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A touch of Zen: Post-translational regulation of the *Leishmania* stress response

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3 **Summary.** Across bacterial, archaeal, and eukaryotic kingdoms, heat shock proteins
4 (HSPs) are defined as a class of highly conserved chaperone proteins that are rapidly
5 induced in response to temperature increase through dedicated heat shock
6 transcription factors. While this transcriptional response governs cellular adaptation of
7 fungal, plant, and animal cells to thermic shock and other forms of stress, early
8 branching eukaryotes of the kinetoplastid order, including trypanosomatid parasites,
9 lack classical mechanisms of transcriptional regulation and show largely constitutive
10 expression of HSPs thus raising important questions on the function of HSPs in the
11 absence of stress and the regulation of their chaperone activity in response to
12 environmental adversity. Understanding parasite-specific mechanisms of stress
13 response regulation is especially relevant for protozoan parasites of the genus
14 *Leishmania* that are adapted for survival inside highly toxic phagolysosomes of host
15 macrophages causing the various immuno-pathologies of leishmaniasis. Here we review
16 recent advances on the function and regulation of chaperone activities in these
17 kinetoplastid pathogens and propose a new model for stress response regulation
18 through a reciprocal regulatory relationship between stress kinases and chaperones
19 that may be relevant for parasite adaptive differentiation and infectivity.
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Introduction

Protozoan parasites of the genus *Leishmania* belong to a group of early branching unicellular eukaryotes of the family of Trypanosomatidae that generate important human morbidity and mortality (Alvar *et al.*, 2012). These pathogens share a unique biology that is different from most other eukaryotes likely as a result of evolutionary constraints linked to their parasitic life style. Most *Leishmania* species display two developmental stages, an extracellular promastigote form that develops in the sandfly mid-gut and that transmits the disease, and an intracellular amastigote form that differentiates and multiplies inside the macrophage phagolysosome of the mammalian host causing the pathologies of leishmaniasis (Figure 1). *Leishmania* encounters dramatic environmental changes during the infectious cycle, some of which have been shown to induce parasite differentiation and stage-specific expression of virulence determinants appropriate for survival in both insect and vertebrate hosts (Zilberstein *et al.*, 1994). In particular, after transmission to the host during a sand fly blood meal and uptake by macrophages, parasites are exposed to various forms of stress, including temperature shift from 26°C to 37°C (for visceral species), host cell cytotoxic activities such as reactive oxidants produced during the phagocytic oxidative burst, and low pH as well as digestive enzymes following the maturation of the parasite-harboring phagosome to a fully acidified phagolysosome through vesicular fusion (Sibley, 2011). Acidic pH and high temperature encountered inside the host cells triggers the developmental transition to the amastigote stage, that is adapted for intracellular survival and proliferation in the hypoxic, glucose-deprived and hydrolytic environment of the phagolysosome (Zilberstein, 2008).

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3 Thus, *Leishmania* has evolved mechanisms of environmental sensing that are able
4
5 to distinguish between the physiology of vector and host during the infectious cycle, and
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7 trigger stage differentiation that adapts their biology to extracellular or intracellular
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9 growth. Significantly, the differentiation signals are largely linked to environmental
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11 stress, raising important questions on the regulation of trypanosomatid HSPs and
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13 chaperones by environmental cues, and their dual role in signal transduction and stage
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15 differentiation on one hand, and protein folding and stress resistance on the other hand.
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21 ***Leishmania* stress protein families.** In all organisms, the response to heat shock is
22
23 regulated by dedicated signaling pathways resulting in increased expression of HSPs.
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25 These proteins are highly conserved along the evolutionary tree and show a wide
26
27 distribution throughout various intracellular compartments. HSPs play crucial roles in
28
29 folding, assembly of newly synthesized proteins, and are implicated in controlling
30
31 protein trafficking, activity and degradation. They are classified into five major families
32
33 on the basis of their molecular weight (HSP100, HSP90, HSP70, HSP40 and small HSP
34
35 (sHSP)), which are conserved in trypanosomatids (Requena, 2012)) and have been
36
37 linked by pharmacological and genetic analyses to parasite stage differentiation,
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39 viability, and intracellular survival. In the following, we briefly summarize the major
40
41 conserved *Leishmania* HSPs family members, which have been discussed in more detail
42
43 in a recent review (Hombach, 2015).
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49 *L. major* HSP100 (LmjF29.1270) shows cytoplasmic localization and is one of the
50
51 few parasite proteins that show a significant increase in stage-specific abundance during
52
53 axenic amastigotes differentiation (Hubel *et al.*, 1995). It is expressed in early *L.*
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55 *donovani* axenic stage development suggesting that it could play a role in amastigote
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57 protein synthesis (Krobitsch *et al.*, 1998). While HSP100 null mutant parasites were
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3 viable at both the promastigote and axenic amastigote stages, they failed to establish a
4
5 productive intracellular infection thus revealing an important function of this HSP in
6
7 parasite virulence (Hubel *et al.*, 1997), likely due to its critical role in exosome-
8
9 dependent immune modulation (Silverman *et al.*, 2010).
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12 The *Leishmania* genomes encode up to 18 HSP90 gene copies (also referred in the
13
14 literature as HSP83, LmjF33.0312 to LmjF33.0365, (Zilka *et al.*, 2001). HSP90 is
15
16 considered as a regulatory HSP given the fact that its main function is chaperoning client
17
18 proteins implicated in cellular signal transduction and cell cycle control. This ATP-
19
20 dependent protein is the center of chaperone complexes known as 'HSP90 foldosomes'
21
22 that are formed through interaction with other chaperones and co-chaperones
23
24 (including HSP70, HSP40, or stress-induced protein 1 (Sti-1)), and are implicated in
25
26 client protein folding (Rohl *et al.*, 2013). The HSP90 interaction with Sti-1 via a
27
28 conserved tetratricopeptide repeat (TPR) domain, and the presence of stage-specific
29
30 foldosome complexes in *Leishmania* have been confirmed by immuno-precipitation and
31
32 functional genetics analysis (Hombach *et al.*, 2014) (Morales *et al.*, 2010).
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37 Only little is known about the *Leishmania* HSP70 protein family. Sixteen HSP70
38
39 genes were reported in *Leishmania*. Aside from LmjF01.0640, all other HSP70 members
40
41 were classified according to their sequence identity to known HSP70s from other
42
43 species into cytoplasmic (LmjF28.2770, LmjF28.2780, LmjF28.2820, LmjF26.1240,
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45 LmjF18.1370), mitochondrial (LmjF30.2460, LmjF.30.2470, LmjF.302480,
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47 LmjF.30.2490, LmjF.30.2550), ER (LmjF28.1200, LmjF.35.4710), and nuclear
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49 (LmjF.26.0900) HSP70 (Louw *et al.*, 2010). BLAST searches using LmjF28.2770 as a
50
51 query allowed us to identify two additional putative HSP70 proteins in the *L. major*
52
53 genome database belonging to the NBD sugar kinase HSP70 actin superfamily
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55 (LmjF32.0190, LmjF.29.1240). In addition, LmjF.29.1240 contains a TPR domain
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3 suggesting its possible role as a co-chaperone (Drini *et al.*, in preparation). HSP70
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5 proteins are highly abundant in *Leishmania* promastigotes (Brandau *et al.*, 1995), and
6
7 interact with several co-chaperones, including Sti-1 and SGT (small glutamine-rich
8
9 tetratricopeptide repeat) (Hombach *et al.*, 2013), (Ommen *et al.*, 2010) and –
10
11 presumably – a large number of HSP40 family members (Folgueira *et al.*, 2007).
12
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14 Limited information is available for the *L. major* HSP40 and sHSP protein families.
15
16 The HSP40 family comprises 66 different members identified by BLAST searches using
17
18 published sequences of *T. cruzi* HSP40s (Folgueira *et al.*, 2007). These proteins regulate
19
20 HSP70 ATP hydrolysis and are responsible for substrate specificity (Requena, 2012).
21
22 Members of the sHSP chaperone family, which are characterized by a molecular weight
23
24 ranging from 12 to 43 kDa, are poorly conserved in all organisms. In the *L. major*
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26 genome database, it has been reported that by sequence homology to *S. cerevisiae*, only
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28 one gene has been found corresponding to HSP20 (LmjF29.2450). Recently, an
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30 additional small HSP, HSP23, was characterized and shown to be critical for *L. donovani*
31
32 survival at elevated temperatures, including intracellular persistence in macrophages
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34 (Hombach *et al.*, 2014). More studies are needed for this class of protein in order to
35
36 further elucidate its regulatory role in stress response (Folgueira *et al.*, 2007).
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44 **Post-transcriptional regulation of the *Leishmania* stress response.** Across all major
45
46 eukaryotes, the cellular response to stress is regulated on transcriptional levels through
47
48 *trans*-acting nuclear factors termed Heat Shock Factors (HSFs) that act as master
49
50 regulators of stress-induced gene expression through binding to defined *cis*-acting heat
51
52 shock element (HSE) inside promoters or enhancers of HSP genes (Bjork *et al.*, 2010)
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54 (Figure 2). This canonical regulatory mechanism of the stress response acting on
55
56 transcriptional levels however does not apply to *Leishmania* and related
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3 trypanosomatids. First, in contrast to most eukaryotes, there are no classical
4
5 transcription factors encoded in the *Leishmania* genome and gene expression occurs by
6
7 highly parasite-specific mechanisms involving poly-cistronic transcription (Das *et al.*,
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9 2008) (Daniels *et al.*, 2010), and generation of mature mRNAs through a trans-splicing
10
11 reaction that is linked to polyadenylation (Michaeli, 2011). Second, as a result of this
12
13 unique expression strategy, the entire genome is largely constitutively expressed and
14
15 HSP gene transcription is not enhanced upon exposure to stress in contrast to other
16
17 eukaryotes (Brandau *et al.*, 1995) (Quijada *et al.*, 1997), a phenomenon that is illustrated
18
19 by recent transcriptome analyses of *L. donovani* exposed to different forms of stress
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21 ((Saxena *et al.*, 2007) (Lahav *et al.*, 2011), Fig. S1A).
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26 However, the steady-state level of various HSP transcripts is regulated by post-
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28 transcriptional mechanisms, including RNA stability and translational control. For
29
30 example, the 3'UTR of *Leishmania* and *Trypanosoma brucei* HSP70 confers increased
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32 transcript stability under stress conditions (Lee, 1998) (Quijada *et al.*, 2000), as does the
33
34 3'UTR of *Leishmania* HSP90 that is also required for preferential translation during
35
36 stress (Zilka *et al.*, 2001) (David *et al.*, 2010). This regulation relies on the interaction of
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38 *trans*-acting RNA-binding proteins that recognize *cis*-acting elements (reviewed in
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40 (Kramer *et al.*, 2011)). In *T. brucei* for example, the zinc finger protein ZC3H11 has been
41
42 identified as a master regulator of trypanosome chaperone mRNA stability through its
43
44 binding to a common AUU consensus repeat motif inside the 3'UTR of HSP70 and other
45
46 stress proteins (Droll *et al.*, 2013). However, post-transcriptional regulation may have
47
48 only little or no effect on stress protein abundance. In fact, no change of the steady-state
49
50 level of the major HSPs was observed in *L. donovani* in response to environmental stress,
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52 in contrast to most other proteins that are less efficiently translated ((Brandau *et al.*,
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54 1995) (Rosenzweig *et al.*, 2008) (Morales *et al.*, 2010), and Figure S1B).
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5 **Post-translational regulation of trypanosomatid stress proteins.** Analysis of *L.*
6 *donovani* promastigote and axenic amastigote enriched phosphoprotein fractions by 2D
7 electrophoresis (2DE) identified a surprising number of HSPs and chaperones and
8 revealed a twofold increase in global phosphoprotein abundance in amastigotes
9 (Morales *et al.*, 2008). A subsequent quantitative 2D-DiGE analysis using the same
10 extracts showed statistically significant differences in abundance for 38% of the
11 detected phosphoproteins (corresponding to 318 out of 831 analyzed spots) with a
12 highly significant enrichment of chaperone function in the axenic amastigote
13 phosphoproteome (Morales *et al.*, 2010). These results were confirmed by recent
14 systems-level studies investigating the *L. donovani* stage-specific phosphoproteome by
15 shotgun phosphopeptide analysis and iTRAQ LC-MS/MS ((Tsigankov *et al.*, 2013)
16 (Tsigankov *et al.*, 2014)), Figure 2B). Cluster analysis of phosphorylation site
17 conservation between *L. infantum* and human revealed a series of phosphorylation sites
18 that are unique to certain *Leishmania* chaperones and HSPs despite their high
19 conservation to respective human orthologs, including S13 in the TPR-repeat protein
20 LinJ.27.2340, S14 in the putative DNAJ heat shock protein LinJ.27.2350, or S433 and
21 S604 in the HSP70 family member LinJ.28.2950.

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The coordinated, stress-induced phosphorylation of major *Leishmania* HSPs and
chaperones at parasite-specific sites suggests a model of parasite stress protein
regulation at post-translational levels and attributes potential important regulatory
function to these unique residues. This possibility has been genetically investigated for
the TPR-domain proteins Sti-1 and CyP40. *L. donovani cyp40*^{-/-} null mutants were viable
in culture but failed to establish intracellular infection suggesting important virulence
function of this co-chaperone (Yau *et al.*, 2014). Null mutant analysis of Sti-1

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2
3 demonstrated the essential nature of this co-chaperone for *L. donovani* promastigote
4 and axenic amastigote viability, which was strictly dependent on the presence of two
5 phosphorylation sites at S15 and S481 as demonstrated by 'plasmid shuffle' analysis
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7 (Morales *et al.*, 2010). Sti-1 phosphorylation was correlated to the formation of
8
9 amastigote-specific foldosome complexes containing ribosomal client proteins (Morales
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11 *et al.*, 2010), suggesting that this post-translational modification may alter chaperone
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13 interactions or regulate the dynamic turnover of foldosome complexes. In contrast,
14
15 despite the dramatic increase in phosphorylation of CyP40 in axenic amastigotes on a
16
17 single serine residue at S274, *cyp40*^{-/-} parasites complemented with an S/A phospho-
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19 site mutated form of CyP40 fully restored WT phenotype thus ruling out a role of CyP40
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21 phosphorylation in intracellular parasite survival *in vitro*. These examples illustrates
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23 that the relevance of stage-specific phosphorylation for chaperone function needs to be
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25 experimentally investigated for each single identified phosphorylation site.
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32 Together these reports point to a novel post-translational mechanism of stress
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34 response regulation in *Leishmania*. By analogy to stress regulation through HSFs in
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36 other eukaryotes, the absence of these factors in trypanosomatids may have been
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38 compensated for by the evolution of protein kinases that regulate chaperone function.
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40 Similar to the tethering of HSFs by HSP70 and HSP90 in higher eukaryotes, *Leishmania*
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42 chaperones may regulate the activity of protein kinases that are released and activated
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44 during environmentally induced stage differentiation, which in turn may enhance
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46 chaperone activities by HSP phosphorylation that sustains stress-induced amastigote
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48 differentiation (Figure 2).
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55 **The putative *Leishmania* stress kinome.** The post-translational model of *Leishmania*
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57 stress protein regulation raises the question on the nature of the corresponding protein
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3 kinases that recognize parasite HSPs and chaperones as substrates. In higher
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5 eukaryotes, exposure of cells to stress leads to a major change in gene expression, with
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7 an increase in the transcription of genes implicated in stress response such as those
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9 encoding heat shock proteins. This change is mainly due to activation of transcription
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11 factors by phosphorylation through Stress-Activated Protein Kinases (SAPKs), key
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13 stress-signaling molecules and members of the MAPK family that sense and responds to
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15 extracellular environmental changes and are characterized by a TGY, TGP or TQY motif
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17 inside their activation loop (Duch *et al.*, 2012). Only two of the 15 *Leishmania* MAPKs
18
19 homologs, MPK4 and MPK12 are revealed as SAPK-like kinase by their TQY motif
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21 (Wiese, 2007). There is no published experimental data confirming that MPK12 is a
22
23 SAPK-like kinase, except for its relatively high sequence identity to p38 (44%, (Wiese,
24
25 2007)). In contrast, MPK4 has been first linked to stress sensing using a transgenic
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27 approach that revealed increased phosphorylation and activity of this kinase during
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29 axenic amastigote differentiation (Morales *et al.*, 2007). Subsequent null mutant analysis
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31 established essential functions in parasite viability, which was restored in *mpk4*^{-/-}
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33 parasites expressing a kinase mutant with attenuated activity (Dacher *et al.*, 2014).
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35 However, these mutants showed defects in pH-induced differentiation of procyclic to
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37 metacyclic parasites, which caused attenuation of intracellular survival thus linking
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39 MPK4 to parasite pH sensing and/or tolerance.
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46 Two other *Leishmania* MAP kinases, MPK10 and MPK7 have been linked to
47
48 stress-regulated parasite differentiation and growth. Transgenic expression of MPK10 in
49
50 *L. donovani* revealed a transient increase in kinase activity during the first 48h of axenic
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52 amastigote differentiation, which is regulated by an auto-inhibitory domain that is
53
54 essential for axenic amastigote viability (Cayla *et al.*, 2014). Transgenic expression of
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56 MPK7, on the other hand, presents a defect in intracellular and axenic amastigote
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3 growth likely due to an enhanced endogenous stress response as judged by over-
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5 phosphorylation of the translation initiation factor eIF2 α and substantial reduction in *de*
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7 *novo* protein biosynthesis (Morales *et al.*, 2010, Lahav *et al.*, 2011). eIF2 α is the
8
9 substrate of a stress-responsive kinase called PERK (protein kinase RNA (PKR)-like ER
10
11 kinase (Cloutier *et al.*, 2012)). In *L. infantum*, PERK phosphorylates eIF2 α in response to
12
13 endoplasmic reticulum (ER) stress (Chow *et al.*, 2011) (Cloutier *et al.*, 2012). Impairing
14
15 PERK leads to a defect in intracellular amastigote differentiation (Chow *et al.*, 2011),
16
17 which provides a further link between *Leishmania* stress signaling and parasite
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19 differentiation.
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23 Finally, two other kinases involved in stress response in mammals that are
24
25 conserved and were experimentally investigated in *Leishmania*. In mammals,
26
27 Sphingosine Kinase 1 (ortholog of SKa) is a crucial node in the response pathway to
28
29 oxidative stress (OS) (Van Brocklyn *et al.*, 2012). *L. major* Sphingosine kinase A (SKa)
30
31 has a similar role as it is required for non-replicative stationary phase promastigotes to
32
33 survive at high temperature, acidic pH and OS, as well as for intracellular amastigote
34
35 proliferation (Zhang *et al.*, 2013). TOR (Target Of Rapamycin) kinases are master
36
37 regulators that adapt protein synthesis and cell cycle to environmental conditions
38
39 (Laplante *et al.*, 2009). In *L. major*, Tor3 is essential for intracellular survival likely due
40
41 to its implication in the parasite response to hypo-osmotic stress and glucose starvation
42
43 (Madeira da Silva *et al.*, 2010).
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49 Despite the publication of the TriTryp kinome (Parsons *et al.*, 2005), the
50
51 identification of *Leishmania* stress kinases by bio-informatics prediction is rendered
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53 difficult due to the evolution of parasite-specific aspects in kinase regulation and
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55 functions. Thus discovery of novel stress kinases needs to rely on functional approaches.
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57 One possible approach is represented by mining existing phospho-proteomics data.
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3 Tsingakov et al. recently identified protein kinases that are phosphorylated early during
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5 *L. donovani* axenic amastigote differentiation and thus may be directly involved in
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7 parasite stress sensing and signaling (Tsigankov *et al.*, 2014), including a MAPK kinase
8
9 (LinJ.05.0390), an ortholog of mammalian AKT1 (LinJ.25.2450) (Konishi *et al.*, 1997)
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11 (Sato *et al.*, 2000) (Koren *et al.*, 2010), a Ca²⁺/calmodulin dependent protein kinase
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13 (LinJ.35.1070), and a CK1 (LmjF35.1010); the latter having been shown to be essential
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15 for intracellular survival (Rachidi *et al.*, 2014) and thus being a prime candidate for
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17 future functional analysis.
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23 **Conclusion.** *Leishmania* amastigote differentiation is triggered by stress signals
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25 encountered inside the fully acidified phagolysosome of the host macrophages, including
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27 low pH and high temperature. The emphasis of *Leishmania* stress protein regulation on
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29 post-translational mechanisms, such as phosphorylation, likely represents an
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31 evolutionary adaptation of trypanosomatid parasites to absence of transcriptional
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33 control, as well as to its intracellular life style that depends on stress-induced, adaptive
34
35 differentiation. This possibility is supported by the observations that inhibition of
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37 HSP90 ATPase activity by geldanamycin or inhibition of cyclophilin co-chaperones by
38
39 cyclosporine A causes spontaneous conversion of promastigotes into amastigote-like
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41 forms in the absence of pH and temperature shock (Wiesgigl *et al.*, 2001, Yau *et al.*,
42
43 2010). In contrast, inhibition of stress protein function in axenic amastigotes is lethal,
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45 demonstrating a dual role of *Leishmania* stress proteins in cellular signaling required to
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47 maintain the promastigote differentiation state, and in stress resistance required for
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49 amastigote survival (Yau *et al.*, 2010). In conclusion, *Leishmania* not only evolved
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51 parasite-specific mechanisms of HSP gene regulation, but also parasite-specific HSP
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3 functions linking the activity of stress proteins to stage differentiation and stage-specific
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5 survival (Krobitch *et al.*, 1999, Hombach *et al.*, 2013)).
6
7

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Figure legends

Figure 1: *Overview of the Leishmania life cycle.* Insect stage procyclic promastigotes proliferate in the alimentary tract of Phlebotomine sand flies (vector) and differentiate into the virulent metacyclic promastigote form in response to environmental acidification. During a sand fly blood meal this form is transmitted to vertebrate hosts, where it develops into intracellular amastigotes in response to acidic pH and high temperature encountered inside the fully acidified phagolysosome of host macrophages.

Figure 2: *Model of stress-response regulation in Leishmania at post-translational level by phosphorylation.* Most eukaryotes regulate stress protein abundance on transcriptional levels through dedicated *trans*-acting Heat Shock transcription Factors (HSFs) that are inactivated in the absence of stress by binding to HSP70 and HSP90, but activated under stress conditions thus enhancing stress protein production by binding to *cis*-acting Heat Shock Elements (HSEs). In *Leishmania*, the constitutive high expression of HSPs, the absence of *trans*-acting factors (TFs), and the coordinated phosphorylation of HSPs in response to stress at parasite-specific residues support a model of a reciprocal regulatory mechanism where the activity of Stress Kinases (SK) is regulated by interaction with HSPs, and *vice versa* HSP activities are regulated by phosphorylation through SKs.

Figure S1: Abundance of stress protein transcripts (A), and stress proteins (B), and phospho-peptides (C) during axenic amastigote differentiation (data from (Lahav *et al.*, 2011) and (Tsigankov *et al.*, 2014)).

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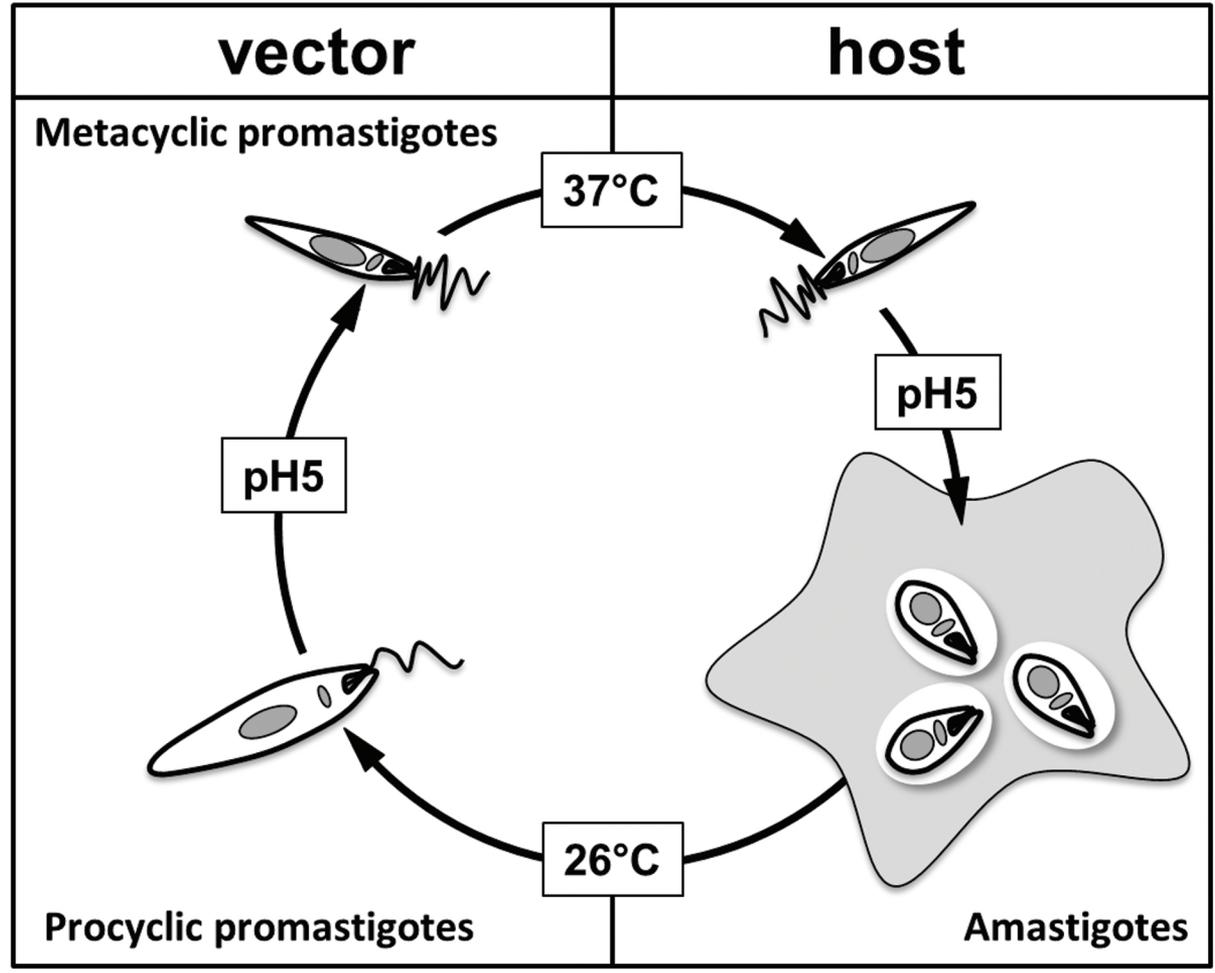
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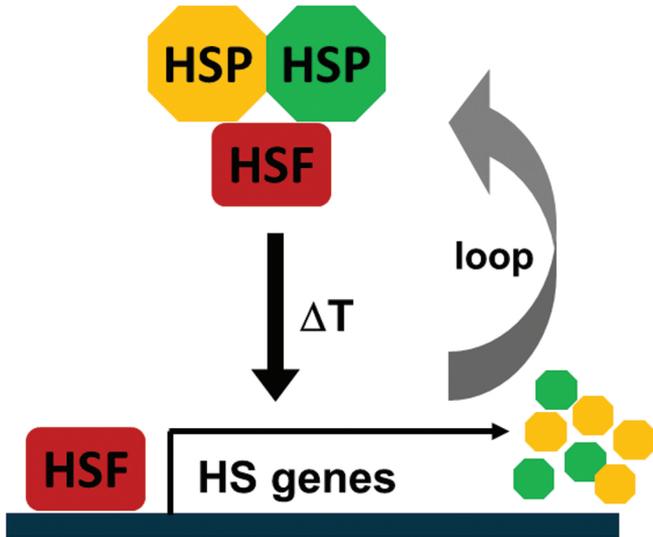
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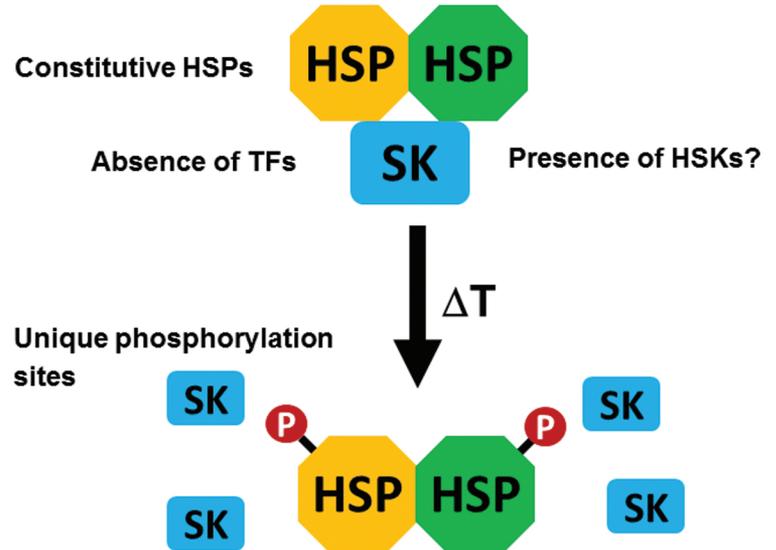
"Classical" eukaryotes



Stress induction of HSP expression

Increased heat tolerance

Leishmania



Stress induction of HSP phosphorylation

Amastigote differentiation

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