The latter three life cycle forms are probably exposed to relatively nutrient restricted environments, although they will have periodic access to sugars, such as sucrose and its digestion products glucose and fructose, following ingestion of plant sap or honeydew by the sand fly (Schlein, 1986). Finally, once metacyclic parasites invade mammalian macrophages, they are targeted to acidic phagolysosomal vesicles that are thought to be relatively rich in amino acids and fatty acids from digestion of proteins and complex lipids within this digestive organelle (Naderer & McConville, 2008), and parasites are also likely exposed to macromolecules prior to their digestion within the vacuole. In addition to these changes in nutrients, the parasites are also exposed to the change from neutral to acidic pH and from ambient temperature to the ~37° temperature of the mammalian host. Despite this apparently plentiful milieu, amastigotes enter a metabolic stringent state in which uptake and catabolism of sugars and amino acids is minimal and replication proceeds at a much slower rate than for promastigotes (McConville *et al.*, 2015). During these developmental transitions, expression of many metabolic enzymes and transporters, or of their mRNAs, is regulated (Alcolea *et al.*, 2018, Inbar *et al.*, 2019), indicating that the parasites apparently sense and respond to these nutrient and metabolic alterations and that the ability to adapt to changes in nutrient composition is probably central to the success of the parasite in surviving pronounced alterations in environmental conditions.

Decades of work on eukaryotes from yeast to humans have revealed common patterns by which cells sense their internal and external status. Broadly, mechanisms can be divided into sensors that reside internally, often within the cytosol, or externally, exposed at the surface of the plasma membrane (Efeyan *et al.*, 2015). One example of a well-studied internal sensor is the GCN2 kinase, which binds to and is activated by uncharged tRNAs, resulting in phosphorylation of the translation initiation factor eIF2α and arrest of translation. Examples of external nutrient sensors (Holsbeeks *et al.*, 2004) include i) some G-protein coupled receptors, ii) carriers that have dual functions as nutrient receptors and transporters, and iii) carrier-like proteins that have lost transport capacity but retain sensing capability. Both classes ii and iii are referred to as ‘transceptors’. One remarkable outcome of the initial genome sequencing projects for *Trypanosoma brucei*, *Leishmania major*, and *Trypanosoma cruzi* (El-Sayed *et al.*, 2005), was the observation that the genomes of these parasites were completely lacking in many of the well-known surface receptor classes common in other eukaryotes, including G-protein coupled receptors and receptor tyrosine kinases. This unanticipated result suggests that these parasites must rely upon intracellular sensors, less conventional plasma membrane sensors such as transceptors, or both, perhaps to a greater extent than most other eukaryotes. Another feature of sensing in kinetoplastid protists that has come to the fore is the recognition that eukaryotic cilia and flagella have central roles as sensory organelles (Wheway *et al.*, 2018), mediating such processes through sensory

proteins located, often selectively, in the ciliary/flagellar membrane. Since *Leishmania* and other kinetoplastid protists are flagellated throughout their life cycles, the flagellum presents itself as a likely organelle for mediating sensing of nutrients and other environmental cues (Fig. 1).

Another notable distinction between kinetoplastid parasites and other eukaryotes is that the genome organization of these protists largely precludes transcriptional regulation of gene expression. Thus, genes are organized in large polycistronic arrays that are transcribed from a single upstream initiation site, requiring that differential regulation must occur at post-transcriptional levels such as mRNA stability, translation, protein stability, post-translational modifications, etc. (Clayton, 2019). In contrast, nutrient sensing in many other eukaryotes often entails transcriptional control of the differentially affected genes. Thus, both the initial nutrient sensors and the mechanisms of response to nutritional availability will often be distinct in these parasites.

Flagellar membrane proteins implicated in sensing

Several studies in recent years have identified flagellar membrane proteins that may be involved in sensing nutrients or other molecular components of the extracellular milieu. One of the first of these to be identified in *Leishmania* was a flagellar glucose/hexose transporter, GT1. Unlike other glucose transporters (GTs), GT1 is selectively targeted to the flagellar membrane (Piper *et al.*, 1995) (Fig. 2A), suggesting by its unique location that it might be involved in glucose sensing. *L. mexicana* promastigotes in which the *GT1* gene had been knocked out by targeted gene replacement achieved a cell density that was higher than wild type or add-back parasites, but instead of entering stationary phase like their wild type counterparts, they underwent a catastrophic loss of viability in dense cultures that had depleted glucose from the medium (Rodriguez-Contreras *et al.*, 2015). Addition of supplemental glucose to the medium greatly blunted the reduction in cell density and allowed the Δ*gt1* null mutants to survive at stationary phase similarly to wild type parasites. These results suggest that GT1 may sense external glucose and send a signal that allows successful transition from logarithmic to stationary phase when glucose is depleted. How such a sensory pathway may work is still unclear, but searching for potential partners that interact with GT1 could identify components of a signal transduction system. Indeed, among the first transceptors identified were the Snf3 and Rgt2 glucose transporter-like proteins from *Saccharomyces cerevisiae* that do not transport glucose but rather bind to and sense this nutrient. These proteins associate with other partner proteins involved in activating signal transduction cascades that control transcription of bona fide glucose transporter genes (Holsbeeks *et al.*, 2004). Of potential significance, the *L. major* TOR kinase, TOR3, is essential for survival of promastigotes when they are starved for glucose (Madeira da Silva & Beverley, 2010) but not when they are starved for amino acids, and a TOR3 signaling pathway might thus be linked to glucose sensing through GT1.

A flagellar aquaglyceroporin, AQP1, was identified in *L. major* and shown to be involved in uptake of water, various small organic solutes, antimonials and arsenicals, and cellular volume regulation (Figarella *et al.*, 2007). Promastigotes overexpressing wild type AQP1 migrated more rapidly up an osmotic gradient than those not overexpressing this protein or

expressing point mutants that were inactive in transport. Hence, this flagellar channel is directly involved in osmotaxis, thus likely playing a sensory role. *Leishmania* parasites experience osmotic gradients while in the sand fly, and it is possible that AQP1 plays a role in migration of the parasites within the insect vector. In addition, amastigotes face osmotic stress within the mammalian host, and since AQP1 is also expressed in that life cycle stage in the flagellar pocket and contractile vacuole/spongiosome complex, this channel may play important physiological roles in both the vector and the mammalian host. It has been demonstrated that phosphorylation of AQP1 by MAPK2 both decreases AQP1 turnover and leads to its redistribution across the entire plasma membrane (Mandal *et al.*, 2012). *L. major MAPK2* knockout mutants recover from hypo-osmotic stress more slowly than wild type parasites. Whether phosphorylation via MAPK2 is part of the molecular mechanism that links AQP1 to osmotaxis has not been investigated.

Arginine is a critical nutrient for *Leishmania* parasites, being a precursor for the synthesis of essential polyamines (Roberts & Axel, 1982) and for protein biosynthesis. Amastigotes must compete with the macrophage to salvage Arg from the macrophage phagolysosome, as i) the host cell uses Arg as a precursor for the synthesis of nitric oxide (NO), a cytotoxic effector employed to kill the parasite within the phagolysosome; ii) in *Leishmania* parasites, Arg is the biosynthetic precursor for trypanothione, the principal reducing agent employed to protect against oxidative damage, including effectors such as NO. *L. donovani* parasites have evolved a system to sense Arg deprivation, which they experience when they enter macrophages (Goldman-Pinkovich *et al.*, 2016). Specifically, one isoform of the AAP3 Arg permease (Shaked-Mishan *et al.*, 2006), AAP3.2, is upregulated, at both the mRNA and protein level, when either promastigotes or amastigotes are deprived for Arg (Goldman-Pinkovich *et al.*, 2016). This upregulation of AAP3.2 increases the Arg transport capacity of the parasites and presumably promotes its survival and its ability to compete for Arg with the host cell. In addition to upregulating expression of AAP3.2, RNA Seq demonstrated the coordinate upregulation of six other mRNAs, five of which encoded other permeases. The authors designated this coordinate regulation as the Arginine Deprivation Response (ADR) due to the initiating Arg deprivation signal, but it is not clear how upregulating the other permeases promotes survival during Arg deprivation. Notably, this ADR was dependent upon the kinase MPK2, implicating a probable phosphorylation cascade in the sensory response.

Subsequently, the *AAP3.2* gene was knocked out by CRISPR-Cas9-mediated gene replacement to test the role of the regulatory response (Goldman-Pinkovitch, bioRxiv, 2019, https://doi.org/10.1101/751610). These knockouts were generated by inserting a linker into the ORF that introduced stop codons, leading to translational termination, but the mutant *AAP3.2* mRNA was still expressed and its response to Arg starvation monitored. Basal Arg transport was retained by the intact adjacent *AAP3.1* gene, which is not regulated by Arg deprivation. Amastigotes of the knockout line were impaired in their ability to grow inside THP-1 macrophages in vitro at a physiological extracellular Arg concentration of 0.1 mM but could be rescued by supplementing the medium with 1.5 mM Arg. Furthermore, two *AAP3.2* null mutants were strongly impaired in their ability to infect the livers of BALB/c mice, confirming that the ADR is critical for robust virulence in *L. donovani*.

AAP3.2 is located in the flagellar membrane as well as in intracellular organelles called glycosomes that enclose multiple metabolic and purine salvage enzymes (Goldman-Pinkovich *et al.*, 2016). Hence, one might postulate that AAP3.2 is a flagellar-associated arginine transceptor that both senses and transports Arg and induces the ADR. Studies with Arg analogs (Pawar *et al.*, 2019) indicate that some substrate analogs that inhibit transport activity do not inhibit Arg sensing or the ADR and that the unmodified amidino group on Arg is essential for sensing but not for transport. These results suggest that the sensory and transport binding sites are distinct; however, they leave open the question of whether these two sites are on AAP3.2 or on different proteins. Furthermore, translational termination knockouts of AAP3.2 were still able to regulate the level of the mutant *AAP3.2* mRNA, which encoded the truncated protein, and of two other mRNAs that are induced by ADR. Hence, AAP3.2 cannot be a sensor that is essential for ADR. A separate study on AAP3 proteins from *L. amazonensis* (Castilho-Martins *et al.*, 2011) has suggested that the parasites may sense both external and internal Arg levels. Hence, it is possible that although ADR regulates expression of the AAP3.2 transporter, the sensor is an intracellular protein.

Proteomic studies of isolated flagella from both *Trypanosoma brucei* (Subota *et al.*, 2014) and *L. mexicana* (Beneke *et al.*, 2019) have identified other candidate flagellar membrane proteins of interest. In *T. brucei* bloodstream forms, a putative Ca^{2+} channel, FS179, is located in the flagellar membrane component (Sanchez *et al.*, 2016) of the flagellum attachment zone (FAZ), the adhesive structure that attaches the flagellum along most of its length to the cell body. Since fluxes in Ca^{2+} often mediate sensory responses (Sanders *et al.*, 2002), it is possible that FS179 plays some role in flagellar-mediated sensing. The ortholog of FS179 in *L. mexicana*, LmxM.33.0480, is located in the FAZ (Fig. 2B), which in *Leishmania* parasites constitutes a discrete spot-like adhesion between the flagellar membrane, near its exit from the flagellar pocket, and the flagellar pocket membrane (Wheeler *et al.*, 2016). Similarly, several transporters and a cNMP-phosphodiesterase (Beneke *et al.*, 2019, Kelly *et al.*, 2020a) are also components of the flagellar membrane, suggesting that some of these proteins could have roles in flagellar sensing. Future studies examining each of these flagellar membrane components will be necessary to determine whether any of them has a sensory role.

cAMP signaling and the flagellum

cAMP is a virtually universal second messenger involved in transmitting information from the exterior to the interior of cells. The role of cAMP and the proteins that mediate its synthesis, adenylate cyclases or ACs, and turnover, phosphodiesterases or PDEs, have been most extensively studied in *T. brucei* (Makin & Gluenz, 2015, Saha *et al.*, 2020, Salmon, 2018), and this work provides a paradigm for exploration of the roles of *Leishmania* flagella in this central signaling pathway. While cAMP signaling has not been explicitly connected with nutrient sensing in *Leishmania*, it is reviewed here as a pathway that is likely involved in environmental sensing in these parasites and for which some molecular components are known. ACs in mammals and many other eukaryotes are integral membrane proteins with 12 transmembrane domains (TMDs) and two conserved AC catalytic domains (Khannpnavar *et al.*, 2020). These ACs are typically regulated by G protein coupled receptors (GPCRs) with 7 TMDs and heterotrimeric G proteins that transmit signals from the GPCRs to the target ACs.

In contrast, the genomes of the TriTryp parasites, *T. brucei*, *T. cruzi*, and *Leishmania* species, do not encode any recognizable GPCRs or heterotrimeric G-proteins (El-Sayed *et al.*, 2005). However, these parasites express a variety (~65 in *T. brucei* and 6 in *L. mexicana*) of single TMD proteins with single conserved AC catalytic domains oriented on the cytosolic side of the plasma membrane and a large hydrophilic extracellular domain. This structure is similar to some mammalian ligand-activated guanylate cyclases (Pandey, 2014) and suggests that the parasite proteins may undergo similar activation by binding of currently unknown ligands to the extracellular domain.

The first such kinetoplastid AC studied was ESAG4 (Paindavoine *et al.*, 1992), encoded by an expression site-associated gene (ESAG) and localized to the flagellar membrane of BF trypanosomes. RNAi knockdown of ESAG4 RNA, and of two of its non-expression site encoded paralogs, generated cytokinesis defects and impaired parasitemia in mice (Salmon *et al.*, 2012a). Studies using expression of a dominant-negative mutant of ESAG4 suggested that active ESAG4 blunts the innate immune response of a murine host by reducing expression of tumor necrosis factor-α (TNF−α) (Salmon *et al.*, 2012b). The model proposed is that phagocytosis of trypanosomes results in ESAG4 being delivered to phagocytes, following lysis of the ingested trypanosomes, thus increasing the intracellular cAMP levels in these host cells. It is further suggested that the augmented cAMP reduces expression of TNF-α at the transcriptional level in those phagocytes and thus promotes parasite survival. In addition several related ACs (AC1, AC2, AC4, and AC6) have been studied in procyclic form (PF) trypanosomes, where they are localized either along the length of the flagellum or concentrated at its distal tip (Saada *et al.*, 2014), and RNAi-mediated knockdown of several of these ACs resulted in increased ‘social motility’ (SOMO) in which parasites showed increased concerted movement (or a hypersocial phenotype in which large streams of parasites migrate in concert and form increased numbers of macroscopic projections on agarose plates) compared to uninduced PFs (Lopez *et al.*, 2015). These results implied that cAMP is involved in regulating SOMO and may play important roles in parasite social motility in vivo as well.

Two PDEs in African trypanosomes, PDEB1 and PDEB2, are associated with the paraflagellar rod component of the flagellar cytoskeleton (Oberholzer *et al.*, 2007). RNAi-mediated knockdown of both proteins simultaneously produced a strong cytokinesis defect in BF trypanosomes and resulted in parasite death in vitro and avirulence in mice. A null mutant of PDEB1 was subsequently shown to be defective for social motility as PFs, being unable to form projecting streams when PFs were plated on agarose (Shaw *et al.*, 2019). Of particular interest, this null mutant was also unable to migrate out of the peritrophic matrix that surrounds PFs in the insect midgut early after infection of the tsetse fly, and it could not complete further development within the insect. Overall, these studies show that cAMP signaling plays central roles in both social motility in vitro and migration of parasites within the tsetse fly and hence is essential for transmission of the parasite by its insect vector.

This body of work in African trypanosomes suggests that interrogation of cAMP signaling in *Leishmania* flagella is likely to uncover critical biological functions potentially linked to flagellum sensing of the extracellular environment. Five clustered genes encoding receptor-like ACs were identified some years ago in *L. donovani*, and two of these proteins, RAC-A

and RAC-B, were functionally expressed in *Xenopus* oocytes (Sanchez *et al.*, 1995). RAC-A exhibited AC activity, RAC-B did not, but co-expression of RAC-B with RAC-A decreased the AC activity of the latter, suggesting that the two proteins may form heterodimers. The genome of *L. mexicana* predicts the existence of 6 such ACs (LmxM.17.0190, 17.0191, 17.0200, 17.0236, 17.0237, and 36.3180). Notably, a recently published flagellar proteome from *L. mexicana* promastigotes (Beneke *et al.*, 2019) found at least one AC, LmxM36.3180, to be enriched in the detergent solubilized fraction from purified flagella, thus suggesting that it is an FM protein (Kelly *et al.*, 2020a). Of interest, a non-flagellar cytosolic protein (LmxM.28.0090) has also been identified as a heme-containing AC that is activated by O_2 (Sen Santara *et al.*, 2013). Gene knockdown and overexpression indicate that this O_2-activated AC is critical for parasite survival under conditions of oxidative stress induced by hypoxia.

Additionally, a putative cyclic nucleotide phosphodiesterase (PDE), LmxM.08_29.2440, was also enriched in the detergent extracted flagellar proteome, and it is predicted to have a single TMD near its C-terminus. Tagging and localization of this protein confirmed that it is present in the flagellum and is concentrated toward the distal tip in many promastigotes (Kelly *et al.*, 2020b) (Fig. 2C). This protein also appears to localize to intracellular vesicles, so whether its biologically relevant activity is exclusively at the flagellum or also elsewhere is currently uncertain. Several PDEs have been studied in *L. major* (Johner *et al.*, 2006) and *L. donovani* (Bhattacharya *et al.*, 2009), but to our knowledge, none of these other PDEs is flagellar in localization.

In summary, a number of components of cAMP signaling in *Leishmania* promastigotes appear to be FM proteins. Given the precedent from *T. brucei*, investigation of the biological functions of these proteins throughout the parasite life cycle, for instance by gene knockout approaches, has the potential to uncover critical roles for cAMP signaling in the flagellum, and we believe such studies should be a high priority. One long standing challenge in both trypanosomes and *Leishmania* is to identify potential ligands for receptor-like ACs and to link such molecules to possible host- and vector-parasite interactions. Given the absence of clues to the identities of such putative ligands and the possibility that they could represent unusual or non-abundant components of the tsetse fly, sand fly or macrophage, it is perhaps not surprising that the resolution of this puzzle has eluded molecular parasitologist to date.

Iron sensing in *Leishmania*

In addition to the role of the flagellar membrane in sensing the environment, other environmental components, including nutrients, are sensed intracellularly. A notable example involves parasite sensing of the critical micronutrient iron, which *Leishmania* parasites require for many conserved metabolic pathways, including electron transport and for activity of the iron-containing superoxide dismutase (FeSOD) that converts superoxide to H_2O_2 and is a major defense against oxidative damage (Mittra *et al.*, 2013). Iron may be acquired either as the metal ion or from external heme, but excessive levels of either substituent are toxic. Hence, these parasites have elaborated sensory mechanisms to increase import of iron and heme under limiting conditions while maintaining their intracellular concentrations at non-toxic levels.

Within the acidic parasitophorous vacuole, where amastigote replication takes place, or within the sand fly, free iron is present in the oxidized and typically protein bound ferric form (Fe^{3+}), but *Leishmania* parasites import water soluble reduced ferrous iron (Fe^{2+}). This oxidation state of iron is first generated at the parasite plasma membrane by the membrane-spanning ferric reductase LFR1 (Flannery *et al.*, 2011), which reduces Fe^{3+} to Fe^{2+} at the cell surface in an NADPH-dependent process, and then imported into the parasite by the ZIP family iron transporter LIT1 (Huynh *et al.*, 2006) (Fig. 3). Once in the parasite cytosol, iron can be translocated into the mitochondrion by the inner mitochondrial membrane transporter, MIT1, a homolog of mitoferrin from yeast and mammals (Mittra *et al.*, 2016), where it can associate with FeSOD and other iron containing proteins. Heme, which cannot be synthesized by *Leishmania* parasites (Chang & Chang, 1985), is imported by two dedicated import proteins, the heme permease LHR1, which is related to the *Caenorhabditis elegans* heme transporter HRG-4 (Huynh *et al.*, 2012), and the major facilitator superfamily protein FLVCRb (Cabello-Donayre *et al.*, 2016). Since conserved cytosolic iron storage proteins have not been identified in *Leishmania* parasites, it is thought that these cells need to regulate the level of intracellular iron through balancing import and export. The major facilitator family protein LIR1 exports iron from the cytoplasm (Laranjeira-Silva *et al.*, 2018), and thus protects parasites from iron overload toxicity.

These pathways of iron regulation are particularly important for amastigotes growing within host cells in the iron-poor parasitophorous vacuole. Null mutants of *LFR1*, *LIT1*, and *LIR1* all show severe growth defects within host cells and attenuated virulence in a mouse model of cutaneous leishmaniasis (Flannery *et al.*, 2011, Huynh *et al.*, 2006, Laranjeira-Silva *et al.*, 2018). The requirement for LFR1 and LIT1 can be complemented by supplementation with iron; when host cells were preloaded with high levels of ferritin, amastigote growth was restored to *Δlfr1* and *Δlit1* parasites. In contrast, iron chelation enhanced the replication of *Δlir1* intracellular amastigotes relative to wild-type parasites (Sarkar *et al.*, 2019), confirming that this permease protects parasites against accumulation of toxic levels of iron. Both of the heme import proteins, LHR1 and FLVCRb, and the mitochondrial iron import protein MIT1, are essential for the growth of promastigotes, and deletion of even one allele of these genes reduced virulence of amastigotes *in vivo* (Cabello-Donayre *et al.*, 2016, Huynh *et al.*, 2012, Mittra *et al.*, 2016).

The levels of these iron transport proteins change in response to iron availability, indicating that parasites sense and respond to iron. The mRNA encoding LFR1, the ferric reductase, is upregulated in response to iron deprivation, and cell lysates made from iron-deprived cells show increased levels of reductase activity (Flannery *et al.*, 2011). Similarly, expression of *LIT1* mRNA is induced when parasites are grown in iron-depleted medium (Mittra *et al.*, 2013). In contrast, the iron export protein LIR1, but not its mRNA, is induced by addition of $FeSO_4$ to the medium and decreased by iron deprivation, consistent with its activity being required under conditions of excess iron (Laranjeira-Silva *et al.*, 2018). The parasite response to iron levels extends beyond the proteins directly involved with import and export. Iron starvation of promastigotes induces differentiation to amastigotes (Mittra *et al.*, 2013). Paradoxically, this response appears to be mediated by induction of LIT1 in response to iron deprivation, resulting in increased intracellular Fe^{2+}, enhanced FeSOD activity, and increased levels of H_2O_2, which acts as an inducer of amastigote differentiation. This

differentiation pathway requires LFR1, LIT1, and MIT1 (Flannery *et al.*, 2011, Mittra *et al.*, 2013, Mittra *et al.*, 2016), and can also be induced by the addition of exogenous H_2O_2.

The mechanisms of parasite iron sensing and subsequent regulation of iron import and export proteins are largely obscure, and this area represents a frontier for future research. However, it seems likely that iron sensing is an intracellular process, because Fe^{2+} is produced at the cell surface and then rapidly imported, and Fe^{3+} is not soluble but is bound to extracellular proteins. The differentiation of promastigotes to amastigotes upon iron depletion is also probably dependent upon increased intracellular iron, resulting from higher levels of LIT1, and subsequent import of Fe^{2+} into mitochondria by MIT1, where it increases the level of the differentiation inducer H_2O_2 (Mittra *et al.*, 2013). While downstream components of nutrient sensing pathways in *Leishmania* are in general poorly understood, the FeSOD is one such component in the iron sensing pathway, being activated by this micronutrient and inducing promastigote to amastigote differentiation by production of H_2O_2.

Purine Sensing in *Leishmania*.

A well characterized example of intracellular nutrient sensing in *Leishmania* involves monitoring the intracellular purine nucleotide pool as part of the purine stress response. Purines are essential macromolecules that i) form the building blocks for DNA, RNA, and various cofactors, ii) act as the principal molecules for storing and transferring energy in all cells, and iii) function as important intracellular and extracellular signaling molecules. Like all known parasitic protists, *Leishmania* are incapable of de novo purine synthesis and must salvage these critical nutrients from their hosts (see (Boitz *et al.*, 2012) for a detailed review). An assortment of secreted and cell-surface nucleases, nucleotidases, and phosphatases convert nucleic acids (RNA and DNA) or nucleotides from the host milieu into substrates for uptake by purine nucleoside and nucleobase transporters. A complex network of intracellular purine salvage enzymes shunt the internalized purine nucleosides and nucleobases into the adenine and guanine nucleotide pools. Because adenine and guanine nucleotides can be readily interconverted, any nucleobase (adenine, guanine, hypoxanthine, xanthine) or nucleoside (adenosine, guanosine, inosine, xanthosine) can serve as the sole purine source for the parasites.

As discussed above, the latter three *Leishmania* life cycle forms in the sand fly vector likely encounter host environments depleted of nutrients, including purines. While purine restriction does not, by itself, trigger differentiation into the infectious metacyclic form, purine depletion is required for efficient metacyclogenesis, both in the sand fly and *in vitro* (Serafim *et al.*, 2012). To survive indeterminate periods of purine scarcity within the host, *Leishmania* have evolved a robust and coordinated stress response pathway. Purine-starved *Leishmania* promastigotes exit the cell cycle, entering a quiescent-like state in which they can persist for >90 days in purine-free culture medium (Martin *et al.*, 2014). This quiescent-like state is accompanied by dramatic remodeling of the transcriptome and proteome, elongation of the cell body, increased motility, and stress granule formation (Carter *et al.*, 2010, Martin *et al.*, 2016, Martin *et al.*, 2014, Shrivastava *et al.*, 2019a, Shrivastava *et al.*, 2019b). The earliest manifestations of the purine stress response are directed at increasing

the acquisition of extracellular purines by upregulating expression of purine salvage enzymes and transporters (Carter *et al.*, 2010, Martin *et al.*, 2014, Ortiz *et al.*, 2010). Regulation of a purine nucleobase transporter, NT3, by purine starvation has been studied in *L. donovani* and shown to be dependent upon a stem-loop regulatory element within the 3'-untranslated region of the mRNA that controls both mRNA translation and stability (Licon & Yates, 2020). Later changes are focused on the establishment of the quiescent-like state, including downregulation of DNA, RNA, and protein synthesis, and upregulation of general stress response pathways, especially those protecting against oxidative stress (Martin *et al.*, 2014).

Adenine nucleotide levels are substantially reduced in purine-starved promastigotes, while guanine nucleotide levels are mostly unaffected at early timepoints, and actually increase over time (Martin *et al.*, 2016). A direct role for alterations of the adenine nucleotide pool in purine sensing was demonstrated using mutants in the purine salvage pathway incapable of interconverting adenine and guanine nucleotides (Martin *et al.*, 2016). The adenine and guanine nucleotide pools were independently depleted by culturing the mutants in medium with specific purine nucleobases that could be incorporated into only one of the two nucleotide branches. Mutants starved for adenine nucleotides, but not guanine nucleotides, responded identically to purine-starved wild type promastigotes, increasing the expression of a suite of known purine-responsive genes, and persisting in a quiescent-like state for at least a month. Normal induction of the purine stress response in the presence of extracellular purines renders direct sensing of extracellular purine availability via a membrane-associated purine receptor unlikely. In contrast, mutants starved for guanine nucleotides, but not adenine nucleotides, failed to upregulate a subset of purine-responsive genes, exhibited an incomplete cell cycle arrest, and experienced a precipitous drop in viability after ten days. This suggested that purine availability is sensed by monitoring the adenine nucleotide pool, and that a reduction in the adenine nucleotide pool is required to instigate the genetic program for long term survival of purine starvation.

The mechanisms of purine sensing and signaling are likely complex and have thus far remained elusive. For example, some purine stress-responsive genes were upregulated normally in mutants starved exclusively for guanine nucleotides, while the expression of others was unchanged, intimating the existence of at least two independent purine sensing/signaling pathways (Martin *et al.*, 2016). An obvious candidate purine sensor and signaling molecule in *Leishmania* is the 5' AMP-activated protein kinase (AMPK), which is highly conserved in eukaryotes and modulates cellular energy status by sensing reductions in the intracellular ATP-to-ADP or ATP-to-AMP ratio (Lin & Hardie, 2018). The substantial reduction in the ATP-to-AMP ratio following five days of purine starvation, but not in earlier timepoints, suggests the possibility that AMPK signaling may play a role in the adaptation to chronic purine starvation (Martin *et al.*, 2016). The potential role of AMPK in the purine stress response, the identification of other purine sensors and signaling pathways, and determining if the purine stress response is operative in amastigotes within host macrophages represent compelling topics for future research.

Summary and Perspectives

Sensing of nutrients is critical for survival of *Leishmania* parasites. Thus, parasites that are null mutants for the flagellar GT1 permease grow to higher maximal cell density in culture than wild type cells but are not able to enter stationary phase and subsequently die with rapid kinetics (Rodriguez-Contreras *et al.*, 2015). This growth expansion followed by catastrophe is reminiscent of similar phenotypes reported for Δ*lit1* null mutants (Mittra *et al.*, 2013) and *LMIT1*/Δ*lmit1* (Mittra *et al.*, 2016) heterozygous null mutants that are deficient for iron uptake. Parasites are also critically dependent on their ability to sense intracellular purine availability. When the Δ*gmpr*/Δ*impdh* dual null mutant of *L. donovani* was cultured in the presence of hypoxanthine as the sole purine, conditions that allowed continued synthesis of adenine nucleotides but not of guanine nucleotides, parasites died (Martin *et al.*, 2016). Lethality ensued because the parasites did not experience a reduction in intracellular adenine nucleotides, the critical signal for sensing purine starvation, even though they were undergoing guanine nucleoside starvation. Failure to sense purine depletion prevented these mutant promastigotes from mounting the protective response exhibited by purine-starved wild type parasites, which induces entry into a palliative quiescent state. Thus, continuing studies mapping mechanisms of nutrient sensing will elucidate processes that are critical for the survival of parasites experiencing hostile environments within their hosts.

There are various other nutrients not covered above that are critical for *Leishmania* parasites, and exploring whether and how their availability is sensed would be of considerable interest. A number of amino acids, including arginine, histidine, phenylalanine, serine, tyrosine, threonine, valine, leucine, lysine, and proline, are considered essential for these parasites (Krasner and Flory, 1971). Heme is an essential nutrient that is not synthesized by these parasites and is transported by the LHR1 transporter (Renberg *et al.*, 2015). Similarly, folates and unconjugated pteridines are not synthesized by *Leishmania* parasites and are salvaged by a variety of transporters (Vickers & Beverley, 2011). Zn^{2+} is another micronutrient that is required by *Leishmania* parasites, but its intracellular level must be controlled to avoid zinc-overload toxicity (Carvalho *et al.*, 2015). This metal ion and others are transported by the ZIP3 permease, and the expression of this transporter is regulated, at the mRNA level, by the availability of Zn^{+2} thus ensuring zinc homeostasis. Additionally, the mRNA for a phosphate transporter from *L infantum* is upregulated ~2-fold by restriction for phosphate (Russo-Abrahao *et al.*, 2013), while starvation for either phosphate or purine upregulates expression of a surface membrane expressed 3'-nucleotidase that cleaves extracellular purine nucleotides and provides both purine nucleosides and phosphate for uptake by the parasite (Sacci *et al.*, 1990). Hence, there are a variety of uptake systems that are likely to respond to sensing of their cognate nutrients that remain to be explored.

A major gap in our knowledge for sensing of nutrients or other environmental cues in *Leishmania* is the paucity of information on the cascades that transmit alterations in nutrient composition to phenotypic changes in the parasite. Recent progress in dissecting two sensory pathways in African trypanosomes provides instructive examples that may be relevant to their phylogenetic cousins. Differentiation of bloodstream resident stumpy form (SF) trypanosomes into procyclic form (PF) parasites within the gut of the tsetse fly vector is promoted by the metabolites citrate and cis-aconitate (Szoor *et al.*, 2020). Work form the

Matthews laboratory has identified two carboxylate transporters expressed in stumpy forms, PAD1 and PAD2, that import these differentiation inducers and hence allow their intracellular sensing. Furthermore, two protein phosphatases that interact with each other, TbPTP1 and TbPIP39, have been implicated as regulators of SF to PF differentiation, and two protein kinases, TbNRKA and TbNRKB, also play a role in differentiation. Of considerable interest, TbPIP39 binds citrate and inhibits its activation of TbPTP1, which in turn increases the level of phosphorylated TbPIP39, thus linking the differentiation inducing metabolite to the downstream signaling components. In the bloodstream, dividing long slender (LS) trypanosomes employ a quorum sensing mechanism that induces differentiation into nondividing SFs at high parasite density (Rojas & Matthews, 2019), developmental forms that are, as indicated above, primed for differentiation into insect stage PFs once they enter a tsetse fly. The long sought stumpy inducing factor, SIF, has been identified recently as oiligopeptides that are probably generated from host proteins as LS parasites lyse and release intracellular proteases. Sensing increasing levels of these oligopeptides requires their internalization by an unusual oligopeptide transporter, GPR89, and a genome-wide RNAi screen has implicated several protein kinases and phosphatases in this signaling pathway as well. Thus, protein phosphorylation seems to play a central role in both SF to PF and LS to SF differentiation, as it does in many eukaryotic signaling pathways. Identification of the molecular components of nutrient sensing pathways in *Leishmania* parasites, possibly by phosphoproteomic and genetic approaches, awaits similar advances.

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Box 1.

Definitions of relevant terms

Promastigotes, including various developmental forms such as procyclics, nectomonads, leptomonads, metacyclics.

Developmental forms of *Leishmania* parasites in the sand fly vector, listed here in order of their emergence following uptake of the blood meal. All of these promastigote forms have extended flagella and are motile. Metacyclics are the infectious forms that are delivered to the vertebrate host by a bite of the infected sand fly.

Amastigotes.

Disease causing stages of the *Leishmania* parasite that live within phagolysosomal vacuoles of vertebrate macrophages. Amastigotes have a short flagellum that barely emerges from the flagellar pocket, and these parasites are non-motile.

Sand fly.

Hematophagous insects, including the genera *Phlebotomus* and *Lutzomyia*, which serve as vectors for *Leishmania* parasites. Parasites initially develop within the insect midgut after ingestion of the parasitized blood meal, but developmental forms of the parasite migrate forward to the mouthparts, where they are delivered to another vertebrate host during acquisition of a second blood meal.

Flagellar membrane.

The component of the surface membrane that covers the flagellum and has a distinct protein and lipid composition from the rest of the plasma membrane. This membrane emerges from the flagellar pocket membrane.

Flagellar pocket.

An invagination of the surface membrane at the point where the flagellum emerges from the cell body. The flagellar pocket consists of a membrane, which is contiguous with both the plasma membrane that surrounds the cell body and the flagellar membrane, and it also encompasses a lumen that is in contact with the extracellular space.

Flagellum attachment zone, FAZ.

This complex structure was first identified in *T. brucei*, where it mediates attachment of the flagellum to the cell body along most of its length. It was subsequently identified in *L. mexicana*, where is constitutes a discrete adhesion between the base of the flagellar membrane and the flagellar pocket membrane. The polytopic membrane protein FAZ5 is a constituent of the flagellar pocket membrane component of the FAZ, whereas the FLA1 binding protein and the Ca^{2+} channel encoded by LmxM.33.0480 are flagellar membrane components of the FAZ.

Transceptor.

A protein that is related in structure to a family of membrane transporters but acts as a signal transduction receptor for a bound ligand. Some transceptors have retained ligand

transport activity as well as signaling capacity, whereas others do not transport ligands but exhibit only sensory signaling function.

Aquaporins, AQPs.

Channel proteins that mediate uptake of water, glycerol, and various other small organic solutes.

Adenylate cyclase, AC.

A membrane bound enzyme that synthesizes cAMP from ATP. In most eukaryotes, adenylate cyclases have 12 transmembrane segments and two intracellular loops containing catalytic domains. AC activity is typically controlled by binding of ligands to upstream G-protein coupled receptors (GPRCs) that activate various heterotrimeric G-proteins, and these activated G-proteins then activate or inhibit AC activity. In kinetoplastid parasites, adenylate cyclases have an intracellular catalytic domain that is conserved in sequence to those of other eukaryotes, but they only have a single transmembrane domain and a large extracellular domain that may bind unknown ligands.

cAMP phosphodiesterase, PDE.

An enzyme that cleaves cAMP to AMP. These enzymes play important roles in controlling the intracellular level of the second messenger cAMP.

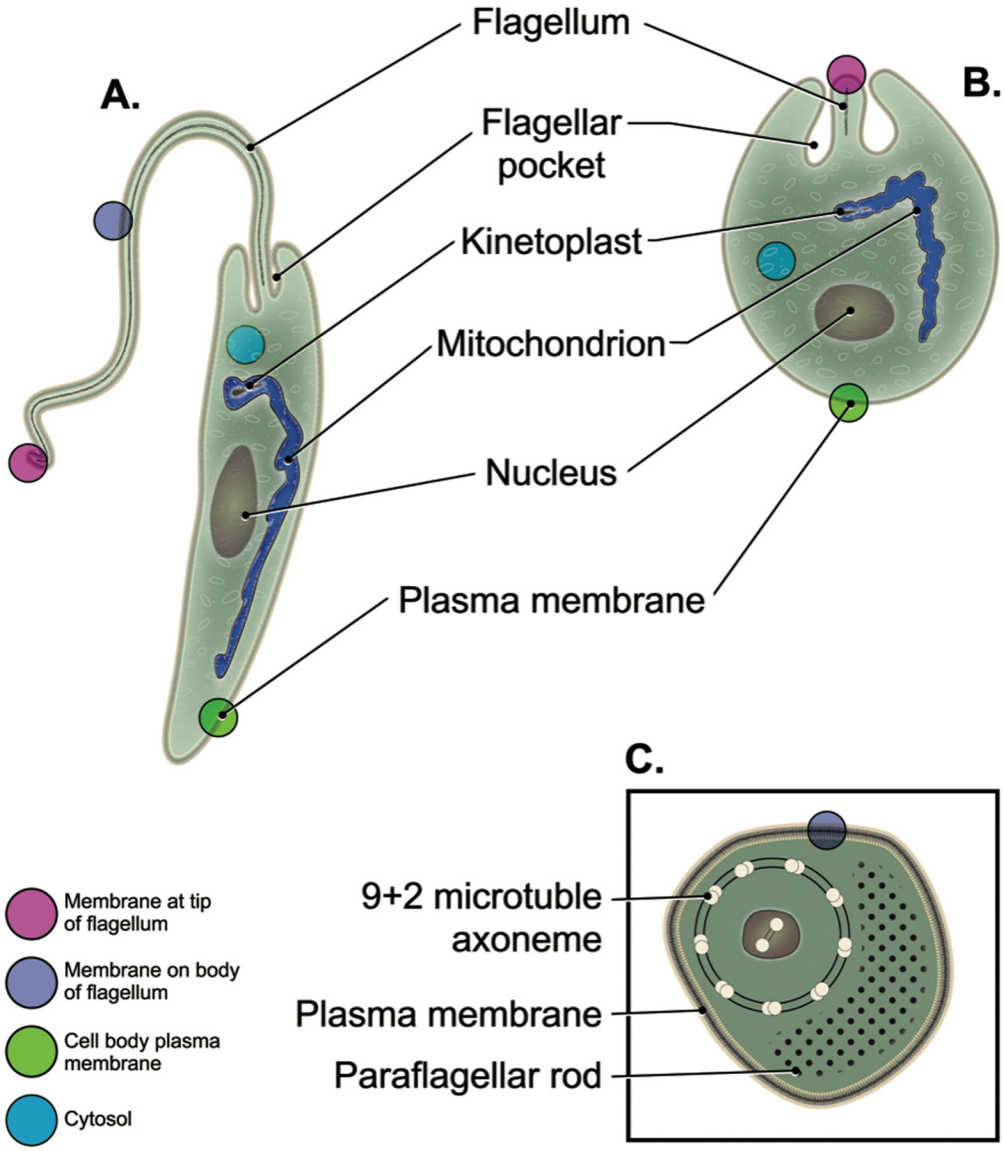

Fig. 1.

Leishmania parasites and locations of potential sensors. A. Insect stage promastigotes have an extended cell body and a long flagellum that exits from the flagellar pocket. B. Intracellular amastigotes have on oval-shaped cell body and a short flagellum that barely emerges from the flagellar pocket. In both life cycle stages, membrane bound sensors could be located at the distal tip of the flagellum (red sphere), along the length of the flagellum (purple sphere), or on the cell body membrane (green sphere), and other sensors could be cytosolic proteins (blue sphere). C. A cross section of the flagellum shows the surrounding flagellar membrane and an integral membrane protein (purple) representing a putative flagellar sensor. Other components of all images are as labeled. This figure was prepared by Laramie Studio, Portland, OR.

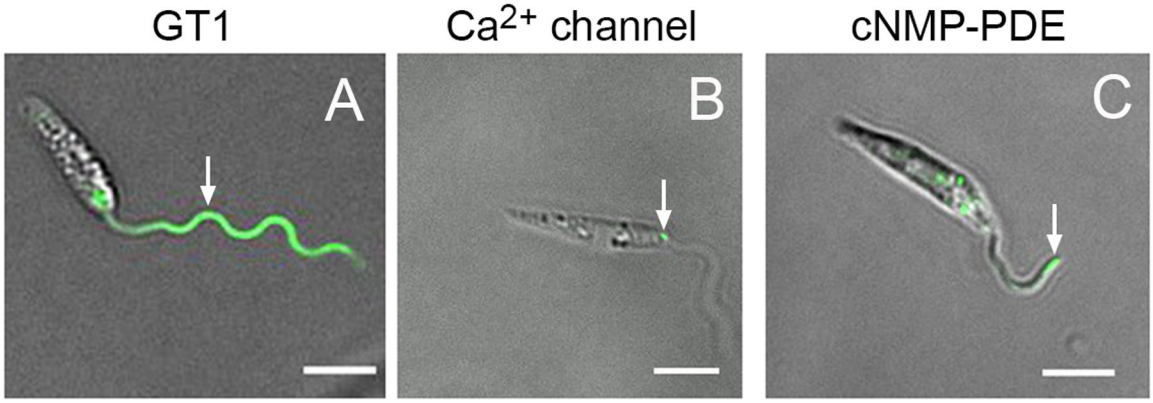

Fig. 2.

Localization of three proteins with potential roles in signaling to different components of the flagellar membrane in *L. mexicana* promastigotes. A) The flagellar glucose transporter GT1, tagged at the C-terminus with GFP, localizes along the length of the flagellum (white arrow). B) The putative Ca^{2+} channel encoded by LmxM.33.0480, fused at its C-terminus to mNeonGreen, is restricted to the flagellum attachment zone (white arrow), a localized adhesion between the flagellar membrane and the flagellar pocket membrane near the base of the flagellum. C) A cNMP-PDE encoded by LmxM.08_29.2440, fused at its C-terminus to mNeonGreen, localizes to the flagellum with a concentration at the distal tip (white arrow) in many parasites. All images are of live parasites imaged in CyGel and represent superpositions of fluorescence and differential interference microscopy. Scale bars are 5 μm.

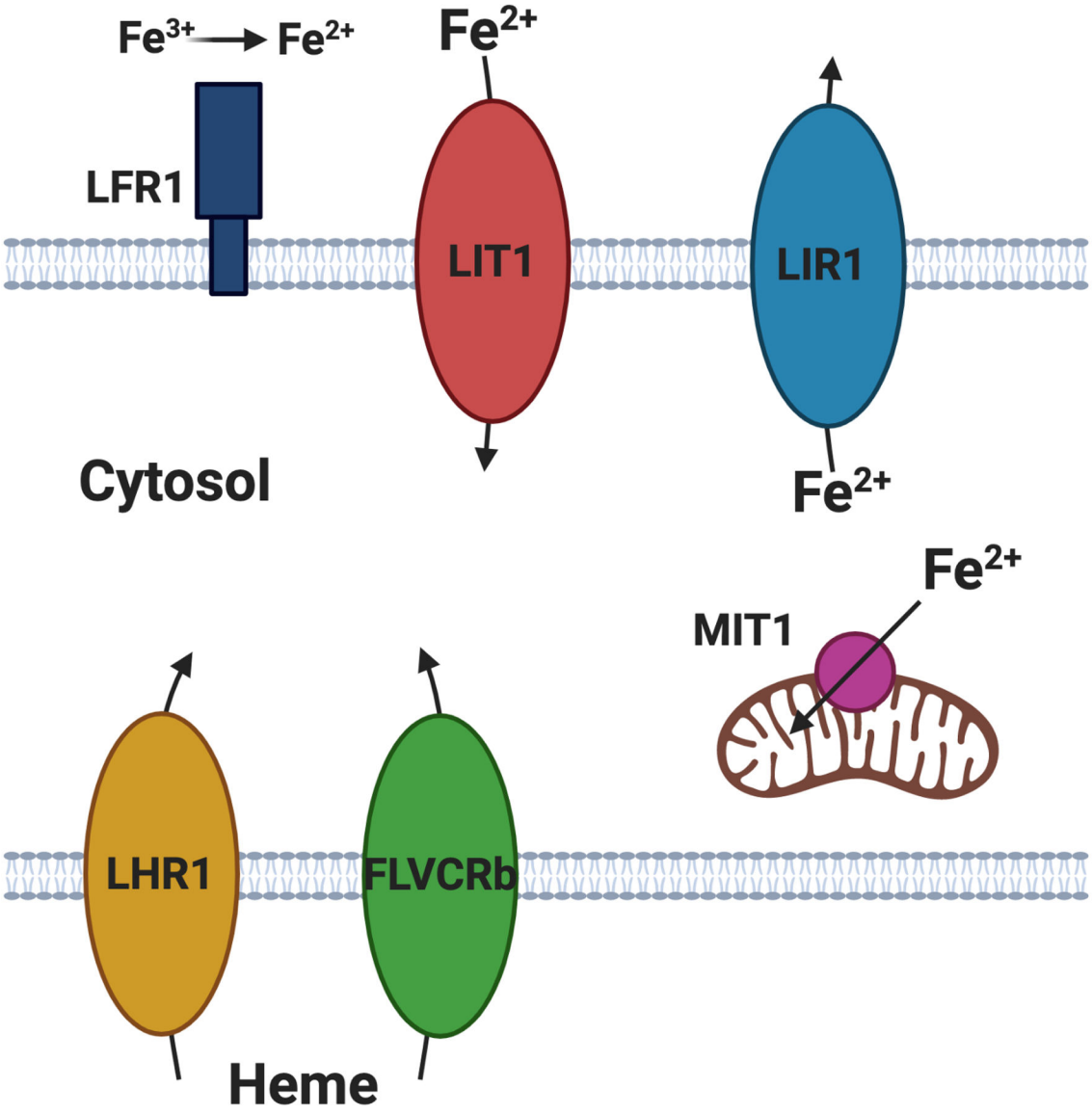

Fig. 3.

Import and export of iron and heme in *Leishmania amazonensis*. Lipid bilayers representing the plasma membrane are indicated at the top and bottom with the cytosol in between, and the mitochondrion is indicated in brown. LFR1 is an extracellularly oriented iron reductase that converts Fe^{3+} to Fe^{2+} at the parasite surface, generating the substrate for the iron permeases. Transporters for import (LIT1) or export (LIR1) of Fe^{2+} across the plasma membrane, for import of Fe^{2+} across the mitochondrial inner membrane (MIT1), and for import of heme across the plasma membrane (LHR1 and FLVCRb) are indicated as either ovals or a circle of different colors. Arrows indicate the direction of transport. Figure created with BioRender.com.