Functional characterization of MKT1 and PBP1 proteins in *Trypanosoma brucei*

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Dedicated to my

Father!

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SUMMARY

In the cytoplasm of a eukaryotic cell, the mRNAs are stored, translated and degraded. The balance between these processes is believed to be determined by multiple interactions between the mRNA and various RNA binding proteins. These interactions in turn are influenced by the gene regulation in response to various changes in the cellular environment.

In *T. brucei,* the regulation of gene expression operates at the posttranscriptional level. ZC3H11, a CCCH zinc finger protein in *T. brucei* is known to specifically bind and stabilize the heat shock mRNAs. Through affinity purification, ZC3H11 was found to interact with MKT1 and PBP1. These proteins caught our interest, as their orthologues are known to exist and interact in yeast. Also, Ataxin-2, the orthologue of PBP1 in mammals is covered in various other studies, owing to its medical relevance. Furthermore, in both yeast and mammals, PBP1 was shown to interact with Poly (A) binding protein (PABP).

During my PhD, I tried to decipher the role of MKT1 and PBP1 in *T.brucei*. I verified the interaction of both these proteins and found them to be essential for survival of bloodstream form of *T.brucei.* Through an extensive yeast two-hybrid analysis, I could show that both PBP1 and MKT1 are necessary for the function of ZC3H11; and PBP1 also interacts with Poly (A) binding protein (PABP). Thus, through my study, I propose a mechanism for action of ZC3H11 where it stabilizes its target chaperone-encoding mRNA by recruiting MKT1-PBP1 complex, which in turns interacts with PABP. The PABP then facilitates mRNA circularization by tethering the 3' end with the 5'cap through its interaction with eIF4E.

Additionally, through interaction partner search by MKT1 yeast two-hybrid library screen, it was found that MKT1 binds to a fair number of RNA binding proteins, some of which also contain a conserved HNPY motif towards their C-terminus. I then validated the importance of this motif in ZC3H11 function and also showed that its presence is sufficient for interaction of MKT1 with CFB1D (another interacting partner of MKT1, identified in the screen). I anticipate that the finding and mechanism proposed for ZC3H11 function, through this study, could perhaps be applicable to other RNA binding proteins, which interact with MKT1.Moreover, I could also show that just like in yeast, MKT1 and PBP1 both co-associate with the translating polyribosomes and PBP1 also relocates to stress granules after nutrient starvation. This is consistent with both these proteins playing roles in transcript regulation following cellular stress.

Overall, through this study I suggest that trypanosome MKT1 acts as a 'hub' assisting in posttranscriptional regulation of various transcripts. I propose that MKT1 confers this role by binding directly or indirectly to a variety of RNA binding proteins thereby upgrading the 'one transcript-one regulator' models for gene regulation.

ZUSAMMENFASSUNG:

mRNA wird im Zytoplasma eukaryotischer Zellen gelagert, translatiert und abgebaut. Das Gleichgewicht dieser Prozesse wird vermutlich über viele Interaktionen zwischen mRNA und verschiedenen RNA-Bindeproteinen (RBP) bestimmt. Diese Interaktionen wiederum werden von der Genregulation als Antwort auf verschiedene Änderungen in der zellulären Umgebung beeinflusst.

In *T. brucei* wirkt die Regulation der Genexpression auf der post-transkriptionalen Ebene. Es ist bekannt, dass ZC3H11, ein CCCH Zinkfingerprotein in *T. brucei*, spezifisch Hitzeschockprotein kodierende mRNAs bindet und stabilisiert. Mittels Affinitätsaufreinigung wurde entdeckt, dass ZC3H11 mit MKT1 und PBP1 interagiert. Diese Proteine sind von Interesse, da ihre Orthologe in Hefe existieren und interagieren. Außerdem wurde Ataxin-2, das PBP1 Ortholog in Säugerzellen, aufgrund seiner medizinischen Relevanz in verschiedenen Studien untersucht. In Hefe und Säugerzellen wurde auch gezeigt, dass PBP1 mit PolyA-Bindeprotein (PABP) interagiert.

In meiner Doktorarbeit versuchte ich die Rollen von MKT1 und PBP1 in *T. brucei* zu entschlüsseln. Ich verifizierte die Interaktion dieser Proteine und fand, dass sie essentiell für die Blutstromform von *T. brucei* sind. Mit Hilfe einer detaillierten Hefe-Zwei-Hybrid (Y2H) Analyse konnte ich zeigen, dass sowohl PBP1 als auch MKT1 für die Funktion von ZC3H11 notwendig sind; außerdem interagiert PBP1 mit PABP in *T. brucei*. Durch diese Studie schlage ich daher einen Mechanismus der Wirkungsweise von ZC3H11 vor, in dem ZC3H11 seine Ziel-Hitzeschock mRNAs stabilisiert, indem es den MKT1-PBP1 Komplex rekrutiert, der seinerseits mit PABP interagiert. PABP erleichtert dann die Zirkularisierung der mRNA mittels Bindung des 3’ Endes mit der 5’ Kappe über die Interaktion mit eIF4E.

Außerdem wurde bei einer Interaktionspartnersuche für MKT1 mittels Y2H gefunden, dass MKT1 mit zahlreichen RBPs interagiert, von denen einige auch ein konserviertes HNPY Motif in Richtung des C-Terminus hatten. Danach validierte ich die Bedeutung dieses Motifs für die Aktivität von ZC3H11 und zeigte außerdem, dass seine Anwesenheit für die Interaktion von MKT1 mit CFB1D (ein weiterer Interaktionspartner von MKT1) ausreichend ist. Ich erwarte dass dieser Fund und der in dieser Studie vorgeschlagene Mechanismus für die Aktivität von ZC3H11, vielleicht auf andere RBPs die mit MKT1 interagieren übertragbar ist.

Desweiteren konnte ich zeigen, dass MKT1 und PBP1 wie in Hefe mit translatierenden Polyribosomen assoziieren und dass PBP1 nach Nährstoffentzug auch in Stresskörnchen relokalisiert. Dies ist konsistent mit einer Rolle dieser beiden Proteine in der Regulation von Transkripten nach zellulärem Stress.

Insgesamt schlage ich in dieser Studie vor, dass trypanosomales MKT1 an einer zentraler Stelle steht, um in der posttranskriptionaler Regulation verschiedener Transkripte auszuhelfen. Ich schlage vor, dass MKT1 diese Rolle durch direkte oder indirekte Bindung zahlreicher RBPs wahrnimmt, und dadurch die „ein Transkript- ein Regulator“ Modelle der Genregulation erweitert.

I.INTRODUCTION

I.1 Trypanosoma brucei: (15-40µm; 1-2µm)

Trypanosoma brucei is a unicellular parasitic flagellate protozoa that belongs to the Trypanosomatidae family of the order Kinetoplastida. The word 'kinetoplast' in the order name refers to the extension of a single mitochondrion, which contains the mitochondrial genome. The kinetoplast is closely associated with the flagellum of the trypanosome and is believed to be an essential part for the regulation of flagella [1]. Kinetoplastida parasites exhibit efficient and diverse biology which incorporates many topics of current interest and study in the broader biological sciences [2].

T. brucei undergoes a complex life cycle as they alternate between Tse-Tse fly vector (procyclic form) and mammalian hosts (bloodstream form). The parasitic nature of *T.brucei* is revealed while it is replicating in the bloodstream of its mammalian host, where it causes Human African Trypanosomiasis: HAT (also known as 'African sleeping sickness') in humans; and Animal African Trypanosomiasis (also called 'Nagana', vernacular meaning useless) in cattles.

The sleeping sickness is mostly lethal and so far there are no satisfactory drugs or vaccine available for its full cure.

The following three, major disease causing subspecies of *T. brucei* have been studied.

- *T. brucei gambiense* - Causes slow onset chronic trypanosomiasis in humans. Mostly found in central and western Africa. A person can be infected for months and possibly years but when the symptoms occur, the disease is already at an advanced stage and is difficult to combat.
- *T. brucei rhodesiense* - Causes fast onset acute trypanosomiasis in humans. Mostly found in southern and eastern Africa. The parasite causes an acute disease that breaks out in few weeks. This form of sleeping sickness quickly leads to clinical symptoms, enabling faster diagnosis.

  Human African trypanosomiasis (HAT) is a disease caused by *T. b. gambiense* and *T. b rhodesiense.*
- *Trypanosoma brucei brucei*- The causative agent of animal African trypanosomiasis commonly found throughout the tropical regions of Africa. *T. b. brucei* is not human infective due to its susceptibility to lysis by Trypanosome Lytic Factor-1 (TLF-1) [3].

However, as it shares many features with *T. b. gambiense* and *T. b. rhodesiense* (such as antigenic variation) it is used as a model for studying human infections in laboratories.

I.2 The life cycle of *Trypanosoma brucei*:

The life cycle of *T.brucei* begins when the vector Tse Tse fly (*Glossina sp*) feeds on the blood meal of its mammalian host. The fly thus injects the parasite, which travels through to the bloodstream of the mammalian host via their lymphatic system. *T.brucei* is referred to as 'bloodstream trypomastigotes' at this stage. The mammalian bloodstream carries and transfers the parasite to different locations inside the body (lymph, spinal fluid) where they increase in number through multiplication by binary fission.

At this stage, if the mammalian host is again bitten by the fly (which feeds on their blood); it transfers the bloodstream trypomastigotes to the fly. The bloodstream trypomastigotes then transform into 'procyclic trypomastigotes' in Tse Tse fly's midgut. They again multiply by binary fission and transform into 'epimastigotes' once they have left the midgut. The epimastigotes then reach the salivary gland of the fly, multiply once again and convert into 'metacyclic trypomastigotes': the infective stage for mammals.

These metacyclic trypomastigotes are again injected into the mammalian host when the Tse Tse takes a blood meal thereby completing the cycle of *T.brucei*.

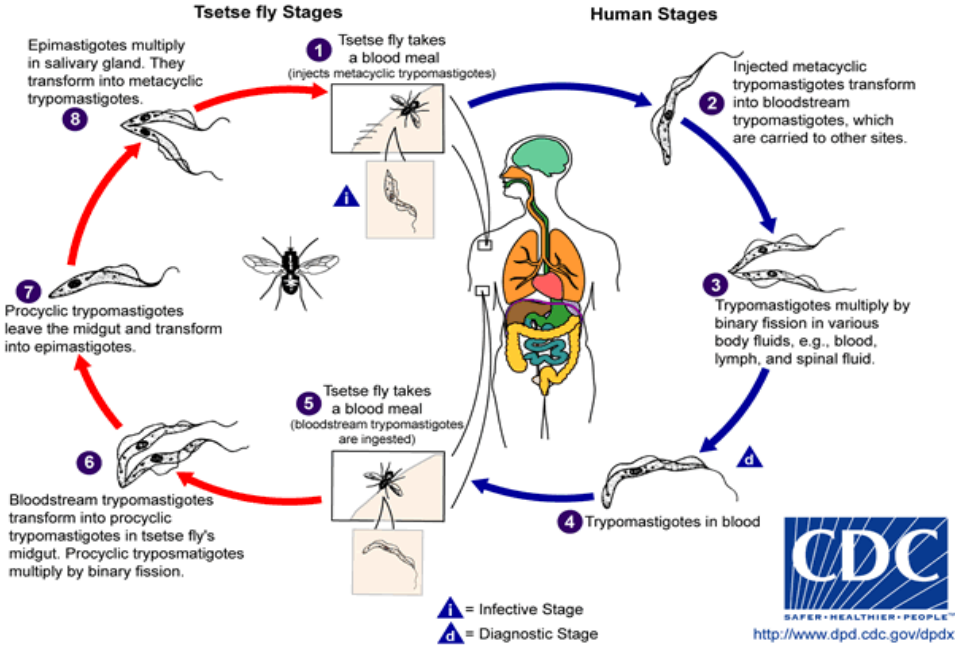


Fig I.2: The transmission cycle of Trypanosoma brucei as given at the webpage of CDC's Division of Parasitic Diseases (http://www.dpd.cdc.gov/dpdx).

I.3 Trypanosomes as a model organism for biological studies:

Although *Trypanosoma brucei* is mostly infamous as a parasite, which causes African sleeping sickness, it still possesses several features to serve as a model organism to study different biological processes. The prime reason for its role as a model organism lies in the fact that it belongs to the Kinetoplastida class of organism, which occupies a very ancient position in the evolutionary tree. Having evolved in a different way than the other routinely studied model organism like *S. cerevisiae, D.melanogaster, H.sapiens* (all of which belong to Opisthokonta group), the Kinetoplastida class offers a diverse and perhaps easier study approach for certain biological processes, which may be difficult to study in the above mentioned Opisthokonta models [4]. For instance *T. brucei* possess an interesting mechanism to regulate its gene expression, which mostly operates at the posttranscriptional level; and exhibit complex patterns of cell morphogenesis to survive in two different organisms during its life cycle. During these developmental stages, they specifically reveal changes in gene expression and metabolism which can be precisely studied by cultivating that particular stage (Bloodstream or Procyclic) of the parasite *in vitro* [5]. Also, unlike other eukaryotes, *T. brucei* contains single (e.g. mitochondria) and unique (e.g. Glycosomes) organelles that undergo various functional and morphological changes during differentiation. This unique feature facilitates the researchers to study organelle biogenesis, turnover and cell division [6] (nicely reviewed in [10]). Further, many biological processes such as RNA editing [7] glycosylphosphatidylinositol (GPI) anchoring [8], trans-splicing [9] and antigenic variation [10] were initially discovered in trypanosomes and were later reported in other eukaryotes.

Moreover, considering *T. brucei* is the only eukaryote reported so far which is capable of synthesizing all three sphingophospholipid classes *de novo*, (namely sphingomyelin, inositolphosphorylceramide and ethanolaminephosphorylceramide), and also as their production is developmentally regulated, *T.brucei* has come up as a novel model organism to study eukaryotic lipid homeostasis particularly with respect to lipid turnover and lipid–protein interactions [5].

Recently, with various ongoing researches related to study of distinct RNP (Ribonucleoproteins) granules containing mRNAs and protein component of P.bodies and Stress granules, *T. brucei* has come forward as a model offering studies related to assembly of these cytoplasmic granules (P bodies and stress granules). This is because, so far, 4 types of RNA granules have been discovered in *T. brucei* (described in details in section I.3.4), which possibly reflects its dependence on posttranscriptional regulation of gene expression. Also, except for SCD6/Rap55, the orthologues of many other essential P-body core proteins, reported in yeast and mammals (such as EDC3, DCP1-DCP2 and Lsm1-7), are absent in

T.brucei. The knockdown of SCD6 by RNAi results in loss of P body [11] and when over-expressed, majority of SCD6 aggregates to multiple granules in the cytoplasm, which recruits other candidate mRNAs-proteins of P bodies and stress granules. [12]. This unique role of SCD6 leading to the formation of RNP granules in *T.brucei* upon over-expression, and the absence of most other core components of RNA granules, promotes *T. brucei* as a model to study the assembly and formation of cytoplasmic RNP granules (P bodies and stress granules).

I.3 Gene regulation in trypanosomes:

I.3.1 Transcription and mRNA splicing:

Unlike the other well studied eukaryotes (like *S.cerevisiae, D.melanogaster, H.sapiens*) which reserve a dedicated promoter for each of their protein encoding gene and where RNA polymerase II is the active enzyme bringing about the gene transcription, the process of gene expression is remarkably different [4]. In Trypanosomes most (but not all!) of the genes are transcribed by RNA polymerase II but in contrast to eukaryotes, no dedicated promoter exist for each of its protein encoding gene [4, 13]. Instead the transcription in trypanosomes is polycistronic, and their transcription by RNA polymerase II leads to a long polycistronic precursor mRNAs (Pre-mRNAs). The pre mRNA comprises more than 100 genes together, whose products are not functionally related [13]. The post transcriptional regulation plays a significant role here in assigning a specific function to each protein-coding exon unit [14]. The adjacent polycistronic transcription units can be convergent or divergent and are spaced apart by regions known as strand switch regions (SSRs)[15]. The specific cleavage of the pre-mRNA takes place in the nucleus. These pre-mRNAs are then co-transcriptionally processed as coupled reactions: *trans*-splicing and 3'-end polyadenylation. During *trans*-splicing a common 39 nt spliced leader (SL) RNA is added to the 5' end of each mRNA. In other words each protein-coding exon is attached to a noncoding spliced leader (SL) miniexon. Like the polycistronic unit, the (SL) RNA is also transcribed by RNA polymerase II, but it has its own dedicated promoter and contains a methylated cap like structure [16]. The 3' end of the mRNA is processed and stabilized by the addition of poly-A tail [13].

While the polycistronic transcription by RNA polymerase II takes place for most of the protein encoding genes, interestingly two stage specific expression of genes encoding for the major surface proteins of trypanosomes take place by RNA polymerase I with its reserved promoter [4].These are the variant surface glycoproteins (VSGs) of the bloodstream stage and EP procyclin of the procyclic (insect) stage and other genes contained on the same

polycistronic units [4]. Additionally, the RNA polymerase I also transcribes ribosomal RNA (rRNA); while transcription of U-RNAs and tRNAs takes place by RNA polymerase III. It is also worthy to mention here that in transgenic trypanosomes, mRNA can also be synthesized by T7 bacterial polymerase and the RNA processing in general is independent of the transcribing polymerase [13, 14].

I.3.2 mRNA degradation in *T.brucei:*

The regulation of gene expression is very essential in the survival of digenetic parasites as they face different environment while shuttling in between their insect vector and mammalian host [17]. As discussed in the previous segment, this regulation mainly acts at a posttranscriptional level mostly by mRNA degradation, mRNA export, translation and formation of cytoplasmic RNA granules [4, 17]. So far, the regulation at the level of mRNA degradation has been widely studied. [4, 14]. In a nutshell, after being transcribed from the polycistronic unit and processing, an mRNA moves to cytoplasm for translation. At this stage, depending on the environment and requirement, the mRNA can be stabilized, stored or degraded. For an mRNA degradation, the CAF-NOT complex is recruited which removes the poly A tail from the 3 ’end. Once the mRNA is deadenylated, it can either be degraded in the 3’ to 5’ direction by the exosome [18] or in the 5’ to 3’ direction by the exoribonuclease XRN1 [4]. For the 5’ to 3’ degradation the *T.brucei* has four homologues of the 5’ to 3’ exoribonuclease XRN1 known as XRNA, XRNB, XRNC and XRND; out of which only XRNA has been shown to be important in mRNA degradation [19].

In an alternative pathway, once out of the nucleus, the mRNA can be directly decapped followed by 5’to 3’ degradation by XRN1. It has already been shown in *T. brucei* that for most mRNAs, deadenylation precedes degradation of the transcript; and knockdown of XRNA preferentially imparts stability to the unstable mRNAs [19]. Recently it was also shown that knock down of either CAF1 or CNOT10, the two essential components CAF-NOT complex in *T. brucei* strongly inhibits the degradation of most of the transcripts [20].

Usually this process is triggered by RNA binding proteins, which specifically bind to the 3’ UTR discussed in details in the following section [15].

I.3.3 The role of RNA-binding proteins in gene regulation:

In all eukaryotes the fate of an mRNA is largely depended on various RNA binding proteins. The *Trypanosoma brucei* genome encodes for a wide variety of RNA binding proteins, consisting of at least 48 Zinc finger (CCCH) domain proteins [21], 74 RRM (RNA recognition motif) domain proteins [22], at least 10 Pumilio domain proteins [23].

These RNA binding proteins bind to the 3' UTR of an mRNA and determines its stability and/or localization [15]. In recent years, use of reporter mRNAs constructs like CAT and luciferase gene have revealed the importance of certain repeats or interesting elements in the 3' UTR of an mRNA, which bring about specific regulation of the reporter gene. [24, 25]. The sequences within the trypanosome mRNAs that mediate degradation are, in some cases, present in form of U-rich elements (URE), which are similar to AU-rich elements (AREs) of mammalian cells [26-28]. For instance, it has already been shown that the mRNAs encoding the glucose transporters THT1 and THT2 are regulated by sequences in their 3' UTR [29]. Also, using the reporter constructs it was shown that sequences in the 3' UTR are responsible for the strong developmental regulation of the phosphoglycerate kinases mRNAs [55]. Further, several RNA binding proteins have been identified in *T.brucei* which confer regulation at the mRNA degradation level. For example DRBD3 [30, 31], PTB2 [31], PUF9 [32], and UBP1/2 [33], impart stabilization to their target mRNA. ZFP3, on the other hand, activates translation [34]. While the mechanism of the regulation brought about by these protein is not known, it has been hypothesized that ZPF3 and PUF9 compete with destabilizing factors [32].

In a recent study in *T.brucei*, ZC3H11, a CCCH zinc finger protein has been found to be a posttranscriptional regulator of heat shock mRNAs. ZC3H11 is essential for survival of bloodstream-form trypanosomes and for recovery of insect-form trypanosomes from heat shock. Using CAT reporter mRNA constructs it is shown that ZC3H11 specifically stabilizes reporter transcripts. Metabolic labeling of ZC3H11 depleted cells by S[35] Methionine, post heat shock showed major decrease in *de novo* synthesis of HSP70 and HSP80 protein in insect stage cells. Also, the Electrophoretic Mobility Shift Assay (EMSA) and Deep sequencing revealed that ZC3H11 specifically recognizes and binds to the AU rich region of 3' UTR of its target mRNA. These observations pointed to a post transcriptional regulation of heat shock genes by ZC3H11 through specific RNA-protein interactions [25].

The advancement in development of novel tools and methods to detect RNA protein interaction have immensely aided in such specific studies. Recently, a global survey of RBP42-bound mRNAs was performed by applying HITS-CLIP technology, which revealed that RBP42 particularly targets mRNAs involved in *Trypanosoma brucei* energy metabolism

[35]. In another milestone study involving the overexpression of a single RNA binding protein RBP6 in cultured noninfectious *T. brucei* culture, the developmental stages of *T. brucei* while it resides in the insect vector during its life cycle, including the production of infectious metacyclic form of parasite (which expresses the VSGs), could be recapitulated.
RNA binding proteins thus have an important role to play in post transcriptional regulation of gene expression and have provided invaluable insights into *T. brucei* biology.

I.3.4 Formation of RNP granules:

RNP (Ribonucleoprotein) granules are microscopically visible, distinct cytoplasmic foci that contain RNA and proteins, which function in storage or decay of an mRNA. Trypanosomes have a large repertoire of different RNP granules, which again comes from the fact that the gene regulation in *T. brucei* is mostly at the post transcriptional level [36] [37].

So far, four different types of RNP granules have been described in *T. brucei* and they are:

a) **P-body-like granules:** These granules in *T. brucei* show the expected increase when polyribosomes are disrupted and they contain similar components to yeast or mammalian P-bodies [38]. But strangely, no orthologue to any enzymes involved in decapping (DCP1, DCP2, and EDC3) have yet been identified in trypanosomes. In fact, even the cytoplasmic Lsm1-7 complex, the likely core component of P-bodies, is absent [39]. The only cytoplasmic Lsm domain protein that is also reported to localize to P bodies, detected in *T. brucei* is SCD6. [38]. SCD6 is also essential for P-body formation [40], and is possibly there to compensate for the absence of the Lsm complex. SCD6, upon overexpression aggregates and parks itself close to the nuclear periphery and recruits both proteins and mRNAs specific to P-bodies and stress granules. The LSm domain of SCD6 is alone sufficient for granule induction and the RGG motif (Glycine-Arginine motif) determines the number and type of granules [40].

b) **Starvation stress granules:** Though similar to P bodies in localization and shape, starvation stress granules are comparatively larger in size. It is suggested that these granules could function in mRNA storage and are temporary, which is evident from the fact that the mRNA returns to the translating state once the stress is over [41].

c) **Heat shock stress granules:** These granules are induced by heat shock mediated translational exit and granule formation is accompanied by increased P-bodies and mRNA decay [38]. Heat shock induced granules are more irregular in shape than P-bodies [38]. However, in *T. brucei* heat shock granule formation does not require the

phosphorylation of eIF2α at the position homologous to that of serine 51 in mammalian eIF2α [42]. Also, the orthologues of TIA-1 and G3BP, the core heat shock stress granule component in mammalian cells [43], are absent in trypanosomes. [38]

d) **Nuclear periphery granules:** Nuclear periphery granules are perhaps a unique category of granules found in trypanosomes [37]. These granules are formed when the trans-splicing is inhibited and they differ from P-bodies as they specifically align around the nuclear periphery. The assembly of nuclear periphery granules seems to be independent of the translation conditions, as they cannot be stained for mRNAs by fluorescent *in situ* hybridization as reported for starvation granules [41]. Perhaps, the mRNA if present are either not accessible for *in situ* hybridization or they are too short lived and are being quickly degraded.

The nuclear periphery granules have been speculated to have a role in the degradation of unspliced RNAs which are constitutively present in trypanosomes and are even capable of entering cytoplasm [37].

I.4 Heat shock response:

Regulation of protein synthesis, sorting, transport and degradation is mandatory to render a tight control and proper functioning of a cell. This quality control of protein is necessary to monitor and facilitate optimal functioning of various organelles and cellular components under normal and adverse conditions. To bring about this control, a group of proteins known as molecular chaperones which includes various heat shock proteins such has Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 are known to exist inside the cell and have been widely characterized in many organisms. In general, in response to an environmental stress, the cells induce the expression of a significant number of genes, particularly those encoding the heat shock proteins (Hsps). The functions of these proteins is to act as a molecular chaperones to correct the misfolded protein, refold the denatured proteins and check the aggregation of proteins by forbidding unusual interactions between amino acid residues.

The immediate effect is brought about by the heat shock transcription factors (HSF). The heat shock factors bind to a consensus near the heat stress inducible promoters known as heat shock element. *Saccharomyces cerevisiae* [44] and *Drosophila melanogaster* [45] express one, mammalian cells three [46] and plants more than 20 heat shock transcription factors[47] . Upon heat shock, certain chaperone proteins associates with the stress affected denatured proteins releasing Heat shock factor (HSF1)[48], that trimerises and translocates from the cytoplasm to the nucleus, which leads to the transcription of heat shock genes [49]. Also the

transcription and translation of housekeeping genes is turned off so that the anabolic energy for the repair process is conserved. An account of events following heat shock stress in different organisms has been fairly reviewed in [50]. In general, heat shock inhibits mRNA splicing in *S. cerevisiae* [51] and mammalian cell lines [52] and causes the formation of stress granules (discussed in details in the previous section) [53, 54]. The formation of stress granules in turn causes a decrease in global translation which is a characteristic event in response to heat shock [55].

In addition, Nucleoli, which are the site of ribosome assembly swell [56] and get impregnated with the deposition of RNA granules and aggregates of ribosomal proteins[57]. Also, a change in morphology and fluidity of cellular membranes has been reported [58]([59]). Heat shock also leads to nuclear accumulation of polyadenylated mRNA in both yeast and mammals [60, 61]. Thus, together in response to these effects, the cells show a cessation in growth and division and finally die [62-64].

As discussed in the above segment it is clear that the heat shock response is a general homeostatic mechanism that protects a cell from the deleterious effects of environmental stress [65]. In this regard it is worthy to mention that, for a wide number of digenetic pathogens, heat shock is a natural event of their life cycle as they have to cycle between a poikilothermic organism and in the mammalian host, a homeothermic organism. Initial analyses of heat-shock gene expression in *Trypanosoma brucei* and *Leishmania major* indicates that heat shock mRNAs are responsible for the adaptation of these parasites to the mammalian host upon infection[66]. Also, increased synthesis of HSP60, HSP70, mitochondrial HSP70 and HSP90 during stage differentiation of *Leishmania donovani* and *Trypanosoma cruzi* have been revealed after intensive proteomic analyses [67, 68]. While as a response to heat shock, most eukaryotes respond by elevating the transcription of their heat shock protein coding gene, the Kinetoplastids including *T.brucei* do not follow this conventional regulation. As mentioned, in these organisms, the heat shock genes are constitutively transcribed and the heat shock specific regulation relies exclusively on post-transcriptional mechanisms [69-71].

Thus, overall when trypanosomes are subjected to heat shock, the transcription by RNA polymerase- II is shut down [71], the trans-splicing of tubulin mRNA is reduced [72], but the trans-splicing of the HSP70 mRNA remains unaffected [73]. Heat shock also causes a defect in growth proliferation that coincides with the down regulation of steady-state levels of most mRNAs. Almost 2 hours post- heat shock, there is a 75% decrease in cellular mRNA level, resulting from a decrease in production and increase in decay [38, 70]. In contrast, the level of HSP70 and HSP83 mRNA remains unaltered and they continue to be translated [38, 70]. The

3′UTR is required to maintain HSP70 mRNA levels at heat shock conditions[70] [69]. Other than that, a major decrease in global protein translation has been observed post heat shock as detected by collapse of polyribosomes and metabolic labeling by S[35] methionine. Heat shock also causes relocation of the ubiquitous PABP and some translation factors in distinct cytoplasmic granules [38] .

I.5 Yeast two-hybrid technology:

Extensive yeast two-hybrid analyses are the heart of this study. The analyses served us in all possible ways by confirming already known putative interactions (pulled down in TAP purification of ZC3H11 protein), identifying novel interacting partners of MKT1 (through genomic library screen), and it also helped us to identify the region/domain responsible for interaction of proteins with MKT1.

The yeast two-hybrid system has been devised to identify genes encoding proteins that physically associate with a given protein *in vivo*. In contrast to other biochemical tools to detect protein-protein interaction, this system is based on a yeast genetic assay in which the interaction of two proteins is measured by the reconstitution of a functional transcriptional activator in yeast [74, 75]. The advantages of this method over other biochemical methods are that the protein-protein interaction is studied *in vivo* in a heterologous organism and this tool is more time and cost efficient. Moreover, through this tool one can also identify/test the role of a particular domain/region involved in the interaction of two proteins.

The basis of the yeast two-hybrid systems relies on the structure of a particular transcription factor that has two physically separable domains. The ‘DNA binding domain’ serves to target the transcription factor to specific promoter sequence located upstream (known as Upstream activating sequence-UAS), and the ‘activation domain’ serves to facilitate assembly of transcription complex, allowing the initiation of transcription. The fact that a functional transcriptional factor can be reconstituted through non covalent interaction of two independent hybrid proteins containing either a DNA binding domain or an activation domain constitutes the basis of yeast two hybrid approach [74]. The hybrid proteins are usually transcriptionally inactive alone or when co-expressed with a non-interacting hybrid protein. However, if the two proteins are meant to interact, when co-expressed, they associate via the interaction between the two fusion protein partners, and become active causing the expression of reporter gene by the specific UAS for the DNA binding domain [74]. Plasmid encoding the fusion protein with the Gal4p DNA-binding domain is referred to as “*bait”* plasmid and the

plasmid encoding the fusion protein with the Gal4p Activation domain is referred to as *"prey"*(Book: Yeast two- hybrid system, Bartel and Fields, 1997, Oxford press).

In our study, we used the Matchmaker GAL4-based two-hybrid assay, where a bait protein is fused to express with the Gal4 DNA-binding domain (DNA-BD), while the prey proteins are fused to express with the Gal4-activation domain (Gal4-AD) such that when bait and prey fusion proteins interact, the DNA binding domain and the DNA activation domain come very near to each other facilitating the activation of transcription of three independent reporter genes, which are ADE2, HIS3, and MEL1 [74, 75]. These three reporter genes MEL1, HIS3, and ADE2 are under the control of three entirely heterologous gal4-responsive promoter elements, which are M1, G1 and G2 respectively. Although the protein-binding sites within each promoter are different, they are still related as all of them contain the same *17-mer*-consensus sequence recognized by Gal4 [76, 77]. Given below is a little more description of the four-reporter gene used in this study.

HIS3 gene encodes for Imidazoleglycerol-phosphate dehydratase, an enzyme catalyzing the sixth step in Histidine biosynthesis[78]. The parent yeast strain in the Matchmaker GAL4-based two-hybrid system™ is unable to synthesize Histidine and is therefore unable to grow on media that lack this essential amino acid. When *bait* and *prey* proteins interact, Gal4-responsive His3 expression permits the cell to biosynthesize Histidine and grows on –His minimal medium.

ADE2 encodes for phosphoribosylaminoimidazole carboxylase, an enzyme that catalyzes the sixth step in the *de novo* biosynthesis of purine nucleotides. The parent yeast strain in the Matchmaker GAL4-based two-hybrid system™ is unable to grow on minimal media that does not contain adenine. However, when both *bait* and *prey* proteins interact, Ade2 expression is activated, allowing these cells to grow on -Ade minimal medium.

MEL1 encodes alpha-galactosidase, an enzyme involved in the catabolic conversion of melibiose to glucose and galactose. As a result of two-hybrid interactions, alpha-galactosidase (MEL1) is expressed and secreted by the yeast cells. Yeast colonies that express Mel1 turn blue in the presence of the chromogenic substrate X-alpha-Gal.

Other than that, in order to select a transformed cell for proper integration and selection of *bait* and *prey* plasmid the transformed cells are selected by growing them in a media lacking Leucine and Tryptophan. Cells harboring bait and prey plasmids are able to grow because the plasmids encodes Tryptophan and Leucine biosynthesis genes respectively, that are otherwise absent from the cell.

Matchmaker GAL4-based two-hybrid system™ parent yeast strain, transformed with correctly expressing bait and prey plasmid is capable of growing in a synthetically defined media lacking Tryptophan and Leucine, commonly known as Double Dropout Media (DDO). This is irrespective whether the bait and prey interact and reconstitute the transcriptional activator. Also the bait plasmid adds a c-myc tag to the encoded protein and a prey plasmid adds an HA tag. Thus, a successfully transformed yeast cell can also be tested for expression of correctly sized protein on a western blot.

However, if the bait and prey code for protein that physically associate with each other, than the yeast cells can grow in a synthetically defined double dropout media additionally lacking histidine or adenine or both (media lacking Tryptophan, Leucine, Histidine and Adenine-Quadruple Dropout (QDO).

Thus, if two proteins under investigation interact, they obviously express the correctly sized bait and prey protein and also grow on a QDO media. While, if they don't interact, they fail to grow on QDO media and just grow on DDO media, expressing the correctly sized bait and prey protein.

It's worthwhile to mention here that at times the bait is a 'self-activator', which means that it is solely capable of allowing the transcription of the reporter gene. So an additional control to rule out this possibility is checking for the interaction of the bait protein under investigation with an irrelevant prey protein from a different organism. One such candidate prey plasmid pGADT7-T which encodes the Gal4 AD fused with SV40 large T-antigen is present in the kit. A self-activating bait protein grows on both DDO and QDO without even interacting with a prey plasmid. Yeast colonies that express Mel1 turn blue in the presence of the chromogenic substrate X-a-Gal.

In another effort to find the putative interacting partners of MKT1, an intensive yeast two-hybrid library screen was performed in this study. The yeast two-hybrid screen was performed in order to find further interactions of MKT1 fused to the GAL4 BD and expressed as bait. To build the library of "prey" trypanosome proteins bearing the activation domain, a randomly sheared trypanosome genomic DNA was used because most of the *T.brucei* genome is coding and only two introns have yet been reported [79]. Approximately $3x10^6$ independent clones were obtained with an insert size ranging from 0.6 - 2.3 kbp. Only one out of 6 clones will contain an insert that is in frame with the selection marker. The prey library was then transfected into yeast expressing MKT1 expressed as bait.

I.6 Functions of MKT1 in yeast:

The MKT1 (Maintenance of yeast Killer Toxin) gene was first defined as a recessive allele present in many laboratory strains of *Saccharomyces cerevisiae* (including the standard lab strain S288C), that results in a loss of M2 double-stranded RNA (viral like particle) at temperatures above 30°C. In other words, Mkt1p was identified as a protein involved in maintenance of yeast killer virus [80, 81].

Yeast (*S. cerevisiae*) is well known to serve as a host to many RNA viruses and prions for their propagation (nicely reviewed in [82]). One such dsRNA virus is known as L-A. The genome of L-A encodes a major coat protein (gag) and its RNA-dependent RNA polymerase (pol), the latter expressed as a gag-pol fusion protein formed by a -1 ribosomal frameshift, a common coding mechanism used by many retroviruses. The L-A resides in yeast along with its dsRNA satellites M1,M2….(several Ms); which encode different secreted protein toxins and are in turn dependent on L-A viral genome for encoding their capsid-coat protein. In other words, L-A virus offers a helper function to these other viral like particles comprising various Ms (M1,M2..) [83] [84]. MKT1 is a non-essential gene in yeast. It was shown that recessive MKT1 allele caused L-A virus to exclude its M2 dsRNA satellite (only M2 not M1) at temperatures above 30°C. It was thus established that MKTI gene product is needed by M2 dsRNA only at temperatures above 30°C and that M2 dsRNA can replicate at temperatures below 30°C even without the MKTI gene product [81].

Moreover, low sporulation efficiency was observed in S288C strain in a quantitative trait locus study of yeast, as compared to wild type; and this decrease was also attributed to MKT1 genotype. However when a critical residue G30 at MKT1 locus in S288C was mutated to D, sporulation efficiency was comparatively increased [85, 86]. The same G30>D mutation was shown to be responsible for poor growth of yeast strain at temperature above 30°C [87], or in high ethanol or low glucose media respectively [88, 89].

MKT1 genotype was reported to make a minor contribution to sensitivity of yeast to some DNA damaging agents [87]. Further, MKT1 was also reported to play a role in mitochondrial genome maintenance and lead to reduced petite colony formation, however the mechanism of this phenotypes is still unknown [90].

Mkt1p is known to be positive regulator of HO endonuclease expression through which the mating type switching occurs in yeast [91] .The most common mode of vegetative growth in yeast is asexual reproduction by budding, where a small bud or daughter cell is formed on the parent cell. The bud then grows and splits into a daughter cell inheriting a part of nucleus from the mother cell. The mother and daughter cells are identical with the only difference that

the mother cells expresses the HO endonuclease gene and are thereby able to switch the mating types [92]. The mating type switching by the HO endonuclease is initiated in the yeast mother cell by creating a double stranded break at the *MAT* locus. The HO gene is transcribed briefly during the cell cycle just preceding the DNA replication and budding.

The HO expression is regulated at the transcriptional level by a negative regulator Ash1. Ash1 is a repressor of transcription of HO gene and it localizes specifically to the daughter cell nucleus [93]. At the post transcriptional level HO expression is regulated by Puf5/Mpt5 [94] which binds to the 3' UTR of HO mRNA and represses HO expression. Mkt1p is also a separate independent player in the post-transcriptional regulation of the HO transcript. The ability of Mkt1p to regulate the translation of HO mRNA is brought about by its interaction with another protein known as Pbp1. Pbp1 stands for ***P**abp1p **b**inding **p**artner*, as it directly interacts with yeast Pabp1p. Loss of Pbp1p or Mkt1p had no effect on the level of HO mRNA but it did decrease its protein level; which indicated that the regulation of HO endonuclease gene by Mkt1p-Pbp1p operates at a post transcriptional level. Also, this regulation was found to be mediated by the 3' UTR of HO mRNA. Further, Mkt1p and Pbp1p co-sedimented with polyribosomes in sucrose gradients, with the distribution of Mkt1p in the polyribosomes dependent on Pbp1p, but not vice versa. This indicates that Pbp1p recruits Mkt1p on the polyribosome and thus both Mkt1p and Pbp1p are required for effective mating-type switching in mother cells [91].

I.7 The functions of Pbp1 and Lsm 12 :

In yeast, Pbp1p was found to exist with both the translating and non- translating pools of mRNA [95, 96]. In general, the C-terminal region of Pab1 is known to be a region facilitating protein-protein interaction and recruiting translation factors or other proteins necessary for the organization of the mRNA ribonucleic acid complex [97]. Pbp1p also binds to poly (A)-nuclease subunit Pan2, and negatively regulates PAN activity [98].

Other interacting partners of Pbp1 in yeast are Dhh1p, Pbp4p and Lsm12p proteins, and all these proteins are known to be associated with the translation machinery [96, 99, 100].

Lsm12p, in yeast contains an RNA-binding Lsm domain and an AD domain; which have been known to control mRNA regulation. Lsm12p associates with Puf3p, which regulates mRNA degradation by binding the 3’ UTR of their target mRNA and enhancing its deadenylation rate [101]. Lsm12p also associates with the transcription factor Stb5, which interacts with the transcriptional repressor Sin3[102].

Under starvation stress in yeast, Pbp1, along with its interacting partners Pabp1, Pbp4, Lsm12 and Dhh1, relocate to the stress granules. Over-expression of Pbp1, Dhh1, and Pab1 also caused growth inhibition in yeast [99].

The orthologues of PBP1 in Human and Drosophila, known as Ataxin-2, is also well studied because of its medical relevance. Human ataxin-2 (ATX2) is a protein coded by spinocerebellar ataxia type 2 gene, SCA2. The SCA2 gene contains a CAG repeat that encodes a polyglutamine stretch in the N-terminal region of ATX2. The polyglutamine stretch of Ataxin-2 is expanded in a disease known as spinocerebellar ataxia, which causes neurodegeneration and ultimately death. It is also associated with an increased risk of amyotrophic lateral sclerosis and Parkinsonism [103] [104-106].

Two recent independent studies in Drosophila have stated Ataxin-2 as a translational activator of the rate-limiting clock component PERIOD (PER). In both the studies it is shown that ATX2 specifically interacts with TWENTY-FOUR (TYF), which is an activator of PER translation. ATX2 forms a complex with TYF and promotes its interaction with polyadenylate-binding protein (PABP) and eukaryotic translation initiation factor 4F (eIF4F), thus presumably promoting per mRNA circularization and translation [107, 108]. Previously, in Drosophila, Ataxin-2 has also been reported to interact with member of Argonaut family and play a role in miRNA silencing [109]. Ataxin-2, just like its yeast orthologue also associates with the polyribosomes and DEAD/H Box RNA Helicase DDX6 [110, 111].

Apart from the role of Pbp1 and Ataxin-2 in regulating the translation of an mRNA forming an association with Pab1 and other proteins, both these proteins have also been reported to play a role in assembly of stress granules. Pbp1 deletion in yeast or siRNA knockdown of Ataxin-2 in mammalian cells leads to significant decrease in stress granule formation under stress [111, 112].

Thus, the above mentioned studies of homologues of MKT1 and PBP1 along with their interaction partners like poly-A binding protein, Dhh1/DDX6 (in both yeast and mammals) and Lsm12 (only in yeast) very clearly indicated their role (individually and/or as a complex) in RNA regulation, and this role seems to be evolutionarily conserved.

I.8 Aim of my thesis:

In order to attain a proper understanding of the dynamics and regulation of mRNA and related proteins, various studies in our lab have been going using *T.brucei* as our model system. In this context, through my study, I tried to decipher the role of MKT1 and PBP1 protein in *T. brucei.* Both MKT1 *(Tb* 927.6.4770) and PBP1 (*Tb* 927.8.4540) were first identified as they were pulled down in the Tandem affinity purification of ZC3H11 [25].

ZC3H11 is a CCCH zinc finger protein in *T. brucei* which is known to specifically bind and stabilize the heat shock mRNAs. The role of ZC3H11 was studied by Dorothea Droll (currently a post doc in our lab).

MKT1 and PBP1 proteins (initially having an unknown function and identity) were found to be homologues of yeast Mkt1p and Pbp1p, hence their name in *T.brucei*. These proteins caught our interest as both of them have been speculated to play a role in maintenance of RNA homeostasis and their homologues are a part of various studies in different organism (discussed in previous sections).

Two other interesting candidates (occasionally mentioned in this study), pulled down in the Tandem affinity purification of ZC3H11 are LSM12 and DHH1. These proteins were also named so owing to their sequence similarity and functional correlation to yeast Lsm12p and Dhh1p.

II. MATERIALS AND METHODS:

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

II.1. Growth and Maintenance of Bacteria:

- **LB broth**

  10 g/L Bacto-tryptone
  5 g/L Bacto-yeast extract
  5 g/L NaCl

pH is adjusted to 7.0 with 5 N NaOH. Autoclaved and Stored at 22°C.

- **LB agar + amp plates**

  Prepared LB broth, then added agar (15 g/L), autoclaved, and cooled to 50°C. Added ampicillin to a concentration of 50ug/ml. Poured plates and stored at 4°C.

- **LB agar + kan plates**

Prepared LB broth, then added agar (15 g/L), autoclaved, and cooled to 50°C. Added kanamycin to 50ug/ml. Poured plates and stored at 4°C.

II.1.1. Cloning

All the steps involved in cloning an insert into the vector (PCR, restriction digestion, ligation, agarose gels, etc), were conducted as described in the TDR/EMBO protocol or as stated in the instruction of the manufacturer. For restriction reactions, enzymes from NEB were applied. In order to blunt a vector, T4 polymerase from NEB was used. For dephosphorylation of linearized plasmids, Antarctic Phosphatase (NEB) was used. For normal control/ colony PCRs GoTaq® DNA Polymerase from Promega was used and for all PCRs for cloning reactions Phusion® High-Fidelity DNA Polymerase from Finnzymes was deployed. To amplify yeast DNA, Q5® High-Fidelity DNA Polymerase (NEB) was used.

II.1.2. Transformation of bacteria and plasmid preparations

50ul of DH5 alpha competent cells were taken and mixed with plasmid and incubated on ice for 10 min. The transformation was done at 42°C in a water bath for 90 seconds followed by chilling on ice for 1 min. The cells were then mixed with 500ul of ΨB medium and incubated at 37°C for 45 mins with shaking. Clones were selected on LB-amp plates (or appropriate antibiotic). Single colonies were grown in LB medium with antibiotic overnight. Plasmids were isolated with the Nucleospin Plasmid kit (Macherey Nagel) and checked by control digest and sequencing.

II.2. Growth and Maintenance of Yeast:

The yeast AH109 strain (Matchmaker 3 System, Clontech) suitable for yeast two-hybrid analysis is grown in YPD media supplemented with Adenine hemi sulphate (YPDA). The transformed wild type strain with bait or prey plasmid is cultured in SD medium containing appropriate dropout mixture.

• **YPD medium**

20 g/L	Difco® peptone (BD company)
10 g/L	Yeast extract (BD company)
18 g/L	Agar (for plates only) (BD company)

Water was added to 885 ml. The medium was autoclaved, and cooled to around 55 °C. Glucose was added to a final concentration of 2% (100 ml of a sterile 20% stock solution).

Additionally for YPDA media, Adenine hemisulfate salt (Sigma) was added to a final concentration of 0.2 % (15ml of 100X stock)

• **SD medium**

Synthetic dropout (SD) minimal medium

6.7 g/L	Yeast nitrogen base without amino acid (Sigma)
20 g/L	Agar (for plates only) (BD company)

Water was added to 800 ml. The medium was autoclaved, and cooled to around 55 °C. Glucose was added to a final concentration of 2% (100 ml of a sterile 20% stock solution) followed by 100 ml of the sterile 10X dropout solution to yield SD/–Trp/–Leu /–Ade/–His media (Quadruple dropout-QDO).

10X Dropout solution

L-Isoleucine	300 mg/L
L-Valine	1500 mg/L
L-Arginine HCl	200 mg/L
L-Lysine HCl	300 mg/L
L-Methionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2000 mg/L
L-Tyrosine	300 mg/L
L-Uracil	200 mg/L

Additionally 10 ml of 100X L- Histidine monohydrate solution (2mg/ml) and 10 ml of Adenine Hemisulphate (2mg/ml) was added to yield SD/–Trp/–Leu media (Double dropout - DDO). All the amino acid supplements were from SERVA.

Stock solutions

- 50% PEG 4000 (Polyethylene glycol; Sigma) Filter-sterilized
- 100% DMSO (Dimethyl sulfoxide; Sigma)
- 10X TE buffer: 0.1 M Tris-HCl, 10 mM EDTA, pH 7.5. Autoclaved.
- 10X LiAc: 1 M lithium acetate (Sigma) Adjusted to pH 7.5 with dilute acetic acid and autoclaved.
- 100mg/ml of Salmon sperm DNA, denatured by boiling for 10mins and chilled on ice for 2mins before use.
- **PEG/LiAc solution (polyethylene glycol/lithium acetate**): Always prepared 10ml of fresh solution prior to use.

	Final concentration	To prepare 10 ml of solution
PEG 4000	40%	8 ml of 50% PEG
TE buffer	1X	1 ml of 10X TE
LiAc	1X	1 ml of 10X LiAc

- **TE/LiAC solution (Tris EDTA, lithium acetate):** Always prepared 10ml of fresh solution prior to use.

	Final concentration	To prepare 10 ml of solution
TE buffer	1X	1 ml of 10X TE
LiAc	1X	1 ml of 10X LiAc

II.2.1.Yeast Transformation

Preparation of Yeast Competent Cells

The LiAc method for preparing yeast competent cells was used for all the transformations. Stored AH109 strain was inoculated in 50ml of YPAD media and incubated at 30° C with shaking for overnight so that the cells go into stationary phase (OD_{600}>1.5). The overnight culture was then transferred into new YPAD medium to produce an OD_{600} = 0.2–0.3. It was then

incubated again at 30°C for 3hr with shaking (200rpm) so that the cells reach a logarithmic phase. After this, the cells were centrifuged at 1000 x g for 5 min at room temperature in a swinging bucket centrifuge. The pellet was washed with water once and resuspended in 1.5ml of freshly prepared, sterile 1X TE/LiAc.

Transformation of Yeast Competent Cells

Mixed 500 ng/ul DNA-BD vector construct and 500ng/ul AD vector construct along with 10ul Salmon Sperm carrier (100mg/ml stock) DNA in an eppendorf tube. Added 100ul of yeast competent cells to each tube and mixed well, followed by addition of 600ul of sterile PEG/LiAc solution to each tube and vortex. The cells were then incubated at 30°C for 30 min with shaking (200 rpm). 70ul DMSO (Sigma) was then added and mixed gently by inversion. Heat shock was then given to the mixture for 15 min in a 42° C water bath followed by sudden chilling on ice. The cells were then pelleted for 30sec at 11000 rpm, supernatant was discarded, and the cells were resuspended in 500ul of 1xTE. These cells were then plated into respective plates (QDO or DDO) and incubated at 30°C for 3-4 days. Positive interactions were indicated by growth on SD/–Trp/–Leu /–Ade/–His plates (Quadruple dropout-QDO).

II.2.2. X-alpha gal assay

The positive colonies were also detected by a blue color change in the colony when spread onto a QDO plate containing 100ul X-alpha gal **(**Carl Roth GmbH) stock (4mg/ml).

II.2.3. Two-hybrid Analysis

Complete or fragments of ORFs were cloned into pGADT7 (GAL4 activation domain vector) and pGBKT7 (GAL4 DNA-binding domain vector) (Matchmaker 3 System, Clontech). The bait has an N-terminally fused GAL4 DNA binding domain and Myc tag, while the prey has N-terminally fused GAL4 activation domain and HA tag. The expression of the fusion protein was confirmed by Western blot analysis. The plasmids were co-transformed pairwise into AH109 yeast strains (Matchmaker 3 System, Clontech). Transformants were selected on four drop-out SD/-Trp/-Leu/-His/-Ade culture plates after 4 days of incubation at 30°C. The positive clones were assayed for α-galactosidase activity, as the colonies turn blue if X-α-gal is present in the QDO plates.

II.2.4. Analysis of library screen candidates

As bait for the library screen, the MKT1 ORF was cloned into the pGBKT7 plasmid. The pGADT7 *T. brucei* genomic library was screened by transformation of AH109 yeast expressing pGBKT7-MKT1. Transformants were selected on QDO medium.

In order to identify the numerous anonymous colonies that came out positive in the MKT1 library screen. The colonies were incubated in fresh QDO liquid media for 2 days with change of media twice. The yeast plasmid was then isolated, grown in bacteria, and the bacterial plasmid was checked by restriction digestion with Hind III, which has two sites in the vector containing the insert. The plasmid, that released one positive insert, was chosen and transfected again in yeast, carrying out a second yeast two-hybrid analysis. If the plasmid contains an insert, which expresses a protein interacting with MKT1, the transformed cells were again capable of growing in QDO plate. If that was the case, then the plasmid was sent for sequencing. The sequence was then identified using BLASTn (NCBI) with the sequence available for *T.brucei* 927 strain available in GeneDB. As the sequence of GAL4AD domain, which precedes the insert, is known, I could exactly locate the region of the target protein, which binds with MKT1.

II.2.5. Plasmid isolation from yeast

The stored colonies were inoculated in SD- QDO medium, incubated at 30°C for overnight with shaking at 200-250 rpm. The cells were then collected by spinning them down at 14000 rpm, the pellet was resuspended in 40ul of remaining medium. 10ul of lyticase (Sigma, 5 units/ul in TE) was added followed by vigorous vortexing. The cells were then incubated at 37°C for 60-90 mins with shaking 200-250rpm. 20ul of 10% SDS was added to each tube and cells were again vortexed for 1min. The sample was then subjected to 3-freeze thaw cycle (-80C for 5 mins each) with vortexing in between. The plasmid isolation protocol by MaCherey Nagel was followed thereon to isolate the yeast plasmid. The plasmid was eluted in 35ul of elution buffer. 20ul of this mixture was taken to transform 200ul of competent cells. After transformation the cells were plated in LB-Amp plates (to obtain the prey plasmid) or LB – Kan plate (to obtain the bait plasmid).

II.2.6. Protein detection

The following antibodies were used in Western blotting: Monoclonal mouse Anti-V5 (Santa Cruz), monoclonal mouse anti c-myc (Santa Cruz), Anti-HA (Santa Cruz). ECL™Anti-Rabbit IgG (GE Healthcare), ECL™Anti-Mouse IgG (GE Healthcare), polyclonal rabbit antibodies anti-myc (A-14, sc-789, Santa Cruz), ECL™Anti-Rat IgG (GE Healthcare),

To find potential homologues of Tb927.8.4540 the protein sequence was analysed by bioinformatics tools such as BLASTp, psi-BLAST and tBLAST. The conserved domains were identified by a SUPERFAMILY search Multiple sequence alignments by MegAlign software using Clustal W algorithm. For *in silico* cloning the SeqBuilder software package from DNASTAR was used.

II.3. Growth and Maintenance of Trypanosome:

While majority of experiments were done in bloodstream form of trypanosomes. For certain stress experiments procyclic form of trypanosome was used. The experiments were done with Lister 427 monomorphic procyclic or bloodstream form parasites expressing the Tet-repressor.

II.3.1. Culturing procyclic form trypanosomes

Procyclic form trypanosomes were cultured in tightly closed flasks in 27°C room. The cell densities were always maintained between $5x10^5$ and $1x10^7$cells/ml in supplemented MEM-Pros. All culture work was performed under sterile conditions in a laminar flow hood. Cryopreservation and thawing was done as for bloodstream cells but in supplemented MEM-Pros medium with 10% glycerol and at a density of about $2x10^6$cell/ml.

MEM-Pros pH 7.4

$CaCl_2$	265mg/l	NaH_2PO_4	0.14g/l
KCl	0.4g/l	HEPES	7.14g/l
$MgSO_4.7H_2O$	0.2g/l	L-Arg-HCl	126mg/ml
NaCl	6.8g/l	L-Cys	24mg/ml
L-His-HCl.H_2O	42mg/l	L-Gln	292mg/l
L-Ile	52mg/ml	L-Tyr	100mg/l
L-Leu	52mg/l	L-Val	46mg/l
L-Lys	73mg/l	L-Pro	600mg/l
L-Met	15mg/l	Adenosine	12mg/l
L-Phe	100mg/l	Ornithine-HCl	10mg/l
L-Thr	48mg/l	L-Try	10mg/l

10ml of MEM non-essential amino acids (Gibco), 10ml of MEM vitamins (Sigma) and 10mg of Phenol red were added to 1l of the medium and the pH was adjusted to 7.4. The medium was filter sterilised, 450ml aliquots were prepared and stored at 4°C.

Supplemented MEM-Pros medium, 500ml

To the 450ml MEM-Pros following components were added:

Heat-inactivated FCS	10% (v/v) (50ml, Gibco)
Hemin	7.5mg/l (1.5ml of stock solution)*
Penicillin/Streptomycin	50U/l (5ml Penicillin-Streptomycin mix, Sigma)

**Hemin stock: 0.25% in 0.1M NaOH, autoclaved and stored at 4°C.*

II.3.2. Culturing bloodstream form trypanosomes

BS cells were cultured in an incubator (Hernaeus instruments) maintained at 37°C and 5% CO_2 in a humidified atmosphere. The caps of the culture flasks were tied loosely to allow gaseous exchange. BS cells were grown in supplemented HMi-9 media. Bloodstream cells were harvested at densities not exceeding $2x10^6$cells/ml. For cryopreservation, cells in the logarithmic growth phase (density between $1\text{-}2x10^6$cells/ml), were spun down and the pellets were resuspended in 1ml of 10% glycerol in HMi-9 media and pipetted in separate cryovials. The vials were wrapped in soft tissue papers and stored overnight at -80°C (to allow slow freezing) and finally transferred to cryoboxes in a liquid nitrogen tank. To restart the culture, cells were thawed at room temperature (RT), mixed with 5ml of HMi-9 media, centrifuged once at 2000rpm (RT), and the pellets were resuspended in 10ml HMi-9 media.

HMi-9 media composition

IMDM* (Gibco)	17.66g/l
$NaHCO_3$ (Roth)	36mM
Hypoxanthine (Serva)	1mM
Na-pyruvate (Serva)	1mM
Thymidine (Sigma)	160mM
Bathocuprosulphonate (Serva)	50mM

Supplemented HMi-9 medium, 500ml

450ml HMi-9 media supplemented with:

Heat inactivated** FCS	10% (v/v) (50ml, Gibco)
Penicillin/Streptomycin	50U/l (5ml Penicillin-Streptomycin mix, Sigma)
L-Cysteine-HCl.H_2O	1.5mM (5ml of stock solution)***
β-mercaptoethanol (Sigma)	0.14% (7.2µl in 5ml)***

* Iscove's Modified Dulbecco's Medium, without supplements

** Foetal Calf Serum (FCS) was heat inactivated by incubating at 55°C for 30 min

*** L-Cysteine-HCl.H_2O and β-mercaptoethanol stock solutions were filter sterilized and stored in 5ml aliquots at -20°C.

Antibiotics	**Supplier**	**Working conc. BS(µg/ml)**	**Working conc. PS(µg/ml)**
Phleomycin	Cayla	0.2	0.5
Neomycin	Gibco	0.5	12
Blasticidin	Invitrogen	5	10
Puromycin	Sigma	0.2	1

II.3.3. Transfection buffers:

- **Amaxa type buffer (from Roditi's lab):**

  For bloodstream trypanosomes

  90mM sodium phosphate, 5mM KCL, 0.15mM CaCl2, 50mM HEPES, pH 7.3

  1-2x 10^7 cells were used per transfection. The cells were mixed with 100ul of Amaxa buffer (or Amaxa type buffer) and mixed with 5-10 ug of DNA. Electroporation was performed using 2mm gap cuvettes with program X-001 in Amaxa® nucleofactor.

- **Zimmerman's Post Fusion Medium (ZPFM):**

  For procyclic trypanosomes

  (132mM NaCl, 8mM KCl, 8mM Na2HPO4, 1.5mM KH2PO4, 1.5mM MgAc x 4 H2O, 90**µ**M 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 CaCl2, 10mM K2HPO4/KH2PO4 pH 7.6, 25mM HEPES, 5mM MgCl2,0.5% Glucose,100**µ**g/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 ug of digested plasmid, 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.

II.4. Co-Immunoprecipitation

For co-Immunoprecipitation (co-IP) experiments, $5x10^7$ trypanosomes were lysed in co-IP-lysis buffer [10mM NaCl, 10mM Tris-HCl, pH7.5, 0.3 % IGEPAL and protease inhibitors (Protease Inhibitor Mixture (EDTA-free); Roche Applied Science] by passing them 5 times through a 27g syringe. Afterwards the cells were centrifuged for 20 min at 4°C, 12000rpm. 50ul of V5 Antibody or c-Myc Antibody coupled agarose slurry were washed 4 times with 1xPBS and once with Co-IP buffer [100mM NaCl, 10mM Tris-HCl, pH7.5 0.3 % IGEPAL and protease inhibitors (Protease Inhibitor Mixture (EDTA-free); Roche Applied Science] at 4°C. The cell supernatant was transferred into a new tube and the salt concentration was adjusted to 150 mM NaCl. A sample of $5x10^5$ cells was taken from the input and 2x Laemmli buffer was added. Beads and cell lysates were incubated for one to 1.5h at 4°C with rotation. A sample equivalent to $5x10^5$ cells was taken from the supernatant and 2x Laemmli buffer was added to it. The beads were washed four times for 5 min at 4°C with salt-adjusted lysis buffer then boiled in 2x Laemmli buffer.

II.5. Tandem Affinity Purification

$5x10^9$ procyclic cells expressing MKT1-TAP (pHD 2326) were used for the TAP experiment. 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 one protease inhibitor cocktail tablet (without EDTA, Roche) was added for 5 ml of lysis buffer. Cells were lysed in a final volume of 4ml lysis buffer by passing 15-20 through a 21-gauge needle. Checked on a glass slide to ensure complete breakage. The lysate was spun at 10,000g for 15 min to remove cell debris. The supernatant was transferred to a new tube. NaCl was added to a final concentration of 0.15 M. Mixed and collected 25 ul aliquot

(start material, #1). 200 µl IgG sepharose bead suspension was transferred into a column (Bio-Rad) and washed with 10ml IPP150. The cleared cell lysate was transferred into the column containing the washed beads and rotated for 1 hrs at 4°C. Elution was done by gravity flow. 25ul of the flow through (IgG flowthrough, #2) was collected for subsequent analysis. The beads were 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 was done in the same column by adding 1ml of TEV cleavage buffer and 100 units of TEV protease (Gibco). The beads were rotated for 1 hrs at 16°C and the eluate was collected by gravity flow. 15ul aliquot was collected (IgG eluate, #3).

II.6. Genomic DNA preparations

Approximately $1x10^8$ trypanosomes (PCF or BSF) were used for genomic DNA (gDNA) isolation. The cells were centrifuged at 2000rpm for 10 min followed by a wash with 1XPBS. gDNA from the cells was isolated using the illustra™ tissue & cells genomic Prep Mini Spin Kit (GE Healthcare, UK), following manufacturer's instructions. The gDNA thus purified was stored at -20°C in small aliquots.

II.7. Polysomes

5×10^8 Bloodstream cells ($d=1 \cdot 10^6$ cells/ml) cells were taken into centrifuge flasks and cycloheximide was added to a concentration of 25 µg/ml. The cells were incubated for 1min at room temperature and then chilled in a dry ice ethanol bath. The cells were then spun down (4°C, 2,300g, 20min), transferred to eppendorf tubes and spun down again at 2,300g for 2min. The pellet was washed twice in polysome buffer (20mM Tris pH:7.4-7.5, 20mM $MgCl_2$, 600mM KCl) containing 200mM sucrose. The pellet was then forced five times through a 27-g needle in ice-cold lysis buffer containing 10 mM Tris–HCl, pH 7.6, 300 mM KCl, 10 mM $MgCl_2$ and protease inhibitors (Protease Inhibitor Mixture [EDTA-free]; Roche Applied Science), 0.4mg/ml Heparin, 1 mM DTT, and 10 µg/mL leupeptin. IGEPAL CA-630 (Sigma) was added to a final concentration of 0.1% and the cells were again forced through the needle five times. For RNAse A control, RNAse A (Sigma) was directly added in the cell lysate to a final concentration of 1mg/ml and incubated for 20 mins at room temperature. The lysate was then layered onto 15%–50% sucrose gradients prepared in polysome buffer and centrifuged at 4°C for 2 h at 40,000 rpm in a Beckman SW60i rotor. Fractions were eluted from the top of the gradient using a Teledyne Isco

(Lincoln, NE) gradient elution system; polysome profiles were obtained by measuring absorbance at 254 nm.

II.8. Immunofluorescence

For the intracellular detection of Myc tagged MKT1 and PBP1, $2X10^6$ of procyclic trypanosomes were sedimented, re-suspended, then fixed in 4% paraformaldehyde (w/v) in 1X PBS for 18 min, sedimented again for 2 min, re-suspended in PBS, and allowed to settle down on a poly-lysine-coated glass surface for overnight. The cells were then permeabilized with chilled methanol (-20°C) for 10 min. Before staining, slides were blocked with 0.5% (w/v) gelatine for 20 min. The cells were then incubated with 1:500 diluted anti c-Myc primary antibody (Santa Cruz Biotechnology, Germany) and 1:1000 SCD6 antibody for 1 h and with a 1:1000 dilution of the second antibody Alexa Fluor 488 goat a- mouse IgG and CY5 goat anti-rabbit for 40 min (Molecular probes). The kinetoplast and the nuclear DNA were then stained with 100 ng/ml DAPI/1X PBS for 10 min.

For stress experiments, heat shock was given to the cells in a water bath at 41°C for 1hour. For glucose starvation, cells were sedimented, washed twice in 1xPBS and then resuspended in 1X PBS for 3 hrs at 27°C. The cells were then sedimented, resuspended and fixed with Paraformaldehyde as described above.

Z-stacks of the cells were taken and the images were deconvoluted (Wiener algorithm) using the Olympus Cell-R microscope.

II.9. Effect of heat shock on protein synthesis

For heat shock experiments, Trypanosomes were subjected to heat-shock at 41°C (for 1 hour) in water bath and harvested immediately for Western blot. To measure protein synthesis (global translation or specific de novo synthesis), $2x10^6$ cells were pelleted, resuspended in 500µl of MEM lacking methionine. After 15mins, [^{35}S] methionine (Amersham, 20µCi) was added and the cells were incubated at 27°C for 20 min. Pelleted cells were washed once (1X PBS+0.5%Glucose) then resuspended in Laemmli sample buffer and subjected to SDS-PAGE. The gel was fixed in 10% acetic acid, 30% methanol solution in water for 45 min, stained with Coomassie followed by de-staining in water. The gel was then incubated for 45 min in En3Hance™ (Amersham), washed in water for another 45mins, dried and exposed for autoradiography.

II.10 List of plasmids plasmids prepared for this study

Table II.10: Table showing the details of the plasmids prepared for this study

Plasmid no.	Description (*T.brucei*)	Backgr-ound vector	Primers used for cloning	Resistance marker
pHD 2166	in situ V5-tagged PBP1	Bla V5	cz3839 ; cz3842	Blastidine
pHD 2285	PBP1_Myc	pHD 1700	cz3936; cz3937	Hygromycin
pHD 2326	MKT1_TAP	pHD 918	cz4507; cz4508	Hygromycin
pHD 2165	PBP1 P2T7	TA blue	cz3856,cz3857	Hygromycin
pHD 2287	PBP1_stem loop RNAi	pHD 1146	cz3876,cz3877	Hygromycin
pHD 2286	MKT1_Stem loop RNAI	pHD 1146	cz3878,cz3879	Hygromycin

Table II.10 continued……

Plasmid no	Description	Primers used for cloning	Resistance marker
pHD2246	pGBKT7 + MKT1	cz4086; cz4087	Kanamycin
pHD2250	pGBKT7 + ZC3H11	cz4068; cz4069	Kanamycin
pHD2252	pGADT7 + PBP1	cz4072; cz4076	Ampicilin
pHD2254	pGADT7 + ZC3H11	cz4068; cz4074	Ampicilin
pHD2255	pGADT7 + MKT1	cz4086; cz4087	Ampicilin
pHD2321	pGADT7 + ZC3H11 1-128	cz4068; cz4524	Ampicilin
pHD2322	pGADT7 + ZC3H11 101-364	cz4525; cz4069	Ampicilin
pHD2323	pGADT7 + ZC3H11-mutated zinc finger (C70S) (amplified from pHD2219)	cz4068; cz4069	Ampicilin
pHD2381	pGBKT7 + LSM12	cz4743; cz4742	Kanamycin
pHD2442	pGADT7 + ZC3H11 101-301	cz4525; cz4649	Ampicilin
pHD2325	pGADT7 + ZC3H11 101-265	cz4525; cz4648	Ampicilin
pHD2324	pGADT7 + ZC3H11 101-202	cz4525; cz4647	Ampicilin
pHD2443	pGADT7 + ZC3H11 101-195	cz4525; cz4677	Ampicilin
pHD2405	pGBKT7 + ZC3H11 186-364	cz4855; cz4857	Kanamycin
pHD2404	pGBKT7 + ZC3H11 200-364	cz4856; cz4857	Kanamycin
pHD2399	pGBKT7 + MKT1 1-494	cz4060; cz4583	Kanamycin
pHD2398	pGBKT7 + MKT1 232-735	cz4749; cz4658	Kanamycin
pHD2402	pGBKT7 + PABP1	cz4735; cz4736	Kanamycin
pHD2403	pGBKT7 + PABP2	cz4756; cz4757	Kanamycin
pHD2429	pGBKT7 + CFB1	cz4991;cz4992	Kanamycin
pHD2428	pGADT7 + CFB1	cz4991;cz4992	Kanamycin
pHD2408	pGBKT7 +ScMkt1p	cz4866; cz4867	Kanamycin
pHD2401	pGADT7 + CFB1#PRRFRHDPY	cz4737; cz4738	Ampicilin
pHD2441	pGADT7 + ZC3H11 101-364(PY to AA)	cz4525; cz4069	Ampicilin

II.11. List of oligos prepared for this study

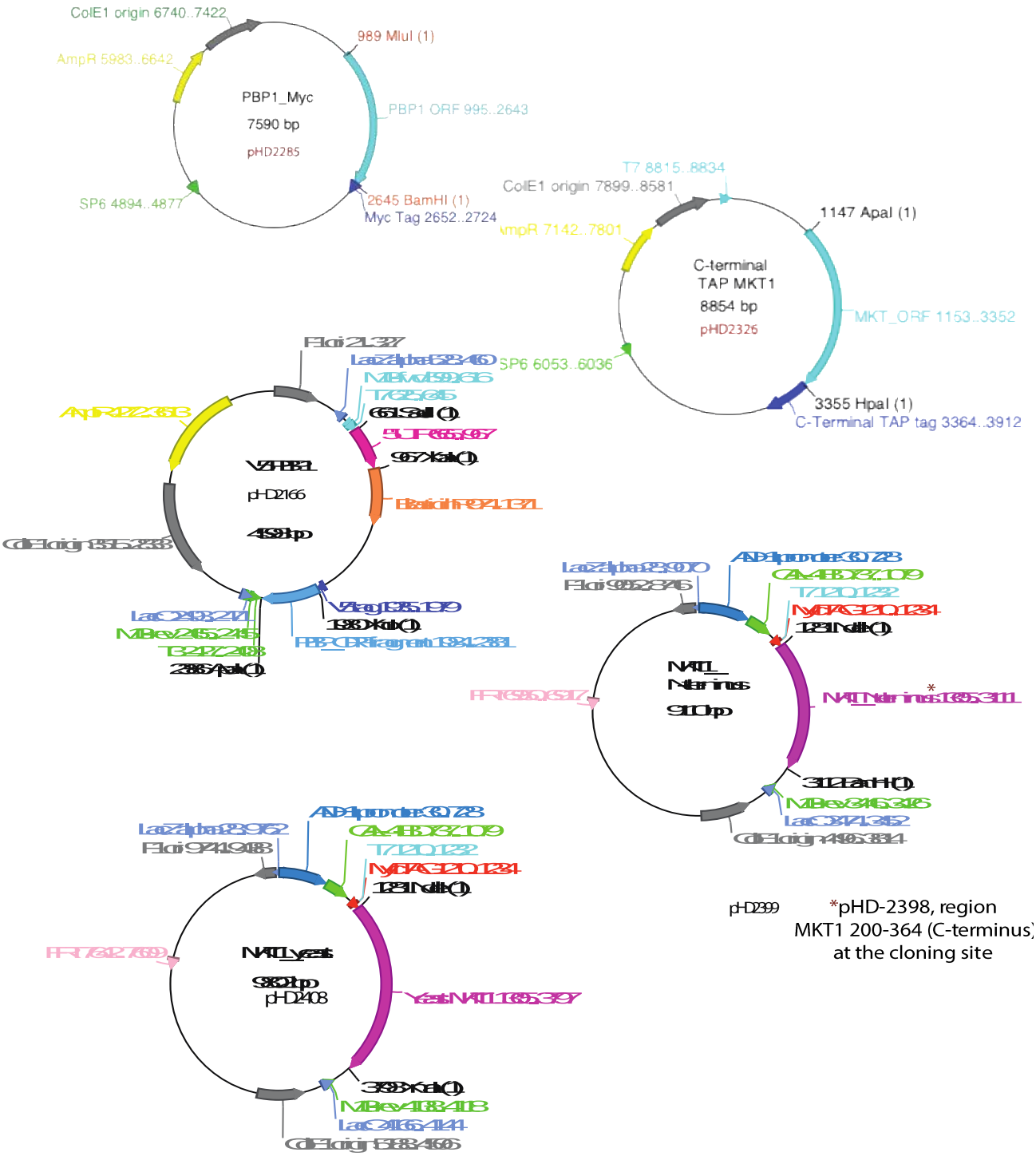
Table II.11: Table showing the details of the oligos prepared for this study.

oligo no.	Description	Restriction	Sequence
cz4072	PBP1 ORF FW	EcoRI	acgaattc**ATG**GAGGGGTCTC
cz4073	PBP1 ORF RV	BamHI	tgggatccATTTCCCAACTCG
cz4076	PBP1 ORF RV	BamHI	aaggatccCTATTTCCCAACTCG
cz4649	(ZC3H11_200 aa)	XhoI	cgCTCGAGACCACCGGTGTTGTTG
cz4648	(ZC3H11_150 aa)	XhoI	cgCTCGAGCTCTTCATTGGCATAC
cz4647	(ZC3H11_100 aa)	XhoI	cgCTCGAGGTTATGGCAGTAGGGA
cz4856	(F_ZC3H11_NoHNPY)	EcoR1	CGgaattcGGATCCATTCGGATTCGTG
cz4855	(F_ZC3H11_+HNPY)	EcoR1	CGgaattcTGCCATAACATTCTTCCAAC
cz4857	(R_ZC3H11_Sal1)	Sal1	CGgtcgacTCACAAGGAAAGAAACATATGC
cz4086	MKT1 (N-terminus_F)	Nde1	aacatatgATGTACCCCCGACACG
cz4749	MKT1 (C-terminus_F)	Nde1	TGcatatgCAGCTCGTTTGAGTGAAGA
cz4735	(PABP1_FWD)	EcoR1	TCgaattcATGACAATCGCTGCACAGGGGGT
cz4736	(PABP1_Rev)	BamH1	GCggatccTTAAGCGCTTGAGGCGTGTACC
cz4756	(PABP2_FWD)	EcoR1	AAcatatgATGGCTGCATTTGCTGCTGCGA
cz4757	(PABP2_Rev)	BamH1	CGggatccCTACATGCCAATGTGACGGTTGAGC

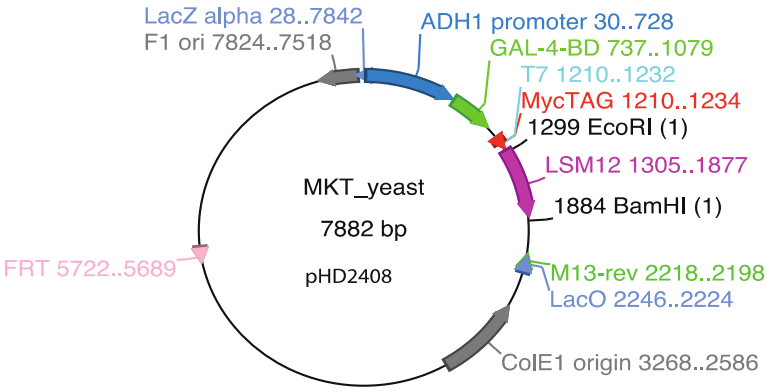
Table II.11 continued…

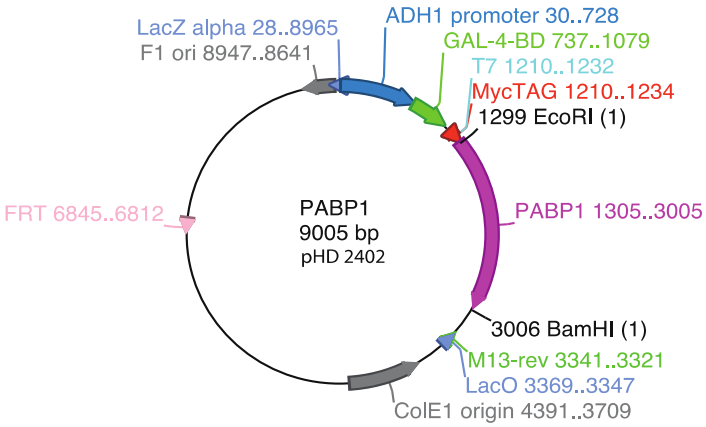
Oligo no.	Description	Restriction	Sequence
cz4991	CFB1-F	EcoR1	GAG GAA TTC ATG TTT TTG AAG GAG GGA
cz4992	CFB1-R	BamH1	ATA GGA TCC CTA AGA TGC CGC TAG TAC G
cz4737	(CFB1D_FRAGMENT_FWD)		aattcGGTGGAGGTCCTAGGCGGTTCCGGCACGACCCGTACc
cz4738	(CFB1D_FRAGMENT_REV)		tcgagGTACGGGTCGTGCCGGAACCGCCTAGGACCTCCACCg
cz4866	Mkt yeast_fwd	Nde1	gcCATATG ATGGCAATCAAGTCATTGGA
cz4867	Mkt yeast_rev	Xma1	CGcccgggTCAGCTAGATAGAGCTGTTGTAGTAA
cz4086	MKT1 ORF FW	NdeI	aacatatgATGTACCCCCGACACG
cz4087	MKT1 ORF RV	BamHI	cgggatccGGATCCTCAATAATATGTTTCTCGC
cz3839	PBP1 5'UTR fragment FW	SacII	gaccgcggCCTCGTAACTAAACTGTC
cz3840	PBP1 5'UTR fragment RV	XbaI	gactctagaACTTTTGATGTTGCCGTC
cz3841	PBP1 ORF fragment FW	XhoI	gacctcgagGAGGGGTCTCATAGCAG
cz3842	PBP1 ORF fragment RV	ApaI	gacgggcccACCAGTCTGATGAGGTTC

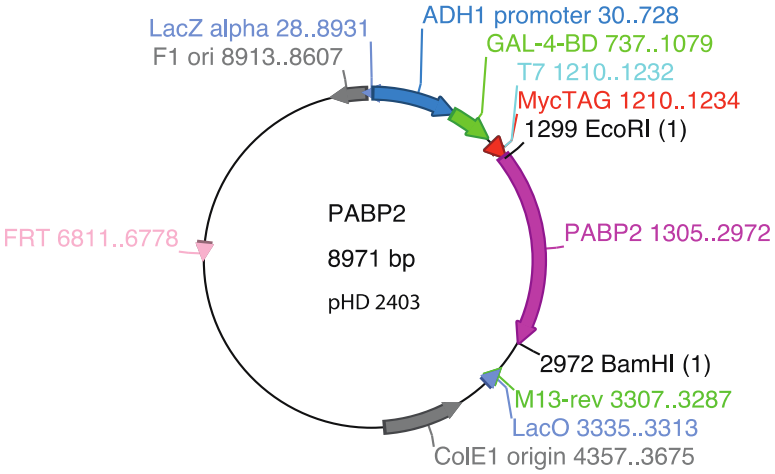
II.12. Vector Maps:

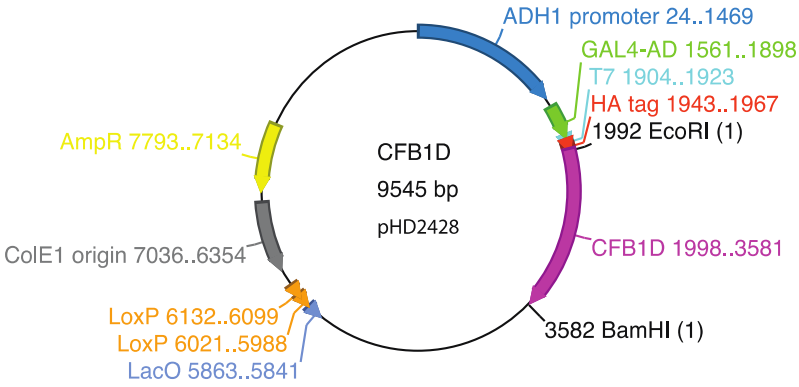


II.12 continued……



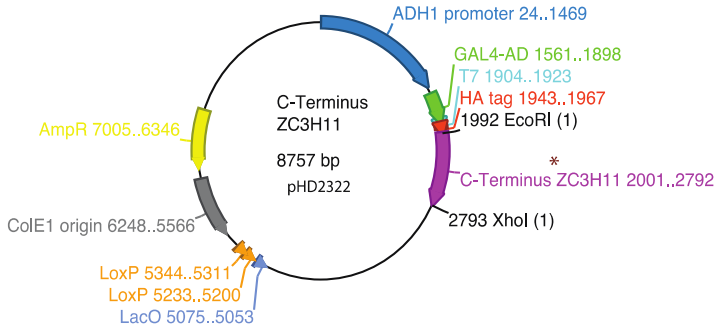






II.12 continued.....

II.12 continued.....

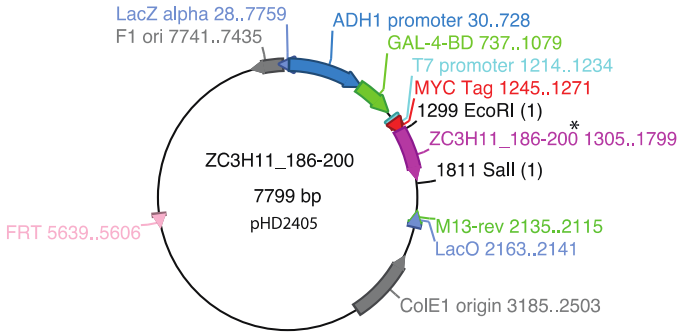


* pHD-2442, region ZC3H11 101-301 at the cloning site

pHD-2325, region ZC3H11 101-265 at the cloning site

pHD-2324, region ZC3H11 101-202 at the cloning site

pHD-2443, region ZC3H11 101-195 at the cloning site



* pHD-2404, region ZC3H11 200-364 at the cloning site

II.13.Web resources:

Kinetoplastid databases:

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

Selection of sequence (fragments) for RNAi:
http://trypanofan.path.cam.ac.uk/software/RNAit.html

NCBI databases:

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

Representative PUF domain sequences:

RCSB Protein data bank	http://www.rcsb.org/pdb/home/home.do

Protein alignment:
CLUSTALW
http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html

Generating printable alignment file:
MegAlign DNA Star

III RESULTS:

III.1. Sequence study of MKT1 and PBP1

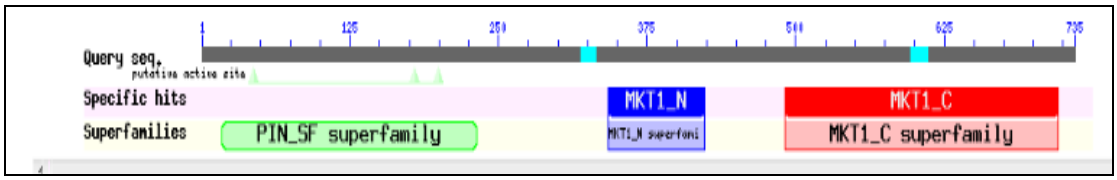
MKT1 *(Tb* 927.6.4770) and PBP1 (*Tb* 927.8.4540) were first identified in TAP purification of ZC3H11 [25]. The BLAST analysis and sequence studies indicate that they are most likely the homologues of yeast Mkt1p and Pbp1p [91]. While MKT1 is well conserved in Kinetoplastids and yeast [91], homologues of PBP1 are also found in humans and have been studied well [95, 111].

The sequence study of MKT1 revealed that MKT1 contains a characteristic PIN domain, which is known to impart nuclease activity to proteins (Fig III.1.1.A). A typical PIN domain contains three nearly invariant acidic residues. The crystal structures show these residues clustered together in the putative active site [113]. An important candidate of protein containing PIN domain is FEN1 [114], the 5'-3' exonuclease of DNA polymerase I. This 5' nucleases is involved in DNA replication, repair, and recombination. It is capable of both 5'-3' exonucleolytic activity and cleaving bifurcated DNA, in an endonucleolytic, structure-specific manner.

The PIN domain, which is at the N-terminus, is well conserved in the various MKT1 homologues, as is the overall protein organization. Indeed, the homologues in mushrooms and other fungi are closer to the trypanosome sequence. However, the residues that impart nuclease activity to FEN1 and other FEN1 like nucleases [114, 115] are absent in all MKT1 homologues considered here (Green dots in Fig III.1.1.B). Thus, retaining a nuclease activity looks quite improbable for these MKT1 homologues. Also, the standard laboratory yeast strain S288C has a G>D mutation in the sequence of Mkt1p that was reported to result in low recovery of yeast cells after stress [81] (Blue arrow in Fig III.1.1.B); however this critical residue (G) is retained in all the other homologues considered here.

Another important residue that has been reported to be responsible for both Fen1 endonuclease activity [114, 115] and Mkt1p effects on *HO* translation [91] is well conserved in trypanosome (Red arrow in Fig III.1.1.B).

A



B

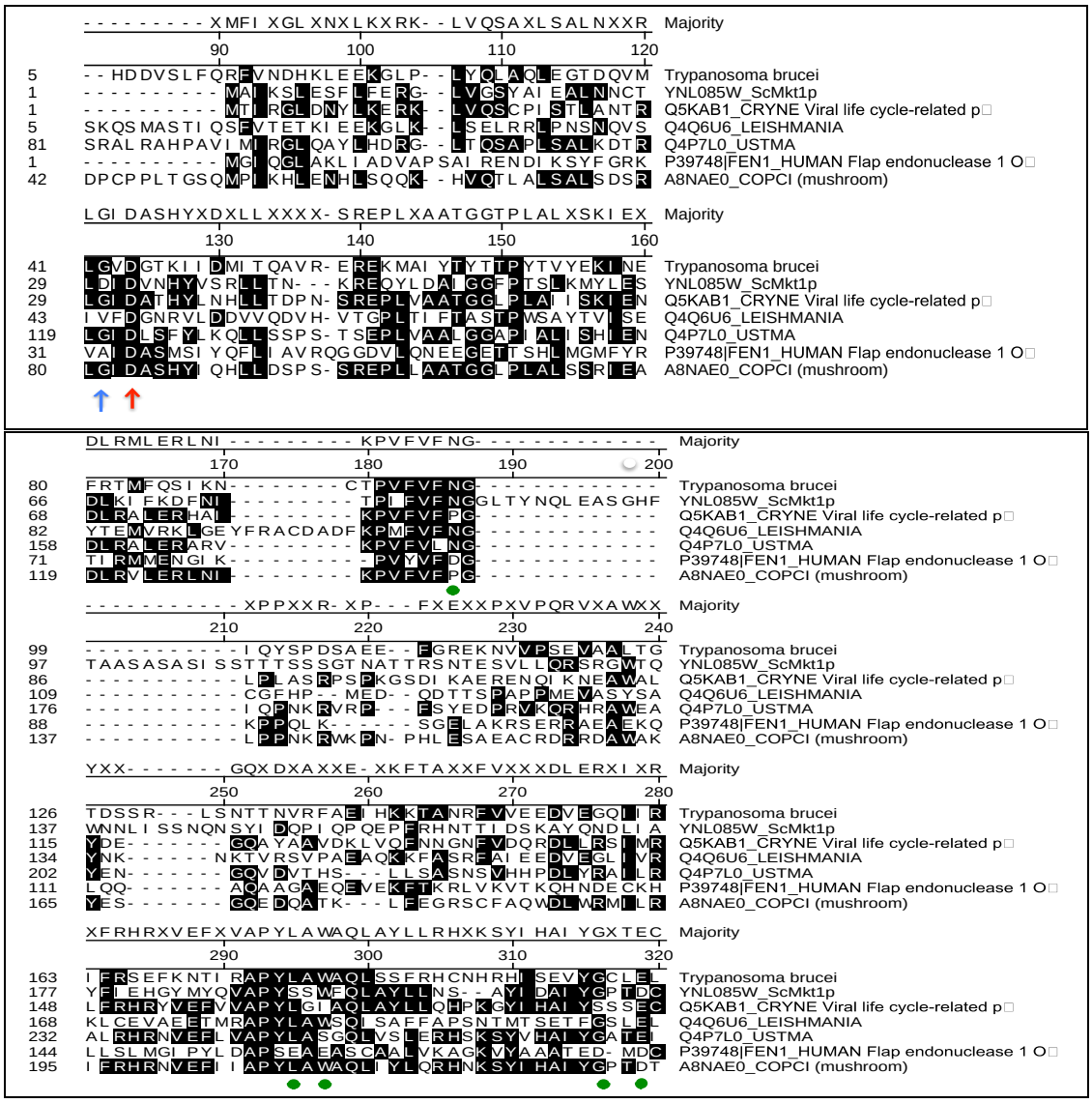
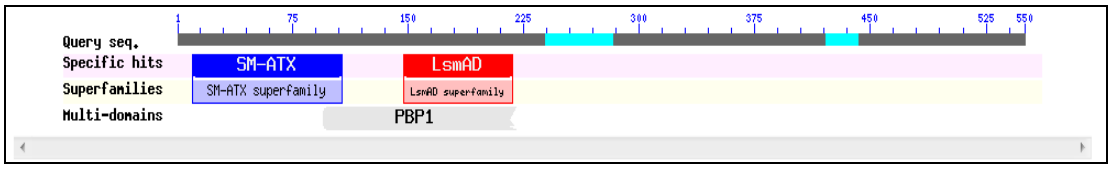


Fig III.1.1: A) The conserved PIN domain in the sequence of MKT1 as shown after CD search (NCBI) B) MKT1 is well conserved in lower eukaryotes. Alignment of MKT1 sequence from different organism: *Saccharomyces cerevisiae*, viral life cycle-related protein-*Cryptococcus neoformans*: CRYNE, Leishmania, *Ustilago maydis* : USTMA, Human flap endonuclease, and *Coprinopsis cinerea*: COPCI. The sequences were aligned by 'MegAlign software' using Clustal W algorithm. Shaded residues (with solid black), match the Consensus exactly.

Further, the sequence study also revealed that PBP1 is a well-conserved protein in eukaryotes. It contains an LSmAD (**LSm A**ssociated **D**omain) domain at the N-terminal end, which its mammalian homologue, Ataxin2 also possesses (Fig III.1.2A). LSm domain is present in proteins, which have role in RNA processing, mRNA degradation or decay [116]. The C-terminus is less well conserved in yeast and trypanosomes, less than 20% identity was found, usually with matching of isolated residues (Fig III.1.2).

A



B

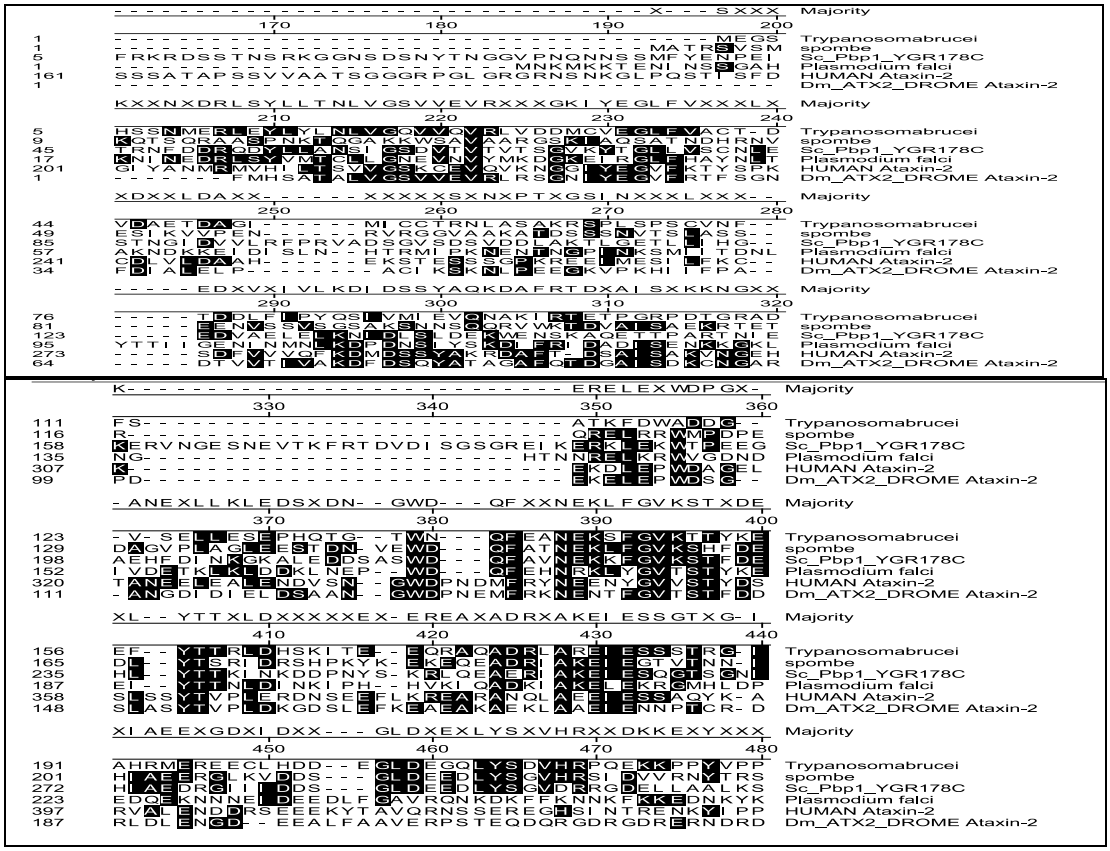


Fig III.1.2: A) The conserved LSmAD domain in the sequence of PBP1 as shown after CD search (NCBI). B) PBP1 is well conserved in lower as well as higher eukaryotes. Alignment of PBP1 sequence from different organism: *S.pombe*, *Saccharomyces cerevisae*, *Plasmodium falciparum*, *Homo sapiens* and *Drosophila melanogaster*. The sequences were aligned by 'MegAlign software' using Clustal W algorithm. Shaded residues (with solid black), match the consensus exactly.

III.2. Expression of PBP1 and MKT1 proteins in BSF and PCF of trypanosomes

The C-terminal Myc tagged overexpression version of MKT1 and the N-Terminal *in-situ* V5 tagged versions of MKT1 (pHD 1983 and pHD 1973 respectively) were made by Dorothea Droll and I used the same cell lines.

To detect the expression PBP1 in BSF and PCF of trypanosomes, one allele was *in-situ* V5 tagged in the endogenous genomic locus yielding pHD 2166. The V5 tag is present at the N-terminal end of the protein. Western blot analysis of the tagged protein confirmed the constitutive expression of V5-PBP1 in BS forms of trypanosomes (Fig III.2. A). Also, an RNA polymerase I promoter driven, ectopically overexpressing, tertracycline inducible cell line with the C-terminal Myc tagged PBP1 was prepared (pHD 2285) and checked on western blot (Fig III.2.B).

1313 wild type cells (without any tagged protein expression) served as the negative control (Fig III.2.A) and expression of aldolase as detected by anti aldolase antibody was used as loading control (Fig III.2.B) for the western blot.

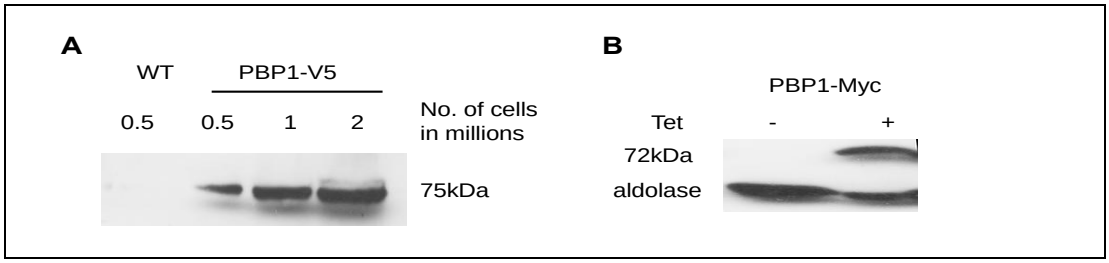


Fig III.2: Detection of protein expression of V5 tagged PBP1 and Myc tagged MKT1. 1313 wild type cells (without any tagged protein expression) served as the negative control (Fig A); and expression of aldolase as detected by anti aldolase antibody was used as loading control (Fig B) for the western blot.

III.3. Both MKT1 and PBP1 are essential for the survival of parasite

While Dorothea identified the lethal RNAi effect of MKT1 in BSF of trypanosomes using P2T7 vector, I verified the effect again by constructing an RNAi vector for MKT1 using the stem loop strategy, which is controlled by RNA Pol I promoter (Fig III.3.B). In this strategy, the same protein fragment (MKT1 or PBP1 described later) fragment is cloned twice (in opposite orientations) on either side of a stuffer sequence. RNAi by stem loop vectors is apparently more regulated and efficient than by the opposing T7 RNA polymerase promoter containing vectors [117].The knockdown of MKT1 in PCF showed severe growth defect for

the survival of the parasite. This was also previously observed using P2T7 RNAi strategy by Dorothea and again verified by me using the stem loop strategy (Fig III.3.B).

I also studied the RNAi knockdown effect on PBP1 using the stem-loop strategy. Both the bloodstream stage and procyclic stage displayed a clear defect in proliferation already after 24 hour of induction of RNAi, followed by cell death (Fig III.3.A).

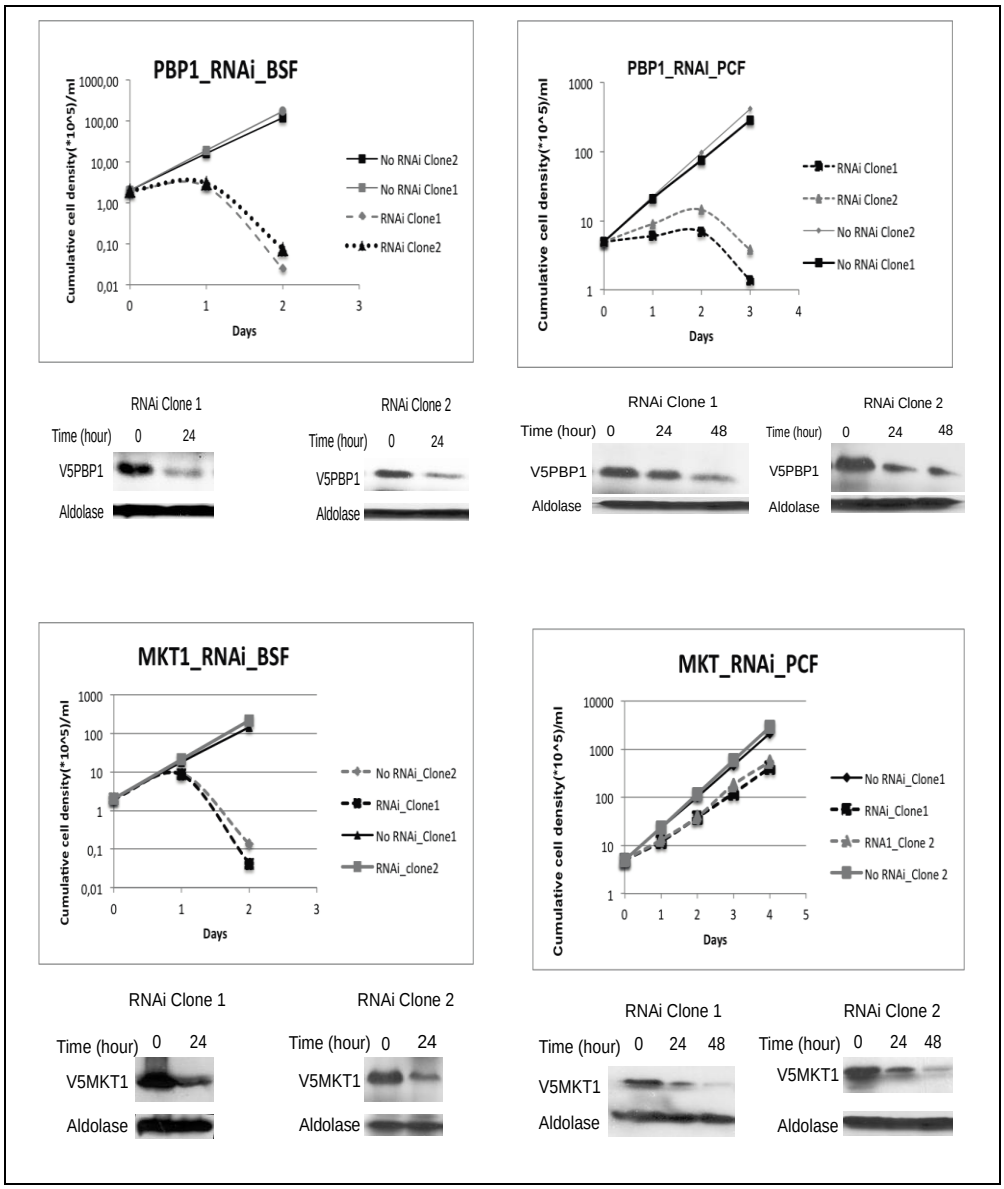


Fig III.3: RNAi knockdown of PBP1 (Fig A); and MKT1 (Fig B) by stem loop strategy, in both Procyclic and Bloodstream form. Knockdown is evident by decreasing protein levels after RNAi is induced by tetracycline on the western blot. Level of aldolase served as negative control

III.4. Interaction and localization of MKT and PBP1:

Previously in the lab the TAP purification of ZC3H11 done by Dorothea, had pulled down MKT1 and PBP1 as the probable interacting partners of ZC3H11. In order to further validate if MKT1 and PBP1 interact with each other *in vivo* like its yeast homologue [91], I did a co-immunoprecipitation analysis using cellines harbouring Myc tagged version of MKT1 and V5 tagged version of PBP1. Thus, when I immnoprecipitated Myc MKT1 from the cell extract with an Anti Myc Agarose beads, V5 PBP1 was coimmunoprecipitated as detected by anti V5 antibody in western blot analysis. The cell extract bearing no Myc tag served as a negative control here, as the probing with anti Myc antibody did not reveal anything and very little V5-PBP1 was seen from this cell extract. This result shows that as reported in yeast the *T. brucei* homologue of PBP1 and MKT1 also interact with each other (Fig III.4.1).

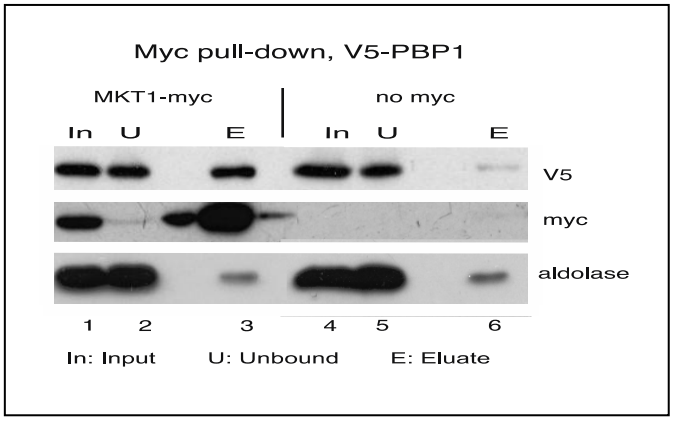


Fig III.4.1: Interaction of PBP1 with MKT1 as detected by co-immunoprecipitation on western blot. Level of aldolase served as a negative control. $5x10^7$ cells were used in total and an equivalent of 5×10^6 cells were collected as input and flowthrough from the lysate before and after binding to the beads.

Further, the immunofluorescence analysis with the Myc tagged version of PBP1 (Fig III.4.2.A) and MKT1 (Fig III.4.2.B) in procyclic form showed that both the proteins localize in the cytosol. The C-terminally Myc-tagged proteins were expressed from a tetracycline-inducible RNA polymerase I promoter, which may result in over-expression. A cytosolic marker Trypanothione reductase was used as a marker for cytosol. These images validate the cytosolic localization of both MKT1 and PBP1 very clearly.

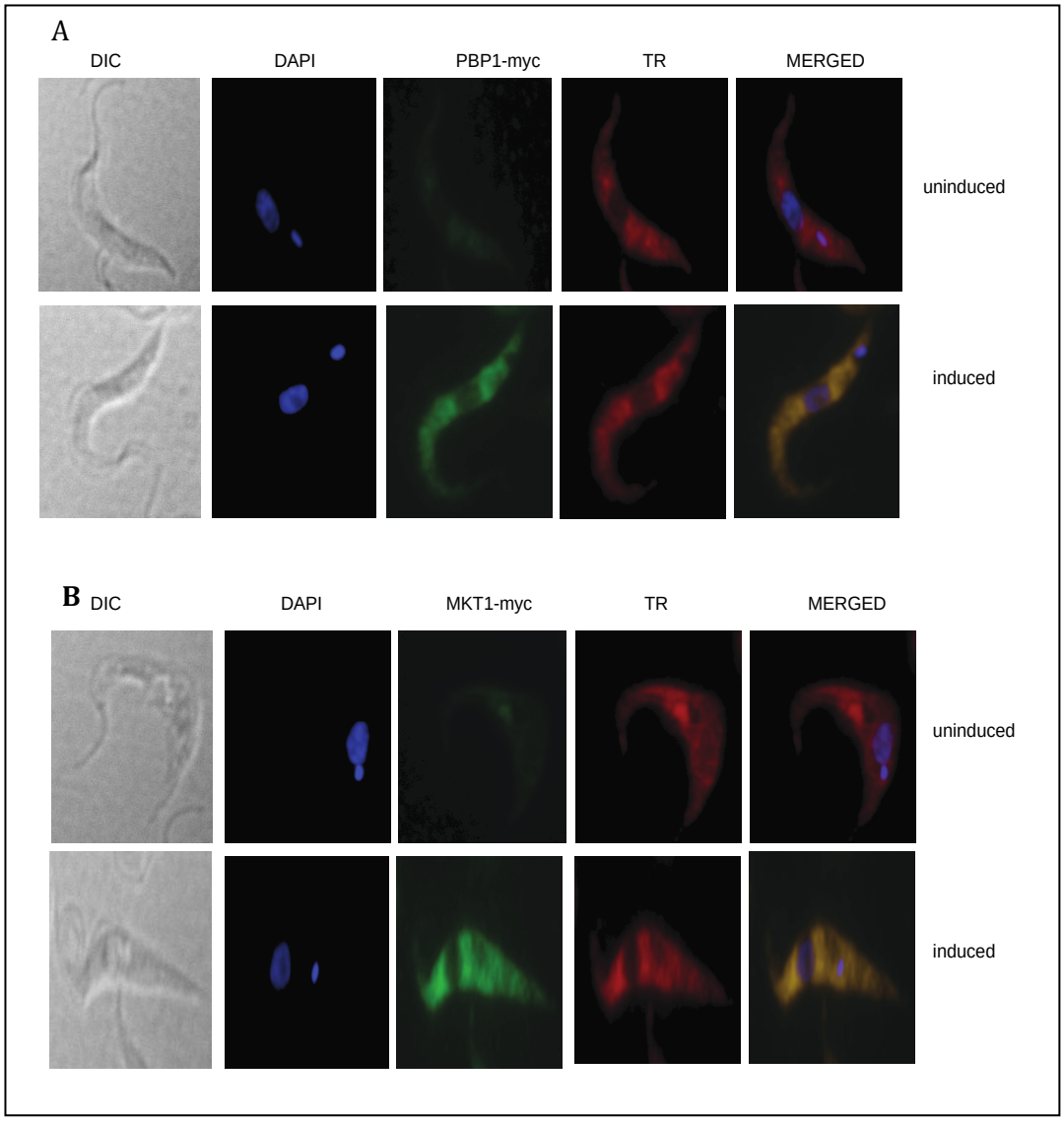


Fig III.4.2: Localization of Myc tagged PBP1 (Fig A) and MKT1 (Fig B) in cytoplasm with and without tetracycline induction. Trypanothione reductase enzyme was used as cytosolic marker.

III.5. MKT1 depletion seems to inhibit global translation:

In order to characterize the role of MKT1 in *T.brucei*, I wanted to address the question if the regulation by MKT1 protein is global or specific. For this I depleted MKT1 by RNAi knockdown for 24 hrs in bloodstream form of *T.brucei*. The western blot clearly revealed depleted level of V5-tagged MKT1 after 24hrs of RNAi knockdown (Fig III.5.1.A). The cells were then pulsed with [^{35}S]-Methionine for 20 min. Proteins were separated by SDS-PAGE, and then stained with Coomassie. The gel was dried and [^{35}S] was detected by autoradiography. The results revealed that the knock down of MKT1 by RNAi triggers a global translation arrest in bloodstream form (BSF) of *T.brucei* (Fig III.5.1.B). The initial RNAi knockdown experiment was conducted by RNAi driven by Bacteriophage T7 promoter (P2T7) (not shown). As the RNAi using P2T7 promoter is very unstable and leaky, a more stable stem-loop RNAi construct (driven by RNA Polymerase I) was designed for MKT1 and the arrest in global translation was again validated. But, since the arrest in translation coincides with cell death, it is difficult to say if the arrest in translation is a primary effect of MKT1 knockdown or it is because result of impaired growth of dying cells.

Arguing that MKT1 is involved in regulating translation, I also checked the polyribosome profile of MKT1 under normal condition and compared it with the cells where MKT1 was knocked down by RNAi. Had it been the case that MKT1 inhibits the initiation of translation, I expect to see a profile with destroyed polyribosomal peaks. But the polyribosome profile was similar for cells with or without knockdown of MKT1 by RNAi (Fig III.5.1.C). This experiment was repeated 2 more times and in all cases the polyribosomal peaks were comparable to those of cells without MKT1 knockdown. This could happen if MKT1 freezes the mRNA on polyribosome and halts the translation at a post initiation phase. Taking into account that knockdown of MKT1 proves lethal for the parasite and looking at these result in an unbiased way, one cannot be sure that the polyribosome profile obtained from the cells with MKT1 knockdown is conclusive (like if it is because of MKT1 regulation of global translation operating at a post-initiation phase) or it's from the cells which has escaped the RNAi or where RNAi isn't very efficient.

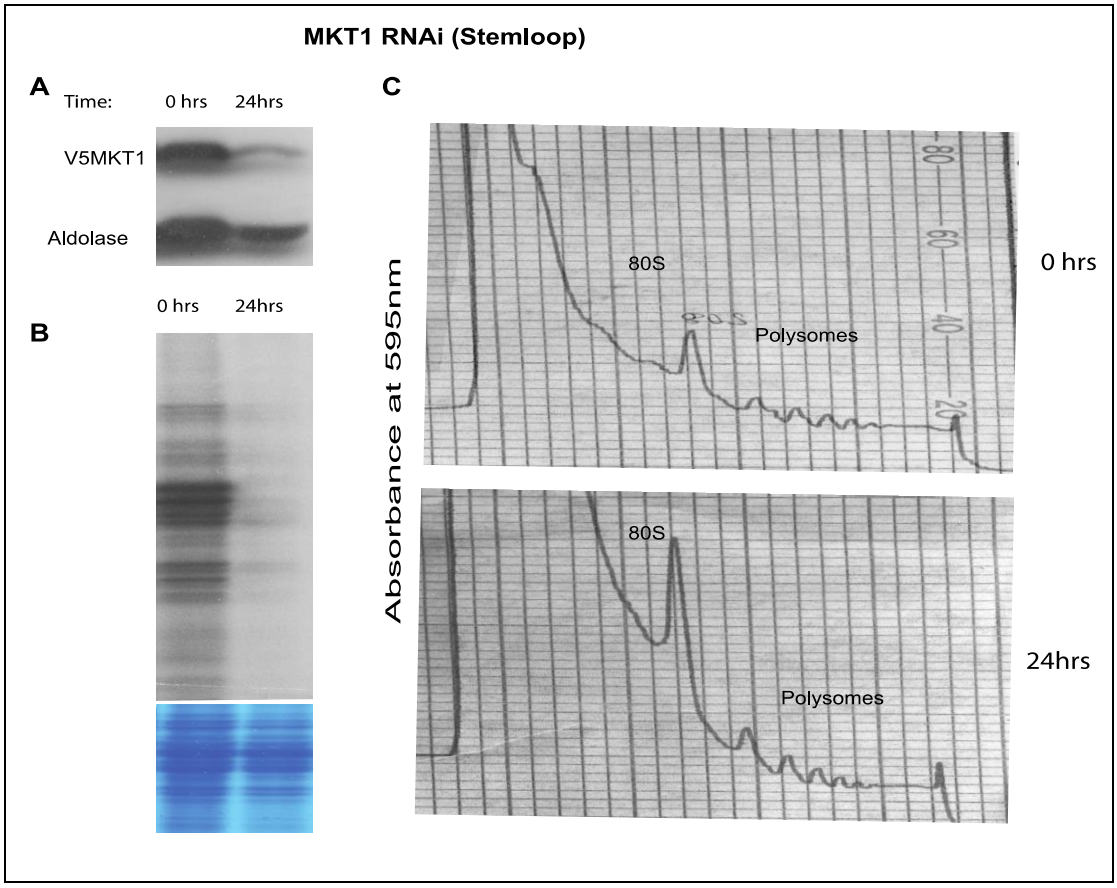


Fig III.5.1: Effect of MKT1 knockdown by RNAi in the bloodstream form. A) Cells showing the depleted level of V5MKT1 after 24hrs of tet induction on a western blot. Aldolase level served as a loading control. B) Metabolically labeled cell lines with and without MKT1 knockdown. Coommassie staining served as a loading control. C) Polysome profile of MKT1 depleted cells after 0 and 24 hrs of RNAi induction.

Thus, in order to probe a little more into this, I compared the translation arrest of MKT1 with ZC3H11 by metabolic labeling, in bloodstream form, because ZC3H11 also shows a growth arrest in 2 days and hasn't been reported to arrest global translation. For this too, I depleted MKT1 or ZC3H11 (in two different cell lines) by RNAi knockdown for 24 hrs, in bloodstream form of *T.brucei*. The cells were then pulsed with [^{35}S]-Methionine for 20 min. Proteins were separated by SDS-PAGE, and then stained with Coomassie. The gel was dried and [^{35}S] was detected by autoradiography. As a result I found that the translational arrest pattern for both ZC3H11 and MKT1 were almost similar (Fig III.5.2). This was again seen at least two more times which got me back to the issue where it was getting cumbersome to identify if the effect in translation are because of MKT1 knockdown or it was a secondary effect due to dying cells or cells with impaired RNAi. This issue was left unresolved at this point because parallelly some other promising results were obtained during the characterization of MKT1 using yeast two hybrid analysis (discussed later).

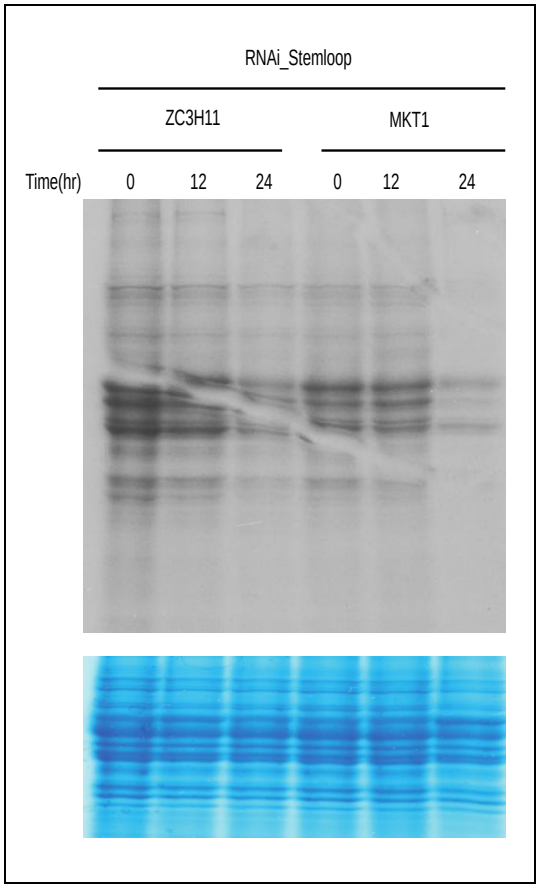


Fig III.5.2: Figure showing metabolically labeled BSF cells after ZC3H11 or MKT1 was knocked down by RNAi. The de novo synthesis of proteins goes down after 24 hrs of RNAi induction, which also overlaps with the time when the cell dies after either of these essential proteins is knocked down in BSF. Coomassie stained gel served as a loading control here.

III.6. RNAi of MKT1 interaction partner ZC3H11, in procyclic form, showed remarkable decrease in the levels of heat shock specific proteins, when subjected to heat shock

Through metabolic labeling with [35S]-methionine, I could show that ZC3H11 depleted cell line has remarkably decreased synthesis of heat shock proteins (Hsp70 and Hsp83), when subjected to heat shock [25]. Thus, this result verifies the finding that ZC3H11 is required for the stress response in *T. brucei*. As previously described [118], a one-hour 41°C heat shock reproducibly decreased *de novo* synthesis of many proteins, as judged by [^{35}S]-methionine labelling (Fig III.6); among those spared were two migrating at about 90 kDa and 70 kDa, which are probably HSP83 and HSP70. This result was similar to that previously seen for insect-stage *Leishmania* [119, 120].

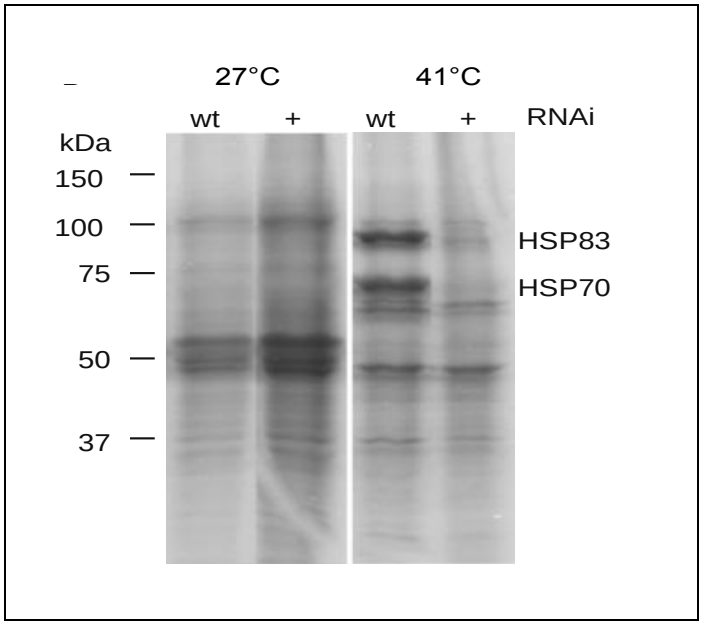


Fig III.6: Figure showing the de novo synthesis of global proteins by metabolic labeling under normal condition and after heat shock (41°C for 1 hrs).

III.7. MKT1 and PBP1 are associated with polysomes

To determine if MKT1-Myc and PBP1-Myc co-sediment with the translation polyribosomes, I individually assayed the two proteins by fractionating cytoplasmic extracts on sucrose gradients and analyzed the fraction by Western Blot. As a result, I could see that both MKT1 (Fig III.7.A) and PBP1 (Fig III.7.C) were found to be associated with the polyribosome. This is because they were detected in the high density fractions of the sucrose gradient which contains the translating polyribosomes. While the ribosomal protein S9 that is abundant in the ribosomal fraction was used as a marker to identify the heavy polyribosomal fractions, a cytoplasmic protein Trypanothione Reductase (TRYR) was used to determine the lighter gradients containing the individual ribosomal subunits and monosomes. Since the Trypanothione Reductase enzyme is found in great abundance in the cells and it appears to seep through the gradient, it was being detected in all the fractions. The profile was obtained using the bloodstream form of the parasite. RNaseA treatment, which destroys the polyribosome, was used as a control here, and a shift in the sedimentation pattern of MKT1 (Fig III.7.B) and PBP1 (Fig III.7.D) from high density to the less density fractions of the sucrose gradient was observed. This suggests that the pattern is RNaseA sensitive, and therefore the MKT1 and PBP1 are probably associated with the polyribosomes.

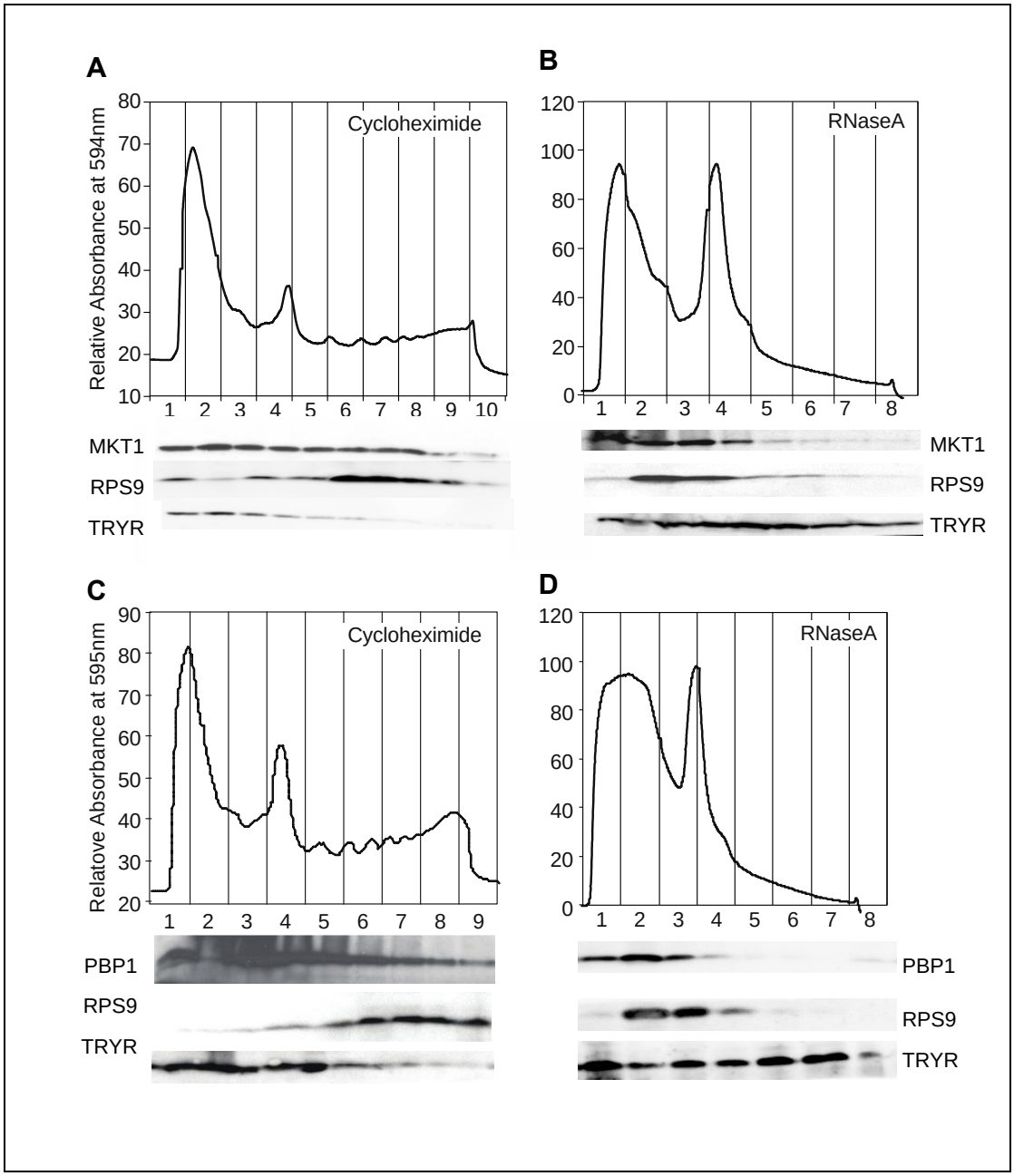


Fig III.7: Polyribosome profile of Myc tagged MKT1 and PBP1 when the mRNA was freezed on the polyribosome during cell harvest by Cycloheximide (Fig A and Fig C respectively). Profile of Myc MKT1 and Myc PBP1 obtained after the polyribosomes were destroyed by RNaseA (Fig B and Fig D respectively). Ribosomal protein S9 acted as a control for fractions containing ribosomes. Cytoplasmic protein Trypanothione reductase (TRYR) was used as a control to detect the cytoplasmic fractions.

III.8. PBP1 and MKT1 are found in stress granules after starvation but not after heat shock:

It has been previously reported for homologues of PBP1 in yeast and Ataxin2 in mammals that they are associated with the stress granules after heat shock stress [99, 113]. In this regard, I tried to see the cellular localization of PBP1 after heat shock stress using procyclic cells. The stress granule marker SCD6 and DHH1 were used as a marker to check if PBP1 co-localizes to them. As a result, while after heat shock, the heat shock granules form containing SCD6, the signal for PBP1 remained in the cytosol like it was under normal condition. The same condition was used using DHH1 as a stress granule marker but again while after heat shock the cells form heat shock granules containing DHH1, PBP1 didn't show any co-localization with DHH1 (Fig III.8.2).

Similar results were seen for MKT1 as well wherein MKT1 did not co-localize with heat shock stress granules containing SCD6 when subjected to heat shock (Fig III.8.1.B).

I further tried to give starvation stress to cells [41]. The starvation stress did lead to granule formation, which not only contained the stress granule marker SCD6, but also PBP1. In other words, in contrast to the normal condition, PBP1 did relocate itself to stress granules when the cells were starved for 3hrs in 1X PBS (Fig III.8.1.A).

When I repeated the same experiment to test for MKT1 localization, I saw that after starvation stress, MKT1 also had granular localization some of which also contained the stress granule marker SCD6. But, the co-localization was not 100 percent for MKT1 as compared to the case of PBP1 and SCD6 (Fig III.8.1.B). A similar observation has also been reported for PABP1 [11] where PABP1 and other translation initiation factors such as eIF4E2 and eIF4E3 were found to be largely but not totally absent from the SCD6 containing nutrient stress granules.

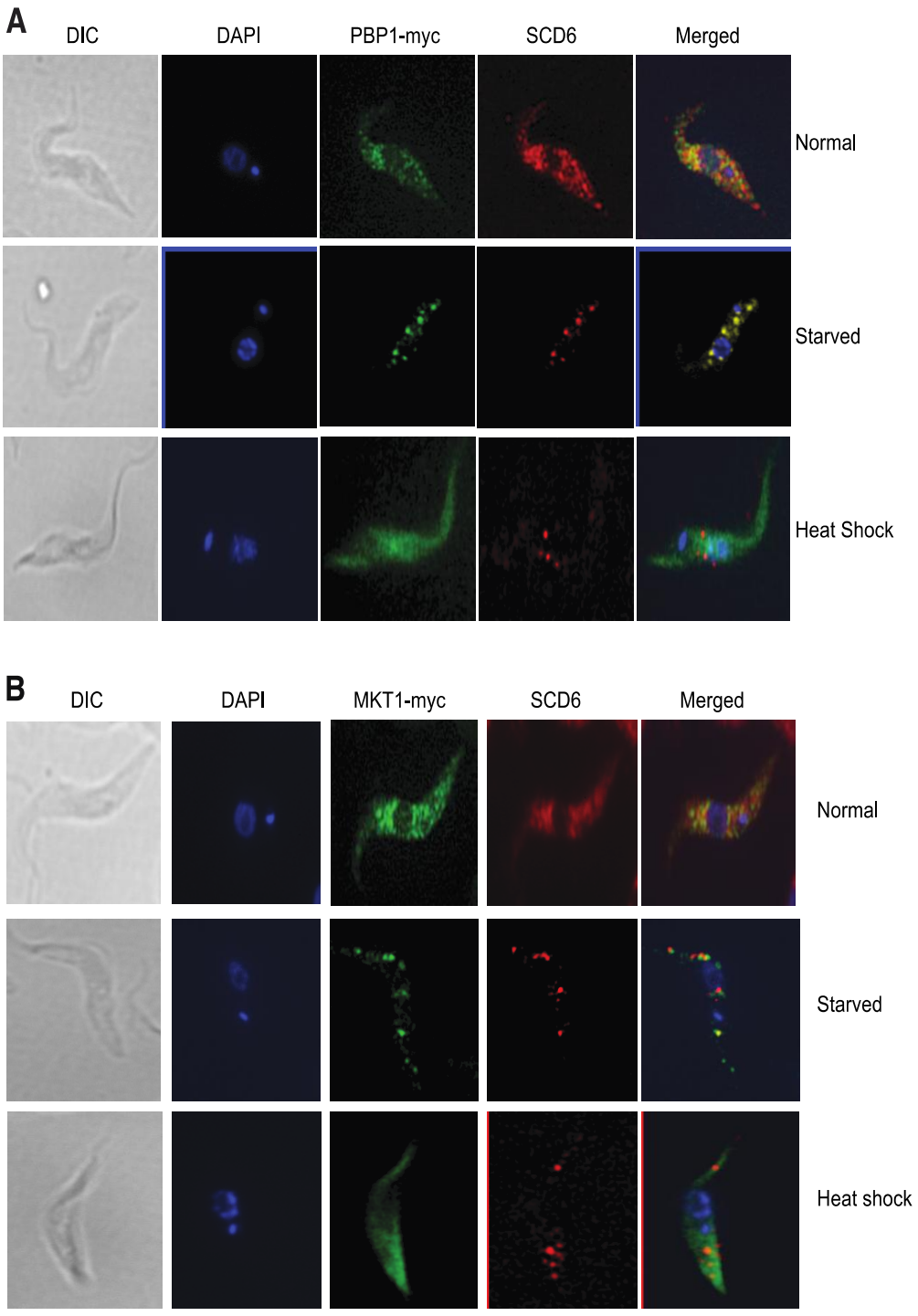


Fig III.8.1: Figure showing localisation of PBP1(A) and MKT1(B) under normal condition (27°C) , after heat shock treatment (41°C for 1 hrs), and after stravtion stress (cells subjected in 1X PBS for 3 hrs at 27°C). The stress granule marker SCD6 was used as marker here.

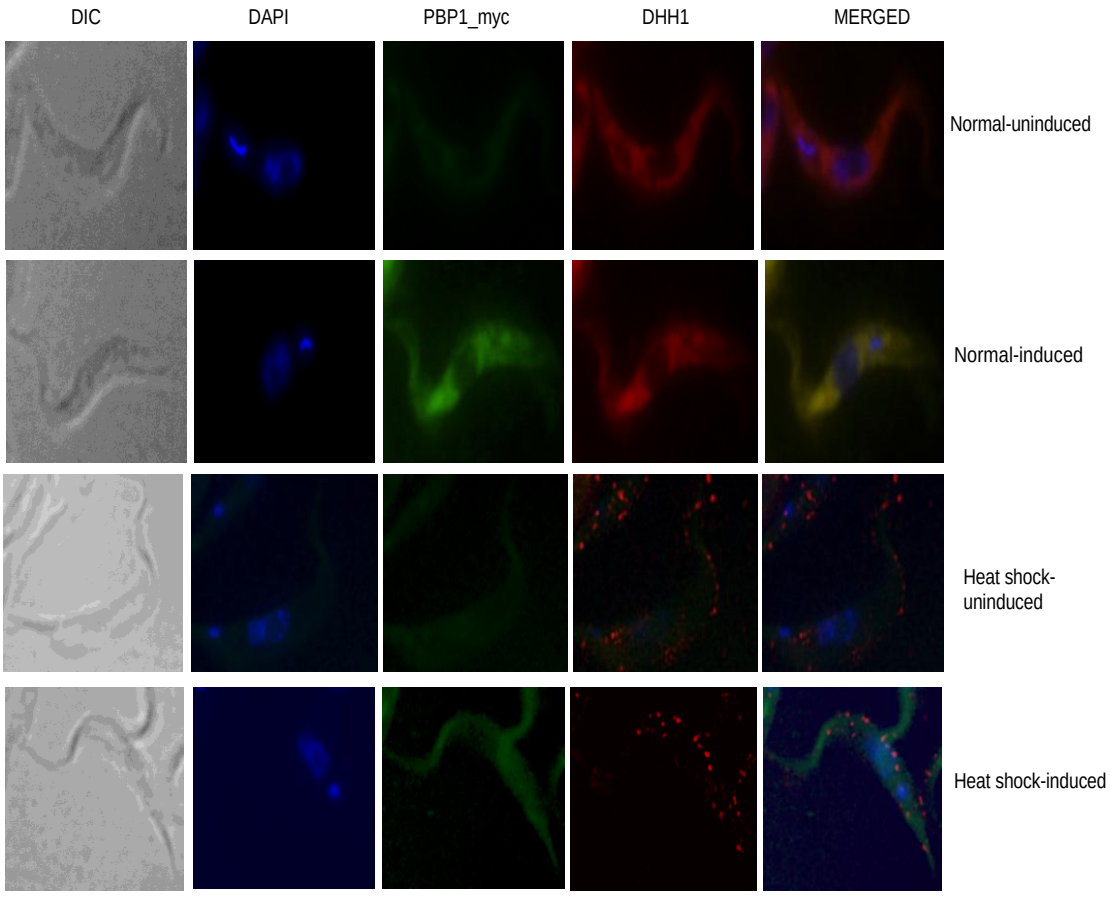


Fig III.8.2: Figure showing localisation of PBP1 under normal condition (27°C) , after heat shock treatment (41°C for 1 hrs). The stress granule marker DHH1 was used as marker here.

III.9. Yeast two hybrid analysis and derivation of interaction domains

III.9.1. Interaction study of MKT1 with other interesting candidates:

In our lab, Dr. Esteban Erben (Post doc, Clayton's lab) has tested the direct interaction of MKT1 with ZC3H11 and PBP1 by yeast two-hybrid analysis. He showed that: (i) MKT1 interacts with ZC3H11, (ii) MKT1 interacts with PBP1, (iii) and ZC3H11 also interacts with PBP1. While MKT1 and ZC3H11 are both functional and informative when expressed as 'Prey' (which contains the activation domain) or 'Bait' (which contains the binding domain) without showing any false self-activation, PBP1 is a self-activator as 'Bait' and is only functional and informative as 'Prey'.

Moreover, in order to determine which region of MKT1 interacts with these proteins, I made two constructs of MKT1 containing only the N-terminal (amino acid no. 1-493) or only C-

terminal fragment (amino acid no. 233-735). The study showed that niether of these fragments are sufficient for interacting with ZC3H11 or PBP1 (Fig III.9.D), which could mean that MKT1 protein folds such that a major region of the protein is involved in interaction with other proteins. Also, in the quest to understand the speculative role of MKT1 in regulating global translation in trypanosome, I also tried to test the interaction of MKT1 as 'Bait' with six translation factors cloned as 'Prey' by my colleague Elisha Muchunga (PhD student, Clayton's lab). The three independent biological replicates showed positive interaction of MKT1 with EF2, eIF5A and ERF1, whereas no interaction with EF1-alpha, ERF3 or eIF-2B (Fig III.9.E). The study further strengthens the argument of MKT1 having involved in influencing translation of proteins, however as these interactions weren't verified *in vivo*, the argument is still questionable.

Additionally, some of the conserved interactions of MKT1 and PBP1 in yeast were verified in trypanosome after they were also pulled down in the TAP purification of ZC3H11 (Ihor Minia and Dorothea Droll). The list includes the well-conserved interaction of PBP1 with poly (A) binding protein PABP1 and PABP2 [11]. PBP1 also showed positive interaction with LSM12 as reported in yeast [41, 99] (Fig III.9.A). Other interactions, which I tested, include the interaction of MKT1 with LSM12, PABP1 and PABP2 all of which turned out to be negative (Fig III.9.B). Other than that, in order to be sure if the Yeast MKT1 is not interfering or influencing the above mentioned tested positive interaction by yeast two hybrid analysis, I tested the interaction of Yeast MKT1 with *T. brucei* PBP1, MKT1 and ZC3H11, all of which were negative (Fig III.9.A). The summary of all these interaction is given in Table III.9.2.a. And the results of alpha galactosidase assay for all these interaction is shown in Fig III.9. In this assay, if the two proteins under the study interact, they activate the transcription of reporter gene *(MEL1)*, which encodes for alpha galactosidase enzyme and enables the auxotrophic yeast two-hybrid strains to grow on nutritionally selective plate containing X- alpha-Gal, and turn blue. X-alpha-Gal is a chromogenic substrate for alpha-galactosidase enzyme. Secretion of this enzyme in response to transcription activation of *MEL1* gene leads to hydrolysis of X-alpha-Gal in the medium causing yeast colonies to develop a blue color.

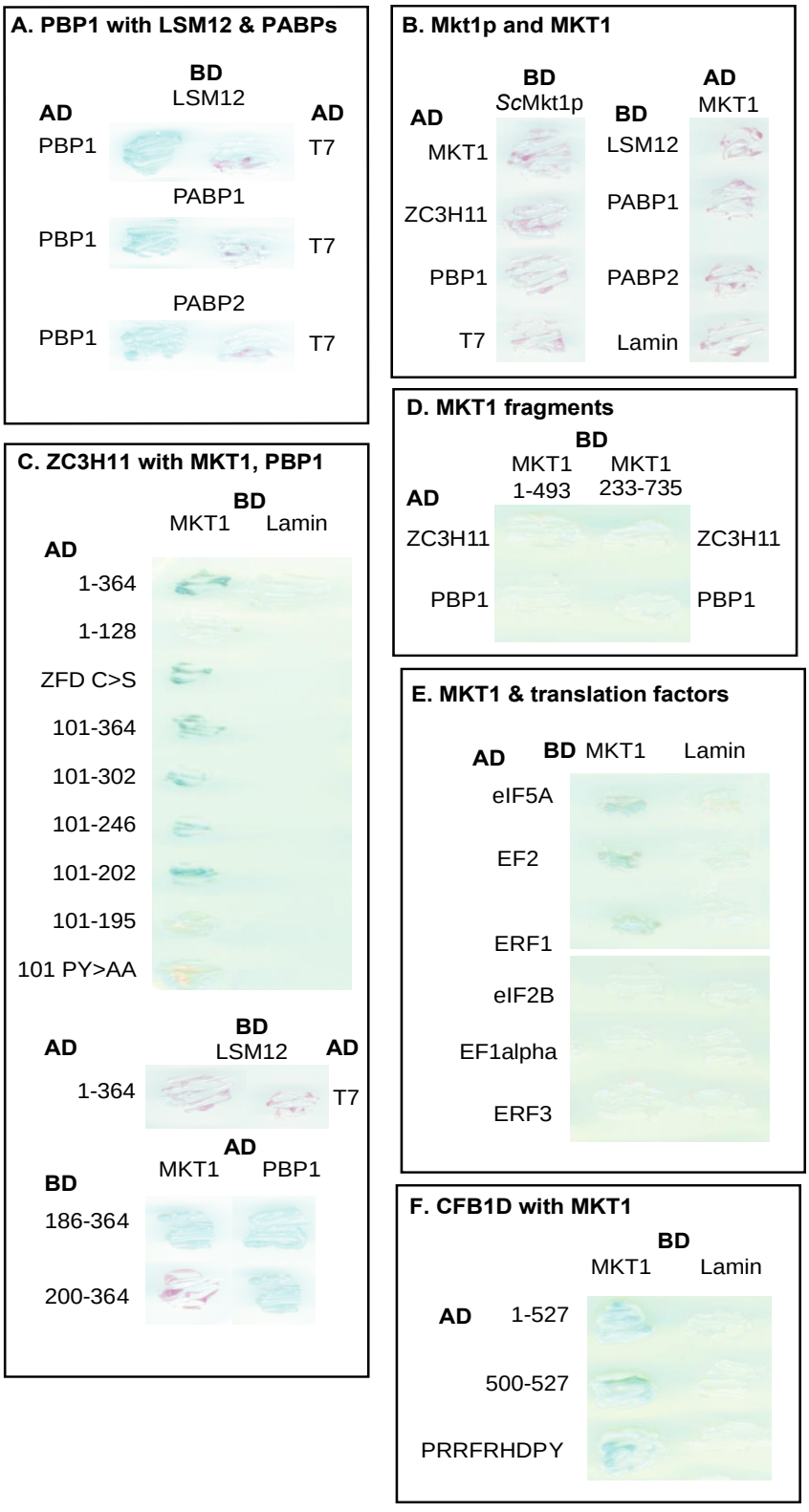


Fig III.9: Figure showing the alpha galactosidase analysis of the yeast two hybrid studies. When two proteins interact, the color of the yeast colony changes to blue as the alpha galactosidase gene gets activated**.**

III.9.2. Identification of putative interaction partners of MKT1 by screening it with the entire genomic library screen:

In order to identify various interacting partners of MKT1, an extensive yeast two hybrid analysis was done by Esteban Erben using MKT1 expressed as Bait (which contains the binding domain) and screening it for interaction with the randomly sheared genomic library [121], expressed as Prey (which contains the activation domain). The screening resulted in around 600 anonymous positive colonies. I manually analyzed 70 colonies by regrowing them under stringent medium conditions, isolating the yeast plasmid, expressing it in E.coli, checking by restriction digestion the two clones for each candidate, selecting the true positive clone by eliminating the false inserts and re-transfecting it in yeast. The analysis yielded 55 positive colonies out of 70, eliminating the false positives. The plasmids of these positive colonies were sequenced and the region of these plasmids, which interacted with MKT1, was determined by aligning them with full-length protein from the TriTryp database. The 55 colonies that had given interactions represented 21 unique protein sequences, with an additional 2 being outside annotated open reading frames (Table III.9.2.b). Considering the principle library screen involved the interaction of MKT1 with randomly sheared genomic DNA acting as Prey, we expect a bias towards the C-terminal end of the Prey protein. Also, the shearing being random, the possibilities of obtaining various fragments of the same protein are also there. Using this argument and obtaining various colonies containing the fragments of the same protein, I could narrow down my interaction region search to the minimum possible region. Interestingly, quite a significant number of interacting partners of MKT1 contained a motif (H/N)(D/E/N/Q) PY in the region, which interacts, with MKT1. The list of all the 55 proteins with their respective interacting region is given in Table III.9.2.b. The presence of (H/N)(D/E/N/Q) PY consensus is also stated there.

The HNPY motif was first found in ZC3H11, where in an independent study to identify the interaction domain of ZC3H11 responsible for interaction with MKT1 by yeast two hybrid, I prepared various truncated versions of ZC3H11 (Fig III.9.C and Table III.9.2.a). The constructs are explained in details with figures and other related results in section III.10 part of the result section (Fig III.10, page 68). In a nutshell, through extensive yeast two-hybrid analysis, I could identify a short region of ZC3H11 containing about 100 amino acids (Construct: 7, Fig III.10, page 68), to be sufficient for interaction with MKT1. This region also contained the conserved consensus of HNPY (PY motif shown in pink). As expected, when I further trimmed this construct beyond the HNPY region from C-terminal end, the protein failed to interact with MKT1 (Construct: 8, Fig III.10, page 68).

Also, as the PY residue was common for all the interacting partners of MKT1 carrying the (H/N)(D/E/N/Q) PY consensus identified through this yeast two hybrid screen, I could further show that the construct of ZC3H11 containing PY to AA mutation did not show any interaction with MKT1 (Construct: 11,Fig III.10, page 68). All the above-mentioned interactions are summarized in Table III.9.2.a and the alpha galactosidase analysis of the yeast two hybrid studies are shown in Fig III.9.C.

Moreover, the importance of this consensus could be further determined when I expressed a highly truncated version of a cyclin F box protein CFB1D [122, 123] (another interacting partner of MKT1 from the yeast two hybrid screen), coding for just 9 amino acids 'PRRFRHDPY' and found that this short truncated version could also interact with MKT1 in yeast (Fig III.9.F). I particularly chose CFB1D as in the manual verification of interacting partners of MKT1, this protein was identified 15 times.

The library screen thus turned out to be very significant in the chase to characterize MKT1 and it gave an exciting new direction to my project.

Table III.9.2.a: Interaction of MKT1 (bait) with various constructs of ZC3H11 (prey) - to identify the exact region responsible for the interaction; and with other interesting candidates.

Construct type	Construct name	MKT1 Bait	MKT1 Prey	PBP1 Prey
Prey	ZC3H11	+		
Prey	ZC3H11 C->S	+		
Prey	ZC3H11 PY->AA	-		
Prey	ZC3H11 1-128	+		
Prey	ZC3H11 101-364	+		
Prey	ZC3H11 101-301	+		
Prey	ZC3H11 101-265	+		
Prey	ZC3H11 101-202 (contains HNPY)	+		
Prey	ZC3H11 101-195 (No HNPY)	-		
Prey	CFB1	+		
Prey	CFB1 500-527	+		
Prey	CFB1 PRRFRHDPY	+		
Prey	EF2	+		
Prey	eIF-5A	+		
Prey	ERF1	+		
Prey	EF1alpha	-		
Prey	ERF3	-		
Prey	eIF-2B	-		
Prey	*Sc* Mkt1	-		
Prey	*Sc* ZC3H11	-		
Prey	*Sc* Pbp1	-		
Bait	ZC3H11		+	+
Bait	ZC3H11 186-364		+	+
Bait	ZC3H11 200-364		-	+
Bait	LSM12		-	+
Bait	MKT1		-	+
Bait	MKT1 1-494			-
Bait	MKT1 232-735			-
Bait	PABP1		-	+
Bait	PABP2		-	+
Bait	CFB1		-	
		ZC3H11 prey		
Bait	LSM12	-		
Bait	MKT1 1-494	-		
Bait	MKT1 232-735	-		

Table III.9.2.b: The list and identity of all the 55 proteins with their respective interacting region that showed positive interaction in MKT1 library screen. The shortest interacting region for the proteins, which came more than once, has been darkened in black. The presence of (H/N)(D/E/N/Q) PY consensus is also stated. The (H/N)(D/E/N/Q) PY motif is highlighted in grey.

Plasmid identity No.	**Accession No. of the target**	**Name of the protein**	**Start in target sequence**	**Full length of target protein**	**Maximum binding length**	**Description**	**Motif**
12A	**Tb927.1.4580**	**CFB1C**	**439**	**527**	**88**		**RFRHDPYVYG**
8E	Tb927.1.4580	CFB1C	434	527	93		RFRHDPYVYG
10E	Tb927.1.4580	CFB1C	400	527	127		RFRHDPYVYG
9A	Tb927.1.4580	CFB1C	379	527	148		RFRHDPYVYG
4A	Tb927.1.4580	CFB1C	375	527	152		RFRHDPYVYG
4E	Tb927.1.4580	CFB1C	358	527	169		RFRHDPYVYG
40	**Tb927.1.4600**	**CFB1D**	**500**	**527**	**27**		**RFRHDPYVYG**
45	Tb927.1.4600	CFB1D	462	527	65		RFRHDPYVYG
50	Tb927.1.4600	CFB1D	403	527	124		RFRHDPYVYG
52	Tb927.1.4600	CFB1D	368	527	159		RFRHDPYVYG
53	Tb927.1.4600	CFB1D	356	527	171		RFRHDPYVYG
54	Tb927.1.4600	CFB1D	356	527	171		RFRHDPYVYG
51	Tb927.1.4600	CFB1D	350	527	177		RFRHDPYVYG
37	Tb927.1.4600	CFB1D	349	527	178		RFRHDPYVYG
58	Tb927.1.4600	CFB1D	299	527	228		RFRHDPYVYG
43	Tb927.1.4650	CFB2	380	476	96		RFRHDPYVYG

Table III.9.2.b continued….

Plasmid identity No.	Accession No. of the target	Name of the protein	Start in target sequence	Full length of target protein	Maximum binding length	Description	Motif
36	**Tb927.3.3960**	**DRBD6A**	**288**	**377**	**89**		**RYTHEPYRSV**
59	Tb927.3.3960	DRBD6A	288	377	89		RYTHEPYRSV
70	Tb927.3.3960	DRBD6A	278	377	99		RYTHEPYRSV
7A	Tb927.3.3960	DRBD6A	271	377	106		RYTHEPYRSV
35	Tb927.3.3930	DRBD6B	201	426	225		CYVHDPYNPS
5E	Tb927.3.3930	DRBD6B	201	426	225		CYVHDPYNPS
61	Tb927.8.1590	UPL3	2450	2548	98	contains a zinc finger type domain	
48	Tb927.5.810	ZC3H11	273	364	91		CVRHNPYCHN
55	Tb927.5.810	ZC3H11	4	364	360		CVRHNPYCHN
10A	Tb927.5.810	ZC3H11	1	362	361		CVRHNPYCHN
57	Tb927.10.5250	ZC3H32	527	655	128		KHTNNPYAFG
44	**Tb927.10.5250**	**ZC3H32**	**372**	**655**	**283**		**KHTNNPYAFG**
11A	Tb927.10.5250	ZC3H32	341	655	314		KHTNNPYAFG
3A	Tb927.10.5250	ZC3H32	25	655	630		KHTNNPYAFG
3E	**Tb927.10.12780**	**ZC3H37**	**198**	**286**	**88**	**Two zinc finger domains**	**FYRHNPY**
39	Tb927.10.12780	ZC3H37	143	286	143		FYRHNPY
34	Tb927.10.12800	ZC3H38	181	280	99		

Table III.9.2.b continued….

Plasmid identity No.	**Accession No. of the target**	**Name of the protein**	**Start in target sequence**	**Full length of target protein**	**Maximum binding length**	**Description**	**Motif**
41	Tb927.10.11760	PUF6	99	843	744		
9E	**Tb927.1.2600**	**PUF9**	**130**	**667**	**537**		**VIQHQPYQLY**
33	Tb927.1.2600	PUF9	27	667	640		VIQHQPYQLY
60	Tb927.1.2600	PUF9	1	667	666		VIQHQPYQLY
68	Tb927.9.11850	SMC1	115	1275	1160	structural maintenance of chromosome 1	
65	Tb927.11.10930	TUBD1	212	489	277		
47	Tb927.11.10930	TUBD1-delta tubulin	442	489	47		
2A	Tb927.5.2820	protein kinase	721	1030	309	kinase domain is first 100AA of N-terminus	#N/A
49	Tb927.11.2830	hypothetical	863	1036	173		
7E	**Tb927.5.1990**	**hypothetical**	**118**	**334**	**216**	**Lots of poly(Q) and very basic**	**#N/A**
69	Tb927.5.1990	hypothetical	118	334	216		
31	Tb927.5.1990	hypothetical	65	334	269		
56	Tb927.8.1290	hypothetical	418	688	270		

Table III.9.2.b continued….

Plasmid identity No.	Accession No. of the target	Name of the protein	Start in target sequence	Full length of target protein	Maximum binding length	Description	Motif
14A	Tb927.6.3090	hypothetical	1294	1621	327	two ARM repeats upstream of interacting region.	#N/A
1E	Tb927.10.840	hypothetical	1380	1719	339		#N/A
67	Tb927.11.3440	hypothetical	65	530	465	P,Q rich protein	
5A	Tb927.10.4010	hypothetical	191	682	491		#N/A
64	Tb927.3.4800	hypothetical	84	619	535		
13A	Tb927.8.1290	hypothetical	370	1106	736		KMQHNPYDRM
38	Tb927.10.15750	hypothetical					
32	Tb927.4.3470	hypothetical					

III.10. ZC3H11 activity in the tethering assay requires the regions that interact with MKT1 and PBP1

It was previously shown in our lab that tethering ZC3H11 to a reporter mRNA resulted in an increase in both mRNA and protein.[25]. Thus we speculated if the activity of ZC3H11 requires the interaction of MKT1 and PBP1.

To decipher this, I made various constructs for ZCH311 and checked them individually for interaction with MKT1 and PBP1. The exact same construct was also tested if it shows a tether effect i.e. the stabilization of target mRNA by artificial tethering.

The results are summarized in Fig III.10. RNA-protein binding analysis of ZC3H11 has been reported earlier [25]. I did all the yeast two-hybrid analysis while Ihor Minia (PhD student, Clayton's lab) did the artificial tethering experiments.

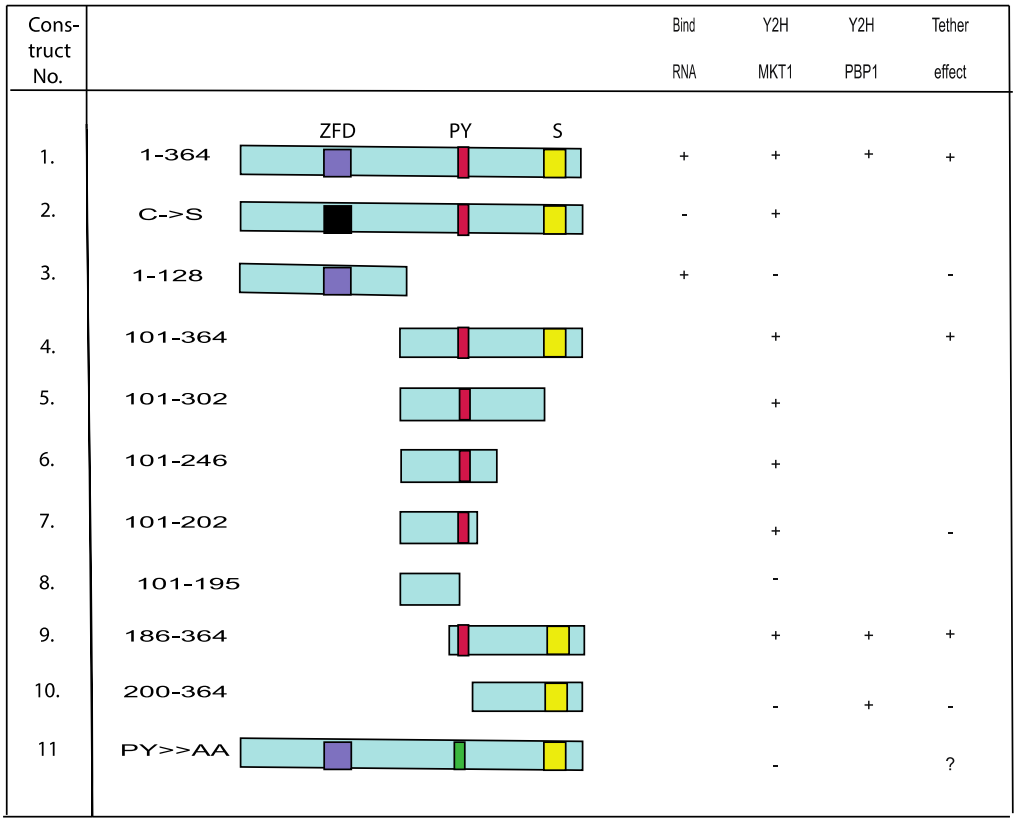


Fig III.10: Figure showing various constructs of ZC3H11: (i) tested for interaction with MKT1 and PBP1 by yeast two hybrid; (ii) if they bind to the RNA; or (iii) if they show a tether effect. ZFD: zinc finger domain (purple square); mutated zinc finger domain, Construct: 2-black square; PY (Proline Tyrosine) motif: pink; Serine rich region: yellow.

As depicted in the Figure III.10, Construct: 1 contains the full length ZC3H11 protein with its characteristic zinc finger domain at its N-terminus, "PY motif" and "Serine rich" region towards its C- terminus. This construct can bind RNA and also interact with both MKT1 and PBP1 (Dorothea Droll and Esteban Erben). Construct: 2 has mutated zinc finger domain so it doesn't bind RNA but still interacts with MKT1. Construct: 3 is a short N- terminus region of ZC3H11. This construct can bind RNA owing to the intact zinc finger domain, but it fails to interact with MKT1 and also fails to show a tethering effect (stabilization of CAT reporter mRNA by ZC3H11). This result hinted that the C-terminus of ZC3H11 binds MKT1 and this region could be responsible for the activity of ZC3H11 rendering mRNA stabilization.

Thus, in order to validate this speculation, I expressed a short C- terminus region of ZC3H11 (Construct: 4) and found it to be able to interact with MKT1. Also, as expected the transcript stabilization activity of ZC3H11 was also restored.

Going with the hypothesis that interaction of MKT1 with ZC3H11 is necessary for ZC3H11 activity, I further tried to trim ZC3H11 from the C- terminal end into three short fragments (Construct: 5, 6, 7), and found all of them to be able to interact with MKT1. This could be possible because even the shortest fragment of ZC3H11 (Construct: 7, amino acid no. 101-202, pHD 2324) contained the PY motif I had suggested earlier to be responsible for interaction with MKT1 protein (Section III.9.2, page 61). To my surprise, this short fragment (Construct: 7) did not allow ZC3H11 activity (stabilization of CAT reporter mRNA by ZC3H11). This indicates that surely MKT1 binding isn't sufficient for ZC3H11 activity (stabilization of CAT reporter mRNA). As reported before (Section III.9.2, page 61), the construct of ZC3H11 lacking HNPY motif (Construct 8), did not interact with MKT1.

The behavior of Construct 7 to be able to interact with MKT1, still forbidding the activity of ZC3H11 initiated another possibility that perhaps the C-terminal region of ZC3H11 was responsible for the function of ZC3H11, and it does so by also interacting with PBP1. So, I then created another construct (Construct: 9), which contained the C- terminal region of ZC3H11 containing the conserved PY motif and checked it for interaction with MKT1 and PBP1. As speculated, indeed this fragment did allow the interaction of MKTI and PBP1 and also facilitated the function of ZC3H11 in the tethering assay (stabilization of CAT reporter mRNA).

So far these results indicated that interaction of ZC3H11 and PBP1 is necessary for ZC3H11 activity. Further, in order to be sure if the interaction of ZC3H11-PBP1 was sufficient, I made another construct of ZC3H11 containing the C- terminus but lacking the

PY motif (Construct: 10). The importance of this PY motif in ZC3H11 to be necessary for its interaction with MKT1 has already been discussed in the previous section (Section III.9.2). Interestingly, not only did this construct fail to interact with MKT1, it also failed to render the function of ZC3H11 and did not stabilize the CAT reporter mRNA. This suggests that binding of PBP1 to ZC3H11 is necessary but not sufficient for ZC3H11. In other words, both MKT1 and PBP1 are required to bind to ZC3H11 and bring about its function in the tethering assay (stabilization of CAT reporter mRNA).

The figure also contains a construct with full length ZC3H11 where the PY motif was mutated to AA (Construct: 11). As discussed in previous section (Section III.9.2), this construct failed to interact with MKT1 and also failed to bring about the activity of ZC3H11 in the tethering assay; thereby establishing again the importance of PY motif for ZC3H11-MKT1 interaction and also the necessity of this interaction for ZC3H11 function in the tethering assay.

III.11. TAP purification of MKT1 to identify putative interaction partners:

A Tandem Affinity Purification (TAP) approach was taken to look for the interaction partners of MKT1. For this, I made a C-terminally, tetracycline inducible, ectopically expressed TAP tagged MKT1 (pHD 2326) and carried out a TAP purification protocol once. But as there wasn't much protein detected in the eluate because of less number of cells used ($5X10^9$) and also, as by that time I had already found out few important putative interacting partners of MKT following the yeast two hybrid library screen, I didn't repeat this experiment anymore.

IV. DISCUSSION:

IV.1. The organization and function of MKT1-PBP1-PABP1 complex seem to be evolutionary conserved:

Trypanosome MKT1 and PBP1 were first identified in the TAP purification of ZC3H11 [25]. This was interesting because the homologues of these two proteins have been previously reported in yeast, where both of them were reported to be directly interacting with each other [91]; and Pbp1p was found to be interacting with poly (A) binding protein Pabp1 [95, 96]

Also, the human and drosophila homologue of PBP1 known as Ataxin2 and Atx2 respectively, have been well studied and were also found to be interacting worth the poly (A) binding protein [110, 111] [107, 108]. Other than that, the interaction of PBP1 with LSM12 too is conserved in yeast and the interaction PBP1-PABP complex with DHH1, a DEAD box RNA Helicase, is also well conserved in yeast and mammals.[99, 111] This shows that indeed the PBP1-PABP1 complex has a major role to play in a cellular system and it may bring about that by its interaction with MKT1, LSM12, DHH1 independently, or together.

Previous works done to decipher the role of Mkt1p1-Pbp1 complex in yeast; or homologues of PBP1 individually in yeast, mammals and drosophila indicate that the function too is well conserved throughout evolution. While in trypanosome, I could show that both MKT1 and PBP1 are essential for the survival of the parasite, yeast Mkt1p and Pbp1 are not essential just like Ataxin2 in mammals [91, 110].

However, many results of this study go hand in hand with the roles of PBP1 in different organism. For instance, *T.brucei* PBP1 (suggested through this study) and its homologues in yeast and mammals were found to be physically associated with translating polyribosome, which indicates that it may take part in regulating mRNA translation [91, 107] [105]. As mentioned above, the interaction of PBP1-PABP complex with DHH1, a DEAD box RNA Helicase, is also well conserved in yeast and mammals under stress [99, 111]. I tried to check the interaction of PBP1 with DHH11 by co-immunoprecipitation but DHH1 being an excessively sticky protein, binds non-specifically to the beads so the result was not conclusive (not shown). However, in a recent study in *T.brucei*, through co-localisation study by immunofluorescence, it is shown that PABP1 and DHH1 do co-localize with each under stress (heat shock and nutrient starvation stress) [11]. In this regard, it's worth mentioning here that in this study I have shown (Result section, Fig III.8.1) that PBP1 relocates itself to starvation stress granules and co-localizes with the stress granule marker SCD6. So it can be hypothesized here that DHH1 also interacts with PBP1 at least as a complex with PABP, just like it has been reported in yeast and mammals [99, 111]. A figure depicting the association

of all these proteins, along with their yeast and mammalian homologues is presented here (Fig IV.1).

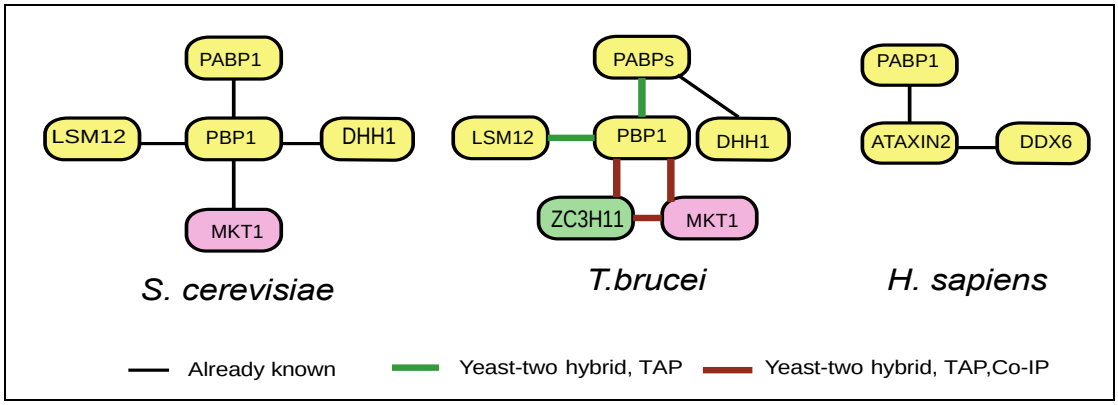


Fig IV.1: Figure showing the T. brucei complex PBP1-PABP1, along with its interacting partners DHH1 conserved in yeast and human. Additionally, the association of PBP1 with MKT1 and LSM12 is conserved yeast.

Moreover, I could also show the relocation of PBP1 into distinct cytoplasmic stress granules (under starvation stress), a feature well conserved in yeast and mammals [99, 111]. When over expressed, PBP1 causes a cessation in growth of the cells, just like it has been reported for orthologues of PBP1 in yeast and mammals [99, 105, 111]. Thus, it could be said that the functional characterization of MKT-PBP1 as a complex or individually is relevant to a certain extent throughout the eukaryotic lineage.

A remarkable observation from this study is that while there is a significant difference in the structure of PBP1 and its homologues in yeast and mammals (Pbp1p and Ataxin2), their role in maintenance of RNA homeostasis are conserved. This is evident from the fact that all of them are associated with the polyribosome under normal condition and relocate to stress granules upon stress. While both Ataxin2 and Pbp1p contain both Lsm and LsmAD domain, Lsm domain is absent in *T.brucei*. In other words, even though PBP1 contains only LsmAD domain, it still retains the function to regulate the mRNA [99, 105, 110].

IV.2. Mechanism of action for ZC3H11:

It has been reported earlier that ZC3H11 specifically recognizes the UAUU sequence in the 3' UTR of certain heat shock mRNAs and stabilizes them [25]. In my studies, I have tried to identify the mechanism of action of ZC3H11 action. Through an extensive yeast two-hybrid analysis, I could find out that the C terminus region of ZC3H11, which is responsible for imparting stabilization to the target mRNA. The results suggest that although binding of MKT1 or PBP1 is necessary, however alone, none of them is sufficient to bring about the stabilization of the target mRNA of ZC3H11. Both MKT1 and PBP1 need to interact with ZC3H11 to lead to stabilization (described in details in result section III.10, page 68). Thus, we propose here that ZC3H11 recognizes and binds to the AU rich region of 3' UTR of its target mRNA and recruits MKT1 and PBP1 which thereby stabilizes the poly (A) tail via interaction with PABP. The attached PABP interacts with the poly (A) tail and/or eIF4E [124]. This could stabilize the interaction with the poly (A) tail; and it might also stabilize mRNA circularization via eIF4E and render it for efficient translation. It is worth mentioning here that unlike in higher eukaryotes, the Poly (A) binding proteins of Leishmania and Trypanosomes interacts with eIF4E instead of eIF4G [124] [15] and circularize the mRNA for efficient translation [125]. Further, this mechanism of transcript stabilization could be extended to any other RNA binding protein, which recruits MKT-PBP1-PABP complex as suggested in the next section.

IV.3. A general mechanism of mRNA regulation at the post-transcriptional level:

The library screen of MKT1 identified 21 unique proteins, which include various zinc finger proteins ZC3H11, ZC3H21, ZC3H31, ZC3H32, ZC3H37 and ZC3H38, two PUF proteins PUF9 and PUF6, DRDB6, CFB, UPL3, SMC1 and TUBD1and 8 hypothetical proteins, out of which at least 8 contained a consensus (H/N) (N/Q/E/D) PY. The PY residues were found to be common in all. The importance of this consensus was validated when I checked a short peptide of CFB1D containing just PRRFRHDPY residues and found that it was able to interact with MKT1. Also, when this critical PY residue was deleted or mutated to AA in ZC3H11, it failed to interact with MKT1. Thus, this couplet residue indeed is necessary for MKT1 to identify and interact with certain interacting partners. Interestingly, all the zinc finger proteins, which came down in this screen, contained this consensus (Figure IV.3.1).

This suggests that MKT1 could possibly act as a posttranscriptional regulator of additional RNA binding proteins and the mechanisms of ZC3H11 action could be regarded as a general mechanism in which MKT1 binds to a specific RNA binding protein and stabilizes it target

mRNA by interacting with PABP via PBP1 and hence the circularization of mRNA (Figure IV.3.2.A)

In another mechanism, the binding of MKT-PBP1-PABP1 complex with an RNA binding protein could also interfere with the circularization of mRNA, which is brought about by the interaction of poly (A) tail and/or eIF4E [126]. This mechanism can also relocate the transcript to stress granules under certain stress conditions (Fig IV.3.2.B) [15, 109].

In an additional study, a high throughput yeast two-hybrid library screen was done by Dr. Esteban Erben to find out potential interaction partners of MKT1. As a result, the screen brought 177 proteins as the potential interaction partners, which included all the 20 targets identified by me in the manual screen except one. The high throughput study identified a deadenylase complex component NOT2 as the interaction partner of MKT1, which indicates the involvement of MKT1 in destabilizing its target mRNA by recruiting CAF1/NOT deadenylase complex. Thus, another hypothetical mechanism of mRNA regulation by recruitment of MKT1-PBP1-PABP1 complex, which further associates with the CAF1/NOT1 complex thereby destabilizing the transcript could be suggested. The deadenylation could lead to the degradation of transcript by exosome and XRN1 in the cytosol or relocation of the transcript post deadenylation to the P bodies for degradation and decay (Fig IV.3.2.C) [4] [15, 127, 128].

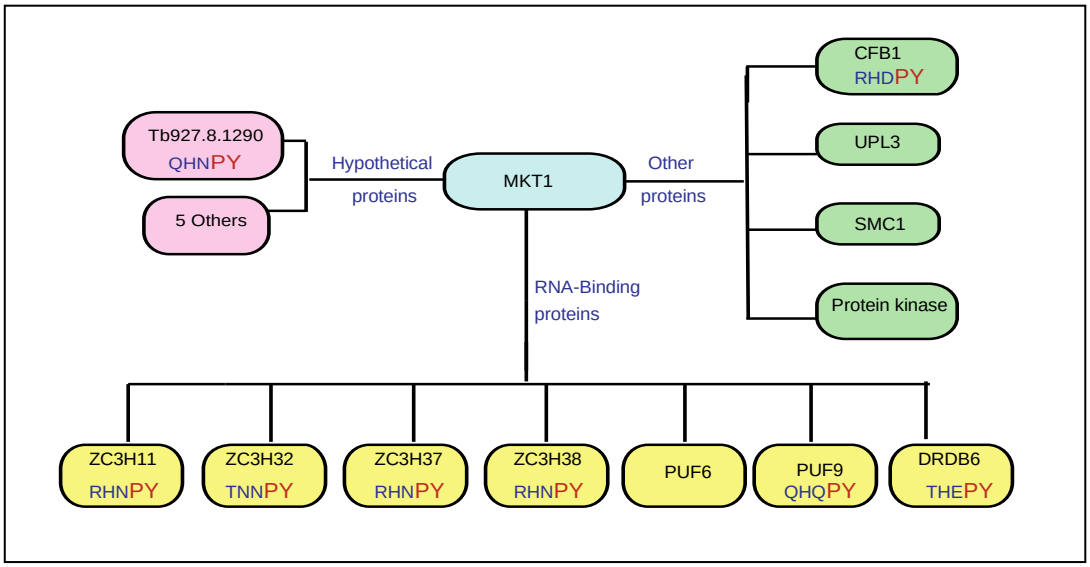


Fig IV.3.1: Figure showing the various interacting partners of MKT1 identified by manually picking up each colony in a yeast two-hybrid library screen. The dominance of RNA binding protein as the interacting partner of MKT1 is insightful. Further, almost 50% of the candidates contain the conserved PY motif.

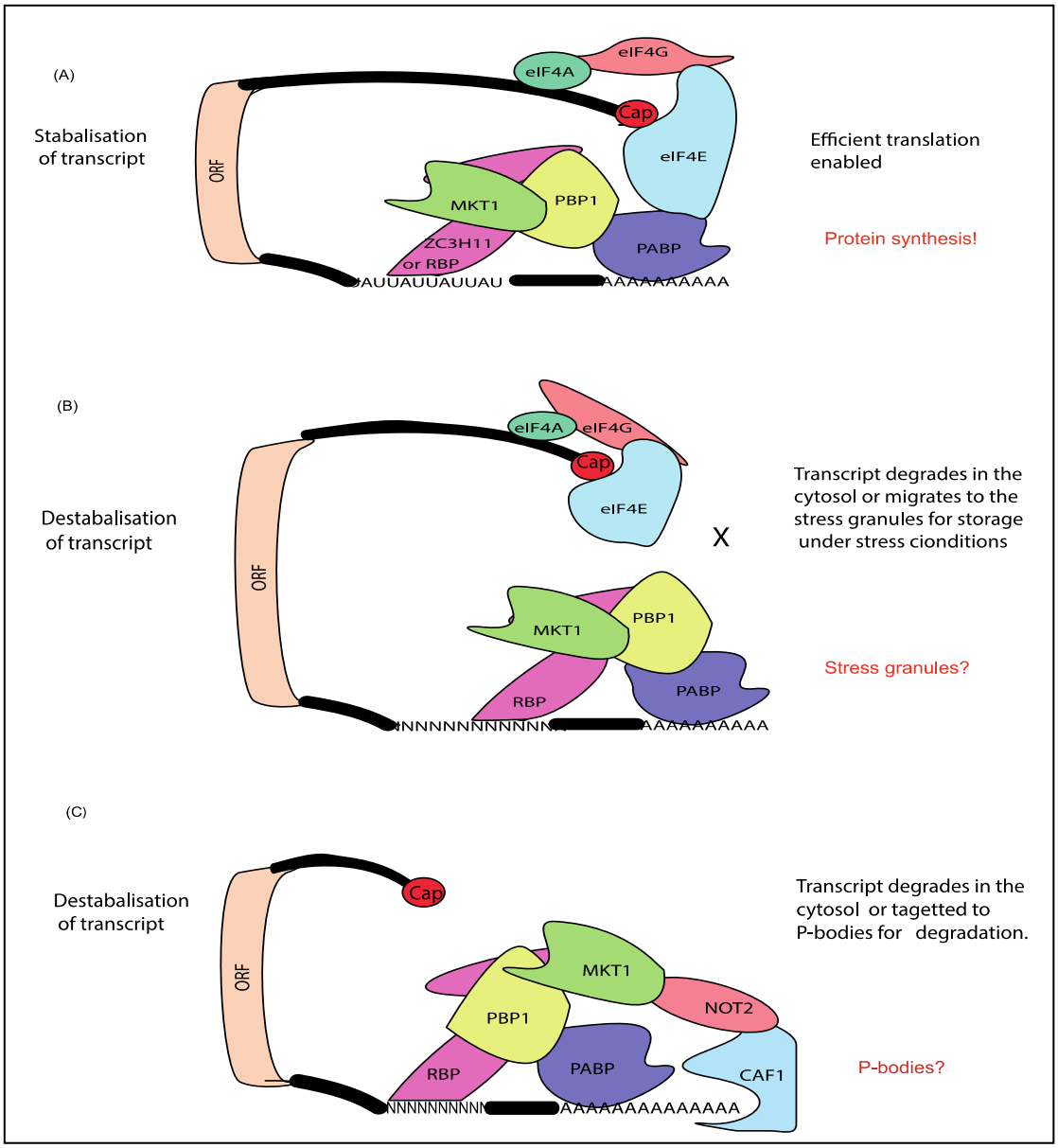


Fig IV.3.2: Hypothetical figure showing possible fate of an mRNA when the MKT-PBP1-PABP1 complex is recruited at the 3' end. The RNA binding protein recognizes specific sequence in the 3' UTR of its target mRNA. The protein then recruits MKT-PBP1-PABP1 complex, which can lead to any of the three fates of the mRNA. A) The PABP1 tethers the 3' end with the 5'cap by interacting with eIF4E and circularizes the mRNA enabling efficient translation. This is exactly the mechanism suggested here for the action of ZC3H11. B) The recruitment of MKT1-PBP1 by RNA binding protein can interfere with the association of PABP1 with eIF4E thereby destabilizing the transcript. This transcript can either be decapped or degraded in the cytosol itself or relocate itself in the stress granules for storage under stress conditions. C) MKT1 interacts with NOT2 which deadenylates the same transcript by recruitment of CAF1/NOT deadenylase complex. Post deadenylation, the transcript can either be degraded in the cytosol or migrate the P-bodies for degradation and decay. Ideas for this figure are adapted from [15, 109, 124, 125, 128].

IV.4. Does MKT1 regulates global translation of proteins?

In yeast, Mkt1p is known to play a role in the post-transcriptional regulation of the HO transcript. The ability of Mkt1p to regulate the translation of *HO* mRNA is brought about by its interaction with Pbp1. Mkt1p and Pbp1p co-sedimented with polyribosomes in sucrose gradients, with the distribution of Mkt1p in the polyribosomes dependent on Pbp1p, but not vice versa. Loss of Pbp1p or Mkt1p had no effect on the level of *HO* mRNA but it did decrease its protein level; which indicated that the regulation of HO endonuclease gene by Mkt1p-Pbp1p operates at a post transcriptional level [91].

A definite role in translation has not been shown for MKT1 in this study. But as MKT1 and PBP1 both showed a similar pattern of sedimentation on sucrose gradient and were found to be associated with the polyribosome, it certainly indicates that MKT1 and PBP1 not only interact with each other, they also have a role in regulating translation of a specific or global transcripts. Finding this result very intriguing, and already knowing about the rapid lethality of the cells where MKT1 is knocked down, I explored the independent role of MKT1 in regulating translation. Indeed, the lack of metabolic labeling by S[35] and a very mild effect on the polyribosome profile, in MKT1 depleted cells hinted in affirming my speculation that MKT1 regulates translation at a post initiation phase. However, as the arrest in global translation, deciphered by lack of metabolically labeling in MKT1 depleted cells somehow overlapped with growth cessation and cell death, it can not be conclusive if the translation arrest is a primary effect of MKT1 knockdown or its because of sick or dead cells (Result section, Fig III.5.1). It is interesting to mention here that I could also show the positive interaction of MKT1 with three translation factors namely EF2, eIF5A and ERF1 through yeast two hybrid analyses but as none of these interactions were tested *in vivo*, so a role of MKT1 in regulating translation could not be confirmed.

IV.5. Localization studies:

It has been widely known in eukaryotes that an mRNA can be temporarily stored by localizing with distinct cytoplasmic granules under different stress conditions. In this regard, extensive localization studies of homologues of PBP1 and its interacting partners under different stress condition have been previously reported; wherein in majority of the studies it has been shown that under certain stress conditions, homologues and certain interacting partners of PBP1 (such as PABP, DHH1, LSM12) relocate into distinct stress granules in the cytoplasm.

For instance, in mammals, Ataxin-2, its interacting partner PABP and DEAD/H-box RNA helicase DDX6, has been found to relocate to stress granules after heat shock [110, 111]. Yeast Pbp1p and Lsm12p, Dhh1p, Pab1p also accumulate in stress granules induced by glucose deprivation [99]. In this context, Trypanosomes also have been shown to contain stress granules after heat shock and starvation [34, 38, 41, 129]. While SCD6, DHH1 and PABP1 have been previously reported to be a component of heat shock induced stress granules [38], recently, they, along with certain translation factors, were also reported to be a component of starvation induced stress granules [11, 12, 34, 129]. In fact, a difference in localization two paralogs of poly (A) binding proteins in trypanosome has been reported recently wherein both the paralogs PABP1 and PABP2 show difference in localization with respect to nutrient stress. While PABP2 was largely found to be a component of nutrient starvation stress granule, PABP1 was largely but not entirely absent, as detected by observing its co-localization with DHH1 (a stress granule marker protein)[11, 12]. A similar but not that drastic difference was seen after heat shock stress, wherein again while both PABP1 and PABP2 formed distinct cytoplasmic granules, their co-localization with stress granule marker DHH1 was a little more for PABP2 as compared to PABP1[11].

In this study, I could show the occurrence of distinct stress granules containing SCD6 or DHH1 after heat shock but neither PBP1 nor MKT1 showed any kind of co-localization with them and remained localized to cytosol. This observation goes with our hypothesis established here because as widely known, stress granules are a sight of mRNA storage during unfavorable condition like heat shock [130, 131] and the mechanism stated here for ZC3H11 function (a heat shock transcript stabilizer [25]) proposes that binding of ZC3H11 to MKT1-PBP1-PABP1 complex is necessary to promote this function, so a halt in translation of MKT1 and PBP1 after heat shock would be inappropriate to bring about this regulation of ZC3H11 function.

In contrast, in this study I could show that when subjected to nutrient stress, both PBP1 and MKT1 did relocate into distinct cytoplasmic granules. While almost all of the PBP1 containing granules co-localized with those of SCD6, MKT1 was largely but not entirely absent from the nutrient starvation stress granules containing SCD6. A similar observation has also been reported for PABP1 [11] where PABP1 and other translation initiation factors such as eIF4E2 and eIF4E3 were found to be largely but not totally absent from the SCD6 containing nutrient stress granules.

Finally, through this study I attempted to characterize the function of MKT1 and PBP1 and found them to assist in the function of ZC3H11 to stabilize the heat shock transcripts. Through the hypothetical models given in (Fig IV.3.2), we speculate that an RNA binding

protein recruits the MKT1-PBP1-PABP1 complex and together they decide the fate of the transcripts (one or many) it binds to. Few studies based on this hypothesis are already going in our lab. For instance, Cornelia Klein (another PhD student) is trying to decipher the role of ZC3H32 (another CCCH Zinc finger protein), which was found to interact with MKT1 in the yeast two-hybrid screen and also TAP purification. So far, she could show that ZC3H32 is an essential zinc finger protein in *T.brucei*, and it destabilizes the reporter mRNA. Further, I also tried to verify the interaction of another Zinc finger protein ZC3H38 and a hypothetical protein *Tb* 927.5.1990 through co-immunoprecipitation. These two proteins were found to interact with MKT1 in the yeast two hybrid screen but were absent from the TAP pull down of MKT1. As a result, it appears that a small portion of both these protein do interact with MKT1. Since both these proteins are not abundant in the cell, in order to have a conclusive result, a new construct with an over-expressed version of ZC3H38 and *Tb* 927.5.1990 needs to be prepared and tested again for interaction with MKT1 through co-immunoprecipitation.

Overall, we speculate MKT1 to act as a 'hub' imparting posttranscriptional regulation to many transcripts. We propose that MKT1 confers this role by binding directly or indirectly to a variety of RNA binding proteins, thereby upgrading the 'one transcript-one regulator' models for gene regulation.

V.LIST OF ABBREVIATIONS:

Amp	ampicillin
APS	ammonium persulphate
ATP	adenosine-5-triosephosphate
bp	base pairs
BME	β-mercaptoethanol
BS	bloodstream form
BSA	Bovine Serum Albumin
BLA	blasticidin
CAT	chloramphenicol acetyl transferase
CIP	Calf intestinal phosphatase
CoIP	Co-immunoprecipitation
DAPI	4',6'-diamino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddH_2O	double distilled water
dGTP	2'-deoxyguanosine 5'-triphosphate
DDO	Double dropout
DMFO	Dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphates
DTT	1,4-Dithiothreitol
E	Eluate
E.coli	*Escherichia coli*
EDTA	ethylenedinitrilo tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
et.al.	and others
EtBr	ethidium bromide

FACS	Fluorescence-activated cell sorting
FCS	foetal calf serum
Fig.	figure
FT	Flow through
gDNA	genomic DNA
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMi-9	Hirumi's modified Iscove's medium - 9
hyg	hygromycin
I	Input
IFA	indirect immunofluorescence assay
IMDM	Iscove's Modified Dulbecco's Medium
IPTG	isopropyl-1-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilo Dalton
kDNA	kinetoplast DNA
kV	kilo volt
LB	Luria-Bertani
MCS	multiple cloning site
µCi	microcurie
MEM	Minimum Essential Medium of Eagle
µl	microliter
µg	microgram
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein
NP-40	nonyl phenoxypolyethoxylethanol
nt	nucleotide
O/N	Overnight
ORF	open reading frame

PAGE	polyacrylamide gel electrophoresis
PARP	procyclic acidic repetitive protein
PBS	phosphate-buffered saline
pBS	plasmid Bluescript
PC	procyclic form
PCR	polymerase chain reaction
pH	-log [H^+]
Phleo	phleomycin
PURO	puromycin
PSB	polysome buffer
QDO	Quadruple dropout
RBP	RNA protein proteins
RDT	recombinant DNA technology
RNAi	RNA interference
RNA pol	RNA polymerase
RNase	ribonuclease
rpm	rounds per minute
RRM	RNA recognition motif
rRNA	ribosomal RNA
RT	room temperature
SDS	sodium dodecyl sulphate
SD	Synthetic dropout
SL	splice leader
spp.	subspecies
SSC	sodium saline citrate
SSR	strand switch region
TAE	Tris-acetate-EDTA
TAP	tandem affinity purification

Taq	*Thermus aquaticus*
TCA	tricarboxylic acid
TE	Tris-EDTA
TEMED	N,N,N,N'-tetramethylethylenediamide
tet	tetracycline
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
U, u	unit
UTR	untranslated region
UV	ultraviolet
Vol	volume
VSG	variant surface glycoprotein
WT	wild type
w/v	weight/volume
v/v	volume/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD	Yeast Potato Dextrose
YPDA	YPD - supplemented with Adenine hemi sulphate
ZPFM	Zimmermann post-fusion medium.

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