Characterising the heat shock response in *Trypanosoma congolense*

Research Masters Thesis

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I, Marianne Aelmans, confirm that the work presented in this thesis is my own and has not been submitted in substantially the same form for the award of a higher degree elsewhere. Where information has been derived from other sources, I confirm this has been indicated in the thesis.

Signed……

Submitted in part fulfilment of the requirements for the degree of Research Masters

1 Abstract

Trypanosoma congolense causes significant economic burden across Sub-Saharan Africa, as it is the causative agent of Animal African Trypanosomiasis (AAT), a wasting disease affecting cattle which currently has no effective pharmaceutical treatment. *T. congolense* is a close relative of *Trypanosoma brucei*, they co-infect the same hosts so have been exposed to similar evolutionary selective pressures, and it is expected they will show similarities in host interactions. While *T. brucei* is a well-studied model organism, very little experimental work has been performed using *T. congolense* as tools have only recently been developed for genetic manipulation, but now is the time to use them to investigate the survival and infection mechanisms of the parasite. One of the major symptoms of AAT is a high fever which *T. congolense* responds to by eliciting the heat shock response, an important virulence factor which allows the parasite to survive in the host. The aim of this project is to characterise the *T. congolense* heat shock response, as understanding the mechanisms involved could pave the way for discovering novel drug targets in this parasite.

It was found that *T. congolense* displays a very different heat shock response to *T. brucei* at both 41 °C and 42 °C, with 10% more cell death but less severe lag in growth in the 24 hours after heat shock. DHH1 and ZC3H11, proteins shown to be involved in heat shock, were successfully fluorescently tagged in BSF cells for both species, and a distinct re-localisation of DHH1 into foci can be seen in *T. brucei* cells upon heat shock but not in *T. congolense*. Flow cytometry analysis of cells in the period after heat shock revealed that both species arrest in G2/M, 4 hours after heat shock for *T. brucei* and 6 hours for *T. congolense*, which may be linked to cell cycle phase differentiation. Overall, *T. congolense* may have a more different heat shock response that *T. brucei* than expected. This is some of the first work investigating specific pathways in *T. congolense* and many techniques were successfully competed for the first time in this laboratory.

Contents

2 Abbreviations

BSF – Bloodstream form

DHH1 – DExD/H-box ATP-dependent RNA helicase 1

LSM12 – SM like 12

PCF – Procyclic form

PABP – Poly-A binding protein

PBP1 – PABP1 binding protein

SCD6 – Suppressor of clathrin deficiency 6

XRNA – Exoribonuclease A

ZC3H11 – Zinc finger CCCH domain containing protein 11

2 Literature Review

2.1 Trypanosomiasis

2.1.1 Impact of HAT and AAT

Trypanosomiasis, also known as sleeping sickness, is a disease present across sub-Saharan Africa which puts many lives at risk. Human African trypanosomiasis (HAT) is caused by the parasites *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense,* and is fatal if left untreated. There are two stages of HAT, stage 1 occurs when parasites are present in the bloodstream of the host, symptoms include intermittent fever and headaches. Stage 2 occurs when the parasite crosses the blood brain barrier and enters the central nervous system, symptoms include a sleep disorder and it will eventually lead to death (Büscher *et al.*, 2017). The parasites can also infect other tissues such as the skin and adipose tissue, which can act as reservoirs of infection (Trindade *et al.*, 2016) (Capewell *et al.*, 2016). The two species of trypanosomes cause different disease, *T. b. rhodesiense* infection leading to a more acute disease that is often fatal within 6 months, and *T. b. gambiense* causes a chronic disease lasting approximately 3 years (Checchi *et al.*, 2008), but can also be asymptomatic for years before symptoms arise. The number of HAT cases have subsided in recent years due to improved drug treatments and increased monitoring of the disease; whilst it is present in 36 countries with 70 million people at risk (Kennedy, 2019), fewer than 3000 cases were reported in 2015 (Büscher *et al.*, 2017) and the WHO has targeted for its eradication by 2030 (Kennedy and Rodgers, 2019).

The more pressing issue now is animal African trypanosomiasis (AAT), a cattle disease that prevents the rearing of cattle across a large area of Sub-Saharan Africa, which impacts the economy and the livelihoods of people living within this region. *Trypanosoma congolense* is the main causative agent, and there has been little research on this species, which explains

the lack of treatment available for the disease. *T. congolense* is only found within the tsetse belt, an area of sub-Saharan Africa where its vector, the tsetse fly, is found. As *T. congolense* has no forms of mechanical transmission to spread without the tsetse fly vector, it is restricted to this area. *T. vivax* is another causative agent of AAT, it can undergo mechanical transmission as well as biological transmission so it is present across a larger region than *T. congolense* and can also be found in south Africa.

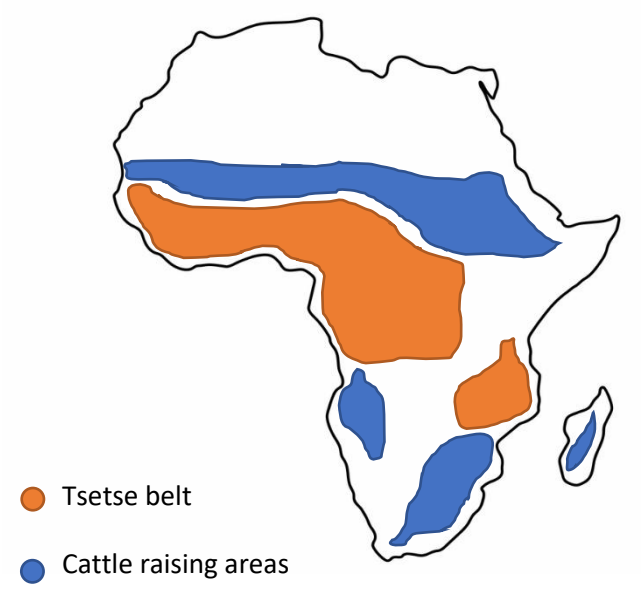

Figure 2.1 The tsetse belt. The region of sub-Saharan Africa where the tsetse fly, vector for *T. congolense*, is found. AAT caused by *T. congolense* is only present in this region which disrupts the cattle rearing industry, causing a significant economic burden.

Image adapted from Yahaya, 2014

AAT is a wasting disease with symptoms including pyrexia, anaemia, weight loss and abortions, it can be fatal if left untreated. How the disease presents in the host varies depending on the causative agent, even different strains of *T. congolense* cause different clinical symptoms, with the Savannah strain causing a more severe disease than the Forest or Killifi strain (Simarro *et*

al., 2012). Ultimately AAT leads to difficulty raising cattle across a large area. This is a financial drain on livestock keepers due to mortality, reduced calving rate, and meat and milk production, it can also cause economic losses for produce farmers because of reduced draft power (Holt *et al.*, 2016). This has been estimated to cause a production loss per cattle from 20% (Holt *et al.,* 2016) to as high as 43.5% (Shaw *et al.,* 2014), and it is estimated there will be a loss of $2.5 billion dollars across the African region over a 20 year period, largely in Ethiopia, Kenya and Uganda (Shaw *et al.*, 2014).

2.1.2 Combatting AAT and HAT

Little has been done in the way of combatting AAT, there are currently 6 drugs commercially available to treat livestock, but the parasites have developed resistance mechanisms against all of them. These drugs are diminazene, homidium bromide/chloride, isometamidium, quinapyramine sulfate/sulfate:chloride, suramin, and melarsomine (Suganuma *et al.*, 2022). The most commonly used trypanocidal drug used in livestock is diminazene, also known as diminazen, this is taken up by the TbAT1 transporter in *T. brucei ,* which has no orthologue in *T. congolense* (Delespaux *et al.*, 2008). The mechanism of how this drug works against *T. congolense* is poorly understood, but is being elucidated, and a recent study showed that it downregulates trypanosome induced cytokine production through disrupting pathways the parasite uses to phosphorylate MAPK and STAT proteins in immune cells (Kuriakose *et al.*, 2019). The resistance mechanisms against diminazene are also poorly understood, it was previously thought that reduced uptake was the cause of resistance, which is the case for many drugs targeting HAT, however it appears that resistant parasites show no changes in uptake, and resistance is linked to reduced mitochondrial membrane potential in resistant parasites, which is likely not the primary cause of resistance (Carruthers *et al.*, 2021).

There are drugs in development to target *T. congolense* and other agents that cause AAT. Preliminary data in mouse models shows that both β-ionine (Aminu *et al.*, 2022) and nitrofutanoin (Suganuma *et al.*, 2022) treatment could reduce parasitaemia and increase survival rates upon *T. congolense* infection. A barrier to getting drugs to the market is that compounds must be appropriate for an agricultural setting, they must be cost efficient and metabolites in the animal must be considered as many drugs are banned for agricultural use due to risks of possible carcinogenicity or genotoxicity when residual metabolites are found in animal products. Other drugs that have been tested in cattle include ethidium and nonindium, which have been shown to be protective to cattle herds in endemic areas (Latif *et al.,* 2019). These are all possible drugs and what is needed is an arsenal of drugs to combat AAT, as resistance is an increasing issue, overall there is a great need for novel drugs to combat AAT, and a better understanding of their mechanisms and the biological processes of *T. congolense.*

An effort has been made to target the tsetse fly vector, in 2000 the Pan African tsetse and trypanosome eradication campaign was established funded by the African development bank (Holt *et al.*, 2016). However, this does not reach many communities and it can be argued that a tailored approach is needed for each. New tools can aid in tsetse control such as insecticide treated screens, insecticide treated cattle, so far the flies have shown no resistance to insecticides (Büscher *et al.*, 2017). There must be integration of both tsetse fly control, and treatment of cattle to begin to control AAT in the tsetse belt.

On the other hand, drug efforts to combat HAT have been successful, as the causative agent in both diseases is so similar, this indicates that with more research, drugs could be developed to combat AAT. Cases of HAT have reduced from 300,000 infected individuals per year and many epidemic outbreaks in the 1990s (Barrett, 1999), to only 977 cases in 2018 (Franco *et al.*, 2020). This was achieved through a unified effort across many African countries initiated by the African heads of state. Active screening by both mobile teams and on health structures

meant cases were detected and treated, this was costly but produced results (Simarro *et al.*, 2014).

Five drugs are routinely used for treatment of HAT, pentamidine and suramin treat stage 1, and melarsoprol, eflornithine and nifurtimox treat stage 2. These are available from the WHO from donors, this accessibility and reduced cost helped reduce cases. Suramin and pentamidine can be used to treat *T. b. rhodesiense* and *T. b. gambiense* stage 1 disease respectively, both need to be injected with multiple doses and have some adverse effects, an ideal drug for stage 1 disease would be oral and only need 1 dose, which would make it easier to treat everyone. Melarsoprol is the first line treatment for *T. b. rhodesiense* stage 2 infection, but it is highly toxic and is fatal for 1 in 20 people that receive the treatment (Blum *et al.,* 2001). Trypanosome resistance to the drug developed in the 1970s and was widespread by the 1990s (Fairlamb and Horn, 2018). Nifurtimox-eflornithine combination therapy (NECT) is the current first line treatment for *T. b. gambiense* infections, which has a cure rate of 95-98% and is less toxic than melarsoprol with a fatality under 1%. Eflornithine muse be administered intravenously for 10 days while nifurtimox is an oral treatment (Priotto *et al.*, 2009). This treatment greatly aided in the reduction of cases we see today but can only be used to treat cases of *T. b. gambiense* stage 2 infection, melarsoprol is still used for *T. b. rhodesiense*. Also, though resistance to both these drugs can be selected for in the lab (Vincent *et al.*, 2010) (Sokolova *et al.*, 2010), suggesting resistance may emerge in the field and another treatment will be needed.

There are other drugs in the pipeline, such as Fexinidazole, which is an oral drug that can treat both stages 1 and 2 of HAT. It was found to be slightly less effective than NECT, at 86.9% efficacy (Kennedy, 2019), but has been approved and added to the arsenal of drugs used to combat disease and can treat *T. b. gambiense* infection. The mode of action for fexinidazole is not fully understood, but it involves interaction with the NTR1 protein, resistance in the lab

has been seen as trypanosomes lose the 3' UTR for this protein, reducing its expression and therefore activity. These trypanosomes can be selected for by increasing selective drug pressure and they showed 20-fold resistance (Slovoka *et al*., 2020) to the drug. This is the same protein that is involved in nifurtimox resistance, so it may be possible that use of both drugs may lead to an increased chance of resistance developing, through cross resistance. However, as cases are currently low, and it is not known if these parasites selected for in the lab would be viable in the field.

Acoziborole is a drug currently undergoing clinical trials, which is a single dose oral treatment for both stage 1 and 2 disease. In phase 1 trials it showed only mild side effects, it is now near the end of phase 2/3 trials and early indications suggest that the drug has good efficacy and safety profiles (Dickie *et al.*, 2020). The drug has been shown to be able to destabilise 92 trypanosome proteins, inhibit polypeptide translation and reduce endocytosis of haptoglobin-haemoglobin within *T. brucei* (Sharma *et al.*, 2022), and it was suggested the mode of action is through preventing protein synthesis leading to inability of the trypanosomes to proliferate. Acoziborole is a benzoxazole class drug, which are recently discovered as a form of trypanosomiasis treatment, some other derivatives may also be able to be used to treat HAT.

HAT is still an issue in some areas, particularly for rural communities. 86% of the cases in 2015 were in the democratic republic of Congo, 5% in the Central African Republic and 2% in Chad (Büscher *et al.*, 2017). Although cases have dramatically decreased, pressure still needs to be applied to prevent epidemics and to achieve eradication. Some barriers include a need for better diagnostic tools, integrating disease control into the health system, maintaining commitment of authorities as cases decrease, civil unrest and treating animal reservoirs for *T. b. rhodesiense* (Büscher *et al.*, 2017). An analysis of the current situation suggested that 10 countries are on track to eliminate infection by 2020 (Franco *et al.*, 2020), but these increased

efforts are still needed in other countries, this analysis also suggests that it is still possible to eliminate the transmission of *T. b. gambiense* by 2030.

2.2 *T. congolense* biology

2.2.1 Life cycles

The most well studied trypanosome is *T. brucei brucei*, this species is routinely used for lab work and genetic tools are effective and reliable. In recent years *T. congolense* culture and genetic tools have been studied in more detail, and routine procedures are being developed (Awuah-Mensah *et al.*, 2021). The life cycle of both species is well understood, and life cycle phases can be cultured in the lab, *T. congolense* is the only species where life cycle phase transitions can be induced by chemicals alone (Coustou *et al.*, 2010). The *T. brucei* life cycle is shown in figure 2.2. bloodstream form (BSF) cells are found in the hosts blood stream, they have a variant surface glycoprotein (VSG) coat that is important for immune evasion. There are waves of parasitaemia, through a system similar to quorum sensing in bacteria, stumpy induction factor (SIF), an oligonucleotide, induces transition to a 'stumpy' form in some cells (Rojas *et al.*, 2019), these cells appear to be more prepared for the transition from human to fly, they arrest in G0/G1 and have an elaborated mitochondrion and can differentiate into the next cell cycle phase when they sense a change in environment (Silvester *et al.,* 2018). When the fly takes a bloodmeal, these cells are taken into the gut, where they undergo differentiation into procyclic form (PCF) cells. These cells have procyclin proteins with either EP or GPEET repeat sequences. These cells multiply in the midgut of the fly and then migrate to the salivary glands, here they asymmetrically differentiate into epimastigote phase cells displaying brucei alanine rich protein (BARP). These cells are able to infect the mammalian host and enter into the bloodstream when the fly takes a bloodmeal (Fenn and Matthews, 2007).

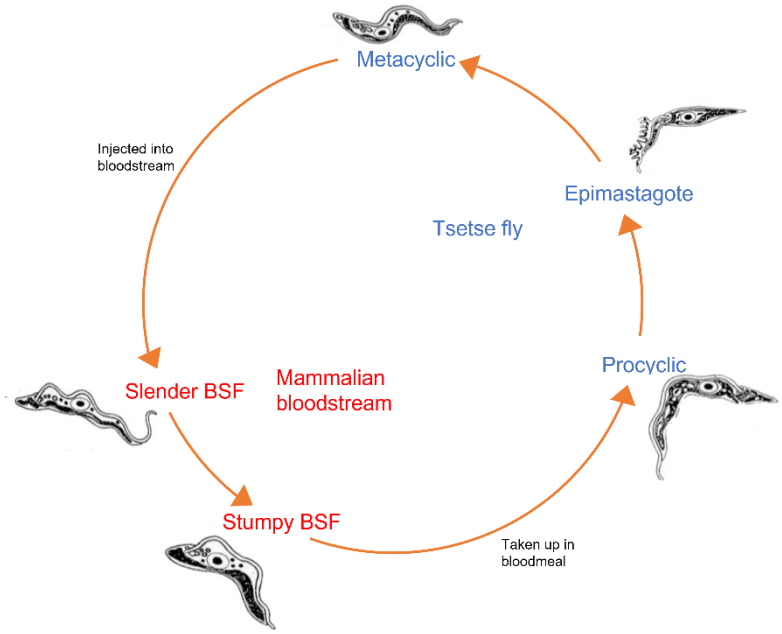

Figure 2.2 The life cycle of *T. brucei*. Metacyclic cells in the fly salivary glands are injected into the bloodstream of the mammalian host when the tsetse fly bites the host. Metacyclic cells differentiate into slender bloodstream form cells. Some of these cells then become stumpy bloodstream form cells which can adapt into procyclic form cells when taken up in the bloodmeal when a tsetse fly bites the host. Procyclic forms in the gut differentiates into Epimastagote which travel to the salivary glands where they differentiate into metacyclic cells.

Image adapted from Jones *et al.*, 2013.

The *T. congolense* life cycle is very similar to that of *T. brucei* , although the route through the tsetse fly differs. Both species initially establish an infection in the midgut of the fly, *T. congolense* then transition into metacyclic phase in the proboscis (foregut) of the fly (Peacock *et al.*, 2012), and then migrate to the salivary glands to be injected into the mammalian host. Another notable difference between these species is that *T. congolense* cells adhere to the vasculature in the host whereas *T. brucei* are free swimming (Banks, 1978). Also *T. brucei* cells invade tissues and cross the blood brain barrier whereas *T. congolense* is strictly intravascular. A major difference in the mammalian host is that *T. congolense* does not show a morphological

change to stumpy form, but the cell cycle does arrest in G0/G1 when the cells reach a high density, these arrested cells encode orthologues to proteins involved in the quorum sensing pathway in *T. brucei* and increase expression of 170 genes compared to cells in log phase. It appears that these arrested *T. congolense* cells may still promote infection or assist in cell survival during transmission into the fly (Silvester *et al*., 2018). These differences may suggest a difference in virulence methods and relationship with the host. Differences in cell biology could also lead to difference in uptake and response to drugs.

T. congolense also displays some different specific surface molecules throughout its life cycle. It has a novel GPI-anchored surface glycoprotein which is only expressed in the epimastigote phase. This was named congolense epimastigote-specific protein (CESP) (Sakurai *et al.,* 2008) and supernatant containing this protein is able to confer plastic adhesive qualities to PCF *T. congolense* parasites, so it may be involved in cells adherence to the proboscis in the teste fly. In the PCF life cycle stage *T. congolense* expresses glutamine and alanine rich protein (GARP) opposed to the BARP protein that *T. brucei* cells express, also the procyclins display a different repeat sequence of EPGENGT, the proteins were named *T. congolense* procyclins (Utz *et al.*, 2006). These are expressed in PCF cells from the midgut of the tsetse fly.

2.2.2 The trypanosome cell cycle

The cell cycle in trypanosomes, as with many aspects of their biology, differentiates from many other eukaryotic cells. They undergo G0, G1, S, G2 and M phases and the associated checkpoints, however they also have sub cell cycles for the kinetoplast and flagellum (Wheeler *et al.,* 2019). In trypanosomes, cytokinesis is separate from mitosis, and occurs afterwards, as opposed to overlapping as it does in many other eukaryotes (Hammarton, 2007). It has been shown that while the nuclear sub cycle is inhibited by knocking down cyclin 2 (CYC2), which is needed to trigger nuclear S phase, the flagellar and organelle sub

cycle still goes ahead, and cytokinesis even occurs, producing abnormal zoid cells (Hammarton *et al.*, 2003). This shows the sub cycles don't always have crosstalk between them, and even cytokinesis is able to occur without the correct nuclear division.

A G1 or G0 cell has 1 nucleus and 1 kinetoplast, so is 1N1K. The order of the cell cycles is that first the kinetoplast goes through S phase and divides into 2, leaving a 1N2K cell. The flagellum and Golgi replicates at a similar time to the kinetoplast (Wheeler *et al.,* 2019). The flagellum extends from the basal body and is attached to the cell body by the flagellar attachment zone, where it meets the cell is an invagination called the flagellar pocket. The flagellum and related cytoskeletal structures form their own division sub cycle. As the kinetoplast divides, the nucleus goes through S phase and then it divides after the kinetoplast, forming a 2K2N cell, after this cytokinesis occurs. The process of the cell cycle is outlined more detail in figure 2.3. Cell types other than those described, such as 2N1K, 2N0K and 2K0N do occur at low levels in a healthy population of cells, these phenotypes are the result of the cell cycles not occurring in the correct order in space and time and are usually unable to divide (Wheeler *et al.,* 2019).

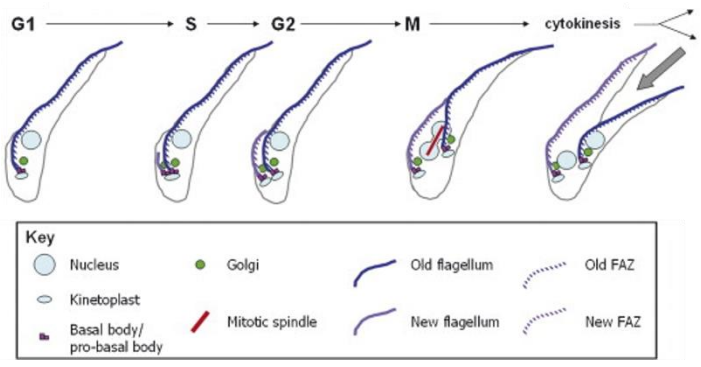

Figure 2.3 Trypanosome life cycle. Trypanosomes undergo G1, S, G2, M phases and cytokinesis. But they also must replicate the kinetoplast and flagellum, which occurs before nuclear division. Once the kinetoplast, flagellum and nucleus have all replicated, the cell will then undergo cytokinesis. FAZ is the flagellar attachment zone.

Image adapted from Hammerton, 2007

Trypanosomes have many proteins analogous to cyclins and cyclin dependant kinases (CDKs) however for many of these proteins function is yet to be elucidated. *T. brucei* displays the same checkpoints at S phase and mitosis and has proteins analogous to the typical metazoan/yeast pathway such as CYC2, 4, 5 and 7 for S phase and cyclin B like cyclins CYC6 and 8 and cyclin related kinase 3 (CRK3) and CRK9 for mitosis (Wheeler *et al.,* 2019). However the complexity and specific interactions are not known. Trypanosomes also don't appear to have orthologues of some checkpoint regulators such as Budding uninhibited by benzimadoles 1 (BUB1) and centromeric histone 3 (CenH3) (Berriman *et al.*, 2005), so have alternative mechanisms for regulating the cell cycle. It is thought that dephosphorylation must play a key role as okadaic acid, which inhibits protein phosphatases PP1, PP2A and PP2B, inhibits kinetoplast segregation on PCF *T. brucei* (Das *et al.*, 1994).

2.2.3 Biological differences between *T. brucei* and *T. congolense*

T. brucei and *T. congolense* have many similarities, including host range and the fact that they both cause AAT in domestic cattle. They are both African trypanosomes, and within this clade of trypanosomes they are evolutionarily similar, as shown in figure 2.3, despite this they display some important differences in their biology. Biological differences include genomic content and pathways for VSG substitution and antigenic variation (Jackson *et al.*, 2012a). Interactions with the host also differ, for example *T. congolense* cells actively adhere to mammalian endothelial cells (Hemphill, 1994), and virulence and transmissibility in both species differs even between isolates of the same species (Gitonga *et al.*, 2017). When inoculated into mice *T. brucei* forms distinct waves of parasitaemia and *T. congolense* forms a lower level endemic infection (Ndungu *et al.*, 2019).

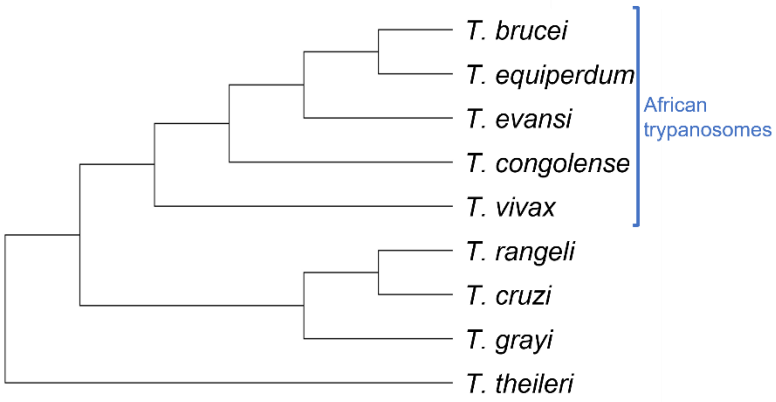

Figure 2.3 Phylogenetic tree showing the African trypanosome clade. Tree shows some species of trypanosomes founds across Africa and South America. Within the African trypanosome clade *T. brucei* and *T. congolense* are closely related, *T. equiperdum* and *T. evansi* are considered by some as subspecies of *T. brucei*. Phylogenetic tree was made using the sequences of the 18S ribosomal DNA from the SSU subunit if the ribosome. The sequences were aligned using ClustalW (Thompson *et al.,* 1994) and a neighbour joining tree was made using MEGA (Kumar *et al.,* 1994) based on the T93 + G model (Tamura and Nei, 1993).

A recent metabolomics study comparing the two species showed that *T. brucei* largely relies on the glycolytic pathway using glucose form the mammalian bloodstream, *T. congolense* consumes less glucose and excretes less pyruvate suggesting it uses other pathways for energy, it may have a more similar metabolome to PCF *T. brucei* cells (Steketee *et al.*, 2021). This means there may be differences in how each species interacts with the host or drugs, which highlights the importance of moving to working with *T. congolense* in the laboratory.

Advances in techniques used for culturing *T. congolense* means that all life cycle stages can now be cultured in the laboratory, and transition between life cycle stages can be chemically induced (Coustou *et al.*, 2010b), which is not possible in *T. brucei* . Recently, techniques for genetic manipulation have been perfected with efficient and reliable transfections and

somewhat reliable RNAi (Awuah-Mensah *et al.*, 2021). The IL3000 strain, which is most commonly cultured in laboratories, is cultured in goat serum at 34 °C (Awuah-Mensah *et al.,* 2021) while *T. brucei* is cultured in foetal bovine serum at 37 °C, these differences are interesting, as both species infect the same hosts, they should be able to be cultured in similar conditions. The differences may represent differences between the IL3000 strain that has been adapted for lab culture compared to the wild type parasite.

2.3 Polycistronic transcription and post transcriptional regulation

Trypanosomes are eukaryotes, however they are evolutionarily distant from many other 'typical eukaryotes'. They belong to the kinetoplastea class, which branched from other eukaryotes such as plants, mammals and yeast very early in eukaryotic evolution (Adl *et al.*, 2012). The defining feature of the kinetoplastids is that they have a circular network of DNA called the kinetoplast as well as the nucleus, which divides independently from the nucleus in the cell cycle, this may be before or after, depending on the species of kinetoplastid. One feature of trypanosomes that is unique is that they display polycistronic transcription, a diagram is shown in figure 2.4, this is more similar to the transcription seen in bacteria than of that seen in other eukaryotes. In trypanosomes genes are arranged in arrays, with open reading frames (ORFs) arranged head to tail with no introns. RNA polymerase 2 transcribes units of approximately 100kb at a time as one mRNA, which is trans-spliced and into individual mRNAs, and these are then capped and poly-adenylated (Clayton, 2019). Translation of each unit is stopped when 2 units converge or when a different polymerase is needed. This occurs simultaneously, so as the mRNA is still being transcribed, the other end is being spliced. This differs from other eukaryotes where each gene has a promoter, and they are transcribed as an individual mRNA.

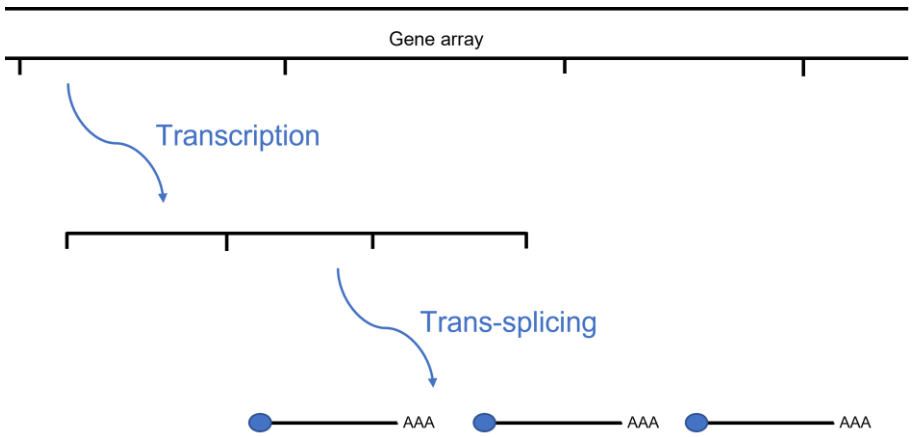

Figure 2.4 Polycistronic transcription in trypanosomes. Gene arrays containing multiple genes are transcribed as one mRNA which is then trans-spliced into individual mRNAs to then be capped and polyadenylated. This happens simultaneously, so as the array is being transcribed mRNAs are being capped and polyadenylated.

Polycistronic transcription has an impact on gene regulation. As individual genes cannot be up or downregulated at a transcriptional level by altering their promoter, and arrays contain functionally unrelated genes, most regulation must occur post transcriptionally (Clayton, 2019). Some of the only examples of transcriptional control are that abundant genes do have multiple copies across the genome so they are more often transcribed, SLRNA is a highly abundant protein which must be transcribed 140 times per hour (Haanstra *et al.*, 2008), compared to twice per hour for many protein coding genes. To achieve this SLRNA genes have nucleosomes phased over them and a specific initiation complex is needed for their transcription (Clayton, 2019). There is also a change in histone formation between life cycle stages, which may affect which genes are translated. In order to compensate for this lack of control at a transcriptional level, trypanosomes have unique aspects of mRNA processing, translation and decay.

Polypyrimidine tracts are common in the 3'UTRs of many genes, these can act as signals for many types of control which influence the mRNA such as subcellular localisation, alternative splicing and mRNA turnover (Rettig *et al.*, 2012). Aspects of translation itself also contribute to control of protein abundance, for example in most organism's codon composition and tRNA abundance affect speed of translation and therefore protein abundance (Hanson *et al*., 2017). There are many post transcriptional modifications which will affect the abundance of mRNAs, shown by the fact that many mRNAs are less abundant than would be expected by their half-lives. For example, the NOT complex deadenylates mRNAs which initiates their degradation (Fadda *et al.*, 2013), and trypanosomes have a novel decapping enzyme (Kramer, 2017).

Also mRNA binding proteins have major effects on the localisation, translation and decay of mRNAs, trypanosomes have over 100 regulatory binding proteins allowing them to fine tune post transcriptional mechanisms for controlling mRNA and protein abundance (Clayton, 2019). Some examples of these mRNA binding proteins include Poly-A binding proteins (PABPs) which bind to the poly-A tail of mRNAs and have been shown to shield the mRNA from degradation and increase translation efficiency (da Costa Lima *et al.*, 2010). Proteins containing ALBA domains affect translation efficiencies of reporter mRNAs and are abundant at high levels in the cell, a procyclic cell can contain 10,000 – 20,000 transcripts (Mani *et al.*, 2011). Pumilo family proteins are other RBP binding proteins, many have unknown functions but PUF9 has been shown to be involved in stabilising mRNAs in late G1 (Archer *et al.*, 2009). Trypanosomes also have 40 zinc finger proteins which are often involved in the differentiation of life cycle stages, for example ZFP1 is present in PCF cells and ZFP2 is present in BSF cells, but both are necessary for the transition between the life cycle stages (Clayton, 2013).

Another interesting family of RNA binding proteins in trypanosomes is the eIF4E family of initiation factors, these bind to the mRNA cap and *T. brucei* has 6 versions of this protein, while yeast cells only have one (Falk *et al*., 2021). It has been shown that the different proteins are

not redundant as they have different roles in the cell, for example eIF4E6 is necessary for BSF cells to survive, as they die when it is knocked out, while eIF4E5 is not necessary in BSF cells, but when it is knocked out these cells differentiate into procyclic that are unable to divide. When eIF4E2 is knocked out, BSF cells survive but grow at a slowed rate and do not display signalling associated with stumpy formation. Overall these proteins appear to be important for cellular differentiation and the increased number of eIF4E proteins is another way trypanosomes display post transcriptional regulation and protein control.

2.4. Heat shock response

2.4.1 Typical eukaryotic heat shock response

When cells are exposed to stress in the form of increased temperatures, they can be damaged as proteins will be misfolded and denatured, this can cause many issues in biological pathways within the cell and may be fatal. In order to survive in increased temperatures, all eukaryotic cells elicit the heat shock response, here the cell goes into survival mode and represses protein production of many proteins, often those involved in growth. Cells will only upregulate proteins needed for survival in high temperatures, like heat shock proteins, which are chaperones to aid protein folding.

The heat shock response in mammalian cells is relatively well understood. Transcription of housekeeping genes such as those involved in the cell cycle and metabolism is repressed, and only vital cells needed for cell survival are transcribed, this includes upregulation of heat shock proteins (HSPs). A translational arrest is triggered by the phosphorylation of eIF2a, which reduces the availability of the eIF2a-GTP-$tRNA^{Met}$ complex which is involved in initiation. This phosphorylation is also necessary for the formation of stress granules. These are granules that appear during stress responses that contain stalled translational pre-initiation complexes, it is thought they are sites of mRNA triage. mRNA move from polysomes to stress granules, where

they are organised, then they may be sent to P-bodies, which are sites of mRNA degradation that also form during stress responses. Both these granules contain 5' exoribonuclease 1 (XRN1), eukaryotic initiation factor 4E (eIF4E) and tristetraprolin (TTP) proteins, but they differ in composition of other proteins (Kedersha *et al.*, 2005).

In total 1,500 genes are upregulated and 8,000 genes are downregulated during the heat shock response in mammalian cells, much of this occurs at a transcriptional level, with translation factors such as heat shock factor 1 (HSF1) and serum response factor (SFR) altering the promoters of individual genes (Mahat *et al.*, 2016) to increase transcription by RNA polymerase II. This is not able to occur in trypanosomes due to their polycistronic nature, so it would be assumed that they have a novel mechanism for initiating the heat shock response.

2.4.2 Initial characterisation of *T. brucei* Heat Shock response

The heat shock response in BSF trypanosomes is an important virulence factor, it is triggered when the cells are exposed to increased temperatures from the fever elicited by the mammalian host immune system and allows the parasite to survive in the bloodstream in these periods, without it they would not be able to survive in the mammalian host. While no work has been carried out on the *T. congolense* heat shock response, the *T. brucei* response has been investigated.

The heat shock response in PCF *T. brucei* was first characterised as a reduction of up to 50% of mRNA transcripts in the cell, with HSP75 and HSP80 remaining steady, this is seen when cells are heated to 41 °C for 1 hour (Muhich *et al.,* 1989). It was later showed, through ^{35}S methionine tagging, that the increase in expression in HSP75 and HSP80 remains until 5 hours after heat shock, and while most mRNAs return to normal rates of expression some are also lowered for up to 5 hours after heat shock, such as tubulin mRNA and other mRNAs that could not be identified (Kramer *et al.*, 2008). There is also a reduction in polysomes 15-30 minutes

into heat shock treatment, this along with reduction of mRNA suggests a growth arrest, where unnecessary pathways are halted and only heat shock proteins and other proteins necessary for the cells survival through increased temperatures are translated and remain active in the cytoplasm. Due to the polycistronic nature of trypanosome transcription, the increase in heat shock related proteins and decrease in total mRNA must occur at a post transcriptional level, through mechanisms such as stabilisation, changes in rates of translation or increased degradation.

Most of the work done on the heat shock response in *T. brucei* has used PCF cells because of convenience and ease of manipulation. The vector of the PCF cells, the tsetse fly, is pliothermic, so maintains its temperature by moving into shade, because of this the internal body temperature in the fly can fluctuate from 20 – 43 °C (Edney and Barrass, 1962). It is possible the heat shock response may differ between PCF and BSF cells, as they have different transcriptomes and experience different temperatures within the respective hosts, the two life stage forms are cultured at different temperatures.

To investigate the mechanisms of post transcriptional modification in these cells and how the reduction in mRNA was achieved, sinefugin treatment was used which inhibits mRNA synthesis by blocking trans splicing. When sinefugin cells are heat shocked, there is a larger decrease in mRNA levels compared to non-heat shocked cells (Kramer *et al.,* 2008), which indicates that there is increased mRNA turnover. It was also shown that cells treated with sinefugin show a more rapid decrease in mRNA levels than those treated with heat shock and no sinefugin, which indicates there is still some mRNA synthesis occurring after heat shock. Overall this would suggest that there are different methods of regulation for different mRNAs, and the pathways involved in regulation during heat shock are complex.

mRNAs of 371 genes are not affected by heat shock, including HSP100, E2 ubiquitin ligases, proteasome subunits and DNA-j domain containing chaperones (Kramer *et al.,* 2008). The

genes that are downregulated are mostly involved in core metabolic functions such as translation initiation factors and tRNA synthesises, also genes involved in cell growth, such as alpha and beta tubulin were downregulated. This is consistent with a growth arrest in the cell where only basic survival mechanisms are maintained.

When *T. brucei* cells are subjected to a heat shock treatment of 41 °C for 1 hour, very few cells die but they experience a lag in growth which returns to normal after 24 hours (Kramer *et al*., 2008). Figure 2.5. shows growth after heat shock at 41 °C in PCF cells vs BSF cells, though it should be noted that (a) shows a change in cell density while (b) shows a change in motility. PCF cells heat shocked for 60 minutes show a lag in growth and at 24 hours there is a significant reduction in cell density, as untreated cells grow to 1×10^7 while cells heat shocked for 1 hour grow to approximately 5×10^6. Cells heat shocked for 90 minutes and 120 minutes shoe an even more significant reduction in growth but appear to return to normal rates by 30 hours. For BSF cells there is a large reduction in growth up to 24 hours after heat shock, as cells only grew to approximately 4×10^6 24 hours after heat shocked compared to 1×10^7. After 24 hours cell growth appears to return to normal. Cells heat shocked for 2 hours completely crash for 48 hours but are then able to recover.

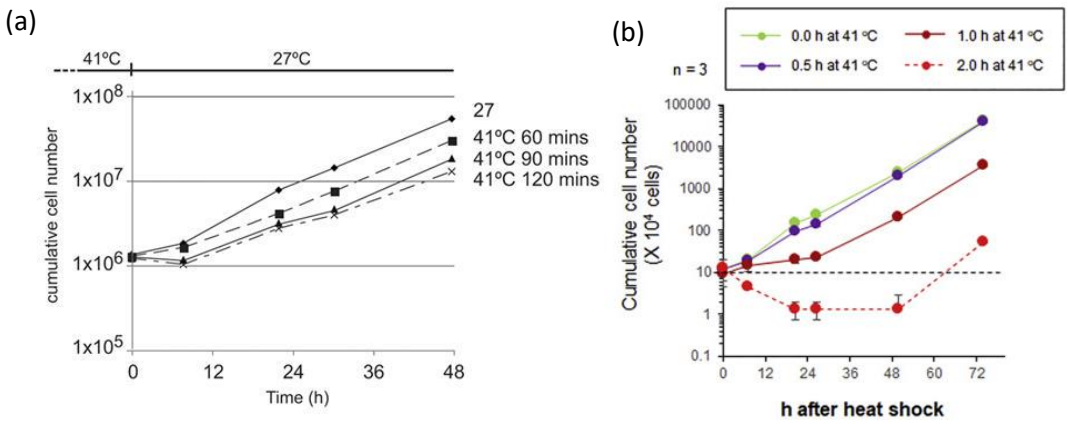

Figure 2.5 Growth arrest during heat shock in (a) PCF trypanosomes and (b) BSF trypanosomes. *T. brucei* cells heat shocked at 41 °C show a delay in growth for at least 24 hours after heat shock.

Images from Kramer *et al.,* 2013 and Ooi *et al.,* 2020.

This lag in growth indicates that cells have been subjected to stress and have had to recover, so it is agreed that cells are initiating the heat shock response. However, 41 °C is physiologically high, cattle can reach up to 42 °C during fever (Bianca, 1963), as well as other animal such as gazelles (Hetem *et al.*, 2012), so while the typical fever induced during AAT is round 36 – 40 °C (Minia and Clayton, 2016), 41 °C is not unreasonable. However, experiments are performed using these conditions for reproducibility and experimental tractability. It is expected that the response initiated by the cells will be the same, even if in the physiological scenario it might be triggered by a lower temperature for longer periods of time. Experiments should be carried out investigating lower temperatures and longer periods of time to ensure that the response being studied is of physiological relevance.

2.4.3 Formation of stress granules in heat shock

Stress granules that form during the heat shock have been visualised in PCF *T. brucei* cells after heat shock treatment and remain for up to 5 hours after treatment (Kramer *et al*., 2008). It is

thought that two types of granules form in the response – heat shock granules and processing bodies (P-bodies). Heat shock granules develop which contain PABP1 and components of the initiation complex, eIF2, eIF3B, eIF4E3 and eIF4E4, and processing bodies increase, these are seen in the cytoplasm in cells that have not been exposed to stress, but increase number when cells are exposed to stress, they contain DExD/H-box ATP-dependent RNA helicase 1 (DHH1), Suppressor of clathrin deficiency 6 (SCD6) and exoribonuclease A (XRNA). Images of these granules are shown in figure 2.6. There is also a novel granule that appeared at the posterior of the cell, containing only XRNA. This is a degradative enzyme, so it is possible these are sites of mRNA degradation. These heat shock granules have been visualised in PCF cells but have not been investigated in BSF cells, further investigation is needed to compare the heat shock response between PCF and BSF cells.

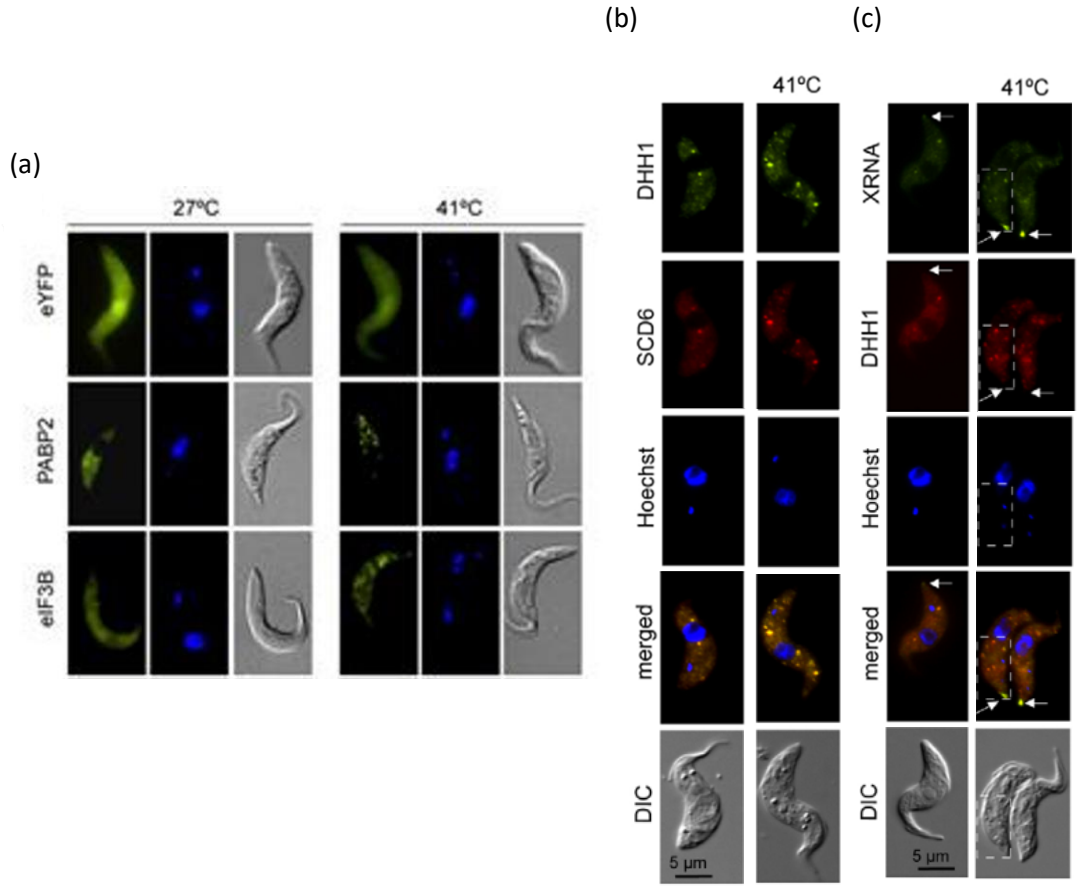

Figure 2.6 (a) Heat shock granules and (b) and (c) P-bodies PCF cells. After 1 hour of heat shock at 41°C, proteins in PCF cells re-localise into heat shock granules and p-bodes. Cells in (a) are PCF *T. brucei* cells expressing eYFP-PABP2 and eYFP-eIF3B (separate cell lines). PABP can be used as a marker for heat shock granules, a type of stress granule that appears after heat shock, other proteins like PABP1 and eIF proteins also re-localise into these granules. Cells in (b) are PCF T. brucei cells expressing both eYFP-DHH1 and SCD6-mChFP and cells in (c) are expressing both mCHFP-DHH1 and XRNA-eYFP. P-bodies are present before heat shock, but the number in each cells increase upon heat shock, DHH1, SCD6 and XRNA are found in these granules, and XRNA also forms a separate novel granule towards the posterior of the cell, shown by arrows.

Images adapted from Kramer *et al*., 2008

The function of these granules is yet to be elucidated. P-bodies are involved in many stress responses, and contain proteins involved in exonucleolytic mRNA degradation, components of the nonsense mediated decay pathway and miRNA associated factors, which would suggest they have a role in mRNA degradation. A later study found that the 2 PABP binding proteins, PABP1 and PABP2 did not localise together during the heat shock response. This study found that PABP1 co-localised with proteins present in heat shock granules and PABP2 co-localised with DHH1 into P-bodies (Kramer *et al.,* 2013). They suggest that P-bodies may be involved in mRNA degradation while heat shock granules are involved in mRNA storage, and PABP proteins binding to mRNAs determine their fate. This study only showed the localisation of PABP proteins and interaction studies were not performed, so theories are only speculative. Trypanosomes and other kinetoplastid have more paralogues of PABP and eIF proteins than yeast and other eukaryotes, while the functions have not yet been determined, it does appear that each have non-redundant roles which may be involved in the additional need for post transcriptional regulation in these cells.

Kramer *et al*., 2008 also proved that the *T. brucei* heat shock response pathway is independent of eIF2a phosphorylation, when the homologous mutation site in *T. brucei* is mutated to a non-phosphorylation site, the heat shock response continues as normal, and cells are able to survive increased temperatures. This proved that while the events seen in heat shock in trypanosomes and typical eukaryotic cells are the same, trypanosomes have a novel pathway for triggering these events.

2.4.4 ZC3H11 is a key regulator of the heat shock response in *T. brucei*

While the response was being characterised the question still remained as to how it is regulated and what pathways are involved. Zinc finger CCCH domain containing protein 11 (ZC3H11), a zinc finger RNA binding protein was shown to be a key regulator of the heat shock

response. Western blotting shows that ZC3H11 is increased during heat shock in both PCF and BSF cells (Droll *et al.*, 2013) and the banding pattern changes upon heat shock. This was shown to be due to phosphorylation of the protein, as the higher band disappears when treated with a phosphatase and can be rescues with phosphatase inhibitors. It is also increased in other treatments that cause stress for the cells, such as puromycin treatment and MG132 treatment. ZC3H11 is expressed at very low levels in none heat shocked cells, which can make analysis difficult, but it can be visualised using TAP which shows it has a cytoplasmic localisation.

RNAi of ZC3H11 shows that it is vital for the heat shock response to be successful. RNAi in BSF cells is fatal which is unfortunate as this means experiments cannot be performed with them. However ZC3H11 RNAi is not fatal in PCF cells, cells grow at a normal rate but then are unable to survive heat shock. There is also a reduction in 72 mRNAs including HSP70, suggesting that ZC3H11 is involved in their stabilisation. Proteins HSP70, HSP83, HSP110, KKBP and HSP 40 no longer persist after heat shock in ZC3H11 RNAi cells, also HSP100 is not induced (Droll *et al*., 2013). A microarray of mRNAs showed that after heat shock in normal PCF cells shows many mRNAs are increased during the heat shock response, comparing with a microarray of ZC3H11 RNAi cells after heat shock showed that 27% of these mRNAs required ZC3H11 in order to be upregulated. This shows that ZC3H11 plays a big role in the stabilisation of mRNAs during heat shock, but that there are also other mechanisms involved.

Myc-tagging showed that ZC3H11 binds to many mRNAs for proteins involved in stress responses, these include 13 proteins that are chaperones for protein refolding and all cytosolic HSPs (Droll *et al.,* 2013). It also binds to some proteins not involved in the stress response such as GPEET. ZC3H11 has been shown to bind to mRNAs via a UAU x4 repeat motif in the 3' UTR and is able to actively stabilise them, which differs from most eukaryotic organisms where an AU rich sequence in the 3' UTR is usually involved in destabilising an mRNA. The mechanism of action of ZC3H11 has been described (Singh *et al.*, 2014), it recruits a complex of MKT1, SM

like 12 (LSM12) LSM12 and PABP1 binding protein 1 (PBP1), which all have homologues in yeast. The suggested model for this complex and how it stabilises mRNAs is shown in figure 2.7. This paper speculated that MKT1 binding to mRNAs may select them for binding to PABP2 and translation using eIF4E1, which may be the case in *Leishmania* where eIF4E1 appears to be involved in translation at higher temperatures (Zinoviev *et al.*, 2011). MKT1 binds to proteins via a (H/N)(D/E/N/Q)PY motif, when this is mutated the interaction between MKT1 and its partners is abolished (Singh *et al*., 2014). Binding partners of MKT1 were identified in a yeast 2 hybrid system screen, while the PBP1 and MKT1 homologues in yeast were tester for, it is possible that other yeast proteins may have affected the interactions seen, so results need further confirmation.

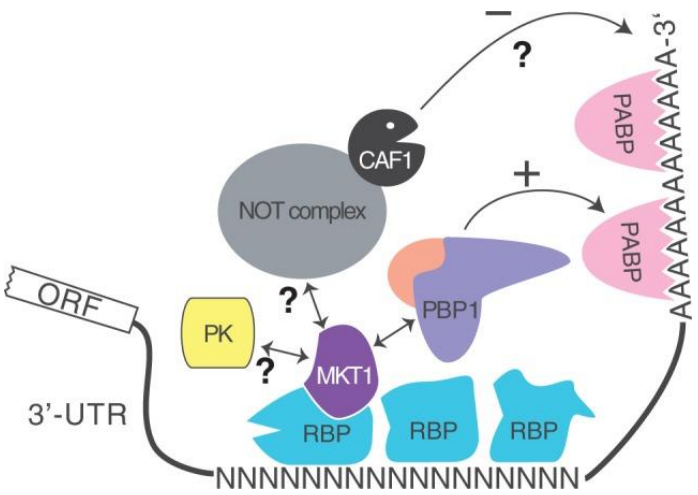

Figure 2.7 Proposed mechanism for ZC3H11 stabilisation of mRNAs. ZC3H11 is an RNA binding protein which recruits MKT1 and PBP1, which then in turn recruit PABP to the mRNA. PABP binds to the poly-A tail. There may then be further interactions involving the chromatin assembly factor 1 (CAF1) and negative on TATA 1 (NOT1) deadnylation complex, various protein kinases and other proteins which stabilise the mRNA.

Image from Singh *et al.,* 2014

The next step is looking into the regulation of ZC3H11, to try and find out how the protein is rapidly increased during the heat shock response. There is no change in ZC3H11 mRNA levels after heat shock, so the increase in protein must be controlled by post transcriptional mechanisms. It is known that treatment with the proteasome inhibitor MG132 increases the abundance of ZC3H11, which suggests it could be degraded in stable cells, however this could also be due to proteasome inhibition causing a stress response in the cell, which in turn increases ZC3H11 abundance. More work would be needed to determine if ZC3H11 is normally degraded by the proteasome, such as finding out if it is ubiquitinylated.

It is likely that ZC3H11 is regulated by a 2-fold system, as the mRNA moves into polysomes during heat shock which would increase translation. There is also stabilisation of the protein, one enzyme that is likely involved is CK1.2. When CK1.2 is depleted using RNAi, it appears that ZC3H11 can no longer be phosphorylated. CK1.2 is inactivated at 41 °C which presents the idea that when cells experience heat shock, CK1.2 is inactivated and its inhibitory phosphorylation on ZC3H11 is removed. The phosphorylation sites on ZC3H11 have been identified as S275 which shows a 16-fold increase during the heat shock response, and S23, S26 and S279 which show increase in phosphorylation to a lesser extent (Ooi *et al.,* 2020). However none of these phosphorylation sites are within the CK1.2 recognition motifs, though it is possible it works through an indirect mechanism.

2.4.5 Dynamic changes in phosphorylation during heat shock

The pathways and sequence of events of the heat shock response have begun to be elucidated with a phosphoproteomic analysis of heat shocked BSF *T. brucei* cells after 1 hour at 41 °C (Ooi *et al.,* 2020). There are 193 heat shock responsive phosphorylation sites and only 20 proteins that change in abundance at this timepoint, which is to be expected due to the need for more post transcriptional changes for a fast response in *T. brucei* . Many of the expected proteins

and their phosphorylation sites were identified such as DHH1 showing an increase of 140-fold at 2 phosphorylation sites, PABP showing an 8 to 16-fold change across its phosphorylation sites and HSP100 showing a 1.2-fold increase in protein abundance, more changes are outlined in table 2.1. However there are some unexpected results such as PABP1 showing no change in phosphorylation, and HSPs 110, 84 and 83 showing no change in abundance or phosphorylation, these HSPs are integral to the typical eukaryotic response, it is possible that changes occur at later timepoints.

Table 2.1 Selected proteins that show increases in phosphorylation heat shock response.

Data from Ooi *et al*., 2020

Protein	Protein ID	Phosphorylation site	Fold change
DHH1	Tb927.10.3990	S84	140
		T82	126
		T22	5
ZC3H11	Tb927.5.810	S275	16
CK1.2	Tb927.5.800	S21	3.5
		S19	2.8
PABP2	Tb927.5.800	S93	16
		T167	8.2
		S212	8.8
		S259	8.1
HSP70	Tb927.11.11330	S464	4.5
		S89	2.5
HSP83	Tb11.v5.0543	S48	3.7

The data in table 2.1 is from immediately after the cells are exposed to heat shock of 41 °C for 1 hour, to gain a better insight into the sequence of events and signalling pathways of the heat shock response other timepoints should be investigated. It is known that the cells still show effects of heat shock up to 5 hours after, such as persistence of heat shock granules and decreased tubulin mRNA abundance (Kramer *et al*., 2008). Most experiments studying heat shock only investigate cells immediately after exposure, but it would be interesting to see what is causing these effects and how the cells respond after heat shock.

2.5 Aims of project

This project will aim to characterise the heat shock in *T. congolense*, using what is known about the mechanisms in the *T. brucei* heat shock response. As these 2 species are evolutionary similar and have the same host range, it is thought their mechanisms in responding to the environment in the host could be conserved. The heat shock response represents an important virulence factor of the trypanosome parasites, as it allows them to survive in the bloodstream of the mammalian host, so in the long-term studying this pathway could lead to the discovery of novel drug targets to alleviate the burden of AAT on people living in across central Africa.

The first aim is to find a temperature and timepoint to study *T. congolense* heat shock in the lab setting by exposing them to different lengths and temperatures of heat shock using a water bath and studying cell death and growth after these treatments. *T. brucei* shows a response to heat shock at 41 °C for 1 hour, and it is expected this will be the same for *T. congolense* as they infect the same mammalian hosts. However the strain of *T. congolense* available for lab culture is incubated at 34 °C, compared to *T. brucei* which is incubated at 37 °C , it is possible this will affect the temperatures that *T. congolense* respond to heat shock.

The arrest after heat shock will also be investigated in both *T. brucei* and *T. congolense* BSF cells. Flow cytometry will be used to visualise the cell cycle stages of the population of cells during the growth arrest, to see if the cell cycle of cells is affected by heat shock and at which point.

Secondly, genetic manipulation will be used to study the changes in localisation and abundance in proteins that have been associated in heat shock. The proteins DHH1, ZC3H11, XRNA, HSP100 and PABP1 will be fluorescently tagged in both *T. brucei* and *T. congolense* BSF cells. Cells will be exposed to heat shock and examined with fluorescence microscopy and flow cytometry to see if P-bodies and heat shock granules form in *T. congolense* cells.

3 Materials and Methods

3.1 Materials

3.1.1 Cell lines

The *T. brucei* 2T1 Lister 427 (Alsford *et al.,* 2005) and *T. congolense* TcoSM IL3000 (Awuah-Mensah *et al.,* 2021), kindly provided by the Wickstead lab in Nottingham, were used for all experiments.

3.1.2 Plasmids

Plasmids pEnNY0, pEnNmSt0-N and pSIS-HHsfG (Wickstead *et al.*, 2010) were kindly provided by the Wickstead lab in Nottingham. These were used for N-terminal and C-terminal tagging of proteins in both *T. brucei* and *T. congolense*.

3.2 *T. brucei* medium

T. brucei 2T1 BSF cells were cultured at 37 °C and 5% CO_2 with 0.5 µg/ml phleomycin (Melford) and 0.2 µg/ml puromycin (Roche). Cells were maintained in logarithmic growth phase and cultured in in 5 - 10 ml of HMTI-11 media, a modified HMI-9 media (Hirumi and Hirumi, 1989).

1 pack of HMI-9 powder (Invitrogen) was combined with 10 g of sodium bicarbonate (Sigma) and 4.5 l ddH_2O, then pH was adjusted to 7.3. This was then filtered through a 0.2 µm filter under sterile conditions and stored in 450 ml aliqiots at 4 °C. Prior to use 50 ml foetal bovine serum (Labtech) and 5 ml L-glutamine (Biosera) were added under sterile conditions.

3.3 *T. congolense* medium

T. congolense TcoSM BSF cells were cultured at 34 °C at 5% CO_2 with 0.25 µg/ml puromycin. Cells were maintained in logarithmic growth phase. Cells were grown in TcoBSF-1T media (Coustou *et al.*, 2010). To make the media, components in table 3.1 were combined, and pH was adjusted to 7.3. Then in the goat serum (Gibco) and glutamine were added under sterile conditions and the media was filtered through a 0.2 µm filtration unit.

Table 3.1 Components of TcoBSF-1T media

Component	**Amount in 500 ml media**
MEM powder (Sigma)	4.8 g
Sodium bicarbonate (Sigma)	1.1 g
HEPES (Melford)	2.98 g
Glucose (Duschefa)	0.55 g
Sodium pyruvate (Sigma)	55 mg
Adenosine (Sigma)	5.3 mg
Hypoxanthine (100mM)	0.5 ml
Thymidine (Sigma)	2.42 mg
Barthocuproinedisuplonic acid (Sigma)	5.65 mg
1-Thioglycerol (neat) (Sigma)	7 µl
ddH_2O	425 ml
Goat serum (Gibco)	75 ml
L-Glutamine (200mM) (Biosera)	5 ml

3.4 Heat shock protocols

10 - 20 ml cells at a density of 5×10^5 cells/ml were placed in falcon tubes in a Clifton NE4 recirculating water bath at the specified temperature and time. Cell density was determined by counting on a haemocytometer and temperature was recorded using an RS digital thermometer.

3.5 Flow cytometry

For flow cytometry samples of fixed cells were made using 1×10^6 cells fixed in 70% methanol, for *T. congolense*, cells were first fixed in 50% methanol, and then increased to 70% after 1 hour to avoid cells bursting. Samples were fixed overnight at 4 °C then cells were washed and resuspended in PBS and DNA was treated with 10 µg/ml RNAaseA (Sigma) and stained using 10 µg/ml propidium iodide (Invitrogen).Samples were then analysed on a Beckman Coulter Cytoflex flow cytometer using Cytoflex software. 10,000 events were recorded and cells events were gated for forward and side scatter to remove doublets, then gated for cell cycle phase depending on the level of PI stain.

3.6 Fluorescence microscopy

To make slides, 5×10^5 2T1 cells were centrifuged for 3 minutes at 1800 g, then washed in PBS. Cells were centrifuged again and resuspended in 25 µl PBS, and placed on a superfrost plus adhesion slide (Epredia). Slides were then left to air dry, and cells were then fixed using 50 µl 4% PFA, after 10 minutes slides were washed with PBS, then were left to dry. Flouroshield mounting medium (Sigma) was then used to stain genetic material with DAPI.

For flagellar staining, cells were fixed in methanol overnight and then washed with PBS, blocked for 45 minutes with PBS + 0.005% tween-20 and 1% BSA, then incubated with L8C4 antibody which targets paraflagellar rod 2 (PFR2) (kindly provided by McKean lab at Lancaster

University) for 1 hour, washed and incubated with anti-mouse antibody (abcam) (Cat # ab150113) for 1 hour, all in a humidity chamber. Then slides were washed and left to air dry, then 1 drop of flouroshield was added.

Slides were stored at 4 °C in the dark, then visualised using a fluorescence microscope. For nuclei and kinetoplast counts, 150-200 cells from each slide were counted from each slide. Z-stack images were taken using a Leica DM RXA2 fluorescence microscope, using Leica FW 4000 software.

3.7 Cloning

3.7.1 PCR

Primers ordered from Sigma, a full table is included in appendix. Gene assemblies Trypanosoma brucei brucei TREU927 and Trypanosoma congolense IL3000 were used. Gene sequences were found by searching the gene of interest on TriTrypDB (https://tritrypdb.org/tritrypdb/app/search?q=TcIL3000_10_3320).

PCRs were performed with GoTaq enzyme, for each primer pair, components of the reaction are shown in table 3.2. For these reactions, the thermocycler was programmed to 94 °C for 2 minutes to denature DNA, variable temperature (see appendix) for 1 minute for primers to anneal and 72 °C for 1 minute for elongation, this sequence was repeated 34 times.

Table 3.2 Components of GoTaq PCR reactions

Reagent	Volume
5 × goTaq buffer (Promega)	10 µl
MgCl (25mM) (Promega)	5 µl
Nucleotide dNTPs (10mM) (Promega)	1 µl
Template DNA	0.5 µl
Taq polymerase (5u/µl) (Promega)	0.25 µl
F-primer (20mM/ml)	2 µl
R-primer (20mM/ml)	2 µl
ddH_2O	29.15 µl

In these reactions, template DNA was gDNA from the relevant species or cell line. For most amplifications this was *T. brucei* 2T1 Lister 427 or *T. congolense* TcoSM IL3000 gDNA, this was extracted as described in section 1.9.

For PCRs amplifying products of over 1000 bp, OneTaq enzyme was used instead, reagents for these reactions are shown in table 3.3. For these reactions, the thermocycler was programmed to 94 °C for 30 seconds to denature DNA, variable temperature (see appendix) for 1 minute for primers to anneal and 68 °C for 2 minutes for elongation, this sequence was repeated 35 times.

Table 3.3 Components of OneTaq PCR reactions

Reagent	Volume
OneTaq Quick load 2x MM with standard buffer (BioLabs)	25 µl
Template DNA	1 µl
F-primer (20 mM/ml)	1 µl
R-primer (20 mM/ml)	1 µl
ddH_2O	22 µl

3.7.2 Agarose gel electrophoresis

1% agarose gel were prepared with 1 in 10,000 SYBR safe (Invitrogen) and 1 × Tris base, acetic acid and EDTA running buffer (TAE running buffer). Samples were run alongside 3 µl 100 bp (0.13 µg/µl) or 1 kb MW ladder (0.1 µg/µl) (both (both Promega) and 6 × purple loading dye was added to colourless samples. Gels were run for approximately 30 minutes at 100 V. Then gels were images on a BioRad Gel Doc EZ imager.

3.7.3 Gel extraction

The desired band from and agarose gel electrophoresis was excised on a UV transilluminator (Syngene) and DNA was extracted using a Thermo Scientific GeneJet Gel Extraction kit according to manufacturer's instructions.

3.7.4 Ligations

Backbones and inserts were ligated together using 0.5 µl T4 DNA ligase (3 u/µl) (Promega), 0.5 µl 10 x rapid ligation buffer (Promega), 0.5 µl vector and 3.5 µl insert. Reactions were left at room temperature overnight.

3.7.5 Transformations

5 µl of plasmid DNA was added to a 25 µl aliquot of high efficiency *Escherichia coli* competent cells (NEB), these were left on ice for 10 minutes. The cells were then heat shocked at 42 °C for 30 seconds and left on ice for 5 minutes. Cells were allowed to recover in 150 µl SOC medium for 30 minutes in a shaking incubator at 37 °C and were then plated out onto LB plates with 100 µg/ml Carbenicillin and incubated at 37 °C overnight.

For blue white screening, 30µl 0.1 mM IPTG (Melford) and 30 µl 20 µg/ml X-Gal (Thermo) was spread on the plates before bacterial cells.

3.7.6 DNA mini-preps

Colonies were selected for minipreps and grown in 3 ml LB broth with 100 µg/ml Carbenicillin overnight. Cell suspensions were then centrifuged at 3500 x g for 10 minutes to pellet bacterial cells. Minipreps were then performed using the Thermo Scientific GeneJet Miniprep kit according to manufacturer's instructions.

3.7.7 RE digest

For digestion reactions 1/10 of total reaction volume of 10 × Cutsmart buffer (NEB) and 1/20 of total reaction volume of each restriction enzyme (NEB) were used. Reactions were incubated at 37 °C for minimum of 2 hours.

3.7.8 Sequencing

Aliquots of the miniprep products were then sent for sequencing at Dundee DNA sequencing services using standard or bespoke primers. The DNA sequence obtained was then compared to genomic sequences obtained from TriTrypDB (Aslett *et al.*, 2009) using ApE software (Davis and Jorgensen, 2022).

3.8 Transfections

3.8.1 Ethanol precipitation

For ethanol precipitation, 1/10 volume sodium acetate 3M pH 5.2 and 2 × volume of 100% ethanol was added to the plasmid DNA, this was then vortexed and left at -20 °C overnight. The next day this was centrifuged for 15 minutes at 20,000 × *g* at 4 °C. Supernatant was discarded and the pellet was resuspended in 2x volume of 70% ethanol, this was centrifuged at 20,000 × *g* for 5 minutes at 4 °C. Supernatant was discarded and the pellet was air dried, then resuspended in 10 µl ddH_2O.

3.8.2 *T. brucei* transfections

For *T. brucei* constructs, 3 µg plasmid DNA was linearised by digesting with *Not*I enzyme and incubating at 37 °C for at least 2 hours. Then ethanol precipitation was performed (see 1.8.1).

2×10^7 cells were grown and maintained in log phase. These were centrifuged at 800 *× g* and resuspended in 100 µl nucleofector solution and supplement (Lonza). Plasmid DNA was added to the cells and they were electroporated using an Amaxa nucleofector II (Lonza) set to programme X00-1. Negative controls were carried out using ddH_2O instead of plasmid DNA. Cells were left to recover at 37 °C in 25 ml media with no drugs for 6 hours. Then drugs were added, as shown in table 3.4, and cells were plated out into 24 well plates with 1 ml of cells in each well.

Table 3.4 Drug concentration used during transfections

Backbone	**Drug**	**Concentration (*T. brucei*)**	**Concentration (*T. congolense*)**
pEnNmSt0-N	G418 (Melford)	2.5 µl/ml	0.5 µl/ml
pEnNY0	Hygromycin (Melford)	2.5 µl/ml	0.4 µl/ml

After 5 days, the cells were screened to find wells with live cells, it was assumed that due to the concentration of cells after the transfection, only one successful clone would be plated into each well, so any population at this stage would be clonal. These were selected and then cultured for further analysis.

3.8.3 *T. congolense* transfections

For *T. congolense* constructs, midiprep was performed to amplify the plasmid DNA before linearisation, as recommended by Awuah-Mensah *et al*., 2021. A QUIAGEN midiprep kit was used, according to package instructions. 10 µg of plasmid DNA was then linearised by digesting

with *Not*I enzyme and incubating at 37 °C for at least 2 hours. Then ethanol precipitation was performed (see 1.8.1).

4×10^7 cells were grown and maintained in log phase. These were centrifuged at 800 *× g* and resuspended in 100µl nucleofector solution (Lonza). Plasmid DNA was added to the cells and they were electroporated using an Amaxa nucleofector II (Lonza) set to programme Z00-1. Cells were left to recover in 25 ml media, with no drugs added, for 6 hours. Then drugs were added, as shown in table 3.4, and cells were plated out into 24 well plates with 1 ml of cells in each wells.

After 10 days, the cells were screened to find wells with single clones (as described in section 3.8.2). These were selected and then cultured for further analysis.

3.9 Genomic DNA (gDNA) extraction

To prepare genomic DNA for amplification of sequences to insert into plasmids, 100 ml of cells at 1×10^6 were centrifuged at 800 *× g* for 10 minutes and the pellet was resuspended in 1 ml PBS. This was centrifuged at 16,000 *× g* for 15 seconds and the pellet was resuspended in 1 ml TRIzol reagent (Invitrogen) and mixed thoroughly. This was incubated at room temperature for 5 minutes and then mixed with 200 µl of chloroform, then incubated at room temperature for 3 minutes. This was centrifuged at 12,000 *× g* for 15 minutes at 4°C and the upper phase containing RNA was removed. 300 µl of 100% ethanol was added and this was incubated for 3 minutes. This was centrifuged at 2000 *× g* for 5 minutes at 4°C and pellet was washed with 1 ml 0.1M Na Citrate, 10% ethanol pH 8.5. This was incubated for 30 minutes at room temperature, then centrifuged at 2000 *× g* for 5 minutes. Then 1.5 ml 75% ethanol was added and incubated for 30 minutes at room temperature. Then it was centrifuged at 2000 *× g* for 5 minutes at 4 °C and the pellet was left to air dry. The pellet was resuspended in 500 µl 8 mM NaOH, centrifuged at 12,000 *× g* for 10 minutes at 4 °C and the

supernatant was kept, it was adjusted to pH 7.5 and EDTA was added to make a final concentration of 1mM, this was stored at 4 °C.

To prepare genomic DNA for PCRs to determine whether transfected cell lines had DNA inserted at the desired genomic locus, 10 ml of a dense culture of cells was centrifuged at 3900 *× g* for 1 minute at 4 °C. Supernatant was removed and cells resuspended in 100 µl nucleofector solution (Lonza) and 1µl 10% SDS. This was incubated at 55 °C for 10 minutes and 50µl 3M $NaOAc_2$ was added. This was incubated on ice for 5 minutes, then centrifuged at 20,000 x g for 4 °C. Supernatant was transferred to a new tube and 200 µl 100% ethanol was added. This was centrifuged again, supernatant was removed and gDNA pellet was allowed to air dry at room temperature. The pellet was then resuspended in 100 µl ddH_2O.

3.10 SDS-PAGE

2×10^6 cells were prepared and resuspended in 20 µl 2 × SDS loading buffer, samples were denatured by boiling at 95 °C for 10 minutes. 15µl of each sample was then run on Mini-PROTEAN TGX precast gel (Biorad). for approximately 1 hour at 150 V alongside 10µl of a precision plus protein dual colour standards ladder (Biorad)

3.11 Western blot

After running an SDS-PAGE, proteins were transferred on to a PVDF membrane (Biorad) using the Trans-Blot Turbo RTA transfer kit (Biorad), according to instructions.

Blots were then blocked for 45 minutes in a 1% milk solution and stained for the protein of interest using antibodies shown in table 3.5. Images were then taken using a BioRad Chemidoc MP with super signal west femto kit ECL substrate (Thermo). for HRP conjugated antibodies and a LI-COR Odyssey FC for fluorescent antibodies.

Table 3.5 Antibodies used for staining western blots

Protein	Primary antibody	Dilution	Secondary antibody	Dilution
mSt-DHH1	α-mCherry (Invitrogen) (Cat # PA5-34974)	1:1,000	α-rabbit IRDye (Invitrogen) (Cat # A32734)	1:10,000
eYFP-ZC3H11	α-GFP (Roche) (Cat # 11814460001)	91:1,000	α-mouse HRP (Sigma) (Cat # 12-349)	1:5,000f
EF1α	α-EF1α (Santa Cruz) (Cat # sc-101035)	1:10,000	α-mouse IRDye (Invitrogen) (Cat # 926-32210)	1:10,000
H3	α-H3 (Abcam) (Cat # NB500-171)	1:2,000	α-rabbit IRDye (Invitrogen) (Cat # A32734)	1:10,000

*All secondary antibodies used were raised in goat

4 Characterisation of heat shock response in bloodstream form trypanosomes

4.1 Aims & Background

In the literature, *T. brucei* heat shock is most often studied using a treatment of 41 °C for 1 hour, after which a lag in growth is observed that lasts for 24 hours and there is a loss of motility but little cell death (Kramer *et al*., 2013;Ooi *et al*., 2020). These observations suggest the cells have undergone stress but have employed the heat shock response and are able to recover, hence why this is chosen as a good point to study the heat shock response. The first aim of this project was to investigate whether *T. congolense* undergoes the same response as *T. brucei ,* and to find the optimal point to study the *T. congolense* heat shock response. To do this, both *T. brucei* and *T. congolense* cells were subjected to heat shock at varying temperatures and times and the lag in growth was investigated. An interesting thing to note about *T. congolense* is that the IL3000 strain that is used for lab culture is cultured at 34 °C, whereas *T. brucei* is cultured at 37 °C, this is likely to affect the temperatures at which the cells undergo the heat shock response. The cell cycle was also investigated in the period of slowed growth after heat shock in both species, to see whether a cell cycle arrest occurred, and at which point in the cell cycle.

4.2 Results

4.2.1 Temperature optimisation

Heat shock in trypanosomes was simulated by placing cells in a water bath at the desired temperature. To ensure that the cells were actually being exposed to this treatment at the correct temperature, a high accuracy thermometer was used to test the temperatures of

water baths over a 2 hour period. The temperature of the water baths was measured to observe any variation over the 2 hours, and the temperature of liquid placed in the water bath was also measured with a probe, to see how quickly samples come to the correct temperature.

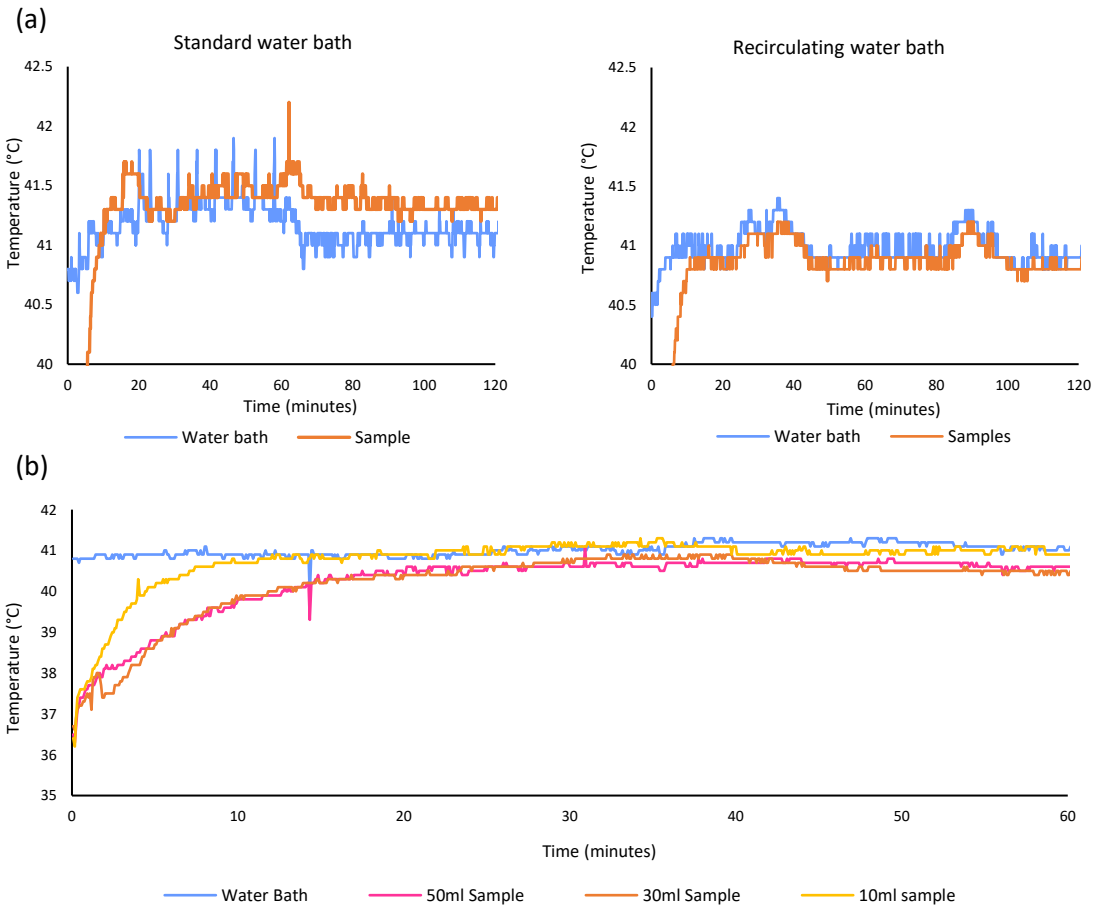

Figure 4.1 Temperature stability of water baths. (a) Comparison of temperature over 120 minutes between a recirculating and non-recirculating water bath, (b) temperature of differing volumes of liquid when placed in the water bath. Temperatures were recorded using a UKRAS calibrated RS dual probe thermometer.

The temperature profiles recorded show that while both water baths have slight oscillations in temperature (Figure 4.1), the recirculating water bath has a much more stable temperature

than the standard, which would affect the consistency of different heat shock treatments. Using the recirculating water bath ensures reproducibility between experiments. While all volumes, from 10 ml - 50 ml do reach the temperature of the water bath within 20 minutes, while 10 ml samples reach the desired temperature faster than 30 ml and 50 ml, the difference was determined to be minor.

4.2.2 *T. brucei* growth after Heat Shock

Initially, the heat shock response in *T. brucei* had to be investigated to be sure that previously seen results were reproducible. *T. brucei* cells were heat shocked for 0.5, 1 and 2 hours at 41 °C and growth was observed for 72 hours.

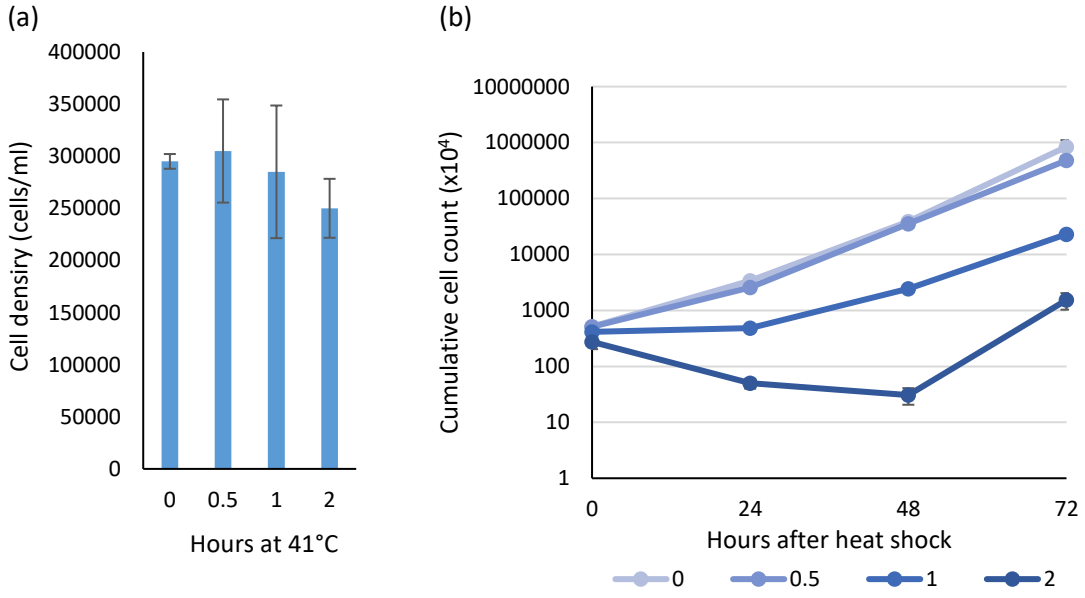

Figure 4.2 Growth of *T. brucei* cells exposed to heat shock at 41 °C. (a) A culture of cells was split into 4 tubes which were exposed to heat shock for 0, 0.5, 1 or 2 hours, cell density was recorded. (b) Cell growth over 72 hours after heat shock. Cell densities were recorded using a haemocytometer. Error bars show mean +/- standard deviation (n=3).

After 0.5 hours of heat shock at 41 °C, cells continue to grow upon return to normal culture conditions. There is little cell death at 41 °C for 1 hour, but then by 2 hours 50% of cells die after heat shock. *T. brucei* cells that are heat shocked at 41 °C for 1 hour show delayed growth for 24 hours after heat shock and then return to the normal rate. Cells heat shocked for 2 hours experience a more severe effect and show a lag in growth for 48 hours, while those heat shocked for 0.5 hours show very little effect. These results indicate that 41 °C for 1 hour is a suitable timepoint to study heat shock as the cells are able to survive but the lag in growth suggests a response is triggered. This also shows that the previously seen results in Kramer *et al.*, 2008 were reproducible with the conditions used in the lab.

To investigate what is happening to the cells during this period of slow growth, and whether the cell cycle was affected, cells were heat shocked and flow cytometry samples were taken every hour for 9 hours. DNA was stained with propidium iodide so that a cell cycle profile could be analysed to see if the lag in growth is linked to an arrest in the cell cycle, and at which point in the cell cycle this was occurring.

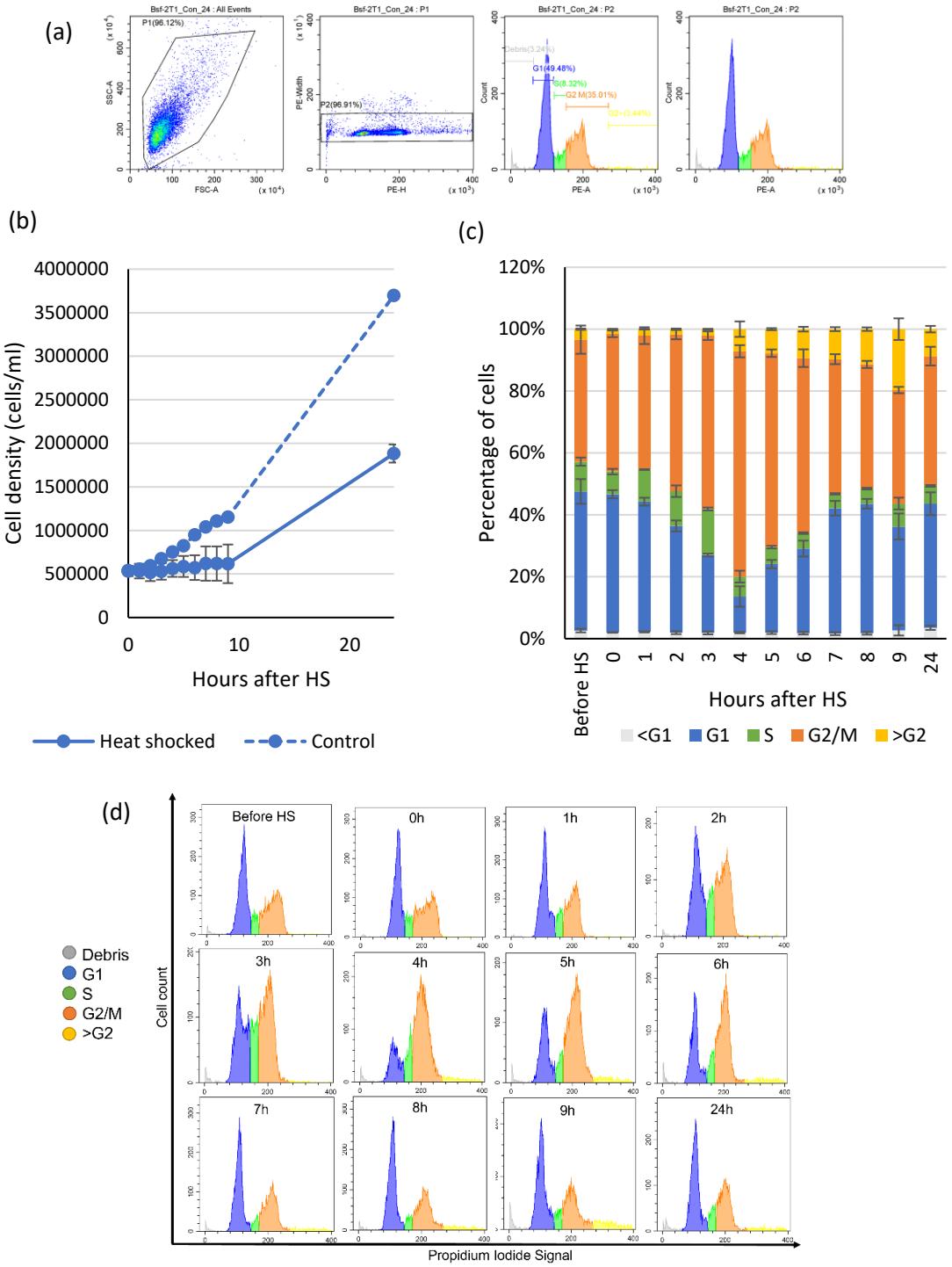

Figure 4.3 *T. brucei* cell cycle arrest after heat shock at 41 °C. Cells were subjected to heat shock at 41 °C for 1 hour, (a) 10,000 events were recorded and gated to remove doublets and the PI level was used to separate cell cycle phases. (b) Cell growth was recorded using a haemocytometer. Samples for flow cytometry were collected at hourly intervals, these were stained with PI and run on a Cytoflex flow cytometer. (c) Averages of 3 experiments and (d) representative cell cycle profiles are shown. Error bars shown standard deviation (n=3), data analysed using Cytoflex software.

After heat shock at 41 °C for 1 hour, *T. brucei* shows a specific and reversible cell cycle arrest (Figure 4.3). At 4 hours cells accumulate in G2/M phase of the cell cycle, indicating that cells have replicated DNA but there may be an issue with division. This experiment was repeated in triplicate, the averages of each experiment are shown in figure 4.3.c, a similar trend is seen in each repeat, with the amount of cells in G2/M peaking at either between 4-6 hours in all repeats.

To examine this arrest further, cells were stained with DAPI and L8C4 antibody that targets the paraflagellar rod (PFR) in the flagellum. This allows more distinction of which point in the cell cycle cells in G2/M were arresting at, as the cell cycle profiles only show the amount of genetic material within each cell. This NK configurations show whether cells that have arrested in G2/M were pre- or post-mitotic. For these counts a dividing kinetoplast (DK) was determined by eye, looking for kinetoplasts in the characteristic 'dumbbell' shape. Something to note is that the PFR is only able to be seen after it enters the flagellar pocket. This may mean in some cases when the flagellum is in its early stages of replication, it could not be seen. This antibody was used as it was available in the lab.

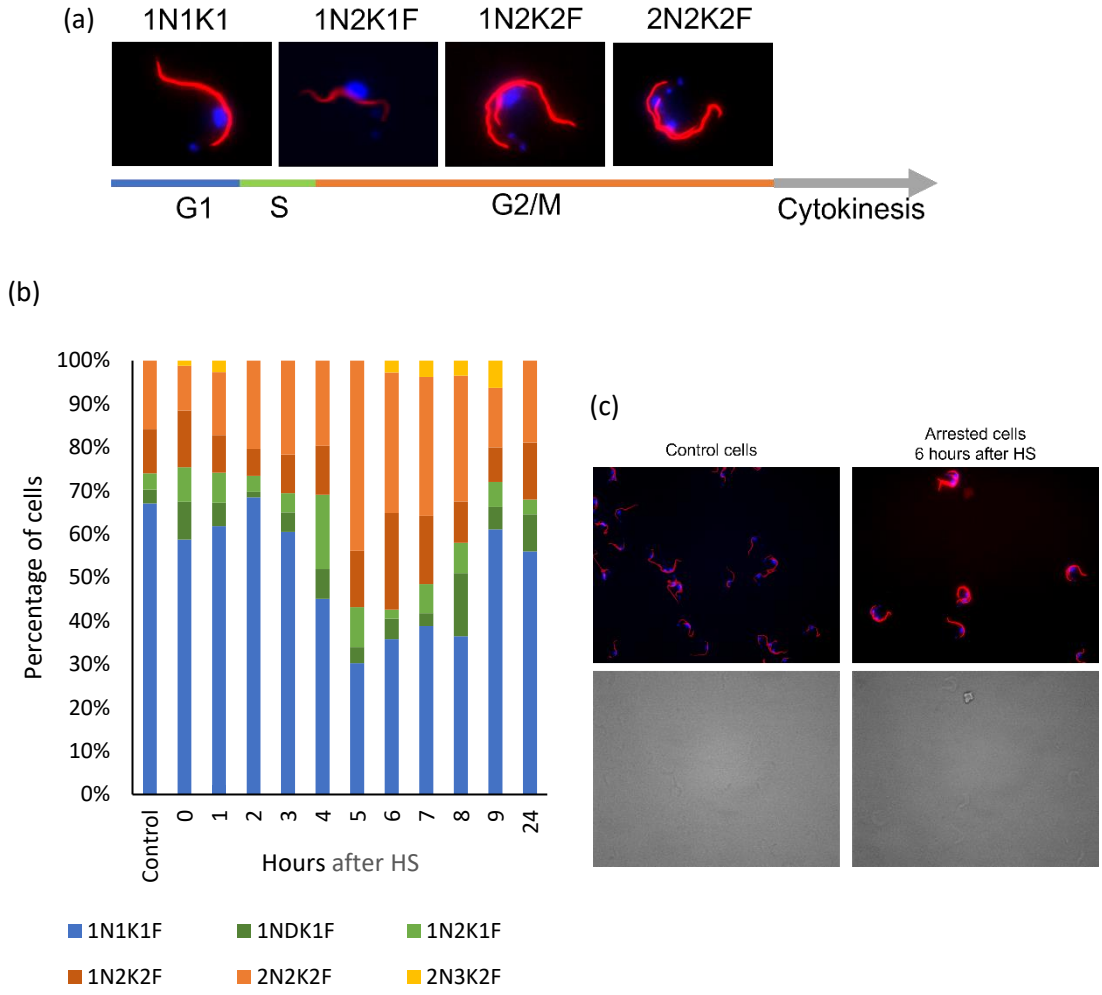

Figure 4.4 IF analysis of cell cycle arrest after heat shock at 41 °C in *T. brucei*. Slides of *T. brucei* cells were prepared at hourly intervals after heat shock at 41 °C for 1 hour, slides were fixed in methanol and stained with α-L8C4 and DAPI. (a) Example of cell types counted is shown, number of nuclei (N), kinetoplast (K) and flagella (F) were counted. (b) Approximately 200 cells from each slide were counted and cell cycle phase was recorded, and (c) images of the arrested cell population were taken using a fluorescent microscope.

The NK configurations show that cells arrested at 5 hours after heat shock, with an increase in 2N2K cells (figure 4.4) which corresponds with 'G2/M' in the cell cycle profiles previously shown, these cells have 4C DNA content but appear to be post-mitosis. At the peak of the arrest there are 45% of cells in 2N2K and 15% of cells in 1N2K2F, both these cell types may be in G2/M, compared to 80% of cells in G2/M in the flow cytometry data (figure 4.3). This may

be because of the number of cells recorded, for NK counts 150-200 cells were counted per timepoint, whereas for flow cytometry a much larger number of cells is counted. The process of fixing cells to slides may also have affected the results. Nevertheless, an arrest in 2N2K is seen, which peaks at 5 hours after heat shock and reverses to normal levels by 24 hours. There was also an increase in unhealthy 2N3K cells, which may suggest a delay in cytokinesis. These cells accumulated in the 9 hours after heat shock and then appeared to not be present at 24 hours.

Next, the cells were treated with a 42 °C heat shock to see how this would affect their growth and the cell cycle arrest seen. The experiments were repeated at this temperature, cells were heat shocked for 0.5, 1 and 2 hours and growth was recorded.

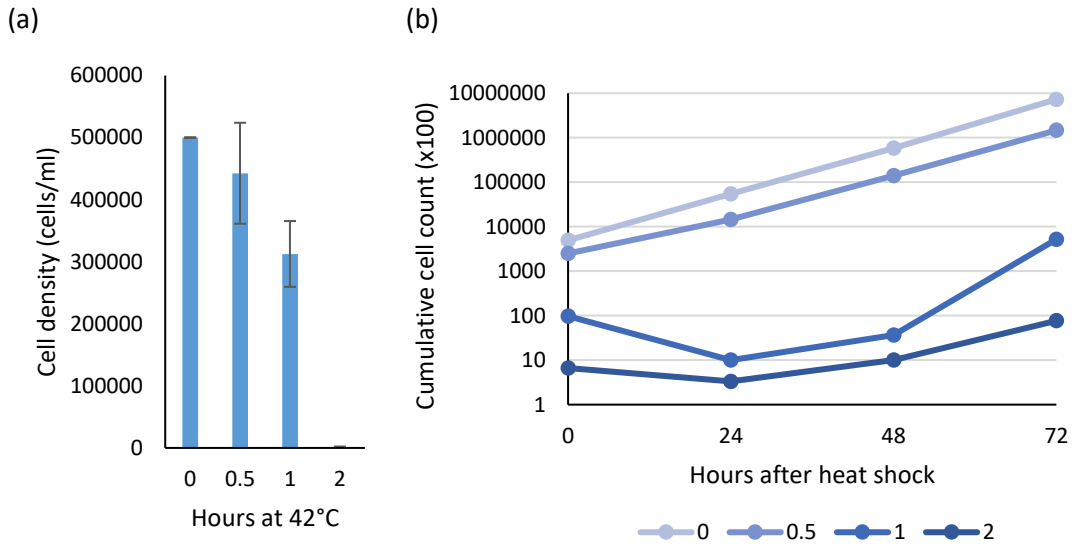

Figure 4.5 Growth of *T. brucei* cells exposed to heat shock at 42 °C. (a) A culture of cells was split into 4 tubes which were exposed to heat shock for 0, 0.5, 1 or 2 hours, cell density was recorded. (b) Cell growth over 72 hours after heat shock. Cell densities were recorded using a haemocytometer. Error bars show standard deviation (n=3).

When the temperature was increased to 42 °C, the cells experienced a larger effect in terms of cell death and lag in growth. Cells heat shocked for 2 hours showed a large reduction in density, with only 0 or 1 live cell visible in each haemocytometer count but at 48 hours had begun to recover and return to a normal growth rate. Cells heat shocked for 0.5 hours showed more cell death than at 41 °C immediately after heat shock but appeared to still grow at the same rate as controls. Approximately 40% of cells died after heat shock, and cells appeared to halt growth and continue to die in the 24 hours after 1 hour of heat shock, before returning to normal growth rate by 48 hours.

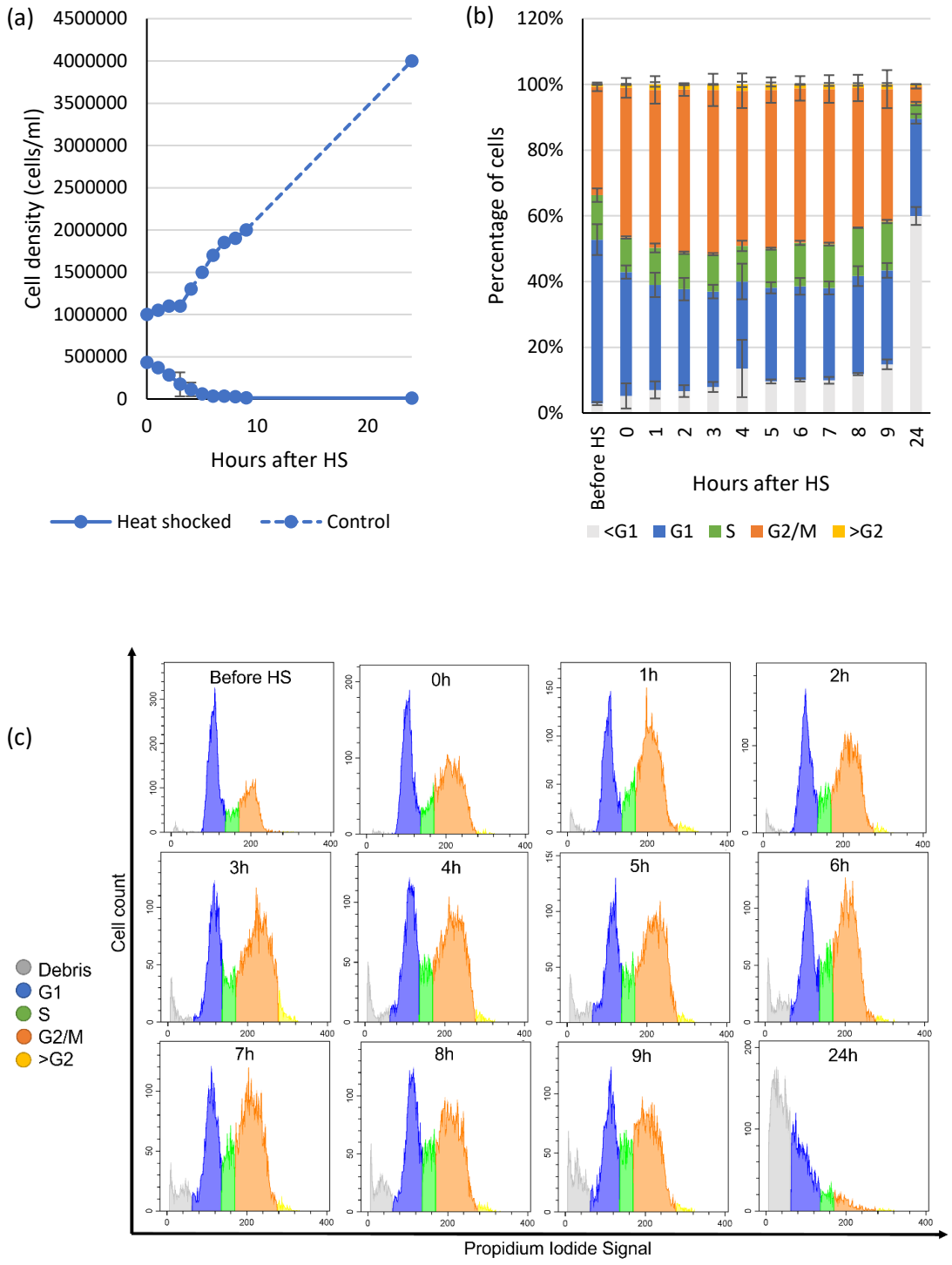

Figure 4.6 *T. brucei* cell cycle arrest after heat shock at 42 °C for 1h. Cells were subjected to heat shock at 42 °C for 1 hour, cell growth was recorded using a haemocytometer (a). Samples for flow cytometry were collected at hourly intervals, these were stained with PI and run on a Cytoflex flow cytometer. Averages of 3 experiments are shown in (b) and representative cell cycle profiles are shown in (c). Error bars shown standard deviation (n=3), data analysed using Cytoflex software.

When *T. brucei* cells are heat shocked at 42 °C, many more cells die, then cell density reduces for the 9 hours after heat shock (figure 4.6), by 24 hours after heat shock the population density has decreased so there are no living cells on the haemocytometer. Looking at the cell cycle phases shows that cells appear to be in an arrested state after heat shock and then remain in this state for the 9 hours when samples were taken. By 24 hours, a large build-up of cell debris and sub-G1 cells was observed. Overall, *T. brucei* cells cannot survive after a heat shock of 42 °C for 1 hour, all samples are extremely unhealthy and cell density reduces so much that no live cells are visible on the haemocytometer. The very few cells able to survive resume growth after 48 hours (figure 4.5).

It would be interesting to perform a NK count on cells heat shocked at 42 °C, and slides were prepared for these counts, however there was too much cell debris on the slides so counting was not possible.

4.2.3 *T. congolense* culture

The strains of *T. congolense* available to culture, IL3000 and its derivative TcoSM (Awuah-Mensah *et al.,* 2021) are grown at 34 °C, lower than 2T1 *T. brucei* cells which are cultured at 37 °C. Other differences in culture conditions are that they are grown in different media, and 2T1 cells are grown in 10% FBS and IL3000 and TcoSM cells are grown in 15% goat sera. This is a curious difference as these are both BSF cells which occupy very similar host ranges, so it would be expected that they could be cultured in the same media at the same temperature. *T. congolense* cells were incubated at 37 °C to see how the increased temperature affects their growth.

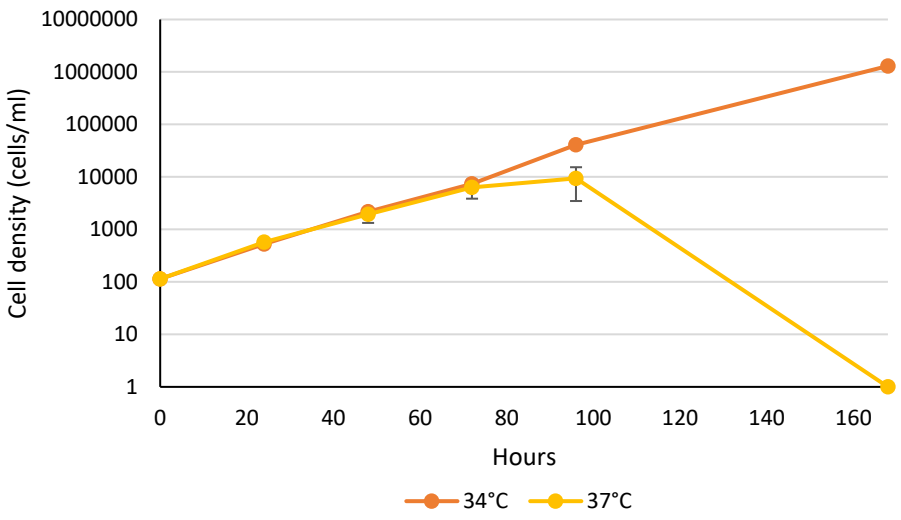

Figure 4.6 *T. congolense* growth at 37 °C. Cell density was counted every 24 hours with a haemocytometer.

T. congolense cells cultured at 37 °C are able to grow at a normal rate for 4 days then some time after this point the population crashes (Figure 4.6). By day 5 growth is severely lagging and by day 7 the whole population dies out.

4.2.4 *T. congolense* growth after heat shock

Next *T. congolense* cells were heat shocked under the same conditions as *T. brucei* , to see if there were any differences in the reactions between the 2 species, and to try to determine an optimum temperature to study the *T. congolense* heat shock response. Cells were heat shocked for 0.5, 1 and 2 hours at 41 °C.

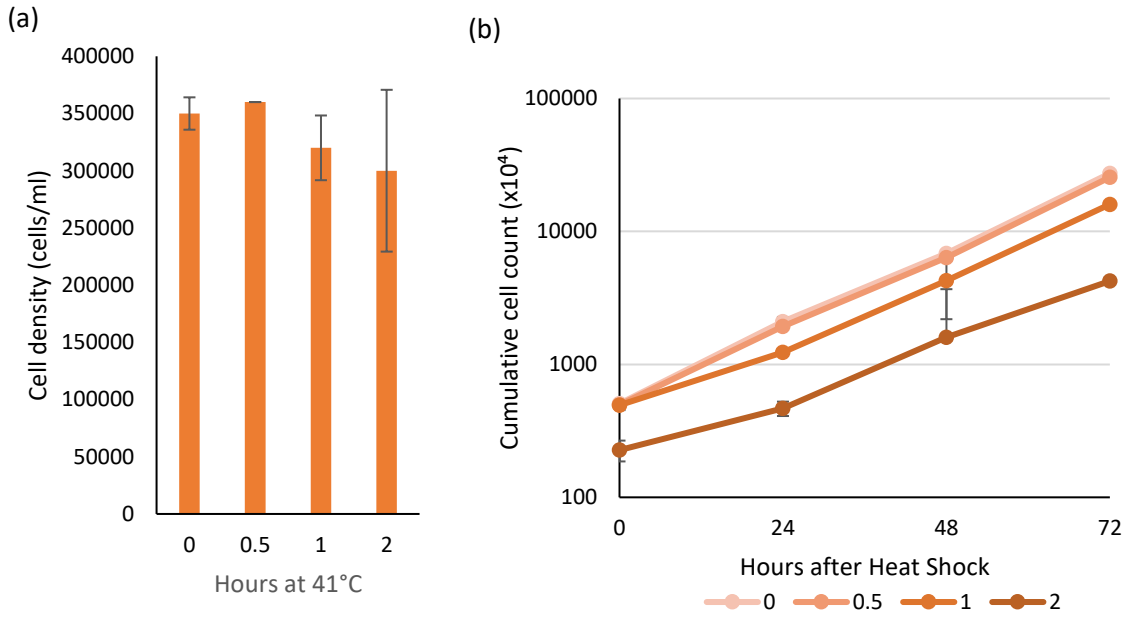

Figure 4.7 *T. congolense* cells exposed to heat shock at 41 °C. (a) Cell density after a heat shock of 0, 0.5, 1 or 2 hours was recorded and (b) cell growth over 72 hours after heat shock. Cell densities were recorded using a haemocytometer. Standard deviations shown (n=3).

At 41 °C, little cell death was seen after 1 and even 2 hours of heat shock treatment, as shown in figure 4.7, cell density only decreased from 5×10^5 cells/ml to 3×10^5 cells/ml after 2 hours of heat shock, although this was variable. When looking into the recovery of cells over 72 hours after heat shock, there was only a slight lag in cells that were heat shocked for 1 hour and even cells heat shocked for 2 hours showed only a slight lag in growth rate.

Next, the cell cycle of these cells was investigated using flow cytometry, cells were stained with propidium iodide and samples were taken every hour after heat shock treatment to investigate the lag in growth.

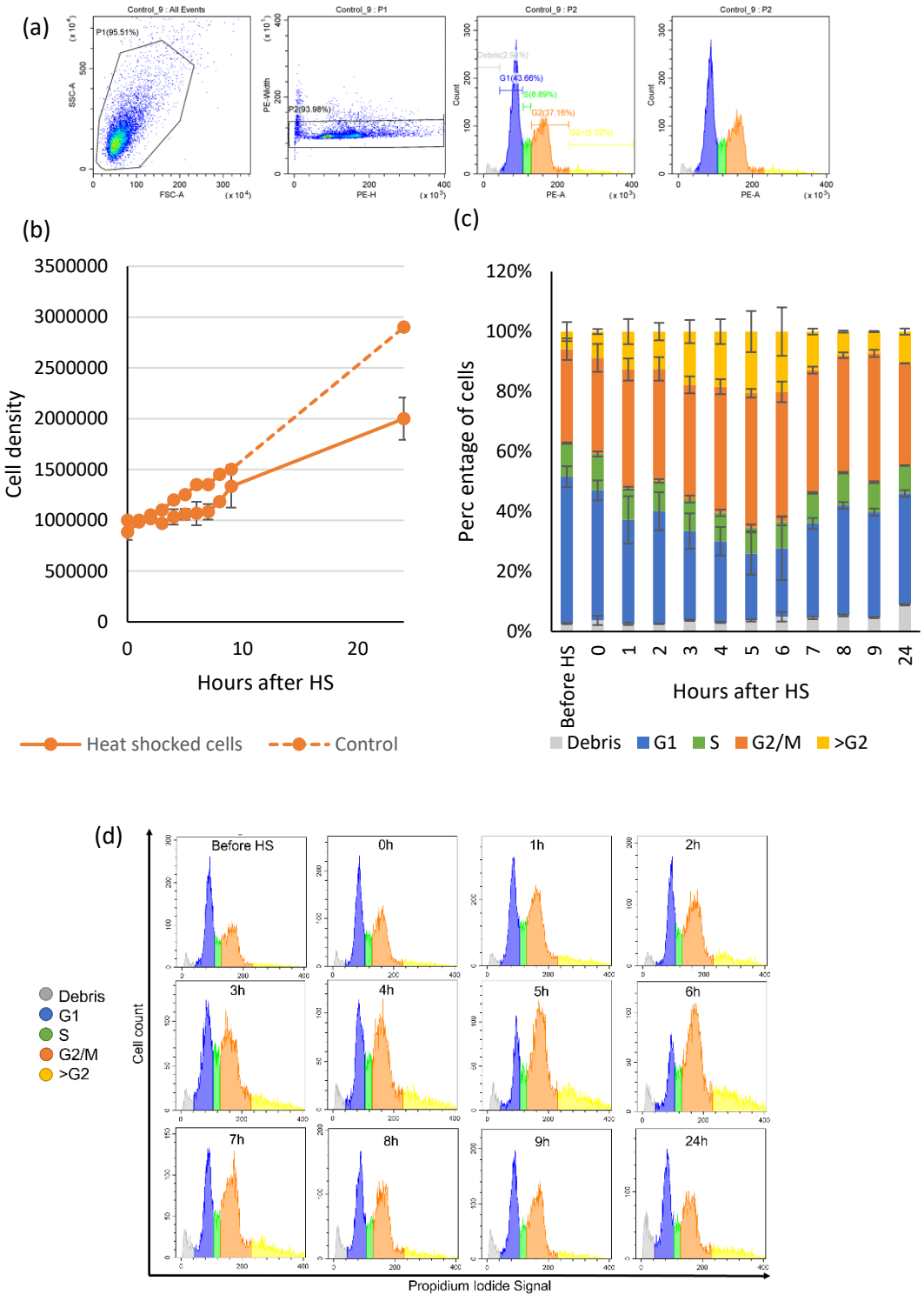

Figure 4.8 *T. congolense* cell cycle arrest after heat shock at 41 °C. Cells were subjected to heat shock at 41 °C for 1 hour, (a) 10,000 events were recorded and gated to remove doublets and the PI level was used to separate cell cycle phases. (b) Cell growth was recorded using a haemocytometer. Samples for flow cytometry were collected at hourly intervals, these were stained with PI and run on a Cytoflex flow cytometer. (c) Averages of 3 experiments and (d) representative cell cycle profiles are shown. Error bars shown standard deviation (n=3), data analysed using Cytoflex software.

T. congolense cells also experience a lag in growth in the hour after a heat shock treatment of 41 °C for 1 hour, 6 hours after heat shock there is an accumulation of cells in G2/M (figure 4.8), which begins to reverse and appears to be back to normal levels by 24 hours. There is also an accumulation of abnormal cells <G1 and >G2, at 24 hours these cells are still present within the sample, suggesting that these cells may not have fully recovered from the effects of heat shock.

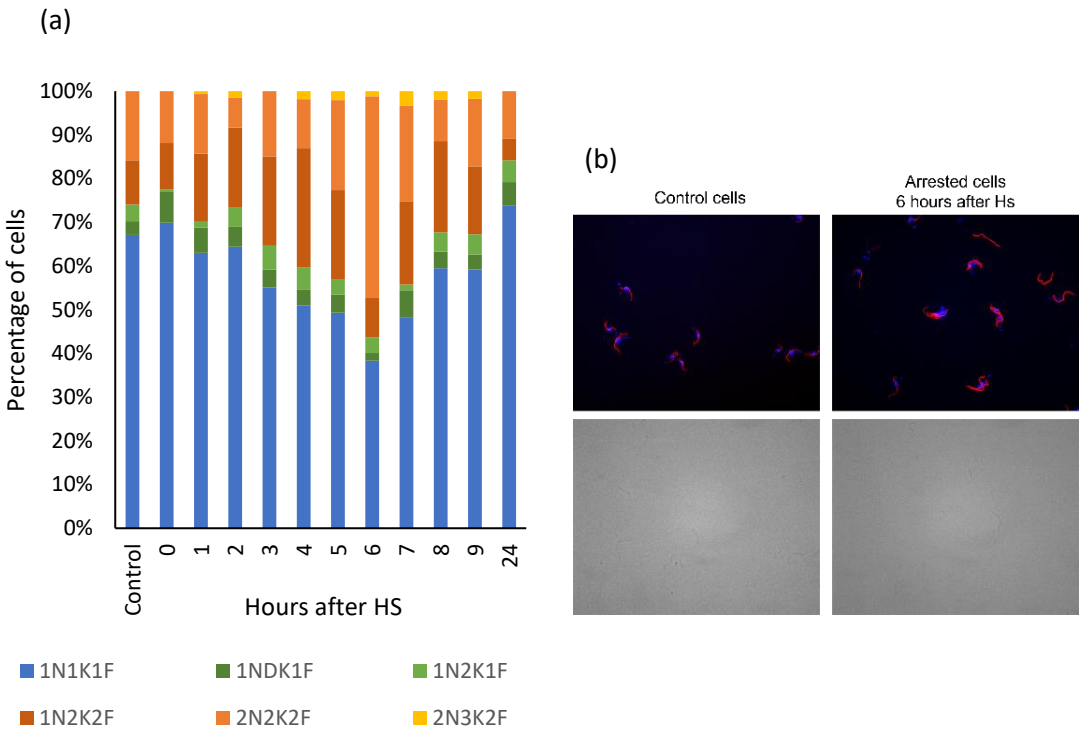

Figure 4.9 IF analysis of cell cycle arrest after heat shock at 41 °C for 1h in *T. congolense*. Slides of *T. congolense* cells were prepared at hourly intervals after heat shock at 41 °C for 1 hour, slides were fixed in methanol and stained with α-L8C4 and DAPI. then approximately 200 cells from each slide were counted and cell cycle phase was recorded, graph shown in (a), and images of the arrested cell population were taken (b).

NK configuration analysis showed that *T. congolense* did experience a change in cell cycle profile at 6 hours after heat shock there is a large increase in the number of cells in 2N2K phase (figure 4.9), and an increase in abnormal 2N3K cells. At the 5 hour timepoint, there were many cells with enlarged nuclei in the 1N2K2F cell cycle phase. There is also an increase in unhealthy cells that have not undergone division correctly, as seen in the IF image in figure 4.9.

Next, as the *T. congolense* cells showed more moderate cell cycle arrest response at 41 °C, experiments were repeated at 42 °C to see how the cells respond to heat shock at a higher temperature, and to determine which temperature would be preferred for further experiments investigating the *T. congolense* heat shock response.

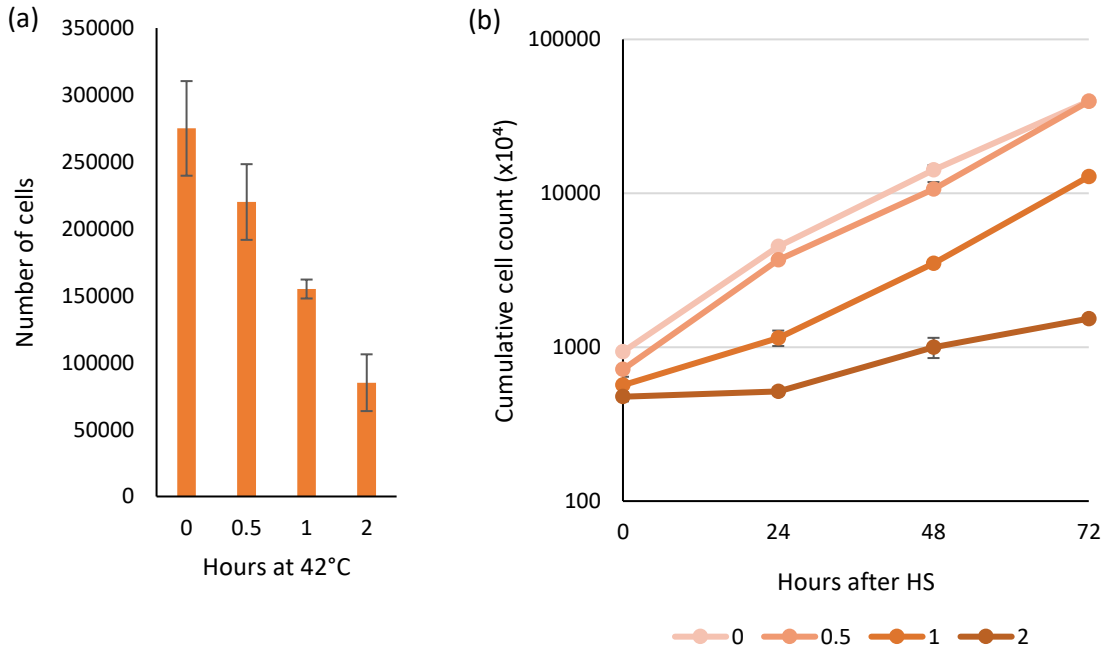

Figure 4.10 Growth of *T. congolense* cells exposed to heat shock at 42 °C. (a) Cell density after a heat shock of 0, 0.5, 1 or 2 hours was recorded and (b) cell growth over 72 hours after heat shock. Cell densities were recorded using a haemocytometer. Error bars show standard deviation (n=3).

Cells heat shocked at 42 °C experienced a more severe response (figure 4.10), those heat shocked for 0.5 hours did not show a significant change in growth after heat shock. Cells heat

shocked at 1 hour showed more of a response, with 40% of cells dying, and growth was reduced for 24 hours after heat shock. Many cells heat shocked for 2 hours died, and growth rate was decreased for 72 hours after heat shock, cells did recover after this point.

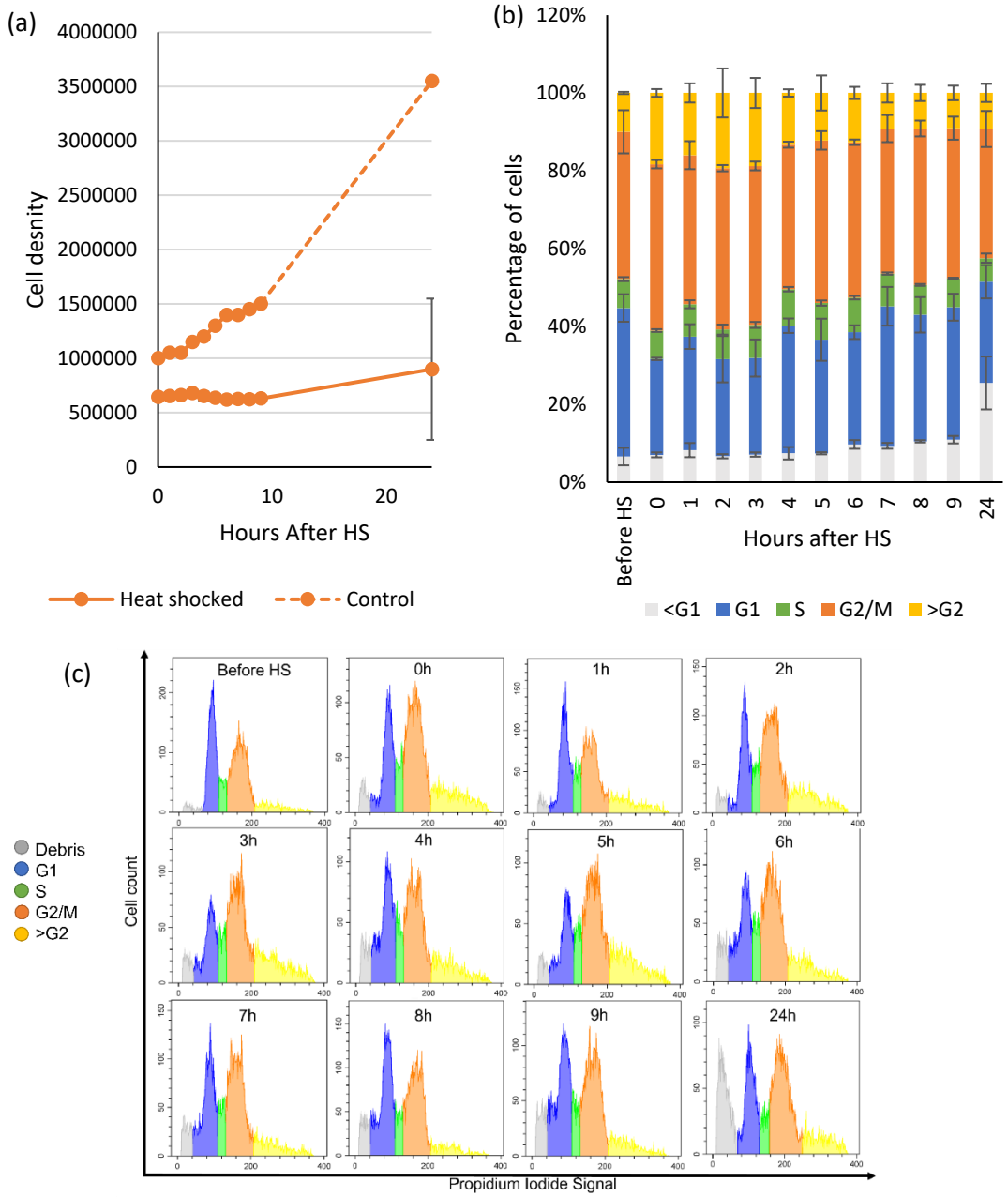

Figure 4.11 *T. congolense* cell cycle arrest after heat shock at 42 °C for 1h. Cells were subjected to heat shock at 42 °C for 1 hour, (a) cell growth was recorded using a haemocytometer. Samples for flow cytometry were collected at hourly intervals, these were stained with PI and run on a Cytoflex flow cytometer. (b) Averages of 3 experiments and (c) representative cell cycle profiles are shown. Error bars shown standard deviation (n=3), data analysed using Cytoflex software.

T. congolense cells heat shocked at 42 °C for 1 hour show a lag in growth for the 9 hours after heat shock but do begin to grow by 24 hours (figure 4.11). They appear to enter an arrest upon heat shock treatment, and at 0 hours have an increased number of cells in G2/M and cells with more DNA content, which remains throughout the 9 hours after heat shock, in this time there is also an increase in cell debris/<G1 cells. By 24 hours, the samples have not returned to normal, there is a large build-up of <G1 cells and still increased >G2 cells, both of these cell types are unhealthy.

4.3 Discussion

4.3.1 Summary of results

The two species *T. brucei* and *T. congolense* were exposed to heat shock at 41 °C and 42 °C to compare changes in growth rate in the period after heat shock. It was expected that these two species would respond in a similar way due to the fact that they infect a similar host range so should have been exposed to the same evolutionary pressures, however this was not the case. While *T. brucei* cells show a clear lag in growth for 24 hours after heat shock at 41 °C, and an irreversible arrest leading to a crash in growth after heat shock at 42 °C, *T. congolense* show a less significant lag in growth at 41 °C and remain growing, albeit at a highly reduced rate, at 42 °C. These initial experiments suggest that *T. congolense* and *T. brucei* do respond differently to heat shock and that different temperatures and timepoints will need to be investigated in order to find optimal conditions to trigger the response.

Table 4.1 Summary of results

HS Treatment	Response	*T. brucei*	*T. congolense*
41 °C for 1 hour	Growth	5% +/- 25% cells die and growth is slowed for 24 hours	5% +/- 10% cells die and slightly reduced growth rate for 24 hours
	Cell cycle arrest	Arrest at 4-5 hours after HS, 70% cells in G2/M	Arrest 6 hours after HS, 45% of cells in G2/M
42 °C for 1 hour	Growth	40% +/- 10% cells die and growth is halted for 48 hours	50% +/- 2% cells die and growth is slowed for 24 hours
	Cell cycle arrest	Severe cell cycle defect, cells die	40% cells accumulate in G2/M, accumulation of <G2 cells

Next, flow cytometry was used to investigate the number of cells in each cell cycle phase in the population in the period after heat shock, where cells exhibit slowed growth. *T. brucei* cells heat shocked at 41 °C showed an accumulation of 70% of cells in G2/M up to 5 hours after heat shock, which then appeared to release, leading to an increase in cells in G1 phase. *T. congolense* cells exposed to the same heat shock treatment showed a later arrest of 40% of cells in G2/M at 6 hours after heat shock but the same transition into G1 afterwards is not seen.

When exposed to heat shock at 42 °C, the flow cytometry results are less clear due to the large amount of cell death in each species. As shown in figures 4.5 and 4.10, approximately half of the cells in a population die when exposed to 1 hour of heat shock at 42 °C. When cells are fixed for flow cytometry there is no distinction between live and dead cells, so the cell cycle profiles include dead cells. There is also a large amount of debris in these samples which may also influence the cell cycle profiles, though some of it can be gated out.

The flow cytometry profiles for *T. brucei* show a large increase in debris which is highlighted at 24 hours, it appears that cells do not lyse immediately upon heat shock, but by 24 hours most of the dead cells have lysed. The results for *T. congolense* suggest an immediate increase in cells in G2/M phase, which remains for all 9 hours after heat shock, at earlier timepoints this likely does not indicate that the cells have moved to G2/M phase as there is not enough time for them to move through their cell cycle. This result may be caused by increased clumping together of cells or cell debris, however a growth arrest must be occurring as cells are none dividing, which is shown in the cell counts.

The *T. congolense* cells heat shocked at both 41 °C and 42 °C appeared to be extremely unhealthy. The arrest seen at 41 °C does not fully resolve by 24 hours, as the population still has an increased number of <G2 and >G1 cells, whereas in *T. brucei* there is a clear arrest at 4-5 hours which fully resolves. This could indicate that though *T. congolense* cells continue to grow after heat shock at 41 °C, there is a defect that leads to cell death, rather than a reversible arrest. At 42 °C cells are clearly unable to divide as the cell number remains the same for 9 hours after heat shock, and it appears cells immediately arrest in G2/M, cells are still extremely unhealthy as seen by the increase in >G2 cells. It is possible that both these temperatures are too high to investigate the heat shock response and are causing too large of a population of unhealthy cells to see a clear arrest and resolution, and the build-up of abnormal cells with DNA content <G1 and >G2 indicates a cytokinesis defect with cells

dividing incorrectly. To investigate further, the cells should be heat shocked at a lower temperature, to see if the same clear arrest and recovery can be seen as in *T. brucei* , which would indicate a successful heat shock response.

4.3.2 Differences between *T. brucei* and *T. congolense* heat shock response

All results indicate that *T. congolense* and *T. brucei* respond differently to heat shock, this difference was unexpected as the two species are so closely related and have an extremely similar host range, with both species predominantly infecting cattle and other ruminants, there should be no large differences in the temperatures each species is exposed to in the wild. These two species commonly co-infect the same host and are so interlinked that proteins isolated from *T. congolense* cells can trigger the quorum sensing dependent stumpy dorm differentiation in *T. brucei* cells (Silvester *et al.*, 2017). Co-infection with *T. congolense* can even accelerate the differentiation to stumpy form in *T. brucei* cells, likely increasing transmission potential and virulence. All of this shows how these two species have been exposed to the same environments and evolutionary pressures. However they do behave differently within the host, so it is possible that this difference is thermotolerance is an indication of the species behaviour in the wild.

The IL3000 lab strain does show some unexpected differences in culture when compared with *T. brucei* . IL3000 is a *T. congolense* Savannah strain that is widely used for lab culture, however when infected into mice it causes a severe disease that is fatal after 1 week (Bengaly *et al.*, 2002), compared to other strains which cause symptoms more similar to AAT and are able to be cleared by the mouse immune system. This could indicate that this strain has adapted drastically in lab culture. It is grown at 34 °C in goat sera, compared to 2T1 *T. brucei* cells which grow at 37 °C in foetal bovine sera. Wild type parasites infect a bovine host, and most mammalian infective parasites are able to be cultured at 37 °C, as this is the

ambient temperature inside the bloodstream. Anecdotally, it is known that some IL3000 cell lines have adapted to growth at 37 °C (Paula MacGregor, Personal communication), so these should be investigated to see if they show the same growth kinetics when exposed to heat shock at 41 °C and at 42 °C, this could show whether the Il3000 cells used in these experiments have adaptions related to the heat shock response.

Another difference between the two species is that they have different cell cycle lengths, *T. brucei* has a doubling time of 6 hours and *T. congolense* has a doubling time of 12 hours. These differences may also account for the difference in response seen, especially the difference in time for the cell cycle arrest, as *T. brucei* cells arrest 4 hours after heat shock and *T. congolense* cells arrest 6 hours after heat shock, this is likely due to the longer time it takes for *T. congolense* cells to move through their cell cycle.

4.3.3 Significance of cell cycle arrest

For the first time, a cell cycle arrest was shown in both *T. brucei* and *T. congolense* cells that have undergone heat shock. Previous work had shown that there is no difference in cell cycle profile 24 hours after heat shock (Droll *et al.,* 2013), but other timepoints had not been investigated. It was known that during the heat shock response there is a halt in protein production especially in pathways such as cell growth and replication, and that there is a lag in growth rate after heat shock (Kramer *et al.,* 2008). This led to the investigation into the cell cycle using flow cytometry, which showed, in figures 4.3 and 4.8, that both *T. brucei* and *T. congolense* arrest in G2/M during heat shock recovery after a heat shock at 41 °C, the arrest peaks at 4 hours in *T. brucei* and 6 hours in *T. congolense*. This difference is likely due to the fact that *T. congolense* has a longer cell cycle.

The flow cytometry data and IF data did not always show the same response, it should be noted that flow cytometry cannot differentiate between cells that are pre-mitotic (1N2K2F)

and those that have undergone mitosis (2N2K), and the purpose of the IF experiments was to try to further determine where in the cell cycle the arrest was taking place. The IF analysis did show an increase in 2N2K cells, which correspond to the G2/M cell cycle phase in the flow cytometry populations, which peaked at 5 hours for *T. brucei* and 6 hours for *T. congolense*. However, the increase in this cell type did not account for the large increase of G2 cells seen in the flow cytometry experiments. There was also an increase in 1N2K2F cells, which could be in either S phase or undergoing mitosis.

This discrepancy has been seen in previous work by Benz *et al.*, 2017, where cells were synchronised by elutriation and then observed at different cell cycle phases, there was always a larger number of cells in G2 seen by flow cytometry than by the karyotyping done using a microscope. Benz *et al*., 2019 found that in a healthy population of BSF *T. brucei* cells enriched in G2/M, 50% of cells appeared at G2/M in flow cytometry but only 20% of these were 2N2K, and 25% were 1N2K. The results in the IF in figure 4.4 show a different distribution of cells, with 45% of cells in 2K2N, out of 73% in G2/M in the flow cytometry data. This higher proportion of G2/M cells in the 2N2K state could be enough to suggest that the arrest seen after heat shock is post-mitotic.

The phenotype of an arrest in G2/M has been shown in *T. brucei* when VSG is depleted in cells. When VSG translation is blocked, a precise pre cytokinesis arrest is seen, with 50% of cells in 2N2K phase 12 hours after blocking VSG22 synthesis. This is linked with depletions in structures related to secretion, namely the Golgi apparatus and endoplasmic reticulum exit sites, and both the Golgi apparatus and endoplasmic reticulum show a distorted morphology (Ooi *et al.*, 2018). As a similar arrest is seen in response to heat shock and VSG depletion, so it may be possible that there is a link between the 2 and VSG is damaged, and production halted during the heat shock response. However as the arrest seen after heat shock, while

being in G2/M, may be a pre-mitotic arrest, more work would have to be done to investigate the similarity of these 2 phenotypes.

It has previously been suggested that the heat shock response may not only be a virulence factor but may also be involved in triggering life cycle phase differentiation in these parasites (Minia *et al.*, 2016). When a metacyclic parasite from the fly is injected into the mammalian host blood stream, it experiences an increase in ambient temperature, from approximately 28 °C in the fly, to 37 °C in the bloodstream. In this way, the cell may sense a change in temperature and trigger differentiation, which would involve an arrest in growth. When parasites differentiate into stumpy form, which is able to infect the tsetse fly vector, an arrest is also seen, however this arrest is at G1. Parasitaemia peaks when the fever is at its highest (Van den Bossche *et al.,* 2005), it could be possible that heat shock is an enhancer for stumpy formation.

It is known that in *Leishmania*, heat shock is directly involved in triggering a differentiation from the promastigote fly infective stage to the amastigote mammalian infective stage (Shapira *et al.*, 1988). *Leishmania* are parasites in the same genus as trypanosomes, they also display polycistronic transcription so share similarities with trypanosomes. In *Leishmania donovani*, the HSP100 protein is required for normal development of amastigotes, the mammalian infective stage of the parasite, to survive in host cells. HSP100 acts as a promoter for amastigote development (Krobitsch and Clos, 1999). HSP83, a key regulator of the *Leishmania* heat shock response, is inactivated with increased temperatures, when this protein is inhibited by geldanamycin, cells arrest in G2 These cells arrest in G2/M (Wiesgigl and Clos, 2017). It was suggested that levels of HSP83 act as a thermometer to detect ambient temperature and trigger life cycle change.

The arrest in G2/M seen in *Leishmania* cells undergoing life cycle differentiation is similar to what was observed in *T. brucei* and *T. congolense* cells after undergoing the heat shock

response. The heat shock response in trypanosomes may be linked to life cycle phase differentiation, as it is in *Leishmania*. However, in *Leishmania* this arrest is triggered in fly infective parasites entering the mammalian host bloodstream and differentiating into mammalian infective cells. The trypanosome cells investigated in this experiment were all BSF cells, which would not be undergoing this temperature change and differentiation. Further investigations could include performing these flow cytometry experiments using PCF cells to confirm if they also display an arrest in G2/M upon heat shock, and also by repeating using pleomorphic BSF cells, which are able to differentiate from slender to stumpy form, to see whether any phenotypic changes or even differentiation coincides with the arrest.

5 in situ tagging of heat shock response proteins in bloodstream form trypanosomes

5.1 Introduction and background

In *T. brucei* PCF cells, heat shock granules and P-bodies have been shown to form in response to heat shock, these are foci that are sites of either mRNA degradation or storage (Kramer *et al.*, 2008). They contain proteins that can be used as markers for these granules, which in PCF cells re-localise within the cytosol to form discrete foci immediately after a heat shock treatment of 41°C for 1 hour. This response has not been shown in BSF cells, so the next aim of this project was to use genetic manipulation to fluorescently tag proteins in BSF *T. brucei* cells that localise in either heat shock granules or P-bodies, or proteins known to be involved in the heat shock response, to be able to observe any changes in their localisation and abundance upon heat shock.

The proteins chosen for in situ tagging were DHH1, XRNA and PABP1 which all re-localise to either heat shock granules or P-bodies in PCF (Kramer *et al.*, 2008), and ZC3H11 and HSP100 which are integral to the heat shock response (Droll *et al.,* 2013; Ooi *et al.,* 2020). The aim was to investigate how their abundance changes during and after the heat shock response.

The heat shock response has not previously been investigated in *T. congolense,* but tools for genetic manipulation have recently been tested and developed (Awuah-Mensah *et al*., 2021). For the proteins above, homologues were found in *T. congolense* which were chosen for fluorescence tagging. Plasmids used for *T. brucei* fluorescent tagging have been shown to successfully work in *T. congolense*, though more plasmid is needed for transfection into trypanosomes. Transfections in *T. congolense* are a relatively new procedure, so optimisation would need to be performed, but it was expected that these constructs would successfully

be able to be created and transfected into *T. congolense* so that the heat shock response could be investigated.

5.2 Results

5.2.1 Proteins selected for tagging

The proteins chosen for investigation are shown in table 5.1. Proteins were chosen that have previously been shown to respond during heat shock in PCF *T. brucei* cells. Homologues were then found in *T. congolense* using TriTrypDB (Aslett *et al.*, 2009), a database of genomes of kinetoplast organisms. Gene assemblies Trypanosoma brucei brucei TREU927 and Trypanosoma congolense IL3000 were used. All of the chosen genes had orthologues in *T. congolense*, however the gene assembly for SCD6 was incomplete, lacking the genes either side of SCD6 which are needed to design primers of the UTRs, so this gene was not chosen for investigation.

Table 5.1 Proteins chosen for fluorescent tagging

Protein name	Gene in *T. brucei*	Gene in *T. congolense*	Sequence identity between species	Response to HS
DHH1	Tb927.10.3990	TcIL3000_10_3320	81.3%	Localises into P-bodies
ZC3H11	Tb927.5.810	TcIL3000_0_37550	52.8%	Regulates response
XRNA	Tb927.7.4900	TcIL3000_7_4080	60.5%	Localises into P-bodies and novel granule
HSP100	Tb927.2.5980	TcIL3000_2_1630	70.1%	Effector of response
PABP1	Tb927.9.9290	TcIL3000_9_3370	75.7%	Localises into HS granules

It was then decided whether genes would be tagged at the N- or C- terminus. To do this previous literature and Tryptag (Dean *et al.,* 2017) data was reviewed. TryTag is a project that aimed to tag every protein in PCF cells in order to show localisation. All proteins were tagged with mNeonGreen and tagging was attempted at the N- and C- terminus, data of successes and failures is provided. As tagging may interrupt the 5' or 3' UTR it can sometimes disrupt the resulting protein, making it non-functional, so TrypTag data is useful to avoid tagging a protein and creating a non-functional product. All chosen proteins, except DHH1,

can be successfully tagged at both N and C terminus according to TrypTag. for DHH1, only N terminal was possible.

When looking at previous literature where these proteins have been tagged, it was found that all had been tagged at both N and C terminal (Kramer *et al.,* 2008; Singh *et al.,* 2014), except PABP1 which was only tagged at the C- terminus, and HSP100 which had not been fluorescently tagged. When choosing whether to tag at the N- or C- terminus for these experiments, previous literature was used as a rational, so for DHH1, ZC3H11 and XRNA, the N- terminus was chosen, and for PABP1, the C- terminus was chosen. When possible, the N-terminus was chosen, as this leaves the native 3' UTR alone, which in trypanosomes is often more important for regulation of the gene, including ZC3H11 which is known to interact with proteins via a motif in the 3' UTR (Singh *et al.,* 2014).

5.2.2 Cloning strategy

Proteins were tagged endogenously with fluorescent proteins eYFP or mStrawberry (mST), using the plasmids pEnNmSt0-N and pEnNY0 kindly provided by the Wickstead lab in Nottingham (Wickstead *et al.*, 2010). The process for tagging using these plasmids is shown in figure 5.1. First, a section from the UTR and ORF of the chosen gene was amplified using PCR. Restriction enzyme cut sites were added in the primers, which can be used to insert these amplified fragments into a plasmid backbone. An additional *Not*I cut site is inserted which was used to linearise the construct. After linearisation, the ORF and UTR fragments flank the construct, and act of sites of homologous recombination into the trypanosome genome, inserting the fluorescent protein and drug resistance cassette in the original plasmid.

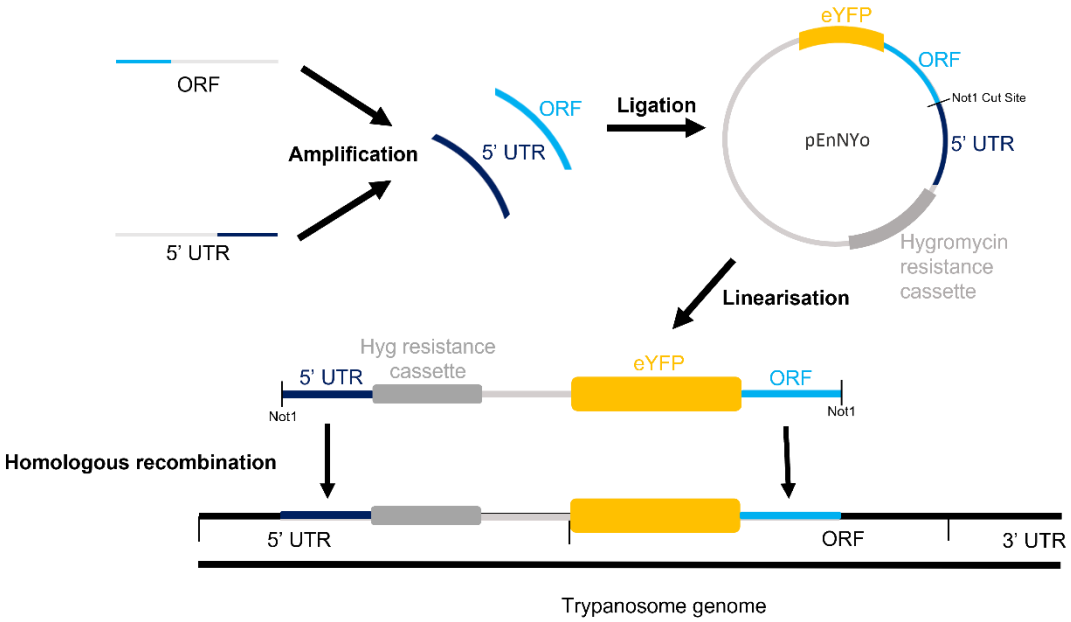

Figure 5.1 Cloning strategy. Sections of the ORF and either 5' of the selected gene were amplified with PCR and ligated into the plasmid, RE cut sites were incorporated into the PCR primers flank the inserts, and a NOT1 cut site was incorporated. NOT1 was then used to linearise the plasmid, so that the ORF and UTR sequences are on the outside. These sections can then homologously recombine into the trypanosome genome, inserting the drug resistance cassette and fluorescent protein sequence.

The cloning strategy for N- terminal tagging using the pEnNYo plasmid is shown in figure 5.1. The other plasmids available were pEnNmSt0-N which contains m-Strawberry fluorescent protein and a hygromycin resistance cassette, this can also be used for N- terminal tagging. Proteins DHH1, ZC3H11 and XRNA were chosen to be tagged at the N-terminal with pEnNY0 and pEnNmSt0,and HSP100, PABP1 and PABP2 were chosen to be tagged at the C terminal with pSIS-HHsfG.

5.2.3 Cloning – N-Terminal Tagging

Primers were designed to flank a section of 300-500 bp in either the ORF, 5' UTR of the protein of interest in either *T. brucei* or *T. congolense*, this size was chosen as recommended by Awuah-Mensah *et al*., 2020. The primers were used in a PCR reaction to amplify the desired inserts from genomic DNA, which were then run out on an agarose gel and bands of the correct size were extracted from the gel. Example of a successful PCR reactions is shown in figure 5.2.

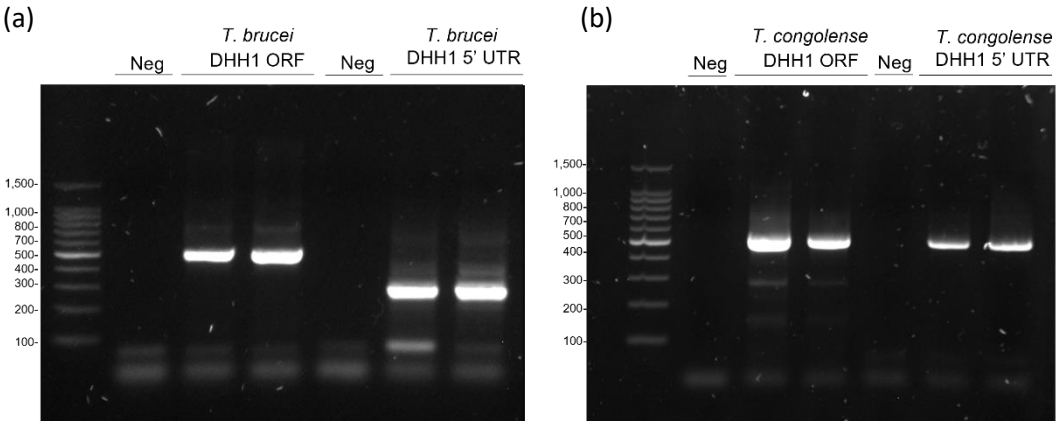

Figure 5.2 Agarose gels of amplified PCR products. Primers were designed to flank a section of the DHH1 5'UTR and ORF in both (a) *T. brucei* and (b) *T. congolense*. These were amplified using GoTaq enzyme and genomic DNA from 2T1 BSF cells or TcoSM cells as a template. Negative controls were performed using no polymerase. Ladder labels are in bp.

The PCR reactions were run on agarose gels as shown in figure 5.2, these inserts are of the expected size, which is 512 bp for the *T. brucei* DHH1 ORF, *T. congolense* DHH1 ORF and *T. congolense* 5' UTR inserts, and 314 bp for the *T. brucei* DHH1 5' UTR insert. These inserts were extracted, ligated into pGEM-T easy and then sequenced and compared to genomic sequences to ensure the no errors had occurred (Fig 5.3). ORF sequences must have no

mutations, or redundant mutations, so as not to affect the sequence of the tagged protein. UTR sequences with some mutations may still be usable, as they just need the sequences to be similar enough to be able to correctly recombine with the genomic sequence and integrate the construct into the correct location in the genome.

(a) *T. brucei* DHH1 ORF

```
Lister_427      ACATTATCTAGAGTAACCGATGATGATTGGAGGGAAGGTTTGAAAGCGCCAACTAAAGAT 60
Insert_1        ACATTATCTAGAGTAACCGATGATGATTGGAGGGAAGGTTTGAAAGCGCCAACTAAAGAT 60
                ************************************************************

Lister_427      GTGCGAAAGAAGACAGAAGACGTGGAGTCTCGGCGTAATGTTACCTTTGAGGAGTATGGG 120
Insert_1        GTGCGAAAGAAGACAGAAGACGTGGAGTCTCGGCGTAATGTTACCTTTGAGGAGTATGGG 120
                ************************************************************

Lister_427      CTCCGACGTGAGCTCCAGATGGGTATCTTCGAGAAGGGCTTCGAACGGCCCAGTCCGGTG 180
Insert_1        CTCCGACGTGAGCTCCAGATGGGTATCTTCGAGAAGGGCTTCGAACGGCCCAGTCCGGTG 180
                ************************************************************

Lister_427      CAGGAGGAGGCCATTCCTGTGGCCCTGCAGGGAAAAGATGTGCTAGCACGTGCCAAAAAC 240
Insert_1        CAGGAGGAGGCCATTCCTGTGGCCCTGCAGGGAAAAGATGTGCTAGCACGTGCCAAAAAC 240
                ************************************************************

Lister_427      GGCACGGGAAAAACGGCTTCCTTCGTCATTCCCGTATTGGAGAAGGTCGACACGCAGTTG 300
Insert_1        GGCACGGGAAAAACGGCTTCCTTCGTCATTCCCGTATTGGAGAAGGTCGACACGCAGTTG 300
                ************************************************************

Lister_427      CCTCATATCCAGGCGCTCCTCATGGTTCCCACTCGCGAACTCGCGCTACAGACGGCTCAG 360
Insert_1        CCTCATATCCAGGCGCTCCTCATGGTTCCCACTCGCGAACTCGCGCTACAGACGGCTCAG 360
                ************************************************************

Lister_427      GTGACAAAAGAGCTTGGCAAGCACATCACCGGGTTGGAGGTTATGGTCACAACTGGTGGA 420
Insert_1        GTGACAAAAGAGCTTGGCAAGCACATCACCGGGTTGGAGGTTATGGTCACAACTGGTGGA 420
                ************************************************************

Lister_427      ACAACACTCCGTGATGATATTTTGCGCCTGCAGAGTGTGGTGCACGTGCTTGTGGCCACA 480
Insert_1        ACAACACTCCGTGATGATATTTTGCGCCTGCAGAGTGTGGTGCACGTGCTTGTGGCCACA 480
                ************************************************************

Lister_427      CCTGGGCGTGCGGTGGATCTCGCTAGTAAAGGGCGGCCGCGGATCCAATACG         532
Insert_1        CCTGGGCGTGCGGTGGATCTCGCTAGTAAAGGGCGGCCGCGGATCCAATACG         532
                ***************************************************
```

(b) *T. brucei* DHH1 5’ UTR

```
lister_427      ACATTATCTAGAGCGGCCGCGTCTACTATAAGAAGGGAAGAAAAATAGTTGTAGACAGCA 60
insert_2        ACATTATCTAGAGCGGCCGCGTCTACTATAAGAAGGGAAGAAAAATAGTTGTAGACAGCA 60
                ************************************************************

lister_427      GATATATACAAAGGGGTAAAAGCAGGAAAGCTACAGGGAAGAGTACAAGTAAGGGAAGCA 120
insert_2        GATATATACAAAGGGGTAAAAGCAGGAAAGCGACAGGGAAGAGTACAAGTAAGGGAAGCA 120
                ***************************** ************************** ***

lister_427      AAAGCATACACTGCGAAGAAGGATTTGCATACCGAAACAAACAATCGGCAACAAAAAGTG 180
insert_2        AAAGCATACACTGCGAAGAAGGATTTGCATACCGAAACAAACAATCGGCAACAAAAAGTG 180
                ************************************************************

lister_427      AAAAGTGTGGGGAAGTGAAGCAGTCTCCATACAAGAATATCACACAACATACGCCCACAG 240
insert_2        AAAAGTGTGGGGAAGTGAAGCAGTCTCCATACAAGAATATCACACAACATACGCCCACAG 240
                ************************************************************

lister_427      TCGCGTATACACCCCCACAGAGAATAATACATCCCAACCTAACAACAGCAACGAATACGG 300
insert_2        TCGCGTATACACCCCCACAGAGAATAATACATCCCAACCTAACAACAGCAACGAATACGG 300
                ************************************************************

lister_427      GATCCAATACG           311
insert_2        GATCCAATACG           311
                ***********
```

(c) *T. congolense* DHH1 ORF

```
IL3000        GTCACTGAAGATGACTGGAAGAAGGGTCTGAACGCGCCAAAGAAGGATGTGCGGAAGAAG  60
Insert_3      GTCACTGAAGATGACTGGAAGAAGGGTCTGAACGCGCCAAAGAAGGATGTGCGGAAGAAG  60
              ************************************************************

IL3000        ACTGAGGATGTTGAGTCTCGTCGTGATGTCACCTTTGAAGAGTATGGCTTGCGCCGTGAG  120
Insert_3      ACTGAGGATGTTGAGTCTCGTCGTGATGTCACCTTTGAAGAGTATGGCTTGCGCCGTGAG  120
              ************************************************************

IL3000        CTTCAGATGGGCATATTTGAGAAGGGCTTCGAACGCCCCAGTCCGGTGCAGGAAGAGGCT  180
Insert_3      CTTCAGATGGGCATATTTGAGAAGGGCTTCGAACGCCCCAGTCCGGTGCAGGAAGAGGCT  180
              ************************************************************

IL3000        ATTCCCGTGGCTTTGCAGGGAAAGGATGTGCTAGCGCGTGCCAAGAATGGTACCGGCAAA  240
Insert_3      ATTCCCGTGGCTTTGCAGGGAAAGGATGTGCTAGCGCGTGCCAAGAATGGTACCGGCAAA  240
              ************************************************************

IL3000        ACCGCATCTTTTGTTATACCTGTCCTGGAGAAGATTGACACGAGTCTCCCTCACATCCAG  300
Insert_3      ACCGCATCTTTTGTTATACCTGTCCTGGAGAAGATTGACACGAGTCTCCCTCACATCCAG  300
              ************************************************************

IL3000        GCGCTGCTGATGGTCCCTACGCGTGAGTTGGCTCTTCAGACCGCGCAGGTGACTAAGGAG  360
Insert_3      GCGCTGCTGATGGTCCCTACGCGTGAGTTGGCTCTTCAGACCGCGCAGGTGACTAAGGAG  360
              ************************************************************

IL3000        CTTGGAAAGCATATCCCTGGTCTCGAGGTGATGGTCACCACCGGCGGCACAACACTCCGT  420
Insert_3      CTTGGAAAGCATATCCCTGGTCTCGAGGTGATGGTCACCACCGGCGGCACAACACTCCGT  420
              ************************************************************

IL3000        GATGATATATTACGCTTGCAGAGCCCTGTGCACGTACTGGTAGCGACTCCGGGTCGAGCG  480
Insert_3      GATGATATATTACGCTTGCAGAGCCCTGTGCACGTACTGGTAGCGACTCCGGGTCGAGCG  480
              ************************************************************

IL3000        GTGGATCTTGCTAGTAAAGG    500
Insert_3      GTGGATCTTGCTAGTAAAGG    500
              ********************
```

(d) *T. congolense* DHH1 5' UTR

```
IL3000     ACATTATCTAGAGCGGCCGCTGTTTCTATTATTGTATGAAATCATCGATTTTCCCCCTCA     60
Insert_4   ACATTATCTAGAGCGGCCGCTGTTTCTATTATTGTATGAAATCATCGATTTTCCCCCGCA     60
           ************************************************************ **

IL3000     AATTGCGAACCAGCTGCACTGGATACAAATGAGGTCAGTTCGCTCCTGCTGCTGTACCCG     120
Insert_4   AATTGCGAACCAGCTGCACTGGATACAAGTGAGGTCAGTTCGCTCCTGCTGCTGTACCCG     120
           *************************** ******************************

IL3000     TTTTCGAGCCAAATGTGTATTTCTTCTTGGATGAAGAAGAAAGAAGAAAAACGAAAAACA     180
Insert_    TTTTCGAGCCAAATGTGTATTTCTTCTTGGATGAAGAAGAAAGAAGAAAAACGAAAAACA     180
           ************************************************************

IL3000     GTGATTATTTTGTGTTTCGCACAACTCTAGTGGAAAATGCAATCAAAAGACAAAAATTTG     240
Insert_    GTGATTATTTTGTGTTTCGCACAACTCTAGTGGAAAATTCAATCAAAAGACAAAAATTTG     240
           ************************************ ********************

IL3000     ACCTAATTCTGCACATCCCTTTATGAATACTAAACTCATGTGAAGTTTCGATAGGTCTAC     300
Insert_4   ACCTAATTCTGCACATCCCTTTATAAATCCTAAACTCATGTGAAGTTTCGATAGGTCTAC     300
           ************************ *** ********************************

IL3000     TAATAAGAAGGAACGTGAGTATTGTAGACGGCCGAGCGAATAAAAGAGAAGCCATAAAAG     360
Insert_4   TAATAAGAAGGAACGTGAGTATTGTAGACGGCCGAGCGAATAAAAGAGAAGACATAAAAG     360
           ************************************************* ********

IL3000     GCACTCTGAAGGAGAAGGTCACATAGCGAATTAAGACAACCGAAACAACATCATACGTGG     420
Insert_4   GCACTCTGAAGGAGAAGGTCACATAGCGAATTAAGACAACCGAAACAACATCATACGTGG     420
           *** ********************************************************

IL3000     GGAAGTTTCCACGGGAAATTCACACATTATACAGTCAGTCACAAATACGTACGTTCCCGC     480
Insert_4   GGAAGTTTCCACGGGAAATTCACACATTATACAGTCAGTCACAAATACGTACGTTCCCGC     480
           ************************************************************

IL3000     GTAGTAAGGAGACCTCAAACCAACAATACCGACGACTACTGGATCCAATACG 532
Insert_4   GTAGTAAGGAGACCTCAAACCAACAATACCGACGACTACTGGATCCAATACG 532
           ************************************************
```

Figure 5.3 Sequencing data for the sequences of the DHH1 gene. Sequences are from the (a) *T. brucei* DHH1 ORF, (b) *T. brucei* DHH1 5' UTR, (c) *T. congolense* DHH1 ORF and (d) *T. congolense* DHH1 5' UTR. Mutations are highlighted. Sequences from the amplified PCR products were inserted into the P-GemT easy vector, then cut out and sequenced, they were compared to the Tb427_100044400 gene in from *T. brucei* Lister strain 427 2018 genome and TcIL3000_10_3320 gene from the *T. congolense* IL3000 genome on TriTrypDB (Aslett *et al.,* 2009). Sequencing performed by Dundee DNA sequencing and services facility.

The sequences of the inserts were amplified using p-GemT easy and digested and sequenced, these were then aligned with the genomic sequence from *T. brucei* lister 427 and *T. congolense* IL300 2018, using the genes found on TriTrypDB (Aslett *et al., 2008*), the strain used for culturing (figure 5.3). There are no mutations in the DHH1 ORF sequences (figure 5.3. a and c), and some mutations in the UTRs (figure 5.3 b and d), highlighted in the sequences. These sequences were determined to be usable as there was no mutations in the ORF sequence and few mutation in the UTR sequence, it is expected that there will be variance in the UTR sequences. They were digested and cut out of the p-GEM backbone and ligated into the pEnNmSt0-N backbone. Cut sites for restriction enzymes *BamH*I and *Xho*I had been incorporated to flank the sequences in the pGEM vector, and these were used to digest the inserts and pEnNmSt0-N backbone.

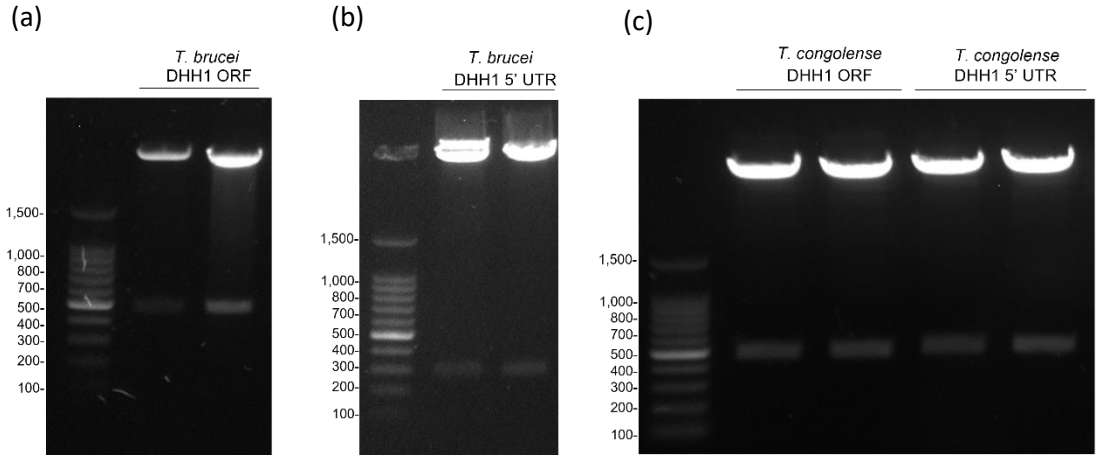

Figure 5.4 Agarose gels of analytical restriction enzyme digests of plasmids containing DHH1 sequences. Inserts are from (a) *T. brucei* DHH1 ORF, (b) *T. brucei* 5' UTR and (c) *T. congolense* DHH1 ORF and 5' UTR. Constructs were digested with restriction enzymes and run on an agarose gel to ensure an insert of the correct size had been successfully ligated into the backbone, the size was expected to be 532 bp for all inserts except the *T. brucei*DHH1 5' UTR, which is 314bp. Ladder labels are in bp.

The UTR was ligated into the backbone, then sent for sequencing to confirm there were no mutations, then the ORF was ligated in, and the final plasmid was sequenced again. Final plasmids were treated with restriction enzymes *Xba*I and *BamH*I or *Xho*I (see appendix) for 2 hours, which cuts out the inserts, these were then run on an agarose gel which confirmed they were the correct size (figure 5.4). For the DHH1 inserts, all were the correct size in *T. brucei* and *T. congolense*. Once confirmed, plasmids were sent off for sequencing a final time. The plasmids could then be prepared for transformations into trypanosomes.

This process was repeated to make constructs incorporating sequences from ZC3H11 and XRNA genes into the pEnNY0 backbone in both *T. brucei* and *T. congolense*. For all completed constructs, and analytical digest was performed by incubating 3 µg of each plasmid with the corresponding restriction enzymes (see appendix) to ensure inserts of the correct size had been ligated into the backbone. The plasmid for XRNA in *T. brucei* could not be completed due to a recurring point mutation occurring in the insert for the ORF of the gene.

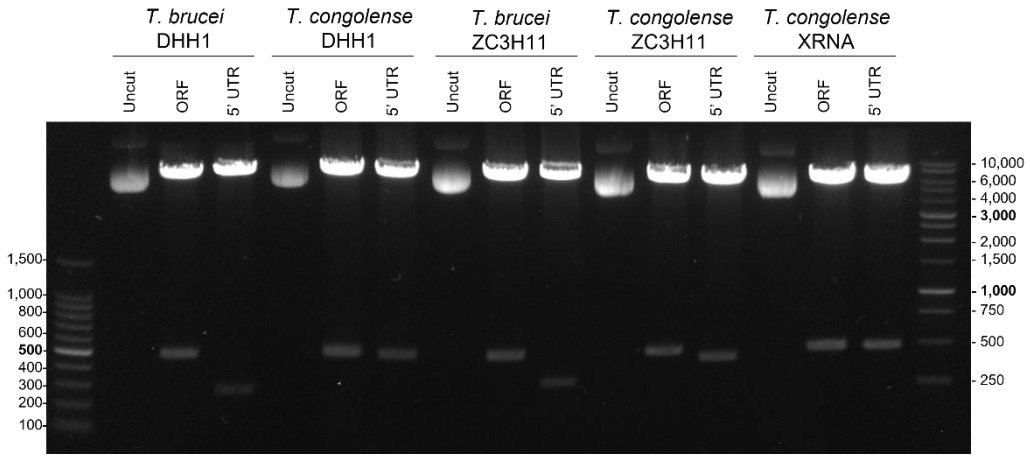

Figure 5.5 Agarose gel of all completed constructs. Undigested and digested constructs were run on an agarose gel to show that inserts of the correct size have been ligated into the backbone. Ladder labels are in bp.

All inserts cut out in the analytical digest (figure 5.5) were the correct size, so these plasmids could be used for transfections into trypanosomes.

5.2.4 Generating cell lines

The N-terminal tagging plasmids were transfected into either 2T1 *T. brucei* or TcoSM *T. congolense* cells to generate genetically modified cell lines. The constructs are linearised with *Not*I restriction enzyme, and the plasmid is arranged in a way that allows the inserted sequences from the 5' UTR and ORF of the gene of interest to homologously recombine with the ORF and UTR in the genome, a diagram is shown in figure 5.6. This fuses a fluorescence protein to the N-terminus of the protein of interest, creating a fluorescent tag to enable its expression to be detected. It also inserts a resistance gene into the trypanosome for whether G418 or hygromycin, so these can be used to select clones where successful recombination has occurred.

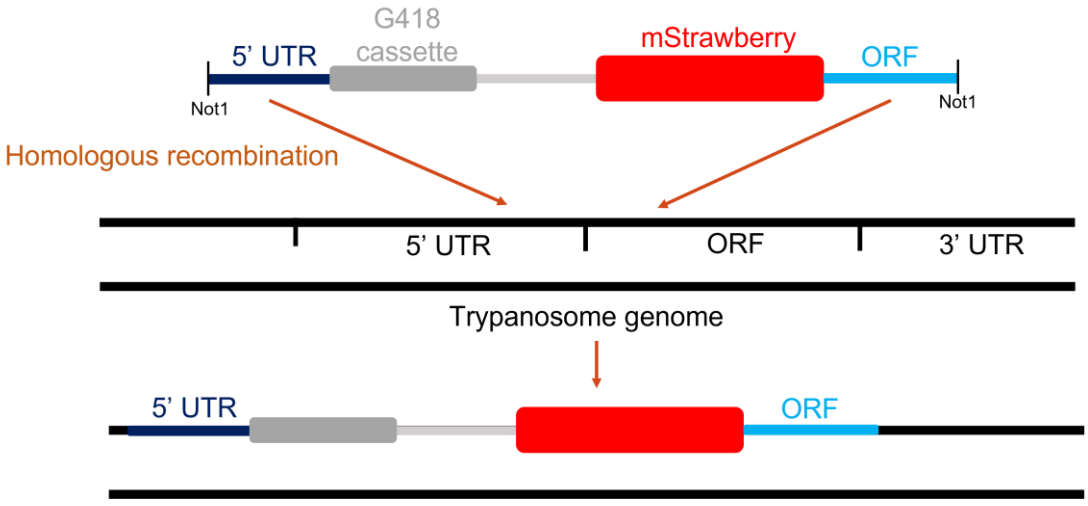

Figure 5.6 Diagram of the linearised plasmid and how it recombines into the trypanosome genome.

Transfections into *T. brucei* cells were performed using standard conditions, but as transfections in *T. congolense* cells have not previously been performed in this lab, conditions had to be optimised. For the first transfection using G418 drug, all the negative control cells, which had not been transformed with any plasmid DNA, were able to survive G418 treatment. Drug treatments were then screened against *T. congolense* cells to determine which drug concentration caused cells to die, results are shown in table 5.2.

Table. 5.2 Concentration of drugs tested for *T. congolense* cells

Drug	**Concentration**	**Time taken for cells to die (days)**
G418	0.02 µg/ml	7
	0.04 µg/ml	3
Hygromycin	0.025 µg/ml	7
	0.05 µg/ml	4
	0.075 µg/ml	3

A concentration of 0.4 µg/ml was chosen for G418 during transfections and selection which was then decreased to 0.2 µg/ml when clones are transferred into flasks. For hygromycin a concentration of 0.5 µg/ml was chosen.

Overall, 4 cell lines were successfully generated through transfections, these are shown in table 5.3. A transfection to generate the cell line for eYFP-XRNA in *T. congolense* was attempted, and clones were seen during the screening phase, however when these were

transferred to flasks the cell lines did not survive. The concentration of hygromycin to use for *T. congolense* transfections must still be optimised.

Table 5.3 Clones generated from transfections

Cell line	Organism	Number of Successful clones	Selection drug	Concentration of drug
mSt-DHH1	*T. brucei*	4	G418	2.5µg/ml
eYFP-ZC3H11	*T. brucei*	4	Hygromycin	2.5µg/ml
mSt-DHH1	*T. congolense*	2	G418	0.2-0.4 µg/ml*
eYFP-ZC3H11	*T. congolense*	4	Hygromycin	0.25-0.5 µg/ml*
eYFP-XRNA	*T. congolense*	0	Hygromycin	0.5 µg/ml

*Different concentrations of drug were used for initial selection and then maintenance of the cell line

5.2.5 Validation of mSt-DHH1 cell lines

Once successful transfections had been performed and clonal populations had been established, cells had to be analysed to confirm that the correct gene had been tagged with a fluorescent protein. Firstly, gDNA was extracted from the GM cell lines and a PCR was performed using a forward primer that binds upstream of the modified gene and a reverse

primer that either binds to a section in the drug resistance cassette in the GM gene or the ORF which represents the wild type gene (Fig 5.7a). It was expected that both products could be able to be amplified from template gDNA from the modified cell lines as the construct should only recombine with one allele of the gene.

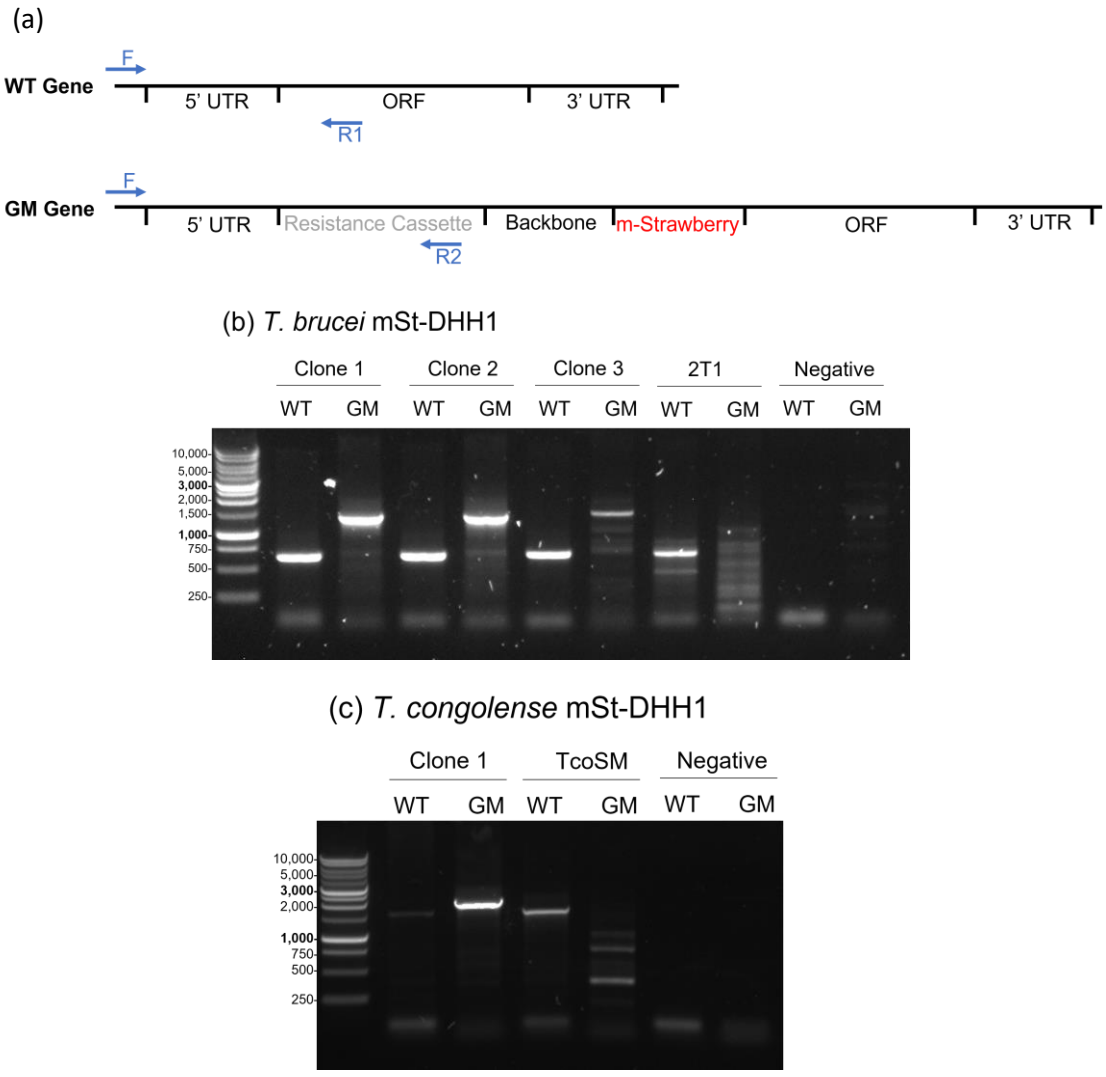

Figure 5.7 PCR amplification of genetically modified DHH1 genes. In order to validate cell lines, primers were designed to amplify sections of the wild type and genetically modified DHH1 gene, (a) schematic shown. (b) For *T. brucei* clones the expected band size for the WT gene is 625 bp and GM gene is 1,370bp, (c) for *T. congolense* clones expected band size for the WT gene is 1,149 bp and GM gene is 2,056bp. Ladder labels are in bp.

All cell lines contained one allele of the wild type gene and one allele of the tagged gene (figure 5.7). Primers were designed to amplify a section of 1,370 bp in *T. brucei* and 2,056 bp in *T. congolense* from the genetically modified gene, containing a sequence from the G418 resistance cassette and directly upstream from the gene. This ensures that the resistance cassette, and plasmid as a whole, has been inserted into the correct location in the genome. All clones were able to amplify this section. All clones also were able to amplify the wild type gene with primers designed to amplify a section containing the 5' UTR and some of the ORF of the DHH1 gene which is 625 bp in *T. brucei* and 1,149 bp in *T. congolense*. As all clones could also amplify this section, it shows that the recombination has occurred in only one allele, as expected. Positive controls using the wild type gDNA were only able to amplify the WT gene as expected, through the TcoSM gDNA had a lower DNA concentration, so the band is faint.

Next, western blots were performed to confirm the expression of DHH1 with m-Strawberry. Whole cell lysates from transfection clones were prepared and then run on an SDS-PAGE gel, these were then transferred, and western blots were stained with α-mCherry antibody to detect the presence of m-Strawberry, as these proteins contain the same epitope that the antibody binds to.

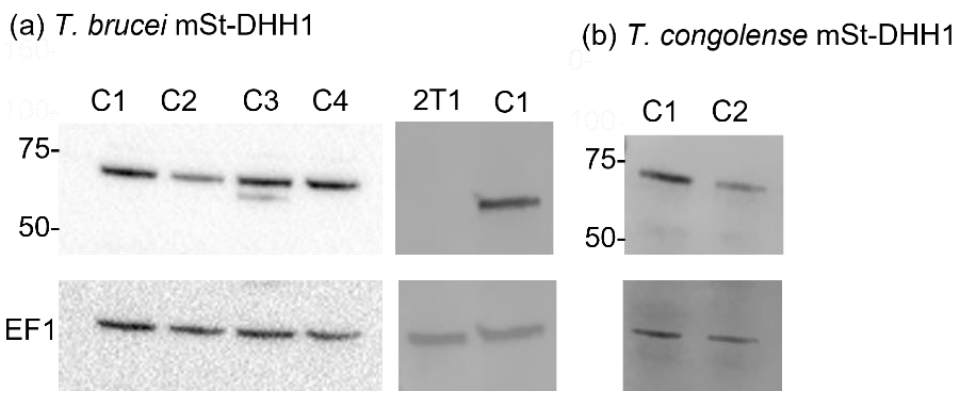

Figure 5.8 Western blots of mSt-DHH1. (a) *T. brucei* mSt-DHH1 cell lines, (b) *T. congolense* mSt-DHH1 cell lines Cell lysates were prepared of all clones expressing mSt-DHH1 in both *T. brucei* and *T. congolense*. Western blots were stained with α-mCherry antibody and fluorescent secondary antibody, then visualised using a LI-COR odyssey FC, or with a HRP secondary and visualised on a chemidoc. EF1α protein was used as a loading control, an α-EF1α primary antibody and fluorescent or HRP secondary antibody was used. Ladder labels are in kDa and C1 refers to clone 1.

The westerns performed show that all clones are expressing a tagged form of DHH1 (figure 5.8). DHH1 tagged with m-strawberry is predicted to migrate to 72 kDa in both *T. brucei* and *T. congolense*, and this is what is seen on the western blots. Clone 3 of the *T. brucei* cell lines has a slightly smaller band which may be a degradation product, clones 1, 2 and 4 were chosen for any further analysis. As for *T. congolense*, both clones are expressing the protein. A control is shown for *T. brucei* , parental 2T1 cells have no band when probed with α-mCherry, unfortunately due to issues with western blots, this was the only control which could be performed.

5.2.6 Validation of eYFP-ZC3H11 cell lines

To validate the *T. brucei* and *T. congolense* cell lines expressing eYFP tagged ZC3H11, PCRs were performed as in 1.6.1. Genomic DNA was extracted from all cell lines and primers were designed to amplify sections of both the wild type and genetically modified gene (Figure 5.9).

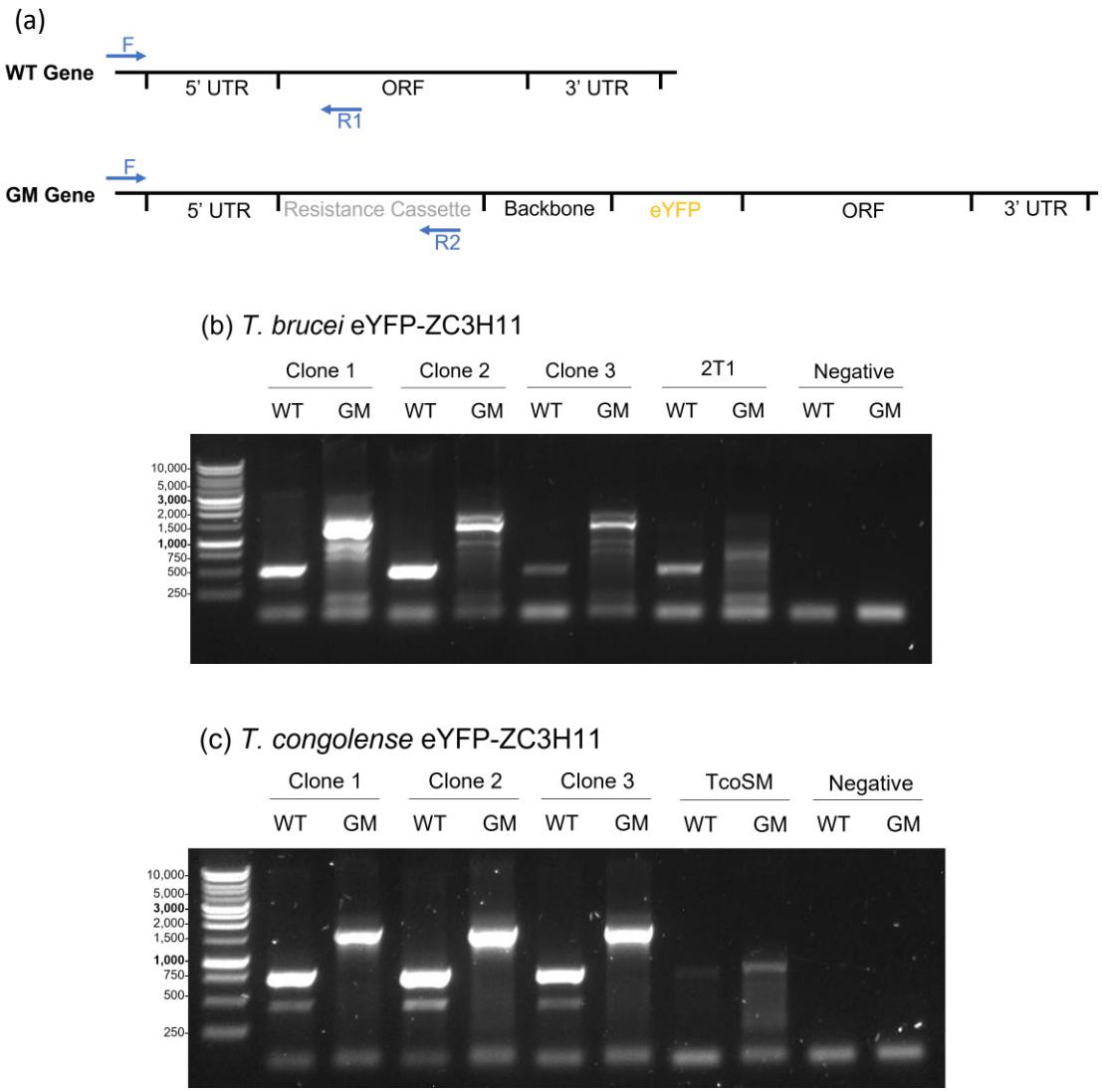

Figure 5.9 PCR amplification of genetically modified ZC3H11 genes. In order to validate cell lines, primers were designed to amplify sections of the wild type and genetically modified ZC3H11 gene, (a) schematic shown. (b) For *T. brucei* clones the expected band size for the WT gene is 512 bp and GM gene is 1,323bp, (c) for *T. congolense* clones expected band size for the WT gene is 783 bp and GM gene is 1,634bp. Ladder labels are in bp.

As sections from both the WT and GM gene could be amplified by PCR (figure 5.9), all cell lines contain both versions of the gene. The target sizes for these amplicons were 1,323 bp in *T. brucei* and 1,634 bp in *T. congolense* for the WT gene and 512 bp in *T. brucei* and 783 bp in *T. congolense* for the GM gene. The parental cell lines, 2T1 and TcoSM contain the WT gene but the GM gene, also negative controls with no gDNA show no amplification. This validates that the plasmid has been inserted into the correct part of the genome as the forward primer in these reactions is upstream of the gene of interest.

Next, western blots were performed using whole cell lysates of cells from each cell lines to detect if the cell lines are expressing the tagged version of ZC3H11. These were run on and SDS-PAGE and transferred to western blots and stained with an α-GFP antibody which should bind to the eYFP tag on ZC3H11.

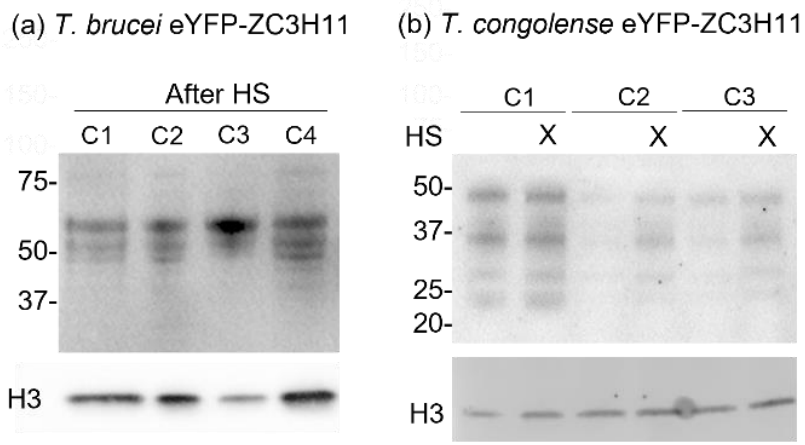

Figure 5.10 Western blots of mSt-DHH1. Cell lysates were prepared of all clones expressing eYFP-ZC3H11in both (a) *T. brucei* and (b) *T. congolense*. Cells were treated with heat shock at 41 °C for one hour so they would increase expression of ZC3H11. Western blots were stained with α-GFP antibody and HRP-conjugated secondary antibody, then visualised using a chemidoc. H3 protein was used as a loading control, an α-H3 primary antibody and HRP-conjugated secondary antibody was used. Ladder labels are in kDa C1 refers to clone 1.

It is known that ZC3H11 is expressed at very low levels in non-stressed PCF and BSF cells, so cells were subjected to heat shock and then whole cell lysates were prepared. A more specific femto ECL substrate kit had to be used to detect any protein on these western blots for both *T. brucei* and *T. congolense*, when this is used, bands can be seen. In *T. brucei* it is expected that eYFP-ZC3H11 will migrate to 65 kDa, the bands on the blot appear to range from 50-60 kDa, there are 3 distinct bands which may represent post translational modifications or degradation of the protein. In *T. congolense* the protein is expected to migrate to 45 kDa, there is a strong band at about 48 kDa and 36 kDa and weaker smaller bands.

5.3 Discussion

5.3.1 Summary of results

Overall 5 constructs were successfully generated and 4 were transformed into trypanosomes. These cell lines are expressing mSt-DHH1 in both *T. brucei* and *T. congolense* and eYFP-ZC3H11 in both *T. brucei* and *T. congolense*, and all were validated through PCR and western blot. These cell lines will be used for further analysis to investigate how these tagged proteins respond during the heat shock response and in the recovery period after.

Table 5.4 Completed plasmids for N-terminal tagging

Protein	Tag	Plasmid	Cell line
DHH1 (*T. brucei*)	m-Strawberry	Complete	Generated
ZC3H11 (*T. brucei*)	eYFP	Complete	Generated
XRNA (*T. brucei*)	eYFP	Not complete	Not generated
DHH1 (*T. congolense*)	m-Strawberry	Complete	Generated
ZC3H11 (*T. congolense*)	eYFP	Complete	Generated
XRNA (*T. congolense*)	eYFP	Complete	Not generated

Due to time constraints the constructs planned for C-terminal tagging could not be created, these are HSP100 and PABP1, which both respond to the heat shock response. In future these constructs should be created and transfected into BSF *T. brucei* and *T. congolense* trypanosomes to see their response.

5.3.2 Optimisation of *T. congolense* transfections

Genetic manipulation in *T. congolense* is a relatively new procedure and this work represents the first time *T. congolense* transfections were attempted and successfully performed in this laboratory. For fluorescent tagging in this project, the same plasmid backbones were used for *T. congolense* as in *T. brucei*, but sections of the *T. congolense* specific-sequences were cloned in, this was done according to the procedures outlined in Awuah-Mensah *et al.,* 2021. For the transfections themselves, the procedure between *T. brucei* and *T. congolense* differs

and even in the literature different procedures are used as outlined in table 5.5, so these had to optimised to find a successful procedure for *T. congolense* transfections.

Table 5.5 Differences in procedures for *T. congolense* transfections

Condition	**Awuah-Mensah *et al.*, 2021**	**Steketee *et al.*, 2021**	**Used in this project**
Plasmid DNA	10 µg	12 µg	10 µg
Number of cells	2-3 × 10^7	4 × 10^7	3.5 × 10^7
Drug concentrations	0.05 µg/ml puro 0.02 µg/ml G418 0.05 µg/ml hygromycin	0.05 µg/ml puro for transfection, 0.025 µg/ml for maintenance 0.04 µg/ml G418 for transfection, 0.02 µg/ml for maintenance	0.05 µg/ml hygromycin, 0.025 µg/ml for maintenance 0.04 µg/ml G418 for transfection, 0.02 µg/ml G418 for maintenance

The first attempt at *T. congolense* transfections was unsuccessful, a plasmid for Tub-B RNAi was used as this has been successfully performed before. This first attempt failed as the X-001 programme was used, and control cells did not die, but also cells couldn't survive for a long period of time in the concentrations of drug used, which was 0.05 µg/ml hygromycin and 0.04 µg/ml G418. After this, drug concentrations used were tested in order to

successfully transfect *T. congolense* cells, as described in table 5.2, once drug concentrations were determined, transfections were attempted again.

The second attempt used plasmids for mSt-DHH1, eYFP-ZC3H11 and eYFP-XRNA fluorescent tagging and the procedure outlined in table 5.5. All transfections successfully generated clones in 96-well plates, however when transferred to flasks, the clones for eYFP-ZC3H11 and eYFP-XRNA were not growing at the normal rate in 0.5 µg/ml hygromycin, and unfortunately the clones for eYFP-XRNA died out. Drug concentration was lowered to 0.25 µg/ml, but still needs to be fully optimised to ensure clones can grow at a normal rate but there is still enough drug to ensure selection.

Overall, successful cloning and transfections were performed in *T. congolense* cells, generating the first genetically modified *T. congolense* cell lines in this laboratory. Though some optimisation is still needed, this was still very successful.

5.3.3 ZC3H11 Western Blots

When ZC3H11 from *T. brucei* cells was visualised on a western blot it formed a banding pattern with 4 distinct bands ranging from 50 – 60 kDa (figure 5.10). This likely represents different phospho-states and possibly degradation products of the protein. In the literature, different banding patterns have been shown for this protein, examples in figure 5.10.

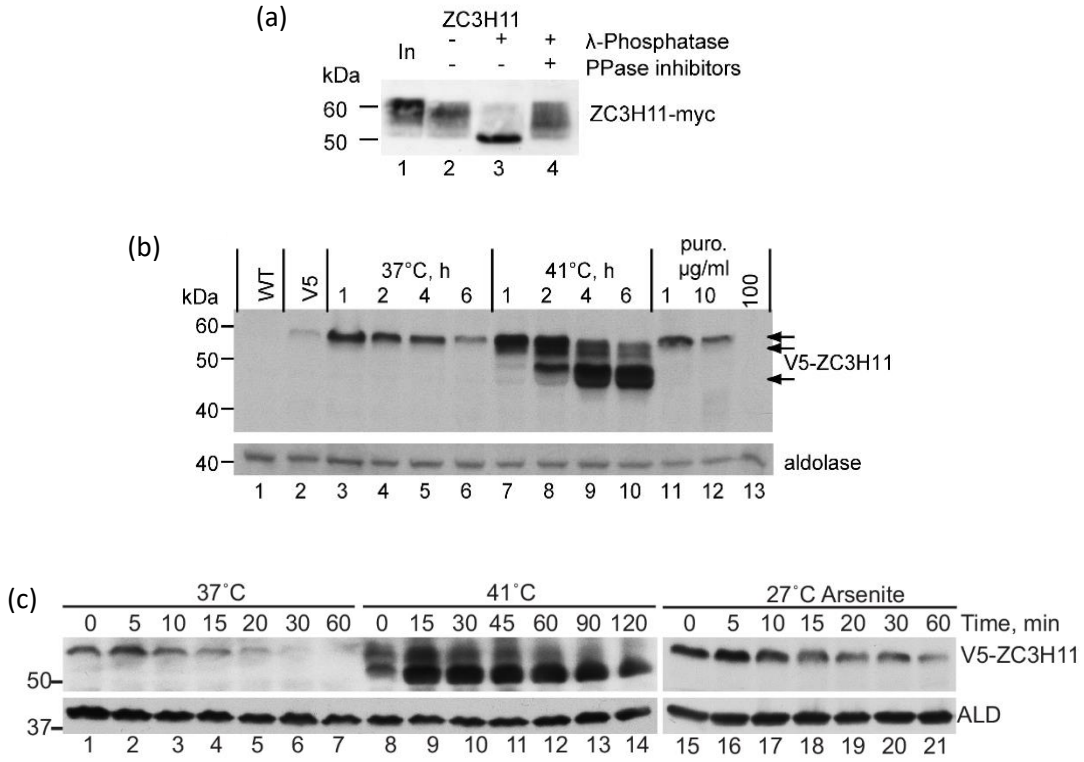

Figure 5.11 ZC3H11 western blots from previous literature. (a) and (b) was adapted from Droll *et al.,* 2013, cells were treated with a phosphatase and phosphatase inhibitor, or heat shock and puromycin treatment, prior to whole cell lysate preparation, then probed for ZC3H11-myc on a western blot. (b) was adapted from Minia and Clayton., 2016, and shows cells heat shocked at 41 °C and treated with arsenite to simulate stress, prior to western blot and probe for V5-ZC3H11.

ZC3H11 with a myc tag or V5 tag has been shown to migrate to 60 kDa, but appears as a smear. When treated with a phosphorylase, the bands collapse into one band at 50 kDa, this can then be returned to the normal state with a phosphatase inhibitor (figure 5.11 a), from this it was concluded that the bands are the protein at different phospho-states, it is known that ZC3H11 has at least 4 phosphorylation sites (Ooi *et al*., 2020). When cells undergo heat shock at 41 °C, this band at 50 kDa can also be seen (figure 5.11 b), so it was suggested the

protein is dephosphorylated upon heat shock. Another experiment (figure 5.11 c) found a similar banding pattern.

The bands seen in figure 5.10 do resemble those seen by Droll *et al*., 2013, though are more defined. ZC3H11 has been tagged with eYFP, which is a much larger protein, so the size of the bands differs. Repeating the phosphatase treatment would be useful to confirm if each of the bands are phospho-states of the protein. This was attempted, but due to difficulties with western blots it could not be completed.

6 Examining the heat shock response using tagged cell lines

6.1 Introduction

Cell lines that express fluorescently tagged proteins involved in the heat shock response, DHH1 and ZC3H11, in both *T. brucei* and *T. congolense* have been successfully created and validated (Chapter 5). Here, these cell lines are used to examine how the tagged proteins respond during heat shock and in the recovery period afterwards.

DHH1 has been shown to re-localise into P-bodies upon heat shock in PCF cells (Kramer *et al*., 2008), but this has not been repeated in BSF *T. brucei* trypanosomes or to our knowledge ever investigated in *T. congolense*. This protein can be examined using fluorescence microscopy to observe any changes in localisation upon induction of heat shock.

ZC3H11 has been shown to increase in abundance upon heat shock and it is usually undetectable in non-stressed cells (Droll *et al*., 2013). The abundance can be investigated using western blotting to quantify the number of proteins in cells and any changes in the isoforms present, by looking at the banding pattern.

Due to time constraints, only initial characterisation was possible and for many experiments additional repeats are needed to be certain of any conclusions. Data has been presented here to stimulate discussion and acts as an early insight into results.

6.2 Results

6.2.1 Re-localisation of DHH1 in BSF *T. brucei*

DHH1 has been shown to re-localise into P-bodies upon heat shock treatment in PCF cells (Kramer *et al*., 2013). Cells under normal conditions showed some foci, but the number increased upon heat shock at 41 C for 1 h and remained increased for up to 4 hours upon return to normal culture after heat shock. To investigate how DHH1 protein responds to heat shock in BSF *T. brucei* and *T. congolense* cells, slides were made before and after heat shock at 41°C for 1h for three different clones. This would show if the same localisation is seen and if the response is consistent across all clones.

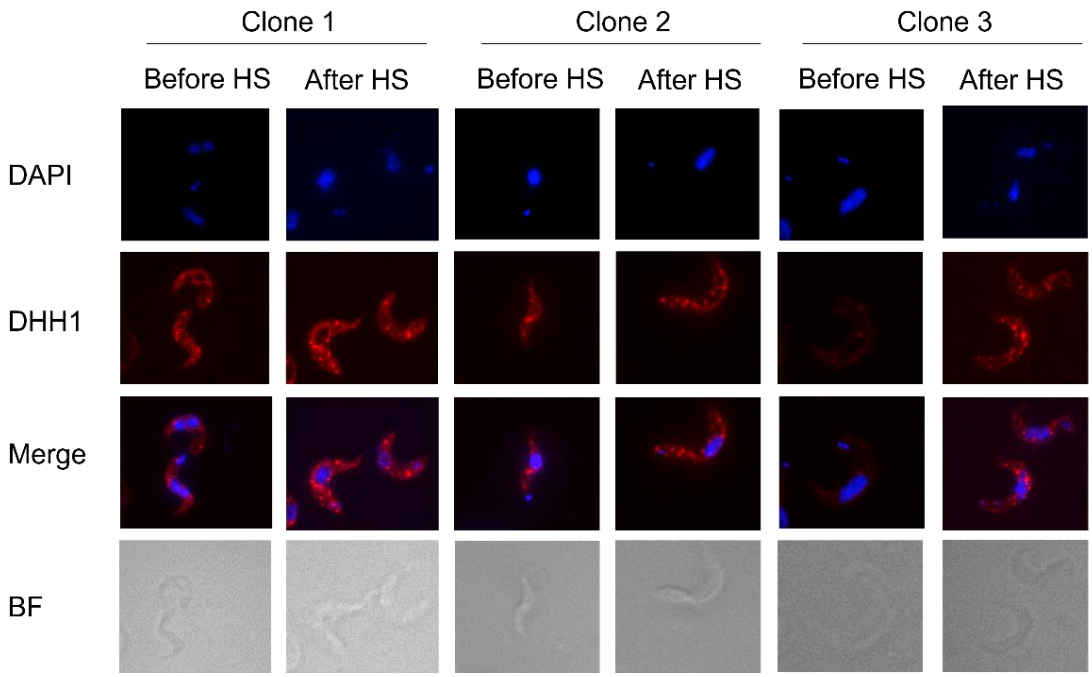

Figure 6.1 *T. brucei* clones expressing mSt-DHH1 before and after heat shock at 41 °C. Cells were exposed to heat shock at 41 °C for 1 hour and slides were prepared with 5 x 10^6 cells, fixed with 4% PFA for 5 minutes and then flouroshield mounting medium was used to stain DAPI. Images were taken using a Leica DM RXA2 fluorescence microscope and edited with ImageJ Fiji software.

In *T. brucei* BSF cells, DHH1 appears to re-localise into locales within the cytoplasm upon heat shock, in cells before heat shock, DHH1 does localise into foci, and upon heat shock, the number of foci appear to increase in all clones (figure 6.1). This consistently happens in all clones, though clone 4 appears to be expressing DHH1 at a lower level in the control cells, however these still re-localise into distinct foci upon heat shock. The cells shown in the image are representative of the population, however there was variation in some slides, so these results need to be quantified by counting the number of foci in each cell to have more confidence in the conclusions.

Next, to investigate how long the punctate localisation persisted, a time course was performed and slides were made every 2 hours after heat shock. As clones showed similar results, clone 1 was used for further experiments.

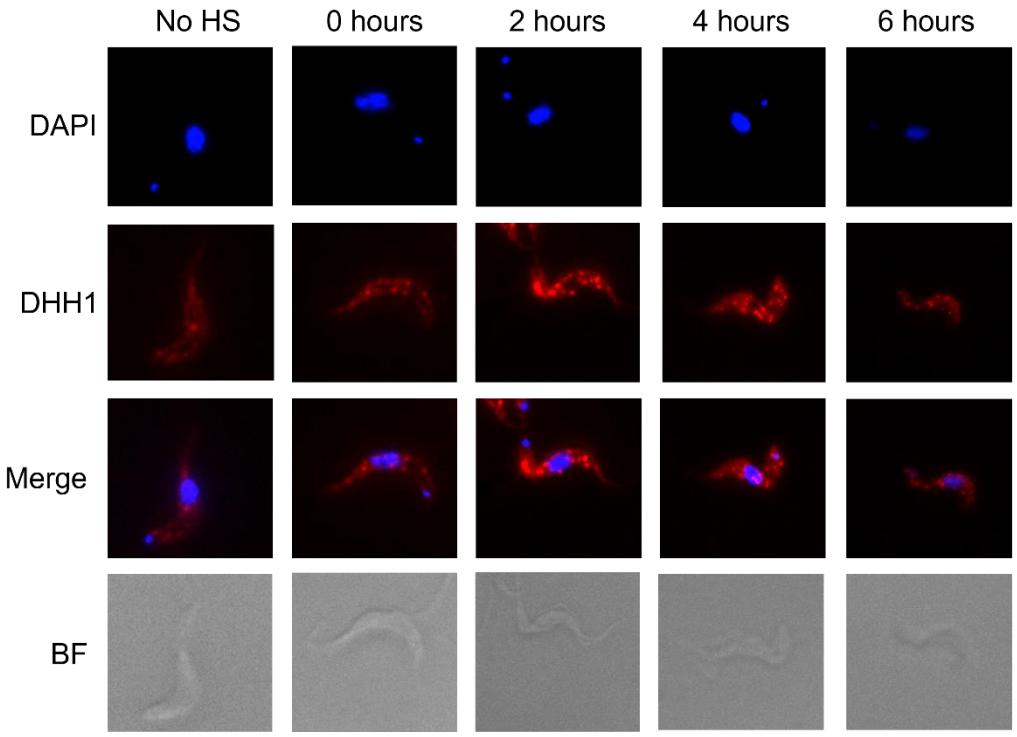

Figure 6.2 *T. brucei* expressing mSt-DHH1 recovery after heat shock at 41 °C. Cells were exposed to heat shock at 41 °C for 1 hour and slides were prepared every 2 hours after heat shock. 5 x 10^6 cells were used per slide, these were fixed with 4% PFA for 5 minutes and then flouroshield mounting medium was used to stain DAPI. Images were taken using a Leica DM RXA2 fluorescence microscope and edited with ImageJ Fiji software.

The phenotype of increased foci containing DHH1, assumed to be P-bodies, was observed to remain in the 6h period where cells are recovering from heat shock (Figure 6.2). At 2, 4 and 6 hours after heat shock most cells in the population still had an increased number of foci. Cells at 2 and 4 hours had an even brighter signal, which could mean the cells are expressing more DHH1, and this appears to decrease at 6 hours. However, this could also be due to variation in the signal seen on the microscope and bleaching of the mStrawberry fluorescent protein.

While localisation of DHH1 has been previously investigated (Kramer *et al.*, 2008), it is not known if the amount of protein increases in the recovery period after heat shock. To quantify the amount of DHH1 in the cells, whole cell lysates were prepared and western blots were performed. Samples were taken for 8 hours after heat shock and also at 24 hours.

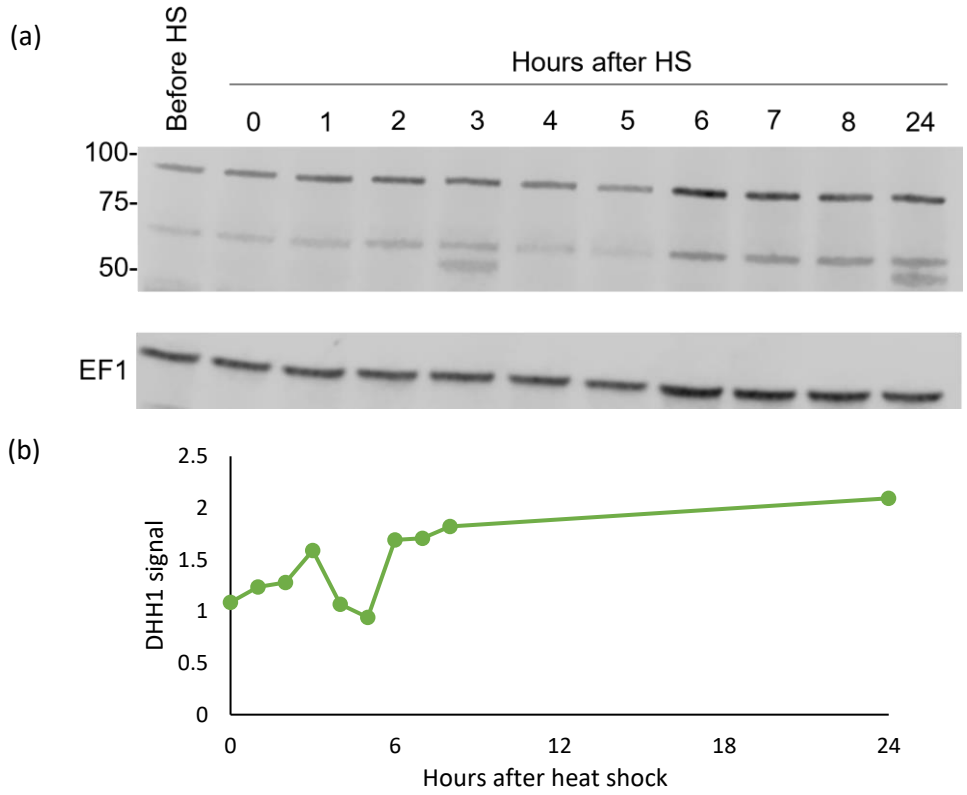

Figure 6.3 Western blot of DHH1 signal during heat shock recovery. *T. brucei* cells expressing mSt-DHH1 cells were heat shocked at 41 °C for 1 hour and whole cell lysate samples were prepared at hourly timepoints, using 2 x 10^6 cells per sample. These were run by SDS-PAGE and transferred to a PVDF membrane. α-mCherry antibody was used as it binds to the mStrawberry tag on the DHH1 protein, and a fluorescent secondary antibody was used, blots were imaged using a LICOR. EF1α was used as a loading control, with α-EF1α primary antibody. The trace in (b) was made by normalising all DHH1 signals to their loading control, and then normalising all to the before heat shock sample, so that a value of 1 is equal to the control sample. Ladder labels are in kDa.

The expected size for mSt-DHH1 is 72 kDa, and there is a band this size on the western in figure 6.3, so this protein was used to make a trace of DHH1 signal in the cells. The trace is normalised so that a signal of 1 represents the signal of the control sample, and anything higher than 1 is an increase from normal levels. This trace suggests that in for 3 hours after heat shock DHH1 signal increases, and then experiences a sharp decrease at 4 and 5 hours. Then the signal increases again and remains increased at 24 hours. The western shows 2 bands at 3 and 24 hours after heat shock which could represent a degradation product. There is an unexpected band at 95 kDa, this was not seen in previous western blots using this cell line (see chapter 5) and is present in all samples including the control. This band was not included in analysis as it was only seen in this experiment.

6.2.2 Re-localisation of DHH1 in BSF *T. congolense*

T. congolense clones expressing mSt-DHH1 were imaged before and after heat shock to see if DHH1 re-localises in a similar way in this species of trypanosome.

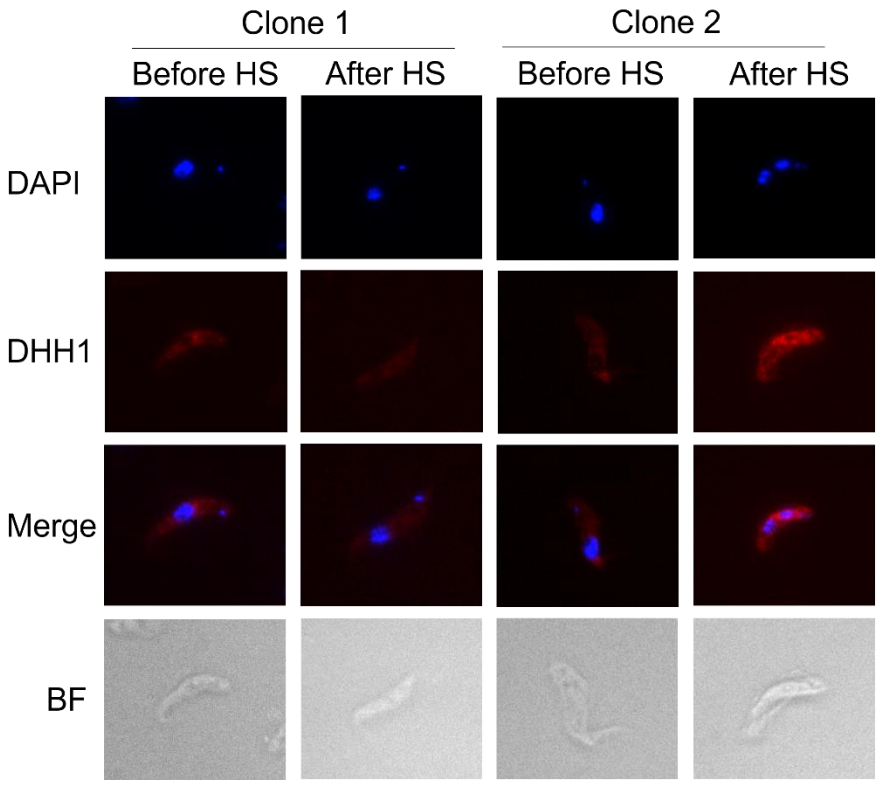

Figure 6.4 *T. congolense* clones expressing mSt-DHH1 before and after heat shock at 41 °C. Cells were exposed to heat shock at 41 °C for 1 hour and slides were prepared with 5 x 10^6 cells, fixed with 4% PFA for 5 minutes and then flouroshield mounting medium was used to stain DAPI. Images were taken using a Leica DM RXA2 fluorescence microscope and edited with ImageJ Fiji software.

In *T. congolense* BSF cells, DHH1 displays a cytoplasmic signal in all clones before and after heat shock (figure 6.4). The response in the 2 clones does not appear to be the same, with clone 1 showing little change and a possible decrease in signal. Clone 2 shows some areas of increased intensity and some limited in foci, but they do not appear the same as the distinct foci seen in *T. brucei* cells. Next, a time course was using clone 1 to see if this signal changed throughout the recovery period after heat shock

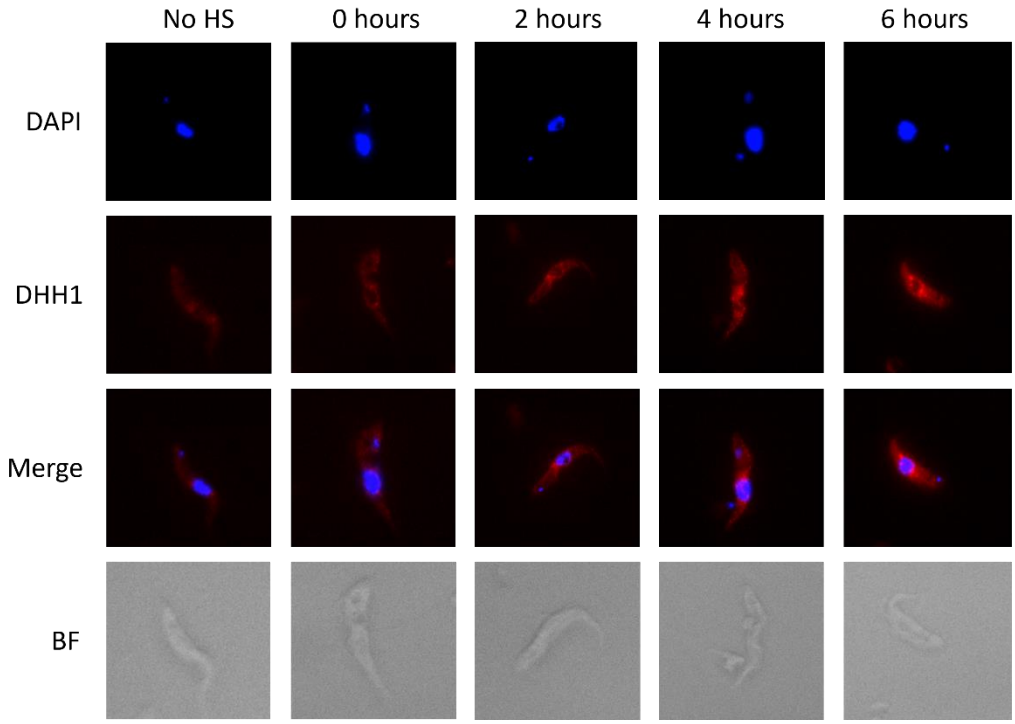

Figure 6.5 *T. congolense* expressing mSt-DHH1 recovery after heat shock at 41 °C. Cells were exposed to heat shock at 41 °C for 1 hour and slides were prepared every 2 hours after heat shock. 5 x 10^6 cells were used per slide, these were fixed with 4% PFA for 5 minutes and then flouroshield mounting medium was used to stain DAPI. Images were taken using a Leica DM RXA2 fluorescence microscope and edited with ImageJ Fiji software.

DHH1 in *T. congolense* cells heat shocked at 41 °C remains mostly cytoplasmic straight after heat shock and in the 6 hours afterwards (figure 6.5). There are some foci seen in all samples, but a cytosolic background can be seen in all images, the number of foci does not appear to increase. However the overall signal does increase in cells at 4 and 6 hours after heat shock, this could be due to an increase in DHH1 in the cells, or could be due to variations in the fluorescent signal picked up by the microscope. Further analysis by western blot will be needed to confirm that this is an increase in protein levels.

6.2.3 ZC3H11 response in BSF *T. brucei*

To investigate levels of ZC3H11 in *T. brucei* cells in the recovery period after heat shock, a western blot was prepared using whole cell lysates. Cells were subjected to heat shock at 41 °C for 1 hour and samples were made every hour for 8 hours after return to normal culture conditions with a final sample made at 24 hours.

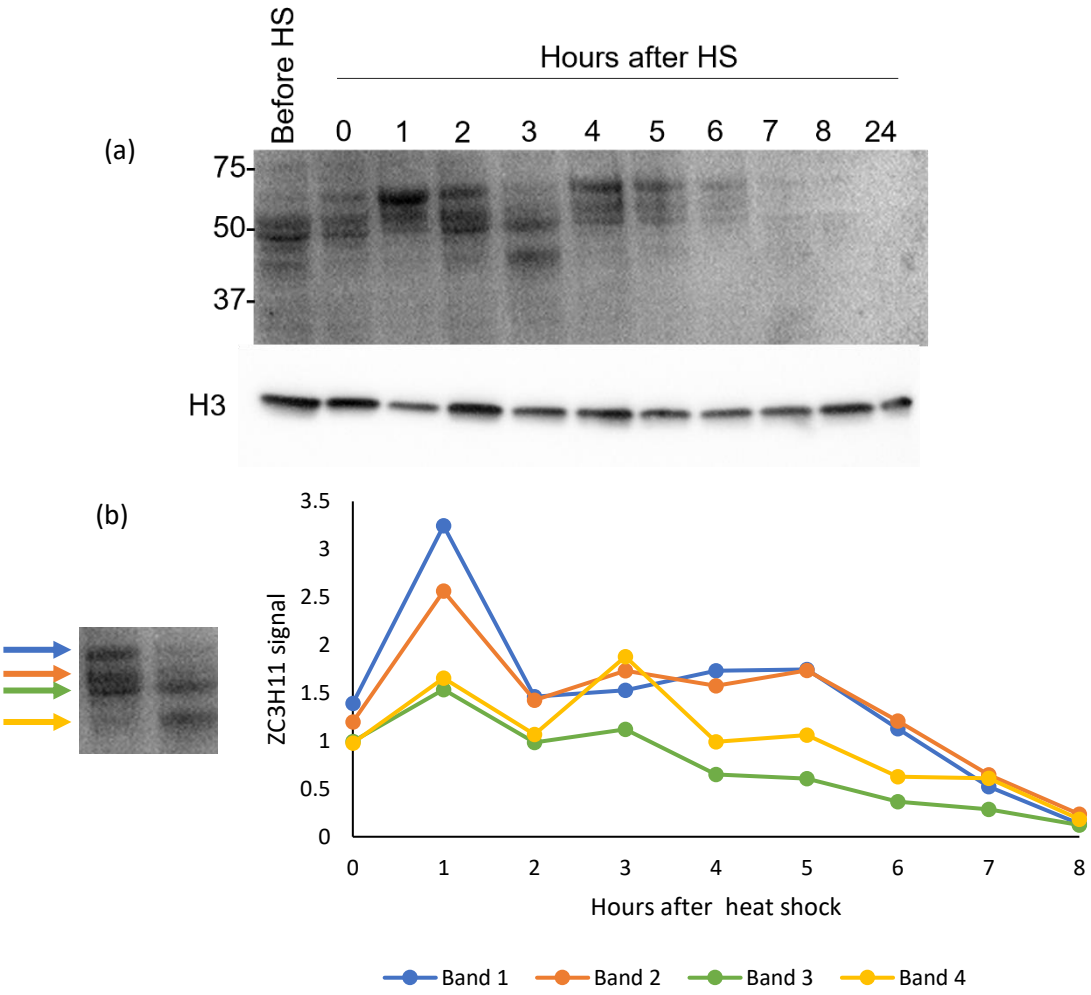

Figure 6.6 Western blot of ZC3H11 signal during heat shock recovery. (a) *T. brucei* cells expressing eYFP-ZC3H11 cells were heat shocked at 41°C for 1 hour and whole cell lysate samples were prepared at hourly timepoints, using 2 x 10^6 cells per sample. These were run by SDS-PAGE and transferred to a PVDF membrane. α-GFP antibody was used as it binds to the YFP tag on the ZC3H11 protein, blots were imaged using a chemidoc. The trace in (b) was made by normalising all ZC3H11 signals to their loading control, and then normalising all to the before heat shock sample, so that a value of 1 is equal to the control sample. Ladder labels are in kDa.

The western blot of ZC3H11 shows a banding pattern with 4 separate bands (figure 6.6), which represent different post translation modification states of ZC3H11 and possibly degradation products. The expected size of eYFP-ZC3H11 is 65 kDa, there is a band at this size, but also 2 smaller bands around 50 kDa and another at 45 kDa. The general trend of the bands suggests and increase of protein and change in phosphorylation state after heat shock, it is particularly odd that the sample before heat shock shows higher protein than 6, 7, 8 and 24 hours after heat shock. It is possible that the protein is actively degraded after the heat shock response, but more repeats would be needed to confirm this.

6.3 Discussion

6.3.1 Summary of results

It appears that DHH1 in BSF *T. brucei* cells does re-localise into foci within the cell, possibly P-bodies upon heat shock, and they remain for 6 hours after heat shock. In *T. congolense* this is not seen, and the signal remains mainly cytoplasmic, although the protein may increase in abundance. The IF images shown in these results are representative of the populations seen on the slides, however in all experiments this will need to be quantified to truly draw conclusions for results. This can be done by counting the number of foci cells contain across a population of cells.

The westerns shown for quantifying DHH1 and ZC3H11 will have to be repeated in triplicate to see if the same response is seen. While the banding pattern in ZC3H11 is similar to what was seen by Droll *et al.,* 2013, experiments should be performed to determine what isoform each band is. Overall the results from the westerns shown are inconclusive, and further repeats and experiments are needed. The results shown in this chapter are only preliminary, but enough of a response is seen to start a discussion.

6.3.2 Response of DHH1

The response of DHH1 during heat shock had previously been shown in PCF cells, it re-localises into P-bodies which remain for 4 hours after heat shock. These granules are present in non-stressed cells, but increase in number upon heat shock (Kramer *et al*., 2008). In BSF cells, there are more foci containing DHH1 in none-stressed cells (figure 6.1), the number of foci increases upon heat shock and this effect remains for at least 6 hours (figure 6.2), and the level of protein also possibly increases during this time. This result was expected as while a change in localisation can happen instantly after heat shock, it would take longer for an increase in protein to take place. It is possible that pathways for increased DHH1 are triggered upon heat shock, which take 4 hours to take effect. The accumulation of foci occurs at the same time as the cell cycle arrest seen in chapter 4, which peaks at 4-5 hours after heat shock in *T. brucei* cells. It makes sense that the accumulation of foci occurs at the same time as a cell cycle arrest, as they are thought to be sites of mRNA storage and degradation (Kramer *et al*., 2008).

In *T. congolense*, this effect is not seen, there are not distinct foci in non-stressed or heat shocked cells, both show a cytoplasmic localisation. This shows that DHH1 protein behaves differently in *T. congolense* cells, different proteins may be involved in P-bodies and the stress response in these cells. It is likely that 41 °C is not the correct temperature to trigger DHH1 re-localisation fully, whether this is because the temperature is too high, and cells are unhealthy or too low, so the heat shock response is not fully induced. DHH1 re-localisation will have to be investigated at different temperatures to determine this.

6.3.3 Analysis of western blots

The western blot results, though flawed, indicate that at 3 hours after heat shock there is a change in both DHH1 and ZC3H11. At this timepoint DHH1 has a band that may be a degradation product, and ZC3H11 shows very different banding, which may also be a degradation product, or a large change in phosphorylation state. This timepoint matches up with the arrest at 4-5 hours seen in *T. brucei*, and it could be theorised that at 3 hours there is an important checkpoint in the response that leads to the arrest at 4-5 hours. The amount of DHH1 appears to reduce at 4 and 5 hours, which would agree with the idea that at 3 hours it is being degraded, however after this it returns to high levels. ZC3H11 returns to the normal banding pattern at 4 and 5 hours, and after that the amount of protein appears to reduce.

More work is needed to confirm the responses of DHH1 and ZC3H11 and truly draw any conclusions. Starting with ensuring no bands appear when probing cell lysates of the parental cell lines 2T1 and TcoSM, and repeating experiments a minimum of 3 times, in multiple clones, this could not be performed due to issues with western blots (see section 7.3.2). Phosphatase experiments should also be performed, as described in section 5.3.3, to be able to determine what the different ZC3H11 bands represent, which will be useful to draw a conclusion of how the protein is responding to the heat shock response.

7 General discussion

7.1 Studying the heat shock response in BSF *T. brucei* and *T. congolense*

The heat shock response is an important virulence factor for trypanosomes, and it is especially relevant for BSF parasites as the heat shock response is triggered to survive the high temperatures the parasites are exposed to during the fever elicited by the mammalian host immune system. Previous work showed that *T. brucei* cells trigger the heat shock response when exposed to 41 °C for 1 hour, there is a lag in growth for 24 hours after heat shock and a halt in protein production and formation of heat shock granules. The key regulator of the response, ZC3H11 has been identified and a mechanism for its action in stabilising mRNAs of proteins needed in the heat shock response was proposed (Singh *et al.,* 2014). Most of this work was done in PCF cells, so this work has begun to characterise this response in clinically relevant BSF *T. brucei* and *T. congolense* cells.

Genetic tools for *T. congolense* have recently been developed (Awuah-Mensah *et al.*, 2021), making this the perfect time to study the biological processes of this species of trypanosome. Much of the previous work has focussed on a broader scale, looking at the systems in *T. congolense* cells, such as their metabolism (Steketee er al., 2021), genome (Jackson *et al.*, 2012b) and gene expression (Silvester *et al*., 2018) . Currently, no work has been published investigating the heat shock response in *T. congolense*.

The aim of this work was to characterise the BSF *T. congolense* heat shock response, and also the BSF *T. brucei* response, and to compare the two. This was done by placing cells in a water bath at 41 °C and 42 °C for varying amount of time to simulate heat shock, and then investigating growth and cell cycle arrest. Then cell lines expressing fluorescent DHH1 and ZC3H11, key proteins involved in the heat shock response, were developed. These were used to investigate the behaviour of these proteins during and after the heat shock response.

7.2 Analysis of results

One major conclusion of the results in chapters 4 and 6 are that *T. brucei* and *T. congolense* may display more differences in the heat shock response than expected. They show different kinetics of growth after heat shock, and while *T. brucei* shows a clear arrest in G2/M, with 75% of cells in this life cycle stage at 4 hours after heat shock, which resolves fully by 24 hours, *T. congolense* shows a less defined arrest, which doesn't appear to fully resolve. It may be that *T. congolense* is unhealthy at 41 °C and 42 °C , shown by the large amount of abnormal cell types (<G1 and >G2), even though the lag in growth is less severe. Overall, more investigation is needed at lower temperatures to try and find a point where we can be sure *T. congolense* is undergoing a successful heat shock response.

An issue with this data is that the strain used, IL3000, does not grow at 37 °C (Coustou *et al.*, 2010) which is the temperature used to culture *T. brucei* cells, and the ambient temperature in the mammalian bloodstream. This could mean an adaption has arisen in this strain during lab culture which caused a change to the heat shock response pathway. There are some IL3000 lines able to grow at 37 °C, which should be investigated.

T. brucei and *T. congolense* cells were genetically modified to tag DHH1 and ZC3H11 proteins with fluorescent tags, allowing western blots and fluorescent microscopy to be performed. In *T. brucei* BSF cells, DHH1 re-localises into foci immediately after heat shock and these foci remain for up to 6 hours. This corresponds with the arrest seen, and it is likely that the foci represent sites of mRNA storage and degradation as the cell shuts down unnecessary pathways, such as growth, which leads to a cell cycle arrest.

T. congolense did not show such a clear re-localisation of DHH1, which is most likely because 41 °C is not the optimal temperature for these cells to trigger the heat shock response, there was some increase in foci, which may represent a partial triggering of the response.

This was the first time this arrest has been investigated. It has been previously theorised that the heat shock response in *T. brucei* may be linked to cell cycle phase differentiation (Minia *et al.*, 2016). It is widely known that cells undergoing stumpy differentiation arrest in G1 (Reuner *et al.*, 1997). *Leishmania* cells arrest in G1 when transitioning from the fly host to the mammalian host (Wiesgigl and Clos, 2001), and it is known that heat shock is a trigger for this differentiation. It could be theorised that heat shock is also linked to differentiation in *T. brucei* . Though it is not the direct trigger, as it is known cells use a quorum sensing like mechanism to trigger differentiation (Silvester *et al.*, 2017), it may be an enhancer for this process. The cell cycle arrest in G2/M shown in section 4 may be linked to differentiation between cell cycle phases.

Another link between heat shock and cell differentiation is that ZC3H11 has been shown to be upregulated in stumpy form cells (Silvester *et al*., 2018). While zinc finger proteins are commonly linked to developmental events in *T. brucei* , ZC3H11 was the only zinc finger protein of those investigated in this study to be upregulated in these cells, and it has otherwise only been linked to the heat shock response. Interestingly, a different zinc finger protein (TcIL3000_0_11070), which is not an orthologue of ZC3H11, was upregulated in *T. congolense* cells, this protein is uncharacterised. This could suggest that ZC3H11 has different functions in the 2 species, the results of this study cannot confirm or deny this, as little analysis was able to perform with the *T. congolense* eYFP-ZC3H11 cell line.

7.3 Limitations

7.3.1 Unexpected differences between *T. brucei* and *T. congolense*

One limitation of this work which took some time to troubleshoot was unexpected differences in *T. congolense* cells. A major difference that was found is that the standard protocol for preparing *T. brucei* samples for flow cytometry could not be used for *T.*

congolense, it led to a very small number of whole cells that could be stained with the PI stain. Many protocols were tried, using Hoechst stain, PFA fixative instead of methanol and testing out different lengths of fixation time. It was eventually found that fixing the cells in 70% methanol causes them to burst. They have to be fixed in 50% methanol to avoid cell lysis, this was then increased to 70% after 1 hour to maintain the quality of the fixed sample.

Another difference is preparing whole cell lysate samples, as seen in figures 5.8 and 5.10, the loading bands for *T. congolense* samples contains less protein than *T. brucei* . For all western samples 2×10^6 cells were used. It is unlikely that *T. congolense* cells express much less H3 and EF1α proteins as these are important housekeeping genes. It is more likely that some cells are lost in the process of preparing samples, but it is not currently known at which stage this occurs. It may be necessary to use more cells to prepare *T. congolense* whole cell lysate samples.

T. congolense transfections also still need to be optimised. The concentration of G418 used to grow transfected cell lines varies in the literature (Steketee *et al*., 2021;Awuah-Mensah *et al*., 2021), and it was found in this work that switching using 0.4 µg/ml for the transfection and clone selection and then 0. 2 µg/ml for maintaining clones worked. A similar method may have to be used for hygromycin, it was found that transfections were successful, and clones could be selected using 0.5 µg/ml, however clones grew very slowly in this concentration of drug. Cells were grown in 0.025 µg/ml, however, even then they did not appear to be growing at the normal rate, different concentrations still need to be tested.

7.3.2 Immunofluorescent tags

For immunofluorescence analysis, DHH1 was tagged with mStrawberry protein. A limitation of using this fluorescent protein is that it bleaches very quickly, while images could be taken of the protein using the Leica DM RXA2 microscope, the brightness of the fluorescence signal

was not always consistent even when imaging cells from the same slides. This causes an issue as it means the signal detected may not be consistent, and an increase or decrease seen in different samples may actually be due to bleaching of the protein, and not represent a change in DHH1 levels in the cells. It has been found that red fluorescent proteins with a similar structure to mStrawberry bleach quickly (Drobizhev *et al.,*, 2022), so it may be necessary to use a different fluorescent protein, such as eYFP or GFP, as these are more stable. For all of the experiments looking into DHH1 localisation, results need to be quantified and repeated to be able to draw any conclusions, the work shown in this project is just an early indication of the proteins response during heat shock.

7.3.2 Western blots

Western blots were used to detect the amount of protein present in cells. ZC3H11 is one of the proteins investigated. As this protein is present in such low levels in cells, it had to be detected using a highly sensitive ECL substrate, used to detect HRP conjugated antibodies. While this substrate worked the first time it was used, problems were encountered later on. When visualising westerns on the chemidoc system, the signal was far too strong and detected every protein in the sample, and caused ghosting of the ZC3H11 bands, an example is shown in figure 7.1.

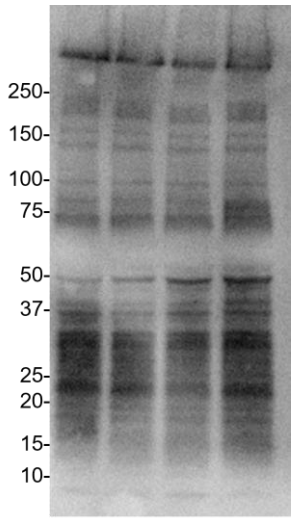

Figure 7.1 Western blot showing ghosting. Whole cell lysates were prepared and run on an SDS-PAGE, then transferred via western blotting. α-GFP antibody was used to bind to the eYFP-ZC3H11 protein, and a femto kit ECL substrate was used for visualisation on a BioRad Chemidoc MP. Ladder labels are in kDa

The western blot shown above had an exposure time of 1 second, dark bands for all proteins appeared straight away, as well as a white band at the expected size of eYFP-ZC3H11 (64 kDa). To try to find the cause of this issue different antibodies and concentrations, blocking times and detection systems were tested but this problem could not be resolved within the timeframe of the project. This meant that no further investigations into the ZC3H11 protein, such as repeating the time course after heat shock, and incubating with phosphatase, could be performed.

7.4 Future work

There are many directions for future work after this project. The main thing that still needs to be investigated is if *T. congolense* exhibits a clear cell cycle arrest and restart, with cells accumulating in G2/M and then moving into G1, at lower temperatures. The experiments should be repeated at 40 °C and possibly lower, to see if this can be achieved. It would also be interesting to see whether PCF cells show this arrest, as most of the previous work on the

heat shock response was done in these cells. The Il3000 strain of *T. congolense* may have adapted in the lab causing a change to its heat shock response, as it cannot be cultured at the typical temperature of 37 °C. To look into what has caused this, the cell cycle of cells grown at 37 °C could be analysed with flow cytometry, to see if there is a growth arrest before the population crash.

When looking at the cell cycle arrest using flow cytometry, one problem is the data is the fact that cells that were alive at the time of fixation cannot be differentiated from dead cells. A live dead stain could be used in to separate the cell cycles of just the live cells in the sample, this could show whether the arrest is a sign of healthy cells that will survive after heat shock, if it is only seen in live cells, or if it is linked to cell death.

When looking at the cells in an arrested state using IF, many cells were in either the 1N2K2F and 1N2K2F stages of the cell cycle. It is known that cells arrest in G2/M but more clarity could be provided through further experiments. One example is using an antibody that distinguishes nuclei undergoing mitosis, such as the α-H3Me$_2$ antibody used b Benz *et al*., 2017. This could further show what is occurring in these arrested cells, and at what point they are unable to continue moving through their cell cycle. It is unknown if this arrest is caused by damage to the cells or is a defensive mechanism that allows them to survive heat shock. Arrested cells may either die off as they are unable to repair the cell damage, or they may be the population that survives heat shock.

In general , this work has shown that looking into the hours after heat shock is important, as the cells will have delayed effects from triggering the heat shock response. Due to the nature of trypanosome gene regulation, with post transcriptional modifications being the dominant mechanism for altering protein levels in response to stress, it will take some time for changes in protein level to take place. Further work into the heat shock response should look into later timepoints, especially at 4 hours after heat shock at 41 °C in *T. brucei* and timepoints

around it, looking into cells just before arrest may highlight pathways needed for cells to survive, that are crucial to the heat shock response.

7.5 Conclusions

Overall, this work is some of the first looking into specific pathways in *T. congolense*. Some of the kinetics of the heat shock response were shown and an unexpected difference between *T. brucei* and *T. congolense* was found. More *T. congolense* cells die when exposed to heat shock at 41 °C and 42 °C, however there is less of a lag of growth in the period afterwards.

An arrest in G2/M was also shown in response to heat shock was also shown in both *T. brucei* and *T. congolense*, this has previously not been investigated. This arrest is important as it supports the idea that the heat shock response may be linked to differentiation of cells, with the ambient temperature of the host being a trigger that cells detect which is part of initiating differentiation. This system is seen in *Leishmania*, and it is possible that this arrest is part of preparing for differentiation of cells.

Two proteins were successfully tagged in both species, and DHH1 shows a clear re-localisation in *T. brucei* cells after a heat shock of 41 °C, but not *T. congolense*, which further suggests that the heat shock response is not being triggered. While lots of work could not be performed due to time constraints, there are many directions to go in from here. Constructs were made that need to be transfected into cells and proteins investigated, and different temperatures and timepoints can still be investigated.

This work represents a success in *T. congolense* culture and genetic manipulation in this lab. This was the first time in the lab that *T. congolense* was analysed using flow cytometry and western blot, and the first successful transfection of this cell line. Many techniques were tested and optimised, which will pave the way for more work moving forward.

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9 References

Adl, S. M., Simpson, A. G. B., Lane, C. E., Lukeš, J., Bass, D., Bowser, S. S., Brown, M. W., Burki, F., Dunthorn, M., Hampl, V., Heiss, A., Hoppenrath, M., Lara, E., Gall, L. Le, Lynn, D. H., McManus, H., Mitchell, E. A. D., Mozley-Stanridge, S. E., Parfrey, L. W., Pawlowski, J., Rueckert, S., Shadwick, L., Schoch, C. L., Smirnov, A. and Spiegel, F. W. (2012) 'The revised classification of eukaryotes', *Journal of Eukaryotic Microbiology*. 59(5), pp. 429–514. doi: 10.1111/j.1550-7408.2012.00644.x.

Aminu, S., Ibrahim, M. A., Dada Chechet, G. and Onyike, E. (2022) 'Chemotherapeutic potentials of β-ionone against Trypanosoma congolense infection: Inhibition of parasite proliferation, anemia development, trans-sialidase (TconTS3 and TconTS4) gene expressions, and phospholipase A 2', *Chemical biology & drug design*. 99(6), pp. 908–922. doi: 10.1111/CBDD.14048.

Archer, S. K., Luu, V.-D., De Queiroz, R. A., Brems, S. and Clayton, C. (2009) 'Trypanosoma brucei PUF9 Regulates mRNAs for Proteins Involved in Replicative Processes over the Cell Cycle'. *PLoS Pathogens.* 5(8), e10000565. doi: 10.1371/journal.ppat.1000565.

Aslett, M., Aurrecoechea, C., Berriman, M., Brestelli, J., Brunk, B. P., Carrington, M., Depledge, D. P., Fischer, S., Gajria, B., Gao, X., Gardner, M. J., Gingle, A., Grant, G., Harb, O. S., Heiges, M., Hertz-Fowler, C., Houston, R., Innamorato, F., Iodice, J., Kissinger, J. C., Kraemer, E., Li, W., Logan, F. J., Miller, J. A., Mitra, S., Myler, P. J., Nayak, V., Pennington, C., Phan, I., Pinney, D. F., Ramasamy, G., Rogers, M. B., Roos, D. S., Ross, C., Sivam, D., Smith, D. F., Srinivasamoorthy, G., Stoeckert, C. J., Subramanian, S., Thibodeau, R., Tivey, A., Treatman, C., Velarde, G. and Wang, H. (2009) 'TriTrypDB: A functional genomic resource for the Trypanosomatidae', *Nucleic Acids Research*. 38(SUPPL.1). doi: 10.1093/nar/gkp851.

Awuah-Mensah, G., McDonald, J., Steketee, P. C., Autheman, D., Whipple, S., D'Archivio, S.,

Brandt, C., Clare, S., Harcourt, K., Wright, G. J., Morrison, L. J., Gadelha, C. and Wickstead, B. (2021) 'Reliable, scalable functional genetics in bloodstream-form Trypanosoma congolense in vitro and in vivo', *PLoS pathogens*. 17(1), e10009224. doi: 10.1371/JOURNAL.PPAT.1009224.

BANKS, K. L. (1978) 'Binding of Trypanosoma congolense to the Walls of Small Blood Vessels*', *The Journal of Protozoology*. 25(2), pp. 241–245. doi: 10.1111/J.1550-7408.1978.TB04405.X.

Barrett, M. P. (1999) 'The fall and rise of sleeping sickness', *Lancet*, 353(9159), pp. 1113–1114. doi: 10.1016/S0140-6736(98)00416-4.

Bengaly, Z., Sidibe, I., Boly, H., Sawadogo, L. and Desquesnes, M. (2002) 'Comparative pathogenicity of three genetically distinct Trypanosoma congolense-types in inbred Balb/c mice', *Veterinary Parasitology*. 105(2), pp. 111–118. doi: 10.1016/S0304-4017(01)00609-4.

Benz, C., Dondelinger, F., McKean, P. G. and Urbaniak, M. D. (2017) 'Cell cycle synchronisation of Trypanosoma brucei by centrifugal counter-flow elutriation reveals the timing of nuclear and kinetoplast DNA replication', *Scientific Reports.* 7(1), pp. 1–10. doi: 10.1038/s41598-017-17779-z.

Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D. C., Lennard, N. J., Caler, E., Hamlin, N. E., Haas, B., Böhme, U., Hannick, L., Aslett, M. A., Shallom, J., Marcello, L., Hou, L., Wickstead, B., Alsmark, U. C. M., Arrowsmith, C., Atkin, R. J., Barron, A. J., Bringaud, F., Brooks, K., Carrington, M., Cherevach, I., Chillingworth, T. J., Churcher, C., Clark, L. N., Corton, C. H., Cronin, A., Davies, R. M., Doggett, J., Djikeng, A., Feldblyum, T., Field, M. C., Fraser, A., Goodhead, I., Hance, Z., Harper, D., Harris, B. R., Hauser, H., Hostetler, J., Ivens, A., Jagels, K., Johnson, D., Johnson, J., Jones, K., Kerhornou, A. X., Koo, H., Larke, N., Landfear, S., Larkin, C., Leech, V., Line, A., Lord, A., MacLeod, A., Mooney, P. J., Moule, S.,

Martin, D. M. A., Morgan, G. W., Mungall, K., Norbertczak, H., Ormond, D., Pai, G., Peacock, C. S., Peterson, J., Quail, M. A., Rabbinowitsch, E., Rajandream, M. A., Reitter, C., Salzberg, S. L., Sanders, M., Schobel, S., Sharp, S., Simmonds, M., Simpson, A. J., Tallon, L., Turner, C. M. R., Tait, A., Tivey, A. R., Van Aken, S., Walker, D., Wanless, D., Wang, S., White, B., White, O., Whitehead, S., Woodward, J., Wortman, J., Adams, M. D., Embley, T. M., Gull, K., Ullu, E., Barry, J. D., Fairlamb, A. H., Opperdoes, F., Barrell, B. G., Donelson, J. E., Hall, N., Fraser, C. M., Melville, S. E. and El-Sayed, N. M. (2005) 'The genome of the African trypanosome Trypanosoma brucei', *Science*. 309(5733), pp. 416–422. doi: 10.1126/SCIENCE.1112642/SUPPL_FILE/BERRIMAN.SOM.PDF.

Bianca, W. (1963) 'Rectal temperature and respiratory rate as indicators of heat tolerance in cattleNo Title', *The Journal of Agricultural Science*, 60(1), pp. 113–120. doi: https://doi.org/10.1017/S0021859600015902.

Blum, J., Nkunku, S. and Burri, C. (2001) 'Clinical description of encephalopathic syndromes and risk factors for their occurrence and outcome during melarsoprol treatment of human African trypanosomiasis', *Tropical Medicine & International Health*. 6(5), pp. 390–400. doi: 10.1046/J.1365-3156.2001.00710.X.

Büscher, P., Cecchi, G., Jamonneau, V. and Priotto, G. (2017) 'Human African trypanosomiasis', *Lancet.* 390(10110), pp. 2397–2409. doi: 10.1016/S0140-6736(17)31510-6.

Capewell, P., Cren-Travaillé, C., Marchesi, F., Johnston, P., Clucas, C., Benson, R. A., Gorman, T. A., Calvo-Alvarez, E., Crouzols, A., Jouvion, G., Jamonneau, V., Weir, W., Lynn Stevenson, M., O'Neill, K., Cooper, A., Swar, N. R. K., Bucheton, B., Ngoyi, D. M., Garside, P., Rotureau, B. and MacLeod, A. (2016) 'The skin is a significant but overlooked anatomical reservoir for vector-borne African trypanosomes', *eLife*. 5(1), e17716. doi: 10.7554/ELIFE.17716.

Carruthers, L. V., Munday, J. C., Ebiloma, G. U., Steketee, P., Jayaraman, S., Campagnaro, G.

D., Ungogo, M. A., Lemgruber, L., Donachie, A. M., Rowan, T. G., Peter, R., Morrison, L. J., Barrett, M. P. and De Koning, H. P. (2021) 'Diminazene resistance in Trypanosoma congolense is not caused by reduced transport capacity but associated with reduced mitochondrial membrane potential', *Molecular Microbiology*. 116(2), pp. 564–588. doi: 10.1111/MMI.14733.

Checchi, F., Filipe, J. A. N., Haydon, D. T., Chandramohan, D. and Chappuis, F. (2008) 'Estimates of the duration of the early and late stage of gambiense sleeping sickness', *BMC Infectious Diseases*, 8(16), pp. 200. doi: 10.1186/1471-2334-8-16.

Clayton, C. (2013) 'The Regulation of Trypanosome Gene Expression by RNA-Binding Proteins Trypanosomes Depend on Post-transcriptional Mechanisms to Regulate Gene Expression', *PLoS Pathogens*, 9(11), e1003680. doi: 10.1371/journal.ppat.1003680

Clayton, C. (2019) 'Regulation of gene expression in trypanosomatids: Living with polycistronic transcription', *Open Biology*. 9(6), e190072. doi: 10.1098/rsob.190072.

da Costa Lima, T. D., Moura, D. M. N., Reis, C. R. S., Vasconcelos, J. R. C., Ellis, L., Carrington, M., Figueiredo, R. C. B. Q. and de Melo Neto, O. P. (2010) 'Functional characterization of three *Leishmania* poly(A) binding protein homologues with distinct binding properties to RNA and protein partners', *Eukaryotic Cell*. 9(10), pp. 1484–1494. doi: 10.1128/EC.00148-10/ASSET/23405719-FE64-4D86-8073-9316BB71F3C0/ASSETS/GRAPHIC/ZEK9990935950007.JPEG.

Coustou, V., Guegan, F., Plazolles, N. and Baltz, T. (2010a) 'Complete in vitro life cycle of Trypanosoma congolense: Development of genetic tools', *PLoS Neglected Tropical Diseases*. 4(3), e618. doi: 10.1371/journal.pntd.0000618.

Coustou, V., Guegan, F., Plazolles, N. and Baltz, T. (2010b) 'Complete in vitro life cycle of Trypanosoma congolense: Development of genetic tools', *PLoS Neglected Tropical Diseases*.

4(3), e618. doi: 10.1371/JOURNAL.PNTD.0000618.

Das, A., Gale, M., Carter, V. and Parsons, M. (1994) 'The protein phosphatase inhibitor okadaic acid induces defects in cytokinesis and organellar genome segregation in Trypanosoma brucei', *Journal of Cell Science*. 107(12), pp. 3477–3483. doi: 10.1242/JCS.107.12.3477.

Davis, M. W. and Jorgensen, E. M. (2022) 'ApE, A Plasmid Editor: A Freely Available DNA Manipulation and Visualization Program', *Frontiers in Bioinformatics*. 4(2), e818619. doi: 10.3389/FBINF.2022.818619.

Dean, S., Sunter, J. D. and Wheeler, R. J. (2017) 'TrypTag.org: A Trypanosome Genome-wide Protein Localisation Resource', *Trends in Parasitology*. 33(2), pp. 80–82. doi: 10.1016/j.pt.2016.10.009.

Delespaux, V., Dinka, H., Masumu, J., Van den Bossche, P. and Geerts, S. (2008) 'Five-fold increase in Trypanosoma congolense isolates resistant to diminazene aceturate over a seven-year period in Eastern Zambia', *Drug Resistance Updates*, 11(6): pp. 205-209. doi: 10.1016/j.drup.2008.10.002.

Dickie, E. A., Giordani, F., Gould, M. K., Mäser, P., Burri, C., Mottram, J. C., Rao, S. P. S. and Barrett, M. P. (2020) 'New Drugs for Human African Trypanosomiasis: A Twenty First Century Success Story', *Tropical Medicine and Infectious Disease,* 5(1), pp. 29. doi: 10.3390/TROPICALMED5010029.

Drobizhev, M., Molina, R. S. and Franklin, J. (2022) 'Multiphoton Bleaching of Red Fluorescent Proteins and the Ways to Reduce It'. *International journal of molecular science.* 23(2), e770. doi: 10.3390/ijms23020770.

Droll, D., Minia, I., Fadda, A., Singh, A., Stewart, M., Queiroz, R. and Clayton, C. (2013) 'Post-

Transcriptional Regulation of the Trypanosome Heat Shock Response by a Zinc Finger Protein', *PLoS Pathogens*. 9(4), e1003286. doi: 10.1371/journal.ppat.1003286.

Edney, E. B. and Barrass, R. (1962) 'The body temperature of the tsetse fly, Glossina morsitans Westwood (Diptera, Muscidae)', *Journal of Insect Physiology*. 8(4), pp. 469–481. doi: 10.1016/0022-1910(62)90079-3.

Fadda, A., Färber, V., Droll, D. and Clayton, C. (2013) 'The roles of 3'-exoribonucleases and the exosome in trypanosome mRNA degradation', *RNA*. 19(7), pp. 937–947. doi: 10.1261/RNA.038430.113.

Fairlamb, A. H. and Horn, D. (2018) 'Melarsoprol Resistance in African Trypanosomiasis', *Trends in parasitology*. 34(6), pp. 481–492. doi: 10.1016/J.PT.2018.04.002.

Falk, F., Marucha, K. K. and Clayton, C. (2021) 'The EIF4E1-4EIP cap-binding complex of Trypanosoma brucei interacts with the terminal uridylyl transferase TUT3', *PLoS ONE*. 16(11), e0258903. doi: 10.1371/JOURNAL.PONE.0258903.

Fenn, K. and Matthews, K. R. (2007) 'The cell biology of Trypanosoma brucei differentiation', *Current Opinion in Microbiology*. 10(6), pp. 539–546. doi: 10.1016/j.mib.2007.09.014.

Franco, J. R., Cecchi, G., Priotto, G., Paone, M., Diarra, A., Grout, L., Simarro, P. P., Zhao, W. and Argaw, D. (2020) 'Monitoring the elimination of human African trypanosomiasis at continental and country level: Update to 2018', *PLoS Neglected Tropical Diseases*. 14(5), pp. 1–18. doi: 10.1371/JOURNAL.PNTD.0008261.

Gitonga, P. K., Ndung'u, K., Murilla, G. A., Thande, P. C., Wamwiri, F. N., Auma, J. E., Ngae, G. N., Kibugu, J. K., Kurgat, R. and Thuita, J. K. (2017) 'Differential virulence and tsetse fly transmissibility of Trypanosoma congolense and Trypanosoma brucei strains', *The Onderstepoort journal of veterinary research*. 84(1), pp 1-10. doi: 10.4102/OJVR.V84I1.1412.

Haanstra, J. R., Stewart, M., Luu, V. D., Van Tuijl, A., Westerhoff, H. V., Clayton, C. and Bakker, B. M. (2008) 'Control and regulation of gene expression: Quantitative analysis of the expression of phosphoglycerate kinase in bloodstream form Trypanosoma brucei', *Journal of Biological Chemistry*. 283(5), pp. 2495–2507. doi: 10.1074/JBC.M705782200/ATTACHMENT/81AFC491-6F68-4E9E-8504-DCED5A1F582B/MMC1.PDF.

Hammarton, T. C. (2007) 'Cell cycle regulation in Trypanosoma brucei', *Molecular and Biochemical Parasitology*. 153(1), pp. 1–8. doi: 10.1016/J.MOLBIOPARA.2007.01.017.

Hammarton, T. C., Clark, J., Douglas, F., Boshart, M. and Mottram, J. C. (2003) 'Stage-specific differences in cell cycle control in Trypanosoma brucei revealed by RNA interference of a mitotic cyclin', *Journal of Biological Chemistry*. 278(25), pp. 22877–22886. doi: 10.1074/JBC.M300813200.

Hemphill, A., Frame, I. and Ross, C. A. (1994) 'The interaction of Trypanosoma congolense with endothelial cells', *Parasitology*. 109(5), pp. 631–641. doi: 10.1017/S0031182000076514.

Holt, H. R., Selby, R., Mumba, C., Napier, G. B. and Guitian, J. (2016) 'Assessment of animal African trypanosomiasis (AAT) vulnerability in cattle-owning communities of sub-Saharan Africa the LCNTDR Collection: Advances in scientific research for NTD control', *Parasites and Vectors*. 9(1), pp. 1-12. doi: 10.1186/S13071-016-1336-5/FIGURES/4.

Jackson, A. P., Berry, A., Aslett, M., Allison, H. C., Burton, P., Vavrova-Anderson, J., Brown, R., Browne, H., Corton, N., Hauser, H., Gamble, J., Gilderthorp, R., Marcello, L., McQuillan, J., Otto, T. D., Quail, M. A., Sanders, M. J., Van Tonder, A., Ginger, M. L., Field, M. C., Barry, J. D., Hertz-Fowler, C. and Berriman, M. (2012a) 'Antigenic diversity is generated by distinct evolutionary mechanisms in African trypanosome species', *Proceedings of the National Academy of Sciences of the United States of America*. 109(9), pp. 3416–3421. doi:

10.1073/PNAS.1117313109/-/DCSUPPLEMENTAL.

Jackson, A. P., Berry, A., Aslett, M., Allison, H. C., Burton, P., Vavrova-Anderson, J., Brown, R., Browne, H., Corton, N., Hauser, H., Gamble, J., Gilderthorp, R., Marcello, L., McQuillan, J., Otto, T. D., Quail, M. A., Sanders, M. J., Van Tonder, A., Ginger, M. L., Field, M. C., Barry, J. D., Hertz-Fowler, C. and Berriman, M. (2012b) 'Antigenic diversity is generated by distinct evolutionary mechanisms in African trypanosome species', *Proceedings of the National Academy of Sciences of the United States of America*. 109(9), pp. 3416–3421. doi: 10.1073/PNAS.1117313109/-/DCSUPPLEMENTAL.

Jones, A. J., Grkovic, T., Sykes, M. L. and Avery, V. M. (2013) 'Trypanocidal Activity of Marine Natural Products', *Marine Drugs.* 11(10), pp. 4058–4082. doi: 10.3390/MD11104058.

Kedersha, N., Stoecklin, G., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fitzler, M. J., Scheuner, D., Kaufman, R. J., Golan, D. E. and Anderson, P. (2005) 'Stress granules and processing bodies are dynamically linked sites of mRNP remodeling', *Journal of Cell Biology*. 169(6), pp. 871–884. doi: 10.1083/jcb.200502088.

Kennedy, P. G. E. (2019) 'Update on human African trypanosomiasis (sleeping sickness)', *Journal of Neurology*. 266(9), pp. 2334–2337. doi: 10.1007/s00415-019-09425-7.

Kennedy, P. G. E. and Rodgers, J. (2019) 'Clinical and neuropathogenetic aspects of human African trypanosomiasis', *Frontiers in Immunology*. 10(1), pp. 39. doi: 10.3389/FIMMU.2019.00039/BIBTEX.

Kramer, S. (2017) 'The ApaH-like phosphatase TbALPH1 is the major mRNA decapping enzyme of trypanosomes', *PLOS Pathogens*. 13(6), pp. e1006456. doi: 10.1371/JOURNAL.PPAT.1006456.

Kramer, S., Queiroz, R., Ellis, L., Webb, H., Hoheisel, J. D., Clayton, C. and Carrington, M.

(2008) ‘Heat shock causes a decrease in polysomes and the appearance of stress granules in trypanosomes independently of eIF2α phosphorylation at Thr169’, *Journal of Cell Science*. 121(18), pp. 3002–3014. doi: 10.1242/jcs.031823.

Krobitsch, S. and Clos, J. (1999) ‘A novel role for 100 kD heat shock proteins in the parasite *Leishmania* donovani’, *Cell Stress & Chaperones*. 4(3), pp. 191. doi: 10.1379/1466-1268(1999)004<0191:anrfkh>2.3.co;2.

Kumar, S., Tamura, K. and Nei, M. (1994) ‘MEGA: Molecular evolutionary genetics analysis software for microcomputers’, *Bioinformatics*., 10(2), pp. 189–191. doi: 10.1093/bioinformatics/10.2.189.

Kuriakose, S., Onyilagha, C., Singh, R., Olayinka-Adefemi, F., Jia, P. and Uzonna, J. E. (2019) ‘TLR-2 and MyD88-Dependent Activation of MAPK and STAT Proteins Regulates Proinflammatory Cytokine Response and Immunity to Experimental Trypanosoma congolense Infection’, *Frontiers in Immunology.*, 10(1), e2673. doi: 10.3389/FIMMU.2019.02673.

Latif, A. A., Ntantiso, L. and De Beer, C. (2019) ‘African animal trypanosomosis (nagana) in northern KwaZulu-Natal, South Africa: Strategic treatment of cattle on a farm in endemic area.’, *The Onderstepoort Journal of Veterinary Research*. 86(1), pp. e1–e6. doi: 10.4102/OJVR.V86I1.1639.

Mahat, D. B., Salamanca, H. H., Duarte, F. M., Danko, C. G. and Lis, J. T. (2016) ‘Mammalian Heat Shock Response and Mechanisms Underlying Its Genome-wide Transcriptional Regulation’, *Molecular Cell*. 62(1), pp. 63–78. doi: 10.1016/j.molcel.2016.02.025.

Mani, J., Güttinger, A., Schimanski, B., Heller, M., Acosta-Serrano, A., Pescher, P., Späth, G. and Roditi, I. (2011) ‘Alba-Domain Proteins of Trypanosoma brucei Are Cytoplasmic RNA-Binding Proteins That Interact with the Translation Machinery’, *PLOS ONE*. 6(7), p. e22463.

doi: 10.1371/JOURNAL.PONE.0022463.

Minia, I. and Clayton, C. (2016) 'Regulating a Post-Transcriptional Regulator: Protein Phosphorylation, Degradation and Translational Blockage in Control of the Trypanosome Stress-Response RNA-Binding Protein ZC3H11', *PLoS Pathogens*. 12(3), e1005514. doi: 10.1371/journal.ppat.1005514.

Minia, I., Merce, C., Terrao, M. and Clayton, C. (2016) 'Translation Regulation and RNA Granule Formation after Heat Shock of Procyclic Form Trypanosoma brucei: Many Heat-Induced mRNAs Are also Increased during Differentiation to Mammalian-Infective Forms', *PLOS Neglected Tropical Diseases*. 10(9), e0004982. doi: 10.1371/journal.pntd.0004982.

Muhich, M. L., Hsu, M. M. P. and Boothroyd, J. C. (1989) 'Heat-shock disruption of trans-splicing in trypanosomes: effect on Hsp70, Hsp85 and tubulin mRNA synthesis', *Gene*. 82(1), pp. 169–175. doi: 10.1016/0378-1119(89)90042-5.

Ndungu, K., Thungu, D., Wamwiri, F., Mireji, P., Ngae, G., Gitonga, P., Mulinge, J., Auma, J. and Thuita, J. (2019) 'Route of inoculation influences Trypanosoma congolense and Trypanosoma brucei brucei virulence in Swiss white mice', *PLOS ONE*. 14(6), e0218441. doi: 10.1371/JOURNAL.PONE.0218441.

Ooi, C. P., Benz, C. and Urbaniak, M. D. (2020) 'Phosphoproteomic analysis of mammalian infective Trypanosoma brucei subjected to heat shock suggests atypical mechanisms for thermotolerance', *Journal of Proteomics*. 219(1), e103735. doi: 10.1016/j.jprot.2020.103735.

Ooi, C. P., Smith, T. K., Gluenz, E., Wand, N. V., Vaughan, S. and Rudenko, G. (2018) 'Blocking variant surface glycoprotein synthesis alters endoplasmic reticulum exit sites/Golgi homeostasis in Trypanosoma brucei', *Traffic (Copenhagen, Denmark)*. 19(6), pp. 391. doi: 10.1111/TRA.12561.

Peacock, L., Cook, S., Ferris, V., Bailey, M. and Gibson, W. (2012) 'The life cycle of Trypanosoma (Nannomonas) congolense in the tsetse fly', *Parasites and Vectors*. 5(1), pp. 109. doi: 10.1186/1756-3305-5-109.

Priotto, G., Kasparian, S., Mutombo, W., Ngouama, D., Ghorashian, S., Arnold, U., Ghabri, S., Baudin, E., Buard, V., Kazadi-Kyanza, S., Ilunga, M., Mutangala, W., Pohlig, G., Schmid, C., Karunakara, U., Torreele, E. and Kande, V. (2009) 'Nifurtimox-eflornithine combination therapy for second-stage African Trypanosoma brucei gambiense trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial', *The Lancet*. 374(9683), pp. 56–64. doi: 10.1016/S0140-6736(09)61117-X.

Rettig, J., Wang, Y., Schneider, A. and Ochsenreiter, T. (2012) 'Dual targeting of isoleucyl-tRNA synthetase in Trypanosoma brucei is mediated through alternative trans-splicing', *Nucleic Acids Research*. 40(3), pp. 1299–1306. doi: 10.1093/NAR/GKR794.

Reuner, B., Vassella, E., Yutzy, B. and Boshart, M. (1997) 'Cell density triggers slender to stumpy differentiation of Trypanosoma brucei bloodstream forms in culture', *Molecular and Biochemical Parasitology*. 90(1), pp. 269–280. doi: 10.1016/S0166-6851(97)00160-6.

Rojas, F., Silvester, E., Young, J., Milne, R., Tettey, M., Houston, D. R., Walkinshaw, M. D., Pérez-Pi, I., Auer, M., Denton, H., Smith, T. K., Thompson, J. and Matthews, K. R. (2019) 'Oligopeptide Signaling through TbGPR89 Drives Trypanosome Quorum Sensing', *Cell*. 176(1–2), pp. 306-317.e16. doi: 10.1016/J.CELL.2018.10.041.

Sakurai, T., Sugimoto, C. and Inoue, N. (2008) 'Identification and molecular characterization of a novel stage-specific surface protein of Trypanosoma congolense epimastigotes', *Molecular and Biochemical Parasitology*. 161(1), pp. 1–11. doi: 10.1016/J.MOLBIOPARA.2008.05.003.

Shapira, M., McEwen, J. G. and Jaffe, C. L. (1988) 'Temperature effects on molecular

processes which lead to stage differentiation in *Leishmania*.', *The EMBO Journal*.7(9), pp. 2895–2901. doi: 10.1002/J.1460-2075.1988.TB03147.X.

Sharma, A., Cipriano, M., Ferrins, L., Hajduk, S. L. and Mensa-Wilmot, K. (2022) 'Hypothesis-generating proteome perturbation to identify NEU-4438 and acoziborole modes of action in the African Trypanosome', *iScience*. 25(11), e105302. doi: 10.1016/J.ISCI.2022.105302.

Shaw, A. P. M., Cecchi, G., Wint, G. R. W., Mattioli, R. C. and Robinson, T. P. (2014) 'Mapping the economic benefits to livestock keepers from intervening against bovine trypanosomosis in Eastern Africa', *Preventive Veterinary Medicine*. 113(2), pp. 197–210. doi: 10.1016/j.prevetmed.2013.10.024.

Sheila, R., Willem, H., Strauss, M., Linda, K., Fick, G., Maloney, S. K., Carl, L., Meyer, R., Shobrak, M., Fuller, A. and Mitchell, D. (no date) 'Does size matter? Comparison of body temperature and activity of free-living Arabian oryx (Oryx leucoryx) and the smaller Arabian sand gazelle (Gazella subgutturosa marica) in the Saudi desert', *Journal of complete physiology*. 182(1), pp 437-449. doi: 10.1007/s00360-011-0620-0.

Silvester, E., Ivens, A. and Matthews, K. R. (2018) 'A gene expression comparison of Trypanosoma brucei and Trypanosoma congolense in the bloodstream of the mammalian host reveals species-specific adaptations to density-dependent development', *PLOS Neglected Tropical Diseases*. 12(10), e0006863. doi: 10.1371/JOURNAL.PNTD.0006863.

Silvester, E., Young, J., Ivens, A. and Matthews, K. R. (2017) 'Interspecies quorum sensing in co-infections can manipulate trypanosome transmission potential', *Nature microbiolology.* 2(11), pp. 1471–1479. doi: 10.1038/S41564-017-0014-5.

Simarro, P. P., Cecchi, G., Franco, J. R., Paone, M., Diarra, A., Ruiz-Postigo, J. A., Fèvre, E. M., Mattioli, R. C. and Jannin, J. G. (2012) 'Estimating and Mapping the Population at Risk of Sleeping Sickness', *PLoS Neglected Tropical Diseases*. 6(10), e1859. doi:

10.1371/journal.pntd.0001859.

Simarro, P. P., Cecchi, G., Franco, J. R., Paone, M., Diarra, A., Ruiz-Postigo, J. A., Mattioli, R. C. and Jannin, J. G. (2014) 'Mapping the capacities of fixed health facilities to cover people at risk of gambiense human African trypanosomiasis', *International Journal of Health Geographics*, 13(4), pp110-116. doi: 10.1186/1476-072X-13-4.

Singh, A., Minia, I., Droll, D., Fadda, A., Clayton, C. and Erben, E. (2014) 'Trypanosome MKT1 and the RNA-binding protein ZC3H11: Interactions and potential roles in post-transcriptional regulatory networks', *Nucleic Acids Research*. 42(7), pp. 4652–4668. doi: 10.1093/nar/gkt1416.

Sokolova, A. Y., Wyllie, S., Patterson, S., Oza, S. L., Read, K. D. and Fairlamb, A. H. (2010) 'Cross-resistance to nitro drugs and implications for treatment of human african trypanosomiasis', *Antimicrobial Agents and Chemotherapy*., 54(7), pp. 2893–2900. doi: 10.1128/AAC.00332-10/ASSET/14A4B2EC-1D72-4469-B028-96BE52923C34/ASSETS/GRAPHIC/ZAC9991091050006.JPEG.

Steketee, P. C., Dickie, E. A., Iremonger, J., Crouch, K., Paxton, E., Jayaraman, S., Alfituri, O. A., Awuah-Mensah, G., Ritchie, R., Schnaufer, A., Rowan, T., de Koning, H. P., Gadelha, C., Wickstead, B., Barrett, M. P. and Morrison, L. J. (2021) 'Divergent metabolism between Trypanosoma congolense and Trypanosoma brucei results in differential sensitivity to metabolic inhibition', *PLOS Pathogens*. 17(7), e1009734. doi: 10.1371/JOURNAL.PPAT.1009734.

Suganuma, K., N'da, D. D., Watanabe, K. I., Tanaka, Y., Mossaad, E., Elata, A., Inoue, N. and Kawazu, S. I. (2022) 'Therapeutic Efficacy of Orally Administered Nitrofurantoin against Animal African Trypanosomosis Caused by Trypanosoma congolense Infection', *Pathogens* 11(3) pp. 331. doi: 10.3390/PATHOGENS11030331.

Tamura, K. and Nei, M. (1993) 'Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees', *Molecular Biology and Evolution*, 10(3), pp. 512–526. doi: 10.1093/oxfordjournals.molbev.a040023.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) 'CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice', *Nucleic Acids Research*. 22(22), pp. 4673–4680. doi: 10.1093/nar/22.22.4673.

Trindade, S., Rijo-Ferreira, F., Carvalho, T., Pinto-Neves, D., Guegan, F., Aresta-Branco, F., Bento, F., Young, S. A., Pinto, A., Van Den Abbeele, J., Ribeiro, R. M., Dias, S., Smith, T. K. and Figueiredo, L. M. (2016) 'Trypanosoma brucei Parasites Occupy and Functionally Adapt to the Adipose Tissue in Mice', *Cell Host and Microbe*. 19(6), pp. 837–848. doi: 10.1016/j.chom.2016.05.002.

Utz, S., Roditi, I., Renggli, C. K., Almeida, I. C., Acosta-Serrano, A. and Bütikofer, P. (2006) 'Trypanosoma congolense procyclins: Unmasking cryptic major surface glycoproteins in procyclic forms', *Eukaryotic Cell*. 5(8), pp. 1430–1440. doi: 10.1128/EC.00067-06.

Vincent, I. M., Creek, D., Watson, D. G., Kamleh, M. A., Woods, D. J., Wong, P. E., Burchmore, R. J. S. and Barrett, M. P. (2010) 'A Molecular Mechanism for Eflornithine Resistance in African Trypanosomes', *PLOS Pathogens*. 6(11), e1001204. doi: 10.1371/JOURNAL.PPAT.1001204.

Wheeler, R. J., Gull, K. and Sunter, J. D. (2019) 'Coordination of the Cell Cycle in Trypanosomes', *Annual Reviews* . 73(1), pp. 133–154. doi: 10.1146/ANNUREV-MICRO-020518-115617.

Wickstead, B., Carrington, J. T., Gluenz, E. and Gull, K. (2010) 'The Expanded Kinesin-13 Repertoire of Trypanosomes Contains Only One Mitotic Kinesin Indicating Multiple Extra-

Nuclear Roles', *PLOS ONE*. 5(11), p. e15020. doi: 10.1371/JOURNAL.PONE.0015020.

Wiesgigl, M. and Clos, J. (2001) 'Heat shock protein 90 homeostasis controls stage differentiation in *Leishmania* donovani', *Molecular Biology of the Cell*. 12(11), pp. 3307–3316. doi: 10.1091/mbc.12.11.3307.

Yahaya, A. (2014) 'Evaluation of risk factors of animal trypanosomiasis in Ghana, and the monitoring of the impact of disease and vector eradication intervention in the upper west region of Ghana' Thesis, University of Mintpellier

Zinoviev, A., Léger, M., Wagner, G. and Shapira, M. (2011) 'A novel 4E-interacting protein in *Leishmania* is involved in stage-specific translation pathways', *Nucleic Acids Research*. 39(19), pp. 8404–8415. doi: 10.1093/NAR/GKR555.

10 Appendix

Appendix 1. Table of all primers for 5' tagging

Primer name	Sequence	Orientation	Target protein ID	Target	Annealing temp (°C)	Product size (bp)	Enzymes
MA1	ACATTATCTAGAGTAACCGATGATGATTGGAGG	F	Tb927.10.3990	DHH1 ORF (*T. brucei*)	49	532	*Xba*I
MA2	CGTATTGGATCCGCGGCCGCCCTTTACTAGCGAGATCCAC	R					*BamH*I *Not*I
MA3	ACATTATCTAGAGCGGCCGCGTCTACTATAAGAAGGGAAGAA	F	Tb927.10.3990	DHH1 5' (*T. brucei*)	45	314	*Xba*I
MA4	CGTATTGGATCCCGTATTCGTTGCTGTTGTTAG	R					*BamH*I *Not*I
MA5	ACATTATCTAGAGTCACTGAAGATGACTGGAA	F	TcIL3000_10_3320	DHH1 ORF (*T. congolense*)	45	532	*Xba*I
MA6	CGTATTGGATCCGCGGCCGCCCTTTACTAGCAAGATCCAC	R					*BamH*I *Not*I
MA7	ACATTATCTAGAGCGGCCGCTGTTTCTATTATTGTATGAAATCATCGA	F	TcIL3000_10_3320	DHH1 5' (*T. congolense*)	46	532	*Xba*I
MA8	CGTATTGGATCCAGTAGTCGTCGGTATTGTTG	R					*BamH*I *Not*I
MA9	ACATTATCTAGAAGCACTGCAACATCTGCAC	F	Tb927.5.810	ZC3H11 ORF (*T. brucei*)	54	532	*Xba*I
MA10	CGTATTCTCGAGGCGGCCGCACACGTTGCATCGGACGACA	R					*Xho*I *Not*I
MA11	ACATTATCTAGAGCGGCCGCAATAAAGCGGACTCGAAAAGAA	F	Tb927.5.810	ZC3H11 5' (*T. brucei*)	46	355	*Xba*I
MA12	CGTATTCTCGAGTTTCAAAACACGTGAATAAACTC	R					*Xho*I *Not*I
MA13	ACATTATCTAGACCCGCAGCACTATCCAAG	F	TcIL3000_0_37550	ZC3H11 ORF (*T. congolense*)	54	532	*Xba*I
MA14	CGTATTGGATCCGCGGCCGCGCTCGCTCGCAGATCCCT	R					*BamH*I *Not*I
MA15	ACATTATCTAGAGCGGCCGCAAGGTTGTTTTTCTTCTCTGTTC	F	TcIL3000_0_37550	ZC3H11 5' (*T. congolense*)	48	494	*Xba*I
MA16	CGTATTGGATCCCCTCCAATGTTGAGTATCTTGT	R					*BamH*I *Not*I
MA17	ACATTATCTAGAGGTGTTCCAAAATTCTTTCG	F	Tb927.7.4900	XRNA ORF (*T. brucei*)	46	532	*Xba*I
MA18	CGTATTGGATCCGCGGCCGCATAACGCTACAGTTTTGCCA	R					*BamH*I *Not*I
MA19	ACATTATCTAGAGCGGCCGCAAATACAAAGGAAAAAAAAGAAGTTG	F	Tb927.7.4900	XRNA 5' (*T. brucei*)	45	532	*Xba*I
MA20	CGTATTGGATCCGTCTACACGTATATATAAACTGTTTCG	R					*BamH*I *Not*I
MA21	ACATTATCTAGAGGGGTTCCAAAGTTTTTCCG	F	TcIL3000_7_4080	XRNA ORF (*T.*	51	532	*Xba*I

MA22	CGTATTCTCGAGGCGGCCGCTACACAACTTTGCAACTTTGCC	R		*congolense*)			*XhoI NotI*
MA23	ACATTATCTAGAGCGGCCGCGAGGTGAGAGGTCAGACACG	F	TcIL3000_7_4080	XRNA 5' (*T. congolense*)	50	532	*Xba*I
MA24	CGTATTCTCGAGGATTTTGGCTCGATACCGTT	R					*XhoI NotI*

*All reactions performed with GoTaq enzyme

Appendix 2. Table of all primers for 3' tagging

Primer name	Sequence	Orientation	Target protein ID	Target	Annealing temp (°C)	Product size (bp)	Enzymes
MA25	ACATTAAAGCTTGCGGCCGCGGAC GGTGGACTTCAGCAAC	F	Tb927.2.5980	HSP100 ORF (*T. brucei)*	54	532	*Avr*II *Not*I
MA26	CGTATTCCTAGGTTCCCATTCGTC ACTGGTAAGC	R					*Hind*III
MA27	ACATTAAAGCTTAGGCGCTGGACA AACAGAAA	F	Tb927.2.5980	HSP100 3' (*T. brucei)*	49	532	*Avr*II *Not*I
MA28	CGTATTCCTAGGGCGGCCGCGA ACGACAAAGAATCTTGGAC	R					*Hind*III
MA29	ACATTAGCGGCCGCACACGCATGG GCGCA	F	TcIL3000_2_1630	HSP100 ORF (*T. congolense)*	57	532	*Avr*II *Not*I
MA30	CGTATTCCTAGGAGAATTCACAG CAGCGCGCT	R					*Hind*III
MA31	ACATTAAAGCTTTTGGTACGGTG GAGC	F	TcIL3000_2_1630	HSP100 3' (*T. congolense)*	47	532	*Avr*II *Not*I
MA32	CGTATTCCTAGGGCGGCCGCCAA TAAAGCGCTCCCTTAAA	R					*Hind*III
MA33	ACATTAAAGCTTGCGGCCGCTGCA CCATATGCATCCTCCTCC	F	Tb927.9.9290	PABP1 ORF (*T. brucei)*	57	532	*Avr*II *Not*I
MA34	CGTATTCCTAGGAGCGCTTGAGG CGTGTACCT	R					*Hind*III
MA35	ACATTAAAGCTTAGTTGATTTTGT GAGTGAAAGT	F	Tb927.9.9290	PABP1 3' (*T. brucei)*	46	532	*Avr*II *Not*I
MA36	CGTATTCCTAGGGCGGCCGCACA AGTAGGATACTTCACGA	R					*Hind*III

MA37	ACATTAAAGCTTGCGGCCGCCCAT GCTGAGGTTACAGCTG	F	TcIL3000_9_3370	PABP1 ORF (*T. congolense)*	52	532	*Avr*II *Not*I
MA38	CGTATTCCTAGGAGAATTTGAGGC GTGCACTT	R					*Hind*III
MA39	ACATTAAAGCTTGGCATATAGTTT ACTGAAGGTG	F	TcIL3000_9_3370	PABP1 3' (*T. congolense)*	47	532	*Avr*II *Not*I
MA40	CGTATTCCTAGGGCGGCCGCCAA ATTACAGAACATGCATGTG	R					*Hind*III
MA41	ACATTAAAGCTTGCGGCCGCCTAT GGGTGGCCTGATGGGT	F	Tb927.9.10770	PABP2 ORF (*T. brucei)*	53	532	*Avr*II *Not*I
MA42	CGTATTCCTAGGCATGCCAATGTG ACGGTTGA	R					*Hind*III
MA43	ACATTAAAGCTTGATCAGCATCACT GCAGCG	F	Tb927.9.10770	PABP2 3' (*T. brucei)*	49	532	*Avr*II *Not*I
MA44	CGTATTCCTAGGGCGGCCGCCATTT CCTTTTCACATTGTCACC	R					*Hind*III
MA45	ACATTAAAGCTTGCGGCCGCGTGG CCTCATGAGTGGGAT	F	TcIL3000_9_4350	PABP2 ORF (*T. congolense)*	55	532	*Avr*II *Not*I
MA46	CGTATTCCTAGGCGCGCGTAGGTG GTTGTTG	R					*Hind*III
MA47	ACATTAAAGCTTACCAGCCTACAAC GCGA	F	TcIL3000_9_4350	PABP2 3' (*T. congolense)*	50	532	*Avr*II *Not*I
MA48	CGTATTCCTAGGGCGGCCGCCCCT TGAAATGTACTTGCATCA	R					*Hind*III

*All reactions performed with GoTaq enzyme

Appendix 3. Primers for PCRs to determine genomic location of insert

Primer Name	Sequence	Orientation	Gene	Target	Annealing temp (°C)	Product size (bp)
MA49	ACATTATCGCACACCTCTGTTTATAG	F	ZC3H11 (*T. brucei*)	Directly upstream of gene		
MA50	CGTATTGTCTTGTAGCGCTCCG	R		WT (ORF)	49	512
MA51	CGTATTCGGACGAGTGCTGGG	R		GM (Hyg cassette)	49	1,323
MA53	ACATTAGCGGGCACACATACATAACT	F	DHH1 (*T. brucei*)	Directly upstream of gene		
MA54	CGTATTTGCAGGGCCACAGGAATG	R		WT (ORF)	52	625
MA55	CGTATTGCTGCGAATCGGGAGCG	R		GM (G418 cassette)	52	1,370
MA56	ACATTATCCTGGAGTTGGTACCTGCC	F	ZC3H11 (*T. congolense*)	Directly upstream of gene		
MA57	CGTATTGCAATGTTCATCTCCGCAGT	R		WT (ORF)	52	783
MA58	CGTATTCGGACGAGTGCTGGG	R		GM (Hyg cassette)	52	1,634
MA59	ACATTACCCAGGTATGTGGGGAAGTAG	F	DHH1 (*T. congolense*)	Directly upstream of gene		
MA60	CGTATTGCGTTCAGACCCTTCTTCCA	R		WT (ORF)	54	1,149
MA62	CGTATTGCTGCGAATCGGGAGCG	R		GM (G418 cassette)	54	2,056

*All reactions performed with OneTaq enzyme