

Characterization of the structure-function features of the R2TP complex of *Plasmodium falciparum*

By

Sifiso Duncan Luthuli

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

In the subject of

Biochemistry

at

The University of Venda

Promoter: Prof. A. Shonhai

Co-promoter: Prof. W. Houry

Submitted on

February 2025

Abstract

Malaria remains a major cause of death worldwide. *Plasmodium falciparum* is responsible for the most severe form of malaria. The malaria parasite life cycle involves stages of development that span across the cold-blooded mosquito vector and a warm-blooded human host. Maintaining protein homeostasis across these physiologically distinct life stages of the parasite is mainly accomplished by a family of proteins termed molecular chaperones that assist in protein folding. Heat shock protein 90 (Hsp90) a molecular chaperone of *P. falciparum* is known to be important for cell development and signal transduction. The protein clientele of this chaperone runs in several hundred and efforts are ongoing to account for all its cellular clients. R2TP complex acts as a chaperone or the assembly of critical complexes in the cell, hence it is implicated in the assembly of the Hsp90 functional complex. Hence, this study aims to characterize the structural-functional features of the R2TP complex of *P. falciparum*. Bioinformatics analysis has revealed unique features of this complex with 3 RUVBL genes as opposed to its orthologues from yeast and humans whom both have 2 Rvb and 2 RUVBL genes, respectively. The study involved the design of antibodies and biophysical characterization of PfRUVBL proteins and their functional features. Biophysical characterization of PfRUVBL1, PfRUVBL2, and yeast Rvb1 was conducted using tryptophan spectrophotometry and limited proteolysis. In addition, surface plasmon resonance (SPR) was used to explore the oligomerization of the proteins. The ATPase activities of PfRUVBL1 and PfRUVBL2 were further investigated. Furthermore, the holdase chaperone activities of the proteins were investigated by monitoring the capability of the proteins to suppress heat-induced aggregation of a model protein, malate dehydrogenase (MDH). *P. falciparum* recombinant proteins showed abilities to bind nucleotides and revealed ATPase activity. In addition, these proteins revealed capabilities to self and hetero associates as suggested by SPR and slot blot assays.

Keywords: Malaria, *Plasmodium falciparum*, Hsp90, R2TP complex, PfRUVBL

Declaration

I, Sifiso Duncan Luthuli hereby declare that the thesis for the Doctor of philosophy in Biochemistry degree at the University of Venda, hereby submitted by me, has not previously been submitted for a degree at this or any other university, and that it is my own work in design and execution and that all reference materials contained therein have been duly acknowledged.



Signature.....Date.....12/08/2025.....

Dedication

I dedicate this thesis to my late father, Mr. Dingaen William Luthuli, my mother, Thenjiwe Luthuli, and my daughters, Sfisomuhle and Snenhlanhla Luthuli.

Preface

This thesis comprises five chapters and the outline for each chapter is provided below.

Chapter One: This is a general introduction encompassing all the background to the suggested broad aim and specific objectives. It also highlights the broad problem statement and spells out the study hypothesis.

Chapter Two: This chapter reports on the *in silico* analysis of the *P. falciparum* R2TP complex.

Chapter Three: This chapter represents the production of specific antibodies and recombinant PfRUVBL1 and PfRUVBL2.

Chapter Four: This chapter reports on the analysis of the interaction of PfRUVBL1 and PfRUVBL2 with ligands.

Chapter Five: This chapter covers conclusive remarks and future perspectives.

Acknowledgments

I would like to thank God for his guidance and for giving me the strength to complete my studies. I am also grateful to my promoter, Prof. Addmore Shonhai, for his devoted support, motivation, and incredible mentorship throughout my study journey. His supervision has helped me to develop a deeper understanding and passion for protein biochemistry.

I would also like to extend my gratitude and appreciation to the following people for their role in my study

- Prof. Walid Houry, University of Toronto (Canada), for his contribution to the study design.
- Dr. Thiago V. Seraphim, University of São Paulo-USP, 13560-970 São Carlos, SP, Brazil, for his contributions during the design of the constructs.
- Dr. Pravin Naicker, CSIR, South Africa, for his technical assistance with LCMS.

I wish to extend further acknowledgment to the:

- CSIR (IBS), for the study bursary
- University of Venda Research Committee (South Africa) for funding the project

Finally, I would like to express my special thanks to the members of the ProBioM research group (South Africa, University of Venda) for their support and contributions. Special thanks are due to the Department of Biochemistry and Microbiology staff members for their support throughout my studies. Lastly, I wish to extend my sincere thanks and appreciation to family and friends who always supported me during my studies.

Table of Contents

Abstract.....	ii
Declaration.....	iii
Dedication... ..	iv
Preface.....	iv
Acknowledgments	vi
Table of Contents	vii
List of Figures.....	xii
List of Tables	xiv
List of Symbols.....	xv
List of Outputs.....	xvi
Chapter 1	1
1.1 Malaria - a public health concern	2
1.1.1 The life cycle of <i>Plasmodium falciparum</i>	3
1.1.2 Malaria chemotherapy.....	5
1.2 Pathophysiology of Malaria	6
1.2.1 Host red blood cell invasion.....	8
1.3 Host red blood cell remodelling.....	9
1.4 The R2TP complex	12
1.5 Heat shock proteins as facilitators of proteostasis in <i>P. falciparum</i>	17
1.5.1 Heat shock protein 70 is a potential R2TP complex chaperone.....	18
1.5.2 Hsp90-Hsp70 organizing protein (Hop) mediate Hsp70 and Hsp90 function ...	20
1.5.3 The R2TP complex cooperate with heat shock 90	21
1.5.4.1 Structural features of <i>P. falciparum</i> Hsp90	24
1.5.4.2 Functional versatility of Hsp90.....	26
1.6 The R2TP complex modulates the function of Hsp90 in assembling complexes.....	27
1.7 Diverse functions of the R2TP/Hsp90 complex	30
1.8 The role of the R2TP complex in parasite	32

1.9 Association of Hsp70 with the R2TP complex.....	33
1.10 RUVBL1 and RUVBL2 AAA+ ATPase are versatile scaffolds for multiple complexes.....	35
1.10.1 The AAA+ protein family features.....	38
1.10.1 Structural organization and functions of AAA+ proteins.....	39
1.11 Study rationale.....	41
1.11.1 Hypothesis.....	41
1.11.2 Objectives.....	41
2.0 Chapter 2.....	44
2.1 Introduction.....	45
2.2 Experimental procedures.....	47
2.2.1 Multi sequence alignment.....	47
2.2.2 Prediction of three-dimensional models of PfRUVBL1, PfRUVBL2 and PfRUVBL3.....	47
2.2.3 Investigation of the interaction between chemical ligands and PfRUVBL1, PfRUVBL2, and PfRUVBL3 using molecular docking.....	47
2.2.4 Virtual ligand screening.....	48
2.2.5 Predicting the interaction network of PfHsp90.....	48
2.3 Results.....	49
2.3.1 Analysis of sequence similarities between PfRUVBL1, PfRUVBL2, and PfRUVBL3 proteins and their human and yeast homologs.....	49
2.3.2 Phylogenetical analysis of PfRUVBL1, PfRUVBL2, and PfRUVBL3.....	55
2.4 Three-dimensional models of PfRUVBL1, PfRUVBL2, and PfRUVBL3.....	56
2.5 Predicted secondary structural features of PfRUVBL1, PfRUVBL2, and PfRUVBL3 proteins.....	58
2.6 <i>In silico</i> analysis of <i>P. falciparum</i> RUVBL proteins and their interaction with ATP.....	59
2.7 <i>In silico</i> recognition of sorafenib by <i>P. falciparum</i> RUVBL proteins.....	62
2.8 <i>In silico</i> analysis of select TPR containing domain proteins of <i>P. falciparum</i>	62
2.9 Three-dimensional homologs models of RPAP3, PfHop, and PfTah1.....	64

2.10 The <i>in silico</i> prediction of the interaction network of PfHsp90.....	65
2.11 Discussion.....	67
3. Chapter three.....	70
3.0 Introduction.....	71
3.1 Materials and Methods.....	73
3.1.1 Peptide design.....	74
3.1.2 Confirmation of antibody specificity and titer optimization.....	74
3.1.3 Confirmation of pQE30/PfRUVBL1, pQE30/PfRUVBL2, and pQE30/PfRUVBL3 DNA construct.....	75
3.1.4 Expression of PfRUVBL1 and PfRUVBL2 in <i>E. coli</i> XL1 blue strain.....	75
3.1.5 Purification of recombinant proteins.....	75
3.1.6 Liquid chromatography-mass spectrometry (LC-MS).....	76
3.1.7 In-gel digestion.....	76
3.1.8 LC-MS analysis.....	76
3.1.9 Expression of PfRUVBL1, PfRUVBL2 in <i>E. coli</i> BB2393 and <i>E. coli</i> BB1553 strains.....	77
3.2 Results	78
3.2.1 Epitope selection.....	78
3.2.2 Proteins hydrophobic scale.....	79
3.2.3 Confirmation of the antibody's specificity and optimization of the antibody's titer.....	81
3.2.4 Construction of plasmids expressing PfRUVBL1, PfRUVBL2, and PfRUVBL3.....	82
3.2.5 Expression and purification of recombinant PfRUVBL1 and PfRUVBL2 in <i>E. coli</i> XL1Blue strain.....	83
3.2.6 Enhanced expression yield and purification of PfRUVBL1 and PfRUVBL2 in <i>E.</i> <i>coli</i> BB2393 strain.....	85
3.2.7 Native purification of PfRUVBL2 using polyethyleneimine (PEI).....	86
3.2.8 Discussion	88
4. Chapter four.....	90
4.0 Introduction.....	91

4.1 Materials and Methods.....	94
4.1.1 Materials.....	94
4.1.2 Determining the nucleotide (ATP/ADP) binding affinity of PfRUVBL1 and PfRUVBL2.....	94
4.2.0 Fluorescence spectroscopic analysis of PfRUVBL1 and PfRUVBL2.....	94
4.2.1 Investigation of nucleotide-dependent conformational changes of PfRUVBL1 and PfRUVBL2 using limited proteolysis.....	95
4.2.2 Investigation of basal ATPase activity of PfRUVBL1 and PfRUVBL2.....	96
4.2.3 Investigation of chaperone-like function of PfRUVBL1 and PfRUVBL2 using MDH aggregation assay.....	96
4.2.4 DNA cleaving assay.....	96
4.2.5 Antiplasmodial activity assay.....	97
4.2.6 Investigation of the direct interaction of PfRUVBL1 with PfRUVBL2.....	97
4.2.7 Determination of PfRUVBL1 and PfRUVBL2 binding affinity to PfHsp70-1 and PfHsp90.....	98
4.2.8 Investigating the capability of sorafenib to stabilise PfRUVBL1 and PfRUVBL2 using thermal shift assay (TSA).....	99
4.3 Results	100
4.3.1 PfRUVBL1 and PfRUVBL2 bind to nucleotides	100
4.4 Nucleotide-binding uniquely alters the tertiary structure of PfRUVBL1 and PfRUVBL2.....	102
4.5 Extrinsic fluorescence spectroscopy.....	103
4.6 Nucleotides regulate the conformations of PfRUVBL1 and PfRUVBL2.....	104
4.7 Sorafenib stabilizes PfRUVBL1 and PfRUVBL2 against heat stress.....	105
4.8 The PfRUVBL1 and PfRUVBL2 ATPase activity is partially inhibited by Sorafenib.....	107
4.9 Antiplasmodial activity.....	109
4.10 PfRUVBL1 and PfRUVBL2 both exhibit holdase chaperone function.....	109
4.11 ATP regulates the cleavage of double-stranded DNA (dsDNA) by PfRUVBL2.....	110
4.12 Self- and hetero- association of PfRUVBL1 and PfRUVBL2.....	111

Characterization of the structure-function features of the R2TP complex of *Plasmodium falciparum*

4.13 The interaction of PfRUVBL1 and PfRUVBL2 is regulated by nucleotides.....	112
4.14 Discussion	117
5. Chapter five.....	122
5.0 Conclusion and future perspectives.....	123
References.....	126
Appendix A.....	156
Appendix B.....	163
Appendix C.....	209

List of Figures

Figure 1.1: Malaria cases report.....	2
Figure 1.2: <i>Plasmodium falciparum</i> life cycle.....	4
Figure 1.3: Merozoite structure.....	7
Figure 1.4: Invasion of the RBC by merozoites	8
Figure 1.5: Representation of organelles of the parasite-infected RBCs.....	10
Figure 1.9: Components of the human R2TP complex.....	13
Figure 1.10: The three-dimensional model showing the domain organization of PfPIHIDI...15	
Figure 1.11: The structural features of PfHsp70-1.....	19
Figure 1.12: The structural features of PfHop.....	21
Figure 1.13.1: The structural features of PfHsp90.....	23
Figure 1.8.2: Schematic of the Hsp90 ATPase cycle.....	24
Figure 1.14: The R2TP complex is a scaffold of Hsp90 in a complex assembling.....	28
Figure 1.15: Association of the R2TP/Hsp90 complex with the mTOR complex.....	30
Figure 1.16: Association of the R2TP complex with Hsp90 and Hsp70.....	34
Figure 1.17: The structural features of ATPases members of the ASCE sub-group of AAA+ proteins.....	39
Figure 2.1.1: Sequence similarity between RUVBL proteins from <i>Plasmodium</i> , human, and yeast homologs.....	53
Figure 2.1.2: Evolutional analysis of malaria parasites RUVBL-like proteins.....	55
Figure 2.1.3: Three-dimensional models of PfRUVBL1, PfRUVBL2 and PfRUVBL3.....	57
Figure 2.1.4: Molecular predictive docking of ATP binding to PfRUVBL1, PfRUVBL2, PfRUVBL3, yRvb1, and yRvb2.....	59
Figure 2.1.5: Molecular docking of Sorafenib to PfRUVBL1, PfRUVBL2, PfRUVBL3, yRvb1, and yRvb2.....	61
Figure 2.2.1: Sequence alignment of TPR domain containing proteins.....	62
Figure 2.2.2: Homology remodelling of RPAP3, PfHop, and PfTah1.....	64
Figure 2.3: PfHsp90 predicted interaction network.....	65
Figure 3.1: Multisequence alignment of PfRUVBL1, PfRUVBL2, and PfRUVBL3 with their yeast and human homologs.....	78
Figure 3.2. Investigation of antibody specificity and optimization of the titer.....	82
Figure 3.3: Expression and purification of PfRUVBL1 and PfRUVBL2 in <i>E. coli</i> XL1 blue strain.....	84

Figure 3.4: Expression and purification of PfRUVBL1 and PfRUVBL2 in <i>E. coli</i> BB2393 strain.....	86
Figure 3.5: Purification of PfRUVBL2 in the presence of solubilizing detergent showed oligomers.....	87
Figure 4.1: Nucleotide-dependent interaction of PfRUVBL1 and PfRUVBL2 in comparison to their yeast homolog Rvb1.....	103
Figure 4.2: The interaction of PfRUVBL1 and PfRUVBL2 with nucleotides and sorafenib alters their tertiary structure.....	105
Figure 4.3: The ANS binding spectra of PfRUVBL1 and PfRUVBL2 inferring tertiary structure perturbation by ATP and ADP.....	106
Figure 4.4: Limited proteolysis confirming nucleotide-induced structural changes in PfRUVBL1 and PfRUVBL2 compared to their yeast homolog yRvb1.....	107
Figure 5.5: Sorafenib stabilizes PfRUVBL1 and PfRUVBL2.....	109
Figure 4.6: PfRUVBL1 and PfRUVBL2 exhibits ATPase activity.....	110
Figure 4.7: PfRUVBL1 and PfRUVBL2 revealed holdase chaperone function.....	112
Figure 4.8: PfRUVBL1 and PfRUVBL2 exhibit DNA cleaving function.....	113
Figure 4.9: Recombinant PfRUVBL1 and PfRUVBL2 demonstrated self and hetero interactions.....	114
Figure 4.10: Interaction of PfRUVBL1 and PfRUVBL2 is regulated by nucleotides.....	115
Figure 4.11: PfHsp70-1 interacts with PfRUVBL1 in a concentration-dependent fashion...	116
Figure 4.12: PfHsp90 interacts with PfRUVBL2 within micromolar range.....	117
Figure 4.13: Interaction of PfHsp90 with small molecular inhibitors.....	118

List of tables

Table 1.1: Major heat shock proteins of <i>P. falciparum</i>	17
Table 1.2: Hsp90 co-chaperones.....	26
Table 1.4: R2TP/Hsp90 complex clients.....	28
Table 1.5: RUVBL1 and RUVBL2 members nomenclature and their species of origin.....	35
Table 2.1: <i>In silico</i> secondary structural features analysis of PfRUVBL1, PfRUVBL2, and PfRUVBL3	58
Table 2.2: Secondary structural features of TPR-domain containing proteins.....	63
Table 3.1: List of plasmid constructs and bacterial strains used for protein expression.....	72
Table 3.2a: Characteristics of selected peptides.....	79
Table 3.2b Peptides Modification.....	80
Table 3.3: LCMS data analyses.....	85
Table 4.2: ATPase activity kinetics.....	110
Table 4.3: Antiplasmodial activity.....	111

List of Symbols

Abbreviations of units and symbol interpretation

% percent

μl microlitre

A₃₂₀ absorbance at 320 nanometres

A₃₆₀ absorbance at 360 nanometres

A₅₉₅ absorbance at 595 nanometres

A₆₀₀ absorbance at 600 nanometres

bp base pair

kDa kilodalton

mM millimolar

μM micromolar

pM picomolar

°C Degree Celsius

ml millilitre

l litre

w/v weight per volume

v/v volume per volume

μg microgram

g gram

kg kilogram

α alpha

β beta

List of Outputs

Journal article

Luthuli, S.D. and Shonhai, A., 2023. The multi-faceted roles of R2TP complex span across regulation of gene expression, translation, and protein functional assembly. *Biophysical Reviews*, 15(6), pp.1951-1965.

Conference/Workshop Proceedings

Luthuli and Shonhai, 2023. Structure-function features of the R2TP complex of *Plasmodium falciparum*. SAYAS, university of Venda.

Luthuli and Shonhai. 2024. Characterization of structure-function features of the R2TP complex of *Plasmodium falciparum*. SASBMB/IUMBMB congress in Polokwane.

Chapter one

Literature review

1.1 Malaria - a public health concern

Malaria is caused by apicomplexan parasites of the genus *Plasmodium*. Fever, anemia, headache, and chills characterize the disease. According to the World Health Organization (WHO, 2023), the COVID-19 pandemic disrupted services that regulate the spread of this life-threatening disease, leading to a marked increase in malaria cases (Figure 1.1). Sub-Saharan Africa disproportionately bears the burden of malaria, representing about 94% of all malaria cases and 95% of deaths in 2022 (Figure 1.1; WHO, 2023). It is crucial to note that just over half of all global malaria deaths occurred in four African countries: Nigeria, the Democratic Republic of Congo, Uganda, and Mozambique (WHO, 2023), all located in the Sub-Saharan region.

During the period from 2019 to 2021, an estimated 13.4 million additional cases were attributed to disruptions caused by the COVID-19 pandemic (WHO, 2023). The incidence of cases in the African region decreased from 373 to 225 per 1000 population at risk between 2000 and 2019. Encouragingly, these cases subsequently declined to 229 per 1000 population post the COVID-19 pandemic (WHO, 2023).

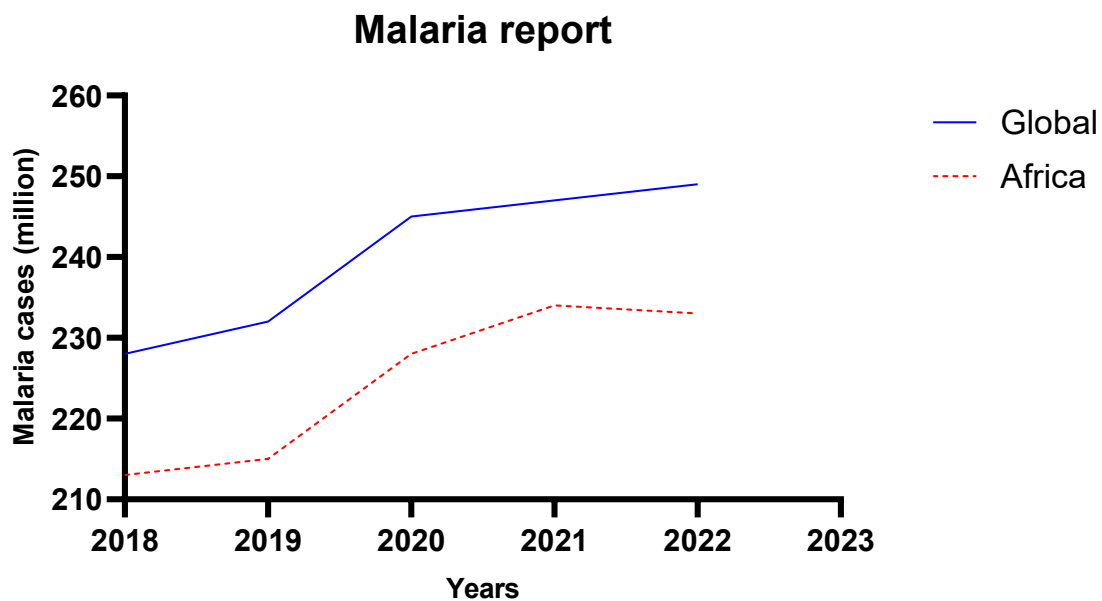


Figure 1.1: Malaria cases report

Malaria is caused by five different *Plasmodium* parasite species: *Plasmodium falciparum*, *Plasmodium ovule*, *Plasmodium vivax*, *Plasmodium knowlesi*, and *Plasmodium malariae* (Tuteja, 2007; Vythilingam *et al.*, 2021). *Plasmodium falciparum* (*P. falciparum*) accounts for most malaria-related deaths (WHO, 2023). It should be noted that infections from mixed

species of *Plasmodium* frequently go undetected unless molecular methods are used to resolve them (Cnops *et al.*, 2011). The reported proportion of mixed infections varies by country and region from 1.2%-20% (Kotepui *et al.*, 2020). In a study conducted in Israel, the incidence of mixed malaria infections among travelers returning from Sub-Saharan Africa was 3.7%, confirming Africa as the most infected region (Manor *et al.*, 2021).

1.1.1 The life cycle of *Plasmodium falciparum*

Malaria parasites are transmitted to humans through the bite of mosquitoes. The vector is primarily a female mosquito from the genus *Anopheles* (Djihinto *et al.*, 2022). The most dominant *Anopheles* vectors in Africa are *Anopheles gambiae*, *Anopheles arabiensis*, *Anopheles coluzzii*, and *Anopheles funestus* (Coetzee *et al.*, 2013). The development of malaria in the blood stage is characterized by episodes of fever during which the body temperature may rise to 41°C. The parasite's adaptive strategy to manage stress emanating from physiological changes is proposed to augment the parasite's resilience associated with its molecular chaperone system (Pavinthra *et al.*, 2004).

The parasite's capability to withstand immune responses poses significant challenges to the global control and elimination of malaria strategies (Sato, 2021). The parasite develops and multiplies within the host organism and enjoys benefits such as a ready supply of nutrients. However, the benefits also require the parasite to give up control and independence to endure conditions experienced by its host.

The life cycle of the main agent of malaria, *P. falciparum*, involves complex stages alternating between mosquito vectors and human hosts (Parham and Hughes, 2015). The cycle begins when an infected female *Anopheles* mosquito bites a human and injects sporozoites into the bloodstream. These sporozoites quickly travel to the liver, where they multiply to several thousands of merozoites (Figure 1.2). This stage is asymptomatic and lasts about 5-7 days, during which the parasite is not directly detectable in the bloodstream (Mo and McGugan, 2018).

Merozoites are then released from the liver cells into the bloodstream, where they invade the red blood cells (RBC). Inside the RBC, merozoites transform through several forms, including the ring stage, trophozoite stage, schizont stage, and gametocytes. Some merozoites differentiate into gametocytes, and these are taken up by another mosquito during a blood meal (Molina-Franky *et al.*, 2022).

Characterization of the structure-function features of the R2TP complex of *Plasmodium falciparum*

In the midgut of a mosquito, gametocytes develop into male and female gametes. After fertilization, zygotes form and mature into motile ookinetes. These ookinetes then penetrate the mosquito midgut wall and continue developing into oocysts on the outer surface of the midgut (Bennink *et al.*, 2016). Thousands of sporozoites develop inside the oocyst over 1-2 weeks and then migrate to the mosquito's salivary glands. Eventually, they are transmitted to the human host during a blood meal.

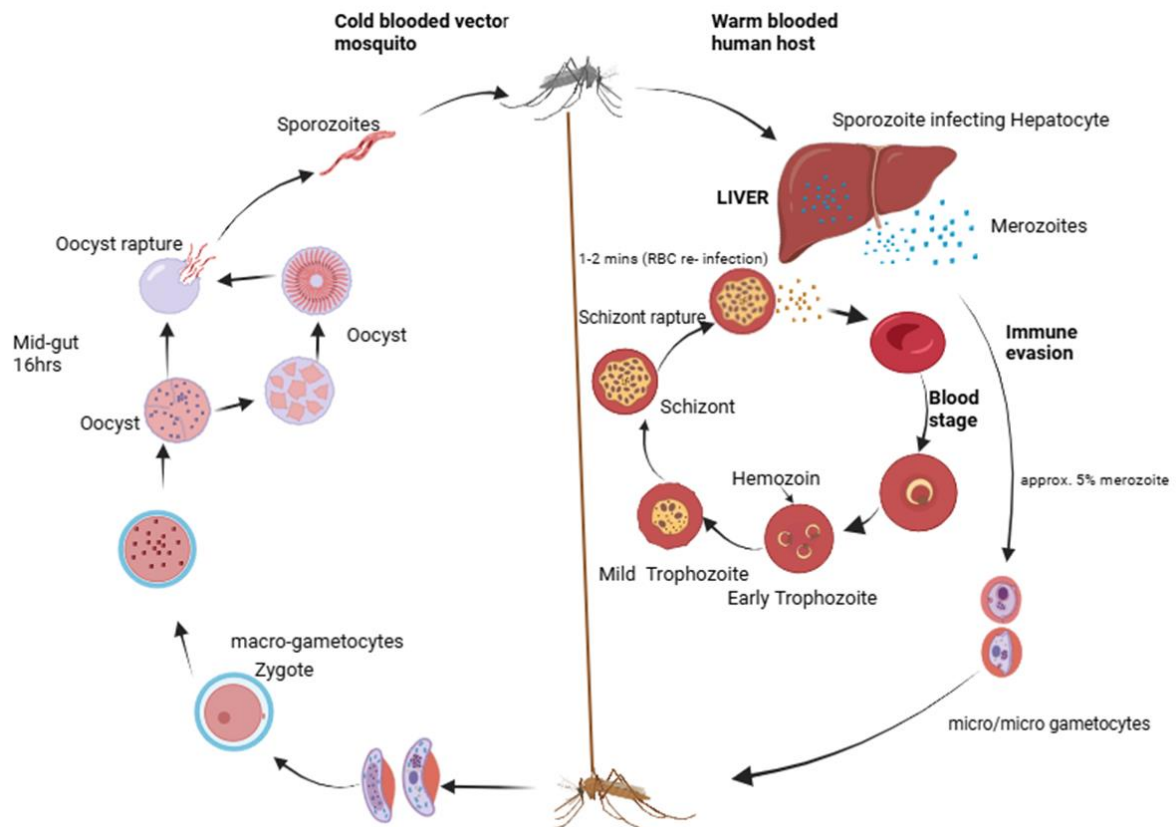


Figure 1.2: *Plasmodium falciparum* life cycle

The parasite *P. falciparum* is injected into the warm-blooded human host as sporozoites during the Anopheles mosquito blood meal. The sporozoites subsequently migrate to the liver and infect hepatocytes. In the hepatocyte, the sporozoites develop and mature into merozoites through schizogony rupture. Merozoites leave the liver to the bloodstream where they infect the RBC. Infected RBCs then burst releasing more merozoites, which infect new RBCs within 48 hours. Less than 8% of merozoites differentiate into gametocytes, which are taken up by the mosquito during blood meal. The sexual reproduction stage occurs through gametocytes that fuse and differentiate in the mosquito midgut to form new sporozoites. (Adapted from Seraphim *et al.*, 2019).

1.1.2 Malaria Chemotherapy

The treatment of malaria is a complex process involving various interventions to eliminate the malaria parasites from the body or alleviate the associated symptoms (Ould Ahmedou Salem *et al.*, 2015). The choice of treatment depends on several factors, including the species of *Plasmodium* causing the infection, the severity of the illness, and any patterns of drug resistance in the region.

Currently, the most effective treatments for uncomplicated malaria caused by *P. falciparum*, prevalent in many malaria-endemic regions, are Artemisinin-based Combination Therapies (ACTs) (Arya *et al.*, 2021). ACTs consist of artemisinin derivatives combined with other drugs such as lumefantrine or amodiaquine, which have proven to be highly effective. The artemisinin component works rapidly to reduce the number of parasites, while the partner drugs eliminate any remaining parasites and help prevent the development of resistance (Arya *et al.*, 2021).

Historically, chloroquine (CQ) was effective against *P. vivax* and *P. malariae*, but widespread resistance has rendered it ineffective against most strains of *P. falciparum*. Despite this, chloroquine is still used in regions where the parasite remains susceptible (Silva-Pinto *et al.*, 2021). In cases of severe malaria or when ACT therapies are unavailable, quinine and quinidine are typically used (Achan *et al.*, 2011). These drugs are often administered intravenously for severe cases and followed by orally administered treatment.

Utilizing Insecticide-Treated bed Nets (ITNs) is a highly effective method of preventing malaria and reducing illness, severe disease, and fatalities in malaria-endemic regions (WHO, 2023). In 2020, forty-six nations successfully organized campaigns to distribute 272 million ITNs (WHO, 2023). Notably, these efforts were initiated before the onset of the COVID-19 pandemic. In 2021, 171 million ITNs were distributed, with 70 million carried over from the previous year (WHO, 2023). Among these, eight nations, including Benin, Eritrea, Indonesia, Nigeria, Solomon Islands, Thailand, Uganda, and Vanuatu, distributed 60% of their ITNs, highlighting commendable progress. However, it is important to note that some countries, such as Botswana, the Central African Republic, Chad, Haiti, India, Pakistan, and Sierra Leone, did not meet their distribution targets (WHO, 2023).

1.2 Pathophysiology of Malaria

The merozoites is a unicellular form of the malaria parasite of about 1.5µm long, egg-shaped, and were first reported to be fluffy sheaths composed of surface proteins (Figure 1.3; Bannister *et al.*, 1975; reviewed by Beeson *et al.*, 2016). The composition and function of merozoites have been reported to promote parasite attachment to RBCs (Beeson *et al.*, 2016). The more pointed end of the merozoite contains the apical complex consisting of the secretory organelles, rhoptries, micronemes, dense granules, as well as an apical polar ring, which acts as a microtubule organising center (Farrow *et al.*, 2011). The wider end of the merozoite consists of the nucleus, plastid organelle, and mitochondria (Figure 1.3). The rhoptries and micronemes contain proteins important for RBC invasion (Beeson *et al.*, 2016). The rhoptries are a pair of electron-dense structures, while micronemes are several small fusiform bodies attached to the rhoptry duct (Figure 1.3). The dense granules are round-shaped structures in all parts of the merozoite cytoplasm (Preiser *et al.*, 2000).

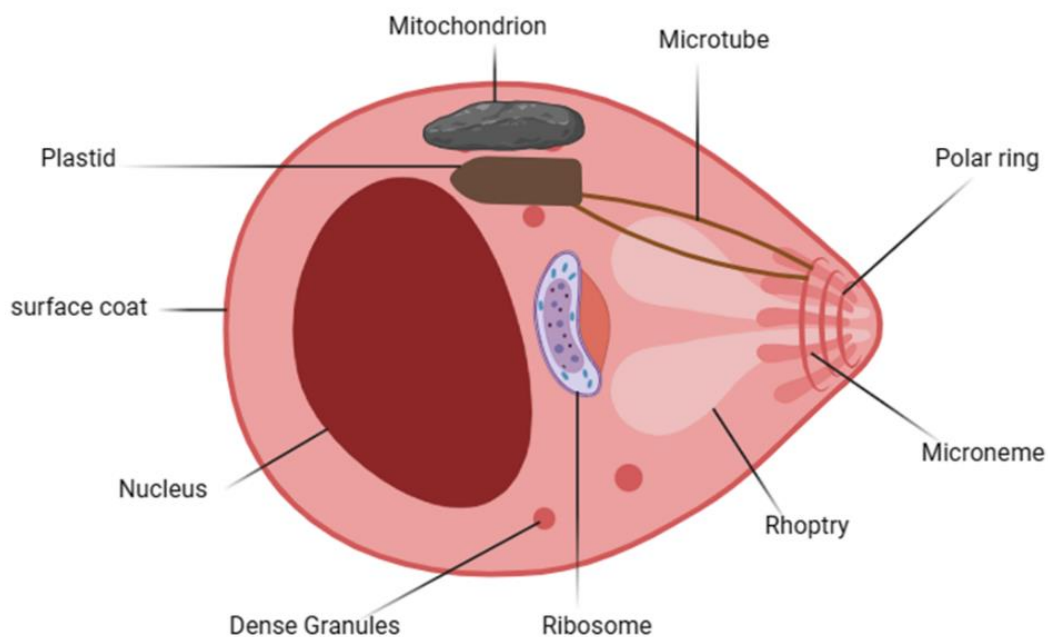


Figure 1.3: Merozoite structure

Diagrammatic representation of merozoites organelles
(Adapted from Bannister and Mitchell, 2009).

The surface coat of the merozoite is composed mainly of glycosylphosphatidylinositol (GPI)-anchored proteins and integral membrane proteins, while peripherally associated surface proteins only contribute a minor percentage (Gison *et al.*, 2006). Various GPI-anchored merozoite surface proteins have been reported and categorized into families, including MSPs

(MSP1, MSP2, MSP4, MSP5, and MSP10) and the 6 cysteine domain proteins (Pf92, Pf38, and Pf12) (Sanders *et al.*, 2005). Studies have shown that microneme/associated cysteine-rich protective antigen (CyRPA) and GPI-anchored microneme antigen (GAMA) travel to the surface of the merozoite before the RBCs invasion (Arumugam *et al.*, 2024).

Physiological changes in malaria-infected patients occur immediately after the destruction of infected RBC leading to the release of merozoites into the bloodstream (Figure 1.4; Piccaluga and Ignatius, 2023). The released merozoites infect healthy RBCs in the circulation, and when RBCs are destroyed by the system, the patient experiences intravascular hemolysis (Piccaluga and Ignatius, 2023). The rupture of the first liver schizont and release of motile merozoites into the peripheral circulation to invade RBCs marks the beginning of symptomatic infection (Zekar and Sharman, 2023). Cerebral malaria and severe malaria with multi-organ dysfunction are associated with excessive tumor necrosis factor production. This could lead to systemic inflammation, endothelial cell activation, and contribute to malaria pathology (Milner, 2018).

Maier *et al* (2009) reported that *P. falciparum* exported proteins interact with RBC membrane proteins and cause significant changes in host cell morphology, physiology, and function. Sequestration of the mature form is induced by its attachment to endothelial cells, blood cells, platelets, and uninfected RBCs. Sequestration of mature infected RBCs by endothelial cells indirectly promotes parasite proliferation, explaining the log-linear increase in parasite burden in nonimmune *P. falciparum* patients (Oelschlegel *et al.*, 2024). The RBC membrane protein 1 (PfEMP-1) is the most virulent agent of *P. falciparum* (Nandhini and Perumal, 2018). PfEMP-1 is expressed on the surface of infected RBCs during their asexual life cycle (Jensen *et al.*, 2019). Sequestration of infected RBCs is thought to be facilitated by the binding of the PfEMP-1 to endothelial receptors (Kraemer *et al.*, 2006).

1.2.1 Host red blood cell invasion

The parasite-host relationship is complicated and involves various aspects. In the human body, RBCs or their corresponding proteins play a crucial role in transporting nutrients and gases (Paul *et al.*, 2022). Mature RBCs have a biconcave and disc-like shape, lacking a nucleus (Dijkman *et al.*, 2021). This structure provides the flexibility needed to navigate the cardiovascular system and an increased surface area for efficient gas exchange, enabling the cells to perform their functions (Paulet *et al.*, 2022).

Parasites have specific mechanisms to attach to and invade host cells or tissues. For instance, *P. falciparum* uses proteins on its surface to attach to and enter RBCs (Figure 1.4; Baruch *et*

al., 1995; Zekar and Sharman, 2023). The adhesins and invasins class of proteins (Wright and Rayner, 2014) control the primary contact between the merozoite and the RBC. The dominant adhesins involved in RBC invasion by *P. falciparum* belong to the RBC binding-like domain (EBL) and reticulocyte binding-like homology family (PfRh) family (Cowman *et al.*, 2012).

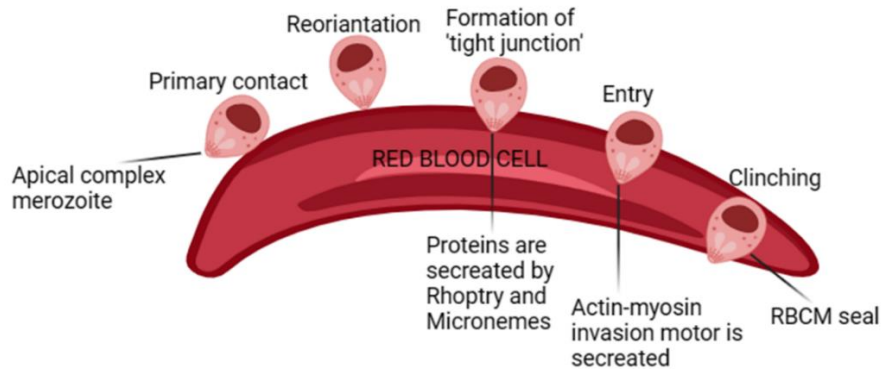


Figure 1.4: Invasion of the merozoite into the RBC

The RBC invasion is a complex step involving the invasion, merozoite reorientation, formation of the juncture, entry, and lastly the RBC sealing. (Adapted from Beeson *et al.*, 2016).

Parasites can alter their surface proteins to evade detection or utilize host nutrients for their growth. The host responds to the presence of the parasites through a combination of innate and adaptive immune mechanisms, including inflammation, production of antibodies, and activation of immune cells aimed at targeting and eliminating the parasites (Hart *et al.*, 2023). Parasites can manipulate host physiology to their advantage. This may involve altering host cell metabolism or immune signalling pathways to create a more favourable environment for their survival. Parasites can cause disease symptoms through various means, such as direct tissue damage, release of toxins, or induction of inflammatory responses. The severity of the disease often hinges on the parasite load, the host's immune response, and the specific characteristics of the parasite-host interaction (Hart *et al.*, 2023).

Parasite-host interactions are diverse and complex, varying significantly depending on the specific parasite and host involved. Parasites often have specialized mechanisms to attach to and invade host cells or tissues. Once inside the host, parasites must adapt to evade host immune responses and utilize host resources for their survival and replication. This adaptation often involves complex molecular interactions between the parasite and the host cell environment (Marzal *et al.*, 2020). Hosts mount immune responses against parasites through both innate and adaptive immune mechanisms. This involves the recognition of parasite-specific molecules (e.g., antigens) by host immune cells and the subsequent activation of

immune responses aimed at controlling and eliminating the parasite (Marzal *et al.*, 2020). Some parasites can manipulate host physiology to enhance their survival. For example, *Toxoplasma gondii*, the causative agent of toxoplasmosis, can alter host cell signalling pathways to evade immune detection and establish chronic infection (Hunter and Sibley, 2012). Understanding these interactions is crucial for developing effective strategies to combat parasitic diseases, including vaccines, antiparasitic drugs, and public health interventions aimed at interrupting parasite transmission cycles (Peymanfar and Taylor-Robinson, 2016).

Vaccines targeting Merozoite Surface Protein-1 (MSP1), specifically the MSP1-19 fragment, have demonstrated the potential to stimulate protective immune responses against the malaria parasite (Green *et al.*, 2020). Ongoing research and innovative techniques, such as combination vaccines and prime-boost strategies, show the potential to overcome obstacles like genetic diversity and immune evasion to develop a successful MSP1-based malaria vaccine (Dutta *et al.*, 2007). MSP1 is a leading candidate for malaria vaccine development due to its crucial role in the invasion of RBC by *P. falciparum* (Holder, 2009). MSP1 is one of the most abundant proteins on the surface of merozoites and is essential for their attachment and invasion of RBC.

1.3 Host red blood cell remodelling

The malaria parasites inside RBCs attempt to survive by inducing several changes in the host cell (Marzal *et al.*, 2020). The development of the parasite within infected RBCs takes approximately 48 hours and is divided into several different stages (Marzal *et al.*, 2020). These stages include (i) the initial contact of the merozoite with the RBC, (ii) the rearrangement of the merozoite that allows the apical end to contact the RBC, and (iii) molecules involved in invasion released from organelles (rhoptry and micronemes), (iv) the RBC membrane is pulled around the parasite to form the parasitophorous vacuole (PV) (Figure 1.5). Subsequently, the actin-myosin entry motor is released, triggering a protease cleavage event, and (v) the RBC membrane is sealed, and the parasite is kept inside (Weiss *et al.*, 2015; Blake *et al.*, 2020).

Following the invasion, the parasite encases itself within the RBC membrane, forming a membrane shield known as the PV (Figure 1.5) (Cowman *et al.*, 2012; Marzal *et al.*, 2020). The PV is a transport compartment for proteins destined for the RBC cytosol (Charpian and Przyborski, 2008). The central cavity of the PV is a small space, approximately 50 nm in diameter as revealed by electron micrographs (Trelka *et al.*, 2000). The structural boundary surrounding the PV is referred to as the parasitophorous vacuole membrane (PVM) (Figure

1.5; Goldberg and Zimmerberg, 2020). This membrane forms a physical barrier that separates the parasite from the RBC cytosol (Sherling and van Ooij, 2016). Additionally, this parasite is associated with the development of membranous structures called the tubulovesicular network (TVN) and Maurer's cleft (Maluf *et al.*, 2020). Maurer's clefts are parasite-derived structures within the host cell cytoplasm that are believed to function as sorting compartments between parasite and RBC membranes (McHugh *et al.*, 2020).

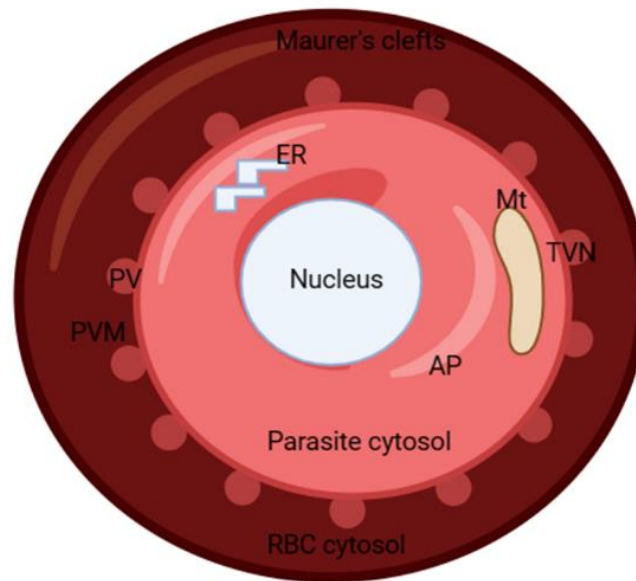


Figure 1.5: Representation of organelles of the parasite-infected RBCs

Definition of abbreviations: ER (endoplasmic reticulum), Mt (mitochondria), PV (parasitophorous vacuole), TVN (tubulovesicular network), and PVM (PV membrane). (Adapted from Deponte *et al.*, 2012).

The organelles play a crucial role in the regeneration of host cells. Specifically, proteins intended for the cell surface are temporarily redirected to the Maurer's cleft (McHugh *et al.*, 2020). The exit of merozoites during the blood stage occurs after the rupture of the parasitophorous vacuole membrane (PVM), followed shortly by the rupture and vesiculation of the RBC membrane (RBCM) (Tan and Blackman, 2021). A recent report suggests that exogenous phosphatidylcholine acts as a promoter for this exit process (Paul *et al.*, 2020). The *P. falciparum* surface anion channel (PSAC) is composed of members of the cell adhesion junction antigen (CLAG) protein family (Gupta *et al.*, 2018). These proteins increase the strength of RBCs and help restore their structure (Mohandas and Gallagher, 2008). Understanding the detailed mechanisms of RBC remodeling by *Plasmodium* parasites is important. It has been demonstrated that anion channels induced by the parasite in infected RBCs are crucial for the parasite's survival and proliferation (Ginsburg *et al.*, 1985). These channels, often referred to as new permeability pathways (NPPs), significantly alter the

permeability of the host cell membrane, allowing the parasite to obtain essential nutrients and dispose of waste products (Ginsburg *et al.*, 1985).

The intracellular environment of the parasite is complex. Due to the lack of nutrients and biological needs, the parasite develops extraordinary survival strategies. Parasites face challenges because mature, terminally differentiated, metabolically compromised RBCs do not provide a system for protein or lipid synthesis (Deroost *et al.*, 2016). The host supplies the parasite with a limited amount of hemoglobin, which the parasite must digest. However, hemoglobin lacks the essential amino acid isoleucine (Divo *et al.*, 1985; Deroost *et al.*, 2016). Research has shown that this amino acid is crucial for parasite growth, and its deficiency causes the parasite to go into hibernation (Babbitt *et al.*, 2012; Prior *et al.*, 2021). Therefore, parasites must modify host cells to survive.

1.4 The R2TP complex

The R2TP complex has been proposed to play a significant role in the development and function of parasites, particularly in the context of *Plasmodium*, *Trypanosoma*, *Leishmania donovan*, and other eukaryotic parasites (Ahmad *et al.*, 2013; Seraphim *et al.*, 2019). The R2TP complex is a multi-protein chaperone complex involved in various cellular processes. It helps in the assembly and stabilization of large macromolecular machines such as RNA polymerase complexes, small nucleolar ribonucleoproteins (snoRNPs), and the PI3-kinase-like kinase (PIKK) family of proteins (Seraphim *et al.*, 2022). The complex plays a crucial role in the biogenesis of ribonucleoproteins and maintaining cellular homeostasis. It consists of four core components: Rvb1, Rvb2, Tah1, and Pih1 (Zhao *et al.*, 2005). In mammals the R2TP core components are associated with an additional subunit of prefoldin (PFDL) and prefoldin-like proteins PFDN2, PFDN6, URH1, UXT, PDRG1, and it interacts with two additional components, namely the RNA polymerase subunit POLR2E/RPB5 and WDR92/Monad (Kim *et al.*, 2013; King *et al.*, 2015). Rvb1 and Rvb2 are highly conserved AAA+ ATPases that provide the energy required for the assembly and disassembly of protein complexes (Paci *et al.*, 2020). Tah1 (tetratricopeptide repeat-containing protein) interacts with Hsp90, linking the R2TP complex to the Hsp90 chaperone system (Houry *et al.*, 2018).

Pih1 (protein interacting with Hsp90) interacts with Tah1 and the client proteins, serving as a scaffold that brings the complex components together (Zhao *et al.*, 2005). The R2TP complex, in conjunction with Hsp90, plays a crucial role in the assembly, stabilization, and regulation of various essential cellular macromolecular complexes. Functions of the R2TP/Hsp90 Complex

include the assembly and maturation of small nucleolar ribonucleoproteins (snoRNPs), which are crucial for ribosomal RNA processing (Boulon *et al.*, 2008). The R2TP/Hsp90 complex assists in assembling and stabilizing RNA polymerase II by ensuring proper folding and integration of its components (Boulon *et al.*, 2012).

In addition, the complex stabilizes and regulates members of the PIKK family, including mTOR, ATR, and DNA-PK, which are involved in key signaling pathways related to cell growth, DNA damage response, and repair (Bhadra and Xu, 2023). The R2TP complex, with Hsp90, recognizes client proteins needing stabilization or assembly. Tah1 facilitates interaction with Hsp90, while Pih1 and Rvb1/Rvb2 handle the client proteins (Pal, 2014). The ATPase activity of Rvb1 and Rvb2 provides the energy required for the conformational changes necessary for the assembly and remodeling of client protein complexes (Nano *et al.*, 2014). The R2TP complex integrates into multiple cellular pathways by interacting with various client proteins and complexes, highlighting its versatility and essential role in cellular homeostasis (Boulon *et al.*, 2012).

The R2TP complex is a multi-protein chaperone assembly involved in various cellular processes. It helps in the assembly and stabilization of large macromolecular machines such as RNA polymerase complexes, small nucleolar ribonucleoproteins (snoRNPs), and the PI3-kinase-like kinase (PIKK) family of proteins (Seraphim *et al.*, 2022). The complex plays a crucial role in the biogenesis of ribonucleoproteins and maintaining cellular homeostasis. It consists of four core components: Rvb1, Rvb2, Tah1, and Pih1 (Figure 1.6; Zhao *et al.*, 2005). Rvb1 and Rvb2 are AAA+ ATPases that provide the energy required for the assembly and disassembly of protein complexes (Paci *et al.*, 2020). Tah1 is a TPR (tetratricopeptide repeat) domain-containing protein that interacts with Hsp90, linking the R2TP complex to the Hsp90 chaperone system (Houry *et al.*, 2018).

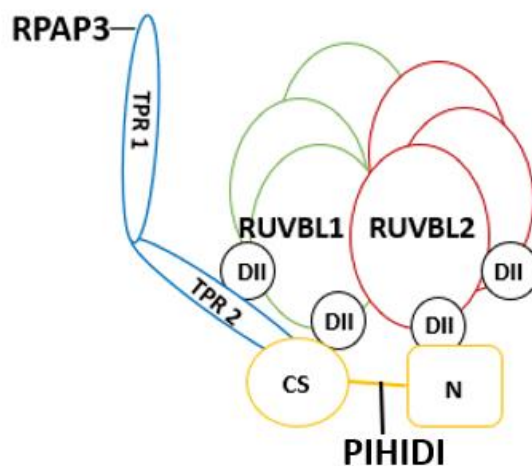


Figure 1.6: Components of the human R2TP complex

Constitute components of the R2TP complex, Rvb1-Rvb2-Tah1-Pih1 (Adapted from Zhao *et al.*, 2005).

1.4.1 Structure-function features of the R2TP complex components

The R2TP complex is involved in diverse cellular activities, and clients loading into the R2TP complex may either interact directly with R2TP components or indirectly via a linker protein (Kakihara *et al.*, 2014). Several confirmed functions that Hsp90 undertakes through its cooperation with the R2TP complex have been reported (Kakihara *et al.*, 2014).

Pih1 and Tah1 serve as lids of the AAA+ hexamers of RUVBL1 and RUVBL2, respectively (Tian *et al.*, 2017), resulting in a dodecamer R2TP complex (Figure 1.6). Pih1 protein is thought to function as a central mediator, which connects the RUVBL1/2 to Tah1 and eventually links the complex to Hsp90 (Pal *et al.*, 2014). It has been demonstrated that Tah1 and Pih1 are Hsp90 interactors (Rivera-Calzada *et al.*, 2017), confirming their role as components of the R2TP complex. PIHIDI/Pih1 is structurally composed of two distinct domains (Figure 1.7) (PIHI-N and PIHI-C). It has been suggested that the PIH-N domain binds phospho-peptide (Pal *et al.*, 2014). The PIH1-C region contains the CS domain previously identified in several Hsp90 complexes (Horejsi *et al.*, 2014; Henri *et al.*, 2018).

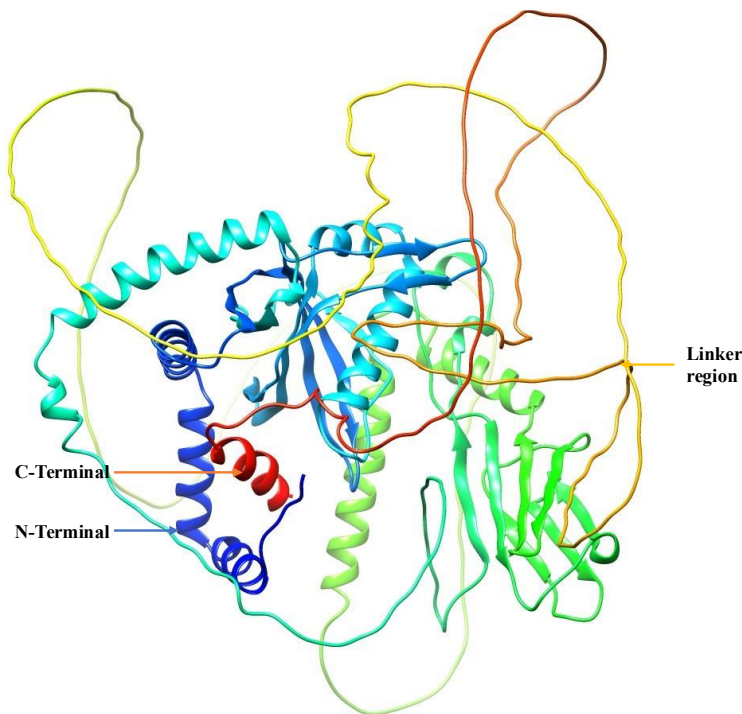


Figure 1.7: The 3D structure model showing the domain organization of PfPIHIDI

Representation of the putative PfPIHIDI (PF3D7_0931700) 3D model, sequence was obtained in the PlasmDB (plasmodb.org) and rendered in PDB RCSB to obtain the CIF file. Discovery Studio (v21.1.0.20298) was used to convert the CIF file to PDB before visualization using the Chimera 1.15 tool.

Pih1 interacts with snoRNP complex and ribosome biogenesis-related proteins such as Rrp43, a component of the exosome, and Nop58 a component of box C/D SnoRNP [small nucleolar ribonucleoprotein complex box C motifs (RUGAUGA) and box D (CUGA)] (Lakshminarasimhan *et al.*, 2016). Nop58 is an essential nuclear protein, and Cwc24 is a zinc finger protein related to pre-U3 snoRNP splicing (Eckert *et al.*, 2010). The human Pih1, also known as PIHIDI, interacts with box C/D snoRNP factor Nop1/fibrillarin, Nop58, Nop56, Tel2, proteins required for PIKKs stability, and WDR92/Monad (Lakomek *et al.*, 2015). The Monad/WDR92 contains WD40 repeats, and this protein is a subunit of the pre-foldin complex and WD40 repeat domain (WDR) protein that is implicated in apoptosis (Saeki *et al.*, 2006). WDR domains are β -propeller domains that serve as protein interaction scaffolds in multiprotein complexes (Schapira *et al.*, 2017).

The central cavity of the WDR domain is structurally unique for different species and hence is deemed a druggable candidate (Schapira *et al.*, 2017). The overexpression of either Monad/WDR92 or RPAP3 in HEK293 cells enhances apoptosis and caspase-3 activation induced by tumor necrosis factor- α (TNF- α) and cycloheximide (CHX) (Patel-King and King, 2016). RPAP3, also known as hSpagh is the human homologue of yeast Tah1 (Morgan *et al.*,

2015). Yeast Tah1 is composed of 111 amino acid residues and has two TPR motifs, whereas RPAP3 consists of 665 amino acid residues and contains six predicted TPR motifs (Taipale *et al.*, 2010). Depletion of RPAP3 significantly reduced the induction of apoptosis, suggesting that Monad/WDR92 and RPAP3 could be modulators of the apoptotic pathway (Saeki *et al.*, 2006). In addition, overexpression of RPAP3 in humans promotes UV-induced cell death, while knockdown of RPAP3 reduces cell death. In contrast, the knockdown of RUVBL2 enhances cell death upon UV treatment (Wang *et al.*, 2024). Furthermore, depletion of PIH1D1 promotes apoptosis and caspase-3 activation induced by doxorubicin in 20S osteosarcoma human cells (Wang *et al.*, 2024). Pih1 is an unstable protein that is degradation-prone but is stabilized by its binding to heat shock protein 90 (Hsp90) and Tah1 (Jiménez *et al.*, 2012). Tah1 is composed of an N terminal domain of about 90 amino acid residues containing two tetratricopeptide repeat (TPR) motifs which are known to mediate the interaction of Hsp90 with its cofactors (Taipale *et al.*, 2010), and the C-terminal segment of about 21 amino acids residue (Pal *et al.*, 2014).

RNA polymerase II (RNAPII) synthesizes capped non-coding RNAs and all mRNAs (Boulon *et al.*, 2010). This enzyme is crucial for regulating gene expression (Fuda *et al.*, 2009). RNAPII comprises 12 subunits, including Rpb1 and Rpb3, which are important for the assembly of RNAPII (Boulon *et al.*, 2010). Tah1 has been shown to directly interact with Rpb1 (the largest subunit of RNAPII) and thus possibly recruit the R2TP complex and prefoldin-like proteins together to facilitate RNAPII complex assembly (Boulon *et al.*, 2010) (Table 3). Tah1 possesses two TPR motifs; one of these recognizes the C-terminal MEEVD motif of Hsp90. The C-terminus of Tah1 is the only requirement for binding to Pih1 (Zhao *et al.*, 2008). This suggests that the TPR-containing proteins, Tah1/RPAP3, interact with proteins such as Hsp90 through the C-terminal MEEVD. Tah1 contains tetratricopeptide repeat (TPR) motifs that mediate the interaction of Hsp90 with its cofactors (Taipale *et al.*, 2010; Gitau *et al.*, 2012; Zininga *et al.*, 2015). Tah1 is the Hsp90 co-chaperone (Eckert *et al.*, 2010). RPAP3 (FLJ21908), also known as hSpagh has been identified as the human counterpart of Tah1 in the R2TP complex in human cells (Morgan *et al.*, 2015). This difference suggests that RPAP3 may have a greater capacity for protein-protein interactions, potentially allowing it to interact with a broader range of proteins or to form more stable complexes (Pal *et al.*, 2014).

RUVBL proteins form a hexamer ring and are implicated in variable cellular processes, including transcription, DNA repair, cellular transformation, cell growth, and cell invasion (Eckert *et al.*, 2010). The importance of RUVBLs in transcriptional control is highlighted by

the fact that they participate in chromatin remodeling as a member of several complexes, such as INO80, SRCAP, Uril, and Tip60. RUVBLs have been reported to interact physically with several transcription factors, including β -catenin, TBP (TATA-binding protein) Myc, E2F1, ATF2 (Activating Transcription Factor2), and H1F1, thereby participating in the transcription control (Eckert *et al.*, 2010).

1.5 Heat shock proteins as facilitators of proteostasis in *P. falciparum*

P. falciparum Heat shock proteins (Hsp) play important roles in parasite survival, stress response, and parasite development. These proteins help the parasite deal with the harsh conditions within the human host and during transmission via mosquitoes (Shonhai *et al.*, 2005; Shonhai *et al.*, 2021). They are vital in the parasite's lifecycle, especially in protein folding, trafficking, and stress response (Cockburn *et al.*, 2014). Not only are these proteins essential for the parasite's survival, but they also present promising targets for the development of new antimalarial therapies and vaccines. Continued research into the specific functions and mechanisms of these HSPs will be crucial in advancing malaria control strategies (Yang *et al.*, 2021). Hsps are a group of proteins that constitute a significant portion of the molecular chaperone protein class. They are mainly responsible for maintaining the proper folding and conformation of other proteins in the cell and are therefore referred to as molecular chaperones. They were named "heat shock proteins" because they were initially discovered in response to the heat stress gene (Ritossa, 1962).

Cells exposed to induced stress stimulate the synthesis of molecules classified according to their molecular sizes in kDa. Major Hsp classes include small heat shock proteins (sHsps), heat shock protein 40 kDa (Hsp40), heat shock protein 60 kDa (Hsp60), heat shock protein 70 kDa (Hsp70), heat shock protein 90 kDa (Hsp90), and heat shock protein 100 kDa (Hsp100) (Table 1.1) (Hartl *et al.*, 2011; Reviewed by Hu *et al.*, 2022). Hsps are involved in maintaining proteostasis, ensuring cell survival. Due to increasing discrepancies in the protein nomenclature for heat shock protein families, a proposal for amendments was made (Kampinga *et al.*, 2009; Reviewed by Hu *et al.*, 2022).

Table 1.1: Major heat shock proteins of *P. falciparum*

Protein family	Function	Approximately monomer size/kDa	Localization	References
Hsp110	Hsp70 nucleotide exchange factor, protein aggregation suppression, possess holdase activity	100-150	cytosol, ER,	Muralidharan <i>et al.</i> , 2012; Zininga <i>et al.</i> , 2016
Hsp100	Facilitate protein folding and dis-assembly of quaternary structures of polypeptide complexes	80-110	apicoplast, mitochondria, PV	Nowicki <i>et al.</i> , 2012; Kedzierska-Mieszkowska and Zolkiewski, 2021
Hsp90	Essential for cell survival and development, involved in cell cycle control and regulating cell signalling pathways	82-96	cytosol, apicoplast, ER, mitochondria	Acharya <i>et al.</i> , 2007; Shahinas <i>et al.</i> , 2013; reviewed by Zininga and Shonhai, 2019
Hsp70	Folding of nascent polypeptides, protein translocation	72-76	ER, cytosol, mitochondria, nucleus, apicoplast, J-dots (host RBC)	Shonhai <i>et al.</i> , 2007; Mabate <i>et al.</i> , 2018; Ahmad <i>et al.</i> , 2024
Hsp60	Involved in the folding of newly synthesized proteins	58-65	apicoplast, mitochondria	Polson <i>et al.</i> , 2018
Hsp40	Stabilizes the interaction between Hsp70 and its unfolded substrate	40-100	cytosol, membranes, apicoplast, Maurer's cleft, ER	Kulzer <i>et al.</i> , 2010; Njunge <i>et al.</i> , 2013
sHsp	Prevent aggregation of unfolded proteins	12-43	cytosol, nucleus	Montagna <i>et al.</i> , 2012

1.5.1 Heat shock protein 70 is a potential R2TP complex chaperone

Hsp70 (DnaK in prokaryotes) protein family is involved in several processes including the correct folding of nascent polypeptides, re-folding of misfolded and denatured proteins, and protein translocation (Rosenzweig *et al.*, 2019). Apart from its regulated expression in response to stress, the phosphorylation of Hsp70 is regulated by physiological stress (Sherman and

Goldberg, 1993; Reviewed by Hu *et al.*, 2022). Thus, phosphorylated Hsp70 interacts more efficiently with substrates, and this up-regulates its chaperone function during cellular stress (Sherman and Goldberg, 1993; Reviewed by Hu *et al.*, 2022).

P. falciparum Hsp70-1 (PfHsp70-1) (PF3D7_0818900) is an essential molecular chaperone involved in protein folding, trafficking, and stress response, making it indispensable for the parasite's survival and development (Shonhai *et al.*, 2005). PfHsp70-1 is involved in protein folding, protection against stress, and the refolding of denatured proteins. It interacts with co-chaperones and other Hsps to maintain cellular proteostasis (Shonhai *et al.*, 2008). PfHsp70-1 also serves as 'holders', keeping or maintaining the peptide extended conformation is fundamental for their translocation across biological barriers (Shonhai *et al.*, 2008; Zininga *et al.*, 2015b). This capability was previously demonstrated in nuclear-encoded proteins destined for the apicoplast of *P. falciparum*. Apicoplast-targeted proteins possess Hsp70 recognition motifs and any mutation on these motifs abrogated trafficking of the proteins to the apicoplast (Rosenzweig *et al.*, 2019). PfHsp70-1 is implicated in the immune evasion strategies of the parasite by modulating the host's immune response (Mutambo *et al.*, 2004). It may interact with host immune cells, and influence cytokine production, and antigen presentation, potentially aiding in parasite survival and pathogenesis (Lu *et al.*, 2020).

PfHsp70 is made up of two main domains connected by a linker: the approximately 44-kDa N-terminal nucleotide-binding domain (NBD) and the approximately 25-kDa C-terminal substrate-binding domain (SBD) (Figure 1.8). The NBD of Hsp70 contains the subdomains IA, IB, IIA, and IIB, which together form a hydrophilic nucleotide binding cleft (Makumire *et al.*, 2021). The two-layered twisted β -sandwich (SBD β) and the α -helical segment (SBD α) make up the SBD. After the substrate binds in the SBD β , the SBD α stabilizes substrate binding and acts as a lid. The linker's hydrophobic character enables it to orient the two domains and control ATP hydrolysis (Chakafana *et al.*, 2021). The main criterion that qualifies protein as an Hsp70 substrate is the presence of large hydrophobic patches (Takenaka *et al.*, 1995; Shonhai, 2021).

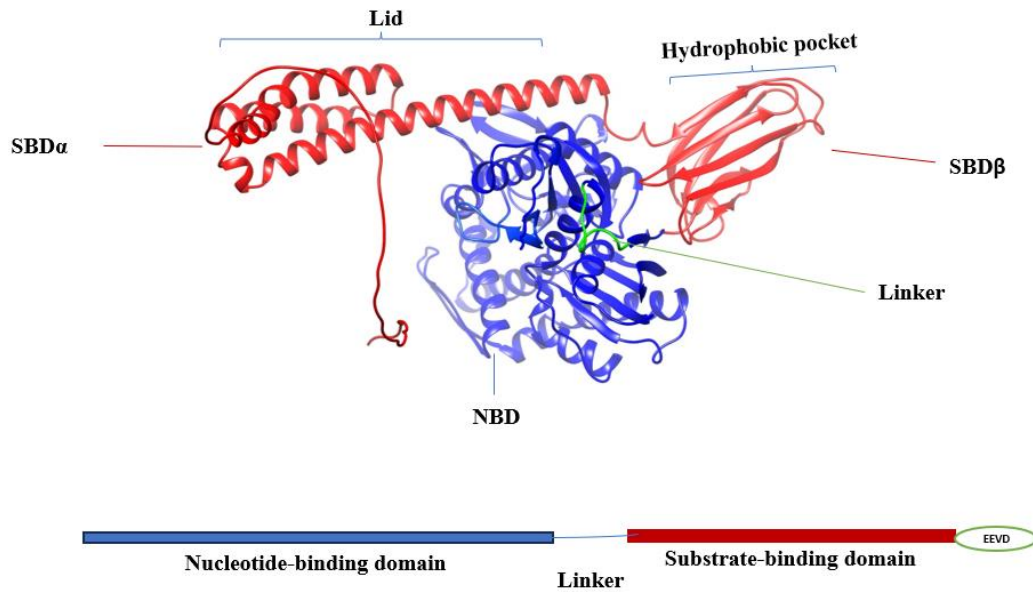


Figure 1.8: The structural features of PfHsp70-1

PfHsp70-1 structure consists of the ATPase domain linked to the SBD through a charged linker. The C-terminal consists of the EEVD motifs for interacting with co-chaperones. Representation of the three-dimensional structure of PfHsp70-1 protein PF3D7_0818900 (PlasmoDB: <https://plasmodb.org/>). Colors denotation: blue color (nucleotide-binding domain), red color (substrate-binding domain), green color (linker). The structure was drawn using the Chimera 1.15 tool (Figure was adapted from Shonhai *et al.*, 2007).

It has been demonstrated that when ATP binds to Hsp70, the protein undergoes enormous structural changes that force it to take on a closed conformation (Mayer and Bukau, 2005; Shonhai, 2021). ATP-bound Hsp70 has a low substrate affinity of 1–10 μM , whereas ADP-bound Hsp70 has a much higher affinity of 1–10 nM (Alderson *et al.*, 2016). Plasmodial Hsp70s tend to bind asparagine-rich peptides (Mabate *et al.*, 2018; Chakafana *et al.*, 2019). For example, PfHsp70-1 demonstrated affinity to the peptide substrate NRNNTG ($K_D = 10.2$ nM) and to GFRNNMYRF peptide ($K_D = 87.7$ nM) (Lebepe *et al.*, 2020).

Co-chaperones are essential partners of Hsp70, assisting in various aspects of its function, including substrate binding, ATP hydrolysis, and folding activity (Laufen *et al.*, 1999). In *P. falciparum*, co-chaperones help Hsp70 perform its roles effectively within the parasite's complex life cycle and stressful environments. Key co-chaperones of PfHsp70-1 include J-domain proteins (Hsp40), nucleotide exchange factors (NEFs), and others like Hsp90 and its co-chaperones (Botha *et al.*, 2007; Bracher and Verghese, 2015). Hsp40 proteins /J-domain proteins (JDPs) act as co-chaperones by stimulating the ATPase activity of Hsp70, which is crucial for its chaperone function. They help in substrate recognition and delivery to Hsp70

(Botha *et al.*, 2007). NEFs facilitate the exchange of ADP for ATP in Hsp70, a critical step that allows Hsp70 to undergo conformational changes necessary for its chaperone cycle (Bracher and Verghese, 2015). The Hsp70-Hsp90 organizing protein (Hop) acts as a scaffold, facilitating the transfer of client proteins from Hsp70 to Hsp90 (Blatch *et al.*, 1999; Gitau *et al.*, 2012).

1.5.2 Hsp90-Hsp70 organizing protein (Hop) mediates Hsp70 and Hsp90 function

Hop, also known as Sti1 (Stress-inducible protein 1), is a co-chaperone that mediates the interaction between Hsp70 and Hsp90, facilitating the transfer of client proteins between these two chaperones (Blatch *et al.*, 1999; Bhattacharya *et al.*, 2020). In *P. falciparum*, Hop (PfHop) plays a crucial role in the maturation and stabilization of various proteins essential for the parasite's survival and pathogenicity. The coordinated action of Hop to facilitate the partnership of Hsp70 and Hsp90 ensures the proper folding and stabilization of key parasitic proteins, which is crucial under the stress conditions encountered in the human host (Acharya *et al.*, 2007). Hop is involved in the parasite's response to heat shock and other environmental stresses, aiding in the protection and recovery of essential proteins during febrile episodes in malaria patients (Gitau *et al.*, 2012). The interaction between Hsp70 and Hsp90, mediated by Hop, is crucial for the development of *P. falciparum* during different stages of its lifecycle, including the erythrocytic stage, where the parasite undergoes rapid growth and replication (Pavithra *et al.*, 2007).

Hop is found in the cytosol under normal growth conditions but translocates to the nucleus under stress (Daniel *et al.*, 2008). Hop consists of three tetratricopeptide repeat TPR1, TPR2A, and TPR2B domains (Figure 1.9). TPR domains are essential for interaction with Hsp70 and Hsp90. Hsp70 and Hsp90 EEVD/MEEVD motifs are necessary for the interaction with Hop. PfHop binds the EEVD motif of PfHsp70-1 through TPR1 (Gitau *et al.*, 2012; Zininga *et al.*, 2015b) while the TPR2A subdomain interacts with Hsp90 (Onuoha *et al.*, 2008). The TPR2B subdomain of Hop strongly binds to Hsp70 adjacent to the TPR2A (bound to Hsp90), handing over the substrate (Röhl *et al.*, 2015; Bhattacharya *et al.*, 2020).

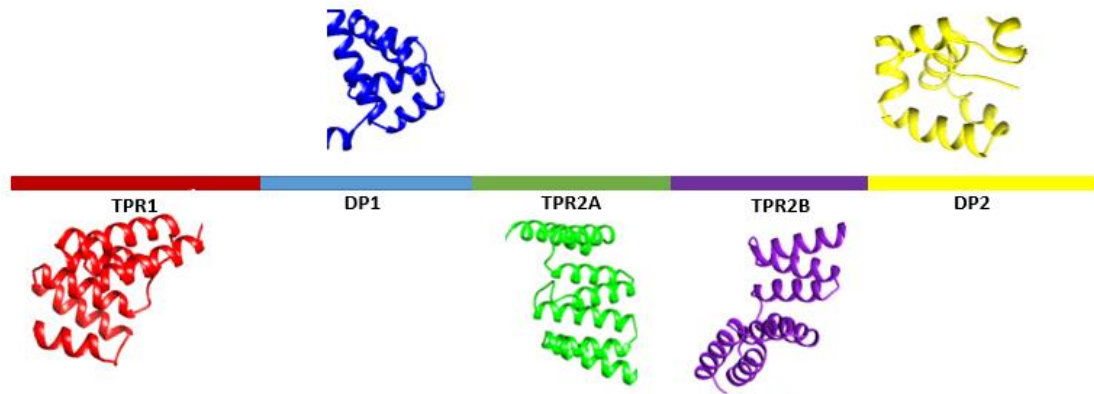


Figure 1.9: Structural features of PfHop

Three-dimensional model of PfHop PF3D7_1434300, showing the five distinct domains (TPR1, TPR2A, TPR2B, DP1, and DP2). The sequence was obtained from the PlasmoDB (<https://plasmodb.org/>) and rendered in PDB RCSB to obtain the CIF file. Discovery Studio (v21.1.0.20298) converted the CIF file to PDB before visualization using the Chimera 1.15 tool. (Adapted from Schmid *et al.*, 2012).

PfHop homolog (PfHop, PF3D7_1434300) is heat-inducible (Gitau *et al.*, 2012). PfHop consists of TPR1, DP1, TPR2A, TPR2B, and DP2 subdomains, of which the TPR1 is connected to TPR2A-TPR2B via a flexible linker DP1 (Figure 1.9; Schmid *et al.*, 2012). PfHop associates with PfHsp70-1 in a nucleotide-dependent manner, and the interaction is facilitated by ADP rather than ATP, suggesting that the low-affinity state of Hsp70 (ATP state) enables the release of the substrate to PfHsp90 (Zininga *et al.*, 2015).

PfHop represents a potential target for antimalarial drugs due to its central role in the chaperone network. Inhibitors that disrupt the interaction between PfHsp70 and PfHsp90 could impair protein folding and function in the parasite, leading to its death (Lackie *et al.*, 2017). Targeting PfHop in combination with other chaperones that it cooperates with might enhance the therapeutic efficacy against malaria by simultaneously disrupting multiple pathways essential for parasite survival (Silva *et al.*, 2020).

1.5.3 The R2TP complex cooperates with heat shock protein 90

Heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone from bacteria to humans. It is essential for the proper folding and maintenance of its client proteins. Hsp90 client proteins are found in both prokaryotes and eukaryotes except for Archae (McClellan *et al.*, 2007; Reviewed by Wei *et al.*, 2024). Over 600 Hsp90 interactors have been identified in yeast, spanning diverse families of cellular proteins, which include transcription factors, steroid hormone receptors, and protein kinases (Zhao *et al.*, 2005, Reviewed by Schopf *et al.*, 2017).

P. falciparum Hsp90 (PfHsp90) is an essential molecular chaperone involved in the folding, maturation, and stabilization of many important proteins, often called client proteins. The interaction between PfHsp90 and its client proteins is crucial for the parasite's survival, especially under stressful conditions such as those encountered during the febrile response in malaria-infected individuals. PfHsp90 assists in the proper folding and maturation of newly synthesized and stress-denatured proteins. This function is vital for maintaining the structural integrity and function of numerous client proteins within the parasite (Shonhai *et al.*, 2011). PfHsp90 is upregulated in response to heat shock and other stress conditions, helping the parasite cope with the human host's hostile environment during malaria infection (Pavithra *et al.*, 2007).

PfHsp90 interacts with several kinases and signaling proteins, stabilizing them and ensuring their proper function (Banumathy *et al.*, 2003). These proteins are essential for the parasite's intracellular signaling pathways and adaptation to environmental changes (Banumathy *et al.*, 2003). Many proteins exported by *P. falciparum* to the host RBC cytoplasm are PfHsp90 clients. These include virulence factors like the knob-associated histidine-rich protein (KAHRP) and members of the *P. falciparum* RBC membrane protein 1 (PfEMP1) family (Acharya *et al.*, 2007). Critical metabolic enzymes in *P. falciparum* rely on PfHsp90 for stability and functionality. This includes enzymes involved in glycolysis and nucleotide synthesis, which are crucial for the rapid proliferation of the parasite (Picard *et al.*, 2002).

Four Hsp90 proteins have been identified in the *P. falciparum* genome: mitochondrial PfTRAP1 (PF3D7_1118200), endoplasmic reticulum PfGrp94 (PF3D7_1222300), and two cytosolic PfHsp90 (PF3D7_0708400 and PF3D7_1443900) (Pallavi *et al.*, 2010). It has been proposed that PfHsp90 expression levels account for a major portion of the entire cellular proteome (Acharya *et al.*, 2007). Among the two PfHsp90 isoforms in the cytosol, only PF3D7_0708400, also known as PfHsp90 α , has MEEVD motifs. PfHsp90 α is expressed during the blood stage of the parasite life cycle and is induced by stress. Both the pre-erythrocytic and erythrocytic stages have been shown to require PfHsp90 (Pallavi *et al.*, 2010).

Targeting *P. falciparum* Hsp90 with specific inhibitors could disrupt the function of its client proteins, leading to the accumulation of misfolded proteins and parasite death. Hsp90 inhibitors, such as geldanamycin and its derivatives, have shown efficacy against *P. falciparum* (Kumar *et al.*, 2018). Combining Hsp90 inhibitors with other antimalarial drugs can enhance

therapeutic efficacy by simultaneously targeting multiple pathways essential for parasite survival (Kumar *et al.*, 2018).

1.5.4.1 Structural features of *P. falciparum* Hsp90

PfHsp90 forms a homodimeric biologically functional unit composed of three distinct regions connected by a flexible linker. Each protomer (monomer unit) contains a highly conserved N-terminal domain (NTD) responsible for nucleotide binding, a middle domain (MD) important for client recognition and ATP hydrolysis, and a C-terminal domain (CTD), which is the primary site responsible for dimerization (Figure 1.10.1; Pearl and Prodromou, 2006; Taipale *et al.*, 2010). Additionally, the CTD contains a conserved ‘MEEVD’ motif that is important for interactions with the tetratricopeptide repeat domain (TPR) class of co-chaperone proteins, which regulate Hsp90 protein activity (Figure 1.10.1; Banumathy *et al.*, 2003).

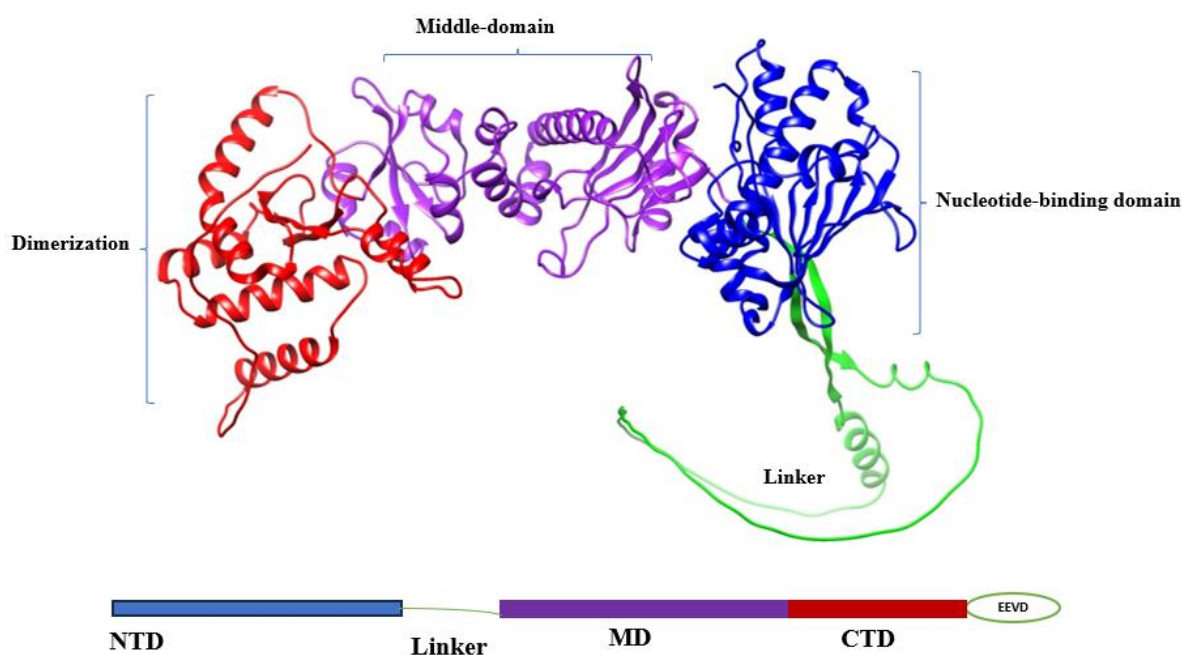


Figure 1.10.1: The structural features of PfHsp90

PfHsp90 three-dimensional model of PfHsp90 protein PF3D7_0708400 (monomer unit) and domains organization N- terminal (ATPase domain) ATP hydrolysis, the linker, M domain (substrate-binding domain), dimerization domain (self-association), C-terminal (MEEVD motifs) (Meyer *et al.*, 2003). Colors denotation: blue colors (NTD), green (Linker), purple (MD), red color (CTD). The protein sequence was obtained from PlasmoDB (<https://plasmodb.org>), and Discovery Studio (v21.1.0.20298) was used to convert the CIF file to PDB before visualization using the Chimera 1.15 tool.

The NTD contains a deep ATP/ADP binding pocket evolutionarily conserved amongst the GHKL (gyrase, Hsp90, Histidine kinases, Mutl) ATPase superfamily (Meyer *et al.*, 2003). The

binding pocket is composed of multiple α -helices and a β -sheet flanking the bound ATP molecule (Prodromou *et al.*, 1997; Prodromou and Bjorklund, 2022). Intramolecular recruitment of the MD on each protomer brings critical residues into contact, forming the required split ATPase active site necessary for ATP hydrolysis (Ali *et al.*, 2006). The N-terminal region has a lid that extends to the nucleotide-binding pocket in the ATP-bound state and holds the nucleotide in place but remains in an open conformation upon ADP binding (Figure 1.10.2; Ali *et al.*, 2006). Nucleotide binding and hydrolysis are directly coupled to large structural rearrangements in Hsp90 that are regulated through interaction with clients and co-chaperone protein (Lavery *et al.*, 2014).

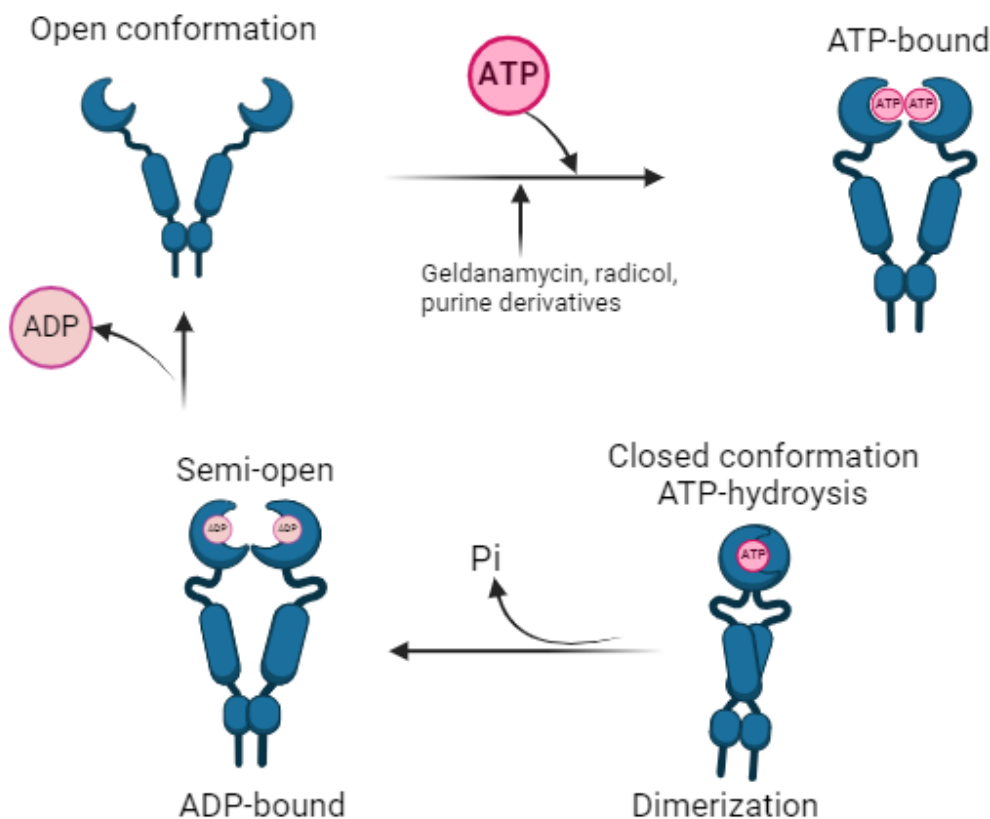


Figure 1.10.2: Schematic of the ATPase cycle of Hsp90

Hsp90 homodimer initially adopts an open V-shape conformation, binding of ATP to the N-terminal ATPase domain induces conformational changes where the lid N-terminal closes and ATP cradles in the nucleotide-binding pocket. This induces dimerization of the N-terminal domains of each protomer, followed by closure of Hsp90 and recruitment of the M-domain for ATP hydrolysis. The dimers dissociate into a semi-open intermediates state with ADP bound, and the release of ADP dissociates the N-termini to allow repetition of the ATP cycle. Common Hsp90 inhibitors bind to the N-terminus of Hsp90 and compete with ATP for binding. The structure was drawn using Biorender (www.biorender.com).

Apo-Hsp90 presents an 'open' conformation where the two protomers form a V-shaped dimer (Vaughan *et al.*, 2010). Nucleotide-binding induces an intermediate state where the N-terminal

lid cradles ATP within the nucleotide-binding pocket, this results in a second repositioning of the N-terminal region favoring its dimerization and recruitment of MD for ATP hydrolysis through a conserved arginine (R380 in yeast) that contacts the γ -phosphate of ATP (Cunningham *et al.*, 2012).

Finally, ATP is hydrolyzed, and Hsp90 reverts to the ‘open’ conformation where the N-terminal domains dissociate, allowing for the repetition of the ATP hydrolysis cycle (Figure 1.10.2) and structural rearrangements in Hsp90 during client refolding are influenced and regulated by a diverse set of co-chaperones (Zuehlke and Johnson, 2010). The co-chaperones have varying effects on ATPase activity and conformational rearrangements in Hsp90 (Dutta *et al.*, 2020). Making Hsp90 folding machinery pattern unique for every protein folding process that involves Hsp90. Post-translational modifications of Hsp90, including acetylation, nitrosylation, phosphorylation, and methylation, have been reviewed (Buchner and Li, 2013).

1.5.4.2 Functional versatility of Hsp90

Hsp90 is essential in *P. falciparum* (Banumathy *et al.*, 2003). Hsp90 cooperates with several co-chaperones (Table 1.2) to drive its diverse cellular processes. Hop is a scaffold protein that facilitates the transfer of client proteins from Hsp70 to Hsp90 (Bhattacharya and Picard, 2021). In *P. falciparum*, as in other organisms, these co-chaperones play an important role in the maturation and stabilization of PfHsp90 client proteins. Hsp90 client proteins are known to be involved in a wide range of physiological events, including cell cycle progression, signal transduction, and transcription regulation. The PfHsp90 machinery is required for clients to correct folding, activation, transport, and degradation (Dutta *et al.*, 2020). Hop domains enable simultaneous binding to Hsp70 and Hsp90, promoting the formation of a functional chaperone complex (Hatherley *et al.*, 2015; Makumire *et al.*, 2020).

p23 stabilizes the ATP-bound state of Hsp90, which is crucial for the chaperoning activity. It also prevents the premature release of client proteins, ensuring proper maturation (Bohen *et al.*, 1995). CDC37 is specifically involved in the maturation of kinase clients. It binds to both Hsp90 and kinase clients, stabilizing the complex and ensuring proper folding and activation of kinases (Table 1.2; Dutta *et al.*, 2022). Aha1 is a co-chaperone that stimulates the ATPase activity of Hsp90, which is essential for the chaperone cycle (Panaretou *et al.*, 2002). This activation leads to conformational changes necessary for client protein processing (Panaretou *et al.*, 2002). FKBP51 and FKBP52 are immunophilins that bind to Hsp90 and regulate its

function. The former and latter have peptidyl-prolyl isomerase activity, which assists in the proper folding of client proteins (Wochnik *et al.*, 2004).

Table 1.2: Hsp90 co-chaperones

Co-chaperone	Function	co-chaperone motif binding Hsp90	Interaction site in Hsp90 domains	Effect on Hsp90 ATPase activity	Reference
Hop/Sti1	Mediates interaction between Hsp90 and Hsp70	TPR2A, TPR2B	N, M, C	Inhibition	Schwarz <i>et al.</i> , 2023
Cdc37	Mediates activation of protein kinase substrates	M, C	N	Inhibition	Eckl <i>et al.</i> , 2016
SGT1/	Client adaptor	CS domain	N	No effect	Kadota <i>et al.</i> , 2010
Pih1	Protein interacting with Hsp90 (R2TP complex member)	CS domain	N	Unknown	Zhao <i>et al.</i> , 2005
TAH1	Interaction with the R2TP complex	TPR	C	Weak inhibition	Morgan <i>et al.</i> , 2015
Aha1	Stimulate ATPase activity	N, C	N, M	Acceleration	Huai <i>et al.</i> , 2005
Ppt1/PP5	Clients' activation	TPR1, TPR2, TPR3	C	No effect	Bellosta <i>et al.</i> , 2005
FKBP51/FKBP52	Peptidyl propyl isomerase	TPR	C	No effect	Wang <i>et al.</i> , 2024
P23/Sba1	Stabilizes, closes, and clamps substrate	N	N, M	Inhibition	Noddings <i>et al.</i> , 2022

Representation of some of the known Hsp90 chaperones

1.6 The R2TP complex modulates the function of Hsp90 in assembling complexes

The R2TP complex plays a crucial role in supporting the function of Hsp90 by assisting in the formation, maintenance, and control of key macromolecular complexes (Boulon *et al.*, 2012). The coordinated actions of its components ensure that Hsp90 can efficiently perform its role as a molecular chaperone, contributing to cellular homeostasis and offering potential

therapeutic targets for various diseases (Houry, 2022). The complex helps in the maturation of various multiprotein complexes, including the RNA Polymerase II subunits, which are responsible for transcribing mRNA (Table 1.4) in eukaryotic cells. RPAP3, a component of the R2TP complex, interacts with RNA Pol II and stabilizes its structure (Puri *et al.*, 2021).

The complex of snoRNPs is essential for the chemical modification and processing of ribosomal RNA. The R2TP assists in facilitating its component assembly by stabilizing the snoRNA (Figure 1.10) and its associated proteins, ensuring correct folding and functionality (Houry, 2022). The most conserved protein in the spliceosome is pre-mRNA splicing factor 8 (PRPF8), which forms part of the U5 small nuclear ribonucleoprotein particle (U5 snRNP) (Galej *et al.*, 2013). PRPF8 has been reported to associate in a complex with EFTUD2 and SNRNP200 as part of the spliceosome in the nucleus (Serna *et al.*, 2021). The assembly and biogenesis of the U5 snRNP and PRPF8 have been reported to depend on the R2TP/Hsp90 machinery complex (Malinova *et al.*, 2017). The PIKK (PI3-kinase-like kinase) family includes key regulators of cellular responses to DNA damage and nutrient availability, such as mTOR, ATR, and DNA-PK (Figure 1.11). The R2TP complex, through its interactions with Hsp90, aids in the assembly and stability of PIKK proteins, ensuring their proper localization and functionality in cellular signaling pathways (Horejsí *et al.*, 2010).

The telomerase complex is essential for the maintenance of telomere length and genome stability. The R2TP complex, with Hsp90, facilitates the proper assembly and maturation of the telomerase complex (Figure 1.11), ensuring its activity and stability in the cell (Venteicher *et al.*, 2008). In addition, the R2TP complex assists in the assembly of the Fanconi Anemia (FA) core complex by stabilizing its components and facilitating their interactions, which is critical for the complex's function in DNA repair pathways (Table 1.4; Rajendra *et al.*, 2014). FA is a rare autosomal recessive genetic disease that leads to impaired response to DNA damage (Rajendra *et al.*, 2014). The FA core complex is involved in DNA repair, particularly in response to DNA interstrand crosslinks (Rajendra *et al.*, 2014).

Characterization of the structure-function features of the R2TP complex of *Plasmodium falciparum*

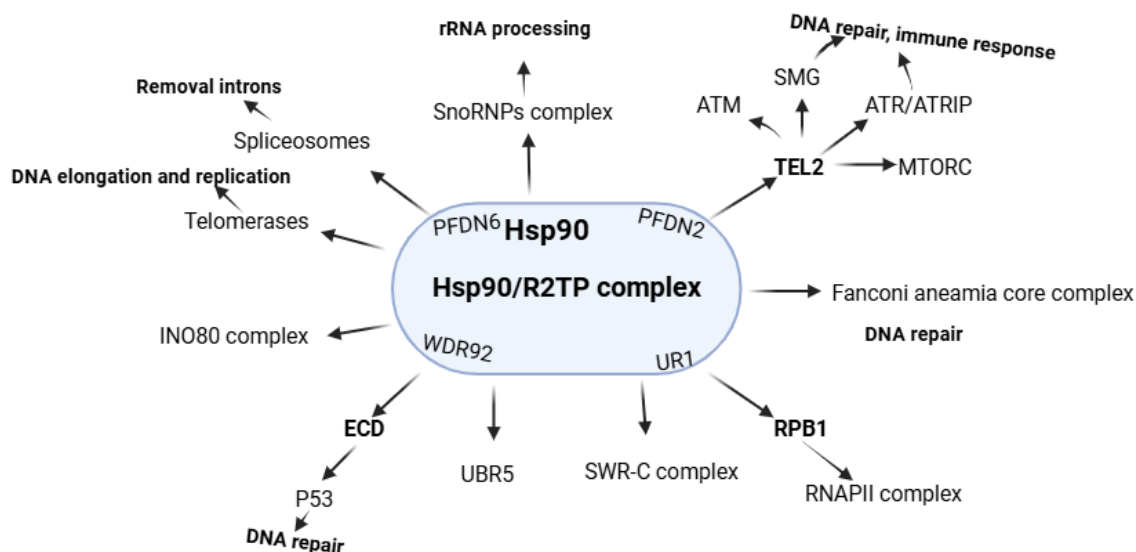


Figure 1.11: The R2TP complex is a scaffold of Hsp90 in a complex assembling

Diagrammatic representation of complex and non-complex clients of Hsp90 that are mediated by the R2TP complex.

Table 1.4: R2TP/Hsp90 complex clients

Client name	Function	Reference
SMG	DNA repair, transcription, translation, splicing, histone mRNA processing	Yamashita <i>et al.</i> , 2001
MTOR	Cellular metabolism, immune response, proliferation	Szwed <i>et al.</i> , 2021
ATR/ATM	DNA repair	Yang <i>et al.</i> , 2006
PIKKs	Stress-induced signalling pathways, cell growth, proliferation	Angira <i>et al.</i> , 2020
Telomerases	DNA replication and elongation	Jafri <i>et al.</i> , 2016
Spliceosomes	Removes introns	Pedrotti <i>et al.</i> , 2014
RNAPII	Transcribe coding and non-coding genes	Schier <i>et al.</i> , 2020
INO80 complex/ SWR complex	DNA replication, DNA repair	Conaway and Conaway, 2009
snoRNA	Cleavage and processing of ribosomal RNA	Henras <i>et al.</i> , 2015

Tip60 complex	Chromatin remodelling, gene transcription, DNA repair	Ikura <i>et al.</i> , 2000
UBR5	DNA repair, transcription, and apoptosis	shearer <i>et al.</i> , 2015
P53	DNA repair	Ozaki and Nakagawara, 2011

Definition of terms in table 1.4: UBR5 (E3 ubiquitin-protein ligase which is a nucleus cellular component), snoRNAs (small nucleolar ribonucleoprotein complex), SWR-C/SWR1 and INO80 are multi-subunit complexes that catalyze the admission and deletion of histone variant, TEL2 (telomere length regulation protein), SMG 1 (suppressor with morphological effect on genitalia 1) and ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3 related)), DNA-PK (DNA-dependent-Protein Kinase).

The R2TP/Hsp90 complex is critical in assembling, stabilizing, and controlling important multiprotein complexes. By serving as a scaffold and providing chaperone activity, this complex, in conjunction with Hsp90, ensures the correct operation and stability of these complexes (Henras *et al.*, 2015). These complexes are indispensable for diverse cellular processes such as transcription, RNA processing, DNA repair, and signal transduction (Houry, 2022). Understanding these interactions offers valuable insights into maintaining cellular equilibrium and identifying potential therapeutic targets for diseases characterized by disruptions in these processes.

1.7 Diverse functions of the R2TP/Hsp90 complex

The R2TP complex involvement in cancer is linked to its role in regulating processes essential for cancer cell survival and proliferation. The complex is involved in the stabilization and activity of several oncogenic proteins and pathways, such as mTOR (Figure 1.12), which is crucial for cell growth and metabolism (Yang *et al.*, 2024). mTOR signalling is responsible for cell growth and proliferation, cell survival, metabolism, and protein synthesis (Tan *et al.*, 2019). mTOR consists of two distinct complexes, referred to as mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2), that are both implicated in multiple diseases associated with cancer development and metabolic alterations (Figure 1.12; Tan *et al.*, 2019). Driscoll et al. in 2016 reported that mTORC2 signalling drives the development and progression of pancreatic cancer.

R2TP assists in the assembly of RNA polymerase II, which is essential for the transcription of genes involved in cell cycle progression and survival. Abnormal transcriptional regulation is a hallmark of cancer (Boulon *et al.*, 2010). The R2TP complex is critical for the biogenesis of small nucleolar RNPs (snoRNPs), which are involved in ribosome biogenesis. Cancer cells

often show increased ribosome biogenesis to meet their high protein synthesis demands (Bizarro *et al.*, 2014). The R2TP complex is implicated in the stabilization of telomerase, an enzyme that maintains telomere length, enabling the unlimited replicative potential of cancer cells (Mao *et al.*, 2024). The R2TP/PFDL complex plays a crucial role in the assembly of complexes of snoRNPs, nuclear RNA polymerases, ZNHIT2, and the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK)-containing subunits (Zhao *et al.*, 2008; Cloutier *et al.*, 2017). The abrogation of the expression of ZNHIT2 and RUVBL2 expression adversely impacted the protein composition of the U5 snRNP, suggesting a function for these proteins in the biogenesis of ribonucleoprotein (Cloutier *et al.*, 2017).

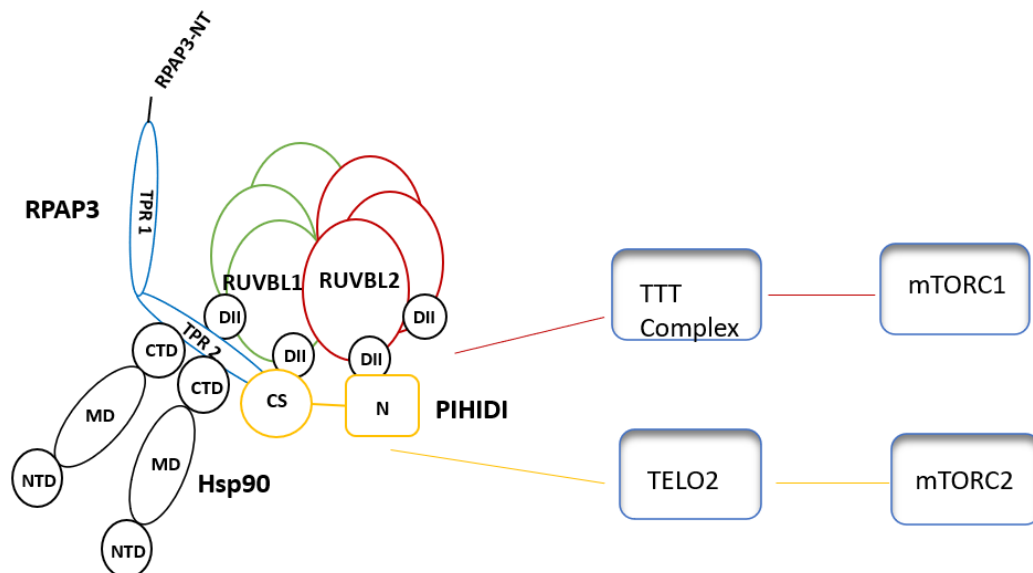


Figure 1.12: Association of the R2TP/Hsp90 complex with the mTOR complex

Direct association of RUVBL1 and RUVBL2 with mTORC1 through the TTT complex had been previously reported (Kim *et al.*, 2013). Furthermore, the association of PIH1D1 with mTORC2 has been reported. (Adapted from Takai *et al.*, 2010).

Both RUVBL1 and RUVBL2 reportedly occur in complex with TTT complex (TELO2-TTI1-TTI2) forming (RUVBL1-RUVBL2-TELO2-TTI1-TTI2) complex (Figure 1.12) (Kim *et al.*, 2013). They are further implicated in mTORC1 dimerization and lysosomal localization (Kim *et al.*, 2013). The stability of the mTOR protein family is regulated by the R2TP/pre-foldin complex through a linker, TELO2. Research suggested that high levels of the R2TP complex might stabilize overexpressed mTOR proteins and contribute to the malignancy of cancer (Kakihara *et al.*, 2019). RUVBL1 and RUVBL2 have been shown to facilitate proteostasis through their capability to enhance aggresome formation and disaggregation of amyloid fibrils

(Zaarur *et al.*, 2015). RUVBL1 and RUVBL2 are further implicated in facilitating the branch migration of holiday junctions, which occurs during homologous recombination (Matias *et al.*, 2006). The interaction of RUVBL1 with γ -tubulin is thought to influence mitosis (Gartner *et al.*, 2003; Ducat *et al.*, 2008).

The most aggressive form of mature B-lymphocyte cancer is diffuse large B-cell lymphoma (DLBCL), commonly found in non-Hodgkin's lymphoma in adults with heterogeneous genetic disorders (Lone *et al.*, 2022). This disorder is perpetuated by B-cells of the germinal centre (GC) and contributes to cancer development attributes such as high cell growth rate and genomic instability (Hanahan, 2012). An increase in cell proliferation makes normal B-germinal center (GCB) cells more susceptible to cancer (Monti *et al.*, 2012). Deregulation of tumor suppressor pathways, including p53 and pRb, is responsible for rapid proliferation and transformation (Giacinti and Giordano 2006). In addition, the function of Rb and p53 is activated by the R2TP complex, suggesting that any alterations in any of the members of the complex can drive cells to malignancy (Lone *et al.*, 2022).

R2TP complex functions as a regulator and suppressor of paramyxoviruses RNA synthesis (Kato *et al.*, 2019). Paramyxoviruses encode six or seven structural proteins and contain control regions at both genome terminals (Li and Pattnaik 1999). The viral genome has been found to associate with ribonucleoprotein (RNP) complexes, which are R2TP complex clients (Lamba *et al.*, 2006). Oncolytic viruses are considered possible anticancer therapeutic agents. One of the prospective oncolytic agents is paramyxovirus (Cattaneo, 2010). It has been shown that the R2TP complex interacted with the paramyxovirus polymerase L protein, and silencing the R2TP complex pathway led to uncontrolled upregulation of mumps virus (MuV) gene transcription but without replication of the host genome (Kato *et al.*, 2019). In addition, the R2TP complex is essential not only in MuV replication but also in regulating host innate immune responses (Kato *et al.*, 2019).

1.8 The role of the R2TP complex in parasites

The R2TP complex has vital roles in various processes within infectious agents. This includes assisting in protein assembly and stability, which aids in the proper folding, assembly, and stability of essential protein complexes crucial for the pathogen's survival and virulence (Lynham and Houry, 2022). The ATPases Rvb1 and Rvb2 provide the energy necessary for these assembly processes through their ATPase activity (Lynham and Houry, 2022). Moreover, the complex regulates gene expression by participating in the biogenesis and assembly of RNA

polymerase II, which is essential for gene transcription. This affects the expression of virulence factors and other important genes in infectious agents (Kato *et al.*, 2019).

The R2TP complex is involved in the stress response of infectious agents, working with HSP90 to ensure the proper function of stress response proteins and complexes (von Morgen *et al.*, 2015). Additionally, it plays a role in ribosome biogenesis, assisting in the proper assembly of ribosomes essential for protein synthesis, which allows pathogens to efficiently produce the proteins needed for their growth and replication (Yu *et al.*, 2019; Seraphim *et al.*, 2022). The complex interacts with nascent protein complexes, helping them attain their functional conformations and integrating them into larger cellular structures. The coordination between Rvb1/Rvb2 and other subunits ensures that the assembly process is tightly regulated and efficient (Martino *et al.*, 2018). Furthermore, the complex contributes to various signaling pathways by aiding in the assembly of key signaling complexes, which can influence the pathogen's ability to respond to environmental cues and host defenses (Kakihara and Saeki, 2014). The R2TP complex also regulates nuclear hormone receptors, influencing the transcriptional response to hormones and other signalling molecules (Huen *et al.*, 2010).

In the context of *Plasmodium falciparum*, the R2TP complex may play a crucial role in the development of the malaria parasite. It could be involved in assembling protein complexes necessary for the parasite's lifecycle and may also contribute to drug resistance by stabilizing and assembling complexes involved in stress responses (Sen *et al.*, 2018; reviewed by Luthuli and Shonhai, 2023). The characterization of the PfR2TP complex may contribute to the understanding of the parasite's biology and the design of antimalarial drugs. Furthermore, the complex may influence the expression of proteins that help the parasite evade the host immune system.

1.9 Association of Hsp70 with the R2TP complex

Hsp70 is a molecular chaperone that is involved in several processes, including protein folding, protein unfolding, assembly and disassembly of protein units, protein translocation, signal transduction, and DNA replication (Mayer and Bukau 2005; Shonhai 2021). Hsp70 function is further regulated by various post-translational events such as cellular stress (heat stress, toxic and oxidative stress, heavy metals, and nutrient deficiency) (Rohland *et al.*, 2022). The chaperone is associated with proteins from initial folding stages to degradation, making members of the Hsp70 family central mediators of cellular proteostasis (Chakafana and Shonhai 2019). Mutations in genes encoding components of the Hsp70 system are linked to

several human diseases, including Parkinson's disease, diabetes mellitus, colorectal cancer, and cardiomyopathy (Nikita *et al.*, 2020).

Although Hsp70 and Hsp90 function independently, they cooperate to fold specific clients such as transcription factors, kinases, and nuclear receptors (Shonhai *et al.*, 2008; Alvira *et al.*, 2014). Hsp90 function is regulated by more than 20 co-chaperones (Pearl and Prodromou, 2006). The co-chaperones bind Hsp90 at various stages of its functional cycle. STI1/Hop (Blatch and Lässle, 1999) is one of its upstream co-chaperones whose function is to clamp Hsp90, thus maintaining its open conformation to allow for substrate transfer from Hsp70 (Moll *et al.*, 2022). Downstream co-chaperones include p23, which maintains the closed conformation of Hsp90, thus allowing clients to find their full fold (Ali *et al.*, 2006). R2TP proteins also play an important role in regulating the ATPase activity of Hsp90, thus promoting client exchange between Hsp70 and Hsp90. In addition, the R2TP complex brings Hsp90 in contact with its assembled clients (Figure 1.13; Zhao *et al.*, 2008).

Cytosol-localized Hsp70 and Hsp90 have a C-terminally located EEVD motif that interacts with Hop through its tetratricopeptide repeat (TPR) domains, TPR1 and TPR2A, respectively (Assimon *et al.*, 2019). Hop possesses nine tetratricopeptide motifs forming three TPR domains named TPR1, TPR2A, and TPR2B, and binds Hsp70 in the TPR1 and TPR2B domains, while the TPR2A domain preferentially binds Hsp90. Spaghetti (Spag) is a co-chaperone found in *Drosophila melanogaster* that binds to both Hsp90 and Hsp70 and has been shown to stimulate Hsp70 activity, emphasizing the role of Hsp70 in the R2TP complex (Benbahouche *et al.*, 2014).

RPAP3 contains two TPR domains that are proposed to bind not only Hsp90 but also Hsp70 (Henri *et al.*, 2018). There's a suggestion that Hsp70 binds to RPAP3 with medium affinity, while the RPAP3-Hsp90 complex is more favored (Henri *et al.*, 2018). Nonetheless, the potential interaction of RPAP3 with both Hsp70 and Hsp90 suggests that this protein acts as a bridge for client exchange between the two chaperones (Figure 1.13; Henri *et al.*, 2018).

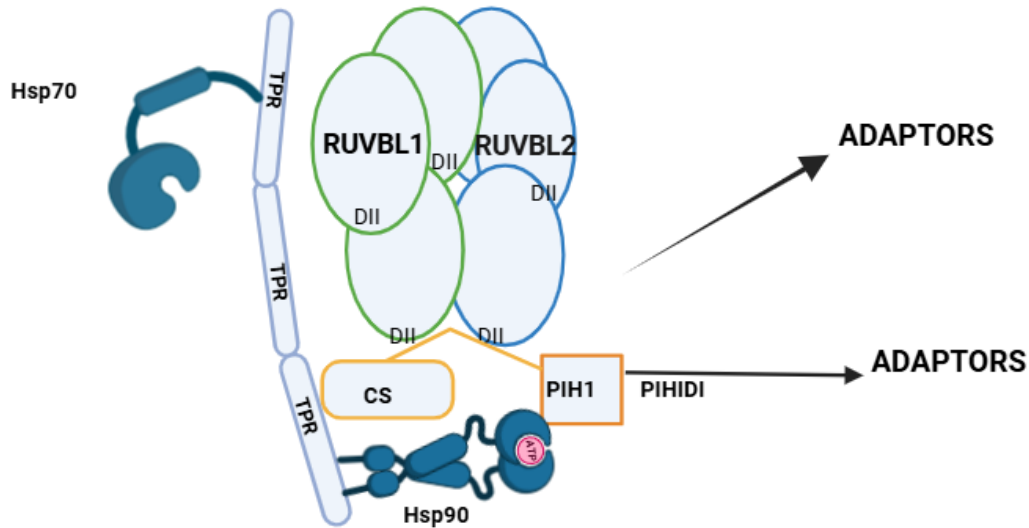


Figure 1.13: Association of the R2TP complex with Hsp90 and Hsp70

NBD (nucleotide-binding domain); NTD (N-terminal domain); SBD (substrate-binding domain); CTD (C-terminal domain); EEVD (motif found in Hsp70 SBD, and Hsp90 CTD); DII (domain two). (The figure was adapted from Henri *et al.*, 2018).

The interaction of TPR domain-containing co-chaperones with Hsp90 and Hsp70 is well established. FKBP8, an immunophilin belonging to the FK506-binding protein family, has one TPR domain and binds more tightly to Hsp90 than Hsp70 (Blundell *et al.*, 2017). A recent study on the regulation of prion formation demonstrated the vital interaction between Hsp70 and Tah1 (RPAP3 homology) in regulating prion propagation. Deletion of *Tah1* was shown to improve the production of prions (Puri *et al.*, 2021). Prion formation relies on chaperone activity to propagate stably *in vivo* (Ramanova and Chernoff, 2009). Hsp70 and its functional partner Hsp104 facilitate the fragmentation of growing amyloid fibrils, thereby exposing more fibril-growing ends and generating more infectious prion seeds (Masison *et al.*, 2009).

1.10 RUVBL1 and RUVBL2 AAA+ ATPase are versatile scaffolds for multiple complexes

RUVBL1 and RUVBL2 are highly similar AAA+ proteins with about 40% sequence identity (Qiu *et al.*, 1998). Their names reflect the species they originate from (Table 1.5). AAA+ proteins typically form hexameric or dodecameric ring structures and are characterized by the AAA+ domain, which contains the highly conserved Walker A and Walker B motifs that are implicated in nucleotide binding and hydrolysis (Walker *et al.*, 1982). They also contain sensors I and II and the arginine finger found in other AAA+ proteins. Human RUVBL1 and RUVBL2 share 43% sequence identity and have a common domain organization (Dauden *et al.*, 2021).

Table 1.5: RUVBL1 and RUVBL2 members nomenclature and their species of origin.

Protein	Alternative name	Origin	Localization	Reference
Pontin	Rvb1	<i>Saccharomyces cerevisiae</i>	Cytosol and nucleus	Zhao <i>et al.</i> , 2005
	RUVBL1	<i>Homo sapiens</i>	Cytosol and nucleus	Qiu <i>et al.</i> , 1998
	TIP48	<i>Saccharomyces cerevisiae</i>	Nucleus	Dauden <i>et al.</i> , 2021
	ECP54	<i>Caenorhabditis elegans</i>	Cytosol and nucleus	Salzer <i>et al.</i> , 1999
	Tip49	<i>Drosophila melanogaster</i>	Nuclear	Bellosta <i>et al.</i> , 2005
Reptin	Rvb2	<i>Saccharomyces cerevisiae</i>	Cytosol and nucleus	Zhao <i>et al.</i> , 2005
	RUVBL2	<i>Homo sapiens</i>	Cytosol and nucleus	Qiu <i>et al.</i> , 1998
	TIH2	<i>Saccharomyces cerevisiae</i>	Nucleus	Lim <i>et al.</i> , 2000
	ECP51	<i>Caenorhabditis elegans</i>	Cytosol and nucleus	Salzer <i>et al.</i> , 1999
	TIP49B	<i>Saccharomyces cerevisiae</i>	Nucleus	Lim <i>et al.</i> , 2000; Dauden <i>et al.</i> , 2021

The insertion domain found in RUVBL1 and RUVBL2 is similar to the helical domain in the AAA+ domain of the unfoldase chaperone, HslU/CipY (Bochtler *et al.*, 2000; Wang *et al.*, 2001). RUVBL1 and RUVBL2 come together to form a hexameric ring and, along with their functional partners Tah1 and Pih1, create a dodecameric complex (Tsaneva *et al.*, 1993; Matias *et al.*, 2006). RUVBL1 and RUVBL2 are conserved across all species, from humans to bacteria (Matias *et al.*, 2006; Houry *et al.*, 2018). Both RUVBL1 and RUVBL2 exhibit ATPase and helicase activities (Nano and Houry, 2013). In eukaryotic cells, RUVBL1 and RUVBL2 are expressed in low abundance compared to other components of several complexes with which

they associate. This suggests that they transiently interact with several complexes (Gallant 2007; Nano and Houry 2013).

Several studies have demonstrated that RUVBL1 and RUVBL2 are associated with various chromatin remodeling complexes, such as the INO80 complex in *Saccharomyces cerevisiae*, *Homo sapiens*, and *Drosophila melanogaster* (Shen *et al.*, 2000; Jónsson *et al.*, 2001; Jin *et al.*, 2005). In addition, the two proteins are thought to be involved in the formation of multi-subunit complexes, including those that are involved in processes that catalyze the deposition and removal of the histone variant, SWR-C complex in *S. cerevisiae* (Morrison and Shen, 2009) and *Homo sapiens* (Kobor *et al.*, 2004). In humans, RUVBL1 and RUVBL2 are implicated in the formation of the acetyltransferase complex (TiP60 complex), a key regulator of genome expression and stability (Kusch *et al.*, 2004; Cai *et al.*, 2005). The AAA+ subdomain of human RUVBL1 has a crystal structure with a central channel large enough to fit single-stranded DNA (ssDNA) but too small to accommodate double-helix DNA. This suggests that the hexamer is associated only with ssDNA (Matias *et al.*, 2006). RUVBL1 and RUVBL2 proteins have been shown to independently form part of the chromatin remodeling complexes INO80 (Shen *et al.*, 2000) and SWR-C complexes (Krogan *et al.*, 2003). Due to their roles in functional protein complex assembly, RUVBL1 and RUVBL2 are expressed in various organs such as the lungs, liver, and colon (Nano and Houry, 2013). Consequently, their functional deficiency results in apoptotic cell death and various diseases, including cancer (Rousseau, 2007; Nano and Houry, 2013).

The formation of complex structures within crowded cells relies on maintaining macromolecules in proper conformations and preventing them from forming non-productive associations until they are prepared to join the correct complex. RUVBL1 and RUVBL2 serve as both chaperones and modules for protein complex assembly. Their chaperone function is regulated by an insertion domain located between the Walker A and Walker B motifs (von Morgen *et al.*, 2015). RUVBL proteins are conserved across various species, including those causing infectious diseases. For instance, the malaria-causing agent *P. falciparum* expresses members of the R2TP family but is unique in expressing three RUVBL proteins and lacking an RPAP3/Tah1 homolog (Seraphim *et al.*, 2019). These findings suggest a unique role for these proteins in certain organisms, making them potential targets for selective drug design efforts. Yeast Rvb1/Rvb2 and human RUVBL1/RUVBL2 each have an AAA+ domain containing conserved motifs, such as Walker A, Walker B, sensor 1, arginine finger, and sensor 2. An insertion domain between the Walker A and Walker B motifs is proposed to bind

DNA/RNA (Matias *et al.*, 2006). The location of this insertion domain within the AAA+ domain resembles that of the helical domain within the AAAA+ domain of the unfoldase chaperone (unfolding and disentangling misfolded polypeptides) HsIU/CipY (Bochtier *et al.*, 2000; Wang *et al.*, 2001). However, currently, there is no molecular understanding of how nucleotide binding and hydrolysis affect the conformation of this domain or the complex.

1.10.1 The AAA+ protein's features

AAA+ proteins are molecular chaperones characterized by a highly conserved Phosphate Loop-Nucleoside Triphosphate (P-loop NTPase) (Erdmann *et al.*, 1991; Gates and Martins, 2020). The NTPase domain consists of an ATPase module of about 200-250 amino acids, which features an $\alpha\beta\alpha$ core. This structure is further divided into a P-loop (Walker A and Walker B). These domains are comprised of two helices arranged in a hairpin-like structure and are oriented in a left-handed manner (Seraphim and Houry, 2020). Members of the AAA+ superfamily are divided into two groups: the kinase group (KG) and the additional strand catalytic E (ASEC) group (Seraphim and Houry, 2020). AAA+ members belonging to the ASCE subgroup are conserved across all species, from bacteria to humans (Snider *et al.*, 2008; Seraphim and Houry, 2020).

The ASCE subgroup, like all P-loop NTPases, possesses a core $\alpha\beta\alpha$ nucleotide-binding domain that exhibits two key functional features: nucleotide-binding (ATP) and hydrolysis ($\text{ATP} \rightarrow \text{ADP} + \text{PO}_4^{3-}$) and is generally referred to as Walker A and Walker B, respectively (Ogura *et al.*, 2004). All ASCE group members also possess a catalytic glutamate (E) residue located within the Walker B motif (Figure 1.14), while those that fall under the kinase-GTPase (KG) group exhibit Walker A and Walker B motifs adjacent to each other (Seraphim and Houry 2020). The Walker A motif is located within the P-loop, which is, in turn, positioned between the β_1 strand and the α -helix. The Walker A motif is responsible for both ATP binding and regulates conformational changes that drive ATP hydrolysis (Ogura *et al.*, 2004). On the other hand, the hydrophobic Walker B motif is located within the β_3 strand and contributes towards ATP hydrolysis and metal ion coordination (Scortecci *et al.*, 2024). The Walker B motif is characterized by the presence of acidic residues (aspartate/glutamate) (Walker *et al.*, 1982; Leipe *et al.*, 2003; Scortecci *et al.*, 2024).

In addition to the Walker motifs, the ASCE members possess the following conserved segments: second region of homology (SRH), arginine finger (R-finger), sensor 1, and sensor 2 (Seraphim and Houry 2020). The SRH is located between the $\beta_4 - \beta_5$ loop. On the other

hand, sensor 2 is located within the third helix of the small α -helical domain and is characterized by either arginine or lysine residues located near the beginning of the $\alpha 7$ segment. The arginine and lysine residues regulate inter- and intra-domain communication of the protein (Neuwald *et al.*, 1999). SRH further acts as a sensor for nucleotide binding and hydrolysis (Guenther *et al.*, 1997). The location of the ATPase sites at the interface of two adjacent AAA+ subunits offers pathways for allosteric signal transmission and subunit coupling (Zeymer *et al.*, 2014).

1.10.2 Structural organization and functions of AAA+ proteins

Typically, an AAA+ protein contains a central β -sheet organized in a $\beta 5$ - $\beta 1$ - $\beta 4$ - $\beta 3$ - $\beta 2$ order that is flanked by α -helices to form an $\alpha\beta\alpha$ sandwich (Seraphim and Houry 2020). Concerning domain arrangement, AAA+ proteins are characterized by the presence of the non-ATPase N-terminal domain, which is followed by either one or two ATPase domains: domain one (DI) and domain two (DII) (Martias *et al.*, 2006; Figure 1.14). The ATP-binding site is located within the DI and extended to the DI-DII linker, also referred to as the middle domain (MD). One of the most notable members of the AAA+ family of proteins is the Clp chaperones/proteases. The Clp family of ATPases possesses three domains: the $\alpha\beta\alpha$ domain I (DI) and the α -helical domain III (DIII) that form the alternating AAA ring, while a central and protruding domain II (DII) represents a specific insertion located in the core AAA+ domains (Aguado *et al.*, 2015). The DI and DIII domains help to form a hexameric ring that serves as a primary feature of AAA+ proteins (Wang *et al.*, 2003). Hexamerization of the protein is crucial for ATP binding and hydrolysis (Ogura and Wilkinson, 2001; Wang *et al.*, 2003; Aker *et al.*, 2007).

Characterization of the structure-function features of the R2TP complex of *Plasmodium falciparum*

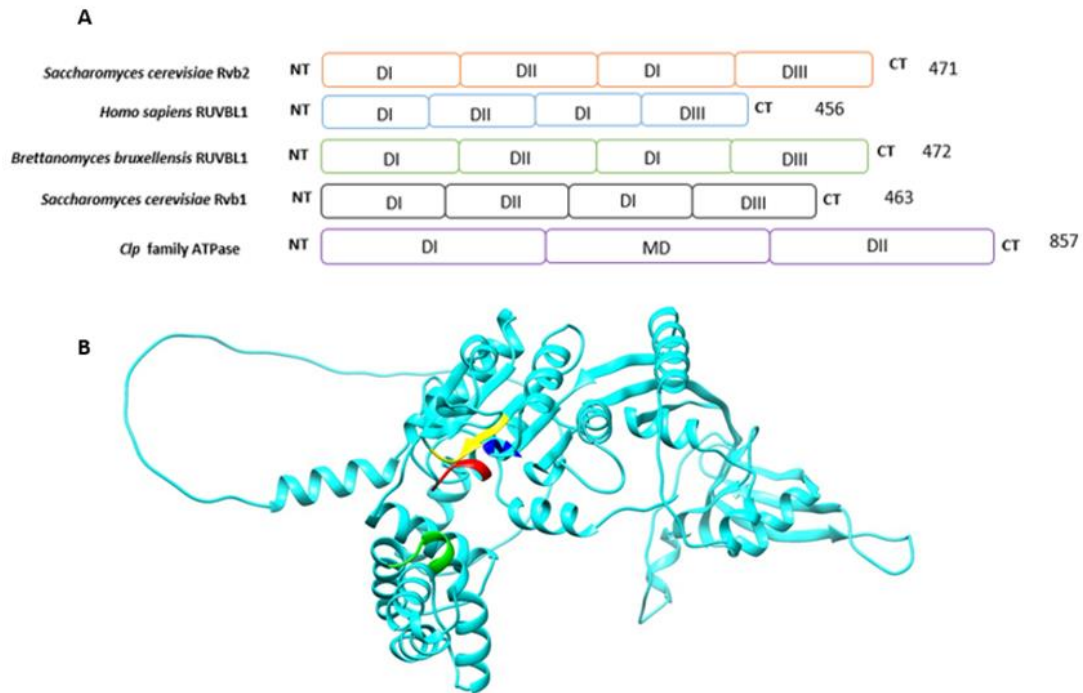


Figure 1.14: The structural features of ATPases members of the ASCE sub-group of AAA+ proteins.

(A) Domain organization of AAA+ proteins, Clp ATPase family of proteins possesses two ATPase domains (DI and DII), while ASCE group members have three ATPase domains (DI, DII, and DIII). Accession codes: *Saccharomyces cerevisiae*: KZV07276.1; *Homo sapiens*: NP_003698; *Brettanomyces bruxellensis*: VUG16836; *Clp B* ATPase: AC073428.1 (B) PfRUVBL1 three-dimensional structure, Walker A (red), Walker B (green), Sensor 1 (purple), and Sensor 2 (yellow). Sequences obtained from PlasmoDB (<https://plasmodb.org>) and structures remodelled using UCSF Chimera 1.15 tool.

AAA+ proteins play a role as transducing elements in various functions in which energy extracted from ATP hydrolysis is used in molecular remodelling events (Snider *et al.*, 2008). Their functions cover a diverse portfolio, including protein unfolding and degradation, peroxisome biogenesis, bacteriochlorophyll biosynthesis, DNA recombination, replication, and repair (Snider *et al.*, 2008). In addition, compared to other P-loop ATPases, AAA+ members possess an additional α -helical sub-domain, which accounts for their unique structure-function features (Hanson *et al.*, 1997). AAA+ proteins mostly occur as oligomers and function as hexameric rings (Hartmann and Vale 1999). The oligomerization of AAA+ proteins is stabilized largely by the binding of these proteins to nucleotides (Ogura and Wilkinson 2001). The AAA+ domain is known to facilitate the oligomerization of proteins into hexameric ring-like structures (Miller and Enemark 2016). The active site is located at the AAA+ domain interface, whose residues interact with residues in a neighboring AAA+ domain to drive oligomerization (Miller and Enemark, 2016). Conformational changes that the

hexameric protein undergoes induce ATPase activity (Kondo *et al.*, 1997; Gates and Martins, 2020).

1.11 Study Rationale

The malaria parasites replicate rapidly within the human host, mainly by synthesizing biomolecules like lipids and proteins (Santiago *et al.*, 2004; Absalon *et al.*, 2016). Proteostasis is crucial for the survival and pathogenicity of the parasite. Hsp90 is a molecular chaperone of *P. falciparum* that plays a significant role in cell development and signal transduction. It has a wide range of protein clients and is vital for the parasite's survival (Banumathy *et al.*, 2003). The co-chaperones of Hsp90 are essential for its functional versatility. There are approximately 700 putative Hsp90-interacting proteins, including two unknown proteins named Pih1 (protein interacting with Hsp90) and Tah1 (TPR-containing protein associated with Hsp90) (Zhao *et al.*, 2005; Weidenauer *et al.*, 2017). Pih1 and Tah1 tightly bind Rvb1 and Rvb2 to form a four-protein complex called R2TP (Rvb1-Rvb2-Tah1-Pih1) (Zhao *et al.*, 2005). This complex serves as an assembly platform that bridges interactions of several proteins (Rivera-Calzada *et al.*, 2017; Martino *et al.*, 2018), thereby driving Hsp90 function in *P. falciparum* and other organisms. Hsp90 has unique co-chaperones that it interacts with for specific processes, and these cooperations may contribute to functional differences between Hsp90 in parasites and humans (Johnson & Brown, 2009). As described above, the R2TP complex is essential for the proper function of various cellular processes. However, its exact role in these processes remains unclear in *P. falciparum*. Therefore, understanding the biochemistry and molecular structure and function provides great advancement to understanding the biological function in various activities that the complex participates in and aid in better targeting the R2TP complex for antimalarial drug discovery.

1.11.1 Hypothesis

Members of the R2TP complex of *Plasmodium falciparum* exhibit structure-function features consistent with their crucial role in the development of the parasite.

1.11.2 Specific Objectives

1.11.3 *In silico* characterization of the structure-function features of the R2TP complex of *Plasmodium falciparum*

Experimental approaches

- i. Multi-sequence alignments were conducted to determine the level of conservation between *P. falciparum* RuvB proteins and their homologs and orthologues
- ii. Homology modelling of *P. falciparum* RuvB proteins was conducted to identify structural similarities
- iii. Structure-based virtual screening was conducted to characterize protein-ligand interaction using molecular docking analysis
- iv. STRING analysis was used to predict PfHsp90 interactome

1.11.4 Antibody and recombinant protein production

Experimental approaches

- i. Peptide design and addition of a carrier protein
- ii. Immunization of peptide
- iii. Serum collection and antibody purification
- iv. Specificity and titer optimization
- i. PfRUVBL1, PfRUVBL2, and yRvb1 recombinant proteins were expressed in *E. coli* strains (*E. coli* XL1 blue and *E. coli* dnaK103) and purified using nickel affinity chromatography

1.11.5 Biochemical characterization of the structure-function features of PfRUVBL1 and PfRUVBL2

Experimental approaches

- i. Tertiary conformations of PfRUVBL1, PfRUVBL2, and yRvb1 were analysed using intrinsic (tyrosine) and extrinsic (1-Anilino-8-Naphthalene Sulfonate, ANS) fluorescence spectroscopy
- ii. The influence of nucleotides (ATP and ADP) on the structural conformations of PfRUVBL1, PfRUVBL2, and yRvb1 was analyzed using a limited proteolysis assay
- iii. Surface Plasmon Resonance (SPR) analysis was used to evaluate nucleotide (ATP and ADP) binding affinity to PfRUVBL1, PfRUVBL2, and yRvb1
- iv. The ATPase activity of PfRUVBL1, PfRUVBL2, and yRvb1 was determined using colorimetric ATP hydrolysis activity assays
- v. Evaluation of the capability of PfRUVBL1 and PfRUVBL2 to suppress induced heat-induced aggregation of proteins *in vitro*

- vi. The capability of PfRUVBL1 and PfRUVBL2 to cleave DNA was investigated

1.11.6 Exploring PfRUVBL1-PfRUVBL2 direct association and their interaction with sorafenib

Experimental approaches

- i. The interaction of PfRUVBL1 and PfRUVBL2 was analyzed using SPR and slot blotting assay (hetero interaction)
- ii. Interaction of PfRUVBL1 with itself and PfRUVBL2 with itself were analysed using SPR (homo interaction)
- iii. The interaction of PfRUVBL1 and PfRUVBL2 with inhibitor sorafenib was evaluated using SPR and thermal-shift assay
- iv. The anti-malarial activity of the compound sorafenib was analysed using an ant-plasmodial activity assay
- v. The interaction of PfRUVBL1 and PfRUVBL2 with molecular chaperones (PfHsp70 and PfHsp90) was analysed using SPR
- vi. The interaction of PfHsp90 with inhibitors geldanamycin and novobiocin was evaluated using SPR and thermal-shift assay

Chapter two

In silico* analysis of the R2TP complex of *P. falciparum

2.1 Introduction

The R2TP complex is an essential protein complex that is involved in assembling and stabilizing various cellular machines, such as RNA polymerase II, small nucleolar ribonucleoproteins (snoRNPs), and other protein complexes that play crucial roles in essential cellular processes (Seraphim *et al.*, 2022). In *P. falciparum*, the parasite responsible for the most severe form of malaria (Sato, 2021), the role of the R2TP complex is yet to be fully understood. It is thought that the R2TP complex of *P. falciparum* is important for the assembly of protein complexes implicated in cellular development (Seraphim *et al.*, 2019; Luthuli and Shonhai, 2023). The R2TP complex assists Hsp90 by linking it to a diverse set of client proteins, thereby facilitating signal transduction and cell division (Seraphim *et al.*, 2019).

P. falciparum has three genes that encode RUVBL proteins (Ahmad *et al.*, 2012). These proteins are homologs of two proteins found in human and yeast systems and belong to the AAA+ family (Luthuli and Shonhai, 2023). Essential features such as Walker A, Walker B, sensor 1, sensor 2, and R-finger motifs are reportedly conserved across all three *P. falciparum* RUVBL proteins (Sen *et al.*, 2018; Luthuli and Shonhai, 2023). ATPases, also known as ATP-dependent molecular machines, play a crucial role in various important processes by utilizing Walker motifs for ATP binding and hydrolysis (Walker, 1998). ATP binding induces conformational changes in RUVBL proteins, which are essential for their function, enabling the assembly of large multiprotein complexes (Matias *et al.*, 2006). Characterization of *P. falciparum* RUVBL proteins is crucial to our understanding of the otherwise complex life cycle of the parasite.

Sorafenib is a multi-kinase inhibitor primarily used as an anti-cancer drug. It targets several intracellular and cell surface kinases involved in tumor cell proliferation and the formation of new blood vessels that supply tumors (Escudier *et al.*, 2007). Sorafenib inhibits the activity of Raf-1, B-Raf, VEGFR (vascular endothelial growth factor receptor), PDGFR (platelet-derived growth factor receptor), and several other kinases, thereby reducing tumor growth and the development of new blood vessels that supply the tumor (Llovet *et al.*, 2008). Sorafenib has been reported to inhibit the *in vitro* proliferation of ATC cell lines and significantly inhibit tumor angiogenesis by inducing endothelial apoptosis in an orthotopic model of thyroid cancer (Kim *et al.*, 2007). Given their diverse function and significant contribution to cancer development, RUVBL proteins are considered highly promising targets for the research and development of cancer treatment drugs (Nano *et al.*, 2020). Sorafenib has been reported to

inhibit the ATPase activity of the RUVBL1/2 complex via their insertion domains (Nano *et al.*, 2020).

Ahmand *et al.* (2013) identified a gene encoding for a TPR-containing protein using bioinformatics analysis. The protein encoded by the PF3D7_1434300 gene contains three TPR domains in its 564 amino acid-length structure, like human RPAP3 (665 amino acid residues). As much as this protein was identified, it was also shown that this protein lacks the RPAP3-specific C-terminal domain (Maurizy *et al.* 2018). A detailed literature survey revealed that this gene encodes for the Hsp70/Hsp90-organizing protein (Hop) (Gitau *et al.*, 2012; Zininga *et al.*, 2015; Silva *et al.*, 2019). PfHop (PF3D7_1434300) is a co-chaperone of PfHsp90 that possesses three TPR motifs (Gitau *et al.*, 2012; Zininga *et al.*, 2015). Since RPAP3/Tah1 homology appears to be a missing member in *P. falciparum*, it is tempting to speculate that PfHop may fulfill this role. PfTah1 (PF3D7_0213500) is a putative ortholog of the yeast Tah1 (TPR-containing protein associated with Hsp90), which is 460 amino acids long and has been proposed to be a member of the R2TP complex (Reviewed by Dutta *et al.*, 2022).

In the current study, the following specific objectives were implemented to establish the structure-function features of the R2TP complex of *P. falciparum*:

- Multi-sequence alignments were conducted to determine the level of conservation between *P. falciparum* RUVBL1, RUVBL2, and RUVBL3 proteins and their homologs and orthologues
- Three-dimensional homology modelling of *P. falciparum* RUVBL1, RUVBL2, and RUVBL3 proteins was conducted to identify structural similarities
- Molecular docking was conducted to characterize *P. falciparum* RUVBL proteins-ligand interaction using molecular docking analysis
- STRING analysis was used to predict PfHsp90 interactome and its association with R2TP complex co-chaperones

2.2 Experimental procedure

2.2.1 Multiple sequence alignment

Multiple sequence alignments were conducted to determine the level of conservation of functional motifs of the malarial parasite PfRUVBL1, PfRUVBL2, and PfRUVBL3 proteins, including the Walker A and Walker B, sensor 1, sensor 2, and arginine finger (Seraphim and Houry, 2022). Sequences were obtained from the PlasmoDB database (www.PlasmoDB.org) and the NCIB database (www.NCIB.org). The retrieved sequences were aligned and shaded using the Bio-edit tool ([www.Bioedit. Software. Informer. com](http://www.Bioedit.Software.Informer.com)), and the aligned sequences had their residues auto-calculated for their similarity and conservation using Bio-edit tool version 7.2.5.

2.2.2 Prediction of three-dimensional structures of PfRUVBL1, PfRUVBL2, and PfRUVBL3 proteins

Three-dimensional models were generated from sequences retrieved in PDB format (www.rcsb.org) and rendered using Chimera 1.15v (Pettersen *et al.*, 2004). The three-dimensional models of PfRUVBL1, PfRUVBL2, and PfRUVBL3 were compared using the superposing tool (Chimera 1.15). The superimposed models were scored based on structural similarities using root mean square derivatives (RMSD) statistics. Secondary structures of PfRUVBL1, PfRUVBL2, and PfRUVBL3 were generated by depositing the amino acid sequences into an online tool PHYRE2 (<http://www.sgb.bio.ic.co.uk/phyre2>; Ahmad *et al.*, 2013).

2.2.3 Investigation of the interaction between chemical ligands and PfRUVBL1, PfRUVBL2, and PfRUVBL3 using molecular docking technique

The molecular docking approach was used to model the interaction between a small molecule (Sorafenib) and nucleotide with PfRUVBL1, PfRUVBL2, and PfRUVBL3. This allowed for the characterization of the behavior of small molecules in the binding site of the target proteins (McConkey *et al.*, 2002). The docking process involved two basic steps: prediction of the ligand conformation, its position, and orientation within these sites (usually referred to as a "pose") and assessment of the binding affinity (Meng *et al.*, 2011). The structures of ATP, ADP, and sorafenib were drawn using the pepdraw tool (www.pepdraw.com), and docking was performed using PyRx-0.8 (www.pyrx.sourceforge.io) and rendered on Discovery Studio visualizer v21.1.0.20298.

2.2.4 Virtual ligand screening

Docking-based virtual screening (DBVS) methods were used to assess computationally the behavior of nucleotide and sorafenib in target binding pockets of PfRUVBL1, PfRUVBL2, and PfRUVBL3. PyRx 0.8 (www.pyrx.sourceforge.io) docking tool was used in this study, which integrates several open-source software, including AutoDock, AutoDock Vina, and Open Babel, to carry out DBVS effortlessly (Kondapuram *et al.*, 2021). Biovia Discovery Studio v21.1.0.20298 was used to view the 3-dimensional structure, residues, and atoms, including inter and intramolecular forces (Trott and Olson, 2010; Akinnusi *et al.*, 2022).

2.2.5 Predicting the interaction network of PfHsp90

The interactome prediction of PfHsp90 was conducted using data downloaded from the STRING v12 ([http:// string-db.org](http://string-db.org)) database. STRING works as a collective database that integrates data collected from several sources, including gene neighbourhood, gene fusions, gene co-occurrence, text-mining, and co-expression (Szklarczyk *et al.*, 2019). The network created displays nodes and edges with a high confidence level of 0.9. The three genomic context prediction channels (neighbourhood, fusion, and gene co-occurrence) are meticulously derived from systematic comparisons of all genomes to assess the implications of past genome rearrangements, gene gains and losses, and gene fusion events. The co-expression channel is firmly grounded in gene-by-gene correlation tests across a substantial number of gene expression datasets (Skrabanek *et al.*, 2021).

2.3 Results

2.3.1 Analysis of sequence similarity between PfRUVBL1, PfRUVBL2, and PfRUVBL3 proteins and their human and yeast homologues

The ASCE subgroup encompasses P-loop NTPases, a core $\alpha\beta\alpha$ nucleotide-binding domain exists, housing two essential functional elements: nucleotide binding (ATP) and hydrolysis ($\text{ATP} \rightarrow \text{ADP} + \text{PO}_4^{3-}$), commonly known as Walker A and Walker B, respectively (Ogura *et al.*, 2004). All members of the ASCE group also contain a catalytic glutamate (E) residue within the Walker B motif (Figure 2.1.1). The kinase-GTPase (KG) group has adjacent Walker A and Walker B motifs (Seraphim and Houry 2020). The Walker A motif is located within the P-loop, between the $\beta 1$ strand and the α -helix (Figure 2.1.1 and Figure 2.1.3).

Multiple sequence alignments were conducted to determine the level of conservation of RUVBL-like proteins from *Plasmodium* species compared to human and yeast orthologues (Figure 2.1.1). The Walker A (GSGKT/S) motif is responsible for both ATP binding and regulation of conformational changes that drive ATP hydrolysis (Ogura *et al.*, 2004). Walker B (DEV/AH), a motif that is hydrophobic and located within the $\beta 3$ strand and contributes to ATP hydrolysis and metal ion coordination (Scorteccie *et al.*, 2024). The Walker B motif is characterized by the presence of acidic residues (aspartate/glutamate) (Walker *et al.*, 1982; Leippe *et al.*, 2003; Kanade *et al.*, 2020). At neutral pH, the two negatively charged amino acids glutamic acid (E) and aspartate (D) are known to be involved in ATP hydrolysis and Mg^{2+} binding, respectively (Seraphim and Houry, 2020).

In addition to the Walker motifs, *P. falciparum* members possess the following conserved segments: second region of homology (SRH), arginine finger (R-finger), sensor 1 (L/MATNR), and sensor 2 (S/CSLRYA) (Seraphim and Houry, 2020, Figure 2.1.1). The SRH is located between the $\beta 4$ and $\beta 5$ loops. On the other hand, sensor 2 is located within the third helix of the small α -helical domain and is characterized by either arginine or lysine residues located near the beginning of the $\alpha 7$ segment. The arginine and lysine residues regulate inter- and intra-domain communication of the protein (Neuwald *et al.*, 1999). SRH further acts as a Sensor for nucleotide binding and hydrolysis (Guenther *et al.*, 1997).

PfRUVBL1 and PfRUVBL3 proteins possess valine residues at position 324, while PfRUVBL2 possesses alanine residues at this position (Figure 2.1.1). This substitution is conserved as both valine and alanine are nonpolar and hydrophobic. In the Walker A motif at residue S¹⁴⁶ (numbering based on PfRUVBL1), serine is replaced by threonine in PfRUVBL3

proteins. This is a conserved substitution since the two amino acids are polar and uncharged (Figure 2.1.1). Asparagine is a non-essential amino acid that plays an important role in protein and nucleotide biosynthesis (Zhu *et al.*, 2017; Chiu *et al.*, 2020). The data showed that this amino acid is conserved in the Walker B motif (Figure 2.1.1).

Furthermore, the findings revealed that asparagine is conserved across all *Plasmodium* species (Figure 2.1.1), implicating the capability of PfRUVBL1, PfRUVBL2, and PfRUVBL3 proteins to bind ATP. Generally, nucleotide-binding and hydrolysis motifs are conserved across *Plasmodium* species. Further, conserved substitution in the sensor 1 motif between L³⁹⁴ to (RUVBL1) to M³⁹⁴ (RUVBL2 and RUVBL3) confirms similarity between RUVBL1 and yeast Rvb1, while human RUVBL1 possesses phenylalanine at this position (Figure 2.1.1). The insertion domain of *Plasmodium* RUVBL1 and RUVBL2 is conserved compared to RUVBL3, suggesting that RUVBL1 and RUVBL2 are closely related. The sequence alignment further showed that the arginine finger located in sensor 1 and sensor 2 is conserved (Figure 2.1.1). Arginine plays a crucial role in ATP interactions, particularly in proteins that bind ATP, such as kinases and ATPases (Pols *et al.*, 2021). Its positively charged side chain can interact with the negatively charged phosphate groups of ATP, stabilizing the binding of ATP in the active sites (Pols *et al.*, 2021).

P.fRUVBL1	1	-----MNIE-----SSR-EKERI
P.bRUVBL1	1	-----MNIE-----SNK-EKERI
P.mRUVBL1	1	-----MNIE-----SNK-EKERI
P.oRUVBL1	1	-----MNIE-----SSK-DKERI
P.vRUVBL1	1	MRCVIEAVLTLPFCITIHVRSTYEALFQSPSTLLLPKMNIIE-----SNR-EKERI
P.kRUVBL1	1	-----MNIE-----SNR-EKERI
P.fRUVBL2	1	-----MEINISNITN-----SSNNKKERV
P.mRUVBL2	1	-----MQINLENINT-----SGDTKKERV
P.oRUVBL2	1	-----MQVSLTNIPS-----SSEAKKERV
P.vRUVBL2	1	-----MQINLASVNT-----SSDAKKERV
P.kRUVBL2	1	-----MQIKLASVNT-----SSDAKKERV
P.bRUVBL2	1	-----MEINLTNISI-----SNDGKKERI
P.fRUVBL3	1	-----MKLEE-----VKDIQKIERI
P.mRUVBL3	1	-----MKLEE-----VKDIQKIERI
P.oRUVBL3	1	-----MKLEE-----VKDIQKIERI
P.vRUVBL3	1	-----MKLEE-----VKDIQKIERI
P.kRUVBL3	1	-----MKLEE-----VKDIQKIERI
P.bRUVBL3	1	-----MKLEE-----VKDIQKIERI
Rvb1	1	-----MVAISEVKENPGVNSSNSGAVTRT
Rvb2	1	-----MSIQTSDPNE-----TSDLKSLSLI
HsRUVBL1	1	-----MKIEEVKS-----TTKTORI
HsRUVBL2	1	-----MATVTATTKVPE-----IRDVTRIERI

P.fRUVBL1	14	SIHSHISGLGLDADGYVTDLFEEENIKKRDMYSEP-----TNKIENNDIKNGTILDPDK
P.bRUVBL1	14	SIHSHISGLGLDADGFVHDVDLDEPQKIY-----EKIEKD--GNLTNGENI
P.mRUVBL1	14	NIHSHISGLGLDADGFVYDINFIAQEKKN-----GKICKNE-QNGITFQNDI
P.oRUVBL1	14	SIHSHITGLGLDMDGFVSDLYFEETKKKK-----NKIEQN--GNGIINYEDI
P.vRUVBL1	54	SIHSHISGLGLDADGFVQDLNFEQKKGKGGKMGLGGQPNGQLNGVTNGQ--PNGATNGHVN
P.kRUVBL1	14	SIHSHISGLGLDADGFVQDLILEQKKGKSGKMG-----VNGMMNGQ--PKGSVIKHAN
P.fRUVBL2	20	NIHSHIKGLGVNTNIYLHENEISLS-----
P.mRUVBL2	20	NIHSHIKGLGVNTNIYLHEEEINLS-----
P.oRUVBL2	20	NIHSHIKGLGVNTNIYMHENDINLT-----
P.vRUVBL2	20	NIHSHIKGLGVNTNIYLHENDVNLT-----
P.kRUVBL2	20	NIHSHIKGLGVNTNIYLHENDVNLT-----
P.bRUVBL2	20	NIHSHIKGLGVNTNIYMHEDDEVNLS-----
P.fRUVBL3	16	GAHSHIRGLGLNDCLDARY-----
P.mRUVBL3	16	GAHSHIRGLGLNDCLDARY-----
P.oRUVBL3	16	GAHSHIRGLGLNDCLDARY-----
P.vRUVBL3	16	GAHSHIRGLGLNDCLDARY-----
P.kRUVBL3	16	GAHSHIRGLGLNDCLDARY-----
P.bRUVBL3	16	GAHSHIRGLGLNDCLDARY-----
Rvb1	25	AAHTHIKGLGLDESGVAKR-----
Rvb2	21	AAHSHITGLGLDENLQPRP-----
HsRUVBL1	16	ASHSHVKGLGLDESGLAKQ-----
HsRUVBL2	23	GAHSHIRGLGLDDALEPRO-----

P.fRUVBL1	68	VDNICNS-----YKLENNNGNYENNKICYNNISDD--EDEVVKNEYKCKGMV
P.bRUVBL1	58	HDNQYN-----EICNMNGDNNIDRSGNEGEE--GKDKIKNLYNCKGMV
P.mRUVBL1	60	THNILLDQVK-----YEEKYNEYDSN-YEASSNGDDSEDDE-VKNFYNCKGMI
P.oRUVBL1	59	SKGLLP-----REKFNGYEDNDDMCSSKADDESNEEVVKNEFNCKGMV
P.vRUVBL1	113	ADGALCNEFTGDGMGGEQEMKANSAPNGYAHTNGECTNPQEKs--DEEVFRNEFYNCKGMI
P.kRUVBL1	65	PDEALCNEFTGNAMEEEQGMKATSELNGYSHTNGECAHPQEEs--DDEVYRNEFYNCKGMI
P.fRUVBL2	44	-----DEKYSMFFDNSCGLV
P.mRUVBL2	44	-----DTRYSIFFDNSCGLV
P.oRUVBL2	44	-----DERYSMFFDNTCGLI
P.vRUVBL2	44	-----DERYSMFFDNSCGLV
P.kRUVBL2	44	-----DERYSMFFDNSCGLI
P.bRUVBL2	44	-----DEKYSMFFDNTCGLV
P.fRUVBL3	34	-----CSEGMI
P.mRUVBL3	34	-----CSEGMI

In silico analysis of the R2TP complex of *P. falciparum*

P. oRUVBL3	34	-----CSEGM
P. vRUVBL3	34	-----CSEGM
P. kRUVBL3	34	-----CSEGM
P. bRUVBL3	34	-----CSEGM
Rvb1	43	-----VEGGFV
Rvb2	39	-----TSEGMV
HsRUVBL1	34	-----AASGLV
HsRUVBL2	41	-----ASQGMV

P. fRUVBL1	112	GQKKAREAAAGIFINLIKEKNIC-KCLLLAGPSGSGKTAIAIAISKEISDESIPFCIFNAS
P. bRUVBL1	99	GQKKAREAAAGIFINLIKEKNIC-KCLLLAGPSGSGKTAIAIAISKEISEEAIPFCIFNAS
P. mRUVBL1	107	GQKKAREAAAGIFINLIKEKNIC-KCLLLAGPSGSGKTAIAIAISKEISEDSIPFCIFNAS
P. oRUVBL1	103	GQKKAREAAAGIFINLIKEKNIC-KCLLLAGPSGSGKTAIAIAISKEISEESIPFCIFNAS
P. vRUVBL1	171	GQKKAREAAAGIFINLIKEKNIC-KCLLLAGPSGSGKTAIAIAISKEISEDSIPFCIFNAS
P. kRUVBL1	123	GQKKAREAAAGIFINLIKEKNIC-KCLLLAGPSGSGKTAIAIAISKEISEDSIPFCIFNAS
P. fRUVBL2	60	GQFKAREASLFLVDLIKQKKLAGKCILLAGPSGSGKSAIAIGISREIN-RKMPFVFLSGS
P. mRUVBL2	60	GQFKAREASLFLVDLIKQKKLAGKCILLAGPSGSGKSAIAIGISREIN-KKMPFVFLSGS
P. oRUVBL2	60	GQFKAREASLFLVDLIKQKKLAGKCILLAGPSGSGKSAIAIGISREIN-KKMPFVFLSGS
P. vRUVBL2	60	GQFKAREASLFLVDLIKQKKLAGKCILLAGPSGSGKSAIAIGISREIN-KKMPFVFLSGS
P. kRUVBL2	60	GQFKAREASLFLVDLIKQKKLAGKCILLAGPSGSGKSAIAIGISREIN-KKMPFVFLSGS
P. bRUVBL2	60	GQFKAREASLFLVDLIKQKKLAGKCILLAGPSGSGKSAIAIGISREIN-KKMPFVFLSGS
P. fRUVBL3	41	GQMSARKAAGIVLRMIKEGRISGRAILLAGQFGTGKTAIAMGIAKALG-EDTPFTHISGS
P. mRUVBL3	41	GQMSARKAAGIVLRMIKEGRISGRAILLAGQFGTGKTAIAMGIAKALG-EDTPFTHISGS
P. oRUVBL3	41	GQMSARKAAGIVLRMIKEGRISGRAILLAGQFGTGKTAIAMGIAKALG-EDTPFTHISGS
P. vRUVBL3	41	GQMSARKAAGIVLRMIKEGRISGRAILLAGQFGTGKTAIAMGIAKALG-EDTPFTHISGS
P. kRUVBL3	41	GQMSARKAAGIVLRMIKEGRISGRAILLAGQFGTGKTAIAMGIAKALG-EDTPFTHISGS
P. bRUVBL3	41	GQMSARKAAGIVLRMIKEGRISGRAILLAGQFGTGKTAIAMGIAKALG-EDTPFTHISGS
Rvb1	50	GQIBAREACGVIVDLIKAKKMSGRAILLAGGISTGKTAIALAISQELG-PKVPFCLVGS
Rvb2	46	GQLQARRAAGVILKMOVNGTLAGRAVLVAGPESTGKTAIAMGVSQSLG-KDVPFTAAGS
HsRUVBL1	41	GQENAREACGVIVELIKSKMAGRAVLVAGPESTGKTAIALAISQELG-SKVPFCPMVGS
HsRUVBL2	48	GQLAARRAAGVVLEMIREGKIAGRAVLVAGPESTGKTAIAMGMAQALG-PDTPFTAAGS

P. fRUVBL1	171	QVYSCEVKKTEILTQYIRKSIGVKIKEIKEVEFEGEVVQIEP----FFDNTYDEQKISYVH
P. bRUVBL1	158	QVYSCEVKKTEILTQYIRKSIGVKIKEIKEVEFEGEVVKLEP----FYDDTYDEKTIISYVH
P. mRUVBL1	166	QVYSCEVKKTEILTQYIRKSIGVKIKEIKEVEFEGEVVKLEP----FYDDTYEEKKISYVH
P. oRUVBL1	162	QVYSCEVKKTEILTQYIRKSIGVKIKEIKEVEFEGEVINIEP----FYDDTYEEKKISYVH
P. vRUVBL1	230	QVYSCEVKKTEILTQYIRKSIGVKIKETKEVEFEGEVTKIEP----FYDDTYEEKKISYVH
P. kRUVBL1	182	QVYSCEVKKTEILTQYIRKSIGVKIKEIKEVEFEGEVTKIEP----FYDDTYEEKKISYVH
P. fRUVBL2	119	EVYSNEIKKTEVILEAFRFSIHIIKIKEEKLVEYEGEVVDMVVEENECLYSLNKAQINAI
P. mRUVBL2	119	EVYSNEIKKTEVILEAFRFSIHIIKIKEEKLVEYEGEVVDMVVEENECLYSLNKAQINAI
P. oRUVBL2	119	EVYSNEIKKTEVILEAFRFSIHIIKIKEEKLVEYEGEVVDMVVEENECLYSLNKAQINAI
P. vRUVBL2	119	EVYSNEIKKTEVILEAFRFSIHIIKIKEEKLVEYEGEVVDMVVEENECLYSLNKAQINAI
P. kRUVBL2	119	EVYSNEIKKTEVILEAFRFSIHIIKIKEEKLVEYEGEVVDMVVEENECLYSLNKAQINAI
P. bRUVBL2	119	EVYSNEIKKTEVILEAFRFSIHIIKIKEEKLVEYEGEVVDMVVEENECLYSLNKAQINAI
P. fRUVBL3	100	EVYSLEMSKTEALTQAFRRSIGVRVKEESEVIEGEVVEIEIEKFNERDINNKNKKLG--K
P. mRUVBL3	100	EVYSLEMSKTEALTQAFRRSIGVRVKEESEVIEGEVVEIEIEKFNERDINNKNKKVG--K
P. oRUVBL3	100	EVYSLEMSKTEALTQAFRRSIGVRVKEESEVIEGEVVEIEIEKFNERDINNKNKKVG--K
P. vRUVBL3	100	EVYSLEMSKTEALTQAFRRSIGVRVKEESEVIEGEVVEIEIEKFNERDINNKNKKVG--K
P. kRUVBL3	100	EVYSLEMSKTEALTQAFRRSIGVRVKEESEVIEGEVVEIEIEKFNERDINNKNKKVG--K
P. bRUVBL3	100	EVYSLEMSKTEALTQAFRRSIGVRVKEESEVIEGEVVEIEIEKFNERDINNKNKKVG--K
Rvb1	109	EIYSVEVKKTEITLMENFRRAIGLRIKETKEVYEGEVTELTPEDAENPLGGYG-KTISHVI
Rvb2	105	EIISLELSKTEALTQAFRRSIGIKIKEETEIEIEGEVVEIQIDRS----ITG-GHKQG--K
HsRUVBL1	100	EVYSTETIKKTEVLMENFRRAIGLRIKETKEVYEGEVTELTPECETENPMGGYG-KTISHVI
HsRUVBL2	107	EIISLEMSKTEALTQAFRRSIGVRVKEETEIEIEGEVVEIQIDRP----ATGTGSKVG--K

P. fRUVBL1	227	ITLKTLEQKKIKIHSSIIYDNLLEKEIQEKDIYIESSHSGNVKRVGKCSLYQDMFDIETD
P. bRUVBL1	214	ITLKTLEQKKIKIHSSIIYENILKEIQEKDVIIYIESSHGLVKRVGRCISLYQDMFDIETD
P. mRUVBL1	222	ITLKTLEQKKIKIHSSIIYENILKEIQEKDVIIYIESSHGIVKRVGKCSLYHDMFDIETD
P. oRUVBL1	218	ITLKTLEQKKIKIHSSIIYENIEKEIQEKDIYIESSHGIVKRVGKCSLYQDMFDIETD
P. vRUVBL1	286	ITLKTLEQKKIKIHSSIIYENIVKEIQEKDVIIYIESSHGLVKRVGKCSLYEDMFDIETD
P. kRUVBL1	238	ITLKTLEQKKIKIHSSIIYENIVKEIQEKDIYIESSHGLVKRVGKCSLYQDMFDIETD
P. fRUVBL2	179	ITLKSVMGSKTLRLAPKIHQIAREKIKIGDVIIYIE TNTGHVKRLGRCNDYAKYDIEFD

P.mRUVBL2	179	ITLKTIVKGSKTLRLAPKIH	EQIVREKIKIGDIIYIETNTG	HVKRLGRCNVYSKEYDIEFD
P.oRUVBL2	179	ITLKTIVKGSKTLRLAPKIH	EQIVREKIKIGDVIYIETNTG	HVKRLGRCNAYSKEYDIEFD
P.vRUVBL2	179	ITLKTIVKGAKTTLRLAPKIH	EQIVREKIKIGDVIYIETNTG	HVKRLGRCNVYSKEYDIEFD
P.kRUVBL2	179	ITLKTIVKGAKTTLRLAPKIH	EQIVREKIKIGDVIYIETNTG	HVKRLGRCNVYSKEYDIEFD
P.bRUVBL2	179	ITLKTIVKGTSLRLAPKIH	EQIVREKIKIGDVIYIEVNTG	HVKRLGRCNTYSKEYDIEFD
P.fRUVBL3	158	MILKTTEMETLYDLGSKMIEA	LQKENITAGDVICIDKGTGKIT	IKGKSFARSKDYDAMD
P.mRUVBL3	158	MILKTTEMETLYDLGSKMIEA	LQKENITAGDVICIDKSTGKIT	IKGKSFARSKDYDAMD
P.oRUVBL3	158	MILKTTEMETLYDLGSKMIEA	LQKENITAGDVICIDKSTGKIT	IKGKSFARSKDYDAMD
P.vRUVBL3	158	MILKTTEMETLYDLGSKMIEA	LQKENITAGDVICIDKSTGKIT	IKGKSFARSKDYDAMD
P.kRUVBL3	158	MILKTTEMETLYDLGSKMIEA	LQKENITAGDVICIDKGTGKIT	IKGKSFARSKDYDAMD
P.bRUVBL3	158	MILKTTEMETLYDLGSKMIEA	LQKENITAGDVICIDKSTGKIT	IKGKSFARSKDYDAMD
Rvb1	168	VGLKSAKGTTLRLDPTIYES	TQREKVSIGDVIYIEANTGAV	KRVGRSDAYATEFDLE
Rvb2	158	LTIKTTDMETIYELGNKMID	GLTKEKVLGADVISIDKASG	KITKLGRSFARSRDYD
HsRUVBL1	159	IGLKTAAGTKQLKLDPSIF	ESLQKERVEAGDVIYIEAN	SGAVKRQGRCDTYATE
HsRUVBL2	161	ITLKTTEMETIYDLGTMIE	SLTKDKVQAGDVITIDKAT	GKISKLGSRFTRARDY
P.fRUVBL1	286	--TFVDLPKGNVHKKKNII	QNTLYDLIDVSNVQP--	KDNILDFLQN-NKSKKTE
P.bRUVBL1	273	--TFVDLPKGNVHKKKNII	QNTLYDLIDVSNVQP--	KDNILNFFQN-AKCKKTE
P.mRUVBL1	281	--TFVDLPKGNVHKKKNII	QNTLYDLIDVSNVQP--	RDNILNFLQN-SKSKKTE
P.oRUVBL1	277	--TFVDLPKGNVHKKKNII	QNTLYDLIDVSNVQP--	KDNILNFLQN-SKSKKTE
P.vRUVBL1	345	--TFVDMPRGNVHKKKNII	QNTLYDLIDVSNVQP--	KDNILNFLQN-SKSKKTE
P.kRUVBL1	297	--TFVDLPKGNVHKKKNII	QNTLYDLIDVSNVQP--	KDNILNFLQN-SKSKKTE
P.fRUVBL2	238	--EYVSLPKGEVHKKKEV	VQQLSLHDIDLANANPTVG	EDLASVLNSYLRFKKTE
P.mRUVBL2	238	--EYVSLPKGEVHKKKEV	VQQLSLHDIDLANANPTVG	EDLASVLNSYLRFKKTE
P.oRUVBL2	238	--EYVSLPKGEVHKKKEV	VQQLSLHDIDLANANPTVG	EDLASVLNSYLRFKKTE
P.vRUVBL2	238	--EYVSLPKGEVHKKKEV	VQQLSLHDIDLANANPTVG	EDLASVLNSYLRFKKTE
P.kRUVBL2	238	--EYVSLPKGEVHKKKEV	VQQLSLHDIDLANANPTVG	EDLASVLNSYLRFKKTE
P.bRUVBL2	238	--EYVSLPKGEVHKKKEV	VQQLSLHDIDLANANPTVG	EDLASVLNSYLRFKKTE
P.fRUVBL3	218	NTLFVQCPEGELQKRKEV	VHTVTLHDIDAINSRT---	QGFLALFSG---DTGEIK
P.mRUVBL3	218	NTNFVQCPEGELQKRKEV	VHTVTLHDIDAINSRT---	QGFLALFSG---DTGEIK
P.oRUVBL3	218	NTNFVQCPEGELQKRKEV	VHTVTLHDIDAINSRT---	QGFLALFSG---DTGEIK
P.vRUVBL3	218	NTNFVQCPEGELQKRKEV	VHTVTLHDIDAINSRT---	QGFLALFSG---DTGEIK
P.kRUVBL3	218	NTNFVQCPEGELQKRKEV	VHTVTLHDIDAINSRT---	QGFLALFSG---DTGEIK
P.bRUVBL3	218	NTNFVQCPEGELQKRKEV	VHTVTLHDIDAINSRT---	QGFLALFSG---DTGEIK
Rvb1	227	--EYVPLPKGEVHKKKEI	VQDVTLHDIDLVANARPQ	GGQDVISMGMQLLKP
Rvb2	218	DTRFVQCPEGELQKRKEV	VHTVTLHDIDAINSRT---	QGFLALFTG---DTGEIR
HsRUVBL1	218	--EYVPLPKGDVHKKKEI	VQDVTLHDIDLVANARPQ	GGQDILSMGMQLMKP
HsRUVBL2	221	QTKFVQCPEGELQKRKEV	VHTVTLHDIDAINSRT---	QGFLALFSG---DTGEIK
P.fRUVBL1	342	NEINKIVYKYVDQGI	AOIPGVLFIDEVHMLDIEC	FITYLNRTLESNLAPV
P.bRUVBL1	329	NEINKIVYKYVDQGI	AOIPGVLFIDEVHMLDIEC	FITYLNRTLESNLAP
P.mRUVBL1	337	NEINKIVYKYVDQGI	AOIPGVLFIDEVHMLDIEC	FITYLNRTLESNLAP
P.oRUVBL1	333	NEINKIVYKYVDQGI	AOIPGVLFIDEVHMLDIEC	FITYLNRTLESNLAP
P.vRUVBL1	401	NEINKIVYKYVDQGI	AOIPGVLFIDEVHMLDIEC	FITYLNRTLESNLAP
P.kRUVBL1	353	NEINKIVYKYVDQGI	AOIPGVLFIDEVHMLDIEC	FITYLNRTLESNLAP
P.fRUVBL2	297	VEINKTVNKFLESG	LAETIPGVLYIDEAHMLDIE	CFSYLNRAIESPLAPI
P.mRUVBL2	297	VEINKTVNKFLESG	LAETIPGVLYIDEAHMLDIE	CFSYLNRAIESPLAPI
P.oRUVBL2	297	VEINKTVNKFLESG	LAETIPGVLYIDEAHMLDIE	CFSYLNRAIESPLAPI
P.vRUVBL2	297	VEINKTVNKFLESG	LAETIPGVLYIDEAHMLDIE	CFSYLNRAIESPLAPI
P.kRUVBL2	297	VEINKTVNKFLESG	LAETIPGVLYIDEAHMLDIE	CFSYLNRAIESPLAPI
P.bRUVBL2	297	VEINKTVNKFLESG	LAETIPGVLYIDEAHMLDIE	CFSYLNRAIESPLAPI
P.fRUVBL3	271	EHIDMKINWQDEKA	EIVPGVLFIDEVHMLDIEC	FITYLNRALESEQSP
P.mRUVBL3	271	EHIDMKINWQDEKA	EIVPGVLFIDEVHMLDIEC	FITYLNRALESEQSP
P.oRUVBL3	271	EHIDMKINWQDEKA	EIVPGVLFIDEVHMLDIEC	FITYLNRALESEQSP
P.vRUVBL3	271	EHIDMKINWQDEKA	EIVPGVLFIDEVHMLDIEC	FITYLNRALESEQSP
P.kRUVBL3	271	EHIDMKINWQDEKA	EIVPGVLFIDEVHMLDIEC	FITYLNRALESEQSP
P.bRUVBL3	271	EHIDMKINWQDEKA	EIVPGVLFIDEVHMLDIEC	FITYLNRALESEQSP
Rvb1	286	QEVNKVAKYIDQGA	EIVPGVLFIDEVHMLDIE	FITYLNRALESNIAPV
Rvb2	271	DQINTKVAEWKEEG	KAEIVPGVLFIDEVHMLDIE	CFSYLNRALEDEFAP
HsRUVBL1	277	GEINKVVKYIDQGA	EIVPGVLFIDEVHMLDIE	CFTYLNRALESSIAP
HsRUVBL2	274	EQINAKVAEWREEG	KAEIVPGVLFIDEVHMLDIE	SFSYLNRALESMDAP

P. fRUVBL1	402	NIKGTN-IISAHGIPVDLLDR	IIIVKTMLYNKEEILQVLKLRCKFERIKIDSEALDYLS
P. bRUVBL1	389	NIKGTN-IISAHGIPVDLLDR	IIIVKTMLYNKEEILQVLKLRCKFENIKIENEALNYLS
P. mRUVBL1	396	-----R	IIIVKTMLYNKEEILQVLKLRCKFEKIKIEREALNYLAD
P. oRUVBL1	393	NIKGTN-IISAHGIPVDLLDR	IIIVKTMLYNKEEILQVLKLRCKFEKIKIENEALNYLAD
P. vRUVBL1	461	NIKGTN-IISAHGIPVDLLDR	IIIVKTMLYNKEEILQVLKLRCKFEKIKIENEALNYLAD
P. kRUVBL1	413	NIKGTN-IISAHGIPVDLLDR	IIIVKTMLYNKEEILQVLKLRCKFEKIKIENEALNYLAD
P. fRUVBL2	357	TVKGTD-NIEFHGIPVDLLDR	IIIKTFPYTLKEIVQILALRAHSEKINISEQGMNYLAK
P. mRUVBL2	357	TVKGTD-NIEFHGIPVDLLDR	IIIKTFPYTLKEIVQILALRAHSEKINISEQGMNYLAK
P. oRUVBL2	357	TVKGTD-NIEFHGIPVDLLDR	IIIKTFPYTLKEIVQILALRAHSEKINISEQGMNYLAK
P. vRUVBL2	357	TVKGTD-NIEFHGIPVDLLDR	IIIKTFPYTLKEIVQILALRAHSEKINISEQGMNYLAK
P. kRUVBL2	357	TVKGTD-NIEFHGIPVDLLDR	IIIKTFPYTLKEIVQILALRAHSEKINISEQGMNYLAK
P. bRUVBL2	357	TVKGTD-NIEFHGIPVDLLDR	IIIKTFPYTLKEIVQILALRAHSEKINISEQGMNYLAK
P. fRUVBL3	331	HIRGTD-YKAHFGIPDLDLDR	TLIIPTYPYKHQDILKILEQRAEEEDVDIDEYAKELLCK
P. mRUVBL3	331	HIRGTD-YKAHFGIPDLDLDR	TLIIPTYPYKHQDILKILEQRAEEEDVDIDDCAKELLCK
P. oRUVBL3	331	HIRGTD-YKAHFGIPDLDLDR	TLIIPTYPYKHQDILKILEQRAEEEDVEIDDYAKELLCK
P. vRUVBL3	331	HIRGTD-YKAHFGIPDLDLDR	TLIIPTYPYKHQDIMKILEQRAEEEDVEIDYAKELLCK
P. kRUVBL3	331	HIRGTD-YKAHFGIPDLDLDR	TLIIPTYPYKHQDIMKILEQRAEEEDVEIDYAKELLCK
P. bRUVBL3	331	HIRGTD-YKAHFGIPDLDLDR	TLIIPTYPYMHEDIMKILEQRAEEEDVEIDFAKELLCK
Rvb1	346	TVRGTEVLSHGVPPDL	ILIVRTLIPYDKDEIRTIERRATVERLOVESSALDILAT
Rvb2	331	KTRGTN-YKSHGIPDLDLDR	SLIITTKSYNEQEIKTILSIRAQEELEVELSSDALDLTK
HsRUVBL1	337	VIRGTEDITSEHGIPDLDLDR	MIIRTMLYTPQEMKQIIKIRAQTEGINTISEEALNHLGE
HsRUVBL2	334	RIRGTS-YQSEHGIPDLDLDR	LIVSTTPYSEKDTKQILRIRCEEEDVMSDAYTVLTR
P. fRUVBL1	461	IGIKCSIRYAIQLLTPAKILSKKKGKKKIDKNITEIVSSIFFD	TKRSTQLLLNDDKNKMY
P. bRUVBL1	448	IGMNCSLRYAIQLLTPAKILSKRKGGKMKISKSIIEIVSSIFFD	TKRSTQLLLNDDKNKMY
P. mRUVBL1	437	IGITCSLRAYIQLLTPAKILSKRKGGKKNISKNIIEIVSSIFFD	TKRSTQLLLNDDKNKMY
P. oRUVBL1	452	IGINCSLRAYIQLLTPAKILSKRKGGKKNKCIIEIVSSIFFD	TKRSTQLLLNDDKNKMY
P. vRUVBL1	520	IGIQCSLRAYIQLLTPAKILSKRKGGKKNKSIVEIVSSIFFD	TKRSTQLLLNDDKNKMY
P. kRUVBL1	472	IGIQCSLRAYIQLLTPAKILSKRKGGKKNKSIIIEIVSSIFFD	TKRSTQLLLNDDKNKMY
P. fRUVBL2	416	IGIQSSLRFAMLLEPSRIATLEGNTIIDIKHIEQADELFMDAKT	SAHRVVDQSNKFEVN
P. mRUVBL2	416	IGIQSSLRFAMLLEPSRIATLEGNTIIDIKHIEQADELFMDAKT	SAHRVVEQANKFEVN
P. oRUVBL2	416	IGIQSSLRFAMLLEPSRIATLEGNTIIDIKHIEQADELFMDAKT	SAHRVAEQSNKFEVS
P. vRUVBL2	416	IGSQSSLRFAMLLEPSRIATLEGNTIIDIKHIEQADELFMDAKT	SAHRVAEQSNKFEVS
P. kRUVBL2	416	IGSQSSLRFAMLLEPSRIATLEGNTIIDIKHIEQADELFMDAKT	SAHRVAEQSNKFEVN
P. bRUVBL2	416	IGIQSSLRFAMLLEPSRIATLEGNTIIDIKHIEQADELFMDAKT	SAHRVADQSNKFEVN
P. fRUVBL3	390	IASSESLRYALHLITLANLVSKKRKATEVTVDVRRVYNLFIDV	KRSTQYLIIEYQNEFMF
P. mRUVBL3	390	IASSESLRYALHLITLANLVSKKRKATEVTVDVRRVYNLFIDV	KRSTQYLIIEYQNEFMF
P. oRUVBL3	390	IASSESLRYALHLITLANLVSKKRKATEVTVDVRRVYNLFIDV	KRSTQYLIIEYQNEFMF
P. vRUVBL3	390	IASSESLRYALHLITLANLVSKKRKATEVTVDVRRVYNLFIDV	KRSTQYLIIEYQNEFMF
P. kRUVBL3	390	IASSESLRYALHLITLANLVSKKRKATEVTVDVRRVYNLFIDV	KRSTQYLIIEYQNEFMF
P. bRUVBL3	390	IASSESLRYALHLITLANLVAKKRKATEVTVDVRRVYNLFIDV	KRSTQYLIIEYQNEFMF
Rvb1	406	MGTETSLRYALQLLAPCGILAQTSNRKEITVNDVN	EAKLLFLDAKRSTKILETSAN-YL-
Rvb2	390	TGVETSLRYSSNLISVAQIAMKRKNNTVEVEDVKRAYLLFLD	SARSVKYQENESQYID
HsRUVBL1	397	IGTKTTIRYSVQLLTPANLAKINGKDSIEKEHVEEISELFYDAK	SSAKILADQDKYMK
HsRUVBL2	393	IGLETSLRYAIQLLTAASLVCRKRKGTEVQVDDIKRVYSLFLD	ESRSTQYMKYQDAFLF

Figure 2.1.1: Sequence similarity between RUVBL proteins from *Plasmodium*, human, and yeast homologs

The residues highlighted represent Walker A (dark red box) and Walker B (purple box), which are implicated in ATP binding and hydrolysis, respectively (Walker *et al.*, 1982). Highlighted motifs are sensor 1 (blue box), sensor 2 (orange box), and arginine finger (red dashed box) (Neuwald *et al.*, 1999; Seraphim and Houry, 2020). Asp amino acid regulates Mg^{2+} (red arrow), and Glu amino acid is also highlighted by a green arrow, a yellow box represents conserved residues, the insertion domain is represented by a dashed purple box, and conserved motifs are highlighted by the dashed green box. Accession numbers: *P. falciparum* RUVBL1 (PF3D7_0809700), *P. berghei* RUVBL1 (PBANKA_1427700), *P. malariae* RUVBL1 (PmUG01_14046600), *P. ovale* RUVBL1 (PocGH01_14037900), *P. vivax* RUVBL1 (PVP01_1430600), *P. knowlesi* RUVBL1 (PKNH_1431000), *P. falciparum* RUVBL2 (PF3D7_1106000), *P. malariae* RUVBL2 (PmUG01_09015600), *P. ovale* RUVBL2 (PocGH01_09012700), *P. vivax* RUVBL2 (PVP01_0906800), *P. knowlesi* RUVBL2 (PKNH_0903600), *P. falciparum* RUVBL3 (PF3D7_1362200), *P. malariae* RUVBL3 (PmUG01_11023900), *P. ovale* RUVBL3 (PocGH01_11018400), *P. vivax* RUVBL3 (PVP01_1109800), *P. knowlesi* RUVBL3 (PKNH_1109100), Rvb1 *Saccharomyces cerevisiae*

(QHB07651.1), Rvb2 *Saccharomyces cerevisiae* (QHB12030), *Homo sapiens* RUVBL1 (NP_003698 XP_944800), *Homo sapiens* RUVBL2 (CAG38538). The analysis was conducted using Bioedit tool (www. Bioedit. Software. Informer. com).

2.3.2 Phylogenetical analysis of *Plasmodium* RUVBL1, RUVBL2, and RUVBL3 proteins

The phylogenetic analysis of the *Plasmodium* RUVBL proteins with their yeast and human orthologues was conducted by comparison of the amino acid sequences. Two distinct clusters were observed in the phylogenetic analysis. The first cluster comprised *Plasmodium* RUVBL3 proteins with human RUVBL2 and yeast Rvb2 (Figure 2.1.2). In addition, *Plasmodium* RUVBL1 and RUVBL2 clustered with human RUVBL1 and yeast Rvb1 (Figure 2.1.2). Further, the *Plasmodium* RUVBL1 and RUVBL2 cluster is subdivided into two closely related clusters of independent *Plasmodium* RUVBL1 and RUVBL2 sub-clusters (Figure 2.1.2). PfRUVBL1 and PfRUVBL2 are closely related to yeast Rvb1, while PfRUVBL3 is more closely related to yeast Rvb2 (Ahmad *et al.*, 2013).

Phylogenetic analysis showed that essential RUVBL protein domains (Walker A, Walker B, sensor 1, and sensor 2) are conserved across species (Figure 2.1.1), suggesting their role in processes such as DNA repair, chromatin remodeling, and ribosome biogenesis (Seraphim *et al.*, 2019). *Plasmodium* RUVBL proteins show significant divergence from their human and yeast counterparts (Figure 2.1.2). This might suggest adaptations unique to the parasite's life cycle, including its interaction with the host or its specific cellular needs, such as stress response during the erythrocytic stages. The analysis indicated a close association between human RUVBL1 and yeast Rvb1 proteins, similar to human RUVBL2 and yeast Rvb2. The divergence observed in yeast and human RUVBL proteins is distinct and unique compared to *Plasmodium* RUVBL proteins. This suggests that distinct functions in these homologs evolved early in the lineage. The comparison of phylogenetic analysis mirrors observation made using sequence alignment data in Figure 2.1.1.

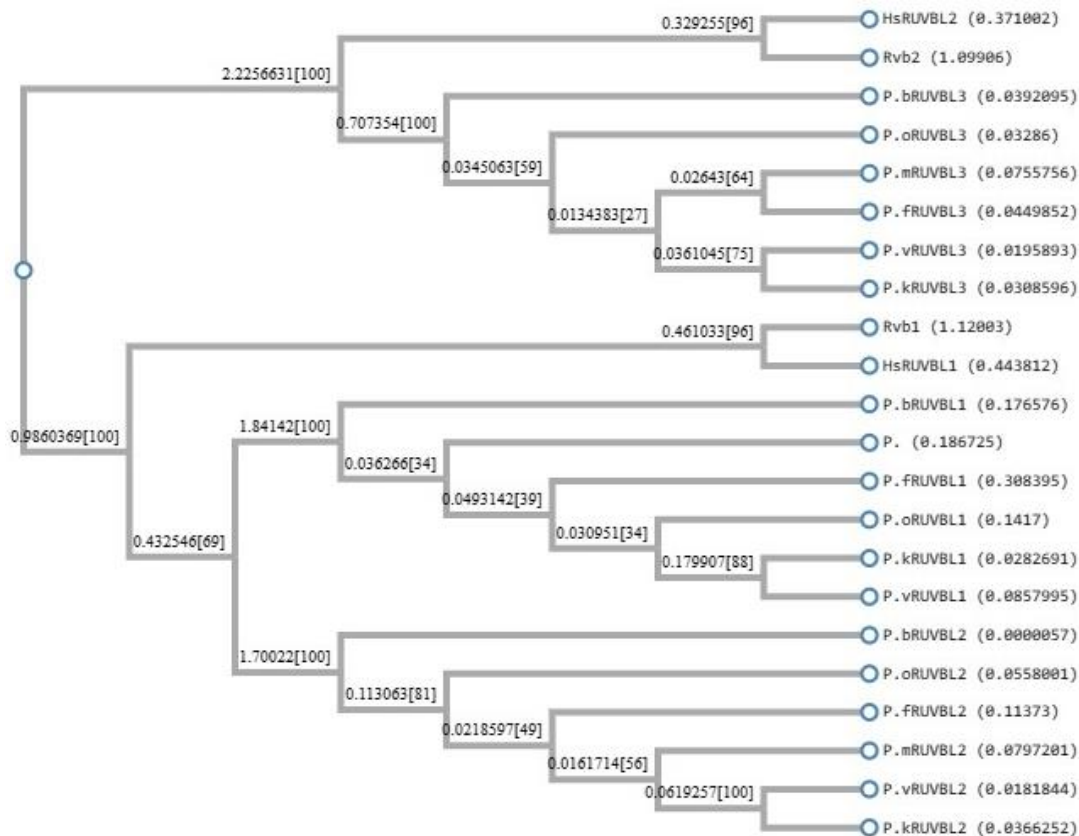


Figure 2.1.2: Evolutional analysis of malaria parasites RUVBL-like proteins

The neighboring joining phylogenetic tree of PfRUVBL proteins and their homologs. Dendrograms were constructed using Clustal Omega EMBL-EBI (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>), and the bootstrap was set at 100. Accession numbers are as described in Figure 2.1.1

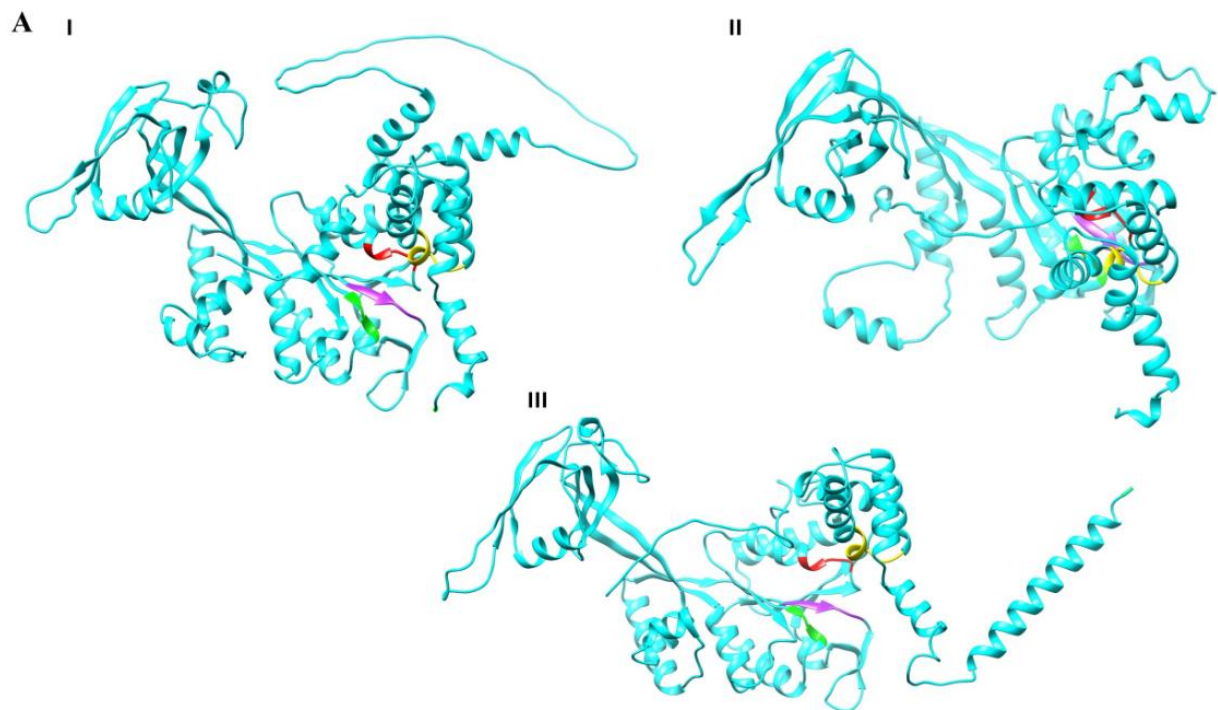
2.4 Three-dimensional homolog models of PfRUVBL1, PfRUVBL2, and PfRUVBL3

The *in silico* structural prediction and superposition of three-dimensional models of PfRUVBL1, PfRUVBL2, and PfRUVBL3 were conducted to ascertain the level of fold similarity. The predicted sequences were used to generate models, and the superposition tool (Chimera 1.15v) was used to predict the degree of identity. Sequence comparison involves the direct approach to identifying homologs (Hubbard, 1997), while three-dimensional models of proteins could be helpful where sequence similarity is low (Murzin *et al.*, 1995). The minimum level of similarity for proteins to be inferred as homologs has been set at 30% (Peason, 2013).

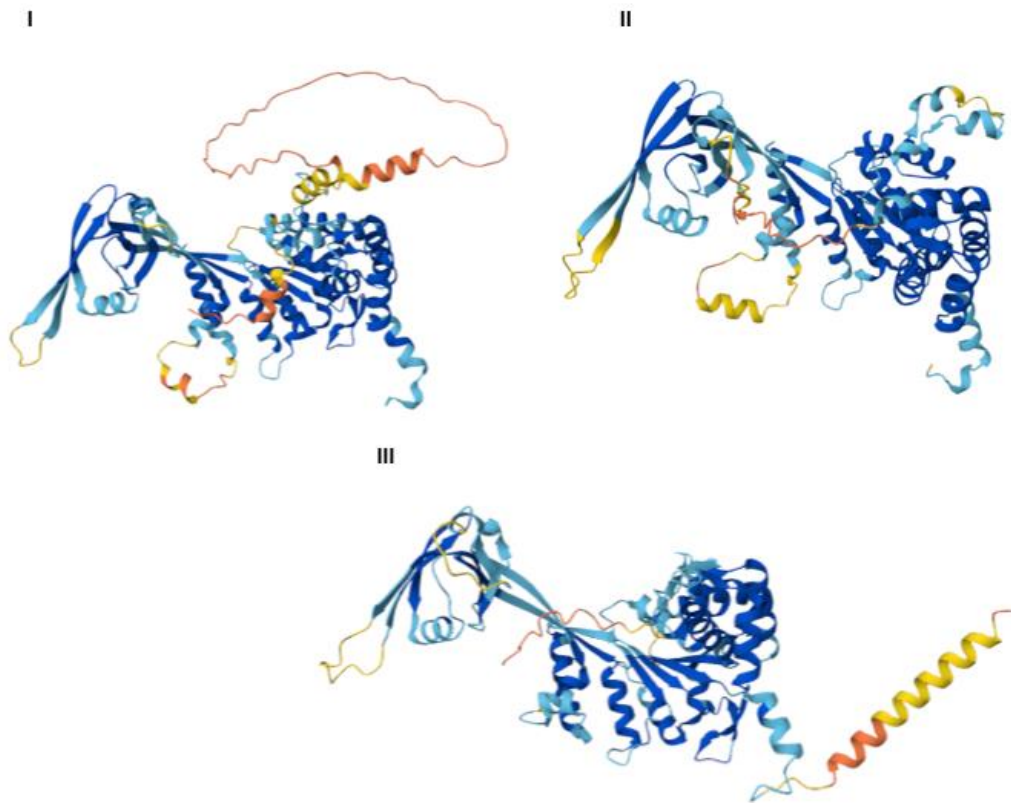
Walker A, Walker B, and sensor 2 motifs were predicted to be predominantly α -helix structures, while sensor 1 is composed of β -sheets (Figure 2.1.3). All AAA+ functional motifs, including Walker A, Walker B, sensor 1, sensor 2, and arginine finger, were predicted to be in similar structural positions in all three *P. falciparum* RUVBL proteins. This may suggest that PfRUVBL1, PfRUVBL2, and PfRUVBL3 have similar functions or similar biological

behavior. Structural super positioning results showed that PfRUVBL1 and PfRUVBL2 share 45% structural identity (Figure 2.1.3). Human RUVBL1 and RUVBL2 share 43% sequence identity and possess ATPase functional domains (Dauden *et al.*, 2021).

The observed structural identity supports observations in the sequence alignment and phylogenetic analysis (Figure 2.1.1; Figure 2.1.2). PfRUVBL1 and PfRUVBL3 share 33% identity, while PfRUVBL2 and PfRUVBL3 share 34% identity (Figure 2.1.3). The high structural identity that these proteins share argues that they are homologs and that PfRUVBL1 and PfRUVBL2 are closely related.



B



C

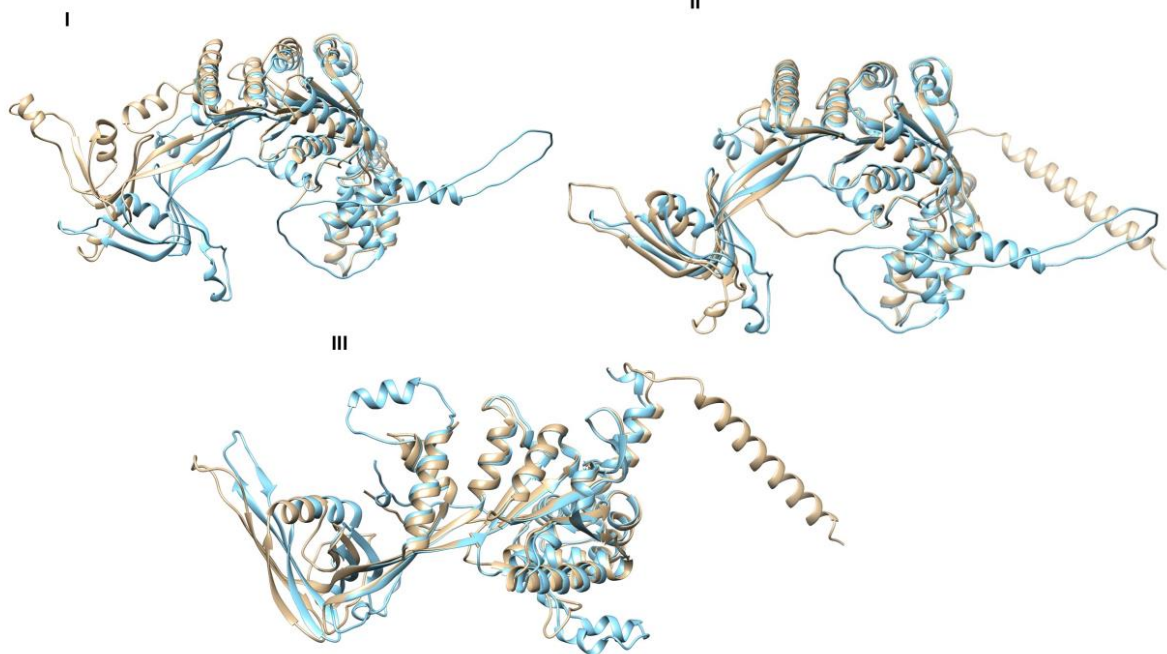


Figure 2.1.3: Three-dimensional models of PfRUVBL1, PfRUVBL2 and PfRUVBL3

Three-dimensional models (A) Walker A (red), Walker B (green), Sensor 1 (purple), and Sensor 2 (yellow). (B) de novo AlphaFold three-dimensional models (C) super positioned structures, (I) PfRUVBL1 (light blue) and PfRUVBL2 (gold) with 45% identity, (II) PfRUVBL1 (light blue) and PfRUVBL3 (gold) with 33.4% identity, (III) PfRUVBL2 (light blue) and PfRUVBL3 (gold) with 34% identity. Sequences obtained from PlasmoDB (www.plasmodb.org) and structures remodelled using the UCSF Chimera v1.15 tool.

2.5 Predicted secondary structural features of PfRUVBL1, PfRUVBL2, and PfRUVBL3 proteins

Primary amino acid sequence folds to form secondary structures that are stabilized by hydrogen bonds in their polypeptide backbone (Banach *et al.*, 2019). The hydrogen bond emanates from the attractive force between the slightly positive hydrogen atom and an electronegative acceptor atom, oxygen (Rehman *et al.*, 2022). Hydrogen bonding is important for structural integrity and stability (Myers and Pace, 1996), secondary structure formation (Kundu *et al.*, 2009), and molecular recognition (Deremble and Lavery, 2005). There are four known folds of proteins: α -helix and β -pleated sheet, beta-turn, and random coil (Table 1, Appendix A).

Disordered regions in proteins refer to segments that do not adopt a fixed or stable three-dimensional structure under physiological conditions. If the entire protein lacks a defined structure, these regions are often called intrinsically disordered regions (IDRs) or intrinsically disordered proteins (IDPs). Disordered regions play crucial functional roles in proteins despite their lack of a stable structure. They are integral to protein-protein interactions, molecular flexibility, conformational changes, post-translational modifications, and the regulation of protein function (Babu *et al.*, 2011; Dunker *et al.*, 2013).

The secondary structures analysis, as predicted by the tool Phyre2, was based on 100% confidence of varying covered regions (Table 1, Appendix B1). Helix structures were predicted to be dominant in all proteins (Table 1). The percentage contribution of the helix was projected to be similar between PfRUVBL2, PfRUVBL3, and Rvb2, while PfRUVBL1 and Rvb1 showed marginal differences. These results mirror observations made using both the sequence alignment (Figure 2.1.1) and phylogenetic analysis (Figure 2.1.2). In *P. falciparum*, disordered regions are essential in enabling parasite proteins to interact with various host proteins and aiding the parasite in evading detection by the host immune system (Guy *et al.*, 2015).

Table 1: Predicted secondary structural features of the R2TP members of *P. falciparum*

Protein	Helix %	B-strand %	Disordered %	Confidence %	Coverage %
PfRUVBL1	28	18	8	100	79
PfRUVBL2	32	21	12	100	93
PfRUVBL3	32	23	9	100	86
RVB1	29	22	6	100	95
RVB2	32	18	12	100	90
RUVBL1	34	14	12	100	87
RUVBL2	31	20	8	100	89

Prediction of *P. falciparum* RUVBL proteins, yeast Rvb proteins, and Homo sapiens RUVBL-like proteins secondary structural features. The analysis was conducted using the Phyre2 tool (www.sbg.bio.ic.ac.uk).

2.6 *In silico* analysis of *P. falciparum* RUVBL proteins and their interaction with ATP

The docking technique was used to predict the binding affinity of ligand ATP to receptor proteins. PfRUVBL1 showed binding to ATP with strong affinity compared to PfRUVBL2 and PfRUVBL3. The residues that form hydrogen bonds with ATP from PfRUVBL1 are all constituents of the Walker A motif (Ser¹⁴⁴, Gly¹⁴⁵, Lys¹⁴⁶, Thr¹⁴⁷), while in PfRUVBL2, no interaction between ATP and Walker A was observed. The interaction between PfRUVBL3 and ATP was observed to be dominantly facilitated by van der Waals forces (Figure 2.1.4).

ATP binding affinity to PfRUVBL1 was observed to be (-8.0 kcal/mol), PfRUVBL2 (-5.6 kcal/mol), and PfRUVBL3 (-6.5 kcal/mol) (Figure 2.1.4). Yeast Rvb1 showed strong ATP affinity (-8.8 kcal/mol) compared to yRvb2 (-7.6 kcal/mol). Rvb1 interacts with ATP through the Walker A motif with residues (Gly⁸⁰, Ser⁸², Thr⁸³, Gly⁸⁴, Thr⁸⁶), while yRvb2 binds ATP with residues distributed between the Walker A and insertion domain.

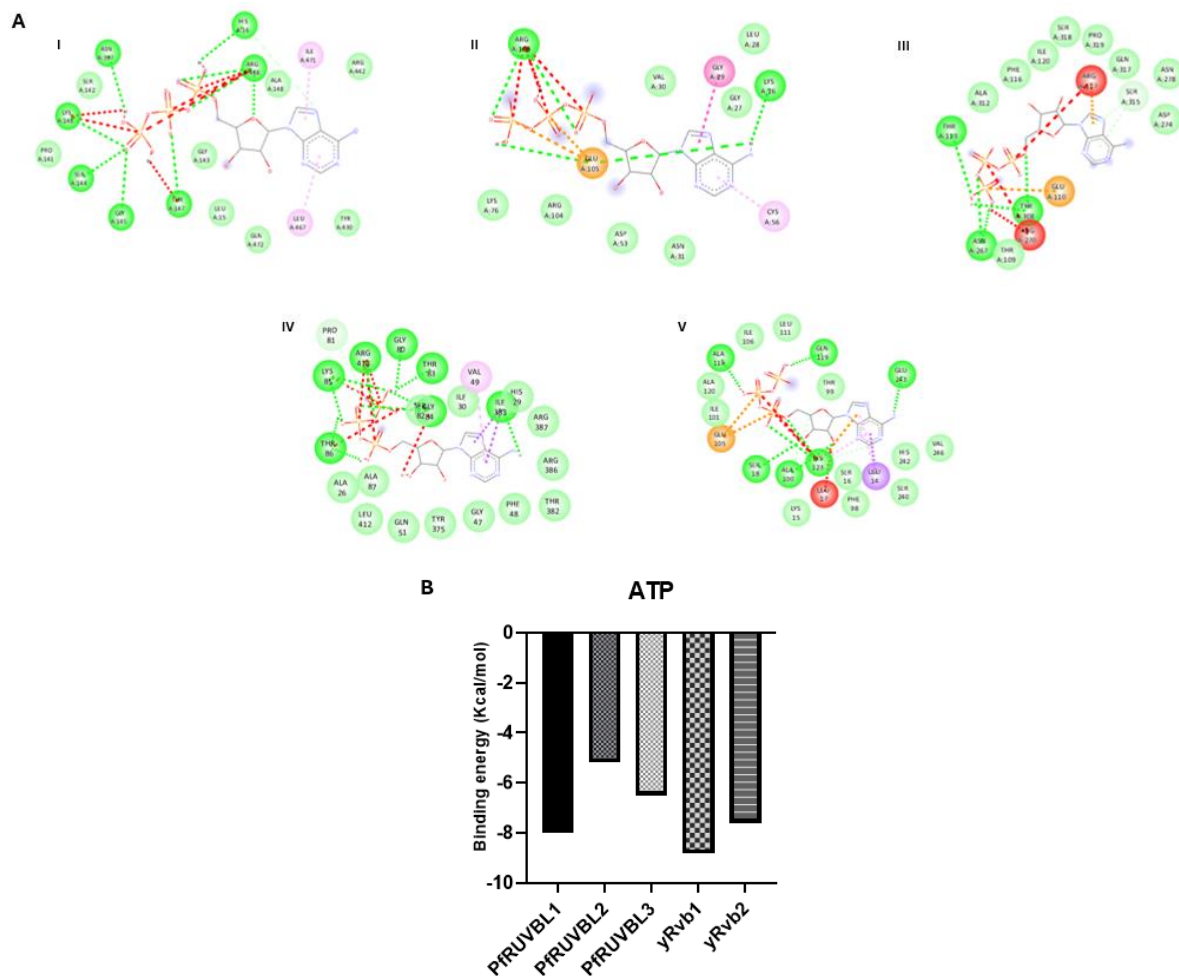


Figure 2.1.4: Molecular predictive docking of ATP binding to PfrUVBL1, PfrUVBL2, PfrUVBL3, yRvb1, and yRvb2

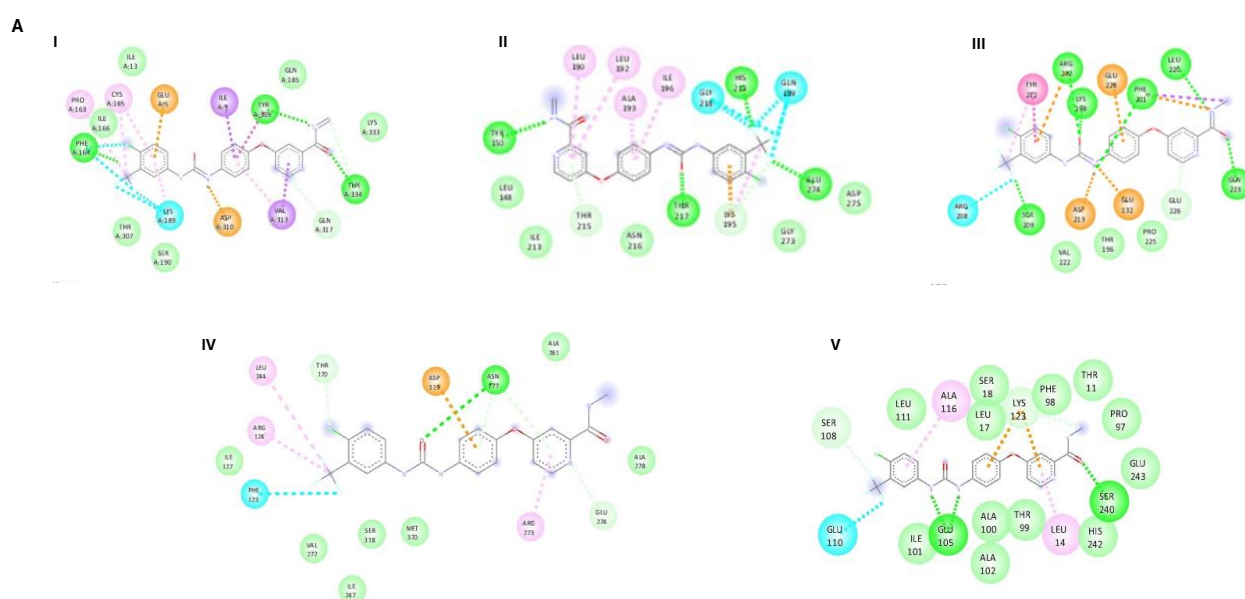
ATP binds to PfrUVBL proteins comparable to yeast orthologs, (A) binding structure showing the residues involved in the interaction, (B) graphic representation of predicted binding energy scores. The analysis was conducted using PyRx (www.pyrx.sourceforge.io) and rendered on Discovery Studio v21.1.0.20298.

PfrUVBL1, PfrUVBL2, and PfrUVBL3 belong to the AAA+ ATPase family. Their activity relies on ATP binding and hydrolysis. ATPases utilize the energy from ATP binding and hydrolysis to bring about structural changes in themselves and the protein complexes they interact with. Therefore, it is important to understand how PfrUVBL1, PfrUVBL2, and PfrUVBL3 interact with ATP to predict the necessary structural changes for their function in the R2TP complex. As anticipated, PfrUVBL1, PfrUVBL2, and PfrUVBL3 exhibit highly conserved ATP-binding domains like their counterparts in other organisms (Figure 2.1.1). Molecular docking results have revealed the key residues involved in ATP binding and hydrolysis.

2.7 In silico recognition of sorafenib by *P. falciparum* RUVBL proteins

In the current study, the binding affinity of sorafenib for PfRUVBL1 (I), PfRUVBL3 (II), PfRUVBL3 (III), yRvb1 (IV), and yRvb2 (V) was investigated using docking-based virtual screening analysis. The findings show that sorafenib binds to PfRUVBL1 and PfRUVBL3 with a strong affinity of -8.6 and -8.5 Kcal/mol, respectively, it binds to PfRUVBL2 with -8.1 Kcal/mol binding affinity (Figure 2.1.5). Sorafenib showed a strong binding affinity on Rvb2 of -9.0 Kcal/mol compared to Rvb1 with an affinity of -7.5 Kcal/mol.

Sorafenib has been identified as an inhibitor of RUVBL2, which inhibits the ATPase activity of the RUVBL1/2 complex (Nano *et al.*, 2020). The residues that sorafenib engages with investigated proteins are mostly located close to the Walker A motif. Most of the observed interactions are facilitated by hydrogen bonding and van der Waals forces (Figure 2.1.5). Hydrogen bonds and van der Waals forces provide most of the interactions that underpin protein folding, structure, and molecular recognition and are potentially significant in drug discovery (Hubbard *et al.*, 2010; Itoh *et al.*, 2019).



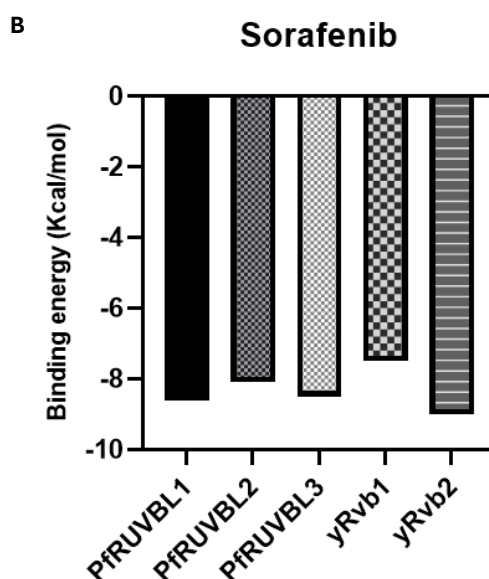


Figure 2.1.5: Molecular docking of Sorafenib to PfrUVBL1, PfrUVBL2, PfrUVBL3, yRvb1, and yRvb2
Sorafenib binds to PfrUVBL proteins comparable to yeast orthologs, (A) binding structure showing the residues involved in the interaction, (B) graphic representation of predicted binding energy scores. The analysis was conducted using PyRx (www.pyrx.sourceforge.io) and rendered on Discovery Studio v21.1.0.20298.

2.8 In silico analysis of select TPR-containing domain proteins of *P. falciparum*

The R2TP complex member that contains TPR domains was suggested to be a gene encoding PF3D7_1434300 using bioinformatics analysis (Ahmand *et al.*, 2013). The protein encoded by the PF3D7_1434300 gene contains three TPR domains in its 564 amino acid-length structure, like human RPAP3 (665 amino acid residues). As much as this protein was suggested, it was also shown that this protein lacks the RPAP3-specific C-terminal domain (Maurizy *et al.* 2018). A detailed literature survey revealed that this gene encodes for the Hsp70/Hsp90-organizing protein (Hop) (Gitau *et al.*, 2012; Zininga *et al.*, 2015; Silva *et al.*, 2019). PfTah1 (PF3D7_0213500) is a putative ortholog of the yeast Tah1 (TPR-containing protein associated with Hsp90), which is 460 amino acids long and has been proposed to be a member of the R2TP complex (Reviewed by Dutta *et al.*, 2022).

In the current study, sequence alignment was used to analyze the degree of conservation between PfHop, hHop, Tah1, RPAP3, and PfTah1 (Figure 2.2.1). The TPR1 domain (based on PfHop) is conserved, with residues implicated in TPR1/Hsp70 interactions being conserved in PfHop, hHop, and PfTah1. The TPR2A domain is fairly conserved with a high level of conservation in residues implicated in TPR2A/Hsp90 interaction (Figure 2.2.1). The TPR2B domain is also fairly conserved with high levels of similar amino acids. The observed similarities may suggest that these proteins serve overlapping roles.

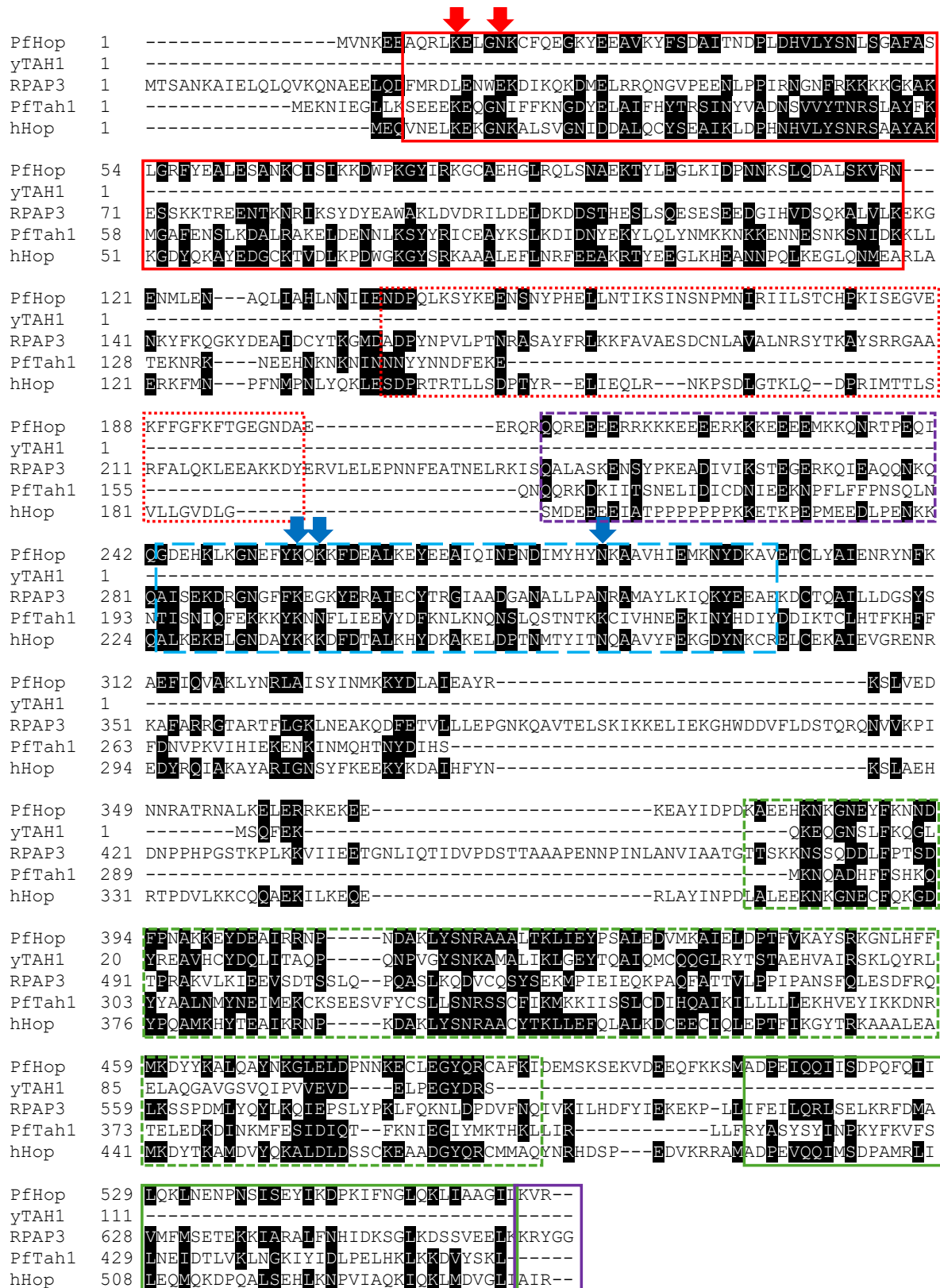


Figure 2.2.1: Sequence alignment of TPR domains containing proteins

Colours denote: TPR1 domain (red solid), TPR2A domain (dashed blue), TPR2B (green dots), DP1 (red dots), DP2 (solid green), NLS1 (purple dots), NLS2 (purple solid). Amino acids are implicated in TPR1/Hsp70 interaction (red arrow) and TPR2A/Hsp90 interactions (blue arrow). Hop *P. falciparum* (PF3D7_1434300), RPAP3 Human (NP_001139547.1), TAH1 yeast (AJQ35178.1), PfTah1

(PF3D7_0213500), hHop (NP_001269581.1). The analysis was conducted using Bioedit (www.bioedit.software.informer.com).

Table 2: Secondary structure features

Protein	Helix %	B-strand %	Disordered %	Confidence %	Coverage %
PfHop	69	0	0	100	57
RPAP3	54	0	6	99.8	20
PfTAH1	47	0	38	99.8	67

Secondary structures prediction revealed that all the proteins are predominated by alpha helix structures. PfHop 100% showed 69% α -helix, RPAP3 reported 54% α -helix, and PfTAH1 revealed 47% α -helix (Table 2, Appendix B2). α -helix are stabilized by hydrogen bonds between the carbonyl oxygen of one amino acid and the amide hydrogen of another, located four residues away. This repetitive hydrogen bonding provides a stable structure without the need for complex interactions with the environment. Alpha helix structures play critical roles in protein-protein interactions, serving as structural motifs that facilitate specific and stable binding (Edwards and Wilson, 2011). Homodimers have longer helical regions with prominent helix-helix pairs (Guharoy and Chakrabarti, 2007). PfTah1 showed a high percentage of disordered regions, and this may be due to the lack of a stable 3D structure, which led to several dynamics during interconversion.

2.9 Three-dimensional homolog models of RPAP3, PfHop, and PfTah1

The matrix 62 was used to evaluate the degree of conservation between PfTah1, PfHop, and RPAP3 (Figure 2.2.2). The iteration of pruning long atom pairs was set not to exceed 2.0 angstroms. Results showed the percentage of identity for RPAP3 and PfTah1 as 0.22%, while the one for PfTah1 and PfHop was 6.09%. The low identity percentage suggests that these proteins are not homologs.

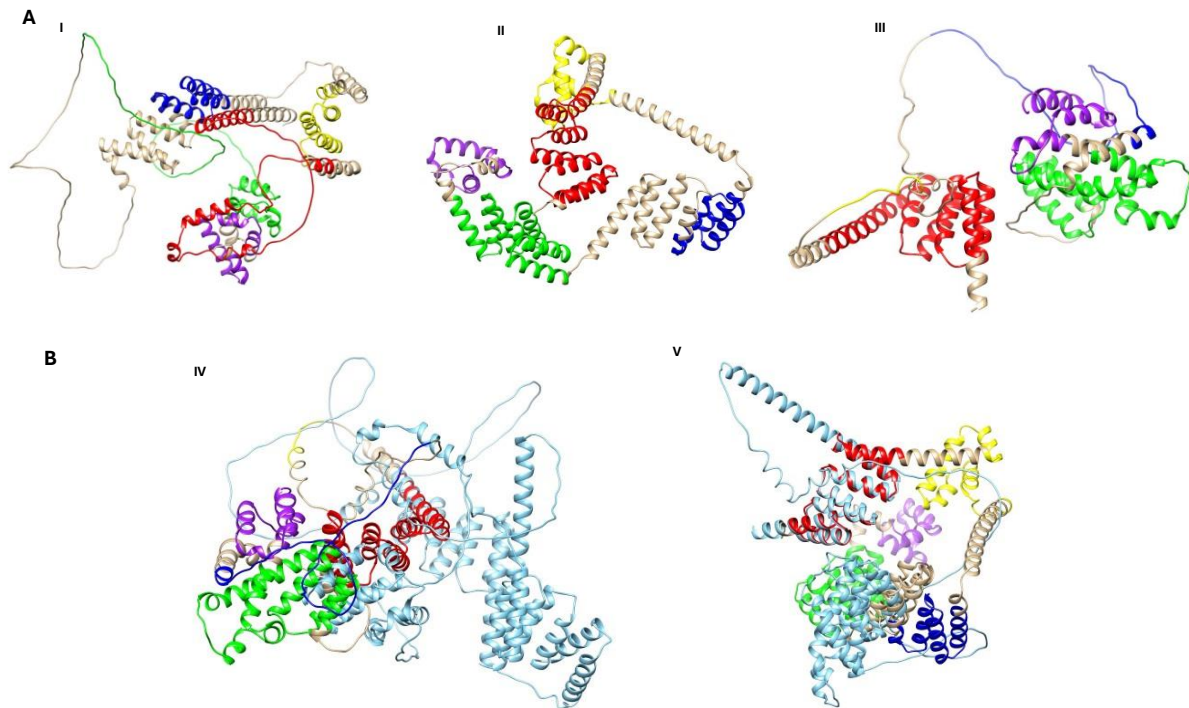


Figure 2.2.2: Homology remodelling of RPAP3, PfHop, and PfTah1

Highlighted are the following motifs: (A) Three-dimensional models of PfTah1 (I), PfHop (II), and RPAP3 (III), also highlighted are functional motifs, TPR1 (red), TPR2A, (blue), TPR2B (green), DP1 (yellow), and DP2 (purple). (B), super-positioned structures RPAP3 and PfTah1 (IV), PfTah1 and PfHop (V), showing structural conformational similarities. Sequences obtained from accession numbers as mentioned in Figure 2.2.1. The sequences were rendered on the UCSF Chimera 1.15 tool.

2.10 The *in silico* prediction of the interaction network of PfHsp90

The PfHsp90 search criteria included at most 20 interactors in the first shell and at most 100 interactors in the second shell. The predicted interactome revealed that PfHsp90 interacts with a wide range of clients with diverse cellular functions (Figure 2.3). These functions include transcription factors, translocation, complex subunits, kinases, ATPases, ribosomal components, mitochondrial proteins, and activators, among others.

PfHsp90 is essential for parasite development and survival (Banumathy *et al.*, 2003). At least 627 putative Hsp90-interacting proteins were identified. Among them, two unknown proteins were named and further characterized: Pih1 (protein interacting with Hsp90) and Tah1 (TPR-containing protein associated with Hsp90) (Zhao *et al.*, 2005). Two *P. falciparum* R2TP complex components were identified by STRING as PfHsp90 interactors, PfTah1 (PF3D7_0213500) and PfPIH1(PF3D7_1235000). In addition, PfHsp70 (PF3D7_0818900) and PfHop (PF3D7_1434300) were also identified (Figure 2.3).

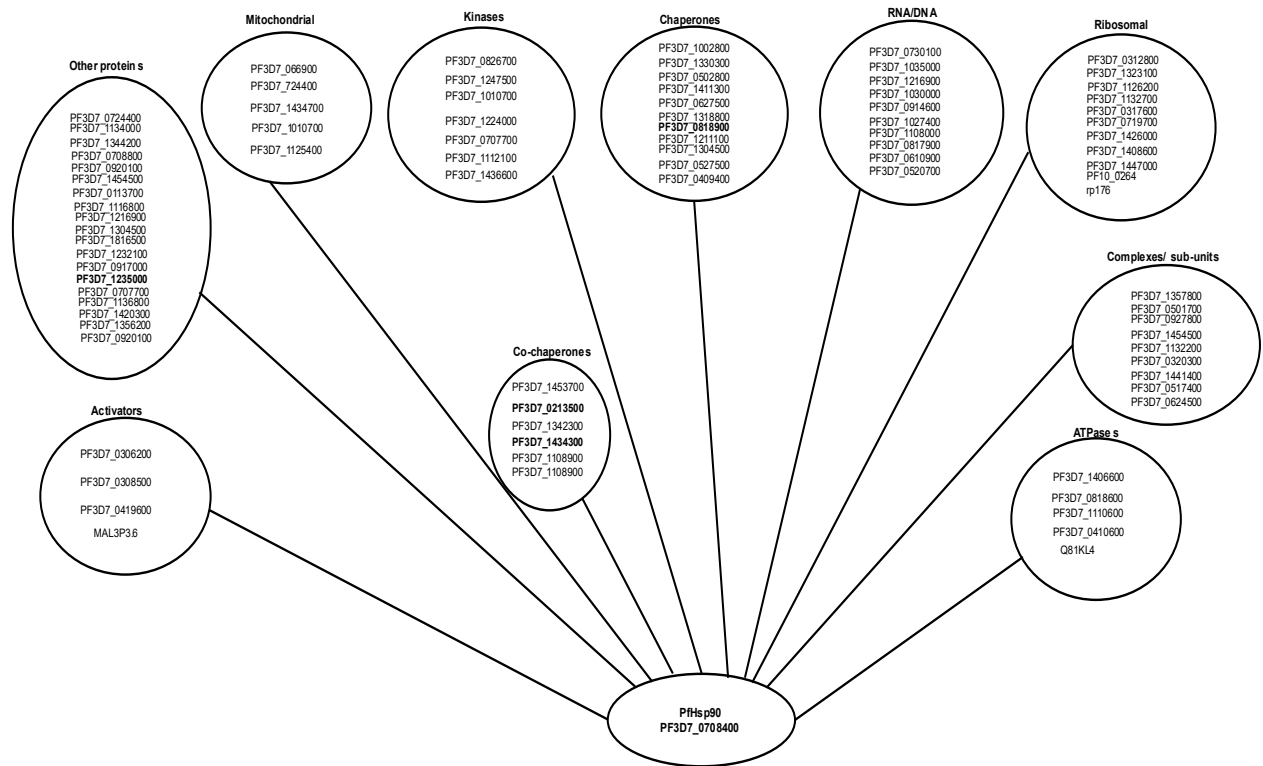


Figure 2.3: PfHsp90 predicted interactors network

Summarised Interaction network of PfHsp90 based on 100 closest partnerships as predicted by STRING. In bold are R2TP complex members (PfTah1, PF3D7_0213500 and PfPIH1, PF3D7_1235000) as well as PfHsp90 folding machinery components (PfHsp70-1, PF3D7_0818900 and PfHop, PF3D7_1434300).

2.11 Discussion

The RUVBL proteins of *Plasmodium* species belong to the AAA+ family and possess all ATPase functional motifs. These motifs, including Walker A, Walker B, Sensor 1, Sensor 2, and R-Finger, are highly conserved across *Plasmodium* species RUVBL proteins. Furthermore, sequence alignment has revealed a close similarity between PfRUVBL1 and PfRUVBL2 proteins compared to PfRUVBL3. This aligns with a study by Sen et al. (2018), which reported that PfRUVBL1 and PfRUVBL2 are closely related homologs with 43% sequence similarity.

In this study, sequence alignment data revealed several conserved substitutions in PfRUVBL1, PfRUVBL2, and PfRUVBL3 with minor variations and substitutions. These substitutions involve polar uncharged amino acids in Walker A, valine to alanine in Walker B, and various hydrophobic amino acids in sensor1. Additionally, a non-conserved substitution was found in sensor2. The conserved substitutions may allow the proteins to retain their functional integrity (French and Robson, 1983). The similarity matrix BLOSUM62 showed the sequence identity between PfRUVBL1 and PfRUVBL2 to be 43%, while PfRUVBL1 and PfRUVBL3 revealed 32% identity, and PfRUVBL2 and PfRUVBL3 had 35% sequence identity.

Phylogenetic analysis reveals that *Plasmodium* species RUVBL proteins are divergent from those of *E. coli* and *H. sapiens*. *Plasmodium* RUVBL1 and RUVBL2 may arise from a common ancestor distinct from RUVBL3, which clusters with *H. sapiens* RUVBL2 and yeast Rvb2 (Figure 2.1.2). This supports a study by Sen et al. (2018) indicating that PfRUVBL1 and PfRUVBL2 are closely related to yeast Rvb1, while PfRUVBL3 is closely related to yeast Rvb2. Sequence alignment and analysis with well-studied yeast and human homologs suggest that all malarial parasite RUVBL proteins may be classified as belonging to the AAA+ family and probably play similar roles in parasite development as observed in their homologs.

Secondary structures arise due to the regular folding of the backbone of the polypeptide chain, facilitated by hydrogen bonding between the main chain carbonyl oxygen and amide nitrogen (Dunker *et al.*, 2013). The study showed that *P. falciparum* RUVBL proteins are mainly made up of α -helix structures, as indicated in Table 1. Proteins with a significant amount of α -helix in their secondary structures facilitate interaction, including protein-protein and protein-nucleic acid interactions, which are processes that are suggested to be facilitated by the R2TP complex (Johene and Thornton, 1995). Physical interactions between proteins, either stable or transient in the form of complexes, are central and important to *P. falciparum* cellular processes (Hillier *et al.*, 2019).

The disordered regions observed in all three *P. falciparum* RUVBL proteins may enable them to adopt multiple conformations and interact with various partners. This flexibility is crucial for signalling proteins, where the same region can bind to different molecules depending on the cellular context (Iqbal *et al.*, 2015). In *Plasmodium falciparum*, disordered regions are essential in enabling parasite proteins to interact with various host proteins and aiding the parasite in evading detection by the host immune system. Intrinsically disordered proteins have various functions, including regions. Numerous intrinsically disordered proteins have been characterized, including kinases, transcription factors, and translation inhibitors (Dunker *et al.*, 2002). Disordered regions of transcription factors and kinases have been associated with protein-protein binding and protein-DNA binding (Dunker *et al.*, 2014). RUVBL1 and RUVBL2 proteins play a dual role in interacting with other proteins to form macromolecular complexes and directly bind to DNA to regulate chromatin structure and DNA repair (Zhao *et al.*, 2005; Seraphim *et al.*, 2019).

The ATP-dependent activity of these proteins reflects the role of this nucleotide in regulating the R2TP complex assembly. Predicting their interaction with ATP will clarify how energy derived from ATP hydrolysis drives these essential cellular processes in the malaria parasite. ATP-binding pockets are common drug targets, particularly for ATPase proteins like PfRUVBL1, PfRUVBL2, and PfRUVBL3. Furthermore, the interaction of sorafenib with PfRUVBL1, PfRUVBL2, and PfRUVBL3 was observed to be facilitated by residues unique to those involved in ATP binding, suggesting that sorafenib is not an ATPase mimic inhibitor. The presence of ATP has been reported to facilitate higher-order RUVBL complexes and stimulate R2TP-mediated complex assembly (McKeegan *et al.*, 2009; Kim *et al.*, 2013).

Sorafenib is primarily known for its role in targeting kinases like RAF and VEGFR in cancer, but its effects on ATP-binding proteins like PfRUVBL1, PfRUVBL2, and PfRUVBL3 could reveal a novel role for it. Research has shown that sorafenib has an IC₅₀ of 6nM for Raf1 kinase and 57nM for p38 α kinase (Namboodiri *et al.*, 2010). This compound has been proposed as a potential inhibitor of the RUVBL2 protein (Nano *et al.*, 2020). Sorafenib, as a kinase inhibitor, binds to ATP-binding pockets in kinases to inhibit ATP-dependent signalling. PfRUVBL1, PfRUVBL2, and PfRUVBL3 are ATPases (Ahmand *et al.*, 2013; Seraphim *et al.*, 2019; Luthuli and Shonhai, 2023), they also possess ATP-binding domains, interesting molecular docking results showed that sorafenib does not interact with Walker domains of *P. falciparum* RUVBL proteins.

PfHop, RPAP3, and PfTah1 are thought to localize in the cytosol (Rodriguez and Llorca, 2020; Gitau *et al.*, 2012). PfHop is proposed to facilitate the cooperation of two prominent cytosolic chaperones, PfHsp70-1 and PfHsp90 (Gitau *et al.*, 2012). This complex is essential for the folding and maturation of proteins, including kinases and steroid hormone receptors (Carrigan *et al.*, 2015). Even though these proteins contain TPR domains, PfHop possesses three TPR domains (TPR1, TPR2A, TPR2B) (Gitau *et al.*, 2012), RPAP3 has 6 TPR domains (Rodriguez and Llorca, 2020), and PfTah1 is predicted to possess three TPR domains. In this study, sequence alignment revealed that PfHop, RPAP3, and PfTah1 contain conserved residues implicated in both PfHsp70 and PfHsp90 interaction. In the TPR1 (based on PfHop), residues lysine (K¹¹) and asparagine (N¹⁵) that are implicated in PfHsp70 interaction are conserved (Figure 2.2.1). In addition, the TPR2A residues lysine (K²⁴⁹, N²⁵¹) that are involved in Hsp90 interaction are also conserved. Both domains had evenly distributed and conserved lysine and asparagine residues (Figure 2.2.1).

Literature has reported that the TPR1 and TPR2B subdomains of PfHop preferentially bind to PfHsp70-1 in its C-terminal (EEVD) motif, while TPR2A prefers to bind to PfHsp90 (Gitau *et al.*, 2012). Previous studies have shown that the RPAP3 carboxylate clamp forms polar contact with the Hsp70 peptide through the Hsp70 EEVD motif (Henri *et al.*, 2018). The exact mechanism by which RPAP3 interacts with Hsp70 is not fully understood, while its interaction with Hsp90 has been demonstrated (Abel *et al.*, 2022). PfTah1 is a putative protein that has not been studied yet. Structurally, RPAP3, PfTah1, and PfHop are predicted to be predominated by α -helix folds, making them ideal for interaction with other biological entities. The PfHsp90 interactome prediction has revealed the two R2TP complex components as part of its diverse and wide range of clients (Figure 2.3). Genomic-wide screening revealed Pih1 and Tah1 as Hsp90 interactors (Zhao *et al.*, 2005). Therefore, it is not surprising that *P. falciparum* genomic screening identified PfTah1 and PfPih1 as PfHsp90 interactors using computational methods (Dutta *et al.*, 2022).

In conclusion, the findings from this study noted that PfRUVBL1, PfRUVBL2, and PfRUVBL3 possess motifs that are essential for ATP binding and hydrolysis. As such, it is imperative to validate whether the recombinant proteins would exhibit capabilities to bind and hydrolyse ATP *in vitro*.

Chapter three

Antibody and recombinant protein production

3.0 Introduction

Following the *in silico* analyses of the *Plasmodium falciparum* R2TP complex discussed in Chapter 2, it became important to produce specific antibodies and to identify an optimal bacterial strain for the expression and production of recombinant PfRUVBL1 and PfRUVBL2 proteins. Despite the availability of other protein production systems, which include mammalian cell culture, baculovirus-insect cell expression system, and others (Legastelois *et al.*, 2016). *E. coli* remains the most used host for producing recombinant proteins, including those of eukaryotes. It is the most well-studied organism for heterologous protein expression (Terpe, 2006), with advantages such as low cost, high growth rate, and well-characterized genome (Huleani *et al.*, 2021). *E. coli* limitations are observed when hosting eukaryotic proteins. Eukaryotic proteins typically fold slowly and often require the assistance of folding chaperones (Oberg *et al.*, 1994). In contrast, *E. coli* transcription and translation are fast and tightly coupled (Francis and Page, 2010). This production rate normally leads to the massive production of partially folded, unfolded, or misfolded and insoluble proteins (Niazi and Magoola, 2023).

Mammalian cell cultures are still important to produce complex molecules with precise glycosylation (O'Flaherty *et al.*, 2020). Mammalian cells are related to human cells in respect to their molecular structures and biochemical characteristics; hence, they are used to produce more than 50% of therapeutic proteins (Zhu *et al.*, 2017). It should be noted that different *E. coli* strains offer advantages in protein expression depending on the specific protein being produced. Large and multidomain proteins as well as membrane proteins often fail to express in *E. coli* or are produced as insoluble/inclusion bodies (Pellizza *et al.*, 2018).

Antibodies are proteins that play a key role in the immune system and are also used as research tools in protein and cell biology (Zhang *et al.*, 2020). Antibodies target proteins in vitro and inside cells, which assist the development of therapies by either using them as drugs or as tools to characterize targeted proteins (Carter and Lazar, 2018). At the protein target level, they offer advantages such as targeting protein conformation, post-translational modification, splice variants, functional epitopes, and cellular components (Roth *et al.*, 2019). Hence, antibodies are vital to molecular biology and protein biochemistry. Antibodies are used to identify, quantify, purify, and characterize specific proteins and contribute to the knowledge of disease mechanisms (Forthal, 2014; Biddle *et al.*, 2024).

Objectives of the study

1. PfRUVBL1, PfRUVBL2, and PfRUVBL3 peptides design for antibody production
2. Peptide immunization and antibody production
3. Confirmation of antibody specificity and titer optimization
4. Heterologous expression and purification of PfRUVBL1 and PfRUVBL2 in various *E. coli* strains

3.1 Materials and Methods

The reagents used in the study are listed in Appendix C. The following antibodies were generated and used in this study: rabbit-raised α -PfRUVBL1 and α -PfRUVBL2 were synthesized and used. α -His antibody, and secondary HRP-conjugated α -rabbit (Thermofischer Scientific, USA), mouse-raised α -DnaK antibodies (Thermofischer Scientific, USA; Makhoba *et al.*, 2016), and secondary α -mouse antibodies (Sigma-Aldrich, USA), rabbit-raised α -PfHsp70-1 (Shonhai *et al.*, 2008) and secondary α -rabbit (Thermofischer Scientific, USA). The plasmid constructs and bacterial strains used in this study are listed in Table 3.1.

Table 3.1: List of plasmid constructs and bacterial strains used for protein expression

Constructs	Description	Supplier/ Reference
pQE30/PfRUVBL1	pQE30 encoding full length PfRUVBL1, Amp ^R	This study
pQE30/PfRUVBL2	pQE30 encoding full length PfRUVBL2, Amp ^R	This study
pPROEX Htb-yRvb1	pPROEX Htb encoding full-length yRvb1, Amp ^R	This study
pQE30/PfHsp70-1	pQE30 encoding full length PfHsp70-1, Amp ^R	Shonhai <i>et al.</i> , 2008
pQE30/PfHsp90	pQE30 encoding full length PfHsp90, Amp ^R	This study
Strains for protein expression		
<i>E. coli</i> XL1 blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacIqZΔM15 Tn10 (Tetr)]	Thermofischer scientific, (USA)
<i>E. coli</i> JM109	F' (traD36, proAB+, lacIq, lacZ-M15), endA1, recA1, hsdR17, (rk-, mk+), mcrA, supE44, e-gyrA96, relA1, - (lac-proAB)	Thermofischer scientific, (USA)
<i>E. coli dnaK103</i>	C600 <i>dnaK103</i> Amp ^R thr:: Tn10	Mayer <i>et al.</i> , 2000
<i>E. coli</i> Δ<i>dnaK52</i>	MC4100 Δ <i>dnaK52</i> :: Cm ^R sid B1	Mayer <i>et al.</i> , 2000

3.1 Methods

3.1.1 Peptide design

MSA was performed to identify specific regions in the recombinant proteins PfRUVBL1 (PF3D7_0809700), PfRUVBL2 (PF3D7_1106000), and PfRUVBL3 (PF3D7_1362200). This was done in comparison to their yeast and human orthologs' sequences to recognize regions that can be targeted by the immune system (Appendix B3). The MSA was performed as described in Chapter Two. Briefly, sequences were obtained from the PlasmoDB database (www.PlasmoDB.org) and UniProt database (www.UniProt.org). The retrieved sequences were aligned and shaded using the Bioedit tool. The variable regions are ideal for producing strain- or variant-specific antibodies as they can evolve rapidly to evade immune detection, making them valuable for targeted antibody production. The epitopes for antibody production are exposed on the surface and accessible to antibodies. The protein hydrophobic scale was employed to accurately predict the optimal surface residues for antibody production (Appendix B4). Hydrophilic residues that were unique for each *P. falciparum* protein were selected for peptide generation (Appendix B4). Conjugation of a carrier protein Keyhole Limpet Hemocyanin (KLH), which helps to improve the immune response, was done by BIOMATIK (USA). Peptide immunization and antibody purification were conducted at Stellenbosch University in Professor Dirk Bellstedt's laboratory.

3.1.2 Confirmation of antibody specificity and titer optimization

To confirm the specificity of the antibody used and to optimize the titer, control proteins, specifically BSA and the stress protein *E. coli* DnaK, were incorporated in the experiment. α -PfRUVBL1 and α -PfRUVBL2 antibodies were utilized at two concentrations: 1:2500 and 1:5000, to assess antibody specificity. The choice of these concentrations was intended to nuance distinctions of both specificity and sensitivity, ensuring accurate measurement of the target proteins without interference from non-specific binding.

The following plasmids were a kind donation from Professor Walid Houry (Toronto, Canada), four *P. falciparum* and four yeast plasmids. The four *P. falciparum* plasmids encode for PfRUVBL1, PfRUVBL2, PfRUVBL3, and PfPIHIDI, and the yeast plasmids were Rvbl1, Rvbl2, Pih1, and Tah1. *P. falciparum* constructs were cloned into the pUCIDT vector, and yeast constructs were cloned into pPROEX (yRvb1) and p11 vectors for the rest. The pUCIDT vector is compatible with the *E. coli* DH5 α strain (Ta Giap *et al.*, 2021), and hence, the construct was sub-cloned into the pQE30 vector, which is more compatible with *E. coli* XL1 blue strains and

other *E. coli* strains. The sub-cloned product plasmids featured *Bam*H1 and *Hind*III restriction sites. The sub-cloning procedure assists in transferring DNA fragments from one vector to another (Taghian and Nickoloff).

3.1.3 Confirmation of pQE30/PfRUVBL1, pQE30/PfRUVBL2, and pQE30/PfRUVBL3 DNA constructs

pQE30/PfRUVBL1, pQE30/PfRUVBL2, and pQE30/PfRUVBL3 cloned products were confirmed through restriction digestion analysis using the *Bam*H1 and *Hind*III enzymes (Appendix A 2) (Thermo Scientific, USA). Subsequently, the resulting products were assessed through 0.8% agarose gel electrophoresis for estimating fragment sizes as previously described (Zininga *et al.*, 2015). In addition, plasmids were confirmed using sequencing (Gene-script, USA) for pQE30/ PfRUVBL1, pQE30/PfRUVBL2, and pQE30/PfRUVBL3 (Appendix A4).

3.1.4 Expression of PfRUVBL1 and PfRUVBL2 in *E. coli* XL1 blue strain

The plasmid constructs used encoded for PfRUVBL1, PfRUVBL2, PfHsp90, PfHsp70-1, and yRvb1. The recombinant proteins were expressed in *E. coli* XL1 blue cells (ThermoFisher Scientific, USA) following a protocol previously described (Shonhai *et al.*, 2008; Gitau *et al.*, 2012). The transformed cells were grown in 2YT broth (1.0% yeast extract, 1.6% tryptone, 0.5% sodium chloride) supplemented with 100 µg/ml ampicillin at 37°C. Protein expression was induced with 1mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), and samples were collected at hourly intervals over 5 hours for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

3.1.5 Purification of recombinant proteins

The purification of the expressed recombinant proteins (PfRUVBL1, PfRUVBL2) and (yRvb1, PfHsp70-1, PfHsp90; Appendix) involved the use of nickel affinity chromatography, following the method outlined by Zininga *et al.* (2015a). For the purification, specific buffers were prepared. The lysis buffer consisted of 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2 mM imidazole, 1 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor, and 1 mg/ml lysozyme. The wash buffer was modified by adding imidazole to a final concentration of 25 mM to the lysis buffer. Elution buffer 1 contained lysis buffer with 125 mM imidazole, while elution buffer 2 contained 250 mM imidazole. Throughout the purification, samples were taken for SDS-PAGE analysis to evaluate protein purity. The purified recombinant proteins were then dialyzed extensively in a dialysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5% (v/v)

glycerol) supplemented with 1 mM 2- β mercaptoethanol using Amicon R centrifugal filter units, following the protocol provided by the manufacturer (Merck, USA).

3.1.6 Liquid chromatography-mass spectrometry (LC-MS)

3.1.7 In-gel digestion

The protein band isolated from the SDS-PAGE was digested from the gel following a previously described method (Shevchenko *et al.*, 2006). Briefly, the proteins were reduced in the gel with 10 mM DTT in 25 mM NH_4HCO_3 for one hour at 60 °C. After cooling to room temperature, 100% acetonitrile was added and incubated for 10 minutes. The supernatant was discarded, and then 55 mM iodoacetamide (IAA) in 25 mM NH_4HCO_3 was added to the gel pieces. The reaction proceeded in the dark for 20 minutes at room temperature. The supernatant was discarded, the gels were dehydrated with 25 mM NH_4HCO_3 in 50% acetonitrile, vortexed, and the supernatant was removed. The gel pieces were dried completely, and freshly prepared trypsin was added. Protein digestion was allowed to proceed overnight at 37 °C. The digestion was quenched by adding 0.1% formic acid, and the samples were dried under a vacuum. For mass spectrometry analysis, the dried samples were re-suspended in 2% acetonitrile and 0.2% formic acid.

3.1.8 LC-MS analysis

Tryptic peptides from each gel fraction were analyzed using a Dionex Ultimate 3000 RSLC system coupled to an AB Sciex 6600 TripleTOF mass spectrometer. Injected peptides were inline desalted using an Acclaim PepMap C18 trap column (75 $\mu\text{m} \times 2 \text{ cm}$; 2 min at 5 $\mu\text{l} \cdot \text{min}^{-1}$ using 2% ACN/0.2% FA). Trapped peptides were gradient eluted and separated on a Waters nanoEase CSH C18 column (75 $\mu\text{m} \times 25 \text{ cm}$, 1.7 μm particle size) at a flow rate of 0.3 $\mu\text{l} \cdot \text{min}^{-1}$ with a gradient of 6-40% B over 15 min (A: 0.1% FA; B: 80% ACN/0.1% FA). The 5600 TripleTOF mass spectrometer operated in positive ion mode. Data-dependent acquisition (DDA) was employed; precursor (MS) scans were acquired from m/z 400-1500 (2^+ - 5^+ charges) using an accumulation time of 100 ms followed by 40 fragment ion (MS/MS) scans, acquired from m/z 100-1800 with 20 ms accumulation time each.

Raw data files (.wiff) were searched with Protein Pilot V5.0 software (SCIEX) using a protein database containing available *Escherichia coli* sequences downloaded from UniProt common contaminants and sequences of recombinant proteins of interest. Trypsin was set as the

digestion enzyme, and cysteine alkylation (iodoacetamide) was allowed as a fixed modification in the search parameters.

3.1.9 Expression of PfRUVBL1, PfRUVBL2 in *E. coli* dnaK103 and *E. coli* Δ dnaK52 strains

The *E. coli* strain BB1553 (MC4100 Δ dnaK52::CmR sidB1) does not have the indigenous DnaK gene, and its growth is hindered at temperatures higher than 30°C due to its compromised folding function (Paek and Walker, 1986). These cells have been extensively utilized as a model for investigating protein folding in living organisms (Suppini *et al.*, 2004; Shonhai *et al.*, 2005). On the other hand, the *E. coli* dnaK103 (BB2393 [C600 dnaK103(Am) thr::Tn10]) cells produce a non-functional truncated DnaK, leading to restricted growth at temperatures below 30°C and sensitivity to cold and heat stress (Mayer *et al.*, 2000; Ncube *et al.*, 2024). The 2YT media was supplemented with 100 μgml^{-1} ampicillin and 35 μgml^{-1} (Δ dnaK52), 100 μgml^{-1} ampicillin and 50 μgml^{-1} kanamycin (dnaK103). Protein expression was induced with 1mM isopropyl- β -D-1-thiogalactopyranoside (IPTG), and samples were collected at hourly intervals over 5 hours for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

3.2 Results

3.2.1 Epitope selection

The sequence alignment of PfRUVBL1, PfRUVBL2, and PfRUVBL3 with their homologous counterparts from yeast and humans revealed both conserved and distinct regions across these proteins, as illustrated in Figure 3.1. Notably, during this analysis, two unique regions were identified in PfRUVBL1: **EENIKKRD MYSE** and **TNKIENNDIKNGTILDDDKVDN**. Similarly, two unique regions in PfRUVBL2, designated as **HENEISLSDEKYSM** and **NISNITNSSNN**, were identified. Lastly, PfRUVBL3 was found to contain one unique region, which is represented by the sequence **KFNERDINNKNK**. Unique epitopes reduce the chances of cross-reactivity with similar proteins, improving the specificity of the antibody. Unique epitopes further reduce non-specific binding.

The sequences identified in the study were carefully selected to design epitope-based peptides. Given that *P. falciparum* is a human parasite, it was crucial to consider human homologous sequences to ensure the specificity of the intended antibody in distinguishing the parasite from the human homolog in the RBC-cultured parasite. In the context of protein structure, globular proteins typically exhibit a hydrophobic core that is stabilized by non-polar amino acids, while their outer surface is primarily composed of hydrophilic amino acids that interact with the surrounding aqueous environment. This characteristic is essential in determining how proteins behave in solution and how they interact with antibodies and other biomolecules. Reflecting on this, evaluating the hydropathy of the selected regions becomes fundamental. Hydropathy analysis can reveal the distribution of hydrophobic and hydrophilic residues across the peptide, enabling the prediction of how well the peptide would perform in a biological setting (Figure 3.2). Such an evaluation is pivotal in guiding the design process to optimize the immunogenic potential of the peptides tailored for antibody development, ultimately enhancing the efficacy of targeted therapeutic strategies against *P. falciparum*.

3.2.1 Multisequence alignment

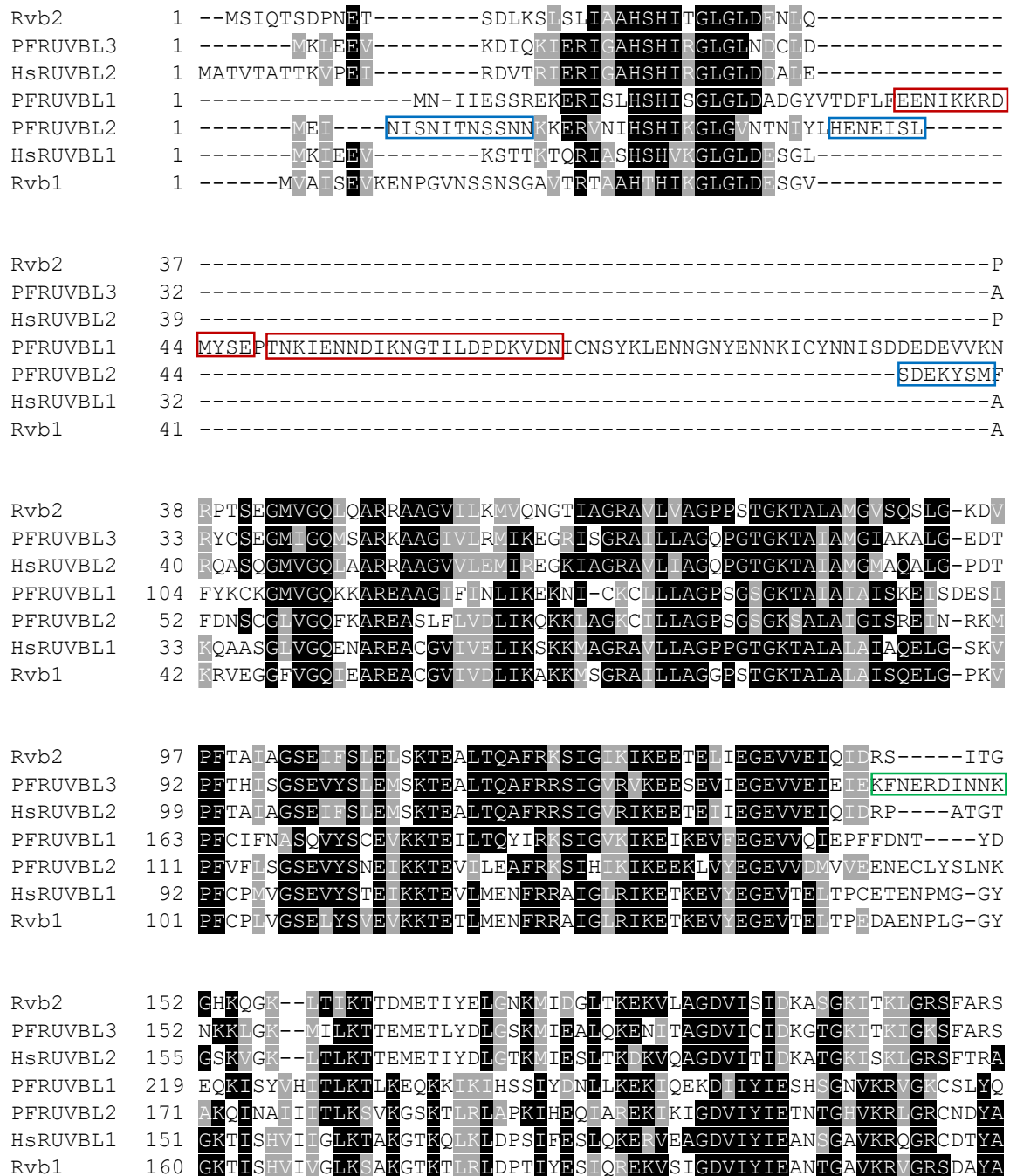


Figure 3.1: Multisequence alignment of PfrUVBL1, PfrUVBL2, and PfrUVBL3 with their yeast and human homologous.

Identification of epitopes using MSA, PfrUVBL1 epitopes are denoted by a red box, PfrUVBL2 epitopes are represented with a blue box, while PfrUVBL3 are circled with a green box. The analysis was conducted using Bioedit tool (www.bioedit.software.informer.com).

3.2.2 Hydrophobic scale of PfrUVBL1, PfrUVBL2, and PfrUVBL3

The hydrophobic scale is an important tool in selecting and optimizing epitopes and improving antibody stability. Selecting more hydrophilic regions (found on the protein surface) increases

the likelihood of generating antibodies that recognize native, folded proteins. Upon analyzing the hydrophobic and hydrophilic characteristics of the PfRUVBL1, PfRUVBL2, and PfRUVBL3 proteins, specific amino acids were identified that exhibit favorable properties for peptide design (Table 3.1, Appendix B4). Hydropathy results indicate that PfRUVBL1 has a low hydrophilic amino acid constituent of 67% compared to PfRUVBL2 with 71% and PfRUVBL3 with 83% (Figure 3.2; Table 3.1).

Table 3.1: Characteristics of selected peptides

PfRUVBL1		PfRUVBL2		PfRUVBL3	
<u>Amino acid</u>	<u>Hydropathy</u>	<u>Amino acid</u>	<u>Hydropathy</u>	<u>Amino acid</u>	<u>Hydropathy</u>
E (Glu)	hydrophilic	H (His)	hydrophilic	K (Lys)	Highly hydrophilic
E (Glu)	hydrophilic	E (Glu)	hydrophilic	F (Phe)	hydrophobic
N (Asn)	Polar and hydrophilic	N (Asn)	Polar and hydrophilic	N (Asn)	Polar and hydrophilic
N (Asn)	Polar and hydrophilic	E (Glu)	hydrophilic	E (Glu)	hydrophilic
I (Ile)	hydrophobic	I (Ile)	hydrophobic	R (Arg)	hydrophilic
K (Lys)	Highly hydrophilic	S (Ser)	Hydrophilic	D (Asp)	hydrophilic
N (Asn)	Polar and hydrophilic	L (Leu)	hydrophobic	I (Ile)	hydrophobic
G (Gly)	hydrophobic	S (Ser)	Hydrophilic	N (Asn)	Polar and hydrophilic
T (Thr)	Highly hydrophilic	D (Asp)	hydrophilic	N (Asn)	Polar and hydrophilic
I (Ile)	hydrophobic	E (Glu)	hydrophilic	K (Lys)	Highly hydrophilic
L (Leu)	hydrophobic	K (Lys)	Highly hydrophilic	N (Asn)	Polar and hydrophilic
D (Asp)	hydrophilic	Y (Tyr)	hydrophobic	K (Lys)	Highly hydrophilic
P (Pro)	hydrophobic	S (Ser)	Hydrophilic		
D (Asp)	hydrophilic	M (Met)	hydrophobic		
K (Lys)	Highly hydrophilic				
Appr. Hydrophilicity %	67	Appr. Hydrophilicity %	71	Appr. Hydrophilicity %	83

The combination of the small antigen with a carrier protein introduces additional epitopes that can further stimulate B cells, leading to enhanced antibody production. This synergistic effect not only improves the recognition of the antigen by immune cells but also results in a more potent and sustained immune response (Karst *et al.*, 2017).

3.2.3 Confirmation of the antibody's specificity and optimization of the antibody's titer

To verify the specificity of the antibodies utilized in the study, control proteins such as BSA and the molecular chaperone *E. coli* DnaK were probed using the antibodies (Figure 3.2). Purification of PfRUVBL1 and PfRUVBL2 resulted in yields of approximately 7.0 µg/L and 6.5 µg/L respectively. The α-PfRUVL1 was able to detect the PfRUVBL1 recombinant protein at a titer concentration of 1:2500 of 5% milk in TBST (Tris-buffered saline with Tween). The α-PfRUVL2 detected the PfRUVBL2 recombinant at a titer of 1:5000 (Figure 3.4). The disparity in the antibody titer responses is likely a result of differences in the composition of hydrophilic amino acids in the peptides designed for each antibody (Table 3.1).

Detection of PfRUVBL1 and PfRUVBL2 recombinant proteins by their respective specific antibodies in the presence of BSA, DnaK, PfRUVBL1, and PfRUVBL2 (depending on the antibody used) proteins in the assay validates their specificity. These results reinforce the specificity of the antibodies, which is defined as the ability of an antibody to bind exclusively to a particular antigen or epitope (Jain and Salunke, 2019). In addition to these findings, it was crucial to establish that the pre-immunization breed exhibited no detectable response to the recombinant proteins tested. The observed absence of response further corroborates that any signals detected during the experiment were attributable solely to the antibodies generated against the injected peptide (Figure 3.2). Such rigorous controls affirm the reliability of the antibody specificity in detecting target antigens in complex biological samples and their potential use in immune-based assays.

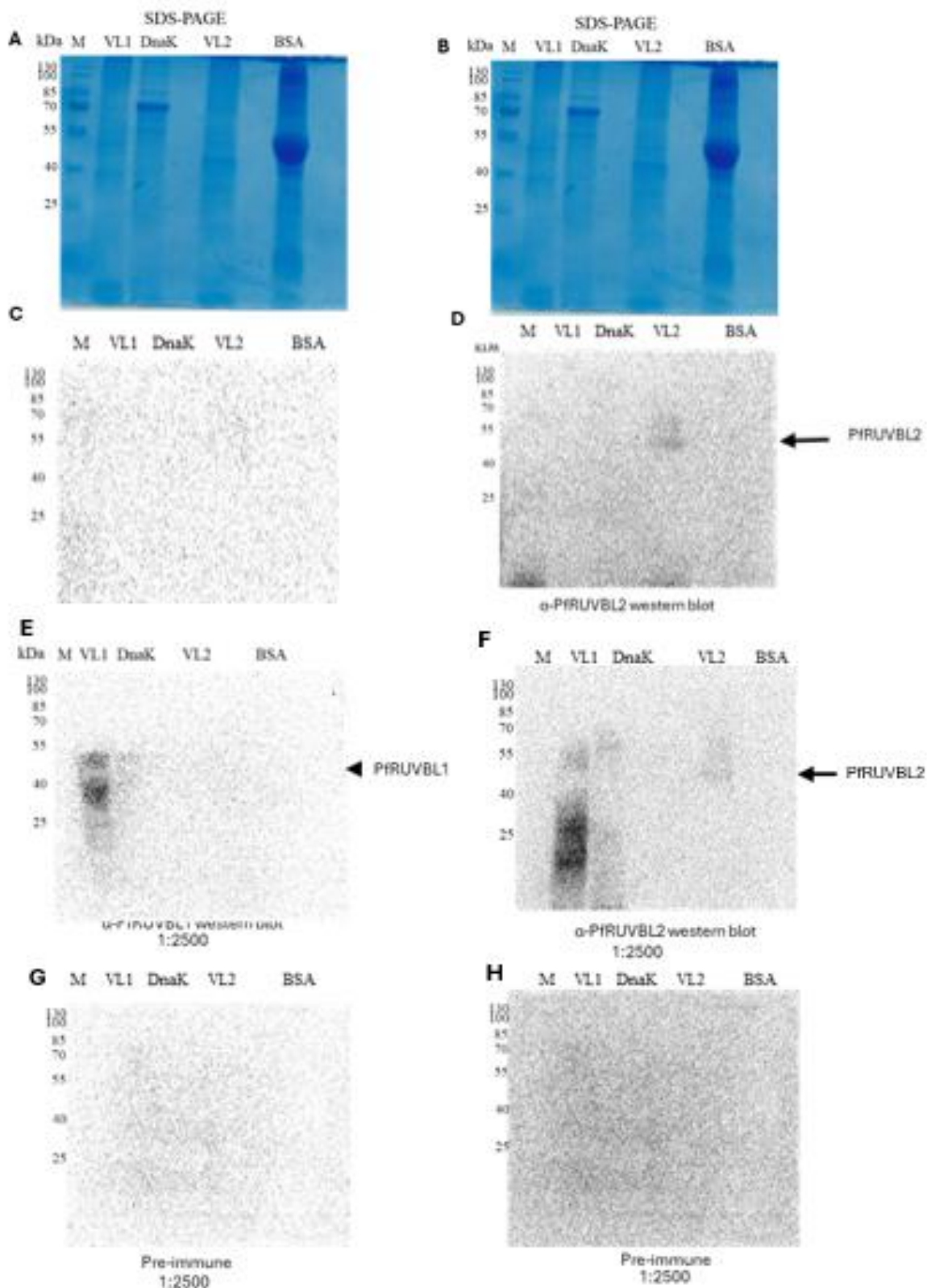


Figure 3.2. Investigation of antibody specificity and optimization of the titer

SDS-PAGE analysis of PfRUVBL1, PfRUVBL2, DnaK, and BSA and western blot analysis. (A/B) SDS-PAGE analysis of recombinant proteins, (C) Western blot analysis using α -PfRUVBL1 (1:5000), (D) Western blot analysis using α -PfRUVBL2 (1:5000), (E) Western blot analysis using α -PfRUVBL1 (1:2500), (F) Western blot

analysis using α -PfRUVBL2 (1:2500), (G) Western blot analysis using pre-immune α -PfRUVBL1 (1:5000), (H) Western blot analysis using pre-immune α -PfRUVBL2 (1:5000).

3.2.4 Construction of plasmids expressing PfRUVBL1, PfRUVBL2, and PfRUVBL3

pQE30/PfRUVBL1, pQE30/PfRUVBL2, and pQE30/PfRUVBL3 plasmid constructs were verified by restriction digest using enzymes *Bam*H1 and *Hind*III and DNA sequencing (Gene-script, USA). The striction digestion of pQE30/PfRUVBL1 plasmid construct with either *Bam*H1 and *Hind*III produced a band of approximately 4988 bp, which corresponds to the size of pQE30 (3461) and PfRUVBL1 insert (1527), pQE30/PfRUVBL2, produced a band size of approximately 4853bp corresponding to pQE30 (3461) and PfRUVBL2 (1392), while pQE30/PfRUVBL3 produced a band approximately 4877 corresponding to pQE30 (3461) and PfRUVBL3 (1416) (Appendix B5).

3.2.4 Expression and purification of recombinant PfRUVBL1 and PfRUVBL2 in *E. coli* XL1blue strain

Recombinant PfRUVBL1 and PfRUVBL2 were expressed in *E. coli* XL1 blue cells and purified using nickel affinity chromatography (Figure 3.3). Expression and purification of proteins were observed in the SDS-PAGE as species around 50 kDa and confirmed using western blotting. The presence of the His-tag fused to an N-terminal cleavable with TEV protease facilitated the native NI-NTA affinity purification. Notably, PfRUVBL2 co-elutes with DnaK, as confirmed by α -DnaK antibodies (Figure 3.3). DnaK contamination affects downstream applications such as protein activity assays. Copurification of proteins with DnaK has been reported to influence experiments that involve unfolding-refolding of the desired protein (Rial and Ceccarelli, 2002).

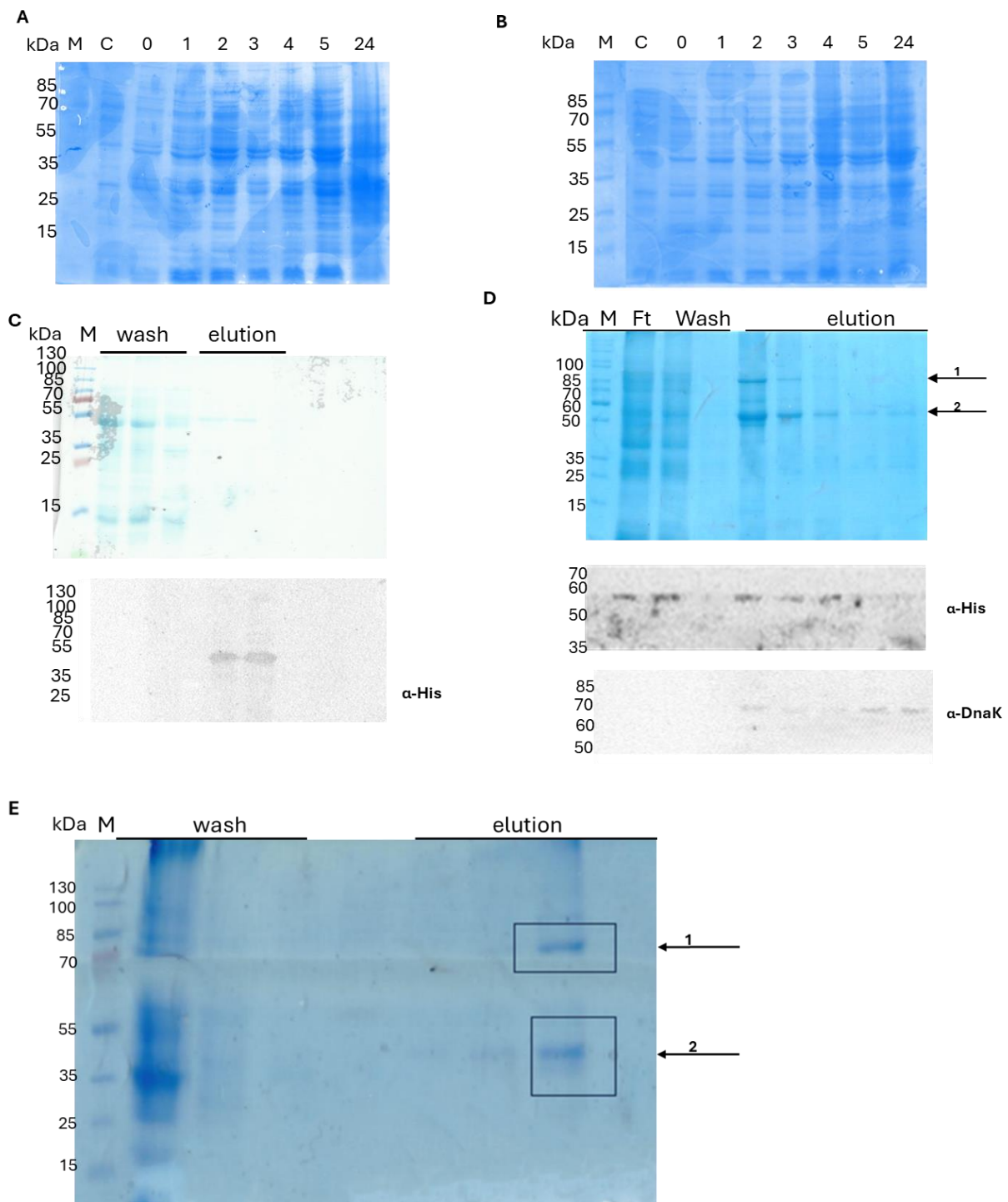


Figure 3.3: Expression and purification of PfRUVBL1 and PfRUVBL2

PfRUVBL1 and PfRUVBL2 were expressed in *E. coli* XL1 Blue cells transformed with pQE30/PfRUVBL1 and pQE30/PfRUVBL2, respectively. SDS-PAGE analysis of expression of PfRUVBL1 (A) and PfRUVBL2 (B), M: molecular marker, C: total extract for cells transformed with a neat pQE30 vector, 0: pre-induction sample, lanes 1-5/24 represent time intervals where each collected at respective hours after IPTG induction. Purification of PfRUVBL1 (C) and PfRUVBL2 (D) using affinity chromatography, M: molecular marker, Ft: flow through, wash for unbound, elution protein sample. (E) SDS-PAGE (gel prepared for LCMS analysis), same gel annotation as in C and D. Band 1 (*E. coli* DnaK); band 2 (PfRUVBL2).

Potential DnaK contaminants on PfRUVBL2 were further analyzed using liquid chromatography-mass spectrometry (LCMS). The study aimed to identify and characterize proteins within specific gel bands (1 and 2) to understand their molecular sizes, which could be inferred from their potential functional roles. LCMS data (Table 3.3; Appendix B18) showed that PfRUVBL2 co-elutes not only with DnaK but also with *E. coli* Hsp90. The ability of chaperones to bind to unfolded proteins or exposed polypeptide chains makes the removal of contamination challenging during protein purification (Morales *et al.*, 2019). On the other hand, the R2TP complex has been shown to associate with Hsp90 during multi-protein complex assembly (Houry *et al.*, 2018; Lynham *et al.*, 2022). R2TP complex interacts with both Hsp90 and Hsp70 in humans (von Morgen *et al.*, 2015; Abel *et al.*, 2022). Hence, the co-elution of PfRUVBL2 and *E. coli* chaperones may be attributed to the co-functional mechanism of these proteins in the parasite.

Table 3.3: Liquid Chromatography mass spectrometric analysis to identify *E. coli* proteins co-eluting with PfRUVBL2

Band 1			
Accession number	Protein name	Specie	Band percentage composition
P0A6Z3	HTPG	<i>E. coli</i> chaperone	84%
P0A6Y8	DnaK	<i>E. coli</i> chaperone	62%
P63284	ClpB	<i>E. coli</i> chaperone	53%
P0CE48	EFTU2	Elongation factor Tu2	41%
Band 2			
PF3D7_1106000	PfRUVBL2	<i>P. falciparum</i> RuvB-like protein 2	91%

3.2.5 Expression and purification of PfRUVBL1 and PfRUVBL2 in *E. coli* dnaK103 strain

E. coli BB2393 is a specialized strain that produces a truncated DnaK that is non-functional (Mayer *et al.*, 2000; Makhoba *et al.*, 2016). The strain is engineered to enhance protein expression, and it carries specific mutations that affect metabolic pathways, codon usage, or reduce the accumulation of toxic metabolic byproducts (Spence *et al.*, 1990). This led to higher yields of PfRUVBL1 and PfRUVBL2 proteins that were free from DnaK contamination (Figure 3.4). The strain is often used for proteins with low expression yield due to codon bias or toxic effects of overexpressed proteins. *E. coli* BB2393 better accommodates non-optimal

codons, which is particularly beneficial for expressing eukaryotic proteins like *Plasmodium* proteins (de Marco *et al.*, 2007). This is important because most eukaryotic genes, such as PfRUVBL1 and PfRUVBL2, have codons that are rarely used in *E. coli*, leading to poor translation. Hence, *E. coli* BB2393 cells address this limitation by improving codon availability and increasing translation efficiency.

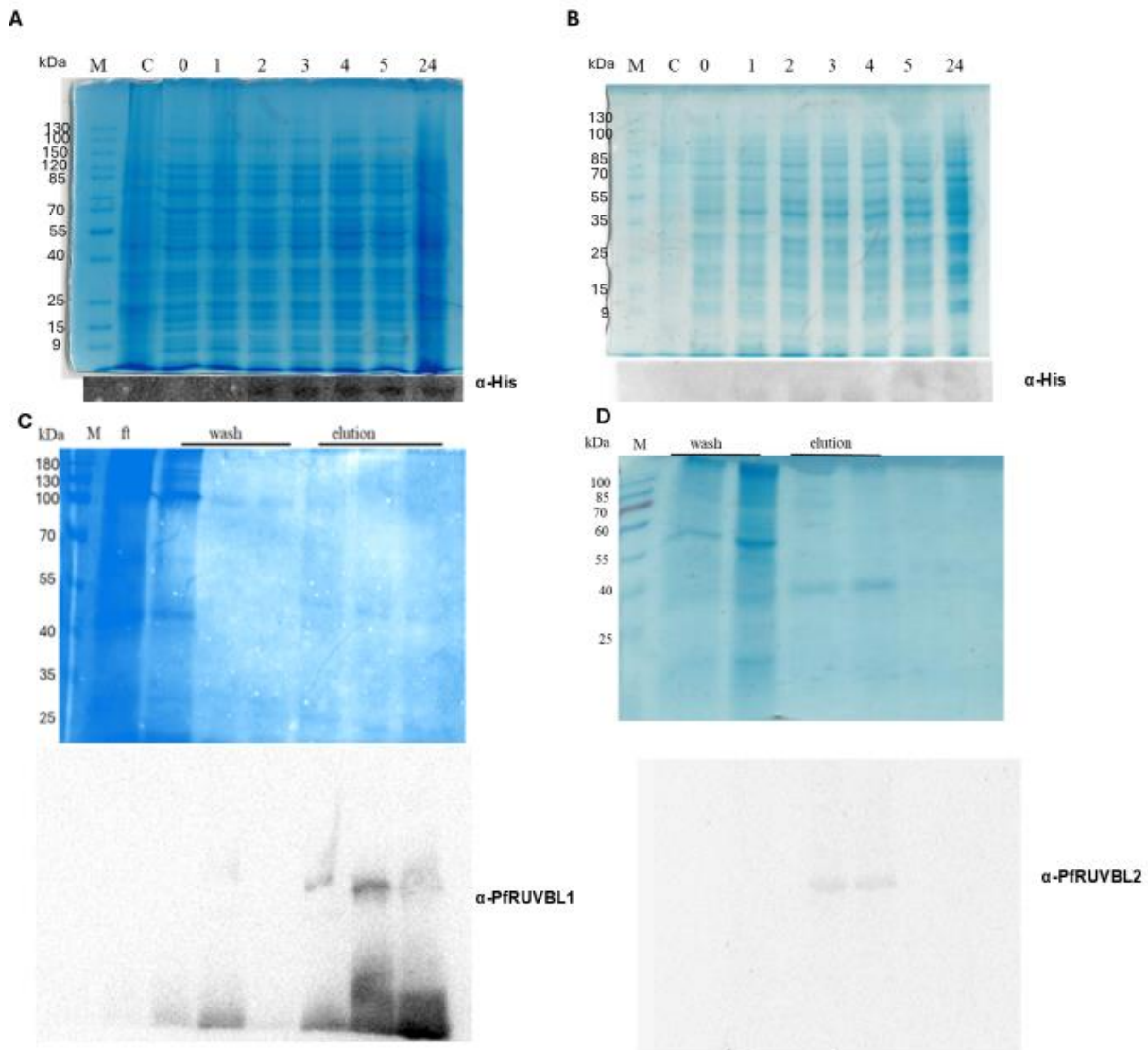


Figure 3.4: Expression and purification of PfRUVBL1 and PfRUVBL2 in *E. coli* BB2393 cells

PfRUVBL1 and PfRUVBL2 were expressed in BB2393 cells transformed with pQE30/PfRUVBL1 and pQE30/PfRUVBL2 respectively. SDS-PAGE analysis of expression of PfRUVBL1 (A) and PfRUVBL2 (B), M: molecular marker, C: total extract for cells transformed with a neat pQE30 vector, 0: pre-induction sample, lanes 1-5/24 represent time intervals where each collected at respective hours after IPTG induction. Purification of PfRUVBL1 (C) and PfRUVBL2 (D) using affinity chromatography, M: molecular marker, Ft: flow through, wash for unbound, elution protein sample. Low panels represent western blot analysis using specific antibodies as labeled.

3.2.7 Purification of PfRUVBL2 using polyethyleneimine (PEI)

PEI is a cationic polymer that binds to negatively charged proteins, and it provides a favourable environment for protein stability (Burgess, 2009; Lubkowska *et al.*, 2023). The principle of flocculation of the PEI is thought to be based on the difference in charge between the polymer and the targeted molecule (Lubkowska *et al.*, 2023). Recombinant PfRUVBL2 expressed in *E. coli* BB2393 strain and lysed in the presence of 5% PEI was purified by affinity chromatography, analyzed by SDS-PAGE, and confirmed by western blot analysis using α -PfRUVBL2 (Figure 3.5). PEI was used to precipitate impurities selectively based on charge. PEI modifies surface charge or form complexes that reduces nonspecific binding and improve elution profile (Jozala *et al.*, 2016). Purification eluted a species of approximately 50 kDa (band 3) however, bands running approximately 100 and 150 kDa (bands 1 and 2) were observed and recognized by the α -PfRUVBL2 antibody (Figure 3.5). It is suggested that these bands represent oligomer forms of PfRUVBL2. Oligomers form the association of multiple protein subunits, which interacts and bind together to create a functional protein. They are essential for many biological processes including enzymatic activity, signal transduction and structural support within the cell.

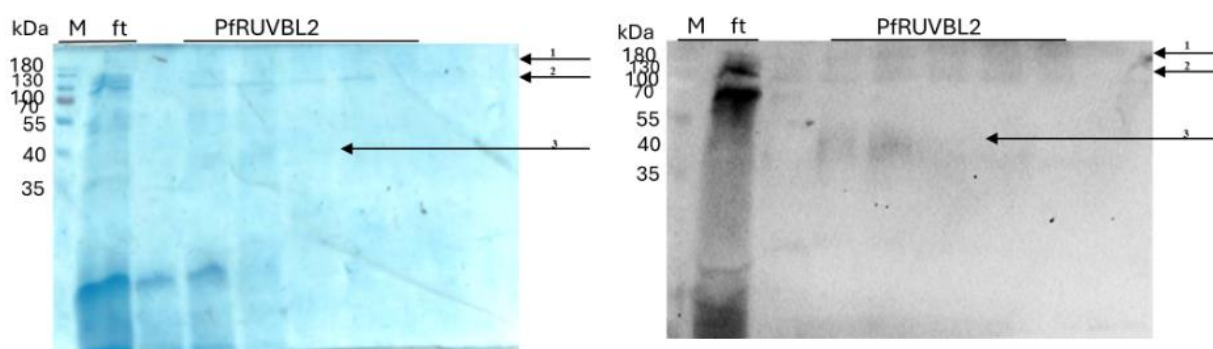


Figure 3.5: Purification of PfRUVBL2 in the presence of PEI showed oligomers

Purification of PfRUVBL2 expressed in BB2393 cells lysed in the presence of solubilizing detergent PEI. M: molecular marker, Ft: flow through. Left: western blot analysis using α -PfRUVBL2 antibody.

The solubility and stability effects of imidazole can be used for the purification of His-tag and poorly soluble recombinant proteins (Hamilton *et al.*, 2003). Imidazole can influence downstream processes that involve sensitive enzyme assays and protein labeling (Tang *et al.*, 2023). Hence, it is suggested to remove imidazole for enzymatic-related assays (Figure 3.6).

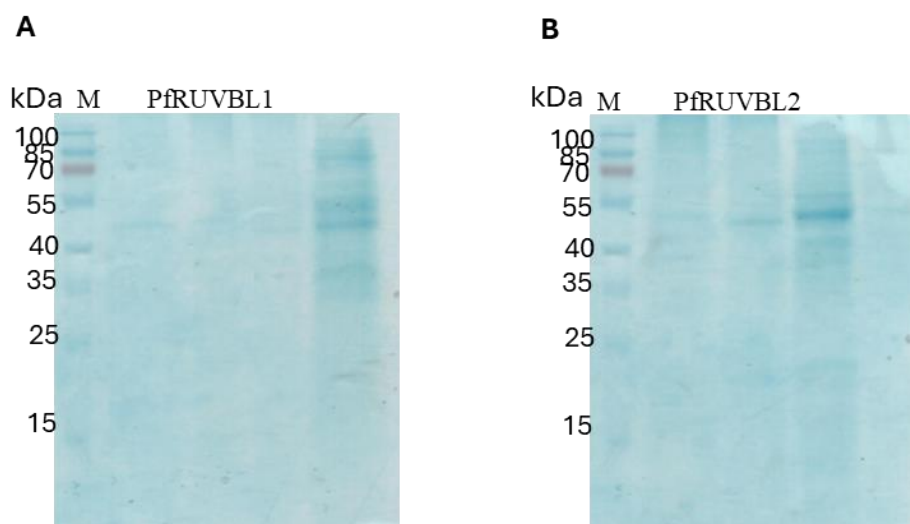


Figure 3.6: Dialysed protein samples

PfRUVBL2 and PfRUVBL1 were successfully dialyzed using an imidazole-free lysis buffer. A PfRUVBL1 and B PfRUVBL2

Table 3.3.1 Protein concentrations

Protein name	Purity (protein concentration)
PfRUVBL1 (batch 1)	2.7 ug/L
PfRUVBL1 (batch 2)	3.5 ug/L
PfRUVBL1 (batch 3)	2.9 ug/L
PfRUVBL2 (batch 1)	3.0 ug/L
PfRUVBL2 (batch 2)	3.3 ug/L
PfRUVBL2 (batch 3)	2.7 ug/L
yRVB1 (batch1)	4.9 ug/L
yRVB1 (batch2)	5.0 ug/L
yRVB1 (batch3)	4.7 ug/L
PfHsp90	6.8 ug/L
PfHsp70-1	7.8 ug/L

3.4 Discussion

The MSA of *P. falciparum* RUVBL proteins against their homologous was useful in identifying unique regions in the targeted proteins. By selecting unique, non-homologous regions, the risk of cross-reactivity with similar proteins is reduced (Figure 3.1), resulting in higher antibody specificity (Altschul *et al.*, 1990). Misleading similarities in non-target proteins can lead to non-specific binding (Guvén *et al.*, 2014). Sequence alignment identified unique peptide sequences, minimizing cross-reactivity risk and improving antibody quality (Sievers and Higgins, 2014). Selected sequences were predominantly hydrophilic, with short hydrophobic stretches (Table 3.1; Figure 3.2). Hydropathy findings assisted in selecting hydrophilic, surface-exposed regions as epitopes for antibody production (Kyte and Doolittle, 1982). Antibodies raised against hydrophilic regions are more likely to recognize the native, folded protein, as these regions are accessible to the immune system in soluble and membrane proteins.

Antibody production is a foundational tool in biotechnology and medicine, with applications ranging from basic research to therapeutic development (Diamos *et al.*, 2020). Advances in recombinant DNA technology have expanded the potential of antibody production, allowing for precise engineering and large-scale production of antibodies tailored to specific needs (Tiller and Tessier, 2015). Producing antibodies for eukaryotic proteins presents several challenges, primarily due to the complexity of these. The main challenges include ensuring proper folding, post-translation modification, and secretion, which are crucial for antibody function (Bhatwa *et al.*, 2021). In the context of this study, recombinant antibodies were produced and used to validate purified recombinant proteins (Rahumatullah *et al.*, 2020). The challenges of RUVBL protein production have been reported (Baron *et al.*, 2016; Sen *et al.*, 2018) Low yield of concentrations between 0.1 – 0.15 mg/ml of both RUVBL1 and RUVBL2 have been reported (Niewiarowski *et al.*, 2010). The intricate roles and interactions of these proteins suggest that their expression must be carefully managed to maintain cellular balance (Baron *et al.*, 2016; Sen *et al.*, 2018)

DnaK is a member of the Hsp70 family of molecular chaperones, which primarily bind misfolded proteins and assist them in achieving the correct folded structure (Shonhai *et al.*, 2007). It is, therefore, plausible that PfRUVBL2 is released from the ribosome in a misfolded state, which may attract DnaK, as observed in this study (Figure 3.4, Table 3.3). To avoid contamination from DnaK, it is recommended to express recombinant proteins in *E. coli* Δ dnaK cells (Morales *et al.*, 2019). The *E. coli* BB2393 strain has enhanced translation efficiency,

improved protein folding, and reduced degradation compared to standard *E. coli* strains (de Marco *et al.*, 2007). Consequently, using *E. coli* BB2393 for the expression and purification of PfRUVBL1 and PfRUVBL2 leads to better-folded proteins with less DnaK contamination.

Proteins contaminated with DnaK can lead to inaccurate results and complicate findings, as heat shock protein (Hsp) contaminants may interfere with downstream processes (Morales *et al.*, 2019). During heterologous protein expression, molecular chaperones tend to bind to the target protein with high affinity, causing the chaperone to remain attached throughout the purification stages (Morales *et al.*, 2019). The Hsp70 molecular chaperone interacts with specific protein sequences that are commonly found in nearly all proteins (Rudiger *et al.*, 1997). These sequences are typically present during protein synthesis or may be exposed after folding in the interdomain regions or loops (Ambrose *et al.*, 2024).

Bands that were isolated through SDS-PAGE were subjected to enzymatic digestion using trypsin and analyzed using liquid chromatography coupled with mass spectrometry (LC-MS). This enzyme selectively cleaves at the carboxyl side of arginine and lysine residues, resulting in a diverse array of peptide fragments that are suitable for mass spectrometric analysis (Wang *et al.*, 2019). The LCMS data confirmed the contamination of PfRUVBL2 with two prominent molecular chaperones, Hsp70 and Hsp90, from *E. coli*. R2TP complex components have been proposed to interact with Hsp90 in humans (Martino *et al.*, 2018; Abeza *et al.*, 2024). RPAP3 homology of PIHIDI in *P. falciparum* mediates the interaction of the R2TP complex with substrate enriched with helical-type domains (Seraphim *et al.*, 2022). The RPAP3 is composed of two TPR domains whereby TPR2 is known to have a higher affinity for Hsp90, and TPR1 is thought to interact with Hsp70 (Henri *et al.*, 2018). It is for this reason that co-eluting PfRUVBL2 and Hsp70 and Hsp90 homologous is suggested to mimic the partnership present in *P. falciparum*.

In conclusion, our findings indicate that PfRUVBL2 assembles into higher-ordered structural formations during native purification processes. PfRUVBL2 functions as a member of the assembly machinery complex. Oligomerization is essential for many biological processes, including enzymatic activity, signal transduction, and structural support within the cell. Oligomerization of RUVBL proteins allows them to form multi-subunit complexes, which is essential for their biological function (Gorynia *et al.*, 2011). Disruption of oligomerization impairs the capability of RUVBL1/2 in complex assembly and may affect cell growth or stress response (Llorca and Houry, 2011). Notably, this protein tends to co-elute with the chaperone

proteins Hsp70 and Hsp90 from *E. coli* when purified using the *E. coli* XL1 strain. This observation suggests potential interactions or functional relationships between PfRUVBL2 and these heat shock proteins. However, this purification challenge can be effectively addressed by utilizing *E. coli* strains that are deficient in the DnaK chaperone, such as the *E. coli* $\Delta dnaK$ strains, including *E. coli* *dnaK103*. By employing these alternate strains, we can minimize unwanted interactions and improve the purity of the PfRUVBL2 isolation, facilitating further studies on its biological functions and structural properties. Over traditional affinity chromatographic purification approaches, size exclusion chromatography can ensure better protein purity.

Chapter four

Analysis of the interaction of PfRUVBL1 and PfRUVBL2 with ligands

4.0 Introduction

PfRUVBL1 and PfRUVBL2 are two ATPase components of the *Plasmodium falciparum* R2TP complex. Structural and biochemical studies on homologs of RUVBL1 and RUVBL2 from humans show that nucleotide binding and hydrolysis induce conformational changes that are essential for the assembly and regulation of multiprotein complexes, including R2TP and various chromatin remodeling complexes (Lopez-Perrote *et al.*, 2012). RUVBL1 and RUVBL2 bind nucleotides through conserved motifs within their AAA+ ATPase domains (Gorynia *et al.*, 2011).

The binding of ATP induces a major conformational change in ATPases, leading to a tightly packed "closed" conformation (Munoz-Hernandez *et al.*, 2019). This transition is crucial, as it contrasts with the "open" conformation induced by the binding of ADP or the absence of any nucleotide altogether (Gorynia *et al.*, 2011; Munoz-Hernandez *et al.*, 2019). The dynamic interplay of these alternating conformations is critical for the activity of the proteins concerned. Nucleotides are known to facilitate protein complexes assembly, disassembly, and remodeling, essential for maintaining cellular homeostasis and executing various biological processes. The ability to transition between distinct functional states allows RUVBL proteins to facilitate protein folding, prevent aggregation, and assist in refolding misfolded proteins, all vital for cellular function and overall organismal health (Nano *et al.*, 2018). When ATP binds, RUVBL1 and RUVBL2 adopt a more stable structural arrangement, forming either hexameric or dodecameric ring complexes. This stable conformation enables RUVBL1 and RUVBL2 to act effectively as scaffolds, providing the necessary structural framework for assembling or remodeling other protein complexes (Gorynia *et al.*, 2011; Yenerall *et al.*, 2020). The ATP-dependent conformational shifts are vital in the context of the R2TP complex and other related assemblies. Conformational changes promote the binding of client proteins and facilitate the release of these proteins or other components within the complex, ensuring efficient functional interactions within the cellular environment. In addition, conformational changes allow proteins to dynamically adjust their shape, exposing or hiding specific binding sites depending on the specific requirement (Weikl and Paul, 2014). This capability is important for the R2TP complex since it functions as a crucial co-chaperone primarily responsible for facilitating the assembly and stabilization of large protein complexes within the cell (Kakihara and Houry, 2012).

RUVBL1 and RUVBL2 are integral to numerous processes that are essential for maintaining cellular integrity and function. These proteins are particularly important in chromatin remodeling, which enables the regulation of gene expression by altering the structure of chromatin (Dauden *et al.*, 2021). Additionally, they play a vital role in DNA damage repair mechanisms, helping to safeguard genomic stability by facilitating the repair of DNA breaks and lesions (Lopez-Perrote *et al.*, 2012). Furthermore, RUVBL1 and RUVBL2 are involved in the assembly of various protein complexes that are necessary for a plethora of cellular activities (Horejsi *et al.*, 2014).

Their functional versatility is largely attributed to their capacity to engage in self-associations, forming homo-oligomers, as well as hetero-associations, forming hetero-oligomers with other proteins (López-Perrote *et al.*, 2020). This ability to create different protein complexes allows them to participate in diverse biological pathways and cellular contexts, underscoring their significance in cellular homeostasis and stress response. The multifaceted roles of RUVBL1 and RUVBL2 have been highlighted in several studies, which support their crucial contributions to cellular functions (Nano *et al.*, 2013; López-Perrote *et al.*, 2020). In the context of *P. falciparum*, protein complex assembly is crucial for its survival and performing essential functions, including invading host red blood cells, establishing its intracellular environment, exporting virulent factors, and chromatin complexes. *P. falciparum* critically relies on the precise regulation of gene expression throughout its life cycle (Hollin and Roch, 2020). The Rhop complexes consist of Clag3, Rhop2, and Rhop3 and are essential for *P. falciparum* gene expression and growth in the infected RBC (Blatch, 2022). Transcription factors and chromatin complexes regulate the development of *P. falciparum* (Bonnell *et al.*, 2024).

RUVBL1 and RUVBL2 interact with Hsp90, a key molecular chaperone, as part of the R2TP complex (Lynham *et al.*, 2022). The DII domain of RUVBL1 and RUVBL2 is essential for this interaction, as it helps recruit and stabilize client proteins that are dependent on the Hsp90-R2TP system for their assembly and maturation (Pal *et al.*, 2021). Research has demonstrated that mutations or deletions in the DII domain impair the ability of RUVBL1/2 to interact with Hsp90, disrupting downstream protein stabilization and complex assembly (Gorynia *et al.*, 2011; Nano *et al.*, 2013). The DII domain is a structural feature that extends outward from the core AAA+ ATPase domain, enabling specific protein-protein interactions and regulating complex formation. The R2TP complex is envisaged to play a similar role in *P. falciparum* as augmented in its yeast and human homologs which assist the parasite with pathogenicity and

survival abilities. Hence, the current study sought to characterize the structure-function features of the *P. falciparum* R2TP complex.

The objectives of the study were to:

- I. Analyze the conformations of PfRUVBL1 and PfRUVBL2 using intrinsic, extrinsic fluorescence spectroscopy and limited proteolysis assay.
- II. determine the nucleotide (ATP and ADP) binding affinity to PfRUVBL1 and PfRUVBL2 using the SPR technique.
- III. Determine the ATPase activity of PfRUVBL1 and PfRUVBL2 using colorimetric ATP hydrolysis activity assays.
- IV. assess the capability of PfRUVBL1 and PfRUVBL2 to suppress heat-induced aggregation of model protein *in vitro*.
- V. Determine the capability of PfRUVBL1 and PfRUVBL2 to cleave DNA.
- VI. Explore the PfRUVBL1 and PfRUVBL2 direct association and their interaction with sorafenib using SPR (surface plasmon resonance), slot blot, and thermal shift assay.

4.1 Materials and Methods

4.1.1 Materials

The reagents and materials used in this study are listed in Appendix C1.

4.1.2 Determining the nucleotide (ATP/ADP) binding affinity of PfRUVBL1 and PfRUVBL2

The nucleotide equilibrium binding affinity assay of PfRUVBL1 and PfRUVBL2 relative to their yeast homology was conducted by SPR analysis using the Bio Navis TM 220A ILVES MP SPR (Bio Navis, Tampere, Finland) system at room temperature. Degassed PBS (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 137 mM KCl, and 20 mM EDTA; pH 7.4). was used as a running buffer (Zininga *et al.*, 2016; Mabate *et al.*, 2018). The proteins (PfRUVBL1 and PfRUVBL2) were each immobilized as ligands onto the gold functionalized three-dimensional carboxymethyl dextran sensor chip (CMD Three-dimension 500L) (Bio Navis, Tampere, USA). Individual proteins were immobilized onto the CMD Three-dimension 500L sensor surface at 25 µg/ml concentration.

Immobilization of ligand was achieved through the activation of the chip surface by an equimolar mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxy-succinimide (NHS) for amine coupling as described by the manufacturer (Bio Navis), Tampere, Finland). Following immobilization, ATP was then injected in series in aliquots of 0, 125, 250, 500, 1000, and 2000 nM at a flow rate of 50 µl/min. Association between ligand and analyte at a steady state was allowed for 1 min, while dissociation was monitored for a total of 2 min. The steady-state equilibrium constant data were processed and analysed using Trace Drawer software version 1.8 (Ridgeview instrument, Sweden). Kinetic evaluations were conducted on sensograms after all six ATP concentrations were fitted using a 1:1 Langmuir model.

4.2 Fluorescence spectroscopic analysis of PfRUVBL1 and PfRUVBL2

The tyrosine fluorescence spectroscopy assay was carried out using a modified protocol from the previously described method (Zininga *et al.*, 2016). The intrinsic (tyrosine) fluorescence assays were used to examine recombinant proteins' tertiary structures. The fluorescence spectra produced were analyzed using a JASCO FP-6300 spectrofluorometer (JASCO Ltd, UK) after being initially excited at 285 nm. The emission spectra were observed between 280-500 nm at a scanning speed of 500 nm/min, and triplicate scans were conducted for each sample, with the results being averaged. The effects of nucleotides on the tertiary structures of recombinant

proteins were confidently determined by exposing the proteins to nucleotides (5 mM ADP/ATP) for 15 minutes before conducting fluorescence readings. The assay was repeated in the presence of 10 μ M sorafenib. Statistical analysis was conducted using a One-Way ANOVA at $p < 0.05^*$; $p < 0.01^{**}$; $p < 0.001^{***}$.

To investigate the fold state of proteins concern, the effect of chemical denaturant on the respective recombinant proteins was assessed. Briefly, 4 μ M of the respective recombinant proteins were allowed to sit for 30 minutes in the presence of varying concentrations of urea (0 - 8 M) before reading the fluorescence. Urea is a potent chaotropic denaturant capable of disrupting the tertiary structure of a protein either by directly destabilizing internal, non-covalent bonds between peptide groups or by modifying the hydrodynamics of the protein structure (Dunbar *et al.*, 1997).

The tertiary structures of recombinant proteins were validated by extrinsic fluorescence using ANS assays as previously described (Achilonu *et al.*, 2014; Maake and Achilonu, 2024). ANS is a small amphipathic dye that is used as a probe to detect hydrophobic pockets on the surface of recombinant proteins (Tiwari *et al.*, 2009). Briefly, 200 μ M ANS was incubated with 4 μ M of the respective protein for a total of 30 minutes at 25°C in the dark. The reaction was monitored by recording fluorescence spectra between 400 – 700 nm following initial excitation at 390 nm. A total of 3 scans were collected and averaged for each sample. In addition, the fluorescence spectra of free ANS were monitored as a negative control.

4.2.1 Investigation of nucleotide-dependent conformational changes of PfRUVBL1 and PfRUVBL2 using limited proteolysis

The limited proteolysis method involves controlled protein exposure to a proteolytic enzyme. The first cleavage of the protein occurs while preserving its tertiary and quaternary structure. Due to the trypsin size, this restricts initial cleavage sites to the outermost regions of the protein subunit surfaces accessible to the enzyme's active site. The limited trypsin proteolysis was conducted following a slightly modified method previously described by Zininga *et al.* (2016). Briefly, 20 μ g of trypsin was incubated with 4 μ M of PfRUVBL1 and PfRUVBL2 individually at 37 °C at various times in the presence of 5mM ATP and ADP. The reaction was terminated by heating at 100 °C for 10 minutes in the electrophoresis sample buffer. SDS-PAGE then separated the reaction products, and after electrophoresis, the gels were stained with silver stain.

4.2.2 Investigation of basal ATPase activity of PfRUVBL1 and PfRUVBL2

The ATPase activities of PfRUVBL1 and PfRUVBL2 relative to their yeast homology were determined using a calorimetric ATPase activity assay as previously described (Zininga *et al.*, 2016; Mabate *et al.*, 2018). Initially, 0.4 μ M of each protein was incubated for 5 hours in HKMD buffer (10 mM HEPES-KOH pH7.5, 100 mM KCl, 2 mM MgCl₂, 0.5 mM DTT) with varying concentrations of ATP (0 – 5 mM). Boiled samples of each protein were used as non-enzymatic control to monitor spontaneous hydrolysis of ATP, while PfHsp70-1 was used as positive control. Three independent assay runs were conducted using different protein batches for each protein. To determine the kinetics for the ATPase activity of each protein, Michealis-Menten plots were generated using GraphPad Prism 6.05.

To further verify the ATPase activity of PfRUVBL1 and PfRUVBL2, the assay was repeated in the presence of 10 μ M Sorafenib, and Sorafenib was found to inhibit the ATPase activity of recombinant human RUVBL1/2 complex *in vitro* (Nano *et al.*, 2020). Statistical analysis was conducted using a One-Way ANOVA at $p < 0.05^*$; $p < 0.01^{**}$; $p < 0.001^{***}$.

4.2.3 Investigation of chaperone-like function of PfRUVBL1 and PfRUVBL2 using MDH aggregation assay

The capability of PfRUVBL1 and PfRUVBL2 to suppress heat-induced aggregation of malate dehydrogenase (MDH) from porcine heart (Sigma-Aldrich, USA) was investigated as previously described (Shonhai *et al.*, 2008; Luthuli *et al.*, 2013). A well-studied *P. falciparum* heat shock protein (PfHsp70-1) was used as a control. Briefly, 0.4 μ M recombinant protein (PfRUVBL1, PfRUVBL2, and PfHsp70-1) were each incubated with equimolar MDH concentration. The respective mixtures were each incubated at 51 °C for 2 hours. The optical density (OD) readings were taken every 5 mins at 340 nM using a spectraMax D3 spectrometer (Molecular Devices, USA). The resulting ODs were then converted to aggregation percentages relative to the aggregation level of MDH (set as 100%), and data was analysed using GraphPad Prism 6.05 software (San Diego, USA).

4.2.4 DNA cleaving assay

The DNA cleavage capabilities of PfRUVBL1 and PfRUVBL2 were examined following a previously described protocol (Sen *et al.*, 2018). In this study, both proteins were prepared at 10 μ M and incubated with 100 ng of double-stranded DNA (pQE30) in a reaction buffer consisting of 20 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 100 mM KCl, 8 mM DTT, 4% sucrose,

and 5 mM ATP. The incubation was performed at 30°C for one hour. To stop the reaction, a 6X loading dye was added, and the samples were subsequently analyzed using agarose gel electrophoresis. Visualization of the DNA bands was achieved using a ChemDoc system from Bio-Rad.

4.2.5 Antiplasmodial activity assay

The test samples were prepared to a 10 mmol/l stock solution in 100% DMSO. Samples were tested as a suspension if not completely dissolved. Further dilutions to the desired starting concentration were freshly prepared in growth media on each occasion of the experiment. The standard antimalarial drugs CQ and artesunate (AT) were used as the reference drug in all experiments. A full dose response was performed for all compounds in a 96-well plate to determine the concentration inhibiting 50% of parasite growth (IC_{50} -value). Test samples were tested at a starting concentration of 10 μ mol/l, which was then serially diluted 2-fold in growth medium to generate the tested concentration range. The same dilution technique was used for all samples. CQ and AT were tested from a starting concentration of 1 μ g/ml. The highest concentration of solvent to which the parasites were exposed was <0.5% and exhibited no measurable effect on parasite viability.

The assay plate was incubated at 37 °C for 72 hrs in a sealed gas chamber under 3% O₂ and 4% CO₂, with the balance being N₂. After 72 hrs, the wells in the assay plate were gently resuspended, and 15 μ l from each well was transferred to a duplicate plate containing 100 μ l of Malstat reagent and 25 μ l of nitroblue tetrazolium solution in each well. Plates were left to develop for 20 mins in the dark, and then the absorbance of each well was quantified using a spectrophotometer at 620 nm wavelength.

The remaining population of parasites at each concentration of the test compound was determined by comparing the absorbance of each well to the absorbance of a well containing the drug-free control. Survival was plotted against concentration, and the IC_{50} values were obtained using a nonlinear dose-response curve fitting analysis via the Dotmatics software platform.

4.2.6 Investigation of the direct interaction of PfRUVBL1 with PfRUVBL2

The binding affinities of both PfRUVBL1 and PfRUVBL2 homo and hetero association were determined using SPR. The analysis was conducted using Bio Navis™ 220A ILVES MP SPR (Bio Navis, Tampere, Finland) system at 25 °C. PfRUVBL1 and PfRUVBL2 were

immobilized as ligands onto CMD three-dimensional chips as previously described (section 5.2). PfRUVBL1 and PfRUVBL2 were individually injected as analytes at 0, 125, 250, 500, 1000, and 2000 nM concentrations and a 50 μ l/min flow rate in each flow channel.

To further verify the interaction between, direct association was investigated using the slot blot technique. Briefly, varying concentrations (0, 0.2, 0.4, 0.6 μ M) of PfRUVBL1 were prepared in PBS buffer (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 137 mM KCl and 20 mM EDTA; pH 7.4). BSA was used as the negative control since it does not interact with PfRUVBL1 refer to section 3.3. PfRUVBL1 was immobilized onto the nitrocellulose membrane by spotting. Blocking was performed using 5% skimmed milk (TBST). The membrane was overlaid with 0.6 μ M PfRUVBL2 overnight and then washed three times with TBST. Primary α -PfRUVBL2 (1:5000, Chapter 3) was used to detect PfRUVBL2, followed by secondary α -rabbits IgG (1:5000) antibody. Detection of bands was performed using ECL and visualized using ChemDoc (Bio-Rad). Assays were repeated in the presence of 5 Mm ATP, ADP, and 10 μ M Sorafenib.

4.2.7 Determination of PfRUVBL1 and PfRUVBL2 binding affinity to PfHsp70-1 and PfHsp90

The binding affinities of both PfHsp70-1 and PfHsp90 with either PfRUVBL1 or PfRUVBL2 were determined using SPR. The analysis was carried out using Bio Navis TM 220A ILVES MP SPR (Bio Navis, Tampere, Finland) system at 25 °C using degassed PBS (4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 137 mM KCl and 20 mM EDTA; pH 7.4). PfRUVBL1 and PfRUVBL2 were immobilized as ligands onto CMD three-dimensional chips, as previously described (section 5.2). PfHsp70-1 and PfHsp90 were individually injected as analytes at concentrations of 0, 125, 250, 500, 1000, and 2000 nM and a flow rate of 50 μ l/min in each flow channel.

The interaction between the ligand and the analyte was permitted to reach a steady state for 1 minute. During this period, the binding dynamics were closely monitored. Following the establishment of the steady state, the dissociation phase was observed for a total of 2 minutes. To analyze and process the steady state equilibrium constant data obtained from these interactions, we utilized Trace Drawer software version 1.8, developed by Ridgeview Instruments based in Sweden. This software facilitated a thorough examination of the binding kinetics and equilibrium to ensure accurate and reliable results.

4.2.8 Investigating the capability of sorafenib to stabilise PfRUVBL1 and PfRUVBL2 using thermal shift assay (TSA)

The TSA uses the phenomenon in which ligand binding increases PfRUVBL1 and PfRUVBL2 heat stability. The assay measures the melting temperature of a protein (T_m), which is the temperature at which there is 50 % denaturation (Huynh and Partch, 2015). TSA determines the shift in melting temperature by changes in light scattering or by fluorescence technique (Groftehaug *et al.*, 2015). Briefly, reaction components were added to the reaction plate on ice (Appendix 10) and mixed 10 times by pipetting with (8X diluted) protein Thermal shift Dye. The plates were sealed with MicroAmp optical adhesive film, span at 1000 rpm for 1 min, and then placed on ice. The TSA was run using the real-time PCR instrument software (Quant Studio™ 5 system), and the experiment run file was set up as outlined (Appendix A10). To calculate the derivative melting temperature (T_m), the region of analysis (ROA) was established using the auto single T_m mode. The mode allows for an automated assessment of the melting temperature by analyzing the thermal profile of the sample, ensuring precise measurements and optimal conditions for determining the T_m value.

4.3 Results

4.3.1 PfRUVBL1 and PfRUVBL2 bind to nucleotides

Based on the *in silico* studies presented in Chapter Two, it is evident that both PfRUVBL1 and PfRUVBL2 are capable of binding ATP. The binding of ATP significantly influences the conformational states of ATPases (Lopez-Perrote *et al.*, 2012). Specifically, RUVBL1 and RUVBL2 utilize Walker A motifs situated within their AAA+ ATPase domains to bind to ATP or ADP (Gorynia *et al.*, 2011). Consequently, establishing the nucleotide-binding affinities of both PfRUVBL1 and PfRUVBL2 was a critical objective of the study (Figure 4.1). Additionally, it was necessary to evaluate the conformational variations induced by nucleotide binding (Figure 4.2). The investigation revealed that both PfRUVBL1 and PfRUVBL2 bind nucleotides in a concentration-dependent manner, achieving an equilibrium at a concentration of approximately 1 μM (Figure 4.2).

The K_D value in SPR is a quantitative measurement of the dissociation equilibrium constant, which represents the concentration of ligands of which 50% of the protein binding sites are occupied (Sparks *et al.*, 2019). The k_a represents the binding of the analyte, while the k_d is the removal of the analyte (Figure 4.1G; Sparks *et al.*, 2019). The χ^2 shows how well a fitted curve matches the experimental data (Onell and Andersson, 2005). The χ^2 can be used as a global measurement for the residual noise, and typically its value should be less than 10 (Figure 4.1G; Zhang and Oglesbee, 2003; Onell and Andersson, 2005).

Notably, the data suggest that PfRUVBL1 and PfRUVBL2 display varied affinity for ATP. PfRUVBL2 displayed a markedly enhanced ATP binding affinity, quantified as $K_D = 1.44 \mu\text{M}$, compared to PfRUVBL1, which exhibited a K_D value of $8.25 \mu\text{M}$ (Figure 4.1G). This disparity underscores the essential role of ATP binding in the functionality of both PfRUVBL1 and PfRUVBL2, suggesting that they are both adept at uniquely binding ATP, which is crucial for their ATPase activity. Furthermore, these binding affinity results align with the molecular docking predictions presented in Chapter 2, section 2.1.4, although the *in vitro* findings indicate a higher ATP affinity for PfRUVBL2 than initially predicted.

Analysis of the interaction of PfRUVBL1 and PfRUVBL2 with ligands

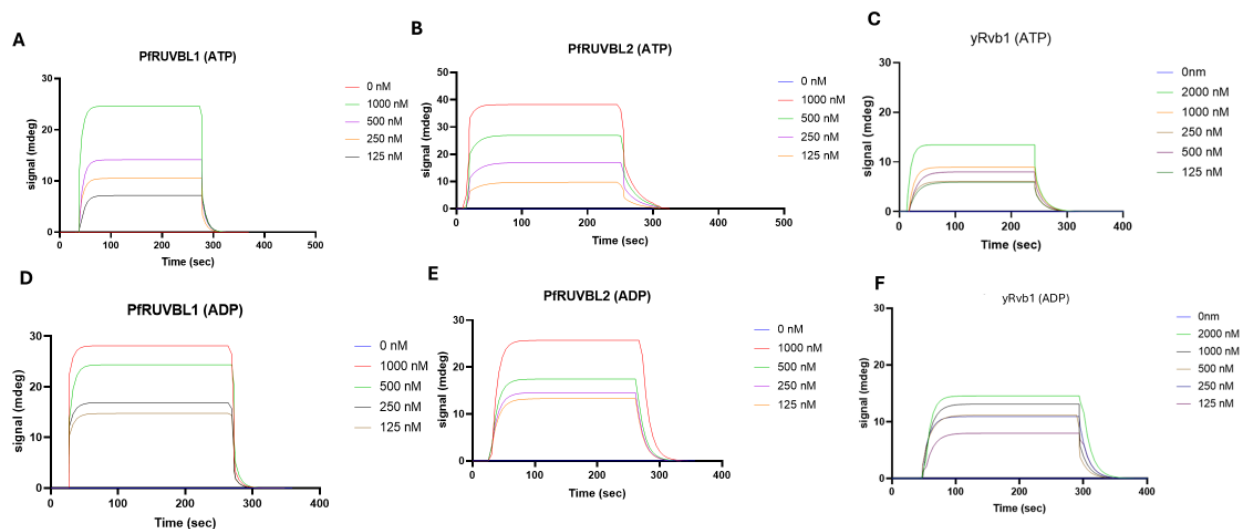


Figure 4.1: Nucleotide-dependent interaction of PfRUVBL1 and PfRUVBL2 in comparative to their yeast homology Rvb1

SPR sensorgrams showing PfRUVBL1, PfRUVBL2 and yRvb1 binding to ATP and ADP at micromolar range, (A) PfRUVBL1 binding to ATP, (B) PfRUVBL2 binding to ATP, (C) yRvb1 binding to ATP, (D) PfRUVBL1 binding to ADP, (E) PfRUVBL2 binding to ADP, and (F) yRvb1 binding to ADP, (G) Binding affinities table.

4.4 Nucleotide-binding uniquely alters the tertiary structure of PfRUVBL1 and PfRUVBL2

Nucleotide-binding to RUVBL1 and RUVBL2 has been shown to induce significant conformational changes in these molecules, as demonstrated in human homologs (Gorynia *et al.*, 2011). Specifically, the conformational alterations that occur upon the binding and hydrolysis of ATP play a crucial role in propelling the functional cycle of these proteins (Garcia-Martin *et al.*, 2024). The effects of ATP and ADP on the tertiary structure of PfRUVBL1 and PfRUVBL2 relative to their yeast homolog, Rvb1 was investigated in the current study. Additionally, the effect of sorafenib, a small molecule inhibitor, on the tertiary conformation of both PfRUVBL1 and PfRUVBL2 was further explored (Figure 4.2).

PfRUVBL1 and PfRUVBL2 demonstrated distinct spectral blue shifts when bound to ATP and ADP, with a notably more pronounced blue shift observed in the ATP-bound state (Figure 4.2). Previous studies have established that RUVBL1 and RUVBL2 tend to adopt a closed conformation in the presence of ATP, while the binding of ADP or the absence of nucleotides leads to a transition to a more relaxed or open conformation (Gorynia *et al.*, 2011; Munoz-Hernandez *et al.*, 2019). PfRUVBL1 underwent a significant blue shift of 6 nM upon ATP binding, mirroring the behavior of the yeast homolog yRvb1. In contrast, PfRUVBL2 exhibited a smaller blue shift of just 2 nM. These differences suggest that PfRUVBL1 and PfRUVBL2 adopt distinct structural conformations relative to their yeast counterpart, highlighting their unique biophysical properties.

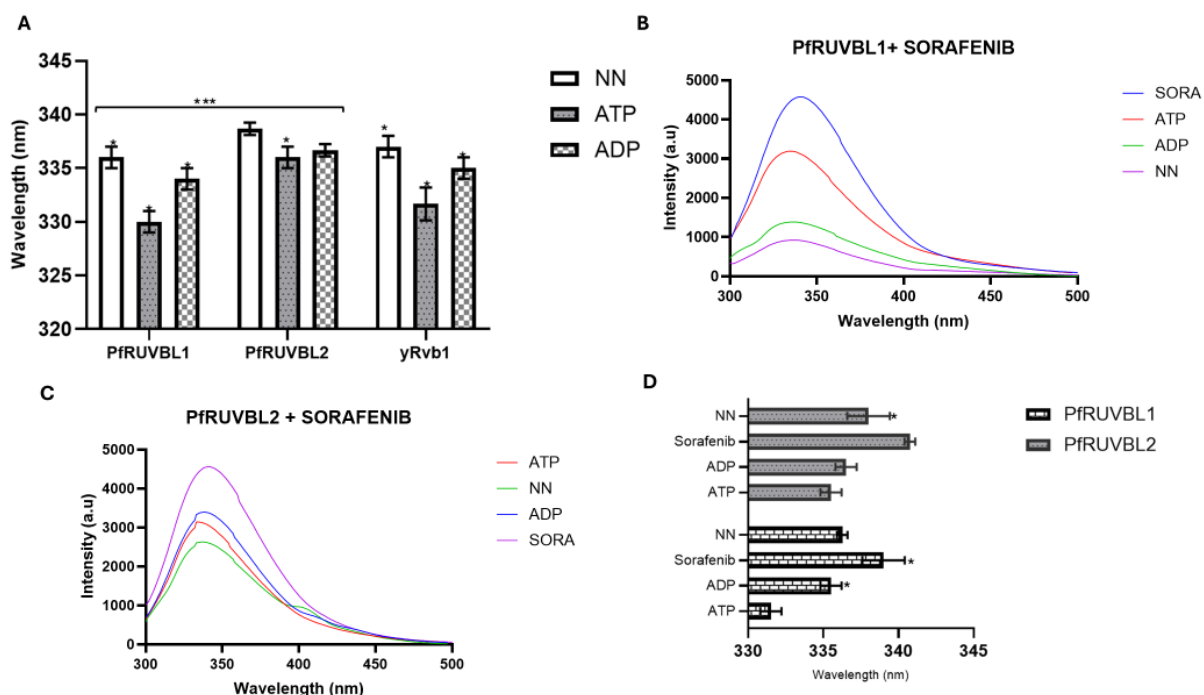


Figure 4.2: The interaction of PfRUVBL1 and PfRUVBL2 with nucleotides and sorafenib alters their tertiary structure The shift emission maxima wavelength for each protein in the presence of ATP, ADP, and sorafenib bound state were calculated and plotted. The assay was conducted in the absence of ligand (NN) in the presence of either 5 mM ATP/ADP or 10 μ M sorafenib. Statistical analyses were conducted on PfRUVBL1 and PfRUVBL2 in the presence and absence of small molecules.

Furthermore, the binding of Sorafenib to PfRUVBL1 and PfRUVBL2 resulted in a notable redshift (Figure 4.2). This observation implies that Sorafenib may induce unfolding behavior in both proteins. The exposure of hydrophobic tyrosine residues in the presence of Sorafenib suggests a significant effect of this small molecule on the folding and conformational stability of PfRUVBL1 and PfRUVBL2, potentially leading to altered functional outcomes for these proteins.

4.5 Extrinsic fluorescence spectroscopy

To further validate the influence of ATP and ADP on PfRUVBL1 and PfRUVBL2 tertiary conformation, the ANS binding assay was conducted (Figure 4.3). ANS binding is known to be influenced predominantly by the cationic charge of macromolecules interacting with hydrophobic areas on proteins (Achilonu *et al.*, 2014). In the experiments, both PfRUVBL1 and PfRUVBL2 exhibited a notable decrease in quantum yield and a redshift in the fluorescence spectrum when ANS was introduced (Figure 4.3). The observed spectral shifts indicate that PfRUVBL1 and PfRUVBL2 attain compact structures and do not possess the

expected non-polar characteristics typically associated with hydrophobic interactions upon binding with ANS.

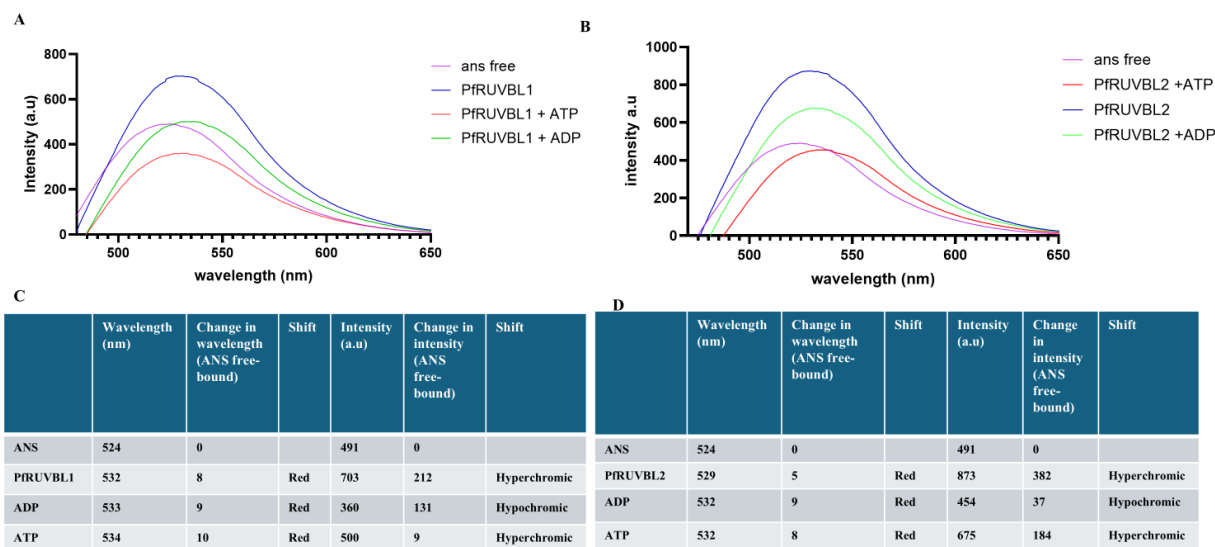


Figure 4.3: The ANS binding spectra of PfRUVBL1 and PfRUVBL2 in the presence of ATP and ADP

The shift emission maxima wavelength for each protein in the presence of ATP and ADP were calculated and plotted. The assay was conducted in the absence of and the presence of 5 mM ATP/ADP, and ANS-free was used as the baseline.

Furthermore, the presence of nucleotides such as ATP and ADP appeared to enhance the compact structure of the proteins (Figure 4.3). This suggests that nucleotides play a significant role in modulating the conformational dynamics of both PfRUVBL1 and PfRUVBL2. The findings from the ANS binding assay are consistent with the results from intrinsic spectroscopy (Figure 4.2), which also indicated a compacted conformation of both PfRUVBL proteins in the presence of nucleotides. This alignment of data suggests a strong relationship between nucleotide binding and the structural characteristics of PfRUVBL1 and PfRUVBL2, particularly regarding their hydrophobic regions.

4.6 Nucleotides regulate the conformations of PfRUVBL1 and PfRUVBL2

The influence of nucleotides on the structural conformation of PfRUVBL1 and PfRUVBL2, compared to their yeast homolog, Rvb1, was further investigated using partial proteolysis in the presence of 5 mM (ATP or ADP). For the proteolytic analysis, trypsin was chosen as the protease due to its exceptional cleavage specificity (Zheng *et al.*, 2017). The proteolysis was performed under carefully optimized conditions to ensure accuracy and reproducibility (Mansuri *et al.*, 2024). The resulting fragments of PfRUVBL1, PfRUVBL2, and their yeast

counterpart yRvb1 were subsequently analyzed using silver-staining techniques, which allowed for the visualization of the generated fragments (Figure 4.4).

Before the initiation of trypsin digestion, the proteins existed in their monomeric forms without any discernible breakdown products (Figure 4.4; Lane C). Following the addition of trypsin, the protein samples were monitored at intervals of 5, 15, and 30 minutes to track the progression of proteolytic cleavage and any conformational changes occurring over time (Figure 4.4). The profiles of fragments that were generated upon the proteolysis of PfRUVBL1 and PfRUVBL2 relative to yeast Rvb1 homolog in the presence of ATP or ADP were different (Figure 4.4). PfRUVBL1, in the presence of ATP, exposes more digestion sites compared to PfRUVBL2. PfRUVBL2 in the presence of ATP remains undigested compared to the presence of ADP, like yeast Rvb1, which remains undigested in the presence of ATP after 30 mins (Figure 4.4). This suggests that ATP and ADP had varied effects on PfRUVBL1 and PfRUVBL2.

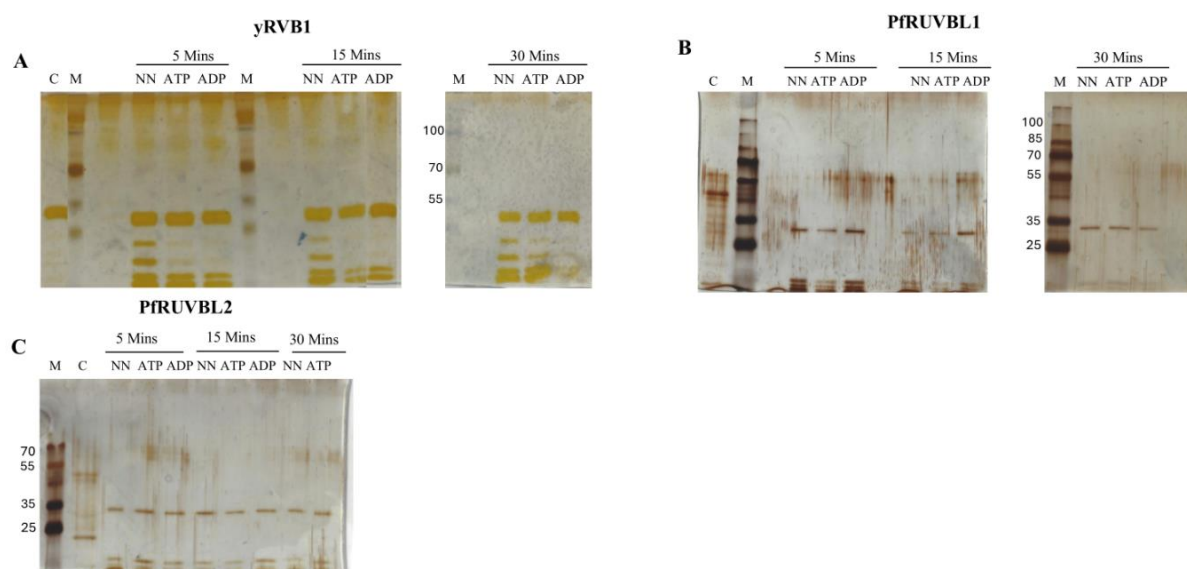


Figure 4.4: Limited proteolysis confirming nucleotide-induced structural changes in PfRUVBL1 and PfRUVBL2

Silver-stained SDS-PAGE showing partial digestion of proteins in a nucleotide-free buffer (NN) and in the presence of 5 Mm ATP and ADP, respectively.

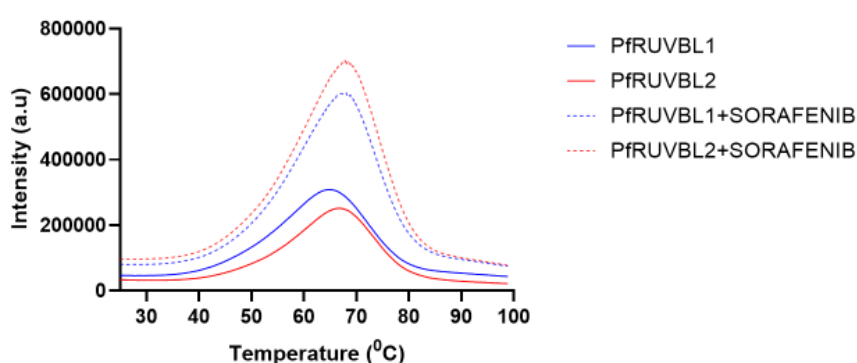
4.7 Sorafenib stabilizes PfRUVBL1 and PfRUVBL2 against heat stress

The melting temperatures of PfRUVBL1 and PfRUVBL2 were assessed through a thermal shift assay (TSA), a sensitive method for evaluating protein stability in the presence of sorafenib as a ligand (Huynh *et al.*, 2020). In the absence of sorafenib, PfRUVBL1 demonstrated a melting temperature (T_m) of 63.02 °C, indicating its baseline thermal stability.

PfRUVBL2 exhibited a slightly higher T_m of 64.5 °C, suggesting that it may possess a greater intrinsic stability compared to PfRUVBL1 (Figure 4.5). During the assay, the fluorescence intensity associated with the melting peak was measured. For PfRUVBL1, in the absence of any ligand, the melting peak was recorded at 300,611 fluorescence units, while PfRUVBL2 displayed a different peak at 241101 fluorescence units. These values serve as reference points for understanding how ligand binding affects protein stability (Grøftehaug *et al.*, 2015).

Upon sorafenib introduction, significant changes in the melting temperatures were observed (Figure 4.5). PfRUVBL1's T_m rose to 68.0 °C, highlighting a notable increase in its thermal stability upon ligand binding. This change was accompanied by an elevated melting peak intensity, recorded at 603201 603,201 fluorescence units, further indicating enhanced interaction with the ligand (Figure 4.5). Similarly, PfRUVBL2 exhibited an even more pronounced stability shift in the presence of sorafenib, with a T_m reaching 69.0 °C. This increase suggests that the ligand effectively stabilizes the protein structure. The melting peak intensity also rose significantly, recorded at 691472 fluorescence units.

A



B

Sample	T_m	Melting Peak
PfRUVBL1	63.016	300611
PfRUVBL2	64.513	241101
PfRUVBL1/Sor	68.105	603201
PfRUVBL2/Sor	69.003	691472

Figure 5.5: Sorafenib stabilizes PfRUVBL1 and PfRUVBL2

The interaction of PfRUVBL1 and PfRUVBL2 was investigated using TSA). (A) fluorescence vs temperature spectrum of PfRUVBL1 and PfRUVBL2 in the presence of ligand sorafenib (B) melting affinity table. Data was analyzed using thermal shift software.

Overall, the results of the thermal shift assay indicate that the binding of sorafenib enhances the stability of both PfRUVBL1 and PfRUVBL2. This is consistent with findings from prior studies (Singh *et al.*, 2018; Huynh *et al.*, 2020) that explored the interactions between sorafenib and similar protein targets.

4.8 The PfRUVBL1 and PfRUVBL2 ATPase activity is partially inhibited by Sorafenib

ATPase activity assay was conducted to investigate the capability of PfRUVBL1 and PfRUVBL2 to hydrolyze ATP. The release of inorganic phosphate was monitored upon ATP hydrolysis. PfRUVBL1 and PfRUVBL2 showed capabilities to bind nucleotides, and nucleotide binding changes their conformation (Figure 4.1; 4.2). Findings from tyrosine fluorescence and limited proteolysis suggest that changes in the protein's conformation are regulated by nucleotides. PfRUVBL1 and PfRUVBL2 belong to the AAA+ ATPases hence it was important to evaluate their ATPase activity. RUVBL1 and RUVBL2 have been reported to possess weak ATPase activity (Nano *et al.*, 2013). Previously, it has been demonstrated that human RUVBL proteins possess low ATPase activity compared to other AAA+ protein and yeast Rvb proteins (Marrione and Cox, 1996; Gribun *et al.*, 2008).

PfRUVBL1 exhibited a K_m value of 1.89 mM, PfRUVBL2 had 1.10 mM K_m , while the complex of PfRUVBL1/2 had 0.5 mM K_m (Figure 4.6; Table 4.2). This suggests that the PfRUVBL1/2 complex has a higher affinity for ATP compared to individual proteins. PfRUVBL1, in the presence of Sorafenib, reported a K_m value of 2.07 mM, while PfRUVBL2 with Sorafenib reported 2.34 mM K_m as determined by Michaelis-Menten plots. The kinetics (Table 4.2) suggest that PfRUVBL1 and PfRUVBL2 ATPase activity is slightly lower than yRvb1 and PfHsp70-1. Sorafenib has a marginal inhibition effect on the ATPase activity of PfRUVBL1 and PfRUVBL2 proteins *in vitro* (Figure 4.6). The differences in the relative ATPase activity (as K_m) were statistically significant between PfRUVBL1 and PfRUVBL2 and in the presence of Sorafenib as observed on One-Way ANOVA ($p < 0.001$; Figure 4.6B).

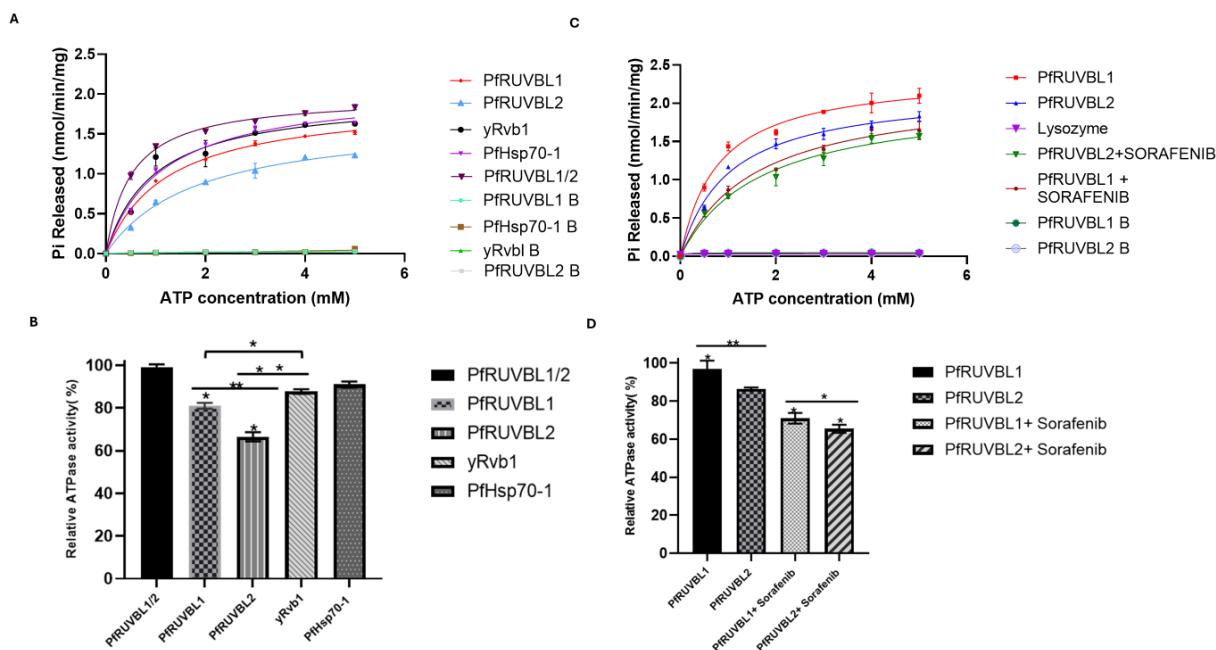


Table 4.2: ATPase activity kinetics

Protein	$V_{\max}$ (nmol/min/mg)	K_m (mM)	Reference
yRVB1	1.970(+/-0.959)	0.954(+/-0.959)	This study
hRUVBL1	0.3153(+/-1.11)	1.290(+/-0.19)	Nardin, 2018
hRUVBL2	1.234(4+/-4.64)	0.70(+/-0.11)	Nardin, 2018
PfRUVBL1	1.948(+/-0.987)	1.886(+/-0.987)	This study
PfRUVBL1/Sor	1.254(+/-0.657)	2.077(+/-1.707)	This study
PfRUVBL2	2.078(+/-0.989)	1.110(+/-0.989)	This study
PfRUVBL2/Sor	1.546(+/-0.987)	2.346(+/-0.987)	This study
PfRUVBL1/2	1.997(+/-0.678)	0.501(+/-0.678)	This study
PfHsp70-1	1.730(+/-0.985)	1.758(+/-0.985)	This study
PfHsp70-1	6.521(+/-0.79)	1.26(+/-0.79)	Mudau, 2022
PfHsp70-1	1.320(+/-5.801)	15.07(+/-0.38)	Chakafana, 2020

Figure 4.6: PfRUVBL1 and PfRUVBL2 exhibits ATPase activity

Evaluation of basal ATPase activity of PfRUVBL1, PfRUVBL2, and PfRUVBL1/2 mini complex. (A) Michaelis plot of the amount of Pi released in the absence of sorafenib, (B) Relative ATPase activity of PfRUVBL1, PfRUVBL2, PfRUVBL1/2, PfHsp70-1, and yRvb1 of Pi released, (C) Michaelis plot of the amount of Pi released in the presence of sorafenib, (D) Relative ATPase activity of PfRUVBL1 and PfRUVBL2 in the presence of sorafenib, Table (4.2) proteins ATPase activity kinetics. The experiments were conducted in triplicate and repeated on three separate occasions using different protein batches to ensure reproducibility. Standard deviations were drawn with results accumulated from different batches and showed marginal differences. Statistical analyses were conducted on PfRUVBL1 and PfRUVBL2 in the presence and absence of small molecules.

4.9 Antiplasmodial activity

The control compounds chloroquine and artesunate are extremely active and showed IC₅₀ values of 9 nM and 4 nM, respectively, in NF54 (Table 4.3). As expected, no significant difference was seen with AT in Dd2; however, CQ showed an increase of > 20-fold against the resistant parasite (Table 4.3). Sorafenib had an insignificant activity with a value of 9.86 µM in the NF54 strain (wild-type drug-sensitive strain) and no activity in the Dd2 strain (drug-resistant strain) (Table 4.3). Phenylthynesulfonamide (PES), an inhibitor of PfHsp70-PfHsp partnership exposed in increasing concentrations (2- 500 µM), registered a modest IC₅₀ of 38.7 µM (Muthelo *et al.*, 2022). In the current study, sorafenib was inactive at 10 µM against both the wild-type and the resistant parasite (Table 4.3). The current study does not exclude the off-target inhibition of RUVBL proteins by sorafenib.

Table 4.3: Antiplasmodial activity

Code	Nf54		Dd2		RI
	Mean	SD	Mean	SD	
SFB	9.860	NA	10.0	NA	ND
CQ	0.009	0.002	0.194	0.011	21.67
AT	0.004	0.001	0.005	0.001	1.00

Table 4.3: Antiplasmodial activity of the compound series against *P. falciparum* Nf54

Data shown are the mean of two evaluations in triplicate and are presented in µmol/L. NA-Not applicable, ND-Not determined.

4.10 PfRUVBL1 and PfRUVBL2 both exhibit holdase chaperone function

The heat-induced aggregation of MDH was conducted to investigate the chaperone-like activity of PfRUVBL1 and PfRUVBL2. Rvb1 and Rvb2 from yeast have been previously reported to possess chaperone activity in the assembly of various complexes (Nano *et al.*, 2013). MDH is an aggregation-prone protein when exposed to elevated temperatures (Shonhai *et al.*, 2008; Luthuli *et al.*, 2013). PfHsp70-1 is known to suppress MDH aggregation (Luthuli *et al.*, 2013; Makumire *et al.*, 2014). Hence, it was used as the control. PfHsp70-1 was able to suppress MDH by more than 50%, which agrees with previous studies (Shonhai *et al.*, 2008; Zininga *et al.*, 2016; Muthelo *et al.*, 2022).

Both PfRUVBL1 and PfRUVBL2 were stable at 51 °C with aggregation percentages of 37 % and 22 %, respectively. PfRUVBL1 was able to suppress MDH aggregation up to 72 % MDH

aggregation, and PfRUVBL2 suppressed MDH aggregation up to 80 % (Figure 4.7). The reduced MDH aggregation activity may demonstrate PfRUVBL1 and PfRUVBL2 holdase chaperone activity, which is essential in multi-protein complex assembly. Significant statistical differences existed in the aggregation pattern of both PfRUVBL1 and PfRUVBL2 and their capability to suppress MDH aggregation (Figure 4.7, $p < 0.001$). This data agrees with observed TSA results, which reported PfRUVBL1 and PfRUVBL2 having different melting temperatures.

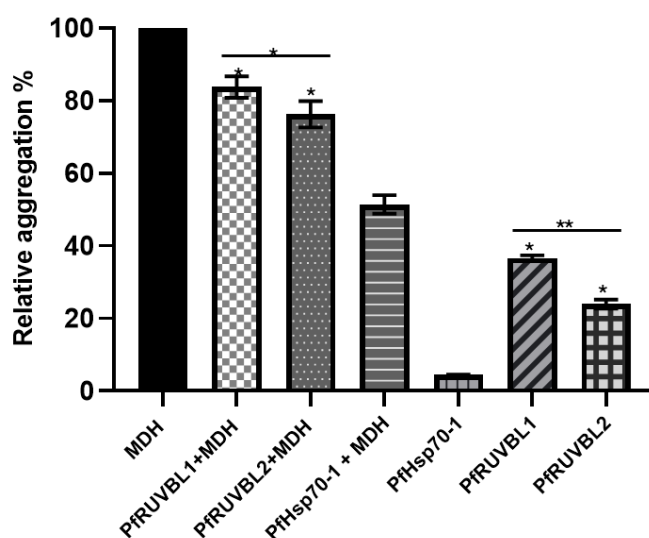


Figure 4.7: PfRUVBL1 and PfRUVBL2 exhibits holdase chaperone function

Heat-induced aggregation of MDH was investigated at 51 °C. The capability of PfRUVBL1 and PfRUVBL2 to suppress MDH aggregation was investigated. The assay was run in the presence of PfHsp70-1 as an assay control. Three independent assays were performed, and results are plotted as a means with standard deviations shown. Statistical analysis was conducted at $p < 0.01^{**}$ using One-way ANOVA.

4.11 ATP regulates the cleavage of double-stranded DNA (dsDNA) by PfRUVBL2

The capability of PfRUVBL1 and PfRUVBL2 to cleave DNA was conducted using dsDNA, and the pQE30 plasmid was exposed to 5 mM ATP (Figure 4.8). Restriction endonuclease *Bam*H1 was used as a control (Newman *et al.*, 1994). The unique insertion domain of PfRUVBL3 has been reported to possess DNA cleavage activity (Sen *et al.*, 2018). PfRUVBL1 partially converted nicked dsDNA into linear form in the absence of ATP, in the presence of ATP, the original form was retained (Figure 4.8). This could suggest that PfRUVBL1 has no activity against dsDNA.

PfrUVBL2 digested supercoiled double-stranded DNA (dsDNA) into nicked dsDNA when ATP was present in the reaction mixture (Figure 4.8). However, when ATP was excluded from the conditions, no activity was detected, suggesting ATP dependence for this conversion process. These findings suggest that PfrUVBL2 possesses inherent DNA cleaving activity that targets double-stranded DNA (dsDNA) and that this activity is likely regulated by ATP, highlighting its potential role in cellular processes involving DNA manipulation and repair.

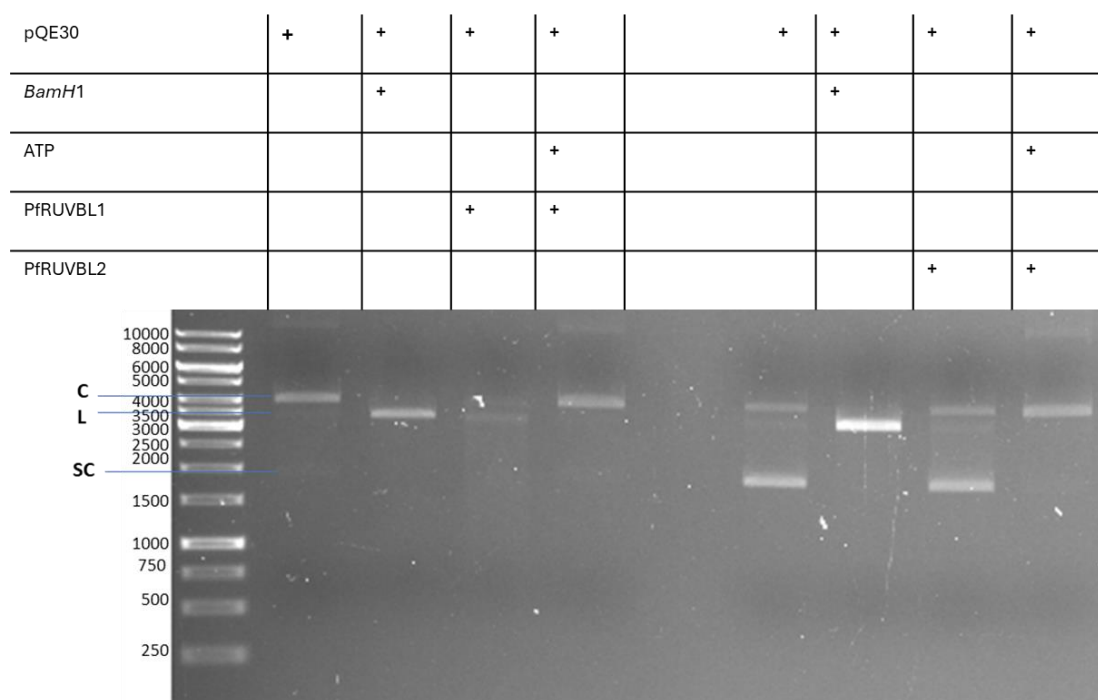


Figure 4.8: PfrUVBL1 and PfrUVBL2 exhibit DNA cleaving function

in vitro analysis of recombinant PfrUVBL1 and PfrUVBL2 cleaving capability. Double-stranded Pqe30 was incubated with PfrUVBL1 and PfrUVBL2 in the presence/ absence of ATP, (C) nicked DNA, (L) linear DNA, and (SC) supercoiled DNA.

4.12 Self- and hetero- association of PfrUVBL1 and PfrUVBL2

The interaction between PfrUVBL1 and PfrUVBL2 and their self-association was examined using surface plasmon resonance (SPR) analysis. The resulting sensorgram indicated that this interaction occurred in a concentration-dependent manner, implying that it is specific (Figure 4.9). The results confirmed that PfrUVBL1 and PfrUVBL2 self-associate and hetero-associate at low micromolar concentrations (Table 5.1 in Appendix C). Research indicates that RUVBL1 and RUVBL2 assemble into hetero-hexameric ring structures (Gorynia *et al.*, 2011; Seraphim *et al.*, 2021). These structures are characterized by an alternating arrangement, highlighting the intricate organization of these proteins.

When PfRUVBL1 was used as both ligand and analyte, it exhibited a dissociation constant (K_D) value of 38.9 μM , compared to the 10.4 μM value recorded for PfRUVBL2 (Table 5.1; Appendix B13). This indicates that PfRUVBL1 has a lower self-associating affinity than PfRUVBL2.

To further investigate the hetero-association between PfRUVBL1 and PfRUVBL2, PfRUVBL2 was immobilized as the ligand on a chip, while equimolar PfRUVBL1 served as the analyte (Figure 5.9 C). The derived sensorgram demonstrated that this interaction was specific and occurred in a concentration-dependent fashion (Figure 5.9 C; Appendix B13). The affinity recorded for this interaction was 102 μM , indicating a specific interaction between the two proteins occurring in a concentration-dependent fashion.

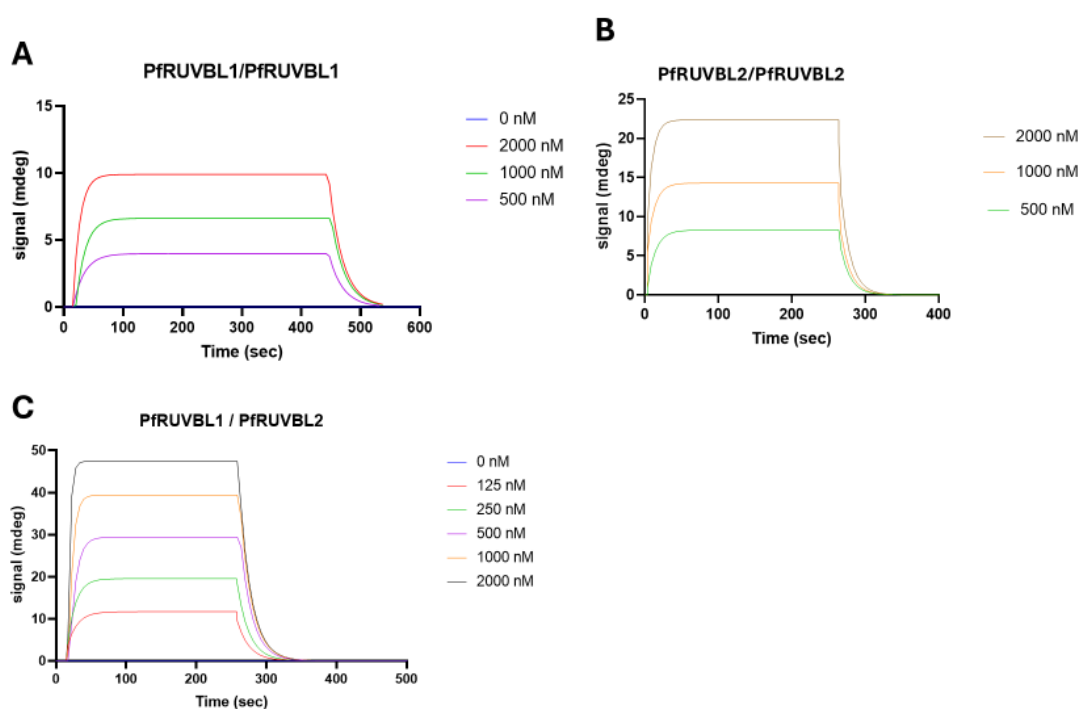


Figure 4.9: Recombinant PfRUVBL1 and PfRUVBL2 demonstrated self and hetero association
SPR sensorgrams for the interaction of PfRUVBL1 with PfRUVBL2. (A) self-association of PfRUVBL1, (B) self-association of PfRUVBL2, and (C) interaction of PfRUVBL1 and PfRUVBL2 in a concentration-dependent fashion.

4.13 The interaction of PfRUVBL1 and PfRUVBL2 is regulated by nucleotides

To further validate the interaction between PfRUVBL1 and PfRUVBL2, a slot blot assay was conducted. In this experiment, the bait protein PfRUVBL1 was immobilized on a nitrocellulose membrane alongside a positive control represented by PfRUVBL2 and a negative control composed of BSA (Figure 4.10). Following the immobilization, the membrane was overlaid

with recombinant PfRUVBL2, allowing for the assessment of protein-protein interactions in the presence of the ligands. Subsequent immunoblotting was carried out utilizing an antibody specific to PfRUVBL2 (Chapter 3). The assay was repeated in the presence of varying ligands (ATP, ADP, and sorafenib). RUVBL1-His₆ and untagged RUVBL2 co-expressed in *E. coli* and co-eluted using metal affinity and fractionated by SEC (Niewiarowski *et al.*, 2010), and this association was facilitated by ATP.

The results revealed that less intense bands were present in the lanes where ADP was included compared to those with ATP and those where no nucleotide was applied, indicating reduced affinity under these conditions (Figure 4.10). Notably, the presence of the inhibitor sorafenib appeared to compromise the interaction between the two proteins, as demonstrated in the immunoblotting results (Figure 4.10). Densitometric analysis of the bands confirmed statistically significant differences in the interaction strengths of PfRUVBL1 and PfRUVBL2 when exposed to the various ligands tested, with a highly significant p-value of less than 0.001 (Figure 4.10 B). This suggests that the type of ligand has a considerable effect on the binding affinity of the proteins in question.

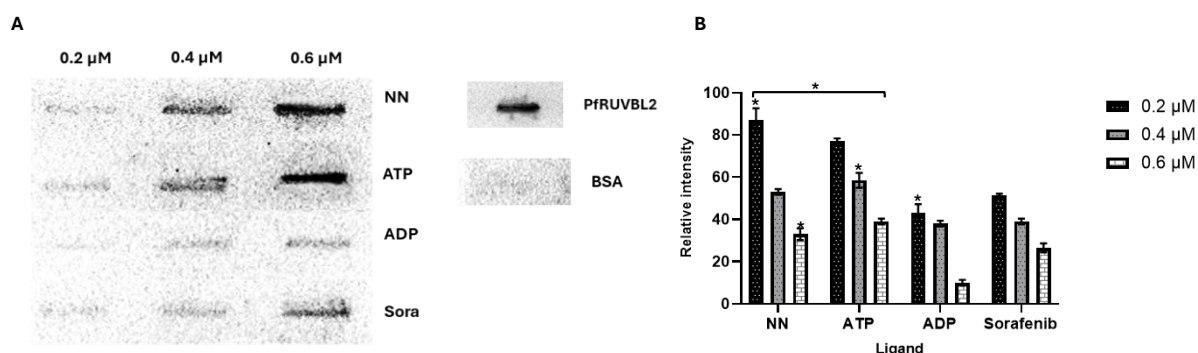


Figure 4.10: Interaction of PfRUVBL1 and PfRUVBL2 is regulated by nucleotides

Various concentrations of PfRUVBL1 (0.2, 0.4, 0.6 μ M) were spotted on the nitrocellulose membrane as the prey protein. BSA (0.6 μ M) was used as a negative control, and PfRUVBL2 0.6 μ M as the positive control. (A) The reaction was conducted in the absence of ligand (NN), presence of ATP, ADP, and sorafenib, and (B) densitometry analysis. Statistical analyses were conducted using One-Way ANOVA ($p < 0.001$).

The SPR technique was further used to validate the interaction between PfRUVBL1 and PfHsp70-1. The sensorgram demonstrates that the interaction between PfHsp70-1 and PfRUVBL1 occurs in a concentration-dependent (Figure 5.11), suggesting that this interaction is specific. The results confirmed that PfRUVBL1 interacts with PfHsp70-1 at a far

millimolar concentration, with a K_D value of 131 mM (Table 5.2, Appendix B14). The recorded kinetics indicate that the interaction between PfRUVBL1 and PfHsp70-1 is relatively weak.

The interaction between PfHsp70-1 and PfHop was assessed as a control, yielding a K_D value of 1.57 μ M in a concentration-dependent manner, which also suggests a specific interaction. Previous studies have described the association between PfHsp70-1 and PfHop using co-immunoprecipitation assays and biochemical methods (Gitau *et al.*, 2012; Zininga *et al.*, 2015b).

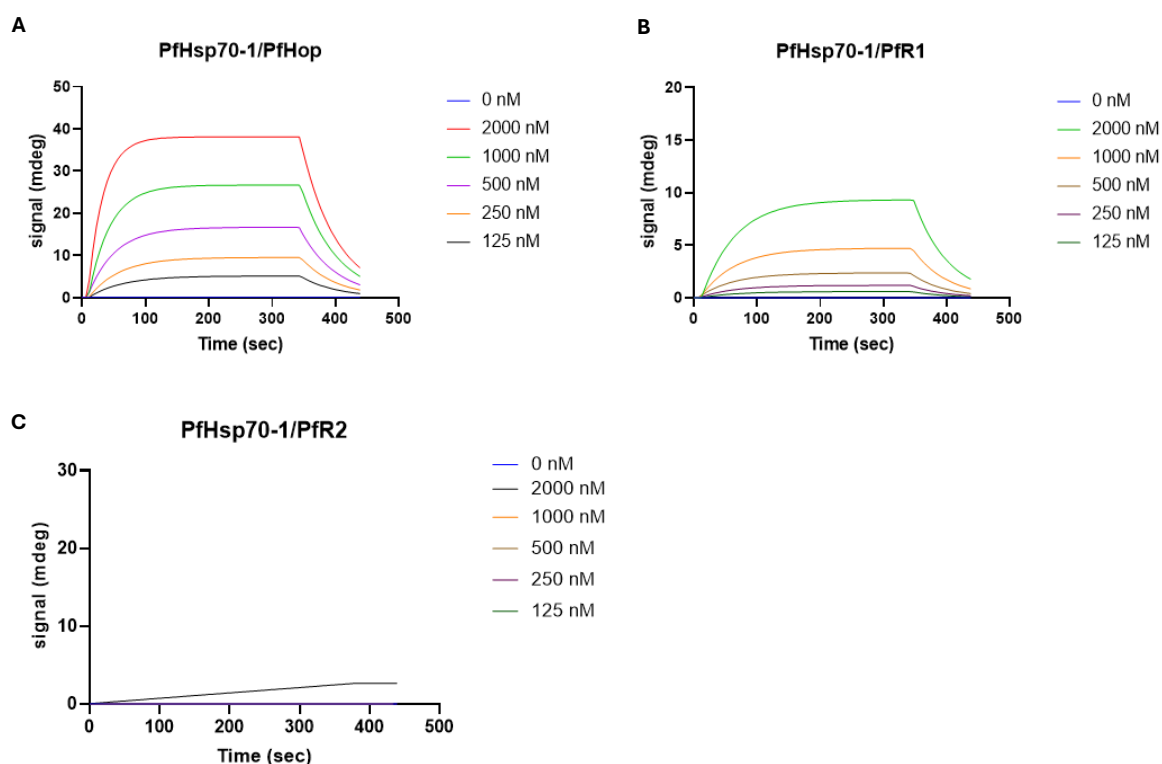


Figure 4.11: PfHsp70-1 interacts with PfRUVBL1 in a concentration-dependent fashion
Sensorgrams representing the interaction of PfHsp70-1 with PfHop (A), PfRUVBL1 (B) and PfRUVBL2 (C) are shown.

Studies have shown that RUVBL2 interacts with HSP90 as part of the R2TP complex in humans (Lakomek *et al.*, 2015). The interaction is normally mediated through co-chaperones such as RPAP3 or PIH1D1. The partnership of RUVBL2 and RPAP3 enhances binding signals between RUVBL2 and Hsp90 (Lakomek *et al.*, 2015; Lynham *et al.*, 2022). The results confirmed the interaction of PfRUVBL2 with PfHsp90 with the micromolar range at low concentrations (125, 250, and 500 nM) (Figure 4.12), with a K_D value of 5.33 μ M (Table 5.2, Appendix B15). The recorded kinetics from sensorgrams indicate that the interaction between PfRUVBL2 and PfHsp90 is specific. There was no observed interaction between PfRUVBL1

and PfHsp90 under the current experimental settings (125, 250, 500, 1000 nM) (Figure 4.12 C).

The interaction between PfHsp90 and PfHop was used as a control, yielding a K_D value of 15.7 μM in a concentration-dependent manner. The interaction between PfHop and PfHsp90 has been previously demonstrated (Gitau *et al.*, 2011; Silva *et al.*, 2020).

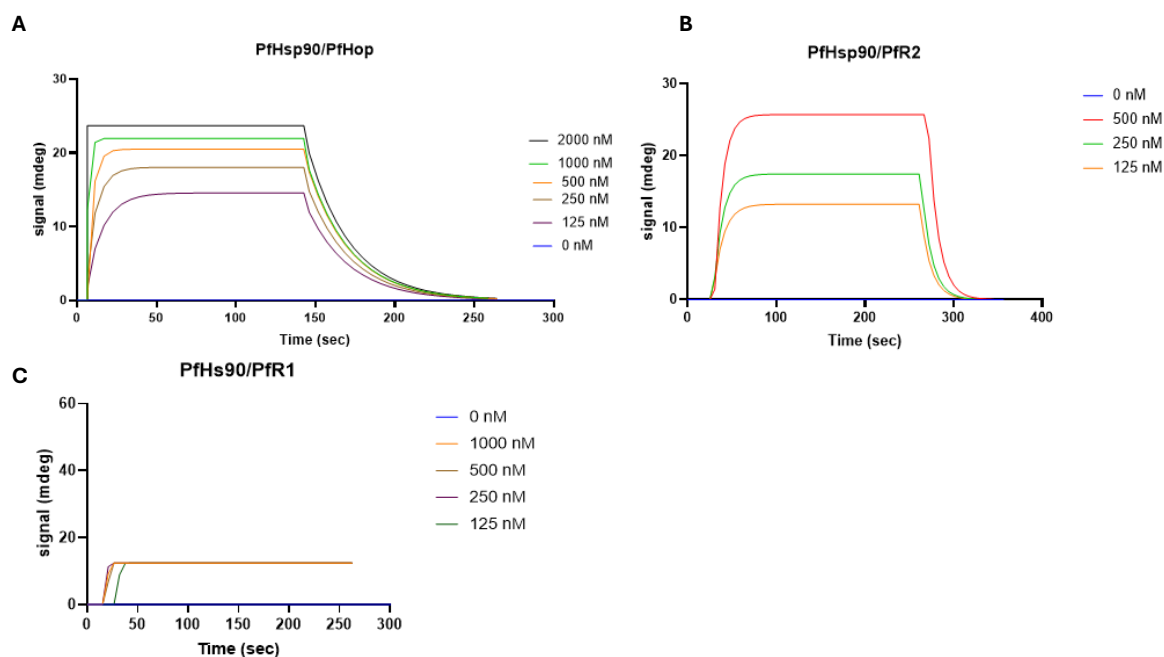


Figure 4.12: PfHsp90 interacts with PfRUVBL2 in the micromolar range

Sensorgrams representing the interaction of PfHsp90 with PfRUVBL2. (A) interaction of PfHsp90 with PfHop, (B) interaction of PfHsp90 with PfRUVBL2, (C) interaction of PfHsp90 with PfRUVBL1.

Sorafenib inhibits tumor cell proliferation and angiogenesis by targeting numerous serine/threonine and tyrosine kinases (Wilhelm *et al.*, 2006). Cancer cells use the Hsp90 chaperone machinery to protect an array of mutated and overexpressed oncoproteins from misfolding and degradation (Whitesell and Lindquist, 2005). Novobiocin and geldanamycin have been reported as Hsp90 inhibitors (Dutta *et al.*, 2019). The current study sought to investigate the binding kinetics of sorafenib to PfHsp90.

The data obtained suggest that PfHsp90 does not interact with sorafenib (125, 250, 500, 1000, 2000 nM; Figure 4.13). As controls, novobiocin and geldanamycin were prepared, and analytes (125, 250, 500, 1000, 2000 nM) were analyzed individually. Novobiocin and geldanamycin yielded K_D values of 56.3 μM and 38.8 μM , respectively (Figure 4.13). The interaction of

PfHsp90 with both novobiocin and geldanamycin was observed to be in a concentration-dependent manner, as shown by sensograms (Figure 4.13).

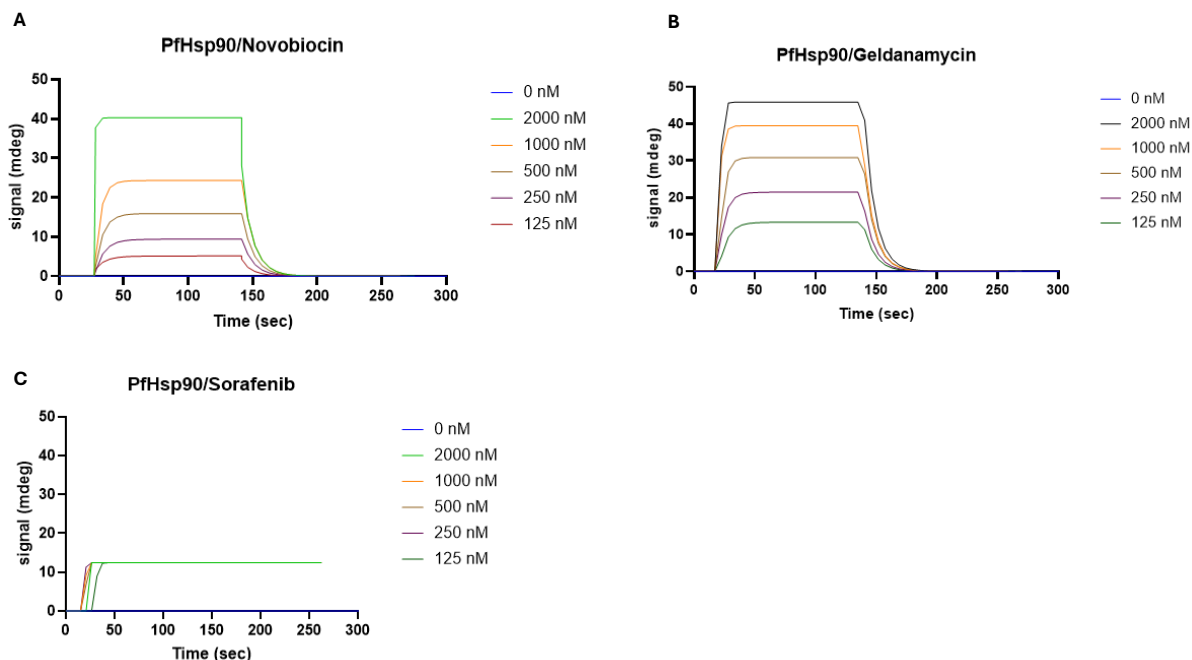


Figure 4.13: interaction of PfHsp90 with small molecular inhibitors

(A) interaction of PfHsp90 with novobiocin, (B) interaction of PfHsp90 with geldanamycin, and (C) interaction of PfHsp90 with sorafenib.

4.14 Discussion

PfRUVBL1 and PfRUVBL2 are integral ATPase components of the R2TP complex in *Plasmodium falciparum*, a critical parasitic organism responsible for malaria. The binding of nucleotides, particularly ATP and ADP, to these ATPases is vital for their structural conformation and functionality, allowing them to participate effectively in various cellular processes (Matias *et al.*, 2006). SPR analysis has provided compelling evidence that both PfRUVBL1 and PfRUVBL2 exhibit nucleotide binding at micromolar concentrations (Figure 4.1). Notably, PfRUVBL2 demonstrated a significantly higher affinity for ATP, with a K_D of 1.44 μM . ATP binding to ATPases control the ATPase rings C- terminal side and regulate cellular assembly (Kim *et al.*, 2015). In contrast, PfRUVBL1 showed a lower affinity, quantifiable by a K_D value of 8.25 μM (Figure 4.1). This marked difference in ATP affinity highlights the crucial role that ATP binding plays in the regulation of the functional activities of both proteins, indicating their specialized capabilities in ATP binding and subsequent ATPase activity.

The underlying mechanism of ATP binding and hydrolysis is thought to be intricately linked to the Walker A motif, a conserved sequence motif that facilitates the coordination of ATP during the cellular processes (Walker, 1986; delToro *et al.*, 2016). Studies have elucidated that the hydroxyl group of serine or threonine residues is critical for coordinating magnesium ions (Mg^{2+}) (Saraste *et al.*, 1990; Shi, 2012). The amino group of lysine is responsible for stabilizing the β - and γ -phosphate groups of ATP, optimizing them for hydrolytic reactions (Shi, 2012).

Structural analyses focused on phi29 have revealed that the C-terminal glycine of the Walker A motif plays an essential role in binding the phosphate groups of the nucleotides, further underscoring the complex's functional dynamics (Mao *et al.*, 2016). Binding affinities obtained here align with earlier molecular docking predictions, although the *in vitro* analyses indicate that PfRUVBL2 possesses a higher affinity for ATP than was initially anticipated. This revised understanding could have significant implications for the functional characterization of these ATPases within the R2TP complex and their potential roles in malaria pathogenesis. The binding of ATP induces a "closed" conformation in which the subunits are tightly packed, while the presence of ADP or the absence of nucleotide results in a more "open" conformation (Gorynia *et al.*, 2011; Silva *et al.*, 2018). The ATP-bound forms of RUVBL1 and RUVBL2 typically form more stable hexameric or dodecameric ring structures, allowing them to function

as scaffolds or remodelers for protein complex formation (Silva *et al.*, 2018). Structural analyses of RUVBL2 suggest that ATP binding triggers domain II motion through interactions with conserved histidine residues in the N-terminal loop (Silva *et al.*, 2018). Mutations or deletions in domain II destabilize the RUVBL1 and RUVBL2 dodecamer complex (Niewiarowski *et al.*, 2010).

Extrinsic and intrinsic fluorescence spectroscopy analyses demonstrated that PfRUVBL1 and PfRUVBL2 exhibit distinct structural conformations compared to their yeast homolog, highlighting their unique biophysical characteristics (Figure 4.2; Figure 4.3). Detailed fluorescence measurements indicated that PfRUVBL1 underwent a significant blue shift of 6 nm upon ATP binding, consistent with the behavior observed in its yeast counterpart, yRvb1. This blue shift suggests an increased burial of the fluorophore, specifically the tyrosine residue, within the protein's three-dimensional structure (Rodrigues *et al.*, 2023). In contrast, PfRUVBL2 displayed a more modest blue shift of only 2 nM upon the same interaction with ATP. This comparatively smaller shift hints at differences in how ATP binding influences the structural dynamics of PfRUVBL2 relative to PfRUVBL1. Moreover, when exposed to the small molecule sorafenib, both PfRUVBL1 and PfRUVBL2 exhibited a notable red shift in their fluorescence spectra. This redshift is indicative of unfolding behavior within the proteins, suggesting that sorafenib may unravel the folded status. The observed redshift implies that the hydrophobic tyrosine residues become more exposed in the presence of sorafenib, reflecting the significant effects of this small molecule on the folding and conformational stability of both PfRUVBL1 and PfRUVBL2. These findings provide critical insights into the structural intricacies of these proteins and their interactions with ATP and other ligands, which could have implications for understanding their parasite biology.

Chromatographic analyses conducted on the Rvb1/Rvb2 complex extracted from *Neurospora crassa* have revealed that this complex predominantly exists in two oligomeric forms: dimers and hexamers (Campanella *et al.*, 2021). Notably, the presence of ATP has been shown to preferentially promote the formation of hexameric structures, whereas the presence of ADP appears to favor the establishment of larger multimeric states, which are likely to represent dodecamer formations (Campanella *et al.*, 2021). Further investigations using fluorescence spectroscopy have provided additional insights, indicating that both PfRUVBL1 and PfRUVBL2 conform to more compact and organized structures when ATP or ADP is present (Figures 4.2 and 4.3). These compact conformations are particularly conducive to facilitating protein-protein interactions, which are crucial for the formation of larger multi-protein

assemblies. The stability of protein oligomers generally hinges on the formation of non-covalent interactions between compatible interfaces. These interfaces are typically stabilized through a combination of hydrophobic interactions, hydrogen bonding, and electrostatic attractions, all of which play a vital role in maintaining the integrity of protein structures (Larsen *et al.*, 1998). This intricate network of interactions not only stabilizes the oligomeric states but also enhances the biological functionality of the protein complexes within cellular environments.

RUVBL1 and RUVBL2 demonstrate low ATPase activity when assessed in isolation (Matias *et al.*, 2006). When these proteins form a complex, however, their ATPase activity significantly increases, indicating that the interaction between RUVBL1 and RUVBL2 enhances their functional capacity (Puri *et al.*, 2007; Niewiarowski *et al.*, 2010). This increase in activity is further regulated by client-induced conformational changes within the DII domain, highlighting the importance of structural dynamics in enzyme function (Dauden *et al.*, 2021). Subsequent ATPase activity assays have shown that ATP exhibits a higher binding affinity when part of the PfRUVBL1/2 complex compared to the individual proteins (Figure 4.6). For instance, when assessing the kinetics of PfRUVBL1 in the presence of the drug sorafenib, the Michaelis constant (K_m) was determined to be 2.07 mM. PfRUVBL2 exhibited a K_m value of 2.34 mM under similar conditions. This indicates that the ATPase activity of both PfRUVBL1 and PfRUVBL2 is somewhat lower than that observed for the yeast homolog yRvb1 and PfHsp70-1, suggesting a relative inefficiency in their ATPase function in the assay context. The relatively low ATPase activity of individual PfRUVBL1 and PfRUVBL2 may imply a functional partnership in these proteins exists. Sorafenib exerts only a marginal effect on the ATPase activity of both PfRUVBL1 and PfRUVBL2 proteins when tested *in vitro* (Figure 5.6). This suggests that while sorafenib interacts with these proteins, it does not significantly inhibit or enhance their ATPase activity within the experimental framework used. Overall, these findings contribute to our understanding of the biochemical properties and regulatory mechanisms of the PfRUVBL1 and PfRUVBL2 proteins in various cellular processes. It would be worth investigating the effect of sorafenib on the PfRUVBL1, PfRUVBL2, and PfRUVBL3 mini-complex, as well as the 2RTP complex assembly/disassembly in the presence of ATP/ADP.

The binding of nucleotides to the proteins Rvb1 and Rvb2 induces significant conformational changes that can influence their interactions with DNA molecules (Campanella *et al.*, 2021). PfRUVBL2 demonstrated capabilities to convert supercoiled double-stranded DNA (dsDNA)

into nicked dsDNA in the presence of ATP within the reaction mixture (Figure 5.8). These observations suggest that PfRUVBL2 may possess intrinsic activity for cleaving DNA, with a specific affinity for double-stranded forms, indicating its potential role in various cellular mechanisms associated with DNA manipulation, replication, and repair processes. Similarly, PfRUVBL3 has also been shown to exhibit DNA cleavage activity; however, this function is influenced by its insertion domain (Sen *et al.*, 2018). Understanding the precise mechanisms by which these proteins interact with DNA and the regulatory factors, such as ATP, involved in their activity can provide valuable insights into their roles in cellular functions and the broader implications in genomic stability and maintenance.

PfRUVBL1 and PfRUVBL2 displayed remarkable stability at a temperature of 51 °C, with aggregation percentages recorded at 37% for PfRUVBL1 and 22% for PfRUVBL2 (Figure 4.7). This indicates that PfRUVBL2 has a superior ability to remain soluble under elevated temperatures compared to PfRUVBL1. MDH aggregation has been previously demonstrated to be suppressed by PfHsp70-1 by more than 50% (Shonhai *et al.*, 2008; Luthuli *et al.*, 2013). PfRUVBL1 was found to effectively suppress the aggregation of MDH by 72%, while PfRUVBL2 demonstrated an even greater capability, reducing MDH aggregation by 80% (Figure 4.7). These significant reductions in aggregation suggest that both PfRUVBL1 and PfRUVBL2 possess holdase activity. This activity is essential for the proper assembly of multi-protein complexes, highlighting the potential roles these proteins play in maintaining protein homeostasis and facilitating protein interactions within cellular environments.

PfRUVBL1 and PfRUVBL2 engaged in both self- and hetero-oligomeric interactions, which have been quantitatively assessed through SPR analysis (Figure 4.9). This analysis revealed that the interaction between these two proteins is not only specific but also exhibits a concentration-dependent behavior, suggesting that varying concentrations lead to different interaction dynamics (Figure 4.9). Further investigations using slot blot assays indicated that the binding of nucleotides significantly influences the interaction between PfRUVBL1 and PfRUVBL2. Specifically, the presence of ADP resulted in less intense signal bands, indicating a diminished interaction, in comparison to the stronger interactions observed when ATP or no nucleotide was present (Figure 4.10). This finding underscores the regulatory role that nucleotide binding plays in modulating the protein-protein interaction. Moreover, the effect of the small molecule inhibitor sorafenib was examined, revealing that it moderately disrupts the interaction between PfRUVBL1 and PfRUVBL2. Immunoblotting results showed a noticeable weakening of the interaction in the presence of sorafenib, further suggesting that this inhibitor

can influence the oligomerization state of these proteins (Figure 4.10). Hence, it suggested that sorafenib may exhibit antiplasmodial activity, which is linked to the disruption of the *P. falciparum* R2TP complex system.

Chapter five

Conclusion and Future Perspective

5.0 Conclusion and Future Perspectives

Hsp90/R2TP complex is not only essential for the folding of newly synthesized proteins, but it also regulates and facilitates the assembly of multi-protein complexes that are essential for the development of *P. falciparum* (Seraphim *et al.*, 2019; Luthuli and Shonhai, 2023). The R2TP complex regulates the formation of complexes such as spliceosome, a multi-component complex composed of pre-mRNA, small nuclear ribonucleoprotein particles, and folding factors (Hossain *et al.*, 2013). The R2TP complex is suggested to regulate cell growth under normal and physiological conditions, which is essential for the survival of the parasite (Boulon *et al.*, 2012). Splicing is an important process in *P. falciparum* that excises intron from its precursor mRNA to generate matured mRNA (Iriko *et al.*, 2009). Dysfunction of spliceosomes has been suggested to lead to hematological malignancies and splicesomopathies (Maciejewski and Padgett, 2012; Griffin and Saint-Jeannet, 2020). In the cell, ATP is required for the assembly of the spliceosome complex (Bowers *et al.*, 2004). Experiments in this study validated that the ATPase member of *P. falciparum* R2TP complex (PfRUVBL1 and PfRUVBL2) binds and hydrolyzes ATP (Figure 4.1; Figure 4.6). The capability of PfRUVBL1 and PfRUVBL2 to bind and hydrolyze ATP is attributed to their Walker A and Walker B motifs (Walker, 1986; Luthuli and Shonhai, 2023). Mutations on these motifs may validate their importance in ATP binding and hydrolysis.

In addition, telomerase is a ribonucleoprotein complex (RNP) essential for the maintenance of chromosome ends in eukaryotes (He and Feigon, 2022). Mutations in telomerase holoenzyme components lead to diseases known as telomere biology disorders, while telomerase upregulation is suggested to be essential for the immortalization of cancer cells (Roake and Artandi, 2020). Hsp90 chaperone function on telomerase depends on R2TP since RUVBL1 and RUVBL2 were reported to interact with TERT (telomerase reverse transcriptase) (Venteicher *et al.*, 2008). Hence, it was important to characterize the function of two higher conserved *P. falciparum* R2TP complex members (PfRUVBL1 and PfRUVBL2) *in vitro*.

Unique sequences of PfRUVBL1, PfRUVBL2, and PfRUVBL3, in comparison with their yeast and human homologs, were selected as epitopes for antibody production using MSA (Figure 3.1). Specific antibodies against PfRUVBL1, PfRUVBL2, and PfRUVBL3 were produced in rabbits (Stellenbosch). All recombinant proteins were expressed and purified successfully in *E. coli* cells (Figure 3.3; Figure 3.5). Both predictive studies and *in vitro* analyses showed that PfRUVBL1 and PfRUVBL2 bind and hydrolyze ATP. These findings support suggestions that they belong to the ATPase family. In addition, it is also suggested that these ATPases contribute

to the R2TP complex compared to their yeast and human homologs. However, studies based on *in silico* work revealed that the R2TP complex from *Plasmodium* spp. is composed of three RuvB-like genes encoding three proteins (Ahmad *et al.*, 2013). It will be interesting to unfold why the parasite requires an additional ATPase compared to other organisms.

In vitro studies examining the ATPase activity and potential interactions of PfRUVBL1 and PfRUVBL2 have yielded some preliminary insights; however, these findings are predominantly isolated from the complex biological context of *Plasmodium falciparum*. Despite this, their specific roles during the different developmental stages of the malaria parasite remain ambiguous and inadequately defined. The absence of comprehensive data that links the ATPase activity of PfRUVBL1 and PfRUVBL2 to stage-specific gene expression patterns, as well as to phenotypes resulting from genetic loss-of-function experiments, poses a significant challenge in evaluating their actual importance in terms of parasite proliferation and virulence. While reverse genetic screens have indicated that these genes may be crucial for parasite survival, the functional studies that could validate and elucidate these roles are notably limited.

Molecules that are involved in complex assembly are expected to possess the ability to stabilize other molecules. Hence, the capability of PfRUVBL1 and PfRUVBL2 to suppress heat-induced aggregation of MDH was observed (Figure 4.7). This evidence suggests that the proteins possess holdase chaperone activity, which is the capability of chaperones to protect their client from aggregating (de Graff *et al.*, 2020). PfRUVBL2 exhibited ATP-dependent DNA cleavage activity against dsDNA (Figure 4.8), which is attributed to the function of the complex in chromatin remodeling. Studies have reported that PfRUVBL3 possesses DNA cleavage activity against dsDNA (Sen *et al.*, 2018). Homologs of PfRUVBL1 and PfRUVBL2 from yeast have been identified as essential components of complexes, including DNA repair, DNA replication, cell cycle, and chromosome segregation (Morrison *et al.*, 2009; Campanella *et al.*, 2019). Hence, it is plausible that PfRUVBL1 and PfRUVBL2 interact with DNA.

RUVBL1 and RUVBL2 are known to assemble into hetero-hexameric ring structures, as demonstrated by previous studies (Gorynia *et al.*, 2011; Seraphim *et al.*, 2021). These structures are notable for their complex and alternating arrangement, which underscores the intricate organizational properties of these proteins. In the current investigation, the specific interactions between PfRUVBL1 and PfRUVBL2 were demonstrated using SPR analysis to elucidate their self-association dynamics (Figure 4.9). The findings from our SPR analysis confirmed the

presence of a direct interaction between PfRUVBL1 and PfRUVBL2. A slot blot assay was conducted to further substantiate these results, which corroborated our SPR findings and reinforced the validity of the observed protein interactions (Figure 4.10). Importantly, the study revealed that the interactions between PfRUVBL1 and PfRUVBL2 are influenced by small molecules such as sorafenib, as well as nucleotides, including ATP and ADP, suggesting a regulatory role that these elements play in the functionality of this protein complex.

The current investigation provides new insights by demonstrating, for the first time, the interaction between two crucial proteins, PfRUVBL1 and PfRUVBL2, and the molecular chaperones PfHsp90 and PfHsp70-1 (Figures 4.11; 4.12). Previous research in the literature has indicated that both PfRUVBL1 and PfRUVBL2 are known to associate with Hsp90 as part of the R2TP complex, a multi-protein assembly essential for the regulation of protein complexes and the assembly of important cellular structures (Lakomek *et al.*, 2015; Seraphim *et al.*, 2019). The mechanisms underlying these interactions often involve co-chaperones such as RPAP3 or PIH1D1, which can enhance the binding signals and stability of the interactions among these proteins (Lakomek *et al.*, 2015; Lynham *et al.*, 2022).

Furthermore, the experimental approach included purifying PfRUVBL2 from *E. coli* XL1 blue cells. Intriguingly, this purification process suggested the potential incorporation of *E. coli* Hsp70 into the protein complex, a finding that was subsequently corroborated through (LCMS) techniques. This observation raises the intriguing possibility that PfHsp90 and PfHsp70-1 may engage directly with PfRUVBL1 and PfRUVBL2, hinting at a complex interplay between these molecular chaperones and the RUVBL proteins that could be vital for understanding their roles in cellular processes.

Research indicates the existence of a putative R2TP complex in *P. falciparum*, which potentially plays a crucial role in assembling essential multiprotein complexes essential for the parasite's development and progression through its lifecycle (Seraphim *et al.*, 2019). This R2TP complex may have significant implications for malaria pathogenesis, as *P. falciparum* must precisely regulate gene expression to navigate its intricate lifecycle and effectively adapt to varying host environments.

To achieve this, the parasite utilizes various tactics, including a highly specialized and streamlined set of transcriptional regulators. These include various transcription factors, chromatin remodeling complexes, and RNA-binding proteins, which collaboratively orchestrate the expression of genes critical for virulence and survival (Vembar *et al.*, 2014;

Subudhi *et al.*, 2023). Homologs of RUVBL1 and RUVBL2 are present within *P. falciparum* and are actively expressed throughout the intraerythrocytic developmental cycle. Disruption in the functioning of these proteins or the associated regulatory pathways could have detrimental effects on the parasite's ability to thrive, impacting both its survival and virulence (Baron *et al.*, 2017). Understanding these complex interactions and mechanisms, researchers can attain deeper insights into how *P. falciparum* influences its host and progresses through different stages of infection, ultimately informing future strategies for malaria control and treatment.

It is crucial to enhance the bulk shift effect in the association and dissociation curves by systematically varying the flow rate. This optimization will allow for a more nuanced understanding of the kinetics involved in the binding processes. By adjusting the flow rate, we can identify how different velocities influence the interactions, potentially leading to improved accuracy in determining binding affinities and rates. This approach can also help in refining experimental conditions to better simulate physiological environments, ultimately providing deeper insights into the mechanisms underlying molecular interactions. Future study includes the localization of the R2TP complex, both as individual members and as the complex, using fluorescence microscopy in the presence and absence of sorafenib to determine any effect sorafenib might have on their localization. Cellular localization aid predicts interaction partners and participation in molecular pathways of the R2TP complex in *P. falciparum*. The late procurement of antibodies, due to technical limitations, prevented the timely execution of parasite-based assays as originally planned.

References

- Abel, Y., Charron, C., Virgiglio, C., Bourguignon-Igel, V., Quinternet, M., Chagot, M.E., Robert, M.C., Verheggen, C., Branlant, C., Bertrand, E. and Manival, X., 2022. The interaction between RPAP3 and TRBP reveals a possible involvement of the HSP90/R2TP chaperone complex in the regulation of miRNA activity. *Nucleic Acids Research*, 50(4) 2172-2189.
- Abéza, C., Busse, P., Paiva, A.C., Chagot, M.E., Schneider, J., Robert, M.C., Vandermoere, F., Schaeffer, C., Charpentier, B., Sousa, P.M. and Bandejas, T.M., 2024. The HSP90/R2TP quaternary chaperone scaffolds assembly of the TSC complex. *Journal of Molecular Biology*, 436(23), 168840.
- Absalon, S., Robbins, J.A. and Dvorin, J.D., 2016. An essential malaria protein defines the architecture of blood-stage and transmission-stage parasites. *Nature communications*, 7(1), 11449.
- Achan, J., Talisuna, A.O., Erhart, A., Yeka, A., Tibenderana, J.K., Baliraine, F.N., Rosenthal, P.J. and D'Alessandro, U., 2011. Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. *Malaria Journal*, 10, 1-12.
- Acharya, P., Kumar, R. and Tatu, U., 2007. Chaperoning a cellular upheaval in malaria: heat shock proteins in *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, 153(2), 85-94.
- Achilonu, I., Siganunu, T.P. and Dirr, H.W., 2014. Purification and characterisation of recombinant human eukaryotic elongation factor 1 gamma. *Protein Expression and Purification*, 99, 70-77.
- Aguado, A., Fernández-Higuero, J. A., Moro, F., and Muga, A. 2015b. Chaperone-assisted protein aggregate reactivation: different solutions for the same problem. *Archives of Biochemistry and Biophysics*. 580, 121–134.
- Ahmad, M., Afrin, F. and Tuteja, R., 2013. Identification of R2TP complex of *Leishmania donovani* and *Plasmodium falciparum* using genome wide in-silico analysis. *Communicative and Integrative Biology*, 6(6), e26005.
- Ahmad, T., Alhammadi, B.A., Almaazmi, S.Y., Arafa, S., Blatch, G.L., Dutta, T., Gestwicki, J.E., Keyzers, R.A., Shonhai, A. and Singh, H., 2024. *Plasmodium falciparum* heat shock proteins as anti-malarial drug targets: An update. *Cell Stress and Chaperones*, 9(2), 326-337.
- Aker, J., Hesselink, R., Engel, R., Karlova, R., Borst, J.W., Visser, A.J. and de Vries, S.C., 2007. *In Vivo* hexamerization and characterization of the Arabidopsis AAA ATPase CDC48A complex using forster resonance energy transfer-fluorescence lifetime imaging microscopy and fluorescence correlation spectroscopy. *PMCID. Plant Physiology*, 145(2), 339-350.
- Akinnusi, P.A., Olubode, S.O., Alade, A.A., Ahmed, S.A., Ayekolu, S.F., Ogunlade, T.M., Gbore, D.J., Rotimi, O.D. and Ayodele, A.O., 2022. A molecular modeling approach for structure-based virtual screening and identification of novel anti-hypercholesterolemic agents from Grape. *Informatics in Medicine Unlocked*, 32, 101065.
- Alderson, T.R., Kim, J.H. and Markley, J.L., 2016. Dynamical structures of Hsp70 and Hsp70-Hsp40 complexes. *Structure*, 24(7), 1014-1030.

- Ali, M.M., Roe, S.M., Vaughan, C.K., Meyer, P., Panaretou, B., Piper, P.W., Prodromou, C. and Pearl, L.H., 2006. Crystal structure of an Hsp90–nucleotide–p23/Sba1 closed chaperone complex. *Nature*, 440(7087), 1013-1017.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403-410.
- Alvira, S., Cuéllar, J., Röhl, A., Yamamoto, S., Itoh, H., Alfonso, C., Rivas, G., Buchner, J. and Valpuesta, J.M., 2014. Structural characterization of the substrate transfer mechanism in Hsp70/Hsp90 folding machinery mediated by Hop. *Nature Communications*, 5(1), 5484.
- Ambrose, A.J., Zerio, C.J., Sivinski, J., Zhu, X., Godek, J., Sanchez, J.L., Khanna, M., Khanna, R., Lairson, L., Zhang, D.D. and Chapman, E., 2024. Human Hsp70 Substrate-Binding Domains Recognize Distinct Client Proteins. *Biochemistry*, 63(3), 251-263.
- Angira, D., Shaik, A. and Thiruvengatam, V., 2020. Structural and strategic landscape of PIKK protein family and their inhibitors: an overview. *Frontiers in Bioscience-Landmark*, 25(8), 1538-1567.
- Arumugam, G., Saravu, K., Kothapalli, P., Nallapati, V.T., Bhat, P., Achari, M., Kulal, N., Ananganallur Nagarajan, S., SL, H. and Kumar, A., 2024. Mosquito prevalence, resting habitat preference, and Plasmodium infection status of anophelines in coastal Karnataka during the declining phase of malaria—an exploratory study. *Parasitology Research*, 123(8), 308.
- Arya, A., Foko, L.P.K., Chaudhry, S., Sharma, A. and Singh, V., 2021. Artemisinin-based combination therapy (ACT) and drug resistance molecular markers: A systematic review of clinical studies from two malaria endemic regions—India and sub-Saharan Africa. *International Journal for Parasitology: Drugs and Drug Resistance*, 15, 43-56.
- Assimon, V.A., Tang, Y., Vargas, J.D., Lee, G.J., Wu, Z.Y., Lou, K., Yao, B., Menon, M.K., Pios, A., Perez, K.C., 2019. CB- 6644 is a selective inhibitor of the RUVBL1/2 complex with anticancer activity. *ACS Chemical Biology Journal*, 14, 236-244.
- Babbitt, S.E., Altenhofen, L., Cobbold, S.A., Istvan, E.S., Fennell, C., Doerig, C., Llinás, M. and Goldberg, D.E., 2012. *Plasmodium falciparum* responds to amino acid starvation by entering into a hibernatory state. *Proceedings of the National Academy of Sciences*, 109(47), 3278-3287.
- Babu, N.J. and Nangia, A., 2011. Solubility advantage of amorphous drugs and pharmaceutical cocrystals. *Crystal Growth & Design*, 11(7), 2662-2679.
- Banach, M., Chomilier, J. and Roterman, I., 2021. Contribution to the Understanding of Protein–Protein Interface and Ligand Binding Site Based on Hydrophobicity Distribution—Application to Ferredoxin I and II Cases. *Applied Sciences*, 11(18), 8514.
- Bannister, L.H. and Mitchell, G.H., 2009. The malaria merozoite, forty years on. *Parasitology*, 136(12), 1435-1444.
- Bannister, L.H., Butcher, G.A. and Mitchell, G.H., 1977. Recent advances in understanding the invasion of erythrocytes by merozoites of *Plasmodium knowlesi*. *Bulletin of the World Health Organization*, 55(2-3), 163.

- Banumathy, G., Singh, V., Pavithra, S.R. and Tatu, U., 2003. Heat shock protein 90 function is essential for *Plasmodium falciparum* growth in human erythrocytes. *Journal of Biological Chemistry*, 278(20), 18336-18345.
- Baron, B.W., Baron, R.M. and Baron, J.M., 2017. Reticulocytes are an enriched source of the RUVBL1 protein. *Acta Haematologica*, 138(3), 162-165.
- Beeson, J.G., Kurtovic, L., Dobaño, C., Opi, D.H., Chan, J.A., Feng, G., Good, M.F., Reiling, L. and Boyle, M.J., 2019. Challenges and strategies for developing efficacious and long-lasting malaria vaccines. *Science Translational Medicine*, 11(474), e1458.
- Bellosta, P., Hulf, T., Diop, S.B., Usseglio, F., Pradel, J., Aragnol, D. and Gallant, P., 2005. Myc interacts genetically with Tip48/Reptin and Tip49/Pontin to control growth and proliferation during *Drosophila* development. *Proceedings of the National Academy of Sciences*, 102(33), 11799-11804.
- Benbahouche, N.E.H., Iliopoulos, I., Török, I., Marhold, J., Henri, J., Kajava, A.V., Farkaš, R., Kempf, T., Schnölzer, M., Meyer, P. and Kiss, I., 2014. *Drosophila* Spag Is the Homolog of RNA Polymerase II-associated Protein 3 (RPAP3) and Recruits the Heat Shock Proteins 70 and 90 (Hsp70 and Hsp90) during the Assembly of Cellular Machineries. *Journal of Biological Chemistry*, 289(9), 6236-6247.
- Bennink, S., Kiesow, M.J. and Pradel, G., 2016. The development of malaria parasites in the mosquito midgut. *Cellular Microbiology*, 18(7), 905-918.
- Bhadra, S. and Xu, Y.J., 2023. TTT (Tel2-Tti1-Tti2) Complex, the co-chaperone of PIKKs and a potential target for cancer chemotherapy. *International Journal of Molecular Sciences*, 24(9), 8268.
- Bhattacharya, K., Weidenauer, L., Luengo, T.M., Pieters, E.C., Echeverría, P.C., Bernasconi, L., Wider, D., Sadian, Y., Koopman, M.B., Villemin, M. and Bauer, C., 2020. The Hsp70-Hsp90 co-chaperone Hop/Sti1 shifts the proteostatic balance from folding towards degradation. *Nature Communications*, 11(1), 5975.
- Bhatwa, A., Wang, W., Hassan, Y.I., Abraham, N., Li, X.Z. and Zhou, T., 2021. Challenges associated with the formation of recombinant protein inclusion bodies in *Escherichia coli* and strategies to address them for industrial applications. *Frontiers in Bioengineering and Biotechnology*, 9, 630551.
- Bizarro, J., Charron, C., Boulon, S., Westman, B., Pradet-Balade, B., Vandermoere, F., Chagot, M.E., Hallais, M., Ahmad, Y., Leonhardt, H. and Lamond, A., 2014. Proteomic and 3D structure analyses highlight the C/D box snoRNP assembly mechanism and its control. *Journal of Cell Biology*, 207(4), 463-480.
- Blake, T.C., Haase, S. and Baum, J., 2020. Actomyosin forces and the energetics of red blood cell invasion by the malaria parasite *Plasmodium falciparum*. *PLoS Pathogens*, 16(10), e1009007.
- Blatch, G. L., Lässle, M., 1999. The tetratricopeptide repeat: a structural motif mediating protein- protein interactions. *Bioessays*, 21, 932-939. Blatch, G.L., 2022. *Plasmodium*

- falciparum* molecular chaperones: Guardians of the malaria parasite proteome and renovators of the host proteome. *Frontiers in Cell and Developmental Biology*, 10, 921739.
- Blundell, K.L.I.M., Pal, M., Roe, S.M., Pearl, L.H., Prodromou, C., 2017. The structure of FKBP38 in complex with the MEEVD tetratricopeptide binding motif of Hsp90. *PLoS One*, 12(3), 10-1371.
- Bochtler, M., Hartmann, C., Song, H.K., Bourenkov, G.P., Bartunik, H.D., Huber, R., 2000. The structures of HsIU and the ATP-dependent protease HsIU-HsIV. *Nature*, 403,800–805.
- .
- Bohen, S.P. 1995. Hsp90 mutants disrupt glucocorticoid receptor ligand binding and destabilize aporeceptor complexes. *Journal of Biological Chemistry*, 270(49), 29433-29438.
- Botha, M., Pesce, E.R. and Blatch, G.L., 2007. The Hsp40 proteins of *Plasmodium falciparum* and other apicomplexa: regulating chaperone power in the parasite and the host. *The International Journal of Biochemistry & Cell Biology*, 39(10), 1781-1803.
- Boulon, S., Bertrand, E. and Pradet-Balade, B., 2012. HSP90 and the R2TP co-chaperone complex: building multi-protein machineries essential for cell growth and gene expression. *RNA Biology*, 9(2), 148-154.
- Bracher, A. and Verghese, J., 2015. The nucleotide exchange factors of Hsp70 molecular chaperones. *Frontiers in Molecular Biosciences*, 2, 10.
- Buchner, J. and Li, J., 2013. Structure, function and regulation of the hsp90 machinery. *Biomedical Journal*, 36(3), 106.
- Burgess, R.R. 2009. Protein precipitation techniques. *Methods in Enzymology*, 463, 331-342.
- Cai, Y., Jin, J., Tomomori-Sato, C., Sato, S., Sorokina, I., Parmely, T.J., Conaway, R.C., Conaway, J.W. 2003 Identification of new subunits of the multiprotein mammalian TRRAP/TIP60-containing histone acetyltransferase complex. *Journal of Biological Chemistry*. 278.
- Campanella, J.E.M., Junior, S.L.R., Kiraly, V.T.R., Gomes, A.A.S., de Barros, A.C., Mateos, P.A., Freitas, F.Z., de Mattos Fontes, M.R., Borges, J.C. and Bertolini, M.C., 2021. Biochemical and biophysical characterization of the RVB-1/RVB-2 protein complex, the RuvBL/RVB homologues in *Neurospora crassa*. *Journal of Biological Chemistry*, 191, 11-26.
- Carter, P.J. and Lazar, G.A., 2018. Next generation antibody drugs: pursuit of the 'high-hanging fruit'. *Nature Reviews Drug Discovery*, 17(3), 197-223.
- Cattaneo, R., 2010. Paramyxovirus entry and targeted vectors for cancer therapy. *PLoS Pathogens*, 6(6), e1000973.
- Chakafana, G. and Shonhai, A., 2021. The role of non-canonical Hsp70s (Hsp110/Grp170) in cancer. *Cells*, 10(2), 254.
- Chakafana, G., Zininga, T., Shonhai, A. 2019b. The link that binds: The linker of Hsp70 as a helm of the protein's function. *Biomolecules*, 9, 543-563.

- Charpian, S. and Przyborski, J.M. 2008. Protein transport across the parasitophorous vacuole of *Plasmodium falciparum*: into the great wide open. *Traffic*, 9(2), 157-165.
- Chiu, M., Taurino, G., Bianchi, M.G., and Bussolati, O. 2021. The role of amino acids in the crosstalk between mesenchymal stromal cells and neoplastic cells in the hematopoietic niche. *Frontiers in Cell and Developmental Biology*, 9, 714755.
- Cloutier, P., Poitras, C., Durand, M., Hekmat, O., Fiola-Masson, É., Bouchard, A., Faubert, D., Chabot, B. and Coulombe, B., 2017. R2TP/Prefoldin-like component RUVBL1/RUVBL2 directly interacts with ZNHIT2 to regulate assembly of U5 small nuclear ribonucleoprotein. *Nature Communications*, 8(1), 15615.
- Cnops, L., Boderie, M., Gillet, P., Van Esbroeck, M. and Jacobs, J., 2011. Rapid diagnostic tests as a source of DNA for *Plasmodium* species-specific real-time PCR. *Malaria Journal*, 10, 1-11.
- Cockburn, I.L., Boshoff, A., Pesce, E.R. and Blatch, G.L., 2014. Selective modulation of plasmodial Hsp70s by small molecules with antimalarial activity. *Biological Chemistry*, 395(11), 1353-1362.
- Coetzee, N., von Grüning, H., Opperman, D., van der Watt, M., Reader, J. and Birkholtz, L.M., 2020. Epigenetic inhibitors target multiple stages of *Plasmodium falciparum* parasites. *Scientific Reports*, 10(1), 2355.
- Conaway, R.C. and Conaway, J.W., 2009. The INO80 chromatin remodeling complex in transcription, replication and repair. *Trends in Biochemical Sciences*, 34(2), 71-77.
- Cowman, A.F., Berry, D. and Baum, J., 2012. The cellular and molecular basis for malaria parasite invasion of the human red blood cell. *Journal of Cell Biology*, 198(6), 961-971.
- Cunningham, C.N., Southworth, D.R., Krukenberg, K.A. and Agard, D.A., 2012. The conserved arginine 380 of Hsp90 is not a catalytic residue, but stabilizes the closed conformation required for ATP hydrolysis. *Protein Science*, 21(8), 1162-1171.
- Daniel, S., Bradley, G., Longshaw, V.M., Söti, C., Csermely, P. and Blatch, G.L., 2008. Nuclear translocation of the phosphoprotein Hop (Hsp70/Hsp90 organizing protein) occurs under heat shock, and its proposed nuclear localization signal is involved in Hsp90 binding. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1783(6), 1003-1014.
- Dauden, M.I., López-Perrote, A. and Llorca, O., 2021. RUVBL1–RUVBL2 AAA-ATPase: a versatile scaffold for multiple complexes and functions. *Current Opinion in Structural Biology*, 67, 78-85.
- .
- De Graff, A.M., Mosedale, D.E., Sharp, T., Dill, K.A. and Grainger, D.J., 2020. Proteostasis is adaptive: Balancing chaperone holdases against foldases. *PLoS Computational Biology*, 16(12), e1008460.
- De Marco, A. 2007. Protocol for preparing proteins with improved solubility by co-expressing with molecular chaperones in *Escherichia coli*. *Nature Protocols*, 2(10), 2632-2639.

- Del Toro-Equihua, M., Velasco-Rodríguez, R., López-Ascencio, R. and Vásquez, C. 2016. Effect of an avocado oil-enhanced diet (*Persea americana*) on sucrose-induced insulin resistance in Wistar rats. *Journal of Food and Drug Analysis*, 24(2), 350-357.
- Deponte, M., Hoppe, H.C., Lee, M.C., Maier, A.G., Richard, D., Rug, M., Spielmann, T. and Przyborski, J.M. 2012. Wherever I may roam: protein and membrane trafficking in *P. falciparum*-infected red blood cells. *Molecular and Biochemical Parasitology*, 186(2), 95-116.
- Deremble, C. and Lavery, R. 2005. Macromolecular recognition. *Current Opinion in Structural Biology*, 15(2), 171-175.
- Deroost, K., Pham, T.T., Opdenakker, G. and Van den Steen, P.E. 2016. The immunological balance between host and parasite in malaria. *FEMS Microbiology Reviews*, 40(2), 208-257.
- Diamos, A.G., Hunter, J.G., Pardhe, M.D., Rosenthal, S.H., Sun, H., Foster, B.C., DiPalma, M.P., Chen, Q. and Mason, H.S. 2020. High level production of monoclonal antibodies using an optimized plant expression system. *Frontiers in Bioengineering and Biotechnology*, 7, 472.
- Dijkman, P.M., Marzluf, T., Zhang, Y., Chang, S.Y.S., Helm, D., Lanzer, M., Bujard, H. and Kudryashev, M., 2021. Structure of the merozoite surface protein 1 from *Plasmodium falciparum*. *Science Advances*, 7(23), e0465.
- Djihinto, O.Y., Medjigbodo, A.A., Gangbadja, A.R., Saizonou, H.M., Lagnika, H.O., Nanmede, D., Djossou, L., Bohounton, R., Sovegnon, P.M., Fanou, M.J. and Agonhossou, R. 2022. Malaria-transmitting vectors microbiota: Overview and interactions with anopheles mosquito biology. *Frontiers in Microbiology*, 13, 891573.
- Driscoll, D.R., Karim, S.A., Sano, M., Gay, D.M., Jacob, W., Yu, J., Mizukami, Y., Gopinathan, A., Jodrell, D.I., Evans, T.J. and Bardeesy, N. 2016. mTORC2 signaling drives the development and progression of pancreatic cancer. *Cancer Research*, 76(23), 6911-6923.
- Ducat, D., Kawaguchi, S.I., Liu, H., Yates III, J.R., and Zheng, Y. 2008. Regulation of microtubule assembly and organization in mitosis by the AAA+ ATPase Pontin. *Molecular Biology of the Cell*, 19(7), 3097-3110.
- Dunbar, J., Yennawar, H.P., Banerjee, S., Luo, J. and Farber, G.K. 1997. The effect of denaturants on protein structure. *Protein Science*, 6(8), 1727-1733.
- Dunker, A.K., Babu, M.M., Barbar, E., Blackledge, M., Bondos, S.E., Dosztányi, Z., Dyson, H.J., Forman-Kay, J., Fuxreiter, M., Gsponer, J. and Han, K.H., 2013. What's in a name? Why these proteins are intrinsically disordered: Why these proteins are intrinsically disordered. *Intrinsically Disordered Proteins*, 1(1), e24157.
- Dutta, D., Chattopadhyay, S., Bagchi, P., Halder, U.C., Nandi, S., Mukherjee, A., Kobayashi, N., Taniguchi, K. and Chawla-Sarkar, M., 2020. Withdrawal: Active participation of cellular chaperone Hsp90 in regulating the function of rotavirus nonstructural protein 3 (NSP3). *Journal of Biological Chemistry*, 295(39), 13696.
- Dutta, S., Tewari, A., Balaji, C., Verma, R., Moitra, A., Yadav, M., Agrawal, P., Sahal, D. and Jarori, G.K., 2018. Strain-transcending neutralization of malaria parasite by antibodies against *Plasmodium falciparum* enolase. *Malaria Journal*, 17, 1-17.

- Dutta, T., Singh, H., Edkins, A.L. and Blatch, G.L., 2022. Hsp90 and associated co-chaperones of the malaria parasite. *Biomolecules*, 12(8), 1018.
- Eckert, K., Saliou, J.M., Monlezun, L., Vigouroux, A., Atmane, N., Caillat, C., Quevillon-Chérueil, S., Madiona, K., Nicaise, M., Lazereg, S. and Van Dorsselaer, A., 2010. The Pih1-Tah1 cochaperone complex inhibits Hsp90 molecular chaperone ATPase activity. *Journal of Biological Chemistry*, 285(41), 31304-31312.
- Eckl, J.M., Daake, M., Schwartz, S. and Richter, K., 2016. Nucleotide-free B-Raf is preferentially bound by Hsp90 and Cdc37 in vitro. *Journal of Molecular Biology*, 428(20), 4185-4196.
- Edwards, T.A. and Wilson, A.J., 2011. Helix-mediated protein–protein interactions as targets for intervention using foldamers. *Amino Acids*, 41, 743-754.
- El Chamy Maluf, S., Icimoto, M.Y., Melo, P.M.S., Budu, A., Coimbra, R., Gazarini, M.L. and Carmona, A.K., 2020. Human plasma plasminogen internalization route in *Plasmodium falciparum*-infected erythrocytes. *Malaria Journal*, 19, 1-10.
- Erdmann, R., Wiebel, F.F., Flessau, A., Rytka, J., Beyer, A., Frohlich, K.U., Kunau, W.H., 1991. *PAS1*, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. *Cell*, 64, 499-510.
- Escudier, B., Eisen, T., Stadler, W.M., Szczylik, C., Oudard, S., Siebels, M., Negrier, S., Chevreau, C., Solska, E., Desai, A.A. and Rolland, F., 2007. Sorafenib in advanced clear-cell renal-cell carcinoma. *New England Journal of Medicine*, 356(2), 125-134.
- Farrow, R.E., Green, J., Katsimitsoulia, Z., Taylor, W.R., Holder, A.A. and Molloy, J.E., 2011, December. The mechanism of erythrocyte invasion by the malarial parasite, *Plasmodium falciparum*. *Seminars in Cell & Developmental Biology*. Academic Press, 22 (9), 953-960. .
- Forthal, D.N., 2014. Functions of antibodies. *Microbiology Spectrum*, 2(4), 10-1128.
- Francis, D.M. and Page, R., 2010. Strategies to optimize protein expression in *E. coli*. *Current Protocols in Protein Science*, 61(1), 5-24.
- French, S. and Robson, B., 1983. What is a conservative substitution. *Journal of Molecular Evolution*, 19, 171-175.
- Fuda, N.J., Ardehali, M.B. and Lis, J.T., 2009. Defining mechanisms that regulate RNA polymerase II transcription in vivo. *Nature*, 461(7261), 186-192.
- Gallant, P. 2007. Control of transcription by Pontin and Reptin. *Trends in Cell Biology*, 17, 187–192.
- García-Martín, C., López-Perrote, A., Boskovic, J. and Llorca, O., 2024. Mechanism of allosteric inhibition of RUVBL1-RUVBL2 ATPase by the small molecule CB-6644. *Cell Reports Physical Science*, 5, 6.
- Gartner, W., Rossbacher, J., Zierhut, B., Daneva, T., Base, W., Weissel, M., Waldhausl, W., Pasternack, M.S., and Wagner, L., 2003. The ATP-dependent helicase RUVBL1/TIP49a associates with tubulin during mitosis. *Cell Motility of Cytoskeleton*, 56, 79– 93.

- Gates, S.N., Martin, A., 2020. Stairway to translocation: AAA+ motor structures reveal the mechanisms of ATP-dependent substrate translocation. *Protein Science*, 29, 407–419.
- Giacinti, C., Giordano, A., (2006). RB and cell cycle progression. *Oncogene*, 25(38), 5220–5227.
- Giap, H.T., Han, P.N., Phuong, T.L.D., Phung, P.T.T. and Van, V., 2021. Cloning, expression and purification of fructosyl amino acid oxidase (FAOX) in *Escherichia Coli*. *Ho Chi Minh City Open University Journal of Science-Engineering and Technology*, 11(1), 20-28.
- Gilson, P.R. and Crabb, B.S., 2009. Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. *International Journal for Parasitology*, 39(1), 91-96.
- Ginsburg, H., Handeli, S., Friedman, S., Gorodetsky, R. and Krugliak, M., 1986. Effects of red blood cell potassium and hypertonicity on the growth of *Plasmodium falciparum* in culture. *Zeitschrift für Parasitenkunde*, 72, 185-199.
- Gitau, G.W., Mandal, P., Blatch, G.L., Przyborski, J. and Shonhai, A., 2012. Characterisation of the *Plasmodium falciparum* Hsp70–Hsp90 organising protein (PfHop). *Cell Stress and Chaperones*, 17, 191-202.
- Goldberg, D.E. and Zimmerberg, J., 2020. Hardly vacuuous: The parasitophorous vacuolar membrane of malaria parasites. *Trends in Parasitology*, 36(2), 138-146.
- Gorynia, S., Bandejas, T.M., Pinho, F.G., McVey, C.E., Vonnheim, C., Round, A., Svergun, D.I., Donner, P., Matias, P.M. and Carrondo, M.A., 2011. Structural and functional insights into a dodecameric molecular machine–The RuvBL1/RuvBL2 complex. *Journal of Structural Biology*, 176(3), 279-291.
- Green, J.L., Wu, Y., Encheva, V., Lasonder, E., Prommaban, A., Kunzelmann, S., Christodoulou, E., Grainger, M., Truongvan, N., Bothe, S. and Sharma, V., 2020. Ubiquitin activation is essential for schizont maturation in *Plasmodium falciparum* blood-stage development. *PLoS Pathogens*, 16(6), e1008640.
- Gribun, A., Cheung, K.L., Huen, J., Ortega, J. and Houry, W.A., 2008. Yeast Rvb1 and Rvb2 are ATP-dependent DNA helicases that form a heterohexameric complex. *Journal of Molecular Biology*, 376(5), 1320-1333.
- Griffin, C. and Saint-Jeannet, J.P., 2020. Spliceosomopathies: diseases and mechanisms. *Developmental Dynamics*, 249(9), 1038-1046.
- Grøftehaug, M.K., Hajizadeh, N.R., Swann, M.J. and Pohl, E., 2015. Protein–ligand interactions were investigated by thermal shift assays (TSA) and dual polarization interferometry (DPI). *Acta Crystallographica Section D: Biological Crystallography*, 71(1), 36-44.
- Guenther, B. D., Onrust, R., Sali, A., O'Donnell, M., and Kuriyan, J. 1997. Crystal structure of the d' subunit of the clamp-loader complex of E. coli DNA polymerase III. *Cell*, 91, 335-345.

- Guharoy, M. and Chakrabarti, P., 2007. Secondary structure-based analysis and classification of biological interfaces: identification of binding motifs in protein–protein interactions. *Bioinformatics*, 23(15), 1909-1918.
- Gupta, S., Singh, S., Popovici, J., Roesch, C., Shakri, A.R., Guillotte-Blisnick, M., Huon, C., Menard, D. and Chitnis, C.E., 2018. Targeting a reticulocyte binding protein and Duffy binding protein to inhibit reticulocyte invasion by *Plasmodium vivax*. *Scientific Reports*, 8(1), 10511.
- Guyen, Z.Z., 2014. Project based learning: A constructive way toward learner autonomy. *International Journal of Languages in Education and Teaching*, 2(3), 182-193.
- Guy, A.J., Irani, V., MacRaid, C.A., Anders, R.F., Norton, R.S., Beeson, J.G., Richards, J.S. and Ramsland, P.A., 2015. Insights into the immunological properties of intrinsically disordered malaria proteins using proteome scale predictions. *PLoS One*, 10(10), e0141729.
- Hanahan D. 2012. Hallmarks of cancer: a 2012 perspective. *Ann Oncol*. 23.
- Hanson, P.I. Roth, R. Morisaki, H. Jahn, R. Heuser, J.E., 1997. Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell*, 90, 523-535.
- Hamilton, S., Odili, J., Pacifico, M.D., Wilson, G.D., Kupsch, J.M., 2003. Effect of imidazole on the solubility of is-tagged antibody fragment. *Hybrid Hybridomics*, PMID, 22(6), 347-355.
- Hart, M.N., Mohring, F., DonVito, S.M., Thomas, J.A., Muller-Sienerth, N., Wright, G.J., Knuepfer, E., Saibil, H.R. and Moon, R.W., 2023. Sequential roles for red blood cell binding proteins enable phased commitment to invasion for malaria parasites. *Nature Communications*, 14(1), 4619.
- Hartl, F.U., Bracher, A. and Hayer-Hartl, M., 2011. Molecular chaperones in protein folding and proteostasis. *Nature*, 475(7356), 324-332.
- Hatherley, R., Clitheroe, C.L., Faya, N. and Bishop, Ö.T., 2015. *Plasmodium falciparum* Hop: detailed analysis on complex formation with Hsp70 and Hsp90. *Biochemical and Biophysical Research Communications*, 456(1), 440-445.
- He, Y. and Feigon, J., 2022. Telomerase structural biology comes of age. *Current Opinion in Structural Biology*, 76, 102446.
- Henras, A.K., Plisson-Chastang, C., O'Donohue, M.F., Chakraborty, A. and Gleizes, P.E., 2015. An overview of pre-ribosomal RNA processing in eukaryotes. *Wiley Interdisciplinary Reviews: RNA*, 6(2), 225-242.
- Henri, J., Chagot, M.E., Bourguet, M., Abel, Y., Terral, G., Maurizy, C., Aigueperse, C., Georgescauld, F., Vandermoere, F., Saint-Fort, R. and Behm-Ansmant, I., 2018. Deep structural analysis of RPAP3 and PIH1D1, two components of the HSP90 co-chaperone R2TP complex. *Structure*, 26(9), 1196-1209.
- Hillier, C., Pardo, M., Yu, L., Bushell, E., Sanderson, T., Metcalf, T., Herd, C., Anar, B., Rayner, J.C., Billker, O. and Choudhary, J.S., 2019. Landscape of the *Plasmodium* interactome reveals both conserved and species-specific functionality. *Cell Reports*, 28(6), 1635-1647.

- Holder, A.A., 2009. The carboxy-terminus of merozoite surface protein 1: structure, specific antibodies and immunity to malaria. *Parasitology*, 136(12), 1445-1456.
- Hollin, T. and Le Roch, K.G., 2020. From genes to transcripts, a tightly regulated journey in Plasmodium. *Frontiers in Cellular and Infection Microbiology*, 10, 618454.
- Horejší, Z., Takai, H., Adelman, C.A., Collis, S.J., Flynn, H., Maslen, S., Skehel, J.M., de Lange, T., Boulton, S.J., 2010. CK2 phospho-dependent binding of R2TP complex to TEL2 is essential for mTOR and SMG1 stability, *Molecular Cell*, 39, 839 -850,
- Hořejší, Z., Stach, L., Flower, T.G., Joshi, D., Flynn, H., Skehel, J.M., O'Reilly, N.J., Ogrodowicz, R.W., Smerdon, S.J. and Boulton, S.J., 2014. Phosphorylation-dependent PIH1D1 interactions define substrate specificity of the R2TP cochaperone complex. *Cell Reports*, 7(1), 19-26.
- Hořejší, Z., Takai, H., Adelman, C.A., Collis, S.J., Flynn, H., Maslen, S., Skehel, J.M., de Lange, T. and Boulton, S.J., 2010. CK2 phospho-dependent binding of R2TP complex to TEL2 is essential for mTOR and SMG1 stability. *Molecular Cell*, 39(6), 839-850.
- Hossain, M.A., Claggett, J.M., Edwards, S.R., Shi, A., Pennebaker, S.L., Cheng, M.Y., Hasty, J. and Johnson, T.L., 2016. Posttranscriptional regulation of Gcr1 expression and activity is crucial for metabolic adjustment in response to glucose availability. *Molecular Cell*, 62(3), 346-358.
- Houry, W.A., 2022. Assembly principles of the human R2TP chaperone complex reveal the presence of R2T and R2P complexes. *Biophysical Journal*, 121(3), 323.
- Houry, W.A., Bertrand, E. and Coulombe, B., 2018. The PAQosome, an R2TP-based chaperone for quaternary structure formation. *Trends in Biochemical Sciences*, 43(1), 4-9.
- Hu, C., Yang, J., Qi, Z., Wu, H., Wang, B., Zou, F., Mei, H., Liu, J., Wang, W. and Liu, Q., 2022. Heat shock proteins: Biological functions, pathological roles, and therapeutic opportunities. *Medical Communications*, 3(3), 161.
- Hubbard, S.J., 1998. The structural aspects of limited proteolysis of native proteins. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, 1382(2), 191-206.
- Huen, J., Kakihara, Y., Ugwu, F., Cheung, K.L., Ortega, J., Houry, W.A., 2010. Rvb1-Rvb2: essential ATP-dependent helicases for critical complexes. *Biochemistry and Cell Biology*, 88, 29-40.
- Huleani, S., Roberts, M.R., Beales, L. and Papaioannou, E.H., 2022. *Escherichia coli* as an antibody expression host for the production of diagnostic proteins: significance and expression. *Critical Reviews in Biotechnology*, 42(5), 756-773.
- Hunter, C.A. and Sibley, L.D., 2012. Modulation of innate immunity by *Toxoplasma gondii* virulence effectors. *Nature Reviews Microbiology*, 10(11), 766-778.
- Huynh, K. and Partch, C.L., 2015. Analysis of protein stability and ligand interactions by thermal shift assay. *Current Protocols in Protein Science*, 79(1), 28-9.

- Ikura, T., Ogryzko, V.V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J. and Nakatani, Y., 2000. Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell*, 102(4), 463-473.
- Iqbal, S. and Hoque, M.T., 2015. DisPredict: a predictor of disordered protein using optimized RBF kernel. *PLoS One*, 10(10), 0141551.
- Iriko, H., Jin, L., Kaneko, O., Takeo, S., Han, E.T., Tachibana, M., Otsuki, H., Torii, M. and Tsuboi, T., 2009. A small-scale systematic analysis of alternative splicing in *Plasmodium falciparum*. *Parasitology International*, 58(2), 196-199.
- Itoh, H. and Inoue, M., 2019. Comprehensive structure–activity relationship studies of macrocyclic natural products enabled by their total syntheses. *Chemical Reviews*, 119(17), 10002-10031.
- Jafri, M.A., Ansari, S.A., Alqahtani, M.H. and Shay, J.W., 2016. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. *Genome Medicine*, 8, 1-18.
- Jain, D. and Salunke, D.M., 2019. Antibody specificity and promiscuity. *Biochemical Journal*, 476(3), 433-447.
- Jiménez, B., Ugwu, F., Zhao, R., Ortí, L., Makhnevych, T., Pineda-Lucena, A. and Houry, W.A., 2012. Structure of minimal tetratricopeptide repeat domain protein Tah1 reveals mechanism of its interaction with Pih1 and Hsp90. *Journal of Biological Chemistry*, 287(8), 5698-5709.
- Jin, J., Cai, Y., Yao, T., Gottschalk, A.J., Florens, L., Swanson, S.K., Gutiérrez, J.L., Coleman, M.K., Workman, J.L., Mushegian, A. and Washburn, M.P., 2005. A mammalian chromatin remodeling complex with similarities to the yeast INO80 complex. *Journal of Biological Chemistry*, 280(50), 207– 41 212.
- Johnson, J.L. and Brown, C., 2009. Plasticity of the Hsp90 chaperone machine in divergent eukaryotic organisms. *Cell Stress and Chaperones*, 14(1), 83-94.
- Jones, S. and Thornton, J.M., 1996. Principles of protein-protein interactions. *Proceedings of the National Academy of Sciences*, 93(1), 13-20.
- Jonsson, Z.O., Dhar, S.K., Narlikar, G.J., Auty, R., Wagle, N., Pellman, D., Pratt, R.E., Kingston, R., Dutta, A., 2001. Rvb1p and Rvb2p are essential components of a chromatin remodeling complex that regulates transcription of over 5% of yeast genes. *Journal of Biological Chemistry*, 276(16) 279– 16 288.
- Kadota, Y., Shirasu, K. and Guerois, R., 2010. NLR sensors meet at the SGT1–HSP90 crossroad. *Trends in Biochemical Sciences*, 35(4), 199-207.
- Kakihara, Y. and Saeki, M., 2014. The R2TP chaperone complex: its involvement in snoRNP assembly and tumorigenesis, *Biomolecules Concepts*, 5, 513-520
- Kakihara, Y., Kiguchi, T., Ohazama, A. and Saeki, M., 2020. R2TP/PAQosome as a promising chemotherapeutic target in cancer. *Japanese Dental Science Review*, 56(1), 38-42.

- Kakihara, Y., Makhnevych, T., Zhao, L., Tang, W. and Houry, W.A., 2014. Nutritional status modulates box C/D snoRNP biogenesis by regulated subcellular relocalization of the R2TP complex. *Genome Biology*, 15, 1-21.
- Kampinga, H.H., Hageman, J., Vos, M.J., Kubota, H., Tanguay, R.M., Bruford, E.A., Cheetham, M.E., Chen, B. and Hightower, L.E., 2009. Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress and Chaperones*, 14(1), 105-111.
- Karst, D.J., Steinebach, F., Soos, M. and Morbidelli, M., 2017. Process performance and product quality in an integrated continuous antibody production process. *Biotechnology and Bioengineering*, 114(2), 298-307.
- Katoh, H., Sekizuka, T., Nakatsu, Y., Nakagawa, R., Nao, N., Sakata, M., Kato, F., Kuroda, M., Kidokoro, M. and Takeda, M., 2019. The R2TP complex regulates paramyxovirus RNA synthesis. *PLoS Pathogens*, 15(5), 1007749.
- Kędzierska-Mieszkowska, S. and Zolkiewski, M., 2021. Hsp100 molecular chaperone ClpB and its role in virulence of bacterial pathogens. *International Journal of Molecular Sciences*, 22(10), 5319.
- Kim, H.J., Kim, N.C., Wang, Y.D., Scarborough, E.A., Moore, J., Diaz, Z., MacLea, K.S., Freibaum, B., Li, S., Molliex, A. and Kanagaraj, A.P., 2013. Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature*, 495(7442), 467-473.
- Kim, Y., Park, J., Joo, S.Y., Kim, B.G., Jo, A., Lee, H. and Cho, Y., 2022. Structure of the human TELO2-TTI1-TTI2 complex. *Journal of Molecular Biology*, 434(2), 167370.
- Kobor, M.S., Venkatasubrahmanyam, S., Meneghini, M.D, Gin, J.W., Jennings, J.L., Link, A.J., Madhani, H.D., Rine, J., 2004 A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. *PLoS Biology*, 2, 131.
- Kondo, H., Rabouille, C., Newman, R., Levine, T.P., Pappin, D., Freemont, P., and Warren, G., 1997. p47 is a cofactor for p97-mediated membrane fusion. *Nature*, 388, 75–78.
- Kotepui, M., Kotepui, K.U., De Jesus Milanez, G. and Masangkay, F.R., 2020. Plasmodium spp. mixed infection leading to severe malaria: a systematic review and meta-analysis. *Scientific Reports*, 10(1), 11068.
- Kraemer, S.M., Kyes, S.A., Aggarwal, G., Springer, A.L., Nelson, S.O., Christodoulou, Z., Smith, L.M., Wang, W., Levin, E., Newbold, C.I. and Myler, P.J., 2007. Patterns of gene recombination shape var gene repertoires in *Plasmodium falciparum*: comparisons of geographically diverse isolates. *BMC Genomics*, 8, 1-18.
- Krogan, N.J., Keogh, M.C., Datta, N., Sawa, C., Ryan, O.W., Ding, H., Haw, R.A., Pootoolal, J., Tong, A., Richards, D.P. and Wu, X., 2003. A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. *Molecular Cell*, 12, 1565-1576.
- Külzer, S., Charnaud, S., Dagan, T., Riedel, J., Mandal, P., Pesce, E.R., Blatch, G.L., Crabb, B.S., Gilson, P.R. and Przyborski, J.M., 2012. *Plasmodium falciparum*-encoded exported hsp70/hsp40 chaperone/co-chaperone complexes within the host erythrocyte. *Cellular Microbiology*, 14(11), 1784-1795.

- Kumar, V., Noor, R.E., Davis, R.E., Zhang, Z., Sipavicius, E., Keramisanou, D., Blagg, B.S. and Gelis, I., 2018. Molecular insights into the interaction of Hsp90 with allosteric inhibitors targeting the C-terminal domain. *Medical Chemistry Communications*, 9(8), 1323-1331.
- Kundu, S., Sengupta, S., Chatterjee, S., Mitra, S. and Bhattacharyya, A., 2009. Cadmium induces lung inflammation independent of lung cell proliferation: a molecular approach. *Journal of Inflammation*, 6, 1-15.
- Kusch, T., Florens, L., Macdonald, W.H., Swanson, S.K., Glaser, R.L., Yates III, J.R., Abmayr, S.M., Washburn, M.P., Workman, J.L., 2004 Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. *Science*, 306, 2084– 2087.
- Kyte, J. and Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology*, 157(1), 105-132.
- Lackie, R.E., Maciejewski, A., Ostapchenko, V.G., Marques-Lopes, J., Choy, W.Y., Duennwald, M.L., Prado, V.F. and Prado, M.A., 2017. The Hsp70/Hsp90 chaperone machinery in neurodegenerative diseases. *Frontiers in Neuroscience*, 11, 254.
- Lakomek, K., Stoehr, G., Tosi, A., Schmailzl, M. and Hopfner, K.P., 2015. Structural basis for dodecameric assembly states and conformational plasticity of the full-length AAA+ ATPases Rvb1- Rvb2. *Structure*, 23(3), 483-495.
- Lakshminarasimhan, M., Boanca, G., Banks, C.A., Hattem, G.L., Gabriel, A.E., Groppe, B.D., Smoyer, C., Malanowski, K.E., Peak, A., Florens, L. and Washburn, M.P., 2016. Proteomic and genomic analyses of the Rvb1 and Rvb2 interaction network upon deletion of R2TP complex components. *Molecular & Cellular Proteomics*, 15(3), 960-974.
- Lamb, R.A., Paterson, R.G. and Jardetzky, T.S., 2006. Paramyxovirus membrane fusion: lessons from the F and HN atomic structures. *Virology*, 344(1), 30-37.
- Larsen, J.O., 1998. Stereology of nerve cross sections. *Journal of Neuroscience Methods*, 85(1), 107-118.
- Laufen, T., Mayer, M.P., Beisel, C., Klostermeier, D., Mogk, A., Reinstein, J. and Bukau, B., 1999. Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones. *Proceedings of the National Academy of Sciences*, 96(10), 5452-5457.
- Lavery, L.A., Partridge, J.R., Ramelot, T.A., Elnatan, D., Kennedy, M.A. and Agard, D.A., 2014. Structural asymmetry in the closed state of mitochondrial Hsp90 (TRAP1) supports a two-step ATP hydrolysis mechanism. *Molecular Cell*, 53(2), 330-343.
- Lebepe, C.M., Matambanadzo, P.R., Makhoba, X.H., Achilonu, I., Zininga, T. and Shonhai, A., 2020. Comparative characterization of *Plasmodium falciparum* Hsp70-1 relative to *E. coli* DnaK reveals the functional specificity of the parasite chaperone. *Biomolecules*, 10(6), 856.
- Legastelois, I., Buffin, S., Peubez, I., Mignon, C., Sodoyer, R. and Werle, B., 2017. Non-conventional expression systems for the production of vaccine proteins and immunotherapeutic molecules. *Human Vaccines & Immunotherapeutics*, 13(4), 947-961.
- Li, T. and Pattnaik, A.K., 1999. Overlapping signals for transcription and replication at the 3' terminus of the vesicular stomatitis virus genome. *Journal of Virology*, 73(1), 444-452.

- Lim, R., Kimata, Y., Hidezumi, O., Tetsuro, K., Noriko, Kikuchi., Tsuneyoshi, H., Kenji, Ko., 2000. The *Saccharomyces cerevisiae* RuvB-like Protein, Tih2p, Is Required for Cell Cycle Progression and RNA Polymerase II-directed Transcription. *Journal of Biological Chemistry*, 275(29).
- Llovet, J.M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.F., De Oliveira, A.C., Santoro, A., Raoul, J.L., Forner, A. and Schwartz, M., 2008. Sorafenib in advanced hepatocellular carcinoma. *New England Journal of Medicine*, 359(4), 378-390.
- Lone, M., Shadang, M., Akhter, Q., Kumar, M., Mallick, S., Gogia, A., Nilima, N., Chauhan, S.S., Mir, R.A., 2022. Components of the R2TP complex correlates with poor prognosis in DLBCL. *Pathobiology*, 89, 146-156.
- López-Perrote, A., Munoz-Hernandez, H., Gil, D. and Llorca, O., 2012. Conformational transitions regulate the exposure of a DNA-binding domain in the RuvBL1–RuvBL2 complex. *Nucleic Acids Research*, 40(21), 11086-11099.
- Lu, K.Y., Pasaje, C.F.A., Srivastava, T., Loisel, D.R., Niles, J.C. and Derbyshire, E., 2020. Phosphatidylinositol 3-phosphate and Hsp70 protect *Plasmodium falciparum* from heat-induced cell death. *Elife*, 9, e56773.
- Lubkowska, A., Dudzińska, W. and Pluta, W., 2023. Antioxidant enzyme activity and serum HSP70 concentrations in relation to insulin resistance and lipid profile in lean and overweight young men. *Antioxidants*, 12(3), 655.
- Luthuli, S.D. and Shonhai, A., 2023. The multi-faceted roles of R2TP complex span across regulation of gene expression, translation, and protein functional assembly. *Biophysical Reviews*, 15(6), 1951-1965.
- Luthuli, S.D., Chili, M.M., Revaprasadu, N. and Shonhai, A., 2013. Cysteine-capped gold nanoparticles suppress aggregation of proteins exposed to heat stress. *IUBMB life*, 65(5), 454-461.
- Lynham, J. and Houry, W.A., 2022. The role of Hsp90-R2TP in macromolecular complex assembly and stabilization. *Biomolecules*, 12(8), 1045.
- Maake, R. and Achilonu, I., 2024. Expression, Purification and Biophysical Characterisation of *Klebsiella pneumoniae* Protein Adenylyltransferase: A Systematic Integration of Empirical and Computational Modelling Approaches. *The Protein Journal*, 43(4), 751-770.
- Mabate, B., Zininga, T., Ramatsui, L., Makumire, S., Achilonu, I., Dirr, H.W. and Shonhai, A., 2018. Structural and biochemical characterization of *Plasmodium falciparum* Hsp70-x reveals functional versatility of its C-terminal EEVN motif. *Proteins: Structure, Function, and Bioinformatics*, 86(11), 1189-1201.
- Maciejewski, J.P. and Padgett, R.A., 2012. Defects in spliceosomal machinery: a new pathway of leukaemogenesis. *British Journal of Haematology*, 158(2), 165-173.
- Maier, A.G., Cooke, B.M., Cowman, A.F. and Tilley, L., 2009. Malaria parasite proteins that remodel the host erythrocyte. *Nature Reviews Microbiology*, 7(5), 341-354.

- Makhoba, X.H., Burger, A., Coertzen, D., Zininga, T., Birkholtz, L.M. and Shonhai, A., 2016. Use of a chimeric Hsp70 to enhance the quality of recombinant *Plasmodium falciparum* s-adenosylmethionine decarboxylase protein produced in *Escherichia coli*. *PLoS One*, 11(3), 0152626.
- Makumire, S., Dongola, T.H., Chakafana, G., Tshikonwane, L., Chauke, C.T., Maharaj, T., Zininga, T. and Shonhai, A., 2021. Mutation of GGMP repeat segments of *Plasmodium falciparum* Hsp70-1 compromises chaperone function and Hop co-chaperone binding. *International Journal of Molecular Sciences*, 22(4), 2226.
- Makumire, S., Revaprasadu, N. and Shonhai, A., 2015. DnaK protein alleviates toxicity induced by citrate-coated gold nanoparticles in *Escherichia coli*. *PLoS One*, 10(4), 0121243.
- Makumire, S., Zininga, T., Vahokoski, J., Kursula, I. and Shonhai, A., 2020. Biophysical analysis of *Plasmodium falciparum* Hsp70-Hsp90 organising protein (PfHop) reveals a monomer that is characterised by folded segments connected by flexible linkers. *PLoS One*, 15(4), e0226657.
- Malinová, A., 2017. The role of pre-mRNA splicing in human hereditary diseases. Univerzita Karlova, Přírodovědecká fakulta.
- Manor, D.U., Grossman, D.T., Vainer, J. and Schwartz, P.E., 2022. A nationwide study of imported *Plasmodium ovale* and mixed infections in Israel 2008–2020. *Journal of Travel Medicine*, 29(4), 192.
- Mansuri, M.S., Bathla, S., Lam, T.T., Nairn, A.C. and Williams, K.R., 2024. Optimal conditions for carrying out trypsin digestions on complex proteomes: From bulk samples to single cells. *Journal of Proteomics*, 3, 105109.
- Mao, Y.Q.K., 2024. The Functions of the Chaperone Complex R2TP in Human Health and Disease. University of Toronto. Canada
- Marrione, P.E. and Cox, M.M., 1996. Allosteric effects of RuvA protein, ATP, and DNA on RuvB protein-mediated ATP hydrolysis. *Biochemistry*, 35(34), 11228-11238.
- Martino, F., Pal, M., Muñoz-Hernández, H., Rodríguez, C.F., Núñez-Ramírez, R., Gil-Carton, D., Degliesposti, G., Skehel, J.M., Roe, S.M., Prodromou, C. and Pearl, L.H., 2018. RPAP3 provides a flexible scaffold for coupling HSP90 to the human R2TP co-chaperone complex. *Nature Communications*, 9(1), 1501.
- Marzal, A. and Garcia-Longoria, L., 2020. The role of malaria parasites in invasion biology. *Avian Malaria and Related Parasites in the Tropics: Ecology, Evolution and Systematics*, 487-512.
- Masison, D.C., Kirkland, P.A., Sharma, D., 2009. Influence of Hsp70s and their regulators on yeast prion propagation. *Prion*, 3(2), 65-73.
- Matambo, T.S., Odunuga, O.O., Boshoff, A., Blatch, G.L., 2004. Overproduction, purification and characterization of the *Plasmodium falciparum* heat shock protein 70. *Protein Expression and Purification*, 33, 214-222.

- Matias, P.M., Gorynia, S., Donner, P. and Carrondo, M.A., 2006. Crystal structure of the human AAA+ protein RuvBL1. *Journal of Biological Chemistry*, 281(50), 38918-38929.
- Maurizy, C., Quinternet, M., Abel, Y., Verheggen, C., Santo, P.E., Bourguet, M., CF Paiva, A., Bragantini, B., Chagot, M.E., Robert, M.C. and Abeza, C., 2018. The RPAP3-Cterminal domain identifies R2TP-like quaternary chaperones. *Nature Communications*, 9(1), 2093.
- Mayer, M.P. and Bukau, B., 2005. Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences*, 62, 670-684.
- Mayer, M.P., Schröder, H., Rüdiger, S., Paal, K., Laufen, T. and Bukau, B., 2000. Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nature Structural Biology*, 7(7), 586-593.
- McClellan, A.J., Xia, Y., Deutschbauer, A.M., Davis, R.W., Gerstein, M. and Frydman, J., 2007. Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches. *Cell*, 131(1), 121-135.
- McConkey, B.J., Sobolev, V. and Edelman, M., 2002. The performance of current methods in ligand–protein docking. *Current Science*, 845-856.
- McHugh, E., Carmo, O.M., Blanch, A., Looker, O., Liu, B., Tiash, S., Andrew, D., Batinovic, S., Low, A.J., Cho, H.J. and McMillan, P., 2020. Role of *Plasmodium falciparum* protein GEXP07 in Maurer's cleft morphology, knob architecture, and *P. falciparum* EMP1 trafficking. *Molecular Biology*, 11(2), 10-1128.
- McKeegan, K.S., Debieux, C.M., Watkins, N.J., 2009. Evidence that the AAA⁺ proteins Tip48 and Tip49 bridge interactions between 15.5K and the related NOP56 and NOP58 proteins during box C/D snoRNP biogenesis. *Molecular and Cell Biology*, 29, 4971-4981.
- Meng, X.Y., Zhang, H.X., Mezei, M. and Cui, M., 2011. Molecular docking: a powerful approach for structure-based drug discovery. *Current Computer-aided Drug Design*, 7(2), 146-157.
- Meyer, P., Prodromou, C., Hu, B., Vaughan, C., Roe, S.M., Panaretou, B., Piper, P.W. and Pearl, L.H., 2003. Structural and functional analysis of the middle segment of hsp90: implications for ATP hydrolysis and client protein and cochaperone interactions. *Molecular Cell*, 11(3), 647-658.
- Milner, D.A., 2018. Malaria pathogenesis. *Cold Spring Harbor Perspectives in Medicine*, 8(1), 025569.
- Mo, A.X. and McGugan, G., 2018. Understanding the liver-stage biology of malaria parasites: insights to enable and accelerate the development of a highly efficacious vaccine. *The American Journal of Tropical Medicine and Hygiene*, 99(4), 827.
- Mohandas, N. and Gallagher, P.G., 2008. Red cell membrane: past, present, and future. *Blood, The Journal of the American Society of Hematology*, 112(10), 3939-3948.
- Molina-Franky, J., Patarroyo, M.E., Kalkum, M. and Patarroyo, M.A., 2022. The cellular and molecular interaction between erythrocytes and *Plasmodium falciparum* merozoites. *Frontiers in Cellular and Infection Microbiology*, 12, 816574.

- Moll, A., Ramirez, L.M., Ninov, M., Schwarz, J., Urlaub, H. and Zweckstetter, M., 2022. Hsp multichaperone complex buffers pathologically modified Tau. *Nature Communications*, 13(1), 3668.
- Montagna, G.N., Matuschewski, K. and Buscaglia, C.A., 2012. Small heat shock proteins in cellular adhesion and migration: evidence from *Plasmodium* genetics. *Cell Adhesion & Migration*, 6(2), 78-84.
- Monti, S., Chapuy, B., Takeyama, K., Rodig, S.J., Hao, Y., Yeda, K.T., Inguilizian, H., Mermel, C., Currie, T., Dogan, A. and Kutok, J.L., 2012. Integrative analysis reveals an outcome-associated and targetable pattern of p53 and cell cycle deregulation in diffuse large B cell lymphoma. *Cancer Cell*, 22(3), 359–372.
- Morales, E.S., Parcerisa, I.L. and Ceccarelli, E.A., 2019. A novel method for removing contaminant Hsp70 molecular chaperones from recombinant proteins. *Protein Science*, 28(4), 800-807.
- Morgan, R.M., Pal, M., Roe, S.M., Pearl, L.H. and Prodromou, C., 2015. Tah1 helix-swap dimerization prevents mixed Hsp90 co-chaperone complexes. *Acta Crystallographica Section D: Biological Crystallography*, 71(5), 1197-1206.
- Morrison, A.J., Shen, X., 2009 Chromatin remodelling beyond transcription: the INO80 and SWR1 complexes. *National Reviews on Molecular and Cell Biology*, 10, 373– 384.
- Muller, P., Ruckova, E., Halada, P., Coates, P. J., Hrstka, R., Lane, D. P., and Vojtesek, B. 2013. C-terminal phosphorylation of Hsp70 and Hsp90 regulates alternate binding to co-chaperones CHIP and HOP to determine cellular protein folding/degradation balances. *Oncogene*, 32, 3101–3110.
- Muñoz-Hernández, H., Pal, M., Rodríguez, C.F., Fernandez-Leiro, R., Prodromou, C., Pearl, L.H. and Llorca, O., 2019. Structural mechanism for regulation of the AAA-ATPases RUVBL1-RUVBL2 in the R2TP co-chaperone revealed by cryo-EM. *Science Advances*, 5(5), 1616.
- Muralidharan, V., Oksman, A., Pal, P., Lindquist, S. and Goldberg, D.E., 2012. *Plasmodium falciparum* heat shock protein 110 stabilizes the asparagine repeat-rich parasite proteome during malarial fevers. *Nature Communications*, 3(1), 1310.
- Muthelo, T., Mulaudzi, V., Netshishivhe, M., Dongola, T.H., Kok, M., Makumire, S., de Villiers, M., Burger, A., Zininga, T. and Shonhai, A., 2022. Inhibition of *Plasmodium falciparum* Hsp70-Hop partnership by 2-phenylthynesulfonamide. *Frontiers in Molecular Biosciences*, 9, 947203.
- Myers, J.K. and Pace, C.N., 1996. Hydrogen bonding stabilizes globular proteins. *Biophysical Journal*, 71(4), 2033-2039.
- Namboodiri, H.V., Bukhtiyarova, M., Ramcharan, J., Karpusas, M., Lee, Y. and Springman, E.B., 2010. Analysis of imatinib and sorafenib binding to p38 α compared with c-Abl and b-Raf provides structural insights for understanding the selectivity of inhibitors targeting the DFG-out form of protein kinases. *Biochemistry*, 49(17), 3611-3618.

- Nano, N. and Houry, W.A., 2013. Chaperone-like activity of the AAA+ proteins Rvb1 and Rvb2 in the assembly of various complexes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1617), 20110399.
- Nano, N., Ugwu, F., Seraphim, T.V., Li, T., Azer, G., Isaac, M., Prakesch, M., Barbosa, L.R., Ramos, C.H., Datti, A. and Houry, W.A., 2020. Sorafenib as an Inhibitor of RUVBL2. *Biomolecules*, 10(4), 605.
- Ncube, H.R., Dali, U., Dongola, T.H. and Shonhai, A., 2024. Author Spotlight: Exploring Heat Shock Proteins in Malaria and Tuberculosis Infections. *Journal of Visualized Experiments*, 205, e66515.
- Neuwald, A.F., Aravind, L., Spouge, J.L., Koonin, E.V., 1999. AAA+: a class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Research*, 9, 27-43.
- Newman, M., Strzelecka, T., Dorner, L.F., Schildkraut, I. and Aggarwal, A.K., 1994. Structure of restriction endonuclease Bam HI and its relationship to *Eco* RI. *Nature*, 368(6472), 660-664.
- Niazi, S.K. and Magoola, M., 2023. mRNA and Synthesis-Based Therapeutic Proteins: A Non-Recombinant Affordable Option. *Biologics*, 3(4), 355-379.
- Nikita, J.G., Wasono, M.A.J. and Satriawan, M., 2020. CO₂ laser photoacoustic spectrometer for measuring ethylene, acetone, and ammonia in the breath of patients with renal disease. *Sensing and Bio-Sensing Research*, 30, 100387.
- Njunge, M, J. H., Ludewig, M., Boshoff, A., Pesce, E.R. and L Blatch, G., 2013. Hsp70s and J proteins of Plasmodium parasites infecting rodents and primates: structure, function, clinical relevance, and drug targets. *Current Pharmaceutical Design*, 19(3), 387-403.
- Noddings, C.M., Wang, R.Y.R., Johnson, J.L. and Agard, D.A., 2022. Structure of Hsp90–p23–GR reveals the Hsp90 client-remodelling mechanism. *Nature*, 601(7893), 465-469.
- Nowicki, Ł., Leźnicki, P., Morawiec, E., Litwińczuk, N. and Liberek, K., 2012. Role of a conserved aspartic acid in nucleotide binding domain 1 (NBD1) of Hsp100 chaperones in their activities. *Cell Stress and Chaperones*, 17(3), 361-373.
- O’Carrigan, B., Lim, J.S.J., Jalil, A., Harris, S.J., Papadatos-Pastos, D., Banerji, U., Lopez, J., de Bono, J.S. and Yap, T.A., 2018. Target-based therapeutic matching of phase I trials in patients with metastatic breast cancer in a tertiary referral centre. *British Journal of Cancer*, 119(8), 922-927.
- O’Flaherty, R., Bergin, A., Flampouri, E., Mota, L.M., Obaidi, I., Quigley, A., Xie, Y. and Butler, M., 2020. Mammalian cell culture for production of recombinant proteins: A review of the critical steps in their biomanufacturing. *Biotechnology Advances*, 43, 107552.
- Oberg, K., Chrnyk, B.A., Wetzel, R. and Fink, A.L., 1994. Native-like secondary structure in interleukin-1. beta. Inclusion bodies by attenuated total reflectance FTIR. *Biochemistry*, 33(9), 2628-2634.
- Oelschlegel, A.M., Bhattacharjee, R., Wenk, P., Harit, K., Rothkötter, H.J., Koch, S.P., Boehm-Sturm, P., Matuschewski, K., Budinger, E., Schlüter, D. and Goldschmidt, J., 2024. Beyond the

microcirculation: sequestration of infected red blood cells and reduced flow in large draining veins in experimental cerebral malaria. *Nature Communications*, 15(1), 2396.

Ogura, T., Whiteheart, S.W., Wilkinson, A.J., 2004. Conserved arginine residues implicated in ATP hydrolysis, nucleotide-sensing, and inter-subunit interactions in AAA and AAA+ ATPases. *Journal of Structural Biology*, 146,106–112.

Ogura, T., Wilkinson, A.J., 2001. AAA+ superfamily ATPases: common structure - diverse function. *Genes Cells*, 6,575–597.

Önell, A. and Andersson, K., 2005. Kinetic determinations of molecular interactions using Biacore—minimum data requirements for efficient experimental design. *Journal of Molecular Recognition: An Interdisciplinary Journal*, 18(4), 307-317.

Onuoha, S.C., Coulstock, E.T., Grossmann, J.G. and Jackson, S.E., 2008. Structural studies on the co-chaperone Hop and its complexes with Hsp90. *Journal of Molecular Biology*, 379(4), 732-744.

Ould Ahmedou Salem, M.S., Mohamed Lemine, Y.O., Deida, J.M., Lemrabott, M.A.O., Ouldabdallahi, M., Ba, M.D.D., Boukhary, A.O.M.S., Khairy, M.L.O., Abdel Aziz, M.B., Ringwald, P. and Basco, L.K., 2015. Efficacy of chloroquine for the treatment of *Plasmodium vivax* in the Saharan zone in Mauritania. *Malaria Journal*, 14, 1-5.

Ozaki, T. and Nakagawara, A., 2011. Role of p53 in cell death and human cancers. *Cancers*, 3(1), 994-1013.

Pal, M., Morgan, M., Phelps, S.E., Roe, S.M., Parry-Morris, S., Downs, J.A., Polier, S., Pearl, L.H. and Prodromou, C., 2014. Structural basis for phosphorylation-dependent recruitment of Tel2 to Hsp90 by Pih1. *Structure*, 22(6), 805-818.

Pal, M., Muñoz-Hernandez, H., Bjorklund, D., Zhou, L., Degliesposti, G., Skehel, J.M., Hesketh, E.L., Thompson, R.F., Pearl, L.H., Llorca, O. and Prodromou, C., 2021. Structure of the TELO2-TTI1-TTI2 complex and its function in TOR recruitment to the R2TP chaperone. *Cell Reports*, 36(1).

Pallavi, R., Roy, N., Nageshan, R.K., Talukdar, P., Pavithra, S.R., Reddy, R., Venketesh, S., Kumar, R., Gupta, A.K., Singh, R.K. and Yadav, S.C., 2010. Heat shock protein 90 as a drug target against protozoan infections: biochemical characterization of HSP90 from *Plasmodium falciparum* and *Trypanosoma evansi* and evaluation of its inhibitor as a candidate drug. *Journal of Biological Chemistry*, 285(49), 37964-37975.

Panaretou, B., Siligardi, G., Meyer, P., Maloney, A., Sullivan, J.K., Singh, S., Millson, S.H., Clarke, P.A., Naaby-Hansen, S., Stein, R. and Cramer, R., 2002. Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1. *Molecular Cell*, 10(6), 1307-1318.

Parham, P.E. and Hughes, D.A., 2015. Climate influences on the cost-effectiveness of vector-based interventions against malaria in elimination scenarios. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370(1665), 20130557.

Patel-King, R.S. and King, S.M., 2016. A prefoldin-associated WD-repeat protein (WDR92) is required for the correct architectural assembly of motile cilia. *Molecular Biology of the Cell*, 27(8), 1204-1209.

- Paul, A.S., Egan, E.S. and Duraisingh, M.T., 2015. Host–parasite interactions that guide red blood cell invasion by malaria parasites. *Current Opinion in Hematology*, 22(3), 220-226.
- Paul, R., Zhou, Y., Nikfar, M., Razizadeh, M. and Liu, Y., 2020. Quantitative absorption imaging of red blood cells to determine physical and mechanical properties. *RSC Advances*, 10(64), 38923-38936.
- Pavithra, S.R., Banumathy, G., Joy, O., Singh, V. and Tatu, U., 2004. Recurrent fever promotes *Plasmodium falciparum* development in human erythrocytes. *Journal of Biological Chemistry*, 279(45), 46692-46699.
- Pavithra, S.R., Kumar, R. and Tatu, U., 2007. Systems analysis of chaperone networks in the malarial parasite *Plasmodium falciparum*. *PLoS Computational Biology*, 3(9), e168.
- Pearl, L.H. and Prodromou, C., 2006. Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annual Review of Biochemistry*, 75(1), 271-294.
- Pearson, W.R., 2013. An introduction to sequence similarity (“homology”) searching. *Current Protocols in Bioinformatics*, 42(1), 3-1.
- Pedrotti, S. and Cooper, T.A., 2014. In Brief:(mis) splicing in disease.
- Pellizza, L., Smal, C., Rodrigo, G. and Arán, M., 2018. Codon usage clusters correlation: towards protein solubility prediction in heterologous expression systems in *E. coli*. *Scientific Reports*, 8(1), 10618.
- Picard, D., 2002. Heat-shock protein 90, a chaperone for folding and regulation. *Cellular and Molecular Life Sciences CMLS*, 59, 1640-1648.
- Piccaluga, P.P. and Ignatius, W., 2023. Pathology of Malaria. In *Malaria-Recent Advances and New Perspectives*.
- Pols, T., Singh, S., Deelman-Driessen, C., Gaastra, B.F. and Poolman, B., 2021. Enzymology of the pathway for ATP production by arginine breakdown. *The FEBS Journal*, 288(1), 293-309.
- Polson, E.S., Kuchler, V.B., Abbosh, C., Ross, E.M., Mathew, R.K., Beard, H.A., da Silva, B., Holding, A.N., Ballereau, S., Chuntharpursat-Bon, E. and Williams, J., 2018. KHS101 disrupts energy metabolism in human glioblastoma cells and reduces tumor growth in mice. *Science Translational Medicine*, 10(454), 2718.
- Preisner, P., Kaviratne, M., Khan, S., Bannister, L. and Jarra, W., 2000. The apical organelles of malaria merozoites: host cell selection, invasion, host immunity and immune evasion. *Microbes and Infection*, 2(12), 1461-1477.
- Prior, K.F., Middleton, B., Owolabi, A.T., Westwood, M.L., Holland, J., O'Donnell, A.J., Blackman, M.J., Skene, D.J. and Reece, S.E., 2021. Synchrony between daily rhythms of malaria parasites and hosts is driven by an essential amino acid. *Wellcome Open Research*, 6.
- Prodromou, C. and Bjorklund, D.M., 2022. Advances towards understanding the mechanism of action of the Hsp90 complex. *Biomolecules*, 12(5), 600.

- Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W. and Pearl, L.H., 1997. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell*, 90(1), 65-75.
- Puri, A., Singh, P., Kumar, N., Kumar, R. and Sharma, D., 2021. Tah1, a key component of R2TP complex that regulates assembly of snoRNP, is involved in de novo generation and maintenance of yeast prion [URE3]. *Journal of Molecular Biology*, 433(13), 166976.
- Putri, D.A.S., Sulistyaningsih, E., Kusuma, I.F. and Dewi, R., 2022. Total Leukocyte Count in *Rattus norvegicus* after Duffy Binding-Like 2 β -Plasmodium falciparum Erythrocyte Membrane Protein 1 Recombinant Protein Injection: The way to a Peptide-based Malaria Vaccine Development. *Biomolecular and Health Science Journal*, 5(2), 71-76.
- Qiu, X., Lin, Y., Thome, K., Pian, P., Schlegel, B., Weremowicz, S., Parvin, J., and Dutta, A., 1998. A eukaryotic RuvB-like protein (RUVBL1) is essential for growth. *Journal of Biological Chemistry*, 273, 27786–27793.
- Rahumatullah, A., Yunus, M.H., Tye, G.J. and Noordin, R., 2020. Applications of recombinant monoclonal antibodies against filarial antigen proteins. *The American Journal of Tropical Medicine and Hygiene*, 102(3), 578.
- Rajendra, E., Oestergaard, V.H., Langevin, F., Wang, M., Dornan, G.L., Patel, K.J. and Passmore, L.A., 2014. The genetic and biochemical basis of FANCD2 monoubiquitination. *Molecular cell*, 54(5), 858-869.
- Rehman, M.A., Fatima, J., Rehman, Z.U., Alomar, S.Y., Sohlab, M. and Hamad, A., 2024. The DFT study of the structural, hydrogen, electronic, mechanical, thermal, and optical properties of KXH3 (X= Ca, Sc, Ti, & Ni) perovskites for H₂ storage applications. *Structural Chemistry*, 1-14.
- Rial, D.V. and Ceccarelli, E.A., 2002. Removal of DnaK contamination during fusion protein purifications. *Protein Expression and Purification*, 25(3), 503-507.
- Ritossa, F., 1962. A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia*, 18(12), 571-573.
- Rivera-Calzada, A., Pal, M., Munoz-Hernandez, H., Luque-Ortega, J.R., Gil-Carton, D., Degliesposti, G., Skehel, J.M., Prodromou, C., Pearl, L.H. and Llorca, O., 2017. The structure of the R2TP complex defines a platform for recruiting diverse client proteins to the HSP90 molecular chaperone system. *Structure*, 25(7), 1145-1152.
- Roake, C.M. and Artandi, S.E., 2020. Regulation of human telomerase in homeostasis and disease. *Nature Reviews Molecular Cell Biology*, 21(7), 384-397.
- Rodriguez, C.F., Llorca, O., 2020. RPAP3 C-terminal domain: a conserved domain for the assembly of R2TP Co-chaperone complexes. *Cell. PMID*, 9(10), 3390.
- Röhl, A., Tippel, F., Bender, E., Schmid, A.B., Richter, K., Madl, T. and Buchner, J., 2015. Hop/Sti1 phosphorylation inhibits its co-chaperone function. *EMBO Reports*, 16(2), 240-249.

- Rohland, L., Kityk, R., Smalinskaitė, L., Mayer, M.P., 2022. Conformational dynamics of the Hsp70 chaperone throughout key steps of its ATPase cycle. *Proceeding of National Academy Science*, 119(4), 810-1073.
- Romanova, N.V. and Chernoff, Y.O., 2009. Hsp104 and prion propagation. *Protein and peptide letters*, 16(6), 598-605.
- Rosenzweig, R., Nillegoda, N.B., Mayer, M.P. and Bukau, B., 2019. The Hsp70 chaperone network. *Nature Reviews Molecular Cell Biology*, 20(11), 665-680.
- Roth, L., Grzeschik, J., Hinz, S.C., Becker, S., Toleikis, L., Busch, M., Kolmar, H., Krah, S. and Zielonka, S., 2019. Facile generation of antibody heavy and light chain diversities for yeast surface display by Golden Gate Cloning. *Biological Chemistry*, 400(3), 383-393.
- Rousseau, B. Menard, L. Haurie, V., Taras, D., Blanc, J.F., Moreau-Gaudry, F., Metzler, P., Hugues, M., Boyault, S., Lemièrre, S. and Canron, X., 2007. Overexpression and role of the ATPase and putative DNA helicase RuvB-like 2 in human hepatocellular carcinoma. *Hepatology*, 46, 1108–1118.
- Rüdiger, S., Germeroth, L., Schneider-Mergener, J. and Bukau, B., 1997. Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *The EMBO Journal*. 1.
- Saeki, M., Egusa, H., Kamano, Y., Kakihara, Y., Houry, W.A., Yatani, H., Noguchi, S. and Kamisaki, Y., 2013. Exosome-bound WD repeat protein Monad inhibits breast cancer cell invasion by degrading amphiregulin mRNA. *PloS One*, 8(7), e67326.
- Salzer, U., Kubicek, M., Prohaska, R., 1999. Isolation, molecular characterization, and tissue-specific expression of ECP-51 and ECP-54 (TIP49), two homologous, interacting erythroid cytosolic proteins. *Biochemistry and Biophysical Science Acta*, 1446, 365–370.
- Sanders, P.R., Gilson, P.R., Cantin, G.T., Greenbaum, D.C., Nebl, T., Carucci, D.J., McConville, M.J., Schofield, L., Hodder, A.N., Yates, J.R. and Crabb, B.S., 2005. Distinct protein classes including novel merozoite surface antigens in Raft-like membranes of *Plasmodium falciparum*. *Journal of Biological Chemistry*, 280(48), 40169-40176.
- Santiago, T.C., Zufferey, R., Mehra, R.S., Coleman, R.A. and Mamoun, C.B., 2004. The *Plasmodium falciparum* PfGatp is an endoplasmic reticulum membrane protein important for the initial step of malarial glycerolipid synthesis. *Journal of Biological Chemistry*, 279(10), 9222-9232.
- Sato, S., 2021. Plasmodium—a brief introduction to the parasites causing human malaria and their basic biology. *Journal of Physiological Anthropology*, 40(1), 1.
- Schapira, M., Tyers, M., Torrent, M. and Arrowsmith, C.H., 2017. WD40 repeat domain proteins: a novel target class. *Nature Reviews Drug discovery*, 16(11), 773-786.
- Schier, A.C. and Taatjes, D.J., 2020. Structure and mechanism of the RNA polymerase II transcription machinery. *Genes and Development*, 34(7-8), 465-488.

- Schmid, A.B., Lagleder, S., Gräwert, M.A., Röhl, A., Hagn, F., Wandinger, S.K., Cox, M.B., Demmer, O., Richter, K., Groll, M. and Kessler, H., 2012. The architecture of functional modules in the Hsp90 co-chaperone Sti1/Hop. *The EMBO Journal*, 31(6), 1506-1517.
- Schopf, F.H., Biebl, M.M. and Buchner, J., 2017. The HSP90 chaperone machinery. *Nature Reviews Molecular and Cell Biology*, 18(6), 345-360.
- Schwarz, K., Baindur-Hudson, S., Blatch, G.L. and Edkins, A.L., 2022. Hsp70/Hsp90 organising protein (Hop): coordinating much more than chaperones. *The Networking of Chaperones by Co-Chaperones*, 81-125.
- Scortecci, J.F., Garces, F.A., Mahto, J.K., Molday, L.L., Van Petegem, F. and Molday, R.S., 2024. Structural and functional characterization of the nucleotide-binding domains of ABCA4 and their role in Stargardt disease. *Journal of Biological Chemistry*, 300(9).
- Sen, U., Saxena, H., Khurana, J., Nayak, A. and Gupta, A., 2018. Plasmodium falciparum RUVBL3 protein: a novel DNA modifying enzyme and an interacting partner of essential HAT protein MYST. *Scientific Reports*, 8(1), 10917.
- Seraphim, T.V., Chakafana, G., Shonhai, A. and Houry, W.A., 2019. Plasmodium falciparum R2TP complex: driver of parasite Hsp90 function. *Biophysical Reviews*, 11(6), 1007-1015.
- Seraphim, T.V., Nano, N., Cheung, Y.W.S., Aluksanasuwan, S., Colleti, C., Mao, Y.Q., Bhandari, V., Young, G., Höll, L., Phanse, S. and Gordiyenko, Y., 2022. Assembly principles of the human R2TP chaperone complex reveal the presence of R2T and R2P complexes. *Structure*, 30(1), 156-171.
- Serna, M., González-Corpas, A., Cabezudo, S., López-Perrote, A., Degliesposti, G., Zarzuela, E., Skehel, J.M., Muñoz, J. and Llorca, O., 2022. CryoEM of RUVBL1–RUVBL2–ZNHIT2, a complex that interacts with pre-mRNA-processing-splicing factor 8. *Nucleic Acids Research*, 50(2), 1128-1146.
- Shahinas, D., Folefoc, A. and Pillai, D.R., 2013. Targeting Plasmodium falciparum Hsp90: towards reversing antimalarial resistance. *Pathogens*, 2(1), 33-54.
- Shearer, R.F., Iconomou, M., Watts, C.K. and Saunders, D.N., 2015. Functional roles of the E3 ubiquitin ligase UBR5 in cancer. *Molecular Cancer Research*, 13(12), 1523-1532.
- Shen, X., Mizuguchi, G., Hamiche, A., Wu, C., 2000. A chromatin remodelling complex involved in transcription and DNA processing. *Nature*, 406, 541-54.
- Sherling, E.S. and van Ooij, C., 2016. Host cell remodeling by pathogens: the exomembrane system in Plasmodium-infected erythrocytes. *FEMS Microbiology Reviews*, 40(5), 701-721.
- Sherman, M.Y. and Goldberg, A.L., 1993. Heat shock of Escherichia coli increases binding of dnaK (the hsp70 homolog) to polypeptides by promoting its phosphorylation. *Proceedings of the National Academy of Sciences*, 90(18), 8648-8652.
- Shi, S.P., Qiu, J.D., Sun, X.Y., Suo, S.B., Huang, S.Y. and Liang, R.P., 2012. A method to distinguish between lysine acetylation and lysine methylation from protein sequences. *Journal of Theoretical Biology*, 310, 223-230.

- Shonhai, A., 2021. The role of Hsp70s in the development and pathogenicity of *Plasmodium falciparum*. *Heat Shock Proteins of Malaria*, 75-95.
- Shonhai, A., Boshoff, A. and Blatch, G.L., 2007. The structural and functional diversity of Hsp70 proteins from *Plasmodium falciparum*. *Protein Science*, 16(9), 1803-1818.
- Shonhai, A., Boshoff, A., Blatch, G.L., 2005. *Plasmodium falciparum* heat shock protein 70 is able to suppress the thermosensitivity of an *Escherichia coli* DnaK mutant strain. *Molecular Genetics and Genomics*, 274, 70-78.
- Shonhai, A., Botha, M., de Beer, T. A. P, Boshoff, A and Blatch, G. L., 2008. Structure- function study of *Plasmodium falciparum* Hsp70 using three-dimensional modelling and *in vitro* analyses. *Protein and Peptide Letters*, 15, 1117-1125.
- Shonhai, A., G Maier, A., M Przyborski, J. and L Blatch, G., 2011. Intracellular protozoan parasites of humans: the role of molecular chaperones in development and pathogenesis. *Protein and Peptide Letters*, 18(2), 143-157.
- Sievers, F. and Higgins, D.G., 2014. Clustal Omega, accurate alignment of very large numbers of sequences. *Multiple Sequence Alignment Methods*, 105-116.
- Silva, N.S., Bertolino-Reis, D.E., Dores-Silva, P.R., Anneta, F.B., Seraphim, T.V., Barbosa, L.R. and Borges, J.C., 2020. Structural studies of the Hsp70/Hsp90 organizing protein of *Plasmodium falciparum* and its modulation of Hsp70 and Hsp90 ATPase activities. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1868(1), 140282.
- Silva-Pinto, A., Domingos, J., Cardoso, M., Reis, A., Benavente, E.D., Caldas, J.P., Conceição, C., Toscano, C., Baptista-Fernandes, T., Clark, T.G. and Mansinho, K., 2021. Artemether-lumefantrine treatment failure of uncomplicated *Plasmodium falciparum* malaria in travellers coming from Angola and Mozambique. *International Journal of Infectious Diseases*, 110, 151-154.
- Snider, J., Thibault, G., Houry, W.A., 2008. The AAA+ superfamily of functionally diverse proteins. *Genome Biology*. 9(4),216-30. Sparks, R.P., Jenkins, J.L. and Fratti, R., 2019. Use of surface plasmon resonance (SPR) to determine binding affinities and kinetic parameters between components important in fusion machinery. *SNAREs: Methods and Protocols*, 199-210.
- Spence, A.M., Coulson, A. and Hodgkin, J., 1990. The product of fem-1, a nematode sex-determining gene, contains a motif found in cell cycle control proteins and receptors for cell-cell interactions. *Cell*, 60(6), 981-990.
- Subudhi, A.K., Green, J.L., Satyam, R., Salunke, R.P., Lenz, T., Shuaib, M., Isaioglou, I., Abel, S., Gupta, M., Esau, L. and Mourier, T., 2023. DNA-binding protein PfAP2-P regulates parasite pathogenesis during malaria parasite blood stages. *Nature Microbiology*, 8(11), 2154-2169.
- Suppini, J.P., Amor, M., Alix, J.H. and Ladjimi, M.M., 2004. Complementation of an *Escherichia coli* DnaK defect by Hsc70-DnaK chimeric proteins. *Journal of Bacteriology*, 186(18), 6248-6253.
- Szklarczyk, D., Gable, A.L., Nastou, K.C., Lyon, D., Kirsch, R., Pyysalo, S., Doncheva, N.T., Legeay, M., Fang, T., Bork, P. and Jensen, L.J., 2021. The STRING database in 2021:

customizable protein–protein networks and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Research*, 49, 605-612.

Szwed, A., Kim, E. and Jacinto, E., 2021. Regulation and metabolic functions of mTORC1 and mTORC2. *Physiological Reviews*, 101(3), 1371-1426.

Taghian, D.G. and Nickoloff, J.A., 1996. Subcloning strategies and protocols. *Basic DNA and RNA Protocols*, 221-235.

Taipale, M., Jarosz, D.F. and Lindquist, S., 2010. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature Reviews Molecular cell biology*, 11(7), 515-528.

Takenaka, I.M. and Hightower, L.E., 1993. Regulation of chicken Hsp70 and Hsp90 family gene expression by transforming growth factor- β 1. *Journal of Cellular Physiology*, 155(1), 54-62.

Tan, M.S.Y. and Blackman, M.J., 2021. Malaria parasite egress at a glance. *Journal of Cell Science*, 134(5), 257345.

Terpe, K., 2006. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology*, 72, 211-222.

Tian, S., Yu, G., He, H., Zhao, Y., Liu, P., Marshall, A.G., Demeler, B., Stagg, S.M. and Li, H., 2017. Pih1p-Tah1p puts a lid on hexameric AAA+ ATPases Rvb1/2p. *Structure*, 25(10), 1519-1529.

Tiller, K.E. and Tessier, P.M., 2015. Advances in antibody design. *Annual Review of Biomedical Engineering*, 17(1), 191-216.

Tiwari, P., Jain, P.K. and Mishra, D. 2022. Characterization and optimization of Recombinant Gm-Csf Protein: Expression and Functional Analysis. *Journal of Advance Zoology*, 43(1), 866-876.

Trelka, D.P., Schneider, T.G., Reeder, J.C. and Taraschi, T.F., 2000. Evidence for vesicle-mediated trafficking of parasite proteins to the host cell cytosol and erythrocyte surface membrane in *Plasmodium falciparum* infected erythrocytes. *Molecular and Biochemical Parasitology*, 106(1), 131-145.

Trott, O. and Olson, A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, 31(2), 455-461.

Tsaneva, I. R., Berndt, M. and Stephen, C. W., 1993. RuvA and RuvB proteins of *Escherichia coli* exhibit DNA helicase activity in vitro. *Proceeding of National Academic Science. USA*, 90, 1315-1319.

Tuikue Ndam, N., Moussiliou, A., Lavstsen, T., Kamaliddin, C., Jensen, A.T., Mama, A., Tahar, R., Wang, C.W., Jespersen, J.S., Alao, J.M. and Gamain, B., 2017. Parasites causing cerebral falciparum malaria bind multiple endothelial receptors and express EPCR and ICAM-1-binding PfEMP1. *The Journal of Infectious Diseases*, 215(12), 1918-1925.

- Tuteja, R., 2007. Unraveling the components of protein translocation pathway in human malaria parasite *Plasmodium falciparum*. *Archives of Biochemistry and Biophysics*, 467(2), 249-260.
- Vaughan, C.K., Neckers, L. and Piper, P.W., 2010. Understanding of the Hsp90 molecular chaperone reaches new heights. *Nature Structural & Molecular Biology*, 17(12), 1400-1404.
- Vembar, S.S., Scherf, A. and Siegel, T.N., 2014. Noncoding RNAs as emerging regulators of *Plasmodium falciparum* virulence gene expression. *Current Opinion in Microbiology*, 20, 153-161.
- Venteicher, A.S., Meng, Z., Mason, P.J., Veenstra, T.D. and Artandi, S.E., 2008. Identification of ATPases pontin and reptin as telomerase components essential for holoenzyme assembly. *Cell*, 132(6), 945-957.
- von Morgen, P., Hořejší, Z. and Macurek, L., 2015. Substrate recognition and function of the R2TP complex in response to cellular stress. *Frontiers in Genetics*, 6, 69.
- Vythilingam, I., Chua, T.H., Liew, J.W.K., Manin, B.O. and Ferguson, H.M., 2021. The vectors of *Plasmodium knowlesi* and other simian malaria in Southeast Asia: challenges in malaria elimination. *Advances in Parasitology*, 113, 131-189.
- Walker, J.E., Saraste, M., Runswick, M.J., Gay, N.J., (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases, and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO Journal*, 1(8), 945-51.
- Wang, J., Song, J.J., Seong, I.S., Franklin, M.C., Kamtekar, S., Eom, S.H., Chung, C.H., 2001. Nucleotide-dependent conformational changes in a protease-associated ATPase HsIU. *Structure*, 9, 1107-1116.
- Wang, Q., Song, C., Li, C.C., 2003. Hexamerization of p97-VCP is promoted by ATP binding to the D1 domain and is required for ATPase and biological activities. *Biochemistry and Biophysics Research Communications*. 300, 253-260.
- Wang, Z., Yang, C., Wang, X., Liao, H., Liu, X., Liu, H., Zhang, M., Zhang, L. and Wang, H., 2024. Knockdown of RUVBL2 improves hnRNPA2/B1-stress granules dynamics to inhibit perioperative neurocognitive disorders in aged mild cognitive impairment rats. *Aging Cell*, 14418.
- Wei, H., Zhang, Y., Jia, Y., Chen, X., Niu, T., Chatterjee, A., He, P. and Hou, G., 2024. Heat shock protein 90: biological functions, diseases, and therapeutic targets. *Medicine Communication*, 5(2), e470.
- Weidenauer, L., Wang, T., Joshi, S., Chiosis, G., Quadroni, M.R., 2017. Proteomic interrogation of HSP90 and insights for medical research. *Expert Review of Proteomics*, 4(12), 1105-1117.
- Weikl, T.R. and Paul, F., 2014. Conformational selection in protein binding and function. *Protein Science*, 23(11), 1508-1518.
- Weiss, G.E., Gilson, P.R., Taechalertpaisarn, T., Tham, W.H., de Jong, N.W., Harvey, K.L., Fowkes, F.J., Barlow, P.N., Rayner, J.C., Wright, G.J. and Cowman, A.F., 2015. Revealing the sequence and resulting cellular morphology of receptor-ligand interactions during *Plasmodium falciparum* invasion of erythrocytes. *PLoS Pathogens*, 11(2), e1004670.

- Whitesell, L. and Lindquist, S.L., 2005. HSP90 and the chaperoning of cancer. *Nature Reviews Cancer*, 5(10), 761-772.
- Wilhelm, S., Carter, C., Lynch, M., Lowinger, T., Dumas, J., Smith, R.A., Schwartz, B., Simantov, R. and Kelley, S., 2006. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nature Reviews Drug Discovery*, 5(10), 835-844.
- Wohnik, G.M., Young, J.C., Schmidt, U., Holsboer, F., Hartl, F.U. and Rein, T., 2004. Inhibition of GR-mediated transcription by p23 requires interaction with Hsp90. *FEBS Letters*, 560(1-3), 35-38.
- World Health Organization, 2023. WHO *guidelines for malaria*, WHO/UCN/GMP/2023.01). World Health Organization.
- Wright, G.J. and Rayner, J.C., 2014. *Plasmodium falciparum* erythrocyte invasion: combining function with immune evasion. *PLoS Pathogens*, 10(3), e1003943.
- Yamashita, A., Ohnishi, T., Kashima, I., Taya, Y. and Ohno, S., 2001. Human SMG-1, a novel phosphatidylinositol 3-kinase-related protein kinase, associates with components of the mRNA surveillance complex and is involved in the regulation of nonsense-mediated mRNA decay. *Genes and Development*, 15(17), 2215-2228.
- Yang, Q., Inoki, K., Ikenoue, T. and Guan, K.L., 2006. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes and Development*, 20(20), 2820-2832.
- Yang, S., Xiao, H. and Cao, L., 2021. Recent advances in heat shock proteins in cancer diagnosis, prognosis, metabolism, and treatment. *Biomedicine & Pharmacotherapy*, 142, 112074.
- Yang, Y., Zhang, G., Su, M., Shi, Q. and Chen, Q., 2024. Prefoldin Subunits and Its Associate Partners: Conservations and Specificities in Plants. *Plants*, 13(4), 556.
- Yenerall, P., Das, A.K., Wang, S., Kollipara, R.K., Li, L.S., Villalobos, P., Flaming, J., Lin, Y.F., Huffman, K., Timmons, B.C. and Gilbreath, C., 2020. RUVBL1/RUVBL2 ATPase activity drives PAQosome maturation, DNA replication and radioresistance in lung cancer. *Cell Chemical Biology*, 27(1), 105-121.
- Yu, G., Zhao, Y., Tian, S., Rai, J., He, H., Spear, J., Sousa, D., Fan, J., Yu, H.G., Stagg, S.M. and Li, H., 2019. Yeast R2TP interacts with extended termini of client protein Nop58p. *Scientific Reports*, 9(1), 20228.
- Zaarur, N., Xu, X., Lestienne, P., Meriin, A.B., McComb, M., Costello, C.E., Newnam, G.P., Ganti, R., Romanova, N.V., Shanmugasundaram, M. and Silva, S.T., 2015. RuvbL1 and RuvbL2 enhance aggresome formation and disaggregate amyloid fibrils. *The EMBO Journal*, 34(18), 2363-2382.
- Zekar, L. and Sharman, T., 2023. *Plasmodium falciparum* malaria, StatPearlsZeymer, C., Fischer, S., and Reinstein, J., 2014. Transacting arginine residues in the AAA+ chaperone, ClpB allosterically regulates the activity through inter- and intradomain communication. *Journal of Biology and Chemistry*, 289, 32965–32976.

- Zhang, X. and Oglesbee, M., 2003. Use of surface plasmon resonance for the measurement of low affinity binding interactions between HSP72 and measles virus nucleocapsid protein. *Biological Procedures Online*, 5, 170-181.
- Zhao, J., Yuan, Q., Wang, H., Liu, W., Liao, X., Su, Y., Wang, X., Yuan, J., Li, T., Li, J. and Qian, S., 2020. Antibody responses to SARS-CoV-2 in patients with novel coronavirus disease 2019. *Clinical infectious diseases*, 71(16), 2027-2034.
- Zhao, R., Davey, M., Hsu, Y.C., Kaplanek, P., Tong, A., Parsons, A.B., Krogan, N., Cagney, G., Mai, D., Greenblatt, J. and Boone, C., 2005. Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. *Cell*, 120(5), 715-727.
- Zhao, R., Kakihara, Y., Gribun, A., Huen, J., Yang, G., Khanna, M., Costanzo, M., Brost, R.L., Boone, C., Hughes, T.R. and Yip, C.M., 2008. Molecular chaperone Hsp90 stabilizes Pih1/Nop17 to maintain R2TP complex activity that regulates snoRNA accumulation. *The Journal of Cell Biology*, 180(3), 563-578.
- Zheng, Y.Z. and DeMarco, M.L., 2017. Manipulating trypsin digestion conditions to accelerate proteolysis and simplify digestion workflows in development of protein mass spectrometric assays for the clinical laboratory. *Clinical Mass Spectrometry*, 6, 1-12.
- Zhu, Y., Li, T., Ramos da Silva, S., Lee, J.J., Lu, C., Eoh, H., Jung, J.U. and Gao, S.J., 2017. A critical role of glutamine and asparagine γ -nitrogen in nucleotide biosynthesis in cancer cells hijacked by an oncogenic virus. *Medical Biology*, 8(4), 10-1128.
- Zininga, T. and Shonhai, A., 2019. Small molecule inhibitors targeting the heat shock protein system of human obligate protozoan parasites. *International Journal of Molecular Sciences*, 20(23), 5930.
- Zininga, T., Achilonu, I., Hoppe, H., Prinsloo, E., Dirr, H. W., Shonhai, A. (2015b). Overexpression, Purification and Characterisation of the *Plasmodium falciparum* Hsp70-z (PfHsp70-z) Protein. *PloS One*, 10, e0129445.
- Zininga, T., Achilonu, I., Hoppe, H., Prinsloo, E., Dirr, H.W. and Shonhai, A., 2016. *Plasmodium falciparum* Hsp70-z, an Hsp110 homolog, exhibits independent chaperone activity and interacts with Hsp70-1 in a nucleotide-dependent fashion. *Cell Stress and Chaperones*, 21(3), 499-513.
- Zininga, T., Makumire, S., Gitau, G.W., Njunge, J.M., Pooe, O.J., Klimek, H., Scheurr, R., Raifer, H., Prinsloo, E., Przyborski, J.M. and Hoppe, H., 2015. *Plasmodium falciparum* Hop (PfHop) interacts with the Hsp70 chaperone in a nucleotide-dependent fashion and exhibits ligand selectivity. *PLoS One*, 10(8), 0135326.
- Zuehlke, A. and Johnson, J.L., 2010. Hsp90 and co-chaperones twist the functions of diverse client proteins. *Biopolymers: Original Research on Biomolecules*, 93(3), 211-217

Appendix

Appendix A: General experimental procedures

A1 Plasmid DNA extraction

Plasmid DNA was extracted using a Thermo Scientific™ Plasmid miniprep Kit according to the manufacturer's protocol.

A2 Restriction digest analysis

Plasmid DNA was digested using *Bam*HI and *Hind*III restriction enzymes following the method described below. The reagents were set up as follows: sterile deionized water (16 µl), 10x restriction buffer (2 µl), and DNA (100-200 ng) 2 µl. The reaction was initiated by adding two units (2 µl) of restriction enzymes. The reaction was allowed to proceed for 1-2 hours at 37 °C. The reaction was terminated by adding 4 µl of 10X DNA loading buffer (0.25% bromophenol blue and 30 % glycerol). The product was then analysed using agarose gel electrophoresis as described in (Appendix A3).

A3 Agarose gel electrophoresis

0.8 % (w/v) agarose gel was prepared and dissolved in 1X TAE buffer (40 Mm Tris, 20 mM acetic acid, and 1 mM EDTA) by heating with frequent agitation. The agarose gel was cooled to 55 °C before addition of ethidium bromide (0.5 µg/ml). The agarose gel was allowed to polymerise for 15 minutes at room temperature. The agarose gel was placed in the electrophoresis chamber and covered with 1X TAE buffer. A volume of 4 µl of 10X DNA loading buffer (0.25% bromophenol blue and 30 % glycerol) was mixed with a 20 µl sample followed by loading into the agarose gel wells. Electrophoresis was conducted at 100 volts for one hour. The gel was then visualized using UV lights (BioRad ChemDoc Bioimaging System, USA).

A4 DNA Sequencing

pQE30/PfRUVBL1, pQE30/PfRUVBL2, and pQE30/PfRUVBL3 plasmids construct were verified by DNA sequencing (Inqaba, RSA) using the forward primer (5'- CCCGAAAAGTGCCTG-3') with a *Bam*HI restriction site and reverse primer (5'- GTTCTGAGGTCATTACTGG-3') with a *Hind*III restriction site.

Reagent	Volume (µl)
Big dye 3.1	1

Appendix

Sequencing buffer	1
Primer (3.2 pmol)	1
Plasmid (100ng)	X
Water (ultrapure)	$10 - (3+x)$

A5 Transformation

A volume of 2 μ l (equivalent to about 10 ng) of plasmid DNA was added into an aliquot of 100 μ l of competent cells. The cells were then incubated on ice for 30 minutes, followed by heat shock at 42 °C for 45 seconds, and immediately placed on ice for 10 minutes. A volume of 900 μ l of 2YT broth was added and then incubated at 37 °C for one hour with gentle agitation. The cells were transferred on 2YT plates containing the desired antibiotics, followed by incubation at 37 °C overnight.

A6 SDS PAGE analysis

Proteins were treated by boiling in SDS sample buffer (0.25% Coomassie Brilliant blue (R250), 2% SDS, 10 % glycerol (v/v), 100 mM Tris, and 1 % β -mercaptoethanol) in a ratio of 4:1 for 5 mins at 95 °C and resolved using 12 % acrylamide resolving gel prepared as shown below (Table A.1). The gel is then transferred into the electrophoresis tank and electrophoresis buffer (25 mM Tris, pH 8.3 250 mM glycine and 0.1% (w/v) SDS) was added. The boiled samples were loaded in the respective wells, and pre-stained protein molecular weight markers (ThermoFisher Scientific, USA) were also loaded. The electrophoresis was performed at 150 volts for one hour using the Bio-Rad Mini protein electrophoresis system (Biorad, U.S.A).

Table A1 Preparation of SDS-PAGE

	5 % Stacking Gel	12 % Separating Gel
30 % Bis/acrylamide	0.235	2.08
1.5 M Tris (pH 8.8)	-	1.25
1.0 M Tris (pH 6.8)	0.437	-
10 % SDS	0.0175	0.05

Appendix

10 % Ammonium persulphate	0.00875	0.025
Distilled water	1.05	1.58
TEMED	0.03	0.0020

A7 Western blot analysis

Proteins were resolved in 12% acrylamide gel as described above (Appendix A.6). Removal of SDS-PAGE gel from the glass plates after completion of the electrophoresis process and cutting off of the stacking gel was done. The Whatman filter papers, gel, two scotchbrite fibre pads, and nitrocellulose were immersed in the buffer and left to equilibrate at 8 °C for 30 minutes. Preparation of the gel for transfer was done as follows: filter paper was placed on a scotchbrite fibre pad; Gel was placed on the filter paper, ensuring no air bubbles were trapped; nitrocellulose was placed over the gel; another filter paper was laid on top of the nitrocellulose, followed by another scotchbrite pad. The transfer of the protein on the nitrocellulose membrane was performed by running at 100 volts for one hour. The membrane was removed from the sandwich and rinsed using a transfer buffer, as well as removal of adhering gel on the nitrocellulose membrane using a cotton swab. The blot was stained with Ponceau stain to determine the success of the transfer, followed by visualizing the band using chemiluminescence. The membrane was blocked in 10 ml of (5 % non-fat milk in TBS) for one hour on a rotary shaker set at 1 rpm. The membrane was washed three times in TBS-Tween for 10 minutes, followed by incubation of the membrane with primary antibody for one hour. Unbound primary antibody was removed by washing the membrane three times using TBS-Tween for 10 minutes each wash. The membrane was incubated with a secondary antibody for one hour, followed by washing of the membrane three times using TBS-Tween.

A8 Chemiluminescent detection

The Thermo Scientific Pierce ECL Western Blotting Substrate is a highly sensitive, nonradioactive, enhanced luminol-based chemiluminescent substrate for the detection of horseradish peroxidase (HRP) on immunoblots.

1. Remove blot from the transfer apparatus and block nonspecific sites with Blocking Reagent for 60 minutes at room temperature (RT) with shaking. If desired, block overnight at 2-8 °C without shaking.
2. Remove the Blocking Reagent and add the primary antibody working dilution. Incubate blot for 1 hour at RT with shaking or overnight at 2-8 °C without shaking.
3. Briefly rinse the membrane in Wash Buffer two times.

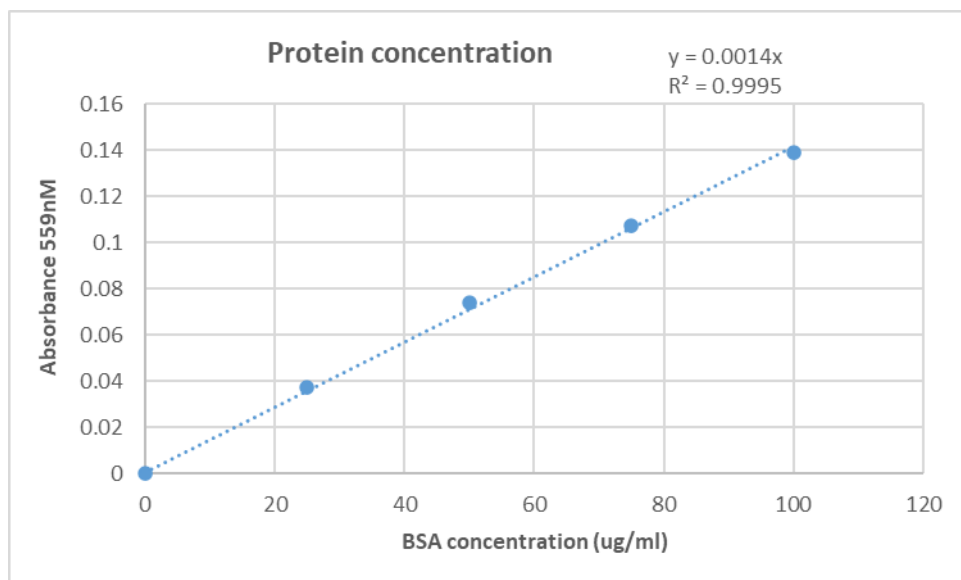
Appendix

4. Wash the membrane by suspending it in a Wash Buffer and agitating for ≥ 5 minutes. Replace the Wash Buffer at least 4-6 times. Increasing the Wash Buffer volume, the number of washes, and the wash duration may help minimize the background signal.
5. Incubate blot with the HRP-conjugate working dilution for one hour at RT with shaking.
6. Repeat Steps 3 and 4 to remove non-bound HRP-conjugate. Note: The membrane MUST be thoroughly washed after incubation with the HRP-conjugate.
7. Prepare the substrate working solution by mixing equal parts of Detection Reagents 1 and 2. Use 0.125 mL Working Solution per cm² of the membrane. Note: For best results, prepare a working solution immediately before use. The working solution is stable for one hour at RT.
8. Incubate the blot with a working solution for one minute at RT.
9. Remove the blot from the working solution and place it in a plastic sheet protector or clear plastic wrap. Use an absorbent tissue to remove excess liquid and carefully press out any bubbles from between the blot and the membrane protector.

A9 Determination of protein concentration using Bradford assay

Protein concentration was determined by Bradford's method (Bradford, 1976). Bovine serum albumin (BSA) standards were prepared using concentrations ranging from 0 to 1 mg/ml in 0.15 M NaCl. Bradford's reagent 200 μ l (Sigma Aldrich, USA) was added to 10 μ l of protein, and the reaction was incubated in the dark at room temperature for five minutes. Absorbance was read at 595 nm using a SpectraMax M3 (Molecular Devices, USA). The recombinant protein was similarly treated, and the protein concentration was determined by extrapolation from the standard curve as indicated in (Appendix B3; Figure B1). The readings were prepared in triplicate, and the average was obtained.

Appendix



A9: Bradford BSA standard curve

A10: Thermal shift assay

Preparation of thermal shift reaction

Prepare a fresh dilution of Protein Thermal shift Dye (1000X) to 8X

Place the reaction plate on ice, then add reaction components to the plate

Prepare the reaction on ice

Components	Volume (μL)
Protein thermal shift buffer	5.0
Protein (0.1 to 1 mg/ml) + ligand (0.5 mM)	12.5
Diluted thermal shift Dye (8X)	2.5
Total volume for each reaction	20

Prepare the control reaction on ice.

Components	Volume (μL)
Protein thermal shift buffer	5.0
Water + 2.0 μl Protein thermal shift control + protein thermal shift control ligand (0.1 mM or 1mM final concentration)	12.5
Diluted thermal shift Dye (8X)	2.5
Total volume for each reaction	20

Appendix

Pipet up and down 10 times to mix well.

Seal the plate with MicroAmp optical adhesive film, spin it at 1000 rpm for 1 minute, then place it on ice.

Using the real-time PCR instrument software, open and set up the experiment run file.

Setup	Setting
Experiment properties	- Experiment type: melt curve - Reagents: Other - Ramp speed: fast or standard
Target properties	- Reporter: ROX - Quencher: None
Plate layout	- Assign targets to all wells in use - Passive reference: None
Run method	- Reaction volume per well: 20 μl - Ramp mode: continuous - Thermal profile: Step 1, Temp: 25°C, Time: 2 minutes Step 2, Temp: 99°C, Time: 2 minutes - Ramp rate: ViiA TM 7 Real-time PCR system: Step 1: 1.6°C/s, Step 2: 0.05°C/s Step 1, step1+, 7500 and 7500 fast - Optical filters: Excitation filter: x4(580$\pm$10) Emission filter: m4(623$\pm$14)

Appendix

A11: Cleaving His-Tag with TEV protease

The standard reaction buffer for TEV protease (Sigma-Aldrich, USA) is 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 1 mM DTT, but the enzyme has a relatively flat activity profile at pH values between 4 and 9 and will tolerate a range of buffers, including phosphate, MES, and acetate. TEV protease activity is not adversely affected by the addition of glycerol or sorbitol up to at least 40% (w/v). TEV protease activity is not inhibited by PMSF and AEBSF (1 mM), TLCK (1 mM), Bestatin (1 mg/mL), pepstatin A (1 mM), EDTA (1 mM), E-64 (3 mg/mL), or “complete” protease inhibitor cocktail (Thermo Fischer). 100 µg of partially pure fusion proteins with a canonical TEV protease recognition site (ENLYFQG) in the linker region (Tropea *et al.*, 2007) is incubated overnight at 4–8 °C in 50 µl of standard reaction buffer (Appendix F2) in the presence of 5.0 µg His7-TEV protease. The reaction products are separated by dialysis using 30K Amicon[®] centrifugal filter units followed by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue.

Phyre2

Email	dunsifiso@yahoo.com
Description	PFRUVBL1_
Date	Thu Aug 26 10:15:23 BST 2021
Unique Job ID	c12e7d6920f4111a

Secondary structure and disorder prediction

Sequence: M N I I E S S R E K E R I S L H S H I S G L G L D A D G Y V T D F L F E E N I K K R D M Y S E P T N K I E N N D I K N G

Secondary structure: [Alpha helix] [Alpha helix] [Alpha helix] [Alpha helix]

SS confidence: [High] [High] [High] [High]

Disorder: [Disordered] [Disordered] [Disordered] [Disordered]

Disorder confidence: [High] [High] [High] [High]

Sequence: T I L D P D K V D N I C N S Y K L E N N G N Y E N N K I C Y N N I S D D E D E V V K N F Y K C K G M V G Q K K A R E A A

Secondary structure: [Alpha helix] [Alpha helix] [Alpha helix]

SS confidence: [High] [High] [High]

Disorder: [Disordered] [Disordered] [Disordered]

Disorder confidence: [High] [High] [High]

Sequence: G I F I N L I K E K N I C K C L L L A G P S G S G K T A I A I A S K E I S D E S I P F C I F N A S Q V Y S C E V K K T

Secondary structure: [Alpha helix] [Beta strand] [Alpha helix] [Beta strand] [Alpha helix]

SS confidence: [High] [High] [High] [High] [High]

Disorder: [Disordered] [Disordered] [Disordered]

Disorder confidence: [High] [High] [High]

Sequence: E I L T Q Y I R K S I G V K I K E I K E V F E G E V V Q I E P F F D N T Y D E Q K I S Y V H I T L K T L K E Q K K I K I

Secondary structure: [Alpha helix] [Beta strand] [Beta strand] [Beta strand] [Beta strand]

SS confidence: [High] [High] [High] [High] [High]

Disorder: [Disordered] [Disordered] [Disordered]

Disorder confidence: [High] [High] [High]

Sequence: H S S I Y D N L L K E K I Q E K D I I Y I E S H S G N V K R V G K C S L Y Q D M F D I E T D T F V D L P K G N V H K K K

Secondary structure: [Alpha helix] [Beta strand] [Beta strand] [Alpha helix] [Beta strand]

SS confidence: [High] [High] [High] [High] [High]

Disorder: [Disordered] [Disordered] [Disordered]

Disorder confidence: [High] [High] [High]

Sequence: N I I Q N I T L Y D L D V S N V Q P K D N I L D F L Q N N K S K K T E I T D K L R N E I N K I V Y K Y V D Q G I A Q I I

Secondary structure: [Beta strand] [Alpha helix] [Alpha helix] [Alpha helix]

SS confidence: [High] [High] [High] [High]

Disorder: [Disordered] [Disordered] [Disordered]

Disorder confidence: [High] [High] [High]

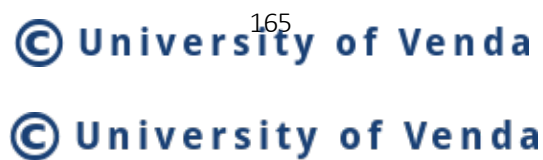
Sequence: P G V L F I D E V H M L D I E C F T Y L N R T L E S N L A P V V I L A T N R G I C N I K G T N I I S A H G I P V D L L D

Secondary structure: [Beta strand] [Alpha helix] [Alpha helix] [Beta strand] [Alpha helix]

SS confidence: [High] [High] [High] [High] [High]

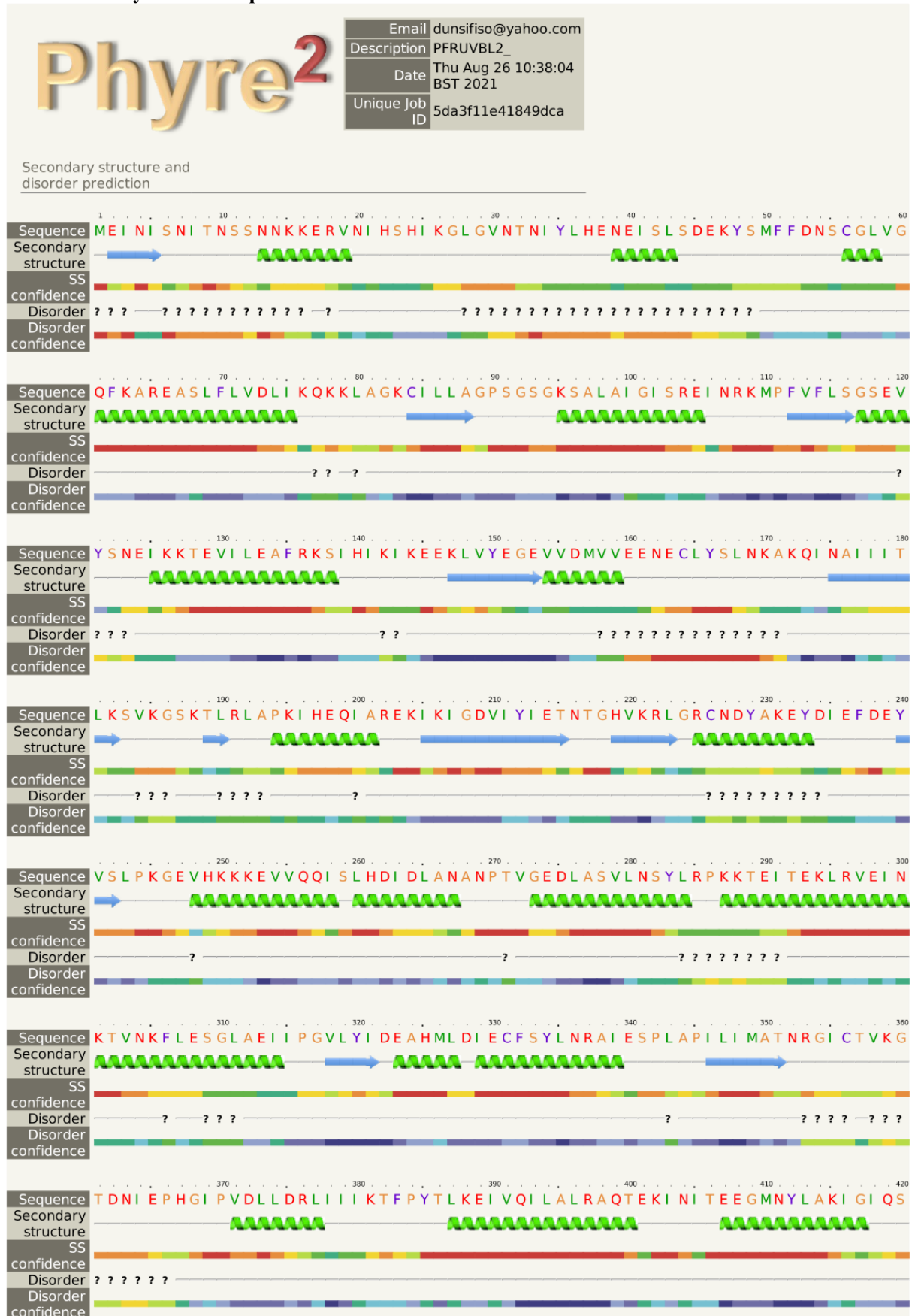
Disorder: [Disordered] [Disordered] [Disordered]

Disorder confidence: [High] [High] [High]

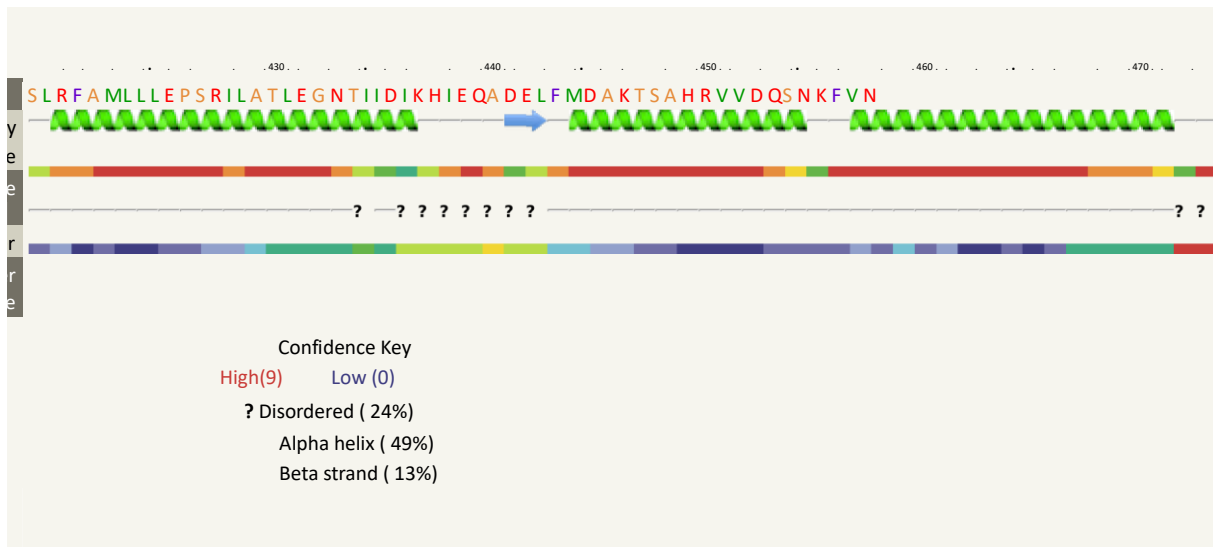


Appendix

B1: Secondary structural prediction of PFRUVBL2

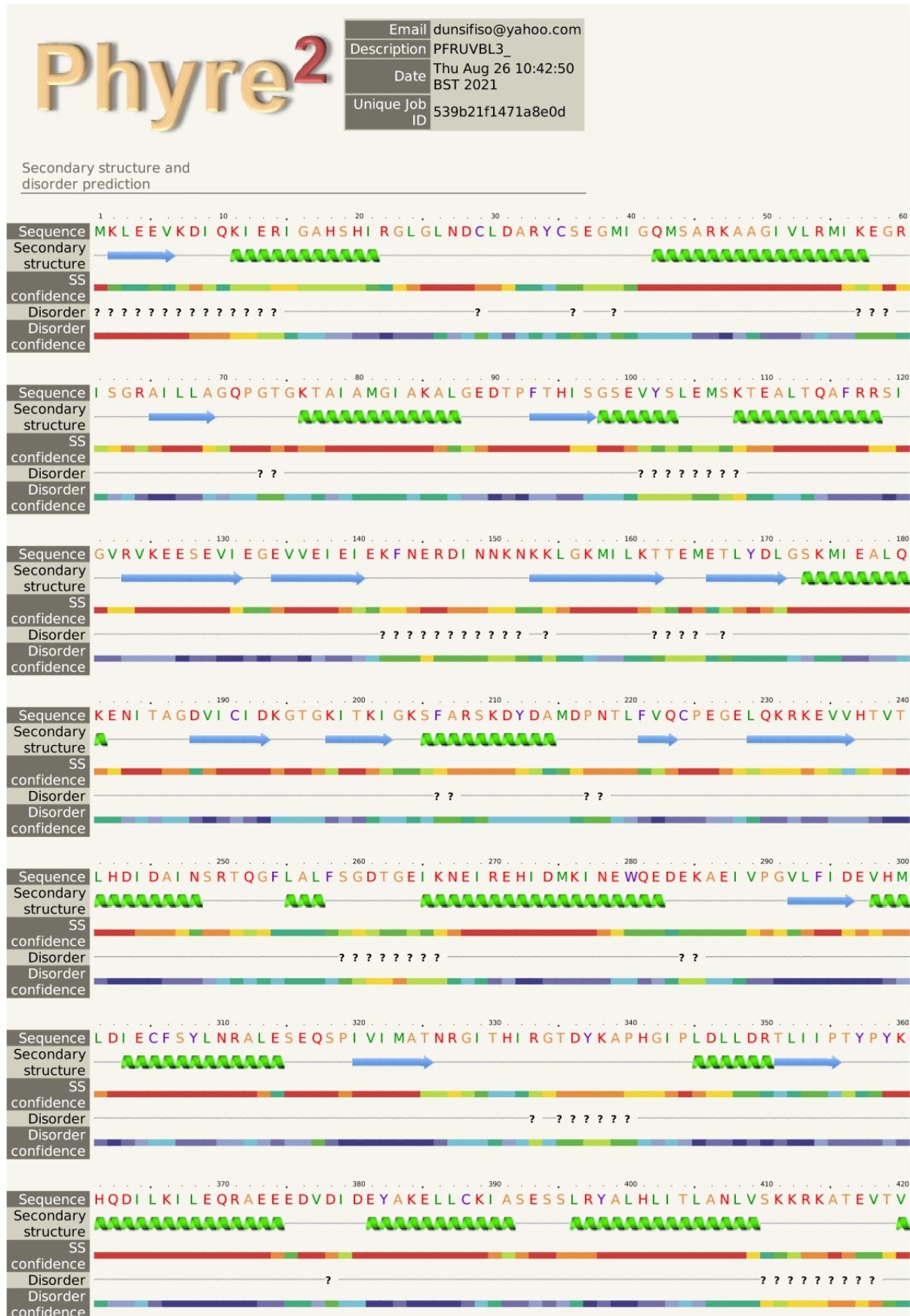


Appendix

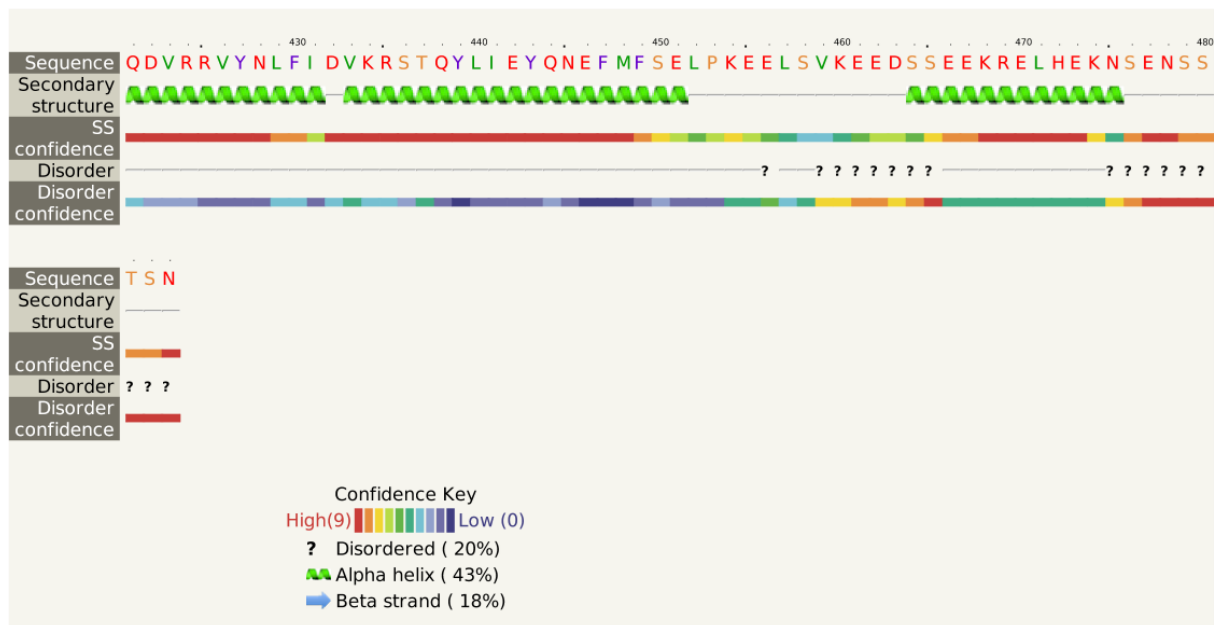


Appendix

B1: Secondary structural prediction of PFRUVBL3

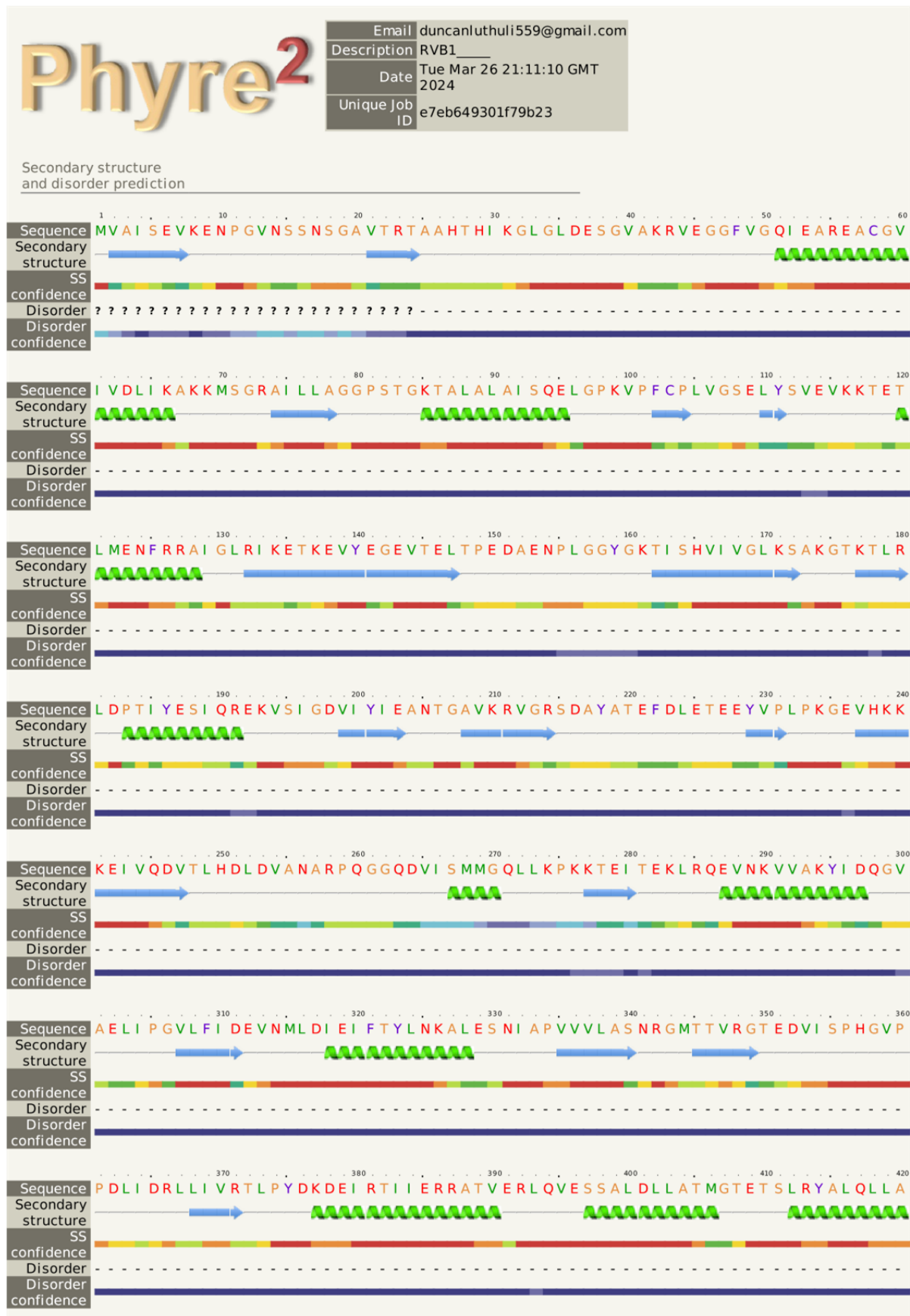


Appendix

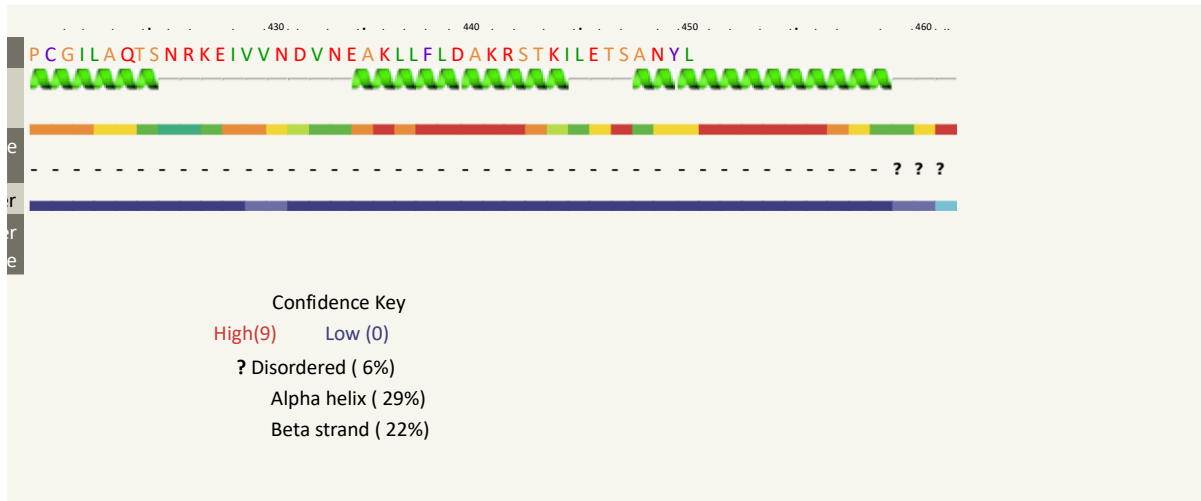


Appendix

B1: Secondary structural prediction of RVB1

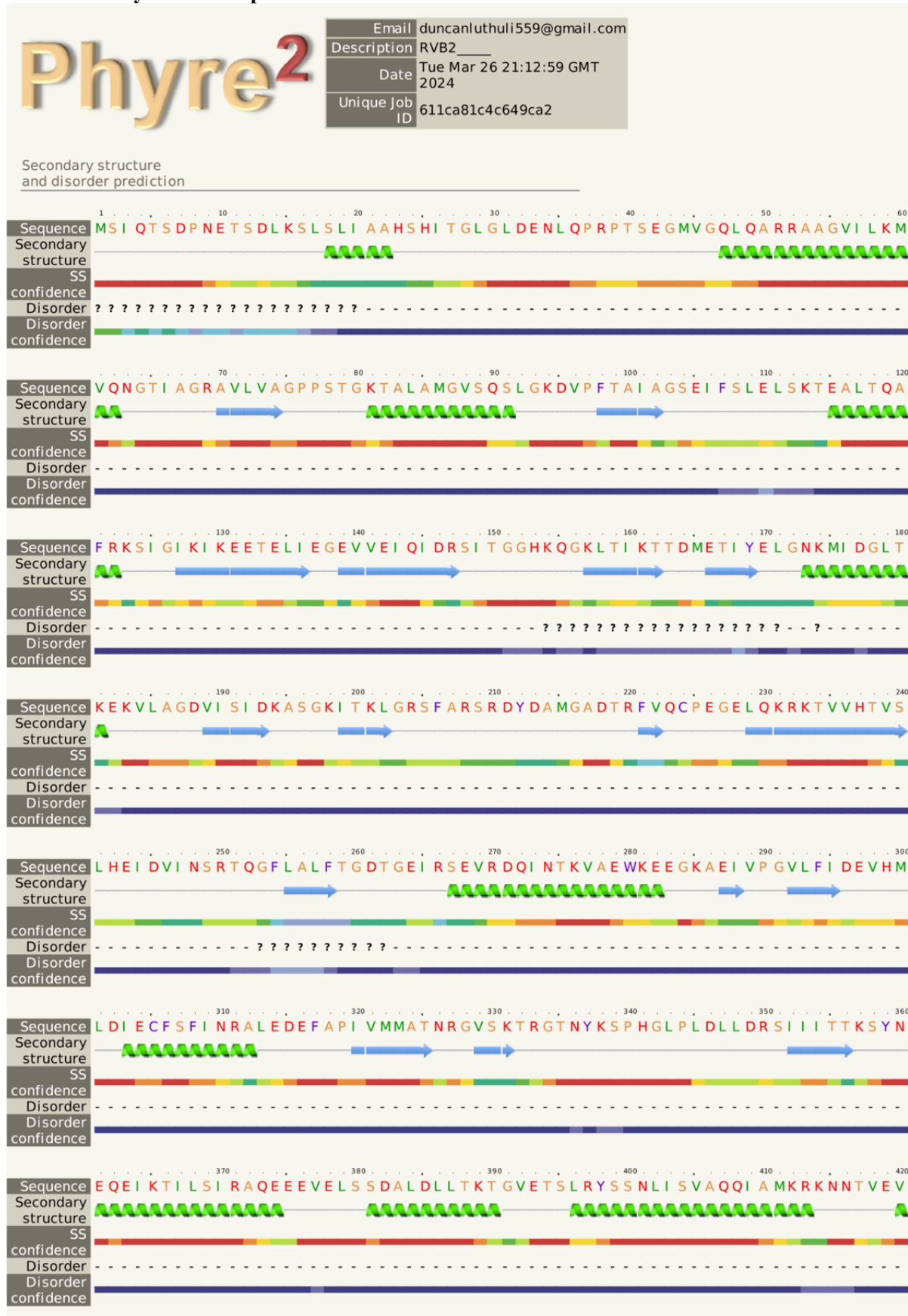


Appendix

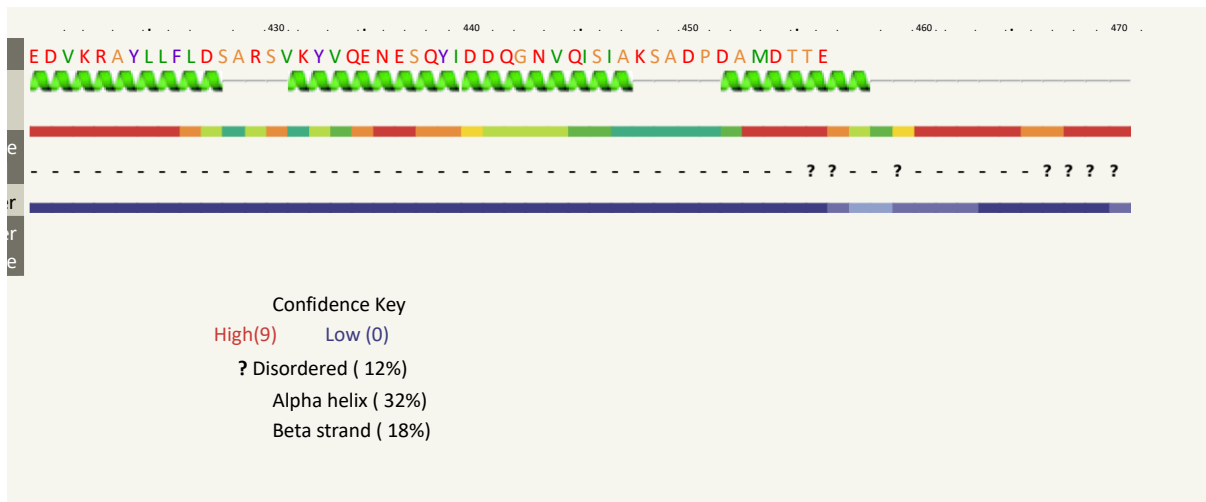


Appendix

B1: Secondary structural prediction of RVB2

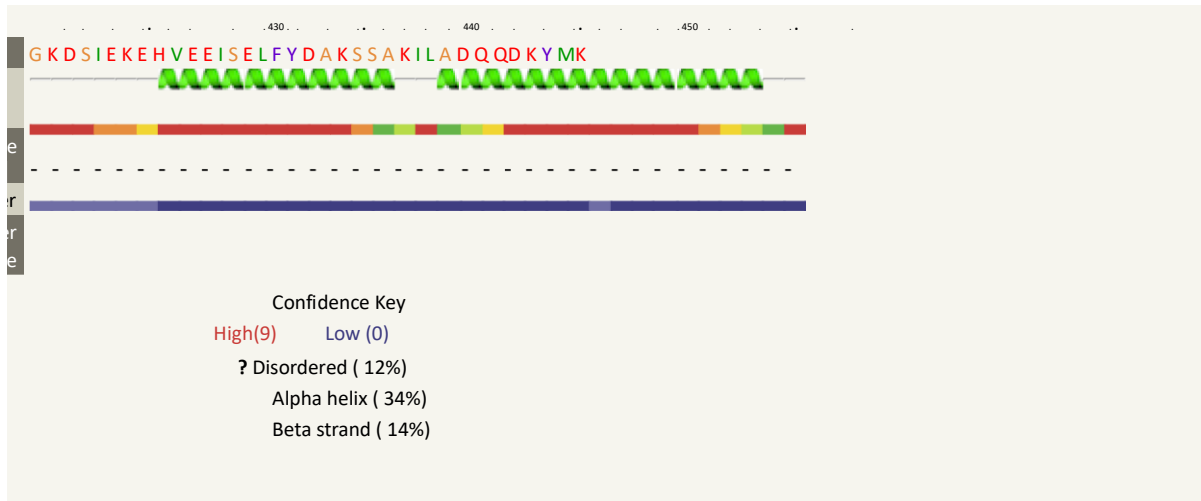


Appendix



[illegible]

Appendix



Email

duncanluthuli559@gmail.com

Description

HsRUVBL2_

Date

Tue Mar 26 21:22:24 GMT 2024

Unique Job ID

739f7da16e3bdf5f

Phyre2

Secondary structure and disorder prediction

Sequence

MATVTATTKVPEIRDVTRIERI GAHSHIRGLGLDDALEPRQASQGMVGQLAARRAAGVVV

Secondary structure

SS confidence

Disorder

???????????????????????????

Disorder confidence

Sequence

E M I R E G K I A G R A V L I A G Q P G T G K T A I A M G M A Q A L G P D T P F T A I A G S E I F S L E M S K T E A L T

Secondary structure

SS confidence

Disorder

Disorder confidence

Sequence

Q A F R R S I G V R I K E E T E I I E G E V V E I Q I D R P A T G T G S K V G K L T L K T T E M E T I Y D L G T K M I E

Secondary structure

SS confidence

Disorder

????????????? ? ?

Disorder confidence

Sequence

S L T K D K V Q A G D V I T I D K A T G K I S K L G R S F T R A R D Y D A M G S Q T K F V Q C P D G E L Q K R K E V V H

Secondary structure

SS confidence

Disorder

Disorder confidence

Sequence

T V S L H E I D V I N S R T Q G F L A L F S G D T G E I K S E V R E Q I N A K V A E W R E E G K A E I I P G V L F I D E

Secondary structure

SS confidence

Disorder

Disorder confidence

Sequence

V H M L D I E S F S F L N R A L E S D M A P V L I M A T N R G I T R I R G T S Y Q S P H G I P I D L L D R L L I V S T T

Secondary structure

SS confidence

Disorder

Disorder confidence

Sequence

P Y S E K D T K Q I L R I R C E E E D V E M S E D A Y T V L T R I G L E T S L R Y A I Q L I T A A S L V C R K R K G T E

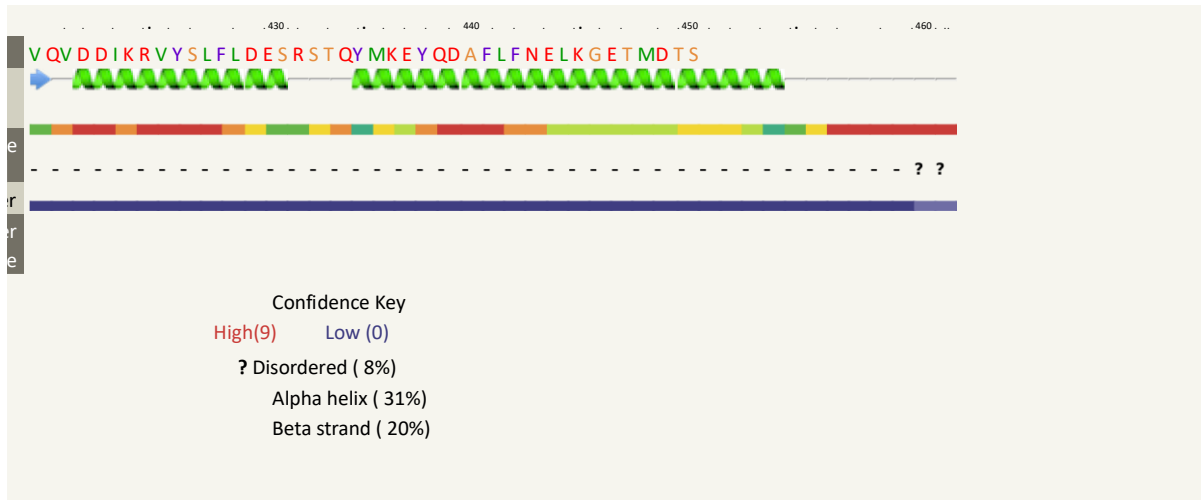
Secondary structure

SS confidence

Disorder

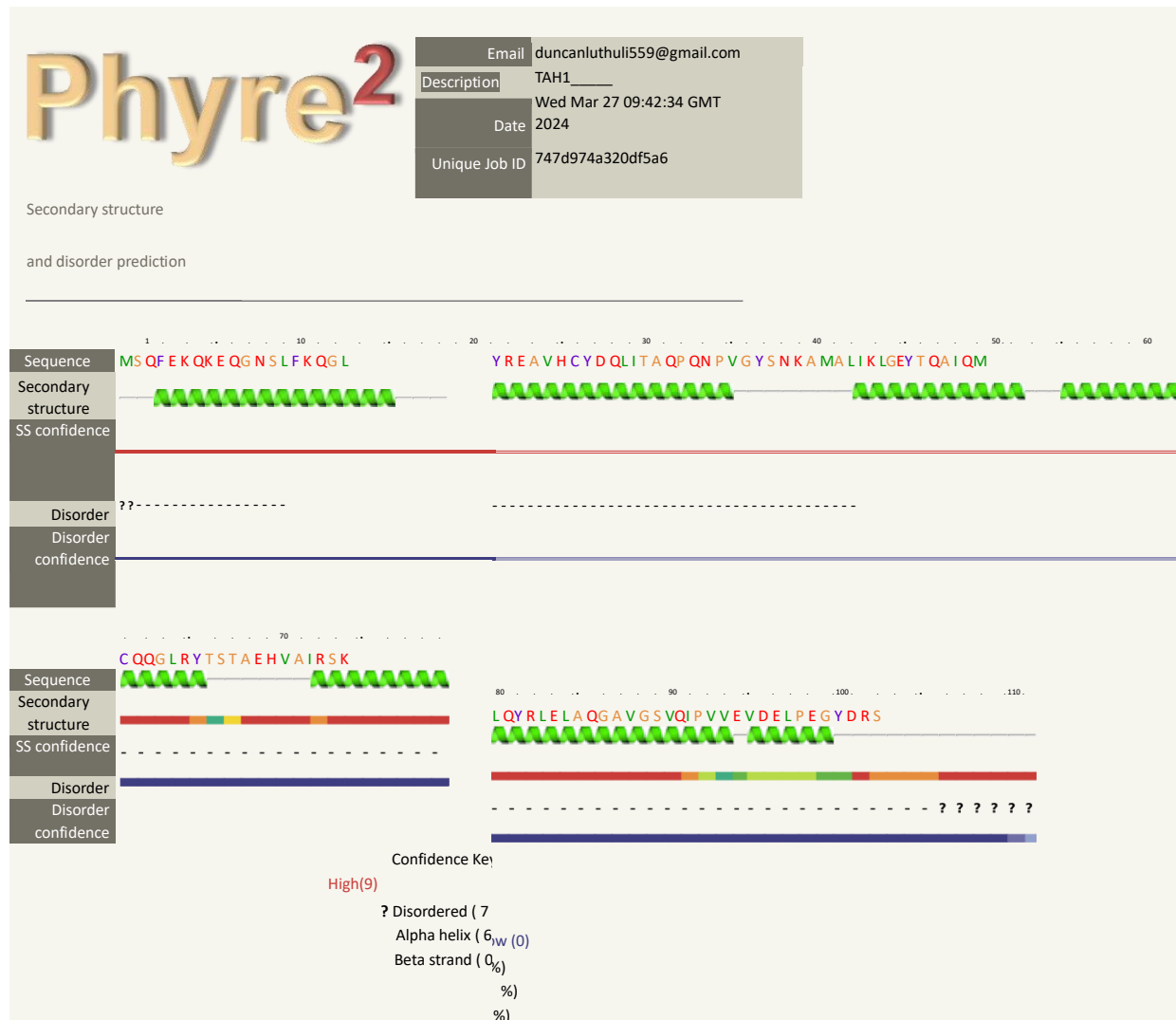
Disorder confidence

Appendix

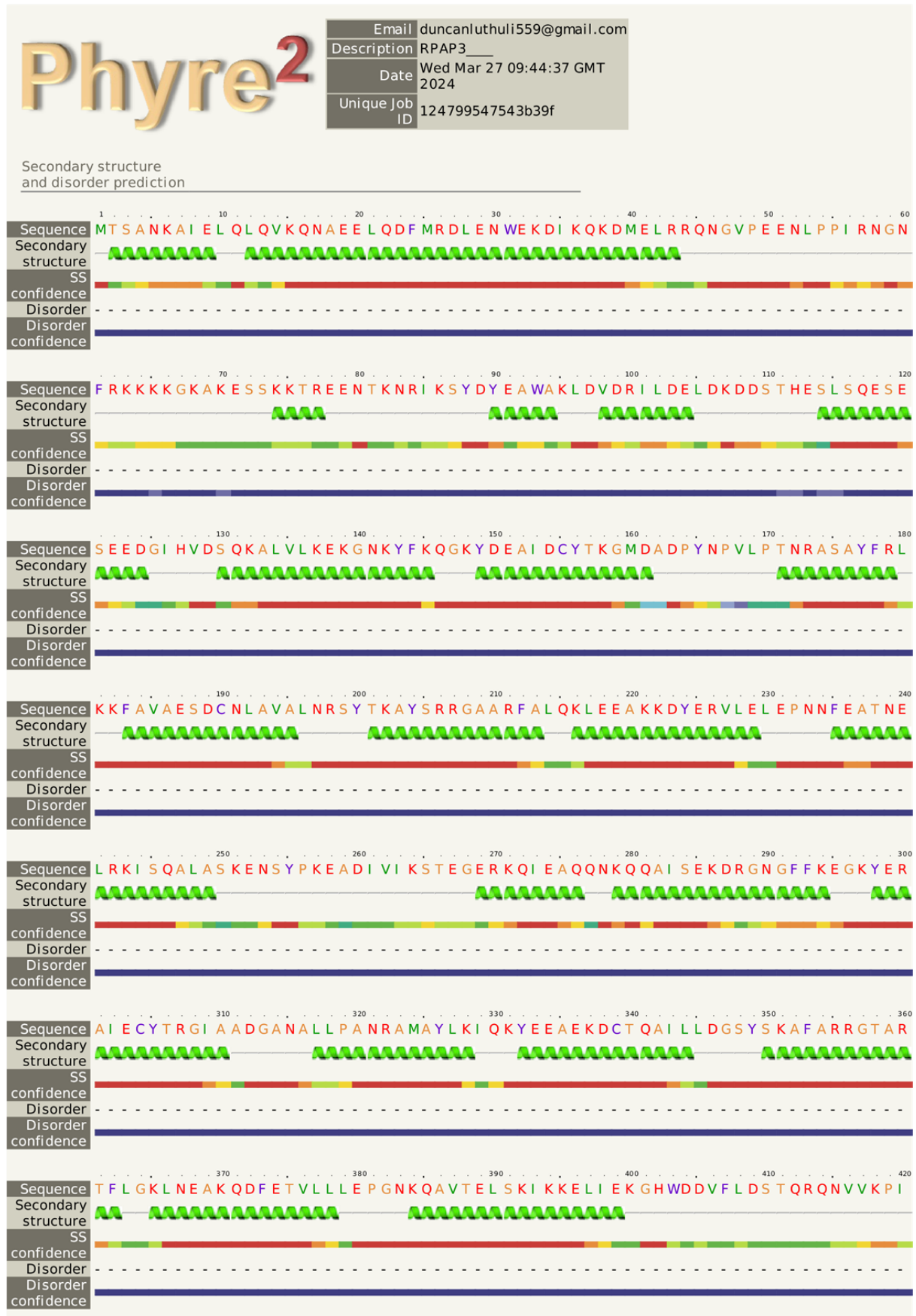


Appendix

B2: Secondary structural prediction of TAH1



B2: Secondary structural prediction of RPAP3



Appendix



B3: Protein interactome network

Human Hsp90 interactors

#node	identifier	node degree
AHSA1	9606.ENSP00000216479	33
AR	9606.ENSP00000363822	28
BAG1	9606.ENSP00000489189	29
BAG2	9606.ENSP00000359727	29
BAG3	9606.ENSP00000358081	32
BAG5	9606.ENSP00000338814	21
BCL2A1	9606.ENSP00000267953	9
BTC	9606.ENSP00000379092	10
CDC37	9606.ENSP00000222005	45
CHORDC1	9606.ENSP00000319255	12
CHUK	9606.ENSP00000359424	16
DNAJA1	9606.ENSP00000369127	35
DNAJA2	9606.ENSP00000314030	26
DNAJA3	9606.ENSP00000262375	19
DNAJB1	9606.ENSP00000254322	41
DNAJC7	9606.ENSP00000406463	26
EGFR	9606.ENSP00000275493	36
ERBB2	9606.ENSP00000269571	29
ERBIN	9606.ENSP00000426632	12
ESR2	9606.ENSP00000343925	18
FKBP4	9606.ENSP00000001008	38

Appendix

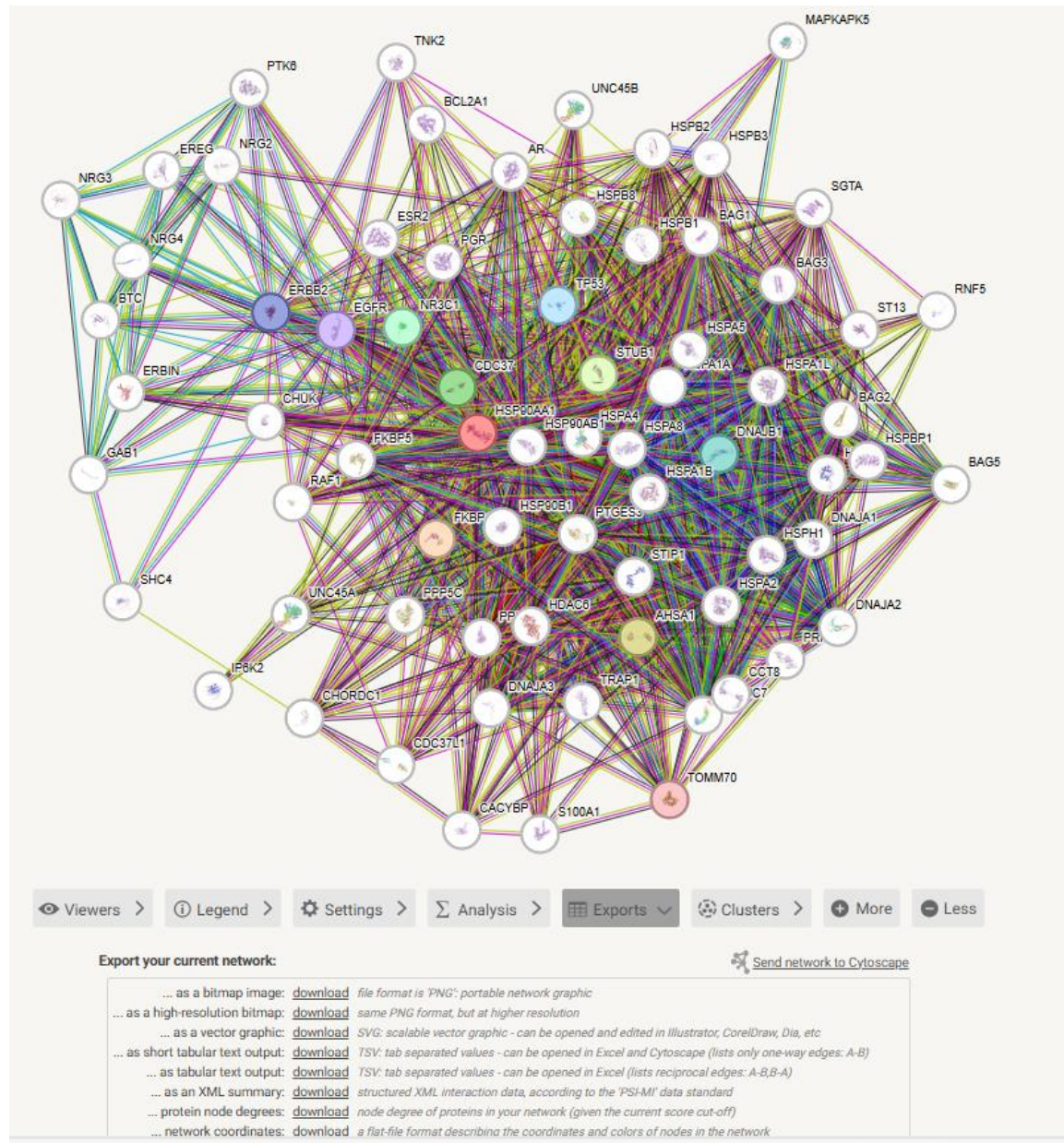
FKBP5	9606.ENSF00000444810	24
GAB1	9606.ENSF00000262995	10
HDAC6	9606.ENSF00000365804	20
HSF1	9606.ENSF00000431512	27
HSP90AA1	9606.ENSF00000335153	58
HSP90AB1	9606.ENSF00000360609	53
HSP90B1	9606.ENSF00000299767	38
HSPA1A	9606.ENSF00000364802	37
HSPA1B	9606.ENSF00000364801	42
HSPA1L	9606.ENSF00000364805	35
HSPA2	9606.ENSF00000378199	34
HSPA4	9606.ENSF00000302961	49
HSPA8	9606.ENSF00000437125	47
HSPB1	9606.ENSF00000248553	30
HSPB2	9606.ENSF00000302476	25
HSPB3	9606.ENSF00000303394	22
HSPB8	9606.ENSF00000281938	26
HSPBP1	9606.ENSF00000255631	34
IP6K2	9606.ENSF00000331103	5
NR3C1	9606.ENSF00000231509	25
NRG2	9606.ENSF00000354910	10
NRG3	9606.ENSF00000361214	9
NRG4	9606.ENSF00000378367	9
PGR	9606.ENSF00000325120	24

Appendix

PPID	9606.ENSPO00000303754	26
PPP5C	9606.ENSPO00000012443	21
PRKN	9606.ENSPO00000355865	19
PTGES3	9606.ENSPO00000482075	40
RAF1	9606.ENSPO00000401888	14
RNF5	9606.ENSPO00000364235	10
S100A1	9606.ENSPO00000292169	10
SHC4	9606.ENSPO00000329668	4
ST13	9606.ENSPO00000216218	24
STIP1	9606.ENSPO00000351646	36
STUB1	9606.ENSPO00000219548	46
TNK2	9606.ENSPO00000371341	8
TOMM70	9606.ENSPO00000284320	18
TP53	9606.ENSPO00000269305	39
UNC45A	9606.ENSPO00000407487	11
UNC45B	9606.ENSPO00000268876	11

Appendix

hHsp90 interactors



Yeast Hsp90 interactors

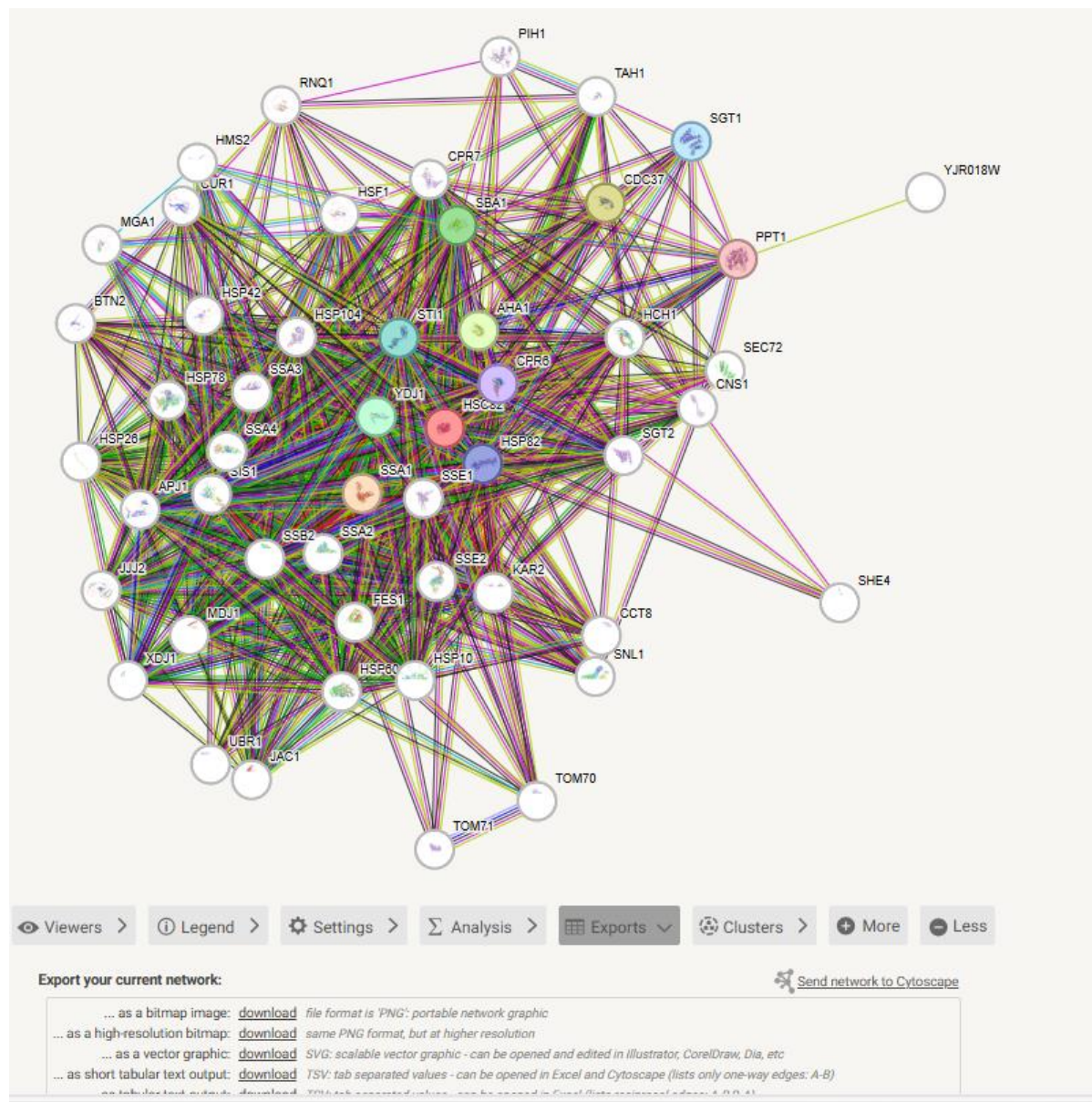
#node identifier	node degree
AHA1 4932.YDR214W	41
APJ1 4932.YNL077W	33
BTN2 4932.YGR142W	22
CCT8 4932.YJL008C	18
CDC37 4932.YDR168W	21
CNS1 4932.YBR155W	22
CPR6 4932.YLR216C	37
CPR7 4932.YJR032W	29
CUR1 4932.YPR158W	20
FES1 4932.YBR101C	32
HCH1 4932.YNL281W	28
HMS2 4932.YJR147W	11
HSC82 4932.YMR186W	48
HSF1 4932.YGL073W	28
HSP10 4932.YOR020C	29
HSP104 4932.YLL026W	37
HSP26 4932.YBR072W	28
HSP42 4932.YDR171W	32
HSP60 4932.YLR259C	30
HSP78 4932.YDR258C	33
HSP82 4932.YPL240C	48
JAC1 4932.YGL018C	20

Appendix

JJJ2	4932.YJL162C	26
KAR2	4932.YJL034W	36
MDJ1	4932.YFL016C	30
MGA1	4932.YGR249W	11
PIH1	4932.YHR034C	9
PPT1	4932.YGR123C	18
RNQ1	4932.YCL028W	15
SBA1	4932.YKL117W	37
SEC72	4932.YLR292C	12
SGT1	4932.YOR057W	11
SGT2	4932.YOR007C	22
SHE4	4932.YOR035C	4
SIS1	4932.YNL007C	40
SNL1	4932.YIL016W	16
SSA1	4932.YAL005C	45
SSA2	4932.YLL024C	40
SSA3	4932.YBL075C	36
SSA4	4932.YER103W	35
SSB2	4932.YNL209W	36
SSE1	4932.YPL106C	43
SSE2	4932.YBR169C	35
STI1	4932.YOR027W	44
TAH1	4932.YCR060W	16
TOM70	4932.YNL121C	12

Appendix

TOM71	4932.YHR117W	5
UBR1	4932.YGR184C	10
XDJ1	4932.YLR090W	25
YDJ1	4932.YNL064C	41
YJR018W	4932.YJR018W	1



Appendix

B4: Antibody production

Immunisation Schedule

Antigen detail: *Plasmodium falciparum* RUVBL 1 peptide, resuspended in 1 mL sterile MilliQ. Adsorption to acid-treated, naked *Salmonella minnesota* R595 performed on days as seen below. Dried protein-bacteria resuspended in 2.5 mL PBS and sonicated before immunisation.

Adsorption:

1. 28/06/2024 Sample from University of Venda
2. 11/07/2024 Sample from University of Venda

Rabbit: Female, Flemish Giant (Roxette)

Date	Day	Dosage (µg)	Blood serum volume (mL)	Comments
01.07.2024	0	± 240	±0.5	Baseline serum (day 0)
04.07.2024	3	± 240	-	
08.07.2024	7	±240	-	
Week off				
15.07.2024	14	±240	-	
18.07.2024	18	±240	-	
22.07.2024	21	±240	-	
Week off				
29.07.2024	28	±240	±0.4	Day 28 serum (check-up)
01.08.2024	31	±240	-	
05.08.2024	35	±240	-	
Week off				
12.08.2024	42	-	±10	Day 42 serum (final)

Appendix

Antigen preparation

Sample detail: *Plasmodium falciparum* RUVBL 1 peptide.

Concentration: 1 mg/mL

1st Adsorption date: 28 June 2024

Adsorption calculation

Need **1** part protein (antigen): **5** parts naked bacteria (acid-treated, naked bacteria previously prepared)

$$\begin{aligned}\text{Vol antigen needed (mL)} &= \frac{0.2 \text{ mg}}{[\text{protein}] \text{mg/mL}} \\ &= \frac{0.2}{1} \\ &= 0.2 \text{ mL} \\ &\sim 200 \text{ }\mu\text{L}\end{aligned}$$

1 mg/mL naked bacteria + 200 μ L RUVbL 1 = **$\pm$ 1.2 mg dry weight**

1.2 mg prepared in **2.5 mL PBS** (pH 7.2) = **0.48 mg/mL**

Therefore, in each **0.5 mL** injection = **$\pm$ 240 μ g** total protein-bacteria

Concentration: 1 mg/mL (same sample as used previously)

2nd Adsorption date: 11 July 2024

Adsorption calculation

Need **1** part protein (antigen): **5** parts naked bacteria (acid-treated, naked bacteria previously prepared)

$$\begin{aligned}\text{Vol antigen needed (mL)} &= \frac{0.2 \text{ mg}}{[\text{protein}] \text{mg/mL}} \\ &= \frac{0.2}{1} \\ &= 0.2 \text{ mL}\end{aligned}$$

Appendix

~ 200 μ L

1 mg/mL naked bacteria + 200 μ L RUVbL 1 = $\pm$ 1.2 mg dry weight

1.2 mg prepared in 2.5 mL PBS (pH 7.2) = 0.48 mg/mL

Therefore, in each 0.5 mL injection = $\pm$ 240 μ g total protein-bacteria

Immunisation schedule

Antigen detail: *Plasmodium falciparum* RUVBL 2 peptide, resuspended in 1 mL sterile MilliQ. Adsorption to acid-treated, naked *Salmonella minnesota* R595 performed on days as seen below. Dried protein-bacteria resuspended in 2.5 mL PBS and sonicated before immunisation.

Adsorption:

1. 28/06/2024 Sample from the University of Venda
2. 11/07/2024 Sample from the University of Venda

Rabbit: Female, Flemish Giant (Jenna)

Date	Day	Dosage (μ g)	Blood serum volume (mL)	Comments
01.07.2024	0	$\pm$ 240	$\pm$ 0.5	Baseline serum (day 0)
04.07.2024	3	$\pm$ 240	-	
08.07.2024	7	$\pm$ 240	-	
Week off				
15.07.2024	14	$\pm$ 240	-	
18.07.2024	18	$\pm$ 240	-	
22.07.2024	21	$\pm$ 240	-	
Week off				
29.07.2024	28	$\pm$ 240	$\pm$ 0.5	Day 28 serum (check-up)
01.08.2024	31	$\pm$ 240	-	

Appendix

05.08.2024	35	±240	-	
Week off				
12.08.2024	42	-	±11	Day 42 serum (final)

Antigen preparation

Sample detail: *Plasmodium falciparum* RUVBL 2 peptide.

Concentration: 1 mg/mL

1st Adsorption date: 28 June 2024

Adsorption calculation

Need **1** part protein (antigen): **5** parts naked bacteria (acid-treated, naked bacteria previously prepared)

$$\begin{aligned}
 \text{Vol antigen needed (mL)} &= \frac{0.2 \text{ mg}}{[\text{protein}] \text{mg/mL}} \\
 &= \frac{0.2}{1} \\
 &= 0.2 \text{ mL} \\
 &\sim 200 \text{ } \mu\text{L}
 \end{aligned}$$

1 mg/mL naked bacteria + 200 μ L RUVBL 2 = **±1.2 mg dry weight**

1.2 mg prepared in **2.5 mL PBS** (pH 7.2) = **0.48 mg/mL**

Therefore, in each **0.5 mL** injection = **±240 μ g** total protein-bacteria

Concentration: 1 mg/mL (same sample as used previously)

2nd Adsorption date: 11 July 2024

Adsorption calculation

Appendix

Need **1** part protein (antigen): **5** parts naked bacteria (acid-treated, naked bacteria previously prepared)

$$\begin{aligned}\text{Vol antigen needed (mL)} &= \frac{0.2 \text{ mg}}{[\text{protein}] \text{ mg/mL}} \\ &= \frac{0.2}{1} \\ &= 0.2 \text{ mL} \\ &\sim 200 \text{ }\mu\text{L}\end{aligned}$$

1 mg/mL naked bacteria + 200 μ L RUVbL 2 = **± 1.2 mg dry weight**

1.2 mg prepared in **2.5 mL PBS** (pH 7.2) = **0.48 mg/mL**

Therefore, in each **0.5 mL** injection = **± 240 μ g** total protein-bacteria

Immunisation schedule

Antigen detail: *Plasmodium falciparum* RUVBL 3 peptide, resuspended in 1 mL sterile MilliQ. Adsorption to acid-treated, naked *Salmonella minnesota* R595 performed on days as seen below. Dried protein-bacteria resuspended in 2.5 mL PBS and sonicated before immunisation.

Adsorption:

1. 28/06/2024 Sample from the University of Venda
2. 11/07/2024 Sample from the University of Venda

Rabbit: Male, Flemish Giant (Sebola)

Date	Day	Dosage (μ g)	Blood serum volume (mL)	Comments
01.07.2024	0	± 240	± 0.5	Baseline serum (day 0)

Appendix

04.07.2024	3	± 240	-	
08.07.2024	7	± 240	-	
Week off				
15.07.2024	14	± 240	-	
18.07.2024	18	± 240	-	
22.07.2024	21	± 240	-	
Week off				
29.07.2024	28	± 240	± 0.5	Day 28 serum (check-up)
01.08.2024	31	± 240	-	
05.08.2024	35	± 240	-	
Week off				
12.08.2024	42	-	± 10	Day 42 serum (final)

Antigen preparation

Sample detail: *Plasmodium falciparum* RUVBL 3.

Concentration: 1 mg/mL

1st Adsorption date: 28 June 2024

Adsorption calculation

Need **1** part protein (antigen): **5** parts naked bacteria (acid-treated, naked bacteria previously prepared)

Appendix

$$\text{Vol antigen needed (mL)} = \frac{0.2 \text{ mg}}{[\text{protein}] \text{ mg/mL}}$$

$$= \frac{0.2}{1}$$

$$= 0.2 \text{ mL}$$

$$\sim 200 \text{ }\mu\text{L}$$

1 mg/mL naked bacteria + 200 μL RUVBL 3 = $\pm 1.2 \text{ mg dry weight}$

1.2 mg prepared in 2.5 mL PBS (pH 7.2) = 0.48 mg/mL

Therefore, in each 0.5 mL injection = $\pm 240 \text{ }\mu\text{g}$ total protein-bacteria

Concentration: 1 mg/mL (same sample as previous)

2nd Adsorption date: 11 July 2024

Adsorption calculation

Need 1 part protein (antigen): 5 parts naked bacteria (acid-treated, naked bacteria previously prepared)

$$\text{Vol antigen needed (mL)} = \frac{0.2 \text{ mg}}{[\text{protein}] \text{ mg/mL}}$$

$$= \frac{0.2}{1}$$

$$= 0.2 \text{ mL}$$

$$\sim 200 \text{ }\mu\text{L}$$

1 mg/mL naked bacteria + 200 μL RUVBL 3 = $\pm 1.2 \text{ mg dry weight}$

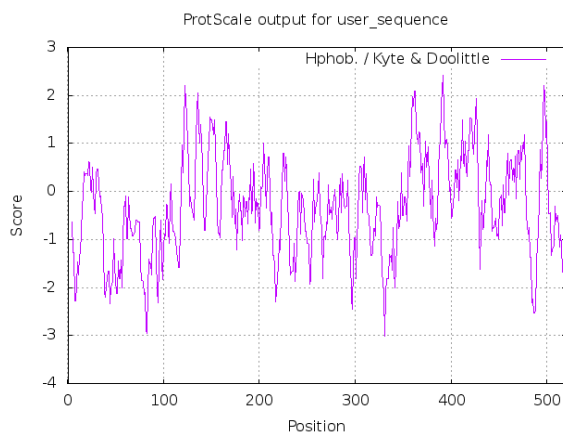
1.2 mg prepared in 2.5 mL PBS (pH 7.2) = 0.48 mg/mL

Appendix

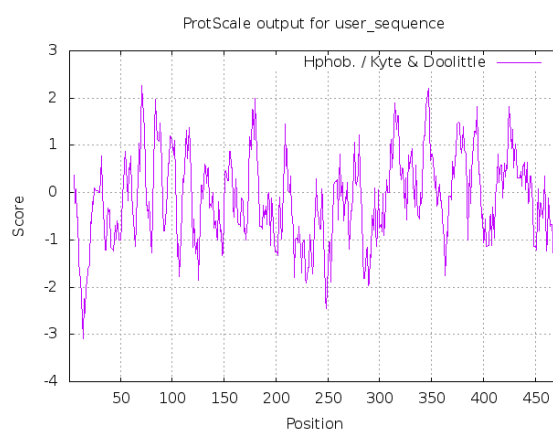
Therefore, in each **0.5 mL** injection = **±240 µg** total protein-bacteria

B5: Amino acid hydrophathy

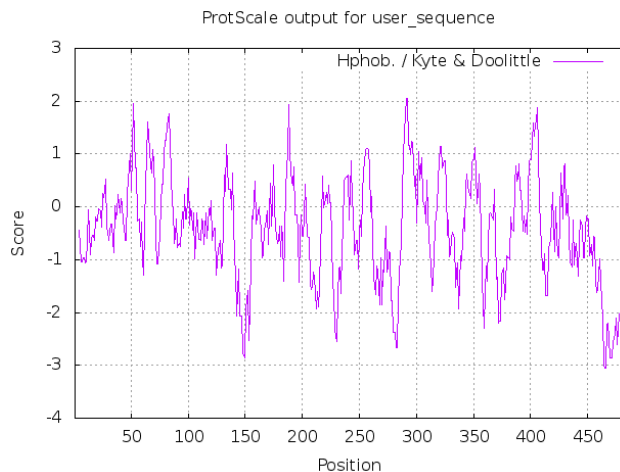
PfRUVBL1



PfRUVBL2



PfRUVBL3



B5: hydrophilic/hydrophobic scale of amino acid residues of PfRUVBL1, PfRUVBL2 and PfRUVBL3

The charts illustrate amino acid hydrophathy scores and sequence positions. The score ranges from (-4.0 to 3). The negative score signifies hydrophilic amino acids and the positive means hydrophobic.

Appendix

Table 3.2 Peptides Modification

Properties	PfRUVBL1	PfRUVBL2	PfRUVBL3
Number of amino acids	15	14	12
Sequence	ENNDIKNGTILDPDK	HENEISLSDEKYSM	KFNERDINNKNK
Purity	90%	90%	90%
Quantity	5mg	5mg	5mg
Modifications	KLH on Cys	KLH on Cys	KLH on Cys
N-terminus	acetylation	free amine	free amine
C-terminus	free acid	amidated	amidated

Appendix

B6: Confirmation of Plasmid constructs by restriction digested

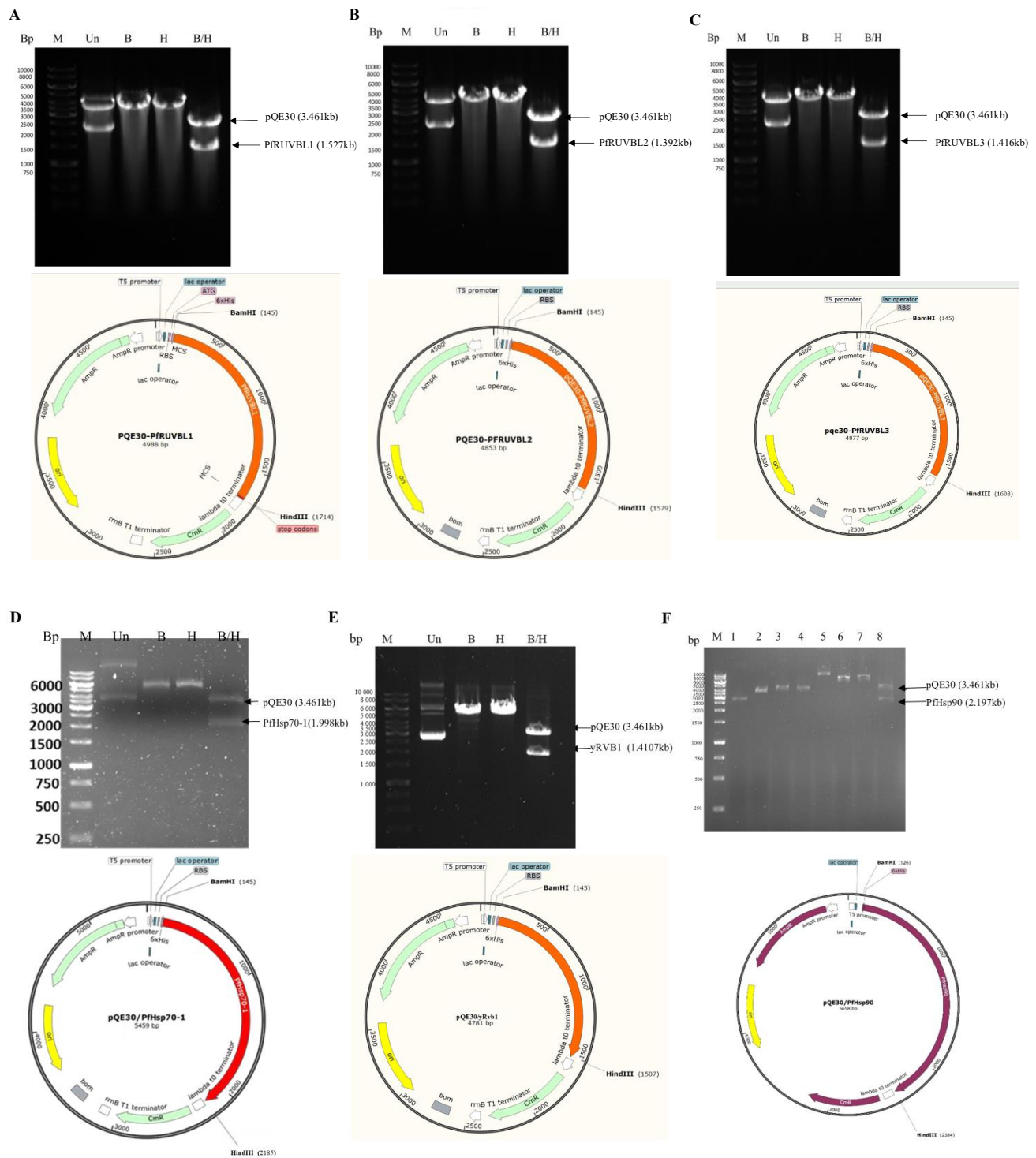
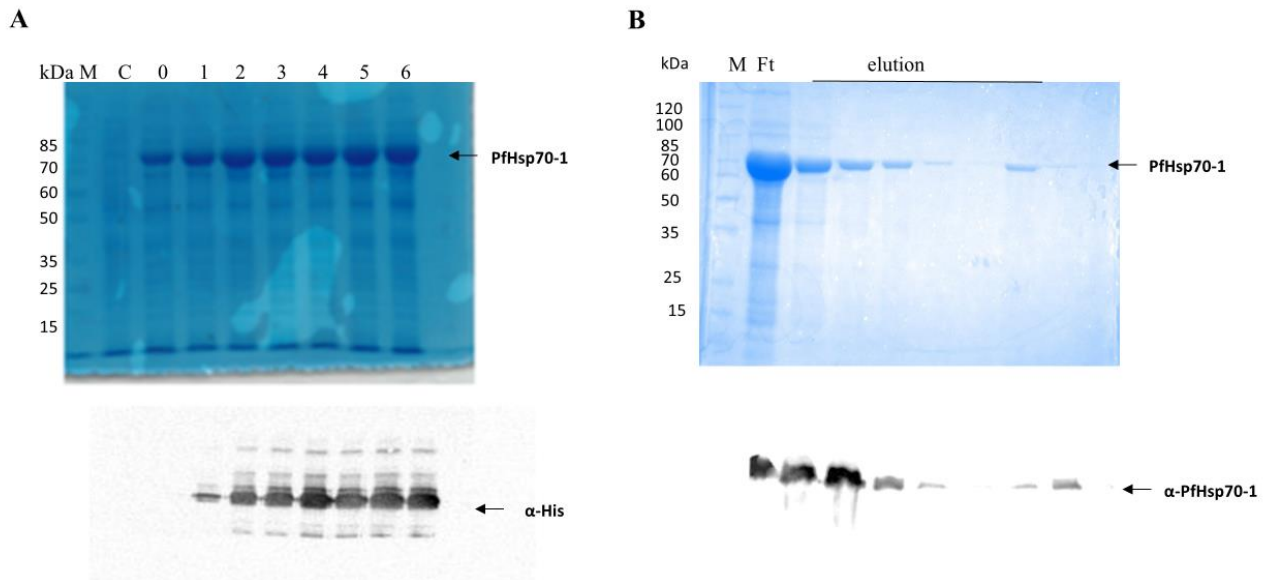


Figure B6. Plasmid maps and restriction agarose gel for PfHsp70-1, yRVB1, and PfHSP90 plasmid constructs
 Restriction analysis of DNA plasmids. (A) Plasmid map and agarose gel electrophoresis of pQE30/PfRUVBL1 showing the *Bam*HI and *Hind*III restriction sites. (B) Plasmid map and agarose gel electrophoresis of pQE30/PfRUVBL2 showing the *Bam*HI and *Hind*III restriction sites. (C) Plasmid map and agarose gel electrophoresis of pQE30/PfRUVBL3 showing the *Bam*HI and *Hind*III restriction sites. (D) Plasmid map and agarose gel electrophoresis of pQE30/PfHsp70-1 showing the *Bam*HI and *Hind*III restriction sites. (E) Plasmid map and agarose gel electrophoresis of pQE30/yRVB1 showing the *Bam*HI and *Hind*III restriction sites. (F) Plasmid map and agarose gel electrophoresis of pQE30/PfHsp90 showing the *Bam*HI and *Hind*III restriction sites.

B7: PfHsp70-1 expression and purification

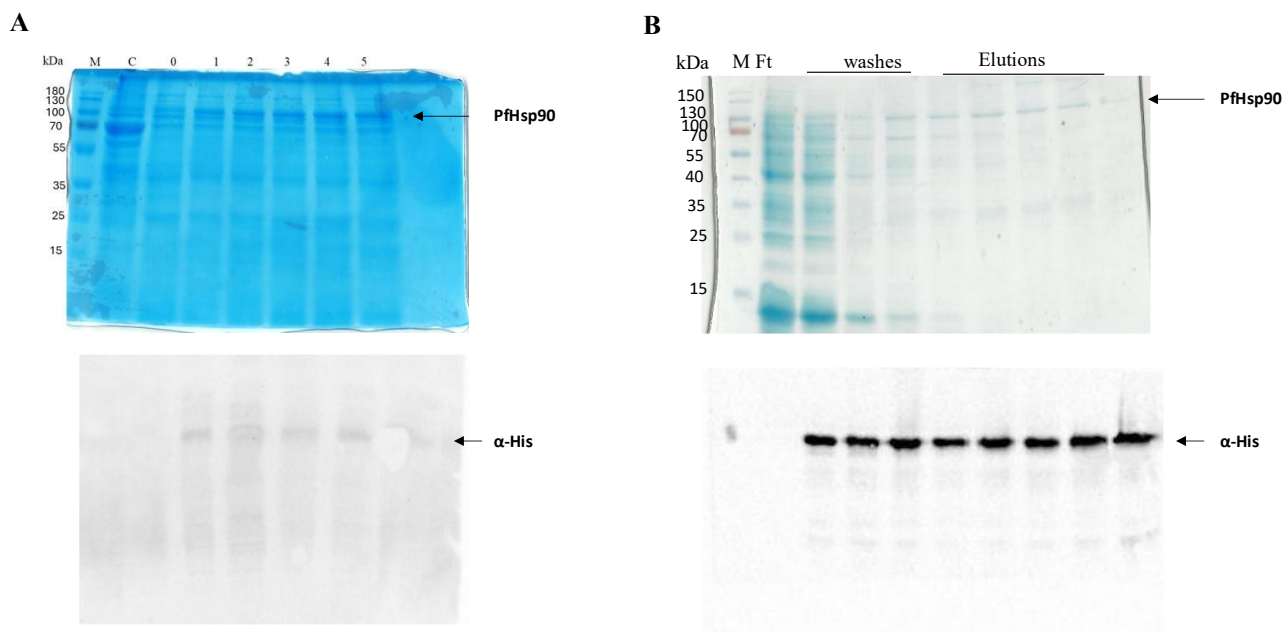


B7: Expression and purification of PfHsp70-1

(A) SDS-PAGE analysis for the expression of PfHsp70-1. M represents the molecular weight marker (in kDa), C represents the pQE30 vector control with no insert. 0 represents the pre-induction samples. Samples 1-6 were hourly samples that were each collected at the respective hour after IPTG induction. Respective immunoblot for expression using α -His antibodies is labelled below expression. (B) Purification samples for PfHsp70-1. Ft: flowthrough, elution: PfHsp70-1 protein. Immunoblot for purification using α -PfHsp70-1 antibodies is labelled below purification.

B8: PfHsp90 expression and purification

Appendix

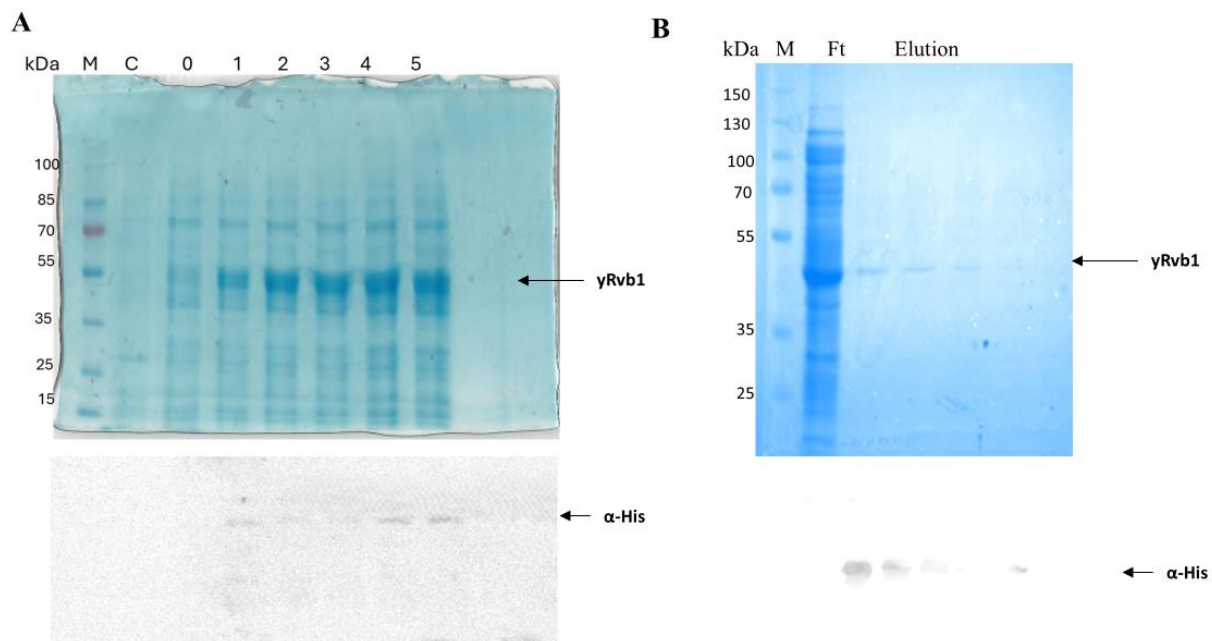


B8: Expression and purification of PfHsp90

(A) SDS-PAGE analysis for the expression of PfHsp90. M represents the molecular weight marker (in kDa), C represents the pQE30 vector control with no insert. 0 represents the pre-induction samples. Samples 1-5 were hourly samples that were each collected at the respective hour after IPTG induction. Respective immunoblots for expression using α -His antibodies are labelled below expression. (B) Purification samples for PfHsp90. Ft: flowthrough, washes are labelled, elutions: PfHsp90 protein. Immunoblot for purification using α -His antibodies is labelled below purification.

B9: yRVB1 expression and purification

Appendix

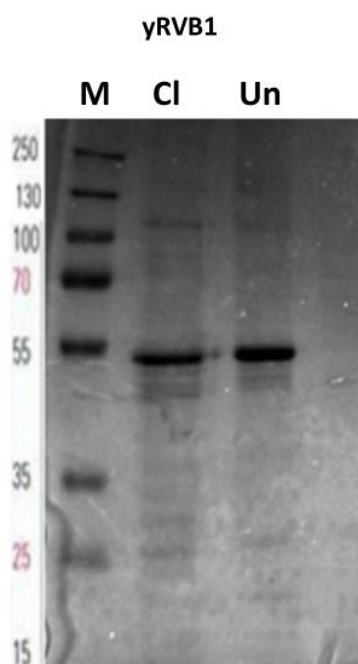


B9: Expression and purification of yRVB1

(A) SDS-PAGE analysis for the expression of yRVB1. M represents the molecular weight marker (in kDa), C represents the pQE30 vector control with no insert. 0 represents the pre-induction samples. Samples 1-5 were hourly samples that were each collected at the respective hour after IPTG induction. Respective immunoblots for expression using α -His antibodies are labelled below expression. (B) Purification samples for yRVB1. Ft: flowthrough, elution: yRVB1 protein.

B10: TEV cleavage of yeast RVB1

Appendix



B9: TEV His-tag cleavage

yRVB1 His-tag cleavage using TEV. M: molecular marker, cl: cleaved protein, Un: un-cleaved protein.

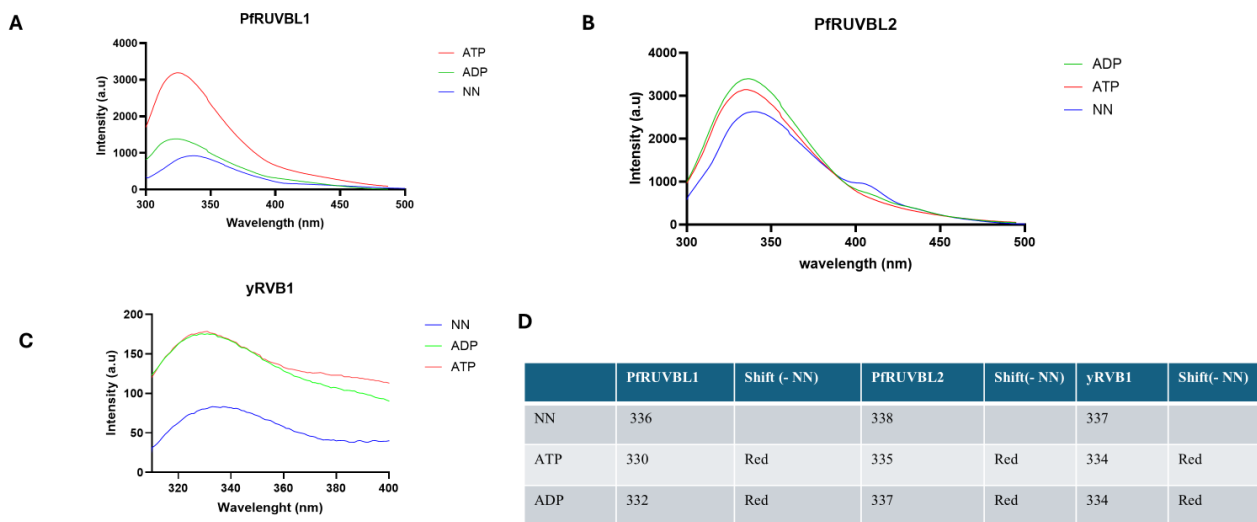
B11: PfRUVBL1 and PfRUVBL2 nucleotide binding kinetic table

Analyte	Protein	K_a	K_d	KD	Chi ²	U-value
ADP	PfRUVBL1	1.70E ⁰⁴	1.27E ⁻⁰¹	7.43E ⁻⁶	2.8	5
ADP	PfRUVBL2	1.68E ⁰⁴	8.94E ⁻⁰²	5.33E ⁻⁶	1.26	6.7
ADP	yRvb1	1.98E ⁰⁴	7.68E ⁻⁰²	3.87E ⁻⁶	1.84	12.3
ATP	PfRUVBL1	1.39E ⁰⁴	1.15E ⁻⁰¹	8.25E ⁻⁶	3.1	8.6
ATP	PfRUVBL2	3.31E ⁰⁴	4.76E ⁻⁰²	1.44E ⁻⁶	9.8	8.9
ATP	yRvb1	4.03E ⁰⁴	6.58E ⁻⁰²	1.63E ⁻⁶	1.34	6.3

B11: ATP/ADP binding to PfRUVBL1, PfRUVBL2, and yRVB1 kinetic table

Appendix

B 12: Nucleotide-binding alters the tertiary conformations of PfRUVBL1 and PfRUVBL2



B12: Tertiary structural analysis of PfRUVBL1, PfRUVBL2 and yRVB1.

Tyrosine fluorescence spectra curves of PfRUVBL1, PfRUVBL2 and yRVB1 in the presence and absence of ATP and ADP. (A) spectra curves of PfRUVBL1, (B) spectra curves of PfRUVBL2 and (C) spectra curves of yRVB1. (D) shifts table.

B13: Urea denaturation

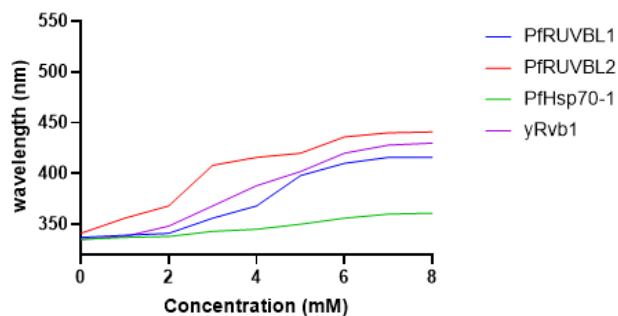


Figure B13: Urea induced changes in PfRUVBL1, PfRUVBL2, PfHsp70-1, and yRVB1 secondary structures

PfRUVBL2 is less stable compared to PfRUVBL1 and yRVB1. PfHsp70-1 is more stable compared to proteins upon urea denaturation.

Appendix

B14: Self and hetero-association of PfRUVBL1 and PfRUVBL2

Analyte	Protein	K_a	K_d	KD	Chi ²	U-value
PfRUVBL1	PfRUVBL1	1.13E ⁰⁶	4.39E ⁻³	3.89E ⁻⁸	8.77	17.5
PfRUVBL2	PfRUVBL2	7.20E ⁰⁵	7.52E ⁻²	1.04E ⁻⁷	6.19	14.2
Analyte	Ligand	K_a	K_d	KD	Chi ²	U-value
PfRUVBL1	PfRUVBL2	5.77E ⁰⁶	5.91E ⁻²	1.02E ⁻⁸	10.1	21

B15: Interaction of PfRUVBL1 and PfRUVBL2 with PfHsp70-1

Analyte	Protein	K_a	K_d	KD	Chi ²	U-value
PfHop	PfHsp70-1	1.18E ⁰⁴	1.79E ⁻²	1.57E ⁻⁶	32	25
PfRUVBL1	PfHsp70-1	1.32E ⁰²	1.73E ⁰²	1.31E ⁻⁵	16.84	47.4
PfRUVBL2	PfHsp70-1	1.26E ⁰⁶	1.22E ⁰⁸	9.71E ⁰¹	11.34	>50

B16: Interaction of PfRUVBL2 with PfHsp90

Analyte	Protein	K_a	K_d	KD	Chi ²	U-value
PfHop	PfHsp90	2.36E ⁰⁵	3.69E ⁻²	1.57E ⁻⁷	38.41	17.4
Analyte	Protein	K_a	K_d	KD	Chi ²	U-value
PfRUVBL2	PfHsp90	1.68E ⁰⁴	8.94E ⁻²	5.33E ⁻⁶	1,26	6,7

B17: PfHsp90 interaction with select small molecular inhibitors

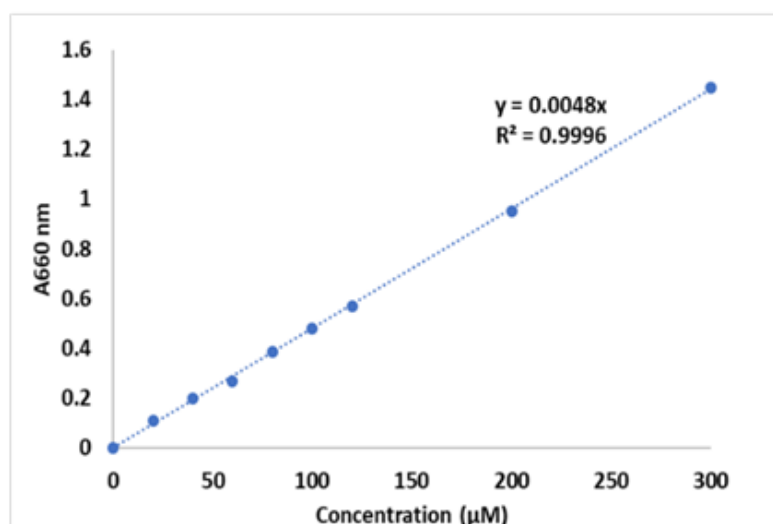
Analyte	Protein	K_a	K_d	KD	Chi ²	U-value
Novobiocin	PfHsp90	2.02E ⁰⁵	1.14E ⁻¹	5.63E ⁻⁷	68	48
Geldanamycin	PfHsp90	2.88E ⁰⁵	1.12E ⁻¹	3.88E ⁻⁷	59	42

Appendix

Sorafenib	PfHsp90	10.67E ⁰³	7.98E ⁻³	9.86E ⁻⁰¹	1170	>50
-----------	---------	----------------------	---------------------	----------------------	------	-----

B18: Phosphate Standard Curve

A



Appendix

B19: LCMS data analysis

Used	Total	%Cov(95)	Accession	Name
112.46	112.46	83.96999836	sp P0A6Z3 HTPG_ECOLI	Chaperone protein HtpG OS=E
68.72	68.72	53.32999825	sp P0A6Y8 DNAK_ECOLI	Chaperone protein DnaK OS=E
43.69	43.69	71.31999731	sp P0CE48 EFTU2_ECOLI	Elongation factor Tu 2 OS=Esch
42.49	42.52	41.69000089	sp P63284 CLPB_ECOLI	ClpB proteases
39.61	39.61	34.16000009	sp P13029 KATG_ECOLI	Catalase-peroxidase OS=Esche
36.65	36.65	38.87000084	sp P00363 FRDA_ECOLI	Fumarate reductase flavoprote
27.46	27.46	31.38999939	sp P0A6F5 CH60_ECOLI	Chaperonin GroEL OS=Escheric
27.13	27.16	24.92000014	sp P06959 ODP2_ECOLI	Dihydrolipoyllysine-residue ac
26.73	26.78	46.93000019	sp P09394 GLPQ_ECOLI	Glycerophosphodiester phospl
23.11	23.11	26.35999918	sp P0AC41 SDHA_ECOLI	Succinate dehydrogenase flavo
21	21	33.50999951	sp P0A836 SUCC_ECOLI	Succinate--CoA ligase [ADP-for
18.76	18.76	81.09999895	sp P68066 GRCA_ECOLI	Autonomous glycyl radical cof
17.65	17.67	23.52000028	sp P22259 PCKA_ECOLI	Phosphoenolpyruvate carboxy
16.92	16.96	46.02000117	sp P0AGE9 SUCD_ECOLI	Succinate--CoA ligase [ADP-for
16.09	16.11	28.47999993	sp P0A9P0 DLDH_ECOLI	Dihydrolipoyl dehydrogenase (
15.72	15.78	20.46999931	sp P0AG67 RS1_ECOLI	30S ribosomal protein S1 OS=E
15.57	15.59	27.88000107	sp P61889 MDH_ECOLI	Malate dehydrogenase OS=Esc
13.99	14	12.80000061	sp P0ABH9 CLPA_ECOLI	ATP-dependent Clp protease A
13.49	13.51	14.76999968	sp P0ADY1 PPID_ECOLI	Periplasmic chaperone PpiD O
12.63	12.64	12.35999987	sp P0A6M8 EFG_ECOLI	Elongation factor G OS=Escher
12	12	58.32999945	sp P0A862 TPX_ECOLI	Thiol peroxidase OS=Escherich
11.83	11.86	20.36000043	sp P0A7Z4 RPOA_ECOLI	DNA-directed RNA polymerase
11.61	11.62	12.95000017	sp P0A853 TNAA_ECOLI	Tryptophanase OS=Escherichia
11.52	11.58	16.80999994	sp P0AD61 KPYK1_ECOLI	Pyruvate kinase I OS=Escherich
11.43	11.46	46.63000107	sp P0A8F0 UPP_ECOLI	Uracil phosphoribosyltransfera
10.98	11	40.7799989	sp P62399 RL5_ECOLI	50S ribosomal protein L5 OS=E
10.9	10.97	32.62000084	sp P0AE08 AHPC_ECOLI	Alkyl hydroperoxide reductase
10.39	10.46	24.16999936	sp P0A9B2 G3P1_ECOLI	Glyceraldehyde-3-phosphate c
10.31	10.33	35.58999896	sp P0AG55 RL6_ECOLI	50S ribosomal protein L6 OS=E
9.4	9.43	16.94999933	sp P0AC38 ASPA_ECOLI	Aspartate ammonia-lyase OS=
8.45	8.46	19.08999979	sp P0A7V0 RS2_ECOLI	30S ribosomal protein S2 OS=E
8.03	8.03	5.311999843	sp P21179 CATE_ECOLI	Catalase HP11 OS=Escherichia c
8	8	6.938000023	sp P27302 TKT1_ECOLI	Transketolase 1 OS=Escherichi
8	8	20.08000016	sp P0AC47 FRDB_ECOLI	Fumarate reductase iron-sulfu
8	8	15.32000005	sp P0AB71 ALF_ECOLI	Fructose-bisphosphate aldolas
8	8	16.71999991	sp P0A870 TALB_ECOLI	Transaldolase B OS=Escherichi
7.92	8	15.54999948	sp P0A6P1 EFTS_ECOLI	Elongation factor Ts OS=Esche
7.88	8.01	27.18999982	sp P38489 NFSB_ECOLI	Oxygen-insensitive NAD(P)H ni
7.77	7.85	13.26999962	sp P0A6K6 DEOB_ECOLI	Phosphopentomutase OS=Escl
7.34	7.45	9.960000217	sp P0A6F3 GLPK_ECOLI	Glycerol kinase OS=Escherichia
6.99	7.06	14.86999989	sp P0A825 GLYA_ECOLI	Serine hydroxymethyltransfera
6.97	7.06	9.555000067	sp P0DTT0 BIPA_ECOLI	50S ribosomal subunit assemb
6.96	7.05	18.65999997	sp P0C8J6 GATY_ECOLI	D-tagatose-1,6-bisphosphate a

Appendix

N	Unused	Total	%Cov(9	Accessi	Name	Species	Peptide
1	52.91	52.91	79.95	sp P0CE48	Elongation	ECOLI	38
2	36.32	36.32	61.17	sp P09394	Glyceroph	ECOLI	30
3	29.96	29.96	38.08	sp P0A6K6	Phosphop	ECOLI	17
4	28.45	28.45	40.29	sp P0A825	Serine hyc	ECOLI	20
5	24.51	24.51	34.79	sp P0A836	Succinate-	ECOLI	13
6	20.25	20.25	16.34	sp P63284	Chaperon	ECOLI	10
8	16.56	16.56	32.17	sp P46853	Uncharact	ECOLI	8
10	14	14	21.53	sp P0A6B7	Cysteine d	ECOLI	7
11	13.98	13.99	21.41	sp P0AE06	Multidrug	ECOLI	7
12	13.77	13.79	13.14	sp P0A6Z3	Chaperon	ECOLI	7
13	13.69	13.76	23.35	sp P0A6J8	D-alanine-	ECOLI	7
14	13.23	13.3	22.36	sp P0A9P6	Dihydrolip	ECOLI	8
15	13.01	13.11	23.56	sp P0A9B7	Glyceralde	ECOLI	8
16	12.98	13.02	48.13	sp P0AE08	Alkyl hydr	ECOLI	7
18	12.82	12.89	25.61	sp P0AGE5	Succinate-	ECOLI	6
19	12.35	12.47	13.15	sp P0A6F3	Glycerol ki	ECOLI	6
20	12.26	12.43	44.88	sp P68066	Autonomo	ECOLI	7
21	11.93	12.01	17.19	sp P19926	Glucose-1-	ECOLI	6
22	11.75	11.83	16.01	sp P0A953	3-oxoacyl-	ECOLI	7
23	10.01	10.01	14.99	sp P0A795	Phosphog	ECOLI	5
24	9.38	9.43	20.25	sp P0A6A7	Acetate ki	ECOLI	5
26	8.31	8.36	11.87	sp Q46807	Putative c	ECOLI	4
28	7.32	7.41	9.135	sp P08200	Isocitrate	ECOLI	4
29	7.32	7.38	17.96	sp P0C8J6	D-tagatose	ECOLI	5
30	7.13	7.2	11.25	sp P0A7Z4	DNA-direc	ECOLI	4
31	6.95	7.02	13.18	sp P75691	Aldehyde	ECOLI	4
32	6.9	6.98	12.91	sp P27248	Aminomet	ECOLI	4
33	6.06	6.06	8.707	sp P0ADA7	Murein hy	ECOLI	3
34	6.03	6.07	6.638	sp P05042	Fumarate	ECOLI	3
36	6	6	8.088	sp P29745	Peptidase	ECOLI	3
37	6	6	9.541	sp P0A6P7	Elongation	ECOLI	3
38	6	6	23.81	sp P0A867	Thiol pero	ECOLI	3
39	5.89	5.99	7.95	sp P0AC37	Aspartate	ECOLI	4
40	5.84	5.94	21.74	sp P12758	Uridine ph	ECOLI	3
41	5.57	5.62	10.58	sp P61889	Malate de	ECOLI	3
42	5.54	5.59	9.259	sp P0A6P5	Enolase O	ECOLI	3
43	5.51	5.59	9.444	sp P0A7I0	Peptide ch	ECOLI	3
44	5.49	5.55	6.081	sp P21507	ATP-deper	ECOLI	3
45	5.44	5.52	5.857	sp Q46807	D-phenylh	ECOLI	3
46	5.25	5.36	6.683	sp P0A745	UDP-N-ace	ECOLI	3
47	4.91	4.99	30.1	sp P0A7R1	30S ribosc	ECOLI	3
58	4	4	33.33	sp P69776	Major out	ECOLI	3
61	3.91	4.08	8.163	sp P39173	Putative gl	ECOLI	3
63	3.77	3.95	7.675	sp P0ACC7	Bifunction	ECOLI	4

Appendix

Appendix C: List of reagents

REAGENT	SUPPLIER
Acetic acid	Merck, Germany
Adenosine triphosphate	Sigma, U.S.A
Agarose	Whitehead scientific, South Africa
Ammonium molybdate	Merck, Germany
Ammonium persulphate	Merck, Germany
Ampicillin	Sigma, U.S.A
Bovine serum albumin	Sigma, U.S.A
Bromophenol blue	Sigma, U.S.A
Calcium chloride	Merck, Germany
Chloramphenicol	Sigma, U.S.A
Coomassie brilliant blue R250	Merck, Germany
Diethiothreitol	Sigma, U.S.A
DreamTaq master mix	Thermo Scientific, U.S.A
Ethidium bromide	Sigma, U.S.A
Glacial acetic acid	Merck, Germany
Glycerol	Merck,
Glycine	Merck, Germany
Imidazole	Sigma, U.S.A
Isopropyl-1-thio-D-galacopyranoside	Sigma, U.S.A
Lysozyme	Merck, Germany
Magnesium chloride	Merck, Germany

Appendix

Methanol	Merck, Germany
Monoclonal anti-His6-HRP antibodies	Sigma, U.S.A
Ni-NTA resin	Thermo Scientific, U.S.A
Nitrocellulose membrane	Pierce, U.S.A
PageRuler Prestained Protein Ladder	Thermo Scientific, U.S.A
Peptone	Merck, Germany
Phenylmethylsulfonyl fluoride	Sigma, U.S.A
Polyacrylamide	Merck, Germany
Polyethylene glycol 2000	Sigma, U.S.A
Polyethylenimine	Sigma, U.S.A
Ponceau S	Sigma, U.S.A
Potassium chloride	Merck, Germany
Potassium dihydrogen phosphate	Merck, Germany
Proteinase-K	Sigma, U.S.A
Rapid ligation buffer	Promega, Germany
Restriction enzymes	Thermo Scientific, U.S.A
Sodium chloride	Merck, Germany
Sodium dodecyl sulphate	Merck, Germany
Sodium hydroxide	Merck, Germany
TEMED	Sigma, U.S.A
Tris	Merck, Germany

Appendix

Tryptone	Merck, Germany
Tween 20	Merck, Germany
Urea	Melford, UK
Yeast extract powder	Merck, Germany
β -mercaptoethanol	Sigma, U.S.A

Appendix