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***LiZIP3* is a cellular zinc transporter that mediates the tightly regulated import of zinc in *Leishmania infantum* parasites**

Sandra Carvalho^{1,2,3}, Rosa Barreira da Silva^{1,2,3,†}, Ali Shawki⁴, Helena Castro^{1,2,3,#}, Márcia Lamy^{1,2,3,§}, David Eide⁵, Vítor Costa^{1,2,3}, Bryan Mackenzie⁴, and Ana M. Tomás^{1,2,3,*}

¹Instituto de Investigação e Inovação em Saúde, Universidade do Porto, 4200 Porto, Portugal

²IBMC - Instituto de Biologia Molecular e Celular, Universidade do Porto, 4150-180 Porto, Portugal

³ICBAS - Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, 4050-313 Porto, Portugal

⁴Department of Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267, USA

⁵Department of Nutritional Sciences, University of Wisconsin-Madison, 1415 Linden Drive, Madison, WI 53706, USA

Summary

Cellular zinc homeostasis ensures that the intracellular concentration of this element is kept within limits that enable its participation in critical physiological processes without exerting toxic effects. We report here the identification and characterization of the first mediator of zinc homeostasis in *Leishmania infantum*, *LiZIP3*, a member of the ZIP family of divalent metal-ion transporters. The zinc transporter activity of *LiZIP3* was first disclosed by its capacity to rescue the growth of *Saccharomyces cerevisiae* strains deficient in zinc acquisition. Subsequent expression of *LiZIP3* in *Xenopus laevis* oocytes was shown to stimulate the uptake of a broad range of metal ions, among which Zn^{2+} was the preferred *LiZIP3* substrate ($K_{0.5} \approx 0.1 \mu M$). Evidence that *LiZIP3* functions as a zinc importer in *L. infantum* came from the observations that the protein localizes to the cell membrane and that its overexpression leads to augmented zinc internalization. Importantly, expression and cell-surface location of *LiZIP3* are lost when parasites face high zinc bioavailability. *LiZIP3* decline in response to zinc is regulated at the mRNA level in a process involving (a) short-lived protein(s). Collectively, our data reveal that *LiZIP3* enables *L. infantum* to acquire zinc in a highly regulated manner, hence contributing to zinc homeostasis.

* Address correspondence to: Ana M. Tomás, Instituto de Biologia Molecular e Celular, Rua do Campo Alegre 823 4150-180 Porto, Portugal, atomas@ibmc.up.pt; Telephone: (+351) 226 074 956; Fax: (+351) 226 098 480.

Helena Castro, Instituto de Biologia Molecular e Celular, Rua do Campo Alegre 823 4150-180 Porto, Portugal, hcastro@ibmc.up.pt; Telephone: (+351) 226 074 956; Fax: (+351) 226 098 480

† Present address: Immunobiology of Dendritic Cells, Institut Pasteur, Bâtiment Metchnikoff, 75724 Paris, France.

§ Present address: IBET, Instituto de Biologia Experimental e Tecnológica, 2780-157 Oeiras, Portugal.

Conflict of interest

The authors declare no conflict of interest

Keywords

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Introduction

Zinc is a fast and flexible ligand exchanger without inherent redox properties. These characteristics make zinc a versatile micronutrient that plays fundamental roles in a multitude of physiological processes (reviewed in Stefanidou *et al.*, 2006, and in Chasapis *et al.*, 2012). The biological protagonism of zinc is evident when one considers that this metal serves as a structural and catalytic cofactor for about 10% of any proteome and, as more recently discovered, as an intra- or extracellular cell messenger (reviewed in Maret, 2013). Consistent with its numerous functions, zinc deficiency leads to severe defects and, ultimately, to cell death (reviewed in Chasapis *et al.*, 2012). However, accumulation of zinc above certain thresholds is also hazardous to cells (reviewed in Plum *et al.*, 2010; Wang & Fierke, 2013). To guarantee that intracellular zinc levels are kept within tolerable limits, organisms are endowed with a sophisticated system of transporters and chelating molecules (*e.g.* metallothionein) that respond to zinc in a coordinated way, adjusting uptake, storage and/or elimination according to the concentration of the metal in the environment and their own individual demands.

Several families of transporters have been shown capable of transporting zinc across the plasma membrane. One of the most important is the ZRT/IRT-like Protein (ZIP) family of divalent metal transporters which often transport, apart from zinc, other metal ions such as iron, cadmium and manganese with either broad or restricted substrate specificity (reviewed in Guerinot, 2000, and in Gaither & Eide, 2001a). The precise mode of function of ZIP proteins has yet to be elucidated. The family encompasses 4 subfamilies, I, II, LIV-1 and gufA, classified on the basis of sequence similarity (reviewed in Gaither & Eide, 2001a), which, in addition to being present at the cell surface, can locate to the membranes of organelles. Nevertheless, it is consensually accepted that ZIP proteins transport metal ions into the cytosol (reviewed in Eide, 2004). Analysis of how ZIP transporters contribute to finely tune the level of zinc that permeates cells has indicated that some of these molecules are positively or negatively modulated by the low and high bioavailability of zinc, respectively, through transcriptional and/or post-transcriptional mechanisms (reviewed in Guerinot, 2000, and in Gaither & Eide, 2001a).

Protozoan parasites of the genus *Leishmania* are agents of leishmaniasis, a group of vector-borne diseases that afflicts approximately 12 million people worldwide and results in severe disfigurement, disability and even death. These parasites cycle between two main stages, the promastigote in the insect vector and the amastigote in mammalian hosts. As in other organisms, zinc is an essential nutrient for parasite replication and infectivity. Illustrating this, several essential *Leishmania* proteins are known or predicted to bind zinc, perhaps the most prominent example being the major surface protease (MSP or GP63), a zinc metalloprotease implicated in several functions along parasite development, and a recognized virulence factor (Olivier *et al.*, 2012). Despite the biological importance of zinc

to *Leishmania* and of its potential toxic effects (Sharquie *et al.*, 1997), no information exists regarding the processes underlying zinc homeostasis in these parasites. That *Leishmania* must adapt to fluctuations in zinc bioavailability appears, however, evident as they often thrive in people with deficits in this metal (Pasa *et al.*, 2003; Van Weyenbergh *et al.*, 2004; Bern *et al.*, 2007; Amini *et al.*, 2009; Mishra *et al.*, 2010).

This study is focused on zinc acquisition in *Leishmania*. In order to advance our understanding of the mechanism employed by these parasites to regulate zinc uptake, we searched the genome of *Leishmania infantum* for ZIP homologues and identified a candidate gene for zinc internalization in this parasite, *LiZIP3*. We report here that *LiZIP3* is a zinc transporter, the first characterized in *Leishmania*. Furthermore, we show that *LiZIP3* enables *L. infantum* to acquire zinc in a tightly regulated way.

Results

The *L. infantum* ZIP3 gene encodes a putative zinc transporter

Analysis of the *L. infantum* genomic database (TriTrypDB, <http://www.tritrypdb.org/tritrypdb/>) identified three putative ZIP members. One of these, *LiZIP1*, encoded by a pair of identical genes (*LinJ.31.3180* and *LinJ.31.3190*), is the orthologue of the *Leishmania amazonensis* ferrous-ion transporter LIT1 described by Huynh *et al.* (2006). The second, *LiZIP2*, specified by a single-copy gene (*LinJ.33.3350*), is a non-characterized protein glossed in the database as a putative cation transporter. The third putative ZIP member is encoded by another pair of identical genes (*LinJ.28.2050* and *LinJ.28.2060*) and annotated as a zinc transporter-like protein. The work reported in this manuscript refers to the last of these molecules, herein designated as *LiZIP3*.

The predicted *LiZIP3* protein is composed of 462 amino acids and has an expected molecular mass of 48.364 kDa and isoelectric point of 6.67. We generated a topology model of this molecule (Fig. 1) using TopPred software (<http://mobylye.pasteur.fr/cgi-bin/portal.py?#forms::toppred>). Specific features of its primary structure led us to consider *LiZIP3* as both a ZIP member and a candidate for zinc transport, namely (i) the presence of a conserved ZIP-family consensus sequence [LIVFA] [GAS] [LIVMD] [LIVSCG] [LIVFAS] H [SAN] [LIVFA] [LIVFMAT] [LIVDE] G [LIVF] [SAN] [LIVVF] [GS] (reviewed in Eng *et al.*, 1998), except that, in positions 4 and 15, a threonine (T) and an alanine (A) substitute for [LIVSCG] and [GS] respectively. These deviations from the consensus sequence do not exclude *LiZIP3* from the ZIP family of transporters according to the PFAM database (Punta *et al.*, 2012); and (ii) like most ZIP members, *LiZIP3* is predicted to possess eight transmembrane domains (TMDs), amino (N)- and short carboxyl (C)-termini exposed to the extracellular milieu or to the lumen of organelles, and a non-conserved domain between TMDs III and IV facing the cytosol (Fig. 1). The latter region contains a HQHG sequence akin to the (HX)_n motif present in most ZIPs and postulated to serve as a metal coordinating site (Eide *et al.*, 1996; Zhao & Eide, 1996a,b; Milon *et al.*, 2006; Nishida *et al.*, 2008; Jacques *et al.*, 2010). Another noteworthy characteristic of the *LiZIP3* sequence, shared with some other ZIP members, is the presence of a long N-terminus, comprising one quarter of the protein and evidencing several histidine-rich clusters. *LiZIP3* contains those amino acid residues previously identified by mutational analysis to be critical for zinc transport activity

(Rogers *et al.*, 2000; Jacques *et al.*, 2010; Taudte & Grass, 2010) (Fig. 1). The *L. infantum* protein also contains amino acid residues previously found to be important for ZIP proteins to transport other metal ions namely iron, manganese and cadmium (Rogers *et al.*, 2000; Jacques *et al.*, 2010) (Fig. 1). In conclusion, sequence analysis indicates that *LiZIP3* is a putative zinc transporter in *L. infantum*.

***LiZIP3* exhibits zinc transporter activity**

As a starting point to analyse the function of *LiZIP3*, we examined the time course of its expression during *L. infantum* promastigote growth by using western blot analysis. To that end, we produced an antibody against a 14-amino acid peptide located in the variable loop between putative TMDs III and IV of *LiZIP3*. The antibody recognized a protein band of 45 kDa, close to the predicted size of the *LiZIP3* nascent peptide (Fig. 2A). This band was undetectable at 0 and 24 h of culture but became more intense from 48 h onwards. This increase in *LiZIP3* expression was accompanied by the progressive depletion of zinc from the culture medium, as determined by monitoring zinc content in the parasite supernatants at each time-point by using atomic absorption spectrometry. Together, these observations suggest that *LiZIP3* is expressed in *L. infantum* in conditions of lower zinc concentrations and support a role of this protein in zinc acquisition.

We indirectly tested the zinc-transport activity of *LiZIP3* by using heterologous complementation in *S. cerevisiae*. The *zap1* (ZHY6) strain, which is defective in high- and low-affinity zinc acquisition (Zhao & Eide, 1997) and is therefore unable to grow in zinc-limiting conditions, was transfected with an expression vector carrying the *LiZIP3* gene (pYES2*LiZIP3*). The ability of *LiZIP3*-complemented yeast to grow in low-zinc medium was next compared with that of the wild type and of the mutant strain transfected with the empty pYES2 vector (Fig. 2B). Both the wild type and the *zap1* strains were able to grow in SD-glucose, a zinc-rich medium; however, when cultured in zinc-limited medium [SD-glucose, 1 mM EDTA (a divalent metal chelator), 0.9 mM ZnCl₂], the *zap1* strain proliferated only if transfected with the *LiZIP3*-containing plasmid. In other words, *LiZIP3* is required for zinc acquisition by this yeast strain. Similar results were observed for the *zrt1zrt2* (ZHY3) strain (Fig. S1), lacking the high- and low-affinity zinc transporters *ScZRT1* and *ScZRT2* (Zhao & Eide, 1996b). Therefore, functional complementation in yeast indicates that *LiZIP3* is an active zinc transporter.

Properties of transport by *LiZIP3*

To more directly establish the properties of *LiZIP3*-mediated transport we expressed *LiZIP3* in RNA-injected *Xenopus laevis* oocytes. In oocytes expressing *LiZIP3* we observed immunoreactivity at or near the cell perimeter that was not present in control oocytes (Fig. 3A). We also observed in all oocytes some intracellular immunoreactivity that was not specific to *LiZIP3* expression.

Since several ZIP family members exhibit broad metal reactivity with metal ions, we examined the substrate profile of *LiZIP3* in *X. laevis* oocytes at pH 7.5 (Fig. 3B). Expression of *LiZIP3* stimulated the uptake of a broad range of transition metal ions compared with control oocytes. The largest absolute increase was, by far, that for ⁶⁵Zn²⁺, whereas modest

increases were detected for $^{109}\text{Cd}^{2+}$, $^{57}\text{Co}^{2+}$, $^{64}\text{Cu}^{1+}$, $^{64}\text{Cu}^{2+}$, and $^{55}\text{Fe}^{2+}$, but not for $^{54}\text{Mn}^{2+}$. These results confirm that *LiZIP3* is a metal-ion transporter with an apparent preference for zinc ions.

We examined the saturation kinetics of *LiZIP3*-mediated zinc transport over the concentration range 0.1–10 μM at pH 7.5 (Fig. 3C). *LiZIP3*-mediated zinc transport data (calculated as the difference between the uptake in *LiZIP3*-expressing and that in control oocytes, and by propagating the error) were fit by Equation 1 (Experimental Procedures), revealing that zinc transport by *LiZIP3* is saturable with modestly high affinity for zinc judged from the zinc concentration at which transport was half-maximal ($K_{0.5}^{\text{Zn}}$) of 0.1 μM . By measuring $^{65}\text{Zn}^{2+}$ over the pH range 5.5 to 7.5, we observed maximal activity at pH 7.5 (Fig. 3D); however, substantial activity remained at pH 6.5 and pH 5.5 whereas mammalian ZIP transporters are more strongly inhibited at lower pH (Pinilla-Tenas *et al.*, 2011; Wang *et al.*, 2012).

LiZIP3* is a zinc uptake transporter in *L. infantum

Having ascertained that *LiZIP3* possesses zinc-transport activity in heterologous systems, we considered its potential role in the uptake of zinc in *L. infantum*. We first examined the subcellular location of the protein in the parasite by immunofluorescence using the anti-*LiZIP3* antibody (Fig. 4A). We found *LiZIP3* to be mostly present at the cell surface, a location consistent with a role in zinc internalization. Importantly, cell-surface localization of *LiZIP3* was observed only when parasites were cultured in the presence of 1 mM EDTA. Supplementation of this culture with 50 μM zinc for 24 h led to the disappearance of the protein from the parasite cell membrane.

As a second approach to verify the involvement of *LiZIP3* in zinc acquisition in *L. infantum*, we measured the amount of zinc internalized during 24 h by parasites overexpressing *LiZIP3* and by controls. The former were obtained by transfection with the pXG*LiZIP3* expression vector and confirmed to express increased levels of *LiZIP3* at the parasite surface by western blotting of biotinylated cell-membrane proteins (Fig. 4B). For this analysis we used parasites at 72 h of growth, *i.e.*, at a point of the growth curve where zinc concentration in the medium is already below detection limit (Fig. 2A). Consistent with being in zinc limiting medium, both parasite lines contained similar zinc levels, as measured by atomic absorption spectrometry (Fig. 4C). Conversely, 24 h after a zinc stimulus (provided by diluting parasites in fresh medium), *LiZIP3*-overexpressing cells presented higher zinc content than controls indicating that they internalized more zinc (1.45 vs 0.67 fmol/parasite). This experiment provided direct evidence that *LiZIP3* functions in zinc uptake in *Leishmania*.

Collectively, data reported here indicate that *LiZIP3* functions as a zinc importer in *L. infantum*. Furthermore, they suggest that expression of this protein is modulated by the extracellular zinc levels.

Expression of *LiZIP3* in *L. infantum* responds specifically to zinc

To more precisely test the effect of zinc on the expression of *LiZIP3* we examined how zinc deprivation affects *LiZIP3* in parasites grown in media containing varying concentrations of

EDTA, by using western blotting. EDTA caused a dose-dependent up-regulation of *LZIP3* expression (Fig. 5A) that was not the result of a nonspecific effect of the chelator since the control protein *L*GAPDH was unchanged. When EDTA-containing cultures were supplemented with zinc, the level of *LZIP3* decreased (Fig. 5B). A similar effect, *i.e.*, induction of *LZIP3* that was reversed upon introduction of zinc, was observed when parasites were grown in the presence of the zinc chelator TPEN (Fig. 5C). These observations strengthen the conclusion that the expression of this protein is regulated according to the concentration of zinc in the medium, and furthermore suggest that parasites are able to sense very small concentrations of zinc. In fact, based on the high affinity of EDTA for zinc, one can assume that the concentration of free zinc in media containing 0.4 mM EDTA plus 50 μ M zinc is very low, most likely within the sub-picomolar range. Nonetheless, these seemingly low concentrations of zinc are sensed by *Leishmania* parasites, which react by down-regulating *LZIP3* expression.

Since *LZIP3* can transport cadmium, cobalt, iron and copper (Fig. 3A) in addition to zinc, we investigated whether *LZIP3* expression is responsive also to a range of metals. For this analysis, promastigote cultures were supplemented with 100 μ M copper or 125 μ M cadmium, cobalt, iron, calcium, nickel, magnesium or manganese, or 50 μ M zinc and incubated for 24 h after which changes in the protein level were assessed by western blotting. Whereas 50 μ M zinc (or even 1 μ M, Fig. S2) strongly down-regulated *LZIP3* levels, no other metal did so, despite their presence at concentrations of 100 or 125 μ M (Fig. 5D). The slight reduction in the intensity of the 45 kDa band extracted from cultures treated with cobalt was likely due to protein aggregation during sample processing as deduced from the observation of high molecular weight bands. Interestingly, the presence of copper and cadmium led to an increase in the level of *LZIP3*. As these two ions were previously reported to inhibit metal transport by some ZIP members (Eide *et al.*, 1996; Grotz *et al.*, 1998; Korshunova *et al.*, 1999; Moreau *et al.*, 2002; Ramesh *et al.*, 2003; He *et al.*, 2009; Taudte & Grass, 2010; Stephens *et al.*, 2011), we considered that their competition with zinc for the transporter may explain the up-regulation of *LZIP3*. We found that copper I and II were each capable of inhibiting zinc transport in oocytes expressing *LZIP3* (Fig. S3A and B); however, the degree of inhibition by copper was modest so other mechanisms may additionally account for the up-regulation of *LZIP3* expression in promastigote cultures.

In short, our results demonstrate that the *L. infantum* ZIP3 protein is a zinc-transporter tightly regulated by zinc but not by other metals. Together with the observation that zinc chelators promote *LZIP3* cell surface expression whereas zinc supplementation induces its loss, our findings also suggest that *LZIP3* is a key component of a cellular homeostatic system that allows the parasite to cope with fluctuations in zinc bioavailability. To gain insight into the mechanisms underlying the zinc-induced modulation of *LZIP3* we investigated the molecular basis for the decrease of this transporter in *L. infantum* during zinc surplus.

***LZIP3* decrease in response to zinc is regulated at the mRNA level**

Rapid endocytosis of ZIP transporters in response to an increase in zinc levels appears to be a conserved mechanism by which cells prevent zinc overload (Gitan *et al.*, 1998; Kim *et al.*,

2004; Mao *et al.*, 2007; Wang *et al.*, 2004). Often, this process is accompanied by the subsequent degradation of the protein (Gitan *et al.*, 1998; Mao *et al.*, 2007) as well as the arrest of ZIP synthesis (Zhao & Eide, 1996a; Weaver *et al.*, 2007). To test whether zinc induces the fast removal of surface-associated *LIZIP3*, we performed cell-membrane biotinylation assays of parasites following metal supplementation. We observed decreased surface-associated *LIZIP3* after 12 h, but not 6 h, supplementation with 50 μ M zinc (Fig. 6A, compare lanes 1, 2, and 3). Such regulation is clearly much slower than the rapid endocytosis (within a few minutes) reported for analogous transporters in response to zinc overload (Gitan *et al.*, 1998; Kim *et al.*, 2004; Mao *et al.*, 2007; Wang *et al.*, 2004). Removal of *LIZIP3* from the membrane was not observed in control parasites that had not been supplemented with zinc (Fig. 6A, lanes 4 and 5). Moreover, the internalization of *LIZIP3* recorded at 12 h was not accompanied by a decrease in the total amount of this protein (Fig. 6B, lanes 2 and 3), indicating that protein degradation also is unlikely to act as a mechanism by which *LIZIP3* may be rapidly modulated. Instead, the early response of parasites to zinc appears to involve the arrest of *LIZIP3* synthesis. This conclusion is supported by the observation that whereas untreated cells accumulated the transporter over 12 h culture (Fig. 6B, lanes 1, 4 and 5), zinc-supplemented parasites did not do so (Fig 6B, lanes 1, 2 and 3). That parasites rapidly react to zinc by blocking *LIZIP3* synthesis is corroborated by the fact that *LIZIP3* mRNA is down-regulated within 1 h of zinc addition (Fig. 6C). Such decrease of mRNA in response to zinc is specific for *LIZIP3* as it was not observed for the two other *L. infantum* ZIP members, *LIZIP1* and *LIZIP2* (Fig. S5). Notably, this mechanism is triggered by concentrations as low as 1 μ M, strengthening the importance of *LIZIP3* mRNA down-regulation for homeostasis of zinc.

A labile protein mediates *LIZIP3* down-regulation in response to zinc

Owing to the fact that transcription in *Leishmania* spp. is polycistronic, these parasites are unable to control gene expression at the transcription level (reviewed in Requena, 2011). Accordingly, one explanation for the observed decay of *LIZIP3* mRNA upon zinc supplementation is that this metal alters the stability of the transcript. To test this hypothesis, we inhibited transcription by using actinomycin D and measured the levels of *LIZIP3* mRNA in the absence or presence of 50 μ M zinc by real-time reverse transcription-PCR (qRT-PCR) at varying time-points. We found a significant reduction in *LIZIP3* mRNA levels in parasites supplemented with zinc for 30 and 60 min relatively to non-treated controls (Fig. 7A), an observation that supports the conclusion that this metal induces the destabilization of the transcript. Next, we measured the effect of zinc on *LIZIP3* mRNA decay in parasites co-expressing *LIZIP3* from its chromosome context and from an episome carrying the *LIZIP3* gene devoid of its own untranslated regions (UTRs). We found that, while the mRNA derived from endogenous *LIZIP3* showed the expected increased degradation rate when zinc was available, that from the episome was not affected by zinc (Fig. S6). These data indicate that the *LIZIP3* UTRs are required for the mRNA destabilization induced by zinc and implicate the presence of elements in those regions responding to zinc concentration.

Short-lived proteins are often involved in regulation of mRNA stability in trypanosomatids (Wilson *et al.*, 1993; Argaman *et al.*, 1994; Graham & Barry, 1996; Quijada *et al.*, 1997; Webb *et al.*, 2005). To determine whether such molecules operate in the decay of *LIZIP3*

mRNA by zinc, we incubated parasites at the late-logarithmic phase of growth in the presence of cycloheximide or puromycin for 2 h to block protein synthesis and allow any short-lived protein to decay. We then added 50 μ M zinc and assessed mRNA levels 1 h later by using qRT-PCR. The results of this analysis revealed that, unlike controls, parasites cultured in the presence of the inhibitors were unable to down-regulate the *LiZIP3* transcript in response to zinc (Fig. 7B and C). These data suggest that zinc induces the expression of a labile protein which, directly or indirectly, causes destabilization of the *LiZIP3* mRNA.

Discussion

The maintenance of an adequate intracellular zinc pool is crucial for normal cell physiology. One of the mechanisms through which this is achieved is by controlling the quantity of metal that is translocated across the cell membrane. This manuscript reports a novel member of the ZRT/IRT-like Protein (ZIP) family, *LiZIP3*, and provides evidence that it enables *L. infantum* to acquire zinc in a highly regulated way.

Support for a role of *LiZIP3* in zinc internalization in *L. infantum* came from several observations. First, this protein was established to be a member of a family of transporters that contribute to elevate the cytosolic zinc content, the ZIP family, with conserved amino acids previously found to be important for the functionality of these molecules. These include several residues which are likely part of zinc binding sites associated to or within the channel through which substrates pass (Rogers *et al.*, 2000; reviewed in Gaither & Eide, 2001a), and a (HX)₂ in the cytosolic variable loop between TMDs III and IV, reminiscent of a (HX)_n cluster which was also proposed to bind zinc. It is possible that, during transport, this histidine-rich motif becomes spatially close to histidines in TMDs IV and V and is, thus, an integral part of the mechanism for zinc translocation across membranes (Milon *et al.*, 2006), either because it facilitates transport (Milon *et al.*, 2006; Jacques *et al.*, 2010) or because it imparts metal selectivity (Nishida *et al.*, 2008). Alternatively, the (HX)_n might bind cytoplasmic zinc functioning instead as a zinc sensor (Mao *et al.*, 2007). A second argument strengthening the proposal that *LiZIP3* enables zinc incorporation in parasites came from analysis in *S. cerevisiae* mutant strains and in *X. laevis* oocytes which experimentally confirmed that *LiZIP3* displays zinc transport activity. Finally, the observation that *LiZIP3* is present at the parasite cell membrane and that *LiZIP3*-overexpressing cells incorporate more zinc than wild type *L. infantum* provided solid evidence that this molecule is implicated in the internalization of zinc into the parasite. Importantly, we found that *LiZIP3* is tightly modulated by the external concentration of this metal, its expression and cell surface location being either induced or repressed when bioavailability of zinc was low or high, respectively. Other metals did not affect *LiZIP3* expression or localization indicating that these effects are zinc specific. These data suggest that *LiZIP3* is important for the parasite to adjust the level of zinc entering the cell at a particular timing. Remarkably, *Leishmania* appear to be able to sense extremely low concentrations of zinc (within the sub-picomolar range), responding to these by regulating *LiZIP3* expression.

Import of metals, as of other nutrients, into organisms is frequently carried out by more than one transporter displaying different affinities. Low-affinity transporters, which are

constitutively expressed or are regulated only to a low extent, enable adequate influx of nutrients when these are plentiful but cannot provide sufficient substrate in case of scarcity. In these conditions, finely regulated high-affinity transporters take over, allowing organisms to maintain their vital activities (Eide, 2012). Our data indicating that zinc specifically modulates expression of *LZIP3* and that such regulation occurs in the face of small changes in zinc bioavailability strongly suggest that this protein functions as a high-affinity zinc transporter in *L. infantum*. This is in line with the estimated $K_{0.5}$ of 0.1 μM for zinc transport in *X. laevis* oocytes expressing *LiZIP3*, similar to that of the high-affinity zinc transporters *ScZRT1* (Zhao & Eide, 1996a), and *ZIP5* and *ZIP6* of *Medicago truncatula* (Stephens *et al.*, 2011).

ZIP proteins can transport zinc and/or other metal ions. The substrate profile obtained in *X. laevis* oocytes indicates that *LiZIP3* can transport Cu^{1+} , Cu^{2+} , Cd^{2+} , Co^{2+} and Fe^{2+} in addition to Zn^{2+} . Although we lack a complete inhibitory profile, the observation that 10-fold excess Cu^{1+} (the metal ion that, after zinc, is most efficiently internalized in *X. laevis*) and Cu^{2+} only modestly inhibited zinc uptake by *LiZIP3* and the larger fluxes observed for zinc than for other metals, suggest that *LiZIP3* is a zinc-preferring transporter. Nevertheless, it is possible that, under zinc-limited conditions in which the protein is maximally expressed at the cell membrane, *LiZIP3* may supply *L. infantum* with cations other than zinc.

As argued above, *LiZIP3* is one factor that allows *L. infantum* to withstand changes in zinc bioavailability. We addressed the zinc-response of this protein by investigating the mechanism behind *LiZIP3* down-regulation upon zinc supplementation. A conserved mechanism operating in such situation involves rapid decrease of surface exposed transporter by induction of its endocytosis, simultaneously to synthesis arrest (Zhao & Eide, 1996a; Gitan *et al.*, 1998; Mao *et al.*, 2007; Weaver *et al.*, 2007). Interestingly, we did not find evidence for accelerated *LiZIP3* endocytosis, only for arrested protein synthesis. Protein internalization occurred only several hours after zinc addition raising the possibility that this is a late regulatory mechanism. In the absence of rapid protein removal from the cell membrane, the question arises of how *Leishmania* cope for over 6 h with excess zinc in their environment. One hypothesis is that *LiZIP3* is promptly inactivated by post-translational modifications (PTMs). Although, up to now, no PTMs directly modulating *ZIP* activity have been reported, *LiZIP3* holds a long histidine-rich extracellular N-terminal extension for which a role in modulation of zinc uptake has been previously proposed (Eide, 2005). The observed tolerance of *L. infantum* to the presence of surface *LiZIP3* for several hours after zinc supplementation can also be explained by increased sequestration of the metal into specific subcellular proteins or sites. In this regard, acidocalcisomes arise as a putative storage organelle as they have been shown to contain zinc (Miranda *et al.*, 2004; Lefurgey *et al.*, 2005). Intake of zinc into these structures is presumably carried out by an acidocalcisome membrane protein previously described in *Trypanosoma cruzi* (Ferella *et al.*, 2008) and with orthologues in *Leishmania*. Finally, we cannot rule out the possibility that a rapidly activated zinc cell exporter contributes to counteract excessive internalization of zinc in the parasite cytoplasm.

The present study reveals that a major mechanism by which zinc negatively modulates *LiZIP3* expression occurs at the level of mRNA stability. This process may involve a short-

lived regulatory protein which interacts directly or indirectly with zinc-sensing element(s) within the gene's UTRs. A likely scenario is that the regulatory protein possesses a zinc-finger motif requiring zinc to become stable and exert its function. Future work should address the identity of this labile protein as well as of the zinc-responsive motif(s) within the *LiZIP3* UTR(s) as this may be central to elucidate how zinc homeostatic responses are controlled in *Leishmania*. In other organisms the binding of specific transcription factors to promoter specific elements provides an important point to coordinate the zinc homeostatic response. In *Leishmania* and other trypanosomatids, which are virtually devoid of transcription regulation, no such mechanism can take place. Nonetheless, recent studies have shown that genes co-expressed specifically in a definite time-point share unique sequences in the untranslated regions of their mRNAs (Haile & Papadopoulou, 2007). It is, thus, reasonable to assume that the zinc-sensing element(s) within the *LiZIP3* UTRs may be common to other genes sharing the same response to zinc and that their expression is controlled by the same protein factor.

In conclusion, by identifying and characterizing *L. infantum* ZIP3, this report uncovers one of the major players that allow *Leishmania* to obtain critical zinc without facing the dangers of zinc overload. This report also provides the first insight into the biology of zinc in a trypanosomatid organism.

Experimental Procedures

Reagents

Unless otherwise indicated, restriction enzymes were purchased from Fermentas and reagents were obtained from Sigma–Aldrich Corp. or Research Products International Corp.

Plasmid construction

To generate the constructs used in this work, the *LiZIP3* ORF was excised by restriction from the p423ADHLiZIP3 plasmid (Supporting Experimental Procedures) and cloned into appropriate plasmids, as detailed next. In the case of the pYES2LiZIP3 construct, *LiZIP3* was cloned into the *Hind*III and *Xho*I restriction sites of the pYES2 plasmid (Invitrogen). To generate pOX(+)*LiZIP3*, *LiZIP3* was obtained by restriction with *Spe*I, Klenow end-filled, then *Xho*I and cloned into the *Eco*RV (Roche) and *Xho*I sites of pOX(+) (Takanaga *et al.*, 2005). Finally, to assemble pXGLiZIP3, the *LiZIP3* ORF was isolated by double digestion with *Hind*III and *Xho*I, Klenow end-filled and cloned into the *Sma*I site of pXG (Ha *et al.*, 1996).

Functional expression in *S. cerevisiae*

The yeast strains used in this work were the following: wild type (DY1457, *MAT α ade6 can1 his3 leu2 trp1 ura3*) and *zap1*⁻ (ZHY6, *MAT α ade6 can1 his3 leu2 trp1 ura3 zap1 ::TRP1*) (Zhao & Eide, 1997). Yeast strains were grown in Synthetic Defined (SD)-glucose medium [0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose] supplemented with the amino acidic auxotrophic requirements. Zinc-limiting medium was obtained by addition of 1 mM ethylenediaminetetraacetic acid (EDTA).

Expression of *LiZIP3* in *X. laevis* oocytes

We performed laparotomy and ovariectomy on adult female *X. laevis* frogs (Nasco, Fort Atkinson, WI, USA) under 3-aminoethylbenzoate methanesulfonate anesthesia (0.1% in 1:1 water/ice, by immersion), following a protocol approved by the University of Cincinnati Institutional Animal Care and Use Committee. Ovarian tissue was isolated and treated with collagenase A (Roche) and oocytes were isolated and stored at 17°C in modified Barths' medium as described (Mitchell *et al.*, 2014). The construct pOX(+)*LiZIP3* was linearized with *Swa*I and used to synthesize RNA *in vitro* using the mMESSAGE mMACHINE/SP6 RNA polymerase transcription kit (Applied Biosystems/Ambion) according to the manufacturers' protocols. Defolliculate stage V–VI oocytes were injected with 50 ng of cRNA and incubated for 6–7 days before being used in functional assays and for immunofluorescence. Noninjected oocytes were used as controls.

Radiotracer uptake assays in *X. laevis* oocytes

We used the radiotracer metals ^{109}Cd (added as CdCl_2) at final specific activity of 28 MBq mg^{-1} , ^{65}Zn (added as ZnCl_2) at 0.1–2.1 GBq mg^{-1} (both obtained from the National Laboratory, Oak Ridge, TN, USA), ^{57}Co (added as CoCl_2) at 2.94 GBq mg^{-1} , ^{55}Fe (added as FeCl_3) at 230 MBq mg^{-1} , and ^{54}Mn (added as MnCl_2) at 24 MBq mg^{-1} (purchased from Perkin–Elmer Life Science Products), and ^{64}Cu (added as CuCl_2) at 2.0 GBq mg^{-1} (Washington University–St Louis, MO, USA). To measure radiotracer metal-ion uptake, oocytes were incubated at room temperature (23°C) in a standard transport medium for 30 min as described (Wang *et al.*, 2012). Media containing iron or copper were supplemented with 100 μM or 1 mM *L*-ascorbic acid or 100 μM *L*-histidine, when indicated in the figure legends. Radiotracer metal-ion uptake was measured over 10 or 30 min, *i.e.*, well within the timecourse of linear uptake (> 2 h) of zinc, copper or iron in oocytes expressing mammalian ZIP orthologs (Pinilla-Tenas *et al.*, 2011; Antala & Dempski, 2012) exhibiting higher fluxes than did *LiZIP3*. Uptake was terminated by rapidly washing the oocytes three times in ice-cold pH-7.5 transport medium. Oocytes were then solubilized using 5% (w/v) sodium dodecylsulfate (SDS) and radiotracer content assayed by liquid-scintillation counting using Scintisafe-30% liquid scintillation cocktail (Fisher Scientific). Saturation kinetics of zinc uptake were obtained by measuring the internalization of ^{65}Zn in the range 0.1–10 μM in control and *LiZIP3*-expressing oocytes. Data were fit by a Michaelis–Menten function (Equation 1) (Illing *et al.*, 2012), for which V^S is the velocity (uptake) of substrate S ($^{65}\text{Zn}^{2+}$), V_{max}^S the derived maximum velocity, S the concentration of substrate S, and $K_{0.5}^S$ the substrate concentration at which velocity was half-maximal.

$$V^S = \frac{V_{\text{max}}^S \cdot S}{(K_{0.5}^S) + S} \quad (\text{Equation 1})$$

Leishmania infantum cultures

Leishmania infantum promastigotes (strain MHOM/MA/67/ITMAP-263) were cultured at 25°C in RPMI 1640 GlutaMAX™-I medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBSi), 50 U mL^{-1} penicillin, 50 $\mu\text{g mL}^{-1}$ streptomycin (all from Gibco)

and 20 mM HEPES pH 7.4. For each assay parasites were first pre-passaged, *i.e.*, they were subjected to 4–5 daily passages in culture medium at 10^6 mL⁻¹, and seeded at 10^6 mL⁻¹ at the beginning of the experiments.

Production of pXG and of pXGLiZIP3 parasites

Promastigotes in the logarithmic phase of growth were electroporated as described elsewhere (Beverley & Clayton, 1993) with 3–15 µg of plasmid DNA (pXG or pXGLiZIP3). Parasites were allowed to recover for 24 h before being selected on agar plates containing 15 µg mL⁻¹ G418 (Sigma). Isolated clones were then cultured in selective liquid medium. The genotype of the transfectants was analysed by Southern blotting of genomic DNAs extracted by the proteinase K method (Blaxter *et al.*, 1988).

Quantification of zinc in *L. infantum* parasites

To analyse the zinc content of pXG- and pXGLiZIP3-transfected *L. infantum* lines, parasites grown in normal medium for 72 h were seeded at 10^6 mL⁻¹ in fresh medium for 24 h. Aliquots of approximately 10^8 parasites, harvested immediately before and 24 h after medium replacement, were washed twice in ice-cold PBS (0.1 M sodium phosphate buffer pH 7.2, 0.15 M NaCl) and pellets dried at 37°C, dissolved in 65% (v/v) nitric acid for 2 h at 65°C and analysed by atomic absorption spectrometry (flame atomization) using the Atomic Absorption Spectrometer PU 9200X (Philips).

Protein extracts and western blotting

Parasites were harvested at 3000 ×g for 10 min at 4°C and washed twice in ice-cold PBS. The pellet was suspended at 6.25×10^8 mL⁻¹ in 4% (w/v) SDS in 50 mM Tris-HCl pH 7.4 containing a cocktail of protease inhibitors. The suspension was subjected to ultrasounds and stored at -70°C until further use. Before loading into SDS PAGE gels, extracts were treated with 4 M urea and 1% (v/v) β-mercaptoethanol, without boiling or heating. Western blotting was performed according to standard protocols. Control proteins were analysed upon membrane stripping and re-hybridization with the appropriate antibodies.

Cell membrane biotinylation

Parasites were harvested, washed twice in ice-cold PBS containing 1 mg mL⁻¹ glucose (PBS-glucose) and suspended at approximately 1.5×10^8 mL⁻¹ in 500 µL of 1 mg mL⁻¹ EZ-Link™ Sulpho-NHS-SS-Biotin (Thermo Scientific) (prepared in ice-cold PBS-glucose). Upon 1 h incubation on ice with constant shaking, parasites were washed in PBS-glucose and cell pellets stored at -70°C. For further processing of samples, these were lysed in 20 µL 4% (w/v) SDS in 50 mM Tris-HCl pH 7.4, containing a cocktail of protease inhibitors, and diluted by adding 80 µL PBS-glucose. Cell lysates were then sonicated, diluted in 700 µL PBS-glucose and further incubated with 30 µL of a 1:10 dilution of High Capacity Neutravidin® Agarose Resin (Thermo Scientific), for 1 h at room temperature with shaking. Next, beads were pelleted by centrifugation at 3000 ×g for 2 min and washed twice in 1 mL PBS. Biotinylated proteins were eluted from the beads by incubation for 2 h at room temperature in Laemli sample buffer containing 50 mM 1,4-dithiothreitol (DTT) and 4 M urea. Eluted proteins were subjected to western blotting, without heating or boiling.

Antibodies

Polyclonal antibodies against *LiZIP3* were produced by the GenScript Corp.'s custom antibody production services. A 14-amino acid peptide (LKDMYGGGAEDGQGG) within the variable region of *LiZIP3* was synthesized with an additional C-terminal cysteine and conjugated to the carrier protein keyhole limpet hemocyanin (KLH). After immunization of rabbits, antibodies were affinity-purified and used in western blotting and immunofluorescence. Sera against the glycosomal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the major surface glycoprotease (GP63) were a gift from Dr. P. Michels and Dr. F. Opperdoes and Dr. M. Wilson, respectively.

Effect of metal concentration on *LiZIP3* expression

To analyse protein expression throughout the days of promastigote culture, parasites were seeded at 10^6 mL⁻¹ in normal culture medium. Cells were counted every 24 h by using a Neubauer chamber, pelleted and subjected to protein extraction. Zinc concentration of the corresponding supernatants was determined by atomic absorption spectrometry, as above. To examine the effect of metal supplementation on *LiZIP3* expression, parasites were grown for 48 h in normal medium prior to the addition of metals in the following concentrations: 50 μ M ZnCl₂, 100 μ M CuSO₄·5H₂O and 125 μ M Fe-citrate, MnCl₂·4H₂O, MgCl₂·6H₂O, CaCl₂, CdCl₂·2.5H₂O, NiSO₄·6H₂O and CoCl₂·6H₂O. Parasites were pelleted after 24 h and subjected to protein extraction. Finally, the impact of metal depletion on *LiZIP3* expression was analysed by growing parasites in the presence of 0–1 mM EDTA or of 10 μ M N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN). All solutions, except for TPEN, were aqueous. TPEN was prepared in 50% (v/v) dimethyl sulfoxide (DMSO).

Indirect immunofluorescence assay

To localize *LiZIP3* in *L. infantum*, parasites were harvested, washed twice in ice-cold PBS, fixed in 4% paraformaldehyde (w/v) and spotted onto polylysine-treated slides. Cells were permeabilized with 0.1% (v/v) Triton X-100 and, after blocking with 1% (w/v) BSA, sequentially incubated with anti-*LiZIP3* and Alexa Fluor® 488-conjugated (Invitrogen) antibodies. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). Slides were mounted in 70% (v/v) glycerol in PBS. We performed immunolocalization of *LiZIP3* in oocytes by using oocytes embedded and freshly frozen in Tissue-Tek O.C.T. (optimum cutting temperature) compound (Sakura Finetek U.S.A., Inc.). Five micrometer sections were cut by using a cryostat (Leica 3050S), collected onto polylysine-treated slides and dried overnight at room temperature. Fixation was performed with pre-cooled acetone (–20°C) for 10 min, after which the acetone was poured off and allowed to evaporate from the tissue. Oocyte sections were blocked with 1% (w/v) BSA and incubated with anti-*LiZIP3* antibody followed by Alexa Fluor® 488-conjugated secondary antibody. Slides were mounted and visualized as described for parasites. Images were acquired with the AxioCam MR ver.3.0 camera (Carl Zeiss) coupled to the AxioImager Z1 microscope (Carl Zeiss) and with the laser scanning confocal microscope Leica TCS SP5 II (Leica Microsystems).

mRNA analysis

mRNA was measured by Real-Time Reverse Transcription-PCR (qRT-PCR). Promastigotes were seeded at 10^6 mL⁻¹ and grown for 48 h prior to any treatment. To study the effect of zinc in the *LiZIP3* mRNA, parasites were incubated for 1 h in the presence of 1 or 50 μ M ZnCl₂. The effect of zinc on the degradation of *LiZIP3* transcript was determined in cells cultured in the presence of 10 μ g mL⁻¹ of actinomycin D and 50 μ M ZnCl₂ for 15, 30 and 60 min. The existence of a labile protein was evaluated on parasites treated with 50 μ g mL⁻¹ cycloheximide or 200 μ g mL⁻¹ puromycin for 2 h and subsequently incubated for 1 h with 50 μ M ZnCl₂. Following the specific treatments, parasites were harvested, washed once in ice-cold PBS and stored at -70°C until being processed for RNA extraction. Total RNA of parasites was extracted using the RNeasy Plus Mini Kit (QIAGEN). Synthesis of cDNA was achieved from 1 μ g of total RNA using Superscript® VILO™ cDNA synthesis kit (Invitrogen). Real-Time PCR was performed using iQ™ SYBR® GREEN Supermix in an iCycler iQ5 from Bio-Rad according to the cycling conditions: an initial step at 95°C for 3.5 min, followed by 40 cycles at 95°C for 30 sec, 59°C for 45 sec and 72°C for 30 sec. The melt curve was obtained by decreasing the temperature by 0.5°C in each of the 10-sec 81 steps, starting on 55°C . Primers were designed to amplify sequences of approximately 200 bp (Table S1). The efficiency of each pair was tested and verified to be similar. Results were normalized by the *L. infantum* cytosolic GAPDH (*LinJ.36.2480*) as endogenous control. Relative expression levels (REL) were calculated according to Equation 2:

$$\text{REL} = 2^{[\text{CT}(\text{LiGAPDH}) - \text{CT}(\text{LiZIP3})]} \times 1000 \quad (\text{Equation 2})$$

Statistical analysis

We used Student's *t* test or one-way analysis of variance (ANOVA) in the analysis of the results obtained with parasites, followed by pairwise multiple comparisons using the Bonferroni post-hoc test. Radiotracer uptake data in oocytes were presented as mean and standard deviation (SD) for *n* independent observations and analysed using two-way ANOVA followed, where appropriate, by pairwise multiple comparisons using the Holm-Šidák test (for which *P* is the adjusted probability). Data were fit by Equation 1 by using the least-squares method of nonlinear regression followed by *F*-tests of the significance of the fit to the model; SE is the standard error of the estimate, and *P* refers to the significance of the fit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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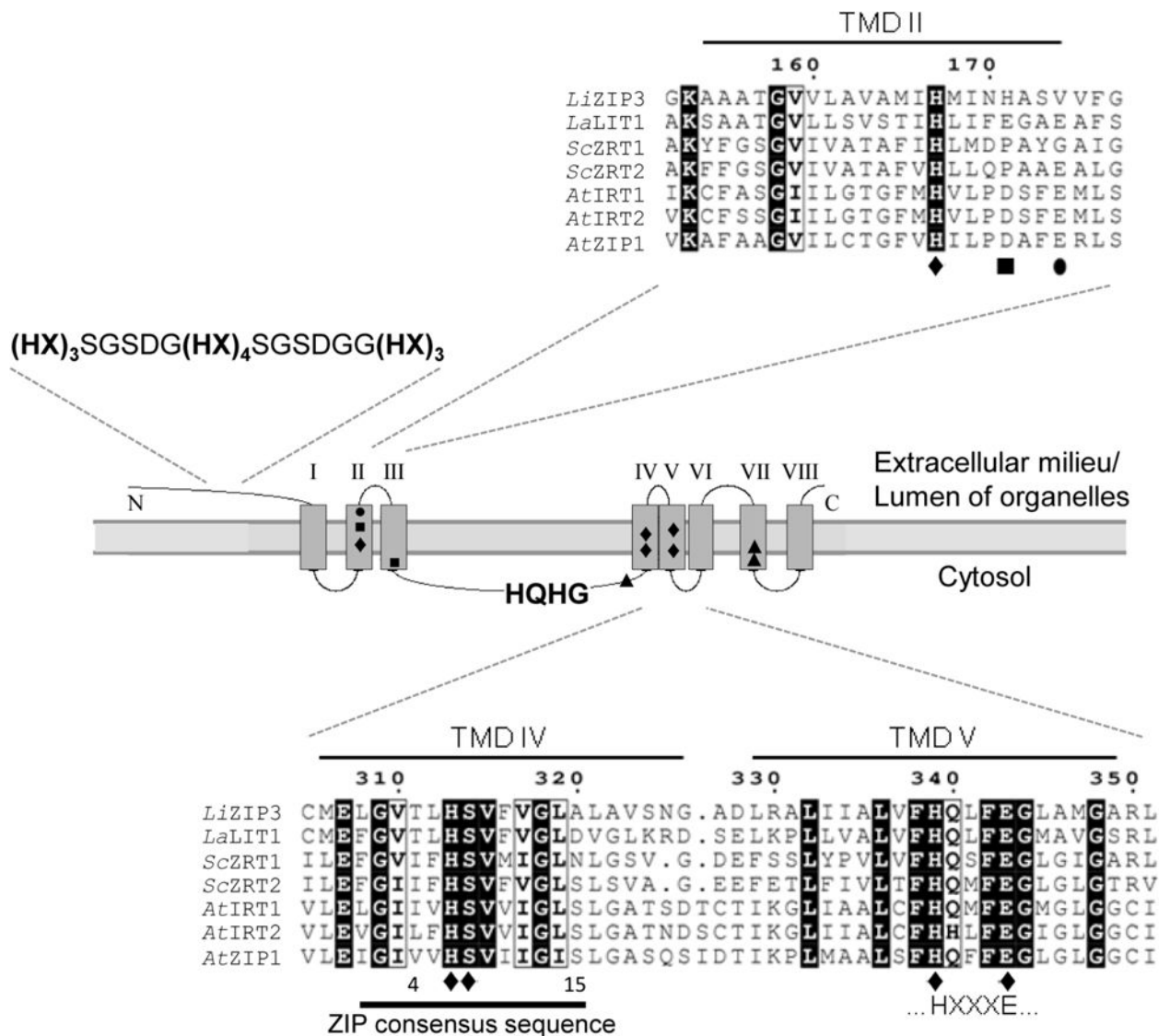


Fig. 1. *LiZIP3* is predicted to encode a member of the ZIP family. Alignment of selected residues of the predicted amino acid sequence of *LiZIP3* with experimentally characterized ZIP members from *L. amazonensis* (*La*), *S. cerevisiae* (*Sc*) and *A. thaliana* (*At*) using MultAlin interface (Corpet, 1988). Also depicted is the topology of *LiZIP3* predicted with TopPred 0.01 (Claros & von Heijne, 1994). Highlighted are the *LiZIP3* transmembrane domains (TMDs) and the residues shown to be important for transport of zinc (●), of iron, manganese, zinc and cadmium (◆), and of iron and manganese (■) in *AtIRT1*, and of iron (▲) in *LaLIT1*. Histidine-rich regions, the ZIP consensus sequence and the HXXXE motif, characteristic of ZIP members, are also represented. Accession numbers: *LiZIP3*, LinJ. 28.2050; *ScZRT1*, CAA96975.1; *ScZRT2*, CAA97701.1; *AtIRT1*, NP_567590.3; *AtIRT2*, NP_001031670.1; *AtZIP1*, NP_187881.1; *LaLIT1* sequence was obtained by modification of the *L. major* orthologue LmjF.31.3060 according to Huynh *et al.* (2006).

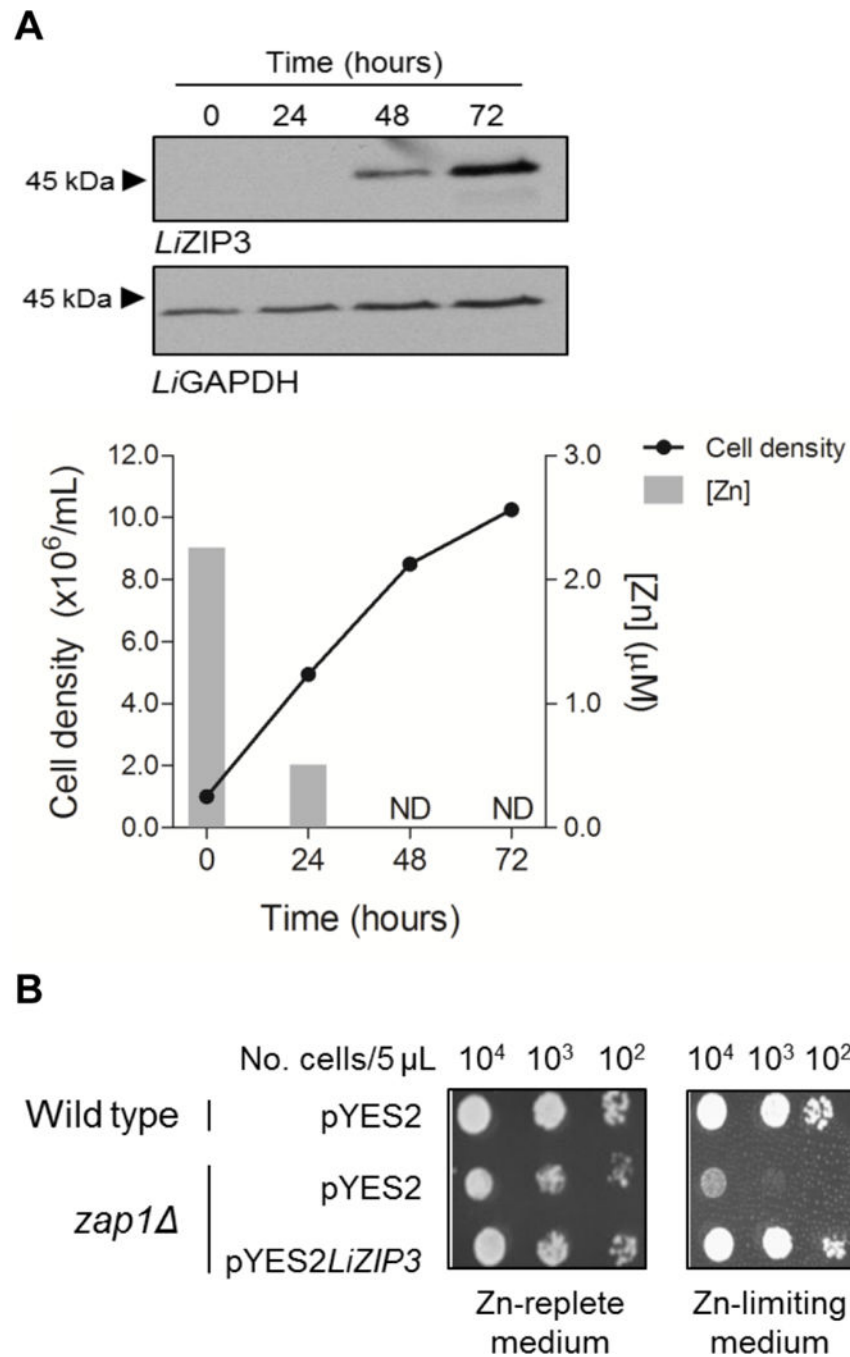


Fig. 2. *LiZIP3* transports zinc

A. *LiZIP3* is expressed in *L. infantum* under low zinc concentrations. Aliquots from a *L. infantum* culture set at 10^6 mL^{-1} were harvested daily and counted to determine cell density. Pellets were subjected to western blotting using an antibody raised against a *LiZIP3* 14-amino acid peptide. A band of approximately 45 kDa corresponding to *LiZIP3* was detected at 48 and 72 h. In parallel, culture supernatants were analysed by atomic absorption spectrometry for their content in zinc. ND, not detected (detection limit = $0.38 \mu\text{M}$).

B. *LZIP3* rescues the deficient zinc acquisition phenotype of the *zap1* yeast strain. The growth of *zap1* bearing pYES3*LZIP3* was compared with that of the same cells and of wild type carrying the empty vector (pYES2) in zinc-replete (SD-glucose) and zinc-limiting media (SD-glucose, 1 mM EDTA, 0.9 mM ZnCl₂).

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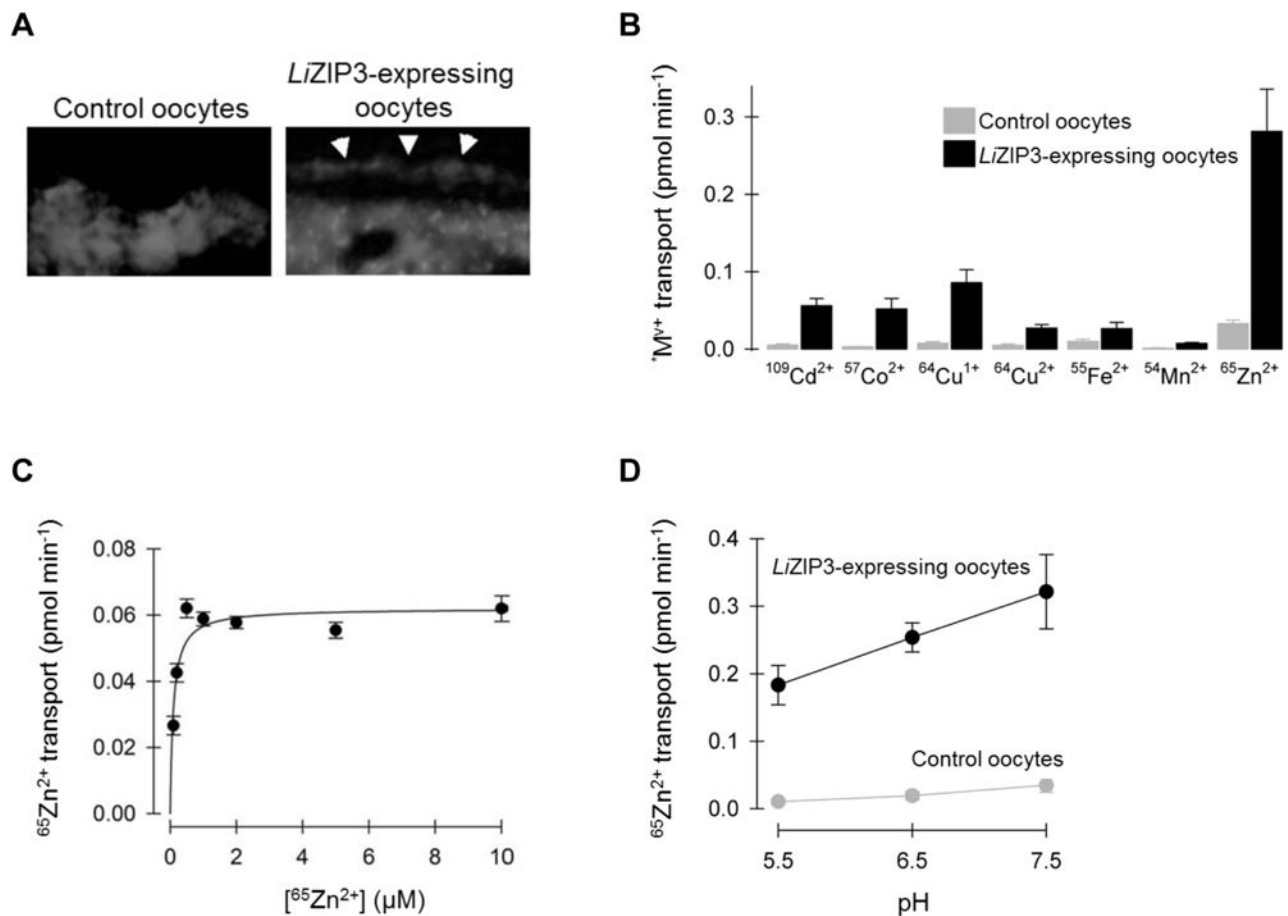


Fig. 3. Properties of *LiZIP3*-mediated metal transport in *X. laevis* oocytes

A. *LiZIP3* RNA-injected *X. laevis* oocytes express *LiZIP3* at the cell membrane.

Immunofluorescence of sections from control and *LiZIP3*-expressing oocytes probed with the anti-*LiZIP3* antibody. Arrowheads point to immunostaining in the approximate position of the cell membrane.

B. Metal-ion substrate profile of *LiZIP3*. Uptake of 5 μM radiotracer metal-ion ($*M^{v+}$, where * represents the radioactive isotope and v the valence) was measured in control oocytes (grey) and oocytes expressing *LiZIP3* (black) at pH 7.5, over 10 min at 23°C. Media containing ⁶⁴Cu¹⁺ or ⁶⁴Cu²⁺ were supplemented with 1 mM *L*-histidine and media with ⁶⁴Cu⁺ or ⁵⁵Fe²⁺ were supplemented with 1 mM *L*-ascorbic acid. Data are mean ± SD of 14–19 oocytes per group. Two-way ANOVA revealed an interaction ($P < 0.001$); *LiZIP3* differed from control for all metals ($P = 0.003$), except for ⁵⁴Mn²⁺ ($P = 0.30$); within *LiZIP3*-expressing oocytes, all metals differed from one another ($P = 0.004$) except ¹⁰⁹Cd²⁺ cf. ⁵⁷Co²⁺ ($P = 0.70$) and ⁶⁴Cu²⁺ cf. ⁵⁵Fe²⁺ ($P = 0.93$).

C. Saturation kinetics of *LiZIP3*-mediated ⁶⁵Zn²⁺ transport at pH 7.5. The uptake of 0.1–10 μM ⁶⁵Zn²⁺ in control oocytes and oocytes expressing *LiZIP3* was measured in standard medium at pH 7.5, over 30 min at 23°C. The *LiZIP3*-mediated component was calculated as the difference between means for control and *LiZIP3*-expressing oocytes and the error (SEM) was propagated by using the root of the sum of squares of SEM (control) and SEM (*LiZIP3*). Data were fit by a Michaelis–Menten function (Equation 1) from which the

following estimates were obtained: $V_{\text{max}}^{\text{S}} = 0.062 \pm 0.01 \text{ pmol min}^{-1}$; $K_{\text{S}0.5} = 0.10 \pm 0.03 \text{ }\mu\text{M}$; $r^2 = 0.84$; $P = 0.004$.

D. Uptake of $2 \text{ }\mu\text{M } ^{65}\text{Zn}^{2+}$ as a function of extracellular pH in control oocytes and in oocytes expressing *LZIP3* measured over 10 min. Data are mean \pm SD ($n = 9\text{--}16$). Two-way ANOVA revealed an interaction ($P < 0.001$); within *LZIP3*-expressing oocytes, each pH differs from one another ($P < 0.001$).

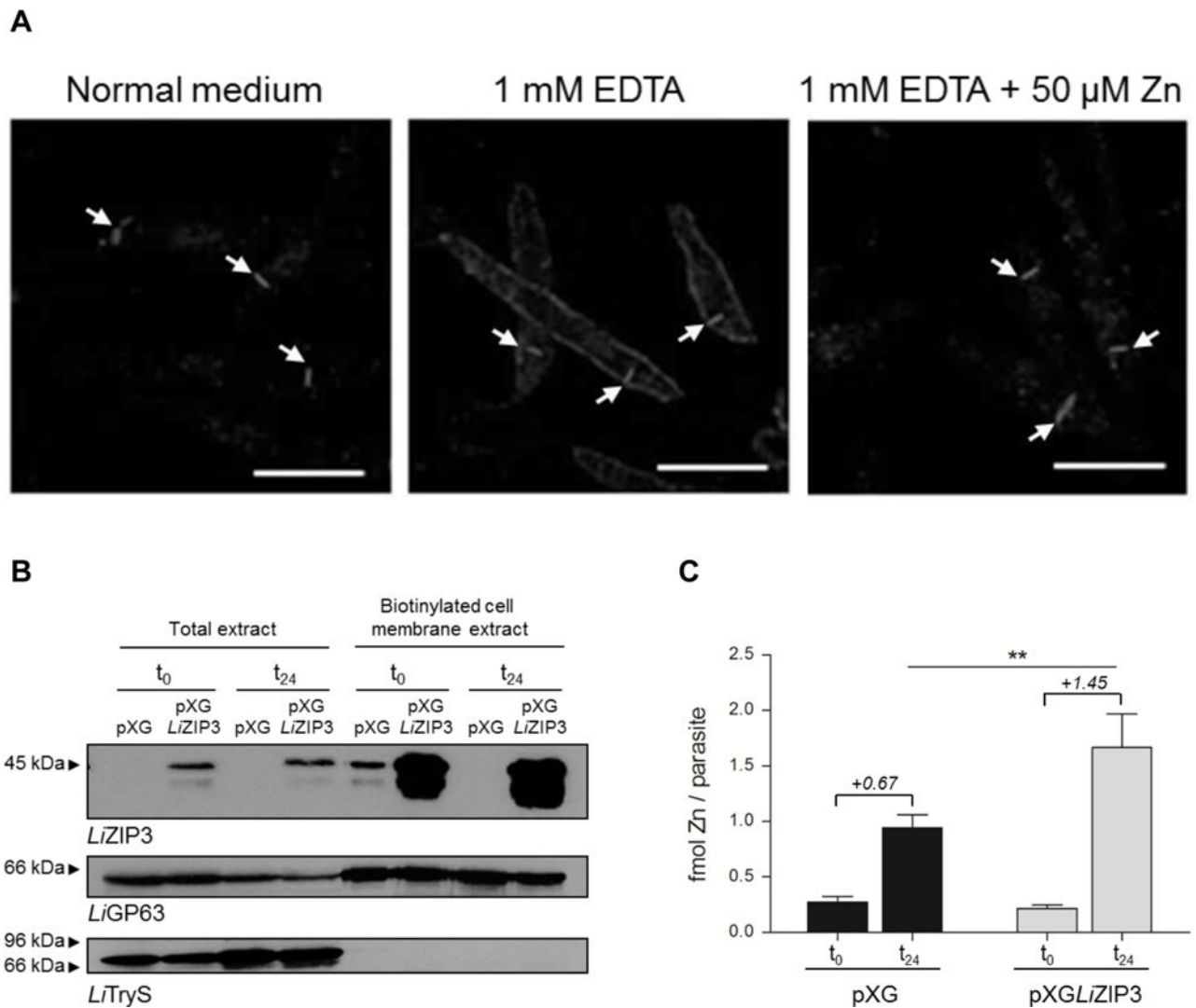


Fig. 4. *LizIP3* is a cell surface zinc transporter in *L. infantum*

A. Zinc restriction stimulates the expression of *LizIP3* at the cell membrane of *L. infantum*. Parasites were grown at an initial density of 10^6 mL⁻¹ in normal medium or in the presence of 1 mM EDTA. At 48 h of growth, 50 μ M zinc were added to an aliquot of the EDTA-treated culture and, 24 h later, *LizIP3* localization was assessed by immunofluorescence using the anti-*LizIP3* antibody. The position of the kinetoplast in each parasite, observed by simultaneously staining samples with DAPI, is indicated by arrows. Scale bar: 5 μ m. B and C. Overexpression of *LizIP3* at the cell surface results in an increased uptake of zinc. B. Control (pXG) and *LizIP3*-overexpressing (pXGLizIP3) parasites with 72 h growth were exposed to a zinc stimulus by dilution to 10^6 mL⁻¹ in fresh medium and cultured for an additional 24 h. The presence of *LizIP3* at the cell membrane, before (t_0) and 24 h after (t_{24}) medium replacement, was analysed by western blot analysis of cell membrane biotinylated proteins (the faster migrating band observed at approximately 38 kDa refers to a protein of unknown identity that is often recognized by the anti-*LizIP3* antibody). Upon stripping, *LiGP63* and trypanothione synthetase (*LiTryS*) (Sousa *et al.*, 2014) were used as controls for cell membrane and cytosolic proteins, respectively.

C. The zinc content of pXG and *LZIP3*-overexpressing parasites was measured by atomic absorption spectrometry at t_0 and t_{24} (mean \pm SD, $n = 3$; asterisks indicate significant differences between pXG and *LZIP3*-overexpressing parasites $**P < 0.01$, Student's *t* test). Numbers in italic refer to the mean of the differences between zinc content at t_0 and t_{24} for each parasite line.

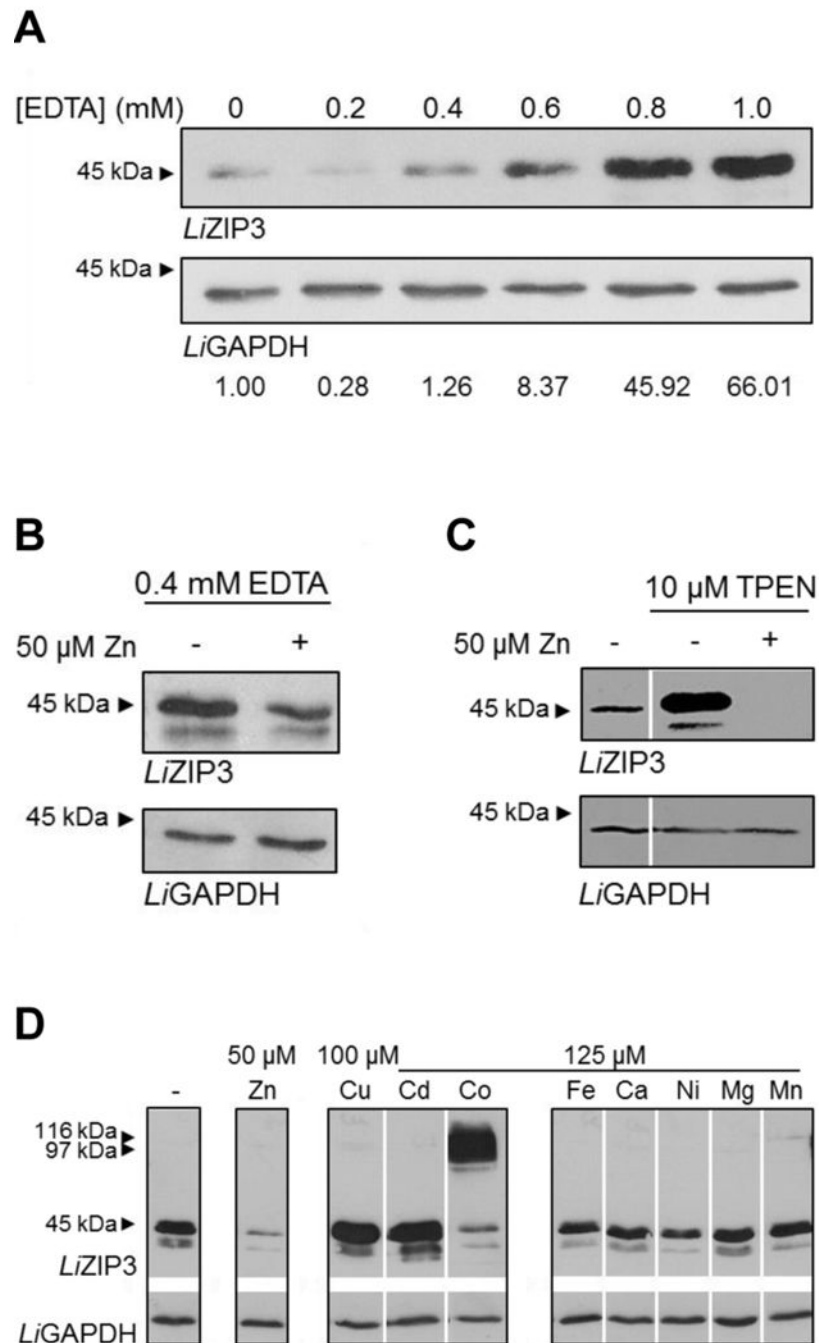


Fig. 5. *LiZIP3* expression in *Leishmania* is modulated by the concentration of zinc in the culture medium

A. Dose-dependent effect of EDTA on *LiZIP3* levels. The effect of the divalent metal chelator on the expression levels of *LiZIP3* was addressed by adding increasing concentrations of EDTA to the culture medium for 48 h. Analysis was performed by western blotting. Values below the blots refer to the densitometry analysis (the intensity of the *LiZIP3* band in the presence of each EDTA concentration was compared to that obtained in the absence of the chelator after loading correction with *LiGAPDH*).

B and C. The presence of zinc in the culture media reduces *LZIP3* expression. The ability of zinc to decrease the *LZIP3* levels in the presence of chelating agents was assessed (B) by supplementing a culture of parasites grown for 24 h in 0.4 mM EDTA with 50 μM zinc or (C) by adding 10 μM TPEN or 10 μM TPEN plus 50 μM zinc to a culture of parasites previously grown for 48 h in normal medium. Twenty four hours upon medium supplementation, parasites were harvested and processed for western blotting.

D. Effect of different divalent metals on *LZIP3* expression. *L. infantum* promastigotes were inoculated in normal medium at 10^6 mL^{-1} and, at 48 h of growth, several divalent metals were added to the cultures for an additional 24 h. The effect of metal supplementation on the level of *LZIP3* expression was analysed by western blotting. Expression of *LiGAPDH* in the same blots was used as loading control in all cases.

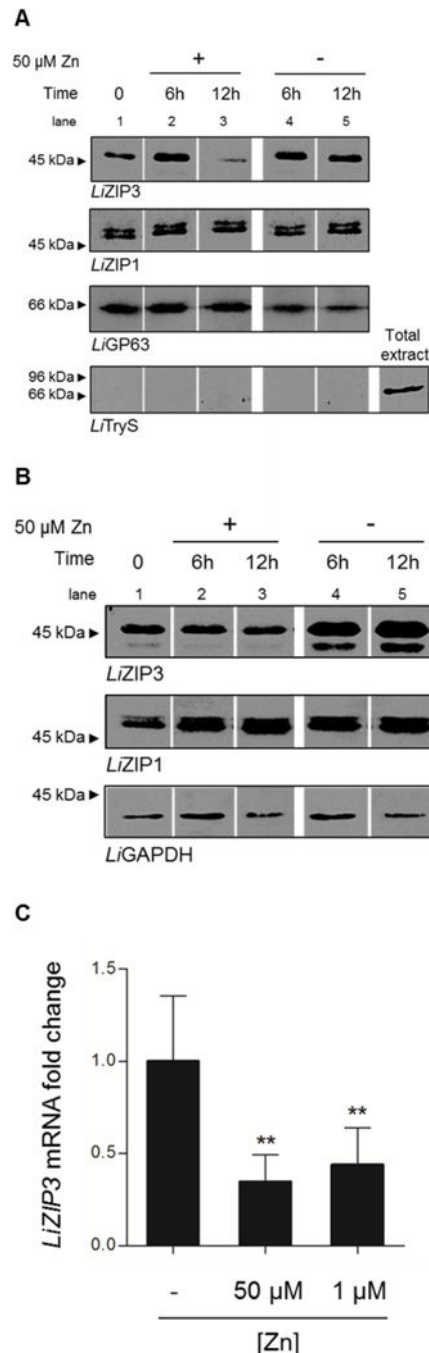


Fig. 6. Reduction of the *LiZIP3* mRNA is an early event on the process of zinc-mediated *LiZIP3* down-regulation

A and B. Zinc-induced decay of surface-associated *LiZIP3* does not result from rapid endocytosis of the transporter but from protein synthesis arrest. A culture of promastigotes grown for 48 h in normal medium was supplemented with 50 μ M zinc for different time-points. Before and 6 and 12 h after the addition of the metal, aliquots were pelleted for (A) cell membrane biotinylation and (B) total protein extraction. *LiZIP3* levels were analysed by western blotting. The specificity of the zinc effect on cell membrane and total *LiZIP3* levels was controlled, upon stripping, with antibodies recognizing other cell surface proteins,

*Li*GP63 and *Li*ZIP1, and soluble *Li*GAPDH. Exclusivity of biotinylation of cell surface proteins is shown with *Li*TryS. (Uncropped blots are in Fig. S4A and B).

C. Zinc supplementation causes a decrease in *Li*ZIP3 mRNA level. Fifty or 1 μ M zinc were added to cultures of promastigotes grown for 48 h in normal medium. Changes in *Li*ZIP3 mRNA caused by metal supplementation were measured by qRT-PCR 1 h upon introduction of the metal in the culture (mean \pm SD, $n = 5$; asterisks indicate significant differences to control, $**P < 0.01$, One-way ANOVA).

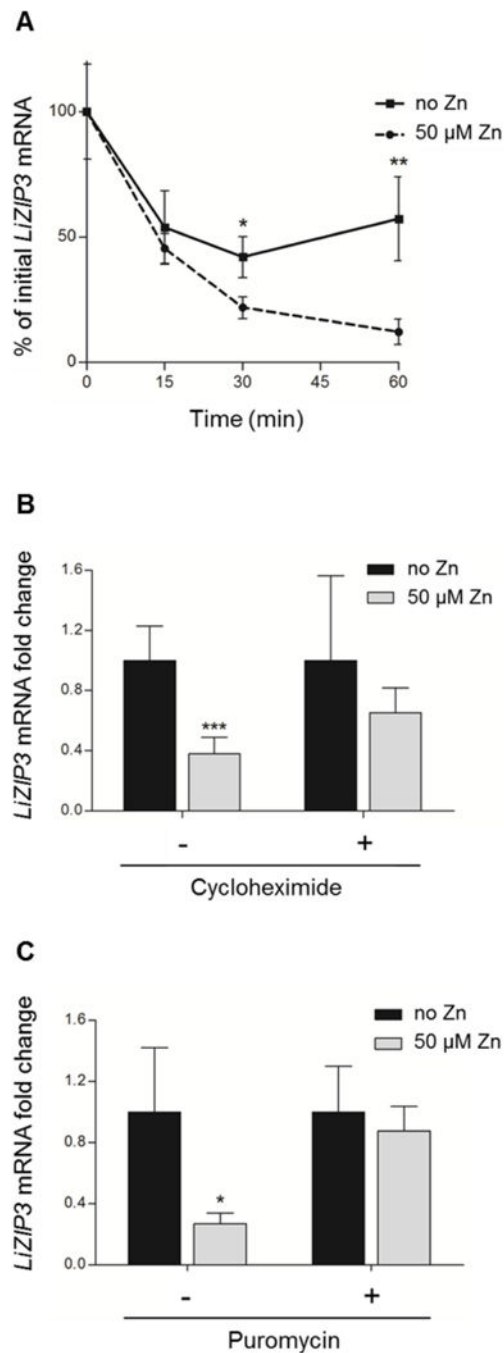


Fig. 7. Expression of *LiZIP3* is regulated by zinc at the mRNA stability level through a process involving a short-lived protein

A. Effect of zinc on the degradation of *LiZIP3* mRNA. Messenger RNA synthesis was blocked with $10 \mu\text{g mL}^{-1}$ actinomycin D and the degradation of *LiZIP3* mRNA in the absence and in the presence of $50 \mu\text{M}$ zinc was accompanied by qRT-PCR (mean \pm SD, $n = 3-4$; asterisks indicate significant differences in each time-point, * $P < 0.05$, ** $P < 0.01$, Student's t test).

B and C. Down-regulation of *LiZIP3* mRNA requires the activity of a labile protein. Promastigotes grown for 48 h in normal medium were incubated with $50 \mu\text{g mL}^{-1}$

cycloheximide (B) or 200 $\mu\text{g mL}^{-1}$ puromycin (C) during 2 h prior to addition of 50 μM zinc for 1 h. Fold changes in *LiZIP3* mRNA were measured by qRT-PCR (mean \pm SD, $n = 5$ for cycloheximide, $n = 3$ for puromycin; asterisks indicate significant differences between cultures not supplemented and supplemented with zinc in each condition tested, *i.e.* with or without cycloheximide or puromycin, * $P < 0.05$, *** $P < 0.001$, Student's *t* test).

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