Histone Lactylation Dynamics in Plasmodium falciparum

Histone Lactylation Dynamics in *Plasmodium falciparum*

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A Thesis in the Field of Biology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2023

Abstract

Epigenetic modifications of the histone are a rapidly unfolding field of research with new posttranslational modifications being uncovered at a rapid pace. Histone lactylation is a newly identified modification associated with metabolism and it has been shown to exist in various organisms including humans and parasites. In some organisms it has shown dynamic changes with response to environmental changes, suggesting a possible mechanism of regulating gene expression profiles in response to environmental factors. In *Plasmodium falciparum* severe infections have a direct impact on environmental conditions, including blood lactate levels. This investigation demonstrates a responsiveness to histone lactylation in *Plasmodium falciparum* at each stage of the intra-erythrocytic development cycle in response to increases in environmental lactate. Additionally, examination of readers and writers of other epigenetic markers led to the investigation of Sirtuin proteins as potential erasers of histone lactylation. Knocking out Sirtuin proteins demonstrates increases in histone lactylation that indicates involvement in the removal of lactate signal from histone proteins.

Acknowledgments

I would like to firstly thank Manoj Duraisingh and all members of the Duraisingh lab for their help and assistance with my research. I've learned so much from them and could not have been able to complete this without their hours and hours of assistance. In particular I would like to thank Manish. Considering the breadth of research that you are involved in, I'm deeply appreciative of the time and effort that you took to walk me through the stages of the research process and for your preparation of samples and hours of tissue culturing. You made any of this possible for me.

I would also like to thank my wife Alyssa! It was so much fun explaining my research and different phases of the process to you. You were always interested and helped me to keep my interest in the process and the topic, especially at times when it continued to drag on and I was ready to be done. Thank you for your support and I love you so much!

I also want to thank my students. Being in school is tough and I hope to become a better teacher in order to help your learning. They inspire me to keep learning every day so I can better serve them.

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Chapter I

Introduction

The single-celled *Plasmodium* parasite is responsible for the infection causing malaria in humans that resulted in an estimated 241 million cases and 627,000 deaths worldwide in 2020. The impacts are especially felt in the developing world, where it is consistently a leading cause of mortality. The majority of these deaths are caused by the *Plasmodium falciparum* species. During the asexual replication in the human erythrocytes, *P. falciparum* undergoes multiple changes at metabolic and gene-expression levels, which drives the rate of asexual proliferation, expression of surface antigens and commitment to the transmissible sexual stages. Changes in epigenetic histone modifications have been shown to play a role in the regulation of these key processes. The *Plasmodium* epigenome has been demonstrated to contain similar eukaryotic posttranslational modifications to histone proteins as well as DNA methylation as those found conserved in higher order eukaryotic species.

The epigenome includes chemical modifications to the histone proteins that interact with DNA or methylation of DNA. These modifications facilitate the transition between the inactive heterochromatin state of DNA and the transcriptionally active euchromatin. There are a variety of classes of epigenetic modifications, however, they share a similar functionality: altering the regulation of protein production either by transcriptional or post-transcriptional regulation (Armstrong et al. 2019). These epigenetic signals produce heritable changes in gene expression without altering the base

pairs of the genetic sequences. Epigenetic modifications were first noticed and studied as far back as the 1940s, however interest in the concepts has exploded in the 21st century. The concepts of epigenetics are being investigated in diverse fields from cancer research to nutritional sciences. The impacts of epigenetics and its role in gene regulation have yet to fully unfold in the academic world.

Histone lactylation is a recently identified form of epigenetic modification. A study by Zhang et al. identified multiple lysines on human and mice histones to be lactylated, which mirrored levels of cellular lactate and glucose. The metabolism of glucose via glycolysis can result in lactate as a byproduct. In a hypoxic environment, cells revert to glycolysis as the sole source of cellular energy and produce excess lactate as a result. Researchers found that exposure to bacterial infection resulted in increases in glycolysis and corresponding increases in lactate levels and histone lactylation, demonstrating lactylation as an important metabolic signal that can affect the epigenome. In addition, similar to histone acetylation, histone lactylation was found to be a positive regulator of gene expression.

This study will aim to investigate the presence and dynamics of histone lactylation in the *P. falciparum* parasite through the intra-erythrocytic developmental cycle (IDC) and in response to various levels of lactate in the growth medium. The parasite will be exposed to different levels of lactate in order to investigate any changes in histone lactylation levels. I will also investigate the temporal nature of histone lactylation, observing the change in histone lactylation across generations after a high initial exposure. This will provide insight into the transgenerational effects of environmental factors which could offer insight into the disease progression of malaria.

In order to observe the potential activity of writers and erasers, several known epigenetic players will be inhibited and the effects on histone lactylation will be observed

Presence and levels of histone lactylation will be monitored using antibody raised against lactylated lysines through western blot analysis. It is expected that histone lactylation will be dynamically modulated in response to levels of lactate and glucose available to the organism. Variances in histone lactylation across the asexual growth cycle of the parasite may better inform how *P. falciparum* dynamically responds to high lactate levels in the severe malaria cases. This project aims to inform future studies to investigate the relationships between environmental lactate levels, histone lactylation and differential regulation of gene expression in *P. falciparum* parasites in severe malaria cases.

Malaria

History of Disease

Malaria is a disease caused by the infection of human erythrocytes by *Plasmodium* parasites and transmitted through the bites of infected female *Anopheles* mosquitoes. The disease has a long history and there is evidence that it has affected humans for thousands of years. Malaria came into public health consciousness in the 19th and 20th centuries and has been a major source of illness and fatality (CDC, 2017). Parasites as the source of malaria were first identified in the 1880s by French army doctor Charles Louis Alphonse Laveran (Scientific American, 1882) and the life cycle of the *Plasmodium falciparum* parasite was confirmed in 1897, identifying it as the source of

malaria (CDC, 2017). Quinine was found to kill the parasite and can still be found as a treatment method today. Identifying mosquitoes as the vector for the disease, governments have focused their global health efforts on the mosquito population control. Use of DichloroDiphenylTrichloroethane (DDT) became widespread, although it has been banned in the US since 1972. In tropical areas with a rainy/dry season cycle, insecticide spray is still used effectively to minimize mosquito populations. Malaria remains a significant issue in Africa and parts of Southeast Asia. In 2021 there were 247 million cases of malaria and 619,000 deaths associated with the disease (WHO, 2022). The African region contained 95% of the cases and 96% of the deaths, with half of all worldwide deaths occurring in four countries alone: Nigeria, Democratic Republic of the Congo, United Republic of Tanzania, and Niger. In the African region, 80% of the deaths occurred in children under 5 years of age. As one of the major sources of child mortality in Africa, malaria and the *P. falciparum* parasite are necessary targets for medical research.

Plasmodium falciparum

Plasmodium is a genus of parasites responsible for the development of malaria. There are five species of *Plasmodium* that can cause malaria in humans: *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi* (CDC). The most dominant strain in Africa remains *P. falciparum* while *P. vivax* is dominant in countries outside of Africa. Phylogenetic analysis of the *P. falciparum* genome indicates the parasite originated in gorilla species and the transmission to humans occurred relatively recently in evolutionary terms (Liu, 2010). After being bitten by an infected mosquito, *P. falciparum* invades red blood cells in the host's body and multiplies over a span of 48 hours.

Symptoms often appear 7-30 days after being bitten by an infected mosquito, usually due to the infection of red blood cells in the bloodstream by the parasite and resulting spread of infectious parasites. Symptoms can include fever, chills, muscle aches, and fatigue, and severe cases can result in anemia, kidney failure, seizures, coma, and death. There are a range of prophylactic malaria measures as well as treatments for malaria, however there have been instances of resistance to medication, highlighting the importance of continual drug development.

Pathogenesis and Current Therapies for Malaria

Early treatment of malaria involved the use of quinine, an alkaloid drug derived from the bark of cinchona plant. It worked as a fairly effective defensive tool in response to malarial symptoms, however often resulted in suppressed infections rather than full clearance. This led to further spread of the disease providing gametocytes that were infective to mosquitoes (Shanks et al. 2016). The parasite growth in host organisms involves a variety of morphological stages and growth cycles, varying in total population from single cells to upwards of populations of 10^6 (Milner et al. 2018). While only a small number of these morphological stages result in clinical disease that we identify as malaria, each stage provides an opportunity for treatment and potential halting of cellular growth and disease progression.

Currently malaria is treated with a wide range of antimalarial medications intended to kill the parasite and stop the progression of the disease. Initially, artemisinin combination therapy (ACT) acts as a first line of defense against uncomplicated malaria and is 90% effective at reducing *P. falciparum* infection. There are a range of other drugs that can be used and have shown effectiveness. Unfortunately, severe malaria can

develop and result in cerebral infection, anemia, acidosis, and other severe complications including renal, vascular, hepatic, and respiratory complications (Autino et al. 2012). These require a range of specific treatments and continued drug research. Additionally, resistance to ACT treatments and partner drugs poses a threat to the progress made against the disease (Plewes et al. 2019). Continued development of defensive drug interventions is necessary for progress against the disease as well as coordinated offensive measures such as insecticide treated bed nets and periodic indoor insecticide spraying.

Life cycle of *Plasmodium falciparum*

Stages of Life

P. falciparum parasites undergo a complex life cycle with several stages and multiple hosts, each exhibiting a different metabolic and gene expression profile. The three main stages are the sporogonic cycle, taking place in the mosquito host, the exo-erythrocytic cycle, and the erythrocytic cycle, both taking place in the infected human host (CDC). The sporogonic cycle details the process in which the parasite multiplies and produces infectious sporozoites. When an infected mosquito inoculates a human with sporozoites, they travel to the liver and undergo the exo-erythrocytic cycle, ultimately producing thousands of merozoites which are released into the blood stream (CDC). The merozoites invade red blood cells (RBC's) and undergo the intra-erythrocytic development cycle (IDC), asexually reproducing to produce more infectious parasites. A single cycle of the IDC takes place over the course of 48 hours. The life cycle in full is shown in Figure 1 (CDC). The IDC that is referenced is shown as part B of the cycle,

displaying the progression from ring stage to trophozoite to schizont and the eventual rupture of the schizont to spread the parasite to other RBCs.

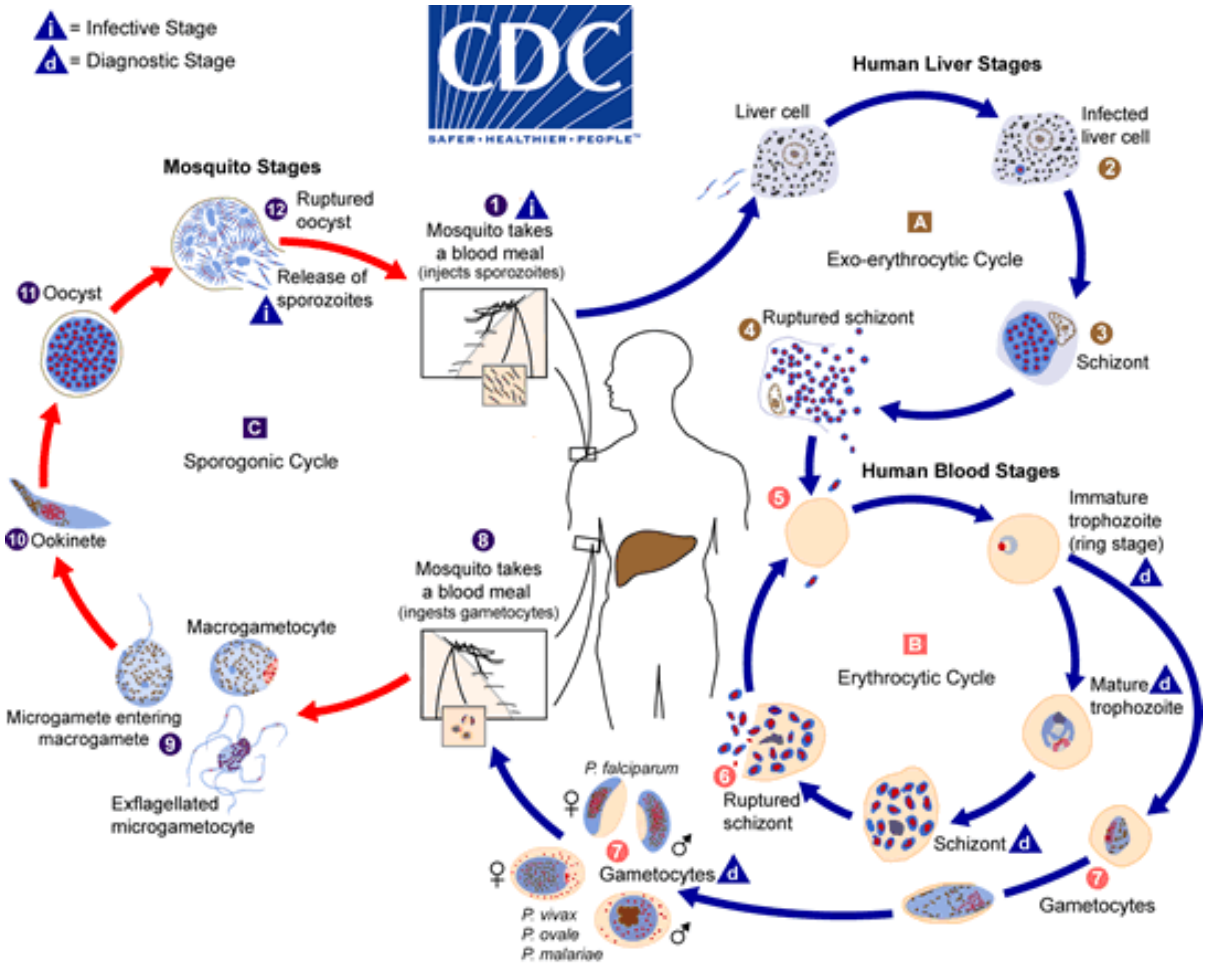

Figure 1. The Life Cycle of Malaria

Malaria life cycle including infection of mosquito host and then development of the parasite in human host. Included is the IDC during which the parasite reproduces asexually and progresses to infect other RBCs. During this stage some parasites switch to sexual reproduction to ensure transmission to their next mosquito host during a blood meal event.

The IDC, and its three stages of parasite development, remains the most enticing target for drug development due to their complex regulation and are the focus of this paper. Upon infecting an RBC, the parasite begins reproducing asexually. This early stage is referred to as the "ring stage" due to its unique morphology and appearance (Tuteja, 2007). During this stage parasite metabolism is highly active, undergoing high rates of glycolysis of imported glucose. *P. falciparum* largely depends on anaerobic respiration, producing up to 100x more lactate than uninfected RBC's (Possemiers et al. 2021). This lactate is excreted into the blood to avoid cytotoxic build up within the infected RBC. The parasite continues growing into the trophozoite stage and then finally the schizont stage, in which it undergoes nuclear division without cytokinesis, producing up to 20 merozoites. The RBC is then lysed and these merozoites are released into the blood to infect further RBC's. Pathogenesis of malarial symptoms are largely associated with the progress of the IDC and symptoms of malaria are a result of parasite progression at this stage. *P. falciparum* has also evolved a wide range of gene expression profiles to evade immune system detection and continue invasion of RBC's. During the course of this process, a small number of merozoites differentiate into male and female gametocytes, converting to sexual stages of growth separate from the asexual growth of the IDC. These gametocytes are necessary and must be absorbed during a mosquito blood meal of an infected human host. Once in an *Anopheles* mosquito, the gametocytes will begin the cycle anew.

Conversion between Sexual & Asexual Stages

The transmission of the malaria parasite requires some of the parasites to switch from asexual reproduction to sexual reproduction in the formation of gametocytes. This

involves a change in the gene expression profile of the parasite. The mechanisms behind commitment to sexual development aren't fully understood, however *in vitro* studies have shown that environmental conditions such as high host parasitemia, levels of metabolites or exposure to drug treatment increase the commitment to sexual reproduction and gametocytogenesis (Stewart, 2022). The genetic mechanisms behind the switch aren't fully understood, although the epigenetically controlled expression of the gene PfAP2-G has been identified as a likely factor responsible for initiation of the transcriptional program for sexual development (Josling et al. 2015). Because of the importance of the conversion between asexual and sexual development cycles in the extension of the parasite's life cycle, epigenetic regulation and control of this change in gene expression remains a key area of research. In order to develop possible therapies, a deeper understanding of these mechanisms is required.

Epigenetics

Overview of Epigenetics

Epigenetics is a field of study investigating factors that lead to changes in gene expression without altering the original DNA sequence. These changes can be influenced by environmental factors such as diet and stress. These epigenetic modifications are reversible and sensitive to environmental factors. It was identified that these modifications persisted through mitosis, suggesting that there were heritable factors affecting gene expression that could be altered by the environment (Deans et al. 2015). The field of epigenetics is expanding and new signals, such as lactylation as discussed in this paper, are being suggested and studied constantly. The most studied mechanisms by

which gene expression is promoted or repressed include DNA methylation, histone modifications, chromatin structure modification, and non-coding RNA (Al Aboud et al 2018). These “switches” can turn gene expression on and off, forming a layer of control over transcription and translation resulting in unique functions of individual cells dependent on environmental signal factors. As we begin to better understand the links between epigenetics and development of phenotypes, scientists increasingly examine the role of epigenetics and disease development. Better understanding the epigenetic profiles of disease with regards to the pathogenesis of disease could allow for more refined development of therapeutic measures.

Of particular interest are post-translational modifications to histone proteins. Figure 2 demonstrates a range of post-translational modifications that can occur on histone proteins (Millán-Zambrano et al. 2022). The most well studied include acetylation and methylation, however the role newly discovered histone modifications are continuously being examined.

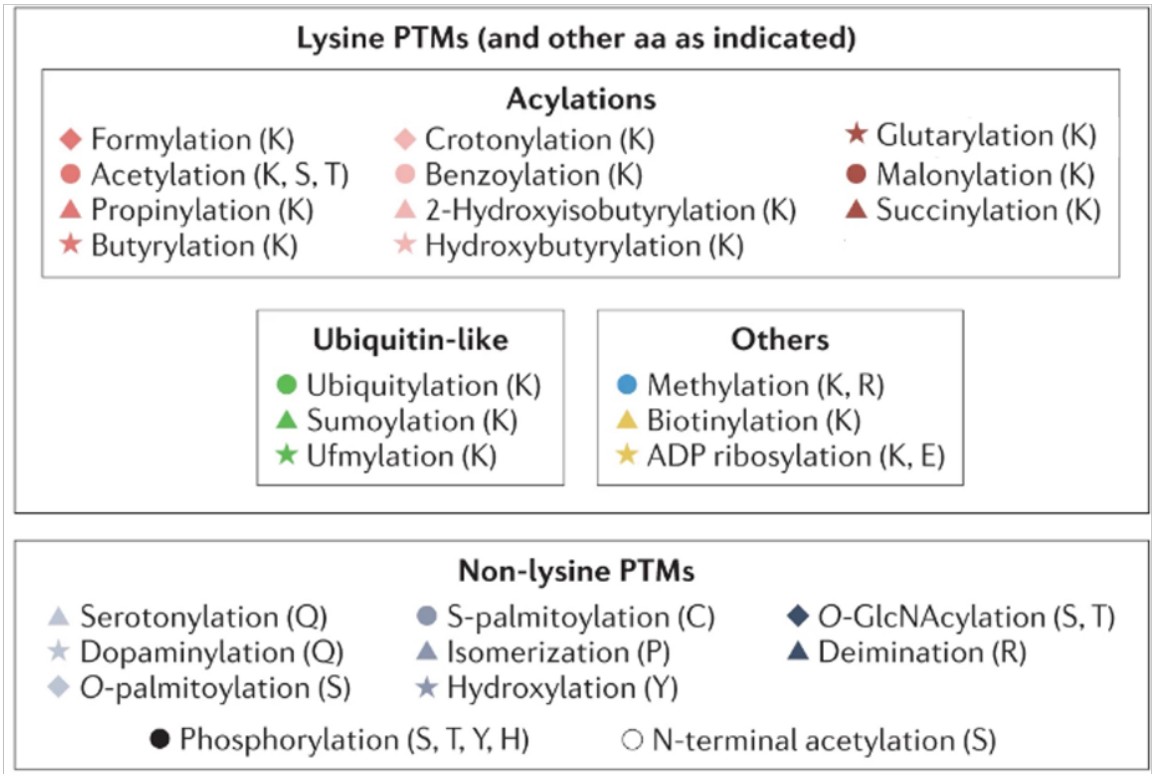

Figure 2. Various Post-Translational Modifications on Lysine Residues in Histones

Lysine residues in histone proteins are modified in a variety of ways. These modifications can impact gene expression by loosening or tightening DNA around the histone protein.

Histone Modification

A histone is a positively charged octamer made up of 2 copies each of histone proteins H2A, H2B, H3, and H4. Negatively charged DNA is packaged around these histone proteins (Shahid et al. 2019). Together they form a nucleosome, one of the basic units of chromatin structure. The aggregation of those nucleosomes form chromatin which make up a chromosome. Chromatin typically exist in two states of structurally formation. As euchromatin, the nucleosomes are separated and the genes are transcriptionally active. As heterochromatin the nucleosomes are condensed and the

genes are transcriptionally repressed and inactive. Modifications to these histone proteins can affect the structure of chromatin, resulting in certain regions of the genome becoming accessible or inaccessible resulting in changes in to gene transcription.

The two most studied histone modifications involve histone acetylation and histone methylation. In acetylation the lysine residues of histone subunits are modified with an acetyl group, changing the charge of the residue and weakening its interaction with DNA and making it available for DNA transcription (Al Aboud et al. 2018). Histone acetylation is typically associated with regions of euchromatin and transcriptional activation while histone methylation is more complex and must be investigated more closely to determine its effect on transcriptional regulation. Methylation can affect both lysine and arginine residues and can include the addition of anywhere from 1-3 methyl groups. Typically, trimethylation of lysine 4 on histone 3 (H3K4me) results in gene activation while trimethylation of lysine 27 on histone 3 (H3K27me3) results in gene repression (Karlic et al. 2010). When investigating these epigenetic markers, it is important to examine the transcriptional activity of the associated genes as well as quantifying protein production of the respective proteins in order to better understand the effect that the epigenetic mark has on gene activity and protein levels.

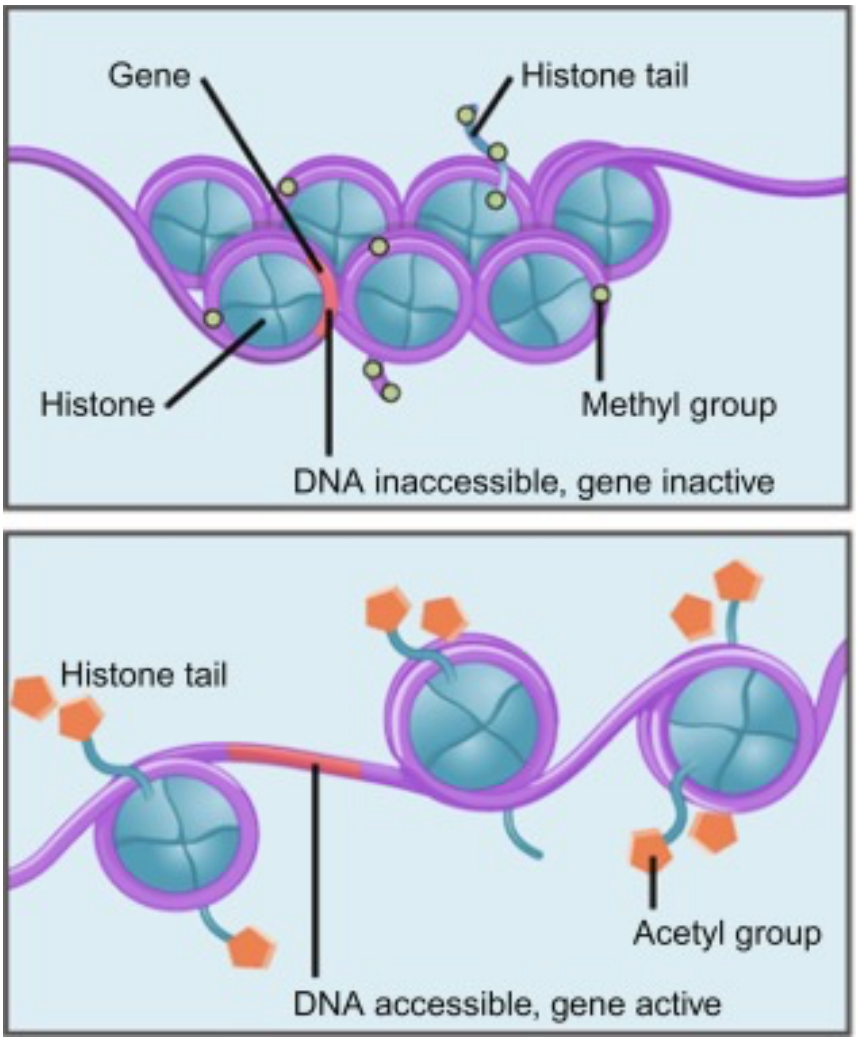

Figure 3. Histone Methylation and Acetylation

Two most commonly studied epigenetic signals include histone acetylation and histone methylation. Methylation is often associated with gene repression and results in tightly packed, inaccessible DNA. This results in gene inactivation. Histone acetylation is often associated with accessible DNA and active gene expression. This chromosome formation is referred to as euchromatin.

Readers, Writers, & Erasers

One of the goals of understanding epigenetics is to identify the proteins involved in altering these modifications. The enzymes are broadly categorized into three groups: readers, writers, and erasers. Readers are protein domains that can recognize epigenetic marks, usually as part of a larger protein complex that will result in enzymatic activity at the site of an epigenetic mark. For example, proteins with bromodomains read histone acetylation markers. Even within the category of bromodomains, there are various functionalities, with specific bromodomains reading specific acetylation marks. Writers are enzymes that add chemical modifications to DNA or histone residues. They can introduce fresh modifications or replicate modifications over the course of mitosis, contributing to the heritability of epigenetic markers. Erasers are enzymes that can remove modifications depending on the requirement of the cell. These enzymes catalyze the erasure of epigenetic marks, reversing the effects of the writers. Together, these various enzymes and reader domains are responsible for transcriptional activation and repression via changes to chromosome formation as one example of this mechanism. Because of these enzymes and their functionality, epigenetic markers allow for broad and dynamic regulation of DNA expression.

Epigenetics and Disease Prevention

Epigenetic epidemiology is a burgeoning field intended to identify and associate epigenetic expression profiles with disease progression or outcome. Cancers have often been associated with changes to genome-wide changes to methylation profiles, activating

cancerous genes and inactivating oncogenes (Feinberg et al. 2018). Better understanding the patterns of epigenetic changes and their associated outcomes with certain cancer types and different cell lines can act as a predictive tool and inform a team regarding how to treat a cancer. Epigenetics can be used similarly in malarial infections. Epigenetic signals can act as diagnostic tools to understand the severity of the infection as well as targets possible mechanisms by which the malaria might grow, reproduce, or avoid detection.

The growth of the malaria parasite is a complex process with a variety of features that affect its development and pathogenesis, both environmental and genetic. Because of this, it can be difficult to identify relevant biomarkers for protection. Outcomes of malaria patients could depend on parasite virulence, infection burden, host immunity and susceptibility to infection, nutrition and gut microbiome, prior experience with antimalarials, or coinfections (Arama et al. 2018). There is nascent research into the effect of the malaria parasite on the hosts epigenome. An example is the hypomethylation and increased expression of ABCB1, a gene responsible for the production of a multidrug resistance transporter protein. By better understanding the changes in parasite gene expression as well as the potential effects on the epigenome of host RBCs, potential pathways for therapeutic or diagnostic tools can be developed.

Epigenetics in Plasmodium falciparum

Var Gene Expression

Var genes are a highly diverse family of genes in part responsible for coding the expression of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). The function of PfEMP1 is to mediate the adherence of infected erythrocytes to vascular tissue,

avoiding destruction by the spleen (Jensen et al. 2020). There are up to 60 *var* genes encoding this protein. At any given time only one variant of PfEMP1 is expressed, however the expressed variant can be changed from one 48 hour IDC growth cycle to another. The ability of the parasite to switch expression between various PfEMP1 variants is a key to the pathogenicity of *Plasmodium* and is a characteristic of the chronicity of untreated parasite infections. Past research has associated histone modifications with variable *var* gene expression. Two marks in particular, H3K9me3 and H3K36me3, have been shown to be devoted to the gene families responsible for *var* gene control (Deistch et al. 2017).

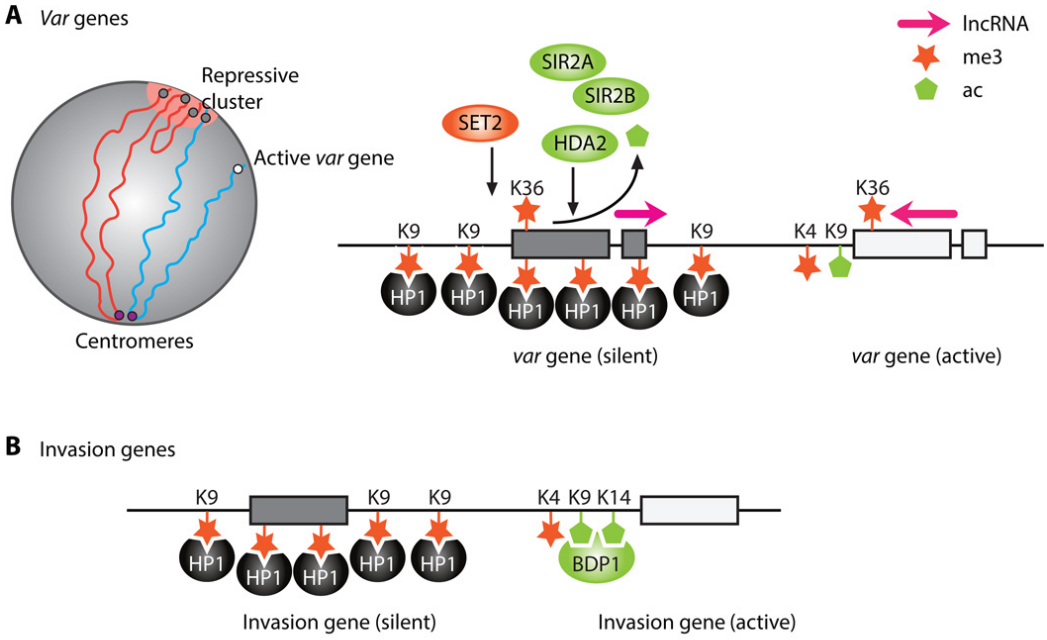

Figure 4. Epigenetic regulation of *Var* genes and invasion genes in *Plasmodium falciparum* (Batugedara et al. 2017)

(A) Var gene regulation is controlled by clustering of H3K9me3 repressive marks responsible for recruiting PfHP1. This results in the formation of heterochromatin and silences the var gene. (B) Some families of invasion genes are regulated via epigenetic mechanisms. In a similar fashion to var genes, H3K9me3 recruits PfHP1 heterochromatin marker.

Experiments showed that modifying expression of writers and erasers for these marks resulted in subsequent disruption of *var* gene expression, associating these marks with stable regulation of these genes (Perez-Toledo et al. 2009). The enzymes in question were histone methyl transferase PfSET2 and histone deacetylase PfHda2. Another experiment studied the effect of knocking out orthologues of the histone deacetylase SIR2. In *Plasmodium* these orthologues are PfSir2a and PfSir2b. When knocked out they appeared to play role in regulating epigenetic signaling on histones. The study of *var* genes offers important insight into the significance of epigenetic control on *Plasmodium* gene regulation.

Epigenetic Control of Sexual Conversion

While commitment to gametocytogenesis does appear to be largely stochastic, there have been *in vitro* studies examining factors that can result in increased rates of conversion. The gene pfg14.748 has been identified as a potential marker for early gametocytes and proteomic analysis identified the proteins encoded by specific genes were found in gametocytes but not in asexual parasites (Josling et al. 2015). Additionally, the transcription factor AP2-G was identified as a regulator of gametocytogenesis (Josling et al. 2015). Proteins in this family are important in developmental stage transitions, potentially acting as a mechanism behind switching between stages of growth. The production of *pfap2-g*, one of the protein products of AP2-G, has been

shown to be regulated epigenetically. Expression of the protein product varied between clonal lines consistent with patterns associated with epigenetic regulation. The gene locus is enriched by *P. falciparum* heterochromatin protein 1 (PfHP1), a highly conserved eukaryotic protein associated with gene silencing (Josling et al. 2015). PfHP1 binds to H3K9me3 and the depletion of the protein will thus lead to an upregulation in expression of *pfap2-g* and a subsequent increase in gametocytogenesis.

Another epigenetic control factor showing signs of involvement is *P. falciparum* histone deacetylase 2 (PfHda2), responsible for erasing acetylation signals from histones. Similar to PfHP1, a decrease in PfHda2 expression results in a subsequent derepression of *pfap2-g* and an increase in conversion of schizonts to gametocytes (Coleman et al. 2014). It's believed that PfHda2 acts upstream of PfHP1 and that it is possible that histone deacetylation is required prior to the functioning of PfHP1. Once the histones are deacetylated and methylated, PfHP1 can bind to H3K9me3 and result in the formation of silenced heterochromatin and the inactivation of *pfap2-g*.

Epigenetic Activity of *Plasmodium falciparum* Sir2

Sirtuin proteins are class III deacetylases widely conserved across life serving an important role in epigenetic control as they remove acetylation signals for histone code. Acetylation signals typically mark euchromatin structures and gene activation, so sirtuin activity is commonly associated with formation of heterochromatin and the silencing of genes. PfSir2 has also been shown to associate with silenced *var* gene expression, implicating the protein with epigenetic control of gene expression (Tonkin et al. 2009). This establishes the factor as a player in gene regulation of *P. falciparum*. PfSir2 has been shown to demonstrate histone deacetylation (HDAC) capability as well as ADP-

ribosyltransferase activity (Merrick et al. 2007), establishing PfSir2 as an important HDAC and regulator of acetylation activity. In vitro analysis using human cell lines indicate that activity of histone deacetylase proteins decrease overall histone acetylation as expected, but also contribute to reduce the levels of histone lactylation (Moreno-Yruela et al. 2022). This finding in human cell lines suggests that PfSir2 could perform a similar function in *P. falciparum*, potentially acting as an eraser of histone lactylation, eventually regulating gene expression.

Metabolic Lactate & Histone Lactylation

Metabolic Lactate & *Plasmodium* Infection

Severe malarial infections are often associated with disruptions to the metabolic environment of the host body. As the body fights a severe malarial infection, it can often result in anemia, hypoglycemia, and hyperlactatemia resulting in lactic acidosis (Possemiers et al. 2021). This severe acidosis disrupts cellular metabolism and while all the effects of this are not fully elucidated, it is often associated with poor outcomes. Infected erythrocytes produce increased levels of lactate and release it into the environment, increasing surrounding levels of lactate. *Plasmodium* derives its energy primarily from the anaerobic glycolysis pathway, in the process converting glucose into lactate. This is likely one of the causes of the increase in lactate levels associated with severe malaria. Other causes of increased lactate production include increased immune response resulting in the destruction of infected and uninfected RBCs. This can result in anemia and decreased oxygen delivery. In addition, parasite sequestration can block blood vessels and result in hypoxia as displayed in Figure 5. These results in increased

levels of anaerobic glycolysis. One examination of sirtuin levels, a protein that often responds to nutritional and metabolic status, indicated that high levels of lactate were associated with high levels of *PfSir2a* expression (Merrick et al. 2012). It's clear that lactate byproduct as the result of malaria growth can affect gene expression and protein expression. One possible mechanism of this expression is direct lactylation of histone proteins themselves, modifying gene expression at the transcriptional level.

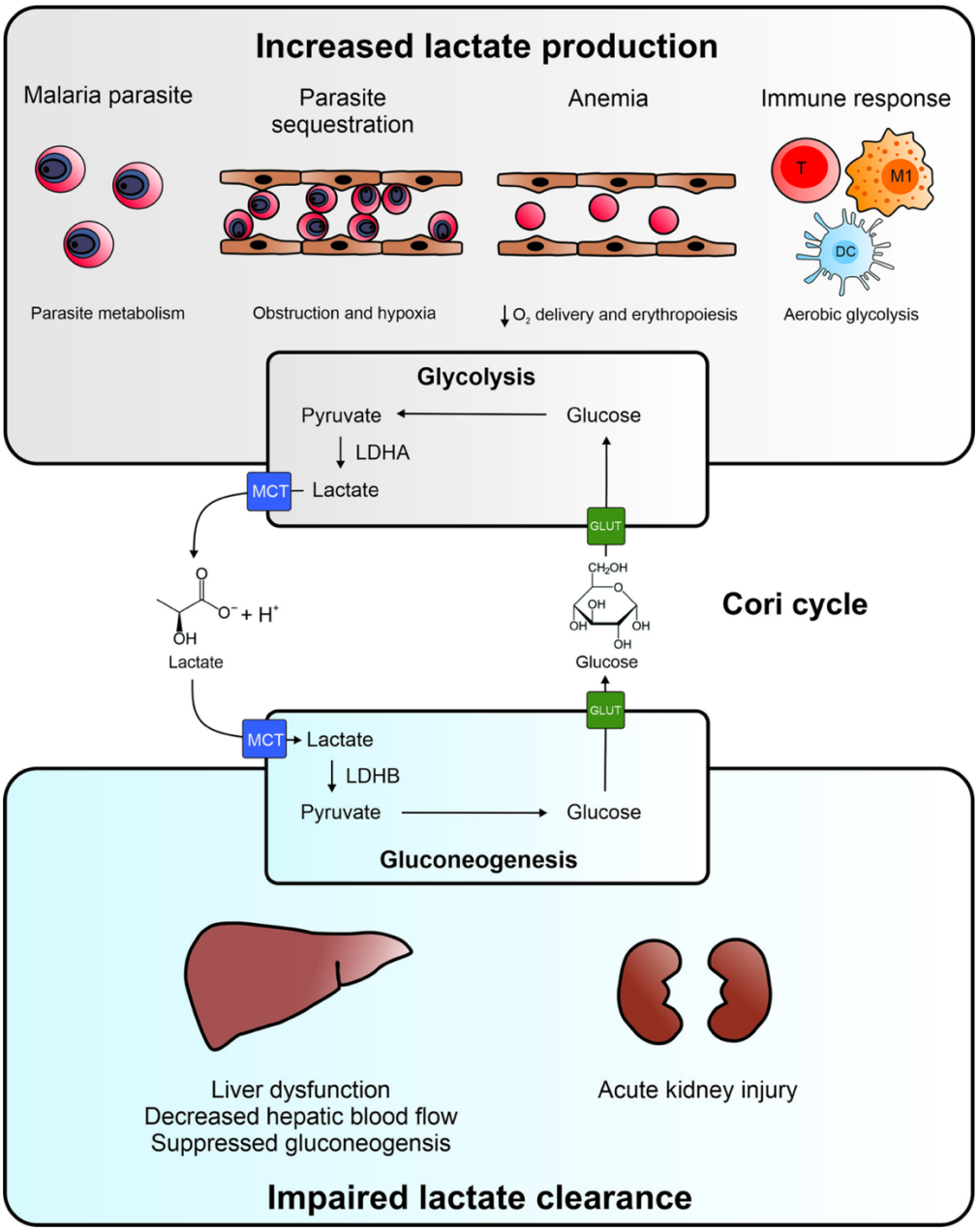

Figure 5. Hyperlactatemia in Malaria Parasite

Some of the causes of increased lactate production as a result of malarial infection. Systemic hypoxia and increased levels of anaerobic glycolysis result in hyperlactatemia. Additionally, parasite infection can affect bodily organs responsible for lactate clearance, resulting in continuation of the high levels of lactate.

Histone Lactylation

Lactylation is a recently proposed histone modification that modifies lysine residues of proteins including histones. It has been demonstrated that both exogenous and endogenous levels of lactate contribute to lactylation. An investigation of histone lactylation showed that levels of histone lactylation (Kla) were impacted by extracellular lactate levels (Zhang et al. 2019). When investigators inhibited lactate production, they observed a corresponding decrease in Kla. A corresponding effect was shown when lactate production was increased. Hypoxic conditions result in increased levels of lactate as glycolysis is used to produce cellular energy. Lactate has often been thought of as a waste product of this process, however, these discoveries suggest it plays a role in gene regulation in response to hypoxic conditions. In order to identify whether Kla had an effect on gene regulation, investigators studied levels of Kla during M1 macrophage polarization. They found that histone lactylation increased in a pattern that was closely correlated with timing (Zhang et al. 2019). Over the course of M1 macrophage polarization, the production of lactate was found to be necessary for histone lactylation dynamics that result in the gene expression promoting an M2-like phenotype, required for maintaining homeostasis.

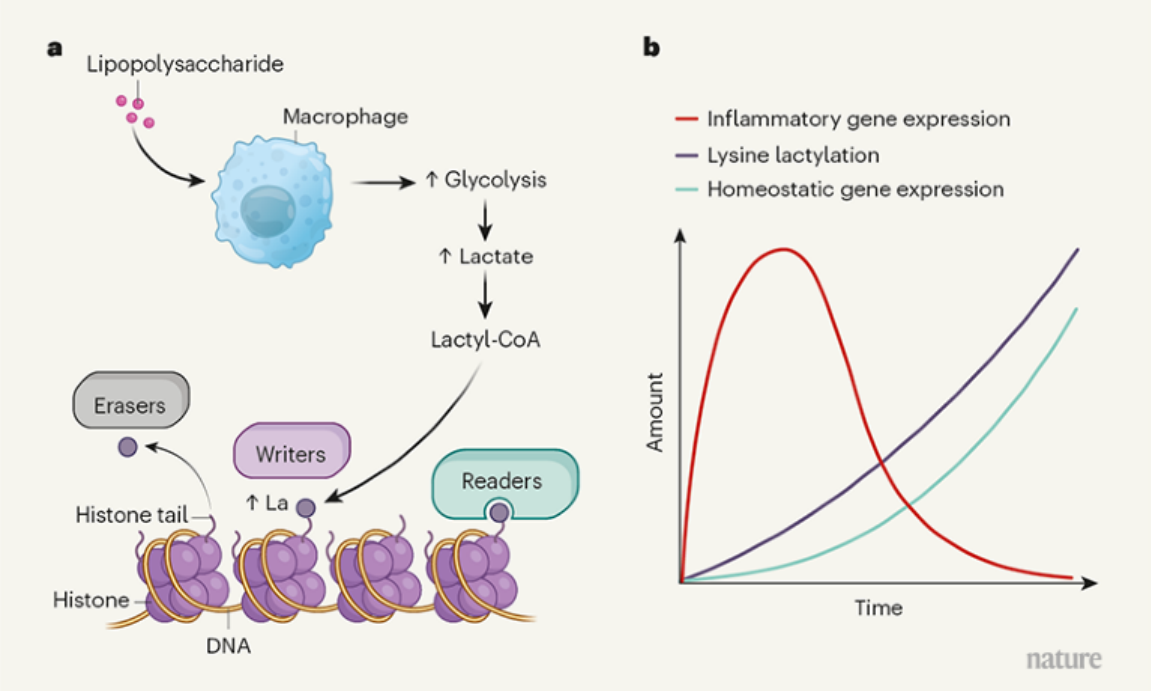

Figure 6. Metabolic Regulation via Histone Lactylation Epigenetics (Izzo et al. 2019)

The mechanism proposed by Zhang et al. suggests that lactate, as a byproduct of glycolysis and in the form of lactyl-CoA, binds to histone proteins and lactylates them. In Zhang's study an increase in lysine lactylation corresponded to a decrease in inflammatory gene expression. Exact effects of histone lactylation are still unknown as well as potential readers, writers, and erasers.

Histone Lactylation in Various Organisms

Since histone lactylation was discovered as a potential epigenetic marker, there have been various organisms in which it has been detected, including several parasites. *Toxoplasma gondii* is a causative agent of toxoplasmosis, infecting the nucleated cells of animals and humans and causing potentially lethal infections, especially in immunocompromised patients. Proteome wide analysis of lysine lactylation resulted in the identification of a range of targets in *T. gondii*, including histone proteins and regulators of metabolism (Zhao et al. 2022). Patterns of histone lactylation showed

patterns of conservation across species between parasite and human however there were also species specific modifications to *T. gondii*. The findings of this study suggests that lysine lactylation acts as regulator of metabolism through direct regulation of metabolic pathways as well as likely impacting gene expression. Another model organism of interest is the protozoan parasite *Trypanosoma brucei*. This parasite causes African trypanosomiasis, often referred to as sleeping sickness. The disease has a high mortality rate and therapeutic efforts have been inadequate in curing or preventing illness. Similar to *T. gondii*, lysine lactylation wad demonstrated across a range of proteins including histones, gene regulators, and metabolic enzymes (Zhang et al. 2021). Several histone sites demonstrated histone lactylation on all of the different subunits. Some of the histone lactylation occurrences demonstrated this conserved nature across humans and mice while *T. brucei* also demonstrated species specific histone lactylation modifications. Zhang also demonstrated that lactylation occurred on histone deacetylases in *T. brucei*, opening the possibility to crosstalk between modifiers of other epigenetic modifications and lactylation signals. Modifiers of post-translational modifications in *P. falciparum* could also be responsible for histone lactylation signal modification.

Lactic acid buildup occurs due to a lack of oxygen and is often a major factor in severe malaria. During the IDC, *Plasmodium* relies heavily on glycolysis to generate energy, producing lactate in the process. Destruction of erythrocytes can disrupt oxygen levels in the blood, further contributing to a hypoxic environment and increasing the levels of anaerobic glycolysis being performed, thus contributing to an increase in lactate. Studies have shown that past a certain lactate threshold, *Plasmodium* switch from the asexual growth of the IDC to a transmissible gametocyte sexual growth cycle. This

switch from asexual to sexual reproduction is regulated by epigenetic mechanisms. However, the mechanism behind the crosstalk of lactate levels and gene expression in *P. falciparum* is still unclear. This study aims to clarify our understanding of histone lactylation dynamics in *P. falciparum* in a range of environmental lactate levels as well as under a variety of parasitemia. Doing so will help direct future research on the mechanisms of histone lactylation and the pathways it helps to regulate.

Hypothesis & Research Aims

Exposure to increased exogenous lactate levels will result in increased histone lactylation in *Plasmodium falciparum* across all asexual stages of the intra-erythrocytic life cycle. Investigation of the temporal dynamics of histone lactylation will demonstrate a responsiveness to changes in histone lactylation that will persist across generations. Histone lactylation will be responsive to modifying known writers and erasers of other histone modifications, demonstrating its function as a potential epigenetic mark for the modification of gene expression.

Specific Aim #1

Hypothesis – Histone lactylation exists in *Plasmodium falciparum* and the histone modification is responsive to changes in exogenous levels of lactate

The existence of histone lactylation has been demonstrated in a variety of organisms at this point, proving its existence as a histone modification. This study aims to be one of the first to demonstrate its existence in the *Plasmodium* parasite, a parasite closely associated with changes to lactate levels in host organisms. The identification of this histone modification in *Plasmodium* introduces a key player in the developing story

of *Plasmodium* variable gene expression. Epigenetic modifications have demonstrated an important role in *Plasmodium's* avoidance of the host immune system as well as changes to the life cycle of the parasite. The proof histone lactylation would be crucial when considering the ability of the parasite to impact lactate levels in the host.

A key factor in this process is proving that lactylation of parasite histones is responsive to environmental lactate. This would suggest that environmental lactate levels have an impact on parasite gene expression via the mechanism of histone lactylation as an epigenetic marker. Culturing parasite in the presence of increase levels of exogenous lactate using ring, trophozoite, and schizont stage parasites will determine responsiveness of parasite to exogenous lactate. Additional insight could be provided by examining various histone lactylation at the different stages of asexual growth to identify key stages of growth that histone lactylation might more directly impact.

Specific Aim #2

Hypothesis – Histone lactylation will respond to changes in expression of suspected erasers of epigenetic markers including *Plasmodium falciparum* sirtuin proteins

Sirtuin proteins are a class of proteins which have demonstrated deacetylase activity across a range of organisms including *Plasmodium falciparum*. The proteins have also demonstrated the ability to remove histone lactylation markers in other organisms. Because of the shared activity across species of the ability to remove acetylation markers, we believe PfSir2 to be a good candidate for investigating its ability to remove lactate markers. We will observe the effects on histone lactylation of knocking out PfSir2 relative to wild type functionality, allowing us to make baseline assumptions of the histone delactylation capability.

Chapter II

Methods

Parasite Culturing

P. falciparum 3D7 (Walter and Eliza Hall Institute) parasites were cultured in human erythrocytes obtained from a commercial source. The parasitic lines lacking PfSir2A and PfSir2B genes were generated in the previous studies and were used in this study to compare histone lactylation status (PMID- 17827348, 19402747). Continuous culture was maintained at 2% hematocrit in RPMI-1640 media (Sigma Aldrich, Cat. No. R6504) supplemented with HEPES, 25 mM; Albumax II, 4.31 mg per ml (Thermo Fisher Scientific); sodium bicarbonate, 2.42 mM; and gentamycin (20 µg per ml). The cultures were incubated at 37 °C in hypoxic conditions (1–5% O2, 5% CO2, with N2 as balance) in modular incubator chambers. The asexual stages of the parasite were synchronized by magnetic affinity purification of schizonts (MACs LS column, Miltenyi, fitted with a 23-gauge needle) as previously described (PMID-32669539).

To vary the lactate concentration, a solution of Sodium L-Lactate salt (Sigma Aldrich, Cat. No. L7022) was exogenously added to the culture. To release the parasites from erythrocytes, 0.15% saponin solution was used. In brief, infected erythrocytes were suspended in 5 ml of 0.15% saponin in PBS and incubated on ice for 10 mins. Additional washes with ice-cold PBS without saponin was given to wash out erythrocytic proteins and parasite pellets were obtained after centrifugation at 12,000 rpm for 2 mins. This process is repeated a minimum of two times and up to three times, until the supernatant is clear indicating that all RBC product has been discarded.

The pellet that remains is then suspended in 10X volume of 1X SDS sample buffer (ThermoScientific, Cat. number J61337.AC) to lyse the parasites. Approximately, 7 μL β-mercaptoethanol was added to 1mL of SDS sample buffer to denature proteins. The parasite pellets were suspended well and then boiled at 90°C for up to 10 minutes to encourage solubilization of the pellet in the SDS sample buffer. The samples were then kept at room temperature, resuspended using pipette and then centrifuged at 13,500 RPM for 10 minutes. The supernatant containing proteins was then transferred to fresh labelled Eppendorf tubes and either used immediately for western blots or stored at -20°C.

Western Blot Protocol

Samples were loaded into SDS-PAGE Tris/Tricine gels (Bio-Rad, Cat. No. 4563066). Tricine buffer has a lower negative charge than glycine, which allows samples to migrate faster (Chevalier, 2011). Its high ionic strength allows more ion movement as well as less protein movement, offering high resolution separation of low molecular weight proteins such as histones. As the molecular weight reference, Precision Plus Protein Dual Color Standards (Bio-Rad, Cat. No. 1610374) were loaded in one of the wells and run along the samples1X Tris/Tricine running buffer (Bio-Rad, Cat. No. 1610744) was used to run the gels. The gel was run at 80 volts for 20 minutes and then increased to 120 volts for the next 45-60 minutes. Proteins from the gels were then transferred to nitrocellulose paper the Bio-Rad Trans-Blot Turbo Transfer System. After successful transfer of the proteins, nitrocellulose blots were incubated in Li-Cor blocking buffer (LI-COR Cat. No. 927-70001) for one hour at room temperature to block the background. After blocking, the blots were incubated with the primary antibody i.e.

Rabbit anti-lactyllysine monoclonal antibody purchased from PTMBio, category #PTM-1401. The antibody was suspended in Li-Cor blocking buffer solution at a concentration of 500ng/mL. The antibody detects and specifically binds to lactylated lysines. As a loading control, Mouse anti-H4 monoclonal antibody (Abcam, Cat. No. ab17036) was used. The blots were incubated for 1-3 hours at room temperature or overnight at 4°C. After primary antibody incubation, blots were washed three times with PBST (0.05% Tween in PBS). The secondary antibodies used were Li-Cor Goat anti-Rabbit IRDye 800CW IgG and Goat anti-Mouse IRDye 680CW IgG secondary antibodies suspended in Li-Cor blocking buffer at 1:10,000 dilution. The nitrocellulose blot was then incubated with the secondary antibody for ~1 hour at room temperature in light protected boxes. At last, blots were washed three times with PBS/Tween and then immediately scanned using an imager.

Gel Scanning

Scanning was performed using a Li-Cor Odyssey CLx Imager, which allows for consistent and accurate digital scans of western blots and rapid analysis of the western blot imaging. Scans were performed at wavelengths of 800nm and 700nm and both simultaneously at a range of intensities dependent on the signal from the western blot. The 700nm wavelength was associated with imaging with the Goat anti-Mouse secondary antibody which was used to image H4 quantities. The 800nm wavelength was used to image the Goat anti-Rabbit secondary antibody which was most frequently associated with lactylation signals. Histone H4 levels were used as loading controls and to normalize lactylation intensities across samples. This acted as the experimental signal and was used to collect results.

Data Analysis

Data analysis was performed using Li-Cor Image Studio software. This software is compatible with the Li-Cor Odyssey CLx Imager as well as older Li-Cor imaging systems such as Fc Imaging Systems, Pearl Imaging System, and C-DiGit Blot Scanner. This software is used for the quantification of Western blot images. The software was used to calculate the relative abundance of signal at wavelengths 700nm and 800nm, corresponding to Histone H4 and lactylation levels. The normalized intensities for all the samples were then plotted as graph using Microsoft Excel or GraphPad Prism.

Chapter III

Results

Existence of Histone Lactylation in *P. falciparum*

Histone Lactylation Occurs in *Plasmodium* Parasite

The first step in our research was to confirm the existence of histone lactylation in *Plasmodium* as a possible epigenetic marker. Histone lactylation has been researched as a novel epigenetic marker and, other than Zhang's discovery in macrophages, has been shown in a variety of organisms. These include rice (Meng et al. 2021) and *Escherichia coli* (Dong et al. 2022), Toxoplasma (Zhao et al. 2022) and Trypanosoma (Zhang et al. 2021). To investigate if histone lactylation is conserved in P. falciparum, we cultured the parasite in human RBCs for several life cycles and performed a western blot analysis using anti-lactyllysine antibody. Experimental procedure for the western blot is outlined in Figure 7. In brief, parasites were harvested and lysed to extract proteins, which were then resolved using Tris tricine gels and probed for lactylation on lysines.

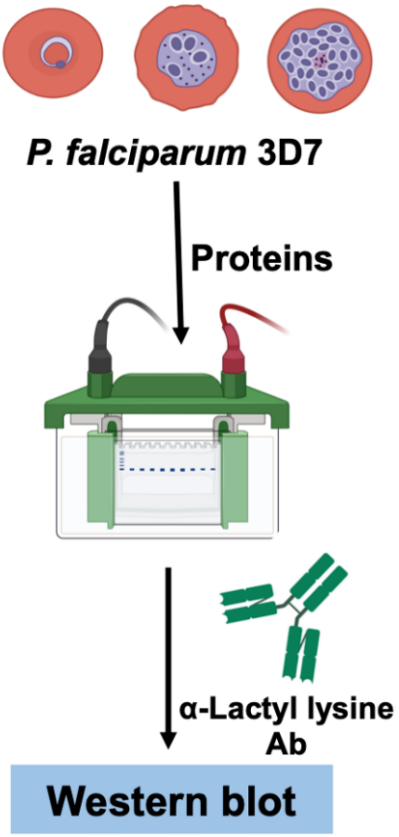

Figure 7. Experimental outline for western blot analysis on P. falciparum proteins using anti-lactyllysine antibody.

Western blot revealed lactylation signals on low molecular weight proteins in the lane loaded with P. falciparum proteins, similar to the molecular weight of histones (Fig. 8). The signals were absent on the host erythrocyte proteins, revealing that lactylation signals were restricted to P. falciparum proteins. In contrast to the lactylome reported in other organisms such as human cells, Toxoplasma and Trypanosoma parasites, lactylation

in *P. falciparum* seems to be enriched or restricted to histones. One investigation into protein lactylation in *Toxoplasma gondii* identified over 900 proteins with lactylated lysine residues in multiple subcellular compartments of the parasite (Yin et al, 2022). The western blot showed lactylated lysine residues on proteins as small as 10 kDa and as large as 250 kDa. In comparison, investigation in *Plasmodium falciparum* identified fewer clearly lactylated proteins (Figure 8). As shown in the figure, a distinct band at ~15 kDa showed a protein that is highly lactylated. As a loading control, anti-H4 antibody was used. The superimposition of lactylation and histone H4 signals suggest that the highly lactylated protein could be histone H4. This suggests that while lactylation may play a variety of roles in other organisms through multiple lactylated proteins, in *P. falciparum* it is enriched on histone proteins.

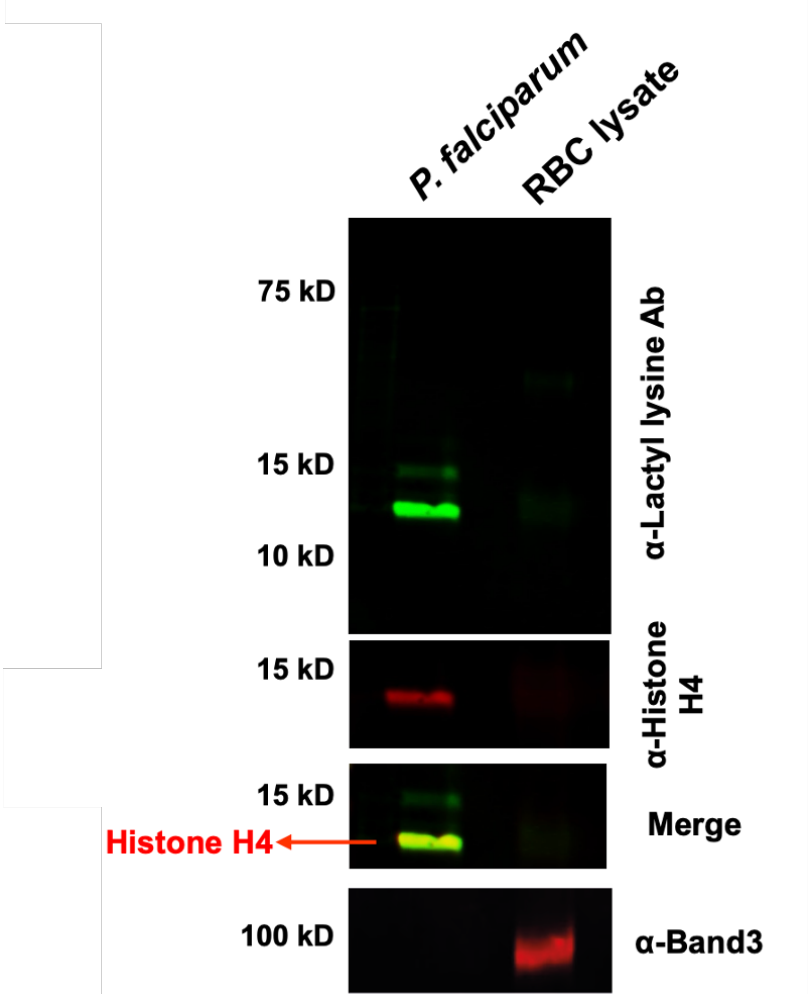

Figure 8. Western blot analysis on *P. falciparum* and host erythrocyte proteins using anti-lactyllysine and anti-Histone H4 antibodies.

The blot revealed presence of lactylated proteins in P. falciparum with Histone H4 as the most abundant lactylated protein.

Histone Lactylation at Various Stages of the Intra-Erythrocytic Development Cycle

Next, we wanted to investigate histone lactylation throughout the stages of the Intra-Erythrocytic Development Cycle (IDC). These include the ring stage, trophozoite stage, and schizont stage. In order to investigate this, infected RBCs were cultured in RPMI media in absence of exogenous lactate and parasites were synchronized as explained later in the method section. Parasites at ring, trophozoite and schizont stages were then isolated and proteins were extracted for western blots in three biological replicates. As shown in Figure 9, the results show histone lactylation to be present in all three asexual stages with slight enrichment during the trophozoite stage. This is interesting as trophozoites are known as the most metabolically active stage of the IDC, producing more lactate, likely resulting in an increase in histone lactylation. Further studies will be needed to investigate the impact of different levels of histone lactylation and its impact on gene expression and parasite biology.

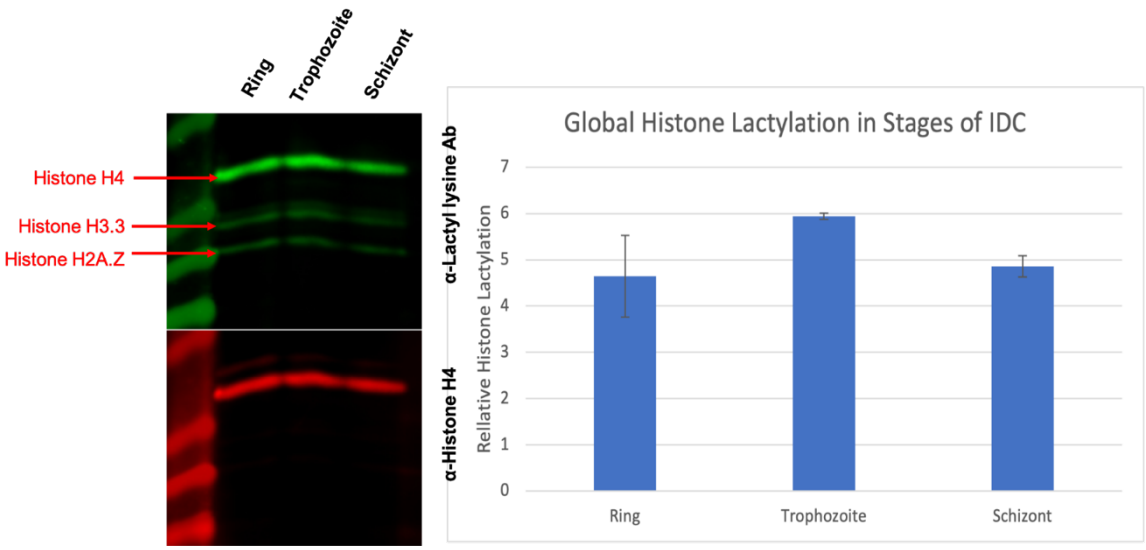

Figure 9. Relative Histone Lactylation at Various Stages of IDC

Analysis of histone lactylation across three stages of IDC in P. falciparum showed presence of lactylation in all three stages tested in the study with marginal increase in the trophozoite stage. Histone lactylation intensity for each stage was normalized using the histone H4 intensity.

Responsiveness of Histone Lactylation to Changes in Exogenous Lactate

Histone lactylation levels are responsiveness to changes in the lactate levels

Once we identified the presence of histone lactylation in *Plasmodium falciparum,* our next goal was to investigate the response of histone lactylation to exogenous lactate levels. It's known that severe malaria results in hyperlactatemia in the infected host. Additionally, hyperlactatemia is associated with high mortality rates in cases of severe malaria. A study by Castaño-Cerezo demonstrated that increased levels of exogenous acetate impacted histone acetylation patterns. This change resulted in a change in gene expression patterns, establishing the connection between environmental factors and

changes to gene expression in parasites. We were focused on uncovering whether histone lactylation behaves in a similar fashion. In order to achieve this *P. falciparum* cultures were incubated in varying levels of exogenous lactate. These included an untreated control sample with an exogenous lactate level of 0.5 mM and experimental cultures incubated at 2mM, 4mM, 8mM, 16mM, and 24 mM of lactate (Figure 10). Normal blood lactate concentrations in healthy individuals can range from 0.5 to 1.5mM, while lactic acidosis is defined as a blood lactate concentration higher than 5mM and can reach as high as 11.2 mM in severe malaria (Wilairatan et al. 2021).

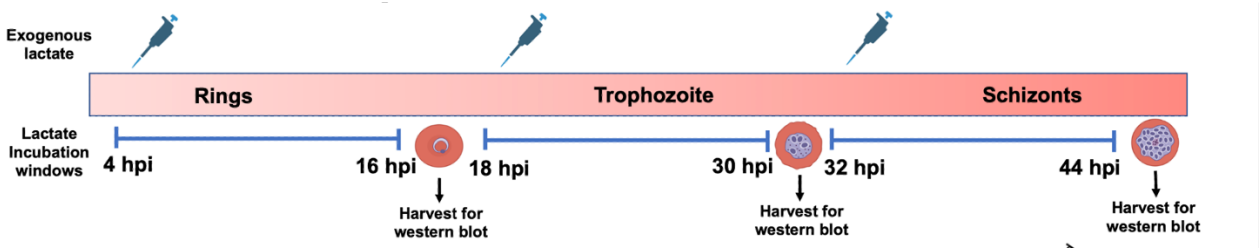

Figure 10. Experimental Setup for Incubation with Exogenous Lactate

Samples were cultured with varying levels of exogenous lactate. Harvest for western blot occurred at three times to test lactylation levels at various stages of asexual growth.

Impact of Exogenous Lactate on Histone Lactylation in Ring Stage Parasites

Analysis of ring stage transcriptome suggests that during this stage biological processes are focused on antigenic variation and virulence. During the early ring a primary virulence factor, stage ring-infected surface antigen 1 (RESA1), is heavily expressed (Bozdech et al. 2003). Members of var gene family, which contributes to antigenic variance are expressed in this stage. The ring stage parasites were cultured in lactate

levels ranging from 0.5 mM to 24 mM for a span of 12 hours and then harvested for western blot analysis. As per in line with our hypothesis, the ring stage parasites showed sensitivity to levels of environmental lactate. As shown in Figure 11, lactylation levels were induced by 1.4-fold and 1.7-fold upon treating with 8mM and 16mM of lactate, respectively. If histone lactylation regulates gene expression in *P. falciparum*, similar to human cells, there is a possibility that genes expressed in ring stage parasites such as var genes, could be regulated in response to varying lactate levels.

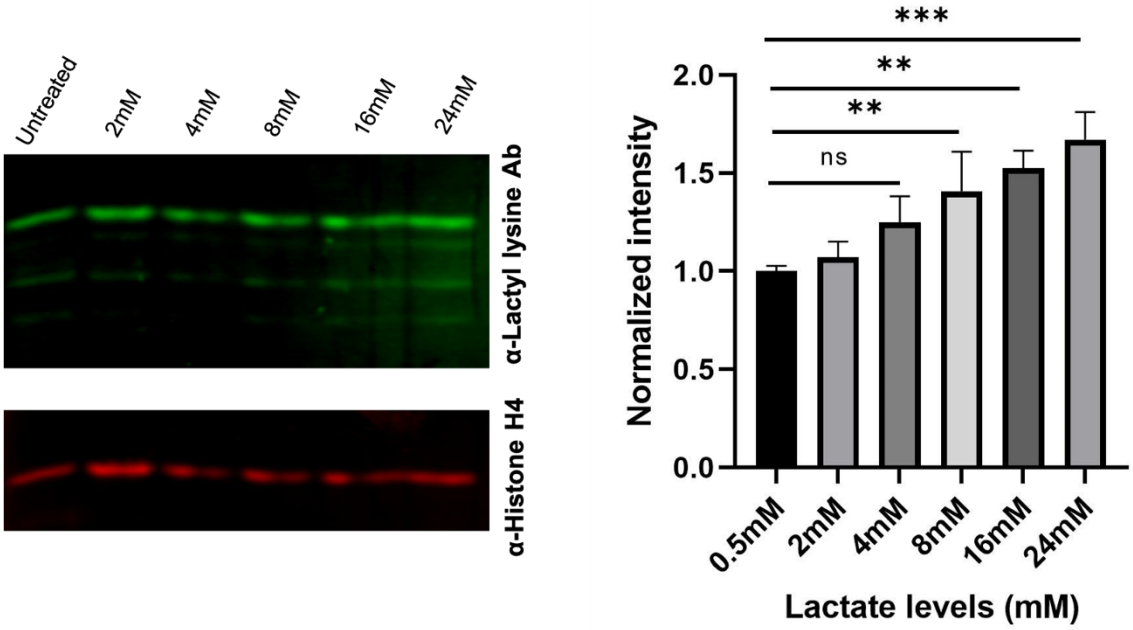

Figure 11. Relative Histone Lactylation of Ring Stage Parasite in Varying Levels of Environmental Lactate

As environmental lactate was increased both strains of P. falciparum displayed corresponding increases in levels of histone lactylation. Western blot images demonstrate this correlated increase in histone lactylation with increases in environmental lactate

Impact of Exogenous Lactate on Histone Lactylation in Trophozoite Stage Parasites

The trophozoite stage of the IDC sees the highest production of ATP as well as consumption of energy sources (Fang et al. 2014). Biomass production is at relatively high levels and is rapidly consuming the cytoplasm of its host red blood cells as it prepares to produce more parasite and continue infected RBCs. As a byproduct of this metabolic activity the trophozoite stage produces and secretes the highest amount of lactate into the surroundings. This high amount of lactate production can contribute to development of lactic acidosis. We harvested trophozoites at 30-32hpi and prepared the samples for western blot analysis using three separate biological replicates of the *Plasmodium falciparum* strain. Unfortunately, western blots for BR1 were unable to be used for analysis but global histone lactylation data for BR2 and BR3 was gathered. Both showed corresponding increases in histone lactylation as exogenous lactate levels increased (Figure 12). At 8mM the strains showed a 1.3-fold increase in histone lactylation and at 24mM they displayed an increase of two times the level of histone lactylation.

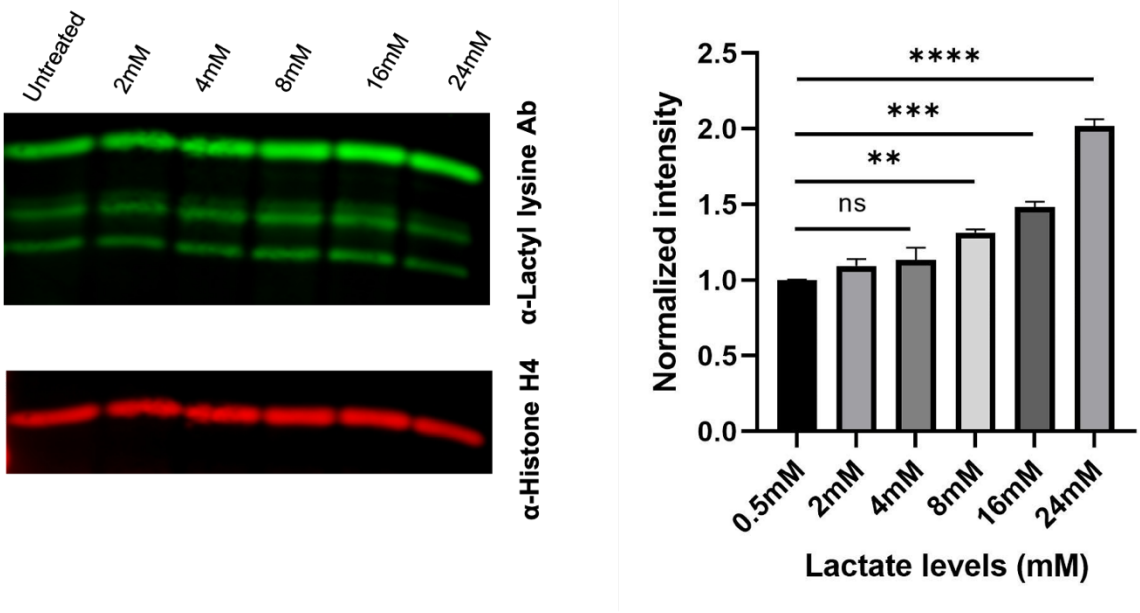

Figure 12. Relative Histone Lactylation of Trophozoite Stage Parasite at Varying Levels of Histone Lactylation

As exogenous lactate increased a corresponding increase in histone lactylation was evident in trophozoite stage parasites.

Impact of Exogenous Lactate on Histone Lactylation in Schizont Stage Parasites

During the schizont stage of the IDC *P. falciparum* is replicating and dividing, producing merozoites in the process of preparing to invade other RBCs in the host. While not as metabolically active as the trophozoite stage, it is still consuming glucose and producing lactate as a byproduct. In the set of experiments examining histone lactylation in schizont stages the fluorescent antibodies used were switched. Both biological replicates that were tested displayed an increase in histone lactylation with increases in exogenous lactate. At 8mM schizonts displayed an increase of 1.3-1.6 fold increase in histone lactylation (Figure 13).

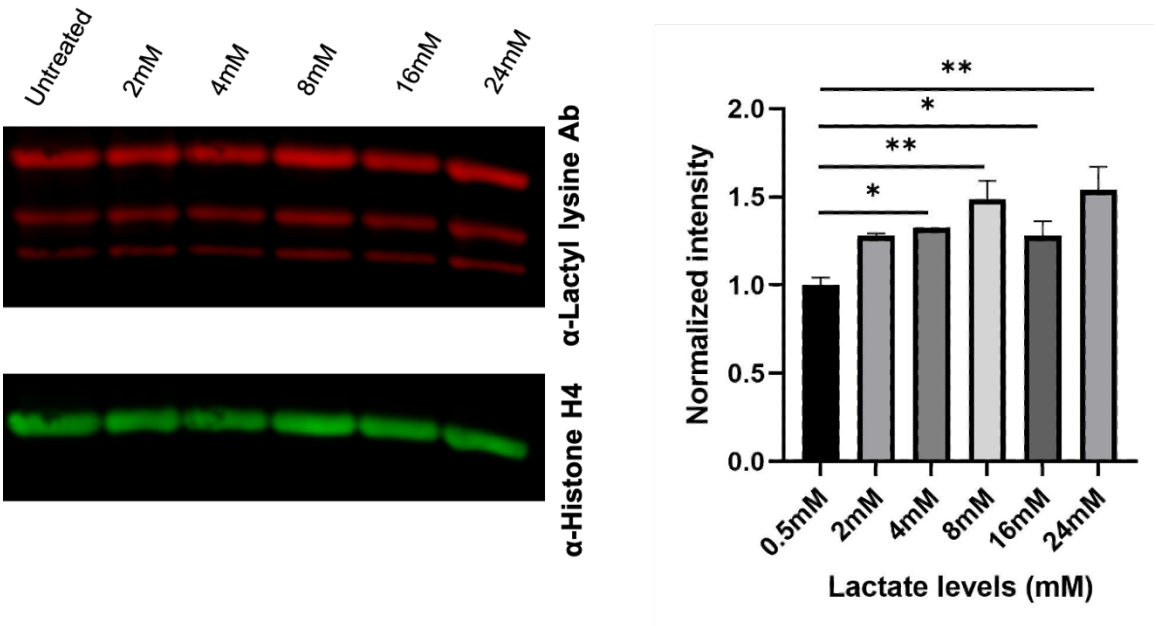

Figure 13. Relative Histone Lactylation of Schizont Stage Parasite at Varying Levels of Histone Lactylation

Increase in exogenous lactate resulted in an increase in histone lactylation in schizont stage parasites.

Incubation with Formate-Nitrite Transporter Inhibitor Increases Histone Lactylation

Formate-nitrite transporters (FNTs) are responsible for transporting small metabolites. In *Plasmodium*, PfFNT was discovered to be the malaria parasite's lactate transporter (Ouwehand et al. 2000) Figure 14. It has often been described as a potential target of therapeutic drugs designed as antimalarials due to its role in managing lactate levels in the parasite (Golldack et al. 2017). Active malarial infections produce large amounts of lactate which could be harmful to parasite growth. PfFNT is responsible for the efflux of lactate from the developing parasite. By disrupting the molecular activity, it could affect the homeostatic environment the parasite requires to survive. By inhibiting

the activity of PfFNT it's theorized that lactate builds up in the parasite. This can kill the parasite likely due to acidification of parasite cytosol (Hapuarachchi et al. 2017). 5-Nitro-2-(3-phenylproplylamino) benzoic acid (NPPB) is a known anion transport inhibitor has been shown to reduce formate and lactate transport in *P. falciparum* by 70% at 100 μM (PMID-25823844, *Marchetii RV et.al.*). To investigate whether inhibition of PfFNT activity leads to an increase in the intracellular lactate levels and histone lactylation, we cultured trophozoite stages of *P. falciparum* parasites in presence of 100 μM NPPB and DMSO control for 6 hours. As shown in Figure 15, the histone lactylation levels were found to be elevated by over 2-folds upon treatment with NPPB, suggesting buildup of intracellular lactate levels upon inhibition of PfFNT and inducing histone lactylation. This is the first study in Plasmodium parasites that shows impact on the epigenome upon inhibition of a metabolite transporter. Further efforts will be needed to uncover the gene expression changes in the parasite associated with buildup of intracellular lactate levels.

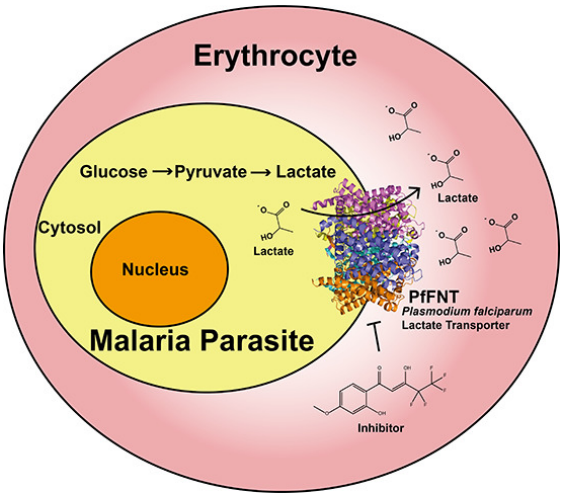

Figure 14. Mechanism of PfFNT and lactose transport in malaria parasite

Plasmodium parasites metabolize glucose into lactate, which is then exported out through transporters such as PfFNT. Inhibition of PfFNT can lead to accumulation of lactate inside the parasite.

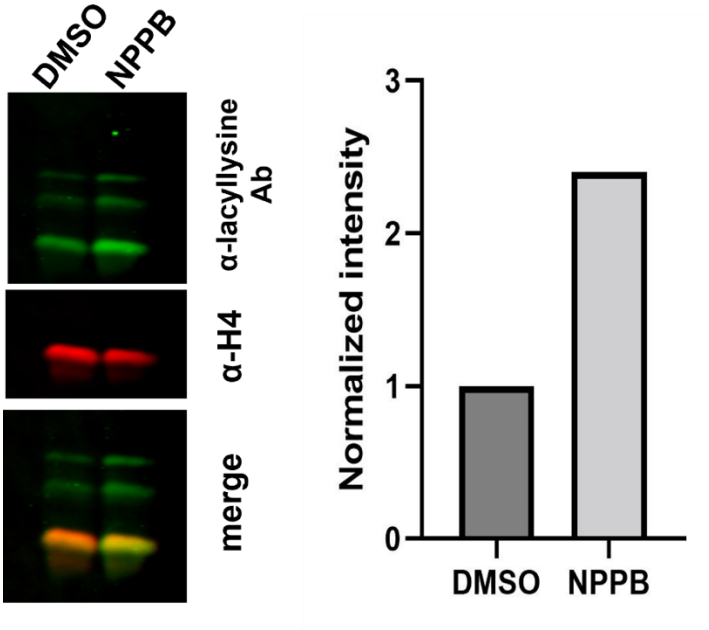

Figure 15. Impact of PfFNT inhibition

Figure 16. Inhibition of PfFNT using NPPB drug resulted in hyperlactylation of histones. When normalized against histone H4 levels, lactylation levels were induced by over 2-folds upon inhibition of PfFNT.

Histone Lactylation Continues to Increase in Extended Exposure to Exogenous Lactate

One goal of this investigation was to study the dynamic nature of histone lactylation in *P. falciparum* with respect to changes in environmental lactate and various stages of the IDC. We were also interested in how quickly histone lactylation might increase or decrease. This would allow us to gauge the timing with which histone lactylation would respond to environmental lactate. The experimental setup included incubating a control treatment in untreated conditions (resulting in an exogenous lactate level of 0.5mM) and an experimental condition of 8mM exogenous lactate. These were incubated for two IDC cycles and cultures were collected at 48 hours and 94 hours. Then

both samples were returned to 0.5mM and cultured an additional cycle of the IDC. This third sample was collected at 118 hours. This would allow us to determine whether histone lactylation remained high despite being in a lower level of exogenous lactate, suggesting heritability of histone lactylation as an epigenetic mark. The data from those samples confirm the hypothesis that increased exposure to higher levels of exogenous lactate will result in increased levels of histone lactylation (Figure 16).

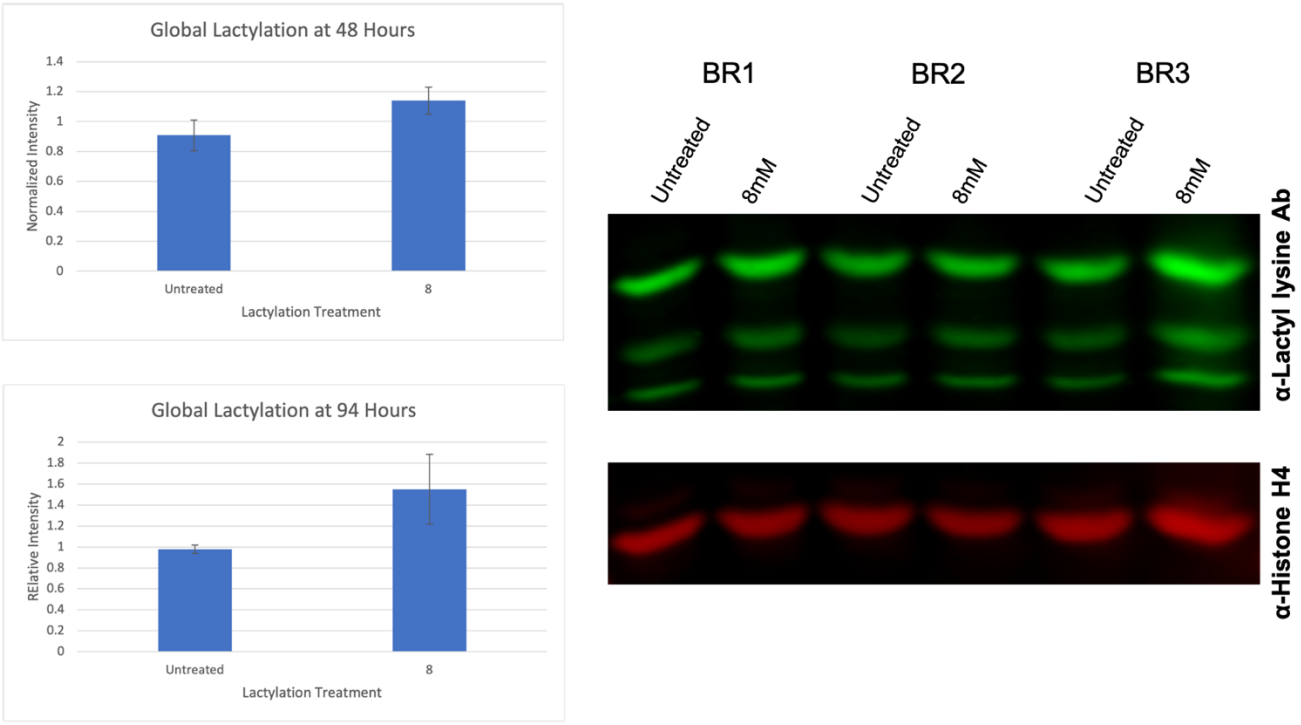

Figure 16. Extended Exposure to High Exogenous Lactate Increased Histone Lactylation

Culture samples collected at 48 hours and 94 hours indicate that extended exposure to high levels of exogenous lactate result in increased histone lactylation.

Delactylation Activity of Histone Deacetylase PfSir2

Knowing that Sirtuins functions as a delactylase in humans, we decided to investigate the delactylation potential of PfSir2 in *P. falciparum*. PfSir2 has been shown to function as a deacetylase and an ADP ribosyltransferase in *Plasmodium* (Merrick et al. 2007), demonstrating potential for modification of epigenetic signals related to metabolic activity. Considering the fact that sirtuin expression is dysregulated in cases of severe malaria, it was determined that investigating PfSir2 activity relative to histone lactylation would be a good indicator of potential involvement. In order to investigate this, two knockout lines were used that were generated in previous studies: ΔSir2A and ΔSir2B. Parasites were cultured under lactate levels of >2mM and trophozoite and schizont stage parasites were collected and processed for western blots (Figure 17). Histone lactylation in wild-type and knock out lines were quantified as shown in Figure 17. We observed increased levels of histone lactylation in the parasites that lack PfSir2B gene. The level of hyperlactylation was higher in trophozoite stage as compared to schizonts, which correlates with the known expression of PfSir2B in the trophozoite stage. In contrast, PfSir2A is known achieve its peak expression in late schizont stages and its deletion did not affect histone lactylation significantly. The results strongly suggests that PfSir2B is one of the histone delactylase. PfSir2B is known to regulate expression of internal chromosome var genes with UpsB promoter type (PMID: 19402747). It is believed that Sirtuins repress expression of var genes through their deacetylase activity. However, it is likely that histone delactylase activity of PfSir2B could contribute to this process.

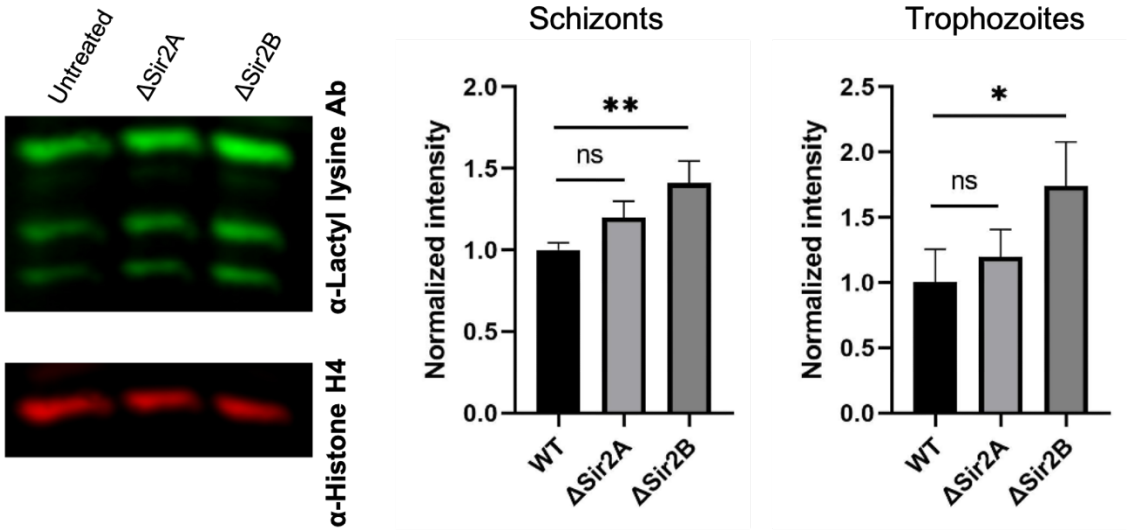

Figure 17. Relative Histone Lactylation in PfSir2 Knockout in Trophozoites & Schizonts

Knockout of PfSir2 resulted in increased histone lactylation in both knockout lines generated. Increases in histone lactylation were seen in both trophozoite and schizont stage parasites with the

Chapter IV

Discussion

This study shows that histone lactylation is a novel epigenetic signal in *Plasmodium falciparum* that is dynamically regulated in response to environmental lactate. We provide evidence that asexual stages of *P. falciparum* are responsive to environmental lactate levels, altered through exogenous supplementation or buildup of intracellular levels.. Additionally, we hypothesize that PfSir2 plays a role in erasure of lactylation marks on histone proteins.

The existence of histone lactylation in *P. falciparum* is consistent with what has been discovered in other parasites with regards to epigenetic signals. Since its discovery in human macrophage cells, this novel PTM has been reported in multiple prokaryotic and eukaryotic organisms, including Toxoplasma and Trypanosoma parasites. It is known that *P. falciparum* utilizes epigenetic mechanisms to modify expression of genes such as *var* genes, which contributes to parasitic antigenicity and avoiding immune clearance. However, the upstream sensors and regulators of the parasitic epigenetic code are not well understood.

Western blot imagery in other species indicates lysine lactylation in a wide range of proteins, and mass spectrometry analyses identified these proteins to be metabolic enzymes, cell signaling molecules and histones. In contrary, we observed lysine lactylation in *P. falciparum* solely on histone proteins. Not only does the mark exist in the parasite, but it seems to change over the course of the IDC, suggesting exogenous lactate may impact gene expression over the course of parasite growth. Lactate levels are likely constantly fluctuating in and around the parasite and host environment and we see

clear changes in histone lactylation as the parasite undergoes growth and development during the IDC.

One of the key factors we wanted to investigate was the responsiveness of histone lactylation to lactate in the environment. In order for histone lactylation to be a dynamic epigenetic modification utilized by the parasite to sense and react to environmental conditions, it would have to be responsive to exogenous lactate. One of the hallmarks of *P. falciparum* infections, especially severe infections, is production of lactate as a result of anaerobic glycolysis and metabolic activity. Upon liver dysfunction, the clearance of lactate in humans can get hampered, which further contributes to lactic acidosis. In this study we have shown that at each stage of the IDC *P. falciparum* histone lactylation is responsive to environmental lactate. As the parasite grows and metabolic activity increases, it produces more lactate and could lead to hyperlactylation of histones. We believe that *P. falciparum* utilizes histone lactylation as one of its tools for understanding its surroundings and growth. It is likely that similar to human cells, histone lactylation in *P. falciparum* regulates expression of genes, which will allow the parasite to gauge metabolic status of the host and alter its biological processes such as asexual proliferation, var gene expression and sexual conversion. The discoveries made in this study provides a platform for other studies focused on identification of specific genes that are epigenetically regulated by histone lactylation.

In order to better understand histone lactylation its important to identify the enzymes responsible for their addition and removal. We investigated Sirtuin enzymes in *P. falciparum* parasites and identified PfSir2B as a key enzyme involved in the process of removing histone lactylation signals. In parasites lacking PfSir2B, a significant increase

in histone lactylation was observed, suggesting it plays a role in erasure of the signal. This possibility is supported by research that suggests sirtuin-like enzymes may play a role in managing metabolic epigenetic signaling in other organisms. Sirtuin genes are known to be overexpressed in severe malaria cases with high lactate levels. It is also possible that *P. falciparum* possesses other histone lactylation enzymes that are species specific that we are unaware of at the moment. Further efforts will be needed to identify any other potential eraser or the writers of this modification.

Histone lactylation is an emerging epigenetic signaling pathway that demonstrates a direct link between organism metabolism and modifications in gene regulation as demonstrated in Figure 18. In *Plasmodium falciparum* it suggests a unique mechanism by which parasite can monitor host environment and respond to potential complications or conditions such as hyperlactatemia. The potential for research regarding therapeutic targets as well as better understanding the mechanisms of severe malarial complications are important and require further research.

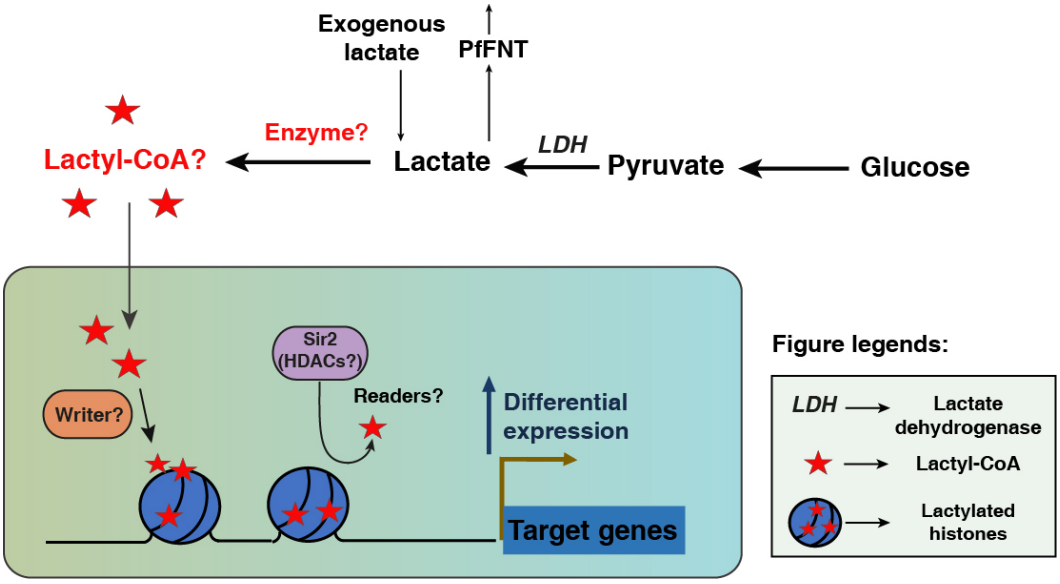

Figure 18. Summary of Lactate Production and Histone Lactylation

A summary figure depicting the process by which glucose metabolism results in lactate production. The intracellular lactate pools are dependent on exogenous lactate levels and export of lactate through PfFNT transporter. The lactate can as a source of histone lactylation through unknown writer enzyme, which can be erased through enzymes such as Sirtuin. Histone lactylation could likely result in differential expression of target genes, linking their expression to lactate levels.

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