

**Antisense Transcription in *Plasmodium falciparum*:
Mechanisms, Functions, and Implications for
Malaria Biology**

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध
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2024

CERTIFICATE

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Abbreviations

WHO : World Health Organisation
RBC : Red Blood Cell
IDC : Intraerythrocytic developmental cycle
AMA 1 : Apical Membrane Antigen 1
RBP : Reticulocyte Binding Protein
RON : Rhoptry Neck Protein
EMP 1 : Erythrocyte Membrane Protein 1
RNAi : RNA Interference
ncRNA : Noncoding RNA
AS : Antisense
AS RNA : Antisense RNA
SAGE : Serial analysis of gene expression
mRNA : messenger RNA
NAT Pair : Natural antisense transcript pair
lncRNA : Long noncoding RNA
VSA : Variant Surface Antigen
IL-10 : Interleukin 10
AP2/ apiAP2 : Apetella 2
RNA Pol II : RNA Polymerase II
ChIP : Chromatin immunoprecipitation
HP1 : Heterochromatin protein 1
GRN : Gene Regulatory Network
RBP : RNA Binding Protein
dsRNA : Double stranded RNA
siRNA : Small interfering RNA
ASO : Antisense oligonucleotides
RISC : RNA Induced Silencing complex
ceRNA : Competing endogenous RNA
NaCl : Sodium chloride

MgCl₂ : Magnesium chloride
EDTA : Ethylenediaminetetraacetic acid
EGTA : Ethylene glycol tetraacetic acid
PIC : Protease Inhibitor Complex
PMSF : Phenylmethanesulfonyl fluoride
KCl : Potassium chloride
SRA : Sequence Read Archive
PBS : Phosphate Buffer Saline
DTT : Dithiothreitol
TCA : Tricarboxylic acid cycle
HAT : Histone acetylation assay
NGS : Next generation sequencing
ORF : Open reading frame
ACN : Acetonitrile
TFA : Trifluoroacetic acid
IAA : Iodoacetamide
CPC : Coding potential calculator
Xist : X inactive specific transcript
dsRIP : Double stranded RNA Immunoprecipitation

Preface

Plasmodium falciparum causes the most severe form of malaria in humans. To complete its life cycle, the parasite undergoes numerous morphological modifications. The parasite is pre-programmed to resist a variety of pressures and can thrive in a variety of settings. Consequently, the pathogenicity of the parasite increases, and antibiotic resistance develops. The underlying biology suggests that gene expression is tightly controlled at the transcriptional, post-transcriptional, and/or translational level. Regulation via epigenetic pathways has also been widely explored. Furthermore, non-coding RNA-mediated gene regulation is a well-studied mechanism, however it is poorly understood in *P. falciparum*. Stranded RNA sequencing was used to investigate the role of antisense transcription in gene regulation. Antisense transcription has been discovered to be a common occurrence. It is found to be significantly deregulated in stress-induced circumstances, implying that it might play an important function. Antisense RNA, unlike sense RNA, associates with specific proteins, according to our findings. It also makes us think about the control that takes place at the chromatin and translation levels. We developed stable transgenic lines to study the likely mechanism of action. We demonstrate that RNA-RNA duplex formation stabilises RNA and reduces the degradation rates of sense and antisense RNA, resulting in an increase in net quantities. We also show that these duplex RNAs are either not translated or cause ribosomal stalling. We demonstrate antisense and duplex RNA attachment with ribosomes, which increases under stress circumstances. At the same time, we're trying to figure out antisense RNA's non-canonical features. Using a combination of in-silico and in-vitro approaches, we discovered that antisense RNA has coding potential. The validity of peptides synthesized from antisense RNA ORFs, as well as the difference in stress conditions, has been established. We're still attempting to figure out what these peptides do. Our findings are expected to provide light on previously undisclosed aspects of *Plasmodium* gene regulation. This could help with aspects of translational research and diagnostic innovation in the future.

Chapter 1

Antisense Transcription in *Plasmodium falciparum*: Mechanisms, Functions, and Implications for Malaria Biology

1. Life cycle of *Plasmodium falciparum*: a detailed view

Plasmodium falciparum is the most lethal species amongst the malaria-causing protozoan species, accounting for the majority of malaria-related illness and death globally. This parasite has a complex life cycle that alternates between human hosts and the female Anopheles mosquitoes, aiding its transmission and pathogenicity (WHO report, 2023).

The life cycle begins when an infected female Anopheles mosquito bites a human during a blood meal, injecting sporozoites into the bloodstream. These sporozoites are the infective sexual forms of the parasite. Once in the bloodstream, sporozoites rapidly travel to the liver and invade liver cells (hepatocytes). Within the liver, they reproduce asexually and develop into schizonts, which can produce thousands of merozoites. When the liver cells rupture, these merozoites are released into the bloodstream. This phase is referred to as the liver stage or exoerythrocytic phase, which transitions into the blood stage or the erythrocytic phase (Enomoto et al., 2012; Venugopal et al., 2020; Tavares et al., 2013; Amino et al., 2006).

During the blood stage, merozoites invade red blood cells (RBCs), where they first develop into asexual ring stages and then differentiate into trophozoites. Further trophozoites mature into schizonts, which divide into merozoites, causing the infected RBCs to burst and release more merozoites into circulation. This cycle of bursting open of RBCs occurs roughly every 48 hours, resulting in the fever and chills characteristic of malaria. Some merozoites will transform into gametocytes, the sexual form of the parasite, which are then available to be ingested by another mosquito during a subsequent blood meal, marking the beginning of the mosquito stage. Importantly, these stages are marked by dramatically different morphological characteristics. Also, there is noted to be a dramatic difference in the gene expression paradigm and metabolic rates throughout the intraerythrocytic developmental stages (Mota et al., 2001; Sturm et al., 2006; Sologub et al., 2011).

To continue the cycle further, when a mosquito bites an infected individual, it takes in gametocytes along with the blood. Once inside the mosquito's gut, these gametocytes mature into male and female gametes, which fertilize to form zygotes. The zygotes develop into ookinetes, which penetrate the mosquito's midgut wall and form oocysts. Eventually, these oocysts rupture, releasing sporozoites that travel to the mosquito's salivary glands, ready to infect another human host. Continuous spread and exposure to this virulence cycle leads to spread of malaria in a population. (Sologub et al., 2011). A schematic representation of *P. falciparum* life cycle is depicted below in Figure 1.

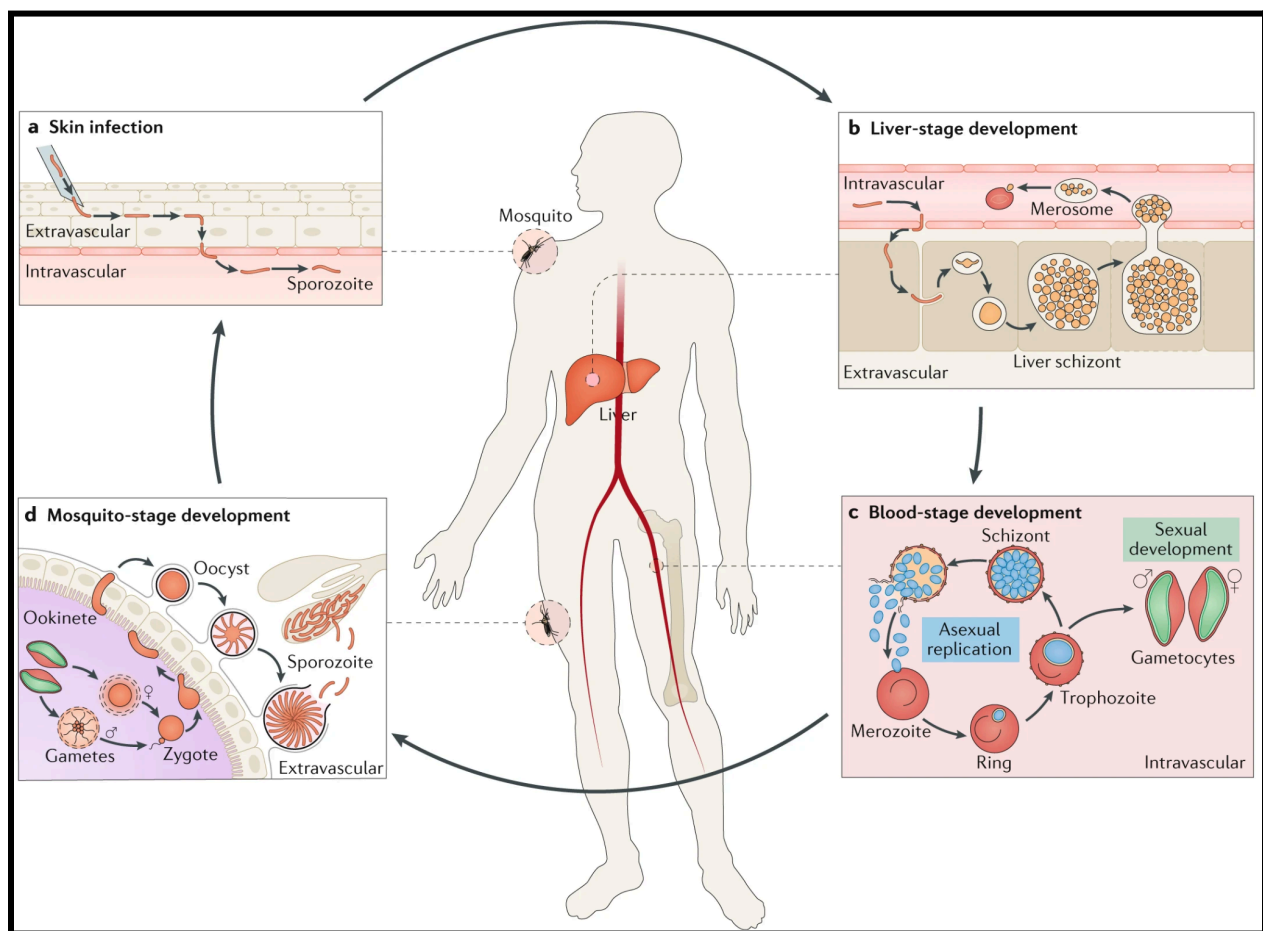


Figure 1: A descriptive *Plasmodium falciparum* life cycle: (a) *P. falciparum* sporozoites are injected into the host during a blood meal by the infected Anopheles mosquito which enter the bloodstream, travel to the liver sinusoids, and then invade a hepatocyte by several transmigration events. (b) Following asexual replication cycle in the hepatocyte, produces a liver schizont with thousands of merozoites. Merozoites, once released, infect red blood cells, kicking off the

intraerythrocytic developmental parasite cycle. (c) After invading a red blood cell, they progress from ring stages to trophozoites and finally to schizonts leading to merozoites. (d) Anopheles mosquito collects gametocytes while feeding on an infected human. Male and female gametocytes lead to gametogenesis and further development within the mosquito's midgut (Adapted from: Venugopal et al., 2020).

1.1 Pathogenicity of *Plasmodium falciparum* and its contributing factors

Plasmodium falciparum is associated with a high pathogenicity rate, which contributes to its rapid spread and the challenge of drug resistance. The pathogenic effects of *P. falciparum* infection arise from several key mechanisms. The major goals for increased pathogenicity are to be able to invade and destroy red blood cells (RBCs). The parasite has evolved specialized surface proteins that facilitate its attachment to and invasion of RBCs, thereby advancing its intraerythrocytic developmental cycle (IDC). The IDC progresses and marks different developmental and morphological stages. This process ultimately results in the destruction of the RBCs, leading to anemia in malaria-infected patients (Thompson et al., 2023).

The **invasion** process begins when the merozoite form of *P. falciparum* (the stage responsible for invasion) attaches to the surface of the RBC. This attachment is mediated by specific surface proteins, including Apical Membrane Antigen 1 (AMA1) and Reticulocyte Binding Protein (RBP). Following this initial attachment, the parasite recognizes and binds to specific receptors on the RBC surface, such as Band 3 and Glycophorin A, which are essential for the subsequent steps of invasion (Wright et al., 2014; Riglar et al., 2011; Harvey et al., 2012; Hanssen et al., 2013; Singh et al., 2010).

The entry of the parasite occurs through receptor-mediated endocytosis, where the merozoite interacts with RBC surface receptors, leading to its engulfment within a membrane-bound vacuole and allowing it to enter the RBC. Additionally, active penetration can occur, in which the parasite releases proteolytic enzymes that degrade host cell membranes, facilitating its entry. This engulfment is mediated via a specialized set of proteins responsible for endocytosis. The Rhoptry Neck Protein (RON) complex plays a critical role in this process. During invasion, RON inserts into the RBC membrane, forming a structure known as the moving junction. This junction

is vital for ensuring tight adhesion of the parasite and successful invasion of the RBC, represented in Figure 2 below (Hanssen et al., 2013; Singh et al., 2010).

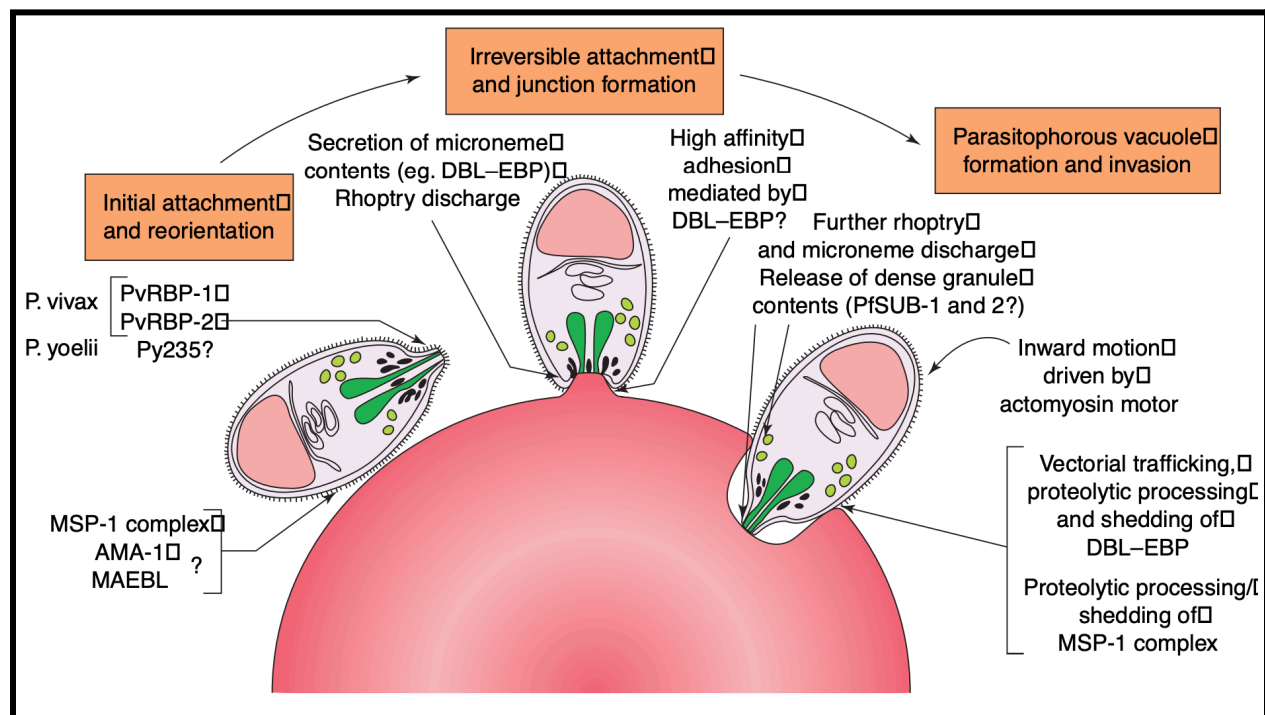


Figure 2: Diagram showing the initial phases of the merozoite's invasion into red blood cells (RBCs) and the functions of the different proteins. The merozoite's several secretory organelles are displayed as pale green (dense granules), black (micronemes), and dark green (paired rhoptries). Adhesive ligands like MSP-1 and other elements of the related protein complex that are stably present on the merozoite surface may be involved in the first, low-affinity interaction with the host cell. Another possibility is that it involves MAEBL or AMA-1, which is secreted onto the parasite surface. Micronemal components like EBA-175 and other DBL-EBPs may be exocytosed upon reorientation, which could trigger tight attachment and junction formation. The parasite may subsequently be driven into the developing parasitophorous vacuole by the impacts of anterior-to-posterior trafficking and/or proteolytic shedding of these and other RBC-binding proteins, which are connected to the activity of a sub-pellicular actomyosin motor. The suggested paradigm is somewhat speculative, the authors would like to emphasize. Apical membrane antigen-1 (AMA-1), Duffy-binding-like erythrocyte-binding protein (DBL-EBP), 175 kDa erythrocyte-binding antigen (EBA-175), and

merozoite surface protein-1 (MSP-1) are some examples of abbreviations (Adapted from: Chitnis et al., 2000).

This invasion leads to **sequestration**, a crucial aspect of the pathogenicity of *Plasmodium falciparum*. This process involves the adhesion of infected red blood cells (RBCs) to the vascular endothelium, particularly in critical organs like the brain and placenta. Refer figure 3 below for representation of the process of sequestration. The adhesion results in the formation of clusters of adhered RBCs that attach to the endothelium while evading recognition by the immune system. Such sequestration contributes to severe complications, including cerebral malaria and placental malaria, both of which present significant health risks (David et al., 1983; Sherman et al., 2003; Craig et al., 2012).

Sequestration is facilitated by the expression of specific proteins, particularly **PfEMP1** (*Plasmodium falciparum* Erythrocyte Membrane Protein 1. These proteins enable the infected RBCs to adhere to endothelial cells. The adherence is mediated through interactions between PfEMP1 on the surface of infected RBCs and receptors on the endothelial cells, such as **CD36**, **ICAM-1**, and **VCAM-1**. This interaction is vital for the sequestration process, leading to reduced circulation within the vasculature and promoting immune evasion (Chakravorty et al., 2008; Cojean et al., 2008).

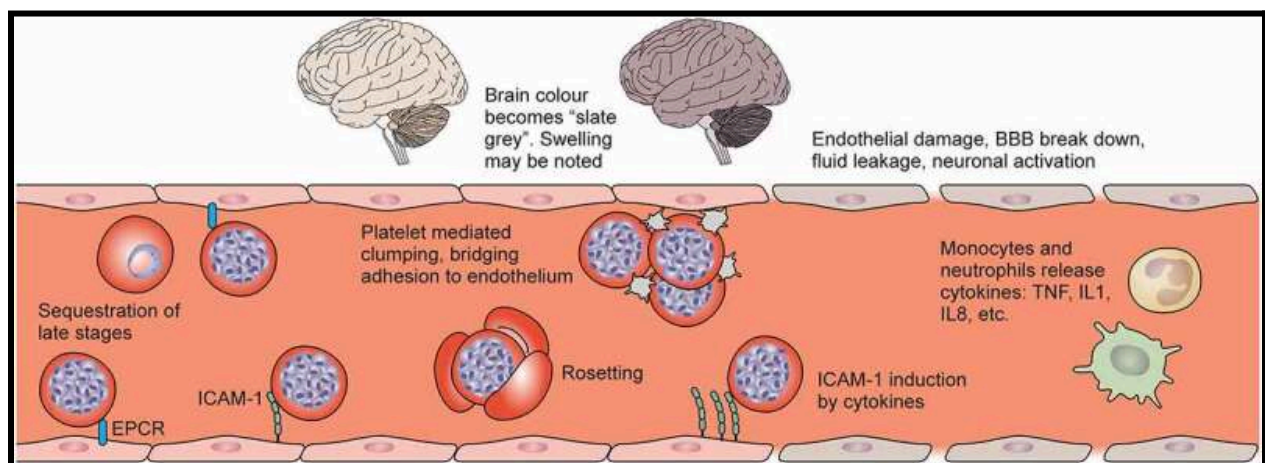


Figure 3 : Sequestration in *Plasmodium falciparum*. In cases of deadly cerebral malaria, the colour of the brain shifts from light tan to a "slate grey" hue when viewed from left to right. The

deep vasculature, particularly the brain arteries, is where mature infected erythrocytes (IEs) hide. Heparan sulphate proteoglycans, ICAM-1, and endothelial protein C receptor (EPCR) are examples of receptors found on endothelial cells. Platelets have the ability to clump IEs and act as a bridge between the endothelium and CD36-binding IEs. By causing monocytes and neutrophils to release cytokines and immune mediators, IE can disrupt the blood–brain barrier and increase the expression of ICAM-1. Leakage of fluid between and through cerebral endothelial cells can activate neurons and cause potentially lethal brain oedema and discolouration (Adapted from: Walker et al., 2023)

Additionally, there is a fascinating mechanism of immune evasion: the parasite undergoes **antigenic variation**, changing the proteins on the surface of infected red blood cells (RBCs) to evade detection by the host's immune system. This crucial ability to escape immune responses significantly contributes to the persistence of the infection. Antigenic variation is primarily driven by the expression of PfEMP1 and the switching of various surface antigens. Other surface proteins, such as RAPs (Reticulocyte-Binding Protein Homologs) and RIFINs (Repetitive Interspersed Family of Proteins), also participate in antigenic variation, further complicating the parasite's immune evasion strategy (Scherf et al., 2008).

Furthermore, **cytokine release** complicates the pathogenicity: The presence of the parasite triggers an immune response that leads to the release of pro-inflammatory cytokines. This inflammatory response can cause significant tissue damage and exacerbate the clinical manifestations of severe malaria. Key cytokines of *Plasmodium falciparum* infections are Tumor Necrosis Factor-alpha (TNF- α), Interleukin-6 (IL-6), Interleukin-10 (IL-10). In severe cases of malaria, particularly cerebral malaria, a "cytokine storm" can occur. This phenomenon is characterized by an overwhelming release of pro-inflammatory cytokines, leading to systemic inflammation, endothelial activation, and increased vascular permeability. The cytokine storm can result in severe complications such as cerebral edema, tissue damage, and multi-organ failure (Angulo et al., 2002; de Roquetaillade et al., 2023).

Lastly, there are also **metabolic disturbances**: The metabolic activities of *P. falciparum* modify the host RBCs, resulting in increased oxidative stress and additional damage to the cells. This disruption in metabolism plays a role in the pathophysiology of malaria. Disruption of Glucose

metabolism (hypoglycemia), Anemia and Iron Metabolism, Lactic Acidosis, Disturbances in Lipid Metabolism, Metabolic Stress and Host Response have been an arena of constant research offering insightful discoveries (Tewari et al., 2022).

These factors contribute to increasing the pathogenicity of the parasite. Key pathogenicity factors are summarised in Figure 4.

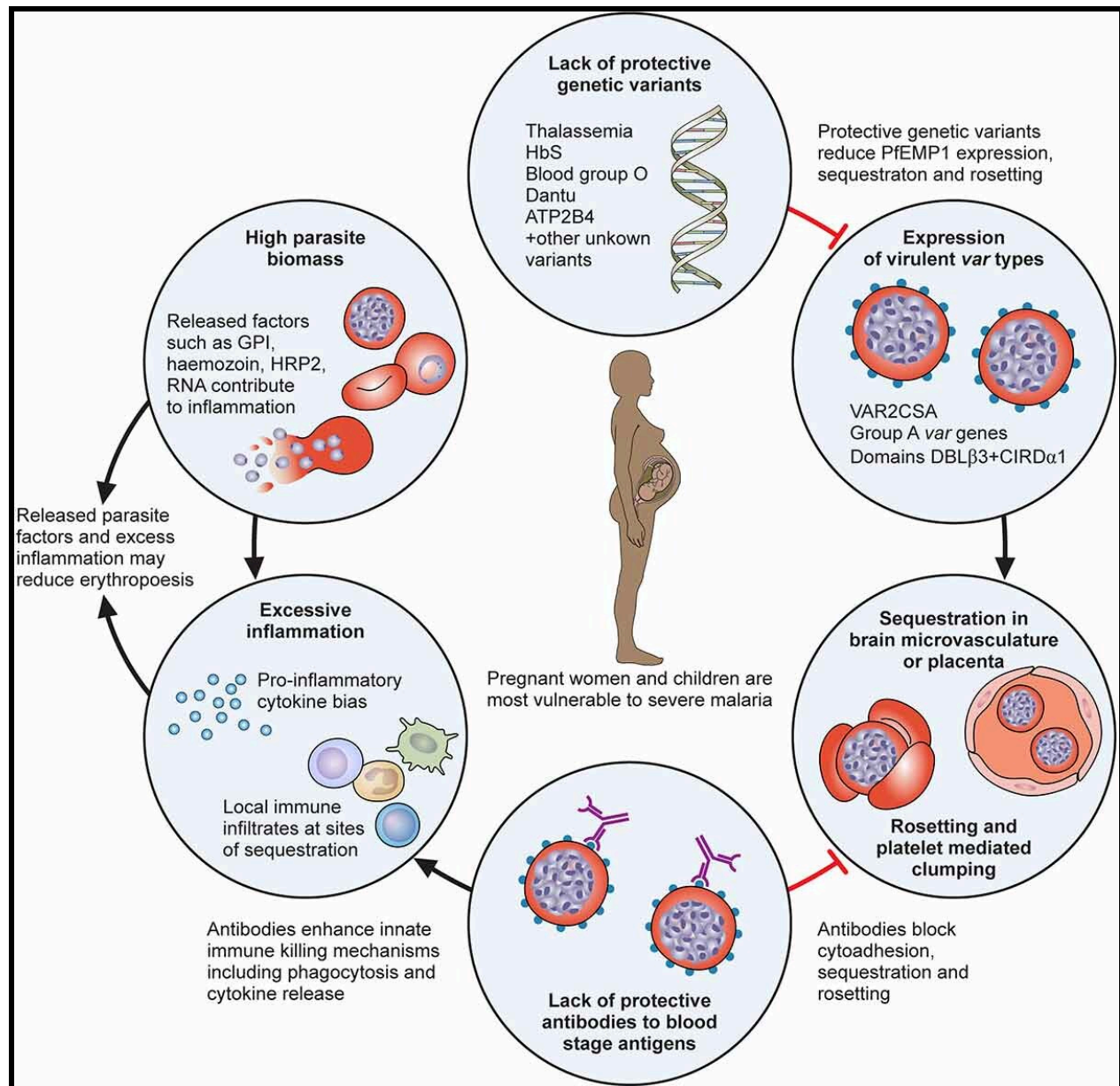


Figure 4: Key pathogenic factors that contribute to severe malaria. In addition to being inflammatory stimulants, high parasite biomass and parasite products released into the

bloodstream during cell lysis may also directly cause severe anaemia by lowering erythropoiesis. Through excessive complement-mediated lysis of uninfected erythrocytes, a significant inflammatory response, including Th1 type cytokine bias and sequestration of innate immune cells, including monocytes, can activate and damage endothelial cells, promote sequestration, and cause severe anemia. Although the primary targets of antibodies are unknown, a deficiency of antibodies directed against IE surface antigens may be a factor in increased parasite biomass and sequestration. The pathophysiology of cerebral malaria is largely dependent on the sequestration of IE in young children's brains, which is controlled by PfEMP1 and enables the parasite to avoid splenic clearance. Poor pregnancy outcomes are closely linked to sequestration in the placenta. PfEMP1 produced from VAR2CSA mediates sequestration in the placenta, while Group A PfEMP1 with domains that bind to ICAM-1 and EPCR is linked to sequestration in the brain microvasculature. PfEMP1 expression, sequestration, and rosetting can all be decreased by protective genetic variations (Adapted from: Walker et al., 2023).

1.2 Clinical Manifestations of Malaria

Infections caused by *P. falciparum* can present a diverse array of symptoms, ranging from asymptomatic cases to severe malaria. Some cases may remain unidentified for years while others may show quick symptoms some of which may include the ones listed below also depicted in **Figure 5** :

- High fever and chills
- Significant anemia
- Jaundice (yellowing of the skin and eyes)
- Splenomegaly (enlargement of the spleen)
- Neurological issues, such as seizures and altered consciousness, particularly in cases of cerebral malaria.

Syndrome	Clinical features	Possible sequence or mechanism of disease
Cerebral malaria	Sustained impaired consciousness, coma, long-term neurological sequelae	Cerebral parasite sequestration; bioactive GPI; pro-inflammatory cytokine cascade; endothelial-cell activation; natural killer T-cell activation; T_H1/T_H2 -cell balance; chemokine production; monocyte, macrophage and neutrophil recruitment; platelet and fibrinogen deposition; CD4 ⁺ , CD8 ⁺ and $\gamma\delta$ T-cell involvement; IFN- γ production; neurological metabolic derangements; possibly hypoxia
Placental malaria	Placental insufficiency, low birth weight, premature delivery, loss of fetus	<i>Plasmodium falciparum</i> EMP1-mediated binding to placental endothelium and syncytiotrophoblast through chondroitin sulphate A and hyaluronic acid; cytokine production; chemokine-mediated recruitment and infiltration of monocytes; intravascular macrophage differentiation
Severe malarial anaemia	Pallor, lethargy, haemoglobin level of 4–6 g per 10 ml	Erythropoietic suppression by toxins and cytokines; increased RBC destruction, owing to parasitization, RBC alterations, complement and immune complex or antigen deposition, erythrophagocytosis, splenic hyperphagism, CD4 ⁺ T cells, T_H1/T_H2 cytokine balance (TNF and IFN- γ versus IL-10)
Metabolic acidosis	Respiratory distress, deep breathing (Kussmaul breathing), hypovolaemia	Molecular mechanisms unknown. Possibly widespread parasite sequestration; bioactive toxins; increased vascular permeability; reduced tissue perfusion; anaemia; pulmonary airway obstruction; hypoxia; increased host glycolysis; repressed gluconeogenesis. Some overlap with shock-like syndrome
Shock-like syndrome (systemic inflammatory-response-like syndrome)	Shock, haemodynamic changes, impaired organ perfusion, disseminated intravascular coagulation	Bioactive toxins; T_H1 cytokines; acute-phase reactants

EMP1, erythrocyte membrane protein 1; GPI, glycosylphosphatidylinositol; IFN- γ , interferon- γ ; IL-10, interleukin-10; RBC, red blood cell; T_H , T helper; TNF, tumour-necrosis factor.

Figure 5: Severe and fatal disease syndromes, clinical features and possible sequence of events in malaria as represented in the table above (Adapted from: Schofield et al., 2005).

In conclusion, understanding the life cycle and pathogenicity of *Plasmodium falciparum* is crucial for developing effective treatments and preventive measures against malaria. Ongoing research is essential for creating vaccines, improving diagnostic methods, and finding new antimalarial drugs to combat this significant global health challenge. The complexities of the parasite's life cycle and its mechanisms of pathogenicity underline the need for continued vigilance and innovation in malaria control efforts.

2. Gene Regulation in Malaria parasite

2.1. Life Cycle Complexity and Gene Regulation

Malaria parasites exhibit a complex life cycle that alternates between two hosts, transitioning through different morphological and physiological stages. At each stage, the parasite must express specific sets of genes to survive and adapt to varying environmental conditions. A stringent pattern of gene expression regulation enables *Plasmodium* parasite to complete its life cycle and also evade numerous stresses posed against it and even develop drug resistance along with continuing its progression through sexual stages. Exemplary research has proven the model of just in time expression of gene expression which elaborates that genes in throughout this process, mRNAs from the majority of genes are transcribed across multiple time points but reach peak abundance only once during the IDC, which is thought to correspond to the time point when its encoded product is most required - this is called ‘just in time expression’ model (Painter et al., 2018). It has also been demonstrated that gene expression is remarkably conserved across all the *Plasmodium* strains (Painter et al., 2018) (Hoo et al., 2016). Recent studies have shown that this process is apparently hardwired and unresponsive to perturbations.

It has also been well documented that there is an absence of RNA interference in the *Plasmodium* parasites as certain important proteins responsible for this machinery are absent from the parasite hence rendering the idea of RNAi mediated gene regulation out of consideration.

To complicate the scenario of plausible aspects of gene regulation it is very well known that the parasite has a peculiar genome majority of which is in the euchromatin stage. Predominantly the genome is composed of AT bases. The AT bias in the genome reaches to 80-85% in the coding regions whereas it can go up to 90-95% AT richness in the non-coding regions. Such extreme AT bias can lead to transcriptional biases and also bring about challenges in gene expression control and regulation. A constitutively open chromatin with AT bias can be a major limitation in transcriptional processes specially for a parasitic system which needs to adapt and multiply quickly in a rapidly changing environment hence making it even more intriguing to understand as to how gene regulation takes place within the parasite.

It has also been very well researched that there is a general paucity of transcription factors in the parasite only known till date are the Apetala2 family transcription factors. To date 20 such factors have been documented. There are also limited ways of gene repression known in the system. Gene repression is limited to only a few repressive epigenetic marks and hence it is largely unclear how genes are turned to response in such an effective manner (Thompson et al., 2023).

While looking at a finer resolution, it is observed that approximately 90% of the *Plasmodium* genome is transcribed generating not just mRNA bearing protein-coding potential but also a vast diversity of non - coding RNA (ncRNA) majority of which are known today to be antisense transcripts in nature (Chappell et al., 2020). But the presence of antisense transcripts during the erythrocytic stages of the parasites was revealed by one of the earliest studies published in 2001 using Serial Analysis of Gene Expression (SAGE) technology by Patankar et al. (Patankar et al., 2001). Further, the extent of this phenomenon was determined by surveying the distribution of sense and antisense transcripts at the asexual transcriptome level, providing the first hints for the possible function of antisense transcription in the *Plasmodium* system (Gunasekera et al., 2004).

Antisense transcripts are transcribed from the strand opposite to that of the sense transcript of either protein-coding or non-scripts are transcribed from the strand opposite to that of the sense transcript of either protein-coding or non- protein-coding genes (Pelechano & Steinmetz et al., 2013). But whether their vast diversity is translated into functional characteristics remains elusive and controversial. However, more and more studies across different model systems are shedding light on their role in the regulation of gene expression (Xu et al., 2018). As a general observation, it has been found along with the increasing complexity of the organism, diversity, and levels of ncRNA scale up while there is not a great change in the protein-coding genes. This implies that they have a crucial role to play in regulating the morphological complexity of the cell (Mattick et al., 2018). The sheer permissiveness, diversity throws limelight on an unexplored dimension of antisense transcription. Recent research utilizing state of art RNA sequencing technologies have shown that *Plasmodium* transcribes more antisense RNA (AS) than any other eukaryotic system where their role in gene regulation has also been well documented (Chappell et al., 2020).

It is intriguing to understand why a parasite that transpires a life cycle of a limited period across two completely different hosts would evolve to produce enormous amounts of non-coding antisense RNA if they had no function within the cell. Under such a paradigm situation the focus is turned towards antisense transcription as a strategy for gene regulation, as its prevalence and importance have been studied through other models and disease systems as well. The significance of this phenomenon is highlighted by the fact that antisense transcripts can act at potentially all stages of gene expression, from transcription to translation to RNA degradation. They are being popularly regarded as regulatory networks that could ‘rewire’ the expression of genes either along with protein complexes or alone could form a self - regulatory circuit that could regulate the expression of their sense counterpart (mRNA) or adjacent genes (NAT pair) (Pelechano & Steinmetz et al., 2013). They have also been implicated in increasing the virulence of the parasite in clinical conditions (Amit-Avraham et al., 2015; Barcons-Simon et al., 2020; Kate M. Broadbent et al., 2015; Kate Mariel Broadbent, 2014; Droll et al., 2018; Epp et al., 2009; Guizetti et al., 2016; Jing et al., 2018; Yin et al., 2020; Boopathi et al., 2013, 2014; Subudhi et al., 2014a, 2014b).

The speculations of long non-coding RNA (lncRNA) being regulators of gene expression in *Plasmodium* arise from the fact that all kinds of conventional gene regulation machinery that operate in other eukaryotic systems are absent in this system. The classical mechanisms of gene regulation like RNA interference, non-homologous end joining are absent in *Plasmodium*. Additionally, gene regulator molecules like microRNA are absent (Baum et al., 2009; Chamnanchanunt et al., 2017; Deitsch et al., 2007; Toenhake & Bártfai, 2019). It has also been shown that an abundance of bidirectional promoter activity leads to the generation of Antisense transcripts although the promoter architecture remains largely uncharacterized especially for Antisense transcription (Chappell et al., 2020). The expression patterns of AS have been shown to strongly correlate with the temporal expression of the adjacent coding mRNA, suggesting that these transcript pairs are co-regulated. It is also believed that AS are not just a byproduct of bidirectional promoter activity but could also have a function in gene regulation, possibly mediated by the binding of regulatory proteins containing an RNA recognition motif (RRM), many of which are encoded in the *P. falciparum* genome (Reddy et al., 2015), but which are largely uncharacterized (Bunnik et al., 2016). Additionally, our study of independently regulated

antisense transcription may help future studies of gene regulation, as seen with the antisense transcript of PfGDV1 which decides the fate of sexual commitment (Filarsky et al., 2018, *Science*). We propose that study of AS would implicate in a clearer understanding of the infection biology.

Gene regulation in response to adaptation to host conditions including stresses perceived by the parasite

Upon entering the human body, *P. falciparum* must adapt to various stress conditions in order to continue its virulence cycle. Some of the common stress that the parasite is able to tide are elaborated below and also summarised in Figure 6 below

- **Temperature Stress:** The parasite endures temperature fluctuations due to the host's fever response, which can impact its development and viability.
- **Nutrient Stress:** The parasite experiences nutrient stress from the fluctuating availability of essential nutrients in the host's bloodstream, necessitating efficient nutrient uptake and utilization mechanisms.
- **Density Stress:** As the parasite population increases, competition for resources and space within the erythrocytes intensifies, which can influence its growth and reproduction.
- **Pharmacological Stress:** Exposure to antimalarial drugs presents a significant challenge. Over time, *P. falciparum* has developed mechanisms to resist these drugs, enhancing its virulence and survival (Rawat et al., 2021).

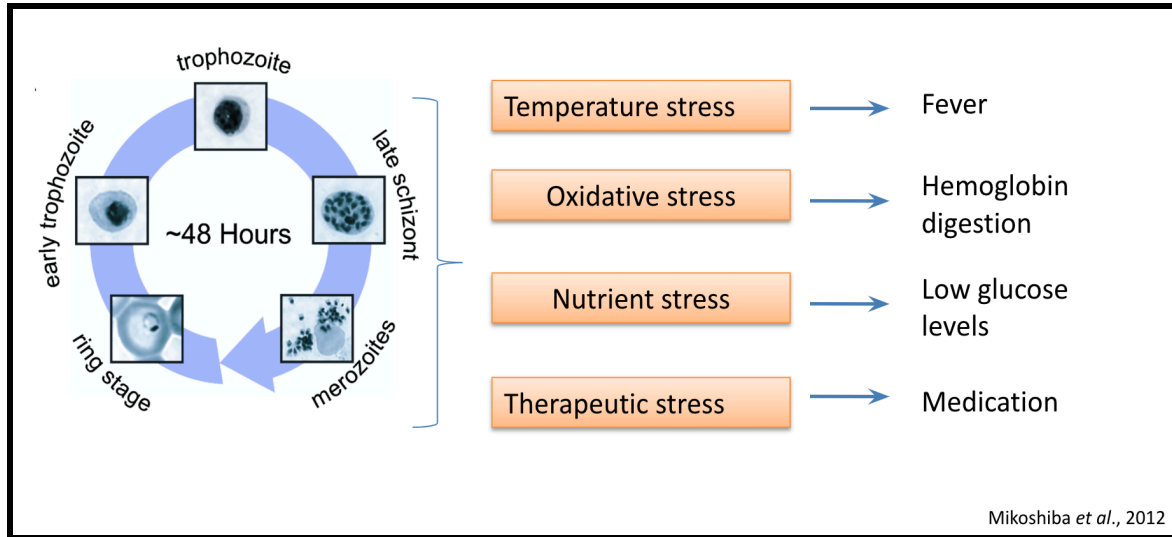


Figure 6: Schematic of stresses faced by the *Plasmodium falciparum*: Namely Temperature stress caused because of fever induction, Oxidative stress which is a result of hemoglobin digestion, Nutrient stress caused by depleting glucose levels within the bloodstream and finally Therapeutic stress caused by introduction of medications during treatment paradigm by medical professionals.

Gene regulation for the purpose of immune evasion

The host's immune system poses a constant threat to the parasite, employing various surveillance mechanisms to detect and eliminate it. For a successful infection, *P. falciparum* has evolved several immune evasion strategies, including **Antigenic Variation**: By frequently altering its surface antigens, primarily through the expression of PfEMP1, the parasite can evade recognition by the host's immune system, prolonging its survival (Deutsch et al., 2017; Scherf et al., 2008; Kyes et al., 2007; Schneider et al., 2023; Michel-Todó et al., 2023; Florini et al., 2022).

Gene regulation in response to transition to sexual stages

For transmission to mosquitoes, *P. falciparum* undergoes sexual differentiation, requiring specific morphological and physiological changes in response to environmental cues. This transition is crucial for the parasite's life cycle, enhancing its chances of successful uptake by the mosquito vector.

In summary, *Plasmodium falciparum* showcases remarkable adaptability, allowing it to thrive in the human host despite facing numerous stresses and immune challenges (Hollin, T., 2020; Russell et al., 2023; Kengne-Ouafo et al., 2023; Prajapati et al., 2020; Liu et al., 2024; Russell et al., 2023). This ability to navigate its environment and evade the immune response is vital for its virulence and continued transmission.

2.2 Antigenic Variation and Immune Evasion in *Plasmodium falciparum*: a detailed introduction

Plasmodium falciparum, the most virulent species of malaria-causing parasites, has developed sophisticated strategies for immune evasion, with antigenic variation being a key mechanism. This allows the parasite to persist in the host despite the immune response aimed at eliminating it. Below is a detailed exploration of how *P. falciparum* utilizes antigenic variation and other strategies for immune evasion. A summary of the variable surface antigens is also provided in Figure 7 below.

a. PfEMP1 Expression

- **PfEMP1 (*Plasmodium falciparum* Erythrocyte Membrane Protein 1):** This is the primary antigen responsible for antigenic variation. PfEMP1 is encoded by a large family of genes known as *var* genes, which can number over 60 different variants (Othman et al., 2023; Hviid et al., 2024; Kinyua et al., 2024; Otoboh et al., 2024; Hviid et al., 2023; Chen et al., 2021).
- **Switching Mechanism:** The parasite can switch between different *var* genes during its life cycle, resulting in the expression of different PfEMP1 variants on the surface of infected red blood cells (RBCs). This process is mediated by genetic recombination and transcriptional regulation, allowing the parasite to alter its surface antigens rapidly (Schneider et al., 2023; Smith et al., 2014; Janes et al., 2011; Mok et al., 2008; Horrocks et al., 2005).

b. Clonal Variation

- **Diversity of Antigens:** Within a single infection, multiple clonal variants of *P. falciparum* can coexist, each expressing distinct PfEMP1 variants (Rovira-Graells et al., 2012; Scherf et al., 2008; Gómez-Díaz et al., 2017; Lavazec et al., 2007; Khattab et al., 2011; Beeson et al., 2002; Howitt et al., 2009). This diversity complicates the immune response, as the host immune system must continuously adapt to recognize new antigens.

C. Other Surface Proteins Involved in Immune Evasion

In addition to PfEMP1, other surface proteins contribute to antigenic variation and immune evasion:

- **RAPs (Reticulocyte-Binding Protein Homologs):** These proteins facilitate the binding of the parasite to reticulocytes and play a role in the invasion of RBCs. Variations in RAPs can also contribute to immune evasion (Baldi et al., 2000; Ghosh et al., 2017; Hollin et al., 2022).
- **RIFINs (Repetitive Interspersed Family of Proteins):** These proteins are involved in immune evasion through their ability to undergo antigenic variation, thereby altering the surface properties of infected RBCs (Dinko et al., 2016; Goel et al., 2015; Petter et al., 2007).

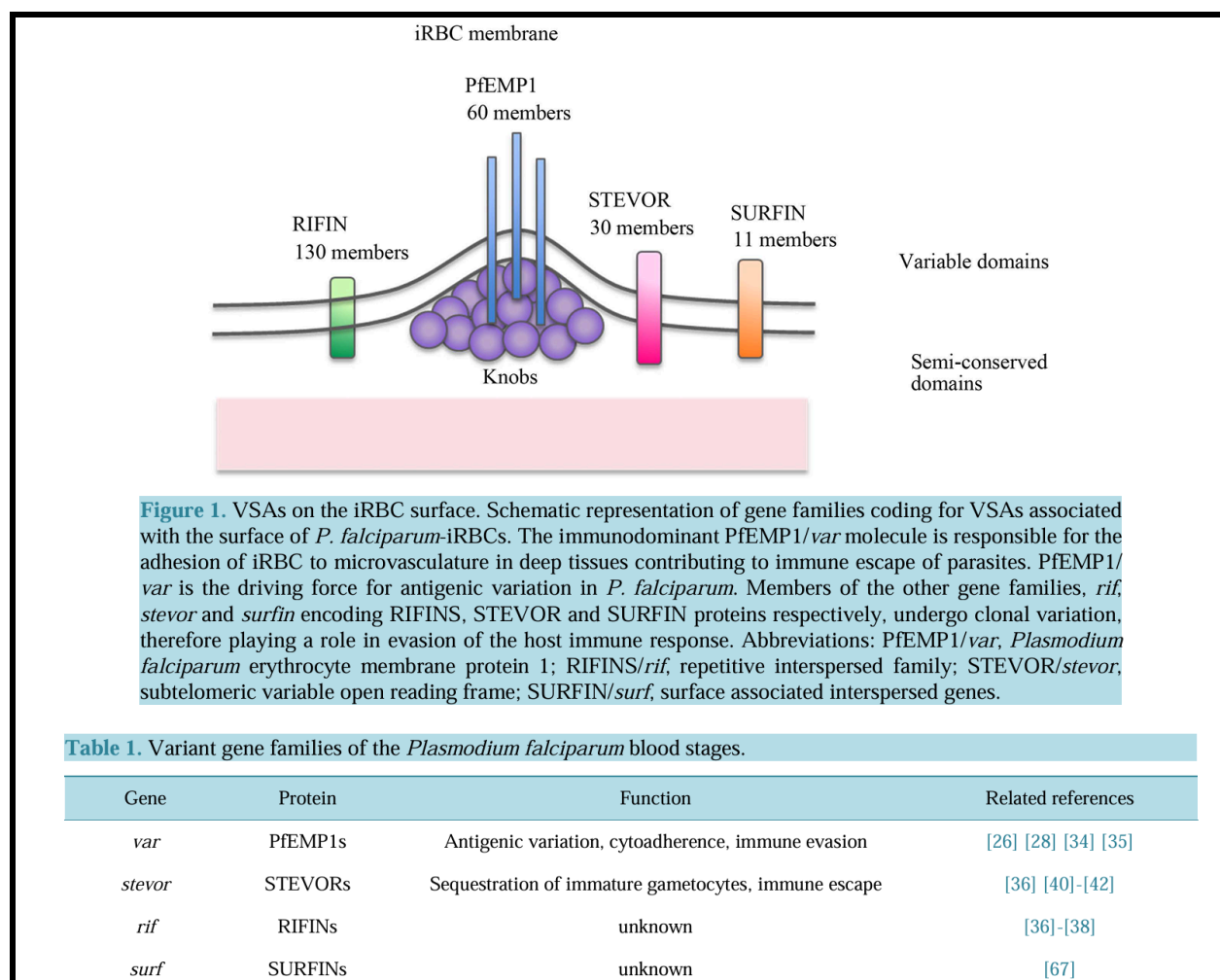


Figure 7: Schematic shows Variable Surface antigens on infected RBCs surface. Schematic of gene families encoding Variant Surface Antigens (VSA) which are associated with the surface of infected RBCs (Adapted from: Dinko et al., 2016)

2.3. Immune Evasion Strategies stem from the underlying pattern of gene expression: a detailed introduction

a. Avoidance of Immune Detection

- **Evasion of Antibody Response:** By changing its surface antigens, *P. falciparum* can evade pre-existing antibodies in the host. This strategy allows the parasite to survive despite the presence of an adaptive immune response (Belachew et al., 2018; Wright et al., 2014; Persson et al., 2008; Saito et al., 2017; Malaguarnera et al., 2002; Schmidt et al., 2015).
- **Sequestration:** Infected RBCs can adhere to endothelial cells in the blood vessels, particularly in the brain and placenta. This sequestration helps the parasite evade immune detection and contributes to severe complications like cerebral malaria (Scherf et al., 2008).

b. Inhibition of Immune Responses

- **Cytokine Modulation:** *P. falciparum* can influence the host's immune response by modulating cytokine release. The parasite can induce the production of immunosuppressive cytokines like IL-10, which can dampen the immune response, facilitating its persistence in the host (Obeagu et al., 2024; Afolayan et al., 2020; Popa et al., 2021). Factors that contribute to giving rise to antibody-independent (T-cell) immunity are shown in Figure 8 below.

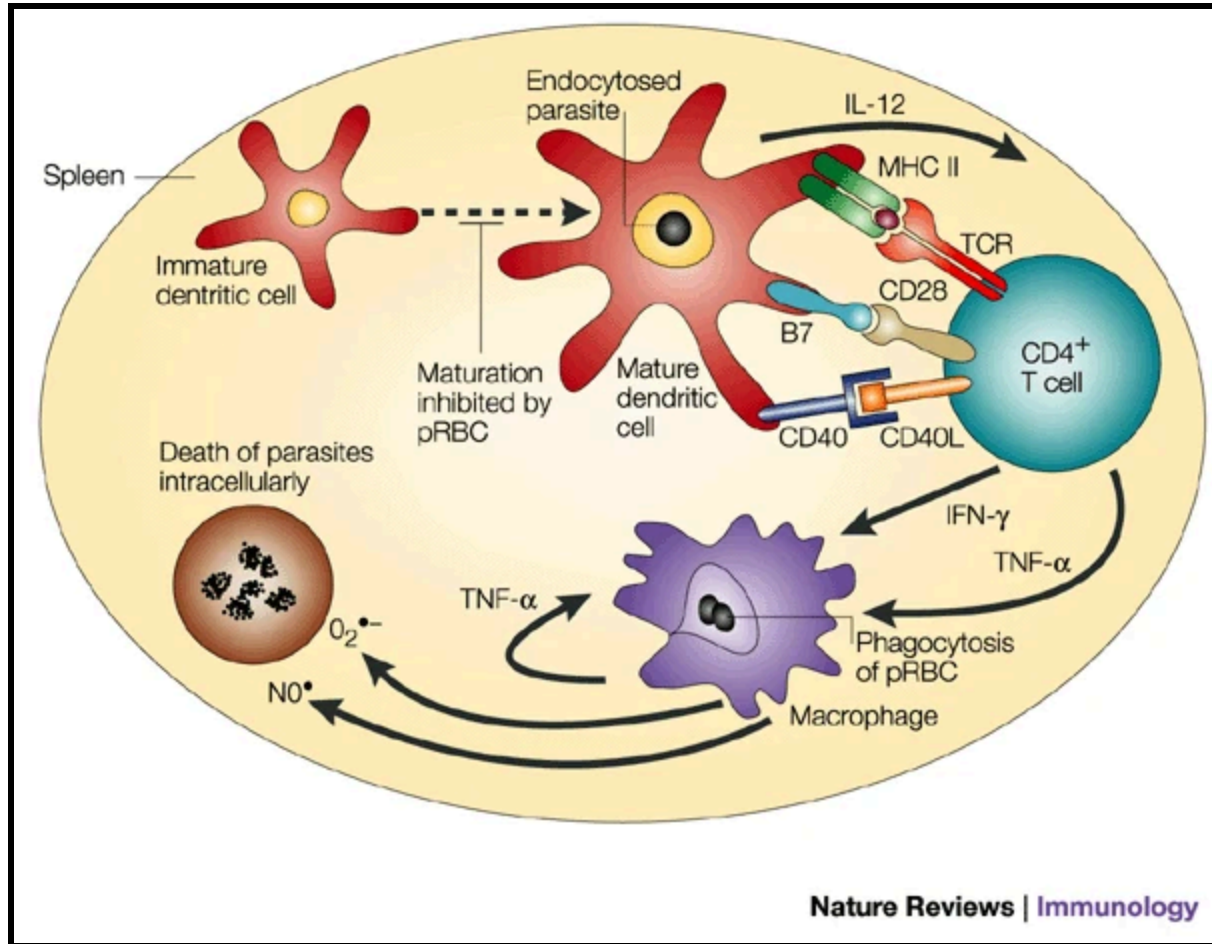


Figure 8: Factors that contribute to giving rise to antibody-independent (T-cell) immunity are shown. Mature dendritic cells first activate CD4⁺ T cells, which in turn triggers macrophage activation, phagocytosis of parasitised red blood cells (pRBC), and the production of cytokines and other minor inflammatory chemicals (including oxygen radicals and nitric oxide). It is believed that the spleen has a major, if not exclusive, role in T-cell immunity. TCR stands for T-cell receptor; TNF-α for tumor-necrosis factor-α; MHC II for major histocompatibility complex class II; IFN-γ for interferon-γ; and IL-12 for interleukin-12 (Adapted from: Good et al., 2001, *Nat Rev Immunol*).

2.4. Drug Resistance generation is a result of underlying genomic alterations: a detailed overview

The development of drug resistance in malaria is partly driven by mutations and alterations in gene expression, particularly in genes involved in the metabolic pathways targeted by antimalarial drugs. For instance, mutations in the *pfcrt* (chloroquine resistance transporter) and *pfmdr1* (multidrug resistance gene) affect the parasite's ability to survive in the presence of chloroquine and other antimalarials (Tuedom et al., 2021; Wicht et al., 2020; Fontecha et al., 2021; Yobi et al., 2021).

Regulation of gene expression can also contribute to adaptive resistance mechanisms, where the parasite alters the expression of transporter proteins or other molecules to reduce drug efficacy (Gil et al., 2021; Wunderlich et al., 2022, Wicht et al., 2020). A summary of the drug resistance pathways which are mediated via changes in gene expression paradigm are listed below:

a. Genetic Mutations

- **Point Mutations:** Mutations in specific genes can confer resistance to particular drugs. For example:
 - **PfCRT (*Plasmodium falciparum* Chloroquine Resistance Transporter):** Mutations in this gene are associated with chloroquine resistance, affecting drug transport into the parasite (Tuedom et al., 2021; Wicht et al., 2020; Fontecha et al., 2021; Yobi et al., 2021).
 - **PfMDR1 (*Plasmodium falciparum* Multidrug Resistance 1):** Variants in this gene can influence resistance to multiple drugs, including mefloquine and artemisinin derivatives (Tuedom et al., 2021; Wicht et al., 2020; Fontecha et al., 2021; Yobi et al., 2021).

b. Increased Drug Efflux

- **Transport Proteins:** Mutations in transport proteins, such as PfCRT and PfMDR1, can lead to increased efflux of the drug from the parasite, reducing its efficacy (Gil et al., 2021; Wunderlich et al., 2022, Wicht et al., 2020).

c. Alterations in Drug Target Sites

- **Target Modification:** Changes in the structure of drug target sites, such as the dihydrofolate reductase-thymidylate synthase complex (DHFR-TS), can lead to antifolate resistance through mutations in the corresponding genes (Pacheco et al., 2020; Wicht et al., 2020; Chaianantakul et al., 2020; Oléfongo et al., 2024; Kamchonwongpaisa et al., 2020).

d. Enhanced Detoxification Pathways

- **Reactive Oxygen Species (ROS) Management:** Some resistant strains exhibit enhanced detoxification mechanisms, allowing them to cope with the oxidative stress induced by drugs like artemisinin (Ribbiso et al., 2021; Gupta et al., 2022; de Villiers et al., 2021).

2.5. Transmission and Sexual Development is promoted via altering gene expression: a detailed overview

Gene regulation plays a key role in the differentiation of asexual blood-stage parasites into sexual forms (gametocytes), which are necessary for transmission to mosquitoes. A finely-tuned regulatory network controls this process, ensuring that the parasite population maintains both asexual forms, responsible for human disease, and gametocytes, which ensure transmission and survival in the mosquito vector (Venugopal et al., 2020).

Recent studies have highlighted transcription factors like *AP2-G* that are crucial for activating the gametocyte development pathway. Understanding these regulators opens new opportunities for interrupting transmission, as blocking sexual differentiation could halt the spread of the parasite (Venugopal et al., 2020; Rea et al., 2018). Refer to Figure 9 below for a detailed description of sexual development in the parasite.

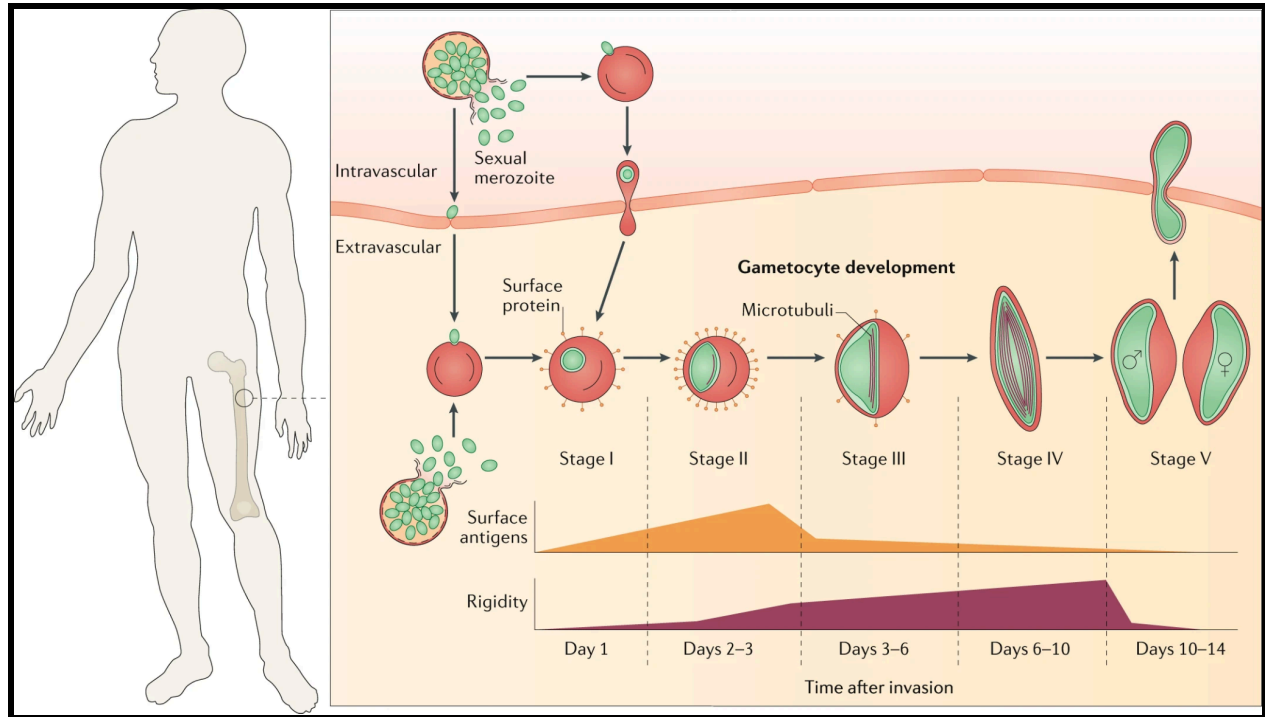


Figure 9: A subset of schizonts commit to the sexual cycle, producing sexual merozoites. Merozoites and young gametocytes (green) home to the bone marrow, leave the sinusoids and enter the parenchyma. Alternatively, the gametocytes form in the parenchyma from committed schizonts. In the bone marrow parenchyma, gametocytes develop from stage I to stage IV. Remodeling of the membrane of the host red blood cell (red) results in transient deposition of surface antigens (orange) and a reversible increase in cellular rigidity (purple). Restored deformability during maturation to stage V gametocytes triggers their release back into the bloodstream, where they can be taken up during another mosquito bite (Adapted from: Venugopal et al., 2020).

2.6. Research and Therapeutic Implications

Gene regulation in malaria provides valuable insights for developing new therapeutic strategies. By identifying key regulators of parasite development, scientists can target specific stages of the life cycle or block vital processes such as immune evasion or transmission. Advances in gene editing technologies, such as CRISPR-Cas9, allow researchers to manipulate parasite genes and study their roles in real-time, offering potential avenues for drug and vaccine development.

Inhibiting key gene regulatory pathways could also prevent the parasite from switching antigens, making it easier for the immune system to recognize and eliminate it. Additionally, understanding the regulatory mechanisms behind drug resistance can aid in designing next-generation antimalarials that circumvent resistance mechanisms.

In conclusion, Gene regulation is central to the survival, adaptability, and pathogenicity of malaria parasites. It governs critical processes such as life cycle progression, immune evasion, drug resistance, and transmission. A deeper understanding of how *Plasmodium* regulates its genes holds the key to developing more effective interventions for malaria control and eradication, including novel drugs and vaccines. Therefore, ongoing research into the molecular mechanisms of gene regulation in malaria parasites remains vital to combatting this global health challenge.

3. Literature Review on Gene Regulation in *Plasmodium* Species

The malaria-causing *Plasmodium* species exhibit complex gene regulatory mechanisms that are essential for their survival and adaptability throughout their life cycle, which alternates between a mosquito vector and a human host. Understanding the molecular details of gene regulation in *Plasmodium* is crucial for uncovering parasite biology and identifying novel therapeutic interventions to control and eliminate malaria. This literature review summarizes the current knowledge on the transcriptional, epigenetic, and post-transcriptional mechanisms governing gene expression in *Plasmodium* species, with a particular focus on *Plasmodium falciparum*.

3.1. Overview of Gene Regulation in *Plasmodium* Species

Gene regulation in *Plasmodium* is stage-specific and occurs in response to the parasite's changing environments as it moves between the mosquito and human host. The parasite has evolved sophisticated strategies to ensure precise control of gene expression during key transitions such as sporozoite formation, merozoite invasion, and gametocytogenesis. These

regulatory mechanisms enable the parasite to adapt to temperature fluctuations, nutrient availability, immune pressures, and other external factors.

Research over the past decade has revealed that *Plasmodium* employs several modes of gene regulation, including transcriptional control, chromatin-based epigenetic regulation, and post-transcriptional modifications. Each layer of regulation plays a specific role in the parasite's developmental progression.

3.2. Transcriptional Regulation in *Plasmodium*

a. Transcription Factors

- Unlike many other eukaryotes, *Plasmodium* has a significantly reduced number of canonical transcription factors. Instead, it relies heavily on a family of unique transcription factors known as **Apetala 2 (ApiAP2)** proteins, which are homologous to plant AP2 transcription factors.
 - **ApiAP2 proteins:** These transcription factors are critical for controlling gene expression during different stages of the parasite's life cycle. For example, **AP2-G** is essential for the transition to gametocytogenesis, marking the switch from asexual to sexual development (Kafsack et al., 2014). Other ApiAP2 proteins regulate the expression of genes necessary for sporozoite development, erythrocyte invasion, and immune evasion (Painter et al., 2011).
- **Stage-Specific Transcriptional Waves:** Studies using transcriptomic profiling methods such as microarrays and RNA sequencing have demonstrated that *Plasmodium* gene expression is highly stage-specific. The work by (Bozdech et al., 2003) was pioneering in showing that gene expression in *P. falciparum* follows a cyclical pattern during the intraerythrocytic developmental cycle (IDC). Genes involved in invasion, replication, and egress are expressed in tightly controlled temporal waves, with transcription being fine-tuned at each phase of the cycle.

b. Promoter Architecture and RNA Polymerase II Activity

- The regulation of transcription initiation is mediated by weak and degenerate promoter sequences in *Plasmodium* species, often lacking the well-conserved TATA boxes seen in other eukaryotes. Nonetheless, promoter regions contain essential **cis-regulatory elements** that recruit the general transcription machinery, including **RNA Polymerase II (Pol II)**, to initiate gene transcription.
- RNA Pol II activity is modulated throughout the life cycle, with Pol II occupancy patterns correlating with stage-specific gene expression (Toenhake et al., 2018). Chromatin immunoprecipitation (ChIP) studies have identified key genomic regions where Pol II binds, providing insights into how transcriptional activation is spatially and temporally regulated.

3.3. Epigenetic Regulation in *Plasmodium*

a. Chromatin Structure and Histone Modifications

Epigenetic regulation plays a significant role in controlling gene expression in *Plasmodium* species. Epigenetic changes are primarily mediated through modifications to histone proteins, which influence chromatin structure and accessibility.

- **Histone Modifications:** The post-translational modification of histones, including **acetylation**, **methylation**, and **phosphorylation**, regulates gene expression by altering the accessibility of DNA to transcriptional machinery. For example, histone H3 lysine 9 acetylation (H3K9ac) is associated with active transcription, while histone H3 lysine 9 trimethylation (H3K9me3) is linked to gene silencing.
- **Heterochromatin Protein 1 (HP1):** HP1 is a key player in the formation of heterochromatin, a densely packed form of chromatin that silences gene expression. In *P. falciparum*, HP1 is essential for silencing the **var gene family**, which encodes **PfEMP1**, a protein responsible for antigenic variation and immune evasion (Flueck et al., 2009).

b. Antigenic Variation and Var Gene Silencing

- *P. falciparum*'s ability to evade the immune system is primarily driven by antigenic variation. This process is governed by the mutually exclusive expression of **var genes**,

which encode different variants of PfEMP1, the primary protein responsible for cytoadherence of infected erythrocytes to the endothelial lining.

- **Epigenetic Silencing:** Only one var gene is expressed at any given time, while the others are epigenetically silenced by heterochromatin marks such as H3K9me3 and HP1 association (Scherf et al., 2008). Epigenetic switching between active and inactive var genes allows the parasite to periodically alter its surface antigen, helping it evade host immune responses.

c. Nucleosome Positioning and Chromatin Remodeling

- Nucleosome positioning and chromatin remodeling further contribute to transcriptional regulation in *Plasmodium*. The parasite's genome is compact, with nucleosomes closely spaced and actively remodeled during the transition between life cycle stages (Ponts et al., 2010). This remodeling allows the parasite to rapidly activate or repress genes in response to external cues such as nutrient availability or host immune factors.

3.4. Post-Transcriptional Regulation in *Plasmodium*

a. mRNA Stability and Degradation

Post-transcriptional regulation in *Plasmodium* plays a crucial role in fine-tuning gene expression by controlling mRNA stability, degradation, and translation.

- **mRNA Decay:** The half-life of mRNAs in *Plasmodium* varies widely, with stage-specific regulation of mRNA stability observed during the IDC and gametocyte stages. For instance, mRNAs encoding proteins no longer needed at specific stages are rapidly degraded (Shock et al., 2007).
- **mRNA-binding proteins (RBPs):** Several RBPs have been identified that regulate the stability and translation of specific mRNAs, helping the parasite to regulate protein production in response to environmental changes (Mair et al., 2010).

b. Non-Coding RNAs and Antisense Transcription

- **Non-Coding RNAs:** While *Plasmodium* lacks canonical RNA interference (RNAi) pathways, non-coding RNAs, including **antisense RNAs (asRNAs)**, have been implicated in the regulation of gene expression. Antisense transcripts are often found overlapping with sense transcripts, potentially regulating the stability, splicing, or translation of corresponding mRNAs (Raabe et al., 2009).
- **Functional Role of Antisense RNAs:** Although the exact role of antisense transcription remains an active area of research, evidence suggests that antisense RNAs in *P. falciparum* may regulate the expression of virulence factors, including *var* genes and genes involved in drug resistance.

c. Translational Control

- **Translational repression:** In addition to mRNA stability, translation is tightly regulated. For example, during the sexual stages in the mosquito, many mRNAs are stored in a translationally repressed state until they are needed for transmission into the human host. This translational control is mediated by RBPs that bind specific sequences in mRNA, preventing ribosome access until the appropriate signal is received for protein synthesis (Mair et al., 2006).

3.5. Gene Regulatory Networks and Systems Biology Approaches

a. Gene Expression Profiling

The use of transcriptomic approaches such as RNA-Seq and microarrays has been instrumental in mapping the gene expression profiles of *Plasmodium* species across different life cycle stages. These datasets have revealed that gene expression in *Plasmodium* is highly regulated and dynamic, with distinct transcriptional programs activated in response to host environments.

b. Systems Biology and Regulatory Networks

- **Gene Regulatory Networks (GRNs):** Systems biology approaches have been used to model **gene regulatory networks (GRNs)** that describe the interactions between transcription factors, chromatin modifiers, and target genes. These models provide

insights into how the parasite integrates multiple layers of regulation to control stage-specific gene expression (Llinás et al., 2006).

3.6. Challenges and Future Directions

a. Lack of RNAi Machinery

Unlike many other eukaryotes, *Plasmodium* species lack the canonical RNA interference (RNAi) machinery. This has posed challenges in studying gene function and regulation. However, the development of alternative gene manipulation techniques such as **CRISPR-Cas9** and conditional knockdown systems has allowed researchers to overcome these challenges and explore gene function in the parasite (Ghorbal et al., 2014).

b. Therapeutic Potential

Understanding gene regulation in *Plasmodium* offers potential therapeutic opportunities. For instance, targeting epigenetic regulators, such as histone-modifying enzymes or nucleosome remodelers, could disrupt the parasite's ability to regulate key genes involved in survival, virulence, and drug resistance.

To conclude, gene regulation in *Plasmodium* species is a multifaceted process that involves transcriptional, epigenetic, and post-transcriptional mechanisms. These regulatory processes allow the parasite to adapt to different environments and hosts, making them essential for parasite survival and virulence. Continued research into these mechanisms, aided by advanced technologies such as CRISPR and systems biology approaches, promises to provide deeper insights into *Plasmodium* biology and identify novel targets for malaria intervention strategies.

4. Overview of Transcriptional and Post-Transcriptional Regulation in *Plasmodium falciparum*

The regulation of gene expression in *Plasmodium falciparum*, the causative agent of the most severe form of malaria, is a complex process that occurs at both the transcriptional and post-transcriptional levels. This tightly regulated system allows the parasite to adapt to different environments, including human hosts and mosquito vectors, as it progresses through its complex life cycle. This overview explores the key mechanisms of transcriptional and post-transcriptional regulation in *P. falciparum* and their role in parasite survival, pathogenicity, and adaptation.

4.1. Transcriptional Regulation in *P. falciparum*

a. Transcription Factors and Regulatory Elements

- *P. falciparum* has a significantly reduced number of classical transcription factors compared to other eukaryotes. Instead, the parasite relies heavily on a family of specialized transcription factors known as **ApiAP2** (Apetala 2) proteins, which are unique to apicomplexan parasites.
 - **ApiAP2 proteins:** These proteins play crucial roles in regulating stage-specific gene expression throughout the parasite's life cycle. For instance, **AP2-G** is essential for the initiation of gametocytogenesis, marking the transition from asexual to sexual forms (Sinha et al., 2014).
 - **Stage-specific transcription:** Different ApiAP2 proteins are active at different stages of development, enabling precise regulation of gene expression that allows the parasite to transition between various stages like merozoites, trophozoites, schizonts, and gametocytes (Siegel, T. N., 2014).

b. Gene Regulatory Elements and Promoters

- Promoter regions in *P. falciparum* contain various **cis-acting elements**, but these elements are often more degenerate than in other eukaryotic organisms. The basic components, such as TATA boxes and transcription initiation sites, are present but exhibit lower conservation.

- Despite the lack of conventional transcription factors, **RNA Polymerase II (Pol II)** remains central to transcriptional initiation and elongation. Its activity is regulated according to the parasite's developmental stage, controlling the transcription of key genes such as those required for red blood cell invasion or immune evasion.

c. Chromatin and Epigenetic Regulation

Epigenetic mechanisms, particularly **histone modifications** and **chromatin remodeling**, play a key role in regulating gene expression in *P. falciparum*.

- **Histone modifications:** The addition of methyl or acetyl groups to histone tails can either activate or repress gene expression by altering chromatin structure. For instance, **histone H3 lysine 9 trimethylation (H3K9me3)** is associated with gene silencing, while **H3K9 acetylation (H3K9ac)** is linked to gene activation. (Scherf et al., 2008)
- **Heterochromatin Protein 1 (HP1):** HP1 is involved in the formation of repressive heterochromatin and plays a role in the silencing of virulence genes such as **var** genes, which encode the immune evasion protein PfEMP1. This silencing is crucial for antigenic variation, which allows the parasite to evade the host immune system.

d. Antigenic Variation and Transcriptional Control

- The **var gene family** is one of the most studied examples of transcriptional regulation in *P. falciparum*. These genes are responsible for the production of **PfEMP1**, a key protein that enables the parasite to evade immune detection by altering the antigenic profile of infected red blood cells.
 - **Monoallelic expression:** Only one *var* gene is transcribed at a time, while the others remain epigenetically silenced. The regulation of this monoallelic expression involves a combination of chromatin remodeling, histone modifications, and nuclear positioning.

4.2. Post-Transcriptional Regulation in *P. falciparum*

a. Alternative Splicing and RNA Processing

Post-transcriptional regulation in *P. falciparum* is an additional layer of gene regulation that determines how transcribed mRNAs are processed, spliced, stabilized, or translated.

- **Alternative splicing:** Although relatively rare in *P. falciparum* compared to other eukaryotes, alternative splicing occurs and contributes to generating protein diversity. Some genes involved in essential functions, such as invasion and metabolism, undergo alternative splicing.
- **RNA processing:** This includes the addition of a 5' cap and poly-A tail, as well as the removal of introns through splicing. The processing of RNA transcripts is necessary for producing mature mRNA that can be translated into proteins (Scherf et al., 2008)

b. mRNA Stability and Degradation

- **mRNA decay pathways:** *P. falciparum* has mechanisms in place to control the stability and degradation of mRNA transcripts, allowing the parasite to rapidly alter gene expression in response to environmental cues, such as temperature changes or the presence of drugs. For instance, mRNAs encoding proteins no longer required for a specific stage of development are degraded through regulated pathways.
- **mRNA half-life:** Studies have shown that the half-life of mRNAs varies significantly across different life cycle stages. Transcripts with shorter half-lives allow the parasite to rapidly shift its protein expression profile, while longer-lived mRNAs provide a stable supply of essential proteins during slower stages of the life cycle.

c. Non-Coding RNAs and Antisense Transcripts

Non-coding RNAs, including **antisense RNAs** (asRNAs) and other regulatory RNAs, are emerging as key players in post-transcriptional gene regulation in *P. falciparum*.

- **Antisense RNAs (asRNAs):** These are transcribed from the opposite strand of protein-coding genes and often overlap with the sense transcript. Antisense RNAs have been shown to regulate gene expression by affecting the stability, translation, or degradation of their corresponding sense mRNAs. For example, antisense transcription plays a role in controlling the expression of *var* genes (Scherf et al., 2008).

- **Regulation by small RNAs:** While *P. falciparum* lacks a canonical RNA interference (RNAi) pathway, small RNAs have been implicated in regulating gene expression post-transcriptionally. Although their exact role is still being investigated, small RNAs could influence processes such as mRNA stability or translation efficiency.

d. Translation Regulation

The regulation of translation—how mRNAs are converted into proteins—is another crucial aspect of post-transcriptional control in *P. falciparum*.

- **Ribosome-associated mechanisms:** Translational control is tightly linked to the life cycle stages of the parasite. In the mosquito vector, for instance, many mRNAs are stored in a translationally inactive state until the parasite enters the human host, where these transcripts are then activated for translation.
- **Protein complexes and translational repression:** Translational repression is often mediated by **RNA-binding proteins (RBPs)**, which bind to specific sequences or structures within the mRNA, preventing ribosome access and thus inhibiting protein synthesis. This form of regulation allows *P. falciparum* to conserve energy and resources by ensuring that proteins are only produced when needed (Scherf et al., 2008).

In conclusion, the regulation of gene expression in *Plasmodium falciparum* involves a complex interplay of transcriptional and post-transcriptional mechanisms. Transcription factors like ApiAP2 proteins, epigenetic modifications, and chromatin remodeling drive stage-specific transcription, while post-transcriptional mechanisms, including mRNA processing, stability control, and translational regulation, allow the parasite to rapidly adapt to changing environmental conditions. Understanding these processes is crucial for uncovering new therapeutic targets to combat malaria, particularly those that could disrupt the parasite's ability to transition through its life cycle or evade the host immune system.

5. Antisense Transcription: Definition and Significance in *Plasmodium falciparum*

Antisense transcription refers to the process by which RNA is transcribed from the DNA strand that is opposite (antisense) to the coding strand of a gene. In other words, while the sense strand of DNA contains the sequence that codes for a protein, the antisense strand acts as the template for the production of non-coding RNA molecules that are complementary to the mRNA (sense RNA) produced from the coding strand (Pelechano et al., 2013; Wagner et al., 1994; Vanhee-Brossollet et al., 1998; Katayama et al., 2005; David et al., 2006; Kampa et al., 2004).

These antisense RNAs do not code for proteins themselves but can play regulatory roles by binding to the sense mRNA and influencing its stability, translation, or degradation (Pelechano et al., 2013). In *Plasmodium falciparum*, the malaria-causing parasite, antisense transcription has gained significant attention due to its potential role in controlling parasite development and survival (Broadbent et al., 2015; Bozdech et al., 2006; Guizetti et al., 2008; Pelechano et al., 2013).

5.1 Definition of Antisense Transcription

- **Mechanism:** Antisense transcription occurs when RNA polymerase transcribes the non-coding strand of DNA, leading to the formation of RNA molecules that can hybridize with the sense strand. This process can result in the formation of double-stranded RNA (dsRNA), which may regulate gene expression by various mechanisms represented in Figure 10 as well (Wery et al., 2011; Guttman et al., 2012; Mercer et al., 2013; Moazed et al., 2009).
- **Types of Antisense RNAs:** Antisense transcripts can include long non-coding RNAs (lncRNAs) and small interfering RNAs (siRNAs), which can modulate the stability, translation, and transcription of target mRNAs (Wei et al., 2011; Guttman et al., 2012; Mercer et al., 2013; Moazed et al., 2009). Refer to Figure 11 below for different orientations and mechanisms of antisense RNA.

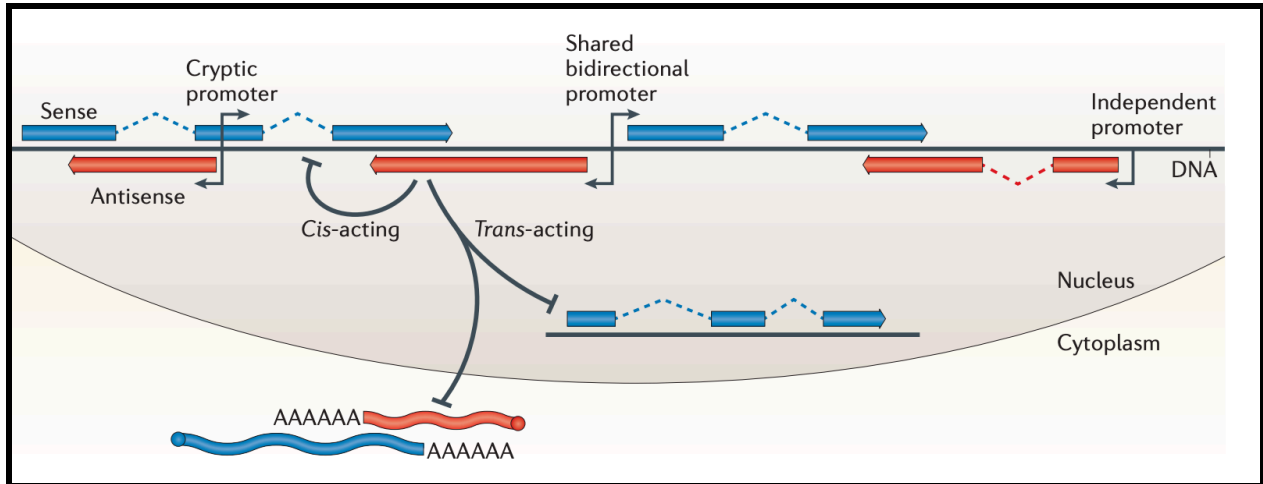


Figure 10: Schematic represents the origin and possible means of interaction of antisense RNA to other RNA molecules within the cell (Adapted from: Pelechano et al., 2013).

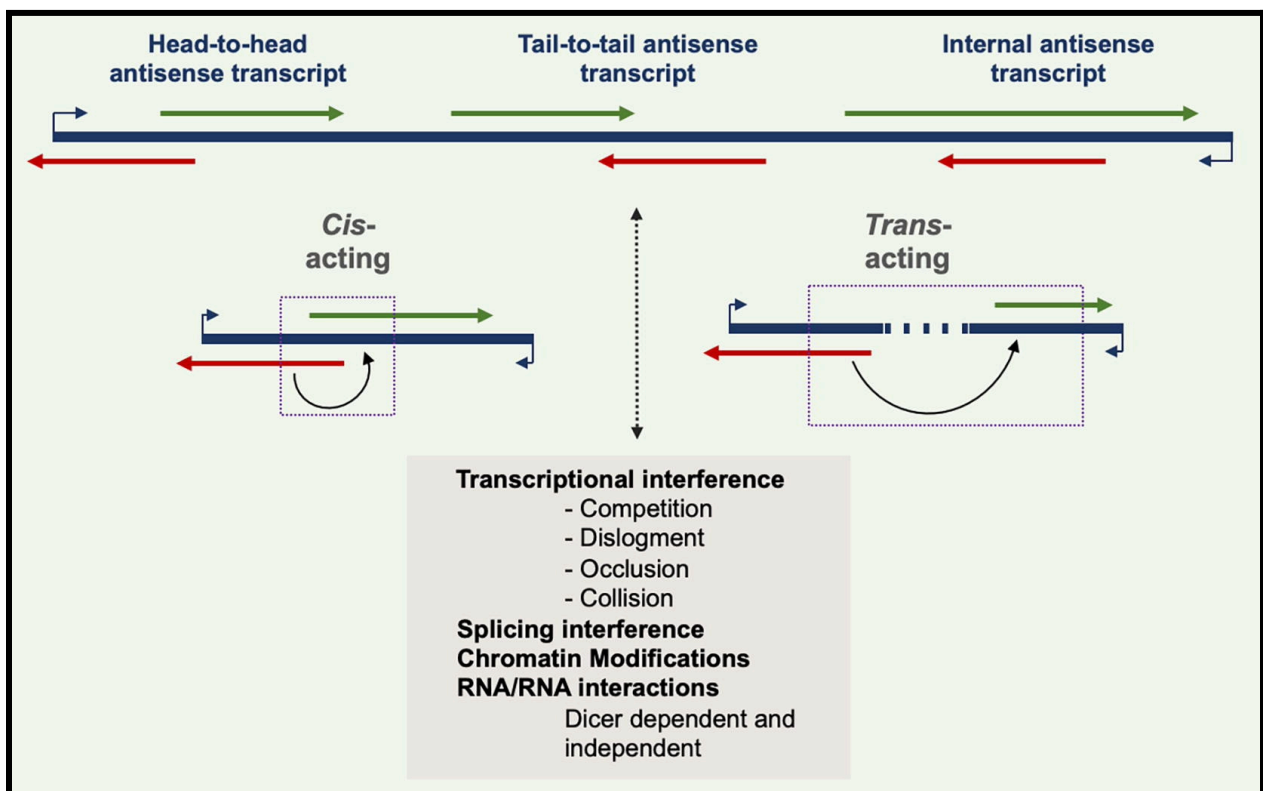


Figure 11: Natural antisense transcript classification and mechanisms of regulation. Sense and antisense pairs can be head-to-head, tail-to-tail or fully overlapped. NATs can regulate genes

in the vicinity (*cis*-NATs) or at large distances in the genome (*trans*-NATs) (Adapted from: Pelechano et al., 2013).

5.2 Significance of Antisense Transcription in *Plasmodium falciparum*

1.Regulation of Gene Expression: In *Plasmodium falciparum*, the regulation of gene expression is critical for managing its complex life cycle, adapting to various host environments, and evading the host's immune system. Antisense RNAs could play an important role in regulating the expression of key genes by plausible pathways:

- Inhibiting or promoting mRNA translation through RNA-RNA interactions.
- Influencing the stability or degradation of specific mRNAs.
- Modulating the timing of gene expression, especially during critical transitions between life cycle stages.

Several studies have shown the operation of these pathways across different systems but no information is available from the *Plasmodium* system (Kawano et al., 2007; Sigova et al., 2013; Neil et al., 2009; Xu et al., 2009; Seila et al., 2008; Core et al., 2008; Kim et al., 2012; Carrozza et al., 2005; Kaplan et al., 2003; Kaplan et al., 2007; Carrieri et al., 2012; Shearwin et al., 2005; Ma et al., 2009; Crampton et al., 2006; Hobson et al., 2012; Palmer et al., 2009; Gelfand et al., 2011; Su et al., 2012; Miura et al., 2008; Faghihi et al., 2008; Faghihi et al., 2010; Ebert et al., 2010; Lasa et al., 2011). By regulating these processes, antisense transcripts can fine-tune the expression of genes required for parasite survival, asexual replication, and transmission to mosquitoes.

2. Stage-Specific Expression: In *P. falciparum*, antisense transcription may play a role in regulating genes that are differentially expressed during various stages of the parasite's life cycle, such as the asexual blood stage, sexual stage, and the liver stage. This regulation is vital for the parasite's adaptation to different environments and hosts (Li et al., 2021; Yin et al., 2020; Chappell et al., 2020; Gunasekera et al., 2004; Siegel et al., 2014; Patankar et al., 2001; Fox et al., 1994; Subudhi et al., 2014).

3. Antigenic Variation and Immune Evasion: *P. falciparum* exhibits antigenic variation, especially in genes like the *var* gene family, which encodes for proteins like *PfEMP1* that help the parasite adhere to host cells and evade immune detection. Antisense transcription has been implicated in regulating the expression of *var* genes, playing a role in "switching" between different variants of *PfEMP1* proteins. This switching enables the parasite to present new surface antigens to avoid immune recognition (Scherf et al., 2008; Othman et al., 2023; Hviid et al., 2024; Kinyua et al., 2024; Otoboh et al., 2024; Hviid et al., 2023; Chen et al., 202; Deitsch et al. W., 2017; Scherf et al., 2008; Kyes et al., 2007; Schneider et al., 2023; Michel-Todó et al., 2023; Florini et al., 2022; Rovira-Graells et al., 2012; Scherf et al., 2008; Gómez-Díaz et al., 2017; Lavazec et al., 2007; Khattab et al., 2011; Beeson et al., 2002; Howitt et al., 2009). Antisense RNA may silence certain *var* genes while activating others, thus ensuring that only one or a few *var* genes are expressed at a time, facilitating immune evasion and increased virulence.

4. Role in Sexual Development and Transmission: Another critical process influenced by antisense transcription in *Plasmodium falciparum* is sexual differentiation, which is essential for the transmission of the parasite to mosquitoes. Specific antisense RNAs have been found to regulate the expression of genes involved in gametocyte development (the sexual stage). These antisense RNAs help control the balance between asexual replication and the development of gametocytes, ensuring successful transmission between human hosts and mosquitoes (Hollin et al., 2020; Russell et al., 2023; Kengne-Ouafo et al., 2023; Prajapati et al., 2020; Liu et al., 2024; Russell et al., 2023; Venugopal et al., 2020, Rea et al., 2018; Josling et al., 2015; Bancells et al., 2019, Rono et al., 2018; Kooij et al., 2007). For example, antisense transcripts can repress genes necessary for the asexual blood stages and promote the expression of genes required for sexual differentiation, thus aiding in the lifecycle transition.

5. Parasite Adaptation and Stress Response: Antisense transcription in *Plasmodium falciparum* also plays a role in how the parasite responds to environmental stress, such as fluctuations in nutrient availability, immune responses from the host, or drug treatment. Antisense RNAs might regulate stress-response genes, helping the parasite adjust its metabolism or life cycle in response to these pressures (Torrentino-Madamet et al., 2011; Anagu et al., 2020; Bunnik et al., 2013; Schneider et al., 2023; Heinberg et al., 2022). Our study leverages on this lesser studied domain in *Plasmodium*.

6. Drug Resistance: Antisense RNA can potentially influence the expression of genes associated with drug resistance. By regulating the expression of genes like *pfcr1* (chloroquine resistance transporter) or *pfmdr1* (multidrug resistance protein), antisense transcription may contribute to the emergence or suppression of resistance phenotypes. Understanding this regulatory mechanism could open new avenues for developing treatments that target antisense transcription pathways to combat drug resistance in malaria (Barker Jr et al., 1996; Subudhi et al., 2014; Chaijaroenkul et al., 2011; Sully et al., 2016).

In Conclusion, antisense transcription in *Plasmodium falciparum* represents an important layer of gene regulation that helps the parasite control key processes such as immune evasion, life cycle progression, and adaptation to environmental stresses. While much remains to be understood about the exact mechanisms by which antisense RNA functions in malaria, it is clear that these non-coding RNAs play a vital role in the parasite's survival and pathogenicity. Targeting antisense transcription pathways holds potential as a novel therapeutic approach for controlling malaria and preventing the spread of drug-resistant strains.

6. Historical Context and Discovery of Antisense Transcription in *Plasmodium falciparum*

The discovery of antisense transcription in *Plasmodium falciparum* unfolded as part of a broader understanding of non-coding RNA regulation in eukaryotes and parasites. Antisense transcription, while known in bacteria and other model organisms, was not widely recognized in *Plasmodium* until relatively recently, as advances in genomic and transcriptomic techniques allowed researchers to probe the complex gene regulation mechanisms in the malaria parasite.

6.1. Early Stages: The Genetic Puzzle of Malaria Parasites

Historically, the study of *Plasmodium falciparum* has been driven by efforts to understand its biology, life cycle, and survival strategies, especially in the face of immune system pressures and

drug treatments. The *P. falciparum* genome, completed in 2002, was a landmark achievement that enabled scientists to explore the genetic landscape of the parasite in greater depth (Gardner et al., 2002). This was the first high-resolution map of the entire genome, and it revealed the presence of many genes involved in antigenic variation, drug resistance, and sexual differentiation.

However, as researchers studied gene expression in *P. falciparum*, they noticed that many genes did not follow the typical linear model of transcription and translation. There was mounting evidence of complex post-transcriptional regulation, particularly in how certain genes were expressed during different stages of the parasite's life cycle. These irregularities suggested that mechanisms beyond classical transcriptional control were at play.

6.2. Emerging Recognition of Non-coding RNAs

In the early 2000s, the study of non-coding RNAs (ncRNAs) across various organisms began to gain traction. ncRNAs are RNA molecules that do not encode proteins but instead regulate various biological processes, often by interacting with messenger RNA (mRNA). Given the increasing interest in ncRNAs in other eukaryotes, researchers began to explore their potential role in *Plasmodium falciparum*.

Advancements in high-throughput sequencing technologies, such as RNA sequencing (RNA-Seq), allowed scientists to examine the parasite's transcriptome in detail. It was through these studies that antisense transcripts—RNAs complementary to coding (sense) RNAs—were discovered across different regions of the *P. falciparum* genome. In particular, research published in 2006 by Bozdech et al. demonstrated the presence of widespread antisense transcription in *P. falciparum*, suggesting a novel regulatory mechanism operating within the parasite's genome.

6.3. Key Studies and Confirmation

The first direct evidence of widespread antisense transcription in *Plasmodium falciparum* came from global transcriptional profiling studies. These studies revealed a substantial number of antisense transcripts associated with both protein-coding genes and regions of the genome not previously known to produce any functional molecules.

Some important early discoveries include:

- **2006:** The study by Bozdech et al. utilized whole-genome microarrays and identified transcripts from the antisense strand of numerous genes across the parasite's life cycle. These antisense RNAs were found in both coding and non-coding regions, suggesting their regulatory potential (Bozdech et al., 2006).
- **2008:** Guizetti et al. showed that antisense transcripts could regulate the expression of antigenic variation genes like the *var* family, which encode *PfEMP1* proteins responsible for cytoadherence and immune evasion. Their findings highlighted that antisense RNA was involved in the monoallelic expression of *var* genes, a critical process that allows the parasite to switch between different antigenic forms to avoid host immune detection.

These studies provided crucial evidence that antisense transcription was not merely a transcriptional "noise" but an integral part of the parasite's gene regulatory network.

6.4. The Role of Antisense Transcription in Life Cycle Regulation

As more research focused on the temporal and spatial expression patterns of genes during different stages of the *P. falciparum* life cycle, it became evident that antisense transcription played a role in regulating key processes such as:

- **Asexual Replication:** Antisense RNAs were found to regulate genes involved in the parasite's rapid multiplication within red blood cells.
- **Sexual Differentiation:** Antisense transcription was linked to genes controlling the development of gametocytes, the sexual forms of the parasite required for transmission to mosquitoes.

One key transcription factor identified in this context was **AP2-G** (Filarsky et al., 2018), which is involved in gametocyte development, refer to Figure 12 for a detailed representation. Studies showed that antisense RNAs might help repress certain genes in asexual stages while allowing them to activate during sexual differentiation, further confirming the regulatory significance of antisense transcription in the parasite (Rea et al., 2018).

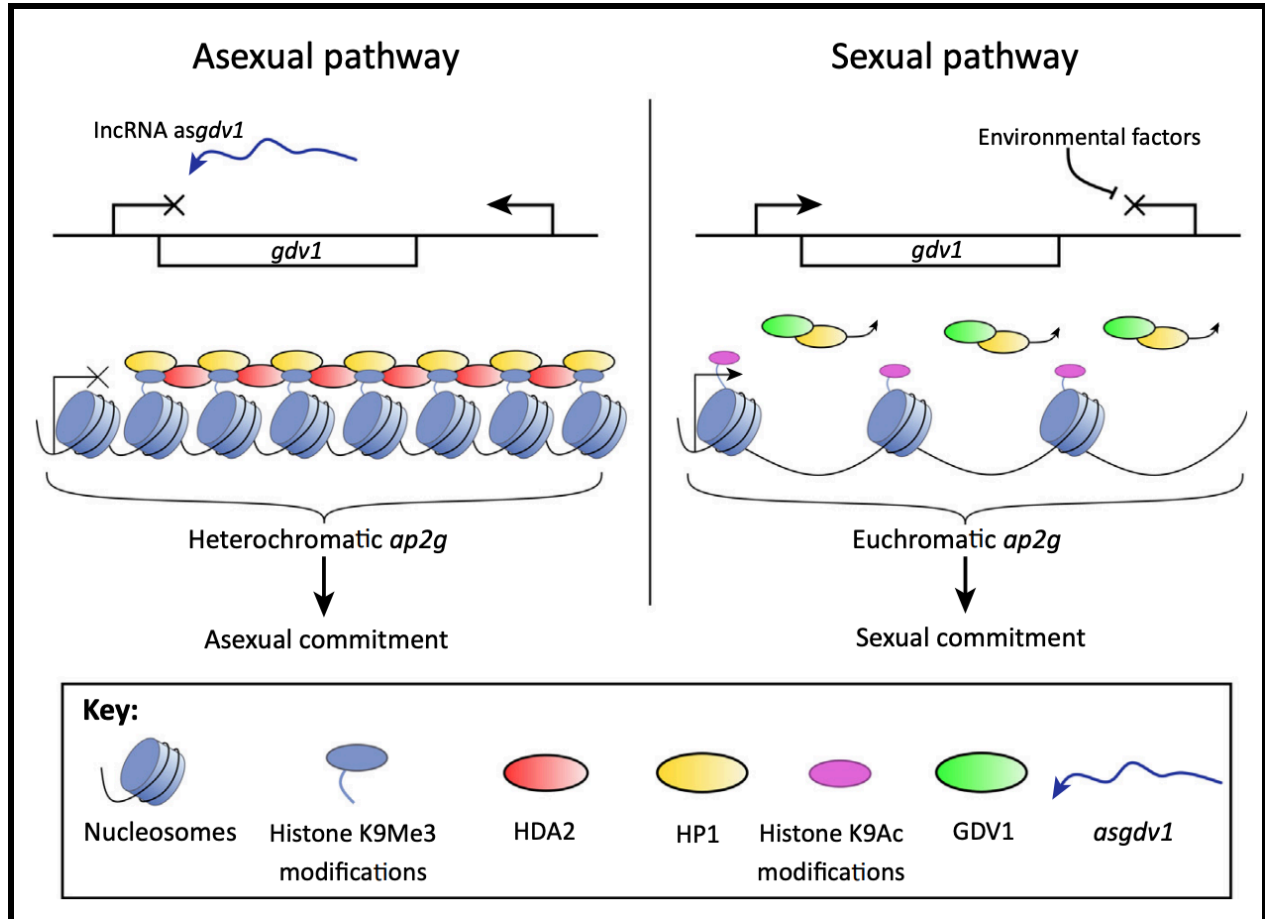


Figure 12: Regulation of Sexual Commitment in Human Malaria. The long noncoding *gdv1* antisense RNA *asgdv1* functions as a negative regulator of GDV1 production in parasites that are developing asexually. Due to H3K9 methylation and deacetylation at numerous loci, including AP2G, HP1 and HDA2 are able to sustain a repressed chromatin state in the absence of GDV1. This keeps AP2G from being expressed, enabling asexual development to continue. As *gdv1* is somehow inactivated in a small group of parasites, either randomly or as a result of transduction of different environmental signals. This permits GDV1 expression and, consequently, the eviction of HP1 from particular loci, including AP2G. The chromatin reverts to a euchromatic state in the absence of HP1 to sustain H3K9 methylation, which causes AP2G expression and the subsequent commitment to sexual development (Adapted from: Rea et al., 2018).

6.5. Mechanistic Insights and Current Research

As of the 2010s, more sophisticated transcriptomic studies provided mechanistic insights into how antisense transcripts function, also shown in [figure 13](#). Researchers uncovered that antisense RNAs in *Plasmodium falciparum* can ([Petter et al., 2015](#); [Villegas et al., 2015](#)):

- **Silence gene expression** by hybridizing with complementary sense mRNA and inhibiting its translation.
- **Regulate gene activation** by interacting with chromatin or transcription machinery, altering the accessibility of the DNA template for gene transcription.

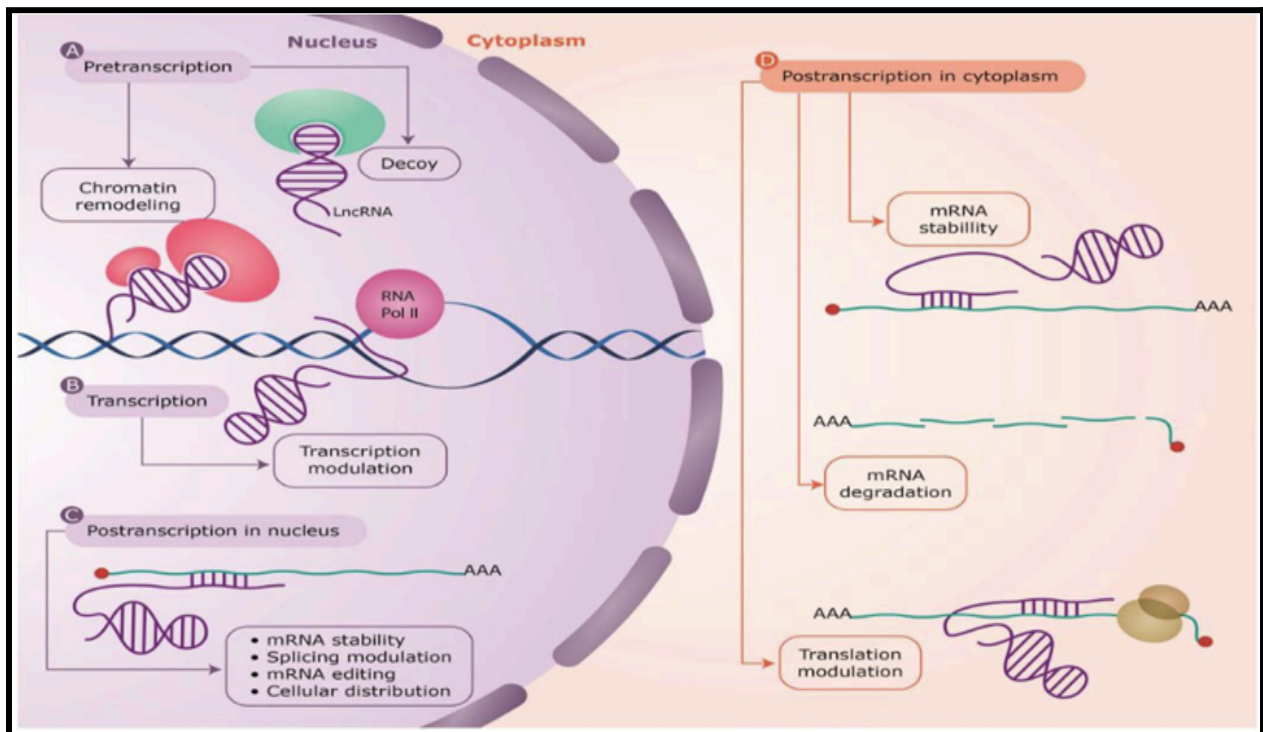


Figure 13: The potential method of action of NATs is depicted in the following figure. The mechanism of action of NATs is influenced by a number of factors. To produce an effect, the transcript's orientation, location inside the cell, stability, and secondary structures all play a part. In the form of transcript development, NATs may be essential at nearly every stage of gene expression. Depending on whether they control gene expression at the transcription start, co-transcriptional, post-transcriptional, or post-translational levels, antisense transcripts may operate in the cytoplasm or the nucleus ([Adapted from Villegas et al, 2015](#)).

These mechanisms were shown to be vital in processes such as antigenic variation, where the controlled switching of *var* genes ensures that only one gene is active at a time. Such findings underscored the parasite's dependence on antisense transcription for immune evasion and survival in the host. **However no mechanism has been proven yet through strategic study and research.**

6.6. Significance for Future Research and Therapeutics

The discovery of antisense transcription has opened new avenues for malaria research, especially in understanding gene regulation at a deeper level. It offers potential therapeutic opportunities:

- **Targeting Antisense Pathways:** Since antisense transcripts play critical roles in gene regulation, targeting these pathways could disrupt vital processes such as immune evasion or drug resistance.
- **Drug Development:** Understanding how antisense transcription affects genes related to drug resistance could help scientists develop new drugs that can either enhance or inhibit these regulatory mechanisms.

To conclude, the historical discovery of antisense transcription in *Plasmodium falciparum* has provided invaluable insights into the parasite's complex gene regulatory network. Initially discovered in the mid-2000s through genome-wide transcriptomic studies, antisense transcription is now recognized as a key regulatory mechanism that controls various aspects of the parasite's life cycle, including immune evasion, sexual differentiation, and drug resistance. As research progresses, targeting antisense RNA pathways offers a promising direction for developing novel antimalarial therapies.

7. Comparison of Sense and Antisense Transcription in *Plasmodium falciparum*

Plasmodium falciparum, the malaria-causing parasite, utilizes both sense and antisense transcription to regulate its gene expression. These two processes are fundamentally different, yet both are critical for the parasite's survival, development, and ability to adapt to host environments. Understanding the comparison between sense and antisense transcription can provide insights into the complex regulatory mechanisms of *P. falciparum*.

7.1. Definition and Process

- **Sense Transcription:** Sense transcription is the conventional process of copying the DNA sequence of a gene to produce messenger RNA (mRNA), which carries the genetic code for protein synthesis. The mRNA is transcribed from the **sense strand** (coding strand) of DNA. This process involves RNA polymerase reading the DNA template, producing a complementary RNA sequence, and processing it for translation into proteins (Pelechano et al., 2013).

Key Steps:

- Transcription of the coding strand into mRNA.
 - mRNA is processed (e.g., capping, splicing, polyadenylation).
 - The mRNA is translated into a specific protein.
- **Antisense Transcription:** Antisense transcription involves the production of RNA from the **antisense strand** of DNA, which is complementary to the sense strand. These antisense RNAs (asRNAs) do not code for proteins but regulate gene expression by interacting with the corresponding sense mRNAs or modulating chromatin structure and transcriptional activity (Pelechano et al., 2013).

Key Steps:

- Transcription of the non-coding (antisense) strand.
- Production of non-coding RNA molecules that are complementary to sense mRNAs.
- These asRNAs can bind to the sense mRNAs, affecting their stability, translation, or degradation.

7.2. Function

- **Sense Transcription:** The primary function of sense transcription is to produce mRNAs that encode proteins necessary for the parasite's biological functions. These proteins are crucial for the parasite's growth, reproduction, survival, immune evasion, and adaptation to environmental changes (Petter et al., 2015).

Examples of genes transcribed via sense transcription:

- **PfEMP1** (Plasmodium falciparum erythrocyte membrane protein 1): A key protein involved in cytoadherence and immune evasion.
- **PfCRT** (Chloroquine resistance transporter): Involved in drug resistance mechanisms.
- **MSP1** (Merozoite surface protein 1): Involved in red blood cell invasion.
- **Antisense Transcription:** Antisense transcription, on the other hand, plays a regulatory role in controlling when, where, and how much of a given gene is expressed (Petter, M., 2015). The antisense RNAs produced can regulate the expression of genes by:
 - **Inhibiting mRNA translation** through RNA-RNA hybridization.
 - **Promoting mRNA degradation**, leading to reduced protein synthesis.
 - **Altering chromatin structure** to either promote or repress gene transcription.
- Example roles of antisense transcription:
 - **Regulation of antigenic variation:** Antisense RNAs regulate the monoallelic expression of *var* genes, controlling which *PfEMP1* variant is expressed to help the parasite evade immune detection.
 - **Sexual differentiation:** Antisense RNAs are involved in regulating genes required for the development of gametocytes, the sexual forms necessary for transmission to mosquitoes.

7.3. Gene Expression Control

- **Sense Transcription:** Sense transcription is the main driver of protein production in *P. falciparum*, directly influencing the parasite's physiology. The expression of sense transcripts is tightly regulated depending on the parasite's stage in its complex life cycle (e.g., liver stage, blood stage, gametocyte stage) to ensure that the necessary proteins are

produced at the right time and place.

Stage-Specific Expression: For example, during the asexual blood stage, the parasite produces proteins necessary for red blood cell invasion and replication, such as MSP1, whereas during the sexual stage, proteins involved in gametocyte maturation are transcribed (Bozdech et al., 2003; Hu et al., 2010; Yamagishi et al., 2014; Tonkin-Hill et al. Q., 2018; Chappell et al., 2020).

- **Antisense Transcription:** Antisense transcription provides a secondary level of control by fine-tuning gene expression. It ensures that the expression of sense transcripts is regulated according to the needs of the parasite at different stages. For example, antisense RNAs can silence certain genes during asexual replication, while allowing their expression during sexual differentiation.

Temporal Control: Antisense transcription helps to regulate the timing of gene expression, ensuring that only the genes necessary for a particular life stage are activated (Horrocks et al., 2009; Patankar et al., 2001; Raabe et al., 2010).

7.4. Mechanisms of Regulation

- **Sense Transcription:** Sense transcription is regulated primarily by transcription factors, promoter regions, enhancers, and chromatin modifications. Transcription factors such as AP2-G bind to specific DNA sequences and recruit RNA polymerase to initiate transcription. Epigenetic modifications, such as DNA methylation and histone modification, also play a role in activating or silencing genes.
- **Antisense Transcription:** Antisense transcription across different systems is known to operate through multiple mechanisms but no such pathways have been proven in Plasmodium yet, refer to Figure 14 for schematic representation of the mechanisms.
 - **Transcriptional interference:** Antisense RNAs can interfere with the transcription of the sense strand by physically blocking access to the DNA or by altering the chromatin state. Refer to figure 15 for types of transcriptional interference and Figure 16 for regulation of its neighboring gene.
 - **RNA interference (RNAi):** Antisense transcripts can bind to their complementary mRNAs, forming double-stranded RNA (dsRNA), which can

trigger degradation by RNA-induced silencing complexes (RISC). **However RNAi is not operating in *Plasmodium* as proven before.**

- **Epigenetic regulation:** Antisense transcription can influence chromatin structure, modifying the accessibility of sense transcripts to transcriptional machinery.

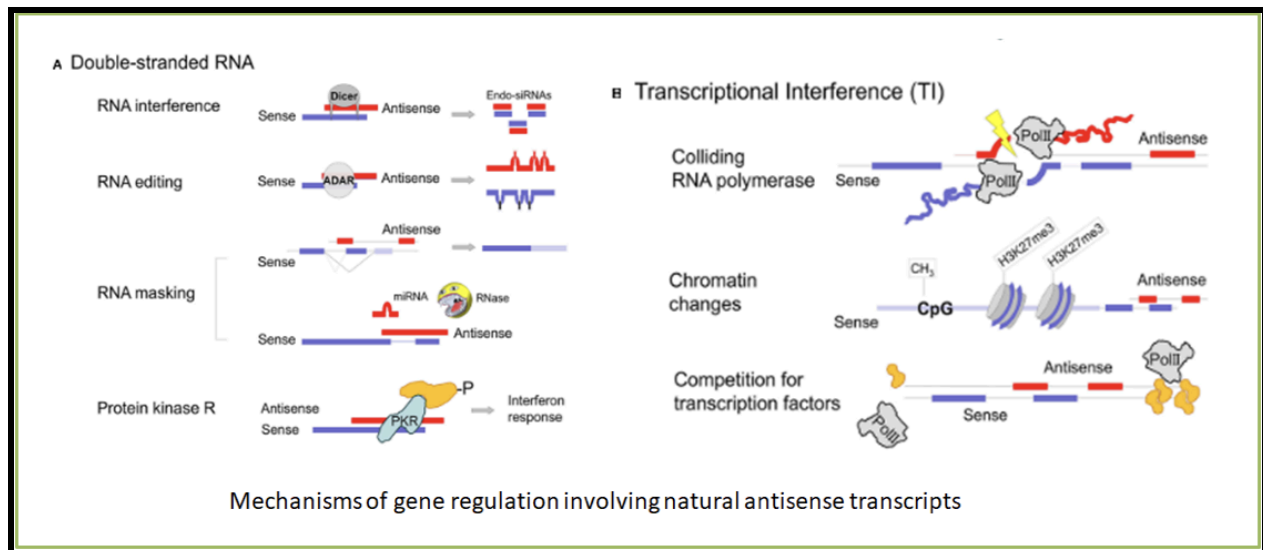


Figure 14: Evidence from other model systems have shown that NATs could potentially hinder the initiation of the transcript via transcriptional interference. During this process, transcription of one gene negatively regulates the expression of another in cis. This has been shown to occur because of promoter competition; assembly of transcriptional machinery at one promoter prevents assembly at the neighbouring promoter. This could also happen via occlusion of transcription binding sites. Epigenetic modifications at the chromatin, DNA or histone level could also contribute to the same. Repression via RNA could also happen because of a unique mechanism of formation of triple RNA- DNA helices. Figure below demonstrates possible ways of transcriptional inhibition (Adapted from: Santos, F., 2022).

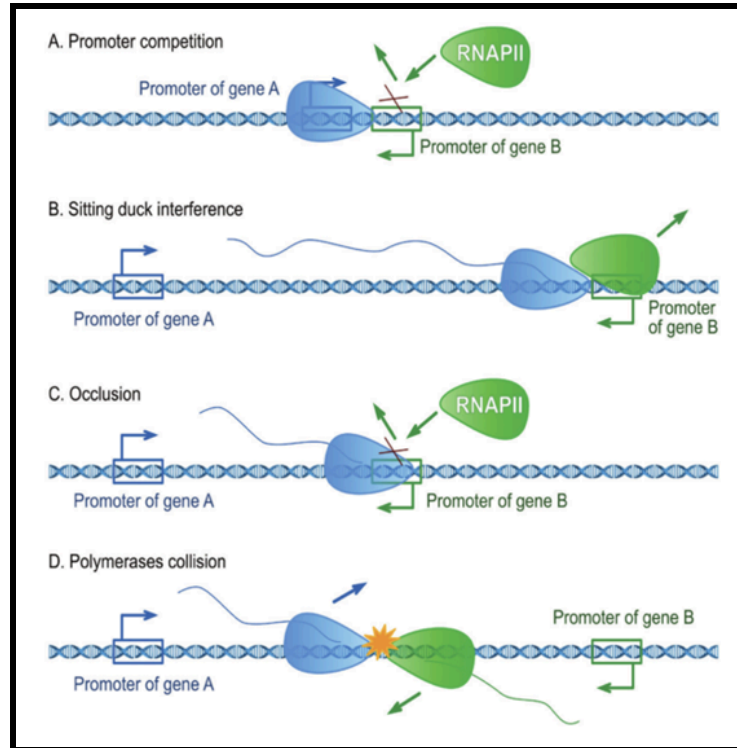


Figure 15: Transcriptional interference that occurs co-transcriptionally could occur due to direct collision of RNA polymerase, in which an elongating polymerase dislodges another firing promoter (sitting-duck interference). Bidirectional transcription from two strong promoters could also lead to interference via collision. While in case of two weak promoters, polymerase pausing could lead to the same. Co- transcriptionally NATs could modulate the isoform structure formation of sense transcript, most probably by affecting alternative splicing. Another possible way could be transcript attenuation, which leads to premature termination of transcription, leading to differential regulation of genes (Adapted from: Santos, F., 2022).

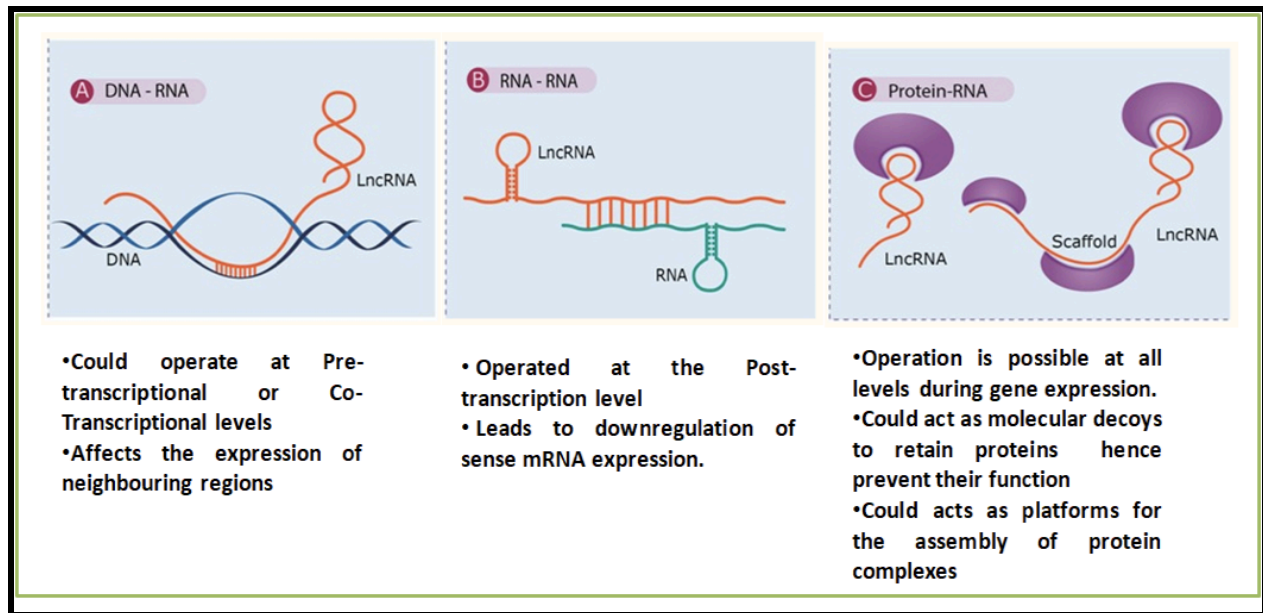


Figure 16: Figure above demonstrates mechanism of actions that could be employed at various steps for regulation of neighboring gene expression. In a popular verse NATs could affect the transcriptional ‘life’ of the sense RNA, which directly implicates in translational control by affecting mRNA isoform. The post transcriptional effect is probably the fastest and more robust because it directly affects the mRNA molecules already synthesized in the cell. This avoids the lag between the alteration of transcription rates and formation of new mRNA concentration. But it is limited in the terms that it requires both sense and antisense transcripts at the same time within the cell. This could lead to both positive and negative effects on the mRNA stability and translation (Adapted from: Santos, F., 2022).

7.5. Examples in *Plasmodium falciparum*

- **Sense Transcription:**

- **Var Genes and PfEMP1:** During sense transcription, the parasite expresses one *var* gene at a time, leading to the production of a specific *PfEMP1* variant that helps the parasite adhere to host cells and avoid immune detection (Epp et al., 2009; Amit-Avraham et al., 2015; Brancucci et al., 2014).

- **MSP1:** This gene is transcribed during the blood stage of the parasite to facilitate invasion of red blood cells, a critical step in parasite replication (Hocking et al., 2022; Kaiser et al., 2004; Gunasekera et al., 2004).
- **Antisense Transcription:**
 - **Regulation of Var Genes:** Antisense transcripts are involved in the regulation of *var* genes, helping to control the switching between different variants of *PfEMP1*, ensuring only one variant is expressed at a time (Hollin et al., 2020; Russell et al., 2023; Kengne-Ouafo et al., 2023; Prajapati et al., 2020; Liu et al., 2024; Russell et al., 2023; Venugopal et al., 2020, Rea et al., 2018; Josling et al., 2015; Bancells et al., 2019, Rono et al., 2018; Kooij et al., 2007).
 - **Gametocyte Development:** Antisense transcripts regulate genes involved in the sexual differentiation process, balancing the transition from asexual replication to the production of gametocytes, which are crucial for transmission to mosquitoes. A solid example of this is AP2-G antisense RNA which is proven to control sexual differentiation (Hollin et al., 2020; Russell et al., 2023; Kengne-Ouafo et al., 2023; Prajapati et al., 2020; Liu et al., 2024; Russell et al., 2023; Venugopal et al., 2020, Rea et al., 2018; Josling et al., 2015; Bancells et al., 2019, Rono et al., 2018; Kooij et al., 2007).

7.6. Evolutionary and Adaptive Significance

- **Sense Transcription:** Sense transcription is the fundamental process by which genes are expressed and proteins are synthesized. In *P. falciparum*, sense transcription allows for the production of proteins that are crucial for survival, reproduction, and adaptation to different environments (human host and mosquito vector) (Werner et al., 2024; Mattick et al., 2023; Lipman et al., 1997; Wight et al., 2013; Werner et al., 2009).
- **Antisense Transcription:** Antisense transcription provides an additional layer of control that enhances the parasite's adaptability. By regulating sense transcripts post-transcriptionally, antisense transcription allows *P. falciparum* to rapidly respond to environmental stresses, immune pressures, or drug treatments. This flexibility is especially important for survival within diverse host environments and for evading the

immune system (Mattick et al., 2023; Lipman et al., 1997; Wight et al., 2013; Werner et al., 2009).

In Conclusion, sense and antisense transcription in *Plasmodium falciparum* work in a complementary manner to regulate gene expression. While sense transcription drives protein production, antisense transcription provides fine-tuned regulatory control, ensuring that genes are expressed at the right time and in response to the parasite's environmental needs. Both processes are critical for the parasite's ability to complete its complex life cycle, evade host immune responses, and adapt to changing conditions. Understanding the interplay between these two forms of transcription offers valuable insights for developing new malaria therapies, particularly those targeting regulatory pathways.

8. Characterizing Antisense Transcription in *Plasmodium falciparum*

Antisense transcription in *Plasmodium falciparum* refers to the process of producing non-coding RNA molecules from the strand of DNA that is opposite to the protein-coding sense strand. The characterization of antisense transcription in *P. falciparum* involves understanding its prevalence, functional roles, and regulatory mechanisms. In recent years, advancements in genomics and transcriptomics have provided significant insights into the nature and functions of antisense RNAs (asRNAs) in this malaria-causing parasite.

8.1. Prevalence of Antisense Transcription in *P. falciparum*

- **Genome-wide Presence:** Antisense transcription is pervasive across the *P. falciparum* genome. Studies have demonstrated that a significant portion of the parasite's genes are associated with antisense RNAs, refer [figure 17](#). For instance, a global transcriptomic study using RNA-sequencing revealed that nearly half of the coding genes in *P. falciparum* exhibit some form of antisense RNA production (Siegel et al., 2014).

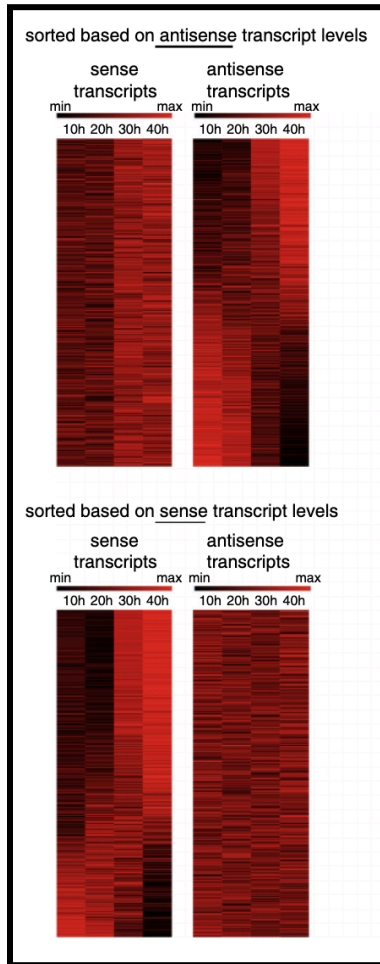


Figure 17: Antisense transcription is abundant and developmentally regulated. Heat map of genes containing sense and antisense transcripts (n =2389 NATs defined with relaxed threshold) sorted based on antisense RNA trends (top panel) and sense RNA trends (bottom panel) (Adapted from: Siegel et al., 2014).

Stage-specific Expression: Antisense transcripts in *P. falciparum* are not uniformly expressed throughout its life cycle, refer Figure 18. Instead, they show stage-specific expression patterns, suggesting their role in regulating gene expression during different phases of the parasite's complex life cycle, such as the asexual blood stage and the sexual gametocyte stage. This indicates that antisense transcription is temporally regulated to meet the changing needs of the parasite (Siegel et al., 2014).

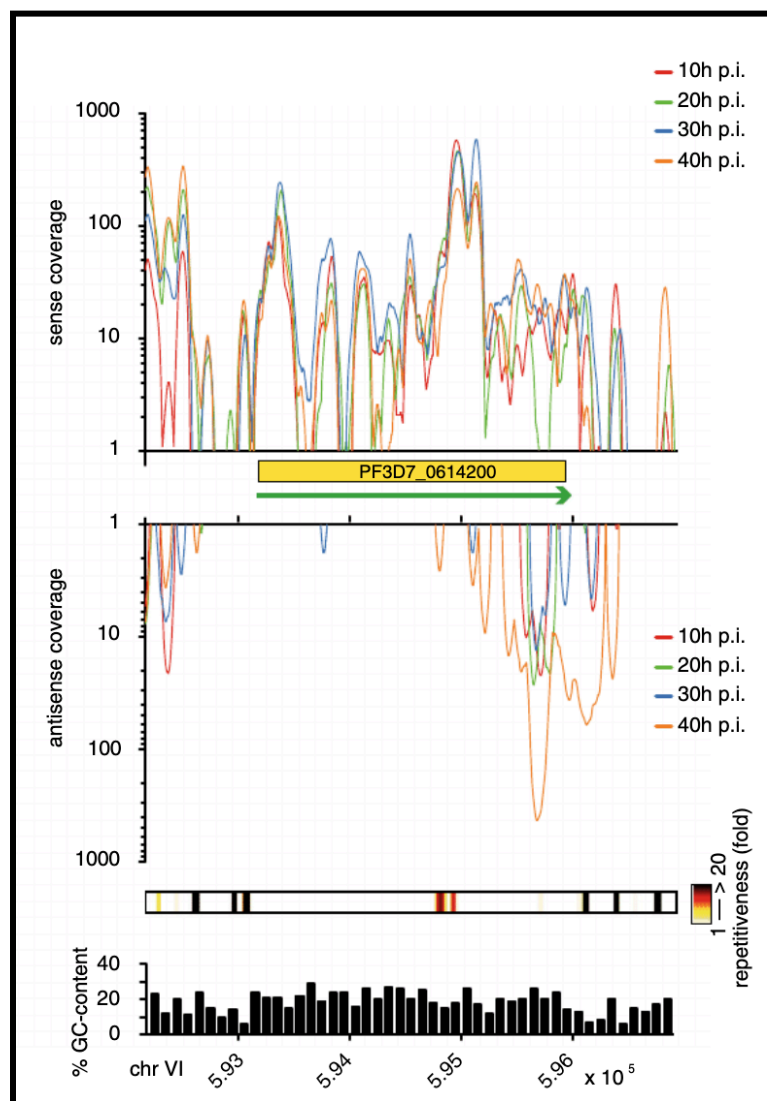


Figure 18: Coverage plot for a gene containing developmentally regulated NATs. Yellow box represents CDS and green arrow indicates direction of transcription (Adapted from: Siegel et al., 2014).

8.2. Key Genes and Loci Involved in Antisense Transcription

- **Antigenic Variation Genes (e.g., *var* Genes):** One of the most well-studied examples of antisense transcription in *P. falciparum* is its role in regulating the expression of *var* genes, which encode *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is a critical factor in the parasite's ability to adhere to host cells and evade the immune system. Antisense RNAs regulate the monoallelic expression of *var* genes, ensuring that only one *var* gene is expressed at a time, thereby facilitating antigenic variation.
- **Gametocyte Development Genes:** Antisense transcription is also implicated in regulating genes involved in the sexual differentiation of *P. falciparum*. Genes such as **AP2-G**, a master regulator of gametocyte development, are controlled by antisense RNAs to balance the parasite's transition from asexual replication to the production of sexual gametocytes, which are necessary for transmission to mosquitoes.
- **Drug Resistance Genes:** Antisense RNAs may also regulate genes associated with drug resistance, such as **pfmdr1** (multidrug resistance gene) and **pfert** (chloroquine resistance transporter gene). Understanding how antisense transcription affects the expression of these genes can provide insights into the mechanisms underlying drug resistance in malaria.

8.3. Possible mechanisms of Antisense RNA Function

- **Transcriptional Interference:** Antisense RNAs can interfere with the transcription of sense RNAs by blocking the recruitment of transcription factors or RNA polymerase to the promoter of the sense gene. This prevents the gene from being transcribed into mRNA, thereby silencing it at the transcriptional level (Santos et al., 2022).
- **RNA-RNA Hybridization:** One of the most common mechanisms of antisense RNA function is through RNA-RNA interactions. Antisense transcripts bind to their

complementary sense mRNA, forming a double-stranded RNA (dsRNA) duplex (Santos et al., 2022). This binding can:

- Inhibit the translation of mRNA into proteins.
 - Promote mRNA degradation by activating the RNA interference (RNAi) pathway.
 - Prevent the splicing of pre-mRNAs, thereby regulating the production of mature mRNA.
- **Chromatin Modification:** Antisense transcription can also influence chromatin structure. It can recruit chromatin-modifying complexes that either open or close chromatin around the sense gene locus, making it more or less accessible to transcriptional machinery. This epigenetic regulation plays a key role in controlling gene expression (Santos et al., 2022).

8.4. Functional Roles of Antisense Transcription

- **Regulation of Antigenic Variation:** In *P. falciparum*, antigenic variation is a crucial mechanism by which the parasite evades host immune detection. Antisense transcription regulates the switching between different *var* gene variants, allowing the parasite to periodically change the proteins displayed on the surface of infected red blood cells. This process enables *P. falciparum* to present new antigens to the immune system, thus avoiding destruction by host antibodies.
- **Control of Sexual Differentiation:** Antisense RNAs are involved in regulating genes that determine whether the parasite continues asexual replication or transitions to the sexual gametocyte stage. This is critical for the parasite's life cycle, as the gametocyte stage is essential for transmission to mosquitoes. By regulating the timing and expression of genes such as **AP2-G**, antisense transcription ensures the parasite can adapt to the need for transmission at the appropriate time.
- **Modulation of Drug Resistance:** Antisense transcription may regulate the expression of drug resistance genes, allowing the parasite to adapt to antimalarial treatments. By controlling the levels of mRNAs associated with drug resistance, antisense RNAs may contribute to the emergence or suppression of resistance phenotypes. Understanding these regulatory pathways offers potential strategies for combating drug-resistant malaria.

8.5. Experimental Techniques for Characterizing Antisense Transcription

Characterizing antisense transcription in *P. falciparum* requires advanced molecular and genomic techniques due to the complex nature of the parasite's genome and transcriptional activity. Some key methods include:

- **RNA Sequencing (RNA-Seq):** High-throughput RNA-Seq has been instrumental in mapping both sense and antisense transcripts across the *P. falciparum* genome. By sequencing RNA from different stages of the parasite's life cycle, researchers can identify and quantify antisense transcripts, as well as their expression patterns in various conditions (Katayama et al., 2005; David et al., 2006; Kampa et al., 2004; Perocchi et al., 2007; Xu et al., 2011; Levin et al., 2010; Xu et al., 2009; van Dijk et al., 2011; Su et al., 2012; Wilkening et al., 2013; Pelechano et al., 2013; Churchman et al., 2011; Xu et al., 2009; Churchman et al., 2012, Guttman et al., 2009).
- **Strand-Specific RNA Sequencing:** This technique allows researchers to distinguish between sense and antisense transcripts. Traditional RNA-Seq does not differentiate between the strands of origin for RNA, but strand-specific methods ensure that researchers can accurately map which transcripts are produced from the sense or antisense strands (Xu et al., 2009; van Dijk et al., 2011; Su et al., 2012; Wilkening et al., 2013; Pelechano et al., 2013; Churchman et al., 2011; Xu et al., 2009; Churchman et al., 2012, Guttman et al., 2009).
- **Chromatin Immunoprecipitation (ChIP):** ChIP is used to study the interactions between antisense transcription and chromatin structure. By targeting specific histone modifications or transcription factors, researchers can identify regions of the genome where antisense transcription is associated with epigenetic changes that regulate gene expression (Pelechano et al., 2013).
- **RNA-RNA Interaction Mapping:** Techniques like CLIP-Seq (crosslinking immunoprecipitation sequencing) or RIP-Seq (RNA immunoprecipitation sequencing) are used to study RNA-RNA interactions, allowing researchers to identify how antisense

RNAs bind to sense mRNAs and regulate their function (Hafner et al., 2021; Zhao et al., 2010).

8.6. Challenges and Future Directions

- **Understanding Mechanisms:** While the presence of antisense transcripts in *P. falciparum* is well-documented, the exact mechanisms by which they regulate gene expression are still not fully understood. Further research is needed to elucidate how antisense RNAs influence chromatin dynamics, RNA processing, and translation in the parasite.
- **Functional Validation:** Many antisense transcripts have been identified, but their specific roles in regulating parasite biology remain to be experimentally validated. Functional studies, such as gene knockdowns or overexpression experiments, will be critical in determining the biological relevance of specific antisense RNAs.
- **Therapeutic Potential:** Given their regulatory importance, antisense transcripts represent potential targets for new antimalarial therapies. Drugs that target antisense RNA pathways could disrupt key processes like antigenic variation or drug resistance, making the parasite more vulnerable to treatment.

In Conclusion, characterizing antisense transcription in *Plasmodium falciparum* reveals a complex and dynamic layer of gene regulation that is crucial for the parasite's survival, adaptation, and pathogenicity. Antisense RNAs play a key role in controlling antigenic variation, sexual differentiation, and possibly drug resistance. Through a combination of advanced genomic techniques and functional studies, researchers are beginning to unravel the intricacies of antisense transcription in *P. falciparum*, with the ultimate goal of identifying new therapeutic strategies to combat malaria.

9. Exploring the possible functional implications of antisense RNA in *Plasmodium falciparum*

Antisense RNAs (asRNAs) in *Plasmodium falciparum* represent a fascinating layer of gene regulation with significant functional implications for the parasite's survival, pathogenicity, immune evasion, and adaptation. Although traditionally seen as non-coding RNA, antisense RNAs serve diverse roles that affect the parasite's ability to thrive in both human hosts and mosquitoes. Here, we explore the functional implications of these antisense RNAs in *P. falciparum* and their contribution to key biological processes, a summary of the same has been provided in [Figure 19](#).

9.1. Regulation of Antigenic Variation

- **Var Genes and PfEMP1 Expression:** One of the most well-characterized roles of antisense RNAs in *P. falciparum* is their involvement in the regulation of *var* genes, which encode *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is a key virulence factor that allows the parasite to evade the host immune system by adhering to host endothelial cells, leading to cytoadherence and sequestration ([Othman et al., 2023](#); [Hviid et al., 2024](#); [Kinyua et al., 2024](#); [Otoboh et al., 2024](#); [Hviid et al., 2023](#); [Chen et al., 2021](#)).
- **Monoallelic Expression Control:** Only one *var* gene is expressed at a time, while the others remain silenced. Antisense RNAs play a crucial role in ensuring this monoallelic expression by interacting with the sense transcripts of *var* genes, potentially affecting the stability and transcription of sense mRNA. This regulation ensures the parasite can continuously switch between different *PfEMP1* variants, avoiding immune recognition ([Dzikowski et al., 2006](#); [Voss et al., 2006](#); [Borst et al., 1999](#); [Dzikowski et al., 2007](#)).
- **Immune Evasion:** By promoting antigenic variation, antisense RNAs allow *P. falciparum* to periodically switch the variant of PfEMP1 presented on the surface of infected erythrocytes, helping it evade host immune responses and prolong infection ([Heinberg et al., 2022](#)).

9.2. Control of Gene Expression During the Parasite's Life Cycle

- **Stage-Specific Expression of Genes:** *Plasmodium falciparum* has a complex life cycle that includes multiple stages in the human host (liver, asexual blood stages, sexual gametocyte stages) and mosquito vector. Antisense RNAs play a regulatory role in controlling the expression of genes required at specific life cycle stages (Li et al., 2021; Yin et al., 2020; Chappell et al., 2020; Gunasekera et al., 2004; Siegel et al., 2014; Patankar et al., 2001; Fox et al., 1994; Subudhi et al., 2014; Filarsky et al., 2018).
- **Asexual vs. Sexual Development:** Antisense RNAs are particularly important in the regulation of genes involved in sexual differentiation. For example, the transcription factor **AP2-G**, which triggers the development of gametocytes (the sexual form of the parasite), is controlled by antisense RNA-mediated mechanisms. Antisense RNAs silence **AP2-G** expression during the asexual stage but allow its expression when the parasite switches to gametocytogenesis. This regulation is essential for balancing asexual replication (which sustains the infection in the host) and sexual development (which is necessary for transmission to mosquitoes (Hollin et al., 2020; Russell et al., 2023; Kengne-Ouafo et al., 2023; Prajapati et al., 2020; Liu et al., 2024; Russell et al., 2023; Venugopal et al., 2020, Rea et al., 2018; Josling et al., 2015; Bancells et al., 2019, Rono et al., 2018; Kooij et al., 2007).
- **Gametocyte Maturation:** Antisense RNAs are also involved in the timing and progression of gametocyte maturation, influencing the parasite's ability to successfully develop into transmissible forms (Filarsky et al., 2018).

9.3. Post-Transcriptional Regulation of mRNA Stability and Translation

- **mRNA Degradation:** Antisense RNAs can form double-stranded RNA (dsRNA) complexes with sense mRNAs, which can trigger mRNA degradation via RNA interference (RNAi) pathways. This process reduces the availability of sense transcripts for translation, effectively silencing the gene at the post-transcriptional level. This mechanism is critical for ensuring that specific proteins are not produced when they are not needed, allowing the parasite to conserve resources and avoid immune detection.

- **Translational Inhibition:** In addition to promoting mRNA degradation, antisense RNAs can inhibit translation by directly binding to sense mRNAs. This blockage prevents the ribosome from initiating translation, reducing the production of proteins. This level of control allows *P. falciparum* to quickly adjust its protein synthesis machinery in response to environmental changes, such as nutrient availability, immune pressures, or the presence of antimalarial drugs.

9.4. Regulation of Epigenetic States

- **Chromatin Remodeling:** Antisense transcription has been linked to epigenetic regulation in *Plasmodium falciparum*. Antisense RNAs can recruit chromatin-modifying enzymes, leading to changes in histone marks and chromatin accessibility. This can either activate or repress gene expression by altering the transcriptional landscape of specific loci. In the case of *var* genes, for instance, antisense transcription is associated with heterochromatic silencing of non-expressed *var* genes while allowing the expression of a single *var* gene at a time.
- **Histone Modification:** Antisense RNAs can induce specific histone modifications that either enhance or suppress the transcription of nearby genes. By influencing chromatin structure, antisense RNAs contribute to the parasite's ability to dynamically switch between different epigenetic states, facilitating adaptation to different environments or stresses.

9.5. Modulation of Drug Resistance Mechanisms

- **Role in Resistance Gene Regulation:** Antisense RNAs are believed to regulate genes associated with drug resistance, such as **pfmdr1** (multidrug resistance gene 1) and **pfert** (chloroquine resistance transporter). Antisense RNAs may affect the expression of these resistance genes by modulating mRNA stability or chromatin structure. This regulation could potentially lead to the emergence of drug-resistant strains when the parasite is exposed to antimalarial treatments.
- **Adaptive Response to Drug Pressure:** Antisense RNA-mediated regulation of resistance genes could provide the parasite with a rapid and flexible response to the

presence of drugs. By quickly adjusting the expression levels of key resistance-related genes, *P. falciparum* can survive in the presence of drug treatments, leading to the development of resistance over time. This makes antisense RNAs a potential target for novel therapies aimed at disrupting the parasite's ability to develop resistance.

9.6. Response to Environmental Stress

- **Heat Shock and Stress Conditions:** Antisense RNAs may help *P. falciparum* respond to environmental stresses such as heat shock (e.g., fever in the host) or nutrient deprivation. By regulating stress-responsive genes at the post-transcriptional level, antisense RNAs allow the parasite to rapidly adapt to unfavorable conditions. For instance, during heat shock, the parasite may reduce the expression of certain proteins through antisense RNA-mediated inhibition, conserving energy for essential survival processes.
- **Hypoxia Response:** *P. falciparum* experiences hypoxic conditions during its blood-stage infection, particularly in sequestration events where infected red blood cells adhere to the vascular endothelium. Antisense transcription may play a role in regulating the expression of genes involved in the parasite's response to low oxygen levels, helping it survive in these stressful environments.

9.7. Potential for Therapeutic Targeting

- **Targeting Antisense RNA Pathways:** The functional significance of antisense RNAs in regulating essential biological processes makes them an attractive target for therapeutic interventions. Disrupting antisense RNA pathways could interfere with key regulatory mechanisms in *P. falciparum*, leading to defects in antigenic variation, gametocyte development, or drug resistance. For instance, drugs that mimic or inhibit antisense RNAs could be used to downregulate genes responsible for immune evasion or drug resistance.
- **RNA-based Therapies:** Antisense oligonucleotides (ASOs) or small interfering RNAs (siRNAs) that target specific antisense RNAs could be designed to alter gene expression in *P. falciparum*. This approach has been successful in other diseases, and applying it to

malaria could lead to the development of novel treatments that exploit the regulatory functions of antisense RNAs.

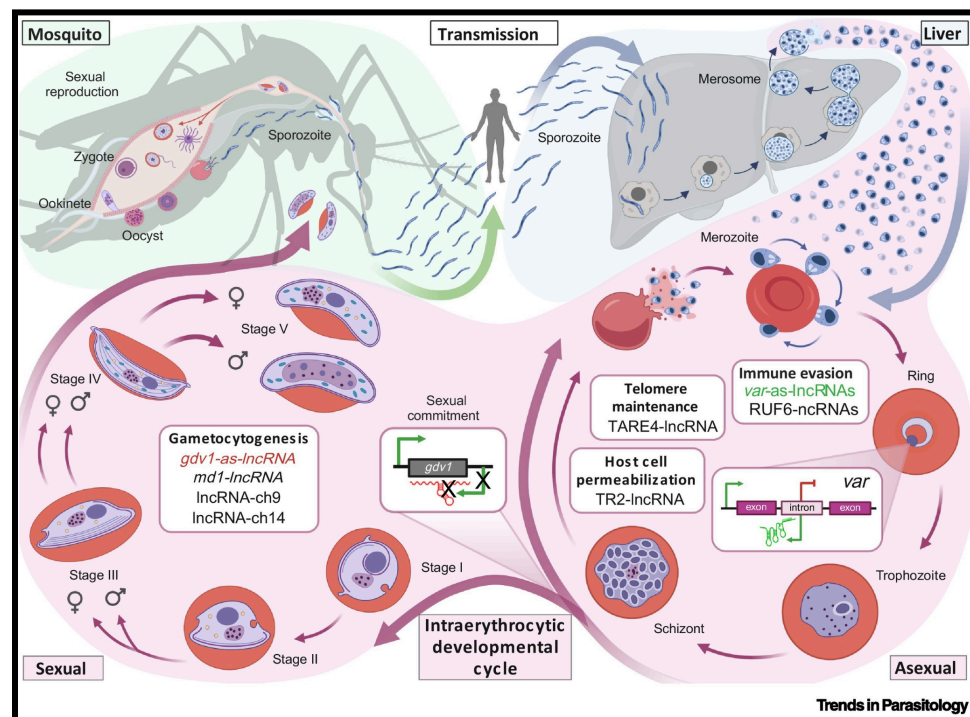


Figure 19: A detailed schematic of roles of antisense transcription in *Plasmodium falciparum* is presented above. The schematic represents the roles of numerous antisense RNA studied so far (Adapted from: Thompson TA., 2023).

Conclusion

The functional implications of antisense RNAs in *Plasmodium falciparum* are diverse and critical for the parasite's survival and pathogenicity. Antisense RNAs regulate essential processes such as antigenic variation, sexual differentiation, gene expression during various life cycle stages, and drug resistance. By modulating mRNA stability, translation, and epigenetic states, these non-coding RNAs offer *P. falciparum* a flexible and adaptive regulatory system. Targeting antisense RNA pathways holds great potential for developing new therapeutic strategies to combat malaria, particularly in the areas of immune evasion and drug resistance. Further

research into the specific mechanisms and functions of antisense transcription in *P. falciparum* will provide deeper insights into how these processes can be exploited for novel treatments.

10. Role of other classes of Non-Coding RNAs in *Plasmodium* Biology

Non-coding RNAs (ncRNAs) play crucial roles in regulating various biological processes in *Plasmodium* species, including gene expression, development, and response to environmental changes. While much of the research on ncRNAs has focused on their functions in other eukaryotes, the unique biology of *Plasmodium* offers fascinating insights into how these molecules contribute to the lifecycle of the malaria parasite. This overview discusses the types of non-coding RNAs present in *Plasmodium*, their functional roles, and their significance in the biology of the parasite.

10.1. Types of Non-Coding RNAs in *Plasmodium*

Plasmodium species express a variety of non-coding RNAs, which can be broadly categorized into several types:

a. Long Non-Coding RNAs (lncRNAs)

- lncRNAs are defined as transcripts longer than 200 nucleotides that do not encode proteins. They can regulate gene expression at various levels, including transcription, splicing, and translation.

b. Small Non-Coding RNAs (sncRNAs)

- This category includes microRNAs (miRNAs) and small interfering RNAs (siRNAs), typically ranging from 20 to 30 nucleotides. While *Plasmodium* does not have a classical RNA interference (RNAi) pathway, some small RNAs have been implicated in regulating gene expression.

c. Antisense RNAs (asRNAs)

- These are transcribed from the opposite strand of a coding gene and can overlap with the sense transcript. Antisense RNAs can modulate the stability and translation of their corresponding sense mRNAs.

10.2. Functions of Non-Coding RNAs in *Plasmodium*

a. Gene Regulation

- Non-coding RNAs play essential roles in the regulation of gene expression in *Plasmodium*. For example, lncRNAs can act as scaffolds for chromatin-modifying complexes or serve as decoys for transcription factors, influencing the transcriptional landscape of the parasite.
- **Antisense RNAs** have been shown to regulate the expression of *var* genes, which are crucial for antigenic variation and immune evasion. The presence of asRNAs can affect the stability and expression levels of their corresponding sense transcripts, allowing the parasite to finely tune the expression of surface antigens (Raabe et al., 2009).

b. Developmental Regulation

- Non-coding RNAs are involved in the developmental transitions of *Plasmodium*, particularly during the complex lifecycle stages. For example, specific lncRNAs have been linked to the regulation of genes important for the transition from asexual replication to sexual differentiation in the mosquito host (Kafsack et al., 2014).
- Research has identified stage-specific expression patterns of lncRNAs, suggesting that they may play roles in orchestrating developmental changes as the parasite progresses through its lifecycle (Sinha et al., 2014).

c. Response to Environmental Cues

- Non-coding RNAs allow *Plasmodium* to respond dynamically to changes in the environment, such as shifts in temperature, nutrient availability, or host immune responses. For example, changes in the expression levels of specific lncRNAs have been

observed during different life stages and environmental conditions, indicating their involvement in adapting to various stressors (Mair et al., 2010).

- Some small RNAs may be involved in the modulation of mRNA stability in response to stress, helping the parasite conserve resources or enhance survival during adverse conditions.

d. Role in Virulence and Immune Evasion

- Non-coding RNAs are crucial for the virulence of *Plasmodium* by influencing the expression of genes involved in immune evasion, such as the aforementioned *var* genes. The expression of different *var* genes at various stages allows the parasite to evade the host immune system through antigenic variation.
- Research has demonstrated that manipulating the expression of lncRNAs can alter the virulence of *P. falciparum*, underscoring their role in pathogenicity (Scherf et al., 2008).

10.3. Mechanisms of Action

Non-coding RNAs exert their regulatory functions through various mechanisms, including:

a. Epigenetic Modifications

- Non-coding RNAs can interact with chromatin-modifying complexes to bring about epigenetic changes that regulate gene expression. For example, lncRNAs can recruit histone-modifying enzymes to specific genomic regions, leading to changes in histone marks that influence transcriptional activity.

b. RNA-RNA Interactions

- Small RNAs can base-pair with target mRNAs, influencing their stability and translation. While *Plasmodium* lacks a canonical RNAi mechanism, some small RNAs have been shown to modulate the expression of specific genes by directly interacting with their mRNAs.

c. Competing Endogenous RNAs (ceRNAs)

- Non-coding RNAs can act as ceRNAs by sequestering common miRNAs or other small RNAs, thereby influencing the expression of target genes indirectly. This competitive interaction can lead to changes in the levels of target mRNAs.

10.4. Current Research and Future Directions

Recent studies have significantly advanced our understanding of the roles of non-coding RNAs in *Plasmodium* biology, but many questions remain. Future research should focus on:

- **Functional Characterization:** Further investigations are needed to elucidate the specific functions of various non-coding RNAs and their molecular mechanisms in regulating gene expression and parasite development.
- **Systems Biology Approaches:** Integrating transcriptomic and genomic data with computational modeling can help identify non-coding RNA networks and their interactions with coding genes and regulatory elements.
- **Therapeutic Potential:** Targeting non-coding RNAs represents a promising avenue for malaria treatment. Understanding how these RNAs regulate key pathways in *Plasmodium* could lead to novel therapeutic strategies that disrupt parasite growth or enhance the host immune response.

In conclusion, non-coding RNAs are emerging as critical regulators of gene expression and biological processes in *Plasmodium* species. Their roles in gene regulation, developmental transitions, environmental responses, and virulence highlight their importance in the complex biology of the malaria parasite. Continued research into the functional roles of non-coding RNAs will enhance our understanding of *Plasmodium* biology and may lead to the identification of new targets for malaria intervention.

11. Mechanisms of Antisense RNA action possible and their relevance for *Plasmodium falciparum*

Antisense RNA (asRNA) refers to RNA molecules that are complementary to a specific mRNA sequence and can regulate gene expression at various levels. The action of asRNAs can occur through several mechanisms, and their significance has been observed across many organisms, including bacteria, plants, and animals. In the context of *Plasmodium* species, particularly *Plasmodium falciparum*, asRNAs are believed to play critical roles in gene regulation, virulence, and lifecycle transitions (Pelechano, V., 2013). Here's an overview of the primary mechanisms through which antisense RNAs exert their effects:

11.1. Base-Pairing with mRNA

a. mRNA Stabilization and Degradation

- **Base-Pairing:** Antisense RNAs can bind to their corresponding sense mRNA through complementary base-pairing. This interaction can lead to the stabilization or degradation of the mRNA, depending on the specific mechanism at play.
- **Stabilization:** In some cases, the formation of an RNA duplex between asRNA and mRNA can protect the mRNA from degradation by exonucleases, thereby enhancing its stability and potentially increasing protein production.
- **Degradation:** Conversely, the binding of asRNA can signal for the degradation of the mRNA, either by recruiting ribonucleases or by promoting the formation of structures that are recognized for degradation by cellular machinery (Pelechano, V., 2013).

b. RNA Induced Silencing Complex (RISC)

In some organisms, asRNAs can interact with components of the RNA-induced silencing complex (RISC), leading to mRNA silencing. The presence of an asRNA can guide RISC to the target mRNA for cleavage or translational repression.

11.2. Transcriptional Interference

Antisense RNAs can inhibit the transcription of their complementary genes by interfering with the transcriptional machinery (Pelechano, V., 2013). This can occur in several ways:

a. Blocking RNA Polymerase Access

By binding to the promoter region or transcription start site of the sense gene, asRNA can physically block RNA polymerase from accessing the DNA template, thereby preventing transcription.

b. Disruption of RNA Polymerase Processivity

If an asRNA binds to the elongating RNA polymerase, it may cause the polymerase to stall, leading to incomplete or inefficient transcription of the sense RNA.

11.3. Regulation of mRNA Splicing and Processing

AsRNAs can influence mRNA splicing patterns, thereby affecting the final mRNA isoforms produced. The interaction of asRNAs with spliceosome components can alter the recognition of splice sites, leading to the inclusion or exclusion of certain exons (Pelechano, V., 2013).

a. Alternative Splicing Modulation

AsRNAs may promote or inhibit the inclusion of specific exons, resulting in different protein isoforms that can have distinct functions or regulatory roles.

b. 5' Cap and Poly(A) Tail Processing

Antisense RNAs can also affect the addition of the 5' cap or poly(A) tail, which are critical for mRNA stability, translation efficiency, and export from the nucleus.

11.4. Formation of RNA-RNA Interactions

AsRNAs can form complex structures with other RNA molecules, leading to regulatory outcomes that affect gene expression:

a. Competing Endogenous RNAs (ceRNAs)

Antisense RNAs can act as competing endogenous RNAs (ceRNAs) by sequestering shared miRNAs, thus influencing the expression levels of both the asRNA and its target mRNA.

b. Molecular Scaffolds

AsRNAs can serve as scaffolds that bring together other regulatory RNAs or proteins, facilitating the formation of ribonucleoprotein complexes that modulate gene expression (Pelechano, V., 2013).

11.5. Epigenetic Regulation

While less common in the context of asRNAs, there is evidence that some antisense transcripts can influence chromatin modifications and epigenetic states of their target genes.

a. Recruitment of Chromatin-Modifying Complexes

AsRNAs can recruit histone-modifying enzymes to the promoter region of target genes, leading to epigenetic changes that affect transcriptional activity. For example, asRNA binding may promote histone acetylation or methylation, altering chromatin structure and accessibility (Pelechano, V., 2013).

11.6. Antisense Transcription in *Plasmodium*

In *Plasmodium falciparum*, antisense transcription has been identified as a significant regulatory mechanism:

- **Var Gene Regulation:** Antisense RNAs have been shown to regulate the expression of *var* genes, which encode the surface protein PfEMP1. This regulation is vital for antigenic variation, allowing the parasite to evade the host immune system (Raabe et al., 2009).
- **Stage-Specific Expression:** The expression of antisense RNAs correlates with different life cycle stages of *Plasmodium*, suggesting that they play roles in the transition between stages, potentially affecting developmental processes and virulence (Pelechano, V., 2013).

In Conclusion, antisense RNAs are versatile regulators of gene expression, influencing various aspects of RNA stability, transcription, splicing, and epigenetic modification. Their action through base-pairing with mRNAs, transcriptional interference, and formation of complex RNA interactions highlights their importance in regulating gene expression in *Plasmodium* and other organisms. Understanding these mechanisms provides valuable insights into the biology of the malaria parasite and may uncover new therapeutic targets for malaria intervention.

12. Examples of Antisense RNA action in other organisms and their relevance to *Plasmodium falciparum*

Antisense RNAs (asRNAs) are widely studied across various organisms, and their mechanisms of action provide valuable insights into gene regulation. Here are some notable examples from different biological contexts, followed by their relevance to *Plasmodium falciparum*. A tabular summary is also available in [Figure 20](#).

12.1. Bacteria

a. Escherichia coli (E. coli)

- **Example:** In *E. coli*, the regulation of the **fimbrial adhesin gene** (fimA) is controlled by the antisense RNA **FimB**. FimB base-pairs with fimA mRNA, preventing its translation and regulating fimbriae expression, which is crucial for bacterial adhesion and virulence ([Schwan et al., 2011](#)).
- **Relevance to *P. falciparum*:** Similar regulatory mechanisms may exist in *P. falciparum* for genes involved in surface antigen expression, such as the *var* genes. Understanding bacterial antisense RNA functions could guide research on the roles of asRNAs in antigenic variation in *Plasmodium*.

b. Salmonella enterica

- **Example:** The **asRNA Sra** inhibits the translation of the **invF** gene, which is involved in invasion and virulence. By regulating *invF* expression, Sra helps the bacteria adapt to environmental changes and host defenses (Kawano et al., 2020).
- **Relevance to *P. falciparum*:** The mechanisms of asRNA action in bacterial pathogens can inform studies on how *P. falciparum* utilizes asRNAs to modulate virulence factors in response to the host environment, potentially leading to new therapeutic strategies.

12.2. Plants

a. *Arabidopsis thaliana*

- **Example:** In *Arabidopsis*, the **NPR1** gene, which regulates plant immunity, is modulated by an asRNA known as **NPR1-AS**. This asRNA can influence the stability and translation of NPR1 mRNA, affecting the plant's defense responses against pathogens (Després et al., 2003).
- **Relevance to *P. falciparum*:** The regulatory principles observed in plant systems may be applicable to understanding how *P. falciparum* regulates its immune evasion mechanisms during infection. Antisense RNAs could modulate genes involved in parasite defense against host immune responses.

12.3. Fungi

a. *Neurospora crassa*

- **Example:** The **ascospore formation** in *Neurospora crassa* is regulated by the asRNA **asc-1**, which controls the expression of genes involved in developmental transitions. The action of asRNA influences the timing and pattern of ascospore formation (Wang et al., 2021).
- **Relevance to *P. falciparum*:** The role of asRNAs in developmental processes in fungi can shed light on the potential for asRNAs in regulating the complex lifecycle stages of *P. falciparum*, including the transition between asexual replication and sexual differentiation.

12.4. Animals

a. Mammals (Human)

- **Example:** In humans, **linc-MYC** is an lncRNA that can act as an asRNA regulating the MYC oncogene. linc-MYC can influence the stability of MYC mRNA, thereby modulating cell proliferation and tumorigenesis (Iaccarino et al ., 2017).
- **Relevance to *P. falciparum*:** The similarities in asRNA regulation of oncogenes in mammals may parallel the regulation of virulence-associated genes in *P. falciparum*. Insights from human studies could help identify similar pathways in the malaria parasite, particularly regarding how asRNAs contribute to growth and survival in the host.

b. *Mus musculus* (Mouse)

- **Example:** In mice, asRNAs have been shown to regulate the expression of genes involved in embryonic development. The **Kcnq1ot1** asRNA modulates the expression of imprinted genes by altering chromatin structure and gene accessibility (Korostowski et al ., 2012).
- **Relevance to *P. falciparum*:** The role of asRNAs in development and imprinting in mammals provides a model for exploring how asRNAs might influence developmental transitions in *Plasmodium* species during their lifecycle stages.

Table 1 | **Examples of functional antisense transcription across all kingdoms of life**

Mechanism of action	Antisense locus	Effects	Species	Refs
DNA methylation	<i>LUC7L</i>	Methylates <i>HBA1</i> promoter CpG island, which represses its expression	Humans	76
	<i>Aim</i>	Regulates <i>Igf2r</i> imprinting by DNA methylation	Mice	77,78
Chromatin modifications	<i>XIST</i> and <i>TSIX</i>	Inactivates X chromosome gene expression	Mammals	2
	<i>ANRIL</i>	Represses the tumour suppressor locus <i>CDKN2B-CDKN2A</i> by both histone H3 lysine 27 (H3K27) methylation and DNA methylation	Humans	57, 80
	<i>BDNF-AS</i>	Represses <i>BDNF</i> by histone modification	Mammals	81
	<i>HOTAIR</i>	Silences the <i>HOXD</i> locus in <i>trans</i> by the recruitment of Polycomb proteins	Humans	3
	<i>COOLAIR</i>	Represses <i>FLC</i> sense gene by H3K4 demethylation and recruits Polycomb proteins, which increase H3K27me3 levels	Plants	85, 86
	<i>COLD AIR</i>	Antisense to <i>COOLAIR</i> ; represses <i>FLC</i> sense gene by the recruitment of Polycomb proteins	Plants	88
	<i>AS</i> to <i>PHO84</i>	Represses <i>PHO84</i> by histone deacetylation both in <i>cis</i> and in <i>trans</i>	<i>S. cerevisiae</i>	4,69
	<i>RTL</i>	Silences transcription of the <i>Ty1</i> retrotransposon in <i>trans</i> through chromatin modification and post-transcriptionally controls its retrotransposition	<i>S. cerevisiae</i>	70, 73
Transcriptional interference	<i>RME2</i>	Represses <i>IME4</i> by transcriptional interference in <i>cis</i> and functions after transcription initiation of <i>IME4</i>	<i>S. cerevisiae</i>	5,99
Isoform variation	<i>ZEB2-AS</i>	Induces exon skipping in <i>ZEB2</i> , which produces an alternative isoform that has increased translation efficiency	Humans	7
Translation efficiency	<i>AS</i> to <i>Uchl1</i>	Increases translation efficiency of <i>Uchl1</i> using a <i>SINEB2</i> domain	Mice	33
	<i>SymR</i>	Decreases translation efficiency of <i>SymE</i> by competing with binding of the 30S ribosome	Enterobacteria	6
RNA stability	<i>BACE1-AS</i>	Increases stability of <i>BACE1</i> by masking an microRNA-binding site	Humans	105, 106
	<i>WDR83</i> and <i>DHPS</i>	Increase their mutual stability by forming a duplex within their 3' untranslated regions	Humans	53

Aim, antisense *Igf2r* RNA; *ANRIL*, antisense non-coding RNA in the *INK4* locus; *AS*, antisense; *BACE1*, β -site APP-cleaving enzyme 1; *BDNF*, brain-derived neurotrophic factor; *COLD AIR*, *COLD-ASSISTED INTRONIC NON-CODING RNA*; *COOLAIR*, *COLD-INDUCED LONG ANTISENSE INTRAGENIC RNA*; *DHPS*, deoxyhypusine synthase; *FLC*, *FLOWERING LOCUS C*; *HBA1*, haemoglobin $\alpha 1$; *HOTAIR*, *HOX* transcript antisense RNA; *HOXD*, homeobox D; *Igf2r*, insulin-like growth factor 2 receptor; *IME4*, inducer of meiosis; *LUC7L*, Putative RNA-binding protein Luc7-like 1; *PHO84*, phosphate metabolism; *RME2*, regulator of meiosis 2; *RTL*, antisense to *LTR* (long terminal repeat); *S. cerevisiae*, *Saccharomyces cerevisiae*; *SymE*, SOS-induced *yjiW* gene with similarity to *MazE*; *TSIX*, *XIST* antisense RNA; *Uchl1*, ubiquitin carboxy-terminal hydrolase L1; *WDR83*, WD repeat domain 83; *XIST*, X inactive-specific transcript; *ZEB2*, zinc-finger E-box-binding homeobox 2.

Figure 20: Table representing different antisense RNA and their functions (Adapted from: Pelechano et al ., 2013)

The study of antisense RNAs in various organisms has revealed diverse mechanisms of action that play critical roles in gene regulation, development, and pathogenicity. The insights gained from these examples can inform research on antisense RNA functions in *Plasmodium falciparum*. Understanding the roles of asRNAs in malaria biology may uncover novel regulatory pathways, enhance our knowledge of parasite adaptation and virulence, and identify potential targets for therapeutic interventions against malaria.

13. Open questions in the field of antisense transcription in *Plasmodium falciparum*

1. What is the mechanism of action of antisense transcription in Apicomplexa?

Even while antisense transcription is well understood in the genomics era, the precise mechanism of action that helps parasites regulate their genes and contributes to their virulence is still unknown. This context's knowledge may be directly applied to the creation of technology that would stop the parasite's growth.

2. Is there a global regulator of AS transcription ?

Understanding whether there is a global regulator for AS transcription that may be controlling the levels of gene expression via antisense transcription in a stress and stimulus-dependent manner is crucial to figuring out the full mechanism of action of antisense transcription. It is still necessary to identify the global regulator in order to determine whether it may be targeted for therapeutic purposes.

3. Does Antisense transcription operate as a global phenomenon ?

In the same way, it is still unclear if AS transcription is a universal phenomena that controls gene expression or if regulation changes in response to different environmental stressors. Even if AS

transcription is a process that is preserved in all Apicomplexan parasites or if certain parasites have developed a unique method of action for gene control is unknown.

4. What is the Biological relevance of antisense transcription ?

The permissive presence of AS transcripts in both patient samples and in vitro culture indicates the physiological significance of AS transcription. Their levels fluctuate according to the parasites' developmental stages; in Plasmodium, it has been noted that NAT levels rise as the parasites grow, and at this point, virulence typically manifests as illness signs. Additionally, when the disease's severity increases, their levels are observed to change. They have also been linked to metabolic pathways whose imbalance has been shown to directly increase the disease's severity. What physiologic significance does AS transcription have in such a situation?

5. What is the relevance of AS transcription in generating virulence of the parasite ?

It has been demonstrated that non-coding RNA controls the expression of virulence genes that are mutually exclusive. Could AS transcripts have a role in var gene switching in this situation? Var gene switching is a crucial factor in cytoadherence, host cell invasion, and immune response evasion. This flipping might be utilised as a possible marker for the parasite-caused disorders if its sequence could be determined.

6. Does AS transcription contribute in generating drug resistance

It is now widely known that parasites are becoming more resistant to drugs all around the world. However, it is currently unclear if AS transcripts contribute to the development of drug resistance in apicomplexa. In vitro cultures of drug-resistant parasites have shown a significantly higher abundance and dysregulation than drug-sensitive parasites. Therapeutics in the form of target-specific alternatives may have a brighter future if such transcripts are shown to be the cause of drug resistance.

13.1. Future perspective

The traditional method of altering gene activity and then analysing the phenotype and genotype is the best way to comprehend the mechanism of action of AS transcription. Furthermore, the understanding gained from researching the mechanism of action of antisense transcription may be applied to the creation of a therapeutic strategy that inhibits the parasite's growth in the most target-specific way possible.

13.2. Lacunae in the field

Since the field's inception, researchers have studied antisense transcription using relatively basic methods. However, there is a wealth of information about the parasite's genome, transcriptome, proteome, and metabolome available in the present state of "omics" research. It demonstrates unequivocally that there is no lack of technology in the modern era to answer queries that could resolve important issues. Understanding the mechanism of action of AS transcription and the global regulator governing the mechanism is still a mystery in the area.

The development of chemotherapeutics and diagnostics may greatly benefit from the solution to this general query. This would be a solution to target the parasite in the most non-intrusive and target-specific way possible without interfering with the host's metabolic processes because the mechanism is inherent to the parasite.

Chapter 2

Chapter 2: Regulatory functions of PfGCN5 in nutrient sensing, stress response and transcriptional regulation in *Plasmodium falciparum*

Introduction

Millions of people die from malaria each year, an infectious disease that can be fatal and is caused by a parasite from the species *Plasmodium*. Even after over a century of attempts to eradicate malaria, it remains one of the most common parasite infections in tropical and subtropical regions of the world. According to WHO (2024), there were 219 million malaria infections and an estimated 435,000 fatalities in 2024. Malaria's clinical manifestations include fever, anaemia, acidosis, and organ failure. The parasite infection may cause cerebral malaria or possibly death if treatment is not received (WHO 2024). The life cycle of *Plasmodium* is intricate and involves both an invertebrate and a vertebrate host. It involves rapid morphological changes and cellular differentiation events that occur at specific times. Human chronic infection may be caused by transcriptional and post-transcriptional control of gene expression (Abel and Le Roch et al., 2019; Coleman and Duraisingh et al., 2008; Karmodiya et al. 2015). Aly et al. (2009) state that the parasite transmits itself to the human host, where the asexual cycle is completed, through the female *Anopheles* mosquito.

The parasites can withstand a variety of stresses during different stages of development, including drug exposure, parasite density, elevated body temperature (fever), and fluctuations in nutrients and nutrition (Becker et al., 2004; Engelbrecht and Coetzer, 2013; Oakley et al., 2007; Pirio et al., 2012; Rawat et al., 2019a; Rosenberg et al., 2009). It is well recognised that *P. falciparum*'s increased virulence and the emergence of drug resistance are directly related to these stressors. It has been abundantly clear in recent years that different protozoan parasites alter and subvert the metabolism and apparatus of their hosts in order to evade immune reactions (Becker et al., 2004; Rawat et al. 2019a; Rosenberg et al., 2009; Blader et al. 2001; Decherf et al. 2004; Lang et al. 2007; Martin and Kirk 2007; Plattner and Soldati Favre 2008; Singh et al. 2007, Goldberg et al. 1990; Kim et al., 2007; Engelbrecht and Coetzer 2013; Oakley et al. 2007; Perca 'rio et al., 2012).

According to a number of studies (Babbitt et al., 2012; Ting et al., 2005), the parasite is dependent on its host for nutrients because it would no longer be able to synthesise them on its own. Furthermore, during the intraerythrocytic life cycle, the malaria parasite is known to derive all of its energy from the glycolysis and degradation of host cell haemoglobin (Zuzarte-Lui 's and Mota 2018; Gardner et al., 2002; Olszewski et al., 2009). Any changes to the host cell or ambient milieu could negatively impact the proliferation and virulence of the parasites, as they are entirely dependent on them for resources (Plattner and Soldati-Favre 2008; Zuzarte-Lui 's and Mota 2018).

The process of invading numerous tissues, evading immune monitoring, and escaping pharmacological pressure may be severely affected by nutritional changes (Billker et al. 1998; Dean et al., 2009; Ke et al., 2015; MacRae et al., 2013; Mancio-Silva et al., 2017; Srivastava et al., 2016). It is well known that during infection, the host's glucose levels can fall to as low as 50 mg/dl, which can lead to conditions of hypoglycemia. Consequently, a mechanism that is effective in sensing and reacting quickly to any changes in the content of nutrients and adjusting to a new environment is needed. This can involve adjusting the programs for differentiation and replication. As *Plasmodium* spp. shift between human and mosquito hosts, their ability to sense, react, and adapt to altering nutritional content is clearly seen (Zuzarte-Lui 's and Mota 2018).

Significantly, these quick changes in nutrition and physiological states within and between different hosts necessitate quick changes in cellular and molecular programming, which can only be accomplished by particular parasites that are primed to support antigenic variation and differential stress response (Rawat et al., 2019b). Therefore, a molecular sensor with information-relaying capabilities may be a tactical development that helps parasites adapt and live in novel surroundings. Our lab's earlier studies revealed that PfGCN5, a histone acetyltransferase, is connected to genes relevant to stress and virulence in *P. falciparum* (Rawat et al., 2019a).

Given that the virulence and survival of parasites are known to be significantly influenced by their metabolism (Mancio-Silva et al., 2017), we sought to comprehend PfGCN5's function in transcriptional regulation under conditions of nutritional stress. Here, we demonstrate that PfGCN5 is linked to genes that are critical for maintaining parasite cellular homeostasis under

conditions of nutritional stress. Therefore, our research indicates that PfGCN5 plays a significant role in the activation of genes in *Plasmodium falciparum* that respond to nutrient stress under nutrient stress conditions.

Materials and Methods

1. In vitro culture of *P. falciparum* parasites

The RPMI1640 medium containing 25 mM HEPES, 0.5% AlbuMAX II, 1.77 mM sodium bicarbonate, 2 grams/liter glucose, 100 microM hypoxanthine, and 12.5 lgml-1 gentamicin sulphate was used to cultivate the *P. falciparum* 3D7 strain at 37°C in Eppendorf system incubator. A 1%–1.5% haematocrit and 5% parasitemia were maintained for the parasites. Fresh O+ human RBC that was obtained from healthy human donors through blood banks was used to cultivate parasites. Double synchronisation was performed using 63% percoll gradient centrifugation and 5% sorbitol. Slides were prepared using a thin blood smear stained with Giemsa, to assess parasite's growth.

2. Nutrient stress induction

Using 5% sorbitol, parasites were closely synchronised at the early ring stage. At late ring stage (*16 hpi), nutrient stress was triggered and continued for 6 h (*22 hpi). RPMI1640 medium supplemented with 25 mM HEPES, 0.5% AlbuMAX II, 1.77 mM sodium bicarbonate, 0.5 g/l glucose, 100 microM hypoxanthine, and 12.5 lgml-1 gentamicin sulphate was used to develop the parasites under conditions of nutrient stress at 37°C.

3. Western blotting and Antibodies

Using saponin lysis (0.15%) at 37°C for 15 minutes, parasites were extracted. Prior to the lysis technique the parasites were cleaned using 1X PBS. The parasites were lysed using a buffer called parasite lysis buffer (10 mM Tris-Cl pH 8.0, 150 mM sodium chloride (NaCl), 0.5% nonyl phenoxypolyethoxylethanol (NP 40), 0.5% sodium deoxycholate, 1.5 mM magnesium chloride (MgCl₂), 0.1 mM ethylene diamine tetra acetic acid (EDTA), 1X protease inhibitor cocktail (PIC), and 1 mM phenylmethylsulfonyl fluoride (PMSF). Three freeze- thaw cycles were

performed using liquid Nitrogen and thawing on ice. This parasite lysate was centrifuged for 30 minutes at 4°C at 13,000 rpm.

Lysate proteins were separated on 7.5 -- 12% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membrane using wet transfer device as a routine protocol in the lab. 5% skimmed milk produced in TBS-T (Tris Buffer Saline with 0.01% Tween-20) was used to block the PVDF membrane. For immunoprecipitation and Western blotting, anti-rabbit IgG (OSB PM035) and previously produced PfGCN5 antibody (Rawat et al. 2019a) were utilized. Purified His tagged PfGAPDH recombinant protein was used to generate the PfGAPDH antibody in house following rabbit injection protocol. As previously mentioned, the PfGAPDH antibody was purified (Rawat et al., 2019a).

For immunoprecipitation and Western blotting, anti-rabbit IgG (OSB PM035) and previously produced PfGCN5 antibody (Rawat et al., 2019a) were utilized. Purified Histagged PfGAPDH recombinant protein was used to create the PfGAPDH antibody (data not shown). As previously mentioned, the PfGAPDH antibody was purified (Rawat et al., 2019a).

4. Immunofluorescence assay

Parasites were fixed with 4% paraformaldehyde (PFA) and 0.00075% glutaraldehyde for 30 minutes at 37°C. Followed by five minutes of permeabilization which was done using 0.1% Triton X-100 in PBS. 1X PBS was then used to wash the parasites thrice. After blocking for one hour at room temperature with 3% BSA, the primary antibody was incubated in 3% BSA for three hours. To get rid of the unbound primary antibody, three PBS washes were carried out. The secondary antibody (Invitrogen, Alexa 647) was incubated for one hour at room temperature on gentle rotation. Before mounting parasites on glass slides using ProLong Gold Antifade with DAPI (Invitrogen), they were cleaned with 1X PBS. Then using a Leica SP8 confocal microscope, pictures were taken.

5. Quantitative RT-PCR (qRT-PCR)

To isolate the RNA, Biorad's TRIzol reagent was used. Using random primers and the ImProm-II Reverse Transcription System (Promega) kit, 2 microgram of DNase free RNA were converted to cDNA. 5'-TGCTGATAA TAAAGGGGCTGC-3' and 5'-AATGGCCATGCAG ACTGTTG-3'

are the PfGCN5 primers used for qRT-PCR. An internal control of 18 s RNA was employed, utilising forward primer 5'-GCTGACTACGTCCCTGCCC-3' and reverse primer 5'-ACAATTCATCATATCTTTCAATCGGTA-3'. For gene-specific qRT-PCR, the following forward and reverse primers were used: PfMyst (GGGGGAAAGAATGAAAACAAAG and GTTATTCCATTCCCCCACTTCT), PF14_0350 (AAGAT TGCCCAGATTGGA and TCGGCTAGCTGAAC CAAAAT), PFA0465c (CGGGCAAAGGAGTTGA TTTA and GCTGGGTTGTTAGAAACTCG), PF13_0131 (AAACAACATCCAAGTGAACACG and CGTCGATTGATTTTATCATTTG), Pf3D7_01030700 (TTTGGAATGAAGGAACAGG and TAATTTGC CCACACAGCAAA), and Pf3D7_1215300 (TACCA GAAAGCGCAACTGAA and CAATTTTAAAGA GGAACCACCA). CFX96 Real Time PCR detection system (Biorad) was used for quantitative PCR. Each experiment included a technical triplicate and was performed over three independent biological replicates.

6. Chromatin immunoprecipitation, library preparation and data analysis

After cross-linking infected RBCs for 10 minutes at room temperature with 1% formaldehyde (Thermo Scientific 28908), 150 mM glycine was added to quench the reaction. The cells were lysed in sonication buffer (10 mM Tris-HCl pH 7.5, 200 mM NaCl, 1% SDS, 4% NP-40, 1 mM PMSF, 1X PIC) after being swelled (25 mM Tris pH 7.9, 1.5 mM MgCl₂, 10 mM KCL, 0.1% NP40, 1 mM DTT, 0.5 mM PMSF, 1x PIC). Covaris S220 was utilised to conduct sonication and produce chromatin with a size of 200–400 bp. Following preclearing, chromatin was treated with an antibody for a whole night at 4C. Subsequently, it was incubated for four hours at 4C with saturated Protein G Sepharose beads. Chromatin was eluted using ChIP elution buffer (1% SDS, 0.1M sodium bicarbonate). For the creation of ChIP libraries, NEBNext Ultra II DNA Library Prep kit was utilised. The Beckman Coulter Agencourt XP beads were used for size selection. Adapter-ligated fragments were purified using Agencourt XP beads (Beckman Coulter) and then PCR-amplified using indexing primers. The Agilent DNA 1000 kit and the Agilent Bioanalyzer 2100 were used to evaluate the electropherograms of the libraries. The libraries were combined at an equimolar concentration, and the Illumina HiSeq 2500 (BENCOS Research Solutions Pvt. Ltd., Maharashtra) was used to sequence 50 bp reads. Using Bowtie2 with default parameters, ChIP-seq data were mapped to *Plasmodium falciparum* 3D7 genome version 37 ([http:// plasmodb.org/plasmo/](http://plasmodb.org/plasmo/)). Peak calling against input control data was performed using

MACS2 peak calling software with default settings. Bedtools were used to annotate the peaks. Log2-fold enrichment [1.7] peaks were employed in the analysis.

7. Data source and analysis

The Sequence Read Archive (SRA) under accession number ID SUB5643208 provided previously published RNAseq and ChIP sequencing data, which were used as a control.

8. PfGAPDH cloning, expression and purification

Using gene-specific primers (Forward primer 50-ACGCATATGATGGCAGTAACAAAACCTTG-30 and Reverse primer 50-ATGCCTCGAGGTTGTTAGTAATGTGTACGG-30), the full length PfGAPDH DNA sequence was amplified from parasite genomic DNA. Using the restriction enzymes NdeI and XhoI, the PCR-amplified fragment was cloned in frame into the pET28a? plasmid vector containing a 6x-His tagged fusion protein. DNA Sanger sequencing was used to confirm cloning. To express proteins, *E. coli* BL21 (DE3) was transformed with the cloned plasmid (pET28a?_PfGAPDH). 0.5 mM isopropyl-1-thio-b-d-galactopyranoside (IPTG) was used to induce expression for five hours at 25°C at an optical density of 0.6 at 600 nm. Sonication buffer (10 mM Tris Cl pH 8.0, 300 mM NaCl, 10% Glycerol, 1X PIC, 1X PMSF) was used to lyse the bacterial pellet. To purify the recombinant PfGAPDH, Ni-NTA beads (Qiagen) were used. Using SDS PAGE, protein purification was examined. Western blotting with Anti-His was used to validate protein expression. Protein was dialysed in the sonication buffer after purification and kept at 4°C. The pGEX-4T1 plasmid-cloned PfGCN5 (HAT and bromodomain) was utilized to express the glutathione S-transferase (GST) fusion protein (Rawat et al. 2019a). Expression was induced for five hours at 25°C using 0.5 mM isopropyl-1-thio-b-d-galactopyranoside (IPTG) at an optical density of 0.6 at 600 nm. Glutathione sepharose 4B beads (GE Healthcare Life Science) were used to purify the protein. For protein elution, a decreased glutathione concentration of 20 mM was utilized. Ni-NTA beads were used in the protein purification process. Various reduced glutathione concentrations were used to elute the protein. Dialysed proteins were kept at -20°C after being purified.

9. Recombinant protein interaction assay

Overnight incubation was conducted using recombinant pure PfGCN5-HAT, Bromodomain (GST tagged), and PfGAPDH (His tagged). As a control, PfGAPDH and GST proteins were also incubated in a comparable manner. For 12 hours at 4C, an anti-GST antibody was added. The proteins were incubated for 4 hours at 4C with slow rotation before Dynabeads Protein G (Invitrogen) beads were added. After incubation, beads were eluted with glycine at pH 2.5 and cleaned with washing buffer (10 mM Tris-cl pH 8.0, 300 mM NaCl, 10% Glycerol, 10 mM Imidazole, 1X-PIC, 1X-PMSF). Western blotting was done after the eluted material was processed on SDS-PAGE. The purpose of the Anti-His antibody incubation was to determine if PfGAPDH was lowered via PfGCN5 protein.

10. Nuclear and cytoplasmic fractionation

In 500 ml of ice-cold lysis buffer (10 mM HEPES pH-7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.65% NP40, 1 mM DTT, 2X PMSF), the saponin-lysed parasite pellet was again suspended. The mixture was then incubated on ice for 30 minutes while being periodically mixed. The mixture was spun for 20 minutes at 4 degC at 13000 rpm. After being separated, the supernatant was designated as cytoplasmic extract. The parasite pellet was cleaned three times by resuspending it in 500 microl of cytoplasmic buffer, spinning it for ten minutes at 13000 rpm, and then discarding the supernatant after five minutes of ice incubation. After that, the pellet was twice cleaned using the same technique as before, but with 500 ml of cold 1X PBS added to 1X PMSF for each wash. The pellet was resuspended in three volumes of nuclear buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM DTT, and 1 mM EDTA) and incubated on ice for 30 minutes after the pellet volume was determined (1 volume). After centrifugation for 20 minutes at 4 degC at 13,000 rpm, the supernatant was extracted and labeled as nuclear extract. Actin served as the cytoplasmic control and histone H3 as the nuclear control.

11. Acetylation assay

Using the fractionation procedure, nuclear lysate was isolated from 3D7 parasite pellets that had been saponin-lysed. PfGAPDH protein that has been recombinant was used in the acetylation assay. Because it was challenging to clone and express full-length PfGCN5 in a bacterial culture, we used a particular antibody to pull down the native PfGCN5 protein. The acetylation experiment was conducted using the pull-down complex that contains PfGCN5. HAT buffer (250

mM Tris-Cl (pH 8), 50% glycerol, 0.5 mM EDTA, 5 mM DTT, 250 mM KCl, 100 mM Na-Butyrate, 1X PIC) was used for the acetylation experiment. The acetylation experiment was incubated for one hour at 30°C. The recombinant PfGCN5 protein's acetylation level was measured with a pan acetyl antibody (Abcam, ab22550).

Results

1. PfGCN5 is upregulated during nutrient stress condition

Previous research conducted by our group has demonstrated that PfGCN5 controls the expression of genes that respond to stress when *P. falciparum* is exposed to artemisinin and temperature stress (Rawat et al., 2019a). To take this study further and to acquire a better understanding of the role of PfGCN5 under nutrient stress, we exposed ring stage parasites (16 h post synchronisation) to a nutrient stress condition (1/4 normal glucose concentration) for 6 hours before harvesting them (Figure 1A). The parasites' cell cycle progression was not affected by the nutrient stress conditions used in the experiment and the same was experimentally validated. The complete elimination of glucose from the culture media resulted in a significant reduction in parasite development, ultimately leading to their death, hence this scheme was abolished.

Science has proven that Histone acetyltransferases activate genes by acetylating histones at the N terminus. Therefore, we investigated the expression level of all known histone acetyltransferases together with PfGCN5 in *P. falciparum* under nutritional stress conditions (Kanyal et al., 2018). Interestingly, PfGCN5 had the greatest increase compared to other histone acetyltransferases (Figure 1B). To better understand global transcriptional dysregulation, we performed RNA-sequencing (RNA-Seq) under both nutrient stress and control settings. A total of 303 genes were discovered to be considerably deregulated ([2 folds]); 191 genes were upregulated and 112 genes were downregulated during nutritional stress conditions (Figure 1C). Furthermore, gene ontology analysis of upregulated genes revealed an enrichment of metabolism-related pathways under nutrient stress conditions (Figure 1D).

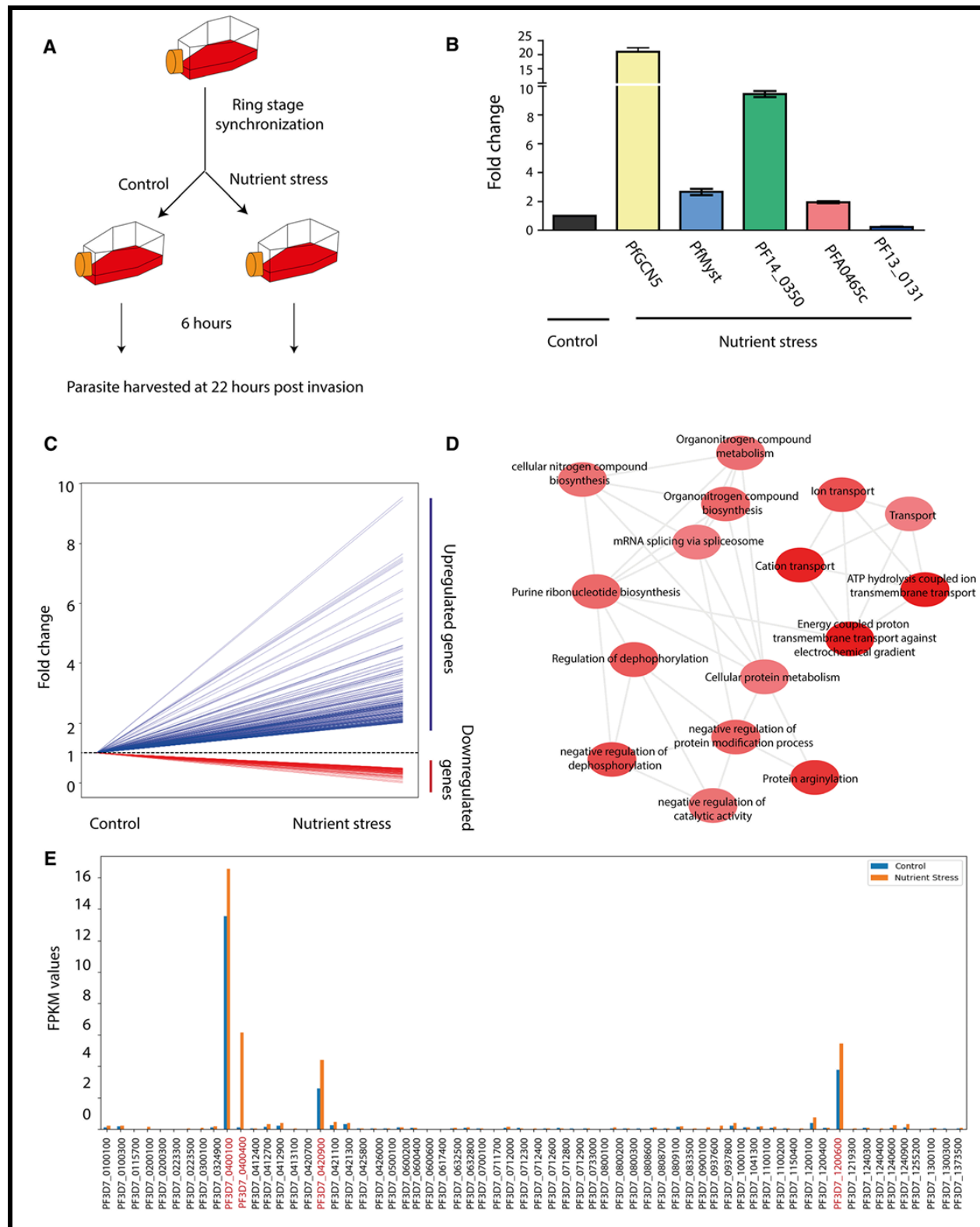


Figure 1: Role of PfGCN5 in *Plasmodium falciparum*. Under conditions of nutritional stress, PfGCN5 is upregulated and global transcription is deregulated. (A) The schematic illustrates how a nutrient stress state is induced to simulate the hypoglycemia seen during a malaria infection. (B) RT-qPCR results under nutrient stress conditions, demonstrating the highest upregulation of PfGCN5 among all known HATs (experiment conducted in three biological replicates using 18S rRNA as an endogenous control). (C) A profile plot showing how gene expression varies under conditions of nutritional stress. Red denotes 112 down-regulated genes and blue denotes 191 up-regulated genes. (D) Purine ribonucleotide biosynthesis, cellular protein metabolism, organonitrogen chemical production, and other key metabolic pathways are over-represented, according to gene ontology analysis of upregulated genes (from RNA seq analysis). (E) Histogram demonstrating var gene transcriptional dysregulation during nutritional stress (Adapted from: Rawat, M., Malhotra, R., et al., 2020).

2. PfGCN5 associates with metabolic genes during nutrient stress condition

Furthermore, we used high-throughput chromatin immunoprecipitation followed by sequencing (ChIP-seq) to analyse the association of PfGCN5 in nutrition sensing. PfGCN5's association with 554 genes, primarily at their gene body and promoter regions under nutrient stress conditions, was shown by a genome-wide study of the protein (Figure 2A). Remarkably, genes linked to immune response and antigenic variation were discovered to be targets of PfGCN5 in the context of nutritional stress (Supplementary Figure 1) as well as control (Rawat et al., 2019a). Under conditions of nutrient stress, PfGCN5 was discovered to be exclusively linked to metabolic genes, suggesting its role in the transcriptional control of metabolic genes (Figure 2B). Next, we examined PfGCN5 bound genes' expression levels in conditions of nutritional stress. It's interesting to note that under stress conditions, the expression levels of only metabolic genes—not all PfGCN5 bound genes—showed a considerable upregulation (Figure 2C). Additionally, we assessed the PfGCN5-bound genes' expression levels with and without garcinol, a PfGCN5 inhibitor.

Figure 2D illustrates how we found that PfGCN5 bound genes, which are elevated in nutrient stress conditions, either failed to upregulate or displayed downregulation when garcinol was

administered. Therefore, our data points to a crucial and causative role for PfGCN5, but they do not rule out the potential that garcinol inhibits other histone acetyltransferases. Furthermore, it was discovered that PfGCN5 occupied the PfGAPDH locus under nutritional stress, demonstrating PfGCN5's direct control of PfGAPDH. All of these findings indicate that PfGCN5 binds to genes involved in metabolism when under nutritional stress and may control the expression of these genes to preserve homeostasis.

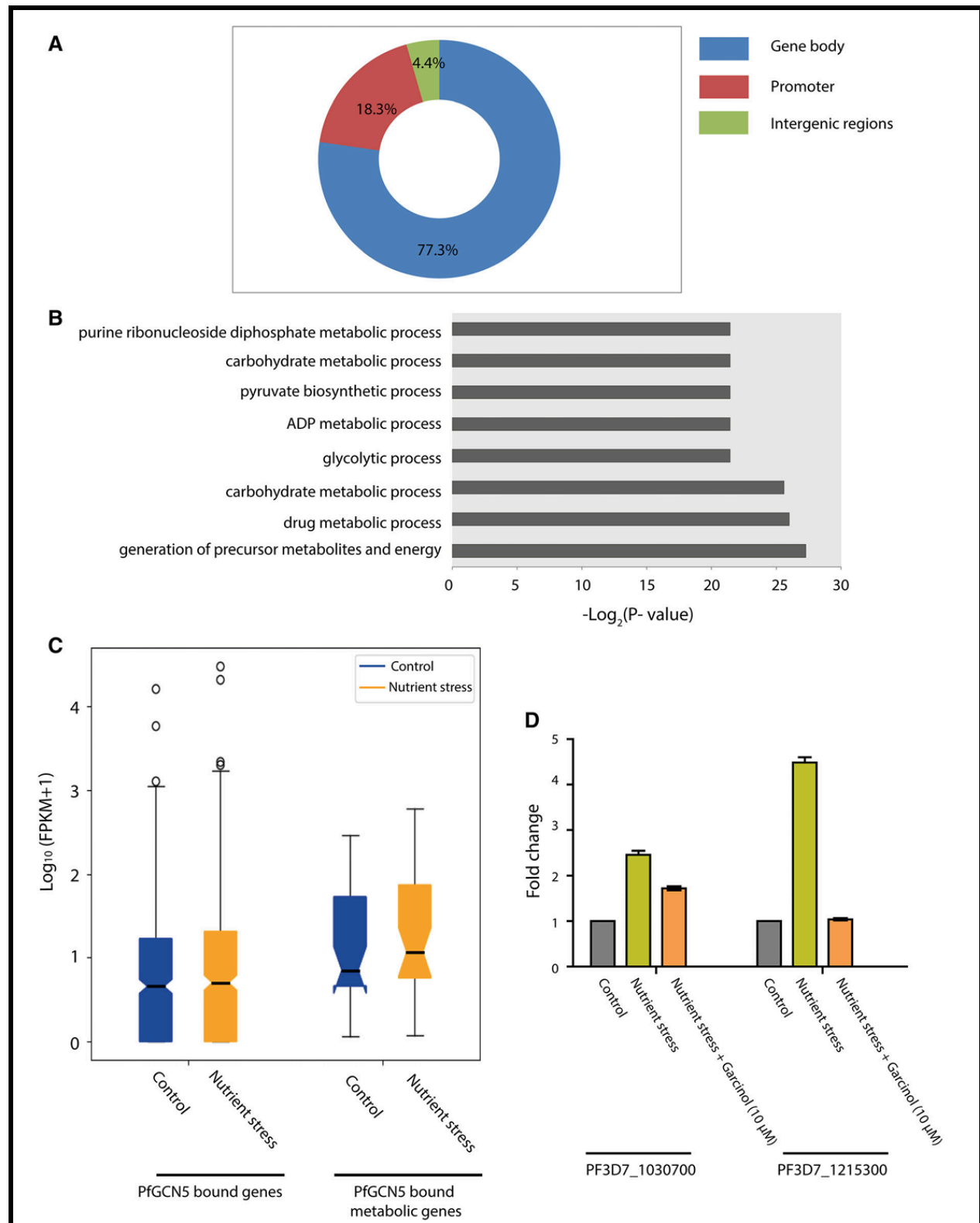


Figure 2: Under conditions of nutritional stress, PfGCN5 binds to metabolic genes. (A) Doughnut chart illustrating PfGCN5's ChIP-seq-based enrichment, primarily over promoter and gene body regions. (B) PfGCN5 binding peak gene ontology analysis shows metabolic gene enrichment. (C) Under nutrient stress conditions, the box and whisker plot indicates a considerable upregulation of PFGCN5 bound metabolic genes relative to all PfGCN5 bound genes. (D) The transcript levels of genes under nutritional stress were evaluated by RT-qPCR with or without the GCN5 inhibitor garcinol (Adapted from: Rawat, M., Malhotra, R., 2020).

3. PfGCN5 interacts with different metabolic enzymes

We also examined PfGCN5's interacting partners to have more understanding of the potential mechanism by which it could be involved in nutrient sensing. Using immunoprecipitation and mass spectrometry (Figure 3A) we previously found that several metabolic proteins have high confidence hits, along with other expected chromatin organisation and stress/stimuli responsive partners (Rawat et al., 2019a). Glycolytic proteins are known to carry out extracellular tasks in parasites, including cell adhesion, immunological response and homeostatic regulation, and nutrition acquisition (Gomez-Arreaza et al., 2014). We questioned whether metabolic proteins in *P. falciparum* work in tandem with PfGCN5 to regulate genes as a side project.

We questioned whether PfGCN5 could acetylate metabolic proteins as previously published acetylome data revealed that a variety of proteins involved in glycolysis are acetylated (Cobbold et al., 2016; Miao et al., 2013). PfGAPDH was identified by our mass spectrometry data as PfGCN5's most reliable interaction partner. Furthermore, research using a plant model system showed that GAPDH translocates into the nucleus after being acetylated by the GCN5 paralog PCAF. There, it binds to the promoters of glycolytic genes and controls the expression of those genes in response to a glucose stimulus (Ventura et al., 2010). Additionally, we discovered that under conditions of nutrient stress, the RNA expression level of PfGAPDH increases ([2 folds]) (Figure 3B). As a result, PfGAPDH was identified as the metabolic protein of interest for additional research.

A

Metabolic pathway	Genes
Glycolysis / Gluconeogenesis	phosphoglycerate kinase hexokinase pyruvate kinase glyceraldehyde-3-phosphate dehydrogenase L-lactate dehydrogenase ATP-dependent 6-phosphofructokinase enolase fructose-bisphosphate aldolase
Pentose phosphate pathway	ATP-dependent 6-phosphofructokinase phosphoribosylpyrophosphate synthetase fructose-bisphosphate aldolase
Fructose and mannose metabolism	hexokinase ATP-dependent 6-phosphofructokinase triosephosphate isomerase fructose-bisphosphate aldolase
Purine metabolism	purine nucleoside phosphorylase pyruvate kinase adenylate kinase phosphoribosylpyrophosphate synthetase inosine-5'-monophosphate dehydrogenase hypoxanthine-guanine phosphoribosyltransferase

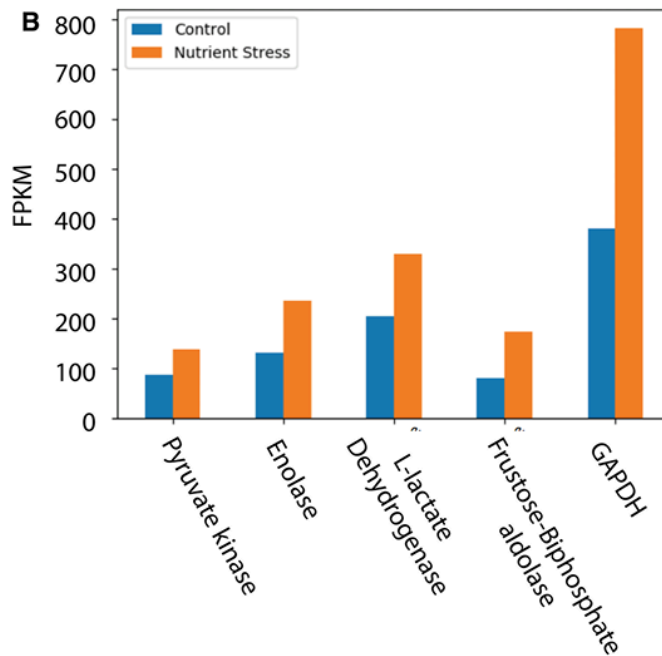
B

Figure 3: PfGCN5 interacts with many enzymes involved in metabolism.

(A) Table listing the PfGCN5 interactomes known to be involved in several metabolic processes.
(B) A histogram illustrating the level of RNA expression of a few metabolic genes under conditions of nutritional stress (Adapted from: Rawat, M., Malhotra, R., 2020).

4. PfGAPDH translocates to the nucleus to perform gene regulatory roles

We used nuclear cytoplasmic fractionation to investigate the distribution of PfGAPDH. As anticipated, we discovered that PfGAPDH was primarily located in the cytoplasm, but surprisingly, under normal circumstances, it was also identified in the nucleus (Figure 4A). Additionally, PfGAPDH protein was shown to be localised in both the cytoplasm and the nucleus according to immunofluorescence analysis using a PfGAPDH specific antibody (Figure 4B). We used recombinant PfGAPDH and PfGCN5 proteins in an in vitro interaction test to further confirm the interaction between PfGCN5 (HAT and bromodomain) and PfGAPDH. It's interesting to note that PfGCN5 and PfGAPDH interacted in vitro (Figure 4C). We chose to investigate if PfGCN5 acetylates PfGAPDH because PfGAPDH was discovered to be acetylated in Plasmodium acetylome data. Remarkably, it was discovered that recombinant PfGAPDH was acetylated when PfGCN5 was present (Figure 4D). In summary, our findings indicate that PfGCN5 interacts with PfGAPDH and acetylates it, potentially within the nucleus. To confirm PfGAPDH's function in the nucleus, more research is required. Direct chromatin interactions may be the mechanism by which PfGAPDH in complex with PfGCN5 regulates gene expression.

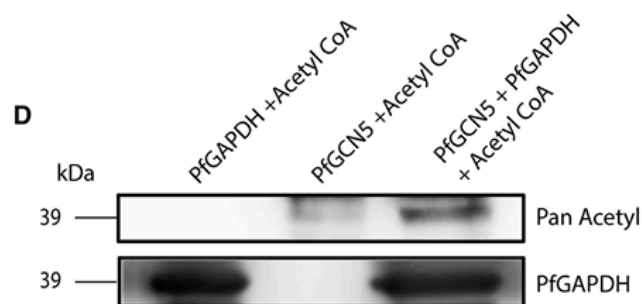
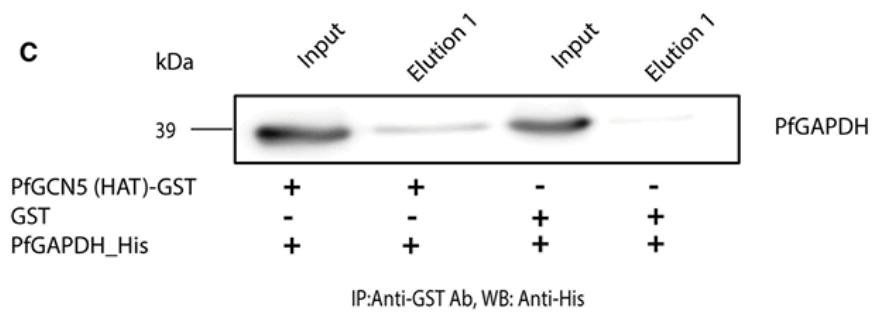
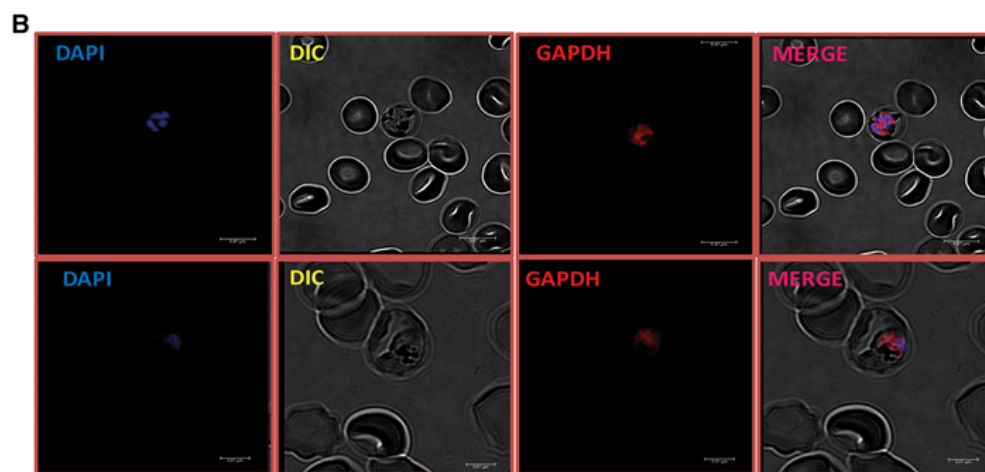
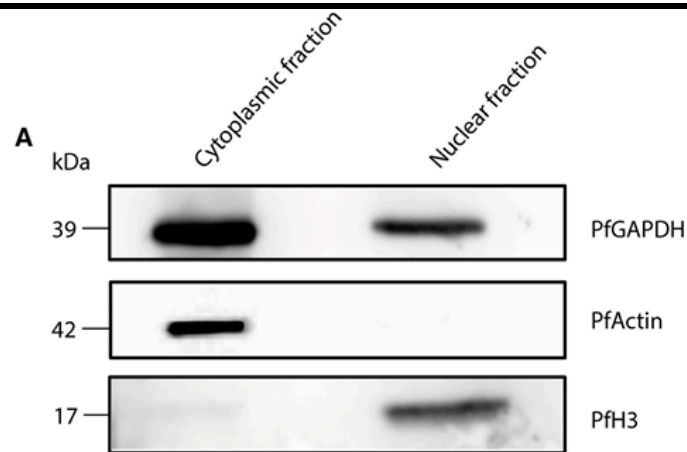


Figure 4: (A) PfGAPDH distribution using nuclear and cytoplasmic fractionation. (A) PfGAPDH was found to be distributed in both cytoplasm and nucleus. Histone H3 and actin are used as nuclear and cytoplasmic controls, respectively. (B) Immunofluorescence analysis showing localization of PfGAPDH in both nucleus and cytoplasm using PfGAPDH specific antibody. (C) In vitro interaction assay of recombinant purified PfGCN5 (GST tagged) and PfGAPDH (His tagged). Interaction was confirmed with Western blotting using Anti-His antibody. (D) In vitro acetylation assay was performed to test whether PfGCN5 acetylates recombinant PfGAPDH protein. PfGAPDH acetylation levels were assessed using pan-acetyl antibody (Adapted from: Rawat, M., Malhotra, R., 2020).

Discussion

Plasmodium falciparum, which solely depends on its hosts throughout its life cycle for their nutrition. The parasite needs nutrients as an energy source for both development and gene expression regulation (Mancio-Silva et al., 2017). Any modulations to the necessary levels of nutrients can have a deleterious impact on both the parasite and the host because infection creates a shared pool of nutrients. Therefore, for the parasites to successfully infect a host, they must be able to quickly detect, react to, and adjust to any changes in the host's nutritional environment. It is clear that the parasites can sense changes in the surrounding environment, such as a drop in glucose levels which may drop to as low as 50 mg/dl. Such a situation of hypoglycemia can be fatal for the host and also challenging for the parasite.

It is unclear how the parasites sense the nutritional condition of their host because classical nutrient sensing pathways are likely absent and the tricarboxylic acid (TCA) cycle is known to have a redundant role during the parasite's intraerythrocytic life cycle. The fact that parasites are known to only use the host cell's hemoglobin for energy catabolism adds to the intricacy. In this case, parasites have to plan low-energy, high-efficiency responses to unexpected changes in the host's nutritional environment in order to minimize the associated costs, which include decreased rates of replication and virulence. The virulence of the parasites has been found to be enhanced by metabolism-related proteins, although this needs global transcriptome rearrangement under unfavorable circumstances.

In this work, we tried to clarify the function of a histone acetyltransferase (HAT) PfGCN5 in the regulation of transcription under conditions of nutritional stress. Under conditions of nutritional stress, we discovered a considerable elevation of PfGCN5 and PfGAPDH transcript levels. It's interesting to note that PfGCN5 has been linked to several metabolic genes that are elevated in situations of nutritional stress as its moonlighting role. Studies on fractionation and localisation revealed that PfGAPDH is found in the nucleus and cytoplasm. Furthermore, it was discovered that PfGCN5 acetylates PfGAPDH in vitro and interacts with it.

Therefore, it's probable that PfGAPDH is further translocated to the nucleus during nutritional stress, where PfGCN5 acetylates it. The expression of metabolic genes may be regulated by acetylated PfGAPDH in combination with or without PfGCN5 (Figure 5). Some of the unresolved issues surrounding PfGCN5 and PfGAPDH mediated transcriptional regulation under nutritional stress conditions would be explored by more thorough research. (i) Does the distribution of PfGAPDH in the cytoplasm and nucleus alter in response to nutritional stress? (ii) Does PfGAPDH control the expression of genes via interacting with the chromatin? (iii) Alternatively, is the PfGCN5-PfGAPDH complex's synergy necessary for the regulation of gene expression? To sum up, deciphering the nutrition sensing processes would reveal the molecular networks governing the parasite's virulence and open the door to disease prevention.

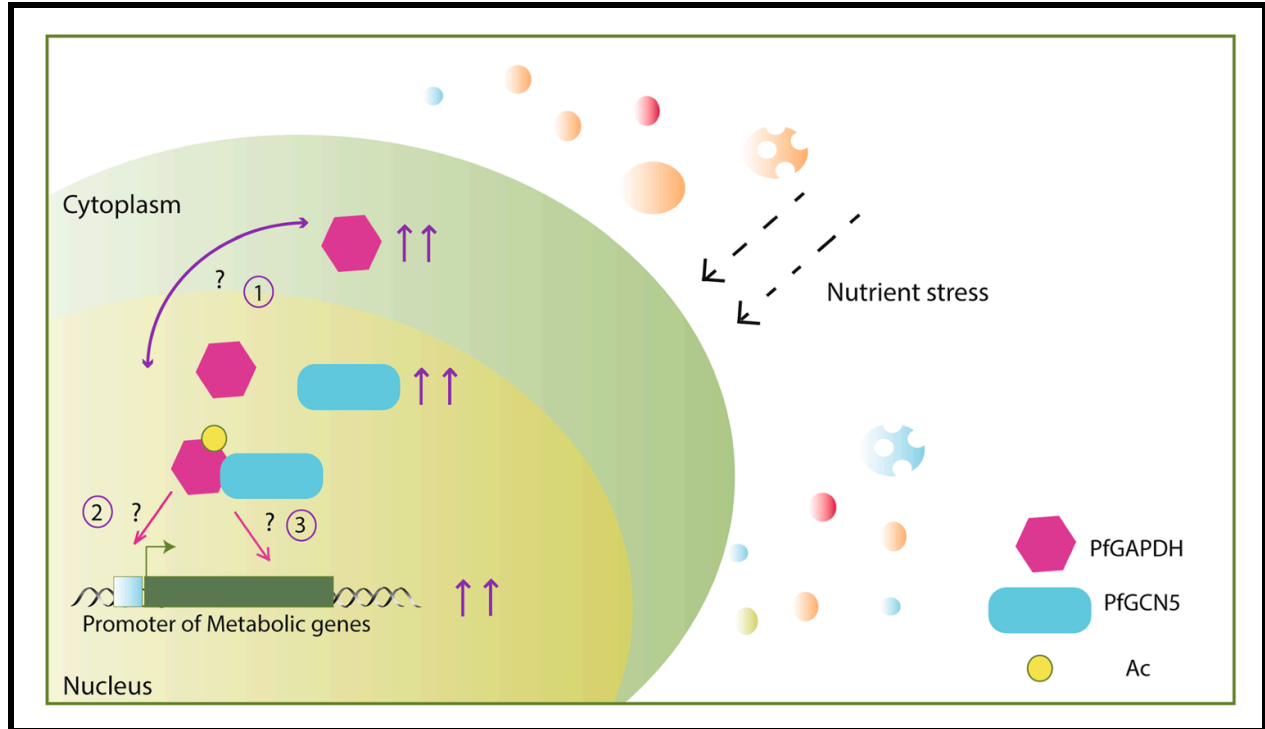


Figure 5: Schematic representation for PfGCN5-mediated transcriptional regulation and nutrition sensing in *P. falciparum* are suggested mechanisms. Under typical growth conditions, PfGAPDH was discovered to be present in both the cytoplasm and the nucleus. Additionally, PfGCN5 most likely interacts with and acetylated PfGAPDH in the nucleus. It's possible that under nutritional stress, the distribution of PfGAPDH in the cytoplasm and nucleus changes (1). The PfGCN5-PfGAPDH complex increases the expression of metabolic genes by binding to their promoters (2) or gene bodies (3) (Adapted from: Rawat, M., Malhotra, R.,2020).

Chapter 3

Chapter 3: Elucidating the non-canonical roles of antisense RNA mediated via antisense RNA translation into small proteins or micro peptides

Introduction

The *Plasmodium* genus, which causes malaria, continues to pose a serious danger to public health worldwide because of the development of artemisinin resistance and increased virulence. Consequently, there is an urgent need to investigate putative mechanistic control routes (White et al., 2014; van der Pluijm et al., 2020). *P. falciparum* has been extensively investigated as a research model organism for malaria sickness; yet, the infection and resistance mechanism remain largely unexplained. This is likely due to *P. falciparum*'s remarkable capacity to adapt and tide numerous stressors, and also develop drug resistance while evading human immunity.

Our understanding of the genome and transcriptome has greatly expanded because of next-generation sequencing methods. Indeed, this has provided insight into the molecular mechanisms controlling gene expression. Nevertheless, these approaches have relied on the sequencing of short or fragmented reads that have been amplified using PCR-based methods, which frequently results in mistakes and hinders the precise identification of full-length transcripts and gene structures, including alternative splicing events. This is particularly problematic in the case of a parasite like *Plasmodium*, whose genome is highly branched and peculiar, as previously discussed (van der Pluijm et al., 2020; White et al., 2014).

New developments in full-length RNA-seq technologies have proven how effective they are, especially when it comes to determining gene architectures and fully examining open reading frames. For transcriptome profiling, these systems are gradually taking the place of short-read RNA sequencing.

These limitations have also affected our understanding of the *Plasmodium falciparum* infection biology because of the genome's highly varied and repetitive sequences, which make short-read sequencing genetic investigations more difficult. Gene fusions, long non-coding RNAs (lncRNAs), alternative polyadenylation, alternative splicing and other gene structural changes all

add to the complexity of the transcriptome, affecting the regulation of gene expression and function (Ma et al., 2018; Yang, M., 2021).

Recent experiments utilizing state of the art technology have demonstrated the significance of non-coding RNAs in the overall biology of Apicomplexan parasites (Li et al., 2020). Numerous studies which have been performed to study non-coding RNA have not yet considered the possibility of gene fusion and recent advancements of full length or long read sequencing have offered some eye opening details into the parasites genomic landscape (Rhoads and Au, 2015; Raabe et al., 2010; Chappell et al., 2020; Liao et al., 2014; Broadbent et al., 2015; Mourier et al., 2008; Siegel et al., 2014).

The long standing knowledge in the field suggests that Open reading frames (ORFs) which are greater than 100 codons in length are thought to be coding sequences that could be translated into proteins for many years (Cabrera-Quio et al., 2016; Yin et al., 2019). Whereas small open reading frames have been described as ORFs which are less than 100 codons in length. These are also generated during the transcriptional stages, but have been disregarded as meaningless or dark matter because functional proteins corresponding to the same could never be identified. Even bioinformatics algorithms have not provided a complete picture on their importance (Frith et al., 2006; Ladoukakis et al., 2011; Yang, M., 2021).

Landmark study performed on Baker's yeast studied the small open reading frames (sORF) for the first time and identified 299 hitherto unannotated sORFs (Kastenmayer et al., 2006). Eventually, it was extrapolated and discovered that many different types of organisms, including bacteria, plants, insects and humans, have sORFs with a high potential for encoding small proteins/ peptides or microproteins (Yang, M., 2021; Cabrera-Quio et al., 2016; Miravet-Verde et al., 2019; Segonzac and Monaghan, 2019; Orr et al., 2020; Hsu and Benfey, 2018; Fesenko et al., 2019; Patraquim et al., 2020; Lee et al., 2015; Ruiz-Orera and Alba, 2019).

Landmark study by Yang, M., 2021 in *Plasmodium* performed PacBio sequencing for in depth study of full length transcriptome to get antisense RNA sequences at the greatest resolution and identified through four algorithms 12,553 potential lncRNAs predicted by four algorithms, the intersection of which were divided into four categories: Antisense (2,023, 55.84%), lncRNA

(1,071, 29.56%), sense-overlapping (394, 10.87%), and sense-intronic (135, 3.73%). They also identified and bioinformatically validated 160 small proteins in *Plasmodium*.

The most recent study which has been published in 2024 examined comprehensive ribosome profiling data sets and genome-wide translated ORFs for five eukaryotes: humans, mice, zebrafish, worms, and yeast (Yang et. al, 2024). To study the likelihood that the encoded peptide is stable in humans they studied their PepScore based on ORF properties (expected length, encoded domain, and conservation). In addition, systematic ectopic expression demonstrated that stable complex-associating microproteins can be produced in overlapping coding sections and 5'/3' untranslated regions of mRNAs in addition to identified noncoding RNAs. Stable non canonical proteins localized to distinct subcellular compartments and follow similar pathways as mRNA originated proteins. Certain peptides, particularly those with moderate PepScores, can be stabilised by inhibiting the proteasomal/lysosomal degradation pathways; however, short peptides with low PepScores cannot have their expression restored, indicating that cellular proteases are directly responsible for their disintegration (Yang, H., 2024). Celebrated research has also shed light on the roles of micropeptides in disease biology such as in various cancers. These studies have also highlighted the potential role of small proteins in diagnosis and treatment (Mohsen, J., 2023; Ge A., 2024; Posner, Z., 2023; Aspden JL, 2014; Hofman, D. A., 2024; Camarena, M. E., 2024).

We postulated that small proteins, or sORFs, probably play a major role in *P. falciparum*'s growth, development, and infection processes in light of the existing findings from other systems such as bacteria, yeast, humans etc (Yang et. al, 2024). Examining if these small proteins come from non-coding areas like antisense RNA piqued our curiosity. Our theory has been that antisense RNA is freely present in the same cell environment as sense RNA. Since they share the same base composition, the translation machinery can easily translate antisense RNA into small proteins, which may play significant roles in enhancing the virulence of the parasite. Examples of these proteins could include virulence-related proteins, metabolic sensing pathways, and cell-to-cell communication.

Further, we decided to integrate our in-house generated Oxford Nanopore RNA sequencing dataset along with the dataset published by Lee et al., 2020 and compare it with the in house

performed mass spectrometry for fractionated small proteins and *Plasmodium* secretome under control and stress conditions. The experiments from this study have been initiated, characterized and validated from the beginning and are in progress currently but nevertheless we have seen some interesting developments and findings which have been presented below. Besides this project shows immense potential that needs to be leveraged on in future.

Materials and Methods

1. Parasite culture and stress induction

Parasites were cultured in the same way as mentioned above in Chapter 2 Materials and Methods section.

2. Stress induction for the parasites

Mixed stage *Plasmodium falciparum* 3D7 parasites (largely at the trophozoite to schizont) were utilised. The batch of parasites were split identically, one batch was subjected to control conditions and grown at 37 deg celsius whereas the other batch was grown at an elevated temperature of 41 deg celsius for the period of 6 hours following which they were harvested using Saponin method (described before) and flash frozen at -80 deg celsius. Elevated temperature was subjected in order to introduce temperature stress to the parasite that mimicked the fever conditions.

3. Parasite Harvesting

Parasites were harvested using the Saponin method and flash frozen for future use.

4. Small protein fractionation using DSC columns

Protein lysate was prepared by lysing the parasites in the parasite lysis buffer (recipe mentioned before) followed by three freeze thaw cycles. This lysate was spun at a high speed of 12,000 rpm for 30 min at 4 deg celsius to collect the supernatant. The supernatant was collected in separate tubes and flash frozen using liquid nitrogen. This frozen supernatant was later lyophilized using the lyophilizer at -83°C and low pressure; this procedure took a couple of hours or till overnight until the entire lysate was in a lyophilized state. This lyophilized protein powder was dissolved in

5%ACN in 0.1% TFA. Later this preparation was passed through the DSC 8 and DSC 18 columns separately as per the manufacturer protocol (Petruschke, H., 2020). In short, Bond Elut C8 cartridges (100 mg, Agilent, USA) were equilibrated with 1 mL of methanol and 2 mL of 0.1% trifluoroacetic acid (TFA) prior to loading 1 mL of protein samples in UT-buffer. Following two washing processes with 1 mL of 0.1% TFA, eluting was done using 1:1 V/V and 3:1 V/V 0.1% TFA:acetonitrile. Following the instructions for in-solution mass spectrometry, the proteins were first eluted and evaporated using SpeedVac, and then they were subsequently proteolytically digested for mass spectrometry.

Following the manufacturer's instructions, a similar procedure was used to elute tiny proteins using DSC 8 columns. This was done in order to try to get more results and sample the tiny proteins as best as possible.

5. Spent media collection for Secretome sampling and processing

The secretome of the parasites was collected during the same stage as the parasite harvesting. The spent media was collected in a separate 50 ml falcon and spun down at high speed to remove an cellular mass following which the supernatant was separated. It was flash frozen and lyophilized and later dissolved in 5%ACN in 0.1% TFA and passed through DSC 8 and DSC 18 columns separately. In solution proteolytic lysis was performed and subjected to mass spectrometry.

6. In solution proteolytic cleavage followed by ReDiMe labeling and mass spectrometry

Three replicates were used to quantify and separate 100 µg of protein-containing lysate. In order to accomplish disulphide reduction, samples were heated on a thermoshaker (60°C; 1 hour; 1,400 rpm) and a 25 mM dithiothreitol- (DTT-) solution was added. The next step was alkylation with 100 mM 2-iodoacetamide, followed by overnight enzymatic digestion with trypsin (1:50 v/v) at 37°C and incubation for 30 minutes at 1,400 rpm.

The ReDiMe labelling procedure was used on the proteolytically cleaved peptides in order to perform quantitative mass spectrometry estimations (Hsu, J. L., 2016; Tolonen AC, 2014). The idea behind is to label proteins differently with heavy and light formaldehyde, which alters their

total mass and allows researchers to examine variations in protein expression across different samples or conditions to be compared.

7. Gradient SDS Page Gel based fractionation

In-house, a gradient SDS PAGE was created by significantly altering our standard procedure. The acrylamide solution was placed bottom-up between the two casting plates, ranging in concentration from higher to lower. 20% Acrylamide solution made up the lowest or first layer that was added, and as we moved upward, we added smaller percentages to the resolution gel, such as 15 to 10 percent. After that, we finally set the stacking gel at the customary concentration of 4% Acrylamide. We can isolate the tiniest proteins (those with an interest range of 20 kDa and below) using this stacking technique without having to worry about losing them during electrophoresis (Figure 1).

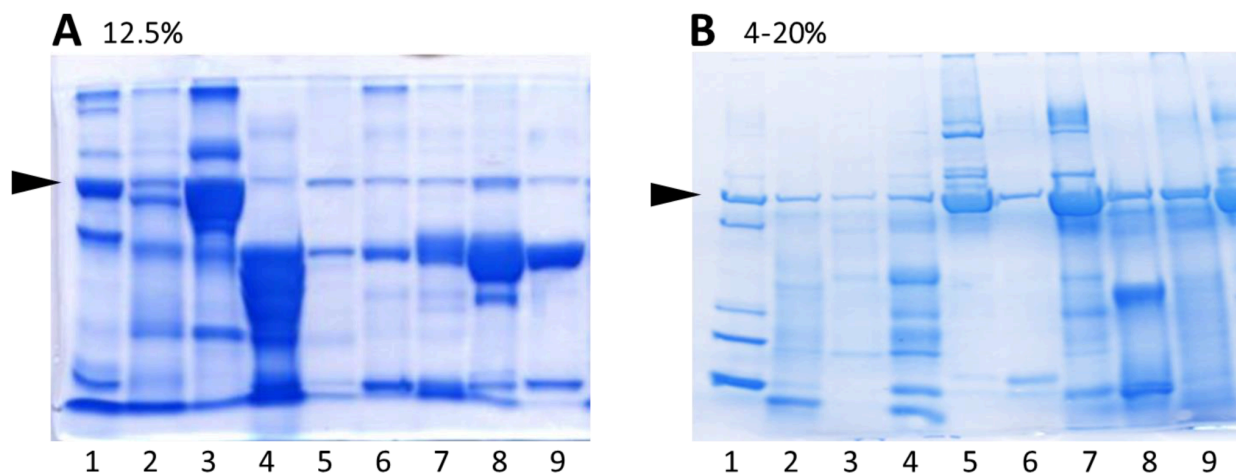


Figure 1: Representation of gradient SDS page fractionation to separate small proteins

8. Centricon based Protein concentration

We tried another method for protein enrichment for proteins smaller than 10 kDa, for which we used Protein concentration columns with a cut off of 10 kDa. We spun the lysate through the concentration columns as manufacturers guidelines, and collected the flow through which consists of proteins of interest. Later we precipitated the proteins using acetone for 20 min at -20 deg celsius. Proteins were digested with trypsin.

9. In-Gel proteolytic cleavage for mass spectrometry

The protein lysate is precipitated overnight at -20°C with acetone 1:5 (v/v) and then centrifuged at 14,000 x g for 10 min at 4°C. The purpose of this step is to load more protein onto fractionation gels and concentrate the protein from bigger sample material. The sample of concentrated protein was put into wells that were twice as big. 100 µg of the protein pellet was resuspended in 100 µL of loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris/HCl, pH 6.8) and then incubated at 90°C for 5 minutes. The material was added to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; 4% stacking gel and 12% separating gel). Electrophoresis was performed on each gel at a rate of 80–120V.

Proteins were stained using Colloidal Coomassie Brilliant Blue G-250 (Roth, Kassel, Germany), and then destaining was done using a freshly made Glacial acetic acid and methanol-based solution. The gel fragments are sliced sterily after full destaining, and we then use a destaining solution based on mass spectrometry.

The addition of 150 ng of Promega's modified porcine trypsin and an overnight incubation period at 37°C were necessary for the In Gel proteolytic cleavage procedure. After adding formic acid, the reaction reached a final concentration of 4%. The mixtures were vacuum centrifuged to remove the supernatant and gel elution solutions (first elution step: 40% (v/v) acetonitrile, second elution step: 80% (v/v)). The peptides were reconstituted using 0.1% formic acid. After being extracted, peptide lysates were dried using a SpeedVac and then dissolved in 0.1% formic acid. The samples are then sent away for in-house mass spectrometry utilising the IDA method for 4-6 hours of run time after completing peptide filtration or cleanup using a C18 column.

10. Bioinformatic analysis

The Mass spectrometry data received from SciEx QTOF was processed through SciEx proprietary Protein Pilot analysis software to get peptides information. Samples were processed at 95% accuracy and compared against Plasmodium falciparum proteome version 60. This would give us the information about known or annotated proteins, but we also wanted to look at the plausible proteome from the non-coding regions.

To study the proteome which is possible from the genome scale we translated the entire genome into all frames of translation. The positive strand was translated in three frames of +1, +2, +3 and the negative strand was translated into -1, -2, -3 frames. The in-silico translated proteome was chopped at every stop codon interval and their unique genomic positions were marked as fasta headers. Later this in-silico generated file was used as a database to fetch peptides found in the in house mass spec data set.

Results

1. Designing the experiment for small protein identification through mass spectrometry

We intended to study the small proteins in depth, for which we decided to go ahead with characterisation of techniques and rely on the technique that offers us greater depth for the small proteins and micropeptides. So, our work begins with characterisation of techniques for small protein mass spectrometry.

We began with performing SDS PAGE gel based gradient fractionation. We adopted the Chen et al. technique and prepared gradient SDS PAGE gels in house. The gels consisted of 4% stacking gel layered on top of 10-20% gradient in the resolving gel. The wells were fused together in pairs to load higher sample volume and concentration. 100 µl of protein lysate was run on the gel and after the run was complete we cut the entire set of proteins below 20 KDa and 10 KDa size. The gel piece was processed for in gel mass spectrometry and the data was analyzed yielding us 35 peptides at 95% confidence interval originating from non-coding regions of the genome.

Secondly, we tried the centricon based small protein enrichment followed by in solution mass spectrometry.

2. In-silico database curation

We intended to create a global scale database through in -silico means in order to map all potential peptides that could be derived from non-coding regions. For the same we translated the

entire genome into all six frames as per the orientation. The positive strand was translated into +1,+2,+3 frames whereas the negative frame was translated into -1,-2,-3 frames. The translated sequences were chopped at every stop codon and the respective genomic positions were listed as fasta headers for each sequence. This global scale file was used to find peptides from the in house generated mass spectrometry data set.

Another way in which we created an in-silico database was by using the publicly available Ribo Seq data by Caro et al. We extracted all the sequences of the antisense RNA that were reported to be associated with the ribosomes. Later we translated them into all possible frames of translation and used this file as another database to map peptides of interest .

We utilized yet another method to find peptides that could be originating from non coding regions, for which we ran tools such as CPC2, CNCI and CPC to find non-coding RNA with potential of translational. We extracted the sequences and translated them accordingly. Yet again this file was used as a database to find peptides.

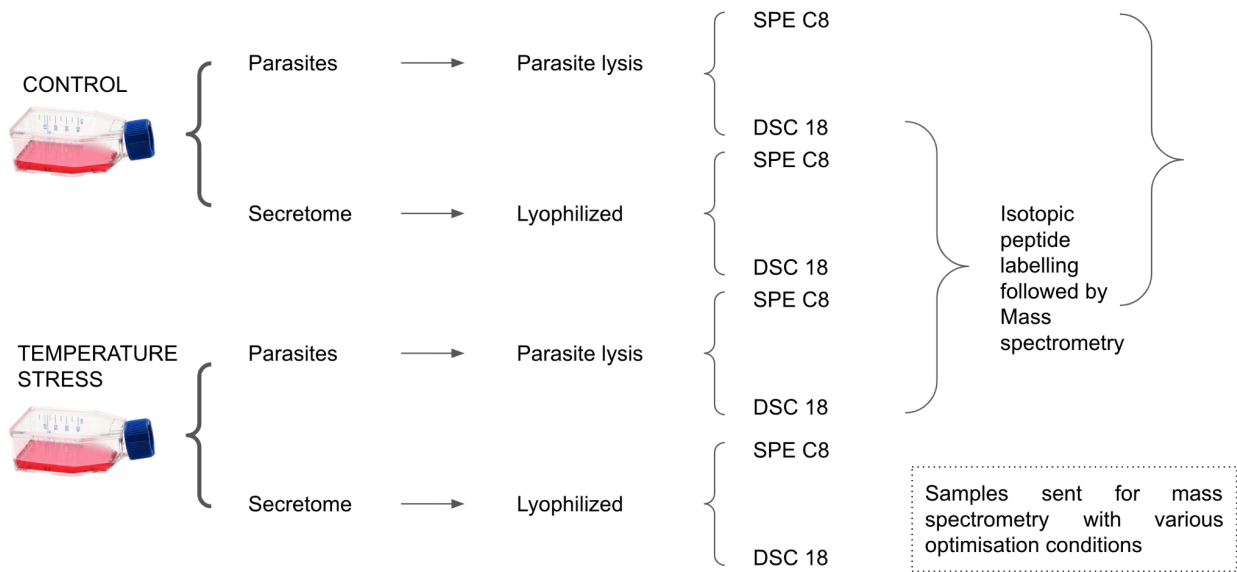
Additionally, we also looked at the small protein published dataset by Yang et al., 2021 and looked at antisense and other ncRNAs. We translated the sequences the same way to use it for peptide matching.

3. Finding the peptides against the in-silico curated database

In order to find peptides present in our in house data sets against the in-silico curated files of possible peptides we used SciEx proprietary software called as Protein Pilot, which helps identify peptides from raw data sets. We used a stringency of 95% accuracy to find peptides. Refer [Figure 2 A,B,C](#) for data on peptides found through our technique. Briefly, Panel A represents the experimental paradigm followed for through identification of small proteins in the parasite. Panel B represents small proteins identified under control condition and their in-silico validation. Panel C represents small proteins found to be deregulated upon temperature stress induced conditions.

Alternatively we also used MaxQuant software for peptide identification at high stringency rates.

A)



B)

Peptide found	Non-coding region from	Confidence	Match with other organisms	RNA detected
NKPMIIKR	PF3D7_1236800	99%	None	Yes
NDPAVILVQK	PF3D7_1347300	99%	None	Yes
NFFYIITSER	PF3D7_1238900	98%	None	Yes
LHFFNDIFYFLF	PF3D7_0523100	99%	None	Yes
IELILFYIK	PF3D7_1010300	99%	None	Yes
IEDYKYCFIK, NIYMYIKK, KNILYMYIYFK	PF3D7_1319400	99%	None	Yes

C)

Annotated proteins validated	Temp/Control condition (H:L ratio)
PF3D7_0714000 Histone H2B variant	1.223765969
PF3D7_1105100 Histone H2B	1.385951042
PF3D7_1102800 Early transcribed membrane protein 11.2	1.926257968
PF3D7_0617800 Histone H2A	1.509264946
PF3D7_1401400 Early transcribed membrane protein 14.1	1.34226203
PF3D7_0320900 Histone H2A.Z	1.345733047
PF3D7_0813300 NPL domain-containing protein, putative	2.52380991
PF3D7_1412500 actin II PF3D7_1246200 actin I	1.391077995
PF3D7_0532100 Early transcribed membrane protein 5	1.126767993
gene=PF3D7_1001500 Early transcribed membrane protein 10.1	1.693037033
PF3D7_0307100 40S ribosomal protein S12, putative	1.060145974
PF3D7_0516200 40S ribosomal protein S11	1.564306974
PF3D7_0423700 early transcribed membrane protein 4	2.159068108

Figure 2: Experimental paradigm and finding small proteins from *Plasmodium falciparum*

(A) Experimental paradigm followed for through identification of small proteins in *P.falciparum*
(B) Small proteins found originating from non-coding regions under control condition and their in-silico validation
C) Small proteins found to be originating from non-coding regions and deregulated across temperature induced conditions.

4. In-silico validation of the peptides

Our next step was to confirm or validate the authenticity of the peptides and their region of origin. We did this step by manual verification means and indeed found the peptides to be originating from the sequence and region mapped. This verification step indeed increased our confidence in the peptides selected (Figure 3 A).

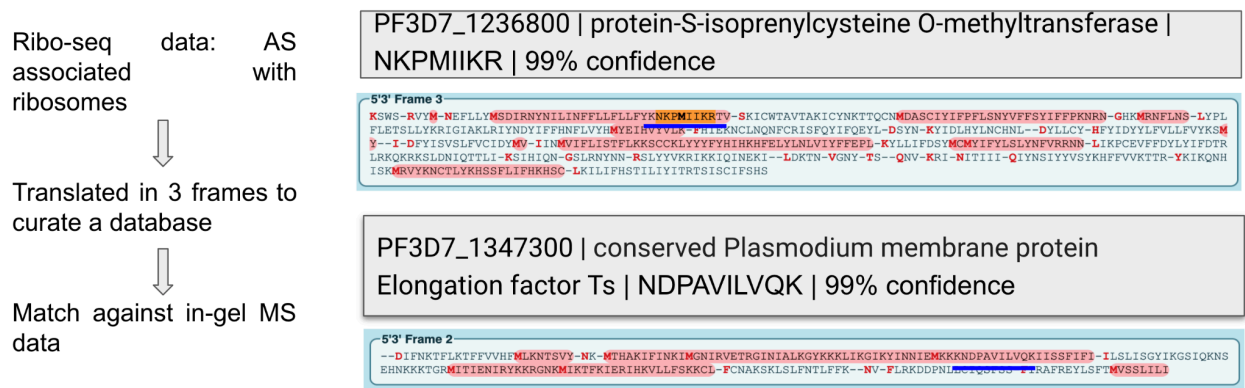


Figure 3: Step-wise representation of in-silico validation of peptides A) Panel on the left hand side represents steps followed for in-silico validation of peptides B) Panel on the right represents validated peptides

5. Experimental validation of the selected peptides

Our next step was to experimentally validate the peptides for which we devised a cloning strategy to overexpress the peptides, if the peptides were to overexpress with the fluorescent protein it would suggest that the peptides have the potential to be expressed and are getting expressed within the cell either under control condition or even across stress induced conditions (Figure 4 A and B).

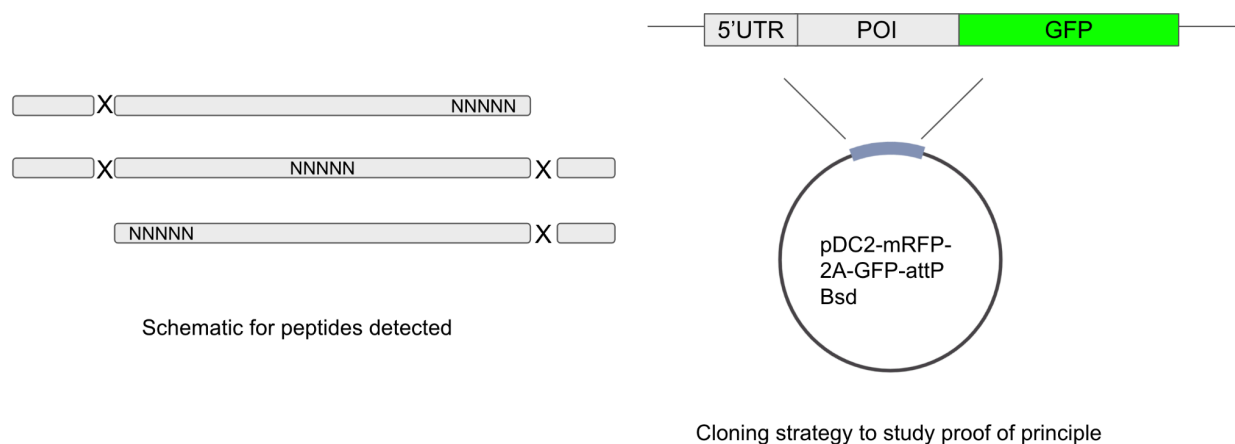


Figure 4: Schematic representation of selection of peptides for experimental validation A) Panel on the left represents selection of candidates for experimental validation based on the presence of interleaving stop codon B) Panel on the right represents cloning strategy for experimental validation.

Work in progress

Efforts are underway to study small proteins in greater detail. Shortlisted small protein candidates need to be cloned in an overexpression vector which would eventually be transfected in the parasites. An expression of downstream linked fluorescent protein would mean that the entire frame got translated and hence the small proteins hold translational potential. The cloning primers and strategy are well in place for future use.

Following in-vitro experiments even utilising the transgenic lines could shed light on their potential roles and we plan to employ detailed in-silico analysis to study small proteins in *Plasmodium*.

Discussion

Plasmodium falciparum has evolved various mechanisms to cause a successful infection even tiding against unfavourable environmental conditions, host immune pressure, drug pressure. As we delve deeper into the ways the parasite is able to tolerate and thrive, we are intrigued even more. Here, we tried to explore the presence of small proteins or proteins originating from the non-coding regions of the genome and their potential implications for the parasite.

We postulate that the non-coding part of the parasite's genome could also be contributing to non-canonical aspects of gene regulation. We hypothesize that the antisense RNA or other non-coding RNA are composed of the same bases as the mRNA and are readily available in the same milieu of the cell to the ribosomal machinery. We hypothesize that these antisense or non-coding RNA could be getting translated themselves into small proteins or micropeptides that may have some regulatory roles or may shed some light on the nature of the parasite's translational machinery.

We begin with standardising methods to isolate and study small proteins, following rigorous rounds of standardisation for the *Plasmodium* system we move ahead with preparing a custom hypothetical library of small proteins through in-silico means. The library created by in-silico analysis consists of numerous potential open reading translational frames from non-coding regions. We map our mass spectrometry candidates across this data set to retrieve further information. The next important step was to verify the authenticity of these peptides which we did through manual methods and keeping stringent cut offs to only work with high confidence candidates. We shortlisted a few interesting candidates where we observed either a stop codon skip or a ncORF. These regions will be cloned in an overexpression vector for transfections in the parasite. Following creation of transgenic lines we intend to study the expression of small proteins and their potential roles for the parasite in conditions such as stress response, cell to cell signaling, drug pressure etc. The work is currently underway and holds promising potential. Leads from this study can elevate our knowledge about *Plasmodium* biology and open doors to newer approaches to tackle this deadly parasite.

Chapter 4

Chapter 4: Antisense transcription mediated gene expression regulation in *Plasmodium falciparum* via the control of transcriptional and translational arcs

Introduction

Malaria is a mosquito borne infectious disease caused by the protozoan parasite *Plasmodium*. The parasite is transmitted by the bite of an infected female Anopheles mosquito. There are five species of *Plasmodium* known to cause Malaria in humans amongst which *Plasmodium falciparum* is the most widespread and lethal. It has been known to inflict a global burden on world health leading to an approximate of 627,00 deaths in 2020 (WHO 2023). The parasite has an extremely complex lifecycle that transpires between two completely different hosts; humans and mosquitoes. The sporozoites are transmitted from an infected mosquito to the humans during a blood meal. These sporozoites home their way towards the liver, where they initiate parasite replication. This stage is called the pre-erythrocytic stage. After a period of incubation these sporozoites give rise to tens of thousands of infectious merozoites. These merozoites start the erythrocytic cycle of invasion where they invade millions of healthy erythrocytes. Within the erythrocytes, the parasites mature from ring, to trophozoites to multinucleated schizonts, leading to mature merozoites. Every 48 hrs the merozoites burst open the erythrocytes and re-infect fresh erythrocytes to continue the cycle. The rupture corresponds to clinical symptoms like fever, chills and nausea (Enomoto et al., 2012; Venugopal et al., 2020; Tavares et al., 2013; Amino et al., 2006; Mota et al., 2001; Sturm et al., 2006; Sologub et al., 2011).

During the intra-erythrocytic cycle a small proportion of parasites may develop into sexual stage male and female gametocytes in order to continue the infection cycle. These gametocytes get picked up or ingested during the mosquitoes blood meal. Inside the mosquito midgut these gametocytes undergo sexual reproduction to form zygotes that subsequently differentiate into a mobile ookinete and subsequently an oocyst. The oocyst grows and produces thousands of sporozoites which migrate to the mosquito salivary glands where they are ready to infect another individual during the subsequent bite (Enomoto et al., 2012; Venugopal et al., 2020; Tavares et al., 2013; Amino et al., 2006; Mota et al., 2001; Sturm et al., 2006; Sologub et al., 2011).

The parasite during its complex and multi-stage developmental cycle is able to undergo distinct morphological and physiological changes in response to environmental fluctuations, all that is tightly regulated by an underlying pattern of gene expression paradigm (Scherf et al., 2008).

Gene expression profiling techniques such as RNA sequencing, nascent RNA sequencing, and single cell RNA sequencing have revealed that the majority of gene expression occurs in a cascade-like and time-dependent manner that is also developmentally regulated (Siegel et al., 2014; Bozdech et al., 2003; Hu et al., 2010; Yamagishi et al., 2014; Tonkin-Hill et al. Q., 2018; Chappell et al., 2020). Despite the fact that the underlying molecular mechanism is largely unknown.

The peculiarities of the *Plasmodium* genome make it even more challenging to explain the stringent gene expression regulation. The parasite's genome is predominantly AT biased and there is a general paucity of sequence specific transcription factors. Only 27 apicomplexan apetala2 (ApiAP2) transcription factors have been identified as specific transcription factors to govern gene expression. These have been known to play specific roles as activators and repressors of transcription (Painter et al., 2011; Sinha et al., 2014). Its genome is known to be in majorly euchromatin stage. Also there are limited ways of gene repression known in the parasite. Interestingly, there is pervasive non-coding RNA transcription observed in the parasite majority being antisense in origin. The current understanding of gene expression regulation highlights the importance of a combination of transcription and post-transcriptional regulatory events (Broadbent et al., 2015; Bozdech et al., 2006; Guizetti et al., 2008; Pelechano et al., 2013). Additionally, epigenetic regulation and genome architecture have been shown to greatly impact gene expression. There is limited understanding for the way gene regulation is entirely regulated to support the parasite's complex life cycle. Also, a thorough understanding of the same is essential to identify ways to therapeutically target the parasite.

With advances in Next Generation Sequencing technologies, attempts have been made to understand the genome, revealing that the transcriptome of an organism is much larger than studied and expected. Across eukaryotes, many non-coding RNAs have been identified and linked to important biological processes. They have been implicated in diseases and have also

been identified as potential therapeutic and diagnostic targets. Interestingly, they have also been identified as key regulators of gene expression and chromatin states across multiple systems. Non-coding RNAs are classified on the basis of their origin, length, overlap with neighboring genes etc. One important class of non-coding RNA called the long non-coding RNA are defined as non-protein coding in nature with a minimum length of 200 bp. Long non-coding RNA (lncRNA) have features similar to mRNA like a 5' cap, a poly A tail and presence of introns. LncRNA have been identified to be expressed in a cell type specific and developmentally regulated way. LncRNA have been known to interact with DNA, RNA and proteins. Owing to this property lncRNA have the potential to regulate gene regulation at the chromatin, transcription, translation and epigenetic levels. LncRNA have been identified to be nuclear and cytoplasmic in origin. Nuclear lncRNA have the potential to regulate transcription by tethering to genomic DNA and modulating long range interactions. By this property they can modulate promoter accessibility or recruit transcription factors or chromatin remodeling enzymes to deregulate transcriptional activity. This way the transcription could either increase or decrease depending upon the recruitment of either histone acetyltransferases or deacetylases respectively. They have also been hypothesized to bring about changes in the protein partners binding to the DNA, hence bringing out changes in the transcriptional status. Additionally, their association to RNA binding proteins and spliceosomal factors have been noted to alter RNA metabolism. To add onto the complexity of lncRNA mediated gene regulation, in the cytosol they have been found to affect the mRNA export rates and stability to eventually affect the translation dynamics (Pelechano et al., 2013).

LncRNA have been found to play critical roles across many eukaryotic systems. In mammals a very well studied case of X inactive specific transcript (Xist) lncRNA mediated X chromosome inactivation during zygotic development. It has been shown that accumulation of Xist lncRNA on the X chromosome recruits histone modifying enzymes to lay repressive epigenetic marks such as H3K9 and H3K27 methylation. This results in heterochromatinization and gene silencing. Other popular examples of lncRNA mediated gene regulation include the HOX transcript antisense (HOTAIR) which has been shown to interact with Polycomb group proteins to silence gene expression. Also, telomeres have been shown to comprise lncRNA such as long telomeric repeats containing lncRNA (TERRA). With thousands of lncRNA identified and

numerous being studied in mammals, our understanding of their role in gene expression regulation is only starting to uncover (Pelechano et al., 2013).

Of great importance, the role of lncRNA in the malarial parasite is slowly coming to limelight. They are emerging as important candidates for parasite's developmental regime. Till date several studies have utilized RT-qPCR, microarray and NGS based technologies to report the presence of lncRNA in *Plasmodium falciparum*. With technological advances, we have come to recognise that thousands of genes in *Plasmodium falciparum* produce lncRNA, of great importance antisense transcription is a prevalent and pervasive phenomenon in the parasite. Antisense RNA have been implicated to play key roles in the parasites' biology like telomere maintenance, sexual development, var gene regulation though their mechanism of action and their role in stress response remains elusive (Othman et al., 2023; Hviid et al., 2024; Kinyua et al., 2024; Otoboh et al., 2024; Hviid et al., 2023; Chen et al., 2021). That's where our study aims to fill the gap and delves into great details to systematically characterize antisense transcription and study its physiological association to stress response. It further takes on to decipher the mechanism of action followed by antisense transcripts for gene regulation.

Materials and methods

1. In-vitro *Plasmodium* parasite culture maintenance

The RPMI-1640 medium supplemented with 25 mM HEPES, 0.5% Albumax, 1.77 mM sodium bicarbonate, 2 grammes/litre D-glucose, 100 uM hypoxanthine, and 12.5 ug/ml Gentamicin was used to cultivate the *Plasmodium falciparum* 3D7 strain in a sterile environment at 37 degrees Celsius with 5% CO₂. 10% parasitemia and a 3% haematocrit were maintained for the parasites. Blood banks provided fresh, healthy O-positive human red blood cells to support the parasite cultivation. As previously mentioned, 5% Sorbitol and 63% Percoll gradient were used to synchronise the parasites at the ring and schizont stages, respectively. Giemsa staining and thin smear preparation were used to track the parasite's growth.

2. Temperature Stress induction

In order to replicate the fever conditions the parasites encountered during the infection cycle in the human host, temperature stress was introduced into the parasites. Using 5% Sorbitol treatment, the parasites were tightly synchronised during the 16 hpi ring stage to produce temperature stress. After synchronisation, the temperature stress induction culture was grown for six hours at a higher temperature of 41 degrees Celsius, whereas the control culture was cultivated at a standard 37 degrees Celsius. The parasites were extracted using the Saponin lysis procedure, which is explained below, shortly after the above-mentioned incubation period. For later use, the parasite pellet was kept at -80 degrees Celsius after being flash frozen in liquid nitrogen. Purezol was used to resuspend parasite pellets that were set aside for RNA isolation.

3. Parasite harvesting protocol

The Saponin lysis procedure was used to extract parasites. The parasite culture was briefly spun down and given a 1X PBS wash. The contaminated RBC pellet was then lysed in saponin and briefly incubated in a water bath at 37 degrees Celsius. The parasites were then removed by rapidly spinning down the lysate and washing it with PBS. At -80 degrees Celsius, the parasite pellet was kept in storage.

4. Cloning strategy

Using gene-specific primers forward and reverse primer, specific regions of the DNA sequence were amplified from parasite genomic DNA. Restriction enzymes were used to clone the PCR-amplified fragment in frame within the vector backbone. Sanger sequencing was used to confirm the cloning. For clone expansion, *E. coli* DH5 alpha was transformed using the cloned plasmid and plasmid isolation was performed using the Qiagen kit for the confirmed clone.

5. Western blotting

Parasites were collected with 0.15 percent saponin. Parasite pellets were rinsed with phosphate buffer saline (PBS). Parasites were lysed in ice-cold parasite lysis buffer (TRIS pH 8.0, 150 mM sodium chloride (NaCl), 0.5% nonyl phenoxyethoxyethanol (NP-40), 0.5% sodium deoxycholate, 0.1 mM ethylenediaminetetraacetic acid, 1.5 mM magnesium chloride (MgCl₂), 1X protease inhibitor cocktail (PIC), and 1 mM phenylmethylsulfonyl fluoride (PMSF). To ensure effective parasite lysis, three freeze-thaw cycles were done with liquid nitrogen. To

remove debris, parasites were spun at $17,949\times g$ for 30 minutes. The supernatant was transferred to another tube. The lysate proteins were separated on 7.5-12% polyacrylamide gels and transferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk and probed with primary antibody overnight at 4°C. After overnight incubation, membranes were washed with 1X Tris-buffered saline and 0.1% Tween 20 (TBST), followed by a 1-hour incubation with the secondary antibody in TBST (1:5000, Biorad). Following the secondary antibody incubation, three washes were performed for 10 minutes each. Clarity Western ECL substrate (Biorad) was used to create the blots.

6. Quantitative RT- PCR

RNA isolation was performed with TRIzol reagent (Biorad). The ImProm-II Reverse transcription system (Promega) was used to synthesise cDNA from 1 µg of DNase-free RNA, following the manufacturer's recommendations. Random primers were employed for cDNA synthesis. Real-time PCR was performed using a Biorad CFX96 Real-Time PCR detection equipment. To account for heterogeneity between samples, 18S rRNA and tRNA synthetase were utilised as internal controls. The expression was quantified using the fluorescence readout of SYBR green dye incorporation into amplification targets (Biorad). Each experiment had technical replicates and three separate biological replicates.

7. FACS sorting

1 ml of parasite culture to be sampled was added to 5 ml of 1X sterile PBS in Beckman FACS sorting tubes to dilute the sample for further analysis. The tubes were then analysed using BD FACSAria sorting equipment. Cleanup, normalization and quality control were performed before analysis of the sample. Besides for gating purposes uninfected RBCs were used. To sort infected cells, DAPI stained infected RBCs were used to create a gate. Sorting was further done on the basis of fluorescent lasers corresponding to GFP (478 nm) and mRFP (594 nm) and for each sample minimum 100,000 events were recorded at slow speed. Sorted cells were collected and added to a 24 well plate well for further proliferation and making a pure line. BD FACSAria proprietary software FloJo was used for analysis.

8. Transgenic generation

Ring stage transfection was carried out using the Biorad Electroporation method. Early stage rings 0-2 hrs post invasion were tightly synchronised using Sorbitol method. The ring stage parasites were pelleted by centrifugation. To achieve a final volume of 800 μ l, 100 μ l parasitised red blood cells were pelleted and resuspended in cytomix containing 150 μ g DNA. In 0.4 cm cuvettes, electroporations were performed with 960 μ f and 0.31 kV settings. In a 25 ml flask, electroporated samples were promptly combined with 10 ml of full culture media and fresh red blood cells to achieve a 1% haemoglobin content. Cultures were kept on necessary drug pressure after 24 hours post transfection. Media change was done every alternate day and the cultures were split and provided with fresh RBCs every 7th day.

9. In vitro transcription

DNA template for specific RNA synthesis was PCR amplified from genomic DNA or complementary DNA while incorporating T7 promoter sequence through forward primer and poly A chain through the reverse primer. The PCR product was purified using a DNA cleanup kit from Qiagen as per manufacturer's instructions. The PCR products were used as templates for in vitro transcription of RNA. T7 polymerase enzyme was purified in house and the buffer (10X) contained 40mM Tris-HCl 7.9, Spermidine 2mM, 6mM MgCl₂·6H₂O, 10mM NaCl, nuclease free water. For a total of 50 μ l reaction, 5 μ l of 10X buffer, 0.5 μ l of each rNTP, 0.5 μ l of 1M DTT, 2.5 μ l T7 polymerase, 1 μ l of RNase inhibitor and 100 ng of template was supplemented. The reaction was set overnight at 37 deg celsius in a thermomixer and the next day RNA was precipitated using Isopropanol.

10. RNA associated protein pull down

For this experiment we followed the guidelines by Gordan et. al, 2015. Briefly, In-vitro transcription was carried out the same way as mentioned before and RNA was obtained with the exception that Biotinylated rNTPs were used in place of unlabelled rNTPs. Biotin RNA labelling mix (11685597910) was procured from Roche for this purpose. This labelled RNA was passed through G50 sephadex columns (Roche 11273973001) and collected in RNase free tubes. Simultaneously, protein lysate or parasite lysate was prepared by freeze-thaw method as mentioned before. Meanwhile, for proper folding of the RNA, the RNA was denatured by heating at 65 deg celsius for 10 min and then cooling down slowly at 4 deg celsius. This RNA

was incubated with the protein lysate at 4 deg celsius for 30 min at slow rotational speed of 10-12 rpm. Next, 150 µl of streptavidin agarose beads were washed thrice with buffer A (150mM KCl, 25mM Tris pH 7.4, 5mM EDTA, 0.5mM DTT, 0.5% NP40, 1X PIC, 1mM PMSF). Later these beads were added to the RNA-Protein lysate mix and were allowed to bind to the biotinylated RNA for 30 min at room temperature on slow rotation. Next, the beads were washed thrice with buffer A and pelleted and on-bead mass spectrometry was performed for identification of interacting proteins.

11. On bead mass spectrometry

We adopted the protocol by Turriziani et.al, 2014. Briefly, to break down the baits and associated proteins, the beads were trypsinised in 60 µL of Buffer 1, which included 2 M urea, 50 mM Tris-HCl pH 7.5, and 5 µg/mL Trypsin. In a thermomixer, digestion was carried out for 30 m at 27 °C while being shaken at 800 rpm. Following the first digestion, the samples were centrifuged for 30 seconds at 7000 rpm, and the supernatant was taken out and put in new tubes with labels. The supernatants were combined after the beads were rinsed twice in 25 µL of buffer 2, which included 2 M urea, 50 mM Tris-HCl pH 7.5, and 1 mM DTT. The samples were placed on the bench and allowed to continue digesting at room temperature for the entire night. The samples were incubated for 30 minutes in the dark after 20 µL of iodoacetamide (5 mg/mL) was added the next morning. Samples were desalted in C18 stagetips after being treated with 1 µL trifluoroacetic acid (TFA) to halt digestion. In short, one tip was made for each sample by inserting a tiny disc of Empore material 3 M into a regular pipette tip. 50 µL of 50% acetonitrile and 0.1% TFA buffer were used to activate the tips, and 50 µL of 0.1% TFA was used to wash them. After adding 100 µL of material to the column, 50 µL of 0.1% TFA solution was used for two washings. Using a syringe or a light centrifugation step, liquid was manually moved through the pipette tip. The liquid can be passed through the tips of both of these processes using a syringe or a (light) centrifuge. Next, 25 µL of 50% acetonitrile and 0.1% TFA buffer were used twice to elute the peptides. This final stage was carried out by hand using a syringe. After evaporating the samples in a Speedvac concentrator, they were re-suspended in 12 µL of 0.1% TFA buffer and subjected to mass spectrometry analysis.

12. dsRIP Seq

We adopted the protocol by Gao et. al, 2021. Briefly, the parasite cells were lysed in dsRIP seq lysis buffer (100 mM NaCl, 50 mM Tris-HCl 7.4, 3mM MgCl₂, 0.5% IGEPAL 630, RNase free water). Following the lysis in 600ul, 5 ul of anti-J2 Ab (1µg/µl) was added and it was allowed to incubate for 2 hrs at 4 deg celsius on slow rotation. Towards the end of the incubation, 25 µl of washed Protein G beads are added. The beads are washed thrice with 500 µl of dsRIP lysis buffer and allowed to incubate for 1 hr at 4 deg celsius with rotation. At the end the beads are washed with dRIP lysis buffer thrice and later resuspended in Trizol to isolate RNA using Trizol method.

13. Ribosome associated RNA sequencing

We largely tweaked the protocol presented by Bunnik et. al, 2013. The control and temperature stress treated parasites were treated with 200µM Cycloheximide at 37 deg celsius for 10 min. Following a 4-minute pelleting process at 660 x g, erythrocytes underwent two rounds of washing in phosphate-buffered saline with 200 µM cycloheximide. 2.2 volumes of lysis buffer (1% (v/v) Igepal CA-360 and 0.5% (w/v) sodium deoxycholate in polysome buffer (400 mM potassium acetate, 25 mM potassium HEPES pH 7.2, 15 mM magnesium acetate, 200 µM cycloheximide, 1 mM dithiothreitol (DTT), and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride HCl (AEBSF)) were added to the pellets, which were left on ice after the final wash. The lysates were centrifuged for 10 minutes at 20,000 x g at 4°C following a 10-minute incubation on ice. To concentrate the ribosomes, the cleared lysates were subsequently placed over a sucrose cushion (1 M sucrose in polysome buffer). For large culture quantities, a Type 70 Ti rotor (Beckman Coulter, Brea, CA, USA) was used to centrifuge 20 ml of lysate on top of 6 ml of sucrose cushion in 26 ml polycarbonate ultracentrifuge tubes for 3 hours at 50,000 rpm at 4°C. The Ribosome pellet collected at the bottom of the tube was resuspended in Trizol and RNA was isolated and processed for Ribodepletion based NEB Ultra II Directional RNA sequencing as per manufacturer protocol.

14. Whole cell RNA sequencing and data analysis

Parasites were taken for RNA isolation after 6 hours of stress exposure. Total RNA was obtained using TRIzol reagent in accordance with procedure. DNase-treated RNA was used for cDNA synthesis. The RNA's quality was confirmed using an Agilent Bioanalyzer 2100. Three biological replicates were pooled to perform RNA sequencing. The samples' cDNA libraries

were created using the NEB Ultra II Directional RNA library preparation kit and NEB Ribodepletion kit was used alongwith. The transcriptome was sequenced in triplicate using the Illumina NextSeq 500 system (2×150 bp read length) in house. FASTQC was used to perform quality control on the RNA-sequencing reads, and the reads were trimmed based on the quality estimations. The quality-verified reads were mapped onto the reference genome (PlasmoDB_v60) using the HISAT2 program (New Tuxedo). After the mapping percentage was verified, the alignment data (SAM format) was converted to its binary equivalent (BAM format) with SAMtools. The BAM files are split into sense and antisense files using a custom script using SAM Flags. The htseq-count feature was utilised to quantify the number of reads mapped to genomic features (genes, exons, etc.). The count data was then utilised to conduct differential gene expression (DGE) analysis and statistical validation with the Deseq2 package in the R computational environment. MA plots are created with the 'R' program (<http://r-project.org/>).

Results

1. Antisense transcription is a prevalent and pervasive phenomenon in *P. falciparum* and not a result of transcriptional noise neither a consequence of open chromatin

Examining the function of antisense transcription in *P. falciparum* was the main goal of the experiment. Understanding the predominance of antisense in *P. falciparum* was a prerequisite for comprehending the same. In order to adequately investigate the contribution from the sense and antisense strands, we conducted strand-specific RNA sequencing under controlled conditions (Figure 1 A). We used a custom script to analyze the raw data after the Illumina platform sequencing in order to deconvolute each gene's sense and antisense transcription contribution. Antisense transcription is a widespread and abundant phenomena in *P. falciparum*, according to our examination of every duplicate. Antisense transcription is a widespread and abundant phenomena in *P. falciparum*, according to our examination of every duplicate. Antisense transcripts are produced by genes over a period of time, as shown by an FPKM value larger than As we continued to deconstruct, we discovered that the *Plasmodium* genome contains ncRNA, asRNA, mRNA, snoRNA, and circular RNA. We compared our datasets to previously published

data sets by Siegel et al., 2014 in order to further rigorously examine our findings. In summary, we discovered that genes that produce antisense overlapped. Crucially, we were able to identify new antisense-producing genes because of our high sequencing depth, many replicates, and sequencing methodology.

Understanding the origin of antisense transcription in *Plasmodium* was our next goal after recognising the widespread and widespread presence of antisense RNA in the parasite. Given that the majority of the parasite's DNA is euchromatin, it was possible to examine if open chromatinness causes erroneous transcription that manifests as antisense. We examined the global sense to antisense transcription correlation originating from each gene in order to investigate the same. The Pearson's coefficient between sense and antisense was what we used. We discovered a worldwide Pearson's coefficient of 0.33 under the control scenario, indicating a more neutral correlation that is neither significantly favorably nor negatively biased (Figure 2 A). This implies that open chromatin does not cause false transcription to produce antisense. Furthermore, we see a group of genes that exhibit either a strong positive or negative association, which further supports the idea that particular antisense synthesis occurs and may even be linked to the way these genes operate during the parasite's life cycle.

We chose the genes that produce the most antisense in order to learn more about the beginning of antisense transcription and if sense and antisense are related phenomena. To further the rigor of our study, we picked the top 5% of antisense-producing genes (also known as reference genes) and used Bedtools' nearest feature to identify their immediate neighbour gene. Based on their transcriptional orientation, we further divided them into Head to Head, Head to Tail, Tail to Tail, and Tail to Head orientations (Figure 1 B). If the head of the reference gene's increasing antisense RNA could overlap with the head of the antisense of its close neighbouring genes, we dubbed it a Head to Head. We chose the genes that produce the most antisense in order to learn more about the beginning of antisense transcription and if sense and antisense are related phenomena. In a similar way, Tail to Head orientation occurs when the head of the antisense of the gene that is directly adjacent to the reference gene overlaps with the tail of the expanding antisense. We define the other orientations in the same way. We learnt about specific facets of antisense synthesis from each of the orientations we used. Based on the intergenic distance

between the reference and neighbour genes, we rank these subsets in ascending order. We also examine the gene profiles and transcription levels of the selected genes. We aimed to determine whether antisense is due to transcriptional noise or runthrough by examining the head-to-head orientation (Figure 1 D). In that instance, the intergenic distance between the neighbouring and reference genes would also be a determining factor. We found that the reference and neighbour genes' synthesis of antisense was entirely unrelated to one another and unaffected by intergenic distance. Additionally, we observed that the transcripts were specifically enriched near the reference gene's 3' prime end, indicating a particular method of transcript synthesis. This led to the conclusion that the creation of antisense is an independent phenomena that is mostly unrelated to transcriptional runthrough.

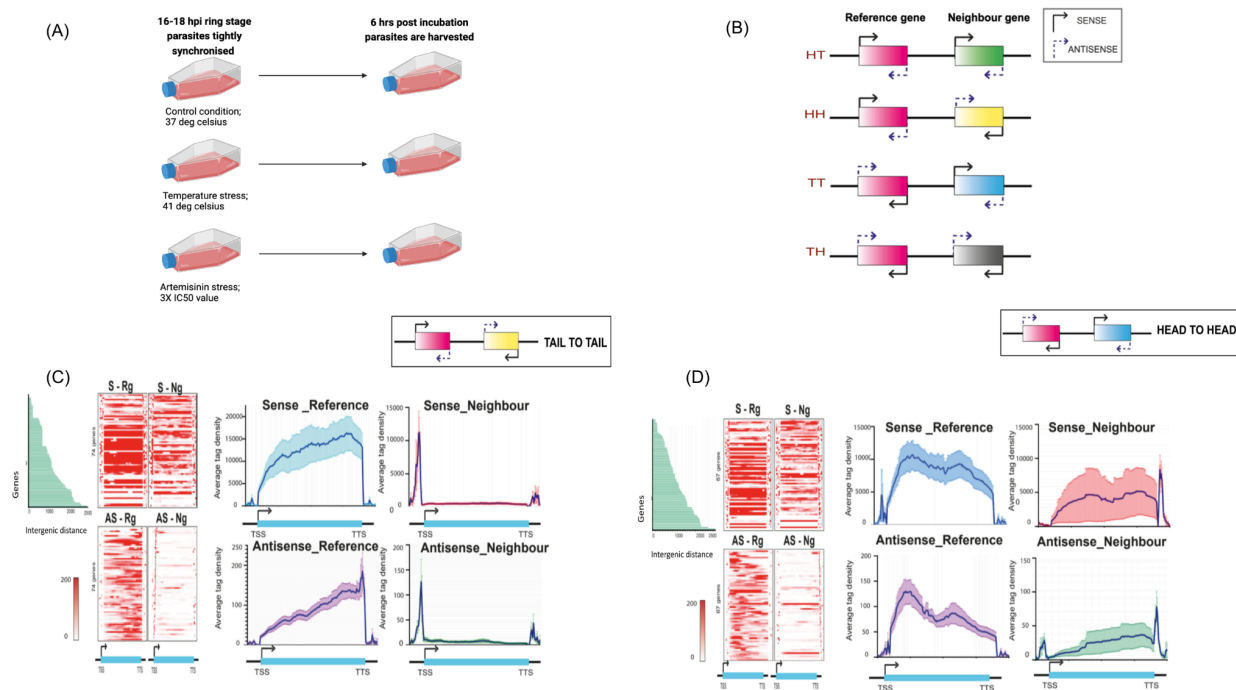
We used the tail to head orientation to better understand whether antisense transcription might be the consequence of divergent transcription (Figure 1 E). As previously stated, our goal in examining this orientation was to determine if divergent transcription contributes to the production of antisense. Here, we once more witnessed the uncoupling of sense and antisense, regardless of intergenic distance. Near the 5' end of the gene body, a more focused enrichment was seen, indicating a particular method of production.

We looked at the final two orientations, head to tail (Figure 1 F) and tail to tail (Figure 1 C), to determine whether antisense transcription is the consequence of transcriptional runthrough. By examining the same, we discovered that either 3' or 5' promoters can govern an independent genesis phenomenon. Thus, it may be concluded that antisense transcription is not caused by runthrough in a significant portion of the genome. However, runthrough has been observed using free direct RNA sequencing techniques such as Oxford Nanopore sequencing and PCR amplification. Recent studies have confirmed this, which is consistent with our own Nanopore data (data not shown here).

We set out to investigate the promoter properties that drive the production of antisense transcripts in order to advance our understanding of the origin of antisense transcription in *Plasmodium*. We planned to investigate the epigenetic environment of antisense promoters in order to achieve this. We do this by using our in-house chip seq datasets, which are created for nearly all epigenetic

alterations related to the promoters. We also use our in-house RNA seq data to identify the top gene candidates that produce antisense. In order to examine only particular antisense products, we chose the genes that produce antisense, which were also confirmed by our Oxford Nanopore data. In order to normalize the impact of transcriptional activity and its connection to histone alterations, we additionally chose an equal number of sense transcript-producing genes with comparable average FPKM. Our study revealed that promoters that generate antisense transcripts are enriched with distinctive histone alterations, such as H3K9ac, H3K14ac, H3K9me3, H3K4me1, H3K4me2. No particular epigenetic mark for antisense is discovered to be present in them. However, compared to sense transcript promoters, their occupancy levels are observed to be lower, implying that the process by which antisense transcripts are generated may be comparable to that of sense transcription (Figure 1 G). Antisense transcription is an autonomous phenomena that is particularly regulated in the parasite, as we have demonstrated thus far.

We also sought to investigate if the reference gene's synthesis of antisense is related to the genes' type or patterns of expression. In order to investigate this, we conducted a gene expression analysis by looking at the FPKM of the genes that are closest to us. We discovered that the closest neighbour genes have the following roles in the parasite that are in concordance with the reference gene's roles.



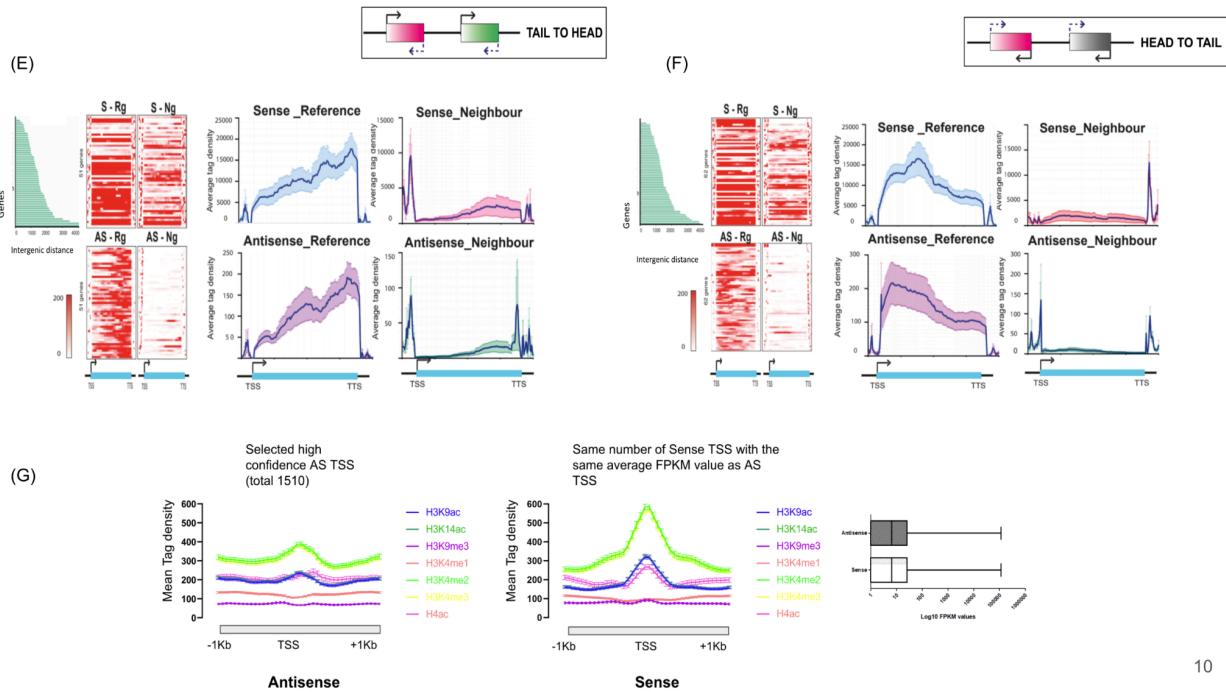


Figure 1: Global characterisation of antisense transcription in *Plasmodium falciparum*. (A) Schematic representation of experimental design for strand specific RNA sequencing under control and stress induced conditions (temperature and artemisinin stress). (B) Diagrammatic representation of different genomic orientations between reference and immediate neighbour gene based on antisense transcription of reference gene. (C) Tail to Tail orientation depicting that antisense transcription is not a result of run through. Bar plot on the left represents the genes in tail to tail orientation arranged in ascending order based on their intergenic distance. Next, the heat map represents the transcription profiles of sense and antisense transcription from reference and neighbour genes. On the right are line plots depicting transcription profiles across the gene body. (D) Head to Head orientation depicting that antisense transcription originates by the action of independent promoters. Bar plot on the left represents the genes in head to head orientation arranged in ascending order based on their intergenic distance. Next, the heat map represents the transcription profiles of sense and antisense transcription from reference and neighbour genes. On the right are line plots depicting transcription profiles across the gene body. (E) Tail to Head orientation depicting that antisense transcription originates by the action of 3' promoter elements. Bar plot on the left represents the genes in tail to head orientation arranged in ascending order based on their intergenic distance. Next, the heat map represents the transcription profiles of sense and antisense transcription from reference and neighbour genes.

On the right are line plots depicting transcription profiles across the gene body. (F) Head to Tail orientation depicting that antisense transcription is not a result of divergent transcription. Bar plot on the left represents the genes in head to tail orientation arranged in ascending order based on their intergenic distance. Next, the heat map represents the transcription profiles of sense and antisense transcription from reference and neighbour genes. On the right are line plots depicting transcription profiles across the gene body. (G) Epigenetic marks occupancy over sense and antisense promoter calculated over same number of genes with same average FPKM.

2. Antisense transcription is a physiologically associated phenomenon in *Plasmodium* parasites associated with stress adaptation in the parasite

It is clear the parasite's highly prevalent antisense transcription is playing crucial roles in the parasitic system. Therefore, our next goal is to comprehend the physiological importance of the parasite *Plasmodium*'s antisense transcription.

We used RNA sequencing under regulated and stress-induced settings to comprehend the physiological significance or relationship of antisense transcription in the parasite. We cultivated tightly synchronised ring stage parasites at a higher temperature of 41 degrees Celsius for six hours rather than 37 degrees in order to replicate the fever conditions created in the human body during the course of infection, which in turn produces elevated heat stress to the parasite. We exposed tightly synchronised ring stage parasites to three times the IC50 value of the Artemisinin medicine for a comparable duration of six hours in order to simulate the pharmacological drug exposure the parasite experiences during the course of infection in the form of medicinal therapy.

Following the previous description, we examined the data sets for the RNA sequencing control condition. We aimed to comprehend the overall pattern of gene expression in both control and stress-induced parasites after the investigation.

First, we examined the patterns of global deregulation seen in both controlled and stress-induced environments. After doing the investigation, we discovered that stress-induced situations result in a general deregulation. When compared to the control condition, it was shown that about 600

genes were upregulated with an FPKM cut off of 2 fold change. Conversely, about 500 genes were downregulated. The mean average plots (MA plots) show the same thing. Understanding the group of genes that were discovered to be activated in situations driven by stress further piqued our interest. To our great surprise, we discovered that genes play a role in immune evasion, virulence, pathogenicity, antigenic variety, and host cell interaction—all of which add to the infection's complexity (Figure 2 B).

Finally, we separated the genes according to the level of antisense synthesis using a general FPKM cut off of 50 in order to determine whether the reference gene's level of antisense production correlates with its physiology. We called the situation high sense - low antisense (HS-LAS) if the sense transcription was higher than the antisense transcription, High antisense - low sense (HAS-LS) if the antisense transcription was higher than the sense transcription, and sense similar to antisense (S=AS) nearly if the sense and antisense production were almost equal. We observed that the majority of housekeeping roles involved the HS-LAS and S=AS categories. Interestingly, however, genes that produce more antisense than sense were linked to functions that increase the parasite's pathogenicity such as immune evasion, cytoadherence, antigenic variation, entry into host and other pathways that increase pathogenicity of the parasite (Figure 2 C).

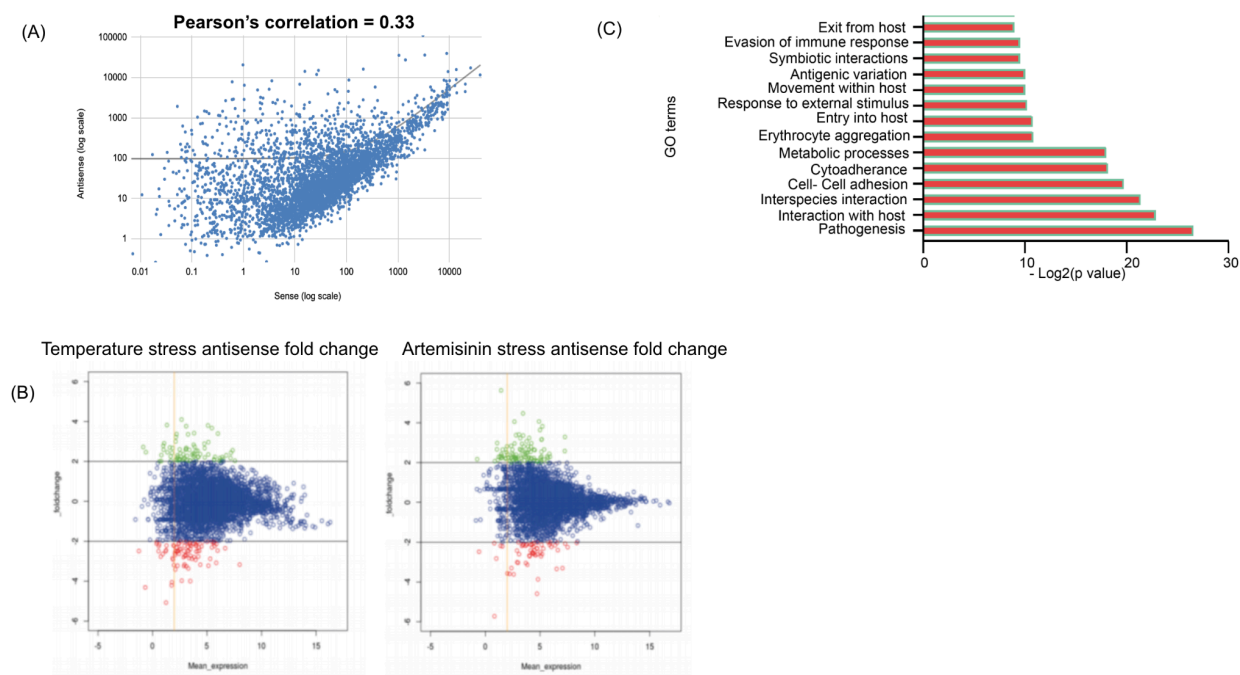


Figure 2: Antisense transcription is deregulated across stress induced conditions. (A) Dot plot represents Pearson's correlation between sense and antisense FPKM values. (B) Mean Average (MA) plots represent fold change and deregulation in antisense transcription upon temperature and artemisinin stress conditions. (C) Histogram representing gene ontology of genes with upregulated antisense across stress induced conditions.

3. Investigate the role of antisense transcription in gene expression regulation for candidate genes PfGCN5 and PfHDAC1

We then looked into how antisense transcription affects the regulation of candidate gene expression. In order to investigate this, we developed a comprehensive strategy that focused on two genes of interest that, according to our RNA sequencing data, are known to create antisense RNA and for which we had laboratory resources. In order to expose the parasites to stress-induced conditions that are comparable to those previously described, we first began our investigation using PfGCN5.

Mapping the strand-specific transcript expression level and the corresponding protein expression was of interest to us. By creating unique strand-specific amplification primers, we used the stranded RT-qPCR approach to investigate the transcript expression level. As previously stated, parasites were cultivated, collected, and examined under three distinct circumstances: control, temperature stress, and artemisinin stress. After the RNA was separated, strand-specific primers were used to create the matching cDNA, which was then used for RT-qPCR. We examined PfGCN5's RNA expression level in each of the three circumstances (**Figure 3 A**).

Comparing the temperature and artemisinin stress conditions to the control condition, we discovered an overall overexpression of RNA. Under both temperature and artemisinin stress, upregulation was noted. Knowing the precise role that sense and antisense transcription play in each illness piqued our interest even more. By creating strand-specific primers, we performed RT-qPCRs based on a stranded technique to investigate the same. After testing and analysis, we discovered that, in comparison to the control condition, sense and antisense transcription was increased by 3 and 30 fold respectively.

We were also curious to find out if the cognate protein levels were impacted by the rise in sense and antisense transcription levels. We used western blotting under both control and stress-induced conditions (temperature and artemisinin stress) to investigate the same. We collected control and stress-induced parasites in the same manner as previously described. Under control settings, we could see protein expression using western blot analysis, however under stress-induced conditions, no protein expression was seen. This is really interesting since we found that stress-induced conditions led to a rise in RNA expression, but this did not correspond to an increase in protein expression; rather, it decreased protein expression. In order to better comprehend this phenomenon, we used transgenic models to determine whether antisense transcription has a role to play in this scenario.

We selected PfGCN5 as the candidate gene for creating transient transgenic models in order to investigate the potential involvement of antisense in this phenomena. Our in-house strand-specific RNA sequencing data and RT-qPCR-based validations confirm that the gene produces both sense and antisense transcripts. Since the full length construct would make overexpression more difficult, we cloned the HAT domain—the functional histone acetylation domain of PfGCN5—into the pARL overexpression vector in the hopes of improving transfection efficiency. To evaluate the impact of either sense or antisense PfGCN5, overexpression transfections were carried out in various combinations. Just sense orientation PfGCN5 was transfected in the first batch, only antisense PfGCN5 was transfected in the second set, and in the third set both sense and antisense PfGCN5 was transfected (Figure 3 C).

Western blotting revealed an increase in PfGCN5 expression following sense construct transfection. We saw a considerable drop in PfGCN5 expression to null upon transfection with an antisense construct. In contrast, we found that sense and antisense co-transfection resulted in less expression than sense transfection alone. According to this investigation, antisense overexpression was linked to PfGCN5 protein depletion, which may be crucial in determining whether antisense RNA influences parasite gene expression. We considered using a method that enables us to develop steady overexpression models rather than transient expression models in

order to better comprehend this. This would enable us to gain a comprehensive and deep understanding of the phenomenon of antisense-mediated gene expression.

We cloned our gene of interest in antisense orientation in the pDC2-mRFP-2A-GFP overexpression vector to produce viable transgenic lines. In order to synthesise antisense HDAC1 RNA upon overexpression, we cloned the entire 1.3 kb PfHDCA1 gene downstream of RFP in an antisense orientation. Because we had previously seen that HDAC1 transcribes small amounts of antisense RNA in its native state and because we had an HDAC1 antibody in the lab that could be used to measure the levels of HDAC1 protein expression after the construct was transfected, we explicitly selected HDAC1 as a prospective gene (Figure 3 D).

We could plainly see from western blotting that when cognate antisense was overexpressed, the quantities of HDAC1 protein disappeared (Figure 3 E). The second biological clone and multiple technical replicates carried out in the laboratory verified the same. To work with a pure population of transgenic cells, we also carried out FACS sorting of the positive population. RFP and GFP positive for just vector control and RFP positive and GFP negative for HDAC1 were used as the foundation for sorting (Figure 3 C).

According to the findings, there may be a role for antisense RNA in translational regulation as cognate antisense overexpression is linked to protein level expression control. Next, we looked at HDAC1's RNA level expression in both the control condition and when HDAC1 AS RNA was overexpressed. We used a strand-specific technique to RT-qPCR in order to investigate the same. As normalised and seen in the control condition, we discovered that HDAC1 sense and antisense RNA rose several times upon HDAC1 AS RNA overexpression. This leads us to believe that overexpression of cognate antisense RNA is linked to changes in RNA expression levels in the cell, indicating that antisense RNA may play a part in transcriptional control. Based on our thorough case study of the GCN5 and HDAC1 genes, we were able to understand that antisense RNA has the ability to interact with the cell at various levels and pathways. Crucially, we discovered that antisense RNA is interacting within the cell and changing transcriptional and translational levels.

We developed a specialised RNA pulldown-based method for *Plasmodium falciparum* 3D7 in order to look into the potential routes at which antisense RNA might be interacting. Using PCR, we amplified the desired gene areas and included the T7 promoter sequence. We made sure to employ biotinylated dUTP marked at position 16 instead of unlabelled rNTPs by using these products as templates for in-vitro transcription. We can use Streptavidin to carry out affinity purification with this method. Therefore, as a control, we chose the cognate sense RNA of three genes that produce substantial levels of antisense RNA. After the target RNA was in vitro transcribed and treated with parasite protein lysate for half an hour, Streptavidin agarose beads were used for pulldown, and on-bead mass spectrometry was performed. As a control for potential background, only beads treated with the protein lysate devoid of RNA were employed. For antisense, sense, and only beads, the same procedure was followed, followed by the on-bead mass spectrometry methodology, peptide cleanup using C18 columns, IDA run for six hours, and data analysis.

The HDAC1, perforin, and cysteine rich protein genes were all analysed. Proteins belonging to the categories of transcription, translation, RNA regulation (histones, DNA-RNA binding proteins, transcription related proteins), protein regulation (ribosome proteins, elongation related proteins), and histone regulation were identified by mass spectrometry for each pulldown. We discovered that both sense and antisense RNA were concentrated in similar pathways. The mass spectrometry data was clean since the single beads that were pulled down were clean and did not bind to any of the lysate's proteins. Unfortunately, we were unable to identify any intriguing hits that may be investigated further as possible global regulators for antisense transcription. Our goal was to examine differently bound proteins among sense and antisense cognate partners. Despite this, we could recognise that antisense RNA has potential and interacts within cells at several levels. This supports our observation that antisense RNA mediated gene regulation may be a very intricate process that differs for different genes and under various circumstances. These findings raise the question of how antisense RNA regulates transcription in the parasite's nucleus or what part it plays in transcriptional regulation.

We postulated that antisense RNA might be causing transcriptional regulation through RNA duplex or base pairing in order to comprehend its function in transcriptional regulation. In order

to investigate this, we devised an experiment to examine the potential function of antisense RNA in the production of RNA-RNA duplexes, which may result in RNA stabilisation and a net increase in RNA quantities by lowering the rates of RNA degradation.

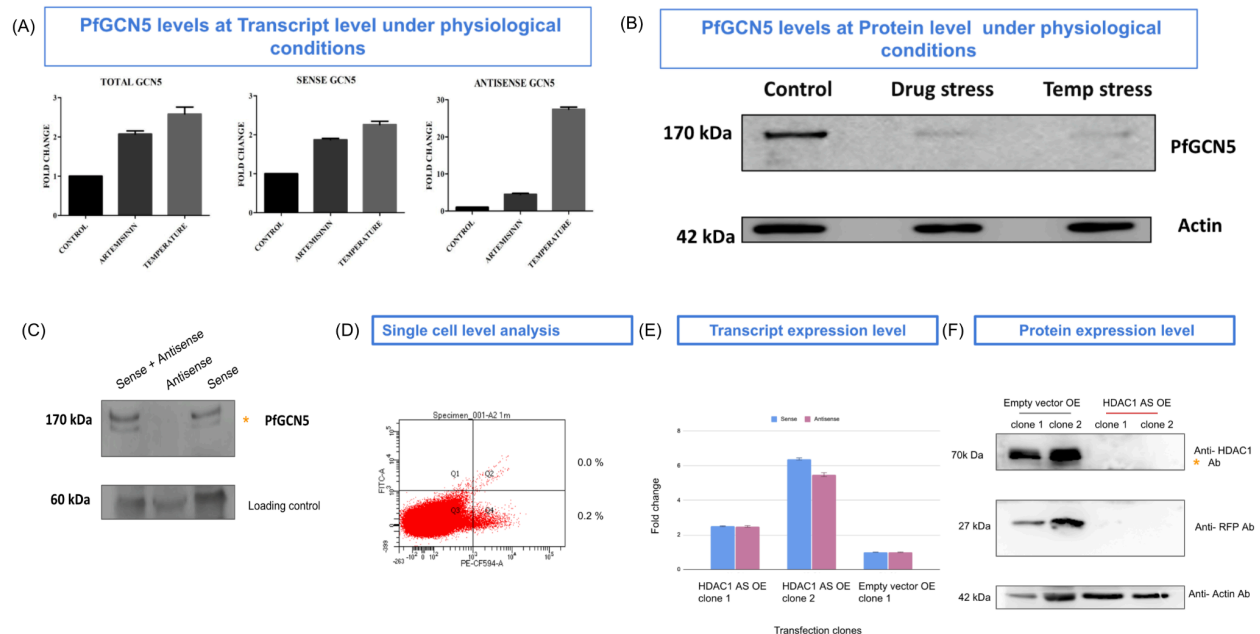


Figure 3: Role of antisense RNA of candidate genes PfGCN5 and PfHDAC1. (A) Box plots represent RT-qPCR data for total PfGCN5 transcription across control and stress induced conditions and also sense and antisense transcription. (B) Western blot represents PfGCN5 protein expression across control and stress induced conditions, probed using in house raised anti PfGCN5 antibody, actin being used as a loading control probed using commercial anti actin antibody. (C) Western blot represents levels of PfGCN5 upon sense and antisense overexpression through Plasmid construct. (D) FACS sorting data for PfHDAC1 stable overexpression line, FACS sorting done on the basis of FITC and GFP channels. (E) Box plot represents RT-qPCR data for sense and antisense PfHDAC1 expression level upon PfHDAC1 antisense stable overexpression through pDC2-mRFP-HDAC1 antisense-GFP construct. (F) Western blot analysis for PfHDAC1 protein level upon antisense overexpression and empty plasmid overexpression used as a control.

4. Investigate the formation of RNA-RNA duplex as a means to RNA stabilization

The following is the experimental paradigm: Using the Sorbitol technique, *Plasmodium falciparum* 3D7 parasites were closely synchronised at the 2-4 hpi stage. Three equal volumes were created from the parasites. Since the first one was seen as a control, it received no treatment. At that precise moment, a third of its volume was taken; this was known as the control 0 hour set. The parasites in the control condition were then also collected six and eighteen hours after being incubated at 37 degrees Celsius. The sense and antisense HDAC1 RNA transfected cells were also isolated at the 6- and 18-hour mark after incubation and treatment. IVT-synthesised RNA sense or antisense HDAC1 RNA was electroporated in the other two sets for the transfected cells, respectively. In order to prevent additional transcription, 25 mg/ml of Actinomycin D was simultaneously administered to both sets. Therefore, the effect we observe would result from the cell's net RNA availability, independent of additional transcription or nascent RNA. Following the harvesting stage, random hexamers were used to create cDNA and the Trizol technique was used to isolate total RNA. RT-qPCR was then used to measure the amounts of sense and antisense RNA in both transfected and control conditions (Figure 4 A and B).

Sense and antisense RNA levels were assessed across transfection sets using the control condition as a normalisation control. At six hours, it was discovered that both sense and antisense HDAC1 were elevated following transfection of sense HDAC1, and vice versa, i.e., following transfection of antisense HDAC1. Similarly, it was shown that sense HDAC1 RNA transfection resulted in a multifold increase in both sense and antisense RNA levels after 18 hours, which was significantly larger than at the 6-hour mark. On the other hand, sense and antisense HDAC1 are upregulated by many times as a result of even antisense RNA transfection. This leads us to the conclusion that the cognate RNA expression level rises regardless of which RNA—sense or antisense RNA—is transfected (Figure 4 C). This is only achievable when the two complementary RNAs are able to base pair with one another, stabilising the pair and significantly lowering its rates of degradation. Greater availability and protection of RNA for just-in-time use—which may be used in adverse conditions—are the outcomes of higher stability and lower degradation rates.

In the larger framework of antisense RNA-mediated gene regulation, this leads us to consider what might be the next stage of duplex RNA production. Does the duplex RNA remain in the nucleus or is it translocated to the cytoplasm? Moving further, like all newly transcribed RNA, the duplex RNA is translocated from the nucleus to the cytoplasm. Duplex RNA is not rendered for the translation process, although freshly transcribed RNA typically goes through translation once it enters the cytoplasm. Because duplex RNA is double stranded in nature, it is either not identified for translation or it can halt translation by blocking the ribosome. To investigate this further we hypothesized that duplex RNA could be translocating to the cytoplasm where they might be stalling the ribosome leading to reduction or complete elimination of translation.

We created a thorough plan to investigate antisense RNA-mediated gene regulation and the potential for ribosome stalling through duplex RNA production. To achieve this, we used sorbitol and percoll double synchronisation to synchronise *Plasmodium falciparum* 3D7 parasites at the 16–18 hpi stage in a carefully planned experiment. Three equal sections of the culture were separated, and each was utilised for three technical replicates of a particular RNA sequencing experiment. After Ribodepletion, whole cell directed RNA sequencing was performed using the first set. There was also a matching set for the heat set condition. The same method as previously described was used to introduce heat stress (Figure 4 E).

We chose to investigate the duplex RNA using a two-pronged strategy. First, we made the decision to use the Illumina platform for dsRIP seq, which stands for double stranded RNA immunoprecipitation followed by sequencing. An anti-J2 antibody, which detects double-stranded RNA and RNA creating a secondary structure of complementary pairing within the RNA sequence, is used to carry out the process (Figure 4 E).

The second strategy involves using ribosome profiling with some adjustments to meet our experimental needs in order to investigate the RNA linked to the ribosomes. The ribosome pellet, which settles at the tube's bottom after ultracentrifugation at 45,000 rpm for 3.5 hours at 4 degrees Celsius in a suitable swinging bucket rotor, is separated by layering cell lysate over a 5–60% sucrose gradient. After being removed from the tube's bottom, the pellet is reconstituted

with Trizol, an RNA isolation reagent. Total RNA is obtained after Trizol exposure, and it is denatured for three minutes at 65 degrees Celsius before being gradually cooled to 4 degrees Celsius, which enables the development of secondary structures and duplexes. As previously mentioned, a split was also utilised for whole cell directed RNA sequencing after ribodepletion. Following the addition of an anti-J2 antibody to bind to duplexes, the complex is subsequently extracted by incubating it with Sepharose G beads. This RNA complex is processed using the sequencing technology of the manufacturer (NEB Ultra II Directional RNA sequencing following Ribodepletion). The data was produced in-house using the NextSeq 550 Illumina platform, and it was examined using a specially created bioinformatic workflow, as previously mentioned.

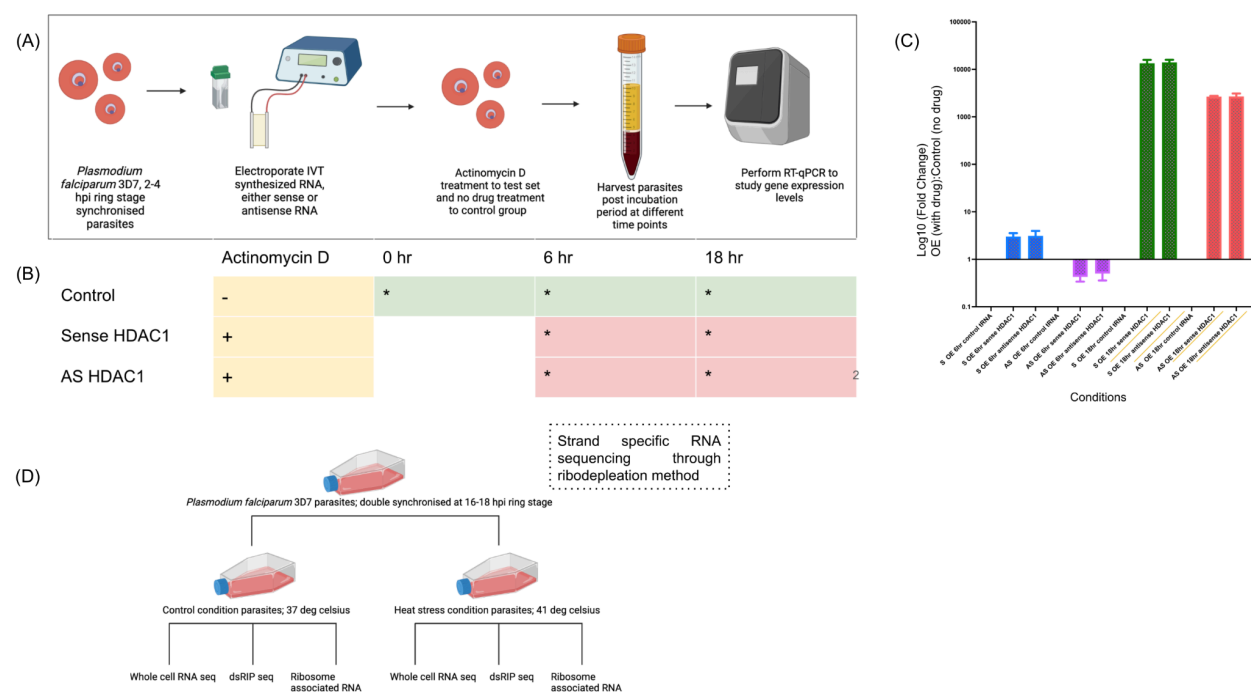


Figure 4: Study of RNA - RNA duplex formation as a means for RNA stabilisation. (A) Schematic represents experimental paradigm for IVT synthesised RNA transfection. (B) Tabular representation of experimental conditions. (C) Box plot represents RT-qPCR data following IVT RNA transfection, suggesting a strong case of RNA duplex formation between sense and antisense RNA leading to RNA stabilisation. (D) Schematic representation of experimental

paradigm to study duplex formation involving whole cell sequencing, dsRIP seq and Ribosome associated RNA sequencing.

5. Duplex RNA associated with ribosomes affect translational status of stress and stimuli dependent genes

The genes whose sense and antisense transcripts occurred together would have formed a duplex pair, according to the same analysis of the Duplex RNA sequencing data. Here, under controlled conditions, we have 668 sense and antisense RNAs in duplex interaction (Figure 5 A). In the duplex under temperature or heat stress conditions, we discovered 766 sense and antisense RNA.

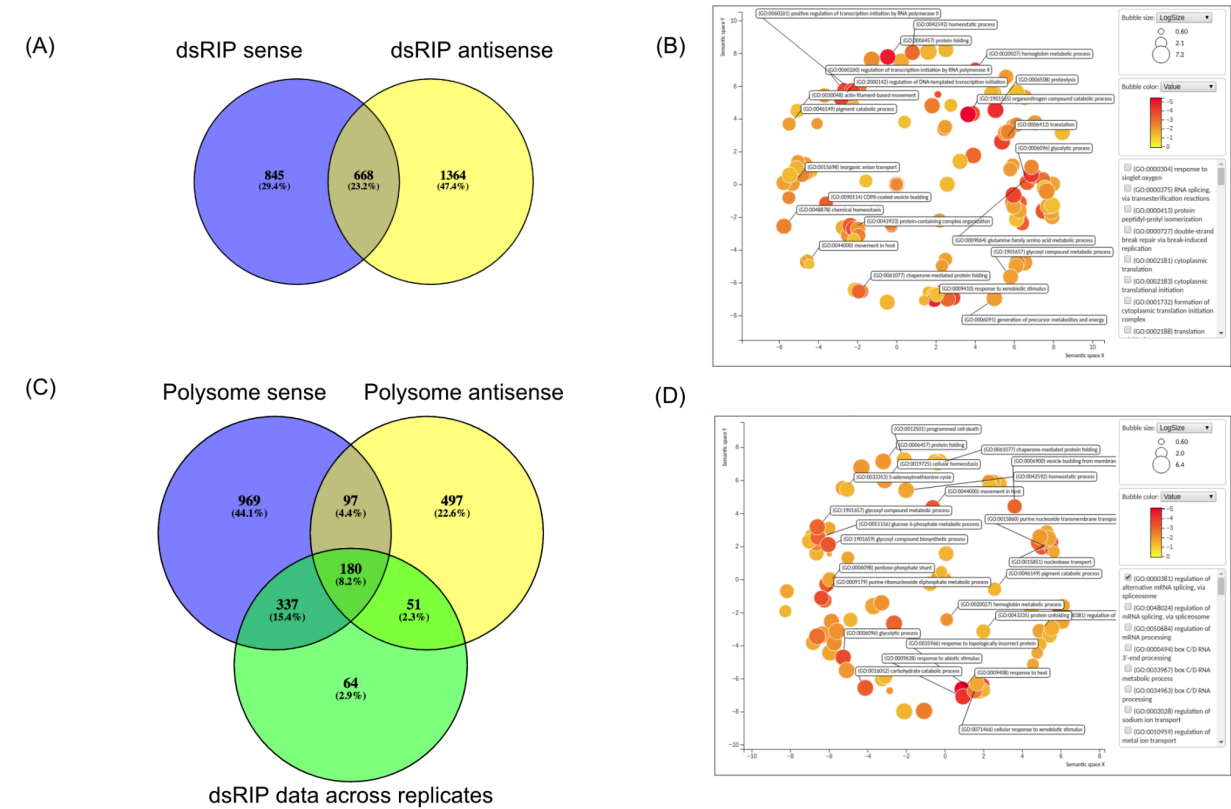
It's interesting to note that 98 new transcripts formed duplexes under stress; these genes may be responsive to stress in a way that results in translational reduction or RNA preservation. Crucially, we discovered that genes that form duplexes under stress-induced conditions are linked to critical pathways like metabolism, transcription, translation, stress-dependent pathways, and stimuli-dependent pathways (Figure 5 B).

In order to determine whether any duplex RNA binds to the ribosome to halt translation, we also attempted to examine RNA's relationship with the ribosome. We discovered that 165 duplex RNA binds to ribosomes and may be preventing translation or stalling polysomes. This relationship is stronger in situations where stress is present. We discovered that this category includes crucial pathways including metabolism, transcription and translation-altering genes, and genes that are dependent on stress and stimulation (Figure 5 C and D).

Further, we looked at the global and comparative levels of antisense RNA at the whole cell global level, dsRIP seq data implying for secondary structures formation and ribosome association of antisense RNA. We found that upon stress induced conditions, a larger proportion of antisense RNA associated to ribosomes and we also found a higher secondary structure or duplex forming tendency upon stress induced conditions (Figure 5 E). This clearly suggests that temperature stress condition induces higher association of antisense RNA to ribosomes which could lead to translational stalling and also formation of duplex which would be a means to

preserve RNA or inhibit their translation.

We also carried out global scale quantitative proteomics through ReDiMe technique across control and temperature stress conditions to see global level protein deregulation, and we found antisense transcripts to be associated with protein downregulation (data not shown).



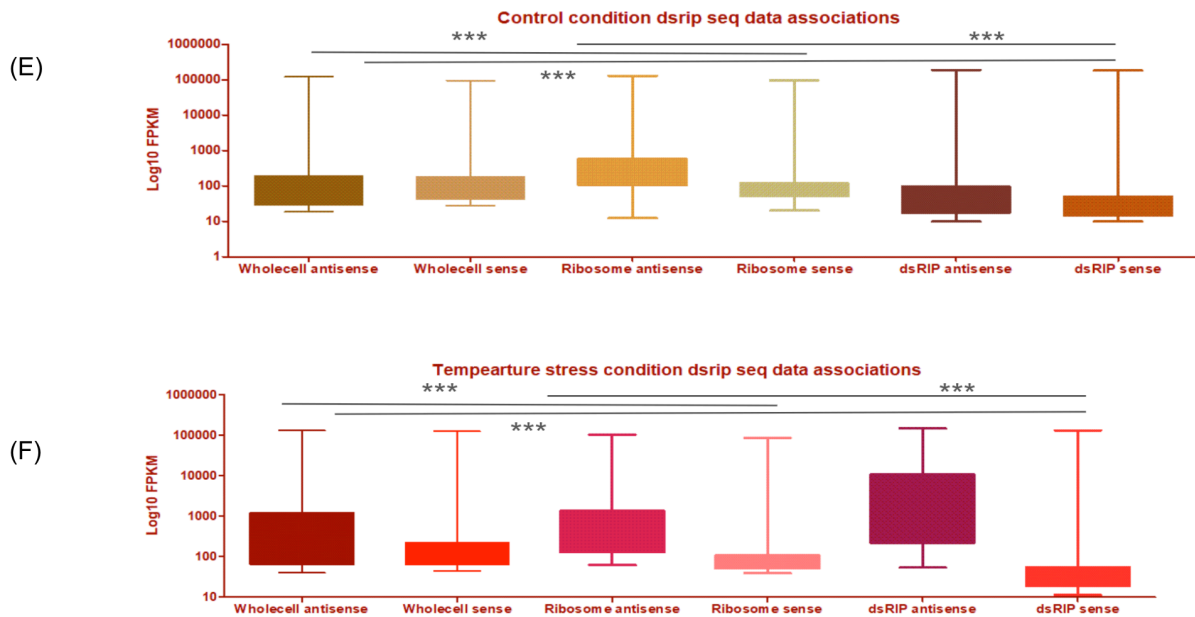


Figure 5: Duplex RNA formation via antisense RNA leads to translation regulation by associating with growing ribosomes. (A) Panel represents the pie chart depicting common genes for which sense and antisense RNA was picked up by sequencing. (B) Panel B representing gene ontology for genes found common in panel A. C) Panel C pie chart represents the duplex candidates which further associate to ribosomes (found from ribosome associated RNA sequencing) (D) Panel D represents gene ontology for duplexes associated to ribosomes. (E and F) Panel E and F shows box plots for FPKM values of duplex forming genes across all data sets suggesting greater association of antisense RNA and their duplex to ribosomes upon temperature stress induced conditions.

6. The working model of our study suggests that antisense RNA mediated duplex formation leads to translation regulation via association with growing ribosomes

This brings us to highlight the working model of our study. We believe that *Plasmodium falciparum* parasites are fast growing cells with active transcription state in the beginning of their lifecycle (ring to trophozoite progression). There is a transcriptional spurt during the early stage and the parasites have to continuously tolerate the ever changing environmental conditions which

are not in the favor of the parasites. Hence, this requires a quick and efficient response mechanism to tide stress and other unfavorable conditions. Our study proves that the parasite follows the Just in time expression model of genes by altering the RNA states. The RNA could be available in the cell in three prominent forms. The first being free RNA that is readily available for post transcriptional modifications, translation and post translational modifications. The RNA could also be rendered in a duplex form that is highly stable and not prone to degradation, hence the RNA could be preserved for later usage or modifications for genes responsive to stress and stimuli or could also be a means to downregulate translation of unrequired genes. The antisense RNA or duplex RNA could also associate to polysomes or ribosomes to either decrease or alter translational rates in favor of the parasite under stress. Hence this mechanism forms a sensitive switch by using duplex RNA as a mode to alter gene expression via an underlying translation mechanism (Figure 6).

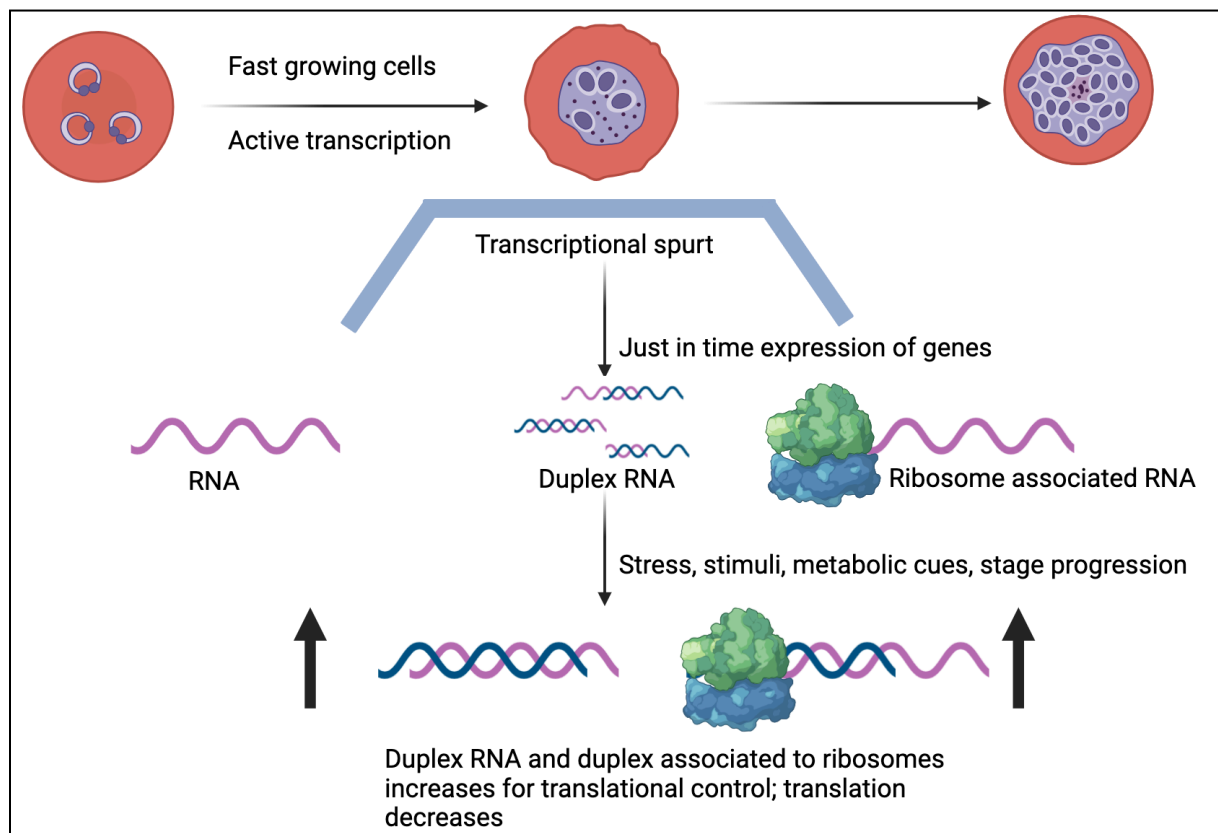


Figure 6: The schematic representation of the working model of our study. The schematic shows that *Plasmodium falciparum* parasites are rapidly developing cells with active

transcription at the start of their lifecycle (ring to trophozoite progression). During the early stage, there is a transcriptional surge, and the parasites must constantly adapt to the ever-changing environmental conditions, which are not favourable to them. As a result, it is necessary to have a quick and efficient response mechanism to tide stress and other adverse conditions. Our findings show that the parasite uses the Just in Time expression model of genes by modifying RNA states. The RNA could be present in the cell in three different forms. The first is free RNA, which is easily accessible for post-transcriptional alterations, translation, and post-translational modifications. The RNA might also be generated in a duplex form that is very stable and resistant to degradation, allowing the RNA to be retained for later use or alterations for genes that respond to stress and stimuli, as well as a mechanism to downregulate translation of unnecessary genes. The antisense or duplex RNA may also bind with polysomes or ribosomes, reducing or altering translational rates in favour of the parasite under stress. As a result, this technique creates a sensitive switch by employing duplex RNA to change gene expression via an underlying translation mechanism.

Discussion

The ability of *Plasmodium falciparum* in causing a successful infection and being able to even develop drug resistance and increase its virulence is dictated by a strong underlying gene expression paradigm. While there are limited ways of gene repression known to regulate an extremely peculiar genome, antisense transcription has gathered distinguished limelight. Primarily, antisense transcripts have been shown to be associated with var gene regulation, sexual development, telomere maintenance, their mechanism of action remains elusive and their role in stress response management remains unexplored. We investigated in this direction by following a systematic and thorough approach. By conducting strand specific RNA sequencing over control and stress induced conditions we firstly showed that antisense transcription is a result of a specific and well regulated transcriptional paradigm and not necessarily a resultant of open chromatin. We further prove through systematic analysis that antisense transcription is not a result of divergent transcription or completely a byproduct of transcriptional runthrough. We also show through epigenetic analysis that antisense transcription promoters carry the same epigenetic marks as sense through their occupancy levels vary. We move further to study the

physiological association of antisense transcription. We show that antisense is highly deregulated upon stress induced conditions. Importantly, antisense transcription is upregulated upon stress induced conditions. Interestingly, the genes which show a pattern of increased antisense transcription contribute to the virulence of the parasite and are involved in pathways such as immune evasion, cytoadherence, antigenic variation, stress and stimuli dependent pathways etc.

Moving further we pick two candidate genes; PfGCN5 and PfHDAC1 and through a series of transgenics creation, RNA and protein estimations we show with confidence that antisense transcripts are associated with increased sense and antisense transcription and cognate protein downregulation. This is an interesting paradox which needs to be studied further. We postulated that antisense RNA leads to stabilization of net RNA via the formation of a stable sense and antisense duplex. This RNA-RNA duplex is highly stable and has a lower degradation rate, hence the net RNA levels increase. To study our hypothesis further we transfected IVT synthesised RNA within the cells and found that irrespective of sense or antisense transfection the cognate RNA's level always increased. Briefly, antisense RNA transfection led to increase in sense RNA level and vice versa was also true, suggesting a strong case of RNA stabilization. So, this explains the case of increased RNA levels upon antisense transfection.

Besides, to study the correlation of decreased protein levels upon antisense transfection we hypothesised that antisense RNA might be associating to ribosomes to stall translation or the duplex formation leads to no translation for those genes as duplexes are not recognised by the translation machinery. To study this further we carried out dsRIP seq to probe RNA-RNA duplex and Ribosome associated RNA sequencing and we found out that indeed duplexes are formed at the global level and these duplexes and also single stranded antisense associate to ribosomes to stall translation. We also carried out global scale quantitative proteomics through ReDiMe technique across control and temperature stress conditions to see global level protein deregulation, and we found antisense transcripts to be associated with protein downregulation (data not shown). Interestingly, we also found that antisense RNA mediated duplex formation and association to ribosomes increases upon stress induced conditions.

Our study certainly uncovers previously undiscovered and undiscussed pathways in *Plasmodium*

falciparum and sheds light on the importance of antisense transcription in *Plasmodium falciparum*. We hope that our study will open doors to newer discoveries and would highlight newer interventions to tackle this parasite.

Chapter 5

Chapter 5: Discussion of the Thesis

Malaria is a major cause of death and morbidity worldwide. *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium knowlesi*, *Plasmodium vivax*, and *Plasmodium malariae* are the five deadly species that cause it in humans. Of them, *Plasmodium falciparum* is the most common and deadly, along with *vivax* sp. Numerous international initiatives are coordinated to reduce the number of cases of malaria and develop preventative strategies, such as vaccinations. It appears that there is hope that *Plasmodium* infections will have significantly decreased by 2035 and that a prophylactic vaccine will be authorised for use. Currently, the final resort is still artemisinin and combination therapy based on artemisinin.

It is well known that the parasite has a very complicated life cycle, moving through several stages and hosts. It is capable of withstanding a wide range of adverse circumstances and changes morphologically. It is well known that the parasite flourishes under stressful environments, even becoming resistant and becoming more virulent. An underlying pattern of strict gene regulation paradigms supports this. The parasite's unusual genome, which is primarily euchromatin in nature and biased towards AT richness, further complicates matters.

The important roles are played by a small group of transcription factors. Although there aren't many known methods for controlling gene expression, recent research using Next Generation Sequencing (NGS) based technologies has demonstrated promise in epigenetic regulation through non-coding RNAs. The majority of the parasite's genes are known to generate antisense transcripts, demonstrating the widespread presence of ncRNA in the parasite's biology. The significance of antisense RNA in controlling critical pathways such as telomere maintenance, var gene regulation, and sexual differentiation has also been brought to light by recent studies. Antisense RNA has been linked to important activities, although its role in stress regulation and the mechanism of action for gene regulation have not yet been identified.

By identifying the role antisense RNA plays in gene regulation, our study fills a knowledge vacuum and recognises the significance of antisense RNAs in *Plasmodium* biology. We provide a thorough methodology to first describe and investigate antisense transcription globally. We are able to decipher the mechanism of action of gene regulation mediated by antisense RNAs by

using candidate genes since we understand the manner of genesis for antisense transcription. We also investigate this further and attempt to determine whether antisense transcription has any new non-canonical characteristics to offer.

I go into more depth about antisense transcription in the first section of my thesis. We discuss the biology of *Plasmodium* and malaria, as well as the major studies of antisense transcription in many kingdoms and its potential significance for the *Plasmodium* system. Additionally, we have clarified what is currently understood about the function of antisense transcription in *Plasmodium*. As we proceed, we go further into the background or set of investigations that made ncRNA come to limelight. We have investigated the significance of antisense RNA in *Plasmodium* biology and comprehensively characterised antisense transcription. Going further, we cover instances from various systems, such as bacteria, plants, fungi, and animals, and provide insight into the potential mechanisms of action that antisense transcription may employ for gene regulation.

I have covered our work that was published in the Journal of Biosciences special issue in the second section of my thesis. The interaction mechanism between PfGCN5 and PfGAPDH in regulating gene expression and nutrition sensing is discussed in the paper. It also draws attention to PfGAPDH's dual role in *Plasmodium*, where it contributes to gene regulation in addition to its metabolic duties. The function of the histone acetyltransferase (HAT) PfGCN5 as a transcriptional regulator in the context of glucose or nutritional stress is discussed in the work.

We demonstrate a substantial upregulation of PfGCN5 and PfGAPDH under dietary stress. PfGCN5 has been linked to several metabolic genes that are activated during nutritional stress, which is an intriguing side effect. Based on fractionation and localisation investigations, PfGAPDH is found in the cytoplasm and nucleus. Furthermore, it was discovered that PfGCN5 acetylates and interacts with PfGAPDH *in vitro*.

PfGAPDH is therefore probably acetylated by PfGCN5 after undergoing further translocation to the nucleus during nutritional stress. The expression of metabolic genes may be regulated by acetylated PfGAPDH, either by itself or in combination with PfGCN5. Some of the unresolved issues with PfGCN5 and PfGAPDH-mediated transcriptional regulation under nutritional stress conditions would be investigated in more detail.

This raises some intriguing queries, such as (i) Does dietary stress alter the distribution of PfGAPDH in the cytoplasm and nucleus? (ii) Does PfGAPDH control gene expression by interacting with the chromatin? Conversely, is the PfGCN5-PfGAPDH complex's synergy necessary for the regulation of gene expression? To sum up, knowing how the parasite senses nutrition can assist in pinpointing the molecular networks that govern its virulence and open the door to disease prevention.

The non-canonical characteristics of *Plasmodium* antisense transcription are then covered in the third chapter of the thesis. According to our theory, antisense transcripts are made up of the same nucleotide bases as sense RNA and are accessible to ribosomes in the same cell environment as mRNA. They certainly have the potential to be translated in theory because of this. Additionally, they have been demonstrated to translate into tiny proteins or micropeptides that may support a variety of cellular processes, including metabolic sensing, export proteins, surface sensing, and cell-to-cell communication. Based on similar hypotheses, we planned to investigate first if tiny proteins derived from ncRNA or antisense RNA regions are present.

We had to carry out a number of standardisation tests in order to eventually be able to track proteins with low molecular weights and abundances, which are typically lost during experimental handling or may not be traced because of technical constraints. By using mass spectrometry, we were able to standardise the techniques for studying tiny proteins. We detected tiny proteins that came from antisense RNA; surprisingly, ribosomal sequencing revealed that these antisense RNAs were also attached to ribosomes. Our next goal was to use bioinformatics to investigate the possible functions of these tiny proteins by analysing their sequences. For instance, a little protein with an antisense origin may bind to particular *Plasmodium* or human receptors or act as a signal peptide.

Our next goal was to proceed with in-vitro characterisation after a comprehensive identification and characterisation of tiny proteins. In order to better understand tiny proteins, we wanted to overexpress them, either with or without an interleaving stop codon, and see if this could translate them. Using the overexpression vector, we aimed to investigate the potential for these peptides to be translated into fluorescently tagged proteins. To achieve this, we cloned intriguing candidates in a pDC2 dual fluorescence vector, both with and without interleaving stop codons.

The expression of the tiny proteins would indicate that the translation machinery of the parasite is well suited to translating such proteins.

Additionally, it would provide us the ability to change the expression level of intriguing candidates inside the cell. The nature of the translational machinery, including its capacity to accept interleaving stop codons and identify antisense RNA as its translation RNA, will also be clarified. We intend to publish this work as a research article after it has been investigated further. This study would open up a new, exciting field of study with potential applications in diagnosis and therapy.

Last but not least, chapter 4 in my thesis identifies *Plasmodium falciparum's* antisense transcription mechanism of action. It provides a very thorough analysis of *Plasmodium* antisense transcription. In order to initially characterise and study antisense transcription on a global scale, we design an extensive set of studies. Using state-of-the-art sequencing technologies based on the Illumina platform, we attempt to comprehend the prevalence of antisense transcription in this study. In our quest to identify the genes in *Plasmodium* that produce the most antisense, we are quite interested to discover that the genes that increase the parasite's pathogenicity and virulence are also linked to the highest antisense transcription.

By resolving several potential genesis scenarios, we are able to comprehend the origins of antisense transcription in *Plasmodium falciparum* and come to the conclusion that it is a phenomenon that is independently regulated and that its correlation with both independent and shared bidirectional promoters is highly significant. Moreover, its development is unrelated to runthrough or divergent transcription. Additionally, we provide a cogent description of the promoter elements and the epigenetic markers they occupy.

Going forward, we explore the phenomena of antisense in greater detail and examine its physiological association by examining its function under stress. Interestingly, we discover that under stress-induced circumstances, antisense transcription is significantly dysregulated. Antisense RNA is significantly upregulated in genes linked to stress, immunological evasion, virulence, and pathogenicity, whereas antisense is downregulated in genes involved in housekeeping. We also confirm this using stranded RT-qPCR methods.

Going forward, we select two candidate genes, PfGCN5 and PfHDAC1, for our investigation in order to determine the mechanism of action for gene regulation. Antisense transcription is linked to enhancing the transcription profile of the homologous gene while reducing or removing its translation, according to in-vitro tests such as RT-qPCR and western blotting as well as the creation of stable overexpression lines. We find that antisense transcription modifies the corresponding gene's transcription and translation using separate techniques and many duplicates. Even while we would naturally anticipate that higher transcription would positively correlate with translation, the opposite is shown, which makes this seem paradoxical. Translation downregulation results from antisense-induced transcription upregulation. As we work to resolve this contradiction, we hypothesise that antisense-sense RNA creates a duplex pair via complementary base pairing.

Due to its strong stability, this duplex is not rendered for RNA breakdown processes. Additionally, the duplex RNA can remain stable within the cell and can also prevent gene translation, which can change the amounts of gene expression. Additionally, the duplex RNA complex can form a sensitive switch that reacts to stimuli and stress. By modifying RNA states and translational levels, this switch can modify the downstream level of gene expression, favoring the parasites. Additionally, we see an increase in duplex creation occurrences in stress-induced situations, indicating that the duplex RNA mechanism is linked to stress avoidance and stimulus detection. It's interesting to note that our duplex RNA seq and ribosome associated RNA sequencing show that the duplex RNA linked to ribosomes also rises during stress.

These intriguing results highlight the increasing significance of antisense transcription and pave the way for more thorough research on the molecular actors driving these pathways. It also creates opportunities for future advancements in detection methods and treatment paradigms. Antisense oligonucleotides have a bright future as both possible vaccine candidates and a paradigm for treatment. We hope that our research will prove pivotal for the same and make a mark in malaria research.

Publication/Manuscripts under preparation

1. Rawat M*, Malhotra R*, Shintre S, Pani S, Karmodiya K (2020) Role of PfGCN5 in nutrient sensing and transcriptional regulation in *Plasmodium falciparum*. J Biosci. 45:11. PMID: 31965989 (*shared first author).
2. Rawat M, Kanyal A, Choubey D, Deshmukh B, Malhotra R, Mamatharani DV, Rao AG & Karmodiya K (2022) Identification of Co-Existing Mutations and Gene Expression Trends Associated With K13-Mediated Artemisinin Resistance in *Plasmodium falciparum*. *Frontiers in Genetics*, 13, Article 824483. <https://doi.org/10.3389/fgene.2022.824483>
3. Malhotra R & Karmodiya K (2024) Elucidating the non-canonical roles of antisense RNA mediated via antisense RNA translation into small proteins or micro peptides (work in progress).
4. Malhotra R & Karmodiya K (2024) Antisense transcription mediated gene expression regulation in *Plasmodium falciparum* via the control of transcriptional and translational arcs (manuscript under preparation).

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