The role of *Tb*ZC3H11 in the trypanosomal heat shock response

Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by
Diplom-Biologin Dorothea Droll
born in Kehl
Oral-examination: September 10th, 2012

Referees: Prof. Dr. Christine Clayton
(ZMBH)
Prof. Dr. Luise Krauth-Siegel
(BZH)

Danksagung

Ich möchte mich herzlich bei Prof. Christine Clayton dafür bedanken, dass Sie meine Doktorarbeit betreut hat. Während sie mir die Möglichkeit gegeben hat eigenständig zu arbeiten stand ihre Tür stets offen für Fragen, Unterstützung und Motivation.
Außerdem möchte ich mich bei meinen TAC-Mitgliedern, Prof Luise Krauth-Siegel und Prof. Lanzer für hilfreiche Vorschläge bedanken.

Besonders herzlich möchte ich mich bei den aktuellen und früheren Kollegen im Clayton-Labor für die tolle Arbeitsatmosphäre bedanken. Die guten Diskussionen, Vorschläge und gegenseitige Unterstützung werde ich immer in guter Erinnerung behalten. Außerdem werde ich natürlich auch die „Movie nights“, Feuerzangenbowle, „Santa-melting“, usw. vermissen.
Vielen Dank an Conny und Diana für das Korrekturlesen.

Table of Contents

Summary

The protist parasite *Trypanosoma brucei* exerts virtually no transcriptional control. Nevertheless, this organism has to adapt its gene expression to different environments, for example the mammalian bloodstream or the midgut of the insect vector. Regulation of gene expression therefore has to rely on post-transcriptional mechanisms. RNA binding proteins are important post-transcriptional regulators, as they can influence their target transcripts' stability, localization and translation.

Members of the CCCH protein family, especially the Tis11 group, are involved in mRNA destabilization and regulation of transcripts containing AU-rich elements in the 3' untranslated region (3'UTR). ZC3H11 is a trypanosome zinc finger protein with one Tis11-like zinc finger domain, which is preceded by a conserved signature.

ZC3H11 protein expression is stabilized upon heat shock or upon inhibition of the proteasome and the protein is phosphorylated.

Depletion of ZC3H11 by RNA interference (RNAi) is lethal in the bloodstream stage of the parasite, but not in normally cultured procyclic (insect stage) trypanosomes. However, depletion in procyclics impairs fitness upon heat shock.

ZC3H11 co-precipitates several heat shock protein and chaperone mRNAs and, using a tethering experiment, it was established that ZC3H11 stabilizes bound transcripts. Furthermore, depletion of ZC3H11 in the bloodstream stage decreases the steady state level of *HSP70* mRNA and its half-life. This effect is mediated by the *HSP70* 3'UTR and more specifically the AUU-repeat-containing part. This AUU-repeat motif was also found to be enriched in the 3'UTRs of the co-precipitated transcripts.

In protein co-immunoprecipitation experiments, an MKT1 homologue was identified as an interaction partner. As yeast Mkt1p interacts with the PABP Binding Protein, PBP1, we propose that the stabilization of target transcripts by ZC3H11 is mediated through this interaction.

My results suggest that ZC3H11 is an important post-transcriptional master regulator of the trypanosome heat shock response.

Zusammenfassung

Der parasitäre Protist *Trypanosoma brucei* übt praktisch keine Kontrolle über die Transkription aus. Dennoch muss dieser Organismus seine Genexpression an verschiedene Umgebungen anpassen, zum Beispiel den Blutstrom in Säugetieren oder den Mitteldarm des Insektenvektors (prozyklische Form). Die Regulation der Genexpression ist daher auf posttranskriptionale Mechanismen angewiesen. RNA-Bindeproteine sind wichtige posttranskriptionale Regulatoren, da sie die Stabilität, Lokalisierung und Translation ihrer Ziel-mRNAs beeinflussen können.
CCCH Zinkfingerproteine, insbesondere die Tis11 Familie, sind an der Destabilisierung und Regulation von mRNAs mit AU-reichen Elementen in der 3'UTR (3' untranslatierte Region) beteiligt. ZC3H11 ist ein trypanosomales Protein mit einer Tis11-ähnlichen Zinkfingerdomäne. Die Expression des ZC3H11 Proteins ist nach Hitzeschock oder Inhibition des Proteasoms erhöht und ZC3H11 ist phosphoryliert.
ZC3H11-Reduktion mittels RNA-Interferenz (RNAi) ist in der Blutstromform, nicht aber in der prozyklischen Form, lethal. Die Reduktion in prozyklischen Parasiten schwächt jedoch die Fitness bei einem Hitzeschock.
Mehrere mRNAs, die für Hitzeschockproteine und Chaperone kodieren, kopräzipitieren mit ZC3H11 und das Protein stabilisiert künstlich gebundene mRNAs. Außerdem verringert *ZC3H11*-RNAi die *HSP70*-mRNA Menge und Stabilität. Dieser Effekt wird von der 3'UTR vermittelt, genauer gesagt von einer Region mit AUU-Wiederholungen. Dieses AUU-Motif war auch in den 3' UTRs der gebundenen Transkripte überrepräsentiert.
Mittels Koimmunopräzipitation wurde ein MKT1-Homolog als Proteinbindepartner identifiziert. Da Mkt1p in Hefe mit dem PABP Bindeprotein, PBP1, interagiert, könnte die Stabilisation der ZC3H11-gebundenen Transkripte über diese Interaktion vermittelt werden.

Meine Ergebnisse legen nahe, dass ZC3H11 ein wichtiger posttranskriptionaler Regulator der trypanosomalen Hitzeschockantwort ist.

1 Introduction

1.1 Trypanosomes

Trypanosomes are protist eukaryotes and belong to the class Kinetoplastida. The parasite species *Trypanosoma brucei gambiense* and *T. b. rhodesiense* cause Human African Trypanosomiasis (HAT), also called sleeping sickness. In most cases this disease is lethal if untreated. Other species, *T. brucei brucei, T. congolense* and *T. vivax* cause the Nagana disease in cattle and pose another economic burden in affected regions in Africa.
Other kinetoplastid species include *Trypanosoma cruzi* and *Leishmania* species that cause the Chagas disease in South America and leishmaniasis in many tropical and subtropical countries.

The transmission cycle of *Trypanosoma brucei*

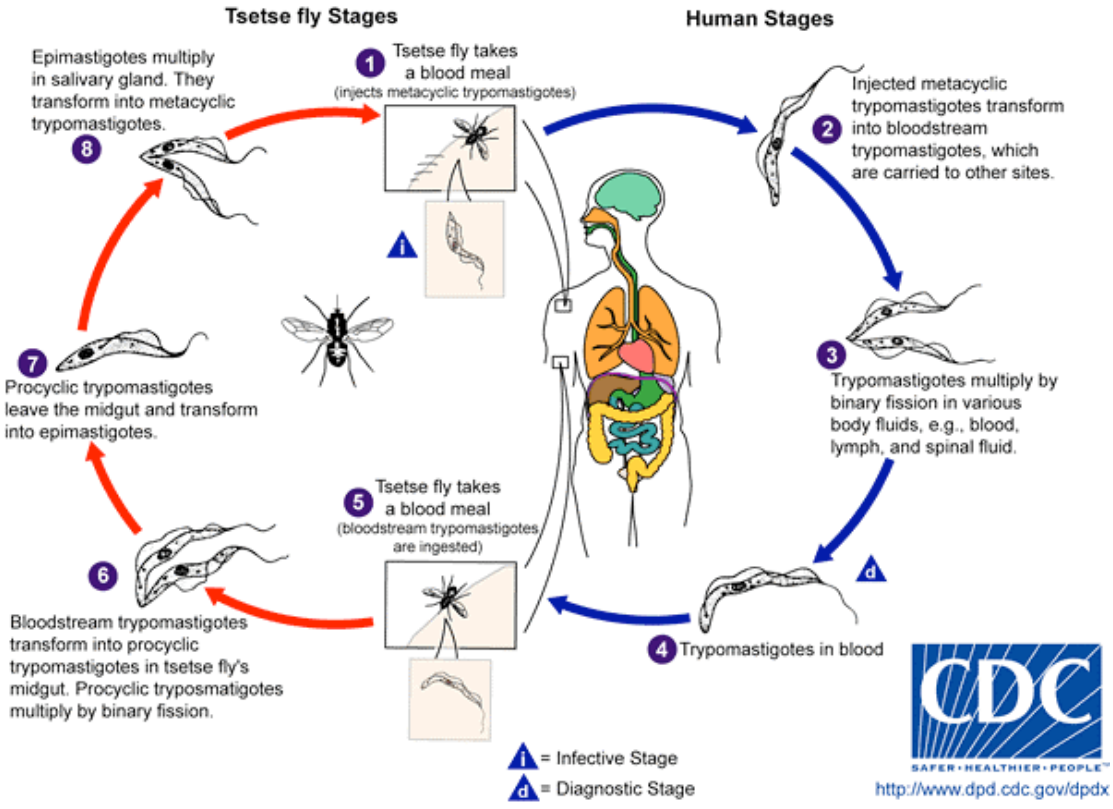


Figure 1.1.1
The transmission cycle of *Trypanosoma brucei* taken from CDC's Division of Parasitic Diseases, http://www.dpd.cdc.gov/dpdx

Trypanosoma brucei is transmitted by an insect vector, the Tsetse fly (*Glossina* spec.). During a blood meal metacyclic trypanosomes are transferred into the mammalian tissue from the salivary gland of the insect and migrate into the bloodstream. In the mammalian host they differentiate to the trypomastigote bloodstream stage. They can again be taken up by a Tsetse fly with a blood meal. In the insect they differentiate to procyclic trypomastigotes and later migrate from the midgut to the salivary gland as epimastigotes. In the salivary gland they develop into metacyclics and a new transmission cycle can begin (see figure 1.1.1).

Trypanosome physiology and peculiarities

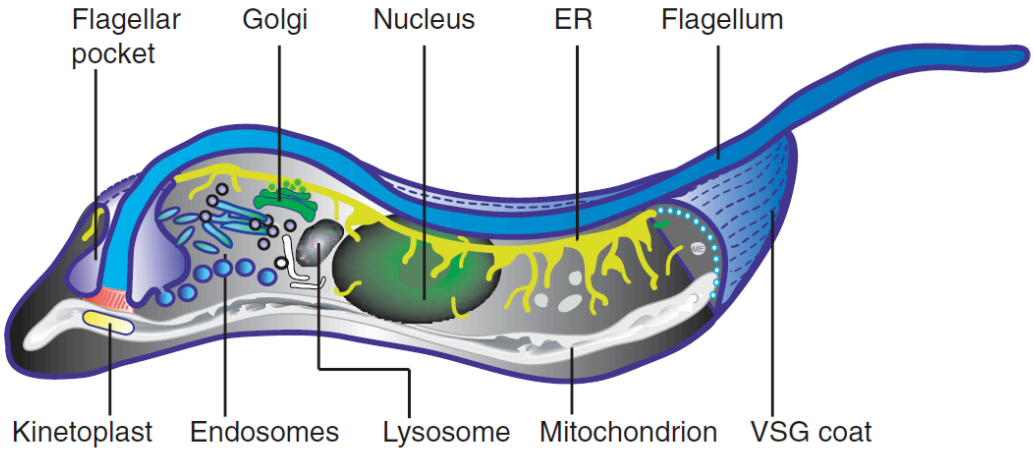


Figure 1.1.2
Cellular architecture of bloodstream form *Trypanosoma brucei*. Taken from (Overath and Engstler 2004).

Apart from their relevance as pathogenic parasites, trypanosomes are also an interesting model organism. As early diverging eukaryotes (Parfrey, Barbero et al. 2006), they share features common in most eukaryotes, but also display unusual peculiarities.
For example they posses a single mitochondrion and the mitochondrial genome has a disc like structure composed of concatenated maxi- and minicircles (Simpson 1987; Shapiro and Englund 1995). This densely packed structure is called the kinetoplast and gave the name to the Kinetoplastida. In the bloodstream stage (with a high glucose environment) the mitochondrion is

repressed and not metabolically important as the parasites' energy is mostly obtained by glycolysis. Another unusual feature is a specialized peroxisome-related organelle, the glycosome, which spatially confines most glycolytic enzymes (Opperdoes and Borst 1977; Michels, Bringaud et al. 2006). In procyclics (the insect stage) energy is obtained by oxidative phosphorylation of amino acids (mainly L-proline) (Bringaud, Riviere et al. 2006).
Another interesting feature of the trypanosomal mitochondrion is RNA editing by insertions or deletions of uridines, which was initially discovered in this organism (Benne, Van den Burg et al. 1986) and has since been found in many variations in diverse organisms (Gray 2012).
GPI-anchored proteins were also discovered in trypanosomes (Masterson, Doering et al. 1989), as the major surface proteins, which make up roughly 10% of the cells total protein content, have a GPI-anchor.
Expression of the bloodstream form specific variant surface glycoprotein (VSG) undergoes a process called antigenic variation. The parasite expresses only one VSG isoform at one time and would eventually be cleared by the immune system, but at a low frequency the parasites switch VSG expression to another isoform, thereby evading the host immune response.

1.2 Regulation of gene expression in trypanosomes

Transcription and processing

Trypanosomes transcribe most protein coding genes with RNA polymerase II. Unlike in most eukaryotes, this is independent of specific promoter sequences and occurs in polycistronic precursor units (Muhich and Boothroyd 1988; McAndrew, Graham et al. 1998).Transcription start sites are marked by histone variants (Siegel, Hekstra et al. 2009).
The individual genes in a polycistronic unit are not obviously functionally related and, even though they are transcribed at the same level, the final expression levels are often different, due to posttranscriptional regulation steps (Clayton 2002).

The polycistronic precursors are processed by *trans*-splicing and coupled polyadenylation to obtain the mature transcript (Ullu, Matthews et al. 1993; Gunzl 2010). *Trans*-splicing is the addition of the 39nt spliced leader (SL) miniexon sequence and was first observed in trypanosomatids (Boothroyd and Cross 1982). Consequently all mature mRNAs begin with the identical SL sequence, which also provides a cap-like structure (Freistadt, Cross et al. 1987). *Cis*-splicing was only experimentally found in two trypanosomal transcripts, poly(A) polymerase (Mair, Shi et al. 2000) and a putative RNA helicase (Jae, Wang et al. 2010).
An additional peculiarity in trypanosomes is that RNA polymerase I not only transcribes ribosomal RNA (rRNA) but also the genes for the major surface proteins (Rudenko, Bishop et al. 1989), VSG and procyclins (in the bloodstream or procyclic stage, respectively). RNA polymerase III transcribes the U RNAs and tRNAs.

Post-transcriptional regulation

Fully processed mRNA is exported to the cytoplasm where its stability depends on the association of various RNA binding proteins (RBPs) or complexes.
Translating mRNA is bound by the eIF4E cap binding complex, which interacts with eIF4G and circularizes the mRNP by interaction with poly(A) binding protein (PABP/Pab1) bound to the poly(A) tail at the 3’ end of the transcript (Amrani, Ghosh et al. 2008).
RNA degradation is initiated by the removal of the poly(A) tail and this is antagonized by the poly(A) binding protein (Milone, Wilusz et al. 2004). After deadenylation by the Caf1/Not complex, degradation can continue from the 3’ to 5’ direction by the exosome (Mitchell, Petfalski et al. 1997).
Following deadenylation, mRNAs can also be degraded from the other end starting with decapping. The trypanosome decapping enzyme has not yet been identified but the decapping activity was found in cell lysates (Milone, Wilusz et al. 2002).
After decapping the exonuclease XRNA, the trypanosome homologue of Xrn1, degrades the mRNA in the 5' to 3' direction (Li, Irmer et al. 2006).

1.3 RNA binding proteins

Control of gene expression on the transcript level can be mediated by *trans*-acting RNA binding proteins, mostly through sequence motifs or structures in the 3'UTRs of the target transcripts. Several RNA binding protein families have already been studied in trypanosomes (Fernandez-Moya and Estevez 2010), these include proteins containing RNA recognition motifs (RRM), PUF domains and CCCH-type zinc finger proteins.

The RRM motif is well represented in Kinetoplastids with over 75 proteins (De Gaudenzi, Frasch et al. 2005). These include proteins known in other organisms such as poly(A) binding proteins, eIF3B or U2AF35 but also many without clear orthologues.

*Tc*UBP1 (uridine binding protein 1) contains single RRM domain and binds to AU-rich elements (ARE) in the 3'UTR of a developmentally regulated mRNA and overexpression leads to decreased levels of this transcript, therefore it appears to be a ARE-destabilizing protein (Di Noia, D'Orso et al. 2000; D'Orso and Frasch 2001). *Tc*UBP1 was also found in a complex with *Tc*UBP2 and poly(A) binding protein. This could possibly destabilize the interaction of PABP1 with the mRNA and lead to degradation (D'Orso and Frasch 2002). In *Trypanosoma brucei* depletion of UBP1 and UBP2 is lethal and overexpression of UBP2 increased the level of several mRNAs, including the CFB1 array. This effect depends on the *CFB1* 3'UTR (Hartmann, Benz et al. 2007). *Tc*RBP3 binds to a set of ribosomal protein RNAs (De Gaudenzi, D'Orso et al. 2003) whereas the *T. brucei* protein *Tb*RBP3 co-precipitated *ZFP1, ZC3H11, CFB1* and transcripts of two hypothetical proteins (Wurst, Robles et al. 2009).

Two other proteins with RRM domains have been studied in trypanosomes: DRBD3 and DRBD4 (aka PTB1/2). DRBD3 was found to bind a number of transcripts that are developmentally regulated (Estevez 2008). Another study showed an involvement in splicing (Stern, Gupta et al. 2009).

Trypanosoma brucei has 11 PUF (Pumilio and FBF) proteins. *Tb*PUF9 is involved in the regulation of transcripts important for cell cycle control (Archer, Luu et al. 2009) whereas *Tb*PUF7 is localized in the nucleolus and depletion affects rRNA maturation (Droll, Archer et al. 2010). *Tb*PUF1 is not essential

(Luu, Brems et al. 2006) and the *T. cruzi* homologue *Tc*PUF6 seems to destabilize its target mRNAs and is associated with DHH1 (Dallagiovanna, Correa et al. 2008).

CCCH zinc finger RNA binding proteins

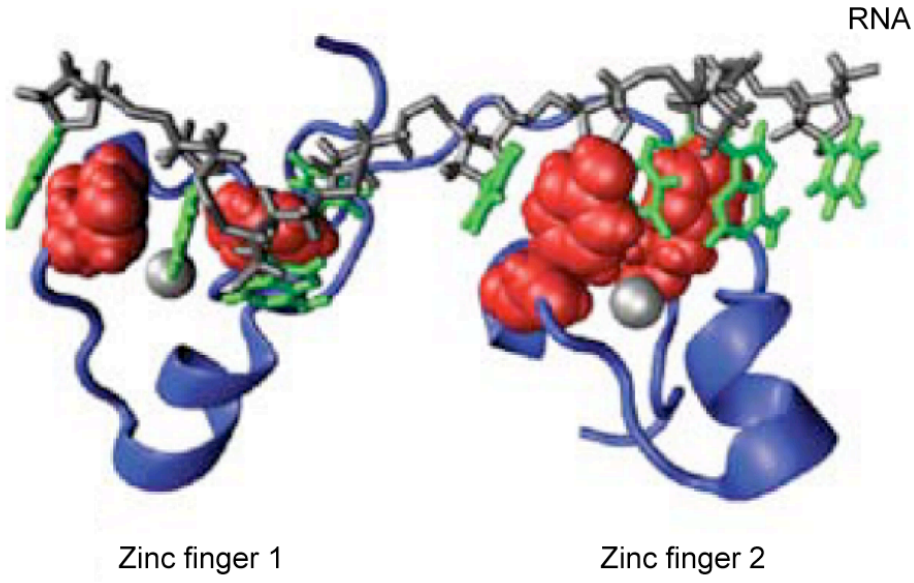


Figure 1.3
NMR structure of TIS11d bound to ARE[9] (Morgan and Massi 2010)
The RNA backbone and bases are shown in gray and green, in blue the protein backbone and in red the protein side chains. The zinc is presented as grey sphere.

RNA binding proteins containing the $CX_8CX_5CX_3H$, or the non-conventional $CX_7CX_4CX_3H$, zinc finger domain, (both abbreviated as CCCH) have been studied in many organisms. The Tis11 (TPA induced sequence 11) family, that contain a tandem zinc finger domain preceded by a signature motif, is the most studied, including for example tristetraproline (TTP) (Varnum, Ma et al. 1991). Tis11 proteins bind target mRNAs via AU-rich elements (AREs: UUAUUUAUU) in their 3'UTRs.
Mammalian TTP (aka TIS11, ZFP36, NUP475) is a destabilizer of various ARE targets, for example TNF-α or GM-CSF (Carballo, Lai et al. 1998; Carballo, Lai et al. 2000).
The yeast Tis11 homologues are Cth1p and Cth2p (Thompson, Lai et al. 1996) and Cth2 has been shown to be involved in ARE mediated decay in

response to iron deficiency (Puig, Askeland et al. 2005) by recruiting Dhh1(Pedro-Segura, Vergara et al. 2008).
Trypanosoma brucei has 48 non-redundant CCCH proteins and most of those have only one single zinc finger domain (Kramer, Kimblin et al. 2010).
The first experimentally studied proteins were *Tb*ZFP1, *Tb*ZFP2 and *Tb*ZFP3 which are all on some level involved in differentiation from bloodstream stage to procyclics (Hendriks, Robinson et al. 2001; Hendriks and Matthews 2005; Paterou, Walrad et al. 2006; Walrad, Paterou et al. 2009; Walrad, Capewell et al. 2012). *Tb*ZFP3 was found to bind to *EP1/GPEET* mRNA (Walrad, Capewell et al. 2012).
*Tc*ZFP1 binds to poly-C oligoribonucleotides *in vitro* (Morking, Dallagiovanna et al. 2004) but also to an AU-rich element found in the 26mer in the *EP/GPEET* mRNA.
Other studies in trypanosomes showed that *Tb*ZC3H20 stabilizes two stage-specific mRNAs (Ling, Trotter et al. 2011), depletion of *Tb*ZC3H18 leads to a defect in differentiation (Benz, Mulindwa et al. 2011) and overexpression of ZC3H13 in procyclics leads to a growth defect (Ouna, Stewart et al. 2012).

1.4 The heat shock stress response

All organisms have to deal with changes in the environment, including changes in temperature. At elevated temperatures protein folding can be compromised and there is a higher demand for chaperones or heat shock proteins (HSPs) in the cell. These can either prevent unfolding or assist in refolding proteins. In most organisms the expression of these genes is activated by transcriptional mechanisms, for example by activation of the eukaryotic heat shock transcription factor HSF (Sorger and Pelham 1988; Pirkkala, Nykanen et al. 2001) or the sigma factor σ^{32} in prokaryotes (Straus, Walter et al. 1987).
The major cytoplasmic chaperone system is the HSP70 system. HSP70 (DnaK) together with the co-chaperone HSP40 (DnaJ) binds to exposed non-native polypeptides and assists in folding, depending on ATP binding and hydrolysis (reviewed in (Mayer and Bukau 2005)). Members of the HSP70 family are found in the cytoplasm, the endoplasmic reticulum (BiP) and in

organelles. The HSP90 system regulates specific client proteins that are involved in signaling processes and there is cooperation with the HSP70 system e.g. via the TPR domain containing protein Sti1 (Hop) (Scheufler, Brinker et al. 2000). Several other TPR domain containing proteins, for example the peptidylprolyl isomerase D (PPID, cyclophilin40) and FKBP-type peptidylprolyl isomerases (FKBP4/51/52), interact with these chaperones. The HSP60 chaperonins (GroEL) are found in organelles and their cofactor is HSP10 (GroES). The HSP100 (AAA+, Clp) family form large hexameric structures and can promote unfolding and degradation of aggregates.

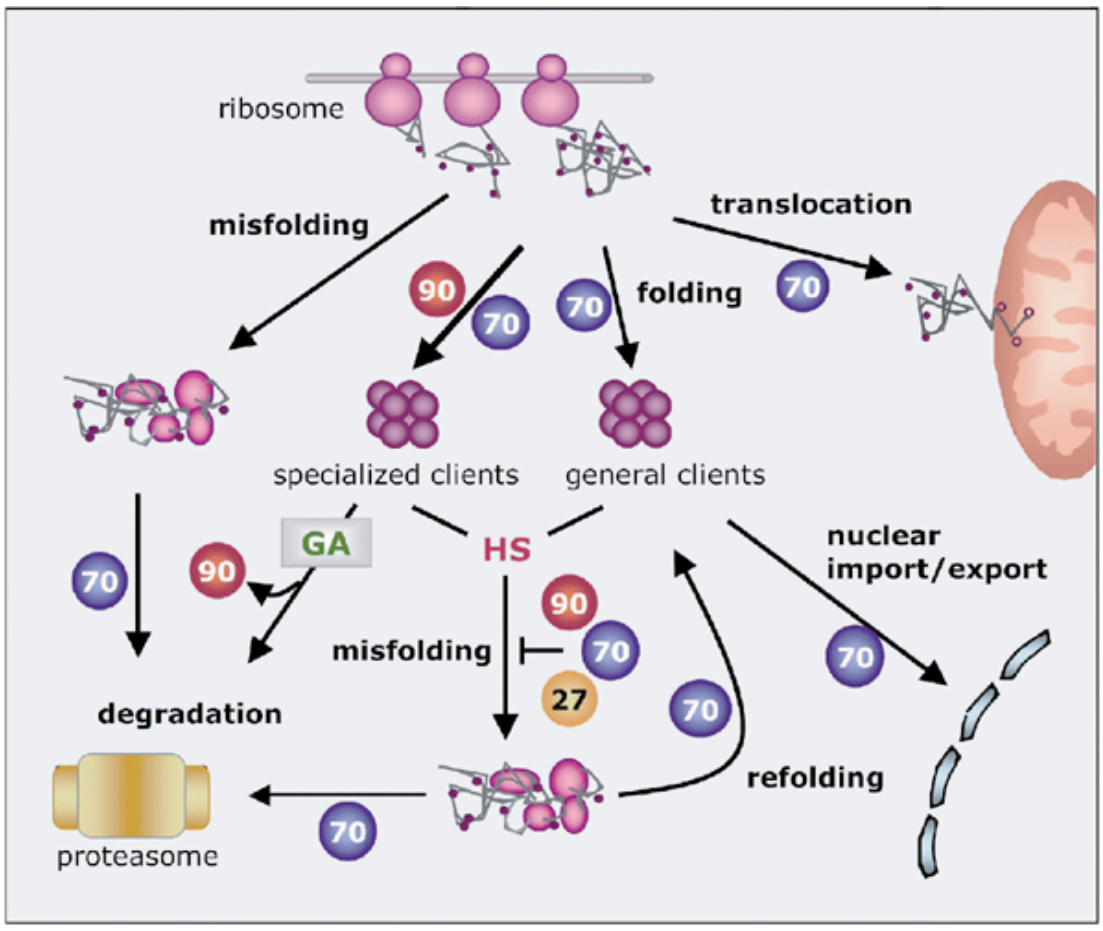


Figure 1.4

The major functions of HSP70 (70, blue circles) and HSP90 (90, red circles) chaperones. Specialized clients (e.g. signaling kinases or HSF1) are held in activation competent states by HSP90, released by addition of the inhibitor geldanamycin and degraded. Taken from (Mosser and Morimoto 2004).

The chaperone system is conserved in kinetoplastids. This was nicely reviewed in (Folgueira and Requena 2007), and the data presented in the following paragraphs was taken from this review. In *Trypanosoma brucei* the major cytoplasmic HSP70 (Tb11.01.3110) is found in a tandem array with five tandem copies (Glass, Polvere et al. 1986). Unfortunately, the HSP70 array was bioinformatically collapsed in the genome assembly. Furthermore the current version also has a frameshift in the CDS compared to the originally published sequence (Glass, Polvere et al. 1986), leading to changes in the C-terminus and eliminating the very conserved terminal EEVD motif.
Several other HSP70 family members are present in the genome, including two identical BiPs (Tb11.02.5500/5450), mitochondrial HSP70s (Tb927.6.3740/3750/3800) and additional putative cytoplasmic HSP70s (Tb11.01.3080, Tb927.7.710, Tb927.7.1030, Tb09.160.3090). HSP110 (SSE) proteins are another HSP70 subfamily and might function as nucleotide exchange factors for canonical HSP70 (Raviol, Sadlish et al. 2006). *T. brucei* has a cytoplasmic (Tb927.10.12710) and an ER resident (Tb09.211.1390) homologue. Genomic analysis showed that the HSP40 (DnaJ) co-chaperone family is very well represented in trypanosomes, with 65 proteins in *T. brucei*. The cytoplasmic HSP83 (HSP90 homologue) genes are also organized in a multicopy array (Tb927.10.10890-10980), and additionally an ER resident (Tb927.3.3580) and a mitochondrial (Tb11.02.0250) HSP90 family gene were identified in the genome.

The heat shock response in trypanosomes

The major difference of the heat shock response in trypanosomes, compared to the “normal” mechanism, is that there is no upregulation of hsp70 transcription upon heat shock (Lee 1995). Heat shock in trypanosomes leads to decreased transcription by Pol II and a disruption in trans-splicing of tubulin mRNA (Muhich and Boothroyd 1988) but not *HSP70* mRNA (Muhich, Hsu et al. 1989).
Due to decreased production and increased degradation, cellular mRNA levels decrease by 75% within 2 hours of heat shock (Muhich and Boothroyd 1988). Even though there is no increase in transcription of hsp70 (Lee 1995),

the *HSP70* and also *HSP83* mRNA levels remain stable during heat shock in contrast to the tubulin transcript (Lee 1998; Kramer, Queiroz et al. 2008). The *HSP70* 3'UTR mediates this effect in *T. brucei* (Hausler and Clayton 1996; Lee 1998) but the mechanism was not known before.

RNA granules

Translation is downregulated during stress conditions and mRNAs can be stored in RNA granules. Mammalian stress granules contain polyadenylated mRNA bound by the 40S subunit, PABP, eIF3, eIF4E/G, eIF2, Dhh1, TTP, Pbp1 and many other factors. The untranslated transcripts can also be degraded, this can be organized in so called processing bodies (P-bodies). These granules share some of the proteins also found in stress granules, e.g. Dhh1, TTP, Scd6, eIF4E, but also decay factors such as the deadenylase complex, the decapping machinery and factors of other decay pathways (Newbury, Mühlemann et al. 2006; Parker and Sheth 2007; Buchan and Parker 2009).

RNA granules have also been observed in trypanosomes (Cassola, De Gaudenzi et al. 2007; Kramer, Queiroz et al. 2008). Upon heat shock the initiation factors eIF4E1, 4E2, 4E3, 4E4, 3B and 2A as well as both PABPs accumulate in granules (Kramer, Queiroz et al. 2008), which share some components with granules formed upon metabolic stress (Cassola, De Gaudenzi et al. 2007). P-bodies in trypanosomes have been shown to contain XRNA, DHH1 and SCD6 (Cassola, De Gaudenzi et al. 2007; Holetz, Correa et al. 2007; Kramer, Queiroz et al. 2008).

2 Materials and methods

Some protocols in this section were taken from previous members of the lab and modified as necessary.

2.1 Trypanosome methods

2.1.1 Bloodstream-form trypanosome cell culture

Monomorphic Lister 427 bloodstream-form trypanosomes were cultured at 37°C in incubators with 5% CO_2 in supplemented HMI-9 medium. The lids of the culture flasks were only loosely fixed to allow air exchange.
Cell densities (determined using a Neubauer counting chamber) did not exceed 1.5x 10^6 cells/ml for experiments.
Cell culture work was done in sterile conditions under a laminar flow hood.

Supplemented HMI-9 medium, 500 ml

HMI-9	(450 ml)
Heat-inactivated FBS	10%(v/v) (50 ml)
Penicillin/Streptomycin	50U/l (5 ml of Penicillin-Streptomycin mix)
L-Cysteine	1.5 mM (5 ml of stock solution)
β-mercaptoethanol	0.14% (7 µl in 5 ml frozen stock)

2.1.2 Procyclic-form trypanosome culture

Monomorphic Lister 427 procyclic trypanosomes were cultured in supplemented MEM-Pros medium in tightly closed cell culture flasks at 27°C at densities between 0.5-8x 10^6 cells/ml. All work was done under sterile conditions in a laminar flow hood.

Supplemented MEM-Pros medium, 500mL

MEM-Pros	(450 ml)
Heat-inactivated FBS	10%(v/v) (50ml)
Hemin	7.5 mg/l (1.5ml of stock solution)
Penicillin/Streptomycin	50U/ml (5ml of stock (5000U/ml), Sigma)

2.1.3 Antibiotics

For selection of transgenic trypanosomes antibiotic resistance genes were used and added in the following concentrations:

Antibiotic	Bloodstream	Procyclic
Phleomycin	1 µg/ml	1 µg/ml
G418	5 µg/ml	15 µg/ml
Hygromycin	15 µg/ml	50 µg/ml
Puromycin	0.2 µg/ml	1 µg/ml
Blasticidin	5 µg/ml	10 µg/ml

All growth experiments were performed in the absence of selective drugs. For inducible expression, tetracycline was added to a final concentration of 200 ng/ml.

2.1.4 Transfection of bloodstream / procyclic trypanosomes

Transfection buffers:
- *Zimmerman's Post Fusion Medium* (ZPFM) for procyclic trypanosomes (132mM NaCl, 8mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4, 1.5mM MgAc x 4 H_2O, 90µM $Ca(OAc)_2$; pH adjusted to 7,0 with NaOH, usually made as a 5x stock, filter-sterilized and stored at 4°C)
- *Cytomix* for bloodstream trypanosomes (2mM EGTA, 120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4/KH_2PO_4 pH 7.6, 25mM HEPES, 5mM $MgCl_2$,0.5% Glucose,100µg/ml BSA, 1mM Hypoxanthine; pH adjusted to 7.6 with KOH, filter-sterilized and stored at 4°C)

1-2x 10^7 cells were used per transfection. The cells were washed twice in the appropriate transfection buffer (see above) and resuspended in 0.5 ml. The cells were mixed with 10 µg of digested plasmid and transferred to a cuvette and electroporated using settings of 1.5 kV and resistance R2 with the BTX electroporation machine. On the next day, the selection antibiotic was added and the cells plated in serial dilution on a 24 well plate. Proliferating clones were picked from the plate several days later and checked for expression of the transgene.
Aliquots of generated cell lines were frozen in medium with 10% glycerol and stored in liquid nitrogen.

2.1.5 Stress conditions

For a heat shock experiment procyclic cells were transferred to a 41°C water bath or the 37°C incubator, usually for 1h or as specified in the experiment. The proteasome inhibitor MG132 (Calbiochem/Merck) was solved in DMSO at a stock concentration of 10 mg/ml. This stock was kept at -20°C and it is not stable for longer periods. For proteasome inhibition MG132 was added directly to the culture to a final concentration of 10 µg/ml (~20µM). Inhibition was usually for 1 hour. Puromycin stress was performed with the drug concentrations as used for selection (chapter 2.1.3).

2.1.6 RNA degradation assay

RNAi was induced one day before the experiment, if applicable. For each time point, 50 ml of bloodstream form cultures at a density of approximately 1x10^6 cells/ml were used. The cells were not centrifuged before the experiment to avoid stress conditions or temperature changes that might affect expression of stress sensitive transcripts.
ActinomycinD was solved in 100% ethanol on the day of the experiment at a stock concentration of 1mg/ml. Sinefungin stocks (1 mg/ml) were prepared with water and kept at -20°C.Centrifugation steps were timed to finish with enough time left for removing the supernatant and stopping the degradation time point by addition of Trifast reagent.
At t=-5 min Sinefungin was added to a final concentration of 2 µg/ml (1:500 dilution) and the culture well mixed.
The t=0 min time point was harvested before addition of ActinomycinD.
After 5min of Sinefungin treatment, 10 µg/ml (1:100 dilution) ActinomycinD was added and well mixed.
Time points were chosen as required, e.g. 0, 15, 30 minutes.

2.1.7 Indirect Immunofluorescent localization assay (IFA)

The immunofluorescence was performed according to the EMBO/TDR protocol.
Rabbit anti-proteinA antibody (Sigma) for TAP-tag detection (1:10000 dilution), the Dhh1 antibody (rabbit, 1:500) and anti-myc antibody (mouse, 1:500) were used.
Either the Olympus CellR microscope or the confocal Leica microscope in the ZMBH microscopy facility were used for image acquisition.

2.1.8 Flow cytometry analysis (FACS)

For each condition an aliquot of approximately 1 x 10^6 cells was washed with 1x PBS and fixed in 70 % ethanol / 30 % TBS overnight at 4 °C. Following fixation, the cells were centrifuged at 4,500 rpm for 10 min at 4 °C, the pellet was resuspended in 500 μl 1x PBS and incubated for 30 minutes at 37 °C with Propidium iodide (30 μg/ml) and RNase A (10 μg/ml). For cell cycle phase determination, 20,000 cells were analyzed using a BD FACSCANTO II flow cytometer (Becton Dickinson Immunocytometry Systems) with a 585/42 band pass filter for the quantification of red fluorescence intensity. The analysis was done using FACSDiva software. For the heat shock experiment no gates were set to exclude dead cells, cell fragments or cell clumps from the analysis.

2.2 DNA methods

2.2.1 Cloning and colony PCR

Enzymes were used with the supplied buffers and according to manufacturers instructions. For the site directed mutagenesis the Phusion site directed mutagenesis kit from NEB (New England Biolabs) was used.
Digests and PCR products were checked on 1% agarose gels prepared with 1xTAE buffer and ethidium bromide.
DNA (and RNA) concentrations were measured using the NanoDrop machine (Peqlab).

2.2.2 Transformation of bacteria and plasmid preparations

Plasmid DNA was added to an aliquot of competent bacteria (DH5 alpha, ~20µl) on ice and incubated for 5 min. The transformation was done at 42°C in a water bath for 60 seconds. Then, the cells were incubated at 37°C with LB medium for 20 min to allow recovery and integration of the plasmid. Clones were selected on LB-ampicillin plates (or appropriate antibiotic). Single colonies were picked and grown in LB medium with antibiotic overnight. Plasmids were isolated with the Nucleospin Plasmid kit (Macherey Nagel) and checked by PCR, control digests or sequencing.

2.2.3 Genomic DNA preparations

3x 10^8 cells were harvested by centrifugation and the pellet resuspended and lysed in 0.5 ml EB buffer (10 mM Tris-Cl pH8.0; 10 mM NaCl; 10 mM EDTA; 0.5% SDS) with addition of 12 µl RNaseA (of 1 mg/ml stock). After one hour of incubation at 37°C, 200µl ice cold ammonium acetate was added and the tube softly vortexed. The precipitated proteins were pelleted by centrifugation and the supernatant transferred to a new tube using a cut of plastic tip to reduce shearing of the DNA. To precipitate the DNA 0.7 sample volumes of isopropanol were added and after gentle mixing the sample was centrifuged until a pellet was visible. This was washed once with 70% ethanol and then again with 100% ethanol. After the final wash the ethanol was carefully completely removed and the DNA shortly dried. Finally the DNA was solved in TE at 37°C. The concentration was measured using the NanoDrop.

2.2.4 Southern blot

To determine the genomic arrangement or the correct integration of the knockout constructs in transgenic trypanosomes, a Southern blot can be used. After analysis of the ZC3H11 locus, BsaBI was chosen as the most informative choice. Genomic DNA from wild type and knockout trypanosomes was isolated and, for each, 10µg of gDNA was digested with a five fold excess of BsaBI (NEB) at 60°C.
After digestion, the DNA was separated on a 0.8% agarose gel with empty lanes between the samples and a size marker. Prior to blotting, the gel was washed in 0.25 M HCl for 15 min to hydrolyze long DNA strands.
Next it was washed for 30 minutes in 1.5 M NaCl; 0.4M NaOH (denaturing) and then twice 15 min in 1.5 M NaCl; 0.5 M Tris-Cl pH7.5 (neutralization).

The Southern blot was assembled like a Northern Blot, and also the hybridization and probes were done accordingly.

2.3 RNA methods

2.3.1 RNA isolation using TriFast

1 ml of TriFast reagent (Peqlab) was added to each cell pellet, and mixed until it completely dissolved. After incubation for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes, 0.2 ml of chloroform per 1 ml of TriFast reagent was added. Now the tubes were vigorously mixed by hand for 15 seconds, and incubated at room temperature for 2 to 3 minutes. Phase separation was performed by centrifugation in the microfuge at full speed for 10 minutes at 4°C. The clear aqueous phase (containing the RNA) was transferred to a fresh tube without disturbing the interphase. An additional wash and phase separation step with chloroform was performed to reduce the phenol contamination. The RNA was now precipitated by mixing with 0.5 ml isopropyl alcohol. After incubation at room temperature for 10 minutes and centrifugation at 12,000 rpm for 20 minutes at 4°C, the RNA precipitate forms a gel-like pellet on the side and bottom of the tube.

After carefully decanting the supernatant, the RNA pellet was washed twice with 1 ml of 75% ethanol. At the end of the procedure, the RNA pellet was briefly dried and then dissolved in 12 µl of sterile water. If necessary, the RNA was incubated at 55°C-60°C to facilitate solving. The RNA concentration was measured with the Nanodrop and the sample diluted if necessary.

2.3.2 RNA agarose gel electrophoresis

5x MOPS buffer (5 mM EDTA, 40 mM NaAc, 0.1 M MOPS (3-[N-morpholino] propane sulfonic acid), pH adjusted to 7

Formaldehyde agarose gel:

H_2O	97 ml
agarose	1.4 g

boil and completely dissolve. After cooling down a little bit add:

5x MOPS	26 ml
formaldehyde (37%)	10 ml

Mix well and pour gel.

2x RNA sample buffer:

10x MOPS	250 µl
37% formaldehyde	300 µl
formamide	1 ml
ethidium bromide	10 µl

small amount of bromphenolblue

Mix the RNA sample (10µg/lane) and also the marker (0.5-10 kb RNA ladder, Invitrogen) with the same volume of loading dye and denature at 65°C for 10 min. Load the samples on the agarose gel and run at 120V in 1x MOPS buffer until the dye is approximately 2 cm from the lower edge of the gel.

2.3.3 Northern blotting and hybridization

The blotting was done for at least 6h with the TurboBlotter™ apparatus (Schleicher&Schuell) using 10x SSC for transfer and the Hybond membrane (GE Healthcare).
After blotting, the RNA was UV-crosslinked to the membrane and then prehybridized (>1h) and hybridized (>4h) at 65°C.

Pre-/Hybridization buffer (10ml)

H_2O	5.9 ml
20x SSC	2.5 ml
10% SDS	0.5 ml
Denhardts solution	1 ml
salmon sperm DNA	100 µl
labeled probe	100 µl

The probes were made from DNA templates using the random primer kit labeling kit (Stratagene) using ^{32}P-alpha dCTP.

oligonucleotides used for probe templates (Northern blot)

cz2827	HSP70 (Tb11.01.3110) probe FW	ggggatccATGACATACGAAGGCGCC
cz3257	HSP70 (Tb11.01.3110) probe RV	ACGACTCCGCAACCTCCT
cz3135	ZC3H11 probe FW	ACATCGTTCCAGAAACTGTGG
cz3136	ZC3H11 probe RV	TATCCGGACTCATTACCAACG
cz2697	CAT probe FW	TACACCGTTTTCCATGAGCA
cz2698	CAT probe RV	CCTGCCACTCATCGCAGTA
cz4577	HSP83 probe FW	TATTGTGAAGAAGGCCCTGG
cz4578	HSP83 probe RV	CTCTTTCATTGCCTTGCACA
cz2581	tubulin probe FW	CCTTTGGCACAACGTCACCACGG
cz2724	tubulin probe RV	TGACTCGCCGCAACCTCGAT
cz3865	NPT probe FW	acggattcGTGGAACAAGATGGATTG
cz3635	NPT probe RV	gccggatcaagcgtatgc
cz4490	spliced leader oligo (anti sense)	CAATATAGTACAGAAACTGTTCTAATAATAGCGTTAGTT
	7SL from pBS plasmid (T7/T3)	

The spliced-leader antisense oligonucleotide was end-labelled using T4-polynucleotide kinase and ^{32}P-gamma ATP. Pre-/hybridization was at 42°C.

oligo labeling reaction

H_2O	6.5 µl
5µM oligonucleotide	1 µl
10x T4PNK buffer	1.5µl
^{32}P-gamma ATP	5µl (50µCi)
T4-PNK	1µl

incubate 1h @ 37°C, then purify with the nucleotide removal kit

oligo-prehybridization solution

H_2O	5.1 ml
20x SSC	3 ml
10% SDS	0.5 ml
Denhardts solution	1 ml
5% Na-pyrophosphate	100 µl
salmon sperm DNA	100 µl

oligo-hybridization solution

H_2O	6.5 ml
20x SSC	3 ml
Denhardts solution	0.2 ml
5% Na-pyrophosphate	100 µl
salmon sperm DNA	100 µl
labeled oligo	100 µl

oligo-wash solution (250ml)

20x SSC	75 ml
5% Na-pyrophosphate	2.5 ml

wash 3x 15 minutes at room temperature and then 10 minutes at 42°C.

2.3.4 microarray

The cell lines used for the microarray experiment were bsf 1313 514 as wild type and bsf 1313 514 1980 for the ZC3H11 RNAi. RNAi was induced for 24h with tetracycline before the cells were harvested, washed once with PBS and frozen in liquid nitrogen until use. The RNA was isolated with the Quiagen RNeasy midi kit, and the RNA concentration measured with the Nanodrop. This RNA was labeled with fluorescent Cy3 or Cy5 dye by Rafael Queiroz, and analyzed on microarrays (TIGR oligoarray) according to his standard protocol.

2.4 Protein methods

2.4.1 SDS polyacrylamid electrophoresis (PAGE)

Cells were harvested and washed once in 1x PBS. Lämmli buffer was added (4x or 2x as needed, containing β-mercaptoethanol), the pellet resuspended and then boiled for 5 min before loading to lyse the cells.
The protein markers used were either Magicmark (Invitrogen) or Precision Plus (Biorad).
SDS polyacrylamide gels (standard recipe) ranged from 10 to 15%, depending on the size of the proteins to be analyzed and were run at 120 V with the Bioroad system in 1x SDS-running buffer.

10x SDS- running buffer (1l)

Tris	30.3 g
Glycine	144.2 g
SDS	10 g

Colloidal coomassie staining
Gels were fixed for 1h before staining over night.
Fixing solution
40% ethanol
10% acetic acid
Colloidal coomassie dye stock (500 ml)
10 ml Coomassie G-250 solution (5% w/v)
50 g NH_4SO_4
6 ml 85% phosphoric acid
fill up with H_2O
Staining solution
200 ml stock
50 ml methanol

If necessary, the signal can be increased with the silver staining kit (Pierce), after complete destaining of the gel background with water

2.4.2 Western blotting and antibody

The SDS-gel was blotted for 1 hour at 100V in Western blotting buffer (SDS running buffer without SDS but with 20% methanol). If needed, the transferred protein was stained with PonceauS solution and scanned. The membrane was blocked in 5% (w/v) milk in TBST for more than 30 minutes before

hybridizing with the respective antibody (in milk-TBST, 1h to ON). After short washes in TBST the appropriate secondary antibody, coupled to HRP (1:2000, GE Healthcare), was added for 1h and the blot then washed again in TBST. The signal was detected using ECL solutions either with X-ray films or with a camera based gel documentation system.

antibody list and dilutions:
α-V5 (AbD-Serotec, mouse 1:2000)
α-myc (Santa Cruz laboratories, mouse 1:2000)
α-aldolase (CC, rabbit 1:50 000).
If necessary the blot was stripped with 25 mM glycine; 1%SDS pH2 for 20 min on a shaker

2.4.3 λ-Phosphatase assay

The protein dephosphorylation assay was performed with the λ-Phosphatase from NEB.
2x 10^7 cells/ sample were harvested and lysed in 45µl lysis buffer. After incubation on ice for 10 minutes, the cell lysate was cleared by centrifugation. 6µl of each 10x reaction buffer and MnCl2 were added to each sample, plus phosphatase (1µl) or inhibitors as required.
Lysis buffer
50 mM Tris, pH 7.5
50 mM NaCl
2 mM $MgCl_2$
0.1% NP-40
+ protease inhibitor (Complete mini, EDTA-free, Roche)
Phosphatase inhibitors used were Sodium Orthovanadate (2mM) and Sodium Fluoride (8mM).

2.4.4 Expression of recombinant protein in *E. coli*

The E.coli strain used was BL21 pRARE and the vector systems pQE-A38 (10x-His, ampicilin) or pET-trx-1b (kanamycin)
The protocols and buffer compositions from the "QIAexpressionist" (Qiagen) were used. Tested conditions included variation of cell densities, IPTG concentration, induction time and growth temperatures.

2.4.5 Tandem affinity purification (TAP)

5x 10^9 procyclic cells, either expressing ZC3H11-TAP (pHD1950) or the empty pHD918 as a control, were used for the TAP experiment. Additionally heat shocked (1 hour at 41°C) ZC3H11-TAP cells were used.

Lysis buffer
10 mM Tris, pH7.8
10 mM NaCl
0.1% IGEPAL

IPP150 IP buffer
150 mM Tris, pH7.8
10 mM NaCl
0.1% IGEPAL

Additionally add one protease inhibitor cocktail tablet (without EDTA, Roche) for 5 ml of lysis buffer.

Break cells in a final volume of 4ml lysis buffer by passing 15-20 through a 21 gauge needle. Check on a glass slide to ensure complete breakage. Spin cell lysate at 10.000g for 15 min to remove cell debris. Transfer the supernatant to a new tube and measure volume. Add NaCl to a final concentration of 0.15 M. Mix and take 25 µl aliquot (start material, #1).

200 µl IgG sepharose bead suspension is transferred into a column (Bio-Rad) and washed with 10ml IPP150. The cleared cell lysate is tranferred into the column containing the washed beads and rotated for 1 hrs at 4°C. Elution is done by gravity flow. 25 µl of the flow through (IgG flowthrough, #2) is taken for subsequent analysis. The beads are washed three times in 10 ml of IPP150 and once with 10 ml of TEV cleavage buffer (IPP150 adjusted to 0.5 mM EDTA and 1 mM DTT). Cleavage is done in the same column by adding 1ml of TEV cleavage buffer and 100 units of TEV protease (Gibco or "homemade"). The beads are rotated for 1 hrs at 16°C and the eluate is recovered by gravity flow. Take a 15 µl aliquot (IgG eluate, #3).

200µl of calmodulin affinity bead suspension is transferred to a column and washed three times with 10ml of IPP150 calmodulin binding buffer (IPP150 with 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$). Three mililiters of IPP150 calmodulin binding buffer and 3 µl of 1 M $CaCl_2$ are added to the 1 ml of eluate recovered after TEV cleavage. This solution is transferred to the column containing washed calmodulin beads and rotated for 1 h at 4°C. Beads are washed three times with IPP150 calmoduling binding buffer. The bound proteins are eluted with 1 ml of IPP150 calmodulin elution buffer (IPP150 with 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1mM imidazole, 2mM EGTA). The eluate is concentrated by TCA precipitation.
The eluates are run on a SDS-PAGE and analysed by MS/MS in the mass spectrometry facility.

2.4.6 Co-immunoprecipitation

Lysis and immunoprecipitation were performed similar to the TAP protocol. To induce expression of V5-ZC3H11, the cultures were treated with MG132 for 1h before the experiment. $4x10^7$ cells were used per sample. The procedure was as for TAP, but the lysis buffer was additionally supplemented with phosphatase inhibitors (Phosphostop, Roche) to obtain a distinct band for ZC3H11 on the Western blot. 50µl anti-myc coupled agarose beads (Biomol) were used for precipitation

2.4.7 CAT assay

To determine the effect of different UTRs on gene expression chloramphenicol acetyl transferase (CAT) reporter constructs were used. The CAT transcript can easily be detected on Northern Blot and the protein expression can be assayed with an enzymatic reaction in the CAT assay. CAT transfers a acetyl group from radioactively labeled ^{14}C butryl CoA to chloramphenicol. After uptake of an acetyl group chloramphenicol will become

water insoluble and move from the aqueous phase of the reaction solution to the upper non-aqueous phase of the scintillation solution (Econofluor-2, Perkin Elmer). With time more and more acetyl-chloramphenicol (and therefore radioactive ^{14}C) will move to the upper phase, which will be measured by the scintillation counter.

Sample Preparation

For one sample ≥10^7 cells were taken and spun down at 2,000 rpm for 10 minutes. Pellets were transferred to a microfuge tube centrifuged at 3,000 rpm for 2 minutes and washed using cold 1x PBS. The cell pellet can be frozen in liquid nitrogen and stored at -80°C.
For lysis 300 µl of CAT assay buffer was added followed by freeze-thawing three times in liquid nitrogen. After centrifugation at 13,000 rpm at 4°C for 3 minutes, the supernatant was transferred to a new tube and can be stored at -80°C until use.

Determination of Protein Concentration

The Bradford assay is used to determine the total protein concentration of a sample. The assay is based on the proportional binding of the Coomassie Blue dye to proteins, Coomassie bound to protein has an absorption maximum at 595 nm. The higher the protein concentration, the higher the absorption at 595 nm will be.
Bradford:
0, 5, 10, 15, 20µg of BSA (0.5 µg/µl stock) in 800µl for standard curve
50 µl of each protein sample +750µl water
all + 200µl Bradford reagent (Biorad), mix, incubate ca. 5 min and measure absorption at 595nm in photometer (manually note values, don't use the Bradford program of the machine since the printer will get stuck)

The CAT Assay

For this assay, equal amounts of protein were taken for each sample and scaled up to 50 µl using 100 mM Tris-HCl, pH 7.8. After addition of 200 µl 100 mM Tris-HCl, pH7.8, 2 µl chloramphenicol (40 mg/ml), 10 µl ^{14}C-butryl CoA (box 21C in -80°C) and finally 4 ml scintillation cocktail (in fume hood, Econofluor-2), the samples were measured in a scintillation counter (Beckman LS6000IC) using program #7, measuring ^{14}C. Samples were measured at appropriate time intervals, every 12 to 16 minutes or as required.
The increase of ^{14}C signal in the scintillation fluid is proportional to the amount of enzyme present in the sample. Therefore after blotting the data the slope in the linear range (before saturation) can be compared between the different samples and the relative CAT protein amount calculated.

2.5 RNA co-immunoprecipitation and deep sequencing

Harvesting cells
2x 10^8 procyclic cells (or double this number for bloodstream form) are used per experiment. To covalently bind the RNA to its protein partner the culture is UV crosslinked at 254 nm (UV Stratalinker™ 2400, Stratagene) prior to lysis. The 2x 10^8 cells are concentrated to 10 ml and spread in a thin layer on a big Petri dish (145 mm radius) and then cross linked with 400 mJ/cm^2 (set machine to 4000 x100 microjoules/cm^2) The cross-linked cells are collected in a 15ml falcon and after pelleting transferred in PBS to an Eppi, spun down and finally snap frozen in liquid nitrogen. The pellet can be stored at -80°C until use.

Immunoprecipitation

Immunoprecipitation buffer IPP150:
150mM NaCl
10mM Tris pH7.5
0.1% IGEPAL

Lysis buffer:
10mM NaCl
10mM Tris pH7.5
0.1% IGEPAL
for a final volume of 5ml buffer (including RNase inhibitors RNasin (Promega) and VRCs, added to each sample directly) prepare 4,5 ml of the above with one tablet of complete mini protease inhibitor (2x) and half a tablet of Phosphostop (1x) (both from Roche)

Per IP 50µl of anti myc-bead slurry (Biomol) are used. Care needs to be taken not to spin the beads to fast to prevent damage; usually spin 1min at 2000rpm. In preparation the beads are washed twice with IPP150, the second wash can be supplemented with RNAse inhibitor. Aliquot (50µl) the beads in labeled Eppis for the IP.

Get the cell pellets from the -80°C freezer and proceed **in the cold room on ice.** Add 30µl RNAsin (Promega), 20µl VRCs (200mM stock) and 450µl lysis buffer to each sample. Lyse the cells by passing 15 times through a 27G needle. Spin down debris for 5min at 6000rpm and then transfer the supernatant into a new tube. Add NaCl to the final concentration of 150 mM (10 mM already in buffer). Add this cleared lysate to the beads and rotate slowly in the cold room for 1 hour.
Transfer the flow through (FT) into a new tube and wash the beads 5 times with 1 ml cold IPP150 (first wash supplemented with some RNAsin (5µl/sample))

Protease digest (to remove covalently cross-linked protein before Trifast)
Add to ~ 50µl of pelleted beads: 1µl 10%SDS, 1µl EDTA, 2µl ProteinaseK (20µg). Add double of everything for 100µl flow through
Incubate at 42°C for 15 min and then proceed with the Trifast procedure with following modifications:
Add 7µl glycogen for precipitation and perform one extra wash with 75% EtOH.

Normally the eluate sample is solved in 25µl and the flow through in 50µl. 10µl of each can be loaded on an denaturing agarose gel for analysis on a Northern Blot.

RNA preparation for deep sequencing
To obtain enough RNA for sequencing, the cell number per sample was scaled up to 6x 10^8 procyclic cells.
The flow through RNA was treated with the Ribominus kit, according to the manufacturers (Invitrogen) instructions to remove ribosomal RNAs.
The library was made in the Bioquant facility by David Ibberson, who also performed the Illumina sequencing.
The reads were aligned to the trypanosome genome and annotated by Abeer Fadda.

2.6 Plasmid list

Plasmid list

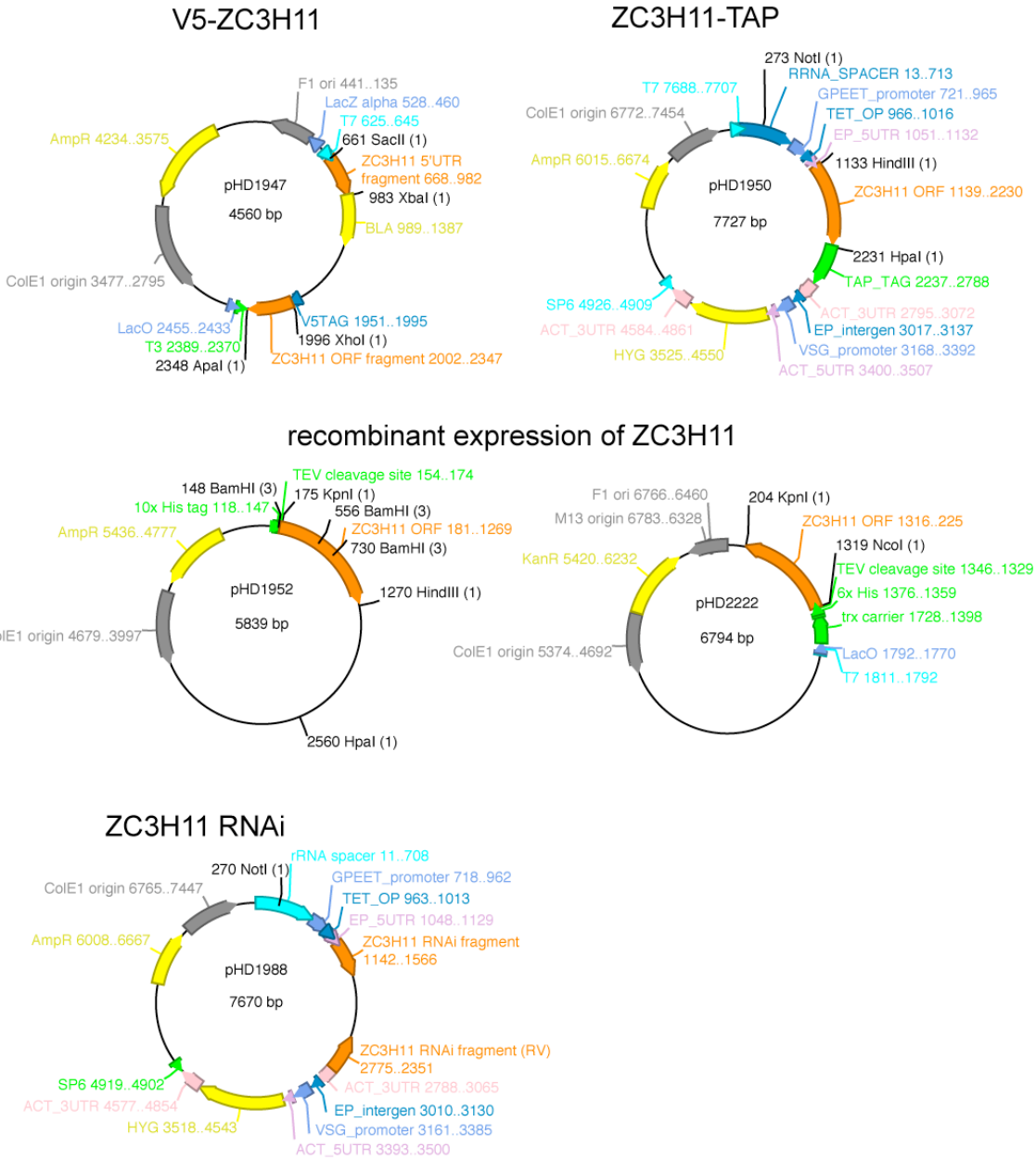
plasmid no.	description	primers used for cloning
pHD1947	in situ V5-tagged ZC3H11	cz3164 to cz3167
pHD1950	ZC3H11-TAP (in pHD918)	cz3155; cz3456
pHD1952	His-ZC3H11 for recombinant protein (in pQE-A38)	cz3024; cz3025
pHD1983	MKT1-myc in pHD1700 (C-terminal tags)	cz3032; cz3033
pHD1987	MKT1 RNAi (p2T7)	cz3052; cz3053
pHD1988	stemloop RNAi targeting ZC3H11 (in pHD1146)	cz3149; cz3150
pHD1991	CAT reporter for tethering, EP 5'UTR and without boxB before actin 3'UTR	P. Delhi
pHD1995	YFP-MKT1 (in p2675, see Carrington vector information)	cz3214, cz3059
pHD1997	ZC3H11 KO plasmid, PAC (in pHD1747)	cz3215 to cz3218
pHD1998	His-MKT1 (in pHD1745, pQE-A38 with polylinker)	
pHD1999	MKT1 RNAi II (p2T7)	cz3219; cz3220
pHD2000	ZC3H11 KO plasmid, BSD	cz3215 to cz3218
pHD2067	ZC3H11-myc, in pHD1700 (C-terminal tags)	cz3155; cz3456
pHD2068	N-terminal fragment of ZC3H11-myc (up to first BamHI site)	
pHD2069	C-terminal fragment of ZC3H11-myc (using internal M101 as start)	cz3457; cz3456
pHD2070	N-terminal fragment of ZC3H11-myc (up to second BamHI site)	
pHD2169	CAT reporter, EP1 5'UTR, 3' truncated actin IGR	
pHD2172	PAC replaced by 2xmyc-NEO fusion resistance in pHD1747 KO vector	cz3866; cz3867; cz3865; cz3860
pHD2194	myc-NPT with HSP70 UTRs, in pHD2172 (HindIII site missing)	cz 141; cz143; cz139; cz3863
pHD2218	myc-ZC3H11 in pHD1701 (N-terminal tags)	
pHD2219	C70S mutant ZC3H11-myc (site directed mutagenesis)	cz4041; cz4042
pHD2222	pET-trx-ZC3H11 (recombinant expression)	
pHD2239	CAT reporter, HSP70 5'UTR, 3' truncated actin IGR	cz3869; cz143
pHD2240	CAT reporter, EP1 5'UTR, HSP70 3'UTR	cz139; cz3868
pHD2241	CAT reporter, HSP70 5'UTR, HSP70 3'UTR	cz3869; cz143; cz139; cz3868
pHD2270	CAT reporter, EP1 5'UTR, 5' part of HSP70 3'UTR	cz139; cz4126
pHD2271	CAT reporter, EP1 5'UTR, 3' part of HSP70 3'UTR	cz4235; cz3868
pHD2272	CAT reporter, EP1 5'UTR, complete actin IGR (BamHI/SalI, from pHD1991)	
pHD2277	CAT reporter for tethering, EP 5'UTR and with 6x boxB before actin 3'UTR	M. Wurst
pHD2299	λN-ZC3H11-myc	cz4480; cz3456
pHD2314	λN-C-termZC3H11-myc	cz4581; cz3456
pHD2318	λN-N-termZC3H11-myc (BamHI digestion of pHD2299)	

2.7 List of oligonucleotides

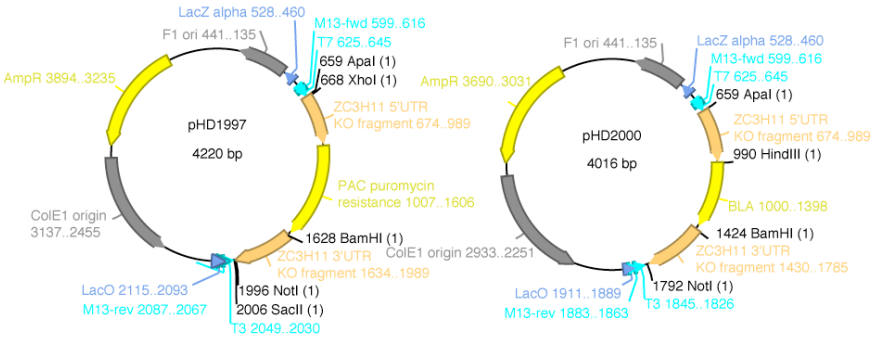
oligonucleotides used for cloning

cz139	HSP70 3'UTR FW (pHD2240, pHD2241 and pHD2270 pHD2194)	BamHI	ctacggatccCAGGTGTATTTCGGACCGGTGTTGCAGT
cz141	HSP70 5'UTR FW (pHD2194)	KpnI	ctacggtaccGATAATGAGCGTTAGTGCT
cz143	HSP70 5'UTR RV (pHD2239, pHD2194)	HindIII	ctagaagcttCAAAGAGGCAGATATTCC
cz3024	ZC3H11 ORF FW (-start) (pHD1952)	KpnI	gacggtaccAGCACTGCAACATCT
cz3025	ZC3H11 ORF RV (pHD1952)	HindIII	gacaagcttTCACAAGGAAAGAAACAT
cz3032	MKT1 ORF FW (pHD1983)	HindIII	gacaagcttATGTACCCCCGACACG
cz3033	MKT1 ORF RV (pHD1983)	BamHI	gacggatccATAATATGTTTCTCGCACTC
cz3052	MKT1 RNAi FW (pHD1987)		gacAGATCTGCATGCgactggccgcttgtagactc
cz3053	MKT1 RNAi RV (pHD1987)		gacGAATTCGTCGACtatcgcgcggactaacttct
cz3059	MKT1 middle RV (pHD1995)		CTCACGCGTgccggggagaacg
cz3149	ZC3H11 stem loop fragment FW (pHD1988)	BglII; SphI	gagaagatctgcatgcATGAGCACTGCAACATCTGC
cz3150	ZC3H11 stem loop fragment RV (pHD1988)	EcoRI; SalI	cggaattcgtcgacATCACACCTCTACGGTTGGC
cz3155	ZC3H11 ORF FW (pHD1950, pHD2067)	HindIII	taataagcttATGAGCACTGCAACATCTGC
cz3164	ZC3H11 ORF fragment FW (pHD1947)	XhoI	taatctcgagAGCACTGCAACATCTGCACC
cz3165	ZC3H11 ORF fragment RV (pHD1947)	ApaI	atatgggcccGTTTCTGGAACGATGTAATCGC
cz3166	ZC3H11 5'UTR fragment FW (pHD1947)	SacII	taatccgcggGTTTATAGAATAAAGCGGACTCG
cz3167	ZC3H11 5'UTR fragment RV (pHD1947)	XbaI	atattctagaCACGTGAATAAACTCTCTGG
cz3214	MKT1 YFP FW +linker (pHD1995)	HindIII	GACAAGCTTCCGCCACCtacccccgacacgatgatg
cz3215	ZC3H11 KO 5' fragment RV (pHD1997/2000)	HindIII	gacaagcttCACGTGAATAAACTCTCTGG
cz3216	ZC3H11 KO 3' fragment FW (pHD1997/2000)	BamHI	gaggarccGTGTGCGCTTGTACGGAAT
cz3217	ZC3H11 KO 3' fragment RV (pHD1997/2000)	XbaI	gatctagaGAGCGAACCGCAAAACATA
cz3218	ZC3H11 KO 5' fragment FW (pHD1997/2000)	XhoI	gactcgagGTTTATAGAATAAAGCGGACTCG
cz3219	MKT1 RNAi_FWII (pHD1999)		AACCGCTAACCGATTTGTTG
cz3220	MKT1 RNAi_RVII (pHD1999)		TGGGGTGGAGTCAAGGTTAG
cz3456	ZC3H11 ORF RV (pHD1950, pHD2067, pHD2299, pHD2314)	HpaI	gacgttaacCAAGGAAAGAAACATATGCAGACC
cz3457	ZC3H11 del. FW (pHD2069)	HindIII	gatcaagcttATCATGGATGGACTCGTGACG
cz3860	Neo ORF RV (pHD2172)	EcoRI	gacgaattcTCAGAAGAACTCGTCAAG
cz3863	Hsp70 3'UTR RV (pHD2194)	SacII	taccgcggATAATGACGGTGGTGGGGAAC
cz3865	Neo -start FW (pHD2172)	BamHI	acggatccGTGGAACAAGATGGATTG
cz3866	myc FW (pHD2172)	HindIII	acaagcttCACATGGGCGAGCAAAAGC
cz3867	myc RV (pHD2172)	BglII	acagatctCAAGTCCTCTTCAGAAATG
cz3868	HSP70 3'UTR RV (pHD2240, pHD2241 and pHD2271)	SalI	gagtcgacATAATGACGGTGGTGGGGAAC
cz3869	HSP70 5'UTR FW (pHD2239)	BamHI	gacggatccGATAATGAGCGTTAGTGCT
cz4041	ZC3H11 site directed mutagenesis FW (pHD2219)		ACAAAGCTGT**ct**AAAAACTTCGTG
cz4042	ZC3H11 site directed mutagenesis RV (pHD2219)		CTTGTAGCGCTCCGCCAAG
cz4126	5' part of HSP70 3'UTR RV (pHD2270)	SalI	gacgtcgacTAGTAGTAGCATCAATGTGTC
cz4235	3' part of HSP70 3'UTR FW (pHD2271)	BamHI	gacggatccGACACATTGATGCTACTAC
cz4480	ZC3H11 ORF FW (pHD2299)	ApaI	tatgggcccAGCACTGCAACATCTG
cz4581	ZC3H11 C-term. fragment FW (pHD2314)	ApaI	tatgggcccATCATGGATGGACTCGTGACG

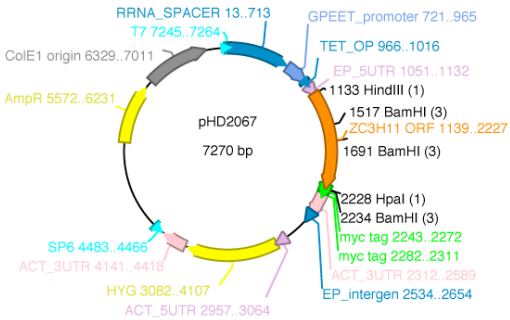
2.8 Plasmid maps

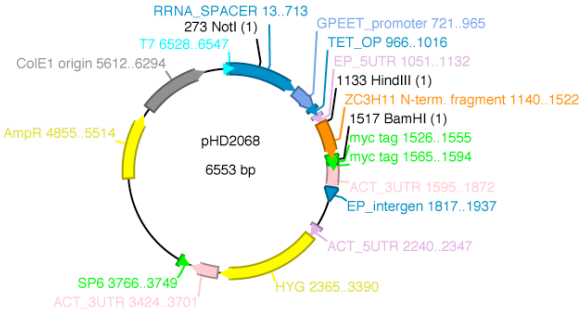


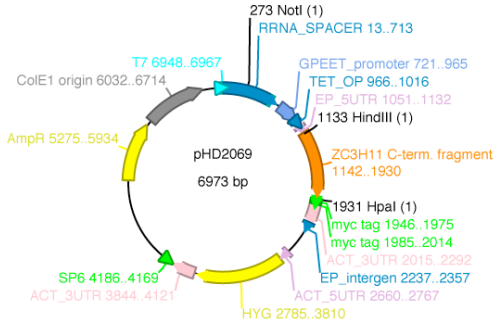
ZC3H11 Knock out

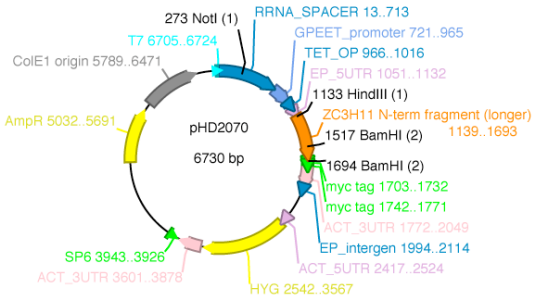


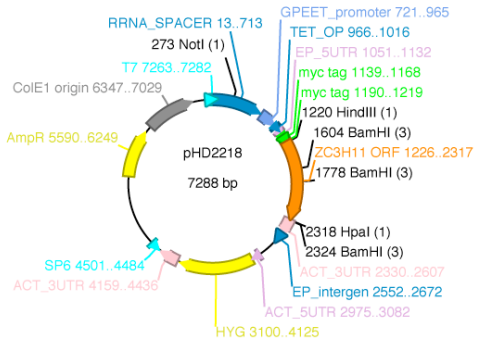
ZC3H11 myc-constructs

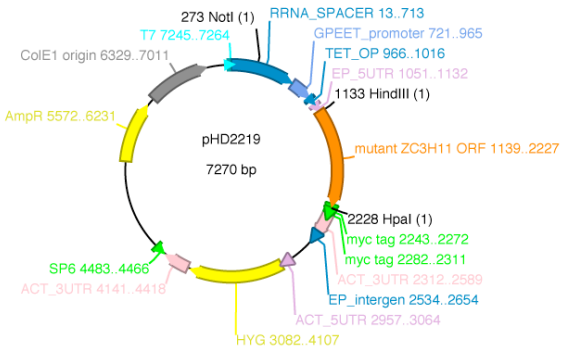




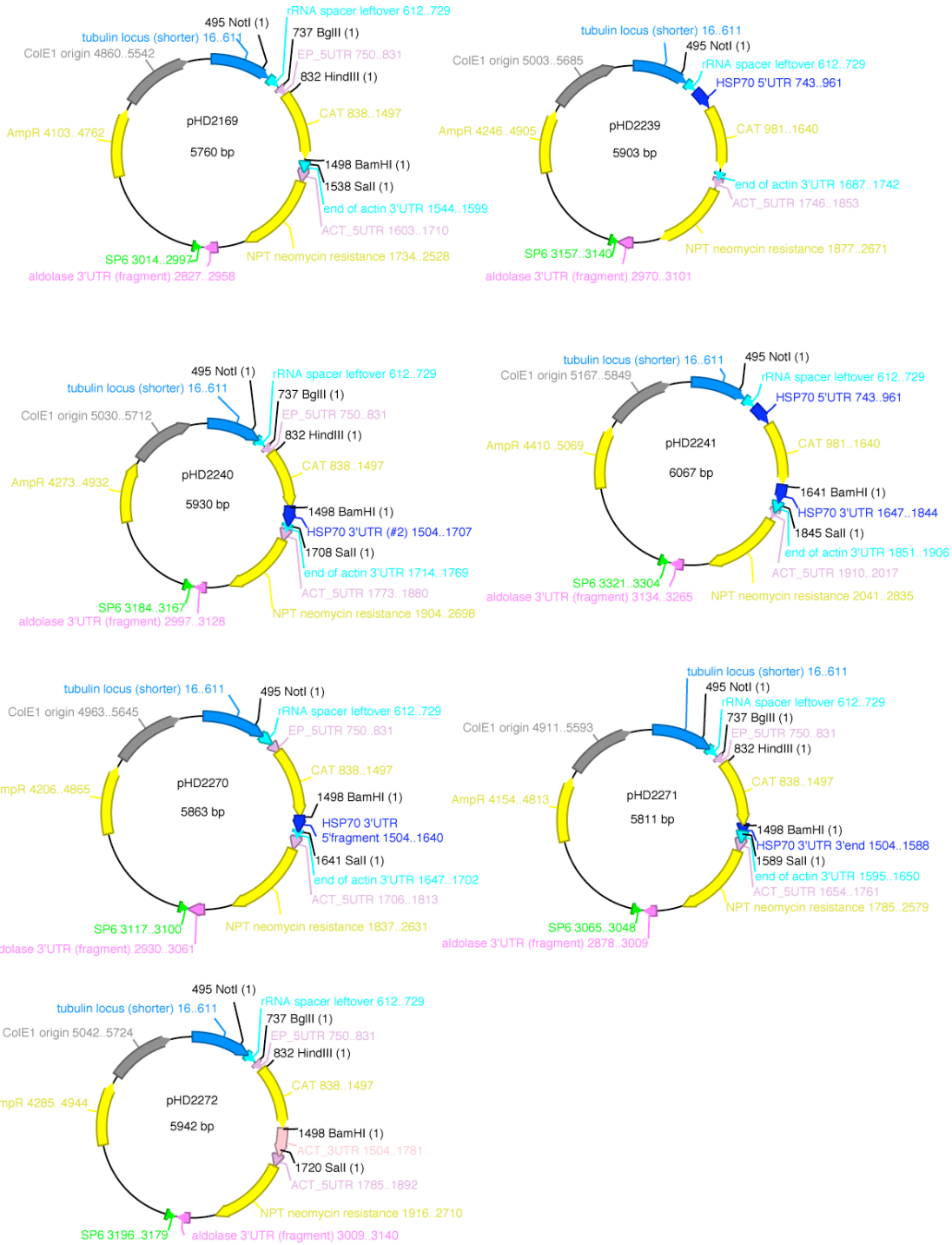




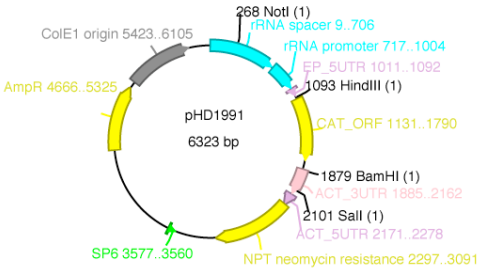


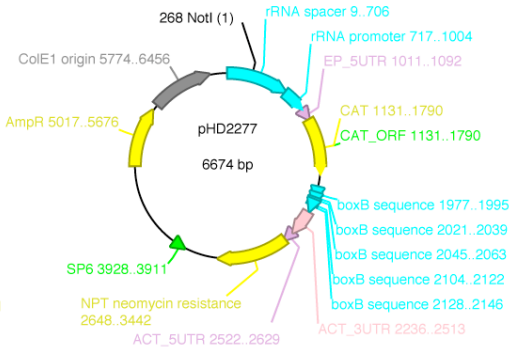


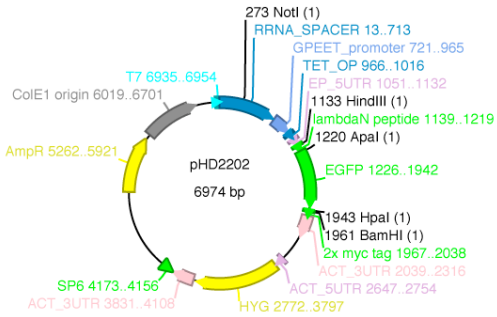
CAT reporter with different 3‘UTRs

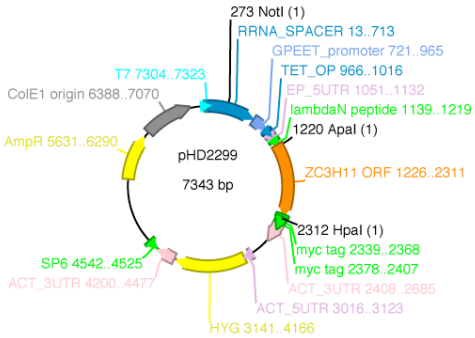


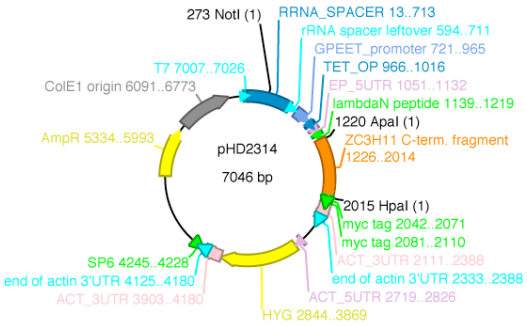
Tethering constructs

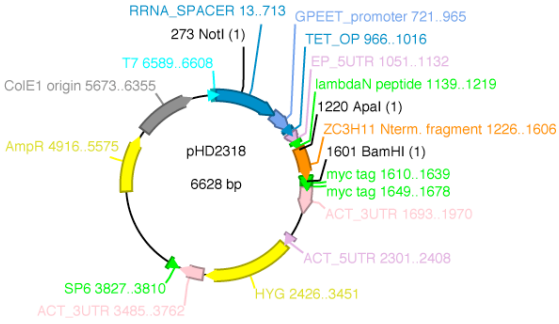


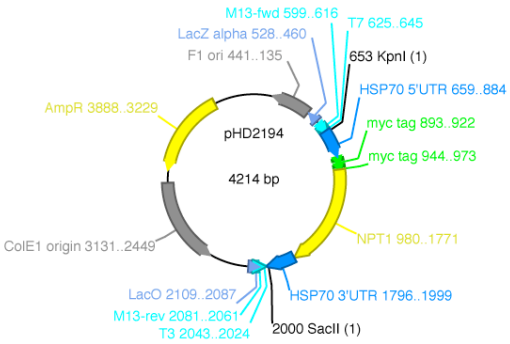






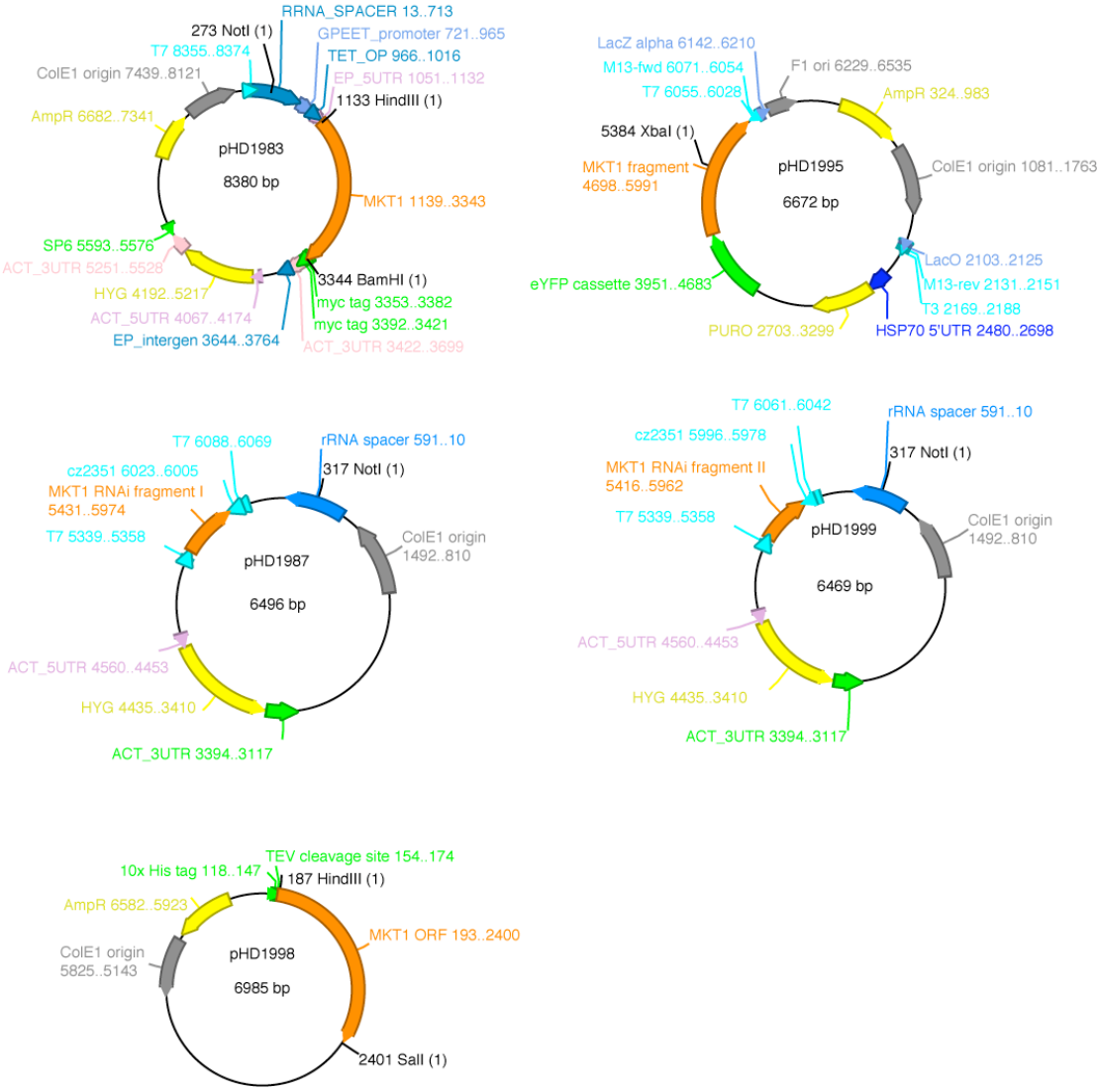






myc-NPT (HSP70 UTRs)

MKT1 constructs



2.9 Web resources

Kinetoplastid databases with expression data and user comments:

TriTrypDB	http://tritrypdb.org
GeneDB	www.genedb.org

NCBI databases, e.g.

BLAST NCBI	http://blast.ncbi.nlm.nih.gov/Blast.cgi

3 Results

3.1 ZC3H11 alignment

As several $C_8C_5C_3H$ RNA binding proteins have been characterized as important RNA-destabilizing proteins in other organisms, e.g. tristetraproline in mammalian cells, we were interested to characterize members in trypanosomes. CCCH proteins of the Tis11 family share a conserved signature motif (K/RYKTEL) preceding their zinc finger domains. The closest matches to this signature were found for *Tb*ZC3H11-13 (see alignment in Figure 3.1 B). In contrast to the Tis11 family, these proteins contain only one zinc finger domain. General information for this gene can be found on TriTrypDB.

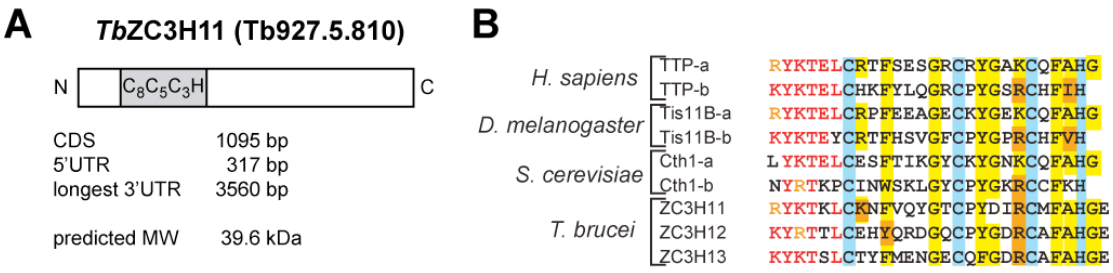


Figure 3.1

A) General cartoon of ZC3H11 and data from the TriTrypDB website.

B) Alignment of the zinc finger domains of several Tis11 family members

The conserved amino acid signature (marked in red) precedes the zinc finger domain (cysteines and histidine shaded in blue). Conserved residues are shaded in yellow and chemically similar ones in orange.

3.2 ZC3H11 RNAi

To determine if ZC3H11 is essential for the parasite, and thus of interest for us, the first experiment performed was the depletion of transcript by inducible RNA interference (RNAi).

RNAi cell lines were generated in both the procyclic and the bloodstream form, using either the p2T7 based pHD1890 plasmid (done by M.Stewart) with opposing T7 promoters or pHD1988 based on the stemloop strategy. The cell

lines obtained for either pHD1890 or pHD1988 showed similar proliferation effects.

The bloodstream stage displayed a clear defect in proliferation already after 24 h of induction of RNAi, followed by cell death (figure 3.2 A). In contrast, there was no effect on procyclic proliferation in normal culture conditions (figure 3.2 B).

The depletion of *ZC3H11* mRNA was verified on a Northern blot, which showed that the RNAi efficiency was similar in both stages (figure 3.2 C). The probe for ZC3H11 detects two bands running at approximately 4 kb and 5.5 kb and both are reduced upon RNAi. Given that the CDS and longest UTR lengths (see figure 3.1 A) sum up to around 5 kb and a poly(A) tail is added, this size could fit the upper band. The shorter transcript probably uses an alternative polyadenylation site as several sites are annotated in the database.

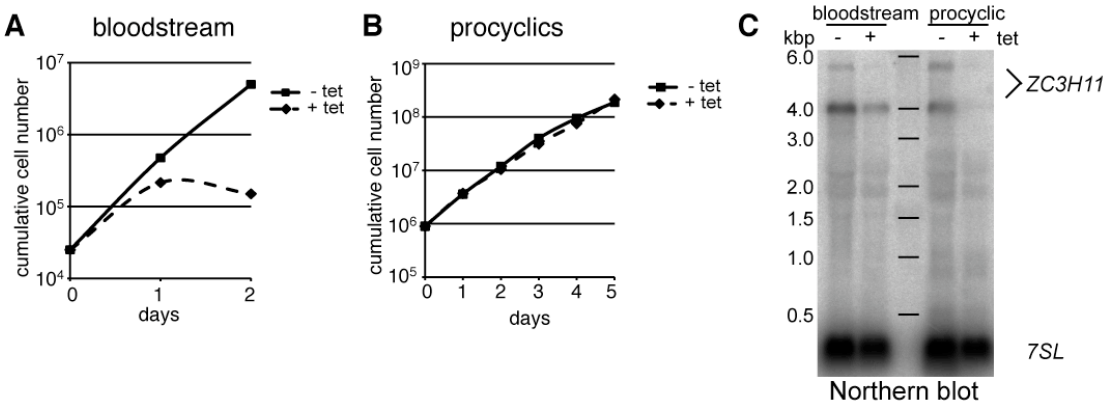


Figure 3.2

A) Effect of ZC3H11 depletion (pHD1988) on bloodstream form parasite proliferation.

B) Effect of ZC3H11 depletion (pHD1988) on procyclic parasite proliferation.

C) Total RNA from RNAi cell lines was extracted and analyzed on Northern blot using probes for ZC3H11 and 7SL (loading control).

3.3 Knockout

To further examine the importance of ZC3H11 for cell survival in both life stages, I attempted to knock out both alleles in the parasite genome by sequentially replacing the ZC3H11 ORF by puromycin and blasticidinS resistance genes (pHD1997 and pHD2000, respectively, figure 3.3 A). Clones were obtained in both life stages by selection for the resistances and the genomic context was checked by Southern blot.

The genomic DNA was digested with BsaBI enzyme, which gives fragments of specific sizes (given in figure 3.3 A). This revealed that for both stages the resistance cassettes integrated, as fragments of the expected size could be observed (figure 3.3 B). Unfortunately, the wild type allele was still present for the bloodstream form, meaning that it is no knockout.

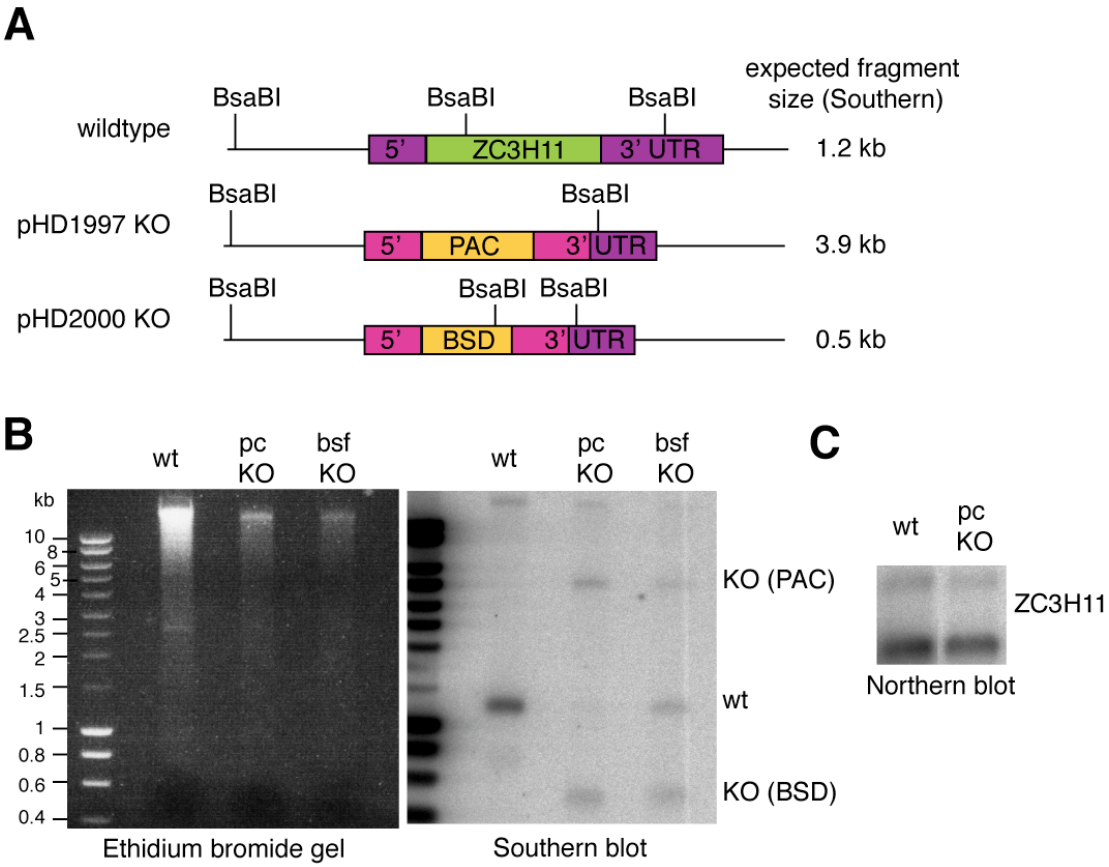


Figure 3.3

A) Schematic representation of the wild type ZC3H11 gene and the knockout constructs in the genomic context. A gene conferring resistance against puromycin (PAC - puromycin N-acetyl-transferase) or blasticidinS (BSD - blasticidin-S deaminase) is each supposed to replace one allele of ZC3H11 by homologous recombination. Restriction sites for BsaBI and resulting fragment sizes are marked.

B) Southern blot. Genomic DNA of wild type and knockout strains was digested with BsaBI and separated on an agarose gel. After blotting the nitrocellulose membrane was probed with the ZC3H11 3'UTR fragment (pink in the schematics).

C) Using a probe for *ZC3H11* ORF, a strong *ZC3H11* mRNA signal can still be detected in the procyclic „KO clone" on a Northern blot.

The procyclic knockout line was therefore also checked carefully and a Northern blot revealed that the mRNA was actually still expressed (figure 3.3 C), so apparently the knockout was not successful either.
After this discouraging attempt, so far no further attempts were made to obtain knockout cell lines.

3.4 Recombinant ZC3H11 protein

Recombinant ZC3H11 was expressed in *E.coli* in order to raise a specific antibody and for possible use in *in vitro* experiments.
The first expression strategy used a 10x Histidine-tag at the N-terminus (pHD1952). In the standard protocol this protein was insoluble with native lysis conditions (S1 in figure 3.4 A). Several variations of expression conditions were tested, but ZC3H11 stayed in the insoluble fraction under native conditions. Nevertheless, denaturing conditions (S2 in figure 3.4 A) allowed purification using Ni-NTA agarose. The purified protein (figure 3.4 B) was dialyzed with PBS, which led to precipitation of the protein. As this, according to the company (Charles Rivers Laboratories), does not interfere with the immunization procedure, the precipitated protein was sent to for immunization of a rat.
The serum was evaluated with trypanosome cell lysates on a Western blot. There was a strong background and no specific band at the expected size (approximately 50-60 kDa, see chapter 3.5 V5-ZC3H11). Different conditions or purification of the antibody from the serum did not improve the result, so it was concluded that this antibody is not useful with the ZC3H11 concentrations found in trypanosomes. The antibody readily detected 25ng of the recombinant protein (not shown), but the exact sensitivity was never determined.

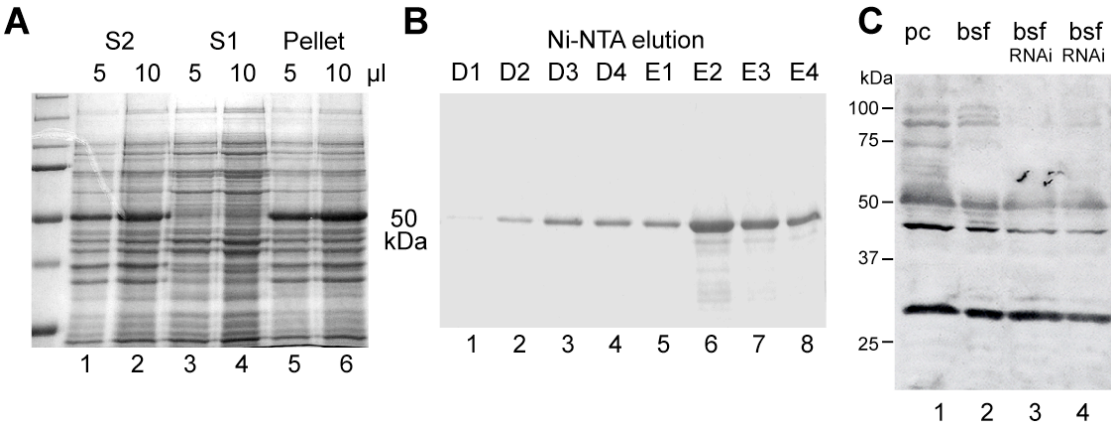


Figure 3.3

A) Initial solubility test of His-ZC3H11. Cells were lysed in native lysis buffer and the soluble proteins (S1) separated from the insoluble fraction by centrifugation. This insoluble fraction was resuspended in denaturing lysis buffer (containing 4M urea) and the solubilized fraction (S2) separated from the still insoluble fraction (Pellet).

B) The eluate fractions from a purification using Ni-NTA agarose in denaturing conditions. Elutions in sequential fractions using buffer D (pH 5.9) and buffer E (pH 4.5).

C) Evaluation of the ZC3H11 antibody using procyclic and bloodstream form cell lysates

Natively purified recombinant protein would be useful for *in vitro* assays, therefore another expression vector was tested with an N- terminal trx-fusion protein that is supposed to assist the solubility of the protein (pET-trx1b; from Krauth-Siegel lab). This fusion protein (pHD2222) was also insoluble and could not be purified in native conditions.

3.5 V5-ZC3H11 expression

Since I did not obtain a usable antibody against TbZC3H11 (see chapter 3.4), one allele was *in situ* V5-tagged in the endogenous genomic locus and used to observe the protein expression. This revealed that the protein is not well expressed in normal culture conditions, both in procyclic and bloodstream stage parasites, as the V5-tagged protein was hardly detectable (figure 3.5 A and B lane 2).

Starving procyclic cells by leaving them at high densities over the weekend led to an increase in the V5-ZC3H11 signal (not shown). Shifting procyclic cells that are normally cultivated at 27°C to 37°C or 41°C for a heat shock also induced V5-ZC3H11 expression within the hour (figure 3.5 A). At 37°C the cells were able to survive and double cell numbers overnight, whereas 41°C quickly led to a decrease in cell motility, followed by death after extended incubation. The apparent molecular weight of V5-ZC3H11 was approximately 60 kDa, which is 20 kDa higher than expected from the sequence (39.6 kDa + 1.6 kDa V5-tag). After prolonged incubation at 41°C (figure 3.5 A, lanes 8 to 10) additional lower bands (50 kDa) appeared which could either be due to removal of post-translational modifications or degradation in the dying cells.

As bloodstream form cells normally grow at 37°C, the heat shock temperature was 43°C. This was rapidly lethal and a large part of the parasites was dead after 1 hour of incubation. However, after 30 minutes an increased V5-ZC3H11 signal can be detected (figure 3.5 B, lane 3).

Subsequently other stress conditions and drugs were tested for induction of ZC3H11 expression. In both life stages inhibition of the proteasome with Lactacystin (not shown) or MG132 (Figure 3.5 C, lanes 1, 2, 8 and 9) or a mild translational stress by low concentrations of puromycin (figure 3.5 A lanes 11 to 13 and B lanes 5 and 6) increased V5-ZC3H11 expression.

Treatment with 0.5 mM arsenite (figure 3.5 C lanes 3 and 4) or 2.5 mM arsenite (not shown) for 2 hours did not induce expression in procyclics. Shifting bloodstream stage cells to 22°C also had no effect on ZC3H11 expression.

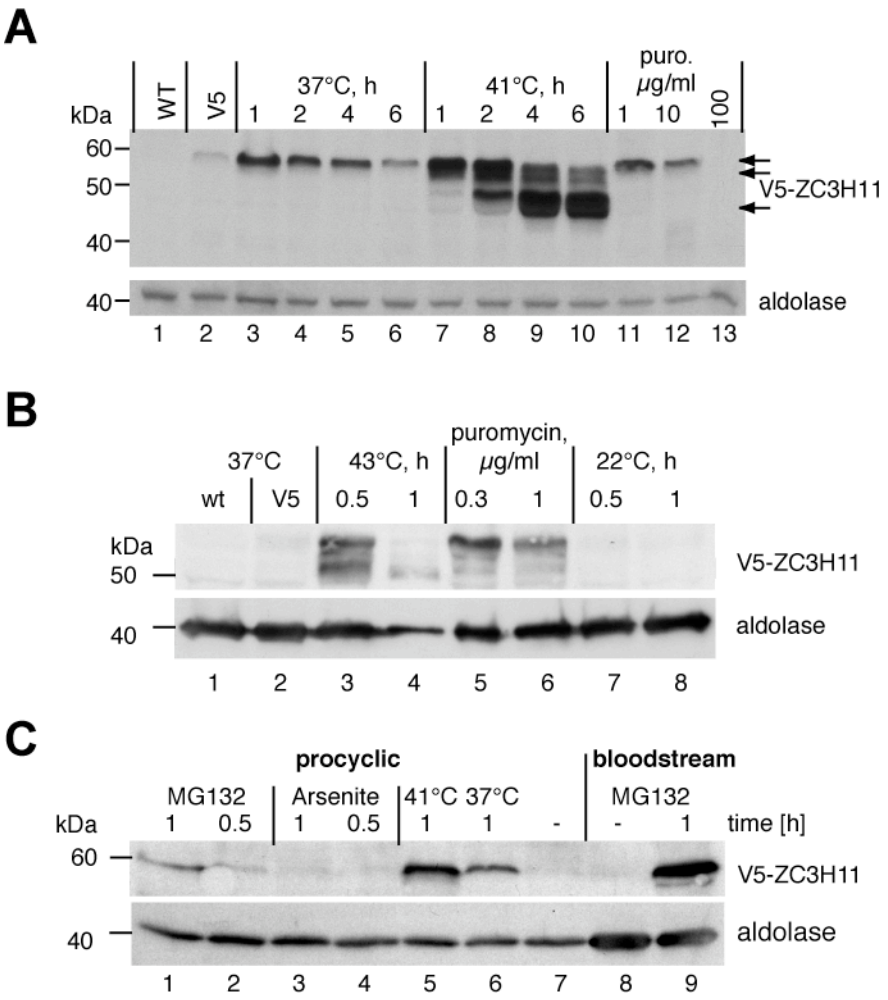


Figure 3.5

A) V5-ZC3H11 expression in procyclic cells. On a Western blot the V5-antibody detects a weak signal in normally cultured cells but the signal increases after heat shock at 37°C or 41°C and after weak translational stress with low concentrations of puromycin. ($1x10^7$ cells/lane)

B) Bloodstream stage parasites were subjected to a heat shock at 43°C, treated with puromycin or incubated at room temperature and expression of V5-ZC3H11 was analyzed on a Western blot. ($1x10^7$ cells/lane)

C) Inhibition of the proteasome by treatment with 10µg/ml MG132 led to increased ZC3H11 expression in both life stages whereas treatment with 0,5mM arsenite had no effect. ($1x10^7$ cells/lane)

3.6 ZC3H11-myc and fragments

In the previous experiments I observed that V5-ZC3H11 migrates at a higher apparent molecular weight than expected. To possibly narrow down the region of the protein responsible for this, the full length protein and several fragments were expressed (figure 3.6 A). These were tetracycline-inducibly expressed with C-terminal myc tags (based on pHD1700) or with N-terminal myc tags (pHD1701).

Both the C-terminally (pHD2067, construct I) and N-terminally (pHD2218, construct VI) tagged full length ZC3H11-myc migrated at approximately 60 kDa (figure 3.6 B and C). Considering that the two myc-tags only give an additional weight of roughly 3 kDa this meant that this construct was also migrating roughly 20 kDa higher than expected, similar to what was already seen for V5-ZC3H11. Interestingly, a construct with a single amino acid change (C70S) to mutate the zinc finger domain (pHD2219, construct V) was expressed as well and it appeared to run as a slightly lower band (figure 3.6 C).

For the N-terminal fragments (pHD2068/2070, constructs II and III) several bands were observed on the Western blot running up to 10 kDa higher than expected. The main band detected for the C-terminal fragment (pHD2069, construct IV) ran at the expected size (30kDa) but a weaker additional band at roughly 45kDa could also be observed. This construct was also apparently expressed at lower levels compared to the N-terminal fragments.

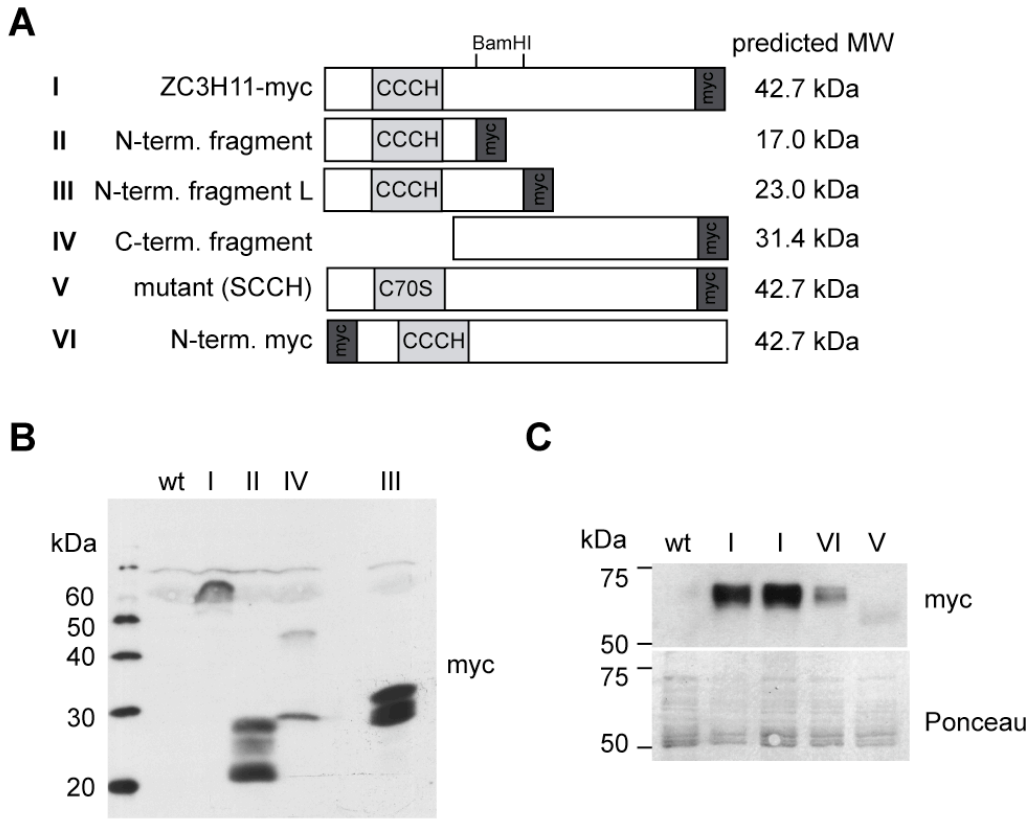


Figure 3.6

A) Schematic representation of the constructs. The zinc finger domain is marked in light and the myc tags in dark grey.

B) Expression of full length ZC3H11 and fragments (constructs I – IV) in bloodstream form trypanosomes detected on Western blot using anti-myc antibody.

C) Expression of C-terminally and N-terminally myc-tagged full length ZC3H11 and the zinc finger mutant (constructs I, V and VI) in procyclics.

3.7 Phosphatase assay

Post-translational modifications of a protein can be an explanation for an unexpected migration pattern on a Western blot and one of the most common modifications is phosphorylation. *Tb*ZC3H11 was not found in the trypanosome phosphoproteome (Nett, Martin et al. 2009) but this could be due to low abundance of the protein. Therefore the myc-tagged ZC3H11 protein and fragments were tested for phosphorylation.

For this, cell lysates were incubated with or without λ-phosphatase and phosphatase inhibitors and the running patterns compared on Western blot.

Phosphatase treatment of the full-length fragment shifted the ZC3H11-myc signal running at roughly 60 kDa to a defined band at 50 kDa (figure 3.7). Addition of phosphatase inhibitors inhibited this shift. It should be noted that compared to the input, the sample incubated without enzyme seemed already more diffuse and shifted to a lower molecular weight, similar to the sample with the phosphatase inhibitors, indicating that some reaction is occurring during the incubation of the lysates without addition of extra phosphatase.
The clones of the zinc finger mutant protein (construct V) did not express as well as the full-length protein (construct I) and the protein migrated at a lower apparent size (compare figure 3.7, lanes 1 and 5). Nevertheless the slightly diffuse signal also collapsed to a defined band upon addition of the phosphatase, but not in the presence of the inhibitors.
The pattern observed for the N-terminal fragment was similar to the full-length protein and seemed to collapse to roughly the predicted molecular weight (17 kDa). Due to the low expression of the C-terminal fragment (construct IV) it was not analysed in this assay. But since multiple close bands were not observed for this fragment and the main band was running at the expected size, in contrast to the N-terminal part it is probably not extensively phosphorylated.
Dephosphorylation of ZC3H11 reduced the apparent molecular weight from around 60 kDa to roughly 50 kDa, which is still 10 kDa higher than expected from the amino acid sequence.

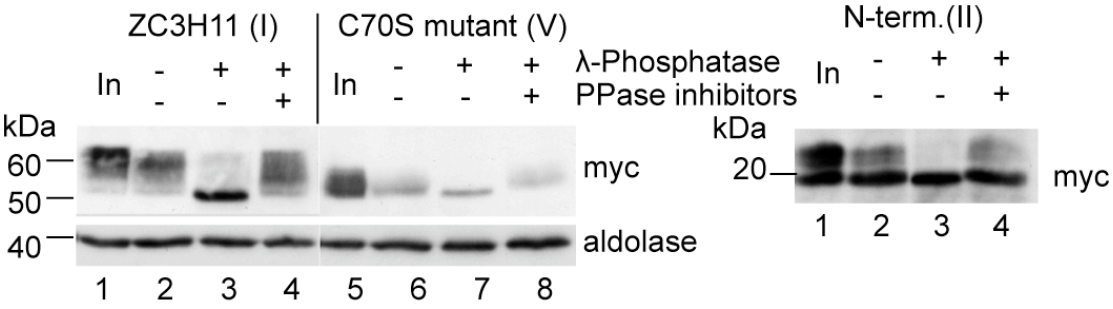


Figure 3.7

Cell lysates of trypanosomes (input: In) expressing ZC3H11-myc, the C70S mutant protein or the N-terminal fragment were incubated in the presence or absence of λ-phosphatase and phosphatase inhibitors (PPase inhibitors) and the migration checked on a Western blot using anti-myc antibody. 5x 10^6 cells were loaded in each lane.

3.8 Microarray

Since the depletion of ZC3H11 has a strong effect on the bloodstream-stage parasite (chapter 3.2), the effect of *ZC3H11* RNAi was further examined by comparing the total mRNA expression profiles of *ZC3H11* depleted (24h induction) and wild type cultures by microarray analysis. The TIGR array, covering most genes with one 70mer oligonucleotide, was used. All steps after isolation of the RNA, including the bioinformatic analysis, were performed by Rafael Queiroz.

Relative to the wild type, 19 transcripts were decreased and only five were increased more than 1.5 fold (table 2.8). Among the decreased transcripts were several that encoded metabolic enzymes. This could be due to a direct effect of ZC3H11 or it could be a secondary effect, e.g. due to the onset of growth inhibition. Remarkably, several chaperone mRNAs were decreased. This was unexpected as chaperones such as HSP70 are constitutively expressed in trypanosomes and had not previously been found to be regulated in microarray studies. The regulation of *HSP70* was verified on a Northern blot (figure 3.8).

Apart from the transcript encoding the major cytoplasmic HSP70 (Tb11.01.3110/ Tb927.11.11330) also a HSP110 homologue (Tb927.10.12710) and co-chaperones like a FKBP family peptidylprolyl cis-trans isomerase containing TPR domains (Tb927.10.16100), a DnaJ (HSP40) protein (Tb927.2.5160) and an HSP20-like protein (Tb11.01.7120/ Tb927.11.15480) were downregulated.

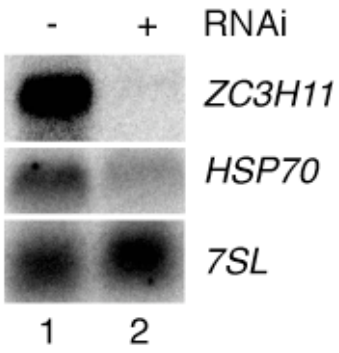


Figure 3.8

ZC3H11 depletion decreases the steady state level of *HSP70*.

microarray: bloodstream form *ZC3H11* RNAi vs. wildtype

GeneDB ID	v4 IDs	Description	p-value	fold change
Tb927.10.1560	Tb10.70.6350	hypothetical protein	0.000	-12.95
Tb927.4.2520		NAD dependent deacetylase (Sir2 family)	0.067	-4.67
Tb927.10.3710	Tb10.70.3660	proteasome activator protein PA26	0.005	-3.73
Tb927.11.10040	Tb11.01.1800	hypothetical protein	0.053	-3.21
Tb927.7.6480		hypothetical protein	0.003	-2.80
Tb927.11.4250	Tb11.02.1750	hypothetical protein	0.052	-2.51
Tb927.10.16100	Tb10.61.0180	PPIase, FKBP and TPR domains	0.005	-2.46
Tb927.5.810		ZC3H11	0.022	-2.37
Tb927.2.1380		leucine-rich repeat protein	0.019	-2.32
Tb927.11.11330	Tb11.01.3110	heat shock protein 70	0.039	-2.24
Tb927.11.11330	Tb11.01.3110	heat shock protein 70	0.067	-2.08
Tb927.9.11600	Tb09.211.2740	Gim5B protein	0.075	-1.88
Tb927.1.3830		glucose-6-phosphate isomerase, glycosomal	0.061	-1.80
Tb927.11.1270	Tb11.47.0022	hypothetical protein	0.162	-1.73
Tb927.8.3530		glycerol-3-phosphate dehydrogenase [NAD+]	0.061	-1.71
Tb927.10.12710	Tb10.389.0880	heat shock protein 110	0.075	-1.64
Tb927.2.5160		chaperone protein DnaJ	0.108	-1.63
Tb927.11.15480	Tb11.01.7120	HSP20-like chaperone	0.282	-1.62
Tb927.10.2020	Tb10.70.5800	hexokinase (HK2)	0.135	-1.60
Tb927.8.2540		3-ketoacyl-CoA thiolase	0.145	-1.54
Tb927.8.480		phosphatidic acid phosphatase protein	0.003	+3.74
Tb927.8.510		hypothetical protein	0.005	+2.61
Tb927.10.10240	Tb10.6k15.0040	procyclin-associated gene 1 (PAG1)	0.099	+1.94
Tb927.5.5260		variant surface glycoprotein (VSG, pseudogene)	0.062	+1.72
Tb927.8.490		hypothetical protein	0.135	+1.56

Table 2.8:
The results of the microarray were filtered for a p-value < 0.3 and a fold regulation > 1.5. The fold change is the relative abundance in ZC3H11 depleted vs. wild type sample. Decrease upon ZC3H11 depletion is marked in orange and increase in green.
In yellow shading are the chaperones that were also found in the RNAIP (chapter 3.13). For convenient comparison with other datasets both v4 and the new v5 gene IDs (relevant for chromosomes 9 to 11) are given as found on TriTrypDB or GeneDB, respectively.

3.9 Half-lives

The decreased steady state level of *HSP70* mRNA upon *ZC3H11* depletion could be due to a destabilization of the transcript. To verify this speculation a RNA degradation assay was performed. Transcription was inhibited with actinomycin D and the remaining mRNA levels checked at several time points (0, 15 and 30 min).
As was seen in the previous experiment, the initial (t0) amount of *HSP70* mRNA was decreased in the *ZC3H11* depleted culture. When we normalized the remaining *HSP70* mRNA to each initial amount it was clear that indeed the half-life decreased and therefore destabilization was the cause for decreased *HSP70* levels (Figure 3.9). The variation of *HSP70* transcript half-

life in different experiments was quite high, both for the wild type and RNAi cell lines, but I reproducibly obtained lower half-lives for the *ZC3H11* depleted cells.

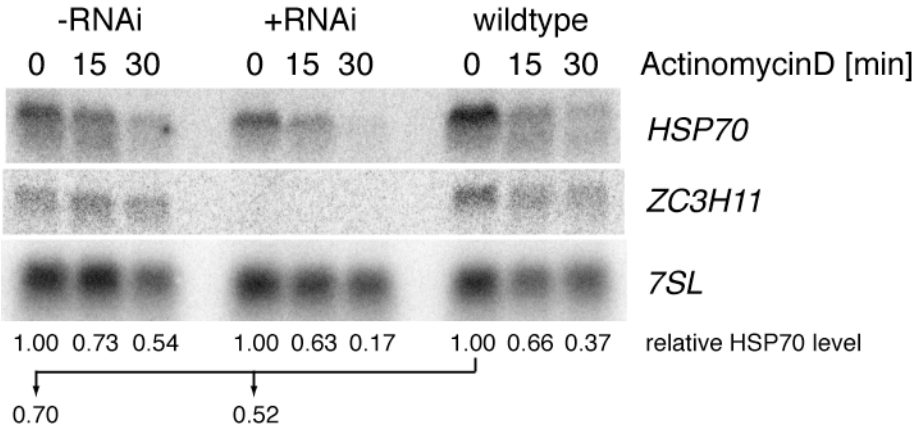


Figure 3.9

At time point 0, transcription was inhibited by addition of actinomycinD in trypanosome cultures with or without ZC3H11 RNAi. Time points were taken after 15 and 30 minutes. The residual levels of *HSP70* mRNA were normalized with *7SL* to the respective input (t0). The relative initial HSP70 levels are marked with the arrows.

3.10 CAT reporter

The effect on HSP70 mRNA abundance and half-life seen upon depletion of ZC3H11 could be mediated by specific signatures in the target transcript. To determine which part of *HSP70* mRNA is responsible, the *HSP70* UTRs were cloned adjacent to the chloramphenicol acetyltransferase (CAT) ORF in a reporter expressed constitutively by RNA polymerase II from the tubulin locus. *CAT* mRNA levels were examined on Northern blot and the protein was measured in the CAT assay.

In the control reporter (pHD 2169) *CAT* is flanked by the *EP* 5'UTR and a truncated actin IGR. These were exchanged with *HSP70* 5', 3' or with both 3' and 5' UTR (pHD2239, 2240 or 2241, respectively) in procyclic and bloodstream-form cells.

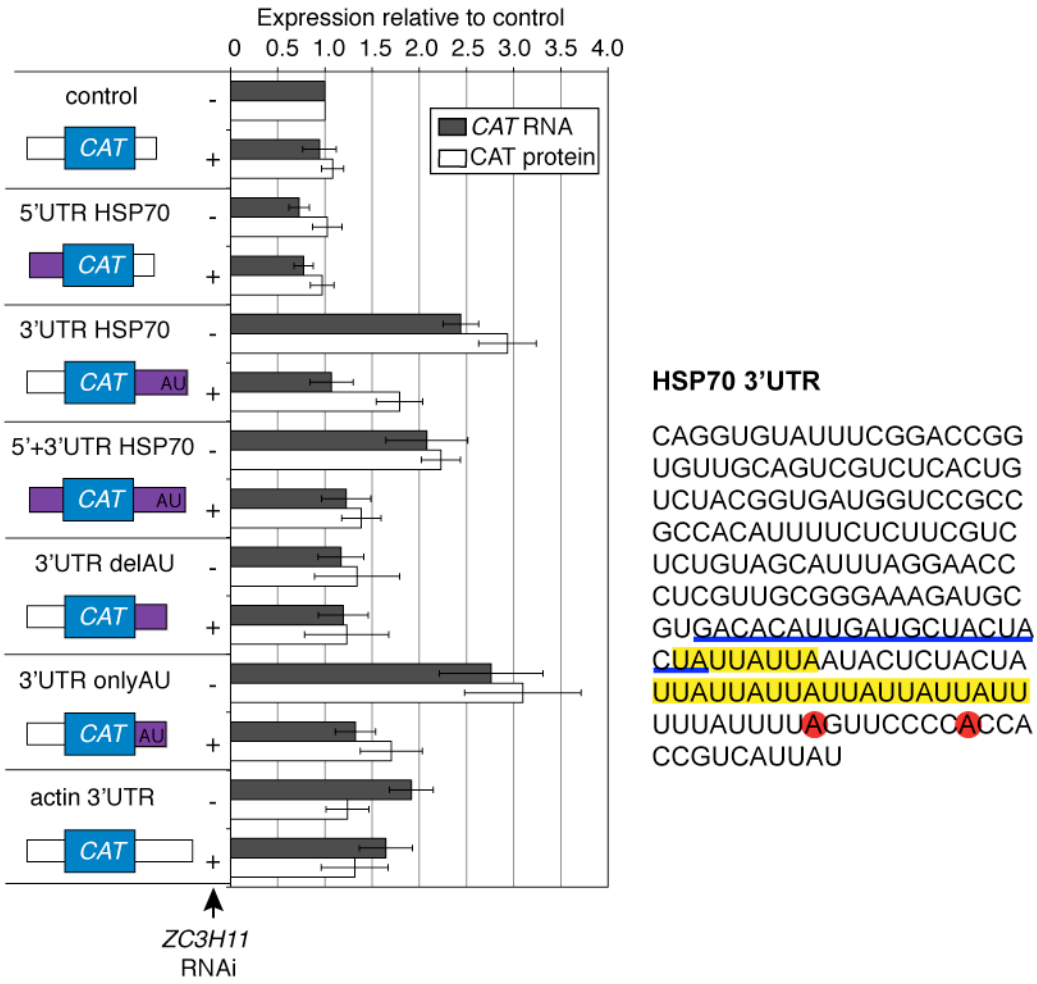


Figure 3.10.1

Expression of CAT reporter constructs in bloodstream form cells with or without ZC3H11 RNAi. Constitutively expressed CAT reporter construct is flanked by *EP* 5'UTR and a truncated actin 3'UTR (both marked in white) in the control. CAT expression in reporter constructs with *HSP70* UTRs (purple) and the effect of induction of ZC3H11 RNAi were compared to the control. *CAT* mRNA levels were determined by Northern blot and protein levels by the CAT assay. The experiments were performed at least in triplicate for each reporter.

In the sequence of the *HSP70* 3'UTR (on the right side) the published polyadenylation sites (Häusler and Clayton 1996) are marked with red circles, the AUU repeats with yellow boxes and the oligonucleotide sequence used to clone the fragments is underlined in blue.

In bloodstream-form cells the levels of CAT protein were generally mirroring *CAT* mRNA levels for all constructs, indicating that there is no specific effect of the UTR sequences on translation efficiency of the reporters. Introducing the *HSP70* 5'UTR did not significantly change CAT expression. In contrast, including the 3'UTR led to an increased expression (~2.5 fold), either with or

without the *HSP70* 5'UTR. In addition, the same cell lines featured inducible *ZC3H11* RNAi, so the effect of *ZC3H11* depletion on reporter expression could be measured. The reporter constructs with the *HSP70* 3' UTR displayed a significant decrease upon induction of RNAi, to a similar level as the level of the control. The CAT expression levels of the control reporter, as well as the *HSP70* 5'UTR reporter, did not change. A construct with the complete actin 3'UTR instead of the truncated version was included as another control (pHD2272). This showed a slightly higher expression level compared to the inital control, but also did not react to *ZC3H11* depletion.
The region responsible for the effect of the *HSP70* 3'UTR was further narrowed down using constructs with two pieces of the *HSP70* 3' UTR, either the 5' ("delAU") or the 3' ("onlyAU") end (pHD2270 and pHD2271, see *HSP70* 3' UTR in figure 3.10.1). The 5' part of the 3'UTR, which does not contain the AUU repeat signature, showed a similar expression to the control and did not react to ZC3H11 down-regulation, while including the 3' part, which consists mostly of AUU repeats, had the same effect as the full *HSP70* 3'UTR.
These results show that in bloodstream-form cells reporters with the *HSP70* 3' UTR, or just the AUU repeat containing part, are destabilized by ZC3H11 depletion.

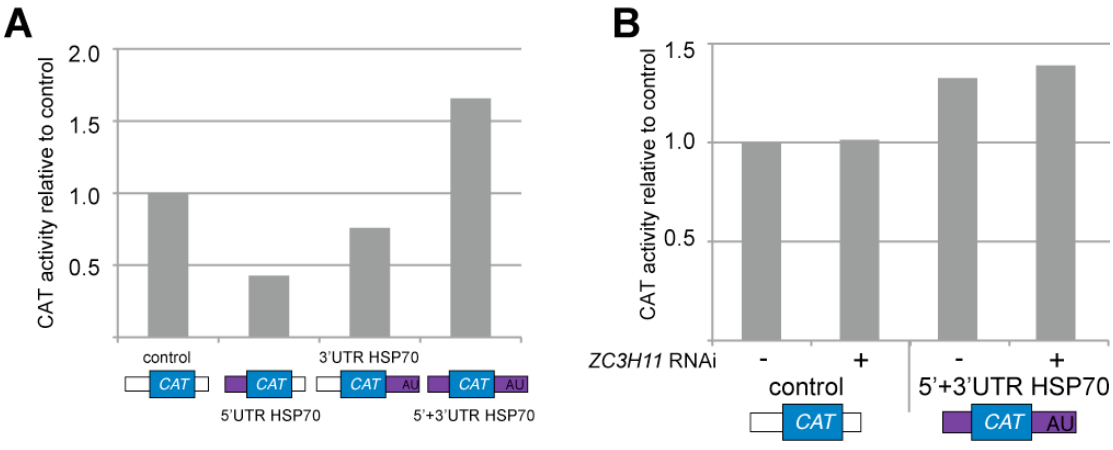


Figure 3.10.2
A) Relative CAT reporter levels in procyclics determined by the CAT assay. Data from one experiment. B) Effect of ZC3H11 RNAi on CAT protein levels in procyclics. Data from one experiment.

The previous experiments were all done in bloodstream-stage cells (where ZC3H11 depletion leads to a proliferation defect) and I also wanted to

examine the situation in procyclic cells. Exchanging the *EP* 5'UTR for *HSP70* 5'UTR led to a reduced reporter expression (~0.4 fold). In contrast to the bloodstream form, the *HSP70* 3'UTR alone did not increase the expression (~0.6 fold) and also the construct with both UTRs did not increase expression as much (1.5 fold) in procyclics cells (Figure 3.10.2, A). This was only done twice and the average is given in the brackets.
ZC3H11 depletion did not affect reporter expression in procyclics, either for the control or the *HSP70* UTRs construct (Figure 3.10.2 B). This experiment was only performed once, but the stabilization mediated by the *HSP70* 3'UTR and also the destabilization observed upon *ZC3H11* depletion in the bloodstream stage does not seem to occur in the procyclic stage. The reporter expression in procyclics was never checked after heat shock, but ectopic expression of ZC3H11-myc did not affect either the control, or the *HSP70* UTRs construct (done once, not shown).

3.11 Myc-NPT1 reporter in HSP70 locus

To gain a better understanding about HSP70 expression in heat shock, a neomycin resistance reporter gene (NPT1) with N-terminal myc-tags was inserted in the HSP70 genomic locus, replacing one copy in the HSP70 array. This construct will be expressed with the *HSP70* UTRs and in the same polycistronic transcription unit. Therefore it should closely mirror HSP70 expression.
After one hour of 37°C heat shock in procyclics, tubulin mRNA levels are unaffected, while at 41°C the levels drastically decrease. In contrast, the *HSP70* and also *NPT1* reporter mRNA show an increase in the mild heat shock and are even stable after the 41°C heat shock. (Figure 3.11 B).
This shows that the reporter is expressed similarly to *HSP70*. As we do not have a specific antibody against the trypanosome HSP70, this myc-NPT reporter protein could be used to visualize the expression in different conditions on a Western blot using commercially available anti-myc antibody.

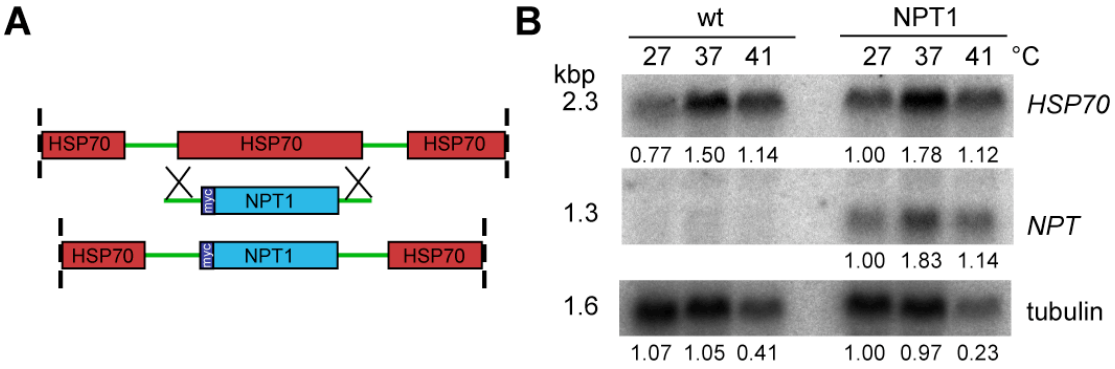


Figure 3.11

A) The NPT1 gene replaces one copy of the HSP70 genes by homologous recombination.

B) mRNA levels of *HSP70*, *NPT* and tubulin in procyclic cells after one hour heat shock at indicated temperatures.

3.12 RNA Co-Immunoprecipitation

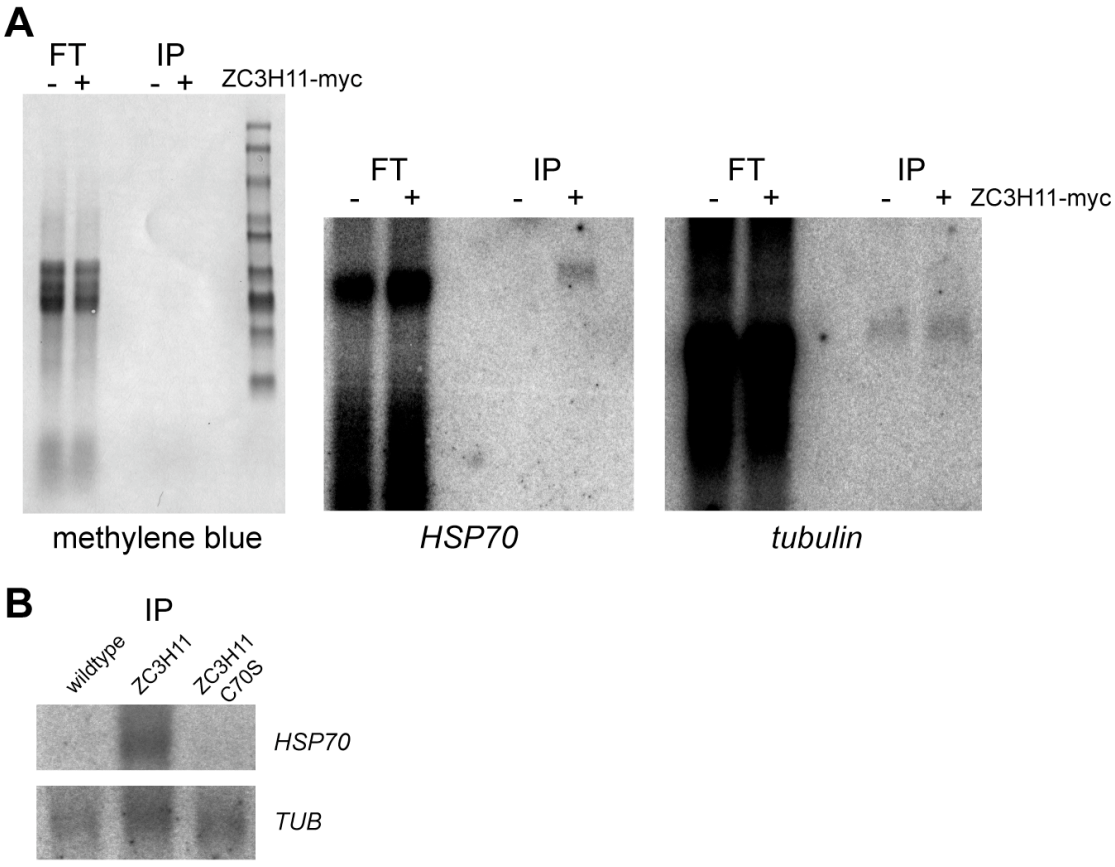


Figure 3.12

A) Northern blot with the flow through (FT) and immunoprecipitated (IP) fractions of a RNA-co-immunoprecipitation. For the flow through sample the equivalent of approximately $8x10^6$ cells and for the eluate sample the equivalent of $1x10^8$ cells are loaded on the Northern blot.

B) The bound fractions of another RNA-IP including the zinc finger mutant cell line.

As CCCH zinc finger proteins are a family of RNA binding proteins, I wanted to identify the mRNA targets of ZC3H11. For this a procyclic cell line ectopically expressing ZC3H11-myc (pHD2067) was used. After precipitation using anti-myc agarose beads, the co-purified RNA in the bound fraction was isolated.
Initially this RNA as well as the bound fraction of a control purification without ZC3H11-myc was checked on a Northern blot and probed for *HSP70* mRNA. This transcript was a suspected target of ZC3H11 due to the decrease observed in the microarray experiment (chapter 3.8) and the decreased half-life upon depletion of ZC3H11 (chapter 3.9). The abundant tubulin mRNA was used as an unspecific control.
During the procedure some degradation was observed (figure smear in the FT) but nevertheless the *HSP70* mRNA was specifically pulled down in the cell line containing ZC3H11-myc (figure 3.12 A). The tubulin control was found at comparable low levels in both the control and the ZC3H11-myc cell line. Additionally the zinc finger mutant protein (C70S) was used in another pull down and this did not co-precipitate HSP70 mRNA (figure 3.12 B). This has to be repeated and it also has to be considered that the expression of this construct was not as good as the endogenous protein (see figure 3.7).

3.13 Deep sequencing

After I determined that the RNA co-immunoprecipitation procedure worked, the experiment was scaled up and RNA samples were prepared for high-throughput sequencing to elucidate the complete spectrum of bound mRNAs. For this the bound mRNA was compared with the unbound mRNA. The unbound sample was treated with the Ribominus kit to remove the extremely abundant ribosomal RNAs. This was not necessary for the bound fraction that did not contain much rRNA, as determined by the methylene blue stain (see figure 3.12 A). These samples were given to David Ibberson at the Bioquant deep sequencing core facility for library construction and sequencing. The resulting dataset was aligned to the genome and bioinformatically analyzed (Abeer Fadda). All transcripts that were enriched more than 3 fold in the

bound versus the unbound fraction and with more than 50 reads in the bound fraction are listed in table 3.13.

27 different genes were enriched in the bound fraction using these criteria. Several multicopy genes, the *HSP83* (the trypanosome HSP90 homologue) array, two identical *HSP60*s on chromosome 10 and the EP procyclins on chromosome 6 (Tb927.6.450/480) gave ambiguous reads therefore only the average is given in the list.

As expected from the previous experiments and the Northern blot control (figure 3.12 A), *HSP70* was found to co-precipitate with ZC3H11 (4.2 fold enrichment). In fact, even though the enrichment was not the highest of the list, it was the most abundant mRNA bound, the second most abundant being *HSP83* followed by alpha and beta tubulin. *HSP83* was also clearly enriched (3.9 fold) while tubulin was not (0.8/0.6 fold). Overall there was a striking enrichment of heat shock proteins and chaperones - 15 out of the 27, thus more than half of the hits belonged to this class. Furthermore putative homologues of all cytoplasmic chaperone classes were found: *HSP70, HSP83, HSP100, HSP110, HSP20*-like and cochaperones DnaJ (HSP40), *STI1* (HOP) and a TPR domain-containing members of each the cyclophilins (putative Cyp40 homologue) and FKBP families. The mitochondrial chaperonin *HSP60* and a glutaredoxin (*GRX2*) were also found. Although the 10kDa heat shock proteins (Tb927.7.1320/1340) gave a lot of reads (~4000) in the eluate, they were only enriched 2.3 fold and so they were below the cutoff for table 3.13.

Most of the other targets were transcripts encoding hypothetical proteins of unknown function, but the procyclic surface protein *GPEET* and *EP* procyclin transcripts of the same region in chromosome 6 were also enriched. Looking closer at the genomic alignments of the reads, I could see that the *EP* reads are probably an artifact due to ambiguous reads shared with the *GPEET* 5' end.

Next, the 3'UTRs of the enriched transcripts were analyzed for motifs using the MotifSampler program (Abeer Fadda). The motif: AUUAUUAUUAUU was found with the highest log-likelihood score. Most of the bound mRNAs in table 3.13 include at least a nine-mer of these AUU repeats (marked in table 3.13), including the *HSP70* 3'UTR (see figure 3.10.1).

RNAs bound by ZC3H11 (RNAIP) eluate reads > 50 enrichment >3

gene ID	new ID	description	eluate reads	eluate rpkm	FT reads	FT rpkm	enrich ment	RNAi effect	AUU repeats
Tb927.8.7540		hypothetical protein	108	33,0	14	1,6	**21,14**	**-1.05**	(+)
Tb927.1.3390		hypothetical protein	212	289,5	49	24,4	**11,85**	**-1.20**	+
Tb927.10.16100		PPIase, FKBP type with TPR domain	3856	3193,1	1038	313,7	**10,18**	**-2.46**	+
Tb11.01.7120	Tb927.11.15480	HSP20-like chaperone	2566	4035,1	727	417,2	**9,67**	**-1.62**	+
Tb927.1.3200		phosphatase-like protein, HSP20-like chaperone	681	761,8	191	78,0	**9,77**	**-1.29**	+
Tb11.57.0002	Tb927.11.2160	hypothetical protein	88	135,6	28	15,7	**8,61**	**+1.14**	+
Tb927.6.510		GPEET2 procyclin	2023	5694,0	836	858,8	**6,63**	**+1.29**	(+)
Tb927.2.5160		chaperone protein DNAj	2853	2495,3	1273	406,4	**6,14**	**-1.63**	+
Tb927.10.780		hypothetical protein	201	407,5	104	77,0	**5,30**	**-1.23**	(+)
Tb927.1.2230		calpain-like protein fragment, putative	543	1137,9	363	277,6	**4,10**	**-1.32**	+
Tb11.01.3110	Tb927.11.11330	heat shock protein 70	12356	6150,7	7999	1453,3	**4,23**	**-2.24**	+
Tb927.6.450/480		EP3-2 procyclin, average	161	415,6	109	103,3	**4,18**	**+1.44**	-
Tb927.5.2940		stress-induced protein sti1	3291	2064,5	2182	499,6	**4,13**	**-1.24**	+
Tb927.10.10890-10980		heat shock protein 83, average	6080	3042,3	4293	784,0	**3,88**	**-1.13**	+
Tb927.7.5400		hypothetical protein	102	63,2	72	16,3	**3,88**	**+1.09**	+
Tb09.160.3530	Tb927.9.4960	hypothetical protein	1946	6538,0	1296	1589,2	**4,11**	**-1.60**	+
Tb927.3.2650		cytochrome c oxidase copper chaperone	258	1079,2	197	300,8	**3,59**	**+1.04**	+
Tb09.211.1350	Tb927.9.9780	PPIase, putative cyclophilin-40	465	434,7	356	121,5	**3,58**	**-1.34**	(+)
Tb927.10.6400/6510		chaperonin HSP60, average	2147	1345,0	1721	393,6	**3,42**	**-1.22**	+
Tb11.01.8750	Tb927.11.16980	chaperone protein DNAj	85	77,2	70	23,2	**3,33**	**-1.11**	+
Tb09.v1.0490	Tb927.9.7890	hypothetical protein	454	1760,0	381	539,1	**3,26**	**-1.33**	+
Tb927.1.1770		glutaredoxin, grx2	57	186,2	48	57,2	**3,25**	**-1.24**	+
Tb927.10.12710		heat shock protein 110	1302	565,6	1108	175,7	**3,22**	**-1.64**	+
Tb927.2.5980		ATP-dependent Clp protease subunit, HSP100	277	108,3	242	34,5	**3,14**	**-1.10**	+
Tb09.211.0040	Tb927.9.7980	hypothetical protein	482	973,5	417	307,4	**3,17**	**+1.07**	+
Tb927.7.4290		HSP20-like chaperone	297	329,5	258	104,5	**3,15**	**-1.18**	+
Tb927.10.8540		chaperone protein DNAj	233	243,9	206	78,7	**3,10**	**+1.12**	+
Tb927.1.2340-2400		alpha tubulin, average	4103	2970,05	14258	3766,82	**0,79**	**+1.42**	-
Tb927.1.2330-2390		beta tubulin, average	5786	4064,59	24691	6332,04	**0,64**	**+1.47**	-

Table 3.13 (on the previous page)
Deep sequencing results of the ZC3H11 RNA-IP. The gene IDs are according to the current version of TritrypDB and the new accession numbers (new ID) for chromosomes 9 and 11 (as used on GeneDB) are also supplied where applicable. “enrichment” is the ratio of the rpkm of the eluate and the unbound flow-through. “RNAi effect” is the data from the microarray experiment for ZC3H11 depletion (see chapter 3.8) and regulation of more than 1.5 fold is highlighted in green. A + for AUU repeats means that at least a nine-mer repeat was found in the 3’UTR of this gene, and for (+) not a perfect nine-mer but similar. Chaperones are marked in yellow shading and the values for the non-enriched tubulin (shaded in red) are given as a control.

3.14 Tethering

RNA binding proteins can affect the target transcripts’ stability or translation. We wanted to determine the effect of physically tethering ZC3H11 protein to a reporter construct. For this a ZC3H11 fusion protein containing an N-terminal lambdaN peptide and C-terminal myc tags (pHD2299) was used (figure 3.14 A). The lambdaN peptide binds to its recognition sequences called “boxB”.
We used cell lines constitutively expressing either a CAT reporter with 5x boxB in the 3’UTR (pHD2277, Martin Wurst) or a control without these sequences (pHD1991, Praveen Delhi).
Upon induction of lambdaN-ZC3H11, expression of the CAT reporter with boxB, but not the one without, was increased (figure 3.14 B). Some leaky expression was observed (figure 3.4 C and D), which probably caused the increased CAT expression even in the absence of tetracycline.

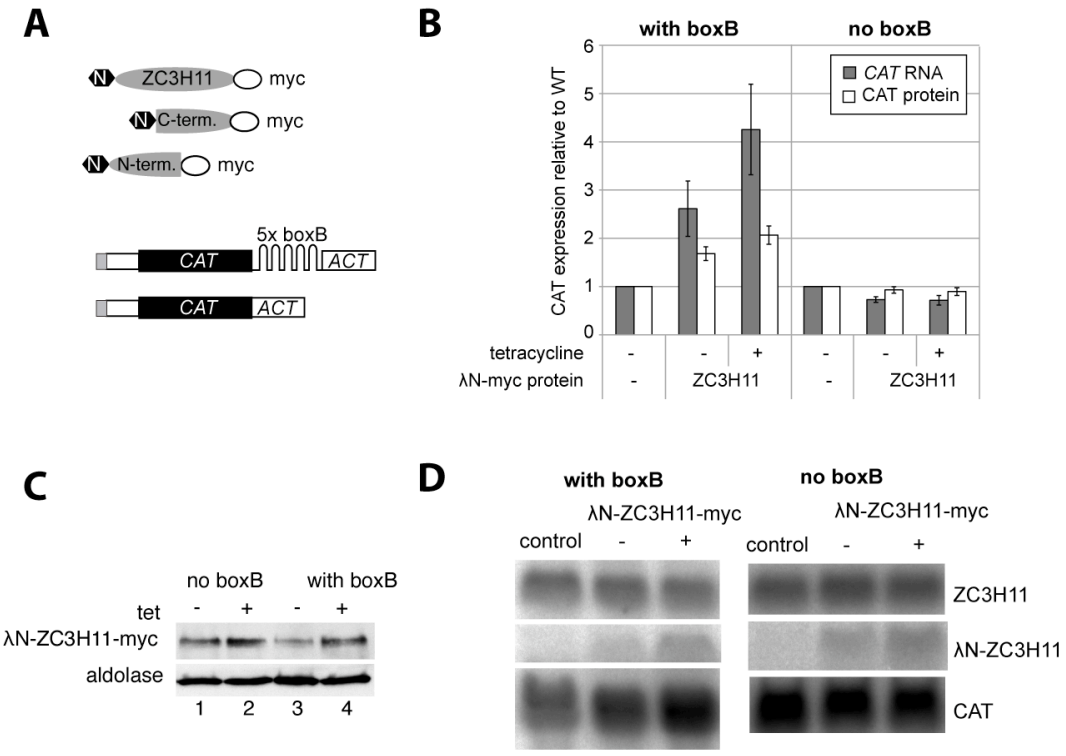


Figure 3.14.1

A) ZC3H11 fusion protein with N-terminal lambdaN-peptide and C-terminal myc tags was inducible expressed in cell lines constitutively expressing a CAT reporter either with or without boxB sequences.

B) CAT mRNA and protein levels were determined by Northern blot or CAT assay. Results of at least three replicates per condition are given with the standard deviation.

C) Western blot showing the expression of ZC3H11 fusion protein.

D) The Northern blot also shows inducible (leaky) expression of the ZC3H11 fusion protein.

Additionally, two fragments of ZC3H11, the N-terminus containing the zinc finger domain (pHD2318) and the C-terminus (pHD2314), corresponding to construct II and IV of chapter 3.6, were expressed for the tethering experiment. The Northern blot, CAT assay and Western blot for the C-terminal fragment was done by the practical student Iker Valle and for the N-terminal fragment by Igor Minia. No effect on reporter expression was observed for the N-terminal fragment but the C-terminal fragment strongly increased reporter expression.

These results showed that ZC3H11 increases the expression of bound transcript and that the C-terminal part is necessary for this effect.

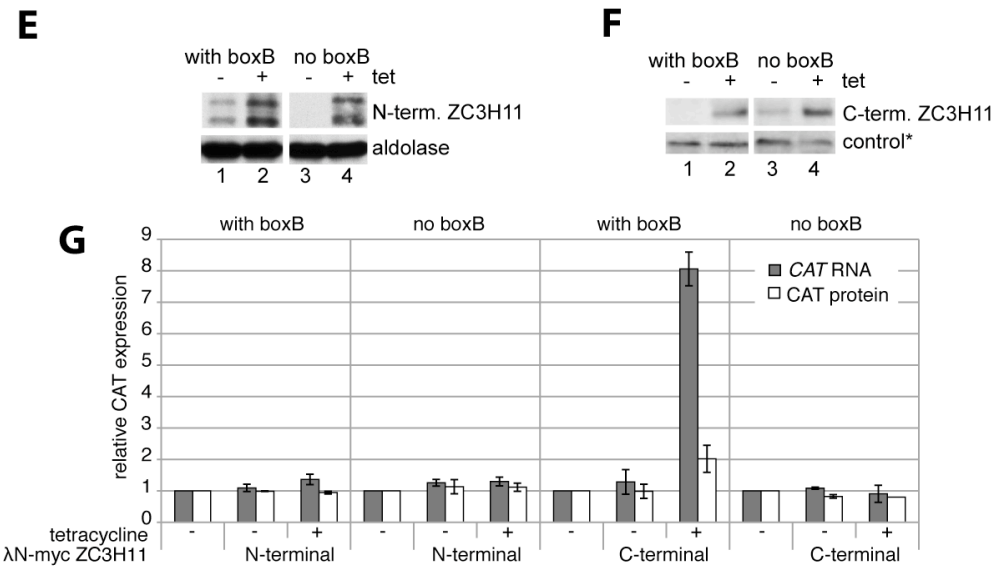


Figure 3.14.2

E) Western blot showing expression of the N-terminal fragment of ZC3H11.

F) Western blot showing expression of the C-terminal fragment of ZC3H11. * In this case a cross-reacting unspecific band was used as a loading control.

G) CAT expression data as in B) in cell lines expressing either the N-terminal or the C-terminal fragment of ZC3H11.

3.15 Heat shock experiment

Since ZC3H11 binds chaperone mRNAs and stabilizes tethered transcripts, we examined its function in the parasites ability to respond to heat shock.
A procyclic strain depleted of ZC3H11 by RNAi was subjected to a heat shock at 41°C for one hour and then returned to 27°C. As this cell line was quite leaky, the RNAi induced cells were compared to the wild type.
Following the recovery for several days a clear impairment was seen for the RNAi cell line both in proliferation (figure 3.15 A) and by FACS (figure 3.15 B). Also the cell motility and morphology was clearly more affected in the ZC3H11-depleted cells. In RNA samples taken directly after the heat shock we could see that in the wild type *HSP70* and *HSP83* mRNA levels were increased (figure 3.15 C and D) as has been previously reported, but this stabilization was lost in the RNAi cell line. In these cells the *HSP70* and *HSP90* mRNA was degraded, like most other mRNAs of the cell, as seen with the spliced leader signal (figure 3.15 C). Interestingly we also noticed that the *ZC3H11* mRNA itself seemed to be stable during the heat shock (figure 3.15 D).

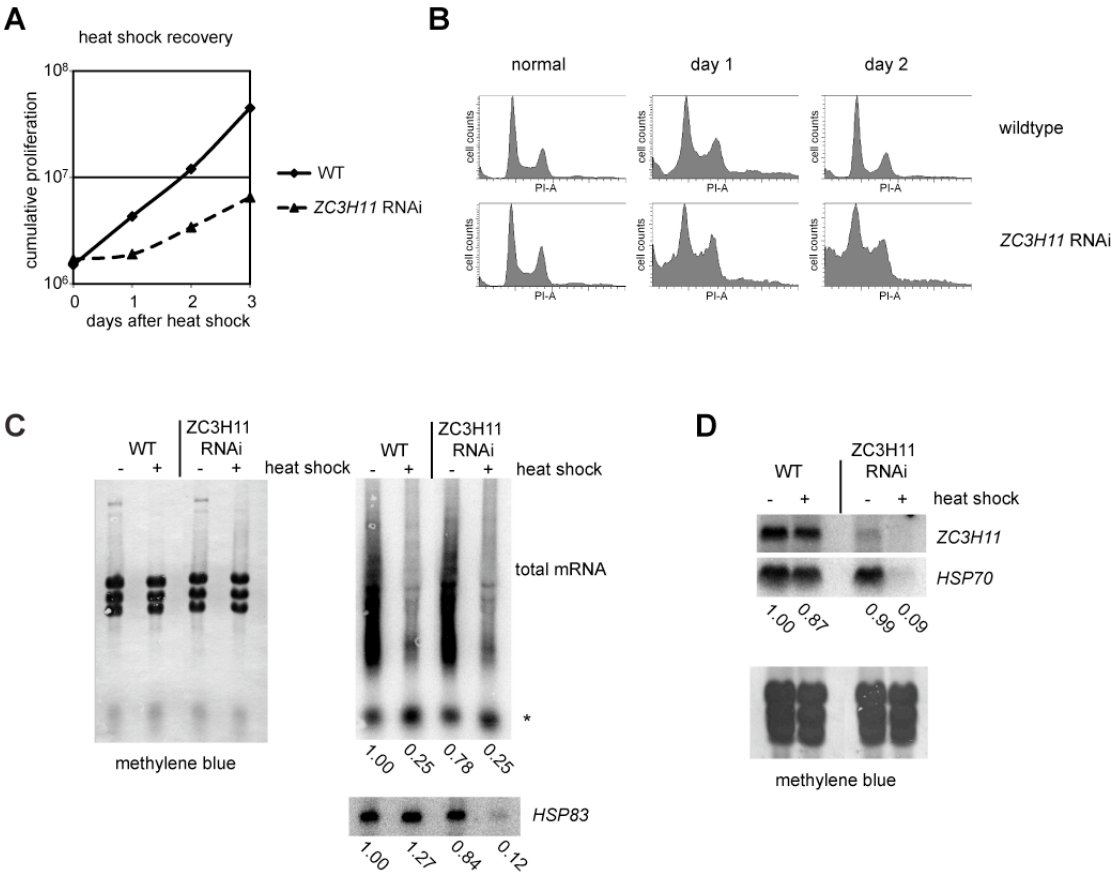


Figure 3.15

A) Procyclic trypanosomes, either wild type (WT) or *ZC3H11* depleted by RNAi, were subjected to one hour of heat shock at 41°C and then returned to 27°C. Cell densities were counted the following days and the cultures diluted accordingly.

B) Trypanosomes at 27°C (normal) or 1 day or 2 days after the heat shock were fixed, stained with propidium iodite and analyzed by FACS.

C) Northern blot using total RNA of the wild type or ZC3H11 RNAi strains with or without heat shock. Total mRNA was detected by hybridization of a spliced leader (SL) antisense oligonucleotide. The * marks the spliced leader precursor. The same blot was then hybridized with a *HSP83* probe and both signals were quantified relative to the wild type sample without heat shock using the methylene blue staining.

D) the same RNA as in C) was analyzed on a different blot for *ZC3H11* and *HSP70* mRNAs. The quantification for *HSP70* is given below the bands.

3.16 Indirect immunofluorescent localization

We next wanted to see the subcellular localization of ZC3H11 by immunofluorescence. We were not able to use the *in situ* tagged V5-ZC3H11 for this purpose, as the V5-antibody did not give a specific signal above the background compared to the wild type. Also the application of stresses that

induced V5-ZC3H11 expression (37°C or puromycin) did not give a satisfactory signal.
Therefore ectopically expressed ZC3H11-TAP (pHD1950) was used for localization. ZC3H11 was clearly excluded from the nucleus and showed an uneven cytoplasmic distribution (figure 3.16).

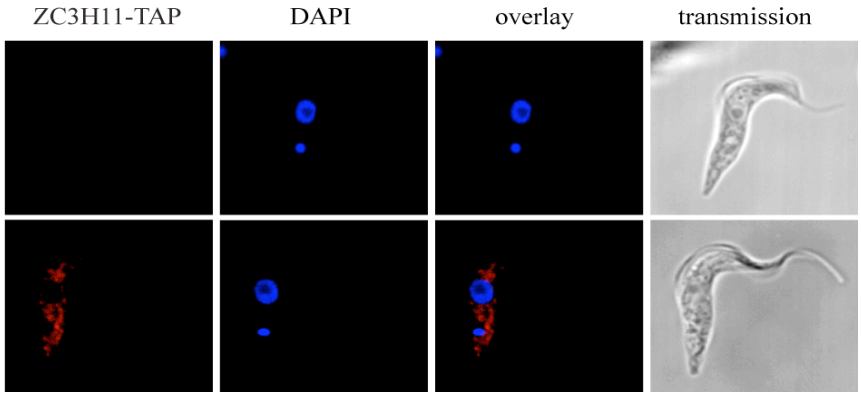


Figure 3.16
Immunofluorescent localization of ZC3H11-TAP in a procyclic cell line. Anti proteinA antibody was used for detection of the TAP tagged protein.

3.17 Tandem affinity purification (TAP)

In order to elucidate the mechanism of ZC3H11 action I looked for protein interaction partners by tandem affinity purification (TAP). The purification was performed with procyclic cell lines expressing either a ZC3H11 fusion protein with a C-terminal TAP-tag (pHD1950) or a control cell line with only the TAP-tag (pHD918). The ZC3H11 cell line was additionally subjected to a heat shock at 41°C to make sure that stress-specific interactions are not missed.
The purification was not very clean as a lot of background bands were seen that were also present in the control eluate. Regions with ZC3H11-specific signal (marked with red bars in Figure 3.17) were cut in gel slices and analyzed by mass spectrometry.
We did not analyze the region between 50 and 60 kDa because there was much unspecific background signal in the control, so only one peptide for ZC3H11 was found in a band cut out at roughly 30 kDa, which should be a degradation product.
The other gel slices contained mostly abundant contaminants that are regularly found in immunoprecipitations, such as chaperones, ribosomal

proteins and abundant metabolic enzymes. Nevertheless, we also found a strong hit (in total 162 peptides) in the one clearly specific band (marked with the arrow in figure 3.17 A). This protein with the accession number Tb927.6.4770 was identified as a putative trypanosome homologue of *Saccharomyces cerevisiae* Mkt1p by using BLAST (29% coverage, E-value 0.002). Comparing the conserved domains we saw a very similar organization (figure 3.17 B), the only domain missing in *Tb*MKT1 being the H3TH domain. 18 peptides of a putative Pbp1p homologue were also found.

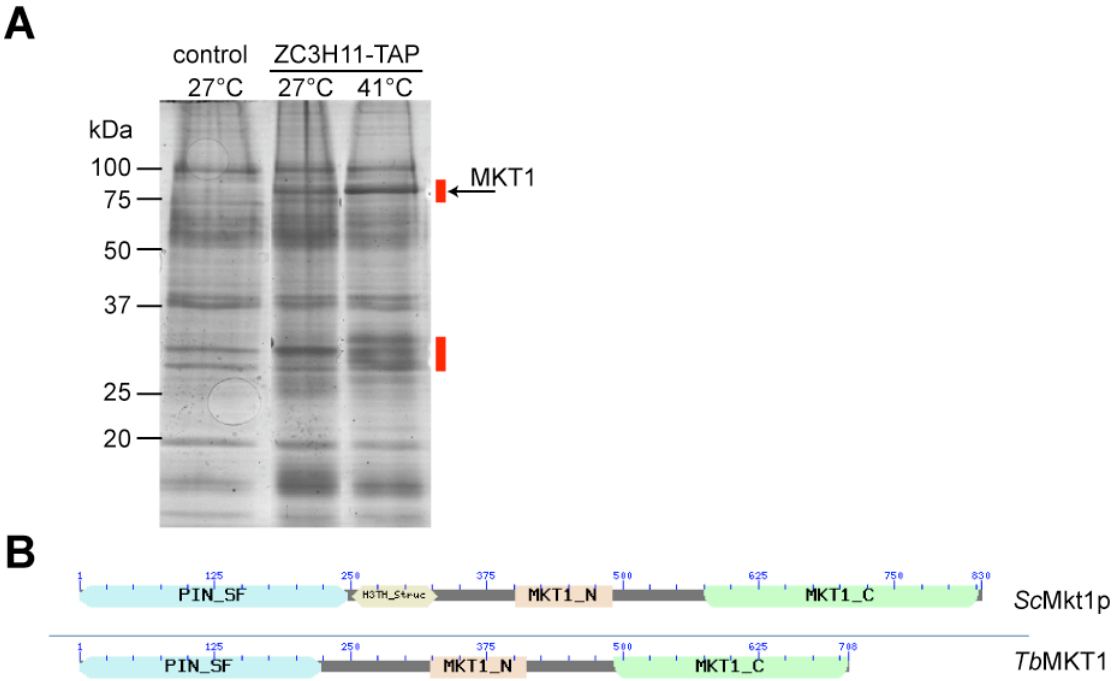


Figure 3.17

A) Syproruby® stained gel of the TAP eluates. Gel slices from the regions marked in red were analyzed by mass spectrometry.

B) Domain structure of *Saccharomyces cerevisiae* and *Trypanosoma brucei* MKT1 proteins from NCBI. PIN_SF (PIN domain of MKT1, cd09858), MKT1_N (N-terminal domain of MKT1, pfam12247), MKT1_C (C-terminal domain of MKT1, pfam12246) H3TH (helix-3-turn-helix of MKT1, cd09902)

3.18 Protein co-immunoprecipitation

To verify the promising putative interaction partner of ZC3H11 found in the tandem affinity purification, *Tb*MKT1, a co-immunoprecipitation was performed using procyclic cells. α-myc agarose beads were used for a pulldown with a cell line expressing V5-ZC3H11 (pHD1947) and MKT1-myc (pHD1983). In parallel, a cell line only expressing V5-ZC3H11 was used as

control. After precipitating MKT1, we observed co-precipitation of ZC3H11. This was not an unspecific binding to the beads as it was not seen in the control, and therefore it depended on the presence of MKT1-myc.
Furthermore, the interaction between MKT1 and ZC3H11 was verified using the Yeast Two Hybrid system (Esteban Erben).
A co-immunoprecipitation of ZC3H11-myc with V5-ZC3H11 was not observed (figure 3.18 B).

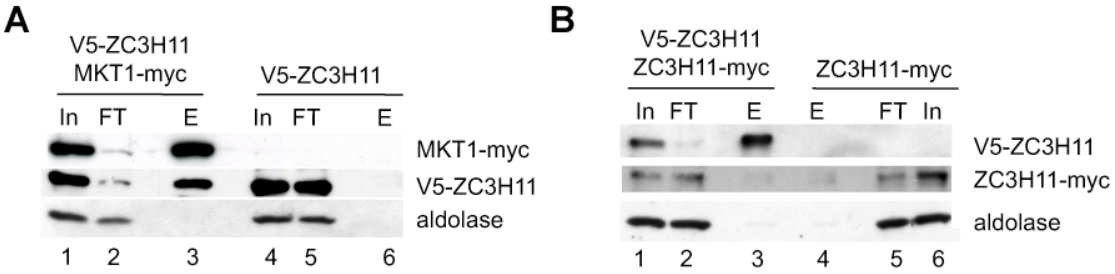


Figure 3.18
A) Co-immunoprecipitation of MKT1-myc and V5-ZC3H11 using α-myc beads and procyclic cells. Input (In), flow through (FT) (both $2x10^6$ cells) and eluate (E, $3.8x\ 10^7$ cells).
B) Co-immunoprecipitation of ZC3H11-myc and V5-ZC3H11 using α-V5 beads and procyclic cells. Input (In), flow through (FT) (both $2x10^6$ cells) and eluate (E, $3.8x\ 10^7$ cells).

3.19 MKT1

As an initial experiment for the study of the interaction partner of ZC3H11, MKT1, the effect of depletion by RNAi (pHD1987) in procyclics and bloodstream stage was analyzed. This revealed a strong proliferation phenotype in the bloodstream forms and a moderate effect on procyclics (figure 3.19 A and B). To exclude any off-target effects a second RNAi construct (pHD1999) with a different target sequence was also used and gave a similar proliferation defect to the initial construct. As these results looked rather promising further experiments were initiated.
Cell lines expressing MKT1-myc (pHD1983) or *in situ* YFP-tagged MKT1 (pHD1995) were generated and used for localization. MKT1-myc showed an uneven, slightly granular, cytosolic signal while the YFP-tagged protein did not appear as granular in the initial experiments. This pattern was reminiscent of the ZC3H11-TAP signal (chapter 3.16). A possible colocalization with DHH1, a component of stress granules, was also examined, but the DHH1 antibody

gave dispersed signal throughout the cell in the preliminary experiment so the conditions have to be optimized in future experiments.

For potential future experiments, recombinant MKT1 with a 10xHis-tag (pHD1998) was cloned. While expression levels in the bacteria were good, in initial experiments it was insoluble.

Since this protein offered a whole new interesting project it was passed on at this point to the next PhD students.

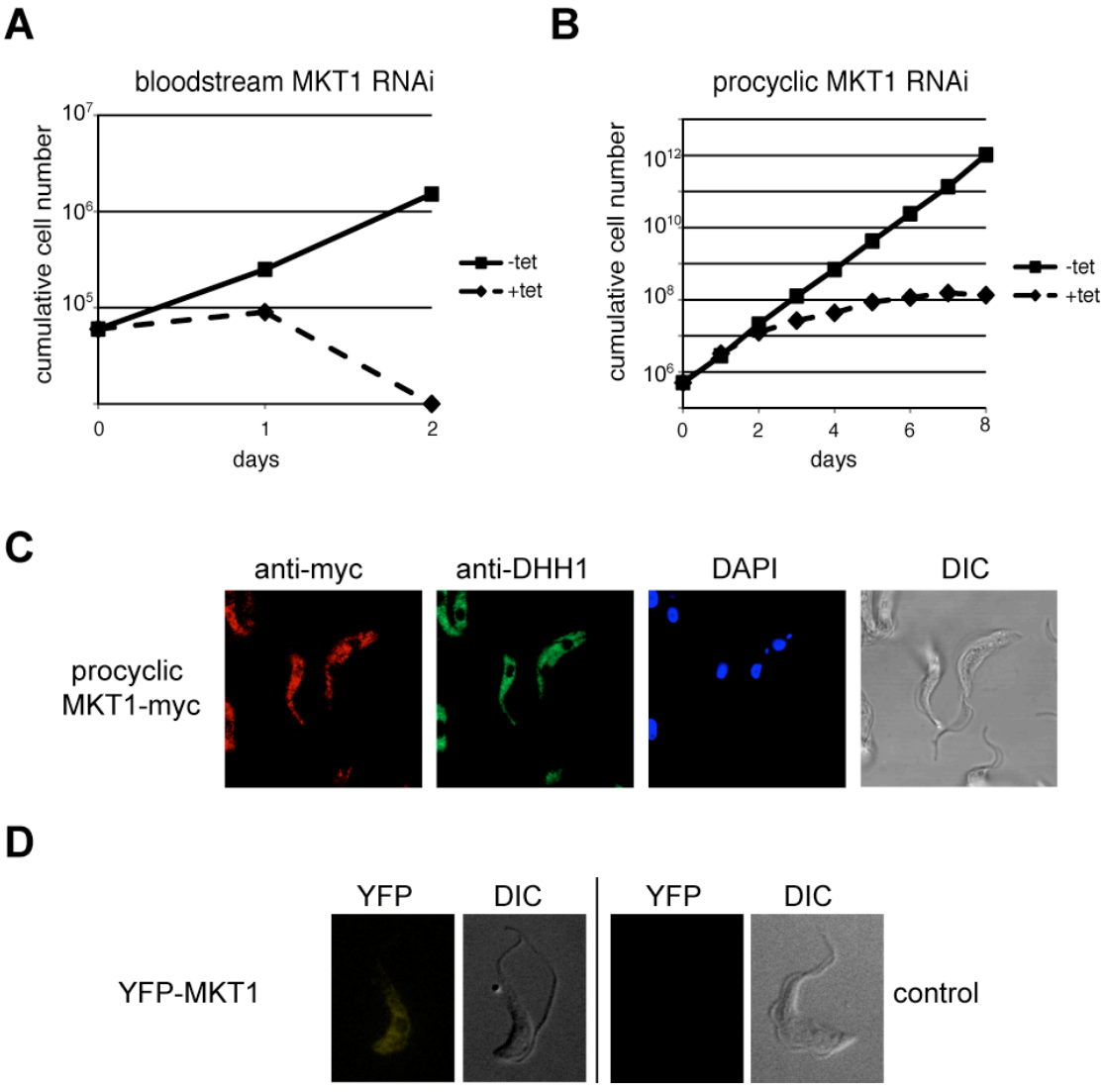


Figure 3.19

A) Proliferation of MKT1 depleted bloodstream-stage cells.

B) Proliferation of MKT1 depleted procyclic cells.

C) Immunofluorescence using myc-tagged MKT1 in procyclics.

D) Fluorescent localization of YFP-MKT1 in bloodstream stage.

4 Discussion

4.1 ZC3H11 expression

During my PhD studies I found that ZC3H11 has an essential function in the bloodstream stage of *T. brucei,* as depletion by RNAi is lethal. Notably this is not the case in procyclics. Either ZC3H11 function is not essential in this stage in culture conditions or its function is taken over by other, procyclic specific, factors. As we could observe a strong effect of ZC3H11 depletion on the procyclics' ability to survive heat shock, it seems more likely that ZC3H11 is needed in specific conditions. I was not successful in generating a ZC3H11 knockout cell line and that could also be an indication that the gene is important, possibly the parasites duplicated the gene.

The observation that ZC3H11 protein expression is apparently very low in normal conditions and induced upon heat shock fits well to the model that the protein is needed in specific conditions. Interestingly, even though ZC3H11 depletion is lethal, the steady state protein levels in the bloodstream stage seem comparable to the very low levels in procyclics. Apparently low amounts are sufficient for its function or ZC3H11 is only transiently induced in response to specific conditions. This would agree with the increased expression after inhibition of the proteasome, indicating that the protein could be constantly turned over under normal conditions and only stabilized upon certain cues. Alternatively, inhibition of the proteasome could be sensed as another stress condition that leads to activation of ZC3H11 expression. There does not seem to be a significant upregulation of *ZC3H11* mRNA upon heat shock, therefore the increased expression is probably due to stabilization of the protein by post-translational modifications, or increased translation. Interestingly, the *ZC3H11* mRNA appears to be stable during heat shock, like the heat shock protein transcripts. This means that potentially translation of this factor could still be active in heat shock conditions, ensuring sufficient levels of ZC3H11 protein.

The phosphorylation status might influence the stability and function of ZC3H11 by regulating interactions with proteins or target RNAs. For example, an upstream signaling kinase could be activated upon stress and phosphorylate ZC3H11, thereby activating the protein.
The observed increase of ZC3H11 protein levels at elevated temperatures could be the key to its physiological function, as it could function as a master regulator of the heat shock response upon activation. To elucidate the exact mechanisms, e.g. the kinase involved and other protein interactions, further studies have to be performed.

4.2 The mRNA targets ZC3H11

In the well-studied mammalian tandem zinc finger proteins the close proximity of the two zinc fingers confers ARE sequence specificity, each binding 4 nucleotides (UAUU) (Morgan and Massi 2010). This sequence-specific RNA binding is mediated by a combination of hydrogen bond interactions between the protein backbone and the bases, and stacking interactions between conserved aromatic residues and RNA bases (see figure 4.2). The length of the linker between the tandem zinc fingers constricts binding to directly neighboring UAUU sequences and therefore AREs (UAUUUAUU).
ZC3H11 has one zinc finger domain that is only expected to specifically interact with four bases, which by itself would not be enough to convey sequence specificity. I have shown that the single zinc finger of ZC3H11 is necessary for RNA binding, as the zinc finger mutant does not pull down the *HSP70* transcript. It is conceivable that ZC3H11 dimerizes, either as homodimer or as a heterodimer with another CCCH protein. Though a co-precipitation of ZC3H11-myc with V5-ZC3H11 could not be observed in a co-immunoprecipitation, the newest yeast two hybrid results (Esteban Erben) indicate that indeed a dimerization of ZC3H11 can occur. Depending on the spacing between the proteins, for example a UAUUAUUAUU recognition could be explained. Repeats of the recognition sequence could raise the binding probability or even allow more than two ZC3H11 proteins to bind. Another possibility is a combined action with another RNA binding protein interaction partner.

Figure 4.2

This superimposed structure of TIS11d zinc finger 1 and 2 bound to RNA was taken from (Hudson, Martinez-Yamout et al. 2004). The RNA is depicted in orange, the protein backbone in light/dark blue, the zinc coordinating side chains in green/yellow and the intercalating side chains in red/pink.

The corresponding residues are marked in the alignment of TIS11d with ZC3H11 using the same color scheme. An asterisk marks residues at positions that have been shown to form backbone hydrogen bonds with the RNA.

Upon ZC3H11 depletion the HSP70 mRNA half-life decreased and I showed that ZC3H11 binds (RNAIP) and stabilizes (tethering) its target transcripts. Furthermore, the CAT reporter data demonstrated that it is the $(AUU)_n$ containing part of the *HSP70* 3'UTR that mediates the stabilization via ZC3H11. This $(AUU)_n$ repeat motif, revealed by analysis of the 3' UTRs of the transcripts bound by ZC3H11, is found in a wide selection of heat shock protein transcript 3' UTRs.

Apart from the heat shock transcripts, the mRNA of the procyclin *GPEET* is also enriched with a high read count. This may be because its 3'UTR also contains some AU-rich parts. As the experiment was performed in procyclics, with ectopically expressed ZC3H11 and without a heat shock, this might not occur in normal conditions. On the other hand, zinc finger proteins in trypanosomes have already been shown to be involved in the stabilization of procyclin mRNA (Hendriks and Matthews 2005; Walrad, Paterou et al. 2009; Walrad, Capewell et al. 2012), so this could also be a relevant target.

Although some mRNA targets were found to be both decreased upon ZC3H11 depletion (microarray) and bound to ZC3H11 (RNA-coIP), the two

datasets did not overlap completely. One reason is that with the microarray we observe transcriptome-wide changes in expression and detect not only direct but also secondary effects of ZC3H11 depletion, for example due to slowed cell growth. On the other hand, the RNA-coIP was performed with procyclics while the microarray was done using the bloodstream form. It is possible that the mechanism of ZC3H11 action is slightly different in those two stages, for example due to different interaction partners or regulatory proteins. Another hint supporting this possibility is that the CAT reporter with *HSP70* UTRs was not affected by ZC3H11 depletion or ectopic expression in procyclics, at least in the absence of heat shock, in contrast to the situation in the bloodstream form.

4.3 A putative mechanism of ZC3H11 action

In trypanosomes ZC3H11 interacts with MKT1, as shown by tandem affinity purification and co-immunoprecipitation. Yeast Mkt1p is involved in translational regulation of transcripts and is found associated with polysomes, dependent on Pbp1 protein (Tadauchi, Inada et al. 2004). Mkt1p contains a PIN domain of the Flap Endonuclease-1 (FEN1)-like family. For both yeast and trypanosome MKT1, the catalytic residues are not conserved and in addition the helix turn helix motif (H3TH), that is thought to interact with DNA in Fen1, lacks an important glycine-rich loop in yeast and is not conserved in trypanosomes. There is no indication for a nuclease function of MKT1 and additionally there is no evidence for direct RNA binding of MKT1, both in yeast and trypanosomes.

Yeast Mkt1p has been shown to interact with Pbp1p (Tadauchi, Inada et al. 2004) and another student in the lab (Aditi Singh) verified that this interaction is conserved in trypanosomes. Homologues of PBP1 are known to interact with poly(A) binding protein (PABP), e.g. the yeast Pbp1p (Mangus, Amrani et al. 1998), mammalian ataxin-2 or drosophila ATX2 (Satterfield and Pallanck 2006). In yeast, Pbp1p also interacts with Lsm12, Pbp4 and Dhh1 (Swisher and Parker 2010). In a new ZC3H11-TAP experiment, repeated by another student (Igor Minia), the complete bound sample was analyzed without cutting out specific bands. In addition to MKT1 and ZC3H11 also PBP1, DHH1 and LSM12 co-purified with ZC3H11, indicating conserved interactions.

The poly(A) binding protein directly interacts with eIF4G which links to eIF4E, circularizing the mRNP. This is thought to enhance translation initiation (Kahvejian, Svitkin et al. 2005; Jackson, Hellen et al. 2010). In trypanosomes tethering of poly(A) binding protein to a transcript strongly increases the targets' expression level. (Delhi, Queiroz et al. 2011; Wurst, Seliger et al. 2012).

Recent results showed, that the C-terminal, but not the N-terminal domain of ZC3H11 interacts with MKT1 (Yeast two hybrid results, Aditi Singh). Since also only the C-terminal part stabilized the reporter expression in the tethering experiment this data would fit to a model in which ZC3H11 function is mediated by its interaction of MKT1.

Taking all this information together, our current model is that ZC3H11, possibly as dimer, acts via the MKT1/PBP1 interaction to stabilize poly(A) binding protein on its target transcripts, thereby stabilizing them.

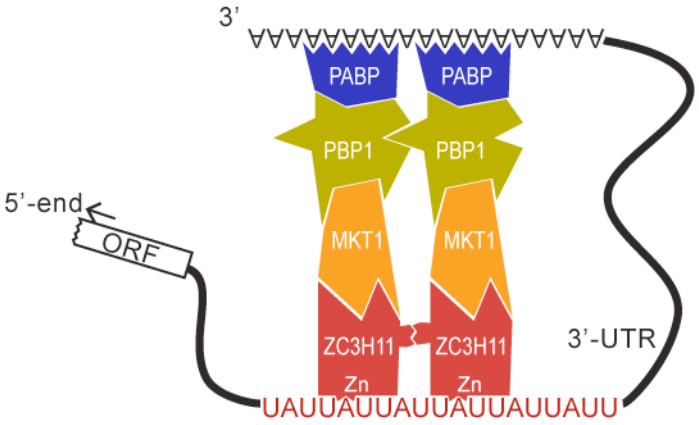


Figure 4.3

A model of the protein interactions mediating ZC3H11 function. The interactions of MKT1 with both ZC3H11 and PBP1 have been verified in trypanosomes.

4.4 The heat shock response in *T. brucei*

In trypanosomes the mechanism of the heat shock response is divergent. There is no significant up-regulation of transcription of heat shock protein genes, but rather a selective stabilization of their mRNAs. It should be mentioned that although the focus lies on transcriptional regulation for most eukaryotes, in yeast it has also recently been shown that regulation of mRNA

stability plays an additional role in the heat shock response (Castells-Roca, Garcia-Martinez et al. 2011).

Our data suggests that ZC3H11 could be a central element in the post-transcriptional heat shock response in *Trypanosoma brucei*. It binds transcripts with the $(AUU)_n$ sequence motif in their 3'UTRs and binding mediates stabilization. This $(AUU)_n$ motif is found in the 3' UTRs of transcripts encoding the major heat shock proteins and their co-factors. Therefore, this motif could allow a concerted regulation of a complete set of heat shock response genes.

It is interesting to note, that these AUU-repeats are also found in the *HSP70* and *HSP83* 3'UTRs of *Trypanosoma cruzi,* but not in *Leishmania major*.

It has been previously shown that also in leishmania the 3'UTR of *HSP83* is mediating the stabilization upon heat shock (Zilka, Garlapati et al. 2001), but in this organism a temperature-sensitive secondary structure was identified in the 3'UTR (David, Gabdank et al. 2010). It seems therefore likely that this related kinetoplastid employs a slightly different regulatory mechanism for control of heat shock gene expression.

Trypanosomes have developed an efficient post-transcriptional heat shock response employing the RNA binding protein ZC3H11 to stabilize heat shock protein transcripts.

5 References

Amrani, N., S. Ghosh, et al. (2008). "Translation factors promote the formation of two states of the closed-loop mRNP." Nature **453**(7199): 1276-1280.

Archer, S. K., V. D. Luu, et al. (2009). "Trypanosoma brucei PUF9 regulates mRNAs for proteins involved in replicative processes over the cell cycle." PLoS Pathog **5**(8): e1000565.

Benne, R., J. Van den Burg, et al. (1986). "Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA." Cell **46**(6): 819-826.

Benz, C., J. Mulindwa, et al. (2011). "The Trypanosoma brucei zinc finger protein ZC3H18 is involved in differentiation." Mol Biochem Parasitol **177**(2): 148-151.

Boothroyd, J. C. and G. A. Cross (1982). "Transcripts coding for variant surface glycoproteins of Trypanosoma brucei have a short, identical exon at their 5' end." Gene **20**(2): 281-289.

Bringaud, F., L. Riviere, et al. (2006). "Energy metabolism of trypanosomatids: adaptation to available carbon sources." Mol Biochem Parasitol **149**(1): 1-9.

Buchan, J. R. and R. Parker (2009). "Eukaryotic stress granules: the ins and outs of translation." Mol Cell **36**(6): 932-941.

Carballo, E., W. S. Lai, et al. (1998). "Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin." Science **281**: 1001-1005.

Carballo, E., W. S. Lai, et al. (2000). "Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability." Blood **95**: 1891-1899.

Cassola, A., J. De Gaudenzi, et al. (2007). "Recruitment of mRNAs to cytoplasmic ribonucleoprotein granules in trypanosomes." Mol Microbiol **65**: 655-670.

Castells-Roca, L., J. Garcia-Martinez, et al. (2011). "Heat shock response in yeast involves changes in both transcription rates and mRNA stabilities." PLoS One **6**(2): e17272.

Clayton, C. E. (2002). "Life without transcriptional control? From fly to man and back again." EMBO J **21**(8): 1881-1888.

D'Orso, I. and A. C. Frasch (2001). "Functionally different AU- and G-rich cis-elements confer developmentally regulated mRNA stability in Trypanosoma cruzi by interaction with specific RNA-binding proteins." J Biol Chem **276**(19): 15783-15793.

D'Orso, I. and A. C. Frasch (2002). "TcUBP-1, an mRNA destabilizing factor from trypanosomes, homodimerizes and interacts with novel AU-rich element- and Poly(A)-binding proteins forming a ribonucleoprotein complex." J Biol Chem **277**(52): 50520-50528.

Dallagiovanna, B., A. Correa, et al. (2008). "Functional genomic characterization of mRNAs associated with TcPUF6, a pumilio-like protein from Trypanosoma cruzi." J Biol Chem **283**(13): 8266-8273.

David, M., I. Gabdank, et al. (2010). "Preferential translation of Hsp83 in Leishmania requires a thermosensitive polypyrimidine-rich element in the 3' UTR and involves scanning of the 5' UTR." RNA **16**(2): 364-374.

De Gaudenzi, J., A. C. Frasch, et al. (2005). "RNA-binding domain proteins in Kinetoplastids: a comparative analysis." Eukaryot Cell **4**(12): 2106-2114.

De Gaudenzi, J. G., I. D'Orso, et al. (2003). "RNA recognition motif-type RNA-binding proteins in Trypanosoma cruzi form a family involved in the interaction with specific transcripts in vivo." J Biol Chem **278**(21): 18884-18894.

Delhi, P., R. Queiroz, et al. (2011). "Is there a classical nonsense-mediated decay pathway in trypanosomes?" PLoS One **6**(9): e25112.

Di Noia, J. M., I. D'Orso, et al. (2000). "AU-rich elements in the 3'-untranslated region of a new mucin-type gene family of Trypanosoma cruzi confers mRNA instability and modulates translation efficiency." J Biol Chem **275**(14): 10218-10227.

Droll, D., S. Archer, et al. (2010). "The trypanosome Pumilio-domain protein PUF7 associates with a nuclear cyclophilin and is involved in ribosomal RNA maturation." FEBS Lett **584**(6): 1156-1162.

Estevez, A. M. (2008). "The RNA-binding protein TbDRBD3 regulates the stability of a specific subset of mRNAs in trypanosomes." Nucleic Acids Res **36**(14): 4573-4586.

Fernandez-Moya, S. M. and A. M. Estevez (2010). "Posttranscriptional control and the role of RNA-binding proteins in gene regulation in trypanosomatid protozoan parasites." Wiley Interdiscip Rev RNA **1**(1): 34-46.

Folgueira, C. and J. M. Requena (2007). "A postgenomic view of the heat shock proteins in kinetoplastids." FEMS Microbiol Rev **31**(4): 359-377.

Freistadt, M. S., G. A. Cross, et al. (1987). "Direct analysis of the mini-exon donor RNA of Trypanosoma brucei: detection of a novel cap structure also present in messenger RNA." Nucleic Acids Res **15**(23): 9861-9879.

Glass, D. J., R. I. Polvere, et al. (1986). "Conserved sequences and transcription of the hsp70 gene family in Trypanosoma brucei." Mol Cell Biol **6**(12): 4657-4666.

Gray, M. W. (2012). "Evolutionary Origin of RNA Editing." Biochemistry.

Gunzl, A. (2010). "The pre-mRNA splicing machinery of trypanosomes: complex or simplified?" Eukaryot Cell **9**(8): 1159-1170.

Hartmann, C., C. Benz, et al. (2007). "Small trypanosome RNA-binding proteins TbUBP1 and TbUBP2 influence expression of F-box protein mRNAs in bloodstream trypanosomes." Eukaryot Cell **6**(11): 1964-1978.

Hausler, T. and C. Clayton (1996). "Post-transcriptional control of hsp70 mRNA in Trypanosoma brucei." Mol Biochem Parasitol **76**(1-2): 57-71.

Häusler, T. and C. E. Clayton (1996). "Post-transcriptional control of hsp 70 mRNA in *Trypanosoma brucei*:." Mol. Biochem. Parasitol. **76**: 57-72.

Hendriks, E. F. and K. R. Matthews (2005). "Disruption of the developmental programme of Trypanosoma brucei by genetic ablation of TbZFP1, a differentiation-enriched CCCH protein." Mol Microbiol **57**(3): 706-716.

Hendriks, E. F., D. R. Robinson, et al. (2001). "A novel CCCH protein which modulates differentiation of Trypanosoma brucei to its procyclic form." EMBO J **20**(23): 6700-6711.

Holetz, F., A. Correa, et al. (2007). "Evidence of P-body-like structures in *Trypanosoma cruzi*." Biochem. Biophys. Res. Commun. **356**: 1062-1067.

Hudson, B. P., M. A. Martinez-Yamout, et al. (2004). "Recognition of the mRNA AU-rich element by the zinc finger domain of TIS11d." Nat Struct Mol Biol **11**(3): 257-264.

Jackson, R. J., C. U. Hellen, et al. (2010). "The mechanism of eukaryotic translation initiation and principles of its regulation." Nat Rev Mol Cell Biol **11**(2): 113-127.

Jae, N., P. Wang, et al. (2010). "Essential role of a trypanosome U4-specific Sm core protein in small nuclear ribonucleoprotein assembly and splicing." Eukaryot Cell **9**(3): 379-386.

Kahvejian, A., Y. V. Svitkin, et al. (2005). "Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms." Genes Dev **19**(1): 104-113.

Kramer, S., N. C. Kimblin, et al. (2010). "Genome-wide in silico screen for CCCH-type zinc finger proteins of Trypanosoma brucei, Trypanosoma cruzi and Leishmania major." BMC Genomics **11**: 283.

Kramer, S., R. Queiroz, et al. (2008). "Heat shock causes a decrease in polysomes and the appearance of stress granules in trypanosomes independently of eIF2(alpha) phosphorylation at Thr169." J Cell Sci **121**(Pt 18): 3002-3014.

Lee, M. G. (1995). "Heat shock does not increase the transcriptional efficiency of the Hsp 70 genes of Trypanosoma brucei." Exp Parasitol **81**(4): 608-613.

Lee, M. G. S. (1998). "The 3' untranslated region of the hsp 70 genes maintains the level of steady state mRNA in Trypanosoma brucei upon heat shock." Nucl. Acids Res. **26**: 4025-4033.

Li, C. H., H. Irmer, et al. (2006). "Roles of a Trypanosoma brucei 5'->3' exoribonuclease homolog in mRNA degradation." RNA **12**(12): 2171-2186.

Ling, A. S., J. R. Trotter, et al. (2011). "A zinc finger protein, TbZC3H20, stabilizes two developmentally regulated mRNAs in trypanosomes." J Biol Chem **286**(23): 20152-20162.

Luu, V. D., S. Brems, et al. (2006). "Functional analysis of Trypanosoma brucei PUF1." Mol Biochem Parasitol **150**(2): 340-349.

Mair, G., H. Shi, et al. (2000). "A new twist in trypanosome RNA metabolism: cis-splicing of pre-mRNA." RNA **6**(2): 163-169.

Mangus, D. A., N. Amrani, et al. (1998). "Pbp1p, a factor interacting with Saccharomyces cerevisiae poly(A)-binding protein, regulates polyadenylation." Mol Cell Biol **18**(12): 7383-7396.

Masterson, W. J., T. L. Doering, et al. (1989). "A novel pathway for glycan assembly: biosynthesis of the glycosyl-phosphatidylinositol anchor of the trypanosome variant surface glycoprotein." Cell **56**(5): 793-800.

Mayer, M. P. and B. Bukau (2005). "Hsp70 chaperones: cellular functions and molecular mechanism." Cell Mol Life Sci **62**(6): 670-684.

McAndrew, M., S. Graham, et al. (1998). "Testing promoter activity in the trypanosome genome: unexpected insights into RNA polymerase II initiation." Exp. Parasitol.

Michels, P. A., F. Bringaud, et al. (2006). "Metabolic functions of glycosomes in trypanosomatids." Biochim Biophys Acta **1763**(12): 1463-1477.

Milone, J., J. Wilusz, et al. (2002). "Identification of mRNA decapping activities and an ARE-regulated 3' to 5' exonuclease activity in trypanosome extracts." Nucleic Acids Res **30**(18): 4040-4050.

Milone, J., J. Wilusz, et al. (2004). "Characterization of deadenylation in trypanosome extracts and its inhibition by poly(A)-binding protein Pab1p." RNA **10**(3): 448-457.

Mitchell, P., E. Petfalski, et al. (1997). "The exosome: a conserved eukaryotic RNA processing complex containing multiple 3'-5' exoribonucleases." Cell **91**: 457-466.

Morgan, B. R. and F. Massi (2010). "A computational study of RNA binding and specificity in the tandem zinc finger domain of TIS11d." Protein Sci **19**(6): 1222-1234.

Morking, P. A., B. M. Dallagiovanna, et al. (2004). "TcZFP1: a CCCH zinc finger protein of Trypanosoma cruzi that binds poly-C oligoribonucleotides in vitro." Biochem Biophys Res Commun **319**(1): 169-177.

Mosser, D. D. and R. I. Morimoto (2004). "Molecular chaperones and the stress of oncogenesis." Oncogene **23**(16): 2907-2918.

Muhich, M. and J. C. Boothroyd (1988). "Polycistronic transcripts in trypanosomes and their accumulation during heat shock: evidence for a precursor role in mRNA synthesis." Mol. Cell. Biol. **8**: 3837-3846.

Muhich, M. L., M. P. Hsu, et al. (1989). "Heat-shock disruption of trans-splicing in trypanosomes: effect on hsp70, hsp85 and tubulin mRNA." Gene **82**(1): 169-175.

Nett, I. R., D. M. Martin, et al. (2009). "The phosphoproteome of bloodstream form Trypanosoma brucei, causative agent of African sleeping sickness." Mol Cell Proteomics **8**(7): 1527-1538.

Newbury, S. F., O. Mühlemann, et al. (2006). "Turnover in the Alps: an mRNA perspective." EMBO reports **7**: 143-148.

Opperdoes, F. R. and P. Borst (1977). "Localization of nine glycolytic enzymes in a microbody-like organelle in Trypanosoma brucei: the glycosome." FEBS Lett **80**(2): 360-364.

Ouna, B. A., M. Stewart, et al. (2012). "The Trypanosoma brucei CCCH zinc finger proteins ZC3H12 and ZC3H13." Mol Biochem Parasitol **183**(2): 184-188.

Overath, P. and M. Engstler (2004). "Endocytosis, membrane recycling and sorting of GPI-anchored proteins: Trypanosoma brucei as a model system." Mol Microbiol **53**(3): 735-744.

Parfrey, L., E. Barbero, et al. (2006). "Evaluating support for the current classification of eukaryotic diversity." PLoS Genet **2**: e220.

Parker, R. and U. Sheth (2007). "P bodies and the control of mRNA translation and degradation." Mol. Cell **25**: 635-646.

Paterou, A., P. Walrad, et al. (2006). "Identification and stage-specific association with the translational apparatus of TbZFP3, a CCCH

protein that promotes trypanosome life-cycle development." J Biol Chem **281**(51): 39002-39013.
Pedro-Segura, E., S. V. Vergara, et al. (2008). "The Cth2 ARE-binding protein recruits the Dhh1 helicase to promote the decay of succinate dehydrogenase SDH4 mRNA in response to iron deficiency." J Biol Chem **283**(42): 28527-28535.
Pirkkala, L., P. Nykanen, et al. (2001). "Roles of the heat shock transcription factors in regulation of the heat shock response and beyond." FASEB J **15**(7): 1118-1131.
Puig, S., E. Askeland, et al. (2005). "Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation." Cell **120**(1): 99-110.
Raviol, H., H. Sadlish, et al. (2006). "Chaperone network in the yeast cytosol: Hsp110 is revealed as an Hsp70 nucleotide exchange factor." EMBO J **25**(11): 2510-2518.
Rudenko, G., D. Bishop, et al. (1989). "Alpha-amanitin resistant transcription of protein coding genes in insect and bloodstream form Trypanosoma brucei." EMBO J **8**(13): 4259-4263.
Satterfield, T. F. and L. J. Pallanck (2006). "Ataxin-2 and its Drosophila homolog, ATX2, physically assemble with polyribosomes." Hum Mol Genet **15**(16): 2523-2532.
Scheufler, C., A. Brinker, et al. (2000). "Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine." Cell **101**(2): 199-210.
Shapiro, T. A. and P. T. Englund (1995). "The structure and replication of kinetoplast DNA." Annu Rev Microbiol **49**: 117-143.
Siegel, T. N., D. R. Hekstra, et al. (2009). "Four histone variants mark the boundaries of polycistronic transcription units in Trypanosoma brucei." Genes Dev **23**(9): 1063-1076.
Simpson, L. (1987). "The mitochondrial genome of kinetoplastid protozoa: genomic organization, transcription, replication, and evolution." Annu Rev Microbiol **41**: 363-382.
Sorger, P. K. and H. R. Pelham (1988). "Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation." Cell **54**(6): 855-864.
Stern, M. Z., S. K. Gupta, et al. (2009). "Multiple roles for polypyrimidine tract binding (PTB) proteins in trypanosome RNA metabolism." RNA **15**(4): 648-665.
Straus, D. B., W. A. Walter, et al. (1987). "The heat shock response of E. coli is regulated by changes in the concentration of sigma 32." Nature **329**(6137): 348-351.
Swisher, K. D. and R. Parker (2010). "Localization to, and effects of Pbp1, Pbp4, Lsm12, Dhh1, and Pab1 on stress granules in Saccharomyces cerevisiae." PLoS One **5**(4): e10006.
Tadauchi, T., T. Inada, et al. (2004). "Posttranscriptional regulation of HO expression by the Mkt1-Pbp1 complex." Mol Cell Biol **24**(9): 3670-3681.
Thompson, M. J., W. S. Lai, et al. (1996). "Cloning and characterization of two yeast genes encoding members of the CCCH class of zinc finger

proteins: zinc finger-mediated impairment of cell growth." Gene **174**(2): 225-233.

Ullu, E., K. R. Matthews, et al. (1993). "Temporal order of RNA-processing reactions in trypanosomes: rapid *trans* splicing precedes polyadenylation of newly synthesized tubulin transcripts." Mol. Cell. Biol. **13**(1): 720-725.

Varnum, B. C., Q. F. Ma, et al. (1991). "The TIS11 primary response gene is a member of a gene family that encodes proteins with a highly conserved sequence containing an unusual Cys-His repeat." Mol Cell Biol **11**(3): 1754-1758.

Walrad, P., A. Paterou, et al. (2009). "Differential trypanosome surface coat regulation by a CCCH protein that co-associates with procyclin mRNA cis-elements." PLoS Pathog **5**(2): e1000317.

Walrad, P. B., P. Capewell, et al. (2012). "The post-transcriptional trans-acting regulator, TbZFP3, co-ordinates transmission-stage enriched mRNAs in Trypanosoma brucei." Nucleic Acids Res **40**(7): 2869-2883.

Wurst, M., A. Robles, et al. (2009). "An RNAi screen of the RRM-domain proteins of Trypanosoma brucei." Mol Biochem Parasitol **163**(1): 61-65.

Wurst, M., B. Seliger, et al. (2012). "Expression of the RNA recognition motif protein RBP10 promotes a bloodstream-form transcript pattern in Trypanosoma brucei." Mol Microbiol **83**(5): 1048-1063.

Zilka, A., S. Garlapati, et al. (2001). "Developmental regulation of heat shock protein 83 in Leishmania. 3' processing and mRNA stability control transcript abundance, and translation id directed by a determinant in the 3'-untranslated region." J Biol Chem **276**(51): 47922-47929.