Protozoan HSP90-heterocomplex: molecular interaction network and biological significance

Running title: Protozoan Hsp90 heterocomplex

Figueras MJ[1], Echeverria PC[2], Angel SO[1,*]

[1]*Laboratorio de Parasitología Molecular, IIB-INTECH, CONICET-UNSAM, Av. Intendente Marino Km. 8.2, C.C 164, (B7130IIWA), Chascomús, Prov. Buenos Aires, Argentina;* [2]*Département de Biologie Cellulaire Université de Genève Sciences III, Geneva, Switzerland.*

*Address correspondence to this author at the Laboratorio de Parasitología Molecular, IIB-INTECH, Av. Intendente Marino Km. 8.2, C.C. 164, (B7130IIWA), Chascomús, Prov. Buenos Aires, Argentina; Tel: +542241430323; Fax: +542241424048; E-mail: sangel@intech.gov.ar

Abstract: The HSP90 chaperone is a highly conserved protein from bacteria to higher eukaryotes. In eukaryotes, this chaperone participates in different large complexes, such as the HSP90 heterocomplex, which has important biological roles in cell homeostasis and differentiation. The HSP90-heterocomplex is also named the HSP90/HSP70 cycle because different co-chaperones (HIP, HSP40, HOP, p23, AHA1, immunophilins, PP5) participate in this complex by assembling sequentially, from the early to the mature complex. In this review, we analyze the conservation and relevance of HSP90 and the HSP90-heterocomplex in several protozoan parasites, with emphasis in *Plasmodium spp.*, *Toxoplasma spp*., *Leishmania* spp. and *Trypanosoma* spp. In the last years, there has been an outburst of studies based on yeast two-hybrid methodology, co-immunoprecipitation-mass spectrometry and bioinformatics, which have generated a most comprehensive protein-protein interaction (PPI) network of HSP90 and its co-chaperones. This review analyzes the existing PPI networks of HSP90 and its co-chaperones of some protozoan parasites and discusses the usefulness of these powerful tools to analyze the biological role of the HSP90-heterocomplex in these parasites. The generation of a *T. gondii* HSP90 heterocomplex PPI network based on experimental data and a recent *Plasmodium* HSP90 heterocomplex PPI network are also included and discussed. As an example, the putative implication of nuclear transport and chromatin (histones and Sir2) as HSP90-heterocomplex interactors is here discussed.

Keywords: co-chaperones; HSP90; *Plasmodium*; protein-protein interactors; network; *Toxoplasma*; trypanosomatids.

1. Introduction

Heat Shock Proteins (HSPs) are highly conserved and widely distributed chaperones, which are divided into subcategories based on molecular weight ranges. Two major classes of HSPs are the 70-kDa HSP70 (70-kDa) family and the HSP90 family (80-100 kDa) [1]. They are abundant in eukaryotic cells and are highly conserved within the three main phylogenetic domains (Bacteria, Archea and Eukarya), which suggests that these HSPs and other molecular chaperones have an important role. Their expression increases when the cell is subjected to stress conditions: increased temperatures, osmotic shock, differences in the pH, UV irradiation, starvation conditions, viral infections, bleedings or fever [2]. For this reason, their role within the cell is thought to help in the response to stress factors, being responsible for what is known as heat shock response or stress response. However, these HSPs are also expressed in high quantities under normal conditions, participating in many cellular processes such as protein folding, unfolding and binding ability [2-3]. Their functions have been implicated in diverse processes including the recovery of misfolded proteins, protein maturation, intracellular transport of proteins, and the regulation of activities of nuclear hormone receptors and other transcription factors, as well as other protein kinases involved in signal transduction and translation control, cell differentiation and development [4-6]. Furthermore, HSP90 has been recently implicated in protein evolution [7].

The conservation of heat shock response in protozoan cells is highly reasonable, since protozoan parasites such as *Leishmania* spp., *Plasmodium* spp. and *Trypanosoma* spp. require a rapid adaptation to changes in temperature associated with transmission from poikilothermic insect vectors to homeothermic mammalian hosts [8]. Other protozoan parasites like *Toxoplasma gondii*, which have a complex life cycle, must also have a sophisticated heat shock response to face different environments and host cell defenses [9]. In addition, protozoan parasites conserve almost all of the molecular chaperone machinery associated with development and pathogenesis recently reviewed by Shonhai et al. [10]. In this review, we focused our analysis in the protozoan HSP90-heterocomplex, also named HSP90/HSP70 cycle and its protein-protein interactors.

2. HSP90 is a highly conserved chaperone with a similar domain organization among organisms

The eukaryotic HSP90 structure is separated into three modules: the N-terminal, the middle and the C-terminal domains. The N-terminal domain, called the ATP binding domain, is connected to the middle client protein binding domain through a "linker" region of variable length [11]. Together, these two domains form a "split" ATPase site, where several chemotherapeutic agents, such as the benzoquinone ansamycin drug geldanamycin (GA) and radicicol (RAD), bind and inhibit the specific chaperone function of this HSP [11-12]. The C-terminal domain is the dimerization domain, which also provides the binding site for several co-chaperone molecules that function with HSP90 as part of a dynamic multi-chaperone complex [4].

In the eukaryotic HSP90, since the linker is longer than the bacterial HSP90 and enriched in charged amino acids, it is named charged linker [11]. *P. falciparum* HSP90 has been shown to have a charged-linker longer than that of human or yeast HSP90, composed of 95, 63 and 56 residues, respectively [13]. The replacement of the human charged linker by the *P. falciparum* version reduces ATPase activity and affects client protein binding [13], thus indicating that this linker could have important functions in giving some specificity to the activity of HSP90 of different species. The analysis of the charged linker of the different protozoan HSP90s other than PfHSP90 has shown that they range from 49 to 65 residues, near the human and yeast versions Fig. (1), indicating that only *Plasmodium* HSP90 seems to have this unique feature. PfHSP90 binds and hydrolyzes ATP more efficiently than human HSP90 [14]. Moreover, the HSP90 inhibitor GA binds to purified PfHSP90 with higher affinity than to human HSP90 [14]. Even though the biological explanation of such particularity is still unclear, this unique feature could be exploited as a drug target [14].

3. *Plasmodium* HSP90

The *P. falciparum* genome encodes three full-length HSP90 genes: HSP90, GRP94 and TRAP1 [15]. In addition, an uncharacterized incomplete Open Reading Frame (ORF) for HSP90 is present [15]. The *P. falciparum* HSP90 (PfHSP90) has 64% of sequence identity with the cytosolic human HSP90. The most conserved domain is the N-terminal ATP-binding domain, with 75% of sequence identity to the N-terminal domain of the human HSP90 [16]. PfHSP90 also contains the EEVD motif in the C-terminal domain –a characteristic hallmark of cytosolic chaperones [16]. As mentioned above, the least conserved

region between PfHSP90 and the human HSP90 is the charged linker region adjacent to the N-terminal ATP-binding pocket [15].

In the mature red blood cell, *P. falciparum* has three stages of development: the ring stage, the trophozoite stage and the schizont stage. PfHSP90 is known to be expressed in all three stages of the parasite life cycle [17]. Inhibition of PfHSP90 function using GA arrests parasite development from the ring stage to the trophozoite stage during the intra-erythrocytic cycle, suggesting that PfHSP90 may play a critical role in this process [16, 18].

PfHSP90 is induced and translocates to the nucleus after heat stress at 41^{o}C [16]. The parasite undergoes repeated episodes of heat shock upon infection: first, when it passes from the female *Anopheles* mosquito (25^{o}C) to humans, and second, when the repeated febrile episodes occur in the patient. When subjected to heat shock at 41◦C and then allowed to recover at 37◦C for 10 h, mimicking febrile episodes, ring-infected erythrocytes show accelerated development from the ring to the trophozoite stage upon receiving a second heat shock as compared to control cultures that have not been subjected to the first heat shock [18]. This effect has been found to be GA-sensitive, suggesting that among the other roles that PfHSP90 might have in the parasite, an important function of this molecular chaperone is as a sensor of environmental conditions such as increased temperature during fever [15, 18].

4. *Toxoplasma* HSP90

Toxoplasma gondii presents four putative proteins with HSP90 signature. One of them is located at the cytosol/nuclei (here TgHSP90) [19]. Another *T. gondii* HSP90, which presents a signal peptide and an HDEL motif, both features of the endoplasmic reticulum HSP90 (GRP96) [20], has been co-immunoprecipitated with parasite histones [21]. The other two proteins with HSP90 signature are the mitochondrial and mitochondrial/chloroplast putative HSP90s [20].

The life cycle of *T. gondii* is very complex, having to alternate between the definitive host, which belongs to the cat family (*Felidae*) [22], and the intermediate host, which could be any warm blooded animal, including birds. The infection is caused by the ingestion of oocysts scattered around in the environment or cysts present in animal tissues. The infection of the intermediate host, including humans, leads to the asexual cycle, which is characterized by two stages, the rapidly growing tachyzoites, and the more slowly dividing bradyzoite contained in tissue cysts. Tachyzoites are responsible for acute

illness and congenital birth defects. By contrast, the bradyzoite form can remain as a latent cyst (especially in neural and muscular tissues) for many years and still be capable of converting into the destructive tachyzoite form if the host immunity wanes [23].

The expression level of TgHSP90 protein increases under stress treatment [19]. By serial analysis of gene expression (SAGE), Radke et al. [24] showed that TgHSP90 mRNA also increases within the first 24 h of bradyzoite development, suggesting that HSP90 mRNA may be an early bradyzoite marker. Taking into account that stressors can trigger parasite differentiation [25], these data link TgHSP90 with parasite development. Interestingly, the subcellular localization of TgHSP90 is also developmentally regulated, being present only in the cytoplasm of tachyzoites and occupying both the nucleus and cytoplasm of bradyzoites [19]. The nuclear localization of TgHSP90 in bradyzoites has been confirmed both in bradyzoites obtained from brain cysts of chronically infected mice and in bradyzoites cultivated in monolayers of host cells [19]. GA treatment of these bradyzoites delays their conversion to tachyzoites, but once the bradyzoite become fully developed tachyzoites, the localization of TgHSP90 changes and is excluded from the nucleus [19].

TgHSP90 is also involved in invasion since GA treatment in extracellular tachyzoites prevents parasite host cell penetration [26]. Ahn et al. [26] also detected TgHSP90 in the extracellular environment, a finding also observed for the *Eimeria tenella* HSP90 chaperone [27]. The interaction between TgHSP90 and some proteins of parasite surface, micronemal or rhoptry organelles, which are involved in invasion [20], should also be taken into account to explain the blocking of invasion due to GA.

5. *Leishmania* and *Trypanosoma* HSP90

Protozoan parasites of the genus *Leishmania* are transmitted by blood-sand feeding sand flies to mammalian hosts, including humans, where they encounter a change in the ambient temperature. Subjecting the promastigotes of various *Leishmania* species to a similar temperature up shift *in vitro* results in the up-regulation of both HSP90 (also known as HSP83) and HSP70 and in a persistent induction of HSP100 [28-30]. In *Leishmania*, HSP90 (HSP83) is not only one of the most abundant proteins, found in the soluble fraction of the cytoplasm and constituting among 2.8% of the cellular

proteins, but is also involved in a variety of cellular processes [29]. In *Leishmania*, as well as in *Trypanosoma spp*., HSP90 is encoded by multiple gene copies organized in a head-to-tail tandem array [29, 31-32]. The number of genes ranges from 6 in *Trypanosoma cruzi* to 17 in *Leishmania major* [33-34]. Apparently, multiple copies of HSP90 would allow the parasite to reach a high synthesis level of the proteins in an organism that relies on post-transcriptional regulation [35]. Furthermore, other two isoforms of HSP90 are also found in *Leishmania* genome. One isoform is a glucose regulated protein 94kDa (GRP94), that localizes to the ER [36-37]. In *Leishmania*, LPG is associated to parasite virulence [36, 38-40]. Since the lack of GRP94 expression has not shown to affect parasite viability, this chaperone was associated to parasite virulence [37]. The other isoform is a single mithocondrial tumor necrosis factor receptor-associated protein 1 (TRAP1) [10]. *T. cruzi* genome also encodes for other HSP90 isoforms: rhree genes have been identified encoding for Grp94 and two genes for TRAP1 [10].

The protozoan parasite *Leishmania donovani* is the main causative agent of visceral leishmaniasis. Its life cycle is characterized by the presence of a proliferative flagellated promastigote stage in the sandfly and a non-motile amastigote stage within a mammalian host [41-42]. The infected sandfly transmits mature, so-called metacyclic promastigotes into the site of the bite of humans or some other mammal, which are phagocytized by antigen presenting cells (APCs) in the skin and transformed into amastigotes within the mature phagolysosomes, spreading around through the lymphatic system [43]. *L. donovani* parasites infect APCs in all visceral organs and cause a debilitating and ultimately lethal disease called Kala Azar or visceral leishmaniasis [43].

HSP90 has been shown to be involved in the control of the cellular differentiation of the protozoan parasite *L. donovani*. Whereas the promastigote can be grown at 25^{o}C and pH 7, the morphological differentiation from promastigote to amastigote stage could be induced by heat shock and media acidification (grown at 37^{o}C, pH 5.5; 37^{o}C and pH 7). Interestingly, this morphological change can be mimicked by growing promastigotes at 25^{o}C, pH7 and blocking the HSP90 function with GA [44-45]. To summarize, disturbances of Hsp90 homeostasis may be considered as a signal to begin differentiation [45]. According to another study, after treatment with GA at higher temperatures, promastigotes exhibit apoptotic morphological changes but not stage differentiation, and the effect is displayed in a dose- and time-dependent manner. In parallel, the populations show a significant increase at the expense of cells in the G0/G1 phase of the cell cycle and a decrease in the S and G2/M phases after GA treatment [46].

182 The protozoan parasite *T. cruzi* is a human pathogen with considerable impact on the health of
183 millions in the Americas [47]. Its life cycle is similar to that of *Leishmania*, being transmitted by the
184 blood-sucking insects of the subfamily *Triatominae* [48]. In *T. cruzi*, the inhibition of HSP90 using GA
185 induces a dose-dependent increase in heat shock protein levels and has been shown to be essential for cell
186 cycle control [49]. In fact, GA-treated epimastigotes and the blood form trypomastigotes have shown a
187 G1 arrest, but, in these cases, the inhibition of HSP90 does not induce parasite differentiation [49]. With
188 these findings, similarly to *Leishmania* HSP90, functional HSP90 is essential for cell division in *T. cruzi*
189 and serves as a feedback inhibitor in the cellular stress response.

190

191 **6. The HSP90/HSP70 cycle is present in protozoa**

192 The function of HSP90 is highly dependent on ATP and on its ATPase activity. HSP90 is also
193 regulated by other proteins, so called co-chaperones. Based on the glucocorticoid receptor (GR) model, a
194 well-studied HSP90 client protein, five proteins involved in the HSP90-heterocomplex cycle have been
195 identified [4]: HSP90, HSP70, HOP (HSP Organization Protein), p23 and HSP40 Fig. (2). In addition,
196 HSP Interacting Protein (HIP), another co-chaperone, has also been detected as part of the HSP90-
197 heterocomplex, becoming an early marker of the HSP90-heterocomplex Fig. (2) [50]. By contrast, the
198 p23 co-chaperone has been detected as a marker of the mature heterocomplex Fig. (2) [51-52]. Another
199 co-chaperone that coordinates the progression of HSP90 chaperone cycle by modulating the ATPase
200 activity of HSP90 is the activator of HSP90 ATPase (Aha1) [53-54]. Besides activating the HSP90
201 ATPase activity, Aha1 has been proposed as a positive regulator of HSP90 chaperone function,
202 whose association with HSP90 is important in driving certain client proteins towards their
203 activated states. Teoricamtne el program alas reenumera. Habria q fijarse si eso altera alguna otra
204 numeración de la letter es de plasmodium!!! Ellos no definen esto que decis!! Sacala pero ojo esta
205 despues.

206 The HSP40 family is classified into type I, II and III [55] depending on their domain/motif
207 organization, and based on findings in *Plasmodium* database, some authors have claimed the existence of
208 type IV as well [56]. Type I and II HSP40s have been associated with the HSP90-heterocomplex [57].

HSP40s type I are represented by human Hdj-2 and yeast Ydj-1, whereas type II are represented by human Hdj1 and yeast Sis1. Sis1 is essential in yeast and in protozoan parasites [58-59].

It is important to mention that other proteins such as the immunophilins (e.g. FBP51, FKBP52 and Cyp40) and Protein Phosphatase 5 (PP5), both containing TPR domains [4], are also involved in the HSP90-heterocomplex cycle. In contrast to Aha1, the immunophilins have a very low stimulatory effect on the ATPase activity of HSP90 but both could act synergistically [60-61]. The immunophilins are in fact peptidyl–prolyl-cis–trans isomerases (PPIases) that are inhibited by immunosuppressive drugs, thus being denominated immunophilins [62-63]. The cytoplasmic dynein forms complexes with HSP90 through immunophilins or PP5, being responsible for the movement towards the nucleus [64-65]. The HSP90-heterocomplexes carrying the client protein can bind one or several immunophilins as well as their homolog, the PP5 protein. PP5 can associate to the HSP90-heterocomplex either in the cytoplasm or the nucleus through its TPR domains [66-67], competing with other immunophilins [68].

Protozoan parasites also present the conserved machinery associated with the HSP90/HSP70 cycle. Almost all protozoan parasites studied present HIP, Ydj1-like, HOP, p23, PP5, FCBP57 and Aha1 (Table 1). It is thus expected that this cycle presents similar behavior and roles in these simple cell units.

7. *Plasmodium falciparum* HSP90/HSP70 cycle

Plasmodium spp. is, together with *T. gondii* and *T. vaginalis*, one of the protozoan parasites that present all of the HSP90-heterocomplex associated co-chaperones described in Table 1. Under normal conditions, PfHSP90 is present in 450-kDa multi-chaperone complexes, as demonstrated by gel filtration analysis of total parasite lysate, co-fractionating and co-immunoprecipitating along with PfHSP70 [16]. Several co-chaperones of HSP90 such as PfPP5, identified by mass spectrometry analysis, have been reported to co-immunoprecipitate with PfHSP90 as well [18, 69]. *P. falciparum* HOP has also been detected as a cytosolic parasite protein interacting with PfHSP90 as part of 400-kDa multicomplex [70-71]. PfHOP has the three TPR domains conserving the most important residues to interact with HSP70 and HSP90 chaperones [71-72]. Recently, the interaction between PfHSP90 and parasite Aha1 has been assessed by the *in vivo* split-ubiquitin assay and the association has been demonstrated *in vitro* by GST pull-down experiments [70]. Similarly, *P. falciparum* p23 and PfHSP90 interaction has been elucidated using recombinant proteins and the GST pull-down approach [72]. As expected, *P. falciparum*

Aha1 and p23 compete for PfHSP90 interaction [70]. The HSP90-heterocomplex is also present in *P. falciparum* and with dynamics and functions similar to those of higher eukaryotes.

Plasmodium falciparum is also sensitive to the immunosuppressive macrolide FK506, suggesting the presence of a FK506-binding protein (FKBP) homolog [73-74]. The first report of an FKBP in this organism was that of Braun et al. [75], who found that it is a cytosolic 35-kDa protein and thus named it PfFKBP35. Monaghan and Bell [74] reported that PfFKBP35 is comprised of a single, N-terminal, FKBP domain and a C-terminal tetratricopeptide repeat (TPR) domain. A PP5 homolog in *P. falciparum* has also been characterized. PfPP5 is a new Ser/Thr protein phosphatase containing three TPRs and a nuclear targeting sequence at its N-terminus [76]. PfPP5 is expressed along the asexual erythrocytic cycle and interacts with PfHSP90 [69].

Plasmodium falciparum presents a large HSP40 family with near 43 members [56]. Among them, a Ydj1/Hdj1-like and a Sis1/Hdj2-like sequence have been identified [56, 77]. The PfYdj1-like protein is a cytosolic chaperone that interacts with parasite HSP70 stimulating the ATP hydrolytic rates of both PfHsp70 and human Hsp70 [56, 78]. The *P. falciparum* putative Sis1 (gene ID PFA0660w) has been shown to be essential for the parasite, having a PEXEL signal that allows importing this chaperone to infected erythrocytes [79].

8. *Toxoplasma gondii* HSP90/HSP70 cycle

The analysis of *Toxoplasma* HSP90-hetercomplex showed the presence of all the 9 components described in Table 1. Some of them are also linked to the existence of at least two different complexes: the early one, formed by the proteins HIP, HSP90 and HSP70, and the late one, formed by at least p23 and HSP90 [19]. Echeverria et al. [19] also observed that HIP is a cytosolic co-chaperone in both tachyzoite and bradyzoite stages. By contrast, p23 was detected in the cytoplasm and nucleus of bradyzoites, while it was present only in the cytoplasm of tachyzoites, following the localization of HSP90 [20, 80]. These data, together with the fact that GA blocks the interconversion in both ways [19], allow hypothesizing that the HSP90 heterocomplex is functional in *T. gondii* and probably has a role during parasite differentiation.

TgFCBP57, an immunophilin that contains an N-terminal FKBP (F) and a C-terminal cyclosporine binding protein (CBP) linked to a TPR domain, has also been characterized in *T. gondii* [81]. Both domains apparently bind to the respective inhibitory drugs (CysA and FK506) and are functional. Knock-down experiments by RNA interference have pointed TgFCBP57 to be essential for its normal growth [81].

Toxoplasma gondii has at least 36 DNAJ-containing sequences [82]. Among them, a putative Ydj1 and Sis1-like HSP40s have been detected. Interestingly, both HSP40s interact with parasite HSP90 and HSP70 ([82], Figueras, unpublished data). Recently, it has been observed that human type I and type II Hsp40 proteins are able to promote the binding of progesterone receptor to the HSP90–substrate interaction cycle but with different affinity, being Ydj1/Hdj2 the one with highest affinity [57]. We have not yet analyzed the interaction affinity of TgSis1 and TgYdj1 with HSP90. Interestingly, preliminary proteomic data would indicate that they pulled down different protein profiles (Figueras, unpublished data). In addition, the role of the human HSP90-heterocomplex in Hepatitis B reverse transcriptase function is associated with Hdj1 (Sis1-like) protein rather than with Hdj2v (Ydj1-like) [83]. TgSis1 and TgYdi1 may be participating in different roles of the HSP90-heterocomplex.

9. The *Leishmania* and *Trypanosoma* HSP90/HSP70 cycle

The *T. cruzi* STI-1/HOP homolog has been recently described and characterized as TcSTI-1 [84]. An interaction between TcSTI-1 and TcHSP70 has been demonstrated through immunoprecipitation assays in epimastigotes. Even though TcSTI-1 displays a cytoplasmic subcelullar localization, also co-localizes to some extent with TcHSP70 around the nucleus [84]. Thus, TcSTI-1 associates with TcHSP70, and TcSTI-1 expression is induced when the parasites are subjected to stress conditions during specific growth phases.

The first evidence of the existence of HOP (also called STI1 and p60) in *Leishmania* was found in a report describing genes that are differentially expressed in *L. donovani* amastigotes [85]. In *L. major*, the synthesis of this protein is up-regulated by heat shock, and the STI/HOP forms a salt-sensitive complex with HSP83 and HSP70 [86]. Its gene product consists of three TPR domains, containing nine TPR motifs. The systematic name corresponding to this *L. major* gene is LmjF08.1110. In the *L. major* genome database, there is also an additional entry (LmjF36.0070) identified as ‘stress-inducible protein

293 STI1 homolog'. However, the encoded protein is shorter than the LmjF08.1110-HOP protein, with a
294 relatively low sequence similarity between both. In *L. donovani*, a *sti/hop* related gene, named *hop-2*
295 (LinJ36_V3.0080), and a gene encoding for a SGT/HIP (LinJ30_V3.2740) homolog have been shown to
296 be dispensable for the growth of promastigotes, for axenic amastigote differentiation and for infectivity
297 towards cultured macrophage-like cells [87]. Both HIP and HOP form high protein complexes, co-
298 immunoprecipitating with HSP90 and HSP70 [87].

299 A BLAST search in the *L. major* genome database was performed using the yeast p23 sequence
300 (GeneDB systematic name YKL117W) as query and the retrieved protein encoded in gene LmjF34.0210
301 was considered as the putative homolog. The sequence homology is highly concentrated in the N terminal
302 moiety (residues 1 to 120), where the HSP90 binding site is found [33]. Another BLAST search made in
303 the *L. major* database using the LmjF34.0210 allowed finding another sequence, the LmjF35.4470, which
304 encodes for a protein with a GM-rich region, a feature present in the subgroup of the p23 proteins [88].
305 Thus, it is highly probable that *Leishmania* has two evolutionary divergent p23 homologs [33].

306 The DnaJ family of proteins in *T. cruzi* presents five Hsp40 co-chaperones (Tcj1, Tcj2, Tcj3,
307 Tcj4 and Tcj6) located in the cytoplasm [59, 89]. Tcj2, Tcj3 and Tcj4 have been identified as Type I
308 HSP40 homologs since they contain the J domain, glycine/phenylalanine (G/F)-rich region and the C-
309 terminal DnaJ domain. Tcj6 is a type II Hsp40 homolog, while Tcj1 is a Type III HSP40 homolog. Olson
310 et al. [90] reported that TcHSP70 has an extremely high ATPase activity, approximately 100 times greater
311 than that of the human HSP70. The *in vitro* basal specific ATP hydrolysis activity (ATPase activity) of
312 His-TcHSP70 has been determined as 40 nmol phosphate/min/mg protein, significantly higher than that
313 reported for other HSP70s, which could be stimulated to a maximal level of 60 nmol phosphate/min/mg
314 protein in the presence of His-Tcj2 [91]. By *in vivo* complementation assays, Tcj2 is able to overcome the
315 temperature sensitivity of the Ydj1 mutant *Saccharomyces cerevisiae* strain JJ160, suggesting that Tcj2
316 may be functionally equivalent to the yeast Hsp40 homolog [91].

317

318 **10. The HSP90-heterocomplex network**

319 Since the Hsp90 machine system is complex in nature, it is expected that its behavior and
320 function cannot be predicted and determined from the analysis of its simplest components. A large

number of genetic, biochemical and proteomic approaches have been generated over the years in the attempt to define the interactome of HSP90 itself and/or of the entire HSP90 machine [92]. Despite these efforts, the global picture of the chaperone-client interactions seems to still be very incomplete. In a recent paper, the Picard lab has shown an entirely different approach that incorporates all of the public datasets, and most importantly, all of the experimentally reported interactions [93]. This database achieved the most comprehensive protein-protein interaction (PPI) network of the human HSP90 molecular chaperone machine to date (available at http://www.picard.ch/Hsp90Int). The predictive power of this database has been demonstrated by showing a role for the HSP90 co-chaperone Aha1 in nucleocytoplasmic transport [93]. This interactome map could be used as a reference, where it is possible to organize and integrate proteomic data from high-throughput analyses to investigate the global roles of the HSP90 machine system.

The *Plasmodium* protein-protein interaction (PPI) map has been determined on the basis of yeast-two hybrid (Y2H) screens [94]. This first study was the platform to go in deep in the malarial chaperone network [95]. This study also combined this Y2H analysis with the computational approaches interlog method, which allows generating a protein network of proteins that are conserved across species [96]. Pavithra et al. [95] took advantage of the existing general chaperone PPI databases to construct the *Plasmodium* chaperone network, and thus allowed covering the whole parasite chaperone machinery. This work gives an interesting example of how the study allows making biological inferences from different proteins, including some hypothetical ones. Regarding the HSP90 interactome, Pavithra et al. [96] observed that HSP90 appears to assist proteins related to the translational machinery and chromatin remodeling, among others. In addition, the interaction of PfHSP90 with a Casein kinase II allowed these authors to suggest that this kinase is involved in regulating PfHSP90 activity, as suggested by previous analysis in which the chaperone phosphorylation is important at functional level during the erythrocyte cycle [16].

The PPI of *Toxoplasma* p23/HSP90 also suggests that HSP90 is involved in glycolysis/ATP generation, translation machinery/protein biosynthesis, chromatin remodeling and stress response [20]. These findings obtained from *P. falciparum* and *T. gondii* studies have been further documented in other organisms [93, 97-100]. The analysis of the PPI of *T. gondii* p23/HSP90 allowed finding another interesting group of proteins that interact with the p23/HSP90 interactome. This group includes the

surface antigen family (SRS), micronemes, dense granules and rhoptry proteins [20], all of which are either surface-anchored proteins or excreted/secreted proteins, and some of which are associated with parasite-host cell invasion. These findings were confirmed in a new study, in which at least 11 rhoptry proteins pulled down with HSP90 (Figueras, unpublished data). These kinds of proteins are also related to the endoplasmic reticulum pathway. It has been recently observed that p23/HSP90 could be associated with vesicles-transport, having an important role for a correct Golgi organelle function [99]. Whether *T. gondii* HSP90 interacts directly with rhoptries, micronemes, dense granules and ER/Golgi secretory proteins or through complexes related to these organelles remains to be clarified. A further analysis of the TgHSP90 PPI network (see below) showed that parasite HSP90 could assist a vesicle-mediated transport protein Fig. (3).

11. Generation of a *T. gondii* PPI network of the HSP90 heterocomplex

The existence of public HSP90 PPI networks and the experimentally reported interactions are a recent kind of approach that constitutes a powerful tool for extending the analysis of the PPI networks of the protozoan HSP90-heterocomplex. This may contribute to understand the biological processes in which this chaperone has a role and to infer the putative functions in protozoan cells. By using the protein query list of *P. falciparum* HSP90 and co-chaperones (Table S1), we searched the *P. falciparum* interactome based on Y2H analysis and thus obtained an interactome of 442 proteins with a total of 10,283 interactions between them. From this interactome, a list of conserved interactions in *T. gondii* interologs brought up a network of 271 proteins with 4373 interactions between them. Then, we added the interactome of 53 proteins with 67 interactions [20] to finally obtain a *T. gondii* experimental and predictive interactome within 317 proteins and 440 interactions between them (Table S2). The result is shown in Fig. (3), where the enriched functional groups are identified through the different color of the nodes.

In order to show some examples of the potential of generating this kind of maps, we noticed that this analysis retrieved some nucleocytoplasmic transport proteins (importin beta-1 and importin subunit beta-3) as HSP90 interactors Fig. (3). The interaction of other nucleocytoplasmic transport-associated proteins with HSP90 was also observed by an improved co-IP-analysis of *T. gondii* HSP90 (Figueras,

unpublished data). This finding is also expected since HSP90 translocates to the nucleus [101], a process that has also been observed in *T. gondii* and *P. yoelii* [19, 102]. It is not clear whether the role of parasite HSP90/p23 is to transport a group of proteins from the cytosol to the nucleus and/or to assist nuclear proteins and factors. Our analysis showed that a group of nuclear proteins, such as chromatin-related proteins and DNA-binding proteins, are important components of the *T. gondii* HSP90 interaction network Fig. (3). In this regard, the PPI network analysis also showed the putative interaction of HSP90-hetercomplex with Sir2 (TGME49_067360; Table S2), a nuclear protein associated to deacetylase activity and silenced chromatin [103], mainly located at telomeric associated sequences (TAS) in *P. falciparum* [104]. Noteworthy, H2A.X, an histone that has been linked to genome silencing in *T. gondii*, that could be located at repetitive putative TAS-like sequences present at chromosome ends, is overexpressed during bradyzoite development, where the nuclear presence of HSP90 and p23 is strong [19-20, 105]. The nexus between HSP90, H2A.X and Sir2 seems to be an interesting line for future investigations.

12. Conclusions & future trends

The HSP90 heterocomplex machinery is highly conserved from protozoan cells to higher eukaryotes, indicating that this complex arose early in evolution, perhaps having an important role in cellular adaptation to different environments and/or developmental processes. Several cell functions like translational machinery, glycolysis, ATP generation, respiration, chromatin modulation, transcription, etc., are processes associated with HSP90 in different species, from protozoan parasites to higher eukaryotes. Although every cell is unique, they use conserved mechanisms in combination with novel proteins and factors to generate the diversity in nature. For these reasons, in some cases, some differences have been found in parasite HSP90 and co-chaperones (e.g. the linker of *P. falciparum* HSP90). In addition, the protozoan parasite HSP90-heterocomplex also involves specific client proteins as it was observed in *T. gondii*, in which specific surface and excreted secreted antigens have been detected as putative TgHSP90 client proteins.

The importance of the HSP90-heterocomplex in this variety of biological processes prompts the need to develop novel and efficient tools to analyze such complex network of interactions. The *T. gondii* PPI-network generated on the basis of *P. falciparum* network and a previous *T. gondii* PPI map have shown that this technique is very powerful and have allowed us to discover new pathways that are

associated with HSP90 and therefore could be blocked by anti-HSP90 drugs. We expect to be able to generate this kind of databases in the future, combining experimental and bioinformatic data of different protozoan parasites, so as to better understand the evolution of different metabolic pathways associated with the HSP90 heterocomplex.

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Conflict of interest

The authors have no conflict of interest to declare.

Legends

Fig. (1). Sequence alignment of the HSP90 linker (HSP90L) region from different protozoan parasites. Bb, *Babesia bovis* (ACV04849); Cp, *Cryptosporidium parvum* (cgd3_3770); Eh, *Entamoeba histolytica* (EHI_102270); Li, *Leishmania infantum* (X87770); Pf, *Plasmodium falciparum* (PF3D7_0708400); Tc, *Trypanosoma cruzi* (AAA30202); Tg, *Toxoplasma gondii*; Tp, *Theileria parvum* (AAA30132); Tv, *Trichomona vaginalis* (TVAG_153560); human (Hu, P07900) and *Saccharomyces cerevisiae* (Sc, CAY86720). The corresponding HSP90 amino acid sequences were aligned by Clustal W (Bioedit program). The linker region of every HSP90 sequence was determined by using the human, *S. cerevisiae* and *P. falciparum* ones, split and all the linker regions were aligned by Clustal W once again. Letters highlighted in black indicate identical residues, whereas letters highlighted in gray indicate similar residues.

Fig. (2). Assembly of HSP90 associated co-chaperones during the HSP90/HSP70 cycle. The role of the HSP90-heterocomplex is to assist the adequate active conformation of the client protein (hexagonal figure). The inactive client protein (oval) is first assembled in an early complex, from which the client protein can undergo two possible pathways: i. assembly of the mature complex or ii. degradation pathway. The HSP90 inhibitors favor the degradation of client proteins. In protozoan parasites, the cdc37 co-chaperone has not yet been identified. Once the client protein adopts its active conformation and is released from the HSP90 mature complex, it is able to carry out its biological functions.

Fig. (3). Interactome map of the HSP90 machine in *Toxoplasma gondii*. Predicted HSP90 machine interologs from *P. falciparum* were obtained and integrated with previously experimentally determined interactors of HSP90 and p23 from *T. gondii*. Colored regions compile the set of proteins enriched for Gene Ontology (GO), and biological processes and functions are indicated in the same colors.

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