Functional Characterization of a Novel Thioredoxin Domain-Containing Protein of the Malaria Parasite Plasmodium

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LOYOLA UNIVERSITY CHICAGO

FUNCTIONAL CHARACTERIZATION OF A NOVEL THIOREDOXIN DOMAIN-CONTAINING PROTEIN OF THE MALARIA PARASITE PLASMODIUM

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN BIOLOGY

BY
RACHEL KOOISTRA
CHICAGO, ILLINOIS
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CHAPTER I

INTRODUCTION

Malaria and \textit{Plasmodium}

In the 2013 World Malaria Report, the World Health Organization (WHO) reported that 97 countries around the world had ongoing malaria transmission, accounting for nearly 3.4 billion people at risk for contracting the disease. There were an estimated 207 million malaria cases in 2012, with approximately 627,000 deaths. 77\% of these deaths occurred in children under the age of five, meaning that malaria killed one child almost every minute during that year. This loss of life took place in spite of $2.5$ billion of international and domestic funding, a number that the WHO suggests is less than half of what is actually needed to control the disease (WHO, 2013).

This enormous toll on society is caused by an ancient, single-celled protozoan parasite of the genus \textit{Plasmodium}. To date, there are five species of \textit{Plasmodium} known to infect humans: \textit{P. falciparum}, \textit{P. vivax}, \textit{P. ovale}, \textit{P. malariae}, and \textit{P. knowlesi} (Oaks, 1991; Singh \textit{et al}, 2004). Of these five, \textit{P. falciparum} is considered to be the most deadly, while \textit{P. vivax} is the most widespread (Li \textit{et al}, 2001). Malaria is a vector-borne disease, and each of these species is transmitted to humans by female mosquitoes of the genus \textit{Anopheles} (Oaks, 1991).
The life cycle of *Plasmodium* can be divided into three different stages: infection, multiplication, and transmission. Infection begins when a mosquito feeds on a naïve host and injects sporozoites into the host’s bloodstream. The sporozoites invade the liver cells, where, over the next 5-15 days, they multiply asexually to produce schizonts, with each schizont containing between 10,000 and 30,000 merozoites. Eventually the infected liver cells rupture, and the merozoites are released into the bloodstream, where they proceed to invade erythrocytes. Within the red blood cell, the merozoite starts an asexual reproduction cycle in which it first matures into a trophozoite and then into a schizont to
again produce more merozoites. When the red blood cell eventually bursts, these merozoites will infect new red blood cells, and the multiplication process will start again (Figure 1). The destruction of an increasing amount of red blood cells eventually leads to the symptoms that characterize the disease malaria.

The transmission phase of the life cycle begins with the formation of male and female gametocytes. When a mosquito takes a blood meal, it ingests gametocytes, which mature into gametes and mate to form zygotes. The zygotes develop into motile ookinetes, which move through the epithelial cells that line the midgut wall and develop into sporozoite-forming oocysts. When the oocyst bursts, the released sporozoites invade the mosquito’s salivary glands, from which they will be injected into another human host, and the life cycle will begin again (Oaks, 1991) (Figure 1).

**Antioxidant Systems of Plasmodium**

Throughout its complex life cycle, the parasite is exposed to challenges and stresses. For example, the majority of parasites ingested by the mosquito will perish within the first 24 hours due to a number of factors, including mosquito-derived digesting enzymes present in the mosquito midgut and the cytotoxic byproducts of their activity (Alavi *et al*, 2003; Arambage *et al*, 2009). Damage may also be done to these parasites by the immune components of the previous vertebrate host (Margos *et al*, 2001). In addition, the mosquito mounts its own immune response against the parasite in the form of antimicrobial peptides as well as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can damage both DNA and cellular targets (Müller, 2004; Fang, 2004).
In order to survive, *Plasmodium* must have the ability to create for itself a redox environment that will minimize the damage caused by these stresses, while at the same time allowing it to continue its own essential metabolic functions (Imlay, 2003). In order to maintain this redox homeostasis, the parasite relies on two main antioxidant systems, the glutathione system and the thioredoxin system (Figure 2), which are also conserved in most eukaryotes (Müller, 2004).

The thioredoxin redox system consists of the oxidoreductase thioredoxin reductase (TrxR) and the small protein thioredoxin (Trx), which supplies electrons to various redox proteins, such as antioxidant thioredoxin-dependent peroxidases (Figure 2). The thioredoxin system transports electrons via reduction-oxidation (redox) mechanisms, a process that depends on thiol-containing cysteines present in the thioredoxin domain of these proteins. In the first step of this system, the enzyme TrxR catalyzes the reduction of oxidized thioredoxin [Trx-(S\textsubscript{2})] (Holmgren, 1979; Kanzok, 2000). This leads to reduction of the disulfide bond in Trx and subsequent formation of two thiol groups in reduced thioredoxin [Trx-(SH)\textsubscript{2}]. These thiol groups can then reduce the disulfide bond in several target proteins, including thioredoxin-dependent peroxidases (TPx) or other disulfide-
containing substrate proteins (Holmgren, 1995). The expanding roles of this redox system in *Plasmodium* are still a topic of study, and more evidence suggests that it may be essential for the parasite. For example, the knockout of thioredoxin reductase has been shown to be lethal to *Plasmodium falciparum* (Krnajski et al., 2002).

**Thioredoxin Superfamily of Proteins**

Thioredoxin and proteins that are reduced by the thioredoxin system are part of a larger group of proteins known as the thioredoxin superfamily. This large family is divided into two main groups, with Group I comprised of proteins that only contain a Trx domain and Group II consisting of proteins that contain a thioredoxin domain along with other domains (Sadek et al., 2003). The thioredoxin domain of these proteins folds into a characteristic three-dimensional structure known as the thioredoxin (Trx) fold (Figure 3). The basic structure of the Trx fold consists of an at least four-stranded beta sheet surrounded by at least two alpha helices (βαβββα) (Martin, 1995; Atkinson and Babbitt, 2009).

![Figure 3. The thioredoxin fold. Shown here is a model of the thioredoxin fold domain of *P. berghei* Trx-1. The basic structure consists of four beta sheets (yellow) surrounded by at least two alpha helices (pink). The active site of Trx-1 is shown. Model generated using SwissModel (Arnad et al, 2006).]
A subset of proteins in the thioredoxin superfamily have been shown to possess redox activity (Atkinson and Babbitt, 2009). In general, redox activity of Trx-like proteins is attributed to the presence of the amino acid cysteine in the active site of the protein (Atkinson and Babbitt, 2009). The classical and widely conserved amino acid sequence, or motif, that was first identified in thioredoxin and is commonly observed in Trx-like proteins consists of two cysteine residues on either side of two other amino acids (CXXC) (Holmgren, 1985). The sulfur-containing cysteine allows the proteins to exist in either a reduced [Trx-(SH)₂] or oxidized [Trx-(S₂)] form (Holmgren, 1995). This enables thioredoxins to reduce substrate proteins through the process of cysteine-thiol disulfide exchange. Briefly, the thiolate of the peroxidatic cysteine of reduced thioredoxin [Trx-(SH)₂] allows for the nucleophilic attack of a disulfide group in a substrate protein, leading to a mixed disulfide. A nucleophilic attack of the deprotonated resolving cysteine then leads to the formation of an oxidized thioredoxin domain (Trx-[S₂]) and reduced substrate protein (Holmgren, 1995). However, more and more variations on this classical active site have been found in thioredoxin fold containing proteins. Some proteins in the thioredoxin superfamily possess only a single cysteine in their active site, while others have no easily discernable active site (Atkinson and Babbitt, 2009).

The phosducin-like family of proteins was one of these groups of proteins in which a catalytic motif was not readily detected (Atkinson and Babbitt, 2009). This family of proteins is broadly conserved, with members appearing in all eukaryotic organisms. Proteins belonging to this family contain a C-terminal thioredoxin domain, but lack the classical CXXC active site (Marchler-Bauer et al, 2009). Phosducin-like
proteins can be organized into three different subgroups (Willardson and Howlett, 2007). Subgroup I consists of phosducin (Pdc) and phosducin-like protein 1 (PhLP1). Subgroup II is represented in humans by two proteins, PhLP2A and PhLP2B, and in lower organisms by a single protein, PhLP2. Subgroup III contains PhLP3, also known as thioredoxin domain-containing protein 9 (TXNDC) or ATP binding protein associated with cell differentiation (APACD). The work presented in this project sheds new light on this subgroup of the thioredoxin superfamily of proteins.

**Studying Plasmodium Mosquito Stages**

As mentioned, *Plasmodium* is faced with particularly stressful conditions during its first 24 hours in the mosquito midgut due to the presence of ROS and RNS (Luckhart *et al*, 1998; Molina-Cruz *et al*, 2008). Once ingested by the mosquito, the parasite gametes leave the host red blood cell, thus switching from an intracellular to an extracellular lifestyle and exposing itself to the harsh environment of the mosquito bloodmeal. The formation of a zygote takes place within an hour of ingestion. Transformation from a zygote to an ookinete, however, forces the parasite to remain in these severe conditions for up to 20 hours (Sinden *et al*, 2004). And while it is hypothesized that members of the thioredoxin family of proteins play a role in defense against this oxidative stress, very little is known about their protective mechanisms (Nickel, 2006). A more thorough understanding of the specific functions of this protein family in ookinetes may provide us with an indication of how to inhibit these proteins and possibly prevent transmission of the parasite.
The mouse malaria parasite *Plasmodium berghei* is an excellent model system for studying the mosquito stages of *Plasmodium* for several reasons. Firstly, since *P. berghei* is not infectious to humans, infected mosquitoes can safely be studied in a lab environment. In addition, research with rodents does not carry the same ethical implications as working with humans or other primates. Importantly, it has previously been shown that all mammalian malaria parasites possess comparable life cycles as well as similar morphological features of the different life stages (Sinden, 1978). The genome organization between rodent-infecting parasites and human-infecting parasites is also conserved (Janse *et al.*, 1994; van Lin *et al.*, 2001). In addition, mammalian malaria parasites have not been found to possess any major differences in their metabolic pathways (Janse and Waters, 1995). And while small differences in the way each species interacts with its host do exist, *P. berghei* still provides us with a very useful starting point from which to research many of the unknown aspects of this parasite.

**Thesis Objective**

While studies in *Plasmodium* have focused on the antioxidant functions of thioredoxins, this group of proteins has been shown to play numerous other roles in other organisms, including protein folding (Ito and Inaba, 2008), cell proliferation (Immenschuh and Baumgart-Vogt, 2005), and signal transduction and transcriptional regulation (Brigelius-Flohe and Flohe, 2011). Despite these findings, some studies estimate that only 5.6% of proteins belonging to the thioredoxin superfamily have been manually associated with a function (Atkinson and Babbitt, 2009). With this in mind, the objective of this project was to characterize a novel thioredoxin domain-containing
protein of the malaria parasite *P. berghei*. The results presented here not only serve to begin to identify the function of this protein in *Plasmodium*, but may also provide new insight into the functions of related proteins in other organisms.
CHAPTER II
MATERIALS AND METHODS

Reagents

All enzymes and enzyme buffers were obtained from Fermentas (Vilnius, Lithuania). Unless otherwise stated, all primary antibodies were obtained from Open Biosystems (Huntsville, AL). All PCR primers were obtained from Fisher Scientific (Fairlawn, NJ).

Agilent Technologies; Santa Clara, CA: BL21 CodonPlus Competent Cells, RNase-It Ribonuclease Cocktail.

Amresco; Solon, OH: 2-mercaptoethanol (BME), glycerol, sodium dodecyl sulfate (SDS), tris.

Bioexpress; Kaysville, UT: agar, fetal bovine serum (FBS), nuclease-free water, tryptone, urea.

Cell Applications, Inc.; San Diego, CA: Bovine insulin.

Electron Microscopy Sciences; Hatfield, PA: Glutaraldehyde, 25% solution.

Enzo Life Sciences; Farmingdale, NY: NADPH tetrasodium salt.

Fisher Scientific; Fairlawn, NJ: ampicillin trihydrate, bovine serum albumin (BSA), calcium chloride (CaCl₂), chloramphenicol, chloroform, deoxycholic acid, dimethyl sulfoxide (DMSO), glacial acetic acid, glycine, hydrochloric acid (HCl), imidazole, isopropyl β-D-1-thiogalactopyranoside (IPTG), kanamycin sulfate, potassium phosphate (KH₂PO₄), methanol, magnesium chloride (MgCl₂), phenol, sodium borohydride, sodium chloride (NaCl), sodium hydroxide (NaOH), sodium phosphate (Na₂PO₄), yeast extract.


Jackson ImmunoResearch Laboratories, Inc; West Grove, PA: Normal Donkey Serum.

Life Technologies; Carlsbad, CA: Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L), Alexa Fluor 555 Goat Anti-Mouse IgG (H+L), Alexa Fluor 555 Goat Anti-Rabbit IgG (H+L),
beta-tubulin monoclonal antibody (mouse), DH5α competent E. coli, DNase I, High Capacity RNA-to-cDNA kit, pRSET-A vector, Quant-iT Protein Assay Kit, Qubit RNA Assay Kit.

*Millipore; Billerica, MA:* cystatin C, Millicell EZ Slide, pepstatin A.

*Molecular Research Center, Inc.; Cincinnati, OH:* Tri-Reagent RT.

*MP Biomedicals; Solon, OH:* 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB).

*Promega; Madison, WI:* dithiothreitol (DTT), pGEM-T Easy Vector System.

*Qiagen; Germantown, MD:* E. coli M15, pQE9 vector, pQE30 vector.

*Sigma-Aldrich; St. Louis, MO:* Bromophenol blue, Coomassie Brilliant Blue R, Dulbecco’s Modified Eagles Medium (DMEM), JM109 Competent Cells, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), paraformaldehyde, penicillin-streptomycin, PIPES, proteinase K, saponin, sodium azide, triton-X 100.

*Thermo Scientific:* 1-Step NBT/BCIP, 6X DNA Loading Dye, DreamTaq PCR Master Mix, Ethidium Bromide Solution, GeneJet Gel Extraction Kit, GeneJet Plasmid Miniprep Kit, GeneRuler 1 kb DNA Ladder, Goat Anti Rabbit IgG Fc Alkaline Phosphatase, HisPur Ni-NTA Resin, lysozyme, Melon Pierce Crosslink Immunoprecipitation Kit, Phusion Site-Directed Mutagenesis Kit, Recombinant Protein A Agarose, Spectra Multicolor Broad Range Protein Ladder, TopVision LE GQ Agarose, Tubulin Beta Polyclonal Antibody (rabbit).

*Whatman; Dassel, Germany:* Reinforced Nitrocellulose Membrane.

**Buffers, Media, and Solutions**

Alkaline phosphatase buffer: 100 mM NaCl, 5 mM MgCl₂, 100 mM tris-HCl.

BRBD80: 80 mM PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.8.

Buffer B: 100 mM NaH₂PO₄, 10 mM tris-HCl. Adjust pH to 8 using NaOH.

Buffer T: 100 mM potassium phosphate, 2 mM EDTA, pH 7.4.

Calcium chloride solution: 60 mM CaCl₂, 15% glycerol, 10 mM PIPES (pH 7). Autoclave or filter sterilize.
Genomic DNA (gDNA) lysis buffer: 40 mM Tris-HCl (pH 8), 80 mM EDTA (pH 8), 2 % SDS, 0.1 mg/mL proteinase K (add just before use).

10 mg/mL insulin: Mix 50 mg bovine insulin in 4 mL 0.5 M Tris- HCl (pH 8). Adjust pH to 2 or 3 using 1 M HCl and then rapidly titrate back to 8 using 1 M NaOH. Add dH2O to 5 mL.

Immunoprecipitation buffer: 50 mM Tris-HCl, 100 mM NaCl, 1% triton-X 100, 0.5% deoxycholic acid, 1% BSA, 0.02% sodium azide, 0.2 mM PMSF, 40 nM cystatin C, 150 nM pepstatin A.

Immunoprecipitation lysis solution: 1% SDS, 100 mM NaCl, 0.2 mM PMSF, 50 mM tris-HCl, 40 nM cystatin C, 150 nM pepstatin A.

Immunoprecipitation wash solution: 50 mM tris-HCl, 100 mM NaCl.

Insulin assay buffer: 20 mM EDTA, 100 mM KH2PO4.

Luria-Bertani (LB) medium: 0.5% yeast extract, 1% NaCl, 1% tryptone.

Nycodenz buffer: 300 mg Tris-HCl, 61 mg CaNa2EDTA · H2O, 110 mg KCl (BDH). Dissolve in 450 mL H2O and adjust pH to 7.5 using 1 M NaOH. Fill to 500 mL and autoclave. Store at room temperature.

100% Nycodenz: 55.2 g Nycodenz (Axis-Shield). Add powder to 200 mL Nycodenz buffer. Autoclave and keep at room temperature. Before use, mix with sterile PBS.

PEM buffer: 100 mM PIPES, 1 mM EGTA, 2 mM MgCl2, pH 6.8.

Phosphate-buffered saline, 10X: 1.37 M NaCl, 27 M KCl, 43 mM Na2PO4, 14 mM KH2PO4.

100X Pyrimethamine Stock Solution (7 mg/mL): 280 mg pyrimethamine, 40 mL DMSO. Dissolve pyrimethamine in DMSO by vortexing for a few minutes until solubilized. The stock solution can be kept at room temperature and diluted in drinking water before use. As the pyrimethamine precipitates when adding water, the pH is lowered by adding HCl to the water. Pyrimethamine will be resolubilized between pH 3-5.

Reservoir buffer, 10X: 0.25 M tris, 2 M glycine.

Schizont culture medium: 16.4 g RPMI without HEPES, 1.75 g NaHCO3, 25 U/mL penicillin/streptomycin. Dissolve all ingredients in 900 mL H2O and adjust pH to 7.2. Fill to 1 L, filter sterilize, aliquot, and store at -20° C.
SDS electrophoresis buffer: 0.125 M tris, 0.96 M glycine, 0.5% SDS.

SDS-PAGE sample buffer, 2X: 0.09 M tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.02% bromophenol blue.

TAE electrophoresis buffer: 242 g tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA solution (pH 8), H₂O to 1 L.

Accession Numbers

PlasmoDB ID numbers for reported genes and proteins are as follows:
Thioredoxin Reductase (PBANKA_082470), Thioredoxin-1 (PBANKA_132090), Trx-469/PhLP1 (PBANKA_120480). GenBank (NCBI) ID numbers are as follows: TXNDC9 (NM_005783.3), Thioredoxin (BC003377.1).

Parasite Maintenance and Mosquito Infections

*Plasmodium berghei* ANKA 2.34 parasites were maintained in Harlan ND4 mice for a maximum of four serial passages and passed through *Anopheles stephensi* mosquitoes.

Cultivation of HepG2 and HeLa Cell Lines

HepG2 cells were provided by Dr. Anwar Khan from University of Illinois at Chicago, and HeLa cells were provided by Father Peter Breslin from Loyola University Medical Center. Cells were maintained in DMEM, 10% FBS, 1% penicillin/streptomycin in a 37° C incubator with 5% CO₂.

Generation of Human and *P. berghei* cDNA

Total RNA from *P. berghei* or HepG2 cells was extracted using Tri-Reagent according to manufacturer’s instructions. Isolated RNA was treated with DNAses I and
subsequently quantified using the Qubit RNA Assay Kit and the Qubit fluorometer. RNA samples were either immediately used for cDNA synthesis or flash frozen and stored at -80°C. cDNA was synthesized from total RNA with the High Capacity RNA-to-cDNA kit using random hexamer primers.

**Isolation and Purification of *P. berghei* gDNA**

A 1 mL sample of mouse blood containing ~20% mixed asexual blood stages was centrifuged at 3000 x g for 2 minutes. The cells were washed once in cold PBS and then resuspended in 1 mL PBS. Saponin was added to a final concentration of 0.05% and the sample was gently mixed. Immediately after lysis was observed, the tube was centrifuged at 6000 x g for 5 minutes. The supernatant was removed and 25 μL of lysis buffer and 75 μL distilled water were added to the pellet. The tube was then incubated at 37°C for 3 hours with intermittent stirring. After this time, 100 μL of distilled water were added followed by addition of 200 μL of phenol (equilibrated with 0.1 M Tris-HCl [pH 7.0]). The solution was mixed well and centrifuged at 2000 x g for 8 minutes. The extraction was performed again with 200 μL of chloroform. 2 μL of RNase-It Ribonuclease Cocktail were added for 30 minutes at 37°C. The extraction was performed again with phenol and chloroform as above.

The gDNA was precipitated by adding one tenth volume of 3 M sodium acetate (pH 5.0) and 2.5 volumes of absolute ethanol. The tube was allowed to sit for 2 hours or overnight at -20°C. The precipitate was centrifuged at 2000 x g for 30 minutes at 4°C and then gently washed once with 70% ethanol and dried in a speed-vac. The pellet was then resuspended in 25 to 100 μL of distilled water, depending on its size.
Polymerase Chain Reaction (PCR) of Target DNA

All PCR reactions were incubated on the Eppendorf Mastercycler ep gradient S thermal cycler.

TXNDC9

The region of the human genome coding for Thioredoxin Domain-Containing Protein 9 (TXNDC9) was amplified using PCR from cDNA obtained from the HepG2 human liver cell line using a forward primer with a 5’ BamHI site and a reverse primer with a 3’ HindIII site. The primers were as follows:

Forward: 5’ CCGGATCCGAAGCTGATGCATCTGTTGACATG 3’
Reverse: 5’ CCTTTCTTTTATACTAAGTCTGAGACTACTACTAATCTTCGAACC 3’

The following reagents: 5 μM forward primer, 5 μM reverse primer, 1:25 diluted cDNA, 1X DreamTaq PCR Master Mix were mixed and brought to a final volume of 20 μL with nuclease-free H₂O. The reaction was incubated on a ThermoCycler on the following program: initial denaturing at 94° C for 10 minutes, then 35 cycles of denaturing at 94° C for 30 seconds, annealing at 60° C for 45 seconds, and elongation at 72° C for 1 minute, with a final elongation at 72° C for 5 minutes.

hTrx

The region of the genome coding for human Thioredoxin was amplified using PCR from cDNA obtained from the HepG2 human hepatocyte line using a forward primer with a 5’ BamHI site and a reverse primer with a 3’ Kpn1 site. The primers were as follows:

Forward: 5’ GGGGATCCGTGAAGCAGATCGAGAGC AAG 3’
Reverse: 5’ CTTGAAGCCACCATTGAATTAGTCTAAGGTACCCC 3’
The following reagents: 5 μM forward primer, 5 μM reverse primer, 1:25 diluted cDNA, 1X DreamTaq PCR Master Mix were mixed and brought to a final volume of 20 μL with nuclease-free H2O. The reaction was incubated on a ThermoCycler on the following program: initial denaturing at 94° C for 10 minutes, then 35 cycles of denaturing at 94° C for 30 seconds, annealing at 60° C for 45 seconds, and elongation at 72° C for 1 minute, with a final elongation at 72° C for 5 minutes.

**PbPhLP1 knockout construct**

The primers for the 5’ region had a 5’ ApaI site and a 3’ HindIII site. The primers for the 3’ region had a 5’ BamHI site and a 3’ XbaI site. The primers were as follows:

5’ Forward: 5’ CC GGGCCC CAATGCCC CAAAACGAAAACAGAC 3’
5’ Reverse: 5’ CCAAGCTT CGAATCTAAATATTCACATCTCCATGTAG 3’
3’ Forward: 5’ CCGGATCCGGCGGAGATAATTTTTCTGAACAAGC 3’
3’ Reverse: 5’ CC TCTAGA CCTCTTCATGATACCTCGATTCAAAGC 3’

The following reagents: 1 μL genomic DNA (gDNA), 1 mM forward primer, 1 mM reverse primer, 1X DreamTaq PCR Master Mix were mixed and brought to a final volume of 25 μL with nuclease-free water. One reaction was set up for each primer pair and incubated in the thermal cycler on the following program: initial denaturing at 94° C for 10 minutes, then 35 cycles of denaturing at 94° C for 30 seconds, annealing at 56° C for 45 seconds, and elongation at 65° C for 45 seconds, with a final elongation at 65° C for 6 minutes.
Site-Directed Mutagenesis

5’ phosphorylated primers were designed to mutagenize PbPhLP1 using the Phusion Site-Directed Mutagenesis Kit. The primers were as follows:

PhLP1-C106S Forward: 5’ AGAAATACTACATGGAGATCGGAATATTTAGATTTCG 3’

PhLP1-C106S Reverse: 5’ ATAAAAATGACAACATACATTTTGTGTTGAATTTTTAC 3’

Plasmid DNA was used as a template. The reaction was set up using reagents from the Phusion Site-Directed Mutagenesis Kit as follows: 10 pg template DNA, 10 mM dNTPs, 1X Phusion HF Buffer, 0.5 μM forward primer, 0.5 μM reverse primer, 1 U Phusion HotStart II DNA Polymerase were mixed and nuclease-free water was added to 50 μL. The reaction was incubated on the thermal cycler on the following program: initial denaturing at 98° C for 2 minutes, then 25 cycles of denaturing at 98° C for 15 seconds, annealing at 60° C for 30 seconds, and elongation at 72° for 2 minutes, with a final elongation at 72° C for 10 minutes. A ligation was then performed to circularize the plasmid. The ligation was set up using the following reagents from the Phusion Site-Directed Mutagenesis Kit: 1X Quick Ligation Buffer, 0.5 μL T4 DNA Ligase were mixed and incubated at room temperature. The ligation reaction was incubated on ice until transformation.

TA Cloning of DNA Fragments into pGEM Vector

The following reagents from the pGEM-T Easy Vector System II: 1X Rapid Ligation Buffer, 25 ng pGEM-T Easy Vector, 3 U T4 DNA Ligase, 1.5 μL solution
containing DNA fragment were mixed and brought to a final volume of 10 μL using nuclease-free water. The reaction was incubated overnight at 4° C.

**Making DH5α and M15 Competent Using CaCl₂**

Cells were grown in 50 mL LB media (plus 0.1 mg/mL kanamycin for M15) to an optical density (OD) of 0.15. Optical density was measured at a wavelength of 650 nm using the Genesys 6 spectrophotometer (Thermo Scientific). The cells were then pelleted at 3500 g for 10 minutes at 4° C. The supernatant was discarded and the pellet was resuspended in 10 mL cold calcium chloride solution. The cells were pelleted at 3500 g for 5 minutes at 4° C. The supernatant was discarded and the pellet was again resuspended in 10 mL cold calcium chloride solution. The cells were incubated on ice for 30 minutes and then spun down at 3500 g for 5 minutes at 4° C. The supernatant was discarded and the pellet was resuspended in 2 mL cold calcium chloride solution. Competent cells were used within 48 hours for transformation.

**E. coli Transformation (DH5α, JM109, M15, BL21)**

5 μL plasmid was mixed with 25 μL of the competent cells in a 1.5 mL microcentrifuge tube. The mixture was incubated on ice for 20 minutes and then heat shocked at 42° C for 20 seconds. The reaction was again placed on ice for 2 minutes before 500 μL LB media were added. The cells were then incubated in a 37° water bath for 1 hour. The transformed cells were spread onto plates containing LB, 1.5% agar plus antibiotics (0.1 mg/mL ampicillin, 0.1 mg/mL kanamycin and ampicillin, or 0.1 mg/mL ampicillin and 35 μg/mL chloramphenicol). In the case of blue/white screening, plates
containing LB, 1.5% agar plus 0.1 mg/mL ampicillin, 0.1 mM IPTG, and 20 μg/mL X-Gal. The plates were incubated overnight in a 37° C incubator.

**Restriction Digests**

Restriction digests were done using the following enzymes: Apal, BamHI, HindIII, KpnI, XbaI. The reactions were set up according to manufacturer’s instructions.

**Ligation of Fragments into Vectors**

Ligations using vectors other than pGEM were set up using the following reagents: 1X T4 DNA Ligase Buffer, 1 μL vector, 16 μL DNA fragment, 0.25 U T4 DNA Ligase. The reaction was mixed and incubated overnight on the Eppendorf Mastercycler ep gradient S at 16° C.

**Protein Expression**

M15 or BL21 cells containing appropriate plasmid were grown in LB plus antibiotic (0.1 mg/mL ampicillin plus 0.1 mg/mL kanamycin for M15, 0.1 mg/mL ampicillin plus 35 μg/mL chloramphenicol for BL21) in a shaking incubator at 37° C (or room temperature for expression of TXNDC9). Optical density (OD) was measured at a wavelength of 650 nm using the Genesys 6 Spectrophotometer (Thermo Scientific) until an OD of approximately 0.5 was reached. IPTG was added to a final concentration of 1 mM to induce protein expression. Cells were grown overnight at 37° C or room temperature while shaking.

**Protein Purification**

The bacterial culture was pelleted by centrifugation at 6000 g for 15 minutes at 4° C and the media was removed. Cells were then resuspended in Buffer B plus 45 mM
imidazole, 10 μM PMSF, and 50 μg/mL lysozyme and shaken at room temperature for 1 hour. Bacteria were sonicated 5 times for 45 seconds and iced for at least 45 seconds between sonications. Cells were then spun down at 35,000 g for 30 minutes at 4 °C. Pellets were discarded and the cell lysate was poured over a Ni-NTA column. 2 mL of Ni-NTA slurry was used for every 500 mL of bacterial culture. The lysate was slowly passed through the column so as to provide adequate time for protein to bind to the beads. The column was then washed and protein was eluted using an imidazole gradient.

Urea Purification

The bacterial culture was pelleted by centrifugation at 6000 g for 15 minutes and the media was removed. Cells were then resuspended in Buffer B with 8 M urea at 5 mL/g of wet weight. This mixture was incubated for two hours at room temperature, and then centrifuged at 10,000 g for 30 minutes at 25 °C. Cell lysate was poured over a Ni-NTA column and was allowed to flow through the column at a rate of approximately 1 drop every 5-8 seconds. After lysate had passed through column, 10 mL of Buffer B with 6 M urea was added. This step was repeated with Buffer B with 4 M urea, 2 M urea, and 1 M urea. The column was then washed with 10 mL Buffer U with 10 mM imidazole and 10 mL Buffer U with 50 mM imidazole. Increased concentration of imidazole in Buffer U were added to elute the protein.

SDS-PAGE

All SDS-PAGE procedures were performed using the Bio-Rad PowerPac Basic and Mini PROTEAN II (Bio-Rad; Hercules, CA). Samples were loaded onto tris-glycine gels immersed in 1X SDS electrophoresis buffer. The Spectra Multicolor Broad Range
Protein Ladder was used to compare protein sizes to known standards. Gels were run at 150 V for approximately 90 minutes.

### DNA Electrophoresis

All PCRs and restriction digests were run on a gel consisting of 1% TopVision LE GQ Agarose in 1X TAE buffer plus 0.5 μg/ml ethidium bromide. For restriction digests, 6X DNA Loading Dye was added to the reactions at a final concentration of 1X. GeneRuler 1 kb DNA Ladder was used to estimate DNA fragment sizes. The samples were run on a DNA electrophoresis cell at 120 V for 30 minutes.

### Construction of Transgenic *P. berghei*

#### Production of Schizonts and Purification

40-80 mL of schizont culture medium were supplemented with 20% Fetal Bovine Serum (FBS) and gassed with 5% CO₂, 3% O₂. A mouse of 3-5% parasitemia was bled, and the blood was mixed with the medium/FBS mixture. The culture was gassed as above. Schizonts were cultured at 37° C while being gently shaken at 50 rpm for 20 hours. The culture was spun at 500 g, and the supernatant was discarded. The blood was layered on top of 55% Nycodenz/PBS and centrifuged at 300 g for 35 minutes with acceleration and deceleration set to 4. The interface containing parasites was transferred into a separate tube. Medium was added to 10-50 mL and the parasites were washed by spinning for 10 minutes at 720 g. The supernatant was removed almost fully, and the schizonts were immediately used for transfection.
Transfection of *P. berghei* with Amaza Electroporation System

2-3 days prior to transfection, a mouse was treated with phenylhydrazine (PH). The mouse was then bled, and the blood was kept at 37°C until use. For transfection, the Nucleofector I electroporator (Amaza Biosystems; Cologne, Germany) and the Human T Cell Nucleofector Kit (Amaza Biosystems; Cologne, Germany) were used. This method was developed by Janse *et al* (Janse *et al*, 2006). 90 μL of Nucleofector solution were mixed with 20 μL Amaza Nucleofector Supplement. 100 μL of this mixture and 1-5 μg of digested plasmid in 10 μL H2O were added to the schizonts. The suspension was rapidly transferred to an electroporation cuvette. Electroporation was performed with program U33. After the ‘OK’ signal appeared, the suspension was mixed with 200 μL of the pre-warmed blood. Merozoites were allowed to invade for 20-30 minutes while shaking at 300 rpm and 37°C in the Eppendorf Thermomixed (Eppendorf; Hamburg, Germany). The parasites were then injected intraperitoneally (*i.p.*) into a naïve mouse.

Drug Selection

Drug selection was started 1 day after infection. Sulphadiazine treatment was applied by giving the mice the drug in drinking water at a concentration of 20 mg/L. Drug pressure was applied for 3 days. Pyrimethamine can be injected *i.p.* or given in drinking water. Here, the drug selection with pyrimethamine was done with drinking water for 3-4 days. WR99210 was injected *i.p.* at a concentration of 4 mg/mL (approximately 10 ng WR99210 per g body weight). 100 μL were given for 3-4 days.
Insulin Assays

A stock solution of 10 mg/mL was made by mixing 50 mg bovine insulin in 4 mL 0.5 M Tris-HCl (pH 8). The pH was adjusted to 2 or 3 using 1 M HCl and then rapidly titrated back to 8 using 1 M NaOH. dH₂O was added to 5 ml. The working solution consisted of 1.75 mg/mL bovine insulin in assay buffer. Each assay had a volume of 600 μL and was read on the Genesys 6 spectrophotometer. 450 μL working solution plus 150 μL of 275-500 mM imidazole was used as a blank in each reaction. The concentration of imidazole in the blank and the control depended on the concentration of the imidazole that was used to elute the protein being tested in the reaction. For each reaction, the working solution was incubated for 3 minutes with DTT added to a final concentration of 1.67 mM, 1.16 mM, or 0.833 mM in a cuvette. The absorbance was read at 650 nm. After 3 minutes, 4-20 μM protein was added, and the contents of the cuvette were mixed every 2 minutes to prevent precipitation from interfering with the beam of light. 20 μM BSA or 150 μL imidazole was used in place of recombinant protein in the control reaction. The reactions were allowed to proceed 30-60 minutes, with readings taken every 30-60 seconds.

Immunoprecipitation of Protein from HeLa Cells

Method 1

The protocol for this method of immunoprecipitation was developed from Kopan et al (Kopan et al, 1996). HeLa cells were grown to confluence in 60 mm dishes. The media was removed, and cells were washed three times with 1X PBS. Cells were lysed using immunoprecipitation lysis solution. After lysis, cellular material was transferred to
a 1.5 mL microcentrifuge tube. Cellular material was sheared by passing the lysate through a 21 gauge needle approximately 10 times. The tubes were then spun at 20,000 g for 15 minutes at 4° C to pellet cell debris. The supernatant was transferred to new 1.5 mL tubes. anti-PbPhLP1 antibodies were added to one tube to a final dilution of 1:600. Immunoprecipitation buffer was added to a final volume of 2 mL. As a control, only immunoprecipitation buffer and cell lysate (no antibodies) were added to a second tube. The tubes were then incubated overnight on a shaker at 4° C.

250 μL of Recombinant Protein A Agarose beads were washed with immunoprecipitation buffer, and 50 μL bead slurry was added to each tube. The tubes were incubated for 3 hours at 4° C while shaking, and then briefly centrifuged to pellet the beads. A bent 28 gauge needle was used to remove liquid from the beads. The beads were resuspended in 1 mL immunoprecipitation buffer and transferred to a new 1.5 mL microcentrifuge tube. The beads were then washed once with 1 mL immunoprecipitation buffer and twice with immunoprecipitation wash solution. After final wash, all the remaining liquid was removed using bent 28 gauge needle. The beads were resuspended in 30 μL 2X SDS-PAGE sample buffer and boiled at 95° C for 10 minutes. The liquid was then run on a tris-glycine gel using the SDS-PAGE method.

**Modifications to Method 1**

Media was removed and plates were washed three times with 1X PBS. 300 μL lysis buffer was added to each plate, and lysate from all plates were combined in a single 15 mL tube. Cells were sheared by passing the lysate through a pipette multiple times. The tube was centrifuged at 12,000 g for 15 minutes at 4° C to pellet cell debris, and the
supernatant was transferred to a 50 mL tube. 12 mL immunoprecipitation buffer was added. One day before harvesting cells, 150 μL bead slurry, 1 mL immunoprecipitation buffer, and anti-PbPhLP1 antibodies added to a final dilution of 1:500 were incubated on a shaker at 4°C overnight. This mixture was added to the 50 mL tube with the cell lysate, and the tube was incubated overnight at 4°C while shaking. The tube was centrifuged briefly to pellet the beads at the bottom of the tube. The supernatant was removed. The beads were resuspended in 1 mL immunoprecipitation buffer and transferred to a 1.5 mL microcentrifuge tube. The tube was centrifuged again to collect the beads at the bottom of the tube, and the supernatant was removed. The resin was washed once with 1 mL immunoprecipitation buffer and three times with wash solution. After all liquid was removed, 30 μL SDS loading buffer was added, and the tube was boiled at 95°C for 10 minutes. The liquid was run on a protein gel as before, and the gel was stained with Coomassie. Bands were sent to be sequenced by mass spectroscopy at the University of Illinois at Chicago.

**Method 2: Pierce Crosslink Immunoprecipitation Kit**

Unless otherwise stated, all reagents used were from Pierce Crosslink Immunoprecipitation Kit. Unless otherwise stated, all centrifugation steps took place at 1000 g for 1 minute.

**Antibody cleanup.** Anti-PbPhLP1 antibodies were purified from serum using the Melon Gel IgG Purification Kit following manufacturers instructions.

**Binding of antibody to Protein A/G Plus Agarose.** 2 mL of 1X Coupling Buffer was prepared for each immunoprecipitation reaction. The bottle of Protein A/G Plus
Agarose was gently swirled to obtain an even suspension. 20 μL of resin slurry was added to a spin column for each reaction. The column was placed in a 1.5 mL microcentrifuge tube and centrifuged. The flowthrough was discarded. The resin was washed twice with 200 μL 1X Coupling Buffer. The bottom of the column was gently tapped on a paper towel to remove excess liquid and the plug was inserted. 10 μg of antibody was prepared for coupling. The volume of antibody was adjusted to 100 μL with sufficient ultrapure water and 20X Coupling Buffer to produce 1X Coupling Buffer. This mixture was added to the spin column, and the screwcap was attached. The column was incubated on a shaker for 1 hour at room temperature. The bottom plug and cap were removed, and the column was placed in a collection tube and centrifuged. The resin was washed once with 100 μL 1X Coupling Buffer and twice with 300 μL 1X Coupling Buffer. The bottom of the column was tapped on a paper towel to remove excess liquid and the plug was inserted.

**Crosslinking bound antibody.** 217 μL DMSO was added to a single tube of DSS to prepare 10X/25 mM solution. The solution was mixed with a pipette until the DSS was completely dissolved, and then diluted 1:10 in DMSO to make 2.5 mM DSS. 2.5 μL 20X Coupling Buffer, 9 μL 2.5 mM DSS, and 38.5 μL nuclease-free water were added to the column and incubated for 1 hour at room temperature while shaking. The bottom plug and cap were removed, and the column was placed in a collection tube and centrifuged. 50 μL Elution Buffer was added and the column was centrifuged. The column was washed twice with 100 μL Elution Buffer and twice with cold IP Lysis/Wash Buffer. The last wash was left in the column, and the column was stored at 4°C until needed.
Cell lysis. The culture medium was removed and each dish was washed once with 1X Coupling Buffer. 500 μL ice cold IP/Lysis Wash Buffer was added to the plates, and the plates were incubated on ice for 5 minutes with periodic mixing. The lysate was transferred to a microcentrifuge tube and centrifuged at 13,000 g for 10 minutes to pellet cell debris. The supernatant was transferred to a new tube and protein concentration was determined using the Quant-iT Protein Assay Kit.

Pre-clear lysate using control agarose resin. For 1 mg lysate, 80 μL Control Agarose Resin slurry was added to a spin column and centrifuged to remove storage buffer. 100 μL 1X Coupling Buffer was added to the column, and the column was centrifuged. 1 mg of lysate was added to the column and shaken at 4° C for 45 minutes. The column was centrifuged. The column was discarded and the flowthrough was saved. The protein concentration was measured using the Quant-iT Protein Assay Kit.

Immunoprecipitation. The column containing resin and antibody was centrifuged to remove IP Lysis/Wash Buffer. 1 mg lysate was added to the column and IP Lysis/Wash Buffer was added to 500 μL. The column was incubated on the shaker overnight at 4° C. The bottom plug and screw cap were removed and the column was centrifuged. The column was washed twice with 200 μL IP Lysis/Wash Buffer. The column was washed once with 100 μL 1X Conditioning Buffer and then placed in a new collection tube. 10 μL Elution Buffer was added to the column, and the column was centrifuged. With the column in the same collection tube, 50 μL Elution Buffer was added and incubated for 5 minutes at room temperature. The column was centrifuged and
the flowthrough was collected. The elution steps were repeated as desired for further samples.

**Sample preparation for SDS-PAGE analysis.** 5X Lane Marker Sample Buffer was equilibrated to room temperature and gently mixed by inverting 5-10 times. Sample buffer was added to elutions to make 1X concentration of sample buffer. DTT was added to 100 mM and samples were boiled at 95° C for 5 minutes. Samples were allowed to cool to room temperature before being loaded on gel. Samples were run using the SDS-PAGE method. The gel was stained with Coomassie, and bands were sent for sequencing by mass spectroscopy at the University of Illinois at Chicago.

**Modifications to Method 2**

Two columns were prepared, one containing lysate, beads, and antibody, and one containing only antibody and beads. 50 μg of anti-PbPhLP1 antibody was used during crosslinking, and wash steps were doubled in order to accommodate larger concentration of protein. The resulting gel was used for a Western blot.

**Western Blots**

**Sample Preparation Using Parasite Lysate**

Cryotubes containing 2 mL of flash frozen blood infected with *P. berghei* was allowed to thaw at room temperature. Blood was transferred to a 50 mL tube. Cryotubes were rinsed with 1X PBS and the rinses were added to the 50 mL tube. 1X PBS was added to a final volume of 20 mL, and the tube was centrifuged at 750 g for 5 minutes. The supernatant was aspirated and the pellet resuspended in 20 mL 1X PBS. The tube was centrifuged at 800 g for 10 minutes. The supernatant was removed, cells were
resuspended in 20 mL 1X PBS, and red blood cells were lysed by adding saponin to a final concentration of 0.01%. The tube was mixed by inversion for 2 minutes and centrifuged at 1800 g for 10 minutes. The supernatant was aspirated and the lysis was repeated. The cells were then resuspended in 20 mL 1X PBS and centrifuged at 1800 g for 10 minutes. The supernatant was aspirated and the cells were resuspended in 1 mL 1X PBS and transferred to a 2 mL microcentrifuge tube. PMSF was added to a final concentration of 60 μM. From this, two 200 μL volumes were transferred to 1.5 mL microcentrifuge tubes. SDS-PAGE sample buffer was added to a final concentration of 1X. BME was added to 0.75% to one of the tubes. Both samples were boiled at 95° C for 5 minutes and centrifuged at 10,000 g for 10 minutes at 4° C. The samples were then run using SDS-PAGE.

**Sample Preparation Using Recombinant Protein**

Desired concentration of protein was used and SDS-PAGE sample buffer was added to a final concentration of 1X. BME was added to a final concentration of 5%. Samples were boiled at 95° C for 2 minutes and iced for 2 minutes before being run using SDS-PAGE.

**Sample Preparation Using Purified Tubulin**

Purified porcine tubulin and PEM buffer were provided by Dr. Holly Goodson at Notre Dame University. Tubulin was diluted 1:1 with PEM buffer. SDS-PAGE sample buffer was added to a final concentration of 1X, and BME was added to a final concentration of 5%. Samples were boiled at 95° C for 2 minutes and iced for 2 minutes before being run using SDS-PAGE.
Transfer from Gel to Nitrocellulose

After electrophoresis, gel was allowed to sit in reservoir buffer for 10 minutes. Nitrocellulose membrane was also incubated in reservoir buffer during this time. The blot was assembled and run while submerged in reservoir buffer using the Bio-Rad Mini Trans-Blot Module (Bio-Rad; Hercules, CA) at 100 V for 1 hour.

Immunofluorescent Staining of Nitrocellulose Membrane

After blotting, the membrane was incubated in blocking buffer (2.5 g milk powder in 1X PBS) for 20 minutes. Blocking buffer was poured off, and, if necessary, membrane was cut if different portions were to be stained using different antibodies. Primary antibodies (rabbit anti-PbPhLP1 or Tubulin Beta Polyclonal Antibody) were diluted using blocking buffer and added to membrane for one hour while shaking. Membrane was washed three times with 1X PBS and then placed in 1X PBS on shaker for 15 minutes. PBS was poured off. The secondary antibody, Goat Anti Rabbit IgG Fc Alkaline Phosphatase, was diluted using blocking buffer and added to membrane for 1 hour while shaking. Membrane was washed three times with 1X PBS. PBS was poured off and BCIP/NBT was allowed to incubate with membrane on shaker until bands started to appear. BCIP/NBT was then removed and alkaline phosphatase was added until membrane was just covered. Membrane was incubated in alkaline phosphatase overnight.

Immunofluorescence

Paraformaldehyde/Glutaraldehyde Fixation

Media was removed and cells were washed once with 1X PBS. 3% paraformaldehyde, 0.25% glutaraldehyde, and 0.2% Triton X-100 in BRBD80 was added
to cells and allowed to incubate for 15 minutes at room temperature. Supernatant was removed, and cells were washed with 1X PBS. Cells were permeabilized by adding -20°C 100% methanol for 10 seconds, and then washed with 1X PBS. 0.5 mg/mL NaBH₄ in 1X PBS was added for 10 minutes. Cells were washed three times with 1X PBS before proceeding to staining.

**Glutaraldehyde Fixation**

Media was removed and cells were washed with 1X PBS warmed to 37°C. BRBD80 plus 0.5% Triton X-100 and 2% glycerol was added to cells for 20 seconds. Glutaraldehyde was added to final concentration of 0.5% and cells were shaken gently for 10 minutes in the solution. The liquid was removed and 0.5 mg/mL NaBH₄ in 1X PBS was added for 7 minutes. Cells were washed three times with 1X PBS and then permeabilized with 0.1% Triton X-100 in PBS for 15 minutes. Cells were washed again three times with 1X PBS before proceeding to staining.

**Antibody Staining**

Cells were blocked for 30 minutes in 0.1% donkey serum in 1X PBS. Cells were then washed three times with 1X PBS. Primary antibodies (rabbit anti-PbPhLP1, anti-beta tubulin monoclonal antibody, or Tubulin Beta Polyclonal Antibody) were diluted in 0.1% donkey serum in 1X PBS and incubated with cells for 1 hour at room temperature while shaking. Cells were then washed three times with 1X PBS. Secondary antibodies (Alexa Fluor 555 Goat Anti-Mouse, Alexa Fluor 555 Goat Anti-Rabbit, or Alexa Fluor 488 Goat Anti-Rabbit) were diluted in 0.1% donkey serum in 1X PBS and incubated with
cells for 1 hour at room temperature while shaking. Cells were washed again three times with 1X PBS.

**Phalloidin Staining**

After permeabilization and antibody staining, phalloidin (provided by Dr. Anwar Khan at University of Illinois at Chicago) was applied at a dilution of 1:60 in 0.1% donkey serum in 1X PBS for 1 hour at room temperature while shaking. Cells were washed three times in 1X PBS.

**Imaging**

Images were acquired on FluoView FV1000 Confocal Laser Scanning Microscope (Olympus). Images were taken in XY plane and compressed around the Z axis. AF 488 was excited by exposure at 488 nm, and AF 555 was excited by exposure at 555 nm, with fluorescent emissions were recorded at 530 nm and 565 nm, respectively.

**Thioredoxin System Assays**

All assays were measured in UV cuvettes using the Genesys 6 spectrophotometer. Unless otherwise stated, all reactions were carried out in Buffer T at room temperature in a final volume of 1 mL.

For all thioredoxin assays, absorbance was measured at 340 nm. 200 μM NADPH and 10-40 μM rPbTrx-1 or hTrx were incubated on spectrophotometer to establish a baseline. 50 nM PbTrxR was added, and the absorbance was read. After the reaction was completed, the protein to be tested was added, and the absorbance was read. Alternatively, the experimental protein was added before PbTrx-1.
Modeling

Comparative models of the PbPhLP1 and hTXNDC9 structures were made using Swiss Model (http://swissmodel.expasy.org; Arnad et al., 2006). The thioredoxin-like domain of Homo sapiens Phosducin-Like Protein 2 (PDCL2) (PDBid: 3EVI) was selected as a template using a protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) against the sequences in the Protein Data Bank. The evolutionary conservation of the structures was investigated using ConSurf (Ashkenazy et al., 2010). The models were visualized using the VMD molecular graphics program (Humphrey et al., 1996).

Phylogenetics

Phylogenetic trees were constructed by Dr. Catherine Putonti at Loyola University Chicago.

Sequence Comparison

The PbPhLP1 and PbPhLP2 amino acid sequences, retrieved from PlasmoDB (Aurrecoechea et al., 2009) were aligned using EBI’s CLUSTALW2 tool online (Larkin et al., 2007). The conserved thioredoxin domain (phosducin (Phd)-like family, Thioredoxin (TRX) domain-containing protein 9 or Phd_like_TxnDC9) was identified within each sequence via NCBI’s Conserved Domain Database (Marchler-Bauer et al., 2009). The sequence identity between the two sequences was calculated based upon the CLUSTALW alignment in BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html).
Identifying Paralogs and Homologs

The PbPhLP1 and PbPhLP2 amino acid sequences were BLASTed against the NCBI nr protein database using blastp, retrieving the 1000 best hits given a word size of two (Altschul et al., 1997). The PbPhLP1 sequence BLAST results all had an e-value less than 0.002; the PbPhLP2 sequence BLAST results all had an e-value less than 0.037.

The BLAST results were downloaded and parsed, using in-house parsers developed in C++, producing FASTA format files. Two FASTA format files were created for the PbPhLP1 and PbPhLP2 searches—the homologous sequences and the orthologous sequences. The homologous files include all 1000 hits for the individual BLAST. These files include orthologous sequences as well as paralogous sequences as there are many copies of the thioredoxin domain within the genomes of all species, not just *Plasmodium*. While the vast majority of the sequences returned for the BLAST searches encompassed more than just the thioredoxin domain, there were several smaller partial sequences, many of which are annotated as thioredoxins or putative thioredoxins.

The second pair of FASTA files created contained only orthologs of PbPhLP1 and orthologs of PbPhLP2. The BLAST of the PbPhLP2 sequence against NCBI’s Protein database identified its paralog, PbPhLP1, with an e-value of $8 \times 10^{-12}$, 68% coverage, and 32% identity. Using this as our threshold, we restricted orthologs to hits with a coverage $\geq 68\%$ and a sequence identity $>32\%$. 50 orthologs were identified for the PbPhLP2 sequence and 140 orthologs were identified for the PbPhLP1 sequence.
Deriving Phylogenetic Trees

Phylogenetic trees were created using MEGA 5.05 (Tamura et al, 2011). Within the MEGA software, the sequences were aligned via CLUSTALW using the default alignment parameters. All phylogenetic trees were derived using the Maximum Likelihood method with 100 bootstrap replications. The trees generated were then saved in NEWICK format and visualized using PhyloWidget (Jordan and Piel, 2008).
CHAPTER III

RESULTS

Preliminary Data

A screen of the Plasmodium genomics database PlasmoDB (Aurrecoechea et al., 2009) for proteins containing a putative thioredoxin domain revealed multiple genes, one of which was a previously uncharacterized gene of the mouse malaria parasite Plasmodium berghei which our lab tentatively named pbtrx-469 (previously PB000469.02.0, now PBANKA_120480). The gene is located on chromosome 12 of the Plasmodium genome and consists of one exon and no introns (Figure 4). The protein consists of two domains, a predicted C-terminal thioredoxin domain and an N-terminal helical domain. The protein sequence lacks any predicted targeting sequence or transmembrane domain, causing us to hypothesize that PbTrx-469 may be cytosolic. In addition, while the protein sequence does contain six cysteines, it lacks the classical CXXC active site. Transcriptome and mass spectrometry data available on PlasmoDB indicated that pbtrx-469 is expressed during all life stages of the P. berghei life cycle (Hall et al., 2005).

Initial work performed in our lab to characterize pbtrx-469 included quantitative real-time RT-PCR (qRT-PCR) to determine the relative expression of the gene (Figure 5a). Expression was examined in blood stages, ookinete cultures, and parasites taken
from the mosquito midgut, and was compared to expression of *P. berghei* Thioredoxin-1 (pbtrx-1). The transcript abundance of *pbtrx-469* was found to be approximately a hundredfold lower than that of *pbtrx-1* in both blood stage samples and mosquito midgut samples, and tenfold lower that *pbtrx-1* in ookinete cultures (Figure 4a). These results indicate that *pbtrx-469* is expressed at low levels when compared with other genes like *pbtrx-1*. In addition, qRT-PCR experiments were completed to examine how the transcript abundance changes during the time that the parasite is within the mosquito
midgut (Figure 5b). The mRNA levels at the 6 hour time point were comparatively low when compared to the profiles of known antioxidant genes (Turturice et al, 2013).

Our lab cloned and expressed PbTrx-469 in a bacterial system. The recombinant protein was purified, and a band at approximately 24 kDa was observed, consistent with the calculated size of recombinant PbTrx-469 (Figure 6).
The purified recombinant protein was then sent out for polyclonal antibody production in rabbits (Open Biosystems, Thermo Scientific). Upon receipt, the antibody-containing serum was tested in a Western blot analysis with recombinant PbTrx-469 (Figure 7). The antibodies produced a prominent band at approximately 24 kDa, suggesting that they primarily bind to recombinant PbTrx-469. As a control, the antibodies were also tested with recombinant PbTrx-1 (Figure 7). No interaction was observed.

![Western blot with recombinant PbTrx-469 and PbTrx-1](image)

**Figure 7.** Western blot with recombinant PbTrx-469 and PbTrx-1. Purified recombinant proteins (6 µg/well) +/- β-mercaptoethanol treatment (βM) were separated on an SDS gel and subsequently transferred onto a nitrocellulose membrane. Blots were probed with either Anti-PbTrx-469 or Anti-PbTrx-1 (1/1000 each) (Kanzok *et al.*, unpublished).

Immunofluorescent assays (IFAs) were then conducted to determine the expression pattern and subcellular localization of Trx-469 in *P. berghei*. The antibodies were used to stain the protein during different stages of the *P. berghei* life cycle, including asexual blood stages such as trophozoites (Figure 8a) and schizonts (Figure
8b), and mosquito stages such as ookinete (Figure 8c), and sporozoites (Figure 8d). In these initial IFA images, it appears that the protein is localized close to the periphery of the parasite cell (Figure 8a-8d), suggesting that PbTrx-469 may be associated with the plasma membrane of the parasite. Interestingly, the anti-PbTrx-469 antibodies seemed to cross-react with components in or close to the membrane of the host red blood cells (Figure 8e). It was unclear whether this signal was due to an unspecific background signal or a more specific cross-reaction with a related protein of the mammalian host cell.

While conducting research with our collaborator Dr. Shin-Ichiro in Japan, we conducted IFAs on human hepatocytes infected with *P. berghei* sporozoites using antibodies against several Trx-like proteins, including PbTrx-469. Interestingly, while anti-PbTrx-1, anti-PbTPx-1, and anti-Pb1CysPrx antibodies were parasite specific, the anti-PbTrx-469 antibodies seemed to be binding to a protein within the human host cells.
as well (Figure 9). The protein appeared to be associated with the microtubular network of the host cell.

The objective of this project was to further characterize this novel thioredoxin domain-containing protein in Plasmodium. Efforts would first be made to characterize \textit{pbtrx-469} using gene knockout experiments. In addition, the presence of the thioredoxin domain within PbTrx-469 caused us to characterize the protein biochemically. The second part of this project was to identify what the antibodies may be recognizing in human cells, and whether or not this target represented a homolog of PbTrx-469.

\textbf{Disruption of the \textit{pbtrx-469} Gene in \textit{P. berghei} Parasites}

To help determine the significance and potential function of PbTrx-469 in the malaria parasite, I aimed to disrupt, or knockout (KO), the \textit{pbtrx-469} gene in \textit{P. berghei}. This project was organized into two parts. My first task was to generate a DNA plasmid or KO construct here at Loyola. The verified construct would then be sent to our collaborator, Dr. Andrew Blagborough, for transfection into \textit{P. berghei} parasites. Dr. Blagborough is a researcher in the laboratory of Dr. Robert Sinden at Imperial College.
London, United Kingdom, and an expert in *Plasmodium* transfection and gene KO studies (Talman *et al.*, 2011).

Since *Plasmodium* is haploid during most of the erythrocytic stages of the life cycle, it is possible to interrupt the function of a gene through a single targeting event (Waters *et al.*, 1997). This is achieved through the process of homologous recombination between a plasmid containing the KO construct and the target gene in the parasite genome (Janse *et al.*, 2006). In general, there are two different methods for targeting a gene in this manner, a double crossover knockout (also known as a replacement knockout) or a single crossover knockout (also known as an insertion knockout) (Ménard and Janse, 1997). I chose to use the former method to KO *pbtrx-469* (Figure 10).

In this method, the construct includes two regions of homology to the gene, and recombination results in the replacement of the entire gene by the selectable marker (Ménard and Janse, 1997). In this approach it is impossible for the parasite to regenerate the wild-type gene. In the single crossover knockout, the construct includes only one region of homology to the gene, and recombination leads to the insertion of the entire KO plasmid. In this case, the entire gene is still present in the knockout parasites, however it is interrupted by a selectable marker. It is therefore possible for the parasite to remove the selectable marker and reform the wild-type (WT) gene (Ménard and Janse, 1997). I therefore chose to use the double-crossover method for this project in order to avoid a reformation of the WT *pbtrx-469* gene.
Generation of the Knockout Construct

I designed PCR primers that amplified two regions of the parasite genome. One region, the 5’ region, coded for a short stretch of the 5’ UTR as well as the beginning of the \textit{pbtrx-469} gene. The other region, the 3’ region, coded for a short stretch of the 3’ UTR as well as the end of the \textit{pbtrx-469} gene (Figure 10). The 5’ region primers were engineered to contain an Apal and a HindIII restriction site, and the 3’ region primers were engineered with a BamHI and a XbaI restriction site. A PCR using genomic \textit{P. berghei} DNA as template resulted in two distinct bands, one at approximately 516 base pairs representing the 5’ region and one at approximately 348 base pairs representing the
3’ region of pbtrx-469 (Figure 11a). Each DNA fragment was TA cloned into the bacterial pGEM T Easy vector (Promega). The resulting plasmids were sequenced and compared to the *P. berghei* genome sequence (PlasmoDB).

A vector designed for targeted KO in *P. berghei*, pBS-DHFR (Donald and Roos, 1993) was kindly provided by Dr. Andrew Blagborough (Figure 10). This vector contains a Dihydrofolate Reductase (DHFR) resistance cassette, which confers resistance to the antimalarial drug pyrimethamine (Waters *et al.*, 1997). The pBS-DHFR vector also contains multiple cloning sites for the insertion of DNA. Following sequence verification, I cloned each region into the pBS-DHFR vector, resulting in the pBS-pbtrx-469-DHFR KO construct (Figure 10).

![Figure 11](image.png)

Figure 11. Generation of pbtrx-469 KO construct. Products obtained were size-fractionated on a 1% agarose/TAE gel. DNA sizes standards appear in lanes labeled L, and the corresponding sizes in bp appear on the left. (a) 5’ and 3’ regions of pbtrx-469 were amplified from *P. berghei* gDNA using PCR. Lane 1 contains the 20 µL PCR reaction for the 5’ region. Lane 2 contains the 20 µL PCR reaction for the 3’ region. (b) The construct was digested and and size fractionated. Lane 1 contains 10 µL of the KO construct digested with ApaI and XbaI.

The resulting plasmids were sent out for sequencing to verify the accuracy of the inserts. In parallel, I performed analytical restriction digestes to ensure that the plasmid
was complete (Figure 11b). Bands at approximately 5000 and 3000 bp were observed, representing the KO insert and the remaining plasmid, respectively. The band at approximately 8000 bp represented undigested plasmid. I performed a large scale plasmid prep and determined DNA concentrations using a NanoDrop 2000 Spectrophotometer (Thermo). I then sent 10 μg of undigested plasmid and 5 μg of ApaI and XBaI digested plasmid to Dr. Andrew Blagborough for the second stage of this project, the transfection of the pBS-\textit{pbtrx-469}-DHFR KO construct into \textit{P. berghei} parasites.

\textit{pbtrx-469}KO Parasites Are Not Viable

As mentioned, Dr. Andrew Blagborough at Imperial College London transfected parasites with the KO construct. After experiments were completed, he provided us with a brief description of his results. Linearized KO plasmid was transfected into purified \textit{P. berghei} schizonts by electroporation using the Nucleofector I electroporator (Lonza AG). Transfected parasites were then injected intraperitoneally (IP) into naïve BLAB c mice. Drug selection with pyrimethamine was started one day after injection. Only transfected parasites that received and maintained the KO plasmid should be able to survive under drug pressure. Drug pressure was maintained for 3-4 days, after which time mice were checked for the presence of parasites. No parasites were observed following the first transfection. Three additional transfections were attempted with the same result. It was concluded that \textit{pbtrx-469} is likely essential for asexual parasite development.

\textbf{Biochemical Characterization of PbTrx-469}

The results from the gene knockout experiments suggested that \textit{pbtrx-469} is essential for \textit{P. berghei}. Yet this extreme phenotype did not allow me to draw any
conclusions about the purpose this gene serves for the parasite. Since PbTrx-469 had previously been cloned and expressed by our lab, I decided to characterize this protein biochemically in order to determine whether or not the Trx domain was important for its function.

Some thioredoxin and thioredoxin-like proteins reduce substrate proteins through the process of cysteine-thiol disulfide exchange (Nickel et al., 2006). The insulin-reduction assay, established by Arne Holmgren (Holmgren, 1979), is a test to determine whether or not a protein possesses redox activity. This assay tests whether the protein is able to catalyze the reduction of the disulfide bonds between the A and B chains of insulin by the redox reagent dithiothreitol (DTT). Reduction of these bonds leads to the precipitation of the two chains. The resulting turbidity of the assay solution can be quantified using a spectrophotometer.

**PbTrx-469 Reduces Insulin in the Insulin-Reduction Assay**

Six or eight μM recombinant PbTrx-469 (rPbTrx-469) was incubated with 1 mM DTT and 26.4 μg bovine insulin in assay buffer. rPbTrx-1 was used as a positive control (Kanzok et al., 2000). A second control containing only DTT and insulin was also used. The reaction was run for 45 minutes. The results show that 6 μM rTrx-469 catalyzes the reduction of insulin at a rate of 0.0038 A/min (Figure 12). Increasing the concentration of rPbTrx-469 to 8 μM resulted in a 1.8 fold increase in the insulin reduction rate. As expected, 8 μM rPbTrx-1 efficiently reduced insulin at a rate of 0.054 A/min. In contrast, no significant reduction was observed in the absence of either rPbTrx-469 or rPbTrx-1 during the time frame of the experiment. These results indicate that PbTrx-469 is redox
active. In addition, since the redox activity of thioredoxin-like proteins is dependent upon cysteine residues, I hypothesized that at least one of the cysteine residues identified in the primary sequence of PbTrx-469 is redox active and part of the putative active site.

**Figure 12. PbTrx-469 exhibits redox activity.** Enzymatic activity of the recombinant protein was determined by adding varying amounts of rPbTrx-469 or rPbTrx-1 to a reaction mixture containing bovine insulin (44 μg/mL) and DTT (1 mM) in a potassium phosphate buffer (100 mM potassium phosphate, 2 mM EDTA, pH 7.4). The reduction of the insulin disulfide bonds was monitored as an absorbance increase at 650 nm in a Genesys6 UV-VIS Spectrophotometer (Thermo).

**PbTrx-469 Exhibits Redox Activity with the Thioredoxin System**

Redox activity of PbTrx-469 in the insulin-reduction assay only indicates the presence of peroxidatic cysteines within the protein. The next step was therefore to test whether PbTrx-469 is reduced by the thioredoxin system, a vital part of the cellular redox network. Testing PbTrx-469 with its endogenous thioredoxin system would provide better information on its potential biochemical and cellular functions.

The thioredoxin system consists of the flavoprotein thioredoxin reductase (TrxR) and the small redox active protein thioredoxin (Trx) (see Introduction, Figure 2). In
addition to cloning and expressing rPbTrx-1, our lab has also recently cloned and expressed rTrxR of *P. berghei* (Turturice *et al*, 2013) and established the redox activities of both enzymes in a thioredoxin assay (Kanzok *et al*, 2000; Haselton *et al*, 2014). PbTrxR utilizes electrons from the universal electron carrier NADPH reduce oxidized PbTrx-1 (Kanzok *et al*, 2002). Since NADPH, but not NADP⁺ exhibits an absorbance at 340 nm, the oxidation of NADPH to NADP⁺ can be measured as a decrease in absorbance using a UV spectrophotometer.

Using the thioredoxin reduction assay, I tested whether or not PbTrx-469 can be reduced by its endogenous thioredoxin system. If PbTrx-469 accepts electrons from the thioredoxin system, I should observe a decrease in absorbance indicating that electrons...
flow from the thioredoxin system to PbTrx-469. An assay was set up in a 1 mL cuvette containing 200 μM NADPH and 20 μM PbTrx-1. The absorbance was measured at 340 nm, giving a reading of 1.45 A, which was in the expected range for the concentration of NADPH used. No change in absorbance was detected as the reaction was allowed to baseline. After 2 minutes, rPbTrxR was added to the assay to a final concentration of 50 nM. As expected, a rapid decrease in absorbance was observed, indicating that rPbTrx-1 was readily reduced by rPbTrxR at a rate of 29.5 μM min$^{-1}$ (Figure 13). The decrease in absorbance ended after all PbTrx-1 was reduced. I then added rPbTrx-469 to the reaction at a final concentration of 10 μM. The system resumed NADPH consumption with an initial rate of 6.5 μM min$^{-1}$ (Figure 13).

I next wanted to determine whether PbTrx-469 was reduced by PbTrxR or PbTrx-1. To determine this, I repeated the experiment by starting the assay with a mix of 200 μM NADPH and 10 μM rPbTrx-469. After 2 minutes of baseline I added 50 nM TrxR. No significant reduction was observed (Figure 13, open circles). After four additional minutes I added 20 μM rPbTrx-1 and observed an immediate rapid decrease in NADPH absorbance, indicating that PbTrx-469 accepts electrons from PbTrx-1 but not from PbTrxR.

To determine the kinetics of the reaction between PbTrx-1 and PbTrx-469, I performed additional experiments and varied the concentration of PbTrx-1 between 10-40 μM while keeping the concentration constant at 10 μM. The kinetic analysis revealed that PbTrx-1 exhibits a $K_M$ of 7.3 μM and a $k_{cat}$ of 1 min$^{-1}$ at a PbTrx-469 concentration of 10 μM (Figure 14).
Modeling of PbTrx-469

Redox activity of Trx-like proteins depends on the strategic position of cysteine residues in the three-dimensional structure of the protein (see Introduction, Figure 3).

Since the insulin-reduction assay and thioredoxin reduction assay both confirmed that PbTrx-469 is redox active, and since this activity requires at least one cysteine residue (Holmgren, 1979), the next step was to determine the redox active cysteine and propose a putative active site for the protein. In collaboration with Ligin Solamen from Dr. Kenneth Olsen’s laboratory in the Department of Chemistry we generated a hypothetical model of PbTrx-469 based on the structure of the related human Phosducin-Like Protein 2 (hPDCL2) (PDBid: 3EVI; Lou et al, 2009) (Figure 15).

This model suggests that the thioredoxin domain of PbTrx-469 consists of five beta strands that form a central beta sheet, surrounded by three alpha helices. In addition,
a putative active site is present at the N-terminal side of helix 2 in the form of a Thr-Arg-Trp-Cys motif. This position coincides with the position of the Cys-Gly-Pro-Cys motif found in classical thioredoxin proteins (Kanzok et al., 2002). Notably, the position of Cys106 of PbTrx-469 corresponds to that of the second cysteine in thioredoxin. In contrast, Thr103 takes the place of the first cysteine in the structure of Trx.

**Role of Cys106 in Redox Activity of PbTrx-469**

To test whether Cys106 in PbTrx-469 is indeed the redox active cysteine, I performed site-directed mutagenesis and mutated Cys106 to a serine (C106S) (Figure 16). I hypothesized that if Cys106 is redox active, its mutation to a serine should result in a significant reduction of redox activity.

**Design of mutagenesis primers and cloning of PbTrx-469**

Using the pQE9-PbTrx-469 expression plasmid as template, I designed two tail-to-tail 5’
phosphorylated primers. The forward (Fwd) primer introduced two point mutations in the codon, thereby changing a TGT (Cys) to a TCG (Ser). Using the Phusion Site-Directed Mutagenesis Kit (Thermo), I performed a PCR that produced a single band at the expected size of 4000 bp, representing the linearized pQE9-PbTrx-469<sup>C106S</sup> expression plasmid. The plasmid was ligated and transformed into competent DH5α cells for sequencing. It was verified that the two mutations were successfully introduced into the new expression plasmid. Confirmed pQE9-PbTrx-469<sup>C106S</sup> plasmids were then transformed into M15 expression cells (Qiagen) for subsequent protein expression as described in Materials and Methods.

![Diagram](image)

Figure 16. Site-directed mutagenesis of Cys106 in PbTrx-469. (a) Sequence of 5’ phosphorylated Fwd primer designed to mutate Cys106 (TGT; yellow) to Ser (TCG; purple). (b) Experimental approach for introduction of point mutations into pQE9-PbPhLP-1 plasmid. PCR of target plasmid using phosphorylated primers yields linear amplified target plasmid with the desired mutation. 5’-phosphorylated primers designed to mutate Cys106 to Ser in pQE9-PbPhLP-1. Adapted from [http://66.155.211.155/nebecomm/products_intl/productF-541.asp](http://66.155.211.155/nebecomm/products_intl/productF-541.asp)

**Expression and purification of PbTrx-469<sup>C106S</sup>.** M15 cells selected for pQE9-PbTrx-469<sup>C106S</sup> were grown in 1 L LB containing ampicillin and kanamycin. Protein
expression was induced via IPTG and recombinant PbTrx-469C106S was purified as described in Materials and Methods. A liter of bacterial culture typically yielded 1-2 mg of PbTrx-469C106S protein. An analytical SDS gel showed a prominent band at approximately 24 kDa, which is consistent with the previously purified wild-type PbTrx-469 as well as the calculated molecular weight of 24 kDa (Figure 17). Another band observed at approximately 10 kDa was assumed to represent proteolysis products.

![SDS gel](image)

Figure 17. Ni-NTA purification of PbTrx-469C106S from M15. Material eluted from the Ni-NTA column was size fractionated on a 4-20% tris-glycine gel and stained in Coomassie blue. Lane L contains 7 μL Thermo Scientific molecular weight standards and the corresponding sizes are indicated on the left. Lanes 1-3 contain 10 μL of material that came down with 50, 75, and 100 mM imidazole, respectively. Lanes 4-8 contain 10 μL fractions of material eluted from the column after applying 500 mM imidazole.

**PbTrx-469C106S** shows significantly reduced redox activity in the insulin reduction assay. Following purification, I first tested potential redox activity of PbTrx-469C106S in the insulin-reduction assay. To this end, I incubated 20 μM PbTrx-469C106S
with 8 mM DTT and 0.79 mg insulin in assay buffer. In a separate assay I used 20 μM wild-type PbTrx-469 instead of PbTrx-469C106S (Figure 18). As expected, 20 μM PbTrx-469 effectively reduced insulin with a rate of reduction of 0.01 A/min for this assay. The insulin reduction activity of PbTrx-469C106S was significantly reduced compared to the wild-type protein, with a rate of reduction of 0.002 A/min. These results indicate that Cys106 in PbTrx-469 is redox active, supporting my hypothesis that it is part of the putative active site.

![Graph showing absorbance over time for C106S and WT proteins.]

**Figure 18.** Insulin assay using recombinant PbTrx-469 and PbTrx-469C106S. In a final volume of 600 μL, the reactions contained 1.31 mg/mL insulin, 0.8 mM DTT, 12.4 mM EDTA, 61.9 mM KH₂PO₄ plus 20 μM of protein. DTT plus 20 μM BSA served as a control. Absorbance was read at 650 nm with readings taken every 30 seconds.

**PbTrx-469C106S is also inactive in the thioredoxin system.** I next aimed to determine whether this mutation would also impact electron transfer between rPbTrx-1 and PbTrx-469. Two assays were conducted in parallel, one using rPbTrx-469 and one using rPbTrx-469C106S (Figure 19). I set up two cuvettes, each containing a mix of 200
μM NADPH and 40 μM rPbTrx-1. rPbTrxR was then added to each cuvette to a final concentration of 50 nM. In each reaction, rPbTrx-1 was rapidly reduced by rPbTrxR at an initial rate of 35 μM min⁻¹. The reactions were followed to completion. I then added 20 μM rPbTrx-469 to the control cuvette and 20 μM rPbTrx-469C106S to the experimental cuvette. In the assay containing rPbTrx-469, NADPH consumption resumed at an initial rate of 3.38 μM min⁻¹ (Figure 19). In contrast, no significant NADPH consumption was observed in the assay containing rPbTrx-469C106S, supporting my hypothesis that C106 is involved in the catalytic mechanism of PbTrx-469.

![Figure 19. Activity of PbTrx-469 and PbTrx-469C106S in thiolredoxin reduction assay. 200 μM NADPH and 50 nM PbTrxR were preincubated in a cuvette. 40 μM PbTrx-1 was added and was rapidly reduced. 10 μM of PbTrx-469 (blue line) or 10 μM of PbTrx-469C106S was added at times indicated by arrows. Oxidation of NADPH to NADP+ was measured at 340 nm on the Genesys6 Spectrophotometer.](image)
**Majority of PbTrx-469 Expressed in *E. coli* is Insoluble**

Purification of PbTrx-469 involved lysing the bacteria that expressed the protein, pelleting the bacteria, and then running the lysate through a Ni-NTA column in order to purify the recombinant protein (see Materials and Methods). Theoretically, the majority of the recombinant protein should be present in the lysate. However, in some cases high level recombinant protein expression in *E. coli* may cause the bacteria to form insoluble protein aggregates known as inclusion bodies (Hartley and Kane, 1988). When this occurs, the protein aggregates must be solubilized from the bacterial pellet, and the proteins refolded.

An SDS-PAGE analysis of the bacterial pellet from purification of PbTrx-469 showed strong bands at the expected molecular weight of the recombinant protein, suggesting that significant amounts of PbTrx-469 may be insoluble and kept in inclusion bodies. A literature review revealed that researchers working on related proteins had encountered similar problems and had used denaturing conditions to extract the protein from the inclusion bodies (Stirling et al, 2006). To achieve a better yield of recombinant PbTrx-469, I therefore used a urea purification method when producing the protein (see Materials and Methods). This method allows for dissolution of the inclusion bodies by destroying all non-covalent interactions. The subsequent and careful removal of the urea then allows for the refolding of the recombinant protein.

SDS-PAGE analyses of elutions collected using our original purification method revealed the presence of multiple bands in addition to the band representing PbTrx-469 (Figure 20a). Thus, we predicted that the urea purification method would also allow for a
better removal of bacterial contaminants. Kinetics of the PbTrx-469 purified under the original conditions are also shown (Figure 20a). An SDS-PAGE analysis of urea purified PbTrx-469 showed fewer bands as well as a higher concentration of protein around the 24 kDa mark (Figure 20b). Enzyme kinetics conducted using urea purified PbTrx-469 showed a lower $K_M$ value, confirming our prediction that the urea purification method produced a protein sample that was both purer and more concentrated.

Figure 20. Urea purification of PbTrx-469. (a) SDS-PAGE of Trx-469 purified under native conditions with corresponding enzyme kinetics. PL=pellet, FT=flow through, W=wash (20 and 50 mM imidazole, respectively). (b) SDS-PAGE of Trx-469 purified under denaturing conditions using 8 M urea. Corresponding enzyme kinetics are shown.
Characterization of Homologs of PbTrx-469

IFA Studies Indicate that Anti-PbTrx-469 Antibodies Recognize a Protein Associated with the Cytoskeleton in Human Cell Lines

The results obtained from previous immunofluorescence assays (IFAs) suggested that antibodies raised against the *P. berghei* protein Trx-469 may cross-react with one or several proteins in the human host cells (see Figure 8). To verify this, I cultured HepG2 cells as well as HeLa cells. After the cells were fixed using glutaraldehyde (see Materials and Methods), I performed double-labeling with anti-PbTrx-469 antibodies and either anti-beta tubulin antibodies or phalloidin, which labels actin. Cells were analyzed using

Figure 21. IFA of human cell lines using anti-PbTrx-469 antibodies. Confocal microscopy images of HepG2 (a-b) or HeLa cells (c-f) stained with (a) anti-PbTrx-469 (green) and Tubulin Beta Polyclonal Antibody (red), (b) anti-PbTrx-469 (red) and phalloidin (green), (c) anti-PbTrx-469 (green), (d) anti-PbTrx-469 (green) and Tubulin Beta Polyclonal Antibody (red) (e) anti-PbTrx-469 (green) and DNA dye TO-PRO-3 (blue), (f) anti-PbTrx-469 (red) and phalloidin (green). Primary anti-PbTrx-469 antibodies were probed with secondary antibody coupled with AF488 (a, c-e) or AF555 (b, f). Primary Tubulin Beta Polyclonal Antibodies were probed with secondary antibody coupled with AF488.
confocal microscopy (Figure 21). In HepG2 cells, the anti-beta tubulin antibodies clearly labeled the microtubule network (Figure 21a). The anti-PbTrx-469 antibodies labeled a target that seemed to be very closely associated with microtubules, since the merged images show a high degree of overlap between the two labeled proteins (Figure 21a). The phalloidin stain clearly labeled the actin network (Figure 21b), however, no significant overlap was observed between the actin network and the target recognized by the anti-PbTrx-469 antibodies (Figure 21b). Similar results were observed in the HeLa cells (Figure 21c-f). The staining of the anti-PbTrx-469 antibodies seemed to be associated with the microtubular (Figure 21c-e) but not the actin (Figure 21f) network. In all IFAs, it appears that the anti-beta tubulin and anti-PbTrx-469 stain colocalize. In contrast, the anti-PbTrx-469 stain and phalloidin stain only colocalized weakly, if at all.

**Phylogenetic Analysis Reveals High Conservation of PbTrx-469 in Eukaryotes**

A cross-reaction such as the one observed in the IFA may be due to the presence of a conserved protein in the two organisms. I hypothesized that the anti-PbTrx-469 antibodies may be recognizing a homolog of PbTrx-469 in human cells. To test whether this may be true, a phylogenetic analysis was performed with Dr. Catherine Putonti. The amino acid sequence of PbTrx-469 was retrieved from PlasmoDB and compared with the NCBI non-redundant (nr) protein database using blastp (Altschul *et al*, 1997) (Figure 22). The BLAST results revealed that homologs of PbTrx-469 are present in most eukaryotes, including organisms such as *Homo sapiens*, *Xenopus laevis*, *Danio rerio*, *Drosophila melanogaster*, *Schistosoma mansoni*, and *Arabidopsis thaliana* (Figure 22b). The degree of sequence similarity between PbTrx-469 and its homologs varied, with some homologs
showing identities as high as 38% in *Homo sapiens* or 41% in *Arabidopsis thaliana* (Figure 22c).

This alignment was used to generate a phylogenetic tree (Figure 22a) demonstrating the relationship between PbTrx-469 and its orthologs in eukaryotic organisms. Some of these homologs (in humans, *Dictyostelium discoideum*, *Ciona intestinalis*, and others) were annotated as phosducin-like proteins. These results caused us to change the annotation of PbTrx-469 to Phosducin-Like Protein 1 (PhLP1) in the *Plasmodium* database PlasmoDB (Putonti et al., 2013).

![Phylogenetic tree showing the relationship between PbTrx-469 and its orthologs in eukaryotic organisms.](image)

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Figure 22. Eukaryotic homologs of PbTrx-469. (a) Maximum Likelihood tree showing PbTrx-469 homologs in model eukaryotic organisms. (b) Sequence alignment of PbTrx-469 and homologs in *Homo sapiens*, *Xenopus laevis*, *Danio rerio*, *Drosophila melanogaster*, *Schistosoma mansoni*, and *Arabidopsis thaliana*. (c) Percent protein sequence identity between PbTrx-469 and homologs.
Interestingly, an analysis of the corresponding literature revealed that homologs of PbPhLP1 are hypothesized to be either directly or indirectly associated with the cytoskeleton in other eukaryotic organisms including *C. elegans* (Ogawa *et al*, 2004), *Arabidopsis thaliana*, and humans (Stirling *et al*, 2006). These reports strengthened our hypothesis that the anti-PbPhLP1 antibodies may cross-react with a protein associated with microtubules in human cells.

The BLAST results included the human homolog, called Thioredoxin Domain-Containing Protein 9 (hTXNDC9), which shares >50% amino acid identity with PbPhLP1 within the thioredoxin domain. I hypothesized that it is very likely that the anti-PbPhLP1 antibodies cross-react with hTXNDC9 in the HeLa and HepG2 cell lines (Figure 21). It might therefore be possible to immunoprecipitate hTXNDC9 from HeLa cells using the anti-PbPhLP1 antibodies.

**Immunoprecipitation to Determine the Target(s) of Anti-PbPhLP1 Antibodies in Human Cells**

I performed immunoprecipitation experiments in order to determine the identities of any proteins recognized by the anti-PbPhLP1 antibodies in human cells. In this technique, antibodies are used to isolate a protein from a solution such as a cell lysate (Bonifacino *et al*, 2001). To this end, the antibodies are attached to a substrate, such as a bead, and then incubated with the cell lysate. Any proteins recognized by the antibodies should bind, while all other proteins flow past. The protein or antibody-protein complex can then be eluted, or immunoprecipitated, separated using SDS-PAGE, and subsequently identified using mass spectrometry.
For this experiment, anti-PbPhLP1 antibodies were incubated with HeLa cell lysate and Recombinant Protein A Agarose beads, which have a high affinity for the constant region of the heavy chain of IgG type antibodies (Kopan et al., 1996). A tube containing only cell lysate and beads, but no antibody, was used as a negative control. Once the protein elutions were run on SDS-PAGE, this control would allow me to distinguish between bands resulting from background and bands resulting from immunoprecipitation by the antibody.

![Image](image_url)

**Figure 23. Immunoprecipitation using anti-PbPhLP1 antibodies.** Material collected through immunoprecipitation was size-fractionated on 10% Tris-Glycine gel and stained in Coomassie blue. Lanes labeled L contain Thermo Scientific molecular weight standards and the sizes are indicated. Lane 1 contains the anti-PbPhLP1 antibody pull-down. Lane 2 contains the antibody only control. Arrows indicate bands that were sequenced. Asterisks indicate bands that were believed to be IgG heavy chain.
The Coomassie stained SDS-PAGE gel of a representative immunoprecipitation experiment is shown (Figure 23). In both the lanes containing the cell lysate sample and the negative control, a prominent band was observed around the 50 kDa mark and was hypothesized to represent the heavy chain of IgG (Figure 23, lane 1 and 2). A previous report had suggested a molecular weight of 26.5 kDa for human TXNDC9 (Stirling et al., 2006). Therefore I hypothesized that a band I observed around the 25 kDa mark may be hTXNDC9 (Figure 23, lane 1). A second band around the 50 kDa mark was present in the lane containing the cell lysate sample (Figure 23, lane 1). This band potentially represented a protein dimer that was immunoprecipitated. I excised both the 25 kDa band as well as the second 50 kDa band and sent them to the University of Illinois at Chicago to be analyzed by mass spectrometry. The analysis revealed that both bands consisted of IgG chains, with the 50 kDa band representing the heavy chain and the 25 kDa band representing the light chain. However, no proteins other than IgG were detected.

I concluded that the high amount of free antibody in the elutions was preventing the detection of bound protein in the pull down. I therefore tested a Crosslink Immunoprecipitation Kit (Pierce) for my subsequent immunoprecipitation experiments. In principle, this kit first irreversibly crosslinks the antibodies to the Recombinant Protein A Agarose beads before the cell lysate is added. Unbound antibodies are removed by washing before the sample cell lysate is added. After antibodies have bound to their respective target proteins, an elution buffer is then added, causing the protein to be released from the beads. The goal is to minimize the amount of unbound IgG present in the elutions.
I repeated my previous experiment with the experimental tube containing cell lysate and anti-PbPhLP1 antibodies crosslinked to beads. The control tube contained only beads and crosslinked anti-PbPhLP1 antibodies. The elutions were split between two separate SDS gels so that one gel could be used for a Western blot and the other gel for Coomassie staining, potential band excision, and subsequent mass spectrometry analysis.

For the Western blot, samples and controls were run in duplicates for subsequent immunoblotting with either anti-PbPhLP1 antibodies or anti-beta tubulin antibodies. Proteins were separated using SDS-PAGE and then transferred to a nitrocellulose membrane (Figure 24). A band at approximately 50 kDa appeared in all four lanes. I concluded that this band corresponds to the IgG heavy chain (Figure 24, lanes 1-4). A band observed at 25 kDa was assumed to represent the IgG light chain (Figure 24, lane 2-3). I also observed several bands in the lane containing the immunoprecipitation sample and that had been incubated with anti-PbPhLP1 antibodies (Figure 24, lane 3). A band seen just below the 50 kDa mark was assumed to be a potential dimer of hTXNDC9. A band appearing at approximately 34 kDa was also identified as a potential protein of interest and was sequenced (Figure 24, lane 3). A band at 55 kDa, which correlates with the molecular weight of both α- and β-tubulin, was present in both experimental lanes and was sequenced, since we hypothesized that tubulin may also be present in the pull down due to an interaction between hTXNDC9 and tubulin (Figure 24, lanes 2 and 4). The band appearing just below the 25 kDa mark in the Western blot was not visible by Coomassie stain and was therefore not sequenced (Figure 24, lane 3). The mass
spectrometry analysis reported that again IgG was the only detectable protein in all bands.

Figure 24. Western blot with immunoprecipitation pull down. Lane 1 contains the antibody only control, immunoblotted with Tubulin Beta Polyclonal Antibody. Lane 2 contains the anti-PbPhLP1 antibody pull-down, immunoblotted with Tubulin Beta Polyclonal Antibody. Lane 3 contains the anti-PbPhLP1 pull-down, immunoblotted with anti-PbPhLP1 antibodies. Lane 4 contains the antibody only control, immunoblotted with anti-PbPhLP1 antibodies. Goat Anti Rabbit IgG Fc Alkaline Phosphatase was used as secondary antibody in all lanes. The membrane was developed using 1 Step NBT/BCIP. Bands corresponding to areas of a similar gel that were sequenced are indicated by arrows. Thermo Scientific molecular weight standards were used, and the corresponding sizes are indicated.

I consulted the troubleshooting guide of the immunoprecipitation kit, and a number of possible solutions were proposed. First, when antibody is detected with the
eluted protein, this may be due to antibodies that were not crosslinked and not removed effectively. Increasing the number of wash steps should help improve this issue. Second, it may also be possible that insufficient amounts of the material used to crosslink the antibodies were added. I planned to implement both of these approaches in subsequent experiments.

However, while these experiments were being conducted, a review of previous data (Figure 21) raised the possibility that the anti-PbPhLP1 antibodies may be cross-reacting with tubulin. My IFA analysis in human cells had suggested a potential association between microtubules and the protein recognized by the anti-PbPhLP1 antibodies. However, my SDS gel analysis of parasite isolates (Figure 27) and immunoprecipitation experiments suggested that the anti-PbPhLP1 antibodies may potentially cross-react with tubulin, which could also explain the staining pattern observed in my IFA images (Figure 21).

**Anti-PbPhLP1 Antibodies Cross-React with Tubulin**

I sent my IFA images to Dr. Holly Goodson, a biochemist at Notre Dame University who is an expert on microtubules (Margolin *et al.*, 2012), for her evaluation. She suggested that I test for a cross-reaction between the anti-PbPhLP1 antibodies and tubulin using a Western blot. She generously provided me with purified porcine tubulin for this purpose.

A Western blot was performed using recombinant PbPhLP1 and porcine tubulin in order to test for the presence of anti-tubulin antibodies in the serum obtained from Open Biosystems, the company that generated the antibodies against our recombinant
PbPhLP1. Antibody development is usually performed in two separate rabbits. Therefore I tested the sera from both rabbits to determine if a contamination with anti-tubulin antibodies occurred in both antibody samples.

15.8 μg of purified tubulin were transferred onto three lanes of nitrocellulose. The lanes were separated and immunoblotted with the anti-PbPhLP1 antibodies from one rabbit (Figure 25, lane 4), the anti-PbPhLP1 antibodies from the second rabbit (Figure 25, lane 5), or the anti-beta tubulin antibodies (Figure 25, lane 3). 0.85 μg of recombinant PbPhLP1 were transferred onto another two lanes of nitrocellulose and separately immunoblotted with both sets of anti-PbPhLP1 antibodies (Figure 25, lanes 1-2).

Figure 25. Western blot of tubulin and PbPhLP1. 15.8 μM purified porcine tubulin (lanes 3-5) and 0.85 μM recombinant PbPhLP1 (lanes 1-2) were size fractionated on a 10% tris-glycine gel. Thermo Scientific molecular weight standards were used, with corresponding sizes indicated. Proteins were electrophoretically transferred to nitrocellulose membrane and immunoblotted with Tubulin Beta Polyclonal Antibodies (lane 3), anti-PbPhLP1 antibodies from first rabbit (lanes 1, 4), or anti-PbPhLP1 antibodies from second rabbit (lanes 2, 5). Goat Anti Rabbit IgG Fc Alkaline Phosphatase was used as a secondary antibody. Membrane was developed using 1 Step NBT/BCIP.
In both lanes containing recombinant PbPhLP1 and immunoblotted with anti-PbPhLP1 antibodies (Figure 25, lanes 1-2), a band was observed at approximately 24 kDa, which is consistent with the molecular weight of rPbPhLP1. This verified that the antibodies, or at least a subset of the antibodies in the sera of each rabbit, detect PbPhLP1. In the lane containing purified tubulin and blotted with the anti-beta tubulin antibodies, a band was observed at approximately 55 kDa, which corresponds to the molecular weight of tubulin (Figure 25, lane 3). A band at approximately 55 kDa was also observed in both lanes containing purified tubulin that were immunoblotted with the anti-PbPhLP1 antibodies (Figure 25, lanes 4-5). These results indicate that both sets of anti-PbPhLP1 antibodies react with rPbPhLP1 (Figure 25, lanes 1-2) as well as tubulin (Figure 25, lanes 4-5). One explanation may be that the serum samples from both rabbits were contaminated with anti-tubulin antibodies. An alternative explanation may be that tubulin and PbPhLP1 share common epitopes that are recognized by the anti-PbPhLP1 antibodies. However, an alignment of PbPhLP1 and *P. berghei* tubulins showed low percentages of amino acid identitites ranging from 7% (alpha tubulin) to 18% (delta tubulin) (Table 1).

<table>
<thead>
<tr>
<th>Name</th>
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Table 1. Identity between *P. berghei* tubulins and PbPhLP1. Amino acid sequences of tubulins of *P. berghei* were aligned with PbPhLP1 using ClustalW. Percent identity between protein sequences is shown.
To test the hypothesis that PbPhLP1 and tubulin share a common epitope, I conducted another Western blot analysis with two samples each of 11.8 μg recombinant PbPhLP1 and 27.5 μg purified tubulin. After transfer to nitrocellulose membrane, the samples were stained with anti-beta tubulin antibodies (Figure 26).

As expected, a band was observed at ~55 kDa in the lane containing purified tubulin (Figure 26, lane 1). In the lane containing rPbPhLP1, a band was observed at ~24 kDa,
consistent with the molecular weight of rPbPhLP1 (Figure 26, lane 3). These results indicate that PbPhLP1 is also recognized by the anti-beta tubulin antibodies. This supports the hypothesis that the two proteins may share similar epitopes.

**Western Blots of Parasite Lysate**

![Western blot image]

Figure 27. Western blot with parasite lysate and recombinant protein. Material was size fractionated on a 4-20% tris-glycine gel and transferred to nitrocellulose membrane. A) Membrane containing parasite lysate was probed with Tubulin Beta Polyclonal Antibodies (lane 1) or Anti-PhLP1 antibodies (lane 2). Molecular markers are shown in lane 3 and corresponding sizes are labeled on the right. Goat Anti Rabbit IgG Fc Alkaline Phosphatase was used as a secondary antibody.

In order to further confirm that the anti-PbPhLP1 antibodies were developed against PbPhLP1, I also conducted a Western blot containing parasite lysate. Two samples of *P. berghei* cell lysate were separated using SDS-PAGE and the resulting gel
was blotted onto a nitrocellulose membrane (Figure 27). The samples were separated and each was exposed to either anti-PbPhLP1 antibodies or anti-beta tubulin antibodies. The banding patterns were compared. The blot that was probed with anti-beta tubulin antibodies showed a single prominent band at approximately 55 kDa, which corresponds to the molecular weight of tubulin (Figure 27, lane 1). In contrast, the blot that was probed with anti-PbPhLP1 antibodies showed two bands at 35 and 50 kDa as well as a stronger band at approximately 25 kDa (Figure 27, lane 2). We assume that the band at approximately 50 kDa represents tubulin, which may consist of a mix of parasite as well as mouse tubulin. It is unlikely that the approximately 25 and 35 kDa bands represent tubulin as they are not seen in the lane that was blotted with anti-beta tubulin antibodies. We hypothesize that the 25 kDa band represents PbPhLP1 and that the 35 kDa band possibly represents mouse APACD, which shares a high homology with hTXNDC9. However, since we were not able to sufficiently resolve the issue of potential cross-reaction of the anti-PbPhLP1 antibodies with tubulin, we decided to use alternative approaches to investigate potential homologs of PbPhLP1.

**Characterization of Human TXNDC9**

I still hypothesized that the anti-PbPhLP1 antibodies recognized a homolog of PbPhLP1 in human cells. It seemed unlikely, given the high degree of identity between PbPhLP1 and its human homolog (see Figure 22), that the antibodies were only recognizing tubulin in the HepG2 and HeLa cells. However, I was unable to draw any conclusions about this hypothetical interaction using either immunoprecipitation or IFA. To extend our analysis of the human homolog of PbPhLP1 I therefore decided to express
recombinant hTXNDC9 to test whether the anti-PbPhLP1 antibodies were able to recognize the human homolog.

**Cloning, heterologous expression, and purification of recombinant hTXNDC9.** I designed PCR primers to amplify the open reading frame of human *txndc9* (NCBI accession number NM_005783.3) from human cDNA generated from HepG2 cells. The PCR resulted in a single band just below the 700 bp marker. This corresponds to the calculated size of the *txndc9* open reading frame (681 bp) (Figure 28). Following sequence verification, I cloned the gene into the bacterial pRSETA expression vector. The six histidines encoded by the pRSETA vector are added to the amino terminus of the protein, thus allowing the recombinant protein to be purified via a Ni-NTA affinity column. The resulting plasmid (pRSETA-hTXNDC9) was sequenced and transformed into BL21-Codon Plus Expression Cells (Agilent).

![Figure 28. Size fractionation of PCR amplified hTXNDC9. Primers designed to flank the sequence of *hxndc9* were used to amplify this region using PCR. The product obtained was size fractionated on a 1% agarose/TAE gel. DNA size standards appear in lane 1, and the corresponding sizes are indicated on the left. Lane 2 contains the 20 uL PCR reaction.](image)
Expression of recombinant hTXNDC9 was conducted following the urea purification protocol and the protein was eluted using an imidazole gradient, as described in the Materials and Methods. Elutions were size-fractionated using SDS-PAGE (Figure 29). Eluates showed a prominent band at approximately 35 kDa which was assumed to represent recombinant hTXNDC9.

Anti-PbPhLP1 antibodies cross-react with recombinant hTXNDC9. A Western blot analysis was performed to determine if anti-PbPhLP1 antibodies cross-react with recombinant hTXNDC. 13.4 μg of recombinant hTXNDC9 was loaded on a gel. 11.8 μg of recombinant PbPhLP1 and 27.5 μg of purified porcine tubulin were also loaded as positive controls. The samples were size-fractionated using SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-PbPhLP1 antibodies (Figure 30). As observed previously, a single band representing tubulin is visible at
approximately 55 kDa in the lane containing purified porcine tubulin (Figure 30, lane 1). A band representing PbPhLP1 appeared at approximately 24 kDa in the lane containing rPbPhLP1 (Figure 30, lane 3). Smaller bands also appeared in the lane and were assumed to represent proteolysis products. A band at approximately 35 kDa was observed in the lane containing recombinant hTXNDC9 (Figure 30, lane 2). This band corresponds to the Coomassie stained band observed during hTXNDC9 purification and thus strongly suggests that the anti-PbPhLP1 antibodies cross-react with hTXNDC9.

Figure 30. Western blot of tubulin, hTXNDC9, and PbPhLP1. Thermo Scientific molecular weight standards were used and the corresponding sizes are indicated on the left. Lane 1 contains 27.5 µg purified porcine tubulin. Lane 2 contains 13.4 µg recombinant hTXNDC9. Lane 3 contains 11.8 µg recombinant PbPhLP1. Proteins were size-fractionated on a 4-20% tris-glycine gel before being electrophoretically transferred to nitrocellulose and probed with anti-PhLP1 antibodies diluted 1:100. 1:100 Goat Anti Rabbit IgG Fc Alkaline Phosphatase was used as a secondary antibody. The membrane was developed using 1 Step NBT/BCIP.
This was not surprising as the *Plasmodium* protein and its human homolog share 55% amino acid identity within the thioredoxin domain (Figure 22c). We also observed that the blot of purified tubulin with anti-beta tubulin antibodies (Figure 30, lane 1) exhibited a relatively weak band when compared with the bands in lanes 2 and 3. We hypothesize that this was due to a deterioration of the purified tubulin sample due to multiple freeze-thaw cycles.

**Conservation of PbPhLP1 active site.** While conducting the phylogenetic analysis of PhLPs (Figure 22), we also investigated whether the homologous proteins contained the same putative atypical active site (Thr-Trp-Arg-Cys) that I had previously proposed for PbPhLP1. We found that all of the *Plasmodium* species as well as the oyster pathogen *Perkinsus marinus* contained the same active site motif of Thr-Trp-Arg-Cys (position 103-106 in PbPhLP1 alignment, Figure 31a). Fifty-six percent of the PbPhLP1 homologs, those in other protozoans, nematodes, insects, and vertebrates, including human, had a putative active site consisting of [Thr-X-Arg-Cys]. Other frequently observed and conserved motifs include [Ser-X1-Arg-Cys], [Thr-X1-X2-Cys], [Ser-X1-X2-Cys], [X1-X2-Arg-Cys], and [X1-X2-X3-Cys]. The fourth position cysteine was found to be conserved throughout the 140 PbPhLP1 homologous sequences examined.

Since I had determined that PbPhLP1 is redox active and accepts electrons from the thioredoxin system (see Figures 12-13), I hypothesized that the presence of this conserved cysteine motif indicates potential catalytic activity of the homologous proteins. In collaboration with Dr. Olsen’s lab we generated a hypothetical model of hTXNDC9 based on the previously published crystal structure of hPDCL2 (PDB ID: 3EVI; Lou *et*
al, 2009) (Figure 31b). The model shows that Cys104 of hTXNDC9 corresponds to the second cysteine in thioredoxin in terms of relative position in the structure and therefore to Cys106 in PbPhLP1 (see Figure 15). This model suggests that Cys104 may be part of a solvent-accessible active site in hTXNDC9. I therefore decided to test the purified recombinant protein for redox activity.

Recombinant hTXNDC9 is active in the insulin reduction assay. I first tested recombinant hTXNDC9 in the insulin-reduction assay. Twenty μM recombinant hTXNDC9 was incubated with 1.16 mM DTT and 0.78 mg bovine insulin in assay buffer. 10 μM of PbTrx-1 was used in a separate reaction as a positive control (Kanzok et al, 2000). 20 μM PbPhLP1 was also used as a positive control. 20 μM BSA was used as a negative control (Figure 32). As previously observed, both PbTrx-1 and PbPhLP1 were
able to catalyze the reaction between DTT and insulin, with rates of 0.455 A/min and 0.03 A/min, respectively (Figure 32). No significant reduction occurred in the presence of BSA. Recombinant hTXNDC9 was also able to reduce insulin at a rate of 0.033 A/min (Figure 32). These results demonstrate that hTXNDC9 effectively catalyzes the reaction between DTT and insulin.

Thioredoxin reduction assay. After observing that hTXNDC9 possesses redox activity, the next step was to determine if this protein is able to accept electrons from the thioredoxin system. Human Thioredoxin (hTrx) was also expressed and purified for this
The oxidation of NADPH to NADP⁺ by the thioredoxin system was measured at 340 nm using the Genesys6 UV-VIS Spectrophotometer (Thermo).

Two hundred μM NADPH and 10 μM hTrx were incubated in a cuvette. The reaction was started when rPbTrxR was added to a final concentration of 50 nM. Recombinant hTrx was rapidly reduced by rPbTrxR (Figure 33). The reaction was followed to completion. After the reaction came to an end, hTXNDC9 was added to a
final concentration of 10 μM. The system resumed NADPH consumption with an initial rate of 7.7 μM min⁻¹ (Figure 33). These results provide evidence that hTXNDC9 is acting like a true member of the thioredoxin system as it is redox active and can receive electrons from thioredoxin.
CHAPTER IV
DISCUSSION

The purpose of this study was to characterize a novel Trx domain-containing protein, known initially as Trx-469, of the rodent malaria parasite *Plasmodium berghei*. In our phylogenetic study, we have identified homologs of Trx-469 in most eukaryotic organisms (Putonti *et al.*, 2013). Homologs in *Homo sapiens, Dictyostelium discoideum, Ciona intestinalis*, and others were annotated as phosducin-like proteins. Since the nomenclature used for these proteins is oftentimes confusing, a table identifying these proteins has been included for the sake of clarity (Table 2).

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</table>

Table 2. Homologs of PbPhLP1. Experimentally characterized homologs of PbPhLP1 with the names used in this manuscript indicated.

Due to the high homology with proteins of the phosducin-like family of proteins we designated Trx-469 as *Plasmodium berghei* Phosducin-Like Protein 1 (PbPhLP1), since it is the first phosducin-like protein to be identified in *Plasmodium*. We identified two additional PhLPs within the *Plasmodium* genome. However, this project focuses on the experimental characterization of PbPhLP1. In response to our publication, the
Plasmodium database PlasmoDB has reannotated orthologs of PbPhLP1 from putative thioredoxins to putative phosducin-like proteins in all Plasmodium species.

In addition to its high level of conservation among eukaryotes, my results also suggested that this gene may play an essential role in the malaria parasite. Four successive attempts to knockout (KO) phlp1 in P. berghei did not produce viable parasites. This strongly suggests that this gene is essential for blood stages of the malaria parasite. This is in contrast to studies in the amoeba Dictyostelium discoideum where the disruption of PhLP3 had no effect while the knockout of PhLP1 caused impairment of G protein signaling and the knockout of PhLP2 was lethal (Blaauw et al., 2003). However, in other organisms RNAi and siRNA knockdown, which, in contrast to a genetic knockout, targets mRNA to reduce the product of a gene rather than completely removing it, led to significant cellular defects. For example, RNAi knockdown of C. elegans C05D11.3 caused abnormal microtubules and defects in cytokinesis (Ogawa et al., 2004). Similar results were obtained from RNAi studies of Arabidopsis thaliana PhLP3 (Castellano and Sablowski, 2008), while siRNA knockdown of mammalian PhLP3 resulted in an elongation of cells and nuclei due to alterations in the cytoskeletal network (Hayes et al., 2011). The results of these studies suggest that siRNA or RNAi may provide more insight into the function of phlp1. However, since RNAi studies are not possible in Plasmodium (Baum et al., 2009), other methods are necessary in order to explore potential roles for this protein in the parasite.

We decided to characterize this protein biochemically due to the presence of the thioredoxin domain in the sequence. My insulin-reduction assay shows that recombinant
PbPhLP1 effectively catalyzes the reduction of insulin by DTT, indicating the presence of at least one redox active cysteine (Holmgren, 1979). PbPhLP1 is the first member of the phosducin-like family of proteins that has been demonstrated to be redox active. While homologs of PbPhLP1 have been expressed by other researchers, the experiments conducted in these studies have focused more on the function of the protein at the cellular level. Human TXNDC9 has previously been expressed as a fusion protein to investigate protein-protein interactions with the chaperonin containing TCP-1 (CCT) in vitro (Stirling et al., 2006). However, this research marks the first time that an enzymatic activity has been demonstrated for any member of the phosducin-like family of proteins.

Although the insulin assay indicates redox activity, it is only useful as an artificial in vitro assay to detect the presence of redox active cysteines in a protein. Significantly, PbPhLP1 also shows activity in the thioredoxin-reduction assay. More specifically, I demonstrated that electrons are passed between PbTrx-1 and PbPhLP1, making PbPhLP1 a potential target for PbTrx-1 in vivo. This activity was observed for proteins purified under both native and denaturing conditions, indicating that denaturing conditions had no adverse effects on the activity of the protein. Furthermore, the kinetic data of native and urea purified PbPhLP1 showed a $K_M$ value of 7.26 $\mu$M with PbTrx-1, a value that is highly comparable to $K_M$ values of *Plasmodium* Trx-1 with other Trx-like proteins (Nickel et al., 2006).

An examination of the amino acid sequence of PbPhLP1 revealed that this protein contains six cysteine residues in its primary sequence. Of these residues, only the cysteine present at position 106 in PbPhLP1 was conserved in all organisms included in
our phylogenetic analysis (Putonti et al., 2013). Furthermore, our hypothetical model of PbPhLP1 in combination with the sequence analysis indicated that Cys106 may be part of a putative TXXC active site that occupies a solvent-accessible position in PbPhLP1. This putative TXXC active site is in the same relative position in the predicted three-dimensional structure as the CXXC active site of Trx, with Cys106 corresponding to the C-terminal cysteine in Trx while Thr103 occupies the position of the N-terminal cysteine. Site-directed mutagenesis of Cys106 significantly reduced the redox activity of PbPhLP1 in the insulin-reduction assay as well as in the thioredoxin-reduction assay, indicating that Cys106 is involved in the redox activity of PbPhLP1. This also supports my hypothesis that this cysteine belongs to the putative active site which we proposed (Putonti et al., 2013).

This putative TXXC active site within the thioredoxin domain is highly conserved in PbPhLP1 homologs in protozoans, nematodes, insects, and vertebrates, including human TXNDC9 (Putonti et al., 2013). While PbPhLP1 possesses six cysteine residues in its structure, sequence analysis showed that hTXNDC9 possesses only three. Yet our hypothetical model of hTXNDC9 placed the conserved cysteine (Cys104) at the same position as Cys106 in PbPhLP1 and the C-terminal cysteine in Trx. We therefore hypothesized that hTXNDC9 may also possess redox activity. This hypothesis was confirmed first in the insulin-reduction assay, where recombinant hTXNDC9 displayed a redox activity similar to that of recombinant PbPhLP1. In addition, hTXNDC9 was shown to accept electrons from its endogenous thioredoxin, and the kinetic values between the Plasmodium and human proteins were comparable. These results represent
an important finding for hTXNDC9, as the possibility of redox activity of the TXNDC9 homolog in *C. elegans*, C05D11.3, has previously been dismissed due to the lack of a characteristic CXXC active site motif (Ogawa *et al.*, 2004). Our results indicate the possibility that all PbPhLP1 homologs may possess redox activity. It may therefore be necessary for potential functions and mechanisms to be reconsidered in light of these findings.

In addition, a previous study of thioredoxin domain containing proteins using computational structure-based approach did not report on any active site motif in members of the phosducin-like family of proteins (Atkinson and Babbitt, 2009). While most of the characterized redox active thioredoxins contain a characteristic CXXC active site motif, it has been shown that a number of thioredoxins or thioredoxin-like proteins either lack the N-terminal or C-terminal cysteine or do not contain an apparent active site (Herrero and de la Torre-Ruiz, 2007). These so-called atypical active sites commonly contain serine or threonine residues in place of one of the cysteines (Atkinson and Babbitt, 2009). This appears to be the case for PbPhLP1 and hTXNDC9, since threonine is present in place of the N-terminal cysteine in both proteins.

We are confident that the reduction of redox activity observed when PbPhLP1 is mutated is due to the absence of the redox active Cys106 rather than a change in the structure of the protein for the following reasons. Stabilizations of protein structures by disulfide bridges rarely occurs in cytoplasmic proteins due to the reducing nature of the cytoplasm (Alberts *et al.*, 2008). Our own observations in *Plasmodium* parasites combined with previous reports on PbPhLP1 homologs indicate an intracellular rather
than extracellular localization of these proteins. Our hypothetical models of PbPhLP1 and hTXNDC9 also suggest that the conserved redox active cysteine is located on the surface of these proteins rather than buried within the structure. It therefore seems unlikely that the mutation of this cysteine to a serine would introduce any sort of significant conformational change in the protein that may account for the decrease in redox activity.

While the results of my investigations of the cellular expression of PbPhLP1 were inconclusive, my observations also suggest that there may be epitopes that are conserved between PbPhLP1 and tubulin. My Western blot analyses show that anti-PbPhLP1 antibodies cross-react with purified tubulin. My IFAs also show that the anti-PbPhLP1 antibodies clearly stain the microtubular network of human cells. In addition, Western blots showed that anti-beta tubulin antibodies cross-react with recombinant PbPhLP1. However, the anti-PbPhLP1 antibodies were also able to recognize recombinant hTXNDC9 on a Western blot, suggesting that at least some of the staining pattern observable in the IFAs may be due to hTXNDC9. These results do not represent the first time that a cross-reaction was observed between cytoskeletal elements and antibodies developed against a member of the phosducin-like family of proteins (Piotrowska and Adler, 2010). Since PbPhLP1 may interact with tubulin in vivo, a further exploration of a common epitope between the two proteins is warranted. However, for the purpose of this project, these results only serve to suggest that the polyclonal PbPhLP1 antibodies are not useful for studies of PbPhLP1. However, considering the possibility of common epitopes between PbPhLP1 and tubulin, a logical future step would be the development of a
monoclonal antibody against PbPhLP1 to advance cellular studies and the investigation of putative interactions between this protein and the cytoskeletal network.

The reason we pursued a possible interaction between PbPhLP1 and the cytoskeleton was that other studies also pointed to an interaction between PbPhLP1 homologs and the cytoskeleton. As mentioned, disruption of this protein in both *C. elegans* and *A. thaliana* led to observable defects in the microtubular network of these organisms (Ogawa *et al*, 2004; Castellano and Sablowski, 2008). In *A. thaliana*, PhLP3 was shown to be required for the microtubule-dependent steps of cell division (Castellano and Sablowski, 2008). It is my hypothesis that the KO of *phlp1* in *P. berghei* may therefore have been lethal because the integrity of the cytoskeletal network had been compromised, ultimately leading to cell death. An alternative method to investigate the cellular function of *phlp1* would be overexpression of the gene. This method has been shown to cause microtubule disassembly and an imbalance of α and β tubulin subunits in a mammalian system (Hayes *et al*, 2011).

My hypothesis that PbPhLP1 is involved in maintenance of the cytoskeleton is based on observations that members of the thioredoxin-like family of proteins can interact with cytoskeletal components. For example, the tubulin heterodimer contains a high number of cysteine residues (20 in the mammalian protein) and may therefore be susceptible to oxidative stress (Lowe *et al*, 2001; Luduena *et al*, 1991). Oxidation of key cysteine residues by reactive oxygen species (ROS) leads to tubulin disulfide formation and subsequent loss of function due to the inhibition of tubulin polymerization (Landino *et al*, 2004). Since ROS are a common occurrence in cells, the microtubules would
require a maintenance and repair system in cells that reduces these destabilizing disulfide bridges. One study reports that the thioredoxin system can maintain the stability of microtubules *in vitro* by keeping the cysteines present in tubulin in a reduced state. (Landino *et al*, 2004). Similar findings were shown for the microtubule-associated proteins tau and microtubule-associated protein-2 (MAP-2) (Landino *et al*, 2004). Peroxynitrite and hydrogen peroxide caused the oxidation in these proteins, leading to a decreased ability to promote microtubule assembly from tubulin subunits. Treating oxidized tau and MAP-2 with the thioredoxin system restored their ability to promote microtubule assembly. These findings show that disulfide bonds present in both tubulin and microtubule-associated proteins may be reduced by the thioredoxin system.

Therefore these studies suggest that the redox activity that we observed for PbPhLP1 and hTXNDC9 may be important for some function involving an interaction with tubulin, a hypothetical interaction supported by previous studies (Blaauw *et al*, 2003; Ogawa *et al*, 2004; Castallano and Sablowski, 2008). In addition, one study on hTXNDC9 suggested that this protein may be involved in the CCT-mediated folding of tubulin subunits (Stirling *et al*, 2006). Further experiments are necessary in order to confirm the findings in this study and also to determine if the activity of this protein plays any role in this putative function.

While KO experiments in *S. cerevisiae* and *D. discoideum* suggested this protein was not essential for the survival of these organisms (Flanary *et al*, 2000; Blaauw *et al*, 2003), we hypothesize that the difference between my KO results and the results from these experiments are due to a difference in the putative active sites of these proteins. *S.*
*cerevisiae* was notably absent from our phylogenetic tree, suggesting that this protein in yeast may not be a homolog of PbPhLP1 or hTXNDC9. In addition, a recently constructed phylogenetic tree rooted on the putative active site of PbPhLP1 placed fungi, including *D. discoideum*, in a different clade of the tree entirely. This suggests that differences in the putative active sites of these proteins may be linked with differences in their roles in their respective organisms. Therefore, *D. discoideum* PhLP3 may not be redox active or have an important function in this organism, while other eukaryotic homologs that are present in different clades than *D. discoideum* in this tree may be redox active and essential for these species. These findings should therefore be taken into account when studying potential functions of these proteins.

In conclusion, this project identifies PbPhLP1 as a highly conserved novel thioredoxin domain-containing, phosducin-like protein that is essential for the malaria parasite *P. berghei*. PbPhLP1, as well as its human homolog hTXNDC9, was shown to be redox active as well as able to accept electrons from the thioredoxin system. This project represents the first biochemical characterization of any phosducin-like protein. The results presented here provide insights into the functional mechanism of these highly conserved proteins.
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