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

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).

Thus, *Leishmania* has evolved mechanisms of environmental sensing that are able to distinguish between the physiology of vector and host during the infectious cycle, and trigger stage differentiation that adapts their biology to extracellular or intracellular growth. Significantly, the differentiation signals are largely linked to environmental stress, raising important questions on the regulation of trypanosomatid HSPs and chaperones by environmental cues, and their dual role in signal transduction and stage differentiation on one hand, and protein folding and stress resistance on the other hand.

***Leishmania* stress protein families.** In all organisms, the response to heat shock is regulated by dedicated signaling pathways resulting in increased expression of HSPs. These proteins are highly conserved along the evolutionary tree and show a wide distribution throughout various intracellular compartments. HSPs play crucial roles in folding, assembly of newly synthesized proteins, and are implicated in controlling protein trafficking, activity and degradation. They are classified into five major families on the basis of their molecular weight (HSP100, HSP90, HSP70, HSP40 and small HSP (sHSP)), which are conserved in trypanosomatids (Requena, 2012)) and have been linked by pharmacological and genetic analyses to parasite stage differentiation, viability, and intracellular survival. In the following, we briefly summarize the major conserved *Leishmania* HSPs family members, which have been discussed in more detail in a recent review (Hombach, 2015).

L. major HSP100 (LmjF29.1270) shows cytoplasmic localization and is one of the few parasite proteins that show a significant increase in stage-specific abundance during axenic amastigotes differentiation (Hubel *et al.*, 1995). It is expressed in early *L. donovani* axenic stage development suggesting that it could play a role in amastigote protein synthesis (Krobitsch *et al.*, 1998). While HSP100 null mutant parasites were

viable at both the promastigote and axenic amastigote stages, they failed to establish a productive intracellular infection thus revealing an important function of this HSP in parasite virulence (Hubel *et al.*, 1997), likely due to its critical role in exosome-dependent immune modulation (Silverman *et al.*, 2010).

The *Leishmania* genomes encode up to 18 HSP90 gene copies (also referred in the literature as HSP83, LmjF33.0312 to LmjF33.0365, (Zilka *et al.*, 2001). HSP90 is considered as a regulatory HSP given the fact that its main function is chaperoning client proteins implicated in cellular signal transduction and cell cycle control. This ATP-dependent protein is the center of chaperone complexes known as 'HSP90 foldosomes' that are formed through interaction with other chaperones and co-chaperones (including HSP70, HSP40, or stress-induced protein 1 (Sti-1)), and are implicated in client protein folding (Rohl *et al.*, 2013). The HSP90 interaction with Sti-1 via a conserved tetratricopeptide repeat (TPR) domain, and the presence of stage-specific foldosome complexes in *Leishmania* have been confirmed by immuno-precipitation and functional genetics analysis (Hombach *et al.*, 2014) (Morales *et al.*, 2010).

Only little is known about the *Leishmania* HSP70 protein family. Sixteen HSP70 genes were reported in *Leishmania.* Aside from LmjF01.0640, all other HSP70 members were classified according to their sequence identity to known HSP70s from other species into cytoplasmic (LmjF28.2770, LmjF28.2780, LmjF28.2820, LmjF26.1240, LmjF18.1370), mitochondrial (LmjF30.2460, LmjF.30.2470, LmjF.302480, LmjF.30.2490, LmjF.30.2550), ER (LmjF28.1200, LmjF.35.4710), and nuclear (LmjF.26.0900) HSP70 (Louw *et al.*, 2010). BLAST searches using LmjF28.2770 as a query allowed us to identify two additional putative HSP70 proteins in the *L. major* genome database belonging to the NBD sugar kinase HSP70 actin superfamily (LmjF32.0190, LmjF.29.1240). In addition, LmjF.29.1240 contains a TPR domain

suggesting its possible role as a co-chaperone (Drini *et al.*, in preparation). HSP70 proteins are highly abundant in *Leishmania* promastigotes (Brandau *et al.*, 1995), and interact with several co-chaperones, including Sti-1 and SGT (small glutamine-rich tetratricopeptide repeat) (Hombach *et al.*, 2013), (Ommen *et al.*, 2010) and – presumably – a large number of HSP40 family members (Folgueira *et al.*, 2007).

Limited information is available for the *L. major* HSP40 and sHSP protein families. The HSP40 family comprises 66 different members identified by BLAST searches using published sequences of *T. cruzi* HSP40s (Folgueira *et al.*, 2007). These proteins regulate HSP70 ATP hydrolysis and are responsible for substrate specificity (Requena, 2012). Members of the sHSP chaperone family, which are characterized by a molecular weight ranging from 12 to 43 kDa, are poorly conserved in all organisms. In the *L. major* genome database, it has been reported that by sequence homology to *S. cerevisisae*, only one gene has been found corresponding to HSP20 (LmjF29.2450). Recently, an additional small HSP, HSP23, was characterized and shown to be critical for *L. donovani* survival at elevated temperatures, including intracellular persistence in macrophages (Hombach *et al.*, 2014). More studies are needed for this class of protein in order to further elucidate its regulatory role in stress response (Folgueira *et al.*, 2007).

Post-transcriptional regulation of the *Leishmania* stress response. Across all major eukaryotes, the cellular response to stress is regulated on transcriptional levels through *trans*-acting nuclear factors termed Heat Shock Factors (HSFs) that act as master regulators of stress-induced gene expression through binding to defined *cis*-acting heat shock element (HSE) inside promoters or enhancers of HSP genes (Bjork *et al.*, 2010) (Figure 2). This canonical regulatory mechanism of the stress response acting on transcriptional levels however does not apply to *Leishmania* and related

trypanosomatids. First, in contrast to most eukaryotes, there are no classical transcription factors encoded in the *Leishmania* genome and gene expression occurs by highly parasite-specific mechanisms involving poly-cistronic transcription (Das *et al.*, 2008) (Daniels *et al.*, 2010), and generation of mature mRNAs through a trans-splicing reaction that is linked to polyadenylation (Michaeli, 2011). Second, as a result of this unique expression strategy, the entire genome is largely constitutively expressed and HSP gene transcription is not enhanced upon exposure to stress in contrast to other eukaryotes (Brandau *et al.*, 1995) (Quijada *et al.*, 1997), a phenomenon that is illustrated by recent transcriptome analyses of *L. donovani* exposed to different forms of stress ((Saxena *et al.*, 2007) (Lahav *et al.*, 2011), Fig. S1A).

However, the steady-state level of various HSP transcripts is regulated by post-transcriptional mechanisms, including RNA stability and translational control. For example, the 3'UTR of *Leishmania* and *Trypanosoma brucei* HSP70 confers increased transcript stability under stress conditions (Lee, 1998) (Quijada *et al.*, 2000), as does the 3'UTR of *Leishmania* HSP90 that is also required for preferential translation during stress (Zilka *et al.*, 2001) (David *et al.*, 2010). This regulation relies on the interaction of *trans*-acting RNA-binding proteins that recognize *cis*-acting elements (reviewed in (Kramer *et al.*, 2011)). In *T. brucei* for example, the zinc finger protein ZC3H11 has been identified as a master regulator of trypanosome chaperone mRNA stability through its binding to a common AUU consensus repeat motif inside the 3'UTR of HSP70 and other stress proteins (Droll *et al.*, 2013). However, post-transcriptional regulation may have only little or no effect on stress protein abundance. In fact, no change of the steady-state level of the major HSPs was observed in *L. donovani* in response to environmental stress, in contrast to most other proteins that are less efficiently translated ((Brandau *et al.*, 1995) (Rosenzweig *et al.*, 2008) (Morales *et al.*, 2010), and Figure S1B).

Post-translational regulation of trypanosomatid stress proteins. Analysis of *L. donovani* promastigote and axenic amastigote enriched phosphoprotein fractions by 2D electrophoresis (2DE) identified a surprising number of HSPs and chaperones and revealed a twofold increase in global phosphoprotein abundance in amastigotes (Morales *et al.*, 2008). A subsequent quantitative 2D-DiGE analysis using the same extracts showed statistically significant differences in abundance for 38% of the detected phosphoproteins (corresponding to 318 out of 831 analyzed spots) with a highly significant enrichment of chaperone function in the axenic amastigote phosphoproteome (Morales *et al.*, 2010). These results were confirmed by recent systems-level studies investigating the *L. donovani* stage-specific phosphoproteome by shotgun phosphopeptide analysis and iTRAQ LC-MS/MS ((Tsigankov *et al.*, 2013) (Tsigankov *et al.*, 2014)), Figure 2B). Cluster analysis of phosphorylation site conservation between *L. infantum* and human revealed a series of phosphorylation sites that are unique to certain *Leishmania* chaperones and HSPs despite their high conservation to respective human orthologs, including S13 in the TPR-repeat protein LinJ.27.2340, S14 in the putative DNAJ heat shock protein LinJ.27.2350, or S433 and S604 in the HSP70 family member LinJ.28.2950.

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

demonstrated the essential nature of this co-chaperone for *L. donovani* promastigote and axenic amastigote viability, which was strictly dependent on the presence of two phosphorylation sites at S15 and S481 as demonstrated by 'plasmid shuffle' analysis (Morales *et al.*, 2010). Sti-1 phosphorylation was correlated to the formation of amastigote-specific foldosome complexes containing ribosomal client proteins (Morales *et al.*, 2010), suggesting that this post-translational modification may alter chaperone interactions or regulate the dynamic turnover of foldosome complexes. In contrast, despite the dramatic increase in phosphorylation of CyP40 in axenic amastigotes on a single serine residue at S274, *cyp40-/-* parasites complemented with an S/A phospho-site mutated form of CyP40 fully restored WT phenotype thus ruling out a role of CyP40 phosphorylation in intracellular parasite survival *in vitro*. These examples illustrates that the relevance of stage-specific phosphorylation for chaperone function needs to be experimentally investigated for each single identified phosphorylation site.

Together these reports point to a novel post-translational mechanism of stress response regulation in *Leishmania.* By analogy to stress regulation through HSFs in other eukaryotes, the absence of these factors in trypanosomatids may have been compensated for by the evolution of protein kinases that regulate chaperone function. Similar to the tethering of HSFs by HSP70 and HSP90 in higher eukaryotes, *Leishmania* chaperones may regulate the activity of protein kinases that are released and activated during environmentally induced stage differentiation, which in turn may enhance chaperone activities by HSP phosphorylation that sustains stress-induced amastigote differentiation (Figure 2).

The putative *Leishmania* stress kinome. The post-translational model of *Leishmania* stress protein regulation raises the question on the nature of the corresponding protein

kinases that recognize parasite HSPs and chaperones as substrates. In higher eukaryotes, exposure of cells to stress leads to a major change in gene expression, with an increase in the transcription of genes implicated in stress response such as those encoding heat shock proteins. This change is mainly due to activation of transcription factors by phosphorylation through Stress-Activated Protein Kinases (SAPKs), key stress-signaling molecules and members of the MAPK family that sense and responds to extracellular environmental changes and are characterized by a TGY, TGP or TQY motif inside their activation loop (Duch *et al.*, 2012). Only two of the 15 *Leishmania* MAPKs homologs, MPK4 and MPK12 are revealed as SAPK-like kinase by their TQY motif (Wiese, 2007). There is no published experimental data confirming that MPK12 is a SAPK-like kinase, except for its relatively high sequence identity to p38 (44%, (Wiese, 2007)). In contrast, MPK4 has been first linked to stress sensing using a transgenic approach that revealed increased phosphorylation and activity of this kinase during axenic amastigote differentiation (Morales *et al.*, 2007). Subsequent null mutant analysis established essential functions in parasite viability, which was restored in *mpk4-/-* parasites expressing a kinase mutant with attenuated activity (Dacher *et al.*, 2014). However, these mutants showed defects in pH-induced differentiation of procyclic to metacyclic parasites, which caused attenuation of intracellular survival thus linking MPK4 to parasite pH sensing and/or tolerance.

Two other *Leishmania* MAP kinases, MPK10 and MPK7 have been linked to stress-regulated parasite differentiation and growth. Transgenic expression of MPK10 in *L. donovani* revealed a transient increase in kinase acivity during the fist 48h of axenic amastigote differentiation, which is regulated by an auto-inhibitory domain that is essential for axenic amastigote viability (Cayla *et al.*, 2014). Transgenic expression of MPK7, on the other hand, presents a defect in intracellular and axenic amastigote

growth likely due to an enhanced endogenous stress response as judged by over-phosphorylation of the translation initiation factor eIF2α and substantial reduction in *de novo* protein biosynthesis (Morales *et al.*, 2010, Lahav *et al.*, 2011). eIF2α is the substrate of a stress-responsive kinase called PERK (protein kinase RNA (PKR)-like ER kinase (Cloutier *et al.*, 2012)). In *L. infantum*, PERK phosphorylates eIF2α in response to endoplasmic reticulum (ER) stress (Chow *et al.*, 2011) (Cloutier *et al.*, 2012). Impairing PERK leads to a defect in intracellular amastigote differentiation (Chow *et al.*, 2011), which provides a further link between *Leishmania* stress signaling and parasite differentiation.

Finally, two other kinases involved in stress response in mammals that are conserved and were experimentally investigated in *Leishmania*. In mammals, Sphingosine Kinase 1 (ortholog of SKa) is a crucial node in the response pathway to oxidative stress (OS) (Van Brocklyn *et al.*, 2012). *L. major* Sphingosine kinase A (SKa) has a similar role as it is required for non-replicative stationary phase promastigotes to survive at high temperature, acidic pH and OS, as well as for intracellular amastigote proliferation (Zhang *et al.*, 2013). TOR (Target Of Rapamycin) kinases are master regulators that adapt protein synthesis and cell cycle to environmental conditions (Laplante *et al.*, 2009). In *L. major*, Tor3 is essential for intracellular survival likely due to its implication in the parasite response to hypo-osmotic stress and glucose starvation (Madeira da Silva *et al.*, 2010).

Despite the publication of the TriTryp kinome (Parsons *et al.*, 2005), the identification of *Leishmania* stress kinases by bio-informatics prediction is rendered difficult due to the evolution of parasite-specific aspects in kinase regulation and functions. Thus discovery of novel stress kinases needs to rely on functional approaches. One possible approach is represented by mining existing phospho-proteomics data.

Tsingakov et al. recently identified protein kinases that are phosphorylated early during *L. donovani* axenic amastigote differentiation and thus may be directly involved in parasite stress sensing and signaling (Tsigankov *et al.*, 2014), including a MAPK kinase (LinJ.05.0390), an ortholog of mammalian AKT1 (LinJ.25.2450) (Konishi *et al.*, 1997) (Sato *et al.*, 2000) (Koren *et al.*, 2010), a Ca2+/calmodulin dependent protein kinase (LinJ.35.1070), and a CK1 (LmjF35.1010); the latter having been shown to be essential for intracellular survival (Rachidi *et al.*, 2014) and thus being a prime candidate for future functional analysis.

Conclusion. *Leishmania* amastigote differentiation is triggered by stress signals encountered inside the fully acidified phagolysosome of the host macrophages, including low pH and high temperature. The emphasis of *Leishmania* stress protein regulation on post-translational mechanisms, such as phosphorylation, likely represents an evolutionary adaptation of trypanosomatid parasites to absence of transcriptional control, as well as to its intracellular life style that depends on stress-induced, adaptive differentiation. This possibility is supported by the observations that inhibition of HSP90 ATPase activity by geldanamycin or inhibition of cyclophilin co-chaperones by cyclosporine A causes spontaneous conversion of promastigotes into amastigote-like forms in the absence of pH and temperature shock (Wiesgigl *et al.*, 2001, Yau *et al.*, 2010). In contrast, inhibition of stress protein function in axenic amastigotes is lethal, demonstrating a dual role of *Leishmania* stress proteins in cellular signaling required to maintain the promastigote differentiation state, and in stress resistance required for amastigote survival (Yau *et al.*, 2010). In conclusion, *Leishmania* not only evolved parasite-specific mechanisms of HSP gene regulation, but also parasite-specific HSP

functions linking the activity of stress proteins to stage differentiation and stage-specific survival (Krobitsch *et al.*, 1999, Hombach *et al.*, 2013)).

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Figure legends

Figure 1: *Overview of the* Leishmania *life cycle.* Insect stage procyclic promastigotes proliferate in the alimentary tract of Phelobotomine 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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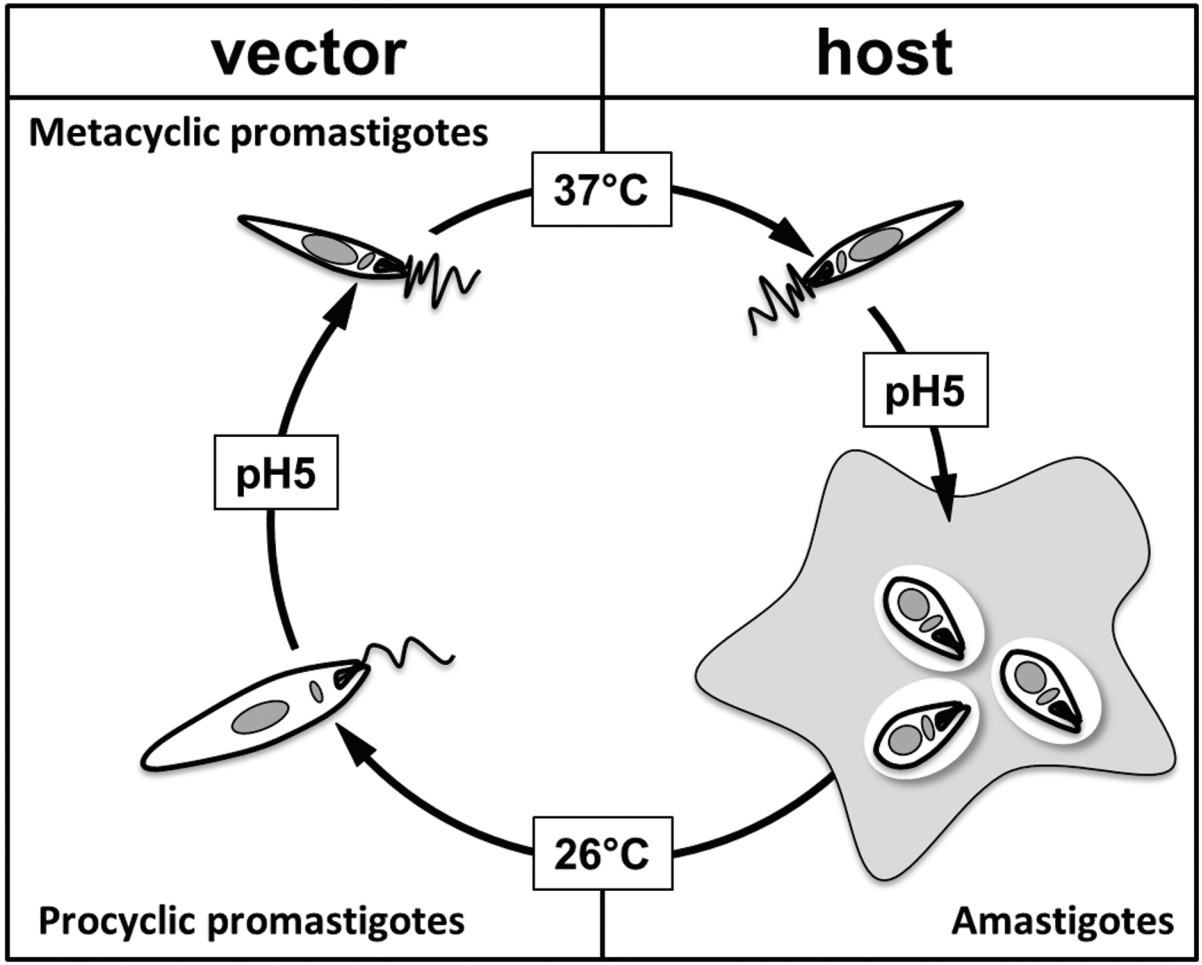
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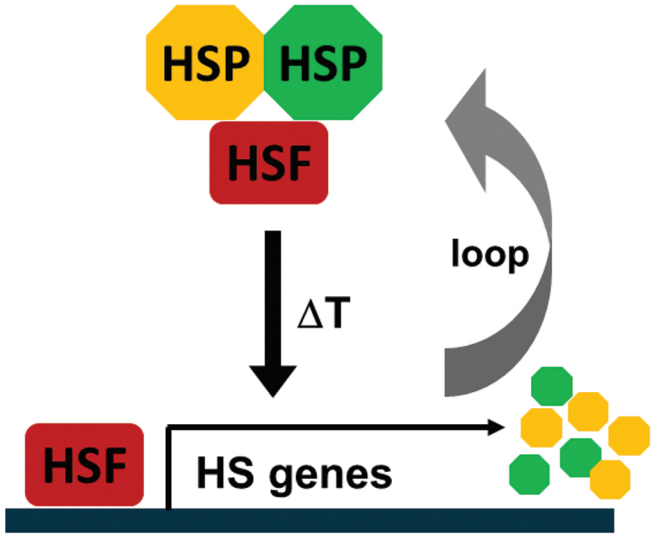
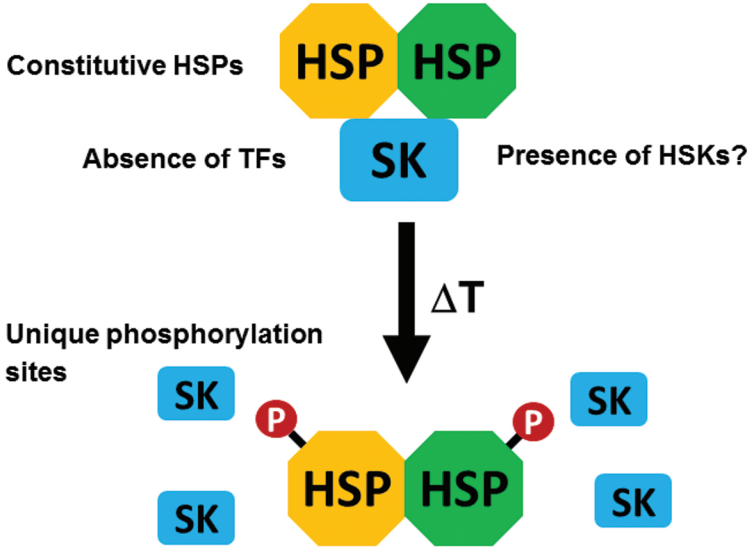


Stress induction of HSP expression

Increased heat tolerance

Leishmania



Stress induction of HSP phosphorylation

Amastigote differentiation