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**Chromatin proteomics reveals a dynamic histone code as novel
druggable system essential to malaria parasite differentiation**

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SUBMISSION DECLARATION



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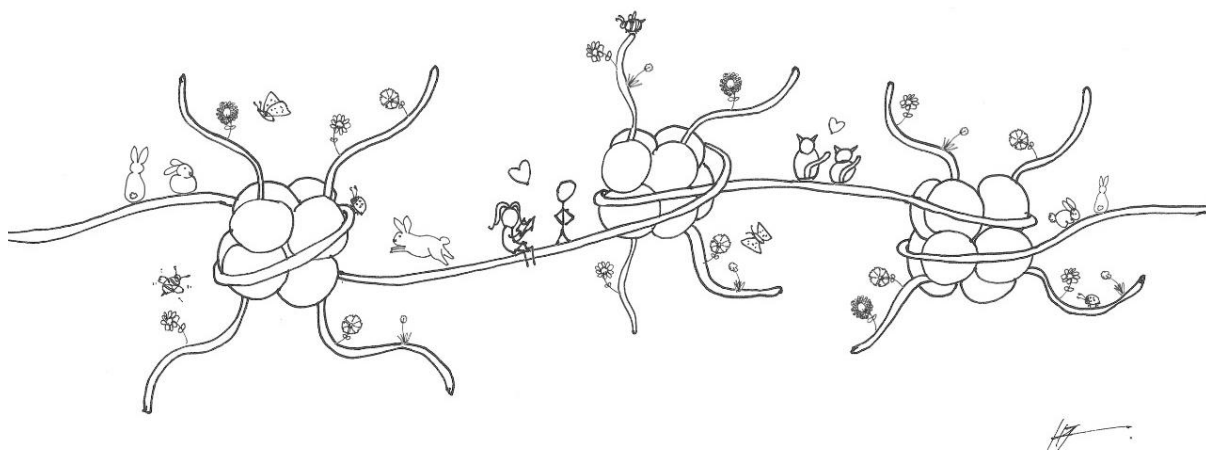
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"The urge to discover secrets is deeply ingrained in human nature; even the least curious mind is roused by the promise of sharing knowledge withheld from others. Most of us are driven to sublimate this urge by the solving of artificial puzzles devised for our entertainment. Detective stories or crossword puzzles cater for the majority; the solution of secret codes may be the hobby of the few." - J. Chadwick, The Decipherment of Linear B

SUMMARY

The malaria causing parasite, *Plasmodium falciparum*, remains one of the deadliest organisms globally. Underlying its unique and complex biology that is divergent from other higher order eukaryotes, lies a weak spot: its epigenome. Histone post-translational modifications (PTMs) are dynamically deposited and removed during the parasite's proliferative asexual intra-erythrocytic developmental cycle and sexual differentiation process to form an overall pattern of histone PTMs that co-exist at specific times. Emerging evidence in eukaryotic gene regulation suggest that histone PTMs do not only function alone but can combine to ultimately signal for a biological outcome that is different from the PTM alone. In this thesis, I explore the combinatorial histone code of *P. falciparum* parasites. Histone PTM combinations are mapped across asexual, early- and late-stage gametocytes to reveal a highly complex "code" that defines each stage. Evidence for the functional relationship between histone PTMs is provided. Since the functional relevance of combinatorial PTMs relies on the effector proteins that bind to them, the proteome associated with histone PTMs that co-exist in the parasite is identified to reveal the association of a unique epigenetic complex that is recruited to each of the PTMs in combination. Lastly, the biological importance of crosstalk between histone PTMs is probed with chemical interference of the effector proteins that establish the combinations. This revealed that gametocytes are particularly susceptible to crosstalk inhibition by proxy, and that arginine methylation is likely critical for gametocyte specific biological processes. Ultimately, this thesis presents fundamental and preliminary evidence of the functional importance of the histone code in *P. falciparum*, a feature that reshapes our understanding of gene regulation in *P. falciparum* well beyond the central dogma of biology.

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Abbreviations

ac	Acetylation
ACN	Acetonitrile
ACT	Artemisinin-based combination therapy
AGC	Automatic gain control
APAD	3-acetyl pyridine adenine dinucleotide
ApiAP2	Apicomplexan-specific Apetala2
ATP	Adenosinetriphosphate
BCA	Bicinchoninic acid
BDP	Bromodomain-containing protein
CenH3	Centromeric H3
ChIP-MS	Chromatin immunoprecipitation followed by mass spectrometry
CID	Collision induce dissociation
CVGE	Clonally variant gene expression
DDA	Data-dependent acquisition
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
DNDM	DNA demethylase
DNMT	DNA methyltransferase
DNMTi	DNA methyltransferase inhibitors
Epidrugs	Epigenetic drugs
ETD	Electron transfer dissociation
FDR	False discovery rate
FIRR	Fragment ion relative ratio
G6PD	Glucose-6-phosphatedehydrogenase
GDV1	Gametocyte development protein 1
GluC	Glutamic acid C-terminus
GO	Gene ontology
GTS	Global technical strategy
HAT	Histone acetyltransferases
HDAC	Histone deacetylases
HDACi	Histone deacetylase inhibitors
HDM	Histone demethylase
HDMi	Histone demethylase inhibitor
HKMT	Histone lysine methyltransferases
HKMTi	Histone lysine methyltransferase inhibitors
HMGB	High mobility group B
HMT	Histone methyltransferase
HMTi	Histone methyltransferase inhibitor
HP1	Heterochromatin protein 1
hpi	Hours post-invasion
HRP	Horseradish peroxidase
I_GC	Immature gametocytes
iBAQ	Intensity-based absolute quantification
IC ₅₀	Half-maximal inhibitory concentration
IDC	Intra-erythrocytic developmental cycle
IMC	Inner membrane complex
IP	Immunoprecipitated
IRS	Indoor residual spraying
ITN	Insecticide-treated mosquito nets
JHDM	Jumonji histone demethylase

JMJC	Jumonji-C domain-containing
lncRNA	Long non-coding RNA
LSD1	Lysine-specific demethylase 1
LysoPC	Lysophosphatidylcholine
M_GC	Mature gametocytes
me1	Monomethylation
me2	Dimethylation
me3	Trimethylation
MS	Mass spectrometry
NAG	N-acetylglucosamine
nanoLC	Nano-liquid chromatography
NAPS	Nucleosome assembly protein
NBT	Nitrobluetetrazoliumchloride
ncRNA	Non-coding RNA
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
PBS	Phosphate buffered saline
PES	Phenazine ethosulphate
ph	Phosphorylation
PHD	Plant homeodomain
pLDH	Parasite lactate dehydrogenase
PRMT	Protein arginine methyltransferases
PTMs	Post-translational modifications
Puf	Pumilio and FEM3 binding factor
rif	repetitive interspersed gene family
RNAi	RNA interference machinery
SAH	S-adenosylhomocysteine
SAHA	Suberoylanilidehydroxamicacid
SAM	S-adenosyl-L-methionine
SET	(Su(var)3-9, Enhancer of Zeste and Trithorax)
StageTip	STopAndGoExtractionTip
stevor	Subtelomeric variable open reading frame gene family
SUMO	Small ubiquitin-like modifier
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TSA	Trichostatin A
TSS	Transcription start sites
ub	Ubiquitination
var	Variant gene family
WCX-HILIC	Weak cation exchange coupled with hydrophilic interaction liquid chromatography
WHO	World Health Organization

Chapter 1 | Introductory framework

1.1 Public health importance of malaria

Malaria is a life-threatening blood illness that has long been a global health issue and continues to be so. In 2019, an estimated 229 million cases of malaria reported, of which ~409 000 cases resulted in death ¹. Malaria is endemic in 87 countries and at least three billion of the world's population is at risk of contracting the disease ¹. Countries in the World Health Organization (WHO) African Region bear the brunt of the disease, accounting for roughly 94% and 95 % of malaria cases and fatalities, respectively. The socioeconomic impacts of malaria therefore place a significant strain on developing countries, where basic health care is typically substandard; there is a close relationship between malaria incidence, parasite transmission, poverty ². Malaria poses a particular threat to the most vulnerable groups, with children under the age of five as the most vulnerable and accounting for 67 % of all deaths in 2019 ¹ and pregnancy associated with increased risks of premature birth, low birth weight, and foetal growth retardation ³.

The WHO Global Technical Strategy for Malaria 2016-2030 (GTS) mandates the reduction of malaria case incidences and mortality rates by at least 40 % by 2020, 75 % by 2025, and 90 % by 2030, compared to a 2015 baseline ¹. Unfortunately, the GTS goals of reducing malaria cases and deaths by 40 % by 2020 was not met, and the current trajectory could lead to a major setback by 2030 ¹. An progress audit performed in 2020 indicated that, although several countries did show a decreased case number decrease since 2015, an alarming increase in malaria cases and mortality rates was observed particularly in the WHO Americas Region, but also in the WHO South-East Asia, Western Pacific, and African regions (Figure 1.1)^{1, 4, 5}. This sketches a worrying scenario and thread towards the GTS goals; a situation exacerbated by the global COVID-19 pandemic. In Africa, the pandemic has worsened the already strained health care systems. This has resulted in the continued implementation of malaria control measures being at risk of continuation, with examples of malaria control ceasing entirely in some areas. This predictively will impact malaria case numbers for the 2020/2021 seasons with a rise in malaria cases pre-empted ^{6, 7}. This clearly emphasises the fragile nature of malaria control strategies and supports continued efforts towards elimination of the disease.

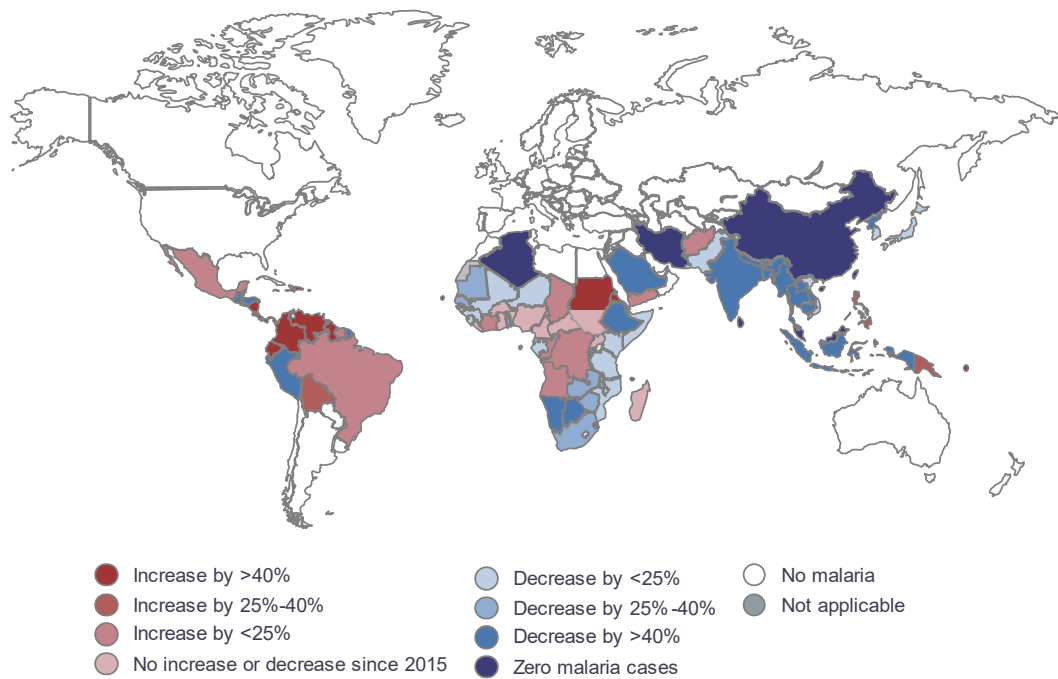


Figure 1.1. World map showing malaria-endemic countries in 2020. Progress towards the WHO Global Technical Strategy 2020 malaria case incidence milestone of at least 40 % reduction from a 2015 baseline is indicated. Figure re-created and adapted from the World Health Organization World Malaria Report 2020.

1.2 Strategies to control the causative agents of malaria

Malaria is caused by infection of parasites from the genus *Plasmodium*, which is transmitted to humans by female *Anopheles* mosquitoes. Of the ~465 *Anopheles* mosquito vectors, ~70 species are competent malaria vectors, with 41 species being regarded as dominant malaria transmission vectors ⁸. In South Africa, *Anopheles arabiensis*, *Anopheles gambiae* and *Anopheles funestus* are most important and wide-spread malaria vectors ⁹. There are six species of *Plasmodium* parasites that infect humans, including *Plasmodium malariae*, *Plasmodium ovale* (with two sub-species recognised: *P. ovale curtisi* and *P. ovale walikeri*), *Plasmodium knowlesi*, *Plasmodium cynomolgi*, *Plasmodium vivax* and *Plasmodium falciparum* ¹. Both *P. malariae* and *P. ovale* are associated with non-severe malaria in humans, while the simian malaria parasites *P. knowlesi* and *P. cynomolgi* have been reported to cause widespread infection of humans in indigenous communities ^{10, 11}. Of all the species, *P. falciparum* is the most prevalent on the African continent and the most lethal *Plasmodium* parasite, causing 99 % of all malaria mortalities ¹. The perfect interplay between *Anopheles* and *Plasmodium* therefore supports disease progression and transmission, and as a result, the majority of intervention strategies are targeted towards eliminating mosquitos' transmission as well as targeting the parasite through prevention and treatment of the clinical pathologies associated with parasite proliferation.

1.2.1 Vector control

Anopheles mosquitos are highly adaptable the local climate, ecology, and human behaviour as evident by their large geographical range and fast evolving behaviour; all of which will influence the success of the vector control strategy applied ¹². Key interventions include the use of insecticide-treated mosquito nets (ITNs), long-lasting insecticidal nets, indoor residual spraying, and larval source management ¹²⁻¹⁴. However, vector control is threatened by the development of insecticide resistance, particularly in *An. gambiae*, most predominantly to the pyrethroids currently used in ITNs ^{1, 15}. This has resulted in alternative, non-insecticide innovations including transgenic mosquitoes carrying antipathogen effector genes, however, these types of strategies carry significant technical, ethical, and legal challenges and far-reaching efficacy has yet to be proven ¹⁶⁻¹⁸.

1.2.2 Parasite control

Accurate diagnosis of malaria can be achieved by detection of *Plasmodium* parasites via microscopy, point-of-care rapid diagnostic tests, polymerase chain reaction and loop-mediated isothermal amplification ¹. Once diagnosed, the main goal of interventions is to reduce the parasite load in patients, thereby alleviating symptoms, and preventing severe malaria and death. Since 2010, the WHO has recommended fixed-dose artemisinin-based combination therapies (ACTs) as the frontline, gold standard for the treatment of malaria ¹⁹⁻²¹. ACTs consist of an artemisinin derivate, which rapidly clears the majority of circulating parasites and relieve symptoms in the patient, combined with a long-lasting partner drug to completely remove any residual parasites after the patient has recovered ¹. However, as was the fate of all the antimalarials historically used, parasite resistance has now developed to artemisinins and its partner drugs ^{22, 23}. This is particularly evident in countries from the Greater Mekong subregion (Cambodia, Lao People's Democratic Republic, Myanmar, Thailand and Vietnam) where a 26 % treatment failure of dihydroartemisinin-piperaquine was seen in 2015 in Vietnam, and resistance to four different ACTs has been reported in Cambodia since ²⁴⁻²⁶. Of great concern is the recent emergence of mutant alleles associated with artemisinin resistance and delayed parasite clearance in Africa ²⁷⁻²⁹.

In a historic moment, the candidate malaria vaccine, "RTS,S/AS01" has recently (October 2021) been recommended by the WHO for administration in children in sub-Saharan Africa ¹. Although in randomised trials the vaccine provided protection against both clinical and severe malaria in children, it remains only ~36 % effective in reducing malaria in children ³⁰⁻³². However, if the vaccine sees large-scale uptake in African countries and is used as a complement to existing interventions, it could drastically reduce mortality rates in the coming

years. Finally, seasonal malaria chemoprevention, intermittent preventative treatment for pregnant women and infants, and mass drug administration are alternative control options that are being explored and developed by the malaria community ^{33, 34}.

One of the main challenges in targeting the parasite remains the complex biology associated with *Plasmodium* development, differentiation, and transmission. To inform future intervention strategies, a continued exploration and thorough understanding of particularly *P. falciparum* parasite biology is essential. Given the distinct biology of each of the multiple life cycle stages, the life cycle of *P. falciparum* represents a viable mining opportunity to support discovery of future therapeutic interventions.

1.3 The complex life cycle of *P. falciparum* parasites

Plasmodia are eukaryotic, endoparasitic protists from the phylum *Apicomplexa*, of which all members are obligatory intracellular auxotrophs that grow and replicate in a specialised compartment within erythrocytes known as the parasitophorous vacuole. *Apicomplexa* are further uniquely characterised by the presence of an apicoplast, a structurally unique organelle similar to chloroplasts. The function of the parasites' life cycle is to produce progeny that undergo a stage differentiation process to ensure continued survival of the specie. Therefore, *Plasmodium* parasites have a complex life cycle involving essentially three events in the human host: an asexual pre-erythrocytic and intra-erythrocytic proliferative cycles to allow population expansion, and a sexual maturation process essential for transmission (Figure 1.2).

Infection is initiated when as few as 100 sporozoites in the salivary gland of a feeding female *Anopheles* mosquito are ejected into the skin of a human as it takes a blood meal ³⁵. After inoculation, sporozoites glide through the dermis into peripheral blood circulation within 30 min and migrate to the liver sinusoids to infect hepatocytes within 2 min, followed by the initiation of exo-erythrocytic schizogony ³⁶. Following ~14 rounds of replication in the liver, mass cytokinesis occurs followed by the release of ~10⁴ of hepatic merozoites from a single sporozoite into the bloodstream in the form of merozoites, parasite-filled vesicles ³⁶⁻⁴⁴.

Blood stage infection is established when these merozoites invade erythrocytes, thus initiating the asexual 48 h intra-erythrocytic developmental cycle (IDC). This intra-erythrocytic environment effectively allows the parasite to avoid the immune system (supported with additional antigenic variation) and mediate sequestration in the microvasculature to prevent splenic clearance ^{45, 46}. A single, haploid merozoite evolves into a ring-stage parasite within 6 h post-invasion (hpi) ^{47, 48}, followed by development into metabolically active trophozoites ⁴⁹.

The latter is characterised by introduction of new permeability pathways that allow for substrate uptake from the surrounding nutrient rich environment and the efflux of metabolic waste products ⁵⁰. *Plasmodium* proteolyses erythrocyte haemoglobin in the digestive vacuole to provide free amino acids (excluding isoleucine) for protein synthesis and to maintain intra-erythrocytic osmolarity ⁵¹. The pigmentation of trophozoite-stage parasites is crystalline haemozoin, which is produced by oxidation of β -haematin. *P. falciparum* is also entirely dependent on the host for purine nucleotides required for DNA replication ⁵². Schizogony commences when the single trophozoite nucleus begins to divide into two daughter nuclear bodies around 33-36 hpi and results in a polyploid, multi-nucleated syncytium ^{48, 53-55}. Erythrocyte rupture occur at 42-48 hpi, releasing 8-24 merozoites per schizont ^{53, 56}. Each released merozoite proceeds to infect a new erythrocyte, allowing for persistent cycles of infection. This coordinated, rapid amplification results in up to 6×10^{10} parasites per infected, untreated, immune-naïve individual ⁵⁷.

1.3.1 *Plasmodium* sexual development

During gametocytogenesis, *P. falciparum* parasites develop through five morphologically, biochemically and physiologically distinct stages in 10-12 days^{59, 60}. Gametocytogenesis in other *Plasmodium* species lasts only ~24-48 h and gametocytes morphologically resemble a round shape that take up the majority of the host erythrocyte volume^{59, 61, 62}. Depending on the temporal expression of factors responsible for sexual differentiation, committed merozoites entering erythrocytes either directly initiate gametocytogenesis (same cycle conversion), or complete another round of asexual replication after which daughter merozoites released from the committed schizont are released to invade erythrocytes and only then initiate gametocytogenesis (next cycle conversion)⁶³.

Stage I to IV gametocytes escape splenic clearance by sequestering in the human bone marrow where they mature to stage V^{64, 65}. Stage I gametocytes resemble the rounded, asexual trophozoite but can be distinguished from these since haemozoin crystals do not form punctate clusters in stage I gametocytes⁶⁶. Stage II gametocytes adopt a lemon-shaped spherical appearance with its mononuclear content. As stage II to stage III transition occurs, the gametocyte (including its nucleus and mitochondria) elongates to a length to width of ratio ~2:1, with one side flattening while the opposite side curves to allow the characteristic D-shape of stage II gametocytes^{67, 68}. This elongation is facilitated by extension machinery that comprises of a membranous structure called the inner membrane complex (IMC) and a dense network of microtubules deposited underneath the parasite plasma membrane^{60, 69}. Stage IV gametocytes are maximally elongated with a crescent banana shape (length to width ratio of 4:1) with aciculate ends. Terminally differentiated, mature stage V male and female gametocytes (micro- and macrogametocytes) retains the crescent shape but now with rounded ends and a length to width of ratio of 3:1; the host erythrocyte is reduced to a thin background layer referred to as a Laveran's bib. Female gametocytes are distinguished from males as they are slightly more curved, have concentrated nuclear material and membrane-bound osmiophilic bodies visible as distinct vesicle-like structures. Stage V gametocytes re-enters the peripheral blood circulation ready for transmission to a feeding mosquito vector.

Within the mosquito midgut, micro- and macrogametocytes egress from the erythrocyte and initiates gametogenesis to differentiate into mature micro- and macrogametes. This is triggered by the drop in temperature (from 37 °C to 25° C), a rise in pH (from 7.4 to 8.0), the mosquito-derived xanthurenic acid and is linked to the mobilisation of intracellular Ca²⁺ stores⁷⁰⁻⁷⁴. The male gametocytes' genome is replicated three times in ~15 min, which ultimately result in eight, motile daughter microgametes^{75, 76}. Each haploid microgamete fertilises a female gamete, resulting in the formation of a diploid zygote that undergoes meiotic

recombination within 3 hours^{72, 77, 78}. The zygote matures into an ookinete, which develops into oocysts where sporogony occurs to produce multiple haploid sporozoite progeny within the cell⁷⁹. After release from the oocysts, approximately 10⁴ sporozoites migrate to the mosquito's salivary glands in preparation for transmission⁸⁰.

Given the complex life cycle, *P. falciparum* has evolved control mechanisms to permit continued transmission and survival. This includes extraordinary control of gene expression at the level of transcription and post-transcriptionally as well as (post)-translational mechanisms, all of which consistently responds to host-environmental signals to allow the parasite to meet the intricate life cycle stage transition requirements.

1.4 Molecular framework of the *P. falciparum* parasite

P. falciparum has one of the most A+T rich genomes yet, characterised at ~81 % A+T-content. The haploid genome is 22.9 Mb in size and packaged into 14 chromosomes⁸¹. Additionally, two extrachromosomal genomes exist - a 35 kb apicoplast genome inherited from photosynthetic algae (the apicomplexan ancestor), and a six kb mitochondrial genome that are both uniparentally inherited⁸²⁻⁸⁴. Of the 5 268 genes in the *P. falciparum* genome, ~62 % are essential for asexual parasite proliferation^{85, 86}.

Like all eukaryotes, *P. falciparum* parasites initiate gene transcription based on molecular cues across a canonical bipartite promoter. Furthermore, transcription is monocistronic (i.e., messenger RNA that can encode only one polypeptide per RNA molecule), performed by RNA polymerase II binding to a TA-dinucleotide initiation element, and dependent on *trans*-acting elements upstream of transcriptional start sites^{87, 88}. Some promoter-proximal and -distal regulatory elements have been linked to stage- or function-specific transcription, implying that distance-independent *cis*-acting elements can regulate genes in *P. falciparum*⁸⁹. Transcription occurs “just-in-time” where transcripts are only produced at specific time points during IDC with ~80 % of genes actively expressed in a monocistronic manner⁹⁰. Of these, >75 % show clear peak expression at a specific time point during the IDC^{90, 91}. By contrast, during gametocytogenesis only 45 % of the genome is actively expressed^{92, 93}, of which ~167 of genes are differentially expressed with the onset of gametogenesis in the mosquito midgut, most notably a group of micro- and macrogamete specific surface antigens genes *pfs25*, *pfs28m*, and *pfs48/45*⁹⁴. A limited repertoire of transcription factors is thought to coordinate this stage-stratified transcriptional control during both the IDC and sexual development. These include the APETALA2/ethylene response factor (AP2/ERF) family limited to the *Plantae* and *Apicomplexa* phyla, and which consist of only 27 members in *Plasmodium*^{95, 96}. As the

only transcription factors in *Plasmodium* to associate to specific *cis*-acting regulatory motifs, *trans*-acting ApiAP2 transcription factors guide transcription of up to hundreds of genes in specific stages of the parasite ⁹⁷. Of these, 21 of the 27 ApiAP2 transcription factors are expressed during the IDC whereas only 15 of the ApiAP2 family members are expressed during gametocytogenesis. ApiAP2 transcription factors can either operate as activators or repressors of gene expression during the life cycle ^{92, 98, 99}. A structured cascade of ApiAP2 expression is evident throughout the parasite's life cycle ⁹², where specific ApiAP2s peak during specific stages. For example, AP2-SP is expressed in sporozoites ¹⁰⁰, AP2-L in liver stages ¹⁰¹, AP2-I during invasion of erythrocytes ¹⁰², AP2-G during asexual parasite differentiation to gametocytes ^{103, 104}, AP2-G2 during gametocytogenesis ¹⁰⁵, AP2-FG in female gametocytes ¹⁰⁶, and AP2-O in ookinetes ¹⁰⁷. Outside of the AP2 family, only two other transcription factors have been described from the myoblastosis family (MYB), MYB1 and MYB2, with the first essential to progression of the IDC ¹⁰⁸. Other *P. falciparum* proteins with DNA binding motifs have been identified, including 37 zinc finger motif proteins and a single β -scaffold protein with a DNA minor groove contact (PF3D7_0109600), which alludes to the presence of other potential transcriptional regulators in *P. falciparum* in the absence of other known transcription factors such as those containing homeodomains or basic leucine zippers ⁹⁹.

The parasite utilises conventional post-transcriptional regulation including mRNA stabilization ¹⁰⁹ and mRNA decay ¹¹⁰, alternative splicing, post-translational modifications, and regulation by non-coding RNA elements ¹¹¹⁻¹¹³. Additionally, strategy-specific translational repression is used to suppress female associated transcripts in a preparatory strategy for transmission ^{114, 115}. This is mediated by DOZI helicase (Development of Zygote Inhibited; an orthologue of DDX6, Dhh1 class DEAD box RNA helicase), CITH (Sm-like factor homolog of CAR-I and Trailer Hitch)¹¹⁶, and Pumilio and Fem-3 binding factor family proteins, which bind to mRNA targets in female gametocytes including known female-specific transcripts for *pfs25* and *pfs28* surface antigens ^{76, 116-121}.

There is compelling evidence that the parasite also uses epigenetic mechanisms, particularly histone post-translational modifications (PTMs), to alter gene expression throughout its life cycle. This entices research into the idiosyncrasies between the parasite's unique epigenetic traits and its long-term survival ¹²²⁻¹²⁵.

1.5 Brief introduction to eukaryotic epigenetic gene regulation

Epigenetic gene regulatory mechanisms modify the chromatin landscape by converting environmental stimuli into changes that influence gene expression in the absence of DNA sequence alterations, ultimately leading to the diversification of phenotypes. Three distinct, but highly interdependent, epigenetic regulatory mechanisms have been noted. This includes DNA methylation, through the action of DNA methyltransferase (DNMT), resulting in chromatin stabilising and inherited 5-methylcytosines ¹²⁶. Secondly, nucleosomal regulation includes the composition of (canonical or variant histones), and modifications to histones (post-translational modifications, PTMs), as well as nucleosome positioning and stability on DNA, all of which influence access to DNA for gene expression. Lastly, RNA-based mechanisms, including long non-coding RNA (lncRNA) and RNA interference factors ^{127, 128}, to target DNA sequences and mostly silence transcription by altering nucleosome positioning, RNA stabilisation and chromatin looping ¹²⁹(Figure 1.3).

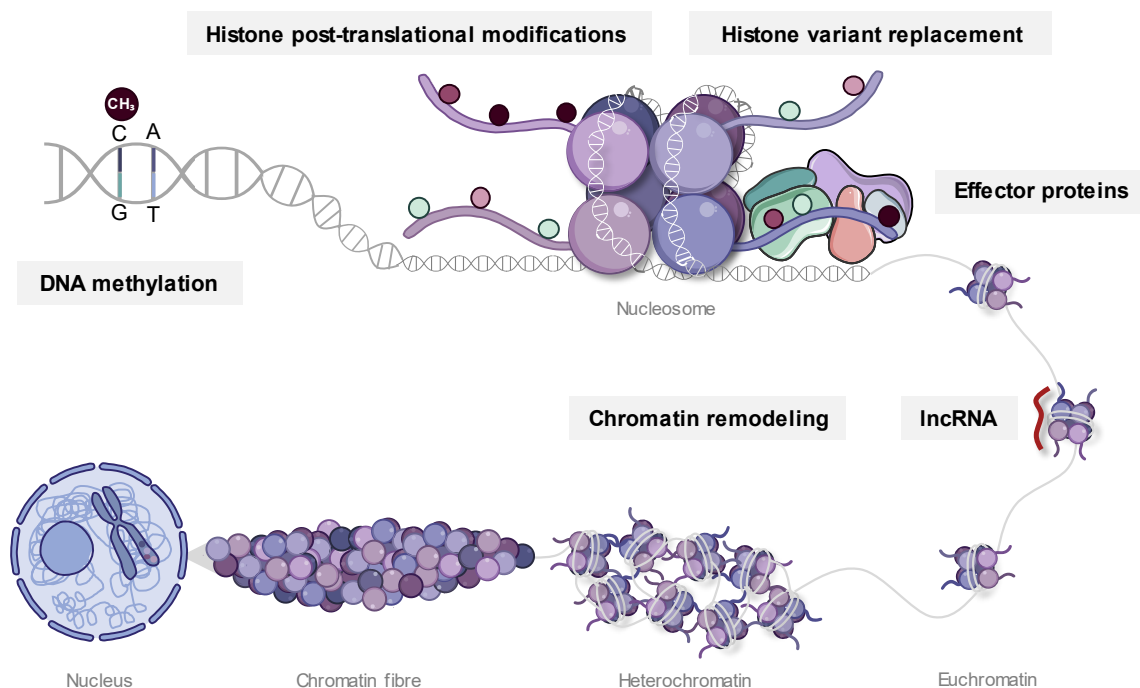


Figure 1.3. Epigenetic gene regulation mechanisms and DNA packaging. Epigenetic gene regulation occurs through methylation of DNA, histone PTMs and through long non-coding RNA (lncRNA) regulation. DNA is wrapped around a histone octamer to form nucleosomes. Nucleosomes are folded to produce chromatin that is packed into chromosomes found the nucleus of the cell.

Histone PTMs represent a core feature underlying cellular differentiation and development in most cell types. The nucleosome unit comprises of 147 bp of DNA wrapped around a histone octamer in approximately 1.65 turns in a left-handed or right-handed superhelix. The histone octamer typically consists of a H3/H4 tetramer and two H2A/H2B dimers that is linked by a

linker histone H1¹³⁰. Covalent chemical modifications on histones' N- (or C-)-terminal tails influence chromatin-dependent processes by functioning as recruiting platforms for effector proteins and by changing the total nucleosomal charge, thereby affecting the histone-DNA interaction^{131, 132}. “Small” histone PTMs including acetylation, methylation, and phosphorylation have been the most extensively studied, while “large” PTMs such as ubiquitylation and SUMOylation (Small Ubiquitin-related MOdifier protein) are less well characterised¹³³.

Acetylation of histones is catalysed by histone acetyltransferases (HAT), which transfers an acetyl from acetyl-coenzyme A to the ϵ -amino on lysine residues. Acetylation is functionally associated with transcriptional activation, as acetylation reduces the positive charge on histone tails which result in a reduced DNA-histone interaction and formation of euchromatin structure¹³⁴⁻¹³⁶ (Figure 1.4). Conversely, the deacetylation of lysine residues by histone deacetylases (HDAC) result in a transcriptionally repressive state^{137, 138}. Mono-, di- and trimethylation of lysine is catalysed by histone lysine methyltransferases (HKMT) and uses S-adenosyl-L-methionine (SAM) as methyl donor¹³⁹. Methylation also occurs on the ω -guanidino group of arginines, catalysed by protein arginine methyltransferases (PRMTs)¹⁴⁰. Methyl group removal is catalysed by lysine demethylases either from the lysine-specific demethylase (LSD1) family or jumonji histone demethylase (JHDM) family^{141, 142}. Methylation is associated with both transcriptional activation and repression, depending on the modified histone residue and the degree of methylation i.e. mono-, di- or trimethylation (Figure 1.4)¹⁴³.

Euchromatic domains are relaxed, 30 nm fibres and facilitate the docking of chromatin binding factors that allow a transcriptionally permissive chromatin state. These domains are often marked with “active” histone PTMs, including histone 3 lysine 9 acetylation (H3K9ac) and H3K4me3. Comparatively, heterochromatin indicates compact, 11 nm fibres inaccessible to DNA binding factors and therefore transcriptionally “repressive”, marked by “repressive” PTMs such as H3K9me3¹⁴⁴.

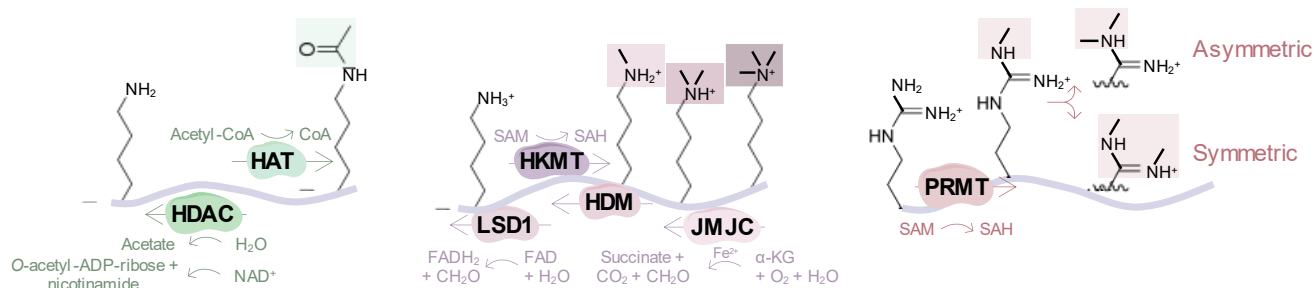


Figure 1.4. Mechanism of acetylation and methylation of histone lysine and arginine residues.

Lysine acetylation is catalysed by HAT that facilitates the transfer of an acetyl group from acetyl-coenzyme A to the ϵ -amino group of the lysine residue, while the removal of acetyl groups is achieved by Zn²⁺-dependent histone deacetylases (HDACs) or by NAD⁺-dependent sirtuins. Methylation of lysine and arginine residues are catalysed by histone (lysine) methyltransferases (H[K]MTs) that require S-adenosyl-L-methionine (SAM) as a methyl donor, producing S-adenosyl-L-homocysteine (SAH). Demethylation is catalysed by histone demethylases (HDMs), lysine-specific demethylase (LSD1), and Jumonji-C containing histone demethylases (JMJC). The oxidation of a methyl group (with FAD or α -ketoglutarate and molecular oxygen as oxidizing agents) is followed by the removal of formaldehyde in LSD1 and JMJC. Protein arginine methyltransferases (PRMTs) transfer a methyl group ω -guanidino group of arginine residues to yield monomethylarginine and symmetric or asymmetric dimethylarginine residues.

Histone PTMs attract epigenetic 'reader' proteins that subsequently recruit chromatin remodellers to catalyse positioning of nucleosomes and induce conformational changes to chromatin via ATP hydrolysis¹⁴⁵⁻¹⁴⁸. Readers bind to histone PTMs via distinct 'reader domains' that include chromodomains (CD) and bromodomain proteins (BDP) that read particular lysine methylations (mostly associated to repressive heterochromatin, e.g., the reader of histone 3 lysine 4 trimethylation, H3K4me3 is a PHD-finger) and acetylation, respectively, Tudor domain containing proteins that read both lysine and arginine methylation and plant homeodomains for lysine trimethylation.

Epigenetic genome regulation in *P. falciparum* parasites is not only extraordinary compared to other higher eukaryotes but contributes significantly to life cycle regulation. Evidence for these conclusions is discussed in the following sections.

1.6 Epigenetic gene regulation in *P. falciparum* parasites

Epigenetic gene regulation in *Plasmodium* is evolutionary diverse from other eukaryotes. The *Plasmodium* epigenome is primarily euchromatic¹⁴⁹, marked with an unusual hydroxymethylation-like modification to cytosine nucleobases¹⁵⁰, lack linker histone H1¹⁵¹, have a short nucleosome repeat length of off ~155 bp (408), does not undergo chromosomal condensation during mitosis⁵³ and does not encode for RNA interference machinery¹⁵².

Histone PTMs underlie many life cycle processes in the parasite, most notably, transcriptional activity, DNA replication, cell cycle progression, host-parasite interactions, and pathogenesis. Although several PTMs have been identified and described in *P. falciparum*, many of the PTMs have not been associated with biological relevance. Here, the focus will be placed on the contribution of acetylation, methylation and phosphorylation as key histone PTMs on canonical and histone variants to inform important biological processes for the parasite.

Histone N-terminal tail PTMs lie at the foundation of critically important biological processes of the parasite, including clonal variant gene expression (CVGE) and commitment to gametocytogenesis. CVGE is fundamentally a bet-hedging strategy involving the stochastic generation of phenotypic diversity, where isogenic parasites under the same environmental conditions can conserve a variant gene (*var* gene) in differential transcriptional states. The *var* gene family consisting of 60 virulence factor members, allowing parasites to evade host immune recognition through switching of these prime immune targets. Epigenetic control of *var* genes involve a combination of repressive histone marks (H3K9me3), activation marks (H3K9ac, H3K4me3), histone variants (H2A.Z, H2B.Z), long non-coding RNA (lncRNA) and localisation to the perinuclear heterochromatin^{153, 154}. In *Plasmodium*, lncRNAs are expressed in a stage-specific manner by maintaining telomere and subtelomeric repetitive sequences in a heterochromatic status¹⁵⁵.

Commitment to terminal cellular differentiation (gametocytogenesis) is associated with induction of an irreversible transcriptional program, mediated by epigenetic mechanisms.^{63, 103, 104, 156, 157} During the IDC, transcription of the *ap2-g* locus is silenced by heterochromatin facilitated by H3K9me3¹⁵⁸, which is bound by heterochromatin protein 1 (PfHP1)¹⁵⁹. PfHP1 acts to protect H3K9me3 from demethylase activity and nucleosomal decondensation and associates with *P. falciparum* histone deacetylase 2 (PfHDAC2) to maintain H3K9me3 and prevent acetylation at this position. Another AP2 member (AP2-HC, AP2-heterochromatin) also form part of this complex¹⁶⁰, although its role in maintaining heterochromatin at the *ap2-g* locus is unknown (Figure 1.5). At around 36 hpi¹⁶¹, the *ap2-g* locus is activated. Gametocyte-development protein 1 (PfGDV1) acts antagonistically against PfHP1¹⁶²⁻¹⁶⁴, potentially impairing maintenance of the PfHP1-H3K9me3 association. Demethylation of H3K9me3 subsequently occur that then allows for the deposition of H3K9ac to enable a euchromatic status for *pfap2-g* transcriptional activation, driven by binding of PfAP2-G itself^{165, 166}.

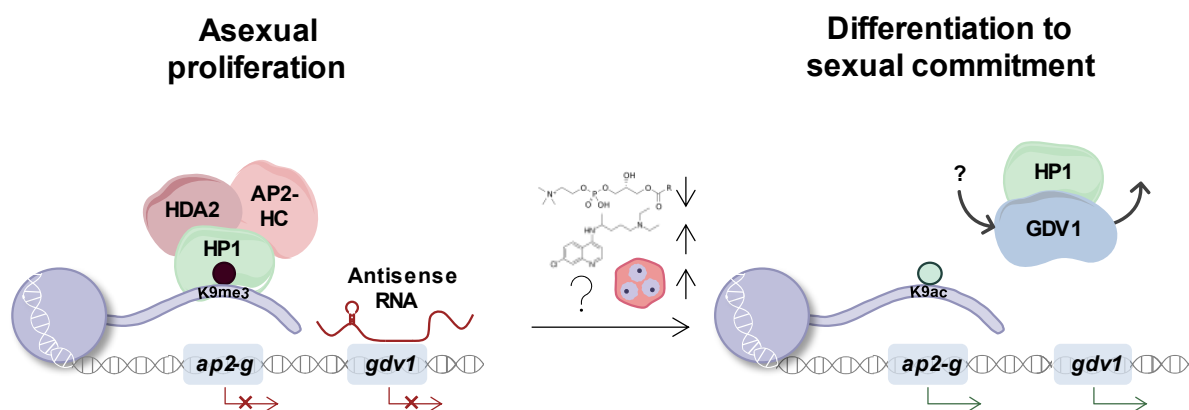


Figure 1.5. Involvement of epigenetic regulation in commitment to gametocytogenesis in *P. falciparum*. In asexual parasites, *ap2-g* is transcriptionally silenced by H3K9me3 bound PfPFHP1 in association with PfHDA and AP2-HC. Additionally, the *gdv1* locus is repressed by *gdv1* antisense RNA. A combination of depleted lysophosphatidylcholine, high parasitaemia (*in vitro*), antimalarial drug presence and a host of other unknown factors trigger the eviction of PfHP1 from the *ap2-g* locus with the assistance of the self-regulation PfGDV1.

Aside of the involvement of epigenetic regulation of CVGE and induction of gametocytogenesis, one of the most characteristic features of the epigenetic landscape is the highly dynamic and differentiated histone PTM profile across various developmental stages of *P. falciparum* ^{151, 153, 154, 167, 168}.

1.7 The histone post-translational landscape of *P. falciparum* parasites

Currently, the majority of PTMs identified on the N-terminal tails of canonical and variant histones in *P. falciparum* involve acetylation, methylation, phosphorylation, SUMOylation and ubiquitination, with the first three the most frequently observed, particularly on H3 and variant histone H3.3, followed by histone H4 (Figure 1.6) ^{153, 154, 167-174}. Ubiquitination and SUMOylation sites and it is thought to be involved in parasite-specific processes such as earmarking stage-specific proteins for degradation (151, 154).

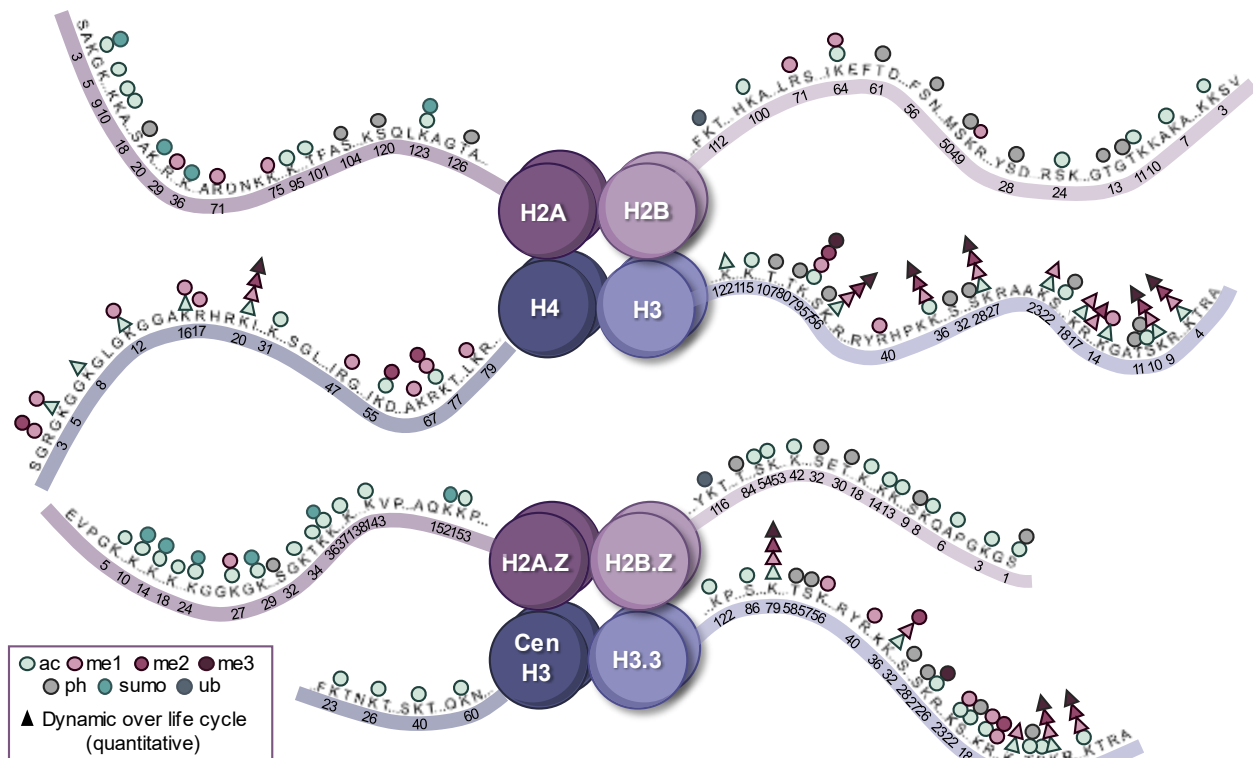


Figure 1.6. The current histone PTM landscape of *P. falciparum* parasites. Map of histone PTMs identified to date in both asexual and sexual life cycle stages in *P. falciparum* parasites combined. The top panel indicates the canonical histones whilst the bottom panel indicates modifications on the variant histones. Histone PTMs that have been quantified across different life cycle stages are shown in triangle shapes (▲), while qualitative PTMs in single or multiple life cycle stages are shown in circles (●). Amino acids (K: lysine, S: serine, R: arginine, T: threonine, A: alanine) are numbered starting from the N-terminal end of histone tails and shown with the corresponding modification(s) including acetylation (○), mono- (○), di- (○) and tri-methylation (●), phosphorylation (○), SUMOylation (●) and ubiquitination (●). Each of the four canonical (H2A, H2B, H3 and H4) and four variant histones (H2A.Z, H2B.Z, H3.3 and centromeric H3 [CenH3]) are shown.

Unlike the human genome which is mostly heterochromatic, *P. falciparum* histones contain primarily euchromatic PTMs during asexual parasite proliferation and contribute to its transcriptionally permissive nature (90% of genes actively transcribed), similar to those of other unicellular eukaryotes¹⁷⁵. Amongst others (Table 1.1), these PTMs include H3K4me1/me2/me3, H3K9ac, H3K14ac, H3K27ac, H3K56ac and H4ac, positively correlated with transcription in trophozoites and schizonts. Although the majority of PTMs that occur in asexual parasites are linked to euchromatin, not all euchromatic PTMs directly correlate with transcription. For instance, H3K4me3 and H3K9ac are widespread across the genome demarcated by H2B.Z in asexual parasites and mark the 5' termini of actively expressed genes. However, only H3K9ac is correlated with transcription^{153, 176}. Modifications typical of heterochromatin include H3K9me3, H3K79me3, H4K20me3, and H3K27me3¹⁶⁷, with H3.3K79me3 and H3K56me1/me2 being abundant in the transcriptionally inactive ring stages.

H4K20me3, interestingly, is highly predictive of transcription in the schizont stage, but does not mark repressive regions in ring stages of the parasite ^{153, 176-179}. H3K9me3 is the most extensively investigated in *P. falciparum* and is enriched to subtelomeric regions of the genome where it has important roles in both asexual parasites and gametocytes in governing CVGE in asexual parasites and commitment to gametocytogenesis ¹⁵⁹.

The transcriptional changes that occur during the IDC and gametocytogenesis are matched by dynamic changes of histone PTMs ^{90, 92, 180}. The shift from the permissive euchromatic status of asexual proliferation to a more heterochromatic nature during gametocytogenesis (151), is reflected by the repression of asexual-specific gene sets that are marked with H4K20me3, H3K27me3 and H3K36me3 (239). H3K9me3 increases in abundance from asexual parasites to early-stage (stage I-III) gametocytes, the latter being largely associated with condensed chromatin during which, relative to asexual stages, a lower abundance of transcripts are produced ¹⁶⁷. Later stages of gametocyte maturation (stages IV and V) show a significant rise in transcriptionally permissive PTMs such as H3K4me1 and H3K18ac, but transcriptionally restrictive PTMs like H3K79me3 remain. Furthermore, mass spectrometry has discovered a subset of gametocyte-specific histone PTMs, which includes H3K36me2, H3K36me3, and H3K79me3 marks exclusive to early- or late-stage gametocytes, whereas H3K9me2 and H3K18me1 were only detected during the IDC. ¹⁶⁷.

While some PTMs in particular are only detected in some of the stages investigated (e.g., H3K9me2, H3K18me1 and H4K20me3 during the IDC ¹⁸¹), euchromatic PTMs including H3K4ac, H3K9me3, H3K36me2, H3K122ac, H4ac and H4K20me3 are conserved across asexual and gametocyte stages of the parasite, albeit present in different relative abundances ¹⁶⁷. This suggests that these PTMs are necessary to maintain the chromatin conformation at specific regions or for constitutive expression of genes important for all stages of parasite development. For example, H3K9ac occupies intergenic regions and is associated with the expression of *var* genes by recruiting PfBDP1 to invasion gene promoters to interact with AP2-I, as PfBDP1 has been found in complex with AP2-I ^{102, 182, 183}. Altogether, this dynamics in PTMs show that the chromatin structure shape-shifts throughout development to accommodate for stage specific gene expression. This points toward the important regulatory role some PTMs might play in other maintaining chromatin structure or by recruiting other effector molecules rather than recruitment of transcription machinery to the sites it demarcates.

The writers and erasers of the above histone PTMs in *P. falciparum* have been investigated and show some divergence from other cells. This includes indications of specificity towards certain life cycle stages as well as specific histone PTMs. Additionally, in some instances, clear indications of essentiality have been obtained, including the important requirement of several histone methyltransferases (Table 1.1), whilst interestingly, most of the demethylases seems to be dispensable to the parasite, which implicates deposition of methylation as a more important process in the parasite or that there is a redundancy between demethylase enzymes. By contrast, both deposition and removal of acetylation marks involve essential histone acetyltransferases (e.g., PfMYST and GCN5) and HDACs (e.g., PfHDAC1, 2 and 3) (Table 1.1).

Histone PTMs are 'read' and 'translated' by recruiting effector proteins to guide downstream nucleosome remodelling and coordinate protein assembly to fine-tune gene regulation¹⁸⁴⁻¹⁸⁶ (Table 1.1). These reader complexes are composed of 'writer' subunits, multiple 'reader' enzymes and/or chromatin remodelling enzymes¹⁸⁷. Several BDP family members for acetylated histone binding have been described, of which all but PfBDP3 are essential to parasite life cycle progression (Table 1.1). The parasite furthermore contains a few chromodomain proteins that are seemingly dispensable except for the clear requirement of PfHP1 and its association to H3K9me3. Amongst the Tudor domain proteins (without writer functions), PfPHD1 and 2 are required by the parasite, with the former involved in the binding of H3K9ac, H3K4me2/me3 and part of a SAGA-like complex (Table 1.1). Interestingly, humans encode for 36 Tudor-domain reader proteins that bind primarily to methylated arginine and to a lesser extent lysine residues while in *P. falciparum*, only two have been annotated, PfTSN and PfSMN (445). PfSMN is the only essential Tudor-domain protein and is increasingly expressed from stage IV gametocytes to ookinetes (273), suggesting a particular biological importance of arginine methylation in these stages.

The observed high degree of connectivity between the diverse, but highly interconnected epigenetic protein modules, as well as the various distinct 'flavours' of each module of the complexes, adds to the amount of complexity that *P. falciparum* employs to distinctly modulate gene expression.

Table 1.1. Catalogue of important chromatin-associated proteins in *P. falciparum*.

Protein	Gene ID	Essentiality	PTM	Note	Reference
WRITERS					
Histone lysine methyltransferases (HKMTs)					
PfSET1	PF3D7_0629700	Dispensable	H3K4	PbSET1 methylate H4R3, H3K4me3 enriched at intergenic regions	Cui <i>et al.</i> , 2008
PfSET2 (SETvs)	PF3D7_1322100	Dispensable	H3K36	<i>var</i> gene silencing	Jiang <i>et al.</i> , 2013; Ukaegbu <i>et al.</i> , 2014
PfSET3 (G9a)	PF3D7_0827800	Essential	H3K9		Voltz <i>et al.</i> , 2010; Lopez-Rubio <i>et al.</i> , 2009
PfSET4	PF3D7_0910000	Dispensable	H3K4	H3K4me3 enriched at intergenic regions	Cui <i>et al.</i> , 2008; Voltz <i>et al.</i> , 2010
PfSET5	PF3D7_1214200	Dispensable	H3K5/K8/K12		Cui <i>et al.</i> , 2008; Voltz <i>et al.</i> , 2010
PfSET6	PF3D7_1355300	Essential	H3K4		Cui <i>et al.</i> , 2008; Voltz <i>et al.</i> , 2010
PfSET7	PF3D7_1115200	Dispensable	H3K4, H3K9, H3K27		Chen <i>et al.</i> , 2016
PfSET8	PF3D7_0403900	Dispensable	H4K20	H4K20me1/me2/me3; PbSET8 methylate H4R23, H3K59	Cui <i>et al.</i> , 2008; Voltz <i>et al.</i> , 2010
PfSET9	PF3D7_0508100	Essential	H4K20		Cui <i>et al.</i> , 2008
PfSET10	PF3D7_1221000	Essential	H3K4	H3K4me1, H3K4me2; active <i>var</i> gene, H3K4me3 enriched at intergenic regions	Volz <i>et al.</i> , 2012
Histone acetyltransferases (HATs)					
PfGCN5	PF3D7_0823300	Defective	H3K9, H3K14	Also have a bromodomain, active <i>var</i> gene	Fan <i>et al.</i> , 2004
PfHAT1	PF3D7_0416400				Cui <i>et al.</i> , 2008
PfMYST	PF3D7_1118600	Essential	H4K5/K8/K12/K16		Miao <i>et al.</i> 2010
Protein arginine methyltransferase (PRMT)					
PfPRMT1	PF3D7_1426200	Dispensable	H3R3	Putative class I, H4R3me	Fan <i>et al.</i> , 2009
PfPRMT4/CAR M1	PF3D7_0811500	Essential		Putative class I	
PfPRMT5	PF3D7_1361000	Dispensable		Putative class II	
ERASERS					
Histone demethylases (HDMs)					
PfJmjC1	PF3D7_0809900	Dispensable	H3K9, H3K36		Cui <i>et al.</i> , 2008; Voltz <i>et al.</i> , 2010
PfJmjC2	PF3D7_0602800	Dispensable			Cui <i>et al.</i> , 2008; Voltz <i>et al.</i> , 2010
PfJmj3	PF3D7_1122200	Dispensable	H3K36		Cui <i>et al.</i> , 2008; Matthews <i>et al.</i> , 2020
PfLSD1	PF3D7_1211600	Dispensable			Cui <i>et al.</i> , 2008; Voltz <i>et al.</i> , 2010
Histone deacetylases (HDACs)					
PfSIR2A	PF3D7_1328800	Dispensable		Transcriptional regulatory protein involved in telomere maintenance and regulation of <i>var</i> gene expression	Merrick <i>et al.</i> , 2010; Mancio-Silva <i>et al.</i> , 2013
PfSIR2B	PF3D7_1451400	Dispensable		Transcriptional regulatory protein involved in telomere maintenance and regulation of <i>var</i> gene expression	Petter <i>et al.</i> , 2011
PfHDAC1	PF3D7_0925700	Essential		Class I histone deacetylase 1	Chaal <i>et al.</i> , 2010
PfHDAC2	PF3D7_1008000	Essential		Class II histone deacetylase 2	Coleman <i>et al.</i> , 2014
PfHDAC3/PfHD A2	PF3D7_1472200	Essential		Class II histone deacetylase, putative, <i>var</i> gene silencing and sexual differentiation	Chaal <i>et al.</i> , 2010; Coleman <i>et al.</i> , 2014
READERS					

Bromodomains: Acetylated histone binding					
PfSET1	PF3D7_0629700	Essential	H3K4	Also has PHD-finger domain	Cui <i>et al.</i> , 2008
PfGCN5	PF3D7_0823300	Defective	H3K4me3	Also has HAT domain, forms part of SAGA-like complex	Hoeijmakers <i>et al.</i> , 2019; Miao <i>et al.</i> , 2021
PfBDP1	PF3D7_1033700	Essential	H2B.ZK3/K8/K13/K14/K198ac H3K18/K27ac, H4K5/K8/K12ac	Erythrocyte invasion	Hoeijmakers <i>et al.</i> , 2019; Josling <i>et al.</i> , 2015; Nyarko <i>et al.</i> , 2020
PfBDP2	PF3D7_1212900	Essential	H2B.ZK3/K8/K13/K14/K198ac H4K5/K8/K12ac		Hoeijmakers <i>et al.</i> , 2019; Josling <i>et al.</i> , 2015; Nyarko <i>et al.</i> , 2020
PfBDP3	PF3D7_0110500	Dispensable			Fleck <i>et al.</i> , 2021
PfBDP4	PF3D7_1475600	Defective			Fleck <i>et al.</i> , 2021
PfBDP5/TAF1	PF3D7_1234100	Essential			Fleck <i>et al.</i> , 2021
PfBDP6/TAF2	PF3D7_0724700	Essential			Fleck <i>et al.</i> , 2021
PHD-finger/ZF-CW domain: Methylated and acetylated histone binding					
PfSET1	PF3D7_0629700	Essential	H3K4		Cui <i>et al.</i> , 2008
PfSET2	PF3D7_1322100	Dispensable			Jiang <i>et al.</i> , 2013; Ukaegbu <i>et al.</i> , 2014
PfSET10	PF3D7_1221000	Essential			Volz <i>et al.</i> , 2012; Schwach <i>et al.</i> , 2012
PfSUMO ligase	PF3D7_1360700	Essential			Reiter <i>et al.</i> , 2016; de Oliveira <i>et al.</i> , 2020
PfLSD1	PF3D7_1211600	Dispensable			Kaur <i>et al.</i> , 2016; Liu <i>et al.</i> , 2018
PHD finger containing protein	PF3D7_0310200	Dispensable			Cowell <i>et al.</i> , 2018
PfPHD1	PF3D7_1008100	Defective	H3K9ac, H3K4me2/me3	Forms part of SAGA-like complex	Hoeijmakers <i>et al.</i> , 2019; Miao <i>et al.</i> , 2021
EELM2 domain containing	PF3D7_1141800	Dispensable	H2B.ZK13/K14/K18ac		Hillier <i>et al.</i> , 2019; Hoeijmakers <i>et al.</i> , 2019
PfPHD2	PF3D7_1433400	Essential			Hoeijmakers <i>et al.</i> , 2019; Miao <i>et al.</i> , 2021
PfZFP	PF3D7_0420000	Dispensable			Ngwa <i>et al.</i> , 2021
Chromodomain-like: Methylated histone binding					
PfHP1	PF3D7_1220900	Essential	H3K9me3	Sexual differentiation, regulation of <i>var</i> family	Bui <i>et al.</i> , 2021; Flueck <i>et al.</i> , 2009; Pérez-Toledo <i>et al.</i> , 2009
PfMYST	PF3D7_1118600	Essential		Also has HAT and ZnF_C2H2 domains, occupy <i>var</i> gene promoter, intergenic regions,	Miao <i>et al.</i> , 2010
Chromo-domain protein	PF3D7_1140700	Dispensable			Hoeijmakers <i>et al.</i> , 2019
PfCHD1	PF3D7_1023900	Dispensable	H3K9me3	chromodomain-helicase-DNA-binding protein 1 homolog, putative	Watzlowik <i>et al.</i> , 2021
14-3-3 – Phosphorylated histone binding					
14-3-3I	PF3D7_0818200	Defective	H3S10, H3S28, H3S32	Binds CDPK1	Jain <i>et al.</i> , 2020; Lasonder <i>et al.</i> , 2021
14-3-3II	PF3D7_1362100	Defective			Meerstein-Kessel <i>et al.</i> , 2021
14-3-3 protein	PF3D7_1422900	Defective			Ukegbu <i>et al.</i> , 2021

YEATS domain: Acetylated and crotonylated histone binding					
YEATS domain-containing protein	PF3D7_0807000	Essential			Sato <i>et al.</i> , 2021
Tudor domain: Methylated histone binding					
PfTSN	PF3D7_1136300	Dispensable		RNA-binding, <i>Staphylococcus aureus</i> nuclease homologue	Counihan <i>et al.</i> , 2021; Hossain <i>et al.</i> , 2008
PfSMN	PF3D7_0323500	Essential		Survival motor neuron-like protein	Hossain <i>et al.</i> , 2008
OTHER CHROMATIN ASSOCIATED PROTEINS					
PfNAPS	PF3D7_0919000	Dispensable		Nucleosome assembly protein	Josling <i>et al.</i> , 2015
PfISWI	PF3D7_0624600	Dispensable		ISWI chromatin-remodeling complex ATPase	Cui <i>et al.</i> , 2010; Bryant <i>et al.</i> , 2020
PfSnf2	PF3D7_0820000	Dispensable		Snf2-related CBP activator, putative	Watzlowik <i>et al.</i> , 2021
PfADA2	PF3D7_1014600			Transcriptional co-activator, associate with PfGCN5.	Fan <i>et al.</i> , 2004
Unknown	PF3D7_1225200	Essential		DNA-binding protein, putative	Hillier <i>et al.</i> , 2019; Hoeijmakers <i>et al.</i> , 2019
PfSF3B3	PF3D7_1234800				Mobegi <i>et al.</i> , 2014
PfHMGB3	PF3D7_1205800	Dispensable		High mobility group protein B3, putative	Lima <i>et al.</i> , 2016
Unknown	PF3D7_1402800	Dispensable			Hoeijmakers <i>et al.</i> , 2019; Miao <i>et al.</i> , 2021
Prohibitin	PF3D7_1014700	Essential			Hoeijmakers <i>et al.</i> , 2019; Singh <i>et al.</i> , 2021
PfAP2-I	PF3D7_1007700	Essential	H3K18ac, H3K27ac		Hoeijmakers <i>et al.</i> , 2019; Santos <i>et al.</i> , 2017
PfSUMO	PF3D7_0505800	Essential			Hamilton <i>et al.</i> , 2014
PfUB	PF3D7_1211800	Essential			Ng <i>et al.</i> , 2017
PfpUB	PF3D7_1365900	Essential			Green <i>et al.</i> , 2020
PfUBC13	PF3D7_0527100	Essential			Maneekesorn <i>et al.</i> , 2021
PfUCH54	PF3D7_1117100	Essential			Karpiyevich <i>et al.</i> , 2019
PfAP2-G2	PF3D7_1408200	Dispensable			Singh <i>et al.</i> , 2021
Unknown	PF3D7_1239800	Essential			Singh <i>et al.</i> , 2021; Baum <i>et al.</i> , 2013; Hoeijmakers <i>et al.</i> , 2019
Chromatin remodeling protein	PF3D7_1104200	Essential		Chromatin remodelling protein	Hillier <i>et al.</i> , 2019, van Biljon <i>et al.</i> , 2019
CAF1/WD40 domain protein	PF3D7_0110700	Essential		Chromatin assembly factor 1 subunit C, putative	Kaushik <i>et al.</i> , 2016
PfCAF1	PF3D7_0501800	Essential		Chromatin assembly factor 1 subunit A	Miao <i>et al.</i> , 2006
SWIB/MDM2 domain protein	PF3D7_0611400	Essential			Vieira <i>et al.</i> , 2016

1.8 Histone PTM combinations: A higher order of gene regulation?

PTMs are rarely found in isolation on N-terminal tails and rather, several histone PTMs coexist in chromatin states to define the biochemical milieu of genomic loci ¹⁸⁸. The combinatorial nature and functional impact of histone PTMs can be described through the 'histone code' hypothesis ^{136, 186}. The key tenet of the histone code hypothesis is that histone PTMs collectively enhance the information content and plasticity of chromatin in relation to the control of all DNA-dependent processes ¹⁸⁹. In recent years, there is mounting evidence to support the notion that different 'proteoforms' of histone tails are linked to different biological outcomes. As such, this greatly impacts our understanding of the chromatin regulatory system as histone PTM combinations, and the associated patterns of interactions between histone PTMs, have been demonstrated to correspond with chromatin states that allow DNA access and modify DNA-histone and histone-histone interactions.

1.8.1. Crosstalk between histone PTMs

The relationship between histone PTMs are defined as the crosstalk between two or more histone PTMs, where the presence or absence of one PTM can positively or negatively influence the writing, erasing, or reading of another PTM ¹⁹⁰. Positive histone PTM crosstalk involves one PTM requiring the presence of another PTM to mediate a biological function, for example, one PTM could increase the occurrence of another PTM, or one PTM requires another PTM for an effector protein to bind ¹⁹¹⁻¹⁹³. In mammalian cells, the interaction between the SAGA complex and H3K4me3 becomes stronger when H3K9 and H3K4 are acetylated on the same N-terminal tail, thereby enhancing transcription ¹⁹⁴. In mouse embryonic stem cells, the combination of H3K9acK14ac specifically correlate to the transcription of a subset of genes ¹⁹⁵. Finally, the combination of H3K4me1K14ac is collectively recognised by ZMYND8 to repress transcription of metastasis-linked genes in cancer cells ¹⁹⁶.

Negative histone PTM crosstalk describes the relationship where one PTM decreases or inhibits the deposition of another PTM or prevents an effector protein from binding. These interactions can also exist when a modification site must be vacant for a factor to bind or another PTM to be written or removed, rendering two histone PTMs mutually exclusive from one another. These include for example where asymmetrical H3R2me2, deposited by PRMT6, inhibits a MLL methyltransferase from trimethylating H3K4 ¹⁹⁷. PRMT5-mediated H3R8me2s antagonises the acetylation of H3K9 ¹⁹⁸, and asymmetrical H3R8me2 blocks the binding of HP1 to methylated H3K9 ¹⁹⁹. Finally, H3S10ph impairs and eject binding of HP1 to H3K9me3, thereby inducing euchromatin formation ^{200, 201}.

The crosstalk between histone PTMs is not limited to PTMs being on the same N-terminal tail but can also occur as trans-histone or even *trans*-nucleosomal events present in chromatin loops^{190, 202}. For example, JMJD2A recognises both H3K4me3 and H4K20me3 found at transcription start sites in stem cells through its double Tudor domain^{203, 204}. Finally, direct BRD4 binding necessitates both H3K9ac and H4K16ac to permit transcription elongation²⁰⁵.

The molecular machinery that governs PTMs and specifies their cellular roles lies at the heart of PTM crosstalk. Apart from having a direct physical effect on chromatin structure, histone PTMs may also function by directing certain downstream activities in chromatin by the selective recruitment of effector proteins or readers that 'dock' onto histone PTMs²⁰⁶⁻²⁰⁸. Differential downstream effects, such as the amount of transcriptional activation, are dependent on the binding of reader proteins. Reader proteins/complexes are sensitive to the histone PTMs that flank the target PTM, e.g., the cooperative binding of TATA-binding protein associated factor-1 (TAF1) binds to diacetylated histone H4 tails more effectively than monoacetylated tails^{209, 210}. The fact that readers with somewhat varied binding and/or signalling capabilities might influence the signalling of another PTM reader or form a unique cellular cascade when combined, suggests that histone combinations provide an extra level of regulation. It is furthermore possible that different PTM combinations reflect cellular metabolic states requiring distinct transcriptional programs^{211, 212}.

1.8.2 Co-existing histone PTMs in *P. falciparum* parasites

The occurrence of 30 co-existing PTMs have been described in *P. falciparum*¹⁶⁷, which include all H3K9 modifications (ac, me1, me2, and me3) with H3K14ac, as well as the same combinations for histone H3.3¹⁶⁷. Other combinations discovered included H3K18me1K23me1, H3K27me1/me2/me3K36me1/me2/me3, and H4ac at K5, K8, K12, and K16. The latter group of PTMs are connected to the poised state of *var* genes when combined with H3K9me3 and was discovered to be more common during gametocytogenesis than asexual proliferation^{169, 213}, implying a poised transcriptional state.

Combinations between di- and tri-methylated H3K27 and H3K36 were particularly stage specific in early-stage gametocytes where H3K36me2/me3 have recently been shown to repress asexual proliferation genes along with repression of genes necessary for commitment to gametocytogenesis (239). Lastly, the combination between H3K18ac and H3K23ac was exclusively enriched in mature gametocytes (stage V). H3K18ac and H3K27ac together dynamically mark regulatory regions of genes expressed during the asexual life cycle^{167, 214}. These combinations have also been validated on variant histone H3.3. Importantly,

combinations of histone PTMs are more abundant in gametocyte stages compared to asexual parasites, with ~60 % of combinations of histone PTMs identified occurring in gametocytes¹⁶⁷. This indicated the possibility that sexual differentiation and development are more reliant on the presence of combinatorial histone PTMs to regulate gene expression.

Evidence is mounting that *P. falciparum* parasites can interpret combinations of PTMs as the parasites encode for various histone readers (see Table 1.1) that exists in multiprotein complexes and could therefore bind to multiple histone PTMs simultaneously¹⁸⁷. Examples include the co-regulation of H3K9ac and H3K14ac by PfGCN5 in *P. falciparum*, similar to what is described in yeast (Figure 1.7)²¹⁵⁻²¹⁷. Additionally, H3K9 and H3K4 methylation co-exists with H3K14ac in *P. falciparum*. PfSET7, a histone methyltransferase (see Table 1.1), specifically methylates both H3K9 and H3K4 only when a pre-existing H3K14ac exists, indicating that the role of H3K9me3K14ac histone PTM combination is specific for PfSET7²¹⁸. This links to H3K9me3-controlled sexual commitment and suggests that the trivalent combination of H3K9me3K4me3K14ac is important for stage-specific epigenetic regulation (Figure 1.7). Interestingly, H3K9me3, PfHP1 and H3K36me3, which associate to heterochromatin and silenced promoters, are mutually exclusive from regions that are marked with H3K4me3 H3K9ac. The histone acetyltransferase, PfMYST, acetylates H4 at K5, K8, K12 and K16, a combination that increases in abundance from trophozoite to schizont stages although H4K8ac has been determined to be the defining PTM from the combination and regulates proliferation and host-parasite interactions regulating chromatin-linked transcriptional changes²¹⁹. Different combinations involving two of the four H4 acetyl PTMs were furthermore particularly abundant in schizonts and moderately abundant throughout gametocytogenesis, suggesting a function is particularly important for schizonts and less so for gametocytes²¹⁹.

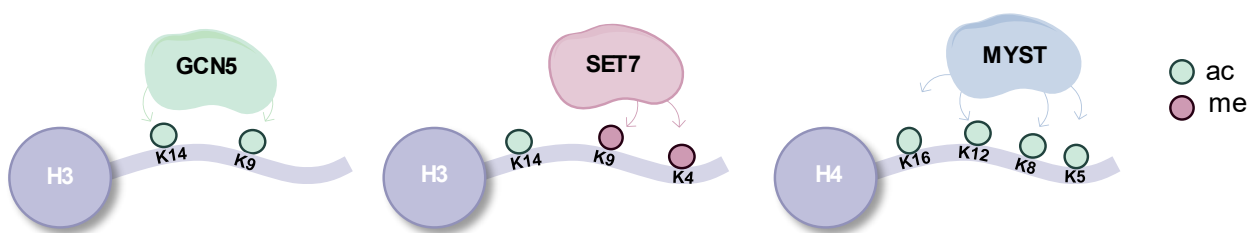


Figure 1.7. Examples of co-existing histone PTMs in *P. falciparum* and the associated writer enzyme. In asexual parasites, H3K9ac and H3K14ac is regulated by PfGCN5, while PfSET7 only methylates H3K9 and H3K4 in the presence of a co-existing H3K14ac. Lastly, PfMYST writes four H4 acetylation's in position H4K5, H4K8, H4K12 and H4K16.

In conclusion, the analysis of single PTMs results in an incomplete picture of the epigenetics regulation in the parasite. Advancing technology to investigate the abundance of combinatorial histone PTM codes, as well as the ability to now identify the readers thereof, might offer a better understanding of which proteins are critical to regulate parasite development and potentially provide treatment options if these were effectively inhibited. A single PTM could occupy different types of chromatin, but a combination of PTMs is more likely to be univocal and therefore more specific if a specific reader is targeted. The potential that different histone PTM combinations could drive a unique epigenetic state in *P. falciparum* is evident (198). However, the vast majority of combinations have not been explored in any systematic form before in *P. falciparum*, although this has now been extensively done in humans^{193, 220-222} and *Caenorhabditis elegans*²²³. Investigation of the underlying mechanisms and fundamental significance of the combinations of histones forming the histone code in *P. falciparum* parasite would fundamentally contribute to the holistic understanding of the essential biology driving development and differentiation of this parasite.

1.9 Hypothesis

This study hypothesises that combinations of histone PTMs drive asexual development and sexual differentiation of *P. falciparum* parasites.

1.10 Aims and objectives

The aim of this study was to define the co-existing histone PTMs landscape of asexual and sexual forms of the *P. falciparum* parasite and identify the proteome associated with specific co-existing histone PTMs to parasite development.

Specific objectives of the study are described as:

1. **To globally identify all co-existing histone PTMs through middle-down proteomics (Chapter 2).** Here, the combinatorial histone PTM landscape was characterised in asexual parasites and immature and mature gametocytes. Stage-specific crosstalk was described that involved novel arginine methylations.
2. **To characterise the proteome landscape associated with histone PTMs that uniquely co-exist in mature stage gametocytes (Chapter 3).** H3K18acK23ac was probed in mature gametocytes to identify its regulatory proteins. Unique epigenetic complex partners were identified, including proteins that interact with the SAGA-like complex and the AP2-G2 transcription factor.
3. **To functionally validate the importance of co-existing histone PTMs by chemical interference of the associated proteome (Chapter 4).** Several HDAC, HAT, HKMT and PRMT inhibitors were tested for their antiparasmodial activity against asexual parasites, immature and mature gametocytes with combinations able to synergise based on perturbation of co-existing histone PTMs.

1.11 Research outputs

Peer reviewed publications:

1. von Grüning, H., Coradin, M., Mendoza, M. R., Reader, J., Sidoli, S., Garcia, B. and Birkholtz, L. (2021) A dynamic and combinatorial histone code drives malaria parasite asexual and sexual development. In review: *Molecular and Cellular Proteomics*. Preprint doi: <https://doi.org/10.1101/2021.07.19.452879>
2. Coetzee, N.*, von Grüning, H.*, Opperman, D. van der Watt, M., Reader, J. and Birkholtz. (2020) Epigenetic inhibitors target multiple stages of *Plasmodium falciparum* parasites. *Sci Rep* **10**, 2355. <https://doi.org/10.1038/s41598-020-59298-4>. * - these authors contributed equally as joint first authors.
3. Connacher J., von Grüning H., and Birkholtz L. Histone modification landscapes as the roadmap for malaria parasite development. Invited review in *Frontiers in Cellular and Developmental Biology: Special Issue "How Epigenetic Mechanisms Contribute to Host-Parasite Interactions*. 2022 – in review.

Conference and seminar proceedings:

Oral presentations:

1. H von Grüning. Invited Plenary. Decoding combinatorial patterns of histone post-translational modifications of *Plasmodium falciparum*. Huck Centre for Malaria Research Seminar at the Pennsylvania State University. State College, United States. 26 September 2019.
2. H von Grüning. Invited Plenary. Decoding combinatorial patterns of histone post-translational modifications of *Plasmodium falciparum*. Department of Biochemistry, Genetics and Microbiology at the University of Pretoria. Pretoria, South Africa. 12 October 2020.
3. von Grüning, H., Coradin, M., Sidoli, S., Garcia, B. A. and Birkholtz, L. Middle-down proteomics reveal crosstalk between histone post-translational modifications during *Plasmodium falciparum* parasite development. BioMalPar XVII: Biology and Pathology of the Malaria Parasite. Virtual. 26 May 2021.
4. von Grüning, H., Coradin, M., Sidoli, S., Garcia, B. A. and Birkholtz, L. Middle-down proteomics reveal crosstalk between histone post-translational modifications during *Plasmodium falciparum* parasite development. Molecular Parasitology Meeting XXXII 2021. Hybrid conference, Woods Hole, Massachusetts, USA). 8 October 2021.

Poster presentations

1. von Grüning, H., Coradin, M., Mendoza, M. G., Sidoli, S., Garcia, B. A. and Birkholtz, L. Decoding combinatorial patterns of histone post-translational modifications of *Plasmodium falciparum*. EMBL BioMalPar VX: Biology and Pathology of the Malaria Parasite. Heidelberg, Germany. 28-30 May 2019.
2. von Grüning, H., Coradin, M., Mendoza, M. G., Sidoli, S., Garcia, B. A. and Birkholtz, L. Decoding combinatorial patterns of histone post-translational modifications of *Plasmodium falciparum*. 30th Annual Molecular Parasitology Meeting. Woods Hole Massachusetts, USA. 14-19 September 2019.
3. von Grüning, H., Coradin, M., Sidoli, S., Garcia, B. A. and Birkholtz, L. Decoding combinatorial patterns of histone posttranslational modifications of *Plasmodium falciparum*. Molecular Parasitology Meeting XXXI. Online. 21-24 September 2020.

Chapter 2 | A dynamic and combinatorial histone code drives malaria parasite asexual and sexual development

The work in this chapter has been included in a manuscript as follows:

von Grüning, H., Coradin, M., Mendoza, M. R., Reader, J., Sidoli, S., Garcia, B. A. and Birkholtz, L. (2021) A dynamic and combinatorial histone code drives malaria parasite asexual and sexual development. *Molecular and Cellular Proteomics* | DOI: 10.1016/j.mcpro.2022.100199

2.1 Introduction

Histone N-terminal tails are reversibly modified by an array of covalent histone PTMs. These alter chromatin structure to fine tune gene expression in most eukaryotes, resulting in changes in cell fate. Although the contribution of individual histone PTMs in specific biological processes are well described^{224, 225}, there is growing evidence to indicate that histone PTMs not only function individually but also act in a concerted manner to direct transcriptional programs that ultimately defines a cell's fate and function¹⁸⁵, such as to proliferate^{193, 226}, differentiate^{227, 228}, or become quiescent^{229, 230}. This association between histone PTMs that work in coordination has been postulated to constitute a functionally-relevant, unique pattern or 'histone code'^{186, 231}.

As an example of the importance of histone PTM crosstalk, the presence of histone H3, serine 10 phosphorylation (H3S10ph) impairs the binding of the effector protein heterochromatin protein 1 (PfHP1) to the well-known repressive PTM, H3K9me3²³². This blocks cellular differentiation in mouse embryonic stem cells^{201, 233}. Such drastic changes in gene regulation and cellular fate can also be effected by combinations of PTMs that include typically less abundant PTMs e.g. H3K9ac and H3K14ac affect H3R8me2^{234, 235}.

P. falciparum is the causative agent of severe malaria in humans, and undergoes rapid rounds of cell division during its asexual replication every ~48 h. A small percentage of asexual parasites differentiate to gametocytes through five distinct stages of development (stages I-V) over ~14 days in *P. falciparum*, after which mature male and female stage V gametocytes can be transmitted to the mosquito vector^{236, 237}. The parasite's chromatin organisation fluctuates between mostly euchromatic in asexual parasites, characterised by transcriptionally permissive PTMs of H3K9ac and H3K4me3^{153, 176, 238}, and more heterochromatic states during gametocytogenesis, marked with H3K9me3, H4K20me3 and extended HP1 occupancy^{167, 239}. However, nuanced distinctions exist between the different developmental stages during gametocytogenesis and underscores the transcriptional differences between the early- (stage II/III) and late-stage (stage IV/V) gametocytes⁹². Typical heterochromatic PTMs such as H3K27me3 (facultative heterochromatin), H3K9me3 (constitutive heterochromatin) and

H3K36me3 (predominantly associated with heterochromatin in *P. falciparum*),^{240, 241} are exclusive to the immature, early-stage gametocytes in *P. falciparum*^{167, 241} whilst more mature stages do contain euchromatic PTMs (H3K4me3 and H4K8ac) in preparation for onwards transmission and gamete formation¹⁶⁷. Several of these individual histone PTMs have been demonstrated to be essential to various biological processes, through gene knockout or chemical perturbation of histone ‘writer’ and ‘eraser’ enzymes^{86, 242}.

Evidence of histone PTM combinations in *P. falciparum* is sparse but includes methylation of H3K4 or H3K9 by the histone lysine N-methyltransferase SET7, only in the presence of already acetylated H3K14²¹⁸. H4K8ac, as a likely regulator of parasite proliferation in asexual parasites¹⁶⁹, is also found in combination with H4K5ac, H4K12ac, and H4K16ac as a result of the acetyltransferase activity of MYST²¹⁹. Quantitative chromatin proteomics alluded to the presence of additional co-existing histone PTMs in *P. falciparum* parasites, with prominent stage specificity and increased presence during gametocytogenesis^{167, 168}.

The majority of co-existing histone PTM pairs are typically identified with a peptide-centric proteomics pipeline, frequently named “bottom-up”. Histones are digested with trypsin or other enzymes that generate relatively short peptides before MS analysis. With this workflow, co-occurrences between histone PTMs can be identified only for those which localise nearby in the amino acid sequence. However, this proteomic approach is not suitable for distal co-occurring PTMs. As such, distinctively modified peptides and co-occurring PTMs cannot be accurately identified and quantified. The development of “middle-down” MS has advanced proteomics and allowed the complexity of PTM combinations to be investigated^{243, 244}. Although technically challenging, middle-down MS allows evaluation of longer histone tails of ~50-60 residues to accurately and simultaneously identify and quantify hundreds of combinatorial PTMs^{245, 246}. With this powerful approach, system-level crosstalk of multiple, interacting co-existing PTMs defined clear, combinatorial histone codes in nematodes²²³, mammalian cells^{193, 220, 221, 245}, cells undergoing epithelial to mesenchymal transition^{245, 247-250} and stem cell reprogramming²⁵¹.

Here, we present the systems-level identification and characterization of the combinatorial histone code of *P. falciparum* parasites, which was generated using quantitative, middle-down proteomics. We identify a comprehensive histone code that is dynamic and has distinct fingerprints in different life cycle stages, implying refined functions to allow different biological outcomes associated with parasite pathology and survival. Several co-existing PTMs are involved in direct crosstalk, indicative of coordinated function, particularly in gametocytes, with the code in immature gametocytes being most connected and mature gametocytes using unique combinations. This is exemplified by the functional association of H3K18acK23ac that

interacts with a unique reader complex in mature gametocytes to enable strategy-specific gene expression. With this first, comprehensive report of a combinatorial histone code in a eukaryotic parasite, we show that *P. falciparum* relies on dynamic interactions between histone PTMs for development and differentiation. This data could serve as a model for the importance of combinatorial histone PTMs in pathogenesis in protista.

2.2 Experimental procedures

2.2.1 *In vitro* cultivation of *P. falciparum* asexual parasites and gametocytes

All *in vitro* experiments involving human blood donors holds ethics approval from the University of Pretoria Research Faculty of Health Sciences Ethics Committee (506/3018 & NAS332/2019) and for working with human malaria parasites from the Faculty of Natural and Agricultural Sciences (180000094). This work abides by to the Declaration of Helsinki principles. Intra-erythrocytic *P. falciparum* parasites (NF54 strain, drug-sensitive) was cultivated in fresh human erythrocytes (either A⁺ or O⁺) in RPMI-1640 culture medium supplemented with 25 mM HEPES (pH 7.5), 0.2 mM hypoxanthine, 0.024 µg/µL gentamycin, 5 µg/µL Albumax II, 23.81 mM sodium bicarbonate and 0.2 % (w/v) D-glucose. Cultures were maintained with daily media change and fresh erythrocyte supplementation at 5 % haematocrit, 2 % parasitaemia under hypoxic conditions (5 % O₂, 5 % CO₂, 90 % N₂) with moderate shaking at 37 °C. Parasites were synchronised to > 90 % ring stages with 5 % (w/v) D-sorbitol. Gametocytogenesis was initiated from a 0.5 % parasitaemia, 6 % haematocrit ring-stage asexual culture by inducing stress through a combination of a drop in haematocrit (to 4 %) and nutrient deprivation (reduced glucose concentrations) ²⁵². After 24 h, parasites committed to gametocytogenesis were further maintained in nutrient-enriched culture media as above, but with the supplementation of 50 mM N-acetyl glucosamine to remove any remaining asexual parasites. Gametocytogenesis was subsequently supported in this media for 10-14 days, with daily media changes and under stationary, hypoxic conditions.

2.2.2 Histone isolation from *P. falciparum* parasites

Histones were isolated and enriched as previously described ^{167, 253}. Briefly, *P. falciparum* parasites (3.0 ± 0.9 % parasitaemia) were released from erythrocytes by incubating with 0.06 % (w/v) saponin, followed by three consecutive washes in 1x phosphate-buffered saline (PBS) with centrifugation at 12 000 xg for 10 min. Nuclei were released from these parasites using a hypotonic buffer containing 10 mM Tris-HCl (pH 8.0), 3 mM MgCl₂, 0.2 % v/v Nonidet P40, 0.25 M sucrose and cOmplete™ EDTA-free cocktail of protease inhibitors. This hypotonic buffer wash was repeated twice, centrifuged at 500 xg, 4 °C for 10 min. The chromatin-containing pellet was subsequently homogenised in a hypotonic buffer lacking Nonidet P40 (10 mM Tris-HCl, pH 8.0, 0.8 M NaCl, 1 mM EDTA, protease inhibitor cocktail) and incubated on ice for 10 min. Histones were subsequently extracted from chromatin and enriched through incubation with 0.25 M hydrochloric acid under continuous mixing for 1 h at 4 °C. The histone-containing supernatant was combined with an equal volume of 20 % trichloroacetic acid for 15 min, on ice and then pelleted at 1 000 xg. The histone-enriched pellet was washed with acetone. A SpeedVac concentrator was used to dry all the samples (SC100, Savant) for storage at -80°C.

2.2.3 Middle-down proteomics for identification of combinatorial histone PTMs

Middle-down MS was performed as before with a few modifications^{223, 250, 254}. Histones (15–50 µg) were suspended in 5 mM ammonium acetate to a final concentration of 0.5 µg/µL (pH 4.0). GluC endoprotease, with specificity to cleave peptide bonds C-terminally at glutamic acid, was diluted to 0.2 µg/µL in the same buffer and added to the histone samples a final concentration of 1:20 GluC enzyme:histone. Histone samples were incubated overnight at room temperature and digestion is blocked by the addition of 1 % formic acid. Trifluoroacetic acid (0.1 % TCA) was added to the digested peptides and desalted using StageTips packed with a solid phase C₁₈ column disk (3M Empore) with porous graphitic carbon (PGC) suspended in 100 % acetonitrile (ACN) on top and washed with 0.1 % TCA. Peptides were eluted by the addition of 70 % ACN and 0.1 % TCA. Histone protein samples were dried and suspended to a concentration of ~2 µg/µL in sample buffer (60 % ACN, 20 mM propionic acid, 1 mM ethylenediamine).

Separation of digested histone peptides was performed by using nanoliter liquid chromatography using an EASY-nLC nanoHPLC (Thermo Scientific, San Jose, CA, USA) equipped with an analytical, weak cation exchange–hydrophilic interaction liquid chromatographic resin (PolycatA, PolyLC, Columbia, MD, USA) column (75 µm ID, 15 cm in length, 1.7 µm in diameter and a 1000 Å porosity). Buffer A was prepared with 70 % ACN and 20 mM propionic acid to adjust the pH to 6.0. Buffer B consisted of 0.1 % formic acid in LC-MS grade H₂O. HPLC gradient was set up for a nonlinear gradient: 0–70 % of buffer B for 2 min followed with 72–85 % buffer B for > 90 min. The gradient was on hold for 5 min at 95 % buffer B to wash the column before sequential sample loading. To minimise sample carryover, blank injections (80–95 % buffer B alone) were run between different samples for at least 20 min.

MS detection was performed using an Orbitrap Fusion MS (Thermo Scientific, San Jose, CA, USA) operated in a data-dependent acquisition (DDA) mode and high mass resolution mode for both MS1 events and MS2 scans for each of the three stages investigated, each for two to three independent biological experiments. The full mass spectrum scan was set at 665–705 *m/z*, as this is the range of the most intense charge states (+8) for histone H3 polypeptides. A charge filter was added to include +8 charge states targeted for fragmentation. The ion transfer tube temperature was set to 300°C and the spray voltage to 2.3 kV. To fragment peptides while retaining PTM information, electron transfer dissociation (ETD) was performed^{255, 256} at a resolution setting of 120 000 (MS1) and 30 000 (MS2). The ETD reaction time was 20 ms for polypeptides with +8 charge states. For high-resolution spectra, three microscans were averaged.

2.2.4 Middle-down proteomics data analysis

Spectra were analysed with Proteome Discoverer (v. 2.4, Thermo Scientific), including Xtract (Thermo) for spectra deconvolution. Three UniProt entries of histone H3 sequences for *P. falciparum* (cross checked with histone sequences obtained from PlasmoDB [<https://plasmodb.org/>, version 43, released 25 April 2019]) were searched using Mascot (v. 2.5, Matrix Science, UK). During individual spectra identification, spectra were filtered on multiple layers: 1) deconvolution discarded all MS/MS ions with no identified charge state; 2) spectra identification was performed using the Mascot algorithm without filters; 3) the Mascot output file was filtered using custom software, ProteoformQuant²⁵⁷, to eliminate all PTM identifications that are not unambiguously supported by MS/MS fragment ions, e.g., a peptide with a modification that could be placed on more than one residue would not pass the filter; 4) for those identifications that pass the ProteoformQuant filter, all mixed MS/MS spectra without unambiguous fragment ions for differential quantification of isobaric peptides are discarded²⁵⁸. Mass tolerance for the precursor was set to 2.1 Da and 0.01 Da for the fragment ions. No missed or non-specific cleavages were considered; only fully digested N-terminal tails were considered. Endoproteinase GluC was used to generate peptides which is a serine protease that cleaves peptide bonds at the C-terminus of glutamic acid residues. Files were searched with the following dynamic modifications: acetylation (K), phosphorylation (ST), mono- and dimethylation (RK), and trimethylation (K). No fixed modifications were considered. The mass tolerance was set to 2.1 Da for precursor ions and 0.01 Da for fragment ions. IsoScale (<http://middle-down.github.io/Software/>) was used to confidently identify and quantify modified peptides. Only c/z fragment ions were allowed. PTMs were only accepted if there were at least one site determining ions on both sides of the assigned PTM. Estimation of FDR is not applicable for middle-down proteomics as peptides are too long to have any decoy match. A list of all peptide sequences identified, and sites of modifications (ambiguous matches are discarded by ProteoformQuant) are all provided in the full datasets in public repositories (see data availability statement below). Peptide identification scores are not applicable.

Interplay scores between bivalent histone PTMs were calculated according to the following equation:

$$Interplay_{PTM1PTM2} = \log_2 \frac{F_{PTM1PTM2}}{F_{PTM1} \times F_{PTM2}}$$

where $F_{PTM1PTM2}$ is the observed relative abundance (F) of the combination divided by the predicted relative abundance of the combination, calculated by multiplication of the relative abundances of the individual histone PTMs. The relative abundance of the individual PTMs was calculated by dividing its intensity by the sum of all modified and unmodified peptides sharing the same sequence. If the interplay score is positive, the PTMs are likely co-dependent

or positively related; a negative interplay score indicates that the PTMs are mutually exclusive. Ring plots were visualised using Cytoscape (v. 3.8.2). Other plots were created using GraphPad Prism 9 and R (R Studio v. 4.0.3).

2.2.5 Experimental design and statistical rationale

The overall aim of this study was to identify combinations of histone PTMs and their theoretical crosstalk patterns on intact histone N-termini across three biologically unique stages of the *P. falciparum* parasite. Two independent biological replicates were analysed for immature, early-stage (stage II-III) and mature (stage V) gametocytes and three independent biological replicates for asexual trophozoite stages. This allowed for assessment of variance in the data, the identification of outliers and normalizing and averaging the relative abundances of proteins identified as a more accurately representative of protein abundance. In all statistical comparisons, *P*-values were corrected for multiple testing according to Benjamini-Hochberg. Statistical analysis was performed using GraphPad Prism v6.00 and R v3.5.3, assuming normal distributions. Unless otherwise noted all error bars represent the standard error of the mean for at least three independent biological replicates.

2.2.6 Data Availability

The middle-down proteomics data generated in this study have been deposited in the Chorus database (<https://chorusproject.org>) and are accessible through project number 1721. All proteomics data generated in this study have been deposited to the ProteomeXchange Consortium (<http://www.proteomexchange.org/>) using the PRIDE partner repository²⁵⁹. These data sets are available with data set identifier PXD030181. The data analysis pipeline meets all MIAPE standards.

2.3 Results

2.3.1 Adapting middle-down proteomics for *P. falciparum* parasites

To investigate the presence of co-existing histone PTMs with middle-down proteomics, we isolated parasites as enriched trophozoites (92 ± 0.9 %), immature early-stage gametocytes (83 ± 3.1 % stage III gametocytes, 14 ± 2.2 % stage II), and mature gametocytes (94 ± 2.9 % stage V), to allow stage-specific differences to be inferred (Figure 2.1).

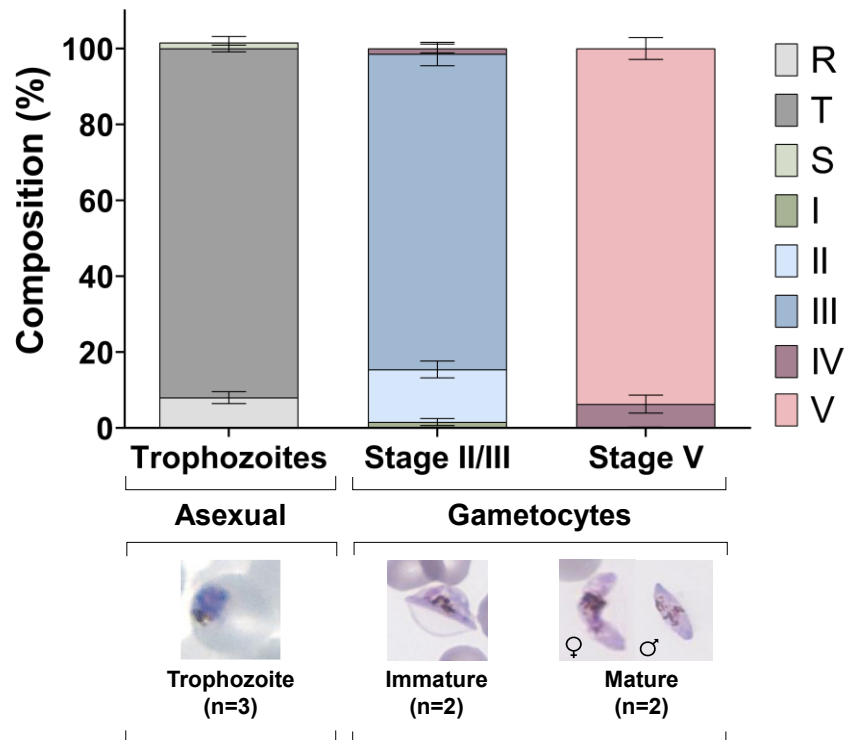


Figure 2.1. Stage composition of *P. falciparum* parasites samples used for middle-down proteomics. The stage composition of the three-biological stage analysed in this study and representative morphology. Trophozoites (mean $\pm$ SEM) samples contained a small percentage of ring (R) and schizont (S) stages, while the stage III samples (mean $\pm$ SD) contained stage I (I), II (II), III (III) and IV (IV), while stage V samples (mean $\pm$ SD) consisted of stage III, IV and V gametocytes. The trophozoites were from three independent biological repeats and gametocyte stages from two independent biological repeats.

All samples yielded $> 300\,000$ cells/sample, which has been optimised to be sufficient to yield the maximum amount of protein for downstream application (Figure 2.2). From the acid-soluble fraction, protein yield was 17 ± 1.1 μ g for the trophozoite population and 58 ± 10 μ g and 70 ± 16 μ g for the immature and mature gametocytes, respectively. This resulted in sufficient yields of acid-soluble nuclear protein fractions containing the histones used for middle-down proteomics.

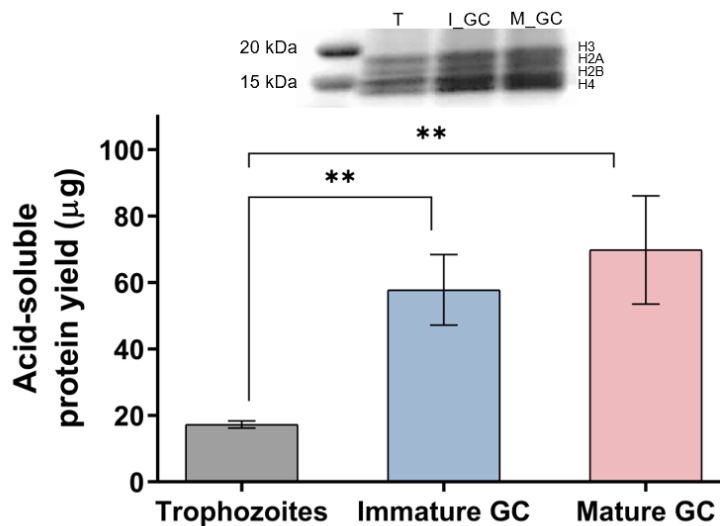


Figure 2.2. The acid soluble protein yield (μg) fractions for each developmental stage. Protein yield was determined for trophozoites, immature gametocytes (GC), and mature stages. Two-tailed equal variance t-test across the stages [$**P < 0.01$], with the average for trophozoites [$n=3$, mean $\pm$ SEM] and average for gametocytes [$n=2$, mean $\pm$ SD]. Histones refer to the isolated acid-soluble protein fraction containing the histone proteins and not to pure isolated histones. SDS-PAGE inset showing the histone fraction proteins, including bands representing histone H3, H2A, H2B and H4.

An optimised middle-down proteomics workflow was established to accurately identify and quantify individual and combinatorial histone PTMs at scale (Figure 2.3)^{254, 260}.

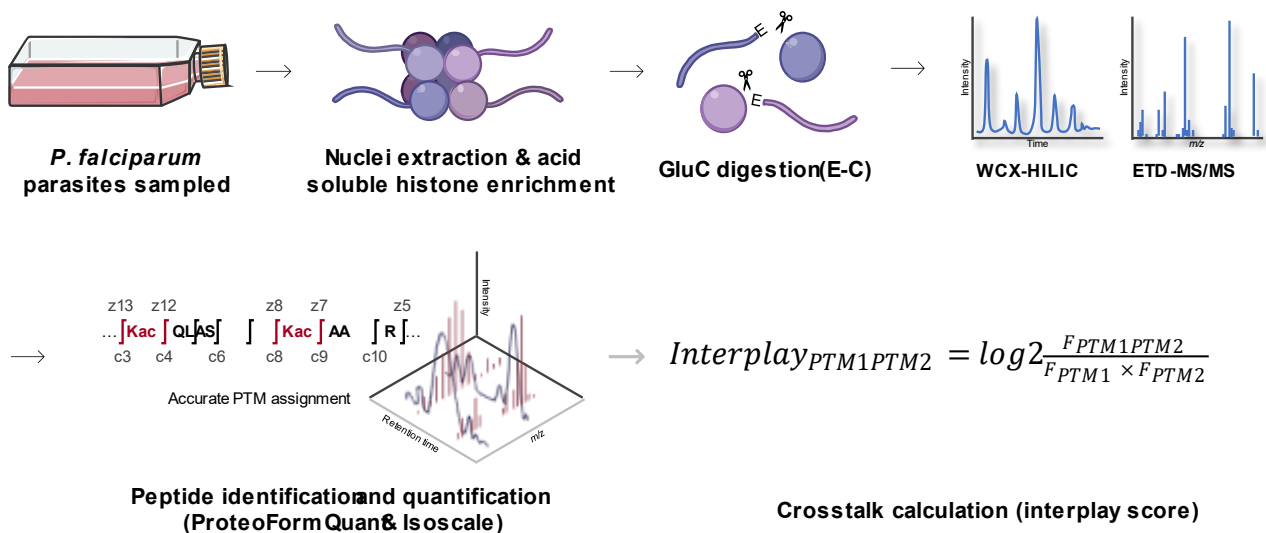


Figure 2.3. Middle-down MS workflow for the analysis of *P. falciparum* parasite. The flask image was adapted from the image from Servier Medical Art (<http://smart.servier.com/>). Servier Medical Art is licensed under a Creative Commons Attribution 3.0 License (CC BY 3.0 license: <https://creativecommons.org/licenses/by/3.0/>).

The native histones from each sample were digested with GluC endoproteinase to produce polypeptide fragments of ~50-60 amino acids in length (≥ 5 kDa) that were separated and identified by high-resolution nano liquid chromatography-MS/MS equipped with a two-column

system consisting of a C18-AQ trap column and a weak cation exchange-hydrophilic interaction chromatography (WCX-HILIC) resin analytical column coupled online with high-resolution tandem mass spectrometry (MS-MS) fragmentation was performed using electron transfer dissociation (ETD). Spectra were identified using the Mascot and peptides, including co-fragmented isobaric species, were quantified using isoScale by employing the fragment ion relative ratio (FIRR) method proposed by Pesavento *et al.* ²⁶¹. Data processing and analysis workflow involved MS spectral deconvolution using Xtract (Thermo Fisher Scientific) followed by database searching using Mascot (Matrix Science, UK) with files generated from PlasmoDB (<https://plasmodb.org/>) and subsequent removal of ambiguously mapped PTMs and stringent quantification (including co-fragmented isobaric species) using IsoScale Slim (<http://middle-down.github.io/Software>). Further data processing and peak extraction were performed with in-house developed tools, ProteoFormQuant and HistoneCoder ^{257, 262}. The relative abundance of an individual PTM (PTM1/2) is calculated by summing the relative abundances of all proteoforms carrying the specific individual PTM. The interplay between two individual modifications is calculated by dividing the observed abundance of bivalent PTMs (FPTM1PTM2) with the predicted frequency of a combinatorial PTM (FPTM1 x FPTM2). FPTM1PTM2 is calculated by summing the relative abundances of all proteoforms carrying both PTMs (Figure 2.3)

Unique PTMs (with a false discovery rate [FDR] <1 %) with sufficient fragment ions were accurately quantified, with a coefficient of variance ≤ 33 % and average Pearson $r^2=0.69$ between biological replicates, allowing comparison of PTM quantities between samples. To ensure differentiation of isobaric peptides as a result of the middle-down proteomics, data-dependent acquisition data were further processed using isoScale Slim ²⁴⁵, which extracts the total fragment ion intensity of histone tail spectra as representative of their abundance, and it discriminates isobaric forms using unique site-specific fragment ions using the principle of the fragment ion relative ratio (FIRR) ²⁶¹. From the accurately identified and quantified histone PTMs per peptide, the co-existing frequency was calculated for the observed presence of combinatorial PTMs (from MS2 level evidence) as a function of predicted co-existence frequencies, referred to as an 'interplay score' ^{261, 263, 264}.

2.3.2 A high-resolution, quantitative compendium of histone PTMs in *Plasmodium* from middle-down proteomics

The high-resolution, comprehensive nature of middle-down proteomics allowed successful identification and quantification of 83 PTMs on histone H2B.Z, H3 and H3.3 (Figure 2.4), as only these histones have GluC cut sites on their N-terminal histone tail ²⁵⁴. Mass tolerance was set at 30 ppm to accurately distinguish between lysine acetylation (42.011 Da) and lysine trimethylation (42.047 Da) as previously determined ²⁵⁸. 72 of the 83 identified PTMs were

confidently quantified; the others were only detected but with a signal insufficient to assess an abundance. As no enrichment was performed for phosphorylation, these could not be quantified. A few PTMs previously identified with H2B.Z (K14ac, T30ac, K42ac), H3 (S10ac, S10ph, S28ph, S32ph) and H3.3 (S10ac, T11ac, S22ac, S28ph, S32ph) could not be confirmed^{167, 168}. Since these PTMs were only previously qualitatively described, their presence remains to be confirmed.

Of the 83 PTMs identified (Figure 2.4), 35 were described for the first time in *P. falciparum* parasites, of which 30 could be accurately quantified. As expected, the rarely observed variant H2B had the least number of PTMs. This included six known PTMs but also one novel PTM (H2B.ZK18me3). Histone H3 contained 40 PTMs of which 33 were accurately quantified, including 13 novel PTMs. These include acetylation and methylation of H3K37, and methylation of several arginine's (H3R8me1/2, H3R26me1, H3R40me2, H3R42me1, and H3R49me1). Several PTMs displayed high relative abundances (> 20 %) in more than two stages (e.g., H3K4me3, H3K14ac, H3R17me1, H3K18me1, H3K37me1, and H3R40me1), with the novel PTM, H3K37me1, highly abundant in immature gametocytes (70 ± 6 %) (Figure 2.4). These changes in abundance levels between stages are also evident for H3K4me3, H3R17me2, and H3R40me1, which increased significantly from trophozoites to immature gametocytes ($P \leq 0.01$, $n \geq 2$). Histone H3.3 contained 37 PTMs (33 quantified) with substantially higher abundances of PTMs modified in both gametocyte stages compared to asexual parasites than seen for H3. Methylation PTMs were again abundant including H3.3K4me1/3, H3.3K18me3, H3.3R17me2 and H3.3R40me1, with H3.3K37me3 significantly increased abundance in mature gametocytes ($P \leq 0.01$, $n = 2$).

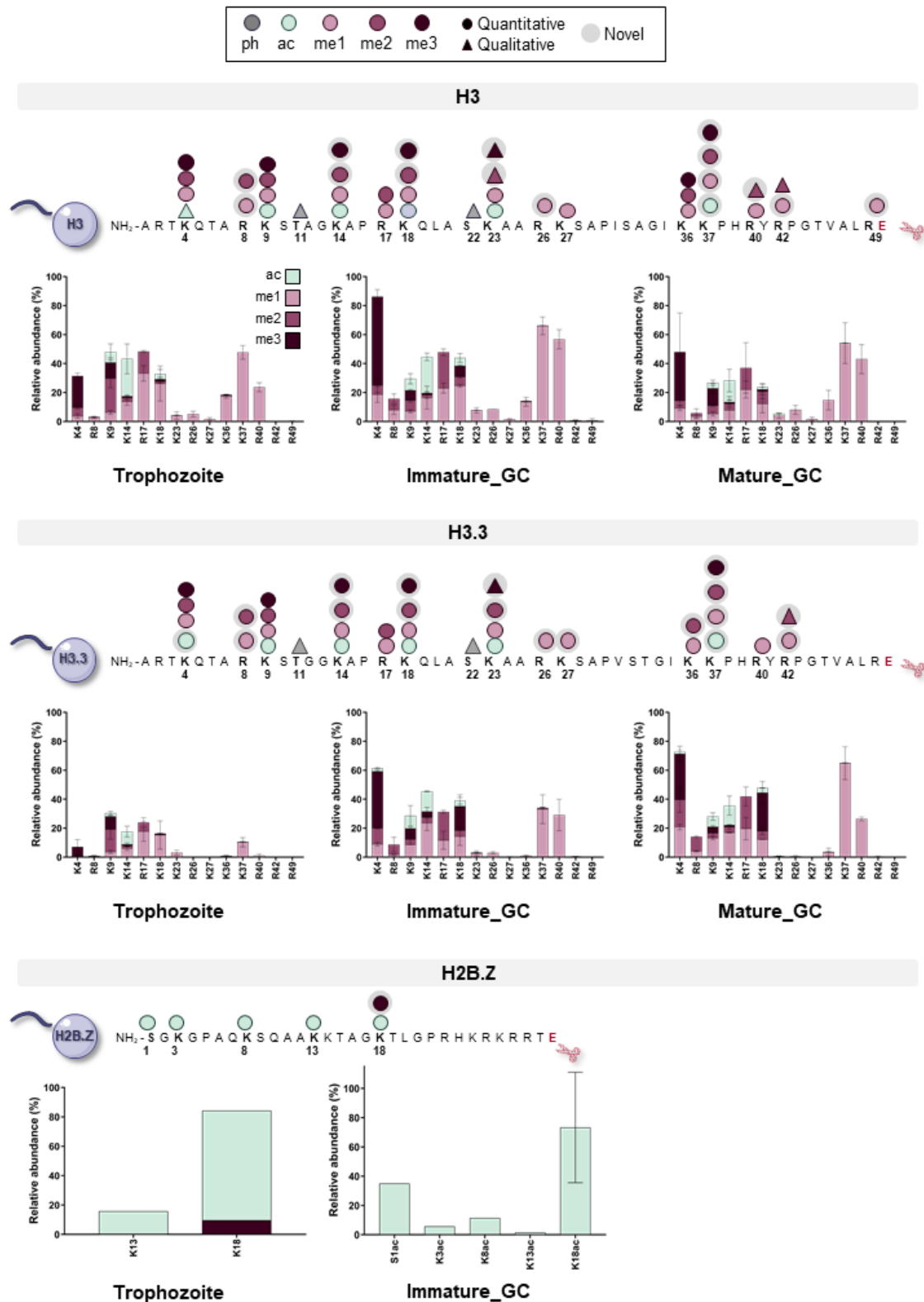


Figure 2.4. The relative abundances of individual PTMs on histone H3 and variant histones H3.3 and H2B.Z from *P. falciparum* trophozoite, immature and mature gametocytes. All individual histone PTM that were detected for histone H3 and H3.3 are indicated, those quantified in circles or only quantitative in triangles. Novel marks are shaded grey. The relative abundances of the individual histone PTMs are indicated as acetylated (teal), monomethylation (me1, light purple), dimethylation (me2, medium purple) and trimethylation (me3, dark purple). Relative abundance is calculated by summing the relative abundances of all the quantified combinatorial codes that included a given PTM. Data are from a single independent biological repeat (mean), except for H2B.ZK18ac in immature gametocytes where relative abundance was derived from two independent biological replicates (mean $\pm$ SD).

Collectively, we demonstrate that middle-down MS identified >80 unique PTMs and quantified 88 % thereof. This includes 30 novel modifications, thereby providing an updated, high-resolution compendium of histone modifications across multiple life cycle stages of *P. falciparum*.

2.3.3 A stage-specific combinatorial histone PTM code exists in *P. falciparum* parasites

The middle-down proteomics dataset was used to identify and quantify co-existing PTMs on H3 and H3.3 across the three life cycle stages of *P. falciparum*. Typically, 2-4 up to a maximum of 7 modifications were present on any given peptide for both H3 and H3.3 (Figure 2.5), similar to what is seen for these two histones in humans and mice ^{244, 265, 266}.

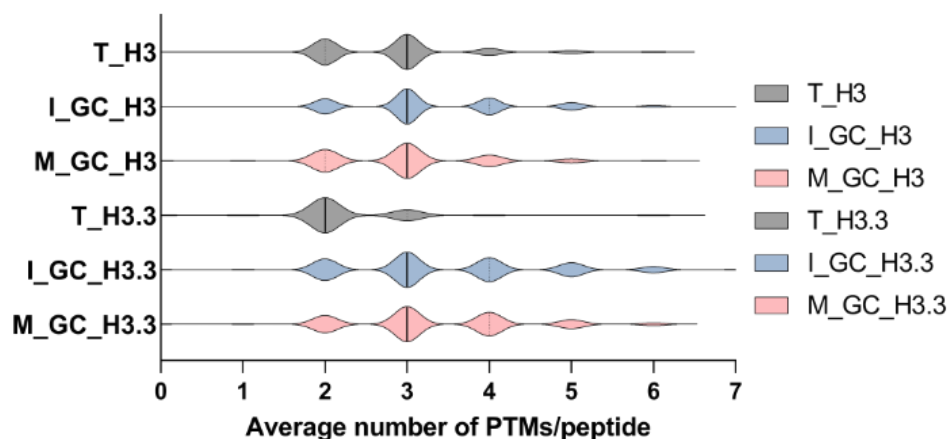


Figure 2.5. The average number of PTMs identified on histone N-terminal peptide tails. Violin plots show the distribution of the number of PTMs that are present on histone H3 and variant H3 tails where the dotted line indicated the upper and lower quartiles and the solid line indicating the median. The lower limit represents an unmodified histone tail. T: Trophozoite; I_GC: Immature GC and M_GC: mature gametocyte.

H3.3 in trophozoites is the exception, with on average only two co-existing PTMs present in trophozoites but as many as seven (e.g., H3K4me1R8me1K9me1K14acR17me1K18acK37me1) in immature gametocytes. Overall, the majority of triple, quadruple and quintuple PTM combinations we observed in gametocytes for H3, implying that additional PTMs might be important for stage development and transitions. Interestingly, stage-stratification was evident in the histone PTM combinations (Figure 2.6). Immature gametocytes displayed the highest proportion of unique combinations (42 %) on H3, with only a minor proportion (9 %) of combinations shared between asexual parasites, immature and mature gametocytes (Figure 2.6). Stage-specific diversity was somewhat less evident for H3.3 (Figure 2.6), with a markedly decreased number of unique co-existing PTMs present in trophozoites for this histone. Unlike with H3, gametocytes harboured the majority of unique combinatorial peptides, suggesting that immature gametocytes

specifically use a subset of combinatorial peptides of H3, but shared combinatorial peptides with mature gametocytes for H3.3.

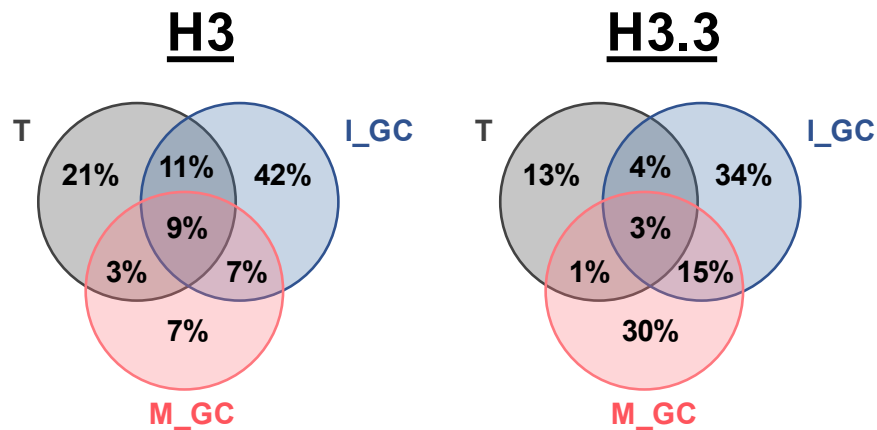


Figure 2.6. Combinatorial peptides shared between trophozoites, immature and mature stage gametocytes on histone H3 and H3.3. T: trophozoite, I_GC: immature gametocyte and M_GC: mature gametocyte.

The stage-stratification was characterised by changes in the identity of the most prevalent combinations (Figure 2.6). Trophozoites are characterised by a large number of combinations involving H3K14ac, H3K4me3, H3K9me2, and novel PTMs H3K37me1 and H3R17me1, with the combinations of K14acR40me1, K37me1R17me1, K4me3R40me1, K14acK37me1 and K4me3K14acK37me1 as most prominent combinations (Figure 2.7). These include the well-characterised, known combination of the archetypical euchromatic PTMs H3K4me3 and H3K9ac^{153, 267}, with H3K14ac due to the coordinated action of SET7²¹⁸ and the histone acetyltransferase GCN5^{215, 268}. In gametocytes, the novel PTMs H3K37me1 and H3R40me1 are involved in the highest number of co-existing PTMs, with further specification seen between immature gametocytes (with frequent interactions with H3R17me1/me2 present) and mature gametocytes (higher connectivity for H3K14ac and H3K4me3 than H3R17me1, Figure 2.8). Combinations involving H3R42me1 occurred exclusively in gametocytes. Arginine methylation may therefore well make up a key component of the histone code in *P. falciparum* parasites along with another histone PTMs, particularly for gametocyte stages.

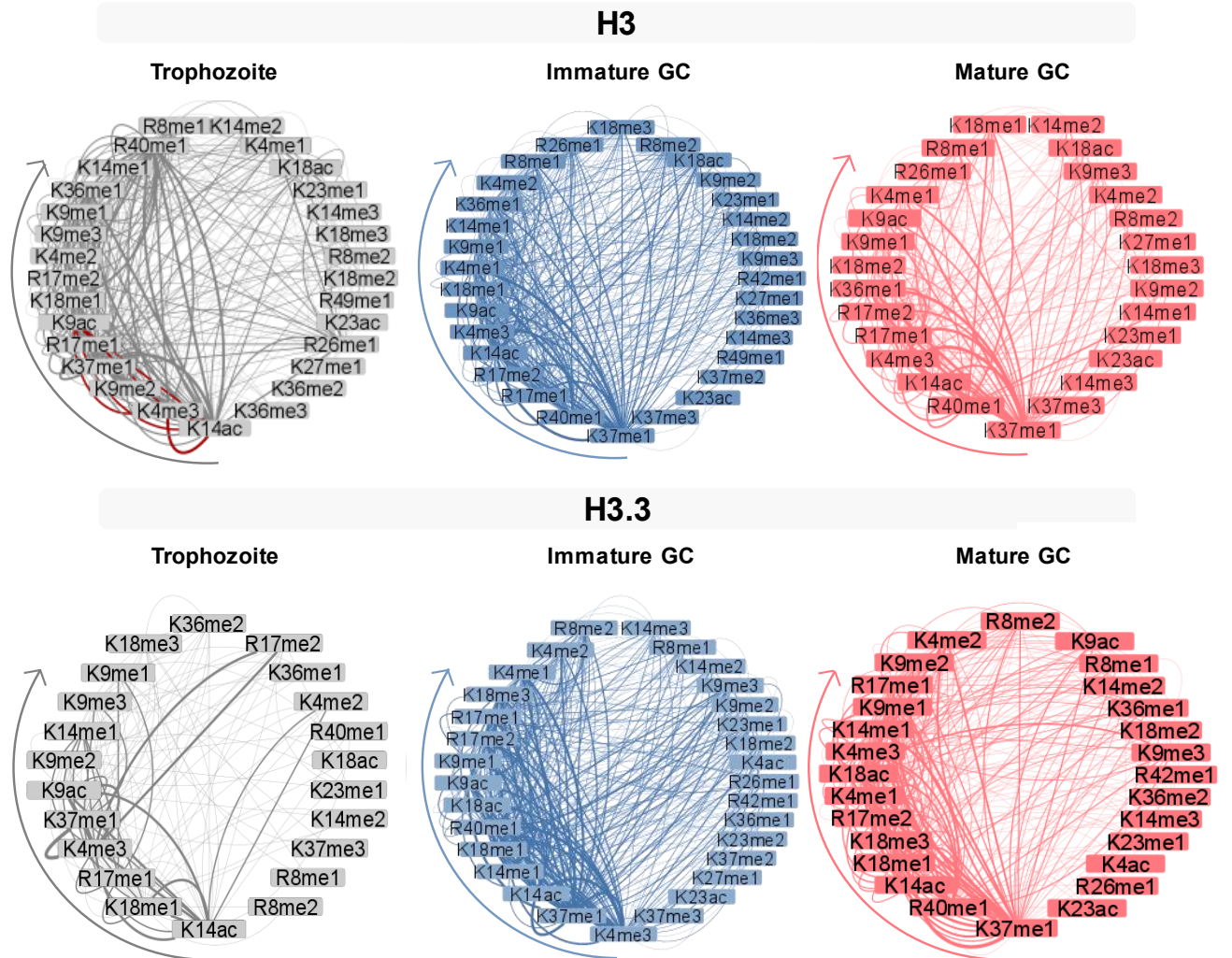


Figure 2.7. Prominent combinatorial histone PTM reorganisation in *P. falciparum* parasites and gametocytes. The co-existing histone PTMs observed on histone H3 and H3.3 for trophozoites, stage III and stage V gametocytes are visualised as ring plots where the nodes are the histone PTMs while the edges represent the connection to another co-existing partner PTM. All combinations are included in the supplementary data. The arrows represent the start of the most connected PTM (left) toward the least connected (right) in a clockwise direction. The co-existence of H3K9ac with H3K4me3, H3K14ac with H3K4me3, and H3K9ac with H3K14ac are highlighted with red edges in the trophozoite stage. The arrows indicate the most prevalent PTM to the least prevalent PTM in co-existence.

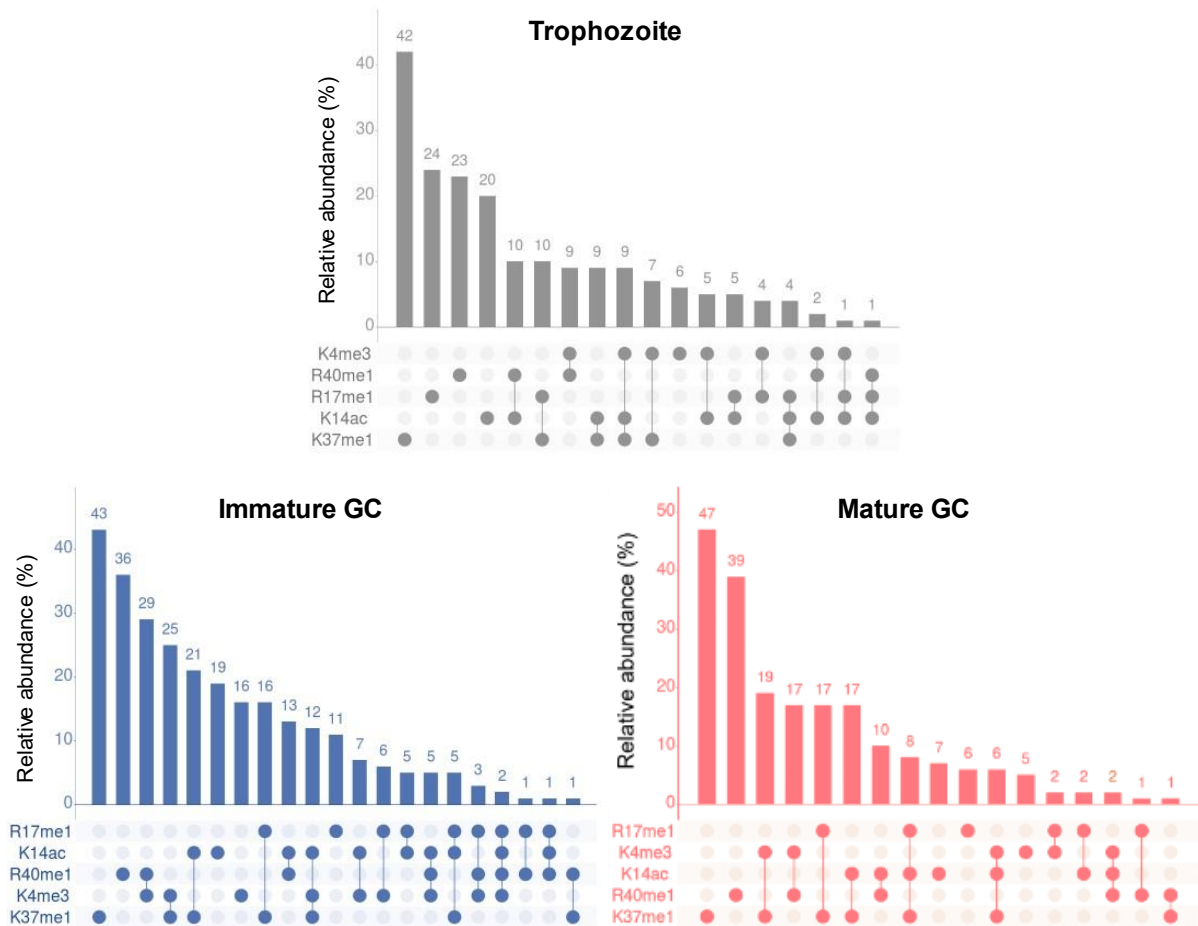


Figure 2.8. Co-existence frequencies of multiple PTMs in *P. falciparum*. UpSetR (<https://jku-vds-lab.at/tools/upset/>) plot showing the co-existence frequencies of multiple PTMs in trophozoites (top panel, grey), immature gametocytes (bottom panel, blue) and mature gametocytes (bottom panel, pink).

The co-existence frequencies indicates that histones are rarely modified by single, individual PTMs. Therefore, multiple histone PTMs, as opposed to individual PTMs, would contribute as a whole to a distinct chromatin structure that could promote gene expression²⁶⁹. Additionally, these histones codes are re-arranged in position and type of modifications in different life cycle stages of the malaria parasite. The most frequent combinations in gametocytes diverge from those in trophozoites, with immature gametocytes associated with the highest number of co-existing PTMs ascribed to the presence of novel PTMs. The parasite, therefore, employs a unique and diverse set of PTM combinations likely to guide stage-specific gene expression or protein-protein interactions.

2.3.4 Histone PTM pairs display unique crosstalk to assert function

The extent and relevance of the influence between pairs of co-existing PTMs were subsequently interrogated by quantifying the co-existing frequency as an interplay score (IS) for bivalent combinations. This provides a metric to predict the likelihood of individual pairs of histone PTMs to either: 1) occur more frequently as co-existing pairs than expected by chance^{258, 263, 270}; or 2) have a negative interplay score to indicate that two individual pairs of PTMs

are observed less frequently than expected by chance. Thus, if two PTMs have a positive interplay score, it indicates that these PTMs likely enhance or disrupt binding of effector proteins therefore share a “co-dependent” relationship to one another. If a negative interplay score is observed, it means that the PTMs act in an antagonistic or mutual exclusive manner from one another, indicating functional independence¹⁹⁰. If two PTMs are randomly and independently deposited on chromatin, they will have an interplay score close to zero.

A third of all co-existing histone PTMs had similar connectivity and co-existing frequency profiles between all three stages of development of *P. falciparum*. The majority of crosstalk combinations were identified for immature gametocytes, followed by mature gametocytes and trophozoite stages (Figure 2.9A). This bias is further apparent where immature gametocytes had a unique set of crosstalk combinations, although the majority of crosstalk combinations were shared among the three stages (Figure 2.9B).

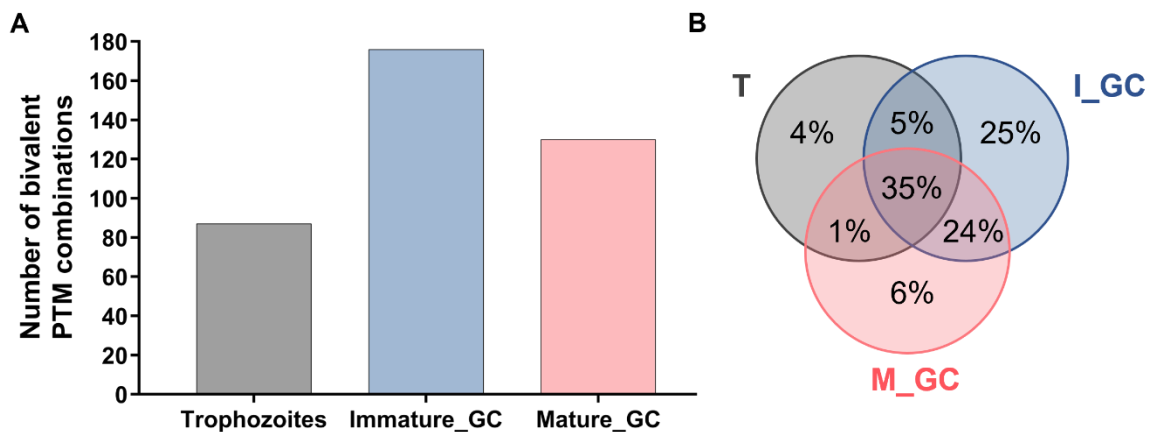


Figure 2.9. The number of bivalent crosstalk combinations characterised. (A) Immature gametocytes (GC) had the highest explained crosstalk for ~180 partner PTMs, followed with mature gametocytes and trophozoites. **(B)** Venn diagram showing the overlap of bivalent combinations between trophozoites (T, grey), immature gametocytes (I_GC, blue) and mature gametocytes (M_GC, pink).

Interestingly, only a small proportion of crosstalk combinations were shared between trophozoites and gametocytes, while the gametocytes stages shared an exclusive set of crosstalk combinations. This result further exemplifies the accompaniment nature of crosstalk in gametocytes. Inspection into the interplay score trends observed for the subset of crosstalk combinations that are shared between all three stages highlighted the increased dynamic nature of crosstalk for gametocytes, while other crosstalk combinations were conserved and maintained throughout the different biological processes trophozoites, and gametocytes performs (Figure 2.10).

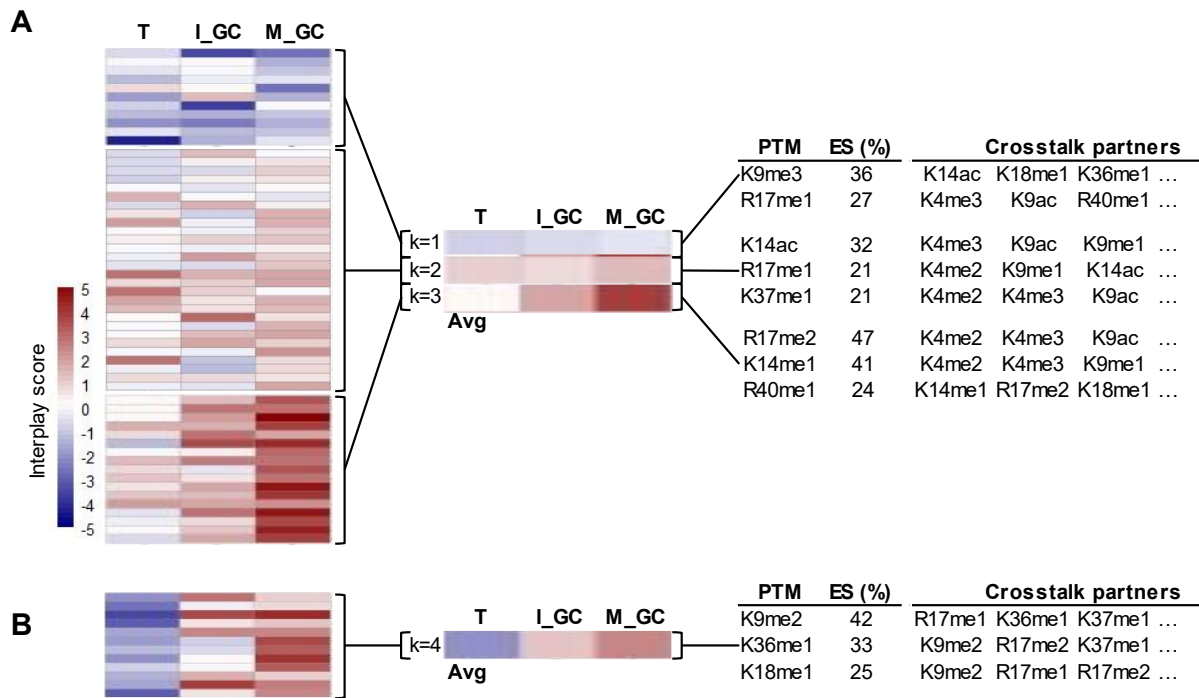


Figure 2.10. Histone modification crosstalk conserved across life cycle stages in *P. falciparum*. (A) Interplay scores for PTM combinations are consistently present in all life cycle stages analysed. Heatmaps show k-means clustering (complete, k=4) of the overlapping 35 % or 68 bivalent combinations and the respective interplay scores shared between all three stages. The average interplay scores are summarised for each cluster. (B) Interplay scores in cluster 4 across the life cycle stages, and PTMs that show enrichment within these clusters are quantified with an enrichment score. An enrichment score percentage (ES %) is shown for the top PTMs that are over-represented in each cluster. The enrichment score is calculated by dividing the total number of times a given PTM is present by the total number of combinations in the cluster. Selected crosstalk partner PTMs are indicated.

Of these, 11 combinations show negative interplay scores throughout development (Figure 2.10A, k=1), which includes multiple combinations of the repressive PTM H3K9me3. For example, this PTM is found on the same histone molecule with H3K14ac significantly more rarely than stochastic co-occurrence in all three stages, implying mutual exclusion and thus opposing biological functions.

The typically euchromatic signal of H3K14ac is therefore negated in the event of H3K9me3, and this contributes to the PfHP1-bound heterochromatic state as described for certain gene sets^{159, 239, 271}. This combination is found also in differentiated stem cells²⁷² where H3K9me3 act as a barrier to cell reprogramming induced in pluripotent stem cells, regardless of the presence of the activating H3K14ac PTM²⁷³. All other combinations with H3K9me3 have negative interplay scores. H3K9me3, therefore, likely act autonomously, independent of association with any other acetylation or methyl PTMs, and not influenced by *P. falciparum* life cycle development. This feature of H3K9me3 is supported in other cell types where H3K9me3 acts to reprogram the identity of various cell types (Figure 2.11)^{274, 275}.

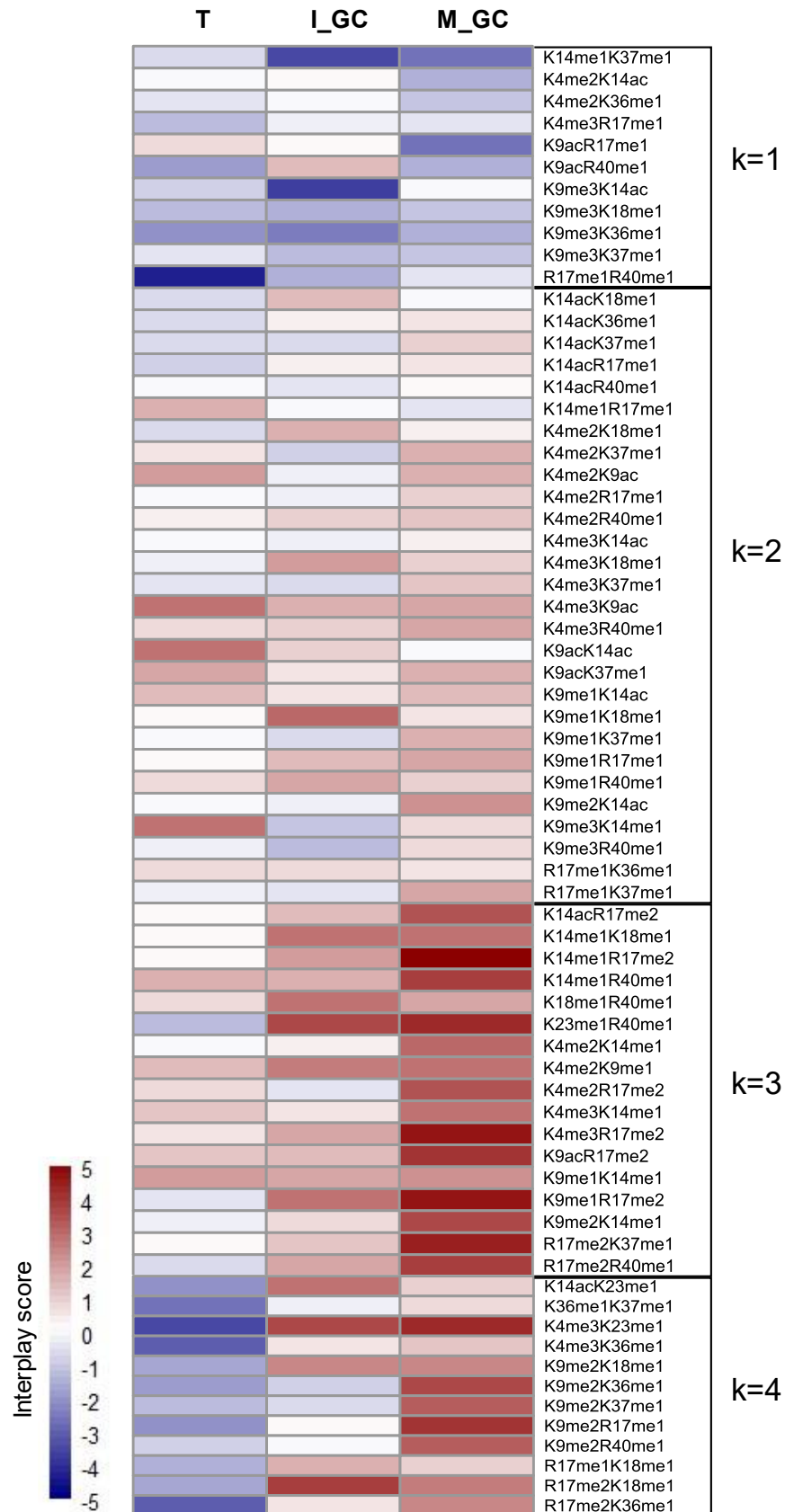


Figure 2.11. Histone modification crosstalk conserved across life cycle stages in *P. falciparum*. Interplay scores for PTM combinations consistently present in all life cycle stages analysed. Heatmap shows k-means clustering (complete, k=4) of the overlapping 35 % or 68 bivalent combinations and the respective interplay scores shared between all three stages. The average interplay scores are summarised for each cluster (cluster 1-4) across the life cycle stages.

Several PTMs (45) display positive interplay throughout development, implying coordinated function or co-dependence including H3R17me2, H3K14me1 and H3R40me1 found in various combinations with partner PTMs, particularly H3K4me2/3 (Figure 2.11). These marks therefore likely never function independently and require interaction with one (or more) PTMs throughout parasite development. Interestingly, a number of these marks show increased positive interplay scores in mature gametocytes, suggesting that these combinations are increasingly critical to these parasites. H3K14ac remains co-dependent on H3K9ac as seen before for asexual parasites^{215, 268} with pronounced dependency evident in gametocytes here (Figure 2.11); indeed, the effector protein, GCN5 is expressed in all three stages investigated including mature gametocytes²⁷⁶.

A set of 12 combinations are found in all three life cycle stages, yet have opposing profile between gametocytes (strong positive interplay scores) and asexual parasites (strong negative interplay) (Figure 2.11). This includes a number of combinations involving H3K9me2, H3K36me1, and H3K18me1. The repressive mark H3K9me2 associates with several other marks (e.g., R17me1, K18me1, K36me1, K37me1, and R40me1), all of which show co-dependency only in mature gametocytes. The strongest differential interplay score between asexual and gametocytes was observed for H3K4me3K23me1. This novel combination is mutually exclusive in trophozoites, supporting the fact that H3K4me3 participates in crosstalk with H3K9ac in asexual parasites¹⁵³. The strong co-dependence of H3K4me3K23me1 is therefore likely important for gametocyte-specific biological processes; the combination of the euchromatic H3K4me3 with H3K23me1 methylation has been associated with heterochromatin in *C. elegans*²⁷⁷. The conserved nature of these co-existing marks across all life cycle stages of *P. falciparum* implies shared importance to parasite biology.

2.3.5 A dynamic histone code describes crosstalk with stage-specific biology in *P. falciparum*

Several PTMs show a crosstalk profile that is uniquely associated with a specific parasite life cycle stage (Figure 2.12). In trophozoites, the majority of the PTM combinations had negative interplay scores, suggesting that these PTMs antagonise each other, and are thus mutually exclusive. Combinations including novel arginine methylation marks like H3K14me2R17me1, show the strongest negative interplay scores (IS=-3.7), suggesting that these marks act with different biological roles from each other in trophozoites (Figure 2.12). H3R40me1 and H3R17me1 mostly show pronounced negative interplay scores for the majority of their interactions in trophozoites. Whilst H3R17me1 is activating as a de-repressor in mammalian cells²⁷⁸, H3R40me1 has only previously been reported in a few organisms including yeast where it is required for efficient sporulation, similar to spermatogenesis in higher eukaryotes

^{279, 280}, while in cancer cells²⁸¹, *C. elegans*²²³ and murine embryonic stem cells, its function is unknown²⁵⁸.

Several important co-dependent interactions with positive interplay scores were identified in trophozoites, the majority of which involved H3K9ac (e.g., H3K9acK14ac IS = 2.8; H3K4me3K9ac IS= 2.9; and H3K9acK36me3) to produce the global euchromatic signal associated with asexual parasites^{153, 176}. H3K9acK36me3 has the strongest positive interplay score (IS=4.3) and both H3K9ac and H3K36me3 have been independently linked to *var* gene transcription^{176, 240, 241, 282, 283}; this co-dependence supports their function in *var* gene expression.

The crosstalk profile in immature gametocytes is more complex, with a larger number of PTMs involved in positive crosstalk, the majority of which involve methylation marks (Figure 2.12). The novel mark H3K14me2 displays the largest number of interactions with positive crosstalk, including that with H3K9ac (IS=6), H3K9me1 (IS=4.9) and with both K18me1/me2 (IS~5). These associations are influenced by the level of methylation (i.e. mono-, di- or trimethylation) to define chromatin states and active genes v. inactive genes within the same locus²⁸⁴⁻²⁸⁶, with H3K14me1 for instance in negative crosstalk with H3K18me2, compared to the positive crosstalk seen for H3K14me2 with H3K18me2. H3K14me2 is likely a repressive mark, similar to the important silencing function of H3K14me3 that marks a set of zinc finger protein genes during trans-differentiation of bone marrow cells into hepatocytes^{287, 288}. The co-dependency with H3K14me2K9me1 (with H3K9me1 as key PTM in the establishment of functional heterochromatin²⁸⁹ and the novel mark H3K14me2K18me1/2, marks H3K14me2 as a key modulator of gene regulation within immature gametocytes, to potentially mediate the establishment of a heterochromatic state.

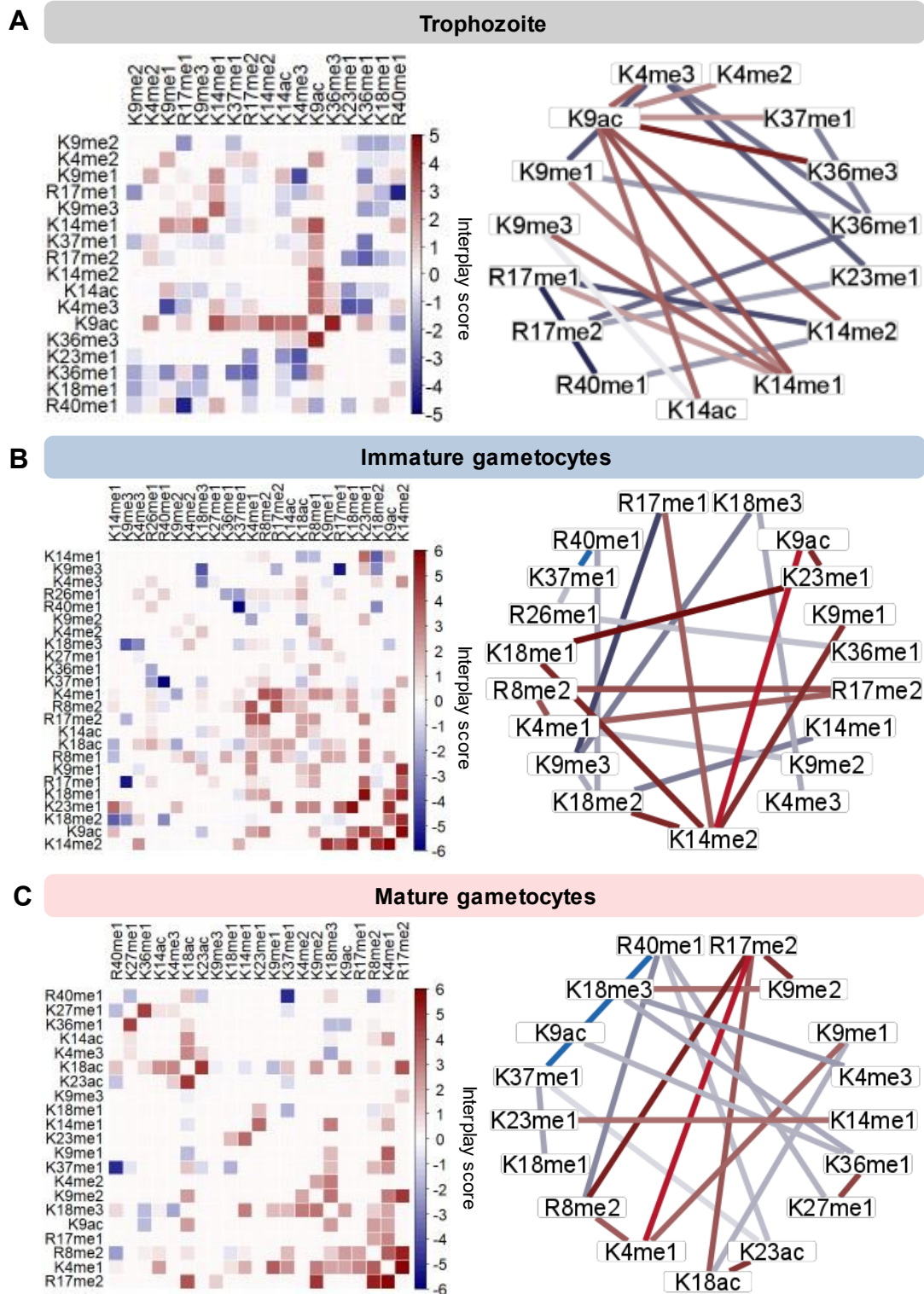


Figure 2.12. Dynamic, stage-stratified histone modification crosstalk in *P. falciparum* parasites. Interplay scores between bivalent PTMs occurring in (A) trophozoites, (B) immature gametocytes, and (C) mature stage gametocytes where the colour intensity of the square is proportional to the interplay score. Ring plots indicate top and bottom 10 bivalent combinations, where the colour of the edges is proportional to the interplay score shown in the heatmap. Interplay scores for trophozoites include the unique 4 % and the overlapping 6 % with immature gametocytes (I_GC) and 1 % with mature gametocytes (M_GC). Interplay scores for immature gametocytes include the unique 25 % and the overlapping 6 % with trophozoites and 25 % with mature gametocytes. Interplay scores for mature gametocytes include the unique 6 % and the overlapping 4 % with trophozoites and 25 % with immature gametocytes.

Amongst the novel arginine PTMs in immature gametocytes, H3R17me2 show positive interplay particularly with H3R8me2 (IS = 4.1, correlated only with active histone PTMs²⁹⁰ and H3K4me1 (IS = 3.6, enhancer associated activation mark²⁹¹, with the latter also positively connected (H3K4me1R8me2, IS = 3.97). H3R17me2 is a typical activation mark in eukaryotes²⁹²⁻²⁹⁴ and the coordination with the two other activation marks indicates that these combinations may be involved in activation processes. Interestingly, changing H3R17 methylation from di- to monomethylation, results in positive interactions with H3K14me2 (IS = 3.59) but also a strong negative interaction with H3K9me3 (IS = -5.33), implying independence of H3R17me1 from H3K9me3. Furthermore, in immature gametocytes, H3R40me1 again shows strong negative interplays, particularly with H3K37me1 (IS = -7) and H3K18me2 (IS = -2.8), confirming its independent action in immatures gametocytes, similar to in trophozoites. Overall, immature gametocytes use highly connected and co-dependent repressive lysine PTMs pairs to induce a more heterochromatic state compared to asexual parasites. However, the majority of arginine PTM coordinates positively and could result in the activation of subsets of genes.

Mature gametocytes have a combinatorial profile similar to immature gametocytes with largely positive interplays scores (Figure 2.12), although the frequency of the combinations somewhat differs from those in immature gametocytes. H3R40me1 remains highly connected in mature gametocytes, again showing independence from other co-existing marks. Activating H3R17me2 is most connected PTM in mature gametocytes, retaining its interactions and co-dependency with other activating marks, H3K18ac (IS = 3.9) and H3K4me1 (IS = 5.9), and with H3K9me2 (IS = 4.8) and H3R8me2 (IS = 5.2). Mature gametocytes are, however, additionally marked by combinations that uniquely and exclusively are present only in this stage of development. This includes co-dependency between H3K27me1K36me1 (IS = 4.3). In embryonic stem cells, H3K27me1 is dependent on H3K36me1 to promote transcription^{262, 295} and an increase in H3K36 methylation to di- and trimethylation negatively impacts H3K27 function and they become mutually exclusive^{296, 297}. A likely explanation for this positive interplay between H3K27 and H3K36 has been proposed to include a situation where methylation of H3K27 by the polycomb repressive complex 2 is inhibited in the case where both sister histones of mononucleosomes carry H3K36 methylation²⁹⁸. In *P. falciparum*, H3K36me2/me3 is repressive to asexual gene sets in immature gametocytes²⁴¹. The exclusive combination of H3K27me1K36me1 in mature gametocytes could therefore be predicative of transcriptional activation of genes required for parasite transmission. Additionally, H3K18ac also is in crosstalk with H3K23ac only in mature gametocytes (IS = 4.7 for H3K18acK23ac). H3K18ac independently associates with active promoters in asexual *P. falciparum* parasites²¹⁴ and human cancer cells, deacetylation of H3K18ac by SIRT7 results

in transcriptional repression ^{299, 300}. Furthermore, the co-dependency between H3K18ac, H3K23ac, and H3R17me2 have been demonstrated to induce transcriptional activation in cancer cells ³⁰¹ and could therefore similarly be critical for stage-specific gene expression exclusively in mature gametocytes. This coordination may be an essential requirement for subsequent gamete formation and fertilization. The histone code in mature gametocytes may therefore result in bistable chromatin to enable a transcriptionally poised state of some genes for rapid fertilization and sexual replication once these mature gametocytes are taken up by a feeding mosquito, and this is mediated by unique co-dependent histone PTM combinations.

2.4 Discussion

Here we present the first systems-level evidence of a comprehensive combinatorial histone code for various life cycle stages of the human malaria parasite, *P. falciparum*. Middle-down proteomics provided high-resolution quantitative data to describe the histone code in this parasite, which could function as a model for other protista. Our data reveal that the combinatorial histone PTM landscape is dynamic, with clear stage-specific differentiation observed, with gametocyte stages more dependent on histone PTM crosstalk than asexual parasites.

Collectively, exemplifies the difference between the asexual replicating parasite and the differentiated non-replicative gametocyte. The histone code of *P. falciparum* asexual parasites resembles that of lower eukaryotes, which has a simple genome organisation and fewer histone PTMs. Given the primal function it performs, elaborate epigenetic gene regulation mechanisms may not be as important to these stages, as most of the genome is in an euchromatic state and actively transcribed during proliferation. Therefore, asexual parasites use less, and mutually exclusive, histone PTMs to regulate key functions such as host immune evasion. However, gametocytes share a similar mechanism of epigenetic gene regulation with other higher-order, multicellular, eukaryotes where chromatin is predominantly condensed and highly regulated to specify the identity and purpose of a cell through multiple histone PTMs. The majority of gametocytes indicate general positive crosstalk between histone PTMs and given the limited number of transcription factors in *P. falciparum* parasites, gametocytes could rather switch to a more complex epigenetic code to impress very specific regulation of its biological processes. This would suggest that at least in some stages of the parasite, epigenetic level gene control is superior to transcriptional level control.

The connectivity within the histone code of *P. falciparum* is characterised by the presence of novel marks, with several new arginine modifications identified. The advances in proteomics technologies such as middle-down proteomics are allowing such robust description of arginine methylation marks ²⁸¹ as observed here, and this contributes to our understanding of the conserved nature of arginine methylation and its key importance to chromatin organisation throughout eukaryotes ²⁹². We show that histone arginine methylation is equally as prevalent and abundant as lysine PTMs in *P. falciparum* and these marks participate in complex crosstalk with one another, particularly in gametocytes. The presence of typically activating marks such as mono- and demethylation of H3R17 ²⁹² and their co-dependence on other marks e.g. H3R8me2 raise interesting questions as to the importance of cooperation in activation of gene sets in gametocytes. This is extended to additional arginine methylation marks including the exclusive nature of H3R42me1 in gametocytes and the highly connected, but independently functioning H3R40me1. It is noteworthy that H3R40me1 is required as

activating mark for spermatogenesis-like processes in yeast ^{279, 280}. These marks could be similarly important for activation of male gamete gene sets, supporting the notion of transcriptionally active 'poised' states in mature gametocytes ⁹² to enable onwards transmission. The importance of arginine methylation in histone PTM combinations to mediate a specific transcriptional outcome in gametocytes is therefore of interest. The parasite genome does contain the necessary machinery for arginine methylation including two putative protein arginine methyltransferases (PRMT1 [PF3D7_1426200] and PRMT5 [PF3D7_1361000]) and a putative histone arginine methyltransferase (CARM1/PRMT4 [PF3D7_0811500]). Indeed, PRMT inhibitors are active against *P. falciparum* parasites ³⁰² and CARM1/PRMT4 is essential in asexual parasites ⁸⁶, supporting the functional importance of these marks. Protein arginine methyltransferase inhibitors are seen as promising anticancer targets ³⁰³ and could be applied in the malaria context for gametocyte-targeting, transmission-blocking compounds.

The connectivity of histone PTMs in the *P. falciparum* histone code is associated with different developmental outcomes, similar to other eukaryotic systems requiring specialization e.g., embryogenesis and stem cell differentiation ^{304, 305}. Importantly, the prevailing heterochromatic mark, H3K9me3, is highly connected but not co-dependent on any other acetylation or methylation mark across all the life cycle stages. In the absence of quantified association between H3S10ph, as co-existing mark impairing PfHP1 binding to H3K9me3 ²⁰¹ in Plasmodia, this indicates that H3K9me3 is likely singularly important to binding of PfHP1 to demarcate heterochromatin in *P. falciparum*. However, gametocytes additionally use other highly connected, but independently acting marks repressive marks such as H3R40me1 and H3K14me2 to likely govern strategy-specific gene inactivation, as has been described for H3K36me2/3 inactivation of gene sets typically only required in asexual parasites ²⁴¹. The observation that repressive marks are highly connected but in the majority of instances show independence or mutual exclusivity to the partner PTMs questions the importance of repression in Plasmodia. The limited set of histone PTMs to enable effective transcriptional repression and induction of heterochromatin could be important to control transcript levels of particular gene sets, e.g., virulence genes in asexual parasites ²⁸³ but are more important during gametocytogenesis. Additional mechanisms including RNA decay may be more influential to regulate transcript levels in general ^{89, 110}.

Connectivity between PTMs in *P. falciparum* is important for coordinated function and activation of euchromatin. Silencing PTMs usually form independent heterochromatic domains (e.g., H3K9me3), but activating marks are frequently found together. The majority of activating PTMs coordinate irrespective of life cycle stages, particularly H3K9acK14ac and explains the euchromatic permissive nature of asexual parasites, enabled by coordinated binding of effector proteins. Novel combinations such as H3K4me3K23me1 and several combinations

with H3R17me1 and H3R17me2 (H3K4me1, H3R8me2, H3K9me2) is pronounced during gametocytogenesis. The H3K9acK36me3 combination is particularly prominent (high positive interplay score, Figure 4.1) and of functional interest since H3K9ac is associated with euchromatin while H3K36me3 is typically heterochromatin associated in *P. falciparum*^{176, 241}. This bivalent combination occurs more frequently than expected in trophozoites, which suggest that they likely have functional relevance for asexual proliferation. Additionally, H3K9acK4me3 co-localise together in a large fraction of the genome in trophozoites but does not seem to correlate with gene expression¹⁷⁶, rather implying a regulatory role in euchromatin maintenance than direct enrichment to regulatory elements for control of gene regulation. Alternatively, the combination could result in recruitment of effector proteins that then can result in subsequent gene regulation events. Additionally, during these stages of unique differentiation of *P. falciparum*, the parasite relies on specific and differentiated combinations, including the unique H3K27me1K36me1 and H3R17me2K18acK23ac combinations seen in only mature gametocytes. The connectivity and crosstalk between histone PTMs are therefore essentially important to establish general euchromatic regions in the parasite's genome across all stages. However, crosstalk of activating marks is more prevalent in gametocytes and requires differentiation in marks used for euchromatin in these stages. This implies the use of specific histone combinations for activation of strategy-specific gene sets to mediate *P. falciparum* transmission.

The histone code in *P. falciparum* is therefore diverse and dynamic to effect different combinations required for proliferation and differentiation. The complex nature of the combinations and changes in the identity of the combinations during stage transition points to this epigenetic level of regulation is a more important level of regulation that can be easily finetuned by variation in the combinations, particularly for gametocytes. With a limited set of effector proteins and only a core of ~5 full effector protein complexes characterised^{187, 306}, the intricacy of the histone code indicates that indeed, combinations of histone PTMs provides the blueprint to ensure differentiation, specificity, and variation to control transcriptional activation of gene sets during *P. falciparum* development.

In conclusion, our study contributes a comprehensive catalogue of histone PTM combinations and provides a foundation for further investigation of the increasingly intricate histone code of the *P. falciparum* parasite.

Chapter 3 | Epi-proteomics reveals a unique chromatin proteome associated with histone post-translational modifications

Part of the work in this chapter has been included in a manuscript as follows:

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3.1 Introduction

Remodelling of chromatin structure is a result of histone post-translational modifications (PTMs), DNA methylation and ATP-dependent enzymes³⁰⁷. A transcriptionally 'permissive' euchromatic state is characterised by highly acetylated histones while a 'repressive' heterochromatin features modified histone modifications and DNA methylation that condenses chromatin. *P. falciparum* uses distinct histone PTMs to control gene expression in both asexual and sexual forms of the parasite, but a different, specialised subset of PTMs is used during sexual gametocytogenesis¹⁶⁷. As a result, in asexual *Plasmodium* parasites, the genome is primarily maintained in a euchromatic state and enriched with histone 3 acetylation in the lysine 9 position (H3K9ac) and a trimethylation of lysine 4 on histone 3 (H3K4me3)^{308, 309}. Heterochromatin is decorated with H3K9me3 coupled with the transcriptional repressor protein heterochromatin protein 1 (PfHP1)^{159, 178, 271}. The latter comprises one of the most important and influential protein-protein-DNA interactions studied in the human malaria parasite, *Plasmodium falciparum* and regulating the bifurcation between asexual proliferation and sexual differentiation. Binding of PfHP1 to H3K9me3 demarcates the *ap2-g* locus and results in condensed chromatin clusters in this region^{159, 271}. As a result, the AP2-G (apicomplexan apetala2) transcription factor is not produced, and sexual development is blocked. Gametocytogenesis commitment is further characterised then by gametocyte development 1 (PfGDV1) protein displacing PfHP1 from the *ap2-g* locus¹⁶³. Taken together, it is evident that histone PTMs and their associated protein interacting partners act in concert to elicit biological events.

Proteins that are involved with chromatin organisation and maintenance have been studied in a wide range of eukaryotes^{227, 307, 310, 311}, although this has been more challenging in *P. falciparum* due to the lack of homology with model eukaryotes. Recently, 1190 candidate chromatin-associated proteins have been identified *in silico* based on chromatin-associated domains identified in 12 model eukaryotic organisms³¹². This included proteins that have parasite specific chromatin associated domains, such the crowded-like family of plant proteins that likely regulates heterochromatin formation in the parasite³¹².

Pull-down assays using synthetic peptides with specific domains revealed that in *P. falciparum*, the variety in which epigenetic reader complexes exists is diverse and include sub-complexes unique to *Plasmodium* ¹⁸⁷. These reader complexes composed of 'writer' subunits, multiple 'reader' enzymes and/or chromatin remodelling enzymes ¹⁸⁷. The observed high degree of connectivity between the diverse, but highly interconnected epigenetic protein modules, as well as the various distinct 'flavours' of each module of the complexes, adds to the amount of complexity that *P. falciparum* employs to distinctly modulate gene expression.

Functional proteomic techniques, such as chromatin immunoprecipitations coupled with MS can provide the protein components of the complexes bound to highly specific regions on chromatin ^{313, 314}. Identifying proteins that are chromatin-associated are challenging since it is necessary to disrupt the integrity of chromatin, while not disrupting the complexes itself. ChIP-MS offer both advantages and disadvantages: it allows the ability to capture native *in vivo* protein-protein interactions in an unbiased fashion without *a priori* information of the type of interactions ³¹⁵. Inevitably, proteins that are nonchromatin-associated are also identified, making it challenging to discern true interactions compared to false-positive results.

The combination of H3K18acK23ac occurs in abundance in mature gametocytes. Furthermore, this unique combination of H3K18acK23ac, and the associated co-existence with H3R17me2 in mature gametocytes, warranted further investigation of the presence of shared effector proteins to promote a biological outcome of transcriptional activation associated with these combinations. Unlike the known H3K9me3 and H3K14ac marks in asexual parasites, H3K18ac and H3K23ac have not been characterised functionally in *Plasmodium*. In embryonic stem cells, H3K18ac promotes mesendodermal differentiation and when in combination with H3K9me3, allows for the activation of nodal target genes through TRIM33 ³¹⁶. H3K18ac further acts as a marker of cancer progression and is localised to transcriptional start sites and enhancers ³¹⁷. Similarly, H3K23ac is an oncogenic marker, where H3K23ac association with TRIM24 regulate, for example, breast cancer development ³¹⁸⁻³²⁰. When in combination with H3K14ac, H3K23ac recruits TRIM24 facilitates glioblastoma progression ³¹⁸. This indicates that histone PTMs, either a single or bivalent PTMs, together with protein associations, can shift transcriptional programs of cells. Investigation into this combination has the potential to improve our understanding of how cell type identity is acquired and maintained.

To identify the protein machinery associate with H3K18ac and H3K23ac marks, we performed quantitative chromatin immunoprecipitation on crosslinked chromatin isolated from mature gametocytes, coupled with quantitative mass spectrometry (ChIP-MS). We identify the proteins associated with the novel histone PTM combination of H3K18ac and H3K23ac, in

transmissible mature gametocyte stages. The results presented here suggest parasite employs different strategies of chromatin regulation during its life cycle, where gametocyte stages a preference for protein associations to histone PTMs as compared to asexual stages. An overlap is observed between chromatin-associated proteins and proteins linked to metabolism, signalling and structure in the parasite. This study ultimately expands our perspective on parasite chromatin biology.

3.2 Experimental procedures

3.2.1 *In vitro* cultivation of *P. falciparum* asexual parasites and gametocytes

Parasite cultures and samples were identical to those in Section 2.2.1, Chapter 2.

3.2.2 H3K18ac and H3K23ac ChIP antibody validation

Using modified and unmodified synthetic peptides (Genscript) designed to contain 17-22 amino acids of the 3D7 histone H3 sequence, the ability of commercially obtained ChIP-grade rabbit monoclonal anti-histone H3 acetyl K18 (Abcam ab177870) and anti-histone H3 acetyl K23 (Abcam ab177275) antibodies to detect modified histone H3 in *P. falciparum* was determined. Modified histone H3 peptides were synthesised to contain acetylation on K18 (STAGKAPR[Lys-Ac]QLASKAAR) and K23 (APRKQLAS[Lys-Ac]AARKSAPI). To evaluate a broader range of specificities, a similar unmodified peptide (STAGKAPRKQLASKAARKSAPI) was included. Each peptide (25 ng) was quantitatively loaded onto nitrocellulose membranes, which were submerged in blocking buffer (5 % skim milk powder in TBS-t containing 50 mM Tris [pH 7.5], 150 mM NaCl, and 0.1 % [v/v] Tween-20) for 30 min followed by 1 h incubation with α -H3K18ac (Abcam, ab119100), α -H3R17me2 or α -H3core (Abcam, ab176842) primary antibody dilutions (1:5000 in TBS-t). Membranes were washed three times in TBS-t and then incubated with HRP-conjugated rabbit α -IgG secondary antibody (1:10000 in TBS-t) for 1 h. (Pierce™ SuperSignal West Pico PLUS Chemiluminescent Substrate was quantified with ImageJ analysis software (1.52a; <http://imagej.nih.gov/ij>)^{321, 322}.

3.2.3 Chromatin immunoprecipitation (ChIP) for analysis of histone PTM-associated proteins

To identify proteins that associate to individual histone PTMs that occur in combination, ChIP-MS was performed using an adapted protocol previously developed³¹⁵. A minimum number of isolated parasites (10^9 cells/mL) was crosslinked with 1 % formaldehyde and subsequently quenched with 125 mM glycine. Crosslinked parasite nuclei were suspended in cold lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, 1 mM DTT, and EDTA-free protease inhibitor cocktail) and transferred to a pre-chilled dounce homogeniser. NP40 was added to a final concentration of 0.25 % and parasites are subsequently lysed with dounce B for ~100 strokes. Sonication shearing buffer (a cocktail of protease inhibitors, 1 % SDS, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA and 100 mM NaCl) were added to the nuclei pellet and sonicated with the BioRuptor UCD-200 (Diagenode, Belgium) for 25 cycles at high power and 30 s intervals. Crosslinks were reversed by incubating the input sample overnight at 65°C. The input sample was suspended to a final volume of 200 μ L with ChIP dilution buffer (0.01 % SDS, 1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 150 mM NaCl, and a cocktail of protease inhibitors). To dilute the

SDS in the dilution buffer, Tris-EDTA buffer (1 M Tris, pH 8.0 and 0.5M EDTA, pH 8.0) was added with RNase to a final concentration of 0.2 µg/µL and incubated for 1 h at 37°C. The chromatin soup was incubated with 1 µg of either anti-H3K18ac or anti-H3K23ac, rotating at 4°C overnight. Protein G magnetic Dynabeads™ (Invitrogen by Thermo Fisher Scientific, Norway) was added and incubated for 2 h rotating at 4°C. A total of 20 % of the eluted chromatin was then retained as the input. The remaining eluted material was then used for the western blot validation of combination histone PTM. The bead-chromatin complex was washed extensively with each of the following buffers with the aspiration of the preceding buffer before the addition of the next buffer: Low salt immune complex wash buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high salt immune complex wash buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl immune complex wash buffer (0.25 M LiCl, 1 % NP-40, 1 % deoxycolate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1) and TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). The beads were suspended in fresh ChIP elution buffer (0.1 M NaHCO₃, 1 % SDS) and the supernatant was collected.

3.2.4 Quantitative mass spectrometry of histone PTM-associated proteins

Briefly, protein samples were mixed with an equal volume of methanol: chloroform (3:1 ratio), followed by a brief vortex step and centrifugation at 4°C. Samples were washed twice with methanol followed by centrifugation at 17 000 xg for 3 min and complete removal of methanol: chloroform. The resulting precipitated proteins were dried at room temperature. All samples were dissolved in a solution containing 6 M urea, 2 M thiourea, and 50 mM ammonium bicarbonate, pH 7-8. Samples were subjected to reduction, alkylation, and digestion in preparation for MS as follows. Samples were incubated at room temperature for 1 h in 5 mM DTT to reduce disulfide bonds, cysteine residues alkylated in 50 mM iodoacetamide, and subjected to Lys-C (1-1.5 µg) and trypsin (10 µg) in-solution digestion at room temperature for 3 h. Samples were then diluted with 50 mM ammonium bicarbonate to lower the urea and DTT concentrations in solution to prevent trypsin inactivation. The samples were sonicated (Sonic Dismembrator Model 100, Fischer Scientific, USA) for 3 cycles of 10 s continuous sonication (output 2) followed by 10 s resting on ice to dissolve the pellets completely. Cysteine residues were alkylated by incubation with 40 mM iodoacetamide. This was followed by overnight digestion with 10 µg trypsin at room temperature. The pH of the trypsin digested samples was adjusted with ammonium hydroxide to ~pH 8-8.3 followed by centrifugation at 17 000 xg for 1 min. StageTip (STop And Go Extraction Tip) clean-up in combination with protein fractionation was performed for all the samples using a dual resin approach, where both Empore™ C18 disks (3 M, USA) and Oligo™ R3 reversed-phase resins were used in combination. This was done to remove unwanted contaminants before MS and to fractionate the samples to ensure optimal protein detection. The column was equilibrated with 1 mM ammonium bicarbonate (pH

8.0), after which the supernatant of each sample was added to the dual resin StageTip and pushed through the column using a syringe (dropwise). All samples were dried down completely under vacuum (SpeedVac concentrator). In preparation for the MS run, 0.1 % formic acid was added to the samples followed by ultrasonic bath sonication for 10 min at 4°C. The samples were centrifuged at 17 000 xg for 10 min, and the supernatant was loaded onto a Dionex™ -LC system (Thermo Fisher Scientific, USA), coupled online with a Q-Exactive HF mass spectrometer (Thermo Scientific, USA). Peptides were loaded into a picofrit 20 cm long fused silica capillary column (75 µm inner diameter) packed in-house with reversed-phase Repro-Sil Pur C18-AQ 3 µm resin. A gradient of 105 min was set for peptide elution from 2-28 % buffer B (100 % ACN/0.1 % formic acid), followed by a gradient from 28-80 % buffer B in 5 min and an isocratic 80 % B for 10 min. The flow rate was set at 300 nL/min. MS method was set up in a DDA mode. The full MS scan was performed at 70 000 resolutions [full width at half maximum (FWHM) at 200 m/z] in the m/z range 350-1200 and an AGC target of 10⁶. Tandem MS (MS/MS) was performed at a resolution setting of 17 500 with a collision induced dissociation (HID) collision energy set to 20, an AGC target of 5x10⁶, a maximum injection time of 100 ms, a loop count of 12, an intensity threshold for signal selection at 10⁴, including charge states 2-4, and a dynamic exclusion set to 45 s.

3.2.5 Chromatin proteomic profiling data analysis

MS raw files were analysed by MaxQuant software version 1.5.2.8. MS/MS spectra were searched against the *Plasmodium falciparum* UniProt FASTA database, containing 33 655 entries. Sequencing grade trypsin (Promega, catalog number V5111) was used to generate peptides. Trypsin is a serine protease that specifically cleaves at the carboxylic terminus of lysine and arginine residues. When proline is at the carboxylic side of lysine or arginine, the peptide bond is almost completely resistant to cleavage by trypsin. Two trypsin missed cleavages were allowed. Carbamidomethyl (C) was set as a static modification. Oxidation (M), as well as acetyl (protein N-term), were selected as variable modifications. Mass accuracy was set at 4.5 ppm for precursor and 0.5 Da for the product mass tolerance. Peptides were filtered for high confidence (FDR<1 %) using a fixed value validator. Intensity-based absolute quantification (iBAQ) enabled for label-free quantification, where iBAQ values are calculated by a MaxQuant algorithm (sum of peak intensities of all peptides matching to a specific protein/number of theoretical peptides). Match between runs was enabled and set to a 1 min window. All samples were run in triplicate for three independent biological replicates. For data analysis, iBAQ values were log₂-transformed and normalised by subtracting to each value the average value of the respective sample. The peptide relative ratio was calculated using the total area under the extracted ion chromatograms of all peptides with the same amino acid sequence (including all its modified forms) as 100 %. For isobaric peptides, the relative ratio of two isobaric forms was estimated by averaging the ratio for each fragment ion with a

different mass between the two species. Next, extracted ion chromatography of those m/z ions with a mass tolerance of 10 ppm was performed. Statistical significance was assessed with a false-discovery rate (FDR) adjusted *P*-value (*P*-value representation * = < 0.05, ** = < 0.005, *** = < 0.0005).

3.2.6 Protein-protein interaction model

STRING v.10 database [string.org, ³²³] parameters were set to include protein-protein interactions based on evidence, sourced from neighbourhood, experiments, databases, co-occurrence, and co-expression. The minimum required interaction score was required to be of high confidence (0.7) with a maximum of 100 proteins interacting the first shell and 100 proteins in the second shell. Data from peptide pulldowns studies were pooled from Singh *et al.*, 2020 and Hoeijmakers *et al.*, 2019 and filtered only to include proteins that were significantly enriched in the respective studies. Finally, protein-protein interactions were derived from a yeast-two-hybrid study (La Count *et al.*, 2005). Taken together, proteins were imported into Cytoscape (version 3.8.2). Proteins were further filtered to include only known chromatin associated proteins and proteins that have homology to known H3K18ac and H3K23ac associated proteins.

3.2.7 Data Availability

All raw files from the ChIP-MS data are freely available on <https://chorusproject.org> at project no. 1730. All proteomics data generated in this study have been deposited to the ProteomeXchange Consortium (<http://www.proteomexchange.org/>) using the PRIDE partner repository ²⁵⁹. These data sets are available with data set identifier PXD030181. The data analysis pipeline meets all MIAPE standards.

3.3 Results

3.3.1 Toward profiling of the histone PTM-associated proteome in *P. falciparum* parasites

To identify and characterise the proteins that associate with histone PTMs, highly synchronised (>80 % stage specificity) mature (stage V) gametocytes were interrogated (n=3, see Figure 3.1 - Chapter 2). Chromatin was isolated from the stage V samples (at 2.92 ± 0.0480 parasitaemia/gametocytaemia (n=3) and immunoprecipitated based on the two histone PTMs under investigation, coupled with high-resolution quantitative nanoLC-MS/MS (Figure 3.1). Using optimised protocols for crosslinking of protein-protein/DNA interactions, nuclei isolation and chromatin shearing, proteins with proximity to specific histone tail modifications can be positively identified. With peak iBAQ, proteins were identified with high confidence (n=3, FDR <1 %) within a sample set, where horizontal comparison between histone PTMs and parasite stages are applicable. The experimental approach allowed for high mass accuracy (4.5 ppm for precursor and 20 ppm for product mass tolerance) and reduced variance between biological replicates, producing robust data which enables for confident evaluation of proteins associated with specific histone PTMs.

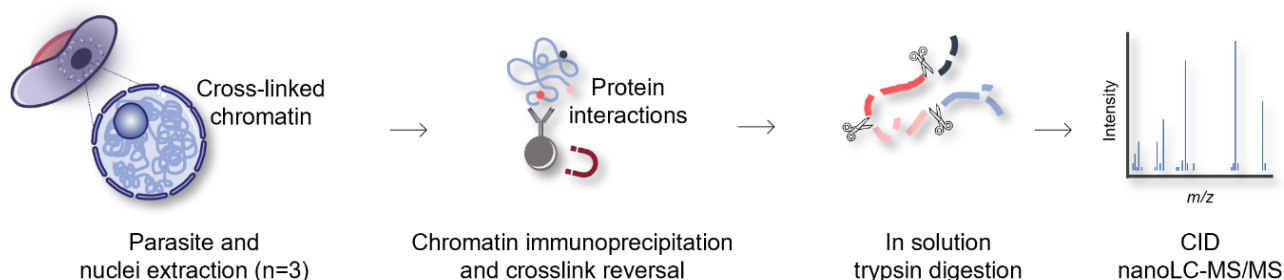


Figure 3.1. Chromatin proteomic profiling of H3K18ac and H3K23ac in mature stage gametocytes. Images were adapted from Servier Medical Art (URL link to the license: <https://creativecommons.org/licenses/by/3.0/>) and changes were made in terms of colour, size, and composition.

The presence of both H3K18ac and H3K23ac as independent PTMs in mature gametocytes were first determined from the same parasite population used for downstream experimentation (Figure 3.2).

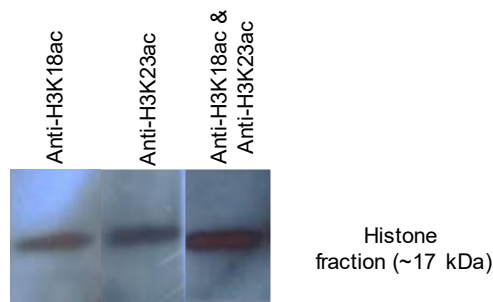


Figure 3.2. H3K18ac and H3K23ac is present in combination in mature stage gametocyte samples prepared for ChIP-MS. The presence of both H3K18ac and H3K23ac PTMs in mature stage gametocytes were confirmed by western blot analysis, showing co-existence in mature stage gametocytes.

Indeed, both PTMs were detected at a high enough abundance to proceed. ChIP experiments rely heavily on the specificity of the antibody used. Since an antibody against H3K18acK23ac is not commercially available, we opted to capture proteins that interact with or are recruited to both H3K18ac and H3K23ac, respectively, in two separate ChIP experiments using antibodies specific to H3K18ac and H3K23ac. Due to a lack of *Plasmodium*-specific antibodies but >99 % sequence similarity between *P. falciparum* H3 and H3 in other organisms, commercial ChIP-grade rabbit α -H3K18ac and α -H3K23ac antibodies were chosen for this work and cross-reactivity and antibody specificity against modified *P. falciparum* H3 was confirmed (Figure 3.3).

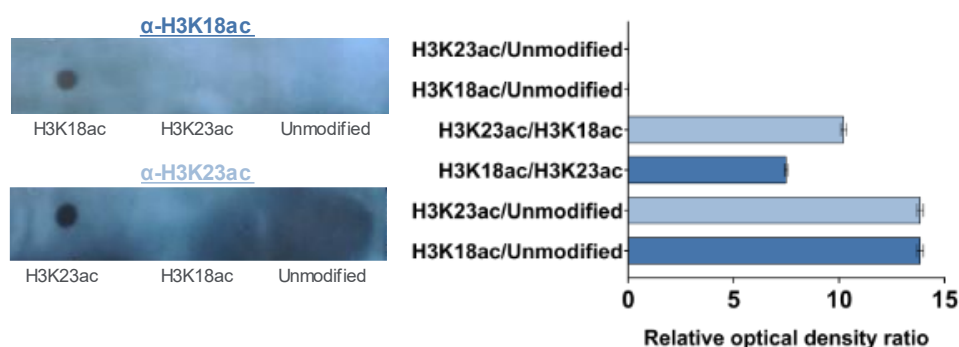


Figure 3.3. H3K18ac and H3K23ac antibody validation showing specificity towards *P. falciparum* H3K18ac and H3K23ac. Validation of antibodies against H3K18ac and H3K23ac for use in ChIP-MS investigations. Dot blot analysis was used to evaluate if the antibodies could detect unmodified (H3K18 and K23) or modified (H3K18ac, H3K23ac) *P. falciparum* H3 synthetic histone peptides. To quantify the ratio at which the antibodies recognise each peptide, the relative optical density was computed.

P. falciparum H3 peptides modified with H3K18ac or H3K23ac had a 7.5- and 6-fold greater specificity, respectively, towards their corresponding modified peptides compared to the unmodified peptide (Figure 3.3). The antibodies were modification-specific, with no cross-reactivity between the H3K18ac and H3K23ac modifications. As a result, these antibodies were used to probe chromatin associated proteins in mature stage gametocytes.

3.3.2 ChIP-MS reveal a H3K18ac and H3K23ac associated proteome

To assess the correlation and variance between biological replicates, raw iBAQ values were Pearson correlated. ChIP-MS data belonging to corresponding replicates were highly correlated with Pearson correlation between all biological replicates, showing R^2 values of 0.84 ± 0.058 ($n=3$), and less correlation (0.31 ± 0.09) to the negative IgG control. This indicated the reproducibility of the data for confident analyses (Figure 3.4A).

The distribution of the iBAQ values obtained for each protein identified either with H3K18ac, H3K23ac were significantly enriched compared to the IgG control, showing that proteins were preferentially enriched in the ChIPed samples rather than the nonspecific IgG samples (Figure 3.4B). The average unique sequence coverage at 14.5 ± 13.1 % with a low number of average unique peptides identified 5.23 ± 7.18 (Figure 3.4C). This is comparable to what was previously found with chemical crosslinking with formaldehyde, as formaldehyde not only induce intermolecular crosslinks between proteins, but also intramolecular crosslinks between residues within the same protein which accounts for the lower sequence coverage as compared to non-crosslinked proteomic studies. Proteins known to be 'false-positives' such as proteins are high in abundance or non-specific binders were also identified but are unlikely to be involved histone PTMs mediated chromatin regulation. On average, ~5 peptides identified per protein (Figure 3.4D) which is comparative to previous ChIP-MS results. Collectively, these experiments form the basis for the quantitative identification of proteins that interact with histone PTMs.

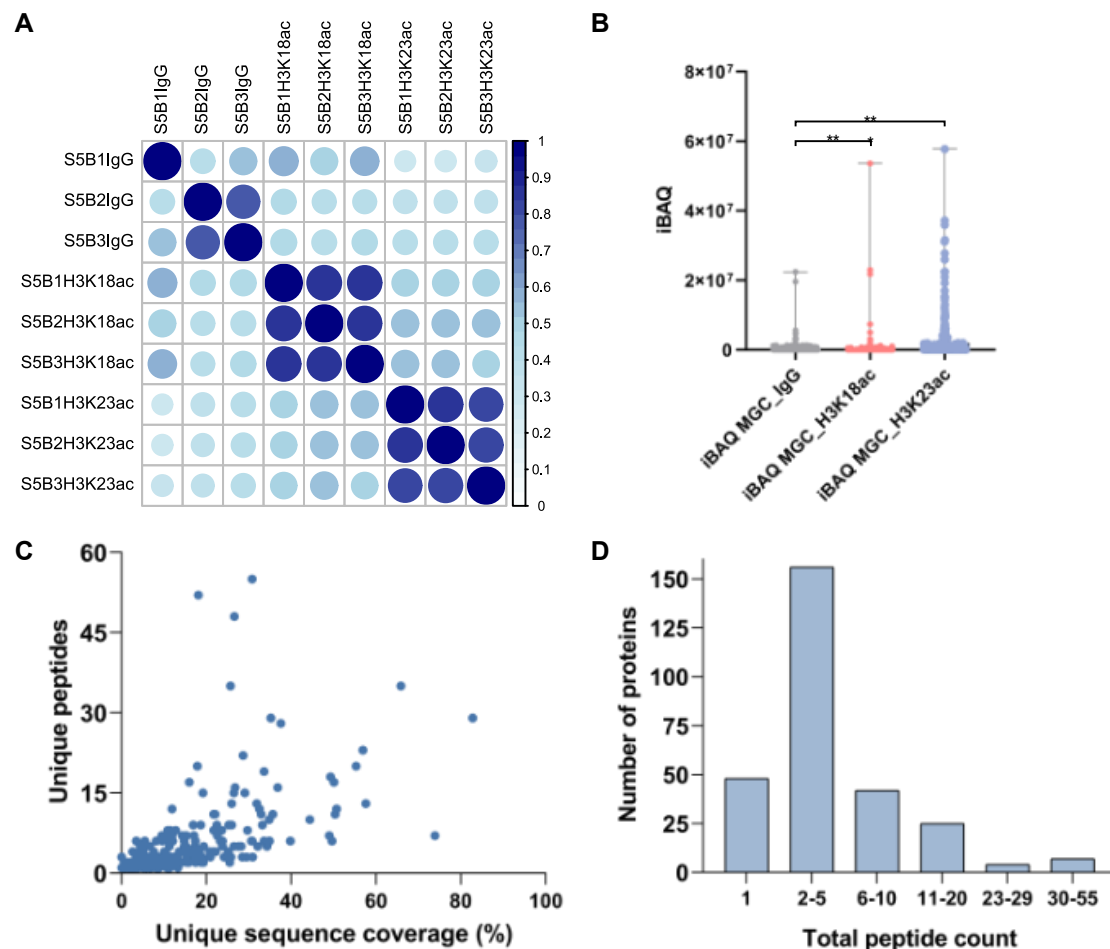


Figure 3.4. Assessment of raw quantitative mass spectrometry data. (A) Pearson correlation coefficient plot of the raw intensity based absolute quantification (iBAQ) values obtained from mass spectrometry of following ChIP. Data are representative of the three independent biological replicates for each histone modification. (B) The raw iBAQ distribution of the proteins identified in the H3K18ac, H3K23ac and IgG ChIP-MS. Each data point represents a protein identified, where data points overlap and fall within other data points. (C) The number of unique peptides, i.e., the number of peptides sequences unique to a protein, is shown along with the unique peptide sequence coverage for each of the proteins identified and quantified. (D) The number of peptides used for the identification and quantification of each protein is shown.

3.3.3. A subset of proteins is shared between H3K18ac and H3K23ac

A total number of 282 *P. falciparum* parasite proteins were identified and quantified, similar to the number of proteins identified with ChIP-MS in embryonic stem cells¹⁹³. We defined proteins significantly associated with H3K18ac and H3K23ac as those with a $\log_2$ fold change ≥ 2 for the ChIP population (crosslinked chromatin preparation) compared to the IgG control ChIP, at an FDR $\leq 5\%$.

Selective enrichment of proteins for H3K18ac and H3K23ac was confirmed by: 1) the significant ($P \leq 0.01$) enrichment of unique peptides for low abundance proteins in the H3K18ac and H3K23ac ChIP preparations compared to the IgG sample preparations; 2) significantly increased abundance of proteins in the histone PTM samples compared to the IgG samples based on relative intensity-based absolute quantification (iBAQ) (Figure 3.4B, P

≤ 0.01) and lastly, 3) the enrichment in the ChIP population for chromatin-associated proteins³¹², the nuclear pore proteome³²⁴, and transcription factor interacting proteins¹⁰⁵. We found three proteins to be strongly enriched for H3K18ac in the ChIP-MS data, compared to 46 proteins for H3K23ac (FDR < 5 %, $\log_2$ fold change ≥ 2 over IgG control ChIP, including AP2-G2 (PF3D7_1408200), a protein with an unknown biological function (PF3D7_1239800), centrin-2 (PF3D7_1446600)(Figure 3.5).

The transcription factor AP2-G2 (PF3D7_1408200), a protein with an unknown biological function (PF3D7_1239800), centrin-2 (PF3D7_1446600) and DnaJ (PF3D7_1002800) were enriched in both PTM ChIPs. Besides the chaperone DnaJ, all of these proteins were previously implicated to be chromatin-associated³¹². The gene for PF3D7_1239800 is refractory to deletion, is likely essential for asexual parasite survival⁸⁶, and is highly expressed in mature gametocytes²⁷⁶. AP2-G2 is also essential for the successful completion of gametocyte maturation and transmission^{105, 325}.

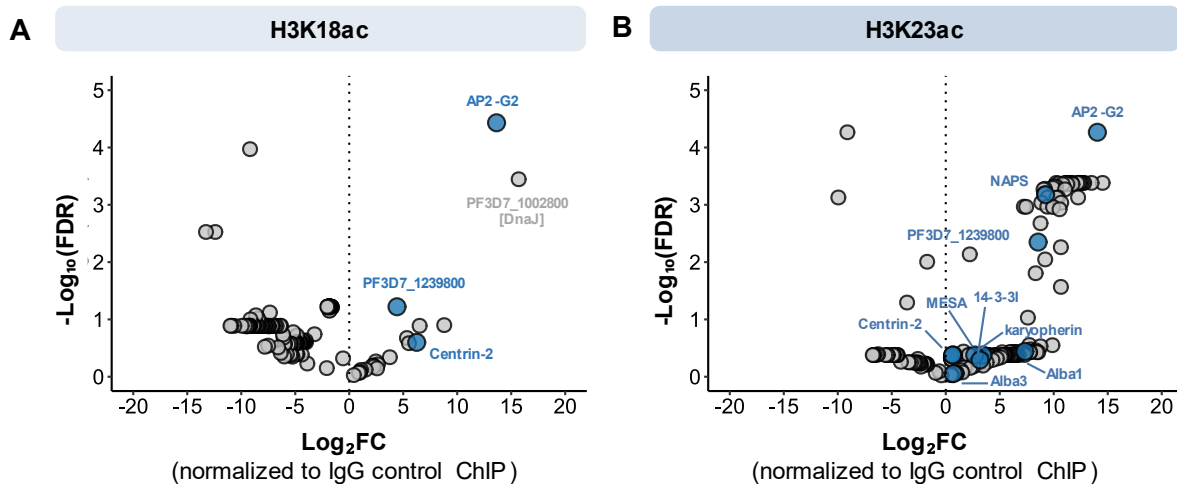


Figure 3.5. ChIP-MS identifies shared and unique proteins associated with H3K18ac and H3K23ac. (A) H3K18ac and (B) H3K23ac-associated proteome enrichment in the ChIP-MS. A total number of 282 proteins were identified in the preparations, with several proteins showing positive $\log_2$ -fold change normalised to the negative control IgG ChIP. Proteins that are shared between the H3K18ac and H3K23ac samples include transcription factor AP2-G2 (PF3D7_1408200), a conserved unknown function protein (PF3D7_1239800), and centrin-2 (PF3D7_1446600) as shown in pink. H3K23ac also includes nucleosome assembly protein (PF3D7_0919000, NAPS), DNA/RNA-binding protein Alba 1 (PF3D7_0814200, Alba1), karyopherin beta (PF3D7_0524000), DNA/RNA-binding protein Alba 3 (PF3D7_1006200), 14-3-3 protein (PF3D7_0818200, 14-3-3I), mature parasite-infected erythrocyte surface antigen (PF3D7_0500800, MESA).

Association of GO annotations to the proteins that were positively enriched (i.e., $\text{FC} > 1$) in both samples indicated the biological processes associated with each histone PTM. Overall, the proteins that are shared between H3K18ac and H3K23ac were associated with chromatin remodelling and were localised to the nucleus of the parasite ($P < 0.05$; Table 3.1). However, each PTM was differentially associated with phosphatidyl metabolism and the mitotic cell

cycle, and calcium and kinase activity in H3K18ac and H3K23ac, respectively. This indicates that although H3K18ac and H3K23ac co-exist and share core proteins, a differential set of effector proteins are exclusively recruited to these PTMs.

Table 3.1. Gene ontology of enriched processes associated with proteins found in H3K18ac and H3K23ac ChIP-MS experiments. Abundant biological processes and cellular compartments associated with proteins are shown per histone PTM based on proteins enriched ($\log_2$ -fold change > 0, FDR < 0.05). Gene Ontology enrichment based on biological processes and cellular compartment, $P < 0.05$) and limited to proteins for which annotation exists.

Sample	Biological process	Cellular compartment
H3K18ac	Chromatin assembly or disassembly, mitotic cell cycle, phosphatidylcholine metabolic process	Nucleus, nucleosome, euchromatin
H3K23ac	Nucleosome and chromatin assembly organisation, cellular calcium ion homeostasis, regulation of lipid kinase activity	Nucleus, nucleosome, methyltransferase complex, nuclear envelope, nuclear membrane

This dataset was subsequently interrogated for the PTM specific protein abundance profiles to identify nuanced differences in the PTM-associated protein abundances.

3.3.4 Chromatin association proteins is characterised by PTM specific protein abundance profiles

Proteins that were identified in H3K18ac and H3K23ac were subsequently clustered based on their relative abundances. To assess the degree of variability observed between the relative abundances between samples and define abundance trends within the samples, k-means clustering was applied. The value for k (i.e., number of clusters or the number of eigenvectors k used for the spectral projection) was chosen using the least sum of squares (Figure 3.6).

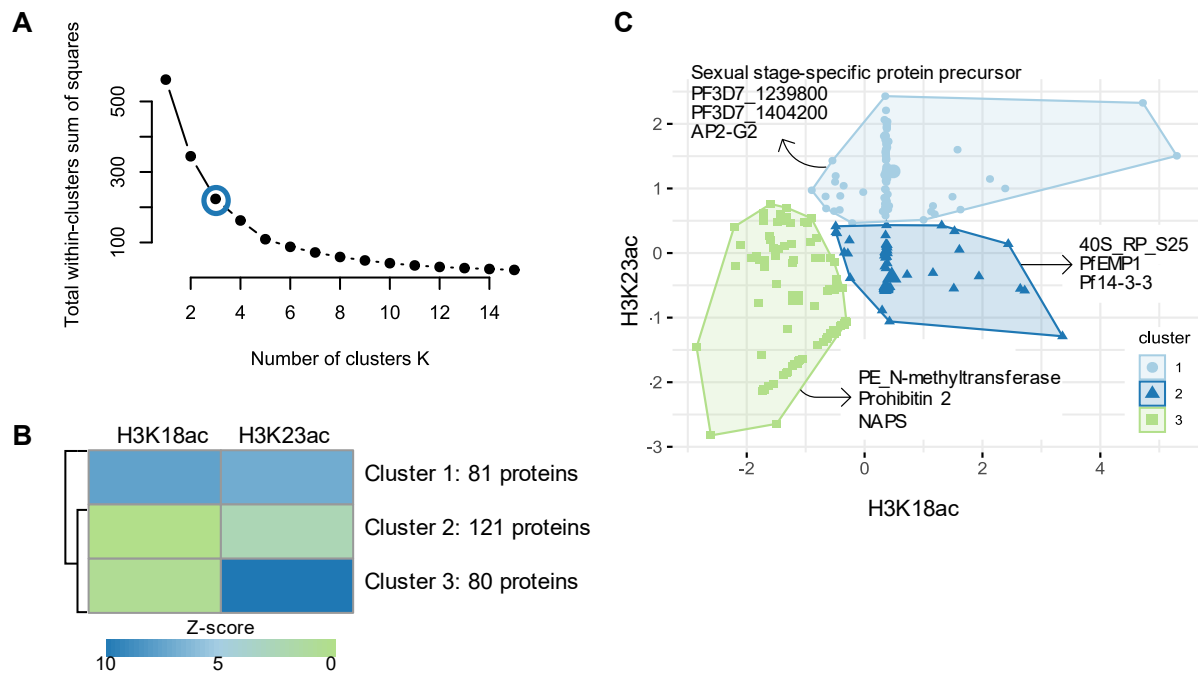


Figure 3.6. PTM-specific protein abundance profiles in mature stage *P. falciparum* gametocytes. (A) Elbow plot showing the optimal number of k-number of clusters as indicated with a blue circle (k=3). (B) K-means clustering of 282 proteins rendered three defined abundance profiles (k=3) as shown for proteins associated with H3K18ac and H3K23ac. (C) Clusters were analysed with principal component analysis, showing two major components explaining most of the variation in the data (x axis [H3K23ac sample protein abundances] 36 % and y axis [H3K18ac sample protein abundances], 19.1 %). The three clusters identified in (B) and the representative protein from each cluster is shown. Representative proteins from each cluster are shown. PE_N-methyltransferase: phosphoethanolamine N-methyltransferase, NAPS: nucleosome assembly protein, PfEMP1: erythrocyte membrane protein 1, 40S_RP_S25: 40S riboprotein S25.

Overall, the clustering process resulted with three main clusters (Figure 3.6), each with a unique abundance signature. Cluster 1 consisted of 81 proteins that indicated a conserved relative abundance between H3K18ac and H3K23ac. These proteins included the previously identified AP2-G2 (PF3D7_7_1408200), a sexual stage-specific protein precursor (PF3D7_0406200) and two abundant unknown proteins, PF3D7_1239800 and PF3D7_1404200 (Figure 3.6C). Cluster 2 contained proteins such as the 40S riboprotein S25, erythrocyte membrane protein 1 (PfEMP1, PF3D7_0632800) and the phosphorylation motif reader protein, Pf14-3-3 (PF3D7_0818200). These proteins were likely false-positives based on their high abundance or were in close-contact with H3K18ac or H3K23ac and were co-precipitated. In all instances, the proteins in cluster 2 had poor Z-scores. Finally, a subset of proteins was uniquely enriched in the H3K23ac sample (Figure 3.6B). This included phosphoethanolamine N-methyltransferase (PF3D7_1343000), prohibitin-2 (PF3D7_1014700) and a nucleosome assembly protein (NAPS, PF3D7_0919000). Taken together, these proteins highlight the shared and unique proteins that are recruited to H3K18ac and/or H3K23ac which provide insight into the possible regulatory proteins that associate to these PTMs and mediate their function.

3.3.5 A possible SAGA-like complex associated with the H3K18acK23ac combination

To further investigate the epigenetic complexes associated with H3K18ac and H3K23ac, protein-protein interaction data were used to assemble a proposed reader complex for these PTMs in mature stage gametocytes, associated with AP2-G2 (Figure 3.7)^{105, 187, 326}.

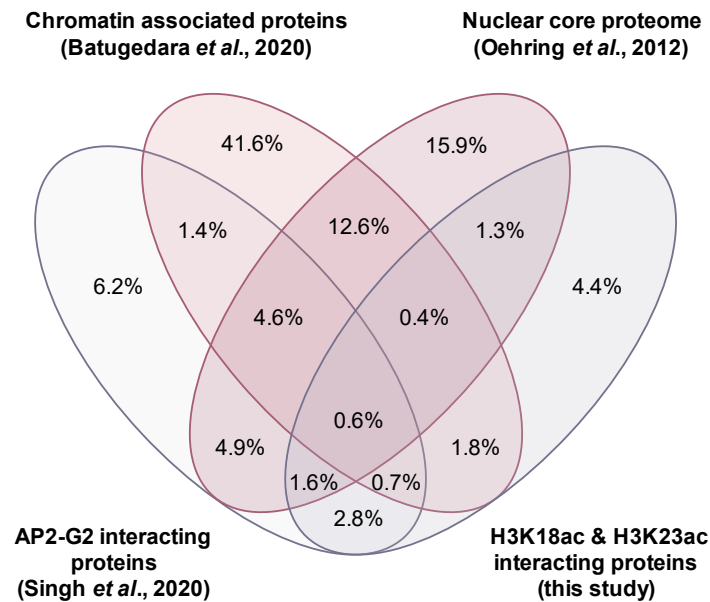


Figure 3.7. Venn diagram depicting the percentage of overlap between previous protein interaction studies. The number of protein overlap between this study and previous chromatin associated pull-down, including a an AP2-G2 peptide pulldown study (Singh *et al.*, 2020)¹⁰⁵, *in silico* predicted chromatin associated proteins from Batugedara *et al.*, 2020³¹² and the nuclear core proteome from Oehring *et al.*, 2012³²⁴. The proteins in the intersection between all four studies (0.6%) were considered for downstream interpretation.

Transcription factors are indeed observed in many histone PTM-associated complexes¹⁸⁷ to recruit additional members of epigenetic complexes, as shown for AP2-I¹⁰². The complex included evidence for the direct interaction between GCN5 (PF3D7_0823300), with acetyltransferase activity, and ADA2 (PF3D7_1014600), pointing towards the involvement of a HAT SAGA-like complex. This was supported by AP2-G2 further associated with the chromodomain-helicase-DNA-binding protein 1 homolog (PF3D7_1023900; CHD1), which in turn associates with PHD2 (PF3D7_1008100), a parasite-specific PHD-finger domain-containing protein with different specificity to its PHD1 partner (unable to bind e.g. H3K4me3,¹⁸⁷). The nucleosome assembly protein (NAPS) and PF3D7_1239800 also have direct interactions with PHD2. In gametocytes, H3K18acK23ac therefore associates with a GCN5-ADA2-PHD2 SAGA-like complex via the tight interaction of AP2-G2, NAPS and PF3D7_1239800 as binding partners to this particular histone combination. Most of these proteins are predominantly expressed in male mature gametocytes compared to female gametocytes, including ADA2, PHD2, ISWI, NAPS, CHD1, and AP2-G2³²⁷, implicating these proteins in downstream sex-specific chromatin structure changes.

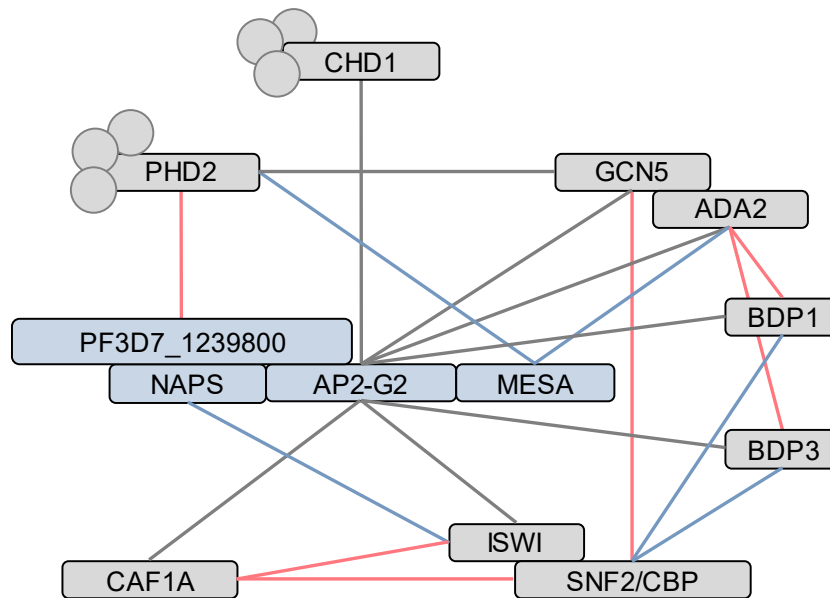


Figure 3.8. A proposed SAGA-like complex that interpret the H3K18acK23ac histone combination. A schematic model of the protein-protein interaction complex manually curated and placed in a network based on proteins as identified in all cross sections from Figure 3.8, in addition to a H3K9acK14ac pulldown (Hoeijmakers *et al.*, 2019)¹⁸⁷, and a previous yeast two-hybrid protein interactions (La Count *et al.*, 2005)³²⁶ and STRING interactions. Shown in grey blocks are interactions that include the transcriptional coactivator ADA2 (PF3D7_1014600, ADA2), histone acetyltransferase GCN5 (PF3D7_0823300, GCN5), chromodomain-helicase-DNA-binding protein 1 homolog (PF3D7_1023900, CHD1), ISWI chromatin-remodelling complex ATPase (PF3D7_0624600, ISWI), Snf2-related CBP activator (PF3D7_0820000, SNF/CBP), chromatin assembly factor 1 subunit A (PF3D7_0501800, CAF1A), and PHD finger protein PHD2 (PF3D7_1433400, PHD2). Grey dots represent other proteins that associate with the respective proteins but are inconsequential. Proteins shown in blue blocks are shared between the H3K18ac and H3K23ac samples include transcription factor AP2-G2 (PF3D7_1408200), nucleosome assembly protein (PF3D7_0919000, NAPS), a conserved unknown function protein (PF3D7_1239800) and mature parasite-infected erythrocyte surface antigen (PF3D7_0500800, MESA). Grey edges represent data from the AP2-G2 and GCN5 interactomes (Singh *et al.*, 2020 and Hoeijmakers *et al.*, 2019); the pink edges represents data from STRING [(STRING: functional protein association networks (string-db.org))]; and the blue edges indicated data from yeast to hybrid study (La Count *et al.*, 2005).

AP2-G2 additionally interacts with other chromatin regulation proteins, including a chromatin assembly factor 1 subunit A (PF3D7_0501800, CAF1A) and an ISWI chromatin-remodelling complex ATPase (PF3D7_0624600, ISWI), which interacts with a Snf2-related CBP activator (PF3D7_0820000). As a result, AP2-G2, H3K18ac, H3K23ac, ISWI chromatin-remodeling complex ATPase (PF3D7_0624600, ISWI), and Snf2-related CBP activator (PF3D7_0820000) share a link. AP2-G2 is associated with H3K18ac and H3K23ac and interacts with ISWI which has a relatively highly homology to TRIM24 (42 % identity), which is a reader of H3K18ac and H3K23ac^{320, 328}. Furthermore, p300/CBP activator is the writer responsible for the deposition of H3K18ac and H3K23ac,^{316, 317, 329} have homology to the *P. falciparum* histone acetyltransferase, GCN5 (PF3D7_0823300, 34 % identity). Furthermore, TRIM24 has been described as CBP-associated proteins³³⁰. This suggests that the SAGA-like complex of *P. falciparum* has as core GCN5/ADA2/PHD2 but in gametocytes, association with the H3K18acK23ac combination includes the additional ISWI/SNF complex effectors. This cooperation between SAGA and SWI/SNF complexes is required to regulate specific

transcriptional responses, as in yeast ³³¹. Since SWI/SNF complex proteins are global nucleosomal organisers that enable the specific binding of selective transcription factors ³³²⁻³³⁴, their involvement could explain the recruitment of AP2-G2.

The involvement of Snf2/CBP further points to functionality for the crosstalk involving the neighbouring H3R17 mark, forming the H3R17me2K18acK23ac coordinating code in *P. falciparum* mature gametocytes. Arginine 17 methylation is achieved systematically: CBP first acetylates H3K18, then H3K23 and this allows the arginine methylase CARM1 to associate with chromatin to methylate H3R17 ^{335, 336}. H3R17me2 is associated with transcriptional activation based on the recruitment of polymerase-associated factor 1 complex to initiate transcription in humans ^{293, 337}, supported by the antiproliferative effects of a specific CARM1 inhibitor on multiple myeloma cell lines ^{338, 339}. This suggests that in *P. falciparum*, this combination could be critical for mature stage gametocytes.

Given the shared core proteins between H3K18ac and H3K23ac and the positive crosstalk observed for this combination (and including R17me2), our data for this combination provides evidence that the combinatorial histone code of the *P. falciparum* parasite can recruit protein complexes unique to a combination to facilitate downstream biological procedures.

3.4 Discussion

It is crucial to understand the proteomic traits associated to histone PTMs involved in proposed stage-specific regulatory processes to make inferences about the best possible approach to target parasite proliferation and transmission of the malaria parasite. Not only do histone post-translational modifications dictate chromatin structure and permissiveness to transcriptional factors, the proteins and protein complexes that bind to the DNA-histone complex and other indirect binding partners reflect if the underlying sequences are to be transcribed, repressed or kept in a poised state. In this study, we aimed to identify proteins that associate to a combination of histone PTMs that indicated high positive crosstalk in mature stages of the *P. falciparum* gametocyte. To achieve this, chromatin immunoprecipitation was coupled with high-resolution quantitative mass-spectrometry. A total of 282 differentially abundant proteins associated with previously determined euchromatic PTMs H3K18ac and H3K23ac. This information aids in the understanding of the unique biological features that underline gametocyte maturation and cell identity.

Interestingly, this study highlights the dynamic interplay between metabolism, signalling and histone PTM mediated chromatin remodelling. Metabolic processes produce cofactors which are used by chromatin-remodelling enzymes³⁴⁰. Histone nucleophilic tails are sensitive to a cellular microenvironment and can be spontaneously covalently modified through non-enzymatic reactions with reactive metabolites^{211, 340}. Additionally, metabolites can serve as signalling molecules to facilitate cell functions in response to the nutrient availability within the host. This feature is highlighted in *P. falciparum* by the repression of gametocytogenesis in response to host lysophosphatidylcholine¹⁶¹. Histone acetylation and methylation can occur through acetyl-CoA and S-adenosylmethionine as donors, respectively³⁴¹⁻³⁴⁴. From this study, enzymes from both methionine/polyamine and phosphatidylethanolamine/choline metabolism were enriched and could suggest that these may serve as possible sources of acetylation and methylation, as these molecules freely diffuse through the nuclear pores and the cytosol. This supports previous work that showed that *de novo* fatty acid synthesis is upregulated in gametocytes^{345, 346}. The exact source of the CoA that is used to modify histones is still unknown. The crosstalk between parasites' metabolic processes and chromatin-mediated gene regulation needs to be further elucidated.

The functional relevance of the connectivity of histone PTMS is underscored by evidence that the associated interacting proteins are also highly connected to include reader and writer proteins, 'flavoured' to specific marks¹⁸⁷. In this manner, the crosstalk in the PTM combinations results in the recruitment of protein complexes to interpret the PTM combinations and allow changes to the chromatin structure. Indeed, we show that the unique co-dependent combination in mature gametocytes, H3K18acK23ac, jointly recruits the

transcription factor AP2-G2, to initiate a SAGA-like complex containing GCN5/ADA2, flavoured with K18ackK23ac-specific effectors. Since co-dependency extends to the triple combination H3R17me2K18ackK23ac, we provide evidence that this combination may indeed functionally associate with a gametocyte-specific SAGA-like complex to mediate stage-specific gene expression exclusively in mature gametocytes, as this combination has been demonstrated to induce transcriptional activation in cancer cells³⁰¹. The identification of AP2-G2 as an immediate binding partner on the H3K18ackK23ac combination suggests that in mature gametocytes, AP2-G2 may act as a transcriptional activator by targeting these histone PTM combinations. Investigations of the specific gene sets controlled by H3R17me2K18ackK23ac and AP2-G2 in mature gametocytes are underway.

Further characterizing the protein complexes that bind genomic loci is a histone PTM defined conformation is critical to understanding the mechanistic view of how the parasite plans and prepares for the next stage of its life cycle. Therefore, the complete repertoire of histone PTMs that occur in combination in the parasite should be resolved, quantified, and interpreted into the biological function the histone code instructs. Ultimately, to fully contextualised future intervention strategies, we need to understand the chromatin dynamics regulating parasite development and survival.

Chapter 4 | Chemical perturbation of combinations of histone post-translational modifications in *P. falciparum* parasites reveals their functional importance

A subset of the work in this chapter has been published as follows:

Coetzee, N.*, von Grüning, H*, Opperman, D., van der Watt, M., Reader, J. and Birkholtz, L. Epigenetic inhibitors target multiple stages of *Plasmodium falciparum* parasites. Nature Scientific Reports | DOI: 10.1038/s41598-020-59298-4.

* = these authors contributed equally as joint first authors of the paper.

4.1 Introduction

Particular histone PTM combinations display a unique stage distribution throughout *P. falciparum* development and differentiation whilst others, although they may occur in both asexual and gametocyte stages of the parasite, have different connectivity patterns and profiles between the stages (151). Of particular interest in trophozoites is the positive crosstalk seen between H3K9ac and H3K14me2, H3K4me3 and H3K36me3. Of these, the bivalent combination of H3K9acK36me3 is particularly prominent as it occurs more frequently than expected in trophozoites and is marked by a highly positive interplay score (Figure 4.1). This combination is of functional interest since H3K9ac is associated with euchromatin while H3K36me3 is typically heterochromatin associated in *P. falciparum*^{176, 241}. H3K9acK4me3 co-localise in a large fraction of the genome in trophozoites but this does not seem to correlate with gene expression^{176, 306}, rather implying a regulatory role in euchromatin maintenance than direct enrichment of regulatory elements for control of gene regulation. Alternatively, the combination could result in recruitment of effector proteins that then can result in subsequent gene regulation events. In *P. falciparum*, K9ac and K14ac is deposited by the HAT PfGCN5 (Table 1.1) whilst K4 can be methylated by any of PfSET1, PfSET4, PfSET6, PfSET7 or PfSET10 as histone methyltransferases (Figure 4.1).

Prominent crosstalk between histone PTMs is especially evident for gametocyte stages where certain histone PTM combinations display above average connectivity and interdependence. In immature and mature gametocytes, the involvement of methylated arginines in crosstalk with other marks remains of unique interest due to their indicated involvement in developmental biology in for instance stem cells³⁴⁷. The association of H3R8me2R17me2 in both immature and mature developmental stages of gametocytes remains intriguing (Figure 4.1), since symmetrical H3R8me2 is typically associated with locus-specific transcriptional activation and repression while H3R17me2 marks actively transcribed promoters²⁹². Readers of methylated arginine residues are primarily Tudor-domain containing proteins. *P. falciparum* encodes for two Tudor-domain containing proteins, of which only one is essential to asexual

parasite survival, and both proteins have not otherwise been interrogated beyond their essentiality for the parasite. Although speculative as such studies have yet to be conducted in the parasite, the contribution of the combinations of H3K9me1R17me2 and H3R8me2R17me2 toward regulation of gene expression in the ‘repressed’ immature gametocytes compared to ‘active’ H3K4me3R17me2 or H3K9acR17me2 in mature gametocytes is of particular interest as H3R17me2 remains conserved in development yet dynamically co-exists with either transcriptionally permissive or repressive PTMs. In both these stages of gametocytes, H3R8me2 is deposited by PRMT5, whilst H3R17me2 would be generated by PRMT4, implying coordinated action between these two methyltransferases to generate the H3K8me2R17me2 combination.

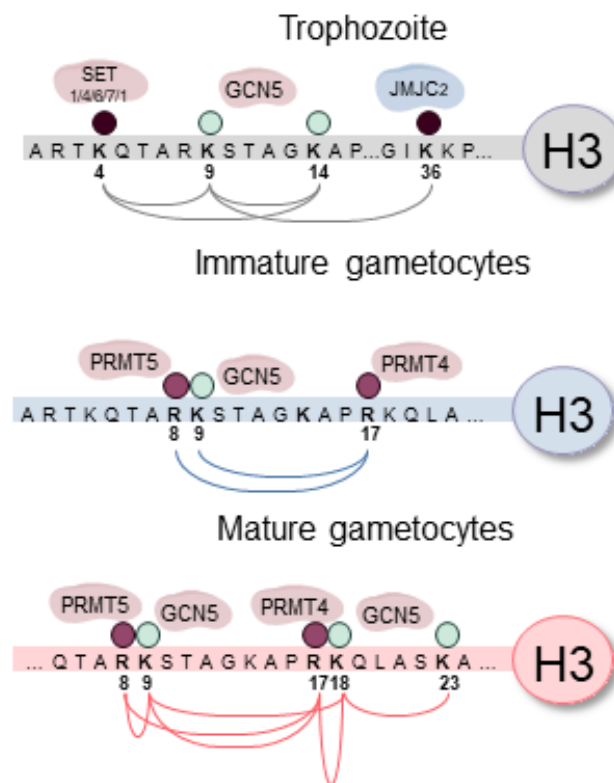


Figure 4.1. Crosstalk between histone PTM combinations in trophozoites, immature and mature gametocytes. Writer proteins of histone PTMs (● ac, acetylation; ● me1, methylation; ● me2, demethylation; ● me3, trimethylation) are shown in pink while eraser protein for H3K36me3 is shown in blue. Interplay scores between histone PTMs are shown for crosstalk pairs with high interplay scores are shown for trophozoite (—), immature (—) and mature (—) gametocytes (GC). PfSET1, PfSET4, PfSET6, PfSET7 or PfSET10 histone methyltransferases deposits H3K4 methylation, while PfSET2 deposits methylation on H3K36 which is removed by PfJMJC1 or PfJMJC3 demethylases. PfGCN5 HAT deposits an acetyl group on H3K9 and H3K14, while PfGCN5* is likely the acetyltransferase responsible for H3K18 and H3K23 acetylation (Chapter 3). The writer for H3K14 methylation is unknown, while protein arginine methyltransferases deposit methyl groups on H3R8 (PRMT5) and H3R17 (PRMT4).

Lastly, the H3R17me2K18ac combination show a distinct co-existence in only in mature gametocytes. The functional relevance of arginine methylation in *P. falciparum* remains unknown, however, H3K18ac accumulates during mitotic phases of the IDC where it

demarcates actively transcribed transcriptional start sites in combination with H3K27ac, PfAP2-I, PfBDP1 and PfH2A.Z ²¹⁴. In mature gametocytes, H3K18ac is predominantly in combination with H3R17me2 and H3K23ac. As seen in Chapter 3, both H3K18ac and H3K23ac possibly recruit a regulatory complex that includes AP2-G2 and PfBDP1 in a SAGA-like complex, suggesting that combinations of histone PTMs have functional relevance to the parasite.

The pronounced importance of these combinations is suggested based on the higher interplay scores (Chapter 2) and involvement of important regulatory proteins with H3K18ac and H3K23ac (Chapter 3), prompted us to study the functional significance of these highly connected PTMs for parasite survival. To interrogate the importance of co-existing PTMs, several unbiased methods are possible, yet not without limitations. Firstly, given that the biological importance of PTMs is mediated by reader proteins, one approach is to mutate the reader protein binding domains to impede their recognition capabilities ³⁴⁸. However, such approach requires conclusive knowledge of the identity of the reader protein, its relative binding affinities, and the possibility that other reader proteins may also recognise the modification or that it might produce off-target effects, which could be misinterpreted. Secondly, site-directed nonsense suppression mutagenesis may be introduced to incorporate a modified amino acid at any position in histone proteins to remove the actual PTM and thereby allow the study of the biological relevance of the modifications ³⁴⁹. However, this approach does not guarantee a homogenous pool of chemically defined histones. Additionally, generating mutant *P. falciparum* parasites are infamously difficult ³⁵⁰, and even if successful, potential lethal effects might prevent downstream studies in mature stage gametocytes as intended here. Thirdly, synthetic peptides can be used to identify the readers of different patterns of PTM combinations but is critically limited since only short peptides can be generated, which is not suitable to characterise the combinatorial readout of multiple and/or distant PTMs (439). Although native chemical ligation and expressed protein ligation can overcome length limit, it is troublesome and time consuming since each peptide segment is manufactured separately or requires that the PTM must be located near the protein termini. In short, results generated with any of the abovementioned methods rely on a reductionist approach to interpret phenotypical outcomes for the parasite.

An alternative means to study the biological importance of combinations of histone PTMs involve chemical interference of crosstalk by inhibiting the function of chromatin associated proteins with epigenetic inhibitors (“epidrugs”). The use of inhibitors that target all three epigenetic protein families—readers, writers, and erasers have been shown to be highly effective to investigate the functional importance of combinatorial histone PTMs in other

cellular systems^{205, 351}. Moreover, using combinations of drugs to target more than one effector protein simultaneously is possible. For example, the combination of DNA methyltransferase inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi) offers a good potential therapeutic effect against cancer³⁵². Additionally, inhibitors that have demonstrated poor activity can be partnered with another inhibitor to enhance their effect³⁵³. In solid tumours, single-agent DNMTi or HDACi therapy has a modest anti-tumour effect, however, combining DNMTi and HDAC inhibition on solid tumours can improve drug sensitivity and induce tumour suppressor gene re-expression³⁵².

Chemical interference of epigenetic modifications is not without limitation. Firstly, selection of such inhibitors to study histone PTMs is based on their selectivity in targeting a single, specific protein. Furthermore, it assumes that a protein homolog exists for *P. falciparum*, and that the homologous protein has the same substrate. Another assumption is that the compound indeed targets the proteins of interest in an exclusive manner to enable inference. Caveats of this strategy include that the effect of a drug is measured by parasite viability and not necessarily due to specific inhibition of their target proteins. However, there is ample evidence of specific targeting of epigenetic modulators by epidrugs in *P. falciparum*, including BIX01294 (G9a inhibitor³⁵⁴, SB939³⁵⁵, JIB04³⁵⁶ and ML324³⁵⁷). Moreover, compounds that target HDACs, HATs, HKMT and histone methyltransferases have been studied in *P. falciparum* parasites and have shown that not only does these compounds represent novel classes of compounds (which will delay resistance development), they are potent inhibitors of multiple stages of the parasite, including parasites that are multidrug resistant³⁵⁸⁻³⁶⁰. PRMTs are also emerging as means to study combinatorial histone codes, with the added benefit of PRMTs being attractive therapeutic targets since it was shown that elevated expression of PRMTs correlated with poor prognosis in cancer patients³⁶¹. Given that PRMTs are involved in transcription, splicing, RNA biology, DNA damage response, and cell metabolism, all of which are disrupted in a variety of cancer forms, they garner considerable interest for their potential inhibitory effects^{303, 362, 363}.

However, epidrugs have not yet been explored in combinations and therefore poses a vast avenue of exploration. Not only does this hold promise to deliver novel combinations with therapeutic applications, but indeed could be a very useful tool to interrogate the functional relevance of co-existing histone PTMs associated with specific biological outcomes in *P. falciparum*. Here, we hypothesised that we could use a combination of epidrugs that target the proteins responsible for deposition or removal of each of histone PTMs that co-exist as defined in Figure 4.1, to study their implied functional relevance and importance for parasite development and differentiation.

4.2 Experimental procedures

4.2.1 *In vitro* cultivation of *P. falciparum* asexual parasites and gametocytes

The same experimental procedure was followed for parasite production and isolation as described in Section 2.2.1, Chapter 2.

4.2.2 Cayman Epigenetics Library screen to identify classes of active epidrugs

SYBR Green I fluorescence was used to determine the activity of compounds against asexual parasites. Drug sensitive (NF54) *P. falciparum* ring-stage parasites (1 % haematocrit, 1 % parasitaemia) were treated with compounds from the Cayman Epigenetics Screening Library (batch number 0466317) at 1 and 5 μ M (for primary screening of inhibitory activity), for 96 h at 37 °C as described^{252, 364}. Chloroquine disulphate (1 μ M) was included as positive drug control. Hit compounds, i.e., compounds that showed activity (>70 % inhibition of parasite proliferation at 5 μ M and >50 % at 1 μ M) were selected for full dose-response evaluation under the same assay conditions as above to determine the inhibitory concentrations of the compounds needed to affect 50 % of the parasite population (IC_{50}). Results were presented as the mean of three independent experiments, each performed in technical triplicates, $\pm$ SEM. Significance was indicated at $P < 0.05$ (*) as determined with a Student's *t*-test. Assay performances were evaluated with Z-factors at >0.7.

4.2.3 SYBR Green I-based fluorescence assay to determine inhibition against the asexual *P. falciparum* parasite stages

In vitro ring-stage *P. falciparum* NF54 parasite cultures (drug sensitive) at 1 % haematocrit and 1 % parasitaemia were treated with epidrugs at the estimated IC_{50} based on previous reports for *P. falciparum* parasites whereas parasites were treated with 1 μ M of GSK3326595 and TP-064 for 96 h at 37° C with chloroquine disulphate (1 μ M, positive drug control) and complete RPMI media (growth control). SYBR Green I fluorescence (as a proxy of DNA replication in viable cells)³⁶⁴⁻³⁶⁶ was measured with using a Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, excitation at 485 nm and emission at 538 nm). Unless otherwise stated, each compound was screened in technical triplicates for at least three independent biological replicates (n=3). A minimum of 9-point curves were evaluated using 2-fold dilution series of each drug individually. From these, the inhibitory concentration of the drug needed to affect 50 % of the parasite population (IC_{50}), was determined (n=3 $\pm$ SEM). For the dose-response curves, data were represented as a percentage of untreated control to determine cell proliferation. Sigmoidal dose-response curves were plotted using GraphPad 5.0, from which the IC_{50} values could be determined. Assay performances were evaluated with Z-factors at

>0.75. All compounds were purchased from MolPort (Latvia) and supplied by Cayman Chemicals (Europe).

4.2.4 Parasite lactate dehydrogenase assay to determine inhibition against the early- and late-stage *P. falciparum* gametocytes

Gametocytes were induced from an asexual *P. falciparum* NF54 parasites culture (see Section 2.2.1, Chapter 2). To determine gametocytocidal activity of compounds against early- and late-stage gametocytes, the parasite lactate dehydrogenase (pLDH) assay was performed^{252, 367}. For the colorimetric detection of a formazan product, this assay uses *P. falciparum* LDH's preference for 3-acetylpyridine adenine dinucleotide (APAD) as a coenzyme in the reaction leading to the conversion of lactate to pyruvate, as opposed to host LDH³⁶⁷. Early- and late-stage (>85 % stage II [early-stage gametocytes]; >70 % stage V and >27 % stage IV [late-stage gametocytes]) *P. falciparum* gametocyte cultures (2 % haematocrit, 5 % gametocytaemia) were treated with compounds at 1 and 5 μ M for dual-point primary screening of inhibitory activity. The controls for this assay included methylene blue (5 μ M, positive control for inhibition), chloroquine (1 μ M, positive control for inhibition) and complete RPMI media (gametocyte viability control). The treated gametocytes were allowed to mature for 72 h at 37 °C, after which a proportion of the culture medium (100 μ L) was replaced, and the plates incubated for a further 72 h. This was followed by the addition of Malstat reagent [0.21 % v/v Triton-100; 222 mM L-(+)-lactic acid; 54.5 mM Tris; 0.166 mM APAD (Sigma-Aldrich, USA); adjusted to pH 9 with 1 M NaOH] and PES/NBT [1.96 mM nitro blue tetrazoliumchloride (NBT); 0.239 mM phenazine ethosulphate (PES)]. The ratio of parasite suspension:Malstat:PES/NBT was kept consistent at 1:5:1.25. The plates were incubated for 40 min in the dark, after which the absorbance was measured with a Multiskan Ascent 354 multiplate scanner (Thermo Labsystems, Finland) at 620 nm. The data were represented as a percentage of the treated control after background subtraction (methylene blue-treated infected erythrocytes samples in which parasite viability was completely inhibited), to determine the percentage inhibition for each compound. From these, the inhibitory concentration of the drug needed to affect 50 % of the parasite population (IC_{50}), was determined ($n=3 \pm$ SEM). For the dose-response curves, data were represented as a percentage of untreated control to determine gametocyte viability. Sigmoidal dose-response curves were plotted using GraphPad 5.0, from which the IC_{50} values could be determined. Assay performances were evaluated Z-factors at >0.75.

4.2.5 Dot blot to determine acetylation and methylation status following epidrug treatment

Mature gametocytes (>85 % stage V, day 10) were treated with either GSK3326595, TP-064, C646, anacardic Acid, or a combination of C646 and TP-064 at their IC_{90} for 24 h before being

sampled. Histones were extracted as described in Chapter 2 (section 2.2.2)¹⁵⁴. Briefly, nuclei were extracted using Dounce homogenization and protease inhibitors in hypotonic lysis buffer (10 mM Tris-HCl [pH 8.0], 0.25 M sucrose, 3 mM MgCl₂, 0.2 percent [v/v] Nonidet P-40). Histones were extracted from resuspended nuclei in Tris buffer (10 mM Tris [pH 8.0], 0.8 M NaCl, 1 mM EDTA, and protease inhibitors) by overnight acid extraction (0.25 M HCl, with rotation at 4 °C) and subsequent precipitation with 20 % TCA. Histone pellets were reconstituted in dddH₂O after being washed with acetone. The BCA Protein Assay Kit (Pierce™, ThermoFisher Scientific) was used to determine the amount of protein in each sample. The chelation of bicinchoninic acid (BCA) with cuprous cations (Cu¹⁺) is detected colourimetrically in this sensitive test. When exposed to an alkaline environment, Cu¹⁺ is produced through the reduction of Cu²⁺^{368, 369}. Absorbance at 562 nm was used to determine the amount of protein that was measured. Thereafter, histones were spotted quantitatively (50 ng per sample dot) on nitrocellulose membranes. Membranes were submerged in blocking buffer (5 % skim milk powder in TBS-t containing 50 mM Tris [pH 7.5], 150 mM NaCl, and 0.1 % [v/v] Tween-20) for 30 min followed by 1 h incubation with α-H3K18ac (Abcam, ab119100), α-H3R17me2 or α-H3core (Abcam, ab176842) primary antibody dilutions (1:5000 in TBS-t). Membranes were washed three times in TBS-t and then incubated with HRP-conjugated rabbit α-IgG secondary antibody (1:10000 in TBS-t) for 1 h. Pierce™ SuperSignal West Pico PLUS Chemiluminescent Substrate was quantified with ImageJ analysis software (1.52; <http://imagej.nih.gov/ij>)^{321, 322}.

4.2.6 Molecular docking of epigenetic inhibitors TP-064 and GSK3226595 into PRMT4 and PRMT5

Molecular docking was performed to predict the interaction between epigenetic inhibitors and PRMTs as potential target. Canonical SMILES for TP-064 and GSK3226595 was used to generate a ligand structure in UCSF Chimera³⁷⁰. The PRMT4 (PF3D7_0811500, also known as CARM1) and PRMT5 (PF3D7_1361000) models selected for docking were obtained from AlphaFold 2.0³⁷¹, which generate protein structure models through an innovative machine learning strategy that employs multi-sequence alignments to combine physiochemical and biological data of the protein of interest to generate a predicted structure with extraordinary precision³⁷¹. Homodimer alignment was performed for PRMT5 using SwissDock with default parameters³⁷². Residues with low quality prediction scores (AlphaFold's per-residue score <70) were trimmed if found in low complexity surface loops, to obtain high-confidence core models that were subsequently prepared for docking using Dock Prep³⁷⁰. Molecular docking were performed for TP-064:PRMT4 and GSK3226595:PRMT5 in SwissDock (278). The scoring function for Gibbs free energy was used to evaluate the most stable binding modes with the ViewDock utility in UCSF Chimera.

4.2.7 Fixed-ratio isobolograms to determine *in vitro* drug interaction

The effect of the combination between anacardic acid, C646, ML324 and BIX01294 (asexual parasites); GSK3326595, TP-064, C646 and BIX01294 (early-stage gametocytes) and between GSK3326595, TP-064, C646 and anacardic acid (late-stage gametocytes) was assessed following the method described by Fivelman, *et al.* ³⁷³. Briefly, six solutions were prepared containing the following fixed molar ratio combinations ratios ([A : B [v:v]): 100:0, 80:20, 60:40, 40:60, 20:80, and 0:100. These solutions were serially diluted. The controls for this assay included chloroquine disulphate (1 μ M, positive drug control) and complete RPMI media containing 0.1 % DMSO (growth control/vehicle control). A synchronous parasite culture containing >80 % rings stages (3 to 10 hpi) was added at a 0.5% parasitaemia and 2 % final haematocrit. IC₅₀ values were determined after a 96 h of incubation using the SYBR-Green I assay. The fractional inhibitory concentration (FIC) of each medication in a given combination was estimated using the formulas $FIC(A) = IC_{50} \text{ of A in combination} / IC_{50} \text{ of A alone}$ and $FIC(B) = IC_{50} \text{ of B in combination} / IC_{50} \text{ of B alone}$ for the arithmetical analysis. A mean $\sum FIC$ of 0.7 represent a synergistic interaction between the tested epidrugs, whereas a mean $\sum FIC$ of 1.3 represent an antagonistic interaction. Interactions with a $\sum FIC$ score of 0.7 to 1.3 are deemed indifferent, or sub- or super-additive. The FIC of each combination was then plotted to obtain the isobologram. The data were obtained in three independent experiments run in triplicate (n=3 $\pm$ SEM). Curves were plotted using GraphPad 5.0.

4.3 Results

4.3.1 Identification of epidrugs targeting different classes of epigenetic modulators in *P. falciparum*

A library of 95 epigenetic targeted anticancer compounds (Cayman Epigenetics Screening Library, Cayman's Chemicals, USA) with known capabilities to inhibit diverse epigenetic modulators in mammalian (human) cancerous cells was evaluated for antiplasmodial activity against multiple *P. falciparum* stages. This was performed to identify compounds that display multistage activity and classes of epigenetic inhibitors that interfere with the function of different epigenetic effector proteins for their possible use to study the effect of disruption of histone PTM crosstalk. The library consisted mostly of HDACi (39 %) and HKMTi (15 %), with the remaining compounds divided into 11 other inhibitor subtypes including targeting of HAT, DNA demethylases (DNMT), DNMT, protein arginine deiminases (PAD), PRMT, bromodomain proteins (BDPs), HDMs, lysine-specific demethylases (LSD), and processes involved in phosphorylation.

Stage-specific evaluation of the activity of the compounds against early- (>85% stage II/III) and late-stage (>95% stage IV/V) gametocytes indicated that the majority of the compounds (76% against asexual parasites, 69% and 82% against early- and late-stage gametocytes) showed no/minimal activity. Although similar hit rates (at >50% inhibition at 5 μ M) were observed between asexual parasites and early-stage gametocytes (24 and 30%, respectively, Pearson correlation r^2 of 0.5), the distribution of compounds displaying moderate activity against early-stage gametocytes were almost double that against asexual parasites (18% vs. 10%). Similar to other chemotypes³⁵⁷, the compounds were the least active against late-stage gametocytes (18% hit rate), matching previous data of activity loss of compounds active against asexual parasites in late-stage gametocytes³⁶⁰. Additionally, the nature of the compounds active against asexual parasites and late-stage gametocytes (and between those active against early- and late-stage gametocytes) showed poor correlation (r^2 of 0.3 and 0.2, respectively), indicating some stage-specificity in the distribution of the compounds active against each stage. A subset of 17 compounds, including previously published compounds like Trichostatin A (TSA), SAHA (suberoylanilide hydroxamic acid) and BIX01294^{354, 374-376} display activity against all life cycle stages tested, confirming the multistage activity of these compounds (Figure 4.2)

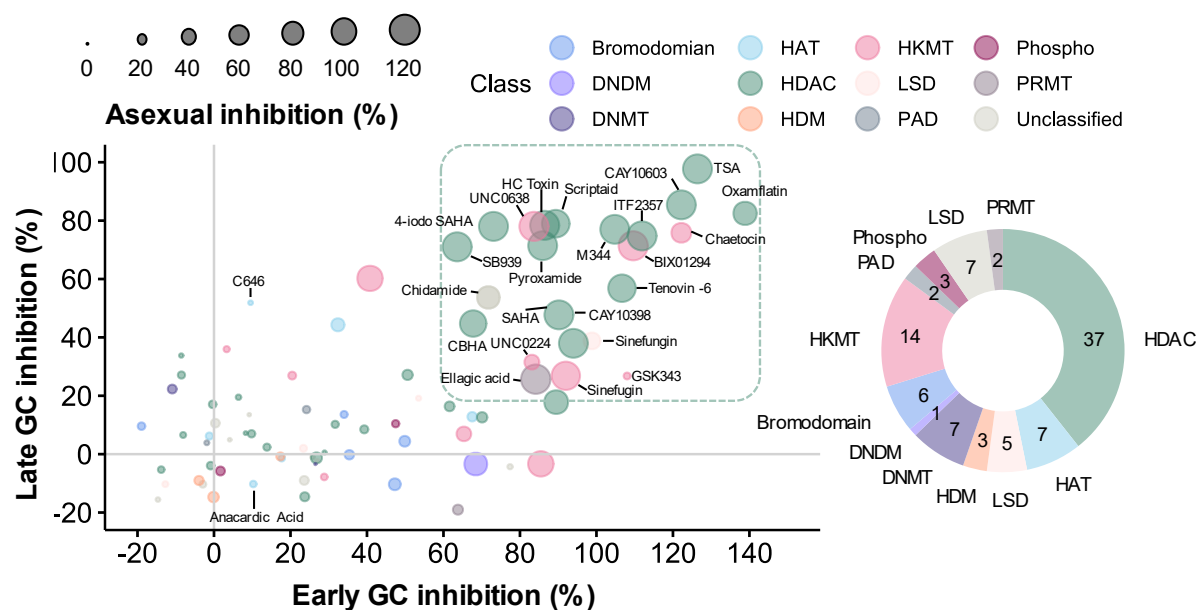


Figure 4.2 Comparative profiling of the Cayman Epigenetics library of drugs screened for inhibition activity against *P. falciparum* parasites. Epi-drug library composition based on inhibitor classification, targeting epigenetic modifiers, with the number of compounds per class indicated. Protein arginine methyltransferase (PRMT), DNA demethylase (DNNDM), DNA methyltransferase (DNMT), histone demethylase (HDM), histone lysine methyltransferase (HKMT), lysine-specific demethylase (LSD), histone acetyltransferase (HAT), histone deacetylase (HDAC). Inhibition at 5 μ M (%) was compared between asexual parasites (circle size; $n=3$) and early & late-stage gametocytes (GC, $n=1$); separated based on the inhibitor type (colour scale corresponding to inhibitor classification as in doughnut chart). Compounds with multi-stage activity is identified in the teal block.

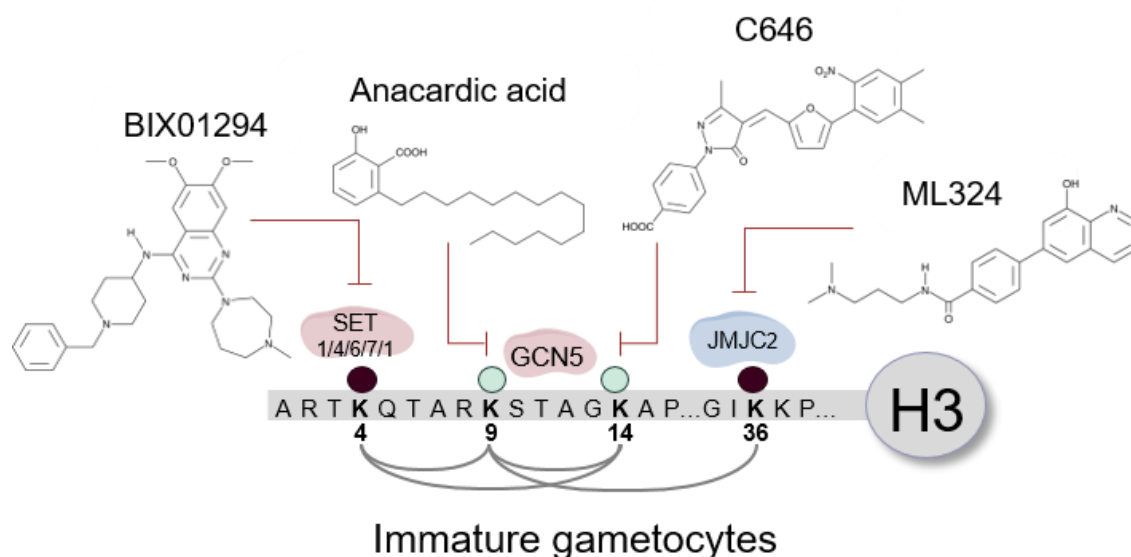
The multistage activity of the 95 compounds was stratified based on the inhibitor class descriptors for these compounds (Figure 4.2). The majority of the active compounds are classified as HDACi and HKMTi and this reflects the fact that these classes are over-represented in the library. All life cycle stages were inhibited (albeit to varying degrees) by HDACi and HKMTi (Figure 4.2). Interestingly, compounds classified in the library as potential DNNDM, DNMT, PRMT and HAT inhibitors were within the 'hit' pool. However, inhibitors in this set targeting BDP, hydroxylation, phosphorylation, and demethylation activities (both histone demethylase and lysine-specific demethylase) were not particularly active (<50% inhibition). HDACi included Scriptaid, HC Toxin, ITF 2357, Tenovin-6, CAY10603, M 344, Oxamflatin, Pyroxamide, TSA, CAY10398, SAHA and Chidamide. HKMTi included Chaetocin, UNC0638 and BIX01294 (Figure 4.2). Within these compounds, some showed distinct dual stage activity against both asexual parasites and early-stage gametocyte (CBHA, Ellagic Acid, SB939, Suberohydroxamic acid, 3-Deazaneplanocin A, (-)-Neplanocin A, 4-iodo-SAHA), or even against both gametocyte stages (UNC1999) (Figure 4.2). An additional subset of compounds is solely active against asexual stages and early-stage gametocytes and include DNNDMi (gemcitabine) and PRMTi sinefungin and S-adenosyl homocysteine (sinefungin/S-Adenosylhomocysteine). PRMTi included in the library were not active against gametocytes.

Additional stage-specificity was evident for early-stage gametocytes (S-Adenosylhomocysteine, 5-Azacytidine, GSK 343, Nicotinamide, JGB1741, Zebularine, CCG-100602) or late-stage gametocytes (3-amino Benzamide, C646, Sirtinol, PCI 34051).

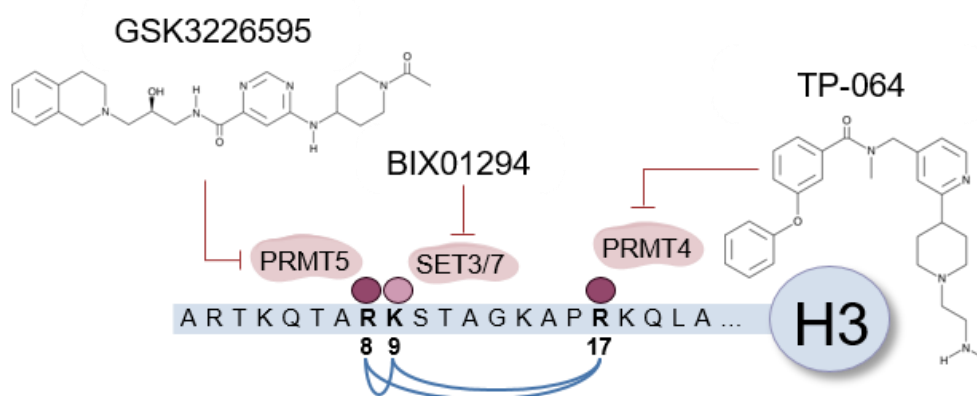
This information was used to select compounds with the ability to target multiple stages of *P. falciparum* development, with the focus placed on compounds that prevent the deposition or removal of histone PTMs. These were subsequently used (in combinations) to preventing the formation of co-existence PTMs. From the drug screen (Figure 4.2), two HATi (C646 and anacardic acid) and one HKMTi (BIX01294) were selected for IC₅₀ determination based on their relative activity and proposed action against target proteins. C646 is an HATi that selectively inhibits p300 and CBP and therefore prevents acetylation of H3K18³⁷⁷⁻³⁸¹. Anacardic acid is a natural chemical derived from cashew nut shells that has been demonstrated to inhibit HAT activity in *P. falciparum* parasites *in vitro*³⁸², with BIX01294 the HKMTi^{354, 383}. In addition, inhibitors that were not included in the Cayman library were also identified for IC₅₀ screening based on their class, specificity, and activity against cancer cells. Because of the substantial interaction between H3K36me3 and H3K9ac in trophozoite stages (Figure 4.1), ML324, a mammalian JMJD2 demethylase inhibitor that has been demonstrated to alter H3K36 methylation in *P. falciparum*, was included^{356, 357}. Two PRMTi, GSK3226595 and TP-064, were lastly also selected for IC₅₀ determination in *P. falciparum* since they both specifically and selectively block methylation of H3R8 and H3R17 by targeting PRMT5 and 4, respectively, and have recently shown potent nanomolar activity against cancer cells³⁸⁴⁻³⁸⁸.

In totality, the compounds selected were used to interrogate the following combinations in asexual parasites: H3K9acK36me3 was interrogated by the combination of anacardic acid/C646 as HATis targeting GCN5, which was combined with ML324 as HDMi³⁵⁷ (Figure 4.3). Additionally, the combination of H3K4me3K9ac was probed by combining BIX01294 as HKMTi with anacardic acid/C646 as HATi³⁸². Finally, the combination of H3K9acK14ac was targeted with both HATi anacardic acid and C646³⁸⁹ (Figure 4.3).

Trophozoite



Immature gametocytes



Mature gametocytes

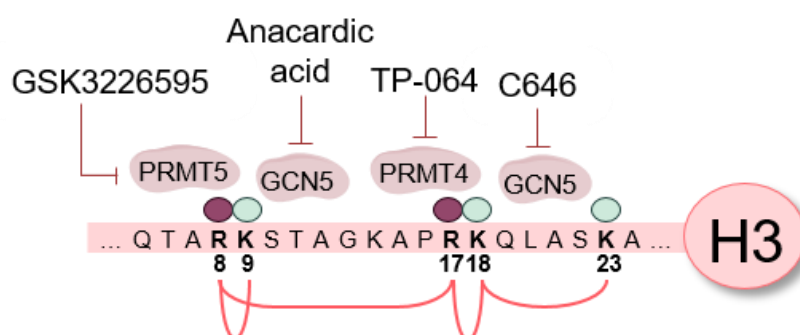


Figure 4.3. Epidrugs selected for to target effector proteins of combinatorial histone PTMs. Histone H3 peptides are shown with corresponding residues for the modification (○ acetylation, ○ monomethylation, ● dimethylation) that will be target using an epidrug shown in purple. Structures are shown for each compound while the curved lines denote crosstalk between histone PTMs. For example, on asexual parasites (top gray peptide), anacardic acid targets PfGCN5 which will directly affect acetylation of H3K9 and H3K14ac, where H3K9ack36me3 showed a strong interaction. Interactions are shown for in immature gametocytes (middle blue peptide) and mature gametocytes (pink bottom peptide).

In immature gametocytes, the combinations of H3R8me2K9me1 and H3K9me1R17me2 was probed by combining BIX01294 as HKTmi of H3K9 methylation with PRMTi GSK3326595 as an inhibitor of PRMT5 and TP-064 as a potent, selective inhibitor of PRMT4³⁸⁷. The H3R8me2R17me2 positive crosstalk was interrogated by combining GSK3226595, a selective reversible inhibitor of PRMT5^{385, 386}, with TP-064.

In mature gametocytes, additional crosstalk between H3R17me2, H3K18ac and H3K23ac was targeted by using TP-064 in combination with C646 or anacardic acid. Interestingly, *P. falciparum* parasites lack a p300/CBP homologue, the target protein of C646. Although the writer enzyme for H3K18ac and H3K23ac in *P. falciparum* is unknown, PfGCN5 and bromodomain proteins 1 and 2 (BDP1, BDP2) exist in complexes with both reader and writing activities that could perform H3K18 and H3K23 acetylation (30). It is likely that a unique complex containing PfGCN5 and members of the SAGA complex associates with H3K18ac and H3K23ac and could therefore be responsible for H3K18ac and H3K23ac writer and/or reader activity. The dose-responses of each of the compounds selected were subsequently determined for asexual parasites, immature (early-) and mature, late-stage gametocytes.

4.3.2 Activity of selected epidrugs against multiple stages of *P. falciparum*

The IC₅₀ of the selected epi-drugs was determined against asexual, early- and late-stage gametocytes of *P. falciparum*. In the HATi class, anacardic acid was slightly more potent at killing all three stages, compared to C646 (Figure 4.4). C646 was the least active compound tested with an IC₅₀ of 60 ± 1.8 µM in asexual parasites, a significant difference (unpaired, two-tailed Student's *t*-test, *n*=3 ± SEM, *P*<0.001) from the IC₅₀ in early-stage gametocytes (IC₅₀: 0.16 ± 0.019 µM), while in late-stage gametocytes, C646 is a potent inhibitor of viability (IC₅₀: 0.087 ± 0.017 µM). As with C646, anacardic acid had a significant (unpaired, two-tailed Student's *t*-test, *n*=3 ± SEM, *P*<0.001) shift in IC₅₀ between asexual parasites and both gametocytes stages, becoming much more potent as differentiation progressed to late-stage gametocytes, from 34 ± 0.28 µM in asexual parasites (similar to a previous report of 30.41 ± 4.54 µM³⁸²), to 0.43 ± 0.11 µM in early-stage gametocytes and finally to 0.082 ± 0.016 µM in late-stage gametocytes (Figure 4.4).

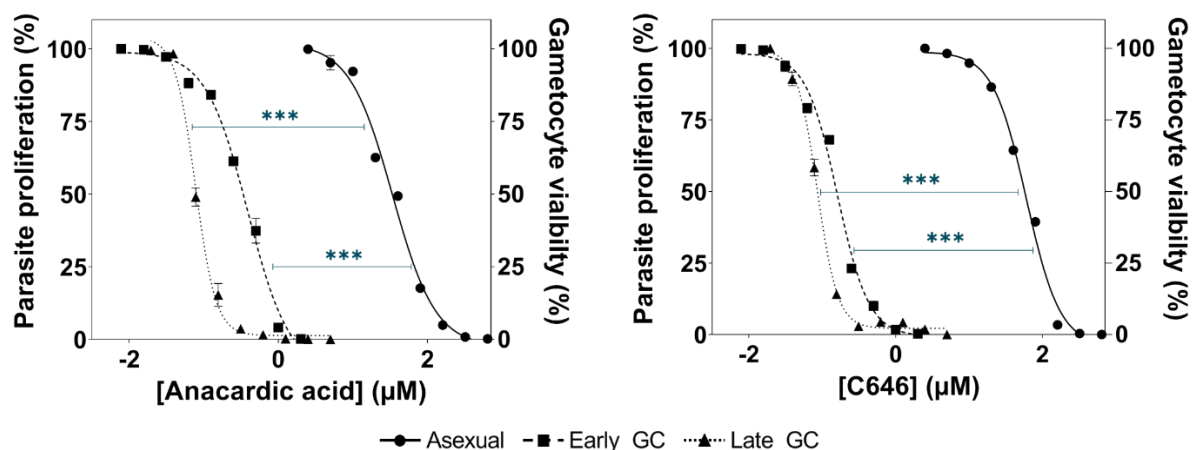


Figure 4.4. *In vitro* activity of HATi anacardic acid and C646 against multiple stages of *P. falciparum*. Compounds were screened using the SYBR Green I-based fluorescence assay to determine dose-response against asexual (—●—) parasites, early (—■—) and late-stage (---▲---) gametocytes (GC). Results are the mean of three independent experiments, performed in triplicate, $\pm$ SEM. Significance is indicated at $P < 0.001$ (***) as determined with an unpaired, two-tailed Student's *t*-test. Error bars fall within symbols where not shown.

From the HKMTi, BIX01294 was >60 times more potent than ML324 in asexual parasites, with an IC_{50} of 0.037 ± 0.001 μ M for BIX01294 compared to 2.4 ± 0.031 μ M ML324 (Figure 4.5), similar to previous reports (352, 355). ML324 had an IC_{50} of 2.3 ± 0.26 μ M compared to 1.2 ± 0.016 μ M for BIX01294 in early-stage gametocytes, while in late-stage gametocytes, ML324 was significantly (unpaired, two-tailed Student's *t*-test, $n=3 \pm$ SEM, $P < 0.001$) more potent than BIX01294 (0.092 ± 0.016 μ M vs. 4.1 ± 0.21 μ M, respectively). BIX012394 indicated a significant (unpaired, two-tailed Student's *t*-test, $n=3 \pm$ SEM, $P < 0.01$) shift in IC_{50} between all three stages investigated and demonstrated the lowest IC_{50} against asexual parasites. By contrast, ML324 had an opposite profile, where it did not show an appreciable difference in IC_{50} between asexual parasites and early-stage gametocytes but had a significant (unpaired, two-tailed Student's *t*-test, $n=3 \pm$ SEM, $P < 0.001$) shift in IC_{50} between asexual parasites and late-stage gametocytes, which was only moderate (unpaired, two-tailed Student's *t*-test, $n=3 \pm$ SEM, $P < 0.05$) between early-stage gametocytes and late-stage gametocytes (Figure 4.5).

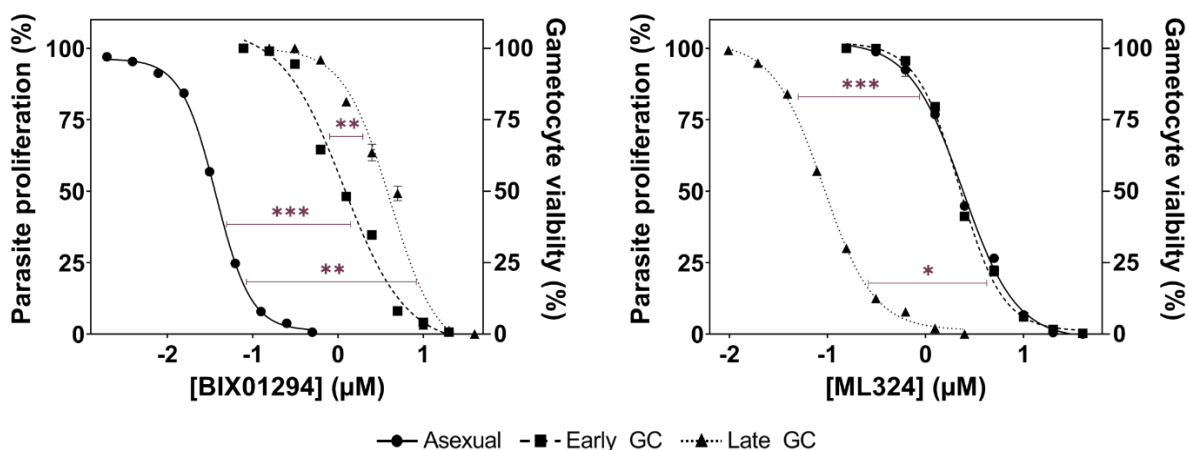


Figure 4.5. *In vitro* activity of HKMTi BIX01294 and ML324 against *P. falciparum*. Compounds were screened using the SYBR Green I-based fluorescence assay to determine dose-response against asexual (—●—) parasites, early (—■—) and late-stage (---▲---) gametocytes (GC). Results are the mean of three independent experiments, performed in triplicate, $\pm$ SEM. Significance is indicated at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) as determined with an unpaired, two-tailed Student's *t*-test. Error bars fall within symbols where not shown.

From the PRMTi class, the biggest differences in observed IC_{50} 's were determined for TP-064 between the three stages. GSK3326595 and TP-064 demonstrated IC_{50} values of 0.31 ± 0.001 μ M and 0.6 ± 0.018 μ M against asexual parasites, where both drugs' IC_{50} values decreased significantly (unpaired, two-tailed Student's *t*-test, $n = 3 \pm$ SEM, $P < 0.01$) in early- and late-stage gametocytes compared to asexual parasites, with GSK3326595 IC_{50} decreasing from 0.11 ± 0.016 μ M to 0.06 ± 0.004 μ M and TP-064's IC_{50} decreasing from 0.14 ± 0.007 μ M to 0.055 ± 0.009 μ M in early- and late-stage gametocytes, respectively (Figure 4.6).

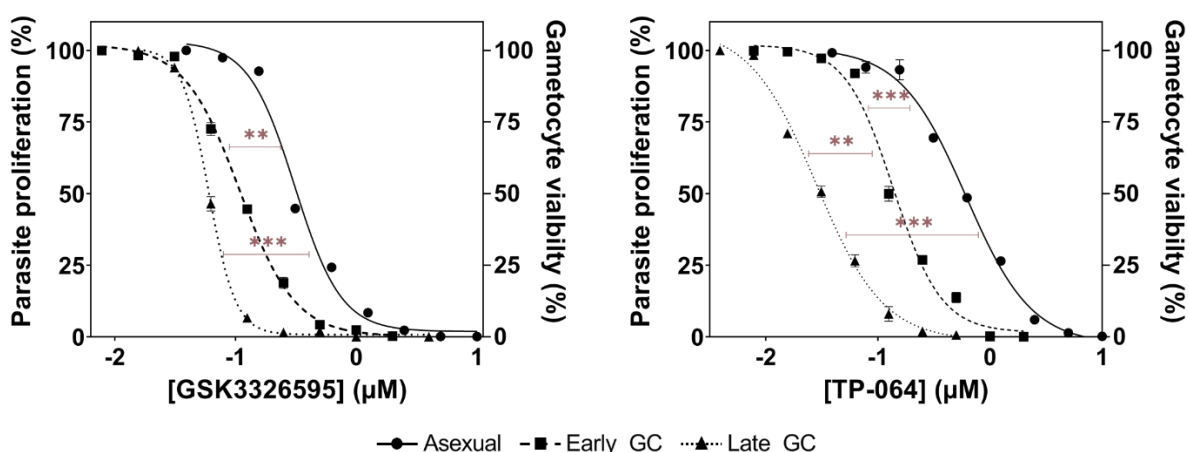


Figure 4.6. *In vitro* activity of PRMTi GSK3326595 and TP-064 against *P. falciparum*. Compounds were screened using the SYBR Green I-based fluorescence assay to determine dose-response against asexual (—●—) parasites, early (—■—) and late-stage (---▲---) gametocytes (GC). Results are the mean of three independent experiments, performed in triplicate, $\pm$ SEM. Significance is indicated at $P < 0.01$ (**) and $P < 0.001$ (***) as determined with an unpaired, two-tailed Student's *t*-test. Error bars fall within symbols where not shown.

The PRMTi indicated sub-micromolar activity ($IC_{50} < 0.63 \mu M$) in all three stages investigated, which indicate the essential nature of arginine methylation for overall parasite survival. Compounds from the HATi class clearly showed higher activity against gametocytes stages compared to asexual parasites ($IC_{50} < 1 \mu M$ vs. $IC_{50} > 34 \mu M$). Interestingly, compounds from the HKMTi indicated opposing activity in asexuals compared to late-stage gametocytes, where BIX01294 were more active against asexual parasites while ML324 was more active against late-stage gametocytes, suggesting that the targets perform critical roles in the respective stages.

4.3.3 Activity of HAT and PRMT inhibitors is associated with hypoacetylation and hypomethylation in mature gametocytes

The selected epidrugs were subsequently evaluated for their ability to modify the acetylation and methylation state of histone PTMs as an indication of their specific mode of action in *P. falciparum*. The ability of the two HKMTi have previously been shown to cause hypomethylation of histone PTMs (373, 352, 354). BIX01294 treatment leads to hypomethylation of H3K9me3 and H3K4me3, whilst ML324 treatments leads to hypermethylation of H3K9 and H3K36 (373, 352, 354). Given the presumed molecular targets and the efficacy of C646, anacardic acid, GSK3326595, and TP-064 against mature gametocytes, these epidrugs were investigated further to see if their inhibitory action was linked to changes in the modification of H3K18ac and H3R17me2, as the two marks under investigation in this study. Treatment of mature gametocytes with the HATi C646 and anacardic acid both resulted in dramatic 33- and 16-fold decreased acetylation of H3K18ac, respectively (Figure 4.7A). Furthermore, a substantial decrease for C646 was not observed when H3R17me2 was probed, possibly indicating specificity of this inhibitor for acetylation. This, therefore, indicates that both these compounds prevent deposition of acetyl at H3K18, possibly by inhibiting PfGCN5.

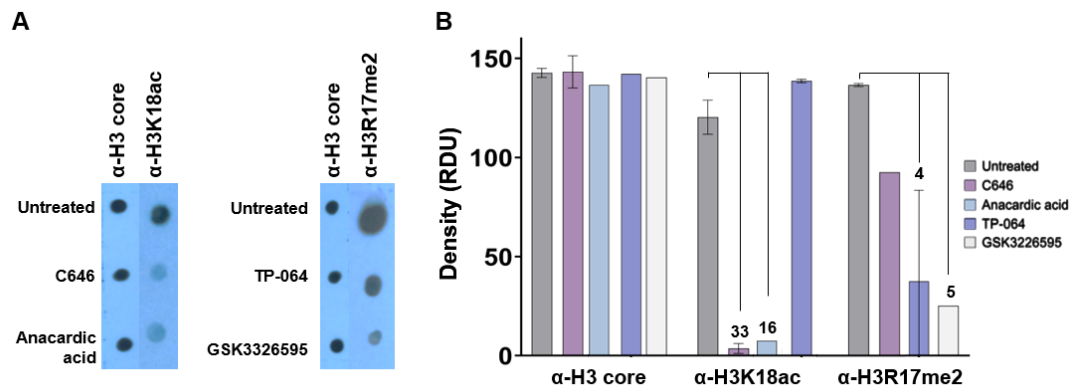


Figure 4.7. Histone acetyltransferase protein arginine methyltransferase inhibitors result in hypoacetylation and hypomethylation of H3K18ac and H3R17me2, respectively. Detection of changes in H3K18ac levels by dot blot analysis (representative blots show) of mature gametocytes (day 10) treated for 24 h at IC_{90} with (A) GSK3326595, TP-064, C646, anacardic acid compared to untreated control gametocytes prepared in parallel. Histones were quantitatively loaded in each instance. (B) Acetylation and methylation levels are expressed as fold changes (treated/parallel untreated control gametocytes) of relative density units (RDU). Data are representative of two independent biological repeats for C646 and TP-064 ($n=2$, mean $\pm$ SD) and only a single biological replicate for anacardic acid and GSK3326595.

Treatment with the PRMTi GSK3326595 and TP-064 resulted in hypomethylation of H3R17me2, with methylation levels 4- and 5-fold lower than the untreated control, respectively (Figure 4.7B). As expected, TP-064 likely inhibited PRMT4, leading to hypomethylation of H3R17me2. These findings also imply that, while GSK3326595 is a PRMT5 inhibitor (H3R8 as substrate which could not be probed in this study as an appropriate antibody was not available) in mammalian cells, it also appears to influence H3R17 methylation in *P. falciparum*.

As PRMT activity and inhibition is poorly explored in *P. falciparum*, the potential of PRMT4 and PRMT5 being potential targets for TP-064 and GSK3326595, respectively, was further explored with molecular docking to predict the interaction between these inhibitors and their respective targets. In the absence of high-resolution crystal structures and homologous structures for PfPRMT4 and PfPRMT5, AlphaFold 2.0 machine learning structures were employed (Figure 4.8).

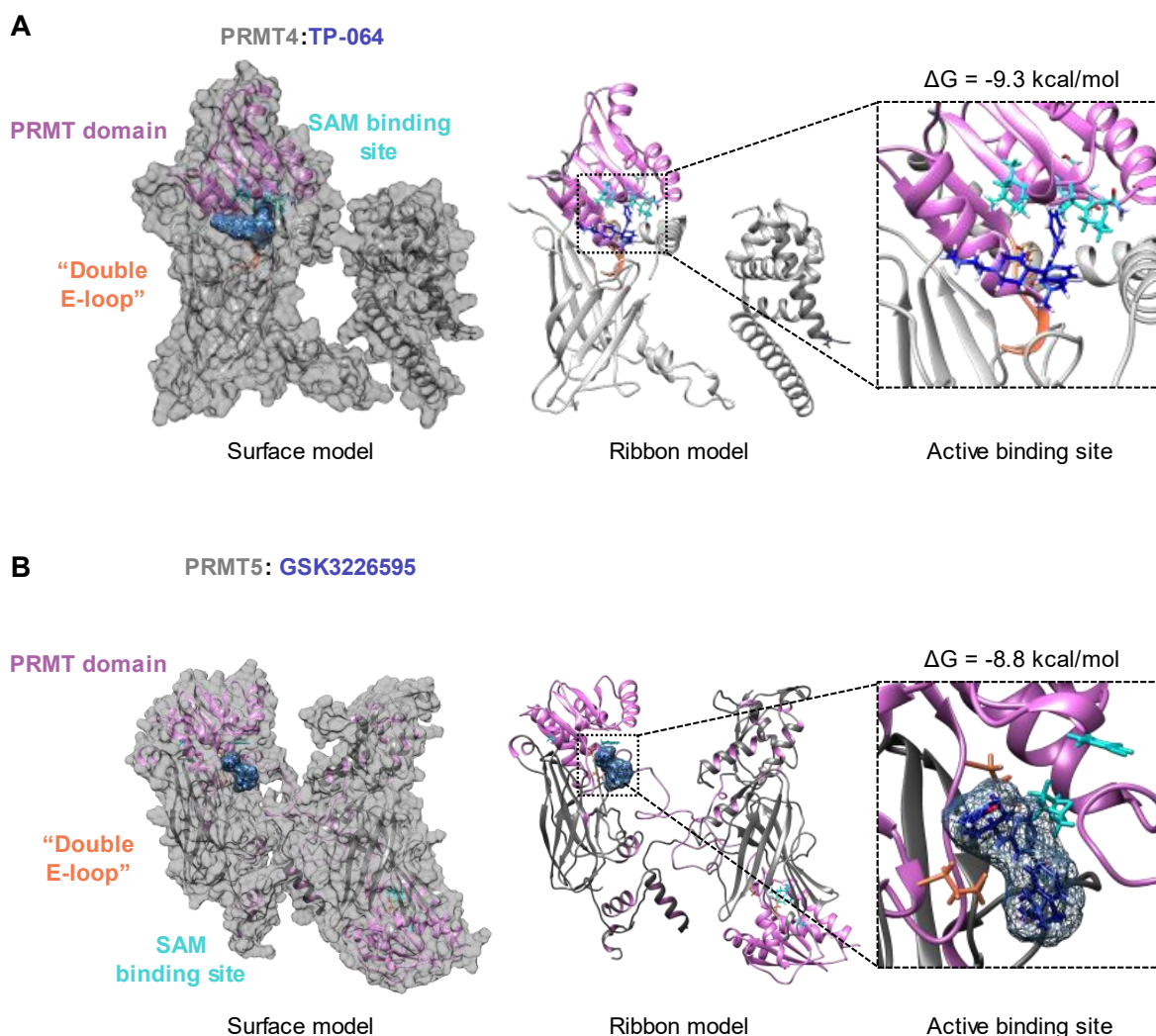


Figure 4.8. Molecular docking prediction of PRMTi into *P. falciparum* PRMT4 and PRMT5. AlphaFold (www.alphafold.ebi.ac.uk) was used to access predicted models for (A) PRMT4 and (B) PRMT5³⁷¹. In UCSF Chimera³⁷⁰, the canonical SMILES were used to create the ligand structures. Dock Prep³⁷⁰ was used to build protein and ligand structures with subsequent docking using SwissDock³⁷². UCSF Chimera was used to visualise the results of the docking. Both PRMT4 and PRMT5's protein arginine methyltransferase domains are shown (plum), with conserved, invariable SAM co-substrate coordinating residues (cyan) and the unique "double E-loop" (coral) to differentiate these sections from the rest of the protein (grey). The Gibbs free energy value (ΔG) of the most favourable binding mode from the cluster, which corresponds to direct blockage of essential amino acid residues, is presented.

The confidence levels for the PRMT domains of the PRMT4 and PRMT5 models obtained from AlphaFold 2.0 indicated high per-residue confidence scores [pLDDT] >90. Predicted PRMT4:TP-064 binding sites were represented by 46 clusters with populations ranging from one to nine members. Of these, 27 clusters demonstrated binding within the PRMT-domain (Phe³⁷⁹ to Val⁸⁸⁷). The second component of the first cluster reflects a TP-064-PRMT binding mode corresponding to the direct obstruction of SAM-binding site residues within the active site (Ile⁵⁵², Cys⁵⁵⁴, Ser⁵⁵⁶, Ser⁵⁵⁷, Ile⁵⁵⁸, Asp⁵⁷⁴, Asn⁵⁷⁵, Leu⁵⁸⁰, Lys⁶⁰⁰, Glu⁶⁴⁵) expected to be crucial for PRMT4 activity with a ΔG -9.3 kcal/mol. When TP-064 is bound to PRMT4, the

bidentate interaction between two conserved glutamate residues (E⁶⁴⁵ to E⁶⁵⁴, "double-E loop") is prevented, which is important for chelating and orienting the guanidine group of the target arginine residue on the histone (Figure 4.8A).

The docking of GSK3326595 into a homodimer PRMT5 resulted in 33 clusters with one to ten members. Two of these clusters indicate that GSK3326595 and the PRMT domain-containing area have a favourable interaction (Phe³⁷⁹ to Val⁸⁸⁷). The binding mode for PRMT5-GSK3326595 with the best interaction has an estimated binding energy of ΔG -8.76 kcal/mol (Figure 4.8B). These molecular docking predictions support the hypothesis that TP-064 and GSK3326595's actions in *P. falciparum* mature gametocytes could be due to PRMT4 and PRMT5 inhibition, respectively. Given PRMTs' widespread biological significance, it's likely that, in addition to the obvious effect on histone N-terminal tail methylation, inhibition of the wide range of PRMT targets it can bind is also responsible for the potent effects reported in mature gametocytes.

4.3.4 Drug interaction between combinations of HATi, HKMTi and PRMTi

Fixed-ratio isobolograms were used to assess the possibility that specific combinations of epidrugs result in inhibition of epigenetic modulators involved in the modification of histone PTM partners that co-exist and display crosstalk³⁹⁰⁻³⁹². This involved several assumptions, including that the epidrugs displayed relative stage-defined activity (Figure 4.4 to Figure 4.6) and that at least for two PTMs in mature gametocytes (H3K18ac and H3R17me2, Figure 4.7), treatment with epidrugs does indeed affect acetyl and methylation status of the histone marks. If the epidrugs does in fact target assumed acetyl-, methyl- and protein arginine transferases involved in establishing the target substrate combinatorial PTMs simultaneously, then a more synergistic result is expected. As such, for isobologram analysis, a threshold value of $\Sigma FIC = 0.5$ is used, where $\Sigma FIC < 0.5$ (with a concave curve) regarded as synergistic and $\Sigma FIC > 0.5$ but with only slight deviation from linear regression as additive or indifferent, moving toward antagonistic if a convex curve is observed, with an $\Sigma FIC > 2$ clearly antagonistic³⁹⁰.

The combination of H3K4me3K9ac in asexual parasites was probed by combining BIX01294 (as HMTi) with anacardic acid/C646 as HATi targeting GCN5³⁸². This combination resulted in a pronounced effect (FICs of 0.58 and 0.67), with the combination with C646 the most pronounced, leaning towards antagonistic. However, the slightly more additive effect seen with anacardic acid supports the known positive interplay between H3K4me3 and H3K9ac (215). Interestingly, the combined use of two HATi (anacardic acid and C646) was additive (FIC 0.62), further supporting the acetylation of both H3K9 and K14 (Figure 4.3). Lastly, the

H3K9acK36me3 combination in asexual parasites was interrogated by the combination of anacardic acid/C646 as HATi targeting GCN5 with ML324 as JMJC2 inhibitor. For both of these combinations, an additive effect was observed with slight curve deviation from the linear line (FICs of 0.53 and 0.45). This again supports and correlates to the positive crosstalk between H3K9ac and H3K36me3. This was specific, as the combination of ML324 with BIX01294, that would target the crosstalk of H3K4 and H3K36 methylation (which does not exist), display variant associations implying that this combination is not important.

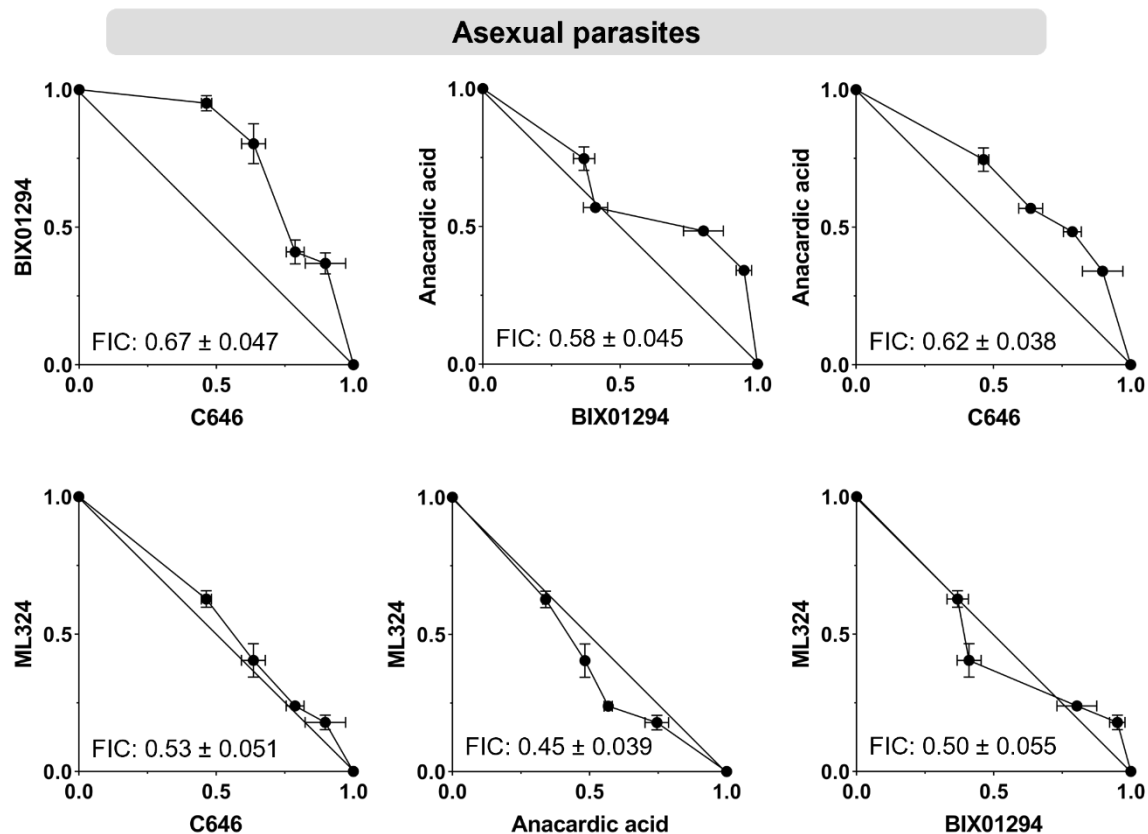


Figure 4.9. Antiplasmodial activity of histone acetyltransferases, histone lysine methyltransferase and protein arginine methyltransferase in combination in asexual *P. falciparum* parasite. Isobolograms show either additive (i.e., slight deviation from linear regression line), antagonistic (convex shape curve) or synergistic (concave shaped curves) interaction between drug interactions in asexual. Plotted values represent ΣFIC_{50} at respective drug combination ratios. The mean $\pm$ SEM FIC data is shown for each combination for three independent biological repeats, performed in technical triplicates.

In early-stage gametocytes, combining BIX01294 as HKTMi with TP-064 as inhibitor of PRMT4 was mostly additive (FIC 0.57), as one would expect if these compounds affect H3K9me2R17me2. However, BIX01294 also displayed some additive nature (FIC 0.59) with the PRMT5 inhibitor GSK3226959, which should exclusively target PRMT5 (H3R8me) – a PTM that is not found in combination with any lysine methylation mark that could be effected by treatment with BIX01294. Therefore, no specific effect could be ascribed to the combination

of BIX01294 with any other compound. However, the H3R8me2R17me2 positive crosstalk in immature gametocytes correlate with a slight synergistic effect other otherwise perfect additive effect between the two PRMT inhibitors, TP-064 and GSK3226595 (FIC 0.49). This supports the important and strong interaction seen between these two arginine methylation marks in immature gametocytes.

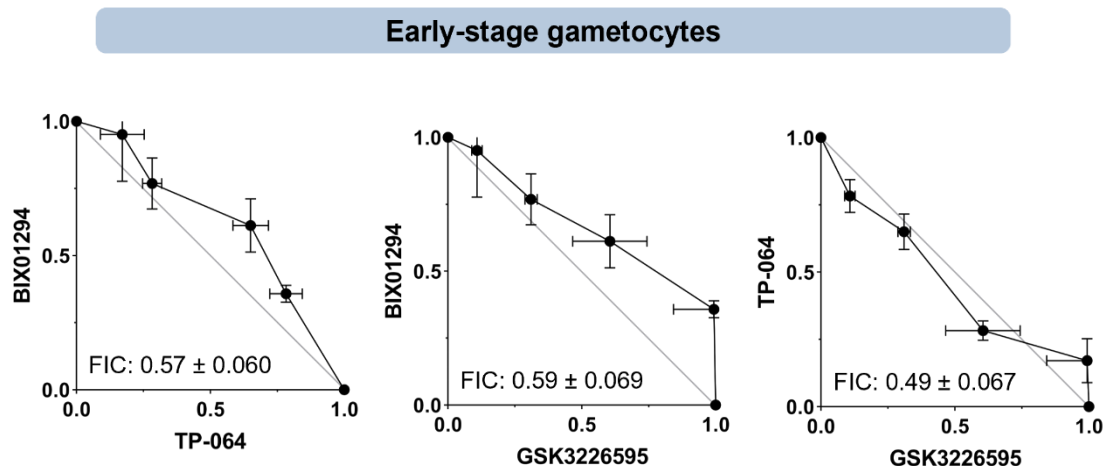


Figure 4.10. Antiplasmodial activity of histone acetyltransferases, histone lysine methyltransferase and protein arginine methyltransferase in combination in early-stage gametocytes *P. falciparum* parasite. Isobolograms show either additive (i.e., slight deviation from linear regression line), antagonistic (convex shape curve) or synergistic (concave shaped curves) interaction between drug interactions in early-stage gametocytes. Plotted values represent $\sum FIC_{50}$ at respective drug combination ratios ($n=3 \pm SEM$). The mean $\pm SEM$ FIC data is shown for each combination.

In late-stage gametocytes, combinations between H3R17me2, H3K18ac and H3K23ac was interrogated. Pronounced synergism was present between GSK332659 and either C646 or TP-064 (FICs 0.25 and 0.33, respectively), suggesting that the function of H3KR8me2 is dependent on H3K18ac and H3R17me2. This reflects the substantial positive crosstalk reported in Chapter 2 between the unusual triple combination of H3R8me2K18acR17me2 (Figure 4.11). Similarly, since C646 and anacardic acid targets PfGCN5 that also deposits K9ac, the synergism that extends to the combination of GSK3226595 and anacardic acid could also explain the crosstalk combination of H3R8me2K9ac. Importantly, the unique combination of H3R18meK18ac in mature gametocytes was supported by the synergism observed between TP-064 and C646 (FIC 0.41, concave curve) (Figure 4.11).

Late-stage gametocytes

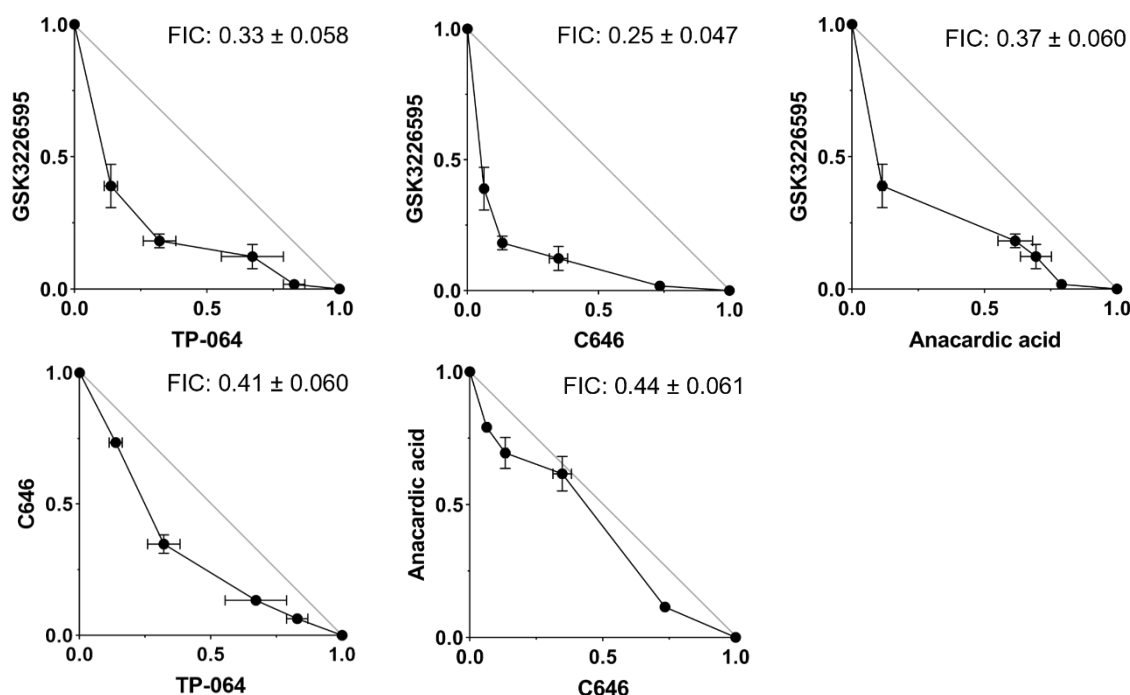


Figure 4.11. Antiplasmodial activity of histone acetyltransferases, histone lysine methyltransferase and protein arginine methyltransferase in combination in late-stage gametocytes *P. falciparum* parasite. Isobolograms show either additive (i.e., slight deviation from linear regression line), antagonistic (convex shape curve) or synergistic (concave shaped curves) interaction between drug interactions in late-stage gametocytes. Plotted values represent $\sum$ FIC₅₀ at respective drug combination ratios ($n=3 \pm$ SEM). The mean $\pm$ SEM FIC data is shown for each combination.

Inhibition of PfGCN5 using both C646 and anacardic acid led to the likely inhibition of H3K23ac crosstalk with H3K9ac or H3K18ac. Overall, late-stage gametocytes were more susceptible to dual inhibition by both a HATi and a PRMTi, indicating that during these stages, arginine histone PTMs in combination with lysine acetylation might act as important regulators. Given that arginine methylation is a key PTM that occur on both histone and non-histone proteins, it was necessary to confirm that treatment with these compounds translated in to hypomethylates H3R17me2 and hypoacetylation of H3K18ac.

4.3.5 Combinations involving PRMT inhibitors directly affect methylation status of *P. falciparum* histone H3

Given that the combination of H3R17me2K18ac indicated possible function crosstalk in Chapter 2, and that parasites treated with TP-064 and C646 were highly susceptible to inhibition when treated in combination, it was necessary to evaluate if the synergism seen in Figure 4.11 is also reflected in changes in the acetylation and methylation status of H3K18ac and H3R17me2 (or their combination), respectively. Mature gametocyte was treated with

C646, TP-064 and a combination of C646 + TP-064 and probed with commercially available H3K18ac, H3R17me2 or a combination of both antibodies (Figure 4.12).

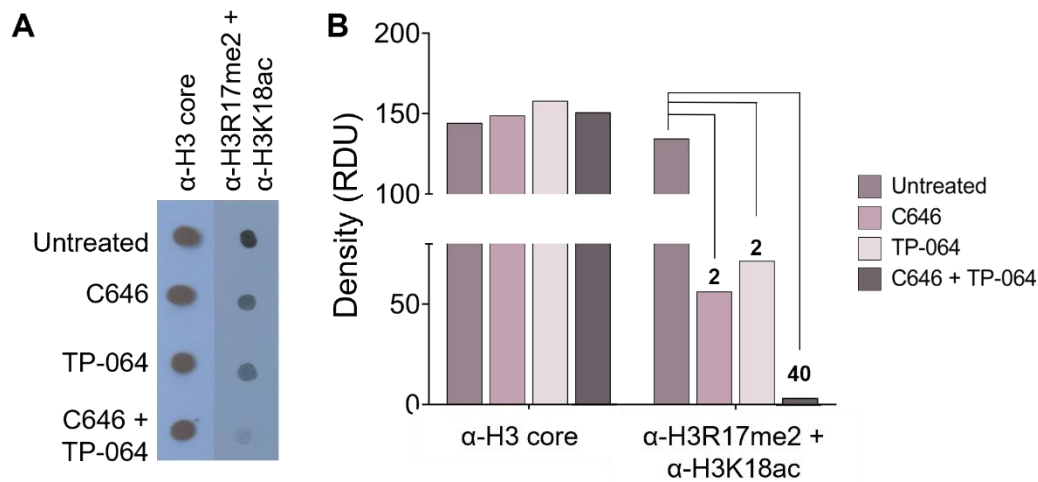


Figure 4.12. The gametocytocidal activity of a combination of histone acetyltransferase and protein arginine methyltransferase inhibitors, C646 and TP-064 is accompanied with the hypomethylation of H3R17me2 and hypoacetylation by H3K18ac. (A) Detection of H3R17me2 and H3K18ac hypomethylation and acetylation by dot blot analysis of late stage (day 10) gametocytes treated for a total of 24 h with IC₉₀ inhibitory concentrations. Histones were loaded quantitatively in each instance. **(B)** Acetylation and methylation levels are expressed as fold changes (treated/parallel untreated control gametocytes) of relative density units (RDU). Data are representative of a single biological replicate.

Treatment with C646 and TP-064 individually led to a >2-fold reduction in H3K18ac + H3R17me2 levels. Similar levels of hypoacetylation and hypomethylation were observed for either C646 or TP-064 treated parasites when probed using both antibodies for H3K18ac and H3R17me2. When parasites were treated with a combination of C646 and TP-064 a considerable hypomethylation (>40-fold) was detected in the level of both H3R17me2 and H3K18ac, compared to untreated levels of H3R17me2 + H3K18ac. Overall, our findings corroborate the hypothesis that the observed co-dependency of H3K18ac and H3R17me2 is reflected in the combined potency of C646 and TP-064, as well as the influence it has on histone methylation and acetylation.

4.4 Discussion

Understanding the epigenetic mechanisms underlying *P. falciparum*'s unique life cycle is important not only for the development of new chemotherapies, but also because the parasite is a model organism with the unique ability to both propagate exponentially and undergo rapid differentiation, a cellular phenomenon important for understanding complex cancers and other cellular features. There is convincing evidence that the epigenome of *P. falciparum* is a druggable component of the parasite's biology that can be exploited^{359, 360, 375, 393-396}. The antiparasmodial action of several chemical scaffolds known to target the epigenetic gene regulatory machinery against asexual parasites, early- and late-stage gametocytes, and the effect of targeting histone PTMs that exist in combination were examined in this study.

The use of this diverse library of epi-drugs was used to prioritise classes and compounds to investigate stage specific crosstalk between combinatorial PTMs. Recognizing the fact that histone-associated epigenetic regulator inhibitors represent more than half of the compounds in the library screened, HDACi and HKMTi remain most active on parasite inhibition as compared to other epigenetic modulator inhibitors. If these inhibitors target the *P. falciparum* homologues of HDACs and HKMTs, the discovery supports the importance of histone acetylation and methylation for parasite gene regulation and survival. The distinct stage distribution and set of PTMs that exhibit crosstalk in the three biological stages explored in Chapter 2 were represented here, with the majority of epidrug combinations resulting in either sub- or sub-therapeutic efficacy. The stage-specific inhibition profiles observed for this wide variety of epi-drug inhibitor classes again support the findings that the parasite makes use of altered epigenetic regulatory mechanisms to differentiate itself during asexual proliferation and sexual differentiation. This suggests that the drug-drug interaction reduces the effect of each drug, implying that the parasite benefited from their inhibition and that other combinations are more important than the one inhibited, or that a signalling pathway was initiated to compensate for the loss of the PTMs, or that these PTMs function independently and that the protein target was not available in the first place, making the drug redundant. In asexual parasites, PTMs are thought to work in a mutually exclusive manner^{153, 176, 179, 216, 240}, making a single epidrug more active than treatment with another epidrug, while gametocytes grow more co-dependent.

Late-stage gametocytes were particularly vulnerable to epidrug combinations, displaying an overall synergism based on the isobolograms. This highlights an important goal to eradicate malaria, i.e., the importance of obtaining drugs that can block or reduce transmission between the human and the mosquito. Stage V gametocyte are more sensitive to inhibition, as it lacks certain pathways to adapt, such as new permeability pathways. Epigenetically, late-stage

gametocytes are prepared and geared toward rapid genome replication with more transcriptionally permissive PTMs, once it has transmitted to the mosquito (151).

Although isobolograms are useful in asserting drug interaction, it is however limited and does not reflect the mode of action, mechanism of action, etc. between the drugs, since 1) the end-point measurement in this case is only cell viability, and 2) the isobolograms' graphical composition is predicated on a constant relative potency. Nonetheless, the concept that two drugs in a sea of other potential interactors, could interact in a way to produce an enhanced effect on the cell is fascinating and can further our understanding of the action of even single drugs. Further studies are required to validate the observed potent killing effect the combination of C646 and TP-064, experimentally validate PfGCN5 as target for C646 and how those correlates to the functional relevance of the specific H3R17me2K18ac combination in late-stage to mature gametocytes.

Using combinations of drugs have the added advantage of therapeutic use, particularly as anticancer therapy, with several compounds being FDA approved for use in acute myeloid leukaemia, B- and T- cell lymphomas and solid tumours ^{353, 397, 398}. Epigenetic inhibitors (“epidrugs”) can be administered as a monotherapy or in combination with other epidrugs to generate a synergistic effect and maximise the therapeutic benefit. Novel treatments against malaria should target the proliferating asexual parasites to alleviate symptoms for the patient, but also a long-lasting compound that is active against the mature-stage gametocytes to block or reduce transmission (440). Here, we show that PRMTi (GSK3326595 and TP-064), HATi (C646 and anacardic acid), and HDMTi (ML324) are potent late-stage gametocyte inhibitors that harbour desirable submicromolar activity against these stages and are therefore ideal candidates to be considered for combination therapy, along with a more potent asexual active compound, such as BIX01294 (HDMI). Another possible favourable candidate partner would be dual-active, inhibiting both asexual and gametocyte stages of the parasite (e.g., the kinase superfamily inhibitors (441)).

Protein arginine methyltransferases, especially PRMT4 and PRMT5, have been the focus on novel target-based drug design studies over the past decade given that their dysregulation is associated with a multitude of cancers and cancer development ^{303, 347, 399-403}. SAM analogues (including the pan methyltransferase inhibitors sinefungin and S-adenosyl homocysteine), substrate peptide analogues, and structures optimised from high throughput screening hits are the three types of PRMT inhibitors that have been reported so far ⁴⁰⁴. Interestingly, PRMTi revealed to be unique compounds with multi-stage activity, with or without a partner drug such

as HATi or HKMTi. Following further experimental validation (including inhibition of male gametocyte exflagellation and transmission decrease), cytotoxicity reduction, and chemical property optimisation to become more stable compounds, these results have the potential to be utilised as lead compounds for antimalarial drug discovery. Since 2018, novel inhibitors discovered have included PRMT4 inhibitors EPZ022302/EZM2302 with an $IC_{50} < 6$ nM with anti-tumour activity *in vivo* and TP-064 ($IC_{50} < 10$ nM against PRMT4 in multiple myeloma and >100-fold selectivity for PRMT4 over other methyltransferases^{338, 387}. Furthermore, the PRMT5 inhibitor, GSK3326595, is the first, and only, PRMT inhibitor currently preparing to undergo phase II clinical trials in patients with lymphomas and solid tumours (clinicaltrials.gov, NCT04676516)^{386, 403, 405, 406}. Common PRMT5 inhibitors are thought to work by inhibiting cellular splicing and causing an isoform transition in MDM4, a major regulator of p53 function, resulting in p53 activation^{361, 400, 407-411}. Molecular modelling shows that TP-064 inhibits PRMT4 and GSK3326595 inhibits PRMT5 with a high level of potency via binding to the SAM and arginine methyltransferase domain binding sites. Since arginine methylation is an epigenetic regulator of transcription and play key roles in pre-mRNA splicing, DNA damage signalling, mRNA translation, cell signalling, and cell fate decision, it will be important to decipher the specific role in *P. falciparum* mature gametocytes.

Finally, the link between the triple combination of H3R17me2, H3K18ac and H3K23ac is established by preliminarily showing that inhibition of PfPRMT4 with TP-064 and PfGCN5 with C646 or anacardic acid have a deadly effect on the parasite, specifically, late-stage gametocytes. These results suggest that these histone PTMs are co-dependent on one another. This is by proxy evident that inhibition of their targets, lead to markedly higher parasite death compared to treatment with the single drug alone. This conclusion is likely due to the methodical approach in which H3R17 gets methylated, as detailed in Chapter 1, in which H3K18ac and H3K23ac are acetylated first, followed by PRMT4 being recruited to methylate H3R17me2^{335, 336}. When C646 prevents H3K18 acetylation, PRMT4 recruitment is prevented. The parasite dies if both are blocked and the triple combination is missing in later stages of the parasite, indicating that the process this combination controls is likely critical to parasite life.

In conclusion, these findings reveal that not only is the *P. falciparum* parasite's histone coding a potent druggable system, but that using epigenetic inhibitors in conjunction improves parasite killing substantially more using single epidrugs. Given the WHO's recommendation for the use of combination drugs in the treatment of malaria, these epidrug combinations are

worth optimising for patient use and inspected to be tested in combination with other classes of compounds.

Chapter 5 | Concluding discussion

Malaria has been, and remains to be, one of the biggest global health concerns. Despite all the available tools and multidisciplinary approaches to combat malaria in a modern day and age, it is the absolute robustness and plasticity of the deadly *Plasmodium falciparum* that has enabled it for millennia to survive and will continue to adapt and survive well beyond humans unless we address its true weak spot. Part of the challenge in developing next generation control strategies has to do with the fact that many peculiarities of the parasite's biology remain ill described or unknown. Understanding gene regulation in the broad context of chromatin-based mechanisms of control is critical to uncover the parasites' secrets to its success in survival. Targeting the development of the parasite rather than a component of mature stage gametocytes can offer better chances to reduce its infectiveness. Drugs that target mature stage gametocytes cells can inhibit them, but hardly prevent their formation (355).

The past two decades in research into chromatin biology of *P. falciparum* has focused on identifying the proteins and factors involved in its life cycle regulation which was largely limited to asexual parasites^{125, 151, 412-415}. Next, focus was placed on revealing the detailed description of individual factors involved and the regulation thereof^{123, 176, 416-419}. This led to two of the biggest breakthroughs in understanding the role epigenetics play in the life cycle regulation of the parasite: antigenic variation, virulence gene expression and silencing, and H3K9me3-HP1 mediated repression of the *ap2-g* locus to prevent commitment to differentiate into gametocytes^{163, 239, 271, 412, 420-422}. Now that there is convincing evidence to support that epigenetic gene regulation is not only fundamental to its development and survival, but it also likely represents its true weak spot. Parasites are highly susceptible to epigenetic machinery inhibition. As a result, I anticipate that the next decade of chromatin biological research in *P. falciparum* will reveal more divergent underlying features of the parasite, beginning with this thesis and deciphering the parasite's histone code. Such an understanding would seem to be a prerequisite for the rational design of next-generation antimalarial therapies.

Remarkably, *P. falciparum* only has a single predicted transcription factor per ~70 protein coding genes, while yeast have one per ~31 genes and humans one per 13 genes^{99, 423, 424}. It is difficult to comprehend how such a limited number of transcription factors can regulate such a A+T-rich genome in a sequence specific manner in the context of an increasingly complex biological system. Part of *Plasmodium* success as a specie is that that it can rapidly change its genome to adapt to its environment using histone modifications as a robust but adaptable matrix, as opposed inducing gain- or loss-of-function mutations to alter gene expression

regulation over multi-generational progeny. This is especially evident in its ability to relative to other organism, rapidly develop drug resistance against front-line chemotherapeutics^{425, 426}. Either transcription factors act in combinations to enable pleiotropic functions, or they are regulated by other protein-protein interactors, or they are post-translationally modified as previously suggested⁴²⁷. An alternative explanation is that a higher order of gene regulation is at play. Regardless of how many transcription factors there are, or their regulation methods, accessibility and binding to DNA is controlled by chromatin structure and nuclear organisation. Gernot Längst and colleagues proposed that the *P. falciparum* nucleosome landscape, transcription factors and protein-protein interactions form part of an equally important and complex regulatory network to guide gene expression throughout its life cycle⁴²⁷. It is therefore critical to understand the contribution of the histone post-translational code to stage specific biology in the parasite. Here, we provide the first and most comprehensive catalogue of histone PTM combinations, and preliminary evidence that this combinatorial histone code is not only functionally important but is also a system that can be used against the parasite in multiple stages of its life cycle.

In Chapter 2 of this thesis, the fundamental information regarding the histone code is provided. Although it is limited to acetylation and methylation of lysine and arginine residues only, it provides and supports existing knowledge that point toward an additional layer of complexity of simple protein modification in describing crosstalk between histone PTMs. Given that histone N-terminal tails were modified with a relative low number of acetylation and methylation compared to the number of sites that can be modified simultaneously, suggest a model where a small number of core PTMs can lead to a large number of different combinations and functional outcomes, a model supported by previous research and theoretical analysis⁴²⁸⁻⁴³¹. Interestingly, some PTMs with assumed opposite function in transcription co-exist together. For example, the combination of H3K27me3 and H3K4me3 co-exist in asexual stages of the parasite are associated with low levels of transcription that mark important developmental genes, keeping them silent but ready to be activated at the onset of developmental programs and cellular differentiation⁴³²⁻⁴³⁶. Such bivalent histone PTMs pinpoint again to the multi-layered sophistication of the histone code in *P. falciparum* parasites.

A key knowledge gap that this thesis addresses is the involvement of other histone PTMs involved in many of the biological processes currently only associated to one PTM, for example, the repression of the *ap2-g* loci by HP1-H3K9me3. There is substantial evidence to support that H3S10ph have negative crosstalk with H3K9me3 by displacing HP1 from H3K9me2/3 sites, H3S10ph disruption causes aberrant mitotic divisions^{201, 232, 437}. Although

H3K14ac does not eject HP1, existing H3K9 PTMs (ac and me2/3) could prevent methyltransferase activity at this site ^{200, 438}. This is also true of mouse stem cells where H3S10ph influence on H3K9me2 has been confirmed by studies that show H3S10ph inhibits the spread of H3K9me2 and other repressive PTMs ⁴³⁷. This suggest that in addition to the known factors involved in commitment to gametocytogenesis, H3S10ph might play an additional critical role *ap2-g* locus expression.

Additionally, the contribution of other canonical and variant histone PTMs, in particular phosphorylation and SUMOylation remains to be interrogated. This is especially true given that certain PTMs or combinations of histone PTMs can define cell cycle compartments, an endeavour that has been particularly challenging for gametocytogenesis ^{54, 439, 440}. For example, accumulation of H4K20me1 marking gene bodies recruit DNA synthesis and origin of replication proteins, a process that is amplified when H4K20 is di- and trimethylated by SET8 which characterised the switch from M-phase to S-phase ⁴⁴¹. Furthermore, interaction between H2A and H4 PTMs peak during M-phase when H3S10 is phosphorylate ⁴⁴². When the writer histone methyltransferase of H4K20 is inhibited with a small molecule inhibitor, it is hypothesised that the hierarchical and sequential acetylation of H4 may act as a compensatory system to protect H4K20me2 from demethylation Because acetylation of neighbouring residues prevents demethylation of H4K20me2, the epigenetic state of the cell is maintained. Simultaneous inhibition of CBP/p300 and SUV4-20 effectively overcomes drug resistance by preventing this compensatory mechanism from functioning ⁴⁴³.

This thesis also highlights the need for future studies to decipher the sequential or hierarchies in writing and erasing histone PTMs. Specifically, the exact mechanism involved and biological importance of the triple combination of H3R17me2K18ackK23ac that is present in stage V gametocytes should be verified. This combination is likely important for gearing the genome and recruiting protein factors to rapidly undergo DNA replication following transmission of the parasite and is established by a likely unique PfGCN5 complex (as seen in Chapter 3). This warrants investigation into the exact impact of this combinations and other stage specific combinations have for the parasite.

The results presented in Chapter 3 highlights an interesting avenue for further interrogation: the interplay between parasite metabolism and epigenetic gene regulation. In addition to known chromatin associated proteins and the transcription factor PfAP2-G2, several metabolic enzymes involved in polyamine and choline metabolism were enriched. The important role of polyamine metabolism (specifically, methionine as a one-carbon methyl donor) has been

demonstrated in DNA and histone methylation, with a known role for lysophosphatidylcholine in commitment to differentiate into gametocytes. Recently (December 2021), acetyl-CoA synthetase has been shown to be an essential, druggable target that alters the acetylation status of histones directly (438). Therefore, the cause and/or effect relationship between nutrient metabolism and the molecular regulatory pathways on an epigenetic scale, and the subsequent role in parasite development remains largely unexplored in the parasite.

In this study we also observed the endpoint result of targeting the combinatorial histone code with combinations of epigenetic protein inhibitors. Since this part lacks the crucial and direct evidence that disruption of relationship between histone PTMs are fundamentally responsible for parasite inhibition, it will be necessarily to meticulously investigate the combinatorial histone code and the point for point, PTM for PTM, importance to the parasite. Notwithstanding, the results showcased a particular and increasing susceptibility of parasites (gametocytes specifically) to protein arginine methylation inhibition. Perhaps, arginine methylation as a post-translational modifications control system is a key feature that is involved in gametocyte specific biology or stage transitions. Arginine methylation has been postulated to be cancers' "best kept secret" ³⁴⁷, and parallels can be drawn between a differentiating gametocyte and cancer development ⁴⁴⁴.

Beyond the cell cycle, histone PTMs can also be used as barcode to identify specific stages of the parasite, a means that would refine stage transitions beyond morphology. Here we see that trophozoites indicated known combinations between H3K4me3K9acK14ac, while although not identified in this particular study, the combination of H3K27me2/me3 and H3K36me2/me3 defines stage II gametocytes ^{167, 241}.

Although our study was limited to combinations on either histone H3 or H3.3, it showed the remarkable feature that the majority of PTMs had a negative crosstalk in asexual parasites, while in gametocytes, most PTMs were dependent on partner PTMs based on their interplay scores. This links back to the fact that asexual parasites are less tightly regulated and have adapted abnormal epigenetic and biological features. On an epigenetic level, these parasites have evolved to have an open chromatin architecture to express most of its genome simultaneously. On a biological level, asexual parasites have evolved a metabolic strategy to maximise its energy generation potential to rapidly expand by dysregulation of aerobic glycolysis (442). Deconvolution of the link between nutrient metabolism and epigenetic gene regulation will be crucial in expanding our knowledge on the *modus operandi* of *P. falciparum* parasites. In contrast to other vector borne blood protozoans, *Plasmodium* is the only genus

that undergoes multiple rounds of asexual schizogony in the blood whereas other hemosporidia only produce gametocytes ⁴⁴⁵. In essence, the strict regulation involved in gametocyte development and maturation contrasts sharply with the promiscuous and seemingly unrestrained asexual stages that serve only to multiply as quickly as possible, foregoing the complexity involved in stage-to-stage transitions during gametocytogenesis.

In conclusion, the histone code of the *P. falciparum* parasite is a refined chromatin 'language' in which different combinations of basic covalent chemical modifications provide dynamic functional outcome underlying one of the most complex life cycle of any organism.

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