Characterization of *Trypanosoma brucei* Sti1 and its interactions with *Trypanosoma brucei* Hsp83 and human Hsp90

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By

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Abstract

Neglected tropical diseases continue to pose global concern due to their impact on health and socio-economic status of developing countries in sub-Saharan Africa. African trypanosomiasis is one of the neglected tropical diseases caused by the kinetoplastid flagellate parasite *Trypanosoma brucei* (*T. brucei*). The disease is fatal if untreated and the toolbox to combat the disease has been plagued with many difficulties such as drug resistance, toxic chemotherapeutics, and cumbersome drug delivery processes. In recent years, the disease has received attention from organizations such as the Drugs for Neglected Diseases initiative (DNDi) in partnership with WHO as well as academia and industry to provide alternatives to the existing drugs as part of a targeted approach to eliminate human African trypanosomiasis by 2030. The life cycle of the *T. brucei* parasite requires that it transitions between a cold-blooded vector (the tsetse fly) and a human host. To survive this extreme environmental change and maintain its infectious cycle, the parasite has evolved an arsenal of tools which include a strong immune evasion technique and a robust molecular chaperone system.

Heat shock protein 90 (Hsp90) is one of the most abundant eukaryotic molecular chaperones that has been extensively studied in many organisms. It is indispensable for maintaining proteostasis in some organisms and its inhibition is currently being explored as a drug target for cancer and other parasitic diseases. In *T. brucei,* cytosolic Hsp90 is specifically referred to as Hsp83 due to variations in the sizes amongst different orthologues. Hsp90 is present in high levels in all stages of the *T. brucei* cell cycle both constitutively and on exposure to stress. To function in the cell, Hsp90 is dependent on co-chaperones, one of which can be found in most organisms, namely, the stress-inducible protein 1 (Sti1). The Hsp90-Sti1 interaction was shown to be crucial for growth in the intracellular kinetoplastid parasite, *Leishmania donovani*. However, this partnership has not been explored in the extracellular parasite *T. brucei*. To analyse the interaction of Hsp90 with Sti1 in *T. brucei*, this study combined *in silico, in vitro* and *in vivo* tools.

In silico analyses of the Hsp90 complement in *T. brucei* revealed the presence of twelve putative *Hsp90* genes, ten of which code for the cytosolic protein and are arranged in tandem in a head to tail fashion on the same chromosome. One gene each was found for the mitochondrial and ER paralogues of Hsp90, similar to all other species analysed. Eight putative co-chaperones specific to *T. brucei* were also discovered: six tetratricopeptide repeat domain (TPR) containing co-chaperones and two non-TPR containing co-chaperones. Structural and evolutionary analysis also confirmed that the domains were conserved across the species analysed. *T. brucei* Sti1 (TbSti1), *T. brucei* cytosolic Hsp90 (TbHsp83) and human cytosolic Hsp90 (hHsp90) were heterologously overproduced in *E. coli* and purified using nickel affinity chromatography. With specific antibodies, the expression and localization of the proteins were confirmed. TbSti1 showed strong affinity to the Hsp90s in the nanomolar range, with higher affinity for hHsp90

compared to TbHsp83. TbHsp83 and hHsp90 showed typical chaperone properties by suppressing the aggregation of thermolabile substrate MDH at equimolar concentrations and both chaperones had potent ATP hydrolysis activity. TbSti1, on the other hand, showed no MDH suppression activity and did not affect the ATP hydrolysis activity of TbHsp83 or hHsp90. *Ex-vivo* experiments using HeLa CRISPR Hop knockout (KO) human cell lines transfected with pcDNA3.1(+)HA-TbSti1 revealed TbSti1 also localized to the cytoplasm. The transfected cells showed a distinct fibroblast-like morphology which was different from the circular morphology seen in the Hop KO untransfected and wild type untransfected cells. Finally, co-immunoprecipitation studies revealed that TbSti1 co-immunoprecipitated with hHsp90. These results show the first characterization of the TbHsp83-TbSti1 partnership in *T. brucei*. The strong association between both proteins suggests a functional role for this partnership in *T. brucei* and could provide an updated context for understanding *Trypanosome brucei* biology.

Declaration

I, Miebaka Jamabo, declare that this thesis is my own unaided work. It is hereby submitted for the degree of Doctor of Philosophy at Rhodes University, Faculty of Science. It has not been submitted before for any degree or examination at any other university.

Miebaka Jamabo

Dedication

This work is dedicated to every young researcher in Nigeria hoping for better infrastructure to conduct quality research.

Acknowledgements

"*It takes a village to raise a child", an* African proverb. Indeed, it took a community of people to achieve success during this PhD journey and I am indebted to every one of you.

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Abbreviations/symbols and their full meaning

Amp^R	Ampicillin resistance (β-lactamase gene)
APS	Ammonium persulphate
AAT	Animal African Trypanosomiasis
ADP	Adenosine diphosphate
Aha1	Activator of Hsp90 ATPase activity 1
ATP	Adenosine triphosphate
~	Approximately
α	Alpha
bp	Base pair
BLAST	Basic Local Alignment Search Tool
β	Beta
B_{max}	maximum number of binding sites
BSF	Blood stream form
BSA	Bovine serum albumin
º C	Degree Celsius
Da	Daltons
DNA	Deoxyribonucleic Acid
DnaK	Prokaryotic Hsp70
DNDi	Drugs for Neglected Diseases initiative
DTT	Dithiothreitol
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
EEVD motif	Glutamate-Glutamate-Valine-Aspartate (Glu-Glu-Val-Asp) motif
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic Reticulum
GRP94	Glucose-regulated protein 94
HAT	Human African Trypanosomiasis
Hsc70	Heat shock cognate protein 70
Hsp	Heat shock protein
Hsp90	90 kDa heat shock protein
Hsp70	70 kDa heat shock protein
HRP	Horse radish peroxidase
H. sapiens	*Homo sapiens*
Hop	Hsp70-Hsp90 organising protein
Kan^R	Kanamycin resistance
K_d	equilibrium dissociation constant
kDa	Kilo Daltons
kbp	Kilo base pair
IPTG	Isopropyl-β-D-thiogalactopyranoside
L. major	*Leishmania major*
MDH	Malate dehydrogenase
MEGA	Molecular Evolutionary Genetics Analysis
µg	Microgram
µl	Microlitre
µM	Micromolar
mg/ml	Milligrams per millilitre
M	Molar
Mb	Mega base

TEMED	N, N, N', N'-Tetramethylethylenediamine
nm	Nanometer(s)
nmol	Nanomoles
NCBI	National Centre for Biotechnology Information
PCF	Procyclic form
PMSF	Phenylmethylsulphonyl fluoride
PP5	Protein phosphatase 5
Pi	Inorganic phosphate
P. falciparum	*Plasmodium falciparum*
PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SGT	Small glutamine-rich TPR-containing protein
SMART	Simple Modular Architecture Research Tool
Spp	Species
Sti1	Stress inducible protein 1
T. brucei	*Trypanosoma brucei*
T. b. brucei	*Trypanosoma brucei brucei*
T. b. rhodesiense	*Trypanosoma brucei rhodesiense*
T. b. gambiense	*Trypanosoma brucei gambiense*
T. cruzi	*Trypanosoma cruzi*
TBS	Tris-buffered saline
TBS-T	Tris buffered saline with Tween
TPR motif	Tetratricopeptide Repeat Motif
TRAP-1	TNF receptor associated protein 1
VSG	Variable surface glycoprotein
V	Volts
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
YT	Yeast- Tryptone media

List of outputs

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1 Literature review and Background

1.1 History and classification of *T. brucei*

For decades prior to the discovery of *Trypanosoma brucei* (*T. brucei*), there was an awareness of a severe wasting disease termed *nagana* in cattle in the sub-Saharan region as well as a deadly sleeping disorder in the same regions. It was only at the end of the 19th century that the cause of *nagana* and sleeping sickness was identified by David Bruce who was studying *nagana* in Zululand when he discovered trypanosomes in the blood of the diseased cattle. Subsequently, the causative parasite was identified as *T. brucei* with successive studies showing it was transmitted by the tsetse fly (Bruce et al., 1914; Vickerman, 1997; Kennedy, 2004, 2013). *T. brucei* is a protozoan parasite transmitted through the bite of the tsetse fly (*Glossina* species) and is responsible for causing Human African Trypanosomiasis (HAT), commonly known as sleeping sickness which is a neglected tropical disease prevalent in sub-Saharan Africa (Kennedy 2004; 2019; Brun et al. 2010). The disease in animals is called *nagana* or Animal African Trypanosomiasis (Holmes, 2013). Although rare, alternative routes of transmission for HAT have been documented such as congenital transmission, sexual transmission, and laboratory accidents (Herwaldt, 2001; Rocha et al., 2004; De Kyvon et al., 2016).

T. brucei is categorized under the genus *Trypanosoma,* the family *Trypanosomastidae* and the order *Kinetoplastida* (Chappuis et al., 2005). Kinetoplastids are a group of flagellated protozoans that are responsible for causing various diseases in humans such as HAT, Chagas disease and some forms of Leishmaniasis (Stuart et al. 2008). These three kinetoplastid pathogens, namely – *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major* (otherwise referred to as TriTryps) have many similarities in their cell biology and thousands of shared orthologues in common but the disease caused by each one is distinct, transmitted by different vectors and they also have unrelated immune evasion techniques in their mammalian hosts (El-Sayed et al. 2005; Stuart et al. 2008). They have special characteristics which includes the presence of a kinetoplast (a form of mitochondrial DNA) and a peculiar way of metabolic compartmentation (Hannaert et al. 2003; Stuart et al. 2008). *T. brucei* is exclusively extracellular, multiplies primarily by binary fission, and as a result of the ease with which it can be cultured and genetically manipulated, it has emerged as a model organism for studying the kinetoplastids (Chappuis et al., 2005; Höög et al., 2010). The parasite (which is about 20-30 μm long and between 1.5 to 5 μm wide) is shaped as a spindle, possesses a nucleus and a mitochondrion which contains the kinetoplast, and has its flagellum running along the entire length of the cell membrane to emerge at the posterior end (Chappuis et al., 2005;

Büscher et al., 2017). The form that exists in the developmental phase of the tsetse fly is the epimastigote while the form that exists in the mammalian host is called the trypomastigote (Figure 1.1) (Chappuis et al., 2005). There are two different morphological trypomastigote forms occurring in the mammalian blood stream, a long slender dividing form which is replaced by a short stumpy non-proliferative form as the parasite multiplies and increases in number (Matthews, 2005).

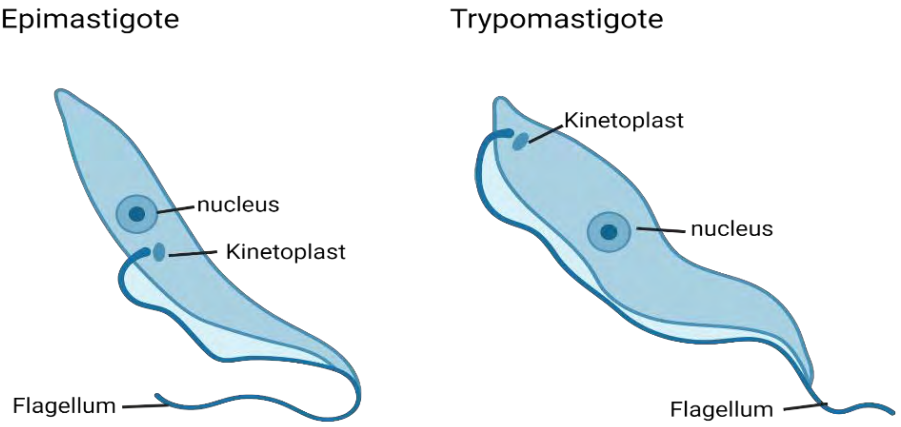


Figure 1.1: Simplified cell architecture of the trypanosome.
Both epimastigote and trypomastigote forms have long flagella but in the epimastigote the kinetoplast is anterior to the nucleus and posterior to the nucleus in the trypomastigote. (Adapted from Sunter and Gull 2016).

T. brucei is known to infect a wide range of domestic and wild mammals, these animals generally serve as reservoir hosts for HAT especially if the parasites they harbour are human-infective parasites (Gibson et al., 2015). *T. brucei* includes three subspecies that are morphologically very similar. The animal infective form, *T. brucei brucei* (*T. b. brucei*) which is responsible for infections in domestic and wild animals; and the human infective forms *T. brucei gambiense* (*T. b.* gambiense) and *T. brucei rhodesiense* (*T. b. rhodesiense*) (Matthews, 2005; Büscher et al., 2017). *T. b. gambiense* is responsible for the chronic progressing form of the disease in humans which is endemic to central and western Africa while *T. b.* rhodesiense causes the acute form of the disease endemic in southern Africa; both human forms can be fatal if untreated (Brun et al. 2010; Simarro et al. 2010; Büscher et al. 2017). The human infective forms though morphologically indistinguishable are heterogenous and can be distinguished using genetic and molecular approaches (Gibson, 2001; Radwanska et al., 2018). *T. b. gambiense* can be specifically identified using

PCR amplification of the gene that encodes for a receptor-like glycoprotein – the *T. b. gambiense* -specific glycoprotein gene involved in human serum resistance (Radwanska et al., 2002). *T. b. rhodesiense* is also characterized by the presence of an exclusive serum resistance associated (*SRA*) gene (Welburn et al., 2001; Radwanska et al., 2002). The SRA protein is important for both species to evade lysis in the human serum (Radwanska et al., 2002; Mabille and Caljon, 2020).

1.1.1 Transmission and life cycle

The human infective *T. brucei* oscillates between two hosts, the tsetse fly, and the human host. Both male and female blood-feeding tsetse fly can cause transmission (Brun et al., 2010). The tsetse fly takes up the bloodstream trypomastigote trypanosome from the blood of the human host while feeding and then multiplies the trypanosome in their midgut, this becomes the replicative procyclic trypomastigote form that enables survival of the trypanosome in this new environment (Urbaniak et al. 2012). After various complex adaptations through the tissues of the tsetse fly, they leave the midgut as epimastigotes and migrate to the salivary gland where they multiply and form the human infective metacyclic trypomastigote, the parasite is then transmitted to another mammalian host upon the bite of this infected fly (Cox, 2004; Büscher et al., 2017) (Figure 1.2). Generally, in a population of tsetse flies, the population carrying the infectious metacyclic form in their salivary glands is less than 0.1% but with its feeding pattern within a 2-3 month lifespan it can infect large numbers of humans and animals (Büscher et al., 2017). Meanwhile, in the human host, after the tsetse fly injects the metacyclic trypomastigote form before its blood meal, the trypanosomes first proliferate in the tissues at the site of the infection (Chappuis et al., 2005). The infected patient exhibits few symptoms immediately after being infected but as the parasite multiplies in the blood and lymphatic vessels (hemo-lymphatic stage), the patient begins to develop fever and rashes. Some of the trypanosomes eventually access and cross the blood-brain barrier to invade the central nervous system (meningo-encephalitic stage) causing neurological breakdown and an altered sleep-wake cycle, hence the name ‘sleeping sickness’, all these can lead to coma and eventual death (Cox, 2004; Barrett and Croft, 2012).

The mammalian bloodstream trypanosomes are pleomorphic as they exist either as the long slender (LS) proliferative forms or the short stumpy (SS) non-proliferative or quiescent forms (Vickerman, 1985; Matthews, 1999; Tyler, 2001). Generally, the dominant population is the long slender form which multiplies rapidly during the early phase of parasitaemia, some of these will transition to the short-stumpy form pre-adapted for life in the tsetse fly vector and will accumulate and ultimately die if not taken up by the vector (Vickerman, 1965; Seed and Wenck, 2003; Schwede et al., 2012). The differentiation to the non-dividing stumpy form is important in regulating parasitaemia and ultimately the virulence of the disease (Tyler et

al., 1997), studies on the differentiation process from the LS to the SS forms have experienced challenges due to the absence of clear regulated markers and this is also complicated by the presence of intermediate forms between both populations (McLintock et al., 1990; Stojdl and Clarke, 1996). One organelle highly elaborated in the bloodstream form differentiation process is the mitochondrion (Tyler et al., 1997; Tyler, 2001). Cytological localization studies using immunofluorescence microscopy was done using various antigens including some heat shock proteins – mitochondria Hsp70 (mHsp70) and Hsp60. The mHsp70 stained the mitochondrion of the SS forms and some LS forms while the Hsp60 stained the mitochondrion of the SS forms but were not clearly detectable in the long slender forms (Tyler et al., 1997)

As the parasite shuttles between the parasite vector and its mammalian host, changing forms from procyclic form (PCF) to bloodstream form (BSF), it also undergoes changes in its gene expression to provide proteins adapted to function in each host (Butter et al., 2013). Numerous transcriptomic and proteomic data have now been published showing above 30% of genes being differentially expressed between both forms of the parasite and this includes genes involved in metabolism (Butter et al., 2013; Trindade et al., 2016). The BSF uses glucose through the glycolytic pathway in the glycosome as they can barely survive in anoxic conditions whereas the PCF makes use of amino acids such as proline and threonine as carbon source through the Krebs cycle in the mitochondrion (Szöör et al., 2014). The most apparent morphological difference seen across the different stages of the parasite is the changing position of the kinetoplast in relation to its central nucleus. In the epimastigote form (in the salivary gland of the tsetse fly), the kinetoplast is located anterior to the nucleus; in the procyclic trypomastigote form, the kinetoplast is located posterior to the nucleus while in the bloodstream trypomastigote the kinetoplast is located even further posterior from the nucleus, almost at the posterior end of the cell (Matthews, 2005). In all stages the flagella is prominent, originating at the flagellar axoneme which is physically linked to the kinetoplast and exits the cell through a flagellar pocket hole (Ogbadoyi et al 2003; Sharma et al. 2009). It is essential for survival of the BSF and responsible for trypanosome motility and attachment to the insect tissues (Buisson and Bastin, 2010).

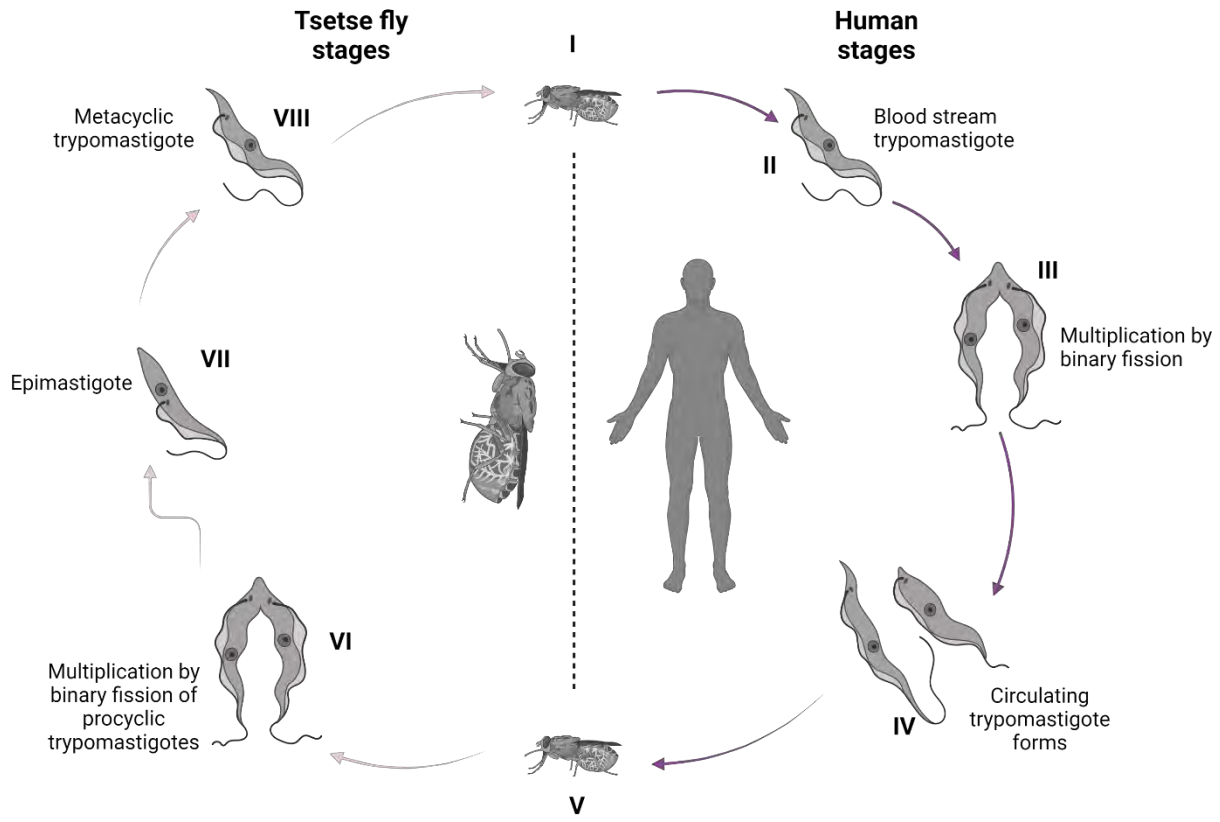


Figure 1.2: Transmission and life cycle of the trypanosome.
The trypanosome is digenetic having to shuttle between the tsetse fly and a mammalian host. 1- The bite of an infected tsetse fly injects the metacyclic trypomastigote into its mammalian host. 2- The injected trypomastigote is transformed into the bloodstream form. 3-4 – The parasite multiplies by binary fission and spreads in the various body fluids. 5- An uninfected tsetse fly becomes infected by taking up bloodstream trypomastigotes during its bloodmeal. 6- The bloodstream trypomastigotes form procyclic trypomastigotes in the midgut multiplying by binary fission. 7-8 - The procyclic trypomastigotes transform to epimastigotes in the salivary gland where they form the metacyclic trypomastigote ready to infect another mammalian host. (Adapted from "CDC - African Trypanosomiasis - Biology" 2020)

T. brucei has been generally shown to reside and multiply in the interstitial spaces, lymphatic system and bloodstream of various tissues of the mammalian host (Losos and Ikede 1972; Kennedy 2013), however, several other tissues have been found to serve as reservoirs for the extracellular parasite *T. brucei* including the skin and adipose tissues (Caljon et al., 2016; Capewell et al., 2016; Tanowitz et al., 2017). Studies have shown, using mice infected with fluorescently tagged *T. brucei* parasites, that the dermis in close proximity to the site of inoculation after the bite of the tsetse fly, retains a small population of the parasite which proliferates and remains highly infectious even in the absence of detectable parasites in the blood (Caljon et al., 2016; Capewell et al., 2016). Also using a mouse model, *T. brucei* parasites were found to assemble in the adipose tissues and these parasites in the adipose tissues show a different metabolic profile compared

to the long slender or short stumpy bloodstream form of the parasites (Trindade et al., 2016). In addition to the skin and adipose tissues, there is also evidence of the parasite residing in the testes leading to sexual transmission as shown in mice (Claes et al., 2009; Biteau et al., 2016). These reservoirs could be an extra layer of protection from the immune response of the host as well as a reason for continuous relapse in infected individuals.

1.2 Human African Trypanosomiasis

Human African Trypanosomiasis (HAT) is endemic to the sub-Saharan African region due to presence of an appropriate habitat for the tsetse fly vector and is prevalent in poverty-stricken regions where survival is dependent on activities such as hunting, farming and fishing (Brun et al., 2010). The chronic infection caused by *T. b. gambiense* accounts for 98% of reported cases and is prevalent in west and central Africa while the acute zoonotic infection caused by *T. b. rhodesiense* is responsible for 2% of the reported cases and is prevalent in eastern and southern Africa (Babokhov et al., 2013). The map in Figure 1.3 shows the geographical distribution of the disease. Both variants of the disease have been reported to exist in Uganda but no evidence of cases of dual infections have been recorded(Picozzi et al., 2005). Human beings are the main reservoirs for the trypanosomes causing *T. b. gambiense* HAT, while animals such as cattle are the main reservoirs of trypanosomes causing *T. b. rhodesiense* HAT (Davis and Kennedy 2000; Kennedy 2013).

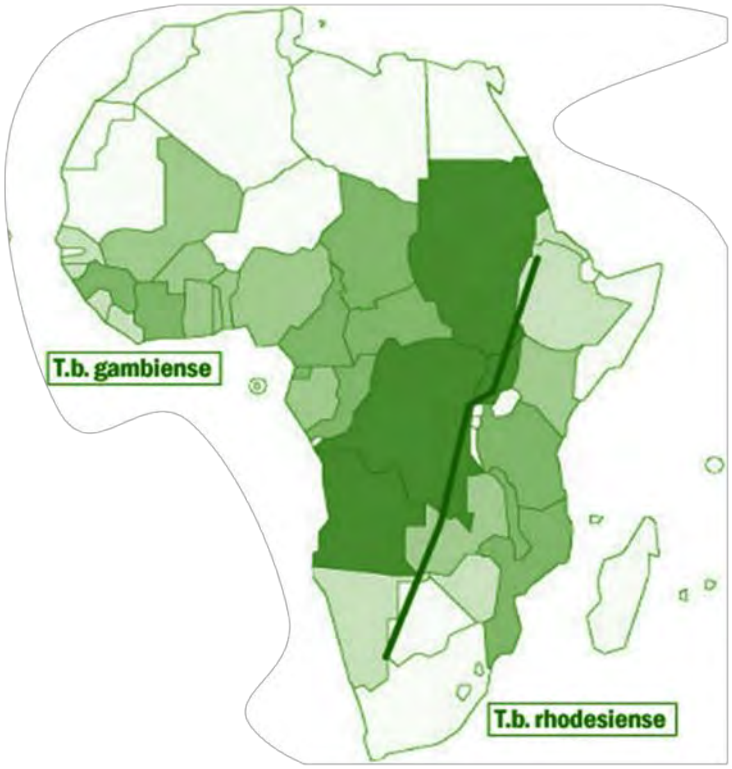


Figure 1.3: Geographical distribution of *T. brucei*
Geographical distribution of *T. brucei gambiense* in west and central Africa and *T. b. rhodesiense* in east and southern Africa (Adapted from Woster 2007)

Following the bite of the infected fly in a mammalian host and the inoculation of the metacyclic form trypanosome, local skin reactions called chancre develops (Barry and Emery, 1984). The development of chancre about three days after the bite occurs before the parasites traverse the lymphatics and blood capillaries and are detected in the blood (Vickerman, 1985). As the parasite traverses all various parts of the mammalian host, the proliferative long slender trypomastigote form multiplies by binary cell fission (Zhang et al., 2019). This proliferative long slender bloodstream form is responsible for the antigenic variation and continuous bouts of infection in response to the IgM released by the mammalian host (Van Meirvenne et al., 1975; Seed, 1977). The cycle continues as the non-proliferative form replaces the long slender form through the vector.

Though binary fission is the well-studied means of multiplication in trypanosomes, sexual reproduction, and genetic exchange in *T. brucei* was first documented in 1986 (Jenni et al., 1986). Subsequently, more studies have been done revealing that genetic exchange occurs in the tsetse fly vector not the mammalian host and it is not an obligatory step of the lifecycle/transmission process (Gibson et al., 2015). The genetic exchange is a true sexual reproduction process involving meiosis and the formation of haploid gametes and

several successful crosses between *T. brucei* strains have been carried out resulting in the generation of new hybrids (Gibson and Bailey, 1994). The meiotic stages were shown to take place from fourteen days post infection when the trypanosomes as epimastigotes have reached the salivary glands (Peacock et al., 2011, 2014). Complete details of the sexual reproduction process are yet to be elucidated in *T. brucei* but it is certain sexual reproduction takes place in trypanosomes and such recombination can create new strains of human pathogens creating a possible risk of future HAT outbreaks (Gibson et al., 2015). These findings that *T. brucei* is a sexual organism point to the fact that the mammalian hosts are just 'vessels' for the trypanosomes to migrate from one insect vector to another as well as serve as long term reservoirs, the insect vector by itself can serve as a host for the parasite (Radwanska et al., 2018).

Over the course of the past century, there have been three major epidemics in Africa: the first epidemic occurred between 1896 and 1906 killing a little less than a million people (Louis and Simarro, 2005; Steverding, 2008). East African countries such as Uganda and Kenya were severely affected by what was then assumed to be *T. b. gambiense* though *T. b. rhodesiense* was yet to be characterized (Hide, 1999). The second epidemic which occurred about ten years later forced the ruling colonial powers to adopt strategies such as continuous surveillance of the population as well as vector control measures; these strategies were effective enough to almost eradicate the disease in fewer than 50 years (Brun et al., 2010). However, as most of the endemic countries started gaining independence, there was a decline in surveillance measures which was also worsened by civil unrest in these countries leading to a resurgence of the disease in numerous countries in central Africa such as Angola, South Sudan and Congo-Zaire (Smith et al. 1998; Moore and Richer 2001; Nieuwenhove et al. 2001; Stanghellini and Josenando 2001). After this peak in the early 20th century, sustained efforts by agencies such as World Health Organization (WHO) to control HAT again included: providing drugs free of charge to affected areas, supporting and strengthening vector/disease control measures, and improving knowledge of the disease (Franco et al., 2014b). These measures have led to reduced reported cases, and from 2018, the total annual cases have been on a steady decline from a thousand with a target of zero incidence of the disease by 2030 (Holmes, 2014; Franco et al., 2020, 2022; WHO, 2021). Apart from the cases mentioned in the endemic regions, a couple of cases have been confirmed in non-endemic regions, most likely from travellers returning from the endemic regions (Simarro et al. 2012; Sudarshi and Brown 2015).

1.2.1 Immunology and antigenic variation

As the parasite has to shuttle between its vector and the mammalian host, its survival and virulence are dependent on various adaptation strategies as it transitions between the mammalian bloodstream and the various compartments of the vector (Matthews 2005; Urbaniak et al. 2012). Humans have resistance to the pathogenic animal trypanosome *T. b. brucei* due to the presence of some innate immunity mediated cytolytic factors present in the high-density lipoprotein (HDL) fraction of the human serum (Seed et al., 1993; Greene and Hajduk, 2016). This cytolytic property is attributed to two fractions- the trypanosome lytic factor 1 and trypanosome lytic factor 2 (TLF1 and TLF2) which contain the protein apolipoprotein L1 (ApoL1) as the main active lytic agent (Raper et al., 1999; Vanhamme et al., 2003; Pays et al., 2006). The human infective subspecies – *T. b. gambiense* and *T. b. rhodesiense* are resistant to the lytic factors and able to survive in the human host using various VSG mechanisms such as the presence of the *SRA* gene in *T. b. rhodesiense* (Van Xong et al., 1998). The lysosomal SRA protein colocalizes with ApoL1 as seen in immunofluorescence studies and is able block the membrane pore forming capacity of ApoL1 in the lysosome, also *T. b. brucei* transfected with *SRA* conferred resistance against the serum trypanolytic factors (Van Xong et al., 1998; Oli et al., 2006). In *T. b. gambiense,* resistance to ApoL1 is achieved with the help of the *T. b. gambiense* specific glycoprotein (TgsGP) which is unrelated to SRA in *T. b. rhodesiense* and TgsGP is also not able to confer resistance on *T. b. brucei* as SRA did (Berberof et al., 2001; Uzureau et al., 2013)

Studies in mice have now shown that the bite of an infected or uninfected trypanosome parasite causes tissue damage and triggers the release of neutrophils to the site of the bite within few hours (Caljon et al., 2018). Neutrophils, as part of the innate immune system possess various pathogen killing abilities (Kolaczkowska and Kubes, 2013; Regli et al., 2017), but some parasites have learnt to exploit these mechanisms. In Leishmania, neutrophils in mice successfully capture the parasites after the bite of an infected sand fly, but these phagocytosed parasites could still spread the infection (Peters et al., 2008; Regli et al., 2017). Furthermore, the depletion of neutrophils showed significant reduction in the number of viable parasites (Peters et al., 2008). For trypanosomes, the viable parasite population in the skin escaped phagocytosis by neutrophils and only parasites with weakened viability were engulfed (Caljon et al., 2018). Neutrophils have therefore been suggested as modulators of parasite infection (Hurrell et al., 2015; Regli et al., 2017).

Production and release of immunoglobulins IgM and IgG is another classic immunological feature associated with trypanosomiasis as a result of B-cell activation (Lejon et al., 2003). Early reported cases of

HAT use the profile of IgM as a parameter to detect the late stage HAT involving the CNS as well as treatment success (Waema et al., 2017) and in non-human primates, the elevated presence of CSF IL-6 also served as a marker for late stage HAT (Waema et al., 2017). In humans, the trypanosome bite leading to the disease HAT also triggers inflammatory components and chemokines such as IL-1 and IL-6, with increase in concentrations of IL-6 and IL-10 consistent with stage progression of the disease (MacLean et al., 2006; Mabille and Caljon, 2020). Subsequently, monocytes, plasma cells, CD4+ and CD8+ lymphocytes access the infection site about 24 hours following the bite of the infected parasite but the functions of these immune complements remain unclear (Mabille and Caljon, 2020).

As previously established, the parasite is extracellular and the bloodstream form in the host is under continuous contact with the immune system of the host, one of its key strategies to escape the immune response of the mammalian host is its antigenic variation (Donelson 2003; Stuart et al. 2008). Antigenic variation refers to the spontaneous process by which trypanosomes keep switching their variant surface glycoprotein (VSG) coat which is present on the surface of their cells. With this tactic they can keep proliferating as they continuously evade the circulating antibodies of their host and this is responsible for the steady bouts of parasitaemia seen in HAT (Turner, 1997; Taylor and Rudenko, 2006; Brun et al., 2010; Babokhov et al., 2013). These circulating antibodies targeting the VSG coat constitute the adaptive immunity (Pinger et al., 2017; Pays and Nolan, 2021) . The VSG coat is usually replaced after about four days following a genetic switch and it only surrounds the bloodstream form (Pinger et al., 2017). As the trypanosome is taken up by the tsetse fly, the VSG coat is replaced with a less dense coat made of procyclins and the parasite only acquires the VSG coat again as it gets into the salivary gland in preparation for infecting another mammalian host (Roditi and Liniger, 2002; Matthews, 2005).

The full genome of the trypanosome was sequenced in 2005 (Berriman et al., 2005) and shown to contain about 9000 to 10 000 genes, 10% of which encode these VSGs (El-Sayed et al., 2000). The parasite expresses only one *VSG* gene at each time point and their sole function is to protect the parasites from lysis by the immune complement system of their host (Taylor and Rudenko, 2006; Brun et al., 2010). Transcription of the VSG occurs at a telomeric expression site (ES) located at the end of the chromosome. Here the parasite is able to replace its coat by recombination events that help change the transcribed gene to a different *VSG* gene or altering the expression site where active transcription is taking place (Borst, 2002; Glover et al., 2013). Though it is reported that one telomeric ES is sufficient to express the VSGs, *T. brucei* has at least 20 expression sites and it can switch from one to the other, further strengthening its antigenic variation against its host (Borst, 2002). Other peculiarities of the VSGs include being transcribed by RNA polymerase I rather than RNA polymerase II which is responsible for transcribing most of the

other protein coding genes in the trypanosome and the transcription of these VSGs occurs in the nucleoplasm (Navarro and Gull, 2001).

Noteworthy is the fact that, though these VSG molecules/antigens can initially stimulate the B and T cell immune response of the host, the immune response is usually targeted at epitopes within the VSG N-terminal which are highly variable and hence incapable of providing protection from the new variants (Black and Mansfield, 2016). Due to this extensive antigenic variation and the inexhaustible repertoire of replacement VSGs, attempts to develop a vaccine based on VSGs is far-fetched and not feasible in spite of intensive research (Cornelissen et al., 1985; Pays, 1995; Stuart et al., 2008a; Holmes, 2013).

1.2.2 Vaccine strategies and vaccine failure

In addition to the trypanosome advantage of evading the human immune response by continuous switching of their VSG coat (Taylor and Rudenko, 2006), trypanosome parasite infection has also been shown to deplete various B cell populations, preventing the formation of B cell memory against the encountered antigens. This in turn weakens the capacity of the host to form long lasting protection (Radwanska et al., 2008; Obishakin et al., 2014; Magez et al., 2020). Antibodies and B cell have been established to play essential roles in trypanosome infection (Campbell et al., 1977; Magez et al., 2008). Early studies in mice infected with the human infective *T. b. gambiense* parasites show suppression of antibody response and polyclonal B cell activation as the hallmark of the infection (Diffley, 1983; Oka et al., 1984). Further studies into the B cell population during trypanosome infections in mice revealed that the spleen was extensively remodeled early into the *T. brucei* infection and there was massive reduction in the B cell population (Radwanska et al., 2008). Furthermore, infection with *T. brucei* in a mouse vaccination model could nullify the vaccine induced protection generated from using a human vaccine against non-related pathogens such as tetanus and diptheria (Radwanska et al., 2008). This ability of trypanosomes to permanently affect the mammalian humoral system as seen in experimental mice models infected with the parasite constitutes a major drawback to vaccine development till date (Radwanska et al., 2008; Magez et al., 2021b).

Previous vaccine approaches have targeted other surface glycoproteins involved in the utilization of nutrients and survival, but these have been unsuccessful for different reasons as some of these molecules are expressed at insufficient levels to induce protective immunity from the host or they are hidden beneath the surface of the VSG coat (Ziegelbauer and Overath, 1992; Magez and Radwanska, 2009; Magez et al.,

2010; Black and Mansfield, 2016). The flagellar pocket has also been described to contain proteins specific to the trypanosome species but vaccine studies in mice and cattle targeting the flagellar pocket showed only partial and in some cases no protection at all (Mkunza et al 1995; Radwanska et al. 2000). With no positive outcome yet in developing a vaccine, drugs have remained the current option for treatment of HAT.

1.2.3 Diagnostics and drug discovery for HAT

HAT caused by *T. b gambiense* results in a prolonged chronic illness that could stretch over many years while HAT caused by *T. b. rhodesiense* is usually acute and fatal within a space of months if untreated (Odiit et al 1997; Checchi et al. 2008). HAT presents symptoms such as lymphadenopathy, headache, anaemia, weakness, fever and cardiac involvement in some cases during the early stage of the disease and for the latter stage, an altered sleep-wake cycle in addition to other neuropsychiatric symptoms is characteristic (World Health Organization, 2013). These symptoms are similar to those of other diseases such as malaria, HIV and tuberculosis and co-infections may even exist leading to misdiagnosis in some cases (Chappuis et al. 2005; Simarro et al. 2012). Diagnosis usually starts with a form of screening to analyse the obvious symptoms presented while bearing in mind the specific parasite species endemic to that region (Kennedy 2004), followed by some form of diagnostic confirmation and finally staging of the disease. Accurate staging and diagnosis of the disease is crucial for chemotherapy given the toxicity of most of the therapeutics available (Louis et al 2001; Chappuis et al. 2005).

Considering the endemic locations and the difficulty of accessing highly sensitive screening techniques in such areas, screening usually begins with a WHO recommended serological test called a CATT (Card Agglutination Test for Trypanosomiasis) (Magnus et al 1978; Miezan et al. 1991). The CATT technique relies on antibody-mediated agglutination to detect trypanosome-specific surface glycoproteins (Migchelsen et al., 2011). This test method is quick, inexpensive and can analyse fresh as well as dried blood samples making it convenient for field use (Noireau et al 1991; Truc et al. 2002). However, CATT is specific for *T. b. gambiense* alone and is inconclusive due to the possibility of misdiagnosis resulting from cross reactivity from other antibodies in cases of infections such as the Epstein-Barr virus (Raffenot et al., 2000), patients therefore still need to undergo a number of confirmatory tests due to the low sensitivity and specificity of the test (Simarro et al. 1999; Radwanska 2010). Trypanosomes, being extracellular parasites can easily be identified microscopically (World Health Organization, 2013; Franco et al., 2014a) and in the event of a positive CATT diagnosis for *T. b. gambiense*, microscopy is needed for confirmation (Manful et al., 2010). Also, for *T. b. rhodesiense,* microscopy is the first step to assess for presence of

parasites in blood or lymph samples, although, due to the morphological similarities between *T. b. gambiense* and *T. b. rhodesiense*, microscopy and geography alone is still insufficient for diagnosis (Deborggraeve and Büscher, 2010; Kennedy, 2013; Franco et al., 2014a). Once parasitological diagnosis has been confirmed, a lumbar puncture needs to be done to access the cerebrospinal fluid (CSF) and determine microscopically if the disease has progressed to the neurological stage before treatment can commence (Sinha et al., 1999; Miézan et al., 2000).

With a continuous need for better diagnostics, a couple of other tests have shown promising results especially for diagnosing HAT. The indirect immunofluorescence antibody-test (IFAT) has been successfully used in population screening in Equatorial Guinea (Simarro et al. 1991; 2006), indirect Enzyme-linked Immunosorbent Assays (ELISAs) have also been used for serodiagnosis of *T. b. gambiense* (Vervoort et al. 1978; Lejon et al. 1998), Polymerase Chain Reaction (PCR) as well as other molecular based tests that detect the DNA/RNA of trypanosomes have also been developed, however some of these alternatives require sophisticated equipment, constant electricity and are mostly tailored for research settings and therefore not readily available in the field or rural areas where preliminary testing is required (Deborggraeve and Büscher, 2010; World Health Organization, 2013). Though the number of cases have reached an all-time low in the past few years, with 663 total cases reported in 2020 (Franco et al., 2022), diagnostics may be impaired by asymptomatic human and animal carriers referred to as 'cryptic parasite reservoirs' and they could be responsible for re-emergence of new cases (Büscher et al., 2018). To combat possible re-emergence post elimination of HAT, a recent highly specific diagnostic ELISA test for *T. b. gambiense* was developed called the g-iELISA (Geerts et al., 2021). The diagnostic test showed promising results in West and Central Africa with similar diagnostic accuracy to previous tests but with less disadvantages, however, it still needs to be evaluated under the prevailing conditions in endemic countries (Geerts et al., 2021).

Drugs are administered for HAT based on the stage of the disease, the oldest drug in use for the first stage of HAT is suramin (Wainwright 2010; Wiedemar et al. 2020). Suramin has been in use for almost a century now for treatment of *T. b. rhodesiense* and other diseases such as river blindness caused by *Onchocerca volvulus* and surra caused by *Trypanosoma evansi* (Hawking, 1958; Brun et al., 2010; Giordani et al., 2016),but due to its inability to cross the blood-brain barrier it is ineffective for stage 2 HAT (Wiedemar et al. 2020). Suramin is injected intravenously with a first dose of 4-5 mg/kg body weight followed by 5 doses weekly of 20 mg/kg body weight (Brun et al., 2010). Suramin has been shown to exhibit slight but reversible adverse reactions such as anaemia, dermatitis, nephrotoxicity and bone marrow toxicity (Anderson et al. 1976; Brun et al. 2010). Suramin is generally not administered for *T. b. gambiense* because of its activity against onchocerciasis which is common in *T. b. gambiense* endemic regions and can cause adverse

reactions in the co-infected patients (World Health Organization, 2013). For *T. b. gambiense,* pentamidine is the usual first line of action (Delespaux and de Koning, 2007). It is given intramuscularly with a dose of 4 mg/kg body weight for 7 days, produces minimal adverse reactions such as hypotension, diarrhoea, nausea and vomiting, but cannot also cross the blood-brain barrier making it ineffective for the second stage HAT (Nok, 2003; Brun et al., 2010; World Health Organization, 2013). In the absence of suramin, pentamidine has also been successfully used in a few cases as treatment for first stage disease caused by *T. b. rhodesiense* (Urech et al. 2011; Simarro et al. 2012).

From the mid-1900s, the foremost drug used as the first line of treatment against stage 2 HAT for *T. b. rhodesiense* and *T. b. gambiense* is melarsoprol (Barrett et al. 2007; World Health Organization 2013). Melarsoprol is synthesized with the addition of dimercaptopropanol, a heavy metal chelator to the arsenic group of melarsen oxide, this is to dampen the toxic effect of the arsenic group making it less toxic but still potent against the parasite (Friedheim, 1949; Babokhov et al., 2013; Fairlamb and Horn, 2018). Melarsoprol is given over a course of 10 days at 2.2 mg/kg body weight, it is administered intravenously and reported to be very painful on administration (Nok 2003; Lutje et al. 2010; Kuepfer et al. 2012). Although melarsoprol is very effective in clearing trypanosomes from lymph or blood in barely 24 hours, severe side effects such as encephalopathy occurs in about 10% of cases and results in fatality in over 50% of these patients (Robertson 1963; Blum et al. 2001; World Health Organization 2013). Other common side effects of treatment with melarsoprol include headache, thrombocytopenia, pyrexia, pruritus and heart failure (Barrett et al. 2007). Eflornithine is a recommended alternative to melarsoprol which gained attention and approval for its anti-trypanosomal activity in the late 20^{th} century, it is quite effective and not as toxic as melarsoprol but also not effective against *T. b. rhodesiense* (Balasegaram et al., 2009; Cullen and Mocerino, 2017). The delivery of eflornithine is however very cumbersome, a dosage of 100 mg/kg body weight is given intravenously every 6 hours for a total of 14 days (Barrett et al. 2007). Reversible side effects usually experienced with eflornithine are myelosuppression, seizures and gastrointestinal disorders which are generally less severe than in cases treated with melarsoprol and thus making it a more feasible option for treatment of *T. b. gambiense* (Priotto et al., 2008). To mitigate the difficulty of administering eflornithine alone, a nifurtimox-eflornithine combination therapy (NECT) has proved to be more efficacious (Priotto et al., 2009). Nifurtimox is an approved treatment for American trypanosomiasis (chagas disease) with adverse neurological and gastro-intestinal effects at high doses (Pépin et al., 1992), but a combination of both nifurtimox and eflornithine treatments in various trials seemed to achieve lower toxicity of both, reduced the length of dosage experienced with eflornithine by 50% and led to higher efficacy in treatment of *T. b. gambiense* (Checchi et al., 2008; Priotto et al., 2008).

Most of these drugs are obviously problematic for usage and/or delivery, while some are plagued with adverse toxic effects as in the case of melarsoprol, eflornithine is difficult to administer, the nifurtimox-eflornithine combination needs to be administered by professionals in a hospital setting, suramin and pentamidine are fully ionized at physiological pH and therefore cannot cross the blood-brain barrier making them insufficient to treat both stages of the disease (World Health Organization, 2013; Cullen and Mocerino, 2017). In addition to these difficulties, there is growing evidence for drug resistance for some of these chemotherapeutics, the nifurtimox-eflornithine combination therapy has shown potential for resistance and treatment failure is as high as 30% for melarsoprol (Ogada 1974; Pepin et al. 1987; Barrett and Croft 2012; Babokhov et al. 2013). Suramin and pentamidine also showed drug resistant phenotype on a tetracycline-inducible RNAi-dependent screen (Alsford et al., 2012; De Koning, 2020)After years of reliance on the NECT combination therapy or melarsoprol to treat second stage HAT, the disease finally received attention in the course of the last decade from organizations such as the Drugs for Neglected Diseases initiative (DNDi) in partnership with WHO as well as academia and industry (Pollastri, 2018). These organizations succeeded in assessing hundreds of compounds for their anti-parasitic activity, one of these compounds called fexinidazole had been shown previously to cure mice with chronic stage 2 HAT (Jennings and Urquhart, 1983). Fexinidazole was initially shelved because it was not commercially viable but was rescued as a promising candidate for treatment of HAT (Pollastri, 2018), preliminary tests carried out in mice where fexinidazole was administered orally at 100 mg/kg a day for 4 days in the case of acute infection and 200 mg/kg a day for 5 days for the chronic infection, completely cured the mice (Torreele et al., 2010). In addition, this short-course oral dose of fexinidazole was found to be well absorbed and tolerated *in vitro* and *in vivo*, showed no genotoxicity in mammalian cells and was effective for both *T. b. gambiense* and *T. b. rhodesiense* and therefore progressed to clinical trials (Torreele et al., 2010; Mesu et al., 2018). Fexinidazole, while still awaiting reports from phase III clinical trials in some countries has been recently approved as the first oral treatment for first and second stage HAT in *T. b. gambiense* (Deeks, 2019). In a trial conducted to assess the safety of fexinidazole in patients with early and late-stage HAT from Democratic Republic of Congo (DRC), fexinidazole given orally was shown to be effective and safe compared to NECT combination therapy (Mesu et al., 2018, 2021; Hidalgo et al., 2021).

The fexinidazole dosage given with a normal meal in human studies was 1800 mg/day for 4 days followed by 1200 mg/day for 6 days, adverse effects associated with fexinidazole include headache, decreased appetite, vomiting, asthenia, dizziness, insomnia and the few deaths recorded in treated cases are reported to be unrelated to the treatment (Mesu et al., 2018; Deeks, 2019). Updated WHO guidelines have now included fexinidazole as the recommended treatment for first stage *T. b. gambiense* HAT and second stage in individuals from six years and older (WHO, 2019; Lindner et al., 2020). The introduction of fexinidazole (FEX therapy) as a major achievement in HAT chemotherapy may pave way for other treatment

combinations with or without fexinidazole (Imran et al., 2022). While this is a first positive outcome in the past few decades for the treatment of HAT, poor compliance studies especially in early stage HAT show the possibility of increased incidence and an overall negative impact in the current decline of reported cases (Das et al., 2021). More options are still needed specially to avoid drug resistance.

T. brucei is an obligate parasite, hence it needs to keep adjusting its cell machinery for maximum functionality as it transitions between the various compartments of both hosts (Agbo et al., 2001; Abbeele et al., 2010). In addition to pressure from the immune system of the host, the parasite is also exposed to varying extreme conditions such as changes in temperature, pH and supply of nutrients and to thrive in this harsh and varied conditions, the parasite needs a proficient protein folding system adaptable across the different environments (Urbaniak et al. 2013; Zininga and Shonhai 2019). Therefore, in addition to the process of antigenic variation peculiar to the trypanosomes for surviving the immune attack of its host, another major toolbox in its arsenal of defences that it must rely on as it transitions and changes environment between the vector and mammalian host is its molecular chaperones (Maresca and Carratù, 1992; Folgueira and Requena, 2007) and these molecular chaperone proteins are being explored as drug targets in other organisms and for other diseases (Whitesell and Lindquist, 2005; Brodsky and Chiosis, 2006; Pallavi et al., 2010; Rochani et al., 2014).

1.3 Molecular chaperones

The term molecular chaperone is used to describe proteins that are involved in proteostasis or protein quality control in the cell. These proteins can interact with other proteins to ensure correct folding, unfolding, turnover rates as well as functionality (Ellis 1990; Hendrick and Hartl 1993; Hendrick and Hartl 1995; Schopf et al. 2017). They are essentially the third-party proteins that help other proteins achieve their active conformations and stabilize them, both in normal physiological cell conditions and when the cells are exposed to stress (Hartl and Hayer-Hartl, 2009). The major set of proteins that are categorized as molecular chaperones are called heat shock proteins (Hsps) which are conserved across species (Lindquist and Craig 1988; Hendrick and Hartl 1993).

Hsps are so named because they were initially identified to be upregulated during conditions of stress such as heat where they function either to prevent proteins from aggregating in such conditions, or to unfold already aggregated proteins and finally they can refold proteins already damaged or target them for degradation (Lindquist and Craig 1988; Hartl et al. 2011; Verghese et al. 2012). These Hsps are usually

located in various cellular organelles, including the cytosol as well as the extracellular environment (De Maio and Vazquez, 2013). Hsps have been classified in various ways across the years according to their molecular weights and relative to their human counterparts: small Hsps/HSPB, Hsp40/DNAJ, Hsp60, Hsp70/HSPA, Hsp90/HSPC and Hsp110/HSPH (Horváth et al. 2008; Kampinga et al. 2009; Hartl et al. 2011). They generally function to enhance proper folding of proteins by binding and protection of hydrophobic residues on their substrate proteins which would have been previously exposed from initial folding or from damage (Mayer 2010; Hartl et al. 2011). Most of these chaperones such as the Hsp60, Hsp70 and Hsp90 families are ATP dependent, they bind and hydrolyse ATP and then use the energy derived from this ATP hydrolysis to facilitate the formation of stable conformations of their substrates (Mayer 2010; Saibil 2013). Chaperones such as Hsp70 and Hsp90 have high affinity for their substrates in their nucleotide-free/ADP-bound state but association and dissociation rates for substrates are high in the ATP-bound state (Obermann et al. 1998; Mayer 2010).

Small Hsps are the least conserved in the chaperone family, their molecular mass ranges between 12 and 40 kDa, they can function independent of ATP and though they don't contain a signal sequence or a transmembrane domain, they are reported to be linked with modulating the fluidity and permeability of the lipid phase of membranes (Horváth et al. 2008; Mchaourab et al. 2009; Kriehuber et al. 2010). The Hsp40s are also called DnaJ proteins due to the presence of their J domains, they are generally known for their function in stimulating the ATPase activity of Hsp70 and they are vast in the trypanosomastids with 65 in *T. brucei* (Folgueira and Requena 2007; Kampinga and Craig 2010; Bentley et al. 2019). Hsp60 or group 1 chaperonin which has been well characterized as GroEL in *E. coli*, is an (un)foldase which is ATP dependent. The homologues of Hsp60 in eukaryotes are located in the mitochondria (Walter, 2002; Horváth et al., 2008). Hsp70, also known as DnaK in *E. coli* is a very well characterized chaperone specific for folding newly synthesized proteins (Boorstein et al. 1994; Horváth et al. 2008). Hsp70 is present in the endoplasmic reticulum (ER) and mitochondria in eukaryotes and carries out some of its chaperone functions by working with other ATP dependent chaperones such as Hsp90 (Requena et al. 2015). Hsp90 is another well characterized ATP dependent chaperone that is abundantly present in the cell under normal conditions (Zuehlke and Johnson 2010; Jackson 2013), beyond their chaperone functions they are mostly required in the activation of specific substrates called 'clients' and are present in the cytoplasm, mitochondria and ER (Zuehlke and Johnson, 2010). The Hsp100 family of chaperones are unique in their ability to disaggregate already aggregated proteins (Barends et al. 2010).

These heat shock proteins, being ubiquitous and critical to the functioning and survival of the cell implies that atypical levels of them are signature signals of various diseases such as cancers and neurodegenerative diseases (Horváth et al., 2008). In malignant cancerous cells, there is abnormal increased expression of one

or more chaperone proteins reflecting the continuous attempt of the chaperones to maintain proteostasis in such cells (Yufu et al. 1992; Kimura et al. 1993; Ralhan and Kaur 1995; Whitesell and Lindquist 2005), overexpression of Hsp70 and Hsp90 in breast cancer correlates with poor prognosis (Yano et al., 1996). Another major function of molecular chaperones is to target old and dysfunctional proteins for degradation (Soti, 2003). However, the accumulation of damaged proteins in aged animals leads to a chaperone overload and decreased potency of chaperones (Nardai et al. 2002), while the levels of Hsc70/Hsp70 remained similar in young and old rats, the amounts of Hsp90α/β were significantly lower in old compared to young rats leading to impaired Hsp90 activity (Conconi et al. 1996; Nardai et al. 2002). So far, heat shock proteins have generally been discussed related to their chaperone functions but with their conservation amongst pathogens, they also act as antigens triggering immune responses against infectious and non-infectious diseases and are therefore involved in immunity (Zügel and Kaufmann, 1999; Van Eden et al., 2007; Moin et al., 2021). Early research in protozoa infections identify heat shock proteins similar to Hsp70 and Hsp90 as antigens in *Plasmodium falciparum* (Jendoubi and Bonnefoy, 1988; Peterson et al., 1988).and in *Trypanosoma cruzi* (Dragon et al., 1987; Engman et al., 1989).

The proteins in these chaperone families do not work alone to achieve active and stable conformations of their substrates, but they usually work in a concerted and systematic manner with other chaperones and co-chaperones, forming a chaperone network of protein-protein interactions (PPI) critical for the functions of these chaperones (Freilich et al., 2018) . These PPI's formed are not static, they link the small Hsps to the larger chaperones via co-chaperones with various degrees of binding affinities, these interactions form the glue for the chaperone network making the folding machinery suitable to carry out their chaperone functions (Mayer and Bukau 2005; Hartl et al. 2011; Brehme et al. 2014; Freilich et al. 2018; Rizzolo and Houry 2019).

1.3.1 Regulation of heat shock proteins

Heat shock proteins have long been established to be upregulated in their expression in response to elevated temperatures and other environmental changes such as change in pH and osmotic pressure (Ploeg et al., 1985). For *T. brucei* and other kinetoplastid parasites, the insect host offers a poikilothermic environment while the mammalian host is homeothermic, this temperature stress triggers heat shock genes and also serves as a signal for differentiation and adaptation to a new environment (Velazques et al., 1980; Graefe et al., 2002). Heat shock proteins are directly involved in parasite differentiation as seen in *Leishmania donovani* (Wiesgigl and Clos, 2001a; Hombach et al., 2013). The application of heat shock for twenty-four

hours *in vitro* induced insect stage promastigote to the mammalian amastigote stage differentiation and this effect could be mimicked by geldanamycin inactivation of HSP90 (Wiesgigl and Clos, 2001a). In *T. cruzi,* inhibition of HSP90 by geldanamycin induced the heat shock response as seen in *Leishmania donovani* but did not induce stage differentiation (Graefe et al., 2002)

The expression of heat shock proteins in eukaryotes is generally controlled by heat-shock transcription factors that bind heat shock elements located in the promoters of heat shock genes and activating their transcription (Droll et al., 2013). Trypanosomes show some distinct molecular mechanisms especially with their RNA biology compared to eukaryotes, transcription for example is polycistronic and carried out by RNA polymerase II, mature RNAs are formed by *trans* splicing and polyadenylation (LeBowitz et al., 1993; Palenchar and Bellofatto, 2006; Michaeli, 2011). Final levels of RNA are achieved with a balance between the RNA synthesis and its degradation and for most trypanosome mRNAs, their rate of degradation is a critical determinant of expression (Manful et al., 2011). Due to these distinct processes, gene expression in kinetoplastids is important and relies on post-transcriptional mechanisms using RNA binding proteins (Clayton, 2002; Clayton and Shapira, 2007; Droll et al., 2013). A distinct group of these RNA binding proteins is characterized by the presence of a zinc finger motif (CCCH type) which binds directly to the RNA and are implicated for their role in controlling gene expression (Lai et al., 2000; Hudson et al., 2004; Kramer et al., 2010; Droll et al., 2013). Proteins containing the zinc finger domain have been identified in *T. brucei* (Hendriks, 2001). *T. brucei*, *T. cruzi* and *L. major* were found to contain forty-eight, fifty-one and fifty-four CCCH proteins respectively in a genome wide in silico screen analysis (Kramer et al., 2010). These proteins are involved in the regulatory process and of great significance in the stage-related differentiation processes.

Some *T. brucei* zinc finger proteins that have been studied and found to be involved in the control of gene expression include - The zinc finger protein ZC3H11 is a cytoplasmic protein conserved in all trypanosomes that regulates their hear shock genes as part of the post-transcription process. It binds selectively to mRNAs encoding chaperones and major Hsps such as HSP70, HSP83, HSP100, HSP110, HSP20, DNAJ1, DNAJ2, and FKBP involved in the stress response (Droll et al., 2013; Minia and Clayton, 2016). The function of ZC3H11 was tested in *T. brucei* procyclic trypanosomes parasites exposed to heat shock, the binding of ZC3H11 to several mRNAs stabilized the mRNAs and *HSP83* mRNA was still actively translated an hour after heat shock. (Kramer et al., 2010; Droll et al., 2013). The role of ZC3H11 in the bloodstream form stage is unclear but its depletion is lethal showing that ZC3H11 is also essential for survival of bloodstream stage *T. brucei* (Droll et al., 2013).

ZC3H32 is another zinc finger domain containing mRNA binding protein which localizes to the cytoplasm and is about twenty times more abundant in the bloodstream than the procyclic form in the *T. brucei*

parasite. The ZC3H32 protein is implied in roles in regulating mRNA translation and degradation in bloodstream-form trypanosomes (Klein et al., 2017). ZC3H30 also binds mRNA and is involved in its degradation. The ZC3H30 mRNA is present in bloodstream and procyclic forms at similar levels (Chakraborty and Clayton, 2018). Under normal conditions, ZC3H30 was found to be dispensable for growth and proliferation in both the bloodstream and the procyclic forms of the trypanosome but depletion of the ZC3H30 made the cells susceptible to cell stress (Chakraborty and Clayton, 2018). TbZFP1, TbZFP2 and TbZFP3 are small proteins (less than 150 amino acids each) with a zinc finger motif conserved in all kinetoplastids and implicated in the differentiation process of *T. brucei* (Hendriks, 2001; Hendriks and Matthews, 2005; Paterou et al., 2006; Walrad et al., 2012). TbZFP1is momentarily enriched during differentiation from the bloodstream to procyclic stage parasite and RNAi depletion of TbZFP2 in the bloodstream form affected the differentiation to the procyclic form (Hendriks, 2001). TbZFP1 is dispensable in bloodstream *T. brucei* cells, but TbZFP1 null mutants were not viable in procyclic forms (Hendriks and Matthews, 2005). Finally, the *T. brucei* zinc finger protein TbZC3H20 is enriched in the procyclic form stage and is also involved in regulating mRNA abundance by stabilizing procyclic form mRNAs (Ling et al., 2011).

1.3.2 The Hsp90 molecular chaperone system

Heat shock protein 90 (Hsp90) is highly conserved, present in bacteria and all eukaryotes but absent in archaea and is one of the most abundant molecular chaperones expressed in the cell, representing approximately 1-2% of the total cell proteins in the cytosol under normal growth conditions (Csermely et al. 1998; Stechmann and Cavalier-Smith 2004; Johnson 2012). Some isoforms of Hsp90 have been shown to be essential for viability, Hsp90β is indispensable in mice for development of embryos (Voss et al. 2000). Mutation of both *Hsp90* genes in yeast affected growth and similar effects were seen in *Drosophila melanogaster* and *Caenorhabditis elegans* (Borkovich et al. 1989; Spence and Georgopoulos 1989; Birnby et al. 2000; van der Straten et al. 1997; Whitesell and Lindquist 2005). In contrast to the effects seen in eukaryotes, HtpG (the bacterial Hsp90 homologue) is expressed at low levels and found to be non-essential in *E. coli* (Bardwell and Craig, 1988). Hsp90 was originally identified as a heat shock protein found in association with the steroid hormone receptor complex (Denis et al., 1988). In addition to its housekeeping chaperone functions such as folding and regulation, it is also involved in a myriad of other functions that cuts across cell cycle regulation, intracellular signalling and cell survival during stressful conditions (Csermely et al. 1998; Picard 2002; van der Straten et al. 1997; Li et al. 2012). Hsp90 is involved in the

activation and proper folding of a specific group of substrates called 'clients', these clients include kinases and steroid receptors are critical in many cellular processes including but not limited to signal transduction, (Richter and Buchner, 2001; Pratt and Toft, 2003). Also, Hsp90 is also implicated in various cancers as biomarkers and for its chaperone role in overexpressed and/or mutated proteins (Whitesell and Lindquist 2005; Kubota et al. 2010; Kazarian et al. 2017).

The approximate molecular weight of Hsp90 ranges from 82-96 kDa but due to its varying molecular weights in different orthologues, Hsp90 is referred to as C62.5 in *E. coli* (Bardwell and Craig, 1987). In yeast there are two almost identical *Hsp90* genes- *Hsc82* and *Hsp82*, the protein Hsc82 is constitutive but also induced to approximately 2-fold at higher temperatures while Hsp82 is induced almost 20 fold at higher temperatures (Borkovich et al., 1989). In *Drosophila*, the homologue of Hsp90 is Hsp83 (van der Straten et al., 1997), Hsp86 and Hsp84 in mice (Young et al. 2001) and Hsp83 in *T. brucei* (Pizarro et al., 2013).

In vertebrate/eukaryotes, there are four confirmed isoforms of Hsp90- the constitutive (Hsp90β) and the inducible (Hsp90α) expressed in the cytosol, a tumour necrosis factor receptor-associated protein 1 (TRAP-1) located in the mitochondria and a glucose regulated protein 94 (Grp94) located in the ER (Whitesell and Lindquist 2005). A fraction of the cytosolic Hsp90 usually localizes to the nucleus and this fraction increases after heat stress (Langer et al. 2003). In addition to the four previously mentioned isoforms, a fifth membrane-associated isoform, Hsp90N which lacks the typical ATP binding domain was previously discovered but later ruled out as a chimera of two genes including HSPC1 (Schweinfest et al. 1998; Chen et al. 2005). The nomenclature for the human Hsp90 isoforms has varied a lot over the years, Hsp90β was once denoted as Hsp86 and Hsp90α denoted as Hsp84 due to Hsp90β being slightly bigger (Csermely et al., 1998). Using the HSPC annotation for Hsp90, HSPC1 refers to Hsp90N/Hsp90AA1; HSPC2 refers to Hsp90α/Hsp90AA2; HSPC3 refers to Hsp90β/Hsp90AB1; HSPC4 refers to GRP94/Hsp90B1/endoplasmin and HSPC5 refers to TRAP-1/Hsp75 (Chen et al. 2005; Kampinga et al. 2009).

Hsp90 is a homodimer, and each monomer has three domains- an approximate 25 kDa N terminal/amino-terminal domain (NTD) where ATP binds, followed by an approximate 35 kDa middle domain (MD) for substrate binding, it also binds ATP and is a site for some co-chaperones to bind (Figure 1.4).

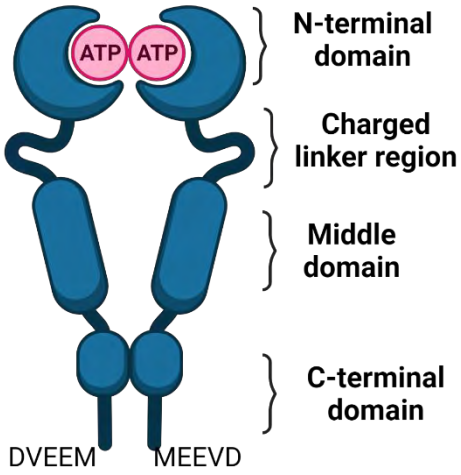


Figure 1.4: Domains of Hsp90.
The N-terminal domain (NTD) where the nucleotide binds is attached by a divergent linker sequence to the middle domain (MD) which is directly attached to the C-terminal domain (CTD). The terminal end of the CTD has a MEEVD motif where the TPR-containing chaperones bind (adapted from Jackson 2013).

The middle domain is directly linked to an approximate 12 kDa C terminal/carboxy-terminal domain (CTD) with a conserved MEEVD sequence (Young et al. 2001; Pearl and Prodromou 2006; Johnson and Brown 2009; Yamamoto et al. 2014). As shown in Figure 1.4, the NTD is separated from the MD by a charged linker sequence and both the NTD and CTD have been implicated to be sites of binding for substrate polypeptides (Young et al. 2001). The CTD is also the site of dimerization of the protein as well as the site of binding for TPR-containing chaperones at the terminal MEEVD motif (Pearl and Prodromou 2006; Jones et al. 2008).

With ongoing studies on Hsp90, it has become obvious that Hsp90 does not function alone, but its activities are regulated by a subset of proteins called co-chaperones. In addition to the regulation by co-chaperones, the chaperone abilities of Hsp90 have been shown variously to depend to a large extent on its capability to bind and hydrolyse ATP (Odunuga et al. 2004) and various structural and biochemical analyses have confirmed the chaperone as an ATPase (Pearl et al. 2008). This ATPase activity which comprises ATP/ADP exchange and ATP hydrolysis is controlled by the co-chaperones of Hsp90 (Odunuga et al. 2004). When ATP binds to Hsp90, a conformational change is induced moving the dimer from an open to a closed conformation which helps to stabilize the interaction of Hsp90 with its substrate and upon hydrolysis of

the ATP the conformation changes again, leading to the release of the substrate and all the co-chaperones involved (Young et al. 2001; Pratt and Toft 2003).

1.3.3 TPR and non-TPR containing co-chaperones

The co-chaperones generally function either by regulating the ATPase activity of Hsp90 or recruiting specified client proteins. Some studies have shown that the co-chaperones Cdc37, p23 and Hop/Sti1 all have an inhibitory effect on Hsp90 ATPase activity (Prodromou 1999; Panaretou et al. 2002; Siligardi et al. 2002; Lee et al. 2012) while Aha1 has an activating effect (Panaretou et al. 2002), and Cpr6 displaces and reverses the inhibitory effect of Sti1 (McLaughlin et al. 2002). The co-chaperones can be divided into two main categories- The TPR containing and non-TPR containing chaperones (Li et al. 2012). A comprehensive list containing the various Hsp90-interacting proteins which includes its co-chaperones is graciously maintained and updated by Didier Picard (listed at **http://www.picard.ch/**). Interactions of Hsp90 with any particular co-chaperone seems to be client and species-specific, also some co-chaperones have been shown to substitute for another as in the case of Cdc37 substituting for Hop in the folding process of protein kinases (Felts et al. 2007; Johnson and Brown 2009; Zuehlke and Johnson 2010).

1.3.3.1 TPR containing co-chaperones

The TPR-containing co-chaperones are a large class of co-chaperones that contain domains with three or more degenerate 34 amino-acid helix-turn-helix tetratricopeptide repeat (TPR) motifs with which they recognize and bind to the C-terminal MEEVD motif of Hsp90 (Lamb et al. 1995; Scheufler et al. 2000; Young et al. 2001). There are at least five TPR-domain containing co-chaperones (Hop, PP5, FKBP52, SGT1, Cyp40 and Cns1) that have been characterized in various organisms and they tend to compete for binding to the MEEVD sequence at the CTD of Hsp90 (Johnson and Brown 2009). Hop/Sti1 contains three TPR domains with three TPR motifs in each domain (Scheufler et al., 2000). It is predicted to have an adaptor function because it links Hsp70 to Hsp90, enabling the transfer of substrates from Hsp70 to Hsp90 in the protein folding process (Odunuga et al. 2004; Makumire et al. 2020). Protein phosphatase 5 (PP5) is a serine/threonine protein phosphatase of the PPP family and exists in all eukaryotes. It has a TPR domain at its N-terminal containing about three to four TPR motifs with which it binds to Hsp90 and a C-terminal

catalytic domain and has been implicated in various signal transduction pathways (Chinkers 2001; Jones et al. 2008). FKBP52 and Cyp40 are members of the immunophilin family, both containing a TPR domain with which they bind to Hsp90 forming separate heterocomplexes and their TPR domain is attached to a peptidyl-prolyl isomerase (PPIase) domain (Owens-Grillo et al. 1995; Young et al. 2001). Small glutamine-rich TPR-containing protein (SGT) contains a TPR domain and a p23-like domain and is involved in cellular quality control (Wunderley et al., 2014). Cyclophilin 7 suppressor (Cns1) in yeast binds to both Hsp70 and Hsp90 through its TPR domain, is essential for cell viability and was shown to activate the ATPase activity of Hsp70 in yeast (Marsh et al. 1998; Hainzl et al. 2004). A TPR-containing protein associated with Hsp90 (Tah1) together with a protein interacting with Hsp90 (Pih1) was discovered in a genome-wide screening to identify Hsp90 interacting partners in yeast (Zhao et al., 2005), further studies into this Tah1-Pih1 complex also suggests a role in Hsp90 ATPase activity and client recruitment (Eckert et al., 2010; Jiménez et al., 2012).

1.3.3.2 Non-TPR containing co-chaperones

The non-TPR containing co-chaperones include Aha1, p23 and Cdc37. Aha1, has a molecular weight of about 38 kDa, binds to the middle domain of Hsp90 and is known for its ability to stimulate the ATPase activity of Hsp90 (Panaretou et al. 2002; Meyer et al. 2003; Seraphim et al. 2013). p23 is said to have intrinsic chaperone properties by itself, it binds the ATP-bound form of Hsp90 and seems to play a role in the dimerization of this domain (Freeman et al.1996; Young and Hartl, 2000). Cdc37 is known for its role in linking Hsp90 to protein kinases, it binds to the NTD of Hsp90 with its middle domain and has also been shown to inhibit ATPase activity of Hsp90 (Kimura et al. 1997; Pearl and Prodromou 2006).

Hsp90 forms multiple complexes with these co-chaperones during its life cycle in the activation of clients and various studies have been carried out to see the progression of the complexes especially given the fact that most of the TPR-containing chaperones have to compete for the same binding sites on Hsp90 (Prodromou 1999). To actively fold a client, it is assumed that Hsp90 first forms a complex with TPR-containing Sti1/Hop which would bind to one monomer of the Hsp90 in its open conformation and inhibit its ATPase activity (Figure 1.5).

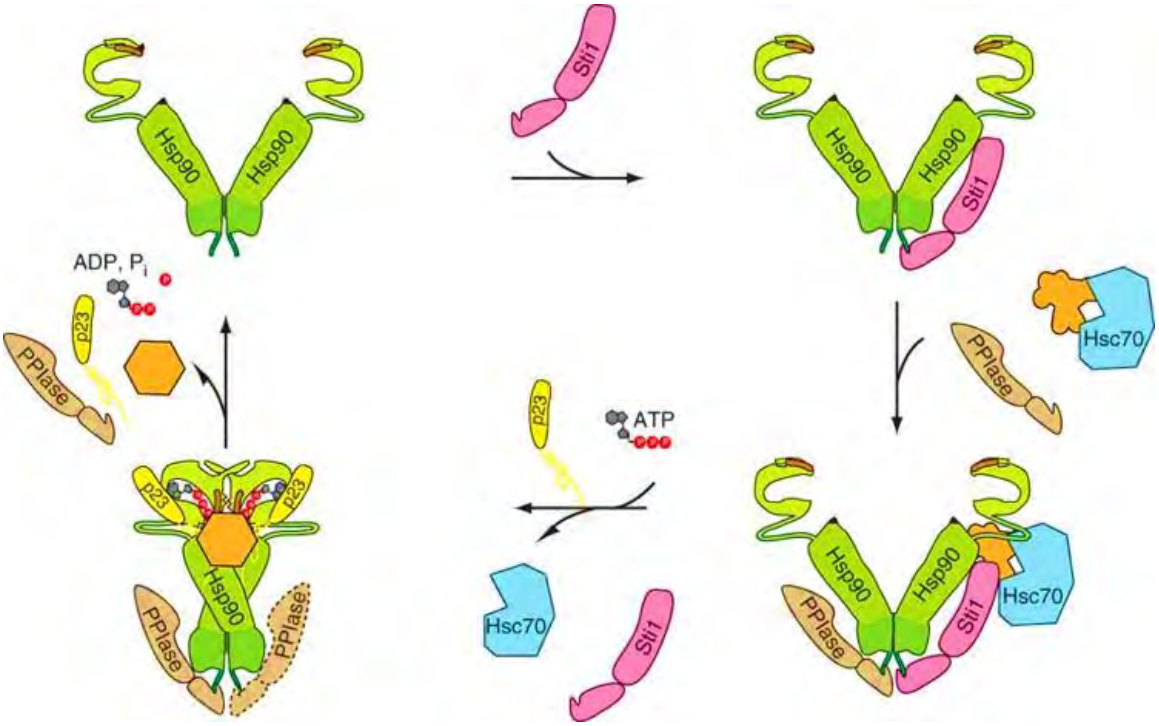


Figure 1.5: The Hsp90 and its co-chaperones cycle model.
Hsp90 exists as a dimer in the open conformation and for the successful folding and release of a client shown in orange, it involves the co-operation of Hsc70 and various co-chaperones starting with Sti1. The process begins with Hsc70 bound to the substrate/unfolded protein, followed by the binding of Sti1/Hop which would serve as the scaffold linking Hsc70 to Hsp90. The immunophilins are added to the complex and in the presence of ATP Hsp90 forms a closed conformation stabilized by p23 completing the foldosome. Upon ATP hydrolysis to yield ADP and P_i, the client is released and Hsp90 returns to its open conformation (adapted from Li et al.2011).

Next, Hsc70 bound to a client attaches to Sti1/Hop and a TPR-containing PPIase attaches to the second monomer of the Hsp90, in the presence of ATP and p23, Hsp90 converts to a closed conformation. In this closed conformation, the binding of Sti1/Hop is weakened, and it is released giving room for another PPIase to attach to the complex. After ATP is hydrolysed, the PPIases, p23 and the now folded client is released and Hsp90 returns to its open conformation (Li et al. 2011). The number of co-chaperones differ in different organisms according to the study carried out by Johnson and Brown 2009**,** no particular co-chaperone was present in all the 19 organisms examined, Hop/Sti1 was the most conserved and prevalent while Cdc37 was the least conserved. Furthermore, all organisms studied contained an orthologue of either Hop/Sti1 or Cdc37.

1.3.4 Stress inducible protein 1 (Sti1)

The co-chaperone Stress inducible protein 1 (Sti1) was first identified and characterized in *Saccharomyces cerevisiae* as a heat inducible protein with molecular weight of about 66 kDa that was required for cellular growth in stressed conditions (Nicolet and Craig, 1989). Subsequently, Sti1 has been identified as highly conserved and its homologues have been characterized in humans (Honoré et al., 1992) and other organisms such as *Leishmania major* (Webb et al., 1997), murine (Lässle et al., 1997), *Caenorhabditis elegans* (Song et al. 2009) and *Trypanosoma cruzi* (Schmidt et al., 2011). Sti1 is also known as Hsp70-Hsp90 organizing protein (Hop) because it was first described in relation to its presence in a complex with Hsp70 and Hsp90 (Smith et al. 1993), it was discovered that Sti1/Hop could bind independently or simultaneously with Hsp70 and Hsp90 and supposedly acted as a bridge connecting Hsp90 to a Hsp70-substrate complex in the assembly of receptor complexes (Chen and Smith 1998). In addition to the signature adaptor function of Hop/Sti1 previously described in progesterone receptor complexes, it is also implicated in homeostatic functions in the cell during heat stress though it has no chaperone function of its own (Nicolet and Craig 1989; Chen and Smith 1998; Song et al. 2009). Other functions include its role in regulating the ATPase activities of both Hsp70 and Hsp90 (Prodromou 1999; Wegele et al. 2003), *Sti1* mutant had reduced lifespan in worms suggesting a role for Sti1 in longevity (Song et al. 2009) and it has been implicated in neuroprotection in mouse neurons (Zanata et al. 2002).

Sti1/Hop is a monomeric protein though previously reported to be a dimer (Prodromou 1999; Yi et al. 2010; Li et al. 2011). It has a conserved structure with three TPR domains (TPR1, TPR2A and TPR2B) widely accepted to be involved in protein-protein interactions and two aspartic acid-proline (DP) domains with a flexible linker between the domains, arranged in the order TPR1-DP1-TPR2A-TPR2B-DP2 (Figure 1.6) (Chen et al. 1996; Scheufler et al. 2000; Odunuga et al. 2004; Yamamoto et al. 2014; Röhl et al. 2015). The N terminal TPR1 domain binds to the C-terminal heptapeptide motif of Hsp70 while the middle terminal TPR2A domain provides a binding site for the C-terminal pentapeptide motif of Hsp90 (Lässle et al. 1997; Demand et al. 1998; Scheufler et al. 2000). A carboxylate clamp is formed as the acidic residues from the EEVD motifs of Hsp70 and Hsp90 bind the basic amino-acid residues within the TPR domains (Scheufler et al., 2000; Flom et al., 2006). The characterization of the C-terminal TPR2B as well as its specific ligand was initially debatable (Odunuga et al. 2004) but it is clearly involved in the interaction of the chaperones, as results from mutations in the various domains show that TPR2A alone does not lead to successful binding of Hsp90 and these three TPR domains may be overlapping and redundant in their functions (Flom et al., 2006, 2007; Schmid et al., 2012; Röhl et al., 2015b). The DP repeats at the C-terminal end are also important

in the Hop-Hsp70-Hsp90 complex as mutations in this region affect binding to Hsp70 and affects recruitment of Hsp90 in the progesterone-receptor pathway complex (Nelson et al. 2003).

Figure 1.6: Domain organization of yeast Sti1.
TPR domains (blue) joined to the DP domains (green) by flexible linkers (adapted from Röhl et al. 2015).

Sti1/Hop predominantly localizes to the cytoplasm under normal growth conditions in the cell, but various sources have reported its presence in the Golgi apparatus, cell surface and nucleus upon heat shock. (Honoré et al. 1992; Lässle et al. 1997; Daniel et al. 2008). A putative nuclear localization signal has been found associated with mSTI1 close to residues identified as phosphorylation sites for cell cycle kinases (Longshaw et al., 2004). This phosphorylation may also be adding another layer of regulation for Sti1/Hop localization as mSTI1 phosphorylated by CKII localized to the nucleus while mSTI1phosphorylated by cdc2 kinase localized to the cytoplasm (Longshaw et al., 2004) and the equilibrium between Hop in its free state and Hop complexed to Hsp90 may also be regulated by Hop phosphorylation (Daniel et al. 2008). Most of these phosphorylation sites that have been characterized are conserved across murine and human species (Bhattacharya and Picard 2021).

Recent discoveries have debunked the role of Sti1/Hop as being indispensable in the interaction between Hsp70 and Hsp90 (Kravats et al. 2018; Bhattacharya et al. 2020). Prokaryotes such as *E. coli* have been established to possess homologues of Hsp70 and Hsp90 but lack identifiable co-chaperones including Sti1/Hop homologue but they are able to interact nonetheless and form complexes stabilized by their client binding (Genest et al., 2015). An *in vitro* study in yeast also identified residues in the middle domain of yeast Hsp90 similar to that of bacteria where Hsp70 can bind directly (Kravats et al., 2018). Human cell lines with Sti1/Hop knocked out were characterized and the cells are able to develop compensatory mechanisms to maintain proteostatic balance (Bhattacharya et al. 2020). Despite these developments, Sti1/Hop has been implicated to be upregulated and promotes the progression of various cancers (Erlich et al., 2007; Kubota et al., 2010); its regulatory activities with Hsp90 has been described to have an impact for survival in cancerous cells, therefore, understanding and controlling its activities is one approach being used as a putative target for cancer therapeutics (Ruckova et al. 2012; Röhl et al. 2015).

1.3.5 Hsp90 and Sti1 as a drug target

With the increasing understanding of the functions of Hsp90 and its role in diseases, numerous small molecule drugs targeting its function and thereby its clients have been identified to be suitable for chemotherapy in cancer and other diseases (Neckers and Ivy 2003; Whitesell and Lindquist 2005). Hsp90 is expressed ~ 10 fold higher levels in tumour cells compared to their normal counterparts, this is because many proteins in these cells depend on the Hsp90 chaperone machinery for their activation and stability and so inhibiting Hsp90 ultimately leads to degradation of these client proteins(Isaacs et al. 2003; Barrott and Haystead 2013; Edkins 2016) . These small molecule inhibitors or chemical probes can act either by inhibiting the enzyme activity of the chaperone or by inhibiting the protein-protein interaction in the chaperone network. So far, many of these inhibitors have progressed to different phases of clinical trials (Barrott and Haystead, 2013; Gestwicki and Shao, 2019). Geldanamycin, a naturally occurring anti-tumour antibiotic (Deboer et al., 1970) was the first Hsp90 inhibitor discovered (Whitesell et al. 1994). It was found that it mimics the ATP/ADP nucleotides and therefore binds Hsp90 at its ATP-binding site (the NTD), inhibiting the ATPase activity of the chaperone and leading to degradation of its clients (Prodromou et al. 1997; Miyata 2005). Geldanamycin and its analogues such as 17-allylaminogeldanamycin (17-AAG) that bind at the NTD have been extensively studied and some have progressed to clinical trials (Miyata 2005; Shrestha et al. 2016). Other Hsp90 inhibitors that bind at the NTD include radicicol which is structurally distinct from geldanamycin and also competes with it for binding (Schulte et al., 1998). Apart from these small molecules that target the NTD, some other molecules such as novobiocin acts in a manner similar to geldanamycin but binds at the CTD (Marcu et al. 2000; Donnelly and Blagg 2008; Chen et al. 2017). Various structural analogues of novobiocin have been developed and they have been shown in various cell lines to exhibit far greater efficacy than novobiocin (Donnelly and Blagg, 2008). Despite the potency of most of these inhibitors in pre-clinical trials, major draw backs come from toxicity (Park et al., 2020). A review on Hsp90 pan-inhibitors showed toxic side effects such as hepatotoxicity and ocular toxicity and these have prevented their FDA approval (Jhaveri et al., 2012; Sanchez et al., 2020; Yu et al., 2022).

Most of these compounds have been studied as inhibitors in the context of cancer therapeutics, however, there are also studies with promising findings to show the efficacy of these inhibitors in parasitic organisms. The dependence on Hsp90 has been tested *in vitro* using the known Hsp90 inhibitors geldanamycin or radicol, in *L. donovani*, this led to a phenotype mimicking the insect to mammalian transition as well as the differentiation of the amastigote from the promastigote stage (Wiesgigl and Clos, 2001b; Roy et al., 2012). *In L. major*, 17-AAG induced abnormal autophagy and eventually led to parasite death (Aloa et al., 2021). Inhibiting Hsp90 in *T. cruzi* led to a heat shock response and halted the cell division process although the

life cycle stage differentiation as seen in *L. donovani* did not take place in *T. cruzi* in response to the inhibition (Graeme et al. 2002) and in *Plasmodium falciparum*, geldanamycin inhibited the growth of the parasite in human erythrocytes (Banumathy et al., 2003).

A panel of known Hsp90 inhibitors was screened against the Hsp90 chaperone in *T. brucei* and explored using biochemical and biophysical techniques to find more potent compounds selective for Hsp90 in *T. brucei* compared to their binding affinity to human Hsp90 isoforms (Pizarro et al., 2013). The authors show that out of the 40 compounds screened, most of which are ATP-competitive inhibitors previously described in literature, about five showed EC_{50} values in sub micromolar range potent against *T. brucei* 427 bloodstream stage parasites and therefore were able to inhibit growth of the parasite *in vitro.* Furthermore, one of the five compounds showed a much higher affinity for TbHsp83 in the parasite compared to Hsp90 of the host. This study provides results from the preliminary stages of potential drug development and provides a basis for more research to confirm the findings and explore the differences between the chaperones and co-chaperones of *T. brucei* compared to those of their hosts.

The co-chaperone, Sti1(Hop), being a major part of the Hsp90 complex, has been implicated in breast and ovarian cancer cells (Whitesell and Lindquist 2005; Sims et al. 2011; Tsai et al. 2012). Hop is therefore being explored as a promising alternative target for the design of inhibitors. This choice of co-chaperones as an alternative target offers more selectivity for the cytosolic Hsp90 chaperone as compared to the Hsp90 inhibitors that target the NTD of all the Hsp90 isoforms (Baindur-Hudson et al. 2015; Edkins 2016). The various inhibitors targeting the Hop interaction with Hsp90 are thought to weaken the downstream folding pathway as well as to inhibit Hsp90 itself (Yi and Regan 2008; Pimienta et al. 2011; Edkins 2016). Various strategies have been employed to design inhibitors to Hop. The first and major approach are small molecules that target the TPR domains in Hop thereby interrupting the binding with Hop and Hsp90 (Pimienta et al. 2011; Horibe et al. 2012). One of these compounds, 1,6-dimethyl-3-propylpyrimido[5,4-*e*][1,2,4]triazine-5,7-dione (C9) was effective in killing breast cancer cells including a drug-resistant subtype (Pimienta et al. 2011). Another set of compounds such as sansalvamide A-amide targets and disrupt proteins that bind at the C-terminal domain of Hsp90 including Hop, but has no effect on ATP hydrolysis (Vasko et al., 2010; Ardi et al., 2011). Premised on these promising findings in human cell lines, and with Hsp90 chaperone being highly conserved across species, the Hsp90-Sti1 interaction could present a viable druggable target in parasitic organisms such as *T. brucei*.

1.4 Knowledge gap and Motivation

The phrase 'neglected tropical diseases' was coined in 1970 by the Rockefeller Foundation to refer to the chronic and crippling diseases caused by the TriTryps, these diseases are usually prevalent in poor and rural communities and so there is no financial incentive for pharmaceutical companies to focus on therapeutics (Bhattacharya et al. 2020). HAT, as previously mentioned is fatal if left untreated, and the tool box for its diagnosis and treatment is old and plagued with various difficulties (Chappuis et al., 2005; Brun et al., 2010; Wastling and Welburn, 2011). The approval of the first ever oral drug (fexinidazole) for treatment of HAT in 2019 is proof of interest/funding in recent years and also a testament to the successful approach of repurposing drugs (Deeks, 2019; Fernández-Prada et al., 2019). Despite the current positive strides made in the search for chemotherapeutics for HAT, the ongoing struggles in vaccine development research(Tabel et al., 2013; Magez et al., 2021a), the continuous problem of drug resistance (Delespaux and de Koning, 2007) and the fear of endemic resurgence of HAT, more satisfactory, easy to deliver and safe chemotherapeutic options are still needed to combat the disease.

T. brucei is a digenetic parasite that shuttles between a vertebrate and invertebrate host during its life cycle (Bhattacharya et al. 2020). The different environments across both hosts exposes the parasite to changing temperature, pH, nutrients and other conditions that cause cellular stress, to ensure its survival and virulence, the parasite relies on the abundantly expressed molecular chaperones that help in promoting folding of proteins and prevent aggregation tendencies during these stressful conditions (Caplan et al. 2007; Requena et al. 2015). The highly conserved Hsp90 molecular chaperone is defined as the hallmark of the stress response in the cell, making up about 2% of the proteins in the cell under normal conditions and indispensable for the growth and survival of many organisms including *T. brucei* (Jones et al. 2008; Johnson and Brown 2009). The presence of numerous co-chaperones complexed with Hsp90 begs the question of which of them is essential or dispensable in the chaperone activities of Hsp90. Some co-chaperones were seen to be specific to obligate parasites such as Sti1, PP5, p23 and Aha1 with Sti1 being the most prevalent in the organisms examined (Johnson and Brown 2009) and therefore likely to play essential roles in the life cycle of these parasites. Inhibitors against Hop have shown promising results as therapeutics in cancer (Yi and Regan 2008; Pimienta et al. 2011; Edkins 2016) and repurposing such drugs against protozoan parasites can be explored.

The successful completion and availability of the TriTryps genomes have offered new possibilities to screen and identify new drug targets. An updated *in silico* analysis of the Hsp70/J protein machinery in *T. brucei* has been conducted exploring the evolutionary, structural, and functional complements of the protein and

its relationship to human and other selected kinetoplastid parasites (Bentley et al. 2019). With the numerous proteomics data published recently on the different life cycle stages of *T. brucei,* there is need for an updated *in silico* analysis of the Hsp90 chaperone complement of *T. brucei.* Apart from *in silico* studies, very little research has been done on Hsp90 in *T. brucei* (Pizarro et al., 2013), also, the co-chaperone Sti1 has been characterized in just two of the TriTryps -*Trypanosoma cruzi*, *Leishmania major*, and *Leishmania donovani* (Webb et al., 1997; Schmidt et al., 2011; Hombach et al., 2013). With the little research that has been done on TbSti1 and the Hsp90-Sti1 partnership in the extracellular parasite *T. brucei,* this study set out to explore both. For a biochemical characterization of TbSti1, this study also explored well characterized mammalian cell lines with human Hop knocked out (Yin et al. 2019; Bhattacharya et al. 2020) as a platform for characterizing TbSti1.

1.5 Hypothesis

The TbHsp83-TbSti1 interaction has unique characteristics similar to the well characterized Hsp90-Sti1 interaction in intracellular parasites and humans and will be important for the biology and functioning of *Trypanosoma brucei*.

1.6 Aim

The aim of this study is to characterize TbSti1 in relation to *Trypanosoma brucei* Hsp83 and human Hsp90

1.7 Broad objectives

1. *In silico* analysis of the Hsp90 chaperone machinery of the African trypanosome, *Trypanosoma brucei.*
2. Biochemical characterization of the *Trypanosoma brucei* co-chaperone TbSti1 and its relationship with TbHsp83 and human Hsp90

1.8 Specific Objectives

1. *In silico* characterization and analysis of the numerous published proteomic data on Hsp90 in *Trypanosoma brucei.*
    - Data mining of Hsp83 and its co-chaperones in *T. brucei*
    - Identification of the human and selected kinetoplastid orthologues of Hsp90 and Hsp83 co-chaperones in *T. brucei*
    - Primary structure sequence analyses
    - Phylogenetic analysis of the evolutionary relationship of Hsp90s in *T. brucei* in relation to human and selected kinetoplastid orthologues of Hsp90
    - Identification of protein domains, protein features, and predicted subcellular localization of Hsp90 and hsp83 co-chaperones in *T. brucei*
    - Prediction of full-length 3D model structure of TbSti1 and its sequence conservation compared to human and other selected kinetoplastids
    - Analysis of the Hsp83 phosphoproteome.
    -

2. Recombinant expression and purification of TbSti1, TbHsp83 and human Hsp90 from *E. coli*
    - Confirmation of all plasmids - pET-30a-TbHsp83.1 plasmid, pET-16b-Hsp90a plasmid, pQE60-TbSti1 and pCDNA3.1(+)_HA-Tbsti1 confirmation using agarose gel electrophoresis.
    - Heterologous production/induction of TbSti1, TbHsp83 and human Hsp90 using 2xYT and terrific broth for maximum yield.
    - Purification of the recombinant proteins from *E. coli* using nickel affinity chromatography.

3. *In vitro* analysis of the interaction between TbSti1 and TbHsp83/Hsp90
    -
    - Qualitative and quantitative interaction assays to investigate the direct binding interaction of TbSti1 with TbHsp83 and human Hsp90 (far western and solid phase binding assay).
    - Investigation of the suppression of aggregation activity of the chaperones TbHsp83 and hHsp90 on malate dehydrogenase (MDH) as substrate in the presence and absence of the co-chaperone TbSti1.

- Investigation of the effect of TbSti1 on the ATPase activity of TbHsp83 and hHsp90.

4. *Ex vivo* analysis of the interaction between TbSti1 and TbHsp83/Hsp90
    - To detect TbSti1 and TbHsp83 in the *T. brucei* parasite lysate and investigate their heat inducible expression.

- Analysis of TbSti1 interaction with Hsp90 and/or Hsp70 by immunoprecipitation from transfected mammalian cell lysates.
- Immunofluorescence assay and microscopy to determine the subcellular localization of TbSti1 when overexpressed in human (HeLa CRISPR Hop knockout (KO)) cell lines transfected with pcDNA3.1(+)_HA-TbSti1.
- Immunofluorescence staining and microscopy to compare the effect of TbSti1 expression in human (HeLa CRISPR Hop knockout (KO)) cell lines transfected with pcDNA3.1(+)_HA-TbSti1.
- Immunofluorescence staining and microscopy to compare morphological effects of TbSti1 expression in in human (HeLa CRISPR Hop knockout [KO]) cell lines transfected with pcDNA3.1(+)_HA-TbSti1.

2 *In Silico* analysis of the Hsp90 chaperone system from the African trypanosome, *Trypanosoma brucei*

2.1 Introduction

TriTryps is an umbrella term for the unicellular protozoan parasites with a distinct mitochondrial DNA called a kinetoplast, the parasites include – *Trypanosoma brucei* (*T. brucei*), *Trypanosoma cruzi* (*T. cruzi*) and *Leishmania major* (*L. major*) responsible for causing African trypanosomiasis, American trypanosomiasis and leishmaniasis respectively (Alcântara et al., 2018; Pita et al., 2019). Though the TriTryps have many shared features, their causative organisms, life cycle progression, target tissues and pathogenesis in mammals differ across each of the organisms (Jackson 2015). *T. brucei* which is a well-studied model of the TriTryps is the organism of interest for this study. It must transition between its parasite vector, the tsetse fly and its mammalian host to complete an infectious life cycle and this transition exposes it to fluctuating environmental conditions (Folgueira and Requena, 2007). To combat this varying conditions, *T. brucei* relies on an arsenal of tools such as its capacity for antigenic variation and its molecular chaperone machinery (Maresca and Carratù 1992; Stuart et al. 2008). At the molecular level, a unique characteristic of gene regulation in trypanosomes is their reliance on polycistronic transcription processed by trans-splicing and there are no known RNA polymerase II promoter and regulatory sequences (Preußer et al. 2012). Due to this reliance on polycistronic transcription which differs considerably from transcription in most eukaryotes, trypanosomes rely on post-transcriptional mechanisms for gene regulation (Preußer et al. 2012). As a result of the complexities of transcription regulation and the obvious differences observed between transcriptomic and proteomic data, trypanosome research in the area of transcription regulation has largely shifted to focus on proteomic studies (Urbaniak et al. 2012; Butter et al. 2013; Goos et al. 2017). The proteome of the nucleus (Goos et al., 2017), nuclear pore (DeGrasse et al., 2008), flagellum (Broadhead et al., 2006; Subota et al., 2014), mitochondrion (Panigrahi et al., 2009), mitochondrial importome (Peikert et al., 2017), mitochondrial membranes (Acestor et al., 2009), glycosome (Colasante et al., 2006; Güther et al., 2014), cell surface (Shimogawa et al., 2015) and life cycle stages (Urbaniak et al. 2012; Butter et al. 2013; Gunasekera et al. 2012) of *T. brucei* have been explored and made available for analysis.

Genetic characterization of the Hsp90 complement in the TriTryps have shown an extensive and expanded number of genes encoding for Hsp90 all arranged in a tandem head to tail fashion and located on a single chromosome (Dragon et al. 1987; Mottram et al. 1989; Hübel and Clos 1996). Folgueira and Requena 2007

identified 17 *Hsp90* genes in *L. major* and 6 in *T. cruzi* after mining the genome of the various databases. Earlier, 10-12 *Hsp90* gene copies had been reported in *T. brucei* (Mottram et al. 1989). In these organisms Hsp90 has been established to function with accessory proteins called co-chaperones, the number of these co-chaperones and their specific functions differ across species (Zuehlke and Johnson, 2010). An added layer of regulation of the chaperone Hsp90 in various organisms comes from post translational modifications (PTMs) (Backe et al., 2020) and in *T. brucei*, the main PTMs include phosphorylation and acetylation (Moretti et al. 2018; Nett et al. 2009) which would be explored in further details in this study. Updated information on the kinetoplastid parasites investigated in this study is found in TriTrypDB (version 46) database (https://tritrypdb.org/tritrypdb/) (Aslett et al., 2010) and a summary of these features are shown in Table 2.1.

Table 2.1: Summary data of selected kinetoplastids used in this study

Species	Strain	NCBI taxon ID	Genome size (Mbp)	Chromosomes	Genes
T. b. brucei	TREU927	185431	35.83	16	11764
T. b. gambiense	DAL972	679716	22.15	11	8082
T. cruzi	CL Brener Esmeraldo-like	_	32.53	41	10596
T. cruzi	Dm28c 2018	_	53.27	_	19112
T. cruzi	Marinkellei strain B7	_	38.65	_	10228
L. major	Friedlin	347515	32.86	36	9378
C. fasciculata	Cf-Cl		32.63	30	9619
Bodo saltans	Lake Konstanz	_	39.86	_	18222

In *T. brucei* an extensive *in silico* analysis of the Hsp70/J protein machinery has been conducted exploring the structural, functional and evolutionary complements of the protein and its relationship to human and other selected kinetoplastid parasites (Bentley et al. 2019). Given the extensive proteomics data now available on the different organelles and life cycle stages of *T. brucei* there is need for an updated *in silico* analysis of the Hsp90 chaperone complement of *T. brucei.* In this study, *in silico* tools were used for a comprehensive structural, functional, and evolutionary analyses of the Hsp90 complement in human and selected kinetoplastid species.

2.2 Objectives

1. Data mining of Hsp90 and its co-chaperones in *T. brucei*
2. Identification of human and selected kinetoplastid orthologues of Hsp90 and its co-chaperones in *T. brucei*
3. Primary structure sequence analyses
4. Phylogenetic analysis of the evolutionary relationship of Hsp90s in *T. brucei* in relation to human and selected kinetoplastid orthologues of Hsp90
5. Identification of protein domains, protein features, and predicted subcellular localization of Hsp90 and its co-chaperones in *T. brucei*
6. Prediction of full-length 3D model structure of TbSti1 and its sequence conservation with human and other selected kinetoplastids
7. Analysis of the Hsp83 phosphoproteome

2.3 Methodology

2.3.1 Database mining, sequence analyses and the determination of the trypanomastid and human orthologues

A BLASTP search using the amino acid sequences of Hsp90 isoforms from the *T. b. brucei* obtained from a previous *in silico* study (Folgueira and Requena, 2007), and the human HSPC2, HSP90AB1/HSPC3, HSP90B1/HSPC4 and HSPC5 isoforms were used as queries on TriTrypDB (version 46) (https://tritrypdb.org/tritrypdb/) (Aslett et al., 2010) and were analysed in order to determine the Hsp90 complement encoded on the *T. b. gambiense* genome, as well as identify new *T. b. brucei* Hsp90/HSPC protein members. The e-value was set at a stringent level of e^{-10} to identify potential Hsp90/HSPC-related sequences for further analysis. Additionally, a keyword search was performed to scan the genome of *T. b. gambiense* for Hsp90/HSPC genes on the TriTrypDB using the search terms: “Hsp90”, “Hsp83”, “heat shock protein” and “molecular chaperone”. The retrieved amino acid sequences from the various keyword searches were screened using Interpro (Integrative protein signature database;

http://www.ebi.ac.uk/interpro/) (Apweiler et al., 2000) which integrates 13 member databases including SMART 7 (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/) (Letunic et al. 2012) and PROSITE (http://prosite.expasy.org/) (Sigrist et al., 2010) for domains annotated by the online servers as "Hsp90". After identification the domains were built by Dr. Stephen Bentley using DOG 2.0 (Domain graph; http://dog.biocuckoo.org/down.php) (Ren et al., 2009).

For identification of *T. brucei* orthologues of selected cytosolic Hsp90 co-chaperones, the protein sequences of the human co-chaperones were used as queries in a BLASTP search on the TriTrypDB. Reciprocal BLASTP was conducted to determine if the identified putative *T. brucei* co-chaperone had the closest match to the equivalent human co-chaperone. The putative amino acid sequences of the co-chaperones from both *T. brucei* subspecies were used as queries in a BLASTP search on the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov), using the default parameters. If the most similar orthologue in the *T. brucei* subspecies was identical to the Hsp90 co-chaperone sequence used as first query, the sequence of the second query was selected as an orthologue. Reciprocal BLASTP was also conducted for the identification of human and selected kinetoplastid orthologues of the putative Hsp90/HSPC proteins from both *T. brucei* subspecies.

2.3.2 Primary structure sequence analyses and homology modelling

Multiple sequence alignment was performed on all Hsp90s in *T. brucei* with human Hsp90s and previously selected kinetoplastids. The amino acid sequences for all selected kinetoplastids were retrieved from TriTryDB (Aslett et al., 2010), while the human Hsp90 sequences were retrieved from the NCBI website (www.ncbi.nlm.nih.gov). sequences were aligned by Clustal ad annotated in Jalview v2.11.14 (Waterhouse et al., 2009). Multiple sequence alignment was also carried out for TbSti1 and Sti1 from human and previously selected kinetoplastids.

The 3D full length structure of TbSti1 and its domains were obtained from AlphaFold DB (AlphaFold Protein Structure Database; https://alphafold.ebi.ac.uk) (Varadi et al., 2022). The models were viewed and annotated in PyMol 2.5 (DeLano, 2002)

2.3.3 Phylogenetic and conserved syntenic analyses

A phylogenetic tree was constructed to analyse the phylogenetic relationship of the Hsp90/HSPC complements in both *T. brucei* subspecies. The full-length amino acid sequences for the Hsp90/HSPC family in the selected kinetoplastid parasites were obtained from TriTryDB (Aslett et al., 2010), and the human protein sequences were obtained from the NCBI website (www.ncbi.nlm.nih.gov). Partial amino acid sequences were omitted from the analysis. Gene ID numbers for the Hsp90/HSPC sequences used in this study are provided in Table 2.2. Multiple sequence alignments for the phylogenetic tree were performed using the inbuilt ClustalW program (Larkin et al., 2007) with default parameters in MEGA-X (Kumar et al., 2018) are shown in Figure 2.1. Maximum likelihood (ML) was utilized to find the best model of evolution and was selected by the Bayesian Information Criterion (BIC) implemented in MEGA-X. The amino acid-based Hsp90/HSPC ML phylogeny was reconstructed using the JTT (Jones-Taylor-Thornton) model matrix (Jones et al. 1992) with gamma distribution shape parameter (G). The ML phylogenetic tree was constructed using MEGA-X (Kumar et al., 2018). The accuracy of the reconstructed tree was assessed using a bootstrap test using 1000 replicates with a pairwise gap deletion mode. The phylogenetic tree for the Hsp90s was unrooted.

Syntenic analysis was conducted to evaluate the conservation of the gene arrangement of the cytosolic *Hsp83* genes in *T. brucei* and selected kinetoplastid parasites. The conserved syntenic regions surrounding the selected *Hsp83* genes were searched by examining the conserved colocalization of neighbouring genes on a scaffold of the *T. brucei* subspecies (*T. b. brucei and T. b gambiense*) and selected kinetoplastid parasites for this study using genome information from TriTryDB. The identities of unknown neighbour genes of the selected *Hsp83* genes were conducted using a BLASTP search on the NCBI database.

2.3.4 Physiochemical properties, protein expression, and the determination of the organelle distribution for the *T. brucei* Hsp90/HSPC complement.

The physiochemical properties, molecular weight (Da) and isoelectric point (pI) of each gene was determined using the compute pI/Mw tool from ExPASy (https://web.expasy.org/compute_pi/) (Gasteiger et al., 2005). Data on the previously reported phenotypic RNA interference (RNAi) knockdown screen, (Alsford et al., 2011), for each member of the Hsp90/HSPC complement and identified Hsp83 co-chaperones were retrieved from TrypsNetDB (http://trypsnetdb.org/QueryPage.aspx) (Gazestani et al.,

2017). The predicted organelle distribution for each protein was searched using the TrypTag microscopy project's online server, (Dean et al. 2017). This project aims at tagging every trypanosome protein with mNeonGreen (mNG) (Shaner et al., 2013) to determine the protein's localization in the cell within the parasite (http://tryptag.org/) (Dean et al.2017). Proteomic data from the mitochondrion (Panigrahi et al., 2009), mitochondrial importome (Peikert et al., 2017), respiratome (Acestor et al., 2009), mitochondrial membranes (outer, intermembrane space, inner and matrix) (Acestor et al., 2009), nucleus (Goos et al., 2017), nuclear pore (DeGrasse et al., 2008), glycosomes (Colasante et al., 2006; Güther et al., 2014), flagellum (Broadhead et al., 2006; Subota et al., 2014) and cell surface (Shimogawa et al., 2015) were also used for the prediction of the organelle distribution for the *T. brucei* Hsp90 complements and Hsp90/ HSPC complements and Hsp83 co-chaperones.

2.3.5 Identification of potential post-translational modification sites for the *T. brucei* Hsp90 proteins

Mass spectrometric information from a collection of relevant databases on *T. brucei* PTMs (Moretti et al. 2018; Nett et al. 2009; Urbaniak et al. 2013; Zhang et al. 2020) for the relevant proteins was retrieved using the previously identified accession numbers. Information on the respective PTMs (modification sites, modification types and modified residue) were obtained and the modified residues were mapped onto appendix Figure 7.4 for all Hsp90 isoforms from *T. brucei* subspecies (*T. b. brucei* and *T. b gambiense*) with orthologues from other kinetoplastids and from human, then analysed for determination of conserved and specific PTMs among the *T. brucei* Hsp90 complements.

2.4 Results and Discussion

2.4.1 Determination of the Hsp90/HSPC complement in *T. b. brucei, T. b. gambiense* and other kinetoplastids

The protozoan parasite, *T. brucei* is comprised of three subspecies, with the genomes of *T. b. gambiense* and *T. b. brucei* already sequenced (Jackson et al. 2010; Gibson 2012).. An *in silico* analysis of the Hsp90/HSPC complement in both *T. brucei* subspecies was conducted to provide an overview of the *T. brucei* Hsp90 family. The nomenclature and format to categorize the *T. brucei* Hsp90 family was adopted from our previous study (Bentley et al. 2019). The orthologue of the cytosolic Hsp90 member in *T. brucei* is termed Hsp83 (Mottram et al. 1989), and thus will be referred to as Hsp83 for the rest of this study. This protein displays variable molecular weight amongst different kinetoplastid protists. However, to underscore whether discussing a protein from *T. b. gambiense* or *T. b. brucei*, the abbreviations Tbg and Tbb respectively, were used in this study. The orthologous relationships of the Hsp90 family from *T. b. brucei* and *T. b. gambiense* to the selected organisms in this study are presented in Table 2.2, and a comprehensive domain organisation of the predicted *T. brucei* Hsp90 proteins is illustrated in Table 2.3.

Table 2.2: The Hsp90/HSPC proteins from *Trypanosoma brucei* with putative orthologues in *T. cruzi, L. major, C. fasciculata, B. saltans* and *H. sapiens.*

	H. sapiens	*T. brucei*	*T. cruzi*[c]	*L. major*	*C. fasciculata*	*B. saltans*		
Name[a]	**Gene ID**[b]	**Gene ID**[b]	**Gene ID**[b]	**Gene ID**[b]	**Gene ID**[b]	**Gene ID**[b]	**Localisation**[d]	**Reference**
HSP90-α/HSPC2 **HSP90-β/HSPC3**	3324 3326	Tb927.10.10890 Tb927.10.10900 Tb927.10.10910 Tb927.10.10920 Tb927.10.10930 Tb927.10.10940 Tb927.10.10950 Tb927.10.10960 Tb927.10.10970 Tb927.10.10980 Tbg972.10.13260 Tbg972.10.13270 Tbg972.10.13280	TcCLB.507713.30 C4B63_113g25 C4B63_113g29 C4B63_113g30 C4B63_113g33 C4B63_84g87 C4B63_84g88 C4B63_84g89 Tc_MARK_3581	LmjF.33.0312 LmjF.33.0314 LmjF.33.0316 LmjF.33.0318 LmjF.33.0320 LmjF.33.0323 LmjF.33.0326 LmjF.33.0330 LmjF.33.0333 LmjF.33.0336 LmjF.33.0340 LmjF.33.0343 LmjF.33.0346 LmjF.33.0350 LmjF.33.0355 LmjF.33.0360 LmjF.33.0365	CFAC1_280011900 CFAC1_280012000	BSAL_87515	CYT NUC FLAGELLAR CELL SURFACE	(Urbaniak et al. 2012; Gunasekera et al. 2012; Subota et al. 2014; Shimogawa et al. 2015; Dean et al. 2017)
GRP94/HSPC4	7184	Tb927.3.3580 Tbg972.3.3850	C4B63_10g439 Tc_MARK_3058	LmjF.29.0760	CFAC1_100018800	BSAL_88715	ER NUC FLAGELLAR CELL SURFACE	(Urbaniak et al. 2012; Gunasekera et al. 2012; Subota et al. 2014;

								Shimogawa et al. 2015)
TRAP-1/HSPC5	10131	Tb927.11.2650 Tbg972.11.2900	TcCLB.504153.310 C4B63_2g430 Tc_MARK_6238	LmjF33.2390	CFAC1_230028300	BSAL_33145	MITO FLAGELLAR	(Panigrahi et al. 2009; Subota et al. 2014; Dean et al. 2017)

[a] The nomenclature for the Hsp90/HSPC proteins from *T. b. brucei*, and *T. b. gambiense* were derived according to Folgueira and Requena (2007).

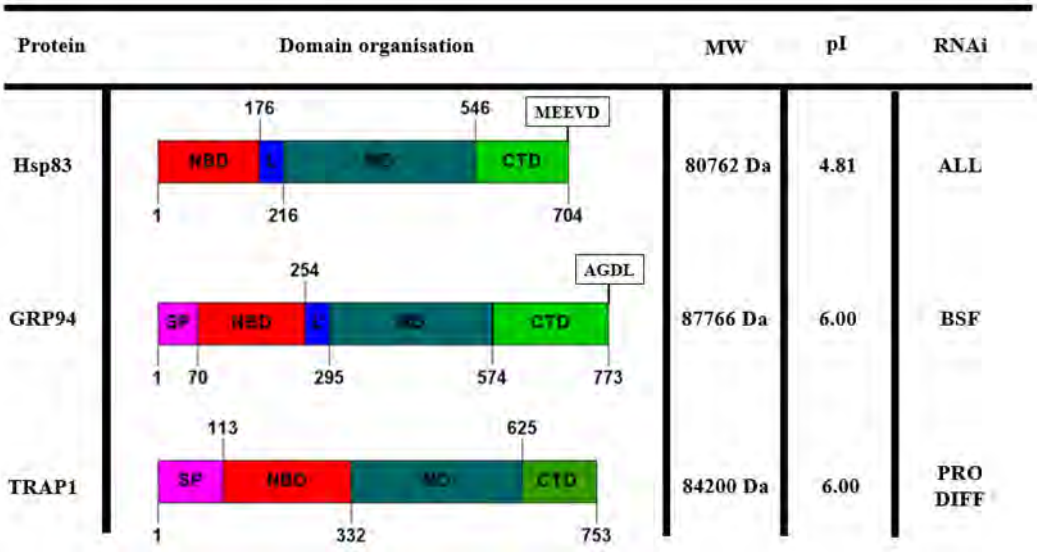
[b] The Gene IDs for the members of the *T. b. brucei* (Tb refers to Tbb), *T. b. gambiense*, *T. cruzi*, *C. fasciculata*, *B. saltans* and *L. major* Hsp90/HSPC protein family were retrieved from the TriTrypDB database (http://tritrypdb.org/tritrypdb/; Aslett et al. 2010). The Gene IDs for the members of the *H. sapiens* Hsp90/HSPC protein family were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/).

[c] The Gene IDs for the orthologues, identified by reciprocal BLASTP analysis, of three strains of *T. cruzi* are listed. *T. cruzi* CL Brener Esmeraldo-like (TcCLB), *T. cruzi* Dm28c 2018 (C4B63), and *T. cruzi* marinkelli strain B7 (Tc_MARK).

[d] Subcellular localizations for the *T. brucei* Hsp90/HSPC proteins were either acquired from using the TrypTag database (http://tryptag.org/; Dean et al. 2017) and/or predicted using various proteomic datasets and online prediction software listed in the materials and methods.

CYT-Cytosol; MITO- Mitochondrion; NUC- Nucleus; ER- Endoplasmic reticulum; GYLCO- glycosomes; FLAGELLAR- Flagellar; CELL SURFACE- Cell surface.

Table 2.3. Domain architecture, protein properties, life cycle expression and RNAi mediated knockdown of the *T. brucei* Hsp90 family.



Each protein sequence is represented by a multi-coloured bar with the numbering on the bottom of the bar indicating the length of the protein in amino acid residues. Protein domains and other associated features that were identified using InterPro (Apweiler et al., 2000) are also shown, and include the signal peptide (SP; pink), N-terminal nucleotide binding domain (NBD; red), linker domain (L; blue), middle client protein-binding domain (MD; turquoise), and a C-terminal dimerization domain (DD; green)

The physiochemical properties, molecular weight (MW) and isoelectric point (pI), for each *T. brucei* Hsp90 protein was calculated using the compute pI/Mw tool from ExPASy (https://web.expasy.org/compute_pi/; (Gasteiger et al., 2005).

Data on the phenotypic knockdown screen using RNAi conducted by Alsford et al. (2011), for Hsp90/HSPC protein member is provided: ALL-required for all life cycle stages; BSF-required for bloodstream form; PRO-required for procyclic form; DIFF-required for differentiation.

Twelve putative *Hsp90* encoding genes were identified in the *T. b. brucei* genome (Table 2.2), which is consistent with previous findings (Mottram et al. 1989; Folgueira and Requena 2007), while 5 putative *Hsp90* encoding genes were identified in this study in the *T. b. gambiense* genome. The reduction in the *Hsp90* gene numbers found in *T. b. gambiense* could be a consequence of the reduced genome size observed in the human infective subspecies (Dero et al., 1987). The intraspecific genomic variation is largely associated with tandem or segmental duplications of genes observed in *T. b. brucei* (Jackson et al. 2010). For the putative *Hsp90* genes identified in this study for *T. b. brucei*, ten of the twelve putative *Hsp90* genes identified were found to be homologous to Hsp83, whereas in *T. b. gambiense*, three of the five putative *Hsp90* genes identified were homologous to *Hsp83* (Table 2.1).

The remaining two *Hsp90* genes found in both *T. b. brucei* (Tb927.3.3580 and Tbg972.3.3850) and *T. b. gambiense* (Tb927.11.2650 and Tbg972.11.2900) showed significant identity to the ER and mitochondrial resident paralogues of Hsp90, GRP94 and TRAP-1 respectively (Table 2.2). This indicates that a single gene copy for *GRP94* and *TRAP-1* is encoded on the genome in both *T. brucei* subspecies. Phylogenetic analysis shows that the *T. brucei* Hsp90/HSPC family is also comprised of 3 distinct Hsp90 groups (Hsp83, GRP94 and TRAP-1), which cluster into clades according to protein sequence and subcellular localisation (Figure 2.1). In contrast to humans with 4 Hsp90 isoforms, there are 3 Hsp90 isoforms (Hsp83, GRP94 and TRAP-1) identified by phylogenetic analysis to be present in all kinetoplastid organisms used in this study (Table 2.2; Figure 2.1).

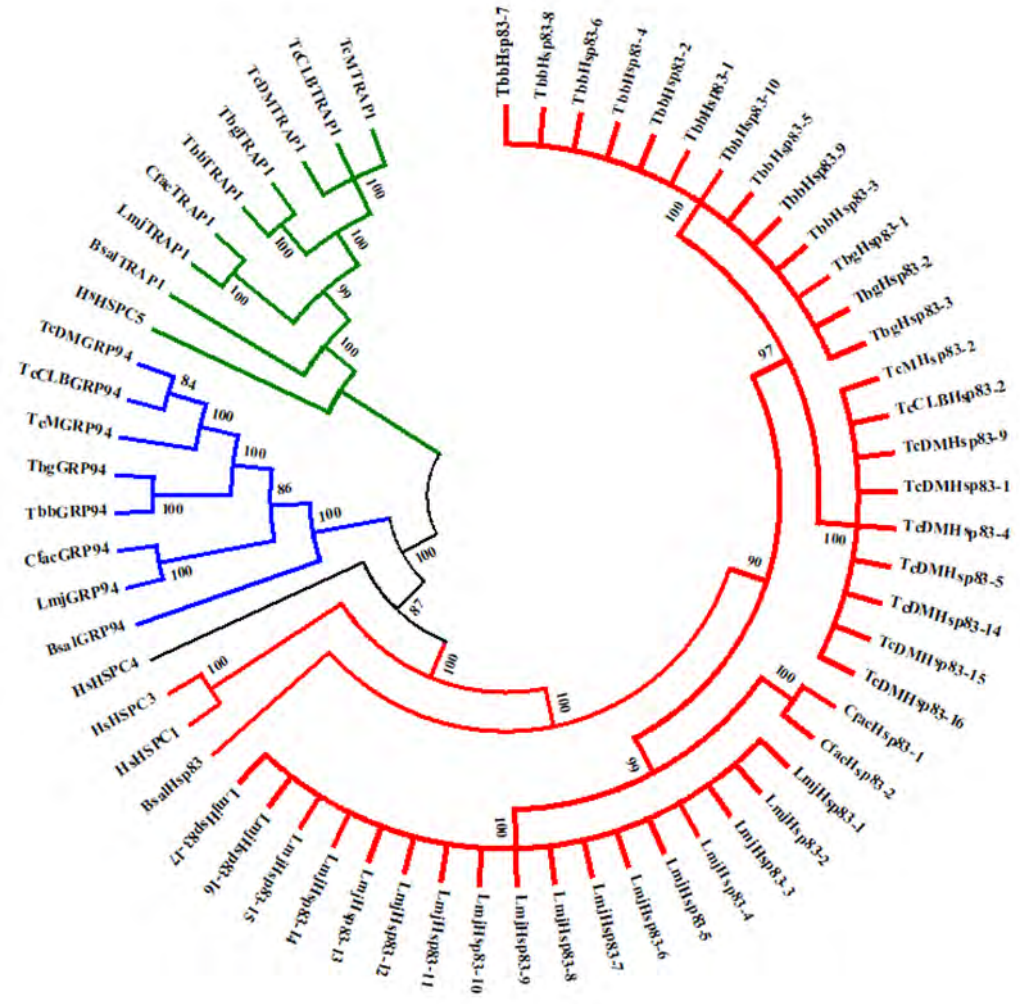


Figure 2.1: Phylogenetic analysis of the Hsp90/HSPC family from *T. brucei* in relation to human and selected trypanosomatids.

Multiple sequence alignment of the full-length amino acid sequences of the *Hsp90/HSPC* gene families in human and selected kinetoplastid parasites was performed using the in-built ClustalW program (Larkin et al., 2007) with default parameters on the MEGA X software (Kumar et al., 2018). The phylogenetic tree was constructed by MEGA 7 using the Maximum-likelihood method based on the Jones–Taylor–Thornton (JTT) matrix-based model of amino acid substitution (Jones et al. 1992) with gamma distribution shape parameter (G). The alignment gaps were excluded from the analysis, and the number of amino acid sites used to construct the tree numbered 572. Bootstrap analysis was computed with 1000 replicates. Accession numbers for the *T. b. brucei* (Tbb), *T. b. gambiense* (Tbg), *T. cruzi* (TcCLB, CL Brener Esmeraldo; TcM, marinkellei strain B7; TcD, Dm28c 2018), *C.*

fasciculata (Cf), *B. saltans* (Bs), and *L. major* (Lmj) Hsp90 amino acid sequences can be found in the Table 2.2. Gene ID numders for human (Hs; *H. sapiens*) HSPC/Hsp90 amino acid sequences are also provided in Table 2.2. The subcellular localisation for Hsp90s is indicated by coloured branches. Red: cytosolic; blue: endoplasmic reticulum; and green: mitochondrion. Scale bar represents 0.2 amino acid substitutions per site.

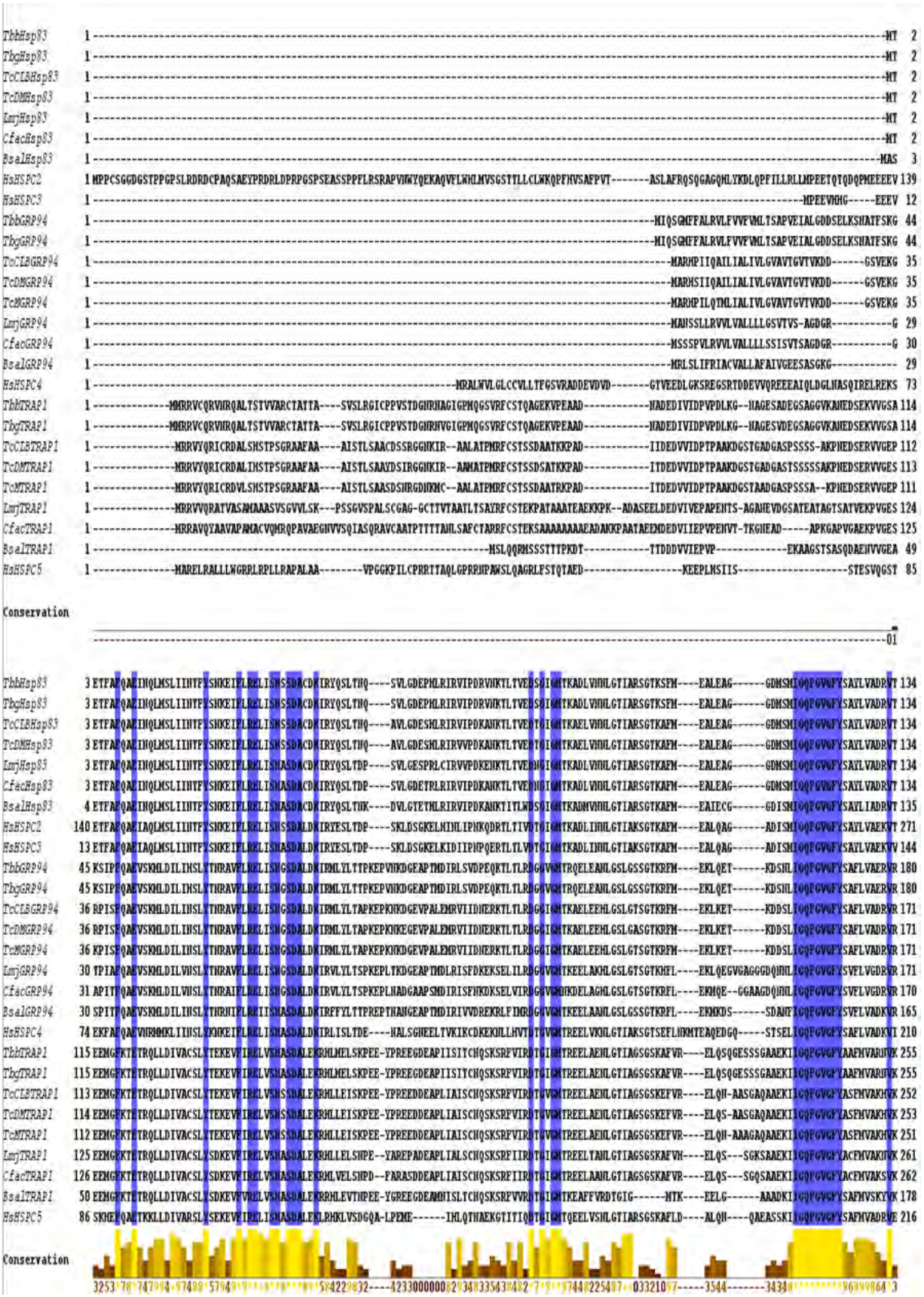
Previous literature reported that eleven *Hsp90* genes are encoded by the *Trypanosoma cruzi* (*T. cruzi*) genome (Shonhai et al., 2011). In this study we included three different *T. cruzi* strains: CL Brener Esmeraldo-like (TcCLB), Dm28c 2018 (C4B63), and marinkelli strain B7 (Tc_MARK) to determine the Hsp90/HSPC complement in the American trypanosome. It was identified in this study that the *T. cruzi* CL Brener Esmeraldo-like strain has two *Hsp90* genes, the Dm28c 2018 strain has nine *Hsp90* genes, and the marinkelli strain B7 has three *Hsp90* genes (Table 2.2). However, this study identified that many of the *Hsp90* genes homologous to *Hsp83* in the three *T. cruzi* strains were found to be partial and/or truncated genes. These partial and/or truncated *Hsp83* genes in this study, were omitted from the analysis. The obvious discrepancy in numbers of genes amongst the *T. cruzi* strains, and its numerous partial and/or truncated Hsp90 sequences has been recently reviewed highlighting the difficulties in *T cruzi* genome analysis (Herreros-Cabello et al., 2020). The first genome sequenced, which is still widely accepted as the main reference, has close to 50% repetitions in its sequence (El-Sayed et al. 2005; El-Sayed et al. 2005) and though newer genomes have been sequenced using short-read sequencing methods as in the case of the *T. cruzi* marinkelli strain B7, these methods lead to the formation fragmented chromosomes due to their inability to create a complete chromosome from their short reads technique (Franzén et al., 2012; Herreros-Cabello et al., 2020). *Leishmania major* (Lmj) contains the largest Hsp90 family with a total of nineteen *Hsp90* genes, seventeen of which were found to be homologous to *Hsp83*, and these findings agree with previous studies (Folgueira and Requena 2007; Shonhai et al. 2011; Requena et al. 2015), and this correlates with the high abundance of the protein being observed in *L. major* and several other *Leishmania spp.* (Brandau et al. 1995). Other trypanosomatids included in this study were the non-parasitic *Bodo saltans* (*B. saltans*) (Deschamps et al., 2011) and the insect infecting *Crithidia fasciculata* (*C. fasciculata*) (Wallace, 1966), which were found to have three and four putative *Hsp90* genes respectively (Table 2.2). Both these trypanosomatids were found to possess genes encoding all three Hsp90 isoforms (Hsp83, TRAP-1 and GRP94), though *C. fasciculata* was identified to possess two *Hsp83* genes (Table 2.2).

Early genomic studies suggest that the human genome contains sixteen *Hsp90* genes (five functional and eleven pseudogenes), which have been categorized, according to the proposed standardized guidelines for HSP nomenclature, into four isoforms under the superfamily name HSPC (Chen et al. 2006; Kampinga et al. 2009). In contrast to the trypanosomatids, humans have two isoforms of Hsp90 localized in the cytoplasm: the inducible form Hsp90α/HSPC2 and the constitutive form HSP90β/HSPC3 (Sreedhar et al. 2004). Phylogenetic analysis has suggested that the two cytosolic isoforms arose from gene duplication, and the organelle Hsp90s (GRP94/HSPC4 and TRAP-1/HSPC5)

developed from a common ancestor (Gupta 1995; Emelyanov 2002; Chen et al. 2005). Hsp83 (Tb927.10.10980) and TRAP-1 (Tb927.11.2650) were identified as phosphoproteins in this study, while kinases are yet to be identified in the ER and little is known about the effect of post-translational modifications on GRP94 (Argon and Simen 1999; Marzec et al. 2012)

2.4.2 Primary structure analysis of the Hsp90 proteins

Primary structure analysis of the cytosolic, mitochondrial and ER Hsp90 proteins was conducted to assess the sequence similarity of Hsp90 across the selected species (Figure 2.2). For the sake of this analysis, just one cytosolic representative of each kinetoplastid organism was used. A complete sequence alignment of all the kinetoplastid cytosolic Hsp90 proteins is provided in Appendix Figure 7.3. Fully conserved (100%) residues of the protein sequences were highlighted across all isoforms of all species. The N terminal ATPase binding domain has the highest number of conserved residues across all isoforms and all species, with this sequence IGQFGVGFY (TbbHsp83 residues 116-124) fully conserved, it will be interesting to explore its specific contribution to the Hsp90 ATPase activity. This finding is similar to previous findings that showed the ATPase domain of TbHsp83 to be ~70% similar to HSPC2 (Pizarro et al., 2013). Other previously identified residues have been found to be conserved. In yeast, a conserved arginine residue in the middle domain (MD) in position 380 (Arg^{380}) was found to be part of the contributing factor to ATP hydrolysis carried out by the NTD (Cunningham et al., 2012). An arginine residue seen in position 375 of TbbHsp83 is conserved across all the isoforms and species. Asp^{79} (D79 in yeast and D93 of human Hsp90) was also identified as a key residue in nucleotide binding (Obermann et al. 1998; Panaretou et al. 1998), a similar residue (D78) in TbbHsp83 was found to be conserved across the species analysed. It would be interesting to see if these residues mirror the same function as experimentally determined in yeast. Compared to all the other cytoplasmic Hsp90s, human Hsp90α (HSPC2) has much longer N-terminal domain with 122 more residues than human Hsp90β (HSPC3). In *T. brucei* the cytosolic Hsp83 has the same terminal pentapeptide (MEEVD) as its human counterparts compared to the other species with different variants, MEQVD in one of the *T. cruzi* strains and in *L. major* and MESVD in *B. saltans.*


TbbHsp83
TbgHsp83
TcCLBHsp83
TcDMHsp83
LmjHsp83
CfacHsp83
BsalHsp83
HsHSPC2
HsHSPC3
TbbGRP94
TbgGRP94
TcCLBGRP94
TcDMGRP94
TcMGRP94
LmjGRP94
CfacGRP94
BsalGRP94
HsHSPC4
TbbTRAP1
TbgTRAP1
TcCLBTRAP1
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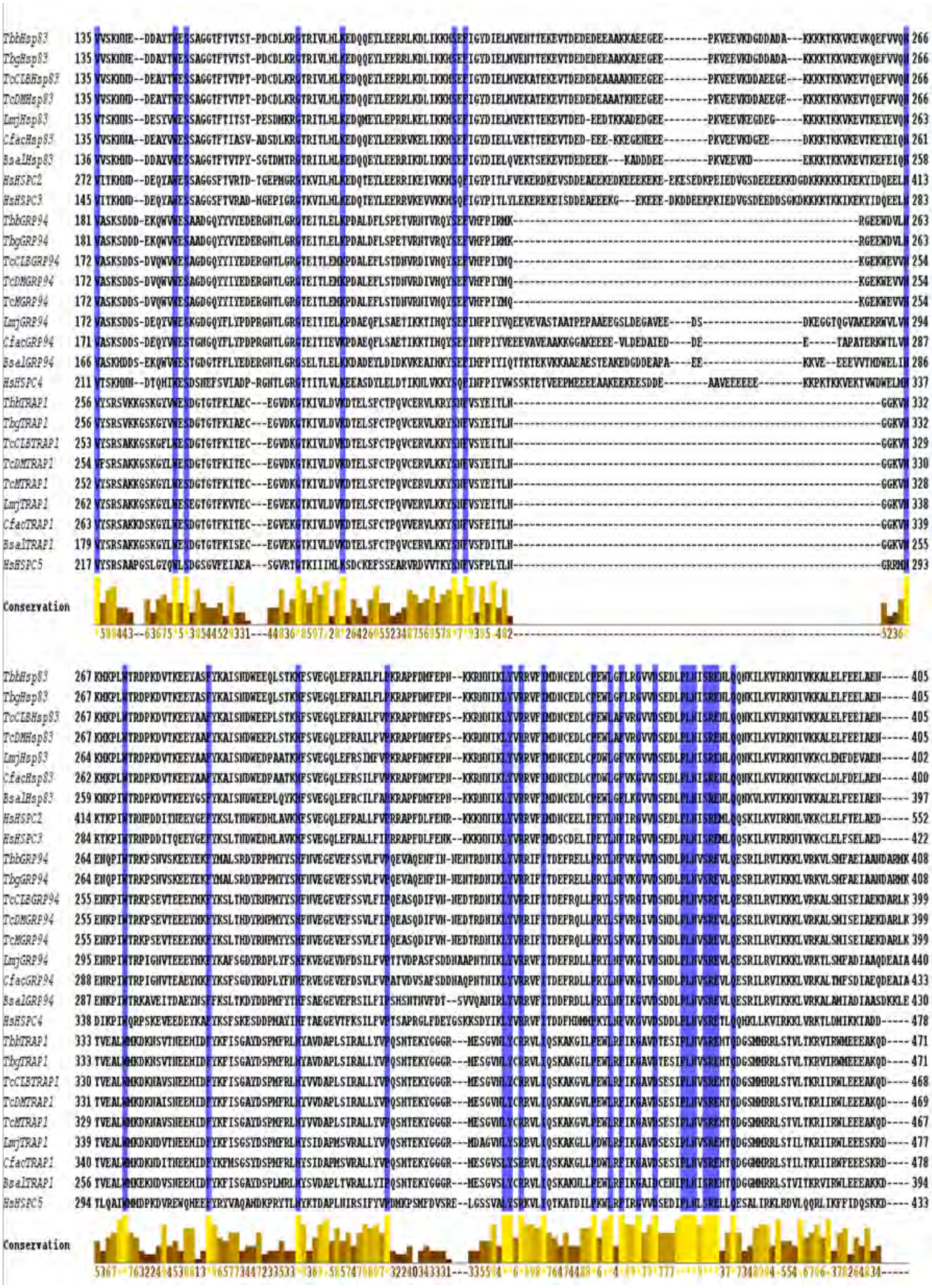
TcMTRAP1
LmjTRAP1
CfacTRAP1
BsalTRAP1
HsHSPC5
Conservation
Conservation


TbbHsp83
TbgHsp83
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CfacHsp83
BsalHsp83
HsHSPC2
HsHSPC3
TbbGRP94
TbgGRP94
TcCLBGRP94
TcDMGRP94
TcMGRP94
LmjGRP94
CfacGRP94
BsalGRP94
HsHSPC4
TbbTRAP1
TbgTRAP1
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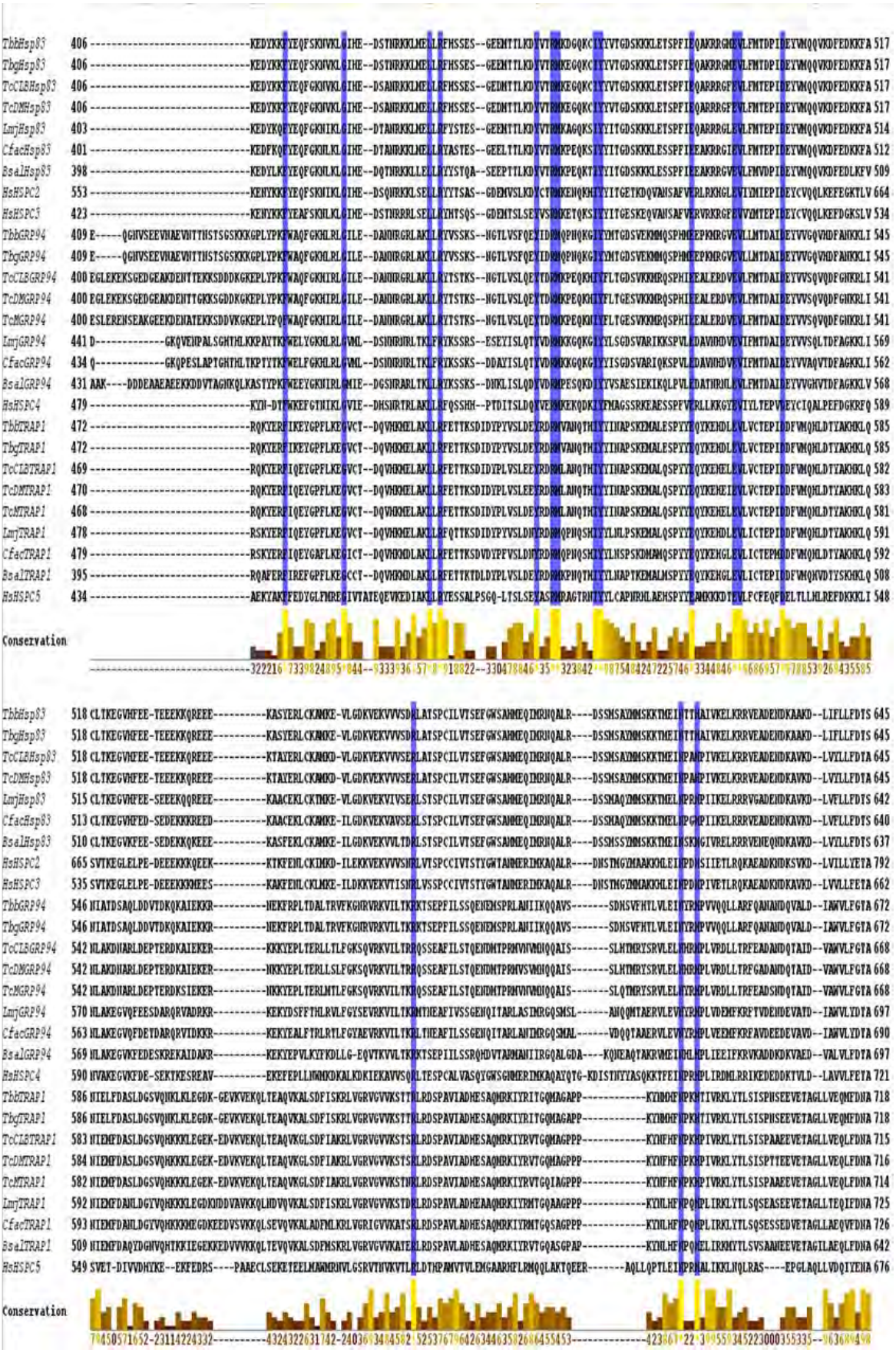
TbbHsp83
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BsalHsp83
HsHSPC2
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TbbGRP94
TbgGRP94
TcCLBGRP94
TcDMGRP94
TcMGRP94
LmjGRP94
CfacGRP94
BsalGRP94
HsHSPC4
TbbTRAP1
TbgTRAP1
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Conservation

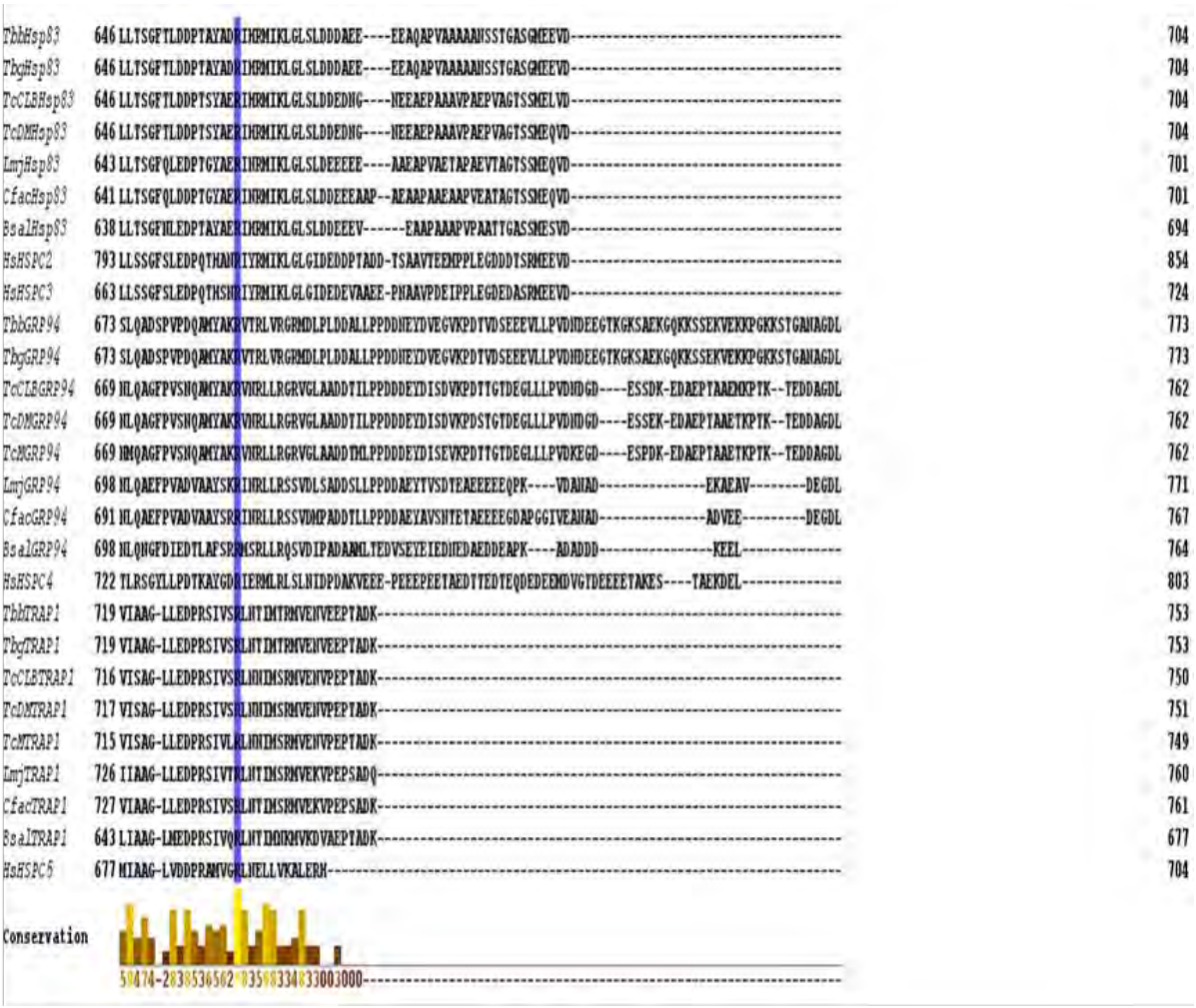


Figure 2.2: Multiple sequence alignment of Hsp90 in selected species.
Cytoplasmic Hsp90 proteins TbbHsp83, TbgHsp83, TcCLBHsp83, TcDMHsp83, LmjHsp83, CfacHsp83, BsalHsp83, HsHSPC2 and HsHSPC3. Endoplasmic Hsp90 proteins TbbGRP94, TbgGRP94, TcCLBGRP94, TcDMGRP94, TcMGRP94, LmjGRP94, CfacGRP94, BsalGRP94 and HsGRP94. Mitochondrial Hsp90 proteins TbbTRAP1, TbgTRAP1, TcCLBTRAP1, TcDMTRAP1, TcMTRAP1, LmjTRAP1, CfacTRAP1, BsalTRAP1 and HsHSPC5. Accession numbers and Gene IDs for all selected species are found in Table 2.2. Fully conserved residues are represented below with gold bars and an * below, as conservation reduces the gold shade darkens with reducing numbers from 9 to 1. For emphasis all fully (100%) conserved residues are highlighted in blue.

2.4.2.1 Hsp83

Hsp83 has been found to be an essential and highly abundant protein, that is encoded by multiple gene copies organized in a head-to-tail tandem array (Folgueira and Requena, 2007). It has been identified in this study and previous studies (Mottram et al. 1989; Folgueira and Requena 2007) that *T. b. brucei* has been shown to encode for ten tandem copies of Hsp83 (Figure 2.2), whereas *T. b. gambiense* genome

encodes three tandem copies of Hsp83 (Figure 2.2). Syntenic analysis revealed that the *TbbHsp83* and *TbgHsp83* genes are both located on chromosome ten in a head to tail orientation, with the same genomic organisation being observed in both *T. brucei* subspecies (Figure 2.2). Like *T. brucei*, a discrepancy in *Hsp83* gene copy numbers was also observed for the three *T. cruzi* strains used in this study (Figure 2.2). Syntenic analysis revealed that the *T. cruzi* Dm28c 2018 (C4B63) strain has 16 tandem copies of *Hsp83*, though nine were partial sequences (Figure 2), whereas both the CL Brener Esmeraldo-like (TcCLB) and marinkelli strain B7 (Tc_MARK) encode for two *Hsp83* genes, with one partial gene each (Figure 2). Syntenic regions surrounding the *Hsp83* genes were found to be virtually conserved across the selected kinetoplastids, with *B. saltans* being the exception (Figure 2.3). Thus, the discrepancy in gene copy number of Hsp83 in the two *T. brucei* subspecies and amongst the kinetoplastid organisms may have arisen from the differences in the life cycle of the kinetoplastids. Datamining of proteomic data revealed that all identified TbbHsp83 (TbbHsp83-1) proteins are present in both life cycle stages of the parasite: the bloodstream stage (BSF) and procyclic stage (PF) and unchanged in protein abundance between the short stumpy (SS) and long slender (LS) phases (Urbaniak et al. 2012; Gunasekera et al. 2012). Protein expression of the TbbHsp83 proteins were reported to be up regulated during the BSF stage (Urbaniak et al. 2012), also, all TbbHsp83 proteins were also present in the cell surface proteome (Subota et al., 2014) and TbbHsp83-10 (Tb927.10.10980) was found in the flagellar proteome (Shimogawa et al., 2015).

The amplification of HSP genes in protozoan parasites has been reported previously (Urményi et al. 2014; Requena et al. 2015; Drini et al. 2016; Bentley et al. 2019), and is considered a means by which the parasites increase chaperone levels to maintain proteostasis under normal and stressful conditions (Wiesgigl and Clos, 2001b). The heat shock response is a highly conserved transcriptional program that in most organisms involves increased heat-shock gene transcription (Nadal et al. 2011). However, in trypanosomatids, control of gene expression occurs almost exclusively at the post-transcriptional level, and HSP synthesis during heat shock depends on regulation of mRNA turnover and translational control (Clayton and Shapira 2007; Requena 2011). In *T. brucei*, post-transcriptional regulation of chaperone mRNAs is facilitated by a zinc finger protein, ZC3H11 (Droll et al., 2013). The mRNA transcript levels of TbbHsp83 in BSF parasites increases >2-fold after heat shock (Ooi et al. 2020) and is stabilized by ZC3H11 to promote the survival of the parasite (Droll et al., 2013). Treatment of *T. b. brucei* BSF parasites with 17-AAG sensitized the parasites to heat shock, as well as caused severe morphological abnormalities and cell cycle disruption (Meyer and Shapiro 2013). Pharmacological inhibition of Hsp83 activity in several *Leishmania spp*. induced morphological and biochemical promastigote-to-amastigote differentiation (Wiesgigl and Clos, 2001b; Bente et al., 2003; Hombach et al., 2013), which mimics environmental triggers such as heat shock and acidic milieu, indicating a pivotal role for Hsp83 in trypanosomatid protists in environmental sensing and life cycle control. Interestingly, treatment of *T.*

cruzi bloodstream trypomastigotes with geldanamycin, induced morphological changes in the parasites but not life cycle progression (Graefe et al., 2002)

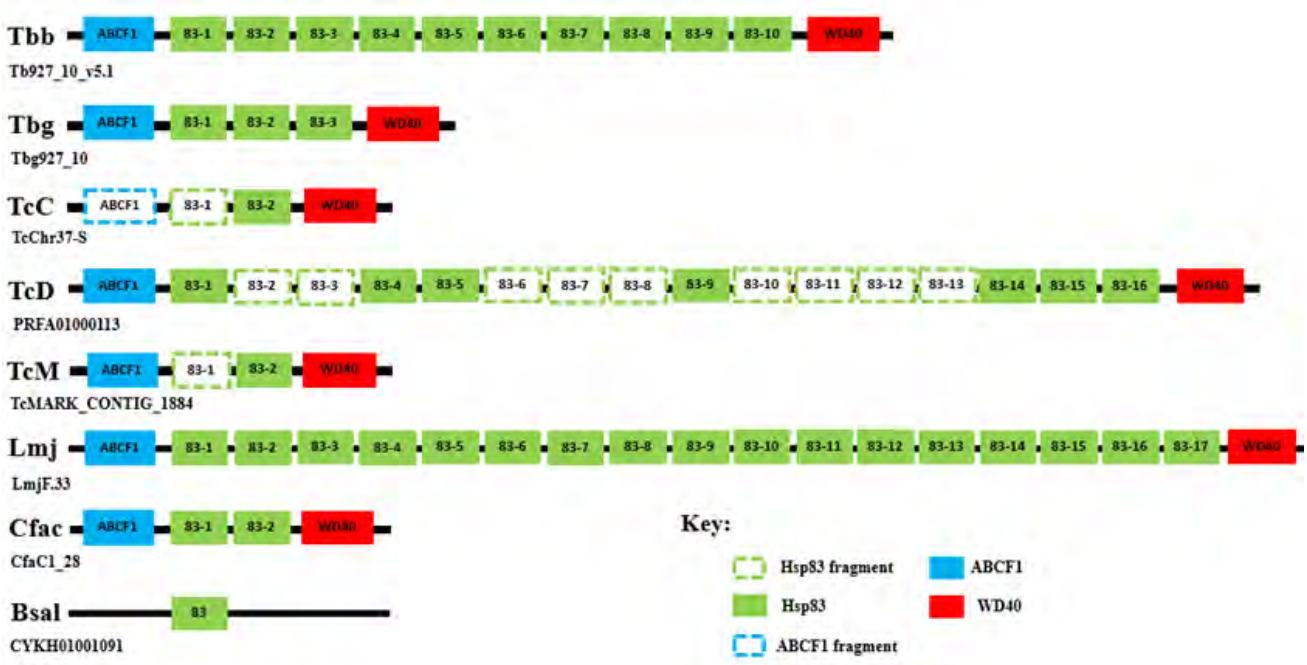


Figure 2.3: Syntenic analysis of the gene arrangement of the *Hsp83* genes in *T. brucei* and selected trypanosomatids.
The conserved syntenic regions surrounding the selected *Hsp83* genes were searched by examining the conserved co-localization of neighbouring genes on a scaffold of the *T. brucei* subspecies, *T. b. brucei* (Tbb) and *T. b. gambiense* (Tbg), and selected trypanosomatids: *T. cruzi* CL Brener Esmeraldo-like (TcCLB), *T. cruzi* Dm28c 2018 (TcD) strain, *T. cruzi* marinkelli strain B7 (TcM), *L. major* (Lmj), *B. saltans* (Bsal) and *C. fasciculata* (Cfac). The genome information used for this study was acquired from TriTrypDB data-base (http://tritrypdb.org/tritrypdb/) (Aslett et al., 2010). The identities of unknown neighbour genes of the selected *Hsp83* genes were conducted using a BLASTP search on the NCBI database. Abbreviations: ABCF1: ATP-binding cassette sub-family F member 1; WD40: WD40-repeat protein.

It would be interesting to investigate if the pharmacological inhibition of Hsp83 has an effect on differentiation amongst the three *T. brucei* subspecies. The monophyletic cluster of the cytosolic Hsp83s suggests a general conservation of function, structure, and sequence in the trypanosomatids Hsp83 homologues (Figure 2.1). The 704 amino acid sequences of the corresponding TbbHsp83 and TbgHsp83 proteins were found to be almost identical (Figure 2.2) and were also found to possess the canonical domain architecture of typical cytosolic Hsp90s (Table 2.3). Despite the overall structural conservation, kinetoplastid Hsp83 proteins possess unique biochemical features which separate them from their human counterparts and can be potentially exploited in selective drug discovery studies. TbbHsp83 from *T. b. brucei* showed ATPase activity which was within the range of other Hsp90 proteins (Pizarro et al., 2013). In comparison to human Hsp90, enhanced ATPase activity has been shown in the Hsp83 orthologue in *Plasmodium falciparum* and *T. cruzi* (Nadeau et al., 1992; Pallavi et

al., 2010). In a study conducted by Pizarro and colleagues (Pizarro et al., 2013), biophysical and biochemical techniques were able to identify three short divergent regions in the TbbHsp83 NBD, that could be targeted for selective pharmacological inhibition of TbbHsp83 over human Hsp90.

It was also interesting to note that the variable charged linker domain which links the NBD to the MD in cytosolic Hsp90s found in higher eukaryotes is also present in *T. brucei*. This region is highly divergent in both length and amino acid sequence among Hsp90 proteins of different species but does not affect Hsp90 function, co-chaperone interaction, and conformation (Hainzl et al., 2009; Tsutsumi et al., 2009, 2012). Hsp90 from many protozoa have extended linkers with the malaria parasite, *Plasmodium falciparum*, exhibiting one of the longest linkers reported thus far (Pallavi et al., 2010). Complementation of human and yeast charged linkers by the *P. falciparum* version reduces ATPase activity and affects client protein binding (Tsutsumi et al., 2009), thus indicating that this linker could provide specificity to the activity of Hsp90 from different species. Therefore, comparative analysis of *T. brucei* Hsp83 proteins with their human counterparts as well as linker swapping experiments will be especially useful in understanding the role of the linker region in *T. brucei* Hsp90 biology, and possible future exploitation as a unique drug binding region.

Post-translational modifications, and particularly phosphorylation of tyrosine, serine, and threonine residues, at multiple sites of cytosolic Hsp90 is a well-known chaperone activity modulator mechanism in many organisms (Miyata and Yahara, 1992; Mollapour et al., 2011; Mollapour and Neckers, 2012; Hombach-Barrigah et al., 2019), Hsp90 steady-state phosphorylation is species-specific relative to the different cellular environments (Mollapour and Neckers, 2012). S53 and S286 were determined to be phospho-modified residues and were conserved within the ten cytosolic TbbHsp83 proteins, while T211, T216, S597 and S694 were conserved in all analysed trypanosomatids in this study (Appendix Figure 7.4) S374 was conserved in both trypanosomatids and humans (Appendix Figure 7.4). The same phospho-modified residues were previously described for the cytosolic Hsp83 orthologue from *L. donovani* (Hombach-Barrigah et al., 2019). Silencing mutations of *L. donovani* Hsp90 T211 and T216 reduced parasite growth, whilst mutation of S594 reduced growth and infectivity (Hombach-Barrigah et al., 2019). The phosphorylation of *L. donovani* Hsp90 at T21 plays a role in the binding of co-chaperones, and mutation of this residue irreversibly inhibited the growth of the promastigote stage (Hombach-Barrigah et al., 2019), however this residue has not been detected as a *T. brucei* phosphorylation site (Cunningham et al. 2008; Mollapour et al. 2011; Hombach-Barrigah et al. 2019). The equivalent site in yeast Hsp90 (T22) was found to be essential for dimerization and ATPase activity (Cunningham et al. 2008). The acetylation sites were predicted for TbHsp83 and mapped as shown in appendix Figure 7.4. The residues conserved amongst the other isoforms were also mapped (appendix Figure 7.4). The predicted N-glycosylation sites, N90, N372 and N612 were conserved in all kinetoplastid and human Hsp90 homologues, whilst N51 was determined to be specific to *T. brucei* Hsp83 (Appendix Figure 7.4). Two ubiquitination sites identified from *T. brucei* Hsp83 (K394 and

K560) were found conserved in all analysed cytosolic Hsp90 isoforms in this study (Appendix Figure 7.4).

2.4.2.2 TRAP-1

The mitochondrial isoform of the Hsp90/HSPC family was first identified in association with the mammalian tumour necrosis factor 1 (TNF-1) protein, hence termed TRAP-1 (Song et al. 1995). It was promptly suggested as a member of the 90-kDa molecular chaperone family due to strong homology with other Hsp90 members (Song et al. 1995). Since then, TRAP-1/HSPC5 orthologues have been identified in a variety of eukaryotic and prokaryotic organisms. This study identified a single entry for a putative *TRAP-1* gene annotated in the genomes of both *T. b. brucei* (Tb927.11.2650) and *T. b. gambiense* (Tbg972.11.2900) (Table 2.2). The selected kinetoplastids in this study were also identified to encode a single copy of TRAP-1 (Table 2.2) which are consistent with previous studies (Folgueira and Requena, 2007), except for *T. cruzi* which was previously stated to encode two TRAP-1 orthologues (Folgueira and Requena, 2007; Shonhai et al., 2011). Phylogenetic analysis indicates a general conservation in trypanosomatid TRAP-1 proteins (Figure 2.1), though little experimental characterization of these geneproducts have been conducted in kinetoplastids. It is predicted that the cellular role of the trypanosomatid TRAP-1 proteins will be orthologous to HsHSPC5, whose major functions is to maintain mitochondrial integrity, modulate mitochondrial metabolism and protect against mitochondrial apoptosis (Altieri et al., 2012). Furthermore, HSPC5 counteracts protein aggregation inside the mitochondria and supports protein folding (Siegelin et al., 2011) leading to healthy, intact mitochondria.

Mammalian TRAP-1 orthologues are localized predominantly in the mitochondrial matrix, where it exists as at least 6 different protein variants resulting from splicing patterns, amino acid additions and/or deletions (Cechetto and Gupta, 2000; Felts et al., 2000). The translation of the main TRAP-1 mRNA generates a precursor protein of 704 amino acids, that contains a putative 59-amino acid, N-terminal mitochondrial import sequence which is removed upon organelle import (Felts et al., 2000; Schleiff and Becker, 2011). It was predicted that both TbbTRAP-1 and TbgTRAP-1 localize in the mitochondria, as the proteins possess a positively charged N-terminal leader sequence. Proteomic and localisation studies confirmed that TbbTRAP-1 localises to the mitochondria (Panigrahi et al. 2009; Dean et al. 2017), but interestingly the protein is also present in the flagella of *T. b. brucei* BSF parasites (Subota et al., 2014) (Table 2.2). The subcellular distribution of TbbTRAP1 during the parasite's life cycle could be related to the shape and functional plasticity of the *T. brucei* single mitochondrion, which undergoes profound alterations to adapt to the different host environments (Osellame et al. 2012). Phenotypic knockdown

of TbbTRAP-1 had a detrimental effect on the survival and fitness of the parasite at the procyclic stage of its life cycle and negatively affected parasite differentiation (Alsford et al., 2011). Thus, *T. brucei* TRAP-1 proteins may be an important modulator of mitochondrial bioenergetics at the procyclic stage, as well play an integral role in parasite pathogenesis.

In terms of PTMs, three phosphorylation sites were found in the middle domain of TRAP-1, with S286 and S363 being specific phosphorylation sites for TRAP-1, though S374 was found to be conserved amongst all the HSP90 proteins analysed (Appendix Figure 7.4). Several amino acids were identified as potential targets for post-translational modifications in human TRAP-1, yet its phosphorylation mechanism remains to be revealed (Altieri et al., 2012). K109, K480 and K601 were found to be specific acetylation sites for TbTRAP-1. Additional TbTRAP-1 acetylation sites on lysine residues were conserved amongst the mitochondrial isoforms from all analysed taxon (Appendix Figure 7.4). Most of these PTMs of Hsp90 and other inferences stated here are yet to be verified experimentally.

2.4.2.3 GRP94

The glucose-regulated 94 kDa protein (GRP94) is a Hsp90 family member residing in the lumen of the endoplasmic reticulum (ER) (Argon and Simen, 1999), where it is involved in the maturation of membrane-resident and secreted protein clients (Marzec et al. 2012). GRP94 is induced in extracellular medium with low glucose levels, it is also referred to as endoplasmin or gp96 with disulfide-bonded proteins as its known substrates (Kang and Welch, 1991; Marzec et al., 2012) *GRP94* is present as a single gene in all metazoa, although the gene is not found in many unicellular organisms such as bacteria, archaea, yeast, and most fungi (Marzec et al. 2012). This study identified a single putative entry for the *GRP94* gene in both *T. brucei* subspecies (Tb927.3.3580 and Tbg927.3.3850 respectively) and the selected trypanosomatids in this study (Table 2.2). These findings are consistent with previous findings for *T. brucei* and *L. major* (Folgueira and Requena, 2007), though previous reports indicated that *T. cruzi* CL Brener Esmeraldo-like strain encodes three GRP94 orthologs (Folgueira and Requena, 2007; Shonhai et al., 2011). However, this study identified that only one GRP94 gene in *T. cruzi* CL Brener Esmeraldo-like strain (TcCLB.506989.190) was found to encode a full-length sequence. The genome of this *T. cruzi* strain needs to be further investigated to determine if these partial sequences of the GRP94 genes (TcCLB.506591.4 and TcCLB.503811.10) are due to sequencing errors.

Both *TbbGRP94* and *TbgGRP94* genes are present on chromosome III and are shown to encode proteins considerably longer in amino acid sequence when compared to Hsp83 (Table 2.3), which is characteristic of GRP94 protein members (Stechmann and Cavalier-Smith 2003; Johnson 2012). GRP94 proteins share structural similarity with cytosolic Hsp90 proteins, though the N-terminus

contains an ER signal peptide while the C-terminal MEEVD peptide is replaced with the KDEL motif that is required for retention in the ER (Argon and Simen, 1999). Sequence analysis of TbbGRP94 and TbgGRP94 indicates that the GRP94 protein shares domain architecture with typical GRP94 proteins including the possession of an N-terminal ER signal peptide. However, a variation in the C-terminal ER retention motif, KDEL, is observed in all the trypanosomatid orthologues of GRP94; AGDL in *Trypanosoma spp*., KEEL in *B. saltans*, EGDL in *C. fasciculata* and all *Leishmania spp.* (Figure 2.1). Phylogenetic analysis indicates that the GRP94 proteins in trypanosomatids could have evolved separately from their mammalian orthologues (Figure 2.1), perhaps to fulfil a specific role within the parasites. Transcriptomic and proteomic studies revealed that TbbGRP94 is expressed at all life cycles with a two-fold increase in protein abundance between the short stumpy (SS) and long slender (LS) stages of the parasite (Gunasekera et al., 2012) . Proteomic studies confirm the presence of GRP94 in flagella and cell surface (Subota et al., 2014; Shimogawa et al., 2015).

In trypanosomatids, the first recognized and characterized *GRP94* gene was in *Leishmania infantum* (*L. infantum*). The GRP94 orthologue in *Leishmania infantum* (*L. infantum*) was shown to localise in the ER and share many of the activities of GRP94s of other eukaryotes (Descoteaux, 2002). Unlike GRP94 in mammalian cells, LinGRP94 is not essential for cell viability and LinGRP94 mRNA is induced developmentally rather than by canonical GRP94-inducing stresses (Descoteaux, 2002). The protein was highly immunogenic during *Leishmania* infection (Larreta et al., 2000, 2002), and essential for lipophosphoglycan (LPG) assembly (Descoteaux, 2002), an abundant surface glycolipid of *Leishmania* promastigotes that is critical to parasite virulence (Yao et al. 2003). Effectively, the critical role of GRP94 in *Leishmania* appears to be adapted to the synthesis of glycoconjugates and directing the host immune response implicating a pivotal role in parasite virulence (Descoteaux, 2002). Though whether this specialized role is conserved in *T. brucei* and other trypanosomatids will need to be elucidated. The function and cellular roles of TbGRP94 should be explored, given the immunogenic and antigenic properties shown by the *L. infantum* GRP94, as this protein could constitute a valuable molecule for diagnostic purposes, and quite possibly a potential candidate for studies of protective immunogenicity. Little is known about the effect of post-translational modifications on GRP94 (Argon and Simen 1999; Marzec et al. 2012).

2.4.3 The *T. brucei* Hsp83 co-chaperone system

In all organisms, Hsp90 is a dynamic protein that undergoes a conformational cycle whose directionality is determined in large part by ATP binding and hydrolysis, together with a cohort of co-chaperones (Panaretou et al. 1998; Prodromou 1999; Johnson and Brown 2009). The Hsp90 chaperone ensemble

can vary in composition depending on the client proteins, but usually includes Hsp70/J-protein, p23, immunophilins, Aha1 and STIP1 (Hop) (Johnson and Brown 2009). The variation in subunit composition across organisms appears to be related to the fact that the function of some Hsp90 co-chaperones may be restricted to specific subsets of client proteins, be required for client protein activation in a species-dependent manner, or made redundant by other co-chaperones (Zuehlke and Johnson, 2010). The Hsp90 chaperone system in intracellular protozoan parasites has been explored in previous studies (Seraphim et al. 2013; Figueras et al. 2014). Thus, using the human and kinetoplastid systems, this study analysed the composition of the *T. brucei* Hsp83 chaperone system. It was determined in this study that *T. brucei* possesses an almost complete set of co-chaperones (Table 2.4), with the only notable absence being cell division cycle 37 (Cdc37). The absence of a gene encoding Cdc37 has also been noted in several intracellular protozoan parasites including *Plasmodium* and *Leishmania spp* (Chua et al. 2014; Figueras et al. 2014; Tatu and Neckers 2014; Hombach-Barrigah et al. 2019). Cdc37 is a co-chaperone that has a specialized and indispensable role in the maturation and/or stabilization of a large subset of protein kinases (Smith and Workman 2009). The absence of Cdc37 in some species shows that clients that are dependent on a specific cochaperone in one species may not require Hsp90 for function in other species, thus the protein kinases in protozoan parasites may have evolved in such a way that the proteins bind a different co-chaperone or are independent of Hsp90 for function. Since little is known about why a protein becomes dependent on Hsp90 for activity or stability, it poses interesting questions on the mechanism by which the maturation and regulation of protein kinases in protozoan parasites is mediated dependent or independent of Hsp83. Exploration of this mechanism may provide a potential avenue for chemotherapeutics since protein kinases are also an attractive drug target in infectious diseases, such as African Trypanosomiasis. The Hsp70/J-protein machinery from *T. brucei* have been explored previously (Bentley et al. 2019). The identified Hsp83 co-chaperones in both *T. brucei* subspecies are listed in Table 2.4, and a comprehensive domain organisation of these predicted proteins is illustrated in Table 2.5. Additionally, the Hsp83 co-chaperones were categorised in this study based on the presence of the TPR domain.

Table 2.4: The Hsp83/HSPC co-chaperones from *Trypanosoma brucei* with their putative orthologues in *T. cruzi, L. major, C. fasciculata, B. saltans* and *H. sapiens*.

	H. sapiens	*T. brucei*	*T. cruzi*[c]	*L. major*	*C. fasciculata*	*B. saltans*		
Name	**Gene ID**[a]	**Gene ID**[a]	**Gene ID**[a]	**Gene ID**[a]	**Gene ID**[a]	**Gene ID**[a]	**Localisation**[b]	**Reference**
A: TPR-containing Hsp83 co-chaperones								
Sti1/Hop	10963	Tb927.5.2940 Tbg972.5.4130	Tc_MARK_9009 C4B63_59g115	LmjF08.1110	CFAC1_020023900	BSAL_57725	CYTO NUC CELL SURFACE (BSF, PF)	(Gunasekera et al. 2012; Urbaniak et al. 2012; Butter et al. 2013; Shimogawa et al. 2015; Dean et al. 2017)
PP5	5536	Tb927.10.13670 Tbg972.10.16800	TcCLB.507993.190 C4B63_4g368	LmjF.18.0150	CFAC1_140007400	BSAL_15705	CYTO (BSF, PF)	(Gunasekera et al. 2012; Urbaniak et al. 2012; Butter et al. 2013; Dean et al. 2017)
Cyp40	5481	Tb927.9.9780 Tbg972.9.5630	TcCLB.506885.400 Tc_MARK_4311 C4B63_2g294	LmjF.35.4770	CFAC1_300099000	BSAL_06490	CYTO FLAGELLAR (BSF)	(Oberholzer et al. 2011; Dean et al. 2017)
DnaJC7/Tpr2	7266	Tb927.10.4900 Tbg972.10.5950	TcCLB.504203.60 Tc_MARK_8493 C4B63_13g112	LmjF.36.0500	CFAC1_250012000	BSAL_30720	CYTO NUC (BSF, PF)	(Urbaniak et al. 2012; Butter et al. 2013; Dean et al. 2017)

FKBP5	2289	Tb927.10.16100 Tbg972.10.19710	TcCLB.511353.10 Tc_MARK_4665 C4B63_157g28 C4B63_171g30	LmjF.19.1530	CFAC1_210025000	BSAL_03610 BSAL_65235	CYTO FLAGELLAR (BSF, PF)	(Gunasekera et al. 2012; Urbaniak et al. 2012; Butter et al. 2013; Subota et al. 2014; Dean et al. 2017)
SGT	6449	Tb927.6.4000 Tbg972.6.3780	TcCLB.511737.10 Tc_MARK_2022 C4B63_18g260	LmjF.30.2740	CFAC1_260051600	BSAL_66445	CYTO FLAGELLAR CELL SURFACE (BSF, PF)	(Gunasekera et al. 2012; Urbaniak et al. 2012; Butter et al. 2013; Subota et al. 2014; Shimogawa et al. 2015; Dean et al. 2017)
B: Non TPR-containing Hsp83 co-chaperones								
p23	10728	Tb927.9.10230 Tb927.10.2620 Tbg972.9.5930 Tbg972.10.3260	TcCLB.509551.70 TcCLB.506407.60 C4B63_2g235 C4B63_47g40	LmjF.35.4470 LmjF.34.0210	CFAC1_300096200 CFAC1_290030000	BSAL_38665	CYTO FLAGELLAR NUC	(Dean et al. 2017)
Aha1	10598	Tb927.10.13710 Tbg972.10.16840	TcCLB.507993.150 Tc_MARK_4860 C4B63_4g357	LmjF.18.0210	CFAC1_140008400	BSAL_15670	CYTO NUC (BSF, PF)	(Gunasekera et al. 2012; Urbaniak et al. 2012; Butter et al. 2013; Dean et al. 2017)

[a] The Gene IDs for the *T. b. brucei* (Tb refers to Tbb), *T. b. gambiense*, *T. cruzi*, *C. fasciculata, B. saltans* and *L. major* Hsp83/HSPC co-chaperones were retrieved from the TriTrypDB database (http://tritrypdb.org/tritrypdb/; Aslett et al. 2010). The Gene IDs for the members of the *H. sapiens* Hsp90/HSPC co-chaperones were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/).

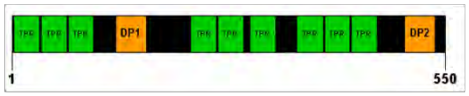
[b] The Gene IDs for the orthologues, identified by reciprocal BLASTP analysis, of three strains of *T. cruzi* are listed. *T. cruzi* CL Brener Esmeraldo-like (TcCLB), *T. cruzi* Dm28c 2018 (C4B63), and *T. cruzi* marinkelli strain B7 (Tc_MARK).

[c] Subcellular localizations for the *T. brucei* Hsp83/HSPC co-chaperone proteins were acquired from using the TrypTag database (http://tryptag.org/; Dean et al. 2017) and/or determined using various proteomic datasets listed in the materials and methods.

CYT-Cytosol; MITO- Mitochondrion; NUC- Nucleus; ER- Endoplasmic reticulum; GYLCO- glycosomes; FLAGELLAR- Flagellar; CELL SURFACE- Cell surface.

Table 2.5: Domain architecture, protein properties, life cycle expression and RNAi mediated knockdown of the Hsp90 TPR (A) and non-TPR (B) co-chaperones in *T. brucei*.

A

Protein	Domain organisation	MW	pI	RNAi
Sti1		62327 Da	6.23	ALL
PP5		53312 Da	6.61	ALL
Cyp40		38094 Da	6.26	BSF DIFF
J52		55926 Da	8.04	BSF DIFF
FKBPL		47604 Da	5.83	BSF DIFF
SGT		45966 Da	4.9	ALL

B

Protein	Domain organisation	MW	pI	RNAi
p23a		18768 Da	4.02	BSF PRO
p23b		21808 Da	4.17	DIFF
Aha1		37612 Da	5.51	BSF

Each protein sequence is represented by a multi-coloured bar with the numbering on the bottom of the bar indicating the length of the protein in amino acid residues. Protein domains and other associated features that were identified using Prosite (Sigrist et al., 2010) and SMART (Letunic et al. 2012)are also shown. The physiochemical properties, molecular weight (MW) and isoelectric point (pI), for each *T. brucei* Hsp90 co-chaperone was calculated using the compute pI/Mw tool from ExPASy (https://web.expasy.org/compute_pi/;Gasteiger et al. 2005). Data on the phenotypic knockdown screen, using RNAi conducted by Alsford et al. (Alsford et al., 2011), for the Hsp90 co-chaperones are provided: ALL-All life cycle stages; BSF- Bloodstream; DIFF- Differentiation; NE- Non-essential; ND-Not determined.

2.4.3.1 TPR-containing co-chaperones

Sti1

Stress-inducible protein 1 (Sti1), also known as Hsp70/Hsp90 organizing protein (Hop or STIP1) in mammals, is one of the best studied co-chaperones in the Hsp90 reaction cycle (Chang et al. 1997; Johnson et al. 1998) as it acts as an adaptor protein, mediating the interaction between Hsp70 and Hsp90 through its TPR domains (Brinker et al. 2002; Odunuga et al. 2003; Baindur-Hudson et al. 2015). Sti1/Hop is a widely conserved Hsp90 co-chaperone and has been annotated and characterized across diverse organisms including several kinetoplastid protists. Initially thought to be an indispensable protein, recent discoveries in yeast and some eukaryotes show that direct interaction can take place *in vitro* between Hsp70 and Hsp90 in the absence of Hop (Kravats et al. 2018; Bhattacharya et al. 2020). A single *Sti1/Hop* gene was found encoded in both *T. brucei* subspecies (Table 2.4), with the amino acid sequence indicating canonical Sti1/Hop domain architecture (Table 2.5). Nine TPR motifs arranged into three TPR domains (TPR1, TPR2A and TPR2B) in addition to two domains rich in proline and aspartic acid (DP1 and DP2) were predicted (Scheufler et al. 2000; Nelson et al. 2003). Molecular modelling was used to predict a full structure of TbSti1 as shown in Figure 2.4. The TbSti1 individual domain structures are structurally conserved showing the typical anti-parallel α-helix structures of the TPR domains (Das 1998; Smith 2004) (Figure 2.4). The TPR domains contain highly conserved residues which form interactions with residues in the C-terminal domain of Hsp90 forming a carboxylate clamp necessary for protein-protein interactions to take place (Odunuga et al. 2003). Sti1 has been shown previously to bind to Hsp90 with its TPR2A domain (Smith 2004; Röhl et al. 2015), the TPR2A conserved residues predicted to be involved in forming the carboxylate clamp include Lys^{229}, Asn^{233}, Asn^{264}, Lys^{301} and Arg^{305} (Odunuga et al. 2003). These residues are conserved as Lys^{230}, Asn^{235}, Asn^{265}, Lys^{302} and Arg^{306} in *T. brucei* and all species analysed (Figure 2.5). Sequence identity showing 100% conservation of the selected kinetoplastids and human Hop are highlighted (Figure 2.5). There are two Hop isoforms in humans, the only difference being Hop1a has more amino acid residues at its NTD start sequence (Figure 2.5)

Both Sti1/Hop orthologues in *T. cruzi* and *L. major* were found to immunoprecipitate with Hsp83 and Hsp70 as well as co-localize with these chaperones in the cytoplasm and/or around the nucleus (Webb et al., 1997; Schmidt et al., 2011). The expression of Hop isoforms was increased in response to different environmental stresses (Webb et al., 1997; Schmidt et al., 2011) with LmjHop being up regulated when the parasites are exposed to heat stress conditions (Webb et al., 1997), whereas only nutritional stress induced expression of TcSti1 in the late growth phase of epimastigotes (Schmidt et al., 2011). The Hsp90-Sti1 com-

plex in *L. major* and *T. cruzi* has been shown to be pivotal to parasite differentiation (Webb et al., 1997; Hombach et al., 2013). Proteomic analysis in *T. brucei* indicates that TbbSti1 is part of the cell surface proteome during the procyclic stage (Shimogawa et al., 2015). Though TbbSti1 is present in both BSF and PCF stages of the parasite, it was more highly expressed in the bloodstream form but unchanged in protein abundance between the short stumpy to long slender transition (Urbaniak et al. 2012; Gunasekera et al. 2012; Butter et al. 2013). These data suggest that the Sti1 orthologue in both *T. brucei* subspecies should function as an adaptor protein for TbHsp83 and TbHsp70s, participating in the foldosome apparatus necessary for maintaining proteostasis, cytoprotection and modulating parasite differentiation.

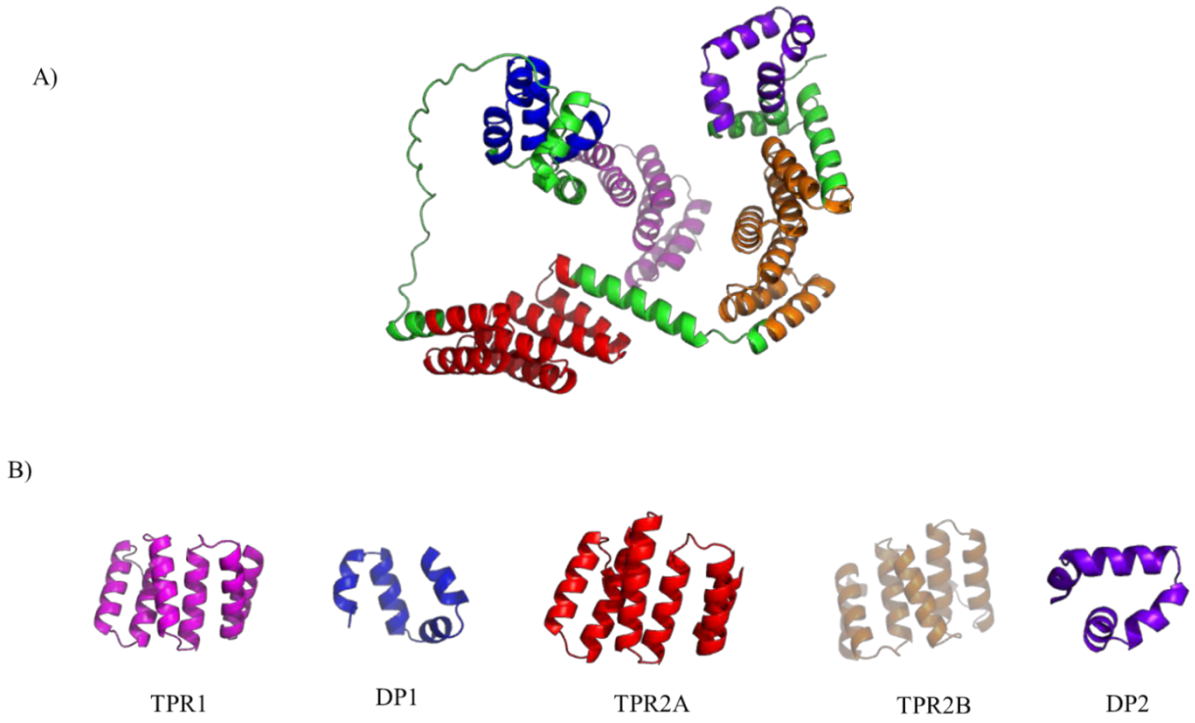


Figure 2.4: Predicted 3D model of TbSti1.
A) Full length cartoon structure of TbSti1 (residues 1-550) B) cartoon structures of various domains of TbSti1, TPR1 (residues 3-104), DP1 (residues 132-171), TPR2A (residues 227-335), TPR2B (residues 362-463) and DP2 (residues 498-537). Full length structure of TbSti1 was predicted using AlphaFold DB (AlphaFold Protein Structure Database; https://alphafold.ebi.ac.uk) (Varadi et al., 2022), individual domains were predicted on Interpro (Integrative protein signature database; http://www.ebi.ac.uk/interpro/) (Apweiler et al., 2000) and cartoon structures modelled and annotated on PyMOL 2.5 (DeLano, 2002).

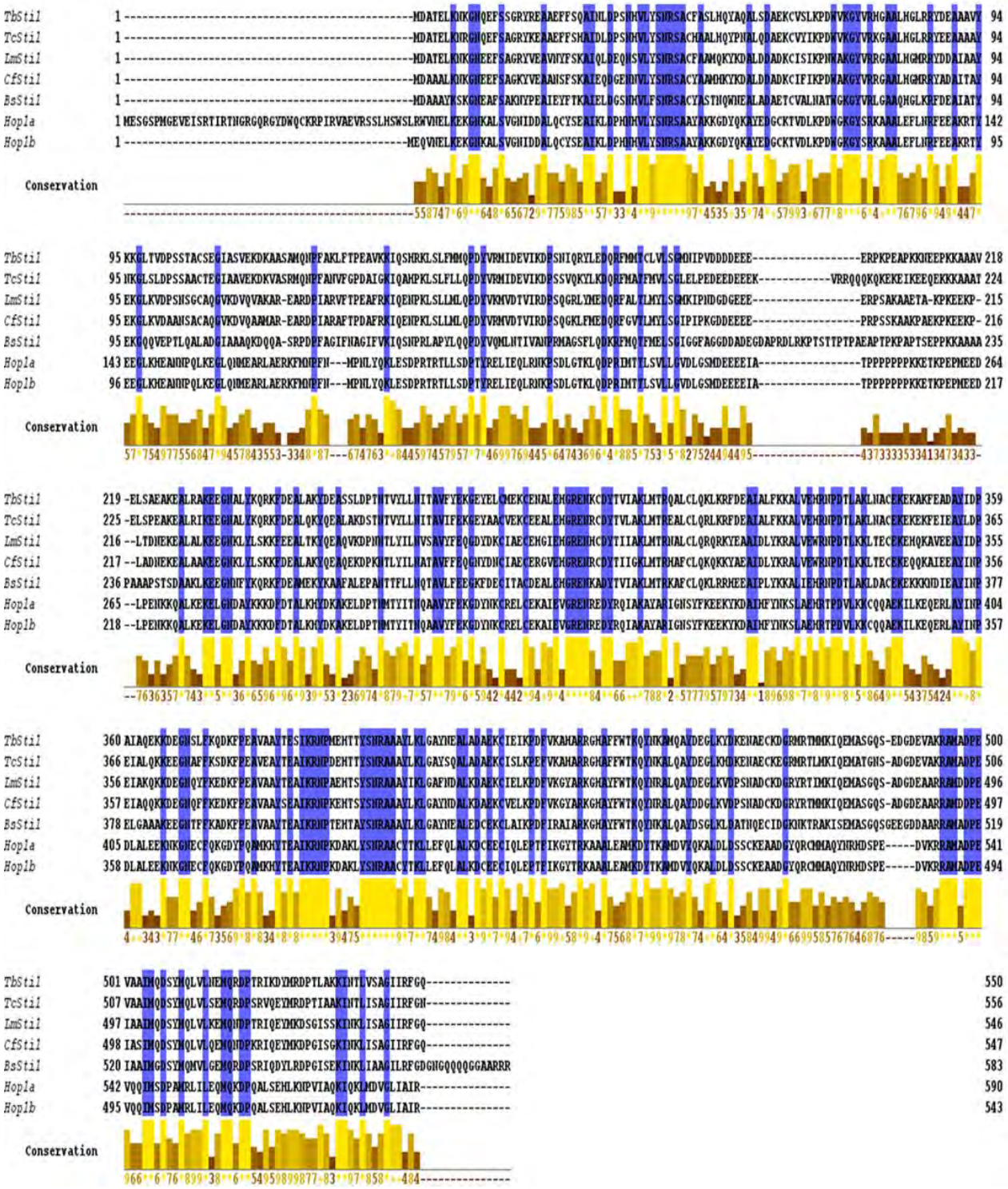


Figure 2.5: Multiple sequence alignment of TbSti1 in selected species.
TbSti1, TcSti1, LmSti1, CfSti1, BsSti1, Hop1a and Hop1b. Accession numbers and Gene IDs for all selected species are found in Table 2.4. Fully conserved residues are represented below with gold bars and an * below, as conservation reduces the gold shade darkens with reducing numbers from 9 to 1. For emphasis all fully (100%) conserved residues are highlighted in blue.

PP5

Protein phosphatase 5 (PP5) is a member of the PPP family of serine/threonine protein phosphatases and it associates with Hsp90 in complexes during client protein maturation (Cohen 1997; Chinkers 2001; Golden et al. 2008). PP5 is characteristically different from other PPP family members in that it possesses an N-terminal TPR domain (Borthwick et al., 2001), which mediates interaction with Hsp90 (Chen et al. 1996). This interaction enables PP5 to modify the phosphorylation status of Hsp90 client proteins (Golden et al. 2008). The gene for PP5 in *T. b. brucei* (*TbbPP5*) has been extensively studied. *TbbPP5* encodes a ~52-kDa protein that possesses the canonical N-terminal TPR domain and phosphatase catalytic domain (Anderson et al. 2006) as shown in Table 2.5. TbbPP5 interacted with TbbHsp83 *in vivo* and co-localized with the chaperone in the cytosol of PRO parasites (Jones et al. 2008). Both TbbPP5 and TbbHsp83, upon heat shock and geldanamycin treatment, accumulated in the nucleus (Jones et al. 2008), indicating that both TbbPP5 and TbbHsp83 translocate to the nucleus when the parasites are exposed to proteotoxic stresses (Jones et al. 2008). TbbPP5 was detected in both BSF and PCF stages of the parasite but upregulated in the procyclic form (Gunasekera et al. 2012; Urbaniak et al. 2012; Butter et al. 2013). Overexpression of TbbPP5 was found to partially negate the effect of geldanamycin treatment on cell growth, which indicates that the co-chaperone enhances the chaperoning function of TbbHsp83 and promotes the folding and maturation process of important regulatory molecules, which facilitate cell growth.

Peptidyl-prolyl cis-trans isomerases (PPIases)

The immunophilin superfamily consists of highly conserved proteins with rotamase or peptidylprolyl cis-trans-isomerase (PPIase) activity that accelerates protein folding by mediating the isomerization of X-Pro-peptide bonds (Galat, 2003; Pratt et al., 2004). The best characterized PPIases belong to two families, the cyclophilin-type (Cyp) and the FKB-506 drug-binding protein type (FKBP) (Steiner and Haughey, 2010). Data mining of the *T. brucei* genome identified that Cyp40 and a putative FKB-506 binding like protein (FKBPL) are present in the extracellular parasite proteome (Table 2.4). Investigation of the domain structure and sequence conservation indicates that both Cyp40 and FKBPL in *T. brucei* display the characteristic two-domain structure of a N-terminal PPIase domain and a C-terminal TPR domain (Table 2.5). Though it must be noted that the C-terminal TPR domain in kinetoplastid Cyp40 underwent substantial evolutionary modification (Yau et al., 2010), thus potentially impacting Cyp40-Hsp83 interactions. Future structure/function studies should explore the effect these modifications have on the isomerase and chaperone activities of the protein in comparison to its human counterpart.

Studies conducted on the Cyp40 orthologue in *L. donovani* have revealed that the protein functions in *Leishmania* stage-specific morphogenesis, motility, and the development of infectious-stage parasites (Yau et al., 2010, 2014). The study conducted by Yau and colleagues (2014) also suggested that LdCyP40 and LdFKBP2 functions in regulating *Leishmania* cytoskeletal dynamics. Given the capacity of CyP40 and FKBP52 to compete for molecular partners (Ratajczak et al. 2003), LdCyP40 may interact with microtubules to promote tubulin polymerization as a means of counteracting LdFKBP52-mediated depolymerization. RNAi-mediated knockdown of both Cyp40 and FKBPL in *T. b. brucei* parasites demonstrated that these proteins are essential at the BSF stage and parasite differentiation with a two-fold upregulation in protein abundance from the short stumpy to long slender parasite stages (Alsford et al. 2011; Gunasekera et al. 2012; Urbaniak et al. 2012; Butter et al. 2013). Proteomic data predicted these proteins to reside in the cytosol and flagellar (Oberholzer et al., 2011; Subota et al., 2014). Together this data indicates that *T. brucei* CyP40 and FKBPL may play essential roles in morphogenesis, motility, and the development of infectious-stage parasites.

J52

The J-protein family is a major subset of co-chaperones for the Hsp70 chaperone machinery, and they are broadly classified into four subtypes (I-IV). The J-protein family from *T. brucei* has been explored previously (Bentley et al. 2019). It was shown in that study that J52 is one of six type III J proteins in *T. brucei* that possesses the TPR domain (others are J42, J51, J52, J53, J65 and J67) (Bentley et al. 2019). J52 is predicted to reside in the cytosol together with J51 and J42 (Bentley et al. 2019). DnaJC7/Tpr2, the human orthologue of J52 was first identified as a cytosolic protein via a two-hybrid screen for interaction with a GAP-related segment (GRD) of neurofibromin. It was reported to encode seven TPR units and possess a domain of high similarity to the DnaJ family (Murthy et al., 1996). Tpr2 also regulates the multichaperone system involving Hsp70 and Hsp90 but in a nucleotide independent manner with Hsp90. DnaJC7 is predominantly thought to be involved in retrograde transport of client proteins from Hsp90 to Hsp70 (Brychzy et al., 2003; Moffatt et al., 2008). Proteomic analysis in *T. brucei* showed J52 to be upregulated in the procyclic form of the parasite (Urbaniak et al. 2012; Butter et al. 2013).

Small glutamine-rich TPR-containing protein (SGT)

The small glutamine-rich TPR-containing protein (SGT) is a co-chaperone involved in a specific branch of the global cellular quality control network that determines the fate of secretory and membrane proteins that

mislocalize to the cytosol (Leznicki and High, 2012; Wunderley et al., 2014). Human SGT is a modular protein characterized by three characteristic sequence motifs, namely an N-terminal dimerization domain, central TPR domain and a glutamine-rich region at the C-terminus (Roberts et al., 2015). The SGT orthologues identified in kinetoplastid protists are atypical (Table 2.4) as these proteins all lack the characteristic glutamine-rich region and contain a substituted region with charged amino acid residues (Ommen et al. 2010). Proteomic analysis in *T. brucei* identified TbbSGT to be upregulated in the procyclic form of the parasite but unchanged in protein abundance between the short stumpy and the long slender BSF, TbbSGT was also part of the flagellar and cell surface proteome (Gunasekera et al. 2012; Urbaniak et al. 2012; Butter et al. 2013; Subota et al. 2014; Shimogawa et al. 2015). The SGT orthologue in *L. donovani* is an essential protein for *L. donovani* promastigote growth and viability (Ommen et al. 2010). LdSGT was shown to form large, stable complexes that included Hsp83, Hsp70, HIP, Hop, J-proteins, and Hsp100 (Ommen et al. 2010), whereas recombinant *L. braziliensis* SGT was shown to interact with both LbHsp90 and HsHsp70-1A (Coto et al., 2018). Therefore, the orthologous proteins in *T. b. brucei* and *T. b. gambiense* may have developed the same activity and assist in the formation of the *T. brucei* Hsp83 chaperone system. Though future studies should be conducted to elucidate SGT-Hsp70/Hsp83 interaction in *T. brucei*.

2.4.3.2 Non-TPR containing Hsp83 co-chaperones

p23

The co-chaperone p23 is a small acidic protein that binds the Hsp90 NBD to stabilize the closed conformation of Hsp90, inhibiting ATPase activity and preventing client protein release from the complex (Young 2000; McLaughlin et al. 2006). In addition to its Hsp90 co-chaperone function, p23 has its own chaperoning activity *in vitro* and can suppress the aggregation of denatured proteins (Bose et al. 1996; Freeman e1996). *In silico* analysis of the genomes of both *T. brucei* subspecies revealed that the parasite possesses two evolutionarily divergent p23 orthologues, and subsequently these orthologous proteins were named p23a and p23b (Table 2.4). The possession of two putative p23 proteins was found to be conserved in all the selected kinetoplastid protists in this study except *B. saltans* (Table 2.4). The Tbp23a and Tbp23b proteins share 28% identity to each other and share 33% and 26% identity respectively to human p23. Additionally, RNAi knockdown of these proteins showed that each p23 protein is essential to parasite viability at specific stages of the life cycle (Table 2.5). The orthologs of these proteins have been explored in two *Leishmania spp*. (Batista et al., 2015). Both proteins in *L. braziliensis* possessed intrinsic chaperone

activity, but they have different client protein specificities; they also inhibit LbrHsp83 ATPase activity to different extents (Batista et al., 2015). Such functional differences might be important in both Hsp90 regulation and in their interactions with client proteins during the life stage transformations of kinetoplastid parasites. However, to support these assertions, more functional and *in vivo* studies of kinetoplastid p23a and p23b proteins are needed.

Aha1

Aha1 has been identified as the primary activator of the ATPase activity of Hsp90 and it acts independently of the other co-chaperones. Homologues of Aha1 have been identified across species from yeast to mammals. Aha1 binds with both its N-and C-terminal domain (Table 2.5) to the NBD and MD of Hsp90 to facilitate the dimerization of the chaperone (Mayer et al. 2002; Koulov et al. 2010; Retzlaff et al. 2010). Data mining of the *T. brucei* genome identified that the parasite encodes for a single *Aha1* gene (Table 2.4). The Aha1 orthologue in *L. braziliensis* (LbrAha1) has been characterized, where it was shown to be a cognate protein that shared several structural and functional properties with the human and yeast orthologues. This suggested similar functional mechanisms among these proteins despite the low degree of conservation in the amino acid sequence (Seraphim et al., 2013). Recombinant LbrAha1 stimulated the weak ATPase activity of recombinant LbrHsp83 by around 10-fold exhibiting a cooperative behaviour according to the model that two LbrAha1 molecules can act on one LbHsp83 dimer (Seraphim et al., 2013). Data from proteomic analysis in *T. brucei* revealed that TbbAha1 is up regulated in the BSF stage of the parasite (Gunasekera et al. 2012; Urbaniak et al. 2012; Butter et al. 2013) as well as being essential to parasite viability at this stage of life cycle (Alsford et al., 2011).

Two other co-chaperones in *T. brucei* had previously been identified, a TPR domain protein identified as Cns1(Tb927.10.11380) and a component of motile flagella 56 (Tb927.9.10490) which is an orthologue of human protein interacting with Hsp90 (Pih1) (Johnson and Brown 2009). Little has been done to explore these two proteins. So far, only the cytosolic Hsp90 has been shown to require the function of co-chaperones, the other forms of Hsp90 function in the absence of co-chaperones (Richter et al. 2007; Masgras et al. 2017).

2.5 Conclusion

The Hsp90 family contains an abundant and essential group of proteins which are highly conserved and implicated in a myriad of cellular functions. Due to their role in cellular proteostasis, they have been implicated in the pathology of many diseases which warrants their targeting as therapeutics (Samant et al. 2012). Previous studies on the Hsp90 complexes of intracellular kinetoplastids such as *Leishmania spp* and *T. cruzi* have been conducted (Shonhai et al., 2011) but not on the extracellular *T. brucei*. Despite the conservation, distinctive differences exist across species and call for further investigation. In this study we report the *in silico* study of the Hsp90 family and its chaperone complement in *T. brucei*. *T. b. brucei* was found to encode 12 putative Hsp90 proteins, 10 of which are cytosolic (Hsp83). Multiple copies of Hsp83 may allow the parasite to reach a high synthesis level of the proteins in an organism that relies on post-transcriptional regulation, and this explains its high levels in the cell even under non-stress conditions (Shonhai et al. 2011; Requena et al. 2015). The expansion of the Hsp90 chaperone complement also reiterates its importance in the biology and functioning of kinetoplastids (Folgueira and Requena 2007; Shonhai et al. 2011; Urményi et al. 2014). Hsp83 was also found present in both stages of the parasite but upregulated in the blood stream form (BSF) but unchanged in protein abundance between the short stumpy and long slender forms of the parasite, this is similar to previous findings of much higher transcripts of Hsp83 in blood stream forms of *T. brucei* reflecting their temperature induced role of differentiation (Ploeg et al. 1985). The upregulation of Hsp83 together with the co-chaperone Sti1in the BSF may be a further indication of their heat inducibility and involvement in cell defence just as seen in Hsp70 (Urményi et al. 2014). Interestingly, GRP94 and the FKBP5 co-chaperone were the only proteins in this study found with higher protein abundance in the long slender BSF compared to the short stumpy form.

Hsp90 has been established to partner with co-chaperones to maintain homeostasis, however, Hsp90 seems to partner with the various co-chaperones as dictated by the client being chaperoned (Radli and Rüdiger, 2017; Sahasrabudhe et al., 2017). This study identified 8 co-chaperones in the *T. brucei* Hsp83 chaperone system which is fewer than the number of co-chaperones in the human system, confirming that the Hsp90 chaperone machinery is species specific (Johnson and Brown 2009). A detailed report for clients in Hsp90 is still largely absent (Roy et al., 2012). Previous studies have indicated that inhibitors targeting Hsp83 have been shown to cure mice of *T. brucei* infection, although the toxicity of inhibitors to Hsp90 in higher eukaryotes is attributed to a functional loss of client proteins and possible cell cycle arrest (Meyer and Shapiro 2013). Most of the identified Hsp90 client proteins in mammals are kinases (Taipale et al., 2012). Despite the fact that most clients for *T. brucei* Hsp90 have not been identified, over 170 protein kinases (about 30% of the number present in their human host), have been recognized (Parsons et al. 2005; Nett et

al. 2009). In addition to being regulated by co-chaperones, Hsp90 is also regulated by various post-translational modifications. Some of these PTM sites have been indicated as potential regulatory sites which affect the binding affinity of inhibitors in PfHsp90 (Pallavi et al., 2010). The *T. brucei* Hsp90, its co-chaperone network, post-translational modifications, and its regulatory mechanisms as well as the subtle structural differences compared to human Hsp90 all provide a context for a Hsp90-targeted therapy in *T. brucei.*

3 Biochemical characterization of Hsp90-Sti1 interaction in *Trypanosoma brucei*

3.1 Introduction

Sti1 has been characterized in other protozoan parasites such as *Plasmodium falciparum* where it mediates the interaction between Hsp70 and Hsp90 (Gitau et al., 2012), LbSti1(LbHop) in *Leishmania braziliensis* interacts with LbHsp90 and inhibits its ATPase activity (Batista et al., 2016), TcSti1 in *Trypanosoma cruzi* was induced by nutritional stress and involved in the differentiation process of the parasite (Schmidt et al. 2011; 2018). However, Sti1 has not been well characterized in *T. brucei*. Our *in silico* findings (Chapter 2) from *T. brucei* confirm the presence of 12 putative copies of the *Hsp90* gene as previously reported (Folgueira and Requena 2007; Mottram et al. 1989), 10 of which code for the cytosolic Hsp90 protein (TbHsp83) and a single *Sti1*gene that codes for the TbSti1 protein which is present in both the procyclic (PCF) and bloodstream form (BSF) of the parasite. The aim of this study was therefore to characterize TbHsp83 and its interaction with TbSti1, using human Hsp90 (hHsp90) as a control. In this study we successfully purified and biochemically characterized Tbsti1 and its interaction with TbHsp83. Understanding this interaction can help distinguish it from the host system and provide an updated context for chemotherapeutic options.

3.2 Objectives

The aim of this study was first to purify recombinant versions of the *T. brucei* and human Hsp90 and the TbSti1 co-chaperone from *E. coli*. Next the biochemical properties of the interaction TbSti1 with TbHsp83 and hHsp90α was analysed.

3.3 Specific objectives

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- Confirmation of all plasmids - pET-30a-TbHsp83.1 plasmid, pET-16b-Hsp90a plasmid, pQE60-TbSti1 and pCDNA3.1(+)_HA-Tbsti1 confirmation using agarose gel electrophoresis.

- Heterologous production/induction of TbSti1, TbHsp83 and human Hsp90 using 2xYT and terrific broth for maximum yield.
- Purification of the recombinant proteins from *E. coli* using nickel affinity chromatography.
- Qualitative and quantitative interaction assays to investigate the direct binding interaction of TbSti1 with TbHsp83 and human Hsp90 (far western and solid phase binding assay).
- Investigation of the suppression of aggregation activity of the chaperones TbHsp83 and hHsp90 on malate dehydrogenase (MDH) as substrate in the presence and absence of the co-chaperone TbSti1.
- Investigation of the effect of TbSti1 on the ATPase activity of TbHsp83 and hHsp90.

3.4 Materials and methods

3.4.1 Materials

Unless stated otherwise, all reagents were purchased from ThermoScientific/Sigma-Aldrich (U.S.A), New England Biolabs (U.S.A), Roche (Germany), Promega (U.S.A), Bio-Rad (U.S.A), Greiner Bio-one (Germany), Merck (Germany) and Santa Cruz Biotechnology Inc (U.S.A). Expression plasmids (pQE60-TbSti1, pET-30a-TbHsp83 and pCDNA3.1(+)_HA-TbSti1) are described in Table 3.1. The rabbit antibodies against *T. brucei* proteins (anti-TbHsp83 and anti-TbSti1) were produced and purchased from GenScript (U.S.A), lot number A313120295 and A313120293 respectively. The pET-16b-Hsp90α plasmid, anti-human antibodies, anti-hHsp90α (Enzo-ADI-SPA-840), anti-hHsp90β (Abcam-ab119833), anti-Hsc70 (StressMarq -SMC 151) and anti-hHop (abcam-ab126724) were kind donations from Professor Adrienne Edkins' lab (Rhodes University, Grahamstown, South Africa). A summary of the strains and plasmid used in this study are in Table 3.1

Table 3.1: Description of strains and plasmids used in this study.

Strain/Plasmids	Description	Source
Strains		
E. coli DH5α	F– *endA1 glnV44 thi- recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15Δ (*lacZYA-argF*) U169, hsdR17 (rK–mK+), λ–	Life Technologies, U.S. A
E. coli JM109	end*A1,* rec*A1,* gyr*A96,* thi, hsd*R17 (rk–, mk+),* rel*A1,* sup*E44, Δ(* lac-pro*AB), [F′* tra*D36,* pro*AB,* laq*IqZΔM15]*	Promega, U.S. A
E. coli M15 (pREP4)	*F-, Φ80ΔlacM15, thi, lac-, mtl-, recA+, KmR*	Qiagen, Germany

E. coli BL21 (DE3)	*F– ompT gal dcm lon hsdSB(rB–mB–)λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS)*	Stratagene, U.S. A
Plasmids		
pQE60-TbSti1	pQE60 encoding TbSTi1, Amp^R	Dr. S. Bentley
pCDNA3.1(+)_HA-TbSti1	pCDNA3.1(+) encoding TbSti1, Amp^R	Dr. S. Bentley
pET-30a-TbHsp83	pET-30a encoding TbHsp83, Kan^R	Paula Macucule-Tinga
pET-16b-Hsp90α	pET-16b encoding hHsp90α, Amp^R.	Dr. Abir Chakraborty

3.4.2 Methods

3.4.2.1 Restriction digestion of plasmid DNA and agarose gel electrophoresis

To verify the integrity of plasmids used in this study (Table 3.1), diagnostic restriction analysis was done using specific restriction enzymes, and visualization and analysis was done with agarose gel electrophoresis. A reaction mix was prepared containing 1 µg plasmid DNA, nuclease free water, 1x buffer and restriction enzymes (20 units per 50 µl reaction) as specified by the manufacturers for single and double digestions (Appendix 7.1.7). The reaction mix was incubated at 37 °C for 16 hours. To visualize the digested products, 0.8% (w/v) agarose gel was prepared in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) with the addition of 0.5 µg/ml ethidium bromide. The restriction digested samples and their controls are loaded after the addition of a 6x DNA loading dye (0.25% [w/v] bromophenol blue, 30% [v/v] glycerol). Electrophoresis took place at 100 V for an hour and was visualized under UV light.

3.4.2.2 Induction studies to assess the production profiles of the recombinant proteins in *E. coli*

Plasmids verified by restriction analysis were used for chemical transformation (Appendix 7.1.4) of competent *E. coli* cells (Appendix 7.1.5) and plated on 2 x Yeast-Tryptone (YT) agar plates (16 g/L tryptone powder, 10 g/L yeast extract, 5 g/L NaCl and 15 g/L agar) with the appropriate antibiotics for each plasmid, namely – ampicillin (100 µg/ml) and kanamycin (50 µg/ml) for pQE60-TbSti1, kanamycin (50 µg/ml) for pET-30a-TbHsp83 and ampicillin (100 µg/ml) for pET-16b-Hsp90α. After successful transformation, a single colony was inoculated into 10 ml of 2xYT broth containing the appropriate antibiotics and cultured overnight at 37 °C with gentle agitation in an incubator. Untransformed competent cells were used as negative controls for each protein. The overnight culture was subsequently diluted 10x with 2 x YT containing appropriate antibiotics for each plasmid and returned to the incubator at 37 °C with agitation (~180 rpm) until mid-log phase (absorbance OD_{600} 0.4-0.6) was attained. A 1 ml pre-induction sample was collected for each protein and expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Post-induction samples (1 ml) were collected hourly at 1, 2, 3, 4, 5 and 16 hours post addition of IPTG for each protein and the cells harvested by centrifugation at 13 000 g for 2 minutes. The supernatant was discarded, and the pellet resuspended in 1x phosphate-buffered saline (PBS) buffer (16 mM sodium phosphate, 150 mM NaCl, 4 mM potassium phosphate, pH 7.5). The volume of 1x PBS used was calculated using the equation -

$$\text{Volume 1x PBS (µl)} = (\text{Absorbance } OD_{600} \div 0.5) \times 150 \times \text{dilution factor (df)}$$

Levels of protein production were assessed using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), and visualization carried out with Coomassie blue staining (Appendix 7.1.9). The confirmation of recombinant protein identities was performed by western blot analysis (Appendix 7.1.10). Specific primary antibodies, rabbit polyclonal anti- TbSti1 (1:2500), rabbit polyclonal anti-TbHsp83, and mouse polyclonal anti-hHsp90β (1:5000) together with their species-specific respective horseradish peroxidase conjugated (HRP) secondary antibodies (1:5000) (Abcam, Cambridge, United Kingdom) were used to confirm the presence of the proteins. Visualization of the protein bands was done using the Clarity™ ECL western blot kit (Bio-Rad, U.S.A) and the images for the western blot and SDS-PAGE were captured on the ChemiDoc imaging system (Bio-Rad, U.S.A).

3.4.2.3 Nickel affinity chromatography for the purification of the recombinant proteins expressed in *E. coli*

The number of hours for incubation after transformation to achieve maximum protein yield was determined from the induction study and expanded to 250 ml broth. Cells were harvested 5 hours post-IPTG induction by centrifugation at 10 000 g, 4 °C for 15 minutes and the resulting pellet resuspended in wash buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM PMSF, 1 mg/ml lysozyme). The suspension was left on ice for 30 minutes and then the lysate was frozen overnight at -80 °C. Frozen lysates were thawed and sonicated followed by centrifugation at 13000 g, 4 °C for 45 minutes, the supernatant containing the soluble proteins were incubated with the cOmplete His-tag purification resin (Roche, Germany) overnight at 4 °C with gentle agitation. Binding is known to occur at near neutral pH and buffers have been severally optimized (Louw et al., 2010),the resins were subsequently centrifuged at 4500 g, 4 °C for 4 minutes to remove unbound proteins and washed at least 4 times in wash buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM imidazole) containing a maximum imidazole concentration of 5 mM. Four elutions were carried out using elution buffer containing 125 mM imidazole (100 mM Tris-HCl, pH 7.5, 300 mM NaCl) with a final elution step after incubating the beads in the elution buffer overnight at 4 °C. The elutions were subjected to extensive dialysis in dialysis buffer (Appendix 7.1.11) and concentrated for 3-4 hours in polyethylene glycol (PEG) 20 000 (Merck, Germany). Protein yield purification was confirmed with SDS-PAGE and western blot as described in 3.4.2.2. Quantification of the protein yield was carried out using Bradford's assay (Appendix 7.1.12) and the proteins aliquoted and stored at -80 °C for future assays.

3.4.2.4 Far western analysis for protein-protein interactions

Protein-protein interactions were detected by far western analysis (Wu et al. 2007; Zininga et al. 2015) with slight modifications. Different concentrations of the prey protein (5 µg, 10 µg and 20 µg), 5 µg of BSA (negative control) and 5 µg of the bait/ligand protein (positive control) were resolved by 10% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Bio-Rad, U.S.A) as in a standard western blot technique. A control membrane was blocked and probed with primary antibody to the prey protein as in a standard western blot procedure. The proteins on the other membranes were denatured and renatured with buffer (100 mM NaCl, 20 mM Tris [pH 7.6], 0.5 mM EDTA, 10% [v/v] glycerol, 0.1% [v/v] Tween-20,

2% [w/v] skim milk powder and 1 mM DTT) containing different concentrations of urea (8 M – 0 M), with a final renaturation step carried out overnight at 4 °C. Membranes were blocked in 5% (w/v) skim milk powder in 1x Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 hour at room temperature. The membranes were incubated with excess of bait/ligand protein (500 µg/ml) in protein binding buffer (100 mM NaCl, 20 mM Tris [pH 7.6], 0.5 mM EDTA, 10% [v/v] glycerol, 0.1% [v/v] Tween-20, 2% [w/v] skim milk powder and 1 mM DTT) overnight at 4 °C with gentle agitation. Another control membrane was incubated in protein binding buffer lacking the bait/ligand protein. Washing off unbound bait/ligand proteins was done three times at 10 minutes each using 1x TBS-Tween buffer (0.1% Tween-20 in 1x TBS) and the membranes were incubated with primary antibody to the bait/ligand protein (1:2500) overnight at 4 °C with gentle agitation. The washing step was repeated three times at 10-minute intervals each using 1x TBS-Tween buffer before the addition of HRP-conjugated secondary antibody (1:5000) for 2 hours at room temperature. A final washing step to remove unbound secondary antibodies was performed before visualization as in standard western blot protocol.

3.4.2.5 Solid phase binding assay for protein-protein interactions

Direct protein-protein interaction was detected with a solid phase binding assay (Hunter et al., 2014) with slight modifications. A volume of 50 µl of 200 µg/ml of the prey protein in buffer A with and without ATP (20 mM Tris-HCl, 150 mM NaCl, pH 7.4, 1 mM ATP, 0.05% [v/v] Tween 20) was used to coat the wells of a high binding 96-well microplate (Greiner Bio-one, UK) for an hour at room temperature with shaking at 50 rpm and then overnight at 4 °C. Next, the wells were blocked with 300 µl 3% (w/v) BSA in buffer A for 1-2 hours at room temperature and serial dilutions of the bait protein (0 – 1000 nM) added overnight at 4 °C. Wells were washed with 200 µl 1% (w/v) BSA in buffer A three times to remove unbound proteins and incubated with specific primary antibodies (50 µl/well) in buffer A (1:5000) to the bait proteins. Primary antibody incubation was done overnight at 4 °C. Another washing step with 200 µl 1% (w/v) BSA in buffer A three times before the addition of appropriate HRP-conjugated specific secondary antibodies (50 µl/well) in buffer A (1:5000) for incubation at room temperature for 1 hour. Washing was carried out with 200 µl 1% (w/v) BSA in buffer A three times and then 100 µl/well of tetramethylbenzidine (TMB) substrate solution (0.1 mg/ml TMB, 0.1% [v/v] DMSO, 0.05 M phosphate citrate pH 5, supplemented with 0.04% [w/v] H_2O_2) added to each well. The reaction was stopped with 50 µl/well 2 M H_2SO_4 and absorbance readings taken at 450 nm.

3.4.2.6 Malate dehydrogenase (MDH) aggregation suppression assays

The ability of the chaperones TbHsp83 and hHsp90 to suppress aggregation of the thermally induced model substrate malate dehydrogenase (MDH) from porcine heart (Sigma-Aldrich, U.S.A.), alone and in the presence of the co-chaperone TbSti1 was investigated with slight modification to previous studies (Nyakundi et al., 2016; Bentley and Boshoff, 2019). The reaction consisted of MDH assay buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.4) which was used as a negative control, 0.72 µM MDH in buffer alone as positive control for aggregation and 0.72 µM MDH with equimolar and sub molar concentrations of the chaperone/co-chaperone proteins (0.72 µM, 0.18 µM and 0.045 µM) in assay buffer. The reaction was carried out in 96-well plates (Greiner Bio-one, Germany) in a total volume of 300 µl. The reaction was placed in an incubator pre-heated to 48 °C and end point absorbance readings taken at 30, 60 and 90 minutes at 360 nm. Calculations were done using the MDH readings to normalize as 100% aggregation.

3.4.2.7 Malachite green ATPase assay

The ATPase activity of the chaperones TbHsp83 and hHsp90 and the co-chaperone TbSti1 was measured by quantifying the amount of inorganic phosphate (Pi) released using a modified malachite green assay protocol (Hoenig et al. 1989). Phosphate standards were made with 100 µM Na_2HPO_4 and a standard curve generated of absorbance vs phosphate concentrations. The equation of the curve was subsequently used to calculate for Pi released in the reaction. To compare the ATP hydrolysis activity of all the proteins, a reaction mix containing the proteins in various concentrations (final working concentration of 0.0625 – 2 µM) was incubated with ATP (final working concentration of 250 µM) in ATPase buffer (20 mM Tris-HCl pH 7.5, 24 mM NaCl, 0.01% [v/v] Triton-X100, 0.5% [v/v] glycerol, 6 mM $MgCl_2$) to a total volume 200 µl. The reaction mixture and phosphate standards were put in a pre-heated water bath at 37 °C for 60 minutes. The reaction was stopped on ice for 5 minutes and equilibrated at room temperature for 10 minutes. A volume of 50 µl of each reaction mix or phosphate standard was dispensed into 96 well plates (Greiner Bio-one, Germany) in triplicates, and 100 µl of freshly made malachite green buffer (which contained a 1: 18:18 ratio of 0. 126% malachite green: 2.6% sodium molybdate: 2.5 N HCl) was added and the reaction stopped with 50 µl 0.1 M sodium citrate buffer (pH 6.6). The green colour developed is representative of inorganic phosphate released. The endpoint absorbance readings were taken at 620 nm, and calculations done using the equation from the standard curve to quantify inorganic phosphate released.

To examine the effect of the co-chaperone TbSti1 on the chaperones, TbHsp83 and hHsp90, the highest concentration of the chaperone proteins from the hydrolysis reaction (2 µM) was incubated with increasing concentrations of TbSti1 (final working concentration of 0.0625 – 2 µM) before the addition of ATP (final working concentration of 250 µM) in ATPase buffer (20 mM Tris-HCl pH 7.5, 24 mM NaCl, 0.01% [v/v] Triton-X100, 0.5% [v/v] glycerol, 6 mM $MgCl_2$) and then incubated in the pre-heated water bath at 37 °C for 60 minutes before proceeding to the malachite green step. The chaperones alone with ATP served as a positive control for ATPase activity.

An ATP titration assay was done by incubating the highest concentration of the proteins from the hydrolysis reaction (2 µM) with different concentrations of ATP (final working concentrations of 7.8 – 1000 µM) and then incubated in the pre-heated water bath at 37 °C for 60 minutes before proceeding to the malachite green step. A Michaelis Menten plot in GraphPad prism 9.0 was used to determine the *V*max and *K*m for each protein.

In all the reaction conditions, calculations were corrected and normalized for spontaneous breakdown of ATP by using the ATPase buffer as blank lacking the proteins.

3.4.2.8 Statistical analyses and Reproducibility

All assays were performed with at least 3 technical replicates from 3 independent purified batches of proteins. Statistical analyses were performed by one/two-way ANOVA or Nonlinear regression (curve fit) in GraphPad prism 9.0 and values below 0.05 were taken as significant.

3.5 Results and discussion

3.5.1 Heterologous production and purification of Sti1, TbHsp83 and human Hsp90

3.5.1.1 TbSti1

The integrity of the bacterial expression plasmid pQE60-TbSti1 and the mammalian expression plasmid pcDNA3.1(+)_HA-TbSti1 were verified using restriction enzymes *Eco*RI/*Hin*dIII and *Nhe*1/*Eco*R1 respectively. Digestion with *Eco*RI or *Hin*dIII linearized the plasmid pQE60-TbSti1 (Figure 3.1 B, lanes 2 and 3) while digestion with *Nhe*I or *Eco*RI linearized the plasmid pcDNA3.1(+)_HA-TbSti1 (Figure 3.1 D, lanes 2 and 3). Digestion of pQE60-TbSti1 with both *Eco*RI and *Hin*dIII produced DNA fragments of ~3367 bp and ~1732 bp corresponding to the bacteria expression plasmid pQE60 and the *TbSti1* coding sequence (Figure 3.1 B, lane 4), while digestion of pcDNA3.1(+)_HA-TbSti1 with both *Nhe*I and *Eco*RI produced DNA fragments of ~5374 bp and ~1715 bp corresponding to the mammalian expression plasmid pCDNA3.1(+) and the *TbSti1*coding sequence (Figure 3.1 D, lane 4)

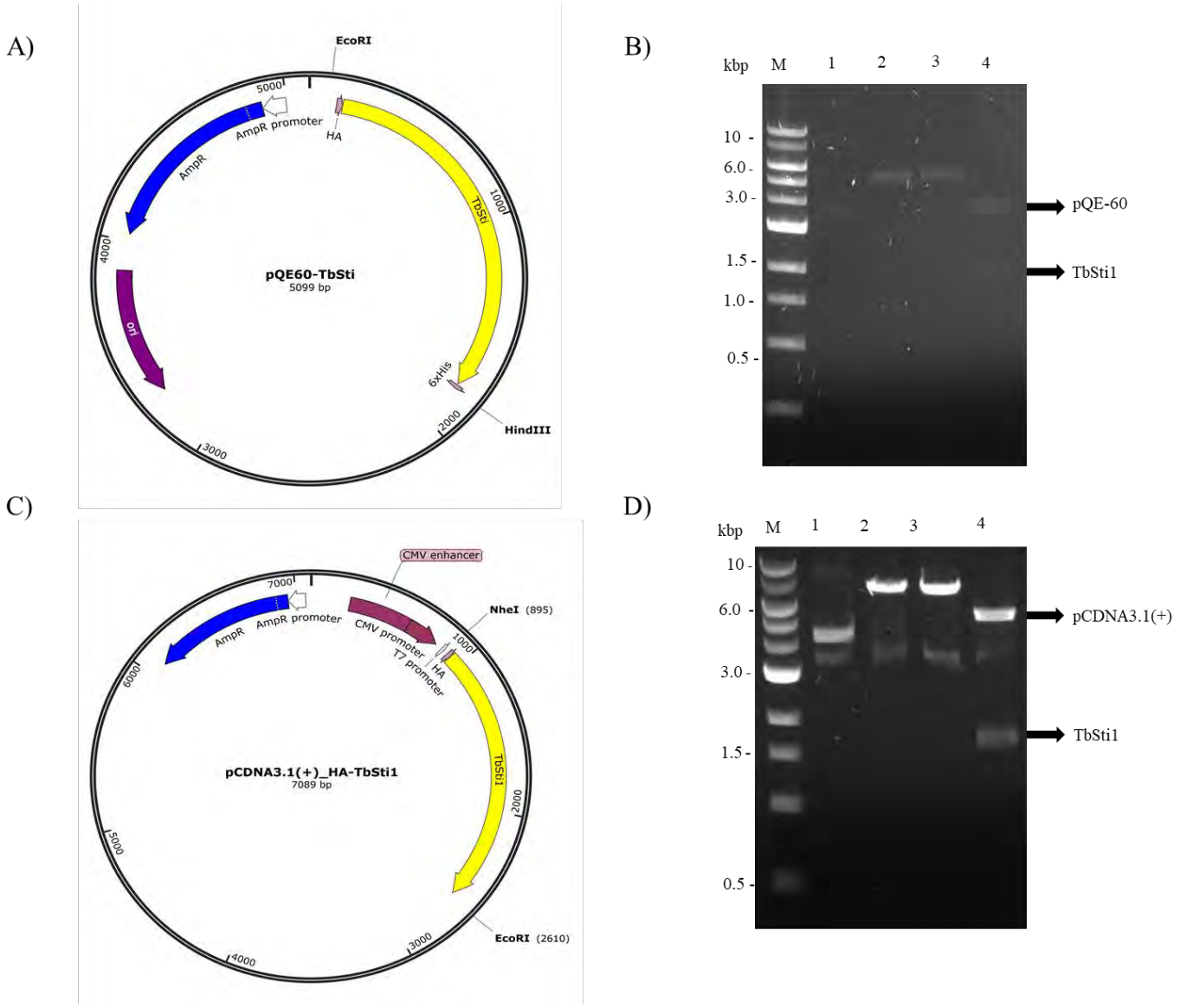


Figure 3.1: Verification of the pQE60-TbSti1 bacteria expression plasmid and the pCDNA3.1(+)_HA-TbSti1 mammalian expression plasmid.
A) Plasmid map of pQE60-TbSti1 indicating the *Eco*RI and *Hin*dIII restriction sites, the *TbSti1* insert in yellow, the HA and His-tag segment upstream and downstream of the *TbSti1* coding sequence respectively. B) 0.8% agarose gel electrophoresis of the diagnostic restriction digest of the bacteria expression plasmid pQE60-TbSti1. Lanes: M – 1kb DNA ladder, lane 1 – unrestricted pQE60-TbSti1, lane 2 - pQE60-TbSti1 restricted with *Eco*RI (~5099 bp), 3 - pQE60-TbSti1 restricted with *Hin*dIII (~5099 bp), lane 4 - pQE60-TbSti1 restricted with both *Eco*RI and *Hin*dIII (~3367 bp and ~1732 bp). C) Plasmid map of pCDNA3.1(+)_HA-TbSti1 indicating the *Nhe*I and *Eco*RI restriction sites, the *TbSti1* insert in yellow and the HA tag segment upstream of the *TbSti1* coding sequence. D) 0.8% agarose gel electrophoresis of the diagnostic restriction digest of the mammalian expression plasmid pCDNA3.1(+)_HA-TbSti1. Lanes: M – 1kb DNA ladder, lane 1 – unrestricted pCDNA3.1(+)_HA-TbSti1, lane 2 - pQE60-TbSti1 restricted with *Nhe*I (~7089 bp), lane 3 - pCDNA3.1(+)_HA-TbSti1 restricted with *Eco*RI (~ 7089 bp), lane 4 - pCDNA3.1(+)_HA-TbSti1 restricted with both *Nhe*I *and Eco*RI (~5374 bp and 1715 bp).

E. coli M15(pREP4) cells were successfully transformed by the bacterial expression plasmid pQE60-TbSti1 with a maximum HA-TbSti1-His protein production 5 hours post-IPTG induction as shown on the 10% SDS-PAGE and western blot analysis (Figure 3.2 A-B). The soluble protein was successfully purified using nickel affinity chromatography and confirmed to be TbSti1 by western blot analysis using a specific anti-TbSti1 antibody (Figure 3.2 C-D). The size of the protein expression bands as well as the eluents from the protein purification correspond to the expected molecular weight of ~63 kDa (Figure 3.2 A-D). Purification yields of ~5 g/L were typically obtained.

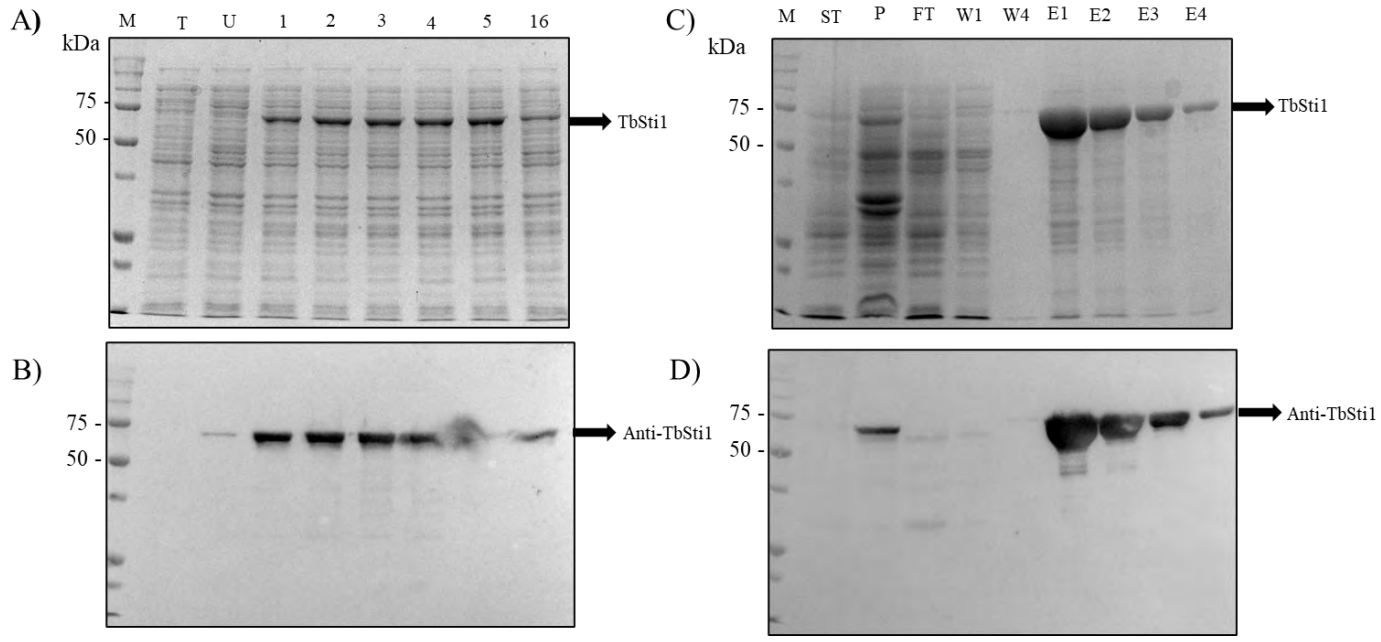


Figure 3.2: Heterologous expression and purification of recombinant HA-TbSti1-His.
A) 10% SDS-PAGE analysis of the expression profile of recombinant TbSti1 in *E. coli* M15 (pREP4) cells visualized using Coomassie blue staining. Lanes: M- Precision Plus Protein™ All Blue Prestained Protein Standard, T- The total cell extract of untransformed *E. coli* M15 (pREP4) cells, U: The total cell extract of *E. coli* M15(pREP4)[pQE60-TbSti1] prior to IPTG induction (uninduced), lanes 1-5- hourly samples one to five hours post 1 mM IPTG induction (total cell extract), lane 16: Overnight induction sample (total cell extract). B) Western analysis of TbSti1 protein expression using anti-TbSti1 antibody C) 10% SDS-PAGE analysis of the purification of TbSti1 using nickel affinity chromatography visualized using Coomassie blue staining. Lanes: M- Precision Plus Protein™ All Blue Prestained Protein Standard, ST- supernatant, P- pellet, FT- Flow-through, W1-W4- Fractions of wash 1 and 4 using native wash buffer (5 mM imidazole), E1-E4- Elutions 1-4 using native elution buffer (125 mM imidazole), D) Western blot analysis of the purification of TbSti1with anti-TbSti1 antibodies.

3.5.1.2 TbHsp83

Verification of the bacteria expression plasmid pET-30a-TbHsp83 was done using restriction enzymes *Nde*I *and Hin*dIII Digestion with *Nde*I or *Hin*dIII linearized the plasmid pET-30a-TbHsp83 (Figure 3.3 B, lanes 1 and 2) Digestion of pET-30a-TbHsp83 with both *Nde*I and *Hin*dIII produced DNA fragments of ~5306 bp and ~2207 bp corresponding to the bacteria expression plasmid pET-30a and the *TbHsp83* coding sequence (Figure 3.3 B, lane 3).

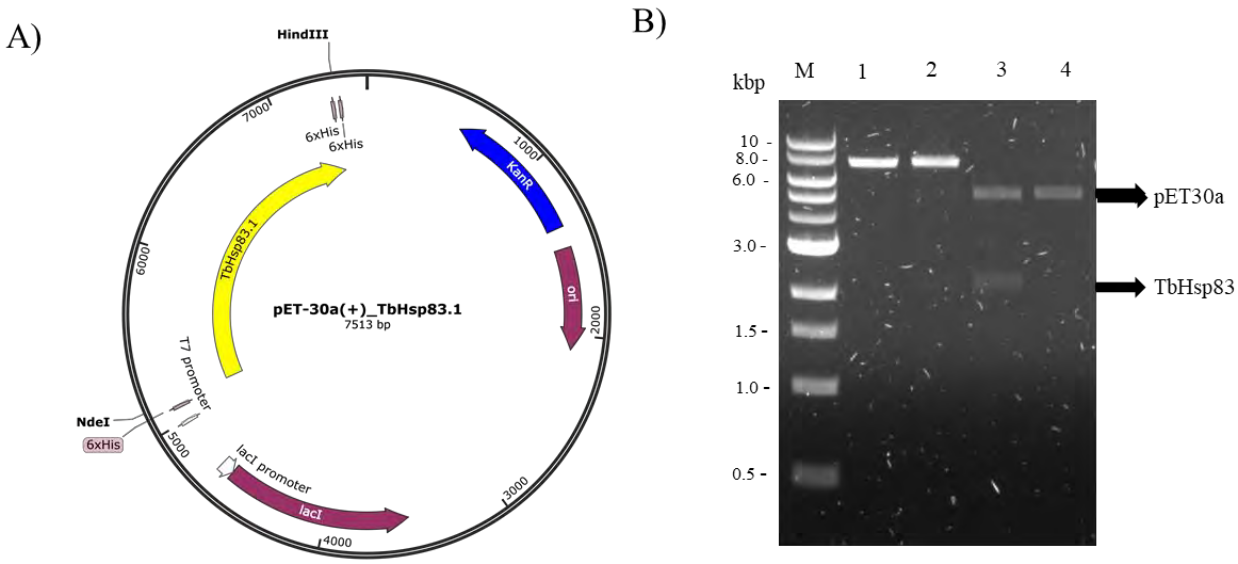


Figure 3.3: Verification of the pET-30a-TbHsp83 bacteria expression plasmid using restriction enzymes digest. A) Plasmid map of pET-30a-TbHsp83 indicating the *Nde*I and *Hin*dIII restriction sites and the *TbHsp83* insert in yellow. B) 0.8% agarose gel electrophoresis of the diagnostic restriction digest of the bacteria expression plasmid pET-30a-TbHsp83. Lanes: M – 1kb DNA ladder, lane 1 – pET-30a-TbHsp83 restricted with *Nde*I (~7513 bp), lane 2 - pET-30a-TbHsp83 restricted with *Hin*dIII (~7513 bp), lane 3 - pET-30a-TbHsp83 restricted with both *Nde*I and *Hin*dIII (~5306 bp and ~2207 bp), lane 4 - unrestricted pET-30a-TbHsp83.

BL21(DE3) cells were successfully transformed by the bacterial expression plasmid pET-30a-TbHsp83 with a maximum TbHsp83-His protein production at 5 hours post-IPTG induction as shown on the 10% SDS PAGE and Western analysis (Figure 3.4 A-B). TbHsp83 was a soluble protein and was successfully purified using nickel affinity chromatography (Figure 3.4 C-D). The specific anti-TbHsp83 antibody produced a band that corresponded to the expected molecular weight of TbHsp83 (~84kDa) (Figure 3.4 B and D). High protein purification yields of about ~5 g/L were typically obtained.

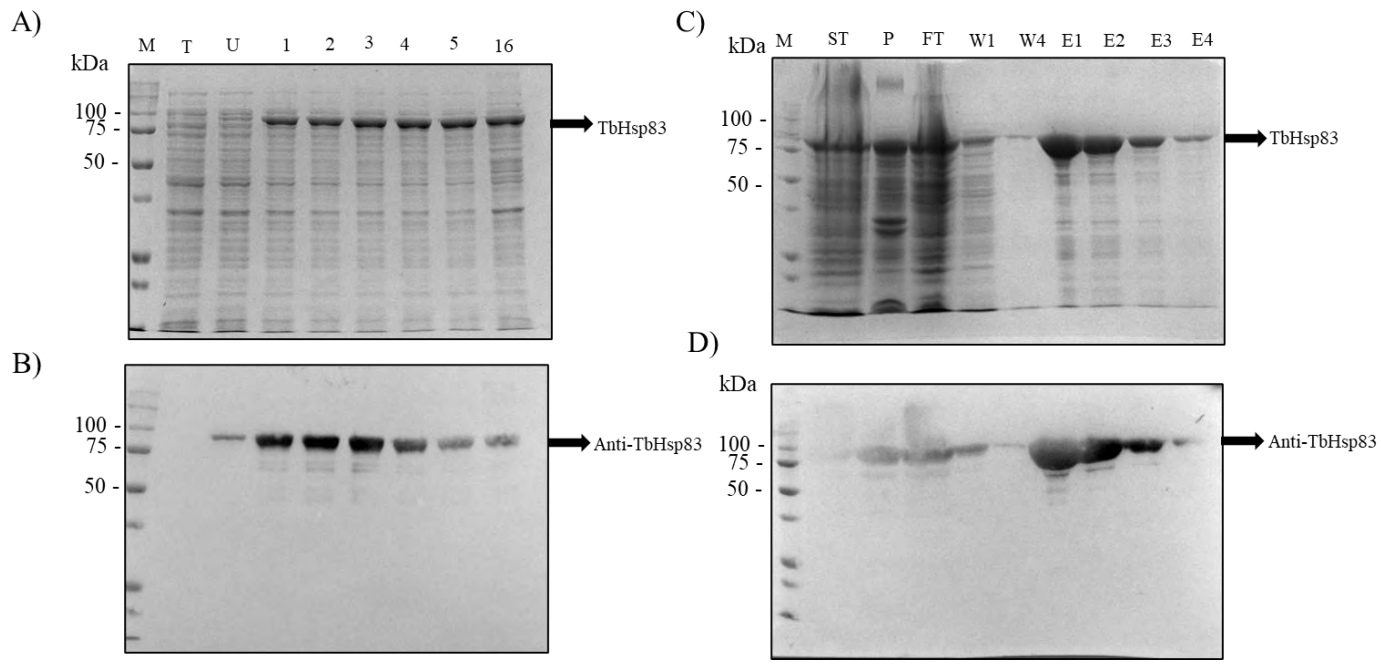


Figure 3.4: Heterologous expression and purification of recombinant TbHsp83-His.
A) 10% SDS-PAGE analysis of the expression profile of recombinant TbHsp83 in *E. coli* BL21(DE3) cells visualized using Coomassie blue staining. Lanes: M- Precision Plus Protein™ All Blue Prestained Protein Standard, T- The total cell extract of untransformed *E. coli* BL21(DE3) cells, U: The total cell extract of *E. coli* BL21(DE3) cells transformed with pET-30a-TbHsp83 prior to IPTG induction (uninduced), lanes 1-5- hourly samples one to five hours post 1 mM IPTG induction (total cell extract), lane 16: Overnight induction sample (total cell extract). B) Western blot analysis of TbHsp83 protein expression using anti-TbHsp83 antibody C) 10% SDS-PAGE analysis of the purification of TbHsp83 using nickel affinity chromatography visualized using Coomassie blue staining. Lanes: M- Precision Plus Protein™ All Blue Prestained Protein Standard, ST- supernatant, P- pellet, FT- Flow-through, W1-W4- Fractions of wash 1 and 4 using native wash buffer (5 mM imidazole), E1-E4- Elutions 1-4 using native elution buffer (125 mM imidazole), D) Western blot analysis of the purification of TbHsp83 using anti-TbHsp83 antibodies.

3.5.1.3 Human Hsp90

Confirmation of the integrity of the bacteria expression plasmid pET-16b-Hsp90α was achieved using restriction enzymes *Bam*HI *and Xho*I. Digestion with *BamH*I or *Xho*I linearized the plasmid pET-16b-Hsp90α (Figure 3.5 B, lanes 2 and 3) Digestion of pET-16b-Hsp90α with both *Bam*HI *and Xho*I produced DNA fragments of ~5706 bp and ~2240 bp corresponding to the bacteria expression plasmid pET-16b and the *hHsp90* coding sequence (Figure 3.5B, lane 4).

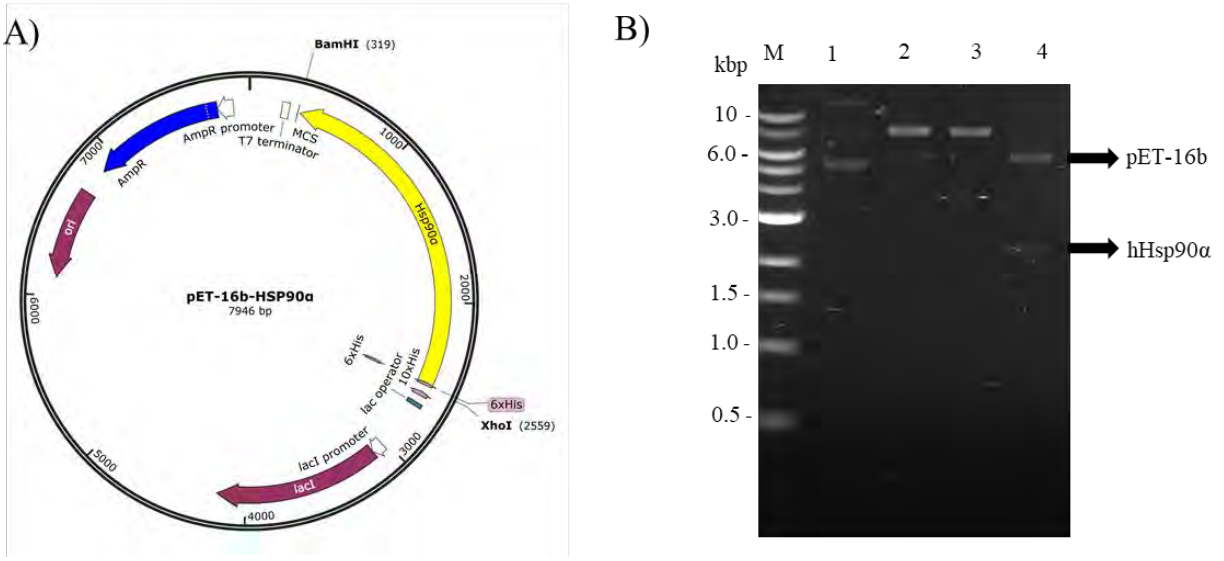


Figure 3.5: Verification of the pET-16b-Hsp90α bacteria expression plasmid using restriction enzymes digest
A) Plasmid map of pET-16b-Hsp90α indicating the *Bam*HI *and Xho*I restriction sites, a His-tag segment downstream of the *hHsp90* coding sequence and the *hHsp90* insert in yellow. B) 0.8% agarose gel electrophoresis of the diagnostic restriction digest of the bacteria expression plasmid pET-16b-Hsp90α. Lanes: M – 1kb DNA ladder, lane 1 – unrestricted pET-16b-Hsp90α, lane 2 - pET-16b-Hsp90α restricted with *Bam*HI (~7946 bp), 3 - pET-16b-Hsp90α restricted with *Xho*I (~7946bp), lane 4 - pET-16b-Hsp90α restricted with both *Bam*HI *and Xho*I (~5706 bp and ~2240 bp).

Production levels of Hsp90α were lower compared to other purified proteins in this study, however a maximum yield was also achieved at 5 hours post-IPTG induction as shown on the 10% SDS-PAGE and western blot analysis (Figure 3.6 A-B). Human His-Hsp90 was a soluble protein and was successfully purified using nickel affinity chromatography (Figure 3.6 C-D). The specific anti-hHsp90α antibody produced bands with size of ~85kDa that corresponds to the expected molecular weight of hHsp90 (Figure 3.6 B and D). Protein yields were typically lower than the yields from TbHsp83-His and HA-TbSti1-His with an amount of less than 2g/L typically obtained.

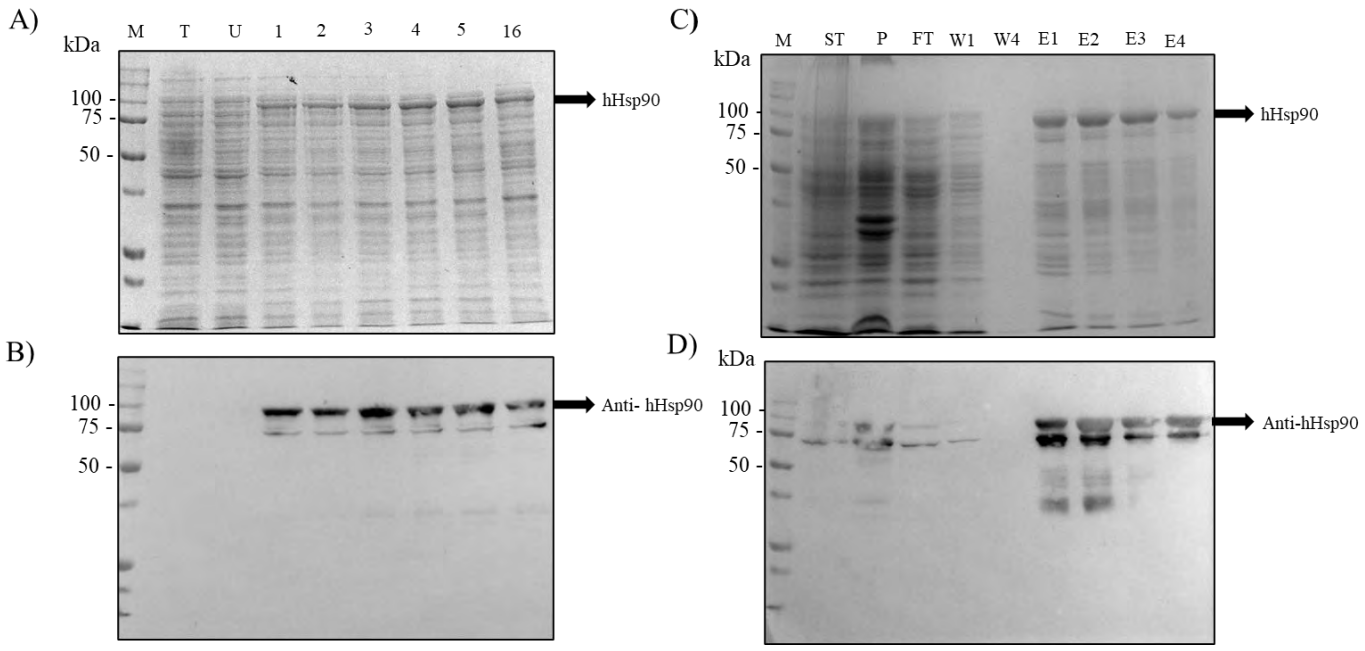


Figure 3.6: Heterologous expression and purification of recombinant His-hHsp90.
10% SDS-PAGE analysis of the expression profile of recombinant hHsp90 in *E. coli* BL21(DE3) cells visualized using Coomassie blue staining. Lanes: M- Precision Plus Protein™ All Blue Prestained Protein Standard, T- The total cell extract of untransformed *E. coli* BL21(DE3) cells, U: The total cell extract of *E. coli* BL21(DE3) cells transformed with pET-16b-Hsp90α prior to IPTG induction (uninduced), lanes 1-5- hourly samples one to five hours post 1 mM IPTG induction (total cell extract), lane 16: Overnight induction sample (total cell extract). B) Western blot analysis of hHsp90 protein expression using anti- hHsp90α antibody C) 10% SDS-PAGE analysis of the purification of hHsp90 using nickel affinity chromatography visualized using Coomassie blue staining. Lanes: M- Precision Plus Protein™ All Blue Prestained Protein Standard, ST- supernatant, P- pellet, FT- Flow-through, W1-W4- Fractions of wash 1 and 4 using native wash buffer (5 mM imidazole), E1-E4- Elutions 1-4 using native elution buffer (125 mM imidazole), D) Western blot analysis of the purification of hHsp90 using anti- hHsp90α antibodies.

3.5.2 Qualitative analysis using far western analysis showed direct binding between TbSti1 and the cytosolic chaperones, TbHsp83 and hHsp90

Far western blotting technique is an *in vitro* technique for studying protein-protein interactions with an advantage for showing direct binding between proteins with information on the size of the binding partners. (Wu et al. 2007). As protein-protein interactions generally depend on the secondary and tertiary structures of the protein which may have been disrupted during the SDS-PAGE preparatory processes. For the far western blotting process, the proteins on the membrane are first denatured with urea or guanidine and then renatured with gradient-reducing urea or guanidine respectively. This helps to recover the secondary and tertiary structures of the protein for effective binding (Wu et al., 2007). Direct binding between recombinant proteins TbHsp83 and hHsp90 with TbSti1 was investigated using far western analysis. In the first

approach, TbSti1 was used as the prey protein (protein immobilized to the membrane) while TbHsp83 and hHsp90 were the bait proteins (proteins in solution) respectively (Figure 3.7 A and B). A second approach involved the reciprocal set up with TbHsp83 and hHsp90 as prey proteins and TbSti1 as the bait protein (Figure 3.7 C and D).

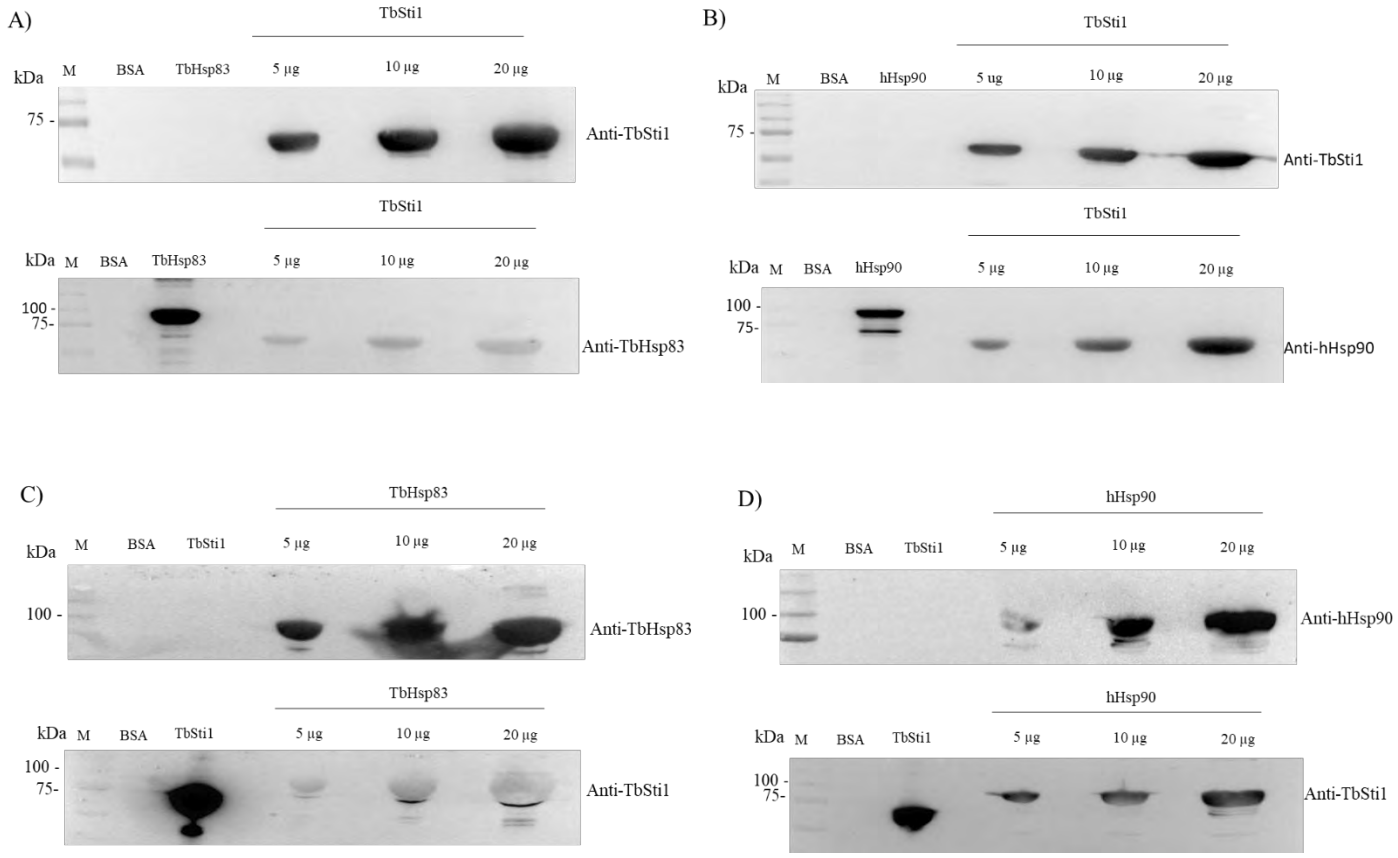


Figure 3.7:TbHsp83 and hHsp90 interact directly with TbSti1.
The direct interaction between purified recombinant proteins TbHsp83/hHsp90 and TbSti1 was explored using far western analysis**.** A) BSA (5µg) as negative control, TbHsp83 (5µg) as positive control and different concentrations of TbSti1 (5µg, 10µg, 20µg) were resolved on a 10% SDS PAGE, transferred to a blot which was probed using anti-TbSti1 (A; top panel). A similar blot was overlayed with TbHsp83 and probed using anti-TbHsp83 (A; bottom panel). B) BSA (5µg) as negative control, hHsp90 (5µg) as positive control and different concentrations of TbSti1 (5µg, 10µg, 20µg) were resolved on a 10% SDS PAGE, transferred to a blot which was probed using anti-TbSti1 (B; top panel). A similar blot was overlayed with hHsp90 and probed using anti-hHsp90α (B; bottom panel). C) BSA (5µg) as negative control, TbSti1 (5µg) as positive control and different concentrations of TbHsp83 (5µg, 10µg, 20µg) were resolved on a 10% SDS PAGE, transferred to a blot which was probed using anti-TbHsp83 (C; top panel). A similar blot was overlayed with TbSti1 and probed using anti-TbSti1 (C; bottom panel). D) BSA (5µg) as negative control, TbSti1 (5µg) as positive control and different concentrations of hHsp90 (5µg, 10µg, 20µg) were resolved on a 10% SDS PAGE, transferred to a blot which was probed using anti-hHsp90α (A; top panel). A similar blot was overlayed with hHsp90 and probed using anti-hHsp90α (D; bottom panel).

A blot containing recombinant TbSti1 (5 µg, 10 µg, 20 µg) as prey protein, negative control protein BSA (5µg) and TbHsp83 or hHsp90 as a positive prey control were probed using anti-TbSti1 which recognized only the TbSti1 proteins (Figure 3.7 A and B; top panels). This confirmed that the anti-TbSti1 antiserum was able to distinguish between TbSti1 and TbHsp83/hHsp90 and that TbSti1 was loaded on to the gel. A similar blot was then incubated with excess of either TbHsp83 or hHsp90 (500 µg/ml) as bait proteins and the binding of the bait to the TbSti1 in the gel detected using specific antibodies to TbHsp83 or hHsp90. Protein bands were observed in the lanes representing the positive controls (TbHsp83/hHsp90) and in increasing concentrations at the positions corresponding to the size of TbSti1 protein (~63kDa) indicating that direct binding had taken place between the chaperones TbHsp83/hHsp90 and the co-chaperone TbSti1 (Figure 3.7 A and B: bottom panels). As expected, no band was observed in the lane for BSA which served as negative control.

The assay was repeated in a reverse order to confirm that the results seen were not due to antibody cross-reactivity but from direct binding of the proteins. The chaperone proteins TbHsp83/hHsp90 (5 µg, 10 µg, 20 µg) were used as prey proteins immobilized to the membrane, negative control protein BSA (5µg) and TbSti1 as positive control. The membranes were probed using anti-TbHsp83 and anti-hHsp90α antibodies which recognized only TbHsp83 and hHsp90 proteins respectively but not TbSti1 (Figure 3.7 C and D; top panels). This also confirms that TbHsp83/hHsp90 were successfully immobilized to the membrane. Similar blots were allowed to interact with excess TbSti1 as bait protein in solution and binding of TbSti1 to the TbHsp83/hHsp90 detected using anti-TbSti1 antibody. Protein bands were observed in the lanes representing the positive controls (TbSti1) and in increasing concentrations at the positions corresponding to the size of TbHsp83/hHsp90 proteins (~84-85 kDa) indicating that direct binding had taken place between the chaperones TbHsp83/hHsp90 and the co-chaperone TbSti1 (Figure 3.7 C and D; bottom panels). As seen in the previous case, no band was observed in the lane for BSA which served as negative control. Taken together, these data suggested a direct interaction between TbSti1 with both the trypanosomal and human Hsp90 isoforms, consistent with other Sti1 isoforms.

3.5.3 Quantitative analysis showed direct binding between the co-chaperone TbSti1 and Hsp90 chaperones, in the presence and absence of ATP

In addition to far western analysis, direct binding was also investigated using a solid phase binding assay. In the first approach, purified TbSti1 in excess (200 µg/ml/~3170 nM) served as the prey protein which was immobilized to the high binding microtiter plate and allowed to interact with the bait proteins,

TbHsp83/hHsp90 in solution at increasing concentrations (0 – 300 nM) in the presence or absence of ATP. Binding was detected using specific primary antibodies to TbHsp83 and hHsp90 and quantifying the increasing absorbance values that corresponded to increasing concentration of the bait proteins. (Figure 3.8 A and B). A non-linear regression analysis (one site-specific binding) was used to determine the K_d (ligand concentration that binds to half the concentration sites at equilibrium) and B_{max} (maximum number of binding sites). In the absence of ATP, the K_d for the binding of TbSti1 and TbHsp83 is 27.17 µM and 38.34 µM in the presence of ATP. An F-test conducted comparing both K_ds gave a P value of 0.62, this suggests that there was no significant difference in binding in the presence or absence of ATP between TbHsp83 and TbSti1 (Figure 3.8 A). The K_d for the binding of TbSti1 and hHsp90 is 3.82 µM in the absence of ATP and 9.61 µM in the presence of ATP. The P value deduced from the F-test in this case was 0.0002, this indicates a significant difference in binding with a higher binding affinity of hhsp90 Tbsti1 is higher in the absence of ATP (Figure 3.8 B). The B_{max} for the TbSti1-hHsp90 and TbSti1-TbHsp83 interaction is approximately 1 in both cases.

The assay was carried out again to compare the binding affinity of both chaperones to TbSti1 and this was done in the absence of ATP. In this case the chaperones served as prey proteins immobilized to the high binding microtiter wells (200 µg/ml/~2380 nM for TbHsp83 and 200 µg/ml/~2350 nM for hHsp90) and allowed to interact with increasing concentrations of Tbsti1 as the bait protein (0 – 300 nM). Binding was detected by probing with anti-TbSti1, and absorbance readings measured and quantified (Figure 3.8 C). Human Hsp90 displayed stronger affinity for TbSti1 with a K_d of 0.68 nM compared to the K_d of 30.58 nM for the TbHsp83-TbSti1 interaction. The P value deduced from the F-test comparing the K_d of both was <0.0001 indicating a significant difference and confirming the stronger binding affinity of the TbSti1-hHsp90 interaction. The B_{max} for both cases were approximately 1 as well. These data show a binding interaction between the chaperones TbHsp83 and hHsp90 with TbSti1.

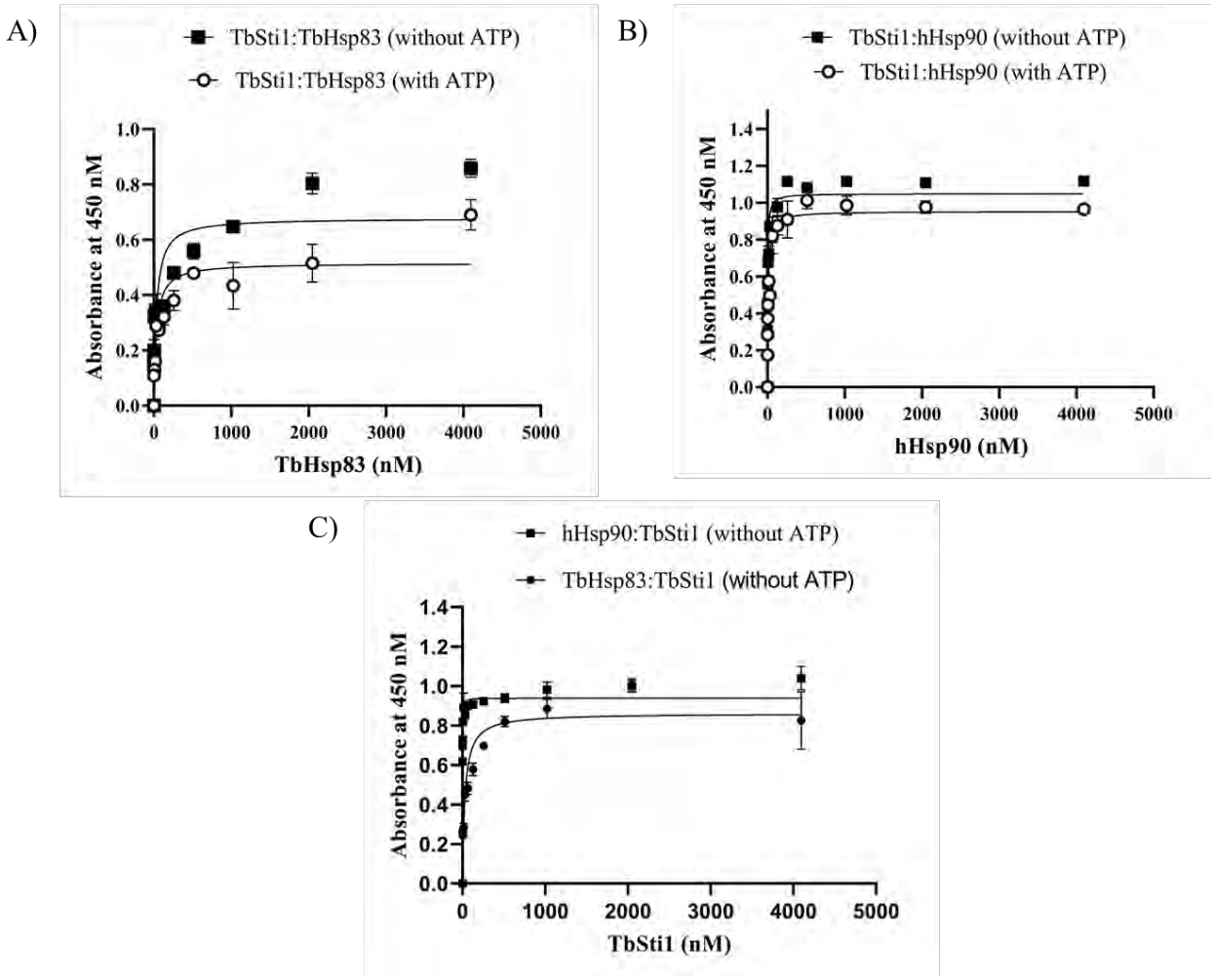
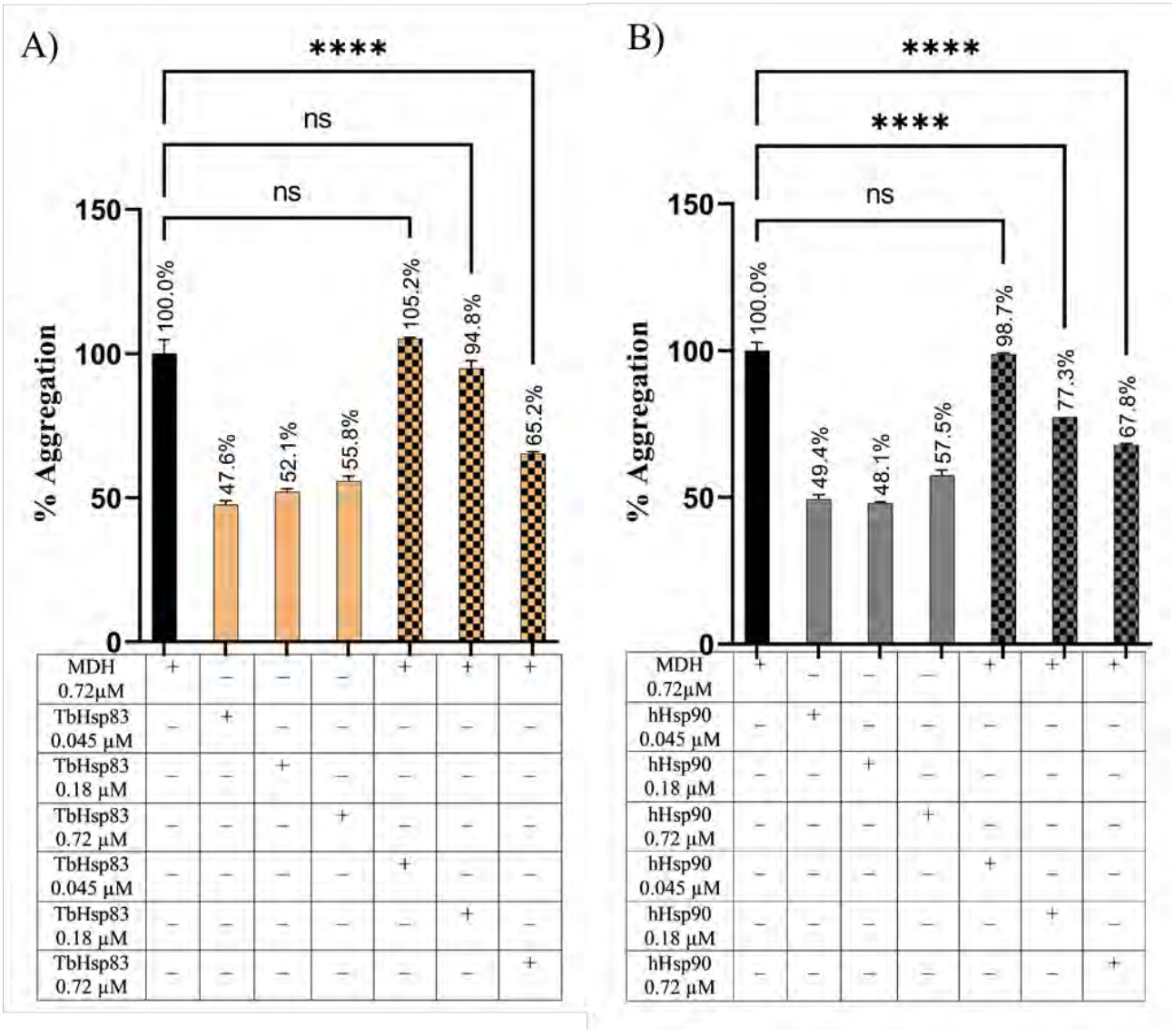


Figure 3.8: TbSti1 binding to TbHsp83 and hHsp90 in the presence and absence of ATP.
A) 200 µg/ml of TbSti1 immobilized to the well interacts with TbHsp83 (0-300 nM) in the presence and absence of ATP. B) 200 µg/ml of TbSti1 immobilized to the well interacts with hHsp90 (0-300 nM) in the presence and absence of ATP. C) 200 µg/ml of TbHsp83/hHsp90 immobilized to the well interacts with TbSti1 (0-300 nM) in the absence of ATP. Detection was done using the respective antibodies and absorbance taken at 450nm. K_d (ligand concentration that binds to half the concentration sites at equilibrium) and B_{max} (maximum number of binding sites) were determined using a non-linear regression analysis (one site-specific binding) in GraphPad prism 9.2.0. Data shown represent averages ±SEM (n=3) from two independent biological replicates.

3.5.4 MDH suppression activity assays

Malate dehydrogenase (MDH) is a substrate prone to aggregation when exposed to high temperatures and therefore a model typically used to assess the holdase activity of molecular chaperones (Shonhai et al., 2008).Analyses were done by comparing the values of the proteins to MDH alone which was taken as 100% aggregation. All proteins alone in the absence of MDH showed significant aggregation. The lower

concentrations of TbHsp83 show no significant MDH aggregation suppression activity but equimolar concentration of TbHsp83 with MDH (0.72µM) led to about 40% suppression of MDH aggregation (Figure 3.9 A). For hHsp90, 0.18 µM and 0.72µM showed significant MDH aggregation suppression activity (Figure 3.9 B) while TbSti1 at all concentrations had no significant suppression effect on the aggregation of MDH as increasing concentration of the protein showed increasing aggregation (Figure 3.9 C). This is consistent with previous findings that Sti1 lacks chaperone activities (Bose et al., 1996). The data shows in summary that the chaperones TbHsp83 and hHsp90 at high concentrations suppress MDH aggregation but TbSti1 has no aggregation suppression activity.



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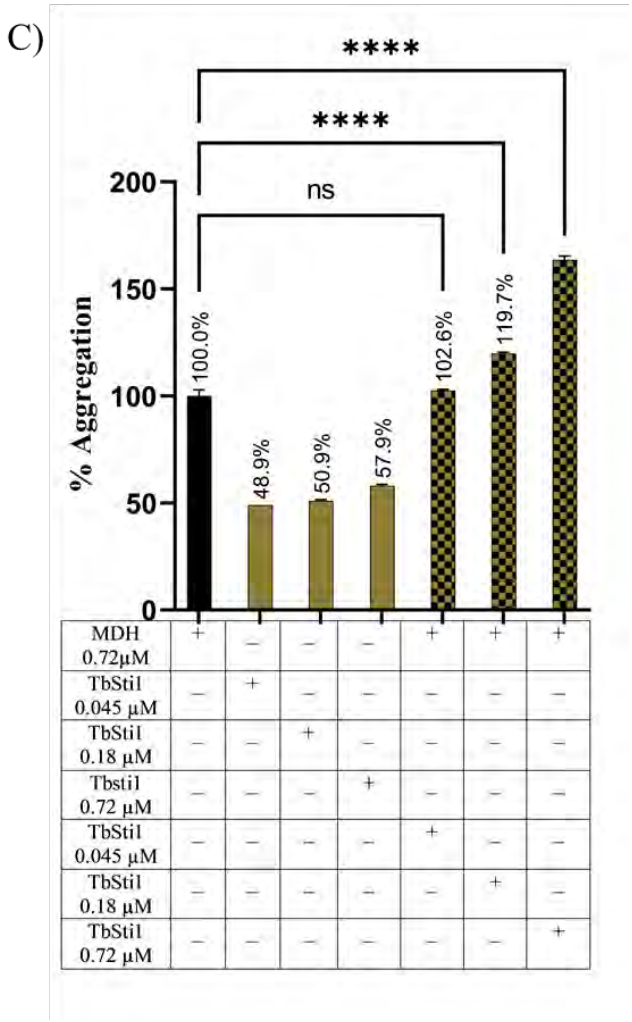

Figure 3.9: MDH suppression activities of TbHsp83, hHsp90 and TbSti1.
The '+' indicates components present in the reaction mixture while '-' indicates components absent, MDH alone is taken as 100% aggregation A) The MDH suppression activities of increasing concentrations of TbHsp83. B) The MDH suppression activities of increasing concentrations of hHsp90. C) The MDH suppression activities of increasing concentrations of TbSti1. Statistics was done using one-way ANOVA, statistical difference is indicated by **** ($P < 0.0001$) and no significance by 'ns'. Data shown represent averages ±SEM (n=3) from three independent biological replicates.

3.5.5 Comparison of ATPase hydrolysis activity of the chaperones TbHsp83 and human Hsp90 and the co-chaperone TbSti1

The basal ATPase activities for the chaperones TbHsp83 and hHsp90 and the co-chaperone TbSti1 was investigated with an optimized malachite green protocol. A total of 250 µM ATP was added to increasing concentrations of the proteins with the highest concentration of 2 µM. All proteins showed varying degrees

of ATP hydrolysis at different concentrations with hHsp90 showing the highest hydrolysis activity across all concentrations (Figure 3.10 A).. The most obvious difference was seen at 2 µM concentration with Hsp83 and hHsp90 showing similar levels of ATPase activity while TbSti1 showed activity comparable to half the activity of Hsp83 and hHsp90.

Michaelis-Menten parameters derived from an ATPase titration assay (Figure 3.10 B) show the following: TbHsp83 (K_m – 403.5 µM, V_{max} - 0.86 µM ATP/min), hHsp90 (K_m – 144.2 µM, v – 0.77 µM ATP/min) and TbSti1 (K_m – 285.4 µM, V_{max} - 0.55 µM ATP/min).

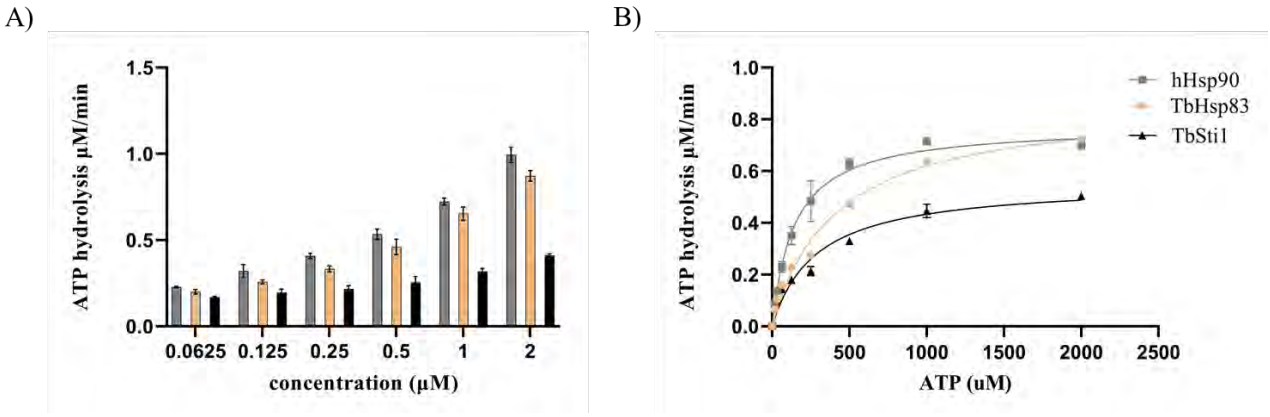

Figure 3.10: Comparison of ATP hydrolysis activity of TbHsp83, hHsp90 and TbSti1.
A) ATP hydrolysis activity comparing increasing concentrations of hHsp90, TbHsp83 and TbSti1 respectively. Multiple comparison statistics was done using two-way ANOVA B) Michaelis-Menten plot of ATP titration activity for hHsp90, TbHsp83 and TbSti1 with increasing ATP concentrations. Michaelis-Menten constants Km and Vmax were determined from a nonlinear regression analysis in GraphPad prism 9.2.0. Data shown represent averages ±SEM (n=3) from three independent biological replicates.

3.5.6 Effect of TbSti1 on the ATPase activity of TbHsp83 and human Hsp90

Previous studies have shown Sti1 to act as an inhibitor to the ATPase activity of Hsp90. Sti1 inhibited the Hsp90 ATPase activity in yeast (Prodromou et al. 1999), PfHop inhibited the ATPase activity of PfHsp90 (Silva et al. 2020) and LbHop inhibited the ATPase activity of LbHsp90 (Batista et al., 2016). However, human Hop had little or no effect on the basal activity of hHsp90 (McLaughlin et al. 2002). Human Hop was shown to undergo conformational changes after binding ATP and displayed ATPase activity comparable to Hsp70 and Hsp90 (Yamamoto et al., 2014). However, there is a possibility this Hop ATPase activity could be from contamination due to the presence of *E. coli* DnaK (Rial and Ceccarelli, 2002). Inhibition of the ATPase activity of Hsp90 by Sti1 is premised on the theory that the binding of Sti1 to Hsp90 prevents the transient dimerization of the Hsp90 N-terminal domain and thereby inhibiting its

ATPase activity (Schmid et al., 2012). To test, the ability of TbSti1 to modulate the ATPase activities of TbHsp83 and hHsp90 was measured by adding equimolar and sub molar concentrations of TbSti1 to 2 µM of TbHsp83 or hHsp90 respectively before the addition of ATP. Excess TbSti1 up to 8 µM was added (data not shown) to compare the findings but increasing concentration of TbSti1 did not show any significant effect on the ATPase activity of either chaperone (Figure 3.11 A and B).

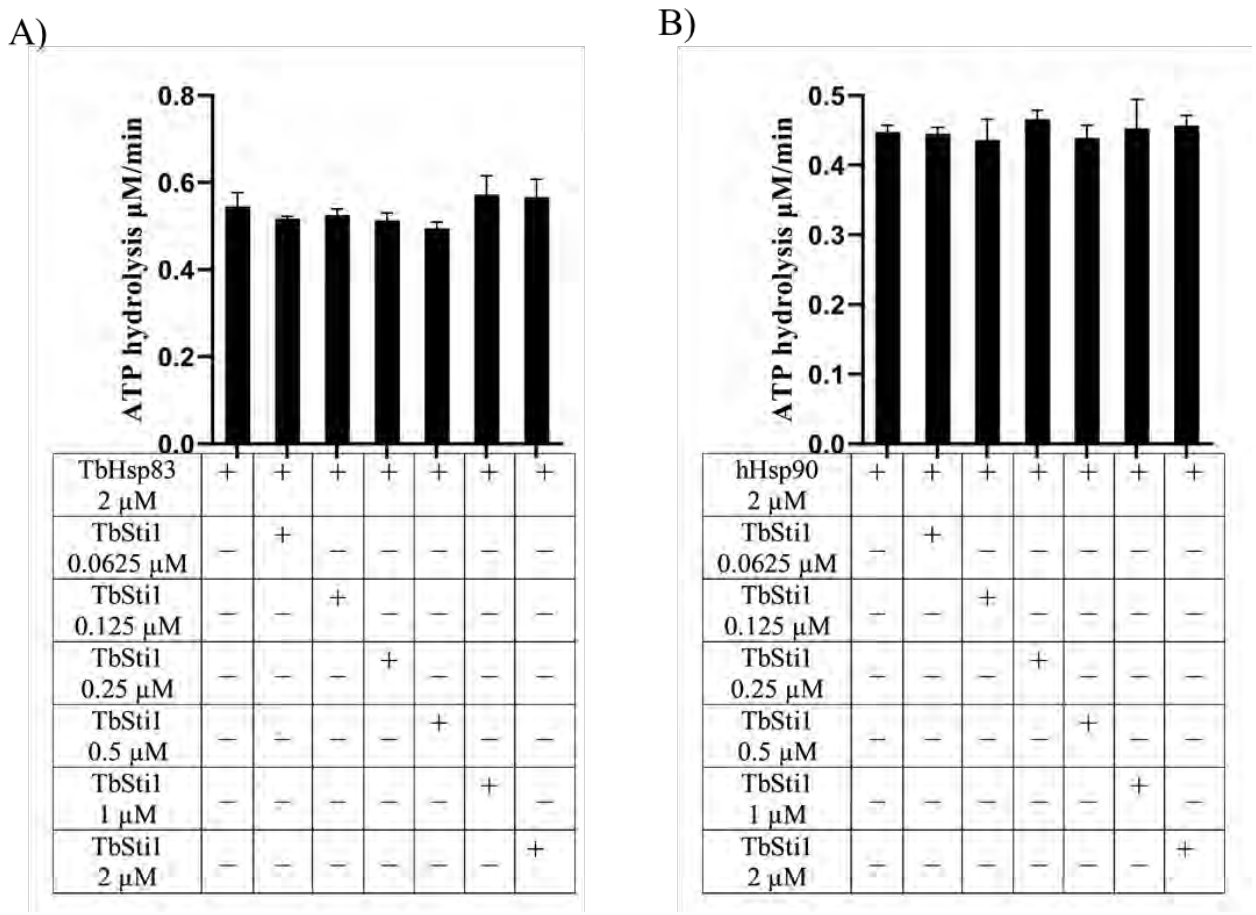

Figure 3.11: Effect of TbSti1 on the ATP hydrolysis activity of TbHsp83 and hHsp90.
The '+' shows components present in the reaction mixture while '-' shows components absent. A) Increasing concentrations of TbSti1 added to 2µM Tbhsp83 in the presence of ATP. B) Increasing concentrations of TbSti1 added to 2µM hHsp90 in the presence of ATP. no statistical distance was seen in all cases, statistics was done using one-way ANOVA. Data shown represent averages ±SEM (n=3) from three independent biological replicates.

3.6 Conclusion

In this study, we biochemically characterized the co-chaperone TbSti1 and its interactions with the molecular chaperones TbHsp83 and hHsp90. Hsp90 has been established as an essential and abundant component of the eukaryotic cell proteome present in both stressed and unstressed conditions (Borkovich et al. 1989; Luengo et al. 2019; Bhattacharya and Picard 2021). In addition to the expansion of the chaperone complement in the eukaryotic proteome, there has also been a noticeable expansion in the accompanying co-chaperones (Brehme et al. 2014; Rizzolo et al. 2017; Klaips et al. 2018). The protein-protein interactions between chaperones and co-chaperones is crucial for maintaining cell proteostasis (Rizzolo et al., 2017; Reidy et al., 2018). The TPR repeat-containing stress-inducible protein Sti1, though found to be non-essential in yeast and some eukaryotic organisms (Chang et al. 1997; Rizzolo et al. 2017; Kravats et al. 2018; Bhattacharya et al. 2020) is a regulatory co-chaperone that interacts with the Hsp90 complex (Li et al. 2012; Rizzolo et al. 2017). One method to confirm the secondary structure composition and the folding state of purified recombinant proteins is circular dichroism (CD) (Greenfield, 2006). Previous studies in *Plasmodium falciparum*, *Leishmania braziliensis* and human Hop reveal the protein to be soluble after purification with a predominantly α-helical protein structure (Carrigan, 2006; Batista et al., 2016; Makumire et al., 2020; Silva et al., 2020). CD spectrometry also revealed PfHop to have a stable conformation at temperatures below 40 °C (Makumire et al., 2020). Biophysical studies may be necessary to confirm the secondary structures and conformations of TbHsp83 and TbSti1 follow a similar pattern to previously described Hop.

A strong interaction between Hsp83 and Sti1 was seen in *Trypanosoma brucei* using a qualitative far western blot analysis and a quantitative solid phase binding assay (K_d - 27.17 μM in the absence of ATP and 38.34 μM in the presence of ATP). Previous studies using isothermal titration calorimetry (ITC) showed a strong binding affinity between Hsp90 and Sti1 in the sub-micromolar range for yeast (K_d - 0.33 μM) (Prodromou et al. 1999), *Leishmania braziliensis* (K_d - 1 μM) (Batista et al., 2016) and human (K_d - 0.69 μM) (Onuoha et al., 2008). Hop is known to preferentially bind the nucleotide-free or ADP-bound form of Hsp90 (Johnson et al. 1998) and as seen in this study, the presence of ATP reduced the affinity of Sti1 to hHsp90 but was insignificant for the binding of TbHsp83.

Hsp90 function and activity is largely dependent on its ATP binding and hydrolysis (Nadeau et al. 1992; Pearl 2016; Schopf et al. 2017). Hsp90 was reported in early findings not to bind ATP and lacked ATPase activity (Nadeau et al., 1992; Jakob et al., 1996). Subsequently, it was established that ATP hydrolysis is essential for Hsp90 activity (Obermann et al. 1998; Panaretou et al. 1998; Grenert et al. 1999), but Hsp90

was shown to possess weak ATPase activity (McLaughlin et al. 2002) and Human Hsp90 has been shown to have a much lower ATPase activity in comparison to PfHsp90 (Pallavi et al., 2010). The hHsp90 ATPase activity also corresponded to previously reported findings (McLaughlin et al. 2002) (Table 3.2). TbSti1 showed ATPase activity which is similar to previously reported human Hop data (Yamamoto et al., 2014). However, because the proteins were purified from *E. coli* there is the possibility that there could be a contamination with bacterial ATPases which might influence the activity. Despite the various ATPase assay techniques employed by various authors, all the reported K_m values are within the range of 100 to 830 µM (McLaughlin et al. 2002) (Table 3.2)

Table 3.2: Comparison of Hsp90 ATP hydrolysis parameters across various organisms.

Organism	K_m (µM)	References
Yeast Hsp82	100	(Panaretou et al. 1998)
Yeast (Hsp82)	172	(Obermann et al., 1998)
Yeast (Hsp82)	830	(Young and Hartl, 2000)
Yeast Hsp90	300	(Weikl et al., 2000)
Human (Hsp90β)	840	(McLaughlin et al. 2002)
E. coli (HtpG)	261	(Owen et al., 2002)
Yeast (Hsc82)	511	(Owen et al., 2002)
Human (Hsp90β)	324	(Owen et al., 2002)
Yeast Hsp90	513	(Rowlands et al., 2004)
P. falciparum Hsp90	611	(Pallavi et al., 2010)
T. brucei Hsp83	360	(Pizarro et al., 2013)
Leishmania braziliensis Hsp90	430	(Silva et al. 2013)
T. brucei Hsp83	486	This study
Human (Hsp90α)	300	This study

Some authors speculate that the inhibition of the ATPase activity of Hsp90 by Sti1 is as a result of the blockage of the nucleotide binding site when Sti1 binds to Hsp90 (Prodromou et al. 1999). It may be a result of the altered conformational changes formed from binding that may be required for ATP hydrolysis to take place (Richter et al., 2003). Our findings suggest that the inhibitory effect of Sti1 on Hsp90 may be species-specific as TbSti1 did not inhibit the ATP hydrolysis of either TbHsp83 or hHsp90 and this also suggests that TbSti1 may be regulating TbHsp83 differently from the canonical inhibition of ATPase

activity as seen in other organisms. However, these are preliminary results and there is a possibility that the basal ATPase activity seen in TbSti1 may have come from DnaK contamination which could have been confirmed by the use of anti-DnaK antibodies.

In addition to its characteristic ATPase activity, Hsp90 also functions as a holdase and can prevent proteins from aggregation (Kampinga 2006; Genest et al. 2019). Light scattering techniques are usually employed to measure the aggregation of proteins and the ability of heat shock proteins to act as holdases to unfold model substrates in conditions of stress (Silva et al. 2013; Nyakundi et al. 2016). Malate dehydrogenase (MDH) (Takahashi-Íñiguez et al., 2016) is a heat-labile protein known to aggregate at temperatures ranging from 45 °C to 48 °C and has been used as a model substrate to test the holdase function of some chaperones (Shonhai et al. 2008; Silva et al. 2013; Nyakundi et al. 2016; Bentley and Boshoff 2019). *Leishmania braziliensis* Hsp90 (LbHsp90) showed potent chaperone activity by its ability to prevent aggregation of MDH or citrate synthase (CS) even at sub-stoichiometric ratios (CS:LbHsp90, 8:1) (Silva et al. 2013). Yeast Hsp90 also substantially suppressed the aggregation of CS (Jakob et al., 1995) and hHsp90 substantially suppressed the aggregation of rhodanese in a dose dependent manner (Young et al. 1997). TbHsp83 in this study showed potent chaperone activity by suppressing the aggregation of MDH at equimolar concentrations of TbHsp83 and MDH. TbSti1, however, didn't suppress MDH aggregation.

In summary, this study shed more light on the interaction between Hsp83 and Sti1 in *Trypanosoma brucei.* Hsp83 bound TbSti1 in the presence and absence of ATP (K_d - 38.34 µM and 27.17 µM respectively). However, and contrary to expected findings, TbSti1 was also able to bind hHsp90 with stronger affinity for hHsp90 (K_d of 0.68 nM) compared to Hsp83 (K_d of 30.58 nM). This highlights the potential similarity between the Sti1 binding sites in both Hsp90 proteins and questions an approach of targeting this interaction as alternative chemotherapeutic approaches. It will be interesting to see if the Sti1 and hHsp90 interaction is similar in other parasites.

4 Characterization of endogenous and recombinant TbSti1 in *T. brucei* lysates and HeLa mammalian cell lines

4.1 Introduction

In silico analysis of the *T. brucei* genome (chapter 2) showed 10 copies of the *TbHsp83* genes (from gene Tb927.10.10890 to gene Tb927.10.10980) with identical amino acid sequences tandemly arranged on chromosome 10, which suggests major roles of this protein in *T. brucei* biology and function. A single copy of *TbSti1* gene was also found in the genome.

The Hsp90 chaperone mechanism has been explored in some trypanomastids. In *Leishmania donovani* (*L. donovani*) Hsp90 inhibition *in vitro* leads to stage differentiation which mimics the temperature induced insect to mammalian stage differentiation (Wiesgigl and Clos, 2001b). In *Trypanosoma cruzi* (*T. cruzi*), Hsp90 inhibition blocked cell division and also induced the heat shock response (Graefe et al., 2002). Although gene silencing of *Sti1* by RNAi in *T. brucei* did not affect cell growth, a single allele knockout of *Sti1* hindered the differentiation process of *T. cruzi* (Schmidt et al. 2018) and the Hsp90/TbSti1 partnership in *L. donovani* was critical for the proliferation of both stages of the parasite (Hombach et al., 2013). The Hsp90/TbSti1 interaction has not been explored in *T. brucei.*

To contribute to our understanding of *T. brucei* biology, this work set out to explore the partnership of TbHsp83 and TbSti1 *in vivo*, the heat shock response of TbHsp83 and Tbsti1 in *T. brucei* lysates, the subcellular localization of TbSti1 by transfection into human HOP knockout mammalian cells.

4.2 Objectives

The aim of this study was to assess the heat inducible expression of the trypanosomal proteins TbSti1 and TbHsp83 as well as characterize TbSti1 transfected into mammalian cell lines.

4.3 Specific objectives

- Investigate the heat inducible expression of TbSti1 and TbHsp83 in *T. brucei* cell cultures.

- Analysis of TbSti1 interaction with Hsp90 and/or Hsp70 by immunoprecipitation from transfected mammalian cell lysates.
- Immunofluorescence assay and microscopy to determine the subcellular localization of TbSti1 when overexpressed in human (HeLa CRISPR Hop knockout (KO)) cell lines transfected with pcDNA3.1(+)_HA-TbSti1
- Immunofluorescence staining and microscopy to compare the effect of TbSti1 expression in (HeLa CRISPR Hop knockout (KO)) cell lines transfected with pcDNA3.1(+)_HA-TbSti1 .
- Immunofluorescence staining and microscopy to compare morphological effects of TbSti1 expression in in human (HeLa CRISPR Hop knockout (KO)) cell lines transfected with pcDNA3.1(+)_HA-TbSti1

4.4 Materials and methods

4.4.1 Materials

Except as stated otherwise, all reagents were purchased from ThermoScientific/Sigma-Aldrich (U.S.A), New England biolabs (U.S.A), Roche (Germany), Promega (U.S.A), Bio-Rad (U.S.A), Greiner Bio-one (Germany), Merck (Germany) and Santa Cruz Biotechnology Inc (U.S.A). pCDNA3.1(+)_HA-TbSti1 (a mammalian expression plasmid for TbSti1 in frame with a HA-tag) (Figure 3.1C) was designed in house and synthesized by GenScript (U.S.A), the rabbit anti-TbHsp83 and anti-TbSti1 antibodies were produced and purchased from GenScript (U.S.A) while the primary antibodies against human – anti-Hsp90α (Enzo-ADI-SPA-840), anti-Hsp90β (Abcam-ab119833), anti-Hsp90αβ (Santa Cruz SC13119) and anti-Hsc70 (StressMarq -SMC 151) were kind donations from Professor Adrienne Edkins' lab (Rhodes University, Grahamstown, South Africa). HeLa Hop KO cells using CRISPR-Cas9 targeting exon 3 in the Hop gene were created and validated by Dr. Jason Sterrenberg from Professor Adrienne Edkins' lab (Rhodes University, Grahamstown, South Africa). Culturing of *T. b. brucei* lysates was done by William Samson from Professor Hoppe's lab (Rhodes University, Grahamstown, South Africa) and mammalian cell culturing was done by Dr. Michaelone Vaaltyn from Professor Edkins lab (Rhodes University, Grahamstown, South Africa).

4.4.2 Methods

4.4.2.1 Detection of TbSti1 and TbHsp83 in parasite lysates in response to heat stress using specific antibodies

The preparation and culturing of bloodstream form *T. b. brucei* laboratory strain parasites was done by William Samson (Hoppe lab, Rhodes University, Grahamstown, South Africa). Bloodstream form *T. b. brucei* Lister 927 variant 221 strain parasites were cultured in filter sterilized complete Iscoves Modified Dulbeccos Media (IMDM) based HM1-9 medium (IMDM base powder, 3.6 mM sodium bicarbonate, 1 mM hypoxanthine, 1 mM sodium pyruvate, 0.16 mM thymidine, 0.05 mM bathocuprone sulphate acid, 10% (v/v) heat inactivated foetal bovine serum, 1.5 mM L-cysteine, 0.2 mM β-mercaptoethanol, pH 7.5) in a humidified chamber at 37 °C with an atmosphere of 5% CO2. Parasites were kept at a density below 2 x 106 cells/ml, their growth was monitored using a Neubauer haemocytometer to count the cell number, after which cells were diluted, according to their density, in the described pre-warmed media. Three samples were prepared per biological replicate, namely – an untreated control sample grown at 37 °C, samples exposed to heat shock at 42 °C for 30 minutes and samples exposed to heat shock at 42 °C for 30 minutes + 4 hours recovery at 37 °C. The pellets were washed with 1x PBS (Phosphate buffered Saline – 16 mM Na_3PO_4, 150 mM NaCl, 4 mM K_3PO_4, pH 7.5), lysed using RIPA + 1% (v/v) Protease Inhibitor Cocktail (PIC from Sigma Aldrich, U.S.A P8340) and incubated on ice for about 25 minutes. The protein concentration of samples was quantified using BCA assay, resolved on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane to be analysed by western blot (Appendix 7.1.10). Anti-TbHsp83 and anti-TbSti1 antibodies (1:5000 each) were used to probe for TbHsp83 and TbSti1 respectively.

4.4.2.2 Immunoprecipitation of human Hsp90 and Hsc70 from mammalian cell lines transfected with TbSti1

HeLa wild type (WT) and HeLa CRISPR Hop knockout (KO) cells were seeded at ~2×10^5 cells per ml. Cells were transfected with either the pcDNA3.1 backbone control or pcDNA3.1(+)_HA-TbSti1 plasmids (2 µg/µl) using the X-tremeGENE HP DNA Transfection Reagent (Roche) as per the manufacturer's instructions. The cells were allowed to grow for 48 hours in a 6-well plate. After discarding the growth medium, Cellytic lysis buffer (Sigma-Aldrich C2978) with Protease Inhibitor Cocktail (PIC Sigma Aldrich, U.S.A) was added and the cell monolayer lysed by scraping and sonicated briefly. A total of 500 µg each

of lysate were added to 10 µl of resin conjugated to HA-antibody (ThermoScientific U.S.A) and left to rotate overnight at 4 °C. The lysate and bead mixtures were transferred to a spin column (SigmaPrep spin column by Sigma-Aldrich U.S.A) and centrifuged at 15000 g for 30 seconds to discard the flow through. The beads were subsequently washed 7 times with 1x PBS-T (Phosphate buffered Saline – 16 mM Na_3PO_4, 150 mM NaCl, 4 mM K_3PO_4, pH 7.5 0.01% Tween 20). Proteins bound to the resin were eluted with 50 µl of 5x SDS-PAGE sample buffer with β-mercaptoethanol by boiling for 5 minutes at 95 °C and centrifuged at 15000 g for 30 seconds. Samples were analysed with western blot (Appendix 7.1.10). Anti-HA antibody was used to detect TbSti1-HA, while anti-Hsp90α and anti-Hsp90β antibodies were used to detect the human Hsp90 proteins and anti-Hsc70 antibody was used to detect human Hsc70.

4.4.2.3 Immunofluorescence assay and microscopy to determine the subcellular localization of TbSti1

The medium from transfected and untransfected HeLa cells as (described in 4.4.2.2) cultured on glass coverslips was discarded and the cells were washed once with sterile 1x PBS and fixed with 4% (w/v) paraformaldehyde in 1x PBS for 10-15 mins at room temperature. Next the slides were washed three times with 1x PBS, permeabilized with 0.1% (v/v) Triton X-100 in 1x PBS for 10-15 mins and blocked with 1% (w/v) bovine serum albumin in 1x PBS for 45 minutes at room temperature. HA-TbSti1 was detected using the specific anti-TbSti1 antibody (1:100) in 0.1% (w/v) BSA/PBS-T solution overnight at 4 °C. Slides were washed twice in 0.1% (w/v) BSA/PBS-T for 5 minutes each and incubated with the corresponding secondary antibody (Alexa Fluor 488 conjugated goat anti-rabbit IgG [Invitrogen]) at a dilution of 1:1000 for 1 h at room temperature in the dark. Control slides from the transfection received secondary antibody but not primary antibody. Slides were washed twice in 0.1% (w/v) BSA/PBS-T for 5 minutes each and a final rinse with Hoechst 33342 (1 µg/ml) performed to stain the nucleus. Finally, slides were mounted using DAKO fluorescent mounting medium and the edges sealed with a clear nail varnish. Imaging was done using 100x objective lens using the Olympus BX43 fluorescence microscope.

The morphological characteristics of the HeLa cells were analysed by comparing the HA-TbSti1 transfected Hop KO cells to untransfected Hop KO cells and untransfected WT HeLa cells. Cell morphology was analysed by staining the membrane with wheat germ agglutination protein conjugated to Alexa Fluor 555 (WGA-555 Invitrogen) and with Hoechst 33342 (1 µg/ml) to stain the nucleus before proceeding with visualization using the Olympus BX43 fluorescence microscope (Olympus microscopy, U.S.A).

4.5 Results and discussion

4.5.1 TbSti1 and TbHsp83 were detected in *T. b. brucei* bloodstream stage parasites

The expression of TbHsp83 and TbSti1 from *T. b. brucei* BSF stage parasites was investigated at permissible temperature conditions (37 °C), after heat shock (42 °C) for 30 minutes and after 4 hours of recovery at 37 °C after the heat shock. Protein expression was assessed by 10% SDS PAGE and western blot analysis of the lysates grown at the different temperatures (Figure 4.1.A-B). 17 kDa Histone loading control was used to achieve equivalent loading. TbHsp83 is an abundant protein in the cell and the anti-TbHsp83 serum detected proteins between 75 kDa and 100 kDa which coincides with the ~90 kDa size of the TbHsp83 protein. Anti-TbSti1 detected a protein with a band size between 50 kDa and 75 kDa which corresponds to the 63 kDa TbSti1 protein (Figure 4.1 A). Densitometric analysis of the protein expression levels between the permissible control, heat shock and recovery expression of each protein did not show significant difference (Figure 4.1 C i and ii). This suggested that TbSti1 and TbHsp83 expressions were not upregulated under the conditions of our heat shock.

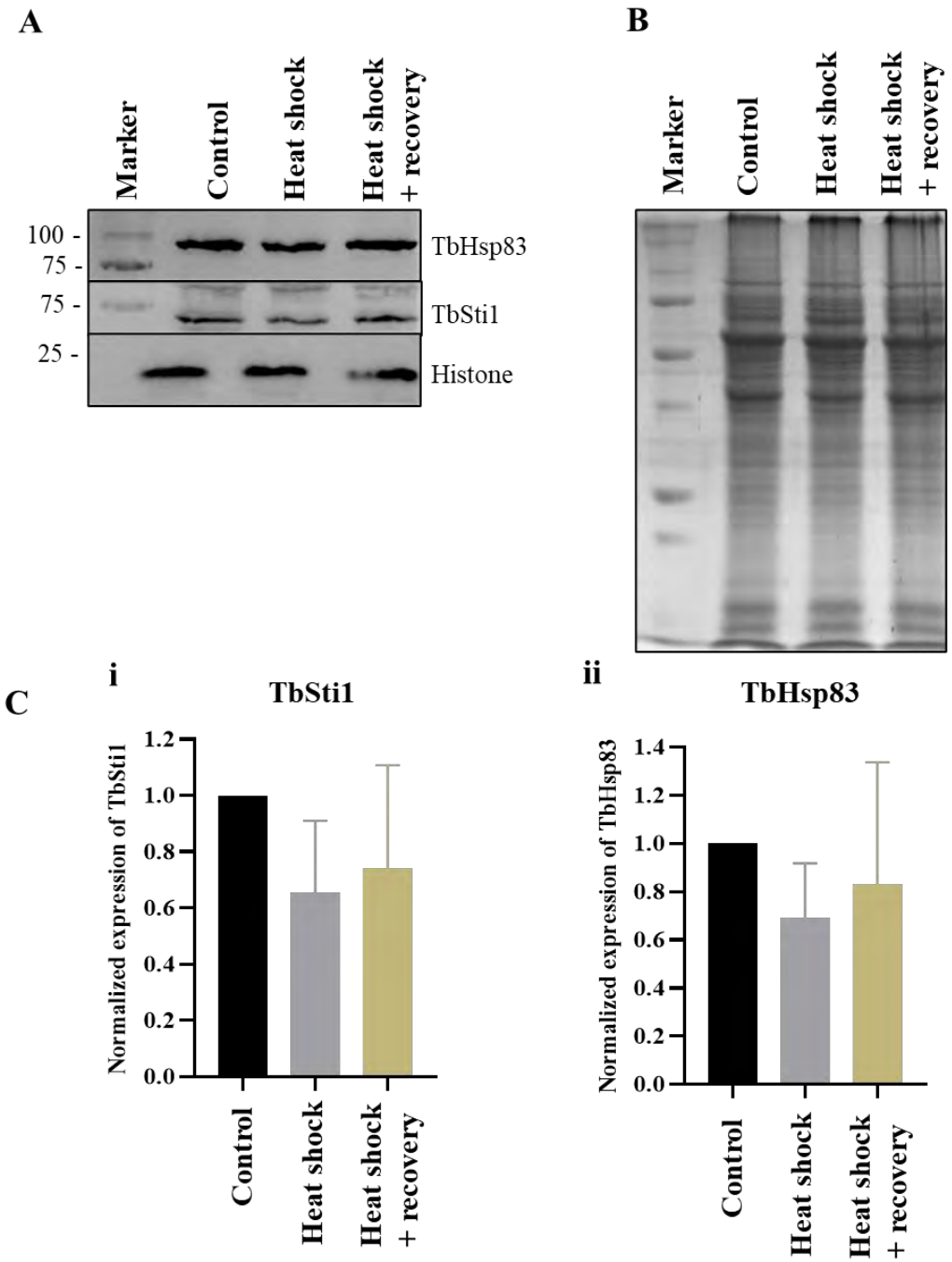

Figure 4.1: Expression of TbSti1 and TbHsp83 in *T. brucei* lysates during heat shock.
Western blot analysis of the expressions of TbSti1, TbHsp83 and Histone H3 loading control for the samples – untreated control grown at 37 °C,, 30 minutes heat shock at 42 °C and 30 minutes heat shock at 42 °C + 4 hours recovery at 37 °C. B) 10% SDS-PAGE analysis of the total protein in *T. brucei* lysates. C) Densitometric analysis of the protein expression levels for i) TbSti1 and ii) TbHsp83. Samples were untreated control cultured at 37 °C, 30 mins heat shock at 42 °C and 30 mins heat shock at 42 °C + 4 hours recovery at 37 °C. Data shown represent averages ±SEM (n=3) from three independent biological replicates.

4.5.2 TbSti1 in a common complex with human Hsp90 and Hsc70 from HeLa Hop KO mammalian cell lysate

Immunoprecipitation of TbSti1-HA from the transfected Hop KO HeLa cell lysates was performed using HA-tag antibody and co-immunoprecipitation of human Hsp90 and Hsp70 determined by western blotting. The HeLa Hop KO cell line was used such that HA-TbSti1 would be the only Sti1 protein expressed. HA-TbSti1 was successfully isolated and both hHsp90 and hHsc70 could be detected in the complex (Figure 4.2). Hsp90β is the constitutive form of the protein and shows a band larger than Hsp90α which is the inducible form. This is consistent with previous findings that Hsp90β is a larger protein than Hsp90α (Csermely et al. 1998; Sreedhar et al. 2004).

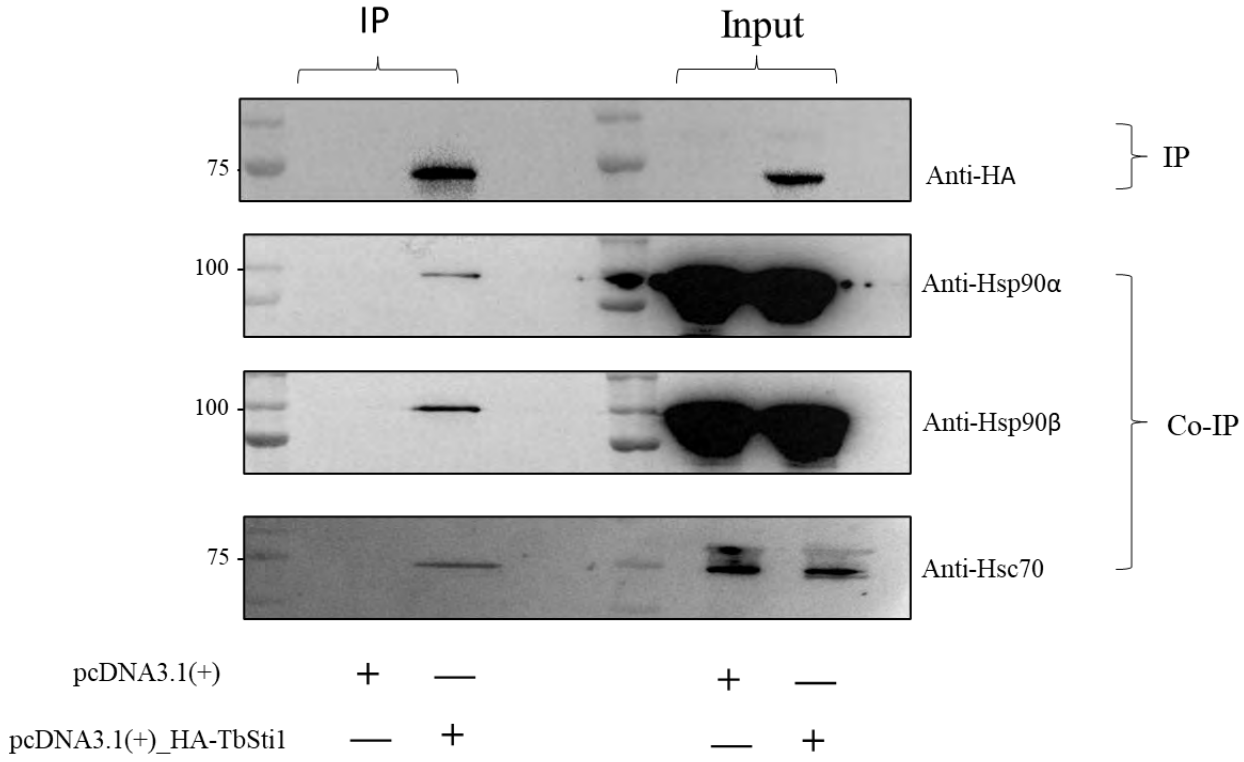

Figure 4.2: Human Hsp90 and Hsp70 can be isolated in complex with HA-TbSti1 from transfected HeLa cell lysates.
HA-tagged TbSti1 immunoprecipitation and co-immunoprecipitation of complexes from whole cell lysates of HeLa Hop KO cells transfected with pcDNA3.1(+)_HA-TbSti1 or pcDNA3.1(+) backbone control. Anti-HA antibody was used to detect HA-TbSti1, anti-Hsp90α/anti-Hsp90β antibodies were used to detect Hsp90α and Hsp90β and anti-Hsc70 was used to detect Hsc70. Data are representative of two independent biological replicates showing the same result.

4.5.3 TbSti1 localizes to the cytoplasm and expression alters mammalian cell line morphology

To assess the subcellular localization of HA-TbSti1, we used indirect immunofluorescence staining of HeLa Hop KO mammalian cells transfected with pcDNA3.1(+)_HA-TbSti1 using a specific anti-TbSti1 antibody. The HeLa Hop KO cell lines was used as this would mean that HA-TbSti1 would be the only Sti1 isoform expressed. The control sample with only secondary antibody but lacking anti-TbSti1 primary antibody showed no detectable fluorescence despite the presence of cells indicated by the nuclear marker. This suggested no background or non-specific fluorescence. A green fluorescence signal corresponding to HA-TbsSti1was observed throughout the cytosol and to a lesser extent around the nucleus. This suggested the presence of HA-TbSti1 in the cytoplasm and in the perinuclear region of the cells (Figure 4.3). In addition, low levels of HA-TbSti1 fluorescence were detected in the nucleus. This staining pattern is consistent with previous findings that Sti1 localizes to the cytosol and co-localizes with proteins in the cytoplasm and/or around the nucleus (Webb et al., 1997; Schmidt et al., 2011; Gitau et al., 2012).

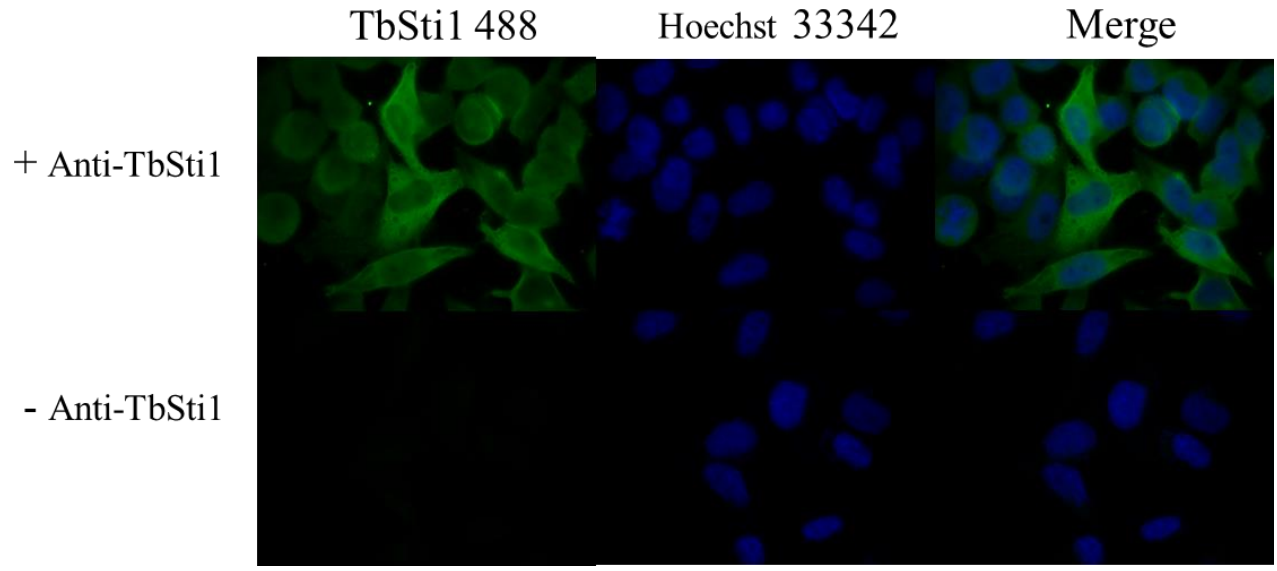

Figure 4.3: Immunofluorescence staining showing cytoplasmic localization of TbSti1 in HeLa Hop KO mammalian cell line.
Top panel probed with anti-TbSti1 primary antibody and Alexa Fluor 488 (green) goat anti-rabbit secondary antibody. Nucleus was stained with Hoechst 33342 (blue). Lower panel was as above but lacking anti-TbSti1 primary antibody, The panel marked merge is an overlay of the HA-TbSti1 and the nucleus panels. Scale bars are 10 μm and images are representative of multiple fields taken at random and display the dominant staining pattern observed.

We also noted that in comparison to the cells transfected with pcDNA3.1(+)_HA-TbSti1 that showed an elongated cell morphology, the cells transfected with the pcDNA3.1 backbone control were less elongated and had a more round morphology (Figure 4.4). This suggested that HA-Sti1 expression altered the morphology of the HeLa Hop KO cells.

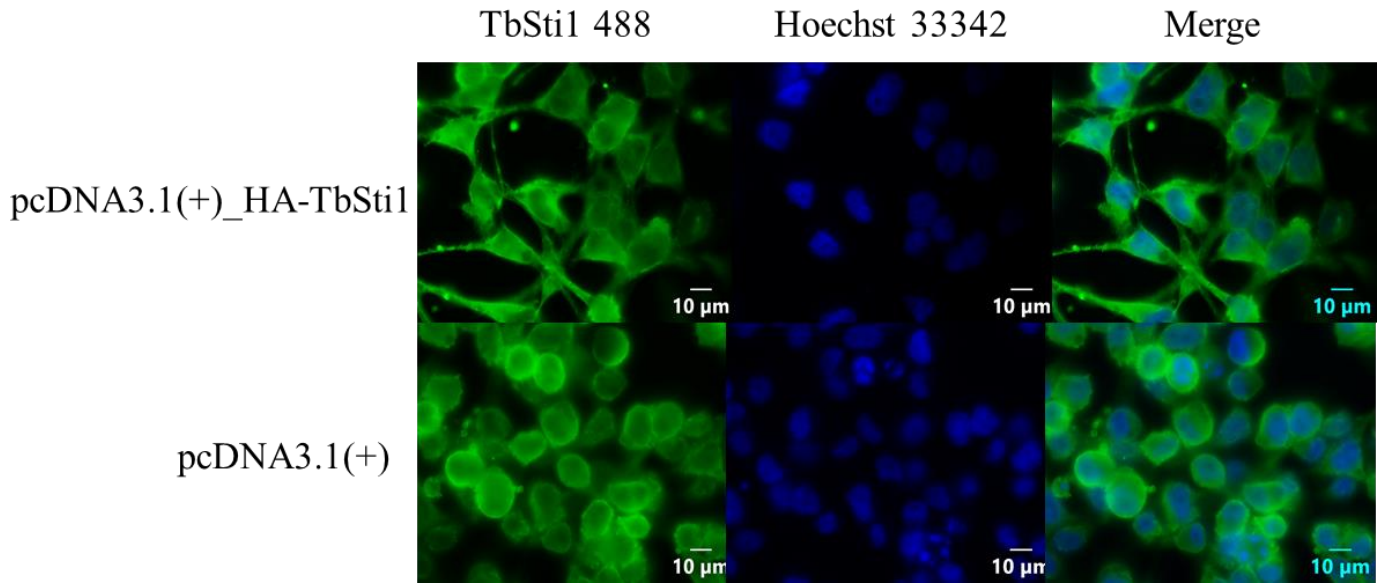

Figure 4.4: pcDNA3.1(+)_HA-TbSti1 transfected HeLa Hop KO cells show different cell morphology from pcDNA3.1 transfected cells.
HeLa cells transfected with pcDNA3.1(+)_HA-TbSti1 and pcDNA3.1 backbone control. Both panels were probed with anti-TbSti1 primary antibody and Alexa Fluor 488 (green) goat anti-rabbit secondary antibody, nucleus was stained with Hoechst 33342 (blue). Scale bars are 10 µM and images are representative of multiple images taken randomly across the slides.

To further corroborate this finding, wheat germ agglutinin conjugated to Alexa fluor 555 dye (WGA-555) was used to stain the cell membrane to analyse the morphological differences in the cells transfected with HA-TbSti1 in comparison to the untransfected and wild type (WT) HeLa cells. The cell membrane contains glycoproteins and glycolipids which contain residues of sialic acid and N-acetylglucosamine that can probed with fluorescent WGA-555 (Chazotte, 2011). The WT and untransfected cells showed a more circular morphology, while the HA-TbSti1 transfected cells were more elongated (Figure 4.5).

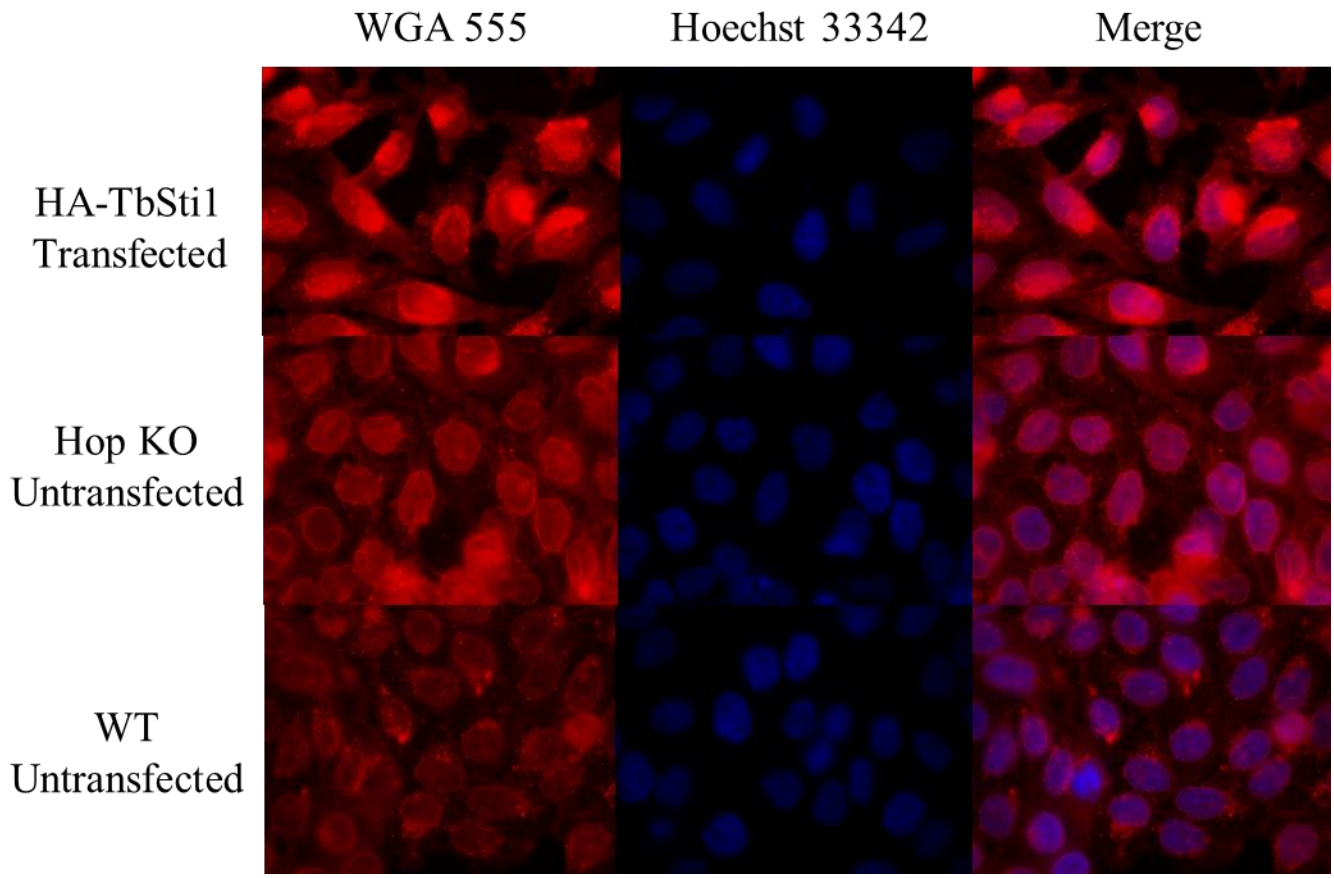

Figure 4.5: HA-TbSti1 transfected HeLa Hop KO cells have a different cell morphology from untransfected Hop KO and HeLa WT cells.
HeLa Hop KO cells transfected with pcDNA3.1(+)_HA-TbSti1 were stained with WGA 555 (red) to highlight their cell morphology, while nucleus was stained with Hoechst 33342 (blue). Hop KO (untransfected) and WT (untransfected) cells were also stained for comparison. Scale bars are 10 µm and images are representative of multiple fields taken at random and display the dominant staining pattern observed.

4.6 Conclusion

In this study we characterized *Trypanosoma brucei* Sti1 (TbSti1) *in vivo* in parasite cultures and *ex vivo* in mammalian cell line models transfected with *TbSti1* to corroborate the findings from the *in vitro* study (Chapter 3). Hsp70/Hsp90 organizing protein (Hop) also known as Stress inducible protein 1 (Sti1) is an abundant protein encoded by a single gene and has been well characterized in yeast and mammals (Nicolet and Craig, 1989; Honoré et al., 1992). *In silico* analysis also confirmed TbSti1 to be coded by a single gene and shares similar domain organization with human Hop. Hop/Sti1 in mouse localized primarily in the cytoplasm but can translocate to the nucleus upon heat shock (Lässle et al. 1997; Daniel et al. 2008; Longshaw et al. 2004). *Plasmodium falciparum* Hop in parasites at the trophozoite stage and *Trypanosoma*

cruzi Sti1 also localized to the cytoplasm (Schmidt et al., 2011; Gitau et al., 2012). To confirm the subcellular localization of TbSti1in the absence of *T. b. brucei* parasites, human cell lines transfected with HA-TbSti1 and grown under basal conditions were analysed by immunofluorescence. Consistent with reports on Hop/Sti1 in other species, TbSti1 predominantly localized to the cytoplasm, perinuclear region and to a lesser extent, in the nucleus (Figure 4.3). Beside the cytosolic localization, immunofluorescence staining on the morphology of cell membrane showed no obvious differences between Hop WT and Hop KO cells, but Hop KO cells transfected with TbSti1 showed a different and distinct fibroblast-like cell morphology (Figure 4.5). This suggests that exogenously expressed TbSti1 may be functional in mammalian cell lines and may alter the morphology of cells due to this intracellular activity. Part of this activity may be mediated by the ability of TbSti1 to interact with human Hsp70 and Hsp90, and hence function in place of human Hop.

Co-immunoprecipitation studies have previously shown PfHop to associate with the PfHsp70 and PfHsp90 complex (Gitau et al., 2012). In this study, both human Hsp90 proteins, the constitutive Hsp90β and Hsc70 and the stress inducible Hsp90α, were in a complex with HA-TbSti1 validating both chaperones as interacting partners. Hop serves as an adaptor protein between Hsp70 and Hsp90, but the Hsp90-Hop complex reduces the affinity and seemingly puts a strain on the Hsp70-Hop complex (Hernández et al. 2002; Gitau et al. 2012). The results using antibodies to Hsp70 showed no bands (data not shown) but antibody to Hsc70, the constitutive form of the protein revealed a band of lower intensity than that of the Hsp90s. The co-immunoprecipitation results provide further proof of TbSti1 as a binding partner of hHsc70 and hHsp90 in cell lines and may have a role in modulating their functions. Furthermore, TbSti1, which possesses ~ 40% sequence similarity to its human counterpart (Hop) showed direct interaction with human Hsp90 *in vitro* therefore it would be interesting in future to determine if TbSti1 could functionally replace Hop in Hop KO mammalian cell lines.

Heat shock expression levels of TbSti1 and TbHsp83 was assessed in bloodstream form parasites exposed to heat shock for 30 minutes. Western blot analysis using specific antibodies directed against both proteins revealed that both proteins are constitutively expressed but did not show significant increase upon a 30-minute heat shock (Figure 4.1). Hsp90 and Sti1 are known stress inducible proteins (Nicolet and Craig, 1989; Lässle et al., 1997). However, TcSti1 was not induced by heat but by nutritional stress in a previous study (Schmidt et al., 2011). Although, TbSti1 was not robustly induced by heat shock after 30 minutes, a longer time exposure of at least one hour may be necessary for a more robust response as seen in *Leishmania major* and *Plasmodium falciparum* (Webb et al., 1997; Zininga et al., 2015) as the effect of other stress conditions on TbSti1 levels could be investigated in future studies.

5 Conclusion and Future Perspectives

The aim of this study was to characterize the co-chaperone Sti1 in *T. brucei* (TbSti1) and its interactions with both TbHsp83 and human Hsp90. To achieve this, *in silico* tools were first employed for bioinformatic analysis of the Hsp90 chaperone machinery in *T. brucei* with side-by-side comparisons to other selected kinetoplastids and the mammalian Hsp90 system. Next, the biochemical characteristics of TbHsp83-TbSti1 was also explored using *in vitro* and *ex vivo* techniques, TbHsp83 showed typical attributes of other characterized Hsp90 chaperones such as its ability to suppress thermally induced MDH. TbSti1 showed no chaperone activities as expected but contrary to previous findings in yeast and other species that Sti1 acts as inhibitor of the ATPase activity of Hsp90 (Prodromou 1999; Batista et al. 2016), TbSti1 in this study showed no inhibitory effect on the ATPase activity of TbHsp83.

A first time in depth *in silico* analysis of the Hsp90 chaperone complement in *T. brucei* was carried out. *T. brucei* is subdivided into 3 subspecies namely – the human infective *T. brucei gambiense* and *T. b. rhodesiense* and the animal infective *T. b. brucei. T. brucei gambiense* and *T. b. brucei* have been sequenced and the information deduced from *T. b. brucei* is similar to *T. b. rhodesiense* as they share the same genetic and biological characteristics (Jackson et al. 2010; Gibson 2012). This study identified twelve putative *Hsp90* genes in *T. b. brucei*, ten of which are identical and code for the cytosolic Hsp83 protein arranged in tandem in a head to tail manner on the same chromosome 10 (Table 2.2, Figure 2.3). This finding is similar to previous findings (Mottram et al. 1989) where the *Hsp83* gene cluster was first cloned and sequenced. An added putative cytosolic *Hsp83* not yet assigned to a chromosome was also identified but it is not clear if it is an addition to the current identified cytosolic proteins or a repetition of one of them and this needs to be further clarified. In *T. brucei gambiense*, five putative *Hsp90* genes were identified three of which are homologous for the cytosolic Hsp83. The extra 2 *Hsp90* genes in both cases code for the mitochondrial (TRAP-1) and ER (GRP94) paralogue of Hsp90. The subcellular localization was further confirmed by phylogenetic analysis with each group (Hsp83, TRAP-1 and GRP94) forming a separate cluster (Figure 2.1). Multiple sequence alignment of all Hsp90 isoforms in all the species analysed showed a conserved sequence (IGQFGVGFY) in the ATPase domain (Figure 2.2), it would be interesting to explore if these residues contribute to the ATPase activity of Hsp90. All the identified TbHsp83 proteins were reported to be present at both stages of the parasite growth (PCF and BSF) but upregulated in the BSF (Urbaniak et al. 2012). The TbHsp83 proteins remained unchanged in abundance in both long slender and short stumpy bloodstream parasites however, the ER isoform GRP94 and the co-chaperone FKBP5 showed a two-fold increase in abundance in the long slender to short stumpy transition (Gunasekera et al., 2012). Many discrepancies in the number of cytosolic *Hsp90* genes were identified across the kinetoplastids and

especially amongst the *T. cruzi* strains, these discrepancies also seen between *T. b. brucei* and *T. b. gambiense* need to be explored further.

The co-chaperone complement of Hsp83 in *T. brucei* was explored. Most well identified co-chaperones were present in *T. brucei* with Cdc37 the obvious absent co-chaperone, the functions of Cdc37 could possibly be carried out by another co-chaperone present in *T. brucei* and this functional replacement could be studied. The Hsp83 co-chaperones present in *T. brucei* were grouped according to the presence of a TPR-domain. Co-chaperones containing TPR domains in this study include Sti1, PP5, Cyp40, J52, FKBPL and SGT while co-chaperones without TPR domains include p23 and Aha1. Nothing is currently known about the presence of co-chaperones in the mitochondria and ER of *T. brucei*, it would be necessary to explore if there are co-chaperones functioning in the mitochondria and ER and if they are absent what mechanisms Hsp90 uses to compensate for their absence.

Multiple sequence alignment showed TbSti1 to possess the conserved TPR domains as in other organisms. The residues that form the carboxylate clamp necessary for binding the chaperones are also conserved (Figure 2.5). It has also been previously established that the TPR2A domain binds to the C-terminal of Hsp90 in plasmodium and other species (Lässle et al., 1997; Zininga et al., 2015), In *Leishmania braziliensis,* various deletion mutants of the domains of LbHop were created to confirm the binding affinity of each domain to Hsp90 (Batista et al., 2016). Such mutational analyses of these domains and the conserved residues in TbSti1 will be necessary to confirm their functions in *T. brucei*. A full-length 3D ribbon image of TbSti1 was shown for the first time together with its characteristic domains (Figure 2.4). The kinome of *T. brucei* has been extensively studied and many of its kinases identified (Parsons et al., 2005) but specific clients of Hsp83 in *T. brucei* have not been identified. *In silico* tools for modelling could be used to predict the clients and the interactome of the *T. brucei* Hsp83 chaperone system.

The recombinant proteins TbSti1, TbHsp83 and hHsp90 as a control were overproduced in *E. coli* and successfully purified using nickel affinity chromatography. All proteins typically yielded high concentrations of ~5 mg/ml after a successful batch of purification. Qualitative (far-western blotting) and quantitative (solid phase binding assay) techniques were used to assess the interaction and binding between TbSti1 and TbHsp83/hHsp90. TbSti1 showed very high binding affinity for TbHsp83/hHsp90 in the nanomolar range, this strong binding affinity is consistent with other organisms where the Hsp90-Sti1 interaction has been characterized (Prodromou et al. 1999; Onuoha et al. 2008; Batista et al. 2016). Though binding between TbSti1 and TbHsp83/hHsp90 occurred in the presence or absence of the nucleotide ATP, the affinity of TbSti1 for hHsp90 was found to be stronger in the absence of the ATP. In a similar study exploring the interaction between PfHop and PfHsp70-1, PfHop interacted more favourably with PfHsp70-1 in the presence of ADP rather than ATP (Zininga et al., 2015). This stronger binding affinity in the absence

of ATP conforms to the supposed model of co-chaperones binding to Hsp90. Sti1 binds to the Hsp90 in the open conformation, the addition of ATP promotes the formation of the closed complex which weakens the binding of Sti1 and facilitates its exit (Li et al. 2011) (Figure 1.5).

The chaperone functions of the proteins were ascertained by carrying out MDH suppression activity assays and ATPase activity assays. The chaperones TbHsp83/hHsp90 showed potent ATPase hydrolysis activity as well as a significant ability to suppress the aggregation of the thermolabile substrate MDH with equimolar concentrations of the protein showing the highest suppression activity. TbSti1 did not suppress the aggregation of MDH and though it showed some level of ATP hydrolysis activity, a key finding in this study is that TbSti1 showed no inhibitory activity on the ATPase activity of TbHsp83/hHsp90. This is contrary to previous findings in other organisms where Sti1 almost completely inhibited the ATPase activity of Hsp90 (Prodromou 1999; Batista et al. 2016; Silva et al. 2020).

To further validate the Hsp90-Sti1 interaction, *in vivo* techniques were used. TbSti1 and Tbhsp83 did not show significant increased expression on exposure to heat stress for thirty minutes in the parasite lysates, a longer time exposure of at least one hour is probably needed for a more robust heat shock response. TbSti1 was successfully transfected into HeLa Hop KO mammalian cell lines and co-immunoprecipitated with hHsp90, this implies that the presence of TbSti1was able to compensate for the absence of human Hop due to their conserved nature. Immunofluorescence studies also confirmed TbSti1 to be a cytosolic protein, but co-localization studies could be done to confirm co-localization of TbSti1 with Hsp83 in the *T. brucei* parasite lysate and in the parasites if accessible.

Though Sti1 has been implicated to have roles in parasite differentiation (Schmidt et al. 2018), the functions of the co-chaperone are varied and include immune and neuronal functions (Webb et al., 1996; Roffé et al., 2010). The canonical adaptor function of Sti1 connecting Hsp70 and Hsp90 (Odunuga et al. 2004; Schmidt et al. 2011) has become debatable in recent years as both chaperones are able to connect directly in the absence of Sti1 in some organisms (Kravats et al. 2018; Bhattacharya et al. 2020). The functions of Sti1 in *T. brucei* has not been explored. In the study on TcSti1 characterization, TbSti1 served as a control and gene silencing by RNAi did not affect growth of the parasite (Schmidt et al. 2018). To study the role of TbSti1 in the viability and general biology of *T.* brucei, RNAi and overexpression studies could be employed to test the role of TbHsp83-TbSti1 interaction in parasite growth and differentiation.

Overall, this study provided the first detailed characterization of Tbhsp83-TbSti1, Sti1 has previously been characterized in other kinetoplastids namely*, Leishmania spp* and *T. cruzi* but not in *T. brucei*. The findings here contribute to the understanding of the chaperone Hsp83 and its interaction with the co-chaperone Sti1.

6 References

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7 Appendices

7.1 General experimental procedures

7.1.1 Yeast-Tryptone (2 x YT) broth growth medium:

Tryptone (Pancreatic digest of casein): 16 g/L

Yeast Extract: 10 g/L

NaCl: 5 g/L

Dissolve in 1L distilled water and autoclave (121 °C and 119 kPa for 30 minutes).

7.1.2 Yeast-Tryptone (2x YT) agar:

2x YT broth growth medium with the addition of 15g bacteriological agar per litre dissolved

in deionised water and autoclaved (121°C and 119 kPa for 30 minutes).

7.1.3 Terrific broth:

Tryptone (Pancreatic digest of casein): 20 g/L

Yeast Extract: 24 g/L

Glycerol: 4 ml/L

Phosphate buffer: (0.017 M KH_2PO_4 + 0.072 M K_2HPO_4) pH 7.4 100 ml/L

autoclave broth and buffer at 121 °C and 119 kPa for 30 minutes, allow broth to cool before adding sterile phosphate buffer.

7.1.4 Bacterial transformation

Relevant competent *E. coli* cells were retrieved from -80 °C freezer and plasmids retrieved from -20 °C freezer. 2 µl of plasmid DNA was added to 50 µl of competent cells and the mixture left to stand on ice for 20 minutes. The mixture was then subjected to heat shock at 42 °C for 1 minute and back on ice for 5 minutes. 1 ml 2x YT sterile broth was added to the mixture and transferred to 37 °C incubator for 1 hour with gentle agitation (~ 180 rpm). After the incubation period ~100 µl of the broth was streaked on agar plates with relevant antibiotics and the rest of the broth centrifuge at 13 000 g for 2 minutes. ~800 µl of the supernatant was removed and the cell pellet resuspended in the remaining 100 µl broth and then streaked

on agar plates with relevant antibiotics and grown overnight at 37 °C. Deionized water was used to replace plasmid DNA for negative controls

7.1.5 Making of competent *E. coli* cells

A colony of transformed cells from each *E. coli* strain of interest was inoculated into 25 ml 2x YT sterile broth with appropriate antibiotics and grown at 37 °C overnight with agitation. The overnight culture was transferred into a total of 250 ml 2x YT sterile broth with appropriate antibiotics and allowed to grow until a mid-log phase of absorbance 0.4-0.6 measured at 600 nm. Using sterile centrifuge tubes, the cells were harvested by centrifugation at 5 000 g for 15 minutes at 4 °C and kept on ice from this point onwards. The cells were resuspended in 10 ml ice-cold 0.1 M $CaCl_2$ and incubated on ice for 20 minutes. The cells were harvested again by centrifugation (as described above) and then resuspended in 5 ml ice cold 0.1 M $CaCl_2$ with 15% (v/v) glycerol. After mixing, the cells were divided into 300µl aliquots and stored at -80°C for further use.

7.1.6 Plasmid mini prep for small scale DNA extraction

A colony of transformed *E. coli* cells with the relevant plasmids of interest was inoculated into 5 ml 2x YT sterile broth with appropriate antibiotics and grown at 37 °C overnight with agitation. Plasmid DNA was extracted from *E. coli* cells using the Promega PureYield™ plasmid miniprep system (Promega corporation, U.S.A) according to the manufacturer's instruction. 3 mls of the bacterial culture were centrifuged for 30 seconds at maximum speed, the supernatant discarded and 600 µl of TE buffer or deionized water added to resuspend the pellet in a 1.5 ml microcentrifuge tube. 100 µl of cell lysis buffer was added and the tube inverted 6 times to form a clear blue solution. Cold neutralization solution was retrieved from 4 °C, 350 µl was added and the solution mixed until a yellow precipitate is formed. The mixture was centrifuged at 13000 g for 3 minutes and the supernatant transferred to a PureYield™ minicolumn followed by centrifugation at 13 000 g for 15 seconds. The flow through was discarded and the minicolumn placed in a PureYield™ collection tube, 200 µl of endotoxin removal wash was added to the tube and then centrifuged for 15 seconds before the addition of another 400 µl of column wash solution. The tubes were centrifuged again for 30 seconds and the minicolumn transferred to 1.5 ml centrifuge tubes. 30 µl of deionized water was added directly to the minicolumn matrix and left to stand for 1 minute followed by centrifugation for 15 seconds and the eluent (plasmid DNA) collected and saved at -20 °C until ready for use.

7.1.7 Plasmid restriction enzyme digest

Plasmid DNA was digested with the desired diagnostic restriction enzymes as described below.

The reaction digest was set up as follows for a total of 20 µl:

2 µl Plasmid DNA (0.5-1 µg/µl)

2 µl 10x restriction buffer

16 µl nuclease free water

The compatibility of the restriction buffers for single and double restriction enzyme digestions were determined as per the suppliers' recommendations. The restriction digest was initiated by adding 1 µg of the desired restriction endonuclease enzyme(s). The reaction was allowed to incubate overnight at 37 ºC. The reaction was stopped by addition of 6x DNA loading buffer (0.25% (w/v) bromophenol blue and 30% (v/v) glycerol). The product was then analysed by agarose gel electrophoresis as described below.

7.1.8 Agarose gel electrophoresis

0.8% (w/v) agarose gel was prepared by dissolving 0.4 g of SeaKem® LE Agarose (Lonza, Switzerland) in 50 ml of 1x Tris-acetate (TAE; 40 mM Tris, pH 7.6, 20 mM glacial acetic acid and 1 mM EDTA) buffer. The suspension was dissolved completely by heating in a microwave and then allowed to cool a bit before the addition of ethidium bromide (0.5 µg/ml). The agarose gel was set in a casting tray and allowed to polymerize at room temperature with comb inserted. The gel was placed in the electrophoresis chamber and covered with 1x TAE buffer. Volume of 7.5 µl of 6x DNA loading buffer (0.25% (w/v) bromophenol blue and 30% (v/v) glycerol) was added to 20µl of sample followed by loading of samples into the wells. Electrophoresis was carried out for 1 hour at 100 V. Agarose gels were visualized using UV light on the Bio-Rad Gel DocTM XR+ Imaging system (Bio-Rad, U.S.A.)

7.1.9 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

Protocol for SDS-PAGE was carried out as previously described (Shapiro et al 1967). Protein samples were first treated by boiling in 5x SDS-PAGE sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.05% bromophenol blue,) in a ratio of 4:1 respectively for 10 minutes at 98 ºC and resolved using 10 % acrylamide resolving gel prepared as shown below (Table A.1). The higher pH of the resolving gel concentrates the proteins before they get to the stacking gel (Brunelle and Green, 2014). The gel is then transferred into the electrophoresis tank and electrophoresis buffer (25 mM Tris, pH 8.3, 250 mM glycine and 0.1% (w/v) SDS) was added. The boiled samples were loaded in respective wells and Precision Plus ProteinTM All Blue Prestained Protein Standard (Bio-Rad, U.S.A.) was also loaded. The electrophoresis was performed at 120 volts for 1 hour 30 minutes using the Bio-Rad Mini-Protean® electrophoresis system (Bio-Rad, U.S.A.).

Table 7.1: Reagents for SDS PAGE.

Reagents (ml)	10% resolving gel	4% stacking gel
Distilled water	4.15 ml	3 ml
1.5 M Tris (pH 8.8)	2.5 ml	_
1.0 M Tris (pH 6.8)	_	1.25 ml
30% Bis/Acrylamide	3.3 ml	0.7 ml
10% SDS	0.1 ml	0.1 ml
10 % Ammonium persulphate	0.1 ml	0.1 ml
TEMED	0.02 ml	0.02 ml

7.1.10 Western blot analysis

Protocol for western blot analysis was carried out as previously described (Towbin et al 1979). Proteins were first resolved by SDS-PAGE as described above and transferred onto nitrocellulose membrane, sandwiched between filter paper and fibre pads, using transfer buffer (20 % [v/v] methanol, 192 mM Glycine, 25 mM Tris) at 100 V for 1 hour, in a Trans-Blot® SD semi-dry transfer cell (Bio-Rad, U.S.A.). Staining of the nitrocellulose membrane in Ponceau-S stain (0.5 % (w/v) Ponceau-S, 1 % (v/v) glacial acetic acid) for 2 minutes allowed for the assessment of the success of the transfer. The membrane was washed with distilled water and incubated in blocking solution comprised of 5% (w/v) fat free milk powder in 1x Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 hour at room temperature and incubated with appropriate primary antibody (1:2500 in blocking solution) overnight at 4°C with gentle agitation (on a rocker). After incubation with primary antibody, the nitrocellulose membrane was washed three times for 15 minutes with 1x TBS containing 0.1% (v/v) Tween 20 (TBS-T). The nitrocellulose membrane was incubated with the appropriate secondary horseradish peroxidase (HRP)-conjugated antibody (1:3000 in blocking solution) for one hour at room temperature and washed three times for 15 minutes with 1x TBS-T. Chemiluminescence-based protein detection were detected on the nitrocellulose membranes using the ClarityTM Western ECL blotting kit as per the supplier's instructions, and the image was captured using the ChemiDocTM XRS+ system (Bio-Rad, U.S.A.).

7.1.11 Buffer exchange by dialysis

After purification, recombinant proteins were subjected to buffer exchange to facilitate the removal of imidazole (used in the elution of the proteins from the cOmpleteTM His-tag purification resin (Roche, Germany) from the protein solution prior to use in *in vitro* work. Eluted proteins were aliquoted into SnakeSkin® dialysis tubing (10 kDa molecular weight cut off) made by tying both ends of a section of dialysis tubing using string. The tubes containing the eluted protein were suspended in 1 litre dialysis buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM KCl, 2 mM MgCl2, 0.5 mM DTT, 10% (v/v) glycerol,), and dialysis was carried out, stirring, overnight (~ 16 hours) at 4 °C. The following day the protein was further dialysed for 6 hours in a fresh volume (1 litre) of the intended assay buffer.

7.1.12 Bradford's assay for protein quantification

Protein quantification by the Bradford's assay was carried out as previously described (Bradford, 1976). In a 96-well plate, 295 µl Bradford's reagent (Sigma-Aldrich, U.S.A.) was added to 5 µl protein samples to be quantified. Along with the samples of unknown protein concentration, a set of bovine serum albumin (BSA) standards of known concentrations (0.1-1.0 mg/ml) were assayed. The samples were allowed to incubate at room temperature for 10 minutes, after which the absorbance of the samples at 595 nm was read using a Powerwave 96-well plate reader (BioTek Instruments Inc., U.S.A.). The absorbance of the BSA standards against BSA concentrations was plotted in a standard curve (Appendix Figure 7.1) to allow for the determination of the concentrations of the recombinant proteins. Any recombinant proteins determined have a higher concentration than the highest BSA concentration (1.0 mg/ml) was diluted and assayed again for accurate concentration determination.

7.2 Supplementary data

7.2.1 Sample Bradford assay standard curve

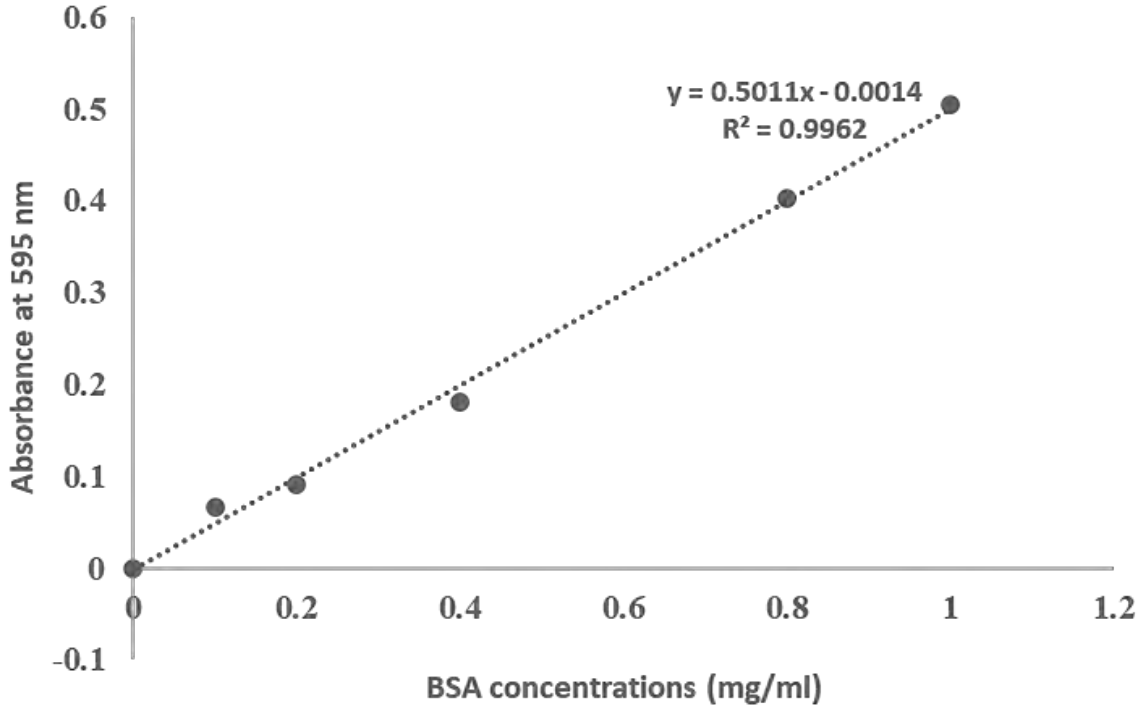


Figure 7.1: Bradford standard curve for protein concentration determination.
Bovine serum albumin (BSA) standards (0.1-1.0 mg/ml) were prepared, and absorbance was read at 595 nm using a Powerwave 96-well plate reader (BioTek Instruments Inc., U.S.A.). The linear equation: y = 0.5011x – 0.0014; R2 = 0.9962 was used to calculate the protein concentration.

7.2.2 Sample Pi standard curve

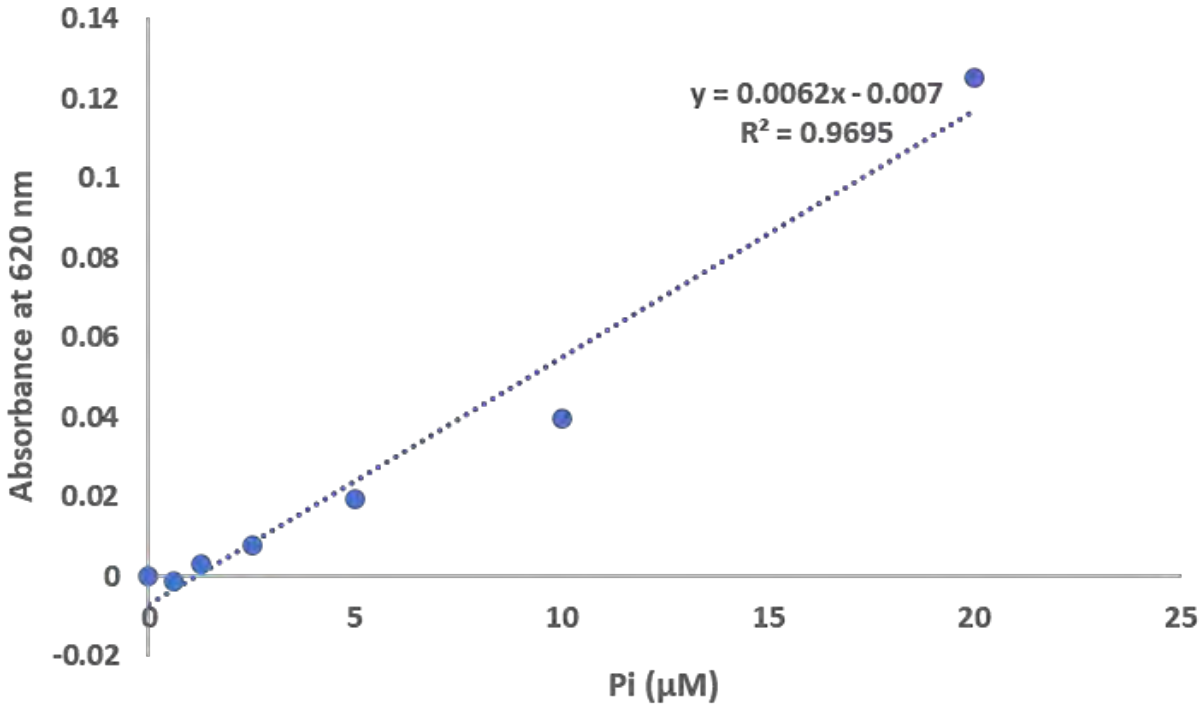
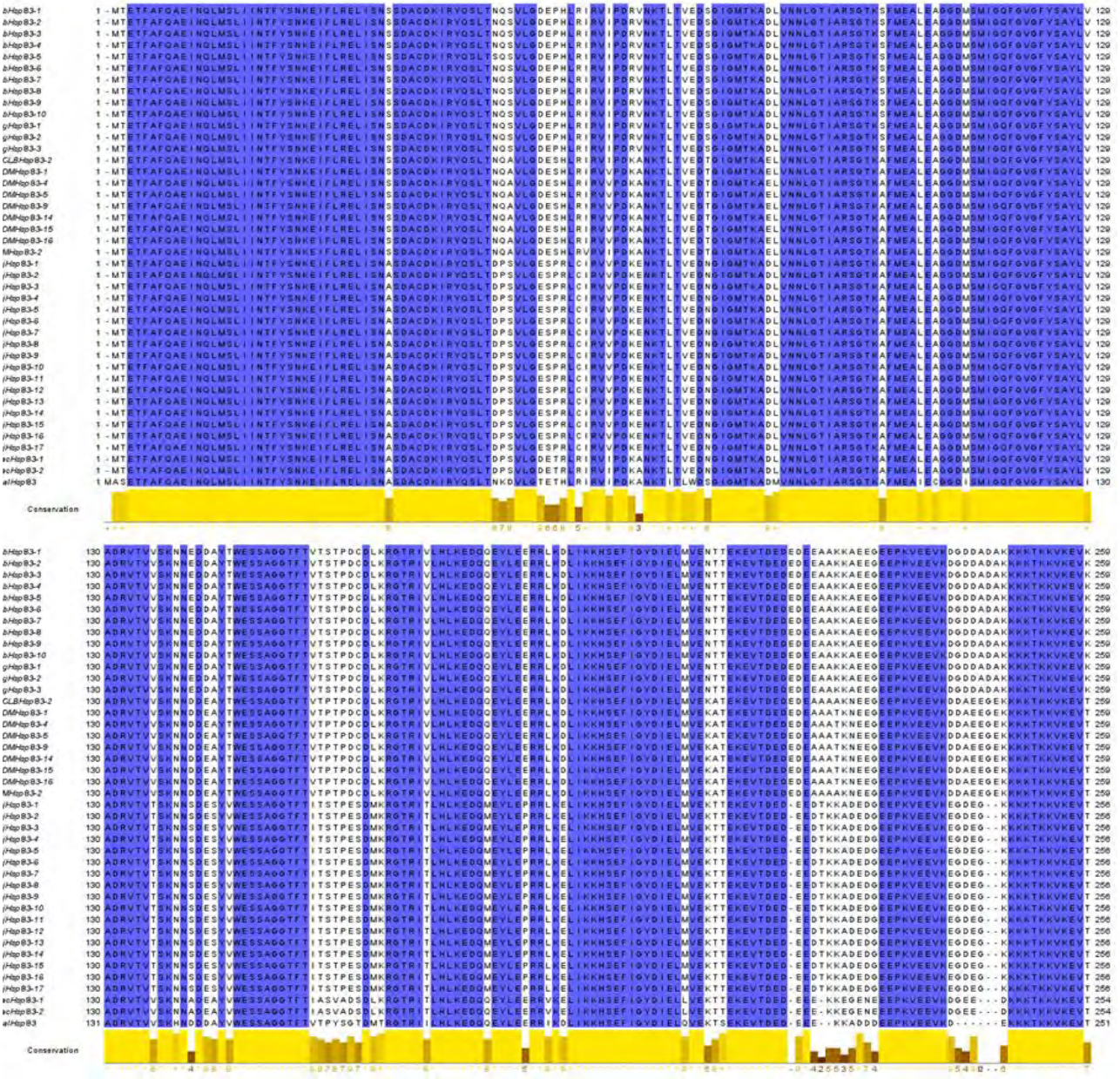
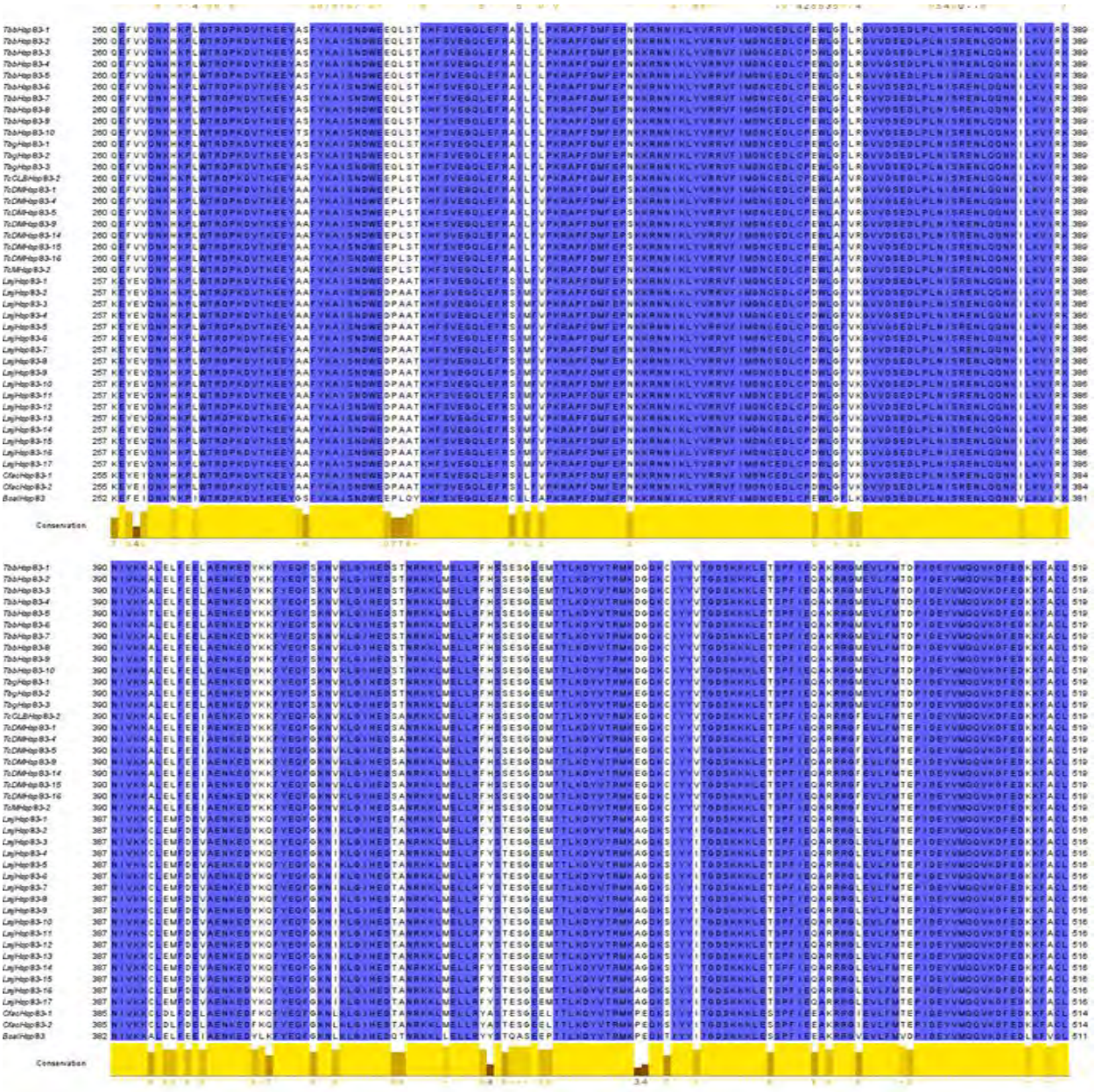


Figure 7.2: Pi standard curve for ATP hydrolysis analysis
Pi standards of concentrations ranging from 0 to 40 µM were prepared, and the absorbance was read at 620 nm using a Powerwave 96-well plate reader (BioTek Instruments Inc., U.S.A.). The linear equation: y = 0.0062x – 0.007; R2 = 0.9695 was used to calculate inorganic phosphate release during ATP hydrolysis.

7.2.3 Multiple sequence alignment for kinetoplastid cytosolic Hsp90s





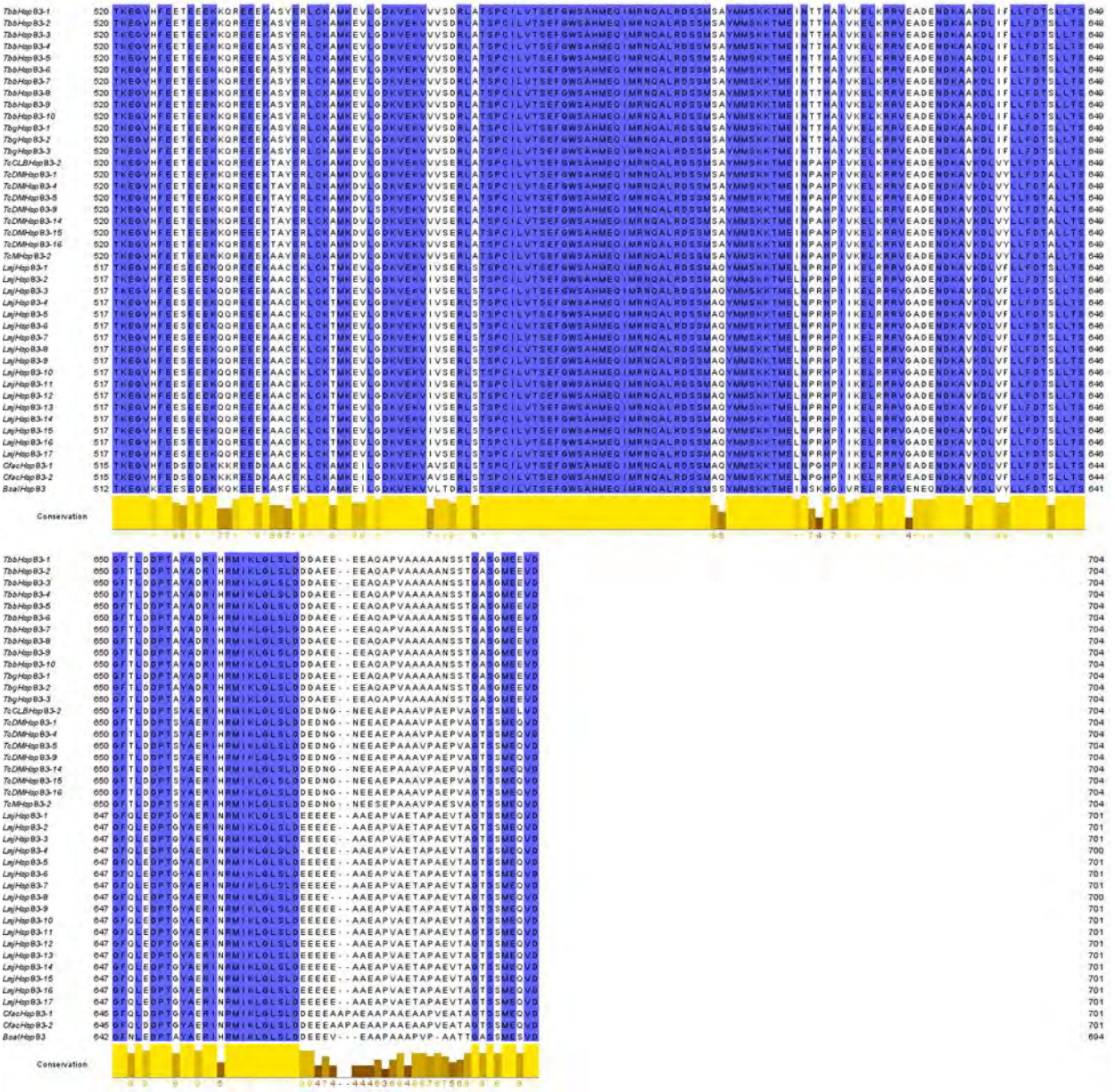
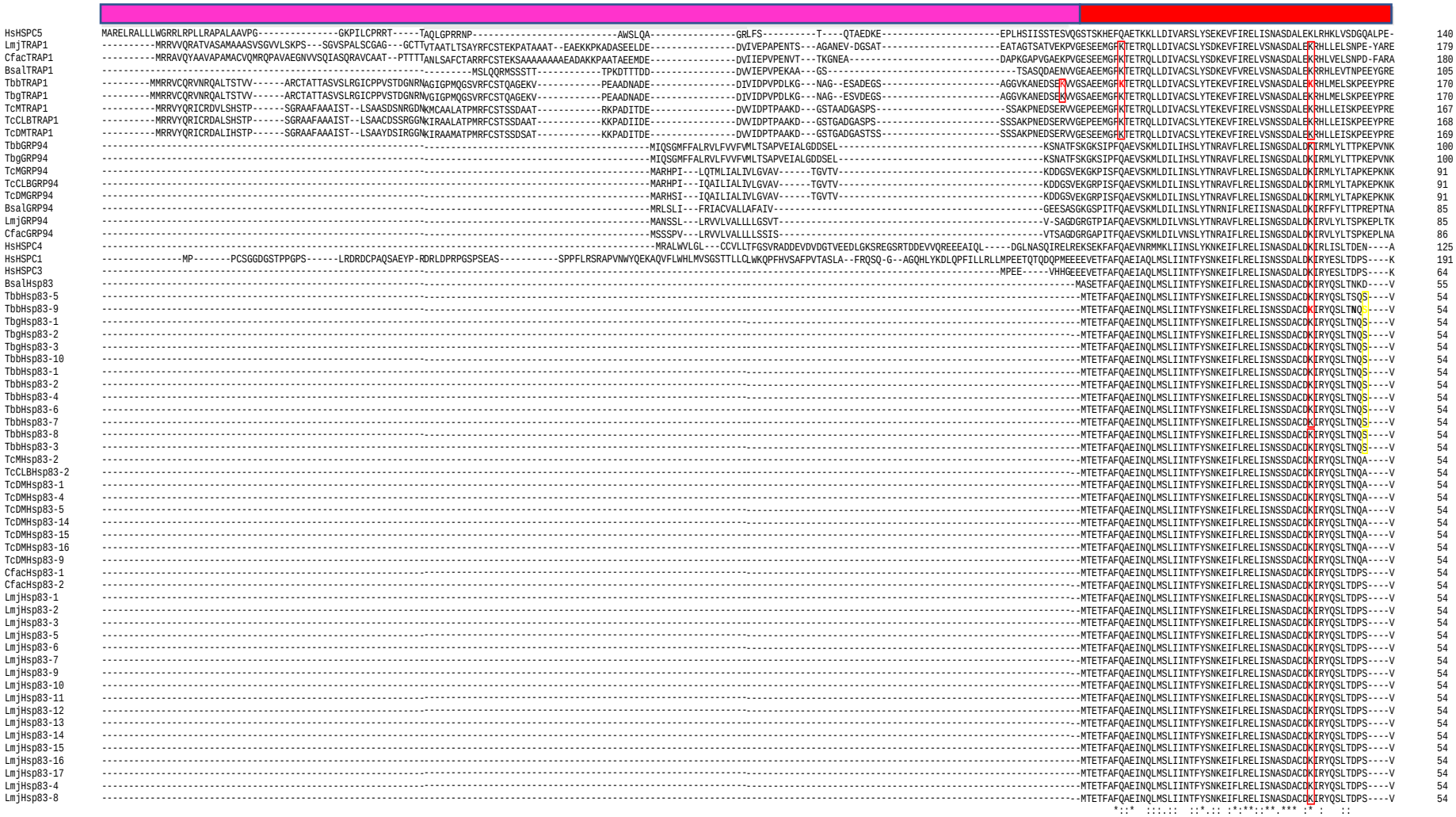
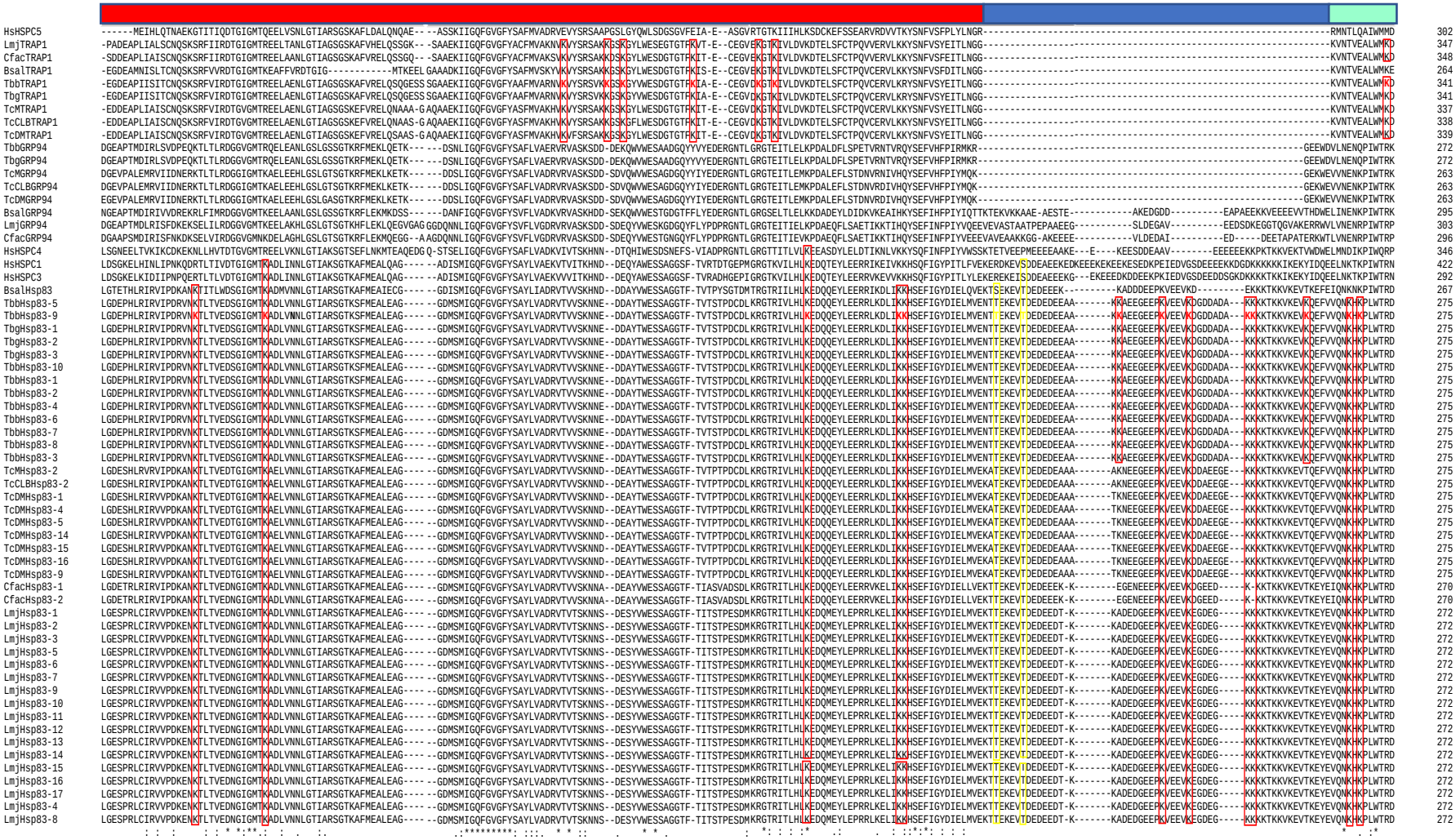
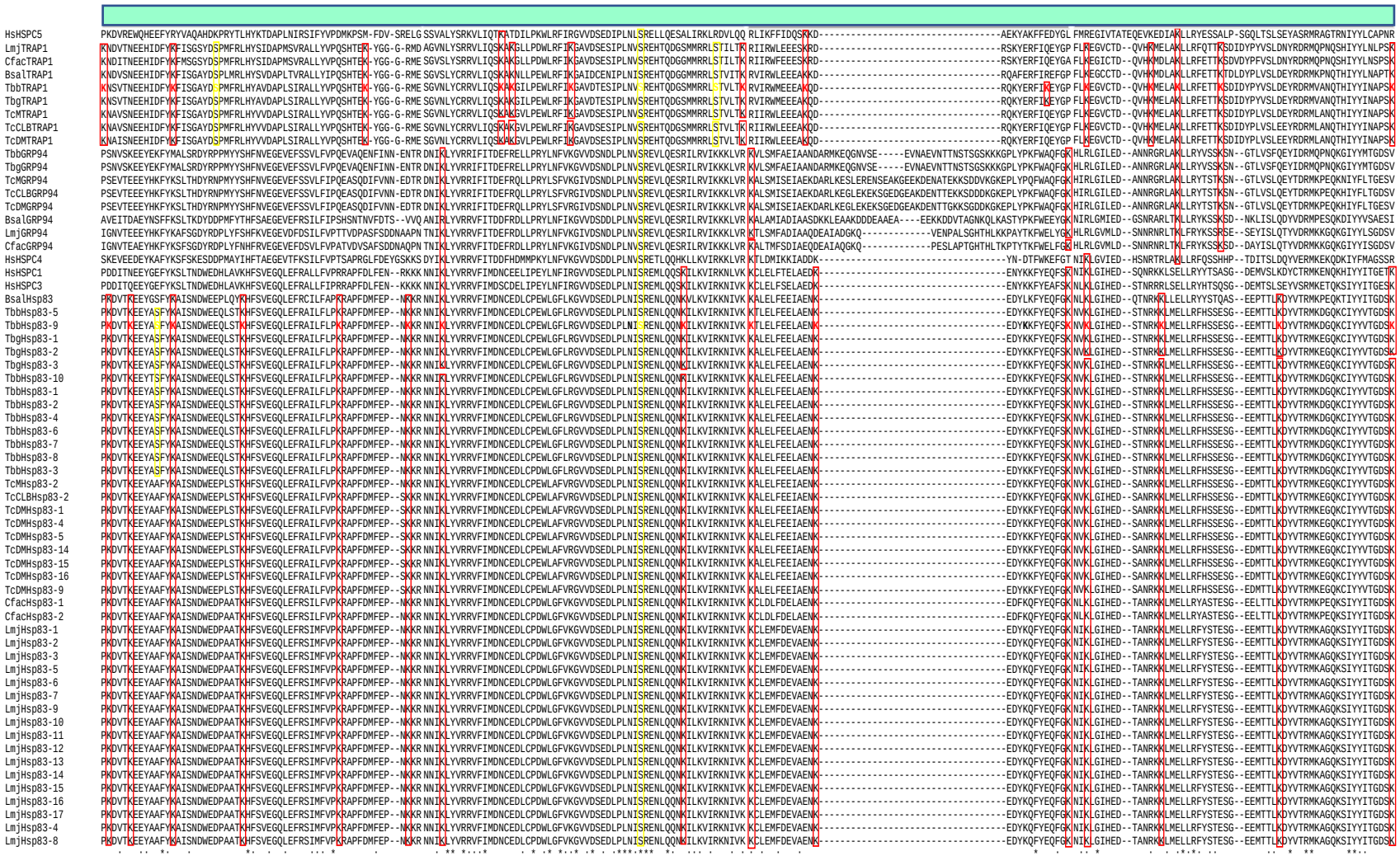


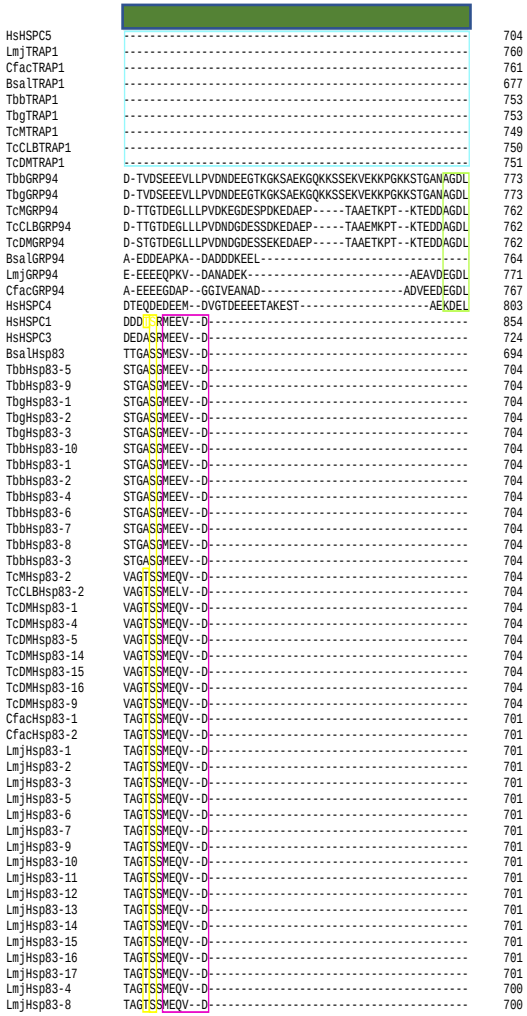
Figure 7.3: Multiple sequence alignment of all cytosolic Hsp90 in selected kinetoplastids.
Cytoplasmic Hsp90 proteins TbbHsp83, TbgHsp83, TcCLBHsp83, TcDMHsp83, LmjHsp83, CfacHsp83 and BsalHsp83 with accession numbers for all selected species in Table 2.2. Fully conserved residues are represented below with gold bars and an * below, as conservation reduces the gold shade darkens with reducing numbers from 9 to 1. For emphasis all fully (100%) conserved residues are highlighted in blue.

7.2.4 Post-translational modifications for cytosolic Hsp90s









Legend

SP

NBD

CLD

MD

CTD

C-terminal tetrapeptide (ER retrieval signal)

C-terminal motif

Acetylation site

Phosphorylation site

Figure 7.4: Post-translational modifications of cytosolic Hsp90

Multiple sequence alignment of the full-length amino acid sequences was performed using the in-built ClustalW program (Larkin et al., 2007) with default parameters in the MEGA X software (Kumar et al., 2018). Degree of amino acid conservation is symbolized by the following: (*) all fully conserved residues; (:) one of the residues is fully conserved and (.) residues are weakly conserved. The C-terminus motifs are empty-boxed in magenta for the cytosolic HSP90 and light blue for the mitochondrial TRAP-1. Residues involved in post translational modifications accordingly with MS PTM's proteomic studies by Nett et al, (2009b) and Zhang et al, (2020) Ooi et al, 2020 are coloured red for acetylation and yellow for phosphorylation. The red and yellow empty-boxed are highlighting conserved modified residues. Accession numbers for the Hsp90/HSPC amino acid sequences used in this study are provided in Table 2.2