The Pumilio-domain protein PUF6 contributes to SIDER2 retroposon-mediated mRNA decay in *Leishmania*

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ABSTRACT

*Leishmania* and other trypanosomatid protozoa lack control at the level of transcription initiation and regulate gene expression exclusively post-transcriptionally. We have reported previously that *Leishmania* harbors a unique class of short interspersed degenerate retroposons (SIDERs) that are predominantly located within 3′-UTRs and play a major role in post-transcriptional control. We have shown that members of the SIDER2 subfamily initiate mRNA decay through endonucleolytic cleavage within the second conserved 79-nt signature sequence of SIDER2 retroposons. Here, we have developed an optimized MS2 coat protein tethering system to capture trans-acting factor(s) regulating SIDER2-mediated mRNA decay. Tethering of the MS2 coat protein to a reporter RNA harboring two MS2 stem-loop aptamers and the cognate SIDER2-containing 3′-UTR in combination with immunoprecipitation and mass spectrometry analysis led to the identification of RNA-binding proteins with known functions in mRNA decay. Among the candidate SIDER2-interacting proteins that were individually tethered to a SIDER2 reporter RNA, the Pumilio-domain protein PUF6 was shown to enhance degradation and reduce transcript half-life. Furthermore, we showed that PUF6 binds to SIDER2 sequences that include the regulatory 79-nt signature motif, hence contributing to the mRNA decay process. Consistent with a role of PUF6 in SIDER2-mediated decay, genetic inactivation of PUF6 resulted in increased accumulation and higher stability of endogenous SIDER2-bearing transcripts. Overall, these studies provide new insights into regulated mRNA decay pathways in *Leishmania* controlled by SIDER2 retroposons and propose a broader role for PUF proteins in mRNA decay within the eukaryotic kingdom.

Keywords: *Leishmania*; SIDER2 retroposons; MS2 coat protein tethering system; Pumilio proteins; PUF6; mRNA decay

INTRODUCTION

*Leishmania* spp. are unicellular eukaryotic pathogens causing a wide spectrum of pathologies in humans ranging from cutaneous to visceral infections (Desjeux 2004). *Leishmania* has a digenetic life cycle alternating between extracellular procystigotes and amastigotes inside the phagolysosome of mammalian macrophages where they replicate and cause disease (Bates and Rogers 2004). Similarly to other trypanosomatids, the *Leishmania* genome is organized in long polycistronic units (Myler et al. 1999; Ivens et al. 2005). In the absence of transcriptional control by RNA polymerase II in trypanosomatids, polycistronic units are transcribed in a constitutive manner and further processed to mature mRNAs through a coordinated 5′-trans-splicing and 3′-polyadenylation cleavage reactions (Papadopoulou et al. 2008; Michaël 2011). In addition, gene expression has been shown to change dramatically throughout the complex life cycles of these parasites (Haile and Papadopoulou 2007; Rochette et al. 2008; Nilsson et al. 2010; Siegel et al. 2010). Regulation of mRNA decay rates through interactions of RNA-binding proteins (RBPs) with cis-acting sequences in 3′-UTRs of trypanosomatid transcripts is central in determining the fate of mRNAs and thus fine-tuning developmental gene expression (McNicoll et al. 2005; Bringaud et al. 2007; Haile and Papadopoulou 2007; Haile et al. 2008; Müller et al. 2010b; Kramer 2012; Clayton 2013). Several RBPs, such as zinc finger proteins, Alba-domain proteins, and Pumilio (PUF) proteins, have been found to regulate mRNA stability in trypanosomatids (Clayton 2013; Dupé et al. 2014).

The genomes of trypanosomatids contain a large repertoire of RBPs, few of which have been characterized to interact with specific sequences in 3′-UTRs. In *Trypanosoma cruzi*, UBPI binds to an AU-rich RNA instability element (ARE) in the 3′-UTR of small mucin mRNAs and promotes RNA destabilization (D’Orso and Frasch 2001). In *T. brucei*, PU9 binds to a consensus sequence in 3′-UTR of transcripts whose function is important in temporal coordination of the kinetoplast and nuclear replication during late S-phase (Archer et al. 2007; Orso and Frasch 2001). In *T. brucei*, UBPI binds to an AU-rich RNA instability element (ARE) in the 3′-UTR of small mucin mRNAs and promotes RNA destabilization (D’Orso and Frasch 2001). In *T. brucei*, PU9 binds to a consensus sequence in 3′-UTR of transcripts whose function is important in temporal coordination of the kinetoplast and nuclear replication during late S-phase (Archer et al. 2007; Orso and Frasch 2001).
The T. brucei ZC3H11 binds to and stabilizes mRNAs encoding chaperones required for protein refolding following heat shock (Droll et al. 2013). DRBD3, a protein containing two RRM domains, plays a role both in splicing and mRNA stability in T. brucei (Estévez 2008; Das et al. 2012). Also, DRBD13 has been demonstrated to negatively regulate mRNAs encoding for cell membrane-associated proteins via interaction with AREs of the target transcripts (Jha et al. 2015). More recently, we showed that the Leishmania infantum Alba3 protein can stabilize the developmentally regulated amastin transcripts specifically in the amastigote stage upon binding to a U-rich element in the 3′UTR (Dupé et al. 2014).

In Leishmania, we have previously identified a large family of extinct retroposon elements termed short interspersed degenerate retroposons (SIDERs) (>2000 copies per genome), predominantly located within 3′UTRs (Bringaud et al. 2007, 2008; Smith et al. 2009). Members of the two major SIDER subfamilies were shown to regulate mRNA turnover in a stage- and species-specific manner (SIDER2 subfamily) (Boucher et al. 2002; McNicoll et al. 2014). More recently, we showed that SIDER2-harboring mRNAs have to be recognized by XRN1-acting factors regulating SIDER2 retroposon-mediated mRNA decay (Bringaud et al. 2007; Smith et al. 2009). More recently, we showed that XRN1 binds to SIDER2-containing reporter RNA. In vivo UV-crosslinking and immunoprecipitation combined with LC–MS/MS studies allowed us to identify several candidate proteins bound to a SIDER2-harboring 3′UTR but not to a 3′UTR lacking SIDER2. Among the candidate RNA-binding proteins with known functions in mRNA decay, the Pumilio-family member (PUF6) was shown to enhance degradation and reduce transcript half-life upon tethering to SIDER2 regulatory sequences. Furthermore, we showed that genetic inactivation of PUF6 leads to an increased stability of endogenous SIDER2-bearing transcripts, supporting a role of PUF6 protein in the SIDER2-mediated mRNA decay process.

RESULTS

Development of an improved MS2 coat protein tethering system for identifying trans-acting factors regulating SIDER2 retroposon-mediated mRNA decay in Leishmania

So far, the N4 peptide has been used successfully to tether RBPs to a particular RNA of interest in the related Trypanosoma species (Delhi et al. 2011; Wurst et al. 2012; Droll et al. 2013; Jha et al. 2014; Singh et al. 2014). Here, we have developed an improved MS2 coat protein tethering system adapted for use in Leishmania to attach RNA-binding proteins (RBPs) specifically to a reporter RNA. A schematic diagram describing this system is shown in Figure 1A. The bacteriophage MS2/R17, MS2 coat protein (MCP) recognizes and binds to a specific stem–loop aptamer termed MS2 stem–loop (MS2 SL) of the replicase open reading frame to suppress its translation (Bernardi and Spahr 1972). This observation was later applied successfully to many fields of RNA biology, including RNA localization, capturing of ribonucleoprotein complexes, mRNA translation, and decay (Keryer-Bibens et al. 2008; Buxbaum et al. 2015). We used the mFold RNA secondary structure prediction software to determine the best flanking sequences required to form a MS2 stem–loop structure. By testing different flanking sequence combinations, we were able to find the most suitable sequence allowing the tandem MS2 hairpin RNA to form in silico (Fig. 1B). Figure 1C displays four LUC–MS2 reporter constructs containing or not a SIDER2 element that were used in this study. In Saccharomyces cerevisiae, it was recently reported that binding of MCP to MS2 SL, inserted into 3′UTR of the genes encoding for QCR8 and PGK1RNA, blocked XRN1 5′–3′ exonuclease activity and led to the accumulation of 3′ mRNA fragments containing MS2 SLs (Garcia and Parker 2015). To ascertain that the insertion of two MS2 hairpin structures downstream from the LUC gene and its interaction with MCP does not alter decay rates of the LUC-SIDER2 reporter transcripts, we compared MS2-LUC–4000 3′UTR along with the non-MS2 RNA, LUC-4000 3′UTR by Northern blot hybridization (Fig. 1D). As we have reported previously, SIDER2 retroposon promotes rapid RNA decay, whereas deletion of SIDER2 from the 3′UTR blocks degradation and causes accumulation of the LUC reporter mRNA (Fig. 1D; Bringaud et al. 2007; Müller et al. 2010b). Here, we show that addition of two MS2 hairpins in the reporter RNA and binding to MCP does not alter degradation by SIDER2 (Fig. 1D). Furthermore, we improved the MS2 pull-down system by optimizing expression levels of MCP. A single copy of MCP was not expressed sufficiently when transfected into L. infantum (data not shown). In the absence
of an effective inducible protein expression system in *L. infantum*, we decided to express MCP episomally to obtain higher levels of expression and consequently sufficient binding to the MS2 SL RNA. Therefore, we generated a tandem MCP construct (tMCP) as MCP binds the hairpin as preformed dimers, thus recruiting two copies of the fused RBP of interest to the tethering site. Bound coat protein dimers interact cooperatively with one another when tandem arrays of hairpins are present (Johansson et al. 1997). Western blot using an anti-HA or an anti-MCP antibody demonstrated a suitable expression of the tMCP protein in our transfectants (Fig. 1E), favoring the coat protein–ligand binding in vivo.

Identification of RNA-binding proteins interacting with a SIDER2 reporter RNA using the MS2 coat protein tethering system

The MS2 coat protein tethering system is a robust in vivo approach to study the functional properties of RBPs by attracting them to any RNA of interest (Keryer-Bibens et al. 2008). To identify RBPs bound to the SIDER2-harboring 3′UTR of the *L. infantum* LinJ.36.4000 transcript, we carried out coimmunoprecipitation (co-IP) studies against the MCP protein combined
with LC/MS-MS analysis. For these studies, we used *L. infantum* parasites coexpressing the PTP-tMCP-HA protein with either the LUC-MS2-4000 3′ UTR (harbors the full-length SIDER2-containing 3′ UTR of LinJ.36.4000 transcript with two MS2 hairpins placed downstream from the LUC reporter gene) or the MS2-LUC-4000 3′ UTR (the two MS2 binding sites were placed upstream of the LUC reporter gene) (Fig. 1A,C). Placing the MS2 binding sites both upstream and downstream, the LUC reporter permits us to eliminate the possibility of occupational effect on SIDER2-interacting proteins imposed by MCP when it binds to MS2 RNA. UV-crosslinking followed by co-IP experiments against the PTP-tMCP-HA protein using an anti-HA antibody coupled to magnetic beads and mass spectrometry analysis revealed a number of candidate proteins, tethered to SIDER2 RNA (Table 1). The presence of PTP-tMCP-HA protein prior and after co-IP studies was verified by Western blotting (Supplemental Fig. S1). Experiments were done with both *L. infantum* promastigotes and axenic amastigotes, but only the results with promastigotes are shown here. A control with a non-SIDER2 3′ UTR (LinJ.39.3990; LUC-MS2-3990 3′ UTR) was also used to assess binding specificity (not shown). Only candidate proteins tethered to the LUC-MS2-4000 3′ UTR mRNA but not to LUC-MS2 or LUC-MS2-3990 3′ UTR (e.g., two negative controls) were considered for further analysis. These include the XRN 5′–3′ exoribonuclease, the deadenylase complex CCR4-NOT1 and NOT5 proteins, an ATP-dependent RNA helicase (Hel3150), a lupus La protein homolog, the Pumilio-family member PUF6, an RNA-binding protein (RBP-0610), and one hypothetical protein (LinJ.29.2040) with no predicted RBP domain(s) (Table 1). NOT1 was excluded from our study as we failed to amplify the corresponding ORF (~7 kb) for cloning purposes. Similarly, NOT5 was excluded from further investigation as we were unable to detect the fusion protein by Western blotting (see Supplemental Fig. S2) and the transfected cells experienced some growth problems.

The Pumilio-domain protein PUF6 causes the highest mRNA destabilizing effect among the candidate SIDER2-interacting proteins once tethered to a SIDER2-harboring reporter RNA

To assess the role candidate SIDER2-interacting proteins may play on LUC-SIDER2 transcript stability, we fused mostly at the C terminus, each of these proteins with the tMCP-HA protein and coexpressed them into *L. infantum* LUC-MS2-4000 3′ UTR or LUC-MS2-4000ΔSIDER2 recombinant strains (Fig. 2A) and proceeded with tethering assays on stably cotransfected cell lines. The expression of the different tMCP-fused candidate proteins was verified by Western blot analysis (Supplemental Fig. S2). No significant variation in the levels of tethered protein expression was observed between LUC-MS2-4000 3′ UTR and LUC-MS2-4000ΔSIDER2 expressing parasites (Supplemental Fig. S2C). LUC-MS2-4000 plasmid copy-number variation among the different cotransfectants was determined by Southern blot hybridization (Supplemental Fig. S3). The use of episomal vectors was our best possible choice at the time as it was technically challenging to delete the entire SIDER2 sequence or the 79-nt signature II from a given 3′ UTR within the genomic locus by homologous recombination. Steady-state levels of LUC mRNA in the different transfectants coexpressing the candidate proteins tethered to the LUC-MS2-4000 SIDER2-bearing reporter RNA were detected by Northern blot hybridization and normalized to the 18S rRNA signal (RNA loading variation) as well as to the LUC-MS2-4000 or LUC-MS2-4000ΔSIDER2 plasmid copy number (slight

| Identified proteins | Accession no. | Peptide no. |
|---------------------|--------------|-------------|
| MS2 coat protein (MCP) | LinJ.25.1920 | 3–4         | 2–4         | 2–4         |
| NOT5 protein | LinJ.21.0880 | 3–5         | 3–5         | 0           |
| CCR4-NOT1 transcription complex subunit 1 | LinJ.16.0400 | 2–4         | 2–4         | 0           |
| XRN 5′–3′ exoribonuclease, putative | LinJ.35.3150 | 2–4         | 2–4         | 0           |
| ATP-dependent RNA helicase, putative | LinJ.17.0610 | 2–4         | 2–3         | 0           |
| RNA-binding protein, putative | LinJ.22.1210 | 2–3         | 2–3         | 0           |
| Pumilio protein 6 (PUF6), putative | LinJ.21.0600 | 2–4         | 2–4         | 0           |
| Lupus La protein homolog, putative | LinJ.29.2040 | 2–2         | 2–2         | 0           |
| Hypothetical protein, unknown function | LinJ.12.0400 | 2–2         | 2–2         | 0           |

*L. infantum* parasites cotransfected with LUC-MS2-4000 or MS2-LUC-4000 and the PTP-tMCP-HA protein were subjected to UV-crosslinking, coimmunoprecipitation, and LC-MS/MS analysis. Results shown here are representative of three independent experiments with 1% false discovery rate (FDR). Only selected proteins bound to the SIDER2-harboring 3′ UTR of the *L. infantum* LinJ.36.4000 transcript but not identified in the PTP-tMCP-HA transfectant (control) are shown here. Proteins identified with >2 peptides and a probability of >95.0% were considered. The number of peptides shown represents the minimum and maximum values found in the triplicate experiments.

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variations were observed between different transfectants; see Supplemental Figs. S3, S4). Normalization of the Northern blot data indicated that among the six candidate proteins investigated here, tethering of PUF6 to the LUC-MS2-4000 mRNA led to the highest effect on RNA fate, with a fourfold decrease in LUC mRNA levels (Fig. 2B). No destabilizing effect was observed upon tethering of PUF6 to the LUC-MS2-4000ΔSIDER2 mRNA (Fig. 2C), indicating that PUF6 has to bind sequences within SIDER2. Two other proteins, RBP-0610 and the hypothetical protein 2040, once tethered to the LUC-MS2-4000 mRNA, resulted in a 2.0-fold and 1.66-fold decrease in LUC mRNA levels, respectively (Fig. 2B). In this study, however, we focused on PUF6 protein, primarily due to its highest destabilizing effect on the LUC-SIDER2 RNA.

Tethering PUF6 protein to a SIDER2-harboring reporter RNA enhances degradation through binding to SIDER2 regulatory sequences

To determine whether PUF6 binds to the second tandem 79-nt SIDER2 signature sequence (SII), shown to be the target of endonucleolytic cleavage (Müller et al. 2010b; Azizi et al. 2017b), we generated another recombinant parasite cell line coexpressing the PUF6-tMCP protein together with the LUC-MS2-4000ΔSII mRNA. This enables us to compare the results of PUF6 tethering to the LUC-MS2-4000ΔSII with PUF6 tethering to LUC-MS2-4000 3'UTR RNAs by Northern blot hybridization. Cotransfectants with the tMCP-HA were also used as controls for comparative studies. LUC mRNA fold-differences were determined following normalization of the hybridization LUC signal intensities to the α-tubulin signal (RNA loading control) as well as to the plasmid copy number (Supplemental Figs. S3, S5). Normalized LUC mRNA values indicated that PUF6 confers a fourfold decrease in LUC-MS2-4000 3'UTR mRNA levels when compared to the control tMCP-HA protein tethered to the same RNA (Fig. 3, left panel). However, PUF6 did not alter LUC mRNA levels when tethered to a reporter transcript lacking SIDER2 (LUC-MS2-4000ΔSIDER2) (Fig. 3, middle panel), as also shown in Figure 2C. Interestingly, we found that once tethered to the LUC-MS2-4000ΔSII RNA, PUF6 does decrease LUC mRNA levels by 2.5-fold (Fig. 3A, right panel) as compared to fourfold whehen tethered to the full-length SIDER2-harboring 3'UTR (Fig. 3, left panel). Altogether, these data suggest that PUF6 binds sequences (or RNA structure) within SIDER2, including signature II, but not exclusively, to accelerate mRNA decay.

We showed that artificial binding of PUF6 to SIDER2 sequences accelerates mRNA decay (Figs. 2, 3). Further, we investigated if decreased mRNA levels upon PUF6 tethering are due to an increased destabilization. To address this question, we measured LUC mRNA half-lives upon tethering of PUF6 to the reporter RNA and compare them with the MCP tethering control. LUC mRNA stability was assessed by Northern blotting on total RNA treated prior with actinomycin D.
pu6 and mRNA decay in leishmania

Genetic inactivation of PUF6 results in increased stabilization of SIDER2-containing endogenous transcripts

To investigate further the role PUF6 plays in SIDER2-mediated mRNA decay, we generated a L. infantum PUF6 null mutant strain. Both PUF6 alleles were successfully replaced sequentially with the hygromycin (HYG) and neomycin phosphotransferase (NEO) resistance genes, as confirmed by Southern blot hybridization (Fig. 6A), indicating that the PUF6 gene is not essential for Leishmania promastigote growth. Next, we evaluated the effect of PUF6 gene inactivation on SIDER2-harboring LinJ.36.4000 and LinJ.08.1220 transcript expression levels by Northern blot hybridization. Both transcripts have been studied extensively in our laboratory (Bringaud et al. 2007; Müller et al. 2010a,b). Interestingly, we observed an increased accumulation of both 4000 and 1220 transcripts by 1.8-fold and 2.6-fold, respectively, in the PUF6−/− knockout (Fig. 6B), which further confirms that PUF6 plays a role in SIDER2-mediated decay.

To examine changes in 4000 and 1220 mRNA stability in the absence of PUF6, we treated L. infantum wild-type and PUF6−/− strains with ActD at indicated time points, and total RNA extracted from these strains was subjected to Northern blot hybridization. Interestingly, half-lives of 4000 and 1220 transcripts were increased by at least twofold in the absence of PUF6 (from 18 min in WT to 32 min in PUF6−/− for 4000, and from 15 min in WT to 34.5 min in PUF6−/− for 1220) (Fig. 6C). These results corroborate our findings that PUF6 contributes to SIDER2-mediated mRNA decay. In another attempt to validate the effect of PUF6 depletion on the stability of 4000 and 1220 transcripts, we treated parasites with cycloheximide (CHX) to inhibit translation elongation and then analyzed total RNA extracted from those cells at various time points post-treatment by Northern blotting. The aim of this experiment was to compare accumulation rates of SIDER2-harboring transcripts between wild-type and PUF6−/− parasites under conditions of global translation inhibition where de novo-synthesis of mRNA decay factors, including PUF6, could be affected. Interestingly, accumulation of both 4000 and 1220 transcripts increased by approximately 2.5- to 3.5-fold on average, after 4–6 h of CHX treatment in PUF6−/− parasites compared to the wild-type strain (Supplemental Fig. S6), suggesting that PUF6 is required for optimal decay of SIDER2-bearing transcripts.
exacerbates RNA degradation but has no effect on an RNA lacking SIDER2. Accordingly, genetic inactivation of PUF6 leads to an increased accumulation and higher stability of *Leishmania* transcripts harboring a SIDER2 element in their 3'UTR. The effect of PUF6 on SIDER2-containing mRNA destabilization is the result of specific binding to sequences within SIDER2 that include, but not exclusively, the second conserved 79-nt signature sequence (signature II) of SIDER2 retroposons, which is the target of endonucleolytic cleavage (Müller et al. 2010b; Azizi et al. 2017b). Indeed, tethering of PUF6 to a SIDER2 element lacking signature II decreases RNA stability albeit to a lesser extent than tethering to the full-length SIDER2 RNA. We have shown recently that SIDER2-mediated mRNA decay is coupled to translation (Azizi et al. 2017a), and our findings here that inhibition of global protein synthesis by cycloheximide increases the accumulation of SIDER2-harboring *Leishmania* transcripts by at least two-to threefold in the PUF6-7′− knockout in comparison to the wild-type strain, suggest that PUF6 contributes to the decay mechanism while associated with SIDER2-bearing mRNAs on translating ribosomes.

The Pumilio proteins are well-known RNA-binding proteins in most eukaryotic lineages. This family has expanded in trypanosomatids with 10 members in *Leishmania* (Luu et al. 2006), whereas only five members were found in the yeast *Saccharomyces cerevisiae* and two in humans. In many organisms such as *S. cerevisiae, Drosophila melanogaster, C. elegans*, and *H. sapiens*, several Pumilio repeats within PUF proteins share the UGUR (R represents a purine) recognition sequence flanked by downstream or upstream sequences often unique to each PUF protein (Wickens et al. 2002; Miller et al. 2008; Ulbricht and Olivas 2008). In all these cases, PUF proteins comprise eight sequence repeats (called PUM repeats) and flanking N- and C-terminal regions (Zhang et al. 1997), out of which repeats 5–8 recognize the UGUR tetranucleotide motif. However, similarly to the yeast Puf1p and Puf2p homologs, the *Leishmania* PUF6 is predicted to encompass 6 PUM repeats (Supplemental Fig. S7), and this suggests that binding via a consensus sequence may be dispensable (Ulbricht and Olivas 2008). At the molecular level, PUF proteins promote translational repression and/or mRNA degradation first by interacting specifically with cis-elements in the 3′UTR of their target mRNAs and through complex interactions with
absence of the SIDER2 element, mRNA decay follows a conventional pattern. The graph shows the half-life of the difference in expression ratios compared to the T0 value and were used in the half-life charts.

Indeed, members of the PUF family bind to 3′ of the PUF family play an important role in mRNA decay. There is increasing evidence in the literature that members of the PUF family bind to 3′ UTRs of various eukaryotic mRNAs and enhance turnover, acting combinatorially with the CCR4–NOT deadenylase complex (Quenault et al. 2011). In humans, PUF proteins, PUM1 and PUM2, recruit the CCR4–NOT complex to stimulate mRNA decay and repress translation (Van Etten et al. 2012). In S. cerevisiae, recruitment of CCR4p, the catalytic subunit of the CCR4–POP2–NOT deadenylase complex, by PUF3p, PUF4p or PUF5p, results in mRNA deadenylation (Goldstrohm et al. 2006, 2007; Hook et al. 2007; Lee et al. 2010). A similar mechanism has been reported in Drosophila, C. elegans, and humans, where recruitment of the deadenylase complex POP2p subunit by PUF proteins stimulates deadenylation of target mRNAs (Goldstrohm et al. 2006; Kadyrova et al. 2007; Suh et al. 2009). Here, MS2 protein tethering to a SIDER2-containing reporter RNA led also to the identification of CCR4–NOT1 and NOT5 deadenylases. However, our previous data support a model for SIDER2-mediated decay, which is deadenylation-independent (Müller et al. 2010b). Therefore, these deadenylases may be recruited to a SIDER2 reporter RNA through their association with PUF6, which specifically binds sequences within SIDER2. In yeast, it has been shown that PUF4p and PUF5p bind to the HO mRNA 3′ UTR, repressing translation, triggering deadenylation and thus mRNA decay, possibly through their association with the decapping activator DCP1p and the DHH1p helicase (Goldstrohm et al. 2006, 2007; Hook et al. 2007). A similar decay mechanism has been proposed in the related trypanosomatid, Trypanosoma cruzi, where PUF6 binds to a subset of mRNAs and its overexpression results in the down-regulation of target transcripts in epimastigotes, possibly through its association with the TcDHH1 helicase (Dallagiovanna et al. 2008). In Leishmania, however, overexpression of PUF6 did not affect steadystate levels of SIDER2-containing transcripts (Supplemental Fig. S8), suggesting that for PUF6 to enhance destabilization of SIDER2 transcripts it has to be artificially tethered to these RNAs. This could imply that PUF6 binds with higher specificity to SIDER2 target mRNAs. Although coimmunoprecipitation experiments against a PUF6-HA protein were not very optimal due to proteolytic degradation of PUF6 under nondenaturing conditions, mass spectrometry studies identified DH11 as one of the PUF6-interacting proteins together with another decay protein, the Dis3-like ribonuclease, an exoribonuclease associated with the human exosome (Staals et al. 2010) (data not shown). Additional preliminary experiments using the BioID method (Roux et al. 2012) as an alternative approach to coimmunoprecipitation, have also led to the identification of the Dis3-like ribonuclease as a PUF6-interacting factor (data not shown). Thus, PUF6 might possibly enhance decay of SIDER2-containing transcripts by facilitating the recruitment of exoribonucleases. Moreover, the fact that PUF6 accelerates mRNA decay only once tethered to SIDER2 sequences, including the second conserved 79-nt signature of SIDER2 retroposons shown previously to be central to endonucleolytic cleavage and degradation (Müller et al. 2010b;
Azizi et al. 2017b), supports the possibility that PUF6 facilitates recognition of SIDER2 by the endoribonuclease.

In summary, this study led to the identification of Pumilio-domain protein PUF6 as the first trans-acting factor shown to interact with SIDER2 regulatory sequences and to accelerate mRNA decay. SIDER2-mediated mRNA decay is initiated by PUF6 binding with specific probes against LinJ.36.4000 and LinJ.08.1220 as well as the α-tubulin probe was used as RNA loading control. Ethidium bromide staining was also included to visualize RNA as an additional loading control. Fold changes of 4000 and 1220 endogenous transcripts in the PUF6−/− knockout relative to the WT are shown here below the blots and were calculated after normalization against the loading controls. Standard deviations are the result of three independent experiments. (C) Stability of SIDER2-harboring LinJ.36.4000 and LinJ.08.1220 transcripts was compared between the WT and PUF6−/− strains following a time course treatment with the RNA Pol II transcription inhibitor ActD. Parasites were collected at indicated time points, and total RNA was extracted and subjected to Northern blotting. Blots were hybridized with the RNA Pol II transcription inhibitor ActD. Parasites were collected at indicated time points, and total RNA was extracted and subjected to Northern blotting. Blots were hybridized with specific probes recognizing the L. infantum SIDER2-bearing transcripts, LinJ.36.4000 (4000) and LinJ.08.1220 (1220). The α-tubulin probe was used as RNA loading control. Ethidium bromide staining was also included to visualize mRNA decay in parasitic protozoa.

**MATERIALS AND METHODS**

**Leishmania culture and transfections**

*Leishmania infantum* MHOM/MA/67/ITMAP-263 (Sereno et al. 2001) promastigotes were cultured in SDM-79 medium supplemented with 10% FCS (Wisent) and 5 μg/mL hemin at 25°C. On average, 10–20 μg plasmid DNA were transfected into *Leishmania* promastigotes by electroporation as previously described (Papadopoulou et al. 1992). To generate stable transfectant cell lines, parasites were selected with 25 μg/mL G418 (Sigma-Aldrich), 10 μg/mL zeocin (Life Technologies), or hygromycin B (Sigma-Aldrich) at 1 mg/mL.

**Tethering constructs**

The parent plasmid pSP72-YNEOαIR described elsewhere (Wu et al. 2000) was used to generate the LUC-MS2-3′UTR constructs. pSP72-YNEOα-LUC-MS2-4000 3′UTR (Y stands for a 92-bp polypyrimidine stretch [Papadopoulou et al. 1994]; α, for the intergenic region of the α-tubulin gene; NEO for the neomycin phosphotransferase gene; and LUC for the luciferase gene) was constructed by PCR amplification of the LinJ.36.4000 3′UTR (Phusion Taq polymerase, Thermoscientific) using primers described in Supplemental Table S1 and subsequently cloned downstream from the LUC gene. The two MS2 hairpins (see Fig. 1B) were placed at the beginning of the 4000 3′UTR via PCR amplification using the forward primer containing the MS2 sequences. To generate LUC-MS2-4000ΔSIDER2, an overlapping fusion PCR approach was used. A similar strategy was used to create LUC-MS2-4000ΔSII lacking only the second 79-nt signature sequence in SIDER2. To construct plasmid pSP72aZEOαMS2-LUC-4000 3′UTR, the MS2-LUC fragment was PCR-amplified and cloned via BamHI–HindIII sites into vector pSP72αZEOα (Papadopoulou et al. 1994). Next, the 3′UTR of LinJ.36.4000 was amplified and cloned into HindIII downstream from the LUC gene. To create the MCP-PTP fusion plasmid, the MS2 coat protein (MCP) tagged with a PTP epitope was first amplified from plasmid pLEW100-PTP-MCP (a generous gift from Dr. Schimanski, Bern, Switzerland) and cloned into XbaI HindIII sites of pSP72αZEOα. Next, the tandem MCP-encoding gene was PCR-amplified and cloned in the HindIII site of pSP72αZEOα-MCP to obtain pSP72aZEOαPTP-MCP harboring two tandem MCP copies. To generate pSP72aZEOα-tMCP, the tandem MCP from the PTP-MCP plasmid was amplified and cloned into the XbaI site of pSP72aZEOα. Genes encoding for candidate proteins interacting with SIDER2 identified using the MS2 tethering system (see Table 1) were cloned upstream of the tMCP-
HA construct. LUC chimeric transcripts and tMCP-fusion genes are all properly 5′-trans-spliced and processed by the *Leishmania enriettii* α-tubulin intergenic region (αIR). In order to make a PUF6 (LinJ.33.1210) null mutant in *L. infantum*, two targeting cassettes comprising either the hygromycin (HYG) or the neomycin (NEO) phosphotransferase genes, flanked by 500 bp DNA fragments at 5′ and 3′ regions of the *PUF6* gene, were generated by overlapping PCR. The nucleotide sequence of all primers used to make the above constructs is indicated in Supplemental Table S1.

**DNA, RNA, and protein manipulations**

DNA extractions were carried out using DNAzol (Life Technologies). Plasmid copy number was estimated by Southern blot hybridization analysis. Approximately 10 µg of DNA from each transfected digest with NdeI was hybridized with a LinJ.36.4000 3′UTR (1 kb) radiolabeled probe. Hybridization intensity signals from plasmid and genomic DNA were measured by a PhosphorImager. A ratio of the signal obtained from the plasmid DNA versus the genomic DNA was used to determine the plasmid copy number in each transfected cell line. Total RNA from parasites was isolated by TRIzol (Life Technologies) according to manufacturer’s instructions and resolved on 1% agarose formaldehyde gels. Northern blots were carried out following standard procedures (Sambrook and Russell 2001). Radioactive DNA probes corresponding to the LUC ORF or to the LinJ.36.4000 3′UTR were synthesized using Klenow fragment DNA polymerase I (New England Biolabs) in the presence of [α-32P]dCTP (PerkinElmer) and random oligonucleotides (NEB) and used in Northern or Southern blots. Western blots were performed from total *L. infantum* lysates equivalent to 2 × 10^6* parasites in 2× Laemmli buffer. HA-tagged proteins were detected using a mouse monoclonal anti-HA tag antibody (Abmgood). The MCP was detected using an anti-MCP rabbit polyclonal antibody (EMD Millipore). Loading control was assessed by rehybridizing the same membrane with a mouse anti-α tubulin antibody (Sigma-Aldrich) or a rabbit anti-NEO antibody (EMD Millipore). Anti-mouse HRP-conjugated, anti-rabbit HRP-conjugated, or anti-goat HRP-conjugated antibodies were used as secondary antibodies. The blots were visualized by chemiluminescence with an ECL+ Western Blotting Detection Kit (GE Healthcare).

**mRNA half-lives and protein synthesis inhibition**

To evaluate the stability of SIDER2-harboring mRNAs expressed as part of episomal vectors or from the genomic locus, we treated mid-log phase *L. infantum* promastigotes with 10 µg/ml of actinomycin D (ActD; Gibco-Life technologies) and 2.5 µg/ml sinfugin (Abcam) to arrest de novo transcription and pre-mRNA trans-splicing, respectively. Sinfugin was added 5 min prior to ActD (Li et al. 2006; Haile et al. 2008). Total RNA was isolated at desired time points and analyzed by Northern blotting. To inhibit global protein synthesis, mid-log phase promastigotes were incubated with 10 µg/ml cycloheximide (Sigma-Aldrich), and at various time points, parasites were collected, total RNA isolated and analyzed by Northern blot hybridization. Following transfer, membranes were exposed to a Phosphorimager, and signal intensity was measured using the ImageQuant 5.2 software.

**Immunoprecipitation and mass spectrometry analysis**

Frozen parasite pellets were immediately resuspended in the lysis buffer comprising 25 mM Tris–HCl pH 7.4, 100 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 0.5% NP40, 5% glycerol, and 1 mM PMSF supplemented with protease inhibitors (Roche). Lysis was completed by 20–30 strokes of a Dounce homogenizer while on ice. Cell debris and insoluble material were separated by 30 min centrifugation at 10,000 g at 4°C. Supernatants were then incubated with Protein G magnetic beads (Thermo Scientific) for 30 min at 4°C in order to reduce nonspecific binding and to eliminate protein binding to the beads. Clear supernatants were further incubated with Protein G anti-HA magnetic beads at 4°C for 4 h on a gentle rotator. Beads were then washed by TBS-0.05% Tween (Sigma-Aldrich) three times (30 sec each) by gentle agitation and subjected to LC–MS/MS analysis as previously described (Padmanabhan et al. 2016). Immunoprecipitation experiments were done on cotransfected parasites as above except that prior to freezing the parasites in liquid nitrogen, they were exposed to 400 ml/cm² UV irradiation on a Stratalinker 2400 UV crosslinker in PBS medium and then immediately harvested and snap-frozen.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.

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