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Apoptosis-like programmed cell death induces antisense ribosomal RNA (rRNA) fragmentation and rRNA degradation in *Leishmania*

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Few natural antisense (as) RNAs have been reported as yet in the unicellular protozoan *Leishmania.* Here, we describe that *Leishmania* produces natural asRNAs complementary to all ribosomal RNA (rRNA) species. Interestingly, we show that drug-induced apoptosis-like programmed cell death triggers fragmentation of asRNA complementary to the large subunit gamma (LSU-γ) rRNA, one of the six 28S rRNA processed fragments in *Leishmania*. Heat and oxidative stress also induce fragmentation of asrRNA, but to a lesser extent. Extensive asrRNA cleavage correlates with rRNA breakdown and translation inhibition. Indeed, overexpression of asLSU-γ rRNA accelerates rRNA degradation upon induction of apoptosis. In addition, we provide mechanistic insight into the regulation of apoptosis-induced asrRNA fragmentation by a 67 kDa ATP-dependent RNA helicase of the DEAD-box subfamily. This helicase binds both sense (s)LSU-γ and asLSU-γ rRNAs, and appears to have a key role in protecting rRNA from degradation by preventing asrRNA cleavage and thus cell death. Remarkably, the asrRNA fragmentation process operates not only in trypanosomatid protozoa but also in mammals. Our findings uncover a novel mechanism of regulation involving asrRNA fragmentation and rRNA breakdown, that is triggered by apoptosis and conditions of reduced translation under stress, and seems to be evolutionary conserved.
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Antisense RNAs (asRNAs) are common in prokaryotes and can mediate a plethora of regulatory processes, including transcription interference, RNA processing, RNA stability or ribosome binding.[1] In eukaryotes, natural astranscripts have established roles in epigenetic silencing, genomic imprinting, alternative splicing, messenger RNA (mRNA) editing, mRNA nuclear transport, stability and translation, as well as in the formation of endogenous siRNAs.[2] The endogenous RNA interference (RNAi) pathway in eukaryotes is one example where asRNA production has a major role in the formation of sRNA–asRNA duplexes recognized by the RNAi machinery.[3] It is becoming clear that asRNAs have an important role in regulating gene expression, but their production and function remain poorly understood.

Leishmania spp. causes a broad range of human diseases known as leishmaniasis. These parasites alternate between two major developmental forms, promastigotes in the insect vector and amastigotes in the phagolysosome of mammalian macrophages. In the absence of typical RNA *pol* II promoter sequences, polycistronic transcription units in *Leishmania* are processed to generate mature mRNAs though coupled 5'-trans-splicing and 3'-cleavage/polyadenylation reactions.[4] Natural asRNAs complementary to the *L. major* Friedlin chromosome 1,[5] or to specific protein-coding transcripts[6,7] and to non-coding developmentally regulated RNAs[8] have been described in *Leishmania*. The existence of astranscripts is puzzling in the absence of any RNAi machinery in *Leishmania*[9] and their putative role in regulating gene expression is still unknown.

Apoptosis, one of the programmed cell death mechanisms, is believed to operate in multicellular organisms to control various physiological processes, but also in response to various stress-induced mechanisms.[10] However, there is now increasing evidence that apoptosis-like programmed cell death (ALPCD) occurs in unicellular organisms[11,12] including *Leishmania.*[13–15] Cell death in *Leishmania* may be helpful in controlling parasite's density in response to limited resources and/or for ensuring propagation only of the cells that are fit to transmit the disease. Interestingly, it has been shown that apoptotic *Leishmania* in the virulent inoculum enhance the transmission and development of the disease.[16] Dying *Leishmania* show typical hallmarks of apoptosis despite the absence of homologs to mammalian key regulatory or effector molecules like caspases.[13,14,17] Various stress conditions, such as nitric oxide,[18] reactive oxygen species,[19] heat stress,[20] starvation,[21] and anti-*Leishmania* drugs

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Abbreviations: ALPCD, apoptosis-like programmed cell death; rRNA, ribosomal RNA; MF, miltefosine; SbIII, trivalent antimony; G418, Geneticin; ss-DNA, single-stranded DNA; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcribed-PCR; 5′-RACE, random amplification of cDNA ends; PBS, phosphate-buffered saline; DTT, dithiothreitol; MS/MS analysis, mass spectrometry analysis
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(e.g., miltefosine (MF) or antimonials)[22–24] have been reported to induce morphological and biochemical features of ALPCD in *Leishmania*.

In this study, we show that induction of ALPCD triggers fragmentation of asRNA complementary to ribosomal RNA (rRNA) in *Leishmania*. asrRNA cleavage is correlated with rRNA degradation and inhibition of general translation. A 67 kDa DEAD-box ATP-dependent RNA helicase is implicated in this process by preventing asrRNA cleavage, and hence protecting rRNA from degradation and *Leishmania* from cell death. Our findings uncover a novel regulatory pathway that seems to be conserved in unicellular protozoa but also in higher eukaryotes.

Results

Natural asRNA complementary to the rRNA is produced in *Leishmania*. To identify small non-coding RNAs in *Leishmania*, we cloned and sequenced small RNAs ($\leq$200 nt) and found that some of these sequences were opposite to the large subunit gamma (LSU-γ) rRNA (data not shown). A unique feature of *Leishmania* and related parasites is that the large 28S rRNA subunit is processed to yield six stable RNA fragments, which include the LSU-α (1840 nt), LSU-β (1570 nt), LSU-γ (213 nt), LSU-δ (180 nt), LSU-ζ (70 nt) and LSU-ε (140 nt).[25] Although asRNAs against few mRNAs[6,7] and non-coding RNAs[8] have been detected in *Leishmania*, the presence of asrRNA species has not been investigated yet in protozoan parasites.

To further search for asrRNA in *Leishmania,* we used strand-specific reverse transcribed-polymerase chain reaction (RT-PCR) and northern blot hybridization with srRNA-specific riboprobes for detecting the 28S, 18S and 5.8S rRNA species. These studies revealed the presence of asRNA complementary to all rRNA species in both developmental forms of *Leishmania* (Figures 1a and b and Supplementary Figure S1). The present study focused on LSU-γ rRNA, one of the six 28S rRNA processed fragments. Strand-specific RT-PCR analysis (Supplementary Table 1) revealed a LSU-γ rRNA PCR product of the expected size (Figure 1a). The asLSU-γ rRNA/sLSU-γ rRNA ratio was estimated by quantitative real-time PCR to be 1/300 (data not shown). asLSU-γ rRNA was also detected in both life stages by northern blot hybridization using the 173 nt single-stranded (ss) DNA probe (Figure 1b). Interestingly, the length of asLSU-γ rRNA was similar to that of the sLSU-γ rRNA (213 nt) (Figures 1b and c). A higher molecular weight band of $\sim$3 kb was also detected by hybridization corresponding most likely to the precursor asLSU-γ RNA (Figures 1b and c), suggesting that asrRNA is processed.

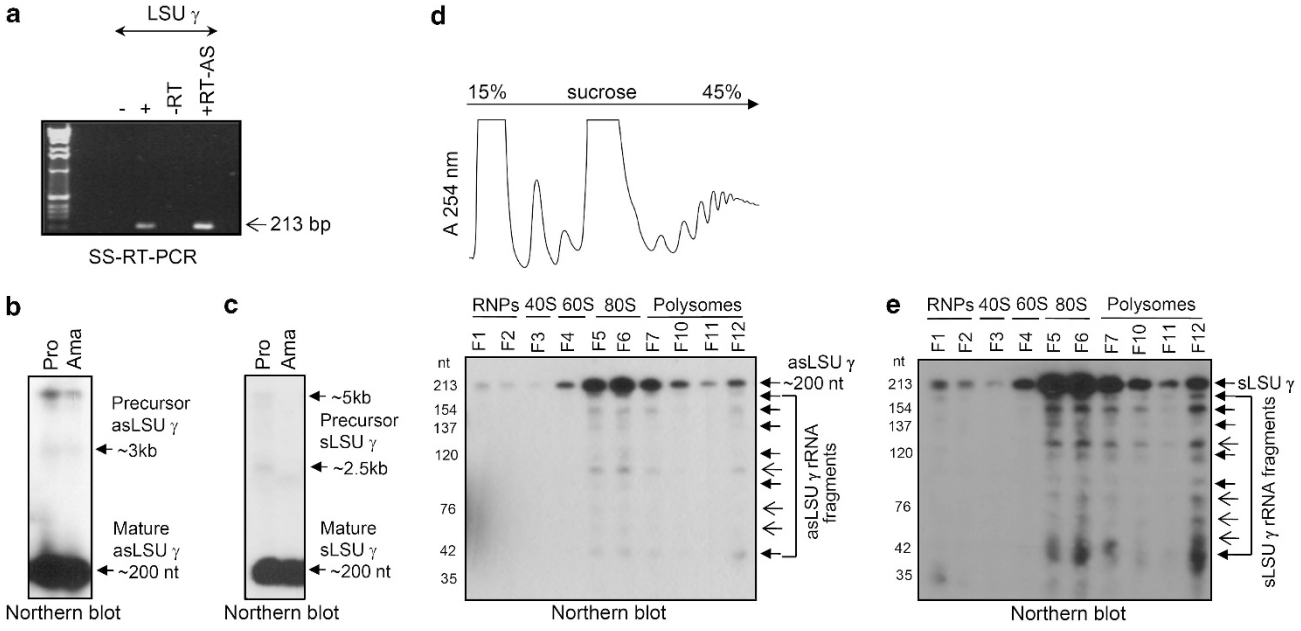

Figure 1 Natural asRNA complementary to the rRNA is produced in *Leishmania* in the context of translating ribosomes. (**a**) asRNA complementary to the LSU-γ rRNA was detected by strand-specific (ss) RT-PCR. The + RT − AS is to detect the asRNA. The − RT reaction is to check for DNA contamination. PCR with genomic DNA (+) was included as a positive control. (**b** and **c**) RNA blots to detect the mature and precursor bands of antisense (as) and sense (s) LSU-γ rRNAs in *Leishmania* promastigote (Pro) and amastigote (Ama) life stages. Total RNA resolved on 1% agarose gel was hybridized with the 173 nt ss-DNA probe corresponding to nucleotides 41–213 of sLSU-γ rRNA and recognizing the asLSU-γ RNA (**b**) or with a 5′-labeled 42 nt oligonucleotide complementary to nucleotides 172–213 of the sLSU-γ rRNA (**c**). (**d**) *Leishmania* promastigote lysates were loaded onto linear 15–45% (w/w) sucrose gradient and fractionated by ultracentrifugation by continuously recording absorbance (A) at 254 nm to separate the 40S and 60S ribosomal subunits from the 80S monosome and polysome fractions (upper panel). Fractionated RNA material corresponding to the small ribonuclear protein complexes (RNPs) (F1 to F2), ribosomal subunits 40S (F3) and 60S (F4), 80S monosome (F5 and F6) and polysomes (F7–F12) was resolved on 10% urea acrylamide gel and analyzed by northern blot hybridization with the 173 nt ss-DNA probe (24 h exposure) (bottom panel). The mature $\sim$200 nt asLSU-γ rRNA is indicated. Smaller asLSU-γ RNA-derived hybridizing fragments are indicated within a bracket. (**e**) The same membrane as in **d** was used for northern blot hybridization after stripping to detect sLSU-γ rRNA using the 42 nt oligonucleotide probe (2 h exposure). sLSU-γ rRNA-derived hybridizing fragments are indicated within a bracket. Plain arrows indicate sLSU-γ and asLSU-γ rRNA-derived fragments of a similar length and open arrows indicate fragments of a different length

asRNA complementary to the *Leishmania* LSU-γ rRNA is enriched in translating ribosomes. To assess whether asLSU-γ RNA is associated with the ribosome, total RNA was sedimented and fractionated in a sucrose gradient to separate the ribosomal subunits from monosomes and polysomes (Figure 1d, upper panel). RNA extracted from each fraction was resolved on a 10% denaturing acrylamide gel and hybridized with the 173 nt ss-DNA probe to detect asLSU-γ RNA. Northern blot hybridization revealed a $\sim$200 nt product corresponding to the mature asLSU-γ RNA enriched in the 60S subunit (F4), the 80S monosome (F5–6) and polysome (F7–12) fractions (Figure 1d, lower panel). Smaller asLSU-γ-hybridizing fragments ranging from $\sim$40–150 nt were also detected in 80S and polysome fractions (Figure 1d, F5–F12, lower panel), suggesting that part of the asLSU-γ RNA is further cleaved. Hybridization with a 5'-end-labeled oligonucleotide probe recognized the mature sLSU-γ rRNA transcript (213 nt) and smaller RNA species, which may correspond to degradation products (Figure 1e). sLSU-γ rRNA and asLSU-γ rRNA fragments were enriched in the same sucrose gradient fractions (Figure 1e, F5–F6 and F7–F12). Sequence of sLSU-γ rRNA-derived fragments confirmed their size from $\sim$35–150 nt (Supplementary Figure S2), corroborating the hybridization data in Figure 1e. Neither sLSU-γ- nor asLSU-γ-derived fragments were detected in the 60S fraction (data not shown), which suggests that fragmentation of sLSU-γ and asLSU-γ rRNAs occurs in the context of the assembling ribosome.

Sequence analysis of several clones corresponding to either the mature sLSU-γ and asLSU-γ rRNAs, or their derived RNA products allowed us to map the 5' and 3' ends of these RNAs. Using 5'-random amplification of cDNA ends (RACE) we found that the 5'-end of the mature asLSU-γ RNA was complementary to the 3'-end of the sLSU-γ rRNA (Supplementary Figures S2 and S3). Mapping of the 3'-end of the mature asLSU-γ RNA showed complementarity to the first nucleotide of the sLSU-γ rRNA (Supplementary Figure S2). Thus, both ends of the asLSU-γ RNA are complementary to those of the sLSU-γ rRNA (Supplementary Figure S2B), indicating that mature sLSU-γ and asLSU-γ rRNAs have the same length (213 nt). Internal cleavages of asLSU-γ RNA generated one nucleotide overhang at their 5'-end with respect to the 3'-end of the corresponding sLSU-γ rRNA fragments ($-$58 (as)/57 (s) and $-$151 (as)/150 (s)) (Supplementary Figure S2A–S2B), suggesting that these RNA molecules may interact before cleavage.

Exposure of *Leishmania* to heat and oxidative stress leading to reduced levels of general translation induce fragmentation of the asLSU-γ RNA. *Leishmania* promastigote to amastigote differentiation is mainly triggered by temperature increase (from 25 to 37 °C) and drop in pH.[26] We investigated whether differentiation signals could modulate asLSU-γ RNA fragmentation. RNA samples isolated either from *L. infantum* promastigotes subjected to heat–stress or from axenic amastigotes treated with H_2O_2 to induce oxidative stress were enriched for small RNAs ($\leq$ 200 nt) and analyzed by primer extension using a primer corresponding to nucleotides 101–118 of LSU-γ rRNA (Supplementary Table 1). Both heat and H_2O_2 stresses triggered a marked increase in asLSU-γ RNA fragmentation over the control (Figures 2a and b). Heat stress significantly reduced global translation as illustrated by polysome-profiling analysis

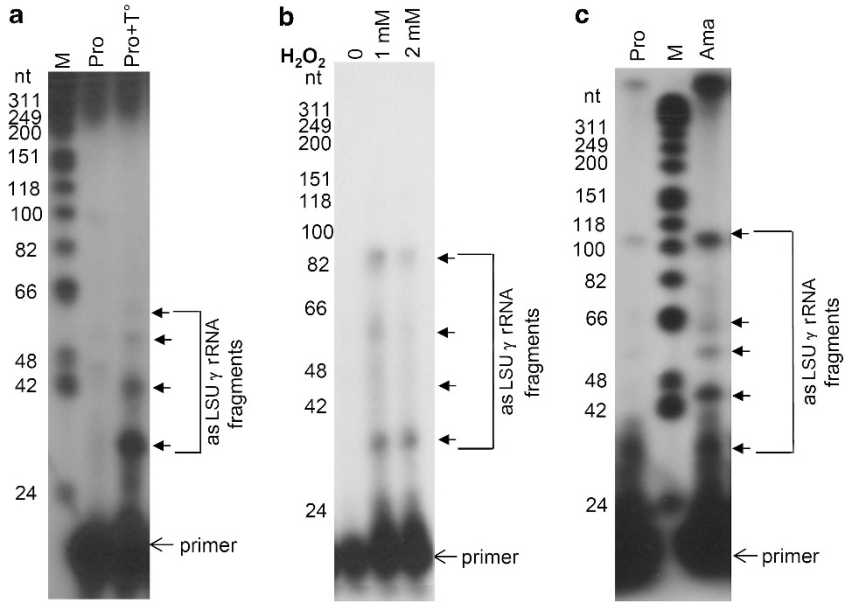

Figure 2 *Leishmania* promastigote to amastigote differentiation and exposure to various stresses induce fragmentation of the asLSU-γ RNA. (**a**) Size-fractionated RNA ($\leq$200 nt) was isolated from *L. infantum* unstressed and heat–stress promastigotes (Pro) and subjected to primer extension analysis using a forward primer corresponding to nucleotides 101–118 of the sLSU-γ rRNA. (**b**) *L. infantum* parasites were treated with 1 mM and 2 mM of H_2O_2 for 8 h and RNA was used for primer extension analysis as in *A*. (**c**) Primer extension analysis of *L. infantum* promastigotes and amastigotes (Ama) as indicated in (**a**). M, the end-labeled DNA ladder (Promega) was used as a reference for molecular size

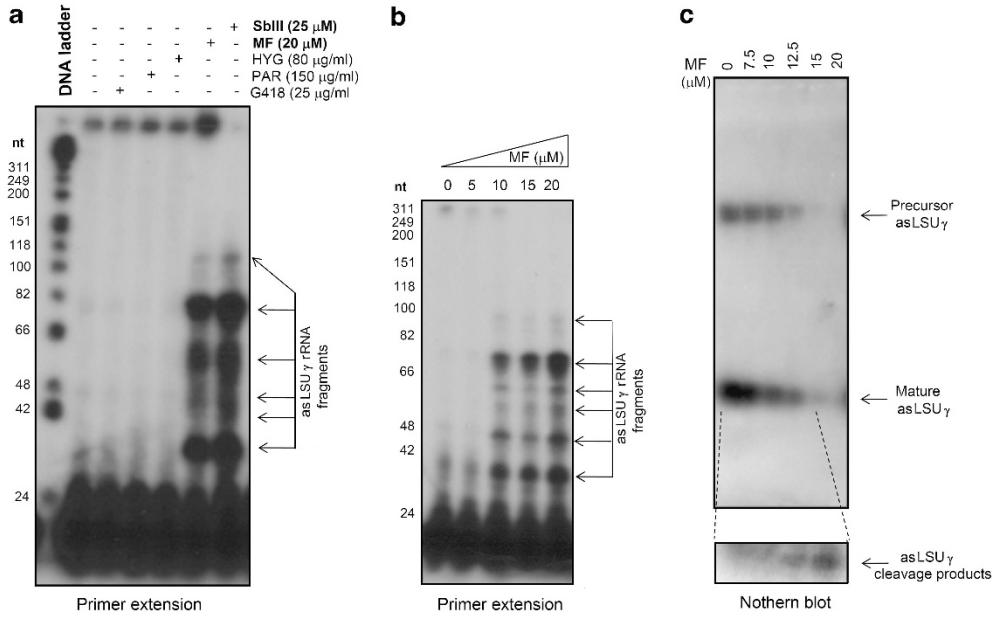

Figure 3 asLSU-γ RNA fragmentation is dramatically increased upon drug-induced ALPCD. (**a**) Primer extension analysis (same primer as in Figure 2a) of total RNA isolated from *L. infantum* amastigotes treated with either G418 (25 μg/ml) or paramomycin sulphate (150 μg/ml) or hygromycin-B (80 μg/ml) or MF (20 μM) and or antimony (SbIII) (25 μM) for 24 h. Arrows indicate the asLSU-γ RNA-derived fragments. End-labeled Decade marker (Ambion) was used as a ladder. (**b**) Primer extension carried out on total RNA extracted from axenic amastigotes treated with increasing concentrations of MF (0–20 μM) for 24 h. Primer extension was performed with different primers that generated in all cases an induced fragmentation pattern for asLSU-γ RNA upon MF treatment (data not shown). (**c**, upper panel) Northern blot analysis of RNA from 0 to 20 μM MF-treated amastigotes for 24 h using the 173 nt ss-DNA probe. (**c**, bottom panel) Overexposed RNA blot to detect asLSU-γ RNA cleavage products accumulated upon increased concentrations of MF

(Supplementary Figure S4A, upper panel) and induced more fragmentation of both asLSU-γ and sLSU-γ rRNAs in comparison with unstressed cells (Supplementary Figures S4A-S4B, bottom panels). Although the fragmentation pattern between heat-stressed and unstressed cells was generally similar, some bands were different, suggesting distinct cleavage events (Supplementary Figure S4A-S4B). Upon heat stress, asLSU-γ and sLSU-γ rRNA fragments were shifted mainly to the monosomes (Supplementary Figure S4A-S4B, F5-7), whereas under physiological conditions these were also detected in heavy polysomes (Supplementary Figure S4A-S4B, F12). Furthermore, *L. infantum* axenic amastigotes shown recently to undergo a reduced translation[27] exhibited more asLSU-γ RNA fragmentation than promastigotes (Figure 2c).

asLSU-γ rRNA fragmentation is dramatically induced upon ALPCD. Exposure of *Leishmania* to H_2O_2[19] or to antileishmanial drugs such as trivalent antimony (SbIII) or MF was shown to induce ALPCD.[23,24] *Leishmania* axenic amastigotes were treated with various drug concentrations for 24 h, and primer extension analysis was carried out on total *Leishmania* RNA. Parasites treated with SbIII or MF demonstrated a dramatic increase in the asLSU-γ RNA cleavage pattern (Figure 3a) in a concentration-dependent manner (Figure 3b). In contrast, other cytotoxic drugs that do not trigger apoptosis in *Leishmania* (e.g., hygromycin-B, paramomycin sulphate and neomycin/Geneticin (G418)) (M Ouellette, personal communication) failed to induce the asrRNA fragmentation process (Figure 3a), even at much higher concentrations (Supplementary Figure S5). Northern blot hybridization revealed that MF-induced asLSU-γ RNA fragmentation (Figure 3b) correlates with reduced levels of both the precursor and mature asLSU-γ RNAs (Figure 3c, upper panel) and with an increased accumulation of smaller RNA products (Figure 3c, bottom panel; prolonged exposure), further supporting that asLSU-γ RNA fragmentation is the result of cleavage reactions.

asrRNA fragmentation upon induction of apoptosis is correlated with extensive rRNA degradation and translation inhibition. Apoptosis in yeast[28] and in mammals[29] triggers rRNA degradation. To test whether induction of asrRNA cleavage during apoptosis has an effect on the degradation of srRNA, we evaluated LSU-γ rRNA expression levels in parasites treated with MF. Interestingly, northern blot hybridization revealed that accumulation of the mature sLSU-γ rRNA is inversely correlated with increasing MF concentrations (Figure 4a, upper panel) and asLSU-γ RNA cleavage (Figure 3b). Primer extension analysis also revealed a decrease of LSU-γ rRNA in axenic amastigotes treated with the apoptosis-inducing agents MF and SbIII, but not with other cytotoxic drugs (Figure 4b), hence corroborating the northern blot data (Figure 4a, upper panel). Thus, degradation of the sLSU-γ rRNA (Figure 4a, upper panel) seems to be related to increased asLSU-γ RNA cleavage (Figure 3a). Consistent with these data, northern blot hybridization on RNA from MF-treated promastigotes showed an increased accumulation of asLSU-γ RNA fragments in the 80S- and polysome-enriched fractions (Figure 4c). MF treatment not only resulted in LSU-γ rRNA degradation but also in the breakdown of all rRNA species

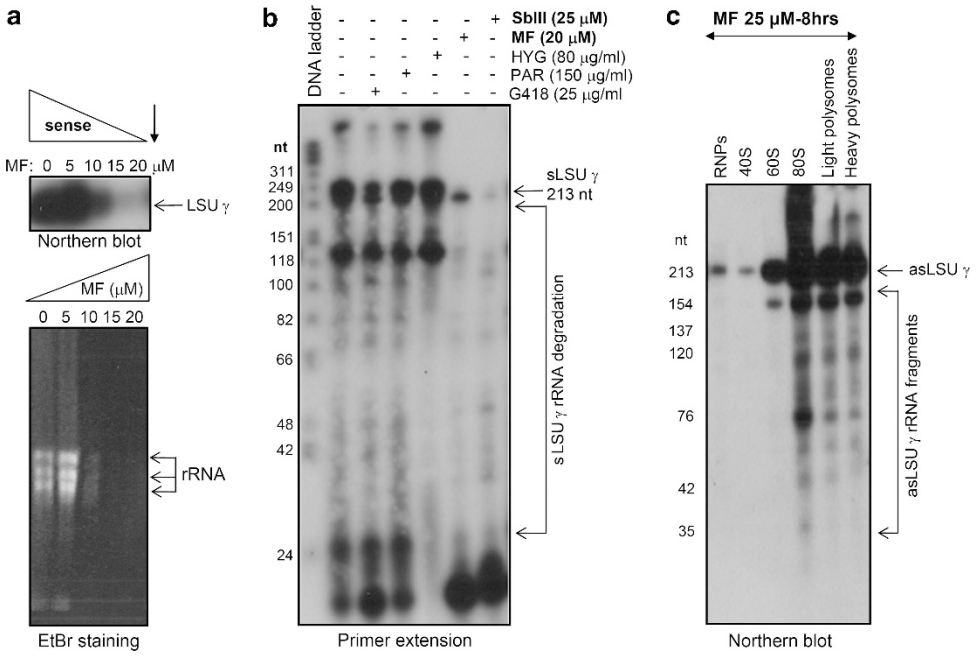

Figure 4 Fragmentation of asLSU-γ RNA upon induction of apoptosis is correlated with extensive degradation of the sLSU-γ LSU-γ rRNA in *L. infantum* axenic amastigotes. (**a**, upper panel) Northern blot analysis of RNA samples extracted from *L. infantum* amastigotes treated with various concentrations of MF (0–20 μM) for 24 h. (**a**, bottom panel) Ethidium bromide (EtBr)-stained RNA gel is shown here. (**b**) Primer extension analysis of *L. infantum* axenic amastigotes treated for 24 h with either G418 (25 μg/ml) or paramomycin sulphate (150 μg/ml) or hygromycin-B (80 μg/ml) or MF (20 μM) and/or Sb III (25 μM). A primer complementary to nucleotides 96–213 of sLSU-γ rRNA was used for the analysis. Arrows indicate the degradation pattern of sLSU-γ rRNA in MF- and SbIII-treated samples. (**c**) Lysates from Leishmania promastigotes treated with MF (25 μM) were fractionated by a 15–45% sucrose gradient. RNA samples were isolated from the respective fractions as indicated in Figure 1d, resolved on a 10% urea acrylamide gel and hybridized with the 173 nt ss-DNA probe to detect asLSU-γ RNA. The asLSU-γ RNA fragments enriched in the 80S and polysome fractions are indicated

(Figure 4a, bottom panel). rRNA degradation following MF treatment had a dramatic effect on global translation as illustrated by polysome-profiling analysis (Supplementary Figure S6).

Next, we investigated whether induction of apoptosis could trigger asrRNA fragmentation and rRNA degradation also in macrophage-derived amastigotes. Primer extension analysis to detect asLSU-γ and sLSU-γ cleavage products was carried out on RNA extracted from *L. infantum* infected THP-1 macrophages treated with MF. Similarly to axenic amastigotes (Figures 3a and 4b), an increased fragmentation pattern of asLSU-γ rRNA was observed in MF-treated macrophages already at 24 h post infection, which was correlated with enhanced degradation of the sLSU-γ rRNA (Figures 5a and b). asLSU-γ fragmentation and sLSU-γ rRNA degradation were further enhanced at 48 and 72 h post infection (Figures 5a and b).

To further investigate the link between asrRNA fragmentation and rRNA degradation, we generated parasite strains overexpressing either the sLSU-γ or the asLSU-γ rRNA (Figure 6a). Overexpression of the asLSU-γ RNA was confirmed by quantitative RT-PCR (qRT-PCR) (Figure 6b). All rRNA species were degraded more rapidly in MF-treated parasites overexpressing the asLSU1.2-RNA (Figure 6c, upper panel). Moreover, primer extension analysis revealed more degradation of the sLSU-γ rRNA in the asLSU1.2-overexpressing *Leishmania* in comparison with the sLSU1.2-overexpressing parasites (Figure 6d). Furthermore, northern blot showed a reduction in the mature asLSU-γ RNA levels in MF-treated asLSU-γ RNA overexpressing parasites (Figure 6c, lower panel) in line with increased asLSU-γ RNA fragmentation under those conditions (Figures 3a and b). Additionally, primer extension kinetics showed that LSU-γ rRNA degradation appears generally at the same time interval than asLSU-γ RNA fragmentation (Supplementary Figure S7).

An ATP-dependent DEAD-box RNA helicase of 67 kDa interacts with both sLSU-γ and asLSU-γ rRNAs to prevent asrRNA fragmentation. To investigate the mechanism of rRNA degradation induced upon apoptosis, we searched for *Leishmania* protein factors binding either to the sLSU-γ or asLSU-γ rRNA using a modified UV-crosslinking method (Materials and Methods). Two major bands of $\sim$67 kDa and $\sim$30 kDa were detected on 15% SDS-PAGE with both the sLSU-γ and asLSU-γ RNA templates (Figure 7a). These bands were gel-excised and analyzed by mass spectrometry analysis (MS/MS) identifying several proteins potentially interacting with the sLSU-γ and asLSU-γ rRNAs (Supplementary Figure S8 and data not shown). We concentrated on LinJ.32.0410 encoding an ATP-dependent RNA helicase of 67 kDa (HEL67) belonging to a conserved subfamily of DEAD-box helicases. The other proteins, including the 30 kDa UV-crosslinked protein will be the subject of a future study. The *Leishmania* HEL67 homolog shares 53% and 38% amino-acid sequence identity with the *Drosophila* Belle and VASA proteins, respectively, and 51% identity with the *S. cerevisiae* Ded1p protein (Supplementary Figure S9).

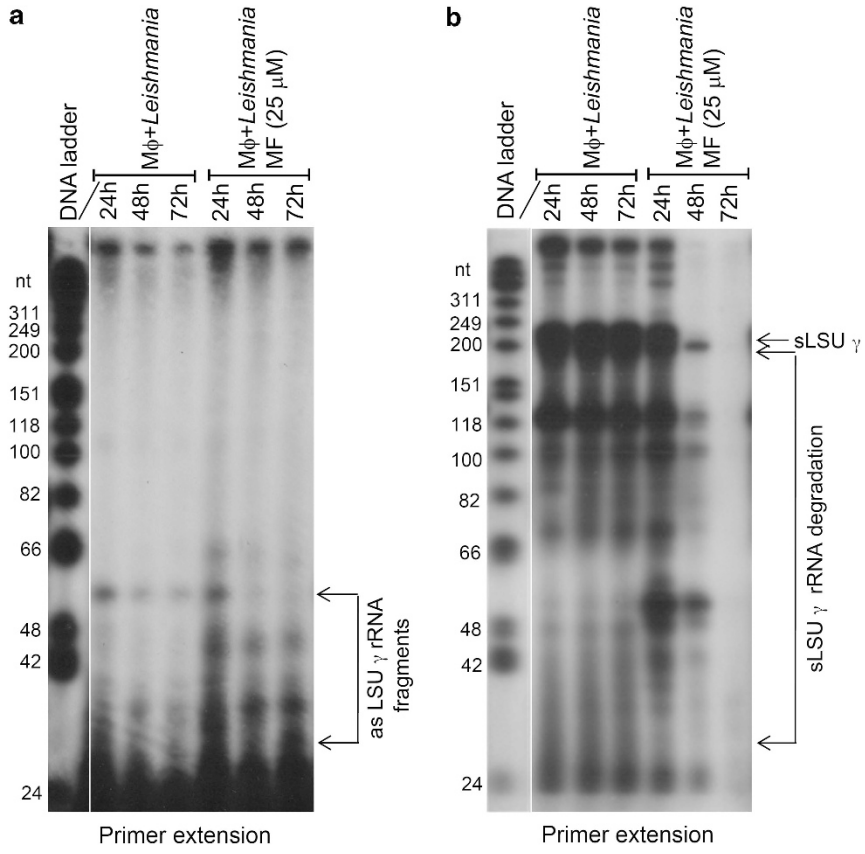

Figure 5 Fragmentation of asLSU-γ RNA upon induction of apoptosis is correlated with extensive degradation of the sLSU-γ LSU-γ rRNA in *L. infantum* macrophage-derived amastigotes. (**a**) Primer extension analysis to detect fragmentation of asLSU-γ rRNA on total RNA extracted from THP-1 human macrophage-derived amastigotes treated with MF (25 μM) for various time points post infection (24, 48 and 72 h) and compared with the untreated control. (**b**) Primer extension analysis of macrophage-derived amastigotes treated with MF as in (**a**) to visualize degradation of sLSU-γ rRNA upon MF treatment

To investigate whether HEL67 protein has a role in asrRNA fragmentation, we generated a *L. infantum* null HEL67$^{(-/-)}$ mutant by replacing the *HEL67* gene by two different selection marker genes (Figure 7b, left panel). Gene replacement was confirmed by PCR (data not shown) and by Southern blot hybridization (Figure 7b, right panel). The lack of the 3.1 kb wild-type *Lin*HEL67 band and the *HYG*- and *NEO*-hybridizing bands of 2.3 kb and 2.1 kb, respectively, confirmed *HEL67* gene inactivation. We also generated an add-back mutant (*Lin*HEL67$^{(-/-)}$REV) expressing the *HEL67* gene into *Lin*HEL67$^{(-/-)}$ background (Figure 7b, left panel). In contrast to wild-type cells, *Lin*HEL67$^{(-/-)}$ promastigotes exposed to elevated temperature (37^{o} C) and acidic pH combined stress demonstrated a marked induction of asLSU-γ RNA fragmentation (Figure 7c, left panel). Remarkably, asLSU-γ RNA fragmentation in *Lin*HEL67$^{(-/-)}$ was as high as in MF-treated parasites undergoing apoptosis (Figure 7c, left panel and 3a, b). This phenotype was rescued in *Lin*HEL67$^{(-/-)}$REV (Figure 7c, left panel). The level of the mature asLSU-γ RNA decreased significantly in *Lin*HEL67$^{(-/-)}$ as estimated by qRT-PCR in comparison with the wild-type or to the add-back mutant (Figure 7c, right panel). Moreover, induction of asLSU-γ RNA fragmentation correlates with a rapid rRNA breakdown in *Lin*HEL67$^{(-/-)}$ (Figure 7d). These findings support a key role of *Lin*HEL67 in preventing asrRNA cleavage and rRNA degradation, hence protecting the parasite from apoptosis.

asrRNA fragmentation is an evolutionary conserved process. Using strand-specific RT-PCR, we also detected asRNA complementary to the LSU-γ rRNA in the related parasite *Trypanosoma brucei* (Figure 8a). Interestingly, asRNA complementary to the 28S rRNA was also detected in the human acute monocytic leukemia THP-1 cell line (Figure 8b). Primer extension analysis revealed that *T. brucei* exposed to H_2O_2, an apoptosis-inducing agent,[19] demonstrated an increasing accumulation of asLSU-γ RNA cleavage products in a concentration-dependent manner (Figure 8c). Similarly, fragmentation of as28S rRNA in human THP-1 cells was markedly enhanced upon H_2O_2 treatment for 24 h (Figure 8d). Induced asrRNA fragmentation was associated with an increase in rRNA degradation (data not shown). Together, these data indicate that natural asRNA complementary to rRNA is also produced in higher eukaryotes and that fragmentation of this asRNA appears to be a stress-induced regulated process.

Discussion

Here, we report that ALPCD triggers fragmentation of asRNA complementary to the LSU-γ rRNA in *Leishmania* that is correlated with rRNA breakdown. Additionally, we provide mechanistic insight into the regulation of this process by an ATP-dependent RNA helicase of the DEAD-box subfamily

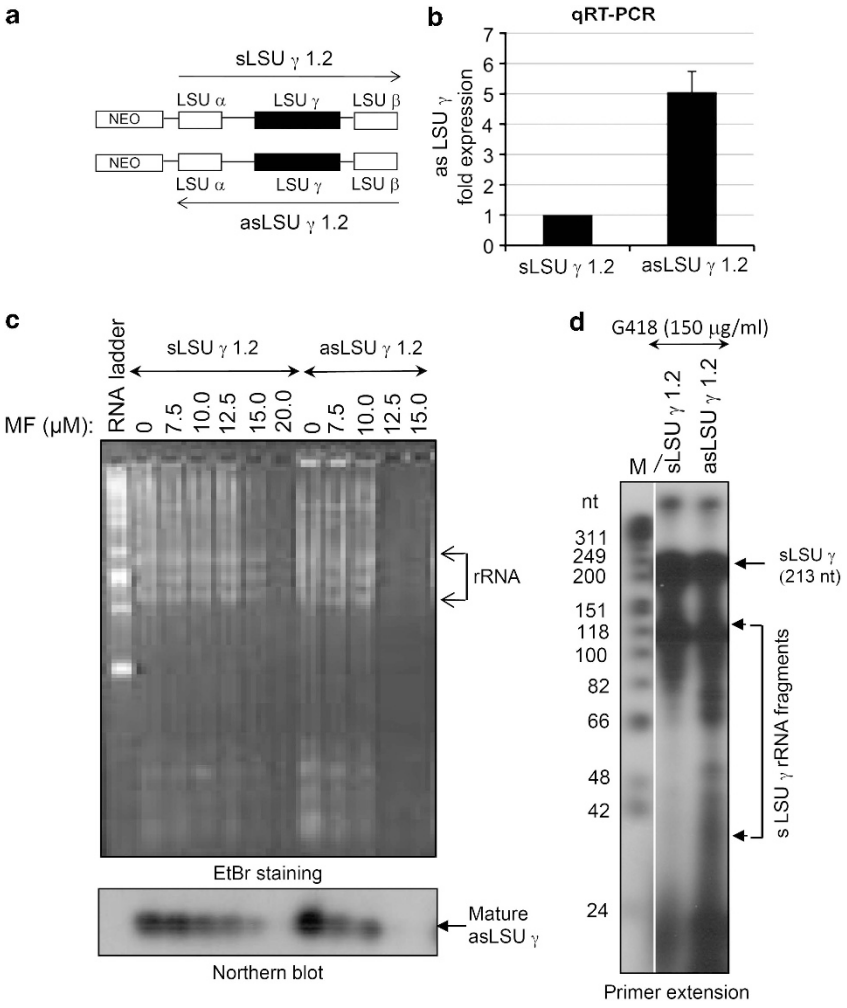

Figure 6 Overexpression of asLSU-γ rRNA stimulates srRNA degradation upon induction of apoptosis. (**a**) Schematic diagram of *Leishmania* expression vectors harboring the full-length LSU-γ (213 bp) and part of the LSU-α and LSU-β in the sense (s) and antisense (as) orientation. (**b**) qRT-PCR to validate overexpression of the asLSU-γ RNA in the asLSU1.2 overexpressor in comparison with the sLSU1.2 overexpressor. (**c**, upper panel) EtBr-stained RNA gel of MF-treated parasites overexpressing either the sLSU1.2 (0–20 μM) or the asLSU1.2 (0–15 μM) rRNA. (**c**, bottom panel) RNA blot with the 173 nt ss-DNA probe recognizing asLSU-γ RNA showing more accumulation of the mature asLSU-γ RNA in the untreated asLSU1.2 overexpressor but increased degradation of this RNA upon MF treatment. (**d**) Primer extension analysis to detect sLSU-γ rRNA and its degradation products in both sLSU1.2- and asLSU1.2-overexpressed strains using a reverse primer complementary to nucleotides 196–213 of sLSU-γ

that interacts with both sLSU-γ and asLSU-γ rRNAs and protects rRNA from degradation by preventing asrRNA cleavage and cell death.

Although few asRNAs against mRNAs[6,7] and non-coding RNAs[8] have been reported previously in *Leishmania*, asRNA complementary to rRNA species has not been investigated yet in protozoan parasites. Recently, in plants, it was shown that overaccumulation of the chloroplast asRNA AS5 is correlated with decreased abundance and inefficient 5S rRNA maturation.[30] Here, we report that *Leishmania* produces natural asRNA complementary to all rRNA species and that this asRNA is associated with the 80S and polyribosomes. We further show that part of the mature asLSU-γ RNA is cleaved into smaller RNA products ($\sim$40–150 nt) and this cleavage is markedly induced upon heat or oxidative stress, conditions where general translation is shown to be significantly reduced (Supplementary Figure S4 and Cloutier *et al*.[27]). srRNA is also fragmented within *Leishmania* translating ribosomes under physiological conditions similarly to bacteria which could degrade rRNA in misassembled ribosome subunits,[31] especially under stress conditions. In *Leishmania,* heat stress leads to increased cleavage of the sLSU-γ rRNA within monosomes, suggesting that most of stress-induced rRNA degradation occurs in assembled ribosomes. Our observations that the mature asLSU-γ RNA is fully complementary to the srRNA, that sLSU-γ and asLSU-γ rRNA-derived fragments overlap to a large extent and that internal cleavages generate one nucleotide overhang might indicate an interaction between srRNAs and asrRNAs before their fragmentation, possibly through endoribonucleolytic activity.

An original finding in this study is that induction of ALPCD triggers a dramatic increase in asLSU-γ RNA fragmentation, which is correlated with an extensive breakdown of rRNA and translation inhibition. Only conditions or drugs known to trigger

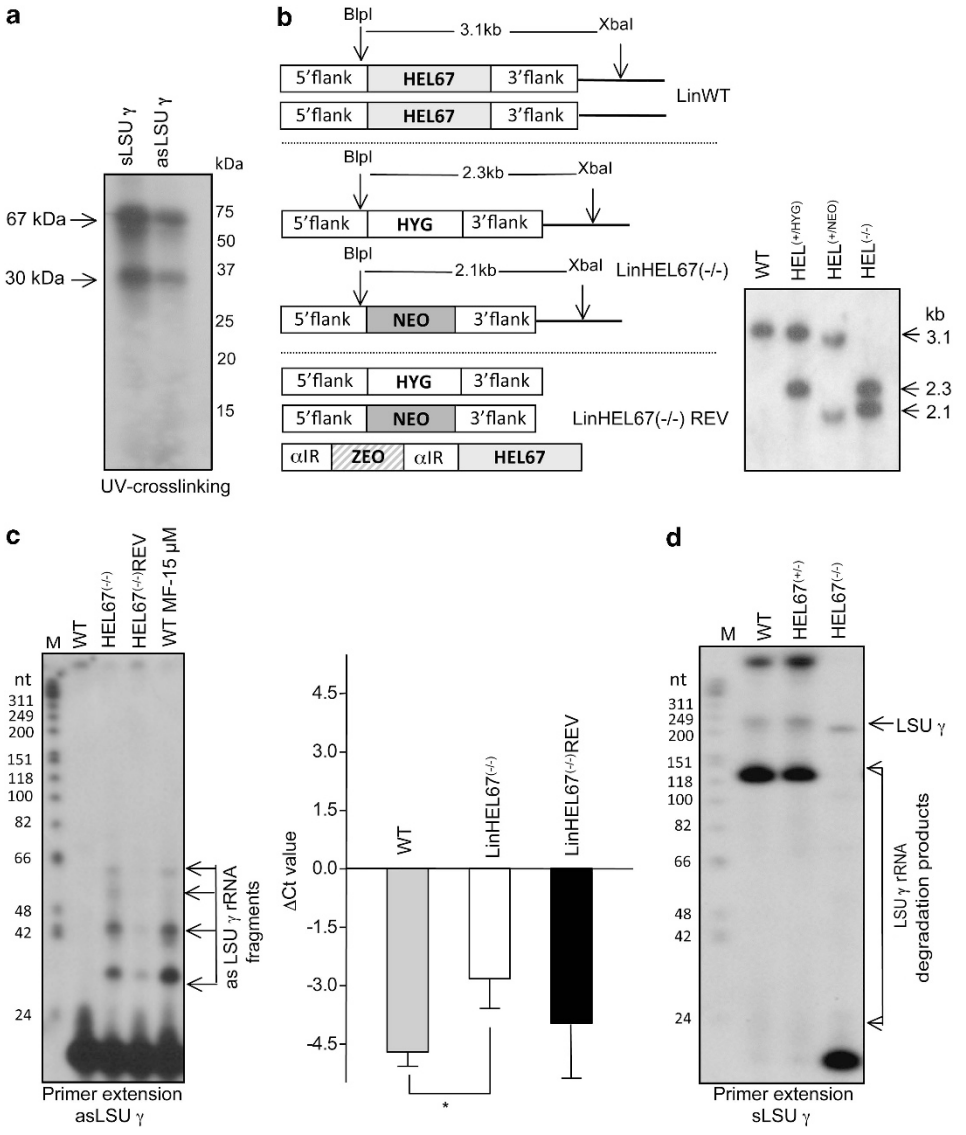

Figure 7 The ATP-dependent DEAD-box RNA helicase HEL67 interacts with both the sLSU-γ and asLSU-γ rRNAs to prevent asrRNA fragmentation. (**a**) A modified UV-crosslinking method was used to identify protein factors bound to *in vitro*-transcribed sLSU-γ and asLSU-γ rRNAs. The 67 kDa and 30 kDa proteins bound to both sLSU-γ and asLSU-γ rRNAs are indicated. (**b**, left panel) Strategy to generate a *L. infantum* null mutant strain (*Lin*HEL$^{(-/-)}$) for the LinJ.32.0410 gene encoding an ATP-dependent RNA helicase of 67 kDa (HEL67). Both alleles of the *HEL67* gene were replaced by the hygromycin phosphotransferase gene (*HYG*) and neomycin phosphotransferase gene (*NEO*) cassettes, respectively, through homologous recombination. An add-back mutant (*Lin*HEL$^{(-/-)}$ REV, **b**, bottom panel) was generated by overexpressing HEL67 as part of the pSPαZEOα-HEL67 vector in the *Lin*HEL67$^{(-/-)}$ mutant background. (**b**, right panel) Southern blot hybridization of genomic DNA digested with *Xba*I and *Blp*I using the *HEL67* 3'-flank sequence as a probe. In *Lin*HEL67$^{(-/-)}$, two hybridizing bands of 2.3 kb (for the *HYG* gene replacement) and 2.1 kb (for the *NEO* gene replacement) were detected but not the 3.1 kb *HEL67* endogenous band. (**c**) Primer extension analysis was performed to detect asLSU-γ RNA fragmentation using the end-labeled forward primer corresponding to nucleotides 101–118 of the LSU-γ rRNA. (**c**, left panel) RNA was extracted from wild-type (WT), *Lin*HEL67$^{(-/-)}$ and *Lin*HEL67$^{(-/-)}$REV *L. infantum* promastigotes subjected to O/N temperature (37 °C) and pH (5.5) stress. MF (15 μM)-treated *L. infantum* axenic amastigotes were used as a positive control for the induction of apoptosis and asLSU-γ RNA fragmentation. (**c**, right panel) SS-qRT-PCR to detect asLSU-γ RNA levels in WT, *Lin*HEL67 $^{(-/-)}$ and *Lin*HEL67$^{(-/-)}$ REV. A primer corresponding to nucleotides 1–18 of sLSU-γ rRNA was used for cDNA synthesis. (**d**) Primer extension analysis using a reverse primer complementary to nucleotides 196–213 of the sLSU-γ rRNA

apoptosis in *Leishmania* could induce asrRNA fragmentation and rRNA degradation. Previous studies in yeast have reported that apoptosis induces specific rRNA degradation and translational arrest.[28] Cleavage of monosome- and polysome-associated 28S rRNA has also been reported in human leukemia cells[32] and in lymphoid cells[29] undergoing apoptosis. A recent report demonstrated that asoligonucleotide-mediated stabilization of endogenous ribozyme-like non-coding rRNAs induced massive cell death via apoptotic and nonapoptotic mechanisms in lung cancer cells.[33] However, asrRNA cleavage has not been reported, yet, as a consequence of apoptosis. We did not observe any significant accumulation of the precursor or mature asLSU-γ RNAs during stress (data not shown), indicating that asrRNA cleavage is central to this regulation. We show that asLSU-γ RNA fragmentation upon induction of apoptosis may directly

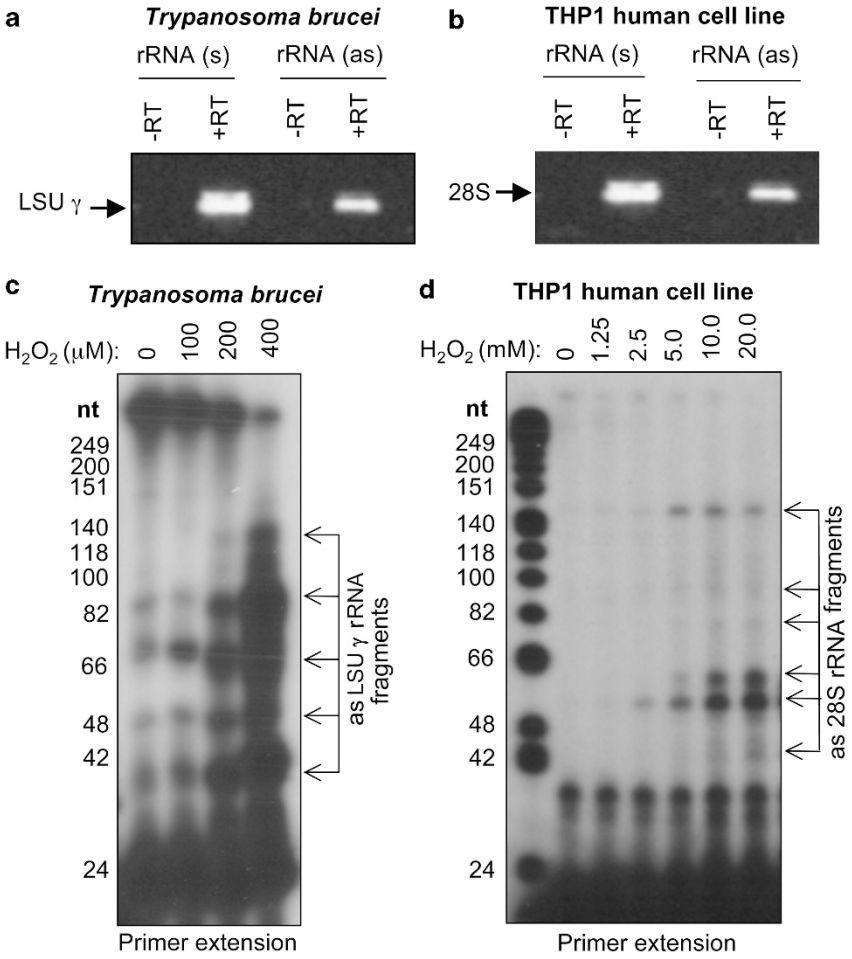

Figure 8 The asrRNA fragmentation process is evolutionary conserved. Single-stranded (SS) RT-PCR was performed to detect asRNA complementary to the LSU-γ rRNA of *Trypanosoma brucei* (**a**) and to the 28S rRNA in the THP-1 human acute monocytic leukemia cell line (**b**) using specific forward primers (see Supplementary Table 1). (**c**) Primer extension analysis using a forward primer complementary to nucleotides 101–118 of the *T. brucei* LSU-γ rRNA to detect asLSU-γ RNA fragmentation in *T. brucei* exposed to H_2O_2 (0–400 μM). (**d**) Primer extension to detect asRNA complementary to the human 28S rRNA in THP-1 cell line treated with H_2O_2 (0–20 mM). A forward primer complementary to nucleotides 1–18 of the human 28S rRNA was used

link to rRNA breakdown. Indeed, ectopic overexpression of asLSU-γ RNA increases accumulation of asLSU-γ RNA fragments and accelerates rRNA degradation upon MF-triggered apoptosis. The fact that rRNA breakdown appears generally at the same time intervals as the asLSU-γ RNA cleavage further favours the possibility that these two events are interrelated.

Another important finding here is the identification of HEL67 that seems to have a protective role in preventing fragmentation of asrRNA and rRNA breakdown through its interaction with sLSU-γ and asLSU-γ rRNAs. The *Leishmania* HEL67 belongs to the superfamily 2-RNA helicases that are ubiquitously involved in various stages of RNA processing and RNP remodeling by promoting ATP-dependent conformational changes and structural transitions.[34] HEL67 shares high sequence identity to the *Drosophila* Belle and VASA DEAD-box proteins,[35,36] and to the yeast Ded1p.[37] Belle promotes mitotic chromosome segregation in *Drosophila* via the endo-siRNA pathway.[35] The germ-line-specific VASA have diverse roles in regulating mRNA translation, germline differentiation, and piwi-interacting RNA-mediated transposon silencing.[36,38] The Ded1/DDX3 protein functions both as a repressor and an activator of translation.[39] A *Leishmania* mutant deficient in HEL67 exhibits a marked increase in asLSU-γ RNA fragmentation when exposed to temperature and acidic pH stress, which is correlated with rapid degradation of the srRNA. Remarkably, the asLSU-γ RNA fragmentation pattern seen in *Lin*HEL67$^{(-/-)}$ is comparable with that induced by MF in parasites undergoing apoptosis. Interestingly, an apoptosis-related phenotype has been reported for a null mutant of the mouse VASA homolog.[36] Increased fragmentation of the asLSU-γ RNA in *Lin*HEL67$^{(-/-)}$ is associated with reduced accumulation of the mature asLSU-γ rRNA, further indicating that HEL67 somehow prevents cleavage of the asrRNA. We propose that accumulation of asrRNA cleavage products may serve as a signal for accelerating rRNA breakdown under conditions of severe stress or apoptosis where global translation is markedly reduced. It remains to be seen, however, how asrRNA cleavage contributes to the degradation of srRNA. Our data support the possibility that under physiological conditions, HEL67 binds to both srRNA and asrRNAs within

translating ribosomes, hence preventing extensive cleavage of the asrRNA and protecting srRNA from degradation. Under conditions of severe stress or apoptosis, translation of HEL67 could be diminished and/or conformational changes within the ribosome could alter HEL67-rRNA interactions, hence allowing RNases to attack the non-translating ribosomes and to initiate rRNA breakdown.

In summary, here we describe for the first time that ALPCD and to a lesser extent stress trigger a regulated process involving fragmentation of asrRNA that is linked to rRNA degradation and translational arrest. This novel mechanism of asrRNA fragmentation seems to be conserved through evolution and it may represent a novel hallmark of apoptosis.

Materials and Methods

Parasite strains and cell culture. *Leishmania infantum* MHOM/MA/67/ITMAP-263, the parental strain for all the parasite lines employed in this study, was cultured as promastigotes in SDM-79 medium (pH 7.3) supplemented with 10% heat-inactivated fetal calf serum (Multicell, Wisent Inc., St-Jean Baptiste, Quebec, Canada) and 5 μg/ml hemin (Sigma, Oakville, Ontario, Canada) at 25°C. *L. infantum* axenic amastigotes were cultured in MAA/20 medium (pH-5.5) at 37°C with 5% CO_2 for 4 days as described.[40] Parasites were subjected to H_2O_2 (0–2 mM) or different drug treatments, including MF (Cayman Chemical, Ann Arbor, MI, USA) (0–40 μM), SbIII (Sigma) (0–100 μM), G418 (Sigma) (0–150 μg/ml), paramomycin sulphate (Sigma) (0–750 μg/ml) and hygromycin-B (Sigma) (80 μg/ml). *T. brucei* procyclics were grown in SDM-79 medium until they reached logarithmic phase. The THP-1 human acute monocytic leukemia cell line was infected with *L. infantum* as previously described[40] but without adding PMA. THP-1 cells were seeded (25×10^6/25 ml) in a 75 ml flask and allowed to grow for 72 h.

DNA constructs and transfections. The vector pSPBT1YNEOα1.2 was constructed as follows. The YNEOα fragment where Y is a 92 bp polypyrimidine stretch, *NEO* the neomycin phosphotransferase gene for resistance to G418 and α the intergenic region of the *L. enriettii* alpha-tubulin gene was amplified from vector pSPYNEOαLUC, and inserted into NotI of pSPBT1.[41] The 1.2 kb fragment harboring the last part of LSU-α (309 bp), the full-length LSU-γ (213 bp) and the first part of LSU-β (414 bp) in sense (sLSU1.2) or antisense (asLSU1.2) orientation was amplified by PCR using specific primers (Supplementary Table 1) and cloned into the *Hin*dIII site of pSPBT1YNEOα. To inactivate the *HEL67* gene of *L. infantum* (LinJ.32.0410) (TriTrypDB; http://tritrypdb.org), *HEL67* 5′- and 3′-flanking regions were amplified from genomic DNA using a PCR fusion-based strategy and fused to the *NEO* and hygromycin phosphotransferase (*HYG*) genes. For overexpressing the *HEL67* gene, the *HEL67* ORF was amplified by PCR and cloned into the *Xba*I and *Hin*dIII sites of vector pSP72αZEOα expressing the zeomycin (*ZEO*) marker. All primer sequences are described in Supplementary Table 1. Purified plasmid DNA (10–20 μg, Qiagen Plasmid Mini Prep Kit, Toronto, Ontario, Canada) was transfected into *Leishmania* by electroporation as described.[41] Stable transfectants were selected and cultivated with either 0.025 mg/ml G418 (Sigma) or 0.080 mg/ml Hygromycin-B (Sigma) or 1 mg/ml zeomycin (Sigma).

DNA and RNA analysis and hybridizations. Genomic DNA of *L. infantum* was extracted using DNAzol (Life Technologies Inc., Toronto, Ontario, Canada) following the manufacturer's instructions. Total RNA was extracted from *L. infantum* after lysis with Trizol (Invitrogen) and analyzed on 1.2% agarose gels. Southern and northern blot hybridizations were performed following standard procedures. Double-stranded DNA probes were labeled with [α-^{32}P] dCTP using random oligonucleotides and the Klenow enzyme (New England Biolabs, Ipswich, MA, USA). The 173 nt ss-DNA probe was used to detect asRNA. A 42 nt end-labeled probe complementary to nucleotides 172–213 of LSU-γ rRNA was used to detect srRNA fragments. Enrichment of small ($\leq$ 200 nt) RNA fraction was carried out using the *mir*Vana miRNA Isolation Kit (Life Technologies Inc.) as described in the manufacturer's protocol.

Strand-specific RT-PCR and qRT-PCR. Total RNA isolated from *L. infantum* promastigotes and amastigotes was purified with the RNeasy kit (Qiagen), treated twice with Turbo DNase (Ambion) and subsequently reverse transcribed using Superscript III reverse transcriptase (Invitrogen) as per the manufacturer's instructions. For strand-specific (SS) RT-PCR, we used forward primers for 28S, 18S, 5.8S, LSU-α, LSU-γ and LSU-β rRNAs (Supplementary Table 1). The resulting cDNA was treated with RNase H (Invitrogen) and PCR was carried out with the same forward and gene-specific reverse primers. The *GAPDH* gene was used for normalization of the qSS-RT-PCR data. Similarly, the RNA from *T. brucei* and the THP-1 cell line was used for SS-RT-PCR with specific primers described in Supplementary Table 1.

Primer extension analysis. Primer extension was performed with the SuperScript III RT kit (Invitrogen). The size of the cDNA fragments corresponds to the number of nucleotides between the labeled primer and the 5′-end of the cleaved LSU-γ rRNA when the reverse transcriptase falls of its template. Primers (Supplementary Table 1) were labeled with [γ-^{32}P] ATP following the polynucleotide kinase protocol (PNK; New England Biolabs). Total RNA was isolated from drug-treated and untreated *L. infantum* promastigote and amastigote and *T. brucei* procyclic samples and used for RT-reactions with labeled forward primers to detect asLSU-γ RNA cleavage products, and reverse primers to detect sLSU-γ cleavage products. RNA from H_2O_2-treated and untreated THP-1 cells was isolated and used for primer extension with a forward primer corresponding to nucleotides 4869–4886 of the 28S rRNA (Supplementary Table 1). The resulting radiolabeled cDNA was resolved on 10% Urea acrylamide gel (Sequagel, National Diagnostics, Atlanta, GA, USA) and visualized by autoradiography. A ΦX174 DNA/HinfI dephosphorylated DNA marker (Promega, Madison, WI, USA) was labeled with [γ-^{32}P]ATP and PNK (New England Biolabs) according to the manufacturer's recommendations and used as a size marker.

5′- and 3′-end mapping of asRNA and sRNA products. asLSU-γ RNA cleavage products were cloned by 5'-RACE. *L. infantum* RNA population of $\sim$200 nt was enriched with the Ambion *mir*Vana miRNA Isolation Kit and ligated with 5'-RACE adapter as per the manufacturer's instructions (First choice RLM-RACE kit; Ambion). Treatments with CIP to remove the 5'-phosphate group and tobacco acid pyrophosphatase to remove the cap structure of mRNAs were omitted. The 5'-RACE adapter-RNA product was converted to cDNA using random hexamer and then PCR-amplified with Taq DNA polymerase (Qiagen) using adapter-specific primer (Ambion) and forward primer (P1) corresponding to nucleotides 1–18 of LSU-γ for antisense and adapter-specific primer and reverse primer (P2) for sRNA (Supplementary Table 1). Nested PCR fragments with 5'-inner primer (provided with the kit) and gene-specific forward primer (P1) (Supplementary Table 1) for asLSU-γ rRNA and reverse primer (P2) and 5'-inner primer for sLSU-γ rRNA were used. To map the 3'-end of asLSU-γ RNA, $\sim$2 μg of total RNA was extended using poly-A polymerase (NEB) in the presence of ATP. The 3'-polyadenylated RNA was converted into cDNA using an oligo-dT primer. The cDNA was used as a template for PCR with oligo-dT and a reverse primer (P2) complementary to nucleotides 96–213 of sLSU-γ rRNA (Supplementary Table 1). The amplified PCR products were cloned into pGEM-T easy vector (Promega) and sequenced.

Polysome-profiling analysis. Approximately 3×10^9 *L. infantum* were treated with 100 μg/ml cycloheximide (Sigma) for 10 min, washed with phosphate-buffered saline, lysed with a Dounce homogenizer and 40 $A_{260\,nm}$ units of the lysate supernatant was layered on top of a 15–45% linear sucrose gradient (10 ml) in gradient buffer (50 mM Tris-HCl pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 3 U/ml RNAGuard (Amersham)) as described.[41] Total RNA was isolated from each fraction following three volumes of ethanol precipitation with 1/10 volume of sodium acetate (3 M, pH-5.2) and 50 μg of glycogen. The RNA was resolved on a 10% Urea acrylamide gel and analyzed by northern blot hybridization to detect sLSU-γ and asLSU-γ rRNAs.

***In vitro* UV-crosslinking studies.** UV-crosslinking of *in vitro*-transcribed radiolabeled sLSU-γ and asLSU-γ transcripts and total *Leishmania* protein lysates (2 mg/ml) was performed as described previously[42] with some modifications. The mixture was transferred to a microplate and UV-irradiated using a Stratalinker UV crosslinker (Agilent Technologies, Santa-Clara, CA, USA) (3×10^5 μJ, 254 nm bulbs) on ice for 15 min. The protein-crosslinked RNA was isolated by Trizol, digested with RNase A, 1 mg/ml (Invitrogen), RNase T1 10 U and RNase V1 (Ambion) and resolved on 15% SDS-PAGE gel. The radioactive bands were cut from the gel, left out for 10 weeks for radioactivity decay and then sent for MS/MS analysis.

Conflict of Interest

The authors declare no conflict of interest.

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