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The response of trypanosomes and other eukaryotes to ER stress and the spliced leader RNA silencing (SLS) pathway in *Trypanosoma brucei*

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Abstract

The unfolded protein response (UPR) is induced when the quality control machinery of the cell is overloaded with unfolded proteins or when one of the functions of the endoplasmic reticulum (ER) is perturbed. Here, I describe UPR in yeast and mammals, and compare it to what we know about pathogenic fungi and the parasitic protozoans from the order kinetoplastida, focusing on the novel pathway the spliced leader silencing (SLS) in *Trypanosoma brucei*. Trypanosomes lack conventional transcription regulation, and thus, lack most of the UPR machinery present in other eukaryotes. Trypanosome genes are transcribed in polycistronic units that are processed by *trans*-splicing and polyadenylation. In *trans*-splicing, which is essential for processing of each mRNA, an exon known as the spliced leader (SL) is added to all mRNAs from a small RNA, the SL RNA. Under severe ER stress, *T. brucei* elicits the SLS pathway. In SLS, the transcription of the SL RNA gene is extinguished, and the entire transcription complex dissociates from the SL RNA promoter. Induction of SLS is mediated by an ER-associated kinase (PK3) that migrates to the nucleus, where it phosphorylates the TATA-binding protein (TRF4), leading shut-off of SL RNA transcription. As a result, *trans*-splicing is inhibited and the parasites activate a programmed cell death (PCD) pathway. Despite the ability to sense the ER stress, the different eukaryotes, especially unicellular parasites and pathogenic fungi, developed a variety of unique and different ways to sense and adjust to this stress in a manner different from their host.

Introduction

The endoplasmic reticulum (ER) is best known for its role in protein processing of nascent secretory proteins, resident lumenal and *trans*-membrane proteins which constitute a third of the proteome (Huh *et al.*, 2003). To accomplish this function, the ER contains chaperones, oxidases, thiol-isomerases and glucosyltransferases that ensure the processing of properly folded proteins and target the mis-folded proteins for degradation (Araki & Nagata, 2012). The unfolded protein response (UPR) is induced when the quality control machinery is overloaded with client proteins or when one of the functions of the ER is perturbed (Ron & Walter, 2007). The UPR has evolved from unicellular eukaryotes to mammals to enhance ER function by induction of genes and production of proteins that are needed to alleviate the ER stress (Travers *et al.*, 2000). If UPR fails in metazoa, apoptosis is induced (Urra *et al.*, 2013). However, the UPR also regulates genes that are not directly related to ER function but have roles in metabolism and inflammation (Fu *et al.*, 2012).

Mounting evidence links ER stress to human diseases as diverse as diabetes, viral infection, Alzheimer's disease, cancer and inflammation (Wang & Kaufman, 2012).

The signaling of the UPR pathway was first elucidated in the yeast *Saccharomyces cerevisiae*, and it starts with the inositol-requiring enzyme (Ire1), an ER-resident *trans*-membrane kinase that is auto-phosphorylated during ER stress, becoming an endo-ribonuclease that catalyzes the removal of an intron of the *Hac1* transcript (Cox & Walter, 1996). This unusual splicing event produces an active transcription factor that belongs to the basic-leucine zipper (bZIP) family. This transcription factor migrates to the nucleus where it binds to many unfolded protein response elements (*UPRE*) present in promoters, such as the promoter of *Kar2* chaperone (Cox & Walter, 1996).

To sense the ER stress through Ire1, ER chaperones within the ER lumen recognize and bind to the hydrophobic regions and truncated glycosylation residues present on unfolded and misfolded proteins (Korennykh *et al.*, 2009). Under normal growth conditions, the ER chaperone, Kar2, binds to Ire1 inhibiting its oligomerization. However, under stress conditions, unfolded proteins accumulate within the ER lumen and interact with Kar2, inducing its release from Ire, and enabling the oligomerization and *trans*-auto-phosphorylation of Ire1.

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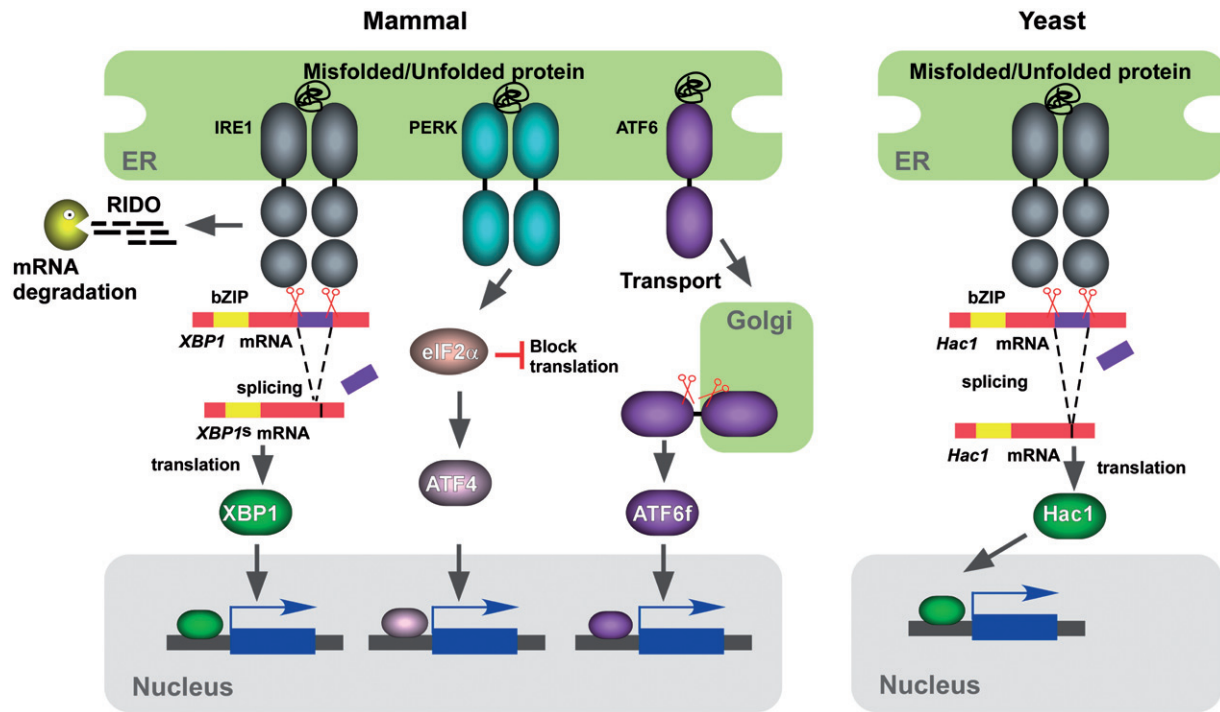
Figure 1. The conventional UPR mechanism in mammals and yeast. ER associated factors that sense ER stress in the presence of unfolded proteins are depicted. The IRE1 is common to yeast and mammals, and leads to splicing of Hac1 or XBP1, which is translated, and translocated to the nucleus to drive transcription of genes that are essential for the ER stress response. (see colour version of this figure at www.informahealthcare.com/bmg).

This oligomerization and *trans*-auto-phosphorylation change the confirmation of Ire1, resulting in an active endonuclease domain that can bind and cleave mRNAs (Gardner *et al.*, 2013) (Figure 1).

Studies in yeast UPR (Travers *et al.*, 2000) revealed that it is not only chaperones and phospholipid synthesis enzymes, that are up-regulated by UPR, but additional functions, such as factors involved in ER-associated protein degradation (ERAD), vesicular trafficking and protein translocation into the ER which relieves the load on the ER.

The UPR in mammals is more complex than that described in yeast, since it contains two additional factors in addition to IRE1 and XBP1 (the paralog of *Hac1*) (Calfon *et al.*, 2002). These include the *trans*-membrane protein with a lumenal domain and cytosolic kinase similar to the PKR kinase that phosphorylates the eIF2α, known as PERK (PKR-like ER kinase) (Harding *et al.*, 1999) and ATF6 (of the activating transcription factor family), which is also an ER-resident stress sensor (Haze *et al.*, 1999; Yoshida *et al.*, 1998). All three pathways (i.e. IRE1, PERK and ATF6) are conserved in metazoa, but the PERK and ATF6 pathways are of lesser importance in invertebrates, including flies and worms (Ryoo *et al.*, 2007; Shen *et al.*, 2001). PERK activation and auto-phosphorylation lead to eIF2α phosphorylation, which inhibits the translation of most mRNAs, but stimulates translation of ATF4 due to the presence of upstream open reading frames (uORFs) present at the 5′ UTR (Lu *et al.*, 2004; Vattem & Wek, 2004). At the initial stage of the UPR, only the translation of mRNAs having uORFs, such as ATF4, is possible (Ventoso *et al.*, 2012). ATF4 is a transcription factor which binds to amino acid response elements (*AARE*s) in target gene promoters to activate transcription (Harding *et al.*, 2003). ATF6 is an ER-localized *trans*-membrane

transcription factor. ER stress releases it from the ER to the Golgi, where it is cleaved by regulated intra-membrane proteolysis (RIP) to liberate the transcriptionally active cytosolic domain that translocates to the nucleus, where it binds to specific sequences in target genes (Baumeister *et al.*, 2005; Li *et al.*, 2000) (Figure 1).

The phenotypes of mice with constitutive deletions of UPR, such as the perk$^{-/-}$ mutant, show evidence of grossly altered ER structure and impaired secretory pathway function affecting the function of endocrine and exocrine cells (Harding *et al.*, 2001). Similar to yeast, mammalian cells respond to ER stress with an up-regulation of genes encoding ER chaperones, ERAD factors, lipid synthesis enzymes and proteins involved in protein trafficking as well as protein synthesis. Subsets of these regulated genes depend on each of the three UPR-specific transcription factors. Although, there is some overlap in genes regulated by these factors, ATF4 controls the transcription of genes involved in protein anabolism and redox defense (Harding *et al.*, 2003), and ATF6 contributes to the regulation of chaperones and ERAD factors (Adachi *et al.*, 2008).

In metazoa, ER stress triggers two distinct outputs of the nuclease activity from IRE1, namely XBP1 splicing (Cox & Walter, 1996) and regulated IRE1-dependent decay (RIDD) (Hollien *et al.*, 2009). The former activates the UPR pathway, whereas the latter selectively degrades a small subset of ER-associated mRNAs and thus shapes the repertoire of proteins translated in ER-stressed cells. Such a cellular response is predicted to reduce the ER load by limiting protein influx, *via* degrading mRNAs encoding for secreted and membrane proteins (Hollien *et al.*, 2009). The RIDD pathway was first discovered in *Drosophila melanogaster* (Hollien & Weissman, 2006) and later confirmed in mammalian cells

(Hollien et al., 2009). RIDD does not occur in S. cerevisiae, but occurs in the fission yeast *Schizosaccaromyces pobe*, which lacks *Hac1*. In *Schizosaccharomyces pombe*, Ire1 initiates the selective decay of a subset of ER localized mRNAs. BiP mRNA is the only mRNA that is cleaved by Ire1 but escapes decay. Truncation of the 3′ UTR stabilizes BiP mRNA, resulting in increased translation of the chaperone (Kimmig et al., 2012) (Figure 1).

ER stress response in pathogenic fungi

The study of the ER response in pathogenic fungi revealed interesting and novel mechanisms that deviate from the conventional UPR mechanism outlined above. Among the pathogenic fungi that are the causative agent of threatening invasive fungal diseases are *Candida* and *Cryptococcus* (Reedy et al., 2007).

Candida glabrata has emerged as an important fungal pathogen in clinical practice, partly because of its decreased susceptibility to anti-fungal therapy. *Candida glabrata* is highly tolerant to ER stress relative to other fungi, such as *S. cerevisiae* (Pfaller & Diekema, 2007). Surprisingly, the canonical UPR mechanism regulated by *Ire1–Hac1* is not conserved in *C. glabrata*. *Candida glabrata* Ire1 does not cleave mRNAs encoding *Hac1* but is involved in RIDD (Miyazaki et al., 2013). The transcriptional response to ER stress in this fungus is mediated by calcineurin signaling *via* the Slt2-MAPK pathway (Miyazaki et al., 2013). Calcineurin mediates the calcium cell survival pathway by regulating intracellular Ca^{2+} homeostasis. ER stress increases Ca^{2+} uptake by stimulating the high-affinity Ca^{2+} channel Cch1–Mid1; calcineurin dephosphorylates the Cch1 subunit of the channel to inhibit Ca^{2+} influx, and therefore prevents cell death (Dudgeon et al., 2008). Slt2 plays a role in the ER stress surveillance (ERSU) pathway that ensures the transmission of only functional ERs to daughter cells during cell division (Babour et al., 2010). Upon ER stress, the ERSU pathway delays ER inheritance and cytokinesis to prevent the death of both mother and daughter cells. In addition, many genes involved in the ribosome activity and cytoplasmic translation are down-regulated in a Slt2-dependent manner during the late phase of the ER stress response in *C. glabrata* (Babour et al., 2010). As noted above, the *Ire1–Hac1* signaling pathway is required for the up-regulation of the ER-resident chaperone, Kar2, in *S. cerevisiae* (Cox & Walter, 1996). In *C. glabrata*, the majority of ER stress-induced genes, including Kar2, are dependent on the calcineurin–Crz1 pathway (Miyazaki et al., 2013). Based on these studies, calcineurin might be an excellent target to improve treatment options for *C. glabrata* infections (Miyazaki & Kohno, 2014).

The second pathogenic fungus whose UPR pathway was elucidated is *Cryptococcus neoformans*. *Cryptococcus neoformans* is an opportunistic fungal pathogen, which causes life-threatening meningoencephalitis in immune-compromised individuals. *Cryptococcus neoformans* is the most commonly isolated clade worldwide, and nearly a million cases of HIV/AIDS-related cryptococcal meningitis occur worldwide every year, causing more than 620 000 deaths (Sorrell, 2001).

Cryptococcus disseminates from the lung through the bloodstream and finds its way to the central nervous system and to the brain, resulting in meningoencephalitis (Sukroongreung et al., 1998). This fungus has an evolutionarily conserved Ire1 as its sole UPR pathway sensor in the ER and is not likely to contain other UPR sensors, such as PERK and ATF6. The *Cryptococcus* Ire1 kinase is highly homologous to that of *S. cerevisiae*. However, *Hxl1* is structurally and phylogenetically distant from yeast *Hac1* or human *XBP1* (Cheon et al., 2011). The expression of *C. neoformans Kar2* is tightly regulated in an Ire1- and Hxl1-dependent manner upon ER stress. *Cryptococcus* contains both evolutionarily conserved and unique UPR components (Jung et al., 2013). Although the *Cryptococcus* UPR pathway regulates ER stress, anti-fungal drug resistance and virulence in an Ire1/Hxl1-dependent manner, Ire1 has also Hxl1-independent roles in capsule biosynthesis and thermal-tolerance (Jung et al., 2013).

Hxl1 appears to be the only bona fide ER stress response transcription factor acting downstream of Ire1, since the expression of spliced *Hxl1* mRNA completely restores wild-type resistance of the *ire1Δ* mutant to ER and cell wall stresses (Cheon et al., 2011).

In addition to its conserved role in the response to ER stress, the *C. neoformans* UPR pathway also controls the thermo-tolerance and virulence of *Cryptococcus* (Cheon et al., 2011). The ability to survive and proliferate at physiological body temperature is an essential virulence factor for most pathogens. Both Ire1 and Hxl1 are required for the growth of *Cryptococcus* at temperatures above 30 °C, and deletion of either gene abolishes its ability to grow at 37 °C. This is likely to be the reason *Cryptococcus* UPR mutants are avirulent (Havel et al., 2011; Kronstad et al., 2008).

In response to ER stress and thermal shock, representative UPR target genes, such as *Kar2, Sec61* (the translocon that mediates the transport of protein across the ER membrane) and *Der1* (involved in ER-associated degradation), were shown to be up-regulated in an Ire1/Hxl1-dependent manner, whereas expression of *Pmt1* and *Pmt4* (protein *O*-mannosyl-transferase) is only dependent on *Hxl1*. The presence of Hxl1-independent Ire1 function also suggests that RIDD may play a role in the ER stress response. It is also tempting to postulate a role of RIDD in host temperature adaptation in *C. neoformans* (Glazier & Panepinto, 2013). These observations strongly suggest that Ire1 has bifurcated signaling branches, one of which includes Hxl1 to execute conserved roles of the UPR pathway, and another that bypasses Hxl1 (Cheon et al., 2011).

Perturbation of the UPR pathway significantly increases Mpk1 phosphorylation levels (under both basal and stress conditions), suggesting that direct or indirect crosstalk occurs between the UPR pathway and the Mpk1 MAPK pathway (Cheon et al., 2011). Crosstalk between the UPR and calcineurin pathways is also likely in *Cryptococcus*. Perturbation of the calcineurin signaling pathway, which is involved in cell wall integrity, thermo-tolerance and virulence in *C. neoformans*, affects *Hxl1* splicing and *Kar2* induction under certain conditions (Cheon et al., 2011). The UPR pathway may also engage in crosstalk with the mRNA degradation machinery in *C. neoformans*. There is transient increase in the abundance of transcripts encoding ER stress proteins in response to host temperature. The transcripts level peaks after one hour and then return to pre-shift level.

On the other hand, mRNA encoding ribosomal proteins are transiently repressed and then come back to normal level. It was demonstrated that mRNA degradation is central in this regulation. In *C. neoformans* mutant lacking either Ccr4, the major deadenylase, or Rbp4, an RNA Pol II subunit, the transient repression of ribosomal proteins transcripts seen after shift to h 37 °C was absent or attenuated (Havel *et al.*, 2011). Thus, the mRNA degradation machinery is an additional level of control for the UPR pathway. The Ire1/Hxl1-dependent UPR pathway serves as a hub in *C. neoformans*, directly or indirectly interacting with other stress-related signaling pathways to coordinate responses to signals (Havel *et al.*, 2011). Recent studies suggest that the UPR pathway could potentially be exploited as a novel drug target for fungal diseases. Hxl1 is an attractive therapeutic target because it is structurally divergent from the host XBP1 transcription factor, and thus Hxl1-specific inhibitors may control the pathogen in the absence of side effects to the host (Cheon *et al.*, 2014). *Cryptococcus* can also serve as an excellent model system to understand the conserved and unique features of the UPR in diverse fungal species (Cheon *et al.*, 2014).

Unique gene expression in trypanosomes and their response to stress

Another interesting group of unicellular eukaryotes with unique UPR pathways is parasites belonging to the order kinetoplastida. These important parasites, also known as the trypnosomatids, include the *Trypanosoma* and *Leishmania* species.

Trypanosoma brucei is the causative agent of sleeping sickness in humans, and Nagana in the livestock. The parasites have two main proliferative stages, the procyclic stage (PCF), which multiplies in the tsetse fly midgut, and the bloodstream form (BSF), which propagates in the mammalian host (Malvy & Chappuis, 2011) (Figure 2). Several morphological and metabolic changes are taking place during transition between the hosts. After two weeks in the midgut of the fly, the PCF moves to the proventriculus which is the terminal portion of the foregut, where the parasites elongate and become epimastigotes. The epimastigotes differentiate to metacyclics which acquire a metacyclic variant surface glycoprotein (VSG) coat. After transmission to the mammalian host *via* the bite of the tsetse fly, the metacyclics transform to slender forms. To sustain infection the slender forms transform to the non-dividing stumpy form that is the form which is infective when entering the fly (Sharma *et al.*, 2009) (Figure 2).

The two other well-studied members of this group is *Leishmania*, which cycles between the mid-gut of the sand fly and the mammalian host (Turco & Sacks, 1991), and *Trypanosoma cruzi*, the causative agent of Chagas' disease which is transmitted to the mammalian host by reduviid bug (de Souza *et al.*, 2010).

Gene expression in trypanosomes is regulated by very unique mechanisms. All mRNAs are transcribed as polycistronic units (Kolev *et al.*, 2010). RNA transcription initiation seems to be controlled by histone marks (Siegel *et al.*, 2009). Beside histone modifications (Moretti & Schenkman, 2013) trypanosomes also present a unique DNA base modification,

β-D-glucopyranosyloxymethyluracil, known as base J. Base J was initially discovered in VSG genes that many except one are silenced during the process of antigenic variation in *T. brucei* (Borst & Sabatini, 2008). However, studies over the years demonstrated the base J has a role beyond antigenic variation, and this modification was localized to domains flanking the polycistronic units of *T. brucei* (Cliffe *et al.*, 2010) and was shown to promote transcription termination in *Leishmania* but not in *T. brucei* (Reynolds *et al.*, 2014; van Luenen *et al.*, 2012).

mRNA processing in trypanosomes differs from this process in other eukaryotes, as in trypanosomes, all mRNAs are *trans*-spliced, while only two *cis*-introns have been identified (Michaeli, 2011). In the *trans*-splicing process, a small exon, the spliced leader (SL) encoded by a small RNA, the SL RNA is donated to pre-mRNA. *Trans*-splicing is coupled to polyadenylation of the upstream gene and the signals for these two processes are spaced ~150 nt apart. An as-yet unidentified factor may exist that links these processes (Michaeli, 2011). The concerted action of polyadenylation and *trans*-splicing is used to separate the mono-cistrons from the polycistronic units (Michaeli, 2011). Post-transcription regulation is very dominant in these parasites and operates at the level of mRNA degradation and translation (Clayton, 2013; Kramer & Carrington, 2011). For most genes, the signals that dictate this regulation are confined to the 3′ UTR (Kramer & Carrington, 2011). The parasites remodel their gene expression while cycling between the two hosts (Rico *et al.*, 2013). During the host transition, parasites need to adapt to drastic changes in pH, temperature, nutrient and oxygen levels. Thus, this tight regulation of gene expression is achieved by utilizing tens of RNA binding proteins (RBPs) that regulate mRNA processing, mRNA stability and translation (Clayton, 2013). Among the RBPs, only a few were shown to affect both splicing and mRNA stability; among these are *T. brucei* PTB1 and PTB2, also known as DRBD3 and DRBD4 (Stern *et al.*, 2009) and hnRNPF/H (Gupta *et al.*, 2013), TSR and TSR1IP (Gupta *et al.*, 2014).

The regulon model was suggested to explain the coordinated gene expression in this family. It was suggested that RBPs coordinately regulate multiple mRNAs by interacting with transcripts containing shared elements (Fernandez-Moya & Estevez, 2010). Most recently, an RNA motif present in the 3′ UTR of genes, such as in genes involved in lysine degradation, inositol phosphate and folate metabolism, was identified in *T. cruzi*. These potential RNA binding sites are enriched with specific motifs, and are present in genes that are differentially expressed during parasite development and stress response (De Gaudenzi *et al.*, 2013).

The most defined and characterized polymerase II promoter in these parasites to date is that of the SL RNA. The promoter consists of a bipartite upstream sequence element (USE) and an initiator element at the transcription start site, while a conventional, albeit divergent, pre-initiation complex drives transcription of the SL RNA gene (Gunzl *et al.*, 1997). SL RNA transcription requires the small nuclear RNA activating protein complex (SNAPc) composed of SNAP50 (also known as tSNAP50), SNAP2 (tSNAP42) and SNAP3 (tSNAP26) (Das *et al.*, 2005; Schimanski *et al.*, 2005). tSNAPc binds to the USE, likely through SNAP2, which

Figure 2. The life cycle of *Trypanosoma brucei*. Scheme illustrating the life cycle of *T. brucei* the parasite that is most extensively discussed in this review. The different life stages in the two hosts are illustrated. The color version of the figures is available online. (see colour version of this figure at www.informahealthcare.com/bmg).
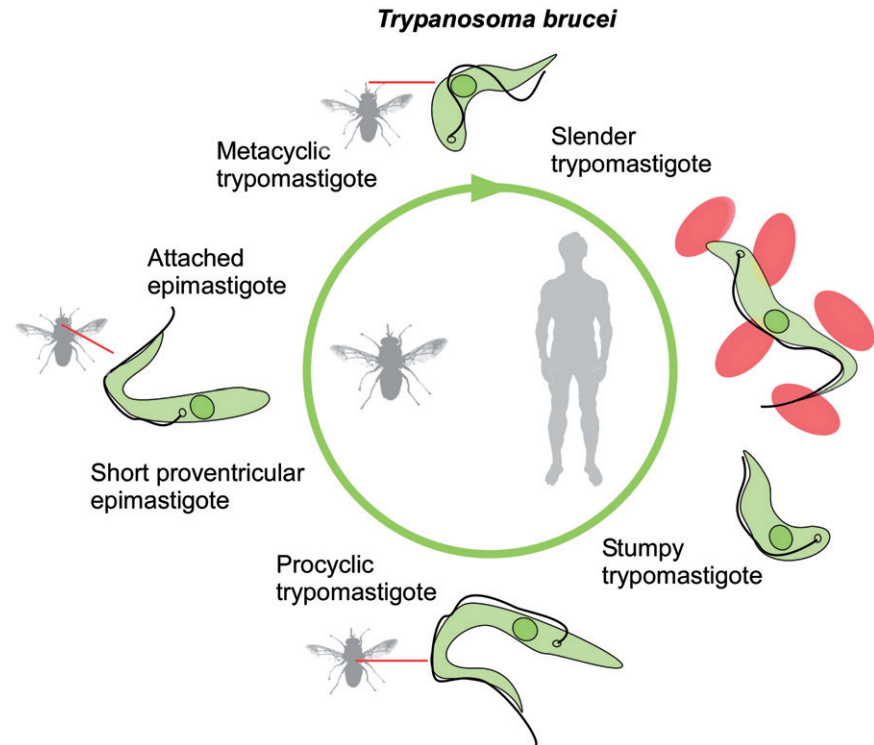
contains a Myb DNA binding domain. tSNAPc is part of a larger protein complex that also comprises trypanosome homologues of the TATA-binding protein (TBP), termed TBP-related factor 4 (TRF4), and transcription factor (TF) IIA (TFIIA) (Das *et al.*, 2005; Schimanski *et al.*, 2005). Moreover, TRF4 (Ruan *et al.*, 2004), TFIIB (Palenchar *et al.*, 2006; Schimanski *et al.*, 2006), TFIIH (Lecordier *et al.*, 2007; Lee *et al.*, 2007) and putative TFIIE homologues TSP1 and TSP2 (Lee *et al.*, 2009) are all required for SL RNA transcription.

Stress response mechanisms in trypanosomatids

Heat shock is the most extensively studied stress response in trypanosomatids. Changes in temperature occur regularly in the life cycle of *T. brucei*, both in the insect vector (because of temperature fluctuations between day and night) and during the rise in temperature when propagating in the mammalian host (Schwede *et al.*, 2011). The response to heat is very rapid and takes place within a few minutes. Reduction in most mRNAs (75%) was observed during heat shock in *T. brucei* resulting from decreased production and increased decay (Kramer *et al.*, 2008). The most extensive studies to decipher the regulation of preferential expression of mRNA encoding for heat shock protein were performed in *Leishmania*. In *Leishmania*, the 3′ UTR of the HSP83 mRNA is sufficient for increased stability and translation upon heat shock, whereas the 5′ UTR has no effect by itself, but does act synergistically with the 3′ UTR (Zilka *et al.*, 2001). In *Leishmania amazonensis*, a *cis*-element sufficient to confer preferential translation upon heat shock was identified in the 3′ UTR of the HSP83 mRNA, and an RNA structure was proposed to change during heat shock and to directly regulate translation (David *et al.*, 2010). In *T. brucei*, heat shock also causes a decrease in polysomes, resulting in changes in cytoplasmic ribonucleoprotein granules (Kramer *et al.*, 2008). Processing (P) bodies containing enzymes of the mRNA degradation pathway are increased. These stress granules contain many of the proteins involved in the initiation of translation. However, XRNA, the cytoplasmic 5′–3′ exoribonuclease that degrades mRNAs upon heat shock, forms a unique focus at the posterior pole of the cell (Kramer *et al.*, 2008). Stress granule formation upon heat shock is independent of eIF2α phosphorylation (Kramer *et al.*, 2008). Stress is known to arrest translation *via* different eIF2α kinases (see below). Interestingly, as noted above, in *T. brucei*, unlike all other eukaryotes, eIF2α is not phosphorylated under heat shock (Kramer *et al.*, 2008).

Recently, it was demonstrated that the *T. brucei* CCCH zinc finger protein ZC3H11 is a post-transcriptional regulator of trypanosome chaperone mRNAs. In procyclic forms, ZC3H11 is required for the stabilization after heat-shock of mRNAs encoding chaperones. Many mRNAs bound to ZC3H11 have a consensus AUU repeat motif in the 3′-UTR. Tethering of ZC3H11 to a reporter mRNA increased reporter expression, showing that it is capable of directly stabilizing mRNA. The study demonstrated that heat shock genes are controlled by a specific RNA-protein interaction (Droll *et al.*, 2013).

Changes in localization of RBP were observed upon induction of stresses, such as heat shock, oxidative stress or starvation. *Trypanosoma cruzi* uridine binding protein 1 (UBP1), a factor that that binds to and destabilizes a specific group of mRNAs, together with its partner, UBP2, migrates to the nucleus under oxidative stress induced by arsenite (Cassola & Frasch, 2009). SR62, a serine–arginine rich protein, and PTB2 translocate from nuclear speckles to the nucleolus upon heat shock in *T. cruzi* (Nazer *et al.*, 2011).

On the other hand, heat shock and starvation promote the accumulation of UBP1, UBP2, the poly(A)-binding proteins PABP1 and PABP2, the RNA-helicase DHH1, the translational repressor SCD6 and several other RBPs in cytoplasmic granules in *T. cruzi* and *T. brucei* (Nazer *et al.*, 2011). The *T. brucei* RNA-binding protein DRBD3 (or PTB1) is part of a RNP complex that changes its subcellular localization and composition upon arsenite or starvation-induced stresses (Fernandez-Moya *et al.*, 2012).

Under physiological starvation conditions in trypanosomes, P bodies contain distinct RBPs bound to mRNA in the form of mRNA granules, in which transcripts are stored and protected from degradation. Other novel types of foci with unknown function that are related to RNA metabolism can be found in these parasites, such as the heat-induced granules (described above), and starvation-induced granules containing transfer RNA halves. Thus, trypanosomes utilize structures to compartmentalize RNPs in the cytoplasm, in an attempt to cope with stressful situations, delaying mRNA translation or degradation. Recent evidence suggests that these cytoplasmic granules are required for survival under adverse growth conditions (Cassola, 2011).

The UPR in nematodes

Like trypanosomes, nematodes process 70% of their mRNA by *trans*-splicing. In contrast to trypanosomes which posses a single SL RNA gene type, in nematodes SL RNA exist in two forms SL1 and SL2. The majority are *trans*-spliced by SL1 and not by SL2. SL2 recipient gene clusters which are similar to bacterial operons may contain 2–8 genes. The operons are transcribed from a 5′ defined polymerase II promoter and gene-specific mRNA are dissected like in trypanosomes from the polycistroinc RNA by concerted action of *trans*-splicing and polyadenylation. SL2 *trans*-splicing requires a sequence between the genes, the Ur element, which base pairs with the 5′ splice site on the SL2 RNA (Spieth *et al.*, 1993). The operons contain primarily genes required for rapid growth, including genes whose products are needed for mitochondrial function and the basic machinery of gene expression. It was suggested that operons allow more efficient recovery from growth-arrested states (Zorio *et al.*, 1994). Thus, although nematodes and trypanosomes process their genes by *trans*-splicing, their gene regulation is very different because nematodes possess extensive transcriptional regulation. It was therefore expected to find in *Caenorhabditis elegans Ire1* which exerts its regulation by activating the transcription of chaperones, as well *perk* homologues (Shen *et al.*, 2001). Interestingly, the study of UPR in *C. elegans* revealed that UPR does not only function autonomously but within the context of a multicellular animal, it exists as a proteostasis network (PN) which is regulated by cell-non-autonomous signaling through specific sensory neurons and by the process of *trans*-cellular chaperone signaling. These newly identified forms of stress signaling control transmits the signal between neurons and non-neuronal somatic tissues to balance chaperones expression in response to environmental UPR stress. *Trans*-cellular chaperone signaling leads to expression of chaperones in somatic tissues, to prevent the spreading of toxic damage. Thus, communication between subcellular compartments and across different cells and even tissues helps to cope with acute ER stress (van Oosten-Hawle & Morimoto, 2014).

Of special interest is also the mechanism that was extensively studied in *C. elegans* of UPR which is activated in the mitochondria. This signaling originates in the matrix of the mitochondria, when the misfolded or unassembled proteins accumulate and exhausts the capacity of the chaperones to cope with the stress. The excessive proteins are digested into short peptides by a proteolytic complex. These peptides are transported by an inner membrane-spanning ATP-binding cassette (ABC) from the mitochondria matrix into the cytoplasm. The released peptides weaken the mitochondrial import. Consequently, a leucine zipper transcription factor ATFS-1 which contains both nuclear localization/export (NLS) and mitochondrial targeting sequences (MTSs) cannot translocate to the mitochondria where it is degraded. The protein therefore translocates to the nucleus, where it activates a broad protective transcriptional program. In the nucleus, ATFS-1 induces mitochondrial protein quality control by transcriptional activation of chaperone and protease genes (Nargund *et al.*, 2012). In addition, ATFS-1 activates the transcription of proteins involved in mitochondrial import, reactive oxygen species (ROS) detoxification. Moreover, a few glycolysis genes are up-regulated by ATFS-1, which shifts the global cell metabolism from respiration to glycolysis further protecting the organism from ROS which is produced by respiration (Nargund *et al.*, 2012).

The UPR in Trypanosomatids

In the absence of transcriptional regulation of individual genes, it could be expected that trypanosomes might not have a mechanism analogous to UPR (described above). It was also argued that trypanosomes may not require a UPR response because these parasites propagate under homeostasis in the host (Koumandou *et al.*, 2008). *Trypanosoma. brucei* BSF cannot compensate for slow-folding VSG production by increasing chaperone production, which is typically of the UPR response, and therefore most of the nascent VSG is degraded by the proteasome (Field *et al.*, 2010).

Indeed, bioinformatic searches failed to detect the key factors of the UPR response including IRE or XBP1 homologues in any trypanosomatids. The answer to whether trypanosomes possess an ER stress–response mechanism came from a study that investigated the mechanism and machinery of protein translocation across the ER membrane in trypanosomes (Goldshmidt *et al.*, 2008). To examine if UPR exists in trypanosomes and is activated by a novel mechanism which is not related to the UPR response in other eukaryotes, cells were exposed to a classic UPR inducer, the reducing agent, Dithiothreitol (DTT), and RNA was subjected to microarray analysis. Inspection of the up-regulated genes suggested that a distinct family of genes were up-regulated. Among these genes are genes involved in the core processes of UPR, such as protein folding, degradation, translocation across the ER, protein sorting, redox balance, and lipid metabolism. Interestingly, other genes, such as genes involved in signal-transduction, RBPs were also increased (Goldshmidt *et al.*, 2010). To examine if these alterations are reminiscent of changes that take place under UPR response of other

organisms, the microarray data were compared to data available for *C. elegans, D. melanogaster* and *Homo sapiens.* The results of this analysis revealed that in trypanosomes the genes mostly affected by DTT treatment are genes involved in protein secretion. Of additional interest is the finding that 35% of the genes whose level was reduced, encode for proteins destined to traverse the ER, i.e. proteins harboring either a signal-peptide or *trans*-membrane domain. These results are reminiscent of the RIDD pathway (described above) (Hollien & Weissman, 2006). Thus, despite lacking a transcriptional network to activate functions essential to cope with ER stress, trypanosomes manage to up-regulate the relevant transcripts induced under UPR in other eukaryotes.

As stated above, the most robust regulatory mechanism in trypanosomes is mRNA stability and preferential translation, which is mediated by the rich repertoire of RBPs (Clayton & Shapira, 2007). It was therefore reasonable to investigate the potential role of RNA stability in regulating the level of mRNA under ER stress. Indeed, mRNA stability of selected mRNAs whose level was increased under DTT treatment was examined, and it was found that the stability of mRNA encoding the chaperone DNAJ, protein disulfide isomerase (PDI), thioredoxin and syntaxin was increased, whereas no change in stability of mRNAs whose level was unchanged during DTT treatment was detected, suggesting that mRNA stabilization is the primary mechanism mediating the enhanced expression of proteins during ER stress (Goldshmidt *et al.*, 2010). The RBPs involved in this regulation are yet to be elucidated. The stabilization of BiP mRNA was also observed in *S. pombe* (Kimmig *et al.*, 2012), thus increase in chaperone mRNA can either be obtained in many eukaryotes by transcriptional regulation or also by mRNA stabilization.

One of the most important branches in the metazoan UPR response is PERK, whose activation leads to phosphorylation of eIF2α, arresting translation (Harding *et al.*, 1999). Studies in *Leishmania* using *in silico* analyses also revealed the absence of proteins involved in transcriptionally mediated UPR, but suggested the presence of both PERK and its target eIF2α and their involvement in the UPR (Gosline *et al.*, 2011). Data demonstrated that stimulation of the UPR in *Leishmania donovani* by treatment with DTT or tunicamycin did not result in up-regulation of the ER chaperone, BiP, but showed increased phosphorylation of eIF2α. It was also demonstrated that *L. donovani* is more sensitive to UPR induction, suggesting that *Leishmania* PERK activation might be a novel target for anti-parasitic drugs (Gosline *et al.*, 2011). Another study performed in *Leishmania infantum* showed that the *Leishmania* PERK is an endoplasmic reticulum (ER) *trans*-membrane protein that largely co-localizes with the ER BiP chaperone. The *Leishmania* PERK catalytic kinase domain undergoes auto-hyperphosphorylation and phosphorylates the translation initiation eIF2α *in vitro* at threonine 166, which is distinct from the Serine 51 that is conserved and is phosphorylated in other eukaryotic eIF2α. PERK is post-translationally regulated specifically in the intracellular stage of the parasite or under ER stress, *via* extensive auto-hyper-phosphorylation. A PERK dominant-negative mutant overexpressing a truncated PERK protein lacking the *N*-terminal luminal domain was shown to

be impaired in eIF2α phosphorylation in response to ER stress or during amastigote differentiation. The lack of eIF2α phosphorylation delays the *Leishmania* differentiation process towards the amastigote form (Chow *et al.*, 2011).

Trypanosoma brucei genome encodes three potential eIF2α kinases (TbeIF2K1 to -K3) (Moraes *et al.*, 2007). TbeIF2K2 is a *trans*-membrane glycoprotein expressed both in procyclic and in bloodstream forms. It is the homologue to the PERK described in *Leishmania*. The catalytic domain of TbeIF2K2 phosphorylates yeast and mammalian eIF2α at Ser51. It also phosphorylates the TbeIF2α at residue Thr169, which corresponds to the *Leishmania* Thr166 (Chow *et al.*, 2011). In both PCF and BSF, TbeIF2K2 is mainly localized in the flagellar pocket, an organelle that mediates exo- and endocytosis in these parasites, but is also found in endosomes (Moraes *et al.*, 2007). However, no evidence exists for the role of this PERK homologue in *T. brucei* ER stress (see below).

Interestingly, in *T. cruzi* phosphorylation of the (eIF2α) takes place during *in vitro* differentiation and under starvation. Tc-eIF2α phosphorylation is critical for parasite differentiation since the overexpression of the mutant eIF2α in epimastigotes abolished metacyclogenesis. Thus, eIF2α phosphorylation is a key step in *T. cruzi* differentiation (Tonelli *et al.*, 2011). The kinase involved in this regulation is the homologue to TbeIF2K1 (Moraes *et al.*, 2007).

Spliced leader silencing – the discovery of a novel pathway in *T. brucei*

In the course of studying the role of the co- and post-translational protein translocation mechanisms across the ER, and exploring the role of the signal recognition particle (SRP) and its receptor SRα, we compared the phenotype of SRα silenced cells to perturbations observed under silencing of SRP proteins in *T. brucei* (Liu *et al.*, 2002; Lustig *et al.*, 2005). Under SRα depletion but not in SRP54 silenced cells, the level of all mRNAs tested was reduced (Lustig *et al.*, 2007). This reduction was a result of inhibition of trans-splicing, due to shut-off in SL RNA transcription. tSANP42 or SNAP2, an SL RNA specific transcription factor, failed to bind to the SL RNA promoter. The process was therefore termed SLS for spliced leader RNA silencing (Lustig *et al.*, 2007) (Figure 3).

SLS was initially discovered in SRα silenced cells, but was later demonstrated in cells silenced for SEC63, a factor that is essential for both post- and co-translational translocation, as well as in cells depleted for the ER translocon, SEC61, the channel through which the proteins traverse the ER (Goldshmidt *et al.*, 2008, 2010). SLS was induced under low pH and in cells treated with DTT or 2-deoxyglucose, which affects glycosylation, and is known to induce UPR (Goldshmidt *et al.*, 2010). SLS is characterized by two hallmarks, the reduced abundance of SL RNA and increased abundance of SNAP2. SNAP2 normally localizes to discrete puncta within the nucleus associated with sites of SL RNA synthesis. However, during SLS, SNAP2 localizes throughout the nucleus because it fails to bind the SL RNA promoter (Lustig *et al.*, 2007).

Induction of SLS leads to programmed cell death (PCD), manifested by appearance of phosphatidyl serine on the cell
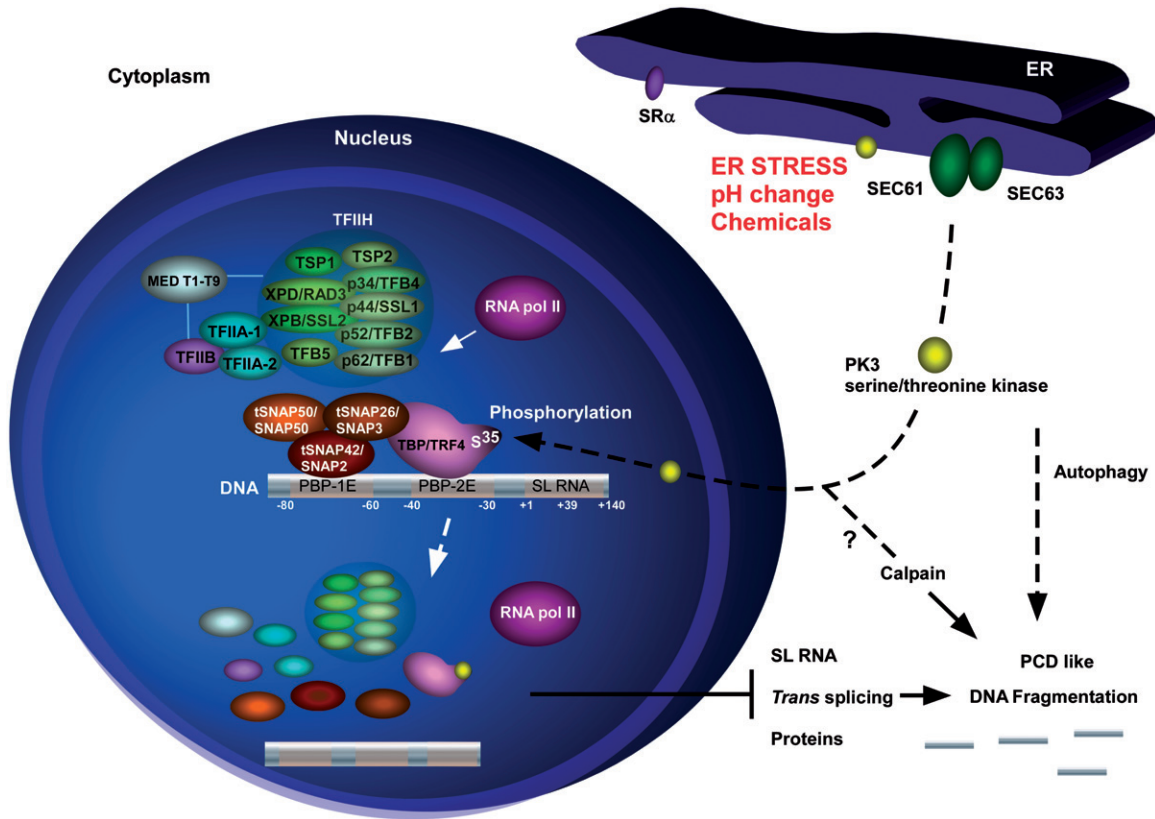
Figure 3. The mechanism of SLS. Upon ER stress induced by chemicals that elicit UPR, pH changes or silencing of factors that are involved in translocation of proteins across the ER, such as SEC61, SEC63 and SRa, the PK3 serine/threonine kinase, which is normally localized to the ER, is auto-phosphorylated and moves to the nucleus where it phosphorylates TRF4 on serine 35, leading to the dissociation of the SL RNA transcription complex (all the factors that are engaged in SL RNA transcription are listed). This leads to dissociation of the pre-initiation complex from the SL RNA promoter, and spreading of the factors in the nucleus. *Trans*-splicing and protein synthesis are inhibited and PCD is activated. The activation of PCD depends on PK3 signaling, and autophagy. (see colour version of this figure at www.informahealthcare.com/bmg).

surface, DNA laddering, chromatin condensation, increased ROS and cytoplasmic Ca^{2+}, and decreased mitochondrial membrane potential (Goldshmidt *et al.*, 2010).

The mechanism of SLS induction

One of the most intriguing questions is how the signal is transmitted from the trypanosome ER to the nucleus to induce changes in the SL RNA transcription complex. To explore the mechanism of SLS and to determine why SL RNA transcription is abolished during SLS, the SL RNA transcription complex was purified using TAP-tagged TRF4, and analyzed by mass-spectrometry. It was found that under SLS induced by *SEC63* silencing, TRF4 undergoes phosphorylation on Serine 35. In addition, a kinase that we termed PK3 and that was annotated previously as TbeIF2K3 co-purified with the SL RNA transcription complex only under SLS. By chromatin immunoprecipitation (ChIP) assay, we demonstrated that under *SEC63* silencing, TRF4 detaches from the SL RNA promoter. In contrast, a serine to glutamate mutant (YFP-TRF4^{S35Q}) remains at the SL RNA transcription site under *SEC63* silencing, suggesting that phosphorylation on this serine is uniquely responsible for the detachment of the SL RNA transcription complex from the promoter under SLS. The PK3 was localized to the ER membrane, but under *SEC63* silencing, the protein is auto-phosphorylated and migrates to the nucleus, where it phosphorylates the TRF4.

PK3 silencing abolished the phosphorylation on TRF4 and perturbed the induction of SLS, as no decrease in SL RNA was observed and the TRF4 remained in the SL RNA transcription site in cell co-silenced for *SEC63* and *PK3*. *PK3* silencing also compromised the PCD induction in *SEC63* silenced cells as evident by the lack of phosphatidyl serine exposure and the absence of the sub-G1 population that is typical of cells dying following *SEC63* silencing. Thus, this study showed that the PK3 kinase transmits the ER stress signal to the nucleus, and provided strong evidence that TRF4 phosphorylation is the main target of this response, leading to disassembly of the RNA pol II transcription pre-initiation complex and cessation of SL RNA gene transcription. In addition, since PK3 activation triggers PCD, we believe that this finding identifies a novel factor involved in *T. brucei* PCD (Figure 3).

SLS and programmed cells death in trypanosomatids

Several publications in the last decade reported that unicellular parasite, such as *Leishmania* and *Trypanosoma* sp., can undergo cell death and possess features that are typical of mammalian apoptosis (Jimenez-Ruiz *et al.*, 2010). Recently, however, this concept was challenged and it was suggested that trypanosomatids cell death might be incidental or considered as unregulated necrosis (Proto *et al.*, 2013). Cell death takes place naturally during the life cycle of *T. brucei*.

In the bloodstream, cells of the *T. brucei* slender form transform into a non-proliferating stumpy from through a quorum sensing mechanism involving the production of stumpy induction factor (SIF) (Vassella *et al.*, 1997). It was suggested that this mechanism on its own is sufficient to achieve sustained infection and efficient transmission (MacGregor *et al.*, 2012). Prostaglandin PGD_2 released from the stumpy form was shown to induce PCD of other stumpy forms thereby avoiding the overpopulation of these parasites (Figarella *et al.*, 2005). However, the sensitivity of *T. brucei* to PGD_2 *in vivo* and the molecular mechanism for the induction is unknown (Proto *et al.*, 2013). Trypanosomes lack caspases that are known execute apoptosis in metazoa (Proto *et al.*, 2013). The trypanosomatid genome encodes several metacaspases, which were shown in plants to have role in PCD (Coll *et al.*, 2010). However, metacaspases do not function in cell death in either *T. brucei* (Helms *et al.*, 2006) nor in *Leishmania* (Castanys-Munoz *et al.*, 2012). It was demonstrated that in *Leishmania* the MCA metacaspase is a negative regulator of amastigote proliferation (Castanys-Munoz *et al.*, 2012).

Cathepsin B proteases were found to be released from lysosomes and were implicated in regulating apoptosis in metazoa, but this is not the case in *Leishmania* (El-Fadili *et al.*, 2011). Of special interest are the calpain-like proteases revealed as factors that function in the signaling of SLS. The protein was up-regulated under *SEC63* silencing and its co-silencing together with *SEC63* abolished SLS (unpublished results). Calpain was shown to function in apoptosis in metazoa (Momeni, 2011).

The recent review mentioned above was skeptical of the existence of apoptosis in trypanosomes claiming that most of the reports of apoptosis in trypanosomatids did not describe the mechanism and the machinery that elicits the death (Proto *et al.*, 2013). However, by co-silencing of *PK3/SEC63* it was possible to uncouple the ER stress from the death signaling. These double-silenced cells maintained the ER stress, as evident by severe defects in processing of the surface GPI-anchored protein, EP; yet, failed to induce SLS. Thus, SLS-induced death is not due to the ER stress *per se* but rather due signaling induced under SLS.

Why has SLS evolved in *T. brucei* as a PCD pathway? It was demonstrated that SLS accelerates the cell death, rapidly eliminating unfit organisms from the population. The apoptotic cell death of SLS-induced cells is a controlled mechanism of cell elimination without liberation of harmful enzymes, such as lysosomal hydrolases, or even cell components that are released from dying cells that can induce inflammation in the host. The altruistic death of the sub-population of these cells is a beneficial strategy of the parasite to quickly eliminate unfit individuals, without damaging the entire population, thereby increasing the chances of survival within the host (Michaeli, 2012). Unlike the cell death of the stumpy form, which is specific to the bloodstream form of the parasites (Figarella *et al.*, 2005), SLS exists in both PCF and BSF (Goldshmidt *et al.*, 2010). SLS activation represents a point of no return.

Small molecules that can activate PK3 can lead the parasite to commit suicide, and thus could be excellent drugs to fight the devastating diseases caused by these organisms.

Conclusions and perspectives

The UPR response, which was first described in *S. cerevisae* and later in mammalian cells, was subsequently identified in many other eukaryotes from flies to worms, as well as in unicellular eukaryotes. In all organisms except trypanosomes, the mechanism involves transcriptional regulation based on the non-conventional splicing of the yeast *Hac1* or the mammalian XBP1, which is spliced by IRE1 following its sensing of the unfolded protein in the ER, resulting in transcriptional activation of chaperones and other ER functions that are crucial for coping with the stress. However, this mechanism is not found in all eukaryotes. An increase in the level of chaperones can also be achieved through alternative pathways, such as a Hac1-independent one, as exemplified in *S. pombe*, where stabilization of the BiP mRNAs is mediated *via* RIDD (Kimmig *et al.*, 2012). In addition, in *Cryptococcus*, the induction of the functions essential for UPR is not only Ire-dependent, and Ire1 may function also in RIDD (Cheon *et al.*, 2011). In trypanosomes, the UPR is not dependent on a specific endonuclease. mRNAs essential for coping with the ER stress are stabilized by an as yet unidentified RBP. Trypanosomes may have a dedicated degradation machinery to eliminate mRNAs encoding membrane and secreted proteins, which might be orchestrated by the interaction of these specific mRNAs with the basal degradation machinery *via* a specific RBP, possibly analogous to the role of *Cryptococcus* Rbp4 that controls stress-regulated mRNAs (Cheon *et al.*, 2011).

Studies in *T. brucei* support the notion that persistent ER stress induces a unique pathway, that of SLS, eventually leading to PCD, similar to apoptosis, which takes place in metazoans under continuous ER stress. SLS-induced cell death is not due only to ER stress *per se*, but also in response to the signaling elicited by PK3. In cells induced for SLS but lacking PK3, the cells die by un-regulated necrosis, rather than by PCD, providing evidence that SLS is indeed a PCD response. The main open question is the identity of the factors that execute PCD in trypanosomes. An unbiased genetic screen using a *T. brucei* RNAi library should reveal additional factors involved in this pathway. Studies are also in progress to biochemically identify proteins associated with PK3 during SLS. Another open question is how PK3, which localizes to the ER membrane, senses the ER stress. It is *via* direct interaction with the translocon and if so with which of the factors.

In sum, the UPR mechanism and machinery seem to differ between pathogen and the host, suggesting these pathways as novel therapeutic targets for developing anti-fungal and anti-parasitic drugs.

Acknowledgements

Declaration of interest

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