Involvement of a Toxoplasma DEAD-box RNA Helicase in Post-transcriptional Gene Regulation

Ahmed Cherry

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Involvement of a *Toxoplasma* DEAD-box RNA Helicase in Post-transcriptional Gene Regulation

By

Ahmed Cherry

A Thesis
Submitted to the Faculty of Graduate Studies through Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2013

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Involvement of a *Toxoplasma* DEAD-box RNA Helicase in Post-transcriptional Gene Regulation

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May 16, 2013
DECLARATION OF ORIGINALITY

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ABSTRACT

RNA helicases are vital components of various pathways for post-transcriptional gene regulation. TgHoDI is a newly identified member of the DEAD-box RNA helicase family in *Toxoplasma gondii*. This study was aimed at characterizing the function of TgHoDI in post-transcriptional regulation during various stages of the parasite life cycle. Through immunofluorescence analyses, TgHoDI was found to colocalize with mRNA aggregates in cytoplasmic foci. Furthermore, colocalization studies of TgHoDI with PABPC3 identified these proteins as components of RNA aggregates known as stress granules. Cycloheximide and arsenite treatment of extracellular parasites revealed the presence of mechanisms involved in the determination of mRNA fate in *Toxoplasma*. Also, TgHoDI was found to functionally compliment the temperature sensitive phenotype in Δdhh1 yeast, validating its role in stress response. These findings confirm that TgHoDI is a vital component in the formation of stress granules and possibly involved in the mechanisms of post-transcriptional gene regulation.
DEDICATION

To my family
Words cannot describe what you mean to me.
I could never have done it without your love and support.
ACKNOWLEDGEMENTS

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thank you for all of your support and I hope that I have helped you as much as you helped me.

I am extremely fortunate for my friends and family. You have supported me throughout the years and it is that support which has allowed me to succeed. Thank you for always understanding when I couldn't be home for dinner, or for missing a family event. You are my life and without you none of this would have been possible. Without your love, my accomplishments are nothing. Thank you, from the bottom of my heart.
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<tbody>
<tr>
<td>AAA+</td>
<td>ATPases Associated with diverse cellular Activities</td>
</tr>
<tr>
<td>AGO</td>
<td>Argonaut</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAG1</td>
<td>Bradyzoite Antigen-1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Bz</td>
<td>Bradyzoite</td>
</tr>
<tr>
<td>CCS</td>
<td>Cosmic Calf Serum</td>
</tr>
<tr>
<td>CDS</td>
<td>Coding Sequence</td>
</tr>
<tr>
<td>CGH-1</td>
<td>Conserved Germline Helicase-1</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>CTE</td>
<td>C-Terminal Extension</td>
</tr>
<tr>
<td>Dcp1a/Dcp2a</td>
<td>Decapping Enzyme 1a/2a</td>
</tr>
<tr>
<td>ddFKBP</td>
<td>Destabilizing Domain</td>
</tr>
<tr>
<td>dFBS</td>
<td>Dialyzed Fetal Bovine Serum</td>
</tr>
<tr>
<td>Dhh1</td>
<td>DEAD/H Homologue-1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Media</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DOZI</td>
<td>Development of Zygote Inhibited</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic Initiation Factor</td>
</tr>
<tr>
<td>ENO2</td>
<td>Enolase-2</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed Sequence Tag</td>
</tr>
<tr>
<td>GPD</td>
<td>Glyceraldehyde-3-phosphate Dehydrogenase</td>
</tr>
<tr>
<td>HFF</td>
<td>Human Foreskin Fibroblast</td>
</tr>
<tr>
<td>HoDI</td>
<td>Homologue of DOZI</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence Assay</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Burtani</td>
</tr>
<tr>
<td>LDH1</td>
<td>Lactate Dehydrogenase-1</td>
</tr>
<tr>
<td>m^7G</td>
<td>7-Methylguanylate</td>
</tr>
<tr>
<td>MCM</td>
<td>Mini Chromosome Maintenance</td>
</tr>
</tbody>
</table>
MEM  Minimum Essential Media Eagle
mRNA  Messenger Ribonucleic Acid
mRNP  Messenger Ribonucleoprotein
NMD  Non-sense Mediated Decay
NTE  N-Terminal Extension
ORF  Open Reading Frame
PABP  Poly(A) Binding Protein
PB  Processing Body
PCR  Polymerase Chain Reaction
PV  Parasitophorous Vacuole
RecA  Recombinase
RFLP  Restriction Fragment Length Polymorphism
Rho  Rhodamine
RIPA  Radioimmunoprecipitation Assay
RNA  Ribonucleic Acid
RNAi  RNA Interference
SAG1  Surface Antigen-1
SDS  Sodium Dodecyl Sulfate
SF  Streptavidin-FLAG
SF1-SF6  Superfamily 1-6
SG  Stress Granules
SNP  Single Nucleotide Polymorphism
SOC  Super Optimal Broth with Catabolite Repression
SSC  Saline-sodium Citrate
ssRNA  Single Stranded RNA
TBST  Tris-Buffered Saline and Tween 20
α-Tub  α-Tubulin
Tz  Tachyzoite
UTR  Untranslated Region
YFP  Yellow Fluorescent Protein
CHAPTER I - LITERATURE REVIEW

1.1 Toxoplasma gondii as a model organism

Toxoplasma gondii is an intracellular parasitic protozoan and serves as a model organism for the study of pathogenic Apicomplexa members including Plasmodium, Neospora, Eimeria and Cryptosporidium [1]. In comparison to other Apicomplexa, T. gondii is easily propagated in vitro using cell monolayers. Also, a high transfection efficiency and the ability to stably express protein epitope tags allows for the study of biochemical properties that are not readily expressed in other organisms. The haploid nuclear genome which contains 14 chromosomes, consisting of approximately $8 \times 10^7$ base pairs [1], allows for the modification or elimination of genes through mutagenesis without allelic complementation. Thorough documentation of mutagenesis and selectable markers allow for the isolation of transformed parasites in gene replacement studies through homologous and random recombination [2]. In addition, the well-established mouse model and the ability to monitor host response has made T. gondii ideal for the study of Apicomplexan biology.

1.2 Life cycle

Toxoplasma is unique in its capability of infecting and replicating in virtually any nucleated avian or mammalian cell [3]. The parasite's life cycle consists of two parts; sexual reproduction within the feline (definitive host) and asexual replication correlating with non-feline infections (intermediate host) [4]. Due to its implication in human disease, the asexual life cycle will be discussed in further detail. The asexual life cycle consists of two distinct stages known as tachyzoite (tz) and bradyzoite (bz), which result in acute or chronic infections, respectively [4]. In the acute tachyzoite stage, parasites are
in a rapid growth form and are capable of active infection. Tachyzoites enter the host cell by penetrating through the host cell membrane and become surrounded by a parasitophorous vacuole (PV). Parasite replication within host cells occurs at a generation time of 6 to 8 hours within the PV through the process of endodyogeny [3]. Endodyogeny is a form of indefinite reproduction in which two progeny form within the parental parasite [5]. The production of a large number of parasites, approximately 64-128, leads to the rupture of host cells (lysis). Parasites then exit the cell, in a process referred to as egress, and are then able to infect neighbouring cells [3]. Under the stress of the host's immunity, tachyzoite to bradyzoite differentiation occurs, resulting in the formation of tissue cysts approximately 7-10 days post-infection [4]. This marks the second asexual stage, which is characterized by slow parasitic growth. Similarly to tachyzoites, bradyzoite parasites divide by endodyogeny, resulting in increased cyst size [4]. These cysts are found primarily in muscle tissue and the central nervous system, where they remain for the entirety of the host's life [3].

1.3 Clonal Lineages of *T. gondii*

*Toxoplasma* consists of three clonal lineages, type I (RH and GT-1), II (ME49) and III (CEP and VEG), which differ in virulence and epidemiological occurrence [6]. Although the strains are divided amongst these three clonal groups, at any given locus the three groups only possess two separate alleles, which are ~98% identical at the nucleotide level [7]. All three strains share the trait of oral infectivity thereby bypassing the need for the sexual stage of the parasitic life cycle [8]. This bypassing of sexual reproduction, as well as the need of a definitive host, limits genetic recombination and hinders the formation of a wider range of genotypes [9]. Furthermore, infection in intermediate hosts
is typically caused by a single strain and therefore recombinant genotypes are unlikely to emerge [8].

Studies by Sibley and Boothroyd (1992) sought to determine the variation in parasitic strains by means of polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLPs) [10]. In the study, 28 isolates from a diverse set of hosts were analyzed using a subset of gene probes previously used as characteristic markers for the genetic and physical mapping of the Toxoplasma genome. An example of such a marker, surface antigen-1 (SAG1), is a single-copy gene encoding the major surface antigen [10]. RFLP analysis revealed that all three strains showed an identical RFLP pattern for the genes studied, indicating the genetic homogeneity among the strains [10]. Consistent with this, the differences among the strains, at the genome sequence level, was determined to be less than 1% [10]. Similar studies conducted by Howe and Sibley (1995), which performed PCR/RFLP studies on 6 independent loci, determined that the percentage of nucleotide divergence between estimated strains was <5% [11]. Furthermore, studies by Cristina et al. (1995) resulted in comparative results when performing RFLP and isoenzyme analysis on 14 parasite isolates [12]. This genetic homogeneity has lead to the conclusion that these virulent strains, I, II and III, were recently derived from a single clonal lineage [13].

The studies conducted provided preliminary analysis of Toxoplasma genome, which has since been fully sequenced by the J. Craig Venter Institute. The completion of the parasitic genome subsequently lead to the assembly of the bioinformatic database referred to as ToxoDB [14]. This database is a culmination of studies conducted by several researchers in the field to provide access to the genomic sequence of all known T.
*gondii* strains [15]. Furthermore, the database contains annotation of the type II strain with analyses of express sequence tags (ESTs), single nucleotide polymorphisms (SNPs) analyses, open reading frames (ORFs) identification, proteomic and microarray studies [15]. Due to the highly similar genomes of the three clonal strains, data exhibited for the type II strain provides an accurate representation of information on the remaining two strains.

Although there is no significant variation at the genomic level, studies in mice have shown that infections by the various clonal lineages result in altered pathogenesis; with type I strains being highly virulent in comparison to the relatively non-virulent type II and III strains [10]. This enhanced virulence of type I is in part due to the over-activation of the Th1 immune response, which is a cell-mediated immunity targeting intracellular pathogens [8]. Furthermore, type I strains share a combination of alleles at many loci, which is not seen in non-virulent strains, indicating acute virulence is a result of genotype variations [8]. Analysis of *T. gondii* strains in human disease has found an increased frequency of type I stains in AIDS patients, however the majority of infections are caused by type II strains [16]. Furthermore, type I has been associated with recurrent ocular toxoplasmosis in immunocompromised patients, suggesting that type I is more pathogenic [17]. Taken together however, most studies in North America and Europe has identified type II strains as the most prevalent cause of human toxoplasmosis in congenital and AIDS patients while type III is largely confined to animals [18, 19].

1.4 Pathogenesis

*T. gondii* is the causative agent of toxoplasmosis which effects a third of the human population [6]. Infection can generally occur by ingestion of contaminated meat,
water or the exposure to oocysts present in infected feces. During an acute acquired infection, individuals exhibit influenza-like symptoms with limited cases of prolonged fever, lymphadenopathy or retinochoroiditis [1]. As a result, clinical manifestations are commonly limited to immunodeficient individuals such as AIDS patients. Among these individuals, intracerebral focal lesions may result in encephalitis, that if left untreated, can be fatal. Congenital toxoplasmosis may also occur and is a result of an acute infection of an expectant mother. The ability of tachyzoite parasites to be transmitted through the placenta, as well as the lack of a fetal immune system, causes infection in the unborn fetus [6]. The severity of congenital infection varies; however, spontaneous abortions and neurological disorders, such as toxoplastic encephalitis and ocular toxoplasmosis may occur [6]. The current drug therapy for toxoplasmosis consists of a drug regimen centered around pyrimethamine in addition to either sulfonamides or clindamycin [1]. The combination of clindamycin and pyrimethamine is used in patients with who do not tolerate sulfonamides [20]. These drugs effectively kill parasites in the tachyzoite stage, however do not target the chronic bradyzoite stage [1]. This, combined with the toxic side effects of these drugs, creates the need for a more effective and long-term clinical treatment. Due to the prevalence of Toxoplasma infection in the human population, it is necessary to understand the developmental mechanisms behind stress response, primarily those involved in parasite differentiation and cyst formation. Analysis of gene expression and regulation which accompanies such cellular processes may allow for the understanding of the underlying mechanisms responsible and assist in the determination of better clinical treatments.
1.5 Gene Regulation

Throughout the parasitic life cycle, a fine balance of transcriptional and translational control occurs to maintain proper levels of gene expression. Gene regulation is fundamental for the coordinate synthesis, assembly and localization of macromolecular structures of the cell, thereby ensuring proper functioning of biological mechanisms necessary for survival [21]. Gene regulation can generally be divided into two levels: (i) transcriptional and (ii) post-transcriptional regulation.

(i) **Transcriptional regulation** ensures that the levels of transcripts are maintained at the necessary levels throughout various growth and developmental stages. For transcription to occur, certain regions of chromosomes (genomic DNA) must first be made accessible to allow for the binding of the transcription protein complex. The accessibility of DNA is altered by a class of chromatin remodeling enzymes, for example those capable of covalently modifying histones. The genome of *Toxoplasma* contains several histone acetyltransferases which were determined to be necessary in the transcriptional regulation of chromatin bound to histone 3 (H3) and histone 4 (H4) [1]. The acetylation of lysine residues on the N-terminal histone tails results in the transcriptional activation and repression by the addition or removal of acetyl groups, respectively [22]. Similarly, the addition of methyl groups to lysine and arginine residues on H3 and H4 has been shown to confer gene activation and silencing in *Toxoplasma*, with five protein methyltransferases and a lysine-specific demethyltransferase homologue identified [1]. Furthermore, several putative proteins were identified in the genome of *Toxoplasma* whose eukaryotic homologues act in histone modification by means of activation,
through phosphorylation, ADP-ribosylation, and ubiquitylation, or repression, by means of sumoylation [1, 23].

Studies by Soldati and Boothroyd (1995) demonstrated that genomic regions flanking ORFs, referred to as cis-elements, were able to confer both characteristic specificity and strength of expression of endogenous protein coding regions [24]. These elements identified in the 5'-flanking region vary in size from a hundred to thousands of nucleotides and have been exploited in the construction of expression vectors, resulting in the high expression of target genomic sequences [24]. Examples of these 5' exploited promoter regions are the tachyzoite-specific expression vectors derived from lactate dehydrogenase 1 (LDH1) or surface antigen 1 (SAG1) and bradyzoite-specific vectors constructed from bradyzoite antigen 1 (BAG1) or enolase 1 (ENO1). Although specific cis-acting elements in the 5'-flanking regions of many genes have been identified, such as the six tandemly repeated 27 base pair repeats directing transcription initiation of SAG1, the exact location of regulatory elements of many genes remains unknown [1].

The coupling of these cis-elements with sequence-specific DNA binding proteins, known as trans-regulating factors, have been shown to control gene regulation of target sequences. This is carried out by the binding of these factors to specific sequences resulting in the promotion or repression of transcription by the activation or inhibition of RNA polymerase, respectively [22]. These factors are classified on the basis of their DNA binding domains which may consist of: (i) a helix-turn-helix, (ii) two cysteine-two histidine zinc finger, (iii) a multi-cysteine zinc finger or the (iv) basic DNA binding domain [25]. Stimulation of transcription results from the assembly of the basal complex, which consists of RNA polymerase II and transcription factors such as transcription
factor IIIB (TFIIB), at specific activation domains present in the promoter of a gene [25]. In comparison, inhibition of transcription by protein factors may function in several mechanisms including: (i) the binding of an inhibitory factor which directly represses a gene, (ii) binding of a factor which interferes with the binding of stimulatory factors or (iii) the quenching of stimulatory factors by binding of inhibitory units [25]. In Apicomplexa, the presence of trans-regulating factors, such as TFIID, and the emergence of proteins containing nucleotide binding domains within the parasitic genome suggesting to the presence of such transcriptional regulation mechanisms in these organisms [1]. Studies by Vaquero et al. (2005) confirmed the action of such trans-regulating factors in *P. falciparum* in which a DNA binding protein, PfMyb1, was determined to be involved in a regulatory network of proteins required for parasite growth [26].

**(ii) Post-transcriptional regulation** is required for the completion of mRNA synthesis and occurs prior to export of transcripts from the nucleus. Three major processes which comprise the post-transcriptional alterations of pre-mRNA within the nucleus are the (i) acquisition of a 5’ cap structure, (ii) splicing out of introns and (iii) addition of a poly(A) tail. After the synthesis of approximately 30 nucleotides, the addition of the 5’ cap structure occurs in a series of steps [27]. First, the pre-mRNA 5'-triphosphate is hydrolyzed to a diphosphate by an RNA triphosphatase. Then, guanylyltransferase catalyzes the addition of a GMP molecule to the recently hydrolyzed nucleotide residing on the 5’ end of the pre-mRNA. In the final step of the reaction, a methyltransferase methylates the N7 position of the GMP, completing the cap structure [27]. Interestingly, both the RNA triphosphatase and the guanylyltransferase remain attached to the mRNA and act to recruit the cap binding complex (CBC) [27]. The CBC is believed to function
in the stabilization of mRNA by hindering the ability of 5'-3' exonucleases to access the mRNA.

The next processing event in eukaryotes is the splicing of non-coding sequences known as introns. Within the pre-mRNA several consensus cis-elements are present which are necessary for the splicing reaction. These elements are the 5' and 3' exon-intron junctions (indicated by |) which are marked by the consensus sequences of AG|GURAGU and YAG|RNNN (R: purine, Y: pyrimidine), respectively [27]. Furthermore, a branchpoint is present approximately 100 nucleotides upstream of the 3' exon-intron junction which contains a highly conserved adenosine [27]. Splicing occurs in two distinct steps and is catalyzed by the spliceosome which is comprised of five small nuclear RNAs assembled with other proteins [27]. These components are essential in the assembly, stabilization and activity of the spliceosome complex, resulting in the production of a pre-mRNA molecule comprised of exons alone.

The final step in nuclear processing of mRNA is the addition of a 3' polyadenylated tail consisting of approximately 200 adenine residues. Prior to the addition of poly(A) tail, the pre-mRNA must be cleaved at a site present between a highly conserved AAUAAA sequence and a uracil or GU-rich motif known as a downstream sequence element (DSE) [27]. This cleavage is performed by two multisubunit protein complexes in association with two essential factors, cleavage factors I and II, which direct cleavage of the pre-mRNA. Poly(A) polymerase, the enzyme catalyzing the addition of the poly(A) tail, is usually required within the cleavage reaction complex and directs the poly(A) addition. Poly(A) binding protein II then binds to the emerging poly(A) tail and also enhances the processivity of the poly(A) polymerase [27]. Once all
three processes are complete, the now mature mRNA can be exported from the nucleus to the cytoplasm where it is capable of either undergoing active translation or becoming translationally repressed.

1.6 Emerging paradigm of Gene regulation, cytoplasmic regulation of mRNA

Once messenger RNA (mRNA) is exported from the nucleus into cytosolic compartments, the regulation of gene expression is further maintained through the processes of (i) translation, (ii) mRNA degradation (decay) and (iii) mRNA storage. The fate of transcripts, whether they are actively translated, degraded or stored is controlled and reflected by the composition of messenger ribonucleoprotein (mRNP) complexes which interact with mRNAs [28]. The flux of mRNP remodeling directs cytoplasmic mRNAs among complexes such as (i) the polysome, for the translation process, (ii) processing bodies (PBs) and (iii) stress granules (SGs) for degradation and storage, respectively [29]. The composition of mRNPs play a vital role in dictating the fate of transcripts with major mRNP remodeling occurring throughout the mRNA cycle. mRNAs may be actively shuttled between each of the three mRNPs, resulting in dynamic alterations in mRNP composition. An illustration of this dynamic cycle is shown below (Figure 1.1).
Figure 1.1 Cytoplasmic regulation of mRNA fate

Cytoplasmic mRNAs are engaged in three possible cellular processes (i) active translation, where polysome structures are formed, (ii) degradation via processing bodies and (iii) storage in stress granules. The image was modified from [30].
(i) Regulation of translation

During translation, the 40S and 60S ribosomal subunits are recruited onto mRNA transcripts by a number of eukaryotic translation initiation factors (eIFs) [31]. The recruitment of factors, referred to as the initiation phase, can be directed in either a 7-methylguanylate (m\(^7\)G) 5′-cap dependent or independent manner. During cap-dependent initiation the m\(^7\)G 5′-cap structure is recognized by the initiation factors eIF4F, eIF4E and eIF3 which then recruit the 40S ribosomal subunit [31]. The dependency on the m\(^7\)G 5′-cap structure allows for fine-tuning of gene expression by enhancing or repressing the assembly and activity of the cap-binding complex through the reversible phosphorylation of translation factors. Conversely, during cap independent translation an RNA structure, referred to as an internal ribosome entry segment (IRES), aids in the assembly of translational machinery at a position close to the initiation codon [31]. In either form of initiation, once the ribosomal subunits are recruited, a downstream scanning for the translation initiation codon (AUG) occurs and translation of a nascent polypeptide is performed. These repetitive rounds of translation initiation, elongation and termination occur in the mRNPs referred to as polysomes [32].

(ii) Regulation of mRNA decay

Cytoplasmic mRNA turnover is a vital aspect of cellular physiology and is a key factor in gene expression. In eukaryotes, the degradation of mRNA occurs by three cellular mechanisms: (i) endonucleolytic cleavage, (ii) specialized quality control pathways or (iii) deadenylation dependent 3′ to 5′ decay [33].

Endonucleolytic cleavage is the degradation of mRNA transcripts without the prior need for transcript deadenylation. In yeast, mRNAs stalled in translation elongation
were shown to be recognized and targeted for endonucleolytic cleavage involving proteins similar to translation termination factors such as the eukaryotic release factor 3 (eRF3) [34]. This type of mRNA cleavage is also responsible for RNA-mediated gene silencing (RNAi), resulting in the formation of 21-23 nucleotide RNA species that are used in the targeting of the RNAi-induced silencing complex [33]. To monitor mRNA biogenesis, quality control mechanisms occur in eukaryotic cells which cause the rapid degradation of aberrant mRNAs [33]. These aberrant mRNAs are identified as those transcripts with defective structures within the 5'-cap, start/stop codons or poly(A) tail. For transcripts exhibiting a premature stop codon, non-sense mediated decay (NMD) occurs which is the process of mRNA degradation without the prior necessity of poly(A) tail shortening [33]. This process is responsible for the reduction of truncated proteins. Similarly, the decay of transcripts with an absence of a stop codon is performed via nonstop decay, which directs target mRNA to the exosome [33].

Deadenylation dependent degradation of mRNA occurs via two pathways, both of which begin by the shortening of the poly(A) tail by the action of deadenylases [33]. In yeast, the first pathway results in the degradation of mRNA in a 3' to 5' direction catalyzed by the exosome. The exosome is a large complex of exonucleases which function in RNA degradative and processing events [33]. In the second pathway, following the shortening of the poly(A) tail, the decapping of the m^7G 5'-cap structure is performed by a holoenzyme consisting of Dcp1p and Dcp2p enzymes. The decapped transcripts are then digested by a 5' to 3' exonuclease [33]. Mutagenesis resulting in the loss of either of the subunits, Dcp1p or Dcp2p, results in a complete loss of decapping in vivo, identifying the necessity of the holoenzyme in this pathway [34]. Furthermore,
Dcp2p was identified as the catalytic subunit required for decapping while Dcp1p acts to stimulate the activity of Dcp2p by increasing substrate interaction [34]. The activity of Dcp1p/Dcp2p results in the release of $m^7$GDP, leaving a product consisting of a 5'-monophosphate bound to mRNA [35]. This product is the preferred substrate for exonucleases, resulting in degradation of transcript. The expression of decapping enzymes and presence of aggregated decapping factors within sub-cellular structures in many organisms suggests that this mechanism is conserved throughout eukaryotes.

Decapping of mRNA is controlled by three major factors; the length of the poly(A) tail, the translation status of the transcript and the ability of mRNA to assemble a decapping complex [34]. The commonality of all these factors is the influence of mRNA-specific binding proteins which regulate decapping by either stimulating or inhibiting the action of enzymes. The action of PABP, specifically its ability to protect the poly(A) tail from degradation, inhibits decapping and subsequent mRNA degradation. PABP is believed to act in decapping inhibition by two discrete mechanisms. Firstly, its physical interaction with eIF4G, a component of the cytoplasmic cap-binding complex, may inhibit decapping by enhancing mRNA translation. Interestingly, this stabilization of the cytoplasmic cap-binding complex alone has been shown to inhibit decapping as well [36]. The second mechanism of inhibition stems from *in vitro* studies which suggested that PABP can bind directly to the cap structure and actively inhibit decapping [37]. The second factor influencing decapping of mRNAs is the assembly of various protein complexes which act to enhance the interaction of the decapping enzyme [34]. For example, complexes in yeast containing Lsm1-Lsm7 proteins, Pat1p and Dhh1p have been shown to enhance the decapping action of the Dcp1p/Dcp2p holoenzyme by aiding
in the proteins recruitment to target mRNA [38]. For decapping to occur, there must be a transition of the mRNA from a translationally active complex to an mRNP complex that lacks translation factors. The dissociation of the cap-binding complex marks the transition from active translation to transcript decay. This is followed by the formation of specific cytoplasmic foci, referred to as processing bodies (PBs), where decapping factors and 5' to 3' exonucleases are recruited [34].

(iii) Regulation via RNA storage in sub-cellular structures

**Processing bodies:** PBs are constitutive cytoplasmic aggregates containing translationally repressed mRNPs. These RNA granules consist of non-translating mRNAs and interacting proteins involved in translational repression, mRNA decay and RNA interference [30]. Transcripts appear to be transiently associated with PBs, and if not degraded, are released from PBs and incorporated back into active translation [28]. Transcripts could be targeted for 5’ to 3’ decay or remain translationally inactive, is dependent on variations in the composition of these PBs [28]. In humans for example, the interaction of Puf family members with the 3' untranslated regions (UTRs) of transcripts and the Pop2p subunit of the Ccr4/Pop2/Not complex (found within PBs), directs transcripts for repression and decay [29]. Similarly, the association of Dhh1 with mRNAs in yeast results in the shuttling of transcripts to PBs [39]. Although the complete composition of PBs has yet to be identified, PBs have core protein components consisting of (i) the decapping enzyme Dcp1p/Dcp2p, (ii) activators of decapping and (iii) a 5' to 3' exonuclease [29]. These core proteins can be referred to as the 5' repression-decay complex.
Prior to PB formation, transcripts are released from the polysomes. The subsequent remodeling of mRNP complexes produces an mRNP containing the 5' repression-decay complex [28]. In accordance with this, ribosomal subunits have not been identified in PBs. The treatment of cells with translation elongation inhibitors, such as lactimidomycin, prevents PB assembly and traps mRNP complexes within the polysome [40, 41]. In comparison, inclusion of 5' repression-decay complexes have been shown to be necessary for PB formation. The recruitment of this complex can occur either by an intrinsic mRNA affinity or through the action of mRNP specific factors [28]. Specific structural or sequence targets within the mRNA transcript may result in the recruitment of decapping factors and subsequent targeting to PBs. Alternatively, 5' repression-decay factors interact with mRNA-specific complexes involved in translation initiation inhibition or mRNA decay, resulting in PB assembly [33]. The importance of the 5' repression-decay complex has been exhibited by studies which show an enrichment of 5' decay factors within PBs as well as an increase in PB formation occurring from over expression of individual factors [28]. Also, depletion of factors which recruit the 5' repression-decay complex cause an impairment in PB assembly [28]. In addition to PBs, SGs have also been shown to play a vital role in mRNA regulation during cellular stress.

**Stress granules:** In contrast to PBs, SGs are transient cytoplasmic aggregates which occur in response to the cellular exposure to an environmental stress. Stresses, such as heat shock, result in a sudden arrest of translation which leads to polysome disassembly [42]. The disassembly and subsequent rearrangement of mRNPs could initiate the formation of SGs, which act as emergency storage locations for mRNAs. The exposure of cells to translation initiation impairment drugs, such as hippuristanol, and the
depletion of initiation factors can induce the formation of SGs [28, 42]. The core components of SGs, which act as markers for all SGs, include components of the small ribosomal subunit, translation initiation factors eIF3 and eIF4 and PABP [42]. The composition of the protein complex confirms that these granules are aggregates of mRNPs stalled in the translation initiation step [28]. Several other classes of proteins have been identified in SGs, including mRNA-binding proteins linked to translational silencing (i.e T-cell internal antigen-1; TIA-1) and RNA decay (i.e argonaute proteins; AGO). However, these proteins are not present in all SGs [42]. According to Anderson and Kedersha (2007), the assembly of SGs has been divided into three distinct stages [42]. First, the stalling of translation initiation and the formation of the 48S mRNP is caused by either drug-induced inactivation of eIFs or stress-induced eIF2α phosphorylation. The formation of SGs is then enhanced by the interaction of SG-nucleators with the 48S mRNP during a process termed primary aggregation [42]. The transcripts then bind to other proteins which undergo protein-protein interactions amongst themselves during secondary aggregation, thereby allowing for the subcellular SG structures to become microscopically visible [42]. The fate of these repressed mRNAs is determined by their association with specific RNA-binding proteins present within the SGs. These transcripts could (i) remain stored, (ii) be reinitiated into translation or (iii) selected for decay. Although the direct observation of mRNA shuttling between PBs and SGs has yet to occur, several proteins such as Dhh1 are shown to be capable of shuttling in and out of these granules [30]. Furthermore, PBs and SGs have been seen to contain the same mRNA species [30]. Intriguingly, throughout this dynamic remodeling process, RNA helicases continually emerge in several stages of translational repression. These
proteins have also been shown to colocalize within both SGs and PBs and affect the stability of these granules [30]. These helicases may alter the composition of mRNPs or promote mRNP formation by their activity to remodel RNA-protein complexes, thereby dictating the fate of transcripts [30]. The subsequent alterations result in granule stability and finally the recruitment of specific factors to mRNPs [30]. These functions suggest the important role of these RNA helicases in mRNA storage, repression and subsequently, gene regulation.

1.7 Helicases

Helicases are nucleic-dependent NTPases capable of unwinding DNA or RNA duplex substrates [43]. Their functions are defined by their ability to translocate directionally along the nucleic acid phosphodiester backbone and to simultaneously unwind double-stranded regions by utilizing energy gained from ATP hydrolysis [44]. Helicases play vital roles in various cellular processes, including replication, translation, ribosome synthesis, RNA metabolism and nuclear export processes [45]. All helicases contain central core domains identified by signature motifs which couple NTP binding, NTP hydrolysis and the conformational changes in protein structure, resulting in DNA or RNA remodeling [44]. These signature motifs include: (i) the conserved motifs, Walker A and B, involved in the binding and hydrolysis of the NTP and (ii) an “arginine finger” that plays a key role in energy coupling to unwinding activity [44].

Using their consensus sequences and signature motifs, helicases, which are generally composed of multiple repeating units, are classified into different superfamilies, families, and subclasses (Fig. 1.2). The first tier of classification is based on the features of their central cores whether they contain (i) two RecA-like domains, (ii) six RecA-like
domains arranged in a (hexameric) toroid or (iii) six AAA$^+$ domains arranged in a
(hexameric) toroid [44]. To further categorize helicase members into different
subfamilies, two additional criteria were used. First, their enzymatic activities are towards
single and/or double stranded nucleic acids as their substrates [44]. The helicase members
that utilize single-stranded nucleic acids as their substrates are identified as α helicases,
whereas those using double stranded nucleic acids as their substrates are β helicases [44].
For example, the members of Superfamily 1 (SF1) are α helicases because they will
engage only single stranded nucleic acids, while the members of SF6 are β helicases [44].
Second criterion used for grouping helicase members is their translocation polarity. When
the helicase members translocate from 3’- to 5’-terminus, they are called type A
helicases. Those, that translocate from 5’- to 3’-terminus, are type B helicases [44]. With
these classification criteria, six helicase superfamilies are formed (Fig. 1.2).
Figure 1.2 Classification of Helicase Superfamilies

Helicases can be classified into six major superfamilies: SF1 and SF2 are composed of two repeating subunits of RecA-like domains while SF3-SF6 helicases are composed of 6 repeating subunits of either RecA-like or AAA\(^{+}\) monomers. Classifications are also based on translocation polarity (3'→5' as Type A or 5'→3' as Type B) and whether the nucleic acid substrate is single (\(\alpha\)) or double stranded (\(\beta\)).
The core domain structure is used in the identification of each helicase superfamily. Specifically, SF1 and SF2 helicases contain a core consisting of a tandem repeat of two RecA-like domains while SF3-6 helicases contain a core consisting of six individual RecA- or AAA+-like domains arranged in a toroid. The common features of these core structures will be further discussed and are illustrated below (Fig 1.3).

(Figure 1.3 Representative core structures of helicase superfamilies. (Left) SF1 and SF2 helicases contain a tandem repeat of RecA-like domains (N- and C-core). An NTP analogue (black) is shown at the interface of the core domains. The Walker A (1) and B (2) motifs are located on the N-core side of the cleft. Motif 6 contains the conserved arginine finger residue required for ATP coupling. (Right) SF3-6 helicases contain a core of six individual RecA- or AAA+-like domains in a toroid. This configuration results in six nucleotide-binding pockets; shown to be occupied with NTP analogues (black). H1 and H2 are equivalent to the Walker A and B motifs which are located on the opposite side of the cleft compared to the conserved arginine finger. This image was modified from [44].
1.8 Main characteristics of helicase superfamilies (SFs) based on their core domains

**Hexamer RecA-like domain:** SF4 and SF5 helicases contain six units of RecA-like domains, which are homologous to the bacterial recombinase-A (RecA) protein, arranged to form a barrel-like structure (Fig. 1.2, right panel) [44]. RecA was first identified in *E. coli* to function in the repair and maintenance of nicked DNA [46]. Among SF3 and SF4 helicase members, each RecA-like monomer contains three domains: (i) an amino domain which functions in RecA polymer formation, (ii) a central domain involved in nucleotide and NTP binding, and (iii) the carboxy domain which facilitates interfilament association [46]. The central domain contains two nucleotide binding regions, one for single and another for double stranded nucleotides, as well as one ATP binding region known as the phosphate binding loop (P-loop) [46]. The RecA-like hexameric structure is responsible for the forming of six nucleotide binding pockets, one per domain interface. It is noted that all members of SF4 and SF5 superfamily have the conserved hexameric core and type B translocation directionality [44]. Interestingly, there is no functional commonality among members. For example, SF4 helicases, which were first identified in bacteria and bacteriophages, share five conserved motifs (H1, H1a, H2, H3 and H4) and act as replicative helicases [47]. SF5 helicases, whose best example is Rho helicase, are functionally responsible for termination of transcription in bacteria [23].

**ATPases Associated with diverse cellular Activities (AAA**⁺**):** The core domain of SF3 and SF6 helicase members contain six subunits of AAA⁺ core domain organized in a toroid (Fig. 1.3, right panel) [44]. Notably, the proteins that contain AAA⁺ domain are also considered as members of P-loop NTPases [48]. In addition, the proteins containing
AAA\(^+\) domain have the Walker A and B motifs which are responsible for nucleotide binding and nucleotide hydrolysis [49]. These AAA\(^+\) domain containing proteins characteristically carries a conversed catalytic glutamate which is not seen in other NTPases [50]. The AAA\(^+\) domain is highly conserved and contains approximately 200-250 amino acid residues [48]. Three critical motifs were described within the AAA+ conserved domain and are known as (i) sensor 1, (ii) sensor 2 and (iii) 'box' sequences [51]. Sensor 1 contains a conserved polar residue, which is asparagine, threonine or histidine, to function as a nucleotide sensor for binding and hydrolysis by interacting directly with the \(\gamma\)-phosphate of ATP [50]. Sensor 2 contains a conserved arginine residue and functions in the binding and hydrolysis of ATP as well as the communication and movement between subunits [50, 51]. Finally, the "box" motifs are believed to function in ATP hydrolysis as well as intersubunit communication. SF3 and SF6 members vary in functions and non-central core structures [51]. SF3 helicases were originally identified in DNA and RNA viruses, such as parvo- and papillomaviruses, and are associated with activities such as origin recognition and unwinding [47]. These SF3 helicase members share four conserved motifs (A, B, B' and C) and exhibit type A translocational directionality [44]. On the other hand, the numbers of SF6 helicase members are limited. To date, SF3 has only the archeal and eukaryotic mini chromosome maintenance (MCM) protein complex and the RuvB proteins as the members of the superfamily [44, 52]. MCM is considered as the main eukaryotic replicative helicase and exhibits both A and B type directionality while RuvB proteins work as a dsDNA translocases which processes Holliday junctions [44].
**RecA-like domain:** SF1 and SF2 helicases contain two identical units of RecA-like domains which are connected by a flexible linker, forming a cleft which contains the ATP-binding site (Fig. 1.2, left panel) [53]. The N-core domain contains the conserved motifs I-III and the Q motif, while the C-core domain is composed of motifs IV-VI [54]. These motifs will be discussed in further detail in Section 1.10. Target nucleic acids become associated opposite the ATP-binding site and forms interactions which spans across both domains (Fig. 1.2, left panel) [44]. As shown by X-ray crystallography and various biochemical studies, the binding and hydrolysis of ATP is facilitated by the closure of the cleft between the two RecA-like domains, resulting in nucleic acid unwinding [55].

The structural features of SF1 and SF2 helicase members have continually been subjected to extensive analyses. For example, with intense structural analyses, the formerly known seven conserved motifs of SF1 were resolved to contain more unique motifs within, which gave rise to two SF1 subfamilies, names SF1A and SF1B. Notable, SF1 members utilize single-stranded nucleic acids as their substrate, while SF2 members could accept single or double stranded nucleic acids are their substrates. SF1 and SF2 exhibit A and B type directionality [44].

1.9 SF2 helicases

SF2 contains the largest number of characterized members which are complex by nature. The major subfamilies are: (i) DEAD-box RNA helicases, (ii) the RecQ-like family and (iii) the Snf2-like enzymes [44]. Uniquely, the members of SF2 subfamilies are capable of using either RNA or DNA as their substrates [53]. More specifically, DEAD-box helicases interact with RNA, while RecQ-like and Snf2-like helicases use
DNA as their substrates. Aside from sharing core structural feature, SF2 helicases contain auxiliary domains on their C- and N- termini to confer specificity or complementary catalytic activities [56]. More specifically DEAD-box RNA helicase subfamily play vital role in cellular activities including the disruption of mRNPs and the remodeling of RNA.

1.10 DEAD-box RNA Helicases

DEAD-box RNA helicases were first described by Gorbalenya et al. (1989), when a group of NTPases were defined based on sequence similarity to the eukaryotic initiation factor 4a (eIF4a) to contain several common sequence elements (Fig. 1.4) [57]. Since then, DEAD-box RNA helicases have been classified as the largest helicase family of SF2. The mode of RNA binding in DEAD-box RNA helicases is highly conserved throughout the family [58]. The two central core domains make a contact to its RNA substrate in a non-sequence-specific manner spanning over any consecutive five nucleotides [45]. The interaction involves exclusively the sugar backbone of the RNA. Bound to the enzyme, RNA is bent in a conformation which differs from those seen in most SF2 helicases [45]. In this single stranded RNA (ssRNA) conformation, the RNA undergoes a sharp bend due to ionic and hydrogen bond interactions with several motifs within domain 1 (Fig. 1.4) [59, 60]. The resulting ssRNA structure inhibits the formation of a double stranded helix, giving rise to RNA unwinding [60]. This local strand separation is unique to DEAD-box RNA helicases, as it abolishes the need for translocation in order for strand separation to occur [45].

Additionally, most DEAD-box helicases contain auxiliary domains known as N-terminal and C-terminal extensions (NTE/CTE, Fig 1.4). These extensions vary in length and composition from one protein to another and have been shown to interact with RNA.
or other proteins [55]. These interactions characterize specific DEAD-box RNA helicases, thereby targeting them for specific physiological functions [58]. Furthermore, these auxiliary domains have been shown to result in protein dimerization, regulate functional properties of the RecA-like core domains as well as contribute additional enzymatic activities [45].

**Figure 1.4 Conserved motifs of the DEAD-box RNA helicase subfamily**

DEAD-box RNA helicases are composed of 13 conserved motifs: motifs I-VI and the Q motif. Motifs interacting with RNA are boxed in white while those involved with ATP are in dark grey. Motif III which is involved in coupling ATP binding/hydrolysis with helicase activity is in light grey. The Q motif is known to be specific to the family of DEAD-box helicases while Motif II contains the conserved D-E-A-D amino acid sequence. NTE and CTE are the auxiliary N- and C-terminal domains, respectively. The domains were resolved from the yeast DEAD-box protein Mss116. This image was modified from [45, 61].
1.11 Structural features of DEAD-box RNA helicases

DEAD-box RNA helicases participate in diverse cellular functions in a wide variety of organisms, ranging from *S. cerevisiae* to *Homo sapiens*. Their functions have been implicated in almost all aspects of RNA metabolism including transcriptional control, nuclear mRNA export, translational regulation and RNA decay [58]. To be classified as a DEAD-box RNA helicase, the protein must harbor 13 conserved motifs (Fig. 1.3). Herein the nature and function of each conserved motif, namely the Q motif, motifs I, Ia, Ib, Ic, II, III, IV, IVa, V, Va, Vb and VI will be addressed [45].

**The Q motif** which was identified upstream of motif I and is comprised of a conserved nine amino acid sequence [62]. The sequence of this motif is as follows: GaccPohlQ; where "a" is an aromatic group, "c " is a charged group, "o" is an alcohol, "h" is a hydrophobic group and "l" is an aliphatic group [45]. This motif is characterized specifically by a conserved glutamine residue which is present in 99% of protein sequences analyzed [62]. Although the invariant glutamine residue is present in many helicases, several other conserved residues within the Q motif are only conserved among DEAD-box proteins. As a result, the Q motif is suggested to be specific to DEAD-box helicases [61]. This motif is necessary for efficient binding of ssRNA and conformational changes occurring due to nucleotide binding and ATP hydrolysis. Therefore it has been suggested that the Q motif acts as a sensor of the bound nucleotide and regulator of subsequent ATPase activity [45]. Furthermore, the base pair of the adenine nucleotide interacts specifically with the conserved glutamine residue, resulting in a strong preference for ATP over other nucleotide triphosphates [45].
**Motif I** (*AxTGxGKT*) is known as the Walker A motif is vital for both ATPase and helicases activity. It was found to function in NTP binding by coordination of a Mg$^{2+}$ ion as well as by forming hydrogen bonds with pyrophosphates of target nucleotides [63]. Mutational studies targeting the alanine, lysine or final threonine residues abolished ATPase activity and reduced the affinity for and rate of ATP hydrolysis [56]. Furthermore, this motif is crucial in regulating protein activity through interactions with the phosphate of nucleotides, motif II, motif III and the Q motif [56].

**Motif II** (*DEAD*) is known as the Walker B motif and is necessary for ATPase activity [63]. Along with the Walker A motif, these motifs are not only present in DEAD-box helicases, but are common to all helicases as well as numerous NTPases. Mutational studies reveal similar results as those seen with the Walker A motif, with abolished ATPase and helicase activity without altered RNA binding [56]. Furthermore, the presence of the amino acid sequence Asp-Glu-Ala-Asp in this motif inspired the name of this family of helicases.

**Motif III** (*SAT*) has been suggested to play a role in coupling ATP binding and hydrolysis with helicase activity [56]. This concept originated due to experimental mutations resulting in interrupted substrate unwinding without affecting ATP binding and hydrolysis. However, the role of this motif remains ambiguous due to mutations in motif III varying the impairment of protein function between members present in the SF1 and SF2 superfamilies [56].

**Motif VI** (*HRIGRTGR*) was determined to function in both ATPase and RNA binding activity and is located at the interface of domains 1 and 2. Mutational analysis resulted in the abolishment of helicase activity through the impairment of ATP binding and
hydrolysis [56]. These studies targeted the conserved histidine, the second arginine as well as the glutamine residues. Furthermore, it was determined that the second arginine indeed interacted with the gamma-phosphate of the ATP, confirming the motif function in the coupling of NTP hydrolysis with helicase activity [56].

**Motif Ia (PTRELA) and Ib (TPGR)** are present in domain 1 and do not participate directly in ATP binding or hydrolysis however, are necessary for RNA binding in association with motifs IV and V [56]. Although motifs I, II and II have been well characterized, the roles of motifs Ia, Ib, IV and V remain ambiguous, as the motifs have been less studied [45]. Studies by Schwer and Meszaros (2000) have shown that motif Ia may also participate in structural rearrangements facilitated by ATP binding and hydrolysis [64]. Motif IV is poorly studied, resulting an unclear consensus sequence for the motif. This motif is found at the bottom of domain 2 and has been suggested to play a role in the binding of ssRNA [56]. Finally, Motif V (TDxxARGID) is suggested to function in RNA-binding in conjunction with motifs Ia, Ib and IV. Studies of this motif have also implicated its role in signal transmission of RNA binding to the ATPase domain and subsequent regulation and hydrolysis of ATP. It was also determined that contacts do occur between motif Va and the ribose sugar, however the role of these contacts is unknown [56].

**1.12 Important homologues of DEAD-box RNA helicases**

With intense studies, there are hundreds of members documented. Herein three members of DEAD-box RNA helicases, whose structural and functional features are highly related to the newly characterized DEAD-box RNA helicase member described in this study, will be reviewed.
Yeast DEAD/H helicase-1 (Dhh1) is one of over twenty DEAD-box proteins identified in *Saccharomyces cerevisiae*. Dhh1 plays a vital role in mRNP remodeling during mRNA metabolism and decay [39]. Mutagenesis analysis has shown that Dhh1 knock-out mutants have (i) prolonged half-life of target mRNAs, (ii) accumulated deadenylated transcripts and (iii) the lack of removal of the 5’ m7G cap [39]. Furthermore, it has been that Dhh1 has the physical association with Pop2p (deadenylase) and Dcp1a in processing bodies [65]. Taken together, these studies strongly suggested the involvement of Dhh1 in mRNA turnover and its specific role in mRNA decay.

It has also been shown that Dhh1 is not only involved in mRNA decay, but also plays a role in the storage of mRNAs during environmental stress [66]. Under glucose deprivation, cellular localization of Dhh1 was within stress granules, therefore indicating a possible role in translational repression which does not involve mRNA degradation [66]. Furthermore, yeast cells whose Dhh1 expression was abolished showed deficient in stress granules formation [66]. Intriguingly, the presence of Dhh1 in both stress granules as well as processing bodies indicates a possible role in the shuttling of mRNAs between the two cellular compartments.

Conserved germline helicase-1 (CGH-1) is a DEAD-box helicase found in *Caenorhabditis elegans* and has been found to function in translational repression as well as mRNA degradation [67]. Biochemical analyses have shown that CGH-1 functions by associating with maternal mRNAs to protect them from degradation. These studies have shown that CGH-1 does not only play a vital role in translational repression of maternal mRNAs by means of storage in germline specific granules (P-granules) during oocyte development but also protects target mRNAs by enhancing mRNA stability [67].
Although the function of CGH-1 has been identified, the mode by which that function is performed remains elusive. A model describing the involvement of CGH-1 in maternal mRNA storage and repression has been hypothesized by Parker and Rajyaguru (2008) and may have implications pertaining to this study.

In this model, CGH-1 is involved in translational repression by forming mRNPs which accumulate in P-bodies. These cellular foci may result in the degradation of target mRNAs by the recruitment of decapping enzymes [67]. This concept is centralized around the ability of CGH-1 homologues, such as Dhh1, to interact with decapping enzymes and other components of P-bodies [66]. Furthermore, CGH-1 would transition mRNAs from P-bodies to stress granules where they may interact with proteins involved in translational activation. The evidence for this is the fact that CGH-1 has been purified in RNA-protein complexes including poly(A) binding protein and other proteins which are known components of stress granules [67].

*Development of zygote inhibited (DOZI)* is a DEAD-box member found in *Plasmodium berghei* and shown to function in translational repression in the female gametocyte [68]. Two specific mRNAs, p25 and p28, which encode proteins necessary for zygote development, were found to be translationally repressed within cytoplasmic foci until after gamete formation and fertilization [68]. Intriguingly, immunofluorescence studies identified the colocalization of DOZI within the same mRNP structures. Immunoprecipitation studies and subsequent analyses identified the interaction of substantial amounts of both transcripts, as well as many other transcripts, with transgenic DOZI protein [68].
Mutagenesis showed that DOZI null mutants exhibited a total loss of development of zygotes into mature ookinetes while maintaining normal production of gametocytes and gametes [68]. Furthermore, Northern analysis and oligonucleotide microarray studies showed the loss of p25 and p28 transcripts as well as the reduction of another 370 transcripts; many of which were involved in oocinete motility and invasion and previously shown to be translationally repressed [68]. Interestingly, 92 transcripts were shown to increase in abundance which may indicate further functions of DOZI in mRNA regulation [68]. Overall, the study identified DOZI as a central player in the repression and regulation of gametocytespecific transcripts. Furthermore, the loss of protein expression severely hindered the ability of the parasite to regulate mRNAs in the female gametocyte.

When the composition of the cytoplasmic foci was further analyzed, sixteen major protein factors were present within these structures, including: (i) PABP, (ii) CAR-I/Trailer Hitch Homolog (CITH), (iii) eIF4E, (iv) enolase and (v) Acetylation Lowers Binding Affinity proteins 1 and 2 (Alba 1 and Alba 2) [69]. These studies suggest that DOZI could act primarily in mRNA storage rather than degradation and is a translational repressor necessary for proper zygote development [69]. Furthermore, the study also suggests that such mRNPs are evolutionarily conserved in protozoans. The regulation of gene expression could be controlled by the formation of mRNPs to ensure the stability and storage of mRNAs.

1.13 DEAD-box RNA helicases in Toxoplasma gondii

During the complex life cycle of T. gondii there is a necessity for mechanisms to be in place which regulate gene expression during the parasitic exposure to various
environmental stresses throughout the continuous rounds of host invasion and egress. The discovery of DEAD-box RNA helicases as vital components in post-transcriptional regulatory pathways in several organisms has thereby lead to the current study of homologous RNA helicases in *Toxoplasma gondii*. Bioinformatic analyses have identified 81 putative proteins with functional implications in gene regulation; 20 of which contain conserved amino acid sequence motifs relating to the DEAD-box RNA helicase family. Another 26 proteins were identified as putative ATP-dependent RNA helicases due to the presence of both ATP and nucleotide binding regions. A summary of the DEAD-box and ATP-dependent RNA helicase genes can be found in Appendix A.

A member of the DEAD-Box RNA helicase family in *Toxoplasma* exhibits a high sequence homology to several highly characterized DEAD-box RNA helicases. These homologues, which were previously discussed above, play vital roles in the post-transcriptional gene regulation of target transcripts in their respective organisms. The amino acid sequence alignment of the proteins is shown in Figure 1.5. The RNA helicase exhibited a sequence similarity of 68% to Dhh1, 67% to CGH-1 and 81% to DOZI. Due to the high sequence homology to DOZI, this RNA helicase will be referred to as TgHoDI (*Toxoplasma gondii* Homologue of DOZI; gene ID Tggt1_089230).
<table>
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<th>Sequence</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>DHH1</td>
<td>N-NNFPNTNNSNDTDROWKTAALNFKKDRFRQTTDDVNLNTKGMTEFDYLYKRELLMGIEFAGGK</td>
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</tr>
<tr>
<td>TgHODI</td>
<td>----HVDDPNNSPEEGWSKKVLPKDNVKEGTPKDTEGDYLRRELLMGIEFAGGK</td>
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**Figure 1.5 Sequence alignment of DEAD-box members with the putative TgHoDI**

The amino acid sequences of Dhh1, CGH-1, DOZI and TgHODI were aligned using the Clustal Omega tool [70]. An asterisk (*) indicates a fully conserved residue, (:) indicates strongly similar properties while (.) signifies a weakly conserved residue. Conserved motifs are highlighted in grey with motif II (D-E-A-D) in green.
The possibility of TgHoDI to be involved in post-transcriptional gene regulation was further suggested in a study by Braun et al. (2010), which attempted to decipher the role of *Toxoplasma* homologue of Argonaute (TgAgo) in RNA silencing. This study identified TgHoDI (referred to by its gene ID 583.m00676) as one of four DEAD-box RNA helicases that immunoprecipitate with the Argonaute silencing complex [71]. Also, studies by Al Riyahi et al. (2006) found that stable knockdown TgAgo clones exhibited an impaired ability to silence target gene expression in comparison to wild type parasites; thereby identifying the involvement of Ago as a vital component in the double-stranded RNA-induced gene silencing in *T. gondii* [72]. The apparent association of TgHoDI with TgAgo, as shown by Braun et al. (2010), may therefore implicate the involvement of TgHoDI in post-transcriptional gene regulation in *T. gondii*.

1.14 Research Hypothesis and Objectives

TgHoDI is hypothesized to be a major player in post-transcriptional gene regulation as a central component of mRNPs in *Toxoplasma* due to its high sequence similarity to several vital DEAD-box RNA helicases as well as its involvement within an mRNA silencing complex. This study is the first to analyze a specific DEAD-box homologue in *Toxoplasma*. Specifically, the research is aimed at the determination of the involvement of TgHoDI in post-transcriptional gene regulation and possible involvement in the translational repression of mRNA.

The specific aims are as follows:

(i) To examine the genetics and expression profiles of TgHoDI through the use of bioinformatic analysis.
(ii) Determination of protein function through the analysis of transgenic parasite clones. These clones will be used in studies including:

    a) Immunodetection of cellular extracts to determine expression of TgHoDI during tachyzoite and bradyzoite stages.

    b) Immunofluorescence analysis for identification of protein localization.

    c) Fluorescent in-situ hybridization for localization with mRNA transcripts.

    d) Colocalization analysis with proteins involved in translational repression.

    e) Determination of alterations in TgHoDI localization as a result of drug treatments.

(iii) Complementation studies involving temperature sensitive yeast strains.
CHAPTER II - RESULTS

2.1 Toxoplasma gondii Homologue of DOZI (TgHoDI)

DEAD-box RNA helicases have been described as vital components in post-transcriptional gene regulation in several organisms. In Toxoplasma, a DEAD-box member, which has the highest sequence homology to the proteins CGH-1, Dhh1 and DOZI, hereby referred to as TgHoDI, was chosen as the subject of this study. This study is aimed at determining the presence of mechanisms to regulate gene expression and the cytosolic mRNA fate in T. gondii. First, bioinformatic analyses were performed using Toxodb.org to uncover essential information pertaining to TgHoDI. The information obtained was subsequently used in the design and construction of recombinant TgHoDI reporter plasmids.

The genomic sequence in the TgHoDI locus spans either 4028bp (type I) or 4037 bp (type II and III), of all 3 parasite strains on chromosome XI. Within the TgHoDI locus 5 exons and 4 introns were found, with the 5 exons encoded by a 2073 nucleotide sequence which also contains UTRs of 339 and 306 nucleotides at the 5’ and 3’ ends, respectively. The locus of TgHoDI is annotated as Tgg1_089230 for type I, TgME49_313010 for type II and TgVeg_097040 for type II parasites [15]. The illustration of the genomic sequence and splicing of TgHoDI for type I Toxoplasma, which will be used in this study, is shown in Figure 2.1. The genomic sequence which spans from 2,642,433 to 2,646,460 on Chr. XI results in a coding sequence (CDS) of 1428 base pairs and encodes a 475 amino acid protein with a calculated molecular weight of 53.462 kDa and an isoelectric point of 8.58. An alignment of the nucleotide and corresponding codon sequence for TgHoDI is shown in Figure 2.2. Furthermore, the
derived amino acid sequence is 475 amino acids in length and similar in all strains with only one amino acid variation observed at the 70th residue (Fig. 2.3, highlighted in red). In type I this residue is an asparagine while in type II and III it is a serine.

**Figure 2.1 Genomic organization of TgHoDI**

The 1.42 Kb coding sequence of TgHoDI is the result of the splicing of 5 exons from the genomic sequence which spans from 2,642,433 to 2,646,460 (4.0 Kb) on chromosome XI. Open boxes represent exons in the genomic sequence while introns are shown as connector lines. The genomic location of exon flanks is illustrated by values above the exon-intron junction; i.e exon 1 spans from 2,645,840 to 2,646,460.
Figure 2.2 Nucleotide and Codon sequence of *T. gondii* DEAD-Box protein TgHoDI

Translation of nucleotide coding sequence to the corresponding amino acid sequence for TgHoDI. Analyses was performed using Nucleotide Amino Acid Alignment (NAP) software. (|) Indicates interruption of ORF prior to last threonine for the addition of sequence tags for functional analysis in the study.
**Figure 2.3 Amino acid alignment for TgHoDI protein in three T. gondii lineages**

The amino acid sequences of TgHoDI for the three clonal strains of *T. gondii* were aligned using the Clustal Omega tool [70]. An asterisk (*) indicates a fully conserved residue, while (.) signifies a weakly conserved residue. Variation in amino acid sequences is highlighted in red while putative phosphorylation sites are in grey [14, 15].
Gene expression profiles revealed mRNA levels of TgHoDI equal in all three clonal strains. However, further analysis of mRNA expression levels of TgHoDI for the different asexual stages was conducted only in the type II (Me49) clonal strain [15]. Due to the high sequence similarity between the three strains, which has been previously addressed, the data obtained may be taken as a representation of expression in the type I (RH) strain used in this study. The transcript levels remain relatively unaltered during intracellular/extracellular conditions as well as during bradyzoite differentiation. Furthermore, variation in mRNA expression was only identified in unsporulated oocysts, which exhibited a decrease in mRNA levels.

Microarray data of all examined samples identified the presence of 110 SNPs, of which 106 were synonymous and four non-synonymous, all within the CDS [15]. Furthermore, SNP analysis of the three clonal lineages identified the presence of 35 SNPs (five in the CDS, two in 5' UTR), only one of which is non-synonymous. Interestingly, no identified SNPs were found for analysis of type II and III parasite lineages. This data correlates with the high amino acid sequence similarity observed throughout clonal strains. Three possible phosphorylation sites were identified through mass-spectrometry at serine residues at the 15th, 20th and 24th amino acid position within type I (RH) strain (Fig. 2.3). These residues are conserved among the three strains and suggest post-translational modifications implicated in protein function. A summary of the variations of TgHoDI in the three strains are presented in Table 2.1.
<table>
<thead>
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<tbody>
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<td>Tgg1_089230</td>
</tr>
<tr>
<td>Chromosomal location</td>
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</tr>
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<td></td>
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<tr>
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<tr>
<td>Isoelectric Point</td>
<td></td>
</tr>
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</table>

**Table 2.1 Summary of Bioinformatic data on TgHoDI in three clonal strains**

Data obtained from ToxoDB database comparing several characteristics of the DEAD-box RNA helicase TgHoDI in *Toxoplasma* [15]. The three clonal strains show high sequence similarity at the transcriptional and translational level with almost identical coding sequence lengths, amino acid composition and isoelectric points.
2.2 Generation of Transgenic *Toxoplasma* strains expressing recombinant tagged TgHoDI at physiological levels

To establish a system allowing for the functional study of TgHoDI, we adopted to C-terminally tag with (a) a streptavidin-FLAG tag (SF; 21 a.a., 8.42 kDa) and (b) yellow fluorescent protein (YFP; 239 a.a., 29.56 kDa). Moreover, to access the level of expression at normal physiological levels, the tag sequence was introduced into the chromosomal locus by homologous recombination (knock-in) in the ΔKu80 parasite strain. This strain was instrumental for the generation of knock-in mutants because the Ku80 protein, which functions in non-homologous end-joining pathways, was removed and therefore the ΔKu80 strain allows for only homologous recombination [73]. The nucleotide sequence encoding the protein tags were inserted prior to the last codon (ACA-threonine) in the open reading frame (Fig. 2.2, indicated by |). Due to the fact that commercial antibodies against DEAD-box proteins in *T. gondii* are unavailable, the presence of an epitope tag would allow for the immunological studies necessary to decipher the possible role of TgHoDI.

To isolate and identify parasite clones stably expressing SF and yellow fluorescent protein (YFP) PCR analyses for the presence of designed sequences within the genomic DNA was performed using four specific oligonucleotide primer sets (Appendix B). Two sets of oligonucleotides either (a+b' and b+b') or (a+c and b+c) allowed for the confirmation of recombinant genomic sequences for TgHoDI-SF and TgHoDI-YFP, respectively (Fig. 2.4). The oligonucleotides (b') and (c) specifically amplified the sequences present within the 5' region of the recombinant protein tags and provided a means of identification of clones by the amplification of recombinant bands at
~3 kb and ~4 kb, corresponding to the introduced nucleotide sequence, which were not present in the parental strain (Fig. 2.4). The use of two oligonucleotides (a) and (b) upstream within the coding region of TgHoDI allowed for the confirmation of integration of recombinant sequences into the genome, rather than transient expression of the recombinant plasmid. The combination of these oligonucleotides with the oligonucleotide (a’), which targets the 3’ region of TgHoDI, were used as internal controls and resulted in bands at ~3 kb and ~3.5 kb for both parental and transgenic strains.

**Figure 2.4 PCR analysis of transgenic T. gondii strains expressing recombinant TgHoDI**

ΔKu80 parasites were genetically modified to contain transgenic sequences allowing for the analysis of endogenous TgHoDI protein. *(Top)* Illustration of transgenic and parental genomic sequences with PCR primer binding regions for the three parasite clones. *(Bottom)* PCR analysis for the confirmation of recombinant sequence integration into the T. gondii genome. Amplification of bands of ~3 and 4 kb in transgenic clones, and not in the parental ΔKu80 strain, confirmed successful tagging of TgHoDI protein.
To monitor the expression of recombinant protein, positive clones and parental strains were analyzed using appropriate antisera (Fig. 2.5). When parasite lysates were analyzed with α-GFP and α-FLAG antisera, bands were observed at ~83.0 kDa and ~61.9 kDa corresponding to the expected sizes for TgHoDI-YFP and TgHoDI-SF protein, respectively (Fig. 2.5). Lactate dehydrogenase-1, ~35 kDa, was used as a loading control in the analyses. This data confirms the expression of recombinant protein and supports the results obtained from PCR analyses.

![Figure 2.5 Western blot analysis for confirmation of transgenic TgHoDI protein](image)

**Figure 2.5 Western blot analysis for confirmation of transgenic TgHoDI protein**

Immunoblot of stable transgenic strains expressing TgHoDI-SF and TgHoDI-YFP protein. The presence of bands at ~61.9 and 80.0 kDa in transgenic strains, and not the parental ΔKu80, confirm successful expression of recombinant TgHoDI-SF and TgHoDI-YFP, respectively. Antisera against lactate dehydrogenase-1, ~35 kDa, was used as a loading control for analyses.

2.3 Gross analysis for newly generated parasite strains in comparison to the parental ΔKu80 strain

2.3.1 Doubling time

To determine the effects of recombinant tagged protein on the growth of *T. gondii* parasites, transgenic clones were analyzed in comparison to the parental ΔKu80 strain. Equivalent number of parasites were allowed to infect HFF monolayers and growth was analyzed for various periods. The number of parasites in a total of 100 vacuoles were counted per study, with analyses performed in triplicate for each of the three clones. After
24 hours it was observed that transgenic and parental clones exhibited similar replication patterns with almost half of the total number of vacuoles containing eight parasites (Fig. 2.6). Approximately 50.3% of ΔKu80 parental vacuoles, 45.7% of SF-tagged and 45.0% of YFP-tagged clones contained 8 parasites. This gives an average doubling time of approximately 8 hours for all monitored clones, which is similar to previously reported values [1]. Similarly, after 36 hours almost half of the total vacuoles analyzed contained 16 parasites, reinforcing the approximation of an 8 hour doubling time. At 36 hours, approximately 50.7% of ΔKu80 parental vacuoles, 46.3% of SF-tagged and 46.7% of YFP-tagged clones contained 16 parasites. The similarity in parasite growth when comparing transgenic and parental strains demonstrates that the addition of protein tags had minimal effect on the ability of transgenic parasites to duplicate.

2.3.2 General protein expression

To determine if the addition of protein tags altered parasite phenotypes, the expression of LDH1 was used to monitor variations in parasite metabolic processes for tachyzoite transgenic strains (Fig. 2.7). The level of expression was analyzed as a percentage of α-tubulin (Tub) expression. α-Tubulin is a structural component of the parasite and therefore was used as a baseline for comparison. For the parental ΔKu80 strain, the relative expression of LDH1 was found to be ~93.6% with SF and YFP strains exhibiting relative expression values of ~91.1 and 81.6%, respectively. These variations of 2% and 12%, for SF and YFP respectively, indicate minimal variation in metabolic processes for the transgenic strains. Furthermore, these values indicate that the size of the tag does not greatly influence the cellular phenotype of the parasites.
Figure 2.6 Growth analysis of transgenic and parental clones at 24 and 36 hours.

Parental (ΔKu80) and transgenic strains were allowed to infect HFF monolayers and the number of parasites were counted per vacuole 24 and 36 hr post-infection. At 24 hours, vacuoles for both parental and transgenic clones were found to contain 8 parasites. At 36 hours post-infection, the majority of vacuoles contained 16 parasites. Analyses were performed in triplicate with standard deviation shown (error bars).
2.3.3 Ability to differentiate

The addition of tags could alter the ability of parasites to undergo tachyzoite to bradyzoite differentiation. We thus used a three day CO2 starvation and alkaline treatment protocol to induce differentiation of newly generated clones in comparison to the parental. Three days post-infection, parasite cultures were harvested and Western blot analyses performed. Antisera against BAG1 were used to determine parasites differentiation to the bradyzoite stage. Once again, expression of BAG1 was compared to the level of Tub expression. Parental ΔKu80 parasites were found to have a relative expression of ~26.4% while transgenic SF and YFP clones exhibited levels of ~59.7% and 64.1% (Fig. 2.7). This translates into a 2.26 and 2.46 times increase in BAG1 expression for SF and YFP strains in comparison to the parental strain, indicating an overall increase in bradyzoite differentiation for transgenic clonal parasites.

To compare the TgHoDI levels between the two asexual stages of Toxoplasma, the relative expression of recombinant protein was analyzed as a percentage of α-tubulin in tachyzoite and bradyzoite stages. In tachyzoite parasites, SF and YFP transgenic clones exhibited relative expression levels of 83.9% and 83.4%, respectively. In comparison, bradyzoite parasites were found to express relative TgHoDI levels of 58.3% and 60.7% for SF and YFP transgenic clones, respectively (Fig 2.7). For tachyzoite parasites there is a calculated 1.43 and 1.37 time increase in TgHoDI expression for the SF and YFP clones when compared to their counterparts in the bradyzoite stage. Due to the lack of commercial antisera against endogenous TgHoDI, expression levels could not be compared to the wild type ΔKu80 strain.
Figure 2.7 Comparative protein expression for determination of possible phenotypic variations between transgenic and parental *Toxoplasma* strains

Parasite lysates were subjected to Western blot analysis and subsequent densitometric analysis to determine the affects of adding a protein tag on parasite cellular processes. Protein densities were compared as relative levels with the structural protein α-tubulin as the baseline for analyses. LDH1 levels are not effected in transgenic clones while bradyzoite differentiation appears to be amplified in these parasite strains. Studies were performed in triplicate with error bars representing standard deviation obtained. Statistical analysis was performed via two-tailed student t-test with p<0.05 with (*) indicating significant variation among analyzed samples.
2.4 Attempt to control the level of TgHoDI via ligand induced degradation

To determine the function of TgHoDI in Toxoplasma, it was necessary to develop a system to alter the level of TgHoDI protein. Phenotypic variations could then be monitored as a function of changes in protein level and function. To establish a system for functional studies, TgHoDI was fused to a ligand-controlled destabilizing domain (ddFKBP, 20.75 kDa). This system provides rapid, reversible and fine-tuned regulation of protein levels due to selective stabilization of the tagged protein by the reversible binding of a synthetic ligand, referred to as Shield-1. In the absence of ligand, the ddFKBP causes degradation of tagged protein because of an inherited instability conferred by the fusion protein from the ddFKBP. The addition of a synthetic ligand stabilizes the ddFKBP and shields it from degradation [74]. The coding sequence of ddFKBP was induced between streptavidin and FLAG to allow the read through translation to give TgHoDI-strep-ddFKBP-FLAG (Fig. 2.8). As with previous recombinant proteins constructed in this study, the tag sequence was introduced into the locus within the parental ΔKu80 parasite strain, resulting in a C-terminally tagged fusion protein (Fig. 2.8).

To identify parasite clones stably expressing tagged protein, positive clonal parasites were analyzed using appropriate antisera (Fig. 2.8). Analysis of parasite lysates with α-FLAG antisera revealed a single band at 74.20 kDa corresponding to the expected sizes for TgHoDI-DDSF (Fig. 2.8). Tub, 50.11 kDa, was used as a loading control in the analyses with TgHoDI-SF (61.9 kDa) as a positive control. This data confirms the expression of recombinant protein in transgenic clonal Toxoplasma strains.
To determine if the fusion of ddFKBP to TgHoDI allowed for fine-tuned manipulation of protein expression, the confirmed transgenic clones were first analyzed in the presence and absence of Shield-1 ligand (1 μM). For this preliminary experiment, transgenic parasites, which were sub-cultured previously in media containing Shield-1, were grown in the absence of ligand for 2 passages (~4 days) and parasite lysates were subjected to Western blot analysis. The Antisera against α-FLAG revealed bands corresponding to TgHoDI-DDSF in both treated and untreated samples (Fig. 2.9). This demonstrated the inability of the ddFKBP to destabilize and cause subsequent degradation, of the recombinant protein. TgHoDI-SF was once again used as a positive control. As conducted previously, the relative density of recombinant proteins was compared as a percentage of α-Tubulin expression. It was observed that the absence of Shield-1 had little effect on the expression TgHoDI-DDSF protein, as demonstrated by relative densities of 66.5% and 63.1% for treated and untreated parasites, respectively. Furthermore, these values are comparable to those obtained for the TgHoDI-SF clones (59.9%) in the study. The results obtained demonstrate an inability of the C- terminally fused ddFKBP to destabilize TgHoDI protein. However, this inability to alter protein levels, which may be attributed to the C- terminal placement of the destabilizing domain on TgHoDI, may hint to protein function in cellular processes. Unfortunately, the results obtained indicate that the destabilizing domain system cannot be used for subsequent phenotypic analysis for variations of protein expression.
ΔKu80 parasites were modified to express a TgHoDI-DDSF fusion protein for the manipulation of protein expression. (Top) Illustration of transgenic coding sequence of TgHoDI-DDSF with ddFKBP flanked by streptavidin and FLAG tags on the N- and C-terminus, respectively. (Bottom) Immunoblot of stable transgenic strains expressing TgHoDI-DDSF protein. Antisera against FLAG identified the presence of a single band at 74.2 kDa in two transgenic clones (1 and 2) confirm successful expression of recombinant protein. TgHoDI-SF, 61.9 kDa, was used as a positive control. Antisera against α-tubulin, 50 kDa, was used as a loading control for analyses.
Figure 2.9 Analysis of transgenic TgHoDI expression in the absence of Shield-1

Transgenic parasite lysates were subjected to Western blot and subsequent densitometric analysis to determine the ability of Shield-1 ligand to stabilize recombinant protein. (Top) Western blot of TgHoDI-DDSF stable clones in the presence and absence of Shield-1 at increasing protein concentrations (right to left). TgHoDI-SF was used as a positive control for the analyses. (Bottom) Densitometric analyses of recombinant protein levels as a function of α-tubulin expression. The absence of Shield-1 does not cause the destabilization of TgHoDI, thereby exhibiting an inability to control protein expression.
2.5 Cellular localization of transgenic TgHoDI-SF

To decipher the role of TgHoDI in *Toxoplasma*, immunofluorescence assays (IFA) were performed to determine the cellular localization of TgHoDI protein utilizing transgenic clones expressing TgHoDI-SF. The localization of TgHoDI will assist in analysis of protein function by revealing cytoplasmic or nuclear association in the parasites. Dhh1 and DOZI, TgHoDI homologues, which function in translational repression, were found to localize within cytoplasmic mRNP granules. It is necessary to determine if TgHoDI exhibits comparable cellular localization, which may imply similarities in cellular function. Intracellular transgenic tachyzoite parasites expressing TgHoDI-SF protein displayed perinuclear labeling with a granular pattern dispersed throughout the cytoplasm (Fig. 2.10). Extracellular parasites showed a distinct pattern with highly localized foci of TgHoDI-SF protein (Fig. 2.10). Numerous foci are observed throughout the parasite cytoplasm, however it appears that distinct aggregates are present at the apical end of the parasites. These observations vary significantly from those for intracellular tachyzoites where TgHoDI appears dispersed throughout the cytoplasm. Furthermore, distinct aggregates appear to be localized surrounding the nucleus, which is not observed in intracellular parasites. Interestingly, these results coincide with previously described observations of cytoplasmic granules induced in extracellular parasites as a response to environmental stress [75]. Bradyzoite parasites displayed a granular cytoplasmic localization, similar to intracellular tachyzoite parasites, however distinct foci were also observed at the basal end of the parasites (Fig. 2.10). Furthermore, cytoplasmic foci appear to be larger than their counterparts in intracellular tachyzoite parasites.
Figure 2.10 TgHoDI localizes to the cytoplasm in intra- and extracellular Toxoplasma

Immunofluorescence assay of transgenic T. gondii clone expressing TgHoDI-SF. Parasites were immunostained with anti-FLAG and Rhodamine anti-mouse for detection of TgHoDI-SF (red) and nuclear staining was performed with Hoechst (blue). (Upper panel) Intracellular transgenic tachyzoite parasites display perinuclear labeling with a granular pattern dispersed throughout the cytoplasm. (Middle panel) Extracellular parasites showed a distinct pattern with highly localized foci of TgHoDI surrounding the nucleus. (Lower panel) Intracellular bradyzoite parasites display a granular cytoplasmic localization with large aggregates at the basal end of parasites. Images were acquired using a 100x oil objective. Scale bars represent 5 µm.
2.6 Fluorescent YFP-tag as an alternative to IFA for the localization of TgHoDI

The utilization of a fluorescently labeled protein would remove the necessity to perform IFA targeting TgHoDI protein. This in turn would simplify analyses by minimizing manipulation of samples and removing the need of multiple fluorescent antigens for ensuing colocalization studies. However, the fusion of a yellow fluorescent protein tag contributes an additional 21 kDa to the recombinant protein, therefore it is necessary to ensure that cellular localization is not altered. In comparison to TgHoDI-SF transgenic clones, TgHoDI-YFP intracellular tachyzoite parasites exhibit the same perinuclear granular cytoplasmic localization (Fig. 2.11). TgHoDI-YFP appears widely dispersed throughout the cytoplasm while being excluded from the nucleus. Furthermore, the highly localized protein aggregates were observed for the extracellular parasites, with distinct foci present at the apical ends and surrounding the nucleus (Fig. 2.11). These results are similar to those obtained for the TgHoDI-SF transgenic clonal parasites. The cellular localization of TgHoDI-YFP for the intracellular bradyzoite parasites, appears to be a combination of both intra- and extracellular phenotypes. Although there are several distinct aggregates of protein at the basal end, TgHoDI also appears to be dispersed throughout the cytoplasm in smaller granular aggregates (Fig. 2.11). These observations imply that in comparison to the SF transgenic clones, the additional size attributed by the increased molecular weight YFP-tag does not alter the cellular localization of TgHoDI, therefore enabling the use of this transgenic clone in further functional studies.
Fluorescent microscopy of transgenic *T. gondii* clone expressing TgHoDI-YFP (*yellow*). After fixation, parasite nuclei were stained with Hoechst (*blue*) and imaged. *(Upper panel)* Intracellular transgenic tachyzoite display the same perinuclear granular localization of TgHoDI protein, as seen previously with TgHoDI-SF. *(Middle panel)* Extracellular parasites display highly localized foci of TgHoDI surrounding the nucleus and at the apical parasitic end. *(Lower panel)* Intracellular bradyzoite parasites exhibit granular cytoplasmic TgHoDI localization with large aggregates at the basal end of parasites. Images were acquired using a 100x oil objective. Scale bars represent 5 µm.
2.7 TgHoDI colocalizes with mRNA granules in extracellular *Toxoplasma*

To determine the possible involvement of TgHoDI in the formation of subcellular structures important to cytosolic mRNA fate, it was necessary to identify the location of TgHoDI in correlation to mRNA and subcellular structures. In *S. cerevisiae* Dhh1 has been shown to colocalize with cytoplasmic mRNA granules after induced environmental stress such as glucose deprivation [66]. As previously described by Lirussi *et al.* (2011), mRNA granules have been found to localize in punctate aggregates within the cytoplasm of extracellular parasites [75]. These aggregates are proposed to play a role in stress response and the translational repression of target transcripts [75].

Fluorescent *in-situ* hybridization (FISH) was performed on extracellular TgHoDI-YFP transgenic parasites using an oligo-dT-biotin probe which was subsequently conjugated to streptavidin-Rhodamine. In the extracellular parasites, mRNA appears to be dispersed throughout the parasite with a granular pattern observed (Fig. 2.12). Several distinct foci of mRNA aggregates appear within the cytoplasm surrounding the nucleus. These results are in agreement with the previous study by Lirussi *et al.* (2011) which identified distinct mRNA foci within the cytoplasm. TgHoDI-YFP localizes to distinct cytoplasmic aggregates excluded from the nucleus (Fig. 2.12). TgHoDI appears to colocalize with the mRNA granules at several distinct foci within the cytoplasm (as indicated by arrows) (Fig. 2.12). The colocalization of TgHoDI with these mRNA granules indicate the possible involvement in cytoplasmic regulation of mRNA. It is necessary to further verify whether TgHoDI is involved in regulation by means of translational repression or mRNA turnover.
Figure 2.12 TgHoDI colocalizes with mRNA granules in extracellular *Toxoplasma*

Fluorescent *in-situ* hybridization was performed extracellular parasite transgenic clone expressing TgHoDI-YFP (*false coloured green*). mRNA was targeted using oligo-dT-biotin which was subsequently conjugated to streptavidin-Rho (*red*). TgHoDI localizes to distinct subcellular foci while mRNA displays a granular localization throughout the cytoplasm. TgHoDI appears to colocalize with mRNA foci at the apical parasitic end as well as other aggregates in the cytoplasm (as indicated by arrows). Images were acquired using a 100x oil objective. Scale bars represent 5 µm.
2.8 TgHoDI colocalizes with the Stress Granule Marker PABPC3

The observation that in extracellular parasites TgHoDI colocalizes with mRNA granules led us to identify whether these structures are involved in mRNA degradation or translational repression. IFA analysis was employed to identify if TgHoDI colocalizes with poly(A) binding protein cytoplasmic-3 (PABPC3). Under normal cellular conditions, PABPs isoforms (i.e PABPC1, PABPC3) bind to the poly(A) tails of mRNA and regulates protein translation and mRNA stability [42]. Under environmental stresses, such as oxidative stress or heat shock, translational arrest is triggered, initiating the disassembly of polysomes [28]. Many proteins involved in normal processing events, such as PABPs, would assume emergency functions and become associated to subcellular aggregates known as stress granules [42]. As a result, proteins such as PABPC3 can be employed as markers to identify SGs under induced stresses [42]. Lirussi et al. (2011) showed an increase in cytoplasmic mRNA granules as a result of chemically induced stress [75]. These granules were also shown to contain aggregates of PABP. Therefore, it can be inferred that the mRNA aggregates observed (Fig. 2.11) are stress granules caused by the stalling of translational machinery, rather than sites for mRNA turnover.

When IFA was performed on extracellular parasites using antisera against PABPC3 in the transgenic TgHoDI-YFP strain, PABPC3 appears to be localized throughout the parasite. Several distinct foci appear dispersed throughout the cytoplasm with a large aggregate present at the basal parasitic end (Fig. 2.13). The localization of PABPC3 mimics that of total cellular mRNA previously observed (Fig. 2.13). Once again, TgHoDI-YFP appears to localize specifically within aggregates in the cytoplasm.
As observed for mRNA colocalization, there were several areas of colocalization between TgHoDI and PABPC3 (as indicated by arrows) (Fig. 2.13).

To verify the results obtained from fluorescent microscopy, which identifies the colocalization of TgHoDI with PABPC3 within these mRNA granules, confocal microscopy was employed. Confocal microscopy allows for a higher resolution as well as the ability to control the depth of field for acquired images. As expected, the colocalization of TgHoDI with PABPC3 is observed at distinct foci within the cytoplasm (Fig. 2.13). This further confirms the colocalization of TgHoDI with PABPC3 implicating TgHoDI as a component in stress granules. Therefore, it can be inferred that TgHoDI may function in the translational repression of transcripts in extracellular parasites during stress response.
Figure 2.13 TgHoDI colocalizes with PABPC3 in extracellular Toxoplasma

Immunofluorescence analysis was performed on the extracellular parasite transgenic clone expressing TgHoDI-YFP (false coloured green). (Upper panel) Fluorescent imaging of antisera against PABPC3 (red) exhibits a granular localization throughout the cytoplasm with distinct foci at the basal parasitic end. TgHoDI appears to colocalize with PABPC3 at the apical parasitic end as well as other aggregates in the cytoplasm (as indicated by arrows). (Lower panel) Confocal imaging of extracellular parasites verify the colocalization observed with traditional fluorescent microscopy. Fluorescent microscopy images were acquired using a 100x oil objective, with confocal images obtained with 63x oil objective. Scale bars represent 5 µm.
2.9 TgHoDI aggregates in extracellular Toxoplasma under Oxidative Stress

To determine if TgHoDI participates in stress response and is a member of RNPs formed under stress conditions, two chemicals, cycloheximide and arsenite were utilized for extracellular parasites. Cycloheximide treatment has been shown to prevent stress granule assembly and promote the disassembly of pre-formed stress granules [76]. Cycloheximide inhibits translational elongation and traps mRNPs into the polysome structure, thereby preventing the translocation of mRNA into stress granules [77, 78]. Lirussi et al. (2011) showed that arsenite treatment of extracellular parasites resulted in increased mRNA granule formation [75]. Arsenite induces oxidative stress which results in inhibition of translation and subsequent increase in granule formation [78].

To determine whether the granule structures in Toxoplasma can be influenced by cycloheximide treatment, extracellular TgHoDI-YFP parasites were treated with cycloheximide (50 µg/mL) for one hour. Parasites were subsequently analyzed using FISH targeting total cellular mRNA or IFA employing antibodies against PABPC3. After cycloheximide treatment, TgHoDI no longer appears to form cytoplasmic foci and is now dispersed throughout the cytoplasm (Fig. 2.14, center panels). For both mRNA and PABPC3, cycloheximide treatment appears to have a minimal effect, with the dispersed granular localization observed again. As previously observed for the control parasites, TgHoDI localized to distinct foci in the cytoplasm (Fig. 2.14, left panels). Both PABPC3 and mRNA appear dispersed throughout the parasite with several granular aggregates observed. An overlay of TgHoDI with either PABPC3 or mRNA exhibit identical results as previously observed with several distinct areas of colocalization.
To determine the effect of arsenite to induce granule formation, extracellular *Toxoplasma* parasites were treated with sodium arsenite (10 µM) for one hour. Once again, parasites were analyzed using FISH targeting total cellular mRNA and IFA employing antibodies against PABPC3. In comparison to the control, extracellular parasites treated with arsenite exhibited an increase in the size and number of TgHoDI aggregates within the cytoplasm (Fig. 2.14, *right panels*). An increase in the abundance of mRNA granules is observed dispersed throughout the parasite. This observation is in agreement with a previously described study by Lirussi *et al.* (2011) identifying the effects of drug treatments on mRNA granule formation in *Toxoplasma* [75]. Interestingly, PABPC3 does not exhibit the same drastic increase in aggregate size and number as seen for both TgHoDI and mRNA (Fig. 2.14). Overlay images of TgHoDI with either PABPC3 or mRNA exhibit similar areas of colocalization as previously observed, indicating involvement of TgHoDI in these mRNPs (Fig. 2.14). These observations demonstrate that TgHoDI is a component of stress induced granules in *Toxoplasma* and therefore may be a vital component in post-transcriptional gene regulation.
Figure 2.14 Treatment of extracellular parasites with chemical agents alters TgHoDI localization

IFA and FISH were performed on the extracellular parasite transgenic clone expressing TgHoDI-YFP (false coloured green). (Upper) Fluorescent imaging of antisera against PABPC3 and oligo-dT (red) for control parasites. TgHoDI-YFP extracellular parasites were treated with cycloheximide (50 mg/mL) (middle panels) or sodium m-arsenite (10 µM) (lower panels). Addition of drug treatments appear to alter cellular localization of TgHoDI. Images were acquired using a 100x oil objective. Scale bars represent 5 µm.
2.10 TgHoDI functionally complements Adh1 in S. cerevisiae

To evaluate whether TgHoDI could possess a comparable function as Dhh1, we sought to determine whether TgHoDI could complement the temperature sensitive (TS⁻) phenotype exhibited by Adh1 S. cerevisiae strains. In yeast, Dhh1 functions in mRNP remodeling during mRNA decay by interaction with target transcripts leading to activation of decapping [68]. Furthermore, under glucose deprivation Adh1 cells exhibited a reduction in stress granule formation, indicating its possible role in translational repression during environmental stress [67]. Knock-out studies by Chang et al. (2003) identified Dhh1 as a non-essential protein in yeast viability, however Adh1 strains exhibited impaired growth at both 30°C and 37°C [65]. The sequence similarity between Dhh1 and TgHoDI (69% identity) lead to the analysis of complementation experiments which may shed light on the physiological function of TgHoDI in Toxoplasma.

The Adh1 strain showed a TS⁻ phenotype at both 30°C and 37°C in comparison to wild-type (wt) which coincides with previously described observations (Fig. 2.15) [65]. The growth defect is seen to a greater extent for analyses conducted for 24 hours in comparison to the 48 hour time point (Fig. 2.15). Adh1 cells transformed with empty plasmid (negative control) showed identical growth to the non-treated Adh1 strain confirming the inability of parental vector to alter cell growth (Fig. 2.15). For the expression of Dhh1, cells were transformed with a low copy centromeric plasmid expressing Dhh1 from its endogenous promoter. At 24 hours, transformed cells were able to completely rescue the TS⁻ phenotype. Cells exhibited growth similar to the wt yeast strains at both 30°C and 37°C and the rescued phenotype was further exemplified after 48 hours in which transformed cells exhibited large colonies (Fig. 2.15).
To express TgHoDI in yeast, cells were transformed with a high copy number plasmid (2μm) expressing TgHoDI under the strong yeast GPD promoter. After 24 hours, transformed Δdhh1 cells exhibited a partially rescued TS− phenotype at both 30° and 37°C in comparison to wt and Dhh1 complemented strains (Fig. 2.15). At 48 hours the ability of TgHoDI to compliment the TS− was better observed as indicated by larger yeast colonies. Although the phenotypic rescue was observed at both temperatures, the ability of TgHoDI to complement yeast growth was observed to a greater extent at 30°C. In comparison to cells transformed with Dhh1, phenotypic complementation with TgHoDI appears to occur to a lesser extent, with fewer, smaller colonies observed. Taken together, these results suggest that TgHoDI may act as a functional homologue to Dhh1 in stress response. However, the deviation in TgHoDI’s ability to complement growth to wild type levels, in comparison to Dhh1, leaves open the possibility that TgHoDI functions in a similar but non-identical manner to Dhh1 in yeast.
Figure 2.15 Expression of TgHoDI complements TS⁻ phenotype of Δdhhl yeast strain

Δdhhl yeast strains were transformed with either an empty vector, Dhh1 or TgHoDI for complementation of TS⁻ growth defect. (Upper panel) At 24 hours, Dhh1 was able to restore growth to wt levels at both 30° and 37°C. At both temperatures, TgHoDI partially complements the growth defect of Δdhhl as demonstrated by smaller colony sizes in comparison to wt. (Lower panel) After 48 hours, Dhh1 is observed to fully complement the phenotypic growth defect at both temperatures.
CHAPTER III - DISCUSSION

TgHoDI belongs to the highly conserved DEAD-box RNA helicases which includes the *C. elegans* CGH-1, *S. cerevisiae* Dhh1 and *P. berghei* DOZI [65, 67, 68]. Over the past several decades, members of this protein family have been implicated in a diverse collection of cellular functions ranging from nuclear mRNA export to post-transcriptional gene regulation [55]. Knock-out studies have linked these proteins to a decrease in organism viability with major changes in cellular pathways involved in stress response and mRNP formation [65, 68]. The presence of these proteins in mRNPs in such an array of organisms indicates the involvement of these proteins as evolutionarily conserved components of mRNPs which function in the stability and storage of mRNAs. The current study is aimed to determine the possible involvement of TgHoDI in post-transcriptional gene regulation in *T. gondii*.

Bioinformatic analysis of TgHoDI indicated the importance of its expression to parasite viability. The 1428 bp coding sequence of TgHoDI encodes a 475 amino acids with a molecular weight of 53.46 kDa. Nine highly characterized motifs of the DEAD-box RNA helicase family were identified in TgHoDI, validating its classification within this subfamily (Fig. 3.1). The amino acid sequence is conserved among the three clonal lineages with only one residue difference observed (Fig 3.1). The asparagine residue in type I is changed to a serine for both type II and III strains. Whether this variation may be implicated in a altered protein structure or function was not deduced in the current study. However, the presence of a single amino acid difference suggests a necessary conservation in protein structure and function. Minimal variations in TgHoDI mRNA levels during intracellular/extracellular conditions as well as during bradyzoite
differentiation indicate its expression is required throughout the various stages of the parasite life cycle. The increase in mRNA levels during oocyst sporulation may suggest an additional role in oocyst development. TgHoDI mRNA levels were found to increase four days into sporulation and remain elevated for the remainder of the parasitic life cycle. Therefore, the expression of TgHoDI during early sporulation may be necessary for the regulation of transcripts throughout the remainder of this developmental stage. The DEAD-box RNA helicase DOZI in *Plasmodium* was found to be vital for the translational repression and regulation of developmental mRNAs throughout the early stages of zygote development [68]. This may suggest that TgHoDI functions similarly in mRNA regulation during oocyst development as DOZI in zygote maturation.

Three conserved serine phosphorylation sites are present in the N-terminal extension, indicating the possibility of post-transcriptional modifications (Fig. 3.1). The presence of these sites in the N-terminal extension, which is known to play a role in DEAD-box RNA helicase specificity and cellular function, may indicate the involvement of post-translational modification in protein function. Similar phosphorylation sites have been identified for other DEAD-box helicases, such as Dhh1, however the role of these sites in post-translational modification and protein function have yet to be determined [79]. Furthermore, in eukaryotes, including *Toxoplasma* and *Plasmodium*, the phosphorylation of eIF2α is a conserved mechanism of stress response which represses global protein synthesis and enhances formation of cytoplasmic mRNA granules [80]. This opens the possibility that similar phosphorylation mechanisms may be acting on TgHoDI during cellular responses thereby altering cellular function.
The amino acid sequence of TgHoDI contains the highly characterized DEAD-box motifs (highlighted in grey). Conserved sequences for these motifs is shown (Top). The three phosphorylation sites believed to be involved in functional modifications are identified on serine residues 15, 20 and 25. The single amino acid variation among the three clonal lineages is present at residue 70 (highlighted in green). Introduction of epitope tags was performed prior to the last threonine residue (shown by *).

Figure 3.1 Summary of bioinformatic data pertaining to TgHoDI amino acid sequence
The transgenic TgHoDI-SG and TgHoDI-YFP strains exhibited similar doubling time in comparison to the parental strain, which showed an approximate replication time of approximately 8 hours for the two transgenic clones, indicating that the introduction of epitope tag sequences did not hinder parasite replication. However, some variation in tachyzoite metabolism was identified when protein levels of LDH-1 were analyzed. Also, the levels of BAG1, a bradyzoite stage-specific marker, was induced in both SF and YFP transgenic clones (Fig 2.7). The change of BAG1 expression, which was found to be statistically significant, suggests that the addition of a C-terminal tag may interfere with the downstream activity of TgHoDI during bradyzoite conditions (Fig. 3.1, indicated by *). Linder \textit{et al.} (2011) identified the importance of N- and C-terminal extensions of DEAD-box RNA helicases for mRNA-protein and protein-protein interactions [55]. The addition of protein tags of ~8.4 and ~29.6 kDa, for SF and YFP respectively, may interfere with the ability of TgHoDI to interact with its partner(s). Such interactions become vital when the parasite was exposed to stress conditions, i.e. bradyzoite growth, whereas such conditions were not found in the tachyzoite growth condition. Thus, no detrimental effect of the protein tags was observed under the tachyzoite conditions.

In the attempt to determine the possible function of TgHoDI, knockdown studies utilizing the destabilizing domain system was employed. However, the addition of the destabilizing domain (ddFKBP) failed to induce TgHoDI degradation (Fig. 2.9). This inability to induce degradation indicates that the C-terminal region of TgHoDI may be involved in protein-protein interactions, thereby shielding the ddFKBP from degradation machinery. For future work, an alternative method that allows for the successful down
regulation of TgHoDI expression would give a better understanding of protein function and this mode of gene regulation.

Both transgenic strains, TgHoDI-SF and TgHoDI-YFP, showed similar cellular localization, which indicates that epitope tag size has minimal effect on protein localization. Also, Huynh and Carruthers (2009) showed that this knock-in of SF or YFP tags at the native locus of a gene does not alter the location of the gene products [73]. The absence of TgHoDI in the nucleus in all life stages may suggest its primary function in the cytoplasm. The punctate cytoplasmic localization observed in both extracellular tachyzoite and intracellular bradyzoite parasites mimics those previously described for both Dhh1 and DOZI [65, 68]. Furthermore, mRNA granules have been recently observed in extracellular parasites are associated with increased parasite viability [75]. In comparison to bradyzoite parasites, the presence of very distinct cellular foci of TgHoDI in extracellular Toxoplasma may indicate a variable stress response mechanism during bradyzoite differentiation. In the bradyzoite stage, the localization of TgHoDI appears to be between extra- and intracellular tachyzoite localization. Therefore, this may suggest that under bradyzoite conditions the localization of TgHoDI was altered due to stress response and that the location of TgHoDI reflects the dynamic of tachyzoite to bradyzoite differentiation.

Mair et al. (2006) described the interaction of DOZI with 92 transcripts, thereby validating its role in translational repression in Plasmodium [68]. In extracellular tachyzoites, several distinct areas of colocalization were observed for TgHoDI with mRNAs which indicates the interaction between the two (Fig. 2.12). This study is the first to show that TgHoDI is an important component in post-transcriptional gene
regulation. In agreement with this, the study by Braun et al. (2010) identified TgHoDI (referred to as 583.m00676) as a member of the TgAgo complex which was subsequently implicated in RNA-induced gene silencing [71, 72]. However, the incomplete colocalization of TgHoDI aggregates with mRNAs, which is prominent at the basal parasitic end, suggests that there is a second protein function which has yet to be identified.

Colocalization of TgHoDI and mRNA granules suggests a possible function of TgHoDI in post-transcriptional regulation. Therefore it is necessary to determine TgHoDI's involvement in mRNA fate. DEAD-box RNA helicases are involved in the storage and degradation of transcripts through the interactions with distinct proteins such as PABPs and decapping enzymes (Dcp1/Dcp2) [39]. TgHoDI's colocalization with PABPC3 at several areas throughout the cytoplasm suggests the involvement of TgHoDI in stress response. The incomplete colocalization of TgHoDI with either stress induced mRNA aggregates or PABPC3 may indicate another function within the cytoplasm (Fig. 2.12). This gives rise to the possibility that TgHoDI functions similarly to Dhh1 in both the repression and degradation of transcripts.

To validate the participation of TgHoDI in the function of stress-induced RNA granules, cycloheximide and arsenite treatment was performed. Extracellular parasites subjected to cycloheximide treatment displayed a complete loss of TgHoDI aggregates in the cytoplasm (Fig. 2.14). These observations are in agreement with previous studies identifying the loss of the human Rck/p54 DEAD-box aggregates after cycloheximide treatment [77]. Interestingly, the localization of TgHoDI post-cycloheximide treatment is very similar to that observed for intracellular tachyzoite parasites (Fig. 2.11). This
observation, in combination with the maintained expression of TgHoDI, can be seen as an indication of multiple cellular functions. The ability of the same DEAD-box protein to be involved in various cellular functions has been observed for Dhh1 [58]. Dhh1 was initially determined to be involved in the decapping complex assembly, however recent studies have shown that it is also implicated in stress response and granules formation under glucose deprivation [39, 65]. Arsenite treatment of extracellular parasites increased the size and prevalence of TgHoDI aggregates within the cytoplasm (Fig. 2.14). Also, an increase in mRNA aggregate size and number is in agreement with previous studies analyzing stress response in extracellular parasites [75]. This increase is directly correlated to the oxidative stress induced by arsenite and therefore centers TgHoDI as a player in stress response.

The alterations in granule formation as the consequence of chemical treatments suggest TgHoDI is able to participate in translational repression during environmental stress. As shown in Figure 3.2 (lower illustration) the presence of distinct foci in untreated extracellular parasites is indicative of the shutdown of active translation and the formation of stress granules. Toxoplasma is an obligate intracellular organism incapable of surviving for extended periods outside of a host [1]. Also, the apparent dispersed localization of TgHoDI in intracellular parasite, which is similar to cycloheximide treated parasites, suggests the absence of translational repression with transcripts becoming incorporated into the polysome. Arsenite treatment has been shown to inhibit translation and induce the formation of stress granules. The exposure to arsenite causes a greater stress response, which lead to the increase in both TgHoDI and mRNA granule size and number. Together, these results suggest that TgHoDI participates in the formation of
stress granules when translation is arrested. However, it cannot be ruled out that TgHoDI may also functions in processing bodies. The presence of TgHoDI in intracellular tachyzoites and the incomplete colocalization with PABP in extracellular parasites suggests this second function of TgHoDI within the cytoplasm. Therefore, subsequent experiments must be performed to determine the involvement of TgHoDI in these granules.

The findings of this study strongly suggests the presence of a conserved post-transcriptional mechanism in Toxoplasma involving TgHoDI. As shown in Figure 3.2 (upper illustration), active translation can be halted by either environmental stress (extracellular parasites) or chemical treatment (arsenite) leading to the formation of stress granules. Also, treatments inducing the stalling of translation machinery (i.e. CHX) leads to the dissociation of stress granules and processing bodies by the trapping of mRNA in the polysome. Furthermore, the findings have shown that the composition of these aggregates are indicative of the fate of mRNA associated in them. This study is the first to characterize such a mechanism in Toxoplasma and provides the framework for future studies to identify the mechanisms involved in stress response and tachyzoite-bradyzoite differentiation.
Figure 3.2 Identified mechanism for post-transcriptional gene regulation in *T. gondii*

(*Top*) A general outline of the identified mechanism for post-transcriptional regulation of mRNA. mRNAs can be involved in either active translation (polysome), storage (stress granules) or degradation (processing bodies). Chemical treatments cause a shift of mRNA localization from one sub-cellular structure to another. The composition of these structures dictates the fate of mRNA involved. Double strike through indicates a lack of component present in the structure. (*Bottom*) Summary of IFA observations of this study. The localization of TgHoDI, which has been identified as a member in mechanisms of stress response, is altered in the different parasite strains and under the treatment of cycloheximide and arsenite.
A hallmark experiment in the analysis of newly identified DEAD-box RNA helicases is to determine whether the protein is capable of complementing the TS⁻ growth defect of Δdhhl cells. TgHoDI partially rescued yeast cell growth at 24 hours, validating its function in stress response. After 36 hours complementation appears to occur to a greater extent which may signify the necessity of increased incubation periods for full complementation to occur. However, the expression of TgHoDI from the strong GPD promoter exemplifies that TgHoDI is required at greater levels in comparison to Dhh1 to complement growth (Fig.2.15). The necessary increase in both incubation time and TgHoDI levels required for complete complementation may suggest that TgHoDI functions in a similar, but not identical, cellular stress response mechanism as Dhh1. Many proteins have been shown to function in stress-specific mechanisms, therefore there is a possibility that TgHoDI is incapable of fully functioning in temperature induced stress [81]. In addition, the variation in TgHoDI's ability to complement the TS⁻ phenotype may be an indication of post-translational modifications, such as phosphorylation. The expression of TgHoDI, which has three possible phosphorylation sites, in yeast would influence the occurrence of these modifications leading to possible alterations in protein function. Similarly, TgHoDI may require Toxoplasma specific factors which may enable proper function to occur. Taken as a whole, TgHoDI has been implicated in post-transcriptional regulation during stress response as a functional analogue to Dhh1 in yeast.
CHAPTER IV

Concluding Remarks and Future Directions

DEAD-box RNA helicases are highly conserved proteins which have been implicated in almost all aspects of RNA metabolism in organisms ranging from yeast to complex eukaryotes. However, in *Toxoplasma gondii* the function of these proteins have not yet been studied. Their implications in post-transcriptional gene regulation remains unknown. The study of DEAD-box RNA helicases may provide the information necessary to reveal how these mechanisms function in post-transcriptional regulation and translational repression.

The current study has provided significant insight into the involvement of TgHoDI in the regulation of mRNA during stress response. This findings show that TgHoDI is a component in stress granules and capable of functioning in a similar manner as the DEAD-box protein Dhh1 in yeast. TgHoDI localization is dynamic and reflects its possible participation in post-transcriptional gene regulation. This study strongly suggests the presence of conserved mechanisms regulating the availability of transcripts between translational machinery and sub-cellular structures (SGs and PBs). These mechanisms are vital in stress mediated repression of mRNA as well as tachyzoite-bradyzoite differentiation. Future work should be performed to determine how TgHoDI expression may affect the stress-induced response in *Toxoplasma*. It will be interesting to determine if the abolishment or over expression of TgHoDI protein will alter the ability of parasites to respond to cellular stress. Furthermore, these studies may have implications in the stage-specific repression of transcripts and therefore may shed light on the mechanisms behind tachyzoite-bradyzoite differentiation. For this to occur, it will be necessary to
determine the involvement of TgHoDI with specific mRNAs and therefore alterations in mRNA levels can be analyzed as a function of TgHoDI expression. In accordance with this, it will be vital to investigate the regulation of total mRNA populations at various levels of TgHoDI expression. The ability of TgHoDI to regulate mRNA in both the sexual and asexual parasitic life stages may further allow for the characterization of protein function. The homologue of TgHoDI in Plasmodium was determined to play a larger role in the sexual component of the parasitic life cycle which may suggest the involvement of TgHoDI in the sexual stage of Toxoplasma.

Other studies must be performed to determine if TgHoDI localization is altered as a function of various environmental stresses. It has been shown that the composition of cytosolic granules changes according to variable stress conditions. It will be necessary to determine the involvement of TgHoDI within these granules during stresses such as nutrient deprivation, temperature variations and several chemical treatments. This will clarify the function of TgHoDI as a central player in a universal mechanism of stress response or a stress-specific response. Furthermore, these colocalization studies must be performed with markers of not only stress granules, but also processing bodies (i.e. Dcp1a). The presence of TgHoDI during the various life stages and treatments in this study suggest a second function which may be implicated in p-body formation.

Moreover, in vitro studies analyzing the function of TgHoDI in RNA unwinding will validate its involvement in mRNP remodeling. However, prior to these studies it may be necessary to determine if post-translational protein modifications such as phosphorylation alter protein function.
CHAPTER V - METHODS

* For materials, please see Appendix B.

5.1 Plasmid construction

pSF-TAP-LIC-HX-Dozi and pYFP-LIC-HX-Dozi

Standard protocol for ligation independent cloning was performed as previously described [82]. To produce clonal parasites with endogenous C-terminal tagging of the TgHoDI protein, a 2.8 Kb genomic sequence of TgHoDI (Toxodb gene ID # TggT1_089230; chrXI: 5,292-8,058) was PCR amplified from the ΔKu80 genome using the oligonucleotide primers TgDozi.LIC.F and TgDozi.LIC.R (Appendix B). This product was T4 DNA polymerase treated to create a 15 base pair 5’ overhang by means of 3’to 5’ exonuclease activity. The pYFP-LIC-HX and pSF-TAP-LIC-HX vectors were linearized using PacI restriction endonuclease, T4 DNA polymerase treated and gel purified. The PCR fragment was cloned into the linearized vectors with the resultant plasmids transformed into E.coli cells and subjected to colony PCR using the TgDozi.LIC.F and TgDozi.LIC.R oligonucleotides. Plasmid confirmation was performed by EcoRI and PvuII restriction endonuclease analyses (see plasmid maps, Appendix C).

HoDI-S-DD-F LIC-HX

To produce clonal parasites with endogenous C-terminal tagging of the TgHoDI protein with the ddFKBP, PCR analysis was performed to amplify the CDS of ddFKBP from pCAG-EGFpd2 plasmid using the oligonucleotide primers cf_FW_S_DD_F and cf_FW_S_DD_R (Appendix B). The plasmid expressing HoDI-SF (pSF-TAP-LIC-HX-Dozi) was digested with SfoI which produced a single cut in between the streptavidin and FLAG tags. The ddFKBP product was then introduced into the linearized pSF-TAP-LIC-HX-Dozi plasmid via the cold fusion method of cloning. This allowed for the CDS of
ddFKBP to be flanked by the Strep and FLAG tags. Plasmid confirmation was performed by ApaLI, PstI and PvuII restriction endonuclease analyses (see plasmid maps, Appendix C).

**pRS424-GPD_TgDozi plasmid**

To place the expression of TgHoDI under the control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter for complementation studies in *S. cerevisiae*, the open reading frame of TgHoDI was excised from the pET-28b_TgDozi expression plasmid by NcoI digestion followed by Klenow Fragment treatment to produce a 5’ blunt end. Linearized vector was then digested with XhoI and gel extracted. In the preparation of the pRS424-GPD plasmid the vector was digested with NcoI, phenol: chloroform extracted and digested with SmaI. The fragment was cloned into the linearized vector and the resultant pRS424-GPD_TgDozi plasmid was subjected to HincII and NdeI restriction endonuclease analyses.

**5.2 Cell and Parasite Culture**

**Human Foreskin Fibroblasts**

Human foreskin fibroblasts were maintained using Dulbecco's Modified Eagle Medium (DMEM) with 25 mM D-glucose and 4 mM L-glutamine supplemented with 10% cosmic calf and 0.5x antibiotic-antimycotic at 37°C in 5% CO₂.

**T. gondii**

Two strains of *T. gondii*, RHΔHX and ΔKu80, were cultured in confluent human foreskin fibroblasts (HFF) using Minimal Essential Media Eagle (MEM), supplemented with 1% dialyzed fetal bovine serum (dFBS) and 0.5x antibiotic-antimycotic.
Transgenic parasites, TgHoDI-SF and TgHoDI-YFP, were cultured in MEM supplemented with 1% dFBS, 2.5x antibiotic-antimycotic, 25 mg/mL mycophenolic acid and 25 mg/mL xanthine.

5.3 Transformation of microorganisms

*E. coli*

Standard protocol performed as previously described [83]. XL1-Blue competent cells were incubated with plasmid DNA on ice for 30 minutes. The cells were then heat shocked at 42°C for 45 seconds and incubated on ice for 2 minutes. Cells were then combined with 500 µL of Super Optimal Broth with catabolite-repression (SOC Medium) and incubated at 37°C for 1 hour with agitation. An appropriate amount of transformation mixtures was then plated on an Luria Bertani (LB) agar plate with appropriate antibiotic and grown at 37°C overnight.

*T. gondii*

Standard protocol performed as previously described [84]. Freshly lysed ΔKu80 parasites (~ 2 x 10^6 parasites) were used for each transfection *via* electroporation using a BTX ECM 630 (1500 volts, 25 Ω, and 25 μF). For each transfection, 20 µg of expression plasmids encoding pSF-TAP-LIC-HX-TgDozi or pYFP-LIC-HX-TgDozi were linearized with PflmI and PmeI, respectively, mixed with harvested parasites in a 400 µL electroporation mixture and electroporated in a 4 mm-gap cuvette. Following electroporation, the parasites were cultured in confluent HFF monolayers grown using MEM with 1% dFBS and antibiotic-antimycotic at 37°C in 5% CO₂. Transgenic parasites were then selected for with MEM supplemented with 1% dFBS, 0.5x antibiotic-antimycotic, 25 mg/mL mycophenolic acid and 25 mg/mL xanthine. Clonal parasites
were then isolated by serial dilution in 96 well plates to obtain a clonal population [84]. Transgenic populations were confirmed by PCR and Western blot analysis.

**S. cerevisiae**

Standard lithium acetate transformation protocol was performed as previously described [83]. Wild-type yeast and YTC444 were grown on YAPD and Leu` synthetic drop-out plates, for the WT and YTC444 respectively, and colonies were selected and grown overnight at 30ºC in YAPD media with agitation. YTC444 yeast was transformed with p414PromDhh1and pRS424-GPD_TgDozi, for expression of Dhh1 and TgHoDI, respectively. Salmon sperm DNA was incubated at 90ºC for 3 minutes, quenched on ice for 2 minutes and combined with 500 µL of plate solution. The solution was then combined with yeast cells and ~1 µg of expression plasmid, mixed and incubated overnight at room temperature. Cell suspensions were then centrifuged at 2,000 rpm for 1 minute and cells were re-suspended in 300 µL of 1x TE. An appropriate amount of transformation was then spread onto Leu`/Trp` plates and incubated at 30ºC for 2-4 days.

5.4 Immunological detection methods

**Western Blot**

Standard protocol as previously described [83]. Lysates of *T. gondii* parasites were prepared by addition of 50 µL of RIPA buffer, sonication for 5 seconds and incubation on ice. Protein concentration was measured using the RC DC Protein Assay according to manufacturer’s instructions (Bio-Rad Laboratories, Cat #500-0119) and absorbance values were taken at 750 nm on the Genesys 10 UV/Visible spectrophotometer. Lysates were denatured using 6x SDS protein loading dye and resolved on a 10% SDS-PAGE for 1 hour and subsequently transferred to a nitrocellulose
membrane. Following transfer, nitrocellulose blots were blocked with 5% (w/v) non-fat skim milk in TBST for a minimum of 30 minutes and incubated with primary antibodies in 2% (w/v) non-fat skim milk in TBST for 1 hour. The primary antibodies used were: mouse anti-Tub (1:5,000), mouse anti-FLAG (1:5,000), rabbit anti-LDH1 (1:2,000), and goat anti-GFP (1:2,500) (Appendix B). Blots were subsequently washed 3 times for 5 minutes with TBST and incubated with specific secondary antibodies for 1 hour. The secondary antibodies used were: horseradish peroxidase (HRP)-goat anti-mouse (1:10,000), HRP-rabbit anti-goat (1:10,000), HRP-goat anti-rabbit (1:10,000). Finally, blots were washed, incubated with Chemiluminescent HRP Substrate Kit (Millipore) and visualized using the FluorChem Q Imager (Alpha Innotech) with AlphaView-FluorChem Q software.

**Immunofluorescence Assay**

HFF monolayers were cultured on coverslips until confluent, infected with ~1 x 10^6 freshly lysed parasites and incubated at 37°C and 5% CO₂ for 24 hours. Cells were rinsed three times in 1x (v/v) phosphate buffer saline (PBS), fixed with 3% (w/v) paraformaldehyde for 5 minutes and permeabilized with 0.25% Triton X-100 in PBS for 10 minutes. Coverslips were then washed in 1x PBS and subsequently incubated in a blocking solution of 5% (w/v) bovine serum albumin (BSA) in PBS with gentle agitation for 1 hour at room temperature. Coverslips were then incubated for 1 hour in a humidity chamber with a specific primary antibody diluted in 2% (w/v) BSA. The primary antibodies used were: mouse anti-FLAG (1:500) and rabbit anti-PABPC3 (1:500). Cells were once again washed in 1x PBS and incubated for 1 hour with secondary antibody diluted in 2% (w/v) BSA. The secondary antibodies used were: Rhodamine (Rho)-
conjugated goat anti-rabbit (1:500) and Rho-conjugated goat anti-mouse (1:500). Cells were then stained with Hoechst stain (100 μM) at room temperature, washed twice with 1x PBS and mounted on glass slides with fluoromount. Imaging was performed on a Leica DMI 6000B inverted fluorescent microscope using HCX PL Apo 100x/1.40-0.70 objective and a Leica DFC 360FX camera in addition to the Leica Application Software (LAS). Confocal imaging was performed on an Olympus 1X81 inverted microscope using Plan Apo-N 60x/1.42 oil objective outfitted with Fluoview 1000 imagine system. Post-acquisition image processing was performed with Adobe Photoshop 7.0.

For extracellular *T. gondii*, ~ 1 x 10⁶ freshly lysed parasites were incubated on coverslips at 37°C and 5% CO₂ and allowed to adhere for 1 hour. Coverslips were rinsed in 1x (v/v) PBS and fixed/permeabilized at 4°C with ice cold methanol for 10 minutes. The remaining protocol was as previously described. Fluorescence *in situ* Hybridization

Freshly lysed parasites (~ 1 x 10⁶) were transferred on coverslips at 37°C and 5% CO₂ and allowed to adhere for 1 hour. Coverslips were washed 1x (v/v) PBS and fixed/permeabilized at 4°C with ice cold methanol for 10 minutes. Parasites were rinsed with 2x SSC buffer 2 times for 10 minutes and incubated with hybridization buffer, ~400ng biotin labeled oligo-dT₅₀ and deionized formamide in a humidity chamber overnight at 37°C. The coverslips were then washed 2 times with 2x SSC for 30 minutes and 1 time in 0.5X SSC for 15 minutes. Parasites were then incubated for 60 minutes at room temperature in Rho-conjugated streptavidin (1:500) in 4x SSC in a humidified chamber. Coverslips were then washed with 2 times with 4x SSC for 10 minutes and 1 time in 4x SSC with 0.01% Triton X-100. Finally, coverslips were stained with Hoechst
stain (100 μM) at room temperature, washed twice with 4X SSC for 5 minutes and mounted on glass slides with fluoromount.

5.5 Assays for functional analysis

Drug treatment

For extracellular *T. gondii*, ~ 1 x 10^6 freshly lysed parasites were incubated on coverslips at 37°C and 5% CO₂ and allowed to adhere for 1 hour. Parasites were pretreated with either 50 µg/mL cycloheximide or 10 µM sodium m-arsenite. Subsequent IFA and FISH analysis were performed as previously stated.

*S. cerevisiae Δdhh1 Complementation and Growth*

To determine the ability of TgHoDI to functionally complement the temperature dependence of the YTC444 yeast strain, transformed cells were grown in 4 mL of YAPD media and incubated overnight at 30°C with agitation. For the expression of Dhh1, cells were transformed with a low copy centromeric plasmid expressing Dhh1 from its endogenous promoter. For the expression of TgHoDI, cells were transformed with a high copy number plasmid (2µm) expressing TgHoDI under the strong yeast GPD promoter. The optical density (OD) was then taken at 600 nm to confirm cells were in the mid-log phase of growth. Cells were subsequently diluted to an OD of ~0.5. Serial dilutions were performed using sterile 1X TE and 3 µL of each dilution was spotted onto YPAD plates. Plates were then incubated upright at 30°C and 37°C for 2 days.
REFERENCES


## APPENDIX A

<table>
<thead>
<tr>
<th>Name of Gene</th>
<th>Chr.</th>
<th>Accession Number</th>
<th>Molecular Weight</th>
</tr>
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<td>97328 Da</td>
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Table A1 DEAD-box and ATP dependent RNA helicases in *Toxoplasma*

Bioinformatic analysis of Dead-box and ATP dependent RNA helicases in *T. gondii* using Toxodb Database [15]. Analyses identified 20 putative proteins with high sequence homology to the DEAD-box RNA helicases with another 26 possible ATP dependent RNA helicases. The chromosomal location, gene ID (in type I strain) and molecular weight are shown.
### APPENDIX B

<table>
<thead>
<tr>
<th>Buffers and Solutions</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electroporation mixture</td>
<td>120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄ (pH 7.6), 2 mM EDTA, 5 mM MgCl₂, 2 mM ATP, 5 mM glutathione.</td>
</tr>
<tr>
<td>Radioimmunoprecipitation assay buffer (RIPA)</td>
<td>25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% w/v Triton X-100, 1% w/v sodium deoxycholate and 0.1% w/v SDS</td>
</tr>
<tr>
<td>6x SDS protein loading dye</td>
<td>12% w/v SDS, 0.06% w/v bromophenol blue, 47% v/v glycerol, 6% v/v 1M Tris-pH 6.8 and 12.5% v/v β-mercaptoethanol</td>
</tr>
<tr>
<td>1x Tris-buffered saline with Tween-20 (TBST)</td>
<td>68 mM NaCl, 8.3 mM Tris-base (pH 7.6) and 0.05% v/v Tween-20</td>
</tr>
<tr>
<td>20x Saline-sodium citrate (SSC)</td>
<td>17.5% w/v NaCl, 9% w/v sodium citrate</td>
</tr>
<tr>
<td>Hybridization buffer</td>
<td>25% v/v 20x SSC, 50% v/v 50% dextran sulfate, 25% v/v yeast tRNA</td>
</tr>
</tbody>
</table>

**Table B1 Buffers and their formula for various experiments in the study**

The composition of buffers used in the study are shown with their corresponding composition. These buffers vary in their application from parasite transformation, protein lysate acquisition and FISH analyses.
<table>
<thead>
<tr>
<th>Antibody Against</th>
<th>Antisera Name</th>
<th>Raised in</th>
<th>Obtained From</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-Tubulin</td>
<td>α-Tub (12G10)</td>
<td>Mouse</td>
<td>E.M. Nelsen; DSHB</td>
</tr>
<tr>
<td>D-tag (FLAG)</td>
<td>α-FLAG</td>
<td>Mouse</td>
<td>Applied Biological Materials (ABM) Inc. (#G191)</td>
</tr>
<tr>
<td>Poly-A Binding Protein C3</td>
<td>α-PABPC3</td>
<td>Rabbit</td>
<td>ABM Inc. (#Y058722)</td>
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<tr>
<td>Green Fluorescent Protein</td>
<td>α-GFP</td>
<td>Goat</td>
<td>Rockland (#17549)</td>
</tr>
<tr>
<td>Lactate Dehydrogenase-1</td>
<td>α-LDH1</td>
<td>Rabbit</td>
<td>Dr. Stephen Parmley; Palo Alto Medical Foundation; Calif.</td>
</tr>
<tr>
<td>Bradyzoite Antigen-1</td>
<td>α-BAG1</td>
<td>Mouse</td>
<td>Dr. Louis Weiss, Albert Einstein College of Medicine, N.Y</td>
</tr>
<tr>
<td>Horseradish peroxidase (HRP) anti-mouse</td>
<td>HRP α-Mouse</td>
<td>Goat</td>
<td>Rockland (#19072)</td>
</tr>
<tr>
<td>HRP anti-goat</td>
<td>HRP α-Goat</td>
<td>Rabbit</td>
<td>Rockland (#17320)</td>
</tr>
<tr>
<td>HRP anti-rabbit</td>
<td>HRP α-Rabbit</td>
<td>Goat</td>
<td>Rockland (#15949)</td>
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<tr>
<td>Rhodamine-conjugated (Rho) anti-rabbit</td>
<td>Rho α-Rabbit</td>
<td>Goat</td>
<td>Rockland (#2495)</td>
</tr>
<tr>
<td>Rho-conjugated anti-mouse</td>
<td>Rho α-Mouse</td>
<td>Goat</td>
<td>Rockland (#610-100-040)</td>
</tr>
<tr>
<td>Rho-conjugated anti-goat</td>
<td>Rho α-Goat</td>
<td>Donkey</td>
<td>Rockland (#3212)</td>
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<tr>
<td>Rho-conjugated streptavidin</td>
<td>Rho α-Strep</td>
<td>N/A</td>
<td>Rockland (#22837)</td>
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</tbody>
</table>

**Table B2 Antisera**

Throughout this study, antisera were utilized in immunodetection studies such as Western blot analysis, IFA and FISH. Antisera name, target protein and organism raised in are present. Also, the company and catalog numbers are presented.
<table>
<thead>
<tr>
<th>Culture Material</th>
<th>Composition</th>
<th>Obtained From</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle Media (DMEM)</td>
<td>Modifications. With: 25 mM glucose, 4 mM L-glutamine and 0.0399 mM phenol red. Without: sodium pyruvate, HEPES and sodium bicarbonate.</td>
<td>Invitrogen (#12100-046)</td>
</tr>
<tr>
<td>Antibiotic-Antimycotic (100x)</td>
<td>10000 units/mL penicillin, 10000 µg/mL streptomycin and 25 µg/mL amphotericin B.</td>
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</tr>
<tr>
<td>Minimum Essential Media Eagle (MEM)</td>
<td>Modifications. With: 2 mM L-glutamine and 0.0266 mM phenol red. Without: HEPES and sodium bicarbonate.</td>
<td>Invitrogen (#61100061)</td>
</tr>
<tr>
<td>Yeast Extract Peptone Dextrose with Adenine (YPAD) plate Solution</td>
<td>2% w/v bacto peptone, 1% w/v yeast extract, 2% w/v D-glucose, 2% w/v bacto agar, 0.001% w/v adenine</td>
<td>N/A</td>
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<tr>
<td>Yeast Plate solution</td>
<td>90% w/v of 45% polyethylene glycol, 10% v/v 1M lithium acetate, 1% v/v Tris-HCl pH 7.5 and 0.2% v/v 0.5M EDTA</td>
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<tr>
<td>Super Optimal Broth with catabolite repression (SOC)</td>
<td>2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 0.05% w/v NaCl, 10 mM MgCl₂, 2.5 mM KCl and 20 mM glucose.</td>
<td>N/A</td>
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<tr>
<td>Luria-Burtani (LB) Agar plate Solution</td>
<td>1% w/v bacto-tryptone, 0.5% w/v bacto-yeast, 1% w/v NaCl, 1 x 10⁻⁴ % w/v antibiotic and 1.5% w/v bacto-agar</td>
<td>N/A</td>
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</table>

**Table B3 Culture Material**

Various culture materials were required for growth of various microorganisms used in the study. The name, composition and company (if applicable) are shown.
### Table B4 Miscellaneous Materials for organism treatment

Material presented were used for the treatment of transgenic parasite clones as well as to maintain HFF monolayers. Material name and acquisition company are shown.

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<td>Xanthine</td>
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<td>Mycophenolic acid</td>
<td>Bio Basic Inc. (#24280-93)</td>
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<td>Sodium m-Arsenite</td>
<td>Sigma (C7784-46-5)</td>
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<td>Cycloheximide</td>
<td>Sigma (C7698-1G)</td>
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<td>Cosmic Calf Serum (CCS)</td>
<td>ThermoFisher Scientific; Hyclone (#H3008704N)</td>
</tr>
<tr>
<td>Dialyzed Fetal Bovine serum (dFBS)</td>
<td>ThermoFisher Scientific; Hyclone (#SH3007903)</td>
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<table>
<thead>
<tr>
<th>Parasite/Yeast Strains</th>
<th>Obtained From</th>
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<td>Human foreskin fibroblasts (HFF)</td>
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<td>RHΔHX, <em>T. gondii</em> type I strain</td>
<td>NIH AIDS Research and Reference Reagent</td>
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<tr>
<td></td>
<td>Program, #2857</td>
</tr>
<tr>
<td>ΔKu80 <em>T. gondii</em></td>
<td>Dr. Vernon B. Carruthers, University of</td>
</tr>
<tr>
<td></td>
<td>Michigan, U.S.A</td>
</tr>
<tr>
<td>Wild-type yeast strain (MATa ade2-1,</td>
<td>Dr. Tien-Hsien Chang; Genomics Research</td>
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<tr>
<td>ura3-1, trp1-1, his3-11, leu2-3,112</td>
<td>Center, Academia Sinica, China.</td>
</tr>
<tr>
<td>can1-100)</td>
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</tr>
<tr>
<td>YTC444 strain (MATa dhh1Δ::LEU2 ade2-1,</td>
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</tr>
<tr>
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<td>Center, Academia Sinica, China.</td>
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### Table B5 Organisms used in study

Organisms used in the study are shown with their corresponding place of acquisition. For purchased materials, the corresponding lot number is given.
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<th>Parasite/Yeast Expression Vectors</th>
<th>Obtained From</th>
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<tr>
<td>pYFP-LIC-HX</td>
<td>Dr. Vernon B. Carruthers, Univ. of Michigan, U.S.A</td>
</tr>
<tr>
<td>pSF-TAP-LIC-HX</td>
<td>Dr. Vernon B. Carruthers, Univ. of Michigan, U.S.A</td>
</tr>
<tr>
<td>p414PromDhh1</td>
<td>Dr. Juana Diez, Universitat Pompeu Fabra, Spain</td>
</tr>
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<td>pRS424-GPD</td>
<td>Dr. Elizabeth Craig, Univ. of Wisconsin, U.S.A</td>
</tr>
<tr>
<td>pCAG-EGFpd2</td>
<td>Dr. Markus Meissner, Univ. of Glasgow, Scotland</td>
</tr>
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<td>Constructed for this study</td>
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<tr>
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<tr>
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**Table B6 Parental plasmids and constructs**

The various vectors, both parental constructs for this study, are shown with their corresponding place of acquisition.
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<tr>
<th>Oligonucleotide Name</th>
<th>Sequence</th>
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<td>5’-TACTTCCAATCCAAATTTAATG CTACGACGTGAATTG TTGATGG-3’</td>
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<tr>
<td>TgDozi.LIC.R (a’)</td>
<td>5’-TCCTCCACTTCCAAATTTTAGC GTAAATTGCTTCGTCAA TCTGG-3’</td>
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<tr>
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<td>5’-CTCAGCAGCGCATGCAACAC-3’</td>
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<td>5’-TGCGGATGAGACCAAGAACC-3’</td>
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<td>cf_RV_S_DD_R</td>
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</tr>
<tr>
<td>dT-Fish</td>
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</table>

Table B7 Oligonucleotides used in the study

The complete list of oligonucleotides, and their corresponding sequences, which were used in this study are shown. All oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT), Coralville, Iowa, U.S.A.
APPENDIX C

**Figure C1 Illustration of genomic TgHoDI sequence for creation of expression plasmids**

An illustration of the genomic sequence of TgHoDI (orange) was acquired using the VNTI software V.10.3.0 (Invitrogen). This was used to assist in the cloning of subsequent expression plasmids for the endogenous tagging of TgHoDI protein. The location of oligonucleotide binding sites for the LIC forward and reverse primers are shown. Also, the binding sites of the validation oligonucleotides used for the confirmation of transgenic clones is shown.
Figure C2 Illustration of TgHoDI-SF transgenic plasmid

An illustration of the expression plasmid for transgenic TgHoDI-SF clones. The genomic sequence fragment of TgHoDI (orange) was acquired by PCR analysis using the TgDozi.LIC.F (sense) and TgDozi.LIC.Rv (antisense) oligonucleotides. The location of streptavidin and FLAG tags are shown. Also, the hypoxanthine-xanthine-guanine phosphoribosyl transferase (HX) cassette, which is used for transgenic parasite selection, is shown (green).
Figure C3 Illustration of TgHoDI-YFP transgenic plasmid

An illustration of the expression plasmid for transgenic TgHoDI-YFP clones. The genomic sequence fragment of TgHoDI (orange) was acquired by PCR analysis using the TgDozi.LIC.F (sense) and TgDozi.LIC.Rv (antisense) oligonucleotides. The location of coding sequence for the YFP tag is also shown (orange). The hypoxanthine-xanthine-guanine phosphoribosyl transferase (HX) cassette, which is used for transgenic parasite selection, is shown (green).
Figure C4 Illustration of TgHoDI-S-DD-F transgenic plasmid

An illustration of the expression plasmid for transgenic TgHoDI-S-DD-F clones for knockdown studies. The coding sequence for Strep-ddFKBP-FLAG was acquired by PCR amplification of pCAG-EGFpd2 vector using the cf_FW_S_DD_F and cf_FW_S_DD_R oligonucleotides. The TgHoDI-SF-TAP.LIC plasmid was linearized by SfoI endonuclease which resulted in a single cut in between the streptavidin and FLAG tags. The amplified ddFKBP CDS was then integrated into the linearized vector via cold fusion. The hypoxanthine-xanthine-guanine phosphoribosyl transferase (HX) cassette, which is used for transgenic parasite selection, is shown (green).
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